Applied Ecology and Environmental Research

International Scientific Journal

VOLUME 19 * NUMBER 5 * 2021

Published: September 30, 2021
http://www.aloki.hu
ISSN 1589 1623 / ISSN 1785 0037
DOI: http://dx.doi.org/10.15666/aeer

IMPACT OF CLIMATIC VARIATIONS ON THE FLOWERING PHENOLOGY OF PLANT SPECIES IN JHELUM DISTRICT, PUNJAB, PAKISTAN

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(Received 14th Feb 2021; accepted 14th May 2021)

Abstract. District Jhelum is located in the extremely diverse province of Punjab, Pakistan, and flowering event in plants is always influenced by the environment. This study was conducted during 2018 to 2020 to investigate the climatic effects on flowering cycle of plants. The main focus of the study was to find out the particular association between flowering phenology of plants and climatic variables. Month-wise phenological response of plants was recorded during frequent field visits at multiple representative microhabitats. The response data is saved as binary data matrix, and mean monthly climatic data is obtained through remote sensing, and analysed by using multivariate analyses like canonical correspondence analysis, hierarchical classification and pseudo-canonical correlation. CCA and Hierarchical classification were applied to assess the importance climatic variations towards the flowering phenological response and potential groups respectively. A total of 404 plant species of 223 genera belonging to 75 plant families were examined. Majority of plant species were found in flowering during the month of March (174 spp.) followed by April (159 spp.) and August (158 spp.), similarly, Summer was the leading season (208 spp.) followed by Monsoon (203 spp.), Spring (181 spp.) and Autumn (157 spp.). CCA results depicted that total variations in the flowering phenology response data were 3.45084, and about 45.6% were explained by the explanatory climatic variables. Wind speed, mean monthly maximum temperature and soil moisture were detected as most influential drivers of flowering phenology in the study area. The current study will be useful for researchers as a major source of knowledge for the conservation of valuable species. Such type of attempts will be supportive to explore the phenological response of plants in various habitats such as forest, hilly, riverine, desert and range lands flora in their future projects.
Keywords: *phenological response, hierarchical classification, canonical correspondence analysis*

Introduction

The word "phenology" stands for the life history of plants (Vashistha et al., 2009). To record phenological response at local and regional scale some modeling tools and remote sensing play significant role (Neil and Wu, 2006). Phenology of plants is recorded through observation during ecological explorations to estimate month wise or season wise data including the last stage of appearance (Meier et al., 2007; Menzel et al., 2006). During documentation of ground truth data, various climatic variables were recorded for comparative data analysis (Badeck et al., 2004). In an ecosystem clear effects of climatic variables on phenological response were determined (Kolb et al., 2007). Climatic and phenological relations were documented by many research studies (Petry et al., 2016). Phenology and climatic conditions are linked to multiple scales (Bertin, 2008), environmental variables can affect the functional aspects of plants in any ecosystem (Parmesan, 2006; Calinger et al., 2013) resulting in close relationships among plant pollinators and plant species (Forrest, 2015; Kharouba and Vellend, 2015), and also among migratory birds and plants (Both et al., 2006). In life of plants, some unpredicted

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DOI: http://dx.doi.org/10.15666/aeer/1905_33433376

circumstances can affect the flowering event such as extreme temperature, day length and humidity, and studies documenting the influence of current climate on phenological events become extremely important because researchers already predicted a remarkable potential change in future climate. The presence or absence of biotic factors such as, grazers and insect pollinators and abiotic factors such as temperature, day length, and rainfall which influence the pattern of phenology (Thomson, 2010).

Various research studies resulted that temperature had significant effect on Phenology of plant species. But it was noted that temperature and phenological effect was not uniform in the World. The reason depicted that there was fluctuation in temperatures from different regions. Each species showed particular effect of temperature on phenology. So, the effect of temperature varied from species to species. In different regions of the World, with altitudinal variations, temperature played a basic role in different phenological response (Luo et al., 2007) (Holway and Ward, 1965; Shen et al., 2015; Luo et al., 2007) (Mooney and Billings, 1960). At different stages of phenology, the plants showed variable response at various temperature (Vashistha et al., 2009). International Panel on Climate Change, stated that a global rise of 0.74 °C in surface temperature results in environmental changes including less snow cover, rise in glacier melting, rise in sea level and variations in environmental temperature, rainfall and wind speed (Change, 2007).

In various regions of the World, climatic variations affected phenological responses greatly. The major climatic factors which influence the phenological pattern among various species are temperature, soil moisture, precipitation and rainfall (Chambers et al., 2013; Liu et al., 2016a, b; Inouye, 2008; Wolkovich and Cleland, 2011; Sun et al., 2015; Shen et al., 2016; Buyantuyev and Wu, 2012; Piao et al., 2019; Ma et al., 2013; Yu et al., 2003; Zhang et al., 2018; Visser et al., 2010; Richardson et al., 2013; Badeck et al., 2004; Zalamea and González, 2008). Globally, various seasons also play an important role in the phenology of plant species (Piao et al., 2019; Wolkovich and Cleland, 2011; Mittermeier et al., 2019; Gordo and Sanz, 2005; Morisette et al., 2009; Chambers et al., 2013; Yang et al., 2017).

Many studies resulted that temperature directly had direct influence on the phenological response among various plants species (Piao et al., 2019; Cleland et al., 2007; Cornelius et al., 2013; Prevéy et al., 2017; Crabbe et al., 2016; Shen et al., 2011; Keenan et al., 2020). Whereas, seasonal environmental variations showed a clear association to flowering period of plants. While phonological period, during life cycle of plants, represent prominent association with temperature. Moreover, in some cases, humidity, soil moisture, soil composition and soil texture influence the plant phenology (Cleverly et al., 2016; Francioli et al., 2018; Nandintsetseg and Shinoda, 2011; Peña-Barragán et al., 2011; Bodin and Morlat, 2006). Soil showed a major effect on the life cycle of plants. Many studies from different regions of the World, revealed the influence of soil factors on phenological pattern of plant species (Pausas and Austin, 2001; Okusanya et al., 2016; Anderson et al., 2012; Tadey, 2020; Tooke and Battey, 2010; Staehlin and Fant, 2015; Hulme, 2011; Cleland, 2007; Godoy et al., 2009; Neil et al., 2010; Lesica and Kittelson, 2010; Khanduri et al., 2008; Chen et al., 2020; Wolkovich and Cleland, 2014; McEwan et al., 2011; Matthews and Mazer, 2016).

The effect of climate and phenological response among large number of plants species was investigated in different geographical regions (Menzel et al., 2006; Parmesan, 2006; Parmesan and Yohe, 2003). Phenological response during spring season were recorded from many decades (Chambers et al., 2013; Schwartz et al., 2013; Ge et al., 2015), while phenological stages were not reported exactly (Menzel et al., 2006; Gill et al., 2015). From terrestrial ecosystems, flowering patterns of plant which played a significant role as

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biological factor are influenced by climate variations (Rosenzweig et al., 2007; Khan et al., 2018; Wang et al., 2018). It is resulted that species with progress in phenology with the rise in temperature will have better chances of survival. Such types of species represented maximum number of flowers, biomass production and vegetation cover. On the other hand, species which do not respond to climate variation faced hazard with short growth period as compared to active competitors (Cleland et al., 2012). As, such types of plant species not responding to temperature changes are facing a rapid decline in their abundance during the previous 150 years (Willis et al., 2008). Many ecologists reported the impact of topography, anthropogenic and climatic changes and possible causes upon various plant species (Khan et al., 2019a, b).

Ecologists should focus on durable and long lasting programming of existing natural resources to assess biodiversity of rich flora from unexplored regions by using multivariate analyses as comprising ordination techniques and hierarchical classification (Khan et al., 2019a, b). Moreover, the district Jhelum, Punjab, Pakistan was still unexplored, mainly relating to plant species indicating phenology and its patterns. As a result, the first ever comprehensive research was conducted to explore the unexplained aims which were

a. to explore the flowering response of angiosperms during the year in different seasons and monthly base
b. to discover the effect of climatic factors on phenological response of the plant species.

The current attempt will convey effective ecological knowledge to the researchers, range land managers, foresters, botanists and ecologists in future studies but also provide many valuable plant species grouping with the phonological response.

Materials and methods

Study area

District Jhelum from Pakistan is located towards North of the river Jhelum and bounded by district Rawalpindi in the North, Sargodha and Gujrat districts lies in the South, Azad Kashmir is situated East, and district Chakwal is located West (Mushtaq et al., 2011; Shah et al., 2013; Majeed et al., 2021). Total population of the district Jhelum is 1.223 million, 71% population lives in rural areas while the remaining 29% population lives in urban area (Altaf et al., 2018). The climatic condition showed that the district is semi-arid, warm subtropical region and is categorized by warm summer and severe winters. Jhelum is a semi-mountainous range, mean annual rainfall is 880 mm per annum while annually temperature in average is 23.6 °C. Jhelum river is compromise up to 247, 102 acres of main land of plains on the other hand 41,207 acres is covered by hills (*Figs. 1* and *2*). The second largest salt mine of the world (Khewra) is in Jhelum which covers an area of 2268 acres (Shah et al., 2013; Hamidov et al., 2016). People of district Jhelum have their diverse mode of life span, culture, traditions, beliefs and have been using indigenous plants for various purposes (Iqbal et al., 2011). The ethnic groups of the area showed a strong linkage with wild plants of cultural and medicinal significance (Majeed et al., 2020).

Floristic and phenological data collection

The research area was floristically explored 2018-2020 (3 years) to record plant species. The main focus was to record the phenological response to climatic changes with reference

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to season and monthly basis. The collected specimens of plant species were tagged with voucher number, pressed, fully dried and finally mounted on the International standard sized sheets of herbarium, following the identification by applying Flora of Pakistan (URL: http://www.efloras.org/) and cross matched with floristic literature (Qureshi et al., 2011; Ali and Nasir, 1989; Ali and Qaiser, 1995). Afterward the initial possible identification of specimens, presently established binomials of each plant species and the family names were copied from the plant list ver. 1.1 (URL: http://www.theplantlist.org/) (TPL, 2013), as proposed by (Khan et al., 2016), to evade any taxonomic mistakes and misperception linked to ordering and placement. Further information comprising local names (Cain and Castro, 1960), were also documented. Frequent field visits were conducted to note phenology and to collect the plant samples from study sites. To record phenological responses of plant species, 171 altitudinal transects (Grids 5×5 km^2) containing 513 samples and 1539 sub-plots were studied by applying stratified random vegetation sampling method. Sub-plots (quadrates) size was 10×10 m for tree layer, 5×5 m for shrub layer and 1×1 m for herbaceous layer (herbs and grasses). The completely prepared voucher specimens were placed in the herbarium of the Department of Botany, University of Gujrat, Punjab, Pakistan for future reference and record. Phenological response of each reported plant species was found out by using the given equation:

$$SFR\ (\%) = \frac{Species\ recored\ during\ floweing\ in\ a\ month}{Total\ plant\ species\ docomented\ in\ study\ area} \times 100$$

where: SFR is monthly-based species flowering phenological response. Likewise, the monthly-based response is used to determine the seasonal based flowering response for each plant species, and this classification include winter season (November to February), spring (March to April), summer (May to August), monsoon (July to September), and autumn season (September to October). While family importance value (FIV) was calculated with given equation:

$$FIV\ (\%) = \frac{Species\ belong\ to\ plant\ family}{total\ plant\ species\ reported\ in\ study\ area} \times 100$$

Climate data collection

In the study area, the climate conditions vary both in temporal and spatial scales. The climate data including environmental precipitation, maximum and minimum temperature, humidity, soil moisture, wind speed, and downward short and long wave radiations (2010-2019 = 10 years) of the study area (Jhelum) was developed from the United States National Centers for Environmental Prediction (US-NCEP), Climate Forecast System Reanalysis (CFSR) by applying climate engine, (https://app.climateengine.org/). The temperature data source was CFSv2 19200 m (1/5-deg) daily reanalysis dataset (NOAA) (*Table 2*).

Statistical analyses

The reported data of phenological response was put in Microsoft excel spreadsheet (plant species vs month-seasons), binary data matrix. Phenology of plant species was recorded monthly. Climatic and phenological data was calculated and linked to remote sensing data created with R statistical package (Ilyas et al., 2013), to produce pairwise correlation, distribution and scatterplots (Khan et al., 2015, 2018). Hierarchical clustering tree for months and seasons (Distance; Correlation, Linkage; Ward) was established and the

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package was named as "pvclust" with R statistical package (Team, 2014). CCA was applied by using Canoco software (Ter Braak and Šmilauer, 2012), to find out the impact of climatic factors to show variations in the data for binary response (Khan et al., 2018).

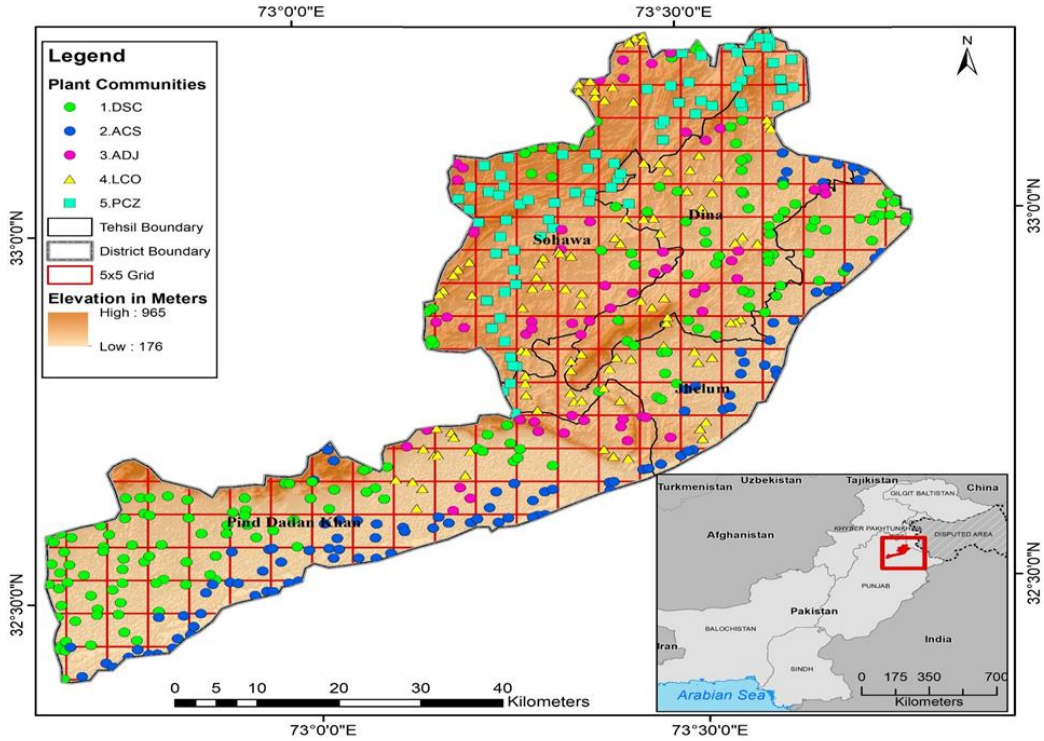

Figure 1. *Map of the study area representing the points of quadrates at different elevations in the district of Jhelum*

Figure. 2. *Landscape representing richness of flora of the study area (a) forest (b) first author identifying plant species (c) view of salt range (d) view of hilly vegetation*

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Results

The record of phenology period of each plant species is a fundamental and important element of such explorations. Reproductive phenological response is permanently interrelated to unique set of climatic variables of any area, thus, assessment of essential climatic factors are needed to lean any potential future climate variation influences.

Floristic classification

A total of 404 plant species were explored including vascular plants belonging to Angiosperms (402 species (99.5%)), Gymnosperms (1 species (0.45%)) and non-vascular Pteridophytes (1 species (1.33%)) including 223 genera and 75 families. Angiosperms were further classified as dicot including 328 species (81.19%), 177 (79.37%) genera and 63 families (84%) while monocot comprised of 74 species (18.32%), 44 (19.73%) genera and 10 families (13.33%) (*Table 1*). The leading plant family was Poaceae (59 spp., 14.6%), followed by Leguminosae (57 spp., 14.11%), Amaranthaceae (27 spp., 6.68%) and Solanaceae (19 spp., 4.7%) (*Fig. 3*), while the leading genus was *Euphorbia* (10 spp., 2.48%), followed by *Brassica* (7 spp., 1.783%), *Heliotropium*, *Acacia*, *Solanum* (6 spp., 1.49% each.) (*Fig. 4*).

Table 1. *Summary of floristic composition in Jhelum district, Punjab, Pakistan*

Phyto-Taxa	Families	Genera	Species
Pteridophytes	1 (1.33%)	1 (0.45%)	1 (0.25%)
Gymnosperms	1 (1.33%)	1 (0.45%)	1 (0.25%)
Angiosperms	73 (97.33%)	221 (99.1%)	402 (99.5%)
Monocots	10 (13.33%)	44 (19.73%)	74 (18.32%)
Dicots	63 (84%)	177 (79.37%)	328 (81.19%)
Total	75 (100%)	223 (100%)	404 (100%)

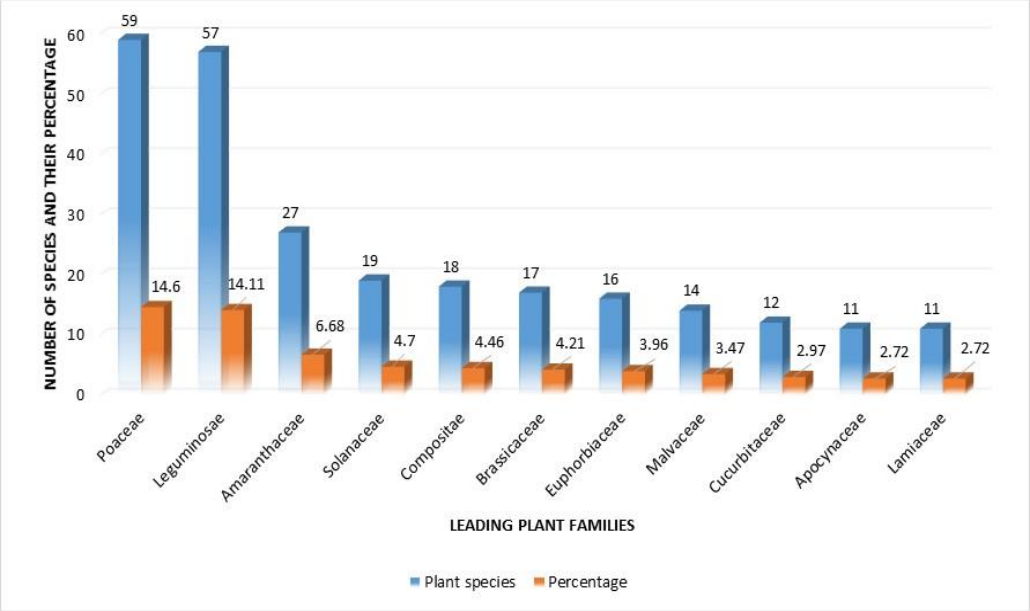

Figure 3. *Graph depicting the leading plant families in the study area*

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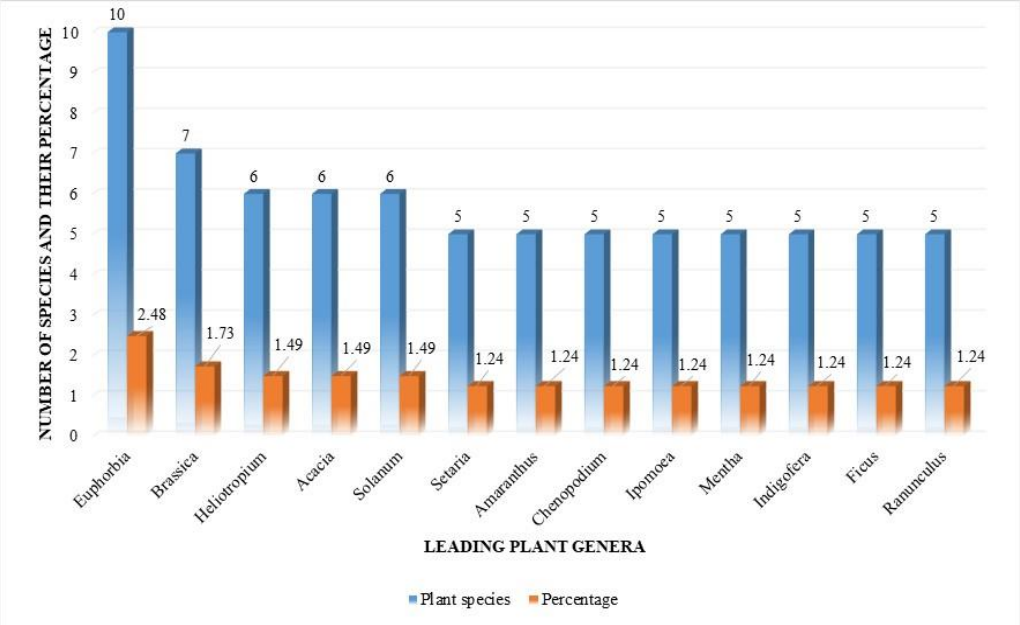

Figure 4. *Graph depicting the leading plant genera in the study area*

With respect to the diverse microhabitats, grassland showed the maximum number of 283 species (70.05% of overall flora), followed by 281 road side species (69.55%), 228 forest species (55.44%) and 223 arable land species (55.2%), rest of micro-habitat resulted waste places with 216 species (53.47%), hilly slope with 209 species (51.73%), shady places with 184 species (45.54%), graveyard with 174 species (43.07), wet land with 129 (31.93%), dry land with 122 species (30.2%), scrubland and home garden, both with 110 species (27.23%), sandy places with 92 species (22.77%) and mountain summits with 34 species (8.2%). An overall habit-wise arrangement of the documented plant species showed four groups. Maximum number of herbs involved 246 species (60.89%), followed by grasses with 59 species (14.6%), shrubs with 50 species (12.38%) and trees with 49 species (12.13%) (*Fig. 5*).

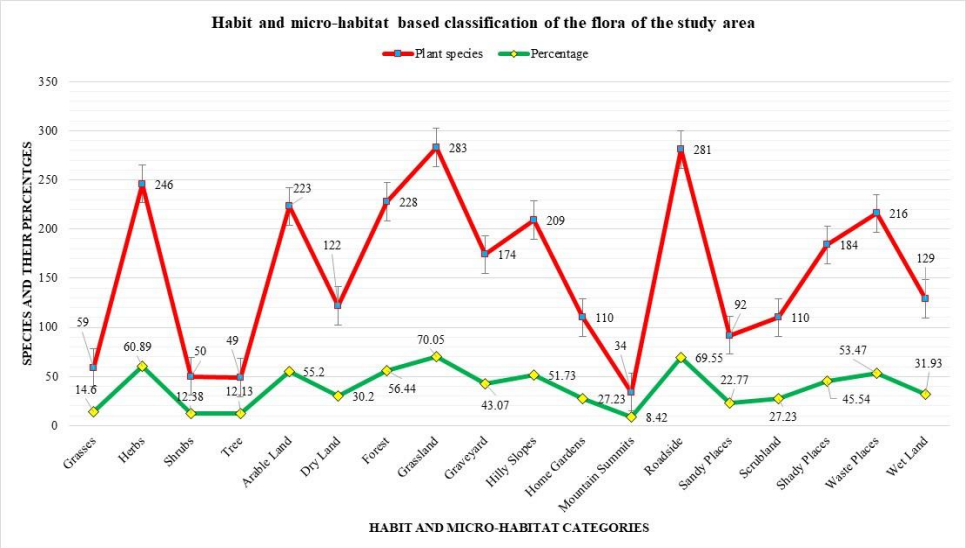

Figure 5. *A graph depicting the results of grouping of vascular plant species into different habitat and micro-habitat categories*

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Flowering phenology and classification

The reproductive phenological response recorded and showed that maximum flowering stage of plant species was during months of March, April and August (174 spp., 43.07%, 159 spp., 39.36% and 158 spp., 39.11%). The minimum phonological response was noted in the month of January and December (5 spp., 1.24%) and November (7 ssp., 1.73%) (*Fig. 6*).

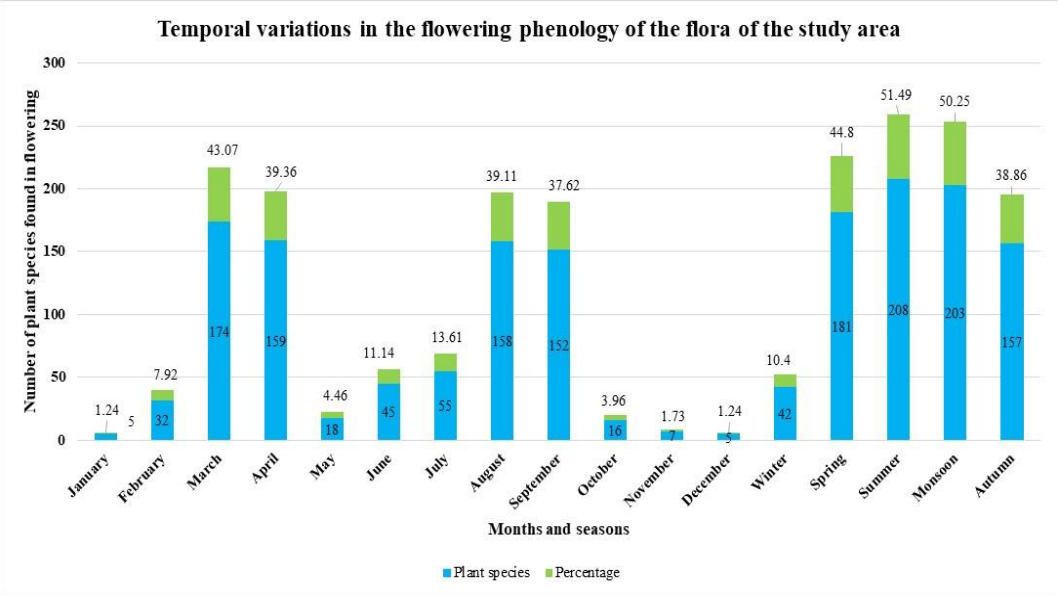

Figure 6. *Graphical representation of temporal variations in the flowering phenology of the vascular plant species*

The reproductive phenology response resulted that the majority of the plant species go through their reproductive phase during March, April, August and September months in a year, while, November to January is not a favored time to arrive into effective reproductive phonological phase due to ecological fluctuations. As far as the beginning time for species phenological response is depicted, most of the plant species started the flowering period in the months of February (32 spp., 7.92%), May (18 spp., 4.45%), June (45 spp., 11.14%) and July (55 spp., 13.61%). While, decline in flowering response with reduced number of plant species occurred in the month of October in 16 spp., 3.96%) (*Fig. 7*).

Leading reproductive phenological response results were shown in the summer by 208 species (51.49%) followed by Monsoon with 203 species (50.25%), during Spring with 181 species (44.8%) and Autumn with 157 species (38.86%). The least phenological response was recorded during Winter in 42 species (10.4%) (*Fig. 8*).

Ordination analysis

With reference to ordination analysis, detrended correspondence analysis (DCA), a unimodal unconstrained model (where as climatic factors were applied for supplementary variables) was designated to pursue the gradient length in the binary compositional phenological response data. The results of presented analysis represented that the gradient length in the response data was above 3 SD (standard deviation of species turnover) for

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the first two DCA axes. Moreover, the response data was binary (1/0), by concluding data on the basis of the given two observations, a constrained uni-modal ordination model such as CCA was used to find out the type variables in the phenological response data described by the recorded predictions, and sort of importance order.

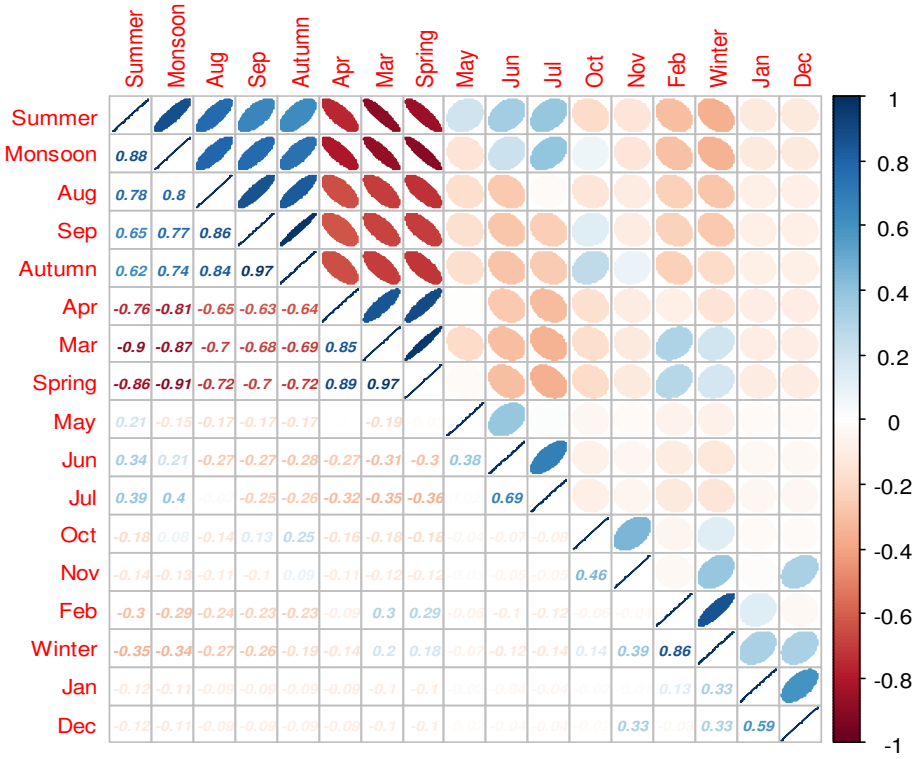

Figure 7. *Correlation plot of months and seasons based on their flowering phenology response*

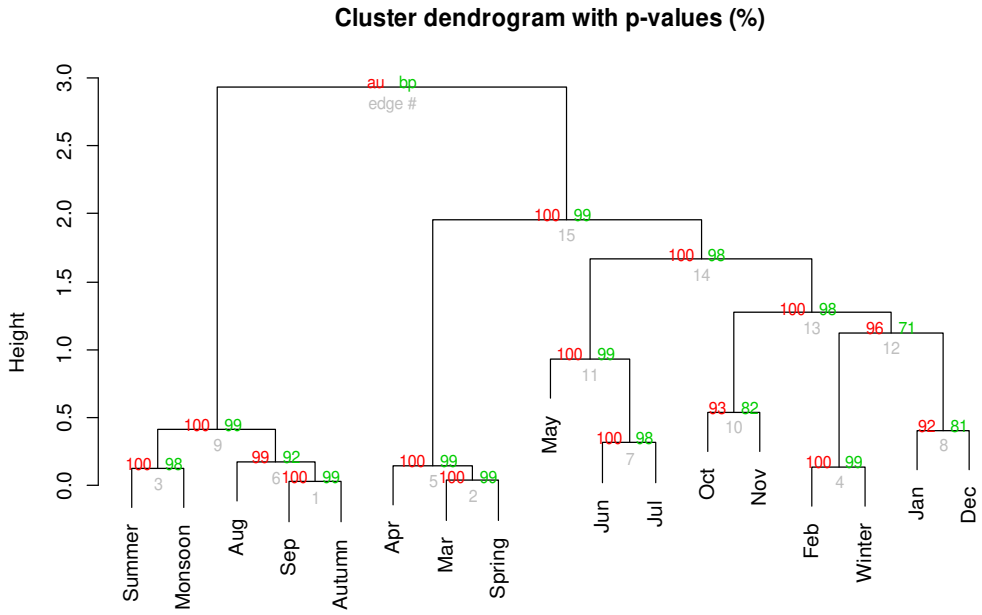

Figure 8. *Hierarchical clustering tree of Months and seasons (Distance: Correlation, Linkage: Ward) with AU/BP% values based on their flowering phenology response*

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The results of Pearson's correlation and its significance showed that overall plant species found in flowering phenological phase in different months is strongly correlated ($r > 0.8$) with mean monthly values of five different climatic variables. These include mean soil moisture ($r = 0.65$), followed by precipitation variable that was found moderately positively correlated ($r = 0.62$), mean specific humidity ($r = 0.60$), long wave radiations ($r = 0.52$), shortwave radiations ($r = 0.49$), mean minimum temperature ($r = 0.46$), mean maximum temperature ($r = 0.40$), and similarly, a strong negative correlation was observed with wind speed ($r = 0.36$) in the study area (*Figs. 9* and *10*).

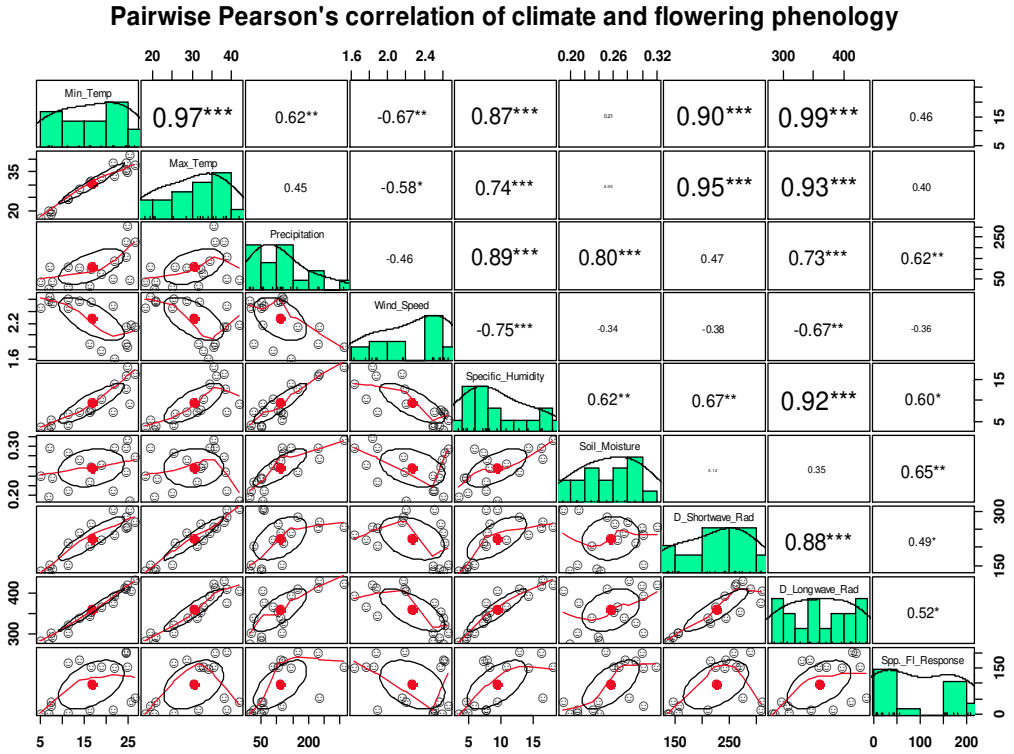

Figure 9. *Graph representing correlation significance. (The distribution of each variable is shown on the diagonal. On the bottom of the diagonal the bivariate scatter plots with a fitted line, and ellipses are presented, while on the top of the diagonal the value of the correlation plus the significance level as stars. Each significance level is linked to a symbol: p-values (0, 0.001, 0.01, 0.05, 0.1, 1) < = > symbols ("***", "**", "*", ".", " ")*

The results showed the interlink age of response (months and seasons) and descriptive (climatic) data. Multi-nonlinearity among climatic variations were determined on the observations within variables of inflation factor (VIFs) assessment of every climatic factors, and a threshold value of < 5 is designated to eliminate the extremely collinear descriptive variations. The ultimate CCA model was included of four types of predictions such as minimum temperature, wind speed, and soil moisture (25 cm below the soil surface) (*Fig. 11*). A total Variations of 3.45084 was noted in the reproductive phenology response data, about 58.85% variations were described by the descriptive variables, and the modified explained variations were 84.68%. The first two CCA axes cumulatively explained about 45.6% variations (*Table 2*). A significantly higher pseudo-canonical correlation ($r > 0.8$) value was recorded for the first three CCA axes which show that the

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nominated predictions were significant factors, and there is no single significant climatic gradient relatively all the four were significant in one way or another (*Table 3*).

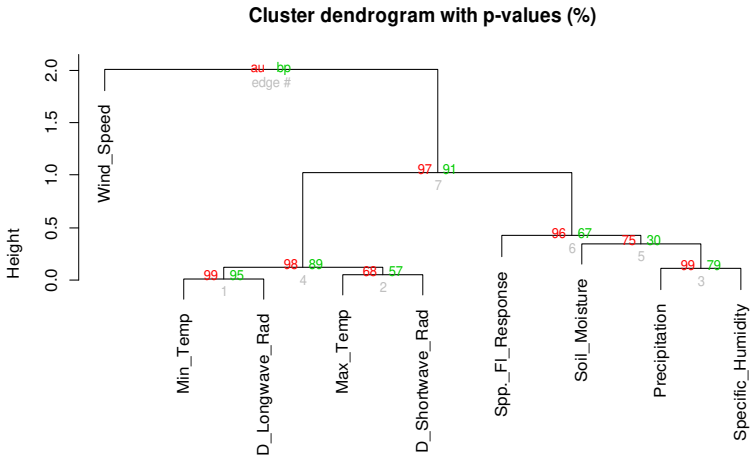

Figure 10. *Hierarchical clustering tree of climate and species flowering response variables (Distance: Correlation, Linkage: Ward) with AU/BP% values (n = 17)*

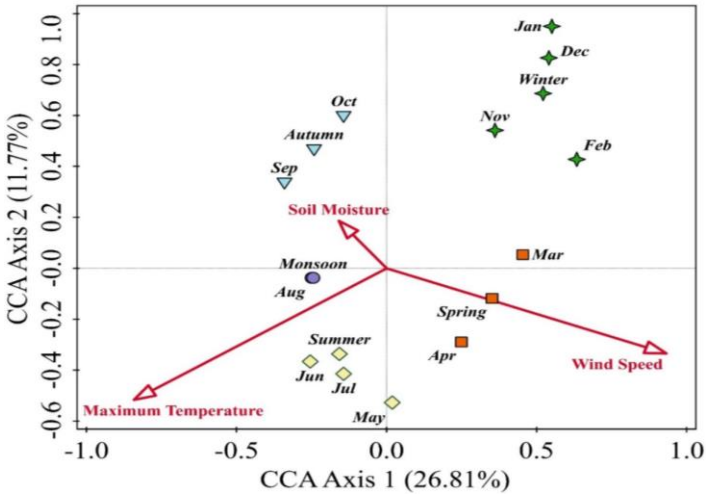

Figure 11. *Canonical correspondence analysis biplot depicting the interrelationships of climate and flowering phenological samples (months and seasons) in the study area*

Table 2. *CCA summary table*

Statistic	Axis 1	Axis 2
Eigenvalues	0.9252	0.406
Explained variation (cumulative)	26.81	38.58
Pseudo-canonical correlation	0.9797	0.8384
Explained fitted variation (cumulative)	58.85	84.68
Total variations	3.45084	
Sum of canonical eigenvalues	1.57358304	
Explained variation %	45.6	
Unexplained variation %	54.4	

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Table 3. *Canonical correspondence analysis numerical results showing the order of importance of studied climatic variables (p-values were corrected by using False Discovery Rate method)*

1. Simple term effects:				
Variable	**Explains %**	**pseudo-F**	**P**	**P(adj)**
Wind speed (M/Sec)	24.7	4.9	0.002	0.0032
Min. temperature °C	24.3	4.8	0.002	0.0032
Downward long wave radiation (W/M^2)	23.4	4.6	0.002	0.0032
Max. temperature °C	22.3	4.3	0.002	0.0032
Specific humidity (g/kg)	21.2	4	0.002	0.0032
Downward shortwave radiation (W/M^2)	15.7	2.8	0.004	0.00533
Precipitation (mm)	10.2	1.7	0.056	0.064
Soil moisture (5 cm; in fraction)	7.6	1.2	0.234	0.234
2. Conditional term effects:				
Wind speed (M/Sec)	24.7	4.9	0.002	0.003
Max. temperature °C	13.3	3	0.002	0.003
Soil moisture (5 cm; in fraction)	7.5	1.8	0.045	0.05

Discussion

Floristic classification and its importance

The study area District Jhelum, Punjab, Pakistan contains hills, Jhelum river flows through it, mostly forest cover, scrub lands, range lands and little part of salt range. The area is unique due to versatile geography, variable ecology and rich soil composition. It was observed that the district contains maximum vegetation cover, species richness and floristic diversity. The conducted study aimed to document the floristic composition of the study area along with diverse features counting flowering phenology and reproductive phenological response of the vascular and non-vascular plant species with respect to basic climatic variables.

In the study area, a total of 401 vascular and 1 non-vascular plant species were recorded. The obtained results of family importance value showed that the leading plant family was Poaceae with 59 species followed by Leguminosae (57 spp.), Amaranthaceae (27 spp.) and Solanaceae (19 sp.) while the leading genus was *Euphorbia* (10 spp.,) followed by *Brassica* (7 spp.), and *Heliotropium*, *Acacia*, and *Solanum* (6 spp. each). The conducted study was similar to the floristic composition of Muzaffarabad district, Azad Jammu and Kashmir, Pakistan published by Khan et al., 2015, who explored that the leading plant family was Compositae (69 spp.), followed by Poaceae (57 spp.), Leguminosae (54 spp.), Lamiaceae (42 spp.) and Rosaceae (29 spp.); whereas the prominent genus was Euphorbia (10 spp.), followed by Cyperus, Ficus, Geranium and Prunus (7 spp. each). Identical discoveries with floristic composition of Qalagai hills, Kabal valley Swat directed by Ilyas et al., 2013, the Poaceae (22 spp.) was the leading plant family followed by Compositae (16 spp.) and Lamiaceae (14 spp.). In the parallel style Shaheen et al., 2015 quantified 65 plant species of 26 families from western Himalayan subtropical forest stands of Kashmir in which Poaceae (8 spp.) was the prominent family followed by Compositae (6 spp.) and Lamiaceae (2 spp.) was typically equivalent to the presented discoveries. Comparable outcomes from Shahbaz Garhi, district Mardan, Pakistan by Khan et al., 2014, showed Poaceae (15 pp.) as the prominent

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DOI: http://dx.doi.org/10.15666/aeer/1905_33433376

family followed by Compositae (14 spp.). The identical survey was documented from district Bagh of Azad Jammu and Kasmir by Tanvir et al., 2014 and reported Poaceae (42 spp.) as the leading plant family followed by Compositae (11 spp.). Khan et al., 2015 recorded Poaceae (54 spp.) as the leading family followed by Compositae (33 app.) and Lamiaceae (23 spp.), and closely match with this study. Khan et al., 2017 described same findings that Poaceae was the prominent family comprised of 20 species followed by Lamiaceae (16 spp.) and Compositae (14 spp.), from Swat Ranizai, district Malakand, Khyber Pakhtunkhwa, Pakistan. Poaceae and Compositae are leading due to widespread ecological amplitude with diverse habitats (Ibrahim et al., 2019).

Traditional uses of 149 species belonging to 60 genera and 16 tribes of 5 sub families of Poaceae were recorded by Majeed et al., 2020, from Punjab Province, Pakistan. Hussain, 2009 documented 120 plant species belonging to 46 families, and detected Poaceae as the leading family with 14 plant species also match with this study. Similar results were presented by Shaheen et al., 2014, from Santh Saroola Kotli Sattian, Rawalpindi, Pakistan, who recorded 106 species, Poaceae family was dominant with 21 spp., followed by Asteraceae (19 spp.), Fabaceae (15 spp.), Euphorbiaceae, Lamiaceae (7 spp., each). Umair et al., 2019 recorded similar results, as 129 plant species belonging to 59 families were examined and Poaceae with 13 plants species was the leading family, from Chenab riverine area, Punjab province Pakistan. Amjad et al., 2016 presented similar results from Nikyal valley, Azad Jammu and Kashmir, Pakistan, who recorded 110 species belonging to 51 families and 98 genera. Poaceae (18 spp.) was the leading family followed by Asteraceae (10 spp.), Lamiaceae (8 spp.) and Fabaceae (7 spp.). Zahoor et al., 2017 investigated 96 plants belonging to 34 families from district Sheikhupura, province Punjab, Pakistan and Poaceae was the dominant family with 16 spp. followed by Fabaceae 15 spp. results were similar to the present study. Plant species of the Poaceae family are not only used as fodder and forage but also contribute substantially to the treatment of various health disorders, particularly in livestock (Majeed et al., 2020).

Climatic determinants of flowering phenology

The flowering response results indicated that majority of plant species flowered during the months of March (43.07%), followed by April (39.36%) and August (39.11%). The minimum phenological response was noted in the month of January (1.24%), December (1.24%) and November (1.73%).

The timing of flowering response as presented above was found highly correlated with the climatic variations (like temperature and monsoon rainfall) of the study area. A constrained unimodal ordination such as CCA was applied to check three predictors including minimum temperature, humidity, and soil moisture (25 cm below the soil surface).

According to the results of conditional (unique) term effect testing, mean maximum temperature was shown as a significant factor of the phenological response followed by soil moisture and wind speed. The majority of plant species are found in flowering stage during July and August months in the Western Himalayan regions of India and Pakistan (Vashistha et al., 2009; Khan et al., 2018), and strikingly match with our findings.

Likewise, the importance of temperature to the plants phenological responses, our results are similar as stated in several explorations (Badeck et al., 2004; Ahas and Aasa, 2006; Estrella and Menzel, 2006; Peñuelas et al., 2009) mostly in higher altitudinal areas of the World.

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DOI: http://dx.doi.org/10.15666/aeer/1905_33433376

Minimum temperature was recorded as significant as maximum temperature in the research area similar to Khan et al., 2018. Furthermore, rainfall was discovered as another main element of the phenological response (Pearson, 2019), the similar influence of rainfall on both spring and fall flowering events was reported from Southeastern United State of America. Our results match with Heydel et al., 2015; and Khan et al., 2018, that is maximum flowering species were recorded during four months (March, April, august, September) due to favorable climatic conditions. Whereas minimum flowering species were documented during three months (January, November and December) due to severe climatic conditions. Many explorations showed that in hilly areas, maximum flowering species were noted due to optimum climatic variables to support the phenomenon (Yadav and Yadav, 2008; Tooke, 2010). So it was assessed that months of May, June and July are appropriate regarding day length and temperature. The highest phenological response of plant species during months of March, April, August and September which can also be linked with maximum rainfall in these months (Summer and monsoon seasons) resulting in higher soil moisture. While, rare plant species were also found in flowering stage in November to January (in winters) due to severity in environmental conditions during mentioned months. Climatic variations might be harmful in general but mainly useful to rare and widespread plant species of this versatile, unique but delicate ecosystem of Jhelum district, Punjab, Pakistan.

Conclusions and recommendations

The research area district Jhelum resulted higher degree of plant species richness features of mostly diverse and rich flora in Punjab, Pakistan. The leading plant family was Poaceae followed by Leguminosae, Amaranthaceae, Solanaceae while the leading genus was *Euphorbia* followed by *Brassica*, *Heliotropium*, *Acacia*, *Solanum*, which proposed that the research area is under heavy anthropogenic pressure and harbors unique climatic environments. As concerned with the reproductive phenological response of the plant species, minimum temperature, wind speed, precipitation and specific humidity are the significant climatic determinants. The study resulted that the temperature is the leading effective feature observing the phenology of the plant species. It was estimated that increase or decrease in temperature showed specific association with pattern of phenology. Response of phenology also showed month and season wise correlation. Suddenly increase in temperature might be dangerous mainly to threatened and widespread flora of the area. The study area needs active supervision and protection strategies done with the participation of the indigenous population. The floristic study and phenology of plant species was explored for the first time. Consequently, the current research serves valuable information in future botanical investigation, and for plant reserve managing and preservation effort in the area. Future research studies should be linked to measurement of continuing climate variations. Under changing climatic conditions, the spread of invasive alien plant species is needed to be controlled to save the ecological niche of indigenous wild flora in the area.

Acknowledgments. This research work is part of the PhD thesis of the first author. Special thanks are due to all study participants of the different local groups who generously shared their knowledge about local names of wild plant species.

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

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_33433376

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DOI: http://dx.doi.org/10.15666/aeer/1905_33433376

APPENDIX

Table A1. *Temporal (2010-2019) variations in the climatic data (Mean ± SD (Min-Max)) of district Jhelum, Punjab, Pakistan*

Months	Min Temp	Max Temp	Precipitation	Wind speed	Specific humidity	Soil moisture	D-Shortwave-Rad	D-Longwave-Rad
January (2010-2019)	5.24±0.81 (3.98-6.53)	18±1.75 (15.81-21.37)	47.74±54.85 (0.4-178.23)	2.49±0.17 (2.29-2.74)	4.07±0.91 (2.59-5.59)	0.24±0.04 (0.17-0.28)	137.35±17.72 (102.79-156.34)	285.19±10.4 (273.15-305.42)
February (2010-2019)	7.32±1.15 (5.24-8.64)	19.3±2.44 (16.42-23.18)	113.9±72.6 (9.08-239.44)	2.66±0.18 (2.44-2.94)	5.83±0.81 (4.75-6.81)	0.3±0.04 (0.24-0.35)	156.06±20.95 (125.65-195.67)	306.07±8.92 (292.52-317.13)
March (2010-2019)	11.36±1.31 (9.96-13.92)	25.3±2.86 (21.53-30.59)	117.59±98.66 (12.1-283.46)	2.62±0.16 (2.39-2.87)	7.2±1.13 (4.92-8.39)	0.29±0.06 (0.2-0.37)	215.35±19.93 (176.04-238.07)	328.84±7.66 (316.34-342.61)
April (2010-2019)	16.49±1.03 (15.04-18.46)	31.99±2.7 (28.93-37.35)	104.04±55.51 (19.16-177.93)	2.58±0.31 (2.34-3.25)	8.21±1.2 (6.72-10.46)	0.26±0.05 (0.16-0.33)	268.88±16.83 (228.62-288.59)	357.86±5.73 (351.28-365.76)
May (2010-2019)	21.48±0.87 (20.15-22.7)	38.79±1.74 (35.16-40.95)	56.02±40.76 (5.83-133.89)	2.51±0.26 (2.14-2.99)	7.59±1.13 (5.96-9.72)	0.21±0.04 (0.16-0.27)	307.76±10.62 (287.96-318.56)	381.49±7.98 (371.25-393.43)
June (2010-2019)	25.49±0.89 (24.24-27.23)	42.06±1.44 (39.4-44.29)	51.78±49.11 (3.66-155.66)	2.19±0.12 (2.05-2.38)	9.38±2.08 (5.88-12.33)	0.19±0.03 (0.15-0.23)	308.9±10.75 (290.16-325.21)	411.44±9.36 (395.77-427.8)
July (2010-2019)	26.53±0.73 (25.73-27.7)	38.17±2.01 (35.78-41.92)	233.75±131.86 (66.36-483.97)	2.16±0.19 (1.9-2.45)	16.48±1.63 (14.26-19.35)	0.28±0.04 (0.23-0.34)	273.07±10.52 (254.7-296.1)	436.86±3.65 (430.46-444.15)
August (2010-2019)	24.82±0.59 (23.95-25.84)	35.46±2.3 (31.81-39.51)	310.41±198 (57.49-741.27)	1.83±0.17 (1.67-2.15)	18.04±1.76 (14.5-19.96)	0.32±0.05 (0.23-0.39)	261.09±13.48 (229.54-281.76)	427.21±4.01 (420.36-434.18)
September (2010-2019)	21.76±0.75 (20.76-23.29)	34.28±2.27 (31.27-37.65)	144.67±128.34 (35.44-413.59)	1.63±0.16 (1.37-1.84)	14.17±2.32 (9.66-16.62)	0.3±0.06 (0.22-0.36)	240.94±12.69 (214.93-256.23)	397.08±8.21 (384.37-411.53)
October (2010-2019)	16.28±0.67 (15.41-17.55)	31.32±2.14 (28.54-34.8)	26.71±22.77 (0.03-58.86)	1.88±0.15 (1.66-2.07)	7.58±1.98 (4.76-11)	0.23±0.06 (0.15-0.3)	208.42±6.36 (199.84-217.49)	345.61±6.99 (336.76-358.26)
November (2010-2019)	11.26±0.52 (10.46-12)	25.01±2.14 (20.85-28.08)	17.43±25.19 (0.05-82.33)	2.49±0.21 (2.18-2.86)	4.68±1.08 (3.06-6.75)	0.21±0.06 (0.15-0.31)	154.49±13.02 (122.82-167.86)	307.63±7.75 (296.51-323.29)
December (2010-2019)	6.79±0.99 (5.65-8.63)	20.34±2.06 (16.7-24.55)	13.85±16.82 (0.01-48.49)	2.6±0.24 (2.28-2.93)	3.4±0.89 (2.08-4.89)	0.2±0.05 (0.14-0.31)	135.96±6.53 (121.15-144.45)	280.97±6.64 (268.85-290.74)

Source: The climate data about precipitation, maximum and minimum temperature, specific humidity, soil moisture, wind speed, and downward short and longwave radiations (2010-2019 = 10 years) of the study area (Jhelum) was acquired from United States National Centers for Environmental Prediction (US-NCEP) Climate Forecast System Reanalysis (CFSR) by using climate engine (https://app.climateengine.org/). The data source was CFSv2 19200 m (1/5-deg) daily reanalysis dataset (NOAA)

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_33433376

Table A2. *Detailed attributes of the floristic elements of Jhelum district, Punjab, Pakistan*

Family	No.	Species	V/No.	Local name	Habit*	Micro-habitats**	Phenology
Pteridophytes and their related species							
1. Pteridaceae	1	*Adiantum capillus-veneris* L.	621/MM//2020	Sarhaj	H	WL	August-September
Gymnosperms							
2. Ephedraceae	2	*Ephedra ciliata* Fisch. & C.A.Mey.	880/MM//2020	Phog	S	DL,FO,GR,HS,RS,SP,SL,WP	March-April
Angiosperms (Monocots)							
3. Araceae	3	*Colocasia esculenta* (L.) Schott	897/MM//2020	Arvi	H	AL,HG	June-July
	4	*Pistia stratiotes* L.	515/MM//2020	Water Cabbage	H	WL	March-April
4. Arecaceae	5	*Phoenix dactylifera* L.	501/MM//2020	Khajoor	T	AL,DL,GL,RS	February-March
5. Asparagaceae	6	*Agave americana* L.	675/MM//2020	Desi kwargandal	S	AL,DL,FO,GL,GR,RS,SP,WP	August-September
6. Cannaceae	7	*Canna indica* L.	867/MM//2020	Ratta phool	H	HG	March-April
7. Commelinaceae	8	*Commelina benghalensis* L.	795/MM//2020	Kani	H	FO,GL,HS,RS,SL,SH,WP,WL	March-April
8. Cyperaceae	9	*Cyperus difformis* L.	776/MM//2020	Chota dheela	H	AL,FO,GL,GR,HS,RS,SL,SH,WP,WL	August-September
	10	*Cyperus iria* L.	573/MM//2020	Murak ghaa	H	AL,FO,GL,GR,HS,RS,SP,SL,SH,WP,WL	August-September
	11	*Cyperus niveus* Retz.	815/MM//2020	Chita dheela	H	AL,FO,GL,GR,HS,RS,SL,SH,WP,WL	August-September
	12	*Cyperus rotundus* L.	663/MM//2020	Murak	H	AL,FO,GL,HS,RS,SL,SH,WP,WL	August-September
9. Juncaceae	13	*Juncus articulatus* L.	546/MM//2020	Dheela	H	FO,GL,HS,WP,WL	August-September
10. Poaceae	14	*Apluda mutica* L.	668/MM//2020	Tachuli	G	AL,DL,FO,GL,GR,RS,SP,WP	August-September
	15	*Aristida abnormis* Chiov.	810/MM//2020	Bara Lumb	G	DL,GL,SP,SH,WP	August-September
	16	*Aristida adscensionis* L.	608/MM//2020	Chitta Lumb	G	DL,GL,SP,SH,WP	August-September
	17	*Aristida mutabilis* Trin. & Rupr.	562/MM//2020	Lumb	G	DL,GL,GR,SP,SH,WP	August-September
	18	*Arundo donax* L.	875/MM//2020	Naru ghaa	G	GL,RS,WP	August-September
	19	*Avena fatua* L.	653/MM//2020	Jai	G	AL,DL,GL,RS,SP,WP	March-April
	20	*Bothriochloa bladhii* (Retz.) S.T.Blake	696/MM//2020	Palwan ghaa	G	AL,GL,HS,RS,SH,WP,WL	August-September
	21	*Brachiaria deflexa* (Schumach.) C.E.Hubb. ex Robyns	632/MM//2020	Moti ghaa	G	AL,GL,HG,RS,SH,WP,WL	August-September
	22	*Brachiaria distachya* (L.) Stapf	622/MM//2020	Seer ghaa	G	AL,GL,HS,RS,SH,WP,WL	August-September
	23	*Brachiaria ramosa* (L.) Stapf	820/MM//2020	Chota kmadi	G	AL,FO,GL,GR,HS,RS,SH,WP,WL	August-September
	24	*Brachiaria reptans* (L.) C.A.Gardner & C.E.Hubb.	654/MM//2020	Para ghaa	G	AL,FO,GL,GR,RS,SH,WP,WL	August-September
	25	*Cenchrus biflorus* Roxb.	692/MM//2020	Chita Dhaman	G	AL,DL,FO,GL,GR,RS,SP,WP	August-September
	26	*Cenchrus ciliaris* L.	734/MM//2020	Kala Dhaman	G	DL,FO,GL,RS,SP,WP	August-September
	27	*Cenchrus pennisetiformis* Steud.	870/MM//2020	Kali Dhamani	G	DL,FO,GL,GR,RS,SP	August-September

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DOI: http://dx.doi.org/10.15666/aeer/1905_33433376

Family	No.	Species	V/No.	Local name	Habit*	Micro-habitats**	Phenology
	28	*Cenchrus setiger* Vahl	704/MM//2020	Kala Dhamani	G	DL,FO,GL,RS,SP,WP	August-September
	29	*Chrysopogon serrulatus* Trin.	586/MM//2020	Jangli jai	G	DL,FO,GL,RS,SP,WP	August-September
	30	*Chrysopogon aucheri* (Boiss.) Stapf	528/MM//2020	Chitta Dhaman	G	DL,FO,GL,HS,RS,SP,WP	August-September
	31	*Cymbopogon jwarancusa* (Jones) Schult.	839/MM//2020	Lamb ghaa	G	DL,GR,RS,SP,WP	August-September
	32	*Cynodon dactylon* (L.) Pers.	660/MM//2020	Khabbal ghaa	G	AL,DL,FO,GL,GR,HS,HG,MS,RS,SP,SL,SH,WP, WL	August-September
	33	*Dactyloctenium aegyptium* (L.) Willd.	559/MM//2020	Khar Madana	G	AL,DL,GL,GR,RS,SP,SL,SH,WP,WL	August-September
	34	*Desmostachya bipinnata* (L.) Stapf	735/MM//2020	Khusa Dab	G	DL,GL,GR,SP,WP	August-September
	35	*Dichanthium annulatum* (Forssk.) Stapf	845/MM//2020	Murgha ghaa	G	AL,FO,GL,GR,HS,HG,RS,SL,SH,WP,WL	August-September
	36	*Digitaria nodosa* Parl.	661/MM//2020	Chota ghaa	G	AL,FO,GL,HS,HG,RS,SH,WP,WL	August-September
	37	*Digitaria sanguinalis* (L.) Scop.	538/MM//2020	Toota ghaa	G	AL,FO,GL,RS,SL,SH,WP,WL	August-September
	38	*Echinochloa colona* (L.) Link	614/MM//2020	Jungli chawly	G	AL,FO,GL,HS,HG,RS,SL,SH,WP,WL	August-September
	39	*Echinochloa crus-galli* (L.) P.Beauv.	731/MM//2020	Sanwari	G	AL,FO,GL,GR,HS,HG,RS,SL,SH,WP,WL	August-September
	40	*Echinochloa stagnina* (Retz.) P.Beauv.	671/MM//2020	Chawly ghaa	G	FO,GL,HS,RS,SH,WP,WL	August-September
	41	*Eleusine indica* (L.) Gaertn.	822/MM//2020	Nika mdhana	G	AL,FO,GL,HS,RS,SH,WP,WL	August-September
	42	*Eragrostis cilianensis* (All.) Janch.	889/MM//2020	Chitti pholi ghaa	G	FO,GL,HS,RS,SL,SH,WP,WL	August-September
	43	*Eragrostis ciliaris* (L.) R.Br.	789/MM//2020	Makni ghaa	G	AL,FO,GL,HS,SL,SH,WP,WL	August-September
	44	*Hordeum vulgare* L.	858/MM//2020	Jao	G	AL,DL,RS	February-March
	45	*Imperata cylindrica* (L.) Raeusch.	775/MM//2020	Baggi sari	G	WL	August-September
	46	*Ochthochloa compressa* (Forssk.) Hilu	529/MM//2020	Tara ghaa	G	DL,HS,RS,SP	August-September
	47	*Oryza sativa* L.	804/MM//2020	Monji, Chawal	G	AL,WL	September-October
	48	*Panicum antidotale* Retz.	508/MM//2020	Bara chawala	G	HS,RS,WL	August-September
	49	*Panicum maximum* Jacq.	524/MM//2020	Bansi ghaa	G	GL,RS,SL,WP,WL	August-September
	50	*Panicum repens* L.	771/MM//2020	Moti khabal	G	AL,GL,GR,HS,RS,WP,WL	August-September
	51	*Panicum turgidum* Forssk.	807/MM//2020	Garam	G	DL,GL	August-September
	52	*Pennisetum divisum* (Forssk. ex J.F.Gmel.) Henrard	637/MM//2020	Desi Garam	G	DL,GL	August-September
	53	*Pennisetum orientale* Rich.	541/MM//2020	Chita ghaa	G	AL,GL,HS,RS,WL	August-September
	54	*Pennisetum glaucum* (L.) R.Br.	852/MM//2020	Bajra	G	AL,DL	June-July
	55	*Phalaris minor* Retz.	784/MM//2020	Dumbi sitti	G	AL,GL,RS,WP,WL	March-April
	56	*Phragmites karka* (Retz.) Trin. ex Steud.	540/MM//2020	Narru	G	RS,WL	August-September
	57	*Poa annua* L.	652/MM//2020	Jangli Jai	G	AL,HS,RS,WP,WL	March-April
	58	*Poa pratensis* L.	557/MM//2020	Sanwak	G	AL,GL,HS,WP,WL	August-September
	59	*Polypogon fugax* Nees ex Steud.	895/MM//2020	Daddi ghaa	G	AL,GL,GR,HS,RS,SH,WP,WL	August-September

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DOI: http://dx.doi.org/10.15666/aeer/1905_33433376

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	60	*Saccharum bengalense* Retz.	582/MM//2020	Kana, Sarkanda	G	FO,GL,GR,HS,RS,SP,SL,WP,WL	August-September
	61	*Saccharum spontaneum* L.	626/MM//2020	Kana	G	DL,FO,GL,GR,HS,RS,SP,SL,WP,WL	August-September
	62	*Saccharum officinarum* L.	558/MM//2020	Ganna phool	G	AL,WL	October-November
	63	*Setaria intermedia* Roem. & Schult.	710/MM//2020	Choti Chawly	G	AL,HS,RS,SH,WP,WL	August-September
	64	*Setaria italica* (L.) P.Beauv.	824/MM//2020	Kangni ghaa	G	AL,GL,GR,WP,WL	August-September
	65	*Setaria pumila* (Poir.) Roem. & Schult.	651/MM//2020	Ban kangni	G	AL,FO,GL,RS,WL	August-September
	66	*Setaria verticillata* (L.) P.Beauv.	898/MM//2020	Bajra ghaa	G	AL,DL,GL,GR,RS,SH,WP,WL	August-September
	67	*Setaria viridis* (L.) P.Beauv.	854/MM//2020	Lumba Kangni ghaa	G	AL,DL,GL,GR,RS,WP,WL	August-September
	68	*Sorghum bicolor* (L.) Moench	601/MM//2020	Jowar, milo	G	AL,GL,RS,WP,WL	June-July
	69	*Sorghum halepense* (L.) Pers.	548/MM//2020	Kmadi ghaa, Baru	G	AL,GL,HS,RS,WL	August-September
	70	*Stipagrostis plumosa* Munro ex T.Anderson	657/MM//2020	Bhalu ghaa	G	AL,GL,HS,WP,WL	August-September
	71	*Triticum aestivum* L.	876/MM//2020	Kanak, Gandum	G	AL,HS,SP	February-March
	72	*Zea mays* L.	803/MM//2020	Makai	G	AL,GL,HS,RS,WL	June-July
11. Typhaceae	73	*Typha domingensis* Pers.	506/MM//2020	Konder	H	WL	October-November
	74	*Typha elephantina* Roxb.	884/MM//2020	Kondar	H	WL	August-September
12. Xanthorrhoeaceae	75	*Aloe vera* (L.) Burm.f.	894/MM//2020	Kawar gandal	H	AL,DL,FO,GR,HG	August-September
	76	*Asphodelus tenuifolius* Cav.	585/MM//2020	Piazi	H	AL,FO,GL,GR,HS,RS,SP,SL,SH,WP,WL	February-March
Angiosperms (Dicots)							
13. Acanthaceae	77	*Dicliptera bupleuroides* Nees	881/MM//2020	Rewari	H	AL,DL,FO,GL,GR,RS,SH,WP	March-April
	78	*Dicliptera verticillata* (Forssk.) C.Chr.	590/MM//2020	Jamni booti	H	DL,FO,GR,HS,RS,SP,SL,SH,WP	February-April
	79	*Justicia adhatoda* L.	574/MM//2020	Baikr	S	DL,FO,GL,GR,HS,MS,RS,SL	February-March
14. Aizoaceae	80	*Trianthema portulacastrum* L.	525/MM//2020	Jangli Sanwak	H	AL,DL,FO,GL,HS,RS,SH,WP,WL	June-July
	81	*Trianthema triquetra* Rottler & Willd.	570/MM//2020	Choti alwati	H	DL,FO,GL,GR,HS,MS,SP,SL,WP	June-July
	82	*Zaleya pentandra* (L.) C.Jeffrey	715/MM//2020	Ratti Hazar dani	H	AL,FO,GL,GR,RS,SL,SH,WL	August-September
15. Amaranthaceae	83	*Achyranthes aspera* L.	802/MM//2020	Puth kanda	H	AL,DL,FO,GL,GR,RS,WP,WL	August-September
	84	*Achyranthes bidentata* Blume	512/MM//2020	Puth kanda	H	AL,DL,FO,GL,GR,RS	August-September
	85	*Aerva javanica* (Burm.f.) Juss. ex Schult.	544/MM//2020	Niki boi	H	DL,FO,GR,HS,SP,SL,WP	February-April
	86	*Aerva lanata* (L.) Juss.	688/MM//2020	Boi	H	DL,FO,GR,HS,SP,SL,WP	May-July
	87	*Alternanthera paronychioides* A.St.-Hil.	724/MM//2020	Chitti pholi	H	DL,FO,GR,HS,MS,SP	August-September
	88	*Alternanthera pungens* Kunth	706/MM//2020	Khaki, Bhakrra	H	FO,GL,HS,RS,SL,SH,WP	March-April
	89	*Alternanthera sessilis* (L.) R.Br. ex DC.	509/MM//2020	Poni booti	H	FO,GL,HS,SL,SH,WP,WL	September-October
	90	*Amaranthus deflexus* L.	542/MM//2020	Jangli Tandla	H	AL,FO,GL,HS,SH,WP	August-September

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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	91	*Amaranthus graecizans* L.	634/MM//2020	Pohli	H	FO,GL,HS,RS,SL,SH,WP	August-September
	92	*Amaranthus retroflexus* L.	673/MM//2020	Aam bathoo	H	FO,GL,GR,HS,RS,SL,SH,WP	March-April
	93	*Amaranthus spinosus* L.	787/MM//2020	Konjel	H	FO,GL,HS,RS,SH,WP,WL	March-April
	94	*Amaranthus viridis* L.	878/MM//2020	Tandla	H	AL,GL,HS,RS,SL,SH,WP	August-September
	95	*Atriplex aucheri* Moq.	678/MM//2020	Loni jhari	S	DL,FO,GL,GR,HS,SL,SH,WP	July-August
	96	*Atriplex dimorphostegia* Kar. & Kir.	736/MM//2020	Loni booti	H	FO,GL,HS,RS,WP,WL	August-September
	97	*Atriplex griffithii* Moq.	551/MM//2020	Lani jhari	S	DL,FO,GL,GR,HS,RS,SP,SL,SH	May-July
	98	*Bassia hyssopifolia* (Pall.) Kuntze	825/MM//2020	Chotti lani, Retli booti	H	DL,FO,GL,GR,HS,RS,SH,WP	March-April
	99	*Beta vulgaris* L.	744/MM//2020	Chukandar	H	AL,GL,HG,RS	March-April
	100	*Chenopodium album* L.	748/MM//2020	Bathoo	H	AL,FO,GL,GR,HS,RS,SL,SH,WP	March-April
	101	*Chenopodium ficifolium* Sm.	552/MM//2020	Jangli Bathoo	H	DL,FO,GL,GR,HS,RS,SH	March-April
	102	*Chenopodium murale* L.	805/MM//2020	Karwa bathoo	H	FO,GL,GR,HS,RS,SL	March-April
	103	*Chenopodium glaucum* L.	887/MM//2020	Ratta Bathoo	H	FO,GL,GR,HS,RS,SH,WP	March-April
	104	*Chenopodium vulvaria* L.	611/MM//2020	Chitta bathoo	H	AL,DL,RS	March-April
	105	*Digera muricata* (L.) Mart.	694/MM//2020	Tandla saag	H	FO,GL,GR,HS,RS,SH,WP,WL	August-September
	106	*Dysphania ambrosioides* (L.) Mosyakin & Clemants	856/MM//2020	Desi Bathoo	H	AL,DL,FO,GL,GR,HS,RS,SL,SH,WP	August-September
	107	*Spinacia oleracea* L.	823/MM//2020	Pallak	H	AL,GL,HG	March-April
	108	*Suaeda acuminata* (C.A.Mey.) Moq.	583/MM//2020	Smandri booti	H	DL,FO,GL,GR,HS,RS,SP,SH	August-September
	109	*Suaeda vermiculata* Forssk. ex J.F.Gmel.	799/MM//2020	Lani booti	S	FO,GL,GR,HS,RS,SL,SH,WP	March-April
16. Amaryllidaceae	110	*Allium cepa* L.	602/MM//2020	Ganda	H	RS	March-April
	111	*Allium sativum* L.	684/MM//2020	Thoom	H	AL,GL,HG	March-April
17. Anacardiaceae	112	*Mangifera indica* L.	580/MM//2020	Aam	T	AL,GL,HG,RS	May-June
18. Annonaceae	113	*Polyalthia longifolia* (Sonn.) Thwaites	677/MM//2020	Ulta ashoq	T	HG,RS	April-June
19. Apiaceae	114	*Centella asiatica* (L.) Urb.	893/MM//2020	Chattri	H	DL,GL,GR,HS,SH,WP	June-August
	115	*Coriandrum sativum* L.	513/MM//2020	Dhaniya	H	AL,GL,HG,RS	March-April
	116	*Daucus carota* L.	843/MM//2020	Gajjar	H	AL,GL,HG	March-April
	117	*Foeniculum vulgare* Mill.	517/MM//2020	Sounf	H	AL,GL,HG	March-April
20. Apocynaceae	118	*Blyttia spiralis* (Forssk.) D.V.Field & J.R.I.Wood	598/MM//2020	Wal-tara	H	FO,HS,SH,WP	March-April
	119	*Calotropis procera* (Aiton) Dryand.	640/MM//2020	Ak	S	DL,FO,GR,HS,RS,SP,WP	March-April
	120	*Calotropis gigantea* (L.) Dryand.	565/MM//2020	Bara ak	S	DL,FO,GR,HS,RS,SP,SH,WP	March-April
	121	*Cascabela thevetia* (L.) Lippold	793/MM//2020	Peeli kanier	T	AL,GL,HG,RS	March-April
	122	*Leptadenia pyrotechnica* (Forssk.) Decne.	883/MM//2020	Khip	S	DL,FO,GR,HS,RS,SP,WP	September-October

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	123	*Nerium oleander* L.	658/MM//2020	Kanair	S	HG,RS	July-August
	124	*Pentatropis capensis* (L. f.) Bullock	853/MM//2020	Tara lee	H	FO,GL,GR,HS,RS,SL,SH,WP	March-April
	125	*Pergularia daemia* (Forssk.) Chiov.	774/MM//2020	Sapni vail	H	FO,GR,HS,RS,SH,WP	February-April
	126	*Pergularia tomentosa* L.	606/MM//2020	Jangli Sapni vail	H	FO,HS,RS,SH,WP,WL	February-April
	127	*Rhazya stricta* Decne.	672/MM//2020	Wlayti Ak	S	DL,FO,GL,GR,HS,RS,SP,SL,SH,WP	August-September
	128	*Tylophora hirsuta* Wight	563/MM//2020	Panjni jhari	S	AL,GL,HG,RS	May-July
21. Berberidaceae	129	*Berberis lycium* Royle.	840/MM//2020	Sumbulu	T	AL,GL,RS	March-April
22. Bignoniaceae	130	*Tecoma stans* (L.) Juss. ex Kunth	806/MM//2020	Peeli jhari	S	HG,RS	August-September
23. Boraginaceae	131	*Cordia myxa* L.	535/MM//2020	Lasura	T	AL,GL,HG,RS	March-April
	132	*Cordia dichotoma* G.Forst.	518/MM//2020	Lasuree	T	AL,GL,HG,RS	March-April
	133	*Heliotropium aucheri* DC.	871/MM//2020	Tara booti	H	DL,FO,GL,GR,HS,RS,SP,SL	March-April
	134	*Heliotropium crispum* Desf.	682/MM//2020	Chitti choli	H	DL,FO,GR,HS,RS,SP,SL,SH,WP	August-September
	135	*Heliotropium curassavicum* L.	507/MM//2020	Lani pata	H	DL,FO,GR,HS,RS,SP,SL,SH,WP	March-April
	136	*Heliotropium europaeum* L.	616/MM//2020	Uth chaaro, Hathi sundi	H	DL,FO,GL,GR,HS,RS,SP,SL,SH,WP	August-September
	137	*Heliotropium strigosum* Willd.	756/MM//2020	Chita koka	H	DL,FO,GR,RS,SP,SL,SH,WP	March-April
	138	*Heliotropium supinum* L.	533/MM//2020	Choti boi	H	DL,FO,GR,HS,RS,SP,SL,WP	March-April
24. Brassicaceae	139	*Brassica nigra* (L.) K.Koch	792/MM//2020	Kala saroon, Kala rayea	H	AL,GL	March-April
	140	*Brassica oleracea* L.	770/MM//2020	Band ghobi	H	AL,GL,HG	February-March
	141	*Brassica deflexa* Boiss.	782/MM//2020	Peela saroon	H	AL,DL,FO,GL,GR,HS,HG,MS,RS,SP,SL,SH,WP,WL	March-April
	142	*Brassica juncea* (L.) Czern.	674/MM//2020	Peela raya	H	AL,GL	March-April
	143	*Brassica napus* L.	629/MM//2020	Shaljam	H	AL,GL,HG	February-March
	144	*Brassica rapa* L.	619/MM//2020	Peeli rayi	H	AL,GL	February-March
	145	*Brassica tournefortii* Gouan	828/MM//2020	Sirmi	H	AL,GL	February-March
	146	*Capsella bursa-pastoris* (L.) Medik.	760/MM//2020	Mirch booti	H	FO,GL,GR,HS,MS,RS,SH,WP,WL	August-September
	147	*Eruca vesicaria* (L.) Cav.	751/MM//2020	Tara Meera, Osoon rayea	H	AL,FO,GL,GR,HS,MS,RS,SL,SH,WP,WL	March-April
	148	*Lepidium apetalum* Willd.	505/MM//2020	Jangli Khoob kalan	H	FO,GL,HS,MS,SH,WP,WL	July-August
	149	*Lepidium didymum* L.	703/MM//2020	Chattri booti	H	AL,FO,GL,GR,HS,MS,SL,SH,WP,WL	March-April
	150	*Lepidium aucheri* Boiss.	873/MM//2020	Choti Lani	H	FO,GL,GR,HS,MS,RS,SL,SH,WP,WL	April-May
	151	*Lepidium sativum* L.	813/MM//2020	Bag bhari	H	HG,RS,SH	March-April
	152	*Malcolmia africana* (L.) R.Br.	780/MM//2020	Chiti phuli	H	DL,FO,GL,GR,MS,RS,SH,WP,WL	March-April
	153	*Raphanus raphanistrum* L.	664/MM//2020	Mongrae	H	AL,GL,HG	February-April

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	154	*Sisymbrium irio* L.	750/MM//2020	Peeli Saroon	H	AL,GL,HS,RS,SH,WP	March-April
	155	*Sisymbrium orientale* L.	826/MM//2020	Jangli Saroon	H	AL,GL,HS,RS,SH,WP	March-April
25. Cactaceae	156	*Opuntia dillenii* (Ker Gawl.) Haw.	699/MM//2020	Chitter thore	H	DL,HS,SP,SL,SH,WP	March-April
26. Cannabaceae	157	*Cannabis sativa* L.	669/MM//2020	Bhang	H	AL,GL,GR,RS,SH,WP,WL	March-April
27. Capparaceae	158	*Capparis decidua* (Forssk.) Edgew.	532/MM//2020	Karir	S	DL,FO,GR,HS,SP	August-September
28. Caricaceae	159	*Carica papaya* L.	904/MM//2020	Papeeta	S	AL,GL,HG	March-April
29. Caryophyllaceae	160	*Stellaria media* (L.) Vill.	796/MM//2020	Chitti booti	H	AL,FO,GL,HS,MS,RS,SL,SH,WP	March-April
	161	*Stellaria persica* Boiss.	511/MM//2020	Sitara booti	H	AL,FO,GL,HS,RS,SL,SH,WP	July-August
	162	*Stellaria decumbens* Edgew.	587/MM//2020	Chitti cona	H	FO	July-August
	163	*Stellaria monosperma* Buch.-Ham. ex D. Don	716/MM//2020	Ghal booti	H	FO,GL,GR,RS,SL,SH,WP,WL	July-August
30. Cleomaceae	164	*Cleome viscosa* L.	578/MM//2020	Zard booti	H	FO,GL,GR,RS,SH,WP,WL	March-April
31. Compositae	165	*Carthamus oxyacantha* M.Bieb.	863/MM//2020	Pholi, Kandiyari	H	DL,FO,GL,GR,HS,RS,SP,SL,WP	August-September
	166	*Cirsium arvense* (L.) Scop.	761/MM//2020	Kandiyari	H	DL,FO,GL,GR,HS,SP,SH,WP	March-April
	167	*Cirsium falconeri* (Hook.f.) Petr.	720/MM//2020	Jhalar Kandiyari	H	DL,FO,GR,HS,RS,SP,SH,WP	September-October
	168	*Echinops echinatus* Roxb.	566/MM//2020	Ont-ktara	H	DL,FO,GL,GR,HS,RS,SP,SL,SH	March-April
	169	*Eclipta prostrata* (L.) L.	847/MM//2020	Bhangra	H	AL,FO,GL,GR,RS,SH,WP,WL	March-April
	170	*Erigeron aegyptiacus* L.	757/MM//2020	Jangli Genda Phool	H	AL,FO,GL,GR,RS,SP,SH	September-October
	171	*Erigeron bonariensis* L.	872/MM//2020	Dodi booti	H	DL,FO,GR,HS,RS,SH,WL	March-April
	172	*Erigeron canadensis* L.	605/MM//2020	Konjel pholi	H	DL,FO,GL,GR,RS,SH,WL	October-November
	173	*Lactuca serriola* L.	609/MM//2020	Bhatal	H	AL,FO,GL,GR,HS,RS,SH,WP,WL	March-April
	174	*Launaea nudicaulis* (L.) Hook.f.	861/MM//2020	Dodak, Dudhkal	H	AL,FO,GL,GR,RS,SH	March-April
	175	*Launaea procumbens* (Roxb.) Ramayya & Rajagopal	821/MM//2020	Dodak, Bhathala	H	AL,FO,GL,GR,RS,SH,WP,WL	March-April
	176	*Parthenium hysterophorus* L.	670/MM//2020	Koka booti	H	AL,DL,FO,GL,GR,RS,SH,WP,WL	March-April
	177	*Silybum marianum* (L.) Gaertn.	553/MM//2020	Ount Katara	H	DL,GL,GR,RS,SL,SH,WP	March-April
	178	*Sonchus arvensis* L.	623/MM//2020	Peeli Bhattal	H	AL,GL,GR,HS,RS,SL,SH,WP	March-April
	179	*Sonchus asper* (L.) Hill	666/MM//2020	Bhattal	H	DL,FO,GL,HS,RS,SL,SH	March-April
	180	*Sonchus oleraceus* (L.) L.	713/MM//2020	Peeli dodhak	H	AL,FO,GR,RS,SH	March-April
	181	*Tagetes erecta* L.	830/MM//2020	Peela Genda	H	HG,RS,SH	July-September
	182	*Xanthium strumarium* L.	764/MM//2020	Puth kanda	H	AL,DL,FO,GL,GR,HS,RS,SH,WP,WL	March-April
32. Convolvulaceae	183	*Convolvulus arvensis* L.	728/MM//2020	Lelli	H	AL,FO,GL,GR,RS,SH,WL	March-April
	184	*Convolvulus prostratus* Forssk.	785/MM//2020	Lehi, Vanvaihre	H	AL,FO,GL,GR,RS,SH,WL	February-April
	185	*Convolvulus lineatus* L.	888/MM//2020	Waja vail	H	AL,FO,GL,GR,RS,SP,SH,WL	March-April

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	186	*Cuscuta reflexa* Roxb.	811/MM//2020	Ashk bail , Ambar bail	H	AL,DL,FO,GR,HS,RS,SL,SH,WP,WL	December-January
	187	*Ipomoea alba* L.	534/MM//2020	Sawer pholi	H	AL,GL,HG,RS,SH,WL	March-April
	188	*Ipomoea carnea* Jacq.	729/MM//2020	Gull e abbasi	S	AL,RS,WL	August-September
	189	*Ipomoea pes-tigridis* L.	903/MM//2020	Chitti chatri	H	AL,DL,GL,GR,HS,RS,SH,WP,WL	August-September
	190	*Ipomoea aquatica* Forssk.	896/MM//2020	Jungli vail	H	AL,FO,GL,GR,RS,SH,WL	February-April
	191	*Ipomoea cairica* (L.) Sweet	604/MM//2020	Jungli lehli	H	AL,FO,GL,GR,HS,RS,SL,SH,WP,WL	July-August
33. Cucurbitaceae	192	*Benincasa hispida* (Thunb.) Cogn.	730/MM//2020	Bari Khakhri	H	AL,GL,HG	June-July
	193	*Citrullus colocynthi*s (L.) Schrad.	638/MM//2020	Tumma	H	AL,DL,FO,GL,GR,HS,RS,SP,SL,SH,WP	May-June
	194	*Citrullus lanatus* (Thunb.) Matsum. & Nakai	755/MM//2020	Dwana, Turbooz	H	AL,GL,HG	July-August
	195	*Cucumis sativus* L.	635/MM//2020	Khera	H	AL,GL,HG	June-July
	196	*Cucumis melo* L.	527/MM//2020	Khakhri	H	AL,GL,HG	June-July
	197	*Cucurbita moschata* Duchesne	718/MM//2020	Paitha	H	AL,GL,HG	June-July
	198	*Cucurbita maxima* Duchesne	702/MM//2020	Ghea	H	AL,GL,HG	June-July
	199	*Cucurbita pepo* L.	709/MM//2020	Kaddu	H	AL,GL,HG	June-July
	200	*Luffa acutangula* (L.) Roxb.	624/MM//2020	Kali Toori	H	AL,GL,HG	June-July
	201	*Luffa cylindrica* (L.) M.Roem.	522/MM//2020	Ghea toori	H	AL,GL,HG	June-July
	202	*Momordica balsamina* L.	712/MM//2020	Jangli kraila	H	AL,GL,HG	May-June
	203	*Praecitrullus fistulosus* (Stocks) Pangalo	809/MM//2020	Tinda	H	AL,GL,HG	June-July
34. Euphorbiaceae	204	*Chrozophora* tinctoria (L.) A.Juss.	850/MM//2020	Chitti Boi	H	DL,FO,GR,HS,RS,SP,SL,WP	May-June
	205	*Chrozophora oblongifolia* (Delile) A.Juss. ex Spreng.	781/MM//2020	Khuri	H	DL,FO,GR,HS,SP,WP	August-September
	206	*Chrozophora plicata* (Vahl) A.Juss. ex Spreng.	656/MM//2020	Chitri booti	H	DL,FO,GR,HS,RS,SP,WP	August-September
	207	*Chrozophora sabulosa* Kar. & Kir.	687/MM//2020	Giri booti	H	DL,FO,GL,GR,HS,RS,SP,WP	August-September
	208	*Croton bonplandianus* Baill.	762/MM//2020	Kala bhangra	H	FO,GL,GR,HS,RS,SH,WP,WL	March-April
	209	*Euphorbia cyathophora* Murray	848/MM//2020	Pathar chat	S	HG,RS,SH	March-April
	210	*Euphorbia helioscopia* L.	892/MM//2020	Choti chattri	H	AL,FO,GL,GR,RS,SH,WP,WL	March-April
	211	*Euphorbia hirta* L.	801/MM//2020	Lal dhudi	H	AL,FO,GL,GR,HS,HG,MS,RS,SL,SH,WP,WL	March-April
	212	*Euphorbia indica* Lam.	837/MM//2020	Dudhi kalan	H	AL,FO,GL,GR,HS,RS,WP,WL	March-April
	213	*Euphorbia prostrata* Aiton	648/MM//2020	Choti it-sit	H	AL,DL,GL,GR,HS,HG,RS,SH,WP	June-July
	214	*Euphorbia thymifolia* L.	819/MM//2020	It-sit	H	AL,DL,FO,GL,GR,HS,HG,RS,SH,WP	June-July
	215	*Euphorbia densa* Schrenk	584/MM//2020	Dhodak	H	AL,DL,FO,GL,GR,RS,SH,WP,WL	June-July
	216	*Euphorbia granulata* Forssk.	874/MM//2020	Ltari booti	H	DL,FO,GR,HS,MS,SP,SL,WP	August-September
	217	*Euphorbia prolifera Buch.*-Ham. ex D.Don	561/MM//2020	Pholi booti	H	DL,FO,GL,GR,HS,MS,RS,SP,SL	April-May

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	218	*Euphorbia serpens* Kunth	779/MM//2020	Boni booti	H	DL,FO,GL,GR,HS,MS,RS,SP,SL,SH	August-September
	219	*Ricinus communis* L.	689/MM//2020	Hernoli	S	AL,DL,FO,GL,GR,HS,RS,SP,SL,WP	August-September
35. Fagaceae	220	*Quercus incana* Bartram	617/MM//2020	Shah baloot	T	AL,FO,GL,HG,RS	August-September
36. Geraniaceae	221	*Geranium rotundifolium* L.	722/MM//2020	Bouni booti	H	AL,FO,GL,GR,RS,SH,WP,WL	March-April
	222	*Geranium lambertii* Sweet	545/MM//2020	Tara booti	H	FO,GL,RS,SH,WP,WL	March-April
	223	*Geranium pratense* L.	707/MM//2020	Asmani pholi	H	AL,FO,GR,HS,RS,SH,WP	March-April
	224	*Geranium pusillum* L.	862/MM//2020	Gana booti	H	FO,GL,GR,HS,RS,SL,SH,WP	August-September
37. Gisekiaceae	225	*Gisekia pharnaceoides* L.	644/MM//2020	Balu ka sag	H	AL,FO,GL,GR,RS,SP,SH,WP	July-August
38. Lamiaceae	226	*Anisomeles indica* (L.) Kuntze	794/MM//2020	Jamni booti	H	FO,HS,RS,SH,WP	March-April
	227	*Mentha longifolia* (L.) L.	698/MM//2020	Jangli poodina	H	FO,GL,HS,SL,SH,WL	May-June
	228	*Mentha arvensis* L.	693/MM//2020	Poodina	H	AL,GL,HG	March-April
	229	*Mentha pulegium* L.	659/MM//2020	Jamni poodina	H	AL,FO,GL,HS,RS,SL,SH,WL	March-April
	230	*Mentha royleana* Wall. ex Benth.	631/MM//2020	Chitta poodina	H	AL,FO,GL,HS,RS,SL,SH,WL	March-April
	231	*Mentha spicata* L.	503/MM//2020	Podina	H	FO,GL,HS,SH,WL	March-April
	232	*Ocimum americanum* L.	890/MM//2020	Danadar booti	H	FO,GR,HS,RS,SL,SH,WP	August-September
	233	*Ocimum basilicum* L.	737/MM//2020	Niazbo	S	HG,SH	August-September
	234	*Salvia aegyptiaca* L.	705/MM//2020	Ksaroo	H	FO,HS,RS,SP,SL,SH,WP	August-September
	235	*Salvia moorcroftiana* Wall. ex Benth.	530/MM//2020	Lapra	H	FO,HS,SL,SH,WP	May-June
	236	*Salvia nubicola* Wall. ex Sweet	841/MM//2020	Hernar	H	FO,HS,RS,SH,WP,WL	August-September
39. Leguminosae	237	*Acacia farnesiana* (L.) Willd.	851/MM//2020	Kabli kikar	T	DL,FO,HS,RS,WP	August-September
	238	*Acacia nilotica* (L.) Delile	783/MM//2020	Kikar	T	DL,FO,HS,RS,SP,WP	August-September
	239	*Acacia catechu* (L.f.) Willd.	514/MM//2020	Wada kiker	T	DL,FO,HS,RS,WP	July-August
	240	*Acacia modesta* Wall.	827/MM//2020	Pholai kiker	T	DL,FO,HS,RS,SP,WP	March-April
	241	*Acacia senegal* (L.) Willd.	618/MM//2020	Wlayti kikeri	T	FO,GL,HG,RS,WP	August-September
	242	*Acacia torta* (Roxb.) Craib	831/MM//2020	Jungli kiker	S	DL,FO,GL,HS,RS	August-September
	243	*Albizia procera* (Roxb.) Benth.	630/MM//2020	Chita Sharin	T	AL,DL,GL,HG,RS,SP	August-September
	244	*Albizia lebbeck* (L.) Benth.	597/MM//2020	Shareen	T	AL,FO,GL,GR,HG,RS,WP	August-September
	245	*Alhagi maurorum* Medik.	610/MM//2020	Kandera	S	DL,FO,GR,HS,SP	July-August
	246	*Arachis hypogaea* L.	877/MM//2020	Moong phali	H	AL,DL,GL,SP	August-September
	247	*Astragalus psilocentros* Fisch.	882/MM//2020	Jangli jantri	H	HS	August-September
	248	*Bauhinia variegata* L.	685/MM//2020	Kachnar	T	AL,GL,HG,RS	March-April
	249	*Butea monosperma* (Lam.) Taub.	772/MM//2020	Wlayti sumbal	T	RS	March-April

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	250	*Cassia fistula* L.	846/MM//2020	Girdi nalli, Amaltas	T	AL,GL,HG,RS	March-April
	251	*Cicer arietinum* L.	577/MM//2020	Choly, chany	H	AL,DL,SP	February-March
	252	*Dalbergia sissoo* DC.	695/MM//2020	Tahli	T	AL,DL,FO,GL,GR,HS,RS,SP,SH,WP,WL	April-May
	253	*Indigofera linifolia* (L.f.) Retz.	714/MM//2020	Lal dani	H	DL,FO,GR,HS,RS,SP,SL,SH,WP	March-April
	254	*Indigofera arabica* Jaub. & Spach	646/MM//2020	Gulabi pholi	H	DL,FO,GR,HS,RS,SP,SL,WP	March-April
	255	*Indigofera sessiliflora* DC.	759/MM//2020	Shareni booti	H	DL,FO,GR,HS,RS,SL,WP	March-April
	256	*Indigofera tinctoria* L.	849/MM//2020	Neeli jhari	S	DL,FO,GR,HS,RS,SL,WP	November-December
	257	*Indigofera trita* L.f.	680/MM//2020	Lal pholi	H	AL,FO,GL,RS,SH,WP,WL	February-March
	258	*Lathyrus aphaca* L.	844/MM//2020	Jangli mattar	H	AL,FO,GL,RS,SH,WP,WL	September-October
	259	*Lathyrus sativus* L.	572/MM//2020	Jangli mattri	H	AL,FO,GL,HS,RS,WP,WL	March-April
	260	*Lathyrus pratensis* L.	818/MM//2020	Peeli vail	H	AL,FO,GL,HS,RS,WP,WL	March-April
	261	*Lens culinaris* Medik.	516/MM//2020	Dal masoor	H	AL,GL,HG	February-March
	262	*Leucaena leucocephala* (Lam.) de Wit	550/MM//2020	Desi shareen	T	AL,GL,GR,HS,HG,RS,WP	March-April
	263	*Medicago sativa* L.	662/MM//2020	Lucen, Losan	H	AL,GL	June-July
	264	*Melilotus indicus* (L.) All.	754/MM//2020	Senji	H	AL,GL,GR,RS,SH,WP,WL	March-April
	265	*Melilotus officinalis* (L.) Pall.	600/MM//2020	Chitti Sinje	H	AL,GL,GR,RS,SH,WP,WL	March-April
	266	*Melilotus messanensis* (L.) All.	790/MM//2020	Patrro	H	AL,GL,RS,WP,WL	March-April
	267	*Parkinsonia aculeata* L.	667/MM//2020	Angrezi kikar	T	DL,FO,GR,HS,RS,SP,WP	August-September
	268	*Pisum sativum* L.	711/MM//2020	Mattar	H	AL,GL	September-October
	269	*Pongamia pinnata* (L.) Pierre	592/MM//2020	Sukh chain	T	AL,GL,HG,RS	August-September
	270	*Prosopis cineraria* (L.) Druce	745/MM//2020	Jand	T	DL,FO,GR,HS,RS,SP,SL,WP	August-September
	271	*Prosopis glandulosa* Torr.	886/MM//2020	Wlayti kikar	T	AL,DL,FO,GR,HS,RS,SP,SL	August-September
	272	*Prosopis juliflora* (Sw.) DC.	547/MM//2020	Phari kikar	T	AL,FO,GR,HS,RS,WP	August-September
	273	*Rhynchosia capitata* (Roth) DC	686/MM//2020	Rawan	H	AL,GL,HG	March-April
	274	*Rhynchosia minima* (L.) DC.	855/MM//2020	Jangli Rawan	H	AL,FO,HS,HG,RS,WP,WL	March-April
	275	*Senna occidentalis* (L.) Link	576/MM//2020	Jangli arwan	H	AL,FO,HS,HG,RS,WP,WL	March-April
	276	*Senna tora* (L.) Roxb.	766/MM//2020	Jantar	H	AL,GL	March-April
	277	*Sesbania sesban* (L.) Merr.	581/MM//2020	Wlayti shareen	T	FO,HS,HG,RS,WP	August-September
	278	*Sesbania concolor* J.B.Gillett	620/MM//2020	Jungli shareen	S	FO,HS,HG,RS	August-September
	279	Sesbania *grandiflora* (L.) Pers.	607/MM//2020	Majandri	T	FO,HS,HG,RS	November-December
	280	*Tamarindus indica* L.	798/MM//2020	Imli	T	AL,GL,HG,RS	December-January
	281	*Trifolium resupinatum* L.	788/MM//2020	Barsan	H	AL,GL	March-April

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	282	*Trifolium alexandrinum* L.	717/MM//2020	Chita Shatala	H	AL,GL	March-April
	283	*Trifolium pratense* L.	556/MM//2020	Gulabi Shatala	H	AL,GL	March-April
	284	*Trifolium repens* L.	579/MM//2020	Shatala	H	AL,GL	February-March
	285	*Trigonella anguina* Delile	568/MM//2020	Jangli meethre	H	AL,DL,FO,GL,GR,HS,RS,SH,WP,WL	March-April
	286	*Trigonella corniculata* Sibth. & Sm.	615/MM//2020	Meethre	H	AL,HG	March-April
	287	*Trigonella foenum-graecum* L.	901/MM//2020	Methra	H	AL,HG	March-April
	288	*Vicia sativa* L.	767/MM//2020	Jangli Rewari	H	AL,FO,GL,HS,SL,SH,WP,WL	March-April
	289	*Vicia bakeri* Ali	549/MM//2020	Daturi	H	AL,FO,GL,HS,RS,SH,WP,WL	August-September
	290	*Vicia faba* L.	725/MM//2020	Lobia	H	AL,GL,HG	December-January
	291	*Vigna mungo* (L.) Hepper	777/MM//2020	Mung dal	H	AL,GL,HG	February-March
	292	*Vigna trilobata* (L.) Verdc.	832/MM//2020	Rawan dal	H	AL,GL,HG	February-March
	293	*Vigna unguiculata* (L.) Walp.	560/MM//2020	Lobia	H	AL,HG	February-March
40. Linaceae	294	*Linum usitatissimum* L.	857/MM//2020	Alsi	H	AL,HG	June-July
41. Lythraceae	295	*Ammannia baccifera* L.	613/MM//2020	Ratta krond	H	AL,DL,FO,GL,HS,RS,SL,WP,WL	August-September
	296	*Ammannia auriculata* Willd.	536/MM//2020	Kandi booti	H	AL,DL,FO,GL,GR,HS,RS,SL,SH,WP	August-September
	297	*Ammannia verticillata* (Ard.) Lam.	902/MM//2020	Nevi kandi	H	AL,DL,FO,GL,GR,HS,RS,WP	August-September
	298	*Lawsonia inermis* L.	833/MM//2020	Mehndi	S	AL,GL,HG	March-April
42. Malvaceae	299	*Abelmoschus esculentus* L. Moench	567/MM//2020	Bhindi	H	AL,HG	June-July
	300	*Abutilon indicum* (L.) Sweet	588/MM//2020	Peela crown	H	FO,GR,HS,RS,SL,SH,WP	March-April
	301	*Abutilon theophrasti* Medik.	543/MM//2020	Janlgi Peela crown	H	FO,GR,HS,RS,SL,WP	March-April
	302	*Abutilon grandifolium* (Willd.) Sweet	765/MM//2020	Gidar booti	S	FO,GR,HS,RS,SL,WP	July-August
	303	*Abutilon hirtum* (Lam.) Sweet	800/MM//2020	Peeli booti	H	FO,RS,SL,WP	March-April
	304	*Bombax ceiba* L.	786/MM//2020	Simbal	T	AL,GL,GR,RS	February-March
	305	*Corchorus depressus* (L.) Stocks	591/MM//2020	Bahu-phali	H	DL,GR,HS,MS,SP,SL	March-April
	306	*Grewia asiatica* L.	721/MM//2020	Falsa	S	AL,GL,HG,RS	February-March
	307	*Malva neglecta* Wallr.	665/MM//2020	Sitara Sunchal	H	AL,DL,FO,GL,GR,RS,SH,WP,WL	March-April
	308	*Malva parviflora* L.	510/MM//2020	Sunchal	H	AL,DL,FO,GL,GR,RS,SH,WP,WL	March-April
	309	*Malva sylvestris* L.	564/MM//2020	Jamni phool	H	HG,RS,SH	April-May
	310	*Malva verticillata* L.	885/MM//2020	Kandi Sunchal	H	AL,GL,GR,RS,SH,WP,WL	March-April
	311	*Malvastrum coromandelianum* (L.) Garcke	642/MM//2020	Khati booti, Peeli booti	H	DL,FO,GL,GR,HS,MS,RS,SL,SH,WP	October-November
	312	*Sida spinosa* L.	719/MM//2020	Jungle maithi	H	FO,GL,GR,HS,MS,RS,SH,WP	March-April
43. Martyniaceae	313	*Martynia annua* L.	575/MM//2020	Gulabi kona	H	FO,GL,GR,HS,MS,RS,SL,SH,WP,WL	March-April

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44. Meliaceae	314	*Azadirachta indica* A.Juss.	633/MM//2020	Neem	T	AL,GL,HG,RS	July-August
	315	*Melia azedarac*h L.	864/MM//2020	Dharaik	T	AL,GL,HG,RS	August-September
45. Menispermaceae	316	*Tinospora sinensis* (Lour.) Merr.	639/MM//2020	Glow	S	AL,GL,HG	April-May
46. Moraceae	317	*Broussonetia papyrifera* (L.) L'Hér. ex Vent.	817/MM//2020	Gul toot	T	HG,RS	March-April
	318	*Ficus benghalensis* L.	593/MM//2020	Desi bohar	T	AL,GL,HG,RS	March-April
	319	*Ficus carica* L.	749/MM//2020	Anjeer	T	AL,GL,HG,RS	May-June
	320	*Ficus religiosa* L.	791/MM//2020	Peepal	T	AL,GL,HG,RS	August-September
	321	*Ficus palmata* Forssk.	879/MM//2020	Desi Anjeer	T	AL,GL,HG,RS	May-June
	322	*Ficus sarmentosa* Buch.-Ham. ex Sm.	521/MM//2020	Wlayti bohar	T	AL,GL,RS	August-September
	323	*Morus alba* L.	866/MM//2020	Safaid toot	T	AL,GL,HG,RS	March-April
	324	*Morus nigra* L.	625/MM//2020	Kala toot	T	AL,GL,HG,RS	March-April
47. Moringaceae	325	*Moringa oleifera* Lam.	700/MM//2020	Sohanjana	T	AL,GL,HG,RS	February-March
48. Musaceae	326	*Musa* × *paradisiaca* L.	645/MM//2020	Keela	H	AL,GL,HG,WL	March-April
49. Myrtaceae	327	*Callistemon lanceolatus* (Sm.) Sweet	643/MM//2020	Bottle bursh	T	HG,RS	March-April
	328	*Eucalyptus globulus* Labill.	554/MM//2020	Saifeda	T	AL,GL,GR,RS,WL	August-September
	329	*Psidium guajava* L.	859/MM//2020	Amrood	S	AL,GL,HG	January-February
	330	*Syzygium cumini* (L.) Skeels	808/MM//2020	Kala jaman	T	AL,HG,RS	August-September
50. Nitrariaceae	331	*Peganum harmala* L.	701/MM//2020	Harmal booti	H	DL,FO,GR,HS,MS,RS,SP	March-April
51. Nyctaginaceae	332	*Boerhavia diffusa* L.	504/MM//2020	Nevi booti	H	AL,FO,GL,HS,RS,SL,SH,WP	July-August
	333	*Boerhavia procumbens* Banks ex Roxb.	649/MM//2020	Itsit	H	FO,GL,HS,RS,WP	June-July
	334	*Boerhavia repens* L.	816/MM//2020	Looni booti	H	AL,FO,GL,GR,HS,RS,SH,WP	August-September
	335	*Bougainvillea glabra* Choisy	520/MM//2020	Rangli bail	S	HG,RS	August-September
	336	*Bougainvillea spectabilis* Willd.	723/MM//2020	Bugal bail	S	HG,RS	August-September
	337	*Mirabilis jalapa* L.	526/MM//2020	Gul-e-Asar	H	HG,RS	August-September
52. Oleaceae	338	*Jasminum grandiflorum* L.	594/MM//2020	Chambeli	S	AL,HG	July-September
	339	*Jasminum sambac* (L.) Aiton	641/MM//2020	Motiya	S	AL,HG	July-September
	340	*Olea ferruginea* Wall. ex Aitch.	746/MM//2020	Kao	S	AL	August-September
53. Oxalidaceae	341	*Oxalis corniculata* L.	732/MM//2020	Peeli booti, Choti lonak	H	AL,FO,GL,GR,HS,RS,SH,WP,WL	February-March
54. Papaveraceae	342	*Fumaria indica* (Hausskn.) Pugsley	537/MM//2020	Papra	H	AL,GL,HS,RS,SH,WP	March-April
	343	*Fumaria vaillantii* Loisel.	899/MM//2020	Shatra papra	H	AL,GL,HS,RS,SH,WP	March-April
55. Pedaliaceae	344	*Sesamum indicum* L.	835/MM//2020	Till	H	AL	March-April
56. Phrymaceae	345	*Mazus pumilus* (Burm.f.) Steenis	691/MM//2020	Chita phol	H	FO,GR,HS,RS,SH,WL	March-April

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57. Plantaginaceae	346	*Veronica anagallis-aquatica* L.	834/MM//2020	Hazar booti	H	AL,FO,GL,HS,RS,SH,WP	March-April
58. Polygonaceae	347	*Persicaria glabra* (Willd.) M.Gómez	523/MM//2020	Hazar dani	H	AL,FO,GL,HS,RS,SP,SH,WP	September-October
	348	*Polygonum plebeium* R.Br.	531/MM//2020	Droonk, Gorakh pan	H	AL,FO,GL,GR,HS,MS,RS,SL,SH,WP,WL	March-April
	349	*Rumex crispus* L.	650/MM//2020	Lonak	H	AL,FO,GL,HS,RS,SL,SH,WP,WL	March-April
	350	*Rumex dentatus* L.	812/MM//2020	Khatkal	H	AL,FO,GL,HS,RS,SL,SH,WP,WL	March-April
	351	*Rumex patientia* L.	860/MM//2020	Khatkal	H	AL,FO,GL,GR,HS,MS,RS,SH,WP,WL	March-April
59. Portulacaceae	352	*Portulaca grandiflora* Hook.	763/MM//2020	Kulfa	H	DL,FO,GL,GR,HS,RS,SP,SL	August-September
	353	*Portulaca oleracea* L.	865/MM//2020	Kulfa lonak	H	FO,GL,HS,RS,SH,WP,WL	August-September
	354	*Portulaca pilosa* L.	829/MM//2020	Lorni booti	H	FO,GL,HS,MS,RS,SH	August-September
	355	*Portulaca quadrifida* L.	753/MM//2020	Lornak booti	H	FO,GL,GR,HS,MS,RS,SL,SH,WP	August-September
60. Primulaceae	356	*Anagallis arvensis* L.	900/MM//2020	Neeli booti, Billi booti.	H	AL,FO,GL,GR,HS,MS,RS,SH,WP	March-April
61. Ranunculaceae	357	*Nigella sativa* L.	740/MM//2020	Kalwanji	H	AL,HG	March-April
	358	*Ranunculus muricatus* L.	773/MM//2020	Chambel booti	H	FO,GL,SH,WP	March-April
	359	*Ranunculus sceleratus* L.	571/MM//2020	Jal Dhania	H	FO,GL,GR,HS,RS,SL,SH	February-March
	360	*Ranunculus arvensis* L.	739/MM//2020	Peela phola	H	DL,GL,HS,SP,SH,WP	March-April
	361	*Ranunculus natans* C.A.Mey.	697/MM//2020	Peela tara	H	FO,GL,GR,HS,SP,SH	August-September
	362	*Ranunculus repens* L.	738/MM//2020	Peela Gullab	H	GL,GR,HS,RS,SH,WP	June-July
62. Rhamnaceae	363	*Ziziphus jujuba* Mill.	726/MM//2020	Bairi	T	DL,FO,GL,GR,HS,HG,RS,SP	August-September
	364	*Ziziphus nummularia* (Burm.f.) Wight & Arn.	612/MM//2020	Jangli bairi	T	FO,GL,HS,RS,SP	August-September
63. Rosaceae	365	*Rosa indica* L.	814/MM//2020	Gulaab	S	HG,RS	March-April
64. Rubiaceae	366	*Galium aparine* L.	589/MM//2020	Wanwair booti	H	AL,FO,GL,GR,HS,RS,SL,SH,WP	June-July
65. Rutaceae	367	*Citrus limon* (L.) Osbeck	869/MM//2020	Nimboo	S	AL,HG	March-April
	368	*Citrus reticulata* Blanco	836/MM//2020	Kino malta	S	AL,HG	September-October
	369	*Citrus sinensis* (L.) Osbeck	743/MM//2020	Musmi malta	S	AL,HG	September-October
66. Salicaceae	370	*Populus alba* L.	778/MM//2020	Sufaid Poplar	T	AL,HG	February-March
	371	*Salix alba* L.	599/MM//2020	Desi bohoer	T	AL,GL,GR,HG,RS	February-April
67. Salvadoraceae	372	*Salvadora oleoides* Decne.	690/MM//2020	Jall, Van	T	DL,FO,GR,HS,SP,WP	August-September
	373	*Salvadora persica* L.	747/MM//2020	Pelo	S	DL,GR,RS,SP,SL,WP	August-September
68. Sapindaceae	374	*Dodonaea viscosa* (L.) Jacq.	768/MM//2020	Sanatha	S	FO,GR,HS,MS,RS,SL,SH,WP	March-April
69. Scrophulariaceae	375	*Verbascum thapsus* L.	891/MM//2020	Gidhar tambaku	H	FO,GR,HS,SP,SH	March-April
70. Solanaceae	376	*Capsicum annuum* L.	742/MM//2020	Shimla mirch	H	AL,GL,HG	June-July
	377	*Cestrum nocturnum* L.	838/MM//2020	Rat ki rani	S	HG,RS	March-April

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	378	*Datura innoxia* Mill.	519/MM//2020	Siah dhatoora	S	FO,GL,GR,HS,MS,RS,SH,WP	August-September
	379	*Datura metel* L.	628/MM//2020	Datura	S	FO,GL,GR,HS,MS,RS,SP,SL,SH,WP,WL	June-July
	380	*Datura stramonium* L.	627/MM//2020	Jangli dhatoora	S	FO,GL,GR,HS,MS,RS,SP,SL,SH,WP,WL	June-July
	381	*Hyoscyamus niger* L.	683/MM//2020	Khoob kalan	H	GL,HS,RS,SH,WP	March-April
	382	*Nicotiana tabacum* L.	679/MM//2020	Tambaku	H	AL,GL,HG	July-September
	383	*Physalis alkekengi* L.	769/MM//2020	Lal cherry	H	FO,GL,HS,SH	June-July
	384	*Physalis minima* L.	733/MM//2020	Jangli rasbari	H	FO,GL,HS,SL,SH	August-October
	385	*Physalis divaricata* D. Don	569/MM//2020	Jungli berry	H	FO,GL,HS,SL,SH,WP	August-September
	386	*Physalis peruviana* L.	502/MM//2020	Jungli berry	H	FO,GL,GR,HS,SL,SH	August-September
	387	*Solanum americanum* Mill.	636/MM//2020	Kainch Mainch, Makao	H	AL,FO,GL,GR,HS,RS,SH,WP	June-July
	388	*Solanum incanum* L.	727/MM//2020	Jangli baingan, Mahokari	S	FO,GL,HS,SH,WP	June-July
	389	*Solanum melongena* L.	681/MM//2020	Baingan	H	AL,HG	June-July
	390	*Solanum nigrum* L.	603/MM//2020	Kanch Manch, Makao	H	AL,DL,FO,GL,GR,HS,RS,SP,SL,SH,WP,WL	March-April
	391	*Solanum surattense* Burm. f.	758/MM//2020	Choti Kandiari	H	DL,FO,GR,HS,MS,RS,SP,SL	October-November
	392	*Solanum tuberosum* L.	596/MM//2020	Allo	H	AL,HG	January-February
	393	*Withania somnifera* (L.) Dunal	555/MM//2020	Aksn, Jangli panair, Akeri	H	DL,FO,GL,GR,HS,RS,SP,SL,SH,WP	March-April
	394	*Withania coagulans* (Stocks) Dunal	741/MM//2020	Jangly chana	S	DL,FO,GL,SP,SH	March-April
71. Tamaricaceae	395	*Tamarix aphylla* (L.) H.Karst.	868/MM//2020	Khagal	T	DL,FO,GL,RS,WP	March-April
	396	*Tamarix dioica* Roxb. ex Roth	676/MM//2020	Khagal, Rukh	S	DL,FO,GL,GR,RS,SP,SL,WP	August-September
72. Verbenaceae	397	*Lantana camara* L.	708/MM//2020	Rangli jhari	S	FO,GL,HS,RS	August-September
	398	*Lantana indica* Roxb.	797/MM//2020	Chitti rangli	S	FO,GL,HS,RS	August-September
	399	*Phyla nodiflora* (L.) Greene	595/MM//2020	Bukand booti	H	FO,GL,MS,SH,WP,WL	August-September
	400	*Verbena officinalis* L.	752/MM//2020	Chandni, Sindhi podina	H	FO,GL,HS,MS,SL,SH,WL	August-September
73. Violaceae	401	*Viola pilosa* Blume	647/MM//2020	Lillio	H	FO,GL,HS,SH,WL	April-May
74. Vitaceae	402	*Vitis vinifera* Linn.	655/MM//2020	Angoor	S	AL,HG	June-July
75. Zygophyllaceae	403	*Fagonia indica* Burm.f.	842/MM//2020	Jawanh booti	S	DL,FO,GR,HS,RS,SP,SL	July-August
	404	*Tribulus terrestris* L.	539/MM//2020	Bhakhra	H	DL,FO,GL,GR,HS,MS,RS,SP,SL,SH,WP	August-September

*; H: Herbs; S: Shrubs; T: Tree; G: Grass; **; AL: Arable Land; DL: Dry Land; FO: Forest; GL: Grassland; GR: Graveyard; HS: Hilly Slopes; HG: Home Gardens; MS: Mountain Summits; RS: Roadside; SP: Sandy Places; SL: Scrubland; SH: Shady Places; WP: Waste Places; WL: Wet Land

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_33433376

GROWTH AND PRODUCTIVITY OF SOYBEAN (*GLYCINE MAX* (L.) MERR.) GENOTYPES UNDER SHADING

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(Received 17th Mar 2021; accepted 19th Jul 2021)

Abstract. The research was aimed to determine the growth of soybean genotypes under shading. This Research was conducted in Malang, East Java, Indonesia. Treatments entailed of two factors, namely level of shading and soybean genotypes, arranged in factorial randomized complete block design with three replications. Level shading entailed of without shading and 50% shading, while soybean genotypes included of Argopuro, Dena 1, Dena 2, Dena 3, Grobogan, Panderman, and Karat 13. The results indicated that the soybean genotype responses to shading were different. Shading reduced leaf area index (LAI), net assimilation rate (NAR), and plant growth rate (PGR), however it increased the specific leaf area (SLA) and leaf area ratio (LAR Dena 1 genotype showed the lowest PGR stress intensity at 6%, followed by Panderman (16%) which are below the average of genotypes stress intensity at 56 DAP (24%). The PGR had significantly positive correlation with seed yield. The average decrease in seed yields of 7 soybean genotypes were 32%, and Dena 1 showed the most tolerant to shading. This proved wih the fact that of Dena 1 which is currently widely planted by the farmers in Indonesia on an intercropping pattern, or under shading of young plantation crops.
Keywords: *intercropping, leaf area, net assimilation, stress intensity, tolerant*

Introduction

In Indonesia, availability of land for soybean (*Glycine max* (L.) Merr.) monoculture cultivation is very limited because most of the land is used for estate plantations and other food crops, especially rice and maize. Therefore, expansion of the soybean planting area can be done by utilizing suboptimal land, especially dry land with intercrop system and among annual estate plant stands. Land available among other plant stands can be in the form of land under plantation stands, forest plants, and young estate plants (0-3 years), as well as land between rows of maize and cassava plants. Shaded lands which can be used for soybean development in Indonesia in the plantation, intercropping with maize, and intercropping with cassava were reaches to 1.7 million ha, 5.5 million ha, and 0.67 million ha, respectively (Mulyani et al., 2009; Statistics Indonesia, 2019).

Harsono et al. (2020) reported that the shade of maize crops above the canopy of soybean in the intercropping with maize (double row with spacing of (40 × 20) cm × 200 cm) and soybean with pplants spacing of 30 cm x 15 cm) at the age of 40 and 60 days were 53-59% and 58-63%, respectively. This condition causes a decrease in soybean yields of 40-44%. In plantation lands, the levels of shade can reach 20-60%, depending on type and age of plantation crops. Shade decreases the photosynthetic rate through reducing the production of ATPs in photosystem II (PSII) reaction center via blocking electron flow rate (Huang et al., 2018; Valladares and Niinemets, 2008).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_33773392

The morphological characteristics of soybean, biomass accumulation and distribution, and yield were significantly affected by shading stress Soybean is susceptible to shading effect of neighboring highstalked maize plants, hence reducing soybean yield (Liu et al., 2010). The characteristics that must be possessed by soybean genotypes in this condition are shading tolerance under shading conditions < 50%. For this reason, soybean plants that are adaptive to shading are needed. Light is a source of energy for plant photosynthesis, and light intensity has an important influence on plant morphology, physiology, and reproduction (Li et al., 2010; Mauro et al., 2014; Wang et al., 2014). In each plant habitat, light intensity varies temporally (seasonally and daily) and spatially. Therefore, plants perform acclimatization and plasticity to overcome the light variation problem (Zhang et al., 2003). Soybean varieties which were exposed to shade stress showed an increased in plant heights and internode lengths, but reduced stem diameters and lignin accumulation in stems (Liu et al., 2018).

Leaf area is a basic component of leaf area index for crop production, which contributes to light interception of the entire plant canopy (Zhu, 2010; Evans, 2013). Hence, leaf area is important for plants to overcome shading. On the other hand, leaves are also involved in avoidance responses to shading. Leaf size is highly dependent on the number and size of cells. However, leaf area is not only determined by the number and size of cells, but also cell division, cell development and the overall tissue of the organ (Gonzalez et al., 2012). Besides being controlled by genetic factors, leaf area is also influenced by environmental factors including light. Shading promotes elongation of petioles and inhibits leaf development. Quantitative methods are used to describe and interpret the performance of all parts of the plant, namely plant growth analysis. Growth analysis is a method used to describe photosynthetic dynamics measured through dry matter production. Plant growth can be measured without damaging the plant, such as measuring plant height or leaf count, but this often lacks accuracy. Dry matter accumulation is more described as a measure of growth because it reflects the ability of plants to bind energy from sunlight through photosynthesis, as well as their interactions with other environmental factors (Sumarsono, 2008).

Shading reduced soybean seed yield 34-55% depending on population density and variety (Liu et al., 2010). The ability of genotypes to respond to shading stress (low light) is different, so that the selection of soybean genotypes that is suitable for the shading environment plays an important role (Polthanee et al., 2011). The objective of this study was to determine the plant growth of seven soybean genotypes under shading condition.

Methodology

Place and time of study

The research was conducted in Kendalpayak Research Station, Malang, East Java, Indonesia, in 2013.

Research design

The research consisted of two factors. The first factor was level of shading, namely without shading or non-shading and 50% shading, while the second factor was seven soybean genotypes, consisted of Argopuro, Dena 1, Dena 2, Dena 3, Grobogan, Panderman, and Karat 13. Placement of treatment was based on a factorial complete randomized block design that was repeated three times.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Each treatment unit was planted on a plot measuring 1.6 m × 3.0 m. Soybean seeds were planted at a spacing distance of 40 cm × 15 cm and two seeds per hole. The 50% shading treatment came from two layers of black paranet mounted at a height of 1.8 m. Shade treatment was applied from planting to harvesting.

Observations were made on the quantitative characters of plants, including total number of leaves per plant, leaf area calculated using a leaf area meter, and seed weight per plot. Observations were carried out destructively every two weeks, starting at the age of 28 days after planting (DAP) to 56 DAP. The method of sample selection was by removing three crops without affected the performance the remaining ones.

Data analysis

The growth analysis was calculated based on the model used by Gardner et al. (1985), namely:

1. Leaf area index (LAI) shows the ratio of the leaf surface to the land area occupied by the plant, calculated by the formula:

$$LAI = \frac{[(LAi + (LAi - 1)]/2}{Ga} \quad \text{(Eq.1)}$$

where:
LAi and LAi-1 = leaf area (cm^2/plant) at Ti and Ti-1, respectively
Ti and Ti-1 = plant age on day i and i-1
Ga = Land area occupied by plants (cm^2)

2. Specific leaf area (SLA) shows the thickness of the leaves based on leaf dry weight, calculated by the formula:

$$SLA = \frac{[(LAi / LDWi)] + [(LAi - 1)/(LDWi - 1)]}{2} \quad \text{(Eq.2)}$$

where:
LAi and LAi-1 = leaf area (cm2/plant) at plant age Ti and Ti-1, respectively
LDWi and LDWi-1 = leaf dry weight (g/plant) at Ti and Ti-1, respectively

3. Leaf area ratio (LAR) is the ratio between the tissue carrying out photosynthesis to the total plant tissue, calculated by the formula:

$$LAR = \frac{[(LAi / TDWi)] + [(LAi - 1)/(TDWi - 1)]}{2} \quad \text{(Eq.3)}$$

where:
LAi and LAi-1 = leaf area (cm2/plant) at plant age Ti and Ti-1, respectively
TDWi and TDWi-1 = total dry weight (g/plant) of Ti and Ti-1, respectively.

4. Net assimilation rate (NAR) shows the net assimilation results, calculated by the formula:

$$NAR = \frac{(TDWi - TDWi-1)}{(T - Ti-1)} + \frac{(\ln LAi - \ln LAi-1)}{(LAi - LAi-1)} \text{ (g/cm}^2\text{/week)} \quad \text{(Eq.4)}$$

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5. Plant growth rate (PGR) shows an increase in plant dry weight per unit land area in certain time intervals, calculated by the formula:

$$PGR = \frac{[(TDWi - TDWi - 1)]/[(T - Ti - 1)]}{Ga} \text{ (g/cm}^2\text{/day)} \qquad \text{(Eq.5)}$$

where:
TDWi and TDWi-1 = total dry weight = leaf dry weight + root dry weight (g/plant) at plant age of Ti and Ti-1, respectively.

The data were analyzed using MSTAT-C statistical software package for variance and continued with the 5% Least Significant Difference (LSD) test to compare the two mean values.

Results and discussion

Leaf area index

The leaf area index (LAI) of the genotypes tested was different in each shading environment (*Table 1*). In general, shading treatment resulted in a reduction of LAI at each observation age, as indicated by shading/non-shading ratio of the mean of all genotypes. At 28 days after planting (DAP), the LAI means were 0.85 and 0.79 mg/m^2/day in non-shading and shading condition, respectively. Not all genotypes showed LAI reduction due to shading, for instance, Argopuro consistently increased LAI of 15%, 31%, and 3% at 28, 42, and 56 DAP, respectively. Panderman also increased LAI of 38% and 5% at 28 and 42 DAP, respectively. These data indicated that at 28 and 42 DAP, there was no effect of shading on LAI conditions of these two genotypes (Argopuro and Panderman). In general, the LAI of each genotype as well as the mean of all genotypes increased with the plant ages (28 – 56 DAP) in both non-shading and shading conditions.

Table 1. *Leaf area index (LAI) and S/NS ratio of seven soybean genotypes in the shading (S) and non-shading (NS) conditions at three plant ages*

Genotypes	Leaf area index (LAI) at plant ages								
	28 days after planting			42 days after planting			56 days after planting		
	Non-shading	Shading	S/NS ratio*	Non-shading	Shading	S/NS ratio	Non-shading	Shading	S/NS ratio
	(mg/m^2/day)		(%)	(mg/m^2/day)		(%)	(mg/m^2/day)		(%)
Argopuro	0.67 ef	0.77 cd	115	1.37 i	1.79 ef	131	1.98 gh	2.04 fg	103
Dena 1	1.08 a	0.76 cde	70	2.46 a	1.78 ef	72	2.91 a	2.85 ab	98
Dena 2	1.06 ab	1.02 ab	96	2.06 b	1.87 de	91	2.48 cd	2.53 c	102
Dena 3	0.73 de	0.46 g	63	1.88 cde	1.13 j	60	2.35 d	1.56 j	66
Grobogan	1.05 ab	0.98 b	93	1.95 bcd	1.87 de	96	2.20 e	1.78 i	81
Karat 13	0.75 de	0.70 def	93	2.02 bc	1.68 fg	83	2.72 b	2.14 ef	79
Panderman	0.61 f	0.84 c	138	1.52 h	1.59 gh	105	1.86 hi	1.84 hi	99
Mean	0.85	0.79	95	1.89	1.67	91	2.36	2.11	90
SD**	0.20	0.19	25.3	0.36	0.26	22.9	0.38	0.45	14.3

*S/NS ratio = shading/non-shading ratio. **SD = standard deviation

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_33773392

Shading caused changes in shading/non-shading ratio of LAI of each soybean genotype. The shading/non-shading ratio of LAI means of 7 genotypes in each plant age were 95%, 91%, and 90% at 28, 42, and 56 DAP, respectively. These data indicated that in this research shading reduced the LAI. This was agree with that of reported by Su et al. (2014) that the LAI of shading soybean plants was reduced by 71.0% in maize-soybean intercropping compared to monocultured soybean crops.

The pattern of changes of shading/non-shading ratio of LAI of each soybean genotype is shown in *Figure 1*. Dena 1, Dena 2 and Dena 3 which are considered tolerant to shading, in this study showed a reduction of LAI in the shading condition less than 100%. Argopuro and Panderman varieties did not show negative effect of shading on LAI, indicated these varieties tolerant to shading based on LAI shading/non-shading ratio.

Increased of LAI in each variety or genotype is as an effort of the plants to increase light capture through increasing leaf area in shading conditions. Leaf area index is the ratio of the leaf surface to the land area where the plant grows to the leaf area as its basic component, which contributes to light interception of the entire plant canopy (Evans, 2013; Zhu et al., 2010).

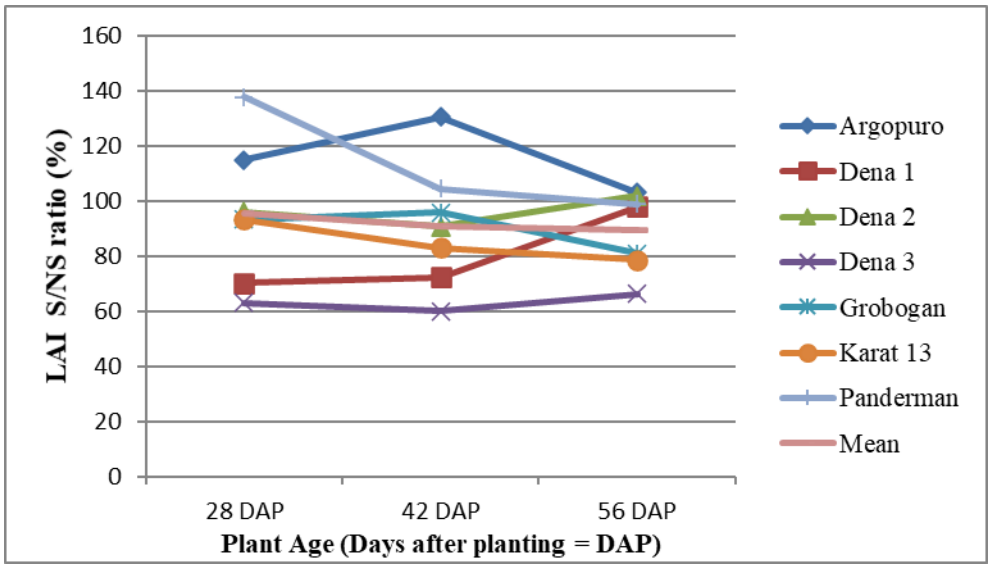

Figure 1. *Shading/Non-shading ratio on leaf area index (LAI) of seven soybean genotypes at three different plant ages*

Leaf area specific

The specific leaf area (SLA) of soybean genotypes tested was different in the two shading environments (*Table 2*). Shading resulted in an increase in SLA of each genotype and the means of 7 genotypes at each observation age. At 28 DAP, the SLA means were 182.0 and 261.6 cm^2/g in non-shading and shading conditions, respectively. The SLA increased as plant grew older, namely at 56 DAP, the SLA means were 361.5 and 428.1 cm^2/g in non-shading and shading conditions, respectively. Overall, shading caused an increase in SLA at various plant ages (*Table 2*).

The rate of reduction or stress intensity of SLA means of 7 genotypes in each plant age ranged from 18.41% at 56 DAP to 49.17% at 28 DAP (*Table 2*), indicated that as

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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plants grew up, the leaves were less sensitive to shading effect. These pattern of SLA stress intensity of soybean genotypes at various ages of observation is presented in *Figure 2*. Almost all of 7 genotypes showed a decreased in SLA stress intensity values as plants grew up, except Grobogan variety that showed an increase value at 56 DAP (*Fig. 2*).

Table 2. *Specific leaf area (SLA) and S/NS ratio of seven soybean genotypes in the shading (S) and non-shading (NS) conditions at three plant ages*

Genotypes	Specific leaf area (cm^2/g) at plant ages								
	28 days after planting			42 days after planting			56 days after planting		
	Non-shading	Shading	S/NS ratio*	Non-shading	Shading	S/NS ratio	Non-shading	Shading	S/NS ratio
	(mg/m2/day)		(%)	(mg/m2/day)		(%)	(mg/m2/day)		(%)
Argopuro	180 ef	262 bcd	146	137 fg	179 b	131	346 de	427 abc	123
Dena 1	205 e	250 d	122	162 cd	196 a	121	425 bc	446 ab	105
Dena 2	193 ef	298 a	154	150 de	194 a	129	409 c	440 ab	107
Dena 3	170 f	199 ef	129	128 g	172 bc	134	336 ef	435 ab	129
Grobogan	179 f	259 cd	145	133 g	169 bc	127	319 f	436 ab	137
Karat 13	175 f	281 abc	160	134 fg	175 b	130	331 ef	362 d	109
Panderman	172 f	286 ab	166	146 ef	193 a	132	363 d	451 a	124
Mean	182.0	261.6	146	141.5	182.5	129	361.5	428.1	119
SD**	12.41	26.25	16.02	11.97	11.32	4.36	40.66	30.23	12.21

*S/NS ratio = shading/non-shading ratio. **SD = standard deviation

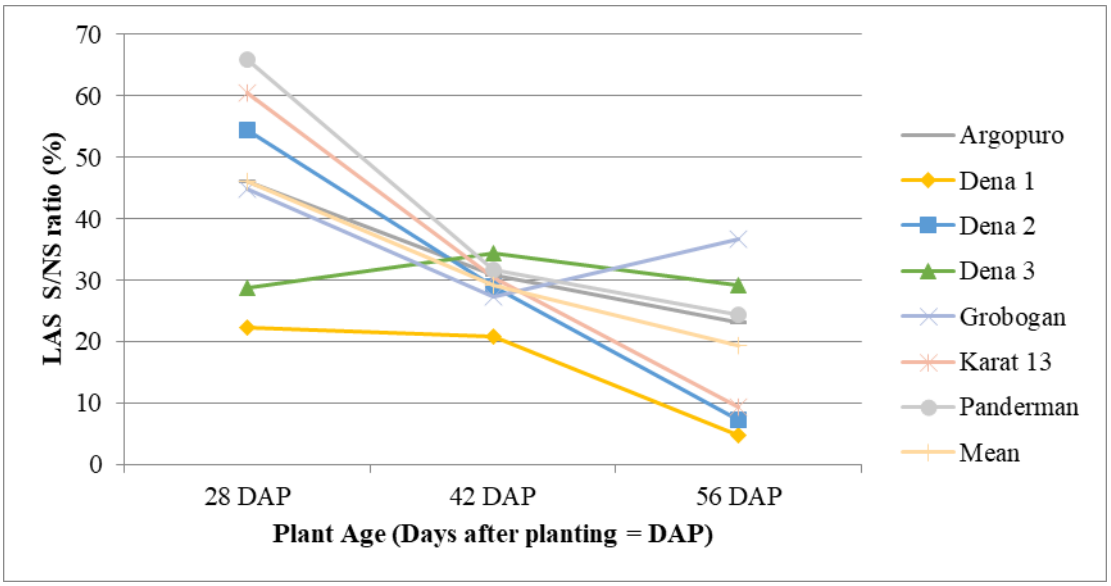

Figure 2. *Shading/Non-shading ratio on specific leaf area (SLA) of seven soybean genotypes at three different plant ages*

Leaf area ratio

The leaf area ratio (LAR) is the ratio between the photosynthetic organs (leaf area) and the photosynthetic product (total dry weight). The higher the LAR value, the greater the photosynthetic product used for the growth of the photosynthetic organ. LAR of

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_33773392

soybean genotypes tested were different in each shading environment. Shading resulted in an increase in LAR of each genotype and the means of 7 genotypes. The highest increase in LAR value occurred at age 28 DAP, followed by age 42 and age 56 DAP i.e. 44.01%, 27.25%, and 13.77%, respectively. In the vegetative phase (aged 28 DAP), there was a sharp increase in LAR values (*Table 3*).

The highest changes in LAR value occurred at age 28 DAP, followed by age 42 and age 56 DAP 44.01%, 27.25%, and 13.77%, respectively (*Table 3*). In the vegetative phase (aged 14 and 28 DAP), there was a sharp increase in LAR values. The pattern of LAR stress intensity of 7 soybean genotypes at various ages of observation is presented in *Figure 3*. All genotypes showed a decreased in LAS stress intensity values as plants grew up until 56 DAP (*Fig. 3*).

Table 3. *Leaf area ratio (LAR) and S/NS ratio of seven soybean genotypes in the shading (S) and non-shading (NS) conditions at three plant ages*

Genotypes	Leaf area ratio (%) at plant ages								
	28 days after planting			42 days after planting			56 days after planting		
	Non-shading	Shading	S/NS ratio*	Non-shading	Shading	S/NS ratio	Non-shading	Shading	S/NS ratio
	(%)		(%)	(%)		(%)	(%)		(%)
Argopuro	115 fg	167 cd	146	82 fg	102 bc	124	62 bcd	66 fg	106
Dena 1	129 ef	162 d	125	93 de	114 a	123	61 cde	65 b	106
Dena 2	124 efg	193 ab	155	83 fg	106 b	127	56 fg	60 de	107
Dena 3	111 g	135 e	121	80 gh	106 b	132	54 g	70 a	129
Grobogan	129 ef	173 cd	134	73 h	96 cd	132	51 h	64 bc	127
Karat 13	119 efg	198 a	167	88 ef	116 a	132	56 fg	64 bc	116
Panderman	111 g	180 bc	163	85 efg	105 b	123	58 ef	62 bcd	107
Mean	119.8	172.5	144	83.5	106.3	127	55.4	63.0	114
SD**	8.07	21.20	18.12	6.21	6.77	4.36	3.98	3.19	10.12

*S/NS ratio = shading/non-shading ratio. **SD = standard deviation

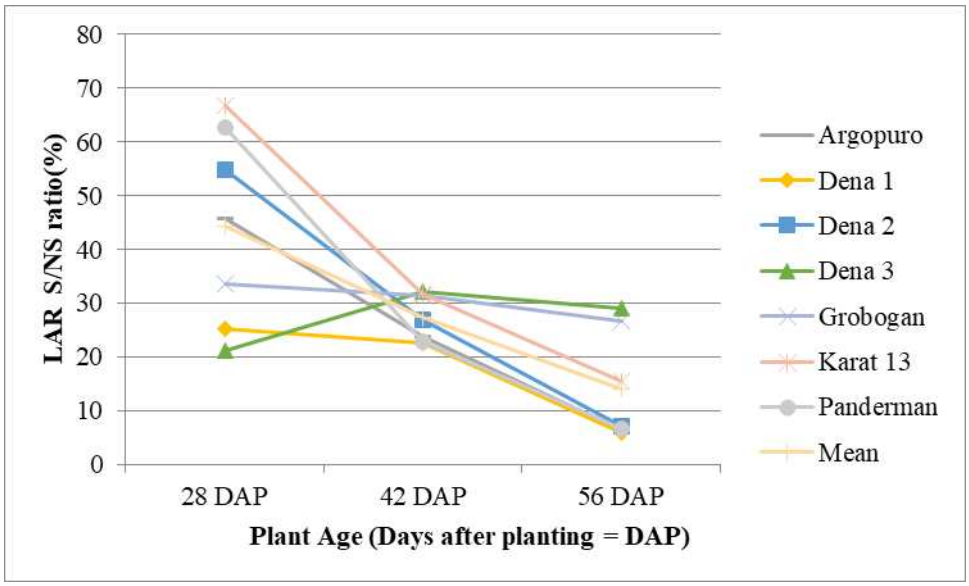

Figure 3. *Shading/non-shading ratio on leaf area ratio (LAR) of seven soybean genotypes at three different plant ages*

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DOI: http://dx.doi.org/10.15666/aeer/1905_33773392

Net assimilation rate

Net assimilation rate (NAR) is the result of net assimilation of the unity of leaf area of time. The NAR value shows a decrease with the increase of plant age. Shading resulted in a decrease in NAR of each genotype and the means of 7 genotypes (*Table 4*). At 28 days after planting (DAP), the NAR means were 16.53 and 10.65 mg/m^2/day in non-shading and shading conditions, respectively. The NAR values decreased as plant grew older (42 and 56 DAP). At 56 DAP the NAR means were 7.50 and 5.94 mg/m^2/day in non-shading and shading conditions, respectively or the decreased were 55% and 46% in non-shading and shading conditions, respectively compared to NAR values at 28 DAP.

Shading caused changes in the NAR or NAR stress intensity of each soybean genotype. The highest mean stress intensity of net assimilation rate (NAR) of seven soybean genotypes due to shading condition at various plant ages occurred at age 28 DAP (35.59%), followed by 24.03% and 20.80% at ages of 42 and age 56 DAP, respectively (*Table 4*). Dena 1, Dena 2 and Dena 3 genotypes which are considered tolerant to shading, in this study showed NAR stress intensity at 28 DAP of 34.57%, 39.32%, and 36.35% which are above the average (31.69%). Grobogan 13 genotype showed the lowest NAR stress intensity of 21.88% followed Karat 13 genotype (31.69%). However, at age 56 DAP, Dena 1, Grobogan and Panderman genotypes showed NAR stress intensity of 16.80%, 12.01%, and 14.93% which are below the average at 56 DAP (19.95%). These 3 genotypes indicated tolerant to shading based on NAR stress intensity at 56 DAP.

Table 4. *Net assimilation rate (NAR) and stress intensity of seven soybean genotypes in the shading (S) and non-shading (NS) conditions at different plant ages*

Genotypes	Net assimilation rate								
	28 days after planting			42 days after planting			56 days after planting		
	Non-shading	Shading	S/NS ratio*	Non-shading	Shading	S/NS ratio*	Non-shading	Shading	S/NS ratio*
	(mg/m^2/day)		(%)	(mg/m^2/day)		(%)	(mg/m^2/day)		(%)
Argopuro	19.27 a	13.11 a	68	8.93 bc	6.29 c	70	10.14 a	7.25 a	71
Dena 1	17.81 a	11.65 b	65	9.37 bc	7.34 bc	78	6.24	5.19 b	83
Dena 2	17.22 a	10.45 b	61	8.61 bc	6.81 c	79	9.13 a	7.30 a	80
Dena 3	15.79 ab	10.05 b	64	13.88 a	9.40 b	68	5.48 c	4.26	78
Grobogan	12.35 c	9.65 bc	78	16.85 a	12.61 a	75	7.56 ab	6.65 a	88
Karat 13	16.32 ab	12.62 b	77	13.34 ab	10.40 ab	78	9.54 a	7.15 a	75
Panderman	16.97 ab	11.02 b	65	12.50 b	9.60 b	77	4.41 c	3.75 bc	85
Mean	16.53	11.22	68	11.93	8.92	75	7.50	5.94	79
SD**	2.15	1.30	6.80	3.08	2.25	4.36	2.20	1.51	5.82

*S/NS ratio = shading/non-shading ratio. **SD = standard deviation

The pattern of changes of NAR stress intensity of each genotype at three ages of observation is presented in *Figure 4*. Almost all of 7 genotypes showed a decreased in LAS stress intensity values as plants grew up, except Dena1 that showed an increase value at 56 DAP**.** This pattern indicated that as plants grew up, the leaves were less sensitive to shading effect.

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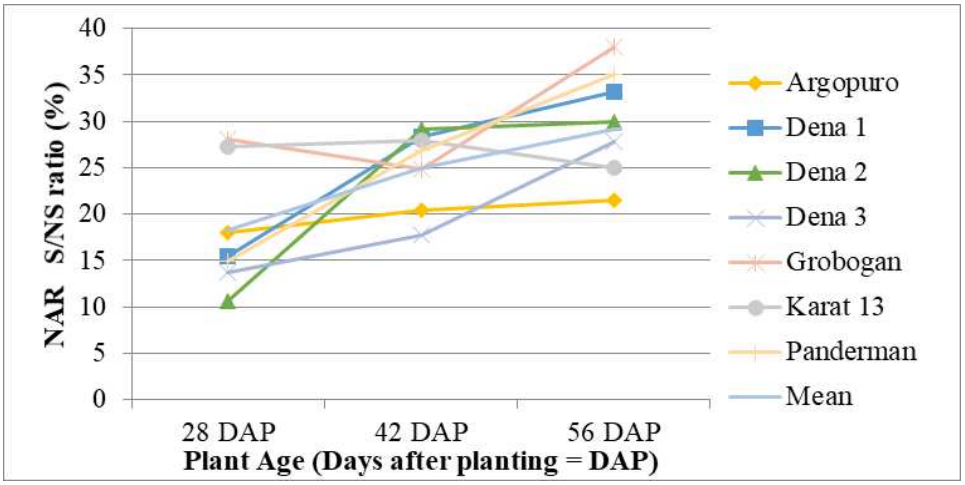

Figure 4. *Shading/non-shading ratio on net assimilation rate (NAR) of seven soybean genotypes at three different plant ages*

Plant growth rate

The plant growth rate (PGR) of each genotype showed differences in each shading environment. Shading resulted in a decrease in PGR of each genotype and the means of 7 genotypes (*Table 5*). At 28 DAP, the PGR means were 0.71 and 0.41 g/cm^2/day in non-shading and shading conditions, respectively. The highest PGR values occurred at 42 DAP then decreased as plant grew older (56 DAP). At 42 DAP the PGR means were 1.31 and 0.99 g/cm^2/day in non-shading and shading conditions, respectively. At 56 DAP the PGR means were 1.08 and 0.80 g/cm^2/day in non-shading and shading conditions, respectively or the decreased were 22% and 14% in non-shading and shading conditions, respectively, compared to PGR values at 42 DAP.

Table 5. *Plant growth rate and stress intensity of seven soybean genotypes in the shading (S) and non-shading (NS) conditions at various plant ages*

Genotypes	Plant growth rate								
	28 days after planting			42 days after planting			56 days after planting		
	Non-shading	Shading	S/NS ratio*	Non-shading	Shading	S/NS ratio*	Non-shading	Shading	S/NS ratio*
	(g/cm^2/day)		(%)	(g/cm^2/day)		(%)	(g/cm^2/day)		(%)
Argopuro	0.62 b	0.30 b	48	0.73 c	0.68 bc	93	1.19 a	0.72 b	61
Dena 1	0.92 a	0.46 a	50	1.36 b	0.96 b	71	1.09 b	1.02 a	94
Dena 2	0.92 a	0.55 a	60	1.05 bc	1.10 b	105	1.36 a	0.95 a	70
Dena 3	0.64 b	0.25 bc	39	1.47 b	0.71 bc	48	0.77 c	0.48 d	62
Grobogan	0.70 b	0.52 a	74	1.95 a	1.39 a	71	1.00 b	0.69 bc	69
Karat 13	0.63 b	0.33 b	52	1.53 b	1.18 a	77	1.15 ab	0.91 a	79
Panderman	0.52 b	0.47 a	90	1.10 bc	0.91 b	83	0.99 b	0.83 b	84
Mean	0.71	0.41	59	1.31	0.99	78	1.08	0.80	74
SD**	0.15	0.12	17.58	0.39	0.25	18.03	0.19	0.18	12.02

*S/NS ratio = shading/non-shading ratio. SD = standard deviation

Plant growth rate (PGR) is the rate of plant weight gain per unit land area per unit time (Gardner et al., 1985). Shading caused changes in the PGR or PGR S/NS ratio of

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DOI: http://dx.doi.org/10.15666/aeer/1905_33773392

each soybean genotype. The highest mean stress intensity of PGR of seven soybean genotypes due to shading condition at various plant ages occurred at age 28 DAP (33.37%), followed by 32.23% and 24.33% at ages of 42 and age 56 DAP, respectively (*Table 5*). Dena 1, Dena 2 and Dena 3 genotypes which are considered tolerant to shading, in this study at 28 DAP showed PGR stress intensity of 28.26%, 18.48%, and 29.69% which are below the average (33.37%). Dena 2 genotype showed the lowest PGR stress intensity of 18.48% followed Dena 1 genotype (28.26%). However at age 56 DAP, Dena 1 genotype showed the lowest PGR stress intensity of 6%, followed by Panderman of 16% which are below the average at 56 DAP (24%). These two genotype indicated tolerant to shading based on PGR stress intensity at 56 DAP.

Seed yield

Shading resulted in decreasing of seed yield/ha average of 7 genotypes by 36.3%. The stress intensity on seed yield due to shading varied for each genotype, by which the higest (60%) showed by Dena 2 variety, while the lowest (26%) showed by Dena 1 variety. The stress intensity on 100 seed weight due to shading showed that the highest (50.6%) was Grobogan 3 variety, while the lowest (7.3%) showed by Dena 2 variety (*Table 6*). In the vegetative phase, plant growth is characterized by the formation of vegetative organs such as leaves, stems and roots (Purcell et al., 2014). Su et al. (2014) reported that in maize-soybean intercropping compared to monocultured soybean crops, the shading effect of maize on soybean caused the seed yield decreased by 32.8%. (Khalid et al., 2019) reported that in maize-soybean intercropping, the seed-yield of 25% shading (T75) was 88% of and non-shading (T0) yield. They suggested that in intercropping planting pattern, the maximum shade density ranges from 20 to 30% to obtain higher seed yield of soybean crop under intercropping-system. The effect of shading on soybean yields were 54.69% lower than those in monoculture respectively, suggested that soybean plants can regulate its morphological characteristics and leaf anatomical structures under different light environments (Fan et al., 2018).

Table 6. *Seed traits and their stress intensity due to shading condition*

Genotypes	Seed yield per ha		
	Non-Shading	Shading	S/NS ratio*
	(ton)		(%)
Argopuro	3.55 b	2.34 e	66
Dena 1	2.06 f	1.52 d	74
Dena 2	2.21 ef	1.32 e	40
Dena 3	2.14 c	1.42 i	45
Grobogan	2.59 d	1.78 gh	69
Karat 13	2.69 h	1.68 d	62
Panderman	2.21 a	1.08 g	51
Mean	**2.49**	**1.59**	**63.7**
SD**	**0.52**	**0.40**	**7.92**

*S/NS ratio = shading/non-shading ratio. **SD = standard deviation

Correlation of stress intensity parameters

Correlation analysis (*Table 7*) showed that the plant growth rate (PGR) was significantly negatively correlated with specific leaf area (SLA) (r = -0.554*) but

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DOI: http://dx.doi.org/10.15666/aeer/1905_33773392

positively correlated with seed yield (r = 0.978**). Leaf area ratio (LAR) showed significantly negative correlation with leaf area index (LAI) (r = -0.643**) but significantly postive correlation with SLA (r = 0.656**). The increase in LAI is related to the plant's efforts to increase light acceptance through increasing leaf area. Verdelli et al. (2012) showed that an increase in light interception was followed by an increase in plant growth rate.

Table 7. *Correlations values among stress intensity parameters*

Characters	LAI	SLA	LAR	NAR	PGR
LAS	0.016				
LAR	-0.643**	0.656**			
NAR	0.089	0.279	0.214		
PGR	0.122	-0.554*	-0.45	0.435	
Seed yield	0.076	-0.658**	-0.514*	0.240	0.978**

* and ** are significantly different at 5% and 1% levels, respectively

The reduction in SLA is associated with an increase in leaf thickness, and is determined by the thickness of the constituent cell tissues, such as upper and lower epidermal cells, and palisade tissue. At low light intensity the plants were only able to develop one layer of palisade tissue while at high light intensity the plants were able to develop two layers of palisade tissue (Sundari et al., 2008). Palisade tissue contains a lot of chloroplasts which are very important in increasing the efficiency of photosynthesis. Shading causes changes in the quantity of light received by soybean plants to decrease, so that it affects the parameters of the analysis of plant growth genotypes. The response of soybean genotypes to shading was different, which was indicated by differences in the growth analysis parameters of each genotype in their respective environments. Lakshmanakumar and Guru (2014) argued that plant growth rate, relative growth rate, net assimilation rate, leaf area index, and leaf specific weight were affected by shading.

Specific leaf area (SLA) is the ratio of leaf area to leaf dry weight, which shows the level of leaf thickness and the efficiency of the leaf area formation process and the greater the SLA value, the thinner the leaf in question. Shading causes an increase in the SLA value which means a decrease in the level of leaf thickness. The results of research by Aragão et al. (2014) showed that the thickness of the leaf tissue was influenced by light intensity, which at high light intensity, the leaf tissue was thicker than at low light intensity. Costa et al. (2010) also reported that leaf thickness increased in plants grown in full sunlight, greater expansion of the abaxial epidermis and spongy parenchyma than in shading grown plants.

The pattern of changes in the SLA due to shading was different (*Fig. 3*). In general, shading increased the SLA of soybean genotypes at different rates of increase between observation times. The increase in SLA indicated that the increase in leaf area was greater than the increase in leaf dry weight. Srikrishnah et al. (2012) stated that plants grown under 50% and 70% shading levels produced higher leaf area and biomass than plants grown under 80% shading. Leaf area is a determining factor in the interception mechanism of light radiation and water, and energy exchange (Peksen, 2007). Thus, it can be stated that increasing SLA is a plant strategy to increase the ability to compete in the shading for light. Devkota and Jha (2010), Gobbi et al. (2011) and Matsoukis et al. (2015) reported that plants grown under shading produced higher SLA than plants grown under full light intensity. Under the shading, the plants have thinner leaves,

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reduced distribution of photosynthate to each cell, so that the cells making up the leaf blade experience a reduction in leaf thickness (Maghfiroh, 2006).

Dena 1 was a soybean genotype that showed relatively stable increase in the LAS value among observation times, except for the age of 56 DAP. The increase in LAS values at age 14, 28, 42 and 56 DAP were 23.43%, 22.30%, 20.75%, and 4.84%, respectively, which means that the leaf thickness level of Dena 1 variety has not changed much in the presence of shading treatment suggesting that Dena 1 variety was adaptive to shading.

The 50% shading increased the leaf area ratio (LAR) value of the soybean genotype (*Table 3*), indicated that the photosynthate yield in the shading was mostly allocated for the formation of photosynthetic organs (leaves). Corre (1983), reported that shading caused an increase in leaf area ratio (LAR) through a reduction in leaf specific weight (LSW) which was associated with a reduction in leaf thickness. The higher the LAR value, the higher the proportion of leaf area which reflects the large area of photosynthesis. According to Costa et al. (2010), increased LAR under shading indicates that plants require a larger leaf area to produce one gram of dry matter than plants that are without shading. Increasing the LAR value of soybean varieties Dena 1 at several times of observation showed stability, with an increase in LAR values of 23.98% (at 14 DAP), 25.27% (at 28 DAP), and 22.65% (at 42 DAP), while increased in LAR values only reached 5.93% at the age of 56 DAP. The increase in LAR in the other six genotypes showed instability which showed that Dena 1 was a soybean genotype that was able to adapt to a shading environment.

The net assimilation rate (NAR) values in the initial period of growth was low, both in the treatment without shading or with 50% shading, then increased in the following period. Öztürk et al. (2014), suggested that plants grown under shading produced low NAR in the initial period of growth and high in the final period of their growth. The reduction in LAB was due to the reduced light received by the leaves because the shading caused the disruption of the photosynthesis process with light as the main energy source in the photosynthesis or assimilation process. The disruption of the photosynthesis process resulted in reduced photosynthate per unit leaf area per unit time. The results of other studies showed that the soybean intercropped with *Populus deltoides x nigra* varieties DN-177, *Acer saccharinum Marsh.,* and *Juglans nigra L.* experienced a reduction in net assimilation by 53.1%, 67.5%, and 46.5% respectively, compared to monoculture soybeans (Peng et al., 2015).

The plant growth rate (PGR) of soybean genotype showed differences in each shading environment (*Fig. 5*). In an environment without shading, Grobogan variety which was classified as early maturity had high PGR at the age of 42 DAP, while Karat 13 which was in the late maturity showed high PGR at 56 DAP. The shaded of Grobogan variety still showed high PGR at the age of 42 DAP, however the PGR decreased with increased plant age. Shaded of Dena 1 and Dena 2 varieties were still able to show high PGR values at the age of 56 DAP. The difference in PGR among genotypes may be due to differences in age among genotypes and also the level of sensitivity of each genotype to different shading stresses. The difference in plant age, caused different growth phases. The growth phase determined the PGR by which maximum PGR occurred in the pod filling phase because the leaf area was maximally developed (Mondal et al*.,* 2012).

Grobogan is classified as an early maturity genotype and susceptible to shading, so it is not able to maintain the PGR in the longer shading stress conditions. Dena 1 and

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Dena 2 varieties were classified as medium maturity and adaptive to shading, so they were able to maintain and even increase PGR in longer shading conditions. Dena 1 and Dena 2 varieties were more efficient in utilizing limited light intensity, compared to the other five genotypes. During its life cycle the plant experiences shading, although only for a short period of time (Valladares and Niinemets, 2008). Active photosynthetic radiation stimulates the light reaction in the photosynthetic process, and is considered to be the main energy source for photosynthesis in shading-tolerant plants (Valladares and Niinemets, 2008; Gommers et al., 2013), and the R:FR ratio is considered to be the most important light factor.

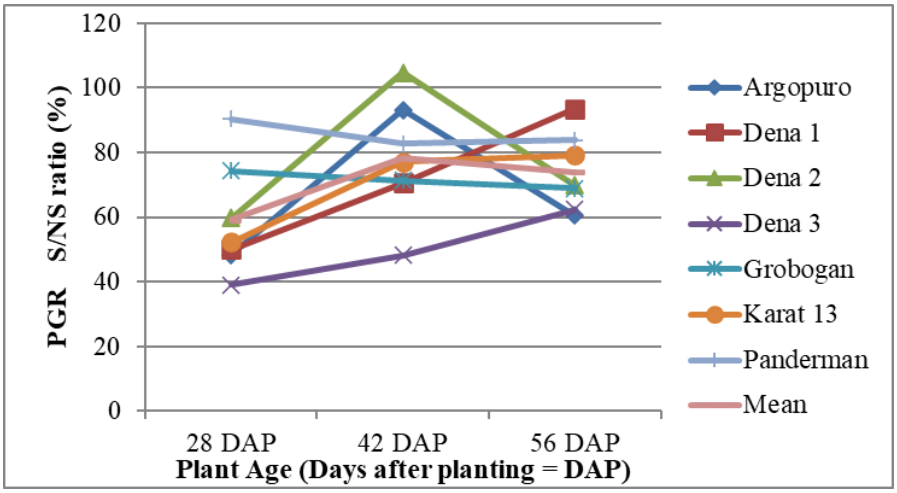

Figure 5. *Shading/non-shading ratio on plant growth rates (PGR) of seven soybean genotypes at three different plant ages*

Plants face shading by implementing two suitable strategies, namely: tolerance and avoidance of shading (Gommers et al., 2013). A tolerant response to shading is achieved through optimization of light capture and utilization, including increased chlorophyll content, specific leaf area, photosystem II:I ratio, and decreased chlorophyll a:b ratio, all of which lead to increased leaf carbon content (Valladares and Niinemets, 2008; Niinemets, 2010; Valladares et al. 2007). Under shading conditions, plants developed relatively larger leaves at the expense of leaf weight per unit area and increased accumulated chlorophyll content per unit leaf weight. This provides a greater opportunity for plants to capture light (Valladares and Niinemets, 2008; Evans et al., 2009; Niinemets, 2010; Casal, 2013; Gommers et al., 2013).

The genotypes used in the study are still grown by farmers until recently. Seed production is conducted by ILETRI to support the accelerated program of increasing soybean production. In 2020, a total of 10,500 kg of Foundation Seeds (FS) class soybeans were produced, respectively, from 9 popular varieties and several other varieties. The figure below shows a total of 16,724 kg of seeds FS were distributed to the seed growers around the country (Balitkabi, 2020).

Conclusion

Soybean genotypes responses to shading were different. Shading reduced leaf area index (LAI), net assimilation rate (NAR), and plant growth rate (PGR), however it

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increased specific leaf area (SLA) and leaf area ratio (LAR). At age 56 DAP, Dena 1 genotype showed the lowest PGR stress intensity at 6%, followed by Panderman at 16% which are below the average of PGR genotypes stress intensity at24%. The PGR had significantly positive correlation with seed yield. Shading resulted in decreasing of seed yield/ha and 100 seed weight as compared to the average of 7 genotypes by 32% and 24.7%, respectively. Dena 1 showed the most tolerant to shading. In the future, it is necessary to investigate the economic feasibility of soybean farming under shading, both with maize and young plantation intercrops.

Acknowledgments. The authors would like to acknowledge the Director General of the Indonesian Agency for Agricultural Research and Development (IAARD) for the research funding, to the Head of ILETRI who have guided of this research, and to technicians for the support during the implementation this research.

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DOI: http://dx.doi.org/10.15666/aeer/1905_33773392

EFFECTS OF MAIZE (*ZEA MAYS* L.) INTERCROPPING WITH LEGUMES ON NITROUS OXIDE (N_2O) EMISSIONS

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(Received 6th Apr 2021; accepted 8th Jul 2021)

Abstract. To investigate the effects of maize/legume intercropping on soil N_2O emissions, six treatments were tested in the North China Plain: maize, M120 (N application rate 120 kg ha^{-1}); maize, M240 (N application rate 240 kg ha^{-1}); soybean (*Glycine max*), SS (120 kg ha^{-1}); maize/soybean intercropping, MS (120 kg ha^{-1}); peanut (*Arachis hypogaea*), PP (120 kg ha^{-1}); and maize/peanut intercropping, MP (120 kg ha^{-1}). The amounts of inorganic nitrogen for the 0–20 cm soil in MS were 24.0%, 5.3%, and 29.3% lower than in SS, M120, and M240, respectively ($P < 0.05$). The total N_2O emissions ranged from 0.41 ± 0.09 to 0.98 ± 0.14 kg ha^{-1}. MP and MS were statistically different at the 95% confidence level, whereas MP produced the least N_2O emissions at 0.41 kg ha^{-1}. The seasonal cumulative N_2O emissions and global warming potential in MS and MP were also significantly lower than those in the other three monoculture treatments ($P < 0.001$). The results demonstrated that maize/legume intercropping can increase the N uptake of crops and reduce the amount of soil inorganic nitrogen and N_2O emissions, thereby, ensuring the sustainability of the agricultural environment.
Keywords: *soybean, peanut, global warming potential, nitrogen rate, cumulative N_2O emissions*

Introduction

Nitrous oxide (N_2O) emissions are essentially derived from the microbial processes of nitrification and denitrification (Kweku et al., 2018). These processes can be influenced by agricultural management practices, such as fertigation, tillage, crop rotation, and cropping systems (Hénault et al., 2012; Nath et al., 2017; Perdomo et al., 2009). Various cropping patterns are being trialed in the North China Plain to reduce N_2O emissions with maize/legume intercropping proving to be promising (Huang et al., 2014). Intercropping prairie cordgrass with Kura clover was shown to effectively reduce fertilizer-derived N_2O emissions and the net global warming potential (Abagandura et al., 2020). A similar suggestion was reported by Senbayram et al. (2016) from intercropping wheat with legumes in Gottingen, Germany. However, global information on the effect of intercropping on N_2O emissions is quite limited.

The presence of nitrogen in the soil through applied fertilizer is the main reason for the increase of N_2O emissions from farmlands (Liu et al., 2019). Therefore, improving the nitrogen use efficiency is an effective method of reducing greenhouse gas emissions.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_33933407

A large number of studies have shown that intercropping can effectively improve the nitrogen use efficiency of the intercropped populations (Yong et al., 2018), increase the nitrogen uptake of crops (Li et al., 2011), and reduce the N_2O emissions (Yu et al., 2019). Studies have shown that the yield of maize/soybean intercropping systems is higher than that of monocultures (Gao et al., 2010; Seran and Brintha, 2010).

Currently, the majority of the intercropping studies focus on interspecific complementarity and competition for nutrients (Gao et al., 2010; Seran and Brintha, 2010), the nitrogen among crop species within intercropping patterns (Qiu et al., 2019), and how to improve the system yield and land equivalent ratio (Dariush et al., 2006). Many studies focused on achieving high yield and high efficiency of crops with only limited information regarding the importance of intercropping techniques for the mitigation of greenhouse gas emissions being reported (Dyer et al., 2012). Although there have been research results on greenhouse gas emissions from intercropping systems, the results are not consistent due to different intercropping patterns and sampling methods.

Few works have reported a relationship between soil N_2O emissions and different intercropping systems. A unique study by Dyer et al. (2012) indicated that, with regard to greenhouse gas (GHG) emissions, both CO_2 and N_2O showed a general trend of greater emission rates in the maize sole crop followed by the soybean sole crop and were the lowest in the intercrops. Thus, intercropping in Pampa, Argentina could be a more sustainable agroecosystem and land management practice. This was the first study to evaluate soil N_2O emissions from a temperate maize/soybean intercropping system.

Intercropping treatments of different varieties of barley and pea also showed the ability to significantly reduce N_2O emissions (Pappa et al., 2011). These studies, and that of Huang et al. (2014), which was conducted in the North China Plain, indicated that intercropping treatments (involving maize and different legumes) could be useful in controlling the N_2O emissions from soils in different agricultural ecosystems. However, some studies showed that maize/legume intercropping had no significant effect on soil N_2O emissions. Vachon (2008) showed that there was no significant difference in the soil N_2O cumulative emissions between corn/soybean intercropping at 1:2 and 2:3 and their single cropping system.

The research on the effect of maize/legume intercropping on N_2O emissions is not sufficient, and the influence mechanism is not clear, and little attention has been paid to the difference of N_2O emissions on the intercropping of maize with different legumes. The objective of this work was to evaluate the effects of maize/legume intercropping on N_2O emissions. The results will aid the development of maize/legume intercropping systems for the mitigation of greenhouse gas emissions and the sustainability of agriculture.

Materials and methods

Study area

The experiment was conducted at the Xinxiang Comprehensive Experimental Station (N35° 14′, E113° 76′, altitude 74 m) of the Chinese Academy of Agricultural Sciences from June to September 2018. The site is located in Qiliying Town, Xinxiang County, Xinxiang City, Henan Province. The station is at the Yellow River Irrigation Diversion Area of Renminshengli Canal in the west of the central part of the Huang-Huai-Hai Plain. This location belongs to the warm temperate continental monsoon climate. The

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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sunshine duration was 2399 h, the annual average temperature was 14 °C, the annual average rainfall was 582 mm, the rainfall from June to September in 2018 was 357.4 mm, the maximum temperature was 39.6 °C, the minimum temperature was 8.8 °C, and the average temperature was 28.4 °C.

The temperature and rainfall during the study period are shown in *Figure 1*. The class of the soil was sandy loam (4.52% clay, 40.27% silt, 55.21% sand) according to world soil resources reference base, the parent material of soil was the sediment after the Yellow River alluvial, and the groundwater depth was more than 5 m (Si et al., 2020). The physical and chemical properties of the soil layer (0–20 cm) of the experimental field are shown in *Table 1*. Before sowing, the total nitrogen of the 0–100 cm soil was 0.9 g kg^{-1}, and the inorganic nitrogen reserves of the 0-100 cm soil were 244.58 kg ha^{-1}.

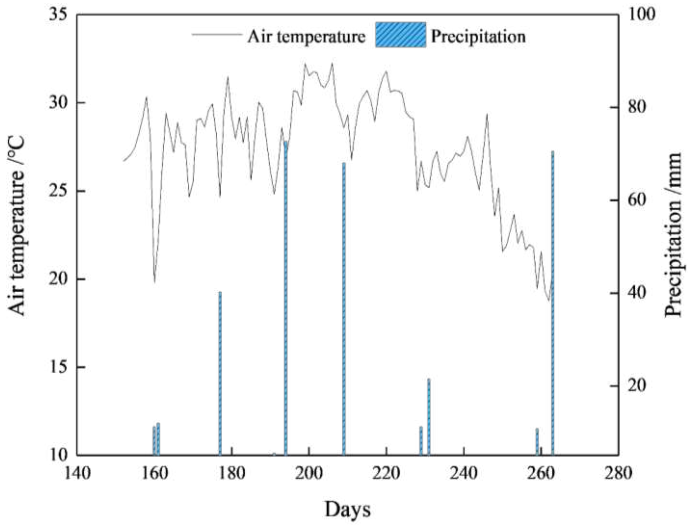

Figure 1. *Air temperature and rainfall during the trial period in 2018*

Table 1. *Soil parameters of the soil at the experimental site*

pH	Organic matter ($g \cdot kg^{-1}$)	Bulk density ($g \cdot cm^{-3}$)	Alkali-hydrolyzed nitrogen content ($mg \cdot kg^{-1}$)	Available K content ($mg \cdot kg^{-1}$)	Available P content ($mg \cdot kg^{-1}$)	Total nitrogen ($g \cdot kg^{-1}$)	Mineral nitrogen ($mg \cdot kg^{-1}$)
8.7	14.4	1.51	90.7	125.9	25.7	0.9	30.2

Experimental design

The experiment consisted of six treatments laid in a Randomized Complete Block Design (RCBD) with three replications. The maize monoculture (M240) treatment with nitrogen (urea) application had a standard rate of 240 kg ha^{-1}. Other treatments included a soybean monoculture (SS), peanut monoculture (PP), maize/peanut intercropping (MP), and maize/soybean intercropping (MS), which received nitrogen (urea) at the rate of 120 kg ha^{-1} considering the potential of legumes for complementing nitrogen supply through fixation (Huang et al., 2014).

To compare and analyze the difference in the N_2O emissions between a monoculture of maize and intercropped population under the same nitrogen application rate, a single cropping of maize with 120 kg ha^{-1} nitrogen fertilizer (M120) was also set up (*Table 2*).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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The maize variety used was "Denghai-605", the peanut variety was "Haihua-1", and the soybean variety was "Jidou-17". Each plot was 7 m wide and 10 m long, planted in a north to south orientation. The row spacings applied for maize and legumes monocultures were 60 and 30 cm, respectively. Each hole received two grains at planting. The detailed configuration (showing both row and plant spacings) of the intercropping pattern is as shown in *Figure 2*.

Table 2. *Description of the treatments applied*

Treatment Code	Description	Nitrogen application rate (kg ha^{-1})
M120	Maize monoculture	120
M240	Maize monoculture	240
SS	Soybean monoculture	120
PP	Peanut monoculture	120
MP	Maize/peanut intercropping	120
MS	Maize/soybean intercropping	120

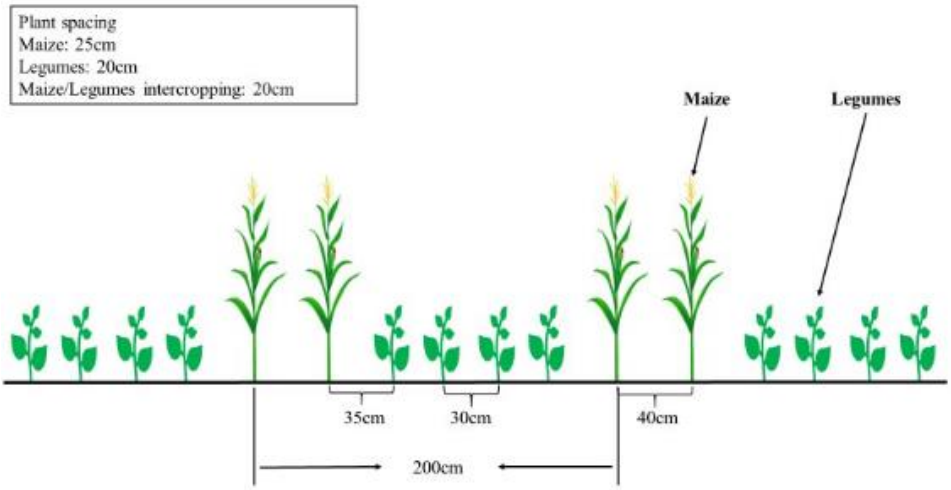

Figure 2. *Sketch of the planting patterns*

The sowing date of maize, soybean, and peanuts was June 10, 2018, while the harvest was on September 15. Before sowing, the application rates of phosphorus and potassium in all the treatments were the same; 105 kg ha^{-1} of K_2SO_4 and 120 kg ha^{-1} of P_2O_5. The nitrogen fertilizer was applied twice with the ratio of base to topdressing of 50:50. The topdressing time was July 12. Irrigation was conducted twice in the whole growth period. The first irrigation date was June 11, and the irrigation method was sprinkler with a quota of 45 mm. The second irrigation date was August 29 when the surface method was used to apply 45 mm.

Sampling and measurement procedures

Sampling and analysis of N_2O gas

Nitrous oxide (N_2O) samples were taken with a static box and analyzed with gas chromatography (Christiansen et al., 2015). Eleven sampling events were conducted

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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during the whole growth period. The size of the static box was length × width × height = 100 cm × 50 cm × 10 cm. The static box was made of an acrylic plate with a thickness of 5 mm. The top of the box was open for plant growth and gas sample collection. The static box was sealed and fixed on each sampling point by the soil sealing method (the static box was designed and installed as shown in *Figs. 3* and *4*).

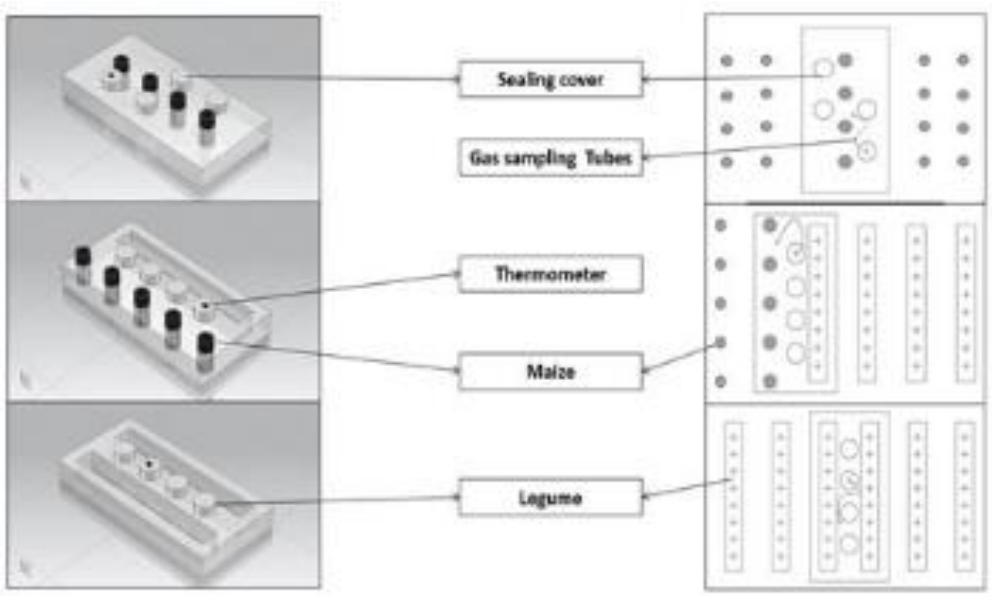

Figure 3. *Experimental layout and positioning of the static box*

Figure 4. *Static box installed at the experimental site*

In *Figure 3*, the left side is the structure diagram of three kinds of static boxes, and the right side shows the orientation of the static box under the three planting styles of single maize, intercrop of maize and legumes, and single legumes. The shaded circles in

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the figure represent Maize and "+" represents soybean or peanuts. Three static boxes were set up in each cell, and the average value of three observations was used. Soil N_2O gas samples were taken every 7 days starting on June 29 (the seedling stage on the 19th day after sowing) until September 27.

In the event of heavy rainfall, the samples were taken on the third day after rainfall to avoid the peak of N_2O emission flux caused by the increase of soil water filled porosity (WFPS). The sampling was conducted from 8:00 to 10:00 a.m., and 30 mL of gas samples (Scheer et al., 2008) were taken at 0, 10, and 20 min after all the covers on the static box were tightened, and the time was recorded with a stopwatch. After sampling, all covers were unscrewed for air circulation in the static box to ensure the normal growth of the crops.

The gas samples collected were analyzed in the laboratory using gas chromatography (model of gas chromatograph: Shimadzu 2010plus). The measuring conditions were as follows: the temperature of the electron capture detector (ECD) detector was 250 °C, the column temperature was 50 °C, the carrier gas was high-purity argon methane gas, and the flow rate was 40 mL min^{-1}. The gas emission flux was calculated using *Equation 1*.

$$F = \frac{M}{V} \times h \times \frac{d_C}{d_t} \times \frac{273}{(273+t)} \quad \text{(Eq.1)}$$

where F is the emission flux of the gas (mg $(m^2 \cdot h)^{-1}$); M is the molecular weight of the gas (g); V is the volume of 1 mol gas in the standard state (L); h is the net height of the sampling box (m); $\frac{d_C}{d_t}$ is the rate of change of gas concentration in the sampling box, 273 is the gas state path constant, and t is the average temperature in the sampling box (°C).

Soil inorganic nitrogen

Soil samples were taken from 0-20 cm to determine the amount of nitrate and ammonium nitrogen. For monocultures, the samples were taken between rows of crops, while for intercropping, the samples were taken between adjacent rows of maize and legume strips, and each sampling point was repeated three times. The fresh soil was extracted with a KCI solution with a concentration of 2 mol L^{-1}. The ratio of soil to water was 1:5. The samples were agitated at 200 R min^{-1} at a constant temperature for 30 min on an oscillator and then filtered. The collected filtrate was determined using an AA3 flow analyzer (SEAL Analytical) (Anning et al., 2021). The formula used to calculate the soil inorganic nitrogen is as follows:

$$SIN = d \times BD(N_1 + N_2) \quad \text{(Eq.2)}$$

where SIN is the soil inorganic nitrogen (kg ha^{-1}); d is the soil depth, taking 20 cm; BD is the soil bulk density (1.51 g cm^{-3}); and N_1 and N_2 are nitrate nitrogen and ammonium nitrogen in the 0-20 cm soil layer, respectively.

Soil water filled porosity

The moisture content of the soil was measured using the gravimetric method, and the water filled pore space (WFPS) was calculated using *Equation 3:*

$$WFPS = \frac{VSWC}{(1-BD/PD)} \quad \text{(Eq.3)}$$

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where *VSWC* is the volumetric soil water content (*VSWC* = soil mass water content × *BD*); *BD* was taken as an average value of 1.51 g cm^{-3}; and *PD* is the soil particle density, taking the value of 2.65 g cm^{-3}.

Soil temperature

During gas sampling, the soil temperatures at 0 and 10 cm were also measured near the static box using a Testo Mini Probe Thermometer, and the average of the two readings was taken.

The cumulative N_2O emissions and the global warming potential

The cumulative N_2O emissions during the whole growth period were estimated using the linear interpolation method as shown in *Equation 4:*

$$TN = \sum(F_{i+1} + F_i)/2 \times (t_{i+1} - t_i) \times 24 \times 10^{-5} \quad \text{(Eq.4)}$$

where *TN* is the total N_2O emissions in the whole growing season (kg ha^{-1}); F_{i+1} is the average N_2O emission flux of the current experiment (μ g $(m^2 \cdot h)^{-1}$); F_i is the average N_2O emission flux from the previous experiment (μ g $(m^2 \cdot h)^{-1}$); and $(t_{i+1} - t_i)$ is the interval between two successive experiments (Li et al., 2013).

Equation 5 was used to calculate the N_2O based global warming potential (Mehmood et al., 2019):

$$GWP\ (N_2O) = TN \times 265 \quad \text{(Eq.5)}$$

where *TN* is the cumulative N_2O emissions (kg ha^{-1}), and 265 is the global warming potential coefficient for N_2O (Stocker et al., 2013).

Statistical analysis of data

The data obtained from the experiments were statistically analyzed by Microsoft Excel, and the significance test was conducted using one-way ANOVA in Spss-23.0 software. The graphs were plotted using Origin Lab software 2018.

Results

Dynamic change characteristics of the soil moisture and temperature

Figure 5 shows the variation trend of water filled pore spaces (WFPS) and the soil temperature under different treatments. For the soil moisture, the soil WFPS of each treatment will peak after rainfall or irrigation; however, the WFPS was generally higher in the M120 treatment. The average WFPS in the M120 treatment was significantly higher than that in other treatments, except for M240, which was similar at ($P < 0.05$) (*Table 3*). In terms of the soil moisture, the average soil WFPS values of maize/legume intercropping (MS) and (MP) were significantly lower than those of monocultures of maize (M120 and M240). However, there was no significant difference in the WFPS between MS and MP nor between PP and SS as shown in *Table 3*.

For the soil temperature, the trend was similar across treatments; however, the general shape initially rose and then decreased with the crop's growth period. At the end

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of the growth period, the temperature dropped to the lowest level (*Fig. 5b*). The average soil temperature of the treatments are also shown in *Table 3*. Generally, there was no significant difference in the soil temperature between intercropping and monocultures.

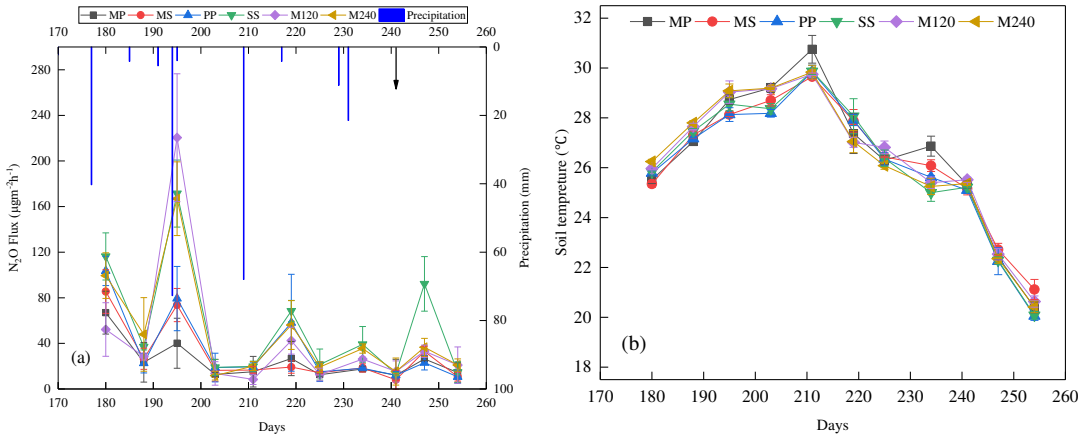

Figure 5. *Changes of the (a) water-filled pore space (WFPS) and (b) soil temperature in different treatments. MP = Maize/peanut intercrop, MS = Maize/soybean intercrop, PP = peanut monoculture, SS = Soybean monoculture, M120 = Maize monoculture (N = 120 kg/ha), M240 Maize monoculture (N = 240 kg/ha)*

Table 3. *The cumulative N_2O emissions, global warming potential, seasonal means of WFPS, temperature N-NH_4^+, N-NO_3^-, and inorganic-N of the soils in different treatments during the trial period*

Treatment	WFPS (%)	Temperature (^{0}C)	N-NH_4^+ (mg kg^{-1})	N-NO_3^- (mg kg^{-1})	SIN (kg ha^{-1})	*TN* (N_2O) (kg ha^{-1})	*GWP* (N_2O) (kg ha^{-1})
MP	44.50 ± 0.99c	26.35 ± 0.15a	3.25 ± 0.19a	7.21 ± 0.76bc	31.60 ± 2.50bc	0.41 ± 0.09c	109.63 ± 23.70c
MS	43.89 ± 1.33c	26.23 ± 0.05ab	3.31 ± 0.09a	6.07 ± 0.77c	28.32 ± 2.60c	0.50 ± 0.02b	132.41 ± 6.46b
PP	46.06 ± 2.51bc	26.03 ± 0.04b	3.20 ± 0.07a	7.70 ± 1.57bc	32.90 ± 4.72bc	0.59 ± 0.06b	155.61 ± 26.16b
SS	45.91 ± 2.11b	26.10 ± 0.07b	3.36 ± 0.22a	8.98 ± 1.48ab	37.26 ± 4.68ab	0.98 ± 0.14a	260.12 ± 36.81a
M120	51.00 ± 1.76a	26.32 ± 0.06a	3.34 ± 0.07a	6.57 ± 0.09c	29.91 ± 0.29c	0.79 ± 0.11a	209.29 ± 28.96a
M240	48.80 ± 2.19a	26.24 ± 0.08a	2.95 ± 0.03b	10.32 ± 1.09a	40.08 ± 3.30a	0.86 ± 0.21a	226.64 ± 55.85a

Different small letters in the same column indicate significant differences at the 0.05 level among treatments

Effects of the different planting patterns on the soil inorganic nitrogen

Table 3 shows the changes in the WPFS, temperature, ammonium, nitrate, inorganic nitrogen, total emissions, and global warming potential (GWP) within the 0-20 cm soil for each treatment. There was no significant difference in the amounts of ammonium in the 0-20 cm soil among treatments with the same amount of nitrogen application. The results of one-way ANOVA showed that the planting patterns had significant effects on the soil nitrate over the whole crop season ($P < 0.05$). The average soil inorganic nitrogen content of the MS treatment was 24.0% lower than SS, 5.3% lower than M120, and 29.3% lower than M240.

On the other hand, the average soil inorganic nitrogen content of the MP and PP treatments were statically similar to M120 but significantly ($P < 0.05$) lower than M240 by 21.1%. The average soil inorganic nitrogen content of the MS treatment was 10.4% lower than MP, and the average soil nitrate nitrogen content in the MS treatment was 15.9% lower than MP, although there were not significant differences in both

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parameters. The average soil nitrate nitrogen content in the MS treatment was 32.5% lower than SS and 41.2% lower than M240 at a significance level of 95%. The single factor analysis of variance showed that the planting pattern comparing monocultures and intercropping had a significant effect on the soil nitrate content of 0-20 cm in the whole growth period ($P < 0.05$).

Effects of different treatments on the soil N_2O emission flux and global warming potential

Figure 6 shows the dynamics of the N_2O emission fluxes. The peak value of N_2O was as a result of rainfall, which increased the soil WFPS. The graph indicates that the N_2O emission fluxes of each treatment had a peak value after rainfall. It can also be seen from *Table 3* that the average N_2O emission fluxes of the MS and MP treatments were lower than those of the other four treatments.

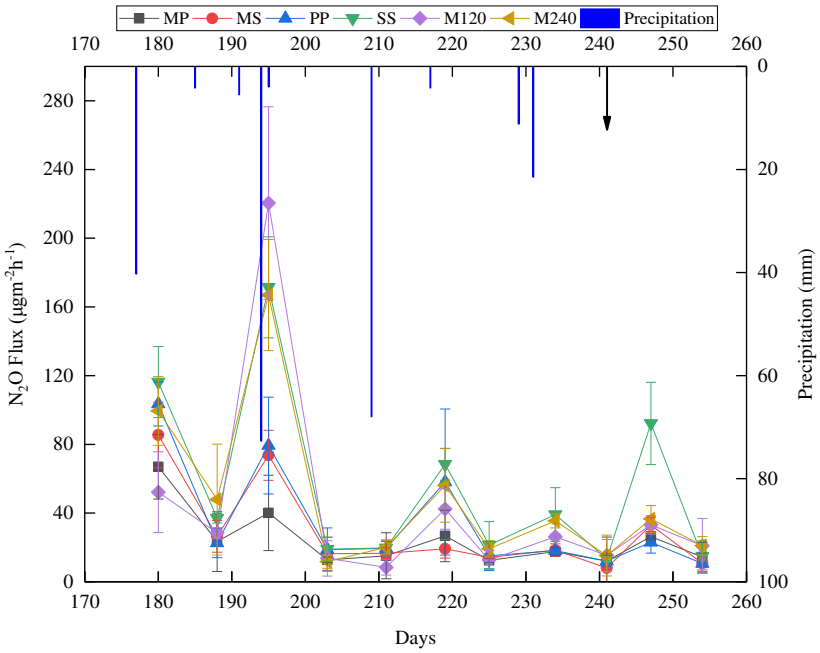

Figure 6. *Dynamics of the soil N_2O emission flux with different treatments. MP = Maize/peanut intercrop, MS = Maize/soybean intercrop, PP = peanut monoculture, SS = Soybean monoculture, M120 = Maize monoculture (N = 120 kg/ha), M240 = Maize monoculture (N = 240 kg/ha)*

Compared with M120, M240, and SS, the average N_2O emission flux of the MS treatment was significantly lower by 32.0%, 39.2%, and 47.4%, respectively ($P < 0.001$). Similarly compared with M120, M240, and PP, the N_2O flux from MP treatments was lower by 43.4%, 49.4%, and 29.6%, respectively. These results indicate that maize legume intercropping significantly reduced the N_2O emission flux of the soil. The difference in the N_2O emission flux between SS and PP was also significant.

From *Table 3*, the MS and MP treatments significantly reduced the cumulative N_2O emissions and global warming potential. The results of one-way ANOVA showed that there were significant differences in the N_2O emissions and global warming potential between MS, MP, and the other four treatments ($P < 0.001$); however, the differences

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among SS, M120, and M240 were not significant. Compared with M120, M240, and SS, the average cumulative emissions of the MS treatment were lower by 36.7%, 41.6%, and 49.1%, respectively. Compared with M120, M240, and PP, the average cumulative emissions of MS treatment were lower by 47.6%, 51.6%, and 29.5%, respectively, and the average cumulative emissions of the MS treatment were 20.8% higher than MP.

Therefore, the maize legume intercropping modes significantly reduced the cumulative N_2O emissions and global warming potential compared with the other monocultures. *Table 4* presents the Pearson correlation coefficients of soil moisture, inorganic nitrogen, and temperature as they affect the average N_2O emissions under different treatments. The correlation coefficients of the soil moisture, inorganic nitrogen, and average N_2O emission flux were different among different treatments; however, they were all positive correlations except for the inorganic nitrogen coefficients in the SS treatment. Additionally, the WFPS showed a consistent significant correlation with N_2O across all treatments.

Table 4. *Pearson correlation analysis between the N_2O, WFPS, soil temperature, and inorganic nitrogen*

Treatment	WFPS (%)	Inorganic N (μg m^{-2} h^{-1})	Temperature (°C)
MP	0.708*	0.740**	-0.17 ^{ns}
MS	0.860**	0.579^{ns}	0.064 ^{ns}
PP	0.737**	0.233 ^{ns}	0.254 ^{ns}
SS	0.860**	-0.027 ^{ns}	0.135 ^{ns}
M120	0.664*	0.165 ^{ns}	0.262 ^{ns}
M240	0.772**	0.248 ^{ns}	0.285 ^{ns}

*Significance ($p < 0.05$), **significance ($p < 0.01$), and ns = no significance

Discussion

Effects of intercropping on N_2O emissions from farmland soil

Intercropping can improve the yield, land equivalent ratio, and resource utilization efficiency of crop systems (Meng et al., 2016). The research results of Chen et al. (2017) on a wheat/garlic intercropped population showed that intercropping increased the wheat yield, and (Huang et al., 2015) concluded that it could also significantly reduce the N_2O emissions from farmland soil in the North China Plain, which is consistent with the results of this study. The highest cumulative N_2O emissions in this study were from the soybean monoculture and maize treatments. The research results of Huang et al. (2019) reported the highest cumulative N_2O emissions from the monoculture of maize treatment which partly agrees with the results of this work.

This inconsistency in conclusions may be due to differences in the planting density, rate of nitrogen fertilizer applied, soil nutrient content, and crop variety in the soybean monoculture treatments of the two studies. In this study, the planting density of soybeans was higher, and the nitrogen application rate was lower; thus, the nitrogen fixation and nitrogen utilization capacity of the monoculture soybeans were changed, resulting in a difference in the soil N_2O emissions between the studies.

The authors in Dyer et al. (2012) also concluded that maize/legume intercropping could reduce greenhouse gas emissions from farmland soils. The average N_2O emission flux of maize/legume intercropping farmland was greater than in the monoculture of

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maize. After a comparative observation, we speculated that this phenomenon may be due to different planting varieties, the planting density, nitrogen use, and the proportion of Gramineae and leguminous crops in the intercropping. Therefore, it is necessary for continuous investigation of different types of intercropping patterns on greenhouse gas emissions to better understand the impacts on the soil carbon and nitrogen dynamics.

Intercropping may change the environmental factors related to soil N_2O production. In this study, the N_2O emissions of monoculture systems was 2.39 times that of intercropping, which may be caused by a higher soil moisture in the monocultures. Soil moisture supports the activity of nitrification and denitrification bacteria in the soil, promoting the formation of nitrate and ammonium, consequently, leading to a significant increase in the N_2O emission rate (Smith et al., 2003). There was a significant and positive correlation between the concentrations of nitrate and ammonium and the N_2O emission rate (Simojoki and Jaakkola, 2000; Avrahami and Bohannan, 2009). The WFPS of intercropping was significantly lower than that of the maize and soybean monocultures, which may be related to the root distribution of the crops in the intercropping system. The researchers in Gao et al. (2009), in a study of the crop root distribution in a maize/soybean intercropping system, found that most of the roots of maize and soybean were distributed in the 0–30 cm soil layer, and the maize roots in the 16–22 cm soil layer could extend laterally to the inter row of soybean strips.

As a result, the water consumption of monoculture system was much higher than that of intercropping. Therefore, the soil moisture status in the intercropping system was lower than that in the monoculture system, which affects the N_2O emissions. There was no significant difference in the soil temperature, nitrate nitrogen, ammonium nitrogen, and inorganic nitrogen between maize monocultures and intercropping with 120 kg ha^{-1} N application, and inorganic nitrogen was significantly lower than for the maize monoculture with 240 kg ha^{-1} N application. The maize monoculture produced higher N_2O emissions compared with the maize/legume intercropping even the N application was reduced to 120 kg ha^{-1}.

Analysis on influencing factors of N_2O emissions from farmland soil

It is generally understood that N_2O is produced in the process of nitrification and denitrification dominated by bacteria (Wunderlin et al., 2012; Zhao et al., 2017). The factors affecting soil microbial activities can directly or indirectly influence the production and emissions of N_2O gas from agricultural soils. These factors include the soil water filled pore spaces (WFPS), soil pH, electrical conductivity (EC) value, temperature, fertilizer use, farming system, and crop planting type (Tang et al., 2016). Soil moisture contents lower than 97% to 84% were found to have the highest correlation with N_2O emissions (Vejan et al., 2016).

However, the soil WFPS in this study were all lower than 84%–86% indicating that intercropping reduced the soil WFPS, which was a major factor in reducing the N_2O emission flux. At the end of the growth period, the amount of inorganic nitrogen in the 0-20 cm soil increased, but the N_2O emission rate decreased. This may be due to the decrease in soil temperature at about 20 ^{0}C. The decrease in temperature became the dominant factor affecting N_2O production, which was verified by Xie and Li (2005). Among the six different treatments in this study, there was no significant difference in the average temperature from the 0-20 cm soil of the intercropping treatments.

From the results, we deduced that the amount of nitrate nitrogen in the 0-20 cm soil was greater than that of ammonium nitrogen, indicating that the soil environment within

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_33933407

the growth period was suitable for nitrification. Inorganic nitrogen is the direct substrate of nitrification-denitrification, and its concentration determines the production and emission process of N_2O (Bai et al., 2017; Liu et al., 2010). Thus, maize/legume intercropping significantly reduced the production and emissions of soil N_2O by reducing the level of soil inorganic nitrogen. From this study, there were significant differences found in the N_2O emissions between maize/peanuts and maize/soybeans.

The average cumulative emissions of the MS treatment were 20.8% higher than MP. However, there were no significant differences in the soil WFPS, inorganic nitrogen, or temperature between MS and MP. Therefore, the difference in gas emissions may be due to the variations in the rhizosphere microbial community caused by different crop species, which will affect the activity of the nitrification and denitrification bacteria (Vejan et al., 2016).

A study by Chen et al. (2018) found that maize/peanut intercropping increased the number of microflora involved in the nitrogen cycle, sulfur cycle, and other beneficial bacteria in the rhizosphere. These microflora promote more of the decomposition and reuse of carbohydrates in the maize/peanut treatments. The researchers also observed a significant reduction in the copies of various genes for denitrification. Similarly, Subbarao et al. (2012) reported that peanut plants released phytochemicals from the roots to inhibit the activities of soil nitrifying microorganisms. Therefore, the difference in the N_2O emissions between MS and MP in the current study is indicative of these findings.

In this study, we focused on the differences in N_2O emissions from cropland soils caused by cropping patterns. The main reason is that intercropping can change the micro-environment of a crop–soil system. Compared with a monoculture, intercropping can significantly affect the composition of the crop-root soil bacterial community, promote soil enzyme activity, and improve the soil nutrient utilization rate (Chen et al., 2018).

We also demonstrated that the intercropping mode had a significant effect on the soil water and heat status. The synergistic utilization of nitrogen in a crop system with the intercropping of cereal and legumes is an important approach to realizing high-efficiency in the utilization of nitrogen (Wang, 2015). Compared with monocultures, maize/legume intercropping improved the nitrogen uptake (Du et al., 2018; Dwivedi et al., 2015), thus, reducing the amount of inorganic nitrogen in the soil, forming a basis for reducing the N_2O emissions from farmland.

Conclusion

The results of this study revealed the contribution of legume intercropping with maize in the reduction of the environmental risks associated with the emissions of N_2O. This understanding will be helpful in ensuring the sustainable production of maize in the study area. Maize intercropping with a legume (peanut or soybean) significantly reduced the cumulative emissions of N_2O compared to the sole cropping of maize, peanuts, or soybeans.

The lowest cumulative emissions of 0.41 kg ha^{-1} were observed in the maize/peanut (MP) treatment, and this was significantly lower than the emissions of maize/soybean (MS). This is despite the fact that the WPFS, inorganic minerals, and nitrate were similar in both the MP and MS treatments. The difference could be attributed to the biological effects of peanut roots on nitrifying bacteria. As our study did not measure

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_33933407

the mechanisms of the microbial community in the production of N_2O, this is suggested for further investigations of the best intercropping combinations to achieve higher sustainability in crop production.

Acknowledgements. Support from labors of the experimental research station of Farmland Irrigation Research Institute of Chinese Academy of Agricultural Sciences, Research team, the Key Laboratory of Crop Water Use and Regulation, Ministry of Agriculture and Rural Affairs/Institute of Farmland Irrigation, Chinese Academy of Agricultural Sciences is gratefully acknowledged. The Authors would like to express appreciation the National Natural Science Foundation of China (NO. 51879267), and the Agricultural Science and Technology Innovation Program (ASTIP), Chinese Academy of Agricultural Sciences.

Conflict of interests: The authors declare no conflict of interests.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_33933407

BIO-ECONOMIC PERFORMANCE OF ORGANIC CUCUMBER (*CUCUMIS SATIVUS* L.) UNDER WOODLOTS-BASED AGROFORESTRY SYSTEMS

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(Received 6th Apr 2021; accepted 8th Jul 2021)

Abstract. The demand for cucumber has risen globally due to its nutraceutical properties and positive health effects. Recently, cucumber has been grown as an associate crop to maximize land-use efficiency. Therefore, this research aimed to find the maximum cucumber yield and economic feasibility under *Albizia lebbeck*, *Melia azedarach*, and *Leucaena leucocephala* woodlots-based agroforestry with mulching. The results revealed that the highest cucumber fruit yield (9.15 tons/ha) was attained by growing under *Albizia lebbeck* with dry water hyacinth mulch. Similarly, the benefit-cost ratio (BCR) was the highest (2.89) cucumber *Albizia lebbeck* woodlot agroforestry, twice as much as mono-cropping production. Thus, it would be concluded that cucumber grown under *Albizia lebbeck* woodlot agroforestry with dry water hyacinth mulch can be a cost-effective production practice for securing higher yields.
Keywords: *mulching, multi-production approach, yield potentiality, benefit-cost ratio, trellis vegetable*

Introduction

The Cucumber (*Cucumis sativus* L.) is an annual trailing vine vegetable belonging to the Cucurbitaceae family. This vegetable is highly offered in the low-fat diet due to its minimum cholesterol content, saturated fat and calories with a higher amount of vitamin-A, vitamin-C, vitamin-K and potassium (Mukherjee et al., 2013). These green fruits produce (0.6 g) protein, (2.6 g) fibre (12 calories) energy, (18 mg) Ca, (0.2 mg) Fe, (0.02 mg) thiamin, (0.02 mg) riboflavin, (0.01 mg) niacin, and (10 mg) vitamin C per 100 g (Rashid, 1999). Additionally, this trellis vegetable frequently produces a higher amount of phytochemicals such as alkaloids, flavonoids, steroids, and tannins commonly used in the cosmetic and pharmaceutical industries (Rajasree et al., 2016). The daily intake of plant-based foods has been well demonstrated to fulfil an essential part in reducing

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_34093429

chronic and degenerative diseases (Hu, 2003; Trichopoulou et al., 2014). However, in recent years, in Bangladesh, people have been intake vegetables 112 g/day/capita (23 g green vegetables, 89 g non-leafy plants), less than the lowest demand of 400 g/day/capita (WHO, 2003). The crop is grown in Bangladesh around 9,593 ha of land and the production 65,499 metric tons, with an average yield of 6.83 tha^{-1} (Anonymous, 2018). This production potential is very poor compare to other cucumber growing countries (Hossain et al., 2018), and their average yield is more than 30 tha^{-1}. Therefore, a significant gap between vegetable demand and supply has been identified to be a critical problem.

The conventional land-use schemes would become more fragile due to enhanced ambient temperature, amounts of CO_2, and other greenhouse gases. The consequence reduced the productive capacity of the system (FAO, 2013). Moreover, the depletion and destruction of forests worsen food insecurity both explicitly and indirectly: directly, by influencing the availability of fruits and timber- and tree-based food items, and indirectly by changing ecological factors important for crop and livestock. (Van Noordwijk et al., 2014). In the last three decades, agroforestry has been actively advocated in tropical and sub-tropical countries as a natural resource conservation technique (Izac and Sanchez, 2001). These multi-production strategies are becoming very popular nowadays due to the diversified production approach (Garrit, 2004). Significant advantages of adopting the strategies are - increased soil fertility, water availability, minimized soil degradation and improved bio-diversity (Chakraborty et al., 2015).

Agroforestry can be attributed to being a multi-functional system that can be ecologically and economically sensitive and addresses socio-economic needs (Sharmin and Rabbi, 2016). The benefits are manifold - compensated losses due to destructions on agriculture and forestry property by natural calamities, enhanced daily-life benefits, tackled rural socio-economic demands, mitigated the problem of climate change and so on (Amin et al., 2017a; Reppin et al., 2020). The harvested products from agroforestry practices (human food, cattle fodder, timber, gum, and construction materials) assist the basic needs (Rahman et al., 2012). Poverty is further alleviated by increasing incomes and involving women in development activities (Rahman et al., 2017).

Multipurpose tree plantations alone or paired with crops may be a successful land rehabilitation method (Maikhuri et al., 2000). The fast-growing small crowns of deciduous nitrogen-fixing trees are the critical factors for sustainable and effective agroforestry practices. In agroforestry practice (*Albizia Lebbeck* L.), the woody perennial contributes to economic aspects (Amin et al., 2017b). As an alternative, (*Leucaena leucocephala* Lam.) is frequently grown in tropical and sub-tropical countries that offer a vast timber, fodder, and healthy food source. The leaves, seeds and fruits extracts of (*Melia azedarach* L.) have been shown a wide choice of therapeutic and pesticide actions against different pathogenic and pest species. These also have anti-inflammatory activity, analgesic, antimalarial activity (Vishnukanta and Rana, 2010), and anticancer (Ntalli et al., 2010).

Teame et al. (2017) stated that organic mulching had a substantial influence on soil moisture content that contributes to the sound development of crops compared to no mulching. Farming with organic mulching eradicated the growth of weed vegetation, lesser crop evaporation rate and created a channel for rainwater infiltration (Yang et al., 2003). Sinkevičienė et al. (2009) reported that organic mulches have plentiful advantages for crop farming by improving the soil chemical, physical, and biological conditions. Sønsteby et al. (2004) found significant potassium and phosphorus percentages in crop

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_34093429

leaves due to the used sawdust. The organic straw mulch also served as a great source of micronutrients in the soil (Sønsteby et al., 2004). Organic grass mulch had the same potentiality (Cadavid et al., 1998). The application of organic mulching on crop production improved plant development, fruit settings and quality yield (Singh et al., 2007; Sharma et al., 2008). Organic material mulching had given some advantages to the root growth, fruit yield, and total soluble solids content (Olfati et al., 2008). Microbial behaviour, which depends on organic material supply, performs a vital role in controlling soil fertility and the transfer of organic matter (Marinari et al., 2007). Soil microorganisms interact quickly to enable the soil to break dormancy and increase mass and spread if conditions are suitable (Xu et al., 2006).

Moreover, in the world food scenario, the food protection concept has to be addressed in technological advancement regarding agricultural research. Direct physical and affordable access to sufficient balanced food satisfying the daily nutritional requirements and food preferences complimenting the wellbeing for everybody at every time can be termed as food protection (World Food Summit, 1996).

The authors have an intriguing interest in addressing the issues mentioned above in this study. In the most petite case, finding out the cheapest environmental-friendly way of enhanced cucumber production pertaining to social, economic, and ecological aspects. The field research aims to select a suitable cucumber production technique considering the growth, yield, and economic potency.

Materials and methods

Research site with soil and climate

The study area was situated in the northern part of Bangladesh under the Agroforestry and Environment Department of Hajee Mohammad Danesh Science and Technology University, Dinajpur. The height of the experimental site was 37.5 metre from the mean sea level (MSL). The research field was followed the old Himalayan Piedmont Plain Area (AEZ No. 01), indicating medium-high land with no water logging condition (Brammer et al., 1988). The soil physical and chemical properties of the experimental field are presented in *Table 1*. The average air temperature (^{o}C), relative humidity (%), rainfall (mm), and sunshine (hrs) of the experimental site are presented in *Fig. 1* from April to July 2020. The maximum air temperature (31.5 ^{o}C) was recorded in May, and the minimum air temperature (19.90 ^{o}C) was noted in April. The relative humidity was found a minimum fluctuation from 83.00% to 92.00%. Instead, the highest monthly precipitation (92.0 mm) was found in July, and the lowest (31.0 mm) was observed in April. In the sunshine, the monthly average variation range from 220.12 hrs to 280.4 hrs.

Plant materials

The woodlots of sixteen (16) years old *Albizia lebbeck*, *Melia azedarach,* and *Leucaena leucocephala* were the experimental fields for the present research work. The trees were arranged in east-west geometry with a 3 metres plant to plant and 3 metres for a row to row difference. At the time of experimentation, the woodlots tree characteristics were showed in *Table 2*. Among the 3 (three) tree species, the mean (n = 12) the plant height, clean bole height, base girth, bole girth, and diameter at breast height (15.0 m, 7.5 m, 118.0 cm, 97 cm, and 31 cm) was recorded from *Albizia lebbeck* woodlot. On the other hand, the *Melia azedarach* woodlot had (17.5 m) plant height, (10.5 m) clean bole

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DOI: http://dx.doi.org/10.15666/aeer/1905_34093429

height, (125.0 cm) base girth, (95.0 cm) bole girth, and (30.3 cm) diameter at breast height. Moreover, the average plant height, clean bole height, base girth, bole girth, and diameter at breast height (21.5 m, 15.5 m, 88 cm, 82 cm, and 26.0 cm) were observed *Leucaena leucocephala* woodlot, respectively. The modern Hybrid Green Bird cucumber variety was the research testing crop (*Table 2*).

Table 1. *The soil physical and chemical properties of the experimental field*

Soil characters	Physical and chemical properties
Sand (%)	63
Silt (%)	32
Clay (%)	5
Textural class	Sandy loam
CEC (meq/ 100g)	8.2
p^H	5.2
Organic matter (%)	1.28
Total nitrogen (%)	0.11
Potassium (meq/ 100g)	0.26
Phosphorus (µg/g)	25.0
Magnesium (meq/ 100g)	0.42
Sodium (meq/ 100g)	0.05
Calcium (meq/ 100g)	1.32
Sulphur (µg/g)	3.2
Boron (µg/g)	0.27
Iron (µg/g)	5.32
Zinc (µg/g)	0.92

Source: Soil Resources Development Institute, Dinajpur, Bangladesh (2020)

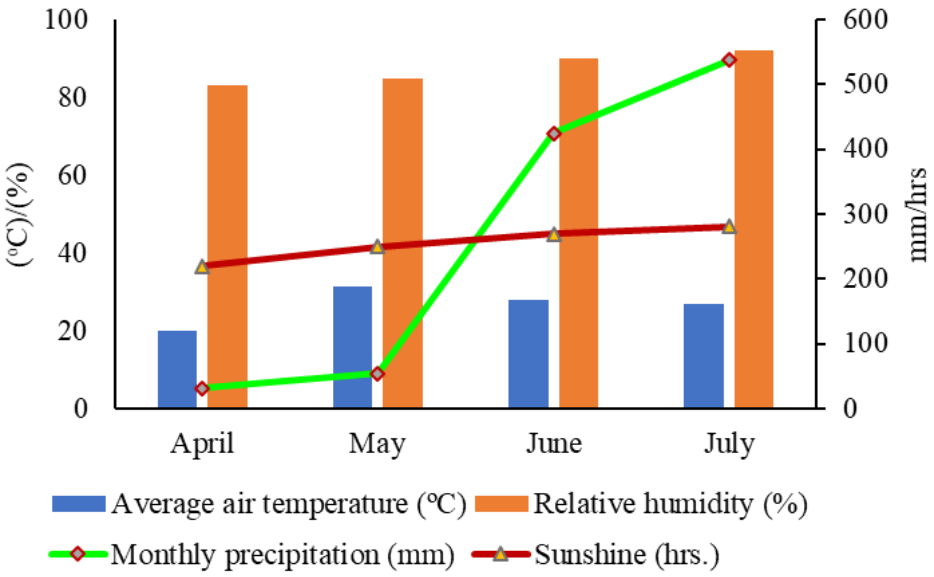

Figure 1. *The experimental site weather data during the period from April to July 2020. Source: Meteorological Station, Wheat Research Center, Noshipur, Dinajpur, Bangladesh*

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DOI: http://dx.doi.org/10.15666/aeer/1905_34093429

Table 2. *The status (mean value) of the woodlot tree species in the research field during the study period (n = 12)*

Woodlots species	Plant height (m)	Clean bole height (m)	Base girth (cm)	Bole girth (cm)	Diameter at breast height (cm)
Albiza lebbeck	15.0	7.5	118	97	31.0
Melia azedarach	17.5	10.5	125	95	30.3
Leucaena leucocephala	21.5	15.5	88	82	26.0

Design of research with treatments

The research was designed following two factors split plot and replicated (3) three times of the treatments. Factor A, four production practice treatments, were arranged in the main field, while factor B, three treatments of the organic mulches, were placed in the sub-plot. So, there were twelve (12) treatment combinations. The total number of experimental plots was 36, and all the plots were the same size, 6.25 m^2. The treatments were as follows- factor A: four production systems T_1= sole cropping of cucumber (control), T_2= cucumber *Albizia lebbeck* woodlot agroforestry, T_3= cucumber *Melia azedarach* woodlot agroforestry, T_4= cucumber *Leucaena leucocephala* woodlot agroforestry, while factor B: M_1= no mulch, M_2= ash mulch, M_3= dry water hyacinth mulch. So, the twelve treatment combinations were (T_1M_1) sole cropping cucumber without mulch, (T_1M_2) sole cropping cucumber with ash mulch, (T_1M_3) sole cropping cucumber with dry water hyacinth mulch, (T_2M_1) cucumber *Albizia lebbeck* woodlot agroforestry without mulch, (T_2M_2) cucumber *Albizia lebbeck* woodlot agroforestry with ash mulch, (T_2M_3) cucumber *Albizia lebbeck* woodlot agroforestry with dry water hyacinth mulch, (T_3M_1) cucumber *Melia azedarach* woodlot agroforestry without mulch, (T_3M_2) cucumber *Melia azedarach* woodlot agroforestry with ash mulch, (T_3M_3) cucumber *Melia azedarach* woodlot agroforestry with dry water hyacinth mulch, (T_4M_1) cucumber *Leucaena leucocephala* woodlot agroforestry without mulch, (T_4M_2) cucumber *Leucaena leucocephala* woodlot agroforestry with ash mulch, and (T_4M_3) cucumber *Leucaena leucocephala* woodlot agroforestry with dry water hyacinth mulch.

Land preparation and crop establishment

The soil of the research field was ploughed by a tractor three times to ensure good tillage. A spade was used to plough the edge of the experimental area. Visibly larger clods were pounded into tiny fragments after ploughing the soil, cleaning the debris and other rubbish for the soil to attain a good cropping condition. The experimental concept was followed in the development of the layout, which was then ready for planting. Every sub-plot had two pits; three (3) cucumber seeds were sown on 14th April 2020 in each pit, apart from 20 cm seed to seed distance. The distance between the pit to the pit was 1.8 metre, where the girth of the pit was 75 cm, and the depth was 10 cm. The seeds were later coated in fine soil by hand. During the cropping season, necessary intercultural operations were carried out to ensure proper growth and production. Just one stable seedling was held to develop in each position five to six days after germination, and the others were discarded. Seedlings that were dead, wounded, or frail were substituted with fresh vigour seedlings from the stock stored. As cucumber is a climbing vegetable, trellises were prepared with bamboo and rope fibre with a height of 150 cm all around in

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_34093429

sub-plots. Weed was present in large quantities in the control treatment. In the plots where weeding was needed, it was done three times. Light irrigation was applied just after sowing the seeds. After 45 days of seed germination, organic mulching materials were applied in the experimental sub-plots with 2.54 cm thickness. Green fruits were harvested at 4-5 days intervals when they attained edible, started from 18th June and was continued up to 16th July.

Data recording parameters

For the growth and production of cucumber, the data was collected in the following heads plant height (cm), leaves plant^{-1}, leaf length & breadth (cm), petiole length (cm), stem girth (cm), main stem internode length (cm), lateral shoots plant^{-1}, fruits plant^{-1}, fruit height (cm), fruit girth (cm), average fruit weight (g), and fruit yield (ton/ha). Additionally, the cost of production, gross and net returns per hectare and the benefit-cost ratio was calculated for economic analysis of cucumber under *Albizia lebbeck*, *Melia azedarach,* and *Leucaena leucocephala* woodlots agroforestry and sole cropping cultivation.

Statistical analysis

Two-factors "Analysis of Variance" (ANOVA) was done using the computer-based MSTAT-C statistical data analysis software. The mean data of treatments and combined effects were separated by the (DMRT) Duncan's Multiple Range Test (Gomez and Gomez, 1984).

Results

The effect of cucumber production systems on growth, yield contributing characters and yield

Cucumber is grown excellently in association with *Albizia lebbeck*, *Melia azedarach,* and *Leucaena leucocephala* woodlots compared to sole cropping (control treatment) (*Table 3*). At the very initial stage (15 DAS), the tallest plant (38.11 cm) was observed in cucumber under *Leucaena leucocephala* (T_4) which was identical to (T_2= 35.67 cm) and (T_3= 34.22 cm). The shortest plant (23.44 cm) was found in the sole cropping of cucumber (T_1). At 30 DAS, the tallest plant (171.60 cm) was recorded in (T_4) cucumber under *Leucaena leucocephala,* which was similar to (T_3= 169.70 cm), and the shortest plant (138.70 cm) was originated in (T_1) sole cropping of cucumber. Finally, the tallest plant (310.40 cm) was noted in T_4, and the shortest plant (260.00 cm) was recorded in T_1 at 45 DAS. The leaves plant^{-1} was perceived significantly maximum (7.33, 44.00, and 93.22 at 15, 30, and 45 DAS) in open field condition (T_1) and the minimum number of leaf plant^{-1} (5.89, 23.67, and 61.44 at 15, 30, and 45 DAS) in cucumber under *Leucaena leucocephala* (T_4). For leaf length of cucumber, the highest leaf length (8.12, 12.96, and 17.87 cm at 15, 30, and 45 DAS) was taken from (T_2) cucumber under *Albizia lebbeck* based agroforestry practice, besides the lowest leaf length (6.53, 12.10, and 15.97 cm at 15, 30 and 45 DAS) was measured from (T_1) sole cropping of cucumber. The leaf breadth was significantly varied due to different woodlots agroforestry practices along with mono-cropping of cucumber. The leaf breadth was calculated the highest (8.83, 15.79, and 22.58 cm at 15, 30, and 45 DAS) in (T_3) cucumber under *Melia azedarach*. In contrast, the lowest leaf breadth (6.63, 13.14, and 18.97 cm at 15, 30 and 45 DAS) in (T_2) cucumber under *Albizia lebbeck*.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_34093429

Table 3. *The effect of cucumber production practices on plant height, number of leaf plant^{-1}, leaf length, and breadth at different DAS*

Treatments	Plant height (cm)			Leaf plant^{-1}			Leaf length (cm)			Leaf breadth (cm)		
	15 DAS	30 DAS	45 DAS	15 DAS	30 DAS	45 DAS	15 DAS	30 DAS	45 DAS	15 DAS	30 DAS	45 DAS
T_1	23.44b	138.7c	260.0d	7.33a	44.00a	93.22a	6.53b	12.10b	15.97c	6.83b	14.12b	20.07b
T_2	35.67a	153.8b	289.3b	6.22ab	28.89b	71.44b	8.12a	12.96a	17.87a	6.63b	13.14c	18.87c
T_3	34.22a	169.7a	270.7c	5.94b	25.00bc	65.11c	7.86a	12.62ab	17.06b	8.83a	15.79a	22.58a
T_4	38.11a	171.6a	310.4a	5.89 b	23.67c	61.44d	6.80b	12.21b	16.00c	8.07a	14.40b	19.96b
CV%	14.65	9.72	2.5	19.63	15.08	2.63	14.27	4.59	3.12	10.91	6.64	2.53

In the column, figures with similar letters or without letters do not differ significantly at the P ≤ 5% level by DMRT

The results of petiole length, stem girth, main stem internode length, and the number of lateral shoots plant^{-1} varied significantly because of different production practices presented in *Table 4*. Notably, the highest petiole length (19.23 cm) was found in cucumber under *Leucaena leucocephala* (T_4), and the lowest petiole length (14.74 cm) was observed in (T_1) sole cropping of cucumber. The stem girth (2.66 cm) was the maximum recorded in (T_4) cucumber under *Leucaena leucocephala*, followed by (2.52 cm) in (T_1). In contrast, the lowest stem girth (2.23 cm) was recorded in (T_2) cucumber under *Albizia lebbeck,* similar to (2.27 cm) in (T_3). The sole cropping cucumber (T_1) gave the highest (5.43 cm) internode length, which was identical to T_2 and T_3, then the lowest (4.31 cm) internode length was calculated in T_4. In the case of the number of lateral shoots, the maximum (8.78) was counted in sole cropping of cucumber (T_1), and the lowest (4.67) was determined in (T_3) cucumber under *Melia azedarach*.

Table 4. *The effect of cucumber production practices on petiole length, stem girth, main stem internode length, and the number of lateral shoots plant^{-1}*

Treatments	Petiole length (cm)	Stem girth (cm)	Main stem internode length (cm)	Number of lateral shoots plant^{-1}
T_1	14.74 d	2.52 a	5.43 a	8.78 a
T_2	18.83 b	2.23 b	4.84 ab	6.67 b
T_3	16.58 c	2.27 b	5.67 a	4.67 c
T_4	19.23 a	2.66 a	4.31 b	4.78 c
CV%	2.01	8.97	13.67	14.38

In the column, figures with similar letters or without letters do not differ significantly at the P ≤ 5% level by DMRT

The number of fruits plant^{-1} was statistically significant by the effect of different cucumber production systems (*Table 5*). Significantly, the highest number of fruits plant^{-1} (10.33) was recorded in the open field, i.e. sole cropping of cucumber (T_1) and the lowest number of fruits plant^{-1} (5.56) was discovered in (T_3) cucumber under *Melia azedarach* based agroforestry practice. Moreover, cucumber fruit length was noted statistically highest (20.02 cm) in (T_2) cucumber under *Albizia lebbeck* based agroforestry practice followed by (T_1) and (T_4) treatments. In contrast, the lowest (17.89 cm) was found in (T_3) cucumber under *Melia azedarach*. The same trend was found for fruit girth as well. The

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
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maximum fruit weight (361.9 g) was calculated in cucumber under *Albizia lebbeck* (T_2) followed by (335.8 g) in T_1 treatment; the minimum (217.8 g) fruit weight was taken in cucumber under *Melia azedarach* (T_3). Furthermore, Fruit yield was measured as the highest (3.56 kg/plot) in (T_2) cucumber under *Albizia lebbeck* followed by T_1 (3.17 kg/plot) and the lowest (1.67 kg/plot) in (T_3) cucumber under *Melia azedarach*. The fruit yield (5.70 tons/ha) was the maximum among the different cucumber production practices in the (T_2) treatment showed in *Fig. 2*.

Table 5. *The effect of cucumber production practices on fruits per plant, fruit length, fruit weight, and fruit yield*

Treatments	Fruits per plant	Fruit length (cm)	Fruit girth (cm)	Average fruit weight (g)	Fruit yield (kg/plot)
T_1	10.33 a	19.11 ab	14.32 ab	335.8 ab	3.17 ab
T_2	9.11 b	20.02 a	15.57 a	361.9 a	3.56 a
T_3	5.56 c	17.89 b	12.03 b	217.8 c	1.67 c
T_4	6.11 c	18.07 ab	14.50 ab	312.7 b	2.96 b
CV%	14.99	10.44	12.88	11.32	13.77

In the column, figures with similar letters or without letters do not differ significantly at the P ≤ 5% level by DMRT

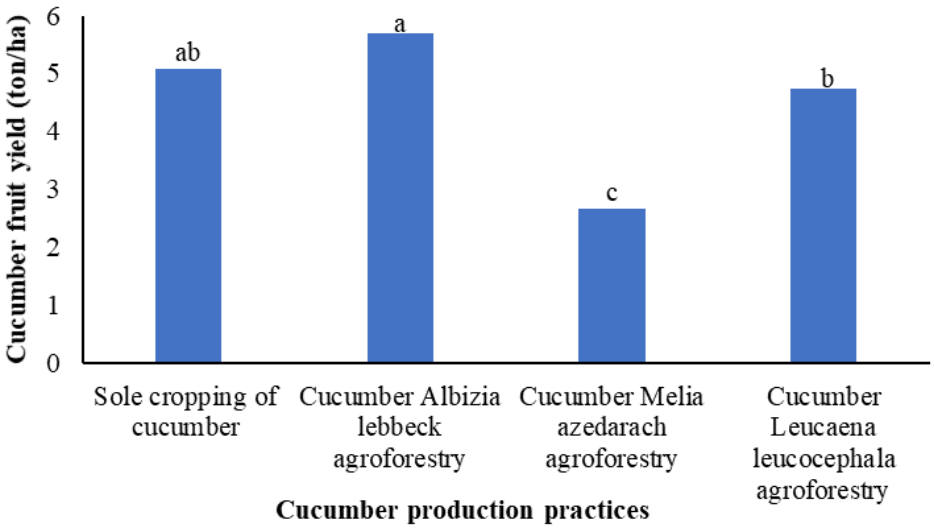

Figure 2. *The effect of cucumber production practices on the fruit yield (ton/ha). In the bar, figures with similar letters or without letters do not differ significantly at the P ≤ 5% level by DMRT*

The effect of organic mulch on the cucumber growth, yield contributing characters and yield

The effect of organic mulch on cucumber plant height, leaf plant^{-1}, leaf length, and breadth was statistically significant; results were shown in *Table 6*. Numerically, the tallest plant (35.33 cm) was recorded in M_2 (ash mulch), and the shortest plant height (31.58 cm) was observed in M_1 (no mulch) control treatment at 15 DAS. At 30 DAS, significantly, the tallest plant (178.8 cm) was noted in M_3 (dry water hyacinth mulch) treatment followed by

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_34093429

M_1 (no mulch), while the shortest plant height (140.3 cm) was noticed in M_2 (ash mulch) treatment. Finally, at 45 DAS, considerably the tallest plant (343.6 cm) was recorded in M_3 (dry water hyacinth mulch) treatment followed by M_1 (no mulch), whereas the shortest plant (228.5 cm) was taken in M_2 (ash mulch) treatment. The leaves plant^{-1} was showed insignificant at 15 and 30 DAS. The maximum leaves plant^{-1} (79.83) was counted in M_2 (ash mulch) treatment, and the minimum leaves plant^{-1} (65.00) was taken in M_1 (no mulch) treatment at 45 DAS respectively. Cucumber leaf length was maximum (7.79 cm, 12.85 cm, and 19.03 at 15, 30 and 45 DAS) recorded in M_3 (dry water hyacinth mulch) followed by M_2 (ash mulch) treatment. On the other hand, minimum leaf length (6.44 cm, 11.89 cm, and 15.83 at 15, 30 and 45 DAS) was found in M_1 (no mulch) control treatment. The same trend of results was observed for leaf breadth at 15, 30 and 45 DAS.

Table 6. *The effect of organic mulch on cucumber plant height, leaf plant^{-1}, leaf length and breadth at different DAS*

Treatments	Plant height (cm)			Leaf plant^{-1}			Leaf length (cm)			Leaf breadth (cm)		
	15 DAS	30 DAS	45 DAS	15 DAS	30 DAS	45 DAS	15 DAS	30 DAS	45 DAS	15 DAS	30 DAS	45 DAS
M_1	31.58	156.2b	275.8b	6.75	30.92	65.00c	6.44b	11.89b	15.83b	6.13b	13.13b	19.01b
M_2	35.33	140.3c	228.5c	6.50	32.00	79.83a	7.75a	12.85a	15.30c	8.03a	14.98a	18.15c
M_3	31.67	178.8a	343.6a	5.75	28.25	73.58b	7.79a	12.68a	19.03a	8.61a	14.88a	23.94a
CV%	14.65	9.72	2.5	19.63	15.08	2.63	14.27	4.59	3.12	10.91	6.64	2.53

In the column, figures with similar letters or without letters do not differ significantly at the $P \leq 5\%$ level by DMRT

The effect of organic mulch on cucumber petiole length, stem girth, main stem internode length, and the number of lateral shoot plant^{-1} was statistically meaningful, and the results are provided in *Table 7*. The highest petiole length (19.38 cm) was noted in M_3 (dry water hyacinth mulch) followed by (16.48 cm) in M_1 (no mulch) treatment, and the lowest petiole length (16.18 cm) was detected in M_2 (ash mulch) treatment. The result of cucumber stem girth was analyzed statistically insignificant because of different organic mulches. The main stem internode length was calculated (5.54 cm), the maximum in M_2 (ash mulch) treatment and the minimum (4.78 cm) in M_3 (dry water hyacinth mulch) treatment similar to M_1 (4.87 cm), respectively. Organic mulch affected the lateral shoot plant$^{-1;}$ as such, the highest lateral shoot plant^{-1} (7.17) was recorded in M_2 (ash mulch) treatment which was identical (6.75) in M_1 treatment. Then again, the lowest number of lateral shoot plant^{-1} (4.75) was observed in M_3 (dry water hyacinth mulch).

Table 7. *The effect of organic mulch on cucumber petiole length, stem girth, main stem internode length and number of lateral shoots plant^{-1}*

Treatments	Petiole length (cm)	Stem girth (cm)	Main stem internode length (cm)	Number of lateral shoots plant^{-1}
M_1	16.48 b	2.43	4.87 b	6.75 a
M_2	16.18 c	2.37	5.54 a	7.17 a
M_3	19.38 a	2.47	4.78 b	4.75 b
CV%	2.01	8.97	13.67	14.38

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_34093429

The cucumber fruits per plant were varied as influenced by organic mulch (*Table 8*). Significantly the highest fruits per plant (11.75) was found in M_3 (dry water hyacinth mulch) treatment which was statistically identical to (11.17) M_2 (ash mulch). Instead, the lowest fruits per plant (4.17) was observed in M_1 (no mulch) control treatment. The fruit length of cucumber was also varied significantly as directly influenced by organic mulch. Substantially, the most extended fruit (21.78 cm) was found in M_3 (dry water hyacinth mulch) treatment, and the shortest fruit length (17.09 cm) was observed in M_1 (no mulch) control treatment which was followed by M_2 (ash mulch) treatment. Fruit girth of cucumber was also varied significantly, the highest fruit girth (16.22 cm) was measured in M_3 (dry water hyacinth mulch) treatment, and the lowest (13.14 cm) fruit girth was taken from M_1 (no mulch) control treatment similar to M_2 (ash mulch). The highest average fruit weight (377.1 g) was recorded in M_3 (dry water hyacinth mulch) treatment, and the lowest average fruit weight (303.6 g) was recorded in M_1 (no mulch) control treatment which was followed by M_2 (ash mulch). The highest cucumber fruit yield (4.38 kg/plot) was observed in M_3 (dry water hyacinth mulch) treatment followed by (3.53 kg/plot) in M_2 (ash mulch) treatment whereas, the lowest fruit yield (2.65 kg/plot) was detected in M_1 (no mulch) control treatment. The fruit yield (7.01 tons/ha) was the highest found in M_3 (dry water hyacinth mulch) treatment compared to other treatments, and the lowest (4.24 tons/ha) was observed in M_1 (no mulch) control treatment (*Fig. 3*).

Table 8. *The effect of organic mulch on the number of cucumber fruits per plant, fruit length, fruit weight and fruit yield*

Treatments	Fruits per plant	Fruit length (cm)	Fruit girth (cm)	Average fruit weight (g)	Fruit yield (kg/plot)
M_1	4.17 b	17.09 b	13.14 b	303.6 b	2.65 b
M_2	11.17 a	17.44 b	13.21 b	322.7 b	3.53 ab
M_3	11.75 a	21.78 a	16.22 a	377.1 a	4.38 a
CV%	14.99	10.44	12.88	11.32	13.77

In the column, figures with similar letters or without letters do not differ significantly at the P ≤ 5% level by DMRT

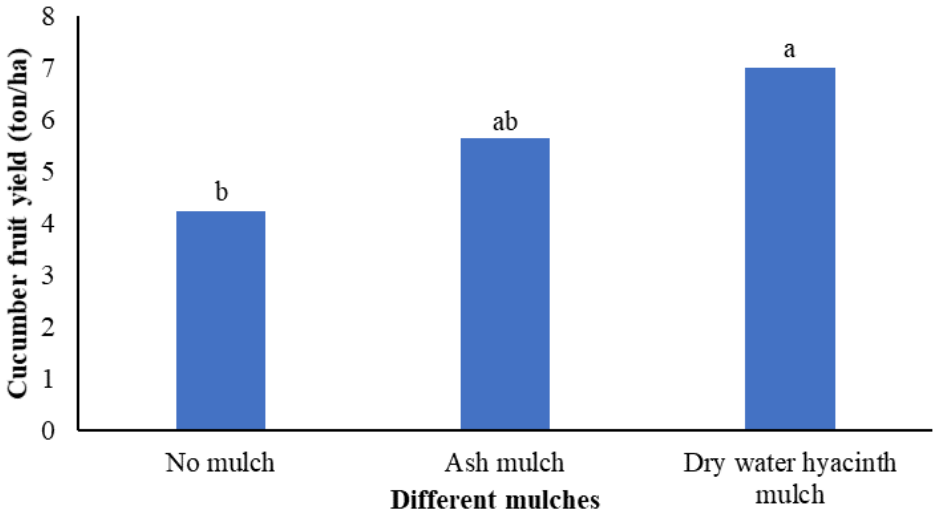

Figure 3. *The effect of organic mulch on the cucumber fruit yield (ton/ha). In the bar, figures with similar letters or without letters do not differ significantly at the P ≤ 5% level by DMRT*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_34093429

The combined effect of cucumber production systems and organic mulch on the growth, yield contributing characters and yield

The combined effect of cucumber production practices and organic mulch on the plant height, leaves plant^{-1}, leaf length, and breadth cucumber was significantly different at 15, 30 and 45 DAS (*Table 9*). The tallest plant (42.33 cm) was observed in T_3M_2 treatment combination followed by T_4M_1 treatment combination, and the shortest plant (20.67 cm) was recorded in T_2M_3 treatment combination at 15 DAS. In the next, at 30 and 45 DAS, the tallest plant (195.00 cm and 441.70 cm) was noted in T_1M_3 treatment combination, whereas the shortest plant (117.30 cm) at 30 DAS in T_2M_2 treatment combination and (204.3 cm) at 45 DAS in T_4M_2 treatment combination. The leaves plant^{-1} at 15 DAS significantly the maximum (7.67) was documented in T_1M_1 and T_1M_2 treatment combinations and the minimum (5.00) in T_3M_3 and T_4M_3 treatment combinations. After that, at 30 DAS, the maximum leaves plant^{-1} (47.33) was calculated in T_1M_3 treatment combination followed by T_1M_1 and T_1M_2 and the minimum leaves plant^{-1} (16.00) T_3M_3 treatment combination. Finally, at 45 DAS, the maximum leaves plant^{-1} (93.70) was observed in T_1M_3 treatment combination, and the minimum leaves plant^{-1} (55.33) was taken in T_4M_3 treatment combination. Leaf length (9.75 and 13.30 cm at 15 and 30 DAS) the highest was noted in T_3M_2 treatment combination, and the lowest leaf length (6.00 and 11.73 cm at 15 and 30 DAS) was found in T_3M_1 and T_1M_2 treatment combination. Finally, at 45 DAS, the highest leaf length (22.60 cm) was collected in T_1M_3 treatment combination, and the lowest leaf length (13.70 cm) was detected in T_1M_1 treatment combination. In the initial stage at 15 DAS, significantly, the highest leaf breadth (10.00 cm) was recorded in T_4M_3 treatment combination, and the lowest leaf breadth (5.37 cm) was measured in T_3M_1 treatment combination. Moreover, at 30 DAS, the highest leaf breadth (16.60 cm) was observed in T_3M_2 treatment combination, whereas the lowest leaf breadth (11.97 cm) was found in T_1M_1 treatment combination. At 45 DAS, the highest leaf breadth (26.47 cm) was calculated in T_1M_3 treatment combination, and the lowest leaf breadth (16.77 cm) was measured in T_1M_1 treatment combination.

The combined effect of cucumber production systems and organic mulch was significantly varied for petiole length, stem girth, main stem internode length, and the number of lateral shoots plant^{-1}; the results epitomized in *Table 10*. The highest petiole length (23.70 cm) was recorded in T_2M_3 treatment combination, and the lowest petiole length (12.93 cm) was calculated in the T_3M_3 treatment combination. The stem girth (2.80 cm) was the maximum collected from T_4M_3 treatment combination, and the minimum (1.97 cm) stem girth was obtained from T_2M_2 treatment combination. The main stem internode length influenced the combined effect of cucumber production systems and organic mulch. The most extended main stem internode length (7.00 cm) was found in T_3M_2 treatment combination followed by (6.03 cm) and observed in the T_1M_3 treatment combination. The shortest main stem internode length (3.87 cm) was recorded in T_4M_3 treatment combination, which was statistically identical to (4.27 cm) found in T_4M_1 treatment combination, respectively. The number of lateral shoots plant^{-1} was the maximum (10.33) was recorded in T_1M_1 treatment combination whereas, the lowest number of lateral shoots plant^{-1} (2.67) was noted in both T_4M_3 and T_3M_3 treatment combinations.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_34093429

Table 9. *The combined effect of cucumber production practices and organic mulch on plant height, number of leaf plant^{-1}, leaf length, and breadth at different DAS*

Treatment combinations	Plant height (cm)			Number of leaf plant^{-1}			Leaf length (cm)			Leaf breadth (cm)		
	15 DAS	30 DAS	45 DAS	15 DAS	30 DAS	45 DAS	15 DAS	30 DAS	45 DAS	15 DAS	30 DAS	45 DAS
T_1M_1	32.00 a-d	154.0cd	267.3de	7.67a	40.67ab	88.67b	7.00b-d	11.97b	13.70h	6.53bc	11.97f	16.77f
T_1M_2	40.67ab	165.7bc	222.3h	7.67a	44.00a	57.33f	6.27d	11.73b	14.87g	6.37bc	12.70ef	16.97f
T_1M_3	34.33a-d	195.0a	441.7a	6.67ab	47.33a	93.7a	7.13b-d	12.60ab	22.60a	7.60b	14.77b-d	26.47a
T_2M_1	25.33b-d	151.7c-e	279.0d	6.67ab	35.00bc	85.67b	6.07d	11.93b	17.23cd	5.77c	13.53c-f	21.43c
T_2M_2	24.33cd	117.3f	235.0g	5.67ab	22.33e-g	62.33e	6.67cd	12.83ab	16.17ef	6.57bc	15.6oab	20.13d
T_2M_3	20.67d	192.3ab	354.0b	6.33ab	29.33c-e	66.33d	6.87b-d	11.87b	20.20b	7.57b	13.23d-f	26.17a
T_3M_1	28.00 a-d	126.7d-f	295.0c	6.33ab	23.33d-g	78.33c	6.00d	11.83b	17.00de	5.37c	14.20b-e	18.10e
T_3M_2	42.33a	154.0cd	252.3f	6.33ab	31.67cd	78.00c	9.57a	13.30a	15.50fg	9.57a	16.60a	17.97e
T_3M_3	32.33a-d	135.3d-f	232.7gh	5.00b	16.00g	39.00g	8.80ab	12.73ab	15.40fg	9.27a	16.57a	20.53d
T_4M_1	41.00ab	192.3ab	261.7ef	6.33ab	24.67d-f	66.67d	6.70cd	11.83b	15.40fg	6.87bc	12.83ef	19.73d
T_4M_2	34.00a-d	124.0ef	204.3i	6.33ab	30.00c-e	62.33e	8.50a-c	13.53a	14.67g	9.63a	15.03abc	17.53ef
T_4M_3	39.33a-c	192.7ab	346.0b	5.00b	20.33fg	55.33f	8.37a-c	13.50a	17.93c	10.00a	15.33ab	22.60b
CV%	14.65	9.72	2.5	19.63	15.08	2.63	14.27	4.59	3.12	10.91	6.64	2.53

In the column, figures with similar letters or without letters do not differ significantly at the P ≤ 5% level by DMRT

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Table 10. *The combined effect of cucumber production practices and organic mulch on petiole length, stem girth, main stem internode length and number of lateral shoots plant^{-1}*

Treatment combinations	Petiole length (cm)	Stem girth (cm)	Main stem internode length (cm)	Number of lateral shoots plant^{-1}
T_1M_1	15.87 e	2.50 a-c	4.97 ab	10.33 a
T_1M_2	17.70 c	2.57 a-c	5.30 ab	8.00 b
T_1M_3	22.93 b	2.50 a-c	6.03 ab	8.00 b
T_2M_1	17.93 c	2.40 a-c	4.83 ab	7.67 b
T_2M_2	16.07 e	1.97 d	5.07 ab	6.67 c
T_2M_3	23.70 a	2.33 b-d	4.63 b	5.67 c
T_3M_1	14.33 f	2.17 cd	5.40 ab	5.33 c
T_3M_2	16.97 d	2.40 a-c	7.00 a	6.00 c
T_3M_3	12.93 g	2.23 b-d	4.60 b	2.67 d
T_4M_1	17.80 c	2.63 ab	4.27 b	3.67 d
T_4M_2	14.00 f	2.53 a-c	4.80 ab	8.00 b
T_4M_3	17.93 c	2.80 a	3.87 b	2.67 d
CV%	2.01	8.97	13.67	14.38

In the column, figures with similar letters or without letters do not differ significantly at the $P \leq 5\%$ level by DMRT

The number of cucumber fruits per plant was significantly influenced by the combined effect of production practices and organic mulch (*Table 11*). Significantly the highest number of fruits per plant (12.33) was recorded in T_1M_3 treatment combination, which was followed by (11.67) in T_2M_3 and (11.33) in T_1M_2 treatment combination. In contrast, the lowest number of fruits per plant (4.33) was documented in T_3M_1 treatment combination. The fruit length of cucumber was found significantly different, significantly the most extended fruit length (23.33 cm) was found in T_2M_3 treatment combination, which was identical to T_1M_3 treatment combination (22.17 cm), and the shortest fruit length (15.80 cm) was recorded in T_3M_1 treatment combination. Similarly, the longest fruit girth (15.83 cm) was found in both T_1M_3, and T_2M_3 treatment combinations, which was identical to T_1M_2 treatment combination (14.87 cm) and the shortest fruit girth (10.30 cm) was recorded in T_3M_1 treatment combination. Average fruit weight was significantly varied, the highest fruit weight (395.30 g) was measured in T_2M_3 treatment combination, which was statistically identical to (373.30 cm) in T_2M_2 treatment combination and (364.70 cm) in T_1M_3 treatment combination and the lowest fruit weight (212.70 g) was recorded in T_3M_1 treatment combination. The fruit yield of cucumber was significantly different because of the combined effect of production systems and organic mulch. Notably, the highest cucumber fruit yield (5.72 kg/plot) was found in T_2M_3 treatment combination, which was indistinguishable from (5.25 kg/plot) in T_1M_3 treatment combination, and the lowest cucumber fruit yield (1.86 kg/plot) was noted in T_3M_1 treatment combination. Furthermore, *Figure 4* provided the cucumber fruit yield (ton/ha). The result showed that the highest cucumber fruit yield (9.15 ton/ha) was observed in T_2M_3 followed by (8.40 tons/ha) in T_1M_3 treatment combination, and the lowest cucumber fruit yield (2.98 tons/ha) was received in T_3M_1 treatment combination (*Fig. 4*).

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Table 11. *The combined effect of cucumber production practices and organic mulch on fruits per plant, fruit length, fruit weight and fruit yield*

Treatment combinations	Fruits per plant	Fruit length (cm)	Fruit girth (cm)	Average fruit weight (g)	Fruit yield (kg/plot)
T_1M_1	6.67 cd	19.30 bc	13.60 b	294.1bc	2.93 cd
T_1M_2	11.33 ab	17.43 c	14.87 ab	328.7 b	4.85 ab
T_1M_3	12.33 a	22.17 ab	15.83 a	364.7 ab	5.25 a
T_2M_1	5.12 d	17.17 c	11.98 cd	256.7 c	3.31 c
T_2M_2	10.67 b	17.00 c	14.21ab	373.3 ab	4.31 b
T_2M_3	11.67 ab	23.33 a	15.83 a	395.3 a	5.72 a
T_3M_1	4.33 e	15.80 d	10.30 d	212.7 d	1.86 d
T_3M_2	5.43 d	16.97 c	11.37cd	271.2 bc	2.18 cd
T_3M_3	6.38 cd	19.73 bc	12.43 c	285.4 bc	2.47 cd
T_4M_1	6.33 cd	17.50 cd	12.83 c	246.9 c	2.89 cd
T_4M_2	7.67 c	16.97 c	13.37 b	312.3 b	3.77 c
T_4M_3	9.33 b	20.90 b	13.95 b	343.6 b	3.88 c
CV%	14.99	10.44	12.88	11.32	13.77

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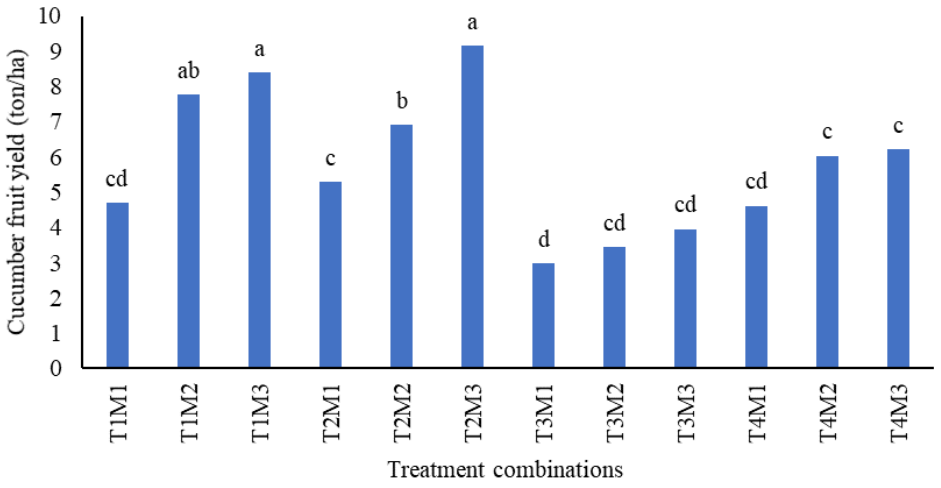

Figure 4. *The combined effect of cucumber production practices and organic mulch on the fruit yield (ton/ha). In the bar, figures with similar letters or without letters do not differ significantly at the P ≤ 5% level by DMRT*

Cost and benefit analysis of cucumber production under Albizia lebbeck, Melia azedarach, and Leucaena leucocephala woodlot-based agroforestry practices and sole cropping

The cost of production, gross & net return, and benefit-cost ratio of cucumber grown under *Albizia lebbeck*, *Melia azedarach*, *Leucaena leucocephala* woodlots agroforestry practices and sole cropping cucumber were calculated according to market prices, and results were displayed in *Table 12* and *Appendix 1*. The values in *Appendix 1* indicated that the total cost of production was the highest (US$ 2342.00 per ha) found in (T_2)

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DOI: http://dx.doi.org/10.15666/aeer/1905_34093429

cucumber under *Albizia lebbeck* based agroforestry practice followed by (T_4) cucumber under *Leucaena leucocephala* based agroforestry practice (US$ 2318.00 per ha). The lowest cost of production was observed in (T_1) cucumber sole cropping (US$ 1892.00 per ha). The highest value of the gross return (US$ 6757.00 per ha) was obtained from (T_2) cucumber under *Albizia lebbeck* based agroforestry practice. In contrast, the lowest value of the gross return (US$ 2095.00 per ha) was acquired from (T_1) cucumber sole cropping. The net return (US$ 4415.00 per ha) was comparatively the highest taken in (T_2) cucumber under *Albizia lebbeck* based agroforestry practice. Simultaneously, the lowest net return (US$ 203.00 per ha) was received from (T_1) cucumber sole cropping. The highest benefit-cost ratio (2.89) was recorded in (T_2) cucumber under *Albizia lebbeck* based agroforestry practice, followed by (2.43) in (T_3) cucumber under *Melia azedarach* based agroforestry practice, and the lowest benefit-cost ratio (1.11) was noted in (T_1) cucumber sole cropping.

Table 12. *The gross return, the net return, and benefit-cost ratio (BCR) of cucumber production under Albizia lebbeck, Melia azedarach, Leucaena leucocephala woodlot-based agroforestry systems and sole cropping*

Treatment	Return (US$/ha)				Gross return ($/ha)	Total cost of production ($/ha	Net return ($/ha	BCR
	Cucumber	*Albizia lebbeck*	*Melia azedarach*	*Leucaena leucocephala*				
T_1	2095.00	……	……	……	2095.00	1892.00	203	1.11
T_2	2345.00	4412.00	……	……	6757.00	2342.00	4415	2.89
T_3	1100.00	……	4471.00		5571.00	2289.00	3282	2.43
T_4	1950.00	……	……	3529.00	5479.00	2318.00	3161	2.36

Note: Market price of cucumber fruit (US$ 0.41/kg), *Albizia lebbeck* (US$ 4.71/Tree/Year), *Melia azedarach* (US$ 5.29/Tree/Year), *Leucaena leucocephala* (US$ 4.11/Tree/Year)

Discussion

The research indicated that the commercial vegetable cucumber would perform significantly as an intercrop under different woodlots-based agroforestry and sole cropping influenced by organic mulch. The cucumber growth was found more prominent under partially shaded conditions compared to open field conditions. The reason for the outcome can be summarized as follows:

The *Albizzia lebbeck* tree provides adequate shade, which aids in retaining water in the cucumber during harvesting. On the other hand, this tree acts as a soil binding agent, which aids in preserving soil properties. Furthermore, the study shows that water hyacinth has a synergistic interaction with *Albizzia lebbeck*. The water hyacinth combination was apparent not only with *Albizzia lebbeck* but also with other trees. Such a result was not observed with the control group. The reason for this is that in a scorching climate, the water hyacinth dries up without shade and is thus unable to show its impact. Mainly the vegetative growth was boosted in cucumber under *Leucaena leucocephala* based agroforestry practice because the transpiration rate was lower. Chauhan et al. (2013) found that regardless of the crop used in the experiment, the transpiration (E) intensity of crops was lowest in the shade, resulting in higher water usage performance in the shade than in the open condition. Furthermore, the dry water hyacinth mulch also enhanced the growth of cucumber effectively. Vidya and Girish (2014) reported that water hyacinth

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
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application in crop fields acted as a facilitator for the crop's growth and development and improved soil fertility.

Moreover, Yaghi et al. (2013) deduced that cucumber production could be enhanced by applying plastic mulch and drip irrigation techniques, although plastic mulching is harmful to the environment. Hence, it would be more environmentally sound to use organic mulch for cucumber production.

For the cucumber reproductive growth, i.e. fruit length, girth, weight, and yield, we found the highest outcome in cucumber under *Albizia lebbeck* based agroforestry practice. Pervin et al. (2015) mentioned that mustard oil was cropped successfully with increased production under the *Albizia lebbeck* wood producing tree agroforestry system. According to Oyebamiji et al. (2017), *Albizia lebbeck* deciduous timber tree had a high potential to improve annual vegetable yield. Lenhard et al. (2013) reported that plants below 70% of shading had higher total rates of chlorophyll, leaf area and weight ratios. The findings showed that both the ash mulch and dry water hyacinth mulch had performed a crucial, active substantial function for cucumber production. According to Ranjan et al. (2017), organic materials would raise soil nutrients, preserves optimal soil temperature, and enhances the physical, chemical, and biological properties of soil.

The cucumber under Albizia lebbeck agroforestry practice with dry water hyacinth mulch combinedly create a valuable production opportunity in the present research. As compared to the conventional cucumber production practice with an average (6.83 ton/ha) fruit yield production (Annon, 2018), our study finds a 25% higher cucumber fruit yield (9.15 ton/ha) (*Fig. 5*).

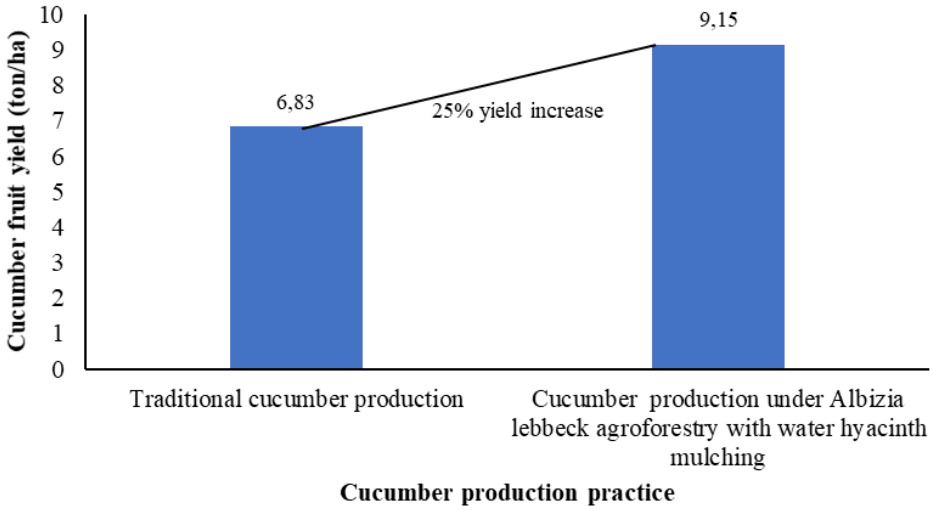

Figure 5. *Cucumber fruit yield (ton/ha) comparison between conventional practice and the present research practice*

From the economic point of view, in the research findings, we found the benefit-cost ratio of cucumber production under *Albizia lebbeck* agroforestry practice (2.89), which was higher than *Melia azedarach* and *Leucaena leucocephala* agroforestry practice along with sole cropping of cucumber. Thus, commercial cucumber production under *Albizia lebbeck* woodlots could bring a considerable return to the cucumber cultivators being the cost-effective solution. Amin et al. (2021) mentioned that the highest benefit-cost ratio (2.14) was found in potato grown under a mango-based agroforestry production system

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that was 20% more than the mono-cropping of crop cultivation. Compared to current farming strategies, this research would come up with a better cost-effective and environmental strategy.

Conclusions

The experimental results reveal that cucumber can be grown successfully under *Albizia lebbeck*, *Melia azedarach,* and *Leucaena leucocephala* woodlots-based agroforestry practice to compare with conventional mono cropping cultivation. Cucumber production was the highest in *Albizia lebbeck* agroforestry practice by applying the dry water hyacinth mulch. This production practice increases yield performance to a higher state than traditional cucumber cultivation. It can be concluded that the cucumber under *Albizia lebbeck* agroforestry practice would enhance the benefit-cost ratio significantly. This production practice is highly beneficial to cucumber cultivating farmers. Furthermore, it is recommended to test cucumber in the vacant space of fruit and forestry tree-based agroforestry practice in future assessment with other kinds of organic mulch.

Acknowledgements. The authors would like to convey optimistic gratefulness to the Agroforestry and Environment Department of HSTU, Dinajpur-5200, Bangladesh. The authors would like to take the opportunity to express their heartiest respect, deepest gratitude, and profound appreciation to all the Faculty of Environmental Management staff (PSU) Prince of Songkla University. They also appreciate the Research and Development section (RDO) of Prince of Songkla University, HatYai, 90110, Songkhla, Thailand. The authors would like to thank Arup Kumar Biswas, deputy director of the Ministry of Power, Energy, and Mineral Resources of the People's Republic of Bangladesh, to assist with the English.

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DOI: http://dx.doi.org/10.15666/aeer/1905_34093429

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_34093429

APPENDIX

Appendix 1. *The production cost of cucumber under Albizia lebbeck, Melia azedarach, and Leucaena leucocephala woodlot-based agroforestry systems along with sole cropping (average of one year)*

Treatment	Input cost									Total input cost (US$/ha)	Overhead cost			Total cost of production (US$ /ha)
	Non-material cost (US$/ha)			Material cost (US$/ha)							Interest of input cost @ 8% for the crop season (US$/ha)	Interest of the value of land (US$ 3529/ha/year) @ 8% for the crop season (US$ /ha)	Miscellaneous cost @ 5% of the input cost (US$ /ha)	
	Trees	Cucumber production	Total non-material cost	Seed	Jute rope	Bamboo stick	Maintenance cost of trees	Initial plantation cost of trees	Total material cost					
T_1	……	945.00	954.00	288.00	78.00	105.00	……	……	471.00	1425.00	114.00	282.00	71.00	1892.00
T_2	168.00	945.00	1122.00	288.00	78.00	105.00	59.00	171.00	701.00	1823.00	146.00	282.00	91.00	2342.00
T_3	169.00	945.00	1123.00	288.00	78.00	105.00	59.00	123.00	653.00	1776.00	142.00	282.00	89.00	2289.00
T_4	171.00	945.00	1125.00	288.00	78.00	105.00	59.00	147.00	677.00	1802.00	144.00	282.00	90.00	2318.00

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_34093429

ALLELOPATHIC EFFECTS OF BLACK NIGHTSHADE (*SOLANUM NIGRUM* L.) ON GERMINATION, GROWTH AND YIELD OF BROAD BEAN (*VICIA FABA* L.) AND COMMON BEAN (*PHASEOLUS VULGARIS* L.)

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(Received 13th Apr 2021; accepted 19th Jul 2021)

Abstract. Germination tests and pot experiments were conducted to explore the allelopathic effect of aqueous extracts of *S. nigrum* on germination, growth and yield of *Vicia faba* (L.) and *Phaseolus vulgaris* (L.). Ten treatments including control were prepared by using extracts obtained from different parts of *S. nigrum,* including roots, leaves, and seeds at 3 concentrations (10, 40, and 60%) to conduct the germination test. The pot experiment was studied by incorporating powder of different plant parts into the soil at 3 concentrations (0.5, 1, and 1.5%). The results of the germination test revealed that the aqueous extracts of roots and leaves significantly reduced germination percentage, radicle, and plumule length of both beans. There was a direct negative relationship between germination test and the plant parts used, including their concentrations. However, seed extract showed a non-significant difference ($p > 0.05$). In the pot experiment, different powders of black nightshade incorporated into the soil decrease plant height, root length, fresh and dry weights. However, the number of leaves was not affected. Also, yield components were decreased with the highest concentrations used. The reduction of germination, growth, and yield of the broad and common beans increased with higher concentrations of root and leaf aqueous extracts, while that of seeds had limited effect. The strongest allelopathic effect of broad and common beans was caused by root extracts of the highest concentration.
Keywords: *aqueous extract, germination test, pot experiment, plant height, yield components*

Introduction

Allelopathy is a form of interaction between plants, exerted through a chemical inhibitor produced by a plant helping to intervene in the growth and development of another one (Zeng et al., 2010). These chemicals with allelopathic potential can be released into the environment, under suitable conditions, in sufficient amounts to affect neighboring plants (Tahir, 2011). Allelopathy has beneficial or harmful effects on plants due to the release of allelochemicals which are secondary metabolites, which are present in all plant tissues including leaves, stems, flowers, roots, and seeds (Mohsin et al., 2016).

Solanaceae plants have the ability of allelopathy, and so that the species of plants control the behavior, form, and amount of allelopathic compounds (Mushtaq and Siddiqui, 2018). The genera of this family, *Withania*, *Solanum*, *Iochroma*, *Nicotiana*, and *Datura* are rich sources of sesquiterpenoid phytoalexins (Elakovich, 1987). The toxic alkaloids present in certain species of the family have given it its vernacular name of nightshade. The genus *Solanum* is massive, contains species distributed in many parts of the world. The non-growth or growth depletion of neighboring plants is caused by allelopathic constituents of this genus (Mushtaq and Siddiqui, 2018). The phenomena have been found in the two biologically active glycoalkaloids, solasonine, and

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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solamargine (Fukuhara and Kubo, 2004). *Solanum nigrum* (L.) is a common and troublesome annual weed in many parts of the world, it is one of the most widespread species in Solanaceae family. *S. nigrum* is a common plant due to its toxic content of Solanine, a glycoalkaloid found in most parts of the plant, with the highest concentrations in the unripened berries (Imad et al., 2017). Although it is considered a rich source of one of the most popular plant poisons, it has also proven to be a pharmacologically prospective reservoir of phytochemicals. Leaves and berries of *S. nigrum* are commonly used in South India for the treatment of gastric ulcers, gastritis, and other gastric problems (Hadi et al., 2017; Chen et al., 2009).

Considering the economic importance, and nutritional facts of leguminous crops, broad and common beans in the world especially in developing countries. The article aimed to determine the effect of aqueous extracts obtained from different parts of the black nightshade, at various concentrations, on the germination, growth and yield of common and broad beans cultivated in Egypt, to assess the plant's allelopathic potential.

Materials and methods

Site of experiments

Two experiments were conducted to study the allelopathic effect of different parts of *S. nigrum,* including germination test and pot experiment. The germination test was carried out in the laboratory of Plant Ecology, Faculty of Science, Zagazig University, Egypt. The pot experiment was carried out in the greenhouse of the faculty under field conditions.

Plant collection

Matured black nightshade was collected at the fruiting stage from an agricultural field west of Zagazig University. The plant was separated into roots, leaves, and seeds then oven-dried and ground into a fine powder using mortar. Broad bean (*V. faba*) and common bean (*Ph. vulgaris*) seeds were obtained from Agricultural Research Centre, Egypt.

Chemical analysis

Quantitative estimation of the four major active principles in root, leaf, and seed powders of black nightshade was carried out. Total alkaloid and saponin were determined according to Obadoni and Ochuko (2001). Total flavonoid content was estimated by the method of Srisawat et al. (2010) and tannin according to Edeoga et al. (2005).

Germination test

This experiment was conducted to explore seed germination, length of the radicle, and plumule of beans under the effect of aqueous extract of roots, leaves, and seeds of black nightshade. Aqueous extract of different plant parts was prepared by adding 20 g of the dried part to 800 ml of distilled water, then put in a magnetic stirrer for 24 h. The extracts were filtered (stock solutions) and stored at 4 °C until use later. From each stock solution, three concentrations were prepared by dilution method (10, 40, and 60%), these concentrations were selected depending on a previous study (Stef et al., 2013).

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Ten seeds of both beans were put sparsely in a filter paper covered glass petri dish (10 seeds/petri dish). Each dish was applied by 5 ml aqueous extract of each plant part, whereas 5 ml distilled water was applied to the control. The treatments were arranged with three replicates in a fully randomized system. The petri dishes were kept in the laboratory at room temperature for 10 days, the germinated seeds were counted to calculate the germination percentage, and the length of radicle and plumule were measured.

Pot experiment

The pots used were 30 cm in diameter, and 30 cm high filled with equal amounts of sieved soil (2:1 v/v clay and sand). The soil was incorporated with 10, 20, and 30 g of plant parts powder representing 0.5, 1, and 1.5% (residue/soil, w/w) respectively. Soil without *S. nigrum* residue represented control. Each pot was planted with 5 seeds of broad and common beans. After emergence, the seedlings were thinned to 3 seedlings per pot. The pots were kept free from weeds and irrigated uniformly. Each treatment was replicated 3 times and pots were distributed in a complete randomized design. At 30 days after sowing, growth parameters like plant height, root length, number of leaves, fresh, and dry weights of plants were recorded. At harvest broad and common beans were taken to determine the yield components like the number of pods/plant, number of seeds/pod, and weight of 100 seeds (g).

Statistical analysis

Data were statistically analyzed using SPSS program version 23. The averages of different groups and standard error were calculated. The two-way ANOVA test was performed for comparison between the different studied factors (i.e., Aqueous extract and treatments) and followed by post hoc test using Duncan multiple range (DMR) test for comparisons between means of groups. The means followed by the same letter in each column are not significantly different from each other at the 5% probability level (p value at 0.05).

Results

Chemical analysis of black nightshade revealed the presence of many bioactive secondary metabolites in all parts of this plant, particularly alkaloids which are responsible for suppressing the growth of other plants (*Table 1*).

Table 1. *Chemical constituents in different parts of black nightshade*

Plant parts	Content (%)			
	Alkaloid	Flavonoid	Tannin	Saponin
Roots	1.68	0.73	0.14	0.30
Leaves	1.39	0.88	0.16	0.28
Seeds	0.962	0.49	0.13	0.27

There was no significant difference between the two leguminous plants in their response to residue and extract of the allelopathic plant for all parameters recorded.

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Germination test

The results presented in *Figures 1* and *2* showed clearly the effects of different aqueous extracts of black nightshade, different treatments, and interaction effects on germination percentage, and length of radicle and plumule of beans. These parameters significantly ($p \leq 0.05$) decreased after being treated with roots and leaves aqueous extracts of black nightshade. However, the reduction in these parameters increased with the concentration of aqueous extracts, and depend on the part used in the extract. According to the displayed data, aqueous extract of seeds was found to be non-significant ($p > 0.05$) for all parameters. The highest inhibitory effects were exhibited by root extract particularly at 60% concentration, compared to control. The lowest seed germination and length of radicle and plumule of broad bean were 68.2%, 2.80 cm, and 3.65 cm respectively for aqueous root extract (*Fig. 1a, b, c*). In common beans, seed germination and length of radicle and plumule significantly lowered to 84.31%, 3.08 cm, and 4.36 cm respectively for those treated with root extract (*Fig. 2a, b, c*).

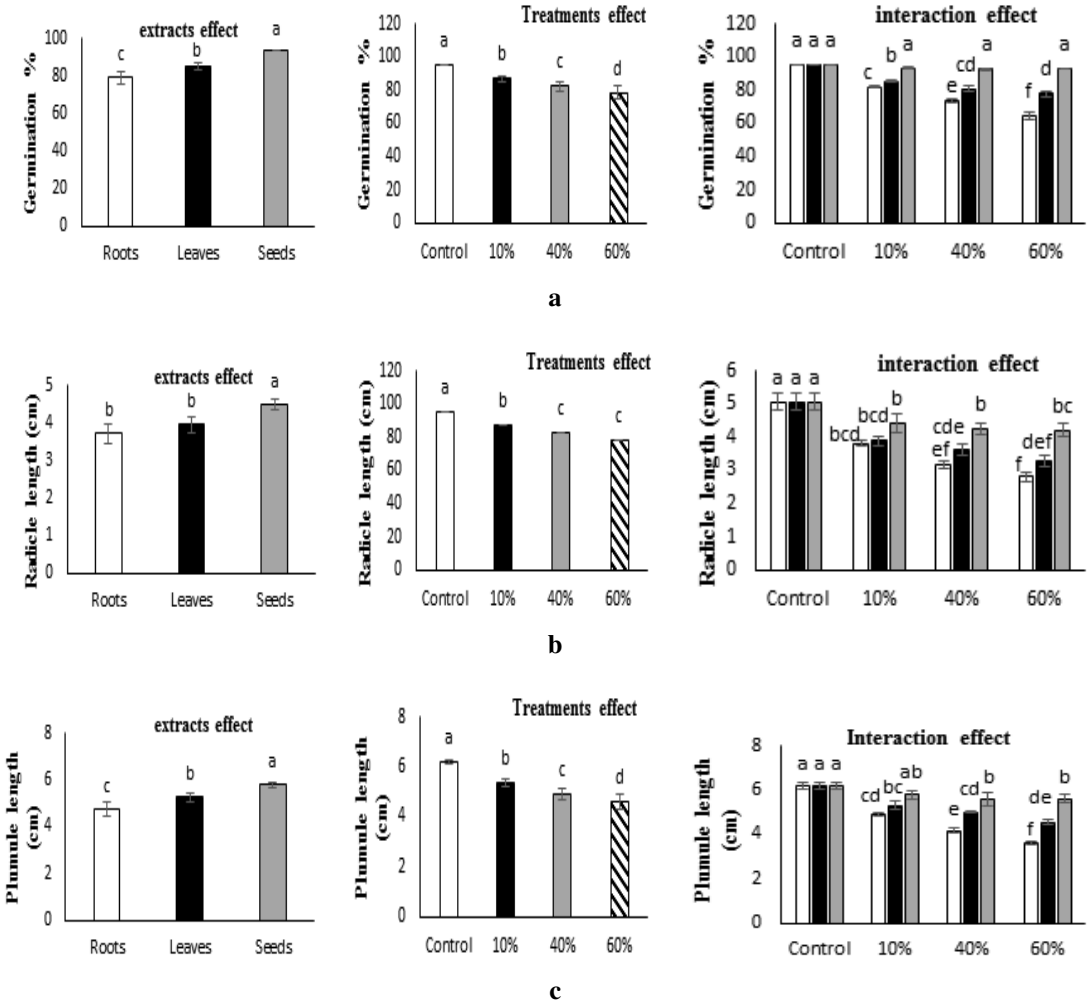

Figure 1. *Effects of black nightshade aqueous extracts, different treatments and interaction effect on broad bean, a: germination percent (%); b: radical length (cm); c: plumule length (cm). Error bars indicating the standard error (SE) of three replicates, are significantly*P < 0.05*

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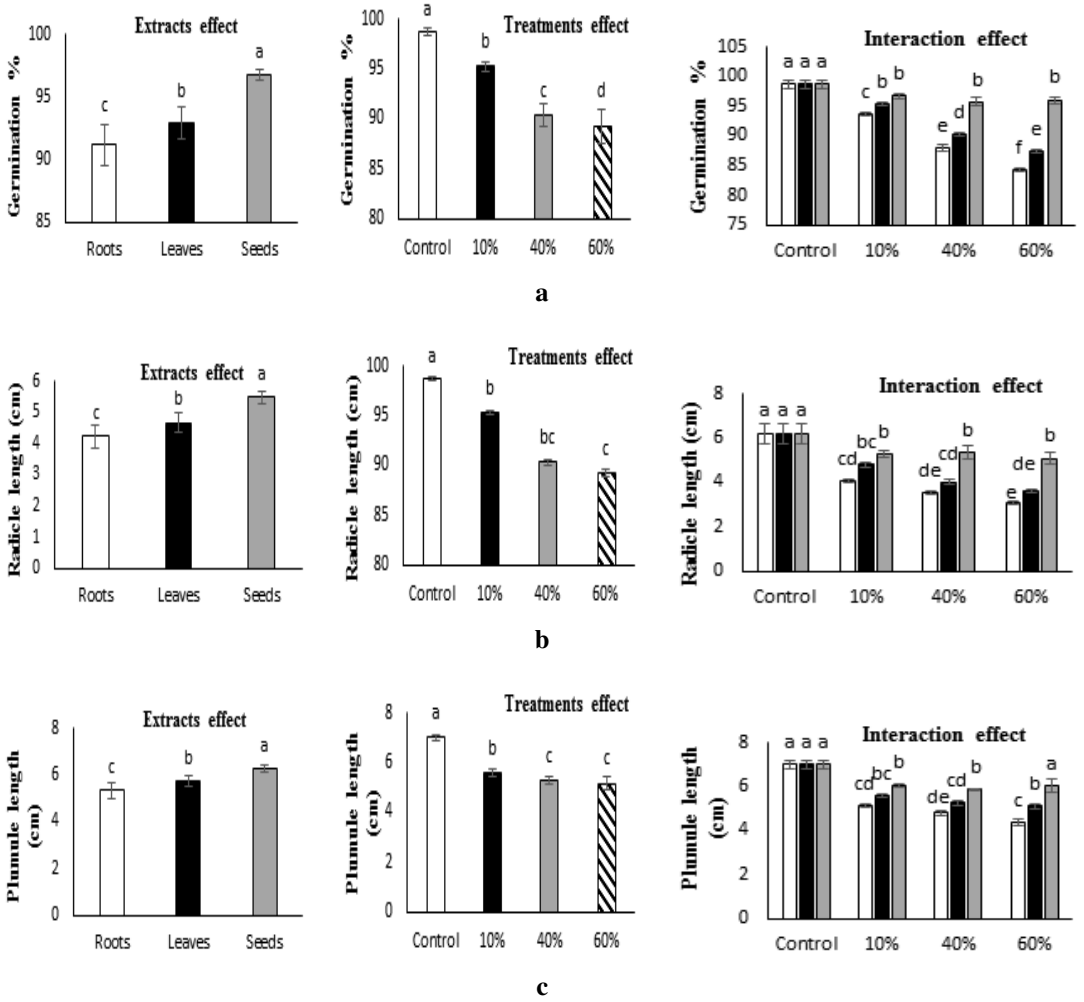

Figure 2. *Effects of black nightshade aqueous extracts, different treatments and interaction effect on common bean, a: germination percent (%); b: radical length (cm); c: plumule length (cm). Error bars indicating the standard error (SE) of three replicates, are significantly*P < 0.05*

Pot experiment

The results presented in *Table 2* and *3* showed the effect of root, leaf, and seed powders incorporated into the soil on growth parameters of beans. All parameters investigated in this study except the number of leaves significantly ($p \leq 0.05$) decreased in both plants treated with different powders of black nightshade.

Relative to control, plant height of both beans significantly decreased, and this reduction was increased as different powders increased in the soil. The greatest reduction in plant height was observed when root powder was added to the soil at 1.5%. The plant height of the broad bean lowered to 27.05 cm (*Table 2*) and the common bean decreased to 26.1 cm (*Table 3*).

The data of root length showed the same trend of plant height, the greatest reduction in root length was recorded when root powder was incorporated into the soil. The root length of broad and common beans in control was 16.23 and 16.53 cm, respectively. The root length decreased to 10.06 cm in the broad bean, and 13.07 cm in the common bean ($p \leq 0.05$).

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DOI: http://dx.doi.org/10.15666/aeer/1905_34313441

Table 2. *Growth parameters of broad bean under the effect of different concentrations of black nightshade residues*

Treatments (%)	Plant height (cm)	Root length (cm)	Fresh wt. (g)	Dry wt. (g)	Leaves no.
R 0.5	29.58 ± 0.32 ef	12.03 ± 0.03 fg	7.44 ± 0.22 de	2.19 ± 0.13 fg	10 ± 0.33 a
R 1	28.55 ± 0.22 g	10.75 ± 0.25 h	6.64 ± 0.17 f	1.81 ± 0.12 g	9.66 ± 0.57 a
R 1.5	27.05 ± 0.12 h	10.06 ± 0.08 h	6.01 ± 0.01 g	1.13 ± 0.08 h	9.66 ± 0.33 a
Mean	30.09 ± 3.09 c	12.27 ± 2.51 c	7.41 ± 1.45 c	2.226 ± 1.03 c	9.91 ± 0.67 a
L 0.5	31.33 ± 0.33 d	13.58 ± 0.25 d	8.12 ± 0.11 c	3.02 ± 0.03 cd	10 ± 0.57 a
L 1	30.18 ± 0.12 e	12.50 ± 0.28 ef	7.98 ± 0.02 cd	2.66 ± 0.07 de	10 ± 0.33 a
L 1.5	29.35 ± 0.19 f	11.54 ± 0.23 g	7.11 ± 0.11 ef	2.28 ± 0.08 ef	9.66 ± 0.33 a
Mean	31.42 ± 2.22 b	13.46 ± 1.87 b	8.19 ± 0.97 b	2.933 ± 0.6 b	10.00 ± 0.74 a
S 0.5	34.50 ± 0.28 ab	15.51 ± 0.28 ab	9.01 ± 0.09 ab	3.32 ± 0.16 ab	10.33 ± 0.66 a
S 1	33.94 ± 0.05 ab	14.50 ± 0.28 ab	8.80 ± 0.1 ab	3.05 ± 0.05 bc	10.33 ± 0.33 a
S 1.5	32.31 ± 0.33 c	12.77 ± 0.39 bc	8.06 ± 0.03 cd	2.80 ± 0.11 d	10.30 ± 0.66 a
Mean	33.90 ± 1.08 a	14.75 ± 1.43 a	8.86 ± 0.63 a	3.231 ± 0.44 a	10.33 ± 0.78 a
Control	34.84 ± 0.31 a	16.23 ± 0.17 ab	9.58 ± 0.34 a	3.75 ± 0.23 ab	10.33 ± 0.33 a

Mean ± standard error based on ANOVA analysis. Means in the same raw followed by the same letter in each column are not significantly different from each other at the 5% probability level (p value at 0.05) according to Duncan Multiple Range Test (DMRT). R: root; L: leaf; S: seed

Table 3. *Growth parameters of common bean under the effect of different concentrations of black nightshade residues*

Treatments (%)	Plant height (cm)	Root length (cm)	Fresh wt. (g)	Dry wt. (g)	Leaves no.
R 0.5	27.55 ± 0.22 ef	14.46 ± 0.15 d	6.76 ± 0.15 def	1.92 ± 0.07 d	7.66 ± 0.33 [a]
R 1	26.87 ± 0.13 g	13.78 ± 0.14 e	6.16 ± 0.09 fg	1.34 ± 0.09 f	8.00 ± 1 a
R 1.5	26.10 ± 0.1 h	13.07 ± 0.07 f	5.67 ± 0.08 g	0.94 ± 0.06 g	7.00 ± 1 a
Mean	27.64 ± 1.57 c	14.46 ± 1.37 c	6.78 ± 1.17 c	1.85 ± 0.89 c	7.58 ± 1.16 a
L 0.5	28.81 ± 0.1 c	15.26 ± 0.13 bc	7.35 ± 0.18 cd	2.30 ± 0.14 c	8.00 ± 0.57 a
L 1	27.87 ± 0.23 de	14.80 ± 0.1 bc	6.91 ± 0.08 de	2.01 ± 0.01 d	7.66 ± 0.33 a
L 1.5	27.18 ± 0.17 fg	14.28 ± 0.15 de	6.51 ± 0.08 ef	1.64 ± 0.11 e	7.66 ± 0.88 a
Mean	28.48 ± 1.16 b	15.22 ± 0.91 b	7.32 ± 0.84 b	2.28 ± 0.62 b	7.75 ± 0.87 a
S 0.5	29.91 ± 0.2 ab	16.15 ± 0.14 a	8.25 ± 0.21 ab	3.03 ± 0.03 ab	8.00 ± 0 a
S 1	29.39 ± 0.23 ab	15.39 ± 0.19 ab	7.77 ± 0.13 bc	2.83 ± 0.1 b	7.66 ± 0.66 a
S 1.5	28.26 ± 0.13 d	15.07 ± 0.07 bc	7.21 ± 0.12 cd	2.46 ± 0.02 c	8.00 ± 0.57 a
Mean	29.40 ± 0.79 a	15.78 ± 0.67 a	7.94 ± 0.61 a	2.88 ± 0.3 a	7.83 ± 0.72 a
Control	30.06 ± 0.19 a	16.53 ± 0.25 a	8.53 ± 0.34 a	3.19 ± 0.07 a	7.66 ± 0.33 a

Mean ± standard error based on ANOVA analysis. Means in the same raw followed by the same letter in each column are not significantly different from each other at the 5% probability level (p value at 0.05) according to Duncan Multiple Range Test (DMRT). R: root; L: leaf; S: seed

Fresh and dry weights of both plants follow the same approach as for the plant height and root length. Relative to control, root powder at concentration 1.5% showed the greatest reduction of fresh weight of both plants. The lowest fresh weight of broad bean

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was 6.01 g while the highest value was 9.58 g observed in control (*Table 2*). In common beans, the lowest fresh weight was 5.67 g while the highest value was 8.53 g which was found in control (*Table 3*). The highest dry weight of broad and common beans were 3.75 and 3.19 g observed in control while the lowest values 1.13, and 0.94 g respectively were found in pots with 1.5% root powder.

The data recorded in beans revealed that the greatest reduction in the previous parameters was observed when roots of black nightshade were applied then the leaves while seeds had limited influence. It is suggested to indicate that the measured properties were not significantly different from the control after treated with seed residues this may be attributed to that this organ has the smallest amount of alkaloid.

For the number of leaves, there was no significant difference between different powders treated with different rates in the soil of both plants ($p > 0.05$). The results showed that the incorporated powders of *S. nigrum* into the soil at rates 0.5, 1, and 1.5% did not affect the number of leaves of broad and common beans.

The results of yield components of beans were recorded in *Table 4*. All applied powders of black nightshade incorporated into the soil (0.5, 1, and 1.5%) significantly decreased all yield components except the seed powder. Root and leaf powders of black nightshade produced lower yield components than control. Therefore, it could be concluded that *S. nigrum* roots and leaves residues (at 0.5, 1, and 1.5%) incorporated into soil caused a reduction in the yield of broad and common beans.

Table 4. *Effect of different residues concentrations of black nightshade on the yield of broad and common beans*

Treatments (%)	Broad bean			Common bean		
	No. of pods	No. of seeds	Wt. of 100 seeds (g)	No. of pods	No. of seeds	Wt. of 100 seeds (g)
R 0.5	5.71±0.19 d	3.42±0.16 cd	79.63±0.77 c	7.91±0.13 b	4.95±0.06 c	35.23±0.12 c
R 1	4.27±0.21 e	2.66±0.11 e	75.86±0.26 d	6.86±0.22 d	4.02±0.18 d	32.56±0.31 d
R 1.5	3.27±0.14 f	1.85±0.10 f	72.38±0.14 e	6.10±0.05 e	3.28±0.17 e	30.66±0.17 e
Mean	5.15±1.64 c	3.06±0.98 c	77.79±4.31 c	7.45±1.14 c	4.43±0.93 b	37.62±0.4 a
L 0.5	6.30±0.25 c	3.80±0.10 bc	81.46±0.4 b	8.17±0.09 b	5.05±0.04 bc	36.13±0.20 b
L 1	5.69±0.21 d	3.08±0.04 de	79.67±0.66 c	7.38±0.19 c	4.28±0.22 d	34.73±0.37 c
L 1.5	4.30±0.15 e	2.18±0.10 f	74.98±0.19 d	6.65±0.18 d	3.79±0.14 de	32.63±0.18 d
Mean	5.91±1.2 b	3.34±0.86 b	79.85±3.29 b	7.78±0.92 b	4.65±0.74 b	37.62±0.4 a
S 0.5	7.05±0.07 ab	4.27±0.12 a	82.33±0.25 a	8.01±0.06 a	5.04±0.17 a	37.90±0.09 a
S 1	6.68±0.24 bc	3.98±0.01 ab	83.08±0.11 a	8.10±0.13 a	5.12±0.27 ab	37.66±0.1 a
S 1.5	6.98±0.21 ab	4.14±0.22 ab	82.44±0.31 a	8.03±0.13 a	4.39±0.27 abc	36.3±0.17 a
Mean	7.02±0.38 a	4.18±0.27 a	83.33±0.39 a	8.96±0.18 a	5.58±0.4 a	37.62±0.4 a
Control	7.36±0.2 a	4.32±0.19 a	83.29±0.21 a	8.93±0.11 a	5.49±0.25 abc	37.62±0.39 a

Mean ± standard error based on ANOVA analysis. Means in the same raw followed by the same letter in each column are not significantly different from each other at the 5% probability level (p value at 0.05) according to Duncan Multiple Range Test (DMRT). R: root; L: leaf; S: seed

Discussion

The high nutritional value and protein content make broad and common beans, belonging to the family Leguminosae, the most important crops in the world, especially in developing countries. Weeds cause a substantial decline in agriculture production

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through direct and indirect effects. Therefore, it is important to determine the effect of these weeds on the growth of crops. Also, *S. nigrum* is distributed in some field crops in Egypt.

According to the displaying data, black nightshade affects germination, growth, and yield of beans negatively. The probable reason for *S. nigrum* potent allelopathic activity may be due to the presence of many bioactive secondary metabolites in this plant, particularly alkaloids which are responsible for suppressing the growth of other plants (Sabh and Ali, 2010). The inhibitory effect on yield and yield parameters is directly proportional to the increase in the concentration used and this might be due to the presence of toxic allelochemicals like alkaloids. According to Girija and Gowri (2008) the leaf, and fruit extracts of *S. nigrum* contain alkaloids, that may suppress the germination, radicle length, and total protein content of *P. sativum*, *E. coracona*, and *T. foenum*.

Eltayeb et al. (1997) reported that solasodine occurs in roots, stems, leaves, and fruits of *S. nigrum*. The lowest levels are registered in stems and the highest in leaves. In vegetative organs, and particularly in roots, absolute amounts of solasodine rise steadily with age but decrease in fruit. From the results obtained, we found that the root of *S. nigrum* had the strongest allelopathic effect on germination, yield, and growth parameters compared to leaves, and seeds. Likewise, Imad et al. (2017) found that the alkaloidal content of plant parts changes during the development of *S. nigrum*, the absolute amount of alkaloid per leaf increased during leaf development, whereas the concentration declined. Both the concentration and the absolute amount of solasodine per fruit decrease with fruit maturation. Generally, Debnath et al. (2016) found that leaves aqueous extract exhibit more allelopathy for seedlings than those from the flowers of *S. sisymbrifolium* (Lam.) on *V. radiata*, *A. mangostanus*, and *B. campestris*, whereas it is less effective on *O. sativa*. However, the leachates extracted at the flowering stage from *N. plumbaginifolia* provided the greatest growth inhibition and biochemical parameters for seedlings of sunflower depending on concentration (Singh et al., 2015). Also, Morais et al. (2013) displayed that ethanol extract and fractions obtained from ripe fruits of *S. lycocarpum* showed allelopathic potential on the growth of onion radicles; inhibitory effect on the growth of hypocotyls, and radicles of lettuce. The results of germination and pot experiments showed a great reduction increased with the concentration of allelopathic plant, this agrees with Bosch et al. (2004) who showed that leaf leachates of *S. mauritianum* (Scop.) reduced germination in *H. stricta* in a concentration-dependent manner. The growth of both beans was suppressed and radicle and plumule length were decreased, these may be attributed to the allelochemicals which disrupt the growth hormones. El-Shora et al. (2015) found that radicle and plumule of fenugreek, a medicinal plant showed a sensitive response to allelochemicals from aqueous extracts which might have interfered with hormones that encourage growth, development, cell elongation, and cell division especially on younger active root tips.

All growth parameters, plant height, root length, fresh and dry weights except number of leaves reduced after applying root and leaf residues in soil. Allelochemicals from leachates of *S. nigrum* might have interfered with photosynthesis leading to drastic changes in the physiology of plants. El-Shora et al. (2018) found that the decline in photosynthesis may have contributed to decreased growth of plants and decreased accumulation of fresh biomass due to decreased water content in the species studied. In this regard, Fikreyesus et al. (2011) reported that tomato root elongation was inhibited

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DOI: http://dx.doi.org/10.15666/aeer/1905_34313441

by extracts of *Eucalyptus camandulensis*, and Gulzar et al. (2014) observed that the root length of *C. album*, *M. alba*, and *N. plumbaginifolia* decreased as the concentration of *C. sophere* increased. The results of the study are consistent with Hamidi et al. (2008) who observed the allelopathic ability of *H. spontaneum* residues incorporated into the soil on the length of seedling, and the dry weight of *T. aestivum*. Also, Ismail and Siddiqui (2011) found that seedling length and weight of *O. sativa* were suppressed by residues of *C. iria*. The greatest reduction in growth parameters was observed at 1.5% concentration residue of *S. nigrum*, and this was consistent with the results of Zohaib et al. (2014) who found that the inhibitory effects of five leguminous weeds against rice caused by the highest concentration of water extracts and residues of these weeds.

Conclusion

Allelopathy can cause substantial decline in the growth and yield of crops. This study was devoted to determining the allelopathic effects of different parts of black nightshade on some leguminous crops, broad and common beans. According to the results of germination and pot experiments, it was found that roots of black nightshade had the highest inhibitory effect on beans. Germination, growth parameters except for the number of leaves and yield exhibited a high level of inhibition by the highest concentration of roots. While seeds showed a limited influence on these parameters. The decline of growth and yield requires further studies on the germinated seeds related to the biosynthesis of secondary metabolites at molecular level. The results confirmed that *S. nigrum* has an allelopathic effect on broad and common beans which depends on plant parts and concentration used in the extract preparation.

Acknowledgements. The authors acknowledge of the Zagazig University, Faculty of Science, Department of Botany and Microbiology for providing laboratory facilities and helping in analytical procedures of the research work.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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EFFECTS OF CADMIUM AND HIGH TEMPERATURE ON CHLOROPHYLL AND MINERAL NUTRIENT CONTENTS IN *Triticum aestivum* L. SEEDLINGS

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(Received 23rd Jul 2020; accepted 10th Jun 2021)

Abstract. Wheat plays a particularly important role in the human diet. Environmental stresses negatively affect wheat development and yield. This study was conducted to determine the effects of high temperature-cadmium interactions on total chlorophyll content, chlorophyll a / chlorophyll b ratio as well as cadmium and mineral nutrient contents (potassium, magnesium, zinc, calcium) in wheat seedlings. Seedlings belong to two varieties of bread wheat (*Triticum aestivum* L.) named Dağdaş and ES-14 were treated with different concentrations of cadmium (Cd) (0, 15, 75 ppm), under different (24/16°C and 40/30°C daytime/night-time) temperature conditions. The total chlorophyll increased in the Dağdaş and decreased in the ES-14 in response to the high-temperature treatment (40/30°C). Cadmium caused a decrease in the Chlorophyll a/b ratio in varieties at high-temperature conditions. The accumulation of cadmium and uptake of other minerals in shoots differed depending on the wheat variety and cadmium-temperature interaction. Cadmium accumulation in shoots increased in response to the cadmium treatments. Cadmium accumulation decreased at the 40/30°C temperature compared to that at 24/16°C temperature. Calcium accumulation increased in shoots as a response to high temperature in both varieties. In both varieties, the potassium content increased in response to cadmium (15 μM) and high temperature.
Keywords: *heavy metal, wheat, Dağdaş, ES-14, heat stress*

Introduction

Climate change and global warming researchers have estimated that a dry and warm climate will affect many regions, including southern Europe recently (Kalefetoğlu and Ekmekçi, 2005). High temperatures can hamper grain production and quality (Gulli et al., 2005). Heat shock can also lead to the development of heavy metal resistance in wheat seedlings (Orzech and Burke, 1988). Soil contamination with heavy metals, such as cadmium (Cd), lead and mercury affects plant growth and development (Qiao et al., 2019). Microelement deficiency affects plant growth (Qiao et al., 2019). Cd can be absorbed easily by different plant parts (Gianazza et al., 2007). Previous research showed that both Cd and high temperatures caused stress in plants, leading to sizable production losses. High concentrations of Cd inhibited plant growth and development, as well as enzyme activity and photosynthetic organs, resulting in reduced photosynthesis (Di Toppi and Gabrielli, 1999). Besides, stress factors affect some transcription factor and genes. TaMYB73, TaERF1 and TaSRG genes' expression

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
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levels increased in the seedlings of two wheat varieties (*Triticum aestivum* L. cv. Ç-1252 and Gün-91) exposed to chromium (Cr) and temperature stress (Ergün et al., 2014). Doğru and Ergün (2021) Dağdaş and Konya 2002 examined Cd and salt interactions in wheat varieties. The research result showed the highest increase in Dağdaş gene expression. High NaCl and Cd concentrations caused an increase in ERF1 expression in the Dağdaş variety. Increased TaSRG expression with Cd application in Konya 2002 variety, probably ıt may be associated with Cd resistance.

Wheat growth and development has declined worldwide because of agricultural areas becoming infertile due to various stresses, including high temperatures, salinity and heavy metal contamination (Öncel et al., 2000; Ergün et al., 2014). The increase in temperatures worldwide, in addition to heavy metal accumulation in the food chain, poses a threat to all living organisms on Earth. Research on the relation between heavy metal contamination and high temperature-induced stress is important for understanding tolerance mechanisms developed by plants growing in regions exposed to stressors. Such research on the mechanisms of heavy metal and high-temperature resistance in plants can aid the selection of heat-resistant and heavy metal-resistant varieties.

The present study aimed to detect the effects of different concentrations of Cd, temperature and temperature–heavy metal (Cd) interactions on the total chlorophyll (Chlorophyll a+b) (Chl) content, Chl a/b ratio, Cd accumulation and mineral (potassium [K], magnesium [Mg], zinc [Zn] and calcium [Ca]) contents in wheat plants.

Materials and methods

Materials

This study was conducted at Hatay Mustafa Kemal University in Turkey. Two varieties of bread wheat (*Triticum aestivum* L. cv. Dagdaş and ES-14) were supplied by the Çukurova University Faculty of Agriculture in Turkey. The Dağdaş - 94 wheat variety has been reported to have higher salt tolerance than the ES-14 variety (Karanlik, 2001). The Dağdaş - 94 wheat variety is known to be resistant to drought, incubation and cold (Öztürk and Aydin, 2017).

Methods

Plant growth conditions

Seeds of the two varieties were germinated between two layers of filter paper in a plant growth cabinet at 24 ± 2°C for 48 h. At the end of this period, seedlings were transferred to pots containing sand and perlite and grown under 24/16°C (daytime/night-time) with 50% humidity in a Percival model plant growth cabinet for 5 d. The seedlings were then transferred to pots containing nutrient solution composed of half-strength Arnon and Hoagland (1940) nutrient solution (pH 5.8).

The experiments were designed according to a completely randomized design with 4 replications. Cultivars are a mean factor, temperature and cadmium doses are split plots on cultivars. The study was conducted under two different temperatures. In the first set of experiments, the plants were grown in nutrient solutions containing Cd at temperatures of 24/16°C (daytime/night-time), which are the optimum temperatures for wheat growth. Chlorine salt of Cd was used as a heavy metal stressor in this study, with three different concentrations (0, 15 or 75 μM) added to the nutrient solution. In the second set of experiments, the same Cd treatments were repeated, but the cabinet

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temperature was increased to 40/30°C (daytime/night-time). The seedlings were grown in these solutions for 5 d. At the end of this period, the plants were harvested. Plant samples were cut from the zone where the root and offshoot parted, and the shoots and roots were harvested separately.

For analyses of chlorophyll content, the fresh samples were determined using a Shimadzu UV-VIS Spectrophotometer.

For analyses of heavy metal and mineral elements (Cd, Ca, K, Mg and Zn), the shoot samples were dried at 70°C for 48 h, and their dry weights were measured. The dry plant samples were digested with sulphuric acid, perchloric acid, nitric acid using the wet decomposition method. The Cd and mineral nutrient concentrations were determined using a Varian Liberty Series II model Inductively Coupled Plasma-Atomic Adsorption Spectrophotometer (Hatay Mustafa Kemal University Antakya Hatay Turkey).

After harvesting the samples, the Chl content in fresh shoots was investigated. The Chl a/b ratio and total Chl (a + b) contents (mg g^{-1} fresh weight (F.W.)) in leaf tissues were determined, according to the method of Arnon (1949), and the corrected values were then calculated according to the method of Porra (2002).

Statistical analysis

The values obtained from the experiments were subjected to analysis of variance (ANOVA) using the general linear models procedure in the SPSS (SPSS Inc. Chicago, Illinois, USA) package program. Statistically significant results were subjected to a Least Significant Difference multiple comparison test ($p < 0.01$).

Results

Total chlorophyll (Chl) content

In the experiments, the total Chl content increased in the Dağdaş variety and decreased in the ES-14 variety in response to the high-temperature treatment (*Fig. 1*). Chl (a / b) ratio 15 µM Cd application, although both temperatures increased ES-14 varieties, did not increase in Dağdaş cultivar at 24/16°C day/night temperature. Dağdaş variety has the lowest total chl at 24/16°C 15 µM, while ES-14 variety increases the total Chl value at 15 µM Cd at both temperatures and decreases at 75 µM Cd.

The chlorophyll a/b ratio

However, the Chl a/b ratio in both varieties decreased in response to the 75 µM Cd treatment at 40/30°C. It also decreased in the ES-14 variety but not in the Dağdaş variety at 24/16°C. The Chl a/b ratio was low in Dağdaş seedlings grown under 24/16°C conditions at Cd concentrations of 15 and 75 µM (*Fig. 2*).

Mineral content

In the present study, there was a close relationship between the Cd concentration and its accumulation in shoots in both varieties ($p < 0.01$) (*Table 1*). More Cd accumulated in the ES-14 variety as compared with that in the Dağdaş variety under 24/16°C conditions ($p < 0.05$). In a previous study on wheat seedlings treated with Cd, more Cd accumulated in root parts than in shoots and seeds. The role of temperature in increasing toxic responses to heavy metals is well known (Li et al., 2011). Roots are the first

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organs to be affected by the accumulation and retention of heavy metals. Heavy metal accumulation together with increasing temperature, affects membrane lipids in the roots and leads to significant inhibition of root growth (Fritioff et al., 2005). In this study, Cd accumulation in shoots increased in the Cd treatments and decreased at high temperature. These findings may be due to Cd accumulation in roots inhibiting root growth, with a subsequent decrease in metal transport to shoots.

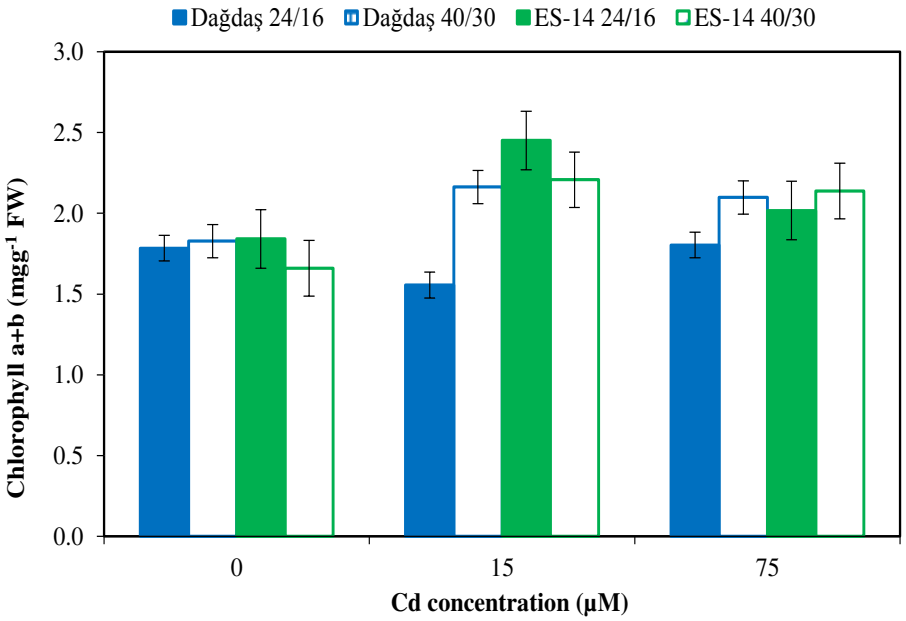

Figure 1. *Effects of cadmium-temperature (24/16°C and 40/30°C) interactions on total chlorophyll (Chl) content (mg g-1 FW) in Dağdaş and ES-14 wheat seedlings*

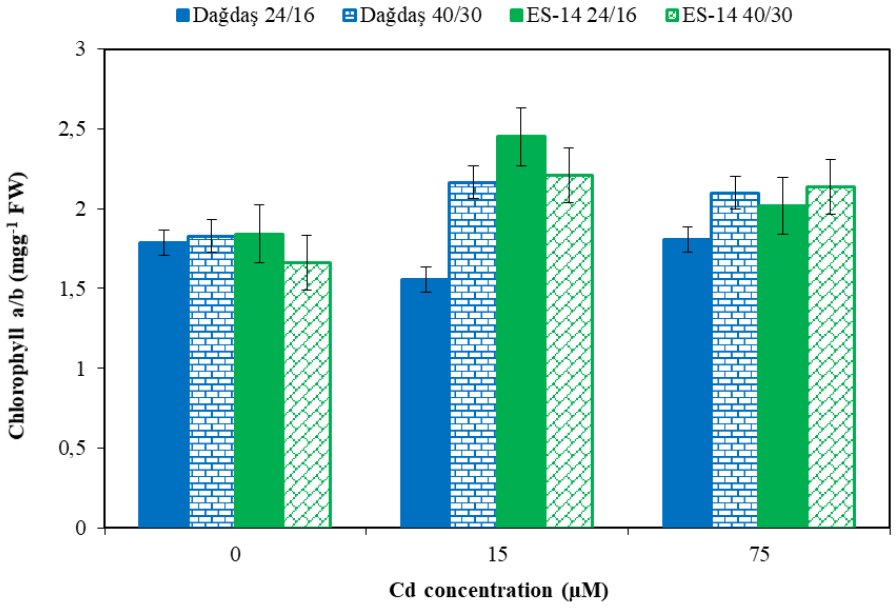

Figure 2. *Effects of cadmium-temperature (24/16°C and 40/30°C) interactions on the chlorophyll a/b ratio in Dağdaş and ES-14 wheat seedlings*

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Table 1. *The effects of genotype and temperature on the minerals (Ca, Cd, K, Mg and Zn) content*

Genotype	Temperatures (^{0}C)														
	Ca (%D.W)			Cd (ppm)			K (%D.W)			Mg (%D.W)			Zn (ppm)		
	24/16	40/30	Avr.	24/16	40/30	Avr.	24/16	40/30	Avr.	24/16	40/30	Avr.	24/16	40/30	Avr.
Dağdaş	0.34 c	0.51 B	0.43 B	37.4 b	15.3 D	26.3 b	0.89 b	0.90 b	0.90 B	0.19 b	0.26 a	0.23	26.2	27.0	26.6
ES-14	0.54 ab	0.55 A	0.55 A	41.7 a	22.1 C	31.9 a	1.11 a	0.93 b	1.02 A	0.22 ab	0.22 ab	0.22	28.8	28.8	28.8
Averege	0.44 B	0.53 A		39.5 A	18.7 B		1.00 A	0.92 B		0.21 B	0.24 A		27.5	27.9	
G	*			*			**			ns			ns		
T	***			***			**			*			ns		
GxT	0.04 ***			1.2 *			0.06 **			0.04 *			ns		
CV	6.07			5.03			5.87			11.9			6.82		

***: p<0.001; **: p< 0.01; *: p< 0.05 and ns: non-significant

In the present study, Ca accumulation in both varieties increased at high temperature. However, the Cd treatment did not appear to influence the Ca content, irrespective of the concentration. More Ca accumulated in the ES-14 variety under optimum temperature conditions as compared with that in the Dağdaş variety under the same conditions ($p < 0.05$). The Ca content in the Dağdaş variety seedlings increased in the high Cd/high-temperature treatment ($p < 0.01$) (*Table 1*). In a previous study, Ca accumulation in shoots of wheat seedlings treated with Cd increased as compared with that in a control Lii 667 variety and decreased in Huabei 45-4 and E81513 varieties (Zhang et al., 2002).

In the present study, the K content in wheat seedlings of the ES-14 variety in the 16/24°C treatment increased as compared with that in the Dağdaş variety under the same temperature conditions ($p < 0.01$) (*Table 2*). The K content in the ES-14 seedlings in the high-temperature/high Cd treatment decreased as compared with that in the control treatment. In contrast, the K content in the Dağdaş variety seedlings increased in the high Cd/high temperature ($p < 0.01$) (*Table 3*). Similarly, Zhang et al. (2002) reported that K accumulation in shoots of wheat seedlings treated with Cd decreased as compared with that in a control Lii 667 variety, whereas it increased in Huabei 45-4 and E81513 varieties.

Table 2. *The effects of cadmium (Cd) and genotype on the minerals (Ca, Cd, K, Mg and Zn) content*

Genotype	Cadmium doses (µM)														
	Ca (%D.W)			Cd (ppm)			K (%D.W)			Mg (%D.W)			Zn (ppm)		
	0	15	75	0	15	75	0	15	75	0	15	75	0	15	75
Dağdaş	0.39 c	0.45 b	0.44 b	0.06 e	22.9 d	56.1 b	0.87 d	0.92 cd	0.91 d	0.22	0.23	0.23	27.3 b	26.3 c	26.2 bc
ES-14	0.55 a	0.53 a	0.56 a	0.06 e	27.0 c	68.5 a	1.07 a	1.02 ab	0.98 bc	0.24	0.22	0.21	30.7 a	29.8 a	25.9 bc
Averege	0.47	0.49	0.50	0.06 C	25.0 B	62.3 A	0.97	0.97	0.94	0.23	0.23	0.22	29.0 A	28.0 AB	26.1 B
Cd	ns			1.27 ***			ns			ns			1.61 **		
GxCd	0.04 **			1.79 ***			0.07 *			ns			2.27 **		
CV	6.07			5.03			5.87			11.9			6.82		

***: p<0.001; **: p< 0,01; *: p< 0,05 and ns: non-significant

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Table 3. *The effects of cadmium (Cd) and temperature on the minerals (Ca, Cd, K, Mg and Zn) content in Dağdaş and ES-14 seedlings*

Genotype	**Temperature (°C)** (Day/night)	**Ca (% D.W)**			**Cd (ppm)**			**K (% D.W)**			**Mg (% D.W)**			**Zn (ppm)**		
		Cd (µM)			Cd (µM)			Cd (µM)			Cd (µM)			Cd (µM)		
		0	15	75	0	15	75	0	15	75	0	15	75	0	15	75
Dağdaş	24/16	0.34 d	0.33 d	0.35 d	0.06	28.6	83.5	0.90 de	0.84 e	0.95 cd	0.17	0.20	0.22	27.3 cd	20.6 f	30.8 b
	40/30	0.43 c	0.57 ab	0.53 ab	0.07	17.3	28.6	0.90 de	0.99 bc	0.86 de	0.27	0.27	0.24	27.3 cd	32.0 bc	21.6 ef
ES-14	24/16	0.52 b	0.53 ab	0.57 ab	0.05	30.9	94.0	1.30 a	1.06 b	0.98 bc	0.24	0.22	0.21	27.2 cd	35.3 a	23.9 e
	40/30	0.58 a	0.53 ab	0.55 ab	0.06	23.0	43.1	0.80 e	0.97 bc	0.98 bc	0.23	0.22	0.21	34.1 a	24.3 de	27.9 bc
Average	24/16	0.43	0.43	0.46	0.05 e	29.7 c	88.8 a	1.10 a	0.95 b	0.97 b	0.20	0.21	0.21	27.3 b	27.9 b	27.3 b
	40/30	0.51	0.55	0.54	0.07 e	20.2 d	35.8 b	0.80 c	0.98 b	0.92 b	0.25	0.24	0.22	30.7 a	28.1 bc	24.8 c
TxCd		ns			1.79	**		0.07	**		ns			2.27	**	
GxTxCd		0.06	**		ns			0.01	**		ns			3.22	**	
CV		6.07			5.03			5.87			11.9			6.82		

**: p< 0,01; *: p< 0,05 and ns: non-significant

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Discussion

It is known that heavy metal and heat stress decreased the chlorophyll content in plants. According to Ergün et al. (2014), total Chl in wheat decreased under combined heat and heavy metal stress, whereas carotenoid levels slightly increased. Chl contents decreased in *T. aestivum* L. seedlings treated with high Cd concentrations under low and high-temperature conditions (Öncel et al., 2000). Hsu and Kao (2003) reported a reduction in Chl in *Oryza sativa* L. TN 1, a Cd-sensitive variety, as compared with that in *O. sativa* L. TNG 67 variety, a Cd-tolerant variety, pointing to Cd-induced toxicity. Shukla et al. (2003) found that the amount of Chl decreased in *T. aestivum* L. seedlings treated with Cd.

Cd causes oxidative damage in plants and competes for cofactors of basic metal ions involved in Chl synthesis (Di Toppi and Gabrielli, 1999). Stobart et al. (1985) reported that Cd inhibited Chl biosynthesis due to its toxic effects. In their study, decreases in the total Chl content were closely related to the type and concentration of heavy metals, with dramatic reductions observed at higher heavy metal concentrations.

In our study, Cd concentrations in both wheat varieties caused a decrease in the amount of Chl. Previous research reported that Cd caused chlorosis by inhibiting the uptake of elements, such as Mg, K, Fe and Ca, which are basic cofactors of the enzymes of photo-system (PS) I and PSII (Shukla et al., 2003). In the present study, Cd caused a distinct decrease in the Chl a/b ratio in both varieties, especially at temperatures of 40/30°C (*Fig. 2*). In a previous study, although the Chl a/b ratio decreased significantly after Cd treatment, the effect was greater on PSII than PSI (Weigel, 1985). In a study on *Phragmites australis* plants treated with Cd, the total Chl content decreased, and Cd-related damage of PSII was higher than that of PSI (Pietrini et al., 2003). In another study, Cd had unfavourable effects on Chl, especially Chl b, causing an increase in the Chl a/b ratio (Ekmekçi et al., 2008).

In this study, the increase in Ca content due to the 40/30, Cd increase in Dağdaş variety wheat seedlings is statistically significant ($p < 0.01$). There is a significant increase in Cd content in both wheat varieties depending on the temperature and the increase in Cd content ($p < 0.01$). While a decrease in K content was observed due to the increasing Cd in the presence of ES-14 at 24/16°C, it is observed that the K content remained the same in Cd (75 µM) content at both temperatures. In Dağdaş variety, Cd (15 µM) dependent increase ($p < 0.01$) at 40/30°C is important, while increasing Cd value at 24/16°C causes a decrease in K content and a significant increase in Cd (75 µM) concentration has been ($p < 0.01$). While the Zn content of the Dağdaş variety decreases at 24/16°C C due to the increase in Cd, it increases in the high Cd concentration ($p < 0.01$), but it was found to increase first and then decrease at 40/30°C ($p < 0.01$). It was stated that the highest Zn content was at Cd (15 µM) application at ES-14 24/16°C, and Cd (15 µM) at 40/30°C in Dağdaş variety ($p < 0.01$). In the present study, there was no significant variation in the Mg content of the wheat seedlings treated with Cd and exposed to different temperatures. However, Zhang et al. (2002) observed that Mg accumulation in shoots of wheat seedlings of E81513 and Huabei 45-4 varieties treated with Cd increased as compared with that in a control and that Mg content decreased only in Lii 667 variety.

In the present study, the highest Zn concentration was detected in ES-14 seedlings treated with 15 µM of Cd at 24/16°C, whereas the lowest concentration (20.6 ppm) was observed in the Dağdaş variety under these conditions ($p < 0.01$) (*Table 3*). However, there was no statistically significant difference in the Zn concentration under the

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DOI: http://dx.doi.org/10.15666/aeer/1905_34433452

different Cd and temperature treatments. Zhang et al. (2002) found that Zn accumulation increased in offshoots of wheat seedlings treated with Cd in Lii 667 and Huabei 45-4 varieties and that it decreased only in an E81513 variety.

Cd affects the permeability of plasma membranes, thereby affecting the nutrient intake of the affected plant (Zhang et al., 2002). Similarly, in this study, the accumulation of mineral nutrients altered, depending on the wheat variety, as well as the Cd concentration and temperature. In previous research, high concentrations of applied Cd reduced concentrations of essential macro- (Mg and S) and micronutrients (Zn, Fe, Mn and Cu) in stems of *Pfaffia glomerata* (Gomes et al., 2013). Besides, interactions between Cd and other nutrients resulted in reduced nutrient uptake and reduced fertility (Zhang et al., 2002). High Cd concentrations caused a significant reduction in K, Mg, Ca, Fe and Zn concentrations in roots and stems of *Juncus effusus* L. (Najeeb et al., 2011).

Conclusion

As a result, it is concluded that high temperature caused a decrease in Cd accumulation in the shoot when caused an increase in Ca and K accumulation. It is concluded that this decrease in accumulation of Cd under the high-temperature stress cause increase in Ca and K uptake. Dağdaş variety used in the study is known that resistant to drought, cold and salinity. In the study, total chlorophyll content was found to be higher in Dağdaş cultivar than ES-14 cultivar. In this case, it is concluded that Dağdaş variety is more resistant to high temperature than the ES-14 variety.

Since the Cd uptake varies considerably at different temperatures, it is necessary to choose species and varieties resistant to Cd toxicity in agriculture, especially in regions with Cd pollution. Crop production has decreased worldwide because agricultural areas have become infertile due to various environmental factors, such as high temperatures, salinity and heavy metal contamination. Understanding physiological and molecular mechanisms is essential to tolerate stress conditions. In today's world where the world population is increasing rapidly, it is necessary to increase new efficiency-enhancing studies to prevent food shortage. Regarding this issue, we think that new studies are needed to take measures against stress and to identify the relevant genes and increase their usability in wheat breeding studies.

New physiological, biochemical and molecular studies on the relationship between heavy metal and high temperatures are needed to improve crop productivity. The present study revealed that interactions between heavy metals (Cd) and temperature may result in the accumulation of Cd and other minerals in shoots, depending on the wheat variety.

Acknowledgements. This study is a Research Project of Dr Nuray ERGÜN, Dr Sema KARANLIK and Dr Murat TİRYAKİOĞLU funded by the Scientific Research Projects Coordinator (BAP) of Hatay Mustafa Kemal University (Project Number:07F0101 2007-2009).

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_34433452

RESPONSE OF COTTON (*Gossypium hirsutum* L.) HYBRİD (F_{5-6}) TO CLIMATIC DIFFERENCES IN TERM OF YIELD AND YIELD COMPONENTS

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(Received 10th Dec 2020; accepted 8th Jul 2021)

Abstract. Cotton yield is affected by environmental conditions. This study was carried out to determine the population means response in eleven hybrids and three check cotton cultivars to climate changes in Antalya-Turkey, in 2017 and 2018. Temperature, relative humidity and precipitation in these years were found to have varied in Antalya. In the May-October period of 2018, the totals of average and maximum temperature were seen higher than in 2017 as opposed to the total of minimum temperature, but the distribution of temperature, relative humidity and precipitation varied according to months. While the genotypes affected by this situation had taller height and more fruiting branches in 2018, they created fewer vegetative branches, had a lower ginning outturn and fewer bolls. On the other hand, while the in Flash cultivar the number of bolls decreased in 2018, in Gloria the loss ratio of bolls minimized. Furthermore, in Sure Grow 125 the seed cotton yield per boll increased and the yield reduced, lower ginning outturn and boll losses were observed that affected fiber yield. As a result of the study, it has been determined that Sure Grow 125 has a high tolerance to climate change and maintains its yield potential and Gloria variety had higher yield at low temperatures, while the hybrids have the genetic potential to increase seed cotton yield in Antalya.
Keywords: *climate change, cotton, fiber yield*

Introduction

Cotton is the most important fiber plant for Turkey as well as for other countries. The rapidly increasing world population and use of natural fiber increase the importance of cotton, while the need of cotton cultivars to special environmental conditions with the global warming limited the production. Turkey ranked as the 7th country in terms of the world cotton production (Kolay, 2019) and this production involves varieties of *Gossypium hirsutum* L. However, climate change in recent years, as in other countries, affects the temperature and precipitation of Turkey. Although a solution is sought for this problem, the genetic potentials of the varieties is of great importance for the solution of possible problems.

Cotton encounters with a lot of biotic and abiotic stress throughout its life and reacts differently with its genetic heritage. These responses can be different depending on the severity of the stress and the stage of plant development. For example, the temperature stress caused by global warming, which is one of the biggest problems of today, affects germination, growth, flowering and boll formation. Moreover, if no precautions are taken, global warming will occur due to the CO_2 increase in the atmosphere by an average of 2.5 °C (1.5 - 4.5 °C) until 2100 (Aksay et al., 2005). This problem will cause melting of polar glaciers, changing day/night temperature difference and precipitation

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_34533465

regime. While plants respond to this situation with field change, germination and lack of development, yield and quality losses. Researchers found that the accumulation of dry matter in cotton occurred at the peak flowering stage and day/night temperature were 30/20 °C (Reddy et al., 1991), and that air temperature, sunlight, soil and relative humidity were also important (Hake and Silvertooth, 1990). Temperatures up to 30-32 °C are effective in boll production but exceeding 32 °C are harmful because they cause a decrease in yield due to square shedding (Unay and Basal, 2005). In addition, Loka and Oosterhuis (2010) reported a decrease in the amount of carbohydrates with respiratory acceleration due to high night temperature.

On the other hand, cotton farming has also been reported to be affected by low temperatures. In particular, temperatures below 10-12 °C cause death by creating a shock effect, while the severity and duration of temperatures of more than 12 °C and less than 18 °C during the germination period, prolonging the growth, development and harvest periods of the plant, delays to pass following stage (Basra, 1999). In contrast, plants can respond to high temperature by controlling stomatal conductivity and stoma number, changing leaf area and leaf number, low temperatures by increasing earliness. Deltapine 41, Africa E5 and Campu cultivars (Gonen, 2017) as well as Melez-1 (Salman et al., 2019) were tolerant to high temperature stresses. Anjum and Khatoon (2003) had reported that *Gossypium hirsutum* L. species were more tolerant than *Gossypium arboreum* L. regarding low temperatures. On the other hand, the period, time, duration and intensity of precipitation affect the germination, growth and development of cotton. While strong and long-term rains that occur after planting reduce germination and seedling emergence, rains occurring during squaring, blooming and boll opening decrease pollination and cause shedding in bolls and contamination in fiber. On the other hand, the precipitation in May and June affects the yield positively (Cetin and Basbag, 2010).

This study was carried out in Antalya to determine the reaction of some cotton genotypes (homozygous and heterozygous, F_{5-6}) with different genetic structure to different climatic conditions.

Materials and Methods

This study was carried out in Antalya-Turkey (36° 53' 48.8" N - 30° 42' 53.4" E, altitude: 39 m) in 2017 and 2018. 11 heterozygote genotypes (Cross-1005, Cross-1006, Cross-1008, Cross-1013, Cross-1019, Cross-1101, Cross-1102, Cross-1103, Cross-1105, Cross-1109 and Cross-1115) were used as plant material with 3 homozygous control cotton (*Gossypium hirsutum* L.) namely Sure Grow 125, Gloria and Flash.

The study was carried out with three replications in randomized complete blocks design in 2017 and 2018. Genotypes were planted on May 8th, 2017 and May 10th, 2018, in 10 meters long rows of 4 in parcels. Distance between rows was 0.70 m, while 0.15 m from plant to plants in one row.

With the planting, 60 kg ha^{-1} of pure nitrogen (N) and 6 kg ha^{-1} pure phosphorus (P_2O_5) were given in the form of compound (20-20-0), and 100 kg ha^{-1} of pure nitrogen (N) was given as urea (46% N) before the second irrigation.

Harvesting was done by hand on October 23rd, 2017 and on October 17th, 2018. While seed cotton yield and fiber yield per hectare were determined from the parcels, the ginning outturn was determined with the following formula (Eq.1) below. Other characteristics were determined over 20 plants in each plot, also. Variance analyses

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_34533465

were done in JMP 13 software with data collected from experiment according to split plots design in randomized complete blocks.

$$\% \text{ Ginning outturn: (Fiber yield/Seed cotton yield)*100} \quad \text{(Eq.1)}$$

The experiment area is sandy-clay, slightly alkaline, salt-free, and is poor in organic matter (1.55%). Climate data of Antalya for 2017 and 2018 differed greatly (*Table 1*). While the first four months of 2018 were hotter, more humid, and rainier than 2017, these values differed in the cotton season (May-October) compared to the months. However, according to long term, while averages of minimum and maximum temperatures were lower in both 2017 and 2018 in the first four months, precipitation was higher. On the other hand, average temperature of 2017 was similar with long-term value.

Moreover, it is understood from *Table 1* that precipitation in the cotton season of 2018 were continued monthly, except August, and 25.2% (139 mm) of total annual precipitation (549.8 mm) fell in the cotton season. According to long term value, 2017 was very dry compared to 2018, but 2018 showed similarity with long term value in term of precipitation in the cotton growing season.

On the other hand, when the data for this period were analyzed, it was observed that the average and maximum temperatures in June and July 2018 were less than in 2017 and but similar August and high in September and October. Furthermore, in total minimum temperatures different were 13.3 ^{o}C the effects of May, July, August and October.

As a result of this study, it was determined that 2017 was drier than both 2018 and long-term averages, while the maximum temperatures for 2017 and 2018 were lower than the long-term average, whereas the minimum temperatures were higher, and 2017 was similar to the long-term value in term of average temperature. The study was analyzed according to the split plot method in a randomized block design, and population means of eleven hybrid combinations were compared with standard-control varieties. In the study, while comparison of genotypes was done with $LSD_{(0.05)}$ (Least significant degree), the yields of genotypes for years were compared with the "t-test" and the following equation (Eq.2) below.

$$\% \text{ Difference: } ((\text{Value}_{2018} - \text{Value}_{2017}) / \text{Value}_{2017}) * 100 \quad \text{(Eq.2)}$$

Results and Discussion

Seed cotton yield (kg ha^{-1})

Seed cotton yields of genotypes differed over the years (*Table 2*). In the cotton season of 2018, the total average and maximum temperature was 3.1 ^{o}C and 14.2 ^{o}C more than 2017, and the minimum temperature was 13.3 ^{o}C less in total. As reported by Salman et al. (2019), heterozygous hybrids and homozygous Gloria responded positively to climate change unlike Flash cultivar while Sure Grow 125 responded negatively that was statistically non-significant. While the seed cotton yield of hybrids increased from 284.8 kg ha^{-1} (2017) to 322.9 kg ha^{-1} (2018), the average of controls decreased from 3179.2 kg ha^{-1} (2017) to 3045.6 kg ha^{-1} (2018), and the average of the region decreased from 3112.9 kg ha^{-1} (2017) to 3082.3 kg ha^{-1} (2018).

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DOI: http://dx.doi.org/10.15666/aeer/1905_34533465

Table 1. *Climate data of Antalya province in 2017 and 2018 with long term average*

MONTHS	Average Temperature (ºC)				Minimum Temperature (ºC)				Maximum Temperature (ºC)				Average Relative Humidity (%)			Precipitation (mm)			
	2017	**2018**	**LT**	**Dif.**	**2017**	**2018**	**LT**	**Dif.**	**2017**	**2018**	**LT**	**Dif.**	**2017**	**2018**	**Dif.**	**2017**	**2018**	**LT**	**Dif.**
January	8.5	10.8	10.0	2.3	0.3	1.7	-4.3	1.4	17.8	20.9	23.9	3.1	68.7	72.2	3.5	56.0	10.8	242.1	-45.2
February	10.4	12.8	10.6	2.4	-1.0	3.4	-4.6	4.4	21.8	21.2	26.7	-0.6	62.0	83.0	21.0	5.0	91.0	154.4	86.0
March	13.1	15.0	12.8	1.9	1.7	6.8	-1.6	5.1	24.4	25.8	28.6	1.4	71.5	78.9	7.4	70.0	94.0	97.2	24.0
April	16.4	18.5	16.3	2.1	4.4	6.7	1.4	2.3	31.6	35.2	36.4	3.6	69.2	68.7	-0.5	27.0	2.0	50.4	-25.0
Total	48.4	57.1	49.7	8.7	5.4	18.6	-9.1	13.2	95.6	103.1	115.6	7.5	271.4	302.8	31.3	158.0	197.8	544.1	39.8
Average	12.1	14.2	12.4	3.5	2.2	7.4	-2.3	5.3	38.2	41.2	28.9	3.0	108.6	121.1	12.5	63.2	79.1	136.0	15.9
May	20.5	23.2	20.5	2.7	12.1	11.9	6.7	-0.2	33.5	35.6	38.7	2.1	73.0	66.2	-6.8	35.0	19.0	32.1	-16.0
June	25.8	25.5	25.3	-0.3	15.5	16.3	11.1	0.8	44.5	38.0	44.8	-6.5	66.4	72.8	6.4	0.0	65.0	10.9	65.0
July	29.4	28.5	28.4	-0.9	18.3	18.2	14.8	-0.1	44.8	43.3	45.0	-1.5	62.0	65.8	3.8	0.0	18.0	4.5	18.0
August	27.9	28.0	28.3	0.1	19.0	17.2	13.6	-1.8	40.3	40.8	44.6	0.5	72.3	71.2	-1.1	0.0	0.0	4.6	0.0
September	25.2	25.9	25.1	0.7	14.7	15.2	10.3	0.5	36.9	40.7	42.5	3.8	72.4	65.1	-7.3	0.0	13.0	18.1	13.0
October	19.7	20.4	20.5	0.7	19.7	7.2	4.9	-12.5	19.7	35.5	38.7	15.8	64.9	67.3	2.3	29.0	24.0	72.1	-5.0
Total	148.4	151.5	148.1	3.0	99.3	86.0	61.4	-13.3	219.7	233.9	254.3	14.2	411.0	408.4	-2.6	64.0	139.0	142.3	75.0
Average	24.7	25.2	24.6	0.5	16.6	14.3	10.2	-2.2	36.6	39.0	42.4	2.4	68.5	68.1	-0.4	10.7	23.2	23.7	12.5
November	14.4	15.7	15.4	1.3	3.1	7.2	0.0	4.1	32.2	31.5	33.0	-0.7	74.0	72.5	-1.5	48.0	57.0	133.6	9.0
December	12.0	11.5	11.6	-0.5	0.8	0.0	-1.9	-0.8	25.9	21.6	25.4	-4.3	81.8	78.0	-3.8	74.0	156.0	265.3	82.0
Total (Year)	223.2	235.7	224.8	12.5	108.6	111.8	50.4	3.2	373.4	390.1	428.3	16.7	838.2	861.5	23.4	344.0	549.8	1085	205.8
Average (Year)	17.2	18.1	18.73	1.0	8.4	8.6	4.2	0.2	28.7	30.0	35.7	1.3	64.5	66.3	1.8	26.5	42.3	90.4	15.8

LT: Long term average (1930-2019), Dif: Difference (2018-2017)

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DOI: http://dx.doi.org/10.15666/aeer/1905_34533465

Table 2. *Analysis of variance of the parameters studied*

Sources	Degree of freedom	Seed cotton yield (kg ha^{-1})	Ginning outturn (%)	Lint yield (kg ha^{-1})	Plant height (cm)	Number of boll per plant (count)	Boll weight (g)	Number of sympodial branches (count)	Number of monopodial branches (count)
Replications	2	4752.83	0.18	935.92	382.93	10.45	1.62	0.44	1.62
Years (Y)	1	7101.67	6.70	1747.52	5809.48	36.51	0.19	28.17	0.06
Genotypes (G)	4	13498.83	10.04	1894.89	167.50	37.86	1.20	5.46	2.48
Y x G	4	10929.05	4.27	1582.84	57.65	16.38	1.03	4.27	0.59
Error	18	41818.84	8.43	6567.25	2003.91	105.61	3.50	12.57	6.15
Total	29	62097.22	57.23	10081.55	32964.48	194.23	6.79	92,48	14.61
CV (%)		15.56	1.68	15.14	9.38	15.87	10.04	5.71	17.80
$LSD_{(0.05)\ Years}$		82.68 *	1.17 *	32.77	18.10 *	4.16 *		1.43 *	
$LSD_{(0.05)\ Genotypes}$		82.68	1.17 *						

CV (%): Coefficient of Variation, $LSD_{(0.05)}$: Least Significant Degree, *: $p<0.05$, **: $p<0.01$, ns: non-significant, p: Probability

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While Gloria cultivar had positive effect on averaged controls from 2742.2 kg ha^{-1} (2017) to 3052.2 kg ha^{-1} (2018), Flash had negative contribution from 3592.1 kg ha^{-1} (2017) to 2904.0 kg ha^{-1} (2018) as Sure Grow 125 from 3203.3 kg ha^{-1} (2017) to 3180.7 kg ha^{-1} (2018). In other words, climate difference had an effect of 13.38% in hybrids, -0.67% in Sure Grow 125, 11.3% in Gloria, -19.2% in Flash, -4.18% in the control mean, and -1% in the region average. This situation may have been caused by genotype x environment dissonance.

While the effect of climate difference on the seed cotton yield was more negative (Chen et al., 2015), Sure Grow 125 was found to be important for both producers and the continuation of the breeding work with the potential to reflect the climate difference to the yield at a minimum rate, and Gloria and hybrids with potential to increase the yield of the region (*Figure 1*).

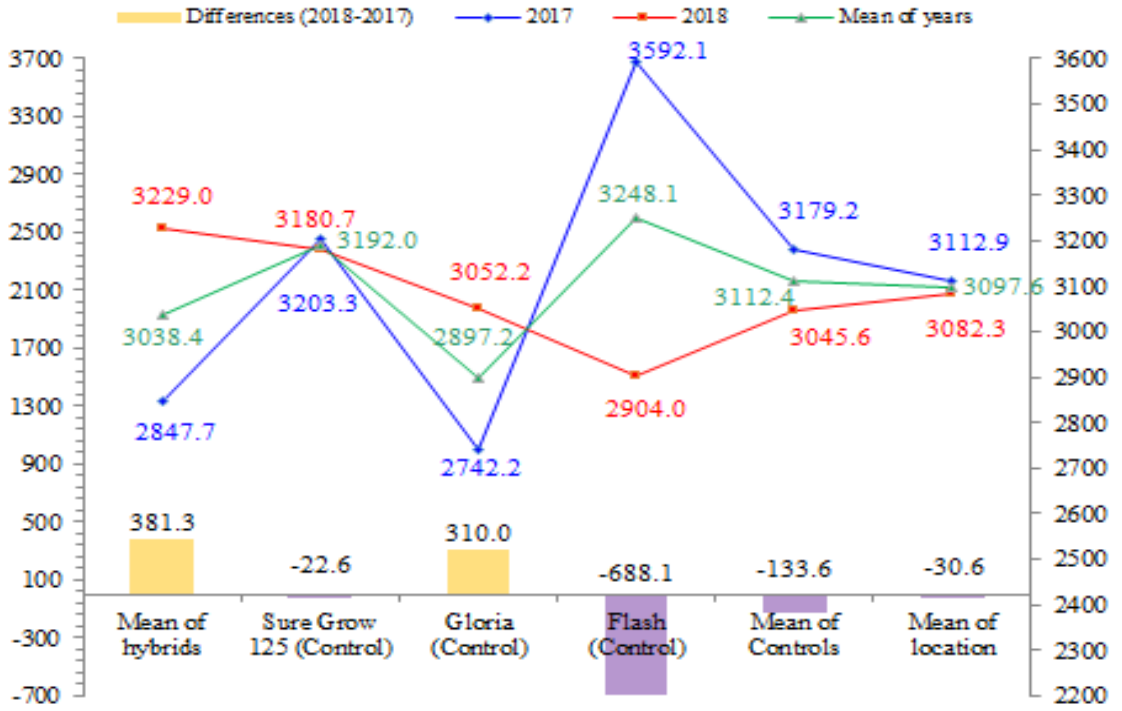

Figure 1. *Seed cotton yield (kg ha^{-1}), average and difference values of the genotypes for years*

Ginning outturn (%)

While genotypes respond to climate change between years by reducing ginning outturn, genotype difference has been found significant (*Table 2 and Figure 2*). The greatest reduction was observed in heterozygous hybrids. Ginning outturn reduced from 42.8% (2017) to 41.0% (2018) in hybrids, from 41.6% (2017) to 39.8% (2018) in the average of controls, from 41.9% (2017) to 39.7% (2018) in the region average.

While Sure Grow 125 was the least affected variety from climate difference and Gloria was the most affected (*Figure 2*), the ginning efficiency decline between years was -8.18% in hybrids, -2.96% in Sure Grow 125, -539% in Gloria, and -5.01% in Flash, -4.33% in the average of controls and -5.25% in the regional average. While climate change reduces ginning outturn in both homozygous and heterozygous

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genotypes, Sure Grow 125 has been found to be important for producers and breeding programs as the least affected variety (*Figure 2*).

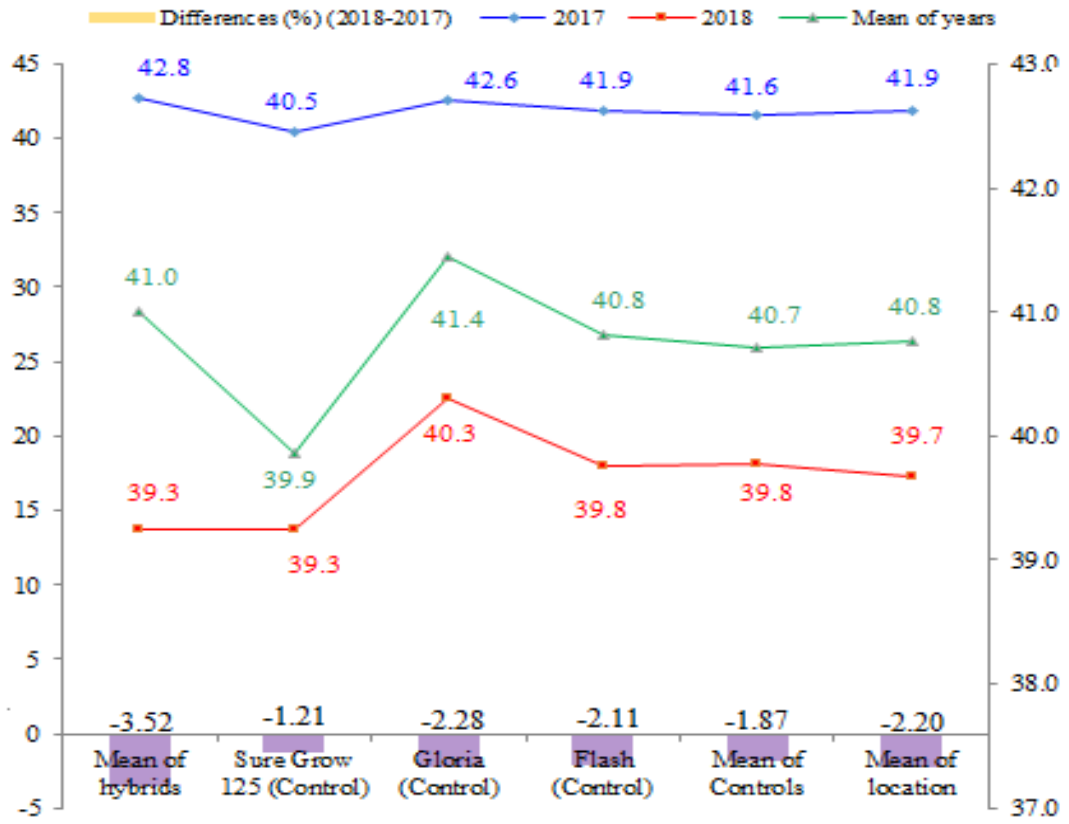

Figure 2. *Ginning outturn (%) average and difference values of the genotypes for years*

Lint yield (kg ha^{-1})

Fiber yield was positively and significantly correlated with seed cotton yield and ginning outturn. In the study where the difference in genotypes and years was significant (*Figure 3*), the fiber yield increased from 122.4 kg ha^{-1} (2017) to 126.9 kg ha^{-1} in hybrids, the average of controls decreased from 132.1 kg ha^{-1} (2017) to 121.1 kg ha^{-1} (2018), and region average decreased from 130.1 kg ha^{-1} (2017) to 122.2 kg ha^{-1} (2018).

Gloria had positive effect on average of controls (from 116.8 kg ha^{-1} (2017) to 122.8 kg ha^{-1} (2018)] while Flash [from 149.9 kg ha^{-1} (2017) to 115.7 kg ha^{-1} (2018)] and Sure Grow 125 [from 129.6 kg ha^{-1} (2017) to 124.7 kg ha^{-1} (2018)] gave negative contribution. The climate difference of the years had positive effect on fiber yield in hybrids (3.68%) and Gloria (5.14%), while Sure Grow 125 (-3.78%), Flash (-22.82%), average of controls (-8.33%) and region average (-6.07%) were negatively affected. Sure Grow 125 was the least affected by climate difference and the control with the highest fiber potential. On the other hand, hybrids had the genetic potential to increase the yield of the region, while the most sensitive variety to climate difference was Flash (*Figure 3*).

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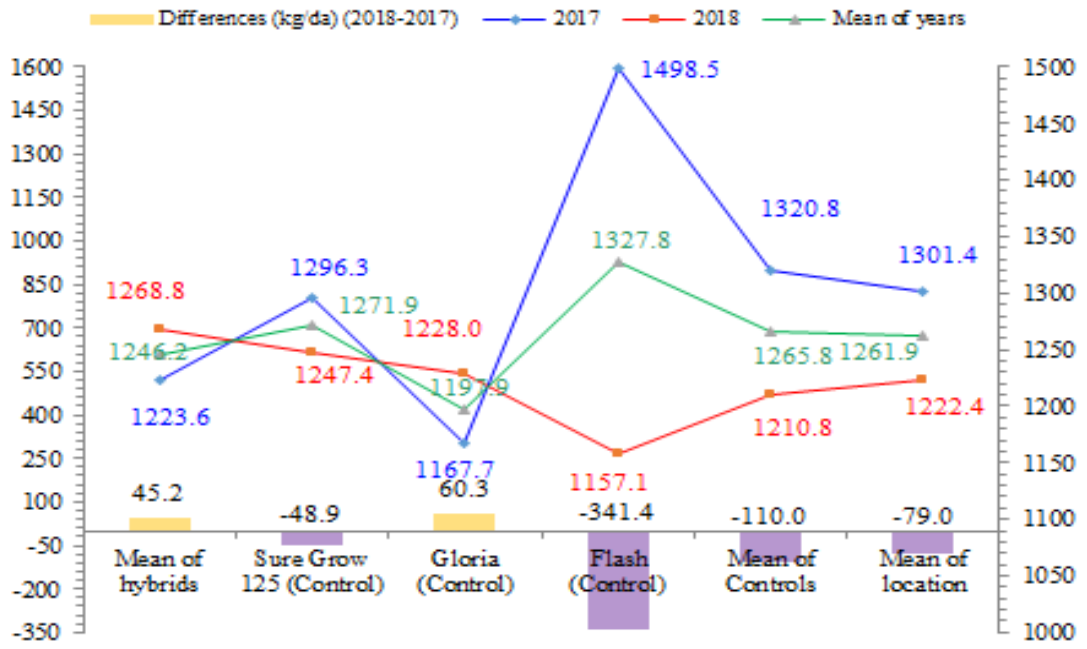

Figure 3. *Fiber yield (kg ha^{-1}) average and difference values of the genotypes for years*

Plant height (cm)

The plant height, which positively correlates with the seed and fiber cotton yields, evaluated climate differences in the direction of vegetative development (*Table 2 and Figure 4*).

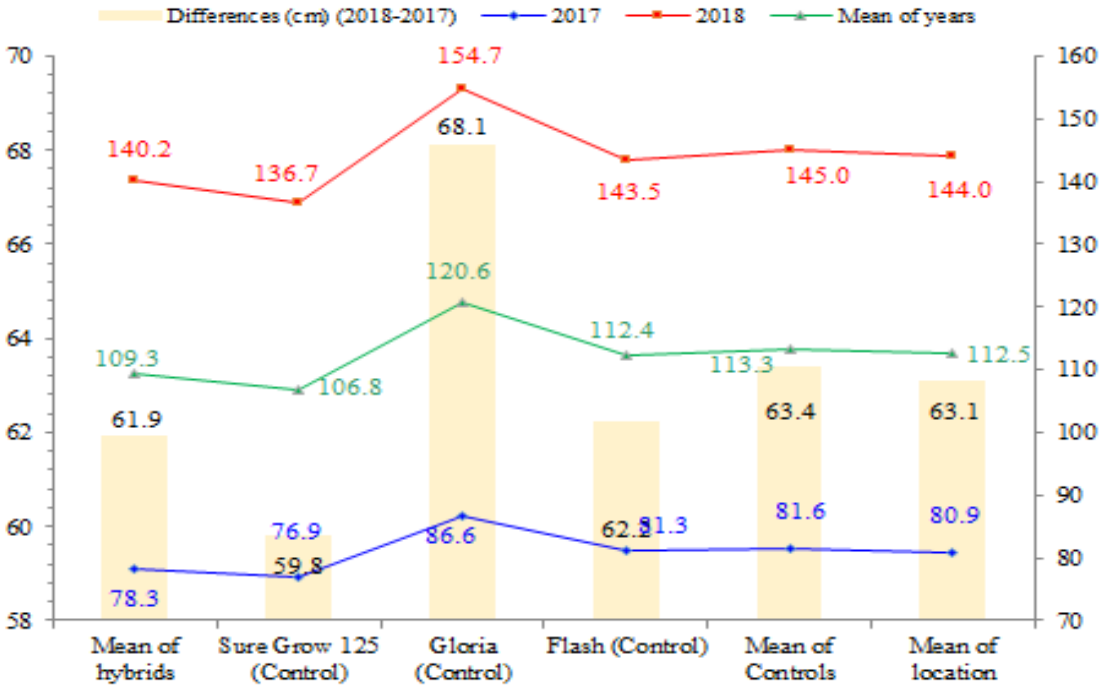

Figure 4. *Plant height (cm), average and difference values of the genotypes for years*

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83 mm precipitation, which fell in July and August 2018, increased atmospheric humidity and caused the temperatures to remain low. Vegetative branches production of plant increased because of average/minimum temperature ratios in June, July and August in 2018 that were incompatible with optimum temperature ratio (30/22 °C) of Reddy et al. (1992). Therefore, the plant height increased from 78.3 cm (2017) to 140.2 cm (2018) in hybrids, from 81.6 cm (2017) to 145 cm (2018) in the control mean and from 80.9 cm (2017) to 144 cm (2018) in the region means (*Figure 4*) but ginning outturn of the region has decreased as well as the seed and fiber cotton yield. While the highest contribution to the average of the controls was from Gloria with 68.1 cm, Sure Grow 125 gave the lowest contribution.

Number of bolls per plant

The number of bolls in a plant is the most important feature that contributes positively to the yield with its boll weight (Worley et al., 1974; Rauf et al., 2006; Srinivas et al., 2014). Since the climatic conditions of 2018 (*Table 1*) encouraged vegetative growth and delayed generative development, the number of bolls of hybrids and varieties decreased.

The number of bolls decreased from 16.1 (2017) to 14.6 (2018) in hybrids, from 16.5 (2017) to 14.0 (2018) on the average of controls, from 16.4 to 14.1 (2017) in the region average that was confirmed by Zhao et al. (2005). While Flash was the biggest contributor to this decrease, Gloria was the most tolerant variety. In other words, climate difference had an impact of -9.32% in hybrids, -11.76% in Sure Grow 125, -3.40% in Gloria, -25.39% in Flash, -15.15% in control mean, and -14.02% in region average. Gloria was found to be important for breeding programs and producers as the most tolerant variety for climate difference (*Figure 5*).

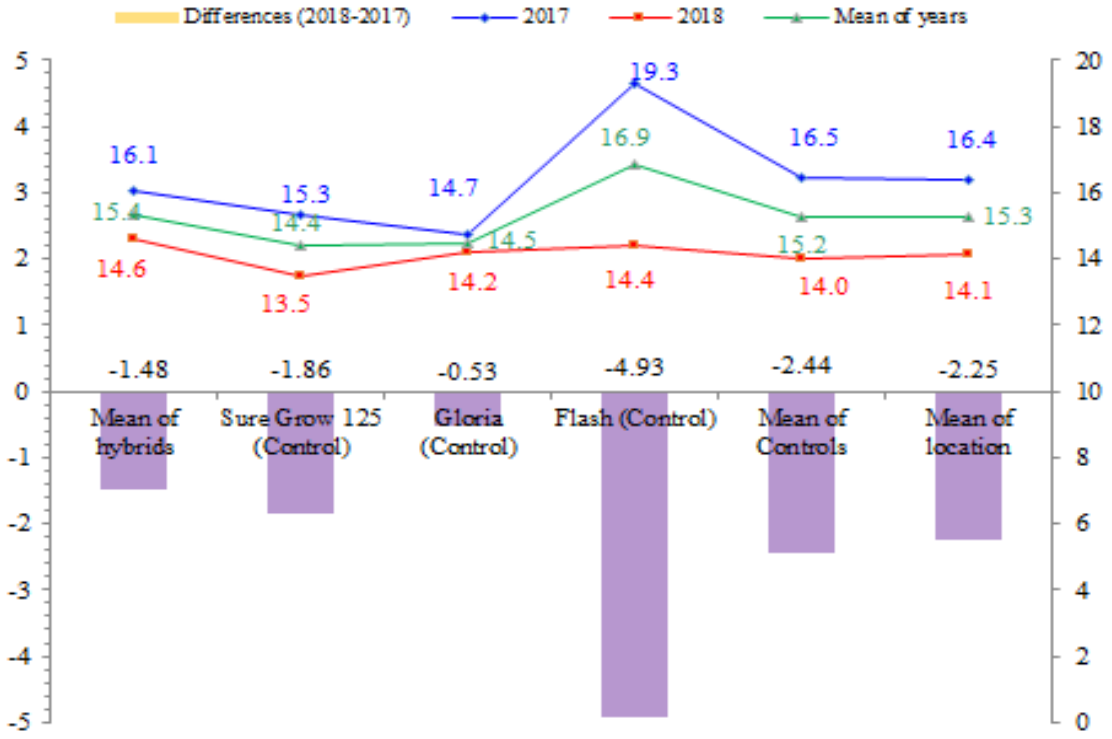

Figure 5. *Number of bolls per plant, average and difference values of the genotypes for years*

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Boll weight (g)

Seed cotton yield per boll is an important feature with a positive correlation with cotton yield (Khalid et al., 2018). The boll weights of hybrids and other controls increased in 2018, except for Sure Grow 125. This was found to be important in terms of reducing the negative impact of climate difference on number of bolls and yield. Boll weight increased from 4.06 g (2017) to 4.28 g (2018) in hybrids, from 4.41 g (2017) to 4.47 g (2018) in control averages, from 4.34 g (2017) to 4.43 g (2018) in region average. Gloria [from 4.07 g (2017) to 4.49 g (2018)] and Flash [4.32 g (2017) to 4.69 g (2018)] had positive effect on average of controls, while Sure Grow 125 [from 4.23 g (2017) to 4.84 g (2018)] had a negative contribution.

In other words, climate difference contributed to the boll weight by 5.42% in hybrids, -12.60% in Sure Grow 125, 10.32% in Gloria, 8.56% in Flash, 1.36% in controls average, and 2.07% in region average. While Sure Grow 125 is the most affected variety of climate change in terms of boll weight, Gloria has been found to be important for breeding programs and producers as the most tolerant variety (*Figure 6*).

Number of fruiting (sympodial) branches

Although the number of fruiting branches is one of the characteristics that positively affect the cotton yield, the number of fruiting branches that sheds bolls is a problem as vegetative growth.

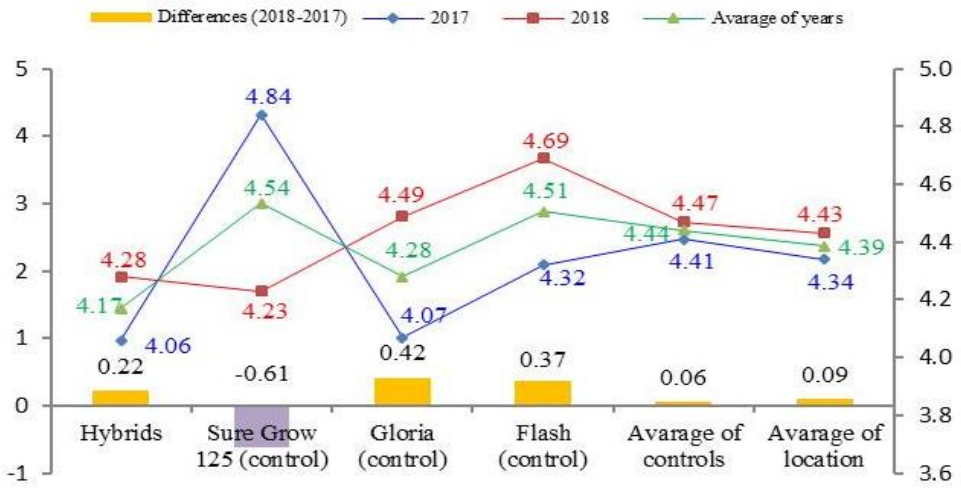

Figure 6. *Boll weight (g), average and difference values of the genotypes for years*

In the study, both homozygous (controls) and heterozygous (hybrids) genotypes increased the number of fruiting branches in 2018 (*Table 2 and Figure 7*), while Flash had the highest number of fruiting branches. Number of sympodial branches increased from 12.9 (2017) to 15.9 (2018) in hybrids, from 13.2 (2017) to 16.2 (2018) in control averages, and from 13.1 (2017) to 16.1 (2018) an average of region that is comparable with Ekinci et al. (2017). In other words, the climate difference contributed the number of fruiting branches as 23.25% in hybrids, 20.8% in Sure Grow 125, 13.98% in Gloria, 33.33% in Flash, 22.72% in the control average, and 22.90% in the region average. While climate change affects Flash variety at most in terms of number of fruiting branches, Gloria was found to be the most tolerant variety for breeding programs and producers (*Figure 7*).

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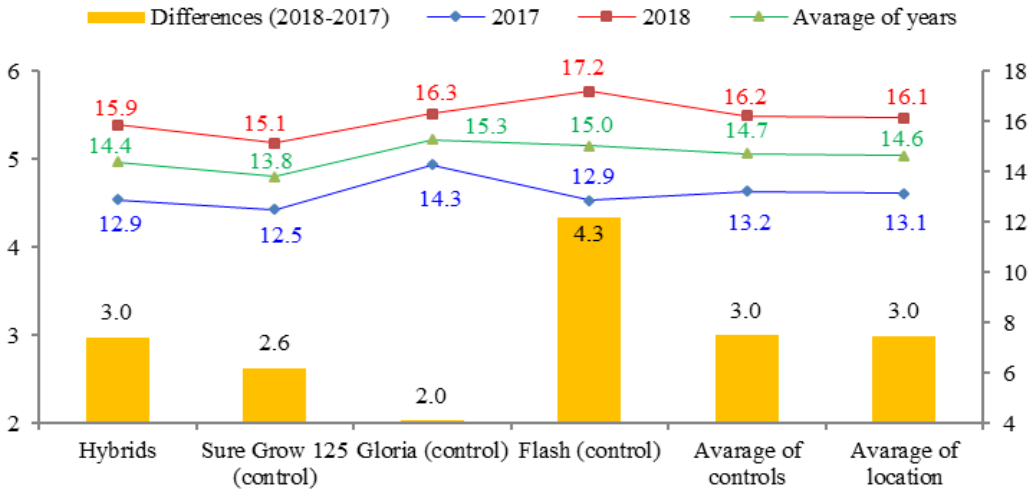

Figure 7. *Number of sympodial branches, average and difference values of the genotypes for years*

Number of vegetative (monopodial) branches

Vegetative branch having bolls affects yield positively, and the non-retainer affects negatively since it is a vegetative shoot. In the study, the number of monopodial branches of other controls and hybrids, except Gloria, decreased in 2018. The number of monopodial branches reduced from 3.77 (2017) to 3.03 (2018) in hybrids, from 3.37 (2017) to 3.14 (2018) in control averages, and from 3.45 (2017) to 3.12 (2018) in region average.

In other words, climate difference contributed to the number of vegetative branches -19.63% in hybrids, -18.73% in Sure Grow 125, 1.84% in Gloria, -5.00% in Flash, -6.83% in the control mean and -9.57% in the region average. While climate difference affects hybrids and Sure Grow 125 the most, Gloria was found to be important for breeding programs and producers as a tolerant variety (*Figure 8*).

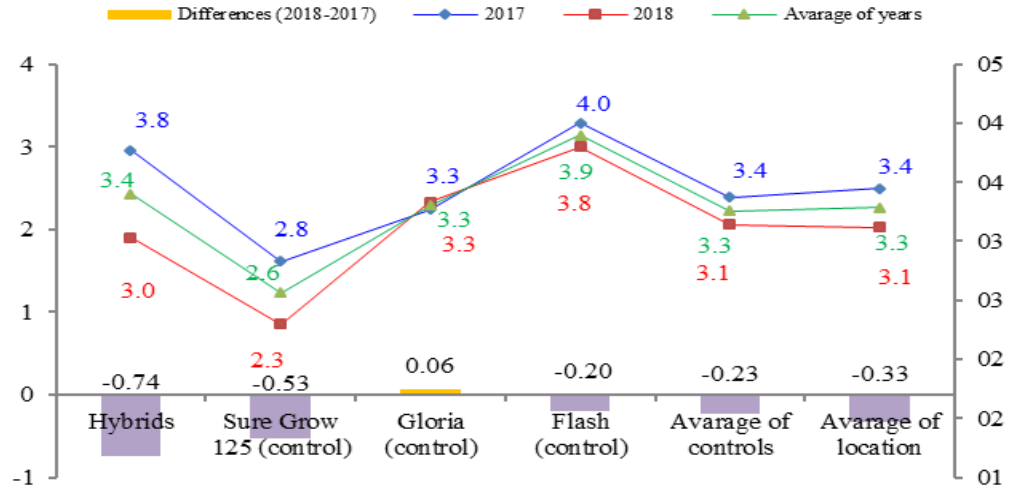

Figure 8. *Number of monopodial branches, average and difference values of the genotypes for years*

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Conclusion

Genotypes differ in terms of ginning outturn compared to the averages of 2017 and 2018. In addition, the differences between years in terms of seed cotton yield, ginning outturn, plant height, number of bolls and number of fruiting branches were also found significant. Hybrids have increased the boll weight and decreased the number of vegetative branches, reducing the potential negative impact of the number of bolls per plant and ginning outturn that are negatively affected by the climate of 2018 on the seed cotton yield and fiber yield. On the other hand, it was determined that the variation continues in hybrids in terms of seed cotton yield, ginning outturn, plant height and number of fruiting branches. Therefore, when suitable plants are selected, the yield of the region may increase. Sure Grow 125, which is one of the controls, is found to be tolerant for the year difference in terms of seed cotton yield, ginning outturn, fiber yield and number of bolls per plant, and is sensitive in terms of boll weight. On the other hand, Gloria was tolerant for fiber yield, number of bolls per plant, number of vegetative branches and had also potential for seed cotton yield, fiber yield, boll weight and number of fruiting branches. Flash was the most sensitive control type in terms of seed cotton yield, ginning outturn, fiber yield, number of bolls per plant and number of fruiting branches. At the end of the study, it has been determined that climate difference will have the least impact on Sure Grow 125, while hybrids have the potential to increase the yield of the region.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_34533465

EFFECT OF NITROGEN ON THE METABOLIC ENZYME ACTIVITY OF LEAVES, PROTEIN CONTENT AND YIELD OF SORGHUM (*SORGHUM BICOLOR* [L.] MOENCH) IN NORTHERN CHINA

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(Received 25th Jan 2021; accepted 14th May 2021)

Abstract. With the improvement of breeding level and mechanization, sorghum planting has achieved the transformation from high-stalk and rare-planting to dwarf and high-density planting in China, but there are few reports on dwarf sorghum under high-density planting condition. Therefore, the aim of this work was to study the effect of different nitrogen doses (0, 100, 200 and 300 kg of nitrogen ha^{-1}) on its metabolic enzyme activity, the leaf area and Chlorophyll, so as to explore changes of yield and protein content of dwarf sorghum under the high-density planting management in northern China. The results of the two-year trials indicated that chlorophyll content, leaf area and the activity of nitrate reductase (NR) and glutamine synthetase (GS) were positively correlated with yield and protein, 200 kg of nitrogen ha^{-1} as the best rate, promoted metabolism enzyme activity, raised leaf area and chlorophyll content, and ensured the highest yield and protein content.
Keywords: *sorghum, nitrogen fertilizer, yield, enzyme, high-density planting*

Introduction

Sorghum bicolor (L.) Moench is the main food and economic crop in arid and semi-arid areas and is widely used in feed, brewing, energy, food processing and other fields (Wang et al., 2015). Nitrogen (N) is a vital component required for the synthesis of chlorophyll and photosynthetic enzymes, which impact the photosynthesis of crops, its application can determine the overall yields of crops (Kaur et al., 2015). In plants, photosynthetic capability and nitrogen metabolism are closely related. Approximately 25% of the energy generated by photosynthesis can be used for nitrate reduction (Khripach et al., 2000; Beevers and Hageman, 1969; Giagnoni et al., 2016). Nitrate is mainly assimilated in plant leaves. The activity of NR is modulated by the photosynthetic electron transport chain (Chow et al., 2015).

At the same time, nitrogen is one of the most important elements in the process of carbon and nitrogen metabolism. NR, glutamate Synthase (GOGAT), GS and glutamate dehydrogenase (GLDH) are the main enzymes that affect nitrogen metabolism, their activity is closely related to soil fertility (Tischne, 2000; Singletary et al., 1990;

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DOI: http://dx.doi.org/10.15666/aeer/1905_34673479

Singaram and Kamalakumari, 2000). The utilization of nitrogen in crops involves the assimilation, transport and reuse of the absorbed nitrogen, in which the assimilating enzymes NR, GS and GOGAT play a key role. Inorganic nitrogen absorbed by sorghum must be assimilated by the plant, and further synthetized into amino acids, proteins and other substances necessary for the organism (Bingham et al., 2012). NR is the first enzyme in the process of nitrogen reduction, the presence of nitrate will promote the activity of NR (Nath and Tuteja, 2016; Lu et al., 2009). GS/GOGAT pathway is the most important pathway for further assimilation of ammonia under normal conditions (Zheng, 2009; Lea and Miflin, 2003).

The catalytic glutamine continues to generate glutamic acid by GOGAT, which involves carbohydrate assimilation and is the key node of carbon nitrogen interaction (Lea and Miflin, 2003). The seed weight increased after overexpression of GOGAT gene in rice, and pointed out that GOGAT played an important role in grain filling (Yamaya et al., 2002). Previous studies on corn (Presterl et al., 2010), wheat (Han et al., 2007; Xiong et al., 2016), rice (Zeng et al., 2007) and other crops showed that NR and GS activities of plants decreased under low nitrogen, but there were differences among different crops. The application of N can be conducive towards the enhancement of the drought resistance of crops by protecting photosynthetic apparatus, activating antioxidant defense systems, and improving osmoregulation, affecting growth and N metabolism (Gou et al., 2017). Most crops require N within an appropriate range that optimally aligns with their physiological requirements, the excessive application of N results in decreased crop yields (Jin et al., 2012). Methods to limit the negative impact of agricultural practices and increasing crop production sustainability has been one of the key agricultural challenges (Ronga et al., 2019; Tilman et al., 2011).

In recent years, sorghum has undergone a transformation from traditional tall-stalk and rare-planting to dwarf and high-density planting (Wang et al., 2011), The height of the main sorghum varieties in the north of Heilongjiang province are between 1.0 and 1.3 m, and the planting density is between 20 -35 $\times$ 10^4 plants/ha (Yang et al., 2015; Shen et al., 2013), which is the area with the largest sorghum production density in China. To the authors' knowledge, the research on dwarf sorghum under high-density planting management was still in a blank stage. In this paper, Keza15, a density-tolerant dwarf sorghum variety, was used as the experimental material. Through the study on the relationships between nitrogen and nitrogen metabolism enzyme activity, the leaf area, Chlorophyll, the regularity of yield and quality formation of high-density cultivation was expounded. It has a great significance to improve the efficiency of nitrogen utilization, to lay a foundation for the reasonable application of nitrogen fertilizer to dwarf sorghum under high-density planting condition.

Materials and methods

Trial materials

Keza15 was obtained from the Keshan branch of the Heilongjiang Academy of Agricultural Sciences (Qiqihar City, Heilongjiang Province, China).

Note: Keza15 was a density-tolerant brewing sorghum hybrid variety, plant height was approximately 100 cm, panicle length was 26.5 cm, the seeds produced reddish-brown oval-shaped grains. Optimal planting density was 300 thousand plants/hectare (*Fig. 1*).

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Figure 1. *Morphological characteristics of Keza15. (A) Seeds, (B) plant, (C) group*

Trial conditions

Field experiments were conducted in an open field at Keshan Branch of Heilongjiang Academy of Agricultural Sciences (48°03′47″N, 125°87′57″E) (Qiqihar City, Heilongjiang Province, China), the experimental soil was a chernozem. The 0-20 cm soil in the plow layer had the following characteristics: organic matter 3.2×10^4 mg·kg^{-1}, pH 6.12, alkali hydrolyzed nitrogen 173 mg·kg^{-1}, available phosphorus 28.8 mg·kg^{-1} and available potassium 307.2 mg·kg^{-1}. The region has a mid-temperate continental monsoon climate, The mean maximum and minimum air temperatures and total rainfall during the cropping cycles (May to September) were 29 and 8.3 °C and 316.5 mm for the year 2016 and 29.3 and 7.8 °C and 357.4 mm for the year 2017, respectively (*Fig. 2*).

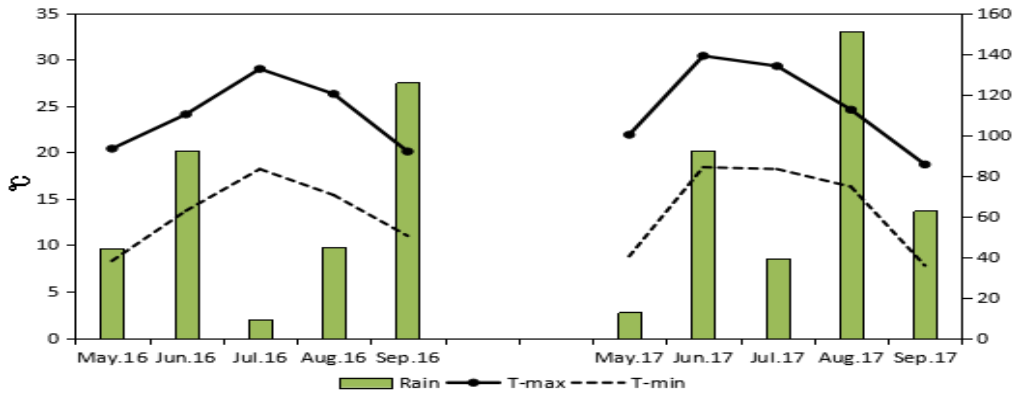

Figure 2. *The mean maximum and minimum air temperatures and total rainfall during the cropping cycles (May to September) recorded in the two growing seasons (2016 and 2017)*

Four nitrogen treatments were set up, and urea was used as the nitrogen source: 0 kg (N0), 100 kg (N100), 200 kg (N200) and 300 kg (N300) of pure nitrogen per hectare in both years.; nitrogen, P and K (P_2O_5 150 kg and K_2O 100 kg per hectare) were applied as seed fertilizer at one time. A randomized block design was used in the experiment with three repetitions. Every experiment repetition was 10 m long, consisting of eight rows with a 0.65 m ridge distance and 300,000 seedlings per hectare, and the management was the same as that of the general production fields.

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Sample handling

Plants of growth uniformity were selected as markers, and the samples were taken at Jointing stage, Flag leaf stage, Loose powder period, Grain filling stage, and at Max mature period.

Determination of leaf area

The leaf area was measured with a CI-203 handheld laser leaf area meter (CID, Inc., Camas, Washington USA).

Determination of chlorophyll

The method of the determination of chlorophyll content was referred to the modified method cited (Wang, 2006). The following formulas were used to calculate the content of photosynthetic pigments, in which V and W indicated the volume of extracting liquid (ml) and the weight of material (g).

$$\text{Chlorophyll a (Ca)} / \text{mg} \cdot \text{g}^{-1} = (12.21A_{663} - 2.81A_{646})\, V/ (1000 \times W) \quad \text{(Eq.1)}$$

$$\text{Chlorophyll b (Cb)} / \text{mg} \cdot \text{g}^{-1} = (20.13A_{646} - 5.03A_{663})\, V/ (1000 \times W) \quad \text{(Eq.2)}$$

$$\text{Chlorophyll} = \text{Chlorophyll a} + \text{Chlorophyll b} \quad \text{(Eq.3)}$$

Determination of nitrogen metabolism related enzyme activity

The activity of NR, GS, GOGAT and GLDH was determined by enzyme-linked immunosorbent assay. The kit was provided by Shanghai Enzyme-linked Biology Co., Ltd. (Shanghai, China), and the determination method was carried out according to the instructions.

Yield measurement

Grain yield and its composition: 20 plants were selected for yield determination. The indices included single ear weight, grain number per ear and thousand kernel weight. The yield was converted by the weight at 14% water content.

Quality measurement

Protein content was measured with a Near infrared quality analyzer (perten DA7200, Swedish).

$$\text{Protein accumulation}(\text{mg.seed}^{-1}) = \text{Protein content}(\%) \times 1000 - \text{grains weight}/1000(\text{mg.seed}^{-1}) \quad \text{(Eq.4)}$$

Analysis software and analysis method

The data were processed by Excel 2013 and were analyzed by SPSS 16.0. Analysis of variance was performed with SPSS 16.0 software, and data from each sampling data were analyzed separately. Means were tested by least significant difference at the $P < 0.05$ level.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_34673479

Results

Effects of nitrogen on leaf area per plant and chlorophyll of sorghum

The results in *Table 1* show that the leaves area per plant was the largest in the loose powder period and the smallest in maturity period. The leaf area per plant of the N0 treatment was significantly lower than that of other treatments. In 2016, the leaf area per plant of N200 treatment were significantly higher than that of N100 treatment. From the beginning of the grain filling stage, the leaf area per plant of the N200 treatment was significantly higher than that of other treatments, and there was no significant difference between the N100 and N300 treatments. In 2017, there was no significant difference of the leaf area per plant of the nitrogen application treatment in the jointing stage and the grain filling stage, the leaf area per plant of the N300 and N200 treatments in the loose powder period were significantly higher than that of the N100 treatment.

Table 1. *Effect of nitrogen on the leaf area per plant*

Years	Treatments	Leaf area per plant (cm^2)				
		Jointing stage	Flag leaf stage	Loose powder stage	Grain filling stage	Wax maturity stage
2016	N0	1830.11±37.65c	2078.61±16.50c	2234.27±39.12c	1994.95±43.67c	1484.96±25.53c
	N100	2273.24±46.41b	2458.95±51.36b	2506.60±50.36b	2414.66±50.09b	1827.45±45.56b
	N200	2433.48±60.62a	2590.64±16.97a	2674.68±42.52a	2631.50±41.53a	1960.99±48.98a
	N300	2475.86±29.89a	2499.38±22.83ab	2588.37±36.80ab	2497.75±10.48b	1815.59±22.22b
2017	N0	1934.98±72.29b	2110.66±46.06c	2291.26±22.45c	2138.35±62.95b	1645.66±22.43c
	N100	2484.13±15.93a	2541.70±21.55b	2706.80±14.15b	2616.15±49.27a	1937.31±28.43a
	N200	2549.97±30.85a	2607.04±4.35ab	2809.66±13.52a	2714.87±28.16a	2012.43±23.06a
	N300	2554.78±24.59a	2643.73±23.66a	2812.57±26.50a	2689.15±25.80a	1813.26±45.57b

Mean ± standard deviation. Values sharing same letters differ non-significantly ($P \leq 0.05$)

The analysis of *Table 2* shows the chlorophyll content of Keza 15 first increased and then decreased with the development of the growth period, and the N0 treatment was significantly lower than the nitrogen treatment. There was no significant difference of the chlorophyll content between N200 and N300 except for the grain filling stage in 2017. The chlorophyll content increased with the increase in the nitrogen application, But the increase of chlorophyll content was not more significant than in N200 treatment.

Table 2. *Effect of nitrogen on chlorophyll content*

Years	Treatments	Chlorophyll content (mg/g)				
		Jointing stage	Flag leaf stage	Loose powder stage	Grain filling stage	Wax maturity stage
2016	N0	3.40 ± 0.21b	3.98 ± 0.05b	4.15 ± 0.05b	3.94 ± 0.04c	1.66 ± 0.04b
	N100	4.93 ± 0.04a	5.05 ± 0.03a	5.22 ± 0.05a	4.30 ± 0.02b	2.54 ± 0.02a
	N200	5.10 ± 0.07a	5.13 ± 0.06a	5.31 ± 0.03a	4.57 ± 0.06a	2.59 ± 0.01a
	N300	5.14 ± 0.06a	5.17 ± 0.04a	5.25 ± 0.04a	4.52 ± 0.04a	2.61 ± 0.03a
2017	N0	4.04 ± 0.11b	4.16 ± 0.03c	4.28 ± 0.06b	3.26 ± 0.04d	1.64 ± 0.02c
	N100	5.12 ± 0.05a	5.25 ± 0.03b	5.69 ± 0.03a	4.36 ± 0.03c	2.63 ± 0.01b
	N200	5.15 ± 0.04a	5.44 ± 0.04a	5.63 ± 0.04a	4.70 ± 0.02a	2.72 ± 0.03a
	N300	5.13 ± 0.06a	5.42 ± 0.01a	5.63 ± 0.05a	4.57 ± 0.03b	2.72 ± 0.02a

Mean ± standard deviation. Values sharing same letters differ non-significantly ($P \leq 0.05$)

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_34673479

Effects of nitrogen on the activity of NR, GOGAT, GS, GLDH in high density sorghum

It can be seen from *Figure 3* that NR activity increases with the increase of nitrogen application, and the nitrogen application treatment in seedling stage is significantly higher than that in non-nitrogen application treatment. From flag picking stage to the end of grouting, there is a significant difference between the four nitrogen treatments. In the early stage of grouting, the activity reaches the peak, the lowest in seedling stage, and the N300 treatment in mature stage is significantly higher than that in N0 and N100.

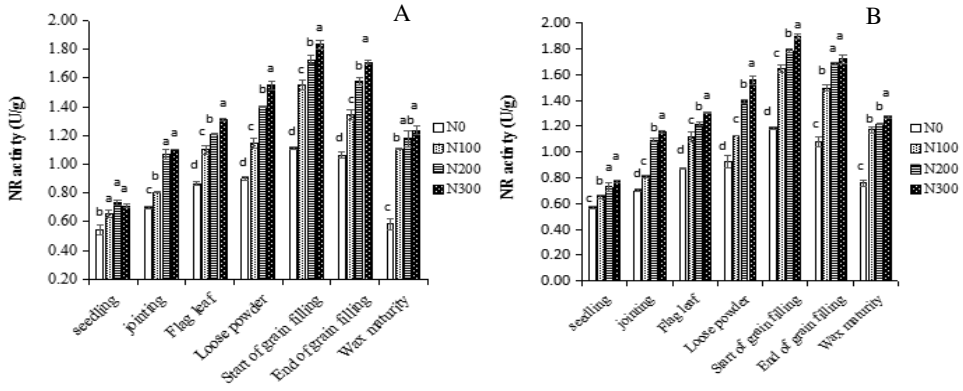

Figure 3. *Effect of nitrogen on NR activity of sorghum leaf (A) 2016, (B) 2017. Different letters indicate different means, according to LSD test ($p < 0.05$)*

It can be seen from *Figure 4* that there was no significant difference in activity of GOGAT among the nitrogen application treatments in the seedling stage, jointing stage and wax mature stage in 2016; there was a significant difference among the treatments in the flag leaf stage; there was no significant difference between the N200 and N300 treatments from the powder stage to the late filling stage, and there was a significant increase in the N200 and N300 treatments compared with the other nitrogen treatments. In 2017, the activity of GOGAT between the N200 and N300 treatments have no significant difference in the jointing stage, late grain filling stage and max maturity stage, but it was significantly higher than in the N0 and N100 treatments; the N300 treatment in the flag leaf stage and loose powder stage was significantly higher than other treatments.

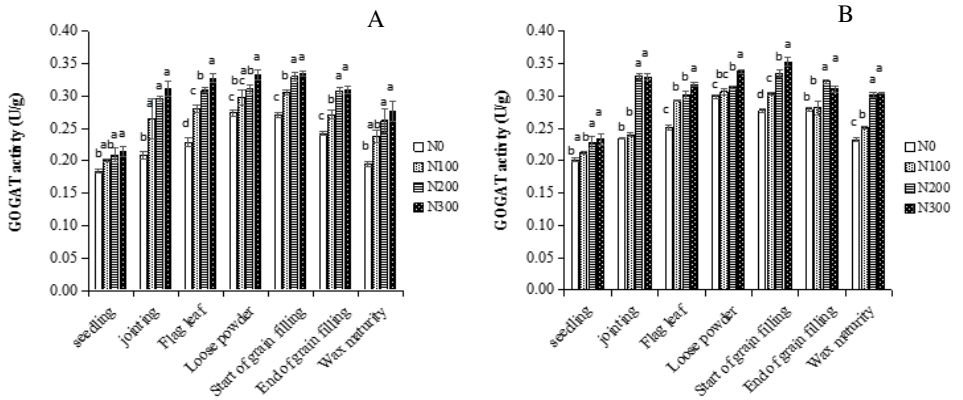

Figure 4. *Effect of nitrogen on GOGAT activity of sorghum leaf (A) 2016, (B) 2017. Different letters indicate different means, according to LSD test ($p < 0.05$)*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_34673479

It can be seen from *Figure 5* that with the increase in the nitrogen level, GS activity increased. In 2016, the N100, N200, and N300 treatments were significantly higher than the N0 treatment except for the seeding stage, and N200, and N300 had no significant difference among the nitrogen treatments except the seedling stage, jointing stage, start of grain filling stage. From the jointing stage, the GS activity of N300 was significantly higher than that of N100. In 2017, the GS activity of the N200 and N300 treatments showed no significant difference in the seedling stage, powder stage and wax maturity stage, but it was significantly higher than in the N0 and N100 treatments. The GS activity of the N300 treatment was the highest and that of the N0 treatment was the lowest.

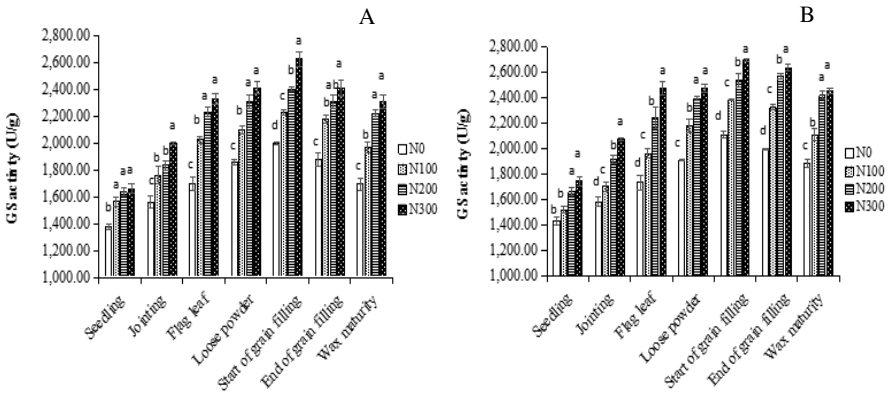

Figure 5. *Effect of nitrogen on GS activity of sorghum leaf (A) 2016, (B) 2017. Different letters indicate different means, according to LSD test ($p < 0.05$)*

It can be seen from *Figure 6* that the activity of GLDH N200 and N300 were significantly higher than that of the N0 treatment. In 2016, the activity of GLDH in the N300 treatment was significantly higher than the other treatments in the jointing stage and early filling stage. The activity of GLDH showed no significant difference between the N200 treatment and the N100 and N300 treatments in the loose powder stage and late filling stage, which were significantly higher than those in the N0 treatment. In 2017, the activity of GLDH in the N300 treatment was significantly higher than in the other treatments. The activity of GLDH showed no significant difference between the N0 and N100 treatments in the powder stage but decreased significantly compared with other treatments. The activity of GLDH in the N200 and N300 were significantly higher than in other treatments at the early filling and wax stages.

Effect of nitrogen on yield and quality

The analysis of *Table 3* shows that in a certain range, the grain weight per ear of sorghum increased with the increase in nitrogen application, but when the nitrogen level exceeded that of the N200 treatment, the grain number per ear no longer continued to increase and began to decline, the change trend is the same as the yield. The 1000-grain weight of the N0 treatment was significantly lower than that of the N100, N200 and N300 treatments, but there was no significant difference between the N100, N200 and N300 treatments.

The effect of nitrogen on sorghum protein increased with the increase in nitrogen application, and there was no significant difference between N200 and N300. It can be seen from the experiment that the effect of nitrogen on protein was the same in different years.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_34673479

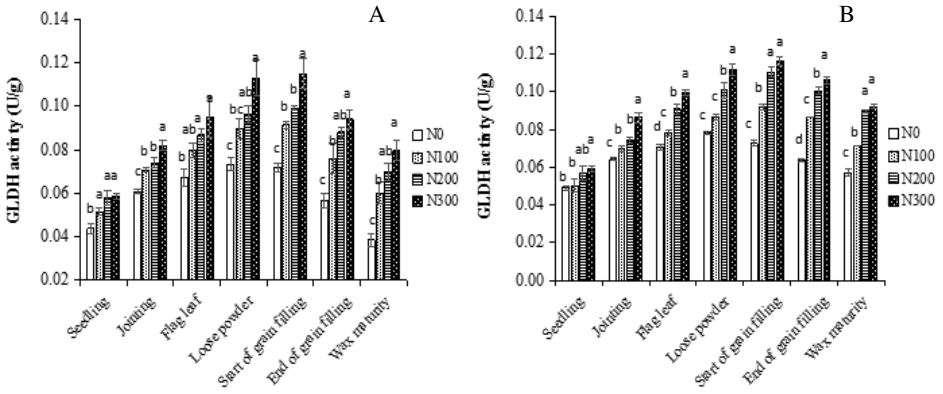

Figure 6. *Effect of nitrogen on GLDH activity of sorghum leaf (A) 2016, (B) 2017. Different letters indicate different means, according to LSD test ($p < 0.05$)*

Table 3. *Effect of nitrogen level on yield and protein accumulation of sorghum*

Years	Treatments	Protein accumulation (mg·grain^{-1})	The grain number per ear (Li)	Thousand grain weight (g)	Yield (kg/hm^2)
2016	N0	1.20 ± 0.01c	1009.00 ± 6.00c	20.44 ± 0.35b	6186.40 ± 118.89c
	N100	2.57 ± 0.05b	1066.00 ± 11.36b	24.30 ± 0.24a	7770.63 ± 27.76b
	N200	2.95 ± 0.05a	1104.70 ± 4.91a	24.53 ± 0.24a	8128.30 ± 95.85a
	N300	2.96 ± 0.06a	1069.00 ± 11.68b	24.15 ± 0.36a	7742.43 ± 97.39b
2017	N0	1.42 ± 0.02c	1018.00 ± 10.21b	20.34 ± 0.45b	6210.82 ± 75.92c
	N100	1.82 ± 0.02b	1087.70 ± 16.41a	25.37 ± 0.58a	8278.23 ± 61.97a
	N200	2.11 ± 0.09a	1069.30 ± 6.39a	25.76 ± 0.58a	8262.74 ± 89.85a
	N300	2.27 ± 0.06a	1002.00 ± 4.51b	25.28 ± 0.41a	7600.17 ± 64.28b

Mean ± standard deviation. Values sharing same letters differ non-significantly ($P \leq 0.05$)

Correlation analysis of enzyme activity, leaf area per plant, chlorophyll content with yield and quality

To find out the relationship between the effects of nitrogen on the physiological and biochemical characters of leaves and the yield and protein of sorghum correlation analysis was carried out. The analysis of *Table 4* shows that the chlorophyll content and leaf area of a single plant were significantly positively correlated with the yield and protein content in different growth periods, and the correlation coefficient is above 0.900. The activity of NR, GOGAT, GS, GLDH NR was positively correlated with protein content for two years.

There was no significant correlation between NR activity and yield at jointing stage and loose powder stage in 2017, but there was a significant positive correlation between NR activity and yield at other stages, and the correlation coefficient was the largest at wax ripening stage. GOGAT activity showed a significant correlation with production in 2016 but showed no significant correlation with production in 2017. GS activity was positively correlated with yield in 2016, there was no significant correlation between the jointing stage in 2017. GLDH content had a significant correlation with yield in 2016-2017, with the largest correlation coefficient in the grouting period.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_34673479

Table 4. *Correlation of enzyme activity, leaf area per plant, chlorophyll content with yield and quality*

Years	Project	Jointing stage		Flag leaf stage		Loose powder stage		Grain filling stage		Wax maturity stage	
		Yield	Protein	Yield	Protein	Yield	Protein	Yield	Protein	Yield	Protein
2016	Chlorophyll	0.938**	0.957**	0.950**	0.977**	0.973**	0.981**	0.902**	0.936**	0.960**	0.975**
	Leaf area	0.926**	0.941**	0.963**	0.976**	0.914**	0.919**	0.956**	0.957**	0.908**	0.939**
	NR	0.760**	0.812**	0.848**	0.896**	0.787**	0.838**	0.810**	0.866**	0.943**	0.961**
	GOGAT	0.750**	0.813**	0.861**	0.890**	0.632*	0.711**	0.821**	0.867**	0.801**	0.811**
	GS	0.733**	0.787**	0.843**	0.886**	0.792**	0.854**	0.832**	0.871**	0.811**	0.862**
	GLDH	0.747**	0.804**	0.674*	0.688*	0.633*	0.702*	0.805**	0.829**	0.778**	0.846**
2017	Chlorophyll	0.932**	0.811**	0.900**	0.883**	0.929**	0.810**	0.902**	0.898**	0.920**	0.866**
	Leaf area	0.880**	0.832**	0.869**	0.907**	0.879**	0.903**	0.871**	0.841**	0.923**	0.609*
	NR	0.539	0.948**	0.726**	0.938**	0.566	0.926**	0.803**	0.930**	0.864**	0.899**
	GOGAT	0.442	0.871**	0.745**	0.922**	0.293	0.780**	0.426	0.721**	0.562	0.914**
	GS	0.487	0.926**	0.51	0.897**	0.684*	0.942**	0.726**	0.984**	0.655*	0.951**
	GLDH	0.371	0.808**	0.482	0.883**	0.469	0.898**	0.730**	0.974**	0.653*	0.952**

ns, no significance; *$p \leq 0.05$; **$p \leq 0.01$

Discussion

Nitrogen utilization is an important physiological activity in plant growth and development. Chlorophyll content, leaf area index, regulation of enzymes have a great impact on nitrogen absorption and utilization (Worku et al., 2012). Leaf senescence and its chlorophyll content, and photoassimilates distribution, affect crop yield and are important parameters that should be evaluated in plant growth and crop yield improvement studies (Ronga et al., 2015; Mosisa and Habtamu, 2007). If too much nitrogen is transported to the grain too early, the nitrogen content in the leaves will be reduced, and the photosynthesis of the plant will be affected, which will lead to a reduction of crop yield (Mi et al., 2012).

It can be seen from the analysis of this experiment that under the condition of dense planting, the leaf area and chlorophyll content of sorghum increased with the increase of nitrogen fertilizer and reached the peak value under N200 treatment. The leaf area and chlorophyll content of N200 and N300 treatments were significantly higher than that of N0 and N100 treatments from jointing stage to loose powder stage, which indicated that the higher nitrogen treatment in this period was beneficial to leaf area and chlorophyll content. The leaf area of N300 treatment was lower than that of N200 treatment (*Table 1*) from the filling grain stage to the wax mature stage. We analyzed that the growth of N300 leaves, and plants was too vigorous, resulting in poor ventilation, the lower leaves could not receive light radiation, resulting in yellowing and wilting under the condition of high density. It may also be due to excessive nitrogen nutrition, resulting in blocked physiological metabolism. From the filling stage to the waxing stage, the chlorophyll content of N200 and N300 was significantly higher than that of N0 and N100 (*Table 2*), which indicated that the higher nitrogen level after the filling grain stage was beneficial to the chlorophyll content of dwarf sorghum. *Table 4* analysis shows that leaf area and chlorophyll content were significantly correlated with yield and protein, and the correlation coefficient was the largest from loose powder stage to filling stage, indicating that leaf area and chlorophyll content will play a positive role in yield and quality of dwarf sorghum in this period.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_34673479

The increase in nitrogen application can promote the activities of NR and GS, the ability of nitrogen absorption and assimilation after flowering, and the content of grain protein (Wang et al., 2002). The activities of enzymes related to leaf nitrogen metabolism are directly affected by the level of soil fertilizer supply (Tischner, 2000). The appropriate amount of nitrogen fertilizer can improve the activities of NR, GS, GOGAT and GLDH in the leaves of maize at the later stage of growth, but excessive application of nitrogen fertilizer will reduce their activities (Zhang et al., 2002; Liu et al., 2007; Geng et al., 2009; Li et al., 2018). In this study, the activities of NR, GS, GLDH and GOGAT in sorghum leaves were significantly reduced by low nitrogen treatment, ranked as N300 > N200 > N100 > N0, indicating that the activities increased with the increase in nitrogen.

The highest value of NR activity in the early stage of grain filling was 103.97%, 137.02%, 135.79% and 157.95% higher than the lowest value of nitrogen treatments in the seedling stage in 2016, and 106.82%, 151.92%, 144.93% and 147.02% higher in 2017. The results showed that lower nitrogen significantly reduced NR activity and the extent of the increase range. The GS activity of Keza15 reached the peak value in the early stage of grain filling, and the difference between the N300 and N0 treatments was 27.78%. Although the activities of GOGAT and GLDH were positively correlated with nitrogen, there were significant differences between the years.

The activity of NR, NIR and GOGAT decreased from flag leaf stage to grain filling stage, while the activity of GS remained stable or even increased, which may be related to the important function of GS in the process of nitrogen reuse (Zheng, 2009; Lea et al., 2003) It was found that the activities of NR, GOGAT, GS and GLDH were kept at a high level from the flag leaf stage to the filling grain stage, and there was no trend of decline, indicating that the application of enzyme activity to nitrogen of dwarf sorghum lasted for a long time.

Increasing nitrogen fertilizer can improve the accumulation of photosynthetic products, improve the use of light energy by sorghum (Wang et al., 2015; Zhou et al., 2016). A suitable amount of nitrogen fertilizer is the key to obtain high yield, high benefit and high nitrogen use efficiency (Liang et al., 2017). The results of the study showed that the yield of sorghum increased first and then decreased with the increase in nitrogen application. The yield of the N0 treatment was significantly lower than that of nitrogen application treatments. Although nitrogen improved the yield of sorghum, too much nitrogen was harmful to the yield of sorghum. When nitrogen exceeded N200, the number of grains per ear no longer continued to increase and began to decrease, change trend and significance are the same as yield change. However, there was no significant difference in 1000 grain weight among different nitrogen treatments. The results showed that the correlation between the yield of sorghum and the number of grains per ear was more significant than that of 1000 grains under the condition of dense planting.

Our research showed that not only leaf area and chlorophyll are important factors to improve yield and quality of high-density planting dwarf sorghum, but also enzyme activity related to nitrogen metabolism is one of the important reasons for high yield. The higher the activity of nitrogen metabolism related enzymes, the stronger the ability of nitrogen assimilation, the more chlorophyll and photosynthetic related enzymes can be synthesized to improve the yield and quality. The higher enzyme activity, green leaf area and chlorophyll content from the filling grain stage to the wax mature stage will play an important role in the formation of yield, which can be used as a reference index for high yield of dwarf sorghum.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Conclusion

Leaf area, chlorophyll content, the activity of NR and GS were the most significant correlation between yield and protein content of dwarf sorghum. The loose powder stage to grain filling stage was an important period for yield and quality formation. The yield of dwarf sorghum was mainly determined by the number of grains per ear, 200 kg of nitrogen ha^{-1} was the most beneficial to yield and protein of dwarf sorghum. It will be our research goal to study the mechanism of nitrogen metabolism of dwarf sorghum under high-density planting condition.

Acknowledgements. This work was supported by the China Agriculture Research System (CARS-06-135-A17)

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DOI: http://dx.doi.org/10.15666/aeer/1905_34673479

ENVIRONMENTAL IMPACTS OF HERBICIDE TOLERANT CROPS AND GLYPHOSATE-BASED HERBICIDES – A REVIEW

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(Received 19th Apr 2021; accepted 19th Jul 2021)

Abstract. Glyphosate is a broad-spectrum, non-selective, contact herbicide, dominating the global pesticide market and the most widely used agricultural chemicals worldwide, to manage pre- and post-emergence weeds. Despite the fact that glyphosate and glyphosate-based herbicides are widely used, and claimed as a "once in a century herbicide", there remains extensive debate on the consequences of glyphosate usage and its impacts on soil, plant, and environmental health, apart from non-targeted vegetation. Though positive effects of glyphosate on agricultural food production, soil conservation and environmental pollution have been put forth by several workers, glyphosate and its negative impacts on the environment, especially its persistence in soils, the emergence of glyphosate-resistant weeds, and its integration into the existing cropping systems in agroecosystems remains a challenge. In this review, we provide updates on glyphosate and glyphosate-based herbicides, and their impacts on the environment, which will be highly useful for researchers and decision-makers to establish policies for glyphosate and glyphosate-based herbicide usage in agriculture.
Keywords: *glyphosate, soil persistence, health impacts, soil functions, food chain, microbial and faunal diversity*

Introduction

Glyphosate (N-(phosphonomethyl) glycine) is a broad-spectrum, nonselective, contact (foliar-applied) herbicide. Glyphosate was commercialized in 1974, and has been widely used to control pre- and post-emergence weeds (grass and broadleaved weeds) in agriculture (Nandula, 2010). Glyphosate inhibits the enzyme 5-1-enolpyruvylshikimate-3-phosphate synthase (EPSPS) involved in the shikimate pathway, leading to build up of shikimate and reducing the synthesis of aromatic amino acids, which are necessary for plant survival (Duke and Powles, 2008). Herbicide-tolerant (HT) crops consistently occupy the largest area of genetically modified (GM) crops, and the most frequently used HT crops are engineered to express *cp4-epsps*, the product of which is not inhibited by the herbicide, "glyphosate" (Duke, 2005). Glyphosate based herbicides (GBHs) are at present the most heavily applied herbicides in the world, and the use of GBHs is likely to rise on the event of approval of Roundup Ready glyphosate-tolerant, worldwide (Benbrook, 2012). The countries that have approved HT include Argentina, Australia, Brazil, Canada, China, Colombia, Costa Rica, EU, Japan, Malaysia, Mexico, New Zealand, Paraguay, Philippines, Singapore, South Africa, South Korea, Taiwan, USA (James, 2003). Depending on weed species, plant type, and other biotic or abiotic factors, the recommended rates of glyphosate vary largely. Consequently, enormous amounts of glyphosate enter the environment every year, and concern has grown over its possible ecological impacts (Myers et al., 2016). Glyphosate, GBHs, and HT crops are expected to affect soil,

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_34813504

plants, environment, and human well-being directly or indirectly (*Fig. 1*). With this background, in this review, we discuss the impacts of HT crops and GBHs on the environment in detail.

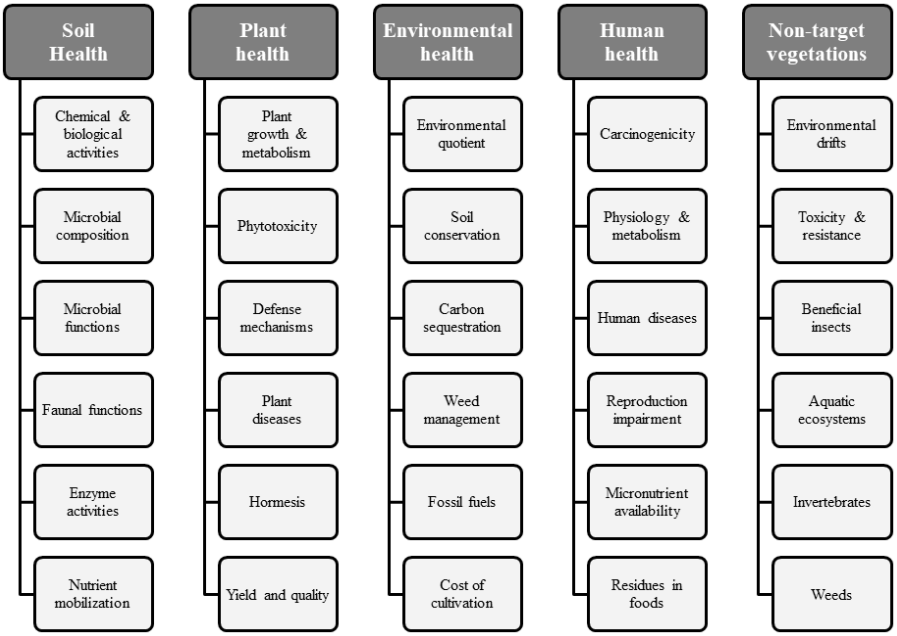

Figure 1. *Possible impacts of herbicide tolerant crops and glyphosate-based herbicides*

Glyphosate in food supply chain and its health impacts

Since the glyphosate is the most widely used herbicide in the environment (Kier and Kirkland, 2013), its long-term use at global scale, not only affect the soil, water, and air, but its entry into the food chain through ingestion are of great health concerns (Bai and Ogbourne, 2016; Torretta et al., 2018) (*Fig. 2*). Glyphosate residues have been detected in several environmental samples such as water, air, food, and feed through drifting, leaching, and surface runoff (Mensah et al., 2012). Furthermore, the drift and dispersal of glyphosate in the soil-water environment can damage-living organisms including aquatic life (Williams et al., 2000; Bailey et al., 2017). Several studies have shown that the absorption constant of the chemical varies between 8 and 377 dm^3 kg^{-1}, depending on the soil characteristics. In water, the half-life of glyphosate shown to vary from a few days to 91 days (Vereecken, 2005; Borggaard and Gimsing, 2008). The time of application also plays a primary role in residue levels in the final product. For example, glyphosate application during harvest has been reported to increase the residue levels in soybeans (Duke et al., 2003; Arregui et al., 2004).

The glyphosate residues were reported from animal feed, animal urine, animal flesh, human food, human milk, and human urine (Acquavella et al., 2004; Borggaard and Gimsing, 2008; Krüger et al., 2013, 2014; Niemann et al., 2015), indicating greater release of glyphosate-salts (isopropyl amine, glyphosate- ammonium, glyphosate-sesquisodium, and glyphosate-trimesium) into the environment, and subsequent entry into food supply chain of increased exposure to glyphosate (Cuhra et al., 2016). In a human exposure study, involving occupationally and para-occupationally exposed subjects, the average urinary levels of glyphosate varied from 0.26 to 73.5 μg/L in

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occupationally exposed subjects. While, the environmental exposure urinary levels ranged from 0.16 to 7.6 µg/L (Gillezeau et al., 2019). Worst case exposure causing acute poisoning (cardiorespiratory toxicity) in adult humans has been reported to be 125 and 5 µg kg^{-1} day^{-1} for glyphosate and aminomethylphosphonic acid (AMPA), respectively (Williams et al., 2000). Several studies have demonstrated the possible entry route of glyphosate in the gastro-intestinal tract of humans and mammals, through inhalation, ingestion, and dermal contact affecting growth, kidney and liver functions, lymphoma, etc. (Peillex and Pelletier, 2020). Glyphosate and GBHs provoke oxidative damage in rats (liver and kidneys) through the disruption of mitochondrial metabolism at exposure levels, which is currently considered safe (Mesnage et al., 2015). Further, studies detected higher levels of glyphosate and AMPA in the tissues of farm animals compared to their usual levels in fat, and increased frequency of kidney disease among the male agricultural workers, who were exposed to heavy GBHs uses (Jayasumana et al., 2014). In vertebrates, glyphosate and GBHs interrupted endocrine-signaling systems and steroid hormones (Thongprakaisang et al., 2013). GBHs are linked to increased risk of developing non-Hodgkin's Lymphoma (NHL) among humans (Schinasi and Leon, 2014). As a chelating agent, glyphosate and GBHs can affect micronutrient availability to living beings, including crops and animals (Johal and Huber, 2009).

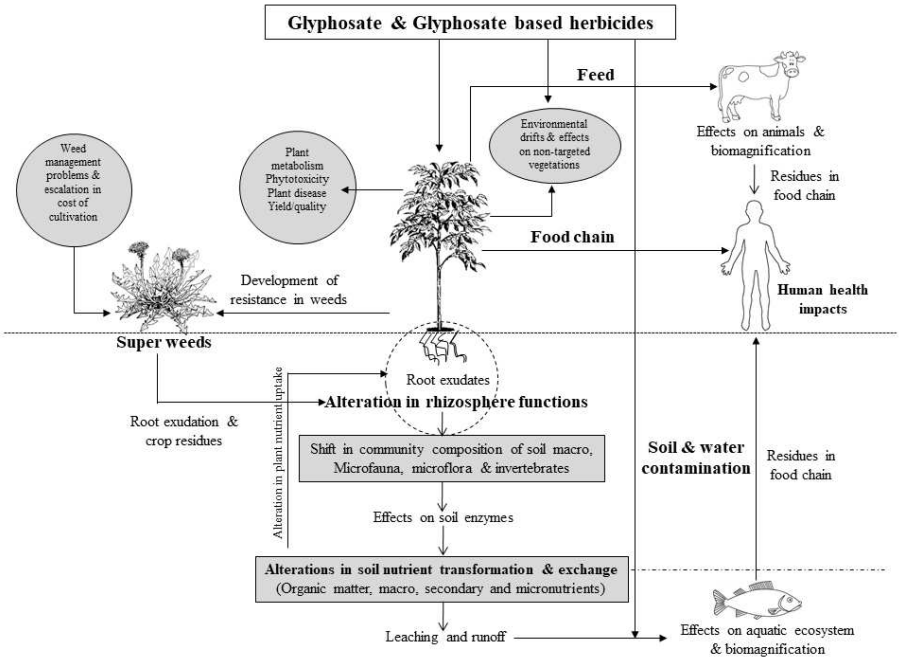

Figure 2. *Environmental fate of glyphosate and glyphosate-based herbicides*

Similarly, in plants, glyphosate altered the functions such as photosynthesis, respiration, and the synthesis of essential aromatic amino acids (Williams et al., 2000; Samsel and Seneff, 2013; Kruger et al., 2014; Bailey et al., 2017). Traces of glyphosate have been recorded in wheat flour, oats, bread (Székács and Darvas, 2012), honey (Rubio et al., 2014), and beers (reuters.com). In Italy, 100 food products based on flour (corn flakes, rusks, pasta, spaghetti) and 26 samples of drinking water showed traces of glyphosate (Corvino, 2015; Test-Salvagente, 2016). Human milk showed glyphosate

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DOI: http://dx.doi.org/10.15666/aeer/1905_34813504

presence in the range of 76 to 166 µ/L. However, this level is considered acceptable by the Environmental Protection Agency (EPA) (momsacrossamerica.com). Traces of glyphosate were detected in 85% of tampons, medical gauze (cotton), panty liners (ecowatch. com; reuters.com). In Germany, human urine samples contained traces of glyphosate in the range of 0.17 to 3.5 µ/L (slowfood.com). Similarly, the children and young people who had worked in the agricultural sector found have higher traces of glyphosate in their urine (foodnavigator.com).

The US Environmental Protection Agency (USEPA) classified glyphosate as a suspected human carcinogen (Category C) in 1985. However, long-term administration studies showed limited evidence of carcinogenicity in animals and inadequate data on humans' carcinogenicity (Rubio et al., 2014). Subsequently, in 1991, EPA included glyphosate in the E category (substances that do not show carcinogenic potential) based on animal and epidemiological studies (www.epa.gov). In 2015, the International Agency for Research on Cancer (IARC) classified glyphosate as "probably carcinogenic to humans", with the insertion of the 2A category (substances with limited evidence of carcinogenicity to humans and sufficient evidence for animals) (IARC, 2015). In 2015, based on the technical assessment of glyphosate by an institution of a member state (German Federal Institute for Risk Assessment-BfR), the European Food Safety Authority (EFSA) concluded that it was "improbable" that the pesticide was genotoxic or carcinogenic to humans (EFSA, 2015). However, subsequent analyses of the toxicological data have concluded that glyphosate is unlikely to pose a genotoxicity or carcinogenic risk to humans (EFSA, 2017; USEPA, 2019). Subsequently, the EFSA proposed new toxicological safety thresholds to improve the control of glyphosate residues in food i.e. (i) Increasing the ADI (Acceptable Daily Intake) or DGA, that is the daily human consumption limit, from 0.03 mg/kg to 0.05 mg/kg, in line with the acute reference dose (ARD), always fixed at 0.05 mg/kg body weight, (ii) The admissible exposure level of the operator (Laeo) was fixed at 0.01 mg/kg of body weight per day (www.efsa.europa.eu). Later, in 2016, the joint expert committee of FAO-WHO on pesticide residues in the environment and food, concluded that "glyphosate is unlikely to lead to carcinogenic risk for humans as a consequence of exposure through the diet" (FAO, 2016).

Persistence, degradation and residual effects

The microbial action is the primary mode of glyphosate mineralization in the soils, and the glyphosate rapidly degrades in the non-sterile than sterile soils, indicating the role of microorganisms in the glyphosate degradation (Borggaard and Gimsing, 2008). In most of the soils, the bulk of glyphosate and its primary metabolite (AMPA) is found on the surface soil (Okada et al., 2016), and the glyphosate does not readily move from most soils to either ground- or surface water (Borggaard and Gimsing, 2008), however, its leaching be faster in the soils with higher macropores (higher content of sand and gravel). On an average, >1% of the glyphosate applied was reported to be lost as runoff. Compared to glyphosate, the AMPA presence in ground/surface water is much higher due to its mobile nature in the soils (Kjaer et al., 2005). GBHs contaminated drinking water via rainwater, surface runoff and leaching into groundwater (Battaglin et al., 2014). Spraying of higher doses of glyphosate above the recommended levels is reported to cause higher runoff. However, glyphosate in surface water was reported to ultimately adsorb onto the soil sediments, where it undergoes biological degradation (Wang et al.,

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2016). Though a small body of literature discusses the bioremediation of soils with high glyphosate content (Zhan et al., 2018), no studies have reported the persistence of glyphosate and AMPA in soils with long-term use in glyphosate-resistant (GR) crops (Duke et al., 2018). Further, the lack of crop yield reduction in soils with long-term usage of glyphosate indicates that if glyphosate has accumulated in such soils, it is not bioavailable as a herbicide (Duke et al., 2018; Reddy et al., 2018).

The glyphosate and its metabolites are readily water-soluble, which makes them difficult to build up or bio-magnify in nature (Duke, 2020). Glyphosate is mineralized via two enzymatic routes in soils; the major one is by glyphosate oxidoreductase, which produces AMPA and glyoxylate (*Fig. 3*). Glyoxylate is a common metabolic compound, whereas AMPA found in the environment comes from the glyphosate degradation and degradation of phosphorus containing detergents (Botta et al., 2009). The second route of degradation is through a carbon-phosphorus (C-P) lyase that produces sarcosine (N-methyl glycine) and inorganic-PO_4. Alternatively, the transformation of glyphosate to AMPA and glyoxylate can also be performed by glycine oxidase (Pollegioni et al., 2011). Both glyoxylate and methylamine was shown to support the growth of microorganisms (Duke, 2011). The glyphosate degradation rate was reported to be faster in aerobic than anaerobic soils, with a half-life value of 1.0 to 67.7 days, and more than 85% of soil-applied glyphosate was reported to be mineralized within the first 44 days, and micromyces were reported to be the main contributor of glyphosate degradation (Alexa et al., 2010). In a broad-range of agricultural soils with different soil properties, 7%-70% of glyphosate degradation occurred in the first 32 days (Nguyen et al., 2018). There was no significant influence of tillage on the degradation of glyphosate, and around 40% of glyphosate applied was reported to dissipate by 3 days in the silt loam soils (Okada et al., 2019). The glyphosate mineralization rate was strongly correlated with soil exchangeable acidity (H^+ and Al^{3+}), exchangeable Ca^{3+} ions, and ammonium lactate-extractable potassium (Mertens et al., 2018).

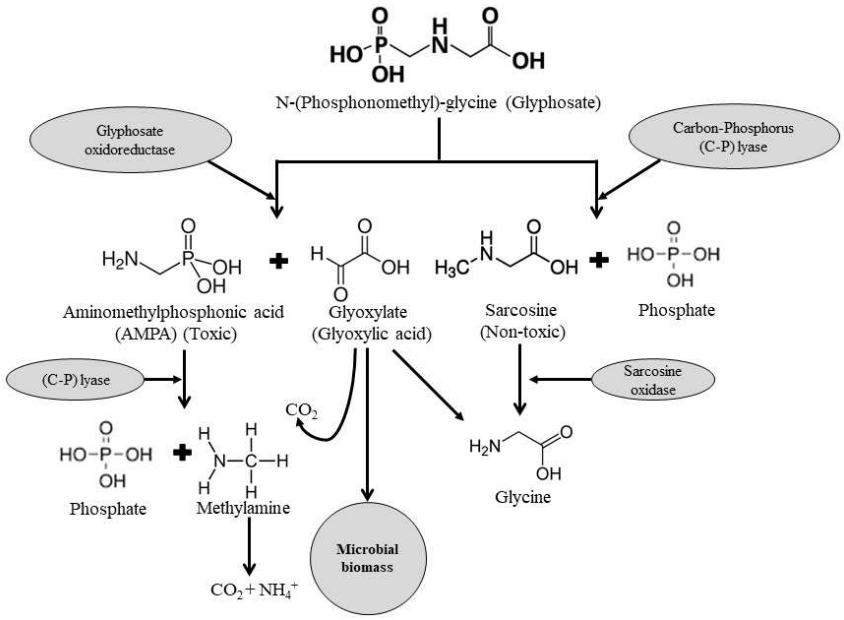

Figure 3. *Degradation pathways of glyphosate*

Glyphosate taken up by plants is reported to exude from roots or leached from plant residues (Laitinen et al., 2007). Glyphosate applied to GR and non-GR canola was

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reported to undergo a slow degradation process, thus increasing its persistence in the sprayed fields (Mamy et al., 2016). Glyphosate and AMPA residues were detected in Roundup Ready soybeans and with other associated crops such as wheat, barley, and vegetable crops (Bøhn et al., 2014). Based on their study on three soybean types (organic, conventional, and genetically modified), Bøhn et al. (2014) reported less total saturated fat and total omega-6 fatty acids in organic-soybeans compared to conventional and GM-Soybeans. Further, high levels of glyphosate (3.3 mg kg^{-1}) and AMPA (5.7 mg kg^{-1}) residues were observed in GM-Soybeans. Though, a non-significant difference in residue decomposition rates of GR-soybean and a counterpart-sensitive soybean, glyphosate application was shown to decrease the decomposition rates at the top soils, but not in the sub-soil (Powell et al., 2009). Further, there were no much differences in ratios of fungal biomass to bacterial biomass in the degrading residues (Powell et al., 2009).

Soil functions, environmental quality and environmental impact quotient

The application of glyphosate to agricultural soil was shown to increase soil respiration by 42%, while there was no clear effect on fluorescein diacetate hydrolysis (an indicator of microbial activity in soils) (Zabaloy et al., 2008). Glyphosate application also stimulate mineralization of native organic matter, carbon and nitrogen mineralization (Eser et al., 2007). There were no significant differences in soil microbial biomass (SMB), microbial respiration and nitrogen mineralization rate in soils with long-term glyphosate exposure (Busse et al., 2001). The application of glyphosate alters the organic carbon content in soils, thus influencing microbial population and their community composition (Imparato et al., 2016). However, a meta-analysis study concluded that the management and environmental factors play a significant role in soil microbial response to glyphosate application (Nguyen et al., 2106).

GBH (Roundup WeatherMax) was reported to affect soil microbial reaction to pesticides such as trifluralin, aldicarb, and mefenoxam+ pentachloronitrobenzene. Where, the soils exposed to glyphosate only exhibited greater cumulative carbon mineralization (Lancaster et al., 2008). Similarly, soil application of glyphosate + diflufenican was shown to inhibit the soil microbial biomass-Carbon (SMBC) and soil enzymes compared to the action of both the herbicides applied individually (Tejada, 2009). Growing GR-cotton, corn, and soybean did not affect the acid or alkaline phosphatase activities (Savin et al., 2009). The application of glyphosate had non-significant effects on soil dehydrogenase activity (DHA) (Zabaloy et al., 2008; Shitha, 2014). Glyphosate applications had a transient effect, without affecting the microbial community, metabolic activity, and soil exoenzyme's activities of the bulk and rhizosphere soil (Jenkins et al., 2017).

Though glyphosate applied to soils was reported to be absorbed by clay minerals, soil organic matter competes for glyphosate adsorption sites, and inhibit its adsorption in clay minerals (Gerritse et al., 1996). Glyphosate was also reported to compete with phosphate, which results in phosphorus runoff after one day of amendment (Sasal et al., 2015). Glyphosate application in soil was found not to effect on exchangeable potassium (available to plant) and non-exchangeable potassium (Lane et al., 2012), and no residual toxicity of K-salt of glyphosate was observed (Kaur and Walia, 2014). Glyphosate as a strong chelating agent, form complexes with minerals and ions, such as calcium, magnesium, manganese, iron, zinc present in soil and water, and subsequently

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make those micronutrients unavailable to plants through immobilization causing nutrient deficiencies (Glass, 1984). Complexing of Mn + glyphosate inside the plant was reported to reduce its bioavailability in soybean (Bott et al., 2008). Alternatively, some complexed ions reach plants through absorption causing ill effects in long-term exposure (Samsel and Seneff, 2013). Likewise, glyphosate application was shown to affect the rhizospheric ratios of manganese-oxidizers to manganese-reducers in the GR-soybeans, resulting in reduced solubility of manganese in soil, and subsequent reduction in plant uptake (Johal and Huber, 2009).

The adoption of GR-crops was reported to reduce herbicide usage (LC_{50}) per hectare by 100 and 500 in soybeans and cotton, respectively (Gardner and Nelson, 2008). Similarly, worldwide cultivation of GR-soybeans, corn, and cotton was found to reduce the environmental impact quotient (EIQ) by 15%, 13%, and 9%, respectively (Barfoot and Brookes, 2014). Consecutively, the EIQ values for GR-soybean, corn, cotton, canola, and sugar beet were reduced to 13%, 13%, 11%, 30%, and 19%, respectively, after two years of cultivation (Brookes and Barfoot, 2018). Though herbicide usage was found to be on the rise in corn, cotton, and soybeans in the United States, the associated herbicide acute hazard quotients declined after the adoption of GR-crops (Kniss, 2017). Among the herbicides used, the glyphosate contributed to 0.1%, 0.3%, and 3.5% of the herbicide chronic toxicity quotients in corn, soybean, and cotton, respectively, suggesting its insignificant role in contributing to the toxicity hazard of its use in agriculture (Kniss, 2017). Further, non-adoption of glyphosate as a herbicide option in agriculture is estimated to increase the EIQ of herbicide use by 0.4% to 11.6% (Brookes, 2018).

Soil conservation and carbon sequestration

At present, tillage is the primary onfarm practice followed to manage weeds in several small and marginal farms. However, long-term tillage practices caused substantial soil loss through erosion and subsequent environmental damage. In addition, soil disturbance and its movement from crop fields to other water-bodies are expected to disrupt ecosystems. Though, use of glyphosate to manage weeds is reported to avoid soil tillage operations, whereby it conserves soil and ecosystems (Cerdeira and Duke, 2006). However, since glyphosate applications kill all weeds, use of GR-crops (including intercrops, cover crops and other cropping system-based crops) became inevitable. Lessing of tillage operations facilitated by the GR crops had led to the minimization of soil loss, environmental pollution and conservation of ecosystems (Duke and Powles, 2009). Though, the impact of reduced- and no-tillage (not ploughed) operations in agriculture on environmental deterioration by adoption of GR-crops or glyphosate application has not been well documented, the greater adoption of reduced- and no-tillage operations due to GR-crops usage, and its effect on the development of GR-weeds, was well studied (Givens et al., 2009). Among several agricultural field operations, tillage was reported to contribute to higher CO_2 production in agricultural systems. Further, the adoption of GR-crops was reported to reduce fossil fuel usage and associated pollution in agriculture, worldwide (Brookes and Barfoot, 2018). Further, recently, there is a stringent competition for agricultural lands to serve several crucial services including food crops, fibre crops, oilseed crops, vegetables, etc. These essential requirements have further worsened the need for lands for intensive agriculture due to recent soil quality loss, land degradation and climatic changes (Balmford et al., 2018).

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Non-adoption of glyphosate in agriculture also increase the demand for additional agricultural land to meet the global crop production (Brookes et al., 2017), which cost both farmers and the public (Duke and Powles, 2008; Duke, 2018).

Development of glyphosate resistance in weeds

Herbicides are one the most important plant protection chemicals, which help reducing labour cost (Travlos et al., 2017). However, increased shortage of work force in agriculture for cultural control of weed species, herbicide usage is on increasing trend in several crops including cotton, corn, and wheat due to the development of herbicide-resistant weeds to commonly used herbicides in agriculture (Travlos et al., 2018). Furthermore, climate change and new cropping systems pose numerous new challenges in weed management in breaking down the development of resistance in weeds (Heap and Duke, 2018; Heap, 2020). Glyphosate is one of the most widely used herbicides globally for both agricultural and nonagricultural applications (Andert et al., 2019) accounting for one-third of the total herbicide usage in agriculture (Székács and Darvas, 2018). The over dependence on glyphosate usage in agriculture has caused the development of weeds resistant to glyphosate (Gonzalez-Torralva et al., 2012; Singh et al., 2020). Presently, around 48 glyphosate-resistant species have been reported worldwide, resulting in low herbicidal efficacy on weeds and higher weed management costs (Heap and Duke, 2018; Heap, 2020). The first case of glyphosate resistance in tall windmill grass (*Chloris elata*) was reported in Cuba (Bracamonte et al., 2017). Fernandez-Moreno et al. (2017a) reported the glyphosate resistance in perennial ryegrass (*Lolium perenne*) and Italian ryegrass (*L. multiflorum*). Glyphosate-resistant weed species were common in four weed families (Poaceae, Asteraceae, Amaranthaceae, and Chenopodiaceae) compared to other primary weed species (Heap and Duke, 2018). The genera *Lolium* (perennial ryegrass), *Chloris* (feathery Rhodes-grass or windmill grass), and *Bromus* (brome grass) in the Poaceae family; *Conyza* (horseweed) and *Ambrosia* (ragweed) genera in the Asteraceae family was shown to be more susceptible to glyphosate resistance. Similarly, *Amaranthus palmeri* (Palmer amaranth), *A. tuberculatus* (roughfruit amaranth), *A. hybridus* (smooth pigweed), and *A. spinosus* (spiny pigweed) species were more prone to glyphosate resistance in the Amaranthaceae family. In the Chenopodiaceae family, the glyphosate-resistant was most prevalent in species *Kochia scoparia* (Mexican fireweed) and *Salsola tragus* (prickly Russian thistle) (Heap and Duke, 2018).

The continued exposure to glyphosate has been shown to develop resistance to glyphosate in several weed species including Buckhorn Plantain (*Plantago lanceolate*), Common Ragweed (*Ambrosia artemisiifolia*), Common Waterhemp (*Amaranthus tuberculatus*), Giant Ragweed (*Ambrosia trifida*), Goose-grass (*Eleusine indica*), Hairy Fleabane (*Conyza bonariensis*), Horseweed (*Erigeron canadensis*), Italian Ryegrass (*Lolium multiflorum*), Johnson-grass (*Sorghum halepense*), Jungle Rice (*Echinochloa colona*), Mexican fireweed (*Kochia scoparia*), Liverseed Grass (*Urochloa panicoides*), Palmer Amaranth (*Amaranthus palmeri*), Ragweed Parthenium (*Parthenium hysterophorus*), Rigid Ryegrass (*Lolium rigidum*), Sour-grass (*Digitaria insularis*), Sumatran Fleabane (*Conyza sumatrensis*), and Wild Poinsettia (Euphorbia heterophylla) (Nandula et al., 2005).

The mechanism of resistance to glyphosate includes (i) Target site resistance (single or multiple base pair alteration, gene amplification or duplication) and (ii) Non-Target

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site resistance (enhanced metabolism, decreased absorption and translocation, sequestration) (Nandula et al., 2017; Heap and Duke, 2018). Dose-dependent development of glyphosate resistance in weeds was also reported. Where, the higher dose was shown to eliminate susceptible populations, resulting in rapid evolution of herbicide resistance in weeds (Heap and Duke, 2018), while, low herbicide dose permits the possibility for outcrossing, cross pollution, and combining in weed populations, which gains glyphosate resistance traits to endure higher glyphosate rates (creeping resistance) (Gressel, 2009; Sammons and Gaines, 2014). The higher level of 3-deoxy-d-arbino-heptulosonate 7-phosphate synthase, involved in the shikimate pathway, is also proposed to be responsible for enhanced carbon flow, which further assisted is imparting the glyphosate resistance (Pline-Srnic, 2006). The over expression of EPSPS gene was reported to be the primary mechanism involved in resistance development to glyphosate herbicide in *L. perenne* (Tani et al., 2016; Yanniccari et al., 2017). Target-site mutations related reduced translocation of glyphosate was reported in beggarticks (*Bidens Pilosa*) (Alcantara-de la Cruz et al., 2016a). Similarly, reduced uptake and translocation in rigid ryegrass (*L. rigidum*) (Fernandez-Moreno et al., 2017b), reduced absorption and translocation in tropical sprangletop (*Lepthochloa virgate*) (Alcántara-de la Cruz et al., 2016b), target-site mutations in *L. perenne* populations (Karn and Jaseniuk, 2017), and target-site and non-target-site resistance in congress grass (*Parthenium hysterophorus*) (Bracamonte et al., 2016) was implicated in resistance development.

Plant physiology and phytotoxicity

In plants, glyphosate usage blocks the synthesis of the aromatic amino acids such as phenylalanine, tyrosine, and tryptophan by targeting the enzyme EPSPS of the shikimic acid pathway. Application of Roundup® to cotton was reported to affect boll distribution and cause abnormality of bolls, which reduces cotton yield, fiber quality, ginning percentage (Viator et al., 2000). Glyphosate application in GR-cotton was shown to reduce plant reproductive attributes such as modifications in floral morphology, pollen viability, and pollination efficiency leading to poor seed setting and greater boll loss (Pline et al., 2003). Glyphosate exposures to crop plants were also reported to alter the characteristics of root exudates, quantitatively and qualitatively affecting their functional roles. In GR-soybean, while the carbohydrates characteristics were not affected by glyphosate application, the amino acids exudation was found to get increased (Kremer et al., 2005).

Hormesis (a stimulatory effect of toxin/herbicide/chemicals on plant growth) is a phenomenon stimulated by the lower concentrations of several herbicides on crop plants. However, sub-toxic concentrations of glyphosate brought a significant change in a plant population of the same species, affecting their growth and development (Brito et al., 2018). Similarly, lower concentrations of glyphosate leaching and runoff from the land to water bodies also stimulate the growth of some algae via hormesis causing eutrophication, however, the glyphosate induced hormesis in algae is generally less (Dabney and Patiño, 2018).

In weed management, application of the recommended rates of glyphosate was not found to affect the mineral composition of GR crops (Duke et al., 2018; Reddy et al., 2018). However, charged minerals such as aluminum and iron oxides act as a binding site for glyphosate in most of the soils (Borggaard and Gimsing, 2008). Glyphosate also

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DOI: http://dx.doi.org/10.15666/aeer/1905_34813504

competes for adsorption sites in soil along with phosphate ions, indicating the significant role of phosphate fertilizers in glyphosate remobilization in soils (Bott et al., 2008). Soils without enough binding sites (example sandy soils) for glyphosate residues cause phytotoxicity due to the presence of unbound glyphosate (Cornish, 1992). Since the glyphosate is an anion at physiological pH, it binds well with most of the divalent metal cations, thus reducing the possibility of phytotoxicity. However, chelating characteristics of glyphosate were linked to phytotoxicity and negative effects on other organisms (Mertens et al., 2018).

Plant defense mechanisms and disease tolerance

Glyphosate herbicide usage in crop plants is implicated in the susceptibility of plants to several diseases through inhibition of EPSPS, which disrupts the shikimic acid pathway, and shikimic acid pathway-derived compounds (phenolics, defense molecules, lignin derivatives, salicylic acid, anthranilic acid, phytoalexins and lignans), that plant syntheses to protect themselves from microbial plant pathogens (Hammerschmidt, 2018). In nutritional aspects, the application of glyphosate was reported to impact plant uptake and transport of micronutrients (Mn, Fe, Cu, and Zn), whose shortage can reduce plant growth and disease resistance (Johal and Huber, 2009). The root infection caused by *Fusarium* spp. in GR-soybean cultivars were found to get aggravated by glyphosate application under controlled and field conditions (Kremer and Means, 2009). Further, glyphosate application also decreased *Pseudomonas* spp, IAA-producing bacteria, and ratio of manganese-reducing to manganese-microbial populations (Kremer and Means, 2009). Glyphosate usage and GR were also linked to sudden death syndrome, in soybean caused by *Fusarium virguliforme* (Njiti et al., 2003).

Though the quantum of glyphosate required to destroy the weeds is considerably low in the presence of plant pathogens (Duke et al., 2018), glyphosate application can be toxic to microbes, particularly rusts (Feng et al., 2005). The drift of glyphosate from sprayed fields be phytotoxic and can decrease the defense mechanism of non-GR plants to plant pathogens (Hammerschmidt, 2018). Glyphosate application to GR soybean was reported not to increase its susceptibility to *Sclerotinia sclerotiorum* (Nelson et al., 2002). Similarly, there were inadequate data to establish a relationship between glyphosate usage and plant diseases caused by *Fusarium* spp (Powell and Swanton, 2008).

Non-target vegetation

Glyphosate is one of the most important herbicides used worldwide on non-GR croplands. Leaching, runoff, and drifting of spray droplets from the treated area are the primary source of glyphosate exposure to non-target vegetations (Duke, 2020). The glyphosate required to cause phytotoxicity in plant species varies considerably depending upon the plant type, glyphosate concentration and its drift levels (Duke, 2020). Glyphosate is documented to have a shorter half-life and low drift potential compared to other herbicides, indicating its safeness on non-target vegetation (Heap and Duke, 2018). Further, the low vapour pressure properties of the glyphosate acid and the isopropylamine salt of glyphosate make them virtually nonvolatile (Duke, 2020). Commonly, the glyphosate that drifts and settling on plant surfaces are either used by the plants as a nutrient source or reaches the soil through rain (Duke, 2020). There

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found to be a minimal plant injury even with aerial spray of glyphosate at minimal distances (Cederlund, 2017). Around 21% of glyphosate and 42% of AMPA was recorded in European surface-soils, where the GR crops were not grown, indicating the drift potential of glyphosate on non-targeted vegetations (Silva et al., 2018). The impact of glyphosate application on non-target plant species has been studied in several crops including peas (*Pisum sativum*) (Orcaray et al., 2012; Zabalza et al., 2017), rice (*Oryza sativa*) (Ahsan et al., 2008), soybean (*Glycine max*) (Hernandez et al., 1999), etc. In most occurrences, glyphosate application was reported to influence the photosynthetic rate and chlorophyll biosynthesis (Zobiole et al., 2012; Serra et al., 2013), photochemical reactions (Vivancos et al., 2011), carbon and nitrogen metabolism (Zobiole et al., 2010; Ding et al., 2011), plant mineral uptake (Cakmak et al., 2009; Zobiole et al., 2010, 2011, 2012), phytohormone synthesis (Sergiev et al., 2006; Miteva et al., 2010), fatty acids and amino acids synthesis (Gomes et al., 2017), secondary metabolite synthesis (Yanniccari et al., 2012). The GBH application also influences the activity of enzymes such as ascorbate peroxidase, catalase, and polyamines (Mkandawire et al., 2014).

Soil microorganisms and their diversity

Glyphosate affect soil microorganisms, their community composition (Kremer and Means, 2009) and their ecological functions, however, most of those effects are found to be minor and transient in nature (Nguyen et al., 2016). Soil microorganisms are capable of using glyphosate as carbon and phosphorus sources (Eser et al., 2007). The effects of glyphosate be transient and minimal on soil microbial population and their functions, as there is no report on yield reductions in GR-crops (Duke and Reddy, 2018). Based on the FAME analysis, glyphosate application to GR-soybean under field conditions had no effect on the rhizosphere and bulk soil community composition (Weaver et al., 2007). Glyphosate usage in GR-corn was shown not to affect the denitrifying bacteria and fungal populations compared with GR-corn and glyphosate sensitive (GS) corn isoline treated with conventional herbicides (Hart et al., 2009). Glyphosate application does not have a significant effect on cotton rhizosphere microbial community composition and their function (Barriuso and Mellado, 2012). There found to no negative effect on root colonization efficiency of Arbuscular mycorrhizal fungi in GR-cotton, corn, and soybean (Savin et al., 2009). However, nitrogen + glyphosate application was reported to negatively affect the growth of AMF as well as the dehydrogenase activity in the silt loam soil (Nivelle et al., 2018). Similarly, the fungal:bacterial ratios were unaffected by glyphosate applications to GR-soybeans (Lane et al., 2012). Pyrosequencing of cloned 16S-rDNA from GR-corn rhizosphere showed no variations in microbial community composition compared to the glyphosate or GTZ (acetochlor + terbuthylazine) treatment (Barriuso et al., 2010). Similarly, long-term studies identified three dominant microbial groups (Proteobacteria, Actinobacteria, and Acidobacteria) in the GR-corn rhizosphere, which indicated a nil or transient effect of glyphosate on these communities (Barriuso et al., 2011). Long-term usage of glyphosate in glyphosate tolerant cropping, resulted in shifts in sub-populations of soil rhizosphere-associated bacterial communities (Xanthamonadales, Acidobacteria), and bacterial exposure to glyphosate was shown to affect their community composition (increased proliferation of glyphosate tolerant bacteria) and down-regulation of carbon metabolism (Newman et al., 2016).

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Glyphosate have direct toxicity on some bacteria, fungi, and protists as these organisms also use the shikimic acid pathway (Feng et al., 2005). Even, mycorrhizal fungi were reported to be sensitive to glyphosate exposure, which affects root colonization efficiency and spore viability (Druille et al., 2013). Higher concentrations of glyphosate inhibit nitrogen fixing potential of cyanobacteria (Bodkhe and Tarar, 2016). The reduction in nodulation efficiency, nitrogen fixation and biomass buildup was reported in GR-soybeans subjected for glyphosate treatment (Zablotowicz and Reddy, 2004). Glyphosate application to GR-soybeans reported to affect leaf chlorophyll, root biomass, plant nitrogen content, nodule biomass and nitrogenase activity compared to the untreated control. However, there are no significant differences between treated and control plants on *nifH* gene abundance (Fan et al., 2017). The combined application of nitrogenous fertilizers and glyphosate does not affect the activities of either ammonia-oxidizing bacteria or archaea and their nitrification activities (Zabaloy et al., 2017). Though the glyphosate application was found to be harmful to microbial populations up to 30 days, the population recovered after 60 days and reached the original level in acidic soil (Kumar et al., 2017) and lateritic soil (Shitha, 2014). The application of higher doses of glyphosate in glyphosate sensitive (GS) pea and triticale was reported to increase the ammonia concentrations in the rhizosphere soil, thus affecting microbial community diversity and richness compared to the control treatments (Mijangos et al., 2009).

Insects and aquatic life

Evidence suggests that GBHs can have an adverse effect on aquatic invertebrate ecology, including amphibian larvae (Cuhra et al., 2013). Simultaneous exposure to GBHs and other stressors has been shown to increase undesirable impacts on fish and amphibians (Jones et al., 2011). Glyphosate concentrations of over 400 μg L^{-1} are possibly toxic to some aquatic species including amphibians and fish (Annette et al., 2014; Braz-Mota et al., 2015). The occurrence of glyphosate in marine ecosystems and its persistence in sea water is also reported (Mercurio et al., 2015). GBHs have negative (phytoplankton and nitrifying community) as well as positive (cyanobacteria) impact on aquatic microorganisms (Vera et al., 2010). The impacts of glyphosate or GBHs can also have differential effects on arthropods, predators and parasites (European Commission, 2002). Honey bees exposure to glyphosate or GBHs at sub-lethal concentrations can impair their behaviour and cognitive capacities (Balbuena et al., 2015). Glyphosate usage in GR soybean and corn was reported to be responsible for the decline in monarch butterflies (*Danaus plexippus* L.) and milkweed (*Asclepias* spp.*)* population (Pleasants and Oberhauser, 2013). However, further studies established that the use of synthetic herbicides for weed management in those crops was responsible for the decline in the population of monarch butterflies, rather than the adoption of GR-crops (Boyle et al., 2019).

Fauna and invertebrates

Exposure to glyphosate has significant effects on earthworm activity and ecological functions, including a decrease in body weight, cocoons and juvenile's population (Gaupp-Berghausen et al., 2015; García-Pérez et al., 2016, 2020). The hatching percentage of *Eisenia fetida* (red wiggler) cocoons was also reported to get reduced

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significantly on exposure to soils treated with Roundup herbicide (Verrell and Van Buskirk, 2004). Glyphosate application also affected the survival rate and cocoon production in *Lumbricus terrestris* (night crawler)*, Octodrilus complanatusas* (large earthworm)*, and Aporrectodea caliginosa* (grey worm) (Stellin et al., 2018). Though earthworms showed glyphosate (1.2 and 2.4 a.i kg ha^{-1}) avoidance, multiplication of earthworms was not affected by glyphosate exposure (Shitha, 2014). Based on litter decomposition studies on GR-soybean and near isoline-sensitive cultivars, protists and nematode populations were not affected by GR-soybean (Powell et al., 2009). Application of GBHs, Roundup, was reported to show a transient effect on the population and functioning of soil fauna enchytraeids and nematodes (Hagner et al., 2019).

Conclusions and future outlook

Since its introduction in 1974, the usage of glyphosate and GBHs has increased approximately 100-fold. Though glyphosate and GBHs affect human and animal health initially, subsequent clinical studies and critical analyses by regulatory bodies concluded that glyphosate is unlikely to cause health risk in humans. However, several world regulatory bodies still raise doubts on impact of glyphosate and GBHs to long-term exposure on human, animals, plants and other non-targets groups, and recommend having more data on its impact on the environment. Nevertheless, several studies have reported the fate of glyphosate in natural ecosystems related to its persistence, degradation and residual effects on crops, there is still a lack of knowledge on glyphosate and GBHs effects on soil, plant (nutrient mobilization, nutrient availability, plant nutrient uptake, phytotoxicity, plant defense, disease tolerance, microbial diversity, faunal activities, enzyme activities, etc.) and environmental quality subjected to its long-term exposure. Further the adoption of multi-cropping systems including intercrops becomes a big question on the event of adoption of HT crops, as the crops which are engineered to express *cp4-epsps* only can survive the glyphosate application. Due to indiscriminate use of glyphosate and GBHs in agricultural lands, there is likely chance to increase the emergence of glyphosate-resistant weeds (Super weeds). However, the available literature infers the ecological effects of glyphosate and GBHs are posing low risk to the environment and the effects are transient. While several studies have reported the negative impact of glyphosate and GBHs on soil, plant, and environmental health, a few also reported the positive effects of glyphosate and GBHs on reduction of greenhouse gas emissions, a decline in the use of fossil fuel in agriculture, enhancement in carbon sequestration, soil conservation, plant growth stimulatory effects, increased yield, reduction in the cost of cultivation, and lesser competition for agricultural lands.

To conclude, though there were extensive studies on the positive and negative impact of glyphosate and GBHs on the environment, the following are the points for consideration with regard to future environmental management with regard to glyphosate usage:

a) Impact on natural enemies of crop plants in different agroecosystems.
b) Impact on natural plant innate defense system against biotic and abiotic stresses.
c) Soil-water-plant relationships, nutrient recycling, nutrient availability and plant uptake under different agro-ecological conditions.

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d) Soil biology and alteration in food-webs including micro, meso, and macro-flora and fauna diversity.
e) Long-term exposure on beneficial microorganisms and their ecological functions.
f) Soil and ground water contamination, biomagnification, entry to the food supply chain in important staple and commercial crops.
g) Impact on fresh and marine ecosystems, biogeochemistry, and nutrient sequestration.
h) Long-term exposure studies on mammals including humans and their risk assessment.
i) Glyphosate residues in crop wastes, its persistence, and their impacts on recycling and agricultural use.
j) Horizontal gene transfer in non-targeted vegetations with regard to use of glyphosate resistant crops.

Conflict of interests. The authors have no conflict of interests.

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DOI: http://dx.doi.org/10.15666/aeer/1905_34813504

ISOLATION OF *TALAROMYCES FLAVUS* FROM ROODEPLAAT DAM AND SCREENING OF ITS SECONDARY METABOLITES IN ARTIFICIAL MEDIA

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(Received 12th Jun 2019; accepted 25th Oct 2019)

Abstract. This paper reports on a divergent approach employing isolation methods and high-resolution mass spectrometry to screen decomposition products that are a result of fungal action on biomass present in aquatic media. Fungi decompose organic matter in surface water through a process which plays a major role in nutrient cycling and maintenance of aquatic life. Moreover, the presence of pathogenic or potentially pathogenic species of fungi in drinking water distribution systems, may cause chronic diseases to consumers and these pathogenic fungi have the potential to produce a variety of secondary metabolites which play important roles in protecting the organism against competition for nutrients and survival. In this paper, the fungus *Talaromyces flavus* was isolated from Roodeplaat Dam in Gauteng province (South Africa) and was identified using sequence analysis of the internal transcriber spacer (ITS) region. Secondary metabolites produced by *T. flavus* grown in liquid culture were extracted and determined using Liquid chromatography-mass spectrometry (LC-MS) and comparative analysis of the secondary metabolite profile was done using *Aspergillus fumigatus* as a reference (ATCC 36607). The influence of nutrients, pH and incubation period on the production of secondary metabolites was demonstrated and validated statistically by principal component analysis (PCA).
Keywords: *fungi, mycotoxins, LC-QTOF-MS, molecular techniques, aquatic life, PCA*

Abbreviations: ITS: internal transcriber spacer; LC-MS: Liquid chromatography-mass spectrometry; PCA: principal component analysis

Introduction

Freshwater fungi produce a diversity of antimicrobial metabolites, which help them compete against other microorganisms (Calvo et al., 2002; El-hasan et al., 2009; Connor et al., 2016). However, some of them produce compounds which are responsible for inflammation and chronic diseases (Hernández-Carlos and Gamboa-Angulo, 2011). Numerous groups of secondary metabolites and bioactive compounds have been isolated from fungal strains collected from diverse environments (Zhao et al., 2010; Swathi, 2013; Imhoff, 2016). A wide range of natural products produced by a variety of fungal species are used for medication, industrial and agricultural purposes (Khan et al., 2014). Some of these compounds are deleterious, while others are beneficial to humankind.

The production of secondary metabolites can be affected by the pH and nutrient levels in the aquatic environment such as carbon and nitrogen sources (Singh et al.,

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2010; Schulthess et al., 2014). The manipulation of growth conditions can lead to the production of various compounds, since most of the natural products are secreted by microbes under specific sets of conditions. The physical parameters such as pH, nutrients, temperature and incubation period can be monitored for the production of diverse structures of compounds for therapeutic uses (Gaden, 2000).The objectives of the study is to investigate the influence of nutrients in the dam water and nuclease-free water by spiking the water sample with isolated fungal *Talaromyces flavus* and the other set with the reference strain of *Aspergillus fumigatus* (ATCC 36607). As well as screening and profiling of the secondary metabolites produced by aquatic fungi in water, using liquid chromatography coupled to quadrupole/time-of-flight mass spectrometry (LC-Q-TOF-MS/MS) and SIMCA software.

Materials and methods

Water sampling

The water samples were collected in November 2015 at Roodeplaat Dam in Gauteng, South Africa. The wall of the dam is 55 m high and has a length of 351 m. Two main sampling sites were selected within the dam, and these were 50 m apart; at each site the samples of water were collected in 1 L clean uninfected bottles at 5 m apart. Two 1 L water samples were selected from the samples points 40 and 50 as indicated in *Figure 1*. These sampling points represent bigger part of the dam. Therefore, water samples were examined for physical and chemical properties such as pH, electric conductivity, temperature and nitrates using a multiprobe (YSI professional plus multiparameter water quality instrument, built in barometer, BOD sensors includes 2-meters cable; 1 or 4 meters on lab pH, ORP and pH/ORP cables, four electrode cells).

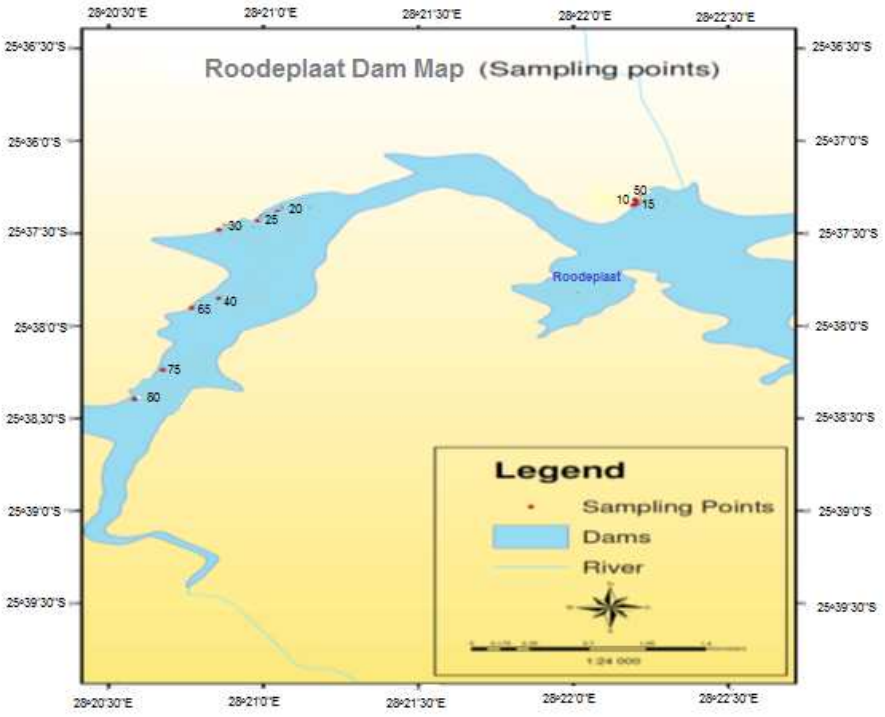

Figure 1. *The sampling points in Roodeplaat Dam*

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DOI: http://dx.doi.org/10.15666/aeer/1905_35053518

Fungal cultures and DNA extraction

Isolation of fungi was obtained through the pour-plate method; using a pipette, about 200 µL of dam water was spread evenly over the surface of three culture plates of potato dextrose agar (PDA) (Sigma-Aldrich, product number P2182). The culture plates were incubated at 25 ± 2 °C for 2 weeks. Cultures were visually examined for colony growth and colour daily. Fungal colonies were examined and then transferred to freshly prepared PDA supplemented with streptomycin sulphate using the hyphal tipping method. The new cultures were then incubated at 25 ± 2 °C. Sub-culturing of mycelia cut from the colony edges continued until pure colonies were obtained. One fungal colony was selected for identification and further study.

DNA extraction

The selected fungal colony was ground with liquid nitrogen using a mortar and pestle and DNA was extracted from 1gram mycelium powder of five-day-old pure fungal cultures using the QIAamp® DNA mini kit (Qiagen). The total quantity of genomic DNA extracted was quantified using the Quantus™ Fluorometer (Promega, USA).

Polymerase chain reaction

Polymerase chain reaction (PCR) was used to amplify the ITS region using the universal primer pair, ITS1F (5'CTTGGTCATTTAGAGGAAGTAA3') and ITS4 (5'TCCTCCGCTTATTGATATGC3'), obtained from Inqaba Biotechnologies, Pretoria, South Africa. The total reaction volume of 25 µL consisted of 1.0 µL of ITS1 forward primer, 1.0 µL of ITS4 reverse primer, 12.5 µL of DreamTaq Green PCR Master Mix (GoTaq®, Green Master Mix, Promega, USA), 3 µL of DNA (template more than 50 ng), and 7.5 µL of nuclease-free water. The PCR setting were as follows, denaturation step of 94 °C for 5 min, proceeded by 35 cycles which were made up of denaturation at 94 °C for 1 min, annealing at 57 °C for 30 s, extension at 72 °C for 30 s, lastly was elongation stage of 72 °C for 10 min. The reaction was finally terminated at -4 °C. Upon completion of the PCR run, a product of 5 µL was loaded on a 1% agarose gel for electrophoresis to confirm the amplification of the target ±500 base-pair fragment in the ITS region. The remainder of the PCR sample was submitted for sequencing at Inqaba Biotechnologies (RSA, Gauteng, Pretoria). The sequence analysis chromatograms were edited with the Chromas software and database similarity matching to identify the fungal strain was done using NCBI BLAST.

Determination of secondary metabolites

One plug (about 3 mm diameter) each of the *Talaromyces flavus* and that of the reference strain *Aspergillus fumigatus* (ATCC 3667) were individually inoculated into 15 mL of potato dextrose broth (PDA) prepared with dam water and nuclease-free water. The supplement of 25% PDA concentration was used to balance nutrients and liquidity of the broth. To determine the secondary metabolites produced by fungi in water, the media were incubated at 28 °C in a shaking incubator maintained at 150 r/min. The samples were collected from the incubator in triplicate after 0 h, 72 h and 7 days (*Table 1*). The pH and conductivity were measured during the collection time. Solid phase extraction (SPE) procedure was used for the extraction of both polar and non-polar compounds. The Oasis® HLB (3 mL) cartridges (Hydrophilic Lipophilic Balance (HLB), Waters Oasis® sample extraction products) were used for the SPE

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procedure. Secondary metabolites were extracted from 10 mL of the broth media. The first step was to condition the solid phase adsorbed with 3 mL of 50% MeOH-H_2O, at the flow rate of approximately less than 3 mL/min. The samples were then introduced into the cartridge at the flow rate of about 8 mL/min. After sample loading the sample was washed with 3 mL of 5% methanol-water. Cartridges were then dried for more than 30 min under vacuum. The analyte was then eluted with 2 x 3 mL of 50% MeOH-H_2O. The extracts were dried using nitrogen gas to near dryness at 30 °C under reduced pressure, and then re-dissolved in 2 mL of methanol and transferred to amber vials. It was then further evaporated to near dryness at 35 °C to concentrate the sample, and subsequently re-dissolved in 500 µL of methanol–water (1:1).

Table 1. *The sample treatments with reference strain and isolated fungi in nuclease-free water (CW) and Dam water (DW)*

Fungi	Water	Treatments (PDA)	Incubation time			Samples		
Aspergillus Fumigatus	Nuclease-Free water	0%	0 h	72h	7 days	0h-A-0%-CW	72h-A-0%-CW	D-A-0%-CW
Aspergillus Fumigatus	Nuclease-Freewater	25%	0 h	72 h	7 days	0h-A-25%-CW	72h-A-25%CW	7D-A-25%-CW
Aspergillus Fumigatus	Dam water	0%	0 h	72 h	7 days	0h-A-0%-DW	72h-A-0%-DW	7D-A-0%-DW
Aspergillus Fumigatus	Dam water	25%	0 h	72 h	7 days	0h-A-25%-DW	72h-A-25%DW	7D-A-25%-DW
Talaromyces Flavus	Nuclease-Freewater	0%	0 h	72 h	7 days	0h-T-0%-CW	2h-T-0%-CW	7D-T-0%-CW
Talaromyces Flavus	Nuclease-Freewater	25%	0 h	72 h	7 days	0h-T-25%-CW	72h-T-25%-CW	7D-T-25%-CW
Talaromyces Flavus	Dam water	0%	0 h	72 h	7 days	0h-T-0%-DW	72h-T-0%-DW	7D-T-0%-DW
Talaromyces Flavus	Dam water	25%	0 h	72 h	7 days	0h-T-25%-DW	72h-T-25%-DW	7D-T-25%-DW
Without Fungi	Nuclease-Freewater	0%	0 h	72 h	7 days	0h-C-0%-CW	72h-C-0%-CW	7D-C-0%-CW
Without Fungi	Nuclease-Freewater	25%	0 h	72 h	7 days	0h-C-25%-CW	72h-C-25%-CW	7D-C-25%-CW
Without Fungi	Dam water	0%	0 h	72 h	7 days	0h-C-0%-DW	72h-C-0%-DW	7D-C-0%-DW
Without Fungi	Dam water	25%	0 h	72 h	7 days	0h-C-25%-DW	72h-C-25%-DW	7D-C-25%-DW

Instrumental analysis using LC-QTOF-MS (Bruker, Bremen, Germany)

The secondary metabolites excreted by fungi were separated in a reversed-phase chromatography (RPC) procedure, using a Waters Acquity UPLC BEH C18 1.7 µm 2.1 X 100 mm column. After separation, secondary metabolites were analysed using LC-QTOF-MS (Impact II system). The analysis was done under positive mode. Secondary metabolites were Analytes of interest eluted with mobile phase A (water acidified with 0.1% formic acid) and B (LC-MS grade methanol acidified with 0.1% formic acid). The sample aliquot of 10 µL was injected into the C18 column and eluted using the gradient method at the speed of 0.2 mL/min.

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Statistical analysis

The influence of nutrients, water type and fungal species on the biosythesis of secondary metabolites was evaluated using principal component analysis from Soft Independent Modelling of Class Analogy (SIMCA) software. The samples were presented in triplicate for each treatment in dam water and the other set in nuclease free water. Related secondary metabolites produced by *Talaromyces flavus* and *Aspergillus fumigatus* (ATCC 36607) during incubation process were assessed in PCA plots.

Results

Identification of fungal species

The pure fungal species of *Talaromyces flavus* was isolated from dam water. This fungus is known as an endophyte however in this study was isolated and identified from a water sample. The PCR results (*Figure 2*) shows a DNA amplification of a PCR band which corresponds to an approximately 500 bp. The PCR bands indicated the presence of fungal DNA in water samples (*Figure 2*). The fungal diagnostic primer pair ITS1F and ITS4 was used in the PCR to confirm the presence of fungal DNA. The PCR product was sent for Sanger sequencing and the obtained sequence was compared with the available sequences on the NCBI database by basic local alignment search tool (BLAST). In addition to the identification done by sequence analysis of the ITS region, the fungal species was also identified morphologically.

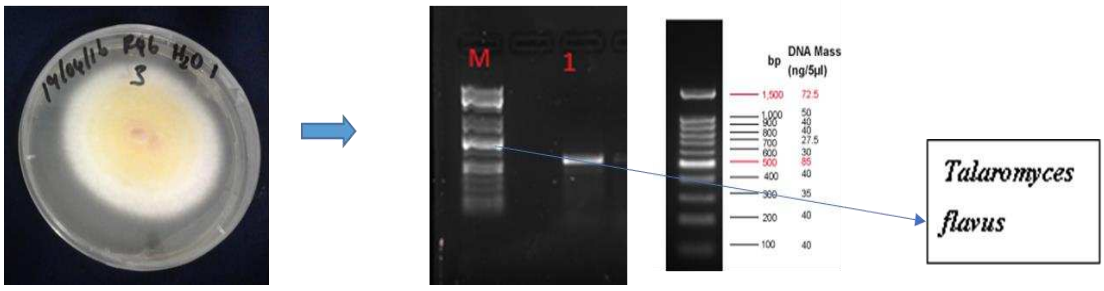

Figure 2. *The pure fungal cultures grown in PDA. Talaromyces flavus Fungal DNA bands in 1% agarose gel. (M) Ladder, (1) water sample 1*

Effect of physical parameters

Upon the introduction of the fungal plugs of *T. flavus* and *A. fumigatus,* the growth media conditions were found to change during the incubation period of 7 days. The changes in the media, pH, conductivity, and incubation conditions have a major role on the production of secondary metabolites. The incubation conditions depend on ecology and physiology of various filamentous fungi. In this study the PDA broth media were incubated at 28 ^{o}C, which is the recommended temperature for *Talaromyces*, *Aspergillus* and *Penicillium* species (Zhu et al., 2014). The influence of PDA nutrient was found to be proportional with the pH of the broth samples. In the absence of nutrients, the pH decreases gradually, while a sharp decrease in pH was observed with an excess amount of nutrients (*Figure 3*). This finding indicates the role played by aquatic fungi in that they utilise the nutrients available in the aquatic environment. The

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nutrients promote high fungal growth which leads to the production of various secondary metabolites.

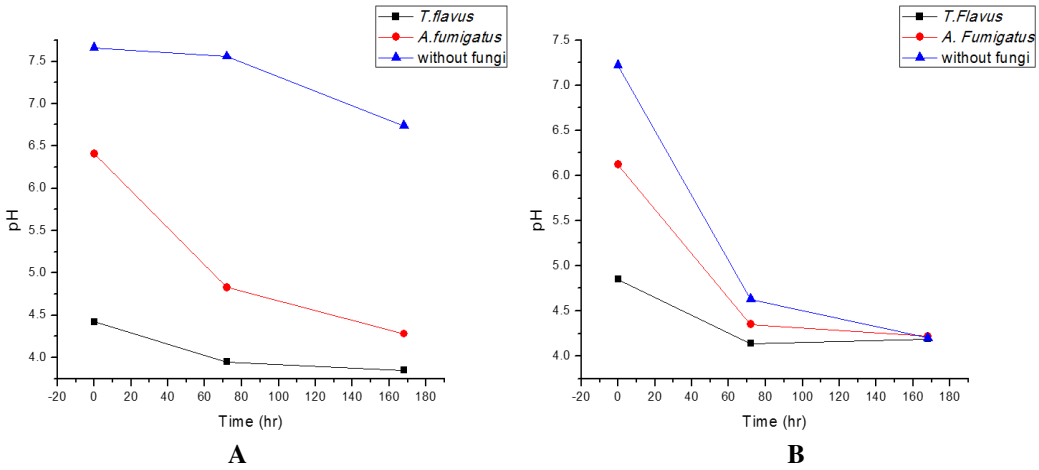

Figure 3. *The relationship between the nutrient level and pH over the incubation period of 7 days. (A) absence of nutrients, (B) excess amount of nutrients*

The decomposition of organic material by fungi has an impact on the nutrient levels in water bodies (Leigh et al., 2009). The trend of the change in pH and conductivity over an incubation period of 7 days is similar for water samples with the fungal plug (*Figure 4A-D*). The growth media without the fungal plug showed a different trend (*Figure 4E,F*); however, the pH of dam water after an incubation period of 7 days was found to be more acidic compared to nuclease-free water, because of the other compounds present in the dam water. The amount of dissolved material is proportional to the electrical conductivity of water. The conductivity was found to be lower in nuclease-free water without the fungal plug because of the lower concentration of ions in the nuclease-free water. The pH was found to decrease between 0 h and 72 h because the fungi consumed many of the nutrients, but the trend changed after 72 h; the pH increases are due to the depletion of nutrients (*Figure 4A*). During the culture period, the pH trend was found to be negatively correlated to that of conductivity of water. The starting pH for dam water was more acidic compared to that of the nuclease-free water with the plug of fungi.

The studies recorded maximum extracts after the incubation period of 7 days. Some changes in the profiling of secondary metabolites were observed after incubating for 72 h and 7 days. There was an increase in the number of compounds released by the isolated fungi (*T. flavus)* and reference strain (*A. fumigatus)* in the media with a higher nutrient level of PDA broth. The SIMCA PCA plots demonstrated the change in the orientation of secondary metabolites during the incubation process. At 0 h there was no significant difference in the natural products produced in dam water and nuclease-free water at various nutrient levels (*Figure 5*). However, the variable with similar properties are coming together.

After an incubation period of 72 h, the groupings of similar compounds were observed in three clusters (*Figure 6*). The samples without fungi are grouped together and the other two clusters are grouped related to nutrient level. Cluster **1** representing the samples treated with 25% PDA nutrients in the presence of *Talaromyces flavus* and

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Aspergillus fumigatus (ATCC 36607). The samples treated with PDA are distinguishable with the sample without treatment, that indicates the difference in compounds produced by fungi. Second group (**2**) showed the samples without treatment forming a cluster because of similar chemical properties and the minimal secondary metabolites produced under this condition. The control samples with and without PDA treated grouped together to form the third cluster **3**.

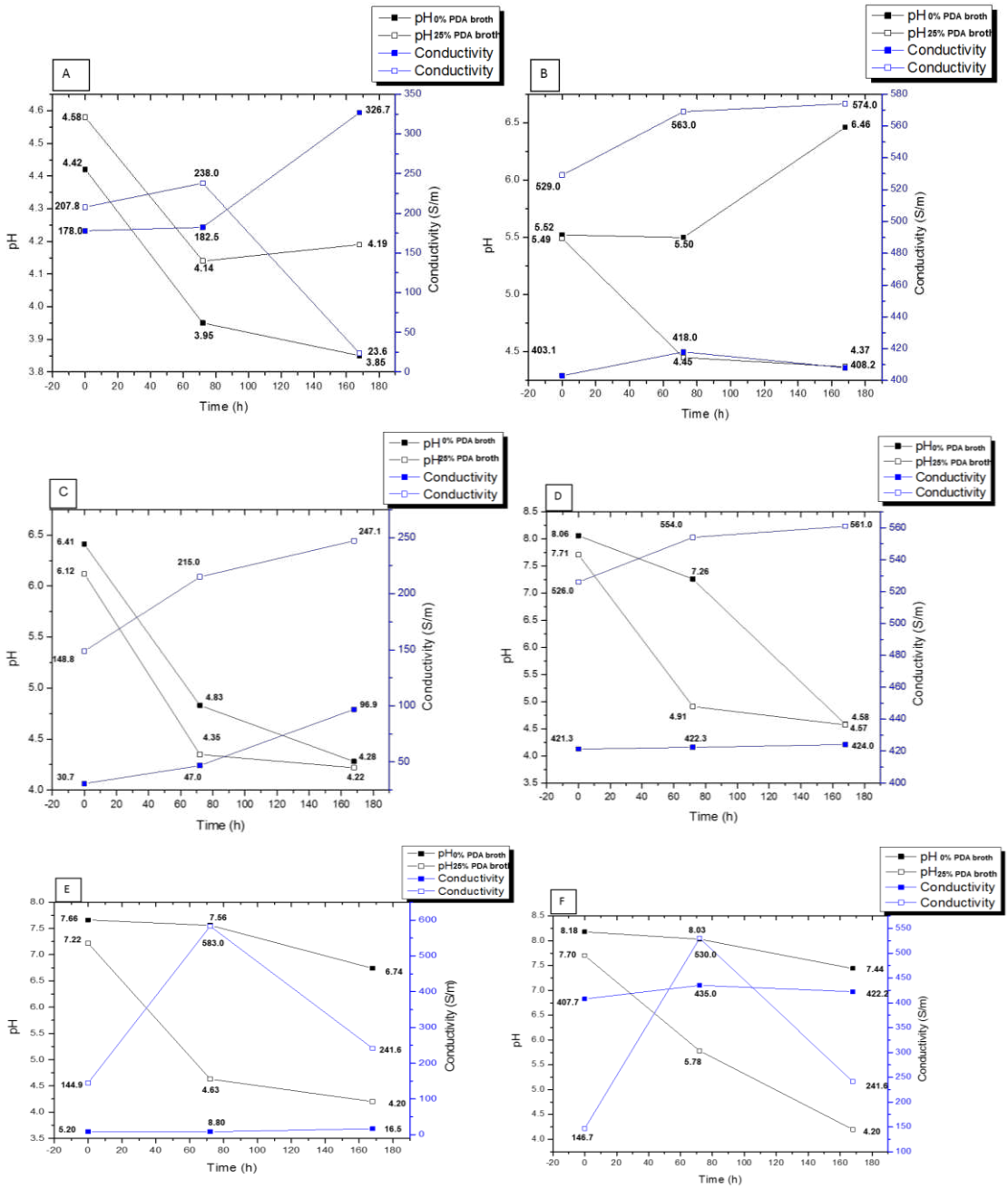

Figure 4. *The effect of fungi on the pH and conductivity of the growth media: (A) T. flavus in nuclease-free water; (B) T. flavus in dam water; (C) A. fumigatus in nuclease-free water; (D) A. fumigatus in dam water; (E) without fungi in nuclease-free water; and (F) without fungi in dam water*

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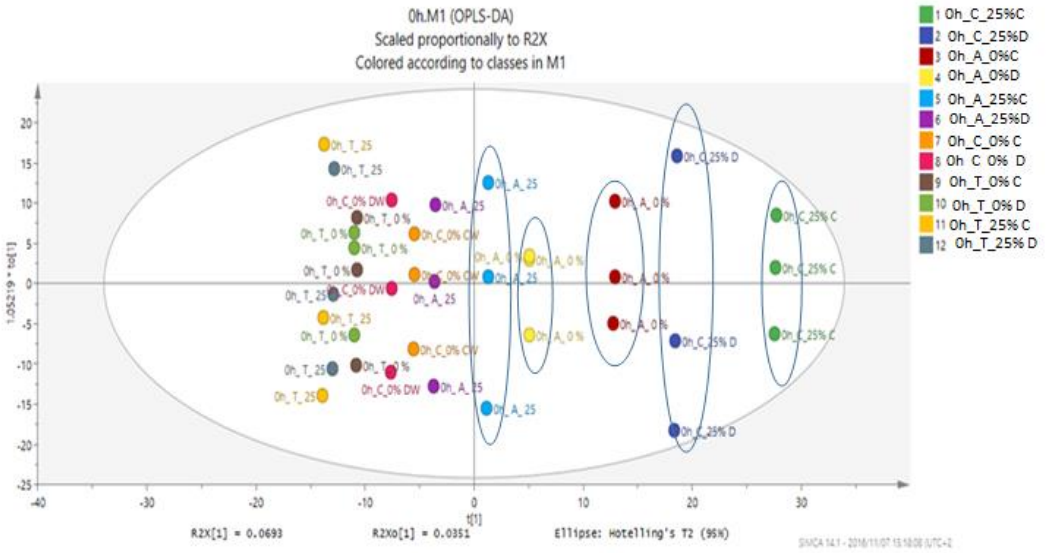

Figure 5. *The principal component analysis (PCA) scatter plots of secondary metabolites produced by fungi after 0 h: (1) without fungi at 25 % PDA in nuclease-free water; (2) without fungi at 25 % PDA in dam wate0r; (3) A. fumigatus at 0 % PDA in nuclease-free water; (4) A. fumigatus at 0 % PDA in dam water; (5) A. fumigatus at 25 % PDA in nuclease-free water; (6) A. fumigatus at 25 % PDA in dam water; (7) without fungi at 0 % PDA in nuclease-free water; (8) without fungi at 0 % PDA in dam water; (9) T. flavus at 0 % PDA in nuclease-free water; (10) T. flavus at 0 % PDA in dam water; (11) T. flavus at 25 % PDA in nuclease-free water; and (12) T. flavus at 25 % PDA in dam water*

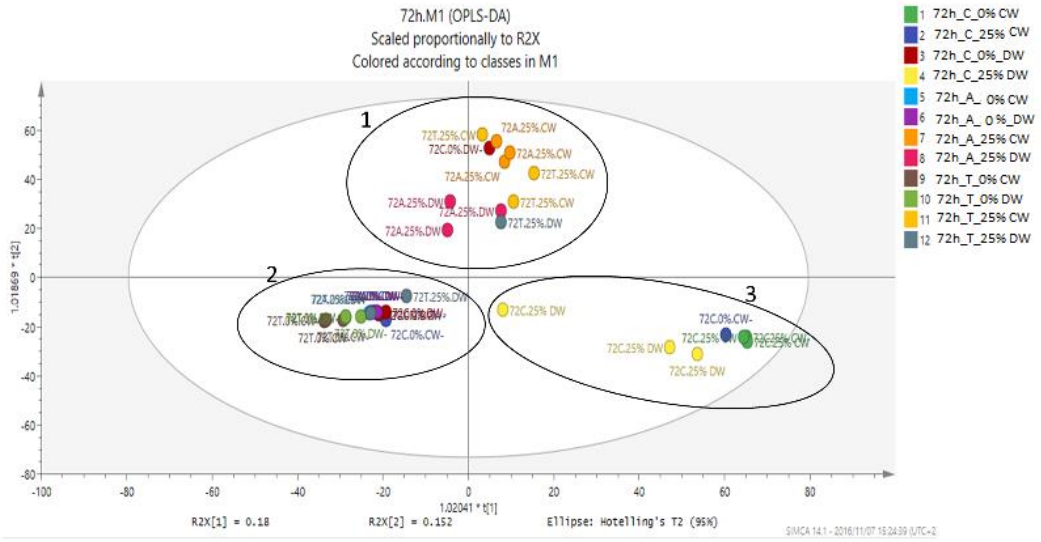

Figure 6. *The principal component analysis (PCA) scatter plots of secondary metabolites produced by fungi after 72 h: (1) without fungi at 0 % PDA in nuclease-free water; (2) without fungi at 25 % PDA in nuclease-free water; (3) without fungi at 0 % PDA dam water. (4) without fungi at 25 % PDA in dam water. (5) A. fumigatus at 0 % PDA in nuclease-free water; (6) A. fumigatus at 25 % PDA in dam water (7) A. fumigatus at 25 % PDA in nuclease-free water; (8) A. fumigatus at 25 % PDA in dam water (9) T. flavus at 0 % PDA in nuclease-free water; (10) T. flavus at 0 % PDA in dam water; (11) T. flavus at 25 % PDA in nuclease-free water; and (12) T. flavus at 25 % PDA in dam water*

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The distribution of secondary metabolites after 7 days showed a significant difference within the groups of samples; however, the stress conditions start to increase when the nutrient levels are no longer high enough to be used by fungi to produce new secondary metabolites (*Figure 7*). Longer incubation showed more interesting results; cluster **1** remained the same after 7 days for samples with fungal plug and 25% PDA treatment. The reorientation observed for cluster **2** and **3** there is an exchange of quadrants. In addition, group **2** showed all the samples without treatment such as control and the samples with fungal plug of *Talaromyces flavus* and *Aspergillus fumigatus* (ATCC 36607). The third cluster is the grouping of samples without fungal plug in the present of PDA treatment.

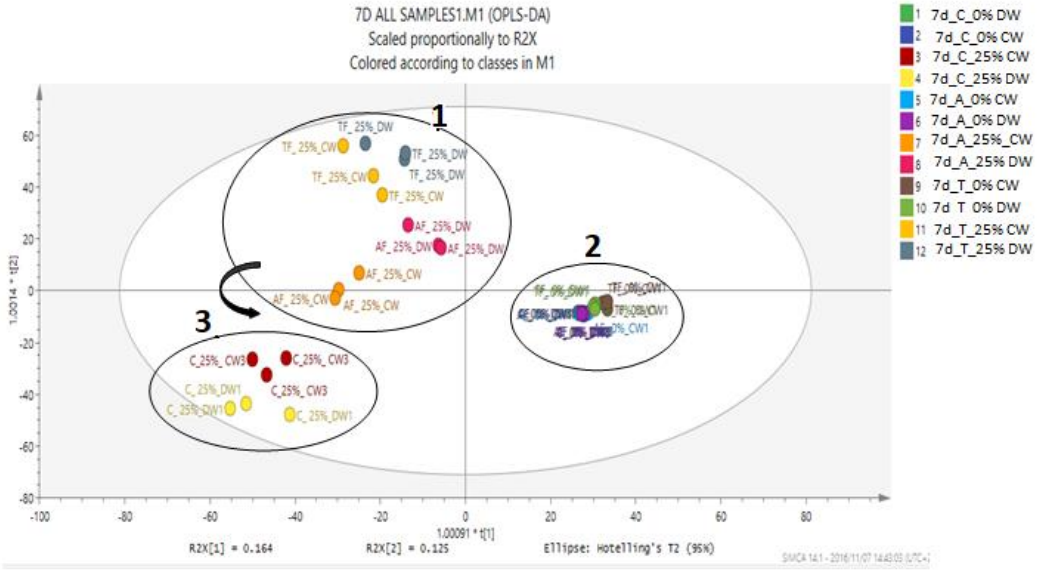

Figure 7. *The principal component analysis (PCA) scatter plots of secondary metabolites produced by fungi after 7 days: (1) without fungi at 0 % PDA in dam water; (2) without fungi at 0 % PDA in nuclease-free water; (3) without fungi at 25 % PDA in nuclease-free water; (4) without fungi at 25 % PDA dam water; (5) A. fumigatus at 0 % PDA in nuclease-free water; (6) A. fumigatus at 0 % PDA in dam water; (7) A. fumigatus at 25 % PDA in nuclease-free water; (6) A. fumigatus at 25 % PDA in dam water; (8) A. fumigatus at 25 % PDA dam water; (9) T. flavus at 0 % PDA in nuclease-free water; (10) T. flavus at 0 % PDA in dam water; (11) T. flavus at 25 % PDA in nuclease-free water; and (12) T. flavus at 25 % PDA in dam water*

Figures 8(1) and *(3)* show the LC/MS chromatograms only for the secondary metabolites produced by *T. flavus* and *A. fumigatus* in nuclease-free water. The different chemical profile of secondary metabolites shows that the nutrients influenced the qualitative production of secondary fungal metabolites. As seen on the LC/MS chromatogram of the sample with high nutrients (*Figures 8(2)* and *(4)*), there are new peaks between retention times of 4 to 8 h with higher intensity. In addition, the incubation period seems to also play a major role in the number of natural products produced by fungi. Comparisons of the profiling chromatogram at 72 h with 7 days' reveal that the fingerprint pattern is the same; however, the intensity is higher after 7 days. New peaks appeared in the chromatogram of the sample with a high nutrient level; this illustrates the important role played by nutrients in the biosynthesis of

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DOI: http://dx.doi.org/10.15666/aeer/1905_35053518

secondary metabolites. It should be noted that the new peaks from dam water could be secondary metabolites, degraded compounds or bio-transformed products due to fungal activities.

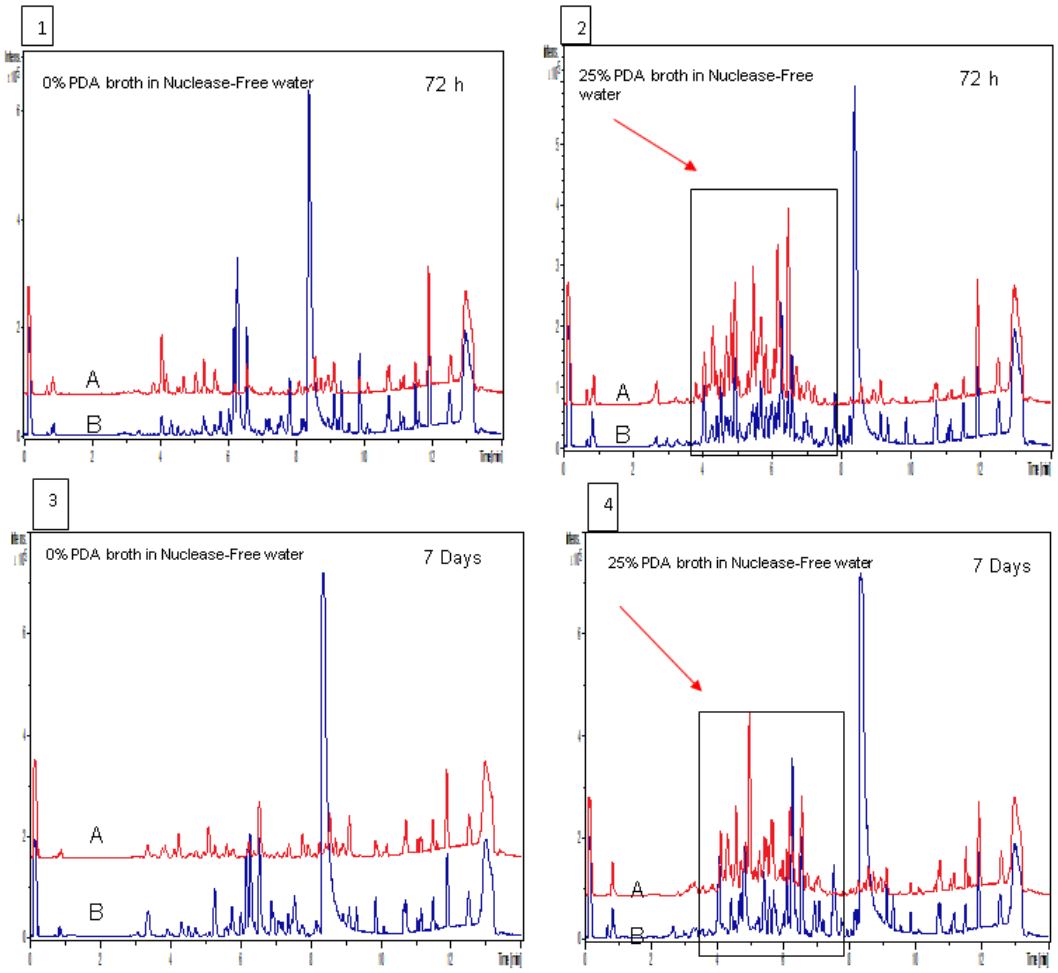

Figure 8. *The LC-MS chromatograms of extracellular secondary metabolites produced by fungi in nuclease-free water: (A) T. flavus; and (B) A. fumigatus*

Discussion

The ITS primers used in this study have been found to have higher sensitivity and produce PCR bands which showed clear amplification of approximately 500 nucleotides fragments (Ferrer et al., 2001). Both the morphological identification and the sequence analysis of the ITS region pointed to *Talaromyces flavus* as the isolated fungus from Roodeplaat Dam.

Talaromyces flavus is commonly found in the soil, plants and major crops such as cotton and potato. It is known as an antagonist that has been used in biological control of some soil-borne and plant pathogens (Ayer et al., 1990; Zhong, 2009; Naraghi et al., 2012). *Talaromyces flavus* has been studied extensively as a biological control of agricultural pathogens by various processes, including parasitism, competition for nutrients and antibiosis (Cordell and Cordell, 2016; Fravel and Roberts, 2016). This fungus is unique and important compared to others due to its extreme tolerance to heat.

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In this study *T. flavus* was isolated from the dam water, while most of the other studies have been identifying this fungus from soil, plants and agricultural commodities.

Screening of *T.flavus* secondary metabolites produced in the artificial media was investigated using dam water and nuclease-free water. As the incubation time increased the growth media were found to be more acidic during the incubation period of 7 days; that was because of the change of nutrients in water. The spike of *T. flavus* and reference strain *A. fumigatus* (ATCC 36607) caused the pH to drop, which means that the fungi utilise dissolved oxygen to break down organic matter, leading to an increase in the amount of dissolved material, hence higher electrical conductivity. However, the conductivity of the broth media prepared with nuclease-free water without the fungal plug showed lower conductivity compared to all other samples. That was expected since nuclease-free water does not contain many compounds compared to the dam water which has a more complex composition.

The availability of aquatic fungal species in surface water mostly depends on the chemical and physical properties of water, since they survive under various favourable conditions (Thakur, 2009). In this study it has been proven that fungal growth influences the quality of the water by changing physical parameters. The pH was found to decrease gradually during the incubation period and the lower pH levels increase the risk of mobilised toxic metals that can be absorbed by aquatic organisms; the change in pH affects many sensitive species in water ecosystems (Barnes et al., 1998). The higher the pH, the higher the solubility effect of some other compounds and elements, which results in the formation of toxic compounds or chemicals that can be easily absorbed by aquatic life (Barrie and Georgii, 1976). Casellato and Said (2006) reported a large number of conidia after 7 days compared to 10 days; this observation is in line with the results reported in this study. The prolonged incubation period increases the stressful environment for microorganisms especially when there are not enough nutrients for survival. The results of this study corroborate the results obtained by Ayer and Racok (1990), who stated that during the incubation period of 5 days the pH of the broth with *T. flavus* decreases.

Cultivation conditions strongly influence the production of bioactive fungal compounds. Parameters such as nutrients, incubation period, pH and temperature are important in the production of secondary metabolites. The study conducted by Schulz et al. on the influence of culture conditions on the production of secondary metabolites by fungi reported observations which are similar to those reported in this present study (Schulz et al., 2008). Both studies found 28 ^{o}C as a recommended incubation temperature for conidia production.

The known secondary metabolites produced by *A. fumigatus* were successfully extracted from both media prepared with dam water and nuclease-free water, indicating that the method for the extraction of secondary metabolites worked well. There is a possibility that some other compounds which are not secondary metabolites may be observed in the dam water; due to biotransformation or biodegradation of substrates in the dam water. In the case of nuclease-free water, the significant difference observed in the PCA plot was without any doubt because of the secondary metabolites produced by the fungi. Significant differences in metabolites were observed in the samples of dam water with varying nutrient levels; the results clearly showed the role of nutrients in the biosynthesis of natural products. The presence of nutrients triggers numerous activities and processes in the complex matrix of dam water because of the many compounds involved.

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DOI: http://dx.doi.org/10.15666/aeer/1905_35053518

Recommendations

- Identification of the secondary fungal metabolites is required for the discovery of novel drugs and useful compounds in pharmaceutical industries. In addition, the proposed biosynthesis mechanism for production of secondary metabolites needs to be explored as it may offer new opportunities for extraction.
- To date, only a few studies are focused on the biological role of aquatic fungi as well as their secondary metabolites in freshwater aquatic ecosystems. That might contribute to the lack of some useful drugs for treatment of various infections or else the toxins that can be harmful to humans and animals. Further research is required for the discovery of novel metabolites from fungi, as well as focusing on removal of toxigenic fungal species from water sources.

Conclusions

This study investigated the conditions that favour the biosynthesis of various classes of secondary metabolites by *T. flavus* and *A. fumigatus.* Every fungal species has its own gene responsible for the synthesis of secondary metabolites; however, the optimum conditions for mycelium growth do not determine the biosynthesis of secondary metabolites. No direct relationship could be established between the rate of fungal growth and secondary metabolite production; it occurs as a specific response to several environmental stress factors, including biotic and abiotic elicitors (the chemical compounds from abiotic and biotic sources that can stimulate stress responses in plants). The regulator gene can be up-regulated or down-regulated under conditions conducive to growth, such as carbon source, nitrogen source, oxidative stress, temperature, and lower pH. The extraction methods was successfully since the known group of secondary metabolites was identified from reference strain (*A.fumigatus* ATCC 36607) culture.

Acknowledgments. We thank Mr Garland More with his assistant on SIMCA software. We are very grateful to the following organizations: University of South Africa, National Research Foundation and Institute for Nanotechnology and Water Sustainability for funding this research.

Compliance with Ethical Standards. The authors declare that there is no conflict of interests regarding the publication of this paper and that the research data included in the manuscript do not involve either human participants or animals.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_35053518

A 99-YEAR CHRONOLOGY OF BLUE PINE (*PINUS WALLICHIANA)* TREE-RINGS CONCERNING INTERANNUAL CLIMATE VARIABILITY IN THE CENTRAL HIMALAYAS OF NEPAL

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

Abstract. We conducted a dendroclimatic study in the Mustang region of the Himalayas in Nepal with aims to develop the tree-ring chronology and to check response of blue pine (*Pinus wallichiana*) radial growth with climate variation in the area. We extracted 120 tree cores at an altitudinal range of 2350 to 2550 m and develop a 99-year long tree-ring width chronology of blue pine which spans from 1913 to 2011.Precipitation and temperature were the main climatic factors limiting the radial growth of *P. wallichiana.* Winter and spring time precipitation was positively correlated to blue pine growth, specifically: precipitation during the previous November ($r = 0.43$, $p < 0.01$), December ($r = 0.39$, $p < 0.05$) and current March ($r = 0.34$, $p < 0.05$). Similarly, there was a positive correlation between growth and September temperature of the current year ($r = 0.398$, $p < 0.05$), while correlation with temperature in previous year October ($r = -0.446$, $p < 0.05$) and December ($r = -0.424$, $p < 0.05$) was negative. Site conditions played a significant role in modulating the effect of precipitation and temperature on radial growth. Longer term regional scale dendrochronological studies are needed to improve our understanding on long-term climate change and its impact on forest growth.
Keywords: *climate change, climatic variations, dendrochronology, Mustang, moisture stress*

Introduction

The Himalaya Mountain region consists of a broad bioclimatic zone (Rai et al., 2019) and is experiencing a rate of warming several times greater than the global average (Shrestha and Aryal, 2011). Small changes in the mean or variability of climatic variables may result in several magnitudes the change in the frequency and intensity of extreme weather events, thereby creating challenging conditions for living organisms to adapt (Stocker et al., 2013). These extreme weather events are expected to alter current and future ecosystem dynamics leading to shifts in species distribution, population structures, vegetation composition, phenology, and length of growing

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DOI: http://dx.doi.org/10.15666/aeer/1905_35193532

seasons (Ziaco et al., 2014; Gaire et al., 2017a). The Himalayan region of Nepal is highly sensitive to the effects of a changing climate. The mean air temperature in Nepal has been increasing at a rate of 0.04 to 0.06 °C/year, which is faster than the global average (Shrestha and Aryal, 2011). Nepal is at a disadvantage in understanding the effects of a changing climate due to limited instrumental weather data (Bräuning, 2004); however, alternative tools such as tree-rings, pollens, lake sediments and ice-cores may be helpful for the reconstruction of past climate (Cook and Kairiukstis, 1990; Gaire et al., 2019). Dendrochronology and its sub-disciplines, such as dendroclimatology, use tree rings to reconstruct yearly variations in climate occurring prior to the interval covered by direct weather measurements (Fritts, 1976; Speer, 2010). Dendrochronology can date the formation of tree rings to an exact calendar year (Speer, 2010). In the last decade, several studies related to tree radial growth response to climate in the Himalaya in Nepal have been conducted (Dawadi et al., 2013; Liang et al., 2014; Gaire et al., 2017; Sigdel et al., 2018). Past studies related radial growth to precipitation (Kharal et al., 2014; Liang et al., 2014; Panthi et al., 2017; Gautam et al., 2021; Aryal et al., 2020), temperature (Gaire et al., 2014; Kharal et al., 2017; Aryal et al., 2018) and a combination of temperature and precipitation (Sano et al., 2005; Sohar et al., 2017).

Conifers are the major species studied under dendrochronological research due to their clearly visible annual ring structure (Gaire et al., 2013; Thapa et al., 2017; Bhandari et al., 2019). More than 60 scientific studies have been carried out in Nepal Himalayas using tree-ring data (Gaire et al., 2013). In Nepal, the longest chronology of a conifer species was for *Tsuga dumosa*, which is 1141 years long ranging from 856 to 1996 (Cook et al., 2003). Blue pine (*Pinus wallichiana* A. B. Jackson), an evergreen tree native to the Himalayan Mountain region, is widely used in multi-aspects dendrochronological research (Jackson, 1994; Gaire et al., 2013, 2019; Shah et al., 2019; Gautam et al., 2020). Schmidt et al. (1999) developed a first master tree-ring chronology for Nepal covering a time span ranging from 1324 to 1997 which included *P. wallichiana* from different regions of western and eastern Nepal. Subsequently, Cook et al. (2003) developed the longest chronology to date of *P. wallichiana,* 694 years ranging from 1303 to 1996, from archeological wood collected from historical structures in Bhratang, Nepal. Similarly, Shah et al. (2019) developed a 175-year long chronology of *P. wallichiana,* ranging in origin from 1840 to 2014, from Kasmir, western Himalaya, India. Yadav and Bhattachryya (1996) used *P. wallichiana* chronology for the study of glacial behavior in the western Himalaya. They concluded that this tree growing in subalpine Himalayan regions would be an excellent candidate to understand past glacier behavior in the region. Recently, Gaire et al. (2019) developed a 405-year long tree-ring chronology of *P. wallichiana* extending from 1611 to 2015 from Dolpo region in western Nepal and reconstructed spring-summer season drought. Similarly, 527 years and 495-years tree–ring chronologies have been developed using *P. wallichiana* from the Bagrot and Astor regions of Karakorum Mountains in northern Pakistan (Asad et al., 2017). For *Abies spectabilis*, Udas (2009) developed chronologies of 107 and 149 years from the two sites in the Mustang district consisting of 46 series from 26 trees. However, there is no study in the Mustang region in the Himalayas focusing only in the Blue pine growth from the natural forest stand. Therefore, to extend the knowledge of tree radial growth as related to past climate in the Himalayas, a study of blue pine (*P. wallichiana*) was conducted in the Mustang district of Nepal.

Materials and Methods

Study area

The study was carried out in Kunjo area of Mustang district (*Figure 1*). Mustang (Latitude: 28.59ºN; Longitude: 83.50ºE) is situated between the Annapurna and Dhaulagiri Himalaya ranges. The distribution of *P. wallichiana* in the Mustang district is noted for its presence at higher elevations which makes it highly suitable for establishing climate-tree growth relationships (Schweingruber et al., 1992). This district is characterized by a predominant rain shadow effect where annual rainfall is less than 200 mm/yr with temperatures ranging from -20 ºC in the winter

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to 26 ºC in the summer (NTNC, 2008). The analysis of weather data from the Jomsom station (1985 to 2016) indicates an overall increasing trend of maximum, minimum and mean annual temperatures by 0.0369 ºC year^{-1}, 0.0067 ºC year^{-1} and 0.0217 ºC year^{-1}, respectively (Adhikari, 2018). Similarly, annual precipitation has also trended upwards at a rate of 3.1823 mm year^{-1}. The year with the highest rainfall (432.1 mm) was recorded in 1995 and the lowest (116.1 mm) was in 1988 (Adhikari, 2018). The *P. wallichiana* forest is located in the southern part of the Mustang district and the associated weather station at Kunjo is in the Thasang Rural Municipality which lies at 2350 to 2550 m in elevation with an annual rainfall of 1200 mm. Trees at the upper boundary of the species distribution are more sensitive to climate variations than trees at lower elevations (Kharal et al., 2014). Therefore, in this study, we collected samples from the upper elevation limit of the forest. The study area is dominated by temperate coniferous and mixed-species forests of *P. wallichiana, Juniperus recurva, Abies spectabilis, Tsuga dumosa and Rhododendron arboreum* as the dominant tree species, and *Cupressus torulosa, Ilex dipyrena, Taxus wallichiana, Betula alnoides* and *Acer* spp. less dominant (Christensen et al., 2009). Geologically, granite and gneiss are the main rock types in the study area which form very coarse textured, acidic and shallow soils. The study sites consist of *P. wallichiana* in pure stands or mixed with other conifers and broadleaves.

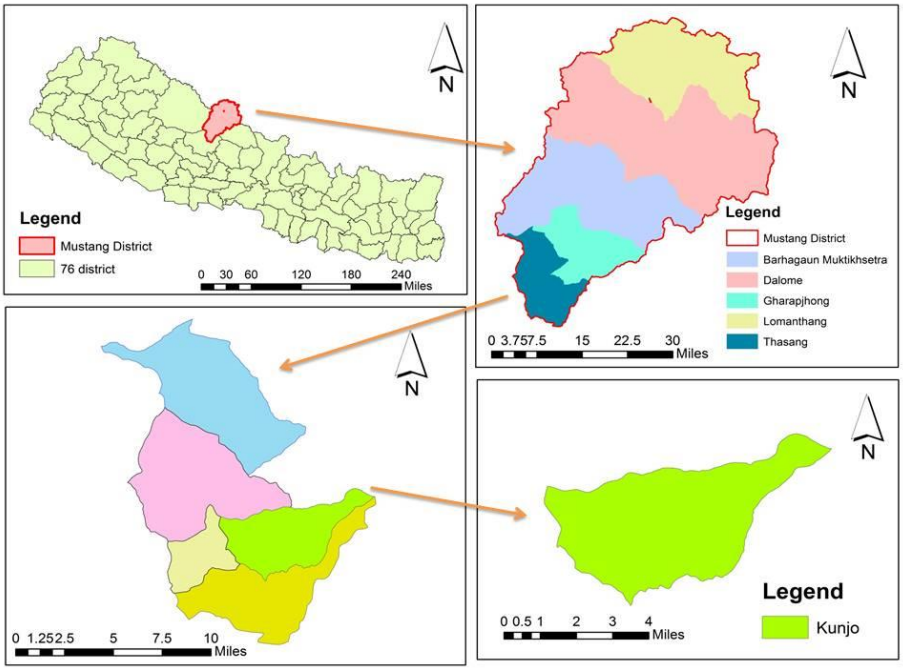

Figure 1. *Map showing the locations of sampling site (Kunjo)*

Study species

Blue pine is a large evergreen tree native to the Himalayan Mountain region, widely distributed at elevations ranging from 1,800 to 3,600 m and occasionally over 4,000 m (Jackson, 1994; Gaire et al., 2019). This species is often found in mixtures with *P. roxburghii* on southern aspects at lower elevations (Jackson, 1994). It is a strongly light demanding, less fire resistant than *P. roxburghii*, and is widely distributed throughout the Himalayas of Nepal. This species is sensitive to climate variation and is therefore well suited to dendrochronology study. Compared to other conifer species in the Himalaya region of Nepal, *P. wallichiana* is relatively less studied (Gaire et al., 2013)

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Field work

Dominant and co-dominant *P. wallichiana* trees were sampled from rocky terrain on southern slopes at elevations ranging from 2350 to 2550 m (*Table 1*). Generally, two cores per tree were extracted at breast height (1.3 m) on opposite sides of the bole (Gaire et al., 2020), parallel to the contour slope. In total, 120 cores from 60 individual trees were collected using a Swedish increment borer following standard techniques (Speer, 2010). The extracted cores were immediately transferred to plastic straw pipes and brought to Dendro-lab for analysis.

Table 1. *Location of meteorological stations and sampling site in Mustang district*

Meteorological stations	Altitude (m)	Latitude	Longitude
Lete	2384	28.38°N	83.36°E
Marpha	2566	28.45 °N	83.42 °E
Jomsom	2744	28.47 °N	83.43 °E

Sampling site (name)	Altitude range (m)	Latitude	Longitude
Kunjo	2350-2550	28.64 °N	83.66 °E

Laboratory work

Core samples were analyzed using established dendrochronological procedures (Speer, 2010). Surfaces of the cores were successively smoothed by hand with a series of sanding papers progressing to finer grits (Gaire et al., 2020). Prior to chronology development, all tree rings in each core samples were counted using a stereo-zoom microscope and dated to the calendar year. Very young series (individual series with less than 50 rings) were discarded from further analysis. Tree-ring widths were measured to the nearest 0.01 mm using the LINTAB measuring system connected to a computer with TSAP-Win associated software (Rinn, 2003). Cross-dating of chronologies corrected errors from false and missing rings or measurement errors to ensure accurate dating of the annual increments. This was done using a visual verification procedure in the TSAP-Win software and further validated through the computer program COFECHA (Holmes, 1983; Grissino-Mayer, 2001). Measured tree-ring series with potential errors identified through COFECHA were double checked prior to removal from further analysis. Finally, a total of 27 series were chosen for chronology development and further analysis, after discarding the very young series and problematic series including those broken or with abnormal growth properties. The software package, ARSTAN, was used to standardize all the tree-ring series (Cook and Holmes, 1986). The purpose of standardization was to: a) remove non-climatic age-related trends from the ring-width series, and b) allow the resulting standardized values to be averaged into a mean value function by adjusting the series for varying growth rates due to differing tree ages and rates of growth. Conventional negative exponential curvilinear or linear regression (any slope) analysis was used to detrend the tree-ring series. This procedure removed age effects due to biological growth trends and other low frequency variations due to stand dynamic features. Then, all detrended series were averaged to chronologies by computing the mean value function. The mean value function concentrates on climate related environment signals and averages out noise from endogenous or exogenous disturbances (Fritts, 1976). For this analysis, a robust bi-weight mean estimation (Mosteller and Tukey, 1977) was used which removed random signals that were related to local disturbances. Finally, a set of three chronologies, were developed: (i) a standard chronology, reflecting variations after removing age effects; (ii) a residual chronology, containing only high-frequency variations after removing autocorrelation from the standard chronology; and (iii) an ARTSAN chronology composed of the residual chronology reincorporated with the pooled auto regression. Chronology statistics such as mean tree-ring width or mean index, standard deviation, standard error, mean sensitivity and autocorrelation were

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calculated. Chronology confidence was estimated by calculating the running Expressed Population Signal (EPS) using a 30-year window with an overlap of 29 years. Wigley et al. (1984) has suggested an EPS value of 0.85 as an acceptable value demonstrating a strong common signal present in a chronology (Cook and Kairiukstis, 1990).

Meteorological data

The climate data at the nearest meteorological stations in Kunjo (Lete 2,384 m, Marpha 2,566 m and Jomsom 2,744 m) were obtained from the Department of Hydrology and Meteorology (*Table 1*). The precipitation data from Lete and Jomsom stations covered the measurement period from 1987 to 2010 and 1969 to 2007, respectively. The temperature data ranges from 1998 to 2007 in Lete and 1961 to 2010 in Jomsom, however, in Jomsom there was 10 years of missing data. Missing temperature and precipitation data from these stations was predicted by using fitting trend line. In order to estimate past climate data, the CRU grid data was used by covering Lete and Jomsom meteorological stations (http://www.cgiar-csi.org/data/climate/item/52-cru-ts-21-climate-database) and sampling site. The climograph of precipitation and temperature of Lete and Jomsom is presented in *Figure 2*.

Climate and tree-ring growth relationships

Both temperature and precipitation were highly correlated among the three meteorological stations of the Mustang district (Kharal et al., 2014). Pearson correlation analysis was used to examine how the climatic variables of monthly mean temperature and total precipitation, influence tree-ring radial growth (Fritts et al., 1971). A climatic window of 16 months starting from the previous year September until the December of the current year was used by following previous tree-ring based studies from the Nepal Himalayas (Sano et al., 2005; Kharal et al., 2017; Gaire et al., 2019). This period of year was selected since climate conditions during the previous and current years can affect the amount of carbon fixed and allocated to tree growth (Grissino-Mayer and Butler, 1993).

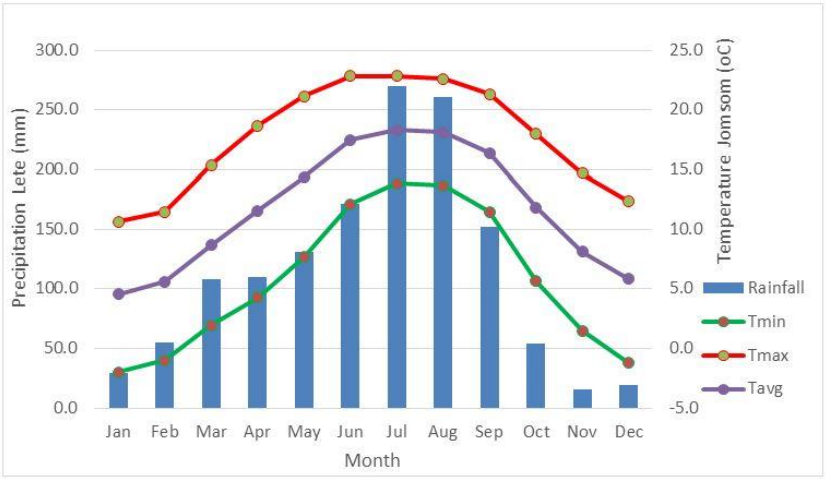

Figure 2. *Average monthly precipitation of Lete and temperature of Jomsom stations*

Results

Chronology statistics

From the 27 well-dated series, a 99-year chronology of *P. wallichiana* spanning from 1913 to 2011 was developed (*Figure 3*). There was a progressive decrease in growth from 1913 to 1925, followed by a four-year increase to 1929 and then again, another cycle to 1943. Comparing this

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growth pattern with the CRU precipitation data during the period of increased growth, August rainfall was found to be higher than in other years. From 1943 onwards, radial tree-ring growth fluctuated at constant rate, with a distinct increase over the period of 1995 to 1998, during which, August rainfall was also found to be above average. This pattern was also seen at the Lete station's rainfall data. Increased radial growth was associated with surplus (above average) rainfall in current year March, and previous year November and December. There are distinct periods of decreased August rainfall in 1925, 1932-1939, 1972, 1985 and 1999 which were associated with reduced radial growth (Graph not shown). While doing comparison with monthly average temperature, a reduced temperature during October through December of the previous year was found to be improved the standard chronology (Graph not shown).

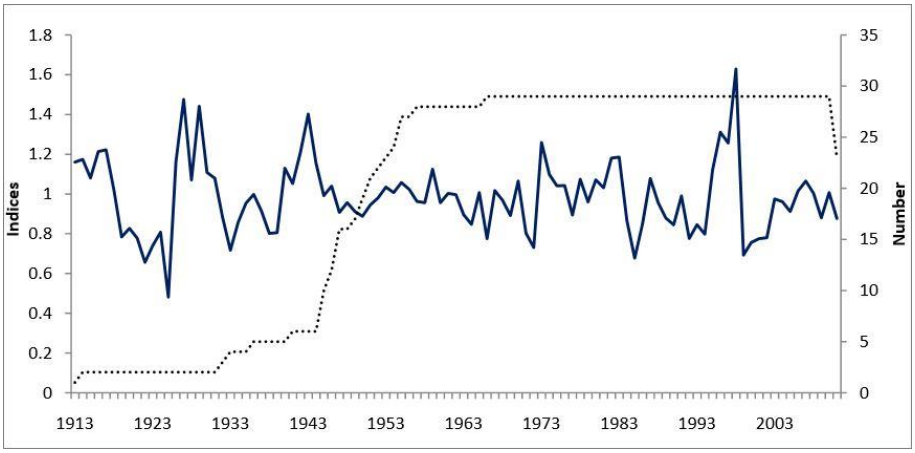

Figure 3. *Tree ring width chronology of Pinus wallichiana. Solid line represents the ring width chronology and dash line represent the sample depth*

The chronology statistics of both standard and residual chronologies were calculated. The correlation with the master series was 0.415 and the mean between trees correlation was calculated to be 0.211 with a standard deviation and error of 0.183 and 0.012, respectively. The mean sensitivity of the standard chronology was found to be 0.145 and that of the residual chronology was 0.16 with a standard deviation of the standard chronology and residual chronology to be 0.183 and 0.164, respectively. The autocorrelation value of standard chronology was 0.394 and that of residual chronology was 0.004. Expressed population signal (EPS) is a measure of verification of representation of the sample to the population with a minimum threshold of 0.85. In this study, the EPS was found to be 0.846 at the 20th sample, 0.852 at the 21st sample, and 0.858 at the 22nd sample. Mean annual growth rate was 3.51 mm with a variance of 0.27. After checking the chronology statistics of standard and residual chronology, we selected the residual chronology for tree-ring and climate relation analysis.

Comparison between CRU and station climate data

Since precipitation data at Lete is limited to 1971 and forward, this is compared with CRU data through correlation analysis and is displayed in *Figure 4*. It was found that mean monthly precipitation correlation of January, March, October, November and December were highly significant whereas April and September were less significant. Similarly, mean monthly temperature correlations of January, February, March, June, July and November were highly significant whereas April was less significant.

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DOI: http://dx.doi.org/10.15666/aeer/1905_35193532

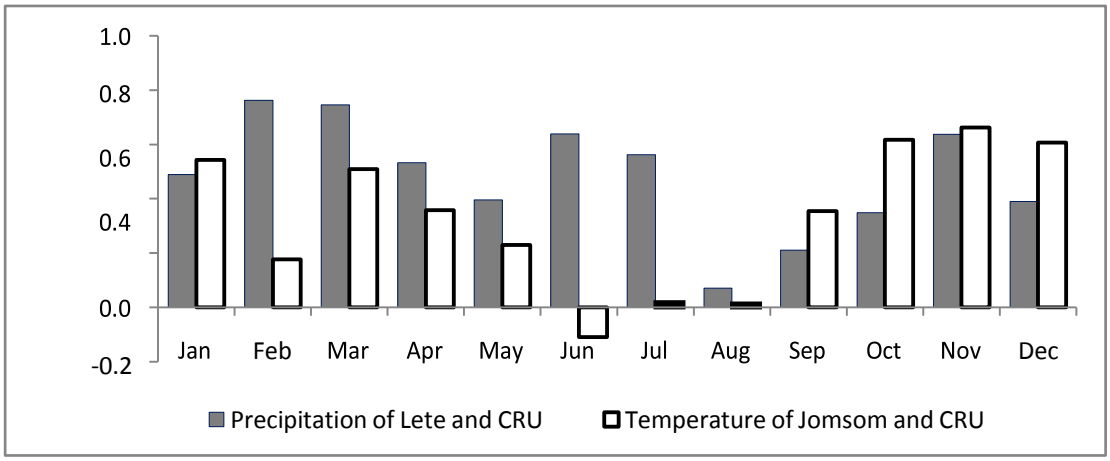

Figure 4. *Correlation between CRU and meteorological station's mean monthly precipitation and temperature data*

Due to a lack of temperature data from Lete prior to 1998, a comparison with Marpha and Jomsom stations was used to fill the data gap (*Figure 5*). Correlation of Lete station's mean monthly temperatures with Jomsom was highly significant for January and February, while less significant with March, April, September, October and November. Although, Marpha station is closer geographically to Lete station, correlation with Jomsom was higher than Marpha and was used to correlate temperature to tree-ring radial growth.

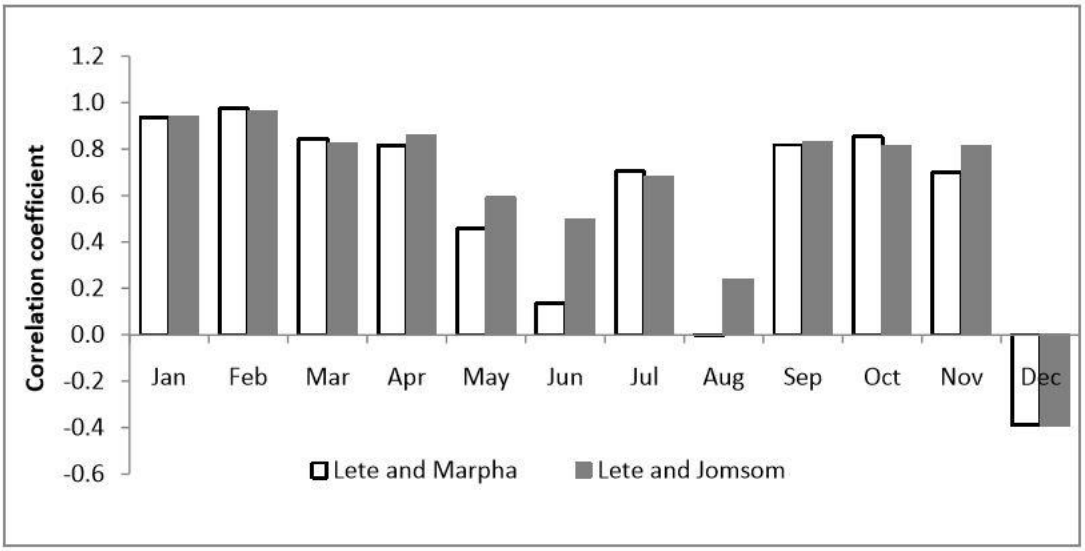

Figure 5. *Mean monthly temperature correlation of Lete with Marpha and Jomsom*

Climate and tree growth relationship

In this analysis, 16 climatic period variables were correlated with the residual tree-ring chronology from 1913 to 2011 (*Figure 6*). *Figure 6* depicts the correlation of residual chronology with mean monthly precipitation and temperature. Tree-ring growth was positively correlated with current year March precipitation ($r = 0.34$, $p < 0.05$) and the previous year's November and December precipitation ($r = 0.43$, $p < 0.01$; $r = 0.39$, $p < 0.05$, respectively) (*Figure 6*). We found a significant negative correlation between the tree growth and temperature at Jomsom station for

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previous year's October and December (0.446, $p < 0.05$ and 0.424, $p < 0.05$, respectively), whereas, a significant positive relationship with current September temperature (0.4, $p<0.05$) (*Figure 6*).

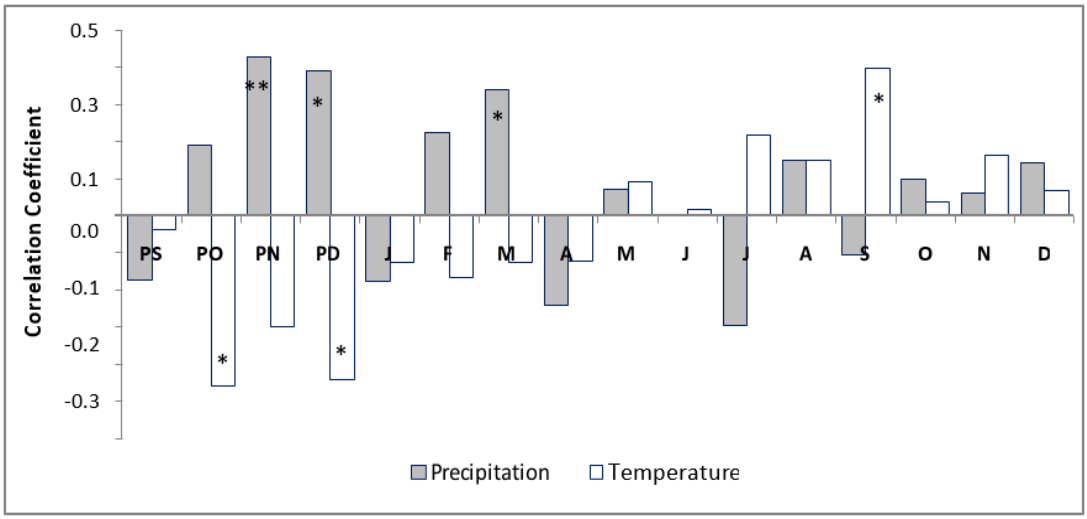

Figure 6. *Correlation of standard chronology with monthly average precipitation and temperature. The PS to D stands for the name of the months starting from previous year September to current year December. Note: ** Significant at p<0.01, * Significant at p<0.05, P: Previous, Month: Initials of each month (J: January, F: February…D: December)*

Discussion

Dendroclimatic potential of Pinus wallichiana from Mustang

We developed a 99-year tree-ring width chronology of *P. wallichiana* dating back to 1913 with a mean sensitivity of 0.145. The chronology statistics of the species depicted its dendroclimatic potential (Fritts, 1976; Speer, 2010). The present study revealed a relatively low chronology mean sensitivity despite being carried out on a harsh southern slope; this may be attributed to a well distributed rainfall with a dry period not exceeding two months (Fritts, 1976). The low mean sensitivity was unexpected, since most of the Mustang region is dry and in a rain shadow with precipitation concentrated to southern areas. However, the mean sensitivity we obtained for blue pine from Mustang region is comparable to that reported for the same species from the Dolpa in the central Himalaya in Nepal (Gaire et al., 2019) and chronology from the northwest Himalaya (Bhattacharyya et al., 1988). In order to get a clear signal of tree-ring radial growth as influenced by weather, areas with human and natural disturbance such as grazing, firewood collection, and selective logging should be avoided. Trees growing on dry sites usually demonstrate higher inter-annual growth variability than trees from temperature limited sites (Liang et al., 2008) which is typical for conifers growing in humid environments (Fan et al., 2009). In the subalpine temperate region, conifers demonstrate a low mean chronological sensitivity under mesic climate conditions when compared to arid sites (Bhattacharyya and Chaudhary, 2003). Most of our tree-ring series were relatively young (all less than 100 years old), that could be another reason for low mean sensitivity (Fritts, 1976).

In this study, we developed a 99-year long tree-ring chronology of *P. wallichiana* extending from 1913 to 2011. This chronology has low autocorrelation, moderate mean sensitivity and a high standard deviation which are considered suitable for dendroclimatic analysis (Fritts, 1976) and the EPS was greater than of 0.85, a commonly used threshold limit (Wigley et al., 1984). In the current study, the mean growth rate was 3.51mm which is greater than the mean annual growth

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rate documented in the Dolpa region of Nepal (Gaire et al., 2019). The low autocorrelation (0.004) indicates that the previous year's growth effect on the current growth is minimal in residual chronology (Speer, 2010) and the EPS value of our chronology is greater than the threshold. These values are similar to those of *P. wallichiana* reported from the different regions in the Himalayas (Asad et al., 2017; Gaire et al., 2019; Shah et al., 2019). Asad et al. (2017) obtained similar chronological statistics from two different locations in western Himalaya in Pakistan, where SD = 0.17 and 0.21, MS=0.14 and 0.13, EPS=0.94 and 0.90. Similarly, Gaire et al. (2019) carried out a similar investigation in the central Himalaya and documented similar chronology statistics: MS=0.156, SD=0.229, EPS (0.955) greater than the threshold with a high SNR (21.23), which indicate that the pine chronologies are suitable for dendroclimatic study.

Climate change impact on Pinus wallichiana

Over the past decade in the Himalayan region, several studies have investigated tree growth response to climate (Gaire et al., 2013, 2017, 2019, 2020; Dawadi et al., 2013; Ling et al., 2014; Kharal et al., 2017; Panthi et al., 2017; Sigdel et al., 2018). Based on published results, tree growth demonstrated a diverse response to climate variation in the Himalaya with some studies indicating that growth was primarily controlled by precipitation at higher elevations (Dawadi et al., 2013; Panthi et al., 2017; Gautam et al., 2021). Some studies showed that growth was primarily controlled by temperature variation (Gaire et al., 2014, 2020; Thapa et al., 2015; Kharal et al., 2017), while others concluded that both precipitation and temperature could significantly influence tree growth (Sohar et al., 2017). Overall, there is general agreement that seasonal climatic variation highly influences the growth of Himalayan conifers (Dawadi et al., 2013).

In order to understand the influence of seasonal variation in precipitation on blue pine tree growth, Shah et al. (2009) combined monthly climate data for pre-monsoon months, i.e. December through April. They reported that monsoon precipitation (July-August) did not play a significant role in limiting the tree growth in the studied eastern Himalaya region. Correlation analysis between monthly mean rainfall and radial growth of *P. eliottii* revealed a significant negative correlation with current year May precipitation and a significant positive correlation with current September precipitation (Harley et al., 2011). Pfeifer et al. (2005) found summer and the preceding autumn (September-October) as the dominant climatic factors controlling tree ring-widths of *P. cembra* in Western Austria.

We found that the growth of blue pine in the Mustang is primarily influenced by the winter and spring season climate and secondly by temperature during late summer. The present study revealed that current September temperature had positive control over the radial growth, whereas October and December temperatures in the previous year had a negative control over the radial growth. The month of September is physiologically active for growth as moisture is generally available owing to monsoon precipitation and groundwater, however low temperatures could limit respiration, photosynthesis, and other biochemical processes essential for growth (Fritts, 1976). Similarly, low temperature in September may cause early cessation of the growth. These environmental conditions are typical in the areas in this study at the upper elevation limit of this species. A high temperature in previous year October and December with a high wind can cause desiccation effect in the temperate and subalpine regions. On the other hand, a high temperature during early winter can promote for melting of snow leading to less snow accumulation to supply water for following growing season. These could be the possible reasons why we obtained positive relations with winter precipitation but negative with the temperature. Temperature effects are most apparent on south-facing and exposed slopes on shallow well drained soils or those with restricted root distributions (Fritts, 1976; Fan et al., 2009). Photosynthesis is sensitive to temperature (Fritts, 1976). Previous studies indicated that winter month temperatures in the previous year were found to have a positive relationship with the radial tree-ring growth of blue pine in Arunachal area in eastern India (November- December) (Shah et al., 2009) and (December-February) Gangotri region in western India Himalaya (Yadav and Bhattacharyya, 1996). This direct relationship between tree growth and winter temperature indicates that

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Himalayan pine trees carry on a significant amount of photosynthesis during warm winters, with stored energy reserves used in the following growing season (Singh and Yadav, 2000; Shah et al., 2019). Similar observations (October and December-January) have been noted in Himalayan pine trees growing at lower altitudes (Yadav and Amalava, 1997).

A tree-ring study was carried out by Udas (2009) in the Mustang district, close to our study area, by using *Abies spectabilis* which showed a negative correlation with previous year December temperatures and current year April, May-June temperatures in line with present study. According to Udas (2009), the negative relationship with winter temperature could be the respiratory loss of carbohydrate reserves during warm winters, thereby, impeding tree growth in the following growing season. Another possible explanation for the indirect relationship of winter temperature with tree growth could be early snowfall in the study area which would act as an insulator on the ground surface thus, protecting the rooting zone from freezing, thereby regulating winter cold stress over short time periods. If trees ceased their annual growth prematurely, then surplus photosynthetic carbohydrates could be stored for immediate growth at the start of the next growing season (Henderson and Grissino-Mayer, 2009).

High moisture during winter and early spring season was found beneficial or positive effect for the blue pine growth in the following growing season. The study area receives precipitation in the form of snow during winter and early spring season. The melting of which can provide continuous moisture supply for tree growth during the growth start in the spring season. A positive winter and spring season moisture signal in the tree growth is widely observed in the dry regions in the Himalayas (Thapa et al., 2015; Panthi et al., 2017; Gaire et al., 2017, 2019). Studies done in the Himalayas also show that tree growth in these regions is limited by moisture availability in the pre-monsoon season and can demonstrate a negative relationship with temperature and a positive one with precipitation (Boragaonkar et al., 1999; Sano et al., 2005; Thapa et al., 2015; Panthi et al., 2017). Similarly, Himalayan fir growing at higher elevations in Manang region is mainly limited by moisture stress rather than by low temperatures (Rai et al., 2019).

To this end, we found that the growth of the blue pine is fluctuating over time in accordance to climate change situation in the area. We found winter and spring season climate has major role for the pine growth in the subsequent growing season. Studies from the Himalayas have indicated higher rate of warming, and higher elevation getting warmer (Shrestha and Aryal, 2011). There is no consistent trend in the precipitation data from the Nepal Himalaya and its trend is increasing or decreasing in different stations (Shrestha and Aryal, 2011). Similar fluctuating situations exist in drought conditions (Panthi et al., 2017; Gaire et al., 2019). If the temperature will be increased continuously with decreasing precipitation, less snow falls or low snow accumulation in the winter and early spring season, it could adversely affect the blue pine forest growth in the study area in Himalayas. However, if there will be an increase in precipitation and snow accumulation concurrent with the warming, and if there will not be winter or spring season drought stress, the pine forest in the Himalayan region can take advantage for its growth in future, and growth of pine will be increased in future in response to climate change.

Conclusion

A century long tree-ring chronology of *P. wallichiana* was developed in the Kunjo area of the Mustang district of Nepal. The present study of *P. wallichiana* tree-ring and climatic relationships indicates that this species is suitable for dendroclimatic study, due to its clear and dateable tree-ring sequences and synchronistic growth patterns. Winter and spring season moisture availability is found as a dominant climatic factor controlling the growth of this species. The dendroclimatic analysis from this investigation has potential to understand long-term dynamics of snowfall (winter precipitation) and climate change effects in the Himalayan region of Nepal. Long-term regional scale chronologies are required to understand current impacts of climate change on forest ecosystems. Continued development of long-term dendroclimatic chronologies in Himalayan conifers are needed to support climate change research and adaptation in the region.

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Acknowledgements. This study was funded by the Strategic Priority Research Program of Chinese Academy of Sciences, Pan-Third Pole Environment Study for a Green Silk Road (XDA 20040301). We thank the Beijing Forestry University, Nepal Academy of Science and Technology, and the Tribhuvan University, Institute of Forestry, Pokhara Campus for providing research facilities. The first author thanks the Chinese Government for the Ph.D. scholarship. We are also grateful to Rajesh Sigdel and all reviewers for their valuable comments and suggestions for improving the quality of this paper.

Conflict of Interests. The authors declare no conflict of interests.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_35193532

EFFECT OF BIOCHAR ON SOIL CADMIUM CONTENT AND CADMIUM UPTAKE OF COTTON (*GOSSYPIUM HIRSUTUM* L.) GROWN IN NORTHWESTERN CHINA

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(Received 27th May 2020; accepted 30th Aug 2021)

Abstract. In order to investigate the effects of biochar on soil cadmium (Cd) content in northwestern China, we used cotton (*Gossypium hirsutum* L.) straw charcoal in a 2-year experiment involving different doses of both biochars (1.5% (C1) and 3% (C2)) and Cd (1 mg·kg^{-1} (H1), 2 mg·kg^{-1} (H2), and 4 mg·kg^{-1} (H3)) in addition to the treatments, control plots were set up with no biochar application. We grew cotton (*Gossypium hirsutum* L.) in pots for each treatment and examined soil pH, Cd forms, and Cd accumulations in aboveground plant organs. Compared to no added biochar, the addition of biochar significantly increased soil pH, but there was no notable difference in soil pH between the C1 and C2 treatments. Biochar significantly decreased soil-available Cd with this effect increasing with higher biochar doses. The lowest amounts of soil-available Cd in all Cd treatments (H0–H3), including the control, occurred later in the study, typically at 90 to 150 days post treatment. In the cotton (*Gossypium hirsutum* L.), Cd preferentially accumulated the most in leaves, then in stems, and then in bolls and the amount of Cd in cotton and soil-available Cd significantly correlated. The addition of biochar promoted the transformation of exchangeable and carbonate-bound Cd to organic matter-bound Cd at 30, 60, and 90 days, and the exchangeable and carbonate-bound Cd to Fe-Mn oxide-bound Cd at 120 and 150 days. In conclusion, biochar addition decreases both Cd bioavailability and the accumulation of Cd in cotton (*Gossypium hirsutum* L.) aboveground organs.
Keywords: *cotton straw biochar, soil pH, Cd forms, Cd bioavailability*

Introduction

Widely found in farmland soils, cadmium (Cd) is a highly toxic heavy metal that biomagnifies through the food chain, thus ultimately endangering human health (Li et al., 2010). Studies proved that metals such as copper, lead, zinc, cobalt, nickel, chromium, and mercury which have been considered as hazardous heavy metals are very toxic elements (Ghassabzadeh et al., 2010). Wastewater, fertilization, and irrigation using wastewater containing Cd are the main sources of Cd pollution in China's soils, contributing to both soil and water pollution in the Xinjiang area of northwest China, where chemical fertilizers containing Cd are used. Such repeated fertilization over many years often leads to an accumulation of heavy metals in the soil and unacceptable amounts of Cd have been found in Xinjiang soil (Wang et al., 2016). When subjected to Cd exposure, plants display shortened root length, decreased chlorophyll amount, and decreased fruit number (Renyuan et al., 2018).

Biochar, a charcoal with a pore structure, high aromatization, and high carbon amount (Aslam et al., 2017), is beneficial in agricultural and environmental contexts, especially since the use of biochar as a soil amendment may potentially help to mitigate global warming (Gaunt, 2008), improve soil quality (Fellet et al., 2011), reduce the bioavailability of organic contaminants (Li et al., 2010), and increase nutrient and water

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DOI: http://dx.doi.org/10.15666/aeer/1905_35333549

retention capacity of soil (Abel et al., 2013; Zheng et al., 2013), thereby increasing crop yield (Zhang et al., 2013). Song et al. (2017) showed that modified walnut shell biochar is a catalyst for the catalytic removal of organic sulfur and arsenic. Other research has shown that biochar decreases the mobility of heavy metals by altering soil pH to control heavy metal mobilization (Tong et al., 2020; Ma et al., 2020; Kim et al., 2015; Zhu et al., 2015). Also, Zhu et al. (2015) demonstrated that 0.5% wine lees-derived biochar decreased exchangeable Cd in soil by 48.14% and Wang et al. (2016) showed that biochar derived from tea branches promoted the growth of *Lolium multiflorum* and reduced antimony and Cd bioavailability.

Cotton *(Gossypium hirsutum* L.*)*, a dominant commercial crop in Xinjiang, was planted in 53.46% of the farmed area and provided 67.3% of the total Chinese cotton harvest (Gaunt, 2008). Long-term, continuous cotton cropping, coupled with the use of agricultural chemicals, have enriched Xinjiang farm soils with heavy metals. Luckily, abundant cotton straw accumulation, common in this large, cultivated area, presents an important opportunity to reduce soil pollution by converting it into biochar and using it as a soil amendment. Therefore, using heavy metal contaminated cotton stalks converted to biochar to both absorb Cd and provide nutrition in cotton field soil would not only reduce the risk of Cd contaminating farmland, but would also usefully reuse Cd-contaminated cotton stalks.

We applied cotton straw biochar to Cd-contaminated, Xinjiang, cotton field soil to examine the effects of biochar on various soil Cd forms and how Cd levels in soil varied over time, as well as cotton's absorption of Cd and how it varied over time. Our results provide basic data and technical reference for controlling Cd pollution in northwestern China farmland soil.

Materials and methods

Soil and biochar collection and their physicochemical properties

We conducted our experiments at the Agricultural College of Shihezi University in Shihezi City, Xinjiang Province, China (86°03′E, 45°19′N) for a two-year continuous remediation. The temperate was of continental climate, with an average annual temperature of 7.5 ~ 8.2 ℃, sunshine duration of 2318 ~ 2732 h, frost-free period of 147 ~ 191 d, annual rainfall of 180 ~ 270 mm, and annual evaporation of 1000 ~ 1500 mm. The heavy metals in the soil of this area come mostly from chemical fertilizers, agricultural chemicals such as pesticides, and long-term continuous cropping. To manage and control potential errors, we used pots filled with loamy soil from test station cotton fields, where cotton had been continuously planted for more than 25 years. Here, because of long-term continuous cropping, the soil had various levels of heavy metals. Before collecting the samples, the depth of the soil sampling was 0-20 cm, we have prepared 800 kg of soil sample we removed soil debris by hand and then air dried the soil before passing it through a 2 mm-mesh sieve, keeping a sub-sample to determine the soil's physicochemical characteristics. The hydrometer method was used to investigate soil particle size distribution, subsequently finding that the soil texture was clay loam (Bouyoucos, 1962), and then used the Walkley–Black method described by Nelson and Sommers (1982) to determine soil organic carbon. We used a soil–water suspension (w/v, 1:2.5), shaken for 1 h, to measure soil pH using a calibrated pH meter (WTW 7110, Weilheim, Germany) (Muhammad, 2019). Total nitrogen (N), phosphorous (P) and potassium (K) concentrations were determined using the Kjeldahl

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DOI: http://dx.doi.org/10.15666/aeer/1905_35333549

protocol (Bremner and Mulvaney, 1982), the Watanabe and Olsen method (1965), and the HF-$HClO_4$-H_2SO_4 digestion method (Page et al., 1982), respectively (*Table 1*).

Table 1. *Basic physical-chemical properties of biochar and soil used in the experiments*

Property	Biochar	Soil
pH	9.50	7.76
Total nitrogen ($g \cdot kg^{-1}$)	0.89	0.46
Total P ($g \cdot kg^{-1}$)	2.54	28.42
Organic matter ($g \cdot kg^{-1}$)	625	14.73
Total K ($g \cdot kg^{-1}$)	8.62	246.83
Total Cd ($mg \cdot kg^{-1}$)	0.021	0.25
Total salinity ($g \cdot kg^{-1}$)	-	3.36
Carboxyl ($mmol \cdot g^{-1}$)	0.20	-
Lactone ($mmol \cdot g^{-1}$)	0.25	-
Phenolic hydroxyl ($mmol \cdot g^{-1}$)	0.21	-

Biochar was prepared using anaerobic pyrolysis of cotton straw at 450 °C for 6 h, with a resultant biochar conversion rate of 37.5% (Parinda et al., 2016). That biochar was then dried, crushed, and screened through a 2-mm sieve and its pH was measured in a 10 $mmol \cdot L^{-1}$ $CaCl_2$ solution (solid: solution = 1:2.5 (w/v)) using a glass electrode and a Corning pH 10 portable pH meter (Acton, MA, USA). We used the same methods to determine total N, total P, total K, and organic carbon concentration as we used for the soil (*Table 1*). Biochar pore structure is illustrated in *Figure 1*.

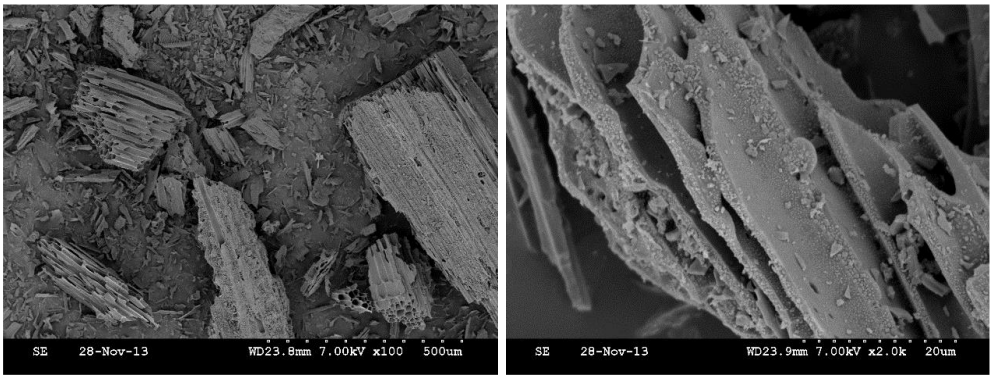

Figure 1. *Representative microporous structure of biochar magnified 100× (500 μm (left)) and 2,000× (20 μm (right))*

Experiment pot design

Before beginning the experiment, the soil was air dried and screened through a 2-mm sieve. We prepared contaminated soil by first dissolving our Cd source, $CdCl_2 \cdot 2.5H_2O$ (2.44 g, analytical reagent, Merck, Germany), in distilled water. We shook the preparation to ensure complete dissolution and then diluted it to 1000 mL, resulting in a 1.2 $g \cdot L^{-1}$ solution of Cd^{2+}. 10, 20, or 40 mL of that stock solution were each mixed with 12 kg soil samples, thus producing experimental samples with 1, 2, and 4 $mg \cdot kg^{-1}$ exogenous Cd^{2+} amount levels. Those levels correspond to 1-, 2-, and 4-times China's

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Level III Soil Environmental Quality standard for Cd. Control soil with no added Cd and the 1, 2, and 4 $mg \cdot kg^{-1}$ Cd samples were named H0, H1, H2, and H3, respectively. Next, we used a plastic container and a spatula to mix biochar into the soil at 1.5% and 3% w/w (equivalent to 23.4 $t \cdot ha^{-1}$ and 46.8 $t \cdot ha^{-1}$), respectively. Biochar levels of 0%, 1.5%, and 3% (mass ratio) were named C0, C1, and C2, respectively. Finally, we made 12 experimental soil treatments by completely mixing either H0, H1, H2, or H3 $CdCl_2$ soils with either C0, C1, or C2 biochar soils on a plastic cloth. For each experimental soil treatment, we put 12 kg of soil into a pot (25 cm × 30 cm), repeating each treatment 5 times for a total of 60 pots. The pots were placed in a randomized block design and maintained in identical conditions for 10 weeks (*Fig. 2*). Each pot received recommended doses of $N–P_2O_2–K_2O$ (180-150-210 $kg \cdot hm^2$ urea, diammonium phosphate, and potassium sulfate, respectively). All the P and K and half of the N was applied before crop sowing and the remaining N was applied after crop establishment.

Figure 2. *Pot culture experiment*

Planting and sample collection

This study was conducted under open air. We first bought seeds of a local cotton variety (LuYan 24) and then disinfected and sterilized (with 2.5% sodium hypochlorite) same-sized seeds. Twenty seeds were planted in each plastic pot and, once they had 3-5 true leaves, seedlings were thinned to 5 per pot and grown for 150 days. To prevent recontamination, we use deionized water for irrigation. We collected soil and leaves and stems plant samples at 30, 60, 90, 120, and 150 days of culture, and collected cotton bells at 60, 90, 120, and 150 days of culture. Soil samples were collected by sampling 3 points in each pot to a maximum soil depth of 20 cm, then the collected samples from each pot were combined and air-dried, the amount of the collected soil sample was

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DOI: http://dx.doi.org/10.15666/aeer/1905_35333549

180 g. Cotton stems, leaves, and bolls were collected and rinsed with deionized water and then weighed on a digital scale to determine each sample's wet weight, before they were oven dried (85 °C) to a constant dry weight.

Tests and assays

We used the Tessier 5 step continuous extraction method (Tessier et al., 1979) to sequentially extract trace metals, including Cd, for subsequent measurement at the end of each step. Each 0.2000 g air-dried soil sample was subjected to the following operations, each designed to extract a fraction of the metals in the soil:

1. Exchangeable: Soil samples were oscillated continuously for 1 h with 1 mol L^{-1} $MgCl_2$ solution (pH = 7).
2. Bound to carbonates: The residue in step (1) was oscillated continuously for 5 h with 1 mol·L^{-1} CH_3COONa solution (pH = 5).
3. Bound to iron (Fe) and manganese (Mn) oxides (Fe-MnO): The residue in step (2) was oscillated continuously for 6 h at 96 ± 3 °C with 0.04 mol·L^{-1} NH_2OH·HCl diluted in 25% acetum solution.
4. Bound to organic matter: The residue in step (3) was oscillated for 2 h at 85 ± 2 °C with 3% H_2O_2 (adjusted to pH = 2 using HNO_3) and 0.02 mol·L^{-1} HNO_3 (volume ratio). Then 3% H_2O_2 (pH = 2) was added, and the mixture was oscillated continuously for 3 h at 85 ± 2 °C. After cooling, the mixture was oscillated continuously for 0.5 h with 3.2 mol·L^{-1} CH_3COONH_4 diluted in 20% HNO_3 (volume ratio).
5. The residue form: Using the subtraction method, after the fractions from the previous 4 extractions were subtracted from the sample, the remaining amount was the residue.

Between each step, the mixture was centrifuged at room temperature for 15 min at 2000 rpm and the supernatant was transferred into a 25 mL centrifugal tube to maintain constant volume. Then we used a Hitachi Z2000 graphite atomic absorption spectrophotometer (Hitachi, SiChuan., Tokyo, Japan) to detect metals in each supernatant sample.

We determined available Cd in the soil by performing diethyline-triamine-penta acetic acid extraction and then testing the extract with a graphite atomic absorption spectrophotometer (Mahanta et al., 2011). We then used microwave digestion-graphite atomic absorption spectrometry to determine Cd levels in the cotton stems, leaves, and bolls (Parinda et al., 2016). Soil pH was determined with the soil pH-potential method, in which soil: water = 2.5:1 (Yan et al., 2000).

In addition, soil water content was determined gravimetrically by comparing the wet and dry weights of soil samples (collected at the end of every cotton growing stage) to determine the amount of soil irrigation (the field moisture capacity of 60%-70%) (Wang et al., 2020).

Data analysis and visualization

The data were compiled in Excel 2016 and two-way analysis of variance (ANOVA) was performed using SPSS 23.0. Multiple comparisons between different treatments were conducted using Duncan's new multiple range test ($\alpha = 0.05$). Charts were drawn using Origin 8.0 (OriginLab, MA, USA).

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DOI: http://dx.doi.org/10.15666/aeer/1905_35333549

Results and analyses

Changes in soil pH

The addition of biochar significantly increased soil pH ($P < 0.05$, *Table 2*), which rose in line with increasing biochar amounts. However, interactions between exogenous Cd and biochar had no significant effects on soil pH ($P < 0.05$) and, as time elapsed, soil pH decreased. The maximum pH at 30 days was in the C2H3 treatment (pH = 8.56), thus showing that added biochar increased pH than those in the C0 treatments (controls, no biochar) ($P < 0.05$). Overall, the experimental groups pH were higher than those of the control groups, but none of them were significantly different ($P < 0.05$). For instance, pH was not significantly different between the C0H0 treatment and the C1H0, C1H1, and C1H3 treatments after 150 days. After 30 days, the C0H0, C0H1, C0H2, and C0H3 treatments pH were 7.48, 7.44, 7.55, and 7.49, respectively, and this trend remained at 60, 90, 120, and 150 days.

Table 2. *Effects of biochar addition on soil pH*

Cd content (mg·kg^{-1})	Biochar (%)	pH				
		30d	60d	90d	120d	150d
H0	C0	7.48b	7.66b	7.54b	7.35b	7.12b
	C1	8.46a	8.24a	8.09a	7.99a	7.46b
	C2	8.49a	8.25a	8.28a	8.16a	7.98a
H1	C0	7.44b	7.56b	7.35b	7.33b	7.33b
	C1	8.48a	8.29a	7.94a	7.46b	7.46b
	C2	8.5a	8.31a	8.26a	8.09a	7.93a
H2	C0	7.55b	7.58b	7.52b	7.35b	7.33b
	C1	8.51a	8.32a	8.24a	7.92a	7.63ab
	C2	8.53a	8.36a	8.37a	8.00a	7.96a
H3	C0	7.49b	7.43b	7.69b	7.34b	7.36b
	C1	8.44a	8.44a	8.30a	8.07a	7.37b
	C2	8.56a	8.49a	8.27a	8.19a	8.03a
Regression analysis (significance)						
Cd content (H)		ns	ns	ns	ns	ns
Biochar ©		**	**	**	**	**
Interaction (H×C)		ns	ns	ns	ns	ns

C0, no added biochar; C1, 1.5% added biochar; C2, 3 % added biochar; H0, no added Cd; H1, 1 mg kg^{-1} Cd added; H2, 2 mg·kg^{-1} Cd added; H3, 4 mg·kg^{-1} Cd added. Different lowercase letters in the same column indicate significant differences ($P < 0.05$) in pH among individual treatments. **, $P < 0.01$; ns, $P \geq 0.05$

Effects of biochar on Cd forms in the soil

Throughout our sampling periods, Cd existed mostly as residue, accounting for 35.24%–63.02% of the total Cd (*Fig. 3*). Compared to C0 (no biochar), the biochar treatments exhibited reduced proportions of exchangeable and carbonate-bound Cd. After 30 days, compared to the C0H0 treatment, the proportion of exchangeable Cd decreased by 1.26% and 2.51% in the C1HO and C2HO treatments, respectively, and the proportion of carbonate-bound Cd decreased by 5.02% and 7.39% in the C1HO and

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DOI: http://dx.doi.org/10.15666/aeer/1905_35333549

C2HO treatments, respectively, while organic-bound Cd increased by 11.57% and 15.21%, respectively. This trend was the same in the H1, H2, and H3 groups (*Fig. 3a*). At 60 days, the proportion of exchangeable and carbonate-bound Cd in the C1H0 and C2H0 treatments decreased by 2.61% and 1.15% (C1H0), and 3.38% and 6.74% (C2H0) respectively, as organic-bound Cd increased by 2.65% and 5.86%, respectively in each treatment. Again H1, H2, and H3 trends for those tests mirrored these findings (*Fig. 3b*). The change of Cd forms after 90 days trended similarly to the 30 and 60-day results, indicating that biochar promoted the transformation of exchangeable and carbonate-bound Cd to organic-bound Cd (*Fig. 3c*). *Figure 3d* and *e* show changes of Cd forms in soils after 120 and 150 days, respectively. At the 120-day mark, the C1H0 and C2H0 treatments had decreased proportions of exchangeable and carbonate-bound Cd (5.01%, 7.69% (C1H0) and 2.37%, 5.52% (C2H0), respectively) and increased proportions of Fe-Mn oxide-bound Cd (9.98% and 18.07%, respectively, *Fig. 3d*).

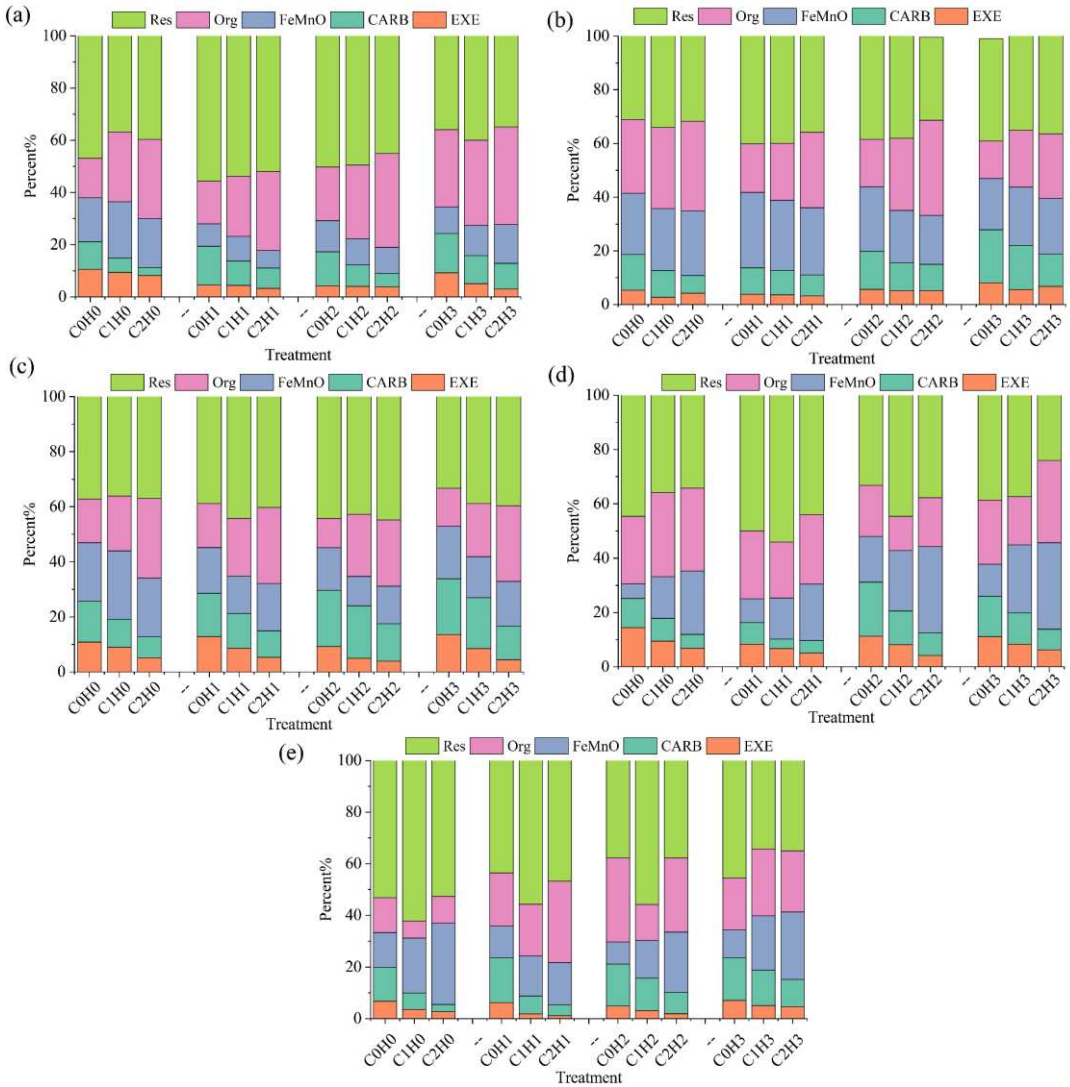

Figure 3. *Effects of biochar on Cd distribution in the soil after 30 days (a), 60 days (b), 90 days (c), 120 days (d), and 150 days (e). Res, Cd residue; Org, fraction bound to organic matter; FeMnO, fraction bound to Fe-Mn oxides; CARB, fraction bound to carbonates; EXE, exchangeable fraction; C0, no added biochar; C1, 1.5% added biochar; C2, 3% added biochar; H0, no added Cd; H1, 1 mg·kg^{-1} Cd added; H2, 2 mg·kg^{-1} Cd added; H3, 4 mg·kg^{-1} Cd added*

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This trend was similar in the H1, H2, and H3 groups, and for all treatments at 150 days (*Fig. 3e*) as well. So, after about 120 days of growth, exchangeable and carbonate-bound Cd in biochar treated soils were being converted to Fe-Mn oxide-bound Cd, instead of to organic-bound Cd, as was seen in earlier periods.

Effects of biochar on available Cd

In our study, the interaction of exogenous Cd with biochar significantly affected the amount of soil-available Cd (*Table 3,* $P < 0.05$). Available Cd increased along with increasing exogenous Cd concentrations and, as time passed, the proportions of available Cd initially decreased and then stabilized. In the C0H0, C0H1, C0H2, C0H3 treatments, available Cd changed little over time, varying from 0.0002–0.0143 $mg \cdot kg^{-1}$, while available Cd in the C1 and C2 groups differed significantly through time ($P < 0.05$). The C0H0 treatment had the least available Cd (0.0808 $mg \cdot kg^{-1}$) at 90 days, but it increased after that. The least available Cd in the C1H0 treatment (0.0702 mg kg^{-1}) was measured at 60 days and it stabilized after that, increasing to 0.0703 $mg \cdot kg^{-1}$ at 150 days. At 90 days, the C2H0 treatment had its lowest available Cd level (0.1107 $mg \cdot kg^{-1}$), but that level later stabilized. So, available Cd in biochar treated soil without added Cd (C1H0 and C2H0) reduced significantly ($P < 0.05$), but available Cd in the biochar treatments with the smallest amount of added Cd (C1H1 and C2H1) were lower than that in the C0H1, no biochar/lowest added Cd treatment ($P < 0.05$). The lowest Cd amount for C1H1 (0.1107 $mg \cdot kg^{-1}$) was measured at 90 days, while the lowest for C2H1 (0.0819 $mg \cdot kg^{-1}$) was at 120 days. After reaching those minimum values, available Cd in both C1H1 and C2H1 stabilized and then decreased. In the H2 groups, available Cd in the C1H2 and C2H2 treatments was lowest at 120 days (0.1732 $mg \cdot kg^{-1}$ and 0.1108 $mg \cdot kg^{-1}$, respectively), Changes in available Cd in the H3 groups trended similarly to those in the H2 groups. Available Cd in the C1H3 and C2H3 treatments was significantly less than that in the C0H3 treatment at 30, 60, 90, 120, 150 days. After 120 days, compared with C0H3, available Cd in the C2H3 treatment had decreased by 82.52% ($P < 0.05$).

Effects of biochar on Cd amounts in cotton plants

Biochar can reduce available Cd in soil by absorption, complexation, and precipitation, thus preventing cotton plants from absorbing Cd. *Figures 4, 5,* and *6* show that cotton leaves preferentially enrich in Cd; however, after adding biochar, cotton's aboveground Cd uptake decreased. In the C0H0 treatment, Cd uptake into cotton leaves, stems, and bolls increased from 0.0737, 0.0144, and 0.0103 $mg \cdot kg^{-1}$ at 30 days to 0.0834, 0.0151, and 0.0114 $mg \cdot kg^{-1}$ at 120 days, respectively, and then stabilized. We observed the same increasing trend in the other H groups. But Cd uptake in the C1 and C2 groups decreased as cultivation time increased. For example, Cd uptake into cotton leaves, stems, and bolls in the C1H3 and C2H3 treatments decreased between 30 days to 150 days ($P < 0.05$) (C1H3: 0.7414, 0.0791, and 0.0538 $mg \cdot kg^{-1}$ to 0.5822, 0.0687, and 0.0519 $mg \cdot kg^{-1}$, respectively, and C2H3: 0.7049, 0.0744, and 0.0496 $mg \cdot kg^{-1}$ to 0.5449, 0.0621, and 0.0471 $mg \cdot kg^{-1}$, respectively). We observed a similar trend in the C1H1, C1H2, C2H1, and C2H2 treatments. In the CIH0 and C2H0 treatments, the lowest uptake of Cd into cotton leaves (0.0563 $mg \cdot kg^{-1}$ and 0.0427 $mg \cdot kg^{-1}$), stems (0.0129 $mg \cdot kg^{-1}$ and 0.0113 $mg \cdot kg^{-1}$), and bolls (0.009 $mg \cdot kg^{-1}$ and 0.0078 $mg \cdot kg^{-1}$) occurred 90 days after biochar addition, corresponding to 30.58%, 49.28%, 15.69%, 26.14%, and 18.92%, 29.73% lower, respectively, than those of the C0 groups

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($P < 0.05$). At the H1 level, Cd uptake into cotton leaves, stems, and bolls after 90 days was the lowest in the C1 treatment (0.1811, 0.0339, and 0.0156 mg·kg^{-1}, respectively), which was 23.01%, 13.96%, and 25.12% lower than in the C0H1 treatment. However, Cd uptake into cotton leaves, stems, and bolls reached its lowest point in the C2H1 treatment after 120 days (0.1551, 0.0300, and 0.0141 mg·kg^{-1}), which were 33.72%, 26.11%, and 32.86% lower than in the control groups. In the CIH0 and C2H0 treatments, the lowest uptake of Cd into cotton leaves (0.4197 mg·kg^{-1} and 0.4034 mg·kg^{-1}), stems (0.0632 mg·kg^{-1} and 0.0601 mg·kg^{-1}), and bolls (0.0385 mg·kg^{-1} and 0.0351 mg·kg^{-1}) decreased by 21.39%, 24.43%, 11.11%, 15.47% and 12.51%, 20.23%, respectively, then uptakes in the C0H2 treatment occurred 90 days after biochar addition. The H3 uptakes trended similarly to H2, showing that biochar reduces the uptake of Cd by cotton, most likely through Cd adsorption.

Table 3. *Effects of biochar on available Cd in the soil at different time points*

Cd content (mg·kg^{-1})	Biochar (%)	Available Cd (mg·kg^{-1})				
		30d	60d	90d	120d	150d
H0	C0	0.0934a	0.0862a	0.0808a	0.0871a	0.1016a
	C1	0.0759b	0.0702b	0.0702b	0.0702b	0.0703b
	C2	0.0682b	0.0598c	0.0439c	0.0439c	0.0439c
H1	C0	0.1641a	0.1573a	0.1518a	0.1498a	0.1493a
	C1	0.1340b	0.1242b	0.1107b	0.1107b	0.1108b
	C2	0.1087c	0.0939c	0.0896c	0.0819c	0.0819c
H2	C0	0.2285a	0.2261a	0.2303a	0.2248a	0.2239a
	C1	0.2051b	0.1935b	0.1761b	0.1732b	0.1733b
	C2	0.1513c	0.1474c	0.1260c	0.1108c	0.1107c
H3	C0	1.1145a	1.1208a	1.1194a	1.1106a	1.1138a
	C1	0.8966b	0.8297b	0.8150b	0.793b	0.7931b
	C2	0.3952c	0.3386c	0.2195c	0.1959c	0.1958c
Regression analysis (significance)						
Cd content (H)		**	**	**	**	**
Biochar (C)		**	**	**	**	**
Interaction (H×C)		**	**	**	**	**

H0, no added Cd; H1, 1 mg·kg^{-1} Cd added; H2, 2 mg·kg^{-1} Cd added; H3, 4 mg·kg^{-1} Cd added; C0, no added biochar; C1, 1.5% added biochar; C2, 3% added biochar; Different lowercase letters in the same column indicate significant differences ($P < 0.05$) in pH among individual treatments. **, $P < 0.01$

Correlations between soil pH, Cd forms, and Cd amounts in cotton

Cd amounts in cotton leaves and stems were significantly correlated with Cd amounts in the soil ($r = 0.977$ and 0.915, respectively; $P < 0.01$) and Cd amounts in cotton leaves, stems, was positively correlated with the amounts of exchangeable and available Cd in the soil (*Table 4*). However, Cd amounts in cotton leaves, stems, and bolls was significantly negatively correlated with the organic-bound Cd amount in the soil ($r = -0.633$, -0.608, and -0.968, respectively; ($P < 0.01$)), as were pH with the amounts of available, exchangeable, and carbonate-bound Cd in the soil ($r = -0.66$, -

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0.543, and -0.555, respectively; $P < 0.01$). Total Cd correlated highly with all Cd forms, while available Cd also correlated highly with both exchangeable and carbonate-bound Cd forms ($r = 0.943$ and 0.716, respectively; $P < 0.01$). Fe-Mn oxide-bound Cd amounts were significantly correlated with organic-bound, but not with available, exchangeable, and carbonate-bound, Cd amounts.

Table 4. *Relationships between soil pH, and Cd contents in the soil and in cotton samples*

Indicators	Soil pH	Soil Cd	Leaf Cd	Stem Cd	Available Cd	EXE	CARB	FeMnO	Org
Soil pH	1								
Soil Cd	0.138	1							
Leaf Cd	-0.126	0.977**	1						
Stem Cd	-0.139	0.915**	0.973**	1					
Available Cd	-0.66**	0.892**	0.797**	0.688**	1				
EXE	-0.543**	0.951**	0.668**	0.871**	0.943**	1			
CARB	-0.555**	0.821**	0.211	0.174	0.716**	0.815**	1		
FeMnO	0.244	-0.642**	-0.265	-0.254	-0.225	-0.191	-0.208	1	
Org	0.254	-0.778**	-0.633**	-0.608**	-0.240	-0.238	-0.209	0.304*	1

Org, Cd fraction bound to organic matter; FeMnO, Cd fraction bound to Fe-Mn oxides; CARB, Cd fraction bound to carbonates; EXE, exchangeable fraction of Cd. **, $P < 0.01$; *, $P < 0.05$

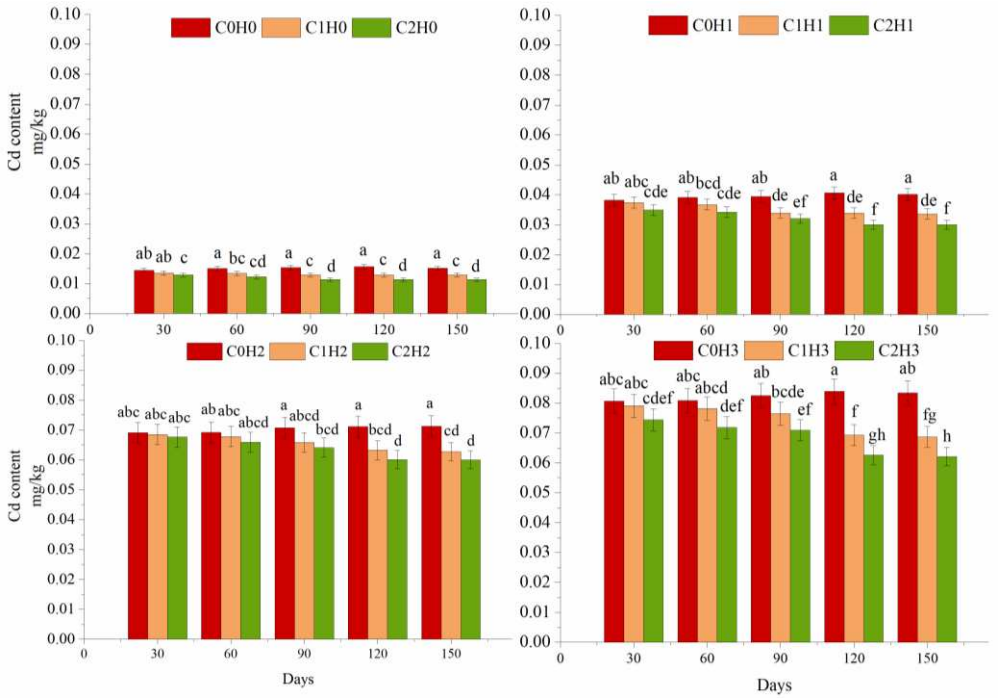

Figure 4. *Effects of biochar on Cd contents in cotton leaves over time. C0, no added biochar; C1, 1.5% added biochar; C2, 3% added biochar; H0, no added Cd; H1, 1 mg·kg^{-1} Cd added; H2, 2 mg·kg^{-1} Cd added; H3, 4 mg·kg^{-1} Cd added. Error bars represent SD, Different letters within the same variety and column indicate significant difference at 0.05 level*

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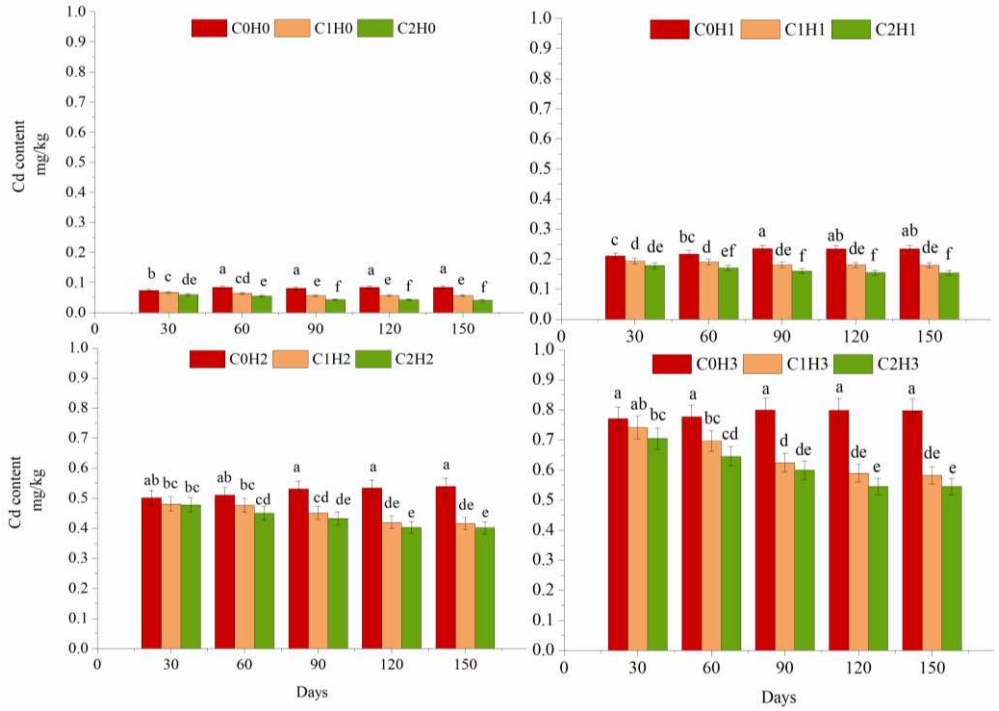

Figure 5. *Effects of biochar on Cd contents in cotton stems over time. C0, no added biochar; C1, 1.5% added biochar; C2, 3% added biochar; H0, no added Cd; H1, 1 mg·kg^{-1} Cd added; H2, 2 mg·kg^{-1} Cd added; H3, 4 mg·kg^{-1} Cd added. Error bars represent SD, Different letters within the same variety and column indicate significant difference at 0.05 level*

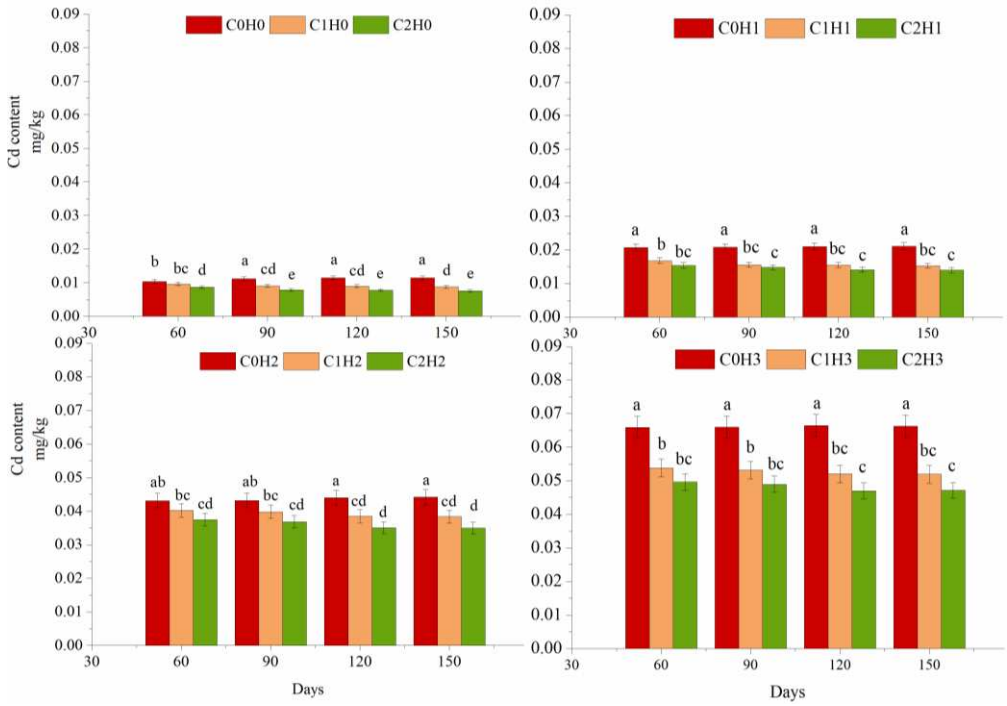

Figure 6. *Effects of biochar on Cd content in cotton bolls over time. C0, no added biochar; C1, 1.5% added biochar; C2, 3% added biochar; H0, no added Cd; H1, 1 mg·kg^{-1} Cd added; H2, 2 mg·kg^{-1} Cd added; H3, 4 mg·kg^{-1} Cd added. Error bars represent SD, Different letters within the same variety and column indicate significant difference at 0.05 level*

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Discussion

Biochar has a rich microporous structure that forms a large specific surface area (Rehman et al., 2021; Ma et al., 2020; Eissa et al., 2019), and its ash contains much soluble calcium, magnesium, potassium, sodium, and other salt-based ions. Biochar added to soil improves the soil salt-based saturation to some extent as salt-based ions interact with both hydrogen and exchangeable aluminum ions. Within this ion-exchange interaction, soil pH increases as the amount of both hydrogen and aluminum ions decrease (Kookana et al., 2011). In our study, the addition of biochar significantly increased soil pH, and pH increases paralleled increasing amounts of added biochar, peaking in the 3% added biochar treatment (0.97–1.47 units relative to the blank). Guo et al. (2017) found that biochar promoted soil sediment formation ($CdCO_3$, Cu $(OH)_2$, and $Pb_5(PO_4)_3OH$) as soil pH increased, thereby reducing the soil Cd amount. In our study, biochar addition also reduced the amount of available Cd in the soil. The more biochar was added, the better the adsorption of soil Cd was. Without biochar and exogenous Cd, soil pH initially increased but then decreased with time. Soil pH also initially increased and then decreased, finally stabilizing with the addition of exogenous Cd without biochar. Irrigation affects the process of soil reduction, ultimately resulting in the consumption of H^+ ions with an accompanying rapid soil pH increase. However, the combined effects of the resultant intermediate product (organic acid) and the final carbonate end product (CO_2) produced during watering causes a gradual decrease in soil pH over time (Yang et al., 2016; Cao et al., 2011).

Availability of heavy metals is an important indicator of heavy metal environmental behavior, and the addition of biochar can effectively reduce that availability. After 2 years, Bian et al. (2014) found that wheat straw biochar continuously reduced the available Cd in paddy soil. By increasing soil pH, biochar promotes ion exchange on its surface (e.g., Ca^{2+}, K^+, and Mg^{2+} in the form of oxides or carbonates) and therefore reduces the mobility of exchangeable and carbonate-bound Cd in the soil (Aslam et al., 2017), an effect that increases along with increasing soil pH (Zhang et al., 2013; Kim et al., 2018). The adsorption of Cd to biochar also plays a non-negligible role in sequestering Cd. In our study, available soil Cd initially decreased and then stabilized, as time passed. The extent of that reduction also differed between the different amounts of exogenous Cd. With no exogenous Cd, the available Cd in the soil decreased to its lowest level at 150 days, but Cd amounts in soil with 1 $mg \cdot kg^{-1}$ added Cd^{2+} were the lowest after 120 and 150 days for treatments with 1.5% and 3% biochar, respectively. As exogenous Cd amounts increased (2 $mg \cdot kg^{-1}$ Cd^{2+} and 4 $mg \cdot kg^{-1}$ Cd^{2+}), the treatments with 1.5% and 3% biochar experienced their lowest Cd amounts at 120 days. We found that the treatments with less added biochar and greater exogenous Cd reached equilibrium quicker than in other treatments, thus indicating that the ability of biochar to adsorb metals decreased over time (Bian et al., 2014). This stabilization at a later period, Kookana et al. (2010) called it “aging”, in which natural organic molecules lead to the “aging” of biochar adsorption capacity, resulted in a balance of available Cd amount in soils (Xu et al., 2017; Mousavi et al., 2010).

Many studies have shown that exchangeable, carbonate-bound, and Fe-Mn oxides of heavy metals are available states and exhibit bioavailability under certain conditions. The effect of biochar on Cd form differs in different regions (Bashir et al., 2017). For example, Zhu et al. (2015) found that biochar decreased exchangeable Cd and increased Fe-Mn oxide-bound Cd in Shenyang paddy soil, while Gu et al. (2018) studied the transformation of Cd form in Hunan Province farmland soils and found that biochar

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decreased exchangeable Cd but increased both organic-bound and residual Cd. Our results showed that, after biochar addition, the proportion of exchangeable and carbonate-bound Cd decreased; organic-bound Cd increased after 30, 60, and 90 days; and the proportion of exchangeable and carbonate-bound Cd decreased at 120 and 150 days, respectively, while the proportion of Fe-Mn oxide-bound Cd increased. The differing results of each study may be because of soil differences and the raw materials used to make biochar (e.g., different proportions of cellulose, hemicellulose, and lignin) between regions result in both inconsistent Cd speciation and effects of biochar on heavy metal Cd forms (Cantrell et al., 2012). However, when soil pH exceeds the zero charge of an Fe-MnO colloid, the charge on the colloidal surface will change from positive to negative, thus increasing the Cd-adsorptive capacity of the colloid and possibly explaining the increase of Fe-Mn oxide-bound Cd (Neumann et al., 2001).

Cd is transported to aboveground plant organs via root absorption. Generally, after biochar addition the Cd amount in those organs decreased, but the effects of each biochar treatment on the accumulation of Cd differed (Zong et al., 2021). Abid et al. (2017) found that Cd amounts in tomato roots were 33% lower than in the control when 1% biochar was added and Jin et al. (2011) showed that Cd amounts in Indian shepherd's purse buds were 76.1%, 82.2%, and 96.3% lower than in the control when 1%, 5%, and 15% chicken manure biochar, respectively, was added. In our study, biochar addition significantly reduced Cd accumulation in cotton, with the largest decrease occurring in the C2H3 treatment (49.28%) and resembling results were published by Rehman et al. (2021). The accumulation and distribution of heavy metals in plants depend on plant species, element species, chemical and biological availability, redox, pH, cation exchange capacity, dissolved oxygen, temperature, and root secretions (Jin et al., 2011). Because of its large biomass, cotton has great tolerance to Cd and the accumulation of Cd, but cotton seedling growth was inhibited when the exogenous soil Cd amount was 20 $mg \cdot L^{-1}$. As their Cd amount increased from 20 $mg \cdot L^{-1}$ to 80 $mg \cdot L^{-1}$, the seedlings' stem and root lengths decreased significantly, but they still grew normally. Given this resilience, cotton is considered a good crop to grow for heavy metal remediation (Jin et al., 2011). Overall, our cotton exhibited good Cd absorption capacity, even without biochar, a result consistent with Ma et al. (2017). In our study, Cd primarily accumulated in cotton leaves, with a maximum uptake in the C0H3 trial (0.7992 $mg \cdot kg^{-1}$). Coincidentally, Cd uptake differed between different parts of various other crops, too. In pakchoi, Cd accumulated more in leaves than in roots and more in leaves than in petioles (Chen et al., 2012), but in maize and wheat, more Cd accumulated in leaves than in grain, and wheat grain enriched in Cd more than corn grain (Wang et al., 2017). In our study, Cd preferentially accumulated in leaves, then in stems and then in bolls. While soil pH did not correlate greatly with total Cd, it did correlate significantly with available Cd, primarily because biochar affected the distribution of heavy metal forms by affecting soil pH (Guo et al., 2017). That correlation showed that available Cd in soil could be directly absorbed by plants, resulting in a significant effect of available Cd on the Cd amount in cotton leaves and stems. Since biochar reduces the availability of soil Cd, it most likely reduces the accumulation of heavy metals in the ground.

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Conclusions

Soil pH increased and available Cd decreased significantly with the addition of biochar, when the Cd dosage was 1 $mg \cdot kg^{-1}$, available Cd in the 1.5% biochar treatment was the lowest at 120 days and at 150 days for the 3% biochar treatment. When Cd dosages were 2 and 4 $mg \cdot kg^{-1}$, soil-available Cd reached minima at 120 days for both the 1.5% and 3% biochar treatments. Exchangeable Cd in the soil was the main source of accumulated Cd in aboveground plant organs. Biochar increased the proportion of organic-bound Cd (30, 60, and 90 days) and Fe-Mn oxide-bound Cd (120 and 150 days), then, biochar promoted the transformation of exchangeable and carbonate-bound Cd to organic-bound and Fe-Mn oxide-bound Cd. Finally, biochar (1.5% and 3%) reduced soil available Cd content and slowed Cd uptake by cotton (*Gossypium hirsutum L.*). The physiological mechanism of biochar on *Gossypium hirsutum* L. growth promotion and Cd stress tolerance is not clear. The next step is to determine the effect of biochar on metabolism and metagenome of *Gossypium hirsutum* L. to determine the role of biochar under heavy metal stress.

Acknowledgments. This study was supported by the National Natural Science Foundation of China (Grant No. 31360301) and the International Cooperation Project of the Ministry of Science and Technology (2015DFA11660) and Major Science and Technology Project of the XPCC (2018AA004, 2018AA005, 2020AB018).

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

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_35333549

CLEAN AGRICULTURE FOR THE SAFE PRODUCTION OF DATE PALM FRUIT (*PHOENIX DACTYLIFERA* L. CV. SEWI) UNDER EGYPTIAN CONDITIONS

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(Received 3rd Jan 2021; accepted 10th Jun 2021)

Abstract. The excessive use of nitrogen fertilization increases soil acidity, and contaminates the environment. It also increases heavy metals concentration in the plant with a serious threat to human health. A wide variety of microorganisms can be used as alternatives to minimize mineral fertilization. This study was conducted during the 2017 and 2018 seasons on 'Sewi' date palm grown under Egyptian conditions, to evaluate the effect of mineral-, organic-, and bio-fertilization on fruit yield and quality, as well as to encourage growers to switch to organic- and bio-fertilization. Fertilization was applied in six treatments; 1) 100% mineral NPK, 2) 100% manure, 3) 100% Azotobacter chroococcum [Az14], Bacillus megatherium var. phosphaticum [B6], 4) Bacillus circulans [B4]), 5) 50% manure + 100% bio-fertilizer, and 6) 50% mineral NPK + 100% bio-fertilizer, plus the control (no fertilization). Results indicated that 50% manure + 100% bio-fertilizer and 50% NPK + 100% bio-fertilizer improved yield and fruit quality. Fertilization with 50% manure + 100% bio-fertilizer led to high values of leaflet area, yield, fruit and flesh weight, soluble solids content, vitamin C, and total sugars, but the lowest fruit firmness. While 50% NPK + 100% bio-fertilizer led to the highest values of new leaves, leaf length, fruit length and diameter, and reducing sugars. Results indicated that non-mineral fertilizers improved yield and fruit quality compared to the mineral ones, and this was also more acceptable to the consumers.
Keywords: *organic fertilizer, SSC, clean agriculture, date palm, fruit quality, Azotobacter chroococcum, bio-fertilizers, Bacillus circulans*

Introduction

Egypt is a subtropical country which lies between 22° and 31° North latitudes and between 25° and 35° East longitudes. Its climate (comprising a mild and wet winter from November to April and a hot and dry summer from May to October) is suitable for the production of many field and horticultural crops (Directorate of Intelligence, 2011). The west delta and the New Valley are the main production regions of semi-dry date cultivars which have moderate moisture content (30-45%) and medium sugar concentrations (45-50%). Typical semi-dry cultivars are 'Siwi', 'Amri', 'Agalni', and 'Saidy' that require about 2700-2900 heat units during the growing season (May-October) with an average daily temperature higher than 80-84°F (27-29 °C). Upper Egypt and the southern parts of the New Valley are the main production regions of dry

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_35513561

date cultivars which have low moisture content (15-20%) and high sugar concentrations (65-70%). Dry date cultivars include 'Sultani', 'Barakawi', 'Abrimi', 'Sakouti', 'Barmatoda', 'Melkabi', 'Gondeila', 'Gargoda', 'Digna', and 'Shamia' that require about 3600-4300 heat units during the growing season (May-October) with an average daily temperature higher than 104- 113°F (40-45 °C) (Riad, 1993).

Date palm, *Phoenix dactylifera* L., is one of the most common domestic tree fruits in the Middle East and North Africa region. Fruit plays a major role in human nutrition (Harhash and Abdel-Nasser, 2010; Sarrwy et al., 2012). The total crop yield in Egypt is estimated to be 1590414 tons of fresh, semi-dry and dry dates (FAO, 2019). Recently, chemical pesticides and fertilizers have been extensively used in tree fruit production (Sarker, 2012), which led to several problems and poor cropping systems. The excessive application of mineral nitrogen fertilizers has increased soil acidity, and contaminated groundwater and the surrounding environment. It has also weakened plant roots making them subjected to several diseases (Ayed, 2002). The price of nitrogen fertilizers has almost doubled during the last 3-4 years, which increases the need for cheaper sources of nitrogen (Andrews et al., 2010). Incorporation of organic matters into soil improved its structure, water retention (Alvarez and Alvarez, 2000), and infiltration rates (Carter, 2002; Bot and Benites, 2005). Organic fertilization may improve the physical and biological characteristics of the soil, and may also serve as a source of mineral nutrients (El-Koumey and Abu–Agwa, 1993; Wander, 2004). The combination of organic and inorganic sources of nutrients is essential to maintain good soil characteristics and to increase nutrient use efficiency (Dev, 2006). The main challenge when converting a conventional farm into an organic one is to have a sufficient N amount that ensures an acceptable crop yield (Hue and Silva. 2000; Galantini and Rosell, 2006; Iqbal, 2012). Recently, biofertilization has an important component of integrated nutrient supply to improve yield and its components through environment-friendly fertilizers (Marozsán et al., 2005; Franche, et al., 2009). Soil microorganisms promote the supply of important nutrients and have a crucial role for overall plant productivity (Garg, 2001; Baca and Elmerich, 2007). Using bio-fertilizers is a better option to reduce agrochemical inputs, and helps maintain soil fertility and strength (Chang. and Young. 1999). An increasing number of farmers are switching to organic and bio-fertilizers since they were found to be gentler on the soil (Cheng and Chung, 2004). Consequently, the value of organic sources has also increased in an increasingly eco-conscious world (Subba Rao, 1982.). The species of Azotobacter are known to fix natural atmospheric nitrogen in soil rhizosphere, thereby contributing towards nitrogen availability for the plants. Azotobacter chroococcum was isolated and used previously as a bio-fertilizer to improve the quantity and the quality of mango crop (Ahmad et al., 2004; Malik, 2009). Soil quality has also improved with the addition of the environment-friendly fertilizers (Young et al., 2003). Bio-fertilizers have also contributed in reducing the negative effect of global warming (Delima, 2012). Microorganisms including Azotobacter, as non-symbiotic nitrogen fixers, were isolated from various ecosystems, and their performance under laboratory and field conditions were tested (Garg, 2001; Baca and Elmerich, 2007; Franche, et al., 2009; Hidayatullah et al., 2018; Abd El-Razek et al., 2018). The enhancement and maintenance of soil fertility through microorganisms is an important goal in sustainable agriculture (Liou and Young, 2002). Hence, several microorganisms can be used as alternatives to chemical fertilizers to minimize the use of mineral fertilizers (Tsai et al., 2004).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_35513561

The aim of the present work was to access the impact of different fertilizers (minerals, organic, bio-fertilizer, and their combinations) on fruit yield and quality of Sewi date palm grown under the Egyptian conditions.

Materials and methods

Microbial strains

Non-symbiotic nitrogen-fixing bacteria, *Azotobacter chroococcum* (Az14), phosphate dissolving bacteria, *Bacillus megatherium* var. *phosphaticum* (B6), and potassium dissolving bacteria, *Bacillus circulans* (B4), were obtained from Bacteriology Lab, Sakha Agricultural Research Station. Pure cultures were maintained on Jensen's medium (Jensen, 1951), modified Bunt and Rovira medium (Abdel-Hafez, 1966), and nutrient agar medium (Atlas, 1997), respectively.

Preparation of inoculum

The sterilized carrier material (peat moss) in sealed bags has aseptically injected with a suitable amount of broth culture (107-108 CFU ml^{-1}). From each inoculum, 3 kg/4200 m^2 was used after mixed with suitable amount of sandy soil of fine clay soil, then strew around the plant.

Plant material

This experiment was conducted during 2017 and 2018 seasons on eighteen- 11 years old 'Sewi' date palms, uniform in size and vigor, grown in sandy loam soil at 7×7 m spacing in a private groove at El-Dakhla Oasis, New Valley Governorate, Egypt. Laboratory work was conducted at the Horticulture Department, Faculty of Agriculture, Kafrelsheik University, Egypt. Soil analysis (*Table 1*) was conducted according to Wilde et al. (1985).

Table 1. *Soil analysis of research field*

Parameters	Before treatment	After treatment
Silt %	5.20	6.00
Clay %	15.30	15.84
Sand %	77.80	72.40
Texture class	Sandy loam	Sandy loam
pH	8.2	7.76
EC (ds\m)	0.38	0.22
$CaCO_3$ %	2.1	2.33
Soluble cations (*meq\L*)		
Ca^{+2}	2.0	1.2
Mg^{+2}	0.8	0.8
Na^{+1}	1.0	0.68
K^{+1}	0.55	0.18
Soluble anions (*meq\L*)		
Cl^{-1}	0.8	0.4
CO_3^{-2}	0.0	0.0
HCO_3^{-1}	0.8	0.8
SO_4	2.75	1.66

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DOI: http://dx.doi.org/10.15666/aeer/1905_35513561

Six fertilization treatments in five replicates (Palm/replicate) were applied as follow; 100% Mineral fertilizer (NPK), 100% Organic fertilizer (manure), 100% Biofertilizer (Azotobacter chroococcum strain EB2), 50% Organic + 100% Biofertilizer, 50% Mineral + 100% Biofertilizer, and the control, as shown in *Table 2*.

Table 2. *Treatments*

No.	Treatments	Mineral fertilization			Organic fertilization (kg/tree/year)	Bio-fertilization (ml/tree/year)
		N (g/tree/year)	P (g/tree/year)	K (g/tree/year)		
T_1	100% Mineral	1200	500	2000	0	0
T_2	100% Organic	0	0	0	100	0
T_3	100% Bio-fertilizer	0	0	0	0	100
T_4	50% Organic + 100% Bio-fertilizer	0	0	0	50	100
T_5	50% Mineral + 100% Bio-fertilizer	600	250	1000	0	100
T_6	Control	0	0	0	0	0

Fertilization type and rate of application

Mineral fertilizer

Nitrogen (N) in the form of ammonium sulphate (20.5% N) was added in three different rates; 400 g × 3 (T_1) and 200 g × 3 (T_5) during the last week of February, last week of April, and last week of May of both seasons. Phosphorus (P) in the form of super phosphate (25.47% P_2O_5) was added in one rate; 400 g ×1 (T_2 and T_6) during the last week of October of both seasons. Potassium (K) in the form of potassium sulphate (50% K_2O) was added in two rates; 500 g × 2 (T_2 and T_6) during the last week of April and the last week of May of both seasons.

Organic fertilizer

Manure contained approximately 0.49% N, 0.17% P, and 0.37% K was added one time in the following rates; 50 kg ×1 (T_2) and 25 kg × 1 (T_4) during the last week of October of both seasons.

Biofertilizer

Azotobacter chroococcum (Az14), *Bacillus megatherium* var. phosphaticum (B6), and *Bacillus circulans* (B_4) bacteria were added in a rate of 70 ml [10^8 cfu/ml] (T_3) at the same time nitrogen and organic fertilization were applied in both seasons (*Table 2*).

Control

No fertilization (T_6).

Parameters

Leaflet area (cm^2) using leaf area meter (LI-COR LI-3100, Nebraska, USA). Yield was estimated as bunch weight per treatment.

Fruit physical and chemical characteristics

Samples of 30 dates were randomly picked from each bunch of each treated palm to determine the physical characteristics of the fruit (weight [g], length [cm], diameter

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DOI: http://dx.doi.org/10.15666/aeer/1905_35513561

[cm], and flesh weight [g]). The same fruit samples were used to estimate soluble solids content [SSC %] of fruit juice using hand-held refractometer; vitamin C (A.O.A.C., 1980); tannins content, determined according to the method of Winton and Winton (1958); total sugars, determined in methanol extract using phenol sulphoric acid method and the percentage was calculated on dry weight basis according to Dubois et al. (1956); and reducing sugars were determined in methanol extract according to A.O.A.C. (1980).

Statistical analysis

Data were analyzed in one-way ANOVA using SAS program (SAS Institute Inc., 2000). Mean comparisons were carried out using least significant difference (LSD) test at $P \leq 0.05$ (Snedecor and Cochran, 1977).

Results

Leaf length, leaf area and number of new leaves

Results presented in *Table 3* showed significant differences between all treatments and the control. Treatment 5 (Organic 50% + Bio 50%) recorded the highest values of leaf length and area during both seasons.

Table 3. *Effect of different sources of fertilizer on leaf length (m), number of leaflet, leaflet area (cm^2) and number of new leaves of "Sewi" date palm during 2017 and 2018 seasons*

Treatments	Leave length (m)		No- leaflet		Leaflet area (cm^2)		No-new leaves	
	2017	2018	2017	2018	2017	2018	2017	2018
100% Mineral (T1)	4.45b	4.68b	170.49a	178.33a	157.79c	173.23b	24.67d	25.33ab
100% Organic (T2)	4.45b	4.73b	166.10a	178.00ab	171.80b	178.59b	21.33d	23.67c
100% Bio-fertilizer (T3)	4.37b	c4.58c	171.09a	178.00ab	139.54d	172.25b	24.67b	23.00c
50% Organic + 100% Bio-fertilizer (T4)	4.40b	4.77b	168.36ab	179.00a	168.73b	176.03b	26.00a	25.67a
50% Mineral + 100% Bio-fertilizer (T5)	4.70a	4.97a	171.54a	181.67a	195.31a	198.87a	22.67c	24.00bc
Control (T6)	4.27b	4.44d	158.16c	174.44b	154.32c	164.45c	21.00d	22.67c
LDS 5%	*0.22*	*0.094*	*3.80*	*3.63*	*7.17*	*6.55*	*1.11*	*1.52*

Means followed by a common letter are not significantly different at the 5% level by LSD

On the other hand, there were significant differences between the control (T6) and all other treatments or in leaflet area during both 2017 and 2018 seasons. Moreover, there were significant differences between T5 and all treatments in regards to the number of new leaves in both seasons. Control treatment recorded the lowest leaf area during 2017 and 2018 seasons.

Yield, fruit and flesh weight, and fruit length and diameter

Control treatment recorded the lowest yield during 2017 and 2018 seasons. Treatment 4 (50% Org + 100% Bio) recorded the highest yield (139.61 kg/palm) in comparison to the control and other treatments during the first season (*Table 4*).

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DOI: http://dx.doi.org/10.15666/aeer/1905_35513561

Table 4. *Effect of different sources of fertilizer on total yield (kg/palm), fruit weight (g) and flesh weight (g), fruit length (cm) and fruit diameter (cm) of "Sewi" date palm during 2017 and 2018 seasons*

Treatments	Yield (kg/palm)		Fruit weight		Flesh weight		Fruit length		Fruit diameter	
	2017	2018	2017	2018	2017	2018	2017	2018	2017	2018
100% Mineral (T1)	116.17b	116.53a	19.47abc	23.60a	17.58a	21.03a	4.45a	4.50a	2.71a	2.71ab
100% Organic (T2)	98.07d	117.89a	19.57ab1	22.24b	16.98ab	20.03b	4.23bc	4.27c	2.64a	2.66bc
100% Bio-fertilizer (T3)	96.83d	106.03bc	18.69bc	22.42b	16.80ab	20.70ab	4.36ab	4.50a	2.70a	2.65c
50% Organic + 100% Bio-fertilizer (T4)	111.96c	105.46b	19.82ab	23.57a	17.35a	21.10a	4.21c	4.27c	2.63a	2.71ab
50% Mineral + 100% Bio-fertilizer (T5)	139.61a	117.59a	20.92a	23.75a	17.73a	21.38a	4.16c	4.36b	2.64a	2.70a
Control	96.33d	52.82c	17.74c	19.56c	15.49b	17.49c	4.14c	4.13d	2.55b	2.61c
LDS	*3.12*	*2.54*	*1.77*	*0.522*	*1.53*	*0.8*	*0.17*	*0.03*	*0.077*	*0.049*

Means followed by a common letter are not significantly different at the 5% level by LSD

All treatments improved fruit weight, flesh weight, and fruit dimensions compared to the control during 2017 and 2018 seasons. Treatment (4) recorded the highest values of fruit weight (20.92 and 17.73 g) and flesh weight (23.75 and 21.38 g) during 2017 and 2018 seasons, respectively. Whereas, treatment 2 (100% Min.) recorded the highest fruit length (4.45 and 4.50 cm) in 2017 and 2018 seasons, respectively.

SSC, vitamin C, tannins, total and reducing sugars

Data in *Table 5* showed significant differences between all treatments and the control in regards to %SSC in both seasons. Control showed the lowest SSC, tannins, and reducing and total sugars during 2017 and 2018 seasons (*Table 5*). Treatment 4 (50% Organic + 100% bio fertilizer) showed the lowest and significant tannins % (0.34%) and the highest total sugars (37.59%) in 2017 season. Treatment T5 (50%Mineral + 100% biofert) recorded the highest content of vitamin C (1.95 and 1.96 mg/100 g flesh weight) and reducing sugars (25.47 and 23.87%) in 2017 and 2018 seasons, respectively. Control recorded the lowest SSC, reducing sugars and the highest tannins in both seasons.

Table 5. *Effect of different sources of fertilizer on SSC (%), vitamin C (mg/100 g fresh weight), total and reducing sugars (%) of "Sewi" date palm during 2017 and 2018 seasons*

Treatments	SSC (%)		Vitamin C (mg/100g fresh weight)		Tannins (%)		Total sugar (%)		Reducing sugar (%)	
	2017	2018	2017	2018	2017	2018	2017	2018	2017	2018
100% Mineral (T1)	25.47c	26.42c	1.47bc	1.53c	0.36c	0.40b	32.81b	32.72c	22.98b	20.99c
100% Organic (T2)	28.53b	30.29b	1.28d	1.43e	0.35c	0.35d	29.71d	36.53ab	20.57c	21.90bc
100% Bio-fertilizer (T3)	26.53c	27.83c	1.40c	1.51d	0.36c	0.38c	29.90d	31.61c	19.87c	20.77c
50% Organic + 100% Bio-fertilizer (T4)	29.43b	29.73c	1.95a	1.97a	0.34c	0.35d	31.35c	35.71b	25.47a	23.87a
50% Mineral + 100% Bio-fertilizer (T5)	31.93a	33.43a	1.55b	1.66b	0.36c	0.34e	35.63a	37.59a	19.54c	23.27ab
Control	22.47d	22.87d	1.52b	1.51d	0.47a	0.45a	25.50e	28.56d	14.97d	18.77d
LDS 5%	*1.63*	*1.85*	0.83	0.015	*0.031*	*0.013*	*1.3*	*1.53*	*1.03*	*1.57*

Means followed by a common letter are not significantly different at the 5% level by LSD

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_35513561

Discussion

The effect of bio fertilization could be highly effective if they have been adopted, and integrated with arming systems (Kennedy, 2004; Banayo et al., 2012; Bhardwarj et al., 2014; Masso et al., 2015). Fertilizers improvement via field experiments is important to test it before being economically feasible (Delima, 2012; Sutton et al., 2013). Trees treated with Bio-fertilizer (T3) is the lowest in most testing characters (leaflet area, yield, bunch, fruit and flesh weight, SSC, and total and reducing sugars) than those treated with mineral and/or organic treatments (T1 and T2), but all treatments were better than the control (T6). Palms treated with 50% Organic + 100 Bio-fertilizer (T4) or 50 Mineral + 100 Bio-fertilizer (T5) were the best compared to all treatments. These results support the findings of Biswas et al. (2000) and Babalola and Glick (2012) that bio-fertilizers were able to stimulate plant growth and increase yield and its components in field experiments. Andrade et al. 1997) and Banayo et al. (2012) reported that yield numerically higher with inorganic fertilization than that with organic or bio- fertilization. Being more soluble, nutrients availability with inorganic fertilizers provides the most N requirement of the plants. On the other hand, bio-fertilizers alone are not sufficient to increase the yield in comparison to the recommended rate of inorganic or organic fertilizers. Bio-fertilizers are only supplied half of the N requirement of the plants. The influence of bio-fertilizers and its combination with organic (T5) or inorganic fertilizers (T6) gave best results than bio-fertilizer alone (T4). Results showed that bio-fertilizers alone could not meet the nutrient requirement of date palms. As cited by Luis and Brown (2003), Banayo et al. (2012) and Sutton et al. (2013), the inorganic fertilizers provided nutrients readily available to the plants, but have negative effects on the environment including soil and groundwater, in addition to other serious threats to human health. They also have direct negative impact on beneficial soil microorganisms. Combining bio-fertilizer with organic fertilizer is slowly and gradually releases nutrients to the plant, improving yield and fruit quality (Mansour, 1998; Banayo et al., 2012). Abdle-Hamid (2002) and Bhardwarj et al. (2014) reported that bio-fertilizers combined with organic or inorganic fertilizers improved yield and fruit quality of olives. Same results were reported in 'Zaghloul' date palm (Osman, 2003), 'Samani' date palms (Elkhayat and Elnoam, 2013), 'Washington' navel oranges (Mostafa, 2002); 'Balady' mandarin (Salama, 2002). Aassy' olive trees (Abd El-Razek et al., 2018); apple (Hidayatullah et al., 2018).

Conclusion

It can be concluded that bio-fertilizer (Azotobacter chroococcum [Az14], Bacillus megatherium var. phosphaticum [B6], and Bacillus circulans [B4]), as a single inoculation is not sufficient for crop growth. Bio-fertilizer in combination with half of the recommended organic or inorganic fertilizer is sufficient to supply 'Sewi' date palm with required nutrients to improve fruit yield and quality under the Egyptian conditions. The activity of this bio-fertilizer in agro ecosystems is neither easily predictable nor always beneficial.

Acknowledgement. The authors extend their appreciation to the Deanship of Scientific Research at King Khalid University for funding this work through R.G.P. 1/219/41.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_35513561

THE EFFECT OF CROPPING METHOD ON THE YIELD, SEED CHEMICAL COMPOSITION AND SEGETAL DIVERSITY OF LENTIL (*LENS CULINARIS* MEDIC.) UNDER ORGANIC FARMING CONDITIONS

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(Received 16th Feb 2021; accepted 14th May 2021)

Abstract. This study evaluated how growing lentil (*Lens culinaris* Medic) with barley (*Hordeum vulgare)* or oats (*Avena sativa* L.) as a supporting plant impacts on its yield, seed chemical composition and segetal diversity in an organic system. The field experiment was conducted over the 2017-2018 period, at the Agricultural Experimental Station in Grabów (Masovian Voivodeship, Poland). The one-factor experiment was set up as a randomized blocks design with four replicates. The study showed that a higher seed yield was obtained under the cropping method, where a supporting crop was used than in sole cropping. In turn, the 1000 seed weight was 4.0% higher in sole cropping. The yield share of lentil grown with oat was lower than with barley. Seeds of lentil grown with cereals contain similar amounts of protein, fat, fibre and phosphorus as those grown in sole cropping. Cropping method of lentil have a significant effect on weed infestation. In both years of the study, the highest weed infestation was observed in lentil grown in sole cropping. Sowing lentil with supporting crops significantly reduced weed infestation.
Keywords: *sole cropping, evaluation, supporting plant, influence, weed infestation*

Introduction

The lentil (*Lens culinaris* Medic) is one of the oldest - apart from pea - cultivated high-protein crops (Piróg et al., 2003). Lentil seeds contain the less antinutritive substances among all legume crops. These are mainly composed of the galactosaccharide group: stachyose, raffinose, and verbascose (Urbano et al., 1995). Lentil is valuable species because of its nutritional quality (Hefnawy, 2011). It is rich sources of complex carbohydrates, proteins, dietary fibers, group B vitamins, mineral components, characterized by high share of exogenous amino acids (especially lysine, leucine, arginine, histidine, valine) (Costa et al., 2006; Joshi et al., 2017; Kowalczyk et al., 2007; Kahraman, 2016; Wang et al., 2008, 2009; Karadavut and Genc, 2010; Hamdi et al., 2012). They are characterized by anti-oxidant properties (Szwejkowska, 2012) and high nutritive value both as human food and fodder for animals (Erskine and Sarker, 2004). The content of nutrients in seeds significantly depends on genetic and environmental factors (Erskine and Sarker, 2004). Moreover, lentil can have a potential role in crop rotation, in particular in organic farms (Gan et al., 2003; Sellami et al., 2019). In spite of many beneficial characteristics of lentil, the acreage of cultivation is quite low. The main reason for this is likely due to its high susceptibility to lodging and low competiveness against weeds (Carr et al., 1995; Chaudhary et al., 2011). One of the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_35633577

methods for limiting lentil against lodging and reducing of weed infestation can be its intercropping with other crop species such as cereal (Duchene et al., 2017; Zawieja, 2006). In organic farming, in which herbicide is prohibited there is important need to seek new solutions for controlling weeds occurrence (Bond and Grundy, 2001). According to several authors (Duer, 2002; Vlachostergios and Roupakias, 2008; Avola et al., 2008) such role can have row intercropping, proper crop rotation, diverse agrotechnic, selection of varieties adapted to soil and climatic conditions with greater competitiveness in relation to undesirable species.

The aim of this study was to evaluate the effect of cropping method on yield, lentil seeds chemical composition and segetal diversity.

Materials and methods

Field experiment and cultivation management

A field study was carried out at the Agricultural Experimental Station in Grabów [51°21′18″N 21°40′09″E] (Masovian Voivodeship, Poland) belonging to the Institute of Soil Science and Plant Cultivation – State Research Institute in Puławy (Lublin voivodeship) (*Fig. 1*). The experiment was conducted over the period of 2017 to 2018. The one-factor experiment was set up as a randomized blocks design with four replicates. The area of a single plot was 35 m^2 and for harvest – 30 m^2. In each year the total number of plots in the experiment was 12. The experiment was established on a Luvisol soil with sandy loam texture classes, belonging to a very good rye complex (www.commons.wikipedia.org). The soil was characterized by the following nutrient content: (mg·100 kg^{-1} soil): P 11.1–13.0; K 15.1–20.4 and Mg 4.0–6.2. Soil pH, as determined in 1 N KCl, was 5.5–6.3. The scheme of the trial included one factor: cropping method of lentil, cultivar 'Anita' [sole cropping (A), intercropping with two cereal species – barley (B) (*Hordeum vulgare* L. - cultivar 'Ella'), oat (C) (*Avena sativa* L. - cultivar 'Bingo')]. The preceding crop was legume/grass mixture. The density (plants·m^{-2}) of lentil in pure cropping was 200, in row intercropping – 100; oat as supporting plant – 250 and barley −150. The row spacing was 20 cm. Cereals were sown separately in the interrows of the lentil crop. In 2017 the lentil seeds were sown in the first 10 days of April, and in 2018 in the second 10 days of April. Mineral fertilization and plant protection products were not applied. The plots were harrowed twice to control weeds in the mixtures. Plants were harvested at the full maturity stage of mixture components in the first 10 days of August. The height of the plants, the height to the first and last pod, the number of pods and seeds on the plant, the weight of seeds per plant, the air dry weight of the stem of one plant and the weight of the pods were determined before harvest. The number and weight of grain per cereal plant, weight of 1000 seeds, and number of production shoots were also determined. The total lentil and cereal seed yield, lentil seed yield, component percentage in yield and weight of 1000 seeds at 14% humidity were determined after harvest. The percentage of species in mixtures was determined after splitting the crop harvested from the whole plot.

Chemical analysis of lentil seeds

Material for chemical analysis was collected at commercial maturity each year. The following parameters were determined in seed samples: N, P (determination by the flow analysis (CFA) and spectrometric detection), K (determination by atomic emission

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DOI: http://dx.doi.org/10.15666/aeer/1905_35633577

spectroscopy (FAES)). Moreover total protein (mineralization in sulfuric acid; determination by the Kjeldahl distillation method), fat content (Soxhlet method) were also determined. The analysis of the chemical composition of the seeds was performed at the Main Chemical Laboratory, accredited by Polish Accreditation Centre (PCA) of the Institute of Soil Science and Plant Cultivation – State Research Institute in Puławy.

Figure 1. *Location of the study site (Public Domain, https://commons.wikimedia.org/w/index.php?curid=89531)*

Weed infestation

Evaluation of weed infestation in lentil crop was performer before harvest using the quantitative-gravimetric method. Analysis deals with the determination of the number, species composition, fresh and dry matter of weeds per sample areas delineated by a 1 x 0.50 m quadrat frame.

For comparison of weed infestation of lentil, the biomass index was determined and calculated for two years (2017 and 2018) according to the formula by Patriquin (1988):

$$biomass\ index = \frac{\text{crop biomass} * 100}{\text{weed biomass} + \text{crop biomass}}$$

The structure of weed composition in the studied crops was also described using two indices: the Shannon-Wiener index (*H'*) and the Simpson dominance index (*SI*). The Shannon's index is an indicator of species diversity. It depends on the number of species and their relative quantitative proportions and is calculated according to Shannon and Weaver's (Zanin et al., 1992):

$$H' = -\Sigma\, pi \ln pi$$

where: *pi* – ratio of weed number the species to the overall weed abundance on each site.

The Simpson index (*SI*) is an indicator described the probability of occurring two individuals of the same species. It takes into account the number of species and the relative abundance of each species and is described by the Simpson model (Zanin et al., 1992):

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DOI: http://dx.doi.org/10.15666/aeer/1905_35633577

$$SI = \Sigma\, pi^2$$

Value ranges from 0 to 1, with values close to 1 indicating a clear dominance of one or more species and a low diversity of the community.

Weather conditions

Throughout the experiment period, weather conditions varied substantially between the years (*Table 1*). In the first year of the study (2017), at the end of the second 10 days of April there was a strong cool down, and at night there was frost, which prevented the sowing of cereal and lentil. In 2017 the highest amount of precipitation was recorded in April, exceeded the long-term average by 77%. In June and the first 10 days of July a small amount of precipitation (32.6 and 9.7 mm respectively) was recorded and it was lower than the long-term average by 54.1 and 65.0% respectively. It had a negative effect on the plants growth and development. In the first 10 days of August there were very small amounts of precipitation (0.9 mm). The average air temperature exceeded the long-term average by 1.4 °C. In 2018, the amount of precipitation in May (97.4 mm) and July (118.5 mm) exceeded the average from multi-years by 70.9 and 41.1% respectively, which favoured the yields of lentil. During April and June the total precipitation was only 65% and 63% of the long-term average, respectively. The average air temperature in this vegetative season exceeded the long-term average by 2.4 °C.

Table 1. *Course of weather conditions during the vegetation periods*

Specification	Month						Sum/Average III-VIII
	March	April	June	July	August	Sept.	
	2017						
Precipitation (mm)	35.8	69.1	34.4	32.6	86.3	55.3	313.5
Air temperature °C	5.7	7.5	13.9	18.1	18.6	19.6	13.9
	2018						
Precipitation (mm)	14.1	25.3	97.4	44.6	118.5	70.6	370.5
Air temperature °C	-0.1	13.3	17.0	18.4	20.4	20.2	14.9
Average precipitation from multi-year* (mm)	30.0	39.0	57.0	71.0	84.0	75.0	356.0
Average air temperature from multi-year* (°C)	1.6	7.7	13.4	16.7	18.3	17.3	12.5

*Mean for 1871-2000

Statistical analysis

The date presented are the mean values from the years 2017-2018, as a result of a similar reaction of the examined plants to different cropping method during two years of the study. The results were statistically analyzed with the use of the variance analysis using Statistica v.10.0 program. Tukey's multiple comparison test was used to compare differences between the means for cropping method while confidence intervals for the means of LSD ($\alpha = 0.05$) were used.

Results and discussion

Species of supporting crop, cropping method and the course of weather conditions had the effect on growth, development and yielding of lentil, oat and barley. Effect of course weather on the lentil yield is confirmed also by Piróg et al. (2003), Biçer and

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Sakar (2004), Filek et al. (2000) and Szwejkowska (2012). The analysis was prepared for two years of the study: 2017 and 2018. In the period of the study the more favorable weather conditions in 2018, allowed to obtain higher total yields of lentils as grown with cereals in sole cropping (*Table 2*).

Table 2. *Total seeds yield of lentil and supporting plant and thousand seeds weight of lentil depending on cropping method (mean ± standard deviation)*

Year	2017			2018			2017-2018		
Cropping method	A*	B	C	A	B	C	A	B	C
	Mean ± SD						Mean		
Seeds yield (t·ha^{-1})	0.57 ±0.07a**	3.12 ±0.12c	1.64 ±0.15b	2.03 ±0.52a	2.74 ±0.10c	2.45 ±0.25b	1.30	2.93	2.04
Weight of 1000 seeds (g)	42.53 ±1.27a	40.98 ±1.22a	41.00 ±4.02a	49.81 ±3.48c	47.63 ±0.85a	47.92 ±3.60b	46.17	44.31	44.46
Number of pods on main steams (per plant)	1.80 ±0.34	1.38 ±0.19	1.73 ±0.23	2.30 ±0.61	1.23 ±0.29	1.43 ±0.84	2.05	1.31	1.58
Number of pods on lateral steams (per plant)	8.60 ±0.50	5.23 ±0.65	6.55 ±0.77	10.23 ±0.32	7.00 ±0.13	7.50 ±0.17	9.41	6.11	7.03
Total number of pods on lateral steams (per plant)	10.40 ±1.70c	6.61 ±0.75a	8.28 ±0.96b	12.43 ±0.09c	8.23 ±0.03a	9.93 ±0.04b	11.42	7.42	9.10
Seeds number per plant	11.30 ±1.77c	6.88 ±1.62a	9.05 ±1.80b	12.87 ±2.15a	8.43 ±2.85b	9.43 ±2.93b	12.08	7.66	9.24
Seed weight per plant (g)	1.55 ±1.11c	0.38 ±0.04a	0.48 ±0.11b	1.75 ±0.32c	0.58 ±0.13a	0.56 ±0.17b	1.65	0.48	0.52
The share of lentil in mixture yield (%)	100.0	2.75	3.25	100.0	11.03	17.80	100.0	6.89	10.53

*A – lentil-sole cropping; B – lentil + oats; C – lentil + barley

**Mean ± standard deviation values followed by different letters are significantly different at $p \leq 0.05$ according to Tukey's honestly significant difference (LSD) test

Higher yield obtained in 2018 was the result of a greater number of pods, seeds number and seed weight on a lentil plant as well as a higher weight of 1000 seeds and grain weight on oat and barley plants (*Table 2*). Independent from the cereal species, the higher yields were found under the cropping methods, where lentil was grown with supporting plants than in sole cropping. In both years of the study, higher yield was provided by growing lentils with oats compared to growing with barley or in sole cropping (statistically significant differences).

Oat were more competitive to lentils than barley, resulting in a lower percentage of legume seeds in mixture with oat (*Table 2*). These results are in agreement with those reported by Nargis et al. (2004), who noted that the highest lentil yields were recorded in sole cropping. Those authors reported also that number and weight of pod per one plant was higher in sole cropping of lentil. Kraska et al. (2020) stated that lentil seed yield grown with oat was lower by 9.4% compared with that of the sole cropped. Gomez et al. (1983) reported that the highest seed yield were obtained in a mixture of lentil with barley or wheat. Similarly, Ahmed et al. (1987) stated that reduction of lentil seed yield grown in mixture with wheat was higher than with cereal crop. But Çiftçi and Űlker (2005) reported that the higher seed yields were lentil was grown with wheat not barley. Those authors noted also that mixed grown of lentil with wheat have a significant effect on plant height, seeds and straw yield, plant population (m^2).

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However, the statistical analysis did not confirm the cropping method to have a significant effect on plant height. Żabiński (2008) reported reaction of lentil on sowing with supporting crop and stated that it depends mainly on properties of the evaluated cultivar. The percentage of seeds yield of lentil grown with supporting crop, regardless cereal species, was significantly lower than in sole sowing.

The lentil grown in sole cropping was characterized by the higher 1000 seed weight, number of pods, number of seeds per pod, seed weight and dry weight stem and siliques of one crop than those grown with supported crop (significant differences) (*Tables 2* and *3*). Lentil grown with supported crops was characterized by the higher settlement of first pod on stem. Cropping method have not any effect on height of last pod settlement and height of plants (*Table 3*). The beneficial effect of intercropping of lentil with oats as a supporting crop on thousand seed weight is confirmed by Kraska et al. (2020). Compared to sole cropping, a significant decrease in plant density was however found (on average by 13.2%). These authors reported that weight of 1000 seeds, number of pods per plant, and first pod height did not differ significantly in the treatments with the lentil varieties. Moreover, these authors stated that lentil cropping method did not differ number of pods from single lentil plant. Nargis et al. (2004) reported that plant height, number of pods and thousand seeds weight were higher in the treatment where a supporting crop was used. According to Vlachostergios and Roupakias (2008) there are large possibilities to increase seed yield of lentil grown under organic farming conditions through appropriate cultivar selection. Rasheed et al. (2008) reported significant correlation between thousand seed weight and seed yield. Positive correlation between seed yield and number of pod per plant and height of plant are confirmed by Amarah et al. (2005), Ayub et al. (2001), Kar et al. (1995), Naseem et al. (1995) and Veerabandhiran and Jahangir (1995). In a study of Lopez-Bellido et al. (2005) number of pods per plant is negatively correlated with number of plants per unit area. According to many authors the appropriate density of plants per unit area determines their proper growth and development and is a guarantee to obtain a high seed yield (Saleem et al., 2012; Ouji et al., 2016). In the study of Kraska et al. (2018, the number of lentil plants per unit area was significant higher (by 15%) in lentil sole cropping.

Table 3. *Stem dry matter of one plant, dry matter of siliques, height to the 1st and the last pod, height to top of plant depending on cropping method (g) (mean ± standard deviation)*

Year	2017			2018			2017-2018		
Cropping method	A*	B	C	A	B	C	A	B	C
	Mean ± SD								
Dry matter of stem per one plant	0.12 ±0.004a**	0.05 ±0.007c	0.05 ±0.006b	0.14 ±0.005a	0.06 ±0.006b	0.06 ±0.008c	0.13	0.06	0.06
Dry matter of siliques	0.03 ±0.004a	0.02 ±0.006a	0.03 ±0.004a	0.08 ±0.002a	0.07 ±0.005a	0.07 ±0.008a	0.05	0.04	0.05
Height to the 1st pod	22.3 ±4.12c	27.6 ±2.55b	24.3 ±1.35a	35.6 ±4.27c	37.8 ±3.25b	38.7 ±2.15a	28.9	32.7	31.5
Height of the last pod	30.0 ±4.14c	31.6 ±2.22b	29.3 ±1.48a	38.6 ±4.14c	39.8 ±2.22b	40.8 ±1.48a	34.3	35.7	35.1
Height to top	30.9 ±4.21c	32.6 ±1.67a	29.7 ±2.84b	42.8 ±4.21c	43.5 ±1.67a	43.8 ±2.84b	36.8	38.1	36.7

*A – lentil-sole cropping; B – lentil + oats; C – lentil + barley

**Mean ± standard deviation values followed by different letters are significantly different at $p \leq 0.05$ according to Tukey's honestly significant difference (LSD) test

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Chemical composition of lentil seeds significantly depended on weather conditions during growing seasons. Course of weather condition in 2017 had the beneficial effect on raising the concentration of protein and fat in lentil seeds, but caused higher fibre content and have a little effect on potassium and phosphorus content (*Table 4*). Intercropping with cereals has no effect on protein, fat, fibre, and phosphorus content (no significant differences) in seeds. Higher content of potassium was noted (significant differences). Intercropping lentil with oats as a supporting crop significantly reduced the content of protein and nitrogen compared to sole cropped lentil. According to Karadavut and Palta (2010) chemical composition of lentil seeds depends on cultivar, type of soil and weather conditions. According to Stacey et al. (2006) and Palta et al. (2010) the content of nutrients component, especially ash, nitrogen, total, fibre, fat and water-soluble protein is feature that vary during growth and development of plant. In the opinion of Stępniak-Sołyga and Wojtasik (2003), a limited amount of precipitation and higher air temperature during the growing season promotes the accumulation of total protein in legume seeds. This was confirmed by Kraska et al. (2018). In study of those authors in the years, when the total precipitation was lowest and the average air temperatures highest, the total protein content in lentil seeds was significantly higher than in the wet and slightly colder year. In turn, Szwejkowska (2012) stated that limited amount of precipitation significantly reduced protein yield from unit area.

Kraska et al. (2018) reported that lentil grown with oat as supporting crop significantly reduced nitrogen, phosphorus, potassium and micro-components (Cu, ZN, Mn, Fe, B) compared to sole cropping. The highest air temperature during growing season and insufficient amount of rainfall significantly decreased the content of micro-components. Özer and Kaya (2010) reported that the seed K content in different lentil varieties ranged from 2.85 to 4.63 g kg^{-1} and P 0.57-1.35 g kg^{-1}. Alghamdi et al. (2014) found that K and P content to be was significantly correlated to the lentil genotype, and this content was from 6.74 to 10.61 g kg^{-1} and from 2.87 to 5.47 g kg^{-1}, respectively.

Table 4. *Concentrations of protein, fibre, fat, phosphorus and potassium in lentil seeds depending on cropping method (g·kg^{-1}s.m.)*

Year	**2017**			**2018**			**2017-2018**		
Cropping method	**A***	**B**	**C**	**A**	**B**	**C**	**A**	**B**	**C**
Content									
Protein	261.3a**	272.5[a]	277.1[a]	254.9[a]	263.1[a]	264.2[a]	258.1	267.8	270.6
Fat	7.1[a]	7.2[a]	6.9[a]	6.7[a]	6.9[a]	6.9[a]	6.9	7.1	6.9
Crude fibre	37.2[a]	36.3[a]	37.1[a]	34.8[a]	35.5[a]	35.3[a]	36.0	35.9	36.2
Phosphorus	5.6[a]	5.6[a]	5.7[a]	5.4[a]	5.3[a]	5.4[a]	5.5	5.4	5.5
Potassium	11.2[a]	11.3[a]	11.6[a]	11.0[a]	11.1[a]	11.6[a]	11.1	11.2	11.6

*A – lentil-sole cropping; B – lentil + oats; C – lentil + barley
**Values followed by a different letter are significantly different ($p < 0.05$)

Lentil is a species that poorly competitor with weeds (Singh et al., 2018). Pawłowski et al. (1990) states the need for the reduction of weeds, which significantly affected the development of legumes and the level of their yield. Cropping method of lentil have a

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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significant effect on weed infestation in crop, expressed by fresh and dry of weeds weight, their number and weed species composition. In both years of the study, the highest weed infestation was observed in lentil grown in sole cropping (*Tables 5-7*). While the lowest weed infestation was noted in lentil grown with barley as supporting crop. Sowing of lentil with barley resulted in increasing competitiveness of lentil against to weeds and reduced weight of weeds by an average of 80%. Intercropping of lentil with oat reduced weed infestation by 38% in 2017 and by 82% in 2018 compared with growing lentil in sole cropping. Fresh and dry matter were significantly less in lentil cropped with barley than with oat. Number of weeds in lentil varied by the cropping method and year of the study. The higher weed infestation was noted in 2017, in which number of weeds was significantly higher than in 2018 (*Tables 6* and *7*). Intercropping of lentil with cereals as supporting crops have effect on weed infestation expressed as number of weeds. In both years of the study the higher weeds number was noted in lentil grown in sole cropping. While sowing lentil with barley was more competitive. Number of weeds in such as cropping method of lentil was less than in sole cropping. Sowing lentil with barley reduced number of weeds species by 16% in 2017 and by 24% in 2018 than in sole cropping. In the study of Kraska et al. (2020) the weed species composition in lentil crop grown in pure sowing and in mixed variety stand was similar. In the study of those authors, sowing lentil with oats reduced number of dicotyledonous weeds from 43 to 39. While it caused the increase in number of monocotyledonous from 6 to 9. Bojarszczuk et al. (2013) and Staniak et al. (2013, 2014) found that mostly dicotyledonous weeds, such as *Chenopodium album, Stellaria media, Capsella bursa-pastoris*, and *Galinsoga parviflora*, were dominant in cereal–legume mixtures. According to Bojarszczuk et al. (2013) and Staniak et al. (2013) weather conditions have the influence on limited of effect mixture. The beneficial effect of mixture was observed in wet years.

Regardless of the lentil cropping method, segetal diversity was similar. The dicotyledonous weeds has the highest percentage in weeds structure. In 2017 depending on cropping method, the percentage of dicotyledonous weeds was from 62% (in sole cropping of lentil) to 83% (in lentil grown with oat) of all weeds. While in 2018 in lentil grown with cereals, monocotyledonous weeds have dominant share (mean 70%). Bojarszczuk et al. (2013), Staniak et al. (2013, 2014) and Bojarszczuk et al. (2017) reported that dicotyledonous weeds were dominant in cereal-legume mixtures.

In 2017, in both experimental treatments, independently of cropping method, *Plantago major*, *Viola arvensis*, *Cirsium arvense*, *Chenopodium album*, and *Erigeron canadensis* were weeds that occurred in greatest number, whereas among monocotyledonous weeds these were *Echinochloa crus-galli* and *Elymus repens*. Moreover, in the lentil sole cropping, 18 weed species was noted, in lentil grown with barley – 17 weed species and in lentil with oat – 14.

In 2018, weed infestation and weed species composition were significantly less than in 2017, as in sole cropping of lentil as grown with cereal as the supporting crops, *Chenopodium album* and *Echinochloa crus-galli* were weeds that occurred in the greatest number. In all treatments 9 weeds species were found. While in the lentil sole cropping, the 18 weed species were noted, in lentil grown with barley – 5 weed species and in lentil with oat – 3.

Jędruszczak et al. (2006) noted 32 weed species in triticale-lupine mixture and 28 weed species in rye with serradella. Staniak and Księżak (2010) noted from 25 to 28 weed species in barley/oat and pea/vetch grown under organic conditions. In the study

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of Kraska et al. (2020), *Echinochloa crus-galli, Galinsoga parviflora, Sonchus arvensis* and *Cheopodium album* were species that occurred in the greatest numbers in lentil crop grown under organic farming conditions. The statistical analysis did not confirm that the lentil cropping method have a significant effect on dicotyledonous weeds number. In turn, it was found that there were significantly less (by 22.4%) monocotyledonous weeds and their dry matter was lower (by 30.5%) in lentil grown with supporting crop.

Table 5. *Fresh and dry matter of weeds ($g \cdot m^{-2}$) depending on lentils cropping method in 2017-2018*

Cropping method	Fresh matter			Dry matter		
	2017	2018	Mean	2017	2018	Mean
A*	817.5**	260.7[c]	539.1	703.4[c]	248.6[b]	476.0
B	546.9[b]	161.9[b]	354.4	151.1[b]	44.1[a]	97.6
C	175.0[a]	55.0[a]	115.0	141.6[a]	43.6[a]	92.6
Mean	513.1	159.2	-	332.0	112.1	-

*A – lentil-sole cropping; B – lentil + oats; C – lentil + barley
**Values followed by a different letter are significantly different ($p < 0.05$)

Table 6. *Weed species composition and number of weeds depending on lentils cropping method the in the first year of the study ($plants \cdot m^{-2}$)*

Weed species	Cropping method		
	A*	B	C
Monocotyledonous weeds:			
Echinochloa crus-galli	74.7	37.3	6.0
Elymus repens	42.7	16.0	3.3
Poa annua	2.0	2.0	-
Setaria pumila	-	-	2.7
Sum of monocotyledonous weeds	119.3	55.3	12.0
Dicotyledonous weeds:			
Anthemis arvensis	1.3	0.7	
Capsella bursa-pastoris	2.0	5.3	6.7
Chenopodium album	58.7	54.0	34.7
Cirsium arvense	39.3	8.7	13.3
Erigeron canadensis	15.3	32.0	8.0
Fallopia convolvulus	2.7	1.3	5.3
Geranium molle	1.3	1.3	0.7
Matricaria maritima L. ssp. *inodora*	0.7	0.7	-
Plantago major	59.3	72.7	9.3
Polygonum aviculare	2.0	2.7	
Polygonum lapathifolium	1.3	-	-
Sonchus arvensis	-	-	2.7
Sonchus asper	0.7	0.7	
Stellaria media	5.3	8.0	7.3
Viola arvensis	7.3	15.3	14.7
Sum of dicotyledonous weeds	197.3	203.3	104.0
Equisetum arvense	1.3	8.0	10.0
Total	317.9	266.6	126.0
Number of species	18	17	14

*A – lentil-sole cropping; B – lentil + oats; C – lentil + barley

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DOI: http://dx.doi.org/10.15666/aeer/1905_35633577

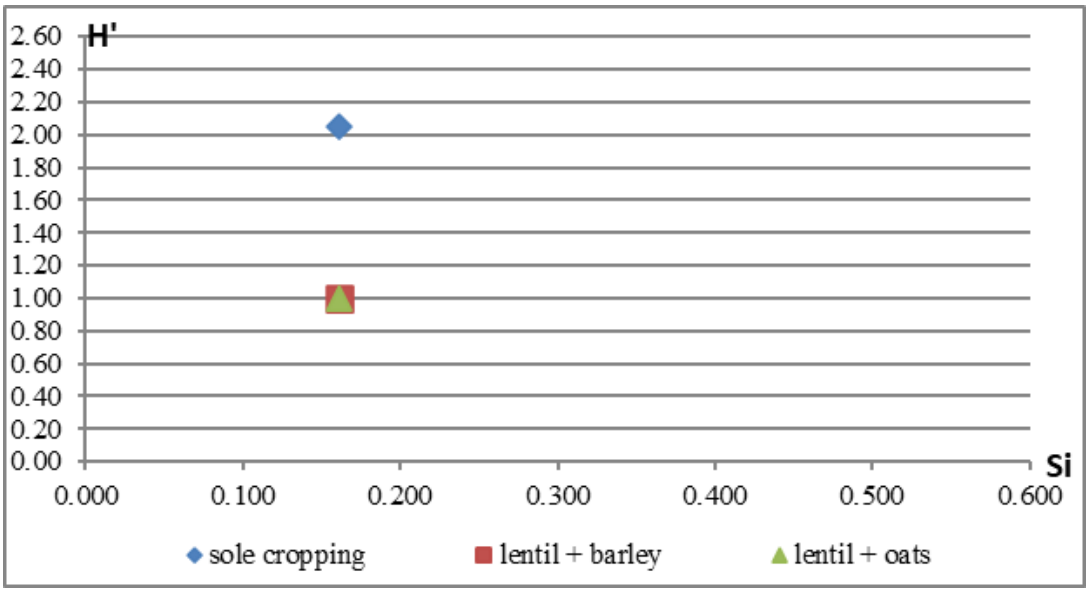
Table 7. *Weed species composition and number of weeds depending on lentils cropping method the in the second year of the study (plants·m^{-2})*

Weeds species	Cropping method		
	A*	B	C
Monocotyledonous weeds:			
Echinochloa crus-galli	10.5	16.5	15.5
Sum of Monocotyledonous weeds	10.5	16.5	15.5
Dicotyledonous weeds:			
Anthemis arvensis	0.5	-	-
Chenopodium album	12.5	3.5	6.0
Cirsium arvense	-	0.5	-
Melandrium album (Mill.) Garcke	1.0	-	
Polygonum persicaria	0.5	-	-
Solanum nigrum	3.5	-	-
Stellaria media	1.0	0.5	2.0
Viola arvensis	1.5	2.5	-
Sum of Dicotyledonous weeds	20.5	7.0	8.0
Total	31.0	23.5	23.5
Number of species	9	5	3

*A – lentil-sole cropping; B – lentil + oats; C – lentil + barley

Segetal diversity expressed by Shannon-Wiener index (*H'*) and Simpson's dominance (*SI*) index was dependent on cropping method of lentil (*Fig. 2*). In both years of the study, the highest segetal diversity was observed in lentil grown in sole cropping (*H'* = 2.048 and 1.414 respectively). No dominance of weed species was noted (*Si* = 0.161 and 0.295). Lentil sole cropping was characterized by the highest number of weed species (18 and 9 units). Significantly less biodiversity index for lentil grown with cereal was found (mean *H* = 1.000 and 0.884).

In both years of the study the highest index of relation between lentil seeds yield and weeds dry matter was in sole cropping, whereas the lowest yields in such as cropping method was noted (*Fig. 3a ,b*). While, the lowest index in lentil grown with oats was noted.



a

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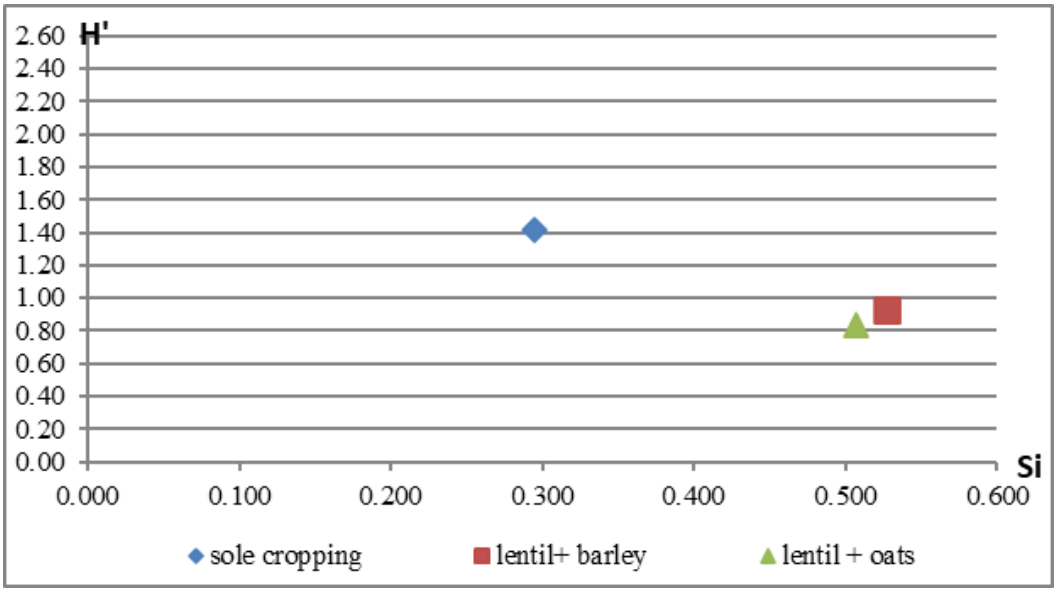
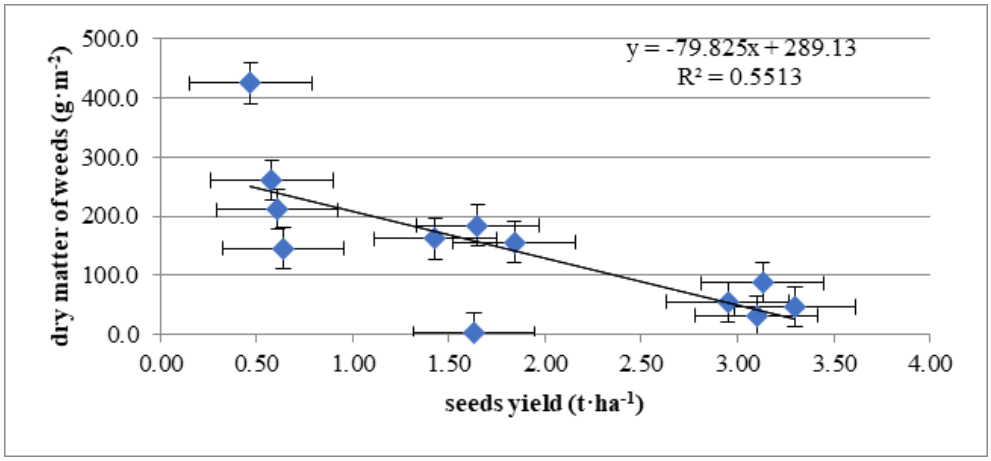
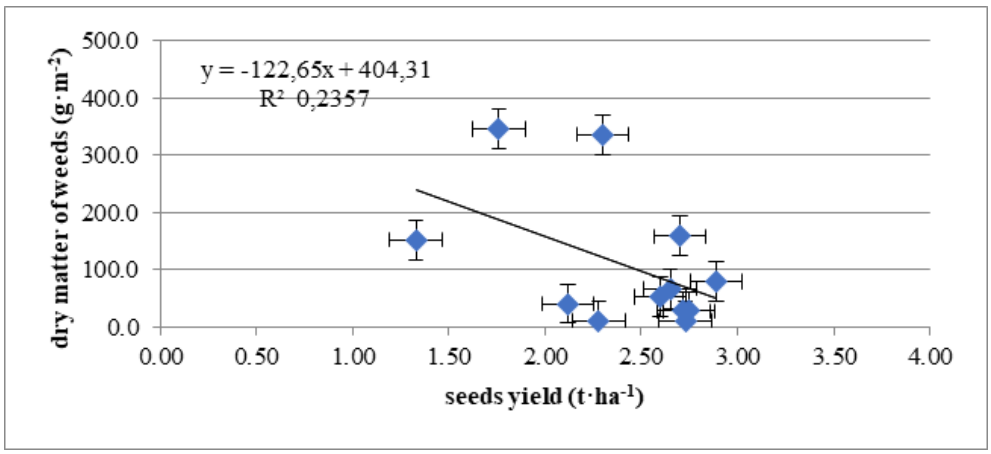


b

Figure 2. *Index of Shannon's diversity (H') and Simpson's dominance (SI) in 2017 (a) and 2018 (b)*



a



b

Figure 3. *Relationship between lentil seeds yield and weeds dry matter in 2017 (a) and 2018 (b)*

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DOI: http://dx.doi.org/10.15666/aeer/1905_35633577

Conclusions

The results of this study reveal that independently of cereal species, the higher yields were obtained, where lentil was grown with supporting plants than in sole cropping. The higher thousand seeds weight, pods number, seeds number and seeds weight on plant was noted in lentil grown in sole cropping than with supporting crops. Lentil seeds grown with cereals was characterized by the similar amounts of protein, fat, fibre and phosphorus as grown in sole cropping. Cropping method of lentil have the significant effect on weed infestation. In both years of the study, the highest weed infestation was observed in lentil grown in sole cropping. Sowing lentil with supporting crops significantly reduced weed infestation. Independently of cropping method, *P. major, V. arvensis, C. arvense, Ch. album, E. crus-galli* were weeds that occurred in the greatest number.

In summary, one condition for using this cropping method is to select a supporting crop and its proportion in the mixture that will help reduce crop lodging, while in the case of low lentil yield, the supporting component largely decreases the risk of total yield loss.

Acknowledgements. This manuscript has not been published or presented elsewhere in part or in entirety, and is not under consideration by another journal. The study design was approved by the appropriate ethics review boards. All the authors have approved the manuscript and agree with submission to your esteemed journal. There are no conflicts of interest to declare.

Conflict of interests. The authors declare no conflict of interests. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_35633577

THE STRUCTURE OF THREATENED VEGETATION IN THE MONTANE TEMPERATE ECOSYSTEM OF PASHAT VALLEY, PAK-AFGHAN BORDER, HINDUKUSH RANGE, BAJAUR, PAKISTAN

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(Received 14th Mar 2021; accepted 19th Jul 2021)

Abstract. The present phytosociological study was conducted during 2017-2020 in Pashat valley, Pak-Afghan border, Bajaur, Pakistan. Fifteen communities were established, 5 for each herb, shrub and tree through the systematic random quadrat method, i.e., 10 m^2 for trees, 5 m^2 for shrubs and 1 m^2 for herbs in the five selected sites. Three communities were documented in the respective sites for trees, shrubs and herbs viz *Pinus-Quercus-Juglans*, *Berberis-Indigofera-Buddleja*, *Rumex-Hyparrhenia-Cynodon* on site I, *Pinus-Olea-Quercus*, *Berberis-Otostegia-Sarcococca* and *Hyparrhenia-Cynodon-Heteropogon* on site II, Olea-Quercus, *Rubus-Otostegia-Berberis* and *Cynodon-Salvia-Cyperus* on site III, *Quercus-Olea-Juglans*, *Sarcococca-Justicia-Otostegia* and *Cynodon-Conyza-Thymus* on site IV and *Quercu-Olea-Ficus, Otostegia-Berberis-Sageretia* and *Apluda-Cynodon-Chrysopogon* on site V. Cluster analysis through PAST and PC-ORD classified it into four groups. Soil samples of the selected sites were physicochemically analyzed. The soil texture ranged from silty to clay loam with basic pH ranging from 7.3-7.8 and low organic matter. Nitrogen (0.036 to 0.044 mg/kg), phosphorus (7.40 to 15.4 mg/kg) and potassium (80 to 120 mg/kg) were among the macronutrients of the soil. Deforestation and soil erosion in the area were the major threats to the phytodiversity.
Keywords: *phytosociology, similarity index, maturity index, species diversity, montane temperate vegetation*

Introduction

Phytosociology is the study of plant assemblages in communities (Dengler, 2016) and it is the best way to learn about structure, habit, niche and habitat of vegetation as well as plant interactions in an ecosystem (Khan et al., 2016). The establishment of plant communities and its composition depend on time and altitude within a region, however, latitude, slope, and precipitation are also key factors (Kharkwal et al., 2005). Phytosociological research is critical for understanding the plant population dynamics and their interactions with biotic and abiotic influences which is the foundation for conservation initiatives, regeneration of degraded areas and long-term forest resource management (Zerwes et al., 2018).

In ecological research, the relationship between abiotic and biotic components of an ecosystem is becoming extremely prevalent (Tavili and Jafari, 2009; Khan et al., 2016). Some species change their growth form, life cycle and development to adopt the changing conditions, resulting in changes in plant populations and thus facilitating the spread of invasive species (Rahman et al., 2016). A quantitative vegetation analysis in a forest ecosystem provides data on species diversity, community organization, niche resources

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_35793600

distribution and species over rate (Mandal and Joshi, 2014). Plants that grow in close proximity have a mutual association with one another and with the surrounding environment (Mishra et al., 1997; Mandal and Joshi, 2014). The quantitative relationship between abundant and rarely growing plant species is an important property of a community. Quantitative analysis of vegetation aims to characterize the vegetation, predict its pattern and classify it in a meaningful way (Braun-Blanquet, 1932; Odum, 1971).

A complex set of both abiotic and biotic factors such as elevation, slope, participation, temperature, deforestation, erosion, trampling overgrazing and developmental works influence on the structure of vegetation of an area (Khan et al., 2011, 2017; Habib et al., 2014; Evangelista et al., 2016; Ali et al., 2018; Mota et al., 2018; Hussain et al., 2019; Bhat et al., 2020; Hailemariam and Temam, 2020; Zaman and Badshah, 2020; Yang et al., 2020). The first and the most important step of any ecological study of an area is to conduct a phytosociological evaluation of the plant population which is essential to understand the function of a community (Warger and Morrel, 1978). A great deal of phytosociological research has been documented in Pakistan. Khan et al. (2016) studied the phytosociology of Indus-Kohistan Pine forests. Haq et al. (2015) documented the vegetation of subtropical forests of district Bataghram. Ali et al. (2015) investigated the vegetation structure in Buner Khyber Pakhtunkhwa in relation to edaphic variables. Ali et al. (2018) studied the vegetation structure and threats to mountain ecosystem of Hindukush rang, Swat Pakistan. In the Koh-e-Safaid range, Kurram, Pakistan, Hussain et al. (2019) summarized seven communities of herbs, shrubs, and trees vegetation. The vegetation dynamics along the altitudinal gradient of Terich valley, Hindukush range, Chitral, Pakistan were studied by Zaman and Badshah (2020). Pashat valley lies in the famous Hindukush ranges of Pakistan having rich phytodiversity which is under high anthropogenic disturbance. The literature review clearly shows that there is no such work done on the Pashat valley which will fill the research gap in the area. There is an utmost need to study the area in phytosociological context which will be helpful for the conservation and sustainable utilization of plant resources of the area and also for future ecological study.

Therefore, the aim of this study was to analyse the structure of plant community in the Pashat valley Bajaur, and to identify the edaphic variables responsible for the establishment of plant communities and their distribution pattern.

Materials and Methods

Study area

Bajaur, a part of the former administrative setup of FATA (Federally Administrated Tribal Area), has been recently merged as a district in the Khyber Pakhtunkhwa province of Pakistan. It lies in the north of the country and nearly one half of its area has a hilly terrain. Some lofty mountains of the Hindu Kush range lie here, in the north and northwest, it shares a 52 km long border with Kunar Province of Afghanistan. The Pashat valley is located in Tehsil Salarzai at 34.45° N to 34.57° N latitude and 71.25° E to 71.35° longitude. The valley has a high peak "the Latai Sar" which is the famous pass for trans-border trade and transportation with Afghanistan. The area has plenty of water resources in spring and stream forms of which the Gaber Chena is the popular picnic spot in summer. The area is also famous for the cultivation of a local variety of rice (*Oryza sativa*) "the Begamai". Its altitude varies from 1300 m to more than 2600 m from sea level (*Fig.*

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DOI: http://dx.doi.org/10.15666/aeer/1905_35793600

1). The weather is moderate in summer but extremely cold in winter due to snowfall. The coldest months are December and January with mean temperature from 5 ℃ to 10 ℃. The hottest month is July with mean temperature which varies 26 ℃ to 40 ℃. The annual rainfall is approximately 500 mm, with 375 mm falling in July and August.

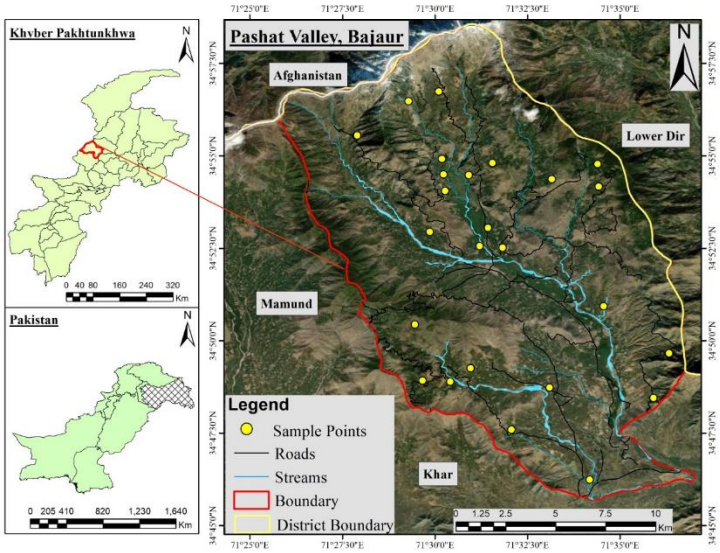

Figure 1. *Map of the study area*

Quantitative analysis of communities

Phytosociological studies were conducted at five different monitoring sites during 2017-2020. At each monitoring site, vegetation was examined using 5 (10 × 10 m) quadrats for trees, 10 (5 × 5 m) quadrats for shrubs, and 15 (1×1 m) quadrats for herbs. The density, cover and frequency of each species were determined using formulae, then converted to relative values to get IV (Importance value) and FIV (Family importance value).

Density: The species density and its relative value were calculated using standard formulae (Oosting, 1956; Hussain, 1989).

$$\text{Density (D)} = \frac{\text{Total number of individuals of a species}}{\text{Total number of quadrates}}$$

$$\text{Relative Density (RD)} = \frac{\text{Number of individuals of a species}}{\text{Total density of all species}} \times 100$$

Cover: The cover and relative cover of species were determined with specified formulae (Cox, 1967; Hussain, 1989).

$$\text{Cover (C)} = \frac{\text{Sum of mid points of a species}}{\text{Total area sampled}}$$

$$\text{Relative cover (RC)} = \frac{\text{Cover of a species}}{\text{Total cover of all species in a stand}} \times 100$$

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DOI: http://dx.doi.org/10.15666/aeer/1905_35793600

Basal Area: Measuring tape is used for diameter of crown and recorded the value in square feet. The basal area was measured through using table of basal area (Cox, 1967; Hussain, 1989).

$$\text{Basal Area (BA)} = \frac{\text{Area of species calculated from circumference at DBH}}{\text{Total sampled area}}$$

$$\text{Relative Basal Area (RBA)} = \frac{\text{Basal area of a given species}}{\text{Total area of all species in a stand}} \times 100$$

Frequency: The frequency and relative frequency of the plants were determined by using the formulae given (Hussain, 1989).

$$\text{Frequency (F)} = \frac{\text{Number of quadrates in which a species present}}{\text{Total number of quadrates taken}} \times 100$$

$$\text{Relative Frequency (RF)} = \frac{\text{Frequency of a species}}{\text{Total frequency of all species}} \times 100$$

Importance value: The relative values of three parameters, namely density, cover and frequency, were added to determine the importance value of a species (Cox, 1967; Hussain, 1989).

$$\text{IV} = \text{RD} + \text{RC} + \text{RF}$$

Family Importance Value: FIV for all recorded families were calculated by adding the importance value of each species from each family.

Index of Similarity (IS): The similarity index was calculated by using Sorensen's index (1948), as revised by Motyka et al. (1950).

$$\text{IS} = \frac{2\text{W}}{\text{A} + \text{B}} \times 100$$

where, 'IS' stands for index of similarity, 'W' stands for the lowest value that both A and B community have in common, 'A' and 'B' stands for species values in community A and B, respectively.

Species Diversity (SD): Simpson-Wiener index of similarity was used to quantify the species diversity in the stands (Simpson, 1961).

$$\text{D} = 1 - \frac{\sum \text{n}(\text{n} - 1)}{\text{N}(\text{N} - 1)}$$

where 'D' represents diversity index, 'N' represents total number of species and 'n' denotes number of a species of an individual.

Maturity index (MI): Maturity index for communities were measured with the method of Pichi-Sermolli (1948).

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DOI: http://dx.doi.org/10.15666/aeer/1905_35793600

$$\text{Maturity Index (MI)} = \frac{\text{Ft}}{\text{N}}$$

where 'Ft' represents the frequency values of all species in a stand and 'N' the total number of species in a stand.

Cluster analysis

PAST application and Principal Component Analysis (PCA) method were used to conduct cluster analysis. It is a classification method for grouping objects that are similar. The dendrogram that emerges is a hierarchical tree like structure. Different biotic communities can be represented by these cluster sampling units.

Soil analysis

The soil samples weighing about 1 kg were taken from the selected sites up to a depth of 15 cm of the area. To make a composite sample, the collected soil samples were thoroughly mixed and stored in polythene bags for physicochemical analysis. For the evaluation of physicochemical properties, the soil samples were analyzed at the Agriculture Research Institute Tarnab Peshawar. Soil texture triangle was used to assess the soil texture (Bouyoucos, 1936; Brady, 1990). The organic matter content was determined using the Walkley (1947) standard process, while pH of the soil was measured by using a portable PHS-25 pH meter (Black, 1965). Following acid-base neutralization, $CaCO_3$ was determined (Rayan et al., 1997). Kjeldahl method (1983) was used to calculate nitrogen content. The concentration sodium (Na) was calculated by using flame photometry, while the concentration of potassium (K) was determined through Olsen and Sommers's process (1982).

Results

Family importance values

The present study was conducted in Pashat valley along Pak-Afghan Border District Bajaur. Five monitoring sites viz. Bagandel, Batwar, Gabar, Saro Wano and Mala Said were recognized. The floristic composition and vegetation structure of the study area is dynamic. In total, 110 plants were identified in sampling units, with 23 species of trees, 32 species of shrubs and 55 species of herbs (*Table 1*). On the basis of importance value (IV), three communities each for trees, shrubs and herbs were documented in the monitoring sites. Seventeen families established the trees communities which is led by family Fagaceae with FIV 249.60 flowed by Moraceae (218.82), Oleaceae (148.95), Pinaceae (146.07), Juglandaceae (120.0), Simaroubaceae (102.27) and the rest of families having FIV value less than 100. Twenty (20) families represent shrubby communities, with Rosaceae dominating with FIV of 185.83, followed by Rhamnaceae (FIV 160.05), Lamiaceae (FIV 142.29), Berberidaceae (FIV 134.40), Papilionaceae (FIV 132.39) Buxaceae (FIV 112. 89) and Acanthaceae (FIV 110.79) while the remaining families have FIV ranges from 85.74 to minimum of 7.08. Similarly, herbaceous families were recorded 25 in which dominant families were Poaceae with FIV (399.28) followed by Lamiaceae (354.30) and Asteraceae (179.67) as shown in *Table 1* and *2*.

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DOI: http://dx.doi.org/10.15666/aeer/1905_35793600

Table 1. *IV for trees, shrubs and herbs species in Pashat valley*

S#	Species	Site-I BT	Site-II BG	Site-III GB	Site-IV SW	Site-V MS
	Trees					
1	*Acacia modesta* Wall.	9.93	15.54	0	16.23	21.21
2	*Ailanthus altissima* (Mill.) Swingle	13.38	17.13	19.62	22.89	29.25
3	*Alnus nitida* (Spach) Endl. Gen.	0	0	16.47	0	0
4	*Celtis aeriocarpa* Decne.	8.94	11.58	0	0	0
5	*Celtis caucasica* Wild.	0	10.05	0	0	0
6	*Diospyros lotus* L.	21.51	16.68	0	0	0
7	*Ficus palmata* Forssk.	16.65	0	19.83	20.64	32.79
8	*Juglans regia* L.	23.64	17.31	20.04	28.53	30.48
9	*Melia azedarach* L.	17.88	0	10.05	17.28	21.48
10	*Morus alba* L.	15.69	15.15	24.12	0	22.05
11	*Morus nigra* L.	9.54	14.58	19.86	0	0
12	*Olea ferruginea* Royle.	21.24	34.23	39.87	28.56	32.97
13	*Pinus roxburghii* Sargent	16.59	19.47	0	16.11	0
14	*Pinus wallichiana* A.B. Jackson.	39.81	54.09	0	0	0
15	*Platanus orientalis* L.	19.14	15.24	22.29	0	0
16	*Pyrus pashia* Ham. ex D. D on.	16.41	0	0	0	0
17	*Quercus baloot* Griffith	0	0	27.57	22.56	0
18	*Quercus dilatata* A. Kern.	0	0	0	21.63	0
19	*Quercus incana* Roxb., Hort. Beng.	30.51	20.34	31.17	48.57	47.25
20	*Robinia pseudoacacia* L.	0	0	17.79	13.89	21.78
21	*Salix acmophylla* Boiss.	0	13.59	16.68	15.45	20.22
22	*Zanthoxylum armatum* DC	19.14	16.56	0	27.75	0
23	*Ziziphus jujuba* Mill.	0	8.43	14.64	0	20.49
	Shrubs					
24	*Andrachne cordifolia* L.	11.88	19.65	12.24	0	0
25	*Berberis lycium* Royle	30.09	32.7	22.32	20.04	29.25
26	*Buddleja crispa* Bth.	23.73	0	0	17.58	20.79
27	*Calotropis procera* (Ait.) Ait.f., Hort.	0	0	7.26	11.46	0
28	*Capparis spinosa* L.	0	0	0	0	7.08
29	*Cestrum elegans* (Brongn. ex Neumann) Schltdl.	0	0	10.98	0	0
30	*Cotoneaster microphyllus* Wall. ex Lindl.	13.35	15.21	10.83	12.42	15.06
31	*Daphne mucronata* Royle	15.81	0	18.36	18.9	21.96
32	*Daphne papyracea* Wall. ex Steud., Nom.	8.4	0	0	0	0
33	*Desmodium elegans* DC.	9.3	16.38	0	0	0
34	*Dodonaea viscosa* (L.) Jacq.	0	13.02	0	18.06	20.64
35	*Hedera nepalensis* K. Koch	9.06	8.85	9.48	0	10.56
36	*Indigofera heterantha* Wall. ex Brandis.	28.56	18.48	19.32	17.19	23.16
37	*Jasminum humile* L.	0	0	13.71	0	0
38	*Jasminum officinale* L.	10.53	0	0	0	0
39	*Justicia adhatoda* L.	16.59	17.07	20.82	32.1	24.21
40	*Lantana indica* Roxb.	0	0	0	11.73	14.16
41	*Mallotus philippensis* (Lam.) Muell.	0	0	0	10.23	0
42	*Maytenus royleanus* Wall. ex Lawson	10.32	0	0	0	0
43	*Otostegia limbata* (Bth.) Boiss	19.71	32.25	26.85	26.67	36.81
44	Reinwardtia trigyna (Roxb.) Planch.	0	11.85	18.99	0	0
45	*Ricinus communis* L.	0	0	12.21	10.71	0
46	*Rubus ellipticus* (Franch.) Thuan.	0	6.99	17.19	0	0
47	*Rubus fruticosus* L.	18.15	18.66	30.3	13.35	0
48	*Rubus niveus* Thunb.	13.92	0	0	0	0
49	*Sageretia thea* (Osbeck) M. C. Johnston	17.91	17.34	21.45	16.05	25.41
50	*Sarcococca saligna* (D. Don) Muell.	22.32	23.88	0	41.28	18.72

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
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S#	Species	Site-I BT	Site-II BG	Site-III GB	Site-IV SW	Site-V MS
51	*Verbena officinalis* L.	0	11.19	0	0	0
52	*Viburnum grandiflorum* Wall. ex Dc.	0	9.03	0	0	0
53	*Vitex negundo* L.	9.96	12.75	0	8.97	16.98
54	*Withania somnifera* (L.) Dunal.	0	0	12.69	0	0
55	*Ziziphus oxyphylla* Edgew.	10.41	14.7	15	13.26	15.21
	Herbs					
56	*Achyranthes bidentata* Blume	0	0	5.19	6.25	0
57	*Ajuga bracteosa* Wall. ex Bth.	10.69	10.58	8.97	11.6	0
58	*Androsace rotundifolia* Hardw.	0	0	9.79	0	0
59	*Apluda mutica* L.	0	15.87	16.8	0	37.56
60	*Argyrolobium roseum* (Camb.) Jaub.	0	0	6.99	0	0
61	*Arisaema flavum* (Forsk.) Schott, Prodr.	6.14	6.95	0	0	0
62	*Asparagus gracilis* Royle.	0	3.52	0	4.99	0
63	*Bromus pectinatus* Thunb. Prodr. Fl. Cap.	0	0	0	0	9.44
64	*Cannabis sativa* L.	0	0	11.29	0	13.78
65	*Carduus edelbergii* Reh. F., K. Danske	0	0	10.19	0	0
66	*Chrysopogon serrulatus* Trin.	0	0	0	0	26.68
67	*Cirsium vulgare* (Savi) Ten.	15.9	0	0	15.61	0
68	*Clematis graveolens* Lindl.	0	0	9.08	0	0
69	*Conyza canadensis* (L.) Cronquist.	15.5	13.63	12.22	24.61	20.26
70	*Cynodon dactylon* (L.) Pers.	15.94	18.69	28.7	26.51	31.4
71	*Cyperus niveus* Retz., Observ.	13.12	10.61	19.82	11.78	13.89
72	*Delphinium ajacis* (L.) Schur	0	10.59	0	0	0
73	*Dicliptera bupleuroides* Nees	0	0	0	10.83	0
74	*Duchesnea indica* (Jacks.) Focke	7.37	9.56	8.65	7.33	9.05
75	*Eragrostis minor* Host	12.14	0	0	13.66	0
76	*Eragrostis papposa* (Roem & Schult.) Steud. Nom.	0	16.65	0	0	0
77	*Erioscirpus comosus* (Wall.) Palla	0	0	0	9.64	0
78	*Filago hurdwarica* (Wall. ex DC) Wagenitz	0	0	0	0	13.04
79	*Filago pyramidata* L.	0	8.62	0	7.12	0
80	*Fimbristylis cymosa* R. Br., Prodr.	0	0	5.19	0	0
81	*Geranium rotundifolium* L.	0	0	8.38	15.28	0
82	*Herniaria hirsuta* L.	0	0	6.34	0	0
83	*Heteropogon contortus* (L.) P. Beauy. ex. Roem & Schult.	11.79	17.81	0	0	0
84	*Hyparrhenia hirta (L.) Stapf*	16.23	21.56	0	12.27	0
85	*Impatiens edgeworthii* Hook. f., Fl. Brit.	0	0	0	11.82	0
86	*Marrubium vulgare* L.	0	0	0	15.63	10.37
87	*Medicago minima* (L.) Grub.	0	0	9.38	0	0
88	*Mentha longifolia* L.	0	0	7.03	0	0
89	*Micromeria biflora* (Buch. Ham. Ex. D. Don) Bth.	13.18	9.83	10.94	10.6	12.86
90	*Nepeta cataria* L.	8.145	8.69	7.22	10.74	9.31
91	*Origanum vulgare* L.	15.8	0	0	0	0
92	*Oxalis corniculata* L.	13.84	13.16	17.38	12.66	14.1
93	*Pennisetum orientale* Rich.	15.1	13.71	0	0	0
94	*Phalaris paradoxa* L.	0	0	0	8.183	0
95	*Plantago lanceolata* L.	11.96	0	0	0	12.79
96	*Pteris cretica* L.	9.92	0	0	0	8.77
97	*Rhynchosia minima* (L.) DC.	0	5.51	0	0	0
98	*Rubia cordifolia* L.	0	13.18	8.52	0	0
99	*Rumex hastatus* D. Don.	23.92	12.57	13.13	0	0
100	*Salvia moorcroftiana* Wall. ex Bth.	0	0	19.84	0	0
101	*Stachys parviflora* Bth.	0	0	0	16.19	16.56
102	*Tagetes minuta* L.	0	11.71	11.26	0	0

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_35793600

S#	Species	Site-I BT	Site-II BG	Site-III GB	Site-IV SW	Site-V MS
103	*Tetrapogon villosus* Desf.	0	12.59	0	0	0
104	*Teucrium royleanum* Wall. ex Bth	15.86	0	11.65	0	15.13
105	*Thymus linearis* Bth. ex Wall.	14.62	10.44	7.26	23.28	11.3
106	*Tulipa clusiana* DC.	12	0	0	0	13.7
107	*Urtica dioica* L.	0	8.49	0	0	0
108	*Verbascum thapsus* L.	11.12	7.89	8.77	0	0
109	*Viola canescens* Wall. ex Roxb.	0	7.57	0	13.39	0
110	*Viola pilosa* Blume	9.73	0	0	0	0

Key: BT-Batwar, BG- Bagandel, GB-Gabar, SW- Saro Wano, MS- Mala Said

Table 2. *Family importance values of Pashat valley*

S#	Family	FIV	S#	Family	FIV
Trees			**Shrubs**		
1	Fagaceae	249.6	15	Oleaceae	24.24
2	Moraceae	218.82	16	Solanaceae	23.67
3	Oleaceae	148.95	17	Asclepiadaceae	18.72
4	Pinaceae	146.07	18	Celastraceae	10.32
5	Juglandaceae	120	19	Caprifoliaceae	9.03
6	Simaroubaceae	102.27	20	Capparidaceae	7.08
7	Meliaceae	66.69	**Herbs**		
8	Salicaceae	65.94	1	Poaceae	399.28
9	Rutaceae	63.45	2	Lamiaceae	354.3
10	Mimosaceae	62.91	3	Asteraceae	179.67
11	Platanaceae	56.67	4	Cyperaceae	84.05
12	Papilionaceae	53.46	5	Oxalidaceae	71.14
13	Rhamnaceae	43.56	6	Polygonaceae	49.62
14	Ebenaceae	38.19	7	Rosaceae	41.96
15	Ulmaceae	30.57	8	Violaceae	30.69
16	Betulaceae	16.47	9	Scrophulariaceae	27.79
17	Rosaceae	16.41	10	Liliaceae	25.7
Shrubs			11	Cannabaceae	25.07
1	Rosaceae	185.43	12	Plantaginaceae	24.75
2	Rhamnaceae	160.05	13	Geraniaceae	23.66
3	Lamiaceae	142.29	14	Papilionaceae	21.88
4	Berberidaceae	134.4	15	Rubiaceae	21.69
5	Papilionaceae	132.39	16	Ranunculaceae	19.67
6	Buxaceae	112.89	17	Pteridaceae	18.69
7	Acanthaceae	110.79	18	Aracaceae	13.08
8	Verbenaceae	85.74	19	Balsaminaceae	11.82
9	Thymelaeaceae	83.43	20	Amaranthaceae	11.45
10	Euphorbiaceae	76.92	21	Acanthaceae	10.83
11	Buddlejaceae	62.1	22	Primulaceae	9.79
12	Sapindaceae	51.72	23	Asparagaceae	8.52
13	Araliaceae	37.95	24	Urticaceae	8.49
14	Linaceae	30.84	25	Illecebraceae	6.34

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_35793600

Communities structure at the monitoring sites

Site I (Batwar)

Plant communities were established at elevation of 2002 m in the North-East of the valley at 34.56° N latitude and 71.29° E longitude. The soil texture was silty, clay loam with 54% silt, 32% clay and 14% sand. The pH of site recorded as 7.4. The soil had nitrogen content of 0.036 mg/kg, phosphorus content of 15.4 mg/kg, and a potassium content of 80 mg/kg. The electrical conductivity was recorded 0.01% and the total soluble salts were 0.19 dsm^{-1}. The $CaCO_3$ content was 9.0%, with just 0.72% organic matter (*Table 3*).

1. Pinus-Quercus-Juglans community (PQJ)

Pinus wallichiana dominated the community with an IV of 39.82 which is associated with *Quercus incana* with IV of 30.51 and *Juglans regia* with an IV 23.66. These were followed by *Diospyrus lotus* (IV 21.51*), Olea ferruginea* (IV 21.24), *Platanus orientalis* (IV 19.16) and *Zanthoxylum armatum* (IV 19.14). In addition to these, *Melia azedarach*, *Ficus palmata*, *Pinus roxburghii*, *Pyrus pashia*, *Morus alba*, *Ailanthus altissimia*, *Acacia modesta*, *Morus nigra* and *Celtis aeropcarpa* had IV value ranging from 17.87 to 8.94 (*Table 1*).

2. Berberis-Indigofera-Buddleja community (BIB)

Berberis lyceum, with an IV of 30.10 uniformly dominated this community. *Indigofera heterantha* with an IV of 28.55, and *Buddleja crispa* with an IV of 23.71, were the other dominant species. *Sarcococca saligna* (IV of 22.30) form mosaic patches in the community due to soil type and topographical variation. The co-dominant species were *Otostegia lambata* (19.73 IV) followed by *Rubus fruticosa* (18.16 IV), *Sageretia thea* (17.92 IV) and *Justicia adhatoda* (16.59 IV). *Daphne mucronata*, *Rubus niveus*, *Cotoneaster microphyllus*, *Andrachne cordifolia*, *Jasminum officinale*, *Maytenus royleanus*, *Vitex negundo*, *Desmodium elegans*, *Hedera nepalensis* and *Daphne papyracea* were the other shrubby members of the community with IV ranges from 15.80 to 8.40 (*Table 1*).

3. Rumex-Hyparrhenia-Cynodon community (RHC)

This community consists of 23 species led by *Rumex hastatus* (IV 23.92) followed by *Hyparrhenia* hirta (IV 16.23), *Cynodon dactylon* (IV 15.94), *Cirsium vulgare* (IV 15.90), *Teucrium royleanum* (IV 15.86), *Origanum vulgare* (IV 15.80)*, Conyza Canadensis* (IV 15.50) and *Pennisetum orientale* (IV 15.10). The remaining species in the community had low IV value ranged from 14.62 to 6.14 (*Table1*).

Site II (Bagandel)

At this site, the plant community established on North and East aspect with elevation of 1895 m at 34.57° N latitude and 71.32° E longitude. The texture class composed of 52% silt, 32% clay and 16% sand. The pH of site recorded as 7.8. The soil had nitrogen content of 0.043 mg/kg, phosphorus content of 14.3 mg/kg and potassium content of 90 mg/kg. Total soluble salts (TSS) were recorded 0.064% and the electrical conductivity (EC) was 0.02 dsm^{-1}. The $CaCO_3$ content was 8.5% with low organic matter 0.86% (*Table 3*).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_35793600

Table 3. *Physicochemical analysis of soil samples of the selected sites of the Pashat valley*

S#	Soil sample	Communities			Physicochemical characteristics									Macro-elements		
					Soil texture				pH	EC	TSS	$CaCO_3$	O.M	N	P	K
		Tree	Shrub	Herb	Clay	Silt	Sand	Textural class		dsm^{-1}	%			mg Kg^{-}		
					%											
1	Site-I (Batwar)	PQJ	BIB	RHC	32	54	14	Silty, clay loam	7.4	0.19	0.061	9.0	0.72	0.036	15.4	80
2	Site-II (Bagandel)	POQ	BOS	HCH	32	52	16	Silty, clay loam	7.8	0.2	0.064	8.5	0.86	0.043	14.3	90
3	Site-III (Gabar)	OQ	ROB	CSC	30	56	14	Silty, clay loam	7.6	0.1	0.032	8.75	0.79	0.039	7.4	110
4	Site-IV (Saro Wano)	QOJ	SJO	CCT	34	50	16	Silty, clay loam	7.3	0.12	0.038	9.25	0.86	0.043	12.8	120
5	Site-V (Mala Said)	QOF	OBS	ACC	28	62	10	Silty, clay loam	7.3	0.14	0.045	7.5	0.89	0.044	13.1	100

Key: E.C= Electrical conductivity, TSS= Total Soluble Salts, O.M= Organic matter, N= Nitrogen, P= Phosphorus, K= Potassium
Key for communities:
Tree: PQJ= *Pinus-Quercus-Juglans*, POQ= *Pinus-Olea-Quercus*, OQ= *Olea-Quercus,* QFJ= *Quercus-Olea-Juglans*, QOF= *Quercus-Olea-Ficus*
Shrubs: BIB= *Berberis-Indigofera-Buddleja*, BOS= *Berberis-Otostegia-Sarcococca*, ROB= *Rubus-Otostegia-Berberis*, SJO= *Sarcococca- Justicia-Otostegia,* OBS= *Otostegia-Berberis-Sageretia*
Herb: RHC= *Rumex-Hyparrhenia-Cynodon*, HCH= *Hyparrhenia-Cynodon-Heteropogon*, CSC= *Cynodon-Salvia-Cyperus*, CCT= *Cynodon- Conyza-Thymus*, ACC= *Apluda-Cynodon-Chrysopogon*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
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1. Pinus-Olea-Quercus community (POQ)

Tree community at site II was dominated by *Pinus wallichiana* with an IV of 54.08. This was followed by *Olea ferruginea* (IV 34.24), next by *Quercus incana (*IV 20.23). *Pinus roxburghii* (IV 19.48*), Juglans regia* (IV 17.33), *Ailanthus altissima* (IV 17.11) and *Diospyros lotus* (IV 16.67) were the next co-dominant plants in the list. In addition to these, *Zanthoxylum armatum*, *Acacia modesta*, *Platanus orientalis*, *Morus alba*, *Morus nigra, Salix acmophylla*, *Celtis aeriocarpa, Celtis caucasica* and *Ziziphus jujuba* had IV value ranging from 16.54 to 8.42 (*Table1*).

2. Berberis-Otostegia-Sarcococca community (BOS)

Berberis lyceum with an IV of 32.71 was found dominant with uniform distribution in the community. The other dominant species were *Otostegia limbata* with an IV of 32.25 and *Sarcococca saligna of 23.87*. The other associated species were *Andrachne cordifolia* (19.67 IV) followed by *Rubus fruticosa* (18.64 IV), *Indigofera heterantha* (18.49 IV) and *Sageretia thea* (17.34 IV). Other shrubby members of the community were *Justicia adhatoda*, *Desmodium elegans*, *Cotoneaster microphyllus*, *Ziziphus oxyphylla*, *Dodonaea viscosa, Vitex negundo*, *Reinwardtia trigyna, Desmodium elegans*, *Verbena officinalis, Viburnum grandiflorum, Hedera nepalensis* and *Rubus ellipticus* had IV between 17.09 and 6.99 (*Table1*).

3. Hyparrhenia-Cynodon-Heteropogon community (HCH)

Grass species were found dominant in this community on the basis of importance values such as *Hyparrhenia hirta* (IV 21.56), *Cynodon dactylon* (IV 18.69) and *Heteropogon contortus* (IV 17.81). The other co-dominant species were *Eragrostis papposa* (16.65), *Apluda mutica* (15.87), *Pennisetum orientale,* (13.71)*, Conyza Canadensis* (13.63) and *Rubia cordifolia* (13.18) (*Table 1*).

Site III (Gabber)

Communities at this site were documented at the North aspect at low elevation of 1553 m at 34.54° N latitude and 71.29° E longitude. The texture class was composed of 56% silt, 30% clay and 14% sand. The pH of site recorded as 7.6. Soil had nitrogen content 0.039 mg/kg, phosphorus 7.4 mg/kg and potassium 110 mg/kg. The electrical conductivity was recorded 0.10 dsm^{-1} and the total soluble salts (TSS) content 0.032%. $CacO_3$ content was 8.75% with just 0.79% organic matter (*Table 3*).

1. Olea-Quercus community (OQ)

Olea ferruginea was found to be dominated with IV of 39.86. Other associated dominant species were *Quercus incana* having an IV of 31.17 and *Quercus baloot* with an IV 27.59. These were followed by *Morus alba* (IV 24.11*), Platanus orientalis* (IV 22.30) and *Juglans regia* (IV 20.05). In addition to these *Morus nigra*, *Ficus palmata*, *Ailanthus altissima*, *Robinia pseudoacacia*, *Salix acmophylla*, *Alnus nitida,* Ziziphus *jujube* and *Melia azedarach* had IV value ranging from 19.86 to 10.04 (*Table1*).

2. Rubus-Otostegia-Berberis community (ROB)

This community was led by *Rubus fruticosus* with an IV of 30.30. The other dominant species were *Otostegia limbata* with an IV of 26.86 and *Berberis lyceum* of 22.33. The

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_35793600

other associated species were *Sageretia thea* (21.45 IV) followed by *Justicia adhatoda* (20.81 IV), *Indigofera heterantha* (19.33 IV) and *Reinwardtia trigyna* (18.98 IV). Other members of the community were *Daphne mucronata*, *Rubus ellipticus*, *Ziziphus oxyphylla, Jasminum humile, Withania somnifera, Andrachne cordifolia, Ricinus communis*, *Cestrum elegans, Cotoneaster microphyllus, Hedera nepalensis* and *Capparis spinosa* had IV ranging from 18.37 to 7.26.

3. Cynodon-Salvia-Cyperus community (CSC)

Cynodon dactylon (IV 28.70), *Salvia moorcroftiana* (IV 19.84) and *Cyperus niveus* (IV 19.82) were the leading representative species of the community. The next dominant species in this community were *Oxalis corniculata* with IV (17.38), *Apluda mutica* (16.80), *Rumex hastatus* (13.13)*, Conyza Canadensis* (12.22) and *Teucrium royleanum* (11.65) (*Table 1*).

Site IV (Saro Wano)

This community was established at 1756 m elevation in the North-West aspect of the study area at 34.53° N latitude and 71.27° E longitude. The soil texture class was composed of 50% silt, 34% clay and 16% sand. The pH of site recorded as 7.3. Soil nitrogen content was estimated (0.043 mg/kg), phosphorus (12.8 mg/kg) and potassium (120 mg/kg). The electrical conductivity was recorded 0.12 dsm^{-1} and total soluble salts (TSS) 0.038%. $CacO_3$ content was 9.25% with low organic matter 0.86% (*Table 3*).

1. Quercus-Ficus-Juglans community (QFJ)

Quercus incana was found to be dominated by IV of 48.57. Other accompanied dominant species were *Ficus palmata* with IV of 28.55 and *Juglans regia* with an IV 28.53. They were followed by *Zanthoxylum armatum* (IV 27.74*), Ailanthus altissima* (IV 22.89) and *Quercus baloot* (IV 22.54). In addition to these *Quercus dilatata*, *Olea ferruginea*, *Melia azedarach, Acacia modesta, Pinus roxburghii*, *Salix acmophylla* and *Robinia pseudoacacia* had IV value ranging from 21.60 to 13.04 (*Table1*).

2. Sarcococca-Justicia-Otostegia community (SJO)

Sarcococca saligna dominated this community uniformly with an IV of 41.29. The other dominant species were *Justicia adhatoda* with an IV of 32.10 and *Otostegia limbata* of 26.68. *Berberis lycium* (20.06 IV) followed by *Daphne mucronata* (18.91 IV), *Dodonaea viscosa* (18.05 IV) and *Buddleja crispa* (17.58 IV) were the other dominant species. Other shrubs within the community were *Indigofera heterantha*, *Sageretia thea*, *Rubus fruticosus, Ziziphus oxyphylla, Cotoneaster microphyllus, Lantana indica, Calotropis procera*, *Ricinus communis, Mallotus philippensis,* and *Vitex negundo* had IV between 17.19 and 8.97 (*Table1*).

3. Cynodon-Conyza-Thymus community (CCT)

The leading species of this community were *Cynodon dactylon* with an IV of 26.51, *Conyza canadensis* with an IV of 24.61, and *Thymus linearis* with an IV of 23.28. *Stachys parviflora* (16.19), *Marrubium vulgare* (15.63), *Cirsium vulgare* (15.61)*, Geranium rotundifolium* (15.28) and *Eragrostis minor* (13.66) were the other co-dominant species in the site (*Table 1*).

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Site V (Mala Said)

The plant communities at this site are established at West aspect at 34.51° N latitude and 71.28° E longitude with an elevation of 1805 m. The soil texture class was composed of 62% silt, 28% clay and 10% sand. The pH of site recorded as 7.3. The calculated macro-element such as nitrogen was 0.044 mg/kg, phosphorus 13.1 mg/kg and potassium 100 mg/kg. The organic matter content was 0.89% and $CacO_3$ 7.50%. The total soluble salts (TSS) were recoded 0.045% and electrical conductivity 0.14 dsm^{-1} (*Table 3*).

1. Quercus-Olea-Ficus community (QOF)

Quercus incana was found to be dominated with IV of 47.25. Other associated dominant species were *Olea ferruginea* having an IV of 32.97 and *Ficus palmata* with an IV 32.79. These were followed by *Juglans regia* (IV 30.4*), Ailanthus altissima* (IV 29.26) and *Morus alba* (IV 22.05). In addition to these *Robinia pseudoacacia*, *Melia azedarach, Acacia modesta, Ziziphus jujuba, Salix acmophylla* had IV value ranging from 21.78 to 20.22 (*Table1*).

2. Otostegia-Berberis-Sageretia community (OBS)

The dominant species in this community were *Otostegia limbata* (IV 36.81), *Berberis lyceum* (IV 29.24) and *Sageretia thea* (IV 25.40). Other co-exiting species in the community were *Justicia adhatoda* (24.20 IV) followed by *Indigofera heterantha* (23.17 IV), *Daphne mucronata* (21.95 IV) and *Buddleja crispa* (20.79 IV). *Dodonaea viscosa*, *Sarcococca saligna, Vitex negundo, Ziziphus oxyphylla, Cotoneaster microphyllus, Lantana indica, Hedera nepalensis,* and *Capparis spinosa* had IV between 20.63 and 7.09 were the other shrubby members of the community (*Table 1*).

3. Apluda-Cynodon-Chrysopogon community (ACC)

This community was dominated by the grass species such as *Apluda mutica* (IV 37.56)*, Cynodon dactylon* (IV 41.40) and *Chrysopogon serrulatus* (IV 26.68 IV). The next associated species were *Conyza canadensis* (20.26), *Stachys parviflora* (16.56), *Teucrium royleanum* (15.13)*, Oxalis corniculata* (14.10) and *Cyperus niveus* (13.89) (*Table 1*).

Index of Similarity (IS)

1. Tree communities

The PQJ community of site I (Batwar) was found to be 6.84% similar to POQ community of Site II (Bagandel) as per Motyka's index of similarity (Motyka et al., 1950). The similarity between Batwar site I community i.e., PQJ and Gabar site III community i.e., OQ was found to be 11.05%. Similarity between the community PQJ and QFJ at Saro Wano, site IV was 10.62%. Similarly, the similarity index between PQJ and QOF communities in Mala Said, site V was 10.38%. Similarity between Bagandel site II, tree community (POQ) and Gabar site III (OQ) was 7.86%. POQ community resembled the tree community QOJ at Saro Wano, site IV by 10.59% and the QOF community at Mala Said, site V by 11.89%. OQ tree community at Gabar site III showed 10.55% similarity with QFJ tree community at Saro Wano site IV, and 11.71% with QOF community at Mala Said site V. QFJ community of Saro Wano site IV shows 12.48% similarity with QOF community at Mala Said site V (*Table 4*).

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DOI: http://dx.doi.org/10.15666/aeer/1905_35793600

Table 4. *Indices of similarity of tree communities*

	PQJ	**POQ**	**OQ**	**QOJ**	**QOF**
PQJ	X	X	X	X	X
POQ	6.84	X	X	X	X
OQ	11.05	7.86	X	X	X
QOJ	8.49	10.59	10.55	X	X
QOF	10.38	11.89	11.71	12.48	X

Key: PQJ= Pinus- Quercus- Juglans, POQ= Pinus- Olea- Quercus, OQ= Olea- Quercus, QFJ= Quercus- Olea- Juglans, QOF= Quercus- Olea- Ficus

2. Shrubby communities

Motyka's index showed minimum similarities among the shrubby communities in the selected sites. The BIB community at site I (Batwar) was 5.79%, similar with the BOS community at site II (Bagandel). At Gabar site III, BIB community was found to be 6.17%, similar to the ROB community. At site IV (Saro Wano), there was a 6.32% similarity between the BIB and SJO communities. At Mala Said site V, the BIB community was 6.54%, similar to OBS community. Bagandel site II, community BOS was 6.11% similar to ROB community at site III (Gabar). The BOS community of Bagandel site II was 7.24% similar to SJO community at Saro Wano site IV. At Mala Said site V, there was a 6.47 % similarity between the BOS and OBS community. The shrub community ROB at Gabar site III was found 7.64% similar to the community SJO at Saro Wano site IV, while the OBS at site V (Mala Said) was found to be 5.94% similar. The shrub community SJO at Saro Wano site IV and the OBS community at Mala Said site V had highest similarity of 8.63% (*Table 5*).

Table 5. *Indices of similarity of shrubby communities*

	BIB	**BOS**	**ROB**	**SJO**	**OBS**
BIB	X	X	X	X	X
BOS	5.97	X	X	X	X
ROB	6.17	6.11	X	X	X
SJO	6.32	7.24	7.64	X	X
OBS	6.54	6.47	5.94	**8.63**	X

Key: BIB= Berberis- Indigofera- Buddleja, BOS= Berberis- Otostegia- Sarcococca, ROB= Rubus- Otostegia- Berberis, SJO= Sarcococca- Justicia- Otostegia, OBS= Otostegia- Berberis- Sageretia

3. Herbaceous communities

RHC community at site I i.e., Batwar was 4.36% identical to HCH community at Bagandel site II among the herbaceous communities. The RHC community was found to be 5.12% similar to CSC community at Gabar site III, and 4.49% similar to the CCT community at Saro Wano site IV. RHC community was 2.97% similar to ACC community at Mala Said site V. The HCH community of Bagendel site II was 5.31% similar to CSC community at Gabar site III, 2.83% to CCT community at Saro Wano site IV and 7.47% to ACC community at Mala Said site V. The CSC herb community of Gabar site III was 3.82% similar with CCT community at Saro Wano site IV and 6.19%

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with ACC community at Mala Said site V. There was 5.46% similarity between the CCT community at Saro Wano site IV and the ACC community at Mala Said site V (*Table 6*).

Table 6. *Indices of similarity of herbaceous communities*

	RHC	HCH	CSC	CCT	ACC
RHC	X	X	X	X	X
HCH	4.36	X	X	X	X
CSC	5.12	5.31	X	X	X
CCT	4.49	2.83	3.82	X	X
ACC	4.97	7.47	6.19	5.46	X

Key: RHC= Rumex- Hyparrhenia- Cynodon, HCH= Hyparrhenia- Cynodon- Heteropogon, CSC= Cynodon- Salvia- Cyperus, CCT= Cynodon- Conyza- Thymus, ACC= Apluda- Cynodon- Chrysopogon

Species diversity (SD)

Simpson' diversity was calculated for the tree communities at site I was 0.992, site II 0.87 site III 0.937, site IV 0.917 and site V 0.911. Similarly, for shrubby community of site I and site II 0.937 each, site III 0.943, site IV 0.919 and site V 0.926. The diversity index for herbaceous communities calculated from 0.906 to 0.961 (*Table 7*). Diversity of species was affected by various abiotic and biotic factors such as elevation, slope, erosion, soil texture, soil structure, climatic condition, grazing, browsing and anthropogenic activities in terms of deforestations. Species diversity is one of the greatest features of plants communities reflecting its structure and composition.

Table 7. *Simpson's diversity indices among the various communities*

S#	Sites	Communities	Simpson's diversity index
		Trees	
1	Site-I	*Pinus-Quercus-Juglans*	0.922
2	Site-II	*Pinus-Olea-Quercus*	0.87
3	Site-III	Olea-Quercus- Quercus	0.937
4	Site-IV	*Quercus-Olea-Juglans*	0.917
5	Site-V	*Quercus-Olea-Ficus*	0.911
		Shrubs	
1	Site-I	*Berberis-Indigofera-Buddleja*	0.937
2	Site-II	*Berberis-Otostegia-Sarcococca*	0.937
3	Site-III	*Rubus-Otostegia-Berberis*	0.943
4	Site-IV	*Sarcococca-Justicia-Otostegia*	0.919
5	Site-V	*Otostegia-Berberis- Sageretia*	0.926
		Herbs	
1	Site-I	*Rumex-Hyparrhenia-Cynodon*	0.957
2	Site-II	*Hyparrhenia-Cynodon-Heteropogon*	0.961
3	Site-III	*Cynodon-Salvia-Cyperus*	0.906
4	Site-IV	*Cynodon-Conyza-Thymus*	0.951
5	Site-V	*Apluda-Cynodon-Chrysopogon*	0.938

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Maturity index (MI)

The maturity index value for the trees, shrubs and herbs communities of the five selected sites ranged from 50.91 to 24.64. The highest maturity indices recorded for tree community at Mala Said site (50.91) and lowest for Bagandel site (36.25). The maturity indices for shrubby communities were highest for Mala Said site (46.0) and lowest for Batwar site (37.89). The herbaceous community at Bagandel site (37.18) had the highest maturity index, while Saro Wano Site (24.64) of the valley had the lowest value (*Table 8*). Value of low maturity indicates the heterogeneity in the plant communities due to its poor adaptation to environmental condition of the area.

Table 8. *Maturity indices of tree, shrubby and herbaceous communities*

S#	Sites	Communities	Maturity Index
		Trees	
1	Site-I	*Pinus-Quercus-Juglans*	48.75
2	Site-II	*Pinus-Olea-Quercus*	36.25
3	Site-III	Olea-Quercus- Quercus	47.14
4	Site-IV	*Quercus-Olea-Juglans*	50.77
5	Site-V	*Quercus-Olea-Ficus*	50.91
		Shrubs	
1	Site-I	*Berberis-Indigofera-Buddleja*	37.89
2	Site-II	*Berberis-Otostegia-Sarcococca*	39.44
3	Site-III	*Rubus-Otostegia-Berberis*	40.55
4	Site-IV	*Sarcococca-Justicia-Otostegia*	40.58
5	Site-V	*Otostegia-Berberis- Sageretia*	46.0
		Herbs	
1	Site-I	*Rumex-Hyparrhenia-Cynodon*	29.27
2	Site-II	*Hyparrhenia-Cynodon-Heteropogon*	37.18
3	Site-III	*Cynodon-Salvia-Cyperus*	28.39
4	Site-IV	*Cynodon-Conyza-Thymus*	24.64
5	Site-V	*Apluda-Cynodon-Chrysopogon*	30.17

Cluster analysis using PAST

Data sets for 110 recorded species across 150 quadrats of five selected sites, classified the plants into four different groups via cluster analysis using PAST software version 4.03 using quantitative values based on the combined IV of each species.

Cluster 1. This group was dominated by tree and shrubby species such as *Quercus incana* with combined IV of 177.84, followed by *Olea ferruginea* with IV of 156.87, *Otostegia limbata* with IV of 142.29, *Berberis lyceum* with IV 134.4. The other associated species were *Cynodon dactylon, Juglans regia, Justicia adhatoda, Sageretia thea, Indigofera heterantha, Apluda mutica, Ailanthus altissima* and *Morus alba* (*Fig. 2*).

Cluster 2. This group was the richest in terms of species diversity dominated by herbs such as *Nepeta cataria* with combined IV 44.11, *Duchesnea indica* 41.96 and *Ajuga bracteosa* 41.83. The other co-dominant associative species were *Stachys parviflora*,

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Cirsium vulgare, *Heteropogon contortus, Pennisetum orientale, Verbascum, Thapsus and Eragrostis minor* (*Fig. 2*). The vegetation of this group was the indicator of moist temperate zone which could survive during the winter season in the form of seeds bank and had the quick regeneration capability during favorable conditions.

Cluster 3. This group had the association of herbaceous and shrubby vegetation dominated by *Rubus fruticosus* with combined IV 80.46 followed by *Rumex hastatus* with IV 50.06 and *Andrachne cordifolia with* IV 43.77. The other co-dominant associated species were *Ziziphus jujuba, Teucrium royleanum, Rubia cordifolia, Ricinus communis, Salvia moorcroftiana, Reinwardtia trigyna, Cannabis sativa* and *Tagetes minuta* (*Fig. 2*).

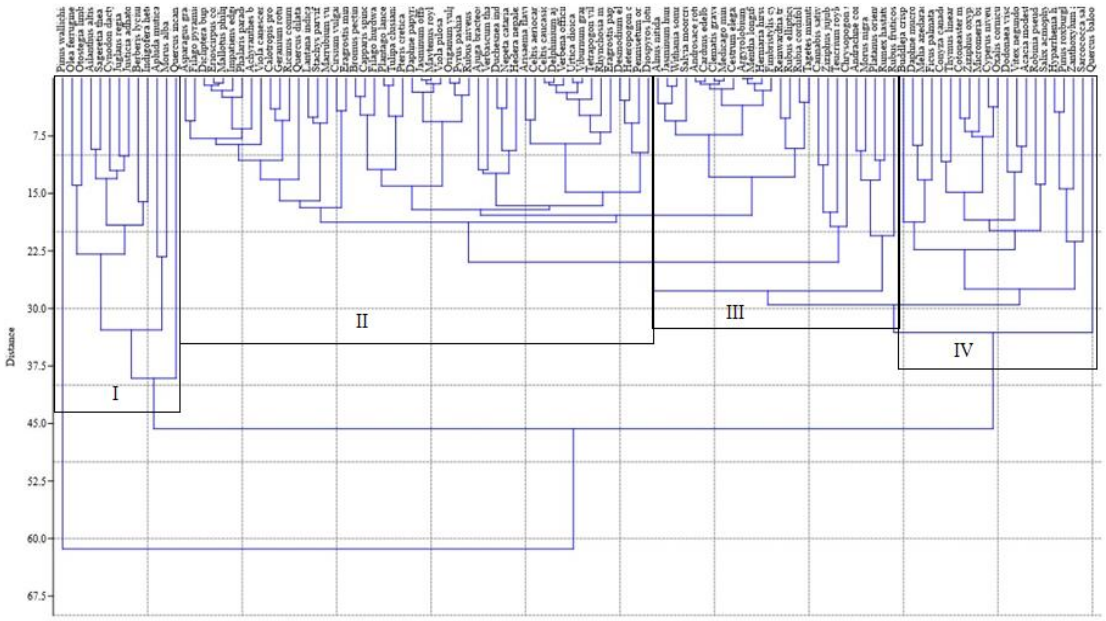

Figure 2. *Cluster dendrogram showing the association on the basis of IV values*

Cluster 4. This group had mixed type of association led by *Sarcococca saligna* with combined IV 106.2, *Ficus palmata* with IV 89.91, *Conyza canadensis* with IV 86.22 and *Oxalis corniculata* with IV 71.14. *Zanthoxylum armatum*, *Acacia modesta*, *Buddleja crispa*, *Micromeria biflora* and *Hyparrhenia hirta* were the other co-dominant allied species (*Fig. 2*).

Principal component analysis using PC-Ord

A PCA biplot was constructed of the plants species under the impact of different sites. In PC-I, species mostly collected from site III, were aggregated in a clumpy manner. In PC-I, *Rubus fruticosa* is located at a maximum distance from the origin of the biplot while *Apluda mutica* is located at the same position in PC -IV, both plants species are strongly negative correlated with each other. On the other hand, *Querqus dilatata*, *Salix acmophylla* and *Viola canescens* are negatively correlated to *Budleja crispa*, *Tulipa clusiana* and *Teucrium stockcianum* placed in PC-II. All plants species aggregated in clumps are strongly correlated along the origin (PC-I and PC-II) as shown in *Figure 3*.

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DOI: http://dx.doi.org/10.15666/aeer/1905_35793600

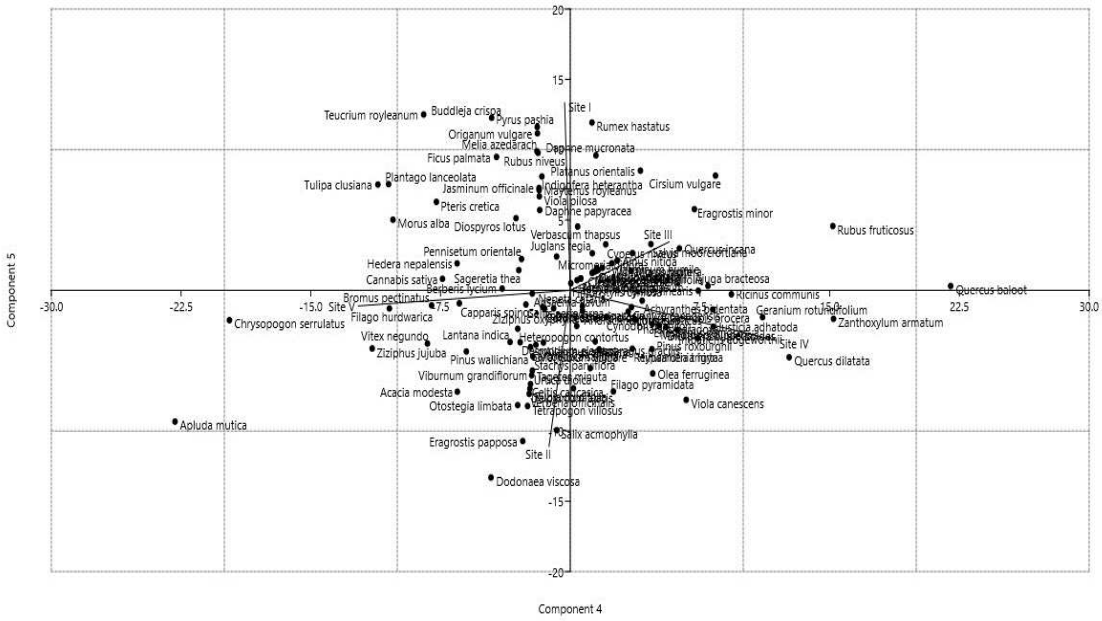

Figure 3. *Principal components analysis (PCA) showing the associations based on IV values*

Discussion

The vegetation structure in the present research was classified into fifteen communities of trees, shrubs and herbs based on importance value (IVs), which is in line with the works of Bokhari et al. (2013), Ali et al. (2015)**,** Ilyas et al. (2015), Siddiqui et al. (2015), Ahmad et al. (2016), Khan et al. (2016), Srivastava et al. (2016), Ali et al. (2018), Hussain et al. (2019), Zaman and Badshah (2020). At Site-I, PQJ-BIB-RHC communities were established for trees, shrubs and herbs. Trees communities in all the monitoring sites of area were under sever anthropogenic stress of deforestation, which had created clumping pattern rather than a uniform. The increase in human population and residential construction work in the area were the main reasons that losses the species in the area. Haq et al. (2015), Ahmad et al. (2016), Khan et al. (2016) and Ali et al. (2018) reported similar communities in their respective study areas. Site-II had POQ-BOS-HCH communities. At the monitoring sites, trees and shrubs communities had erratic pattern. The key deriving force that have an effect on the vegetation structure in both sites I and II are soil erosion. The indicator herbaceous species of eroded soil like *Achyranthes bidentata, Cannabis sativa, Cyperus niveus, Plantago lanceolata, Rumex hastatus, Salvia moorcroftiana* and *Verbascum thapsus* were grow well. Khan et al. (2010), Akhtar and Bergmeier (2015), Ilyas et al. (2015), Ahmad et al. (2016) and Khan et al. (2016) reported similar communities with different IVs. OQ-ROB-CSC communities dominated site III. This site had a thick and scattered community of *Quercus* that was mixed with *Olea. Quercus* community of the area were found disturbed due biotic stress, especially the deforestation. At Site VI, QOJ-SJO-CCT communities were established. Due to deforestation at the North-East aspect, clumpy pitches were found more than the North-West with uniform dispersion. The herbaceous community were under the influence of overgrazing that disturbed the species composition. At Site V, QOF-OBS-ACC communities were documented. The edaphic factors are crucial in determining vegetation patterns since each species in a community has a unique response to them. The soil texture

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of the selected sites was silty to clay loam with high content nutrients and had more water that lead to the development of dense vegetation. The nitrogen concentration in site II, IV and V were found high that improves the growth of herbaceous species. The content of phosphorous was found very low in site III but the pH value is slightly alkaline that may effect on the phosphorus availability to plants. To grow in such condition, the plants may secrete phosphatase enzyme and some organic components to reduce soil pH. This is in line with the findings of Schuur and Matson. (2001), Phillipse, (2003), Wang et al. (2014), Cambrolle et al. (2015) and Zhang et al. (2017). The present study recorded some of endangered, threatened and rear species in the area. The critically endangered species included *Caralluma tuberculata*, *Eremurus himalaicus* and *Nannorrhops ritchiana* while *Arisaema flavum, Bergenia ciliata, Myrtus communis, Solanum surattense*, *Teucrium stocksianum* and *Vitis jacquemontii* were the endangered species. *Ajuga bracteosa*, *Foeniculum vulgare and Jaglans regia* were the vulnerable species. These species were ethnomedicinally used by the local community and hakims for treatment of various diseases. *Nannorrhops ritchiana* which is locally known as 'Mazari' is one of regionally critically endangered species whose leaves were used in making of handicraft such as mats, hand fans, baskets, hats, brooms and trays (Abdullah et al., 2020). Another threatened species is *Juglans regia*, commonly known as 'Ghoz,' whose wood is widely used in furniture production while its bark is utilized as a tooth brush and in traditional cosmetics. The vegetation structure of tree species was found under the anthropogenic activities of local for the extraction wood. The woody species like *Quercus baloot, Quercus incana* and *Olea ferruginea* were found to be decreasing due to its consumption as fuel wood and slow regeneration capacity. The main cause of biodiversity loss in the area is rapid population growth of human which has resulted in the development of roads, buildings and overharvesting of plants.

Conclusion

The results of vegetation structure in 150 sampling units at 5 monitoring sites are presented in this study. The soil analysis covers 9 parameters with different properties at each location. Cluster analysis PAST and PC-Ord software were used which classified the vegetation into four groups based on quantitative values. Variations in edaphic factors, moisture and temperature causes spatial variation in plant communities at the different monitoring sites. The study area vegetation is threatened, and it must be conserved before it is too late. The impact of anthropogenic activities such as over grazing deforms the herbaceous vegetation structure. Increased agriculture practices also affect the overall forest cover. During the harsh winter season, local residents cut the woody plants which not only decreases the species density in the communities but also speed up the soil erosion. The woody and shrubby plants such as *Olea ferruginea*, *Pinus wallichiana, Pinus roxburghii, Quercus baloot, Quercus incana, Berberis lycium, Buddleja crispa*, *Daphne mucronata, Indigofera heterantha*, *Sageretia thea, Viburnum grandiflorum* and *Ziziphus oxyphylla* are under extreme pressure for domestic uses. To combat the effects of deforestation, afforestation projects need to be initiated in the area. The alternative sources for fuel and timber should be provided to protect the vegetation diversity. For regeneration of understory species, a proper grazing management program such as moderate and rotational grazing routine should be ensured. Overpopulation, agricultural practices, developmental work, habitat degradation, deforestation and overgrazing were identified as the main biotic pressures threatening phytodiversity in the study area. As a

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DOI: http://dx.doi.org/10.15666/aeer/1905_35793600

result, it is necessary to protect this phytodiversity for future generations by involving local communities, conservationists, Government and Non-Government Organizations (NGOs). Likely, a comprehensive program must be designed for the measurement of threatened plants diversity and their conservation.

Acknowledgments. This work is a part of the Ph.D. thesis of the first author. The authors acknowledge the assistance of laboratory staff of the Agriculture Research Institute Tarnab Peshawar for physicochemical analysis of soil samples.

Conflict of Interests. The authors declare that they have no competing financial interests.

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DOI: http://dx.doi.org/10.15666/aeer/1905_35793600

THE ECOLOGICAL AMPLITUDE OF GIANT CANE (*ARUNDO DONAX* VAR. *VERSICOLOR*) UNDER DIFFERENT WATER LEVEL GRADIENTS

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(Received 15th Mar 2021; accepted 19th Jul 2021)

Abstract. Water level of wetland, one of the most affected ecological factors by climate change, are dominant forces controlling wetland plant growth and distribution in freshwater ecosystems. To analyze the effect of the water level gradients (-60.74-60 cm) on *Arundo donax* var. *versicolor* seedlings in terms of their growth characteristics (base stems diameter, plant height, and biomass), leaf characteristics (leaf length, width, area, number, and moisture content), photosynthetic pigment (chlorophyll, carotenoids), antioxidant enzymes activity (superoxide dismutase, peroxidase and catalase), soluble protein, malondialdehyde and root activity. We determined and quantified the response relationship between *A. donax* var. *versicolor* and water level based on the Gaussian model. The result showed that underground biomass and Chl a + b were more sensitive indexes to water level. The Chl a + b was more sensitive to the flooded water depth (61.37 cm), but the underground biomass was influenced by the groundwater depth (-59.09 cm). The result also showed that the ecological amplitude of the water level for *A. donax* var. *versicolor* was -59.09 to 61.37 cm, and the optimal range was -13.87 to 10.44 cm. Therefore, we concluded that it could be a suitable species for ecological restoration of the shallow wetland.
Keywords: *Arundo donax var. versicolor, indoor potting method, ecological amplitude, Gaussian Model, response mechanism*

Abbreviations: *A. donax* var. *versicolor*: *Arundo donax* var. *versicolor*; SOD: superoxide dismutase ($U.g^{-1}.min^{-1}$); POD: peroxidase ($U.g^{-1}.min^{-1}$); CAT: catalase ($U.g^{-1}.min^{-1}$); MDA: malondialdehyde ($mg.g^{-1}$); Chl-a: chlorophyll a ($mg.g^{-1}$); Chl-b: chlorophyll b ($mg.g^{-1}$); Chl a + b: total chlorophyll content ($mg.g^{-1}$); Chla/b: chlorophyll a/b; Car: carotenoid ($mg.g^{-1}$)

Introduction

Due to the influence of climate change, severe weather such as drought and heavy precipitation occurs frequently (Hirabayashi et al., 2013), which leads to water level fluctuation in wetlands (Coops et al., 2003). These environmental conditions strongly affect the survival and functioning of wetland plants (Li et al., 2020; Short et al., 2016), and wetland plants have gradually formed their unique survival strategies (Pan et al., 2019) through long-term growth and adaptation to the environment. The adaptation of wetland plants to hydrological processes is not only manifested in the zonal distribution characteristics of water level gradient (Garcia-Baquero et al., 2016; Yin et al., 2010) but also the mechanism of the supply-demand relationship between plant growth and development and water conditions. There is an ecological threshold (Zhao et al., 2007) for the impact of water conditions on wetland plants, namely when water conditions exceed the plant's tolerance range, the plants will grow poorly or even die because they

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DOI: http://dx.doi.org/10.15666/aeer/1905_36013620

cannot properly adjust to water level changes. The change of wetland water level directly affects the composition, diversity, and succession of wetland plant species (Luan et al., 2013). Wetland plants along the water level gradient response research are important to maintain native biodiversity and to design management strategies appropriate for wetland (Magee and Kentula, 2005; Wen et al., 2012). Different water level gradients will affect the soil moisture, air, and affect the physical and chemical properties of soil and the process of vegetation growth environment development, leading to soil environmental change, ultimately affecting the spatial distribution and ecological characteristics of vegetation (Cao et al., 2015).

Arundo donax var. *versicolor* (*A. donax* var. *versicolor*), which is widely introduced and cultivated as a garden foliage plant in China, is a perennial herb in the genus *Arundo* (Xu et al., 2020). Its plant height can reach several meters, the root system is highly developed, the stem is straight and tidy, the leaves are long and narrow with yellow and white stripes, and the color changes with the season. Because of its strong breeding capacity, flooding resistance, high ornamental value, and many other characteristics, it is widely used in artificial wetland, garden landscape, sewage treatment, papermaking, and other fields.

Previous studies on *A. donax* var. *versicolor* mainly focused on garden landscape (Liu et al., 2014), seedling cultivation (Ye and Li, 1994; Zhang et al., 2005), purification of sewage (Xie and Wang, 2009a, b) and photosynthetic characteristics (Yu et al., 2014). In recent years, some studies have explored the effect of drought and rewatering on it (Xu et al., 2020), and its influence on enrichment and migration of heavy metals (Guo et al., 2020; Zhuang et al., 2020). However, studies on water level ecological amplitude of *A. donax* var. *versicolor* have not been conducted, as well as the response of their growth and physiological characteristics to the water level gradient. Our search in terms of the variation of water level to discuss the response of their growth and physiological characteristics to the water level gradient, and explain the adaptive mechanism of the distribution along the water level gradient of *A. donax* var. *versicolor*. Also, we explore the optimal water level ecological amplitude for *A. donax* var. *versicolor*, which could offer a theoretical basis for the application of *A. donax* var. *versicolor* in the restoration of degraded wetland ecosystems.

Materials and methods

Experimental materials

The stems of *A. donax* var. *versicolor* with uniform size and grow well were collected in May 2018 from Aixi Lake National Wetland Park (115.996333°E, 28.695937°N) in Jiangxi Province, China. The stems were cut into 5 cm segments, each segment had a normal side bud, and planted in plastic pots (35 x 26 x 13 cm). The average weight of substrates of each pot was 8 ± 0.5 kg, the stems were evenly buried depth 2 cm of soil, and each pot pre-cultured 9 stems. After 30 days of pre-culture, the side buds of stems grew into seedlings, and those with similar growth status were selected for subsequent experiments. The substrates of the experiment were sandy loam mixing evenly soil with sand (volume ratio of 2:1), which had been naturally air-dried and sieved through a 2 mm sieve. Basic characters of the substrates were analyzed before the experiment (organic matter content: 39 g/kg; total nitrogen (TN): 18 g/kg; pH: 5.4; field water capacity: 30.38%).

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DOI: http://dx.doi.org/10.15666/aeer/1905_36013620

The soil in the substrates is swamp soil collected from Aixi Lake National Wetland Park. The water quality indexes of the test water are as follows (*Table 1*).

Table 1. *Average condition of water quality during experiment. Water quality parameters including total nitrogen (TN), (total phosphorus) TP, ammonia nitrogen (NH_4^+-N), pH and dissolved oxygen (DO)*

Index	Value
TN	1.046 mg/L
TP	0.033 mg/L
NH_4^+-N	0.048 mg/L
pH	6.71
DO	7.18 ml/L

Experimental design

The experiment was conducted in the glass greenhouse of the Key Laboratory of Poyang Lake Wetland and Watershed Research, Ministry of Education, Nanchang, Jiangxi Province, in China. The pre-cultured *A. donax* var. *versicolor* seedlings were transplanted and treated with different water levels. The water level gradient was set up to 16 levels: -60.74 cm, -54.39 cm, -41.67 cm, -28.95 cm, -22.60 cm, -16.24 cm, -9.88 cm, -3.52 cm, 0 cm, 0.29 cm, 10 cm, 20 cm, 30 cm, 40 cm, 50 cm and 60 cm. The double pot method was used for positive and negative value treatment. Plastic pots and basins were put into plastic buckets with a height of 70 cm, upper diameter of 57 cm, and lower diameter of 45 cm for the experiment. Many small holes were drilled in the bottom of each plastic pot, and they were plugged with gauze to make it permeable but block the soil in the pot. The experimental equipment was built shown in *Figure 1*.

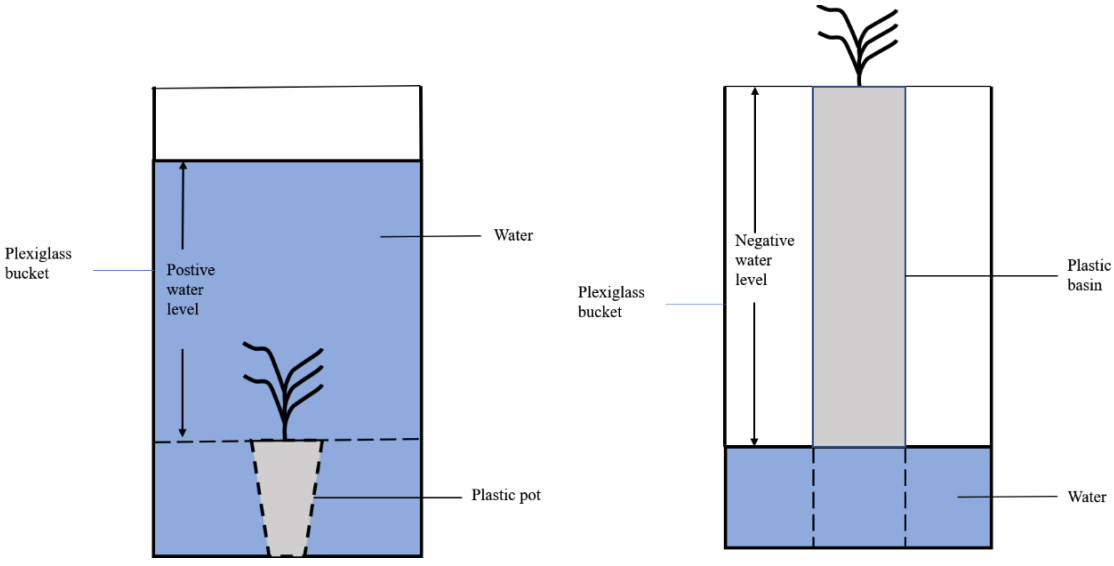

Figure 1. *The simulated experimental equipment of water level. Above treatments all use the soil surface as the interface. When the water level gradient is higher than the soil surface, it is usually called water level (Left), which is a positive value; when it is below the soil surface, it is usually called groundwater depth, which is a negative value (Right)*

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DOI: http://dx.doi.org/10.15666/aeer/1905_36013620

Three replicated treatments (three pots) were set in the experiment, with 9 seedlings in each pot. When measuring indexes, three seedlings were measured in each pot and repeated three times. To avoid the influence of temperature and solar radiation, the temperature and sunshine duration of glass greenhouse is artificially controlled by air conditioning and sunshade. The average temperature was 23.4 ± 5 ℃, and the sunshine duration was 12 h every day in the summer. Water changes in each group were observed every day after the experiment began, and water was added to reach the set value. Because the indoor simulation experiment receives less disturbance, no variation in soil surface was detected during the experiment. Other experiment conditions are the same, so any differences in response among *A. donax* var. *versicolor* between different treatments could be considered as the result of water level changes.

Indexes determine

Growth indexes measure

After the experiment began, plant height, base stems diameter, leaf length, leaf width, leaf number, and leaf area were measured every 7 days (calculated by the coefficient method (Hu, 2015)). Using meter stick measures the plant height. The base stem diameter was measured with vernier caliper and the leaf length and width were measured with a ruler, and *Equation 1* was used to calculate leaf area.

$$S=K\times L\times W \quad \text{(Eq.1)}$$

where: S is the blade area, L is the blade length, W is the blade width and K is the coefficient. The coefficient of this type of blade is 1/3.

After the experiment, we need to carefully dig the seedlings from the experiment pot, flush it with distilled water, then use absorbent paper to absorb surface moisture. Separating plant root, stem, and leaf parts with scissors. Using tape to measure root length, using electronic balance to weigh the fresh weight of root, stem, and leaf. Deactivation of enzymes for root, stem, and leaf at 105 ℃ for 20 min, and drying them at 75 ℃ for 48 h to constant weight, then measuring their dry weight. The biomass, leaf moisture content, and root-cap ratio were obtained by *Equations 2, 3,* and *4*.

$$B_t=B_a+B_u \quad \text{(Eq.2)}$$

$$R=B_u/B_a \quad \text{(Eq.3)}$$

$$M_l=\frac{(M_f-M_d)}{M_f}\times 100\% \quad \text{(Eq.4)}$$

where: B_t is total biomass, B_a is aboveground biomass, B_u is underground biomass, R is root-cap ratio, M_l is leaf moisture content, M_f is fresh leaf mass, M_d is dry leaf mass.

Measurement of physiological indexes

Leaf samples were respectively collected on days 0, 25, 50, 75, and 100 after starting the experiment, and the sampling site was the middle part of the plant. Then determine the content of various photosynthetic pigments, soluble protein, Malondialdehyde (MDA), the activity of Superoxide dismutase (SOD), Peroxidase (POD), and Catalase (CAT). Root

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DOI: http://dx.doi.org/10.15666/aeer/1905_36013620

activity was determined on day 100 after treatment. Repeat 3 parallel measurements for each index. The index determination method is Principles and Techniques of Plant Physiological and Biochemical Experiments prepared by Wang (2006).

The content of photosynthetic pigments, including chlorophyll a (Chl-a), chlorophyll b (Chl-b), chlorophyll a + b content (Chl a + b), chlorophyll a/b content ratio (Chl a/b), and carotenoid (Car), was measured by 95% absolute ethanol extraction. Grind and extract photosynthetic pigments with 95% ethanol, and then filter the extract into a 25 mL brown volumetric flask for constant volume. With 95% alcohol as the control group, the absorbance of chlorophyll a and chlorophyll b were measured at wavelengths of 665 nm and 649 nm, respectively, and the absorbance of carotenoids was measured at 470 nm. The content of soluble protein and MDA were measured by Coomassie brilliant blue-G250 ratio Color method, thiobarbituric acid method, and root activity was determined by the triphenyltetrazolium chloride method (TTC). The activities of SOD, POD, and CAT were measured by nitrogen blue tetrazole method, guaiacol staining method, and ultraviolet absorption method respectively.

SOD can inhibit the reduction of nitrogen blue tetrazole (NBT) under light conditions, the activity of SOD can be measured. The riboflavin can be reduced by light, and the reduced riboflavin is easily oxidized under aerobic conditions to generate superoxide anion free radicals, and then superoxide anion free radicals can transform the NBT. The azole is reduced to blue methylhydrazone, which has a maximum absorption of 560 nm. In addition, superoxide dismutase has a scavenging effect on superoxide anion free radicals, thereby inhibiting the formation of methylhydrazone. Therefore, after the reaction is completed, the enzyme activity can be judged by the color of the reagent to calculate the enzyme activity. It is known that inhibition of 50% photochemical reduction of NBT as an enzyme activity unit, SOD is calculated according to *Equation 5*.

$$S = \frac{(A_{CK} - A_E) * V}{A_{CK} * W * V_t * 0.5} \quad (Eq.5)$$

where: S is the activity of SOD (U/g), A_{CK} is absorbance of control group, A_E is the absorbance of the sample tube, V is the total volume of sample solution (mL); V_t is the sample dosage (mL) at the time of determination, W is the fresh weight of the sample (g).

Peroxidase (POD) has high activity in plants and is closely related to photosynthesis and respiration. Under the action of POD, hydrogen peroxide reacts with guaiacol to make the enzyme solution dark brown. The dark brown product has a higher absorbance at 470 nm, so in this study, the absorbance change at 470 nm wavelength was measured. The POD activity is calculated by *Equation 6*.

$$P = (\Delta A_{470} * V_T) / (W * V_S * T * 0.01) \quad (Eq.6)$$

where: P is the activity of POD (U/(g.min)$^{-1}$), ΔA_{470} is changes in absorbance during the reaction time, V_T is the total volume of sample solution (mL), W is the fresh weight of the sample (g), V_S is the sample dosage at the time of determination (mL), T is reaction time (min).

Hydrogen peroxide (H_2O_2) was absorbed strongly at a wavelength of 240 nm, catalase (CAT) can decompose hydrogen peroxide, so that the absorbance of the reaction solution decreases with the reaction time. The catalase activity can be measured according to the rate of change in absorbance. The CAT activity is calculated by *Equations 7* and *8*.

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$$C=\frac{\Delta A_{240}*V_T}{0.1*V_1*W*T} \quad (Eq.7)$$

$$\Delta A_{240}=A_{S0}-\frac{A_{S1}+A_{S2}}{2} \quad (Eq.8)$$

where: A_{S0} is the absorbance of the control group, A_{S1} and A_{S2} are the absorbance of the sample group, V_T is the total volume of the enzyme solution extract, V_1 is the volume of the enzyme solution for determination, T is the determination time, W is the fresh weight of the sample.

Calculation of water level ecological amplitude

The Gaussian Model (Zhang, 2011) was adopted to calculate the water level ecological amplitude. Generally, the relationship between plant species and the environment conforms to the Gaussian model. Namely, a certain environmental factor has a beneficial effect on plant growth, but after threshold, it will inhibit plant growth and development. *Equation 9* was used to calculate ecological amplitude.

$$y=cexp[-0.5(x-u)^2/t^2] \quad (Eq.9)$$

where: y represents an index of biological characteristics of species; c is the maximum of y; x is the value of the environmental factor, u is the optimum ecological amplitude of plants; and t is species tolerance. Generally, ecological amplitude of plants were [u-2t,u + 2t], and optimum ecological amplitude of plants were [u-t,u + t].

Statistical analysis

Analyzing the data of growth and physiological indexes under different stress treatments by Microsoft-Excel 2010 software. Origin 2019b software was used to draw charts and fit the data to obtain the fitting function relationship of the growth and physiological indexes of *A. donax* var. *versicolor*.

Results

Effects of different water levels on morphology characteristics, leaf characteristics, and biomass of A. donax var. versicolor

The morphological characteristics of *A. donax* var. *versicolor* varied nonlinearly with the water level. The plant height of roughly showed a rising trend with the increase of water level. The base stems diameter and length of the root system firstly increased and then decreased with the increasing water level. The plant height reached its peak of 107.18 cm at a water level of 50 cm and then decreased. The base stems diameter reached a maximum value of 5.52 mm at the water level of -9.88 cm. The root length reached the maximum at the water level of -16.24 cm, which was 83.06 cm. Fitting the water level with the plant height, basal stem, and root system length by quadratic curve fitting. From the fitting effect, only the base stem diameter conforms to the Gaussian model and is normally distributed, $R^2 = 0.8108$ (*Fig. 2*).

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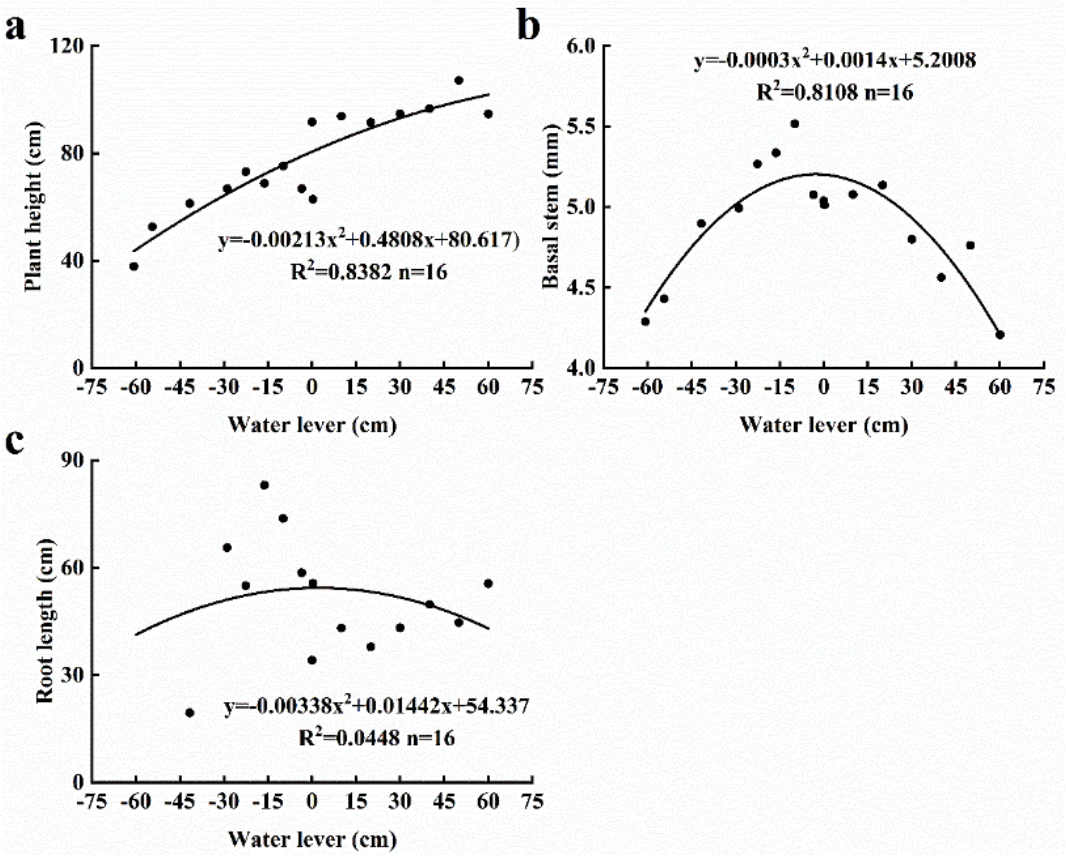

Figure 2. *The A. donax var. versicolor growth characteristics vary with the different water level gradient*

Effects of different water levels on A. donax var. versicolor leaf characteristics

The variation of leaf morphological characteristics showed a nonlinear trend under different water levels. The length, width, number, and area of leaves of *A. donax* var. *versicolor* firstly increased and then decreased with the increase of water level. The leaf moisture content increased as the water level increased. The leaf length reached its maximum of 33.8 cm at the water level of -22.60 cm and decreased from the peak. At the water level of -9.88 cm, the blade width has a significant peak of 1.89 cm. At the water level of 50 cm, the leaf number reached the maximum, which was 14.2. The leaf area reached an obvious peak value of 20.04 cm^2 at a water level of 0 cm and decreased at both ends of this water level. The leaf moisture content roughly showed a rising trend with the increase of water level and reached its maximum of 77.41% at the water level of 40 cm. The quadratic curve was performed for water level and leaf morphological characteristics. From the perspective of fitting effect, blade length ($R^2 = 0.7181$), blade width ($R^2 = 0.715$), leaf number ($R^2 = 0.8282$), and leaf area ($R^2 = 0.7401$) were in line with the Gaussian model and normally distributed (*Fig. 3*).

Effects of different water levels on A. donax var. versicolor biomass

The biomass of *A. donax* var. *versicolor* varied nonlinearly with water level changes. The aboveground biomass, underground biomass, total biomass, and root-cap ratio increased first and then decreased with the increasing water level. The aboveground

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biomass reached a peak of 5.50 g.plant^{-1} at the water level of 50 cm and then decreased. The underground biomass at a water level of 0 cm reached the maximum value, which was 4.57 g.plant^{-1}. The total biomass at the water level of -9.88 cm reached the highest value of 9.19 g.plant^{-1}. The quadratic curve fitting was performed for water level and variety of biomass. For fitting effect, aboveground biomass (R^2 = 0.7185), underground biomass (R^2 = 0.7836), and total biomass (R^2 = 0.8439) were in line with the Gaussian model and normally distributed, the fitting curve of root-cap ratio has a low fitting degree so that does not accord with the normal distribution (*Fig. 4*).

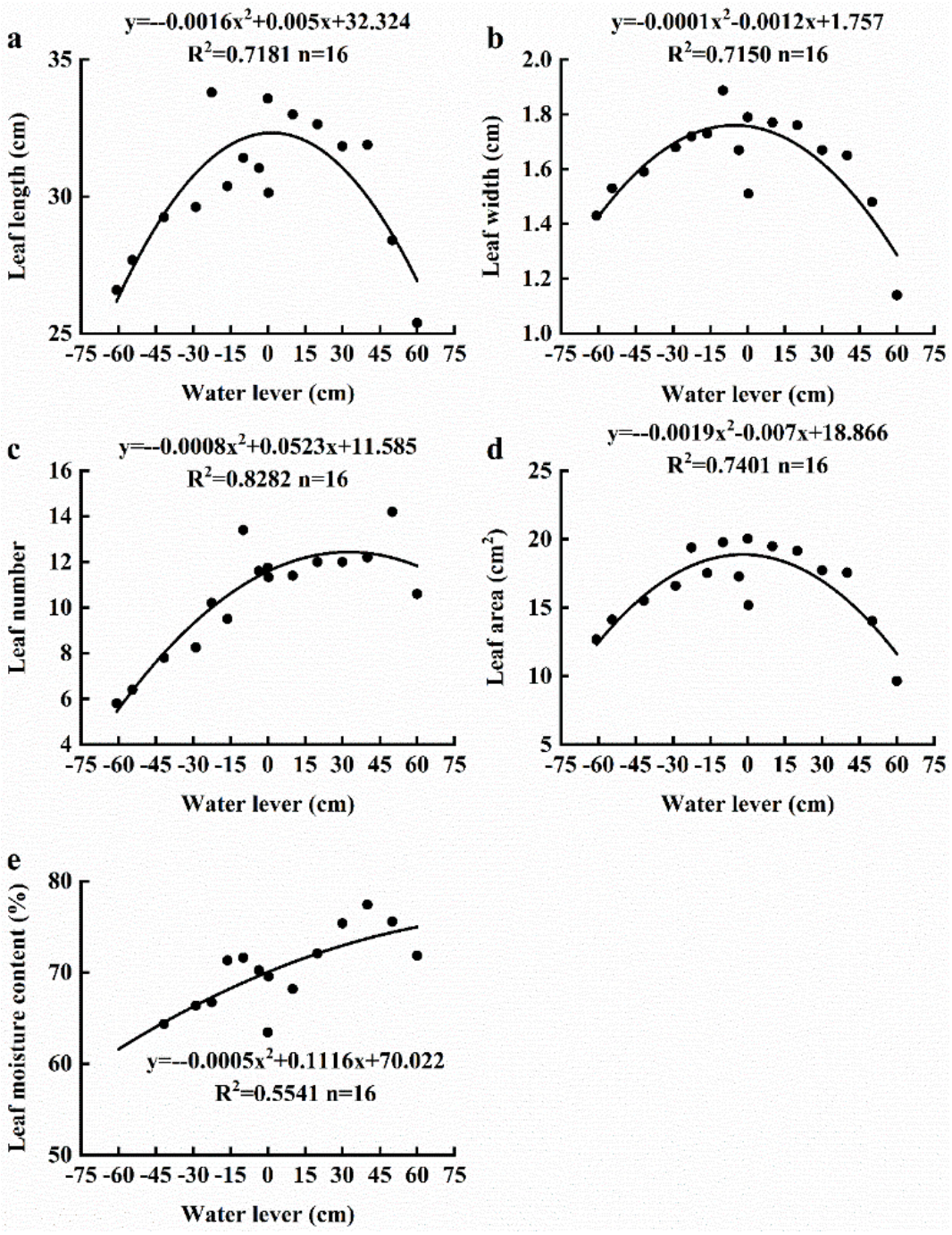

Figure 3. *The A. donax var. versicolor leaf characteristics vary with the different water level gradient*

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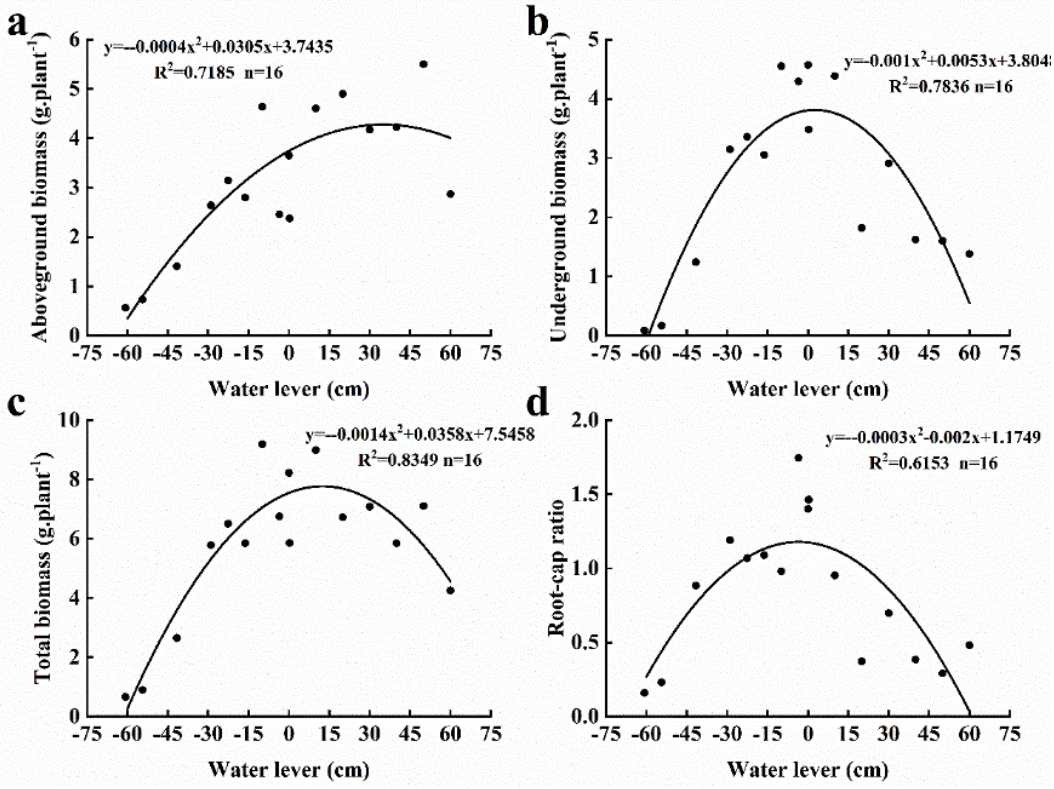

Figure 4. *The A. donax var. versicolor biomass varies with the different water level gradient*

Effects of different water levels on A. donax var. versicolor photosynthetic pigment

The contents of Chl-a, Chl-b, and Chl a + b in *A. donax* var. *versicolor* first increased and then decreased with the increasing water level. The Chl a/b roughly increased with the increasing water level, and the contents of Car generally decreased with the increase of the water level. The content of chlorophyll reached its peak at the water level of -3.52 cm, which was 1.07 $mg.g^{-1}$, and decreased after the maximum value. The content of Chl-b reached a maximum value of 0.40 $mg.g^{-1}$ at the water level of -16.24 cm. The content of Chl a + b reaches its maximum value of 1.44 $mg.g^{-1}$ at the water level of -3.52 cm and has an obvious peak. The water level and photosynthetic pigment content were fitted with a Quadratic curve. From the fitting effect, Chl a/b and Car were not normally distributed. Chl-a (R^2 = 0.7558) and Chl a + b (R^2 = 0.7455) are in line with the Gaussian model and normally distributed (*Fig. 5*).

Effects of different water levels on A. donax var. versicolor antioxidant enzymes activity

The antioxidant enzyme activity of *A. donax* var. *versicolor* varied nonlinearly with the increasing water level. The activity of SOD, POD, and CAT showed a roughly decreasing trend first and then an increasing trend with the increasing water level. The activity of SOD reached its lowest value of 122.86 U. $(g.min)^{-1}$ when the water level is 0.29 cm. The activity of POD has a minimum value of 44.93 U. $(g.min)^{-1}$ when the water level is 0 cm. The activity of CAT has an obvious bottom, which is 1.89 U. $(g.min)^{-1}$ at 10 cm. The water level was fitted with SOD, POD, and CAT by quadratic curve fitting. From the fitting effect, the fitting degree of antioxidant enzyme activity was low and did

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not conform to the normal distribution, so the fitting effect was not good that failing to meet the conditions of the Gaussian equation (*Fig. 6*).

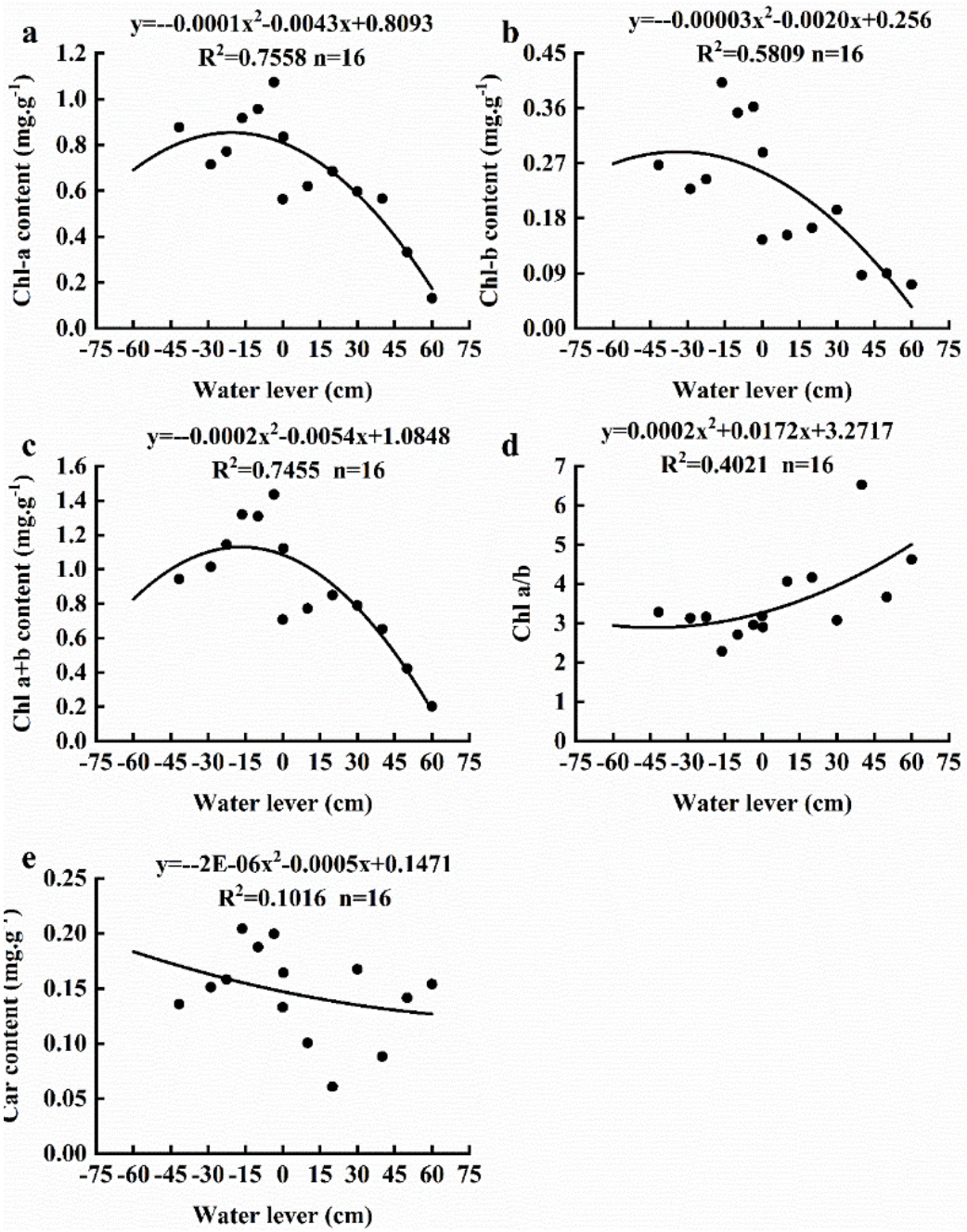

Figure 5. *The A. donax var. versicolor photosynthetic pigments vary with the different water level gradient*

Effects of different water levels on soluble protein and MDA of A. donax var. versicolor

The soluble protein, MDA, and root activity of *A. donax* var. *versicolor* varied nonlinearly with the water level. The soluble protein and MDA decreased first and then increased with the increasing water level, and the root activity roughly increased with the

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water level. When the water level was 0 cm, the content of soluble protein reached the lowest value of 2.17 $mg.g^{-1}$. The content of MDA has an obvious minimum value, which is 9.23 $mg.g^{-1}$ at 0.29 cm water level. And the root activity increased with the water level raised then reached its maximum value of 0.075 mg. $(g.h)^{-1}$ when the water level reached 50 cm. The water level was fitted with soluble protein, MDA, and root activity by a quadratic curve. From the fitting effect, all indexes did not conform to normal distribution and the fitting effect was poor so that failing to meet the conditions of the Gaussian equation (*Fig. 7*).

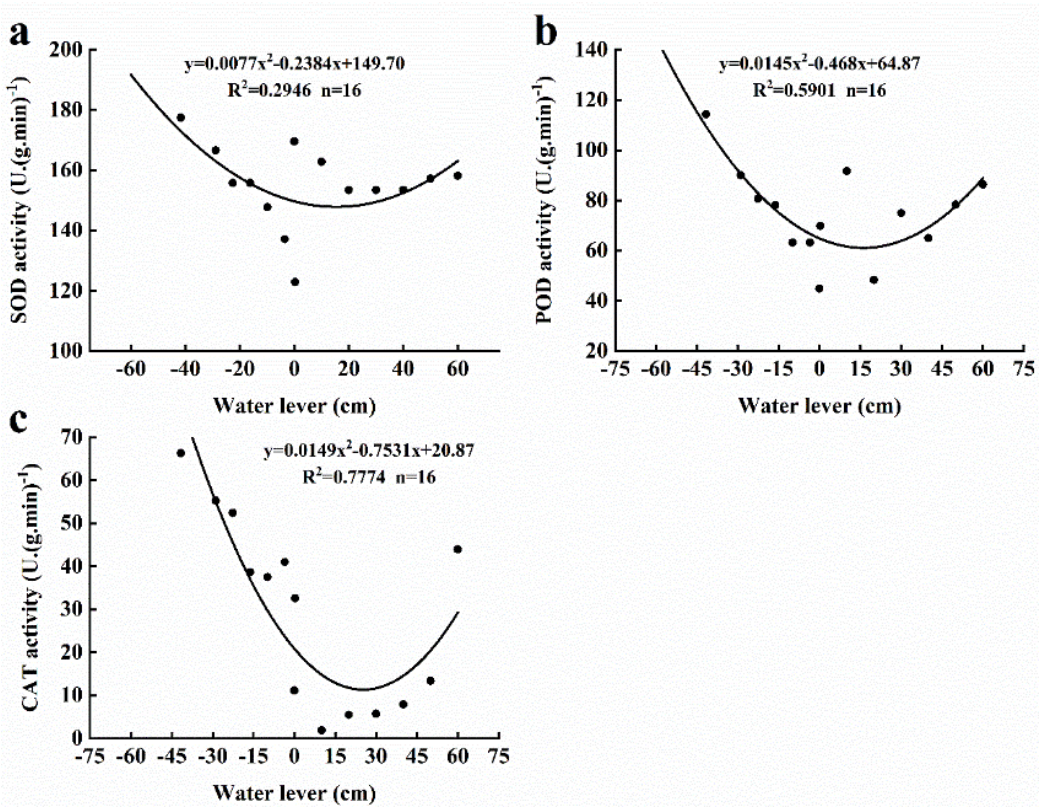

Figure 6. *The A. donax var. versicolor antioxidant enzyme activity varies with the different water level gradient*

The water level ecological amplitude of A. donax var. versicolor

According to the changes in the growth and physiological characteristics of *A. donax* var. *versicolor* under different water level gradients, fitting the water level with base stem diameter, leaf length, leaf width, leaf number, leaf area, aboveground biomass, underground biomass, total biomass, Chl-a, and Chl a + b by a quadratic curve. The fitting equation formula used was $y = ax^2 + bx + c$ (where x represents the water level, cm) (*Table 2*). In terms of the transformation relationship between the fitted quadratic curve and the Gaussian model, the Gaussian regression equation of each index can be acquired (*Table 3*).

According to the fitted Gaussian equation and by using mathematical analysis methods, the optimal water level interval and the limit water level interval of each index can be obtained. Taking the intersection of optimal water level interval and limit water level interval of each index, namely, the lowest value takes the maximum value of the lower

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limit of each interval, and the highest value takes the minimum value of the upper limit of each interval. The obtained intersection is the water level ecological amplitude of *A. donax* var. *versicolor*, and optimal water level ecological amplitude is about [-13.87, 10.44] cm, limit water level ecological amplitude is [-59.09, 61.37] cm (*Table 4*). The result indicates that *A. donax* var. *versicolor* could be grown well when the water level between -13.87 cm to 10.44 cm. However, provide that water levels of more than 61.37 cm or during a time of less than 59.09 cm, The plant cannot maintain normal growth and reproduction. In the process of the experiment, the content of Chl a + b roughly is 0 at the water level of 60 cm, the underground biomass close to 0 at the water level of -60.74 cm, which is very close to the limit water level of *A. donax* var. *versicolor*. The results showed that underground biomass and Chl a + b were more sensitive indexes to water level changes during the growth and development of *A. donax* var. *versicolor*. The Chl a + b was more sensitive to the flooded water depth (61.37 cm), but the underground biomass was influenced by the groundwater depth (-59.09 cm).

Table 2. *The fitting equations between water level and base stem diameter, leaf length, leaf width, leaf number, leaf area, aboveground biomass, underground biomass, total biomass, Chlorophyll a (Chl-a), total chlorophyll (Chl a + b)*

Index	Fitting equation	R^2
Base stems diameter (mm)	$y = -0.0003x^2 + 0.0014x + 5.2008$	0.8108
Leaf length (cm)	$y = -0.0016x^2 + 0.005x + 32.324$	0.7181
Leaf width (cm)	$y = -0.0001x^2-0.0012x + 1.757$	0.7150
Leaf number (cm)	$y = -0.0008x^2 + 0.0523x + 11.585$	0.8282
Leaf area (cm^2)	$y = -0.0019x^2-0.007x + 18.866$	0.7401
Aboveground biomass ($g \cdot plant^{-1}$)	$y = -0.0004x^2 + 0.0305x + 3.7435$	0.7185
Underground biomass ($g \cdot plant^{-1}$)	$y = -0.001x^2 + 0.0053x + 3.8048$	0.7836
Total biomass ($g \cdot plant^{-1}$)	$y = -0.0014x^2 + 0.0358x + 7.5458$	0.8349
Chl-a ($mg \cdot g^{-1}$)	$y = -0.0001x^2-0.0043x + 0.8093$	0.7558
Chl a + b ($mg \cdot g^{-1}$)	$y = -0.0002x^2-0.0054x + 1.0848$	0.7455

Table 3. *Gaussian regression equations of growth and physiological indexes of A. donax var. versicolor. Indexes including base stem diameter, leaf length, leaf width, leaf number, leaf area, aboveground biomass, underground biomass, total biomass, Chlorophyll a (Chl-a), total chlorophyll (Chl a + b)*

Index	Gaussian regression equation
Base stems diameter (mm)	$y = 5.2024\exp[-0.5 (x + 2.3334)^2/65.84352]$
Leaf length (cm)	$y = 32.3279\exp[-0.5 (x-1.5625)^2/71.07212]$
Leaf width (cm)	$y = 1.7551\exp[-0.5 (x-1.5625)^2/71.07212]$
Leaf number (cm)	$y = 12.4398\exp[-0.5 (x-32.6875)^2/62.3493]$
Leaf area (cm^2)	$y = 18.8725\exp[-0.5 (x + 1.8421)^2/49.83192]$
Aboveground biomass ($g \cdot plant^{-1}$)	$y = 4.3249\exp[-0.5 (x-38.125)^2/51.9912]$
Underground biomass ($g \cdot plant^{-1}$)	$y = 3.8118\exp[-0.5 (x-2.65)^2/30.872]$
Total biomass ($g \cdot plant^{-1}$)	$y = 7.7773\exp[-0.5 (x-12.7858)^2/37.26662]$
Chl-a ($mg \cdot g^{-1}$)	$y = 0.8555\exp[-0.5 (x + 21.5)^2/46.24732]$
Chl a + b ($mg \cdot g^{-1}$)	$y = 1.1213\exp[-0.5 (x + 13.5)^2/23.93752]$

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Table 4. *The water level ecological amplitude of A. donax var. versicolor calculated by Gaussian Model*

Index	c	u	t	[u-t,u + t]	[u-2t,u + 2t]
Base stems diameter (mm)	5.20	-2.33	65.84	[-68.18, 63.51]	[-134.02, 129.35]
Leaf length (cm)	32.33	1.56	71.07	[-69.51, 72.63]	[-140.58, 143.71]
Leaf width (cm)	1.76	1.56	71.07	[-69.51, 72.63]	[-140.58, 143.71]
Leaf number (cm)	12.44	32.69	62.35	[-29.66, 95.04]	[-92.01, 157.39]
Leaf area (cm^2)	18.87	-1.84	49.83	[-51.67, 47.99]	[-101.51, 97.82]
Aboveground biomass ($g \cdot plant^{-1}$)	4.32	38.13	51.99	[-13.87, 90.12]	[-65.86, 142.12]
Underground biomass ($g \cdot plant^{-1}$)	3.81	2.65	30.87	[-28.22, 33.52]	[-59.09, 64.39]
Total biomass ($g \cdot plant^{-1}$)	7.78	12.79	37.27	[-24.48, 50.05]	[-61.75, 87.32]
Chl-a ($mg \cdot g^{-1}$)	0.8555	-21.5	46.25	[-67.75, 24.75]	[-113.99, 70.99]
Chl a + b ($mg \cdot g^{-1}$)	1.12	-13.5	23.94	[-37.44, 10.44]	[-88.37, 61.37]
Calculation results (cm)				[-13.87, 10.44]	[-59.09, 61.37]

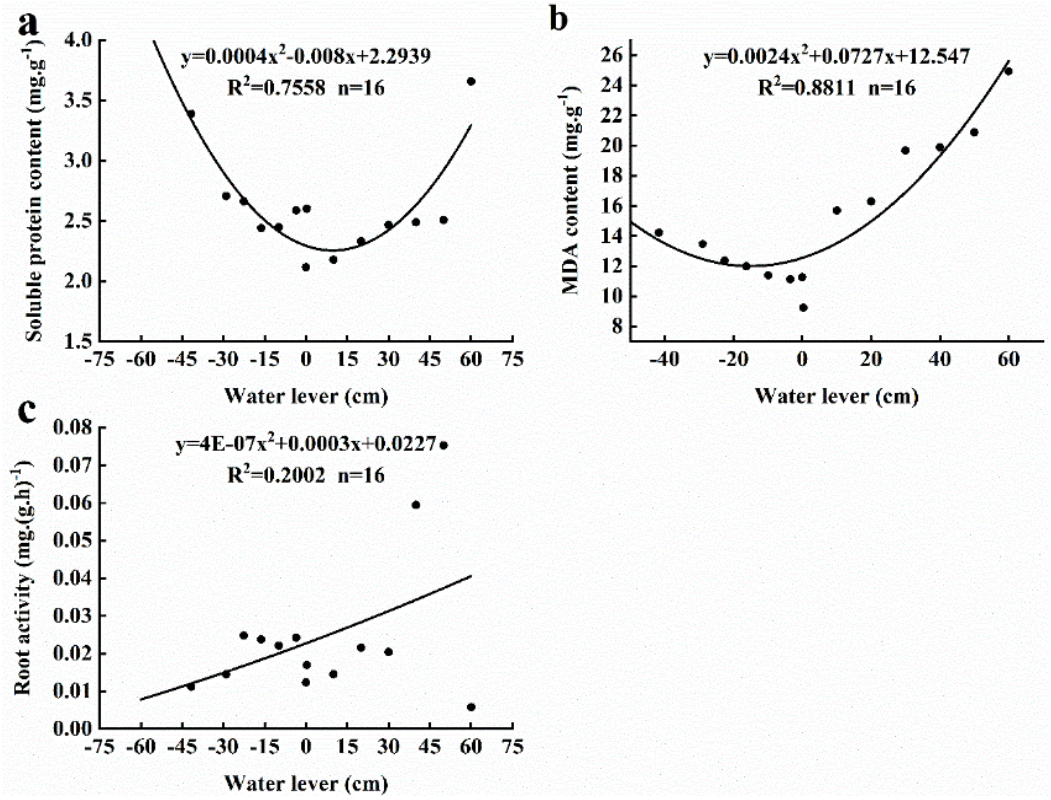

Figure 7. *The A. donax var. versicolor soluble protein, MDA, and root activity vary with the different water level gradient*

Discussion

Responses of morphology to water level changes

The research results showed that base stems diameter, root length, leaf length, leaf width, leaf number, leaf area, aboveground biomass, underground biomass, total biomass, and root-cap ratio all increased first and then decreased with the increasing water level,

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and reached their maximum value at the water level of -22.60 to 0 cm. But the plant height, root length, leaf moisture content generally show a trend of increasing with the rise of water level. Due to its special hollow structure, *A. donax* var. *versicolor* can adapt to environmental pressures more effectively (Okada et al., 2009). Drought restricts plant metabolism leads to poor growth (Cruz et al., 2019; Voronin et al., 2019), therefore, when the water level increases to optimum water level, the morphological indexes of plants increase accordingly. Feng has compared the two groundwater levels of *Carex cinerascens*, 10 cm, and 20 cm, and found that 10 cm groundwater level was overall more suitable for plant growth resulting in higher stem height and biomass (Feng et al., 2020). Under flooded anaerobic conditions, emergent plants will adjust their survival strategies, such as developing shallow roots, reducing underground biomass, and increasing aboveground biomass to accelerate the growth of plant height. The root-cap ratio increases with the decrease of underground biomass and the increase of above-ground biomass. In addition to adjustment of biomass allocation, the plant height and basal stem also changed, and the plants become more slender. The change of plant morphology helps it alleviate the anaerobic condition by absorbing more oxygen transport to roots. The plant leaves also respond significantly to drought and flooding stress, which are mainly manifested as leaf shedding, wilting, and reduced area. The reasonable explanation is that under stress conditions, the nutrients in the leaves are consumed for respiration and cannot be effectively supplemented, leading to the decrease of the leaves' biomass. It is conducted that appropriate drought was conducive to the growth and biomass accumulation of *A. donax* var. *versicolor*. However, all morphological characteristics decreased with the decrease or increase of water level. It indicates that in the environment of excessive drought and flooding, *A. donax* var. *versicolor* would reduce individuals to adapt to adversity, which is similar to the conclusion of the study of *Carex cinerascens* (Yang et al., 2015).

Responses of chlorophyll, antioxidant enzyme activity to water level changes

Our studies indicate that the contents of Chl-a and Chl a + b increased first then decreased with the increasing water level and reached their maximum at the water level of -3.52 cm. The photosynthetic pigment is the most important material component of plant photosynthesis. Our result shows that severe drought and flooding will inhibit the synthesis of photosynthetic pigment and reduce the chlorophyll content of plants, which is consistent with some previous research (Chen et al., 2010; Parolin et al., 2010). Flooding is known to affect photosynthesis through changes in stomatal conductance, many wetland species initially close stomata in response to soil flooding (Li et al., 2010). The reasonable explanation for the decrease in chlorophyll content under long-term flooded conditions is that due to oxygen deficiency, this anaerobic condition will inhibit the synthesis of photosynthetic pigments in wetland plants, and ultimately lead to premature leaf senescence (Zhang et al., 2020). In addition to hypoxia, some scholars believe that lack of nitrogen leads to plant leaf death (Li et al., 2006). Along with the inhibition of plants growth, the suppression of photosynthesis is also a typical response to drought stress (Chaves et al., 2007; Voronin et al., 2019). In normal circumstances, the transpiration of plants is relatively strong, but plants under drought stress have to close their stomata to reduce water loss. Decreased stomatal conductance will affect a series of physiological effects, including the synthesis of photosynthetic pigments. Therefore, tomata closure may be the main factor resulting in the reduction of photosynthetic pigments under drought and flooding.

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To resist the damage caused by high levels of active oxygen caused by drought, plants have evolved enzymatic and non-enzymatic active oxygen scavenging systems to maintain the optimal active oxygen balance required for cell metabolism (Gill and Tuteja, 2010; Hou et al., 2021). The key enzymes include SOD, CAT, and POD. SOD (Alscher et al., 2002) is the first line of defense against active oxygen damage in the plant's antioxidant system, converting superoxide-free radicals into H_2O_2. CAT can remove H_2O_2, which converts it into oxygen and water through a catalytic reaction (Willekens et al., 1997). POD can assist in removing H_2O_2 produced by the SOD disproportionation reaction (Reddy et al., 2004). Our study result indicates that antioxidant enzyme activity decreases first and then increases with the increasing water level, the lowest value reached when the water level was roughly -9.88 - 0 cm. In this condition, the *A. donax* var. *versicolor* can maintain normal growth and development and produces less active oxygen. However, in severe drought and deeper submergence water depth, active oxygen of plants produces excessively (Arbona et al., 2008; Tahkokorpi et al., 2007), so that activates the antioxidant enzyme system in the *A. donax* var. *versicolor*. Then antioxidant enzyme activity will increase and eliminate the damage that active oxygen for the plants (Li et al., 2013b; Rangani et al., 2018). We found that SOD, POD, and CAT all show an upward trend under stress consistent with the antioxidant enzyme study results about *Carex duriuscula*. Their study result indicates that antioxidant enzymatic activity as components of the drought tolerance mechanism in *Carex duriuscula* (Hou et al., 2021).

Responses of MDA, soluble protein, and root activity to water level changes

Flooding stress causes a low light environment, restricts gas diffusion, increases lipid peroxidation of leaf cell membranes, damages the protective enzyme system in the body, degrades chlorophyll, accumulates malondialdehyde content, and decreases photosynthetic rate (Pan and Xue, 2012). MDA is the final product of membrane lipid peroxidation. It is usually used as a lipid peroxidation indicator to indicate the degree of cell membrane lipid peroxidation and the strength of the plant's response to adversity stress (Li et al., 2013a). In our study, when the water level is too low or too high, the MDA content is at a relatively high level and its lowest value at 0 cm water level. The response of MDA concentration of *Phragmites communis* seedling to flooding has been studied, and it increases with flooding and flooded duration (Bai et al., 2012), which is similar to us. The soluble protein as a kind of osmotic adjustment substance can be actively accumulated to protect the cell membrane and reduce peroxide damage to plants when under stronger stress (Guan et al., 2015). The soluble protein content reached the minimum value at a water level of 0 cm, and the content of soluble protein decreased first and then increased with the increase of water level. It suggests that the stress degree for *A. donax* var. *versicolor* is relatively small when the water level at 0 cm, but the cell membrane was damaged under flooding and drought stress. During a severe drought and flood degree, the soluble protein content of *A. donax* var. *versicolor* was high. The results showed that soluble protein played an important role in reducing peroxidation damage. The root activity roughly shows an upward trend with higher water levels. It shows that the root activity is inhabited under drought conditions. The response of soluble protein and root activity to drought stress was similar to other herbaceous plants, in distance, the study of the responses of typical annual herbaceous plants to drought stress (Xi et al., 2021).

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The water level ecological amplitude of A. donax var. versicolor

The results indicated that the water level ecological amplitude of *A. donax* var. *versicolor* was [-59.09 cm, 61.37 cm], the optimum water level was [-13.87 cm, 10.44 cm]. Namely, it cannot normally grow when the water level is below 59.09 cm or higher than 61.37 cm. For the *A. donax* var. *versicolor*, low water level is the most suitable water level. The result also shows that the plant has poor waterlogging resistance and stronger drought resistance. It is consistent with *A. donax* var. *versicolor* waterlogging resistance studies done by Xu Jie (Xu and Tu, 2014). There are also many studies on other helophyte plants. For example, the optimal ecological water level of *Carex lasiocarpa* was found to be 13.45 to 29.78 cm (Luan et al., 2013). The most suitable ecological amplitude of water level of *Calamagrostis angustifolia*, *Carex lasiocaropa* and *C. pseudocuraica* were: [4.46 cm, 20.04 cm], [8.30 cm, 28.40 cm] and [40.87 cm, 48.71 cm] (Xue et al., 2020). The *Phragmites australis* as a common species for studying physiological response models to global change has been found that the survival, physiology, and growth of *Phragmites australis* are less affected by submersion than many other wetland plants. That is, it has a wider ecological amplitude of water level.

By comparing their ecological amplitude, we found that *A. donax* var. *versicolor* is more drought-resistant than *Carex lasiocarpa*, but its ability to resist waterlogging is worse. The result can provide some reference for the cultivation and propagation of *A. donax* var. *versicolor* in wetland parks in the future.

Conclusions

This research through the indoor pot method and setting different water level gradients, observing and studying the growth and physiological indexes of the *A. donax* var. *versicolor* under different water level conditions, and exploring its water level ecological amplitude. The results showed that underground biomass and Chl a + b were more sensitive indexes to water level changes during the growth and development of *A. donax* var. *versicolor*. The Chl a + b was more sensitive to the flooded water depth (61.37 cm), but the underground biomass was influenced by the groundwater depth (-59.09 cm).

Calculated by the Gaussian model, the water level ecological amplitude of *A. donax* var. *versicolor* was [-59.09,61.37] cm, and the optimal water level was [-13.87,10.44] cm. The results suggest that when the water level was lower than about -59.09 cm or higher than 61.37 cm, the seedlings could not grow normally, and the most suitable area for *A. donax* var. *versicolor* was the shallow water. Therefore, the plant could be a suitable species for wetland ecological restoration and garden landscaping.

The study obtained the water level ecological amplitude of *A. donax* var. *versicolor* based on different static water levels. However, some seasonal lakes and wetlands (e.g. Poyang Lake in China) water level fluctuates greatly (Wang et al., 2013), the short-time water level variety has a huge impact on the wetland plants. Therefore, future research on the water level of *A. donax* var. *versicolor* should focus on quantitative its tolerance to water level fluctuations, it is the key to further proving *A. donax* var. *versicolor* can successfully be used for wetland ecological restoration.

Acknowledgments. This research was supported by the National Natural Scientific Foundation of China (42061021), and the Natural Scientific Foundation of Jiangxi Province (20181BAB203021).

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DOI: http://dx.doi.org/10.15666/aeer/1905_36013620

Conflicts of competing interests. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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DOI: http://dx.doi.org/10.15666/aeer/1905_36013620

SUSTAINABILITY OF *CITRUS RETICULATA* L. BLANCO. AND *MANGIFERA INDICA* L. IS RELATED TO ANTIOXIDATIVE DEFENSE UNDER SPATIAL AND TEMPORAL FLUCTUATIONS OF SOIL AND IRRIGATION WATER

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(Received 18th Mar 2021; accepted 29th Jul 2021)

Abstract. Spatial and temporal variability for the quality of surface and ground irrigation water and its impact on soil characteristics were assessed for local farms of *Citrus reticulata* L. Blanco. (Citrus) and *Mangifera indica* L. (Mango). Electrical Conductivity (EC), Potential Hydrogen (pH), Sodium (Na^+), Calcium magnesium (Ca^{+2} + Mg^{+2}), Chromium (Cr), Lead (Pb), Nickel (Ni), Zinc (Zn), Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD) were determined. Ground water at an aquifer of (30-40 cm) (0.57 and 0.88 mg L^{-1}), and two depths of soil profiles (0.97 and 2.29 mg kg^{-1}) had more Cr and Pb, respectively. Biochemical characteristics of the species varied spatially and temporally except the chlorophyll content. Lipid peroxidation via MDA production became evident. An enhanced activity of enzymatic Superoxide dismutase (SOD), Catalase (CAT), Peroxidase (POD) and non-enzymatic components (Ascorbic acid, Carotenoids, Phenols, Sugars) was noticed but the higher resilience in mango can be attributed to non-enzymatic defense. The bioaccumulation of Cr and Pb declined markedly ($P \leq 0.001$) in species from roots to aerial tissues. Citrus pulp contained a significant ($P \leq 0.001$) amount of Cr, and Pb. The study suggested strict environmental monitoring for biodiversity and conservation. However, metal accumulation in the edible portion of citrus seemed to be of great concern for food safety.
Keywords: *water and soil pollution, spatial and temporal variability, citrus and mango, heavy metals, food security and safety*

Introduction

Agriculture serves as the backbone of the economy for many countries of the world as it contributes considerably to their GDP (Leoveanu-Soare et al., 2020). The practice of irrigation has been a central feature of agriculture for over 5,000 years. Like many other countries, Pakistan is also an agricultural country where most of the population relies on arable land for their livelihood. The cultivated area covers 22.68 million hectares out of which 25 percent is rainfed (Chaudhry, 2017).

Multan (30^0 11' 44" North, 71^0 28' 31" East) is an ancient city located in Southern Punjab, Pakistan. It is situated on the main trade route at the convergence of two main rivers; Chenab and Ravi. The area holds a traditional significance for agriculture despite arid climate with an annual rainfall 180 mm with summer and winter temperatures 45°C and 4°C, respectively. Owing to high temperature and rate of evapotranspiration during summer months (May to October), irrigation other than precipitation becomes crucial. Thus, canal (surface) tube well (ground) water is used for irrigation, the latter being ascent through mechanical pumps. Several industries have been set up in the city that include fertilizer, vegetable oil / ghee, textile and tanneries. The effluent or discharge from these industries loaded with several contaminants containing heavy metals is directly thrown into canals or water courses without any treatment. Moreover, land fill

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_36213641

by industrial and domestic wastes in peri-urban and rural areas not only contaminate soil but also ground water (Nai et al., 2021).

Citrus reticulata L. Blanco. (Citrus) and *Mangifera indica* L. (Mango) are the prime fruits of this region thus generate major revenue for the country through export and as such contributes towards socio-economic growth of the region (Randhawa et al., 2014). The production of citrus and mango in the world ranks 10^{th} and 6^{th} with 2.3 and 1.0 million-tons yield, respectively, during 2016 (GOP, 2016). The country earned US$ 21 million from the export of mango alone (Naz et al., 2014). Main mango producing areas in Pakistan are Punjab and Sindh with 1.2 and 0.5 million tons yield, correspondingly (Rehman et al., 2015) while citrus is mostly grown in Multan, Sahiwal and Sargodha regions.

C. reticulata Blano. belongs to the family Rutaceae and is commonly known as "Kinnow" in Pakistan. The prominent aroma and taste make it popular commodity having essential dietary requirements. About 100 ml of Kinnow juice contains 20-25 mg of Vitamin C (Noor, 2015). *M. indica* L. has tropical distribution (Muniraja et al., 2019) and is a member of Anacardiaceae known as "the king of fruits". Popular edible portion has special quality attributes such as nutrition, aroma, sweetness, taste and brix with therapeutic significance.

During the past two decades, rapid industrialization and urbanization resulted in significant pollution of water, air and agricultural soil. Consequently, a considerable decline in the production of these fruits has been reported (Gupta et al., 2020) due to several types of contaminants including both essential and non-essential elements. Although essential elements play a pivotal role for growth of plant species but non-essential trace elements like Chromium (Cr), Cadmium (Cd), Mercury (Hg), and Lead (Pb) are phytotoxic. Owing to their non-biodegradable nature they can persist ubiquitously in the environment (Sabir et al., 2015). These metal elements tend to accumulate in several plant organs that poses serious threat to production and human health consequent upon their consumption (Hu et al., 2017). Agricultural soils when irrigated with waste water get adversely contaminated with such elements. Hence, plants grown on such soils can bioaccumulate a substantial amount of these metals in their different tissues. The soils, plants and the products made thereof have been shown to be highly contaminated therefore is of immense public concern as far as food safety is concerned (Dotaniya et al., 2017). The accumulation of undesirable contents in edible plants parts also deteriorate aroma, taste and sometimes completely spoil the fruits thus result in substantial economic losses.

The productivity also declines because several vital processes associated with growth and development are considerably affected by the presence of metal contaminants in environment (Mahfooz et al., 2020). These contaminants are of hazardous nature as they induce the production of Reactive Oxygen Species (ROS) leading to oxidative damages to several major macromolecules and cellular entities in plants (Ibrahim et al., 2015). The extent of such damage is assessed via quantity of Malondialdehyde (MDA) owing to an affirmative relationship between its amount and oxidative stress (Tirani and Hoghjou, 2019).

Nevertheless, some plant species possess innate capabilities to withstand several environmental constraints (Boquete et al., 2021). In order to cope with oxidative stress, some plausible mechanisms have been evolved in plants. For scavenging of ROS, enzymatic; Super oxide dismutase (SOD), Catalase (CAT) and Peroxidase (POD) (Amjad et al., 2020) and non-enzymatic antioxidants; ascorbic acid, carotenoids, and

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DOI: http://dx.doi.org/10.15666/aeer/1905_36213641

phenolics and soluble sugars (Medveckiene et al., 2020) serve as ubiquitous and common defense in plants.

Keeping in view the above situation, the present study was aimed to reveal relationship between spatial and temporal variation in the quality of irrigation water, soil contamination and bioaccumulation of metal content in the selected fruit species to address food security and safety. The study will serve as a model for citrus and mango growing regions of the world with similar constraints. The foremost objective of the study was to reveal possible underlying biochemical and antioxidative system in these two species as well as to determine metal contents in the edible fruit parts to ensure food safety as per standards already reported (WHO, 2012).

Material and Methods

Study area

Site 1 (Aziz Farm) and site 2 (Nasim Farm) were selected for both fruits from Multan, Punjab, Pakistan depicted in *Figures 1* and *2,* respectively. The choice of these sites was based on a wide range of fluctuating features such as cold winters with ground frost having temperature around 4°C, hot summers and aridity with an average rainfall up to 186 mm during monsoon. Global Positioning System (GPS) was used to record the location of each sampling site as given under.

Several standard procedures were carried out for the analyses of water, soil and plant material and a summary of protocols followed are appended in *Table 1*.

Figure 1. *Location of site1 (Aziz Farm) (30°17'16.6"N, 71°28'25.0"E)*

Figure 2. *Location of site2 (Nasim Farm) (30°18'00.9"N, 71°39'01.7"E)*

Water and soil sampling

Irrigation water samples were collected from their sources (canal and tube well) While soil samples were collected at the depths of 0-10 cm and 10-20 cm at both study sites during summer (May to October) and winter (November to March) in 2017 by using standard sampling procedures by (ICARDA Manual, Estefan et al., 2013). The water samples were collected in clean food grade plastic bottles and soil was collected in polyethylene bags, labeled appropriately then were carried back to the Environment and Evolutionary Laboratories (EEL), Institute of Pure and Applied Biology, Bahauddin Zakariya University, Multan for different physico-chemical and metal analysis.

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DOI: http://dx.doi.org/10.15666/aeer/1905_36213641

Table 1. *Summary analysis of water, soil and plant samples with respective methodology*

Parameters	Methodology/protocols followed
pH	Richards (1954)
EC	Richards (1954)
BOD	Wei (2002)
COD	Jirka and Carter (1975)
Na^{+}, Ca^{+2} + Mg^{+2}	Richards (1954)
Cr, Ni, Pb, Zn	Arora et al. (2008)
Chlorophyll (*a* & b)	Lichtenthaler (1987)
MDA	Cakmak and Horst (1991)
Enzymatic antioxidants	
Superoxide dismutase (SOD)	Giannopolitis and Ries (1977)
Catalase (CAT)	Chance and Maehly (1995)
Peroxidase (POD)	Chance and Maehly (1995)
Non-enzymatic antioxidants	
Ascorbic acid	Keller and Schwager (1977)
Total Carotenoids	Arnon (1949)
Total phenolic contents	Ainsworth and Gillespie (2007)
Soluble Sugars	Prud'homme et al. (1992)

Plant tissues collection

Roots, shoots, leaves and fruits from citrus and mango trees were taken from three fully grown trees during their respective fruiting seasons during June for mango and December, 2017 for citrus from selected sites by employing standard protocols of biochemical analysis. Three fully grown leaves were randomly chosen across the canopy of each tree starting from the base to top.

Preparation of soil and plant assays

For soil digestion, Aqua Regia method (Chen and Ma, 2001) of digestion by using hot plate was used. While the plant tissue samples were digested by employing acid digestion mixture method (Moore and Chapman, 1986).

Pulp extraction and metal analysis

The citrus and mango fruit samples from each orchard were weighed and pulp was separated by passing through a pulper. The obtained pulp was weighed and polyethylene bags were used for packing, labelled and kept in refrigerator at 4^{0}C for 72-96 hours.

Statistical analysis

Each value in the data was across three replications that was used to calculate mean and standard error (±S. E). For biochemical analyses of the plant samples, each tree was considered as an individual and three leaves were taken from each tree. Thus, a single sample is comprised of 9 leaves and each procedure was repeated three times. The data for species, sites, seasons and water sources were then subjected to a Two-Way Analysis of Variance accordingly using MS Excel 2019 and STATISTIX v8.1 in order to determine significant difference between factors separately for each parameter. Least Significant Difference (LSD) between means was calculated by applying Tukey's test (Tukey, 1949) using 5% level of probability.

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DOI: http://dx.doi.org/10.15666/aeer/1905_36213641

Results

Water and soil quality parameters

Canal water samples had the highest pH during summer in comparison to ground water which exhibited higher value for pH during winter at site 1. However, the reverse was true for site 2 with more pH of canal water during winter (*Table 2*). ANOVA for pH of irrigation water showed significant ($P \leq 0.05$) contrast for pH regarding irrigation water sources at both sites (*Table 3*). pH for soil (0-10 cm) depth presented in *Table 4* clearly indicated high pH of the citrus rhizosphere during summer at both sites. The findings were comparable for pH at 10-20 cm soil depth at site 2 (*Table 5*). Statistical analysis (*Table 3*) revealed highly significant ($P \leq 0.001$) contrast for pH regarding seasons and species at both depths of both sites.

Table 2. *Mean values (± S.E) for physico-chemical and heavy metal contents of irrigation water samples collected for surface (canal) and ground water (tube well) during summer (May to October) and winter (November to March), at Site 1 (Aziz Farm) and Site 2 (Nasim Farm), Multan, Punjab, Pakistan*

Parameters	Site 1				Site 2			
	Summer		Winter		Summer		Winter	
	Surface water	Ground water	Surface water	Ground water	Surface water	Ground water	Surface water	Ground water
pH	8.40 ±0.15	6.73 ±0.09	8.09 ±0.03	7.48 ±0.41	8.40 ±0.15	6.73 ±0.09	8.09 ±0.03	7.48 ±0.41
EC (dS m^{-1})	0.46 ±0.00	0.70 ±0.06	0.45 ±0.02	0.66 ±0.02	0.46 ±0.00	0.70 ±0.06	0.45 ±0.02	0.66 ±0.02
BOD (mg l^{-1})	75.03 ±0.06	76.42 ±0.22	80.33 ±0.26	81.58 ±0.22	75.03 ±0.06	76.42 ±0.22	80.33 ±0.26	81.58 ±0.22
COD (mg l^{-1})	179.42 ±0.34	181.10 ±0.24	180.84 ±0.48	181.84 ±0.24	179.42 ±0.34	181.10 ±0.24	180.84 ±0.48	181.84 ±0.24
Na^{+} (mg l^{-1})	31.97 ±1.44	34.96 ±7.37	32.43 ±2.76	28.75 ±3.51	31.97 ±1.44	34.96 ±7.37	32.43 ±2.76	28.75 ±3.51
Ca^{+2} + Mg^{+2} (meq l^{-1})	4.06 ±0.10	5.95 ±0.82	4.42 ±0.16	6.12 ±0.13	4.06 ±0.10	5.95 ±0.82	4.42 ±0.16	6.12 ±0.13
Cr (mg l^{-1})	0.35 ±0.06	0.35 ±0.05	0.35 ±0.01	0.33 ±0.01	0.35± 0.06	0.35 ±0.05	0.35 ±0.01	0.33 ±0.01
Ni (mg l^{-1})	0.22 ±0.03	0.45 ±0,03	0.21 ±0.01	0.44 ±0.01	0.22± 0.03	0.45 ±0,03	0.21 ±0.01	0.44 ±0.01
Pb (mg l^{-1})	0.44 ±0.07	0.56 ±0.03	0.42 ±0,03	0.55 ±0.01	0.44± 0.07	0.56 ±0.03	0.42 ±0,03	0.55 ±0.01
Zn (mg l^{-1})	0.82 ±0.12	1.28 ±0.07	0.72 ±0.05	1.17 ±0.01	0.82± 0.12	1.28 ±0.07	0.72 ±0.05	1.17 ±0.01

Each mean is across n number of replications (n=3)

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DOI: http://dx.doi.org/10.15666/aeer/1905_36213641

Table 3. *Summary of Analysis of Variance for water, soil and plant tissues for various attributes*

Water Parameters	Water							
	Site1		Site 2		Site1		Site 2	
	Sources		Seasons		Sources		Seasons	
	MS	F	MS	F	MS	F	MS	F
pH	3.876	*	0.140	ns	3.172	*	0.969	*
EC	0.001	***	0.151	ns	0.006	***	0.052	ns
Na^{+}	0.357	ns	24.796	ns	4.977	ns	224.36	***
Cr	0.0003	ns	0.0003	ns	0.180	***	0.00008	ns
Ni	0.158	ns	3.00	***	0.024	*	0.00036	***
Pb	0.046	***	0.00068	ns	0.039	***	0.001	ns
Zn	0.621	***	0.033	ns	0.187	***	0.002	ns
BOD	5.187	***	82.111	***	2.210	***	16.170	***
COD	4.272	***	3.674	***	4.826	***	0.018	ns
Ca^{+2}+Mg^{+2}	9.666	ns	0.210	**	1.026	***	1.098	***

Soil Parameters	Soil															
	(Depth 0-10 cm)								(Depth 10-20 cm)							
	Site 1				Site 2				Site 1				Site 2			
	Seasons		Species		Seasons		Species		Seasons		Species		Seasons		Species	
	MS	F	MS	F	MS	F	MS	F	MS	F	MS	F	MS	F	MS	F
pH	0.054	***	0.010	***	0.345	***	0.107	***	0.180	***	0.072	***	0.700	***	0.353	***
EC	0.662	ns	11.642	***	0.456	***	2.707	***	0.297	ns	13.041	***	0.307	***	0.007	ns
Na^{+}	9.95	ns	7897.8	***	94.3	***	13263	***	34.5	ns	20758.4	***	3.32	ns	9366.6	***
Cr	0.014	***	0.036	***	0.007	***	0.019	***	0.016	***	0.092	***	0.010	***	0.024	***
Ni	0.012	***	0.006	*	0.01	ns	0.05	ns	0.027	ns	0.033	*	0.187	**	0.0003	ns
Pb	0.019	***	0.187	***	0.014	***	0.030	***	0.004	ns	0,780	***	0.010	ns	0.269	***
Zn	0.030	**	0.004	***	0.010	***	0.030	**	0.019	*	0.187	**	0.014	*	0.030	*

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DOI: http://dx.doi.org/10.15666/aeer/1905_36213641

Metals	Plant tissues															
	Roots				Shoots				Leaves				Pulp			
	Sites		Species		Sites		Species		Sites		Species		Sites		Species	
	MS	F	MS	F	MS	F	MS	F	MS	F	MS	F	MS	F	MS	F
Cr	182.4	***	632.94	***	1237.87	*	8220.44	***	0.423	ns	267.3	***	1.36	ns	2483.3	**
Ni	266.5	*	178.89	**	425.39	**	2654.83	*	49.23	ns	440.3	**	0.023	ns	520.60	*
Pb	382.2	*	1509.32	*	18.9	*	13794.5	*	182.25	***	342.25	**	4.69	ns	2320.03	*
Zn	26.52	**	42.25	***	13.14	*	12.78	**	3.361	ns	14.69	ns	38.5	ns	61091.8	***

MS= mean square, F= F value, ***, **, * = Significant at 0.001, 0.01 and 0.05 % level of probability, ns = non-significant by ANOVA, double factor with replication

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_36213641

Table 4. *Mean values (± S. E) for physico - chemical and heavy metal contents of soil samples collected at depth (0-10 cm) from rhizospheres of two fruit species (C. reticulata and M. indica) irrigated with two different water sources during summer (May to October) and winter (November to March) at Site 1 (Aziz Farm) and Site 2 (Nasim Farm), Multan, Punjab, Pakistan*

Parameters	Site 1 (Soil depth 0-10 cm)			
	Summer		Winter	
	C. reticulata	*M. indica*	*C. reticulata*	*M. indica*
pH	8.26±0.01	8.25±0.02	8.17±0.01	8.06±0.03
EC (dS m^{-1})	3.51±0.06	2.17±0,01	4.61±1.02	2.01±0.06
Na^+ (mg l^{-1})	182.89±13.30	128.80±0.27	181.93±0.35	133.40±0.53
Cr (mg l^{-1})	0.55±0.01	0.66±0.00	0.62±0.00	0.73±0.00
Ni (mg l^{-1})	0.82±0.01	0.86±0.01	0.88±0.01	0.93±0.001
Pb (mg l^{-1})	2.04±0.15	1.28±0.05	2.11±0.01	1.35±0.00
Zn (mg l^{-1})	0.61±0.01	0.55±0.01	0.69±0.00	0.67±0.01
Site 2 (Soil depth 0-10 cm)				
pH	8.40±0.03	8.25±0.02	8.10±0.06	7.87±0.01
EC (dS m^{-1})	2.60±0,03	3.46±0.03	2.12±0.06	3.16±0.01
Na^+ (mg l^{-1})	130.11±0.90	57.39±1.62	129.49±0.27	69.23±0.74
Cr (mg l^{-1})	0.64±0.00	0.71±0.01	0.68±0.00	0.77±0.01
Ni (mg l^{-1})	1.12±0.12	1.25±0.01	1.18±0.00	1.31±0.00
Pb (mg l^{-1})	1.65±0.06	1.41±0.02	1.69±0.00	1.42±0.00
Zn (mg l^{-1})	0.63±0.00	0.72±0.00	0.68±0.00	0.79±0.01

Each mean is across n number of replications (n=3)

Table 5. *Mean values (± S. E) for physico - chemical and heavy metal contents of soil samples collected at depth (10-20 cm) from rhizospheres of two fruit species (C. reticulata and M. indica) irrigated with two different water sources during summer (May to October) and winter (November to March) at Site 1 (Aziz Farm) and Site 2 (Nasim Farm), Multan, Punjab, Pakistan*

Parameters	Site 1 (Soil depth 0-10 cm)			
	Summer		Winter	
	C. reticulata	*M. indica*	*C. reticulata*	*M. indica*
pH	8.60±0.03	8.51±0.02	8.42±0.00	8.20±0.01
EC (dS m^{-1})	3.58±0.06	2.02±0.02	4.42±0.99	1.81±0.01
Na^+ (mg l^{-1})	202.01±18.87	119.82±0.27	206.54±0.96	122.36±0.48
Cr (mg l^{-1})	0.72±0.00	0.91±0.02	0.81±0.00	0.97±0.01
Ni (mg l^{-1})	1.01±0.06	1.12±0.06	1.11±0.00	1.21±0.00
Pb (mg l^{-1})	2.22±0.08	1.82±0.04	2.29±0.00	1.67±0.00
Zn (mg l^{-1})	0.78±0.01	0.51±0.01	0.84±0.00	0.61±0.01
Site 2 (Soil depth 0-10 cm)				
pH	8.60±0.03	8.40±0.02	8.20±0.03	7.80±0.09
EC (dS m^{-1})	2.35±0.01	2.29±0.04	2.02±0.03	1.98±0.04
Na^+ (mg l^{-1})	118.99±9.00	60.73±2.01	117.66±0.62	64.17±0.35
Cr (mg l^{-1})	0.51±0.01	0.42±0.00	0.57±0.00	0.48±0.00
Ni (mg l^{-1})	1.13±0.11	1.13±0.01	1.39±0.00	1.37±0.00
Pb (mg l^{-1})	1.72±0.11	1.42±0.03	1.78±0.01	1.48±0.00
Zn (mg l^{-1})	0.64±0.00	0.74±0.01	0.71±0.01	0.81±0.01

Each mean is across n number of replications (n=3)

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Table 2 revealed a temporal variation for EC in ground water of the two sites. Analysis of Variance (*Table 3*) depicted a highly significant ($P \leq 0.001$) variability for EC regarding irrigation sources but no temporal contrast at both sites. Electrical conductivity (EC) was greater in citrus root zone during winter at both soil depths of the two sites (*Tables 4 and 5*). Analysis of variance (*Table 3*) clearly indicated that soil rhizosphere of the species was highly significant ($P \leq 0.001$) for EC at both depths at site 1 but at site 2, soil depths possessed a significant ($P \leq 0.001$) temporal disparity for EC.

A higher level of cations; sodium (Na^+) and calcium, magnesium ($Ca^{+2} + Mg^{+2}$) was recorded in tube well water during summer and winter, respectively at site 1but the most elevated values for both cations were measured in canal water during summer at site 2 as presented in *Table 2*. Analysis of Variance (*Table 3*) depicted highly significant ($P \leq 0.001$) contrast for Na^+ regarding seasons at site 2. Statistical analysis also showed marked differences ($P \leq 0.01$) for $Ca^{+2} + Mg^{+2}$ regarding seasons at site 1 but at site 2, ANOVA revealed highly significant ($P \leq 0.001$) disparity for $Ca^{+2} + Mg^{+2}$ regarding sources and seasons. An elevated level of Na^+ was noticed at soil depth (0-10 cm) for citrus rhizosphere during summer at site 1 (*Table 4*) but more Na^+ was found at 10-20 cm of soil during winter (*Table 5*). The statistical analysis (*Table 3*) for Na^+ depicted significant ($P \leq 0.001$) contrast for rhizosphere from two soil depths at both sites.

An elevated demand for biological and chemical oxygen was measured in ground water during winter at site 1 and 2, respectively (*Table 2*). Analysis of Variance (*Table 3*) for BOD and COD showed significant ($P \leq 0.001$) temporal variability as well as disparity between irrigation sources at site 1 but at site 2, water sources were significantly ($P \leq 0.001$) variable for BOD and COD.

Heavy metals (Cr, Ni, Pb, Zn) in irrigation water showed (*Table 2*) higher concentration in ground water during summer except Zn with elevated levels during winter. Overall site 2 exhibited more metal contents. Statistical analysis (*Table 3*) revealed significant ($P \leq 0.001$) variability for Cr regarding water sources. Whereas, ANOVA for Ni depicted significant ($P \leq 0.001$) temporal variability at both sites. Analysis of Variance also depicted that the irrigation sources were highly significant ($P \leq 0.001$) for Pb and Zn. A greater concentration of chromium (Cr) was recorded in mango root zone at soil depth 0-10 cm during winter at site 1 (*Table 4*) but more Cr was detected in deeper soil layers (*Table 5*). Analysis of variance (*Table 3*) for Cr revealed highly significant ($P \leq 0.001$) spatial and temporal variability. A much higher level of nickel (Ni) was found at soil depth 0-10 cm for citrus rhizosphere during winter at site 1 (*Table 4*). At site 2, citrus rhizosphere at 10-20 cm soil depth had an elevated concentration of Ni during winter (*Table 5*). ANOVA (*Table 3*) revealed a significant ($P \leq 0.05$) contrast for Ni regarding rhizospheres of fruit species from both soil depths at site 1. Whereas, at site 2 (10-20 cm soil depth), ANOVA depicted significant ($P \leq 0.001$) temporal variability for Ni. Greater concentrations of lead (Pb) were recorded in citrus root zones at both soil layers at site 1 during winter (*Tables 4 and 5*). Statistical analysis (*Table 3*) revealed significant ($P \leq 0.001$) spatially and temporally variable Pb content. Mean values for Zn presented in *Table 4*, depicted its higher level in citrus rhizosphere at 0-10 cm depth during winter at site 1. On contrary, more Zn was found in mango root zone at 10-20 cm depth at site 2 (*Table 5*). Analysis of Variance (*Table 3*) revealed a significant ($P \leq 0.001$) disparity for Zn for species rhizosphere, seasons and soil depths.

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Biochemical attributes of plant species

Chlorophyll '*a*' (*Figure 3)* exhibited the highest levels in citrus foliage at site 1 in contrast to mango leaves which had higher value for this pigment at site 2. *Figure 3* also depicted a distinct difference for chlorophyll '*b*' in the leaves of two species at site 1. It was also observed that chlorophyll '*b*' was distinctively higher in citrus leaves. Yet at site 2, no marked contrast was observed for chlorophyll '*b*'.

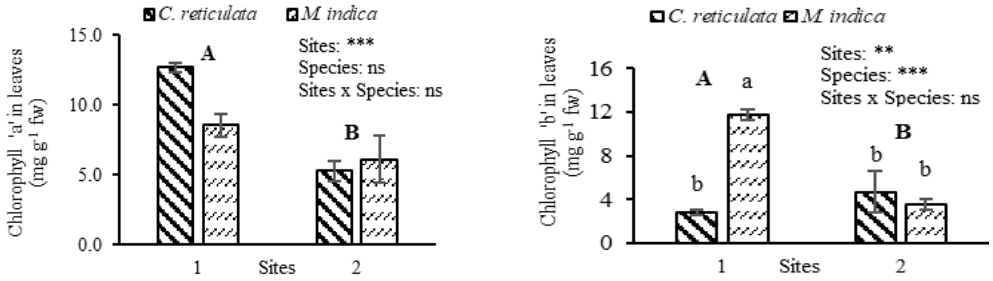

Figure 3. *Mean values for Chlorophyll ('a' and 'b') in leaves of two fruit species (C. reticulata and M. indica) sampled during respective fruiting seasons at Site 1 (Aziz Farm) and Site 2 (Nasim Farm), Multan, Punjab, Pakistan. The comparison between sites with different letters A and B and for species a & b showed a significant contrast. Each mean is across three individual trees with three foliage samples. Each column corresponds to mean value and each vertical line represents ± S.E. Different letters on each column showed significant difference by LSD (Tukey's Test) at 5% level of probability. ***, **, * = Significant at 0.001, 0.01 and 0.05 % level of probability, ns = non-significant by ANOVA, double factor with replication*

Extent of oxidative damage

Mean values presented in *Figure 4* revealed greater production of MDA in mango leaves. Although, MDA production was higher in mango foliage but it did not possess a distinct contrast from citrus. Similarly, the species were invariably different spatially. Analysis of variance (*Figure 4*) depicted that foliage had no noticeable difference between sites for MDA content. However, the foliage of the two species differed significantly ($P \leq 0.05$) for MDA content.

Enzymatic antioxidative activity

An enhanced activity for SOD was noticed that was considerably higher for citrus (*Figure 4*). A distinct variability for the activity SOD was observed in the two species from both sites. Though differential activity of CAT was noticed but the biosynthesis of the enzyme was insignificantly variable in the species (*Figure 4*). Hence, ANOVA (*Figure 4*) did not reveal any marked contrast for CAT activity for the foliage of the species. Conversely, at site 2, a profound contrast was observed for CAT level in the leaves of two species. With regard to POD, both species have indistinctive responses from both sites.

Non enzymatic antioxidative activity

A highly profound contrast was observed for ascorbic acid content in the leaves of two species but mango foliage had noticeably higher level for ascorbic acid. Despite variable mean values, no marked disparity became evident among the foliage of two

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species for ascorbic acid content at site 2 (*Figure 5*). Statistical analysis for ascorbic acid revealed that the sites and species varied ($P \leq 0.001$) considerably. Similarly, more carotenoids were found in mango leaves at site 1 (*Figure 5*) but a spatial contrast was evident. Furthermore, citrus foliage had lower amount of carotenoids as compared to mango leaves which did not exhibit any marked contrast between two species from the both sites. Analysis of Variance depicted significant ($P \leq 0.01$) spatial variability and the species also differed considerably ($P \leq 0.01$). A greater level of phenols (*Figure 5*) was recorded in mango foliage than citrus at both sites hence a spatial variability became evident. However, by contrast higher level of soluble sugar was recorded in citrus foliage than mango at both sites but the two species had a distinct contrast at site 1 (*Figure 5*). ANOVA for soluble sugars and phenols depicted significant ($P < 0.001$) contrast among species.

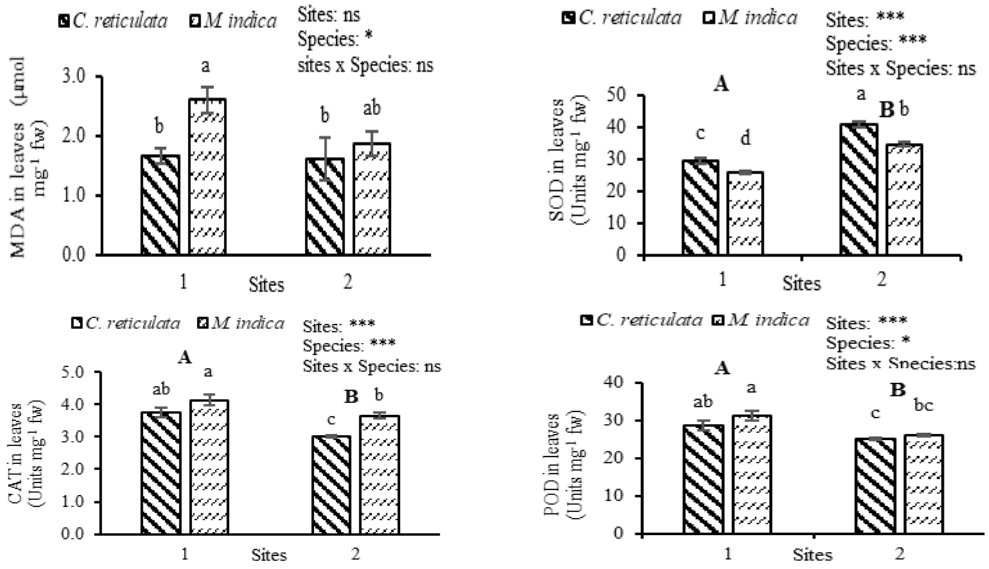

Figure 4. *Mean values for MDA, SOD, CAT, POD in leaves of two fruit species (C. reticulata and M. indica) sampled during respective fruiting seasons at Site 1 (Aziz Farm) and Site 2 (Nasim Farm), Multan, Punjab, Pakistan. The comparison between sites with different letters A and B and for species a & b or without common letter showed a significant contrast. Each mean is across three individual trees with three foliage samples. Each column corresponds to mean value and each vertical line represents ± S.E. Different letters on each column showed significant difference by LSD (Tukey's Test) at 5% level of probability. ***, **, * = Significant at 0.001, 0.01 and 0.05 % level of probability, ns = non-significant by ANOVA, double factor with replication*

Heavy metals (Cr, Ni, Pb, Zn) transport and accumulation in plant tissues

A substantial amount of metals was accumulated in roots and shoots of citrus plants as compared to mango which had more amount of metals in the foliage (*Figure 6*). Citrus fruit pulp has remarkably higher Cr, Ni and Pb but traces of these metals were not found in the edible portions of mango. On contrary, Zn was found in the pulp of both fruit species but was much higher in citrus than that of mango. ANOVA (*Table 3*) depicted that Cr in roots, shoots and leaves was highly significant ($P \leq 0.001$) regarding

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DOI: http://dx.doi.org/10.15666/aeer/1905_36213641

species. Pb in leaves and Zn in pulp showed marked ($P \leq 0.001$) significance regarding sites and species, respectively.

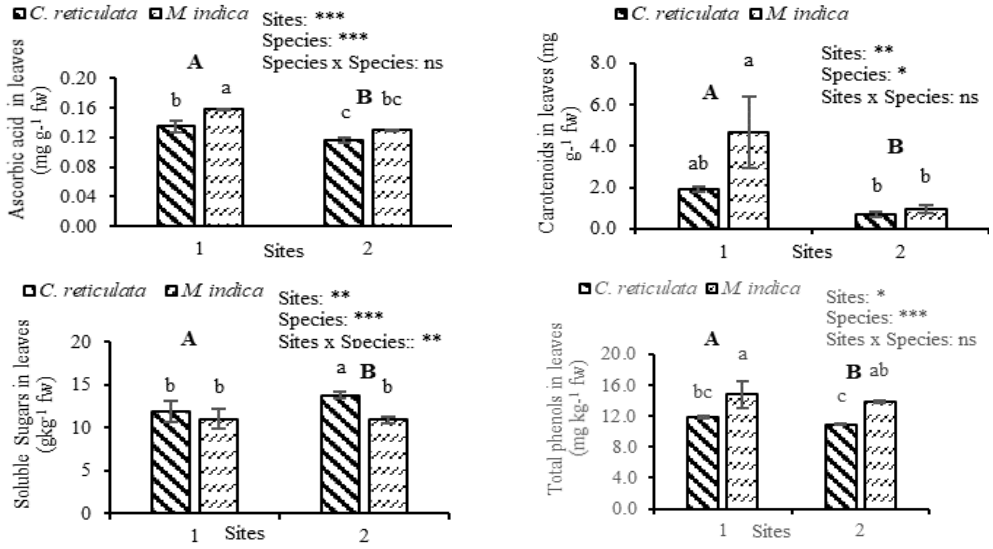

Figure 5. *Mean values for non-enzymatic (ascorbic acid, carotenoids, soluble sugar, total phenolics) activities in leaves of two fruit species (C. reticulata and M. indica) sampled during respective fruiting seasons at Site 1 (Aziz Farm) and Site 2 (Nasim Farm), Multan, Punjab, Pakistan. The comparison between sites with different letters A and B and for species a & b or without common letter showed a significant contrast. Each mean is across three individual trees with three foliage samples. Each column corresponds to mean value and each vertical line represents ± S.E. Different letters on each column showed significant difference by LSD (Tukey's Test) at 5% level of probability. ***, **, * = Significant at 0.001, 0.01 and 0.05 % level of probability, ns = non-significant by ANOVA, double factor with replication*

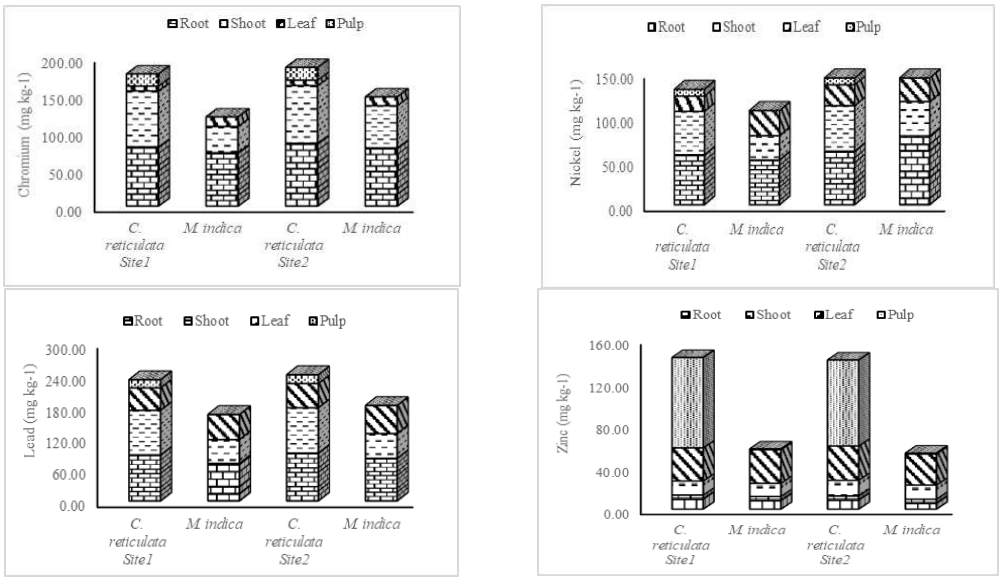

Figure 6. *Mean values for heavy metals (Cr, Ni, Pb, Zn) in (C. reticulata and M. indica) plant tissues (root, shoot, leaves, pulp) sampled from Site 1 (Aziz Farm) and Site 2 (Nasim Farm), Multan, Punjab, Pakistan. Each mean is across three individual trees with three samples for each plant tissue*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_36213641

Discussion

The study primarily focused on the quality of irrigation water, changes in soil properties with particular reference to metal contamination and its impact on socio-economic aspects and food safety. It is well documented that irrigation water whether rain or surface water plays an imperative role for recharging and quality of underground water (Zhang et al., 2016). The recharging ability and quality are important determinants for ground water to suggest its safe consumption (Hussain et al., 2017) because it influences soil properties.

The current study indicated that temporal variation affected the ground water quality and soil properties from the selected sites. Among various water quality parameters, pH is of prime consideration as far as solubility of minerals and metal uptake is concerned. pH of canal irrigation water was high during summer but the potential hydrogen value for ground water was more during winter. The reason for this variation can be attributed to various types of effluents that are thrown into canals by industries during peak working months of summer. However, less evaporation rate during winter months resulted in more deposition of substances in soil and ground water. The findings of our study are in lines to the results documented by Patel et al. (2015) who reported comparable results for high pH of waste water because of several dissolved substances. As far as soil pH is concerned, it influences several factors that alter the soil properties and plant growth as well as it governs nutrient availability and metal uptake by plants (Eid et al., 2018). The soil pH also showed spatial and temporal variability. According to Onwuka and Mang (2018) seasonal change of temperature affects the radiant energy of soil. This change in energy also caused significant disturbance in biochemical and physico-chemical attributes of soils, thus alter decomposition rate, release of minerals organic and inorganic components of soil.

Electrical Conductivity (EC), Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) were found to be higher in ground water during winter. Periodic un and inundation of canals and leaching of salts to ground water seemed to be a possible reason for changes in EC. When water pumps are installed at moderate depth of soil, a significant amount of dissolved salts are mixed with water that upon irrigation can change soil properties. Although soil texture is an important aspect in this regard but the two sites did not possess any marked disparity because sandy loam texture of the soil at two sites. Hussain et al. (2017) provided similar basis for changes in quality of surface and ground water. Application of irrigation water with high pH and EC for a long time significantly increase the EC of soil which might be due to increased exchangeable complexes in soil and certain reactions of alkaline hydrolysis. These findings are in line with the results of Romic et al. (2008) who attributed the same reasons. Further, higher BOD might result from characteristics and types of substances/pollutants that specifically allow multitude kinds of aerobic microflora thus more BOD.

The cations (Na^+ and Ca^{+2} + Mg^{+2}) remained higher in canal water during summer. An elevated soil temperature in summer season resulted in more evaporation from soil surface leaving behind more salts thus more pH, EC and BOD (Tian et al., 2020). Soil Na^+ was higher during summer in citrus root zone but more was found in deeper soil layer with temporal variability. More levels of metals (Cr, Ni and Pb) in surface water during un-inundations of canals seems due to the absence of dilution factor.

With regard to heavy metals in ground water, more metals were found during summer with greater concentration of Cr. The higher levels of Cr in ground water can

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be explained in terms greater solubility of the metal. However, bioavailability and phytotoxicity depends on various soil factors and Cr speciation (Cr III and Cr VI) but in the absence of any special transporters for both forms, the metal mostly retained by the plant roots and in soil. An increasing tendency was observed for Cr in deeper soil layers of mango rhizosphere. Other metals (Ni, Pb and Zn) also followed an increasing trend in citrus root zone at soil depth (10-20 cm). Interestingly, all metals showed the increasing level from upper to deeper soil layer thus, showing the transfer of metals into deeper soil layers. The root morphology of the two species is also distinct that governs the extent of metals in the soil thus, different concentration of Cr, Pb, Ni and Zn in rhizosphere of the species.

The results of the study indicated an affirmative relationship between metal contents of the irrigation water and soil. The greater metal concentration in the deeper soil layer can pose serious threat owing to non-biodegradable nature and contact with plant roots. Dotaniya et al. (2017) also worked out for a relationship between quality of irrigation water and soil contamination. These workers also ascribed water contamination owing to presence of Cr in soil and vice versa.

Tripathi and Chaurasia (2020) demonstrated greater levels Pb in irrigation water from sites where extensive use of fertilizers and pesticides was administrated. The source of Ni pollution seems to be several vegetable oil/ghee industries throwing waste water or landfills (Lu et al., 2016). The use of metal pipes and gradual corrosion due to high pH of water may also result in higher Ni content of water and soil depending on the source of irrigation water. Pb concentration in soil was consistently higher and its persistence and transport in the deeper layers of soil became evident. Findings of the study are in contrary to the results documented by Cecchi et al. (2008) who reported decreasing level of Pb with increasing soil depth. However, behavior of Pb in the soil is governed by complex biogeochemical factors (Punamiya et al., 2010) including pH, cation exchange capacity, soil minerals, microbial flora, quantity of Pb in the soil and the type of plant species growing in the area. Nonetheless, polluted irrigation water mainly causes contamination of agricultural soils (Chaoua et al., 2019).

Zinc (Zn) as an essential element which plays a pivotal role for various metabolic activities in plants (Hafeez et al., 2013) and is the most mobile element to be reached to aquifer. Sandy loam soils can allow percolation more Zn since soil act as sink for Zn hence an efficient uptake by plants becomes crucial. However, Zn uptake is not closely related to its concentration in the soil (Ernst and Nelissen, 2000) but the availability depends on soil pH and P status. The bioaccumulation of Zn in root cells and underlying mechanisms of its uptake are not completely known but its transport across the plasma membrane is mediated by a transport system and intracellular high-affinity binding sites (Gupta et al., 2016). Zn transporter family is comprised of ZIP (ZRT, IRT-like protein) are thought to be the primary transporters involved in its uptake. Thus, its accumulation in water and soil, subsequent transport and bioaccumulation in plants varied from other essential and non-essential metal ions (Guerinot, 2000). Nonetheless, the transport and bioaccumulation of Zn in edible part of the fruits remained of dietary significance. The results of the study are in accordance with the findings of Belkhiri et al. (2018).

The photosynthetic machinery comprising of green pigments chlorophyll (*a* and *b*) can serve as main predictors to signify net carbon assimilation via sunlight absorption by these molecules (Gruber et al., 2019). A species varied in space and time for chlorophyll *a* and *b*. However, the lowest content of chlorophyll (*a*) was found in mango leaves while chlorophyll (*b*) was highly masked in citrus leaves. The amount of

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
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chlorophyll decreases in relation to environmental disturbances where metal contaminants have widely been reported (Houri et al., 2020) to cause biodegradation of these molecules or replacement of metal ions with central magnesium thereby changing the chemistry of photosynthetic pigments.

MDA is a broadly used as a marker and index of oxidative damage that is destruction of biological membranes (Shahid et al., 2017). Therefore, Lou et al. (2017) have investigated MDA to assess physiological damage under various stressful conditions. The analysis of assays from the leaves of citrus and mango revealed more MDA content but the foliage of former species with greater extent of lipid peroxidation. It is highly likely that this oxidative damage is due to the presence of exceeding amount of heavy metals in the growth medium. Nonetheless, differential survival ability of the species allows them to mitigate metal stress through altered biochemical pathways for cellular defense. This adaptability of the living system that controls and regulates the generation of several enzymatic components (SOD, CAT, POD) and some non-enzymatic metabolites (ascorbic acid, carotenoids, phenolics and soluble sugars) are important (Shah et al., 2020). An increased activity of SOD in citrus leaves but more production of CAT and POD in mango foliage indicated oxidative defense in both species However, enzymatic components can work more efficiently if their biosynthesis occur in a co-current manner. Biosynthesis of CAT and POD in citrus seemed to be insufficient to mitigate oxidative damage. However, a sequential production of CAT followed by POD can scavenge free radicals thereby protecting cellular membranes in the mango leaves.

Ascorbic acid (AsA) is a vital, non-enzymatic antioxidant having considerable ability to scavenge ROS (Bilska et al., 2019). The presence of elevated levels of ascorbic acid in mango foliage seemed to mitigate ROS thus, protecting the species against heavy metals present in environment. Similarly, a group of pigments called carotenoids are important components and are capable to destroy ROS, eliminate the excited triplet state of chlorophyll hence play a defensive role for chloroplasts integrity by inhibiting cracking of its membranes (Handa et al., 2018). *M. indica* innately possess several secondary metabolites in different plant parts including carotenoids thus seems to have a better defense where an affirmative relationship was observed between resilience and biosynthesis of carotenoids (Ahmad et al., 2010).

Phenolic compounds also have the same ability. These components make the species less deterrent to insects and pest attack. Again, *M. indica* is characterized by the presence of many phenolic compounds that are species specific and are genetically controlled. Nevertheless, a confirmatory relationship between environmental constraints and phenolics seems to provide a better resilience to the species. Thus, high contents of phenols in the mango family are considered as selective advantage for superior survival ability (Sharma et al., 2019) for various abiotic and biotic stresses.

Likewise, sugars regulate many physiological processes such as respiration, seed germination, flowering, senescence, and act as the most efficient osmo-regulator under various environmental stresses (Sami et al., 2016). In the present study, sugar metabolism did not differ considerably among fruit species. Therefore, non- enzymatic antioxidants other than soluble sugars seemed to have greater impact against heavy metal stress.

Since, all plants have the natural ability to extract metal from the roots to their aerial parts that successively decreases from below ground to above ground but the least contents are found in the fruits and seeds. Nevertheless, these limits are recommended by WHO (2012) to ensure food safety.

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DOI: http://dx.doi.org/10.15666/aeer/1905_36213641

A differential pattern of metal bioaccumulation in plant parts of the two species from two sites became evident. The species and sites exhibited a striking contrast ($p \leq 0.001$) for the bioaccumulation of different metals in plant parts except for the amount of metal in pulp where sites had shown no variability for all four metals. Similarly, Zn content in the foliage in both species and sites had no marked variation (*Table 3*). All plant tissues; roots, shoots and leaves, ($p \leq 0.001$) and edible part/ pulp ($p \leq 0.01$) in the two species varied considerably. However, the two sites differed significantly for metal contents except for pulp. The bioaccumulation and subsequent translocation of Cr was root > shoot < leaves < fruit pulp that is a gradual decline for translocation to above ground plant parts. The behavior of Ni and Pb uptake was comparable to that of Cr despite structural differences of these ions and differential transport mechanism (Chen et al., 2009). On the other hand, Zn transport and accumulation were strikingly different.

In plants, Zinc is of immense importance in fruit setting, quality and yield (Nasir et al., 2016). Zinc transport from root to fruit pulp is of dietary significance particularly in the absence of any visible phototoxic symptoms in fruit species that are characteristically associated with supra optimal levels of Zn. A number of health disorder are associated with Zn deficiency but fruit pulps exhibited ample amount of this essential element (Rahimi et al., 2017).

The detection of Cr in citrus pulp signifies its bioaccumulation in edible portion is parallel to many studies (Omoyajowo et al., 2017). On the other hand, transport and bioaccumulation of Cr in mango appeared to be restricted hence the pulp remained metal free which is in line with Ogunkunle et al. (2014) who reported complete absence of Cr in various fruit species. Likewise, higher levels of Pb citrus pulp found consistent with other researchers (Saleh et al., 2017) who well reported the presence of Pb in a number of fruits across the globe. However, no Pb was found in mango pulp which is advantageous. A number of workers (Pereira et al., 2014; Sajib et al., 2014; Vollmannova et al., 2015) also reported the absence of Pb in many fruits such as *Psidium guajava* (guava), *Litchi chinensis* (litchi), *Butia capitata* (pindo palm) and *Rubus idaeus* (raspberry) owing to the natural ability or specific mechanism for limited transport for least bioaccumulation of metals in edible portion.

The differential occurrence of heavy metal contents in the pulp of two species can be explained in the light of distinct phenology of the two species. With regard to mango, the development of fruit encompasses only a few months as lax panicle (characteristic inflorescence) appeared during March and fruits became ready to harvest from May onwards depending on the variety. However, picking and packaging is completed by August. Thus, the exposure time of the fruit to the growth medium is limited. On the other hand, the floral development in citrus is initiated soon after the picking of fruit during October, each year. The fruits development is initiated within a few weeks after flowering. However, the fruits remained on the plants till maturity in the next season. This long-term exposure of the fruit to medium may result in the uptake and transport of more metals in edible parts of the fruit.

Conclusion

The results of the study signified spatial and temporal variability in the quality of irrigation water and soil characteristics. Though, the two sites did not possess much contrast presumably due to the absence of geographical isolation and similar interacting

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DOI: http://dx.doi.org/10.15666/aeer/1905_36213641

environmental variables. Nevertheless, soil and ground water quality appeared to be altered by the quality of surface water.

The photosynthetic biomolecules varied in species but the trait appeared to be genetically controlled. The ROS production and subsequent enzymatic and non-enzymatic oxidative defense seems to exist in both species but carotenoids and phenols provided more selective advantage to mango to scavenge metal stress.

The study depicted bioaccumulation of heavy metals in edible portion of the citrus only without any potential threat to mango as far as food safety is concerned. Differential metal contaminants can definitely be attributed to phenological traits of the two species. The study suggested strict environmental monitoring for the biodiversity and conservation in the region. At present, quality of water and soil did not seem to be of potential risk but if mounting pollution continues, it can affect sustainability with drastic ecological and socio-economic impacts. The current study signified that functional proteomics and genomics should be addressed for future studies to reveal insights of underlying molecular mechanism/s.

Acknowledgements. The work presented is the part of Ph.D thesis of the first author. The help rendered by Mr. Muhammad Khalid Rasheed, Scientific Officer, Soil and Water Testing Laboratory, Agriculture Department, Multan is greatly acknowledged for physioco-chemical analysis of soil and water samples.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_36213641

COLD STRATIFICATION BREAKS *THALICTRUM UCHIYAMAE* NAKAI SEED DORMANCY VIA PROTEOMIC CHANGES

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(Received 22nd Mar 2021; accepted 19th Jul 2021)

Abstract. This study was conducted to obtain basic data related to the seeds, germination conditions, and physiological changes in the seeds of *Thalictrum uchiyamae* Nakai (Seoul Meadow Rue). The seed was elongated and tapering at both ends, and its length and width were 1.810–2.744 and 0.576–0.989 mm, respectively. When the seeded seeds were planted immediately, the germination rate was less than 5% at 15/6°C or 20/10°C, but after cold stratification for 8 weeks at 5°C, the germination rate exceeded 80% at 30/20°C, 20/10°C, and 15/6°C. Thus, it was confirmed that *T. uchiyamae* seeds are morphophysiologically dormant. The physiological changes in the seeds during cold stratification were investigated in terms of proteomics; the number of activated proteins increased from 330 to 353 after cold stratification. We confirmed that the disappearance of or decrease in tunicamycin and phytochrome is associated with the breaking of dormancy in *T. uchiyamae* seeds.
Keywords: *morphophysiological dormancy, phytochrome, proteomics, Ranunculaceae, tunucamycycin*

Introduction

Dormancy is a peculiar characteristic of seeds; it prevents germination and the subsequent growth of seedlings under unsuitable ecological and environmental conditions (Bewley et al., 2006). Seed dormancy can be divided according to the region and mode of dormancy (Fenner et al., 2005). Baskin and Baskin (2004) classified dormancy types into physiological, morphological, morphophysiological, physical, and combined dormancy.

Seed germination is mainly determined by the plant hormones abscisic acid (ABA) and gibberellin (Razem et al., 2006; Weiss and Ori, 2007). It is not limited thereto, and the presence/absence or activity of various proteins is widely influenced by physiological changes within the seed during germination (Satoh and Esashi, 1979). Proteomics is the large-scale study of proteins, which are an important component of living organisms owing to their various functions (Blackstock and Weir, 1999). It involves the analysis of proteins that are produced, lost, or modified by various systems of an organism. Thus, proteomics supports functional genomics, which analyzes use of genetic information by functional

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_36433654

distinction, according to the situation (Anderson et al., 2016). Nevertheless, plant proteomics is still in an early stage and is focused on intracellular proteomes and protein complexes, for example, proteins in the plasma membrane and chloroplast (Rouquie et al., 1997; Bae et al., 2003; Park, 2004). Seed proteomics focuses on seed development, drying tolerance, germination, qualitative and quantitative changes in the proteome during dormancy, changes in vitality, and responses to environmental factors. It has been mainly applied to study model species such as *Arabidopsis* and important economic crops such as corn and rice (Weiss and Ori, 2007; Kim et al., 2008; He and Yang, 2013; Wang et al., 2015). Through protein profiling, mechanisms of physiological changes in seeds such as metabolism, cell cycle, signaling, and protein-related pathways can be elucidated (Wang et al., 2012).

Ranunculaceae members are distributed worldwide, with 43 genera and 2346 species accounting for approximately 0.8% of all flowering plants (Christenhusz and Byng, 2016). The seeds of most Ranunculaceae members are desorbed from the parent plant along with the immature embryo. Accordingly, dormancy in most Ranunculaceae members has been reported to be morphophysiological, which requires additional physiological treatments, such as gibberellin treatment and cold stratification, to break dormancy and allow immature embryo development and germination (Hepher and Roberts, 1985; Walck et al., 2000).

Thalictrum L. is one of the largest taxa of the family Ranunculus, and 120–200 species are distributed in most temperate regions (Pajeva et al., 2004). There are several studies on the pharmacological effects of *Thalictrum* species, because they possess several compounds, such as alkaloids, that exhibit anticancer and antibacterial activities (Chen et al., 1993; Pajeva et al., 2004). *Thalictrum uchiyamae* Nakai. (Seoul meadow rue) is a perennial herb that grows in various parts of Korea; they mostly grow among wet rocks and in semi-shaded organic soil. The plant grows to a height of approximately 50 cm, blooms with white-purple flowers in June and July, and bears semi-elliptical fruit in August and September (Park and Park, 2008). Young shoots are edible and are mainly used for gardening and ornamental purposes. *Thalictrum uchiyamae* is considered a useful medicinal plant in the Orient, because its rhizomes and roots contain high amounts of alkaloids such as thalicarpine, which reduce inflammation and act as diuretic and antibacterial agents (Bang, 1982; Lee, 1984; Kim, 1993).

Thalictrum uchiyamae was known to be endemic to the Korean Peninsula, but it was excluded from the revised endemic plant list in 2017, following the confirmation of its distribution in Japan (Iwatsuki et al., 2006; Chung et al., 2017). Nevertheless, countries are strengthening their bio-sovereignty with the international implementation of the Convention on Biological Diversity and the Nagoya Protocol. It is apparent that *T. uchiyamae*, which has a high value as a horticultural and medicinal resource, is still a priority consideration for Korea's bio-sovereignty (Paik, 1999; Oh et al., 2005). The pharmaceutical effects of *T. uchiyamae*, such as its antibacterial activity, have been well studies (Lee and Lee, 1982; Lee, 1984); furthermore, *T. uchiyamae* seed germination has been studied in external soil (Lee et al., 2018). Uniform propagation of *T. uchiyamae* in the laboratory is required for mass proliferation, continued use, and utilization of its pharmacological effects.

In physiological studies of seeds, storage proteins, stress responses, and proteomic changes due to germination have been extensively studied in crops such as beans and rice (Yang et al., 2007; De La Fuente et al., 2011; Badowiec and Weidner, 2014). However, physiological studies of seeds of wild plant have been limited to *Aconitum* or *Podophyllum* (Dogra et al., 2013; Rana and Sreenivasulu, 2013).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_36433654

The main purposes of this study were to analyze the dormancy type of *T. uchiyamae* seeds and to establish uniform germination conditions for *T. uchiyamae* seeds in the laboratory. Additionally, we performed proteomic analysis to elucidate the physiological changes, mainly proteomic changes, in seeds during dormancy breaking and to identify the associated proteins.

Materials and methods

Seed collection and characterization (plant material)

Fruits of *T. uchiyamae* were collected in Mt. Gaji, Cheongdo-gun, Gyungsangbuk-do, South Korea, in September 2013. Mature seeds were dried at room temperature for 1 month and stored at 4°C until use. The 1000-seed weight was measured using an electronic scale. The seed shape, size, and color and embryo length were measured using a Leica MZ16FA stereomicroscopic zoom microscope (Leica, Germany). Furthermore, the embryo:seed ratio (E:S ratio) at each germination stage were calculated (Vandelook et al., 2007).

Germination of seeds

For cold stratification, the seeds were treated at 5°C for 2, 4, 8, and 12 weeks, and then incubated at three temperature conditions: 15/6°C, 20/10°C, and 30/20°C with a 14-h photoperiod in a Bio Multi Incubator (LH-30-8CT, NK System Co., Japan). As controls, untreated seeds were also simultaneously placed in the incubator. In all experiments, the seeds were placed on 8% (w/v) agar medium in a Petri dish (90 mm in diameter) with three replicates of 30 seeds. Germination was recorded daily for up to 30 days, and the seeds were considered germinated when the radicle was 1 mm long. Final germination percentage (FGP) was compared to the mean germination time (MGT), germination index (GI), and final germination date (FGD) (Scott et al., 1984).

$$MGT = \Sigma(TiNi) / N \quad \text{(Eq.1)}$$

$$GI = \Sigma TiNi / S \quad \text{(Eq.2)}$$

where, Ti: number of days after sowing, Ni: number of seeds germinated on day i, N: total number of germinated seeds, S: number of seeds sown.

Protein extraction and electrophoresis

Untreated and chilling-treated seeds were used for protein extraction. Total soluble proteins were extracted from 50 seeds under each condition using the P-PER® Plant Protein Extraction Kit (Thermo Scientific, USA). Elution buffer was replaced with cell lysis solution, containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 2.5% (w/v) DTT, and protease inhibitor (Roche, USA), for electrophoresis. Protein concentrations were determined using the Bradford method and rechecked by 1-D electrophoresis on 12% 1D sodium dodecyl sulfate-polyacrylamide gel. For 2D-analysis, 700 µg of each protein was loaded onto a 3–10 pH IPG strip (Amersham, USA), and 2D-separations were performed on 12% SDS-PAGE gel. The gels were fixed and stained with Coomassie brilliant blue G-250 solution and de-stained with acetic acid (ProteomeTech Inc., South Korea).

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Protein analysis

2D pattern matching with protein spots was performed using 2D Platinum software version 5.0 (GE Healthcare, USA). Proteins with more than two-fold changes between the control and treatments were compared. Gel digestion of protein spots was performed, and then LC-MS/MS analysis was conducted at ProteomeTech (Seoul, South Korea) to identify proteins. Peptide spectra of LC-MS/MS were identified against NCBI using MASCOT software (Matrix Science, London, UK).

Data analysis

A completely randomized design with three replications was used. Data were analyzed using the two-way ANOVA with the SPSS statistical package, version 17.0. Treatment means were compared using Tukey's HSD mean separation at the 1% probability level.

Results

Seed morphology

The seeds were 1.810–2.744 mm in length, 0.576–0.989 mm in width, and the weight of thousand seeds was 0.601–0.811 g (*Table 1*). The seeds were elongated and tapering at both ends, and they were dark brown to black (*Fig. 1A, Table 1*).

Table 1. *Characteristics of the fruit and seed of T. uchiyamae used in this study*

Fruit type	Seed shape	Seed length (mm)	Seed width (mm)	1,000-seed weight (g)	Embryo type	E:S ratio*
Achene	Elongated and tapering at both ends	1.810–2.744	0.576–0.989	0.601–0.811	Rudimentary linear	0.15

A rudimentary linear embryo was observed in *T. uchiyamae* seeds (*Fig. 1A*). The E:S ratio revealed that the undeveloped embryo occupied less than 15% of the seed (*Fig. 1B*).

Germination after cold stratification

Matured seeds of *T. uchiyamae* did not germinate at 30°C/20°C; only 3.3% of the seeds germinated at 20°C/10°C and 15°C/6°C during 4 weeks (*Fig. 1C*). Unlike the untreated seeds, the seeds treated at low temperatures germinated (*Table 2*). When the period of cold stratification was shorter than 2 weeks, the germination rate at 15°C/6°C was higher than that at other germination temperatures. At a cold stratification period of more than 4 weeks, seeds at warm temperatures, such as 20°C/10°C and 30°C/20°C, presented a higher germination rate than those at 15°C/6°C. Particularly, final germination was more than 80% at warm germination temperatures after cold stratification for 8 weeks.

The embryo of *T. uchiyamae* seeds did not develop with cold or warm stratification (*Fig. 2*); however, a rapid development of embryos was observed at a warm germination temperature after cold stratification (*Fig. 3*). The results showed that the germination temperature and cold stratification period play a major role in the germination of *T. uchiyamae* seeds.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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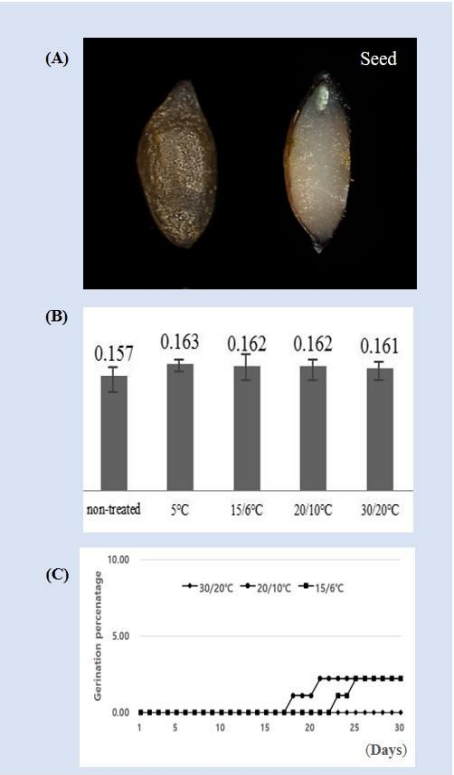

Figure 1. *Basic characteristics of T. uchiyamae seeds. (A) Seed and cross section containing rudimentary embryo. (B) Embryo/seed ratio when incubated for 1 month at each temperature (C) Germination rate graph at each temperature for the untreated*

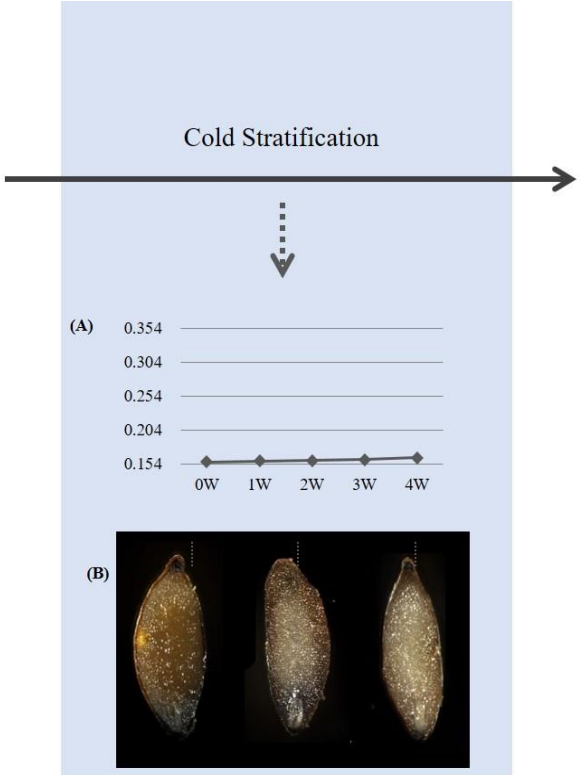

Figure 2. *Embryo was not grown during cold stratification. (A) E:S ratio depends on cold stratification period. (B) Pictures of the embryo at week 0, 2, 4 after cold stratification*

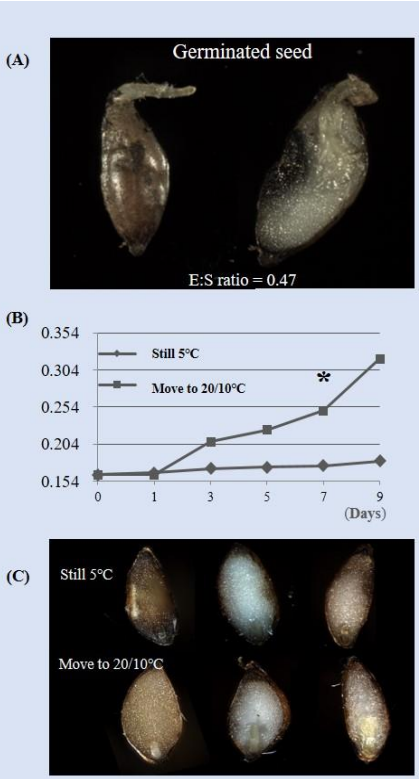

Figure 3. *Embryo development after cold stratification. (A) The radicle appeared after cold stratification. (B) E:S ratio at different temperatures after 4 weeks of cold stratification. (C) Pictures of the embryo on day 1, 5, 9 at 5 or 20/10℃ after 4 weeks of cold stratification. * Start day of germination*

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Table 2. *Seed germination depends on the cold stratification period and germination temperature. The letters beside numeric values indicate significant differences by Tukey' HSD test. CS period, cold stratification period; GT, germination temperature; FGP, final germination percentage; MGT, mean of germination time; FGD, final germination date*

CS period (weeks)	GT (°C)	FGP (%, ±SD)	MGT (days, ±SD)	FGD (day)
0	30/20	0.00	-	-
	20/10	3.33±0 a	23.00±6.24 ab	30
	15/6	3.33±0 a	26.00±3.61 a	30
2	30/20	57.30±1.2 b	11.18±0.98 e	17
	20/10	78.70±1.2 cde	13.73±0.31 cde	27
	15/6	74.70±4.0 bcd	22.67±1.41 ab	30
4	30/20	94.70±0.6 e	13.65±1.65 cde	28
	20/10	90.70±1.2 de	11.20±0.48 de	21
	15/6	64.00±1.7 bc	19.38±1.45 abc	27
8	30/20	85.30±2.3 de	10.29±0.77 de	23
	20/10	90.70±0.6 de	9.46±1.08 e	18
	15/6	84.00±1.7 de	17.86±0.46 cde	24
12	30/20	89.30±1.2 de	6.78±1.02 e	17
	20/10	89.30±1.5 de	9.32±0.22 e	19
	15/6	86.70±0.6 de	12.08±0.47 cde	23

FGP and MGT data are presented as mean and standard deviation (SD)

Proteomic changes in the germination stage

To examine the physiological changes in the seeds induced by cold treatment, the changes in seed proteins were compared through 2D electrophoresis. In total, 330 proteins were detected in the untreated seeds and 353 proteins in the cold-stratified seeds; all proteins were found to be expressed between pH 4 and 8; their molecular weights ranged between 15 and 60 kD.

A total of 47 altered proteins were found based on the differences (two-fold or higher) in the protein expression pattern between the untreated and cold-stratified seeds. The expression of 7 proteins increased and that of 27 proteins decreased; 4 proteins appeared and 9 proteins disappeared (*Fig. 4*). Among the proteins with differences, we identified 16 proteins with the greatest changes, namely, 2 increased, 7 decreased, 4 appeared, and 3 disappeared proteins (*Supplementary Table 1*). Specifically, we confirmed the disappearance of tunicamycin (Spot 1) and reduction in phytochrome (Spot 31) (*Fig. 5*).

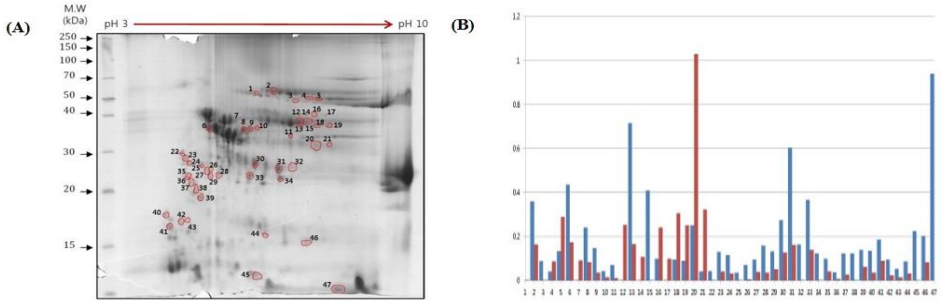

Figure 4. *Proteins whose expression altered after cold stratification. (A) Fotrty-seven proteins whose expression was altered at pH 6–8 were identified (molecular weight 40–70 kD) using 2-D SDS-PAGE. (B) Quantitative comparison of proteins whose expression had altered before and after cold stratification*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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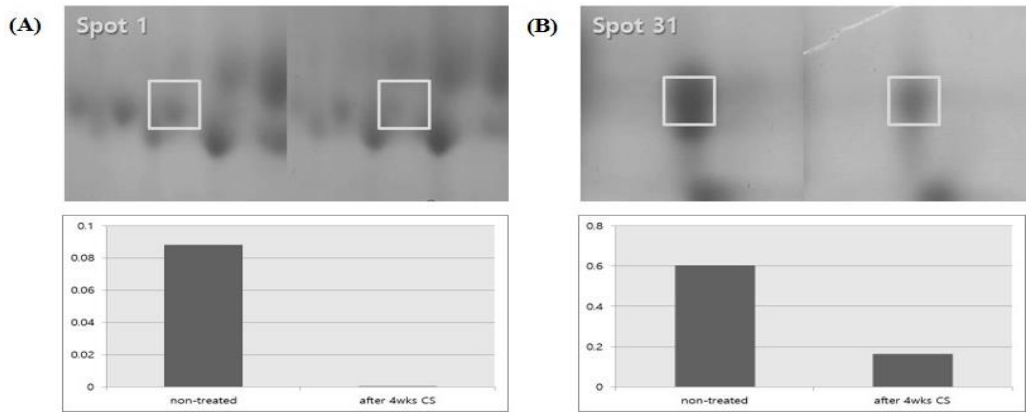

Figure 5. *Changes in spots 1 and 31 after cold stratification. (A) Spot 1 disappeared after cold stratification, (B) The intensity of Spot 31 rapidly decreased during cold stratification*

Discussion

Narrow, elliptic, or fusiform, dark brown to black seeds of *T. uchiyamae* are known to belong to the section physocarpum (Ghimire et al., 2016). Martin's seed internal classification is based on the fact that some Ranunculaceae seeds contain an undeveloped linear embryo surrounded by the endosperm (Martin, 1946). It is known that immature seeds (without a fully developed embryo) of Ranunculaceae members shed from the mother plants, and some species, especially those that shed their seeds in the spring or/and summer, are known to have morphologically undeveloped embryos (Earle, 1938; Tamura and Mizumoto, 1972; Engell, 1995).

Dormancy of seeds with undeveloped embryos is classified as follows: 1) morphological dormancy (MD), when the embryos do not grow to a certain size; 2) physiological dormancy (PD) caused by the physiological inhibition of the embryo; and 3) morphophysiological dormancy (MPD), coexistence of morphological and physiological dormancy (Nikolaeva, 1969). Seeds with MD generally germinate at the germination temperature under wet conditions at 4 weeks. PD of seeds is broken by resolving the physiological inhibition mechanism with cold or warming treatment. Accordingly, MPD is broken when the treatments of both MD and PD are applied; thereafter, the embryos grow and the seeds germinate (Baskin and Baskin, 2004).

Embryo development at all germination temperatures was not achieved at 30 days (data not shown); therefore, the dormancy type of *T. uchiyamae* seeds is MPD, which has to be broken for the transition of physiological mechanisms and embryo growth.

MPD is classified into a simple or complex type based on the temperature of embryo growth. In addition, classification types based on physiological dormancy depth; non-deep, intermediate, and deep exist (Baskin and Baskin, 1998). Here, the embryos did not grow during cold stratification, but the physiological inhibition mechanism was broken, and complete development of the embryo was achieved after the seeds were moved to a warm temperature. According to these results, the dormancy type of *T. uchiyamae* is non-deep, simple MPD. The seeds of *Thalictrum mirabile* are also known to have non-deep, simple MPD (Wang et al., 2012), thus supporting our result.

The seeds of plants that sprout in the spring, such as *Aruncus dioicus* and *Primula sieboldii*, are commonly dormant in winter, and cold stratification is the usual treatment to break their dormancy (Salisbury and Ross, 1985; Hong et al., 2006; Song et al., 2015).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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It is thought that continuous chilling and wet conditions affect germination (Bewley and Black, 1994). Other studies have reported that the optimum germination temperatures in many spring- or summer-germinating species are high (20°C–30°C) after chilling (Baskin and Baskin, 1987; Matsuo and Kubota, 1988). Thus, this result shows that 1) more than 4 weeks of cold stratification is needed to break dormancy and 2) warm temperatures are necessary for seeds of *T. uchiyamae* to germinate.

Tunicamycin (Spot 1) induces protein accumulation and endoplasmic reticulum stress, and is known as a major protein involved in apoptosis (Reis et al., 2011; Barba-Espín et al., 2014). Here, it disappeared after cold stratification (*Fig. 5A*). In contrast to the decay and apoptosis observed in many untreated seeds, the disappearance of tunicamycin induces a reduction in contamination due to the risk of cell death. As a result, seeds under low-temperature conditions can maintain a germination-ready state.

Phytochrome (Spot 31) is known to be involved in the activity of genes required for germination stimulation by breaking seed dormancy (Shin et al., 2016). In the present study, its expression decreased during cold stratification in the dark (*Fig. 5B*). This suggests that there would be a delay in seed germination at cold temperatures until optimal warm germination conditions are reached.

Conclusion

The seeds of *T. uchiyamae* have an immature embryo during desorption, and the dormancy of seeds can be effectively broken through cold stratification under dark conditions. Our findings showed that *T. uchiyamae* seeds have morphophysiological dormancy, which can be broken at a low temperature; embryo development can occur at appropriate warm temperature for germination. During pre-treatment (low temperature under dark conditions), changes in seed proteins, in particular, rapid changes in tunicamycin and phytochrome were observed.

In this study, we could not elucidate the correlation between the physiological changes in the seed and the mechanism of germination. Further research on seed dormancy and germination should be conducted to identify physiological mechanisms, thereby enabling us to clarify the germination conditions of *T. uchiyamae* seeds.

Acknowledgements. This work was supported by the National Research Foundation of Korea Grant funded by the Korean Government (NRF-2019R1I1A2A01062559) and the project “Ex-situ Conservation of Forest Plant Seeds in Korea” (KNA1-2-29).

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_36433654

APPENDIX

Supplementary Table 1. *Change in protein spots between cold-treated and non-treated seeds*

Spot Label	Protein name	Organism	Theor.MW (KDa)	Theor.p*I*	No.of queriesmatched	Score	Expression	Sequence of the matched proteins	NCBI accession No.
1	Tunicamycin induced 1, partial	*Aquilegia coerulea*	33.2	8.62	1	22	Disappeared	K.EAIVKGLGFQTK.I	gi|700256739
3	Not detected						Disappeared		
6	Ribulose-1,5-bisphosphate carboxylase	*Syntriandrium preussii*	51.8	6.18	4	18	Decreased	K.DDCNVNSQPFMRWR.D K.WSPELAAACEVWKEIK.F R.GGLDFTKDDMNVNSQPFMR.W R.GGLDFTKDDMNVNSQPFMR.W	gi|224830705
7	Maturase K, partial	*Aquilegia oxysepala*	27.3	6.83	2	36	Appeared	K.YLIILANDCNPDCNPKK.F K.YLIILANDCNPDCNPKK.F	gi|209489530
9	Not detected						Decreased		
11	Not detected						Decreased		
12	Hypothetical protein OsI_04965	*Oryza sativa* Indica Group	42.2	7.7	1	62	Appeared	R.VCTVHANCCVGLENKVLDLK.N	gi|218189628
13	Hypothetical protein L484_020505	*Morus notabilis*	19.2	7.05	1	60	Decreased	R.HGANFATGGSSIR.L	gi|703146348
14	Predicted protein	*Micromonas* sp. RCC299	252.4	5.59	1	61	Appeared	K.DGSLDLASAQKQLK.D	gi|255076851
15	PREDICTED:GDSL esterase/lipase At5g14450-like	*Cucumis sativus*	41.9	6.36	1	67	Disappeared	R.HGANFATGGSTVR.K	gi|449446714
17	PREDICTED:uncharacterized protein LOC103978659 isoform X1	*Musa acuminata* subsp. *malaccensis*	177.5	6.93	1	59	Appeared	R.QSALTVADADFLR.V	gi|695012108
20	Not detected						Increased		
21	HypotheticalproteinMIMGU_mgv1a004589mg	*Erythranthe guttata*	56.5	5.71	1	61	Increased	K.NMKNNLIPSSPMK.V	gi|604341217
26	Not detected						Decreased		
31	Phytochrome A, partial	*Pteridophyllum racemosum*	49.6	5.87	4	34	Decreased	K.NMGSAASLVMAIVSNEGDEEEEASGPSQPQK.K K.NMGSAASLVMAIVSNEGDEEEEASGPSQPQK.K K.NMGSAASLVMAIVVNEGDEEEEASGPSQPQKK.K K.NMGSAASLVMAIVHNEGDEEEEASGPSQPQKK.K	gi|13383438
33	Not detected						Decreased		

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_36433654

WATER QUALITY OF THE KLANG RIVER, SELANGOR, MALAYSIA AND HEAVY METAL REMOVAL USING PHYTOREMEDIATION

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(Received 23rd Mar 2021; accepted 12th Aug 2021)

Abstract. In this study, nine physicochemical parameters including dissolved oxygen, biochemical oxygen demand, chemical oxygen demand, total suspended solids, ammoniacal nitrogen, conductivity, temperature, pH and heavy metal contents from five sampling stations were measured to determine the water quality index (WQI) of the Klang River, Selangor, Malaysia. The WQI of the first and second sampling stations were classified as classes II and III, respectively. Biological analysis of total coliform and fecal coliform bacteria shows that three sampling stations were highly polluted with 17,500, 11,300 and 10,700 CFU/100 mL of these bacteria. The efficiency of phytoremediation in removing heavy metals (*i.e.* As, Cd, Cu, Mg, Fe, Pb, Zn and Hg) was determined using *Pistia stratiotes* and *Lemna minor* L. Phytoremediation using *Pistia stratiotes* was able to reduce the concentrations of As, Cd, Cu, Fe, Pb, Zn, Mg and Hg to 96.62%, 95.65%, 60.38%, 61.67%, 99.24%, 32.97%, 53.23% and 96.59%, respectively. *Lemna minor* L. was able to remove approximately 10.97%, 83.33%, 35.75%, 39.97%, 100%, 15.32%, 23.81% and 90.91% of As, Cd, Cu, Fe, Pb, Zn, Mg and Hg, respectively. Both plants were able to reduce the heavy metal contents in river water samples at the end of the treatment day.
Keywords: *water treatment, Pistia stratiotes, Lemna minor L., water pollution, aquatic plants*

Introduction

Water pollution has had negative impacts on the environment and human health. The pollutants can come from point sources such as industrial and domestic wastes, while pollutants from non-point sources include agriculture activities and urban runoff (Banch et al., 2020; Harun et al., 2020). Rapid development has contributed to a high amount of human wastes, including local, industrial, commercial and transportation wastes which ends up in rivers (Khataee et al., 2012). In addition, the increase in human population density and the development of industries nearby rivers and coastal areas have increased the pollutant inputs and deteriorated the water quality of the surrounding area (Jindal and Sharma, 2011; Hanafiah et al., 2018a; Harun et al., 2020). Urban rivers have also become polluted due to the discharge from sewage treatment plants as well as overflowing sewage caused by rainfall, leading to fecal contamination which was a major concern in the river near the town area. Nonetheless, industrial and household wastes which were discharge directly or through leakages in the sewage systems flowed into water sources thus causing excessive pollution of surface and underground water (Manikam et al., 2019). Contaminated discharges and effluents from anthropogenic activities have resulted in severe degradation of river water quality (Kamarudin et al., 2019). A large number of rivers were polluted, to the extent that the rivers are not rehabilitate, and the access to a

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DOI: http://dx.doi.org/10.15666/aeer/1905_36553677

clean and safe water supply has become a challenge for the government to overcome (Ghazali and Hanafiah, 2016; Ashraf and Hanafiah, 2019; Aziz and Hanafiah, 2020). Accordingly, water pollution issue has received increasing attention globally in recent years (Lin et al., 2015).

Malaysia is no exception in facing an environmental issue related to water pollution (DOE, 2018; Hanafiah et al., 2018b). Klang River which is located in Klang Valley is a river flows through Kuala Lumpur and Selangor in Malaysia and eventually flows into the Straits of Malacca. It is approximately 120 km (75 mi) in length and drains a basin of about 1,288 km^2 (497 sq. mi). Rapid development has lessened certain stretches of the river to the point that it resembles a large storm drain in some places (Banch et al., 2020) contributing to flash floods in Kuala Lumpur, especially after heavy rain. The two most important tributaries are Selangor River and Langat River. There were two major dams at the upstream of Klang Valley namely Batu Dam and Klang Gates Dam, however, these dams have been polluted due to the untreated sewage and industrial wastes. Sewage goes straight into the river due to inadequate water piping not being linked to the sewage concentration pipes. The vast industrialization, urbanization and rapid economic development in Kuala Lumpur have increased the levels of pollution in the rivers, especially Klang River suffers the most since it flows through the state of Selangor and Kuala Lumpur. The pollution mainly caused from the development which raises numerous environmental concerns along the Klang River. Meanwhile, human activities have caused a substantial hydrological deformation (Safauldeen et al., 2019). The hasty urbanization has directed to both the increasing request for water consumption and the increasing levels of river water pollution in Malaysia (Banch et al., 2019a; Al-Raad et al., 2020).

Nevertheless, water treatment is also crucial to improve water quality, so that the water can be more acceptable to be consumed for various purposes (Sun et al., 2012). Among a number of water treatment methods that have been developed, phytoremediation is one of the alternative technologies that can be implemented to reduce contaminants in river water (Hanafiah et al., 2020). Phytoremediation is a nature-based treatment which uses plants as a phytoremediation agent and it has potential benefits in restoring a balance in stressed environment (Aziz et al., 2020). It is an emerging low-cost technology, non-intrusive and aesthetically pleasing using the remarkable ability of green plants to metabolize various elements and compounds from the environment in their tissues (Selamat et al., 2014). Phytoremediation technology is applicable to a broad range of contaminants, including metals and radionuclides, as well as organic compounds like chlorinated solvents, polycyclic aromatic hydrocarbons, pesticides, explosives and surfactants (Ng and Chan, 2016). The objectives of this study were to determine the water quality of Klang River based on the physical, chemical and biological characteristics and to determine the removal rate of contaminants using *Pistia stratiotes* (water lettuce) and *Lemna minor* L. (duckweed) as phytoremediation agents. *Pistia stratiotes* and *Lemna minor* L. are invasive floating aquatic macrophytes which have the ability in a remediation of diverse chemical pollutants (Jayasri and Suthindhiran, 2017; Schwantes et al., 2019).

Materials and Methods

Sampling and Laboratory Analysis

The first sampling station (ST1) was at Jalan Jelatek, near the fire and rescue station. Second sampling station (ST2) was conducted at Jalan Datuk Keramat, near the railway station and car wash shops. The third sampling station (ST3) was at Jalan Gurney, near

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the restaurants complexes. Fourth sampling station (ST4) was at Jalan Sungai Baru which located near the residential complexes and the fifth sampling station (ST5) was at Jalan Dang Wangi which located near the markets and shops. The coordinates of five sampling stations are provided in *Table 1*. The samplings were conducted two times with a total of three samples were taken at site for replication to get a more accurate and precise results, with a volume of 100 ml per sample. Six parameters were taken to determine the water quality index for Klang River which were dissolved oxygen (DO), biochemical oxygen demand (BOD), chemical oxygen demand (COD), total suspended solid (TSS), ammoniacal nitrogen (NH_3-N) and pH. Other parameters like conductivity, temperature, heavy metals and coliform bacteria have also been taken into account.

Table 1. *The coordinates of the five sampling stations*

Stations	Coordinate
ST1	3° 9' 52.3872"N 101° 44' 4.1496"E
ST2	3° 9' 50.7816"N 101° 43' 27.9804"E
ST3	3° 9' 57.2832"N 101° 43' 1.5744"E
ST4	3° 9' 44.6832"N 101° 42' 38.7468"E
ST5	3° 9' 15.0948"N 101° 42' 2.6244"E

In order to monitor and assess the water quality of river system, Water Quality Index (WQI) has been widely applied which involves the classification of rivers or river segments into classes of quality in a descending order (Asman et al., 2017; Ashraf and Hanafiah, 2017; Harun and Hanafiah, 2018). The index is a numeric expression used to transform a large collection of water quality data into a single index number, which represents the water quality level. A river with high WQI value reflects that the water body is in good condition and vice versa (Suratman et al., 2015). WQI consisted of six parameters, namely DO, BOD, COD, SS, NH_3-N and pH. The WQI of Klang River was determined using in the *Eq. 1*:

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

where,
SIDO = Sub-index for DO; SIBOD = Sub-index for BOD; SICOD = Sub-index for COD; SIAN = Sub-index for AN; SISS = Sub-index for SS; and SIpH = Sub-index for pH.

The reading of DO was taken on the first day and then the samples were kept in a 20°C incubator for five days. After five days, the DO reading was taken again. The calculation of BOD (*Eq. 2*) and TSS (*Eq. 3*) are as follows:

$$\text{BOD} = \text{DO (reading on the first day)} - \text{DO (reading on the fifth day)} \quad \text{(Eq.2)}$$

$$\text{Total suspended solids (mg/L)} = \frac{A-B}{V} \text{ x } 1000 \text{ mL} \quad \text{(Eq.3)}$$

where,
A = Weight of filter paper after filtration (weight of filter paper + dried residue), mg; B = Weight of filter paper before filtration (weight of filter paper), mg; V = Volume of filtered water sample, mL.

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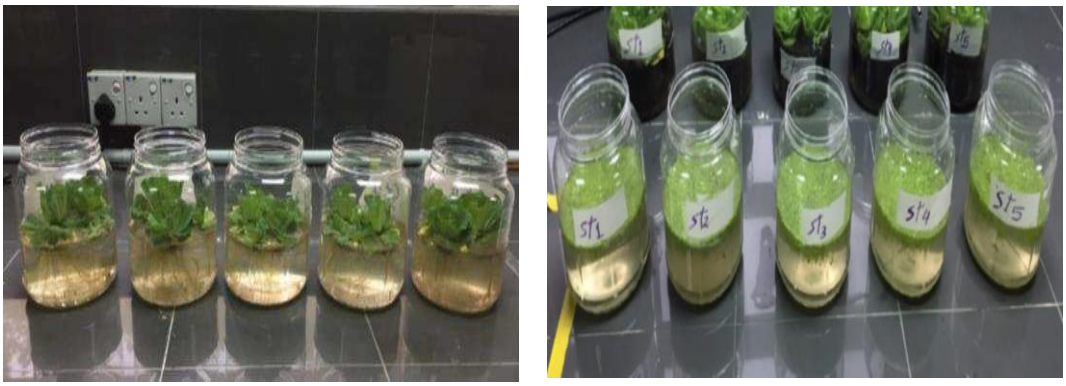
DOI: http://dx.doi.org/10.15666/aeer/1905_36553677

Phytoremediation

The collected *Pistia stratiotes* and *Lemna minor* L. were put on a filter paper to remove excess water and were then transferred into a 5 L plastic containing water sample from different sampling stations. Before the plants were transferred, the water characteristics were determined by analyzing the heavy metals content such as Pb, Zn, Mg, As, Cd, Fe, Al and Li (Ugya, 2015). After 7, 14 and 21 days, the water was analyzed to get the value of its characteristics. *Figure 1a* and *1b* show the growth of *Pistia stratiotes* and *Lemna minor* L. in the laboratory. The parameters before phytoremediation was noted as initial value, while the value after phytoremediation was indicated as final value. All the analysis was done using the methodology according to APHA (1995) and APHA (1998).



(a) (b)

Figure 1. *The growth of a) Pistia stratiotes and b) Lemna minor L. in the laboratory*

Statistical Analysis

Statistical analysis was performed using the statistical packages of IBM SPSS software (version 22, New York, US, 2020). Statistical significant difference analysis was carried out by using one-way ANOVA test to show the P values of each parameter between the five sampling stations.

Results and Discussion

Water Quality Index

Nine parameters namely DO, BOD, COD, TSS, NH_3-N, conductivity, temperature, pH and heavy metals were measured to determine the water quality of Klang River. All parameters for five sampling stations were compared with the water quality index (WQI) of the Department of Environment (DOE).

All samples were collected at two times sampling where the first sampling was conducted during the rainy season and the second sampling was conducted during the dry season. Overall, the average value of WQI for ST1, ST2, ST3, ST4 and ST5 were 74% (class III), 75% (class III), 76% (class III), 81% (class II) and 73% (class III), respectively. *Table 2* shows the average value of WQI at five sampling stations.

Most stations were classified in class III of WQI except for ST4 which is in a class II. According to the classification of the river water in WQI, class III indicated that water was slightly polluted with extensive treatment required such as coagulation process,

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DOI: http://dx.doi.org/10.15666/aeer/1905_36553677

flocculation, sedimentation, filtration and disinfection. Class II (ST4) considered as slightly polluted and a conventional treatment was required. The main sources of pollution at the sampling site was due to various land use activities such as malls, condominium residential, commercial activities, industrial factories, municipal sewers, wet market, sand mining and landfill which affected the water quality of Klang River. *Figure 2* shows the average value of all parameters for five sampling stations at two sampling times.

Table 2. *The average value of WQI for five sampling stations*

Station	DO (mg/L)	BOD (mg/L)	COD (mg/L)	NH_3-N (mg/L)	TSS (g/L)	pH	Temp. (°C)	Conductivity (µs/cm)
ST1	8.305	5.015	49	1.62	0.005	6.6	23.755	183.4
ST2	10.86	5.625	7.75	2.475	0.018	6.6	24.21	187.35
ST3	7.645	4.52	16	2.195	0.006	6.5	23.735	181.2
ST4	7.12	4.595	22.15	1.215	0.010	6.545	23.46	179.75
ST5	7.53	4.92	57	1.095	0.015	6.6	24.195	218.75

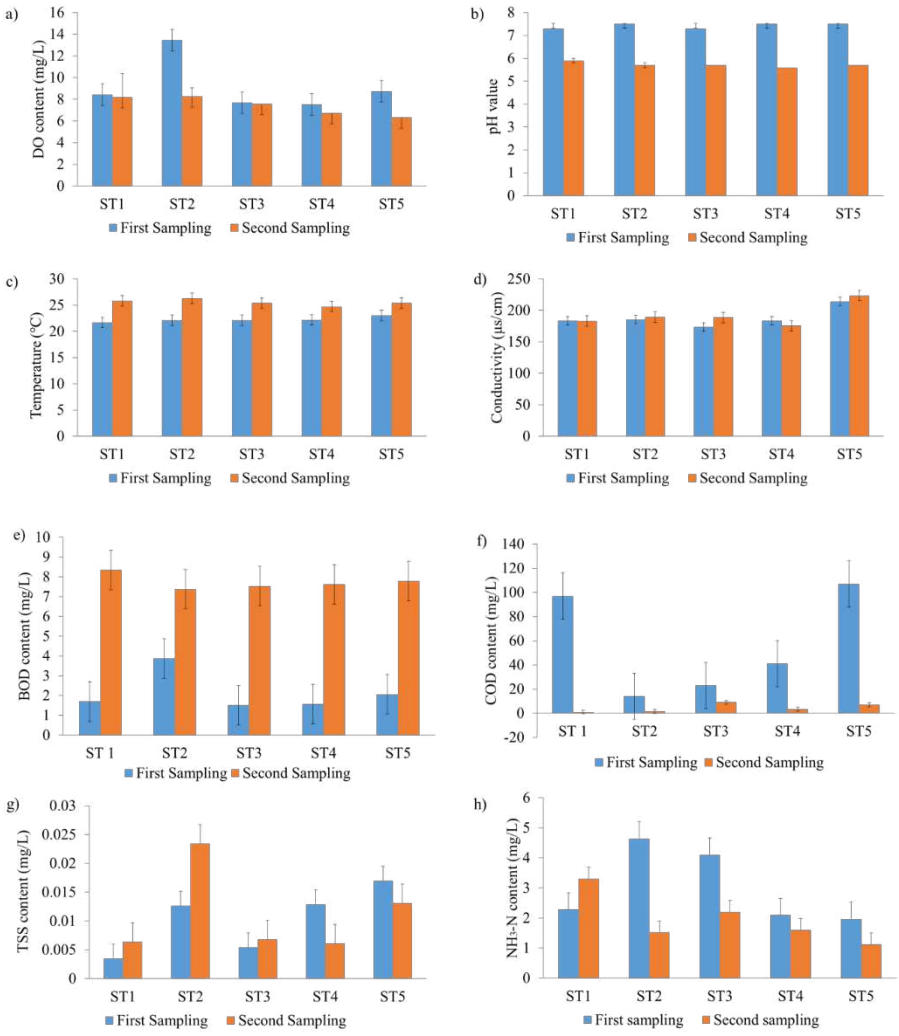

Figure 2. *Average value of a) DO, b) pH, c) temperature, d) conductivity, e) BOD, f) COD, g) TSS and h) NH_3-N for five sampling stations at two sampling times*

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The average of DO value at all five stations were ranged between 7.12 mg/L to 10.86 mg/L. ST2 has the highest average of DO value among the five sampling stations, which was about 10.86 mg/L. While the average DO at ST4 was the lowest with a value of 6.12 mg/L. Most stations were classified in class I (>7 mg/L), except for ST4 and ST5 (during the first sampling) which were in class II. All average values were statistically analyzed using one-way ANOVA test and there was no significant difference of DO between two sampling times for all stations with *p*-value = 0.369 (>0.05) and with a total standard deviation (SD) of 1.8233. According to Ismail and Salim (2013), DO content was affected by the presence of organic and inorganic materials in the water.

At the first sampling the pH for ST1 was 7.3, while at the second sampling, the pH was 5.9. For ST2, the pH values at the first and second samplings were pH 7.5 and 5.7, respectively. The pH value for ST3 was 7.3 at first sampling and was 5.7 at second sampling. ST4 has a pH value of 7.5 at first sampling and showed a minimum pH at second sampling with a value of 5.59. The pH value at ST5 was 7.5 at first sampling and 5.7 at second sampling. All sampling stations during the second sampling was acidic with a pH value of below 6 and based on the WQI, the pH of the five sampling stations in second sampling were classified in class III, which was between 5 and 6. At the first sampling, all five stations have a pH of more than 7, which was alkaline and were classified in class I. Based on the statistical analysis using ANOVA, it was indicated that there was high significant difference in pH between the five sampling stations on the first and second sampling times. All stations in the first sampling were recoded alkaline, meanwhile in second sampling showed an acidic pH of water with *p*-value $p<0.05$ and the total SD was 0.91. The acidity of pH during second sampling might be due to the solidifiers and hardens of organic materials from non-seasonal human activities such as restaurants and cafeterias nearby which produced food wastes, plant residues and animal residues.

For ST1, the minimum temperature value was between 21.7 °C for first sampling and for second sampling was 25.81 °C. In the ST2, the average temperature was between 22.1 °C for first sampling and 26.31 °C for second sampling. For ST3, the average temperature was between 22.1 °C at first sampling and 25.37 °C for second sampling. Average value for ST4 was between 22.2 °C for first sampling and 24.72 °C for second sampling. ST5 was ranged between 23 °C for first sampling and 25.39 °C for second sampling. Based on one–way ANOVA analysis, there was higher significant difference in temperature between the two samplings and within stations. The *p*-value of all samples $p<0.05$ of temperature with the total SD of 1.81.

The average value range for conductivity was between 179.75 µs/cm and 218.75 µs/cm. The conductivity for most of the sampling stations were slightly higher during the second sampling as compared to the value of the first sampling. According to the WQI, it can be deduced that the conductivity of all five sampling stations in both sampling times were classified in class I (<1000). Based on the one-way ANOVA test that has been conducted, there was no significant difference in the conductivity between sampling stations and between the two times sampling, with $p= 0.62$ (>0.05) and a SD of 16.87. The higher conductivity values during the second sampling in the dry season was due to the fact that the water carried all the elements that help to increase the conductivity such as iron and cadmium, which were considered as high conductivity of the same elements.

The average BOD for the two times sampling were between 4.52 mg/L (ST3) to 5.62 mg/L (ST2). The BOD values for all sampling stations at two sampling times were

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DOI: http://dx.doi.org/10.15666/aeer/1905_36553677

classified as class III according to WQI. From the statistical analysis using one-way ANOVA test that has been performed, it can be interpreted that there was high significant difference between two sampling times with *p*-value <0.05, while there was a significant difference between sampling stations with *p*-value = 0.01 and a total SD of 4.08. During dry session at second sampling, the BOD value was higher due to the decayed plants and animal's wastes especially at ST5 and also because of the presence of anthropogenic effluents including faeces, urine, detergents, fats, oil and grease (Banch et al., 2019b). Faeces contain organic and biological contamination and if it drains into water without being treated first, it can increase the risk of water diseases (Wahab et al., 2019). Rainy season during the first sampling resulted in lower BOD value because of a good oxidation in water as well as due to an increased volume and flow rate of the river.

The average value of COD recorded at all sampling stations for two sampling times were between 7.75 mg/L which was at ST2, to 57 mg/L at ST5. According to WQI classification, ST2 was classified in class I (<10 mg/L), while ST3 and ST4 were in class II (10-25 mg/L). ST1 was classified as class III (25-50 mg/L) and ST5 was classified as class IV (50-100 mg/L). Based on the statistical analysis, there was a significant difference in COD value between two sampling times with a *p*-value = 0.027, especially at ST1 and ST5, and there was a significant different between each sampling station with a *p*-value = 0.03 and the total SD was 39.69. COD was the amount of oxygen required to oxidize organic chemicals found in the water. High COD occurs due to the high organic and inorganic materials presence. When the concentration of organic matter in water increased, the COD value was also increased. Increase of chemical organic materials in water samples at ST1 and ST5 due to the disposal of solid waste into water was one of the contributors to the decline in water quality and DO which indicated by the presence of organic and inorganic materials.

The average TSS for all sampling stations at two sampling times was between 0.005 g/L to 0.041 g/L. Most stations were classified as class I (<25 mg/L) according to WQI, except for ST2, it was classified as class II (25-50 mg/L). From the one–way ANOVA analysis, it was found that there was no significant difference in TSS between the two sampling times for all stations with a *p*-value = 0.342 ($p>0.05$) and the SD = 0.006. The total SD for all stations was 0.02. High TSS content was due to the land development activities, mining and erosion activities ranging from mud, waste mineral, fine particles of sand silt and clay. High TSS can affects the metabolism and physiology of various organisms such as fish and aquatics organisms. They were products of run off TSS which increased with the increased of rainfall. Substances presence in the air also affected the rainfall characteristics. Water containing suspended solids and organic matter generally showed high turbidity. Heavy rainfall as in first sampling was the important factor for the reduction of contamination in the atmosphere (Kemker, 2014).

The average of NH_3-N for all sampling stations was range between 1.54 mg/L to 3.15 mg/L. The average NH_3-N value of ST2 shows the highest value of 4.65 mg/L which was at the first sampling. The average value of NH_3-N at ST5 was the lowest with a value of 1.12 mg/L at the second sampling. For ST1, ST2 and ST3, the NH_3-N were classified in class V (>2.7 mg/L) and ST4 and ST5 was classified in class IV (0.9 – 2.7 mg/L). One-way ANOVA analysis shows no significant difference in NH_3-N between two sampling times with a *p*-value of 0.153 ($p>0.05$), and there was no significant within stations with a *p*-value = 0.134 ($p>0.05$) and the total SD was 1.1583. High concentration of ammonium nitrogen in water can threaten aquatic life, especially in terms of respiration rate and increases pulse rate according to Halim et al. (2017). The higher value of

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ammonium nitrogen and other organic substances in water was most probably due to the untreated domestic sewage, waste materials and faeces.

Heavy Metals Content

A total of 19 heavy metals content including aluminum (Al), Arsenic (As), Barium (Ba), Beryllium (Be), Calcium (Ca), Cadmium (Cd), Cobalt (Co), Chromium (Cr), Copper (Cu), Iron (Fe), Potassium (K), Lithium (Li), Magnesium (Mg), Manganese (Mn), Sodium (Na), Nickel (Ni), Lead (Pb), Zinc (Zn) and Mercury (Hg) were measured during two sampling times at all five sampling stations of Klang River (*Table 3*).

Table 3. *Heavy metals concentration (ppb) at five sampling stations for two sampling times*

Metals (ppb)	ST1		ST2		ST3		ST4		ST5	
	First sampling	Second sampling	First sampling	Second sampling	First sampling	Second sampling	First sampling	Second sampling	First sampling	Second sampling
Al	17.91	23.31	56.73	7.54	12.14	7.21	6.89	4.13	26.69	14.01
As	50.81	74.37	51.12	64.25	51.86	65.85	47.42	50.26	44.2	40.49
Ba	104.8	230.08	109.21	249.71	101.5	263.08	106.43	284.69	124.83	287.24
Be	0.02	0.05	0.01	0.02	0.01	0.01	0.01	0.02	0.03	0.019
Ca	3454.99	2973.44	3681.42	2914.67	3617.17	3054.07	3880.95	3506.33	4741.87	4194.6
Cd	0.05	0.35	0.02	0.11	0.019	0.23	0.03	0.06	0.03	0.05
Co	0.23	0.21	0.12	0.14	0.11	0.12	0.13	0.13	0.19	0.21
Cr	3.52	3.29	4.8	2.55	3.81	2.33	3.87	2.43	3.65	2.27
Cu	2.03	2.36	1.44	2.65	1.91	2.28	1.27	1.93	2.12	1.6
Fe	115.9	354.58	125.21	287.05	107.81	313.02	86.86	363.63	110.44	249.54
K	5355.36	9901.15	5544.69	9887.01	5545.81	8330.59	5886.77	9638.96	6078.4	12388.8
Li	1.09	1.25	1.21	1.25	1.16	1.23	1.23	1.31	1.63	1.61
Mg	1635.42	2459.74	1742.29	2721.56	1700.41	2239.62	1924.03	2626.13	1977.16	2444.02
Mn	1.51	0.85	3.81	0.21	0.95	0.11	1.35	0.33	3.03	0.09
Na	16326.7	19628.8	17167.7	20686.9	17216.3	19397.8	18520.3	22116.1	18065.1	21763.8
Ni	1.43	1.79	1.57	1.47	1.22	1.32	1.32	1.52	1.66	2.3
Pb	1.6	0.03	0.47	0.37	0.08	1.32	0.07	0.01	0.08	0.02
Zn	30.24	34.55	29.15	12.37	22.79	35.52	21.67	16.12	21.32	14.1
Hg	0.75	0.88	0.19	0.25	0.16	0.26	0.11	0.16	0.35	0.56

Table 3 shows the average of heavy metals content at the five sampling stations for two sampling times. The unit used for heavy metals concentration in this study was 1 ppb which equal to 0.001 mg/L.

The average of Al concentrations during the two sampling times were 20.61 ppb, 32.14 ppb, 9.68 ppb, 5.52 ppb and 20.35 ppb for ST1, ST2, ST3, ST4 and ST5, respectively. The concentration of Al classified by WQI as class II (<0.06 mg/L). For As, the mean concentration value of two sampling times at ST1 was 62.591 ppb (0.0626 mg/L) which shows a maximum value and ST5 has the lowest mean concentration of As which was 42.346 ppb. The mean concentration of As classified by WQI class as class III (>0.05 mg/L). The mean concentration of Ba of two sampling times were 167.44 ppb, 179.47 ppb, 182.29 ppb, 195.56 ppb and 206.04 ppb for ST1, ST2, ST3, ST4 and ST5, respectively. It was found that the maximum mean concentration of Ba was at ST5 and the lowest was at ST4. The mean concentration of Ba classified by WQI as class I (<1 mg/L). For Be, the mean concentration of two sampling times was 0.041 ppb,

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_36553677

0.02 ppb, 0.015 ppb, 0.021 ppb and 0.025 ppb for ST1, ST2, ST3, ST4 and ST5, respectively. The mean concentration of Be classified by WQI as class I (<0.05 mg/L). It was indicated that, in this study, the mean concentration of Be was the lowest among the 19 heavy metals. The mean concentration of Ca which was the third highest after Na and K during two sampling times has the value of 3214.22 ppb at ST1, 3298.05 ppb at ST2, 3335.62 ppb at ST3, 3693.64 ppb at ST4 and 4468.24 ppb at ST5. The highest mean concentration of Ca was at ST5 and the lowest was at ST1. The mean concentration of Ca classified by WQI as class I.

The mean concentration of Cd of two sampling times has the second lowest value. The value was 0.21 ppb, 0.06 ppb, 0.13 ppb, 0.05 ppb and 0.05 ppb for ST1, ST2, ST3, ST4 and ST5, respectively. The mean concentration of Cd classified by WQI was at class I (<0.001 mg/L). For Co, the mean concentration value at ST1 was 0.23 ppb (0.00023 mg/L), ST2 = 0.13 ppb, ST3 = 0.12 ppb, ST4 = 0.13 ppb and ST5 = 0.21 ppb. The mean concentration of Co classified by WQI as class I. For Cr, the mean concentration value of two sampling times was 3.41 ppb, 3.68 ppb, 3.07 ppb, 3.16 ppb and 2.97 ppb for ST1, ST2, ST3, ST4 and ST5, respectively. Maximum mean concentration of Cr was recorded at ST2 and the lowest was at ST5. The mean concentration of Cr classified by WQI as class I (<0.05 mg/L). Cu mean concentration of two sampling times at ST1 was 2.20 ppb, ST2 = 2.05 ppb, ST3 = 2.10 ppb, ST4 = 1.60 ppb and ST5 = 1.86 ppb. The mean concentration of Cu classified by WQI as class I (<0.02 mg/L). For Fe, the mean concentration value of two sampling times was 235.24 ppb, 206.13 ppb, 210.42 ppb, 225.25 ppb and 179.99 ppb for ST1, ST2, ST3, ST4 and ST5, respectively. The mean concentration of Fe classified by WQI as class I (<1 mg/L). K has the second highest mean concentration after Na which the value for ST1, ST2, ST3, ST4 and ST5 was 7628.26 ppb, 7715.85 ppb, 6938.2 ppb, 7762.87 ppb and 9233.6 ppb, respectively. The mean concentration of K classified by WQI as class I.

The recorded mean concentration of two sampling times for Li was 1.17 ppb, 1.23 ppb, 1.20 ppb, 1.27 ppb and 1.63 ppb for ST1, ST2, ST3, ST4 and ST5, respectively. The mean concentration of Li classified by WQI as class I. Mg mean concentration of two sampling times showed the values of 2047.58 ppb at ST1, 2231.9 ppb at ST2, 1970.02 ppb at ST3, 2275.08 ppb at ST4 and 2210.68 ppb at ST5. The mean concentration of Mg classified by WQI as class I. For Mn, the mean concentration value was 1.18 ppb, 2.02 ppb, 0.53 ppb, 0.84 ppb and 1.57 ppb for ST1, ST2, ST3, ST4 and ST5, respectively. Maximum mean concentration of Mn was at ST2 and the lowest mean was at ST3. The mean concentration of Mn classified by WQI as class II (<0.1 mg/L). Na has the highest mean concentration among the 19 heavy metals. The recorded values for ST1 was 17977.8 ppb which was the lowest mean, ST2 = 18927.3 ppb, ST3 = 18307.1 ppb, ST4 = 20318.2 ppb, which was the highest value and ST5 = 19914.5 ppb. The mean concentration of Na classified by WQI as class I. The mean concentration value for Ni was 1.61 ppb, 1.52 ppb, 1.28 ppb, 1.42 ppb and 1.98 ppb for ST1, ST2, ST3, ST4 and ST5, respectively. The mean concentration of Ni classified by WQI class as class II (<0.05 mg/L). For Pb, the mean concentration value of two sampling times at ST1 was 0.82 ppb, ST2 = 0.42 ppb, ST3 = 0.70 ppb, ST4 = 0.05 ppb and ST5 = 0.05 ppb. The mean concentration of Pb classified by WQI as class III (<0.02 mg/L).

Zn has the mean concentration values of two sampling times of 32.40 ppb, 20.76 ppb, 29.16 ppb, 18.90 ppb and 17.71 ppb for ST1, ST2, ST3, ST4 and ST5, respectively. The mean concentration of Zn classified by WQI as class I (<0.4 mg/L). For Hg, the mean concentration recorded for ST1 was 0.89 ppb which was the highest value, for ST2 was

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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0.26 ppb, ST3 was 0.27 ppb, for ST4 was 0.17 ppb which was the lowest value, and ST5 was 0.57 ppb. The mean concentration of Na classified by WQI as class III (0.0001 mg/L). Most of the sources of heavy metals were from wastes and effluents that were being discharged into the river (Awotedu and Ogunbamowo, 2019). The high concentration of heavy metals was primarily owing to the industrial pollution. Heavy metals in the urban atmosphere were mainly derived from industrial activities (i.e. mining, smelting and fossil fuel combustion), traffic emissions (i.e. vehicle exhausts and the products of wear from tires, brake linings and bearings) and natural minerals sources, forest fires and oceans (Geiger and Cooper, 2010; Ismail and Hanafiah, 2019). According to Tunca et al. (2017), heavy metals have a significant threat to human health as it can accumulate in living organisms. The results showed that most of the heavy metals detected were in satisfactory with the standard for water and packaged drinking water as stated in Drinking Water Quality Standards for Malaysia by DOE.

Total Coliform and Fecal Coliform

The present of total coliform bacteria and fecal coliform, *E. coli* in Klang River was in the range of 102 to 105 cfu/100mL. The coliform bacteria group consist of several genera of bacteria belonging to the family Enterobacteriaces including *Enterobacter, Escherichia, Shigella, Salmonella, Protus* and *Klebsiella*. Most bacteria of this group mainly live in water from digestive system of human and animal, which can be as an indicator for polluted water with domestic untreated wastewater. An appropriate value of water sample was serially diluted with sterile normal saline in order to get countable colonies. Colony count of fecal coliform by bacterial counter chamber as shown in *Figure 3a* Luria agar and *Figure 3b* Luria broth.

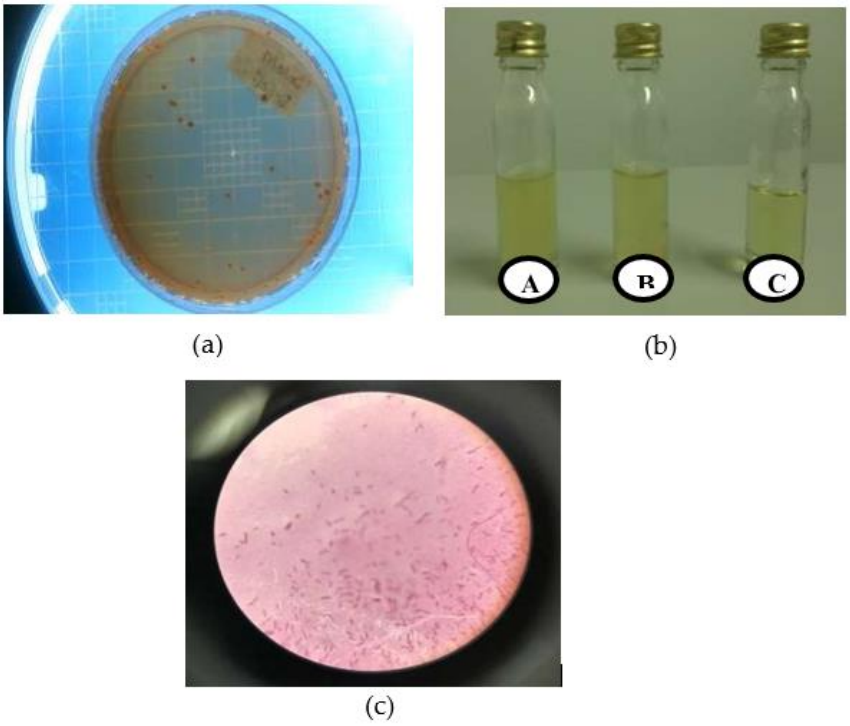

Figure 3. *Fecal coliform (E. coli) on (a) Luria agar and (b) Luria broth where E. Coli diluted in, A) 10-1, B) 10-2, C) negative; and (c) Gram negative short rod E. coli under light microscopy*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_36553677

Figure 3b showed the growth of fecal coliform (*E. coli*) in diluted 10-1, 10-2 and negative (control Luria broth only) as A, B and C, respectively. Total coliform bacteria presented in water samples at ST1, ST2 and ST3 was higher than in the water samples at ST4 and ST5. Range of total coliform in the Klang River was from 7 x 102 CFU/100 mL to 2.5 x 104 CFU/100 mL. The total coliform for ST1 was about 17500 CFU/100 mL, ST2 was 11300 CFU/100 mL and ST3 was 10700 CFU/100 mL, which showed a higher count of total coliform among other stations as shown in *Figure 4*. The total coliform at ST4 was about 4900 CFU/100 mL and ST5 was 4200 CFU/100 mL. According to INWQS for Malaysia, the level of total fecal coliform for recreational water is 400/100 mL and total coliform is 5000/100 mL which classified as class II.

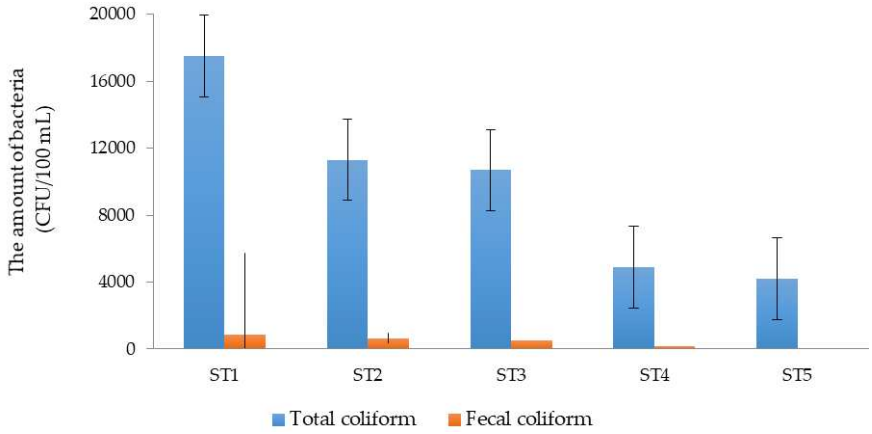

Figure 4. *Total coliform and fecal coliform at all stations*

Not all coliform is associated with the intestinal tract, some genera occur widely in nature (e.g. Enterobacter spp. associated with plant materials) while some fecal coliform may flourish in diverse environments outside the intestinal tract. However, a high number of total coliform in the Klang River may indicated the present of other pathogenic microorganisms that may cause illness. Coliform bacteria have been used as indicator of unsanitary conditions in water and foods for over century. The one-way ANOVA test showed that there were significant differences (p<0.05) for total coliform concentrations in the water between five stations and Klang River was classified in class III for ST1, ST2 and ST3 based on the INWQS.

Fecal coliform bacteria, which belong to Enterobacteriaces, were presented in large number in the feces and intestinal tracts of human and other warm-blooded animals, and can enter river water from human and animal's wastes. If a large number of fecal coliform bacteria (over 400 colonies/100 milliliters (mL) of water sample) are found in water, it is possible that pathogenic microorganisms are also present in the river water. *E. coli*, a gram negative short rod with negative oxidase reactions was chosen as an indicator for fecal pollution (*Figure 3c*). *E. coli* was presented at all time of sampling at Klang River indicating that the water was contaminated by fecal materials from humans. The number of fecal coliform presence in the river varies, some were not detected at 103 dilutions at ST4 and 5 and to maximum value of 3.4 x 103 CFU/100 mL. Stations with higher count of *E. coli* were ST1, ST2 and ST3 as shown in *Figure 4*.

According to INWQS for Malaysia, the level of fecal coliform for recreational water is 400/100 mL and total coliform is 5000/100 mL which classified as class III. ST1, ST2

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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and ST3 of Klang River were acceded the standard permitted level determined by DOE Malaysia. *E. coli, Salmonella* and other Enterobacteriaces bacteria were also found in the river with higher level especially at ST1, ST2 and ST3. ST4 and ST5 were classified as class II. Statistical analysis of coliform which tested by one-way ANOVA test showed there were significant differences with *p*-value, $p = 0.03$ for fecal coliform concentrations in the water between five stations with the SD of 0.26. Meanwhile there were no significant differences between the sampling times of all stations with $p>0.05$. In the study conducted by Balleste et al. (2020), they found that fecal pollution was caused by humans and animals wastes, domestic effluent, improper sanitation systems and land use of agricultural area.

Samples of ST1, ST2 and ST3 were more polluted by total and fecal coliform bacteria and not suitable for activities related to body contact or for fish farming. All stations received untreated human wastes which was discharged from condominiums, small manufactures, restaurants and malls complexes nearby. This may cause the higher count of total and fecal bacteria at these areas. Swimming and fishing in a river water with high levels of fecal coliform bacteria will increase the chance of developing illness (fever, nausea, diarrhea or stomach cramps) from pathogenic entering the fish body and human body through the mouth, nose, ears or injury of skin. Diseases and illnesses that can be contacted in water with high fecal coliform counts include typhoid fever, hepatitis, gastroenteritis and dysentery and ear indications. Fecal coliform, like other bacteria, can usually be killed by boiling water or by treating it with chlorine. Washing thoroughly with soap after contact with contaminated water can also help prevent indications according to APHA (1998).

Phytoremediation by Pistia stratiotes and Lemna minor L.

There were eight high toxic heavy metals which have been tested in the phytoremediation of river water samples using *Pistia stratiotes* and *Lemna minor L.* The value was taken on the 0, 7, 14 and 21 days of experiment. *Figures 5* and *6* show the heavy metals concentration in the phytoremediation test using *Pistia stratiotes* and *Lemna minor L.,* respectively.

Based on the figures, *Pistia stratiotes* and *Lemna minor* L. can reduced the concentration of heavy metals in the river water samples from day 7 until day 21 at each sampling station. The average of initial value of As was ranged between 40.49 ppb at ST5 and 74.37 ppb at ST1. The As concentration in the river for all stations was ranged from 3.89 ppb at ST5 to 5.11 ppb at ST1 after 7 days of treatment with *Pistia stratiotes*. On day 14 of the experiment, the concentration of As was reduced to as low as 2.26 ppb at ST2 and the value was continue to decrease to 2.17 ppb on day 21. It was found that there was a significant decreased of As concentration after 7 days. However, the phytoremediation became slower after 14 days at all stations. The statistical analysis showed that there was a significant difference of As level between stations and there was also a significant difference between sampling times and all the stations were classified as Class II. The standard deviation (SD) for ST2 and ST3 was 0.57 and 0.61, respectively which show a high significant with a *p*-value of $p<0.05$. For the phytoremediation by *Lemna minor* L., the As concentration ranged was from 40.26 ppb at ST5 to 67.55 ppb at ST1 on day 7 of treatment. The average level of As was decreased slightly to 67.23 ppb (0.067 mg/L) according to WQI with a standard of 0.05 mg/L at ST1 and to 40.05 ppb at ST5 after 14 days of phytoremediation. After 21 days of phytoremediation the As value was slightly decreased to 40.003 ppb at ST5 while the value was 66.211 ppb at ST1. It

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_36553677

can be deduced that, the As concentration was slightly decreased at all stations. The statistical analysis showed that there was a significant difference of As level between stations and there was no significant difference between sampling times and all the stations was categorized as Class II. The SD for ST1 and ST3 was 3.73 and 3.07, respectively which show a high significant with a *p*-value of $p = 0.001$.

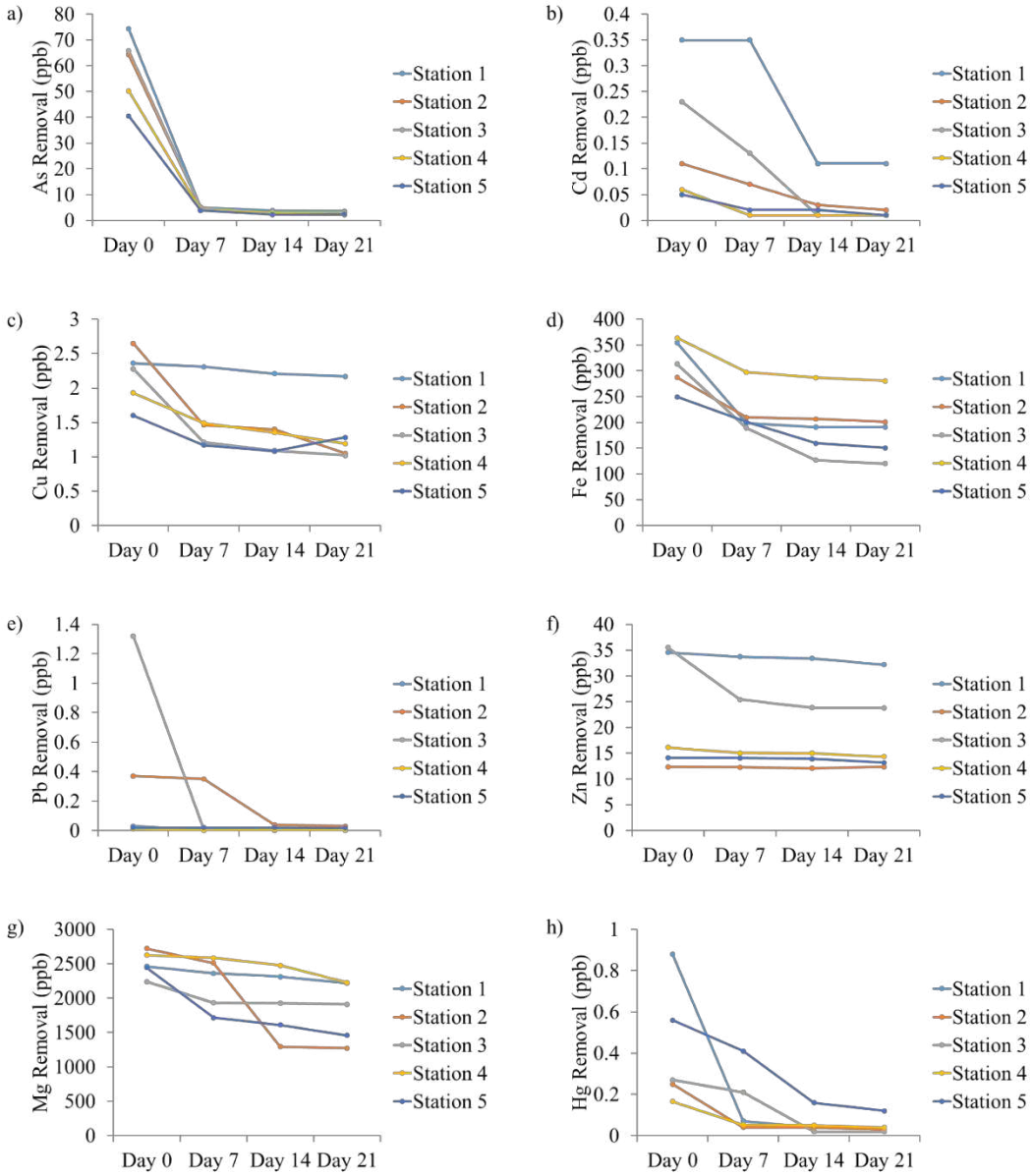

Figure 5. *Heavy metals removal for a) As, b) Cd, c) Cu, d) Fe, e) Pb, f) Zn, g) Mg and h) Hg by Pistia stratiotes*

The average of initial concentration of Cd was ranged between 0.05 ppb at ST5 to 0.35 ppb at ST1. After 7 days of treatment with *Pistia stratiotes*, the Cd concentration reduced to 0.01 ppb at ST4 and to 0.35 (0.0007 mg/L) at ST1. The average level of Cd was 0.011 mg/L according to WQI. On day 21, the lowest value of Cd was recorded at ST3, ST4 and ST5 which was 0.01 ppb. Statistical analysis showed that there was a

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_36553677

significant difference of Cd level between all stations which the *p*–value was $p = 0.023$. Meanwhile at all stations there was no significant difference between sampling times with *p*-value of $p>0.05$ and all stations was classified as Class III. It was noticed that there was a high decreased of Cd after phytoremediation treatment by *Pistia stratiotes* for all stations. For the phytoremediation by *Lemna minor* L., on day 7, the Cd concentration for all stations was ranged from 0.04 ppb at ST4 to 0.35 ppb (0.0007 mg/L) at ST1. The average level of Cd was 0.011 mg/L according to WQI. Based on the statistical analysis that has been performed, it was indicated that there was a significant difference of Cd level between all stations which the *p*–value was $p = 0.034$. Meanwhile at ST1, there was a significant difference between sampling times with a *p*-value of $p<0.05$ and all stations was classified as Class III especially after 14 days of treatment. It was found that there was a high decreased of Cd after phytoremediation for all stations.

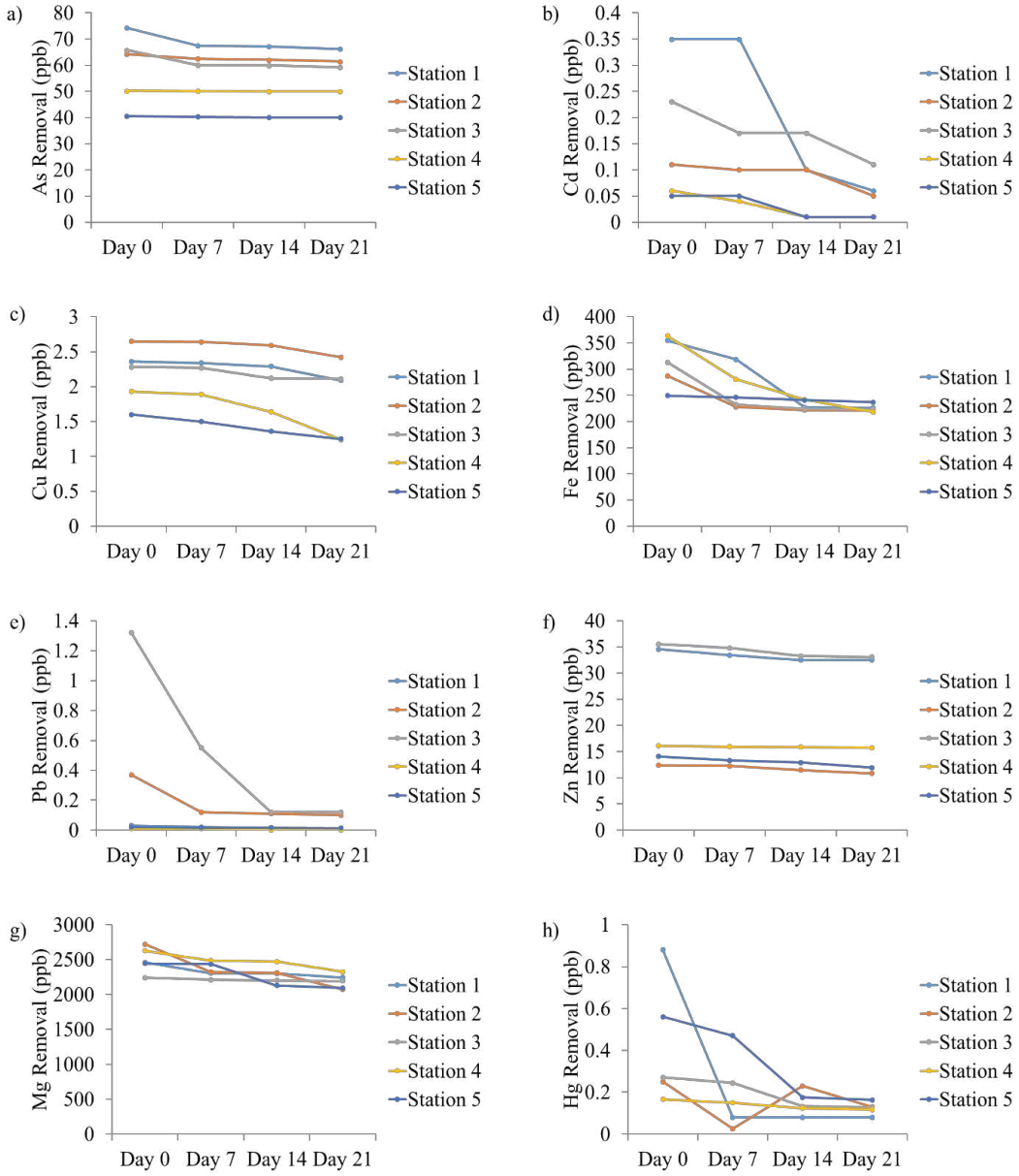

Figure 6. *Heavy metals removal for a) As, b) Cd, c) Cu, d) Fe, e) Pb, f) Zn, g) Mg and h) Hg by Lemna minor L.*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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The concentration of Cu has a ranged of initial value between 1.60 at ST5 and 2.65 at ST2. In the phytoremediation by *Pistia stratiotes*, the concentration of Cu showed a decreasing value on day 7, 14 and 21 for all stations. Cu concentration was reduced to between 1.02 ppb at ST3 to 2.17 ppm (0.0023 mg/L) at ST1 after 21 days of treatment. The average level of Cu was 0.012 mg/L according to WQI which was classified as class III. The results of statistical analysis showed that there was a significant difference of Cu levels between stations with p-value = 0.001 and a standard deviation of 0.59 and there was no significant difference between sampling times with $p>0.05$. *Lemna minor* L. was also able to reduce the concentration of Cu in the river water samples for all stations. On day 21 of treatment, the concentration of Cu was reduced to as low as 1.24 ppb which at ST4. The average level of Cu was 0.012 mg/L according to WQI which was classified as class III. It can be deduced that the phytoremediation by *Lemna minor* L. showed a slightly decreased in the average level of Cu concentration from day 7 until day 21. Based on the statistical analysis conducted, it was found that there was a significant difference of Cu levels between stations with p-value = 0.002 and the total SD was 0.46. There was also a significant difference between sampling times with $p<0.05$.

By means of Fe, the average initial concentration for all stations was between 249.54 ppb (ST5) and 363.63 ppb (ST4). The treatment of river water samples with *Pistia stratiotes* showed that the concentration level of Fe was slightly decreased from day 7 until day 21. After 21 days of treatment, the concentration of Fe at all stations was ranged from 119.99 ppb at ST3 to 280.33 ppb (0.280 mg/L) at ST4. According to WQI, the average level of Fe content should be 1 mg/L and classified as class II. The one-way ANOVA test showed a significant difference between the Fe concentrations for all stations especially samples for ST1, ST2 and ST3 compared to others stations with p-value of $p = 0.001$ and the total SD of 0.21. The Fe concentration in water samples was also slightly decreased from day 7 until day 21 when treated with *Lemna minor* L. The concentration of Fe was reduced to a lowest value of 218.27 ppb at ST4 and highest value of 237 ppb at ST5 after 21 days of treatment. The average level of Fe was 1 mg/L according to WQI and classified as class II. According to the one-way ANOVA test that has been performed, it showed that there was a significant difference between the Fe concentrations for all stations especially for ST1, ST2, ST3 and ST4 compared to ST5 with p-value $p = 0.001$ and the standard deviation was 47.41.

For Pb, the recorded average of initial concentration of Pb for all stations was between 0.01 ppb at ST4 and 1.32 ppb at ST3. In the phytoremediation by *Pistia stratiotes*, it was indicated that the concentration level of Pb was decreased on day 7, 14 and 21 of treatment. The Pb concentration for all stations was ranged from 0.001 ppb at ST1 to 0.03 at ST2 after 21 days. The average level of Pb was 0.02 mg/L according to WQI which indicates that the river was not polluted by Pb. The statistical analysis by one-way ANOVA test showed that there was no significant difference between the five stations and phytoremediation times with a p-value of $p>0.05$. All stations were classified as Class II. As for the treatment by *Lemna minor* L., the level of Pb concentration was also decreased from day 7 until day 21. After 21 days of treatment, the Pb concentration at ST4 showed the lowest final value which was 0 ppb. The average level of Pb was 0.02 mg/L according to WQI which indicates that the river was not polluted by Pb. The statistical analysis by ANOVA test showed that there was a significant difference between ST2 and ST3 for phytoremediation times with p-value $p<0.029$ but there was no significant for other stations. All stations were therefore reported to be classified as Class II.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Zn has the average of initial concentration for all stations of 12.37 ppb at ST2 and 35.52 ppb at ST3. For the phytoremediation by *Pistia stratiotes*, it was found that Zn concentration was slightly decreased from day 7 until day 21. On day 21, the Zn concentration for all stations was ranged from 12.35 ppb at ST2 to 32.17 (0.03 mg/L). The average level of Zn was 0.4 mg/L according to WQI which indicated that the river was not polluted by Zn. The range value of Zn in the river water recorded in all stations were classified as Class III. The one-way ANOVA test showed that there was no significant difference between the samples with sampling times for five stations with *p*-value $p = 0.085$, while there was a significant difference between stations with a *p*-value of $p = 0.002$ and the total SD was 16.44. The level of Zn concentration was also decreasing during the treatment with *Lemna minor* L. The value of Zn concentration for all stations recorded on the 21 days of experiment was ranged from 10.85 ppb at ST2 to 33.06 ppb (0.03 mg/L) at ST3. According to WQI, the average level of Zn was 0.4 mg/L which indicated that the river was not polluted by Zn. Based on the reported Zn concentration, the river water area at all stations can be classified as Class III. Statistical analysis using one-way ANOVA test showed a significant difference between the samples for five stations with the *p*-value of $p<0.05$ and the total SD was 0.1. There was also no significant difference between sampling times with p-value, $p>0.05$ and total SD of 10.44.

As for Mg, it was recorded that the average of its initial concentration for all stations was between 2444.02 ppb at ST5 and 2626.12 ppb at ST4. *Pistia stratiotes* was able to reduce the concentration of Mg in the phytoremediation of river water samples in this study. After 21 days of treatment the Mg concentration was reduced to 1272.81 ppb at ST2 (which indicated the lowest value) and the value was approximately 2225.88 ppb (2.23 mg/L) at ST4 (the highest value among the five stations). The average level of Mg was more than 5000 mg/L according to WQI which therefore indicated that the rivers in this study were in class I. The statistical analysis of one-way ANOVA test showed that there was no significant difference between the five stations with a *p*-value of $p>0.05$ and there was a significant difference between sampling times with $p = 0.002$ and the total SD was 170.8, with slight effect of phytoremediation for this plant on Mg value. The Mg concentration also showed a reduction in its value when being treated using *Lemna minor* L. After 21 days of treatment, the Mg concentration for all stations was ranged from 2073.44 ppb at ST2 to 2326.94 ppb (2.33 mg/L) at ST4. The average level of Mg was more than 5000 mg/L as standardized in WQI. Hence, the rivers in this study can be classified as class I. The one-way ANOVA test showed that there was no significant difference between the five stations with a *p*-value of $p = 0.86$ and there was a significant difference between sampling times with $p = 0.002$. The total SD was 109.08, with slight effect of phytoremediation for this plant on Mg value.

Hg has the average of initial concentration which range between 0.17 ppb at ST4 and 0.88 ppb at ST1. The phytoremediation of river water samples by *Pistia stratiotes* showed a decreasing in the concentration of Hg on day 7, 14 and 21 of treatment. The final Hg concentration recorded a value from 0.02 ppb at ST3 to 0.12 ppb (0.00012 mg/L) at ST5. The average level of Hg was 0.004 mg/L according to WQI. Thus, it was indicated that the water river was not polluted with Hg and all stations was classified as Class III. Based on the statistical analysis that has been performed, there was a significant difference between the five stations with a *p*-value of $p = 0.008$. The total SD was 0.1 and there was a significant difference between sampling times with a *p*-value of $p = 0.04$. When treated with *Lemna minor*, the Hg concentration was slightly decreased from day 7 until the end

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_36553677

of experiment. On day 21, the Hg concentration was reduced to a ranged of 0.08 ppb at ST1 to 0.16 ppb at ST5. According to WQI, the average level of Hg was 0.004 mg/L. Thus, it was indicated that the water river was not polluted with Hg and all stations can be classified as Class III. The parametric one-way ANOVA test showed that there was a significant difference between the five stations with *p*-value, $p = 0.02$ while the total SD was 0.13 especially for ST4 and there was no significant difference between sampling times with *p*-value $p = 0.109$.

Figure 7 shows the heavy metals removal percentage in five sampling stations by *Pistia stratiotes* and *Lemna minor* L.

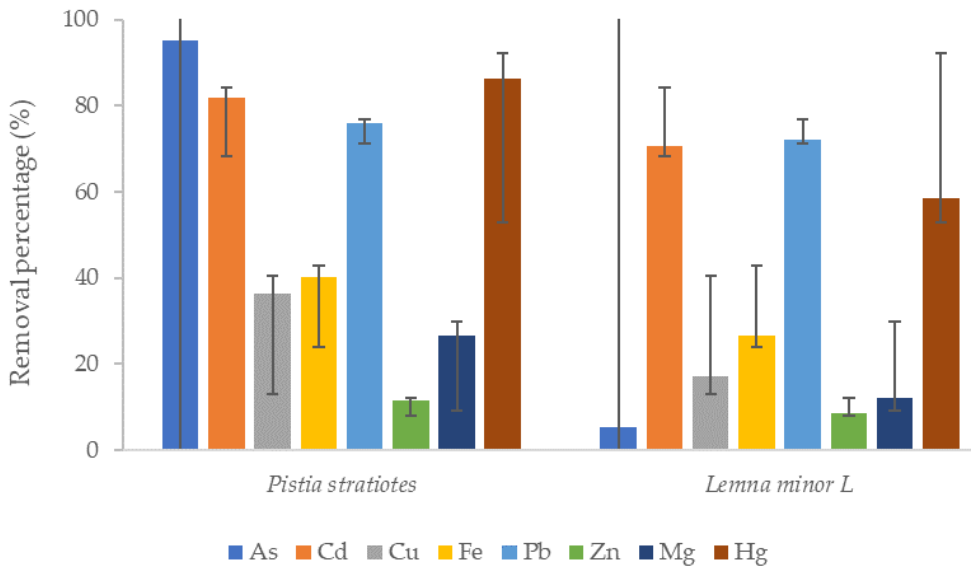

Figure 7. *Reduction percentage of heavy metals by Pistia stratiotes and Lemna minor L.*

As shown in *Figure 7*, it was demonstrated that *Pistia stratiotes* was able to reduce the concentration of As, Cd, Cu, Fe, Pb, Zn, Mg and Hg up to 96.62%, 95.65%, 60.38%, 61.67%, 99.24%, 32.97%, 53.23% and 96.59%, respectively. As reported by Tabinda et al. (2020), *Pistia stratiotes* was able to accumulate about 91.5% of Cu and 77.3% of Cr after 30 days of treatment. In a study performed by Rodrigues et al. (2020), the removal percentage of Zn by *Pistia stratiotes* reached a maximum value of 72% after 7 days of culture in 1.8 mg/L of Zn. Those studies found that higher accumulation of metals was observed in the roots of the plant. This is because *Pistia stratiotes* has a fibrous root system which help the plant to tolerate high concentrations of heavy metals. According to Akhtar et al. (2017), *Pistia stratiotes* can removed heavy metals without caused any damage to the metals. *Pistia stratiotes* has a rapid growth rate which make it able to accumulate high amount of nutrients to grow (Nizam et al., 2020). As stated by Hanafiah et al. (2018b), the ability of *Pistia stratiotes* in heavy metals and nutrients accumulation making this plant suitable to be used in phytoremediation treatment of contaminated wastewater.

While *Lemna minor* L. removed up to approximately 10.97%, 83.33%, 35.75%, 39.97%, 100%, 15.32%, 23.81% and 90.91% of As, Cd, Cu, Fe, Pb, Zn, Mg and Hg, respectively (*Figure 7*). Bokhari et al. (2016) reported that *Lemna minor* was more effective in accumulating Pb other than other metals, which was similar with the results

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DOI: http://dx.doi.org/10.15666/aeer/1905_36553677

obtained from the current study. However, Daud et al. (2018) found a maximum value of reduction for Cu which was 91%. *Lemna minor* is widely distributed throughout the world and can usually be found in ponds, swamps and ditches which were rich in nutrient content (Hazmi and Hanafiah, 2018; Hanafiah et al., 2019). *Lemna minor* L. can adapt extreme climatic and weather conditions such as drought, flood, frost, heat wave and inundation due to its physical characteristics (Ekperusi et al., 2019). *Lemna minor* L. can survive up to one months in drought. The plant also able to tolerate flood and areas where there is a high amount of water, for instance, it can be submerged for more than 120 days (Mohedano et al., 2012), making it perfect for wetlands because it can consume high amount of water. According to Radic et al. (2010), Lemna minor has been recommended for wastewater treatment because this plant is more tolerant to cold, easily harvested and also has a rapid growth rate, thus, it can be a better alternative for monitoring heavy metals content. *Table 4* shows the findings from several previous studies on the phytoremediation of heavy metals by *Pistia stratiotes* and *Lemna minor* L.

Table 4. *Phytoremediation of heavy metals by Pistia stratiotes and Lemna minor L.*

Type of plant	Heavy metals	Removal rate (%)	Duration	Authors
Pistia stratiotes	Cu, Cr	91.5, 77.3	30 days	Tabinda et al. (2020)
	Zn	72	7 days	Rodrigues et al. (2020)
	Pb, Cu	70.7, 66.5	30 days	Aurangzeb et al. (2014)
	Ca, Mg, Na, K	57, 55, 43, 54	60 days	Kumar et al. (2017)
	Cd	27.1	28 days	Kodituwakku and Yatawara (2020)
Lemna minor L.	Pb, Cd, Cu, Ni	97, 94, 94, 99	31 days	Bokhari et al. (2016)
	Cu	91	14 days	Daud et al. (2018)
	Cu, Ni, Pb	58, 68, 62	10 days	Yilmaz and Akbulut (2011)
	K	91.32	20 days	Mishra et al. (2012)
	Ca, Mg	45.7, 32.3	30 days	Farid et al. (2013)

It can be deduced that *Pistia stratiotes* reduced higher percentage of most heavy metals concentration as compared to *Lemna minor* L. Nonetheless, both *Pistia stratiotes* and *Lemna minor* L. plant have a very strong phytoremediation ability in heavy metals removal during the incubation days inside water pot in the laboratory. The absence of sunlight, spaces of growth, less oxygen, reduction of nutrition and other environmental factors after 14 days of growth have affected the plant capability to remove heavy metals from river water samples in the laboratory (Rezania et al., 2015; Suelee et al., 2017). Both *Pistia stratiotes* and *Lemna minor L* treatments achieved some reduction in heavy metals concentration, but samples were too variable within each site to be considered as significant at the real replication on site. Heavy metals uptake by the same plants showed well-marked cultivar differences in successive growing seasons in another field experiment in the Czech Republic. *Pistia stratiotes* and *Lemna minor* L. were best known as rhizofiltrator and phytoextractor due to their ability to accumulate more heavy metals in the roots when being contacted with water for 7 to 14 days (Barchanska et al., 2019; Ekperusi et al., 2019).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_36553677

Conclusions

Monitoring programs with frequent water samplings and determination of physiochemical parameters may representatively provide the status of the surface water quality. Overall, the WQI of the river water samples for rainy period and dry period was classified in classes II and III, respectively. Both plants, *Pistia stratiotes* and *Lemna minor* L. were a good phytoremediation agent especially in removal of heavy metals between 7 to 14 days of incubation. ST1, ST2, and ST3 of Klang River were high polluted by total coliform bacteria byF 17500, 11300 and 10700 CFU/100 mL, respectively. The authorities must be aware of the implications and limitations of benchmarking using the INWQS and WQI, so that river water quality preservation efforts can be executed seamlessly. More importantly is the effective utilization of these methods by the responsible agencies and parties involved in watershed management.

Based on the findings of the present study, it is recommended that phytoremediation plants like *Pistia stratiotes* and *Lemna minor* L. be planted on both sides of the river by placing simple barrier to locate the plant and keeping it in place to optimize heavy metals uptake. Future studies can be conducted to explore more on the pollutants uptake mechanism in terms of toxicity, tolerance towards heavy metals and accumulation of heavy metals in the plants. Besides, the study of phytoremediation by these plants should be carried-out in a longer time length as the time required for plant cultivation as well as plant acclimatization would take a very long time, especially the plant cultivation which would take up to at least few months. It also suggested to increase water tests for total and fecal coliform during the two sessions in different stations in Klang River and other rivers to reduce the pollutions and infection by this pathogenic bacterium. Cooperation from various authorities in Klang River basin management, flood mitigation, and environmental improvement through river basin management training, workshops, forums and regional technical assistance should be improved.

Acknowledgements. Marlia Mohd Hanafiah was financed by the UKM research grants (GUP-2020-034 and DIP-2019-001).

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DOI: http://dx.doi.org/10.15666/aeer/1905_36553677

DETERMINING OPTIMAL DRIP-IRRIGATION VOLUMES AFTER WETTING FOR MULCHED DRY-SEEDED COTTON (*GOSSYPIUM HIRSUTUM L.*) DURING THE SEEDLING STAGE USING HYDRUS-3D

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(Received 29th Mar 2021; accepted 19th Jul 2021)

Abstract. Cotton (*Gossypium hirsutum L.*) is an important cash crop in the Xinjiang Autonomous Region of China where agricultural development is severely hampered by the scarcity of water resources and increasing soil salinity. We developed a new method of establishing cotton crops to maintain crop yields while reducing water use and suppressing salinity. The method consists of dry seeding after wetting with drip irrigation, without the traditional winter or spring irrigation. We verified that the HYDRUS-3D software package could successfully simulate the distribution of water and salinity at the seedling stage in 2009 and used it to determine the irrigation volumes during the seedling stage in 2010. The observed and simulated results were in accordance, and soil salinity did not exceed the salinity threshold in the root zone, indicating that HYDRUS-3D could accurately simulate water and salt transport and was useful for designing appropriate irrigation regimes. A double drip line with single mulch and a low emitter discharge should be applied in dry seeding and after wetted under moderate soil salinity. In addition, in order to help local agricultural production, we chose some impact factors (emitter spacing, emitter discharge and salinity content) based on the current local situation, evaluated the factors' effects on water and salinity distribution, irrigation uniformity (Christiansen uniformity coefficient, *CU*) and leaching rate (L_r), in order to determine the optimal irrigation volume at the seedling stage using by HYDRUS-3D.
Keywords: *cash crop, water-saving technology, water and salt transport, salinity threshold, computer software model*

Introduction

Xinjiang has the largest area of cotton (*Gossypium hirsutum L.*) production in China, where cotton is an important economic crop. It is classified as a salt-tolerant crop, but its tolerance is both limited and variable at different growth stages (Sharif et al., 2019; Long et al., 2019). Different growth stages of cotton have salinity thresholds, above which growth is adversely affected. Jia et al. (1987) reported that cotton growth was seriously suppressed at a salinity of 5 g kg^{-1}. Sun et al. (2009a) reported that thresholds > 4.2 and > 3.3 g kg^{-1} at depths of 20 cm and 40 cm, respectively, during the seedling stage had an impact on cotton yield. Dong et al. (2009) found that seedling emergence was 40% and seedling survival rate was 30% at salinities > 4 g kg^{-1} in a

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_36793702

coastal, saline cotton field in Shandong province. Wang et al. (2010) found that the salinity threshold was 5.8 g kg^{-1} for cotton seedlings in the southern part of the Xinjiang Uygur Autonomous Region of China. Feng et al. (2011) reported threshold values of 5.03 g kg^{-1} at budding and 9.8 g kg^{-1} at flowering, with more suitable values of 2.77 and 5.84 g kg^{-1}, respectively. Sun et al. (2009b) found that a salinity of 2.8 dS m^{-1} had no effect on cotton yield at bolling. These results indicate that the threshold of salt tolerance of cotton is < 6 g kg^{-1}. Ramoliya and Pandey (2002) found that cotton was particularly sensitive to salinity during the seedling stage. High salinity can inhibit seed germination, emergence, growth. Highly saline soils can reduce nutrient uptake via ionic imbalances and competition, factors that lead to crop stunting, accompanied by lower quality and yield. Soil salinity must therefore be maintained at levels below the salt-tolerance threshold, particularly during the seedling stage.

Xinjiang is a typical arid and semi-arid region in China with scarce water resources. Increasing urban and industrial water demands is decreasing the availability of water for agriculture. Efficiency measures are required to address this problem. Drip irrigation with plastic mulch is one of the best methods to conserve water and increase field crop yields (Zhang et al., 2017; Zong et al., 2020; Qin et al., 2016; Yuan et al., 2019; Ospanbayev et al., 2017). The technique can regulate soil temperature, decrease soil salinity and enhance water-use efficiency (Cook et al., 2006; Li et al., 2017a; Filipović et al., 2016; Hu et al., 2018; Ning et al., 2015). Some studies have reported that cotton roots were mostly distributed in the upper 0.4 m of soil when drip irrigation was applied, and most roots were within the upper 0.2-0.3 m (Wei et al., 2002; Liu et al., 2011). Agricultural practices in the study region for increasing water content and decreasing salinity in the soil include winter and spring surface irrigation in early November and mid-March, with volumes of 3000 and 1500 m^3 ha^{-1}, respectively. Irrigating at these times has advantages, but winter and spring irrigation can also raise the water table, increase deep water percolation and increase soil salinity. A new method has been developed to save water and ensure cotton emergence, and the most meet requirement water and salinity threshold during the seedling stage: dry seeding followed by drip irrigation with mulching during establishment, without winter and spring irrigation. This method can improve cotton yield, emergence rate, soil temperature and water conservation (Wang et al., 2006, 2012). Irrigation volume, however, is usually based on experience and can lead to the waste of freshwater and a decrease in emergence rate. These problems must therefore be solved.

Various empirical, analytical and numerical models have been developed (Philip, 1968; Warrick, 1974; Moncef et al., 2002; Kandelous et al., 2008; Dabral et al., 2012; Saxena et al., 2018; Nouri et al., 2019; Kilic, 2020; Karimi et al., 2020) and are widely used. Most of these studies, however, used either planar or axi-symmetrical two-dimensional models (Nazari et al., 2020; Ghazouani et al., 2019; Shiri et al., 2020; Karandish and Šimůnek, 2018). Drip irrigation, though, presents a fully three-dimensional flow problem, especially when two adjacent wetting patterns begin to overlap (Kandelous et al., 2011). The confluence of wetting fronts is common, and cotton is always planted in the overlap zone (the zone irrigated by both of a pair of adjacent drippers), hence knowledge of the distributions of water and salinity in the zone is very important for achieving high crop yields. The optimal irrigation volume must therefore be known to ensure efficient crop production and soil conservation. Numerical modeling using HYDRUS(2D/3D) software (Šimůnek et al., 2008), which is

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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widely used to simulate the movement of water, heat and/or solutes in two or three dimensions in variably saturated porous media, was thus used in this study. (He et al., 2018; Grecco et al., 2019; Haghnazari et al., 2020). Cote et al. (2003) used HYDRUS-2D software to analyze soil water and solute transport under subsurface drip irrigation. Karandish and Šimůnek (2018) studied the soil water movement under single point source drip irrigation. Zhang et al. (2018) studied the variation of horizontal diffusion radius and vertical infiltration depth of point source infiltration in clay loam soil under different emitter discharge, initial soil moisture content and volume mass, and the results showed that the horizontal and vertical diffusion of water in Latosol had an exponential relationship with drip irrigation time and positive correlation with flow rate. Zhao et al. (2018) established the dynamic model of soil water movement under the condition of point source drip irrigation and carried out numerical simulation according to the theory of unsaturated soil water movement. Fan et al. (2020) established the mathematical model of soil water movement and solute movement of buried point source and used the model to describe the distribution law of water and fertilizer movement of sandy loam and loam under the condition of subsurface drip irrigation. Shan et al. (2019) established the mathematical model of emitter flow rate of point source drip irrigation, analyzed the change process of each factor in the model with the emitter flow rate, and determined the main factors affecting the emitter flow rate design of point source drip irrigation based on the equivalent cylindrical wetted body model. To sum up, HYDRUS(2D/3D) software has been widely applied for different crops, such as almond, citrus, wine grapes, maize, eggplant, olive orchards and cotton (Phogat et al., 2020; Scognamiglio et al., 2019; Autovino et al., 2018) and also employed to desgin optimal irrigation strategies (Karandish and Šimůnek, 2019). Therefore, we decide to employed the HYDRUS(2D/3D) software to conduct research. The objectives of this study were to: (i) evaluate the accuracy and usefulness of the HYDRUS-3D model, (ii) determine optimal irrigation volumes under dry seeding and wet-establishment conditions using the model and (iii) provide a reference for designing suitable drip-irrigation schedules and system arrangements.

Materials and methods

Experimental site

Field experiments were conducted in 2009 and 2010 at the Management of Irrigation Station of BaZhou District in Korla County, China (41°35′N, 86°10′E, 903 m a.s.l.). The region is classified as a warm-temperate arid zone with a continental climate. The mean annual precipitation is approximately 58 mm (Liang et al., 2019), most of which falls between June and August. Mean annual evaporation from a free-water surface is 2273-2788 mm (Li et al., 2018). The long-term mean annual temperature is 10.5 °C, with a maximum of 43.6 °C and a minimum of -9.4 °C (Liang and Shi, 2021). Annual total sunshine is 3036 h, and the annual frost-free period is 188 d (Chen et al., 2018). The emergence period of cotton generally lasts 10-15 days (from sowing to emergence), which requires low temperature of 12.1-13.2 ℃, effective accumulated temperature (≥12 ℃) of 57.3-1172 ℃ and active accumulated temperature (≥10 ℃) of 88.2-463.2 ℃. The seedling stage (from emergence to budding) generally lasts 30-40 days, requiring low temperature of 16.9-18.2 ℃, effective accumulated temperature (≥19 ℃) of 32.2-323.0 ℃ and active accumulated temperature (≥10 ℃) of 331.6-1411.6 ℃. The bud

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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stage (from budding to flowering) generally lasts 25-30 days, requiring low temperature of 12.8-18.1 ℃, effective accumulated temperature (≥20 ℃) of 16.1-189 ℃ and active accumulated temperature (≥10 ℃) of 608.2-1061.6 ℃. The flowering and Bolling stage (flowering to boll opening) generally lasts 50-70 days, requiring low temperature of 15.4-18.7 ℃, effective accumulated temperature (≥20 ℃) of 34.6-324.5 ℃ and active accumulated temperature (≥10 ℃) of 1114.6-1636.4 ℃ (Su et al., 2015).

The soil is sandy loam textured consisting 48% sand, 46% silt and 6% clay in the upper 0.6 m of the profile (Li et al., 2019). The average bulk density is 1.56 g cm^{-3} and the average field capacity is 0.255 cm^3 cm^{-3} in the upper 0.6 m of the profile (Tan et al., 2018). The mineral content of the water used for irrigation is 0.8 g L^{-1}.

Experimental design

Three treatment combinations were each applied to three 13.5 × 10 m (135 m^2) plots, for a total of nine plots, in both 2009 and 2010. Each plot in 2009 comprised nine beds, each supplied with water by a single irrigation pipe, in which four rows of cotton seeds were planted 0.1 m apart, with 0.2 m between rows (*Fig. 1*) and covered with plastic mulch. The emitter spacing was 0.3 m, and the discharge rate of the emitters was 3 L h^{-1}. Each plot in 2010 also had nine beds, each with four rows of cotton that were covered with plastic mulch, but double (lateral) irrigation pipes were used. The emitter spacing was 0.3 m, and the discharge rate of the emitters was 1.8 L h^{-1}. The lateral pipes were 0.6 m apart, and two rows of cotton plants were planted 0.1 m apart and 0.1 m either side of each pipe, with a gap of 0.4 m in the center of the bed between each double row. The leaf area index (LAI) of cotton was determined by dividing the total actual leaf area by land area at the initial growth stage. The length and width of each leaf on 5 medium-level growing plants were recorded every 5 days and the products calculated as leaf area. Then actual leaf area was obtained by summing up all the leaf areas and converting the sum using a correction coefficient of 0.73 (Su et al., 2015). The average initial soil water contents in the plots were 0.21 and 0.20 cm^3 cm^{-3} and the salinities were 7.3 and 8.3 g kg^{-1} in the 0-40 cm layer in 2009 and 2010, respectively. The salinity content was moderate (Hafsi et al., 2017). Soil samples were collected from four layers (0-10, 10-20, 20-30, and 30-40 cm) at two locations (0 and 30 cm from an emitter, and in the center of the overlap region between two emitters, respectively) in each plot. The electrical conductivity (*EC*) of soil-solution extracts (1:5 soil–water) was measured with a DDS-307A conductivity meter (Shanghai Precision & Scientific Instrument Inc., Shanghai, China). Salinity thresholds are usually expressed in g kg^{-1} in China, so we determined the relationship between g kg^{-1} and dS m^{-1} as follows. A 1:5 soil–water mixture was shaken for 3 min and filtered. Sixty milliliters of filtrate was placed in a porcelain dish and heated in a water bath. The filtrate was then oven-dried in the dish for 4 h, cooled for 30 min, and weighed. The filtrate was dried for a further 2 h and reweighed, checking that the two measurements were equal. The solid residue was then mixed with 15% hydrogen peroxide, heated in a water bath, and the other operations were repeated. The relationship between S and $EC_{1:5}$ is given by *Equation 1*.

$$S = 4.088EC_{1:5} \qquad R^2 = 0.997 \qquad \text{(Eq.1)}$$

where S is the salinity (g kg^{-1}) and $EC_{1:5}$ is the measured conductivity (dS m^{-1}).

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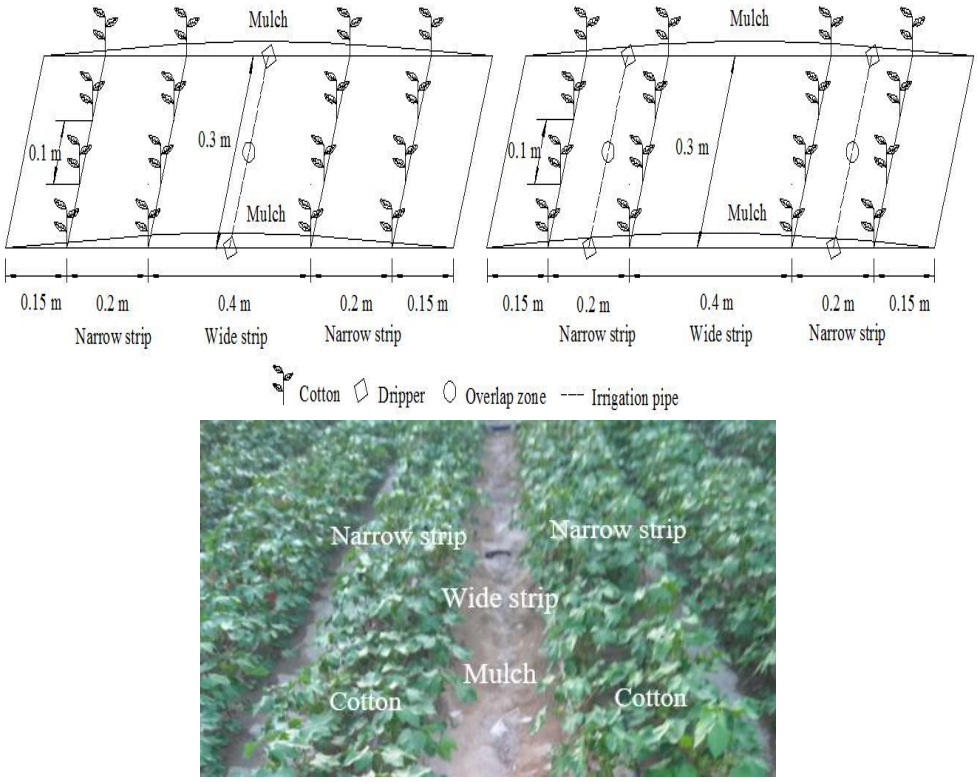

Figure 1. *Schematic layout of the lateral pipes and cotton plants in each bed in the experimental plots*

Irrigation volume and fertilizer application

Cotton seeds were planted on 26 April 2009 and on 28 April 2010. During the seedling stage, the irrigation volumes were 750 m^3 ha^{-1} on 30 April 2009 and 750 m^3 ha^{-1} on 2 May 2010. A compound fertilizer, consisting of urea 375 kg ha^{-1}, ammonium phosphate 300 kg ha^{-1}, potassium sulfate 300 kg ha^{-1} and farm manure 215 kg ha^{-1} was incorporated into the soil before planting. Fertilizer was also applied during cotton growth every two irrigation cycles (every 7 d) at rates of urea 45 kg ha^{-1} between budding and the start of bolling and urea 75 kg ha^{-1} thereafter to the bolling peak. From the end of the seedling stage to the boll stage, spray insecticides were applied to cotton fields every 5-7 days to prevent cotton diseases and insect pests. Cotton was toppled at the end of July to control the growth of the main stem and ensure the formation of cotton yield.

Numerical modeling

Water-flow simulation

The equation governing flow for these conditions is given by the following modified form of Richards' equation (*Eq. 2*):

$$\frac{\partial \theta}{\partial t} = \frac{\partial}{\partial x_i}\left[K\left(K_{ij}^{A}\frac{\partial h}{\partial x_j} + K_{iz}^{A} \right)\right] - S \qquad \text{(Eq.2)}$$

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where θ is the volumetric water content (cm^3 cm^{-3}), h is the pressure head (cm), t is time (min), x_i (i = 1, 2, 3 for a three-dimensional model) are spatial coordinates (cm), S is the sink term (cm^3 cm^{-3} min^{-1}), K_{ij}^A and K_{iz}^A are components of the dimensionless anisotropy hydraulic K^A (dimensionless) and K is the unsaturated hydraulic-conductivity function (cm min^{-1}) given by conductivity tensor (*Eq. 3*):

$$K\left(h,x,y,z\right)=K_s\left(x,y,z\right)K_r\left(h,x,y,z\right) \tag{Eq.3}$$

where K_r is the relative hydraulic conductivity (dimensionless) and K_s is the saturated hydraulic conductivity (cm min^{-1}).

The soil hydraulic properties were specified based on the van Genuchten model (*Eqs. 4–6*):

$$\theta(h)=\begin{cases}\theta_r+\dfrac{(\theta_s-\theta_r)}{(1+\left|\alpha h\right|^n)^m}, h<0\\ \theta_s, \qquad\qquad h\geq 0\end{cases} \tag{Eq.4}$$

$$K(h)=\begin{cases}K_sS_e\left[1-\left(1-S_e^{1/m}\right)^m\right]^2, \ h<0\\ K_s, \qquad\qquad\qquad h\geq 0\end{cases} \tag{Eq.5}$$

$$S_e=\frac{\theta-\theta_r}{\theta_s-\theta_r} \tag{Eq.6}$$

where θ_s is the saturated water content (cm^3 cm^{-3}); θ_r is the residual water content (cm^3 cm^{-3}); α, n and l are shape parameters, with m = 1-1/n; and S_e is the effective saturation.

Salt-transport simulation

Solute transport within the soil profile, which is controlled by both infiltration and diffusion, can be described by the advection-diffusion equation (*Eq. 7*):

$$\theta\frac{\partial c}{\partial t}=\frac{\partial}{\partial x_i}\left(\theta D_{ij}^w\frac{\partial c}{\partial x_j}\right)-\frac{\partial q_i c}{\partial x_i} \tag{Eq.7}$$

where c is the solute concentration in the liquid (g L^{-1}), D_{ij}^w is the effective dispersion coefficient tensor in the soil matrix (cm^2 min^{-1}) and q_i is a component of the fluid flux density.

Root uptake

The water-stress response-function model of Feddes et al. (1978) was used to account for water stress, and the threshold slope model of Khosla (1996) was used for salinity

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stress. Parameters of the water-and salinity-stress response functions were obtained from the literature (Feddes et al., 1978; Khosla, 1996; Rahneshan et al., 2018; Azad et al., 2018). A multiplicative model was used to account for the combined effects of water and salinity stress (Kumar et al., 2021). Cotton roots were sampled at a regular network of sampling points and measured using DT-SCAN (Chen et al., 2020). Root distributions of 57, 38 and 5% at depths of 10, 20 and 30 cm, respectively, were assumed based on the analysis the results. HYDRUS does not allow a temporally variable root zone, so a constant root distribution was used during the simulations (Han et al., 2015).

Initial and boundary conditions

Measured soil water contents and salinity were used as the initial conditions in the flow domain. Temporally variable boundary conditions under the mulch were applied for evaporation, transpiration, and precipitation were obtained from meteorological data. Daily precipitation and reference evapotranspiration (ET_o) were recorded by a weather station within 30 m of the experimental field. The daily crop potential evapotranspiration (ET_p) was calculated by *Equation 8:*

$$ET_p = K_c \cdot ET_o \tag{Eq.8}$$

where K_c is the crop coefficient (0.45) at the seedling stage (Liu et al., 2013).

The ET_p consists of potential transpiration (T_p) and potential evaporation (E_p), which are described by *Equation 9* (Mahey et al., 1984; Campbell and Norman, 1989).

$$\begin{aligned} T_p &= (1 - e^{-k \cdot LAI}) ET_p \\ E_p &= ET_p - T_p \end{aligned} \tag{Eq.9}$$

where k is the radiation-extinction coefficient (0.58 for cotton; Srinet et al., 2019) and LAI is the leaf area index (0.05-0.20 at the seedling stage; Su et al., 2015).

The variable-flux boundary condition for irrigation was based on the length of daily irrigation. Water volumes coupled with irrigation timing were used to determine the input values for the variable-flux boundary condition used as the drip-irrigation source in the HYDRUS-3D simulation. The atmospheric boundary condition was used for bare soil. A constant-flux boundary condition was used along boundary elements representing the emitter during the application of water in simulations of the field experiment. The constant boundary fluxes represented the corresponding measured fluxes of the field experiments. The flux was calculated by dividing the discharge rate by the ponded-surface area of the boundary that represented an emitter in the HYDRUS model, because the HYDRUS model cannot describe changes in the ponded-surface area. We thus chose a constant value that was measured and calculated using the Bresler (1978) equation for 2009 and 2010, respectively. We used the equation in 2010 because the measured and calculated radii of the ponded-surface area in 2009 were 6.8 and 7.7 cm, respectively. The emitter boundary became a zero-flux boundary after each irrigation. Zero-flux boundary conditions were also used for all three dimensions both during and after irrigation. A zero-flux condition was also used along the soil surface, because evaporation could be neglected due to the use of plastic mulch during irrigation. A free-drainage boundary condition was applied along the lower boundary.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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HYDRUS-3D uses the Galerkin finite-element method to solve the governing water-flow equation and the advection-diffusion equation. The transport domain was a set of rectangular parallel pipes (170 cm long, 30 cm wide and 150 cm deep). Running the HYDRUS-3D model required specifying the hydraulic parameters θ_s, θ_r, K_s, α, n, l, D_L (Longitudinal Dispersivity) and D_T (Transverse Dispersivity) (*Table 1*).

Table 1. *Estimated soil hydraulic parameters in 2009 and 2010*

	θ_r ($cm^3\ cm^{-3}$)	θ_s ($cm^3\ cm^{-3}$)	α (cm^{-1})	n	K_s ($cm\ d^{-1}$)	l	D_L (cm)	D_T (cm)
2009	0.0311	0.411	0.019	1.6	24.91	0.5	35.4	5.3
2010	0.0391	0.420	0.016	1.8	30.24	0.5	30.0	6.7

Statistical analysis

The performance of the model simulation was evaluated using two statistical indices. The mean absolute error (*MAE*) and the root mean square error (*RMSE*) quantified the differences between the observations and simulations and were calculated as *Equations 10* and *11,* respectively.

$$MAE = \frac{1}{N}\sum_{i=1}^{N}\left|P_i - O_i\right| \quad \text{(Eq.10)}$$

$$RMSE = \sqrt{\frac{1}{N}\sum_{i=1}^{N}(P_i - O_i)^2} \quad \text{(Eq.11)}$$

where P_i is a simulated value, O_i is an observed value and N is the total number of observed values.

Results and discussion

Soil water content (SWC) and salinity

SWC varied spatially, decreasing horizontally with distance from the drippers and vertically increasing with depth because most of the roots were distributed in the upper soil in both 2009 and 2010 (*Figs. 2* and *3,* respectively). Average SWCs at the end of the seedling stage were 0.22 and 0.18 $cm^3\ cm^{-3}$ in 2009 and 2010, respectively. Average SWCs at the end of the seedling stage were 80 and 70% of the field capacity in 2009 and 2010, respectively. Chen et al. (2019) found that a reasonable SWC ranged from 60 to 80% of the field capacity at various stages, indicating that the irrigation volume was able to meet the water requirements of the crop. SWC was higher in 2009 than in 2010, perhaps salinity was higher in 2009 and exceeded the threshold for cotton at the seedling stage, thereby decreasing root uptake. Ramos et al. (2011), and Bazihizina et al. (2017) also found that higher salinity could decrease soil osmotic potential, reduce root uptake and thus increase SWC.

Soil salinity also varied over time in both 2009 and 2010 (*Figs. 4* and *5,* respectively), perhaps because evapotranspiration and root uptake increased. Salinity also increased horizontally with distance from the dripper and vertically with depth

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because the salt moved into deeper soil with the water. From irrigation event finished to the end of seedling stage, the soil salinity content increased by 55%, 28%, 22%, 9% and 57%, 29%, 22%, 13% for 10 cm, 20 cm, 30 cm, 40 cm in 2009 and 2010, respectively. Li et al. (2018) also found that the upper soil TDS climbed up faster driven by their more pronounced transpiration through root water uptake. The reason is that cotton root mainly distribution in the upper soil layer and as evapotranspiration effects progressively prevailed, soil water started to transport towards the upper soil, it is result that the water was consumed and salinity left on the upper soil.

Average salinities at the end of the seedling stage were 6.3 and 5.8 g kg^{-1} in 2009 and 2010, respectively. Salinity was higher in 2009 than 2010, perhaps because the experimental layout differed between 2009 and 2010, including number of drip lines and emitter discharge. Chen et al. (2019) found that salt accumulation in the root zone was lower with a double than a single drip line under the same volume of irrigation. Emitter discharge could also account for the higher salinity in 2009 than 2010. A lower rate can limit surface ponding and improve the movement of water vertically but not horizontally, but a higher rate will have the opposite effect. Ghazouani (2019) found that increasing the rate of discharge increased the horizontal size of the wetted area and decreased the depth of wetted soil in the same soil type. Che et al. (2021) reported that a lower emitter discharge could leach salinity more than a higher rate. The optimal drip-irrigation layout should thus have a double drip line and a low emitter discharge to ensure that the cotton does not suffer from salinity stress.

Water movement and solute transport can be complicated problems with drip irrigation due to three-dimensional infiltration (before the wetting fronts overlap) and to both two- and three-dimensional infiltration (after the wetting fronts overlap). The overlapped zone varied widely with the crop planted, so the distributions of water and salinity were also criteria for evaluating reasonable irrigation volumes. The distribution and change of water and salinity in the overlap zone was similar to under dripper. SWC and salinity were 0.21 and 0.18 cm^3 cm^{-3} and 6.5 and 5.9 g kg^{-1} in 2009 and 2010, respectively (not shown in figure). SWC was thus sufficient, and the salinity did not exceed the threshold and provided a suitable environment for cotton growth. The irrigation volume was also reasonable in 2010. The HYDRUS model is thus a feasible tool for designing optimal irrigation strategies.

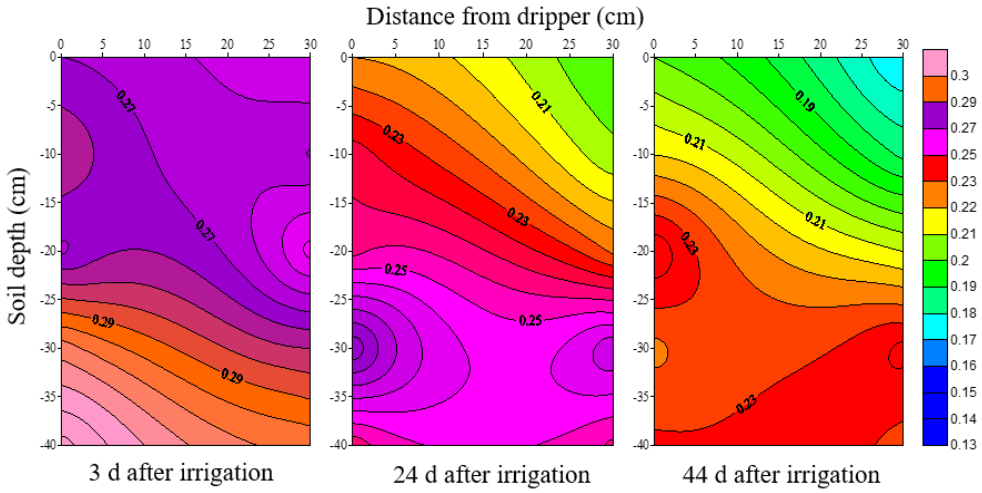

Figure 2. *Variations of soil water content after single irrigation over time in 2009*

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DOI: http://dx.doi.org/10.15666/aeer/1905_36793702

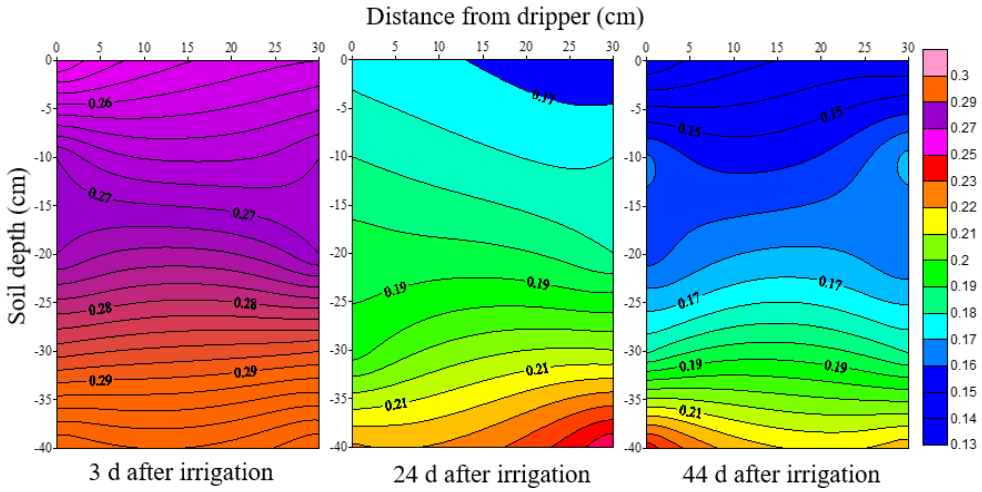

Figure 3. *Variations of soil water content after single irrigation over time in 2010*

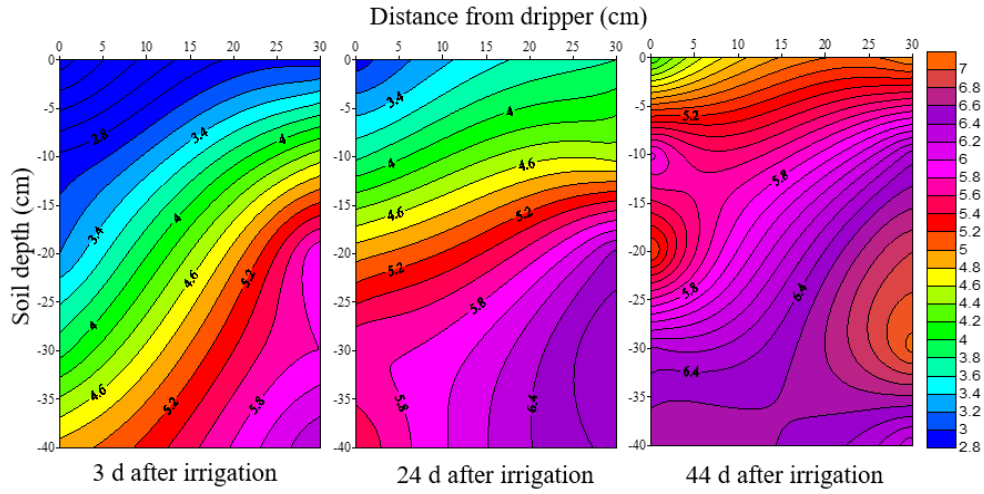

Figure 4. *Variations of soil salinity after single irrigation over time in 2009*

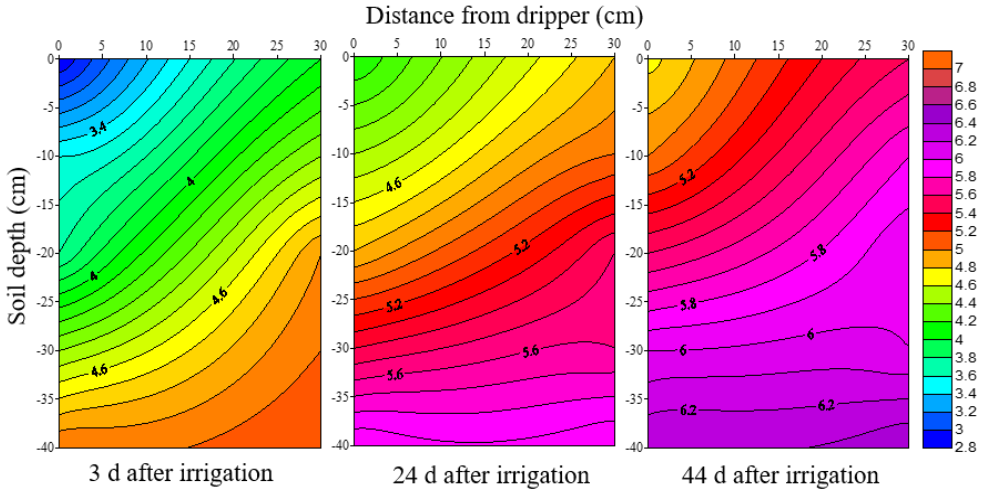

Figure 5. *Variations of soil salinity after single irrigation over time in 2010*

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DOI: http://dx.doi.org/10.15666/aeer/1905_36793702

Model performance

RMSE is an index of the accuracy of model simulation. *RMSE* has the advantage of expressing the error in the same units as the variable, so it has often been used to compare simulated and measured parameters (Li et al., 2017; Xie et al., 2020; Chen et al., 2020). The lower the *RMSE*, the more accurate the simulation. *MAE* is another widely used index for evaluating the accuracy of model simulation (Chen et al., 2019). *RMSE* ranged from 0.008 to 0.022 cm^3 cm^{-3} for SWC and from 0.29 to 0.5 g kg^{-1} for salinity, and *MAE* ranged from 0.005 to 0.011 cm^3 cm^{-3} for SWC and from 0.24 to 0.6 g kg^{-1} for salinity (*Tables 2–5*). These values confirmed the strong relationship between measured and simulated SWC and salinity and indicated that the model could successfully simulate water movement and solute transport.

Table 2. *Statistical comparison of measured and simulated soil-water contents at different positions in 2009*

Position	Depth (cm)	3 d		24 d		44 d	
		Observed	**Simulated**	**Observed**	**Simulated**	**Observed**	**Simulated**
Wide strip	10	0.280	0.275	0.238	0.226	0.215	0.210
	20	0.274	0.283	0.243	0.234	0.239	0.220
	30	0.301	0.290	0.279	0.242	0.223	0.234
	40	0.312	0.294	0.211	0.251	0.231	0.245
Narrow strip	10	0.271	0.275	0.206	0.224	0.184	0.200
	20	0.275	0.286	0.224	0.232	0.210	0.216
	30	0.280	0.289	0.264	0.242	0.237	0.230
	40	0.298	0.293	0.244	0.251	0.228	0.240
Statistical analysis	*RMSE* (cm^3 cm^{-3})	0.009		0.019		0.012	
	MAE (cm^3 cm^{-3})	0.010		0.018		0.011	

Table 3. *Statistical comparison of measured and simulated salinities at different positions in 2009*

Position	Depth (cm)	3 d		24 d		44 d	
		Observed	**Simulated**	**Observed**	**Simulated**	**Observed**	**Simulated**
Wide strip	10	2.84	2.84	3.76	3.44	5.84	4.64
	20	3.20	3.64	4.92	3.80	5.08	4.80
	30	3.92	4.08	5.80	4.16	6.32	5.80
	40	4.88	4.48	5.40	4.80	6.60	6.20
Narrow strip	10	4.62	3.96	4.52	4.16	6.36	5.80
	20	6.00	5.04	6.48	5.20	6.80	6.50
	30	6.20	5.32	6.80	5.72	7.04	6.60
	40	6.40	5.60	6.64	6.00	6.40	6.80
Statistical analysis	*RMSE* (cm^3 cm^{-3})	0.63		0.72		0.50	
	MAE (cm^3 cm^{-3})	0.54		0.60		0.50	

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Table 4. *Statistical comparison of measured and simulated soil-water contents at different positions in 2010*

Position	Depth (cm)	3 d		24 d		44 d	
		Observed	Simulated	Observed	Simulated	Observed	Simulated
Wide strip	10	0.264	0.254	0.191	0.20	0.178	0.174
	20	0.272	0.260	0.200	0.210	0.174	0.187
	30	0.280	0.274	0.220	0.218	0.200	0.203
	40	0.302	0.280	0.240	0.230	0.223	0.219
Narrow strip	10	0.270	0.260	0.175	0.196	0.141	0.174
	20	0.275	0.268	0.180	0.206	0.151	0.187
	30	0.288	0.274	0.209	0.217	0.160	0.203
	40	0.300	0.288	0.219	0.228	0.233	0.210
Statistical analysis	*RMSE* (cm^3 cm^{-3})	0.008		0.014		0.022	
	MAE (cm^3 cm^{-3})	0.005		0.011		0.017	

Table 5. *Statistical comparison of measured and simulated salinities at different positions in 2010*

Position	Depth (cm)	3 d		24 d		44 d	
		Observed	Simulated	Observed	Simulated	Observed	Simulated
Wide strip	10	4.44	4.32	4.8	4.41	4.5	5.00
	20	4.92	4.76	5.5	4.82	5.8	5.21
	30	5.03	5.10	5.70	5.24	6.00	5.64
	40	5.10	5.40	6.01	5.68	6.60	6.42
Narrow strip	10	3.53	3.68	4.20	4.00	5.21	4.81
	20	3.74	4.16	4.81	4.48	5.52	5.23
	30	4.42	4.67	5.42	4.96	6.00	5.68
	40	5.10	5.21	5.68	5.44	6.20	6.00
Statistical analysis	*RMSE* (cm^3 cm^{-3})	0.29		0.44		0.36	
	MAE (cm^3 cm^{-3})	0.24		0.39		0.32	

Accurate model simulation and the analysis of simulation errors are crucial for designing suitable irrigation regimes and for ensuring the applicability of the model (Shan et al., 2019). The analysis of our results identified several criteria for accurate simulation. The HYDRUS (2D/3D) model can not currently describe root growth, so a constant value must be chosen. The distribution of roots can control the dynamics of water and solutes, so these processes must be understood. Understanding root growth and distribution may play a vital role in simulating the dynamics of water and salinity throughout all growth stages. Future versions of the model must incorporate root development to minimize the simulation error. *LAI* determines the accuracy of T_p, and the relationship between T_p and *LAI* is positive but the relationship between T_p and E_p is

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negative. T_p is overestimated if *LAI* is overestimated and E_p is underestimated, and the simulation may lead to water stress in the soil. T_p is underestimated if *LAI* is underestimated and E_p is overestimated, and the simulation may lead to salinity stress in the soil. Ning et al. (2021) found that overestimation of evaporation can lead to high salinity near the surface, which caused osmotic stress. *LAI* is small for cotton at the seedling stage, and choosing zero in the simulation produced good results (Li et al., 2019). *LAI* in our study, however, was small at the initial seedling stage but increased by the end of the seedling stage, so we could not choose zero. Selecting zero may affect the accuracy of the simulation, so we selected values from 0.05 to 0.20 during the seedling stage and obtained satisfactory results. *LAI* varies with growth stage and should be measured carefully and often at various stages using advanced equipment.

The accuracy of the simulation results was affected by the input parameters, so knowing the effects of the input parameters and using suitable methods of measurement or calculation are necessary to ensure that the results are sufficiently reliable and can play a vital role in the design of rational irrigation schedules. Chen et al. (2019) demonstrated that the inability of HYDRUS-2D to accurately predict all scenarios was not a limitation of the model but a limitation of our understanding of the parameters, such as actual ET, water uptake by plants and root growth and distribution.

Effects of examined various factors on the optimal irrigation volume

Optimal irrigation schedule and irrigation system determined by emitter discharge, emitter spacing, salinity content, soil textural and so on. Optimal irrigation volume not only can meet seedling emergency water content and salinity threshold, but also ensure crop demand water and not exceed salinity threshold during the seedling stage. In order to providing guide for local production, based on the local conditions we selected some factors, additional simulations were conducted with HYDRUS-3D, and evaluation irrigation uniformity (Christiansen uniformity coefficient, *CU*) and leaching rate (L_r) using the information presented in *Table 6*.

Table 6. *Parameters used in additional HYDRUS-3D simulations*

Case	Soil type	Emitter spacing (cm)	Emitter discharge (L h^{-1})	Irrigation volume	Water content (cm^3 cm^{-3})	Salinity (g kg^{-1})
Ⅰ	Sandy loam	30	1.6, 2.4, 3.2	10	0.15	8
Ⅱ		20, 30, 40	1.6	10	0.15	8
Ⅲ		30	1.6	10	0.15	6, 8, 10

Emitter discharge

Emitter discharge play an important effect on water movement and salinity transport. *Figure 6* showed that the water content and salinity distribution under the same irrigation volume. The water content is decrease with distance from the emitter and depth increase, but the salinity the opposite. Compared to among the various emitter discharge, emitter discharge bigger and water content higher in horizonal, but inverse for in vertical. As for salinity distribution, emitter discharge bigger and salinity content lower in horizonal, but inverse for in vertical. The *CU* value were 0.757, 0.728 and 0.705 for emitter discharge 1.6 L h^{-1}, 2.4 L h^{-1}, 3.2 L h^{-1}, respectively, we concluded that the *CU* decrease with emitter discharge increase, and the relationship between *CU*

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and emitter discharge (E_d) has a followed power function ($CU = 0.7948E_d^{-0.102}$, $R^2 = 0.9983$). Wang et al. (2020) found that the *CU* decrease with emitter discharge increase at the same irrigation volume and emitter spacing. Leaching rate results were shown in *Table 7.* According to *Table 6* results, we found that smaller emitter discharge favor to pushing salinity to deeper soil, and the relationship between L_r and emitter discharge (E_d) has a followed linear function ($L_r = 0.1842E_d^{-0.071}$, $R^2 = 0.9938$). Wang et al. (2012), Liu et al. (2012), and Martynenko, et al. (2020) also showed that smaller emitter discharge is more favorable to soil movement and salinity leaching in vertical direction. *Figure 7* showed that water and salinity distribution at the end of seedling stage determined on optimal irrigation volume was 720 m^3 hm^{-2}, 1050 m^3 hm^{-2} and 1275 m^3 hm^{-2} for emitter discharge 1.6 L h^{-1}, 2.4 L h^{-1}, 3.2 L h^{-1} by using HYDRUS-3D, respectively. Both irrigation volume (V) and emitter discharge (E_d) can describe linear function relationship ($V = 346.88E_d + 182.5$, $R^2 = 0.988$). Based on *Figure 7,* it is shown that the soil water content and salinity can meet crop demand for growth. Above all, in order to ensure the crop health development, smaller emitter discharge should be priority to consideration.

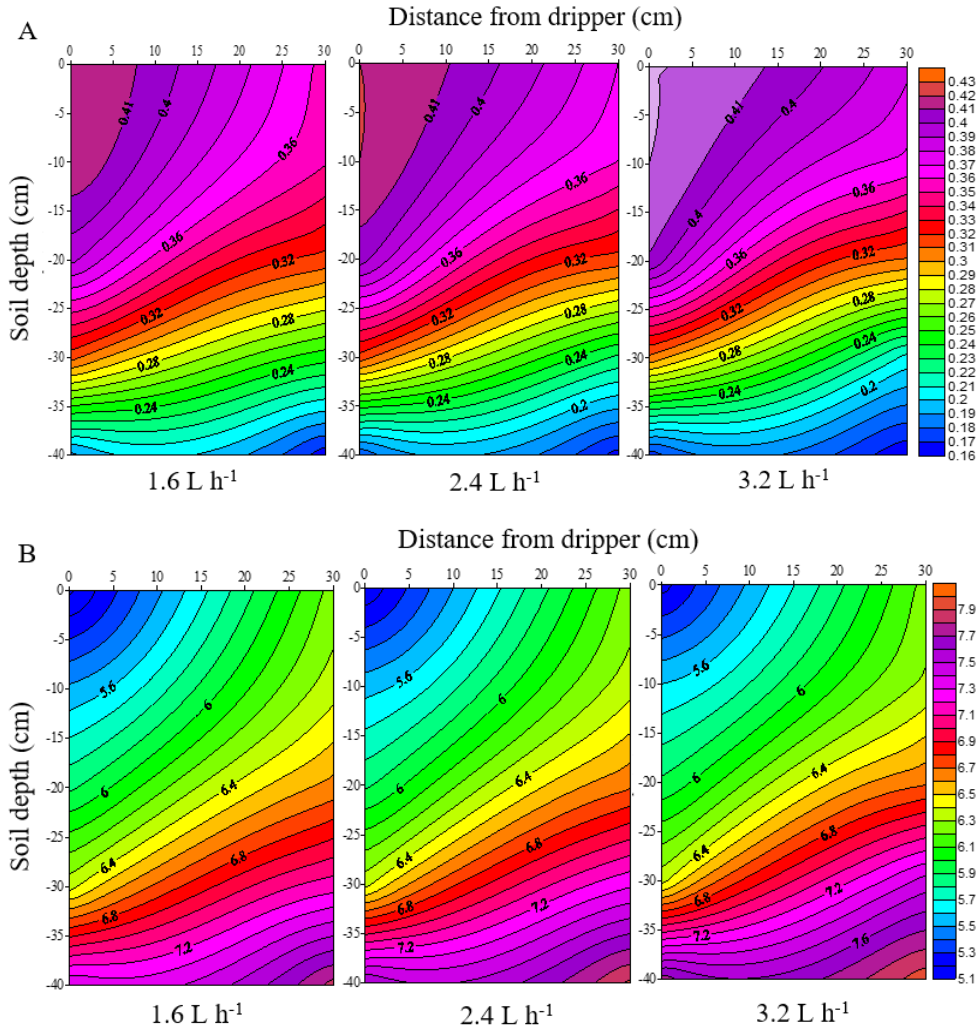

Figure 6. *Distribution water content (A) and salinity content (B) at different emitter discharge after irrigation finished*

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Table 7. *The leaching rates at various emitter discharge after irrigation finished*

Depth (cm)	Emitter discharge (L h^{-1})		
	1.6	**2.4**	**3.2**
10	0.292	0.289	0.280
20	0.230	0.229	0.225
30	0.169	0.163	0.156
40	0.055	0.035	0.021
Leaching rate in the soil profile	0.184	0.176	0.170

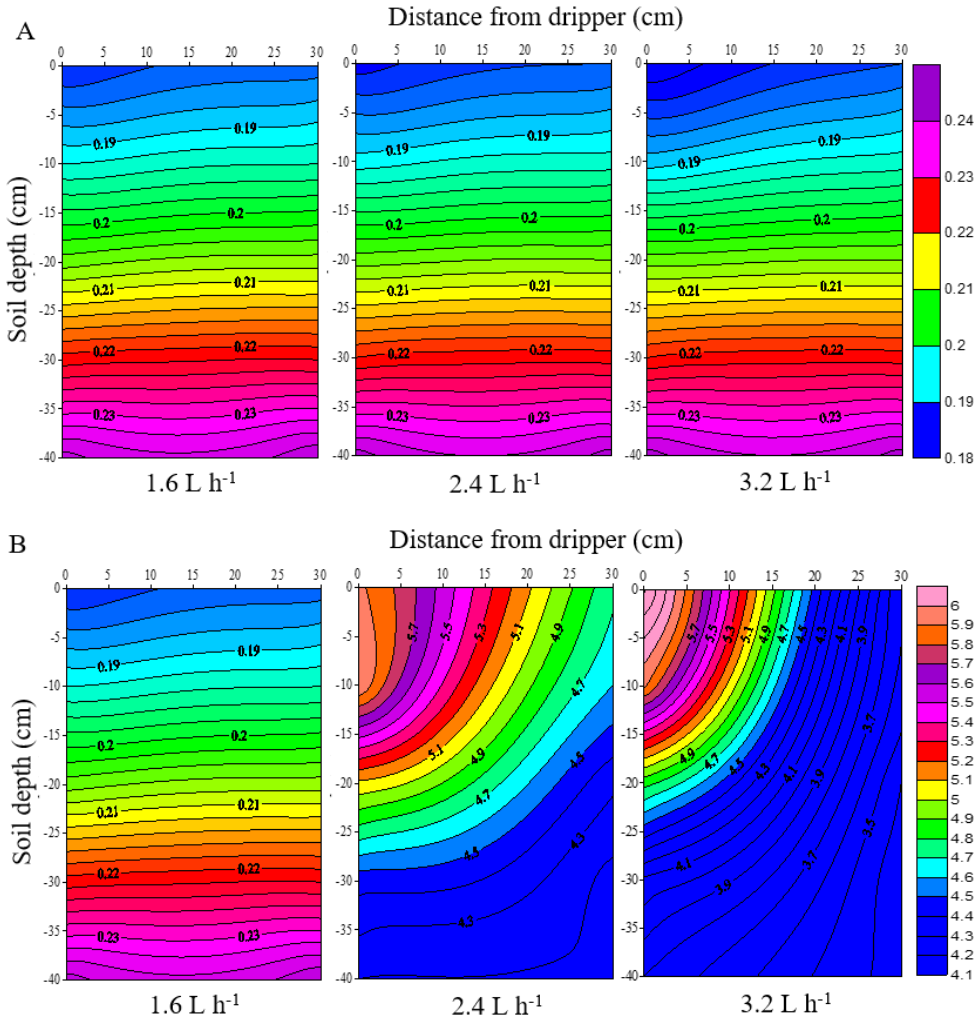

Figure 7. *Distribution water content (A) and salinity content (B) at the end of seedling stage under optimal irrigation volume with various emitter discharge*

Emitter spacing

Emitter spacing is an important part of drip irrigation system and also an important factor effecting on water movement and salinity transport. *Figure 8A* and *B* showed that the simulation results of water and salinity distribution under emitter spacing 20 cm,

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30 cm and 40 cm after irrigation is finished, respectively. According to *Figure 8,* it can be seen that the water content order is emitter spacing 20 cm > 30 cm > 40 cm in horizonal direction and in vertical direction. For the distribution of salinity content, the law is the opposite. The main reason is that the spacing is smaller, the wetting front confluence quickly, this will make the wet front advance fast, the wetting range is larger, the leaching effect is well. Li et al. (2017b) also showed that smaller emitter spacing more beneficial to salt leaching. The *CU* value were 0.886, 0.757 and 0.679 for emitter spacing 20 cm, 30 cm, 40 cm, respectively, we concluded that the relationship between *CU* and emitter spacing (E_s) has a followed power function relationship ($CU = 2.8E_s^{-0.384}$, $R^2 = 0.9998$). Hu et al. (2018) showed that small emitter spacing more better improve *CU*. Leaching rate results shown in *Table 8,* it is illustrated that the leaching rate decreases with depth increased, as well as emitter spacing increases. Both leaching rate (L_r) and emitter spacing (E_s) can be described power function ($L_r = 1.921E_s^{-0.692}$, $R^2 = 0.9993$). *Figure 9* showed that optimal irrigation is 555 m^3 hm^{-2}, 720 m^3 hm^{-2}, 840 m^3 hm^{-2} for emitter spacing 20 cm, 30 cm, 40 cm, respectively. The relationship between irrigation volume (V) and emitter spacing (E_s) has followed linear function ($V = 14.25E_s + 277.5$, $R^2 = 0.9918$). Take the *CU* and L_r into account, smaller spacing should be selected.

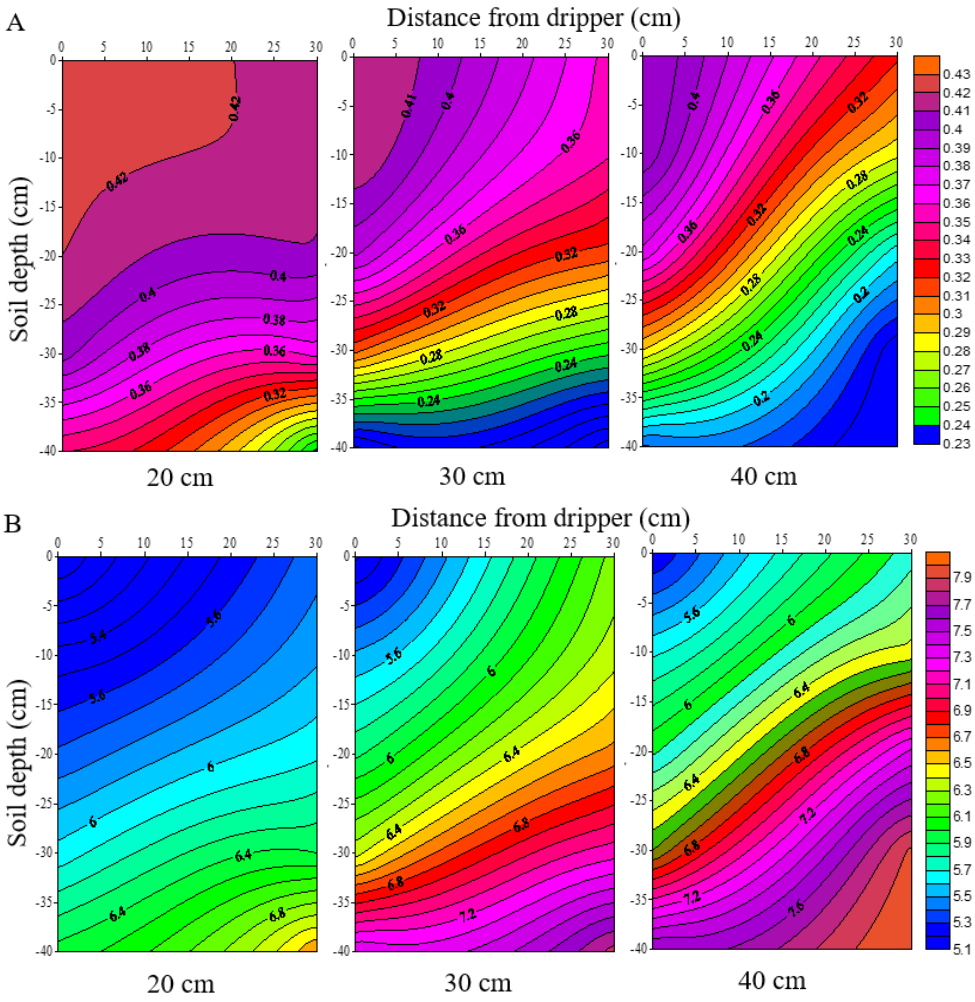

Figure 8. *Distribution water content (A) and salinity content (B) at different emitter spacing after irrigation finished*

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Table 8. *The leaching rates at various emitter spacing after irrigation finished*

Depth (cm)	Emitter spacing (cm)		
	20	**30**	**40**
10	0.321	0.292	0.280
20	0.269	0.230	0.184
30	0.229	0.169	0.115
40	0.16	0.055	0.032
Leaching rate in the soil profile	0.241	0.184	0.149

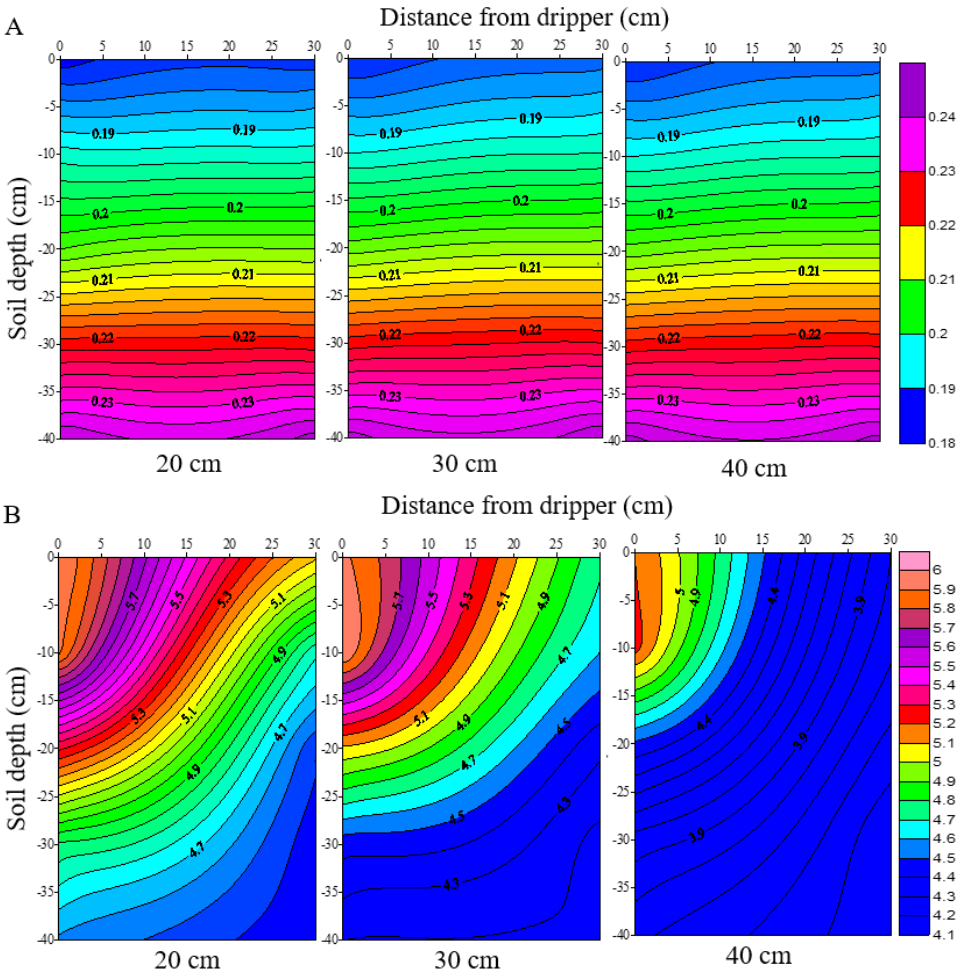

Figure 9. *Distribution water content (A) and salinity content (B) at the end of seedling stage under optimal irrigation volume with various emitter spacing*

Salinity content

Salinity content can impact on the crop whole development stage and yield, and it is necessarily to consideration factor in design optimal irrigation schedule (Ning et al., 2021). The water content decreased with far away from emitter and depth increase (*Fig. 10A*), but as for salinity the law was opposite (*Fig. 10B*). The *CU* value were almost the same 0.757 for salinity content 6 g kg^{-1}, 8 g kg^{-1}, 10 g kg^{-1}, respectively. The results were similar to Long et al. (2019), thus we concluded that the salinity has no

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significance on *CU*. The leaching rate decreased with depth increase, but leaching rate increased as initial salinity content increased (*Table 9*). Both leaching rate (L_r) and initial salinity content (S) could be described power function ($L_r = 0.0075S^{1.50}$, $R^2 = 0.970$). *Figure 11* showed that optimal irrigation was 450 m^3 hm^{-2}, 720 m^3 hm^{-2}, 1050 m^3 hm^{-2} for emitter spacing 6 g kg^{-1}, 8 g kg^{-1}, 10 g kg^{-1}, respectively. The relationship between irrigation volume (V) and Salinity (S) had followed linear function ($V = 14.25S + 277.5$, $R^2 = 0.9967$).

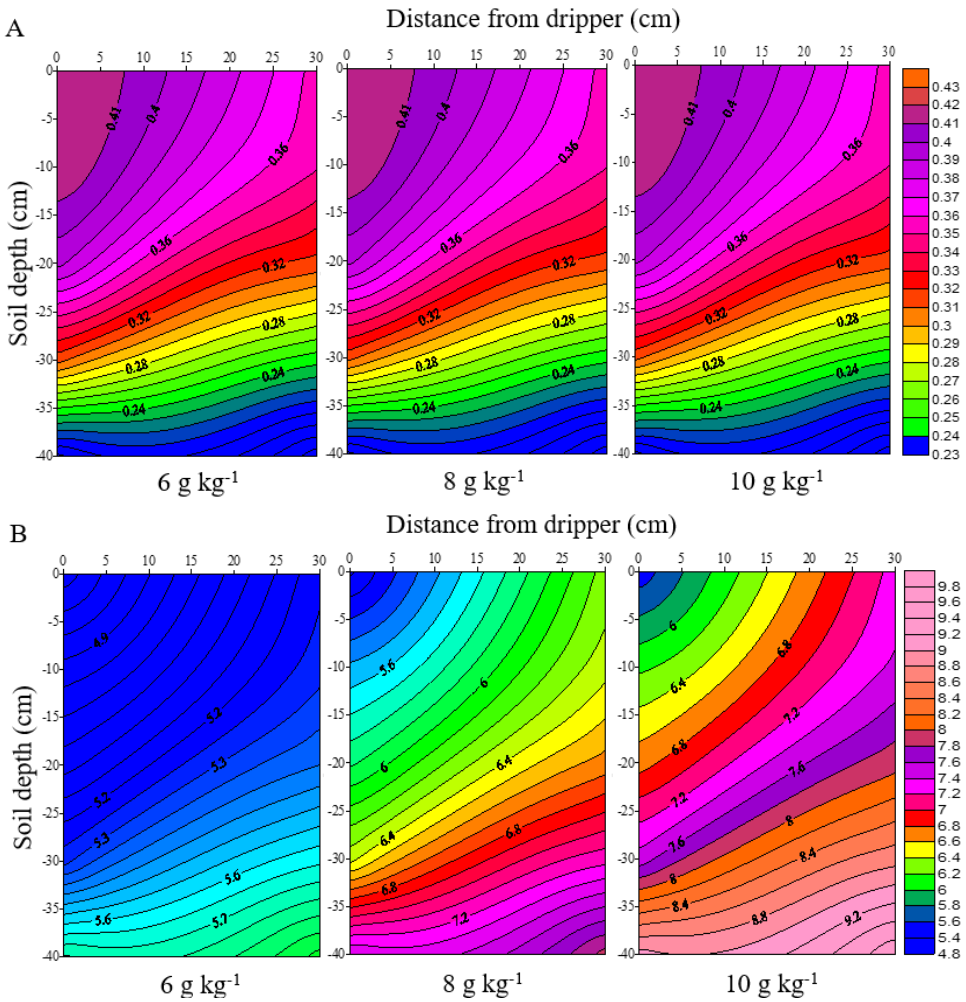

Figure 10. *Distribution water content (A) and salinity content (B) at different salinity content after irrigation finished*

Table 9. *The leaching rates at various initial salinity content after irrigation finished*

Depth (cm)	Salinity content (g kg^{-1})		
	6	8	10
10	0.17	0.292	0.362
20	0.134	0.230	0.284
30	0.098	0.169	0.207
40	0.03	0.055	0.067
Leaching rate in the soil profile	0.064	0.184	0.227

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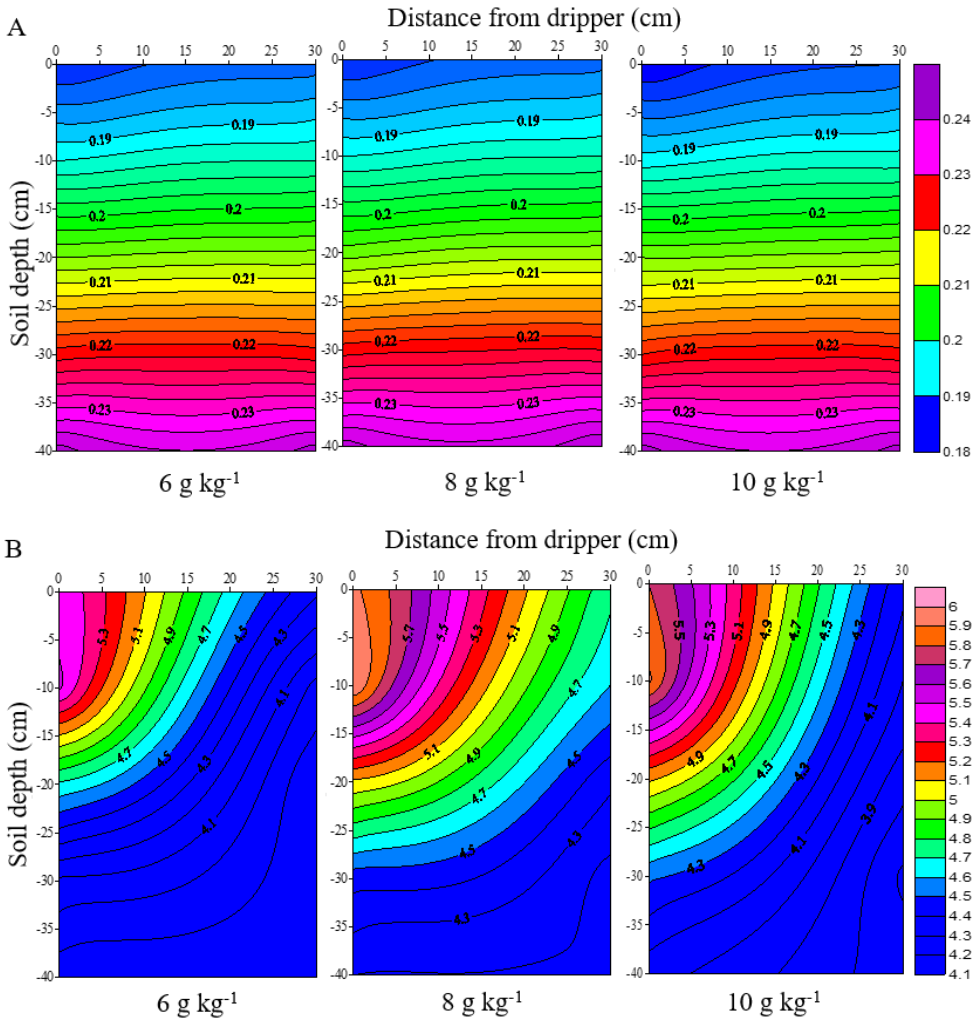

Figure 11. *Distribution water content (A) and salinity content (B) at at the end of seedling stage under optimal irrigation volume with various salinity content*

Conclusions

Improving water-use efficiency and constraining secondary soil salinity are effective measures for overcoming the limitation of agricultural development in arid and semi-arid regions. New technology, suitable system schemes and suitable irrigation regimes are essential for achieving these two goals. We found that dry seeding and planting after pre-germination can significant save water compared to conventional winter and spring irrigation. Based on the salinity results, the suitable schedule is double line with one row, and emitter discharge should not choose large under soil salinity medium degree. HYDRUS-3D can become a useful tool for obtaining information about water movement and solute transport and for designing suitable irrigation regimes. Some problems need to be addressed before the technology can be better applied, such as accounting for variable soil texture and temperature and accommodating other crops. The HYDRUS model could also be improved in the future by incorporating root distribution and development. It is hoped that the research results can provided some value reference for designing robust irrigation system and irrigation schedule. Besides improving the function of the model,

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DOI: http://dx.doi.org/10.15666/aeer/1905_36793702

some key factors should be considered such as temperature. We will comprehensively consider water, salt, the temperature in the future study, not only in the seedling period but in the whole growth period. Reasonable irrigation volume is applied based on soil water stress threshold value, soil salinity stress threshold value, and temperature stress threshold of cotton at various growth stages.

Acknowledgements. This work was supported by the Major Special Science and Technology Project of Xinjiang Province (2020A01002-1).

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_36793702

ASSESSMENT OF GENETIC DIVERSITY AMONG SOYBEAN (*Glycine max* (L.) Merr.) GENOTYPES MAKING USE OF AGRO-MORPHOLOGICAL BASED ON NUTRITIONAL QUALITY TRAITS

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(Received 29th Mar 2021; accepted 19th Jul 2021)

Abstract. Present study aims to assess the genetic diversity of 30 soybean genotypes based on agronomic and quality parameters using near infrared spectroscopy. Oribi, Crawford, BD 1601, Egret, B 66 S 41, B 66 S 387, Dundee, B 66 S 37 and Santa Rosa flowered earlier than B 66 S 256, Solar 12 and B 66 S 8. Yeluanda, 15/06/2012, B 66 S 31 had the highest fibre contents and height. DB 1601 had the highest number of seeds per pod. Oribi had the highest number of branches with PR 165-52 and B 66 S 24 having the highest yields. Dundee and N69-2774 were associated with high oil content while Ex RHOD, R 5-4-2 M, N69-2774 and DB 1601 were associated with both ash and stearic acid. Crawford, Egret and B 66-387 accessions were associated with palmitic acid. B 66 S 8 had the highest oleic acid content. PR 165-52 and B 66 S 8 contained the highest amounts of oil. Multivariate analysis helped us understand important agronomic and genetic traits when selecting soybean genotypes.
Keywords: *agronomic, multivariate analysis quality, parameters, principal component analysis, variation, traits*

Introduction

Genetic diversity based on agronomic traits is one of the oldest and most commonly used methods. It holds the advantage of providing a direct, simple, rapid and inexpensive way of characterising varieties. Phenotypic characterisation is generally viewed as the best determinant of taxonomic classification and agronomic value of crop plants and this makes it the most classical approach when coming to characterisation (Cholastova and Knotova, 2012). Phenotypic characterisation is also important for processes such as development, production and marketing of varieties. Govindarao (2010) stated that it is important to register a new cultivar that is distinct from other existing cultivars in markets where plant breeders' rights exist due to the fact that different cultivars could be identified based on phenotypic descriptors.

The phenotypic characteristics that are commonly used to assess genetic variability in soybean include plant morphology, seedling, seed quality and seed morphological characteristics. Numerous studies have explored the significance of phenotypic characterisation in estimating genetic diversity in soybean (Chen and Nelson, 2004; Ibidunni et al., 2020). A study was conducted by Hamzekhanlu et al. (2011) have studied 34 mutant lines including one control cultivar and detected the variability for number of grains per plant, number of pods per plant, number of leaves per plant, number of nodules per plant, nodule dry weight, 100 seed weight, plant dry weight (shoot dry weight), root

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_37033716

dry weight, harvest index and seed yield per plant. The genotypes were then clustered into four groups.

Furthermore, Manjaya and Bapat (2008) carried out a study observing the genetic variation during the characterisation of 55 soybean varieties using phenotypic traits that included: days to 50% flowering, days to maturity, plant height and number of branches per plant, number of pods per plant, number of seeds per plant, 100 seed weight and yield per plant. Antalikova et al. (2008) used 52 morphological and agronomic characters to find variability for the traits measured on the 52 studied genotypes. In another study involving phenotypic characterisation of 139 soybean genotypes Iqbal et al. (2008), revealed quite a number of significant differences between all the traits that were assessed.

The Asian Vegetable Research and Development Centre (AVRDC), United States and Pakistan studied the genetic diversity of 92 soybean genotypes and they found high coefficient of variations (CVs) coupled with wide ranges on leaf area (44.8%), number of branches per plant (31.7%), pods per plant (29.5%), 100 seed weight (39.0%) and grain yield per plant (46.6%). The findings showed a high level of diversity among the studied genotypes. Interestingly, the genotypes were classified into three distinct groups with the Pakistan germplasm forming its own cluster. Ojo et al. (2012) performed a similar study on 42 genotypes and found seven clusters. The study revealed that the 100 seed weight, number of pods per plant, pod yield per plant and seed yield per plot accounted for the greatest phenotypic variation, implying that there was broad diversity.

Phenotypic traits play a very important role in crop improvement and genetic diversity studies although they may be altered or influenced by the environment. Selection based on these traits is still widely practiced and will continue to play a significant role in estimating diversity among various genotypes using ANOVA in crop research. Results exhibiting high CVs and significant differences present high scope for selection. Furthermore, the clustering patterns obtained from phenotypic data, in respect of the number of clusters generated and genotypes contained in a cluster help to show diversity and the relatedness. The objectives of this study were to determine the presence of genetic diversity among the soybean genotypes using agro-morphological and nutritional quality traits.

Materials and methods

Study site

The experiment was conducted at the Agricultural Research Council – Grain Crops, South Africa located at 26°44′43.16″S–27°04′47.71″E with an altitude of 1340 metres above sea level. Thirty soybean genotypes that are sourced and maintained by the Agricultural Research Council were grown in under controlled conditions in a growth chamber until the 4th leaf stage. Day and night temperatures of the growth chamber were kept constant at 29 °C, and plants were irrigated to field capacity every fourth day depending on soil moisture depletion. Two seeds of each accession were planted in a 5 litre pot containing locally obtained loamy soil. This procedure was replicated three times for each genotype. *Table 1* shows the list of accessions obtained from Agricultural Research Council-Grain Crops gene bank used in the study. Soybean seed was analysed for nutritional quality using the DA 7250 (Perten Instruments) which checked for the following nutritional quality traits: moisture, ash, fibre, linoleic acid, linolenic acid, palmitic acid, stearic acid, and oleic acid.

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DOI: http://dx.doi.org/10.15666/aeer/1905_37033716

Table 1. *A list of accessions obtained from Agricultural Research Council-Grain Crops gene bank used in the study*

No.	Origin/place of collection	Accession name	Growth habit	No.	Origin/place of collection	Accession name	Growth habit
1	Unknown (ARC-GCI)	69 S 7	Indeterminate	16	Zimbabwe	Oribi	Determinate
2	Unknown (ARC-GCI)	B 66 S 31	Indeterminate	17	Unknown (ARC-GCI)	ND 85	Determinate
3	Unknown (ARC-GCI)	Lee Ex RHOD	Indeterminate	18	USA	AGS 239	Determinate
4	China	Columbia M 8 A	Semi-determinate	19	Unknown (ARC-GCI)	61 S 156	Determinate
5	Unknown (ARC-GCI)	IBIS	Indeterminate	20	Brazil	Santa Rosa	Indeterminate
6	USA	R-5-4-2 M	Indeterminate	21	Unknown (ARC-GCI)	B 66 S 41	Determinate
7	Unknown (ARC-GCI)	Egret	Indeterminate	22	Unknown (ARC-GCI)	Egret	Determinate
8	Unknown (ARC-GCI)	15/06/2012	Indeterminate	23	Unknown (ARC-GCI)	Crawford	Determinate
9	Unknown (ARC-GCI)	Dundee	Indeterminate	24	Unknown (ARC-GCI)	B 66 S 37	Indeterminate
10	Unknown (ARC-GCI)	Solar 12	Determinate	25	Unknown (ARC-GCI)	B 66 S 387	Indeterminate
11	USA	Hawkeye	Indeterminate	26	Unknown (ARC-GCI)	B 66 S 24	Indeterminate
12	USA	N69-2774	Indeterminate	27	Unknown (ARC-GCI)	B 66 S 256	Indeterminate
13	Asia	Maksura	Determinate	28	USA	Kahala	Semi-determinate
14	USA	DB 1601	Determinate	29	Unknown (ARC-GCI)	B 66 S 8	Indeterminate
15	USA	Yeluanda	Indeterminate	30	USA	PR 165-52	Indeterminate

Experimental layout and management

This study was conducted during the 2017/2018 growing season. The field has a well-drained sandy loam soil that had a pH ranging between 5.3 and 5.5. Selective pre-emergence herbicide was applied immediately after planting, subsequently; post-emergence herbicide was applied 31 days after sowing. Thirty soybean genotypes were planted in single rows of 3 m, with intra-row and inter-row spacing of 75 cm and 10 cm, respectively, using randomized complete block design, replicated three times. Plants were irrigated to field capacity once or twice a week using sprinklers depending on the soil moisture.

Data collection

Data were collected on the 4th of January 2018 according to the Standard Key Descriptor Lists for Characterizations for soybean (IBPGR, 1984).

Agronomic attributes

Agronomic parameters were measured according to soybean descriptor list (IBPGR, 1984). The number of days to 50 percent flowering that was calculated as days from

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DOI: http://dx.doi.org/10.15666/aeer/1905_37033716

planting to when 50 percent of the plants in each plot have flowered, days to maturity which are days calculated as days from planting to the day when 90 percent of the pods within the plot have dried, plant height was measured from the ground surface to the tip of the growing point using meter ruler over five randomly selected plants at maturity and recorded in centimetres, number of pod per plant which are all the pods per plant harvested, counted and averaged over three plants, pod length of three randomly selected pods per line were measured using a ruler and average length per pod expressed in centimetres, number of seeds per pod are the total number of seed in each pod was counted and averaged over three pods, hundred seed weight are hundred randomly selected good seeds counted and weighed in grams using a digital scale, seed weight is the after threshing of dried pods from each net plot and the seeds were weighed using digital weighing scale and expressed in grams, nodes at flowering are number of nodes at 50 percent flowering stage and lastly, the branch number per plant which are the number of branches counted from the main stem per plant.

Qualitative attributes

The following qualitative traits were measured according to soybean descriptor list (IBPGR, 1984):

Descriptors	**Scale**
Flower colour was recorded using the Munsell Colour Chart using the following	1= White 2 = Yellow 3 = Red 4 = Purple
Stem types were recorded as follows;	3 = Determinate 5 = Semi-determinate 7 = Indeterminate
Leaflet shape was recorded as follows;	3 = Narrow 5 = Intermediate 7 = Broad
Pubescence was recorded	As either “present” or “absent” on the pods
Pubescence density was recorded as follows;	3 = Sparse 5 = Semi-sparse 7 = Normal 9 = Dense (stem/leaves)
Pubescence colour were recorded as follows;	1 = Grey 2= Light brown 3 = Brown (tawny)
Pubescence type was recorded as either	1 = Erect 2 = Semi-appressed 3= Appressed 4 = Curly 5 = Retrorse tip
Corolla colour was recorded as follows; Data was collected from 3 to 5 randomly selected plants within each row	1 = White; 2 = Purple throat; 3 = Purple

Data analysis

Analysis of variance was conducted using PROC GLIMMIX, SAS version 9.4 (PROC GLIMMIX SAS Institute 2013). The Tukey's Student Range Test was used to separate means that were significantly different at P=0.05 as described by Steel and Torrie (1980).

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DOI: http://dx.doi.org/10.15666/aeer/1905_37033716

The principal component analysis (PCA) based on the linear correlation between variables and loading factors was used in multivariate analysis. Agromorphological data were subjected to multivariate data analysis using principal component analysis (PCA – XLSTAT, 2015) to identify and evaluate the groupings between the variables following the description.

Results

Agro-morphological diversity

Quantitative data

The early flowering genotypes, BD 1601, B 66 S 37, Oribi, Crawford, B 66 S 41, Egret, B 66 S 387, Dundee and Santa Rosa, took less than 85 days to flower. In contrast, B 66 S 256, Solar 12 and B 66 S 8 took more than 100 days to flower. Lee Ex RHOD, PR 165-52, Hawkeye, Yeluanda, ND 85, Kahala, IBIS and 61 S 156 genotypes were intermediate and took 86 to 90 days to flower (*Figure 1*).

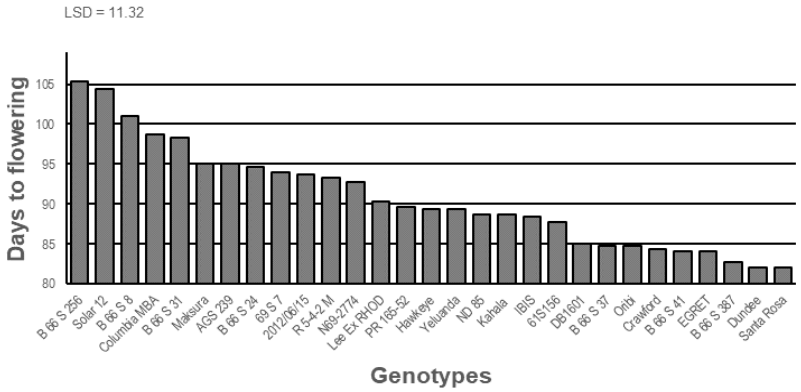

Figure 1. *Number of days to 50% flowering of 30 genotypes of soybean*

About 63% of the evaluated genotypes had three seeds per pod, only Solar 12 had only two seeds per pod (*Figure 2*) and the remaining genotypes had less than two seeds per pod.

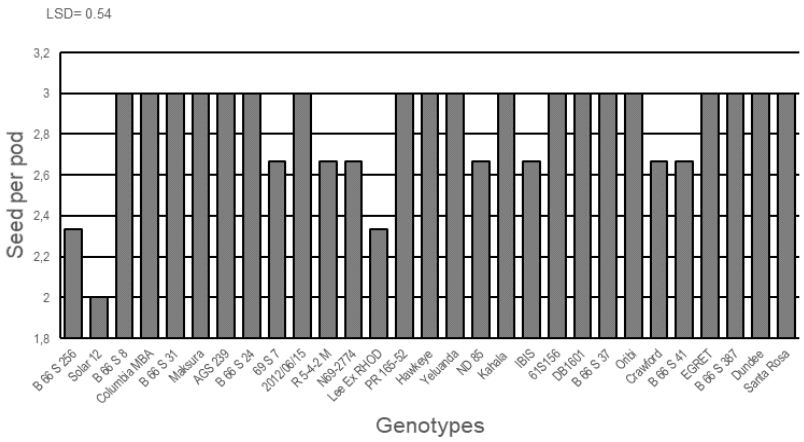

Figure 2. *Seed number per pod of 30 soybean genotypes evaluated*

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DOI: http://dx.doi.org/10.15666/aeer/1905_37033716

Qualitative data

The qualitative data of the thirty soybean genotypes grown in 2017/18 season is presented in *Table 2*. All genotypes studied matured with erect pubescence type. The studied soybean genotypes were dominated by 86.7% of dense and 13.3% of normal pubescence density. Most of the genotypes had 60% of the grey and 20% of brown and light brown pubescent colours. The corolla colours varied between purple and white with 73% of the genotypes being purple and the remainder were white. The genotypes showed different leaf shapes including narrow, intermediate and broad leaves with 46.7% of the genotypes having intermediate leaves, 43.3% had narrow leaves whereas 10% of the genotypes had broad leaves.

Table 2. *Qualitative traits of 30 soybean genotypes recorded in the study*

Genotype	Maturity period	Flower colour	Stem determination	Pubescence	Pubescence type	Pubescence density	Pubescence colour	Corolla colour	Leaflet shape
69 S 7	Early	Purple	Indeterminate	Absent	Erect	Dense	Grey	Purple	Narrow
B 66 S 31	Early	White	Indeterminate	Absent	Erect	Dense	Grey	Purple	Intermediate
Lee Ex RHOD	Early	White	Indeterminate	Absent	Erect	Dense	Brown	White	Narrow
Columbia M8A	Early	Purple	Semi-determinate	Absent	Erect	Dense	Brown	Purple	Broad
IBIS	Early	Purple	Indeterminate	Absent	Erect	Normal	Grey	Purple	Narrow
R 5-4-2 M	Early	White	Indeterminate	Absent	Erect	Dense	Grey	White	Intermediate
Egret	Early	Purple	Indeterminate	Absent	Erect	Dense	Grey	Purple	Narrow
15/06/2012	Early	Purple	Indeterminate	Absent	Erect	Dense	Grey	Purple	Intermediate
DUNDEE	Early	Purple	Indeterminate	Absent	Erect	Dense	Brown	Purple	Narrow
Solar 12	Early	Purple	Determinate	Absent	Erect	Dense	Grey	Purple	Intermediate
Hawkeye	Early	White	Indeterminate	Absent	Erect	Normal	Light brown	Purple	Narrow
N69-2774	Early	White	Indeterminate	Absent	Erect	Normal	Grey	White	Narrow
Maksura	Early	White	Determinate	Absent	Erect	Dense	Grey	Purple	Narrow
DB 1601	Early	Purple	Determinate	Absent	Erect	Dense	Grey	Purple	Broad
Yeluanda	Early	Purple	Indeterminate	Absent	Erect	Dense	Grey	Purple	Intermediate
Oribi	Early	White	Determinate	Absent	Erect	Dense	Grey	White	Intermediate
ND 85	Early	White	Determinate	Absent	Erect	Normal	Light brown	White	Narrow
AGS 239	Early	Purple	Determinate	Absent	Erect	Dense	Brown	Purple	Intermediate
61 S 156	Early	White	Determinate	Absent	Erect	Dense	Grey	Purple	Intermediate
Santa Rosa	Early	Purple	Indeterminate	Absent	Erect	Dense	Light brown	Purple	Narrow
B 66 S 41	Early	Purple	Determinate	Absent	Erect	Dense	Brown	Purple	Narrow
Egret	Early	Purple	Determinate	Absent	Erect	Dense	Grey	Purple	Intermediate
Crawford	Early	Purple	Determinate	Absent	Erect	Dense	Light brown	Purple	Intermediate
B 66 S 37	Early	Purple	Indeterminate	Absent	Erect	Dense	Grey	White	Intermediate
B 66 S 387	Early	White, Purple	Indeterminate	Absent	Erect	Dense	Brown	White	Narrow
B 66 S 24	Early	White	Indeterminate	Absent	Erect	Dense	Grey	White	Intermediate
B 66 S 256	Early	Purple	Indeterminate	Absent	Erect	Dense	Grey	Purple	Intermediate
Kahala	Early	White	Semi-determinate	Absent	Erect	Dense	Light brown	Purple	Intermediate
B 66 S 8	Early	Purple	Indeterminate	Absent	Erect	Dense	Grey	White	Narrow
PR 165-52	Early	White	Indeterminate	Absent	Erect	Dense	Light brown	Purple	Narrow

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DOI: http://dx.doi.org/10.15666/aeer/1905_37033716

Diversity of nutritional quality traits

The nutritional quality traits of soybean genotypes were analysed using analysis of variance. Significant differences ($P \leq 0.05$) were observed among the soybean genotypes based on ash content and oleic acid. Ash content varied between 4.8 and 6.6% whereas oleic acid ranged between 15.4 and 27.8% (*Figure 3*). The genotypes AGS 239 and Yeluanda had relatively higher ash content. Higher oleic acid contents were recorded for genotypes B 66 S 387, followed by B 66 S 8 and Hawkeye and the lowest was genotype DB 1606 (*Figure 4*).

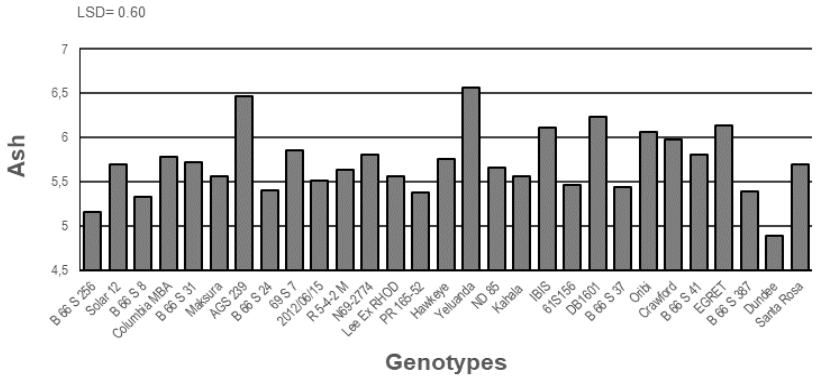

Figure 3. *Ash content percentage of 30 genotypes of soybean evaluated*

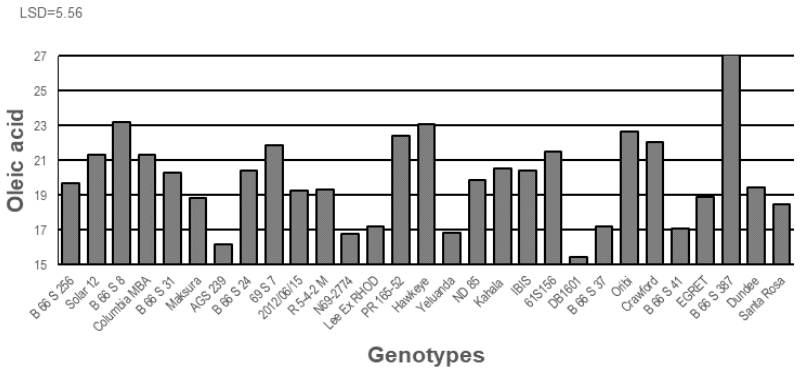

Figure 4. *Oleic acid content of 30 genotypes of soybean evaluated*

Principal component analysis

Principal component analysis (PCA) of agro-morphological and quality traits of soybean. *Table 3* shows the agronomic data that were subjected to a principal component analysis, which revealed that three most important PCs contributed 21.3%, 14.9% and 9.1% to the total variation. The traits that contributed the most variation in the first PC were number of branches per plant, pod number per plant, pod weight before threshing and yield per plant for agro-morphological traits. In the second PC, the traits that were responsible for contributing the most variation were oil, oleic acid, protein, ash, linoleic acid and stearic acid. The largest contributors to variation in the third PC were moisture content, protein content, days to 50% flowering and plant height.

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DOI: http://dx.doi.org/10.15666/aeer/1905_37033716

Table 3. *Factor loadings of the three PCs based on agronomic and quality traits*

Traits	Factor loadings		
	F1	**F2**	**F3**
DFW	-0.048	0.288	-0.499
PHT	-0.278	0.264	-0.450
BNP	0.505	0.023	-0.011
PNP	0.932	-0.114	0.055
PBT	0.941	-0.016	0.045
SNP	0.017	-0.049	0.119
SDP	0.953	-0.147	0.019
YDP	0.937	-0.029	0.010
PDL	-0.022	0.140	-0.066
PDW	0.175	-0.006	-0.390
Protein	0.243	-0.578	-0.537
Oil	-0.096	0.841	0.271
Moisture	0.108	0.346	0.711
Ash	-0.245	-0.580	0.202
Fiber	-0.393	0.379	-0.276
Linoleic acid	-0.318	-0.588	0.234
Linolenic acid	-0.023	-0.300	-0.348
Oleic acid	0.293	0.701	-0.224
Palmitic acid	-0.014	-0.219	0.056
Stearic acid	-0.281	-0.562	0.159
NFW	-0.003	0.066	0.228
Eigenvalue	4.470	3.148	1.920
Variability (%)	**21.287**	**14.992**	**9.142**
Cumulative (%)	21.287	36.279	45.422

Days to 50% flowering=DFW; Plant height=PHT; Number of branches/plant=BNP; Pod number/plant=PNP; Pod weight before threshing=PBT; Seed number per/pod=SNP; Seed number/plant=SDP; Yield/plant (g) =YDP; Pod length (mm) =PDL; Pod width (mm) =PDW; Nodes at flowering=NFW

Principal component biplots

Figure 5 shows that number of branches per plant and pod weight before threshing were highly significant and highly correlated with plant height, days to 50% flowering, nodes at flowering, pod length as well as oil, fiber, oleic acid and moisture; whereas they were negatively correlated with pod number per plant, seed per plant, ash, linoleic acid, linolenic acid, palmitic acid, stearic acid and ash. However, was also positively and significantly associated with pod width. Protein was highly significant and positively correlated with palmitic acid, linolenic acid, linoleic acid as well as stearic acid but negatively correlated fiber. It was also significant and positively correlated with seed number per pod. Seed number per pod was highly significant and negatively associated with pod weight per plant, days to 50% flowering, but positively correlated with pod number per plant. Seed number per pod was highly and positively associated with seed number per plant and protein. Pod number per plant was significant and positively correlated with protein. Pod number per plant before threshing was highly and significantly associated with pod width.

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DOI: http://dx.doi.org/10.15666/aeer/1905_37033716

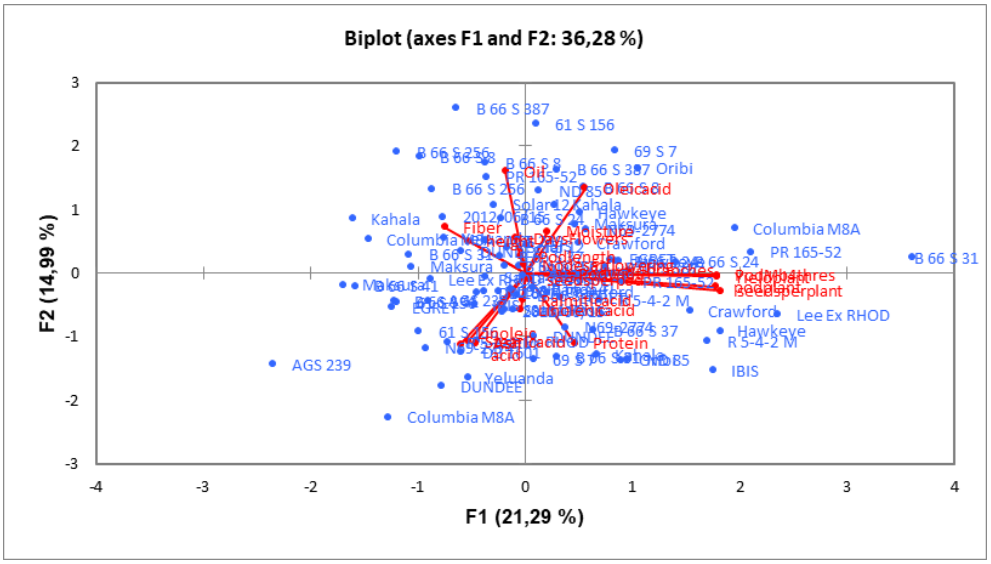

Figure 5. *A Principal Component Biplot depicting agro-morphological and nutritional quality traits of 30 soybean accessions*

Correlation analysis among phenotypic and quality traits

The phenotypic and quality traits were analysed using correlation coefficients. The association of the traits were reported based on the 5% significance level. Plant height was positively correlated with days to flowering. Pod number per plant was positively correlated with plant height and branch number per plant. Pod weight before threshing recorded a positive correlation with plant height, branch number per plant and pod number per plant. Seed number per pod was positively associated with plant height, branch number per plant and pod number per plant. Seed yield per plant was positively associated with plant height, branch number per plant, pod number per plant and seed number per pod. Pod width recorded a negative correlation with seed number per pod. Number of nodes at flowering was negatively correlated with palmitic acid. Oil content recorded a positive correlation with days to flowering and a negative one with protein. Moisture content showed a negative association with protein content and a positive association with oil content. Ash was positively correlated with plant height, seed yield per plant and oil content, and negatively associated with pod weight before threshing. Fiber recorded a positive correlation with plant height, number of branches per plant, seed number per plant, seed yield per plant and oil content and also showed a negative correlation with pod number per plant and pod weight before threshing. Linoleic acid was negatively correlated with days to flowering and positively correlated with oil and ash. Linoleic showed a negative association with moisture content and positive association with linoleic acid. Oleic acid correlated positively with seed yield per plant, oil content and ash and negatively with linoleic acid. Palmitic acid correlated positively with ash and fibre and negative with linoleic and oleic acids. Stearic acid showed a positive correlation with oil content and linoleic acid and a negative correlation with oleic acid. On leaf shape 43.33% were narrow, 46.67 intermediates and 10% were indeterminates. Pubescence density varied at 86.67% for the erect types and 13.33% for the appressed types. Sixty percent of the stems were determinate, 6.67% semi- determinate and 33.33% indeterminate.

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DOI: http://dx.doi.org/10.15666/aeer/1905_37033716

Relationship between the traits

The majority of soybean cultivars that are grown and consumed throughout the world today exhibit yellow or white seed coats, whereas the majority of known accessions of the wild progenitor, *G. soja*, have black or, rarely, brown seed coats. Soybean cultivars should also have chemical composition and qualitative traits that are in need and particularly easy to grow and maintain especially for beneficial aspects either for personal consumption or financial benefits.

The principal component biplot analysis was not only able to identify specific traits contributing to yield but also to identify the out-performing varieties, as this helps to select new lines with these traits. Soybean varieties with higher plant height, more oil content, more branches and pods, grains, fibre, and early flowering days obtained higher yield. Dundee and N69-2774 were associated with high oil content. According to the PCA biplots Lee Ex RHOD, R 5-4-2 M, N69-2774 and DB 1601 were associated with both ash and stearic acid as illustrated on *Figure 5*. Crawford, Egret and B 66 S 387 accessions were associated with palmitic acid. Yeluanda, 15/06/2012, B 66 S 31 had the highest fibre and height. Maksura, N69-2774 and Crawford were associated with both moisture and days to flowering. B66 S8 had the highest oleic acid content. DB 1601 had the highest number of seeds per pod. PR 165-52 and B 66 S 8 contained the largest oil content. High node set at flowering was observed in 15/06/2012 while high pods per plant were observed in Crawford and Lee Ex RHOD. B 66 S 387 and Oribi had the highest number of branches while PR 165-52 and B 66 S 24 had the highest yields per plant.

Discussion

Genetic diversity of germplasm collections can be determined using a traditional cluster analysis; which is easy and effective. This type of method studies the genetic diversity by forming core subsets while grouping accessions according to similar characteristics into one homogenous category. Relationships of accessions are grouped according to similar units and thus make it easy to understand them better and easily translatable. The evaluation of genetic diversity among germplasm is highly required in any hybridization program as it aids in improving the genes of the most diverse lines as well as promoting the use of genetic variations. It is imperative for a breeder to obtain information on genetic diversity and relationships among breeding materials in order to improve the efficiency of a crop, this helps with efficiently managing and conserving germplasm resources. De Chavez et al. (2017) recently studied the diversity among soybean accessions in Philippines reporting a vast diversity. Another study conducted by Khatab et al. (2012) reported the presence of genetic diversity among soybean genotypes using ago-morphological descriptors. Hamzekhanlu et al. (2011) studied 34 mutant lines including one control cultivar and found variability for ten quantitative traits in soybean. A study finding significant differences among all the assessed phenotypic traits was reported by Iqbal et al. (2008).

The assessment of morphological traits with the aim of studying the genetic diversity and classification of existing germplasm material is a traditional method that is low level but can also be a powerful taxonomic tool that can be used to group germplasm prior to their characterisation particularly using more precise marker technologies. Li et al. (2020) observed soybean varieties with higher plant height, more nodes of main stem, branches, pods, grains, and 100-grain weight, or longer growth periods may have higher yield. This concurs with Achina et al. (2019) who observed that the significant attributes include the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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number of pods per plant, seeds per pod, and seed weight, which determine the seed yield. From the research, DB 1601 had the highest number of seeds per pod while PR 165-52 and B 66 S 24 had the highest yields per plant.

In this study, the nutritional quality traits showed diversity among the studied accessions where the oil and protein contents were within the range recorded in other studies (Shi et al., 2010). The oil content showed a range from 13.16% to 15.41%, while protein ranged between 33.29% and 35.71% with Dundee and N69-2774 having the highest high oil content. In order to select accessions with good qualities for hybridization and conservation, breeders will find the diversity among the accessions very beneficial. In a study conducted by Mazid et al. (2013), it was highlighted that the analysis of genetic diversity is very crucial for identifying parents that helps to achieve long-term selection gain.

The study revealed that traits that contributed to the most variation were number of branches per plant, pod number per plant, pod weight before threshing, seed number per plant, and yield per plant, protein and oleic acid. High number of pods per plant was observed in Crawford and Lee Ex RHOD. High oleic acid content was recorded for genotype B 66 S 387, followed by B 66 S 8 and Hawkeye. All genotypes studied matured early with erect pubescence type. The studied soybean genotypes were dominated by 86.7% of dense and 13.3% of normal pubescence density. Most of the genotypes had 60% of the grey and 20% of brown and light brown pubescent colours. The corolla colours varied between purple and white with 73% of the genotypes being purple and the remainder were white. Breeders in the direct and indirect programmes can be provided with information of the traits that were tested for correlation and traits that are desirable in order to select and use these genotypes with significance concurrently. Yield improvement programs thrive better when a breeder understands the relationship between the component of traits and yield as this will aid in making the best selection of desirable genotypes for these programs.

Plant height, days to flowering, plant branches, pods weight before threshing, and pod length concur with the results reported by a study carried out by Malek et al. (2014) where there were positive significant correlations. Machikowa et al. (2007) argue that early maturing varieties tend to have lower yield while late flowering is associated with higher soybean yield. Therefore, the extension of days to flowering of current early varieties, as well as the development of new varieties with a longer vegetative period, may result in higher yielding varieties. These results mean that when selecting genotypes that are high yielding for a breeding programme, these characters should be given more preference and emphasis as the best selection criteria. Kato et al. (2019) observed that the seed yields of semi-determinate and indeterminate lines were higher than that of the determinate ones; and that of the semi-determinate lines was marginally lower than that of the indeterminate lines. The research also highlighted that lodging score of semi-determinate varieties was smaller than that of indeterminate varieties because the main stem length of the semi-determinate varieties was shorter than that of the indeterminate varieties. Anand and Torrie (1963) stated that seed yield always showed a positive correlation with other desirable yield traits which indicates that the increase in one trait would result in the increase of the other; that is, simultaneous increase or decrease of both traits would be easy. Soybean genotypes showing high yields indicate a strong positive correlation between seed yield and other traits and would be fairly easy to identify together with high number of pods per plant.

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The statistical method that is commonly used in populating genetics in order to identify the genetic distribution and structure across a geographical and ethnic is also known as the principal component analysis (PCA) (McVean, 2009). It is further explained that the reason for this type of analysis is to evaluate each and every variable that forms part of the variation that is available among the studied genotypes (Mofokeng and Mashingaidze, 2018). This type of method aids in differentiating the important and less important traits within a studied group by grouping them accordingly.

Conclusion

Using agro-morphological and quality traits, some of the most important soybean cultivars were observed. These included Dundee and N69-2774 having the highest high oleic acid and oil contents. DB 1601 had the highest number of seeds per pod while PR 165-52 and B 66 S 24 had the highest yields. There were positive correlations showed between most of the assessed traits in relation to one another and this will be very helpful in assisting in the combined improvement of these traits by selecting ones that were found to have a positive and high correlation, as well as easily measurable phenotypic traits, although most were found to have highly significant and positive correlation with seed yield per plant. The nutritional quality traits also varied significantly among the accessions. In order to achieve a successful selection of parents for breeding and transgressive segregation, studying and knowing the presence of genetic diversity can be very useful. The aim of the present investigation was to characterise the genetic diversity of the available germplasm, determine gene action controlling grain yield and estimate the breeding gains that have been realized since the inception of the breeding programmes. The specific objectives of the study were successfully accomplished as shown above in the study.

Soybean is one of the most important leguminous crops grown globally. Understanding the genetic diversity and its interaction with the environment is of paramount importance in developing cultivars considering farmer's preferred traits. The multivariate analysis was able to reveal the relationship between the genetic diversity, agronomic and nutritional composition of selected soybean genotypes. For a successful breeding programme to function a complete understanding of the genetic diversity of the crop is required. Better knowledge of the genetic similarities and dissimilarities of breeding material could aid breeders and curators in maintaining genetic diversity, sustain long-term selection gain and conserve the germplasm. Monitoring the genetic diversity among genetic resources of elite breeding material could make crop improvement more efficient by the directed accumulation of favoured alleles thus reducing the amount of material to be screened.

Acknowledgements. This project was supported by the Central University of Technology, Free State Research Grant Scheme, National Research Foundation fund, and Agricultural Research Council- Grain Crops for the use of their facilities.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_37033716

ECOLOGICAL DIVERSITY OF SPINY BROOM (*CALICOTOME VILLOSA* (POIR.) LINK) AND SWEET BROOM (*GENISTA × SPACHIANA*) OF THREE DIFFERENT PROVENANCES: CHEMICAL COMPOSITION, SECONDARY COMPOUNDS OF SHRUB LEAVES AND RESPONSES TO DROUGHT STRESS

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(Received 30th Mar 2021; accepted 19th Jul 2021)

Abstract. In the first stage, levels of primary and secondary compounds, nutritive value of foliage from two fodder shrubs spiny broom (*Calicotome villosa*) and sweet broom (*Genista spachiana*) growing spontaneously in central and northern Tunisia were evaluated in order to study the ecology of species in the country. Feed components were determined by proximate analysis, while phenolic and tannin compounds were also analyzed. The foliage of *C. villosa* and *G. spachiana* is rich in protein (between 36 and 51%). These two species present good protein values and acceptable levels of secondary compounds that may make a choice forage for ruminants, especially during the lean season. In the second stage the study was to examine the germination characteristics of *C. villosa* and *G. spachiana* under controlled conditions with different levels of salt and water stress. Seeds were sown in NaCl solution at different concentrations: 0, 3, 6, 9, 12 and 15 g/l. The water-stress treatments used were 0, -0.03, -0.1, -0.7, -1 and -1.6 MPa obtained by adding PEG_{6000}. *C. villosa* and *G. spachiana* can withstand salinity up to 15 g/l of salt (31% at 37% germination at 15 g/l) and also tolerate higher levels of PEG_{6000} (30% - 40% germination at -1.6 MPa).
Keywords: *environments, nutritional value, germination, drought, salinity*

Introduction

The Fabaceae family (e. g. Legumes) is the most important family among dicotyledons. It contains the largest number of species useful to humans, whether food, industrial or medicinal. According to Judd et al. (2002), fabaceae species are generally herbaceous, shrubs, trees or climbing plants. The leaves are generally alternating, composed of pinnate leaves. Many species of Fabaceae have therapeutic properties and are used in traditional medicine (*Calicotome villosa* and *Genista spachiana*). *Calicotome villosa* (Poir.) Link subsp intermedia belongs to the Fabaceae family. This species is a thorny shrub that can grow up to 2 m tall (Boughalleb et al., 2019). The woody perennials particularly the rangeland shrubs as main source of sustenance and ecological significance. There is urgent need for sustainable utilization

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_37173735

of shrub diversity through scientific conservation as well as the management (Rathore et al., 2011). Fabaceous species are rich in flavonoids with a percentage of (24.24%) and the Genista genus is the richest. In general, the aerial parts contain a higher content of flavonoids. Alkaloids are present to a considerable degree in Fabaceae with a percentage of (18.18%) and the Genista genus is the richest. The aerial parts of Fabaceae species are more widely used and richer in bioactive substances (Berrabah and Chemissa, 2017). The economic importance of these species is significant. Indeed, spiny broom (*Calicotome villosa*) and sweet broom (*Genista spachiana*) are important food and fodder plants and drugs used in therapy. In Tunisia, C. *Villosa* is of great interest for the rehabilitation of degraded ecosystems, food for goats and camelids, and for the use of its essential oils in phytotherapy. Indeed, recent findings revealed. that the index of livestock acceptability for C. *Villosa* is considered as palatable (Gamoun et al., 2018). The ethnobotanical study of the Fabaceae family shows that the latter occupies an important place in traditional medicine because of its therapeutic indications. *C. villosa* leaves are the richest in protein: 33.70% (Berrabah and Chemissa, 2017). C. *villosa* have led to the isolation and identification of flavone glucosides, alkaloids, and anthraquinones from the leaves and flowers (Pistelli et al., 2003) flavone glucosides, steroids, and chalcone from the stems (Alhage et al., 2018), flavonols and alkaloids from the seeds (Elkhamlichi et al., 2017) and C. villosa is a source of natural antioxidants with high industrial value and could be used as a potential food source (Boughalleb et al., 2019). *Calicotome villosa* and *Genista spachiana* are classified as vulnerable in Tunisia and they are of great interest for the rehabilitation of degraded ecosystems, food for goats and camelids, and for the use of its essential oils in phytotherapy (Mechergui et al., 2017). These authors found that the tolerance of the two species to salinity and water stress allows them to bea source of food for goats and camelids during drought (Mechergui et al., 2017). The two species *Calicotome villosa* and *Genista spachiana* can be used as pasture for animals: grass, fruit, tree and shrub leaves. Moreover, the use of these fodder trees and shrubs is an old practice well known to livestock producers. This type of fodder is an important and often indispensable part of livestock feed in developing countries. Tunisia has a significant fodder deficit due to the degradation of the grazing lands. To address this deficit, the planting of forage shrubs is a renewable resource that can provide regular standing biomass throughout the year, as it can be. Tunisia is a north African country and climatically is divided into north and south parts. Southern part of the country is covered by the desert and is accounting for three quarters of the country. One of the promising options for restoration of degraded regions in southern Tunisia is to use native shrub and tree species that have multiple functions in the ecosystem. Several species of the legume family (Fabaceae) are of a high interest due to their adaptation to arid and semi-arid environments, nitrogen fixing capacity and ability to grow in poor soils (Ibanez and Passera, 1997). In the first stage, the approach in this study is to improve the quality of the basic ration by selecting the most nutritious shrub species that can be planted, since the low nutritional value of the available forage is one of the main factors limiting animal productivity. In order to make optimal use of the nutritional potential of this natural vegetation, it is necessary to know its chemical composition, the purpose of this study, which dry matter to determine the primary and secondary components and examine the hemolytic power of saponins in the leaves of nine forage shrubs, in order to improve ruminant breeding, which is a real development issue.

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In the second stage, successful establishment of plants largely depends on successful germination. Seed germination behavior in relation to thermal and salt stress is a very important determinant of the colonization capacity of a species (Ungar, 1995). Tolerance to salinity during germination is critical for the establishment of plants growing in saline soil of arid regions (Khan and Gulzar, 2003; Ungar, 1995). Increased salinity leads to a reduction and/or delay in germination of seeds of both halophytes and glycophytes. Failure of germination in saline soils often is a result of high salt concentrations of salts in the seed-planting zone. Seed germination under saline conditions occurs after high precipitation and rain apports (Khan and Ungar, 1996; El-Keblawy, 2004; Huang et al., 2003; Redondo et al., 2004). The detrimental effect of salinity and osmotic stress are generally less severe at optimum germination temperature (Gorai and Neffati, 2007; Tlig et al., 2008; Gorai et al., 2009; Maraghni et al., 2010).

The present study was undertaken to understand the fodder quality of *Calicotome villosa* and *Gesinta spachiana* leaves and the seed germination requirements of different provenances of these two species under salt and water stresses.

In this study, we wanted to answer the following specific questions: (i) nutritional value of spiny broom (*Calicotome villosa*) and sweet broom (*Genista spachiana*) for livestock; (ii) seed germination of spiny broom and sweet broom under different levels of NaCl-salinity and osmotic stress induced by polyethylene glycol (PEG_{6000}); and (iii) how do the osmotic levels of NaCl and PEG_{6000} induced water stress of the seed's environment affect seed germination?

A detailed knowledge of germination responses may provide: new insights into factors controlling plant recruitment and survival in drought-prone regions and with such adaptation strategies, these species be used to rehabilitate degraded and very stress full areas and constitutes a valuable solution to the lack of animal feed especially in dry rangelands due to the nutritional value.

Materials and methods

Fodder quality of Calicotome villosa and Gesinta Spachiana leaves

Samples were collected in spring of 2018 (May) from Meknassi which is located in center of Tunisia, Bouhedma national park which is located in south of Tunisia (*Fig. 1*) and of the Cap Bon forest, which is located in northeast of Tunisia. The locations and characteristics of sampled sites are (*Fig. 2; Table 1*). The choice of this site is motivated by the biodiversity of the floral procession and the fairly high pastoral pressure encountered there. Leaf samples of each of the two species were collected from the shrubs during spring season of the year. Leaf samples were taken from 10 shrubs per species, the latter being about 200 m apart from each other. On average, a sample of one kilogram of leaves per species was dried for 48 h in a ventilated oven set at 50 °C. This temperature prevents the denaturation of the plant's chemical compounds (Makkar and Singh, 1991). The experiments were conducted using three replicates.

Chemical composition of shrub leaves

All samples of the plant material collected were analysed for dry matter (DM) and mineral matter (MM) contents according to AOAC (1990) procedures. The total nitrogenous material was determined by the Kjeldahl method (ISO 1997). The parietal

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constituents (neutral detergent, NDF - cellulose with neutral detergent; acid detergent, ADF-lignocellulose; and acid detergent lignin, ADL-lignin) were determined according to the method of Van Soest et al. (1991), while the crude fibre was determined by the Weende method (1967). The experiments were conducted using three replicates.

Figure 1. *Calicotome villosa in Bouhedma national park (Tunisia)*

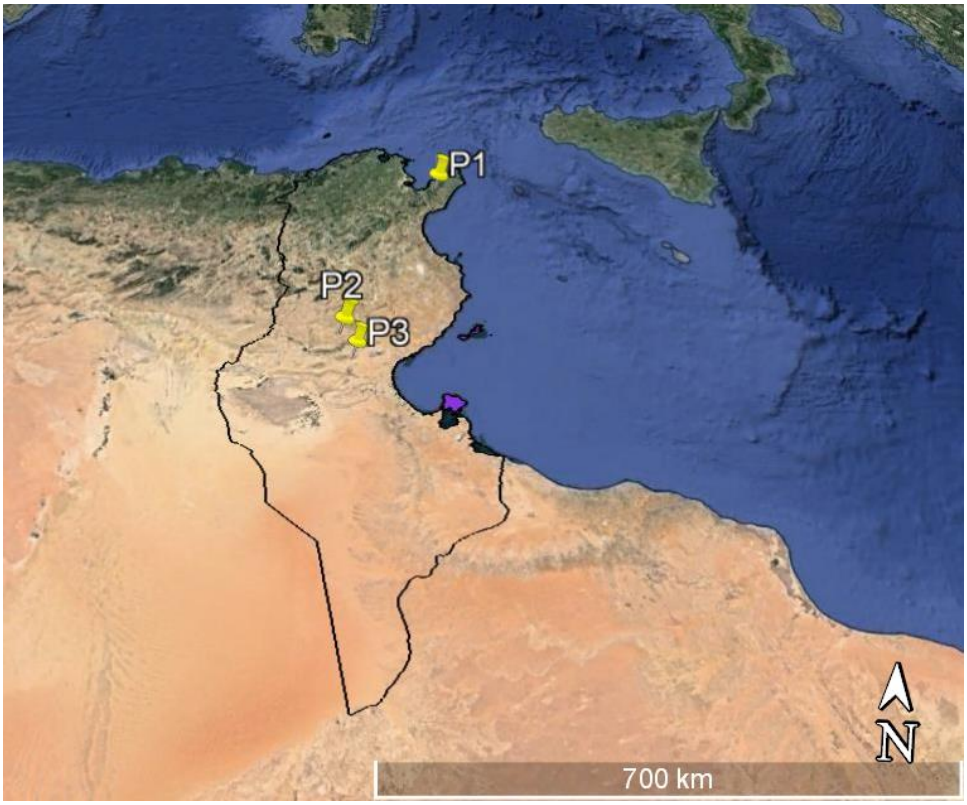

Figure 2. *Tunisia map, location of the populations of spiny broom (Calicotome villosa) and sweet broom (Genista spachiana); P1: Genista spachiana of Rtiba; P2: Calicotome villosa of Meknessi; P3: Calicotome villosa of Bouhedma*

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Table 1. *Location and main ecological traits of the populations of spiny broom (Calicotome villosa) and sweet broom (Genista spachiana)*

Species	Locality	Bioclimatic zone	Latitude	Longitude	Altitude (m)	Rainfall mm/year	Soil
Calicotome villosa	Meknassi	Upper arid	34° 35ˈ N	9° 34ˈ E	150	200-250	Silty, loam, very stony
	Bouhedma	Upper arid	34° 28ˈ N	9°38ˈ E	200	150–200	Calcareous with rock outcrops, very stony
Genista spachiana	Rtiba	Lower semi arid	36°53ˈ N	10°45ˈ E	50	400–500	Deep silty sandy loam

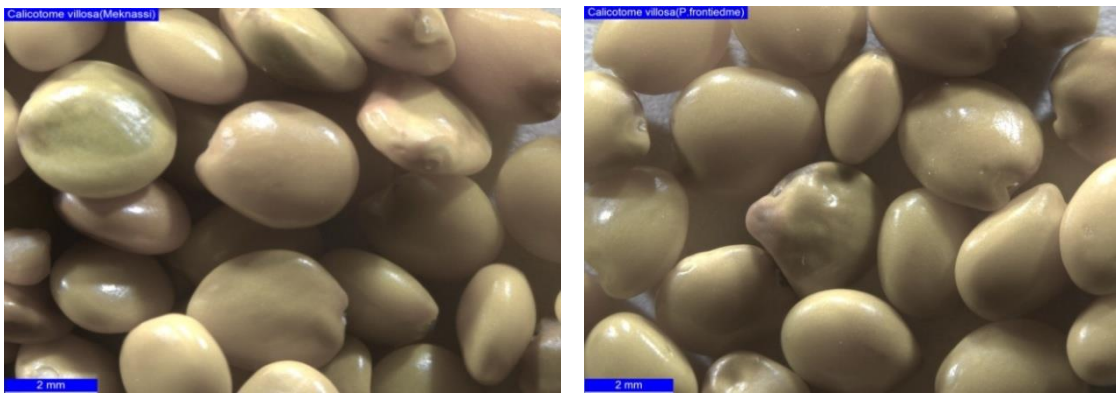
Determination of secondary compounds

Total phenols, measured by the Folin-Ciocalteu reagent, and total tannins were analyzed according to the procedure described by Makkar et al. (1993). The results are expressed in tannic acid equivalent per kilogram of dry matter. Condensed tannins were determined by oxidation in the butanol-HCl reagent in the presence of a ferric reagent using the technique of Porter et al. (1986). The experiments were conducted using three replicates.

Morphology of Calicotome villosa and Genista spachiana and germinative capacity under salinity and water stress

Seeds harvesting of Calicotome villosa and Genista spachiana

The *Calicotome villosa* seeds are harvested in summer of 2018 (July) from two sites in central Tunisia, Meknassi and Bouhedma national park (*Fig. 3*). This zone belongs to the arid bio-climate with an average temperature of 25 to 35 °C and a rainfall of 100 to 350 mm. The soil type is limestone for meknassi and sand for Bouhedma. *Genista spachiana* seeds are harvested in summer of 2018 (July) from the north of Tunisia in Rtiba area (*Fig. 4*), it is located in the sub-humid bio-climate and characterized by a temperature of 15 to 25 °C and a rainfall of 500 to 700 mm and a sandy soil. Seeds were extracted from fruits while soaking in water for 24 h. Healthy seeds were sterilised with benlate (1 g/l) for 20 min followed by 50% sodium hypochlorite for few minutes and rinsed thrice with distilled water.



a **b**

Figure 3. *Seeds of Calicotome villosa of Bouhedma national park (a) and Calicotome villosa of Meknassi (b)*

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Figure 4. *Seeds of Genista spachiana of Rtiba (Cap bon in Tunisia)*

Germinative capacity under salinity and water stress

Seeds were placed in Petri dishes on perlite containing a polyethylene glycol solution (PEG_{6000}) to germinate under osmotic potentials of 0 (control sample), -0.03, -0.1, -0.7, -1 and -1.6 MPa and kept in incubator at 25 °C. Seeds were placed to germinate under NaCl induced salt stress, at 0 (control sample), 3, 6, 9, 15 and 15 g/l. Seeds were germinated using 5 mL of NaCl or Polyethylene glycol (PEG_{6000}) induced solutions. Both osmotic were used to test salt stress and drought stress, which are two key environmental factors where these species study in northern and southern Tunisia. Germination was evaluated daily for 30 days. For each type of stress, the experiment was conducted in completely randomized design, using five replications of 20 seeds comprising 6 concentrations of NaCl salt and 6 concentrations of osmotic potential. Seeds were considered to be germinated when the root length reached the seed length and shoot length reached half of the seed length (Mechergui et al., 2017). The number of germinated seed was counted every day for 30 days. A seed was considered germinated when the emerging radicle elongated 2 mm above the seed surface (Redondo-Gómez et al., 2007).

Germination expressions

Germination of seeds in both the species were counted daily for a period of 30 days. Mean time to germination (MTG) was calculated following the formula given below:

$$MTG = \sum n_i \times d_i / n \qquad \text{(Eq.1)}$$

where "n" is the total number of germinated seeds during the test, "n_i" is the number of germinated seeds on day "d_i" and "i" is the number of days during the germination period of 30 days (Yousheng and Sziklai, 1985). Cumulative germination percentage (GP %) was evaluated daily and the final value was obtained after 30 days and Kotowski's coefficient (CV) was calculated using the formula given below.

$$CV = \sum(n \times Jn) / \sum n \qquad \text{(Eq.2)}$$

where "n" is the total number of germinated seeds during the test, "Jn" is the number of days during the germination period (between 0 and 30 days).

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Statistical analysis

The data of the different parameter values were subjected to the analysis of variance ANOVA with SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Tukey test was used to estimate significant differences between means (Mechergui et al., 2017).

Results

Content of primary and secondary compounds in the sheets

Nutritional characterization of species

The average chemical composition of the pastoral species studied is reported in *Table 2*. The dry matter content of the shrub leaves studied ranged from 27.98% (*Calicotome villosa* of Meknessi) to 41.25% (*Genista spachiana* of Rtiba) with very significant differences between species. The mineral content which is important at *Genista spachiana* of Rtiba (16.25%). *Genista spachiana* of Rtiba is distinguished from other species by its high total nitrogen content (51.87%). Overall, the composition of the parietal fraction of the substrates studied shows a very different composition ($P < 0.05$). The shrub species richest in NDF is *Genista spachiana* of Rtiba (61.98%DM) (*Table 2*). The mineral content which is high at *Genista spachiana* of Rtiba (41.25%) respectively varies very significantly. The one-way ANOVA showed that dry matter (DM), mineral materials (MM), total nitrogenous materials (MAT), crude fibre (CB), neutral detregent fiber (NDF), acid detregent fiber (ADF), and acid detregent lignin (ADL) varied significantly among the three species ($P < 0.001$). The differences between chemical composition are induced by the diversity of the species in the hand and influenced by the environmental conditions in the other hand.

Table 2. *Primary chemical composition of leaves of shrub species (% of dry matter: DM)*

Species	DM	MM	MAT	CB	NDF	ADF	ADL
Calicotome villosa of Meknessi	28.9 ± 5.69^{b}	8.6 ± 1.09^{f}	36.5 ± 5.88^{ab}	19.6 ± 3.69^{e}	36.6 ± 6.25^{a}	26.4 ± 6.25^{c}	21.5 ± 4.26^{d}
Calicotome villosa of Bouhedma	30.6 ± 4.29^{d}	12.6 ± 3.13^{g}	45.9 ± 7.19^{b}	26.5 ± 3.27^{f}	48.6 ± 8.46^{a}	39.9 ± 8.75^{bc}	28.5 ± 3.22^{de}
Genista spachiana of Rtiba	41.2 ± 6.85^{cd}	16.2 ± 2.43^{g}	51.8 ± 8.97^{ab}	31.1 ± 5.29^{ef}	61.8 ± 10.26^{a}	42.7 ± 8.34^{c}	34.5 ± 4.66^{e}
Level of significance	*P < 0.001*	*P < 0.001*	*P < 0.001*	*P < 0.001*	*P < 0.001*	*P < 0.001*	*P < 0.001*

DM: dry matter; MM: mineral materials; MAT: total nitrogenous materials; CB: Crude fibre; NDF: Neutral Detergent Fiber; ADF: Acid Detergent Fiber; ADL: Acid Detergent Lignin

Anti-nutritional substance content of leaves in shrub vegetation

The average richness of the pastoral species studied in phenolic compounds and saponins is reflected in *Table 3*. The average content of total phenols and total tannins shows significant variability between the species studied. *Calicotome villosa* from Meknassi is the species richest in phenolic compounds and total tannins with 76.25 g tannic acid equivalent/kg DM (*Table 3*). The condensed tannin content ranged from 10.68 to 15.24 g leucocyanidin equivalent/kg DM. It is the weakest for *Genista spachiana* of Rtiba. The hydrolysable tannin content of shrub species is generally high, especially in *Calycotum spinosa* of Meknassi (63.58 g/kg DM). *Calicotome villosa* de Bouhedma is the most abundant species in saponin with an average of 10.32 g diosgenin equivalent/kg DM. The hemolytic activity of saponins is an indicator of their biological effect. *Genista spachiana* of Rtiba saponins have the most intense hemolytic activity

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(*Table 3*). We recorded a strong positive correlation between the condensed tannin content and mineral matter content ($r = 0.71$; $P = 0.001$) and between the ADF lignocellulose content and the total phenol content) ($r = 0.93$; $P = 0.001$), as well as between the saponin and dry matter content ($r = 0.68$; $P = 0.001$). It is important to note that the hemolytic activity of saponins is positively correlated with the content of condensed tannins ($r = 0.78$; $P = 0.001$) and with minerals ($r = 0.86$; $P = 0.001$). The one-way ANOVA showed that total phenols (PhT), Total tannins (TT), condensed tannins (TC), saponins (sap), hydrolysable tannins (TH), and hemolytic activity of saponins (AH) varied significantly among the three species ($P < 0.01$). The differences between anti-nutritional substance are induced by the diversity of the species in the hand and influenced by the environmental conditions in the other hand.

Table 3. *Anti-nutritional substance content of leaves in shrub vegetation*

Species	PhT	TT	TC	Sap	TH	AH
Calicotome villosa of Meknessi	112.2 ± 15.36^{a}	76.2 ± 2.35^{b}	15.2 ± 2.48^{d}	7.5 ± 2.98^{ef}	63.5 ± 5.41^{bc}	8.6 ± 2.77^{e}
Calicotome villosa of Bouhedma	125.3 ± 14.24^{a}	74.2 ± 4.12^{b}	11.2 ± 3.44^{d}	10.3 ± 1.20^{de}	59.3 ± 9.68^{c}	9.6 ± 1.33^{f}
Genista spachiana of Rtiba	98.6 ± 8.65^{a}	65.9 ± 4.15^{b}	10.6 ± 2.35^{de}	6.6 ± 1.02^{f}	48.6 ± 6.55^{c}	19.2 ± 3.55^{d}
Level of significance	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$

PhT: Total phenols (in g equivalent tannic acid/kg DM); TT: Total tannins (in g equivalent tannic acid/kg DM); TC: Condensed tannins (in g equivalent leucocyanidine/kg DM); Sap: Saponins (in g equivalent diosgenin/kg DM); TH: Hydrolysable tannins (g/kg DM); AH: Hemolytic activity of saponins (%)

Digestibilities and energy and nitrogen values of forage shrub leaves

The forage value of the foliage of the shrubs studied varies from 0.94 UFL/kg DM for *Calicotome villosa* of Meknessi to 1.09 UFL/kg DM for *Genista spachiana* of Rtiba for whom the MAD are 287.89 (*Table 4*). These shrub leaves are characterized by a calculated average digestibility of organic matter ranging from 55.32 for *Calicotome villosa* of Meknessi to 71.95% *Genista spachiana* of Rtiba. *Calicotome villosa* leaves are the richest in digestible nitrogenous matter (296.6 g/kg DM). The one-way ANOVA showed that Digestibility (MO) and energy (UFL and UFV) and nitrogen values (MAD) of fodder shrub leaves varied significantly among the three species ($P < 0.001$). The differences between digestibilities and energy and nitrogen values are induced by the diversity of the species in the hand and influenced by the environmental conditions in the other hand.

Effects of salt stress on germination

The effect of salinity on germination of *Calicotome villosa* and *Genista spachiana* seeds are presented in *Table 5*. Germination in distilled water was the highest. However, it decreased significantly with an increase in NaCl concentrations (*Table 5*). Seeds germinated rapidly in distilled water during the first five days and decreased gradually with increase in salinity, the highest germination was recorded (82%) in distilled water of *Genista spachiana* of Rtiba and the lowest (31%) corresponding of 15 g/l NaCl from *Calicotome villosa of Bouhedma*. There was a strong negative relationship between germination and salinity. The Germination velocity calculated using the Kotowski index (equation 2) showed that the rate decreased with an increase in salinity (*Table 5*). Results of ANOVA showed that salt stress (NaCl treatments) had a significant effect on germination percentage (*Table 6*) and on mean germination time calculated using equation (2). Germination was significantly reduced by high NaCl levels and there were no great differences in final germination percentage between 3 and 9 g/l, thus

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germination percentage was reduced with increasing NaCl to levels above 12 g/l. A two-way ANOVA of the germination rate indicated a significant effect of salinity but not an interaction between species and salinity (*Table 6*). The differences between germination capacity under salt stress are induced by the diversity of the species in the hand and influenced by the environmental conditions in the other hand.

Table 4. *Digestibility and energy and nitrogen values of fodder shrub leaves*

Species	Digestibility MO (%)	UFL	UFV	MAD
Calicotome villosa of Meknessi	55.3 ± 2.25^{b}	0.9 ± 6.35^{c}	0.9 ± 0.58^{cd}	286.3 ± 12.35^{a}
Calicotome villosa of Bouhedma	61.3 ± 3.35^{b}	1.0 ± 4.16^{c}	1.0 ± 0.65^{d}	296.6 ± 13.23^{a}
Genista spachiana of Rtiba	$71.9 \pm 5.62b$	1.0 ± 6.87^{c}	1.0 ± 0.25^{d}	278.8 ± 22.15^{a}
Level of significance	*P < 0.001*	*P < 0.001*	*P < 0.001*	*P < 0.001*

UFL: Feed Unit Milk/kg DM; UFV: Feed Unit Milk/kg DM; MAD: Digestible nitrogenous matter (g/kg DM)

Table 5. *Mean percentage germination of Calicotome villosa and Genista spachiana seeds after transfer from 0, 3, 6, 9, 12 and 15 g/l NaCl at 25 °C*

Species	NaCl (g/l)					
	0	3	6	9	12	15
Calicotome villosa of Meknassi	75 ± 10.8^{a}	60 ± 3.1^{b}	49 ± 7.6^{cd}	50 ± 6.1^{c}	47 ± 2.5^{e}	32 ± 7.5^{f}
Calicotome villosa of Bouhedma	78 ± 13.7^{a}	51 ± 8.2^{b}	48 ± 6.8^{c}	41 ± 5.3^{cd}	35 ± 4.7^{ef}	31 ± 7.9^{f}
Genista spachiana of Rtiba	82 ± 15.1^{a}	61 ± 7.9^{b}	60 ± 4.4^{bc}	56 ± 6.5^{d}	42 ± 3.7^{e}	37 ± 5.3^{ef}

Different letters indicate significant difference among treatment means ($p < 0.05$; Tukey test)
^{abc}Values in the same row with the same superscript are not significantly different (*P > 0.05*)

Table 6. *Two-way ANOVA of the effects of salinity (S), Species (T), and their interaction on germination characteristics of Calicotome villosa and Genista spachiana*

Variable	Characteristics of germination	*F-value*	*P-value*	Signification
Species	Germination percentage	1.985	0.145	NS
	Kotowski coefficient	0.591	0.557	NS
	Mean time to germination	4.976	0.009	**
Concentration	Germination percentage	11.673	0.000	***
	Kotowski coefficient	12.503	0.000	***
	Mean time to germination	29.097	0.000	***
Species × concentration	Germination percentage	0.225	0.993	NS
	Kotowski coefficient	0.234	0.992	NS
	Mean time to germination	1.259	0.270	NS

Significant difference from control at **P < 0.05, **P < 0.01, *** P < 0.001* by Tukey's test. NS = not significant

Effects of osmotic potential on germination

Osmotic potential significantly (*P < 0.001*) affected the percentage of germination of *Calicotome villosa* and *Genista spachiana* (*Table 7*). Germination percentage decreased significantly with an increase in osmotic potential. Seeds germinated rapidly in distilled

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water during the initial days. Germination rate decreased with increasing levels of osmotic potential (*Table 7*). The highest germination percentage was recorded with distilled water followed by 0, -0.03, -0.1, -0.7, -1 and -1.6 MPa. However, at -0.03 MPa *Calicotome villosa* seeds from Meknassi had a germination rate higher than control. Germination percentage and osmotic potential are negatively correlated in both the species. The differences between germination capacity under osmotic potential are induced by the diversity of the species in the hand and influenced by the environmental conditions in the other hand.

Germination index

The Kotowski *index* showed that the rate decreased with an increase in osmotic potential. In all the provenances of *Calicotome villosa* and *Genista spachiana*, water stress had a significant effect ($P < 0.001$) on germination, mean germination time (MGT) and the Kotowski coefficient (*Table 8*). Germination percentage and speed decreased with decrease in water potential. Germination percentage for *Calicotome* seeds from Meknassi, the highest among all treatments (79 was at 0.03 MPa). Also, germination percentage exceeded 50% at -1 MPa. A two-way ANOVA indicated a significant effect of osmotic potential on germination rate, but not for the interaction between osmotic potential and species germination percentage (*Table 8*).

Table 7. *Mean percentage germination of Calicotome villosa and Genista spachiana seeds at 0, -0.03, -0.1, -0.7, -1 and -1.6 MPa at 25 °C*

Species	Osmotic potential (MPa)					
	0	**-0.03**	**-0.1**	**-0.7**	**-1**	**-1.6**
Calicotome villosa of Bouhedma	78 ± 4.1^{a}	65 ± 10.8^{ab}	65 ± 8.9^{ab}	56 ± 3.6^{c}	41 ± 9.4^{d}	30 ± 4.7^{e}
Calicotome villosa of Meknassi	74 ± 4.8^{ab}	79 ± 4.3^{a}	65 ± 6.8^{b}	56 ± 6.5^{c}	43 ± 8.7^{d}	38 ± 7.5^{e}
Genista spachiana of Rtiba	78 ± 4.0^{a}	65 ± 6.8^{ab}	55 ± 4.7^{b}	48 ± 8.7^{c}	42 ± 9.5^{cd}	40 ± 9.6^{d}

Different letters indicate significant difference among treatment means ($p < 0.05$: Tukey test)
^{abcde}Values in the same row with the same superscript are not significantly different ($P > 0.05$)

Table 8. *Two-way ANOVA of the effects of Osmotic potential (S), Species (T), and their interaction on germination characteristics of Calicotome villosa and Genista spachiana*

Variable	Characteristics of germination	*F-value*	*P-value*	Significance
Species	Germination percentage	0.570	0.568	NS
	Kotowski coefficient	3.782	0.027	*
	Mean time to germination	5.040	0.009	**
Concentration	Germination percentage	14.051	0.000	***
	Kotowski coefficient	6.330	0.000	***
	Mean time to germination	14.731	0.000	***
Species × concentration	Germination percentage	0.436	0.924	NS
	Kotowski coefficient	0.766	0.661	NS
	Mean time to germination	2.340	0.019	*

Significant difference from control at $*P < 0.05$, $**P < 0.01$, $*** P < 0.001$ by Tukey's multiple tests. NS = not significant ($P > 0.05$)

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Discussion

Content of primary and secondary compounds in the sheets

Nutritional characterization of species

Despite the very wide use of concentrated feed in animal nutrition, coarse fodder remains the basis of ruminant feed intake. Spontaneous vegetation, particularly woody vegetation, is an important contribution to meeting the needs of the ruminants that live there. To make optimal use of the nutritional potential of this natural vegetation, it is necessary to know its nutritional value. The season influences the nutritive and anti-nutritive components of forage species in the Himalayan region (Katoch et al., 2012). Seed size can be used as a parameter for predicting germination and seedling growth rate both in nursery and field conditions (Mohamed et al., 2018). The dietary value of a forage is generally judged on the basis of its content of potentially digestible nutrients (mainly energy, nitrogen and minerals) and on the presence of undesirable compounds such as lignin. The foliage of the ten identified shrubs had an DM content of less than 50%, which confirm DM the results of González-Andrés and Ortiz (1996) who observed the same trend with Mediterranean shrub species. This results in the availability of forage shrubs, which regularly provide standing biomass throughout the year, unlike herbaceous species that dry up in summer. Indeed, analyses of the chemical composition of shrub leaves have shown low average dry matter levels, which makes them a water-rich foodstuff, which does not dry out in summer (lean season), and also remains present during the winter period, these two periods being critical for livestock for which herbaceous vegetation is very limited, making them a year-round food resource. The mineral content is medium to high for some shrub leaves (*Calicotome villosa* de Meknassi (8.67%), *Calicotome villosa* de Bouhedma (12.65%) and *Genista spachiana* de Rtiba (16.25%). Different results were recorded by Boubaker et al. (2004) for shrub leaves from northwest Tunisia, for which low mineral values were recorded (2.8-6.4% DM). According to Spears (1994), the concentration of mineral elements in plants varies greatly with soil type, climate and stage of maturity. The majority of shrub leaves sampled by Boubaker et al. (2004) in northwestern Tunisia did not exceed 10% DM in MAT, as did those analyzed in arid (Silva-Pando et al., 1999) or semi-arid (Ben Salem, 2000) areas. The MAT content is highest in *Genista spachiana* (41.25% DM). Indeed, leguminous fodder and shrubs are very often used as livestock feed in many parts of the world, mainly because of their high protein content (Ammar et al., 2004). Given the high MAT content, the use of these legumes is indicated as a protein supplement to poor quality fodder and fibrous by-products. In our case, the MAT content of the species in the first two groups is higher than the minimum level of 7-8% DM required for rumen functioning to ensure maximum metabolic activity and adequate feeding of ruminants (Van Soest, 1994; Norton, 2003). According to Boughalleb et al. (2019) total phenols of C. *Villosa* ranging from 34.6 to 45.1 mg GAE/g DW. Flavonoids varied from 21.4 to 34.1 mg QRE/g DW. High-maintained storage protein content (50.2% DW) occurred in C. *villosa* seeds. Globulins were the major proteins (47.6% of total proteins). Condensed tannins may play an important role in the defense system of seeds that are subjected to oxidative damage induced by abiotic factors (Troszynska and Ciska, 2002). In legume seeds, tannins are considered as antinutritional compounds (Mohan et al., 2016). Boughalleb et al. (2019) showed that the total condensed tannins was registered 0.15 ± 0.01 CTE/g DW. These values remained higher compared to those obtained for the leguminous L. *Sativus* (0.001–0.004 CTE/g DW) (Rybiński et al.,

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DOI: http://dx.doi.org/10.15666/aeer/1905_37173735

2018) and similar to those reported for the extract of *Prosopis farcta* (0.15 mg CE/g DW) by Lajnef et al. (2015).

Anti-nutritional substance content of leaves in shrub vegetation

The total wall contents of forage shrub leaves (NDF) in our study, which ranged from 36 to 61% DM, are generally comparable to those reported by other authors (Makkar and Singh, 1991; Ammar et al., 2005. However, there are some differences to note. This high NDF fraction rate could be explained by the environmental conditions in the semi-arid region. Indeed, Pascual et al. (2000) indicate that high temperatures and low precipitation tend to increase the parietal fraction (NDF) and decrease the soluble content of plants. As for the results of the lignin concentrations of these different samples, they remain high for all species and have good forage values ranging from 0.94 to 1.09 UFL and from 0.91 to 1.06 UFV). Tannins are anti-nutritional substances that are involved in defence mechanis DM, they protect the plant against attacks by pathogenic microorganis DM (fungi and bacteria) and herbivorous predators (animals or foliar insects), they therefore have a very important agronomic advantage (Rira, 2006). Their presence may also reflect a response to stress (scarcity of rainfall, unfavourable soil quality associated with an increase in tannin levels). The large variation in tannin content in woody species has been observed by many researchers (Kaitho et al., 1998). In the study of chemical composition of *Genista aspalathoides* from North Western Tunisia, Selmi et al. (2020) showed that the species contains 34.1% of dry matter (DM), 94.1% organic matter (OM), 2.75% crude protein (CP), 2.37% fat and 5.79% crude fiber (CF), 36.1% ADF, 45.1% NDF, 30.3% ADL, 21.5 mg GAE/g DM (Polyphenols), 2.79 mg QE/g MD (Flavonoïds), and 5.82 mg CE/g MD (Condensed tannins). Similar results were found by Selmi et al. (2018).

Digestibilities and energy and nitrogen values of forage shrub leaves

The results of the total concentration of condensed tannins show significantly different ($P < 0.001$) and low values compared to those obtained in the work of Ammar et al. (2004, 2009) and Alvarez et al. (2005) for shrub leaves from northern Spain. Kumar and Vaithiyanathan (1990) report beneficial effects of condensed tannins in ruminant feeds (<50 g/Kg DM) as they promote the absorption of amino acids in the small intestine by protecting them from the effects of gastric juice. Zimmer and Cordesse (1996) also report that the same dose of tannins extracted from chestnuts improves the quantitative degradation of protein in sheep. Thus, the species studied, based on their moderate content of total and condensed tannins, may have a nutritional advantage for the animal. The chemical composition of the foliage of forage plants varies between species, this may be largely due to genotypic factors because the accumulation of nutrients in plants is a specific property (Minson, 1990) that varies between species and genera. To our knowledge, our work is the first to focus on the determination of saponin content and hemolytic activity of local shrub species. This content varied between 7 and 10 g diosgenin equivalent/kg DM. The planting of fodder shrubs in order to exploit their leaves may present, in Tunisia, an interesting alternative to face the problem of fodder deficit. In this context, the choice of species to be planted must be based both on the determination of forage values (based on the determination of the primary chemical composition) of the leaves and on the content of secondary compounds. For example, *Calicotome villosa* from Meknassi and Bouhedma and

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DOI: http://dx.doi.org/10.15666/aeer/1905_37173735

Genista from Rtiba have a high content of MAD and high UFL, UFV values, but it has high tannin contents that reduce the availability of nutrients in this species to the animal. In the study of chemical composition of *Calicotome villosa* in Algeria, Mebirouk-Boudechiche et al. (2015) showed that the species contains 282 g/kg of dry matter (DM), 73.9 g/kg MS of mineral matter (MM), 337 g/kg MS of total nitrogenous matter (MAT), 17.6 g/kg DM of crude fibre (CF), 119.43 g eq. tannic acid/kg MS of total phenols, 83.68 g eq. tannic acid/kg DM of total tannins.

Effects of salt stress on germination

In arid ecosystems, salinity and moisture availability are among the main factors influencing germination. Indeed, the study of germination patterns of plant species would increase our understanding of their ability to colonize marginalized areas in contrasting environments (Tlili et al., 2019).

Salinity stress can affect seed germination through osmotic effects (Welbaum et al., 1990) and by ion-toxicity (Huang and Reddman, 1995). More than 50% of the seeds of *Calicotome villosa* and *Genista spachiana* germinated at lower salt stress (9 g/l) and at the lower water potential (-1 MPa). This suggested that these species can germinate under low water availability. In study of effect of salinity Mechergui et al. (2017) showed that *Calicotome villosa* and *Genista spachiana* can tolerate salinity of up to 15 g/l of salt (31–37% germination at 15 g/l) and also tolerate drought (30–40% germination at −1.6 MPa. This tolerance of water potential (-0.8 MPa) was also observed in other Fabaceae and desert species like Retama raetam (Youssef, 2009). Generally, salt stress affected the germination capacity and speed of Calicotome and Genista seeds, these results agree with these of Lachiheb et al. (2004). In our study, seed germination percentage was higher in NaCl than in PEG at the same water potential. Some He et al. (2009) demonstrated that NaCl and PEG adversely affected germination, but NaCl had a less inhibitory effect on seed germination than an iso-osmotic solution of PEG. In contrast, Katembe et al. (1998) found that higher concentrations of NaCl (-1 MPa) were more inhibitory to germination of two Atriplex species (A. halunus and A. numelaria). Seeds of Calicotome villosa and Genista spachiana responded in two characteristic ways to salinity: first, germination was reduced, second at very low concentrations, germination was stimulated. Although higher salinity generally decreases germination, the detrimental effect of salinity is less severe at the optimum germination osmotic potential. Salt stress decreased both the rate and percentage of germination of Calicotome villosa and Genista spachiana. Several other studies revealing those halophytes, as well as glycophytes, are sensitive to salt during the germination stage (Katembe et al., 1998; Khan et al., 2002; Gorai and Neffati, 2007; Gorai et al., 2011). Considering the percentage of seeds that germinated at -1 MPa and 12 g/l, we conclude that these two fabaceae species are well adapted to germinate under conditions of water and salt stresses. These abiotic stresses are typical of the environments in which they grow. Where it grows and lives. Arid lands of Tunisia are widely affected by desertification, which is caused particularly by the degradation of the vegetation cover, deforestation and drought. According to Tlili et al. (2019) under control conditions (0 MPa), the highest percentage of germination was reached for A. mollis. However, germination decreased significantly at −0.75 MPa (NaCl and PEG). It is generally assumed that halophytes germinate better at low than at high salinities, but this varies according to the species (Ungar, 1995). Previous studies on Atriplex species, including A. micrantha (Liu et al., 2007; Yan and Wei, 2014), A. halimus (Muñoz-

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Rodríguez et al., 2012) and A. portulacoides (Muñoz-Rodríguez et al., 2017) found a salt tolerance in their germination responses and an inhibitory effect of the bracteoles.

Effects of osmotic potential on germination

The high ability of *Calicotome villosa* and *Genista spachiana* germinate over a wide range of environmental conditions provides an opportunity to contribute to future reforestation. *Calicotome villosa* seeds from Meknassi had a higher rate of germination at -0.03 MPa than at 0 MPa compared with non-stressed seeds. Seed germination percentage and the Kotowski coefficient generally decreases as soil water potential decreases (Evans and Etherington, 1990). Germination of three deciduous semi-shrubs of Artemisia was inhibited severely in PEG_{6000} solutions at −1.2 MPa (Tobe et al., 2000). An increase in osmolality of PEG_{6000} solutions results in decreasing both the percentage and rate of germination *Calicotome villosa* and *Genista spachiana*, indicating that water stress inhibits germination, which is in agreement with the germination behaviour of most species (Tobe et al., 2000; Gorai et al., 2009; Maraghni et al., 2010). It can be concluded that seeds of *Calicotome villosa* and *Genista spachiana* germinate have the ability to tolerate salt stress after exposure to NaCl solutions and osmotic potential concentrations. Further investigations are necessary to understand the early establishment of this species under field conditions and to determine if there are differences between the seed germination stage and early seedling growth in response to salinity and drought stress. These results corroborate the findings obtained by Aiazzi et al. (1992) showed that seeds of *A. cordobens* is had drought-induced dormancy at low temperature, but at 30 °C seeds may lose viability. In contrast, Mandana et al. (2017) found that A. *halimus* is highly tolerant to water stress with a final germination of ca. 31% at−1.5 MPa as compared to salt stress with only a few seeds that germinated (3%). These authors suggested that A. *halimus* seeds were more tolerant to salinity than water stress, which would help in using this species in rehabilitation programs of degraded lands. Sharma (1976) suggested that among semi-arid plant species most of Atriplex species decreased their germination when seeds exposed to less negative osmotic potential (−1.5 MPa). William et al. (1998) showed that imbibition processes, germination and root elongation of A. *prostrate* were inhibited at low osmotic potential (−1 MPa). These traits were more inhibited by salinity than by water stress at−1 MPa. This result is not consistent with study of Tlili et al. (2019) for A. *mollis*.

Conclusions

The use of fodder shrub leaves in the feeding of ruminants is an interesting food alternative given the fodder deficit encountered in Tunisia and the increase in the prices of raw materials formulating concentrated feed. Indeed, the nitrogen and energy values of these shrub leaves can cover the needs of ruminants for a long period of the year and especially during the lean season. Given the overgrazing proble DM in the study area, it would be interesting to start planting some shrubs. In this context, this study may be of interest in DM of the choice of species to be planted. Thus, chemical analysis revealed that the total nitrogen content of the leaves of the species studied is interesting. This makes it possible to consider the leaves of this shrub as an additive nitrogen supplement with fodder or fibrous by-products, however, its high content of tannins and total phenols should be taken into account by combining certain products or applying certain

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methods to deactivate them: use of polyethylene glycol (PEG), essential oils, drying.... *Genista spachiana* is can tolerant to salt stress up to NaCl concentration of 12 g/l. Though germination rate in *Calicotome villosa* and *Genista spachiana* decrease with increasing concentrations of PEG, seeds of the two species germinated to 30 and to 40% at -1.6 MPa. Thus, it is concluded from the study that both the species can tolerate water and salt stresses very well. Further it is also inferred that *Genista spachiana* provenance from Rtiba is more tolerant than *Calicotome villosa* provenance to water stress with a germination rate of 40% at water potential of -1.6 MPa. In order to progress in this field, it would necessary to analyze total phenolics and total tannins content of the different plant tissues, detect other inhibitory compounds and carry out bioassay in the presence and absence of tannin-complexing agents, such as PEG, to estimate tannin activity.

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

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_37173735

EFFECTS OF AN EIGHT-YEAR NITROGEN AND PHOSPHOROUS ADDITION ON LEAF PHOTOSYNTHESIS AND CHEMISTRY OF MATURE *CASTANOPSIS SCLEROPHYLLA* TREES IN SUBTROPICAL CHINA

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(Received 1st Apr 2021; accepted 19th Jul 2021)

Abstract. How long-term nitrogen (N) deposition affects plant growth in N-rich subtropical forest ecosystems, and whether there are interactive effects between N and phosphorous (P) addition on physiological performances remain unclear. To address these questions, an eight-year nutrient addition experiment was conducted to examine the chronic effects on trees in a secondary *Castanopsis sclerophylla* forest in subtropical China. Our key results showed that (1) Long-term N deposition could stimulate mature *C. sclerophylla* growth mainly by increasing the net photosynthetic rate (*Pn*), soluble sugars (*SS*) and total non-structural carbohydrates (*NSCs*). Furthermore, there were significant interactive effects on growth between N and P addition. (2) Leaf chlorophyll a + b, *SS* and total *NSCs* under N, P and NP addition were all increased in two years (except the chlorophyll a + b in 2020 and total *NSCs* in 2019). The positive impacts of P or NP addition on leaf chlorophyll a + b, *SS* and total *NSCs* were stronger compared to N addition. (3) Compared to N addition, P addition had a positive stimulation on leaf *Pn*. These conclusions demonstrated that P addition either alone or together with the addition of N have positive effects on the physiological performances of mature *C. sclerophylla* trees.

Keywords: *nitrogen deposition, photosynthesis, soluble protein, nonstructural carbohydrate, nitrogen and phosphorus interaction*

Introduction

Due to intensive human activities in recent decades (e.g., agricultural intensification, fuel combustion, automobile exhaust emissions), the global nitrogen (N) deposition rate has increased dramatically and is expected to double from the current level by 2050 (Yu et al., 2019; Liu et al., 2019, 2016). Considering that N is the limiting nutrient for plant growth, development and photosynthesis, increased N deposition can provide a new source of fertilizer for plant growth (Güsewell, 2004; Li et al., 2016; Luo et al., 2015), but conversely, can modify biogeochemical cycles (Lu et al., 2014), change ecosystem structures and functions (Cao et al., 2019), and even lead to local extinction of plant species (Gotelli and Ellison, 2002; McClean et al., 2011). Phosphorous (P) is another limiting nutrient affecting plant growth. In contrast to deposited N which has multiple pathway and exhibits high mobility, P originates from fewer sources and has less mobility (Mahowald et al., 2008). Plant N: P stoichiometry can be used to determine

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_37373751

plant adaptation and feedback in response to environmental change. In N-saturated ecosystems, mainly in subtropical and tropical forests, excess N inputs may decrease soil labile P fractions and cause ecological N:P stoichiometry imbalance (Hui et al., 2020; Chen et al., 2018). Although the increasing recognition of soil P availability in regulating N deposition effects on subtropical and tropical forests, the direct experiment of how tree growth and physiology affected by P addition interact with N deposition is still insufficient.

N influences the carboxylation capacity and electron transport rates in photosynthesis (Evans, 1989). Thus, the effects of N addition on plant photosynthesis have been well recognized. However, the results have been inconsistent in different tests, giving both positive (Liang et al., 2020; Liu et al., 2019), neutral (Talhelm et al., 2011) and negative responses (Mao et al., 2017). In N-limited ecosystems, for temperate forests in particular, N deposition can increase photosynthetic capacity and simulate plant growth (Shi et al., 2020). In addition to the universal concept that leaf P is an essential element related to photosynthesis (Crous et al., 2017), P deficiency can affect light-use efficiency (Conroy et al., 1986). However, it remains unclear whether P addition can alleviate the effect of N-induced P limitation on plant photosynthesis.

As necessary components of proteins, amino acids and chlorophylls, N and P are involved in synthesizing various metabolites (Liu et al., 2018a). Thus, either N or P addition can influence leaf biochemical composition and metabolic processes. According to previous studies, excess N input can stimulate the formation of chlorophyll, soluble protein, and free amino acids (*FAA*) in plant tissues (Fritz et al., 2012), which can disorder N metabolism subsequently. Non-structural carbohydrates (*NSCs*) are the main substrates for both primary and secondary plant metabolism, can provide a buffer when plant photosynthesis is insufficient (Hartmann and Trumbore, 2016). N and P addition can affect plant photosynthetic processes and the concentration of *NSCs* including soluble sugar (*SS*) and starch (*ST*) (Liu et al., 2016; Mo et al., 2020). At present, numerous experiments have investigated the effects of increased nutrients supply on tree physiological traits, including leaf gas exchange, biochemical components concentration and carbon (C): N: P stoichiometry. These experiments usually use pulse additions on seedlings or understory shrubs over a relative short timescale (Wang et al., 2019; Mao et al., 2017), which may not indicate the true chronic effects on mature trees.

Castanopsis sclerophylla, a dominant species in evergreen broad-leaved forests, is widely distributed in subtropical areas of eastern Asia and has high ecological and economic value (Shi et al., 2011). This species plays a crucial role in maintaining the function and stability of local ecosystems (Zhang et al., 2007). Over the past few decades, the subtropical areas of eastern China have been affected by human activities, including enhanced N deposition that clearly affects many aspects of its ecosystems. In this study, changes in plant growth, leaf gas change, nutrient status, and chemical traits in a *C. sclerophylla* secondary forest with 8-year N deposition and P addition were investigated. Given the status of local soil as P-limitation (Teng et al., 2018; Han et al., 2005), our objectives were to address the following questions: (1) How does long-term N addition affect the growth, leaf photosynthesis and chemistry of the mature *C. sclerophylla*? (2) whether or not there are interactive effects between N and P addition on physiological performances.

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Material and methods

Study site

The study site (30°01′47″N, 117°21′23″E) was located in Chizhou, south Anhui Province, China. It has a humid subtropical monsoon climate with an average temperature of 16 °C, and an average rainfall of 1521 mm. The soil is thin (between 70 and 100 cm depth) and has a clay-loam texture. The original vegetation was severely destroyed before the 1960s and restored by planting *Castanopsis sclerophylla* (Lindl.) Schott around 1965. This forest is now dominated by *C. sclerophylla* with a diverse sub-canopy of various tree species.

Experimental setup

In June 2012, twelve permanent plots were established (15 m × 15 m, 10-m wide buffer zone). The diameter at breast height (*DBH*) in 2012 showed no significant difference between plots. The experiment included four treatments with three replicates in a randomized block design. The treatments were: control (CK), N addition (100 kg N ha^{-1} $year^{-1}$), P addition (50 kg P ha^{-1} $year^{-1}$) and NP addition (100 kg N ha^{-1} $year^{-1}$ + 50 kg P ha^{-1} $year^{-1}$). N was applied as NH_4NO_3, and P as $Ca(H_2PO_4)_2$. Nutrients were dissolved in 20 L water and evenly sprayed into the corresponding plots near the soil surface using a backpack sprayer every 2 months from June 2012. Control plots only received 20 L water. The buffer zone of 10-m surrounding the plots was also fertilized.

Growth and photosynthesis measurements

In January 2019 (T_1) and December 2019 (T_2), we measured *DBH* of all individuals using a diameter ruler to calculate the breast growth rate (GR_{DBH}):

$$GR_{DBH} = \left(DBH_{T2} - DBH_{T1}\right) / DBH_{T1} \quad \text{(Eq.1)}$$

In July 2019 and July 2020, while constrained by the health status of suitable leaves, at least four trees were randomly selected from each plot for the photosynthesis measurements. We used a 20m-long sea fishing rod with a blade attached to the end (CF140-20, Gaoding, Co., Ltd., China) to collect the fully-extended branches in the canopy (>15 m). During measurements, branches were put into big bucket full of water to avoid water loss. The light response curve measurements were conducted on fully expanded healthy leaves using a Li-6400 portable photosynthesis system (Li-Cor, USA). The chamber CO_2 concentration was 380 μmol mol^{-1}, light intensity was 2000 μmol m^{-2} s^{-1}, and leaf temperature was consistent with atmospheric temperature. After acclimating to the cuvette environment, leaf photosynthetic light response curve was measured in fifteen steps of light intensity, starting at 2000 and decreasing to 1800, 1500, 1300, 1100, 900, 700, 500, 400, 300, 200, 150, 100, 50 and 0 μmol m^{-2} s^{-1}. Leaf photosynthesis was monitored to ensure reaching a stable state at each light intensity before data were recorded. The light response curves were fitted using the equation of the collect nonrectangular hyperbolic model (Ye, 2007).

The tendency of respiration rate (R_d) is given by:

$$R_d = -\alpha LCP \quad \text{(Eq.2)}$$

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The light compensation point (*LCP*) is given by:

$$LCP = \frac{-(\gamma R_d - \alpha) - \sqrt{(\gamma R_d - \alpha)^2 - 4\alpha\beta R_d}}{2\alpha\beta} \quad \text{(Eq.3)}$$

The light saturation point (*LSP*) is given by:

$$LSP = \frac{\sqrt{(\beta + \gamma)\beta} - 1}{\gamma} \quad \text{(Eq.4)}$$

The apparent quantum yield (*AQE*) is given by:

$$AQE = \alpha \frac{1 + (\gamma - \beta)LCP - \beta\gamma LCP^2}{(1 + \gamma LCP)^2} \quad \text{(Eq.5)}$$

where α, β, and γ are coefficients which are independent of light intensity.

In this study, the light-saturated net photosynthetic rate (*Pn*), transpiration rate (*E*), instantaneous water use efficiency (WUE_i) and stomatal conductance (g_s) were measured at 1500 μmol m^{-2} s^{-1} light intensity. The leaf colour (*SPAD*) was measured with a chlorophyll meter (SPAD-502P, Yunnong, Co., Ltd., China).

Leaf pigments, soluble protein and free amino acid concentrations

After the leaf *Pn* measurement, leaves were picked off and immediately put into zip-lock bags with ice packs in a plastic bucket, and then stored at -20 °C freezer until leaf soluble protein (*SP*) and *FAA* measurements were conducted. The collected samples were cleaned with deionized water, and the surface water was sucked dry with filter paper. About 0.20 g fresh leaves were homogenized with a small amount of $CaCO_3$ and quartz sand, containing 3 ml 95% (v/v) ethanol, filter and constant volume to 25 ml. The Chlorophyll a (*Chl a*) and Chlorophyll b (*Chl b*) were determined at 665 nm and 649 nm using a microplate reader (Epoch 2, BioTek Instruments, Inc., USA).

SP and *FAA* were extracted according to Xu and Zhou (2006). Fresh leaves were homogenized with 10 ml of 50 mM sodium phosphate (pH = 7.8), containing 2 mM ethylenediaminetetraacetic acid (EDTA) and 80 mM L-Ascorbic acid. The supernatants were collected for *SP* and *FAA* by centrifugation for 20 min at 15000 r min^{-1}. The *SP* was determined according to Bradford (1976). The *FAA* was measured with an amino acid auto analyzer (Hitachi 835-50, Hitachi Ltd., Japan) according to Barnett and Naylor (1966).

Leaf structural traits, nutrient status and NSCs

Leaf area was measured using a Li-3100A leaf area instrument (Li-Cor, USA). Specific leaf area (*SLA*) was calculated as leaf area divided by leaf dry mass. After the leaf pigments, *SP* and *FAA* measurements, leaf samples were deactivated at 105 °C for 30 min, and then oven-dried at 60 °C to determine their dry mass. Oven-dried leaves were pulverized with a Mill (BO-500S1, Boou, Co., Ltd., China), then finally passed through a 100-mesh sieve. N and P concentration per leaf dry mass (*Nmass* and *Pmass*)

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were measured using a sulfuric acid/hydrogen peroxide digest. The leaf powder samples (0.100 g) were put into a 50 ml conical flask, where 3 ml distilled water and 5 ml sulfuric acid were added to dissolve the liquid at 240 °C about 3 h using a Graphite heating plate (CB-2, Keheng, Co., Ltd., China). The leaf N and P concentrations were analyzed with an element analyzer (CleverchemAnn, Germany). Leaf N concentration per leaf area (*Narea*) was calculated as *Nmass* divided by *SLA*. Photosynthetic N-use efficiency (*PNUE*) was calculated as follows:

$$PNUE = Pn / Narea \times 10 \quad \text{(Eq.6)}$$

The total *NSCs* was the sum of *SS* and *ST*. Leaf *NSCs* was analyzed by standard anthrone-colourimetric method. *SS* were extracted from 50.0 mg powder sample with 4 ml 80% (v/v) ethanol. The extraction was incubated in a water bath at 80 °C for 30 min. The supernatants were collected for *SS* concentration by centrifugation for 5 min at 3000 $r \cdot min^{-1}$. The residue was extracted two more times as described above. To measure *ST*, briefly, after cooling to room temperature, the residue of the ethanol extraction was extracted two times with 2 ml $HClO_4$ followed by colourimetric analysis with anthrone/sulfuric acid. The *SS* and *ST* were analyzed at 625 nm using a microplate reader (Epoch 2, BioTek Instruments, Inc., USA).

Statistical analysis

Statistical analyses were performed using SPSS 20.0 (SPSS, Inc., USA). Before analysis, the data were tested for normal distribution and homogeneity of variances. Multiple comparisons were conducted using Ducan's test to evaluate the differences in variables between four treatments. We conducted two-way ANOVA to evaluate the interacting effects between N and P addition. A p value of less than 0.05 was considered statistically significant.

Results

Tree growth, specific leaf area and leaf nutrient

The GR_{DBH} under N, P and NP addition were promoted (25.35%, 19.25% and 32.86% when compared with CK, respectively; *Fig. 1a*). We did not detect significant effect of N, P addition and their interaction on *SLA* in both 2019 and 2020.

N addition had no significant effect on *Nmass* and leaf *N/P* in 2019, while it increased *Nmass* in 2020 (*Fig. 2a*). P and NP addition increased *Pmass* and decreased leaf *N/P* (14.67 and 10.68 when compared with 27.06 for the CK, respectively) in 2020, but they had no significant effect in 2019 (*Fig. 2c*). Two-way ANOVA analysis found that no significant interactive effect between N and P on *Nmass*, *Pmass* and *N/P* in 2019 (*Table 1*).

Leaf pigments and photosynthesis

P and NP addition all increased *Chl a* and *Chl b* in 2020, and N addition increased *Chl b* in 2019. Furthermore, *C. sclerophylla* possessed a lower *Chl b* under N addition when compared to P and NP addition in 2019. Meanwhile, both *Chl a* and *Chl b* under all treatments were much higher in 2020 than in 2019. Thus, a similar pattern was found

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for leaf *Chl a + b* (*Fig. 3*). Two-way ANOVA analysis found that significant interactive effects between N and P on leaf *Chl b* in both two years and *Chl a + b* in 2020 ($p < 0.05$), whereas it had no effect on leaf *Chl a* (*Table 1*).

Table 1. *Results (F and P values) from two-way ANOVA analysis for the effects of N addition, P addition and their interactions on foliar chemical traits*

Source of variation	Time	N addition		P addition		NP addition	
		F	*p*	F	*p*	F	*p*
SLA	July 2019	0.078	0.787	6.502	**0.034**	0.034	0.858
	July 2020	2.543	0.149	1.989	0.196	0.488	0.504
Nmass	July 2019	0.526	0.489	2.397	0.160	1.489	0.257
	July 2020	0.008	0.929	6.043	**0.039**	31.408	**0.001**
Pmass	July 2019	0.159	0.700	5.360	**0.049**	2.465	0.155
	July 2020	5.785	**0.043**	83.537	**0.000**	0.019	0.894
N/P	July 2019	1.227	0.300	6.129	**0.037**	3.886	0.084
	July 2020	2.537	0.150	43.659	**0.000**	0.162	0.697
Chl a	July 2019	1.559	0.247	9.195	**0.016**	0.065	0.805
	July 2020	15.655	**0.004**	56.718	**0.000**	2.212	0.175
Chl b	July 2019	74.324	**0.000**	183.414	**0.000**	9.247	**0.016**
	July 2020	27.186	**0.001**	60.350	**0.000**	15.856	**0.004**
Chl a + b	July 2019	29.279	**0.001**	84.669	**0.000**	3.193	0.112
	July 2020	20.420	**0.002**	62.228	**0.000**	5.509	**0.047**
SS	July 2019	18.557	**0.003**	14.349	**0.005**	1.050	0.336
	July 2020	1.421	0.267	108.209	**0.000**	23.988	**0.001**
ST	July 2019	0.130	0.727	4.813	0.060	2.004	0.195
	July 2020	0.003	0.957	3.859	0.085	0.117	0.741
NSCs	July 2019	8.308	**0.020**	11.300	**0.010**	0.061	0.811
	July 2020	1.211	0.303	109.157	**0.000**	20.082	**0.002**

SLA: specific leaf area; Nmass: nitrogen content per leaf dry mass; Pmass: phosphorus content per leaf dry mass; Chl a: chlorophyll a; Chl b: chlorophyll b; SS: soluble sugar; ST: starch; NSCs: nonstructural carbohydrates. Significant differences ($p < 0.05$) are shown in bold

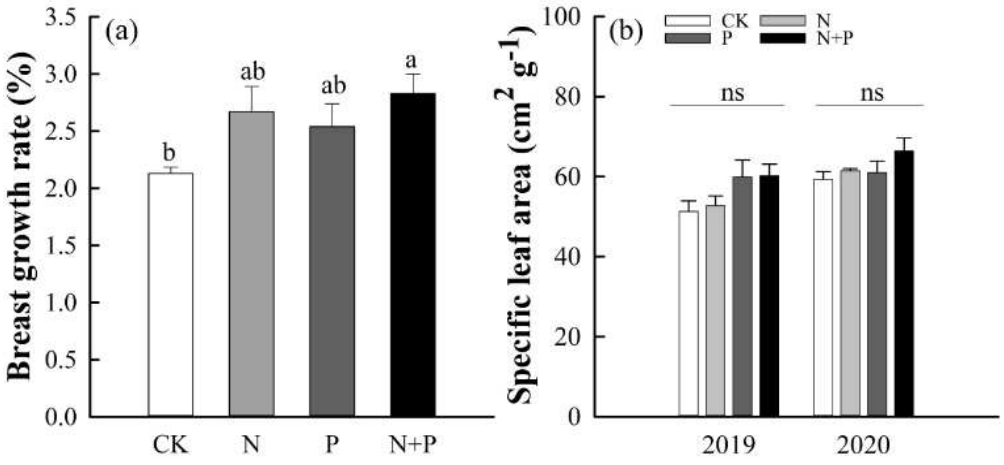

Figure 1. *Effects of N and P addition on breast growth rate (a), specific leaf area (b) of mature Castanopsis sclerophylla trees. Values are mean ± SE (n = 3). Lower case letters indicate significant differences among treatments at the p < 0.05 level in each year*

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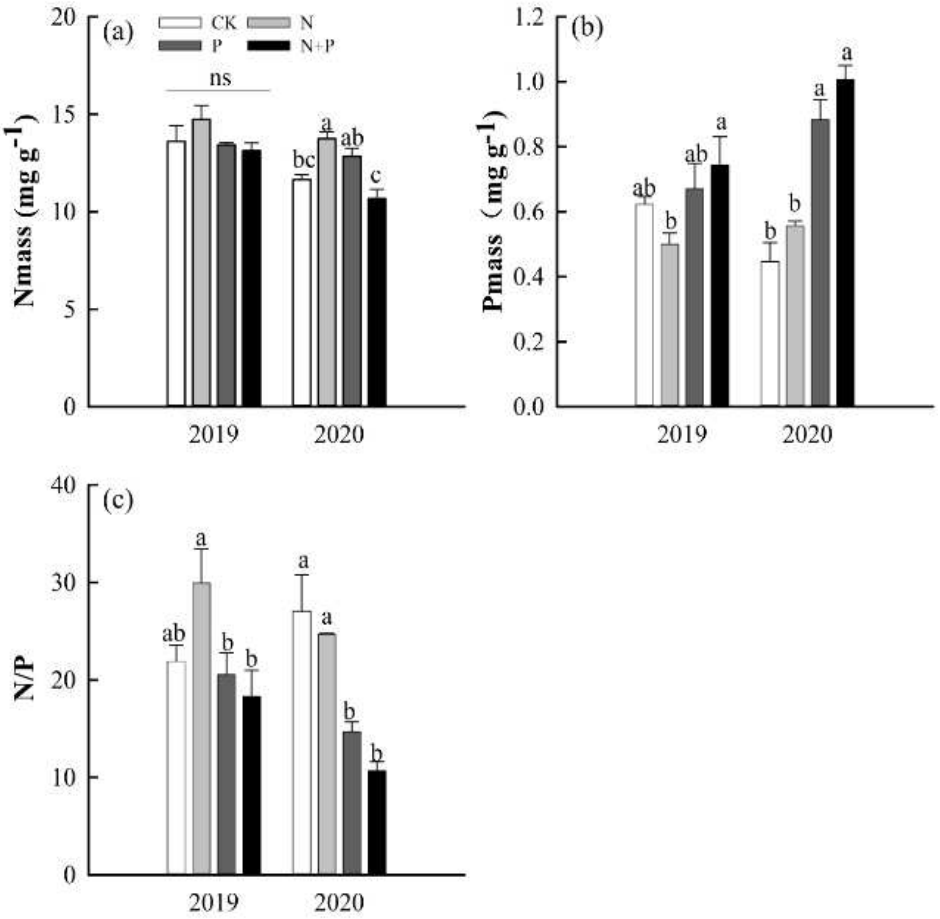

Figure 2. *Effects of N and P addition on leaf N content per unit leaf dry mass (a), leaf P content per unit leaf dry mass (b) and leaf N:P ratio (c) of mature Castanopsis sclerophylla trees. Values are mean ± SE (n = 3). Lower case letters indicate significant differences among treatments at the p < 0.05 level in each year*

There was no significant difference in *LSP*, *LCP*, *AQE* and R_d under N, P or NP addition when compared to the control ($p > 0.05$) (*Table 2*). However, N, P and NP addition increased leaf *Pn*, g_s and *SPAD* ($p < 0.05$). P and NP addition increased leaf *PNUE* and *E* ($p < 0.05$). The WUE_i increased with N and P addition ($p < 0.05$), but slightly decreased with NP addition ($p > 0.05$) (*Table 2*).

Table 2. *Comparisons in parameters from light-response curves between treatments*

Treatment	*LSP* µmol m^{-2}s^{-1}	*LCP* µmol m^{-2}s^{-1}	*AQE* mol mol^{-1}	R_d µmol m^{-2}s^{-1}	*Pn* µmol m^{-2}s^{-1}	*PNUE* µmol g^{-1}s^{-1}	*E* mol m^{-2}s^{-1}	WUE_i µmol mol^{-1}	g_s mol m^{-2}s^{-1}	*SPAD* mg g^{-1}
CK	724.0 (166.6)	81.3 (17.6)	0.032 (0.005)	2.40 (0.21)	8.03c (0.21)	4.09b (0.15)	2.67b (0.18)	3.03c (0.14)	0.112c (0.004)	37.54c (0.18)
N addition	676.0 (93.2)	41.3 (10.4)	0.055 (0.014)	2.07 (0.15)	11.66b (0.22)	5.22b (0.24)	2.88b (0.04)	4.05a (0.03)	0.138b (0.003)	44.85b (1.37)
P addition	804.0 (48.2)	49.3 (8.1)	0.048 (0.008)	2.47 (0.66)	14.38a (0.13)	6.86a (0.50)	4.37a (0.03)	3.29b (0.01)	0.185a (0.002)	55.60a (1.86)
NP addition	1070.7 (187.0)	66.7 (16.7)	0.032 (0.004)	1.97 (0.18)	12.22b (0.14)	7.68a (0.76)	4.06a (0.04)	3.01c (0.01)	0.182a (0.004)	45.84b (0.75)

Values are the mean ± (SE); n = 3. Lower cases letters indicate significant differences among treatments at the p < 0.05 level

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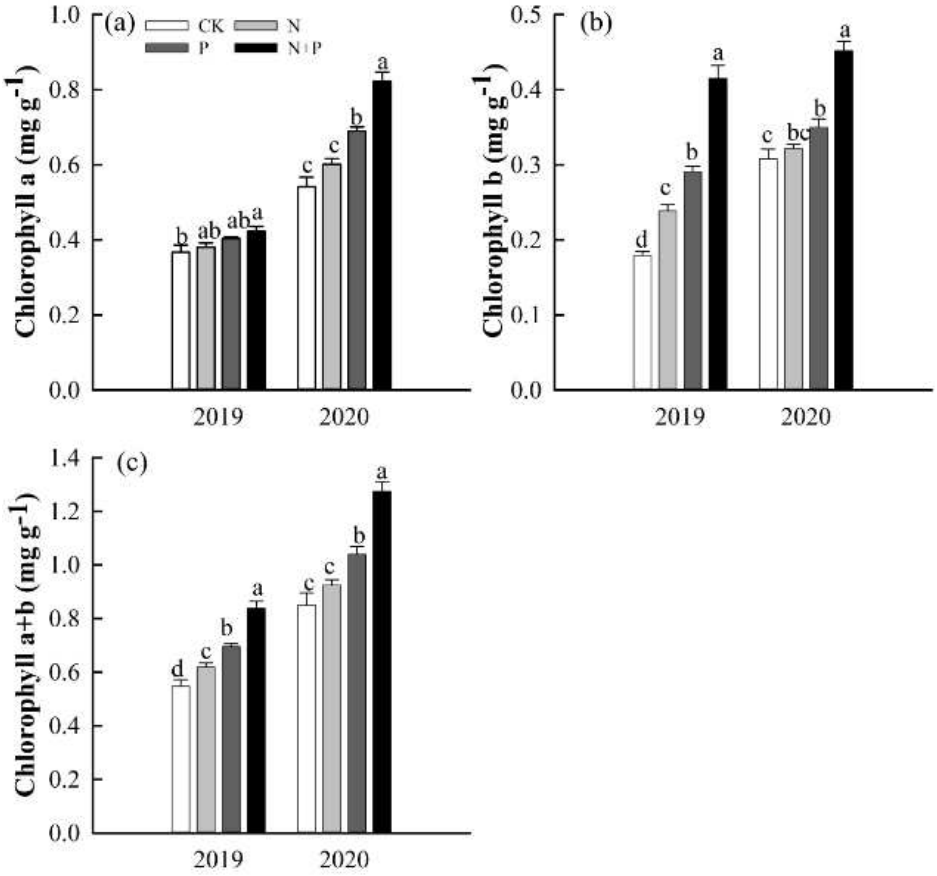

Figure 3. *Effects of N and P addition on concentration of chlorophyll a (a), chlorophyll b (b) and chlorophyll a + b (c) in leaves of mature Castanopsis sclerophylla trees. Values are mean ± SE (n = 3). Lower case letters indicate significant differences among treatments at the p < 0.05 level in each year*

Leaf soluble protein, free amino acid and non-structural carbohydrates

In 2020, N and P addition greatly increased leaf *SP*, while P addition decreased leaf *FAA* of *C. sclerophylla* ($p < 0.05$). NP addition had no influence on leaf *SP* but increased the *FAA* of *C. sclerophylla* leaves ($p < 0.05$). Two-way ANOVA analysis found that significant interactive effect between N and P on leaf *FAA* (*Table 3; Fig. 4*).

N, P and NP addition increased leaf *SS* and total *NSCs* during the experiment, with the exception of N addition that had no influence on total *NSCs* in 2019. There was no detectable change in leaf *ST* in both two years. Two-way ANOVA analysis found that significant interactive effects between N and P on leaf *SS* and *NSCs* in 2020, whereas it had no effect on leaf *ST* (*Table 1; Fig. 5*).

Discussion

Response of growth, specific leaf area and leaf nutrient

In this study, the GR_{DBH} under N addition was 25.35% higher than control ($p = 0.059$, *Fig. 1a*). Interestingly, Tian et al. (2017) found 3.4 years N deposition declined growth of small *Castanopsis eyrei* in a subtropical forest, but it had no significant effect on median

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DOI: http://dx.doi.org/10.15666/aeer/1905_37373751

and large trees. In a meta-analysis, Yue et al. (2017) found that the plant growth could be suppressed by N-induced P limitation. N addition can enhance the availability of N in soil. P addition could relieve soil P deficiency, and consequently promoted the plant growth. Moreover, we found that NP addition posed a positive effect on plant growth. Therefore, it is not surprising that the GR_{DBH} was increased under long-term nutrient addition. No significant difference of *SLA* was found under nutrient addition in two years (*Fig. 1b*). This result was consistent with the findings of Mao et al. (2017), which found long-term N addition has no significant difference of *SLA*.

Table 3. *Results from two-way ANOVA analysis for the effects of N addition, P addition and their interactions on tree growth and physiological traits*

Source of variation	Time	N addition		P addition		NP addition	
		F	Sig.	F	Sig.	F	Sig.
GR_{DBH}	July 2020	5.717	**0.044**	2.696	0.139	0.519	0.492
Protein	July 2020	0.039	0.849	0.247	0.633	20.351	**0.002**
FAA	July 2020	43.442	**0.000**	6.591	**0.033**	31.957	**0.000**
SPAD	July 2020	1.452	0.263	58.169	**0.000**	51.940	**0.000**
LSP	July 2020	0.649	0.444	3.056	0.119	1.343	0.280
LCP	July 2020	0.672	0.436	0.058	0.816	4.300	0.072
AQE	July 2020	0.149	0.710	0.149	0.710	5.357	**0.049**
R_d	July 2020	1.319	0.284	0.002	0.964	0.053	0.824
Pn	July 2020	16.898	**0.003**	373.241	**0.000**	262.392	**0.000**
PNUE	July 2020	4.178	0.075	29.913	**0.001**	0.108	0.751
E	July 2020	0.258	0.625	218.283	**0.000**	6.852	**0.031**
WUE_i	July 2020	25.858	**0.001**	28.983	**0.001**	81.092	**0.000**
g_s	July 2020	12.097	**0.008**	321.954	**0.000**	21.011	**0.002**

GR_{DBH}: the breast growth rate; FAA: free amino acid; SPAD: leaf color value; LSP: light saturation point; LCP: light compensation point; AQY: apparent quantum yield; R_d: respiration rate; Pn: light-saturated net photosynthetic rate; PNUE: photosynthetic nitrogen use efficiency; E: transpiration rate; WUE_i: instantaneous water use efficiency; g_s: stomatal conductance. Significant differences ($p < 0.05$) are shown in bold

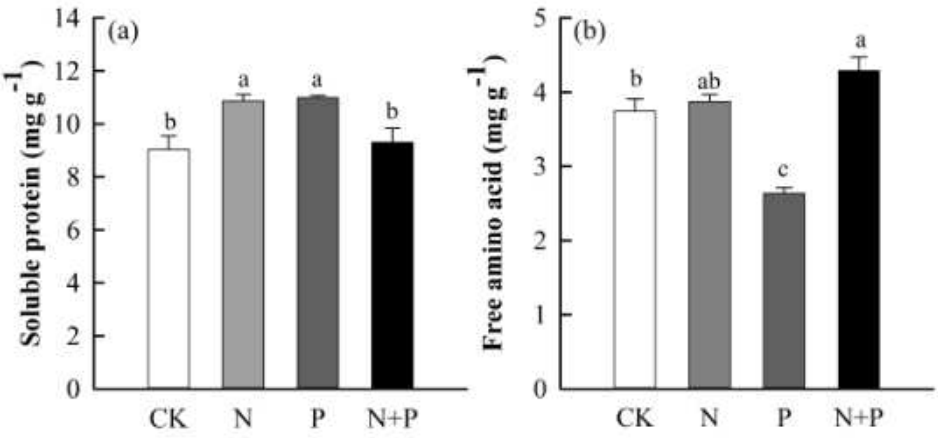

Figure 4. *Effects of N and P addition on concentration of soluble protein (a) and free amino acids (b) in leaves of mature Castanopsis sclerophylla trees. Values are mean ± SE (n = 3). Lower case letters indicate significant differences among treatments at the p < 0.05 level in 2020*

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DOI: http://dx.doi.org/10.15666/aeer/1905_37373751

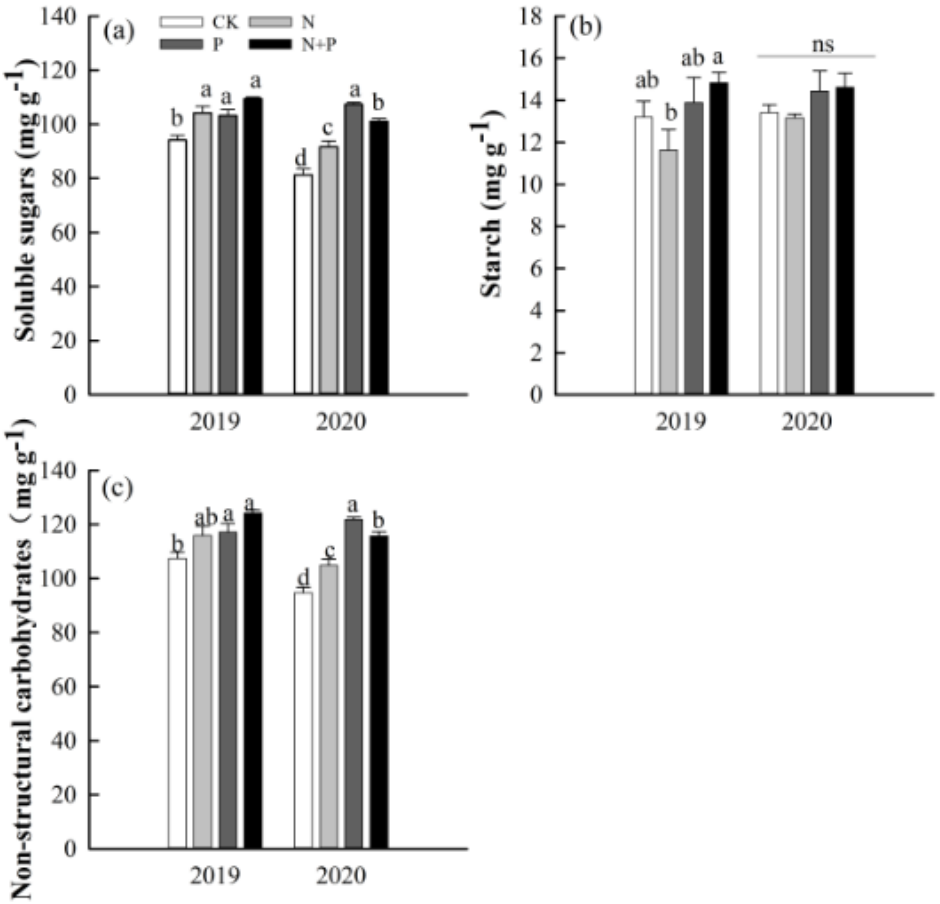

Figure 5. *Effects of N and P addition on concentration of soluble sugars (a), starch (b) and non-structural carbohydrates (c) in leaves of mature Castanopsis sclerophylla trees. Values are mean ± SE (n = 3). Lower case letters indicate significant differences among treatments at the $p < 0.05$ level in each year*

Plant *N/P* can indicate the limitation between N and P. Previous study found the *N/P* < 14 and *N/P* > 16 may amount to N-limited and P-limited (Koerselman and Meuleman, 1996). According to Reich and Oleksyn (2004), the leaf *N/P* in temperate tree species was around 19.9. Our study found that this forest had a relatively higher leaf *N/P* (21.91 and 27.06 at 2019 and 2020, respectively; *Fig. 2b*) compared with temperate tree species. One of the most consistent responses of plants to high N deposition is higher leaf N contents, indicating that N accumulates in plants when excess atmospheric N is absorbed (Talhelm et al., 2011; Mao et al., 2018). In the present study, *Nmass* was increased under N addition in this forest in 2020 (*Fig. 2a*). The increases of *Nmass* prove that plants in N-rich ecosystems can still take up the excessive N supply (Mao et al., 2018; Wang et al., 2019; Gurmesa et al., 2016). Under prolonged nutrient addition, based on the responses of *Pmass* to N addition in both two years, N addition likely did not inhibit P resorption in mature *C. sclerophylla* in this subtropical forest. In addition, P and NP addition significantly increased *Pmass* in 2020. Meanwhile, *Pmass* was much higher in 2020 than in 2019 (*Fig. 2c*). It could be attributed to plant leaves continue to absorb the external phosphorus to diminish the long-term P limitation (Mayor et al., 2014). The leaf *N/P* was significantly or

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DOI: http://dx.doi.org/10.15666/aeer/1905_37373751

marginally decreased under P and NP addition, indicating that P input may alleviate the soil P deficiency and leaf *N/P* imbalance, which induced by excessive N. Furthermore, plant leaves can be affected by environmental changes, the divergent responses of *Nmass*, *Pmass* and leaf *N/P* under N, P addition and their interaction between two years might be attributed to completely different precipitation in July 2019 (160.31 mm) and July 2020 (797.72 mm). The total monthly precipitation was provided by the Shitai Meteorological Bureau.

Response of leaf photosynthesis

In most cases, the *LSP*, *LCP*, *AQE* and R_d were unaffected by nutrient addition (*Table 3*). However, the *Pn* of *C. sclerophylla* was significantly increased under nutrient addition. There are several possible reasons for the increases leaf photosynthesis in this N-rich subtropical forest ecosystems. First, as the critical photosynthetic pigment, leaf *Chl* concentrations were greatly enhanced under P and NP addition. Furthermore, there is often a positive relationship between *SPAD* and *Chlorophyll* (Samdur et al., 2000). We found the leaf *SPAD* was also significantly increased under nutrient addition, and leaf *Chl* was also increased. Therefore, nutrient addition increased leaf *Chl* and *SPAD*, indicating an enhanced photosynthetic capacity. Second, we found that *Pn* increased in line with g_s and *E*. A significant increase of g_s under nutrient addition may positively affect the CO_2 uptake and CO_2 photosynthetic assimilation efficiency, which were significantly correlated with the leaf *Pn* (Li et al., 2018). Third, we also observed increased *Nmass* and *Pmass* under N, P addition or their interaction. Previous studies found that leaf N and P is essential to photosynthetic machinery and have strong influences on the Rubisco carboxylation and electron transport (Evans, 1989; Conroy et al., 1986). Consequently, increases in *Nmass* and *Pmass* were always accompanied by increases in photosynthesis.

Furthermore, we found a relatively stronger stimulation on leaf *Pn* by P addition compared to N addition. This could be attributed to higher leaf g_s, *SPAD* and photosynthetic enzymes (soluble protein in this paper). *C. sclerophylla* exhibited higher *PNUE* under P and NP addition than CK, which can reflect nutrition-related physiological traits of leaves. There is a previous evidence that increased N and P availability can improve *PNUE* (Hidaka and Kitayama, 2009). Such phenomenon could mainly be ascribed to increase of the *Nmass*, *Pmass* and *Pn* under long-term N and P addition.

Response of leaf soluble protein, free amino acid and non-structural carbohydrates

In this subtropical forest, similar to the change of *Nmass*, leaf N allocated to *SP* or *FAA* enhanced consistently with N addition in 2020 (*Fig. 5*), indicating that long-term N addition may result in increases in leaf N assimilation. Increases in leaf N owing to excessive N input are always accompanied by increases in protein, total *FAA*, and/or chlorophyll in previous studies (Bubier et al., 2011; Mao et al., 2018). Besides, the highest *SP* under P addition may be associated with enhanced photosynthetic enzyme activities, given that the *Pn* with P addition was the highest compared with N and NP addition. Compared to N and P addition, however, leaf *SP* under NP addition was lower (*Fig. 5a*). The decline in *SP* might result from the observed significant increases in *FAA* (*Fig. 5b*). Until now, the information about N metabolism in subtropical forests is still insufficient. Our results were consistent with findings in temperate forests. Furthermore,

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DOI: http://dx.doi.org/10.15666/aeer/1905_37373751

the consistent increase of N compounds in leaves indicates that the increased leaf N might be reserved as pigments and *SP* or *FAA* of *C. Sclerophylla* in this N-rich subtropical forest.

As the immediate products of plant photosynthesis, *NSCs* variations can indicate the regulation of plants C metabolism to environmental stress (Liu et al., 2018b; Xiao et al., 2017). Our long-term experiment indicated that nutrient addition significantly increased the leaf *SS* and total *NSCs* (*Fig. 4a,c*). Such a phenomenon may be attributed to higher photosynthesis, which accelerated the C assimilation (Liu et al., 2019). However, our study found that N addition posed a slight negative effect on leaf *ST* in both two years (*Fig. 4b*). As a substance for osmotic adjustment and respiration, *SS* are the primary substrates for plant growth (Hartmann and Trumbore, 2016). It is noteworthy that July was the fast-growing season of *C. sclerophylla*, when the leaf *Pn* cannot maintain high respiration and fast-growing, the leaf *ST* may be degraded to leaf *SS*. Moreover, the higher *SS*, *ST* and total *NSCs* under P and NP addition compared to N addition might indicate that P input can promote the C assimilation ability of *C. sclerophylla* by a higher leaf *Pn*.

Conclusions

This study investigated the response of plant growth, leaf nutrient status, N metabolites and *NSCs* under long-term N and P addition in a secondary *Castanopsis sclerophylla* forest. Results showed that long-term N deposition can promote leaf *Pn*, *SS* and *NSCs* to stimulate mature *C. sclerophylla* growth (GR_{DBH}). Significant interactive effects between N and P were detected in GR_{DBH}, leaf chlorophyll a + b, *SS* and total *NSCs* in both two years, but not in *SLA*, *Nmass* and leaf *ST*. Furthermore, despite the leaf chlorophyll a + b, *Pn*, *SS* and total *NSCs* were increased under N, P and NP addition (except the chlorophyll a + b in 2020 and total *NSCs* in 2019), the positive impacts of P or NP addition on these parameters were stronger compared to N addition. These conclusions indicated that N and P addition have positive interactive effects on the physiological performances of mature *C. sclerophylla*.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_37373751

SUPPLEMENTATION AND OPTIMIZATION OF GROUNDNUT SHELL IN THE DIET OF CONFINED UNSEXED MIXED CATTLE BREED DURING THE WINTER SEASON IN NORTH WEST PROVINCE, SOUTH AFRICA

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(Received 30th Mar 2021; accepted 10th Jun 2021)

Abstract. This study was conducted determine the effect of groundnut shell (GNS) on growth rate, body condition score (BCS) and blood mineral status of confined unsexed mixed cattle breed during the winter season in North West province, South Africa. Thirty-five cattle were divided into five groups (T1, T2, T3, T4, and T5) and each group assigned to GNS at 0, 500, 700, 1000 and 1200 gkg^{-1} feed in a completely randomized design. Cattle were fed basal diet (blue buffalo grass; BBG) and results showed that BBG and GNS were moderate in nutrients. Cattle on diet T3 had improved ($p<0.05$) live weight, average daily gain and BCS. Animals on diets T3 and T4 had increased ($p<0.05$) plasma magnesium and cobalt value than those on diets T1 and T2. Cattle offered diet T3 had increased ($p<0.05$) plasma manganese, iron, carbon, copper and chromium concentrations when compared to those on other four diets. GNS supplementation had no effect ($p>0.05$) on plasma zinc and selenium. GNS supplementation had quadratic effect ($p<0.05$) on plasma minerals. It is concluded that GNS contains moderate amount of essential minerals and therefore suitable for use in cattle production during the winter season in North West Province of South Africa.
Keywords: *ruminant, performance, blood minerals, agro-byproduct, quadratic function*

Introduction

The development of the smallholder cattle industry as an animal protein source for human diets in South Africa has received little attention. Livestock improvement demands efficient use of available feed resources. Factors like agronomic practices, feed processing technologies, and genetic variations have been observed to influence the nutrient (mineral) value of feed raw materials (Khan et al., 2017). Literature is scanty on the mineral status of confined indigenous cattle under a communal grazing system during the dry (winter) season in South Africa or the grass they grazed. Seasonal variability has been reported to affect the minerals status of cattle due to changes in nutrient composition of pasture and their availability (Malau-Aduli et al., 2003; Mokolopi and Beighle, 2006). The long period of dryness usually experienced during the winter season often aggravates mineral deficiency in cattle and this call for detailed studies before a mineral supplement can be recommended. Communal farmers are unwilling to give mineral lick to their cattle for several reasons, of which the most prominent is cost. It is therefore desirable to develop alternative mineral supplement sources in smallholder cattle production especially during the dry season.

Groundnut (*Arachis hypogea* L) shell one of such novel feed mineral resources that has no direct value in human diet. Shell is a by-product of the groundnut processing industry after removing the seed and comprises about 21-29% weight of the whole nut (van Doosselaere, 2013; Davis et al., 2016). The shell is high in lignin which calls for

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_37533772

proper processing before their use in animal ration. Studies have shown that GNS contain 0.50% crude protein (CP), 59.0% crude fibre (CF), 2.50% ash and 4.43% carbohydrates (Abdulrazak et al., 2014). According to Atasie et al. (2009), GNS is rich in minerals such as Na (42.00 mg/100 g), K (705.11 mg/100 g), Mg (3.98.00 mg/100 g), Ca (2.28 mg/100g), Fe (6.97 mg/100 g), Zn (3.20 mg/100 g) and P (10.55 mg/100 g).

The objective of this study, therefore was to ascertain the nutrient content of GNS and the impact of its supplementation on the growth rate, body condition scores and blood mineral status of unsexed mixed cattle breed fed intensively during the dry (winter) season in North West province, South Africa. The optimal GNS supplementation level that improved LW, ADG, BCS and blood mineral indices will be modelled using quadratic regression model.

Materials and Methods

Study area

This study was conducted in Mogosane village of Mafikeng, North West province (*Figure 1*) during the months of May to July 2017. This town has an above-average rainfall of 300 – 700 mm annually and the summer falls between August and March in which the temperatures may range between 22^0C and 34^0C, while the winter arrives around May to July being dry and cool. The average winter temperature is about 16^0C which can decline below 2^0C and can rise to 20^0C.

Figure 1. *Map of Mafikeng.*
Source: https://www.sa-venues.com/maps/northwestprovince/mafikeng.php

Experimental procedure

Kraals were constructed to have five pens with each pen housing seven (7) cattle. This subdivision aid to prevent the animals from roaming around and missing up in order not to disturb the experimental design. All the biosecurity measures adapted in the current aligned to the guidelines of the Ethics Committee of the University of South Africa and

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_37533772

the South African Animal Disease Act 35, of 1984. Thirty-five (35) unsexed mixed cattle breed weighing between 237.63 kg and 278.00 kg were selected from a herd that is exclusively on communal grazing with no mineral supplement at Mogosane village, Mafikeng and randomly assigned to 5 treatments (T1, T2, T3, T4 and T5) with 7 animals per treatment in a completely randomized design. All the cattle were ear tagged. The animals in control (T1) group received a basal diet supplemented with GNS at 0 g/kg feed while those in groups T2, T3, T4 and T5 received basal diet supplemented with GNS at 500, 700, 1000 and 1200 gkg^{-1} feed during the feeding periods, respectively. Feed and water were provided *ad libitum* to the cattle.

Data collection

Samples of BBG and GNS samples were dried in a forced-air oven at 70°C for 48 h and thereafter, milled and sieved via a 2-mm screen (Retsch Zm100 grinder; Glen Mills Inc., Clifton, NJ) and stored in Fisherbrand sterile sampling bags (Fisher Scientific, Pittsburgh, PA). The milled samples were subjected to proximate analysis to determine their CP, CF, ether extract (EE), moisture content (MC), ash and nitrogen-free extracts (NFE) following the standard procedures (AOAC, 2002). All the proximate values were analyzed in triplicates and results reported in percentages. Metabolizable energy (ME) was calculated using the prediction equation reported by Pauzenga (1985) as follows: ME = 37 × CP % + 81.8 × EE % + 35.5 × NFE %. Mineral composition of BBG and GNS were determined using inductively coupled plasma optic emission spectroscopy (PerkinElmer, Waltham, MA) as described (Richter et al., 2012). Blood samples were aseptically collected from the jugular vein the four (4) randomly selected animals in each treatment group at the end of the 8^{th} week of the study. As described by Richter et al. (2012), Jugular blood samples for mineral analysis were collected into vacuum tubes containing the anticoagulants potassium EDTA after restraining the animal. Sera were harvested by centrifugation at 1000 rpm for 10 minutes and later transferred into plastic tubes and stored for mineral analysis. Thereafter, the sera were transported to the laboratory for determination of the mineral content. Upon analysis, plasma samples were vortexed, then diluted to a ratio of 1:20 with 1% nitric acid. Blood samples were analyzed for mineral concentrations using inductively coupled plasma mass spectroscopy (PerkinElmer, Waltham, MA) as described by Pogge et al. (2012). Mean initial live weights (LWs) of the cattle were recorded at the first week of study. Thereafter, mean LW per cattle in each pen were taken on a weekly basis and the total LW were divided by the total number of cattle in the pen to get the mean LW of the cattle. The determined LWs were employed to compute the ADG of the cattle. BCS was determined in a scale of 1 to 5 following the procedures of Wildman et al. (1982). This depends on a visual and tactile appraisal of the body fat reserve in the back and pelvic regions.

Data analysis

The Statistical Analysis System procedure of SAS 9.4 (SAS Institute, 2010) was used and data collected were analyzed by one-way analysis of variance method to ascertain the influence of GNS supplementation on blood mineral concentrations of South African indigenous cattle. Duncan's Multiple Range (DMR) test was done for multiple means comparison and the differences between means were considered statistically significant at $p<0.05$. Data generated on the proximate and mineral composition of BBG and GNS were subjected to descriptive statistics [means, standard deviation (SD) and coefficient of variation (CV)] to establish the reference values of the different parameters measured.

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The supplementation related responses in body weight, body condition scores and blood mineral content to GNS supplementation in confined South African indigenous cattle fed intensively during the winter season were modeled using the following quadratic regression model equation:

$$Y = a + b_1x + b_2x_2 \quad \text{(Eq.1)}$$

where
Y = Live weight, body condition score and blood mineral parameters;
a = the Y-intercept;
b = coefficient of quadratic optimization equation;
x = Groundnut shell supplementation levels and $-b_1/2b_2$ = x value of GNS for optimum response. The quadratic equation was fitted to the experimental data by means of the nonlinear model procedure of SAS (2010). The choice of the quadratic regression model is because it fitted the model and the probability level for significance is 5%.

Results

Nutrient composition of BBG and GNS

The mean total ash, CP, CF, EE, MC, NFE and ME value of the BBG and GNS are given in *Table 1*. The SD values across BBG and GNS were low and ranges between 0.03 – 1.59 and 0.03 – 3.74, respectively. *Table 2* showed the macro-mineral profiling of BBG and GNS. BBG yielded 2.12 g/100 g Ca, 0.37 g/100 g P, 0.23 g/100 g Mg, 0.62 g/100 g K, 0.26 g/100 g S, 19.00 g/100 g Na and 1.03 g/100 g Cl, while GNS yielded 0.36 g/100 g Ca, 0.36 g/100 g P, 0.34 g/100 g Mg, 2.23 g/100 g K, 0.14 g/100 g S, 0.05 g/100 g Na and 0.18 g/100 g Cl. SD values across the macro-mineral values for the BBG and GNS were low and ranged between 0.02 – 1.77 and 0.01 – 0.15, respectively. The mean micro-mineral concentrations of the BBG and GNS were found between 1.85 and 25.01 mg/100 g Mn, 2.59 and 0.32 mg/100 g Fe, 1.82 and 2.67 mg/100 g Cu, 6.12 and 5.04 mg/100 g Zn, 60.25 and 43.87 mg/100 g C, 3.18 and 1.95 mg/g Se, 0.89 and 0.25 mg/100 g Cr, 7.08 and 0.25 mg/100 g Co and 3.25 and 3.20 mg/100 g V, respectively (*Table 3*). Standard deviation values across macro-mineral values were low and ranged 0.12 – 3.95 for BBG and 0.02 – 2.50 GNS.

Table 1. *Proximate analysis (%) of basal diet and GNS*

Parameters	Basal diet				GNS			
	Mean	SD	Min. Value	Max. value	Mean	SD	Min. value	Max. value
Total ash	1.51	0.03	1.48	1.55	2.50	0.03	2.46	2.53
CP	9.09	1.03	7.87	10.4	11.67	0.59	12.01	12.16
CF	18.18	1.46	16.80	20.20	49.00	3.74	45.00	54.00
EE	1.48	0.06	1.39	1.53	1.50	0.04	1.45	1.54
Moisture	15.00	0.82	14.00	16.00	8.00	0.82	7.00	9.00
Carbohydrate	69.74	1.59	68.53	72.00	35.33	2.06	33.00	38.00
ME (Kcal/kg) *	2839.92	8.02	2829.89	2849.52	1714.21	21.17	1696.55	1743.98

*Calculated

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DOI: http://dx.doi.org/10.15666/aeer/1905_37533772

Table 2. *Macro-mineral value of basal diet and GNS measured in dry weight (g/100 g)*

Minerals	Basal diet				GNS			
	Mean	SD	Min. Value	Max. value	Mean	SD	Min. Value	Max. value
Ca	2.12	0.30	1.76	2.50	0.36	0.06	0.31	0.42
P	0.37	0.02	0.35	0.40	0.36	0.03	0.34	0.40
Mg	0.23	0.02	0.21	0.25	0.34	0.05	0.29	0.40
K	0.62	0.02	0.60	0.65	2.23	0.15	2.02	2.43
S	0.26	0.03	0.24	0.30	0.14	0.01	0.13	0.15
Na	19.00	1.77	16.5	20.42	0.05	0.02	0.03	0.07
Cl	1.03	0.04	1.00	1.08	0.18	0.02	0.16	0.20

Table 3. *Micro-mineral value of basal diet and GNS measured in dry weight (mg/100 g)*

Minerals	Basal diet				GNS			
	Mean	SD	Min. value	Max. value	Mean	SD	Min. value	Max. value
Mn	1.85	0.17	1.62	2.01	25.01	1.45	23.6	27.01
Fe	2.59	0.38	2.19	3.10	0.32	0.11	0.20	0.46
Cu	1.82	0.12	1.68	1.98	2.67	0.28	2.40	3.05
Zn	6.12	0.12	5.98	6.40	5.04	0.86	4.16	6.20
C	60.25	3.95	58.85	67.79	43.87	2.50	40.40	46.21
Se	3.18	0.21	2.99	3.47	1.95	0.20	1.68	2.14
Cr	0.89	0.18	0.65	1.08	0.25	0.02	0.22	0.27
Co	7.08	1.33	5.32	8.55	0.25	0.04	0.20	0.30
V	3.25	0.56	2.71	4.02	3.20	0.28	3.00	3.60

Growth rate, condition score and blood mineral characteristics of cattle

Data on the effect of GNS supplementation on LW, ADG, body condition score (BCS) and plasma macro-mineral concentrations of indigenous cattle managed intensively during the winter season in North West province of South Africa are shown in *Figures 2, 3, 4 and Table 4*. Cattle on diet T3 had improved ($p<0.05$) growth rate and body condition score when compared to those on other 4 diets starting from the 4th week of the study. Mean Ca, Mg, P, K, Na, Cl and S value were 3.256 mg%, 0.609 mg%, 0.308 mg%, 4.799 mg%, 15.599 mg%, 6919.8 mg% and 48.177 mg%, respectively, while the CV was ranged from 11.20 to 58.50%. Cattle fed diet T2, T3, T4 and T5 had higher ($p<0.05$) plasma Ca when compared with cattle on diet T1. Cattle on diets T3 and T4 had significantly ($p<0.05$) increased plasma Mg concentration when compared to those on T1 and T2 diets. However, cattle on diets T1 and T2 had similar ($p>0.05$) plasma Mg. Plasma P and K concentrations of cattle fed diet T1 was significantly ($p<0.05$) lower than those fed diets T3, T4 and T5, but had similar value with those fed diet T2. Cattle on diets T3, T4 and T5 had the highest plasma Na values which differed significantly from those on diet T1. Our result also revealed that cattle on diets T1 and T2 had similar ($p<0.05$) plasma Na which differed significantly from those on diets T3 and T4. Furthermore, our results showed statistical ($p<0.05$) effect of GNS supplementation on plasma Cl and S among the dietary groups.

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DOI: http://dx.doi.org/10.15666/aeer/1905_37533772

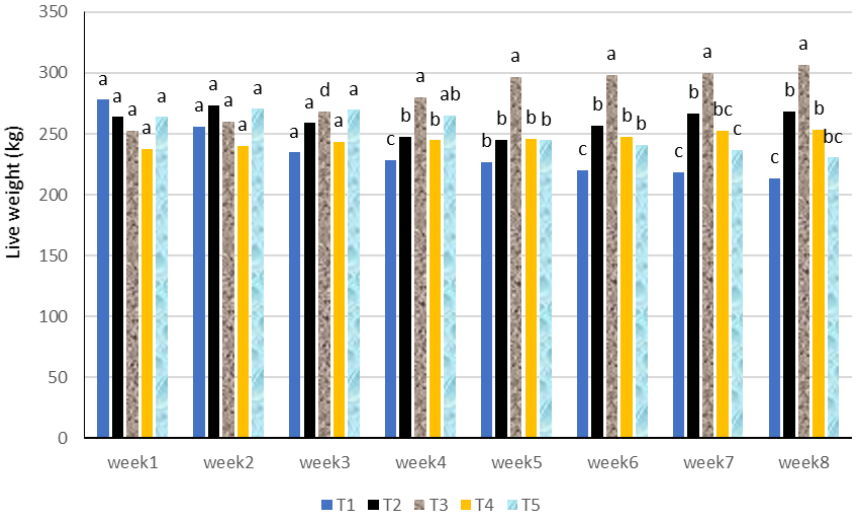

Figure 2. *Live weight of unsexed mixed cattle breed on fed basal diet supplemented with GNS.*
[a,b,c]Bars sharing different letters are significant at p<0.05

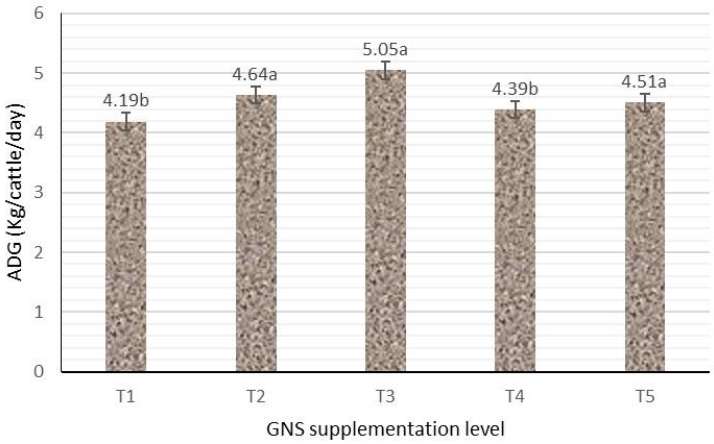

Figure 3. *ADG value of unsexed mixed cattle breed fed basal diet supplemented with GNS.*
[a,b]Bars sharing different letters are significant at p<0.05

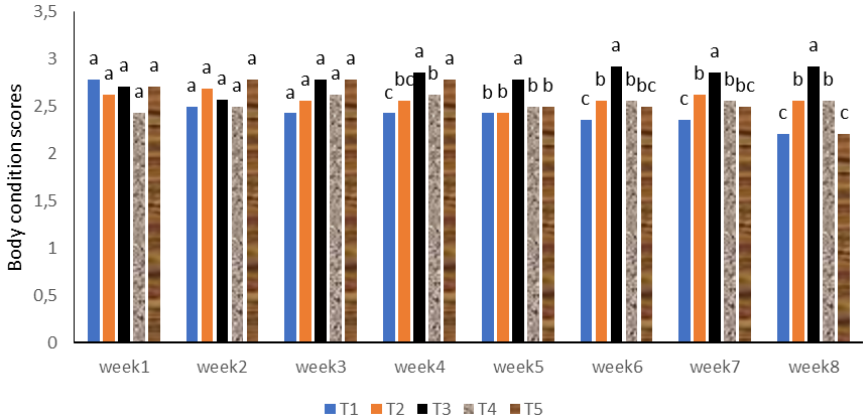

Figure 4. *Body condition scores of indigenous cattle on fed basal diet supplemented with GNS.*
[a,b,c]Bars sharing different letters are significant at p<0.05

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DOI: http://dx.doi.org/10.15666/aeer/1905_37533772

Table 4. *Blood macro-mineral content (mg%) of cattle fed diets supplemented with GNS*

Variables	GNS supplementation levels (g/kg feed)					Mean	SEM	p-value	%CV
	T1	T2	T3	T4	T5				
Ca	2.479^{a}	2.930^{c}	4.383^{b}	3.736^{b}	2.751^{c}	3.256	0.223	0.0001	24.10
Mg	0.206^{d}	0.270^{cd}	0.433^{a}	0.354^{b}	0.279^{c}	0.609	0.022	0.0001	31.70
P	0.231^{c}	0.314c	1.116^{a}	0.756^{b}	0.627^{b}	0.308	0.050	0.0001	58.50
K	3.614^{d}	3.900^{cd}	6.186^{a}	5.514^{ab}	4.785^{bc}	4.799	0.373	0.0001	22.40
Na	12.414^{a}	13.759^{ab}	19.880^{c}	17.326^{c}	14.614^{bc}	15.599	1.247	0.0015	19.40
Cl	6359.4^{c}	6573.6^{bc}	8106.0^{a}	7308.4^{ab}	6251.6^{c}	6919.8	522.3	0.0390	11.20
S	44.847^{bc}	48.443^{b}	58.693^{a}	47.443^{b}	41.457^{c}	48.177	0.531	0.0220	14.30

^{a,b,c,d}Means within rows sharing different letters are significant at $p<0.05$. SEM: Standard error of the mean; CV: Coefficient of variation

Results of the effect of GNS supplementation on plasma micro-mineral concentrations of indigenous cattle managed intensively during the winter season are presented in *Table 5*. Mean Mn, Fe, Cu, Zn, C, Se, Cr, Co and V concentration were 0.0456 mg%, 0.1206 mg%, 0.0216 mg%, 0.0014 mg%, 756.68 mg%, 0.0022 mg%, 0.0218 mg%, 0.2238 mg% and 0.074 mg%, respectively while the coefficient of variations (CV) was ranged from 11.20 to 58.50%. Indigenous cattle offered diet T3 had higher ($p<0.05$) plasma Mn, Fe and C value than those on diets T1, T2, T4 and T5. However, cattle fed diets T1, T2, T4 and T5 had comparable ($p>0.05$) plasma Mn, Fe and C value. Cattle on diet T3 had higher ($p<0.05$) plasma Cu concentration than those on the other 4 diets. However, cattle offered diets T1 had the lowest ($p<0.05$) plasma Cu value than those on diets T3, T4 and T5, but similar to those on diet T2. Cattle fed diets T1, T2, T3, T4 and T5 had similar ($p>0.05$) plasma Zn and Se concentration. Higher ($p<0.05$) plasma Co was recorded in cattle fed diet T3 followed by those fed diets T4 and T5 when compared with those on diet T1 and T2. However, cattle fed diets T1 and T2 had similar ($p>0.05$) plasma Co level. Animals offered diet T3 had increased ($p<0.05$) plasma Cr than those fed diet T5 but similar ($p>0.05$) to those on diets T1, T2 and T4. Cattle fed diet T5 had significantly ($p<0.05$) reduced plasma V when compared to those on diets T3 and T4. However, cattle on diets T1, T2 and T4 had similar ($p>0.05$) plasma V.

Table 5. *Blood micro-mineral contents (mg%) of cattle fed diets supplemented with GNS*

Variables	GNS supplementation levels (g/kg feed)					Mean	SEM	p-value	%CV
	T1	T2	T3	T4	T5				
Mn	0.031^{b}	0.032^{b}	0.100^{a}	0.035^{b}	0.030^{b}	0.0456	0.009	0.0001	66.90
Fe	0.103^{b}	0.111^{b}	0.176^{a}	0.114^{b}	0.099^{b}	0.1206	0.008	0.0001	26.10
Cu	0.008^{d}	0.010^{d}	0.042^{a}	0.028^{b}	0.020^{c}	0.0216	0.001	0.0001	64.60
Zn	0.001	0.001	0.003^{a}	0.001	0.001	0.0014	0.401	0.5228	21.90
C	642.70^{b}	702.5^{b}	990.1^{a}	805.4^{b}	642.7^{b}	756.68	54.56	0.0003	19.30
Se	0.002^{a}	0.002^{a}	0.003^{a}	0.002^{a}	0.002^{a}	0.0022	0.001	0.5238	20.30
Cr	0.021^{ab}	0.022^{ab}	0.026^{a}	0.022^{ab}	0.018^{b}	0.0218	0.002	0.0001	13.10
Co	0.026^{d}	0.027^{cd}	0.433^{a}	0.354^{b}	0.279^{c}	0.2238	0.022	0.0001	19.80
V	0.072^{bc}	0.074^{abc}	0.088^{a}	0.077^{ab}	0.059^{c}	0.0740	0.005	0.0093	13.90

^{a,b,c,d}Means within rows sharing different letters are significant at $p<0.05$

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DOI: http://dx.doi.org/10.15666/aeer/1905_37533772

Optimization function

Results of the effect of GNS supplementation on optimal plasma GR, BCS, Ca, P, Mg, K, Na, Cl and S level in cattle managed intensively during the winter season in North West province of South Africa are shown in *Table 6 and Figures 5 to 14*. GNS was noticed to have a significant quadratic effect on optimal LW, ADG, BCS and plasma K level with a quadratic value of 199.8 + 43.968 GNS – 6.9597 GNS^2, r^2 = 0.5519; 3.574 + 0.7804 GNS – 0.1236 GNS^2, r^2 = 0.5522; 2.1714 + 0.3166 GNS – 0.0482 GNS^2, r^2 = 0.5662 and 1.119 + 2.5333 GNS – 0.3563 GNS^2, r^2 = 0.7191 with the optimum GNS supplementation level being 315.88, 328.42 and 355.0 g/kg feed, respectively. Similar quadratic effect were observed for plasma Ca (0.3648 + 2.2659 GNS – 0.3551 GNS^2, r^2 = 0.7901), plasma P (-0.5544 + 0.8031 GNS – 0.1133 GNS^2, r^2 = 0.6523), plasma Mg (-0.0206 + 0.2459 GNS – 0.0371 GNS^2, r^2 = 0.8086), plasma Na (4.814 + 7.992 GNS – 1.1992 GNS^2, r^2 = 0.7395), plasma Cl (4328 + 2139.9 GNS - 348 GNS^2, r^2 = 0.7063) and plasma S (30.179 + 16.649 GNS – 2.9046 GNS^2, r^2 = 0.7415) level with optimum GNS supplementation being 319.05, 354.41, 331.40, 333.22, 307.46 and 286.60 g/kg feed, respectively (*Table 4.6*). Coefficient of determination (r^2) was ranged from 55.19% to 80.86%. However, the level of GNS needed to optimize plasma K was higher than the level needed for optimizing the growth rate (GR), body condition score (BCS), Ca, P, Mg, Na, Cl and S.

Table 6. *Optimal GR, BCS and plasma micro-mineral level of cattle on GNS supplementation*

Variables	Optimization Equation	r^2	Optimal X - level	Optimal Y- level	P-value
LW	$Y = 199.8 + 43.968x - 6.9597x^2$	0.5519	315.88	269.24	<0.0001
ADG	$Y = 3.574 + 0.7804x - 0.1236x^2$	0.5522	315.70	4.8058	0.0001
BCS	$Y = 2.1714 + 0.3166x - 0.0482x^2$	0.5662	328.42	2.6913	0.0002
Ca	$Y = 0.3648 + 2.2659x - 0.3551x^2$	0.7901	319.05	3.9795	0.0001
P	$Y = -0.5544 + 0.8031x - 0.1133x^2$	0.6523	354.41	1.4231	0.0001
Mg	$Y = -0.0206 + 0.2459x - 0.0371x^2$	0.8086	331.40	0.3869	0.0001
K	$Y = 1.119 + 2.5333x - 0.3563x^2$	0.7191	355.0	5.6220	0.0001
Na	$Y = 4.814 + 7.992x - 1.1992x^2$	0.7395	333.22	18.1296	0.0015
Cl	$Y = 4328 + 2139.9x - 348x^2$	0.7063	307.46	7617.64	0.0390
S	$Y = 30.179 + 16.649x - 2.9046x^2$	0.7415	286.60	54.047	0.0220

r^2: Coefficient of determination; p: probability; GR: growth rate; BCS: body condition score; LW: live weight; ADG: average daily gain; GNS: groundnut shell

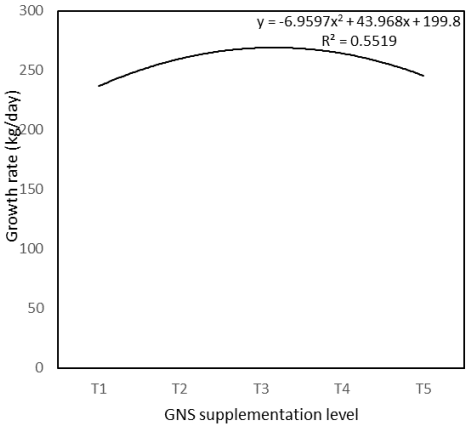

Figure 5. *Estimation curve of the optimal live weight of unsexed mixed cattle breed fed basal diet supplemented with GNS*

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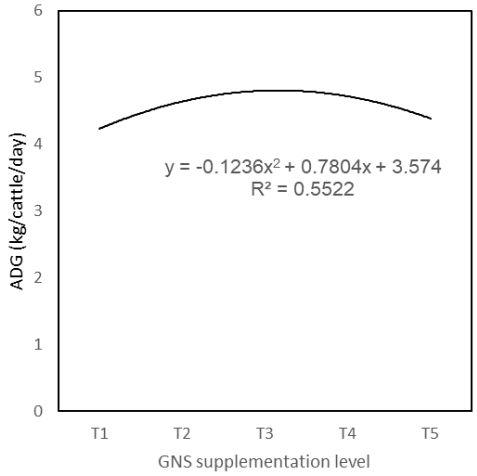

Figure 6. *Estimation curve of the optimal ADG of unsexed mixed cattle breed fed basal diet supplemented with GNS*

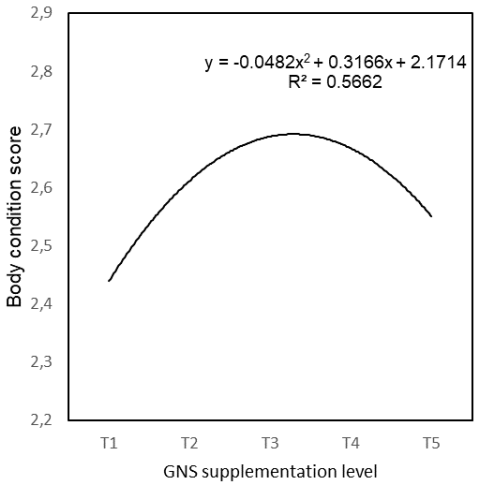

Figure 7. *Estimation curve of the optimal BCS of unsexed mixed cattle breed fed basal diet supplemented with GNS*

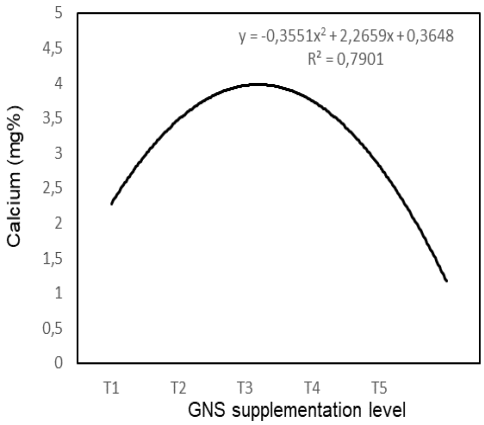

Figure 8. *Estimation curve of the optimal plasma Ca of unsexed mixed cattle breed fed basal diet supplemented with GNS*

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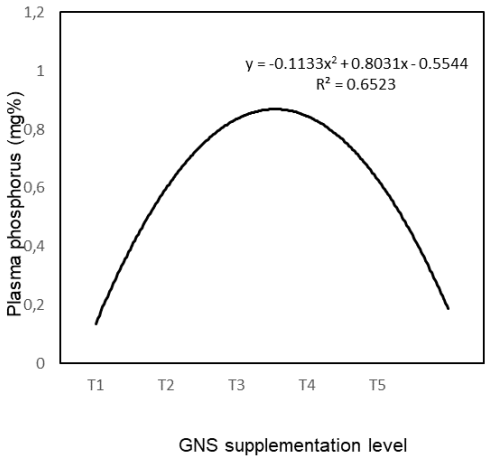

Figure 9. *Estimation curve of the optimal plasma P of unsexed mixed cattle breed fed basal diet supplemented with GNS*

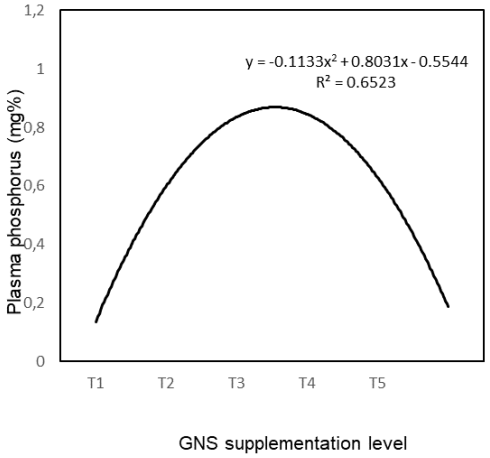

Figure 10. *Estimation curve of the optimal plasma Mg of unsexed mixed cattle breed fed basal diet supplemented with GNS*

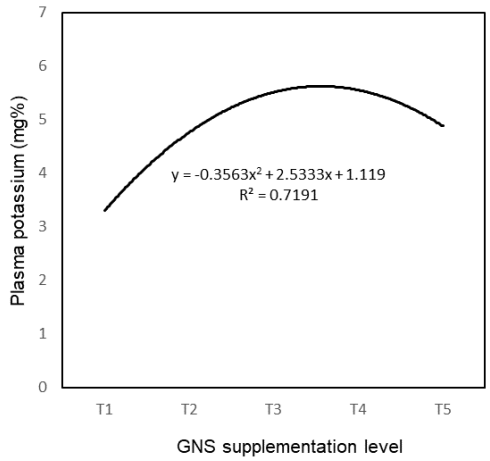

Figure 11. *Estimation curve of the optimal plasma K of unsexed mixed cattle breed fed basal diet supplemented with GNS*

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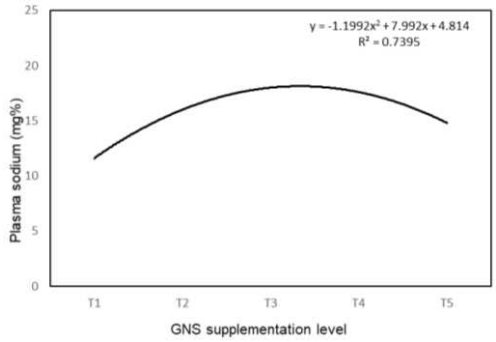

Figure 12. *Estimation curve of the optimal plasma Na of unsexed mixed cattle breed fed basal diet supplemented with GNS*

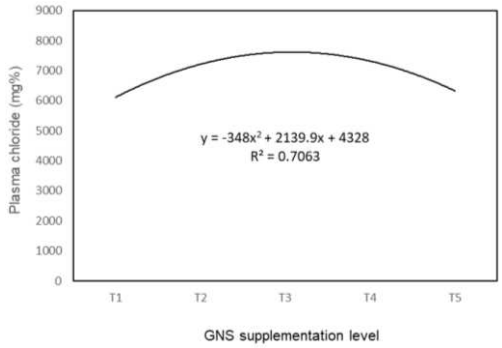

Figure 13. *Estimation curve of the optimal plasma Cl of unsexed mixed cattle breed fed basal diet supplemented with GNS*

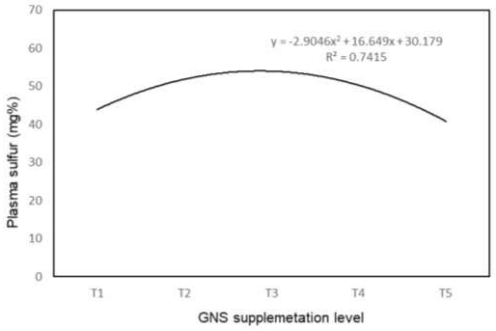

Figure 14. *Estimation curve of the optimal plasma S of unsexed mixed cattle breed fed basal diet supplemented with GNS*

Results of the effect of GNS supplementation on optimal plasma Mn, Fe, Cu, Zn, C, Se, Cr, Co and V level in cattle managed intensively during the winter season in North West province of South Africa are shown in *Table 7 and Figures 15 to 23*. GNS was observed to have a quadratic effect ($p<0.05$) on optimal plasma Co level with a quadratic value of -0.3446 + 0.3563 GNS – 0.0455 GNS^2, $r^2 = 0.6946$ with the optimum GNS supplementation level being 3.9154 g/kg feed. Similar quadratic effect were observed for plasma Mn (-0.0272 + 0.0622 GNS – 0.0104 GNS^2, $r^2 = 0.4045$), plasma Fe (-0.0356 + 0.0736 GNS - 0.0124 GNS^2, $r^2 = 0.5376$), plasma Cu (-0.024 + 0.0325 GNS - 0.0047

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GNS^2, $r^2 = 0.6257$), plasma carbon (267.16 + 403.42 GNS – 65.521 GNS^2, $r^2 = 0.7130$), plasma Cr (0.0146+ 0.0071 GNS – 0.0013 GNS^2, $r^2 = 0.8153$) and plasma V (0.0484 + 0.0256 GNS – 0.0046 GNS^2, $r^2 = 0.8172$) level with optimum GNS supplementation being 299.04, 296.77, 345.74, 307.86, 273.08 and 078.26 g/kg feed, respectively (*Table 7*). Coefficient of determination (r^2) was ranged from 35.71 to 80.72. However, the level of GNS needed to optimize plasma potassium was higher than the level needed for optimizing the Ca, P, Mg, Na, Cl and S. GNS supplementation had no quadratic effect ($p>0.05$) on optimal plasma Zn (-0.0012 + 0.0022 GNS - 0.0004 GNS^2, $r^2 = 0.5893$) and plasma Se (0.0012 + 0.0009 GNS - 0.0001 GNS^2, $r^2 = 0.3571$).

Table 7. *Optimal plasma micromineral level (mg%) value of cattle to GNS supplementation*

Variable	Equation	r^2	Optimal X level	Optimal Y level	p-value
Mn	$Y = -0.0272 + 0.0622x - 0.0104x^2$	0.4045	299.04	0.0658	0.0001
Fe	$Y = – 0.0356 + 0.0736x - 0.0124x^2$	0.5376	296.77	0.1092	0.0001
Cu	$Y = - 0.024 + 0.0325x - 0.0047x^2$	0.6257	345.74	0.0322	0.0001
Zn	$Y = - 0.0012 + 0.0022x - 0.0004x^2$	0.5893	275.00	0.0018	0.5228
C	$Y = 267.16 + 403.42x - 65.521x^2$	0.7130	307.86	888.14	0.0003
Se	$Y = 0.0012 + 0.0009x - 0.0001x^2$	0.3571	450.00	0.0008	0.5238
Cr	$Y = 0.0146 + 0.0071x - 0.0013x^2$	0.8153	273.08	0.0243	0.0001
Co	$Y = -0.3446 + 0.3563x - 0.0455x^2$	0.6946	391.54	0.3529	0.0001
V	$Y = 0.0484 + 0.0256x - 0.0046x^2$	0.8172	78.26	0.0840	0.0093

r^2: Coefficient of determination; p: probability

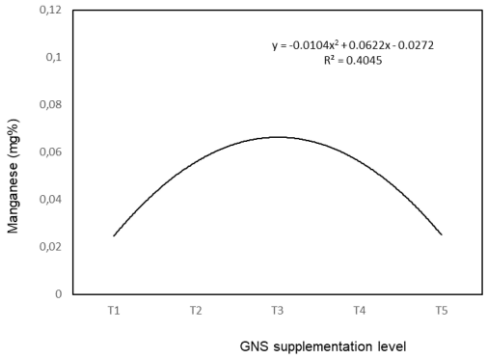

Figure 15. *Estimation curve of the optimal plasma Mn of unsexed mixed cattle breed fed basal diet supplemented with GNS*

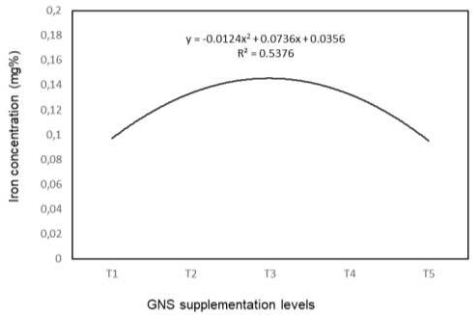

Figure 16. *Estimation curve of the optimal plasma Fe of unsexed mixed cattle breed fed basal diet supplemented with GNS*

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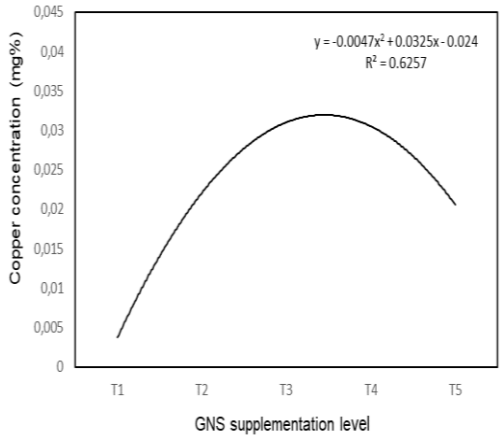

Figure 17. *Estimation curve of the optimal plasma Cu of unsexed mixed cattle breed fed basal diet supplemented with GNS*

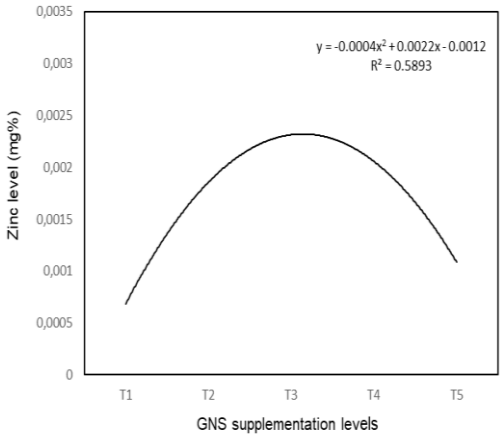

Figure 18. *Estimation curve of the optimal plasma Zn of unsexed mixed cattle breed fed basal diet supplemented with GNS*

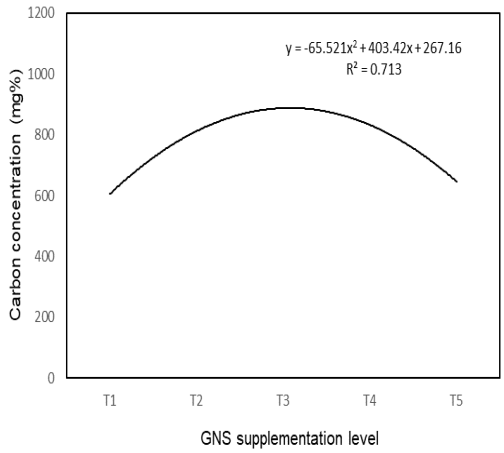

Figure 19. *Estimation curve of the optimal plasma C of unsexed mixed cattle breed fed basal diet supplemented with GNS*

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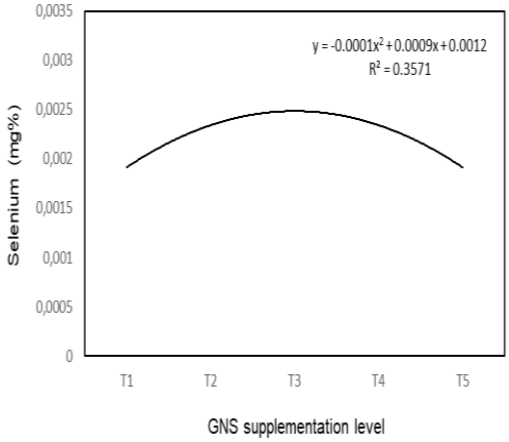

Figure 20. *Estimation curve of the optimal plasma Se of unsexed mixed cattle breed fed basal diet supplemented with GNS*

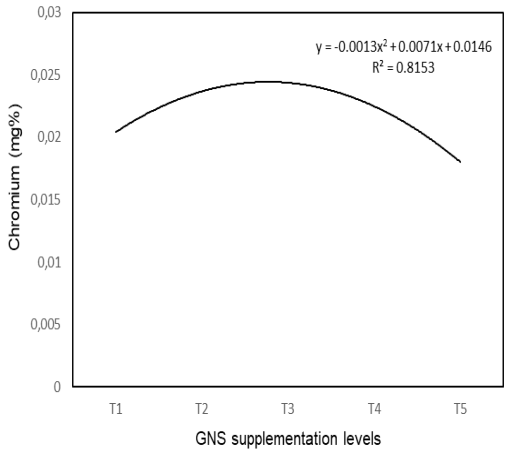

Figure 21. *Estimation curve of the optimal plasma Cr of unsexed mixed cattle breed fed basal diet supplemented with GNS*

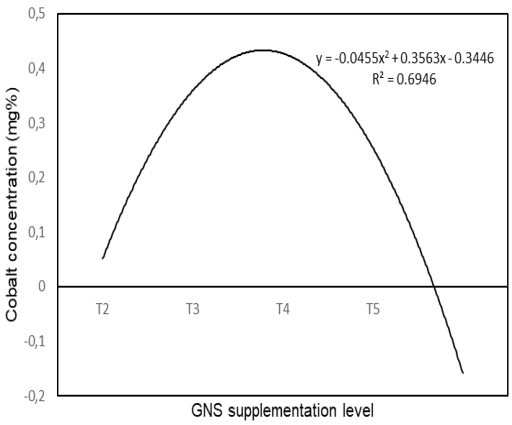

Figure 22. *Estimation curve of the optimal plasma Co of unsexed mixed cattle breed fed basal diet supplemented with GNS*

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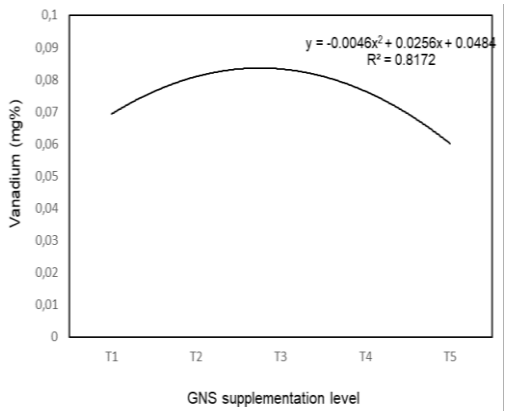

Figure 23. *Estimation curve of the optimal plasma V of unsexed mixed cattle breed fed basal diet supplemented with GNS*

Discussion

Nutrient composition of basal diet and GNS

Studies have shown that biophysical and biochemical fractions are employed in the determination of uptake and availability of nutrients in a test feedstuff (Okoli et al., 2009). The feed value of feedstuff a function of its nutrient content, digestibility and palatability and consequently its associative effects with other feeds (Smith, 1992). Therefore, a proper understanding of the biophysical and biochemical fractions in all feed ingredients is essential (Okoli et al., 2009). The results of the nutrient biochemical component of GNS showed that GNS is rich in fibre but moderate in ash and protein. The 2.5% reported for ash in the present experiment is in harmony with the earlier findings of Abdulrazak et al. (2014) who observed a similar value of 2.5%, but lower than 4.26% reported by Perea-Moreno et al. (2018), 5.3% reported by Khan et al. (2017) and 7.79% reported by Akinfemi et al. (2012) for groundnut shell. The observed discrepancy in these results could be attributed in part to analytical methods adopted or soil type where the crop was harvested. The presence of fibre and ash (minerals) in groundnut shells imply that GNS could be used as a source fibre and mineral supplement for ruminants. The low moisture content of the GNS samples revealed that they can be easily dried to reduce the incidence of microbial attacks. Results of proximate biochemical analysis of GNS showed that crude protein (11.67%) was higher than the range of 4.43 - 7.39% reported by other investigators (Akinfemi et al., 2012; Abdulrazak et al., 2014).

The moisture content (8.8%) reported in the current study was slightly lower than 5.97% obtained for GNS by Perea-Moreno et al. (2018). The carbohydrate value (35.33%) was higher than the range of 18.10 - 25.57% reported by Abdulrazak et al. (2014) and Khan et al. (2017), while the ether extract content (1.50%) was slightly lower than 2.00% and 6.31% obtained by Khan et al. (2017) and Akinfemi et al. (2012), respectively. The crude fibre content reported in the present study was 17% lower and 47% higher than the values of 59.0% and 26.15% earlier reported by Abdulrazak et al. (2014) and Akinfemi et al. (2012), respectively in a similar experiment for groundnut shells. The disparity in the proximate results of the present study with the results of other authors may be due to environment and soil type differences, which has been reported to affect the nutrient composition of feedstuffs (Mecha and Adegbola, 1980; Melesse et al., 2012; Ogunbosoye et al., 2015). Standard deviation (SD) values across the values

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analyzed were narrow showing reliability of these mean values as reference values for the groundnut shell.

The results of the proximate composition of the basal diet (blue buffalo grass) as shown in *Table 4.1* revealed that blue buffalo grass is an excellent source of nutrient (fibre, protein and energy) but low in ash content. Published data on the proximate composition of blue buffalo grass is lacking in the literature, hence there is no data to compare our findings with. The ash value (1.51%) recorded in the present study for blue buffalo grass (*Cenchrus ciliaris*) was lower than 7.54 – 7.71% and 2.0 -18.18% reported for Napier grass (*Pennisetum purpureum*) in South Africa and Nigeria by Rambau et al. (2016) and Okaraonye and Ikewuchi (2009), respectively. The choice of comparing our results with Napier grass in this study instead of other grasses used in ruminant feeding can be explained by the fact that it is high in nutrients (Rambau et al., 2016). The results of crude protein and ether extract value recorded in the present analysis for blue buffalo grass were lower than 13.16 – 15.80% crude protein and 3.25 – 3.63% ether extract reported by Rambau et al. (2016). The disparities in nutrient values could be linked to their genetic make-up. Therefore, forages with higher crude protein content are more nutritive. The moderate fibre level in blue buffalo grass is an indication that it can be easily digested and utilized efficiently by the rumen microbes. Standard deviation values across the values analyzed were narrow showing reliability of these mean values as reference values for the blue buffalo grass.

Mineral composition of basal diet and GNS

Minerals are vital nutrients with specific functions required in small amounts in the body (Gafar and Itodo, 2011) and their absence in the diet can lead to a significant reduction in growth and reproduction (McDonald et al., 1995; Gafar and Itodo, 2011). Generally, ash content gives an indication of the minerals present in feed material. The current study revealed that groundnut shells contain an appreciable quantity of ash an indication that it can serve as a source of mineral for ruminants. Aspects of the mineral profile of groundnut shell reported in the present study were higher than 0.042 g/100 g Na, 0.705 g/100 g K, 0.039 g/100 g Mg, 0.023 g/100 g Ca and 0.011 g/100 g P and lower than 6.97 mg/100 g iron and 3.20 mg/100 g zinc recorded by Atasie et al. (2009). The Cu (2.67 mg/100 g), Zn (5.04 mg/100 g) and Fe (0.32 mg/100 g) were higher than 0.002 mg/100 g, 0.003 mg/100 g and 0.115 mg/100 g, respectively, reported by Grandawa (2014). Slight variations were noticed between the values recorded in the present study for Ca, P, Mg, Na and K and the values of 0.644 g/100 g, 0.134 g/100 g, 0.253 g/100 g, 0.004 g/100 g and 0.782 g/100 g obtained for the same parameters by Akinfemi et al. (2012) for GNS. The results show that the values returned for Ca, Na, K, Mg, P, Zn and Cu obtained in this research for blue buffalo grass were higher than 0.34, 0.06, 1.74, 0.02, 0.012 g/100 g, 1.77 mg/100 g, and 0.77 mg/100 g recorded for the same parameters in Napier grass (Rambau et al., 2016). However, the value recorded for Mn and Fe in the present study was far lower than 17.3 mg/100 g and 63.21 mg/100 g returned for the same parameters in Napier grass (Rambau et al., 2016). The disparity may be partly linked soil type (Zewdu et al., 2003) and genetic differences.

LW, ADG, BCS and blood mineral value of cattle

The investigation revealed that cattle on diet T3 had improved LW, ADG and BCS. The improved LW, ADG and BCS in cattle fed diet T3 could partly be explained by enhanced utilization of the diet. Results revealed that GNS may have ability as a feed

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supplement for confined communal cattle. This is an agreement with the earlier reports of Atasie et al. (2009) and Abdulrazak et al. (2014) that GNS is moderate in essential nutrients. Blood samples are often analyzed with the aim of ascertaining the mineral status of animals. Blood is used as an index of animal nutritional status due to problems in determining mineral status from diet evaluation (Ogbuewu et al., 2015). The biologic availability of dietary minerals is usually at variance and hard to predict due to mineral availability can be influenced by chemical form and source of the mineral as well as the interactions among dietary constituents. The significantly high plasma Ca and Na obtained in indigenous cattle fed diets T2, T3, T4 and T5 indicate the high ability of the experimental animals to absorb these minerals from the feed. Phosphorus, the second most abundant mineral in the body after calcium is required for energy production, skeletal development and maintenance, cell growth and differentiation, muscle tissue building, membrane formation and maintenance of osmotic and acid-base balance (Suttle, 2010). The improved plasma phosphorus level recorded in indigenous cattle on diets T3, T4 and T5 is a pointer that experimental diets are not deficient in dietary phosphorus. This also means that diets T3, T4 and T5 are rich in phosphorus. The significantly increased plasma Mg and K level in cattle fed diets T3, T4 and T5 is an indication that magnesium and potassium level in the diet is not compromised. This further supports the results of our mineral analysis which revealed that groundnut shells and basal diet are rich in macro-minerals. Increased chloride level for the group in diets T3 and T4 shows that the level of this mineral in the treatment diets are not the same to that of the control, meaning that diets T3 and T4 are high in chloride. The significantly increased value of plasma sulfur in the cattle fed diet T3 compared to the control and the groups fed diets T2, T4 and T5 could be attributed to diet, source and chemical form of the mineral, and interactions among dietary constituents which have been reported to influence blood mineral composition (Suttle, 2010).

Utilization of blood metabolite provides an immediate indication of an animal's nutritional status and is recommended when assessing the effects of a diet on the performance of animal in the short to medium term (Pambu-Gollah et al., 2000). Blood serum mineral and electrolyte values in the current study were unaffected by the dietary treatments. The micro-mineral analysis revealed that the Mn, Fe, Cu, C, Cr, Co and V level of the indigenous cattle progressively increased with increasing levels of groundnut shell supplementation up to 700 g/kg feed (diet T3) and started decreasing before it started falling. Cu, Zn, Fe and Se concentrations recorded in the present experiment were below the reference value of 0.8 mg% (copper and zinc), iron (1.3 mg%) and 0.08 mg% (Se) reported for a healthy finishing cattle (Davy et al., 2019). In contrast, the Mn value obtained in this experiment was higher than the values of 0.006 mg% reported for healthy cattle by Davy et al. (2019) in California, USA. The statistically increased plasma Mn, Fe, Cu and Cr concentrations obtained in group fed diet T3 compared to the cattle in the control group (T1) indicate better utilization of the diet. The observed variations on the blood mineral values recorded in this experiment with the values reported by Davy et al. (2019) in the USA could be attributed in part to soil type, breed and sex of the animals used.

Optimization model

Data on the use quadratic regression model to ascertain the optimal GNS supplementation level that optimize growth performance indices and blood mineral traits in cattle are not available. The results of the influence of GNS supplementation showed that GNS had a quadratic action on growth performance (LW, ADG and BCS) and plasma

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macro-minerals (Ca, P, Mg, K, Na, Cl and S) and micro-minerals (Mn, Fe, Cu, C, Cr, C and V) with a p value of <0.0001 to 0.0002, 0.0001 to 0.0390 and 0.0001 - 0.0093, respectively. LW, ADG and BCS were optimized at 315.88, 315.70 and 328.42 g GNS/ kg feed. The results of the plasma Ca, P, Mg, K, Na, Cl and S were optimized at 319.05, 354.41, 331.40, 355.0, 333.22, 307.46 and 288.60 g GNS/ kg feed, respectively. On the other hand, GNS supplementation at 299.04, 296.77, 345.74, 307.86, 273.08, 391.54 and 78.26 g/kg feed optimized plasma Mn, Fe, Cu, C, Cr, Co and V. Plasma Ca, P, Mg, K, Na, Cl and S were optimized at varying GNS levels and the reasons for these variabilities is not fully known. However, this implied that cattle need varying levels of GNS supplemented diets to meet their mineral requirements. The values for coefficient of determination for growth performance indices were ranged 55.19% to 56.62%, while macro-minerals were ranged 65.23.9% to 80.86% which indicate high strength of GNS supplementation on plasma Ca, P, Mg, K, Na, Cl and S in cattle using quadratic function. Also, the coefficient of determination values for plasma micro-minerals were ranged 53.76 - 81.72% except for plasma manganese which had 40.45%. This may imply moderate strength of GNS supplementation on blood Mn concentration. The statistical optimization effect of GNS supplementation on blood macro-minerals and aspects of micro-minerals is a pointer that their values can be predicted at given supplementation level of GNS added in ration. The comparable changes in plasma Se and Zn may indicate that the influence of GNS supplemented diet was the same. However, further study on the effect of GNS supplementation in blood mineral parameters in cattle is desirable.

Conclusion

Groundnut shell is a rich source of dietary fibre and essential minerals and can be used as an excellent crude fibre and mineral sources in cattle diets. This is a pointer that GNS possesses the ability to mobilize and store important minerals in its shells. GNS supplementation influenced production parameters and blood mineral values in unsexed mixed cattle breed. GNS supplementation up to 700 g/kg feed in cattle ration encouraged digestion and absorption of minerals from the digestive tracts. The low to a moderate coefficient of variation of mean blood mineral concentrations obtained in this study established the fact that these values could serve as standard blood mineral value for cattle fed groundnut shells supplemented the diet in the study area.

The optimal response pattern for production parameters (LW, ADG, BCS), blood macro-minerals (Ca, P, Mg, K, Na, Cl, S) and aspect of blood micro-mineral (Mn, Fe, Cu, C, Cr, Co, V) parameters measured in unsexed mixed cattle breed were influenced by the groundnut shell supplementation. The results of the quadratic optimization model revealed that no single GNS rate optimized production data and blood minerals in the present study, meaning that groundnut shell supplementation level for optimal response depended on the production parameter and blood mineral parameter in question. Furthermore, the results of the optimization function revealed that GNS supplementation rate for optimal production parameters and blood minerals was achieved at a rate below 450 g/kg feed. Hence, the feeding program for optimal production parameters and blood mineral levels in unsexed mixed cattle breed must consider the parameter under consideration. However, further research is needed to ascertain the influence of sex on the production indices and blood mineral profiles of unsexed mixed cattle breed under confined system fed BBG supplemented with GNS during the winter season in North West province, South Africa.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_37533772

PHOTOSYNTHESIS, STOMATAL CONDUCTANCE, ENDOGENOUS HORMONES AND ORGANIC ACID SYNERGISTIC REGULATION IN LEAVES OF RICE (*ORYZA SATIVA* L.) UNDER ELEVATED CO_2

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(Received 9th Apr 2021; accepted 8th Jul 2021)

Abstract. To study the photosynthesis, stomatal conductance, endogenous hormones and organic acid regulation in leaves of rice seedlings under elevated CO_2, rice were exposed to different CO_2 concentrations (400±20 μmol mol^{-1}, AA; 600±20 μmol mol^{-1}, LEC; 800±20 μmol mol^{-1}, HEC) for 7 days. The results showed that: The elevated CO_2 increased IAA (Indole-3-acetic acid, IAA), ZT (Zeatin, ZT) and GA_3 (Gibberellins A_3, GA_3) contents significantly ($p<0.05$), which regulated the increasing P_n (Light-saturated net photosynthesis rate, P_n) and Fv/Fm (Maximum quantum yield of PSII photochemistry, Fv/Fm); Gs (Stomatal conductance, Gs) and Tr (Transpiration rate, Tr) were all decreased significantly ($p<0.05$), while ABA (Abscisic acid, ABA) content also decreased, but JA (Jasmonic acid, JA) content increased significantly ($p<0.05$) under EC. Perhaps, the movements of stoma were connected with JA in some degree, but not ABA; IAA/ABA, GA_3/ABA and ZT/ABA were all increased under EC, indicating the promoting effects of photosynthesis. JA/GA_3 decreased under LEC, but increased under HEC, which regulated the elevated Pn under LEC, and severe closure of stomata under HEC. The contents of oxalic acid, tartaric acid, acetic acid, malic acid, lactic acid, fumaric acid and succinic acid significantly decreased ($P<0.05$) in leaves, but contents of citric acid showed no significant difference with AA under HEC, indicating that the TCA cycle rate slowed down.
Keywords: *elevated CO_2, photosynthesis, Gs, ABA, JA, malic acid, fumaric acid*

Introduction

The accumulating CO_2 level has increased over two-fold since the industrial revolution (Guo et al., 2017). This resulted mainly from emissions of fossil fuel burning and net land use change (IPCC, 2013). As the carbon donor of plant photosynthesis, the increase of atmospheric CO_2 concentration is beneficial to the photosynthesis of plants, but decreases stomatal conductance and transpiration rate of leaves (Wang et al., 2019). Stomata play a major role in controlling CO_2 uptake for photosynthesis and water release by transpiration (Tanaka et al., 2013). Endogenous hormones of plants are micro-signal molecules involved in their metabolism. The guard cells are sensitive to environmental factors such as light, temperature, water and CO_2, which in turn means that they transmit information through changes in plant endogenous hormone levels, such as ABA which is always believed to be an important hormone that decreases the stomatal aperture (McAdam et al., 2011). Study have shown that in addition to ABA receptors, there are also IAA receptors in the guard cells, and the effect of high auxin concentrations on stomatal closure may be related to the regulation of anion channels on membrane of guard cells (Marten, 1991). In recent years, as new endogenous hormones, JAs and SA has received extensive attention. JA can promote stomatal closure and prevent stomatal opening in orchids and other plants (Gehring et al., 1997). JA and its

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
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precursor linolenic acid (linolenic acid, LA) can reduce the transpiration rate of tomato (Herde et al., 1997). SA (Salicylic acid, SA) also can decrease the stomatal aperture under certain conditions (Manthe et al., 1992). Plant responses usually regulated by the balance of different hormones, not just the concentration of a single hormone. Synergistic or antagonistic interactions among the different hormone groups add to the complexity of the hormonal system in higher plants (Wilhelm, 2015). Recently, physiologists of plant have provided lots of examples of links between activities of various hormones, that is called ‘‘crosstalk’’, such as JA is involved in crosstalk with SA, auxin, GA and ABA. Common organic acids include citric acid, oxalic acid, acetic acid, fumaric acid, lactic acid, malic acid, succinic acid, tartaric acid, etc., which can regulate various physiological metabolic processes of plants (Dakora and Phillips, 2002). Most of the organic acids come from the tricarboxylic acid cycle (TCA), including some intermediates of glyoxylic acid cycle and can reduce the toxicity of plants to a certain extent (Javed et al., 2017; Fu et al., 2018). At present, most studies focus on organic acids secreted by roots, there are few studies on the changes of organic acids contents in plants and under elevated CO_2 environment, so the objective of this study was to measure stomatal conductance, changes of endogenous hormones (including ABA, GA_3, IAA, JA, SA and ZT) contents and organic acids (including oxalate, tartarate, malate, lactate, acetate, citate, succinate and frumarate) contents of rice seedlings (*Oryza sativa* L.) leaves, so as to know the physiological mechanism response of plants to changed atmosphere environment and provide theoretical basis for the plant growth and crop cultivation in the future.

Materials and Methods

Plant materials and treatments

The uniform and healthy rice seeds (Beijing 2, which has been widely planted in Liaoning province, China) were sterilized, rinsed, and germinated in the dark. The germinating seeds were transferred to beakers containing Hoagland solution (Hoagland and Arnon, 1950). Seedlings were maintained in an artificial climate chamber (14/10 h light/dark period, 28/22℃ day/night, 80% relative air humidity and 800 μmol m^{-2} s^{-1} photosynthetic photon flux density) until to the two-leaves-stage. Then the rice seedlings were divided into three groups: (1) AA, rice seedlings cultured under CO_2 concentration of 400±20 μmol mol^{-1}. (2) LEC, rice seedlings cultured under CO_2 concentration of 600±20 μmol mol^{-1}. (3) HEC, rice seedlings cultured under CO_2 concentration of 800±20 μmol mol^{-1}. All the rice seedlings were cultured for 7 days, then were measured for gas exchange parameters, Chlorophyll a fluorescence parameter (Fv/Fm), hormones contents and organic acids contents. Three repeats were selected randomly in each pot (3 pots/treatment, a total of 9 repeats/treatment).

Growth parameters measurements

Plant height and taproot length were measured using a scale. The sampling plant was divided into the aboveground part and the underground part from the stem base, and the fresh weight was weighed. The plants that have been weighed fresh were placed in an oven at 120℃ for 6 hours, and then placed at 80℃ for 2 hours, and dried until the weight does not change. The dry weight was weighed. Each treatment group was repeated for 3 groups, and 10 plants were selected from each group for average value.

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Gas exchange parameters measurements

Light-saturated net photosynthesis rate (Pn), transpiration rate (Tr) and stomatal conductance (Gs) were recorded under saturated light between 09:00 and 11:00 by a portable photosynthesis system (LI-6400, Li-Cor Inc., Lincoln NE, USA).

Chlorophyll a fluorescence measurements

Chlorophyll a fluorescence parameter (Fv/Fm, maximum quantum yield of PSII photochemistry) was measured after 20-minute dark-adaption of leaves, using a portable fluorometer (Handy-PEA, Hansatech, England).

Extraction and analysis of hormones

Rice seedling leaves were homogenized by methanol and cross-chain polyvinyl pyrrolidone (PVPP) in an ice tray. The homogenate was immersed and extract in a refrigerator at 4℃ for 12 hours, then centrifuged for supernatant. The residue was extracted for 3 times using the same method and the supernatant is merged. The supernatant was placed in a 4℃ incubator (dark) to blow dry. The dry sample was dissolved with 100% methanol then centrifuged for supernatant. The supernatant was firstly filtered through 0.45 μm nylon filters and secondly through 0.22 μm nylon filters, then stored at 4℃. Samples were analyzed using an Agilent 1200 HPLC equipped with a reverse phase C18 column (250×4.6 mm).

The column was operated at 35℃. Isocratic Elution was carried at a flow rate of 0.8 mL/min. The injection volume was 20 μL, with methanol/acetic acid/distilled water (45:0.8:54.2, v/v/v) as the mobile phase. Chromatograms were acquired at 254 nm with standards for ABA, ZT, SA, IAA and GA_3 (purchased from Sigma Aldrich).

The column was operated at 30℃. Isocratic Elution was carried out at a flow rate of 0.3 mL/min. The injection volume was 20 μL, with methanol/formic acid/distilled water (65:0.035:34.965, v/v/v) as the mobile phase. Chromatogram was acquired at 254 nm with standard for JA (purchased from Sigma Aldrich) (Jensen and Junttila, 1982; Hou et al., 2008).

Extraction and analysis of organic acids

0.5 g of rice seedlings leaves were put into a mortar, and 3ml of distilled water was added to grind them to the homogenate at the same time, treated with ultrasonic for 30 min (25℃), then water bath for 15 min (75℃), centrifugally take the supernatant. Filtered supernatant in a disposable filter with an aperture of 0.45 μm, and then filtered it with an aperture of 0.22 μm, and stored it in a refrigerator with a temperature of 4℃. Samples were analyzed using an Agilent1200 HPLC equipped with a reverse phase C18 column (250×4.6 mm).

The column was operated at 35°C. Isocratic Elution was carried at a flow rate of 0.8 mL/min. The injection volume was 20 μL, with 0.01 mM H_2SO_4/methanol (96:4, v/v) as the mobile phase. Chromatograms were acquired at 210 nm with standards for oxalate, tartarate, malate, lactate, acetate, citate, succinate and frumarate (purchased from Sigma Aldrich) (Lodi and Rossin, 1995; Zhen et al., 2000).

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Statistical analysis

ANOVA was carried out to analyze all sets of data using SPSS 20.0 computer package and the Tukey test at 5% probability Levels were used to compare the means. Sample variability is given as the standard deviation (S.D.) for presentation.

Results

Effects of elevated CO_2 on plant growth parameters

The plant height and root length were significant ($p<0.05$) increased by 12.3%, 9.3% and 10.7%, 7.1%, respectively under LEC and HEC compared with AA. The fresh weight of the aboveground part significant ($p<0.05$) increased by 10.9% and 12.1%, and the fresh weight of the underground part significant ($p<0.05$) increased by 50.7% and 70.3% under LEC and HEC, compared with AA. Compared with AA, the underground dry weight of was significant ($p<0.05$) increased by 57.6% and 80.4%, and the dry weight of aboveground part was significant ($p<0.05$) increased by 19.1% and 27.4% under LEC and HEC (*Fig. 1*).

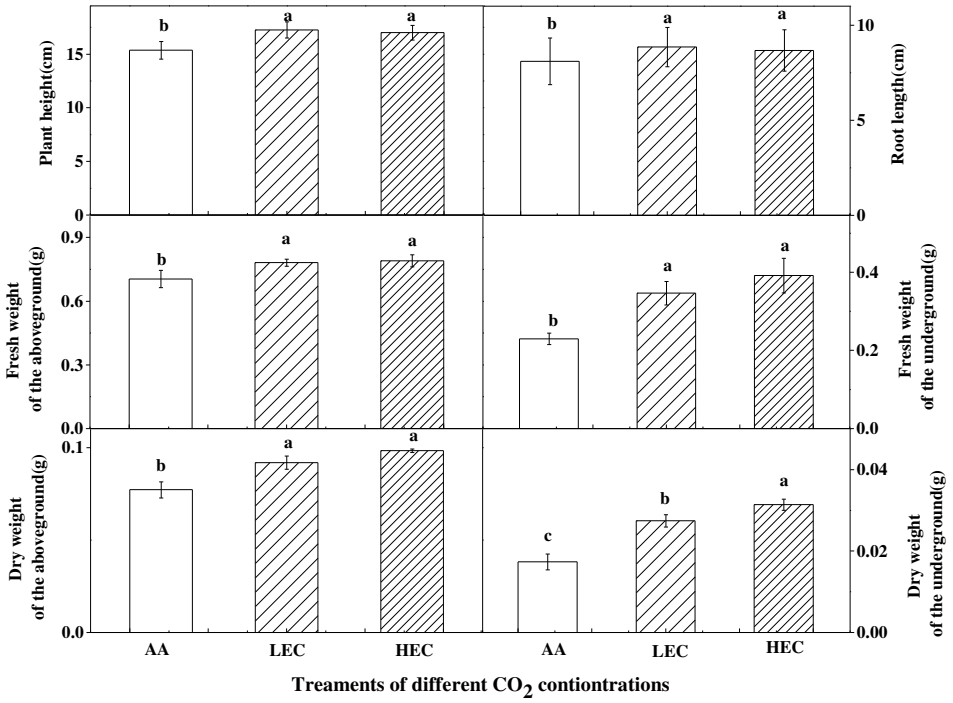

Figure 1. *Effects of CO_2 treatments on plant growth parameters of rice seedlings. The rice seedlings were cultured under different CO_2 concentrations (400±20 μmol mol^{-1}, AA; 600±20 μmol mol^{-1}, LEC; 800±20 μmol mol^{-1}, HEC). The bars indicated standard error. The significance differences were marked as abc at $P < 0.05$ (Tukey test). The following figures are consistent with this figure. (n = 30)*

Effects of elevated CO_2 on Pn, Fv/Fm, gs and Tr

P_n was significantly ($p<0.05$) increased under LEC and HEC compared with AA. The maximum difference (about 19.1%) was recorded under LEC; Fv/Fm was only

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significantly ($p<0.05$) increased under LEC, but not HEC compared with AA. LEC and HEC decreased Gs and Tr significantly ($p<0.05$) compared to the control, and Gs and Tr were lower under HEC than under LEC (*Fig. 2*).

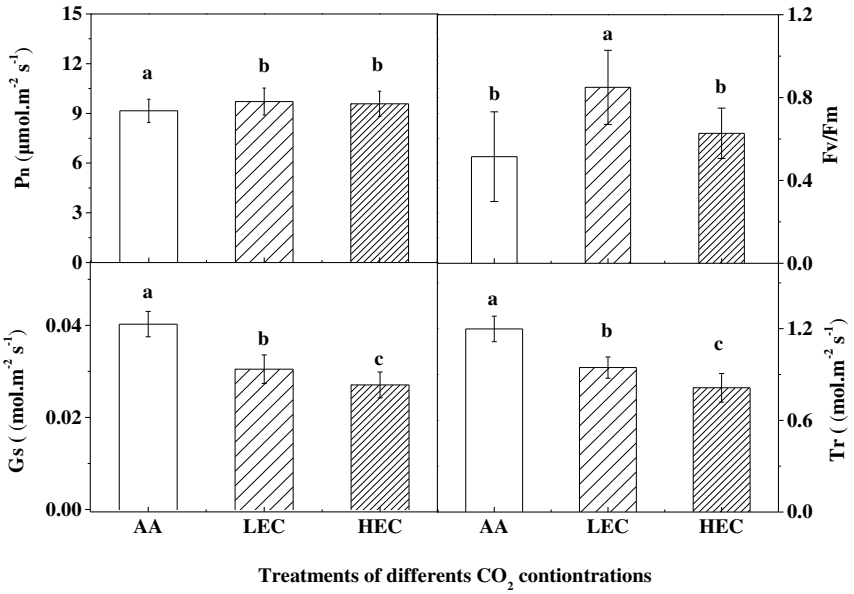

Figure 2. *Effects of CO_2 treatments on Pn, Fv/Fm, Gs and Tr of rice seedlings. Light-saturated net photosynthesis rate, Pn; Maximum quantum yield of PSII photochemistry, Fv/Fm; Stomatal conductance, Gs; Transpiration rate, Tr. (n=9)*

Effects of elevated CO_2 on endogenous hormones contents

Compare to control, ZT, GA_3 and JA contents were increased significantly ($p<0.05$) under LEC and HEC, but IAA content increased significantly ($p<0.05$) only under LEC. The maximum value of ZT, IAA and GA_3 contents were under LEC. There are no significant different of SA content under every treatment. Compared with AA, ABA content decreased (about 36.3%) significantly ($p<0.05$) under HEC (*Fig. 3*).

Effects of elevated CO_2 on ratio of endogenous hormones

IAA/ABA, GA_3/ABA, ZT/ABA, JA/ABA and JA/SA all increased under elevated CO_2, compared to control. The maximum value of IAA/ABA and GA_3/ABA were under LEC, while the maximum value of ZT/ABA, JA/ABA and JA/SA were under HEC. JA/GA_3 decreased under LEC and increased under HEC, compared with AA (*Fig. 4*).

Effects of elevated CO_2 on organic acids contents

In leaves of rice seedlings, compared with AA, there were no significant difference of oxalic acid, citric acid, tartaric acid and acetic acid contents under LEC, but contents of malic acid, lactic acid, fumaric acid and succinic acid significantly decreased ($P<0.05$) by 20.1%, 28.9%, 24.7% and 37.5%, respectively. Under HEC, contents of oxalic acid, tartaric acid, acetic acid, malic acid, lactic acid, fumaric acid and succinic acid significantly decreased ($P<0.05$) by 0.9%, 22.2%, 32.0%, 25.0%, 33.5%, 33.2% and 58.5%, respectively, but contents of citric acid showed no significant difference with AA (*Fig. 5*).

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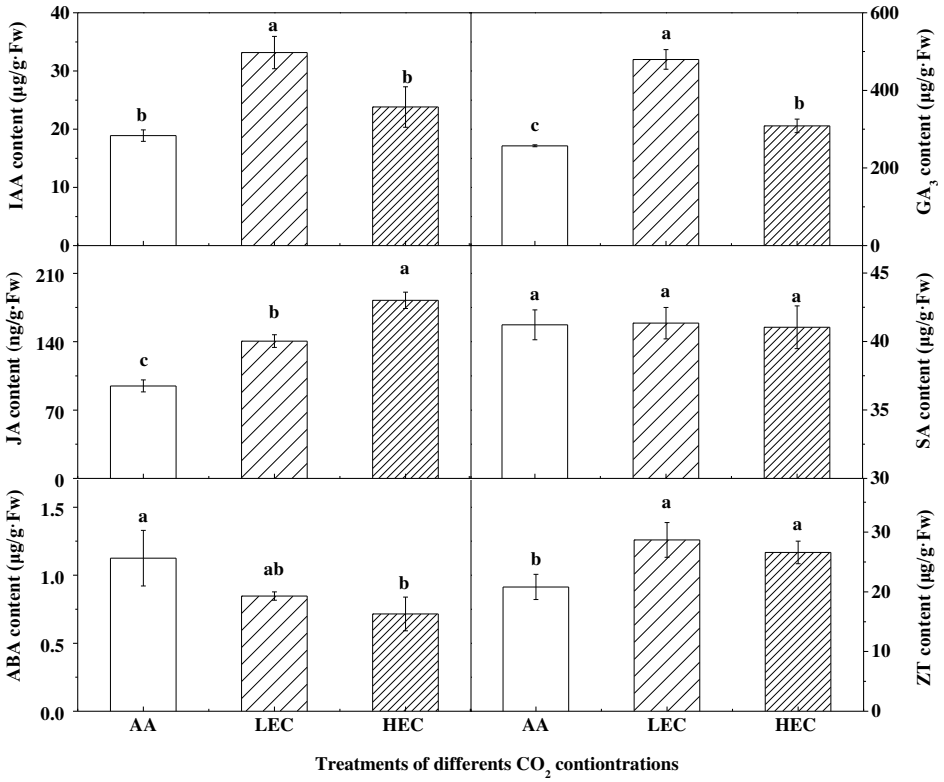

Figure 3. *Effects of CO_2 treatments on the contents of endogenous hormones in leaves of rice seedlings. (n=9)*

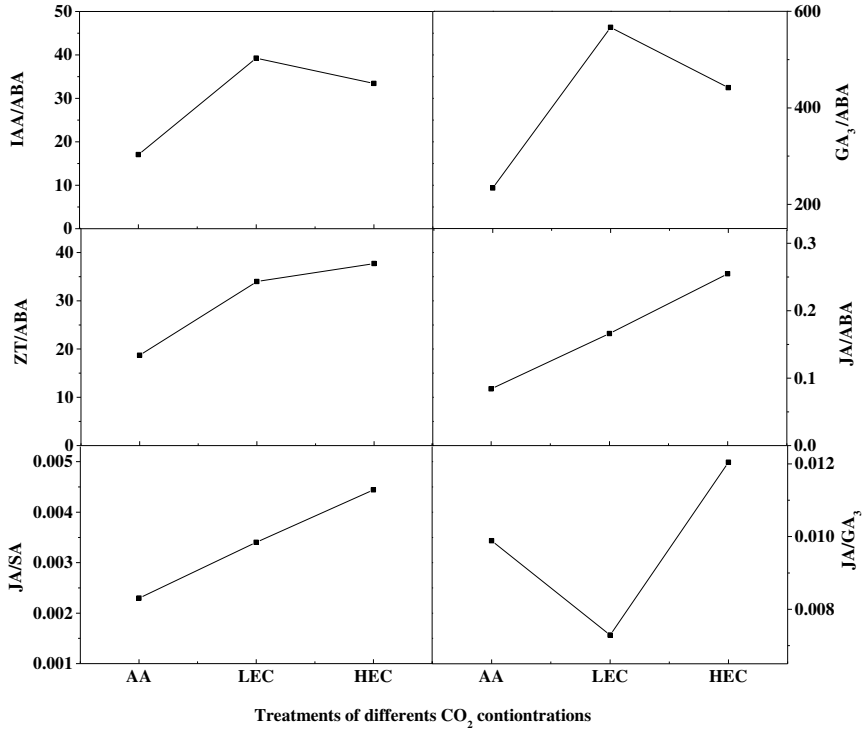

Figure 4. *Effects of CO_2 treatments on endogenous hormones ratios in leaves of rice seedlings. (n=9)*

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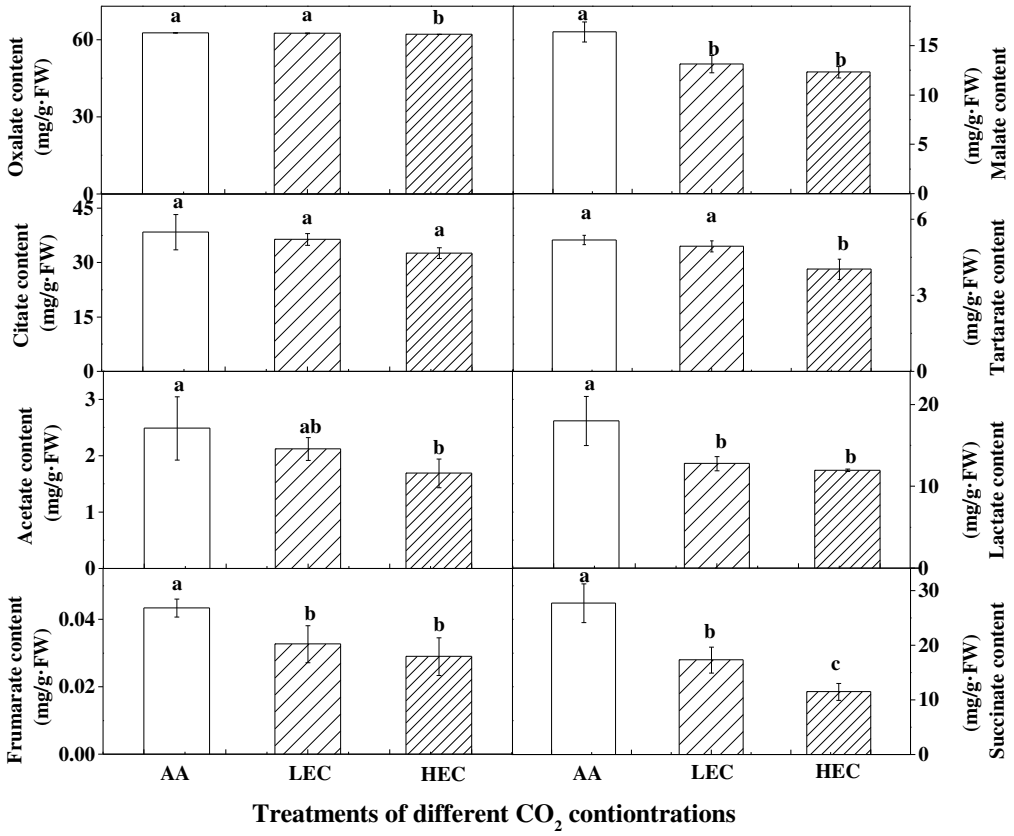

Figure 5. *Effects of CO_2 treatments on the contents of organic acids in leaves of rice seedlings. (n=9)*

Discussion

Growth parameters of rice seedlings under elevated CO_2

Plant growth parameters are the most intuitive measurement indexes to reflect the external characteristics of plants. High concentration of CO_2 can promote the growth of root and shoot of plant seedlings, thicken leaves, increase crop yield, water use efficiency and biomass (Kimball et al., 1995; Ainsworth, 2008). This study showed that compared with the control, plant height, root length, fresh weight and dry weight of rice seedlings under LEC and HEC were significantly ($p<0.05$) increased, which was consistent with the previous research results (Cure and Acock, 1986). This may be because in high CO_2 environment, the growth of rice roots is promoted to varying degrees, which can increase the contact area with nutrients, resulting in a significant increase in water and nutrients absorbed, and speed up the transport of water and inorganic salts from underground parts to aboveground parts, thus increasing plant height, root length, fresh weight and dry weight (Rogers et al., 1996).

Stomatal conductance and photosynthesis changes under elevated CO_2

It has been reported that high CO_2 can stimulate Pn in C_3 plants (Panigrahi et al., 2016; Lahive et al., 2017). We have also reported elevated CO_2 increased Pn and Fv/Fm of *Q. mongolica* leaves generally in two growing seasons, but decreased Gs and Tr slightly (Wang et al., 2019). However, the short-term CO_2-sensing mechanism in guard

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cells to elevated CO_2 is still unknown. In this study, P_n was significantly ($p<0.05$) increased under LEC and HEC, and the maximum difference (about 19.1%) was recorded under LEC, while Fv/Fm also only significantly ($p<0.05$) increased under LEC, but not HEC, compared with AA. That means the light reaction of photosynthesis was not promoted more under high CO_2 concentration. Elevated CO_2 decreased Gs and Tr significantly ($p<0.05$), and the values were lower under HEC than under LEC. That means high CO_2 contentions can cause the partly closure of stomata of rice leaves.

Endogenous hormones contents change under elevated CO_2

Environmental signals can modulate plant responses to the changed environments through changes in hormone concentrations and ratios. Endogenous hormones of plant are trace signaling molecules involved in plant metabolism, they can also regulate the whole process of plant growth and development, including the stomatal conductance. In this study, ZT, GA_3 and JA contents were all increased significantly ($p<0.05$) under elevated CO_2, IAA content also increased significantly ($p<0.05$), but only under LEC. ABA content decreased (about 36.3%) significantly ($p<0.05$) under HEC. There is no significant difference of SA contents under every treatment.

We have studied, elevated CO_2 increased IAA, GA_3 contents, but decreased ABA content (Li et al., 2011). IAA is the predominant auxin in most plants, with higher levels in young growing tissues (Bartel, 1997). It has also been reported that IAA content was elevated 56.6% in leaves of tomato seedlings when the CO_2 concentration changed from 350 to 800 μmol/mol (Wang et al., 2009). Since P_n and Fv/Fm were increased under the elevated CO_2 in our study. Photosynthetic products, such as glucose can induce IAA synthesis pathways *in Arabidopsis thaliana* through sugar signals (Sairanen et al., 2012). GA_3 can also regulate plant growth through integrating multiple signals, and GA_3 in leaves can also be increased under elevated CO_2 (Teng et al., 2006). In our study, IAA and GA_3 contents were all increased under LEC compared with control, but decreased under HEC compared with LEC. That may be resulted in the unchanged Pn between LEC and HEC in rice leaves. Moreover, Fv/Fm even showed no significant change between AC and HEC. Tazoe and Santrucek (2015) reported that stomatal closure induced by elevated CO_2 might be mediated by ABA levels, in our study, high CO_2 contentions caused the partly closure of stomata of rice leaves, but ABA content decreased (about 36.3%) significantly ($p<0.05$) under HEC. That showed CO_2-mediated stomatal closure does not require ABA, which are the same with some researches (Koornneef et al., 1982; Léon-Kloosterziel et al., 1996). In addition, the elevated CO_2 can increase Pn and promote the accumulation of sugars, that maybe make the negative regulation of ABA signaling pathways (Tsai and Gazzarrini, 2014).

JA and SA can participate in the regulation of diverse processes in plants, including growth, photosynthesis and reproductive development (Tariq et al., 2010; Claus, 2015). The partly stomatal closure regulated by JA has been found in some kinds of plants, such as *vicia faba* and *barley* (Tsonev et al., 1998; Liu et al., 2007). In this experiment, JA contents increased, but Gs and Tr decreased significantly under LEC and HEC, compared with AA. Maybe, the movements of stoma connected with JA in some degree, but not ABA. Some study has showed that the increasing levels of SA can increase in the growth of the plants (Tariq et al., 2010). The SA-enhanced growth of the plants might be associated with the regulatory effects of SA on cell growth and division (El-Tayeb, 2005). Moreover, SA inhibits the breakdown of ribulose-1,5-bisphosphate carboxylase/oxygenase, rubisco, in association with the changes in net photosynthetic

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rate, and content of sugars (Chandra and Bhatt, 1998). But other study's results showed that SA induced growth reduction (Manthe et al., 1992). In our study, there were no significant change of SA contents showed, even though, Pn had increased significantly under elevated CO_2, compared with control.

Cytokinins regulate the synthesis of pigments and structural proteins necessary for the formation of the chloroplast thylakoid system and the photosynthetic system, also act as respiration stabilizers, and as a kind of endogenous cytokines, ZT can promote effects of cell division, budding and lateral shoot formation, enhancement of metabolic sinks, as well as inhibition of apical dominance and root development (Kamínek, 2015). In our study, Pn and ZT all increased significantly ($p<0.05$) under EC, and Fv/Fm increased under LEC. It is the same with the research have showed the increasing photosynthetic activity, the leaf area and the quantum efficiency of the PS II photochemistry induced by cytokinins (Moura et al., 2017). While, the increase in transpiration rate (E) or stomatal opening by cytokinins has been reported in leaves of some plant species (Pospíšilová and Dodd, 2005), but in our study, Gs was decrease under EC, that maybe resulted in the different species of plants.

Endogenous hormones Ratios changes under elevated CO_2

Plant growth regulators can be used highly flexibly for the ‘‘fine-tuning’’ of crop plants under largely uncontrollable and unpredictable environmental conditions. ‘‘True’’ plant growth regulators interfere directly with the plant’s hormonal status. Synergistic or antagonistic interactions among the different hormone groups add to the complexity of the hormonal system in higher plants (Wilhelm, 2015). Usually, IAA, ZT and GA_3 are growth-promoting hormones, and ABA is growth-inhibiting hormone. In our study, IAA/ABA, GA_3/ABA and ZT/ABA were all increased by the elevated CO_2, indicated the promoting effects of photosynthesis and growth, that consistent with the results of our previous studies (Li et al., 2011). We also find IAA/ABA and GA_3/ABA were higher under LEC than HEC, that maybe result in the unchanged Pn between LEC and HEC.

JA is involved in cross-talks (links between activities of various hormones) with ABA, SA and GA (Claus, 2015). In our study, the increased JA/ABA ratio under EC maybe result in the increased Pn and decreased Gs, the unchanged SA content may result in the increase of JA/SA ratio under EC. Researches showed GA-mediated growth, accompanied by weakening of JA-mediated growth inhibition and defense responses. We can find that JA/GA decreased under LEC but increased under HEC, which perhaps because the elevated Pn under LEC, and the severe closure of stomata under HEC.

Organic acids contents changes under elevated CO_2

Organic acids are directly or indirectly involved in other metabolic processes, including carbon and nitrogen metabolism, regulating cytoplasmic pH and osmotic potential, and balancing the charge of excess absorption of cations (López-Bucio et al., 2000; Igamberdiev, 2018). Due to the central role of organic acids in cell metabolism, their synthesis and concentration are strictly controlled. In the cytoplasm, the concentration of organic acids is relatively stable. But in vacuoles, organic acid concentrations often vary with the environment in response to nutrient availability and metabolic activity (Gerhardt et al., 1987). In our study, the contents of organic acids decreased in leaves of rice under EC.

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DOI: http://dx.doi.org/10.15666/aeer/1905_37733787

Some organic acids (citric acid, succinic acid, fumaric acid, malic acid) are present in all cells and are intermediates in the TCA cycle. In our result, the content of citric acid in leaves of rice seedlings decreased compared with AA, the difference is not significant. The increases of CO_2 concentrations promote plants photosynthesis, while chloroplast is the place of photosynthesis. The metabolism of organic acids in plants is related to IDH (isocitrate dehydrogenase, IDH), and the increase of chloroplast activity may lead to the increase of NAHP-IDH distribution, thus promoting the decomposition of citric acid (Hodges et al., 2003; Lemaitre et al., 2007). In addition, citric acid can be secreted from the cytosol to the rhizosphere, preventing excess citric acid from accumulating in vacuoles (Langlade, 2002).

This study showed that succinic acid contents in leaves of rice seedlings decreased under HEC. Succinic acid is produced by SSA (succinic hemialdehyde, SSA) following the action of SSADH (succinate hemialdehyde dehydrogenase, SSADH), a key enzyme in the GABA (γ-aminobutyric acid, GABA) metabolic pathway involved in plant defense against environmental stress (Michaeli et al., 2011; Podlešáková et al., 2018). However, the increase of CO_2 concentration promotes the growth and development of plants, so SSADH perhaps is kept at a low level, that may result in the decrease of succinic acid synthesis.

Studies have shown that fumarate appears to behave as both a temporary carbon sink for photosynthate similar to starch (Pracharoenwattana et al., 2010). In carrot cells growing on malate as the carbon source, it was discovered that the malate was first converted into fumarate by a fumarase secreted from the cells and then fumarate was taken up and used for growth (Kim and Lee, 2002). In this study, the contents of fumaric acids in leaves decreased significantly under EC, possibly because fumaric acids acted as a temporary carbon sink of photosynthetic product for plant growth, which result the according decrease of fumaric acid contents under EC.

Malic acid, as an important intermediate, is involved in a series of physiological mechanisms in plants (Fernie and Martinoia, 2009; Santelia and Lawson, 2016). Malic acid also plays an important role in the opening and closing of leaf stomata (Raschke, 2003; Lee et al., 2008). In common, malic acid can decrease the stomatal aperture. In this study, malic acid contents of rice seedlings decreased under EC, which may be related to the change of stomata. In another, the decrease of malic acid contents perhaps because the more malic enzymes in chloroplasts of plants, which can catalyze malic acid in cells to produce CO_2 through decarboxylation to provide NADPH for C3 cycle, so as to improve the photosynthetic efficiency of plants (Rangel et al., 2010).

The metabolism of oxalic acid may be related to the photorespiration glycolic acid pathway and the metabolism of ascorbic acid, isocitrate and oxaloacetic acid (Franceschi, 1987; Horner et al., 2000). It is generally believed that leaves are important sites for the synthesis of oxalic acid (Wagner and Loewus, 1973). Studies have shown that oxalic acids synthesis is related to photorespiration and inhibition of photorespiration can block further oxalic acid accumulation (Fujii, 1994). In this study, oxalic acid content decreased significantly under HEC, possibly because the high CO_2 inhibited the photorespiration of rice. The accumulation of lactic acid in mature root tissues and the secretion of protons from cytosol to rhizosphere in hypoxic environment not only induced the changes of metabolism and pH value of rhizosphere, but also acted as the role of acidification and detoxification and preventing the accumulation of excess lactic acid in vacuole (Xia and Roberts, 1994; Langlade, 2002); glycolic acid oxidation pathway is a special glycolytic pathway in rice root system. Under the condition of

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
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insufficient oxygen supply in rice root, part of acetyl-CoA in root does not enter TCA cycle, but forms acetic acid. Acetic acid is catalyzed by glycolate oxidase to form glycolic acid, and then oxalic acid and formic acid are formed. Meanwhile, reducing substances in the root are oxidized to ensure the physiological function of the root. In our study, there was no low O_2 environment, so the lactic acid and acetic acid contents decreased significantly under EC respectively, which may be an adaptation to the CO_2 environment. Tartaric acid can detoxify, promote the growth of plants under environmental stress (Chen et al., 2017; Riaz et al., 2018). However, the increase of atmospheric CO_2 concentration itself can promote the plant growth, it does not need too much tartaric acid to assist the growth and content of tartaric acid decreased significantly under HEC.

Conclusions

In the present study, rice seedlings leaves were used to study the changes of photosynthesis, stomatal conductance, endogenous hormones and organic acids contents under elevated CO_2. The main results show: Under EC, P_n and Fv/Fm were increased, but Gs and Tr were decreased significantly ($p<0.05$), the partly closure of stomata in rice leaves were induced. IAA, ZT and GA_3 contents were all increased significantly ($p<0.05$) under EC, which resulted in the increased Pn. ABA content decreased under HEC, but JA content increased significantly ($p<0.05$) under EC, Maybe, the movements of stoma connected with JA in some degree, but not ABA. There are no significant changes of SA contents under EC. In our study, IAA/ABA, GA_3/ABA and ZT/ABA were all increased under EC, indicated the promoting effects of photosynthesis and growth. The increased JA/ABA under EC maybe result in the increased Pn and decreased Gs. JA/SA increased under EC, and JA/GA decreased under LEC but increased under HEC, which perhaps because the elevated Pn under LEC, and the severe closure of stomata under HEC. The contents of oxalic acid, tartaric acid, acetic acid, malic acid, lactic acid, fumaric acid and succinic acid significantly decreased ($P<0.05$) in leaves of rice seedlings under HEC. The variation of organic acids may be a mechanism of plant response to high CO_2 environment. The contents of organic acids in the TCA cycle decreased, such as malic acid and fumaric acid, indicating that the TCA cycle rate slowed down. And the organic acids, those with detoxifying effects, such as tartaric acid and oxalic acid significantly decreased ($P<0.05$) under HEC. In the future study, the intrinsic molecular mechanism changes of endogenous hormones and organic acids regulation should be concerned. Studies on its metabolome and transcriptome are ongoing.

Acknowledgements. This work was founded by the National Natural Science Foundation of China (31600314), the Department of Education of Liaoning province Foundation (LZD201901, LZD202004), Innovation training program for college students (202106039).

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DOI: http://dx.doi.org/10.15666/aeer/1905_37733787

COMPARING FACTORS CONDITIONING LANDSCAPE CHANGE DURING VOLCANIC ERUPTION AT VIRUNGA NATIONAL PARK, EAST AFRICA

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(Received 10th Apr 2021; accepted 12th Aug 2021)

Abstract. The change on Virunga National Park (VNP) landscape due to climate change, anthropogenic activities and natural disasters calls for appropriate monitoring and management policies. This study aimed to assess and compare the factors conditioning landscape change during volcanic eruption at VNP located in the East Africa from 1990 to 2018. Land use and land cover Landsat 5 images of 1990 and 2004, and Landsat 8 images of 2018 were analyzed by the supervised maximum likelihood classification techniques. The calculation of Normalized Difference Vegetation Index (NDVI) and its relationship with precipitation and temperature referred to the same images. Remote Sensing (RS) and Geographic Information System (GIS) facilitated data analysis. The results highlighted human activities as key drivers to landscape changes recorded in the central and northern parts. The southern part recorded high extent of changes which transformed 8.36 Km^2 of the park into new lava flow linked to 11.6 Km^2 of grassland and almost 1 Km^2 of shrubland changed into lava plains. The two volcanoes are associated with volcanic plume and gas emission, which mainly cause NDVI perturbation. This study can serve as a baseline in the conservation and management of protected areas and reduction of volcanic effects on national parks.
Keywords: *landscape change, NDVI, rainfall and temperature, Virunga National Park, volcanic eruption*

Introduction

In 1972, the World Heritage Convention was approved to facilitate the conservation in the continuity of the most valuable cultural and natural resources of the world (Meskell, 2013). The convention's goal is to protect resources that possess significant universal values that rise above national boundaries and deserve safeguarding for the benefit of humanity. There are more than 190 countries that are co-signers to the convention, devoting to protect the 1,121 world heritage sites. Out of these properties, 213 are Natural World Heritage Sites (NWHS), adorned for their exceptional natural glamour, biological essence and biodiversity conservation (Edwards Jr. et al., 2018; Lin et al., 2020). NWHSs serve as crucial sources of water, prevention of natural disasters such as landslides or floods, job creation and income generation from tourism and recreation, and provision of habitat to wildlife while the varying human and natural events threaten this natural asset (Osipova et al., 2014).

For example, two most active volcanoes in Africa are Nyiragongo and Nyamuragira located to the south of VNP registered recent eruption. Between 1977 and 2002, Nyiragongo had flank eruptions that led to injuries and deaths, which ultimately triggered socio-economic and humanitarian disasters (Tazieff, 1977; Vaselli et al., 2010; Cuoco et al., 2013). Nyamulagira's lava flow field located in the VNP covers more than 1,100 km^2.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_37893803

Nyamulagira's flank eruptions are associated with the burning of vast hectares of the protected forest and its lava flow destroyed 10-13% of Goma, which rendered about 120,000 persons homeless, and affected the regional economy (Allard et al., 2002; Baxter and Ancia, 2002; Tedesco et al., 2002). Additionally, both Nyamuragira and Nyiragongo emit acidic gas flumes that are highly concentrated with Sulphur dioxide (SO_2) (including ash) which contributes to the acidic rainfall affecting the surface water quality, crops, vegetation, human infrastructure, and human health (Vaselli et al., 2010; Cuoco et al., 2013; Balagizi et al., 2018).

According to Mitchell et al. (2015) and Rolo et al. (2018), the volcanic eruption causes fragmentation of a landscape which generates both negative and positive effects. For instance, Ibáñez et al. (2014) researched the impact of landscape fragmentation on vegetation and reported significant positive effects resulted from the edge effects and fragment size. Negative responses to edge effects were vital for survival, density, colonization, and richness. The study of Mitchell et al. (2015) evaluated the correlation between ecosystem services and landscape integration. The researchers claim that disintegration can have both negative and positive impacts on service flow, although it generally affects the ecosystem service supply negatively. The effects of disintegration are a result of landscape fragmentation being able to facilitate and intersect the movement of substance, energy, organisms, and persons across landscapes (Mitchell et al., 2015). Notably, landscape fragmentation contributes to diminishing the mean forest patch size and increasing the density of the forest patch, which consequently affects the species habitat (Andren, 1994; Estoque et al., 2018).

Volcanic eruptions change the landscape through constructive (pyroclastic deposits, lava flows, and domes) and descriptive (caldera creation and flank failure) processes. More specifically, volcanic eruptions are destructive to plant life and also alter the physical properties land, for instance, porosity, chemistry, and permeability (Mutaqin et al., 2019; Michellier et al., 2020). Previous studies (Marijnen, 2018; Christensen and Arsanjani, 2020) on VNP focus on the effects of volcanic particles on vegetation but omitted the impact of volcanic eruptions on the change on VNP's landscape. The literature indicates lack of implications of lava flow, volcanic emissions, and volcanic plume deposits on the wildlife and their habitat, mainly chimpanzees living on the side of Nyamuragira's lava plains in the Tongo forest.

Remote sensing technology remains to be a key data source for various environmental sciences and activities monitoring, for instance, assessment of change in forest cover (Desclee et al., 2013; Binsangou et al., 2018; Pareta and Pareta, 2019). In addition, land use/land cover (LULC) maps resulting from remote sensing imageries provide valuable information for capturing landscape structure and spatial formation (Estoque et al., 2018; Orimoloye et al., 2019). The authors applied Remote Sensing techniques to assess the impact of volcanic eruption to landscape change at Virunga National Park, East Africa. Satellite images were taken on 18th October, 1990; 26th October 2004 and finally 6th November, 2018.

Methods and Materials

Description of study area

This study considered the Virunga National Park (VNP) located in the East Africa at 0^0 55'S, 29^0 10' E, in the Albertine Rift Valley situated in the eastern Democratic Republic of Congo. It ranges from 2,230 ft in the Semliki River valley to 16,762 ft in altitude. From

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DOI: http://dx.doi.org/10.15666/aeer/1905_37893803

south to north, it encompasses about 300 Km and covers an area of 7,900 km^2. The area is mainly along the international boundaries with Rwanda and Uganda in the eastern part (Crawford and Bernstein, 2008). The VNP (*Figure 1*) shares border with the Democratic Republic of Congo, Uganda and Rwanda.

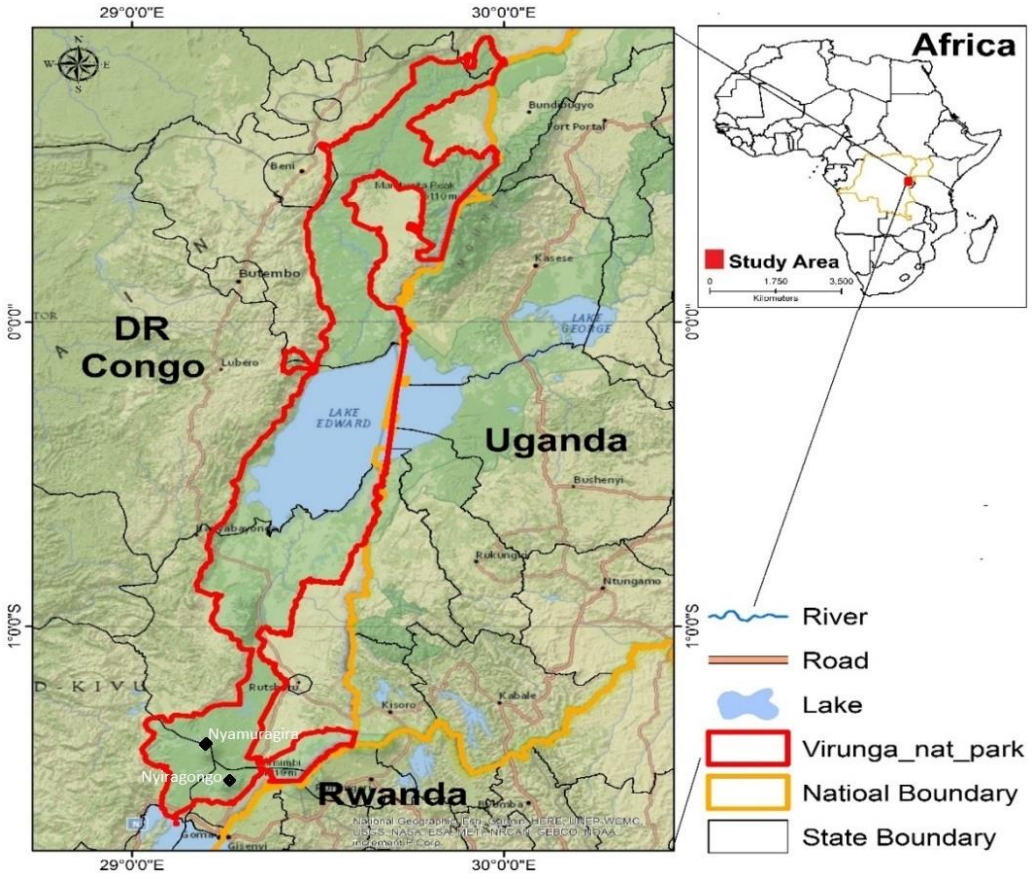

Figure 1. *Map indicating the location of the study area*

Datasets collection and Analysis

For this study, the authors utilized remote sensing satellite images in order to assess the LULC of the area in the years of 1990, 2004, and 2018. These Landsat images were acquired from: https://glovis.usgs. gov/. The 1990-2004 and 2004–2018 images were related to the spatio-temporal landscape changes in VNP and chimpanzee population in the Tongo forest, part of VNP. This period covered both before and after volcanoes major events during the 28 years (Nyiragongo in 2002 and Nyamuragira in 2010-2011).

The analysis of LULC changes of VNP was made by using both Landsat5 images of 1990 and 2004 along with Landsat 8 images of 2018. The LULC classified images were separately classified into seven classes namely: bare area, cropland, forest, grassland, shrubland, water, and wetland. The LULC images were classified with reference to the supervised maximum likelihood classification techniques (Orimoloye et al., 2019). The method necessitates the digitization of training places for every LULC session based on the prior knowledge, and uses the training locations to train and ultimately categorize pixels into the images (Estoque et al., 2018). Atmospheric correction was done using below equation.

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$$p_\lambda = \frac{[(L_\lambda - L_{p1\%}) * \text{pi} * d^2]}{[T_v * (ESUN * cos(z) * T_z + E_{down}) \quad]} \quad \text{(Eq.1)}$$

were p_λ is the planetary reflectance (Unitless), L_λ is the cell value as spectral at sensor radiance, d is the earth-sun distance (astronomical unit), ESUM is the mean solar exo-atmospheric irradiances, T is the atmospheric transmittance (Unitless), E is the emissivity and z is the local sun elevation angle.

Moreover, the study evaluated the Normalized Difference Vegetation Index (NDVI) by utilizing the same Landsat images. However, different bands were used in order to explain difference of the spectral responses of vegetation at the near-infrared and red bands (Mind'je et al., 2019). The values of NDVI were calculated by using the equation below.

$$NDVI = \frac{\rho_{nir} - \rho_{red}}{\rho_{nir} + \rho_{red}} \quad \text{(Eq.2)}$$

The NDVI varies between −1.0 and + 1.0.

However, the authors recognized the fact that NDVI can be affected by vegetation health, seasonality effects, atmospheric effects, and topography, for instance, sun angle (DeRose et al., 2011). Therefore, climatic variables of precipitation and average temperature were used to determine NDVI-precipitation and temperature relationships (Ndayisaba et al., 2017) by using the equation below. The dark object subtraction technique was applied to achieve surface reflectance and full atmospheric correction as described using *Equation 2* (Song et al., 2001).

$$S = \frac{n\sum_{i=1}^{n} X_i Y_i - \sum_{i=1}^{n} X_i \sum_{i=1}^{n} Y_i}{n\sum_{i=1}^{n} X_i^2 - \left(\sum_{i=1}^{n} X_i\right)^2} \quad \text{(Eq.3)}$$

where S is the slope, Y_i and X_i are the dependent variable values and the independent variable in the i^{th} year, respectively, and n is the total number of years considered by the study. Generally, if slope = 0, the dependent variable shows stability. On the other hand, if the slope>0, the variation dependent variable values indicate an increasing trend, whereas when the slope is <0, the dependent variable variation implies a decreasing trend.

The above data sets were collected and analyzed by using both Remote Sensing (RS) and Geographic Information System (GIS) and their corresponding maps are presented in the following section.

Classification accuracy assessment

Furthermore, the authors considered the fact that accuracy exposes the exactness between a truthful standard expected and the image classified of unidentified quality, and that when only using general classes, the user of the map cannot make observations about a selected point on the map with precision (Rwanga and Ndambuki, 2017). Also, it is practical to increase the accuracy of any classification in the GIS environment by integrating numerous classes instead of a specific feature or by decreasing the number of details. More specifically, lower precision is associated with higher accuracy (MacLean and Congalton, 2012). Therefore, in order to ensure the accuracy of the processed image, the authors performed an accuracy assessment.

Furthermore, in order to indicate the impact of volcanic eruption on the landscape change and its wildlife habitat mainly chimpanzee in Tongo forest, the largely affected

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DOI: http://dx.doi.org/10.15666/aeer/1905_37893803

by change on landscape. This exercise was completed by referring to secondary sources which considered the change on the number of chimpanzee, area fragmentation due to landscape change resulting volcanic eruption. All sources used for this track of chimpanzee change were provided in the reference list.

Result and Discussions

Spatial patterns of land use and land cover between 1990 and 2018

The results of the study in *Figure 2A* and *Table 1* indicated that in 1990, the forest occupied the most substantial areas in all the seven land feature types, with about 40% (3119 Km^2) of the entire land cover in the area. The smallest percentage was occupied by wetland at 0.18% (13.9 Km^2) of the total area (*Figure 2A*). In 2004, about 0.75% of forests declined compared to 1990. However, cropland increased by 1.18%, while water and grassland declined by 0.5% and 0.027%, respectively (*Figure 2B* and *Table 1*). Finally, the bare area increased by 0.21%, while shrubland and wetland decreased by 0.034%.

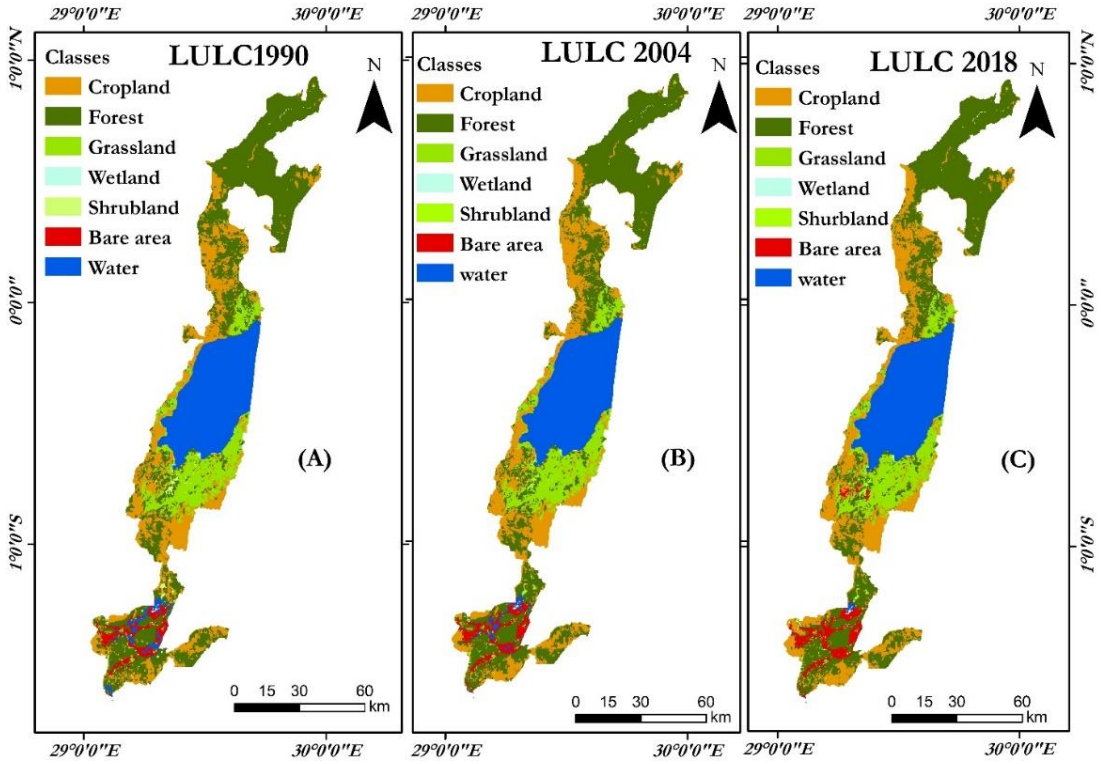

Figure 2. *Land use and land cover at Virunga National Park 1990 (A), 2004 (B), 2018 (C)*

Generally, there is a higher landscape change between 1990 and 2004. From 2004 to 2018, the area occupied by water and forest declined by about 0.56%, while grassland decreased by 0.12%. Cropland increased by 0.41%, and the bare area increased by 0.77%, shrubland increased by 0.042% while the wetland area rose by 0.015% (*Figure 3*). Between 1990 and 2018, there was an increase of cropland, shrubland, and bare area by 1.59%, 0.008% and 0.97%, respectively (*Figure 3*). While forest declined by 1.29%, grassland, wetland, and water decreased by 0.15%, 0.018%, and 1%, respectively.

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Table 1. *LULC change matrix from 1990 to 2018 (Km^2)*

		LULC 2004							
	Classes	**Cropland**	**Forest**	**Grassland**	**Wetland**	**Shrubland**	**Bare Area**	**Water**	**Grand Total**
LULC 1990	**Cropland**	**1667.54**	89.73	0	0	0	0.1	0	**1757.37**
	Forest	180.33	**2936.24**	2.57	0.09	0.29	0.38	0.0000021	**3119.9**
	Grassland	1.33	3.61	**879.16**	0	0	0	0	**884.11**
	Wetland	0	2.76	0	**11.22**	0	0	0	**13.97**
	Shrubland	0	4.18	0	0	**53.13**	0	0.1	**57.41**
	Bare Area	0	2.23E-06	0	0	0	**217.21**	0.0000012	**217.21**
	Water	0.29	26.04	0.28	0	1.33	16.06	**1725.02**	**1769.02**
	Grand Total	**1849.49**	**3062.56**	**882.01**	**11.31**	**54.75**	**233.74**	**1725.12**	**7818.99**
		LULC 2018							
	Classes	**Cropland**	**Forest**	**Grassland**	**Wetland**	**Shrubland**	**Bare Area**	**Water**	**Grand Total**
LULC 2004	**Cropland**	**1825.15**	14.74	0	0	0	9.6	0	**1849.49**
	Forest	57.11	**2992.34**	1.9	1.24	1.52	8.36	0.1	**3062.56**
	Grassland	0	0.57	**869.85**	0	0	11.6	0	**882.01**
	Wetland	0	0.1	0	**11.22**	0	0	0	**11.31**
	Shrubland	0	0.1	0	0	**53.7**	0.86	0.1	**54.75**
	Bare area	0	0.0000063	0	0	0	**233.74**	0	**233.74**
	Water	0	11.21	0.48	0.1	2.85	29.46	**1681.03**	**1725.12**
	Grand Total	**1882.26**	**3019.05**	**872.22**	**12.55**	**58.07**	**293.62**	**1681.22**	**7818.99**
		LULC 2018							
	Classes	**Cropland**	**Forest**	**Grassland**	**Wetland**	**Shrubland**	**Bare Area**	**Water**	**Grand Total**
LULC 1990	**Cropland**	**1645.58**	101.43	0.76	0	0	9.6	0	**1757.37**
	Forest	235.16	**2869.4403**	3.71	1.33	1.81	8.36	0.1	**3119.9**
	Grassland	1.33	4.18	**867**	0	0	11.6	0	**884.11**
	Wetland	0	2.85	0	**11.12**	0	0	0	**13.97**
	Shrubland	0	4.28	0	0	**52.08**	0.86	0.19	**57.41**
	Bare Area	0	0.0000084	2.70E-07	0	0	**217.21**	0	**217.21**
	Water	0.19	36.87	0.76	0.1	4.18	45.99	**1680.93**	**1769.02**
	Grand Total	**1882.26**	**3019.05**	**872.22**	**12.55**	**58.07**	**293.62**	**1681.22**	**7818.99**

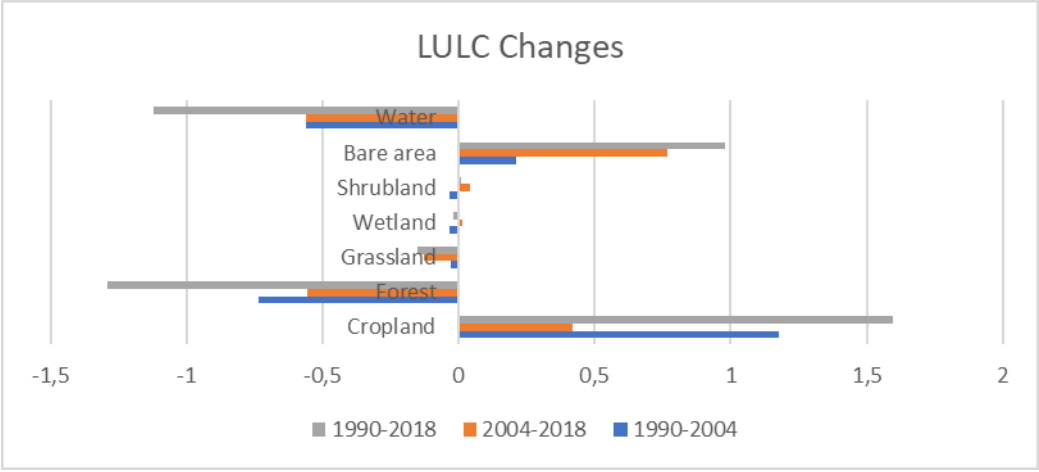

Figure 3. *LULC changes of the VNP, for the periods 1990-2004, 2004-2018 and 1990-2018 in percentage*

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DOI: http://dx.doi.org/10.15666/aeer/1905_37893803

As indicated in *Figure 3*, changes in LULC for the period 1990-2004 was more significant than the change for the period 2004 to 2018. Notably, changes were common across the classes in the study. The bare area increased by 0.21% from 1990 to 2004 and by 0.76% for the period 2004 to 2018 (*Figure 3*).

The expansion of the bare area was mainly found in the southern VNP, where more than 98% of the bare area is situated (*Figure 2A,B and C*). It was noted that the period 1990-2004 showed significant landscape changes in all three parts of the park. This can be associated to the fact that, both the north and central parts faced civil war in DRC between 1996 and 2003 which rendered the park management inactive and contributed to illegal charcoal industry, poaching, and other deforestation activities that have increased (Crawford and Bernstein, 2008). In addition, it is also during the same period that the Soco International PLC began taking a serious interest in oil exploration around the park, especially in the central part along with subsistence agriculture, illegal logging industry and mining across the park (Schiffman, 2016; Christensen and Arsanjani, 2020).

Furthermore, during the same period (1900-2004), the Rwanda Genocide against Tutsi created almost 2 million refugees who settled in the southeast part of the park likely practicing illegal poaching, deforestation, mining, and charcoal production (Blackie et al., 2015). The impacts of this change in landscape affected the conservation in the park, for example: the dramatic drop in the elephant populations that led to dense ligneous vegetation invading the remaining savannahs (Zhang et al., 2006). The results in *Figure 3* indicated that between 2004 and 2008, the bare area increased. Generally, a forest can be changed into a bare area as a result of both natural and human factors. Similarly, in the study area, the bare area is mainly situated in the southern VNP, which holds two of African's most active volcanoes (*Figure 2*). The period 2004 -2018 showed an increase in the bare area of 90% in the southern part of VNP (*Figure 2*). The bare area increase is mainly due to Nyamuragira's major eruption (2010-2011) that turned its vegetation zones into lava plains classified as bare areas (Smets et al., 2010). This eruption of Nyamuragira eruption (2010-2011) transformed 8.36 Km^2 of the forest, 11.6 Km^2 of grassland, and almost 1 Km^2 of shrubland into lava plains between 1990 and 2018 (*Table 1*).

As indicated in *Table 2*, the accuracy measurements were conducted to ascertain the exactness of each classified image (1990, 2004 and 2018). The image classification of the study area revealed that the overall accuracy value is 91.3%, and the kappa coefficient value is 0.88. The accuracy estimation revealed 100% producer accuracy for both water and bare areas. Further, the results in *Table 2* showed that the forest and cropland have about 91.45% and 88.49% producer accuracy, respectively. In contrast, grassland, wetland, and shrubland have 81.45%, 36.6%, and 98.65%, respectively (*Table 2*). The estimated accuracies for the information obtained from the satellite images were judged satisfactory, and they have revealed the accuracy between a truthful standard expected and the classified image of unidentified quality.

Spatio-temporal distribution of NDVI between 1990 and 2018

The results in *Figure 4* revealed a considerable decrease in the greenness within the national park since 1990.The yellow shade represented very scarce vegetation (0.16667-0.3334), and the brown spots represented non-vegetated areas. In the northern part of the study area, the vegetation started to diminish along the river in 1990 (*Figure 4A*). By 2004, the decline in the vegetation happened on the hill along the border of the park. The steady deterioration of the vegetation cover can be primarily attributed to increase in substance agriculture and charcoal production (Christensen and Arsanjani,

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2020). Accordingly, as shown in *Figure 4C*, in 2018, the entire region suffered a drastic reduction of vegetation cover. For example, in the hunting zones of Rutshuru, 90% of the total surface area was entirely degraded (*Figure 4*).

Table 2. *LULC change confusion matrix between 1990 and 2018*

	Cropland	**Forest**	**Grassland**	**Wetland**	**Shrubland**	**Bare area**	**Water**	**User Accuracy**
Cropland	**392**	32	15	5	1	0	0	88.09%
Forest	20	**460**	3	4	1	0	0	94.26%
Grassland	21	9	**101**	4	0	0	0	74.81%
Wetland	9	1	5	**8**	0	0	0	34.78%
Shrubland	1	1	0	1	**151**	0	0	98.05%
Bare Area	0	0	0	0	0	**182**	0	100%
Water	0	0	0	0	0	0	**102**	100%
Producer Accuracy	88.49%	91.45%	81.45%	36.36%	98.65%	100%	100%	

Overall accuracy: 91.3%, Kappa statistics: 0.88

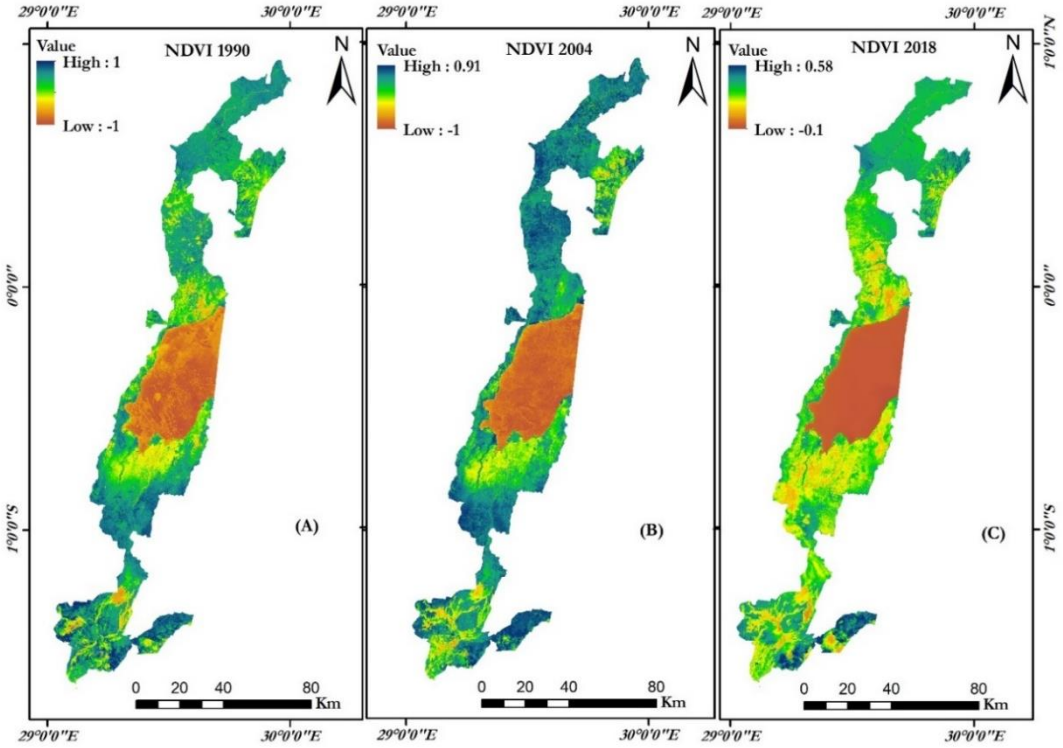

Figure 4. *Spatial and temporal NDVI distribution at Virunga National Park in (A) 1990, (B) 2004 and (C) 2018*

As shown in *Figure 4*, NDVI values within VNP dropped from 0.967742 to -0.123094. Such a drop suggests that the slightly forested areas probably became grasslands or shrubland. The densely vegetated areas observed to the western park are absent in 2018. Similarly, Jones (2019) observes that there are more mildly forested areas and less

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vegetation in the same region. Moreover, the results in *Figure 4C* revealed a significant loss of highly dense forests in the southern part of the study area. The volcanoes located to the east of the national park showed that highest vegetation decline between 1990 and 2018. It can be noted that the vegetation decline, southern part (*Figure 4C*) is mainly due to the volcanic activity that takes place almost every year (Smets et al., 2010) and have severely affected the dense forest where the heads of trees were partly burned by acid rainfalls and volcanic plume (Vaselli et al., 2010). The coral trails seen near the volcanoes are recently dried lava flows (*Figure 4C*). A perturbation event was observed from 1990 to 2018 over a vast region of the southern part of the study area, with a North East -South West spatial distribution (*Figure 4B and C*).

The spatial distribution estimate ranged between (-0.123094 to 0.583163) across Virunga National Park (*Figure 4C*). Lava flow, gas emission, and volcanic plume contribute to the intensity of the NDVI perturbation pattern, as argued by other previous studies (Grainger et al., 2013). The spatial distribution of the perturbation promoted by a volcanic eruption (Lava flow and Volcanic plume deposit) from Nyiragongo and Nyamuragira activities during the period 1990-2018 were noted by observing sudden changes in NDVI temporal dynamics (*Table 3*). The NDVI perturbation pattern was observed in a North-East (NE) and South-West (SW) part of the park (*Figure 4C*). This likely agrees with Cuoco et al. (2013) that the Southwest of Mount Nyamuragira records the highest intensity of volcanic plume dispersion. Equally, Wauthier et al. (2009) pointed the same location as the direction of lava flow originating from the Nyiragongo volcano in January 2002. The NE spatial distribution also coincide with the direction of Nyamuragira lava flows for the 2001 and 2002 eruptions as mapped by Smets (2010) and Li et al. (2018).

Table 3. *The LULC classes and their corresponding ranges of NDVI values*

LULC classes	NDVI range 1990	NDVI range 2004	NDVI range 2018
Cropland	0.3–0.6	0.2–0.7	0.15–0.45
Forest	0.4–0.96	0.3–0.92	0.4–0.56
Grassland	0.1–0.61	0.2–0.55	0.08–0.38
Wetland	0.06–0.5	0.0–0.4	0.05–0.4
Shrubland	0.2–0.4	0.18–0.55	0.1–0.4
Bare area	**0.0**–0.3	0.01–0.36	0–0.22
Water	-0.2–0.10	-0.5–-0.02	-0.1–0.19

In addition, the results in *Figure 4* showed that the southeast part of the park for the period 1990-2004, NDVI perturbation was previously associated at a large extent. This again, can be associated to the reason that the Rwanda Genocide against Tutsi created more than 1 million refugees. The displacement Rwandan refugees to build settlements within the park contributed to illegal poaching, mining and deforestation (Kingston, 2017). Thereafter, the authors performed the NDVI Change detection as illustrated in *Figure 5*. It was noticed that the annual change of the trend in NDVI showed that the maximum vegetation cover for each year was 0.440708, while the minimum was -0.296286. The detection of changes was obtained by using the annual trends in NDVI multiplied by the significance value (p-value). The calculation in *Figure 5* showed that 2.7% significantly improved, 4.7% slightly improved, while 42% of the study area showed stable or no change areas. Also, *Figure 5* showed that 35.7% was slightly degraded, and 14.8% was significantly degraded. Significant degradation was mainly

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DOI: http://dx.doi.org/10.15666/aeer/1905_37893803

observed in the central parts of the park where the population of the fourteen fishing villages increased between 1990 and 2018. The southwest part of the park was also significantly degraded due to volcanic eruptions. Nyamuragira's lava plain is a significant contributor to the degradation apart from the direction of Nyamuragira and Nyiragongo plume, ash, and gases.

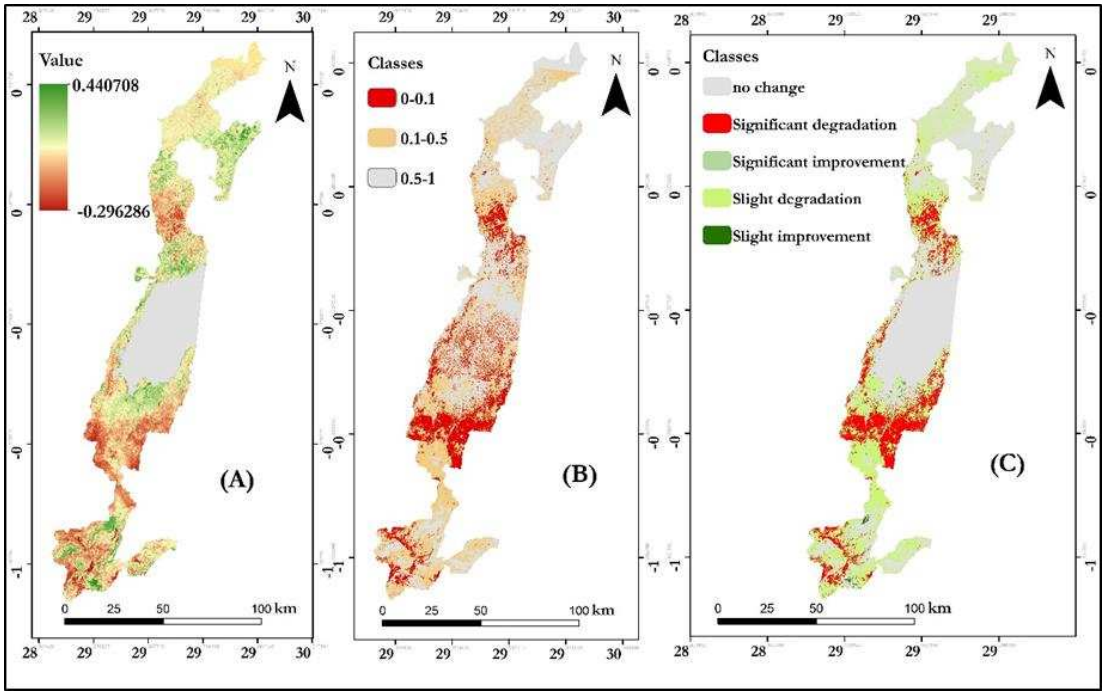

Figure 5. *a) Annual change trends in NDVI, b) Significance (P-Value) of the change trends in NDVI, c) The overall trends classified in into five types: significant improvement, slight improvement, stable or no vegetated, slight degradation and significant degradation*

NDVI - precipitation and temperature relationship

In order to isolate the effects of volcanic eruption in correlation with other environmental aspects that might disturb the state of the NDVI, it is required to find out the contribution of climate variables on the landscape (Ford et al., 2013). For this study, NDVI, precipitation and temperature relationships enabled the authors to isolate the impact of volcanic eruption on the national park's landscape, mainly in one component.

The results in *Figure 6* A indicate that the NDVI-precipitation correlation was positive in the north zone of the VNP, which suggests that the increase of rainfall was proportionally increasing with the NDVI. However, for the southern part, the correlation was negative, which reveals that the rise in precipitation does not correlate with the increase in NDVI. The main direction of volcanic plume represents the negative correlation. Notably, lower NDVI is detected for a pixel compared to the expected value for the same type of vegetation at the same elevation after experiencing rainfall (Balagizi et al., 2018).

Regarding the correlation between temperature and NDVI, the results in *Figure 6B* exhibited a positive correlation between temperature and NDVI. The increase of temperature affected the NDVI positively in the northern, central, and southern parts of the study area. The results implied that the temperature did not affect the vegetation

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DOI: http://dx.doi.org/10.15666/aeer/1905_37893803

greenness for the period between 1990 and 2018. Therefore, the decline of NDVI in the south of the park was likely attributed to two reasons including: (i) the lava flow that changed the forest zones into lava plains (*Figure 2*) and (ii) volcanic plume deposits and volcanic gases emission that diminished the vegetation greenness. The volcanic plume and gases are previously reported, are mainly oriented to the West and South-West from the location of Nyamuragira and Nyiragongo volcanoes in the southern part of our study area due to the dominant regional winds (Vaselli et al., 2010; Balagizi et al., 2018). The NDVI-Precipitation and temperature relationship results isolated the effects of volcanic eruption on the landscape fragmentation from the impact of climatic variables effects.

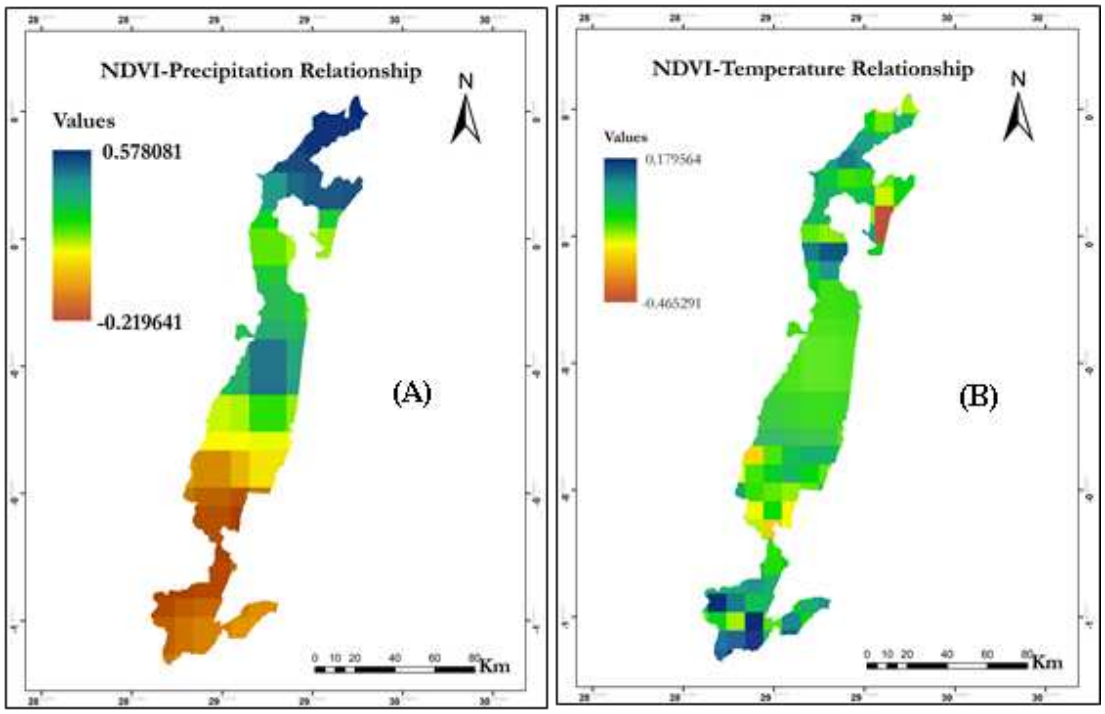

Figure 6. *NDVI-precipitation (A) and NDVI temperature Correlation (B)*

Landscape change and Chimpanzee's habitats fragmentation

Chimpanzees occupy the Tongo forest (10 Km^2) located to the north of the Nyamuragira's lava flow south of the VNP. Since the 1938-40 eruption, flank eruptions have dominated the Nyamuragira activity (Mukendi et al., 2013). Lava flows from these eruptions have gradually reduced the densely forested area. When Nyamuragira erupted, new volcanic surfaces were created by lava and replaced the habitats of chimpanzees in the Tongo forest and other prevailing wildlife along the volcano's lower slopes (*Figure 2C*). As such, the population of Chimpanzees in the Tongo forest became vulnerable to the Nyamuragira eruption events. In 1990, the number of chimpanzees in the Tongo was approximate 50, but as a consequence of habitat loss, the number had declined to 32 in 2005 (van Leeuwen et al., 2020). Based on the results of this this study (*Figure 2C*), it can be noted that the dense forest, which is habitat to chimpanzees, was destroyed by Nyamuragira eruptions.

In addition, it is reported that the lava flows built a new landform (lava plains) at the same time destroying the chimpanzee's habitats zones. The volcanic plume deposit and

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DOI: http://dx.doi.org/10.15666/aeer/1905_37893803

gas emissions affected the chimpanzee's food, as argued by Balagizi et al. (2018). During the 2010-2011 events, almost 40 chimpanzees were surrounded by lava, and the plants that they consume became coated with abrasive volcanic ash. Emissions of lava flows, toxic gases, and forest fires, resulting from volcanic eruption severely affected VNP's Chimpanzees population that had already been isolated by habitat destruction. During the period of this study, it was noticed that the chimpanzees residing along the slopes of Nyamuragira were threatened by the eruptions, especially the January 2010 eruption (Cuoco et al., 2013). In addition to the direct impact on mortality, further loss of habitat due to these eruptions reduces the chimpanzee population.

Comparison between anthropogenic activities and volcanic eruption disaster in the park

The results in *Figure 2* and *Table 1* showed that anthropogenic activities were the main challenge faced by VNP during the study's period. There was a significant change in the landscape for the northern and central parts of the park that are not affected by the volcanic eruption. For the study's period (1990-2018), the national park lost 235.16 km^2 of forest and 1.33 Km^2 of grassland that changed into cropland (*Table 1*). Also, 60% of the forest loss that changed into agriculture is located in the North and central VNP while 40% forest loss was shown in the south. The forest portion that turned into the bare area in the south was estimated at 8.36 Km^2 (*Table 1*). Only 0.05% of the forest portion changed into a bare area in the north and central part while 99.5% of forest turned into bare area in the south. During the same period, the grassland portion that the national park lost due to the volcanic eruption was 11.6 Km^2 (*Table 1*). This showed that anthropogenic activities affected the national park more than ten times than volcanic eruptions did. However, the difference is that for the southern part where the forest is destroyed by lava, it will take much time for the vegetation cover to colonize, as discussed by Liao et al. (2018). The NDVI perturbation also is permanent in the southwest because of the persistent gas emission from the volcanoes (Vaselli et al., 2010). Thus, the volcanic eruption had a significant contribution to the VNP landscape change, but it is not the main challenge facing by the park.

Conclusion

This study aimed to compare the factors conditioning the landscape change during volcanic eruption at Virunga National Park located in the East Africa. Remote Sensing and GIS were the main tools used to collect and analyze relevant data (Landsat images) and climate factors (rainfall and temperature) ranging from1990 to 2018. The results revealed that the north and central parts changed in response to human activities. The civil wars in the DRC, illegal charcoal production, expansion of cropland within the park were the key drivers. Nyiragongo and Nyamuragira's lava flows changed vegetation zones into lava plains which led to land cover forest (8 Km^2), grassland (11 Km^2), and shrubland (1 Km^2). This then, formed a new land (lava plains), then the habitat of chimpanzees was lost, and their volcanic plume and permanent gas emission were the leading causes to NDVI perturbation in the southwest region of VNP. However, precipitation and temperature did not contribute to NDVI perturbation in the southern VNP. Although volcanic eruption changed the landscape in the south, the largest part of land loss resulted from anthropogenic activities, especially in the north, central and southeast VNP. The developments were consistent with the fact that VNP lost 235.16 Km^2 of forest and

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_37893803

1.33 Km^2 of grassland that changed into cropland during the period under investigation. While during the same period the land cover loss due to volcanic eruption was only 8 Km^2 of forest and 11.6 Km^2 of grassland; Therefore, it good to sustain the conservation, improvement, and rehabilitation of the VNP. Incessant observation of land changes in the VNP is also necessary to keep updating management planning. Future research and remote-sensing based activities can utilize this study as a reference considering that it is new for the VNP. Furthermore, year by year LULUC change detection at the study area is suggested to see if human activities do not influence change on volcanic park landscape compared to natural factors.

Funding. This study was supported by the Second Tibetan Plateau Scientific Expedition and Research program (No.2019QZKK0401) and the National Key Research and Development Program of China (No.2016FYC0503306).

Data Availability Statement. The source of all the data used in this study is provided in the manuscript.

Acknowledgements. The authors express their gratitude to the United State Geological Survey (USGS) for making available satellites imageries, the University of Chinese Academy of Sciences scholarship. The authors are grateful to Xinjiang Institute of Ecology and Geography for enabling environment for research and professor Yang Zhaoping for the supervision of this work.

Competing interests. The authors declare no conflict of interests.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_37893803

FERTILIZER ADDITION INDUCES A CASCADE OF PLANT COMMUNITY RESPONSES IN A MEADOW

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(Received 13th Apr 2021; accepted 19th Jul 2021)

Abstract. Fertilizer addition can improve soil nutrients in degraded grasslands, change plant community composition, and promote community productivity. An experiment was established in 2014 near Hulunbuir Grassland Ecosystem National Field Observation Station at Hulunbuir in Inner Mongolia, China, using L14 (3^4) orthogonal test with 14 treatments totally, to compare the change of plant community productivity and plant diversity during three years of fertilization. We monitored the dynamic responses of aboveground biomass, plant species composition, species richness, Shannon diversity, Pielou's evenness after nitrogen (N), phosphorus (P), and potassium (K) addition once a year from 2014 to 2016. Compared to the control treatment of no additional fertilizer, we found that, (1) The fertilization combination of N (274 kg ha^{-1}), P (350 kg ha^{-1}), and K (57 kg ha^{-1}) had a greater effect on biomass after the third year of fertilizer addition; (2) Gramineae accounted for a major portion of biomass in 2016, whereas the proportion of other species declined or disappeared; and (3) the longer the fertilization treatments went on, the lower Shannon diversity and Pielou's evenness turned out to be. All these responses are strongly interrelated in a cascade of changes. The interrelationships between fertilizers and different plant communities attribute the change not only to climate shift but also to time.
Keywords: *nitrogen fertilizer, phosphorus fertilizer, potassium fertilizer, community biomass, species diversity*

Introduction

Arid and semi-arid lands cover nearly one-third of the terrestrial land surface and may be highly sensitive to anthropogenic nutrient inputs (Zhou et al., 2018). Nitrogen (N), phosphorus (P), and potassium (K) are macro nutrient elements constraining plant productivity in many grassland ecosystems. In recent decades, due to the high intensity of human activities, including the expansion of agriculture and fertilizer application, the content of N in the soil is changing on varying degrees (Yahdjian et al., 2011). N is

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_38053821

considered to be the second most limiting factor for plant growth in arid lands globally after water. Unlike most other nutrients, which generally are derived from the breakdown of rocks, nitrogen is derived from the atmosphere. Plants that form symbioses with nitrogen-fixing bacteria, which can acquire nitrogen from its vast atmospheric reservoir, are widespread in natural grasslands and well-managed pastures. The effects of N addition are altering plant community structure including plant productivity and biomass allocation (Gough et al., 2000; Plassmann et al., 2009; Han et al., 2011). Additionally, the nutrients such as phosphorus, potassium, and micronutrients constrain the activity of biological nitrogen fixers (Vitousek, 2015). Simultaneously, nitrogen-phosphorus co-limitation could reflect a nitrogen-induced increased in the production of nitrogen-rich phosphatase enzymes that render phosphorus more available (Pegtel et al., 1996; Houlton et al., 2008; Bai et al., 2017). The principle of grassland fertilization is not only based on the nutritional needs of the plant and the ability of providing nutrients for the soil, but should be also considered the effects and benefits of fertilization. Due to the diverse types of grassland soils in different regions, various types and concentrations of fertilizer will impact changes in grassland productivity differently. Therefore, the definition of fertilizer concentration levels are relative and there are no limits (Bai et al., 2010). Many studies focus on the effect of fertilization on biomass or nutrients of artificial grassland, but only few studies focus on the effect of biomass and species composition in natural grasslands (Blaxter et al., 2009; Xu et al., 2015). Both organic manure and chemical fertilizer applied on the soil of natural grassland can significantly increase grasses growth, but there is a difference in their effects on species composition (Eek et al., 2001; Jiang et al., 2013). A long-term location fertilization study in China indicated that a single application of nitrogen fertilizer on grassland could promote the development of grass, but was not favourable to the growth of legumes. Compared to nitrogen fertilizer, phosphorus fertilizer was more beneficial to the growth of legumes, and potassium fertilizer could promote the photosynthesis of plants and improve cold resistance and disease resistance of plants. Therefore, the balanced application of N, P, K fertilizer has positive effects on grassland yield and quality (Nie et al., 2012). Plant diversity plays an important role for grassland ecosystem functions and is a key for controlling productivity with changing climate (Hector et al., 1999; Zhang et al., 2018). When the diversity of plants is reduced, the buffering effect of the grassland will be reduced and therefore, the service function of the ecosystem is threatened. As a result, ecologists are continually concerned of the relationship between fertilization and plant diversity (Huberty et al., 1998; Richardson et al., 1999; Zeng et al., 2009). There may be positive, negative, or no significant correlations between fertilization and species richness, but thus far no definitive conclusion has been determined (Rajaniemi, 2002; Hejcman et al., 2007; Hautier et al., 2014; Leimer et al., 2015).

The Hulunbuir Meadow steppe is located in Northeast China. It is one of three world-famous grasslands, with an area of 1.08×10^4 km^2. The grassland ecosystem provides fodder for livestock by grazing or mowing. The plant productivity depends on climatic conditions such as temperature and precipitation. The decomposition rate of soil organic matter slowly is constrained mainly by low temperature, which leads to trapping of the nutrient in forms unavailable to the plant. One of the macro-nutrients N, P, and K is add to the soil in short-term, it could generally increase primary productivity and soil organic matter, but excessive fertilization may result in an imbalance of soil and plant nutrients, soil acidification, and toxic effects to plants. This leads to

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_38053821

uncertainty in the overall outcome for plant communities (Warman and Cooper, 2000; Pan et al., 2014). Therefore, the appropriate types and concentrations of fertilizers are the key factor for grassland improvement. Most studies on fertilizer for grassland pay more attention to comparing one or two types at different levels (Oomes, 1990). In order to analyze the effects of different fertilization regimes on the development of natural grassland ecosystems, we tested biomass, plant species composition, and plant diversity under various fertilizer treatments in the Hulunber *Leymus chinensis* Meadow steppe over a three years' period. This study was designed to address the following three questions: (i) How does short-term N, P, K fertilizer addition affect aboveground net primary productivity? (ii) Which is optimal option of fertilizer combination for improvement of grassland yield? (iii) How does species diversity change with optimal fertilization levels in different years?

Materials and methods

Study sites and experimental design

The fertilization experiment was conducted near Hulunbuir Grassland Ecosystem National Field Observation Station, China (49.2313N, 120.0247E). The station, which was built in 1997 by the Chinese Academy of Agricultural Sciences, is located in the core area of Hulunbuir Meadow steppe. The average altitude of this area is 650 m.a.s.l. The climate is continental semi-arid with a dry and cold winter and a rainy summer. The mean annual temperature is 0 °C. The annual precipitation is about 250-350 mm, of which about half falls between June and August. The frost-free period is 85 to 155 days. The type of soil is chestnut soil. The vegetation is dominated by *Leymus chinensis* (Trin.) Tzvel., *Vicia amoena* (Fisch.ex DC.), *Thalictrum squarrosum* (Steph.), *Pulsatilla turczaninovii* (Kryl. et Serg.), *Stipa baicalensis* (Roshev.), and *Cleistogenessquarrosa* (Trin.) Keng, and accompanied by *Carexduriuscula* (C. A. Mey.), *Allium bidentatum* (Allium L.), and *Artemisia tanacetifolia* (Linn.). Prior to 2014, the grassland was irregularly grazed by cattle and the site was mowed once a year in autumn.

In order to prevent grazing, the fertilization experiment was started at a permanent area within an enclosure in 2013, whereas the mowing regime also occurred within the enclosure. The plots had similar plant community composition and structure before fertilizing. Fourteen 6 × 10 m plots were randomly placed with an average separation distance of about 2 m in May 2014. An L14 (3^4) orthogonal test was chosen to test their effects on plant community biomass, plant species composition, species richness, Shannon diversity, and Pielou's evenness. The orthogonal test is an efficient measurement to assay the comprehensive effect of multiple factors, finding the dominant factors and the best combination of levels for them with the least experimental trials, enhancing the reproducibility of the experimental results (Montgomery, 1991). The L14 (3^4) orthogonal test for the experiment was to reduce workload, while it could arrange fourteen treatments with three factors and their four levels each, and test the interactions between factors if they exist. The local soil nutrient survey results in 2013, the total N content in 0-30 cm soil was 2.86 $g \cdot kg^{-1}$, the total P was 0.49 $g \cdot kg^{-1}$ and the total K was 22.96 $g \cdot kg^{-1}$. Comprehensive consideration the soil survey result and local traditional fertilization, selecting fourteen plots (T1 to T14) with nitrogen fertilizer (N), phosphate fertilizer (P), and potassium fertilizer (K), and four fertilization concentration levels for each fertilizer (*Table 1*). The concentrations of four N treatments were 0, 91, 183 and 274 kg ha^{-1} (hereafter denoted as N1, N2, N3 and N4, respectively), four P

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_38053821

treatments were 0, 175, 350, 525 kg ha^{-1} (hereafter denoted as P1, P2, P3, and P4, respectively), and four K treatments were 0, 28, 57, 85 kg ha^{-1} (hereafter denoted as K1, K2, K3, and K4, respectively), three replicates for each of the fourteen plots (hereafter denoted as T1-T14, respectively). The fertilizer was broadcast on the surface by hand once a year, and the date of fertilization was in early June every year.

Table 1. *L14 (3^4) orthogonal test for the combinational effect trial and fertilization amounts of Nitrogen (N), Phosphorous (P), and Potassium (K) applied*

Plot number	Treatment	Fertilization amounts of N, P, K		
		CON_2H_4 (kg ha^{-1})	$CaP_2H_4O_8$ (kg ha^{-1})	K_2SO_4 (kg ha^{-1})
T1	N0P0K0	0	0	0
T2	N0P2K2	0	350	57
T3	N1P2K2	91	350	57
T4	N2P0K2	183	0	57
T5	N2P1K2	183	175	57
T6	N2P2K2	183	350	57
T7	N2P3K2	183	525	57
T8	N2P2K0	183	350	0
T9	N2P2K1	183	350	28
T10	N2P2K3	183	350	85
T11	N3P2K2	274	350	57
T12	N1P1K2	91	175	57
T13	N1P2K1	91	350	28
T14	N2P1K1	183	175	28

The urea ($N \geq 46.4\%$), calcium superphosphate ($P_2O_5 \geq 16\%$), potassium sulfate ($K_2O \geq 51\%$) were applied for nitrogen fertilizer (N), phosphate fertilizer (P), and potassium fertilizer (K) respectively with artificial fertilization

Biomass sampling

Except for surface litter, the total biomass of all plants was harvested from 0.5 × 0.5 m quadrats in August of each year between 2014 and 2016 with three repetitions randomly selected in each plot (*Fig. 1*). Different species were separately cut to the ground level using scissors in each square area. All species were separately weighed after drying at 65 °C for 24 h in the oven. The plant community biomass was the total amount of all plants biomass in each plot (Sala et al., 1988).

Plant species composition and plant diversity

In each plot, we randomly selected three 0.5×0.5 m quadrats for the investigation of plant species composition and plant diversity. Every August was chosen to test because of the peak number of plant species biomass at this time. Plant species composition was calculated using the percentage of the species weight in the community. The richness was defined as the number of species per square area. The diversity index was calculated using the Shannon-Wiener diversity index (Shannon, 1949). Evenness of species was calculated using Pielou's evenness index (Pielou, 1966). During the three years, 62 plant species were identified within the study area, and divided into 6 plant

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DOI: http://dx.doi.org/10.15666/aeer/1905_38053821

functional groups, including 9 Poaceae, 8 Fabaceae, 4 Ranunculaceae, 1 Cyperaceae, 11 Asteraceae, and 29 miscellaneous species or forbs (*Table 2*). We have selected cumulative important values $IV \geq 5$ to be classified as, Poaceae, Fabaceae, Ranunculaceae, Cyperaceae, Asteraceae; other important values (IV) < 5 were classified as miscellaneous species or forbs. The cumulative important values (IV) of different populations were calculated and classified according to the morphology of the different plants (Bu and Jiang, 2014).

The cumulative important values (IV):

$$IV = \frac{RD + RH + RM}{3} \quad \text{(Eq.1)}$$

where: RD = relative density, RH = relative height, RM = relative dry mass.

Figure 1. *The photo of experiment site and taking sample*

Table 2. *Classification of functional group*

Plant functional groups	Plant species
Gramineae	*Leymus chinensis*
	Stipa baicalensis
	Achnatherum sibiricum
	Calamagrostis epigeios
	Poa pratensis
	Koeleria cristata
	Cleistogenes squarrosa
	Bromus inermis
	Festuca ovina
Leguminosae	*Astragalus adsurgens*
	Vicia amoena
	Thermopsis lanceolata
	Astragalus melilotoides
	Astragalus hsinbaticus
	Oxytropis myriophylla
	Melissitus ruthenica
	Melilotus dentate

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DOI: http://dx.doi.org/10.15666/aeer/1905_38053821

Ranunculaceae	*Pulsatilla turczaninovii*
	Thalictrum squarrosum
	Clematis hexapetala
	Caltha palustris
Cyperaceae	*Carex duriuscula*
Asteraceae	*Artemisia dacunculus*
	Artemisia tanacetifolia
	Scorzonera austriaca
	Serratula centauroides
	Heteropappus altaicus
	Artemisia annua
	Tanacetum parthenium
	Tripolium vulgare
	Lxerisson chifolia
	Artemisia frigida
	Saussurea amara
Miscellaneous species or forbs	*Allium bidentatum*
	Allium tenuissimum
	Lilium pumilum
	Veratrum nigrum
	Allium ledebourianum
	Allium prostratum
	Adenophora stenanthina
	Adenophora paniculata
	Viola verecumda
	Chenopodium glaucum
	Orobanche coerulescens
	Swertia pseudochinensis
	Gentianopsis barbata
	Gentiana squarrosa
	Galium verum
	Saposhnikovia divaricata
	Bupleurum chinense
	Dontostemon dentatus
	Lepidium apetalum
	Dianthus chinensis
	Linaria vulgaris
	Veronica longirolia
	Cymbariadahurica
	Iris ventricosa
	Potentilla acaulis
	Potentilla verticillaris
	Potentilla bifurca
	Potentilla nudicaulis
	Potentilla tanacetifolia

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Statistical analysis

We used one-way ANOVA to examine the main effects of fertilizer addition. Prior to analysis, we confirmed that our response variables met normality and equal variance assumptions. To test whether the impacts of fertilizer addition on community biomass, plant species composition, species richness, Shannon diversity, and Pielou's evenness differed across time, repeated analysis of variance (ANOVA) was performed with fertilizer addition as the between-subjects factors and years as the within-subject factor.

Based on the design and character of the orthogonal experiment, the effect of the nitrogen fertilizer gradient on biomass, species richness, Shannon diversity and Pielou's evenness were analyzed by using the same fertilizer gradient of phosphate fertilizer and potassium fertilizer. Nitrogen fertilizer was used as follows T1 (N0P0K0), T2 (N0P2K2), T3 (N1P2K2), T6 (N2P2K2), and T11 (N3P2K2); phosphate fertilizer was used as follows T1 (N0P0K0), T4 (N2P0K2), T5 (N2P1K2), T6 (N2P2K2), and T7 (N2P3K2); and potassium fertilizer was used as follows T1 (N0P0K0), T8 (N2P2K0), T9 (N2P2K1), T6 (N2P2K2), and T10 (N2P2K3). The average of no fertilizer (N0P0K0) and nitrogen-free fertilizer (N0P2K2) was used for the initial gradient of nitrogen fertilizer.

Data were subjected to analysis of variance (ANOVA) using SPSS 20.0 software (SPSS statistical package, Chicago, IL, United States). An alpha of 0.05 was used for statistical significance. If sources were significant, they then underwent an LSD test with significance again declared at the 0.05 level.

Results

Environmental conditions during the fertilization experiment

During the fertilization study, the average of growing-season monthly air temperature from May to August was similar in all three years, but there was a great difference in rainfall (*Fig. 2*). The average of growing-season rainfall in 2014 was 82.28 mm, 78% and 114% higher than that in 2015 and 2016 respectively. Especially in July, the rainfall in 2014 was four times more than that in 2015 and 2016, while June in 2014 was twice higher than that in 2015 and 2016 respectively.

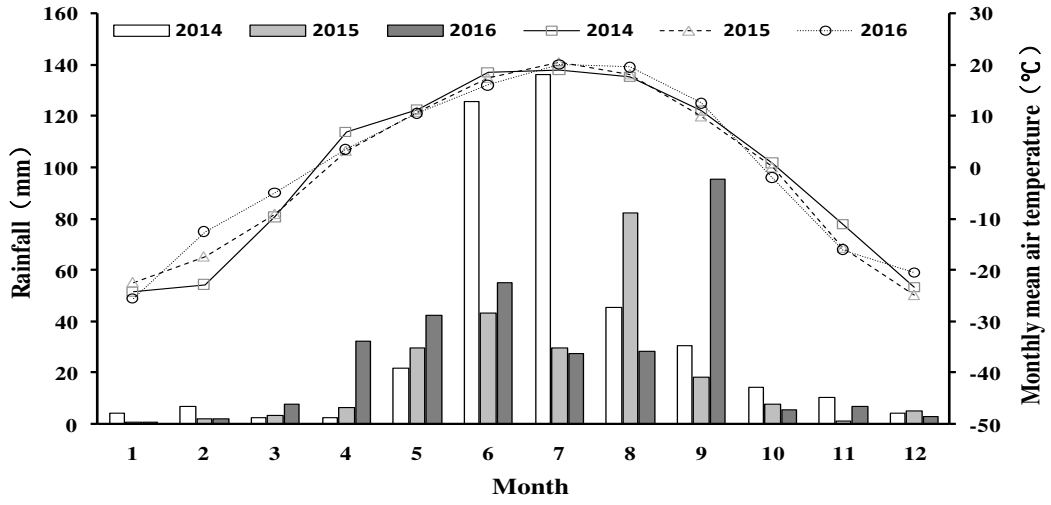

Figure 2. *Monthly mean air temperature (lines) and rainfall (bars) received during the study period from 2014-2016 at the experiment site near Hulunbuir Grassland Ecosystem National Field Observation Station*

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Plant community biomass

The effect of years and fertilizer treatments strongly influenced the total biomass of the community from 2014 to 2016 (*Fig. 3*). A positive effect of fertilizer on biomass emerged from T10 in 2014, was significantly higher than T1 (control), T4, T7, T8, and T9 ($P < 0.05$). This effect was short-lived. Except for T2, other treatments were significantly higher than T1 in 2015 ($P < 0.05$). Interestingly, a cumulative effect of the community biomass was more significant in 2016, which was the third year of fertilizer. T11 was the highest biomass in 2016 and higher than others except for T9 ($P < 0.05$). From the year difference analysis, the same treatment from 2014 to 2016 was significantly different in T1, T10, T12, and T13. There was no obvious difference between T10 and T11 in 2014 and 2015, but T11 was significantly higher than T10 in 2016 ($P < 0.05$). It indicated that T11 was the better fertilizer effect during the three years. The analysis shows that all fertilizer treatments performed better than the control (T1), so it could be concluded that fertilization plays a positive role in grassland productivity.

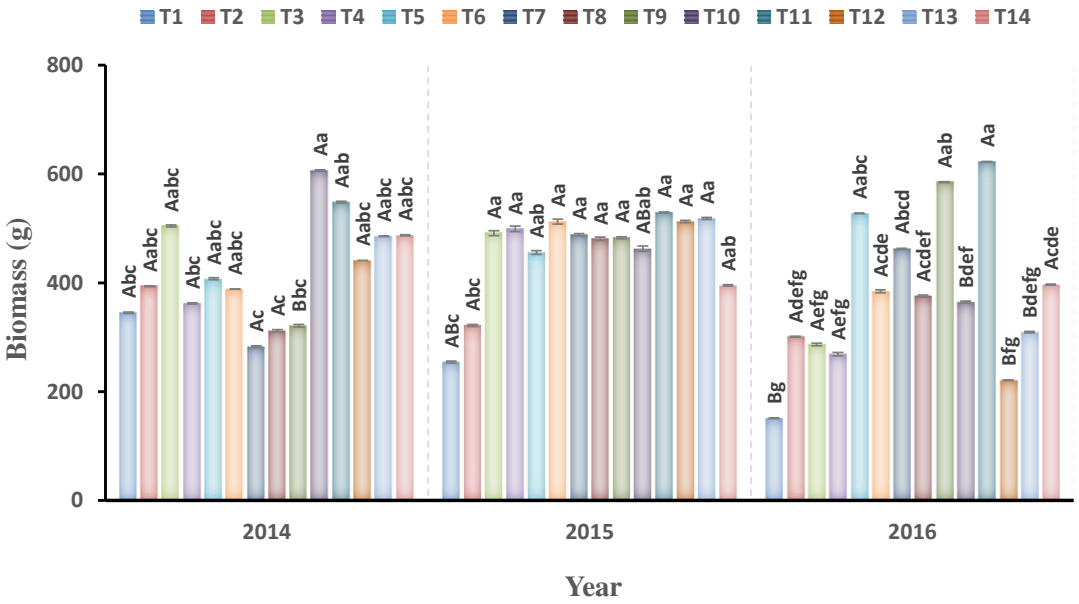

Figure 3. *Effects of different fertilization treatments (Table 1) from 2014 to 2016 on total plant community biomass. The vertical bars represent the standard errors. Different upper-case letters indicate significant difference between years in the same treatment and lower-case letters indicate significant difference between treatments in the same year according LSD test at* $P < 0.05$

The mono-fertilizer effect was analyzed and the difference in partial treatments was significant (*Fig. 4*). In 2014, which was the first year of fertilization, there was only clear effect on K fertilizer at different levels. The community biomass increased with increasing the concentration of K when N and P fertilizer was the same fertilizer level. The trends of N, P, and K were similar in 2015. Except for N0 (P2K2), the biomass of all fertilizer treatments was significantly higher than the control (N0P0K0). Significant effects on biomass at the different level of N, P and K were also observed in 2016, which was the third year of fertilization. The biomass increased with N level increasing

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and N3 the highest one ($P < 0.05$). P1 (N2K2) was the highest one, P1 (N2K2) and P3 (N2K2) were significantly higher than P0 (N2K2) and the control ($P < 0.05$). The low P concentration met the needs of plant growth for phosphorus. This phenomenon is more obvious in terms of potassium fertilizer. Low K concentration was significantly higher than other levels of K ($P < 0.05$).

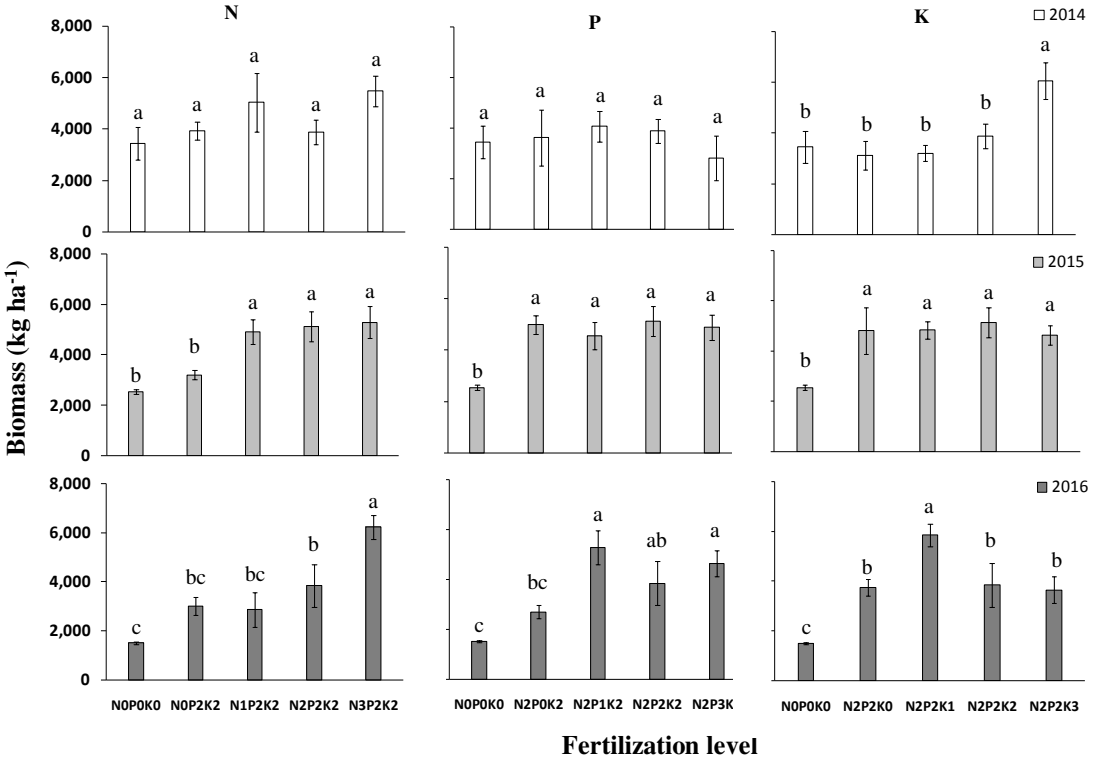

Figure 4. *Total community biomass for different N, P, K levels at August fertilization in 2014, 2015, and 2016. N0P0K0 represents no fertilizer, N0 (P2K2), N1 (P2K2) and N3 (P2K2) represent that different concentration changes of nitrogen under the same phosphorus and potassium concentration. The same mean as phosphorus and potassium. The vertical bars represent the standard errors. Different letters indicate significant difference between treatments in the same year according LSD test at P < 0.05. NS represents no significant differences*

Plant species composition

The relative biomass of the functional groups in different treatments exhibited a significant effect on Gramineae for the three years (*Table 3*). In 2014, the Gramineae achieved the highest percentage of biomass (*Fig. 5*). In 2015, the dominance of miscellaneous species or forbs was gradually promoted, and Gramineae was replaced as the second proportion of biomass and the proportion of other species also decreased to varying degrees. In 2016, the Gramineae achieved the highest percentage again and the proportion of Cyperaceae increased in different treatments compared to 2015. The proportion of miscellaneous species or forbs declined significantly in 2016, Leguminosae and Ranunculaceae even disappeared in most treatments (*Table 3*). Except for Gramineae and Cyperaceae, some species in all treatments disappeared and the highest biomass of Gramineae was still T11.

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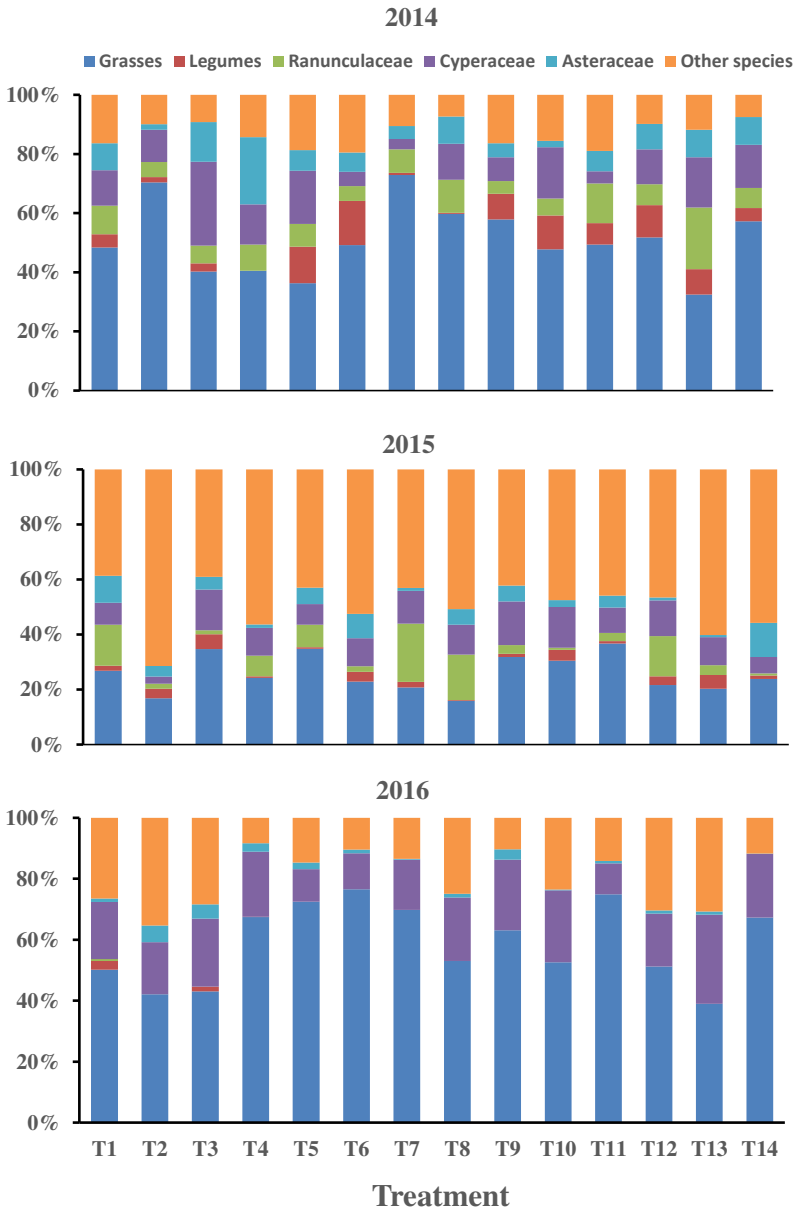

Figure 5. *Treatment (Table 1) effects on aboveground biomass of functional groups of plants in August for 2014, 2015 and 2016*

Table 3. *Results (P-value) of repeated measures ANOVA for biomass of the functional groups within fertilization treatments*

Biomass	Gramineae	Leguminosae	Ranunculaceae	Cyperaceae	Asteraceae	Miscellaneous species or forbs
Treatment (T)	< 0.001	0.495	0.894	0.692	0.958	0.991
Year (Y)	< 0.001	< 0.001	< 0.001	0.672	< 0.001	0.01
T* Y	0.001	0.784	0.99	0.504	0.839	0.998

Species richness, Shannon diversity and Pielou's evenness of plant community

Except for 2015, there were significant differences of species richness between 2014 and 2016, and T11 and T12 being the maximum in 2014 and 2016, respectively

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(*Fig. 6A*). It was also observed that T11 in 2014 was significantly higher than in 2015 and 2016 ($P < 0.05$). In 2015, T12 was significantly higher than the other two years *(Fig. 6a*). From the single fertilizer and year effect analysis, the significantly differences of N, P and K were observed (*Fig. 7*). In addition to the medium concentration of N (N2P2K2) and K (K2N2P2), other concentrations of N and K in 2014 were significantly higher than that in 2015 and 2016 ($P < 0.05$). The medium concentration of N, P, and K reached the maximum value in 2016. Similarly, a significant change in Shannon diversity during the three years was found ($P < 0.05$). The peak value of Shannon diversity was T13 in 2016 and no significant difference on T13 between 2014 and 2016 (*Fig. 6B*). The Shannon diversity of fourteen treatments in 2014 was no different or higher than that in 2015 and 2016 (*Fig. 6b*). This phenomenon was more obvious in the single fertilizer effect (*Fig. 7*). No matter which fertilizer and concentration, Shannon diversity for most treatments were higher in 2014 than that in 2015 and in 2016 ($P < 0.05$). From fertilizer concentration analysis, the control (N0P0K0) was always higher than other concentration of N, P and K during the three years. N0 (P2K2) was the lower one in 2014 and N2 (P2K2) became the lowest in 2016, while P3 (N2K2) in 2016 replaced P0 (N2K2) in 2014 to become the lowest ($P < 0.05$). For the concentration of K, the high concentration (K3N2P2) was always lowest during the three years ($P < 0.05$). In 2015 and 2016, the significant differences of Pielou's evenness between treatments appeared. T9 in 2015 and 2016 were higher than partial treatments and no significant change between the two years (*Fig. 6C*). For the trend of year effect analysis, Pielou's evenness was similar to Shannon diversity (*Fig. 6c*). No matter which fertilizer and concentration, Pielou's evenness in 2014 were no differences or significantly higher than that in 2015 and 2016 ($P < 0.05$). There was no difference in Pielou's evenness in different N concentration in 2014 and 2015, but the difference appeared in 2016 and N1 was the lowest. Pielou's evenness in different P concentration was similar to N, and the lowest value was P1 in 2015 and 2016. The commonality of different K concentrations within the three years was that high concentration (K3N2P2) was always the lowest value ($P < 0.05$).

Discussion

The plant community biomass response to fertilization

The plant community biomass has an obvious change during the three years, as well as the less biomass of some treatments in 2016 than the other two years when severe changed in rainfall. It suggests that effects of fertilizer on plant community biomass are mediated, at least in part, by changes in rainfall (Yu et al., 2015). Many previous studies observed that these divergent effects of fertilizer addition are mediated by many factors which vary in time and space, including but not limited to fertilizer rate, the traits of the species composing the community and soil moisture and climate (Yu et al., 2015; Brooks, 2003; Li et al., 2010). Although the biomass of some treatments in 2016 was lower, it could be seen that the difference in treatments in 2016 was more significant. T11 was the highest one in 2016 and it was still the best combination with the drought conditions between 2015 and 2016. Regardless of the amount of rainfall or scarcity between 2014 and 2016, the fertilizer effect continued to exists during these three years. The biomass of the fertilized plots was always higher than that of the non-fertilized one (T1). These could explain the addition of different concentrations of fertilizers applied that often led to different results in plant community biomass (Su et al., 2012; Zhou et

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al., 2018). Until 2016, our results also revealed that high concentration of nitrogen fertilizer (N3P2K2) was optimum based on the maximum biomass resulting from nitrogen fertilizer level, the low concentration of phosphorus (P1N2K2) and potassium (K1N2P2) were also optimum after the three years fertilization. These results do indicate that the growth of plants in the grassland with additional fertilizer would be greater which agrees with previous research results (Bai et al., 2010). However, there is a threshold of grassland productivity response to fertilization (Bai et al., 2010). As the amount or the period of fertilizer has increased, the amount of biomass did not increase or even decrease until it plateaued.

Plant species composition response to fertilization

Fertilizer addition has commonly been observed to change plant species composition and response of plant community members may dictate different responses to fertilizer addition (Gough et al., 2000; Hautier et al., 2014; Su et al., 2012). Nutrient limitation is a widespread phenomenon in grassland ecosystems. The responses of plant community to fertilizer addition are also contingent upon environmental conditions, especially moisture (Yu et al., 2015; Brooks, 2003; Su et al., 2012; Yahdjian et al., 2011; Zhou et al., 2018). With increasing levels of fertilizer addition, it benefits the dominance of alien annual plants and possibly promotes the invasion of new species in semi-arid regions, at the expense of natives (Brooks, 2003). In our study, the Gramineae became the dominant proportion again in 2016 and some species disappeared. This finding was consistent with previous studies (Zhou et al., 2018). This might suggest that the current dominants possess traits that allow them to benefit from, or showing effects after a period of time, while rare species do not. These responses are probably explained by a combination of differing N, P and K acquisition strategies, fertilizer-use efficiency and maximum growth rates of these different functional types (Wamelink et al., 2009).

Species richness, Shannon diversity and Pielou's evenness of plant community response to fertilization

Diversity including species richness, Shannon diversity, Pielou's evenness and other dimensions commonly lead to enhanced productivity through the mechanism of complementarity (Hejcman et al., 2007; Yahdjian et al., 2011). Species losses and declines in species richness per unit area are commonly induced by fertilizer addition (Su et al., 2012; Clark et al., 2013). In our study, compared to the control, the richness of partial fertilizer treatments decreased significantly, but the highest value of richness appeared in T12 in 2016 (*Fig. 6a*). Regardless of N, P and K, the higher values were always in the fertilizer treatments in 2015 and 2016 (*Fig. 7*). However, there is a significant decline in the value of partial fertilization treatment compared to control. The reasons for the difference from other studies may be that P and K were applied together with N in this experiment but not as a sole treatment like in many other studies. Another reason was that our experiment was short-time and more time was required to fully demonstrate plant community effect. In contrast, the lowest value of Shannon diversity occurred in fertilized treatments, while the values with unfertilized treatment were always higher than some fertilized treatments. As the fertilizer year increased, the values of the same treatment in 2016 were all lower than that in 2014. This is a good illustration of the theory that the longer the fertilization treatments lasted, the lower Shannon diversity became. This phenomenon was also obvious in Pielou's evenness.

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These results do correspond with those obtained in several published studies and document a decreased in species diversity with fertilizer effect (Bobbink and Willems, 1993; Kahmen et al., 2002; Moog et al., 2002).

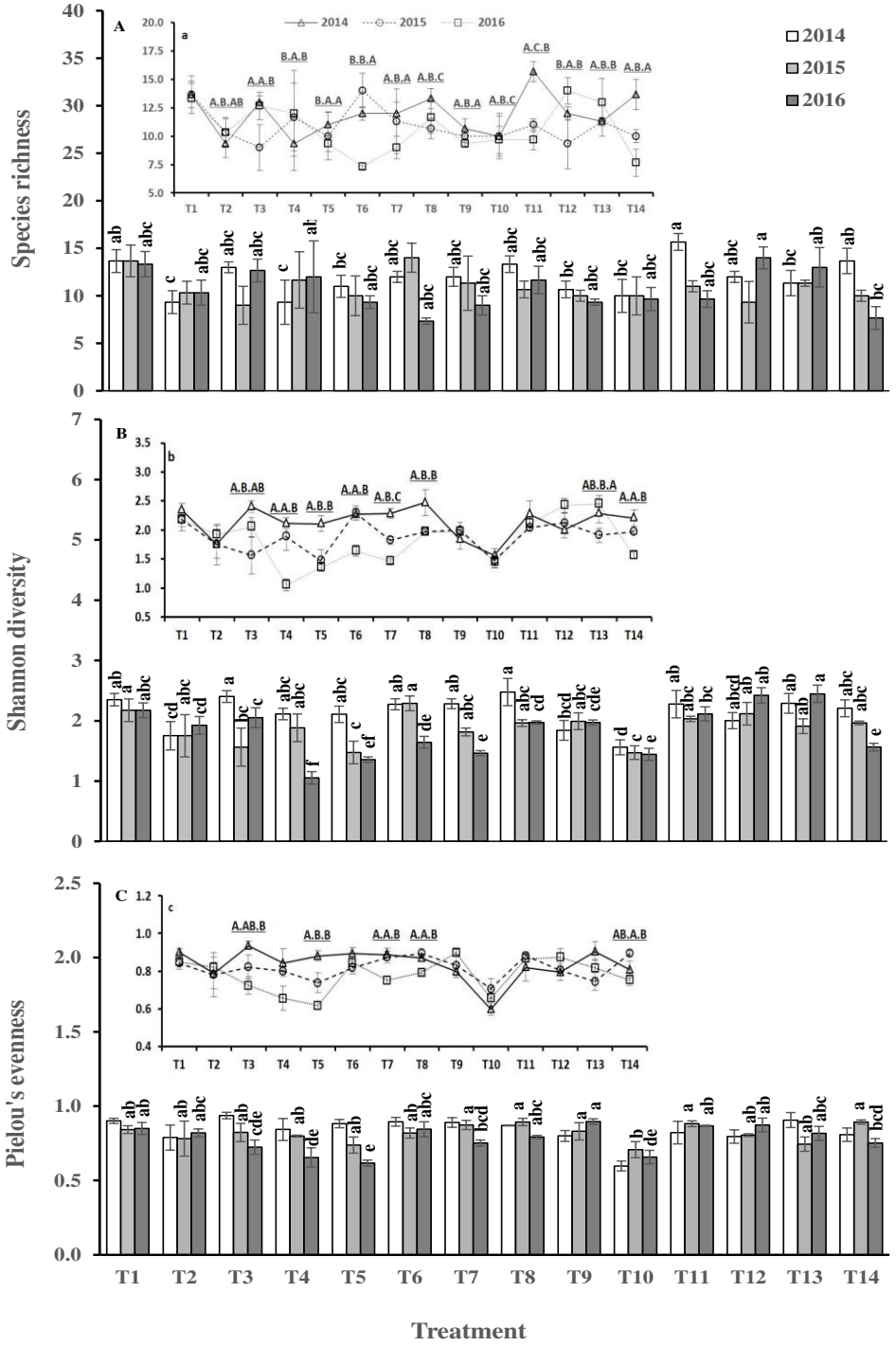

Figure 6. *Treatment (Table 1) and year (2014, 2015 and 2016) effects on species richness, Shannon diversity and Pielou's evenness of plant community in August. Figure (A, B, C) represent treatment effects and different lower-case letters indicate significant difference in treatments (bars). Figure (a, b, c) represent year effects and different upper-case letters indicate significant difference in years (lines) according LSD test at P<0.05. The vertical bars represent the standard errors*

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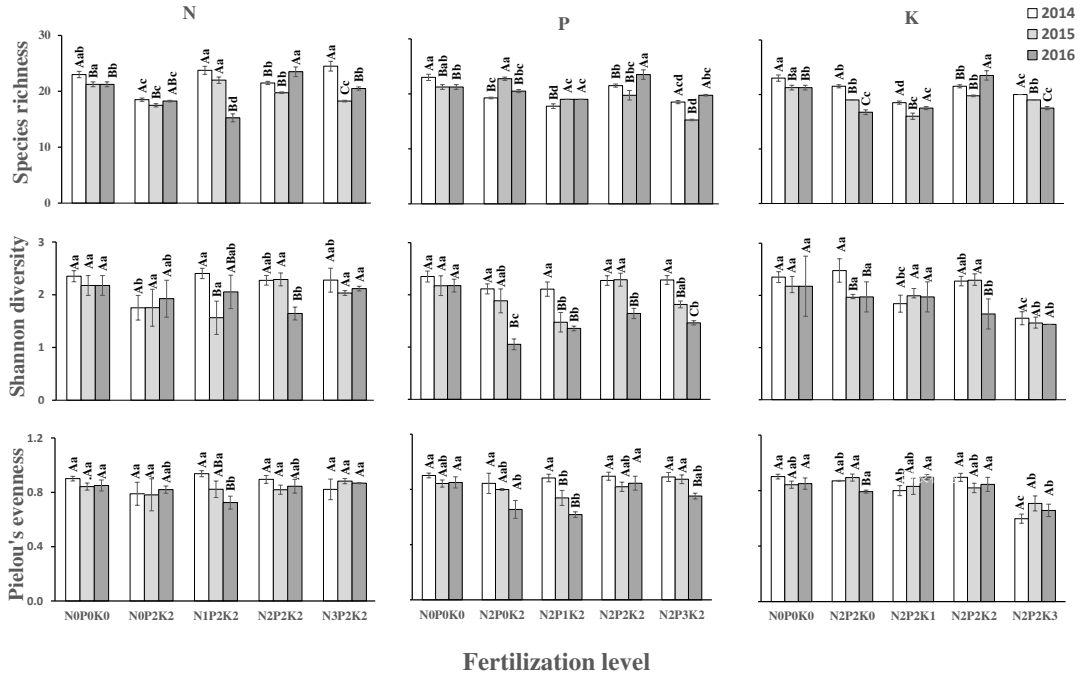

Figure 7. *Species richness, Shannon diversity and Pielou's evenness for four different fertilization levels by year of August fertilization (2014, 2015, and 2016). N0P0K0 represents no fertilizer, N0 (P2K2), N1 (P2K2) and N3 (P2K2) represent that different concentration changes of nitrogen under the same phosphorus and potassium concentration. The same mean as phosphorus and potassium. The vertical bars represent the standard errors. Different letters indicate significant difference between treatments in the same year according LSD test at P<0.05*

Conclusion

Based on the result, we recommend the combination of N (274 kg ha^{-1}), P (350 kg ha^{-1}), and K (57 kg ha^{-1}), and annually to maximize plant community productivity from the semi-arid grassland in Hulunbuir Inner Mongolia, China. Gramineae accounted for the major proportion of biomass in the third year of fertilization, whereas the proportion of other species declined or disappeared. The longer the fertilization treatments went on, the lower Shannon diversity and Pielou's evenness turned out to be. All these responses are strongly interrelated in a cascade of changes. We further establish that effects on plant community biomass are additively influenced by the dose of fertilizer applied and duration of application. In our study, the effects of the year were consequently significant on many indicators. It could indicate that some other factor could have influence on the plant community indicators. Therefore, we conclude that water control will be carried out the original design, to simulate the impact of different precipitation on the experimental results of this study in the future.

Acknowledgements. The authors gratefully acknowledge the technical support of the staff of the Hulunbuir Grassland Ecosystem National Field Observation Station. Special thanks go to Jingzhong Dai and Jing Yao for support during the data collection and regular management of the experimental plots. The authors also thank Shijie Lv (Inner Mongolia Agricultural University) for valuable input to the manuscript. This work was supported by China National Public Welfare Industry (Agriculture) Research Project [grant number 2013060] and China National Key Research and Development Program Project [grant number 2016YFC0500603].

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DOI: http://dx.doi.org/10.15666/aeer/1905_38053821

ASSESSMENT OF THE GENETIC DIVERSITY BETWEEN TWO VARIETIES OF WONDERBOOM (*FICUS SALICIFOLIA* VAHL.) BY USING SIMPLE SEQUENCE REPEAT (SSR) MARKERS

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(Received 13th Apr 2021; accepted 12th Aug 2021)

Abstract. This study aims to determine the genetic diversity of *Ficus salicifolia* varieties collected from (Tamanrasset) Southern Algeria. Currently, little is known about the genetic variability between two varieties "Teloukat" and "Eucalyptoïdes". Thirty-nine fig trees were examined by using six simple sequences repeat (SSR) markers. In total 64 alleles were observed in the samples of two varieties. The number of alleles per locus ranged from seven to eighteen, with an average of 10.66 alleles per locus. The mean values of heterozygosities observed and expected were 0.61 and 0.74 respectively. The positives Fis (Wright inbreeding coefficient) value obtained with (MFC2, MFC8, LMFC26, LMFC30 and FSYCO1) loci indicated deficiency of heterozygote. Considering the PIC (polymorphic information content) results, 5 markers (MFC2, MFC8, LMFC26, LMFC30 and FSYCO1) were classified as highly informative (PIC > 0.5). The phylogenetic diagram performed using the Unweighted Pair Grouping Method with Arithmetic average (UPGMA) showed significant intraspecific polymorphism between the two varieties which was confirmed by Factorial Correspondence Analysis (FCA).
Keywords: *Ficus salicifolia, Tamanrasset, SSR markers, heterozygote, UPGMA, intraspecific polymorphism*

Introduction

Ficus (figs) is a genus which includes economically important fruit species (*Ficus carica*), present worldwide, with 750 species in total from Ficus genus occurring throughout tropical and subtropical regions, almost of which are evergreen trees, shrubs or lianas producing the latex and enclosed inflorescences (Berg, 1989).

In Southern Algeria (Tamanrasset), the Ficus genus is commonly represented by *Ficus salicifolia* Vahl., an endemic species of central Sahara (Ozenda, 1977), which is highly fragmented and rarely exceeds 30 individuals making them locally endangered. It develops intermediate growth forms between shrubs and tree, with small (10 to 20 mm diameter) ripe dark red figs which are edible for humans and animals, two varieties are cultivated "variety Teloukat" and "variety Eucalyptoïdes" (Battandier and Trabut, 1912). Both grow at high altitudes (1400 to 2700 m) along Rockies flaws ravines under arid conditions (temperature from 16 to 30 °C, rainfall less than 50 mm per year).

Several homonyms and synonyms of these trees are used by aboriginals, which make their characterization very difficult, because there is not yet real recognition or information on their ecological or genetic divergence (Sahki and Sahki, 2004). Currently, little is known about genetic diversity of *Ficus salicifolia* varieties except

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_38233835

morphological characters description which are still unreliable for the unambiguously identification of two varieties (Teloukat and Eucalyptoïdes). In addition, variation in phenotypic characters does not necessarily reflect the genetic diversity of the population (Bagavathiannan et al., 2010). Further, most morphological traits are influenced by environmental factors (Teoman et al., 2017). In this investigation, we refer to groups of trees in natural habitats to assess their genetic diversity.

Since, objectivity is essential for the identification; morphological traits can be unreliable for genetic plant identification and characterization (Ercisli et al., 2008). To preserve and improve existing plant genetic resources under harsh conditions, it will be helpful to evaluate genetic diversity and molecular markers permits a more rapid and reliable approach, so, they offer numerous advantages over conventional alternatives based on morphological traits; these markers are stable and detectable in all plant tissues, regardless of environmental conditions (Leal et al., 2010; Do Val et al., 2013).

Previous studies have showed that DNA marker technologies provided an effective tool for the conservation of fig genetic resources, such as random amplified polymeric DNA (RAPD) (Dalkilic et al., 2011; Baziar et al., 2018), restriction fragment length polymorphism (RFLP) (Khadari et al., 2005), amplified fragment length polymorphism (AFLP) (Baraket et al., 2011) and single sequence repeat (SSR) (Ferrara et al., 2016; Fu et al., 2017). To study the genetic diversity, the SSR markers were used regarding their highly polymorphic, co-dominant and abundance in the genome (Fu et al., 2017; Hladnik et al., 2018). Microsatellite markers have been widely used for the analysis of genetic diversity and they play an essential role in all aspects of plant selection; as: apple (*Malus domestica)* (Pikunova et al., 2018); apricot (*Prunus armeniaca*) (Köse et al., 2017); peach (*Prunus persica*) (Licea-Moreno et al., 2019); olive (*Olea europaea ssp europaea*) (Ben Mohamed et al., 2017) and (*Olea europaea* ssp *laperrinei*) (Baali-Cherif and Besnard, 2005). SSR markers are also widely used for the evaluation of the diversity studies and characterization of Fig species trees (Khadari et al., 2004; Essid et al., 2015; Boudchicha et al., 2018).

The apparent ambiguity in the taxonomic classification of two varieties not distinct morphologically (Teloukat and Eucalyptoïdes), directed us to the use of molecular markers; therefore, the aim of this present study is to determine the genetic diversity within individuals of *Ficus salicifolia* enabling to establish the relationship between the two varieties. On the other hand, to define a conservation strategy, the genetic diversity of this species has to be investigated.

Materials and methods

Plant material

Leaves samples of thirty-nine individuals of *Ficus salicifolia* (*Fig. 1*) of both varieties: (Eucalyptoïdes and Telloukat) were collected randomly from a natural habitat of six zones (*Table 1; Appendix 1; Fig. 2*) located in Ahaggar (Tamanrasset: 22°57’42” north, 5°11’51” east) in the central Sahara of Southern Algeria.

Molecular markers

DNA extraction

Total genomic DNA was extracted as described by Khadari et al. (2004) using 200 mg of leaves dried in silica gel, according to the DNeasy Plant Mini Kit (QIAGEN,

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Courtaboeuf, France) optimized by adding 1% polyvinylpyrrolidone (PVP 40.000) to the buffer AP1.

DNA was quantified using spectrometric method (Sambrook et al., 1989): the absorbance was recorded at 260 nm wavelength (Spectrophotometer UV mini-1240, Shimadzu Japan). The relative purity of extracted DNA was estimated after electrophoresis with 0.7% agarose gel stained by 0.2 $\mu g.l^{-1}$ Ethidium Bromide and immersed in 90 mM Tris-Borate, pH 8 and 10 mM Ethyldiamine Tetra-acetic Acid (EDTA), the DNA samples were visualized under ultraviolet light**.**

SSR markers

The 6 SSR markers were selected due to their polymorphism level, the high quality of amplification reproducibility and transferability, since five SSR markers were developed for a common fig (*Ficus carica* L): LMFC26, LMFC30 (Giraldo et al., 2005), MFC2 (Khadari et al., 2001) and MFC8, MFC12 (Achtak et al., 2009), and a marker FSYCO1 was developed for *Ficus sycomorus* (Ahmed et al., 2007). The details of each marker are shown in *Table 2*.

Figure 1. *Morphology of Ficus salicifolia in arid zones (Tamanrasset). A: External morphology of the tree. B: morphological appearance of some vegetative (leaves, shoots) and reproductive (fruits) parts*

Table 1. *List of individual fig trees with GPS data*

Local name of collection zones	Individuals number	Code of trees	Altitude (m)	Latitude/longitude
TIT	10	from Fs01 to Fs10 Variety Teloukat	From 1081 to 1088	22°57’46”/05°09’76”
IH-AGHI	05	from Fs11 to Fs15 Variety Teloukat	From 1093 to 1095	23°04’10”/05°12’85”
IN-HOUTER	01	Fs16 Variety Eucalyptoides	1455	23°04’73”/05°22’85”
TADADINE	03	from Fs17 to Fs19 Variety Eucalyptoides	From 1315 to 1350	22°49’42”/05°57’33”
IN-ZEBIB	18	from Fs20 to Fs37 Variety Teloukat and Variety Eucalyptoides	From 1425 to 1459	22°48’36”/05°37’49”
AMEZEDJINE	02	from Fs38 to Fs39 Variety Teloukat	From 1162 to 1165	22°36’96”/05°24’46”

Fs, Ficus salicifolia

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_38233835

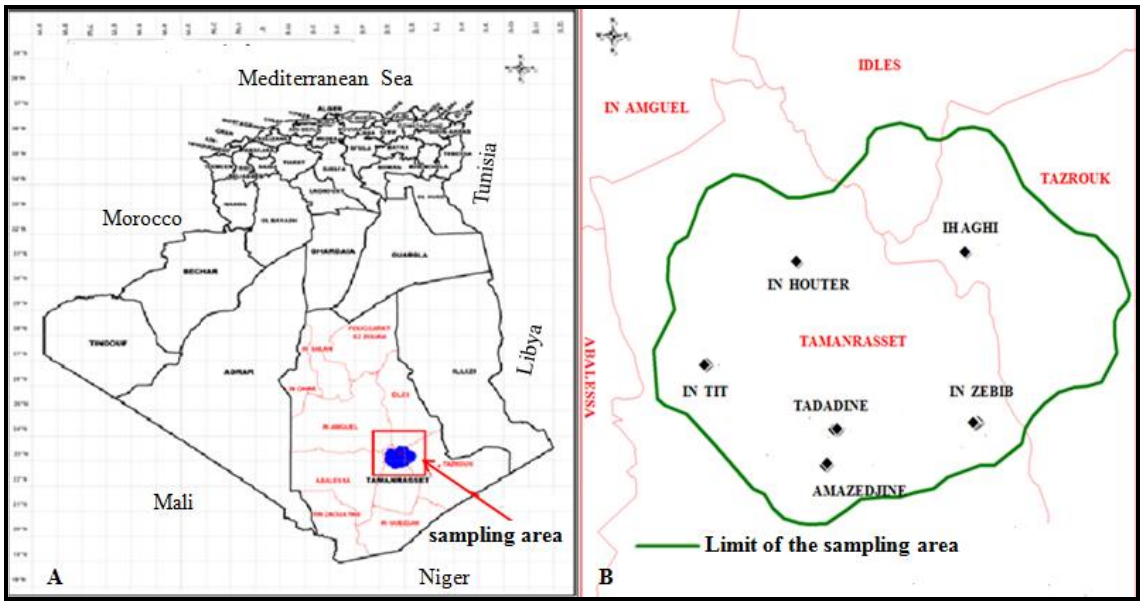

Figure 2. *Map showing location of sampling areas, in Tamanrasset (southern Algeria). A: Algeria within north Africa on a globe (www.Alamy.com). B: Stations of collection*

Table 2. *Fig SSR primer sequences used*

Microsatellite primers	Repeat motif	Primer sequences 5' to 3' (F) (Forward)	Primer sequences 5' to 3' (R) (Reverse)	Tm (°C)
MFC2	$(AC)_{18}(AT)_7$	GCTTCCGATGCTGCTCTTA	TCGGAGACTTTTGTTCAAT	55
FSYCO1	$(A)_{16}(GAAA)_8$	CAAATGAAAAACACAAATTTGCCAA	TGCAAGTACTAATTCCTCTGCCGTG	55
LMFC26	$(TC)_{11}$	ATGTTATAGTTGAGTGAGGATAA	AAATAGTGGATCTTGCATGT	55
MFC8	$(CA)_9TA(CA)_{14}(TA)_6$	GTGGCGTCGTCTCTAATAAT	TATTCTATGCTGTCTTATGTCA	50
MFC12	/	TATCACGGCGGTCTAACTCTGC	CTCCTCATCCCCCTCCCAACT	50
LMFC30	$(CT)_{14}$	TTGTCCGTTTCTTATACAAT	TCTTTTTAGGCAGATGTTAG	55

PCR amplification and genotyping procedure

Amplification was carried out using the PCR conditions of fig studies (Khadari et al., 2004; Achtak et al., 2009) by conducting polymerase chain reaction (PCR) amplification in a total volume of 20 µL containing PCR buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1% Triton X-100 (Perkin Elmer, Milan, Italy) and 0.02% gelatin], 20 ng of fig genomic DNA, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 1 to 4 pmol for dye-labeled primers [fluorescent phosphoramidites FAM, HEX (MWG Biotech, Courtaboeuf, France) or NED (Applied Biosystems, Courtaboeuf, France) at the 5' position], and 2 to 8 pmol for unlabeled, 1 U of Taq DNA polymerase (Sigma-Aldrich, Lyon, France).

Amplification reactions were performed in a thermocycler (MJ Research, Watham, MA). The PCR conditions were as follows: after 5 min denaturation at 94 °C, 35 cycles were performed with 30 s denaturation at 94 °C; 1 min annealing at 55 °C; 1 min elongation at 72 °C; and a final extension step of 7 min elongation at 72 °C. PCR products (2 µl) was mixed with 7.9 formamide and 0.1 µl Gensize 400HD (Rox Size Standard; Applied Biosystems) and were separated using capillary electrophoresis on an ABI prism 3130 XL automatic DNA sequencer (Applied Biosystems).

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DOI: http://dx.doi.org/10.15666/aeer/1905_38233835

Electopherograms were then analyzed with the Gene Mapper 3.7 software (Applied Biosystems). Allele peak profiles were detected at each locus and genotype, manually reviewed and final sizes were rounded to the nearest full number representing the final called allele length.

Data analysis

For each SSR locus, alleles were detected and identified by allele size in base pair (bp) and the number of alleles per locus (A). Different indices of genetic diversity were estimated for each locus: observed heterozygosity (Ho), expected heterozygosity (He), and the Wright inbreeding coefficient (Fis) were computed using the software Genetix 4.5 (Weir and Cockerham, 1984; Belkhir et al., 2004). Polymorphic Information Content (PIC) was calculated using CERVUS 3.03 (Marshall, 1998).

Individuals in relationships were represented by dendrogram constructed from Nei's standard genetic distances (Tamura and Nei, 1993); this dendrogram was generated using Unweighted Pair-Group Method using Arithmetic average (UPGMA) (Saitou and Nei, 1987) and was constructed using the software Clustering Calculator (Brzustowski, 2002). The Factorial Correspondence Analysis (FCA) was also recorded on individual plant using Darwin 3.6 software (Perrier et al., 2003).

Results

Simple sequence repeat polymorphism

A total of 64 alleles were amplified and the number of alleles per marker ranged from five to eighteen with an average of 10.66 alleles and amplification fragment sizes between 135 and 294 nucleotides (*Table 3*).

Table 3. *Locus name and genetic parameters of the South-Algerian fig trees*

Locus	N	Size range	Ho	He	Fis	PIC
MFC2	10	140-166	0.59	0.76	0.171	0.74
FSYCO1	13	135-166	0.79	0.85	0.052	0.84
LMFC26	6	217-242	0.36	0.62	0.272	0.58
MFC8	18	147-206	0.47	0.88	0.433	0.88
MFC12	5	151-174	0.82	0.54	-0.538	0.45
LMFC30	12	264-294	0.65	0.75	0.125	0.72
Mean	10.66	/	0.61	0.73	0.085	0.70

N, Number of alleles; Ho, Observed heterozygosity; He, Expected heterozygosity; Fis, Wright inbreeding coefficient; PIC, Polymorphic information content

The highest number of alleles (18) was detected at the MFC8, that corresponds with the highest values of the genetic diversity parameters (He = 0.88, Fis = 0.43, PIC = 0.88), whereas the lowest number (5 alleles) was obtained for the MFC12.

The values for observed (Ho) and expected (He) heterozygosities ranged from 0.36 to 0.82, and from 0.54 to 0.88 respectively, the average genetic diversity (He = 0.74) was higher than (Ho = 0.61); according to the Hardy-Weinberg (HW) equilibrium, heterozygote excess (Ho > He) was observed for the MFC12 locus, whereas a

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DOI: http://dx.doi.org/10.15666/aeer/1905_38233835

deficiency of heterozygote (Ho < He) was observed in MFC8, MFC2, LMFC26, LMFC30 and FSYCO1 loci.

The wright inbreeding coefficient (Fis) values, could be explained by heterozygous deficiency due to positive values of MFC8, MFC2, LMFC26, LMFC30 and FSYCO1 loci, and negative value of MFC12 locus (-0.538), indicating heterozygote excess (*Table 3*).

The average value of PIC (polymorphic information content) for the primer sets was 0.70, ranging from 0.45 for MFC12 to 0.88 for MFC8. Considering the PIC results, 5 markers (MFC2, MFC8, LMFC26, LMFC30 and FSYCO1) showed PIC values greater than 0.5, therefore, these primers were classified as highly informative and have power for analyzing the genetic variability of *Ficus salicifolia.*

Genetic relationship between two varieties of Ficus salicifolia

To represent the relationships between two varieties of *Ficus salicifolia*, a cluster analysis was used to plot a dendrogram based on Nei's genetic distance (*Fig. 3*): five main clusters are evident.

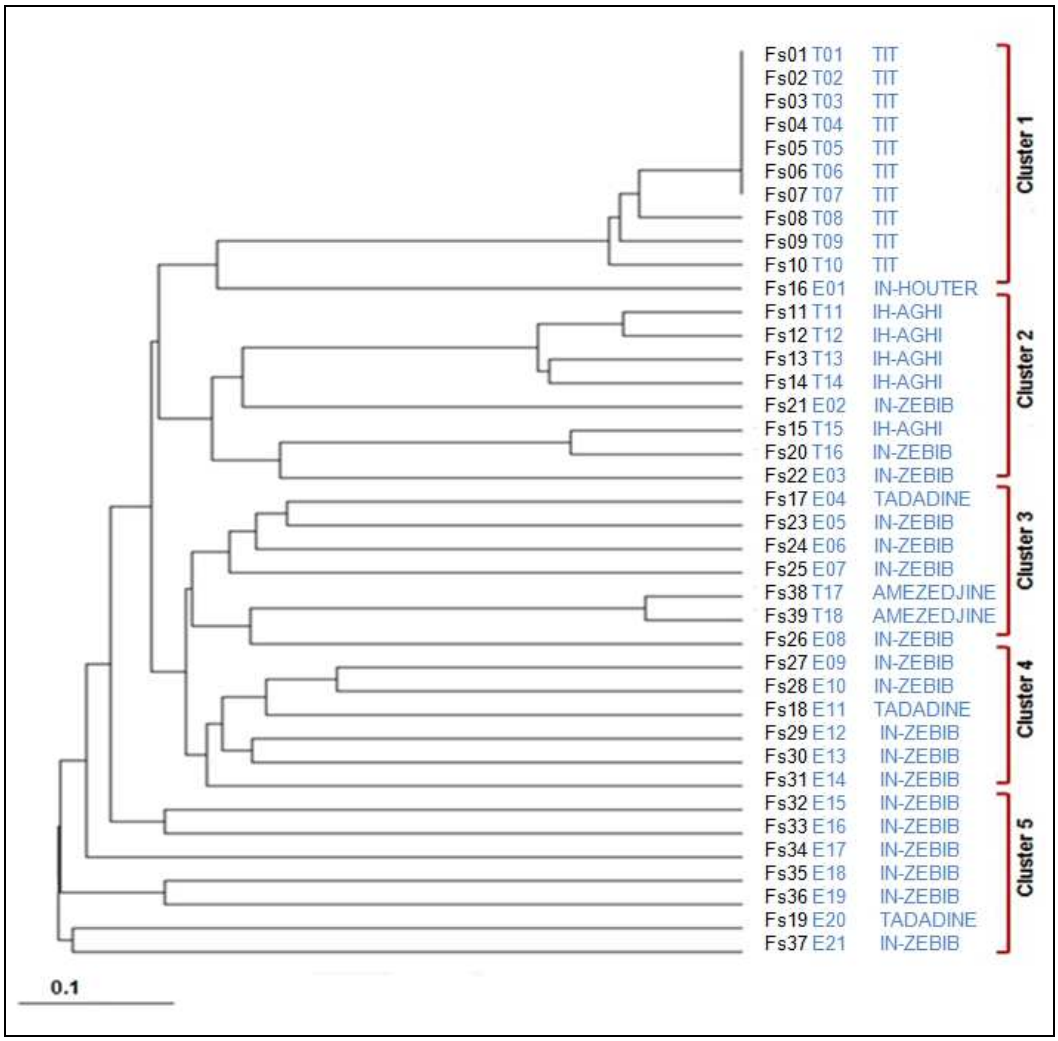

Figure 3. *UPGMA (unweighted pair-group method by arithmetic average) dendrogram based on Nei's genetic distance obtained from analysis of simple sequence repeats data for 39 south Algerian fig trees. Fs, Ficus salicifolia; T, Teloukat; E, Eucalyptoides*

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The first cluster grouped all individuals of *Ficus salicifolia,* variety Teloukat located at Tit (Fs01, Fs02, Fs03, Fs04, Fs05, Fs06, Fs07, Fs08, Fs09, Fs10), only one individual of the variety Eucalyptoïdes (Fs16), located at In-Houter. The trees Fs01 to Fs07 have the same genotype, possibly linked to vegetative propagation.

The second cluster consisting of all individuals of the variety Teloukat located at Ih-Aghi (Fs11, Fs12, Fs13, Fs14, Fs15) and at In-Zebib (Fs20), with 2 individuals of the variety Eucalyptoïdes at In-Zebib (Fs21, Fs22).

The third cluster included the variety Eucalyptoïdes from Tadadine (Fs17) and from In-Zebib (Fs23, Fs24, Fs25, Fs26), and then the variety Teloukat from Amezedjine (Fs38, Fs39).

The fourth cluster formed by all individuals of the variety Eucalyptoïdes located at In- Zebib (Fs27, Fs28, Fs29, Fs30, Fs31) and (Fs18) at Tadadine.

The fifth cluster containing all individuals of the variety Eucalyptoïdes located at In Zebib (Fs32, Fs33, Fs34, Fs35, Fs36, Fs37) and (Fs19) at Tadadine.

Figure 3 showed that the variety Teloukat was represented by short branches of the dendrogram, but the variety Eucalyptoïdes existed in the long branches.

To elucidate genetic relationships among the 39 fig genotypes, the Factorial Correspondence Analysis (FCA) confirmed information derived from the UPGMA clustering, so, the 2 dimensional scatter plot of factorial correspondence analysis coordinates for the first and second axis, showed a clear separation of the 2 varieties of *Ficus salicifolia* (Teloukat and Eucalyptoïdes) (*Fig. 4*); however the variety Tellouket was represented by two groups, probably related to the geographical origin of the genotypes.

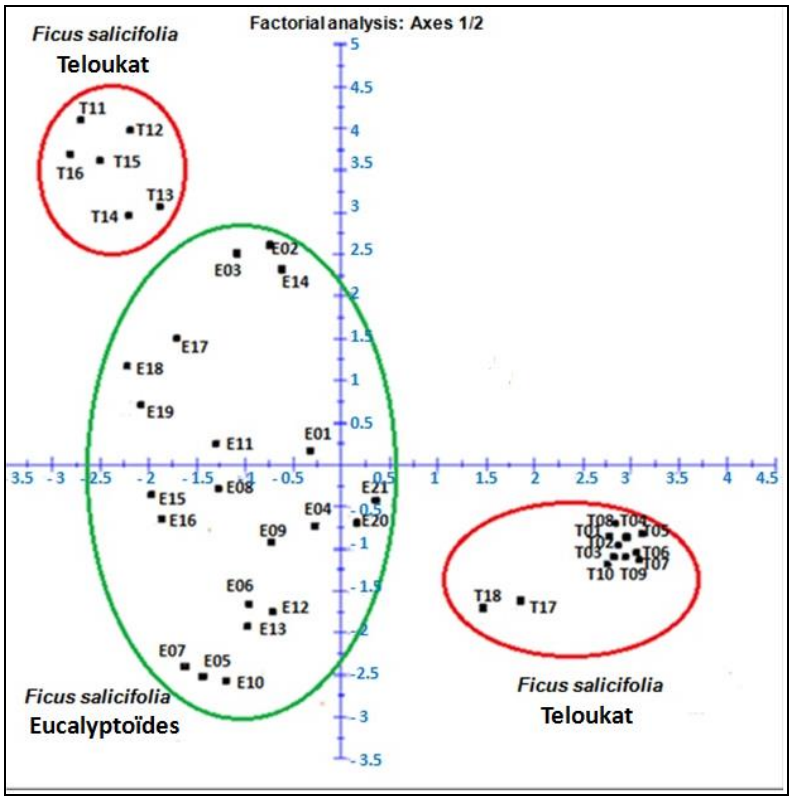

Figure 4. *Factorial coordinate analysis (FCA), obtained by SSR markers of 39 south Algerian Fig trees. T, Teloukat; E, Eucalyptoides*

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Discussion

The microsatellite markers were developed from different species of Ficus: *Ficus carica* (Khadari et al., 2001; Giraldo et al., 2005), *Ficus inspida* (Vignes et al., 2006), *Ficus sycomorus* (Ahmed et al., 2007), *Ficus hirta* (Zheng et al., 2015), *Ficus tikoua* (Zhang et al., 2016) and *Ficus virens* (Fu et al., 2017).

The high transferability of microsatellite markers developed from different subgenus of Ficus for *Ficus citrifolia* and *Ficus eximia* confirm the general applicability of Ficus microsatellite primers to this very large genus (Nazareno et al., 2009). Recently, Ikten et al. (2018), indicated a high level of transferability between *Ficus carica* and related Ficus species which provides growing number of SSR for *Ficus carica*; this would have important benefit for species with genomic studies are limited. Moreover, Hladnik et al. (2018) have studied the diversity parameters of selected microsatellites loci for use cultivar identification and fig genetic resources investigations.

In this study, we have made an attempt to use the utility of SSR markers in studying genetic diversity of species of figs, the performance of these markers was evaluated using various parameters such as observed heterozygosity (Ho), expected heterozygosity (He), Wright inbreeding coefficient (Fis) and Polymorphic information content (PIC).

The SSR loci applied in our analysis detected an average of 10.66 alleles, per locus in 39 individuals fig (*Ficus salicifolia*), which was higher than that found in SSR developed from the *Ficus citrifolia* 7.3 alleles per locus and *Ficus eximia* 6.4 alleles per locus (Nazareno et al., 2009), than from the *Ficus carica*: 5 alleles per locus (Achtak et al., 2009), 4.89 alleles per locus (Ferrara et al., 2016) and 4.2 alleles per locus (Leal et al., 2010). Giraldo et al. (2008) observed a low polymorphism within an ex situ Spanish figs collection, with 3.9 alleles per SSR locus; similarly, Teoman et al. (2017) also found 3.56 alleles per SSR locus within Turkish male and female fig genotypes.

Recently, Boudchicha et al. (2018) published a value of 3.59 alleles per locus within Algerian fig cultivars (*Ficus carica*). The variation in the number of alleles in fig species might be related to the difference in the SSR markers studied, as well as the number of samples and their geographic location (Lopes et al., 2004).

The 6 microsatellites loci used in this work were effective for the characterization of south Algerian fig species with high genetic diversity Ho = 0.61. Achtak et al. (2009) reported that the average Ho value was 0.54 for the characterization of Moroccan cultivars figs; however Tunisian germplasm presents a high genetic diversity with Ho = 0.62 (Ben Abdelkrim et al., 2015). Heterozygosity values of *Ficus citrifolia* and *Ficus eximia* reported by Nazareno et al. (2009) were respectively 0.67 and 0.69.

Moreover, the analysis of European and Asian fig cultivars showed a lower level of diversity with Ho = 0.44 (Ikegami et al., 2009), similar results were obtained to characterize a collection of Turkish male and female fig genotypes with Ho = 0.45 (Teoman et al., 2017) and Algerian fig cultivars (*Ficus carica*) with Ho = 0.46 (Boudchicha et al., 2018), similarly, Giraldo et al. (2008) also determined low polymorphism within an ex situ Spanish collection figs with Ho = 0.41.

According to the Hardy-Weinberg equilibrium (HWE), heterozygote excess (Ho > He) was observed for the MFC12 locus, and a deficiency of heterozygote (Ho < He) was observed in MFC2, MFC8, LMFC26, LMFC30 and FSYCO1 loci.

The average of polymorphism information content (PIC = 0.70) is relatively the same compared with that of previous studies (Ikegami et al., 2009; Teoman et al., 2017).

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Fis values which is a measure of the deviation of genotypic frequencies from panmixia in population in term of heterozygous deficiency or excess, showed that loss of heterozygosity at just one microsatellite (MFC12). Fixation index values give an idea in terms of the inbreeding coefficient and individual differences.

This finding shows that most of the genetic diversity is caused by the difference between the two varieties of *Ficus salicifolia* as leaf morphology for instance: (the variety Teloukat: the leaves are oblong lanceolate, more or less strung at the base; the variety Eucalyptoides: the leaves are narrowly lanceolate, rounded or sub-attenuated at the base). According to our results, the SSR markers genotyping were in line with the phenotypic identification. The two groups of this variety observed might be linked to the geographical origin of the genotypes (Essid et al., 2015). However, for some groups, the individuals of the same variety were not well separated, probably due to the pollination of this species which is related to the pollinating insect (Jousselin et al., 2003), or to technical errors of the method of analysis used (absence of specific markers).

In addition, in natural habitats, *Ficus salicifolia* has substantially regressed with effects of climate changes; therefore, long term persistence of this taxon requires urgent preservation.

The microsatellite markers used in this study are highly polymorphic and efficient in revealing the level of genetic diversity studied; results from this investigation well demonstrate the capacity of SSR markers to separate genotype on 2 varieties from *Ficus salicifolia* (Teloukat and Eucalyptoïdes).

Conclusion

This study reports for the first time the characterization of two varieties of *Ficus salicifolia*, endangered fig species of southern Algeria. A high intra-specific polymorphism recorded by the microsatellite markers suggests their use to study the genetic diversity and the relationship between the different varieties and species of fig trees. This technique offers the ability to detect extensive polymorphisms by its simplicity and rapidity; furthermore, it is useful, to explore the genetic diversity of this important tree using other molecular markers.

Acknowledgements. This study was supported by General Direction of Scientific Research and Technological Development (DGRSDT) with the High Ministry of Research and Study (MESRS) of Algiers. We thank the members of Research Unit (UMR) 1098, Plant Development and Improvement DAP), and those of International Center for Agronomic Research for Development (CIRAD) Montpellier, France for their help in performing genetic study. Also, we thank all researchers of Research Laboratory of Arid Zones (LRZA), University of Sciences and Technology, Houari Boumediene, (USTHB/Algeria); University of Blida I for their help and especially guides of National Institute of Forest Research of Tamanrasset in collecting plant material.

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APPENDIX

Appendix 1. *List of individual fig trees with GPS information*

Local name of collection zones	Number of trees	Code of trees	Code of varieties	Altitude (m)	Latitude	Longitude
TIT	10	Fs01	T01	1086	22°57’54”	05°09’73”
		Fs02	T02	1085	22°57’54”	05°09’73”
		Fs03	T03	1086	22°57’54”	05°09’73”
		Fs04	T04	1081	22°57’54”	05°09’73”
		Fs05	T05	1084	22°57’54”	05°09’72”
		Fs06	T06	1081	22°57’55”	05°09’72”
		Fs07	T07	1084	22°57’38”	05°09’81”
		Fs08	T08	1081	22°57’38”	05°09’81”
		Fs09	T09	1088	22°57’55”	05°09’73”
		Fs10	T10	1082	22°57’55”	05°09’73”
IH-AGHI	05	Fs11	T11	1093	23°04’10”	05°12’85”
		Fs12	T12	1095	23°04’10”	05°12’84”
		Fs13	T13	1094	23°04’10”	05°12’84”
		Fs14	T14	1093	23°04’10”	05°12’84”
		Fs15	T15	1094	23°04’10”	05°12’85”
IN-HOUTER	01	Fs16	E01	1455	23°04’73”	05°22’85”

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DOI: http://dx.doi.org/10.15666/aeer/1905_38233835

TADADINE	03	Fs17 Fs18 Fs19	E04 E11 E20	1315 1330 1350	22°49’42” 22°49’42” 22°49’43”	05°57’33” 05°57’32” 05°57’33”
IN-ZEBIB	18	Fs20 Fs21 Fs22 Fs23 Fs24 Fs25 Fs26 Fs27 Fs28 Fs29 Fs30 Fs31 Fs32 Fs33 Fs34 Fs35 Fs36 Fs37	T16 E02 E03 E05 E06 E07 E08 E09 E10 E12 E13 E14 E15 E16 E17 E18 E19 E21	1431 1425 1435 1441 1456 1449 1433 1428 1451 1446 1459 1428 1454 1452 1458 1440 1448 1447	22°48’17” 22°48’36” 22°48’16” 22°48’42” 22°48’43” 22°48’57” 22°48’28” 22°48’42” 22°48’43” 22°48’41” 22°48’43” 22°48’42” 22°48’43” 22°48’43” 22°48’44” 22°48’35” 22°48’57” 22°48’26”	05°37’22” 05°37’13” 05°37’20” 05°37’27” 05°37’26” 05°37’04” 05°37’34” 05°37’22” 05°37’28” 05°37’89” 05°37’27” 05°37’22” 05°37’24” 05°37’26” 05°37’31” 05°37’61” 05°37’04” 05°37’94”
AMEZEDJINE	02	Fs38 Fs39	T17 T18	1162 1165	22°36’96” 22°36’96”	05°24’46” 05°24’46”

Fs, *Ficus salicifolia,* T, Teloukat; E, Eucalyptoides

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_38233835

EFFECTS OF DIFFERENT CHEMICAL AND ORGANIC FERTILIZERS ON PLANT PROPERTIES OF COTTON (*GOSSYPIUM HIRSUTUM* L.) UNDER NON-SALINE AND SALINE SOIL CONDITIONS

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(Received 14th Apr 2021; accepted 29th Jul 2021)

Abstract. This study was conducted to investigate the effects of organic and chemical fertilizers (diammonium phosphate, DAP) on by physiological development of two cotton (*Gossypium hirsutum* L.) varieties grown under non-saline and saline soil conditions. It was carried out in 2019 at plant research laboratory of Vocational School Technical Sciences in Harran University, Turkey. Chlorophyll content, plant height (cm), root length (cm), fresh root weight (g), dry plant weight (g) and dry root weight (g) of Candia and Lima cotton varieties were determined to evaluate the effects of fertilizers. The layout of the pot experiment was randomized block with three replications. In non-saline soil; the lowest chlorophyll value (17.83) was recorded for the control, while the highest chlorophyll value (32.64) was obtained under the chemical fertilizer (DAP) treatment. In saline soil; the lowest chlorophyll value (20.33) was obtained in chemical fertilizer treatment, and the highest chlorophyll value (24.11) was determined from vermicompost treatment. The lowest plant height (27.83 cm) in non-saline soil was measured in chemical fertilizer application, while the highest plant height (33.00 cm) was recorded in cattle manure application. The shortest root length (13.66 cm) in non-saline soil was obtained from the control, and the highest root length (16.00 cm) was measured in chemical fertilizer.
Keywords: *cotton (Gossypium hirsutum L.), non-saline and saline soil condition, organic and chemical fertilizer, physiological properties*

Introduction

Cotton (*Gossypium hirsutum* L.) is a very important raw material for the textile industry in Turkey and the world. Cotton sector report published by the National Cotton Council in 2019 revealed that 2.57 million tons of seed cotton was produced during 2018/2019 growing seasons in Turkey, and 38% of the seed cotton composed of fiber cotton, which was equal to 976 thousand tons of fiber cotton. Cotton fiber sufficiency ratio of Turkey is around 50% (Anonymous, 2020). Cotton is an industrial plant produced under non-saline soil conditions in semi-arid regions. Cotton plant needs plenty of water and fertilizer during vegetative and generative periods. However, excessive use of fertilizers and irrigation water may lead to an increased soil salinity, which inhibits germination, retards root and stem growth and the development of vegetative parts. Approximately, 20% of arable agricultural lands and 50% of the irrigated lands were affected by salinity (Zhu, 2001). Reclamation and eliminate the harmful effects of saline soils are very difficult and costly. Salt stress is one of the most hazardous abiotic stress factors that limit plant growth. Therefore, efforts should be focused on obtaining salinity tolerant plant varieties.

Salinity in agricultural lands due to the different irrigation systems adversely affects plant growth by osmotic, ionic and oxidative stresses (Munns et al., 2008; Flowers and Colmer, 2008). Cultivation of high yielding varieties in addition to optimizing the growing conditions is another important factor in increasing the cotton production in

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_38373851

Turkey. The optimum germination of seeds, ensuring optimum plant density per unit area and adequate are needed to obtain high yield and quality in cotton (Fujikura et al., 1993; De Villiers et al., 1994). Soil salinity decreases the vegetative development of cotton (Qadir and Shams, 1997), while low salt concentrations can increase growth (Pessarakli and Tucker, 1985; Gorham, 1996). Reclamation of soil salinity by conventional leaching is cost intensive and dependent on the availability of water (Laudicina et al., 2009). Therefore, varieties that are resistant to salt stress should be cultivated. Salt stress reduces the leaf area and the development of root and above-ground parts in cotton (Saghir et al., 2002). Salt concentration reaching 12 EC decreased the germination rate and plant height of cotton plants (Phogat et al., 2001). Similarly, germination rate decreased at 252 meq L^{-1} NaCl level and seedling growth was prevented even at lower concentrations (Casenave et al., 1999). Salinity is one of the most important problems of the agricultural lands in the world and threatens the cotton production areas (Saghir et al., 2002). Degradation of 10 million ha land in the world every year due to the salinity reveals the extend of the problem (Kwiatowski, 1998). Excess level of salts in soils affects around 95 million ha agricultural land in the world (Szabolcs, 1994). In agricultural production; rational and efficient management of plant, soil and water increases the yield per unit area. Excess use of irrigation water will cause rising the groundwater, consequently salt concentration which will restrict plant growth. High salt concentration of soil adversely affects the physiological development of cotton plant.

Insufficient precipitation and high evaporation are the major causes of salinity in arid and semi-arid regions. In addition, improper irrigation can cause soil salinity especially in bad drainage conditions (Ergene, 1982). Limited land used in agricultural production and the continuous increase in food demand necessitates more efficient use of existing arable lands. Therefore, the improvement and productive use saline soils is extremely important (Woods, 1996). The aim of this study was to investigate the responses of different cotton varieties grown in non-saline and saline conditions to chemical and organic fertilizers and to observe different growth parameters of cotton plants starting from the germination of cotton seeds. In this study, salinity tolerance of some cotton varieties in non-saline and saline conditions were determined. This study was also aimed to determine salt-resistant cotton varieties and fertilizer combinations and to recommend varieties and treatments that show high performance to chemical and organic fertilizer applications in both non-saline and saline conditions.

Materials and methods

Materials

Delineated seeds of Candia and Lima cotton cultivars were used as plant material of the study**.** Solid farm fertilizer (Biofarm Fertilizer) and vermicompost (Ecosolfarm) was used as organic fertilizer, and di ammonium phosphate (DAP; 18N-46P_2O_5-0K_2O) was used as chemical fertilizer. Fertilizer doses applied were 2000 kg ha^{-1} solid farm manure, 1500 kg ha^{-1} vermicompost, and 300 kg ha^{-1} DAP. No fertilizer applied to the control plots.

Methods

The lay out of the pot experiment was randomized plots with three replications. This study was carried out in Şanlıurfa, Turkey. The study consisted of the germination test

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DOI: http://dx.doi.org/10.15666/aeer/1905_38373851

and characterization of vegetative growth parameters. Candia and Lima cotton varieties were used for salinity tolerance test in non-saline and saline soils. The study was carried out in the plant laboratory of Sanliurfa Technical Sciences Vocational School of Harran University twice, the first one was in April and the second one was in September 2019. Germination test was conducted under laboratory conditions according to guidelines of ISTA. The surfaces of seeds were sterilized with 70% ethanol for 30 s to prevent contamination of cotton seeds, and kept in pure water for surface sterilization. The seeds were then kept in 2% sodium hypochlorite solution for 3-5 min, and surface sterilization was completed by washing 2-3 times in deionized distilled water. Surface sterilized seeds were sown in pots containing the fertilizers (*Fig. 1*).

Figure 1. *Pots in which the research was conducted (A) saline soil (B) non-saline soil*

Fertilizers were applied at the doses specified by the manufacturers given on the packages. The soils used in the study were taken from a non-saline agricultural field and a saline area. non-saline soils had a pH of 7.82, electrical conductivity (EC) was 3.03 ms cm^{-1} and lime and organic matter contents were 10.96 and value is 0.88%, respectively. Saline soils had a pH of 7.20, EC was 3.74 ms cm^{-1}, lime and organic matter contents were 26.62 and 3.74%, respectively. The pots consist of a radius of 12.5 cm and a height of 20 cm. The soils were passed through a 2 mm sieve and filled into free draining plastic pots with 5 kg soil. The pots were irrigated after sowing to ensure germination of seeds. Water requirement of the plants was observed and the pots were irrigated twice a week with distilled water (pH close to 7). The temperature of the growing environment was set to 27 ± 1 °C per day (Reddy et al., 2004; Salvucci and Crafts-Brandner, 2004). The lighting of the growing environment was set to 14 h light and 10 h dark. Sunlight and fluorescent light were used for the luminous phase. Plant growth was monitored from seed sowing to the squaring period. Plants whose cotyledon leaves completely reached the soil surface were counted. Plants were harvested 12 weeks after the emergence.

Total chlorophyll content (%), plant height (cm), plant root length (cm), overall fresh weight (g), fresh root weight (g), general dry weight (g) and root dry weight (g) of cotton plants were determined. The chlorophyll content (SPAD values) was measured on the leaves of selected plants using a SPAD meter (CCM 200 Plus).

Data obtained in the study was subjected to variance analysis (ANOVA) using JMP 13.0 software to compute the differences of measured variables between the treatments. Analysis of variance was performed by using the averages of the growth parameters

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DOI: http://dx.doi.org/10.15666/aeer/1905_38373851

obtained during the carding period, which is the 60th day of cotton plant development. Following the ANOVA, means were grouped by using least significant difference test (LSD) at 95% probability level.

Results and discussion

The result of variance analysis indicated statistically significant differences among the characteristics of cotton varieties grown in non-saline and saline soils (*Table 1*). Germination under non-saline soil conditions was achieved in all fertilizer applications and plant characteristics were determined when plants reached a certain physiological development. The results recorded under saline soil conditions showed that germination could not be achieved in all fertilizer applications. Germination and physiological development in saline soil were obtained only in chemical fertilizer and vermicompost treatments.

Total chlorophyll content

Total chlorophyll content of plants grown in pot conditions was measured using SPAD device. The SPAD values measured in cotton development stages were significantly different. High chlorophyll content during the squaring period when the plant has the highest physiological development is an expected situation for cotton plants. Chlorophyll values of cotton cultivars grown under non-saline soil conditions were not significantly different (*Table 1*). The chlorophyll content between fertilizer applications was significantly different ($P < 0.01$). The lowest mean chlorophyll value (17.83) was obtained in control and the highest total chlorophyll value (32.64) was recorded in DAP application. The effects of cultivar × fertilizer interaction on total chlorophyll content was statistically significant ($P < 0.01$). The lowest total chlorophyll value (17.44) in variety × fertilizer interaction was obtained in Candia variety * control treatment, while the highest chlorophyll value (37.90) was measured in Candia variety × DAP interaction. Total chlorophyll value in saline soil condition was not significantly different between cotton varieties grown with different fertilizer applications. Total chlorophyll value recorded in different fertilizer treatment was significantly different ($P < 0.01$). The lowest total chlorophyll value (20.33) was obtained in DAP fertilizer application and the highest total chlorophyll value (24.11) in vermicompost treatment. The difference in total chlorophyll content in cultivar × fertilizer interaction was statistically significant ($P < 0.01$). The lowest total chlorophyll value (18.60) was obtained in Candia variety × DAP interaction, while the highest total chlorophyll value (24.63) was recorded in Candia variety × vermicompost treatment. The results indicated no significant difference in total chlorophyll content between the varieties. The differences in chlorophyll values among the fertilizer applications is meaningful. In addition, the chlorophyll content of cotton plants grown in non-saline soils is higher than that measured in saline conditions. High SPAD values obtained in applications with high nitrogen doses indicate higher photosynthesis rate and organic material production. Plants tend to photosynthesize more in non-saline soil conditions and in optimum available nutrition content. Shankar et al. (2020) reported that SPAD values ranged between 41 and 45 with nitrogen application at 60, 90, 120 and 150 kg ha^{-1} doses. The increase in SPAD values with the increase nitrogen doses is in accordance with our findings. Similarly, Doğru and Canavar (2019) stated that soil salinity reduced the pigment content and photosynthetic activity of plants and consequently plant growth

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DOI: http://dx.doi.org/10.15666/aeer/1905_38373851

was adversely affected. In addition, studies using the electron microscope showed that salt stress causes some changes in the chloroplast structure and reduces photosynthetic activity (Mitsuya et al., 2000). Our findings are in agreement with the decrease in total chlorophyll and protochlorophyllide of Greviela arenaria compared to control under saline conditions reported by Kennedy and Filippis (1999), decrease in total chlorophyll and chlorophyll content of tomato reported by Khavari-Nejat and Mostofi (1998), and decrease in chlorophyll a and b and total chlorophyll content of Bruguiera parviflora reported by Alamgir and Ali (1999). Maxwell and Johnson (2000) also revealed that change in photosynthetic pigment biosynthesis is the most obvious effect of salt stress on plants. Bhagwat et al. (2020) carried out a study using different cotton varieties under severe saline conditions, and reported poor germination and subsequent abnormal plant development indicated by low membrane stability (%), leaf relative water content (%), chlorophyll content and photosynthesis. Their findings stating the decreases in carotenoid content, perspiration rate and stomatal conductivity are substantially consistent with our findings. In addition, the findings of Castilo (2011) who reported that stomatal conductance, photosynthesis and carbohydrate formation decreased under excessive salt concentration are also agreement with our findings. Fertilizer applications in saline soil conditions and the cotton varieties used in the experiment did not provide full physiological development. These results are contradicting with the findings of Hemphill et al. (2006) who reported that cotton plant is a relatively resistant to salt tolerance, has the potential to perform physiological development in saline conditions.

Table 1. *Effects of fertilizers applied in non-saline and saline soil conditions on total chlorophyll content*

Properties	Fertilizers	Non-saline soil			Saline soil		
		Varieties			Varieties		
		Candia	Lima	Mean	Candia	Lima	Mean
Chlorophyll	DAP	37.90a	27.40cd	32.64	18.60d	22.06c	20.33
	Cattle manure	29.73b	27.66c	28.70	-	-	-
	Vermicompost	25.50d	25.37d	25.43	24.63a	23.60b	24.11
	Control	17.44e	18.23e	17.83	-	-	-
	Means	27.64	24.66	26.15	21.61	22.83	22.22
	CV (%), LSD	CV (%), 4.39 LSD (Variety), ns LSD (Fertilizer), 1.44** LSD (Variety × Fertilizer), 2.04**			CV (%), 1.02 LSD (Variety), ns LSD (Fertilizer), 0.36** LSD (Variety × Fertilizer), 0.51**		

There is no significant difference between the mean values in the same letter group compared to LSD (5%). ns: not significant. *, **: Important at $P < 0.05$ and $P < 0.01$ level of significance

Plant height

Plant height was not significantly different between cotton varieties under non-saline soil conditions (*Table 2*). The effects of fertilizer treatments on plant height was statistically significant ($P < 0.01$). The lowest mean plant height (27.83 cm) in fertilizer applications was measured DAP application and the highest plant height (33.00 cm) was recorded in cattle manure application. The effects of variety × fertilizer interaction on plant height was not statistically significant. The difference in mean plant height between the variety and fertilizer applications was not significant in saline soil

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conditions. The height of plants grown in saline soil conditions were shorter compared to the plant height in non-saline soil. Saline soil environments are not suitable for plant growth, therefore, the plants cannot grow as under optimum conditions. Castilo (2011) also indicated that salinity is an important abiotic stress factor that has a negative impact on plant growth. The highest plant height was recorded in cattle manure application in non-saline soils. Similarly, Jackson et al. (2003) reported that organic fertilizers improve physical and chemical properties of soils as well as increase the number, diversity and activity of microbial communities. Our results are in agreement with the findings of Khaliq et al. (2006) who stated that organic and microbial fertilizers increased the nutrient uptake of plants. Similar to our study, Barrick et al. (2015) investigated the effect of salinity on growth and some of characteristics of cotton lines grown under organic and conventional conditions. The results showed that cotton lines significantly affected by stress condition including the salinity, and salt applications reported to have negative effects on all properties examined, except chlorophyll content.

Table 2. *Effects of fertilizers applied in non-saline and saline soil conditions on plant height*

Properties	Fertilizers	Non-saline soil			Saline soil		
		Varieties			Varieties		
		Candia	Lima	Mean	Candia	Lima	Mean
Plant height (cm)	DAP	24.33	31.33	27.83c	24.00	27.00	25.50
	Cattle manure	32.33	33.66	33.00a	-	-	-
	Vermicompost	30.33	33.33	31.83ab	26.00	23.66	24.83
	Control	25.66	32.00	28.83bc	-	-	-
	Mean	28.16	32.58	30.37	25.00	25.33	24.16
	CV (%), LSD	CV (%), 9.40 LSD (Variety), ns LSD (Fertilizer), 3.55* LSD (Variety × Fertilizer), ns			CV (%), 16.18 LSD (Variety), ns LSD (Fertilizer), ns LSD (Variety × Fertilizer), ns		

There is no significant difference between the averages in the same letter group compared to LSD (5%). ns: not significant. *, **: Important at $P < 0.05$ and $P < 0.01$ level of significance

Root length

Root length was not statistically different between the cotton varieties grown in non-saline soil conditions (*Table 3*). However, the difference in root length between fertilizers was statistically significant ($P < 0.05$). The lowest root length (13.66 cm) among fertilizer treatments was recorded in control and the longest root length (16.00 cm) was measured in DAP application. The effect of variety × fertilizer interaction on root length was statistically significant ($P < 0.01$). The lowest root length (13.00 cm) value was measured in Lima variety × control interaction, while the longest root length (18.00 cm) was recorded in Lima variety × control interaction. The mean root length recorded for cotton varieties and fertilizers was not significantly different in saline conditions. The result can be attributed to restriction of root and above ground plant part growth under saline conditions. Abdel-Aziz (2019) investigated the effects of humic acid and bacillus bacteria on some physiological and productivity characteristics of cotton plants and reported that fertilization methods had a significant effect on plant growth ratio (g/m^2/week). Similarly, the findings reported by Carter (1975), Munns (2002), Pitman and Läuchli (2002), and Sharma and Goyal (2003) are in accordance

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with the harmful effects of salinity on germination and vegetative growth particularly in arid and semi-arid climates. Ashraf (2004), Mittler (2006), and Neumann (1997) also investigated the salt resistance of plants. The findings of aforementioned researchers are in agreement with our findings that soil salinity and local environmental conditions may cause a serious problem in cotton production. In contrast to our findings, Amjad et al. (2002) reported that root lengths were not affected by low salt density and the root length did not change with the increase in salt content. In addition, Jafri and Ahmet (1994) and Leidi (1994) stated that root length increased at low salt concentrations. The differences in response of plants to root growth can be attributed to the differences in soil and climatic conditions under which the experiments conducted, cotton varieties and management practices.

Table 3. *Effects of fertilizers applied in non-saline and saline soil conditions on root length*

Properties	Fertilizers	Non-saline soil			Saline soil		
		Varieties			Varieties		
		Candia	Lima	Mean	Candia	Lima	Mean
Root length (cm)	DAP	16.00ab	16.00ab	16.00	12.73	16.00	14.36
	Cattle manure	15.66abc	13.66cd	14.50	-	-	-
	Vermicompost	13.33cd	18.00a	15.66	17.33	15.33	16.33
	Control	14.33bcd	13.00d	13.66	-	-	-
	Mean	14.83	15.08	14.95	15.03	15.66	15.34
	CV (%), LSD	CV (%), 9.32 LSD (Variety), ns LSD (Fertilizer), 1.73* LSD (Variety × fertilizer), 2.45**			CV (%), 11.85 LSD (Variety), ns LSD (Fertilizer), ns LSD (Variety × Fertilizer), ns		

There is no significant difference between the averages in the same letter group compared to LSD (5%). ns: not significant. *, *, **: Important at $P < 0.05$ and $P < 0.01$ level of significance

Fresh weight (g)

The difference in fresh weight under non-saline soil conditions was not significant between the cotton varieties. Similarly, the effect of fertilizer treatments on fresh weight was not significant. Lack of significant difference in fresh weight between the cultivars and among the fertilizers used may be related to physiological and environmental effects.

The difference in fresh weight in saline conditions was not statistically significant between the cultivars. The effect of fertilizers on fresh weight under saline conditions was statistically significant ($P < 0.01$). The variety × fertilizer interaction in saline conditions had a statistically significant impact ($P < 0.01$) on fresh weight variety of cotton plants. The lowest fresh weight (13.68 g) in fertilizer treatments under saline conditions was recorded in vermicompost application and the highest fresh (16.81 g) weight was obtained in DAP application (*Table 4*). The lowest fresh weight (13.00 g) in variety × fertilizer interaction was obtained in Lima variety × vermicompost application, while the highest fresh weight (17.33 g) was recorded in Lima variety × DAP application. The fresh plant weight was at a certain level in non-saline soil conditions, however, plant could not grow under saline conditions as compared to non-saline soil conditions. Saline soils do not have optimal conditions for plant growth; thus, vegetative growth did not occur at a desired extent. Similar to the results reported in this

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study, Taghipour and Salehi (2009) reported that application of calcium chloride and sodium chloride to 12 barley varieties significantly decreased fresh weight in all barley varieties. In contrast, some researchers stated that some plant varieties are resistant to high salt concentrations. In addition, Basal (2010) observed significant differences in several characteristics of 15 cotton genotypes grown under increased salt levels. Nirmala (2011) also reported vegetative development of cotton genotypes without showing salt stress. Fresh weights of cotton varieties under different salt concentrations decreased, while fresh weight increased in non-saline soil conditions (Avcı et al., 2020). The findings of Abdel-Aziz (2019) are compatible with our results. The researchers who investigated effects of humic acid and bacillus bacteria on some physiological and productivity properties of a cotton variety, indicated that fertilization methods increased the fresh weight. However, our findings were not in agreement with the results reported by Khenifi et al. (2011) who investigated the effects of salt stress on vegetative growth of cotton genotypes.

Table 4. *Effects of fertilizers applied in non-saline and saline soils on fresh weight (g)*

Properties	Fertilizers	Non-saline soil			Saline soil		
		Varieties			Varieties		
		Candia	Lima	Mean	Candia	Lima	Mean
Fresh weight (g)	DAP	15.53	17.63	16.58	16.30b	17.33a	16.81
	Cattle manure	18.44	15.86	17.15	-	-	-
	Vermicompost	18.60	17.46	18.03	14.36c	13.00d	13.68
	Control	21.80	14.36	18.08	-	-	-
	Mean	18.59	16.33	17.46	15.33	15.16	15.24
	CV (%), LSD	CV (%), 15.40 LSD (Variety), ns LSD (Fertilizer), ns LSD (Variety × Fertilizer), ns			CV (%), 1.33 LSD (Variety), ns LSD (Fertilizer), 0.32** LSD (Variety × Fertilizer), 0.44**		

There is no significant difference between the averages in the same letter group compared to LSD (5%). ns: not significant. *, **: Important at $P < 0.05$ and $P < 0.01$ level of significance

Fresh root weight (g)

The increase in soil content of soils adversely affects the root growth and fresh and dry root weights (Munns, 2002). Fresh root weight under non-saline soil conditions was significantly different ($P < 0.01$) between the cotton cultivars. The difference in fresh root weight between the fertilizer treatments was statistically significant ($P < 0.01$). In addition, a statistically significant difference ($P < 0.01$) was found in variety × fertilizer interaction. Fresh root weight of Candia variety was higher than that of the Lima variety. The difference in fresh root weight between the cotton varieties may be attributed to the genetic structure, environment and environment × genotype interaction. The lowest mean fresh root weight (0.92 g) in different fertilizer treatments was recorded in cattle manure application and the highest fresh root weight (1.10 g) was obtained in vermicompost applications. The lowest fresh root weight (0.71 g) in variety × fertilizer interaction was obtained in Lima variety × control treatment, while the highest fresh root weight (1.25 g) was recorded in Lima variety × vermicompost treatment. The differences in fresh root weight under saline soil conditions was not statistically significant between the cotton varieties, but a statistical difference

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($P < 0.01$) was found among the fertilizers used. In addition, the effect of variety × fertilizer interaction on fresh root weight was statistically significant ($P < 0.01$). The lowest fresh root weight (0.70 g) among different fertilizer treatments was obtained in vermicompost application, while the highest fresh root weight (0.83 g) was recorded in DAP application. The lowest mean fresh root weight (0.68 g) in variety × fertilizer interaction was obtained from Lima variety × vermicompost interaction and the highest mean fresh root weight (0.86 g) was recorded in Lima variety × DAP interaction (*Table 5*). Fresh root weight of cotton varieties decreased with the increase in salinity. The root development of cotton plants increased in parallel to the progress in vegetative development period of the cotton plants. The roots are the first plant parts to interact with salt due to contact with the soil, and are therefore directly affected by the salt concentration of soils. The highest fresh root weight in this study was obtained in vermicompost application. The results are in agreement with the findings of Jackson et al. (2003) who stated that organic fertilizers improved physical and chemical properties of the soil, and also increased the number, diversity and activity of microbial communities in the soil. Our results are in agreement with the findings of Khaliq et al. (2006) who indicated that organic and microbial fertilizers increased the nutrient uptake of plants. The results of Reinhardt and Rost (1995a, b), Raia and Azimov (1988), Meloni et al. (2001), and Ashraf and Ahmad (2000) were in accordance with our results.

Table 5. *Effects of fertilizers applied in non-saline and saline soils on fresh root weight (g)*

Properties	Fertilizers	Non-saline soil			Saline soil		
		Varieties			Varieties		
		Candia	Lima	Mean	Candia	Lima	Mean
Fresh root weight (g)	DAP	0.97c	0.94c	0.96	0.81b	0.86a	0.83
	Cattle manure	1.07b	0.78d	0.92	-	-	-
	Vermicompost	1.09b	1.25a	1.10	0.72c	0.68d	0.70
	Control	1.25a	0.71d	0.98	-	-	-
	Mean	1.09	0.89	0.99	0.77	0.77	0.70
	CV (%), LSD	CV (%), 4.06 LSD (Variety), 0.07** LSD (Fertilizer), 0.05** LSD (Variety × Fertilizer), 0.07**			CV (%), 1.90 LSD (Variety), ns LSD (Fertilizer), 0.02** LSD (Variety × Fertilizer), 0.02**		

There is no significant difference between the averages in the same letter group compared to LSD (5%). ns: not significant. *, **: Important at $P < 0.05$ and $P < 0.01$ level of significance

Dry plant weight (g)

Dry plant weight under non-saline soil conditions was significantly different ($P < 0.05$) between the cotton varieties (*Table 6*). The difference in dry plant weight among fertilizer treatments was statistically significant ($P < 0.01$). In addition, the difference in dry plant weight for variety × fertilizer interaction was also statistically significant ($P < 0.01$). The lowest dry plant weight (2.44 g) in cotton varieties grown under non-saline soil conditions was recorded in Lima variety and the highest dry plant weight (3.11 g) was obtained from Candia variety. Mean dry plant weight of Candia variety was higher compared to dry plant weight of Lima variety. The differences in dry plant weight between the varieties is a genetic feature. The lowest mean dry plant weight (2.48 g) fertilizer treatments was obtained in cattle manure application, while the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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highest mean dry plant weight (3.13 g) was recorded in vermicompost application. The lowest mean dry plant weight (1.90 g) in variety × fertilizer interaction was obtained in Lima variety × cattle manure interaction and the highest mean dry plant weight (3.55 g) was recorded in Candia variety × control interaction. The difference in dry plant weight under saline conditions was not statistically significant between the cotton varieties. The effects of fertilizer treatment and variety × fertilizer interaction on dry plant weight were statistically significant ($P < 0.01$). The lowest mean dry plant weight (1.93 g) among the fertilizer treatments was recorded in vermicompost application and the highest mean dry plant weight (2.38 g) was obtained in DAP application. The lowest mean dry plant weight (1.83 g) in variety × fertilizer interaction was obtained from Lima variety × vermicompost interaction, while the highest mean dry plant weight (2.46 g) was recorded from Lima variety × DAP fertilizer interaction. The results reported by Turan (2000), Revathi and Arumugam (2015), and Soares et al. (2018) revealed that dry plant weight decreased with the increase in salt content of the soils. Our results are similar to the findings of Soyergin (2003) who indicated that manure application affected the net mineralization (C/N) rate and organic fertilizers provide a suitable environment for soil fertility by increasing the soil organic matter in long-term as well as available plant nutrients in short and medium terms. Our findings are consistent with the finding of Abdel-Aziz (2019) who investigated the effects of humic acid and bacillus bacteria on some physiological and productivity characteristics of a cotton variety and stated that fertilization methods significantly affected the dry plant weight. Avcı et al. (2020) studied the effects of salt applications on dry plant weight and reported a decrease in dry plant weight with the increasing salt content of soils.

Table 6. *Effects of fertilizers applied in non-saline and saline soils on dry plant weight (g)*

Properties	Fertilizers	Non-saline soil			Saline soil		
		Varieties			Varieties		
		Candia	Lima	means	Candia	Lima	Means
Dry Plant weight (g)	DAP	2.76c	2.67c	2.71	2.30b	2.46a	2.38
	Cattle manure	3.07b	1.90d	2.48	-	-	-
	Vermicompost	3.09b	3.17b	3.13	2.02c	1.83d	1.93
	Control	3.55a	2.02d	2.78	-	-	-
	Mean	3.11	2.44	2.77	2.16	2.15	2.15
	CV (%), LSD	CV (%), 4.92 LSD (Variety), 0.38* LSD (Fertilizer), 0.17** LSD (Variety × Fertilizer), 0.24**			CV (%), 1.92 LSD (Variety), ns LSD (Fertilizer), 0.05** LSD (Variety × Fertilizer), 0.05**		

There is no significant difference between the averages in the same letter group compared to LSD (5%). ns: not significant. *, **: Important at $P < 0.05$ and $P < 0.01$ level of significance

Dry root weight (g)

Dry root weight under non-saline soil conditions was significantly different ($P < 0.05$) between the cotton cultivars. The dry root weight of cotton varieties was significantly different ($P < 0.01$) between the fertilizers used. In addition, the effects of variety × fertilizer interaction was statistically significant ($P < 0.01$). The lowest root dry weight (0.46 g) was recorded in Lima variety and the highest root dry weight (0.59 g) was recorded in Candia variety. The dry root weight in Candia variety was

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_38373851

higher than that of the Lima variety. The difference in dry root weight between the varieties is related to genetic properties of the cotton varieties. In addition, environmental and cultural factors may have an effect on the difference in dry root weight. The lowest root dry weight (0.47 g) among the fertilizers use, was obtained from cattle manure application and the highest dry weight (0.59 g) was obtained in vermicompost application. The lowest dry root weight (0.36 g) in variety × fertilizer interaction was recorded in Lima variety × cattle manure interaction and the highest dry root weight (0.67 g) was obtained in Candia × control treatment. In terms of variety × fertilizer interaction, the lowest root dry weight (0.36 g) was obtained from Lima variety × cattle manure interaction and the highest root dry weight (0.67 g) was obtained from Candia × control interaction. The dry root weight of cotton varieties under saline conditions was not significantly different. In contrast, the dry root weight among the fertilizers used was significantly different ($P < 0.01$). In addition, a statistically significant difference ($P < 0.05$) was found in variety × fertilizer interaction. The lowest dry root weight (0.37 g) in the fertilizers used was recorded in DAP application, while the highest dry root weight (0.45 g) was obtained from vermicompost application. The lowest dry root weight (0.35 g) in variety × fertilizer interaction was recorded in Lima variety × vermicompost interaction and the highest root dry weight (0.46 g) was obtained from Candia variety × DAP interaction (*Table 7*). Adverse effects of soil salinity on root growth and dry root weight have also been reported by Reinhardt and Rost (1995a, b), Raia and Azimov (1988), Meloni et al. (2001), Ashraf and Ahmad (2000) and Munns (2002).

Table 7. *Effects of fertilizers applied in non-saline and saline soils on dry root weight (g)*

Properties	Fertilizers	Non-saline soil			Saline soil		
		Varieties			Varieties		
		Candia	Lima	Mean	Candia	Lima	Mean
Dry root weight (g)	DAP	0.52c	0.50c	0.51	0.46a	0.45b	0.45
	Cattle manure	0.58b	0.36d	0.47	-	-	-
	Vermicompost	0.58b	0.60b	0.59	0.39c	0.35d	0.37
	Control	0.67a	0.38d	0.53	-	-	-
	Mean	0.59	0.46	0.52	0.42	0.40	0.41
	CV (%), LSD	CV (%), 5.01 LSD (Variety), 0.043* LSD (Fertilizer), 0.02** LSD (Variety × Fertilizer), 0.04**			CV (%), 0.98 LSD (Variety), ns LSD (Fertilizer), 0.22** LSD (Variety × Fertilizer), 0.19*		

There is no significant difference between the averages in the same letter group compared to LSD (5%). ns: not significant. *, **: Important at $P < 0.05$ and $P < 0.01$ level of significance

Conclusion

The results of the study revealed that the highest total chlorophyll content and plant root length was recorded in chemical (DAP) fertilizer application, and the highest plant height was measured in cattle manure application. The fertilizers used had no significant effect on the fresh plant weight. The highest fresh and dry root weight values were recorded in Candia variety and vermicompost application. The results showed that the highest values of plant properties in general under non-saline soil condition were recorded in Candia variety × vermicompost interaction. In saline soil conditions; plant

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germination and development occurred only in DAP DAP and vermicompost applications, and all observations and measurements were carried out in these treatments. The highest total chlorophyll content was obtained in vermicompost application. The highest plant height value was recorded in cattle manure application. Plant root length was not significantly different between the fertilizers used. The highest fresh plant weight, fresh root weight, and dry plant weight values were recorded in the DAP application. The highest dry root weight was obtained in vermicompost application. The highest values of plant properties examines in saline soil conditions were mostly recorded in DAP application. The values of plant properties examined in saline soil conditions were lower compared to those in non-saline soil conditions. The results concluded that saline soil conditions have adverse impact on germination, physiological development and yield of the cotton varieties. The use of organic fertilizers in saline soils positively affected the development of the root part of the plant. For this reason, the use of organic fertilizers in saline soils should be encouraged and more comprehensive studies should be carried out in this area.

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DOI: http://dx.doi.org/10.15666/aeer/1905_38373851

CRITICAL REVIEW OF THE ENVIRONMENTAL INVESTIGATION ON SOIL HEAVY METAL CONTAMINATION

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(Received 15th Apr 2021; accepted 19th Jul 2021)

Abstract. Soil contamination by heavy metals has become a severe environmental issue in the world due to rapid development of urbanisation, industrial, mining, agricultural and natural processes, and chemical compounds. Reliable and quality results quantify the adverse effects of these factors. A precise and cost-efficient study depends on adequate background research, a well-planned sampling design and strategy, quality data, appropriate selection and implementation of analytical techniques and investigation. The investigation methods for heavy metal soil or land contaminations drive decision making and remediation which is very expensive. Therefore, this study offers comprehensive and comparative review on data organisation and treatment; guidelines, legislation of heavy metals; data analysis and investigation methods. The primary objectives of the review are to discuss the various stages involved in the investigation of heavy metals/land for site engineers and environmental scientists. Data analysis methods include exploring contamination indices, statistical and multivariate statistical analysis methods, interpolation techniques, geostatistical estimation, simulation, and combined methods. Strengths, weaknesses and the application scopes of these methods and the resulting models used are critical for success in environmental modelling.
Keywords: *data analysis methods, contamination indices, multivariate analysis, geostatistical simulation, and spatial interpolation methods*

Introduction

Heavy metal contamination in soil or land has become an increasingly common and serious problem and threat to every country of the world due to rapid development of technology, economy, public awareness, and society. In Europe, mineral oil and heavy metals are the main contaminants contributing by 50% to soil contamination. The management of contaminated sites is estimated to cost around six billion Euros annually (Panagos et al., 2013). In the past, soil contamination was not considered as important as air and water pollution, because it was often with wide range and was more difficult to be controlled and governed than air and water pollution. However, in recent years the soil contamination in developed countries has become an essential issue thus, more and more attention is paid to this issue which has become a significant topic of environmental protection worldwide (Su et al., 2014). Characteristics of heavy metal contamination of soils include wide distributions, strong latency, irreversibility, remediation hardness, high cost and complex heavy metal contamination. In the world's top ten environmental events, two events have been related to heavy metal contamination (Yang and Sun, 2009). These are soil, air, and water pollutions, caused by heavy metals, which are a serious threat to almost every country. Heavy metals have been effectively used by humans for thousands of years. Although, several adverse health effects of heavy metals have been known for a long time, exposure to heavy metals continues and is even increasing in some parts of the world, particularly in less developed countries (Jarup, 2013).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_38533878

Heavy metals constitute an ill-defined group of inorganic chemical hazards, and at contaminated sites Pb, Cr, As, Zn, Cd, Cu, Hg and Ni are found most commonly. Source of heavy metal contamination in soil may be classified into two categories, natural and anthropogenic. The spatial distribution of naturally originating heavy metals is highly heterogeneous and different concentrations exist in different soils. Heavy metals have been used for thousands of years in a large variety of industrial products, which have been deposited for a long time as waste. The main anthropogenic sources of heavy metals are agricultural activities, metallurgical activities, mining operations, energy production, transportation, micro-electronic products, and waste disposal. They are found in different forms such as gaseous, particulate, aerosol/aqueous solid and emanate both diffuse and point sources. Literature research showed that exposure of human health from contaminated soil by heavy metals is directly implicated as ingestion, inhalation, skin contact and dermal absorption. Human health is also indirectly affected through contaminating the food, water, and atmosphere. Different contaminants originate different negative effects on human health and environment depending on their properties. These are dispersion solubility in water, bioavailability, carcinogenicity, bioaccumulation, and so on.

Sampling efficiency and representatives in soil contamination by heavy metals are affected by various factors which include sampling design and strategy, sampling location, depth, density, sampling stages and methods. These factors have been widely studied by many researchers (e.g. Coşkun et al., 2006; Davis et al., 2009; Maas et al., 2010; Sun et al., 2010; Wang and Lu, 2011; Lu et al., 2012; Shan et al., 2013; Kelepertzis, 2014; Haung et al., 2015; Mihailovic et al., 2015; Zhou et al., 2016; Moore et al., 2016). The factors optimisation and economic cost of soil sampling are typically analysed by geostatistical techniques and Geographic Information System (GIS) integrated multivariate statistical methods.

Toxic levels of heavy metals may be various in different countries because of different cultures and different protection methods of environment and commonly health. Thus, a large variation in environmental and human health regulations and their effects for heavy metal contaminants in soil may be observed trough the world. Regulations in the developed countries for soil contamination with heavy metals may be guidance and useful to investigate risk assessment and decision-making for the developing countries. Therefore, total concentration levels of heavy metal contaminants for soil quality guidelines and the protection of environmental health in the United Kingdom, the European Community, the Netherlands, Canada, and Australia are presented in *Tables 1-4,* respectively. There are also many environmental laws and regulations for metal contaminants in soil around the world including USA, Germany, Japan, China, Singapore, and Malaysia. For example, there are several federal and state sets of regulations and standards in the United States of America (USA). The most widely recognised methodology for risk assessment of an environmental contaminant developed by US Environment Protection Agency (USEPA, 2011). A wide variation in standards, regulations and their effects were observed throughout the world. In summary, most current legislations are still based on the total concentrations of contaminants in soil. Consequently, these regulations and limits may act as a guideline to purpose risk assessment methodologies, model tools, and exposure scenarios, especially for the developing or less developed countries.

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DOI: http://dx.doi.org/10.15666/aeer/1905_38533878

Table 1. *Heavy metal guideline in soil contaminated land exposure assessment (CLEA, 2009)*

Heavy metals	Function of land use	CLEA soil guideline value (mg/kg)
Pb	Residential with home grown produce	200
	Residential without home grown produce	310
	Allotments	80
	Commercial	2300
Cr VI	Residential with home grown produce	21
	Residential without home grown produce	21
	Allotments	170
	Commercial	49
Cr	Residential with plant uptake	130
	Residential without plant uptake	200
	Commercial and industrial	5000
As	Residential with home grown produce	37
	Residential without home grown produce	40
	Allotment	49
	Commercial	640
Cd	Residential with home grown produce	22
	Residential without home grown produce	150
	Allotment	3.9
	Commercial	410
Hg	Residential	10
	Allotment	26
	Commercial	26
Ni	Residential	130
	Allotment	230
	Commercial	1800

Table 2. *Heavy metal soil and sediment guideline values in the Netherlands (The Ministry of Housing, 2011)*

Heavy metals	Target value (mg/kg)	Intervention value (mg/kg)
Pb	85	530
Cr	100	380
As	29	55
Zn	140	720
Cd	0.8	12
Cu	36	190
Hg	0.3	10
Ni	35	210

The key to effective quality assessment of soil contamination by heavy metals is in the use of investigation methods. There is currently a wide arrange of investigation methods used to evaluate soil contamination. A discussion of the advantages and limitations of different soil contamination assessment methods such as contamination indices, statistical analysis, spatial interpolation techniques, geostatistical methods and combined methods is presented. Contamination indices are the most widely used significant tools for the comprehensive evaluation and the grade of soil contamination. Many authors previously described several indices which are defined for evaluation of the degree of soil

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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contamination in recent publications (Wu et al., 2014; Kovalska et al., 2018). In this study, different aspects, and significant characteristics of the indices such as the similarities and differences, comparisons, advantages and disadvantages were briefly evaluated. This ensures the selection of appropriate indices in the environmental study of different soils.

Table 3. *Soil and quality guidelines for the protection of environmental health values in Canada for land use (Canadian Council of Ministers of the Environment, 2010)*

Heavy metals	Agricultural (mg/kg)	Residential/park land (mg/kg)	Commercial (mg/kg)	Industrial (mg/kg)
Pb	70	140	260	600
Cr	64	64	87	87
As	12	12	12	12
Zn	200	200	360	360
Cd	1.4	10	22	22
Cu	63	63	91	91
Hg	6.6	6.6	24	50
Ni	50	50	50	50

Table 4. *Heavy metal levels in soil in Australia (Department of Environment and Conservation, 2010)*

Heavy metals	Ecological level (mg/kg)	Residential/garden (mg/kg)	Residential/apartments/flats minimum soil access (mg/kg)	Parks/recreational/playing fields area (mg/kg)	Commercial/industrial (mg/kg)
Pb	600	300	1200	600	1500
Cr III	400	120000	48000	240000	60000
Cr VI	1	100	4000	200	500
As	20	100	400	200	500
Zn	200	7000	28000	4000	35000
Cd	3	20	80	40	100
Cu	100	1000	4000	2000	5000
Hg	1	15	60	30	75
Ni	60	600	2400	600	3000

Soil contamination prediction requires frequent use of statistics. Statistical analysis has been used for a long time to address soil contamination as univariate or classical statistics and multivariate statistical analysis. Univariate statistical tools present several facilities including improving understanding of data and soil contamination, providing data quality, organising, and grouping data information, and making inferences and estimations. Multivariate statistical analysis was not alone widely used in environmental studies. However, recently, the use of multivariate statistical analysis integrated with GIS have been successfully studied in the identification of metal sources, assessment of metal behaviour, soil quality, mapping of metal spatial distribution in regions (Saby et al., 2009; Lu et al., 2012; Shao et al., 2014; Haung et al., 2015; Zhou et al., 2016; Ali et al., 2016; Moore et al., 2016; Gabarron et al., 2017). GIS is a system designed to capture, store, manipulate, analyse, manage, and present all types of geographical data (ESRI, 1994). GIS is increasingly used as the most comprehensive tools for life and industry including mapping, environmental impact analyses, geological and mining studies, hydrology, archaeology, rural and urban planning, disaster management and mitigation, crime statistics, health and medical resource, management, transportation planning, agricultural

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DOI: http://dx.doi.org/10.15666/aeer/1905_38533878

applications, climate and meteorology, telecom and network services, and many other areas. GIS provides spatial data entry, management, and retrieval, analysis, and visual functions.

Geostatistics contains different methods based on regionalised theory and stationary for the analysis, estimation and simulation of data correlated in space or time. Geostatistics was initially developed for mineral resource estimation and geological modelling (David, 1977; Isaaks and Srivastava, 1989; Goovaerts, 1997; Rossi and Deutsch, 2014), and later enhanced for spatial analysis of environmental issues (Burges and Webster, 1980; Goovaerts, 1999; Webster and Oliver, 2007; Oliver, 2010). A major aspect of geostatistical modelling is to quantitatively measure spatial variability by subsequent estimation and simulation.

Traditional interpolation or Inverse Distance Weighting (IDW) and geostatistical interpolation or Kriging methods have been increasingly used to estimate the spatial distribution of contaminants in soil for 1990's years (Zhang et al., 1995; Steiger et al., 1996; Journel, 1998; Meirvenne and Goovaerts, 2001). However, these methods have smoothing effects, results in less variance in the estimation than in the observed data. Recently, on the other hand, geostatistical simulations, the most commonly used Sequential Gaussian Simulation (SGS), have overcome the limitations intrinsic in conventional and kriging-based interpolation techniques. SGS reproduce original statistics, histograms and variograms of the spatial variability for the data without smoothing effects. SGS are the most frequently applied in mining industry and environmental studies (Goovaerts al., 1996; Soares, 2001; Meirvenne and Goovaerts, 2001; Pereira et al., 2001; Franco et al., 2006; Ersoy et al., 2008; De Almedia, 2010; Qu et al., 2013; Rossi and Deutsch, 2014; Garcia-Lorezo et al., 2014; Albuquerque et al., 2017; Zhang et al., 2017; Ersoy and Yünsel, 2018).

This review fills a knowledge gap in soil contamination by heavy metals. There are many review publications available that describe only single issue and provide few or no guidelines necessary focusing on practical applications of the environmental research. Descriptions, comparisons, advantages and disadvantages and integrations of the investigation methods or models for soil contamination by heavy metals to use in the spatial distribution, risk analysis and decision making are presented. The paper outlines soil guideline limits and characteristics of heavy metals, establishes investigation and application methods to soil contamination, explores data organisation and treatment and factor affecting the performance of the application methods, demonstrates validation test of estimation and simulation. The workflow approach of the review for contaminated site characterisation is presented in *Figure 1*. This study is presented to describe all important issues in an environmental study based on the workflow except for sampling issues and analytical techniques. These points help to evaluate results, risk assessment, and finally decision making and remediation for responsible authorities. The review may be used by a wide range practitioners, environmental scientists and engineers, and others involved in soil contamination by heavy metals in the world.

Investigation methods used in the examination of soil contamination by heavy metals

Contamination indices

Contamination indices are currently and widely used for the assessment of soil contamination. They also evaluate soil quality and the prediction of future ecosystem

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_38533878

sustainability especially for agricultural purposes. Moreover, the indices provide to determine the source of heavy metals, natural processes, or anthropogenic activities. The most widely applied indices are critically summarised from literature at the following. Uses, advantages, disadvantages and related references are given in *Table 5*. This tabulation is the key assessment of soil contamination by heavy metals. A recent study related to the description of a wide spectrum of contamination indices can be found in Kowalska et al. (2018).

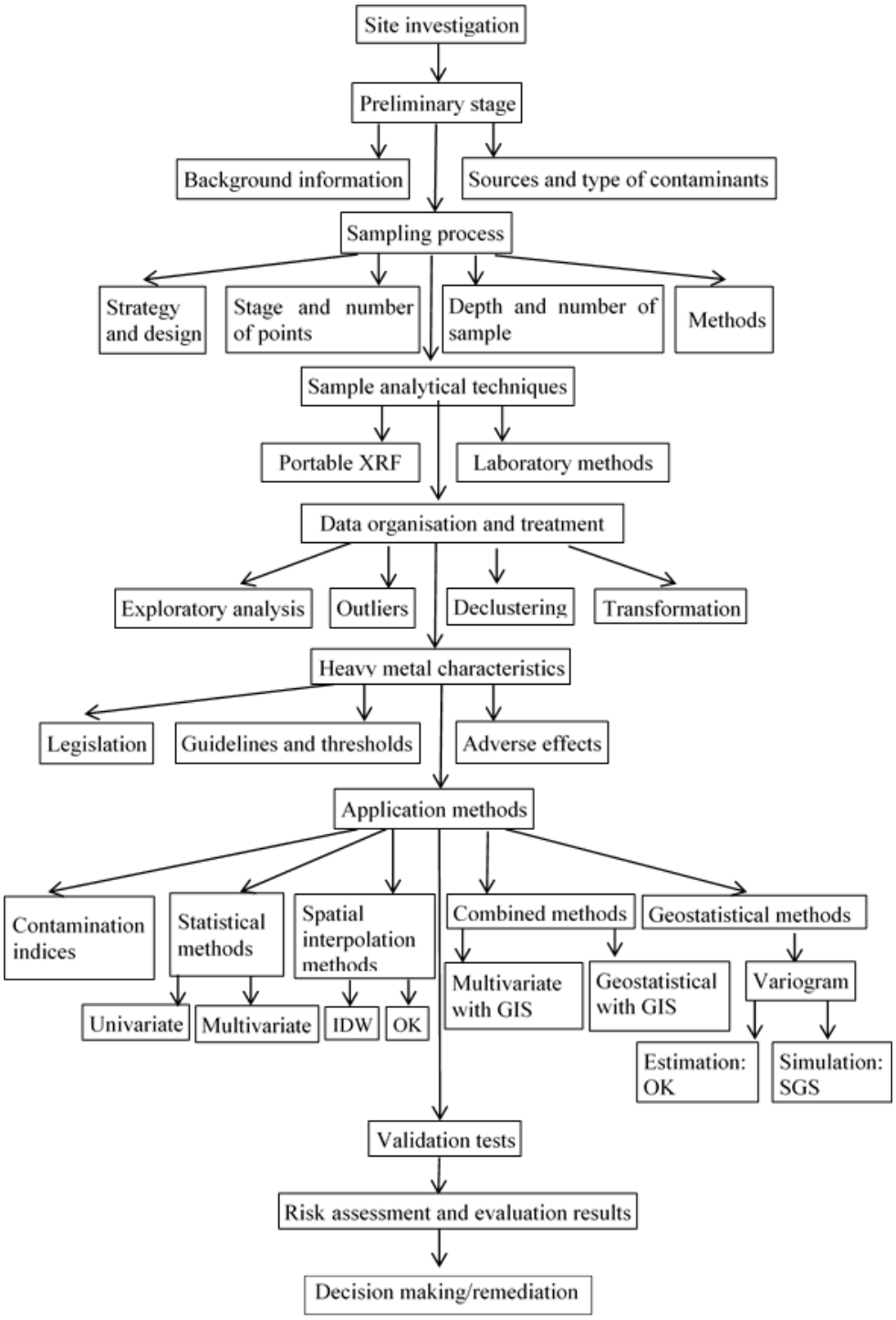

Figure 1. *Site investigation methods workflow in soil contamination by heavy metals*

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Table 5. *The main characteristics of the most widely used contamination indices in soil contamination by heavy metals*

Index	Use extent	Advantages	Disadvantages	References
Igeo	• Simple, easy and most widely used • Contamination degree of single heavy metal • GB	• The comparison of the current and previous contamination • 1.5 multiple factors reduce lithogenic effect • Correct scale	• Bad GB selection, bad results • Ignores natural geochemical changes • Natural variability in GB	Chen et al., 2015 Karim et al., 2015 Sayadi et al., 2015 Wang et al., 2005 Su et al., 2014
PI	• Contamination degree of single heavy metal • Easy and widely use • GB	• Correct scale	• Ignores natural variability • Improper selection GB, wrong results	Begum et al., 2014 Chen et al., 2015 Karim et al., 2015 Sayadi et al., 2015
EF	• Identification of heavy metal origin • Comparison of heavy metal concentrations	• Predicts heavy metal origin and anthropogenic effect • Evaluation of the contamination by single heavy metal • Reduces heavy metal variability • Correct scale	• Results depend on GB selection • Assessment of uncontaminated contents	Inengite et al., 2015 Karim et al., 2015 Thabet et al., 2014 Sayadi et al., 2015 Varol., 2011 Wang et al., 2005
CF	• Soil quality • Toxic materials	• Single for each metal • Containing the difference between sample and reference values • Easy and direct application • Correct scale	• Ignores the variability of natural process and presence of heavy metals • No GB • Previous reference value necessary before contamination	Hakanson, 1980 Inengite et al., 2015
PIsum	• Evaluation of contaminants group for all contamination	• Integrates all heavy metals • Comparing for contamination in different soil • Depend on PI values	• Ignores the variation of natural process and the presence of heavy metals • Selection of GB is important • Missing of correct scale	Hakanson, 1980 Inengite et al., 2015
PLI	• Evaluation of degree of contamination • Easy and widely used	• Integrates a number of heavy metals • Comparing of contamination in different soil • Depends on PI values • GB use	• Related GB • Ignores natural process and presence of heavy metals	Karim et al., 2015 Thabet et al., 2014 Pejman et al., 2015
ExF	• Contaminated site point • All soil evaluation • Easy use	• Integrates all heavy metals	• Not widely used • Ignores natural process • No correct scale	Babelewska, 2010

GB: geochemical background value

Geoaccumulation Index (Igeo) was first introduced by Müller (1969). It provides the assessment of soil contamination by heavy metals depending on its content in a horizon by comparing differences between current and background concentrations. Igeo is defined by *Equation 1:*

$$Igeo = \frac{Log2\ Cn}{1.5\ GB} \quad \text{(Eq.1)}$$

where Cn is the measured concentration of the heavy metal and GB is the geochemical background value of the heavy metal. 1.5 is a constant, providing for an analysis of the variability of the data due to natural processes. Igeo values has been classified into seven quality classes (Müller, 1969; Li et al., 2014).

Single Pollution Index (PI) is used to assess greatest heavy metal accumulation in soil. It is expressed as follows (*Eq. 2*):

$$PI = \frac{Cn}{GB} \quad \text{(Eq.2)}$$

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where Cn is the content of heavy metal and GB is the geochemical background value.

Enrichment Factor (EF) is a measure of the effects of heavy metal concentrations by anthropogenic activity in soil (*Eq. 3*). It is computed in the following formula (Sutherland, 2000).

$$EF = \frac{sample\,{}^{Cn}/_{Lv}}{background\,{}^{GB}/_{Lv}} \quad \text{(Eq.3)}$$

where Lv (sample) is the concentration of the reference element in the soil. GB is the value of geochemical background. Reference metals are Fe/Al/Ca/Ti/Sc/Mn. If the EF value is less than 1.5, there is no heavy metal contamination in soil occurred with natural processes. If EF is more than 1.5, the heavy metal contamination formed as a result of anthropogenic processes (Elias and Gbadegesin, 2011).

Contamination Factor (CF) measures the content of heavy metal from the surface of the soil and values of pre-industrial reference levels defined by Hakanson (1980). CF is calculated by *Equation 4:*

$$CF = \frac{Cm}{Cp-i} \quad \text{(Eq.4)}$$

where Cm is mean content of heavy metal of at least five samples and $C_{p\text{-}i}$ is pre-industrial reference value.

Sum of Contamination (PI_{sum}) is defined as the sum of all determined concentrations of heavy metals, given by Gong et al. (2008). PI_{sum} is computed using *Equation 5:*

$$PI_{sum} = \sum_{i-1}^{n} PI \quad \text{(Eq.5)}$$

where PI is calculated values of single pollution index, n is the number of total heavy metals.

Pollution Load Index (PLI) is used for the sum of assessment of heavy metal contamination in soil (Varol, 2011). PLI is determined from *Equation 6:*

$$PLI = \sqrt[n]{PI_1 \times PI_2 \times PI_3 \times \ldots\ldots PI_n} \quad \text{(Eq.6)}$$

where n is the number of studied heavy metals and PI is calculated values of the single pollution index.

Exposure Factor (ExF) is used to measure the greatest heavy metal accumulation in the study area (*Eq. 7*). This is calculated from the following formula (Babelewska, 2010).

$$ExF = \sum \frac{C_n - C_{av}}{C_{av}} \quad \text{(Eq.7)}$$

where Cn is the content of heavy metal at the sampling point, C_{av} is average content of heavy metal in the soil profile.

Other indices of less use are Nemerow Pollution index, ($PI_{Nemerow}$: Gong et al., 2008); Average Single Pollution Index (PI_{avg}: Gong et al., 2008), Vector Modulus of pollution Index (PI_{vector}: Gong et al., 2008), Background Enrichment Factor (PIN: Caeiro et al., 2005), Multi Element Contamination (MEC: Adamu and Nganje, 2010), Contamination Security Index (CSI: Pejman et al., 2015), Probability of Toxity (MERMQ: Pejman et

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DOI: http://dx.doi.org/10.15666/aeer/1905_38533878

al., 2015), Degree of Contamination (C_{deg}: Hakanson, 1980), Potential Ecological Risk (RI: Hakanson, 1980), Modified Degree of Contamination (mCd: Abrahim and Parker, 2008). These are well documented in the literature (e.g. Kowalska et al., 2018).

Evaluation of geochemical background and contamination indices

The selection and identification of proper reference values for uncontaminated soil is a key task which results in precisely assessing soil contamination by heavy metals, because overall quantitative assessment methods are dependent on reference values of background concentrations (Desaules, 2012). There are many background definitions and related terms in literature. These definitions and applications of background values in environmental geochemistry are discussed and well documented in the literature (e.g., Reimann et al., 2005; Wu et al., 2014). The following important points can be briefly given:

- No specific global and regional background levels of heavy metals can be described. Because natural and anthropogenic effects are different in different regions.
- The levels of background concentrations are based on the area and its scale.
- Background value is a range and not absolute value due to heterogeneity of the environment.
- Natural background may vary in earth crust due to human activities.

Selection of proper geochemical background (GB) is significant in the evaluation of heavy metal contamination (Varol, 2011). Application of various GB provides a more precise investigation of contamination index values. This may be based on the possibility of the contamination of individual sites (Karim et al., 2015).

Two types of GB were classified as reference and local (natural) (Kowalska et al., 2016). The average content of heavy metals can be changed due to local heterogeneity and soil type which may be described with the reference geochemical background (RGB). Local geochemical background (LGB) is the occurrence of natural process which is not affected by human activity (Reimann et al., 2005).

RGBs do not contain natural variability (Xu et al., 2015). Use of RGB is not always possible to recognise natural and anthropogenic effects (Kowalska et al., 2016). However, RGB provides global or regional models of heavy metal contamination (Karim et al., 2015). Calculation of contamination indices needs RGB for many purposes.

LGB contains heavy metal content in rocks and the average content of samples and considers a definite level of human activity (Karim et al., 2015). LGB application is recommended for individual sites under the effect of natural activities and anthropogenic impact (Kierczak et al., 2016). However, LGB may change significantly through lithogenic processes, and its level should be evaluated within geologically homogeneous area (Kowalska et al., 2018). Consequently, literature argued that RGB and LGB values can be used to have complete knowledge (Reimann and de Caritat, 2017; Kowalska et al., 2016).

Many literature research studies have demonstrated that selection of contamination indices are used for different purposes including contamination degree, heavy metal source, potential risk of heavy metal accumulation, ecological risk, the scale of total concentration (e.g. Dung et al., 2013; Guan et al., 2014; Baran et al., 2018). These

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_38533878

criteria are used in the calculation of contamination indices which are based on GB values (e.g. Igeo and EF), data (e.g. CF), and heavy metal content in the soil (e.g. ExF). Although there are clear similarities between contamination indices, they differ from each other due to the effects of several factors.

Igeo and PI are the most accurate and widely used to assess the level of contamination (Begum et al., 2014; Karim et al., 2015; Sayadi et al., 2015). The indices provide to compare previous and present contamination and to have correct scale.

EF makes difference between contamination sources of anthropogenic activities and natural processes (Kowalska et al., 2016). EF identifies low concentrations of heavy element variability (Karim et al., 2015). RGB values have frequently been used in the calculation of EF like Igeo and PI. Heavy metal concentration levels of the sample and reference values are mostly described by concentration variability.

The calculation of CF does not need GB (Li et al., 2016). However, CF distinguishes proportion difference between single heavy metal contamination and previous industrial reference values. CF ignores the variability of natural activities (Varol, 2011).

PIsum and PLI are used for overall soil contamination assessment. These indices are similar to PIavg, PIvector and PIN, which are applied to similar purposes. Their uses are easy and simple. They exhibit reasonable levels of heavy metal contamination (Inengite, 2015). The main weakness of them has individual scale.

Consequently, appropriate selection of contamination indices is based on the degree of contamination, purposes of use, soil type (e.g., farmland, forest, and urban site). Understanding knowledge of contamination index is a basic key task for environmental management, risk of environmental exposure, agricultural practises, ecosystem protection, identification of natural and anthropogenic sources.

Multivariate statistical analysis methods

Multivariate statistical analysis is often used for identifying sources of heavy metal contamination (Mostert et al., 2010). The Methods consist of principal component analysis (PCA), cluster analysis (CA), Pearson correlation analysis, factor analysis (FA), multiple linear regressions (MLR). PCA is the most frequently used multivariate statistical analysis method to reduce data dimension. This technique derives to determine the variance in the data with a small number of independent variables referred to principal components (Boruwka et al., 2005). The relationship between metal fractions and physical chemical properties is determined by PCA. Varimax rotation is applied to minimise the number of variables with a high loading on each component and operates the assessment of results (Mico et al., 2006). In another way, an orthogonal transformation technique is used to obtain the first principal component showing for the highest variance in the observed data. An eigenvalue decomposition in matrix is constructed with the highest eigenvalue which is the principal component of the data (Hou et al., 2017). Consequently, it is important to adequately treat and organise the data for multivariate statistical analysis. Appropriate transformation is necessary.

CA is the second most used multivariate statistical analysis method in the literature (Hou et al., 2017). Variables of the data set are divided into groups of similar features. CA algorithms minimise and maximise inter group variability. CA is used to confirm PCA results for soil contamination by heavy metals.

Other less commonly used methods are Pearson correlation, FA and MLR. Pearson Correlation analysis makes linear correlation between two variables. It corresponds a

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_38533878

correlation coefficient ranging from -1 to 1. -1 represents perfect negative linear correlation, 0 refers to no correlation and 1 indicates perfect positive correlation. Pearson correlation is useful for PCA and CA. Hou et al. (2017) pointed out that Pearson correlation is not a multivariate statistical analysis technique. Because it explains only single pairs of variables at a time. FA proposes to reduce data set dimension like PCA, but mathematical methods of them are different. FA uses a discrete model to provide n variables within latent variables (n > m) whereas PCA does not account the model (Jaliffe, 2002). FA is often used in human behaviour study related to the environment (Hou et al., 2017). However, FA has rarely been used for soil contamination of heavy metal (Romic and Romic, 2003). MLR has seldom been used for spatial distribution of heavy metals in soil. MLR combined PCA was used by Ali et al. (2016) for quantifying the origin of heavy metals.

In conclusion, combined geographic information system (GIS) and multivariate analysis have been recently used by increasing number of studies for the assessment of spatial distribution of heavy metals to quantify soil quality in regional scale (e.g., Huang et al., 2015; Lin et al., 2016; Moore et al., 2016; and Zhou et al., 2016). GIS is a compilation of computer hardware, software, spatial and non-spatial data, and users designed to efficiently capture, store update, manipulate, analyse, and display all forms of geographically referenced information. GIS software is interoperable, supporting many data formats used in the infrastructure life cycle. Its technology provides a central location to conduct spatial analysis, over by data, and integrate other applications or systems. The recent development of GIS is to capture digital data in the field and provide more efficient transfer from field to office. GIS technology is changing fast and moving from mainframe computer to workstation and to desktop-based PC systems. GIS is driven by jurisdictional, purpose or application requirements. Most phases of infrastructure life cycle are commonly affected and enhanced by the enrolments of GIS.

Spatial interpolation methods

Interpolation is the process of estimating the values of interest variables at unsampled areas. Spatial interpolation methods differ from classic modelling approaches since spatial methods provide knowledge about the geographic position of the sample point. In the spatial interpolation sampling points closer to each other, exhibit good correlations and more similarities than the points further away. In this study from literature review (e.g. Li and Heap, 2014; Xi et al., 2011; Hou et al., 2017) the most frequently used spatial interpolation methods, inverse distance weighting and ordinary kriging (OK) were reviewed.

Inverse distance weighting (IDW)

IDW is based on a linear combination of data set. The main advantages of IDW are fast and easy use, directly interpolation (*Table 6*). Thus, the method most widely used for environmental and mining studies. The important weakness of IDW is that it does not account a particular model of spatial correlation for the variables being interest. The interpolating equation is given as follows (*Eq. 8*):

$$Z_{xy} = \frac{\sum_{i=1}^{n} Z i d_{xyi}^{-\beta}}{\sum_{i=1}^{n} d_{xyi}^{-\beta}} \quad \text{(Eq.8)}$$

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where Z_{xy} is the estimated value at an interpolated point, z_i is the control value for the i_{th} sample point, n is the total number of observed points used in interpolation, d_{xyi} is the distance between Z_{xy} and Zi, and β is on exponent described by the user. As the distance increases the weight decreases and weighting power incorporates the weight decreases while the distance increases. The accuracy of IDW may be improved by selecting the optimal neighbouring points and exponent value to generate optimum arrangement between observed data and the prediction. Many soil quality survey studies revealed that integrated IDW with GIS and multivariate statistical analysis have been used in several regions quantifying soil contamination of heavy metals (e.g. Haung et al., 2015; Lee et al., 2006 and Zhang, 2006).

Non-geostatistical rarely used other spatial interpolation methods are nearest neighbours, triangular irregular network related interpolations, natural neighbours, regression models, trend surface analysis, thin plate splines, regression tree, local polynomial, and radial basis functions.

Kriging

Kriging is the geostatistical method that is the most widely used among spatial interpolation methods for spatial distribution in soil. Kriging is produced from regionalised variable theory and dependent on stochastic spatial variation model. Confidence intervals for the values of variables at unsampled locations are estimated by kriging. A linear combination of the observed values with weights gives the kriging predictor. There are many types of kriging that include simple kriging (SK), ordinary kriging (OK), factorial kriging, dual kriging, indicator kriging (IK), disjunctive kriging, model-based kriging. These refer to univariate kriging type, whereas universal kriging (UK), SK with varying local means, kriging with external drift, simple cokriging (SCK), OK, standardised ordinary cokriging, principal component kriging, collocated cokriging, kriging with strata, multivariate factorial kriging, IK with an external drift, indicator cokriging and probability kriging are classified as multivariate kriging types (Li and Heap, 2014).

Kriging equation is given as follows (*Eq. 9*):

$$Z(B) = \sum_{i=1}^{n} \lambda z(xi) \quad \text{(Eq.9)}$$

where Z(B) is the estimated area, λi is weight and Z(xi) is sample value. Ordinary kriging (OK) is the most frequently applied technique among the kriging types for environmental, geological and mining studies. Advantages and weakness features of OK is given in *Table 6*. The main characteristics of OK can be presented at the following points:

- OK is the best linear unbiased estimator.
- OK estimates unsampled locations in studied site
- OK measures estimation errors and uncertainty
- OK minimise the variance of the data. Its variance is based on data values. The error variance is poorly correlated with actual estimation error. Thus, kriging variance may not be used alone as a measure of local uncertainty.
- OK provides spatial structure
- OK requires variogram construction before operating spatial interpolation process. Thus, OK is significantly affected by variogram parameters such as nugget effect, sill, range, variogram model or shape, search radius, number of

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neighbouring measurements. Sufficient data and appropriate distribution of data are necessary for variogram building.

- The biggest weakness of OK has smoothing effects. Interpolated surface is smooth which can cause low values to be overestimated and high values to be underestimated. This most probably resulted in the high contamination risk area. Underestimated and low risk are clean area overestimated.
- Error assessment of OK is based on variogram structure, distribution of data points and size of interpolated blocks.
- If data are sufficient and appropriate distribution to compute variogram, OK will provide a well interpolator for sparse data.
- OK does not require knowledge of the mean over the region of interest and operates under simple stationarity assumptions.
- OK is a robust estimator due to only requiring local stationarity.

Many case studies demonstrated that combined multivariate statistical analysis with kriging analysis can be a reliable and useful tool to determine spatial distribution and source of heavy metals, to quantify soil quality (Maas et al., 2010; Lu et al., 2012; Shao et al., 2014; Cai et al., 2015; Gabarron et al., 2017).

Geostatistical simulations

Simulation is defined as imitations of conditions. Simulation generates an equally probable realization representing spatial distribution of heavy metals and measuring of uncertainty of the area being studied. Exploratory statistics such as mean, median, variance, coefficient of variation, standard deviation, skewness, and kurtosis; histogram; the variogram (spatial dispersion variance) of the original data information are reproduced by simulations on real scale. Simulated realizations are constructed on a fine grid. Simulation characteristics of soil contamination play a key role in sampling strategy and designing, planning, decision making, implementation risk and scheduling in site assessments. Significant parameters can be derived from the distribution of local uncertainty such as exploratory statistics and probability of exceeding value or threshold limit. Thus, a simulation process is a significantly more completed model than the single estimated block or point model.

Simulations provide a variety of purposes including study of element concentration continuity, optimising sampling for advanced investigation, assessment of soil contamination estimation methods, site (environmental) planning, risk evaluation (e.g. financial) and any integration of the aims given here.

There are two types of geostatistical or stochastic simulations, unconditional and conditional. Unconditional simulation is simply an application of the general Monte Carlo Technique that simulate values and are generated with a particular covariance function and variogram. Several simulation techniques exist for practitioners. Four methods are common in use; they are sequential Gaussian simulation (SGS), simulated annealing, and simulation by turning bands and lower-upper decomposition. The first three methods can generally be conditional and lower-upper decomposition is often used for unconditional (Webster and Oliver, 2007).

SGS is the most widely used technique in environmental studies for site assessment particularly to quantify risk and quality of soil (Goovaerts, 2001; Qu et al., 2013; Zhang et al., 2017; Ersoy and Yünsel, 2018). Sequential indicator simulation, direct sequential

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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simulation, and sequential Gaussian cosimulation or joint simulation are an extension of SGS simulation models of several continuous variables and based on SGS algorithms. A growing number of many environmental researchers have also used these applications for soil contamination by heavy metals (Huang et al., 2015; Franco et al., 2006; Ersoy and Yünsel, 2019). Because applications of SGS are simple, flexible, and fast; thus, SGS is briefly reviewed here (*Table 6*). SGS algorithm can be found in the literature in details (Journel and Alabert, 1989; Deutsch and Journel, 1998).

Table 6. *Main characteristics of IDW, OK and SGS*

Method	Inverse distance weighted (IDW)	Ordinary kriging (OK)	Sequential Gaussian simulation (SGS)
Advantages	• Fast and easy use • Direct interpolation • Widely used	• Best linear unbiased estimator • Measures estimation errors/uncertainty • Estimates unsampled locations • Provide spatial structure • Robust estimator • Local stationary • Knowledge mean of the region studied • Most widely used • Good variogram, good estimation	• No smoothing effect • Quantify uncertainty • Probabilistic map present risk assessment • Reproduce statistics, variogram, histogram and contour plots • Maps show contaminated and uncontaminated areas • Assessment of spatial structure • Evaluation of sampling strategy and design • Most frequently used
Weakness	• Does not measure errors • Smoothing effect • Performance based on size of search area • Select of weighting parameters • Neighbouring points	• Smooth effects on results • Bad variogram, bad estimation • Minimise variance lower than data variance • Performance based on variogram • Quality of data • Size of interpolated blocks	• Great tutorial, expertness and experience necessary • Long-time computerising • More trial and error • Reproductions and number of realisations • Results depend on sampling process • Data organisation and treatment • Neighbouring and search parameters • Block characteristics • Variogram and its parameters

A schematic diagram (*Fig. 2*) exhibits the basic and summary steps involved in the process of SGS. The main advantages of SGS include:

- SGS does not have smoothing effects unlike traditional interpolation methods such as kriging, IDW. SGS ensures to evaluate exactly the high and small values in the data.
- SGS generates maps representing an equally probable spatial distribution and to quantify uncertainty of heavy metals for site exploration.
- SGS produces maps showing contaminated areas and uncontaminated areas across the site.
- SGS is a probabilistic approach that provides probabilistic maps; exhibits a different description of regions into safe and hazardous.
- SGS reproduce descriptive statistics, histogram, variogram and contour plots of spatial characteristics. These are correlated with the same spatial characteristics of original data. This refers to validation tests of SGS.

In conclusion, literature studies demonstrated that risk and quality assessment in decision making should not be based on only kriging estimates. SGS should be operated in uncertainly assessment especially soil contamination with heavy metals. Because SGS ensures local variations in values of a contaminant particularly including design and strategy, estimation procedures, site planning and any risk assessment (e.g. financial).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_38533878

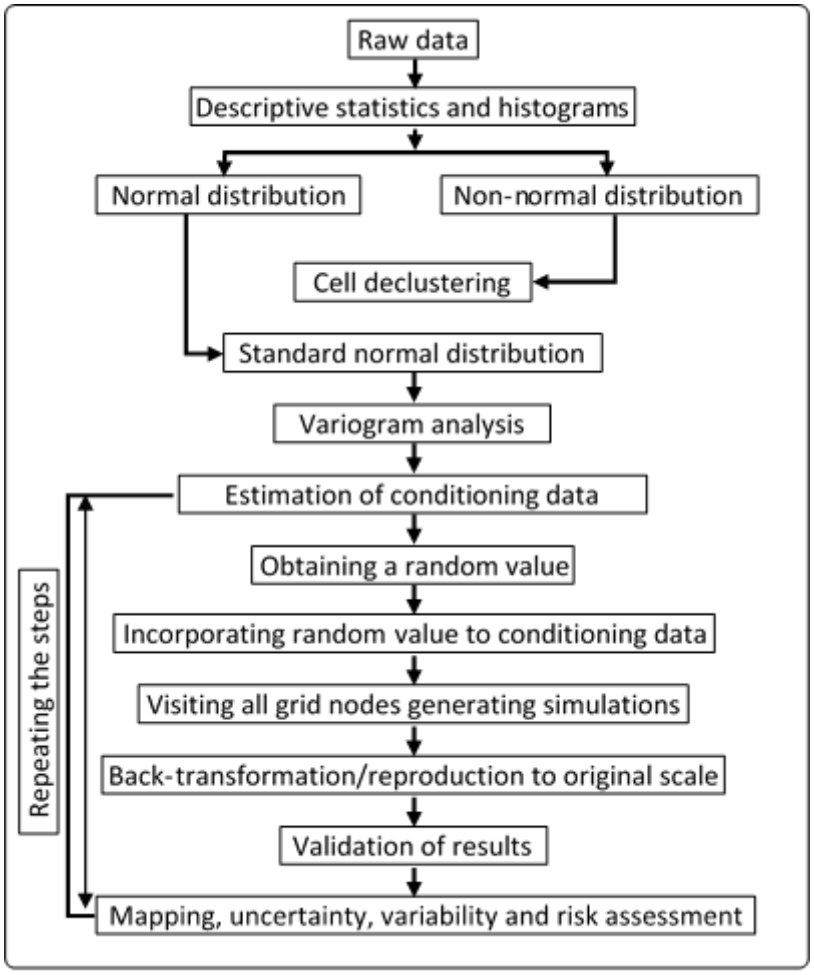

Figure 2. *Schematic diagram showing the basic steps in the process of SGS*

Factors affecting performance of investigation methods (estimation and simulation)

Various factors affect the performance of estimation and simulation methods (Isaaks and Srivastava, 1989; Burrough and Mcdonell, 1998; Zimmerman et al., 1999; Schloeder et al., 2001; Verly, 2005; Wang et al., 2005; Wu et al., 2006; Stahl et al., 2006; Hengl, 2007; Li and Heap, 2011; Xie et al., 2011; Rossi and Deutsch, 2014). These factors can be classified into four groups including sampling process, data organisation and treatment, variogram modelling and model variogram parameters, and cross validation. The factors were usually encountered in the literature.

Sampling process

The quality of soil contamination estimates is dependent on the available data based on the quality of sampling procedures. If the samples are not representative to form sample bias which will directly affect the final contamination estimate the result will not be reliable and accurate.

The estimate performance is generally measured using errors (Sinclair and Blackwell, 2002; Li and Heap, 2011, 2014; Rossi and Deutsch, 2014). There are no perfect measurements (Neufeld, 2005). The relatively large mass of a sample is reduced

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_38533878

to a small subsample from which a few grams are taken to make chemical analysis. There must be a difference between subsample content, the original sample and the analysed (assay) sample. The difference refers to sample error. Rossi and Deutsch (2014) presented that there are two forms of error. One is present due to the intrinsic properties and the material being sampled. The other comes from inappropriate sampling procedures and preparation. In the literature, there are several errors in measurement that are most commonly used including fundamental error, increment delimitation error, increment extraction error, mean or average variance, coefficient of variation, mean absolute error, mean squared error, root mean squared error, relative mean absolute error and relative root mean square error. Fundamental error results from constitution heterogeneity of the material being sampled. This error is random with a mean of zero. However, delamination and exaction errors are mean of non-zero, the errors resulted from improper sampling, and thus bias is related to the sampling procedure.

A variety of issues related to sampling process need to be considered sample collection, handling, preparation, and analysis. However, in this review sampling density or size and sampling design are focused factors especially in environmental studies because these factors were met in previous literature.

Literature studies have usually argued that when the sample density increased, the errors decreased. But as this reached a threshold number of sample density, collected addition of further samples does not improve the performance of estimation methods (Li and Heap, 2008, 2011). However, size of study site plays a significant role on sampling density. Different researchers studied different scales for small survey area or for larger survey area. When sample density is big enough, most estimation methods generate similar results (Burrough and Mcdonell, 1998). Estimation methods produce better results as the sample density increases (Isaak and Srivastava, 1989; Stahl et al., 2006). The effects of sample density on the errors are based on the type of estimation methods (Hengl, 2007; Li et al., 2014; Li and Heap, 2011, 2014). In practical applications, after a number of threshold samples further increase in sample size may not significantly contribute to the accuracy of the estimations and simulations. If sample size does not reach the threshold, it will still be a critical factor. Thus, the impacts of the sample density are controlled by data organisation and treatment as discussed below.

Data organization and treatment

Data is a key factor to influence on the performance of estimation and simulation methods. Major factors related to data quality are summarised here including exploratory data analysis, outliers and declustering.

Exploratory data analysis

The descriptive tabulated and graphical forms have been used to characterise data nature and quality since about 1940. The descriptive summary statistics include mod, median, mean, standard deviation, variance, and standard error of mean, coefficient of variation, skewness, and kurtosis. Histogram, probability, and quantile-quantile plots are graphical applications to assess data distribution. These statistical tools are very useful to construct data quality for several reasons, including understanding of the data and soil contamination, to summarise information, to provide inferences, estimations, and simulations.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_38533878

Normal distribution of the input data can significantly affect estimation and simulation accuracy. If the data are not normally distributed, lognormal transformation is frequently used e.g., lognormal kriging. Other transformation methods may also be applied to obtain normal distribution, resulting in Gaussian kriging and multi-Gaussian kriging (Cressie, 1993). SGS requires normal sore transformation of the original data with zero mean and unit variance.

Histogram is the most basic statistical tool used in exploratory data analysis. Three factors should be considered: arithmetic or logarithmic scaling, arithmetic scaling is appropriate, whereas logarithmic scale clearly represents highly skewed data distribution; range of data; and number of boxes. The mean value is influenced by outliers; the median is affected by missing data. If coefficient of variation is higher than 2 the distribution should be combined with high and low values together.

All data values are given on the probability plot. Different statistical populations can be interpreted. Probability plot is also useful to determine data distribution, straight line on arithmetic scale presents a normal distribution; a straight line on logarithmic scale corresponds a lognormal distribution.

Outliers and declustering

Outliers are extreme values (a small number of very low or very high values) inconsistent with the majority of data values; outliers have significant influence on descriptive statistics and measures of spatial continuity. The extreme values should be removed from the data. There are different ways to identify the outliers: many geostatistical techniques require a transformation of the data that reduce the effect of outliers. Probability plots are useful to check extreme values. Another method is cutting (capping) for identification of outliers. Cutting values higher than cutting threshold (outlier threshold) can regulate to the outlier threshold itself. Outliers can come dramatically to increase the nugget variance of experimental variogram which would mislead us. If outliers are suspected, they should be removed by the identifying technique as explained above.

It is difficult in some situations that whether those values are outliers or not. In such cases, called outliers should be retained. Dowd (1984) and Genton (1998) recommended using the robust variogram estimators which reduce the effects of outliers.

Data are rarely collected randomly. Soil data commonly have relatively high concentration in source zone, thus histogram of raw concentrations is biased. The effect of clustering should be removed to obtain unbiased histogram and summary statistics. Declustering techniques are applied to provide the true form of the spatial distribution and descriptive statistics. Three declustering methods are most commonly used in literature: cell declustering (Journel, 1983), the nearest-neighbour declustering and polygonal declustering (Isaak and Srivastava, 1989). These techniques are well documented in the related references.

Variogram

Estimation (e.g. kriging) and simulation (e.g. SGS) are based on experimental variogram modelling. The variogram parameters consist of lag distance, nugget effect, variance, sill, range, and fitting model. Their definitions can be found in most geostatistical textbooks and software (e.g., Chiles and Delfiner, 2012; Geovariances, 2014; GSLIB: Deutch and Journel, 1998). Oliver and Webster (2014) showed that

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_38533878

reliability of the experimental variogram is affected by several factors including sample size, lag interval and bin width, marginal distribution of the data, anisotropy, and trend.

Accuracy of the experimental variogram is based on the size of the sample. Generally, more data represent more reliability. Webster and Oliver (2014) pointed out that variograms computed from less than 100 data are unreliable. When the sampling interval decreases, the size of the sample increases. Sampling interval provides the applicability of the experimental variogram. If the sampling interval is wider than the correlation range of the study, pure nugget effect will occur in the experimental variogram. This result in estimation is not reliable. Grid sampling is very appropriate in the field to produce well estimation maps and simulation realizations. However, if the grids are coarse, the process may lose the short-range variation significant for the variogram. The solution is that extra samples in the grid should be collected.

Choosing lag distance and bin width significantly affects judgement of the experimental variogram. If lag distance and bin width is short, there will be many variogram models which subject to wide error. In contrast, there will be few models with large lag distance and bin width. In practise, the experimental values should be selected plausibly.

Another important factor influencing variogram is data distribution. Variances incredibly increased as the data positively skewed. Histograms, box-plots and descriptive statistics should be computed to determine the data distribution. Many soil data are strongly and positively skewed in the literature, skewness value of data is greater than 1, and the data will be transformed to logarithms. If the skewness value is between 0.5 and 1, transformation to square roots will make normal distribution. Outliers cause seriously skewed distribution of the data as discussed earlier.

In many cases, data variation is not isotropic, displays anisotropy evidence. Practitioners may ignore or not to detect anisotropy make model improperly. Thus, biased estimations or simulations may emerge. Both directional and omnidirectional experimental variograms of variable/variables should be analysed separately. If the directional variogram reveals anisotropy, we should narrow direction angle to state its expression and to provide the direction of maximum continuity.

Trend can be defined as gradual variation in spatial data. In practise, it is difficult to decide on the trend. Detection of global trend is done by making map from the data with proper software in an easy manner. The map presents gradual continuous variation. Experimental variogram shows increasing changes more steeply when the lag distance increases. We can confirm the presence of trends mapping residuals. Thus, the main direction of the trend can be identified. The experimental variogram is constructed in the direction perpendicular to the principal direct of the trend.

In environmental studies, environmental authorities require predictions of contaminants in blocks that are appropriate size for remediation. Block size is significant for farmer applications of agricultural chemicals such as lime, fertilisers, and pesticides. Oliver and Webster (2014) pointed out that block size is typically 24 m in Europe. In practise, block kriging is more suitable than point estimates (punctual kriging) because block kriging variance is typically much less than punctual kriging variance.

Mapping software uses a moving window based on a chosen size of neighbourhood block in a study area. Although there are no rules to describe the estimation neighbourhood, the following guidance is given by Oliver and Webster (2014):

- If there is good (dense) data, the variogram will have a negligible nugget variance. The radius of the neighbourhood can be chosen close to the range.

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- If a variogram has a large nugget variance, the radius of the neighbourhood can be chosen higher than the range.
- Minimum and maximum number of neighbourhood nearest data to the target is usually recommended 7 and 25, respectively.
- If the data are sparse and unevenly distributed, the neighbourhood is divided into octants. Each octant has at least two data points.
- If the data are irregularly scattered, the neighbourhood will be moved to predict a field of values for mapping.

Cross validation for kriging

Cross validation can be performed to cross-check the candidate models and data by statistical and graphical results. Mean error (ME), mean standardised squared error (MSSE) and mean squared deviation ratio (MSDR- the mean of squared errors (MSE) divided by the referring Kriging variances) is calculated. Perfectly the mean error should be zero, thus Kriging results are unbiased, even poorly produced model. The MSE should be at minimum level, and is composed of good statistics; however, the MSE will not determine a true model. The MSDR is the most testing criteria, it should be 1. If the MSDR is close to 1, this kriging model should be chosen.

Verification of graphical results are the base map, scatter diagram of observed data versus estimated value, histogram of the standardised estimation errors (SEE) and scatter diagram of standardised estimation error versus estimated value. Outliers are depicted on the base map. Scatter plots of observed data versus estimated value show conditional bias and variability. This indicates that true and estimated data should match exactly with each other. Nearly all frequencies of SEE should be equal zero or around zero. Scattered points must be distributed around zero within acceptable limits in graph of estimated value versus SEE. More information related to cross validation of kriging results may be obtained from literature (e.g. Ersoy et al., 2004; Webster and Oliver, 2007).

Cross validation for simulation

Verification of the simulation process can be carried out using a number of tests including summary statistics, histograms, variograms and contour maps. Summary statistics of the simulated data are compared to summary statistics of the raw data. The comparison should be reasonable in good agreement. The histograms of the observed data should be quite similar to the histograms of the simulated realizations. It should be noted that each histogram from produced realizations (e.g., hundred simulated realizations) must be randomly chosen. Simulated data or reproduction variograms are compared to the variograms of the observed data. The comparison should be a good reproduction of spatial variability. The contoured map of the simulated mean data and countered observed data should be presented. The simulation mean values should plausibly reproduce the intrinsic charter of the observed data. More details for validation of simulation results can be found in Ersoy and Yünsel (2019).

Conclusions

The main conclusions of the study are the following points:

- Adequate background research and information identifying sources and types of contaminants should be gathered in preliminary stage for site investigation.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_38533878

- Adequate and good quality data are essential for the assessment of soil contamination by heavy metals.
- Good quality data are controlled by data organisation and treatment such as exploratory data, outlier analysis and declustering.
- There are wide ranges of variations in regulations and soil threshold limits (or heavy metal concentration guideline in soil) for different countries due to different politics, culture, and objectives. Briefly, the most regulations are still dependent on the total concentrations of heavy metals in soil.
- Literature studies demonstrated that there are some similarities and differences between contamination indices. Strengths and limitations of the indices have been compared. Igeo and EF are most commonly and universally applied for a range of contamination. The selection of appropriate index is a key task to understand the degree of contamination, the use of soil, and the aims of contamination indices characteristics. Geochemical background plays a significant role which is based on specific site and scale of contamination assessment. The contamination indices combined with multivariate statistical analysis provide discrimination between natural and anthropogenic heavy metals in soils.
- Multivariate statistical analysis is a better tool than classical univariate statistics identifying sources of heavy metal contamination. Literature research revealed that an integration of multivariate statistical analysis, GIS and geostatistical methods can be accurate and reliable to characterise spatial distribution of heavy metals and to determine their origin.
- Simulation and estimation have different purposes. Simulation provides local variations of a variable examined. Estimation has effect on environmental planning to identify contaminants on scale that physical dispersion can be achieved.
- In theory, simulation is superior to OK, because of its conditional expectation, whereas OK is conditionally biased. In general, performance of kriging techniques is better than interpolation methods (e.g., IDW) or geostatistical methods. However, conventional interpolation methods and kriging techniques have smoothing effects, whereby small values are typically overestimated, while high values are underestimated. Kriging estimates that local error variance is at a minimum level, less than original data variance, thus generally contains bias as a measure of reliability. This has a negative effect on soil contamination or environmental risk assessment. The solution is geostatistical simulation that overcomes kriging and other interpolation methods. SGS is most commonly used for spatial distribution, uncertainty, and risk assessment of heavy metals in soil contamination. The literature review revealed that risk assessment in decision making should not be dependent only kriging estimates. SGS should be carried out in uncertainty assessment, typically heavy elements contamination in soil. Because simulation process results in local variations in values of heavy elements particularly including sampling design and strategy, estimation procedures, site planning and risk assessment (especially financial).
- Performances of geostatistical estimation and simulation are affected by many factors including sampling process, data organisation and treatment, variogram and its parameters; block, search area and neighbourhood characteristics. These factors have been critically and comparatively reviewed.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_38533878

- Multivariate simulation should be applied for soil contamination to create maps assessing uncertainty and representing an equally probable spatial distribution of the heavy metals in soil for future study. These maps will show contaminated and uncontaminated areas in the study site for future studies.
- It is also recommended for future research studies that sequential co-simulation integrated with local singularity analysis is effective, powerful, and useful tool to generate maps for risk analysis and to quantify uncertainty of soil contamination by heavy metals. Uncertainty quantification of soil contamination is a key issue for decision making in environmental analyses.

Acknowledgements. The reviewers and editors are sincerely appreciated for their valuable and constructive comments. This study received no specific grant from any funding agency in the public, commercial, or not-profit sectors.

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DOI: http://dx.doi.org/10.15666/aeer/1905_38533878

EFFECTS OF POTASSIUM HUMATE ON COTTON (*GOSSYPIUM HIRSUTUM* L.) GROWTH AND YIELD AND SOIL SALINITY UNDER FILM-MULCHED DRIP IRRIGATION WITH BRACKISH WATER IN NORTHWEST CHINA

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(Received 21st Apr 2021; accepted 19th Jul 2021)

Abstract. Soil salinity and poor soil structure are the main limitations of the agricultural economy in arid areas. The objectives of this study were to investigate the effects of different potassium humate amounts on cotton growth, seed cotton yield, water use efficiency and soil salinity under film-mulched drip irrigation with brackish water. The results showed potassium humate affected salt accumulation and the salt accumulation rate of potassium humate treatments (2.5, 5, 10, 15, 20, and 30 kg·ha^{-1}) within 0-40 cm soil depths was 63.0%, 67.9%, 68.9%, 70.5%, 75.4%, and 100% lower than the untreated soils. Potassium humate application significantly enhanced the proportion of soil macro-aggregates and the mean weight diameter of water-stable aggregates. Potassium humate significantly increased cotton stem diameter, boll number per plant, plant height, and leaf area index compared to the control. Furthermore, the cotton yield and water use efficiency increased significantly. The potassium humate application amount of 20.5 kg ha^{-1} was recommended as an optimal potassium humate amount of saline soils from the point of view of water saving. The potassium humate application amount of 30 kg·ha^{-1} was recommended as an optimal potassium humate amount of saline soils from the point of view of improving soil quality.
Keywords: *salt leaching efficiency, soil aggregates, mean weight diameter, water use efficiency, growth degree-days*

Introduction

Cotton is the most important renewable natural textile fiber worldwide and the world's sixth-largest source of vegetable oil (Shareef et al., 2018; Zhao et al., 2020). Therefore, optimizing crop productivity is important for sustainable food, feed, fuel, and fiber supplies for the growing human population (Watts et al., 2017). With economic development and population growth, the demand for fresh water resources is gradually increasing (Du et al., 2020).

Water for irrigation is a major limitation to agricultural production in the Xinjiang region. The Xin Jiang region in Northwest China is one of the most important cotton producers. The cotton plantation area in Xinjiang is 1.8×10^6 ha, accounting for 54% of China's total cotton planting area (Kuang et al., 2018). It produced 451×10^4 t of cotton (*Gossypium hirsutum* L.) in 2014, accounting for 73% of China's total cotton production (Tian et al., 2017). Meanwhile, surface water evaporation caused by high temperatures results in a severe water shortage in southern Xinjiang leading to soil salinization, a lowered survival rate for crops, and slow development of local agriculture (Fang et al., 2019). Brackish water can bridge the water supply gap. However, increased use of brackish water for irrigation exacerbates the soil salinization problems, and reduce crop yield (Mahmoodi-Eshkaftaki and Rafiee, 2020; Sekhon et al., 2020).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_38793895

Moreover, soil salinity is one of the most widespread soil degradation processes worldwide and China has the third-largest area of saline-alkali soil (Nan et al., 2020). The global area of saline-alkali soils is approximately 900 million ha (Rath et al., 2019). In China, approximately 3.67 million ha of soil, which represents 4.88% of the total available land across the country, is threatened by salt (Li et al., 2014). The accumulation of salt can directly decrease soil nutrient efficiency by inhibiting microbial mineralization activity in saline soil (Rath and Rousk, 2015). Additionally, salinity can also indirectly affect soil nutrient cycling and efficiency by destroying soil physical structure (Lakhdar et al., 2009; Zhang et al., 2015). High salinity, soil structure degradation, and nutrient deficiencies are the three characteristics of saline-alkali soil that inhibit plant growth and decrease crop yields (Zheng et al., 2018). Thus, the improvement of saline-alkali soil has attracted widespread attention.

Potassium widely exists in peat, lignite and weathered coal, an organic macromolecule with good biological activity. Potassium humate has been reported as a practical and economical option to improve degraded land resources. Furthermore, potassium humate is a common organic fertilizer that can improve soil structure and influence soil microbial activity, thus improving salt-affected soil fertility (Ouni et al., 2014). Khaled et al. (2011) studied the effects of the application of humic acid gradient on soil nutrient content, soil properties and maize growth in saline-alkali land, and found that application of 2 $g \cdot kg^{-1}$ soil humic acid reduced maize's absorption of nitrogen and increased the dry matter weight of maize under salt stress. Ahmed et al. (2020) informed that the application of potassium humate could reduce nitrogen loss in soil. Izhar et al. (2020) found that the application of humic acid can improve crop yield and phosphorus uptake in calcareous soils. Saidimoradi et al. (2019) explored that inclusion of humic acid in the nutrient solution of hydroponically grown strawberry improved plant responses to salinity. Nonetheless, further study is still needed to understand better the effects of potassium humate on cotton in arid-saline soil under conjunctive brackish water irrigation. The present study was conducted to exploit the potential of potassium humate as a treatment for low quality soil and water resources used in cotton production in the Xinjiang region of Northwest China. The primary objectives are to investigate: (1) cotton plant height, leaf area index, aboveground biomass, and yield; (2) soil salinity; (3) the optimal application amount of potassium humate.

Materials and methods

Site description

Field studies were conducted during 2019 at the Bazhou Irrigation Experiment Station (N41°45'20.24", E86° 8'51.16", 901 m) in Korla City, Xinjiang Province, Northwest China. Korla is warm temperate zone with an arid continental arid climate. It has a 226 d frost-free period with 3036 h sunshine hours. The average annual potential evaporation is 2278.2 mm. During the cotton growing season, the long-term average annual temperature and precipitation are 22.5 °C and 70 mm. Characteristics of the daily weather conditions, including daily precipitation and average air temperature, are shown in (*Fig. 1*). The weather data were obtained from the field using an automatic meteorological station. The average temperature in the cotton growth period (April-September) was 24.2 °C, and the total precipitation in this period was 64.4 mm.

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The soil was classified as sandy loam with an average particle size distribution of approximately 41.4% sand, 54.4% silt and 4.2% clay (USDA, 2020), with an average soil bulk density of 1.54 $g \cdot cm^{-3}$, a pH of 8.75, 76.8 $mg \cdot kg^{-1}$ of total organic matter, 3.92 $mg \cdot kg^{-1}$ of total nitrogen, 31.1 $mg \cdot kg^{-1}$ of available phosphorus of and 72.0 mg kg^{-1} of available potassium. The groundwater depth is over 7 m. The electronic conductivity (EC) of groundwater is 2.73-2.95 $ms \cdot cm^{-1}$. The total dissolved solids of groundwater is 2.2 $g \cdot L^{-1}$. A comprehensive list of the and the chemical properties of groundwater is given in *Table 1*.

Table 1. *The chemical properties of groundwater during the cotton growing season*

Properties	pH	HCO^{3-} ($g \cdot L^{-1}$)	Cl^{-} ($g \cdot L^{-1}$)	SO_4^{2-} ($g \cdot L^{-1}$)	Ca^{2+} ($g \cdot L^{-1}$)	Mg^{2+} ($g \cdot L^{-1}$)	K^{+} ($g \cdot L^{-1}$)	Na^{+} ($g \cdot L^{-1}$)
Value	7.38	0.401	0.335	1.110	0.227	0.149	0.029	0.369

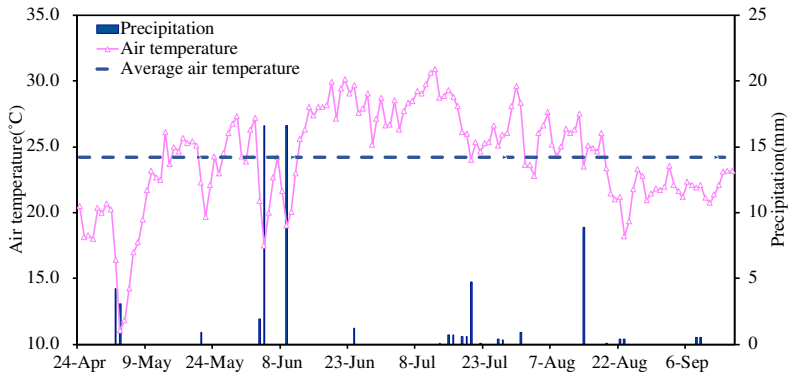

Figure 1. *Daily air temperature and precipitation during the cotton growing seasons*

Experimental treatments and design

A drip irrigation system under film mulch was selected as the method of cultivation. The experiments consisted of seven application levels (0, 2.5, 5, 10, 15, 20, and 30 $kg \cdot ha^{-1}$) of potassium humate. The CK, HA1, HA2, HA3, HA4, HA5 and HA6 treatments represented 0, 2.5, 5, 10, 15, 20, and 30 $kg \cdot ha^{-1}$ of potassium humate, respectively. The water-soluble potassium humate was uniformly sprayed on the soil surface of each plot, and then plowed from the surface to 30 cm using a rotary cultivator. Therefore, the depth of the soil layer mixed with biochar was 0-30 cm. The treatments were replicated three times in a randomized block design. The irrigation schedule for cotton in Xinjiang is shown in *Table 2*.

A 16 mm diameter inlaid, thin-walled, labyrinth drip line was used for irrigation. The average discharge of the emitters was 30 cm, while the cotton plants along each row were spaced 10 cm apart. Water meters and ball valves were installed to control the amount of water applied to each plot. Each field plot was 5.6 m wide and 10 m long.

Urea and potassium dihydrogen phosphate were used as fertilizer, which were applied to the fields at 14 different times. Except for the early cotton growth, each fertilizer application occurred by drip irrigation during the middle of each irrigation stage. Differential pressure tanks with 25 L capacity were used for fertilizers. The solid fertilizers were dissolved in the water one day before irrigation.

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Table 2. *Irrigation scheduling for cotton in Xinjiang*

Irrigation time	Irrigation date	Cotton growing stage	BBCH scale	Irrigation amount (mm)
1	18-June	Seedling and squaring stage	1-59	30
2	23-June			30
3	29-June			30
4	4-July			30
5	11-July			30
6	16-July	Flowering stage	60-69	30
7	21-July			30
8	26-July			30
9	1-Aug	Bolls and boll-opening stage	71-88	30
10	6-Aug			37.5
11	11-Aug			37.5
12	16-Aug			37.5
13	21-Aug			37.5
14	26-Aug			37.5
15	31-Aug			30
Total				487.5

Cotton was planted following the cultivation mode of plastic film mulching with short rod dense planting (*Fig. 2*). The system was installed with a row configuration of 20 cm + 40 cm + 20 cm (narrow-wide-narrow). Two driplines were installed for four rows under a 1 m-wide film. The distance between the two films was 60 cm.

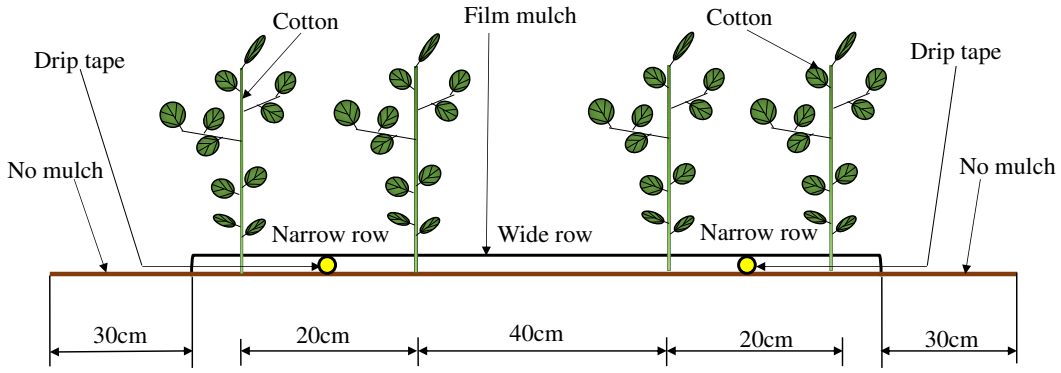

Figure 2. *The relative positions of row spacing, dripline, and plastic film*

Measurements and calculations

Leaf area and leaf area index

At the seedling stage, bud stage, flowering phase, fluid phase and boll-opening stage, four plants were randomly selected from each plot, and the length and width of each leaf on the plants were measured using a tape measure to obtain the leaf area (Kumar et al., 2020).

The green leaf area plant of the plant was calculated using the following equation:

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$$LA = \sum_{i}^{n} a_i \times b_i \times 0.703 \quad \text{(Eq.1)}$$

where LA represents the leaf area for the single cotton plant (cm^2), a_i (cm) and b_i (cm) were length and width of one green leaf, n is the number leaf per plant and 0.703 is the correction factor for the cotton crop (Tan, 2018).

The leaf area index (LAI) was then calculated as follow (Wang et al., 2018; Watson, 1937):

$$LA = LAI / S_O \quad \text{(Eq.2)}$$

where LA_T represents the total area of the leaf (cm^2), and S_O represents the occupied land area (cm^2).

Aboveground dry matter accumulation

At the seedling stage, bud stage, flowering phase, fluid phase and boll-opening stage, four plant were randomly selected from each plot. The leaves, stem, and bolls were placed into an oven at 105 °C for 30 min and then dried at 75 °C to a constant weight.

A logistic regression model was fitted to describe cotton aboveground biomass (Khan et al., 2017).

$$AM = \frac{AM_{MAX}}{1 + e^{(a - b*GDD)}} \quad \text{(Eq.3)}$$

where GDD (°C) is the growing degree-days after sowing, AM (g) is the aboveground biomass, AM_{max} (g) is the maximum aboveground biomass and a and b are the constants to be assessed.

Growing degree-days (GDD), are frequently used to describe the timing of biological processes. The basic equation as follows (Mcmaster and Wilhelm, 1997):

$$GDD = \left[\left(T_{MAX} + T_{MIN}\right)/2\right] - T_{BASE} \quad \text{(Eq.4)}$$

where T_{MAX} and T_{MIN} are daily maximum and minimum air temperature, respectively, and T_{BASE} is the base temperature of cotton (10 °C).

Based on *Equation 1,* the following equations were calculated:

$$GDD_1 = \frac{a - \ln(2 + \sqrt{3})}{b} \quad \text{(Eq.5)}$$

$$GDD_1 = \frac{a - \ln(2 - \sqrt{3})}{b} \quad \text{(Eq.6)}$$

$$V = \frac{b * AM_{max}}{4} \quad \text{(Eq.7)}$$

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$$GDD_3 = \frac{a}{b} \quad \text{(Eq.8)}$$

where V (g·°C) is the highest aboveground biomass rate, GDD_3 (°C) is the growing degree days of largest aboveground biomass accumulation, which initiates at temperature GDD_1 and terminates at GDD_2.

Crop evapotranspiration calculations

During the growth of cotton, crop evapotranspiration (water consumption) could be calculated via the water balance equation as follows (Zhou et al., 2019):

$$ET_c = P + I + G + \Delta W - R_0 - F \quad \text{(Eq.9)}$$

where ET_c is crop evapotranspiration also called water consumption (mm); P is precipitation in the growing period (mm); I is irrigation (mm); G is groundwater recharge (mm); ΔW was calculated as change in soil water storage in the 0-100 cm soil layer from sowing to maturity (mm); R_0 is surface runoff (mm); F is deep percolation (mm). The water table in the experimental-area was below 7 m. Rainfall was very scarce during the growing cotton period; hence the G, R_0, and F could be negligible in this research, respectively. Therefore, the equation can be written as follows:

$$ET_c = \pm\Delta W + P + I \quad \text{(Eq.10)}$$

Soil salinity content and salt accumulation

Soil samples were collected to measure soil salinity content at a 10 cm interval from 0 to 40 cm and at a 20 cm interval from 40 to 100 cm with using an auger (5 cm diameter). All collected soil samples were air-dried, sieved through a 1 mm sieve, and then were used for preparing dilute soil extract solutions based on extract solutions based on extracts with a 1:5 soil–water ratio. Salt concentrations were inferred from the measured electrical conductivity (EC) values (Tan et al., 2018).

$$SC = EC_{1:5} \times 4.25 \quad \text{(Eq.11)}$$

where SC is the soil salinity content ($g \cdot kg^{-1}$) and the number 4.25 is a conversion factor used to convert $EC_{1:5}$ extract to mass salt per unit mass of soil.

The salt balance in an irrigated field zone over a time interval is related to the salt inputs and outputs (Ning et al., 2020).

$$\Delta S = S_2 - S_1 = S_R + S_I + S_G + S_F - S_C - S_P - S_D \quad \text{(Eq.12)}$$

where ΔS (g) is the change of salt storage in the soil; S_1 (g) is the salt content in the soil before sowing; S_2 (g) is the salt content in the soil after harvesting; S_R, S_I, S_G and S_F are salt concentration during precipitation, irrigation, groundwater, and fertilizer; S_C is the salt uptake by the crop; S_P is the total removal of salts by absorption, precipitation and transformation; and S_D is the salt carried away by underground drainage or seepage. The

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groundwater level of the experimental field is 7 m and So S_G, S_P, S_D equals cero. The S_C and S_P are also small and negligible.

Soil aggregate stability

Moreover, at harvesting stage, soil samples were collected from each experimental plot with an auger in 0-15 cm soil layer, and passed through a 2-mm sieve and soil samples were analyzed by wet sieving method (Nie et al., 2018). Water-stable aggregates were classified as the three different aggregate size fractions: 2-1 mm, 1-0.25 mm and < 0.25 mm fractions. Each fraction was weighed to calculate the dry aggregate stability expressed by mean weight diameter (MWD). The MWD was calculated from the mass fraction of soil remaining on each sieve as (Sheehy et al., 2015):

$$MDW = \sum_{i=1}^{n} A_i * W_i \quad \text{(Eq.13)}$$

where A_i is the mean diameter of the three aggregate size classes; W_i is the mass proportion of aggregate size classes remaining on each sieve.

Data analysis

The value of each indicator was the mean of three replicates per treatment, and the SPSS statistics v.22 (IBM, Inc, Chicago, IL, USA) was used to perform analysis of variance. All pair-wise comparisons of the treatment means were performed using the least significant difference (LSD) test with significance determined at the 5% level.

Results

Soil salinity

We defined the desalination rate as $DR = \Delta S / S_i \times 100\%$. In this study, the salt accumulation rate of CK, HA1, HA2, HA3, HA4 and HA5 treatments were 40.3%, 14.9%, 12.9%, 12.5%, 11.9% and 9.9% for the 0-40 cm depth range, respectively. The desalination rate of HA6 treatment was 42.7% for 0-40 cm the depth range. At the 0-100 cm depth range the deposition rate of CK, HA1, HA2, HA3, HA4, HA5 and HA6 treatments were 56.4%, 14.2%, 39.4%, 58.2%, 14.6%, 12.1% and 9.8%, respectively (*Table 3*).

Water-stable aggregates and mean weight diameter (MWD)

The proportion of soil micro-aggregates (<0.25 mm) was significant lower in HA2, HA3, HA4, HA5 and HA6 treatments than that of the CK ($P < 0.01$). However, potassium humate treatments significantly increased the proportion of large soil macro-aggregates (2-1 mm) and small soil macro-aggregates compared with the CK (1-0.25 mm) ($P < 0.01$). This showed that application of potassium humate could enhance formation of soil macro-aggregates (0.25-2 mm). Moreover, the mean weight diameter of soil aggregates was significantly ($P < 0.01$) higher in potassium humate treatments than that of the CK, which indicated application of potassium humate increased the stability of soil aggregates. Hence, potassium humate application was effective in improving the quality of saline soil (*Table 4*).

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Table 3. *Salt accumulation and salt-leaching efficiency at 0-40 cm and 0-100 cm soil depth for different potassium humate application treatments*

Soil depth (cm)	Treatments	S_i (g·m^{-2})	ΔS (g·m^{-2})	Accumulation rate (%)	Desalination rate (%)
0-40	CK	425.8 ± 26.3f	287.6 ± 7.5b	40.3 ± 0.02a	-
	HA1	2238.7 ± 39.3b	393.5 ± 13.8a	14.9 ± 0.01b	-
	HA2	552.2 ± 20.6e	82.1 ± 57.1e	12.9 ± 0.08b	-
	HA3	297.7 ± 44.9g	42.7 ± 32.0e	12.5 ± 0.1b	-
	HA4	1715.7 ± 35.6c	232.1 ± 45.1c	11.9 ± 0.02b	-
	HA5	1546.2 ± 37.3d	170.8 ± 17.1d	9.9 ± 0.01b	-
	HA6	3643.9 ± 36.5a	-1089.8 ± 10.8f	-	-42.7 ± 0.01d
0-100	CK	872.2 ± 77.5E	1129.9 ± 135.0A	56.4 ± 0.05A	-
	HA1	6224.9 ± 277.6B	1033.0 ± 16.9A	14.2 ± 0.01CD	-
	HA2	1557.1 ± 50.3D	1015.1 ± 35.5A	39.4 ± 0.01B	-
	HA3	698.8 ± 53.2E	875.9 ± 77.2B	20.2 ± 0.03A	-
	HA4	4440.9 ± 163.9C	763.1 ± 59.8B	14.6 ± 0.01C	-
	HA5	4307.4 ± 253.2C	594.8 ± 110.4C	12.1 ± 0.02CD	-
	HA6	7518.7 ± 245.7A	818.0 ± 77.4B	9.8 ± 0.01D	-

Data are mean of the three replicates. The different lowercase letters indicate significant differences between treatments of 0-40 cm depth at $P < 0.05$ level according to the LSD test; the same letters are not significantly different at $P > 0.05$ level according to the LSD test. The different capital letters indicate significant differences between treatments of 0-100 cm depth at $P < 0.05$ level according to the LSD test; the same letters are not significantly different at $P > 0.05$ level according to the LSD test

Table 4. *Effects of different potassium humate amounts on water-stable aggregates distribution and mean weight diameter (MWD) in 0-15 cm soil layer*

Treatments	2-1 mm (%)	1-0.25 mm (%)	<0.25 mm (%)	MWD (mm)
CK	15.44 ± 1.36e	37.31 ± 0.97de	47.25 ± 0.41a	0.53 ± 0.02f
HA1	16.22 ± 1.16e	36.53 ± 1.69e	47.25 ± 2.54a	0.53 ± 0.02f
HA2	19.35 ± 0.75d	39.03 ± 0.92cde	41.62 ± 1.67b	0.59 ± 0.02e
HA3	21.48 ± 1.34cd	40.23 ± 1.04bcd	38.29 ± 2.38b	0.62 ± 0.03d
HA4	23.71 ± 0.61bc	42.21 ± 1.03bc	34.09 ± 0.76c	0.66 ± 0.01c
HA5	26.20 ± 1.63ab	43.48 ± 2.93b	30.32 ± 3.13d	0.70 ± 0.03b
HA6	27.43 ± 2.51a	47.30 ± 2.71a	25.26 ± 1.68e	0.74 ± 0.03a
Source of variance				
Potassium humate	**	**	**	**

Data are mean of the three replicates. The different letters indicate significant differences between treatments at $P < 0.05$ level according to the LSD test; the same letters are not significantly different at $P > 0.05$ level according to the LSD test. ** indicates significant differences between treatments at $P < 0.01$

Plant stem diameter, plant height, leaf area index and aboveground biomass

Stem diameter increased first and then stabilized with the increase of growing degree days. Stem diameter increased rapidly between 742 °C and 1096 °C. The stem diameter in each treatment was as follows: HA5 > HA4 > HA1 > HA2 > HA6 > HA3 > CK. The

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maximum stem diameter was obtained in HA5 treatment. HA5 treatment significantly increased the stem diameter compared to CK ($P < 0.05$). HA5 treatment increased stem diameter by 9.3%, 10.5%, 6.4%, 8.2%, 1.3% and 10.7%, compared to CK, HA1, HA2, HA3, HA4 and HA6, respectively (*Fig. 3a*). Potassium humate treatments significantly affected plant height ($P < 0.05$). Plant height increased first and then stabilized with the increasing of growing degree days. The plant height in HA1, HA2, HA3, HA4, HA5 and HA6 treatments increased 6.22%, 12.98%, 16.93%, 19.48%, 18.82% and 20.65%, respectively, compared to CK treatment (*Fig. 3b*). The maximum plant height was obtained in HA6 treatment.

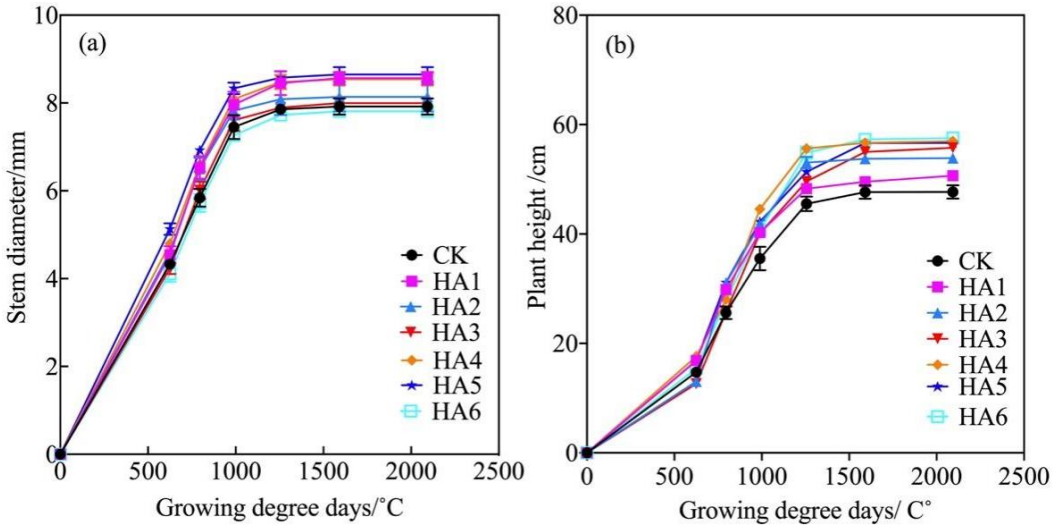

Figure 3. *Effect of potassium humate application on stem diameter and plant height during the cotton growth period. Bars are the means + one standard error of the mean (n = 3)*

The leaf area index increased first and then decreased with the increase of growing degree days. Leaf area index in HA1, HA2, HA3, HA4, HA5 and HA6 treatments significantly increased 0.73%, 4.3%, 7.8%, 7.6%, 11.7% and 16.8%, respectively, compared to CK treatment ($P < 0.05$). The maximum leaf area index was obtained in the HA6 treatment (*Fig. 4a*). The aboveground biomass accumulation increased following a normal logistic function by growing degree days (*Fig. 4b*). The aboveground biomass in HA1, HA2, HA3, HA4, HA5 and HA6 significantly increased 5.96%, 10.13%, 19.73%, 25.53%, 30.28% and 36.29%, respectively, compared to CK treatment ($P < 0.05$). The maximum aboveground biomass was obtained in HA6 treatment.

Simulation of biomass accumulation

Simulation of aboveground biomass with growing degree days (GDD) was assessed using *Equation 3* and the result are shown in *Tables 5* and *6*. The logistic function was followed by aboveground biomass accumulation as a sigmoidal growth pattern, although they differed in equation coefficients among treatments. Calculation by *Equations 4–6* showed the beginning and termination at fast accumulation growing degree-days for cotton aboveground biomass accumulation. HA6 began the fast accumulation growing degree days at 1002.0 °C and ended at 1677.5 °C, and maximum (0.09 $g \cdot °C^{-1}$) rates at 1339.8 compared with other treatment (*Table 6*).

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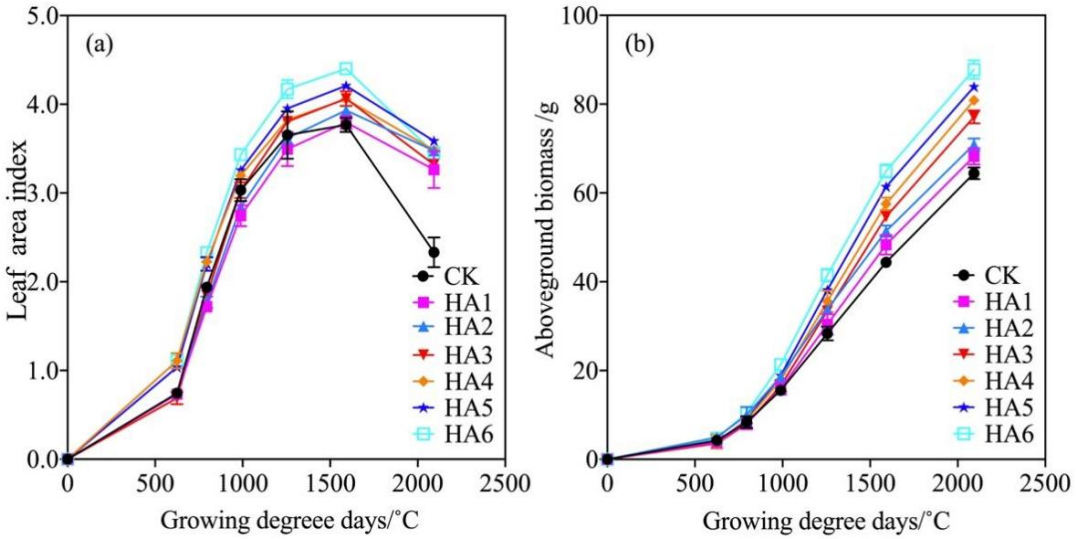

Figure 4. *Effect of potassium humate application on leaf area index and aboveground biomass during the cotton growth period. Bars are the means + one standard error of the mean (n = 3)*

Table 5. *Parameters (AM_{max}, a, b) of the logistic model of dry matter accumulation for each treatment*

Treatments	AM_{max}/g	a	b
CK	70.29 ± 2.26e	4.7448 ± 1.30b	0.0034 ± 0.0010b
HA1	72.81 ± 1.45de	5.0743 ± 0.35a	0.0037 ± 0.0002ab
HA2	75.84 ± 1.39d	4.7309 ± 0.18ab	0.0035 ± 0.0001ab
HA3	81.54 ± 1.49c	5.3981 ± 0.20a	0.0039 ± 0.0002ab
HA4	85.61 ± 1.20b	5.2951 ± 0.40a	0.0039 ± 0.0011ab
HA5	87.86 ± 1.41b	5.4497 ± 0.22a	0.0041 ± 0.0002a
HA6	92.06 ± 2.65a	5.2251 ± 0.19a	0.0039 ± 0.0002ab

Data are mean of the three replicates. The different letters indicate significant differences between treatments at $P < 0.05$ level according to the LSD test; the same letters are not significantly different at $P > 0.05$ level according to the LSD test

Table 6. *Parameters (GDD1, GDD2, V, GDD) of the logistic model of dry matter accumulation for each treatment*

Treatments	GDD_1/°C	GDD_2/°C	V/g·°C^{-1}	GDD/°C
CK	1008.2 ± 176.7a	1782.9 ± 29.0a	0.060 ± 0.022b	1395.6 ± 30.3a
HA1	1015.5 ± 48.9a	1727.4 ± 39.9a	0.067 ± 0.005ab	1371.4 ± 40.3a
HA2	975.4 ± 19.3a	1728.0 ± 10.3a	0.066 ± 0.003ab	1351.7 ± 10.1a
HA3	1046.4 ± 11.3a	1721.8 ± 17.1a	0.079 ± 0.004ab	1384.1 ± 6.5a
HA4	1020.0 ± 65.2.8a	1695.4 ± 10.1a	0.083 ± 0.026ab	1357.7 ± 12.5a
HA5	1008.0 ± 15.5a	1650.4 ± 10.0a	0.090 ± 0.004ab	1329.2 ± 4.3a
HA6	1002.1 ± 11.1a	1677.5 ± 18.8a	0.090 ± 0.004a	1339.8 ± 8.5a

Data are mean of the three replicates. The different letters indicate significant differences between treatments at $P < 0.05$ level according to the LSD test; the same letters are not significantly different at $P > 0.05$ level according to the LSD test

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Boll weight, boll number, seed cotton yield and water use efficiency

Boll weight of HA1, HA2, HA3, HA4, HA5 and HA6 increased 5.1%, 11.2%, 7.8%, 11.2%, 16.7% and 12.8%, respectively, compared to CK treatment. The boll number per plant in the HA5 treatment was significantly higher than those in the other six treatments ($P < 0.05$) (*Fig. 5a*). Boll number of HA1, HA2, HA3, HA4, HA5 and HA6 increased 8.6%, 9.4%, 16.7%, 15.2%, 24.5% and 15.9%, respectively, compared to CK treatment. Seed cotton yield in the HA5 treatment was significantly higher than those in the other six treatment ($P < 0.05$) (*Fig. 5b*). Seed cotton yield of HA1, HA2, HA3, HA4, HA5 and HA6 increased 13.3%, 19.7%, 23.4%, 24.8%, 37.2% and 26.8% (*Fig. 5c*), compared to CK treatment. Water use efficiency of HA1, HA2, HA3, HA4, HA5 and HA6 increased 14.8%, 15.5%, 14.0%, 15.5%, 23.4% and 16.9%, compared to CK treatment (*Table 7*).

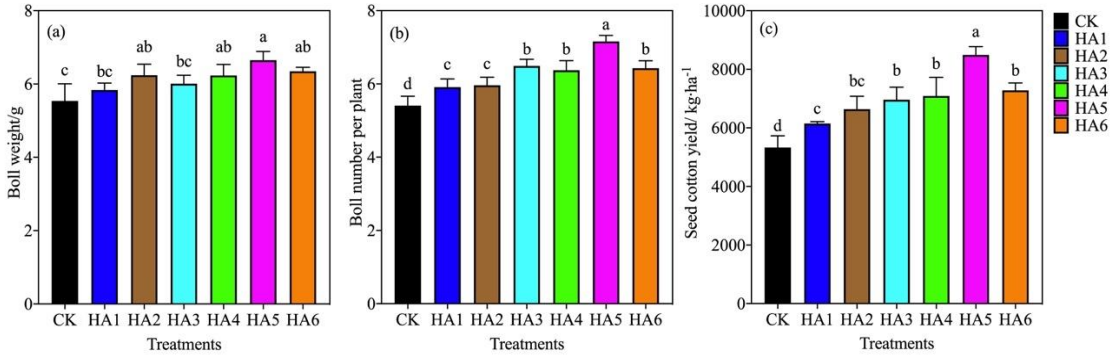

Figure 5. *Effects of different amounts of potassium humate on the boll weight, boll number and seed cotton yield. Bars are the means + one standard error of the mean (n = 3). Different letters above the bars indicate a significant difference at P < 0.05 according to an LSD test*

Table 7. *Water use efficiency and yield of cotton under different potassium humate treatments*

Potassium humate treatment	P (mm)	I (mm)	ΔW (mm)	ET_c (mm)	Y (kg·ha^{-1})	WUE (ka·ha^{-1}·mm^{-1})
CK	64.4	487.5	68.1 ± 6.3a	620.0 ± 6.3a	6058.2 ± 366.8c	9.8 ± 0.5c
HA1	64.4	487.5	44.7 ± 4.2b	596.6 ± 4.2b	6859.4 ± 262.4b	11.5 ± 0.4b
HA2	64.4	487.5	47.3 ± 3.5b	599.2 ± 3.5b	6942.7 ± 398.3ab	11.6 ± 0.6b
HA3	64.4	487.5	44.9 ± 2.5b	596.8 ± 2.5b	6776.1 ± 446.7b	11.4 ± 0.7b
HA4	64.4	487.5	43.9 ± 3.3b	595.8 ± 3.3b	6921.6 ± 412.2ab	11.6 ± 0.6b
HA5	64.4	487.5	46.6 ± 3.5b	598.5 ± 3.5b	7653.2 ± 570.0a	12.8 ± 0.9a
HA6	64.4	487.5	63.9 ± 4.0a	615.8 ± 4.0a	7277.3 ± 262.3ab	11.8 ± 0.4ab

Data are mean of the three replicates. The different letters indicate significant differences between treatments at P < 0.05 level according to the LSD test; the same letters are not significantly different at P > 0.05 level according to the LSD test

The relationship between potassium humate application and seed cotton yield could be described with a quadratic curve (*Fig. 6*); thus, a quadratic regression equation was

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DOI: http://dx.doi.org/10.15666/aeer/1905_38793895

established (*Table 8*). Seed cotton yield did not increase under a potassium humate application level of 30 kg·ha^{-1}.

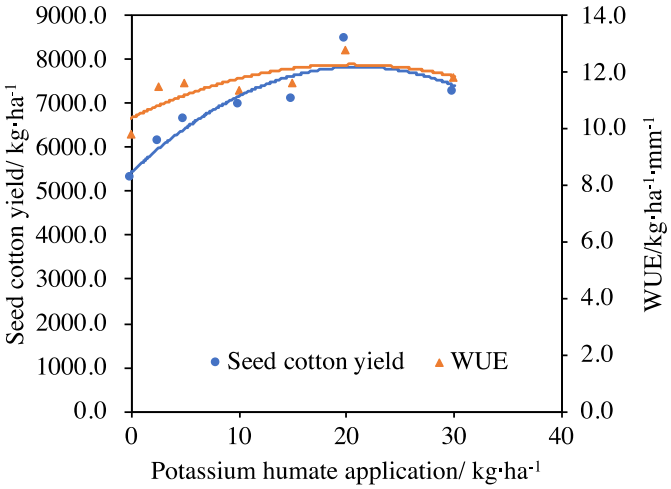

Figure 6. *Relationships between seed cotton yield, irrigation water use efficiency and potassium humate application*

Table 8. *Regression equations between potassium humate application, seed cotton yield, and WUE*

Response variable Y	Regression equation	R^2
Seed cotton yield/Y1	$Y1 = -5.3724X^2 + 226.87X + 5446.2$	$R^2 = 0.85$
WUE/Y2	$Y2 = -0.0045X^2 + 0.1847X + 10.392$	$R^2 = 0.64$

Discussion

Dry (arid and semi-arid) and soil saline regions are characterized by water scarcity. In these regions, saving irrigation water and sustainable soil use with no significant loss of crop yield is an urgent necessity. Studies have demonstrated that the potassium humate application can enhance plant growth and production and salinity control of the rhizosphere (Liang et al., 2007; Imbufe et al., 2005), and our results concur with those findings. Our results showed that application of potassium humate at 2.5, 5, 10, 15, 20, and 30 kg·ha^{-1} could improve seed cotton yield by 13.3%, 19.7%, 23.4%, 37.2% and 26.8%, respectively, and increase, WUE by 14.8%, 15.5%, 14.0%, 15.5%, 23.4% and 16.9%, compared to CK treatment (*Table 6*). The average amount of irrigation for all treatments (CK, HA1, HA2, HA3, HA4, HA5 and HA6) was 487.5 mm during the entire cotton growing season. High seed cotton yield in potassium humate treatments was 6859.4-7653.2 kg·ha^{-1}. Correspondingly, high water use efficiency was 9.8-12.8 ka·ha^{-1}. In contrast, the amount of irrigation required by local farmers was approximately 600 mm during the entire cotton growing season, and the seed cotton yield obtained (without using potassium humate in field) was about 6700 kg·ha^{-1}. Thus, the water use efficiency of local farmers was 11.1 kg·ha^{-1}. Compared to the local irrigation practice without potassium humate, water use efficiency applied potassium humate in the cotton field was 1.15 times higher. The application of potassium humate could save up to 13% water. Grant et al. (2017) showed that cotton yield decreased with

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DOI: http://dx.doi.org/10.15666/aeer/1905_38793895

apparent over-irrigation in soil with low water holding capacity. Therefore, local farmers who did not have access to efficient water irrigation system obtain lower than optimal cotton yield. Thus, we recommended that local farmers use potassium humate in the cotton field to save irrigation water and achieve optimal cotton yield.

The soil with well-stabilized soil aggregates structure had the excellent ability to storing nutrients and soil water (Angers et al., 1992; Liang and Shi, 2020). In current study, potassium humate significantly increased the proportion of soil macro-aggregates, which implied potassium humate could promote the formation of macro-aggregates in saline soil under film mulched drip irrigation. Similar results were also found by Mostafa (2011). Bongiovanni and Lobartini (2006) reported that humic matter, considered as a persistent cementing agent, is involved in stabilizing microaggregates. These microaggregates are bound into macroaggregates, due to the effect of transient binding agents (polysaccharides derived from plants and microorganisms and temporary binding agents (fungal hyphae, fine roots, bacterial cells) (Tisdall and Oades, 1982; Odades, 1993). Particulate organic matter (POM) improves the soil aggregation since it can form an organic core surrounded by clay, silt particles, and aggregates (Six et al., 2004). This was an explanation that potassium humate resulted in the increase of macro-aggregates. In the saline alkaline soil, Ca^{2+} is gradually replaced by Na^{+} at soil exchangeable sites, which process caused degradation of soil structure (Dai et al., 2019). Wu et al. (2021) reported that the correlation between exchangeable Ca and MWD was positive, and the correlation between exchangeable Na^{+} and MWD was negative. In this study, greater MWD was in the treatments with potassium humate rather than CK, suggesting that saline potassium humate improved the stability of water-stable aggregates.

Liu et al. (2020) reported that humic acid improved could reshape the microstructure of macroaggregates by replenishing organic matter, which enhanced salt leaching. In current study, the salt accumulation rate of none application of potassium humate on the balance with 0-40 cm and 0-100 cm soil layer in brackish water irrigation treatment was higher than potassium humate treatments (*Table 3*). The HA6 treatment could leach the soil salinity with 40 cm soil layer.

The potassium humate application played an important role in decreasing the soil salinity, improving seed cotton yield, and water use efficiency. The optimal application amount of potassium humate requires taking these components into account. In experiments presented here, the relationship between cotton yield and the amount of potassium humate and between WUE and the amount of potassium humate followed quadratic curves (*Fig. 6*). The determinate coefficients of the curves were 0.85 and 0.64 for cotton yield and WUE, respectively. The potassium humate production function (*Fig. 6*) for different treatments showed that the highest yield and WUE were obtained at a potassium humate amount of 21.1 $kg \cdot ha^{-1}$ and 20.5 $kg \cdot ha^{-1}$, respectively.

Conclusions

Our findings indicate that in saline soils, application of potassium can improve cotton growth, yields, water use efficiency while at the same time potentially reducing salt accumulation and improving soil macro-aggregates. The highest seed cotton yield and water use efficiency was obtained at potassium humate application amounts of 20 kg ha^{-1} in this study. The highest salt leaching efficiency was obtained at potassium humate application amount of 30 $kg \cdot ha^{-1}$ in this study. The accumulation of salt was

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DOI: http://dx.doi.org/10.15666/aeer/1905_38793895

very small at potassium humate application amount of 20 kg·ha^{-1}. According to the results of regression equation between seed cotton yields, water use efficiency and potassium humate application amount, the highest yield and WUE were obtained at a potassium humate amount of 21.1 kg·ha^{-1} and 20.5 kg·ha^{-1}, respectively. The potassium humate application amount of 20.5 kg·ha^{-1} was recommended as an optimal potassium humate amount of saline soils from the point of view of water saving. The potassium humate application amount of 30 kg·ha^{-1} was recommended as an optimal potassium humate amount of saline soils from the point of view of improving soil quality. Exploring the physiological and ecological functional potential of potassium humate has great theoretical and practical importance for improvement of saline soil and increasing agricultural efficiency. Collectively, the results of this research might encourage farmers to include potassium humate in their soil management practices due to the enhanced yield observed in the present study. However, effects of potassium humate on the relationship between seed cotton yield, nutrient uptake and nutrient use efficiency under brackish water irrigation need to be further evaluated.

Acknowledgements. This work was supported by the key program by the National Natural Science Foundation of China (grant number 41830754) and the National Nature Science Foundation of China (grant number 51679190, 41907010).

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_38793895

EVALUATION OF MANGROVE REHABILITATION AFTER BEING DESTROYED BY CHEMICAL WARFARE USING REMOTE SENSING TECHNOLOGY: A CASE STUDY IN CAN GIO MANGROVE FOREST IN MEKONG DELTA, SOUTHERN VIETNAM

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(Received 21st Apr 2021; accepted 12th Aug 2021)

Abstract. In this study, we used satellite data (optical and radar) to evaluate mangrove rehabilitation in an area that has been completely destroyed by the use of herbicides and other chemical agents by the U.S military in the Can Gio Biosphere Reserve in Mekong Delta, Southern Vietnam. We used the Landsat satellite imagery series to analyse the land cover change, and the results showed that the coverage of the mangroves in this study area has gradually increased over the years: 9.54% in 1972, 34.46% in 1988, 46.34% in 1995, 51.76% in 2000, 54.27% in 2005, 56.93% in 2010, 59.91% in 2015 and 63.70% in 2020. The overall accuracy is 92.76%, and the average accuracy is 85.47%. We used a combination of images taken from ALOS-2 PALSAR-2 and Landsat satellites to build models that detect the structural parameters of mangrove forests. These models can explain mangrove structural parameters such as the basal area of 91% ($R^2 = 0.91$), the height of 60% ($R^2 = 0.60$), the density of 52% ($R^2 = 0.52$) and the biomass of 91% ($R^2 = 0.91$). This study aims to provide results to study area for better management, monitoring, conservation as well as sustainable development of mangrove forests.
Keywords: *mangrove restoration, herbicide, spraying, U.A. air force, Landsat, ALOS-2 PALSAR-2, management, monitoring, conservation, sustainable development, UNESCO/MAB.*

Introduction

Mangrove ecosystems cover a large part of wetland areas and are often distributed in the coasts of the tropical and subtropical regions (Rouse et al., 1974; Hong et al., 1988; Kuenzer et al., 2011; Salem et al., 2012). As a strictly limited habitat, true mangroves are home to about 50 species globally, which belong to 16 families. However, they have many useful functions for mankind (Marchand et al., 2008). The key products and services provided by the mangrove ecosystems include wood products (e.g., fibres, timber, fuelwood and charcoal), non-wood resources (e.g., food, fruits, wildlife, mariculture, capture fishery, honey, chemicals and medicine) and ecosystem and environmental services (e.g., extending the shorelines, coastal protection, eco-tourism, blue carbon storage and CO_2 sequestration) (Lugo et al., 1974; Hong et a., 1999; Duke et al., 2007; Vo et al., 2015; Veettil et al., 2019). Thus, the mangrove ecosystem contributes significantly to the livelihoods, well-being and security of coastal communities (Donato et al., 2011; Murdiyarso et al., 2015; Giri, 2016; FAO, 2020).

The total area of mangroves in the world is estimated at between 138,000 km^2 and 200,000 km^2 and distributed among 128 countries and territories. However, mangroves

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_38973930

are widely distributed mainly (approximately 75%) in 15 countries (Duke et al., 2007; Salem et al., 2012; Giri, 2016; FAO, 2020), and more than 90% are located in the developing countries (Duke et al., 2007). Unfortunately, mangroves are now declining at alarming rates, even faster than inland tropical forests (Salem et al., 2012; Giri et al., 2007, 2016; Chen et al., 2019). According to Duke (2007), the rate of global mangrove loss is 1–2% per year (Duke et al., 2007). Up to about 80% of these human-driven losses occurred in Southeast Asia (Giesen et al., 2007; Goldberg et al., 2020; FAO, 2020). The main causes of mangrove forest decline are increased population pressure in the coastal areas; industrial zone developments in coastal areas; illegal felling; expansion in acreage for agriculture, urbanization and residential land; and shrimp farming (Duke et al., 2007; Salem et al., 2012; Murdiyarso et al., 2015; FAO, 2020).

The mangroves in Vietnam are divided into four main zones from North to South by geographical location and the Can Gio mangrove forest lies in the fourth zone, which has the most diverse species composition and size of mangroves. Vietnam has 36 true mangrove plant species (Hong et al., 1999). Currently, Vietnam has about 169,000 hectares of mangroves, a decrease of 60% as compared to the 1940s (MARD, 2015). South Vietnam mangrove forests occupy 250,000 ha, as reported by Cuong (1964), which are distributed as follows: Ca Mau Peninsula has 150,000 ha, the Can Gio and Phuoc Hoa region have 40,000 ha and the rest have 20,000 ha (Ross, 1974), it covers nearly 80% of the mangroves in Vietnam (Hong et al., 1999).

The mangrove ecosystem is being increasingly recognised and considered as a significant one while creating the goals for national and global development and environments, such as for sustainable development activities and agreement on climate change (Marchand et al., 2008; Murdiyarso et al., 2015; Giri, 2016; FAO, 2020).

Due to the unique nature of the role of mangrove ecosystems. So, over the past decades, many international organizations, investment funds, NGOs and countries have invested millions of dollars for the conservation and restoration of mangroves in the world, and they consider it as an important measure in coastal management (De Leon et al., 1999; Brown et al., 2014; Ha'apio et al., 2018; Hai et al., 2020). However, there have been many unsuccessful or failed projects on mangrove restoration in India (Lewis, 2005), the Philippines (Primavera et al., 2008), the Ha Tinh province of Vietnam (Hong, 1993), northern Vietnam (Seto et al., 2007), etc. This is due to many reasons, mainly that most of these mangrove restoration projects lack data to evaluate the long-term success of the project and even assess the natural conditions before project implementation (Marchand, 2008). This has led to unsuccessful and indirectly wasteful mangrove rehabilitation projects.

There is a growing need for detailed mangrove forest maps for good monitoring and management of mangrove ecosystems (Vaiphasa et al., 2006). However, a detailed mangrove map is not easy to develop because mangroves are difficult to access (Vaiphasa et al., 2006).

Today, there is increasing advancement in technology, satellites and sensors and more accessibility to remotely sensed data with high temporal and resolution imagery. Some of these are available for free, along with strong computer systems (hardware), image processing techniques and new classification algorithms (software) (Giri, 2016; Kuenzer et al., 2011; Pham et al., 2019; Pham et al., 2020). It can be affirmed that remote sensing is a perfect alternative to traditional mangrove mapping methods based entirely on the field because it allows information gathering from the forbidden

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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environment of the mangrove forest, which otherwise, logically and practically, would be difficult to investigate (Vaiphasa et al., 2006).

Studies on the application of remote sensing data in mangrove ecosystems have been reviewed by authors such as Kuenzer et al. (2011), Giri (2016), Guo et al. (2017), Wang et al. (2018) and Pham et al. (2019) over the last two decades and have showed that a majority of the studies used optical and radar data groups while only a few studies used lidar, hyper-spectral data because of difficulty in accessing the data.

The optical satellite images, such as IKONOS, QuickBird, Landsat, SPOT, ALOS ASTER-2 Sentinel 2 and MODIS, are often used to understand the Difference Vegetation Index (DVI), Normalised Difference Vegetation Index (NDVI), Perpendicular Vegetation Index (PVI), Ratio Vegetation Index (RVI), etc. This is done using computational subtraction, division and rational transform groups and applying it while processing images from the red spectral band (R) and near-infrared band (NIR). The vegetative index describes the greenness of the leaves and the relative density and health of vegetation (Toderas et al., 1970; Rouse et al., 1974; Haas et al., 1975; Crippen, 1990; Green et al., 1998). Some impressive studies on mangroves, which use optical satellite data have been conducted. MacDonald (1999) used SPOT and Landsat images to study the rehabilitation of wetlands, including mangroves. Giri et al. (2007) used multi-time data from Landsat 2, 4, 5 and 7 to track mangrove variability in the Sundarbans in Bangladesh and India. Kamthonkiat et al. (2011) used ASTER data to assess the damage and subsequent rehabilitation of mangroves in southern Thailand. Kamal et al. (2016) used vegetation indexes from Landsat and ALOS AVNIR-2 satellite imagery in Moreton Bay of Australia and Karimunjawa island of Indonesia. Oostdijk et al. (2018) used a leaf area index (LAI) to monitor regional mangrove rehabilitation on the east coast of Florida. Chen et al. (2017) used NDVI from time-series Landsat 7/8 and Sentinel-1A imagery in Google Earth Engine cloud computing platform to analyse and create the mangrove forest map of China at 30 m spatial resolution, and it has an accuracy of more than 95%. Long et al. (2011) used Landsat and ISODATA clustering techniques to map the spatial distribution and areal extent of mangrove forests in the Philippines. Luong et al. (2015) Nguyen et al. (2018) used SPOT for analysis of an impact of succession in mangrove association and changes in mangrove forests in Vietnam. Valderrame et al. (2018) used NDVI from SPOT5, Landsat 8 and Sentinel-2 for mapping mangrove forest in Mexico.

In radar images, the most commonly used data are X, C and L bands from satellites such as RADARSAT-1 SAR, ENVISAT ASAR, ALOS-1/2 PALSAR-1/2 and Sentinel-1. Since the early 90s, many studies have demonstrated that the backscatter data is correlated with the structure of the forest and used to build models to estimate forest biomass (Toan et al., 1992). Mougin et al. (1999) checked the correlation between the multi-frequency and multi-polarization from AIRSAR data in P-, L- and C bands with mangrove structural parameters such as height, diameter, density and basal area, which were collected from 12 locations at different periods from the mangrove forest in Guiana, France. They found that most forest parameters with radar data were potentially correlated (Mougin et al., 1999). Recent studies show that Cougo et al. (2015) used Radarsat 2 data to study the mangroves of southern Brazil, while Kovacs et al. (2013) used ALOS PALSAR for monitoring the biophysical parameter of the *Avicennia germinans* forest in the Mexican Pacific. Hong et al. (2015) applied band TerraSAR-X SAR data for the southern mangrove area of Florida, USA; Luong et al. (2019), Pham et al. (2019) and Pham, M.H et al. (2020) used ALOS-2 PLSAR-2 to estimate the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_38973930

mangrove biomass in Vietnam in their respective studies. Nesha et al. (2020) operated with ALOS-2 PALSAR-2 for the mangrove biomass in East Kalimantan, Indonesia. Lucas et al. (2020) combined multiple remote sensing data for the structural characterization of mangrove forests on the west coast of Peninsular Malaysia.

In general, the above studies that used remote sensing images for mangrove monitoring faced the following issues: (i) lack of reliable field data; (ii) some studies used optical satellite images to assess the area or/and the area change of mangrove forests only; (iii) some studies used radar satellite data while often focusing on analysing the structure of mangroves; (iv) some studies did not focus on both optical and radar satellite image data; (v) there was a lack of studies that monitored mangrove rehabilitation over a long period, especially in cases of forest restoration, where mangroves are completely absent or have been destroyed by herbicide in chemical warfare, as in our case study. Therefore, the existing databases are insufficient to evaluate mangrove rehabilitation (area, structure and speed) on an accurate scientific basis. Therefore, the objectives of this study are to overcome the limitations indicated above.

Study area

Geographic location: Can Gio is one of 24 districts of Ho Chi Minh City (HCMC) in Vietnam. The Can Gio mangrove forest lies entirely within the Can Gio district of HCMC. The area lies in the geographic co-ordinates between 10°22'14" N – 10°40'00" N Latitude and 106°46'12'' – 107°00'59" E Longitude. It is about 65 km south of the HCMC centre and covers an area of over 75,000 ha (Tri et al., 2000; Tuan et al., 2002) (*Fig. 1*).

Topographically: Can Gio mangrove forest forms a basin with a minimum altitude range of 0 m – 1.5 m, in the northeastern sector of the forest, with downward inclines from the east, south and west (Tuan et al., 2002; Nam et al., 2014).

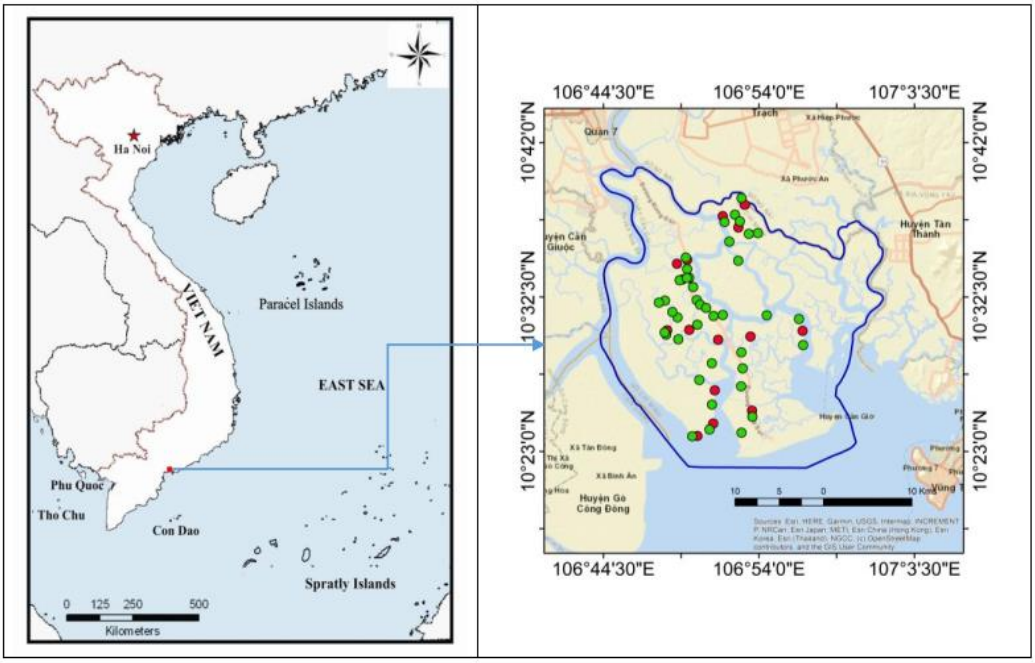

Figure 1. *Location map of the study area, Can Gio Biosphere Reserve in HCMC and its boundary (blue polygon). Distribution of sample plots (training plot is green circle and validation plot is red circle) are also shown over the study area*

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Soil: The Can Gio mangrove forest developed out of a comparatively recent brackish swamp, as the alluvium from the Sai Gon and Dong Nai rivers created. The soil types in Can Gio can be found four mains are saline soil, saline soil, with low alum content, saline soil, with high alum content and soft sandy soil, with mud deposits at the seashore (Tri et al., 2000; Tuan et al., 2002; Nam et al., 2014).

Climate: There are two seasons and the area is affected by equatorial monsoons; rainy season: from May to October and dry season: from November to April.

Temperature and humidity: The yearly average temperature is 25.8 °C. The daily average temperature amplitude is 5–7 °C. During the rainy season, humidity ranges from 79%–83%, with a maximum of 83% in September. In the dry season, humidity ranges from 74%–77%, with a minimum of 74% in April (Tuan et al., 2002; Nam et al., 2014).

Tidal regime: The Can Gio mangrove forest lies in a zone with a bi-diurnal tidal regime (i.e., two ebb and flow tides per day). Tidal amplitudes range from about 2 m at mean tide to 4 m during spring tides. However, the two daily high and low tides differ in height. Maximum tidal amplitudes, in the region of 4.0 – 4.2 m are the highest observed in the whole of Viet Nam (Tri et al., 2000; Tuan et al., 2002; Nam et al., 2014).

Mangrove forests in the Can Gio district before the onset of chemical warfare (30 April 1975)

According to Cuong (1964), before the war, the flora at Can Gio consisted of 34 species and was divided into two communities of salt-water (17 species) and brackish-water plants (17 species) (Cuong, 1964). The Can Gio mangrove forest covered an area of 40.000 ha; the canopy was dense with trees over 25 m tall and 25–50 cm wide (Cuong, 1964; Ross, 1974).

The Second Indochina War by the U.S. military in Vietnam, which used toxic chemicals, was the direst in human history. Between 1961 and 1971, the U.S. sprayed nearly 20 million gallons of Agent Orange and other herbicide across South Vietnam in an attempt to kill the vegetation that hid Vietnamese soldiers (Ross, 1974; Thomas, 1974).

According to Hong (2001), the mangrove ecosystem in Can Gio was completely destroyed (Hong, 2001). Besides that, chemical warfare has had a very serious and long-term impact on human health and the ecological environment (Schecter et al., 1995; Westing, 1983; Falk, 2015). The defoliant was sprayed on mangrove forests and other vegetation, which not only defoliated and killed plants but also destroyed the habitats of other organisms (Ross, 1974; Thomas, 1974; Nham Tuyet and Johansson, 2001; Koniuszewski, 2016).

Although trees remained standing after the spraying, they were dead. These dead trees remained in Can Gio for a short duration before the local people collected them and left the barren soil. The species *Rhizophora apiculate* and *Rhizophora mucronata* virtually disappeared. The trees that remained were small groups of *Ceriops tagal* and *Excoecaria agallocha* in a state of regeneration along the waterways; in tidal flooded areas, *Avicenniaceae* was present; on higher land, *Phoenix paludosa, Acrostichum aureum, Gymnanthera nitida, Derris trifoliata, Azima sarmentosa, Pluchea indian* and *Clerodendrum inerme* survived with a maximum height of only 2 m, while in other areas, nothing was to be seen other than shrubs of *Acanthus spp., Derris trifoliata, Finlaysonia obovate* and *Acrostichum aureum*, etc. (Tuan et al., 2002). Besides that, severe coastal erosion was seen along rivers and canals due to a lack of protective mangrove tree belts (Nam, 1994).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_38973930

Mangrove forests in the Can Gio district after the onset of chemical warfare (30 April 1975)

On 7 August 1978, the Ho Chi Minh City (HCMC) People's Committee signed Decision No. 165/QD-UB, which set out to establish Duyen Hai Forest Plantation, belonging to the HCM Forest Department, with the main mission to provide a base to concentrate their mangrove forest ecosystem reforestation efforts. Following the suggestions by the Forest Department, *Rhizophora apiculate* was selected for the afforestation as this species is a native and fast-growing tree and would thus be able to restore forest cover at the fastest rate. It also has the highest commercial value (Tuan et al., 2002). After 1978, HCMC planted more than 20,000 ha of *Rhizophora apiculata* in the Can Gio District to restore the mangrove ecosystem and ensured that the mangrove forest would serve its dual functions of providing wood fuels and construction materials and protecting the environment (Tuan et al., 2002).

After 22 years of rehabilitation and development owing to the efforts of the Ho Chi Minh city committee and people, the Can Gio forest became the largest replanted mangrove area in Vietnam with a beautiful natural landscape and diverse flora and fauna. This led to its recognition as an International Mangrove Biosphere Reserve by MAB/UNESCO Committee on 21 January 2000 – the first biosphere reserve in Vietnam (Tuan et al., 2002).

Data and methods

Satellite dataset

In this study, we used four optical satellite imagery types – Landsat 1 (1972), Landsat 5 (1988, 1989, 1995, 2000, 2005), Landsat 7 (2002, 2010) and Landsat 8 (2015, 2020) – for the dynamic analysis of the mangrove cover in the study area. All the satellite images were carefully selected with not much difference in the observed time of the season and month, without any cloud cover and after ensuring they were of the best quality. The details on the Landsat satellite image series used in this study are shown in *Table 1*.

We also used the Advanced Land Observing Satellite-2 Synthetic Aperture Radar (ALOS-2 PALSAR-2), launched in 2014 by the Japan Aerospace Exploration Agency (JAXA), which operates in L-band radar and collects very high spatial resolution data. Two scenes from ALOS-2 PALSAR-2 data in 2020 with 10 m pixel resolution and version 2.1 were selected. The details on the ALOS-2 PALSAR-2 data used in this study are shown in *Table 2*.

Prep-processing image

Processing of optical data: A series of processing was carried out before the image classification as-is: radiometric calibration as converted digital numbers (DN) value into spectral radiance and then converted into top-of-atmosphere reflectance (Chander et al., 2009; Potapov et al., 2020) and removed the gap on the Landsat 7 image by "Landsat_gapfill.sav" tool. Geometric correction; Image to map rectification; Image to Image registration. Mosaic and cut images were used according to the boundaries of the study area to reduce the size before classifying. For landcover change analysis, we used the post-classification comparison method based on supervised classification. In this study, we have chosen the NDVI, because it is one of the physiological parameters that

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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correlate with the photosynthetic activity of vegetation. It is a good index extracted from optical remote sensing data and is widely used (DeFries et al., 1995; Xie et al., 2008; Luong et al., 2016; Thuy et al., 2020). The vegetative index that was selected for the landcover classification was the Normalised Difference Vegetation Index (NDVI) and was calculated as a ratio between the RED band and NIR band values using the following formula:

$$\mathrm{NDVI} = \frac{\mathrm{NIR} - \mathrm{RED}}{\mathrm{NIR} + \mathrm{RED}} \quad \text{(Eq.1)}$$

In *Equation 1:* RED is the red band and NIR is the near-infrared band.

Table 1. *Landsat data used in this research*

No.	Type	Scene ID	Part/ row	Observation time	Band used (Pixel resolution)
1	Landsat 1	p134r53_1m19721215	134/53	12/15/1972	Band 3, 4 (57 m)
2	Landsat 5	LT05_L1TP_124053_19890125_20170205_01_T1	124/53	1/25/1989	Band 3, 4 (30 m)
3	Landsat 5	LT05_L1TP_125053_19880114_20170210_01_T1	125/53	1/14/1988	Band 3, 4 (30 m)
4	Landsat 5	LT05_L1TP_124053_19950110_20170110_01_T1	124/53	10/1/1995	Band 3, 4 (30 m)
5	Landsat 5	LT05_L1TP_125053_19950202_20170110_01_T1	125/53	2/2/1995	Band 3, 4 (30 m)
6	Landsat 5	LT05_L1TP_124053_20010315_20161212_01_T1	124/53	3/15/2001	Band 3, 4 (30 m)
7	Landsat 5	LT05_L1TP_124053_20050105_20161129_01_T1	124/53	1/5/2005	Band 3, 4 (30 m)
8	Landsat 5	LT05_L1TP_125053_20050317_20161126_01_T1	125/53	3/17/2005	Band 3, 4 (30 m)
9	Landsat 7	LE07_L1TP_124053_20020310_20170131_01_T1	124/53	3/10/2002	Band 3, 4 (30 m)
10	Landsat 7	LE07_L1TP_124053_20100127_20161215_01_T1	124/53	1/27/2010	Band 3, 4 (30 m)
11	Landsat 8	LC08_L1TP_124053_20150218_20170412_01_T1	124/53	2/18/2015	Band 4, 5 (30 m), Band 8 (15 m)
12	Landsat 8	LC08_L1TP_125053_20150209_20180523_01_T1	125/53	2/9/2015	Band 4, 5 (30 m), Band 8 (15 m)
13	Landsat 8	LC08_L1TP_124053_20200115_20200127_01_T1	124/53	1/15/2020	Band 4, 5 (30 m), Band 8 (15 m)
14	Landsat 8	LC08_L1TP_125053_20200207_20200211_01_T1	125/53	2/7/2020	Band 4, 5 (30 m), Band 8 (15 m)

Table 2. *ALOS-2 PALSAR-2 data used in this research*

No.	Scene ID	Observation time	Polarization (pixel resolution)	Observation angle
1	ALOS2314580190-200320	2020-03-20	HH, HV (10 m)	Off-nadia[deg]: 36.2
2	ALOS2314580200-200320	2020-03-20	HH, HV (10 m)	Off-nadia[deg]: 36.2

Processing of radar data: In this study, we used satellite images from ALOS-2 PALSAR-2. The digital number (DN) values of the ALOS-2 PALSAR-2 images in both the HH and HV polarizations were calibrated by calculating the backscattering intensity using *Equation 2*:

$$\sigma^{\circ} = 10 * \log_{10}(\mathrm{DN}^2) + FC \quad \text{(Eq.2)}$$

In *Equation 2:* σ° is the sigma naught backscattering intensity and *CF* is the calibration factor, which is currently set as −83 (JAXA, 2014).

In this study, we also used eight texture values from the ALOS-2 PALSAR-2 image including, Contrast, Correlation, Dissimilarity, Entropy, Homogeneity, Mean, Second Moment and Variance (Haralick et al., 1973; Anys et al., 1994; Luong et al., 2016).

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DOI: http://dx.doi.org/10.15666/aeer/1905_38973930

Field survey and structure calculations

We carried out a field measurements campaign during July 2020, and each sample plot had a size of 500 m^2 (20 × 25 m). Geographical coordinates (latitudes and longitudes) were identified at the centre of each plot using a GPS instrument. All trees larger than 5 cm in diameter (at 1.3 positions) were measured. The measuring instruments used were Criterion RD1000 Laser (Diameter at breast height-1.3 m position of tree/$D_{1.3m}$) and Trupulse 360B Laser (Height from stump to the top of the crown/H). Other instruments such as tape rope and digital camera were also applied. The authors used the plus average method for parameters measured (diameter, height and density) in each sample plot and convert to per hectare Vo Van Hong et al., 2006).

Field above ground biomass (AGB) and woody volume (V) estimation

We used allometric *Equations 3–11* for calculating the above-ground biomass (AGB) according to previous studies by (Ong et al., 2004; Fromard et al., 1998; Clough and Scott, 1998; Binh., 2007; Hoan et al., 2009; Komiyama et al., 2005; IPCC, 2003) in *Table 3*.

Table 3. *Allometric equations used for AGB mangroves species in the study area*

Species	Allometric equations	References	Equation
Bruguiera gymnorrhiza	$AGB = 0.186 * D^{2.31}$ $(R^2 = 0.99)$	Clough and Scott (1989)	(Eq.3)
Bruguiera parviflora	$AGB = 0.168 * D^{2.42}$ $(R^2 = 0.99)$	Clough and Scott (1989)	(Eq.4)
Xylocarpus granatum	$AGB = 0.0823 * D^{2.59}$ $(R^2 = 0.99)$	Clough and Scott (1989)	(Eq.5)
Avicennia germinans	$AGB = 0.140 * D^{2.40}$ $(R^2 = 0.97)$	Fromard et al. (1998)	(Eq.6)
Rhizophora appiculata	$AGB = 0.235 * D^{2.42}$ $(R^2 = 0.98)$	Ong et al. (2004)	(Eq.7)
Sonneratia caseolaris	$AGB = 0.199 * \rho^{0.90} * D^{2.22} (R^2 = 0.99)$	Komiyama et al. (2005)	(Eq.8)
Ceriops decandra	$AGB = 0.208 * D^{2.36}$ $(R^2 = 0.96)$	Cao Huy Binh (2007)	(Eq.9)
Lumnitzera racemosa	$AGB = 0.74 * D^{2.32}$ $(R^2 = 0.99)$	Hoan et al. (2009)	(Eq.10)
Common equation	$AGB = 0.25 * \rho * D^{.592} (R^2 = 0.98)$	Komiyama et al. (2005)	(Eq.11)

In allometric *Equations 3–11:* AGB is the aboveground biomass of a tree in kilograms (kg); D is the diameter at breast height (1.3 m) in centimeters (cm); ρ is the wood density (tons dry matter/m^3 fresh volume) (IPCC, 2003)

The woody volume (V) of each tree was calculated by using *Equation 12* which uses the basal area of a tree at breast height (G) in squared meters (m^2), total tree height (H) in meters (m) and the conversion factor (F).

$$V = G * H * F \quad \text{(Eq.12)}$$

In *Equation 12:* V is the woody volume (m^3); G is the basal area of tree at breast height 1.3 m in squared meters (m^2); H is the total tree height (H) in meters (m), and F is the conversion factor (F).

A total of 60 sample plots were used in this study. We have randomly selected 42 sample plots for training data and 18 sample plots for validation data. The summary of mangrove inventory parameters in the study area is described in *Table 4*. The location distribution of the sample plots in the study area is shown in *Figure 1.*

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Table 4. *Parameters of the mangrove structure from sample plots in the study area*

No.	Parameter	Minimum	Maximum	Mean	Standard deviation
1	Diameter (cm)	8.30	24.40	17.11	3.29
2	Height (m)	7.89	18.70	14.44	2.11
3	Density (tree/ha)	280	1430	680	243
4	Biomass (ton)	36.22	398	153.44	243.11
5	Woody (m^3)	13.45	263.86	109.97	67.34

Land cover classification

In this study, we have applied Circular No. 33/2018/TT-BNNPTNT (MARD, 2018) from the Ministry of Agriculture and Development of Vietnam. It has adopted the classification criteria of the UNESCO (1973) and Thai Van Trung (1998) for the division of the landcover at the Can Gio Mangrove Biosphere Reserve into two main classes and six sub-classes such as Forest class (Class 1. Very rich forest; Class 2. Rich forest; Class 3. Medium forest; Class 4. Poor forest) and sub-classes (Class 5. Other land and Class 6. Water body) (Luong et al., 2019). We have also temporarily divided the mangrove biomass level in this study area into four classes (*Table 5*).

Table 5. *The criterion for the classification of woody volumes and biomass forest classes*

No.	Main-class	Sub-class	Forest classification criterion	
			Woody volumes (m^3/ha)	biomass forests (ton/ha)
1	Forest	Very rich forest	> 300	> 300
2		Rich forest	200-300	200-300
3		Medium forest	100-200	100-200
4		Poor forest	0-100	0-100
5	Non-forest	Other land	0	0
6		Water body	0	0

Assessing the classification accuracy: A confusion matrix or error matrix is usually used as the quantitative method of characterising image classification accuracy. It is a table that will describe correspondence between the object classification results from satellite images and the ground truths with GPS points recorded. The Kappa test statistic assesses interclassifier agreement and is applied in assessing the classification accuracy of two classifiers, a neural network and a decision tree model on the same data set, therefore, the higher the kappa coefficient, the more accurate the classification of forest vegetation from satellite images (Fitzgerald et al., 1994). At a minimum, a Kappa Coefficient of Agreement should be attached to any resultant classification of satellite imagery (Jager et al., 2000).

Results

Land cover map and area distribution in the Can Gio Biosphere Reserve

1972

The land cover map based on the supervised classification of Landsat 1 in 1972 is given in *Figure 2* and the results of the statistical analysis of the landcover are shown in *Table 6*.

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DOI: http://dx.doi.org/10.15666/aeer/1905_38973930

At this time (1972), the very rich forests, the rich forests and the medium forests did not appear in this area, only poor forests with an area of 7,158.15 ha (9.54%) were spotted.

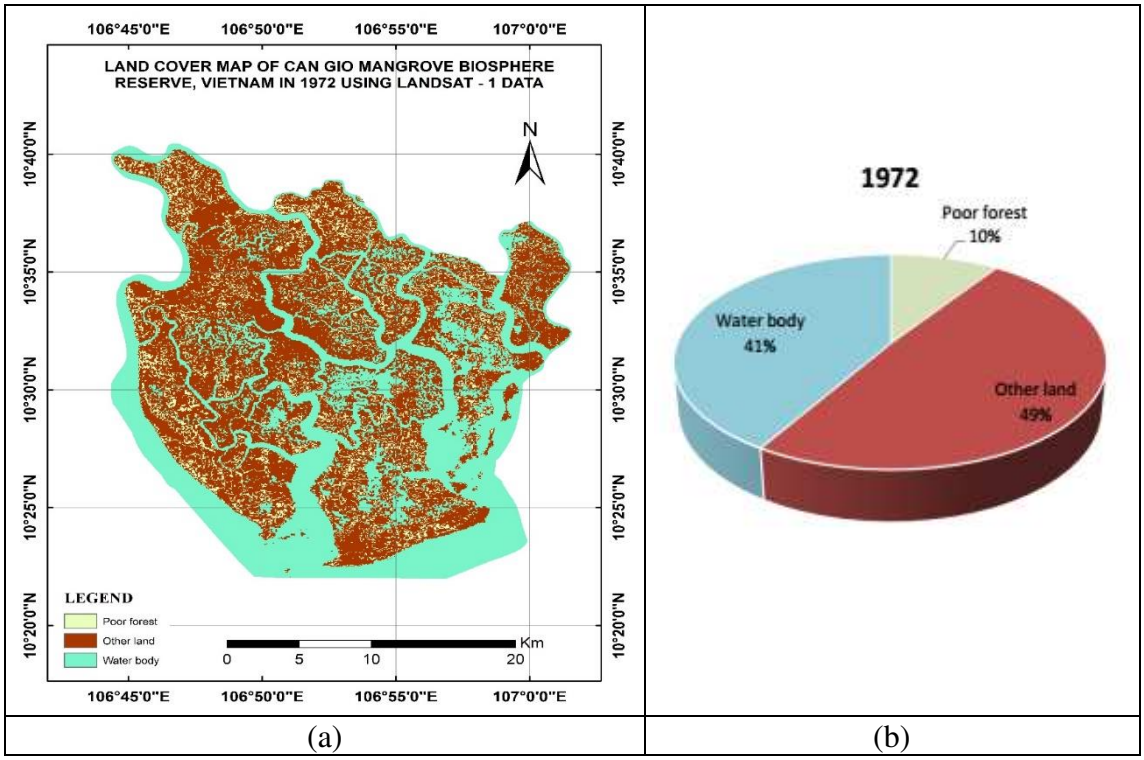

Figure 2. *(a) Land cover map and (b) area distribution in the Can Gio Biosphere Reserve in 1972*

Table 6*. Statistics on the land cover area in the Can Gio Biosphere Reserve in 1972*

No.	Main-class	Sub-class	Area	
			ha	%
1	Forest	Very rich forest	0	0
2		Rich forest	0	0
3		Medium forest	0	0
4		Poor forest	7158.15	9.54
		Sub-total	***7158.15***	***9.54***
5	Non-forest	Other land	36851.22	49.10
6		Water body	31039.39	41.36
		Sub-total	***67890.61***	***90.46***
		Total	**75048.76**	**100.00**

1988

The land cover map based on the supervised classification of Landsat 5 in 1988 had given in *Figure 3* and the results of the statistical analysis of the landcover are shown in *Table 7*.

The statistical results from the classified land cover map in 1988 using Landsat 5 show that the medium forest area is 6695.46 ha (8.92%), the poor forest area is

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DOI: http://dx.doi.org/10.15666/aeer/1905_38973930

19170.00 ha (25.54%), the other land area is 19480.14 ha (25.96%), and the water body area is 29703.16 ha (39.58%).

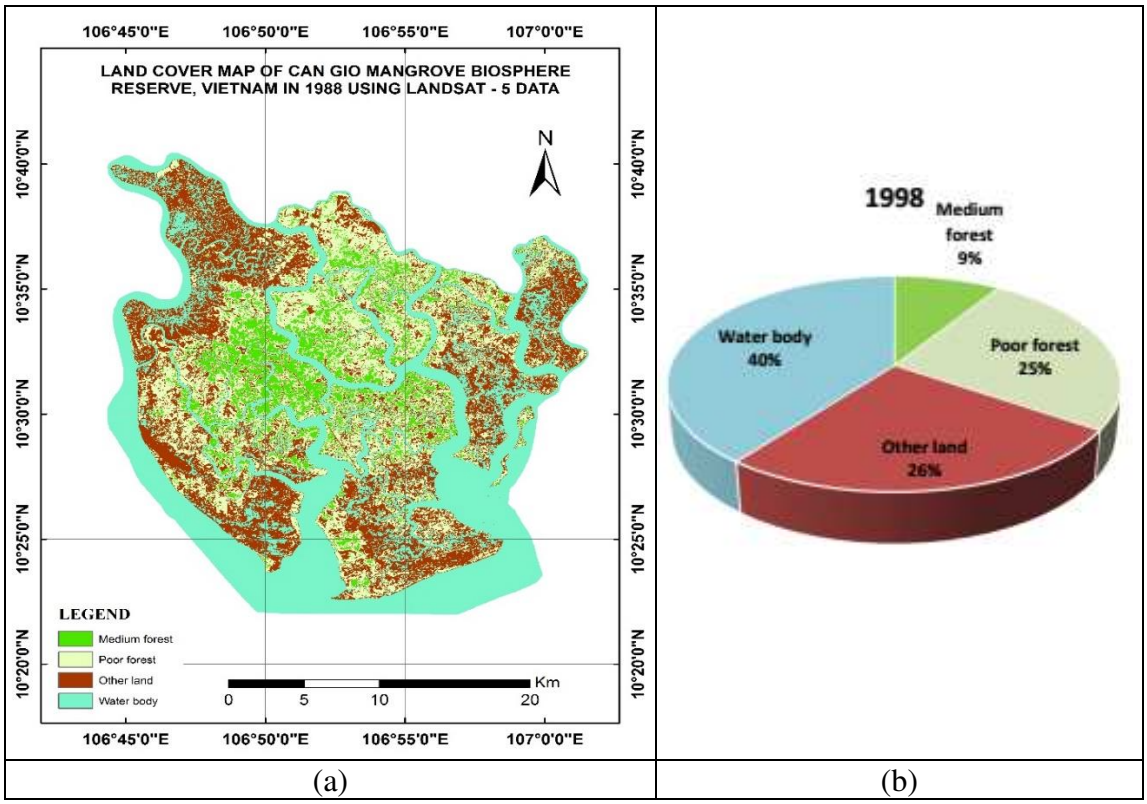

Figure 3. *(a) Land cover map and (b) area distribution in the Can Gio Biosphere Reserve in 1988*

Table 7. *Statistics on the land cover area in the Can Gio Biosphere Reserve in 1988*

No.	Main-class	Sub-class	Area	
			ha	%
1	Forest	Very rich forest	0	0
2		Rich forest	0	0
3		Medium forest	6695.46	8.92
4		Poor forest	19170.00	25.54
		Sub-total	***25865.46***	***34.46***
5	Non-forest	Other land	19480.14	25.96
6		Water body	29703.16	39.58
		Sub-total	***49183.30***	***65.54***
		Total	**75048.76**	**100**

1995

The land cover map based on the supervised classification of Landsat 5 in 1995 is given in *Figure 4* and the results of statistical analysis of the landcover are shown in *Table 8*.

The statistical results from the classified land cover map in 1995 by using Landsat 5 show that the rich forest area is 8995.05 ha (11.99%), the medium forest area is

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13205.43 ha (17.60%), the poor forest area is 12578.85 ha (16.45%), the other land area is 12345.67 ha (16.76%), the water body area is 27923.76 ha (37.21%).

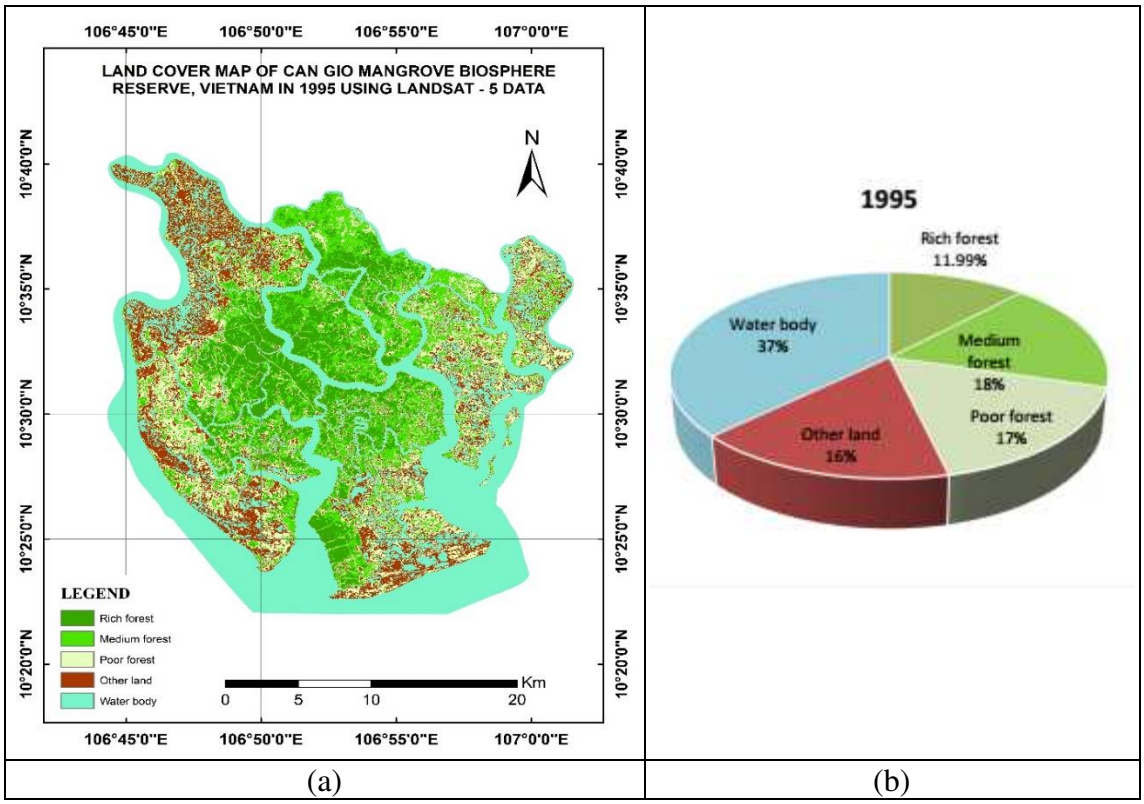

Figure 4. *(a) Land cover map and (b) area distribution in the Can Gio Biosphere Reserve in 1995*

Table 8. *Statistics on the land cover area in the Can Gio Biosphere Reserve in 1995*

No.	Main-class	Sub-class	Area	
			ha	%
1	Forest	Very rich forest	0.00	0.00
2		Rich forest	8995.05	11.99
3		Medium forest	13205.43	17.60
4		Poor forest	12578.85	16.76
		Sub-total	***34779.33***	***46.34***
5	Non-forest	Other land	12345.67	16.45
6		Water body	27923.76	37.21
		Sub-total	***40269.43***	***53.66***
		Total	**75048.76**	**100.00**

2000

The land cover map based on the supervised classification of Landsat 5 in 2000 is given in *Figure 5* and the results of statistical analysis of the landcover are shown in *Table 9*.

The statistical results from the classified land cover map in 2000 using Landsat 5 indicate that the very rich area is 1625.58 ha (2.17%), the rich forest area is 15122.61 ha (20.15%), the medium forest area is 11724.88 ha (15.62%), the poor forest area is

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10375.16 ha (13.82%), the other land area is 8530.93 ha (11.37%), and the water body area is 27669.60 ha (36.87%).

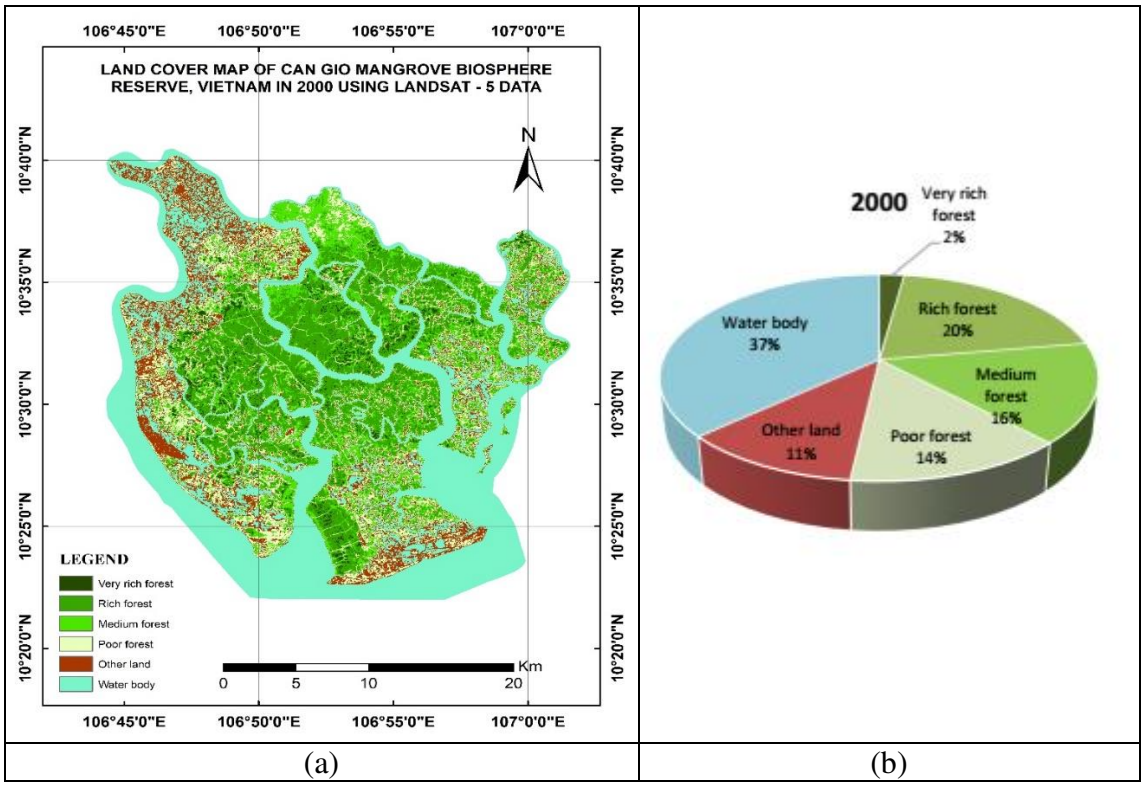

Figure 5. *(a) Land cover map and (b) area distribution in the Can Gio Biosphere Reserve in 2000*

Table 9. *Statistics on the land cover area in the Can Gio Biosphere Reserve in 2000*

No.	Main-class	Sub-class	Area	
			ha	%
1	Forest	Very rich forest	1625.58	2.17
2		Rich forest	15122.61	20.15
3		Medium forest	11724.88	15.62
4		Poor forest	10375.16	13.82
		Sub-total	***38848.23***	***51.76***
5	Non-forest	Other land	8530.93	11.37
6		Water body	27669.60	36.87
		Sub-total	***36200.53***	***48.24***
		Total	**75048.76**	**100.00**

2005

The land cover map based on the supervised classification of Landsat 5 in 2005 is given in *Figure 6* and the results of the statistical analysis of the landcover are shown in *Table 10*.

The statistical results from the classified land cover map in 2005 using Landsat 5 indicate that the very rich forest area is 4892.91 ha (6.52%), the rich forest area is 15382.35 ha (20.50%), the medium forest area is 11888.77 ha (15.84%), the poor forest

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area is 8567.19 ha (11.42%), the other land area is 6850.80 ha (9.13%), the water body area is 27466.74 ha (36.60%).

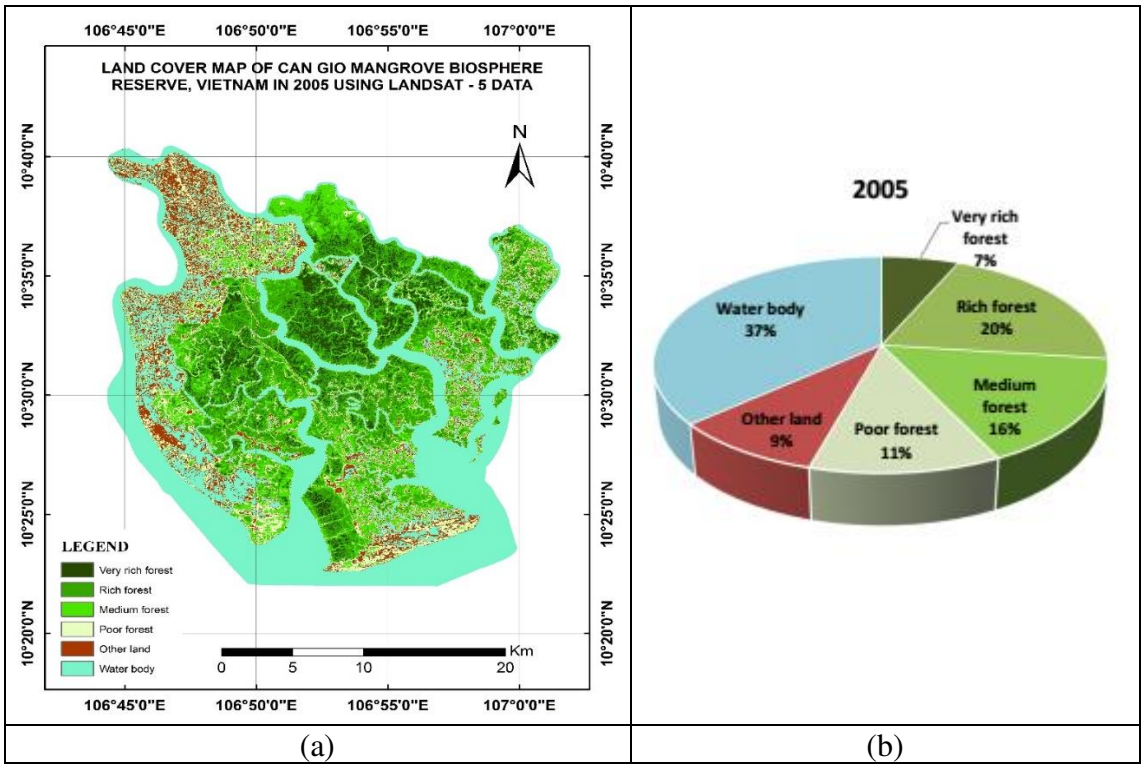

Figure 6. *(a) Land cover map and (b) area distribution in the Can Gio Biosphere Reserve in 2005*

Table 10. *Statistics on the land cover area in the Can Gio Biosphere Reserve in 2005*

No.	Main-class	Sub-class	Area	
			ha	%
1	Forest	Very rich forest	4892.91	6.52
2		Rich forest	15382.35	20.50
3		Medium forest	11888.77	15.84
4		Poor forest	8567.19	11.42
		Sub-total	***40731.22***	***54.27***
5	Non-forest	Other land	6850.80	9.13
6		Water body	27466.74	36.60
		Sub-total	***34317.54***	***45.73***
		Total	**75048.76**	**100.00**

2010

The land cover map based on the supervised classification of Landsat 7 in 2010 is given in *Figure 7* and the results of the statistical analysis of the landcover are shown in *Table 11*.

The statistical results from the classified land cover map in 2010 from Landsat 7 indicate that the very rich forest area is 9433.27 ha (12.57%), the rich forest area is 15121.80 ha (20.15%), the medium forest area is 11152.53 ha (14.86%), the poor forest

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area is 7016.67 ha (14.86%), the other land area is 5.312.70 ha (7.08%), the water body area is 27.011.79 ha (35.99%).

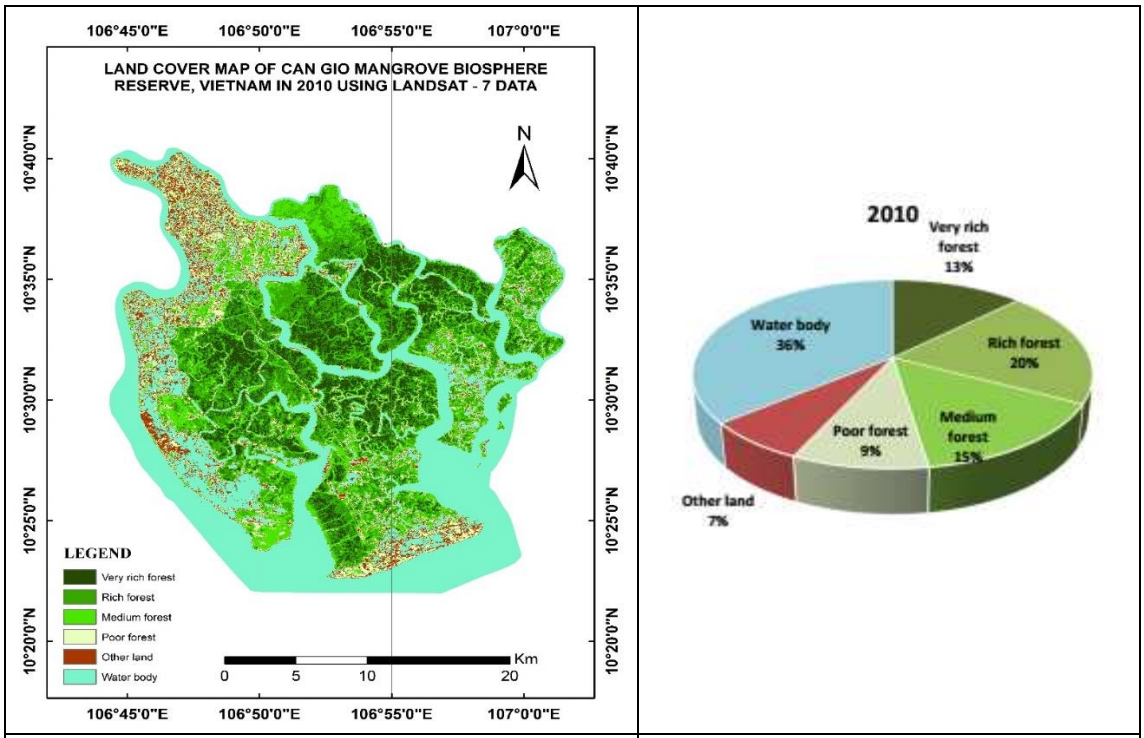

Figure 7. *(a) Land cover map and (b) area distribution in the Can Gio Biosphere Reserve in 2010*

Table 11. *Statistics on the land cover area in the Can Gio Biosphere Reserve in 2010*

No.	Main-class	Sub-class	Area	
			ha	%
1	Forest	Very rich forest	9433.27	12.57
2		Rich forest	15121.80	20.15
3		Medium forest	11152.53	14.86
4		Poor forest	7016.67	9.35
		Sub-total	***42724.27***	***56.93***
5	Non-forest	Other land	5.312.70	7.08
6		Water body	27.011.79	35.99
		Sub-total	***32324.49***	***43.07***
		Total	**75048.76**	**100.00**

2015

The land cover map based on the supervised classification of Landsat 8 in 2015 is given in *Figure 8* and the results of statistical analysis of the landcover are shown in *Table 12*.

The statistical results from the classified land cover map in 2015 from Landsat 8 indicate that the very rich forest area is 10161.18 ha (13.54%), the rich forest area is

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18728.36 ha (24.96%), the medium forest area is 9355.57 ha (12.47%), the poor forest area is 6717.47 ha (8.95%), the other land area is 4474.71 ha (5.96%), the water body area is 25611.48 ha (34.13%).

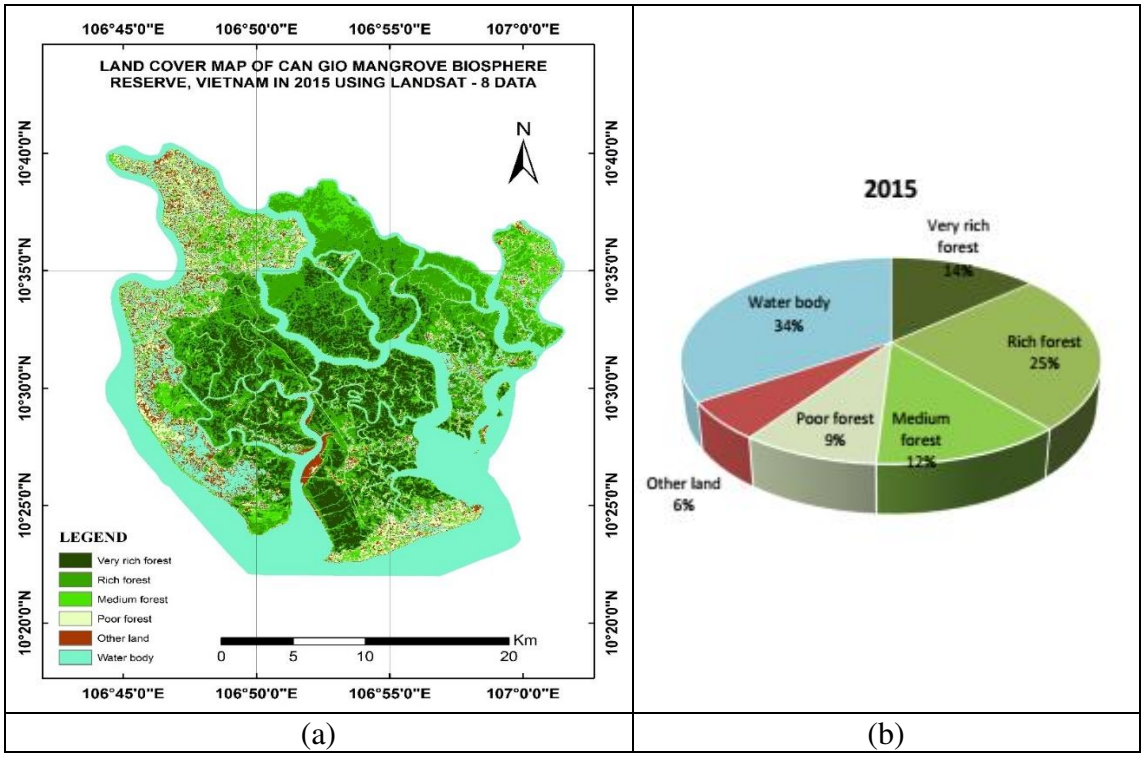

Figure 8. *(a) Land cover map and (b) area distribution in the Can Gio Biosphere Reserve in 2015*

Table 12. *Statistics on the land cover area in the Can Gio Biosphere Reserve in 2015*

No.	Main-class	Sub-class	Area	
			ha	%
1	Forest	Very rich forest	10161.18	13.54
2		Rich forest	18728.36	24.96
3		Medium forest	9355.57	12.47
4		Poor forest	6717.47	8.95
		Sub-total	***44962.57***	***59.91***
5	Non-forest	Other land	4474.71	5.96
6		Water body	25611.48	34.13
		Sub-total	***30086.19***	***40.09***
		Total	**75048.76**	**100.00**

2020

The land cover map based on the supervised classification of Landsat 8 in 2020 is given in *Figure 9* and the results of statistical analysis of the landcover are shown in *Table 13*.

The statistical results from the classified land cover map in 2020 from Landsat 8 show that the very rich forest area is 11994.93 ha (15.98%), the rich forest area is

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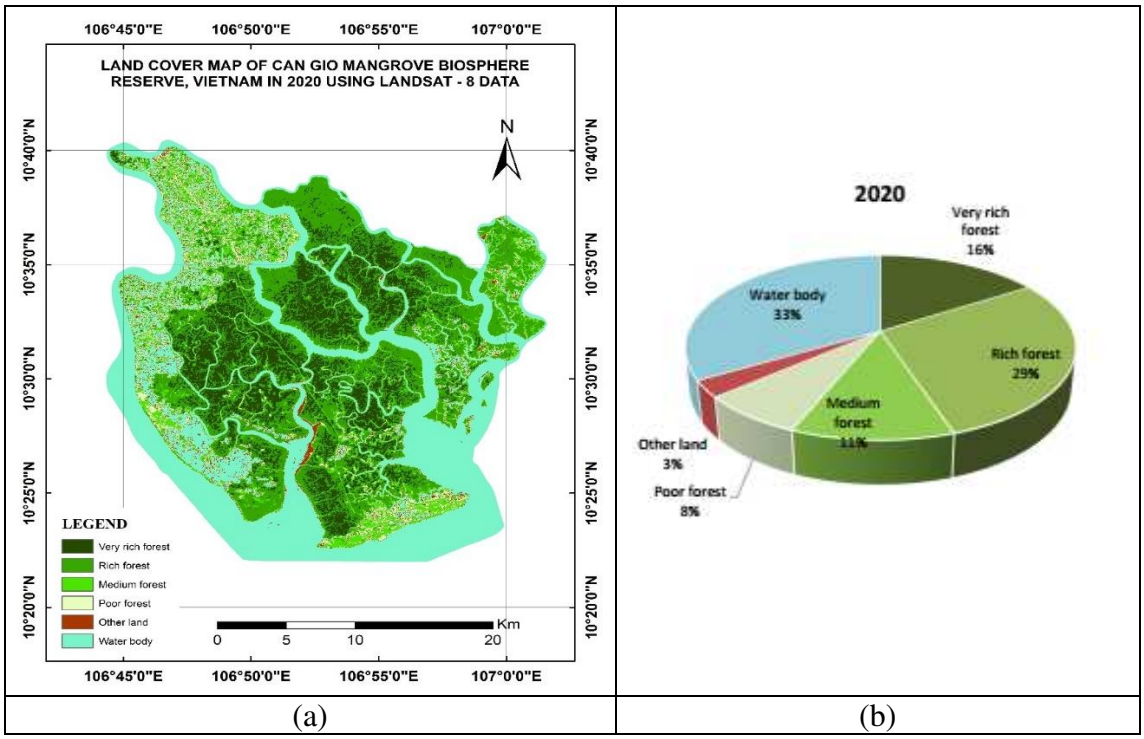
21659.20 ha (28.86%), the medium forest area is 8572.88 ha (11.42%), the poor forest area is 5578.09 ha (7.43%), the other land area is 2198.27 ha (2.93%), the water body area is 25045.39 ha (33.37%) (*Table 14*). The classification accuracy assessment is based on the confusion matrix. The overall accuracy is 92.76% and the average accuracy of 85.47%. Kappa statistics (K^) is 89.99%.



(a) (b)

Figure 9. *(a) Land cover map and (b) area distribution in the Can Gio Biosphere Reserve in 2020*

Table 13. *Statistics on the land cover area in the Can Gio Biosphere Reserve in 2020*

No.	Main-class	Sub-class	Area	
			ha	%
1	Forest	Very rich forest	11994.93	15.98
2		Rich forest	21659.20	28.86
3		Medium forest	8572.88	11.42
4		Poor forest	5578.09	7.43
		Sub-total	***47805.10***	***63.70***
5	Non-forest	Other land	2198.27	2.93
6		Water body	25045.39	33.37
		Sub-total	***27243.66***	***36.30***
		Total	**75048.76**	**100.00**

Land cover area change in the Can Gio Biosphere Reserve

For a detailed assessment of forest changes in this study area, we have tried to select the satellite data with two-time intervals of 5 years and similar to the time of year satellite image acquisition and drop in the period of a national forest inventory of

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Vietnam, every five years. Therefore, in this study, we have selected Landsat satellite image data of 1995, 2000, 2005, 2010, 2015 and 2020 for mangrove change analysis. In this study, we also included temporary regulations for the criteria of positive and negative mangrove forest changes such as: (i) Non-change is the area unchanged; (ii) Negative area changes including; very rich forest (VRF) to rich forest (RF), very rich forest (VRF) to medium forest (MF), very rich forest (VRF) to poor forest (PF), very rich forest (VRF) to other land (OL), very rich forest (VRF) to water body (WB) along with rich forest (RF) to medium forest (MF), rich forest (RF) to poor forest (PF), rich forest (RF) to other land (OL), rich forest (RF) to water body (WB); poor forest (PF) to other land (OL), poor forest (PF) to water body (WB); (iii) Positive area change including; rich forest (RF) to very rich forest (VRF), medium forest (MF) to very rich forest (VRF), medium forest (MF) to rich forest (RF); poor forest (PF) to very rich forest (VRF), medium forest (MF) to rich forest (RF); other land (OL) to very rich forest (VRF), other land (OL) to rich forest (RF); other land (OL) to medium forest (MF), other land (OL) to poor forest (PF); water body (WB) to very rich forest (VRF), water body (WB) to rich forest (RF), water body (WB) to medium forest (MF), water body (WB) to poor forest (PF), water body (WB) to other land (OL).

The statistical results of the land cover area change in the Can Gio Biosphere Reserve, HCMC, Vietnam over the periods such as 1995–2000, 2000–2005, 2005–2010, 2010–2015 and 2015–2020 are shown below.

1995–2000

Statistics from the land cover change matrix have shown that the non-change area is 5076.09 (6.76%), the negative area change is 17196.39 (22.91%), and the positive area change is 52776.28 (70.32%). The detailed statistical results of the land cover area changes in the Can Gio Biosphere Reserve (CGMBR), HCMC, Vietnam over the period from 1995 to 2000 are shown in *Figure 10* and *Table 14*.

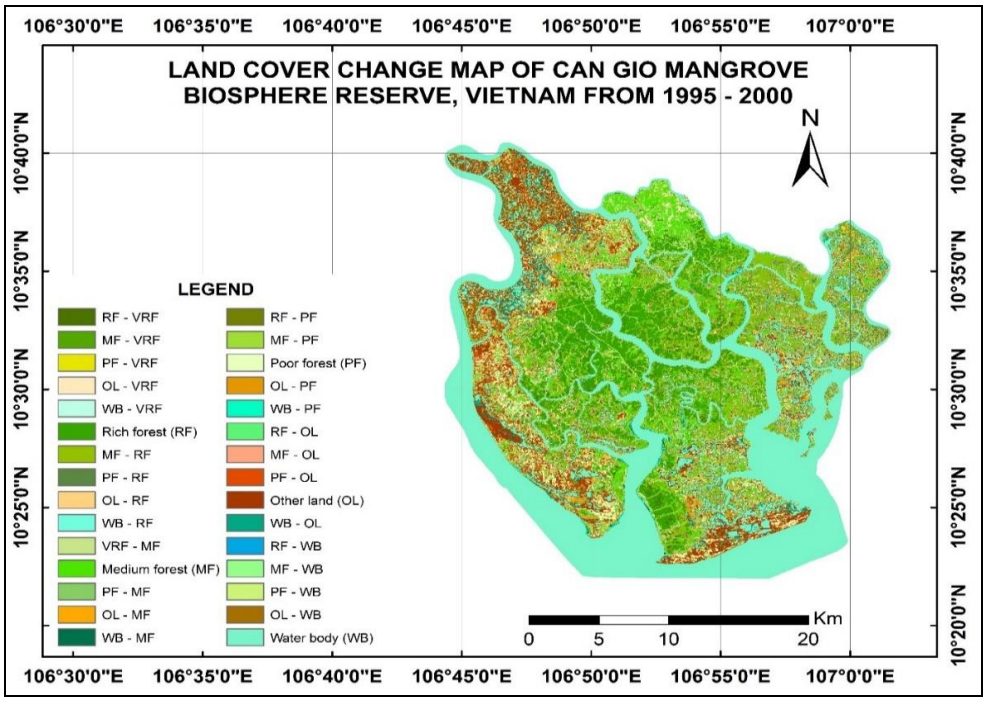

Figure 10. *Land cover change map of the Can Gio Biosphere Reserve from 1995–2000*

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Table 14. *The direction of land cover changes in the CGMBR from 1995–2000 (units: ha)*

No.	Class	Area	
		ha	%
1	Non-change area	5076.09	6.76
2	Negative area change	17196.39	22.91
3	Positive area change	52776.28	70.32
	Total	**75048.76**	**100.00**

2000–2005

Statistics from the land cover change matrix have shown that the non-change area is 49155.12 (65.50%), the negative area change is 5765.04 (7.68%), and the positive area change is 20128.60 (26.82%). The detailed statistical results of the land cover area changes in the Can Gio Biosphere Reserve, HCMC, Vietnam over the period from 2000 to 2005 are shown in *Figure 11* and *Table 15*.

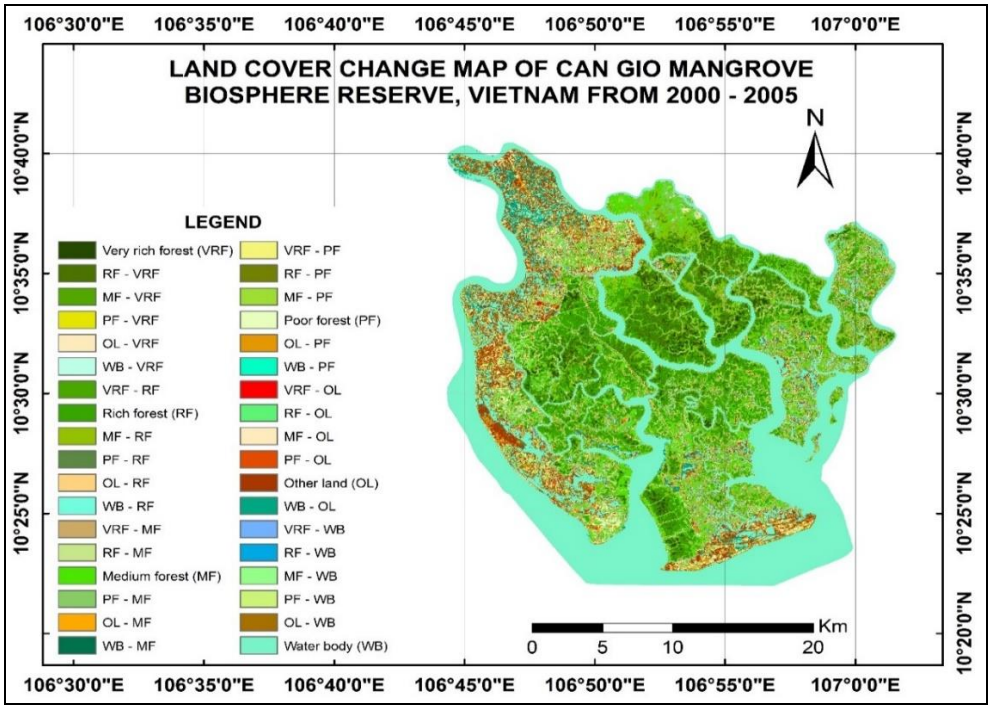

Figure 11. *Land cover change map of the Can Gio Mangrove Biosphere Reserve from 2000–2005*

Table 15. *The direction of land cover changes in the CGMBR from 2000–2005 (units: ha)*

No.	Class	Area	
		ha	%
1	Non-change area	49155.12	65.50
2	Negative area change	5765.04	7.68
3	Positive area change	20128.60	26.82
	Total	**75048.76**	**100.00**

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2005–2010

Statistics from the land cover change matrix have shown that the non-change area is 48464.64 (64.58%), the negative area change is 7094.16 (9.45%), and the positive area change is 19489.96 (25.97%). The detailed statistical results of the land cover area changes in the Can Gio Biosphere Reserve, HCMC, Vietnam over the period from 2005 to 2010 are shown in *Figure 12* and *Table 16*.

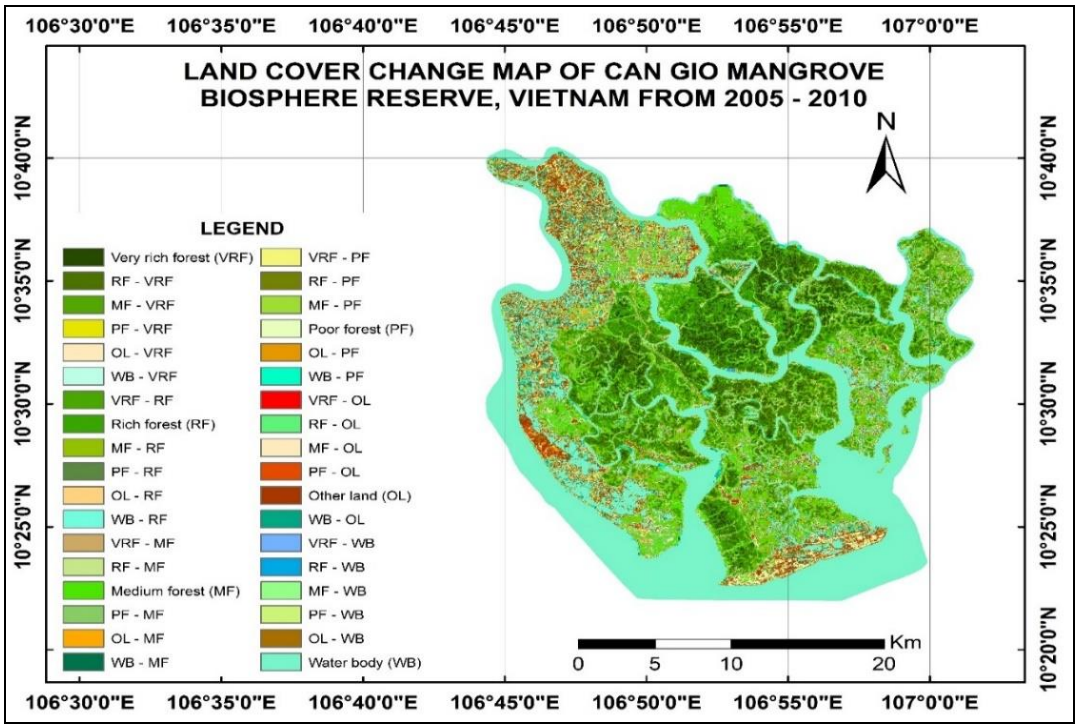

Figure 12. *Land cover change map of the Can Gio Mangrove Biosphere Reserve from 2005–2010*

Table 16. *The direction of land cover changes in the CGMBR from 2005–2010 (units: ha)*

No.	Class	Area	
		ha	%
1	Non-change area	48464.64	64.58
2	Negative area change	7094.16	9.45
3	Positive area change	19489.96	25.97
	Total	**75048.76**	**100.00**

2010–2015

Statistics from the land cover change matrix have shown that the non-change area is 46234.62 (61.61%), the negative area change is 8647.20 (11.52%), and the positive area change is 20166.94 (26.87%). The detailed statistical results of the land cover area changes in the Can Gio Biosphere Reserve, HCMC, Vietnam over the period from 2010 to 2015 are shown in *Figure 13* and *Table 17*.

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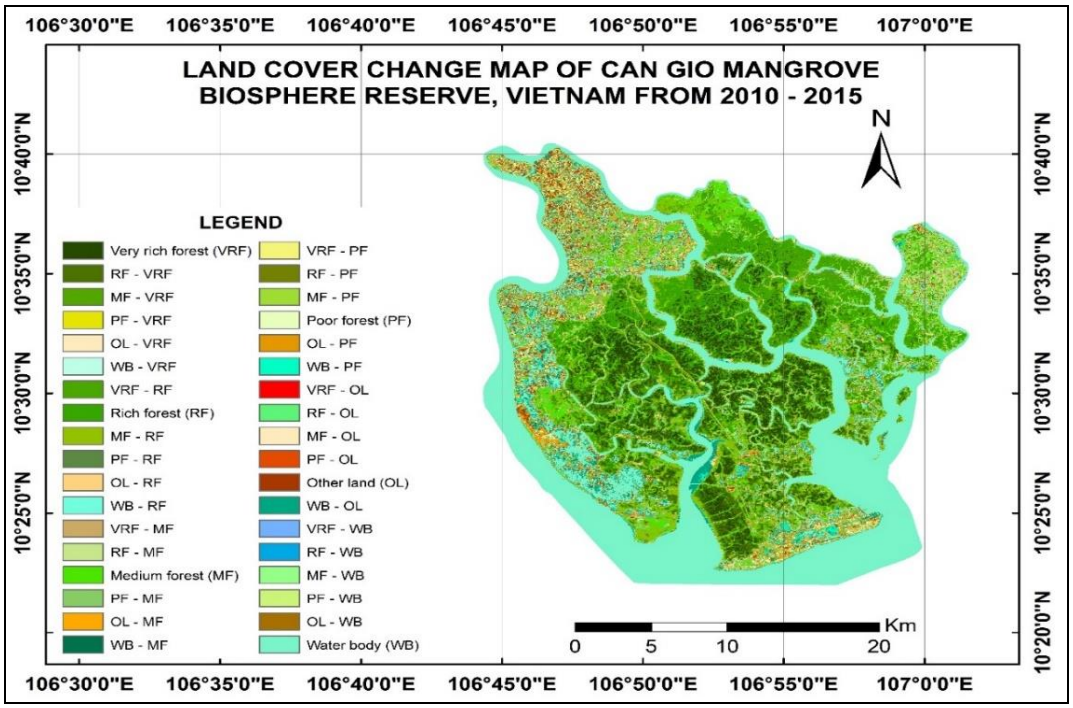

Figure 13. *Land cover change map of the Can Gio Mangrove Biosphere Reserve from 2010–2015*

Table 17*. The direction of land cover changes in the CGMBR from 2010–2015 (units: ha)*

No.	Class	Area	
		ha	%
1	Non-change area	46234.62	61.61
2	Negative area change	8647.20	11.52
3	Positive area change	20166.94	26.87
	Total	**75048.76**	**100.00**

2015–2020

Statistics from the land cover change matrix have shown that the non-change area is 50787.58 (67.67%), the negative area change is 5759.35 (7.67%), and the positive area change is 18501.84 (24.65%). The detailed statistical results of the land cover area changes in the Can Gio Biosphere Reserve, HCMC, Vietnam over the period from 2015 to 2020 are shown in *Figure 14* and *Table 18*.

Table 18. *The direction of land cover changes in the CGMBR from 2015–2020 (units: ha)*

No.	Class	Area	
		ha	%
1	Non-change area	50787.58	67.67
2	Negative area change	5759.35	7.67
3	Positive area change	18501.84	24.65
	Total	**75048.76**	**100.00**

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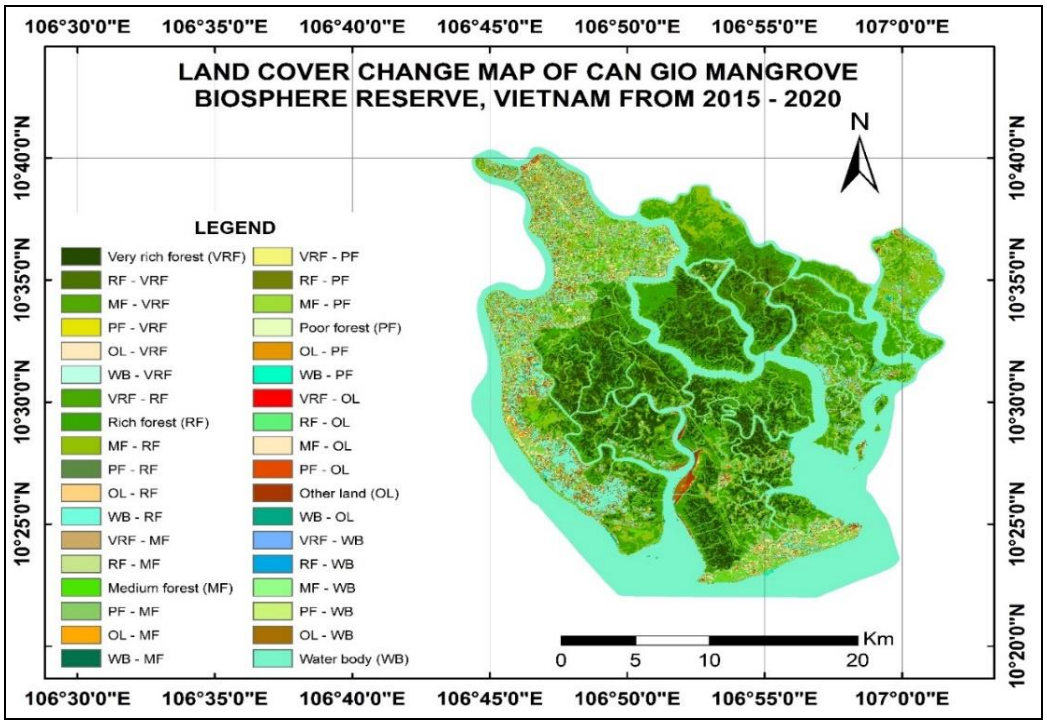

Figure 14. *Land cover change map of the Can Gio Mangrove Biosphere Reserve from 2015–2020*

Detection of parameters of mangrove structures using satellite data

Data such as the backscatter density of HV, HH polarization from the ALOS-2 PALSAR-2 image and NDVI from Landsat 8 image, were extracted for the analysis of the relationship with the parameters of mangrove structure such as basal area (G), height (H), density (N) and biomass (AGB).

Using single-satellite sensor

The sensitivity of NDVI value from Landsat 8 (NDVI_LS8) image to different parameters of mangrove forest (G, H, N and AGB) was statistically analysed using simple linear regression models, (a) Polynomial model (black colour), (b) Linear model (red colour) and (c) Exponential model (blue colour). The coefficient of determination (R^2) and root mean square error (RMSE) were used as the metrics for evaluating the relationships. A total of 12 models were analysed (*Fig. 15*). The result of the relationship between basal area (m^2/ha) and Normalised Difference Vegetation Index from Landsat 8 (NDVI_L8) using polynomial model, linear model and exponential model is ($R^2 = 0.58$), ($R^2 = 0.56$), ($R^2 = 0.61$) equivalent (*Fig. 15.1*).

The result of the relationship between height tree (m/ha) and NDVI using the polynomial model, linear model and exponential model is ($R^2 = 0.30$), ($R^2 = 0.30$), ($R^2 = 0.30$) equivalent (*Fig. 15.2*). The result of the relationship between density (tree/ha) and NDVI using the polynomial model, linear model and exponential model is ($R^2 = 0.17$), ($R^2 = 0.17$), ($R^2 = 0.19$) equivalent (*Fig. 15.3*). The result of the relationship between biomass (ton/ha) and NDVI using polynomial model, linear model and exponential model is ($R^2 = 0.65$), ($R^2 = 0.60$), ($R^2 = 0.63$) equivalent (*Fig. 15.4*). The best results obtained with each parameter of mangrove structures from NDVI are shown in *Table 19*.

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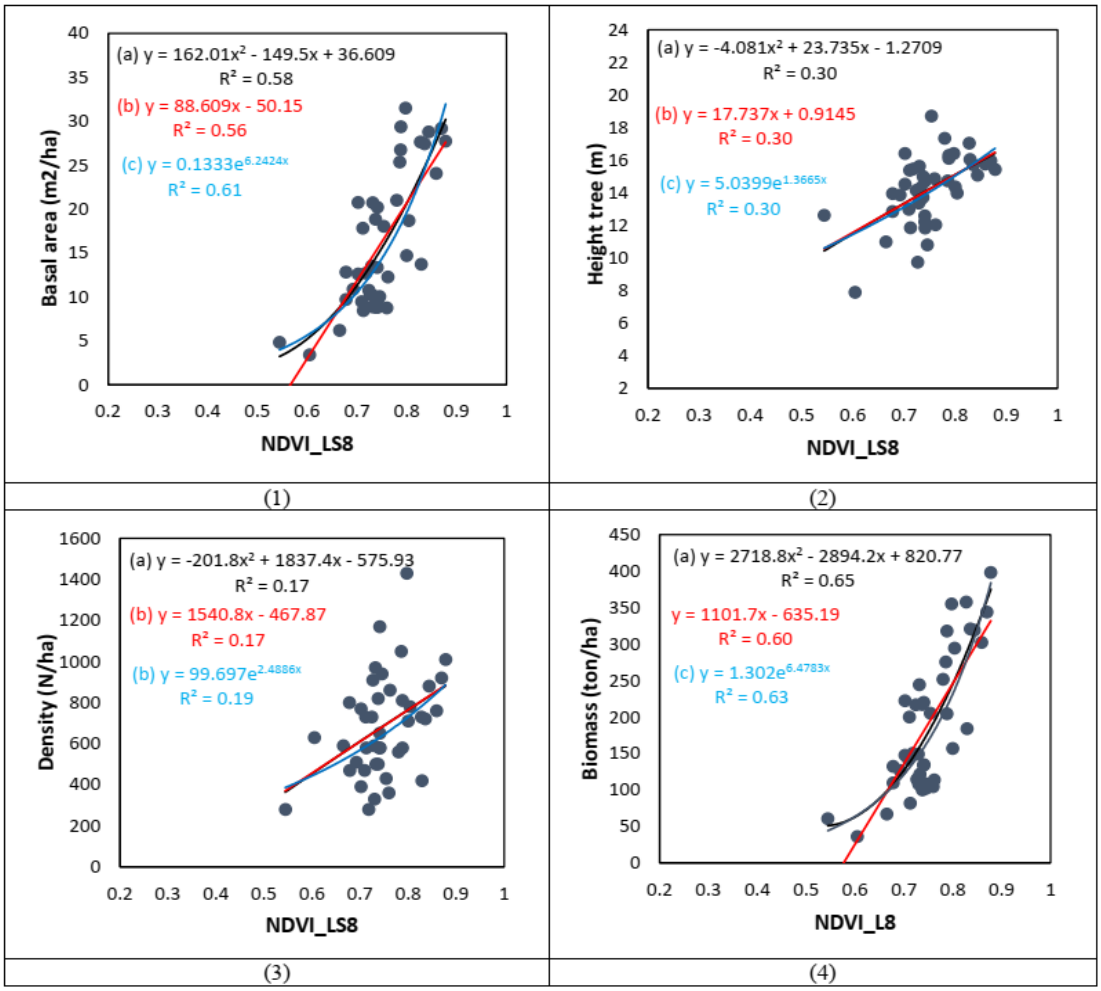

Figure 15. *The relationship between parameters of mangrove structure and NDVI values; (1) Basal area & NDVI, (2) Tree height & NDVI, (3) Tree density & NDVI and (4) Biomass & NDVI*

Table 19. *The best results obtained with each parameter of mangrove structure from NDVI value (Landsat 8)*

No.	Model	R^2	RMSE	Ref. figure
1	Basal area (m^2/ha) = 162.01*NDVI^2 - 149.5*NDVI + 36.609	0.58	3.30	15.1.(a)
2	Height tree (m) = -4.081*NDVI^2 + 23.735*NDVI - 1.2709	0.30	1.50	15.2.(b)
3	Density (tree/ha) = 99.697$e^{2.4886x}$	0.19	199	15.3.(a)
4	Biomass (ton/ha) = 2718.8*NDVI^2 - 2894.2*NDVI + 820.77	0.65	32.89	15.4.(a)

Similarly, the sensitivity of backscatter density (HH) to different parameters of mangrove forest (G, H, N and AGB) was statistically analysed using simple linear regression models, (a) Polynomial model (black colour), (b) Linear model (red colour) and (c) Exponential model (blue colour). A total of 12 models were analysed (Fiure 16). The result of the relationship between basal area (m^2/ha) and HH backscattering intensity (σ^o) using polynomial model, linear model and exponential model is

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($R^2 = 0.34$), ($R^2 = 0.29$), ($R^2 = 0.22$) equivalent (*Fig. 16.1*). The result of the relationship between height tree (m) and HH backscattering intensity (σ^o) using polynomial model, linear model and exponential model is ($R^2 = 0.17$), ($R^2 = 0.17$), ($R^2 = 0.16$) equivalent (*Fig. 16.2*). The result of the relationship between density (tree/ha) and HH backscattering intensity (σ^o) using polynomial model, linear model and exponential model is ($R^2 = 0.07$), ($R^2 = 0.01$), ($R^2 = 0.01$) equivalent (*Fig. 16.3*). The result of the relationship between biomass (ton/ha) and HH backscattering intensity (σ^o) using polynomial model, linear model and exponential model is ($R^2 = 0.42$), ($R^2 = 0.37$), ($R^2 = 0.27$) equivalent (*Fig. 16.4*). The best results obtained with each parameter of mangrove structures from HH polarization are shown in *Table 20*.

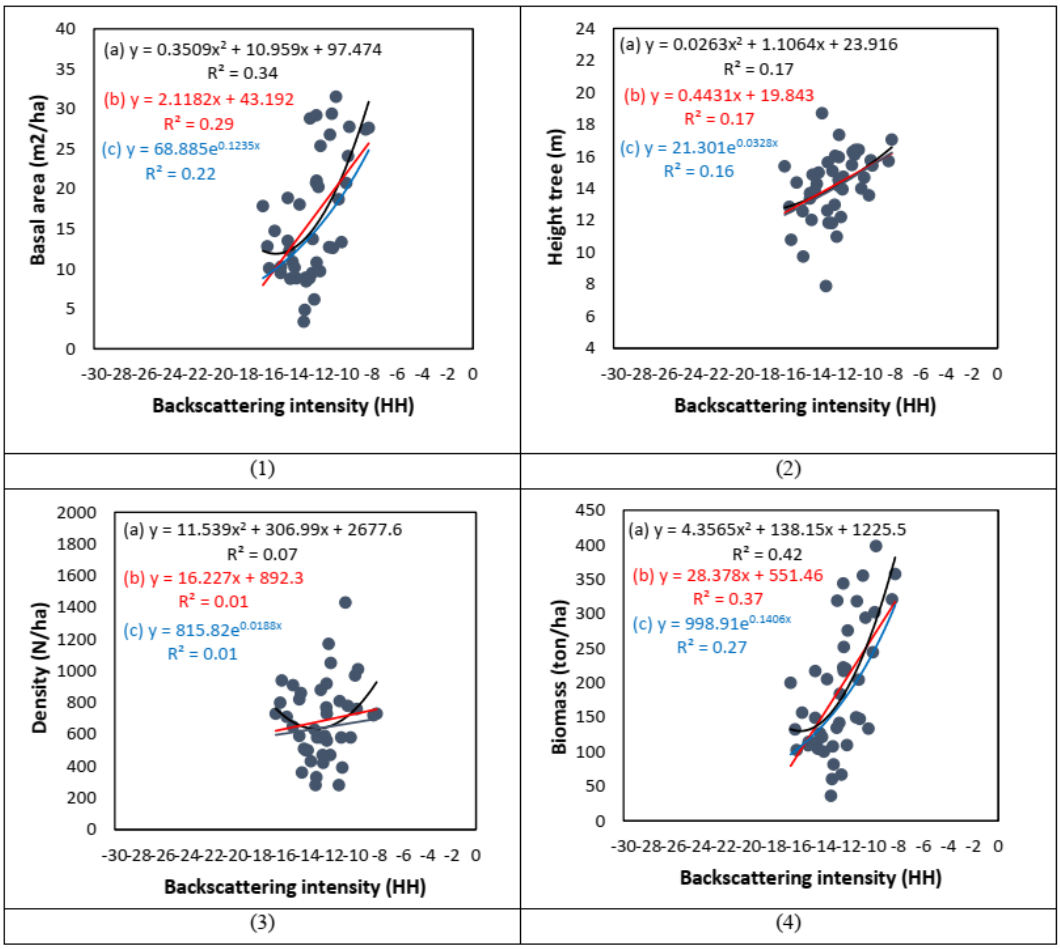

Figure 16. *The relationship between parameters of mangrove structure and HH polarization; (1) Basal area & HH, (2) Tree height & HH, (3) Tree density & HH and (4) Biomass & HH*

Table 20. *The best results obtained with each parameter of mangrove structure from HH backscattering intensity*

No.	Model	R^2	RMSE	Ref. figure
1	Basal area (m^2/ha) = 0.3509*HH^2 + 10.959*HH + 97.474	0.34	5.18	16.1.(a)
2	Height tree (m) = 0.0263*HH^2 + 1.1064*HH + 23.916	0.17	1.78	16.2.(b)
3	Density (tree/ha) = 11.539*HH^2 + 306.99*HH + 2677.6	0.07	228	16.3.(a)
4	Biomass (ton/ha) = 4.3565*HH^2 + 138.15*HH + 1225.5	0.42	54.50	16.4.(a)

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DOI: http://dx.doi.org/10.15666/aeer/1905_38973930

After the previously mentioned analysis, the relationship between the backscatter density (HV) and the parameters of mangrove structure (G, H, N and AGB) was also analysed. The sensitivity of backscatter density (HV) to different parameters of mangrove forest was statistically analysed using simple linear regression models, (a) Polynomial model (black colour); (b) Linear model (red colour) and (c) Exponential model (blue colour). A total of 12 models were analysed (*Figure 17*). The result of the relationship between basal area (m^2/ha) and HV backscattering intensity (σ^o) using polynomial model, linear model and exponential model is ($R^2 = 0.81$), ($R^2 = 0.74$), ($R^2 = 0.66$) equivalent (*Fig. 17.1*). The result of the relationship between height tree (m) and HV backscattering intensity (σ^o) using polynomial model, linear model and exponential model is ($R^2 = 0.50$), ($R^2 = 0.45$), ($R^2 = 0.43$) equivalent (*Fig. 17.2*). The result of the relationship between density (tree/ha) and HV backscattering intensity (σ^o) using polynomial model, linear model and exponential model is ($R^2 = 0.34$), ($R^2 = 0.16$), ($R^2 = 0.13$) equivalent (*Fig. 17.3*). The result of the relationship between biomass (ton/ha) and HV backscattering intensity (σ^o) using polynomial model, linear model and exponential model is ($R^2 = 0.77$), ($R^2 = 0.72$), ($R^2 = 0.65$) equivalent (*Fig. 17.4*). The best results obtained with each parameter of mangrove structures from HV polarization are shown in *Table 21*.

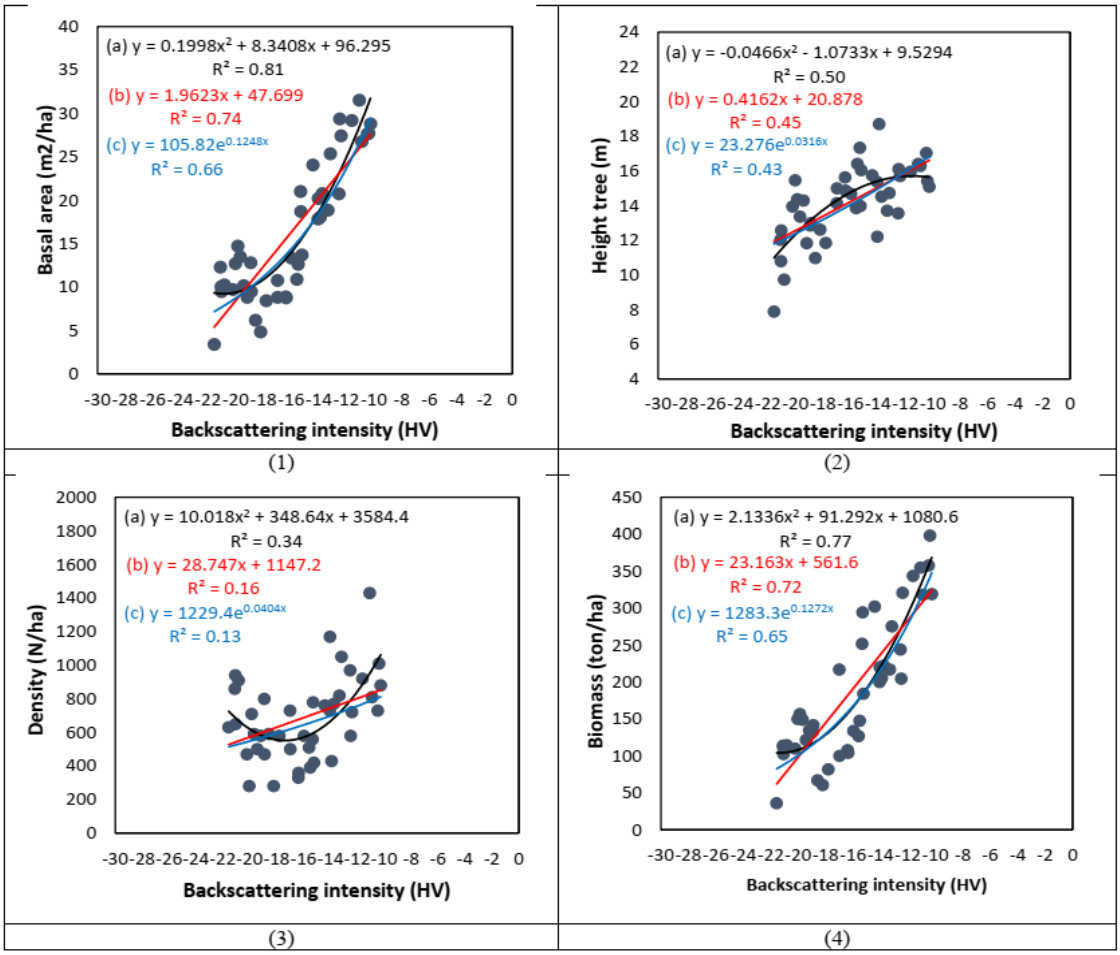

Figure 17. *The relationship between parameters of mangrove structure and HV polarization; (1) Basal area & HV, (2) Tree height & HV, (3) Tree density & HV and (4) Biomass & HV*

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DOI: http://dx.doi.org/10.15666/aeer/1905_38973930

Table 21. *The best results obtained with each parameter of mangrove structure from HV backscattering intensity*

No.	Model	R^2	RMSE	Ref. figure
1	G (m^2/ha) = 0.1998*HV^2 + 8.3408*HV + 96.295	0.81	1.49	17.1.(a)
2	Height tree (m) = -0.0466*HV^2 - 1.0733*HV + 9.5294	0.50	1.07	17.2.(b)
3	Density (tree/ha) = 10.018*HV^2 + 348.64*HV + 3584.4	0.34	162	17.3.(a)
4	Biomass (ton/ha) = 2.1336*HV^2 + 91.292*HV + 1080.6	0.77	21.61	17.4.(a)

Using combined satellite sensors

We combined the HV backscattering intensity (σ^o) and eight textures (contrast, correlation, dissimilarity, entropy, homogeneity, mean, second moment, variance) from ALOS-2 PALSAR-2 and spectral (NDVI) from Landsat 8. The sensitivity of the spectral/backscattering to different parameters of mangrove forest was statistically analysed using multiple linear regression models. The coefficient of determination (R^2) and root mean square error (RMSE) were used as the metrics for evaluating the relationships. A total of 4 models were analysed, and these models can explain mangrove structural parameters such as the basal area of 91% (R^2 = 0.91, RMSE = 0.71), the height of 60% (R^2 = 0.60, RMSE = 0.86), the density of 52% (R^2 = 0.52, RMSE = 118) and the biomass of 91% (R^2 = 0.91, RMSE = 8.46) by satellite data (NDVI, HV backscattering intensity and eight textures) the results obtained with each parameter of mangrove structure, in this case, are shown in *Table 22*.

Table 22. *The results obtained with each parameter of mangrove structure using NDVI value and textures and backscattering intensity from HV polarization*

No.	Model	R^2	RMSE
1	Basal area (m^2) = -326.43 + 1.55*HV + 38.90*NDVI-1.57--1.57* HV_Mean-1.02* HV_Variance + 14.60* HV_Homogeneity-0.16*HV_Contrast + 8.32* HV_Dissimilarit + 100.83* HV_Entropy + 1320.82* HV_SecondMoment + 6.75* HV_Correlation	0.91	0.71
2	Height (m) = 31.65 + 0.24HV + 11.05*LS8_NDVI + 0.46*HV_Mean + 0.22*HV_Variance-2.32*HV_Homogeneity-0.14* HV_Contrast + 0.98*HV_Dissimilarit-11.51* HV_Entropy-99.34* HV_SecondMoment-1.40* HV_Correlation	0.60	0.86
3	Density (tree/ha) = -18422.71 + 20.29*HV + 1114.47*LS8_NDVI-59.32* HV_Mean-60.52* HV_Variance + 981.75* HV_Homogeneity + 3.93* HV_Contrast + 223.83* HV_Dissimilarit	0.52	118
4	Biomass (ton/ha) = -4580.94 + 16.00*HV + 618.47*LS8_NDVI--2.56* HV_Mean + 1.18* HV_Variance + 701.63* HV_Homogeneity-15.78* HV_Contrast + 202.59* HV_Dissimilarit + 1118.49* HV_Entropy + 12928.88* HV_SecondMoment-38.33* HV_Correlation	0.91	8.46

Discussion

The results present a general, detailed and clear overview of the mangrove area as well as the dynamic analysis of the mangrove cover in the study area for nearly 5 decades (1972–2020). It was discovered and recorded using the image series from

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DOI: http://dx.doi.org/10.15666/aeer/1905_38973930

Landsat satellites such as Landsat 1 (1972), Landsat 5 (1988, 1995, 2000, 2005), Landsat 7 (2010) and Landsat 8 (2015, 2020). The research results are as follows:

In 1972, this study area did not show the very rich forests, the rich forests and the medium forests; only poor forests' status was indicated with an area of 7,158.15 ha (9.54%) because the mangrove forest was destroyed by the US military's chemical warfare (1965–1970). The land cover information from our results were not very different from the traditional survey results. The forest covers about 6.3% of the natural land area of Can Gio district; the vegetation in the remaining area was in a very poor condition, and it consisted of saplings of *Lumnitzera racemosa, Excoecaria agallocha, Ceriops tagal* and *Ceriops decandra*. In other areas, nothing was to be seen other than shrubs of *Acanthus spp., Derris trifoliata, Finlaysonia obovata, Acrostichum aureum*, etc., with a maximum height of 2 m (Hong, 2001; Tuam, 2002).

In 1988, after 16 years of afforestation (plantation started in 1978), medium mangroves appeared, with an area of 6695.46 ha (8.92%), together with the poor forest area, which was 19170.00 ha (25.54%), increasing the total forest cover in the study area to 34.46%.

In 1995, the recorded area of rich forest was 8995.05 ha (11.99%), with the medium forest at 13205.43 ha (17.60%) and the poor forest at 12578.85 ha (16.76%), increasing the total forest cover in the study area to 46.34%.

In 2000 (recognised by MAB/UNESCO as the first biosphere reserve in Vietnam), after 22 years of restoration, a very rich forest area was recorded at 1625.58 ha (2.17%); the rich forest was 15122.61 ha (20.15%), the medium forest was 11724.88 ha (15.62%) and the poor forest was 10375.16 ha (13.82%), increasing the total forest cover in the study area to 51.76%. At this time, the medium and poor forest areas were lower than in 1995 because some of these forests were converted to rich and medium forests.

In 2005, the very rich forest area was recorded at 4892.91 ha (6.52%); the rich forest area was 15382.35 ha (20.50%), the medium forest area was 11888.77 ha (15.84%), and the poor forest area was 8567.19 ha (11.42%), which increased the total forest cover in the study area to 54.27%. At this time, the mangrove forest was relatively stable in the area as compared to the period before 2000.

In 2010, the very rich forest area was recorded at 9433.27 ha (12.57%); the rich forest area was 15121.80 ha (20.15%), the medium forest area was 11152.53 ha (14.86%), and the poor forest area was 7016.67 ha (9.35%). At this time, the area of the rich forest increased by about 20% as compared to 2005, leading to an increase in the total forest cover of the study area to 56.93%.

In 2015, the very rich forest area was recorded at 10161.18 ha (13.54%); the rich forest area was 18728.36 ha (24.96%), the medium forest area was 9355.57 ha (12.47%), and the poor forest area was 6717.47 ha (8.95%). Overall, the forest cover increased to 59.91%.

Finally, in 2020, the very rich forest area was 11994.93 ha (15.98%); the rich forest area was 21659.20 ha (28.86%), the medium forest area was 8572.88 ha (11.42%), and the poor forest was 5578.09 ha (7.43%). The forest cover in the Can Gio Biosphere Reserve now reached 63.70%.

The results of the analysis based on the change of each mangrove forest type are as follows: for the poor forest type, it was 9.54% in 1972, 25.54% in 1988, 16.76% in 1995, 13.82% in 2000, 15.84% in 2005, 9.35% in 2010, 8.95% in 2015 and 7.43% in 2020. The area of poor forests increased from 1972 to 2005 and then decreased from 2010 to 2020 because some parts of the poor forests were converted to better forests

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_38973930

such as medium, rich and very rich forests. The medium forest type was missing in 1972 and increased to 8.92% in 1988, 17.60% in 1995, 15.62% in 2000, 15.84% in 2005, 14.86% in 2010, 12.47% in 2015 and 11.42% in 2020. This type of forest underwent little change (except in 1998) because the mangrove forest area conversion in positive and negative ways was in balance. Regarding the rich forest type, the data is follows: 1972 (did not appear),1988 (did not appear), 1995 (11.99% with 8995.05 ha), 2000 (20.15%), 2005 (20.50%), 2010 (20.15%), 2015 (24.96%) and 2020 (28.86%). This mangrove forest had a stable area from 2000 onwards, accounting for more than 20% of the total area. The very rich mangrove forest did not appear from 1972 to 1995. In 2000, a small area of 1625.58 ha (2.17%) was recorded, which increased to 6.52% in 2005, 12.57% in 2010, 13.54% in 2015 and 15.98% in 2020. This type of mangrove forest area increased significantly from 2000 to 2010, but from 2010 to 2020, it did not change much because of the following reasons: (i) over maturation of the forest; (ii) the forest was not thinned for 42 years after plantation and (iii) the phenomenon of ageing, along with pests, tropical storms etc. (Diele et al., 2013; Nam et al., 2014).

The analysis of the trend of land cover changes in the area at different times of the study is as follows: (i) the non-change areas (ha and %) are 5076.09 ha/6.76% (1995–2000), 49155.12 ha/65.50% (2000–2005), 48464.64 ha/64.58% (2005–2010), 46234.62 ha/61.61% (2010–2015) and 50787.58 ha/67.67% (2015–2020); (ii) the negative area changes (ha and %) are 17196.39 ha/22.91% (1995–2000), 5765.04 ha/7.68% (2000–2005), 7094.16 ha/9.45% (2005–2010), 8647.20 ha/11.52% (2010–2015) and 5759.35 ha/7.67% (2015–2020); (iii) the positive area changes (ha and %) are 52776.28 ha/70.32% (1995–2000), 20128.60 ha/26.82% (2000–2005), 19489.96 ha/25.97% (2005–2010), 20166.94 ha/26.87% (2010–2015) and 18501.84 ha/24.65% (2015–2020).

In general, the coverage of the mangroves in the study area gradually increased over the years as follows: 9.54% in 1972, 34.46% in 1988, 46.34% in 1995, 51.76% in 2000, 54.27% in 2005, 56.93% in 2010, 59.91% in 2015 and, finally, 63.70% in 2020.

To detect the parameters of mangrove structures using satellite data to find the best detecting model of forest parameters, this study conducted the research in the following different ways. First, we used a single-satellite sensor. Performance of NDVI value from Landsat 8 to detection of mangrove forest parameters are as follows: NDVI and basal area ($R^2 = 0.58$, RMSE = 3.30), NDVI and height tree ($R^2 = 0.30$, RMSE = 1.5), NDVI and density ($R^2 = 0.19$, RMSE = 199) and NDVI and biomass ($R^2 = 0.65$, RMSE = 32.89); HH backscattering and basal area ($R^2 = 0.34$, RMSE = 5.18), HH backscattering and height ($R^2 = 0.17$, RMSE = 1.78), HH backscattering and density ($R^2 = 0.07$, RMSE = 228) and HH backscattering and biomass ($R^2 = 0.42$, RMSE = 54.50); HV backscattering and basal area ($R^2 = 0.81$, RMSE = 1.49), HV backscattering and height ($R^2 = 0.50$, RMSE = 1.07), HV backscattering and density ($R^2 = 0.34$, RMSE = 162) and HV backscattering and biomass ($R^2 = 0.42$, RMSE = 54.50). The values of NDVI and HH backscattering are not highly effective in estimating the structure of mangrove forests. HV backscattering is better, but the detection of the density of trees is very low.

We used a multi-layered linear regression algorithm to improve the modelling of the mangrove structures by combining the multiple variables of NDVI values from Landsat 8 and HV backscattering, including Contrast, Correlation, Dissimilarity, Entropy, Homogeneity, Mean, Second Moment and Variance from ALOS-2 PALSAR-2. In this way, the combined models can improve the explanation of the mangrove structural

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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parameters such as the basal area of 91% ($R^2 = 0.91$, RMSE = 0.71), the height of 60% ($R^2 = 0.60$, RMSE = 0.86), the density of 52% ($R^2 = 0.52$, RMSE = 118) and the biomass of 91% ($R^2 = 0.91$, RMSE = 8.46) by satellite data. These results improved when compared with previous similar studies of Luong et al. (2016), Nguyen et al. (2016) and Luong et al. (2019) and some other studies such as Vu et al. (2014), Hamdan et al. (2014), Thuy et al. (2020).

In this study, the limitation lies in the lack of assessment of the herbicide content at the respective mangrove changes assessment periods, so it has not been able to evaluate the interaction effects between the rehabilitation of mangroves and herbicide concentrations in the soil here. This study also did not compare with the restoration of mangrove forests of other areas in Vietnam as well as other countries and territories around in the world. In addition, there was no comparison of the recovery of other tropical forest types. These will be our future research interests.

Conclusions

The challenges of climate change today are increasingly unpredictable and requires managers of forest resources, including mangroves, to gain appropriate silvicultural solutions for restoring, managing, conserving and sustainable development of the mangrove forests.

Currently, in Vietnam, a national forest inventory is conducted every 5 years, usually by the Institution of Forest Inventory and Planning (FIPI), and mangroves are classified as a single class, called “mangrove forest”. This national forest inventory was not divided into classes such as very rich forest, rich forest and poor forest as in our study, so the data are incomplete and have no much helpful to assess the status and quality of mangroves. Therefore, this study also recommends that the assessment and monitoring of mangroves should be done together with the assessment of the area, quality and structure of mangroves because when there is sufficient information, such as on area and structures, it is meaningful to the silviculturist.

The results from this study are clear proof that the mangroves in Can Gio are well and successfully recovered since being completely destroyed by the spraying of herbicides and other chemical agents by the US military (1961–1965), through data from Landsat satellite images series of the periods (1972–2020) and it is being well managed.

At present, most of the mangrove area in the Can Gio Biosphere Reserve is over 40 years old, with very high tree density, but not once thinned as required by silvicultural measures. As a consequence, the trees do not receive enough nutrients and adequate living spaces, and diseases and pests, such as the anobium borer, lead to the deterioration of the quality of mangroves. Therefore, thinning is a necessary silvicultural measure to facilitate good forest growth at this time.

Today, there is increasing advancement in technology, satellites, sensors, so expanded accessibility of remotely sensed data with other high temporal and resolution imagery. Some are available for free, with strong computer systems, so satellite image processing speed quickly can completely meet the above requirements. Therefore, we propose to conduct mangrove monitoring in this study area with a frequency of once time per year.

Although, mangrove ecosystems that differ in their location of distribution will differ in terms of salinity, depth and flow regime, etc. However, according to some studies of

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_38973930

Ross (1974), Lugo et al. (1974), Salem et al. (2012), FAO (2020), the mangrove forests of South Vietnam are similar, or nearly so, in their species composition to the well-known mangrove communities of Malaysia, Indonesia, Australia, Africa, Mexico and the United States. So, we hope the results of this study are not just crucial for this study area, which is the Can Gio Biosphere Reserve, Vietnam but also be suggestive for other regions of the world with similar natural conditions for better management, monitoring and conservation along with sustainable development of mangrove forests.

Acknowledgments. The authors are grateful to project No. VAST01.07/20-21 from the Vietnam Academy of Science and Technology (VAST) for financial support of this research.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_38973930

MODEL FIT AND THE ACCURACY OF METHODS PREDICTING BODY WEIGHT FROM BODY MEASUREMENTS IN INDONESIAN BALI CATTLE (*BOS JAVAINCUS* D'ALTON, 1823) POPULATION

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(Received 28th Apr 2021; accepted 12th Aug 2021)

Abstract. This paper aimed to provide a reliable method to predict Bali cattle's body weight (BW). In total 1051 records were obtained which comprised of BW, Chest Girth (CG), Body length (BL) and Whither Height (WH) data from three age groups (weaning, yearling and mature). Data were analyzed separately for each sex and age groups. The predicted BW data were derived from two linear models and conversion from cattle weight measure tape. The model with CG, BL and WH as predictor has better model fits than the model with only CG. Both models have reliable prediction ability indicated by low RMSE (6.623 - 18.684) and CV-RMSE values of less than 25%. Utilizing measuring tape as prediction tool is not recommended due to its poor performance (CV-RMSE > 25%). Bali cattle is a distinct cattle species with unique characteristics; hence, linear model is a suitable method to predict the BW for further purposes.
Keywords: *cattle body weight, reliable prediction, Indonesian native cattle, linear model fit*

Introduction

Applying linear models to predict cattle weight based on their body measurements is a common practice in livestock industry. Studies revealed that employing linear regression model to predict body weight based on their chest girth (CG) has a high model fit; indicated by the coefficient of determination values (R^2) of more than 60% in crossbred dairy cattle in Kenya (Lukuyu et al., 2016), brown-swiss cattle in Turkey (Ozkaya and Bozkurt, 2009) and also in Ethiopian oxen (Goe et al., 2001). Other predictor variables such as body length (BL) and whither height (WH) were also reported to be informative in explaining the variation in cattle's body weight (Heinrichs et al., 1992; Lukuyu et al., 2016; Ozkaya and Bozkurt, 2009).

The goodness of fit for linear models in predicting livestock's body weight based on their body measurements is usually represented by the coefficient of determination (Gunawan and Jakaria, 2007; Lukuyu et al., 2016; Ozkaya and Bozkurt, 2009). It indeed tells us about how good a model is in explaining the variation of the data; however, it does not tell us about the predictive ability of the model. In order to achieve an accurate prediction model, it needs to be tested; an option is by employing cross validation technique. It is a method in which dataset was partitioned into training and test sets

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_39313943

iteratively; the training set was used to build prediction model and the test set was used to validate the model and then to estimate the accuracy of the model prediction (Efron and Gong, 1983; Kohavi, 1995; Schaffer, 1993).

Another method of obtaining the predicted body weight is by utilizing cattle measuring tape (Rondo®); which basically also an application of linear model with CG as the predictor variable. This measuring tape is widely used by livestock practitioner in various countries in Asia (Samosir and Hakim, 2016; Wangchuk et al., 2018). However, its accuracy in predicting body weight of Bali cattle is yet to be estimated.

Bali cattle (*Bos javanicus*) is an Indonesian native cattle species. It is originated from wild Banteng which was first domesticated in the isle of Bali (Copland, 1996; Mohamad et al., 2009; Sutarno and Setyawan, 2015). Most of Bali cattle were reared in semi-intensive farming system (Sari et al., 2016) and mostly owned by smallholder farmers with 2-5 cattle per household (Martojo, 2003). Considering this condition, using body measurements as productivity indicator is preferred by both farmers and the distributors. The reason is mainly for the sake of ease of practice, especially when the access to weighing scale is limited. The ability to accurately predict the cattle's body weight is essential in order to avoid the underestimation of the cattle's economical value as well as in aspects related to veterinary services specifically in administering the correct dose of drugs to the livestock (Machila et al., 2008).

Although methods in predicting body weights based on the body measurements in livestock are common, the level on the fitness and predictive ability vary, depend on the breed or species, sex, age as well as environmental factors. It is thus, specific models need to be developed for different livestock commodities with different production systems. Bali cattle are genetically distanced from two other more commonly found cattle species namely *Bos taurus* and *Bos indicus* (Mohamad et al., 2009); hence, in this study we aimed to distinctively build a body weight prediction model specifically for this species.

Materials and methods

Data collection

Data were collected from the progeny test population at the Bali cattle Breeding Center (BPTU-HPT Plulukan, Singaraja, Bali; 8.4268° S, 114.8639° E) to minimize the chance of having systematic environmental effects. In this facility, mature and fertile female cattle were kept in paddocks with 30 individuals per colony. During the month of September – November, one tested bull was moved in into each paddock to mate naturally with the females. Hence, every year, in this breeding center, the calves were born within approximately the same time period. The obtained data comprised of body weight (BW), body length (BL), chest girth (CG) and wither height (WH). The measurements were conducted on cattle at weaning (age 205 days ± 30 days); yearling (age 365 days ± 30 days) and mature (age > 547 days).

The cattle were weighed with electric weighing scale for livestock with maximum capacity of 2000 kg to the closest 500 g. Rondo® tape was used to measure the CG (in cm) as well as to obtain the instantly predicted body weight (in kg), by observing the opposite side of the tape of the CG value. CG value was obtained as the circumference of the chest behind the front shoulders. BL was measured as the distance from the highest point of the shoulders to the pin bone; and WH was measured as the distance from the ground to the highest point of the withers (Lukuyu et al., 2016). Data with

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DOI: http://dx.doi.org/10.15666/aeer/1905_39313943

missing values, outliers and any anomalies were removed. In total there were 447 records for weaning age (245 male and 202 female); 376 records for yearling age (202 male and 174 female) and 228 records for mature age (126 male and 102 female).

Data analysis

Data analysis were conducted separately for each age group (weaning, yearling and mature) and sex group (male, female and overall) resulting in total of nine subsets of data. Summary statistics of the observed variables are presented in *Table 1*. T-test with $\alpha = 0.05$ were performed to test the difference between the male and female cattle body weight and body measurements. There were two prediction approaches used in this study: 1) linear regression models and 2) body weight prediction based on the Rondo® measuring tape. Prior to model building, Principal Component Analysis (PCA) biplot was used to visualize the correlations among the observed variables.

Table 1. *Summary statistics of the observed variables*

Traits	Number of observations	Mean ± standard deviation			
		Body weight (Kg)	Chest girth (cm)	Body length (cm)	Whither height (cm)
Weaning					
Male	245	87.54 ± 14.32[a]	105.48 ± 10.46[a]	82.53 ± 7.11[a]	87.34 ± 7.37
Female	202	81.42 ± 13.45[b]	103.11 ± 7.13[b]	81.31 ± 5.61[b]	86.79 ± 4.52
Yearling					
Male	202	124.28 ± 16.52[a]	121.13 ± 6.09[a]	91.38 ± 6.39[a]	95.64 ± 4.87[a]
Female	174	110.81 ± 7.66[b]	108.51 ± 7.51[b]	88.25 ± 7.08[b]	92.32 ± 4.74[b]
Mature					
Male	126	199.61 ± 50.69[a]	145.10 ± 13.30[a]	109.08 ± 9.78[a]	110.76 ± 6.84[a]
Female	102	163.00 ± 34.83[b]	134.63 ± 10.45[b]	101.61 ± 6.41[b]	103.72 ± 5.20[b]

Two basic linear models were built to predict Bali cattle's body weight (BW) based on body measurements at different age and sex groups. The first model (*Model 1*) is a simple linear regression with CG as the predictor. The second model (*Model 2*) is a multiple linear regression with all body measurements (CG, BL and WH) as the continuous independent variables. The models read:

$$BW_{ijk} = \beta_{01ij} + \beta_{11ij} CG_{ijk} + \varepsilon_{1ijk} \quad \text{(Model 1)}$$

$$BW_{ijk} = \beta_{02ij} + \beta_{12ij} CG_{ijk} + \beta_{22ij} BL_{ijk} + \beta_{32ij} WH_{ijk} + \varepsilon_{2ijk} \quad \text{(Model 2)}$$

where i = weaning, yearling, mature; j = male, female, overall; $k = 1,2,\ldots,n_{ij}$. ε_{hijk} is the error term for individual k in model h for the age group i and sex group j. β_0's is the intercept. β_1's, β_2's, and β_3's are the regression coefficient for CG, BL, and WH respectively. In total eighteen models were analyzed. The coefficient of determination (R^2) and the standard error of prediction (SEP) were estimated for each model as the parameter of model fitness. Validations were conducted to the eighteen models by means of Leave One Out Cross Validation (LOOCV).

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DOI: http://dx.doi.org/10.15666/aeer/1905_39313943

We applied both models (*Models 1* and *2*) to all nine data subsets (based on three age groups and three sex groups); in total 18 equations were made. For each of the nine group, we also obtained predicted BW values from Rondo® measuring tape. To evaluate the prediction quality, we calculated the Root of Mean Squared Errors (RMSE), both as estimated value and as Coefficient of Variation (CV) presented in percentage (%); and AIC (Akaike Information Criterion) which specifically performed to compare *Models 1* and *2* within each subset of data.

Data were analyzed using R programming language (R Core Team, 2020). The R package 'caret' (Kuhn, 2012) was employed for running the LOOCV; package 'tdr' (Lamigueiro, 2018) was used to estimate the predictive ability metrics and as crosscheck for the estimates obtained from LOOCV with 'caret'. Graphical data visualization was assisted by 'ggplot2' (Wickham, 2016) and 'factoextra' (Kassambara and Mundt, 2020) packages.

Results and discussion

Summary statistics of body weight and body measurements

Body weight and body measurements data at weaning were collected at age range of 175 – 235 days; yearling was at age range of 335 – 400 days, whereas mature age was collected above the age of 547 days or 1.5 years (*Table 1*). Male cattle were significantly heavier ($P \leq 0.05$) during weaning age compared to the female cattle. They also had significantly bigger builds in terms of CG and BL when compared to the female cattle. This trend was also consistent for yearling and mature age groups.

Bali cattle is relatively small when compared to *Bos indicus* and *Bos taurus*. The mean weaning weight (WW) of male and female cattle in this study is within the range of Bali cattle WW reared in different locations in Indonesia which were between 64.4 – 83.9 Kg (Martojo, 2003); but lower compared to the breeding stocks of the same institution where the data was obtained, which were 87.00 – 90.48 kg for female cattle and 88.51 – 98.92 Kg for male cattle (Sari et al., 2016). Yearling weight (YW) of the cattle in this study were also within the normal range of 99.2 – 14.33 Kg (Martojo, 2003; Sari et al., 2016). The mature weights (MW) in our study on average were 199.61 and 163.00 kg for male and female cattle, respectively. This MW values are lower than other studies, which mentioned the mean of mature weights were ranged between 200 to 300 kg (Lindell, 2013; Martojo, 2003). The average cattle's age at the collection of MW data in this study was 650 days or around 1.9 years; difference in measurement age might contribute to the variation of MW.

The difference in body weight and body measurements between sexes became larger as they get older (*Fig. 1*). From this figure, it is clearly visible that although there are still increases in body weight after the cattle matured (age > 1. 5 years; yellow dots), but the body measurements of CG, BL and WH were relatively constant. However, from weaning to yearling the increases in CG, BL and WH are still observable. These results are reasonable as bone structure and body conformation change during animal's growth due to hormonal and physiological reasons (Ford and Klind, 1989).

Model building

In total eighteen linear regression models were built to predict body weight based on body measurements. The most common and most important body measurement variable

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for body weight prediction is CG as it represents the circumference of the cylindrical shape of cattle's body. *Model 1* in this study used CG as the independent variable as suggested by earlier studies (Abdelhadi and Babiker, 2009; Gunawan and Jakaria, 2007; Kashoma et al., 2011; Lukuyu et al., 2016; Ozkaya and Bozkurt, 2009; Tisman et al., 2015; Vanvanhossou et al., 2018).

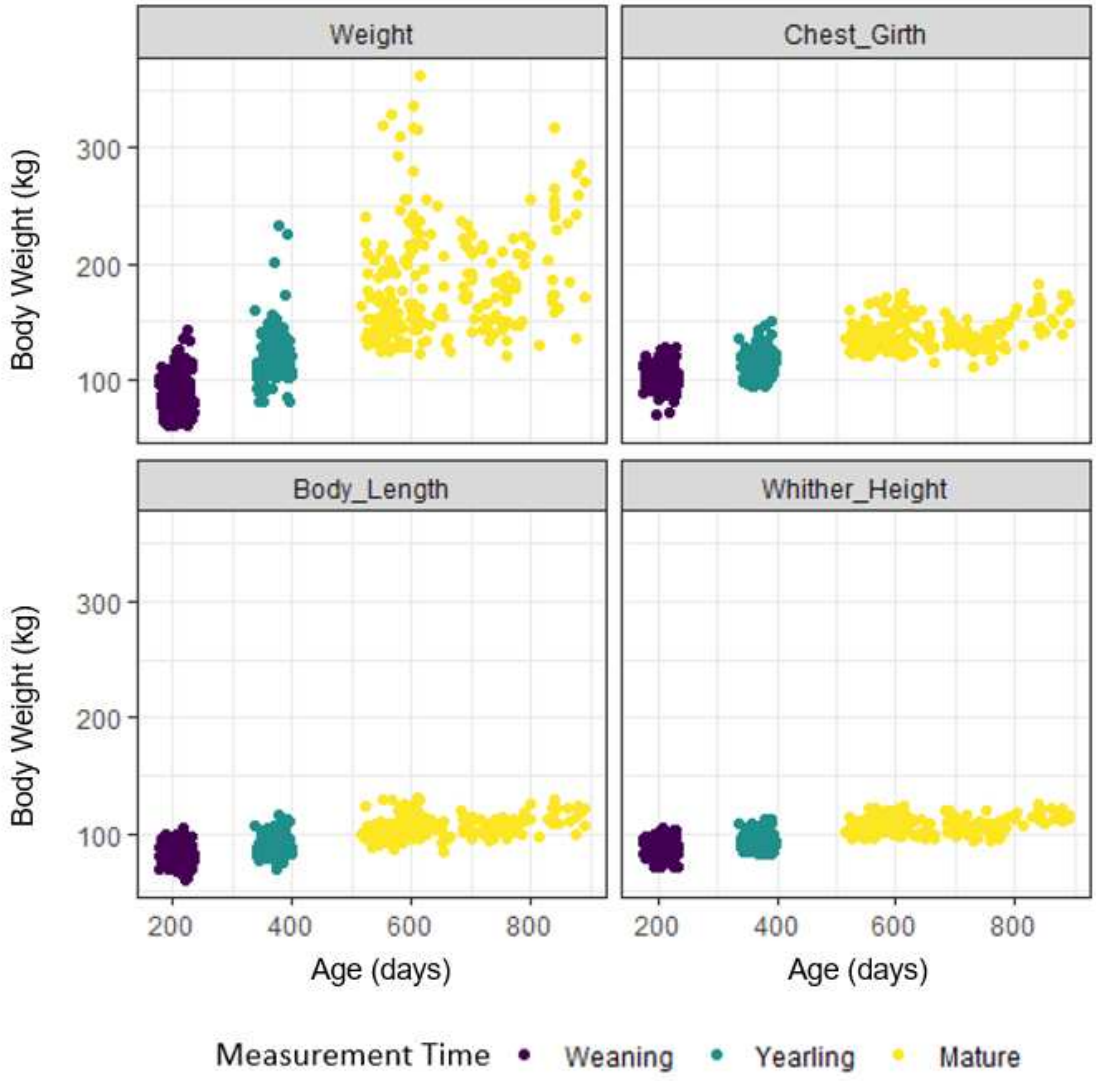

Figure 1. *The distribution of body weight and body measurement variables across measurement time*

Model 2 incorporates all the body measurements (CG, BL and WH) in a multiple linear regression. Both BL and WH shares low positive correlation with BW (*Fig. 2*); however, the inclusion of these variables might improve the model's fitness and predictive ability (Gunawan and Jakaria, 2007; Sahu et al., 2017).

We visualized the correlations among the observed variables in our study with the aid of a PCA-biplot. PCA-biplot contained information regarding the PCA score and the loading plot; the smaller the angle between vectors on the same side of the plot showed high positive correlation while larger angle showed less correlation (Ott et al., 2010).

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DOI: http://dx.doi.org/10.15666/aeer/1905_39313943

The result of PCA analysis showed that the principal component 1 (PC1) explained 90.70% of the total variances whereas PC2 explained 5.20%; hence, these principal components explained sufficient amount of variance to visualize the correlations among explanatory variables in the data without losing much information. *Figure 2* showed that body weight had the highest positive correlation with CG. Studies also suggested that the correlation coefficient between body weight and CG were high; with values between 0.84 – 0.90 in Bali cattle (Gunawan and Jakaria, 2007; Paputungan et al., 2018), 0.57 – 0.80 in Sahiwal cattle (Sahu et al., 2017), 0.92 – 0.95 in Somba cattle (Vanvanhossou et al., 2018) and 0.93 – 0.94 in Tanzanian Shorthorn (Kashoma et al., 2011). The widely used Rondo® measuring tape is also based its prediction on CG value (Machila et al., 2008; Wangchuk et al., 2018).

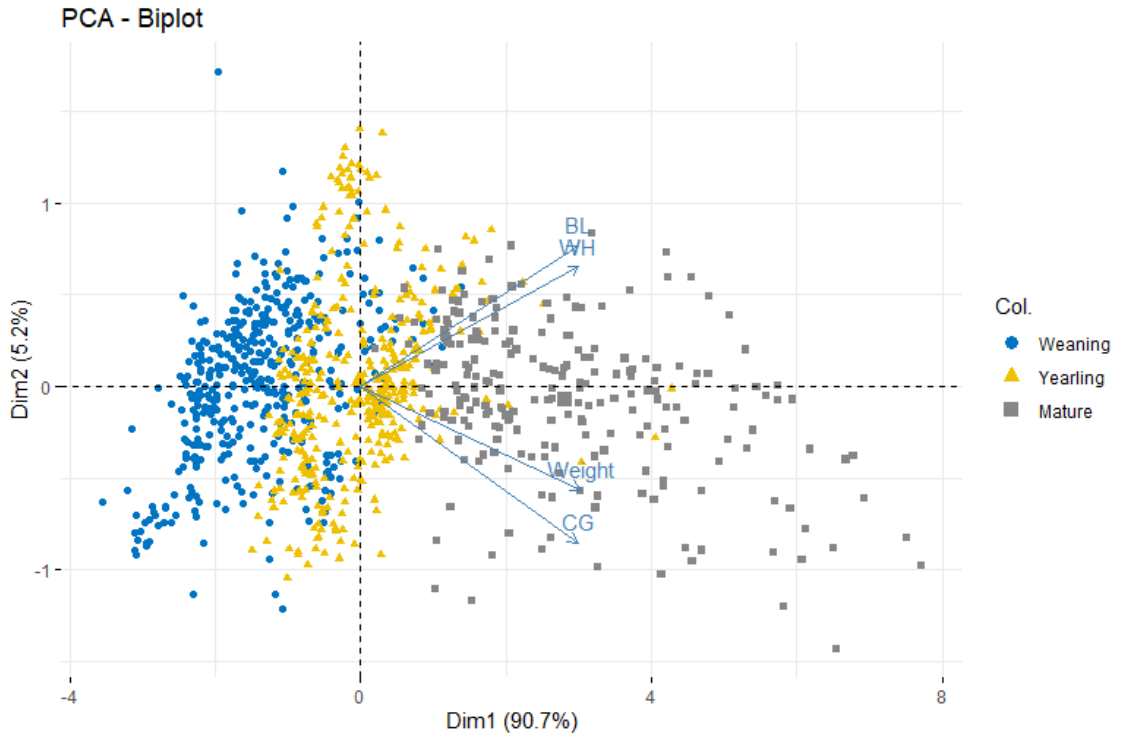

Figure 2. *PCA-biplot to show the correlation between body weight and the body measurements in different observation time.*

Linear models to predict body weight from body measurements

The linear regression models of body weight on body measurement variables for the three age and the three sex groups are presented in *Table 2*. Internal validity checks were performed by testing for outliers with residual QQ plots for both lowest and highest values in each subset of data. Homoscedasticity was also tested with the plots of fitted-residual values. The results (not shown) indicated that there were no outliers and there were randomly spread residual variances in all subsets of data. The parameter estimates of the linear models along with their standard error of predictions (SEP) are presented in *Table 2*.

Standard error of prediction measures the dispersion of predicted values from the known values; hence it indicates how precise the prediction equation is (Hennig and

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Cooper, 2011; Hinton, 2014). In this study, two models were tested on each data subset; and the model with the smaller SEP is considered as giving more precise prediction (Hennig and Cooper, 2011). The results in *Table 2* showed that across all sexes and age groups, *Model 1* with only CG as predictor variable have lower SEP compared to *Model 2* with CG, BL and WH as predictor variables. The adjusted coefficient of determinations (*Table 3*), however, were higher in *Model 2* in all equations. Equations in *Model 2* indeed explained more variances in the data subsets compared to *Model 1*; but *Model 1* gave more precise predictions than *Model 2*.

Table 2. *Linear models to predict body weight from body measurements*

Equation number	Factors	Linear regression equations	SEP*
	Male		
1	Weaning	BW = 13.627 + 0.700CG	0.858
2		BW = -48.521 + 0.398CG + 0.519BL + 0.587WH	2.734
3	Yearling	BW = -107.629 + 1.915CG	1.737
4		BW = -146.975 + 1.434CG + 0.636BL + 0.413WH	4.027
5	Mature	BW = -298.887 + 3.435CG	5.563
6		BW = -337.483 + 2.464CG + 1.475BL + 0.168WH	10.406
	Female		
7	Weaning	BW = -70.835 + 1.477CG	0.822
8		BW = -103.054 + 1.184CG + 0.401BL + 0.343WH	3.608
9	Yearling	BW = 92.625 + 0.1676CG	0.585
10		BW = 12.218 + 0.209CG + 0.075BL + 0.751WH	1.926
11	Mature	BW = -254.947 + 3.105CG	3.254
12		BW = -326.738 + 2.625CG + 0.668BL + 0.660WH	6.483
	Overall		
13	Weaning	BW = -13.678 + 0.943CG	0.628
14		BW = -70.118 + 0.611CG + 0.553BL + 0.526WH	2.191
15	Yearling	BW = 3.815 + 0.991CG	0.690
16		BW = -78.103 + 0.759CG + 0.483BL + 0.693WH	1.983
17	Mature	BW = -287.439 + 3.352CG	3.095
18		BW = -332.577 + 2.545CG + 1.139BL + 0.302WH	6.234

*Standard error of prediction

The performance of the predictive methods

Both the linear models (*Models 1* and *2*) were subjected to cross validation procedure to compare their performances in predicting cattle's body weight based on the body measurement variables. This approach was taken in order to produce the predictive values from each model to be compared with the observed values in the dataset. Statistical metrics were estimated based on comparing the predicted versus the observed values (*Table 3*).

The adjusted coefficient of determination (R^2) of *Model 1* of male cattle is lowest in weaning data (0.258) and highest on mature data (0.835). In mature data of male cattle, the R^2 of *Models 1* and *2* were similar, whereas in weaning data, *Model 2* performed

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DOI: http://dx.doi.org/10.15666/aeer/1905_39313943

much better than *Model 1*. On the other hand, both *Models 1* and *2* performed poorly when applied on the yearling data of female cattle with R^2 of 0.210 and 0.283 respectively. The models were considered as moderately explaining the variance in the weaning and yearling data and highly explaining the variance in mature data subset in all three sex groups.

Table 3. *The fitness and accuracy of the prediction models and Rondo® tape*

Prediction methods	**R^2adj[1)]**	**RMSE[2)]**		**AIC[3)]**
Linear models		**Value**	**CV[4)] (%)**	
Equation number*				
1	0.258	15.094	17.243	1930.207
2	0.494	13.347	15.247	1838.451
3	0.496	12.223	9.835	1572.096
4	0.577	11.208	9.018	1538.369
5	0.811	22.315	11.179	1141.032
6	0.835	21.122	10.582	1125.910
7	0.610	8.523	10.467	1436.918
8	0.650	8.193	10.062	1416.903
9	0.210	7.692	6.941	1202.348
10	0.283	6.623	5.977	1150.142
11	0.865	12.949	7.944	813.224
12	0.882	12.278	7.533	802.152
13	0.367	12.723	15.008	3343.222
14	0.528	11.496	13.560	3314.384
15	0.383	11.760	9.963	2915.187
16	0.547	10.124	8.576	2801.023
17	0.850	18.684	10.197	1928.153
18	0.869	17.543	9.574	1952.510
Rondo tape				
Male				
Weaning	0.721	25.308	28.987	-
Yearling	0.544	34.758	27.967	-
Mature	0.816	67.323	33.728	-
Female				
Weaning	0.593	22.668	27.839	-
Yearling	0.398	23.061	20.811	-
Mature	0.868	50.859	31.202	-
Overall				
Weaning	0.675	24.148	28.529	-
Yearling	0.441	29.919	25.345	-
Mature	0.854	60.513	33.026	-

[1)]Adjusted coefficient of determination; [2)] Root of Mean Squared Error; [3)] Akaike Information Criterion; [4)] Coefficient of Variation; *Refers to *Table 2*

The overall dataset is the total data regardless of sex. Results of R^2 in *Table 3* showed that *Model 1* fit poorly in weaning and yearling data (0.367 and 0.383), but it

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DOI: http://dx.doi.org/10.15666/aeer/1905_39313943

performed well when applied in mature data (0.850). *Model 2* has moderate fitness in weaning and yearling data but high fitness in mature data.

There are, however, explanations on why our fitness estimates were deviated from most of the references which mentioned that linear regression model including CG, which is a variable closely correlated with body weight, normally yielded in high R^2 (Gunawan and Jakaria, 2007; Kashoma et al., 2011; Paputungan et al., 2018; Vanvanhossou et al., 2018). Bali cattle as the object of our study, has not been subjected to any well-designed selection program; thus, their vast genetic variation has yet to undergo any intense selection procedures (Widyas et al., 2017). Although the data were obtained from Bali Cattle Breeding Center, but the currently running breeding program was a conventional and very outdated one; hence, the breeding program's parameters (if existed) are less informative and reliable. The cattle in this study lived in a free-range system; where a colony of cattle stayed in a paddock of pasture with an open shelter (Gunawan and Jakaria, 2011; Widyas et al., 2017). The shelter was also functioned as the place for additional food and water aside from the grasses within the pasture paddocks. In this type of production system, monitoring every cattle's feed consumption is almost impossible. Such free-range cattle production system also introduced natural competition for the resources; especially when their availability was limited; hence, this contributed to higher variation in the cattle's performances.

Weaning to yearling is the most crucial growth period for cattle. Weaning Weight (WW) was measured when the calves were weaned from their mothers (±7 months old or 205 days). This was a phase where the calves were very vulnerable because they must adapt from milk to solid feed. The adaptation ability, of course, may vary among individuals and stress is a common occurrence during this period. On the other hand, yearling weight (YW) was measured at the age of 12 months or around 5 months after the cattle were weaned. The body weight and body measurements data obtained at yearling were thus very dependent on the ability of the individual to cope and adapt with the condition after weaning. The production system and the resources within the system lead to high variation in the performance of Bali cattle (Lindell, 2013); hence, causing the normally well-performed linear models to be less optimal in this population's data. However, after the cattle reached mature age (above 1.5 years) the trend changed, and the structure of the mature dataset are more similar with what commonly occur in this type of study; showed by increases in model fitness parameter.

RMSE is a statistical metric to measure a model's predictive ability which can be obtained by applying cross validation procedure on a dataset. The bias introduced by RMSE estimate is lower compared to the other parameters obtained without cross validation; it is also more robust for smaller dataset (Suparman, 2012). This metric indicates the absolute fitness of the models and could be a representation of their predictive ability. The value of RMSE can only be compared between models applied on the same data. In this study, *Model 2* where all of the body measurement variables (CG, BL and WH) were included, gave predicted values with higher accuracy compared to *Model 1* where only CG was used. The value of the RMSE does not represent anything because it depends on each dataset within which the models were trained (Kohavi, 1995; Schaffer, 1993). Hence, we introduced the coefficient of variation of the RMSE (CV-RMSE) as a measure of errors between predicted and observed data. CV-RMSE is calculated by normalizing the RMSE by the mean of the observed body weight. The value of CV-RMSE below 25% is considered as having a good model fit and reliable predictive ability (Ruiz and Bandera, 2017). In our study the CV-RMSE

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DOI: http://dx.doi.org/10.15666/aeer/1905_39313943

were ranged between 5.977 – 17.243% which made both *Models 1* and *2* were good predictors of cattle's body weight.

To evaluate which model is best in the prediction of cattle's body weight we employ Akaike Information criterion (AIC). This procedure estimates the measure of similarity between models for the same data (Burnham et al., 2011). The best model is the one with the lowest AIC. The results in *Table 3* showed that *Model 2*, with CG, BL and WH as independent variables always had better predictive performance compared to *Model 1* within the same dataset. Further the difference AIC values (ΔAIC) are more than 10 which suggest that the less performed model has no substantial support in the data and deserve no further consideration (Burnham et al., 2011; Wolfinger, 1996).

Rondo® tape is a measuring band in which one side has the unit of cm to measure CG, whereas the subsequent side written the body weight predictive values in kg. We have yet to find information on how the conversion from CG to body weight was made for this tape. Despite this fact, however, this tool is widely used in Indonesia and in Asia (Wangchuk et al., 2018; Widi et al., 2014) due to its practical use. We built a dataset of the predicted body weight based on the conversion of observed CG to body weight using the Rondo® tape and estimate the predictive ability metrics (*Table 3*). The result showed that although there are some high correlation values between the body weight prediction based on the tape versus the observed body weight, but at the same time the RMSE values are considerably high when compared to the prediction using linear models. The CV-RMSE are higher than 25% for all subset of data, suggesting that this tape is less reliable as a predictive tool.

We also calculated the difference between predicted and observed values and plot them against the CG data (*Fig. 3*).

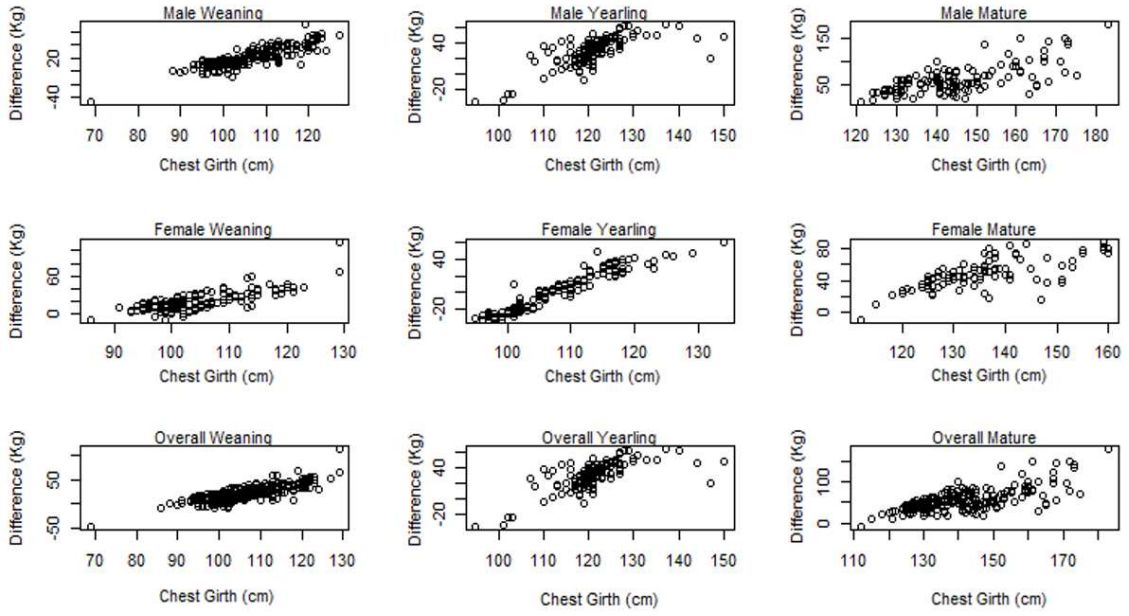

Figure 3. *Relationship between the deviation from observed values and cattle's chest girth*

It is clear to see that CG had high positive correlation with the difference; thus, the larger the CG, the predicted body weight deviated even further from the observed body weight in all subsets of data. Based on this finding, we do not recommend using

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Rondo® tape as a tool to predict Bali cattle's body weight due to its poor accuracy. There are risks of severe over or under-estimation of body weight of Bali cattle which will lead to biased prediction of the performance and economic value of the cattle, inaccurate dose of drug administration, and, if this tool is used in scientific study, it will introduce biased that affect the results of the research.

Conclusions

Bali cattle is a unique cattle species with distinct characteristics and has large genetic distance with the other bovine species. It is why the commonly used practical approaches in predicting body weight based on the body measurements might not be accurate. Rondo® tape is not recommended to be used as body weight prediction tool for these cattle. However, linear models incorporating body measurement variables yielded promising performance in predicting the body weight of this cattle.

Acknowledgement. This project was funded by Master Thesis Grant scheme provided by Directorate General of Higher Education with contract number 113/UN27.21/HK/2020, and the project was coordinated by Dr. Sigit Prastowo.

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DOI: http://dx.doi.org/10.15666/aeer/1905_39313943

GENOME-WIDE ANALYSIS OF DNA METHYLATION PATTERNING IN ALFALFA (*MEDICAGO SATIVA* L.) UNDER MUTAGENIC TREATMENT USING BISULFITE-SEQUENCING (BS-SEQ)

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(Received 28th Apr 2021; accepted 20th Aug 2021)

Abstract. DNA methylation is an important type of epigenetic modification that plays crucial roles in many biological processes. To investigate the epigenetic effect mechanism of ethyl methanesulfonate (EMS) mutation treatment on alfalfa, the whole genome DNA methylation profile of normal growth alfalfa and EMS mutation treatment alfalfa were analyzed by whole-genome bisulfite sequencing (WGBS) technology. The results showed that there were three main types of alfalfa DNA methylation: mCG, mCHH and mCHG; after alfalfa was mutagenized, the rate of methylation at the C site increased, and the methylation rates of CHH, CG and CHG all increased. In addition, DNA methylation mainly occurs in the CG sequence; a total of 8707 DMRs were detected in the differential methylation region (DMR) identification study. Among them, there were 5221 and 3486 hypermethylated DMRs and hypomethylated DMRs after alfalfa mutagenesis. Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses revealed some DMGs related to the occurrence of physiological metabolic processes. Finally, we found that 33 DMGs, including MTR_2g015550, MTR_6g088795, MTR_4g011180 and MTR_0034s0170, are more likely to be involved in plant carbon metabolism, nitrogen metabolism pathways, various amino acid metabolisms and glucose metabolism.
Keywords: *genome-wide DNA methylation, alfalfa, ethyl methane sulfonate, differentially methylated region, methylation profile*

Introduction

Alfalfa (*Medicago sativa* L.) is known as "the King of Forage" because of its high yield, high quality and wide adaptability in the livestock industry. The "Document No. 1" of the Central Government clearly stated: "Accelerate the development of the forage industry, support the cultivation of alfalfa and silage, carry out a combination of grain-feeding and planting, and promote the coordinated development of the ternary planting structure of grain, cash crops, and forage." To develop and grow the forage industry, we must first solve the problem of supplying a large number of high-quality forage. However, the bottleneck of alfalfa industry development in China is mainly the lack of resistant germplasm and high-yield variety resources. High-quality alfalfa species still depend on foreign imports. With the development of animal husbandry in China, animal husbandry in different regions is developing and growing, which has higher requirements for alfalfa yield and quality. China's animal husbandry is mainly concentrated in the northeast and western regions. However, there is a problem of low overwintering rate of alfalfa in the northeast and other alpine regions, which seriously affects the yield and quality of alfalfa and reduces the economic benefits of growers. In

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_39453957

the northwest and other saline alkali areas, the poor salt-tolerance ability of domestic alfalfa also seriously affected the yield of alfalfa, resulting in the low quality and small quantity of domestic alfalfa. In view of the above problems, this study used mutation method to treat alfalfa in order to screen out different resistant or high-yielding variant strains, and analyze the epigenetic characteristics before and after the mutation to provide a theoretical basis for enriching alfalfa germplasm resources. Therefore, it is necessary improve the quality of domestic varieties through improving and enriching forage germplasm resources by using innovative scientific and technological means. Practice has proved that the use of artificial mutation technology is one of the important means of enriching crop germplasm resources, creating new materials and breeding new species (Mashinsky et al., 2001). Mutation breeding has become a method widely used by breeding scholars. In the study of biological effects, mutagenesis mainly studies the changes of cell phenotypes or physiological and biochemical characteristics caused by mutagenesis (Singh et al., 2006; Li et al., 2013; Shi et al., 2010), but in the existing reports, there is a lack of mutagenesis studies on plant epigenetics.

DNA methylation has the function of protecting the genome from exogenous inserted sequences and regulating gene expression in plants, and at the same time, it can control gene expression, maintain genome stability, and play an important role in heterosis. In recent years, with the rapid development of second-generation sequencing technology, genome-wide DNA methylation research has also developed rapidly, and its research has played an important role in many fields such as biology, medicine, agriculture, and the environment (Xue, 2017). Studies by Mirbahai and Grafi (Mirbahai et al., 2014; Grafi. 2011) showed that plant cytosine DNA methylation changes under stress conditions, indicating that plants are involved in DNA methylation in the process of resisting stress environments. Kim et al. (2013) found that radiation can cause changes in plant DNA methylation: the level of genomic DNA methylation in wild-type *Arabidopsis* decreases with increasing dose of gamma radiation. Studies by Shi Jinming et al. (2009) and Zhao et al. (2016) showed that the epigenetic effect of dried rice seeds under heavy ion radiation was significant, and the rate of methylation change of cytosine was higher than that of demethylation.

At present, genome-wide DNA methylation research is mainly concentrated in the fields of human diseases and livestock and poultry. In terms of crops, there are many studies on the methylation changes of single genes or specific sites, but there are few studies on the analysis of the DNA methylation status and methylation patterns of mutational stress alfalfa at the whole genome level. Previous studies have shown that whole-genome bisulfite sequencing (WGBS) is the most comprehensive of the existing methods. In this study, we investigated DNA methylation profiles of alfalfa before and after EMS mutagenesis during the seedling stage using WGBS technology. Our research systematically analyzed the molecular differences of alfalfa tissue methylation levels before and after mutagenesis. In addition, our findings will advance knowledge and understanding of the alfalfa methylome.

Materials and methods

Plant material and mutation treatment

Longmu 806, a main alfalfa variety planted in Heilongjiang Province, P. R. of China. It was selected at the Heilongjiang Province Animal Husbandry Research Institute.

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Ethyl methanesulfonate (EMS) treatment (Shen et al., 2018): Select 300 full-fledged mature alfalfa seeds, treat with concentrated sulfuric acid for 5 min, and then rinse with distilled water multiple times. The seeds were soaked in phosphate buffer solution (100 mmol • L^{-1}, pH 7.0) at 4 ℃ for 12 h, and the seeds were completely submerged in water to swell. Phosphate buffer solution was used to prepare a 0.4% EMS solution (preliminary tests have screened out the most treatment concentration) (Shen et al., 2018; Shen, 2018). The seeds were treated at room temperature for 15 h under dark conditions, and then repeatedly washed with distilled water to remove the residual EMS solution on the surface of the seeds. Untreated seeds were used as controls, and three experimental replicates were set for each experimental treatment.

DNA extraction, WGBS library construction and sequencing

Collect a mixture of leaves with consistent growth and development at the three-leaf stage, 10 strains as a sample, and the test samples were control group (A34_1A) and EMS treatment group (A34_2A). The construction of whole-genome bisulfite sequencing (WGBS) library mainly includes the following four aspects: the genomic DNA was extracted by CTAB method; Genomic DNA was interrupted into 300-700 bp fragments by Bioruptor Pico ultrasound; and then bisulfite conversion was performed using the EZ DNA Methylation Gold Kit; the Accel-NGS® Methyl-Seq DNA Library Kit is used to build single-stranded DNA libraries. After library quality detection, pair-end sequencing was performed with an Illumina XTen (Illumina, San Diego, CA, USA) sequencer, and the specific method was referred to the literature (Guo et al., 2017). All operations were conducted following the Wuhan Kangtest Bioinformation Technology Co., Ltd.'s recommended instructions.

Filtering, comparison and analysis of sequencing data

The peak signal produced by the Illumina HiSeq was transformed into base sequence by base calling as Raw Data or Raw Reads. The Raw Reads were then filtered for subsequent information analysis to ensure the quality of information analysis, including the removal of reads that have adapters and filtration of reads with more than 10% N content or more than 50% low quality bases. The final filtered data are called clean reads.

The sequencing reads need to be aligned with the reference genome (the reference genome selected *Medicago truncatula*, derived from ftp://ftp.ensemblgenomes.org/pub/release-33/plants/fasta/medicago_truncatula/dna/Medicago_truncatula.MedtrA17_4.0.dna.toplevel.fa.gz) before conducting the methylation analysis. Bismark software was used to perform a comparison of the alignments of bisulfite-treated reads to a reference genome using the default parameters (Krueger, 2011).

Estimating methylation levels and the identification of DMRs

To detecting the different methylated C sites in a region, we defined C_i as the number of supporting methylation reads at a single C site, T_i as the number of supporting unmethylation reads at a single C site, i as the position of C. The methylation level of a C site was counted as follows (Schultz, 2012):

$$\text{Methylation level of C site} = C_i / (C_i + T_i) \times 100 \quad \text{(Eq.1)}$$

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Use swDMR software (version 1.0.0) to detect DMR (Yang, 2020). swDMR uses a sliding window (sliding window size: 1000 bp; step length: 100 bp) to detect DMR. Fisher's exact test was used for two samples in this study.

Enrichment analysis of DMR gene KEGG pathway

Pathway enrichment analysis takes KEGG Pathway as the unit (Young, 2010), and applies hypergeometric test to find the pathways that are significantly enriched in DMR-related genes compared with the background of the entire alfalfa genome. KOBAS (Mao, 2005) was used to perform gene pathway enrichment analysis on the gene list where DMR is located, and the pathways with pathway enrichment P-value (or corrected *P*-value) less than 0.05 and the number of genes in this pathway of the query gene were more than 2 were screened out (Xie et al., 2011).

Results

Analysis of alfalfa whole genome DNA methylation sequencing data

A total of 21.46 G and 17.91 G raw bases were generated on average for the A34-1A and A34-2A respectively by using the Illumina XTen sequencing platform. After data filtering, approximately 43 G of Clean reads were obtained (*Table 1*). Use the software SOAPnuke (version 1.6.0) to align the clean reads obtained from the A34-1A and A34-2A samples to the alfalfa genome sequence. The alignment rates are 90.40% and 88.72%, respectively. The average sequencing depth of the whole genome is 50.71× and 42.93× respectively.

Table 1. *Statistical results of sequencing data and clean reads map to reference genome results*

Samples	Raw reads (G)	Clean reads (G)	Mapped reads (G)	Mapped rate (%)	Average depth (×)
A34_1A	21.46	21.44	19.38	90.40%	50.71×
A34_2A	17.91	17.89	15.87	88.72%	42.93×

The characteristics of genome-wide methylated cytosine C in alfalfa

This study found that 32.00% and 35.79% of C sites in the control sample (A34_1A) and EMS sample (A34_2A) were methylated, respectively. There are three main types of DNA methylation at the C site: mCG, mCHG and mCHH. The number and composition ratio of these three types reflect the characteristics of the whole genome methylation of a specific species. Among them, the methylation ratio of C site of A34_2A is higher than that of A34_1A (*Table 2*). The results of the distribution ratio of CG, CHG and CHH in methylated C bases show that the proportion of mCHH sites is the highest (all above 63%) after alfalfa mutagenesis, the frequency of mCG locus and mCHG locus decreased in turn (*Fig. 1*).

Analysis of sequence characteristics near methylated C in CG, CHG and CHH

We extracted the 9 bp sequence near the methylation to study the sequence features near the methylation site. In the alfalfa genome, for CG, we define sites with a methylation level greater than 75% as hypermethylated sites, and sites with

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less than 75% as hypomethylated sites; for CHG and CHH, methyl sites with a level of greater than 25% are defined as hypermethylated sites, and sites with a level of less than 25% are defined as hypomethylated sites (Ryan et al., 2009). As shown in *Figure 2*, the frequency of the bases in the upstream and downstream of pyrimidine was similar, and the sequences of the three motifs of CG, CHG and CHH have no obvious preference.

Table 2. *The number and proportion methylated C in alfalfa*

Samples	Context	Covered	Methylation	C site methylation ratio
A34_1A	C	35259145	11282721	32.00
	CG	3145374	2306626	73.33
	CHG	4641999	1821643	39.24
	CHH	27471772	7154452	26.04
A34_2A	C	25214733	9025061	35.79
	CG	2307826	1747850	75.74
	CHG	3354013	1467649	43.76
	CHH	19552894	5809562	29.71

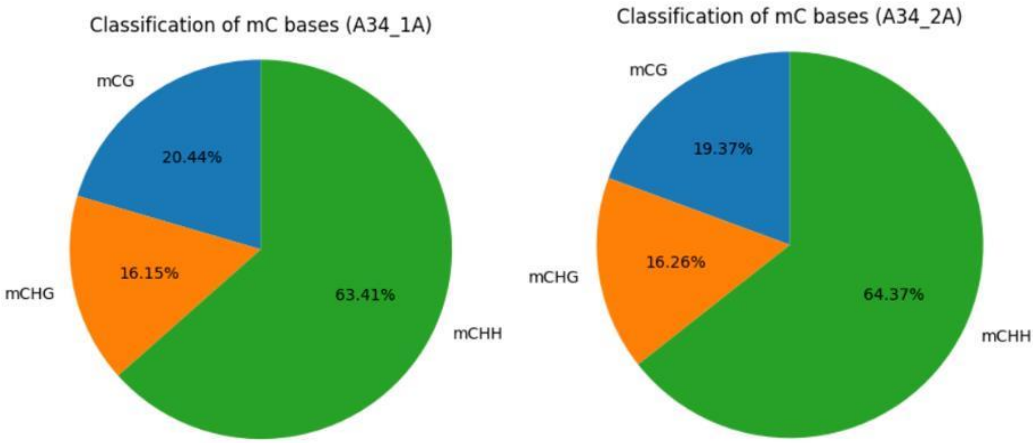

Figure 1. *The distribution of mCG, mCHG and mCHH in all methylcytosine*

Analysis of alfalfa whole genome DNA methylation level

To study the changes in the whole genome methylation level of alfalfa mutagenesis treatment and control treatment. The experiment performed a statistical analysis of the methylation levels of CG, CHG and CHH sequences in the whole genome (*Fig. 3*). The most CG sites in the alfalfa genome were in a state of 80-100% high methylation level, or are not methylated, regardless of the mutagenesis treatment or the control treatment. This is because CG methylation is the most abundant type of DNA methylation (Kanehisa, 2016). It was found that among all methylated cytosine sites, the degree of methylation of CHG and CHH was relatively low, and the methylation level was mainly distributed below 40%. The change trend of CHG sites was in the range of 20-100%, and the change trend was gentle. CHH sites were generally un-methylated or distributed in the 20-40% range, and the methylation level was relatively low; while the methylation level of CG at 80%-100% was higher than that of CHG and CHH. It

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showed that methylation of alfalfa genome mainly occurs at CG sites, and this trend was also a characteristic of alfalfa genome DNA.

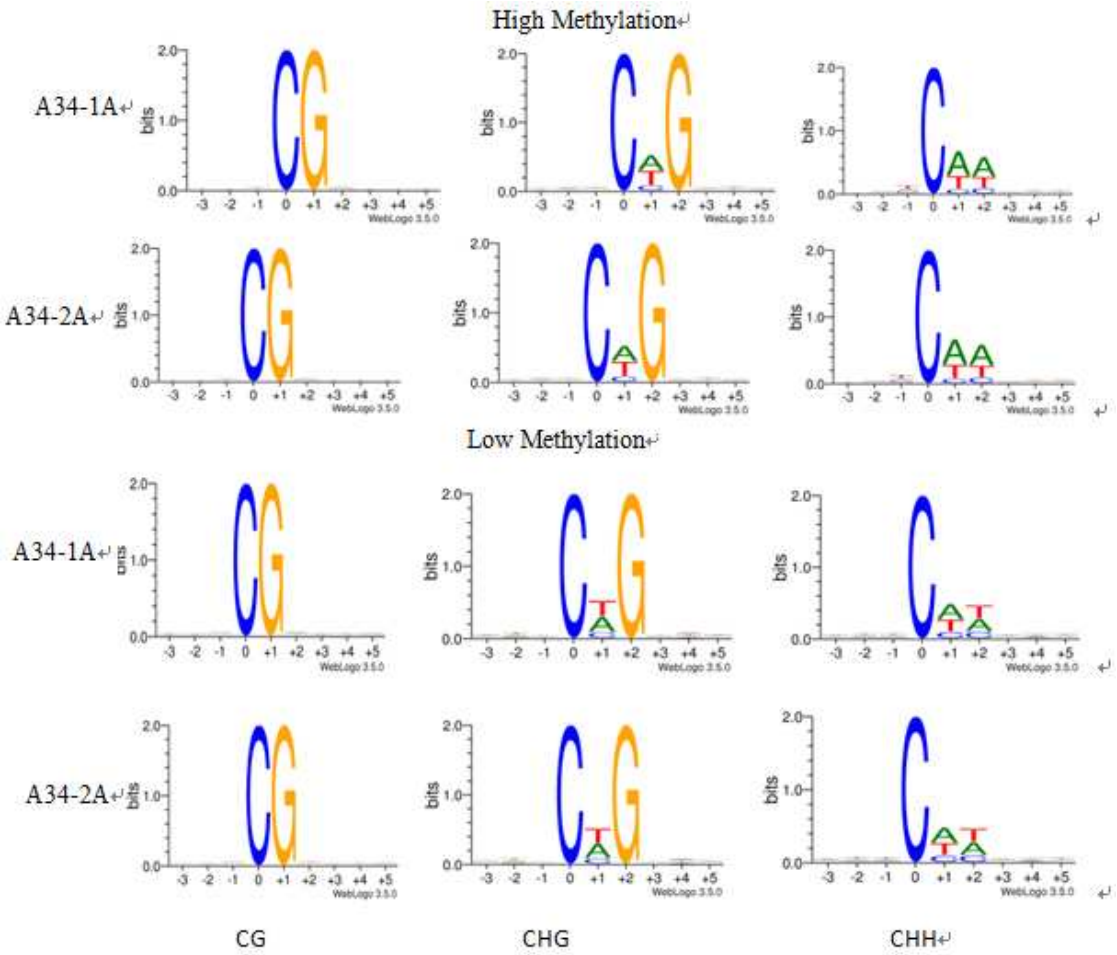

Figure 2. *Methylation preferences in 9 bp spanning CG, CHG, and CHH methylcytosine sites. H = A, C or T. The abscissa is the base number of the methylation site, the total height of each position is the sequence conservation of the base, which represents the relative frequency of the base at that position*

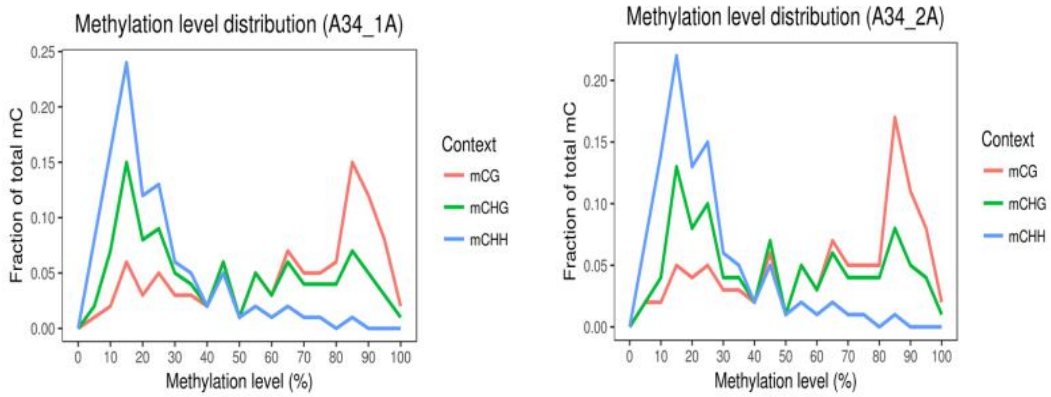

Figure 3. *The methylation levels of CG, CHG and CHH sequences*

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DNA methylation levels of different functional regions

We divided all mC into specific gene features: promoter, 5'UTR (Untranslated Regions), exons, introns and 3'UTR. The methylation levels were evaluated in these functional regions. The methylation levels obtained according to *Equation 1* is shown in *Figure 4*, the trend of methylation levels in the specified regions of the two treatments were similar, and the methylation levels for the CG type were higher than those for the CHG and CHH types. The CG methylation level of many gene regions increased after mutagenesis. It shows that mutagenesis may promote the methylation process of certain sites. Moreover, the results of this study showed that the methylation level of intron was the highest during the mutagenesis of alfalfa, followed by the exon and the promoter regions.

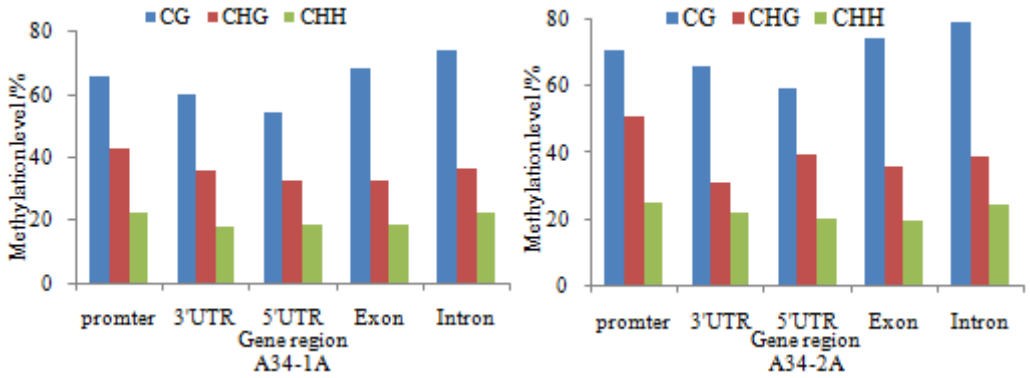

Figure 4. *Methylation level of various types of C in different gene regions*

DMRs analysis for the control and EMS mutagenesis treatments

A total of 8707 DMRs were detected in A34_1A and A34_2A on the difference of methylation regions (*Table 3*). There were 5221 hyper-DMR and 3486 hypo-DMR during the mutagenesis of alfalfa. The CG, CHG and CHH sequences all have higher rates of hypermethylation than that of hypomethylation. The highest rate of hypermethylation was the CHH sequence, which is 64.38%, and the lowest proportion of hypomethylation was CG sequence, which was 41.42%. The comparison of DMR in A34_1A and A34_2A showed that the methylation level generally increased after EMS mutagenesis.

The results of the study on the distribution of DMR in different regions of genes were shown in *Figure 5*. For all methylation types, the ratio of DMRs located in introns and promoters were the highest except for those in distal intergenic regions. This indicates that the promoter and intron regions have a high level of methylation and may participate in the process of methylation regulation.

Table 3. *Details of DMR during A34-1A VS.A34-2A*

Type	Hyper–DMR	Hypo–DMR	Hyper ratio	Hypo ratio
CG–DMR	1635	1156	58.58	41.42
CHG–DMR	1556	1207	56.32	43.68
CHH–DMR	2030	1123	64.38	35.62
Total	5221	3486	59.96	40.04

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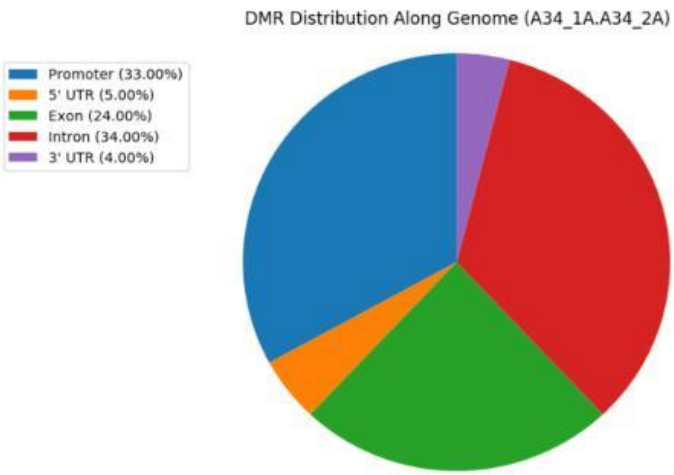

Figure 5. *Proportion of DMR of different sequence types in the genome region*

KEGG pathway enrichment analysis

To probe changes in the methylation status of gene functions after mutagenesis in alfalfa. KOBAS was used to carry on the analysis of gene pathway enrichment on the gene list where DMR is located. Screen the pathways whose pathway enrichment P-value is less than 0.05 and the number of query genes in this pathway is greater than 2 (Xie et al., 2011). The KEGG analysis revealed that there are 20 pathways ($P < 0.1$) were enriched, of which the main enrichment was in starch and sucrose metabolism, sugar metabolism pathways include galactose metabolism, fructose and mannose metabolism, amino acid sugar and nucleotide sugar metabolism, nitrogen metabolism, Pyrimidine metabolism, Oxidative phosphorylation, Arginine and proline metabolism, etc. (*Fig. 6*). There are 33 genes involved in these metabolisms (*Table 4*). The above research results indicate that the differentially methylated genes of alfalfa after mutation may be closely related to the physiological and biochemical metabolic processes such as carbon metabolism and amino acid metabolism.

Discussion

DNA methylation is the main feature of the epigenetic regulatory mechanism that plays an important role in the regulation of gene expression (Yan et al., 2017). As an important epigenetic modification method, DNA methylation can cause heritable changes in gene expression and play an important regulatory role in the growth and development of plants. The level of DNA methylation varies significantly in different plant species, as well as between different species of the same species. Previously, some studies have been conducted to describe DNA methylation for alfalfa (Russo et al., 2013; Barboni et al., 2011; Colosimo et al., 2009; Russo, 2007), but few reports analyzed from the alfalfa mutagenesis genome-wide methylation pattern (Cao et al., 2016). Mutagenesis can cause changes in the organism's genome (Xu et al., 2006; Li et al., 2007), expression group (Hwang et al., 2014), proteome (Wang et al., 2008), and metabolome (Zhang et al., 2011; Kim et al., 2012). Analyzing the changes of alfalfa genomic methylation level before and after mutagenesis can help to study the expression regulation of functional genes and the molecular mechanism of alfalfa

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DOI: http://dx.doi.org/10.15666/aeer/1905_39453957

adaptation to adversity under mutagenic stress. In this study, we used WGBS to investigate the DNA methylation profiles of the genome in leaf tissues between A34_1A and A34_2A to discover the methylation changes before and after mutagenesis. Further correlation analysis indicated that several DMR-related genes were most likely involved in alfalfa physiological resistance.

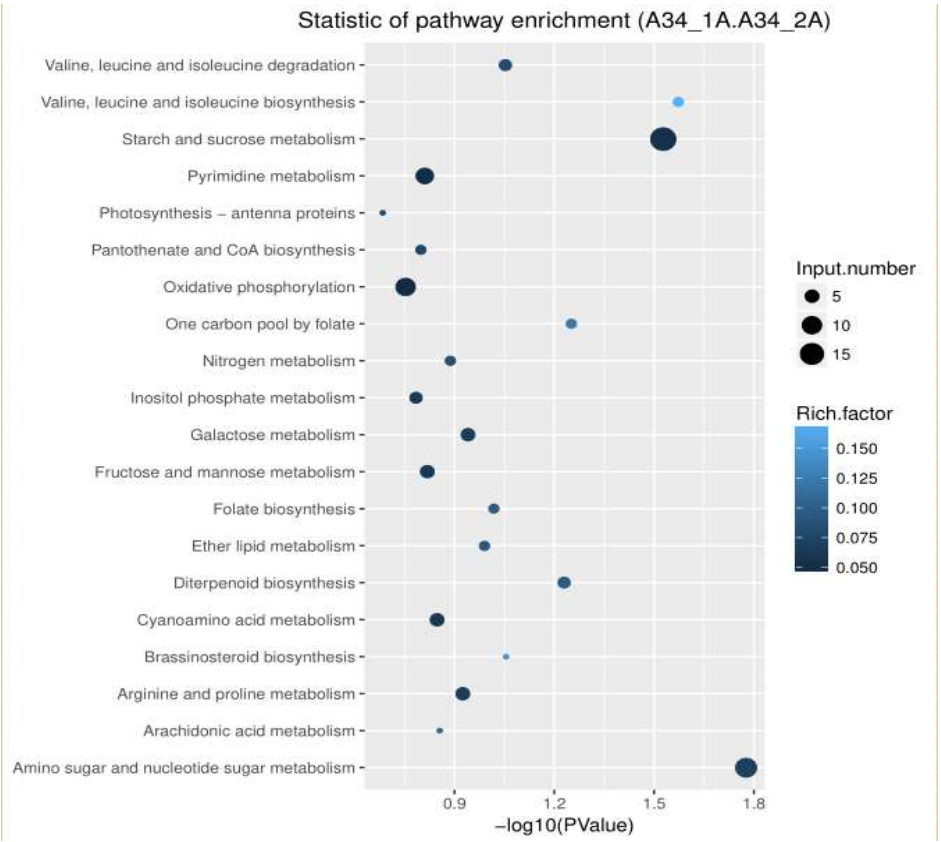

Figure 6. *The enrichment analysis of DGEs in the comparison of A34-1A VS.A34-2A*

Table 4. *The pathways and genes involved in physiological metabolism*

Sample	Pathways	P value	Number	genes
A34-1A VS. A34-2A	Amino sugar and nucleotide sugar metabolism	0.0167	12	MTR_2g015550|MTR_6g088795|MTR_0046s0180|MTR_6g029470|MTR_2g006790|MTR_4g131760|MTR_0354s0040|MTR_8g044160|MTR_3g064490|MTR_0034s0060|MTR_5g021760|MTR_4g070430
	Valine, leucine and isoleucine biosynthesis	0.0267	3	MTR_4g011180|MTR_3g103580|MTR_7g033125
	Starch and sucrose metabolism	0.0297	18	MTR_0034s0170|MTR_7g012840|MTR_4g120280|MTR_4g036685|MTR_4g081530|MTR_4g066590|MTR_2g006790|MTR_4g131760|MTR_0354s0040|MTR_7g113910|MTR_6g088795|MTR_3g064490|MTR_1g015970|MTR_8g032250|MTR_0046s0180|MTR_2g015550|MTR_6g088810|MTR_4g070430

In the *Arabidopsis* methylation group, both the upstream and downstream methylated cytosines have sequence specificity. The preference for the second base upstream of CG for cytosine C is 13 times higher than the preference for adenine A in the case of high methylation levels. At low methylation levels, the upstream of the CHG motif is not

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biased and the downstream always follows a methylated cytosine C. In which, the most obvious phenomenon is that the CHH motif always follows the TA combination, but the second base upstream of the CHH motif is rarely adenine A. (Cokus et al., 2008). Previous studies have shown that there is no preference analysis for plant methylation except for the specificity of upstream and downstream sequences of methylated cytosine in *Arabidopsis*. In this study, the methylation levels of methylated cytosine mC sites were divided into two groups in the alfalfa genome: High Methylation and Low Methylation. The frequency of the bases is similar, and there is no obvious preference for the sequences of the three motifs of CG, CHG and CHH.

DNA methylation level plays an important role in plant development (Zhong et al., 2013). In this study, the proportion of CG-type methylated cytosines was 73.33%-75.74%, and about 30% of methylated cytosines were non-CG types (mCHG, mCHH), indicating that methylation of the alfalfa genome mainly occurred at the CG position point. This is consistent with the findings of Shi et al. (2014) that the DNA methylation of CG site is more than that of CNG site for mature stage rice under heavy ion irradiation treatment. The results of the study showed that during the mutagenesis of alfalfa, the methylation level of the intron region was higher, which may be caused by the involvement of introns in the regulation of gene expression and the prevention of abnormal transcription of intron sequences. These results were consistent with previous reports (Zemach et al., 2013). In addition, related research shown that CNG site methylation plays an important role in plant growth and resistance to external stress (Zhuang. 2008; Liang et al., 2014; Xiao et al., 2006). Kim et al. (2013) found that after gamma irradiation, the DNA methylation of CNG sites in *Arabidopsis* genome was higher than that of CG sites. These differences may be caused by different stages of crop development.

The comparison of the DNA methylation of A34_1A and A34_2A showed that the overall methylation level of A34_2A showed an upward trend. In the 8707 DMRs obtained, CG, CHG and CHH all had hypermethylation, and the methylation was mainly in the CG sequence. We found that the DNA methylation level in the functional element regions of the genome, promoters, introns and other elements did not change much before the alfalfa mutagenesis, but after the EMS mutagenesis, the CG methylation levels of many gene structures has increased, which indicates that there are multiple sites of methylation during the mutagenesis process, which will affect the expression of genes.

DNA methylation can change the physiology and metabolism of plants, promote or inhibit the metabolism and conversion of certain energy substances in the process of plant growth, thereby affecting the vitality of plants and helping plants resist adversity (Lauria et al., 2011). In our previous research, EMS mutagenesis treatment was used to explore the physiological and biochemical characteristics of alfalfa and its effect on alfalfa stress resistance. The results showed that the physiological indexes of alfalfa stress resistance were significantly improved after the mutation. The effect of mutagenesis on improving plant stress resistance was revealed at the physiological and biochemical level (Shen et al., 2018). In this study, the effect of mutagenesis treatment on alfalfa methylation was conducted. Through path enrichment analysis, it was found that there were 33 differentially methylated genes in the three physiological pathways related to stress after alfalfa mutagenesis. We will validate those DMR-related genes from this study in different stages of growth development in the future. The results of this study will further revealed the mechanism that mutagenesis is contribute to increase

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alfalfa resistance at the molecular level. It provides a valuable reference for the study of alfalfa resistance breeding by using mutagenesis technology.

Conclusion

In this study, WGBS technology was used to study the whole genome DNA methylation map of alfalfa mutated by EMS. The levels and patterns of DNA methylation before and after mutation were analyzed, and DMRs/DMGs that may be related to stress physiology were found. The results will help to better understand the epigenetic regulation of alfalfa before and after mutation stress. In the future, DNA methylation and transcriptome association analysis will be used to identify specific up-regulated or down regulated genes, and explore the relationship between methylation and gene expression, so as to provide theoretical basis for epigenetic research of alfalfa.

Acknowledgements. This research was supported by the doctoral research funding of Yichun University (210-3360119010). The authors are grateful to the Institute of Atomic Energy Utilization of Chinese Academy of Agricultural Sciences for providing technical support for this study.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_39453957

ANTIBIOTIC RESIDUES IN FOOD CHAINS; IMPACT ON THE ENVIRONMENT AND HUMAN HEALTH: A REVIEW

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(Received 2nd May 2021; accepted 29th Jul 2021)

Abstract. The antibiotic residues in the food chain are a growing public health concern due to their involvement in the development of antimicrobial resistance, mutagenicity, carcinogenicity, hypersensitivity, bone marrow suppression, and disruption of gut microbiota. The indiscriminate use of antibiotics for the treatment of diseases and improved animal production results in the deposition of these residues in milk, eggs, and meat although their use is not highlighted for the foods consumed by human beings. Moreover, the antibiotics consumed in the clinical settings and animal production are excreted into the environment at a large scale which may adversely disturb the terrestrial and aquatic ecosystems. The matter can become more momentous soon because the production of food animals at an industrial scale will significantly increase the use of antimicrobials. The problem caused by these antibiotic residues in the food chain is two-fold; the direct toxicity to humans and the possibility of the emergence of resistant bacterial strains ultimately leading to the failure of antibiotic therapy. Present article critically analyses the factors contributing to the presence of antibiotic residues in the food chain and their implications and perilous impact on consumers and proposes the possible ways to reduce the antimicrobial residues in the food.

Keywords: *antimicrobial residues, microbiota, antimicrobial resistance, ecosystem, maximum residue limits*

Introduction

Natural, synthetic, as well as semisynthetic drugs which can kill or inhibit the growth of microorganisms, can be termed antibiotics (Catteau et al., 2018). Antibiotics are the most effective group of drugs to treat bacterial infections in both humans as well as in animals by acting specifically on their targets (*Figure 1*). Following the discovery of penicillin in 1928, thousands of antibiotics were produced for animals, plants, and human use. Initially, these antibiotics were employed in clinical and veterinary settings only for the therapeutic management of certain infections; later, the drugs were also used as growth promoters especially in livestock and poultry industries. Antibiotic usage was prohibited in Europe in 2006, however, their use in livestock, poultry, and agriculture

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_39593977

sectors is still common in many parts of the world including China and India (Gonzalez Ronquillo and Angeles Hernandez, 2017; Cowieson and Kluenter, 2019).

Figure 1. *Various groups of antibiotics*

It is estimated that the global usage of antibiotics was around 63,151 tons in 2010 for food animals and aquaculture. Additionally, it is anticipated that the amount will further increase by 67% until 2030 with the highest consumption in India, Brazil, Russia, and South Africa (Van Boeckel et al., 2015). This irrational use of antibiotics in agriculture, humans, and animals resulted in the accumulation of antibiotics residues in the natural ecosystem and environment that can pose harmful effects such as the development of antimicrobial resistance. Many other public health concerns have been raised in regard to the antibiotic residues in the food chain and environment. For example, the ingestion of antibiotic residues could alter the human microbiota and promote resistance among the human normal bacterial flora. Moreover, the unwarranted use of antibiotics leads to their accumulation in the tissues of the food animals as residues and which ultimately become part of the food chain. Therefore their use is prohibited by the health, as well as food regulatory authorities (Landers et al., 2012).

The present study aimed to provide a comprehensive view of health risks associated with antibiotic residues in the food chain and environment including the effects on the emergence of antibiotic-resistant bacteria and to discourse the information gap and provide recommendations to reduce the antibiotic residue in the food chain to avoid the potential hazards to human, animal and environmental health. Here we summarize the studies on the relationship between the use of antimicrobials for the growth promotion in food-producing animals and the development of resistance in bacteria. Further, we focused on the presence and detection method of various antibiotics in foods of especially of animal origin. Moreover, the sources of antibiotics in foods from different origins are summarized. Further, the impact of antibiotic residues on the consumers is summarized.

Antimicrobial residues

Synthetic and semisynthetic antimicrobials are used in veterinary and human medicine for the treatment and control of diseases and can be administered topically, orally, and parenterally. Furthermore, they play an important role in promoting the growth of food

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_39593977

animals (Barton, 2000). Antimicrobials can deposit in tissues of the body as residues and it takes some time for residues to be excreted or metabolized. The amount of these residues can be higher especially when these animals are consumed by the humans during their medication or soon after the medication withdrawal (Tollefson and Miller, 2000). Antibiotic residues can adversely affect human health by various processes such as damaging effects on the organs, antibiotic-resistant genes, and bacteria, direct toxicity to consumers (Kirchhelle, 2018). Several drugs such as nitrofurazone, ipronidazole, fluoroquinolones, chloramphenicol, furazolidone, and dimetridazole are illegal to use in food-producing animals. Antimicrobial residues are used therapeutically as well as prophylactically to promote growth and control diseases. The approved drugs for veterinary use have legally approved maximum residue limits (MRLs) for the parent drugs or their metabolites in the food products from the treated animals through assessment of safe concentration for consumers (Okocha et al., 2018).

Sources of antibiotics in food

The studies have suggested that antibiotics contaminate all kinds of human food products such as vegetables, livestock, aquatic products, and poultry products i.e. eggs, meat, and milk (Van Boeckel et al., 2015). Furthermore, it is considered that antibiotic residues can accumulate in aquatic products as these drug residues have been reported from various aquatic environments (Liu et al., 2017). The antibiotics and antibiotics residues being used as organic fertilizers in manure become part of vegetables (Azanu et al., 2016). Consequently, there are two main sources of antibiotic residues in food. Firstly, antibiotics are used for growth promotion as well as control of disease and to improve the efficacy of food in humans. Secondly the accumulation of these drug residues among the food animals living in an environment contaminated with antibiotics (Manyi-Loh et al., 2018). The sources and pathways of antibiotic residues in the food chain have been shown in *Figure 2*.

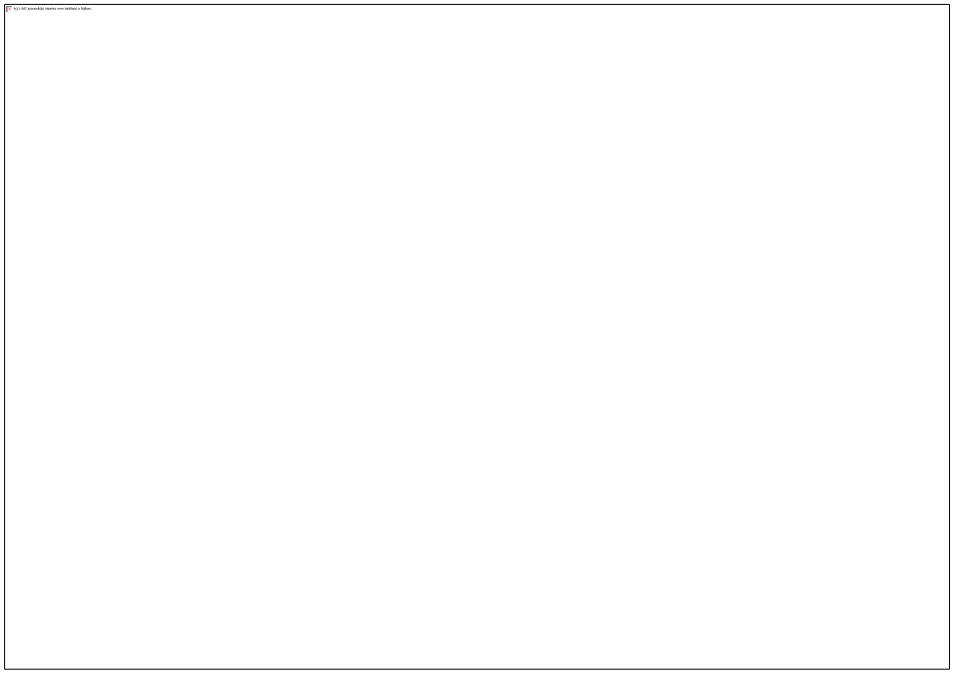

Figure 2. *Sources and pathways of antibiotic residues in the food chain*

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DOI: http://dx.doi.org/10.15666/aeer/1905_39593977

Antibiotics residues in food

Antibiotic residues accumulate in the blood as well as in other tissues, after administration of high concentrations of antibiotics however the use of non-medicated fodder in animals eradicates these residues from the blood as well as tissues of these animals. The high concentration of antibiotics administered to animals through injection or animal-derived feed as well as due to storage, water, and transportation during processing may result in the contamination of animal-origin foods (Nisha, 2008). Furthermore, feces can also contaminate food through fecal recycling, particularly vegetables are contaminated as feces are being used as a fertilizer (Phillips et al., 2004; Darwish et al., 2013). Antibiotics are widely used in fish farming and antibiotic residues are accumulated within human body systems following the use of contaminated fish that can adversely affect human health (Aarestrup et al., 1998). Reducing antibiotic residues in food heat treatment or cooking is important because very few raw products are used (Katz and Brady, 2000). The antibiotic residues accumulate in food products, for example, milk and meat, and several edible products although the low concentrations of antibiotic residues are considered as safe. In contrast, some antibiotics are harmful to human health, and their use is prohibited for example chloramphenicol. Milk is vital as well as beneficial food because it is a source of proteins in every age of people. Widespread use of antibiotics for treatment of mastitis in lactating cows and dry cow therapy leads towards accumulation of antibiotic residues in milk.

Antibiotics residues in livestock and poultry products

For poultry and livestock, antibiotics play a major role in the control and therapy of infectious diseases. Furthermore, antibiotics are commonly used as food additives to promote growth, improve food quality, and in maintenance of health in poultry and livestock industries. Consequently, antibiotic residues obtained from those animals are accumulated in food products and adversely affect human health. Furthermore, different kinds of food products are classified in poultry and livestock, for example, milk, eggs, chicken, pork, and beef, and three possible routes can contribute to the contamination of these products (1) through intrauterine and intramammary infusion or topical contact (2) when injected directly i.e. subcutaneous, intramuscular (IM) or intravenous (IV) for prophylaxis and treatment of disease (3) direct intake of water and feed in which antibiotics accumulate when used as an additive for growth promotion (Mitchell et al., 1998). Initially, antibiotic usage in the veterinary field was started for control and treatment of disease but nowadays these are widely used as additives for growth promotion. It has been observed that antibiotics are widely used as feed additives and fatteners for broilers to improve their growth and for better protection. Moreover, antibiotics also promote growth by decreasing various mechanisms such as toxin formation, nutrients waste, and the immune system's activity (Nisha, 2008). Antibiotics that are widely used for prophylaxis, growth promotion, and treatment in food animals are sulfamethoxazole, enrofloxacin, benzylpenicillin, tylosin, amoxicillin, trimethoprim, oxytetracycline, ampicillin, and streptopenicillin, while amikacin, neomycin, enrofloxacin, doxycycline, tetracycline, tilmicosin, and colistin sulfate are used for promotion of growth in the poultry (Chattopadhyay, 2014). A study has reported that virginiamycin; a food additive used for the livestock to enhance growth was found in the excreta of the treated animals. Additionally, the manure from the excreta serves as

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DOI: http://dx.doi.org/10.15666/aeer/1905_39593977

fertilizer owing to its soil binding capacity, it results in the contamination of water supplies (Tasho and Cho, 2016).

Antibiotics residues in the biological systems

The presence of narrow and broad-spectrum antibiotics in different environmental samples has been reported in various studies (Sarpong and Miller, 2015). The antibiotics are excreted by humans and animals which can contaminate the sewers and then to rivers as well as seas. For example, fluoroquinolone, sulfamethoxazole, and ofloxacin were detected from seawater of Hailing Island, Belgian harbors, and Laizhou Bay, respectively. Furthermore, sulfonamides and tetracycline were also detected in the discharge of wastewater treatment plants (Gao et al., 2012; Larsson, 2014). Several antibiotics are detected in hospital wastes, for example, beta-lactams, macrolides, lincomycin, fluoroquinolones, trimethoprim, sulfamethoxazole, and sulfonamides (Meena et al., 2015). It has been reported that ofloxacin, trimethoprim, sulfamethoxazole, and ciprofloxacin have been detected in municipal wastewater. Several antibiotics are used as feed additives in aquaculture industries. For example, sulfonamides, oxytetracycline, erythromycin, florfenicol, and sarafloxacin, are found in aqua samples. Five different compounds were detected in water samples in China i.e. ofloxacin, sulfonamides, norfloxacin, erythromycin, and amoxicillin with detection rates up to 65 to 7722 ng/l. Oxytetracycline and tylosin were found rarely in wastewater while lincomycin, chlortetracycline, and sulfamethazine were detected frequently, furthermore to detect antibiotic residues from dairies effluents (Kumar et al., 2019).

Antibiotics and their residues are frequently excreted and contaminate natural environments. Different sources contribute to this pool of antibiotic residues, for example, animal husbandry, dairies, veterinaries, domestics, poultry, discharge of hospital waste, animal excreta, pharmaceutical plants, and municipal waste (Pruden et al., 2013; Obayiuwana et al., 2018). Several antibiotics are used in the agriculture industry to enhance fish farming, livestock growth, and beekeeping. The antibiotics and their metabolites are discharged particularly from the feces of the poultry animals and contaminate the natural environment (Hong et al., 2018). These residues and the metabolites pollute water and soil indirectly, for example, a polyether antibiotic i.e., the Monensin antibiotic used to promote the growth of animals in the dairy farms was found to spread to the natural resources (Mutiyar and Mittal, 2014). Although, the consumption of antibiotics is very low in plants than animals the residues were found in water used in agricultural land resulting in the contamination of the agricultural field (Piña et al., 2018). Few antibiotics have low molecular weight such as sulfonamides, aminoglycosides, nitrofurans, quinolones, macrolides, rifamycins, amphenicols, beta-lactams, tetracyclines, phosphonates, and lincosamides, therefore they dissolve readily in water bodies which results in longer persistence of antibiotics (Krzeminski et al., 2019). Antibiotics that are used in livestock can also contaminate the fields by manure and these antibiotic residues are absorbed in the soil and reach the groundwater (Ishikawa et al., 2018). Furthermore, when expired drugs are directly discarded in the sewage grid, these drugs can accumulate in landfills which increases contamination (Akici et al., 2018).

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Antibiotics ecotoxicity and impact on bacteria

The antibiotic residues are mixed with freshwater through soil erosion and rain (Mutiyar and Mittal, 2014). Antibiotics act on both target organisms (bacteria) as well as on non-target organisms, for example on zooplankton, freshwater algae, and fish (Kummerer, 2009). The adverse effects of monensin on species richness have been reported (Hillis et al., 2007). Furthermore, fluoroquinolones are harmful to prokaryotes as compared to eukaryotes that are commonly present in hospital effluents and the long-term use of fluoroquinolone has shown harmful effects on human health as well as high genotoxicity (Brown et al., 2006; Ao et al., 2018). Antibiotics affect directly and indirectly the bacterial populations as their excessive use may lead to the development of resistance (Haller et al., 2002; Grenni et al., 2018). The presence of antibiotic residues in effluents results in contamination of seawater in the USA, Greece, China, Italy, Germany, Turkey, and Belgium. It has been reported that sulfamethoxazole, clarithromycin, roxithromycin, and erythromycin were detected from 153 samples of beach water from the Northern Adriatic Sea, San Francisco Bay, Baltic Sea, Dardanelles, Pacific Sea, Mediterranean, and Aegean Sea (Nodler et al., 2014). Based on biological, physical, and chemical properties antibiotics have different effects on organisms (Yelin and Kishony, 2018). The antibiotic, particularly those being used in the poultry and veterinary industry, increases the chance of survival of bacteria under antibiotic stress due to selection pressure which results in the evolution of multi-drug resistant (MDR) strains. These MDR strains are widely reported in the soil and aquatic milieus (Esiobu et al., 2002; Brown et al., 2006). It has been reported that β-lactams and aminoglycosides are less persistent in the environment as compared to fluoroquinolones as well as sulfonamides. In case of insufficient sunlight, the tetracyclines can persist and accumulate for a longer time. Furthermore, ciprofloxacin at 25,000 ng/l and norfloxacin at 5000 ng/l have shown to affect the DNA of genetically modified *Salmonella typhimurium* strains resulting in mutations (Ao et al., 2018).

Use of antibiotics in foods

Antibiotics are widely used in animals for many advantages covering carcass quality, promotion of growth, animal health, and cost-effective production (Van Boeckel et al., 2015). Antibiotics are the commonest drugs and are widely being used in animals as prophylactic and therapeutic agents for the management of infectious diseases. These antibiotics have played an important role in the prevention and cure of certain important infections caused by *Escherichia coli, Campylobacter fetus, Enterococcus, Leptospira, Streptococcus suis,* and *Salmonella.* Antibiotics enhance growth rate by altering the motility of the gut, by thinning the mucous layers in the gut, by decreasing waste nutrients, immune system activity, and formation of toxins, and by providing favorable conditions for beneficial intestinal microbes to destroy harmful bacteria (Darwish et al., 2013). The body weight of animals increases up to 4–5% that receive antibiotics compared to those which are grown in the absence of these drugs (Witte, 1998). In veterinary medicine, several groups of antibiotics are used for these purposes, for example, lincosamide, aminoglycosides, ansamycins, and glycopeptides, β-lactams i.e. cephalosporin and penicillin, trimethoprim, Sulfonamides, quinolones, nitrofurans, tetracyclines (Cháfer-Pericás et al., 2010a). Few drugs or and their combinations are used as prophylaxis for different plant diseases for example streptomycin-oxytetracycline is used to control halo blight disease in beans and few other bacterial diseases in tomatoes, potatoes, cherries, tobacco, and peppers. Several drugs are used to inhibit trees from bacterial diseases such as

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aminoglycosides and tetracyclines. The studies have also reported the uptake of antibiotics by the vegetables such as corn, radish, carrot, and cabbage which were irrigated with the contaminated water (Kang et al., 2013; Bassil et al., 2013; Chowdhury et al., 2015).

Detection of antibiotic residues

Chromatographic and screening methods including the immunological and enzymatic methods are used to detect antibiotic residues. Microbiological methods such as microbial agar diffusion tests or inhibition of acid production by starter organisms were also used for the detection of antibiotic residues in food (Chowdhury et al., 2015). Antibiotic residues of sulphanilamide, streptomycin, ciprofloxacin, and tetracycline in meat samples were detected by three techniques i.e. HPLC, ELISA, and TLC. HPLC showed different antibiotic residues concentration i.e. 14.6%, 8.3%, 88.8% and 41.1% for tetracycline, ciprofloxacin, sulphanilamide and streptomycin respectively. ELISA detected that 25.3%, 56%, 18%, and 34% samples were positive for tetracycline, ciprofloxacin, sulphanilamide, and streptomycin, respectively, while TLC showed different concentrations i.e. 14.6% tetracycline, 21.4% ciprofloxacin, 92.5% sulphanilamide, and 29.4% streptomycin (Ramatla et al., 2017). In milk samples, the antibiotic residues were detected in 1960 for the very first time using the chromatographic technique. The analysis showed that sulfonamides tetracyclines, fluoroquinolones, and aminoglycosides have 12.64%, 14.01%, 13.46%, and 36.54% antibiotic concentration respectively (Molina et al., 2003). The concentration of various antibiotic residues in different foods are shown in *Table 1*.

Toxic effects of antibiotics residues in food

Antibiotics residues produce many toxic effects; however, the most common manifestation of various drugs is allergic reactions. These hypersensitivity reactions are observed in the case of tetracyclines, penicillin, and aminoglycosides (Katz and Brady, 2000). The effects caused by the long-term use of antibiotics on human health are still unknown. Furthermore, it is observed that β-lactams have few toxic effects on human health, these are mainly involved in eliciting allergic reactions (Davies and Davies, 2010). For instance, it has been reported that tetracycline causes several peculiar reactions like allergy, phototoxic dermatitis, and skin rashes (Yates and deShazo, 2003). Among the individuals sensitive to the penicillins group, the residues of penicillin in milk resulted in allergic reactions (Dewdney et al., 1991; Demoly and Romano, 2005). Another drug streptomycin has significant side effects, for example, it affects vestibular mechanisms in the inner ear which causes the loss of balance, neurotoxic effects on newborns, fever, and skin rashes. The allergic response to the macrolides metabolites modified by the hepatic cells can cause liver injury (Dewdney et al., 1991). Residues of chloramphenicol in foods can cause fatal blood dyscrasia in individuals (Settepani, 1984).

Carcinogenic effects

Carcinogenic residues bind covalently to several intracellular components, for example, glycogen, glutathione, deoxyribonucleic acid (DNA), ribonucleic acid (RNA), proteins, and phospholipids, and show latent hazards (Beyene, 2016). It has been reported that Chloramphenicol residues present in food causes cancer (Demoly and Romano, 2005, Nisha, 2008).

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Table 1. *Detection methods for the determination of concentrations of antibiotic residues in different food items*

Detection Method	Sample Type	Number of samples	Antimicrobial Agents	Concentrations (µg/L or µg/kg)	Reference
LC-qTOF-MS	Fish	53	Multiple	6.9 −148.4	(Wang et al., 2017b)
	Chicken	18	Multiple	0.1 −49.6	
	Pork	17	Multiple	0.3 −27.0	
	Shrimp	18	Multiple	0.4 −66.9	
LC-MS/MS	Milk (Cow)	22	Fluoroquinolones	0.34-333	(Tang et al., 2009)
	Raw milk (Fresh)	25	Sulfapyridine Lincomycin	1.77 11.3	(Han et al., 2015)
	Fish (market)	9	Oxytetracycline	Upto 60	(Cháfer-Pericás et al., 2010b)
	Fish (farmed)	20	Oxytetracycline	59	(Cháfer-Pericás et al., 2011)
	Fish (fresh water)	443	Aminoglycosides	5-125	(Gbylik-Sikorska et al., 2014)
	Fish (freshwater)	13	Multiple	0.4 −38.5	(Wei et al., 2014)
	Fish (freshwater)	128	Erythromycin	2390	(Zhao et al., 2015)
	Fish (farmed)	75	Norfloxacin	15	(Yipel et al., 2017)
	Fish (farmed)	193	Enrofloxacin	>50	(Guidi et al., 2017)
	Fish	116	Sulfonamides	Upto 1634	(Song et al., 2017a)
	Fish	116	Fluoroquinolones	Upto 47108	(Song et al., 2017b)
	Fish	31	Enrofloxacin	Up to 2200	(Chen et al., 2018)
	Wild fish (Marine)	21	Multiple	22-500	(Liu et al., 2018)
	Pork	10	Ampicillin ceftiofur	20 400	(Huang et al., 2016)
	Chicken	156	Sulfonamide	2500-2700	(Yamaguchi et al., 2015)
	Meat (Pork)	100	sulfamethazine	11 −1600	(Ngoc Do et al., 2016)
	Eggs	22	Multiple	Up to 30.3	(Wang et al., 2017a)
	Eggs	111	enrofloxacin	1.7 −1485	(Yamaguchi et al., 2017)
	Vegetables	4	Chlortetracycline	0.1 −532	(Hu et al., 2010)
	Vegetable Crops	5	Multiple	Up to 46.4	(Hu et al., 2010)
	Leafy Vegetables	15	Tetracycline	Up to 8.84	(Yu et al., 2018)
	Fish and Meat (Raw)	18	Erythromycin	58 −87	(Berrada et al., 2008)
	Pork	51	sulfacetamide sulfaquinoxaline	1.2 3.3	(He et al., 2017)
	Egg	51	Sarafloxacin	0.98	
EIA	Fish	20	Oxytetracycline	2.1 −152	(Cháfer-Pericás et al., 2011)
	Chicken	127	Quinolones	30.8 (mean value)	(Er et al., 2013)
	Beef	104	Quinolones	6.64 (mean value)	(Er et al., 2013)
	Raw meat (chicken, beef, and pork)	150	sulfanilamide	14.2 −1280	(Ramatla et al., 2017)

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DOI: http://dx.doi.org/10.15666/aeer/1905_39593977

Detection Method	Sample Type	Number of samples	Antimicrobial Agents	Concentrations (µg/L or µg/kg)	Reference
SNAP® Test	Vegetables	11	Multiple	<10	(Kang et al., 2013)
	Raw milk (fresh)	199	Sulfonamides	<16.3	(Zheng et al., 2013)
HPLC	Milk (cow and goat)	269	sulfamerazine	12.2	(Chung et al., 2009)
	Milk	328	Penicillin-G	15.2 (mean value)	(Olatoye et al., 2016)
	Milk	140	Ampicillin Amoxicillin	0.5 802	(Khanal et al., 2018)
	Raw meat (chicken, beef, and pork)	150	Sulphanilamide Tetracycline Streptomycin Ciprofloxacin	20.7–82.1 41.8–320.8 65.2–952.2 32.8–95.6	(Ramatla et al., 2017)

Disruption of the normal flora

Different bacteria that are part of our normal flora act as a bridge to prevent the entry of pathogens and causing diseases. Antibiotics may kill few important species and will decrease the number of bacteria, for example, broad-spectrum antibiotics such as streptomycin, tylosin, and flunixin in animals while metronidazole, vancomycin, and nitroimidazole in humans affect gastrointestinal diseases and disruption of normal flora (Cotter et al., 2012). The study has shown that the antibiotic residues present in the milk may alter the drug resistance status of the gut microflora during long-term exposure (Chand et al., 2000). Another study has reported the induction of antimicrobial resistance in human intestinal flora after the intake of meat products containing antibiotic residues that resulted in an outbreak of diarrhea (Landers et al., 2012). The intake of these residues entering enter the intestinal tract of humans interacts with nearly 800–1000 bacterial species and approximately 7000 different strains (Jernberg et al., 2010). Although 95% of these bacterial species are beneficial for humans, the remaining bacterial species are opportunistic and can be harmful (Ben et al., 2019). In the human intestinal tract, a microecological balance is maintained between bacteria themselves and the bacteria and the human body, with a predominance of Firmicutes and Bacteroidetes, and a lesser number of Proteobacteria, Actinobacteria, Verrucomicrobia, and many other phyla some of which have not even identified or isolated (Arumugam et al., 2011). Various studies have highlighted the fact that exposure to antimicrobial agents is associated with significant changes in the intestinal microbial population and composition. This effect have a broader spectrum on the microbiota community rather targetting any particular bacterial species (Blaser, 2016). This is quite evident that the antimicrobial therapies resulted in compositional changes of gut microflora with an increasing number of Firmicutes and a reduction in Bacteroidetes. Further, these therapies also resulted in the emergence of antimicrobial-resistant bacterial strains which might be able to persist in the gastrointestinal tract for years (Jernberg et al., 2010; Andersson and Hughes, 2011; Pérez-Cobas et al., 2013). The subsequent imbalance of intestinal microflora may lead to the proliferation of opportunistic pathogens and different diseases for example pseudomembranous colitis, colorectal cancer, and other intestinal disorders (Damman et al., 2012). This scenario can be even worse in case these opportunistic bacteria develop

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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multiple antibiotic resistance and evolve into superbugs, which can be a significant risk to individuals as well as wider society.

Teratogenic effect

Any chemical agent or drug that produces a harmful effect on a fetus or embryo during gestation is termed a teratogen. As a result, congenital disorders that influence functional, as well as structural integrity, take place (Beyene, 2016). For example, when benzimidazole and anthelmintic are administered at an early stage of pregnancy they produce toxic effects on the embryo, furthermore, the drug of oxfendazole i.e. benzimidazole has a mutagenic effect. Enrofloxacin; a fluoroquinolone antibiotic that inhibits the bacteria by targeting the DNA gyrase has shown to be teratogenic for the embryos of rabbits and rats (Guzmán et al., 2003).

Reducing antibiotic residues for safer animal food products

The appropriate use of the drug in veterinary settings results in healthy animal food. However, the higher level of antibiotic residues in the food can adversely affect human health. Multiple animal-related factors including age, breed, body condition and sex, various drug-related factors like dosage of the drug, type of drug formation, and route of administration have possible effects on the pharmacokinetics as well as antibiotic residue levels in meat, eggs, milk, and other edible tissues (Moreno and Lanusse, 2017). The concentration of drug residue in tissues depends upon its several physicochemical properties which play a key role in the regulation of drugs via cell membranes, for example, lipid solubility and acidic or basic properties of drugs. Drugs with highly lipid-solubility enter the extra and intracellular tissue compartments efficiently through passive diffusion, however poorly lipid-soluble drugs remain extracellularly (Lees and Toutain, 2012). It has been observed when drugs are administered through animal ears it helps to prevent the accumulation of drugs in edible animal tissues. Furthermore, various animal products are not consumed raw and the thermal processes such as boiling, sterilization, frying, steaming, pasteurization, evaporation, co-distillation, and roasting can lead to the loss or decrease in the drug residues (Đorđević and Đurović-Pejčev, 2016). Other processes can also help to reduce the drug residues in food products such as storage time, different pH levels, and fermentation.

Reducing antibiotic residues in milk and milk products

Residues of the veterinary drug in milk are an important concern to the consumers, processors, farmers, and milk regulatory agencies as milk or milk products are commonly used by everyone (Shaker and Elsharkawy, 2015). Before consumption, animal products go through vigorous heat treatment that causes protein denaturation, water loss, changed pH, and fat degradation which helps to alter chemical structures, the quantity of drug residue, toxic well as the pharmacological effects (Hsieh et al., 2011). For example, it has been reported that heating milk by sterilization, pasteurization or ultra-heat treatment (UHT) helps to reduce drug residues in the milk. It was observed that the concentration of tetracycline decreased by 30% whereas the concentration of oxytetracycline decreased by 40% by using the ultra-heat treatment, furthermore, the concentration of tetracycline is reduced by 98% using the sterilization process. Macrolides residues can be reduced by

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up to 93% by heat therapy (Zorraquino et al., 2011). During yogurt production through heat treatment, lactic acid helps in the degradation of nafcillin, oxacillin, dicloxacillin and cloxacillin slightly, while precipitated proteins help to decrease residues of penicillin (Grunwald and Petz, 2003). Furthermore, concentrations of trichlorfon, dimethoate, and fenthion were reduced when two starter cultures were added during yogurt preparation (Regueiro et al., 2015). However, a total of 62 antimicrobial agents were tested for heat stability at 56°C (30 minutes) and 121°C (15 minutes) and was found that the aztreonam, azlocillin, oxacillin, and mezlocillin were remarkably heat-stable (Traub and Leonhard, 1995).

Reducing antibiotic residues in eggs

When drugs are given to laying hens, their metabolic products are stored as residues in the components of the egg i.e. albumin and yolk. Raw eggs are generally used after heat treatment and refrigeration. Heat therapy promotes protein denaturation, dehydration, and pH changes that help to decrease chemical formulation, residue quantity, and modify the solubility of residue. As a result, the concentration of tetracycline residues decreased by 52% and 47%, whereas the concentration of enrofloxacin decreased by 69% and 58% after boiling and frying eggs respectively (Ezenduka et al., 2011). However, when eggs were boiled at 100°C for 15 minutes the antibiotic residues i.e. concentration of chlortetracycline was reduced by 61%, ciprofloxacin by 87%, and enrofloxacin by 93%, while other residues such as chlortetracycline by 20–22%, and sulfanilamide by 44-49% were reduced when eggs were refrigerated at 10 °C for 4 weeks (Alaboudi et al., 2013). It was observed that the number of aflatoxin residues was significantly reduced after incorporation of *Bacillus subtilis* biodegradation preparations in the poultry diet which resulted in the biotransformation of toxin and inhibition of intestinal absorption of toxin and consequent reduction of toxin residues in the eggs (Jia et al., 2016).

Reduction of drug residues in meat

Inadequate biosafety methods and improper veterinary drugs may cause a decrease in the quality of meat and the accumulation of drug residues in meat. Several thermal treatments or cooking are essential before the consumption of animal food products. For this purpose, several methods such as fat loss, denaturation of proteins, altered pH, and water loss have been used that aids in modifying the chemical structure, solubility, and concentration of drug residues. It has been observed that after cooking the meat, the concentration of doxycycline was reduced (Javadi, 2011), and changing the pH levels significantly decreased the level of oxytetracycline. For example, oxytetracycline's muscle concentration decreased approximately 53.6% after roasting and 69.6% after boiling, and after changing pH, oxytetracycline reduced up to 67.7%, 53.2%, and 34.3% following boiling, microwaving, and roasting (Vivienne et al., 2018). Furthermore, when pork and chicken are degraded with various heat treatments it significantly reduced oxytetracycline concentration. Furthermore, it has been observed that the concentration of sulfonamide residues decreased up to 38–40% after roasting and 38–40% after boiling in chicken meat (Furusawa and Hanabusa, 2002).

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Management of antimicrobial residues; a food safety perspective

The recommendations by various international organizations have insisted on the wiser use of antimicrobial agents in clinical, veterinary, and agriculture to protect public health. The world health organization (WHO) recommended that the national authorities for agriculture, veterinary, pharmaceuticals, and other stakeholders should focus to disregard the use of antimicrobial agents as growth promoters. Moreover, the antibiotics should be administered among the food animals only when the use is justified and prescribed by the veterinarian especially the third and fourth generation cephalosporins and fluoroquinolones (Economou and Gousia, 2015).

Animal health must be improved to decrease the requirement of antibiotics in food animals through biosafety and biosecurity measures and disease prevention by effective vaccination, use of probiotics, and good hygiene practices. The use of antibiotics must be for therapeutic purposes rather than growth promotion based on bacterial cultures and antibiotic susceptibility testing and clinical experience. The first choice should be narrow-spectrum antibiotics while choosing antimicrobial agents. Further, the veterinarian professional bodies should establish guidelines on a national level for the proper usage of antibiotics among different food animals, by indicating the first, second, and last choices for the management of bacterial infections. Moreover, the economic incentives favoring the inapt prescription of antimicrobial agents should be abolished (Economou and Gousia, 2015; Norris et al., 2019).

An effective surveillance system for antibiotic resistance among commensal and zoonotic bacteria, as well as the bacteria obtained from different foods and food animal reservoirs, is essential to understand the emergence of antibiotic resistance and provide the data for risk assessment and implementation of targeted interventions. The comprehensive surveillance system includes the collection of data followed by analysis and reporting to monitor the temporal trends for the usage of antibiotics in people and food animals and to monitor antibiotic resistance among the bacteria isolated from humans, veterinary and foods (Ferri et al., 2017).

Conclusions

Although the specific regulations are limited due to the lack of consensus on the safer concentrations of antibiotic residues in the environment concerning the development of resistance, The WHO emphasizes the policies for the improvement of pharmaceutical waste management and to minimize the antibiotic residues in the environment. Despite the new medicines in the USA and Europe need environmental risk assessments by considering the ecological impacts of drugs, the medicines already available in the market have not undergone these valuations. These assessments do not address the potential of the drug in resistance implications, rather emphasize the ecological toxicity. The extensive use of antibiotics in animal feed for growth promotion poses a significant threat to public health due to its impact on the development of multidrug-resistant bacterial strains. The stringent control must be adapted to avoid the excessive use of these agents accompanied by the development of alternative measures to safeguard human health and to keep the available antibiotics effective for future clinical implications.

The continuous surveillance of antimicrobial resistance in the human, zoonotic and environmental bacteria is a precondition to understanding this phenomenon that can provide risk assessment data for the evaluation and implementation of targeted interventions. Awareness and communication at national and international levels are

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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necessary for the rational use of antimicrobials in the food chains. These relevant target audiences must be identified including the decision-makers, agriculture, health and veterinary professionals, media, and the public, and should be informed with evidence-based information for their guidance, decisions, and choices. The knowledge gaps still exist in the precise understanding of antibiotic resistance and its implications in food safety. The studies must focus on the quantitative analysis of the disease burden caused by resistant bacteria. These studies will further contribute to assessing the magnitude of the problem and will assist in the risk assessment the designing cost-effective protocols to counteract this menace.

Funding. This research received no external funding.

Conflicts of Interests. The authors declare no conflict of interests.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_39593977

THE IMPACT OF TURF COLORANTS AND OVERSEEDING PRACTICES ON THE DORMANT BERMUDA GRASS (*Cynodon dactylon* L.)

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(Received 4th May 2021; accepted 12th Aug 2021)

Abstract. This research was carried out between May 2016 and February 2018 on the experimental field of Bayindir Vocational Training School at Ege University, Izmir, Turkey. The research study was a pioneer attempt in a Mediterranean area, conducted in two consecutive steps. In the first step, turf color performances of five different turf colorants were tested on a dormant Bermuda grass (*Cynodon dactylon* L.) stand. Dormant Bermuda grass was overseeded with eleven different varieties of cool-season turf grasses in the second step of the study and color, texture and turf quality traits together with the rate of weed invasion and cover were determined. As the results of the study, SP Green, Ecolor Koyu and Ecolor Acik were identified as the brands demonstrating the greatest visual impact on the plots where the turf colorants were applied. Additionally, *Poa pratensis* cultivars were found to be completely inadequate among the tested varieties to compete with Bermuda grass during the summer months, *Agrostis stolonifera* demonstrated the most successful performance in the second year compared to other cool-season turf cultivars tested in the research.
Keywords: *bermudagrass, cool-season turf grass, turf quality, dormancy, turf management, overseeding*

Introduction

In the Mediterranean climate zone, high temperatures and drought stresses affect cultivated plants significantly. The turf grass species used in landscape projects should be selected in accordance with the ecological requirements. *Cynodon dactylon*, *Cynodon transvaalensis*, *Zoysia japonica*, *Paspalum vaginatum, Stenotahrum secundatum* and *Buchleo dactyloides* turf species are often favoured for this purpose due to their high drought resilience and dense coverage. On the other hand, these species go into dormancy in winter and lose their green appearance until the arrival of spring, an aspect which is considered undesirable for aesthetic purposes (Salman and Avcioglu, 2010; Kir et al., 2019).

Turf managers have traditionally overseeded bermudagrass during the fall to maintain a playable desirable and aesthetically appealing turf surfaces throughout the dormancy. This goal, however, is achieved with some difficulty. Maintenance throughout the winter and transitioning the playing surface from a cool-season grass back to a warm-season grass can be challenging. However, overseeding is not the only way to maintain a green turf during the winter. Applying colorant to dormant Bermuda grass is an alternative to overseeding during winter. Although turf colorants are a cost-effective alternative to overseeding, in addition to providing green color, turf colorants absorb heat which helps Bermuda grass emerge from dormancy earlier in the spring. However, there is limited information available about colorant brands and appropriate application rates.

For turf managers in the transition zone, overseeding warm-season grasses with a cool-season species is a common practice to maintain aesthetics and playability during the winter season (Mazur and Rice, 1999). Many golf course superintendents and sports

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_39793989

turf managers rely on this cool-season species for color while Bermuda grass is dormant in late fall, winter and early spring. In the spring, as soil temperatures warm and Bermuda grass resumes growth, cool-season turfgrass are removed by herbicide or by cultural practices such as low mowing, nitrogen fertilization, core aerification, dethatching, topdressing and dragging, which favour the growth and competitiveness of Bermuda grass.

One of the aims of this study was to investigate the effects of some different turf colorants on Bermuda grass stands, to evaluate the turf performances of some cool-season turf species practised in overseeding in autumn, winter and spring period and to determine which cultivars gave successful results in the second year as a result of cool-season grass competition with Bermuda grass (*Cynodon dactylon* L.) in the summer period.

Materials and Methods

The research was conducted in an open research field of Ege University's Bayindir Vocational Training School situated in Izmir, Turkey, (38º20.12'N- 27º67.14'E) at an altitude of 105 metres. The study field was arranged in May 2016 and observations continued until February 2018. The research field was under the effect of typical Mediterranean ecology (*Table 1*). The climatic data indicates that the precipitation levels from May to September were insufficient for the development of turfgrasses. Before seedbed preparation, the experimental plots were equipped with a permanent water pipeline system based on rotary sprinklers. During the summer season, supplemental sprinkler irrigation has been carried out when necessary to prevent visual wilt of the turf. The irrigation was achieved every morning based on 7 – 10 mm/m^2 approximately.

Table 1. *Climatic data of research years and long year averages*

Months	2016			2017			2018			1980-2016		
	TP (mm)	MT (ºC)	ARH (%)	TP (mm)	MT (ºC)	ARH (%)	TP (mm)	MT (ºC)	ARH (%)	TP (mm)	MT (ºC)	ARH (%)
Jan.	-	-	-	206.4	5.4	80.5	106.5	7.3	83.7	125.0	8.4	69.3
Feb.	-	-	-	20.6	8.9	77.0	103.8	10.9	86.7	72.5	8.5	64.4
Mar.	-	-	-	38.9	12.4	76.2	90.1	14.1	75.6	100.9	11.4	60.6
Apr.	-	-	-	56.8	16.0	65.5	12.6	20.0	62.6	64.0	15.0	57.2
May	-	-	-	18.1	21.2	59.6	63.8	23.2	61.8	42.1	20.0	52.1
June	-	-	-	44.7	26.0	57.0	-	-	-	5.2	25.6	40.9
July	-	-	-	0.0	29.8	44.7	-	-	-	6.8	27.4	37.9
Aug.	2.1	29.5	52.4	2.8	29.5	48.8	-	-	-	1.4	27.0	40.9
Sep.	38.7	24.3	54.3	1.2	24.1	54.8	-	-	-	4.8	24.1	4.6
Oct.	0.4	18.4	64.3	43.5	17.8	64.9	-	-	-	21.5	20.3	49.6
Nov.	70.2	12.1	73.1	59.2	11.8	78.7	-	-	-	89.4	13.5	58.9
Jan.	12.7	5.2	72.7	95.3	10.3	85.0	-	-	-	176.1	10.6	67.4
X/∑				587.5	17.8	66.1				709.7	17.7	53.7

TP: total precipitation MT: mean temperature; ARH: average relative humidity; X/∑: mean/total

The research field soil was analysed in terms of physical and chemical properties through the soil analysis laboratory of Ege University, Plant Nutrition and Soil Department Laboratory (*Table 2*). The results indicated that the soil characteristics were aligned with most specifications for grass growth except K, Ca, Mg and Zn content which were at amounts slightly below the required concentrations.

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Table 2. *Soil characteristics of the research field*

Soil Characteristics	Sample Depth (cm)		Soil Characteristics (ppm)	Sample Depth (cm)	
	0-20	20-40		0-20	20-40
pH	5.83	7.43	Available P	2.54	0.88
Total Salinity (%)	0.030	0.030	Available K	40	150
$CaCO_3$ (%)	0.82	2.27	Available Ca	1300	2400
Sand (%)	80.16	73.84	Available Mg	34	174
Silt (%)	18.00	20.72	Available Na	60	40
Clay (%)	1.84	5.44	Available Fe	12.75	11.6
Texture	Loam	Loam	Available Cu	0.35	0.54
Org. Material (%)	2.27	0.83	Available Zn	0.28	0.74
Total N (%)	0.092	0.050	Available Mn	6.84	5.04

The base warm-season turfgrass for the research was Bermuda grass (*Cynodon dactylon* L.) cultivar 'Tifsport'. Over seeded cool-season turfgrasses were *Lolium perenne* 'Sun', 'Double', 'Stolawn', 'Ringles' and 'Strawinsky'; *Festuca arundinacea* 'Lexington' and 'Titanium LS'; *Poa pratensis* 'SR 2100' and 'Evora' and *Agrostis stolonifera* '007' and 'Cobronova' were sown. The overseeding research pattern was a randomized block design with four replications. In total 44 (11×4) plots were arranged; each plot was 2 m^2 (1 m × 2 m), totalling 88 m^2. There was 50 cm distance between the plots; the total research area being 178.5 m^2.

The soil was prepared and Bermuda sod was laid in May 2016. In October, the turf was deeply mowed and verticutting was applied before overseeding. Sowing rates were 50 g/m^2 for *Lolium perenne* and *Festuca arundinacea*, 20 g/m^2 for *Poa pratensis* and 10 g/m^2 for *Agrostis stolonifera*. *Poa pratensis* and *Agrostis stolonifera* due to their small seed sizes they were sown with sand without top dressing, while *L. perenne* and *F. arundinacea* seeds were top dressed and rolled firmly. For ant control, Pestban 2 Dust (2% chlorpyrifos) was applied. The plots were irrigated by sprinkling when necessary.

The turf colorants practised on the turf plots included both foreign (SP Green, Ever Green, Grassline) and domestic (Ecolor Koyu, Ecolor Acik) brands. The turf colorant experiment was conducted in a randomized block design with four replications; composed of 24 (6×4) plots including control plots. Each plot was 1 m × 2 m = 2 m^2, totalling 48 m^2.

The turf colorants were applied in 03 December 2016 when the Bermuda grass was fully dormant. The consistencies of the colorants were arranged in accordance with the brand instructions for 8 m^2. Grassline (1.5 liter colorant + 7.5 liter water + surfactant = 9 liter), SP Green (1.5 liter colorant + 6 liter water + surfactant = 7.5 liter), Ecolor Koyu (1 liter colorant + 6 liter water + surfactant = 7 liter), Ecolor Acik (1 liter colorant + 6 liter water + surfactant = 7 liter) and Ever Green (1 liter colorant + 7 liter water + surfactant = 8 liter) were used as indicated.

Before the overseeding, Inpul 75 WG herbicide (60 g/ha dose + 45 L water) was applied to the Nut Grass (*Cyperus rotundus*). After the overseeding, Entec 26 (26% N – 31% S) fertilizer was added (5 g/m^2 N) monthly. Finally, from May onwards, Malvin XL 035 FS fungicide was used (2500 cc/ha) to control *Pythium* infection. Mowing was carried out weekly to a height of 2.5 cm while paths were cut to 1 cm. Throughout the research study, the following traits were determined on both the individual plants and plots as a whole.

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DOI: http://dx.doi.org/10.15666/aeer/1905_39793989

FieldScout GreenIndex+ Turf App was used to monitor the effects of turf colorants between December and March. For the overseeding experiment, color tests were carried out on a scale of 1 to 9 with 1 indicating yellow, 3 light yellowish-green, 5 green, 7 dark green and 9 very dark green (Morris and Shearman, 1998). The observations were repeated monthly between December and April, and again in July, October and February.

By examining leaf texture, measurements were taken monthly between December and April, then again in July and October. The visual rating of texture was based on a 1 to 9 rating scale with 1 equalling fine and 9 equalling coarse. General appearance scores were classified on a scale of 1-9 with 1 being very unpleasant and 9 very pleasant (Morris and Shearman, 1998). Weeds were scored on a scale of 1 to 9, with 1 indicating an excessive amount of weed invasion and 9 complete absences (Beard, 1973). Covers were scored on a scale of 1 to 9, with 1 being very sparse and 9 very dense.

Statistical analysis was conducted by using TOTEM STAT Statistical program (Acikgoz et al., 2004). The randomised complete block design of the overseeding experiment was evaluated with two replications and with a single replication for the turf colorant experiment. Probabilities equal to or less than 0.05 were considered significant. If, TOTEM STAT indicated differences between treatment means an LSD test was performed to separate them.

Results and Discussion

Color

The color values of each turf colorant are given below (*Table 3*). The turf colorant, month and turf colorant x month interactions were statistically significant.

Table 3. *Color values (1-9 point scale) of turf colorants on dormant Bermuda grass*

Turf Colorants	Color (2016 – 2017)				
	December	**January**	**February**	**March**	**Mean**
Grassline	7.6 d	4.7 e	4.5 d	4.8 c	5.41 e
SP Green	10.0 b	9.9 a	8.7 a	9.1 a	9.22 a
Ecolor Koyu	10.4 a	8.7 b	8.3 b	8.3 b	8.93 b
Ecolor Acik	9.8c	8.1 c	7.8 c	8.3 b	8.51 c
Control plot	1.6 e	1.5 f	1.4 f	3.4 e	1.97 f
Ever Green	10.2 b	5.0 d	3.5 e	3.9 d	5.66 d
Mean	8.27 a	6.32 b	5.70 b	6.30 c	
LSD (5%)	turf colorant: 0.07 month: 0.06 turf colorant x month: 0.15				

The results showed that mean highest color value was reached in December (8.27), with SP Green (9.22) and followed by Ecolor Koyu (8.93) displaying the highest scores for individual turf colorants. In comparison, the lowest mean color value was recorded in February (5.70) while the control plot means (1.97) showed a significantly lower score than plots with turf colorants. In the study, color values of the plots applied turf colorants were higher than those of control plots in March (*Table 3*).

Ratings for colorant-treated turf plots declined with time but were always better than the untreated control (Younger and Fuchigami, 1958) observed a similar response on Bermuda grass (*Cynodon dactylon*) colorant studies conducted in California. Untreated control Bermuda grass remained straw brown during the study. Turfgrass colorant

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DOI: http://dx.doi.org/10.15666/aeer/1905_39793989

treatments improved visual quality ratings when compared to the untreated control. The color value is a significant criterion for turf swards, whereas due to the agronomical and physiological features visual quality is also important (Williems et al., 1993; Braun et al., 2015). The turf colorants practised in this experiment was successful in improving the visual appearance of the turf surfaces although some color degradation did take place by the time. These findings were similar to the results of studies of (Sherman et al., 2005; Briscoe et al., 2010; Braun et al., 2017; Biber and Gokkus, 2020).

Bermuda grass is usually overseeded in the fall with perennial ryegrass or other cool-season grasses to provide green color, a uniform surface, and tolerance to wear while it is dormant. The color values of 11 different cool-season turfgrass cultivars overseeded on Bermuda grass is given in *Table 4* and *Figure 1*. The results indicated that the cultivar, month and cultivar x month interaction were statistically significant. The highest mean color value was achieved equally by the *Lolium perenne* cultivars, 'Sun', 'Ringles' and 'Strawinsky' (7.0), whereas the lowest mean color value was detected on the *Poa pratensis* cv. 'Evora' (6.1). The highest color value was also obtained in April (9.0) and the lowest in February in the second year (2.6).

Table 4. *Color values (1-9 point scale) of cool-season turfgrass cultivars overseeded on Bermuda grass*

		2016			**2017**				**2018**	
	Cultivars	**Dec.**	**Jan.**	**Feb.**	**March**	**Apr.**	**July**	**Oct.**	**Feb.**	**Mean**
Lolium perenne	Sun	8.0 a	7.7 b	7.9 b	8.7 b	9.0 a	7.8 a	6.0 e	1.2 f	7.0 a
	Double	7.9 b	7.8 a	7.8 c	7.8 g	7.8 f	7.6 c	6.0 e	1.2 f	6.7 b
	Stolawn	7.3 e	7.5 c	7.5 d	7.6 h	7.0 g	7.6 c	6.0 e	1.2 f	6.4 d
	Ringles	7.8 c	7.4 d	8.0 a	8.8 a	9.0 a	7.7 b	6.0 e	1.2 f	7.0 a
	Strawinsky	8.0 a	7.7 b	8.0 a	8.2 d	8.6 b	7.8 a	6.0 e	1.6 d	7.0 a
Festuca arundinacea	Lexington	7.6 d	4.6 i	7.2 e	8.4 c	9.0 a	7.5 d	6.2 d	1.2 f	6.5 c
	Titanium LS	7.3 e	4.3 j	7.0 f	8.0 e	8.5 c	7.5 d	6.4 c	1.4 e	6.3 e
Poa pratensis	SR 2100	6.0 f	5.4 e	7.5 d	7.9 f	8.2 d	7.6 c	6.2 d	3.0 c	6.5 c
	Evora	6.0 f	5.3 g	7.2 e	7.6 h	8.0 e	7.6 c	6.0 e	1.0 g	6.1 f
Agrostis stolonifera	007	5.0 g	5.0 g	6.8 g	6.8 I	6.6 h	7.8 a	6.8 a	8.2 a	6.6 c
	Cobronova	5.0 g	4.9 h	6.5 h	6.4 j	6.4 i	7.7 b	6.5 b	7.0 b	6.3 e
	Mean	6.9 e	6.1 f	7.4 d	7.8 b	8.0 a	7.7 c	6.2 g	2.6 h	-
	LSD (5%)	cultivar: 0.02			month: 0.02		cultivar x month: 0.06			

Figure 1. *General view of the overseeded trial area in March 2017*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_39793989

The weather temperature rising above 10 ºC breaks winter dormancy and encourages green tissue development of Bermuda grass (Beard, 1973; Acikgoz, 1994; Avcioglu, 1997). The Bermuda grass emerging from dormancy in May affected the results obtained in July and October. The cool-season turfgrasses were less capable of competing with Bermuda grass during the summer season and by winter in the second year; many cultivars had been rendered almost absent in their plots. However, the average score for *Agrostis stolonifera* '007' (8.2) and 'Cobronova' (7.0) appeared to demonstrate that they were the most resilient of the cultivars surviving the competition period and performing the highest color values in October and February.

Turf color, being indicative of the healthy development of turf crops and the ratio of high photosynthetic activity, is a convenient feature for evaluating the turfs (Martiniello and Andrea, 2006). For this reason, the green color tone increases rapidly in the plants that are able to perform optimum growth and development, which is especially important for turf grasses (Beard, 1973; Avcioglu, 1997). Our turf color results were confirmed by Rossini et al. (2019) and Volterrani and Magni (2004a,b)'s findings.

Texture

The "texture" feature of turfs is symbolized by the width of the leaf blades of turf grasses (Beard, 1973), is a feature that enhances the appearance of the turf plots and exhibits the "fine" texture structure in narrow-leaved grasses. A medium-fine to medium texture, ranging from 1.5 to 3 mm in width is generally preferred for most turf grass uses (Beard, 1973). The texture scores of eleven different cool-season turfgrass cultivars overseeded on Bermuda grass are given in *Table 5*. The results indicated that the cultivar, month and cultivar x month interactions were statistically significant. The highest texture value was observed on *Festuca arundinacea* 'Lexington' and 'Titanium LS' (6.5) plots which compared to the considerably lowest texture value of *Agrostis stolonifera* cultivars (4.1-4.2). The peak mean value was obtained in March and April (6.3) as a result of this favourable vegetation season, while the lowest mean value was recorded in July (3.1) and October (2.4) due to the influence of Bermuda grass which was dominantly present throughout the summer season.

Table 5. *Texture values (1-9 point scale) of cool-season turfgrass cultivars overseeded on Bermuda grass*

		2016	2017						
	Cultivars	**Dec.**	**Jan.**	**Feb.**	**March**	**Apr.**	**July**	**Oct.**	**Mean**
Lolium perenne	Sun	4.3 d	4.4 c	5.2 f	5.3 g	5.3 g	3.0 c	2.5 a	4.3 c
	Double	4.5 b	4.5 b	5.4 e	5.4 f	5.5 f	3.0 c	2.5 a	4.4 b
	Stolawn	4.4 c	4.4 c	5.2 f	5.3 g	5.3 g	3.0 c	2.5 a	4.3 c
	Ringles	4.2 e	4.4 c	5.0 g	5.1 h	5.1 h	3.0 c	2.5 a	4.2 d
	Strawinsky	4.2 e	4.4 c	4.9 h	5.1 h	5.1 h	3.0 c	2.5 a	4.2 d
Festuca arundinacea	Lexington	6.5 a	7.2 a	8.0 a	8.9 a	9.0 a	3.4 a	2.5 a	6.5 a
	Titanium LS	6.5 a	7.2 a	8.0 a	8.9 a	8.9 b	3.4 a	2.5 a	6.5 a
Poa pratensis	SR 2100	3.2 f	4.3 d	5.5 d	6.1 e	6.2 e	3.2 b	2.5 a	4.4 b
	Evora	3.0 g	4.1 e	5.5 d	6.2 d	6.2 e	3.2b	2.5 a	4.4 b
Agrostis stolonifera	007	2.3 h	2.8 f	6.0 c	6.4 c	6.4 d	2.8 d	2.0 c	4.1 e
	Cobronova	2.3 h	2.8 f	6.2 b	6.6 b	6.6 c	3.0 c	2.3 b	4.2 d
	Mean	4.1 d	4.6 c	5.9 b	6.3 a	6.3 a	3.1 e	2.4 f	-
	LSD (5%)	cultivar: 0.03			month: 0.02		cultivar x month: 0.07		

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DOI: http://dx.doi.org/10.15666/aeer/1905_39793989

It was concluded that there are texture differences among the varieties of different cool-season turfgrass types tested in the study. *Festuca arundinacea* has genetically a rough or moderately rough texture depending on the different cultivars. It is a coarse or rough textured grass which displayed clearly these characteristics mentioned in this part of our study. Avcioglu (1997) and Volterrani and Magni (2004a) stated that new *Festuca arundinacea* cultivars with fine texture were improved in many countries since old rough-textured *Festuca arundinacea* cultivars were not being required by customers. Our texture score results were similar to the results of Baker and Jung (1968), Avcioglu (1997) and Salman et al. (2019).

Turf Quality

Since the warm-season turfgrasses are in yellowish-brown-coloured during dormancy period in winter, only the data collected in autumn-winter-spring seasons. The turf quality trait as the composite of colour, uniformity and texture traits is a widely used criterion to define the overall performances of turfs in turf management practices. The turf quality values of eleven different cool-season turfgrass cultivars overseeded on Bermuda grass are given in *Table 6*. The results indicated that the cultivar, month and cultivar x month interaction were statistically significant.

Table 6. *Turf quality (1-9 point scale) values of cool-season turfgrass cultivars overseeded on Bermuda grass*

		2016			2017			2018		
	Cultivars	**Dec.**	**Jan.**	**Feb.**	**March**	**Apr.**	**July**	**Oct.**	**Feb.**	**Mean**
Lolium perenne	Sun	7.4 a	7.5 b	8.0 a	8.7 a	9.0 a	8.4 b	7.2 c	1.2 e	7.2 a
	Double	7.4 a	7.6 a	7.8 b	8.7 a	9.0 a	8.2 c	7.2 c	1.2 e	7.1 b
	Stolawn	7.4 a	7.6 a	7.8 b	8.5 c	8.7 c	8.2 c	7.2 c	1.2 e	7.1 b
	Ringles	7.3 b	7.5 b	8.0 a	8.7 a	9.0 a	8.2 c	7.2 c	1.4 d	7.2 a
	Strawinsky	7.4 a	7.6 a	8.0 a	8.6 b	8.3 d	8.4 b	7.2 c	1.4 d	7.1 b
Festuca arundinacea	Lexington	5.6 c	5.8 c	6.7 c	8.2 d	9.0 a	8.0 d	7.2 c	1.2 e	6.5 e
	Titanium LS	5.5 d	5.7 d	6.6 d	8.2 d	8.9 b	8.0 d	7.2 c	1.2 e	6.4 f
Poa pratensis	SR 2100	4.3 g	4.5 g	4.5 g	6.6 g	7.2 g	8.0 d	7.2 c	2.5 c	5.6 g
	Evora	4.0 h	4.1 h	4.2 h	6.0 h	6.2 h	8.0 d	7.2 c	1.1 f	5.1 h
Agrostis stolonifera	007	4.4 f	4.6 f	5.4 f	7.7 e	8.2 e	8.5 a	8.0 a	7.8 a	6.8 c
	Cobronova	4.6 e	4.8 e	5.6 e	7.5 f	7.6 f	8.4 b	7.6 b	6.5 b	6.6 d
	Mean	5.9 g	6.1 f	6.6 e	7.9 c	8.3 a	8.2 b	7.3 d	2.4 h	-
	LSD (5%)	cultivar: 0.03			month: 0.03		cultivar x month: 0.08			

The results revealed that the highest value was obtained in *Lolium perenne* 'Sun' (7.2) and 'Ringles' (7.2) compared to the lowest valued *Poa pratensis* 'Evora' (5.1) and 'SR 2100' (5.6) both of which displayed scores below the acceptable level. Similarly, the appearance value peaked in April (8.3) and decreased in February (2.4) in the second year.

In December 2016, the turf quality values of the varieties of *Lolium perenne* and *Festuca arundinacea*, which had larger seeds, were higher than the varieties of other species. Turf quality values of all varieties increased until April while Bermuda grass became dormant in July, the grass quality values of all plots were close to each other. In February 2018, highest quality values were recorded in *Agrostis stolonifera* varieties. All overseeded cool-season grasses lost competition with Bermuda grass during the summer

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_39793989

period and could not exist in plots by the second year, except *Agrostis stolonifera* cultivars.

When the total turf quality data of the first year of the study were evaluated, *Lolium perenne* cv. and *Festuca arundinacea* cv. displayed much better performance than the *Poa pratensis* cultivars.

Weed Invasion

The weed invasion scores of cool-season turf cultivars overseeded on Bermuda grass is given in *Table 7*. The results indicated that the cultivar, month and cultivar x month interactions were statistically significant.

Table 7. *Weed invasion (1-9 point scale) values on cool-season turfgrass cultivars on Bermuda grass stand*

		2016			2017			2018		
	Cultivars	**Dec.**	**Jan.**	**Feb.**	**March**	**Apr.**	**July**	**Oct.**	**Feb.**	**Mean**
Lolium perenne	Sun	9.0 a	9.0 a	8.8 b	9.0 a	9.0 a	8.9 b	9.0 a	8.8 c	8.9 b
	Double	9.0 a	9.0 a	8.9 a	9.0 a	9.0 a	8.9 b	9.0 a	8.8 c	8.9 b
	Stolawn	9.0 a	9.0 a	8.9 a	9.0 a	9.0 a	8.9 b	9.0 a	8.9 b	9.0 a
	Ringles	9.0 a	9.0 a	8.8 b	9.0 a	9.0 a	8.9 b	9.0 a	8.8 c	8.9 b
Festuca arundinacea	Strawinsky	9.0 a	9.0 a	8.5 e	8.9b	8.8 b	9.0 a	9.0 a	8.8 c	8.9 b
	Lexington	9.0 a	9.0 a	8.9 a	9.0 a	9.0 a	9.0 a	9.0 a	8.8 c	9.0 a
	Titanium LS	9.0 a	9.0 a	8.9 a	9.0 a	9.0 a	9.0 a	9.0 a	8.8 c	9.0 a
Poa pratensis	SR 2100	9.0 a	9,0 a	8.6 d	8.5 c	8.3 c	9.0 a	9.0 a	8.8 c	8.7 c
	Evora	9.0 a	9.0 a	8.2 f	7.9 d	6.1 d	9.0 a	9.0 a	8.8 c	8.4 d
Agrostis stolonifera	007	9.0 a	9.0 a	8.9 a	9.0 a	9.0 a	8.9 b	9.0 a	9.0 a	9.0 a
	Cobronova	9.0 a	9.0 a	8.7 c	9.0 a	9.0 a	9.0 a	9.0 a	8.9 b	8.9 b
	Mean	9.0 a	9.0 a	8.7 d	8.8 c	8.7 d	8.9 b	9.0 a	8.8 c	-
	LSD (5%)	cultivar: 0.02			month: 0.02		cultivar x month: 0.07			

The mean weed invasion value (8.4-9.0) of cool-season grass cultivars was significant. *Lolium perenne* 'Stolawn', *Festuca arundinacea* 'Lexington' and 'Titanium LS' and *Agrostis stolonifera* '007' plots contained the highest weed invasion scores (9.0) while *Poa pratensis* 'Evora' had the lowest (8.4). The peak mean value (9.0) was reached in December, February and October while the lowest mean value was recorded in February and April (8.7). Overall results were found to be rather high. Since the weed presence on lawns is an undesirable trait, only turf species capable of competing successfully with weeds can sustain the desirable turf appearance (Avcioglu, 1997; Salman et al., 2011). The weed infestation data of the cultivars used in the study were generally high. The dense texture of bermudagrass during summer period and its high competitive capacity contributed these results. Cover data of different cool-season grass cultivars tested in the study also supports our findings here.

Cover

The cover scores of 11 different cool-season turfgrass cultivars overseeded on Bermuda grass are given in *Table 8*. The results displayed that the cultivar, month factors and the cultivar x month interaction were statistically significant.

The cover scores were recorded for 8 different months. *Lolium perenne* 'Sun', 'Double' and 'Stolown' cultivars reached the highest value as 7.7, while *Poa pratensis* 'Evora' (5.7) and 'SR 2100' (6.0) demonstrated the lowest.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_39793989

Table 8. *Cover values (1-9 point scale) of cool-season turfgrass cultivars overseeded on Bermuda grass*

		2016			2017				2018	
	Cultivars	**Dec.**	**Jan.**	**Feb.**	**March**	**Apr.**	**July**	**Oct.**	**Feb.**	**Mean**
Lolium perenne	Sun	8.1 b	8.2 b	8.3 c	8.6 b	9.0 a	9.0 a	9.0 a	1.2 e	7.7 a
	Double	8.4 a	8.4 a	8.4 b	8.6 b	9.0 a	9.0 a	9.0 a	1.2 e	7.7 a
	Stolawn	8.0 c	8.4 a	8.5 a	8.7 a	9.0 a	9.0 a	9.0 a	1.2 e	7.7 a
	Ringles	7.7 d	8.2 b	7.7 e	8.6 b	9.0 a	9.0 a	9.0 a	1.4 d	7.6 b
	Strawinsky	7.5 e	7.7 c	8.0 d	8.6 b	9.0 a	9.0 a	9.0 a	1.4 d	7.5 c
Festuca arundinacea	Lexington	5.5 g	6.7 e	6.9 g	8.2 d	9.0 a	9.0 a	9.0 a	1.2 e	6.9 d
	Titanium LS	5.6 f	6.7 e	6.9 g	8.2 d	9.0 a	9.0 a	9.0 a	1.2 e	6.9 d
Poa pratensis	SR 2100	2.5 j	4.8 g	5.6 h	7.2 e	7.5 d	9.0 a	9.0 a	2.4 c	6.0 e
	Evora	2.5 j	4.6 h	5.3 i	7.1 f	7.2 e	9.0 a	9.0 a	1.0 f	5.7 f
Agrostis stolonifera	007	3.5 i	6.2 f	7.3 f	8.4 c	8.8 b	9.0 a	9.0 a	8.0 a	7.5 c
	Cobronova	5.0 h	6.8 d	7.7 e	8.2 d	8.6 c	9.0 a	9.0 a	6.5 b	7.6 b
	Mean	5.8 f	7.0 e	7.4 d	8.2 c	8.6 b	9.0 a	9.0 a	2.4 g	-
	LSD (5%)	cultivar: 0.03			month: 0.03		cultivar x month: 0.09			

The cover scores varied between 2.4 and 9.0 during 8 months while only *Agrostis stolonifera* was able to compete with Bermuda grass during summer and produce sufficient cover for the second year of the experiment. *Lolium perenne* and *Festuca arundinacea* cultivars with their large seeds were advanced in early sprouting and high tillering stage which allowed them to cover the ground rather rapidly (Salman et al., 2019). These findings were similar to the comments of Acikgoz (1994), Deniz (2018) and Salman et al. (2019). Barton (1997) stated that *Lolium perenne* establishes very rapidly and is included in grass mixtures to provide a quick cover. Many research workers, studying under Mediterranean conditions reported that *Lolium perenne* is a proper turf grass to be included in mixtures (Beard, 1973; Acikgoz, 1994; Avcioglu, 1997; Biber and Gokkus, 2020).

In the first year of the study, the development of *Poa pratensis* cv. and *Agrostis stolonifera* cv. were slow to develop, so they maintained low levels of cover trait. While varieties of *Poa pratensis* exceeded 7 points in March, *Agrostis stolonifera* cultivars reached these scores in February. When the cover data of February in the second year of the study were examined, we determined that all other varieties except *Agrostis stolonifera* were very low in performance. In the second year of the study, cultivar 007 variety of *Agrostis stolonifera* showed much better cover score.

Conclusions

The color values of all turf colorants were sufficient when compared to the control plots and they (SP Green, Ecolor Koyu and Ecolor Acik) all sustained their color successfully. The color values of *Festuca arundinacea*, *Lolium perenne* and *Poa pratensis* were higher than *Agrostis stolonifera* which was more successful in spring than in winter. The textures of all the cool-season turfgrasses were found to be coarse, showing the highest value with *Festuca arundinacea* cultivars. Turf quality value was high in spring, except *Poa pratensis* cultivars. *Lolium perenne* and *Festuca arundinacea* demonstrated the most successful cover in spring while *Poa pratensis* cultivars had limited performance. *Lolium perenne* and *Festuca arundinacea* cultivars demonstrated

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_39793989

the most successful recovery performance. *Agrostis stolonifera* cultivars performed well at competing with Bermuda grass over the summer and had higher thinning scores.

We are suggesting applying turf colorant will reduce costs and increase the performance of coloring in harsh conditions and growth difficulties. Besides, we recommend performing *Agrostis stolonifera* cultivation could enhance covering and decrease numerous times of cultivations and application costs annually.

Acknowledgements. Research study (16-BAMYO-001) founded by Ege University Scientific Research Projects Coordination.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_39793989

IMPACTS OF ENVIRONMENTAL VARIABLES ON MACROINVERTEBRATE FUNCTIONAL FEEDING GROUPS AND BIODIVERSITY IN A MULING RIVER WETLAND FROM NORTHEAST CHINA

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(Received 6th May 2021; accepted 29th Jul 2021)

Abstract. Muling River is the fifth-largest river in Heilongjiang Province, and it is also the main feeding river to the Ussuri River which is the boundary river of China and Russia in Heilongjiang Province northeast China. The Muling River basin is in the south of Sanjiang Plain. Macroinvertebrate samples were collected using a D-frame net and Shannon-Wiener index was calculated in terms of abundance. A total of 158 genera or species macroinvertebrates were collected from the 28 sampling sites and classified into six functional feeding groups including 61 gatherers / collectors, 42 predators, 22 scrapers, 14 shredders, 11 filterers / collectors and 8 omnivores. The correlation and relationship between environmental variables and macroinvertebrate functional feeding groups were explored using Pearson analysis and redundancy analysis. Temperature was associated with macroinvertebrates abundance, and nutrients were the main influence factors in Muling River basin.
Keywords: *Muling River basin, macroinvertebrate, community structure, influencing factors, RDA*

Introduction

Functions of river ecosystem research mostly carried out based on the traditional classification of species. However, recent studies have shown that ecosystem functions are mainly subject to the diversity of functional traits, i.e. the distribution of functional traits and the spatial-temporal pattern of abundance (Elliott and Quintino, 2010). Functional traits are sensitive to environmental changes and play a key role in the study of the relationship between biodiversity and ecosystem functions. Functional diversity based on biological traits is closely related to ecosystem processes and is the key to understand ecosystem and community functions (Jiang et al., 2018). Macroinvertebrates were widely used to monitor the damage of aquatic ecosystem, and they are also an

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_39914006

important part of aquatic food web, which is the basis of nutrient cycle and ecological balance of ecosystem (Mangadze et al., 2016). Many factors have caused damage to the aquatic ecosystem in the Muling River basin, and the importance of monitoring water quality in the Muling River basin through macroinvertebrate is self-evident. The species characteristics of functional groups are more closely related to the environment, which can more directly reflect the ecological process of the ecological environment affecting aquatic communities, and better understand the water ecosystem and its biodiversity (An et al., 2017; Liu et al., 2019). In the river ecosystem, the functional diversity of macroinvertebrate can better reflect the ecosystem functions than community structure. Many studies have shown that substrate type and aquatic vascular plants are affecting the growth and functional group distribution of macroinvertebrate (Hubler et al., 2016; Ding et al., 2017; Kaskela et al., 2017).

Muling River is the fifth-largest river in Heilongjiang Province, and it is also the main feeding river to the Ussuri River which is the boundary river of China and Russia (Li et al., 2016). The approximately length of the Muling River is 834 km with annual water flows of 2.35 billion m^3. The river flows through five counties or cities of Muling, Jixi, Jidong, Mishan and Hulin from the south to the northeast of Heilongjiang Province (Li et al., 2015). Upstream of the river is characterized by temperate continental climate with a hot summer rainy and long cold winter. The annual average precipitation in the upstream is 530 mm and mainly occurs from July to September. In the midstream, the climate is temperate and semi-humid monsoonal with annual average temperature of 3.1°C (-18°C ~ 21°C). The annual precipitation is 522 mm and the frost-free period is 149 days. At downstream area, the climate is characterized by temperate continental monsoonal. In recent years, with the aggravation of agricultural non-point source pollution, industrial discharge pollution and urban living pollution in Muling River Basin, the water quality of Muling River is deteriorating, which has had a negative impact on the local people's production and life.

This study aimed to collect macroinvertebrate fauna, and explore the relationships between macroinvertebrate functional feeding groups and environmental variables in the wetland environments of Muling River basin.

Materials and methods

Study area

Muling River basin located in the south of Sanjiang Plain with an area of 18427 km^2, and it is the fifth-largest river in Heilongjiang Province northeast of China, which is the main feeding river to the Ussuri River the boundary river of China and Russia (*Fig. 1*). According to the manual of inland waters fishery natural resources investigation (Zhang and He, 1991), and principles to the requirement of sampling sites, in combination with climatic characteristics and natural form of Muling River basin, 28 sampling sites were selected from upstream, midstream to downstream (*Table 1*).

Environmental variables data sampling

Samples were collected 3 times from 28 sampling sites of Muling River basin in May (spring), July (summer) and September (autumn) periods in 2015. Water transparency (SD) and water depth (WD) were measured in the field using a Secchi disk and graduated portable staff gauge, respectively. Electric conductivity (EC), dissolved oxygen (DO), pH

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DOI: http://dx.doi.org/10.15666/aeer/1905_39914006

and water temperature (T) also measured in the field using a portable multi-probe (YSI 6600, YSI Inc., USA). We used the Chinese standard methods proposed by Ministry of Environmental Protection of People's Republic of China (Standard, 2002) to determine the concentration of total nitrogen (TN), total phosphorus (TP), N:P ratio (N:P), ammonium nitrogen (NH_4^+-N), nitrate nitrogen (NO_3^--N), chemical oxygen demand (COD_{Mn}).

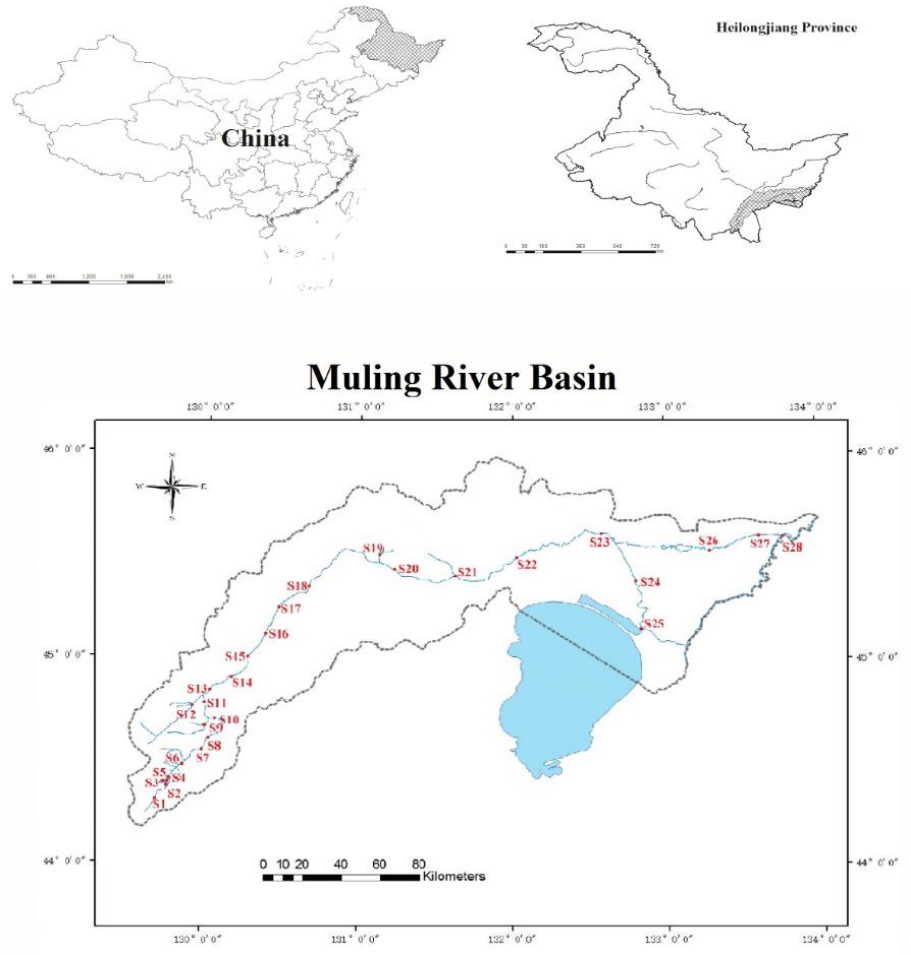

Figure 1. *Location of 28 sampling sites in Muling River basin*

Macroinvertebrate data sampling

Three random subsamples were collected at locations of 1 m^2 at each sampling site by using a D-frame net (0.25-mm, 60 mesh). All macroinvertebrate samples were composited into a single sample, preserved in 75% ethanol and transported to the laboratory for identification. In the laboratory, all samples were sorted on white porcelain pans, identified, and counted with a light stereomicroscope (10×, Leica Microsystems, German). All individuals were identified to genus or species using appropriate identification guides (Morse et al., 1994; Epler, 2001). Taxa were divided into six functional feeding groups according to Cummins (1974) and Duan et al. (2010): predators (PR), omnivores (OM), gatherers/collectors (GC), filterers/collectors (FC), scrapers (SC) and shredders (SH) (Cummins, 1974; Duan et al., 2010).

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DOI: http://dx.doi.org/10.15666/aeer/1905_39914006

Table 1. *The sampling stations and coordinates in Muling River basin*

Sampling sites	Latitude (N)	Longitude (E)
S1	44°01′48″	130°11′24″
S2	44°03′36″	130°10′48″
S3	44°03′00″	130°09′36″
S4	44°04′12″	130°10′48″
S5	44°04′48″	130°10′48″
S6	44°11′24″	130°15′36″
S7	44°13′12″	130°15′00″
S8	44°13′48″	130°15′36″
S9	44°21′36″	130°16′48″
S10	44°24′36″	130°19′12″
S11	44°28′12″	130°14′24″
S12	44°28′12″	130°12′36″
S13	44°29′24″	130°13′48″
S14	44°34′48″	130°19′48″
S15	44°40′12″	130°26′24″
S16	44°53′24″	130°30′36″
S17	45°00′00″	130°32′24″
S18	45°04′48″	130°40′12″
S19	45°18′00″	131°00′36″
S20	45°18′00″	131°03′36″
S21	45°20′24″	131°31′48″
S22	45°27′00″	131°52′12″
S23	45°42′00″	132°25′12″
S24	45°35′24″	132°36′36″
S25	45°19′48″	132°48′36″
S26	45°44′24″	132°57′00″
S27	45°45′36″	133°06′00″
S28	45°58′12″	133°40′12″

Statistical analyses

Variation of environmental variables and abundance of functional groups in different sampling periods were analyzed using One-way ANOVA in SPSS 19.0 software. Before analysis, the data was lg(x+1) transformed to manage variance heterogeneity and ensure the data is normally distributed. In this study, the gradient length of the first ordination axis was 0.404 in the detrended correspondence analysis (DCA). Therefore, redundancy analysis (RDA) with Monte Carlo simulations (499 permutations) ordination based on unimodal method was selected to analyze the relation by using CANOCO for Windows 4.5 software (Microcomputer Power, New York, USA). Pearson correlation analysis was carried out to confirm the significant relationships between environmental variables and the abundance of functional feeding group. Cluster analyses was conducted using the PRIMER 7 software package (Clarke and Gorley, 2015).

Diversity of macroinvertebrate FFGs was represented by Shannon-Wiener index (Shannon and Wiener, 1949) as follows:

$$H' = -\sum_{i=1}^{s} P_i \log_2 P_i \qquad \text{(Eq.1)}$$

where, S is the number of FFGs within the given sample; and P_i is the percentage of FFGs i in the total number of individuals.

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Results

Environmental variables

Among all sampling sties, natural gradients (e.g., dissolved oxygen and temperature) and nutrient indicators (e.g., total phosphorus, N:P ratio, ammonium nitrogen and nitrate nitrogen) were not significantly different ($p > 0.05$), but water transparency, water depth, electric conductivity, total nitrogen and chemical oxygen demand were significantly different ($p < 0.01$) (*Table 2*).

Table 2. *One-Way ANOVA of environmental variables and macroinvertebrate FFGs abundance. Data are average values (with SE). Environmental variables: water transparency (SD), water depth (WD), electric conductivity (EC), dissolved oxygen (DO), pH, water temperature (T), total nitrogen (TN), total phosphorus (TP), N:P ratio (N:P), ammonium nitrogen (NH_4^+-N), nitrate nitrogen (NO_3^--N), chemical oxygen demand (COD_{Mn}). Macroinvertebrate FFGs: predators (PR), omnivores (OM), gatherers/collectors (GC), filterers/collectors (FC), scrapers (SC) and shredders (SH). F-value and P-value from One-way ANOVA by post-hoc test using Tukey HSD ANOVA*

	2015May	**2015Jul.**	**2015Sep.**	***F***	***p*-value**
Environmental variables					
SD (m)	0.35(0.05)	0.32(0.07)	0.48(0.07)	10.418	0.000**
WD (m)	2.72(0.76)	3.13(1.04)	3.02(1.04)	75.232	0.000**
EC (ms/cm)	0.15(0.01)	0.15(0.01)	0.21(0.02)	2.472	0.002**
DO (mg/L)	7.45(0.29)	8.73(0.29)	7.49(0.56)	1.676	0.052
pH	7.42(0.12)	7.03(0.26)	7.99(0.06)	1.903	0.021*
T (℃)	14.81(0.47)	22.26(0.55)	6.89(0.43)	0.215	0.862
TN (mg/L)	1.73(0.14)	1.99(0.21)	1.62(0.16)	2.662	0.001**
TP (mg/L)	0.6(0.05)	0.69(0.04)	0.36(0.03)	0.456	0.986
N:P	3.86(0.56)	3.13(0.35)	6.56(1.6)	0.727	0.815
NH_4^+-N (mg/L)	0.22(0.02)	0.35(0.04)	0.13(0.01)	0.704	0.839
NO_3^--N (mg/L)	0.58(0.07)	1.52(0.5)	0.28(0.03)	1.143	0.329
COD_{Mn} (mg/L)	3.8(0.13)	3.98(0.1)	4.06(0.12)	3.410	0.000**
FFGs abundance					
PR (ind./m^2)	25.75(3.47)	46.75(2.68)	24.54(2.6)	0.614	0.916
OM (ind./m^2)	12.14(3.03)	5.68(0.85)	5.82(1.1)	0.701	0.842
GC (ind./m^2)	60.14(5.94)	114.54(6.51)	36.46(2.92)	0.351	0.998
FC (ind./m^2)	10.93(2.6)	11.86(2.02)	6.5(1.22)	1.615	0.065
SC (ind./m^2)	38.93(12.36)	30.46(4.67)	17.75(2.22)	2.335	0.004**
SH (ind./m^2)	6.68(1.66)	16.5(2.11)	11.54(2.62)	0.754	0.787
Total (ind./m^2)	154.57(14.29)	225.79(8.52)	102.61(6.2)	0.535	0.946
Shannon-Wiener (*H'*)	1.69(0.06)	1.85(0.04)	2.03(0.04)	0.566	0.961

*$P<0.05$, **$P<0.01$

Macroinvertebrate functional feeding groups

During the sampling periods, a total of 13523 macroinvertebrate individuals belonging to 46 families 158 genera or species were identified from the study area, consisting of 61 gatherers/collectors, 42 predators, 22 scrapers, 14 shredders, 11 filterers/collectors and 8 omnivores (*Appendix A*). All FFGs, total abundance and Shannon-Wiener index were not significantly different ($p > 0.05$), while SC group was significantly different ($p < 0.01$) (*Table 1, Fig. 2*). Highest abundance of total macroinvertebrate was observed in summer, while the maximum value of Shannon-Wiener index presented in autumn (*Fig. 3*).

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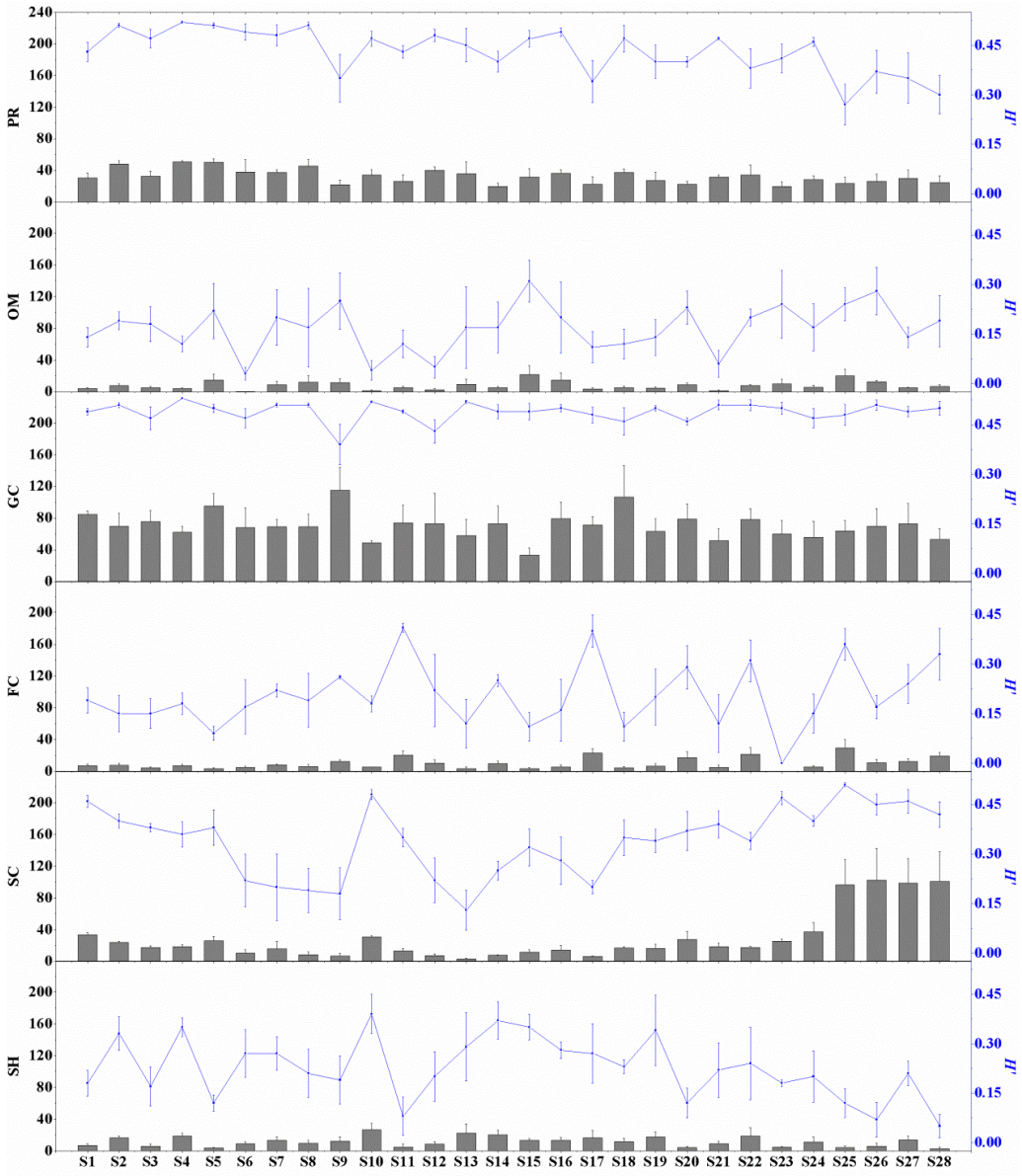

Figure 2. *Macroinvertebrate FFGs abundance (ind./m^2) among sampling sites. Macroinvertebrate FFGs: predators (PR), omnivores (OM), gatherers/collectors (GC), filterers/collectors (FC), scrapers (SC) and shredders (SH)*

Correlation analysis

Correlation analysis indicated that temperature was associated with all FFGs abundance ($p < 0.01$ or $p < 0.05$). By contrast, SD was negative significantly correlated with group FC ($p < 0.05$) and SH ($p < 0.05$) and DO displayed negative correlations with group PR ($p < 0.05$) and SH ($p < 0.01$). The pH value negatively correlated with group GC ($p < 0.01$) and SC ($p < 0.01$). However, WD, TN, TP and NH_4^+-N were only positive significantly correlated with one group, such as PR ($p < 0.05$), SH ($p < 0.01$) and GC ($p < 0.01$), respectively. While N:P ratio was negative significantly correlated with group GC ($p < 0.05$). COD_{Mn} positively correlated with group PR ($p < 0.01$) and SH ($p < 0.01$) and negatively correlated with group SC ($p < 0.05$).

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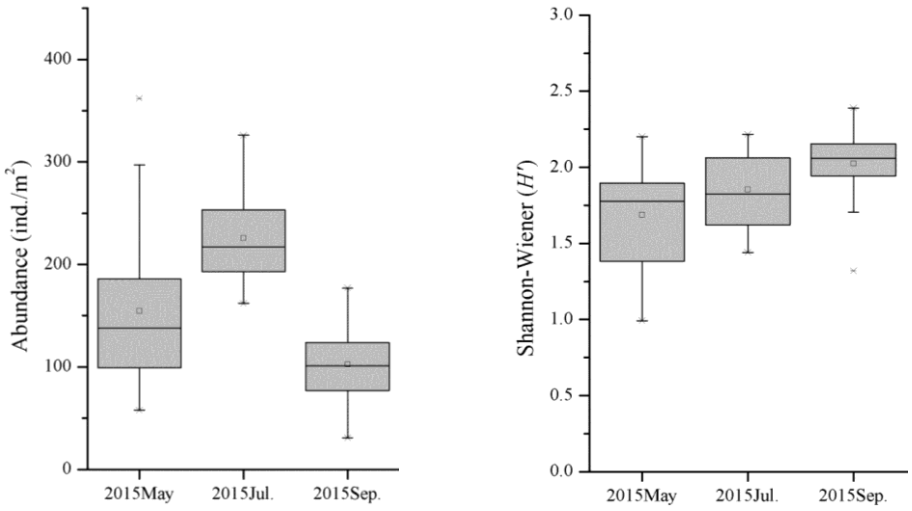

Figure 3. *Boxplots of macroinvertebrate abundance and Shannon-Wiener index among seasons*

On the other hand, environmental variables of WD, DO and N:P positively correlated with biodiversity index (*H'*) of PR, SH and GC groups, respectively. TP was both negatively correlated with group GC and SC biodiversity index. COD_{Mn} was positively correlated with group PR biodiversity index, while negatively correlated with group OM (*Table 3*).

Table 3. *Correlation (Pearson) analysis between functional feeding groups abundance (ind./m²), Shannon-Wiener index (H') and environmental variables. Some variables without any significant correlation were not shown. Environmental variables: water transparency (SD), water depth (WD), electric conductivity (EC), dissolved oxygen (DO), pH, water temperature (T), total nitrogen (TN), total phosphorus (TP), N:P ratio (N:P), ammonium nitrogen (NH_4^+-N), nitrate nitrogen (NO_3^--N), chemical oxygen demand (COD_{Mn}). Macroinvertebrate FFGs: predators (PR), omnivores (OM), gatherers/collectors (GC), filterers/collectors (FC), scrapers (SC) and shredders (SH)*

	Abundance						***H'***				
	PR	**OM**	**GC**	**FC**	**SC**	**SH**	**PR**	**OM**	**GC**	**SC**	**SH**
SD				-0.162*		-0.153*					
WD	0.153*						0.220*				
DO	-0.162*					-0.223**					0.260*
pH			-0.343**		-0.218**						
T	0.477**	0.255**	0.562**	0.303**	0.211**	0.319**					
TN						0.333**					
TP			0.271**						-0.224*	-0.230*	
N:P			-0.155*						0.228*		
NH_4^+-N					0.214**						
COD_{Mn}	0.242**				-0.202**	0.280**	0.274*	-0.218*			

*P<0.05, **P<0.01

RDA analysis

Redundancy analysis (RDA) revealed clear clusters of sampling sites by macroinvertebrate abundance and environmental variables (*Fig. 4*), with several outliers (S2, S25 and S28). The results of Monte Carlo test revealed that the first canonical axis

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and all canonical axes were significantly different (F = 13.781, p = 0.002; F = 2.247, p = 0.004, respectively), indicating associations between macroinvertebrate FFGs and environmental variables existed. The first two axes of FFGs correlations to environmental variables were 0.91 and 0.761, which combined explained 87.4% of FFGs-environment relationship. In RDA biplot, TN and N:P had high inflation factors. Group SC and OM mainly impacted by T and NH_4^+-N at S26 and S27, and group GC and PR positively correlated with EC, WD and SD at S3, S4, S5, S20 and S24. Meanwhile, group SH has a positive correlation with TN, N:P and NO_3^--N at S6, S15, S21, S22 and S23. We also found that pH, DO and COD_{Mn} were the main factors at S7~S14, S16, S17 and S19.

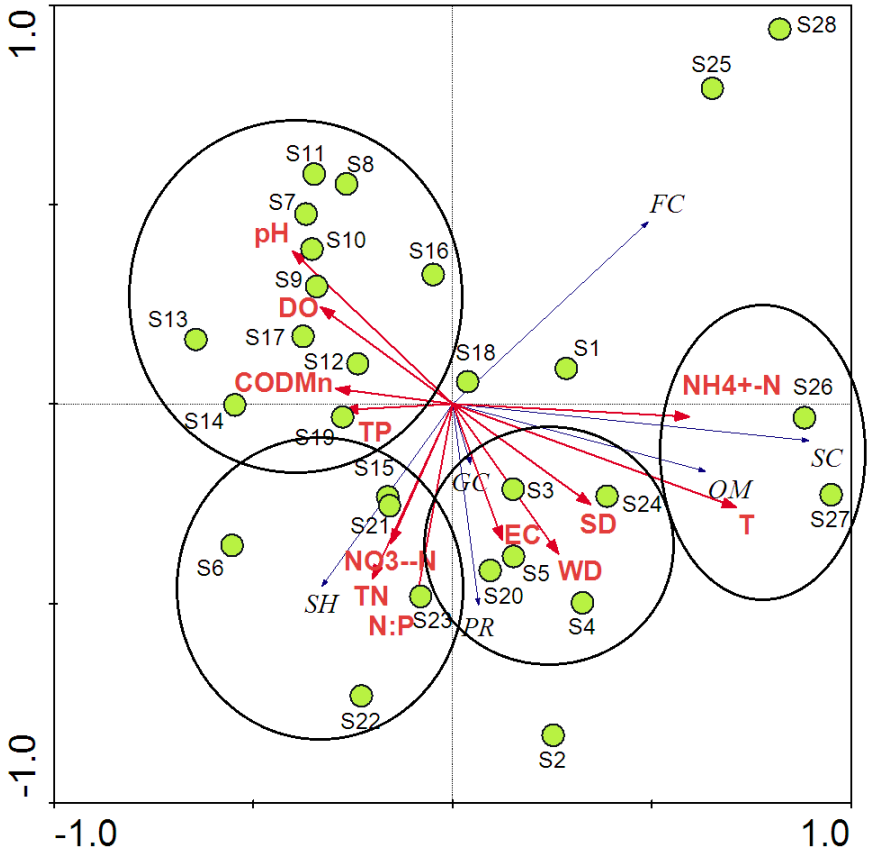

Figure 4. *RDA biplot of macroinvertebrate FFGs abundance and environmental variables with sampling sites in Muling River basin. Environmental variables: water transparency (SD), water depth (WD), electric conductivity (EC), dissolved oxygen (DO), pH, water temperature (T), total nitrogen (TN), total phosphorus (TP), N:P ratio (N:P), ammonium nitrogen (NH_4^+-N), nitrate nitrogen (NO_3^--N), chemical oxygen demand (COD_{Mn}). Macroinvertebrate FFGs: predators (PR), omnivores (OM), gatherers/collectors (GC), filterers/collectors (FC), scrapers (SC) and shredders (SH)*

Discussion

Functional groups can respond to changes in living environment and have a certain impact on ecosystem functions (Jia and Du, 2014). The difference of abundance is the result of habitat filtering, that is, the rank character with higher abundance can be considered as the character with better adaptability to regional environment (Menezes et al., 2010). Ecosystem function is essentially dependent on the functional group of the

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species, and which has become a powerful and reliable method to study the dynamic change of community by functional characters (Hooper and Vitousek, 1997; Diaz et al., 2004; Jiang et al., 2008). The large difference in spatial pattern of functional groups is the response to environmental changes and the tradeoff between different functions.

Globally, changes in land use, especially loss of riparian forests, can lead to a reduction or change in the structure, function and diversity of macroinvertebrate in some river basin (Allan, 2004; Benstead and Pringle, 2004; Jinggut et al., 2012). Once the riparian zone lacks the shelter of riverside forest, the sun will direct to the water surface and cause the water temperature to rise. Because the water temperature is close to the heat-resistant limit in tropical areas, some species of macroinvertebrate adapted to cold water cannot survive (Irons et al., 1994; Boyero et al., 2012). Moreover, the decrease of leaf litter is the main food source of shredders, which will block the growth and development of group SH and make the aquatic ecosystem unbalanced, and ultimately affect the structure and function of the ecosystem (Liu et al., 2019). Serious soil erosion in Muling River Basin, soil and water loss in riparian zones causes large amounts of sediment to enter rivers. The surface of river sediment is covered by muddy soil, which affects the growth of algae (Jia et al., 2009; Jiang et al., 2018). At the same time, these sediments will also adhere to the surface of the body, trachea, and gill of the macroinvertebrate, which leading to the disappearance of macroinvertebrate (Magbanua et al., 2013).

In this study, we demonstrated the impacts of environmental variables on macroinvertebrate feeding functional groups. No significant differences in the FFGs were observed along season gradient, except group SC (*Table 1*). Mollusk (group SC) dominated at sampling sites of S25~S28 in the downstream of river, which close to floodplain wetlands along Ussuri River. Guan et al. (2017) sampled macroinvertebrate assemblages along Wusuli River (upstream, midstream, and downstream), and agreed with the emerging theory suggesting that aquatic invertebrate assemblages in floodplain wetlands should change longitudinally along a river's length and be affected by lateral connectivity of floodplain habitats with main river channels (Guan et al., 2017). Wu et al. (2017) found that snails could be possess several attributes that should make them useful as potential environmental indicators in Sanjiang Plain, and the certain snail species may provide a robust and rapid indicator of environmental impacts in freshwater in Heilongjiang Province of China (Wu et al., 2017). Next year, Guan et al. (2018) also confirmed that the snails (Mollusca: Gastropoda) can rapid assessments of wetland condition using aquatic invertebrates simple effective in northeastern China (Guan et al., 2018).

Macroinvertebrate community structure is usually determined by the physical structure and complexity of the habitat (Rennie and Jackson, 2005). Aquatic vascular plants play an important role in structuring macroinvertebrate species and selecting species related to functional groups dynamics and feeding habits (Valinoti et al., 2011; Gleason et al., 2018). The distribution of macroinvertebrate is also determined by vegetation type, especially the structure and growth form of aquatic vascular plants (Rennie and Jackson, 2005). Aquatic vascular plants affect the underwater climate and chemical properties by absorbing and releasing chemical substances (such as nutrients and antagonistic substances) (Valk and Arnold, 2010). However, as the growth of aquatic vascular plants in northern China is mainly affected by seasonal temperature changes, dominant communities can only be formed in summer and autumn (Liu et al., 2019).

In spring, the farmland near the Muling River basin contains a lot of nutrients (nitrogen and phosphorus) in the sediment of pesticide and chemical fertilizers. Chen et al. (2019)

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studies have shown that nitrogen can enter the water body through fish secretion and excretion (Chen et al., 2019). Nitrogen-containing nutrients in the water body are absorbed by algae growth and carried down together through surface runoff to provide sufficient nutrients for the growth of plankton. Meanwhile, greatly increased the number of plankton which as the source of food for macroinvertebrate, such as group SH positively correlated with TN (*Table 2*).

Moreover, iron, as an element affecting chlorophyll synthesis in plants, is also a trace element needed for phytoplankton growth (Zhang, 2015). Trace element copper is an indispensable metal element for the metabolism of microelements and plants in cell membranes, which can affect the growth of plankton (Zhang et al., 2014). Hydrology is considered the paramount environmental control of freshwater wetlands, with temporary drying being a major constraint on aquatic insects (Wu et al., 2019). The movement group (Liu et al., 2019) of macroinvertebrate could be considered as a new method for monitoring and evaluating water quality in Muling River basin for further studies in the future.

Conclusions

During the three times sampling in Muling River basin, we collected 13523 macroinvertebrate individuals belonging to 46 families 158 genera or species were identified from the study area, consisting of 61 gatherers/collectors, 42 predators, 22 scrapers, 14 shredders, 11 filterers/collectors and 8 omnivores. All FFGs, total abundance and Shannon-Wiener index were not significantly different. Total abundance of macroinvertebrate was higher in summer and biodiversity index was higher in autumn. We found that temperature was associated with all FFGs abundance and nutrients were the main influence factors in Muling River basin. Therefore, controlling the input of nutrients is the key to the ecological environment and aquatic biodiversity protection of the Muling River basin in the future.

Acknowledgements. This study was supported by The central government supports the reform and development fund projects of local colleges and universities “Research on integrated technology innovation of sustainable utilization of cold water fish resources industrialization” (2020GSP14), and Key research topics of economic and social development in Heilongjiang Province (20309). Liaoning science and technology major special plan "Liaoning important marine fish efficient green production mode research and development and demonstration" project (2020JH1/10200002), Dalian Science and Technology Innovation Fund Project (2019J12SN64), and Agricultural finance special project "Investigation of fishery resources and environment in key waters of Northeast China". The authors are grateful to the people that helped with all aspects of the fieldwork.

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DOI: http://dx.doi.org/10.15666/aeer/1905_39914006

APPENDIX

Appendix A. *Macroinvertebrate community structure and functional feeding groups during the sampling periods. Symbols: relative abundance + (<1%), ++ (1–1.9%), +++ (>2%)*

Family	Genera or species	FFGs	2015 May	2015 July	2015 Sep.
Corixidae	*Corixa substriata*	PR	++	++	+
	Hesperocoixa distani	PR		+	
	Hesperocorixa kirkaldy	PR	+		
	Sigra distanti	PR		+	
Tipulidae	*Hexatoma* sp.	PR	+		
	Nippotipula sp.	SH	+	++	+++
	Erioptera sp.	GC	+	+	+
	Pilaria sp.	PR		+	
Chironomidae	*Diplocladius* sp.	GC	++	++	+
	Heterotrissocladius sp.	GC		+	
	Eukiefferiella fuldensis	GC		+	
	Synorthocladius semivirens	GC		++	
	Orthocladius roussellae	GC		+	
	Orthocladius sp.	GC		+	+
	Orrhocladius thienemanni	GC		+	
	Orrhocladius vaillanti	GC		+++	
	Thienemannia gracilis	GC	+	+	++
	Chironomus kiiensisTokunaga	GC	+++	+++	++
	Chironomus flaviplumus	GC	+++	+	+
	Chironomus dorsalis	GC		++	
	Chaetocladius sp.	GC		++	
	Chironomus plumosus	OM	+	+	++
	Cryptochironomus maculipennis	PR	++	++	+
	Parachironomus arcnatus	PR	+	++	+
	Eukiefferiella fittkaui	GC	++	+++	++
	Eukiefferiella fuldensis	GC	+	++	+
	Eukiefferiella sp.	GC		++	
	Eukiefferiella gracei	GC		+	
	Parakiefferiella sp.	GC		+	+
	Dicrotendipes nigrocephalicus	GC	++	++	
	Dicrotendipes pelochloris	GC	++	+++	+
	Dicrotendipes tamaviridis	GC	++	+	
	Dicrotendipus lobifer	GC		++	
	Smittia sp.	GC	+++	++	+
	Pseudosmittia sp.1	GC	+		+
	Pseudosmittia sp.2	GC		+	++
	Polypedilum nubeculosum	SH	+	+	
	Polypedilum laetum	SH	+	++	
	Polypedilum sordens	SH	+	+	++
	Polypedilum scalaenum	SH		+	
	Polypedilum nubifer	SH			

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Family	Genera or species	FFGs	2015 May	2015 July	2015 Sep.
	Cricotopus vierriensis	SH			+++
	Cricotopus bicinctus	SH			+
	Zalutschia sp.	SH		+	
	Apsectrotanypus sp.	PR	+++	+	++
	Glyptotendipes pallens	FC		+	
	Glyptotendipes gripekoveni	FC		+	
	Glyptotendipes tokunagai	FC		+	
	Stictochironomus maculipennis	OM	+		+
	Stictochironomus akizukii	OM	+++	+	+
	Stictochironomus sp.A.	OM	+++	+	++
	Stictochironomus sp.B.	OM	+	+	+
	Stictochironomus caffrarius	OM			
	Paracladopelma undine	GC	+	+	+
	Paracladopelma nigritula	GC	+	+	+
	Chironomus anthracinus	GC	++	+	++
	Harnischia fuscimana	GC	++	+	
	Micropsectra chuzeprima	GC	+	+	++
	Tanypus sp.	PR		+	+++
	Tanypus villipennis	PR	+	+	
	Cladotanytarsus vanderwulpi	GC		+	
	Tanytarsus mendex	FC	+	+	++
	Tanytarsus chinyensis	FC	+++	+	+
	Tanytarsus signatus	FC	+		
Ephemeridae	*Ephemera shengmi*	GC	+	+	
	Ephemera nigroptera	GC	+		
Heptageniidae	*Heptagenia* sp.	SC	++	+	
Ephemerelliae	*Ephemerella nigra*	GC	+	+	
	Ephemerellidae serratella	GC		+	
	Ephemerella fusongensis	GC	+		
Baetidae	*Baetis* sp.	GC	+	+	
	Baetis thermicus	GC		+	
Leptophlebiidae	*Leptophlebia* sp.	GC		+	
	Paraleptophlebia sp.	GC		+	
	Thraulus sp.	GC		+	
Siphlonuridae	*Ameletus montanus*	GC		+	
Potamanthidae	*Potamanthidae* sp.	GC	+		
Chloroperlidae	*Alloperla sapporoensis*	PR	+	+	
	Alloperla nikkoensis	PR	+		
Pteronarcyidae	*Pteronarys* sp.	PR		+	
Pelidae	*Paragnetina* sp.	PR	++	+	
	Cyamia sp.	PR	+	+	
	Agnetina sp.	PR		+	
Perlodidae	*Hydroperla japonica*	PR			
Peltoperlidae	*Perlomyer* sp.	SH	+	+	
Taeniopterygidae	*Doddsia iaponica*	SH		+	
Hydropsychidae	*Hydropsyche* sp.	FC	+	+	+

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Family	Genera or species	FFGs	2015 May	2015 July	2015 Sep.
	Hydropsyche nakaharai	FC	+++		
Hydroptilldae	*Hydroptila* sp.	SC	+	+	+
Polycentropodidae	*Polycentropus* sp.	FC	+	++	++
Goeridae	*Goera ramosa*	SC	+	+	++
	Goera kyotonis	SC	+	+	
Stenopsychidae	*Parastenopsyche* sp.	GC		+	
Rhyacophilidae	*Rhyacophila* sp.	PR	+	+	
Limnephilidae	*Apatania* sp.	SC	+	+	+
	Neophylax sp.	SC		++	+++
	Stenophylax koizumii	SH		+	
	Glyphotaelius admorsus	SH	++		
	Stenophylax koizumii	SH	+		
Libellulidae	*Epiophcetia superstes*	PR	+	+	++
Petaluridae	*Tanypteryx pryeri*	PR		+	++
Macromiidae	*Macromidae* sp.	PR		+	
Libellulidae	*Hydrobasileus* sp.	PR	+	+	
Comphidae	*Davidius nanus*	PR		+	+
	Cercion sieboldii	PR		+	
	Gomphus postocularis	PR			+
	Ictinogomphus sp.	PR		+	
Gomphidae	*Anisogomphus* sp.	PR	+	+	
Lestidae	*Lestes* sp.	PR		+	
Agriidae	*Nenrobasis* sp.	PR		+	+++
	Calopteryx cornecia	PR			+
Dytiscidae	*Cybister japonicus*	PR	+	+	+
Noteridae	*Noterus* sp.	PR		+	
Carabidae	*Chlaenius* sp.	PR		+	++
Hydrophilidae	*Hydrophilus acuminatus*	PR	+	+	
Glossiphoniidae	*Helobdella nuda*	PR	+	+	+
	Batracobdella paludosa	PR	++		+
	Glossiphonia heteroclita	PR	++	+	+
	Parabdella quadrioculata	PR		+	+
	Glossiphonia complanata	PR			
	Glossiphonia lata	PR		+	++
	Whitmania sp.	PR		+	++
Tubificinae	*Limnodrilus hoffmeisteri*	GC	++	++	++
	Limnodrilus claparedeianus	GC	+	+	++
	Limnodrilus helveticus	GC	+	+	+
	Limnodrilus udekemianus	GC	++		
	Limnodrilus amblysetus	GC	+	+	+
	Aulodrilus bretscher	GC		+	+
	Aulodrilus pigueti	GC		+	+
	Branchiura sowerbyi	GC	++	+	+
	Tubifex tubifex	GC	++	+	+
	Spirosperma nikolskyi	GC		+	+++
Enchytraeidae	*Henlea* sp.	GC		+	

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DOI: http://dx.doi.org/10.15666/aeer/1905_39914006

Family	Genera or species	FFGs	2015 May	2015 July	2015 Sep.
Naididae	*Nais variabilis*	GC	++	+	+
	Nais communis	GC	+	+	+
	Nais simplex	GC	+	+	
	Slavina sp.	GC	+		+
	Dero sp.	GC	+		
Melaniidae	*Semisulcospira amurensis*	SC	+++	++	++
	Semisulcospira cancellata	SC	++	+	+
Viviparidae	*Bellamya purrificata*	SC	++	+	++
	Viviparus chui	SC	+	+	+
	Cipangopaludina Chinensis	SC	++	+	+
	Cipangopaludina ussuriensis	SC	+	+	+
Hydrobiidae	*Parafossarulus striatus*	SC	++	+	+
Lymnaeidae	*Lymnaea stagnalis*	SC	++	+	+
	Radix auricularia	SC	+	+	++
	Radix plicatula	SC	+	+	+
	Radix swinhoei	SC	++	+	+
	Radix ovata	SC	++	+	+
	Radix lagotis	SC	++	+	+
	Galba pervia	SC	+++	+	+
	Galba truncatula	SC	+	+	+
Planoribidae	*Polypylis hemisphaerula*	SC	++	+	+
Unionidae	*Unio douglasiae*	FC	+	+	+
	Lanceolaria grayana	FC	+	+	+
Palaemonidae	*Exopalaemon modestus*	OM	+	+	+
	Palaemon sinensis	OM	+	+	+

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_39914006

DYNAMIC CHANGE OF COMPOSITION AND FUNCTIONS OF FLORA ADAPTING TO RAPID URBANIZATION: A CASE STUDY OF HANGZHOU, CHINA

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(Received 7th May 2021; accepted 12th Aug 2021)

Abstract. Rapid urbanization has changed urban ecological environments and affected local biodiversity. An analysis of the existing literature examining changes in urban biodiversity may help with urban planning and biodiversity conservation. The present paper analysed the dynamic change of plant diversity in Hangzhou over the past four decades of rapid urbanization. It was found that although the number of plant species increased from 1,565 in 1981 to 1,799 in 2020, 101 plant species were lost. At the same time, 18 invasive species of plants and 52 ornamental plant species were acquired, with the largest increase observed in perennial herbs as a result of urban greening. A comparison of the respective Ellenberg's Indicator Values (EIV) of lost and acquired species reveals a larger increase in the number of species for sun plants than for shade plants. The latter did not increase with the rapid increase in urban green areas. A significant increase in the number of thermophilous plant species was observed, which is closely related to global warming and the heat island effect in cities. The number of aquatic plants also increased due to urban wetland landscaping. Prioritizing land conservation in urban planning is more conducive to producing and maintaining stable urban ecosystems and practicing sustainable urban development.
Keywords: *plant diversity, Ellenberg's Indicator Value, ecotype, acquired species, endangered plant, urban ecosystems, urban planning*

Introduction

Urban areas are the ecosystems that have been subjected to the highest degree of control by humankind (Grimm et al., 2000); its ecological processes are controlled by the complex interaction of both social-economic factors and natural environmental factors (Kaye et al., 2006), of which human designs and activities have substantially changed localized settings. This is particularly true in recent decades when rapid urbanization has led to significant changes in urban environments. Such indicators of change include the high concentrations of gaseous, granular and photochemical pollutants in the air (Zhang et al., 2012), impaired descent of water due to ground hardening (Vico et al., 2013), soil compaction (Bijoor et al., 2012), high concentrations of heavy metals in soil (Chen et al., 2013), habitat fragmentation (Angold et al., 2006), nitrogen deposition, the urban heat island effect, high concentrations of CO_2 (Lovett et al., 2000; Gregg et al., 2003; Loughner et al., 2012), and human interventions (Niinemets et al., 2008), amongst others.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_40074023

As a result, urban flora has evolved, with significant dynamic changes found in plant diversity (Pickett et al., 2001; Sukopp, 2002; McKinney, 2006; Kowarik, 2011).

Urbanization is a long process, during which urban plants may gradually develop characteristics that allow them to adapt to their environment. Therefore, an analysis of changes in urban plant diversity in different periods will inform our understanding of the relationship between urbanization and the change in floristic composition, and the driving forces behind the change in floristic composition. This study will be helpful for urban development and planning. There have been studies in Europe investigating urban changes in floristic composition over long periods of time (Landolt, 2000; Kent et al., 2001; Chocholouskova and Pyšek, 2003; Sal'nikov and Pilipenko, 2005; Lososová and Simonová, 2008). One study of plant changes in Brussels conducted over 50 years (Godefroid, 2001) found that although the number of plant species had not changed significantly, the number of alien species had increased significantly. Human activities have changed the distribution area of many plant species; for instance, some species growing on farmlands and woodlands or in swamps either decreased or disappeared altogether. One piece of research on plant changes in Frankfurt over a period of 200 years showed that the number of species is decreasing as urbanization increases. The most important driving forces behind species loss are the use of fertilizers and herbicides, the abandonment of woodlands and pastures, and the destruction of wetlands. Any plant species that have increased are typically introduced due to the ornamental function of gardens (Gregor et al., 2012). Although there have been studies on the plant change response to urbanization, not all such studies are comprehensive and the historical plant data collected in these studies are not complete for various reasons (Sudnik-Wojcikowska, 1987). Very few relevant studies can be found outside Europe as there is a lack of historical data available and only a small number of cities have historical records of their flora. This has led to difficulties in studying urban plant diversity over longer time periods. This is particularly so in China, which lacks systematic and complete data historical records on urban plants. China is currently in the midst of rapid urbanization, with its urban plant diversity having already changed in terms of composition and function. An in-depth understanding of the influence of rapid urbanization on urban plant diversity may have referential value for the future of urban planning and regional development.

Ellenberg indicator values (EIV) record the adaptation for all plants in Western Central Europe on a scale of 1-9 based on seven ecological factors: light, temperature, moisture, soil reaction, soil fertility, continentality, and salinity. These values are widely used to describe trends in the changing ecological adaptability of plants in the urban areas of Central Europe (Godefroid, 2001; Gregor et al., 2012; Domina et al., 2018; Salinitro et al., 2019). Based on the study of plant diversity changes in some cities in this region, it is found that urbanization will lead to the increase of shade-tolerant, thermophilic, and fertilizer-loving plant species. Such changes are closely related to the increase in green urban spaces, urban temperature, and nitrogen deposition. Pärtel et al. (1996) determined the habitat range of 14 plant communities in Estonia according to the Ellenberg indicator values (EIV) of vascular plants in the city. Besides Europe, no other region has complete Ellenberg indicator values (EIV), so it is not possible to compare different regions. In China, there is a lack of systematic research on the ecological adaptation of plants. Combined with the current research results, Ellenberg indicator values (EIV) of five ecological factors (including light, temperature, moisture, soil reaction and soil fertility), for more than 4000 plant species were recorded for more than 4000 plant species (Song

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_40074023

et al., 2013). The first two indicators are related to climate, whilst the last three are related to soil properties.

In the last four decades, the city of Hangzhou conducted two comprehensive surveys on urban plant diversity on two occasions (1981 and 2017) and compiled plant diversity inventories. After 2017, scholars went on to identify more plant species in the region and further generate relevant data. Due to these efforts, Hangzhou has become a perfect case for studying the relationship between rapid urbanization and plant diversity evolution. The present paper carries out an analysis of the changes in floristic composition over the past four decades in Hangzhou to understand: (a) the changes in plant diversity and composition in Hangzhou's rapid urbanization and their driving forces; (b) the changes in introduced plants and rare and endangered plants because of rapid urbanization, as well as the role urbanization plays in composition of urban plant diversity; (c) the changes in plant ecotypes and their ecological demand by using Ellenberg's Indicator Values (EIV). This analysis is expected to have reference value for future urban planning.

Materials and Methods

The study area

Hangzhou (118°20′-120°37′, 29°11′-30°34′), the capital city of Zhejiang Province, is in East China's Yangtze River Delta area. The city has witnessed rapid economic development and urbanization since 1980. It covers a total area of 16.85 thousand km^2, among which urban areas cover a total of 8.289 thousand km^2. Over the last 40 years, its population has soared, with its permanent resident population growing from 5.2073 million in 1981 to 10.36 million in 2020. The city is famous for its business and tourism, with its total GDP ranking 9th in China. The annual average temperature ranges from 15.8℃ to 18.5℃. The city receives an average annual rainfall of between 1100 and 1600 mm. The highest point in the city has an altitude of 1787 m. The forest coverage rate in the city totals 66.8%. During the city's rapid transformation, Hangzhou's zoning and areas have seen many changes. At present, there are 10 districts in Hangzhou, as Fuyang City and Lin'an City merged into Hangzhou as urban districts in 2015 and 2017, respectively. However, in the 1980s, the investigation of plant species in Hangzhou did not cover these two areas. In order to ensure a consistent research area, the plant data for Lin'an District and Fuyang District are not included in the present paper (*Fig. 1*).

Figure 1. *The study area and scope*

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DOI: http://dx.doi.org/10.15666/aeer/1905_40074023

Data sources and processing

Data sources

Data on the number of plant species are mainly derived from HBH (1982) and Yu et al. (2017). The numbers of newly identified plant species and their distribution in the region in the years 2017-2020 are also collected (Chen et al., 2019; Ding et al., 2019; Li et al., 2021) to form the dataset for 2020. In addition, the classification of several plant species is adjusted by referring to Flora of China (Wu et al., 2013) and the latest research achievements in plant classification (Xie et al., 2019; Chen et al., 2021; Chi et al., 2021). Hence, the plant inventories in the two periods are obtained.

Flora analysis

The floras are classified in different periods according to the areal–types of Chinese genera of seed plants (Wu, 1991) and pteridophyte (Zang, 1998). Invasive plant species are determined according to Ma (2013) and Yan et al. (2019). Meanwhile, any rare or endangered plants and national key preserved wild plants are determined by Fu (1992) and NFA (1999).

Growth form analysis

By referring to the plant growth form classification designed by Song et al. (2015), the growth forms of plant species are simplified into two levels. Level-I growth forms include Tree (T), Shrub (S), Herb (H), Vine (V) and Epiphyte (Ep). Level-II growth forms of Tree, Shrub and Vine are further divided into Evergreen Tree (ET) and Deciduous Tree (DT), Evergreen Shrub (ES) and Deciduous Shrub (DS), Evergreen Vine (EV) and Deciduous Vine (DV) according to whether they are deciduous or not, respectively. Herb is further divided into Annual Herb (AH) and Perennial Herb (PH) according to the life cycle.

Ecotype determination

According to the Ellenberg Indicator Value (Ellenberg et al., 1991) and Song et al. (2013), all of the ecological factors were classified as one of nine levels with numerical ranges also given. Due to the lack of systematic studies looking into the ecological value of plants in China, we selected five ecological factors including light, temperature, moisture, soil reaction and soil fertility to classify plants in terms of indicator values, which were recorded by Song et al. (2013) (*Table 1*). As EIVs for some plant species were not available in the literature, the indices were calculated by referencing known species growing in the same habitat.

Table 1. *Gradations of plant ecological indicator values (Song et al., 2013)*

Levels	1	2	3	4	5	6	7	8	9
Light	Full shadow	Between 1 and 3	Shadow	Between 3 and 5	Half shadow	Between 5 and 7	Half light	Light	Full light
Temperature	Frigid	Sub-frigid	Cool temperate	Mid-temperate	Warm-temperate	Sub-warm torrid	Warm-torrid	Sub-torrid	Torrid
Moisture	Super xerophyte	Between 1 and 3	Xerophyte	Between 3 and 5	Mesophyte	Between 5 and 7	Hygrophyte	Between 7 and 9	Hepophyte
Soil reaction	Extremely acidic soil	Between 1 and 3	Acidic soil	Between 3 and 5	Weakly Acidic soil	Between 5 and 7	Neutral soil	Between 7 and 9	Alkaline soil
Soil fertility	Extremely poor soil	Between 1 and 3	Poor soil	Between 3 and 5	Mid-rich soil	Between 5 and 7	Rich soil	Super-rich soil	Extremely rich soil

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Data calculation and analysis

The data were statistically analyzed through Excel 2010 software and difference significance analysis conducting using SPSS17.0 software (Wu et al., 2014).

Results

Changes in floristic composition

In 2020, 1799 species of plants were registered in the research area, belonging to 184 families and 845 genera. 234 new species belonging to 6 families and 106 genera were found, which is a significant increase compared to 1981 (*Fig. 2*). The most common species found were in Gramineae, followed by Compositae, Cyperaceae, Leguminosae, and Rosaceae, respectively. The top ten families in terms of amount did not change between 1981 and 2020; however, the number of species of all the families (except Gramineae) increased over this period, with the largest increases found in Compositae, Cyperaceae and Liliaceae (*Fig. 3*).

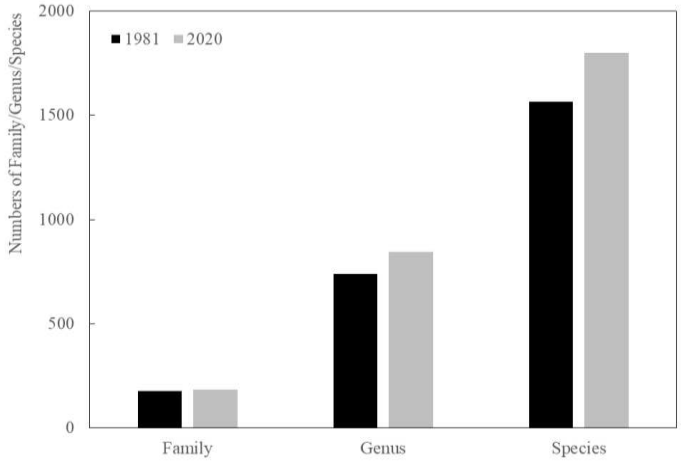

Figure 2. *The number of plant families, genera and species in 1981 and 2020*

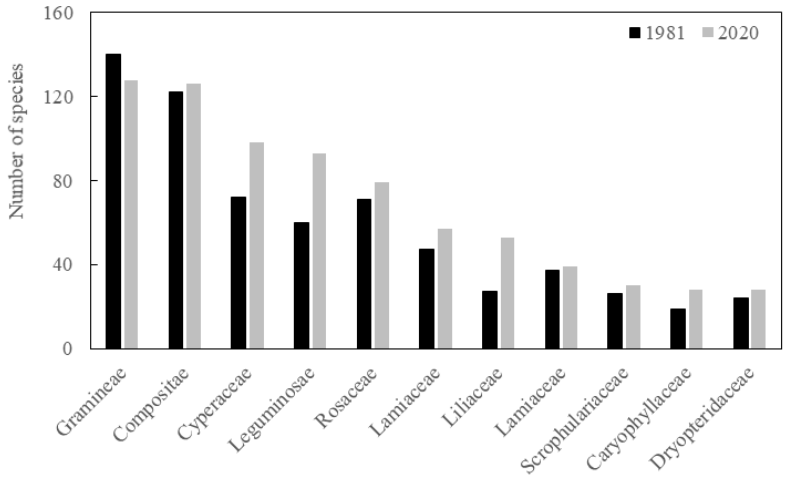

Figure 3. *The top ten families with the most species in 1981 and 2020*

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Although 101 species of plants were lost between 1981 and 2020, 438 new species were found. 1386 species of plants were recorded in both periods and whilst 11 genera of plants were lost between 1981 and 2020, 117 new genera were found. In total, 728 genera of plants were recorded in both periods (*Fig. 4*). Some species were introduced through urban greening, leading to enriched plant diversity in the city. Statistics show that 433 species of ornamental plants were registered in 2020, an increase of 52 from 1981 (*Table 2*).

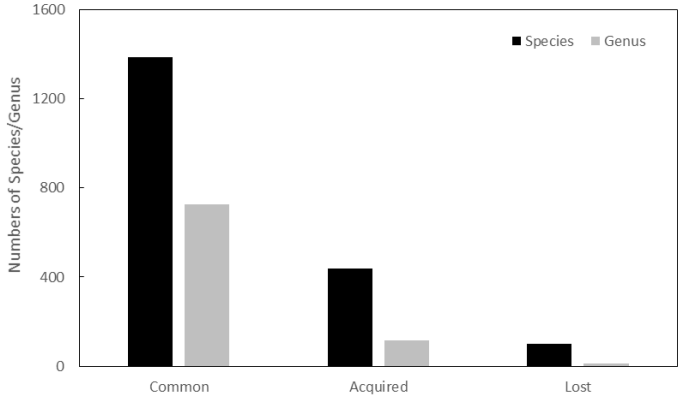

Figure 4. *The number of species and genera of common, acquired, and lost plants in 1981 and 2020*

Table 2. *Changes in plant growth forms between 1981 and 2020*

Growth form		All plants		Ornamental plants	
Level-I	**Level-II**	**1981**	**2020**	**1981**	**2020**
Tree (T)	Evergreen Tree (ET)	72	85	40	43
	Deciduous Tree (DT)	145	164	72	75
Shrub (S)	Evergreen Shrub (ES)	83	100	56	58
	Deciduous Shrub (DS)	131	153	45	50
Vine (V)	Evergreen Vine (EV)	27	38	7	9
	Deciduous Vine (DV)	105	128	7	8
Herb (H)	Perennial Herb (PH)	874	979	130	164
	Annual Herb (AH)	126	149	24	26
Epiphyte (E)		2	3	0	0
Total		1565	1799	381	433

Comparison of flora

A comparison of flora in 1981 and 2020 reveals that the 117 acquired genera of plants belong to 14 areal types. 20 genera were found to belong to the Pantropic type, which is the most common, followed by 14 genera belonging to the Old World Temperate type. The 11 lost plant genera belong to 7 areal types, including 2 belonging to the Pantropic type and 2 to the North Temperate type (*Fig. 5*).

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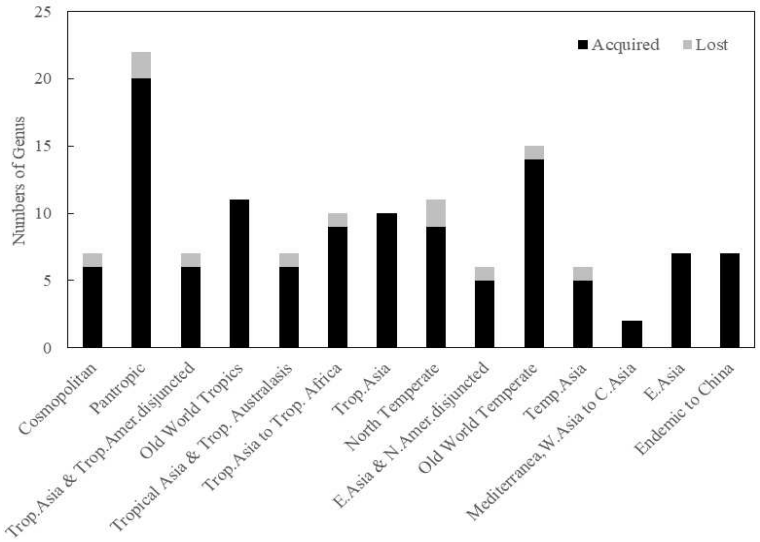

Figure 5. *The areal types of lost and acquired plant genera in 1981 and 2020*

70 invasive species were found in 2020, which is 18 more than in 1981. Most of the invasive species are from Tropical America, with 36 registered in 1981 and 47 in 2020, accounting for 61.1% of the total number of new invasive plant species. Only one invasive plant from Oceania was recorded (*Fig. 6*).

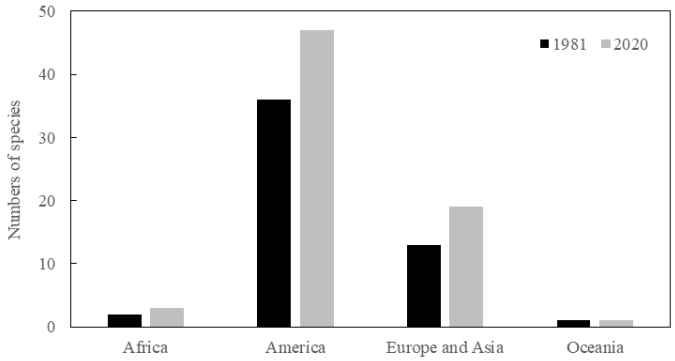

Figure 6. *The number and source of invasive plants in 1981 and 2020*

In 2020, the research area recorded a total of 20 rare or endangered plants and national key preserved wild plant species. Compared to 1981, 15 species were found in new distribution sites. However, the historical distribution sites of 15 species had disappeared. The number of existing distribution sites increased for 9 species, including 3 species that were newly found in the research area. The number of existing distribution sites decreased for 8 species, including *Pterygopleurum neurophyllum* which disappeared from the research area (*Table 3*).

Changes of growth form and ecotype

Perennial herbs have the largest number of species among all plants (979), followed by deciduous trees (164). Compared with 1981, the growth form of all plants in the

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research area saw an increase in 2020. The largest increase was found in perennial herbs, which had 105 new types. In 2020, the number of common ornamental plant species in the research area increased by 52 compared with 1981, including 34 perennial plants, which account for 65.38% of the total increase (*Table 2*).

Table 3. *The number of distribution sites of rare and endangered plants and national key preserved wild plants*

Species	Common English names	HDS	VS	NDS	EDS
Ardisia violacea	--	2	1	0	1
Brachystachyum densiflorum	--	1	1	1	1
Changium smyrnioides	Radix changii	5	0	4	9
Changnienia amoena	--	0	0	1	1
Celastrus orbiculatus	Oriental bittersweet	1	0	0	1
Ceratopteris thalictroides	Water fern	0	0	3	3
Emmenopterys henryi	--	1	1	1	1
Fagopyrum dibotrys	Wild buckwheat	5	1	8	12
Gelidocalamus marmorea	--	0	0	2	2
Glycine soja	Wild soybean	4	3	15	16
Isoetes sinensis	--	4	4	2	2
Machilus chekiangensis	--	2	1	0	1
Mosla hangchowensis	--	7	4	1	4
Ormosia henryi	--	6	4	1	3
Phoebe chekiangensis	--	4	1	3	6
Pteroceltis tatarinowii	Wingceltis	1	1	1	2
Pterygopleurum neurophyllum	--	1	1	0	0
Trapa incisa	Water chestnut	5	3	1	3
Yulania cylindrica	--	1	1	1	1
Zelkova schneideriana	Chinese Zelkova	4	2	0	2

HDS: historical distribution sites, the number of plant distribution sites recorded in historical documents; VS: vanishing sites, the number of plant distribution sites recorded in the historical documents for plants not found in the 2020 survey; NDS: new distribution sites, the number of newly discovered plant distribution sites in 2020; EDS: existing distribution sites, the total number of existing plant distribution sites

A comparison of the ecotype of acquired and lost species reveals that in terms of light demand, more lost species are found to be between Half shadow and Half light (Level 5-7), whilst most of the acquired species are found between Half light and Full light (Level 7-8), increasing by 106 and 109, respectively. Together they account for 49.1% of the total increase. A large increase of 160 species was also found between Shadow and Half shadow (Level 3-5), accounting for 36.5% of the total increase (*Fig. 7A*). Torrid plants have also seen a substantial increase. Plants between Warm-temperate and Sub-torrid (Level 5-8) account for 84.5% of the total increase. A similar trend is found for lost species. However, four Sub-frigid (Level 2) plants were lost (*Fig. 7B*). In terms of moisture, there was a reasonable increase in plant species belonging to Mesophyte (Level 5-6), accounting for 69.6% of the total increase. Xerophyte plants (Level 3-4) and Hepophyte plants (Level 9) also increased sharply (*Fig. 7C*). Plants with weakly acid soil (Level 5-6) experienced the largest increase, whilst plants with alkaline soil (Level 8) saw a mild increase. More lost plants were found to be in neutral soil (Level 7) (*Fig. 7D*). Plants with mid-rich soil (Level 5-6) have been acquired and lost the most, accounting for 74.3% and 63% of their respective totals. Plants with poor soil (Level 3-4) were also acquired mildly (*Fig. 7E*).

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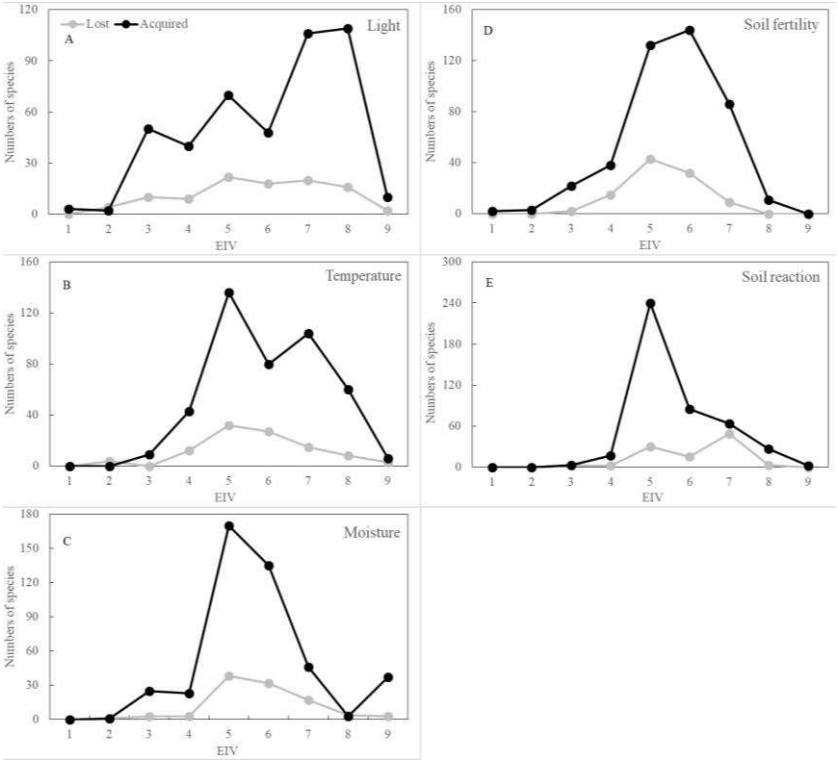

Figure 7. *EIV for acquired and lost plants in 1981 and 2020*

The acquired species prefer higher light, higher temperature and higher soil fertility and there were significant differences compared with those of the lost species. However, there was no significant difference in water demand. In response to the soil reaction, the acquired species prefer acidic soil (*Table 4*).

Table 4. *Comparison of ecological requirements of acquired and lost plants in 1981 and 2020*

Ecological factor	Lost	Acquired	Significance acquired/lost
Light	5.75±1.74	6.23±1.78	**
Temperature	5.69±1.43	5.98±1.35	*
Moisture	5.76±1.21	5.62±1.31	-
Soil reaction	6.17±1.07	5.40±0.83	**
Soil feritily	5.30±0.89	5.55±1.22	*

** Significance of t-test $p < 0.01$; * Significance of t-test $0.01 < p < 0.05$; – Significance of t-test $p > 0.05$

Discussion

Significance of complete historical plant data

Literature on flora can be of great help in analyzing changes in plant diversity and trends in biodiversity development (Knapp et al., 2010). There have been a number of studies conducted looking at the effects of long-term urban environmental changes on biodiversity, such as those studying periods of over 100 years (Fudali, 1995) or 200 years (Gregor et al., 2012). The study on urban biodiversity with the longest time span was conducted by Knapp et al. (2010), which studied urban plant functional characteristics in central Europe over nearly three centuries and analyzed the ecological characteristics of

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urban plants in different time periods. The study proved that plants could adapt and respond to urbanization and urban environments. Meanwhile, biodiversity development trends can differ across different time spans. For example, over a shorter span, urban plant diversity may improve (Grimm et al., 2008), which is similar to our findings; however, Godefroid's study of Brussels over nearly 50 years (Godefroid, 2001) revealed a trend of decreasing plant diversity. Whilst plant diversity gradually decreases over longer time spans (Tait et al., 2005), Salinitro et al.'s study (2019) arrived at an opposite finding, which may be due to the complexity of the factors affecting plant diversity evolution. Changes in the urban environment brought about by rapid urbanization such as ground hardening and increased concentrations of heavy metal in the soil can impair plant growth and development. Nevertheless, other changes such as enhanced urban green areas, improved habitat heterogeneity and targeted human interventions and can provide favorable conditions for plant growth. These factors influence the dynamic change and distribution pattern of urban plant diversity to different degrees in different stages of urban development. Moreover, it takes enormous amounts of time and energy to conduct comprehensive surveys on plant diversity data; as such, this arduous task cannot be properly accomplished in many cities (Niemelä, 1999; Godefroid, 2001). The data used in some studies are often incomplete (Sudnik-Wojcikowska, 1987), which can also lead to different findings. By using comprehensive data on plant species during the rapid urbanization phase of the research area, this study more clearly presents the dynamic changes in urban flora composition during the rapid urban development on a medium time scale.

Changes in plant diversity and influencing factors

Our study has revealed a significant change in the plant diversity of the research area over the past four decades. Urban biodiversity is greatly shaped by human activities, such that human decisions and behaviors impact urban biodiversity (Hope et al., 2003; Wu et al., 2011). Hangzhou has been experiencing very rapid urbanization since 1980, with significant changes taking place in its landuse patterns. For instance, the land used for construction increased from 6.85% in 1985 to 37.84% in 2017 (Yang et al., 2017). The resident population in the research area reached 10.36 million in 2020. The city also welcomed 208 million travelers in 2020 (HBS, 2021). All of these factors have had a substantial impact on local plant diversity.

Over the past four decades, 101 plant species have been lost and 438 plant species have been acquired in the region, with the latter including 52 species of ornamental plants. Many factors have contributed to the disappearance of plant species, such as agricultural intensification, nitrogen input, wetland degradation, construction activities and herbicide use. Among them, the leading factor appears to be changes in land use (Gregor et al., 2012), such as decreased arable land and forests and an increase in built-up areas. The loss of plant species is also related to the way that land is used. In the three decades between 1985-2017 alone, the area of urban arable land converted into construction land and the area of forest converted into construction land reached 1008.63 ha and 949.21 ha, respectively, leading to the loss of some species that originally inhabited the arable land or forest.

Another important factor contributing to enhanced urban plant diversity is that many foreign species are introduced as ornamental plants in urban greening (Thompson et al., 2003) at a rate and amount that far exceed the loss of native species (Sax and Gaines, 2003). It should be noted that this does not completely concur with our findings. In spite

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of the significant increase in plant species in the research area, the number of acquired ornamental plants only accounts for 11.9% of the acquired total. Apparently, the added plants are mostly newly found species distributed in this region, which is most likely a result of the massive efforts in large-scale plant resource investigation in the region in recent years. Although historical surveys are already relatively adequate, omissions are still inevitable. Therefore, it is necessary to conduct in depth supplementary surveys over the longer term. The same work has been carried out in many cities across China and fruitful results have been achieved (Ma, 2008; Ding and Jin, 2017).

Invasive plants have also seen a significant increase in the research area. The number of invasive plant species increased by 34.6% in 2020 compared to 2018, which has already impacted local ecosystems (Wang, 2008; Xie et al., 2012). Invasive plants prefer dry, alkaline and warm places that are rich in nutrients and have sufficient sunlight; such conditions typically result from human beings cutting down trees and piling up construction materials, as well as global warming (Godefroid, 2001). The fragmentation of urban landscapes has also increased the risk of invasive species invading (Thomas et al., 2001). Research has found that most invasive plants come from warm areas in tropical America (Sukopp, 1997) and higher temperatures in urban areas and fragmented ecologies provide favorable conditions for invasive plants. Urban greening and horticultural exchanges have introduced a large number of species. One typical example is *Solidago canadensis* (Wang, 2008), which was introduced to China as an ornamental plant and has since caused serious invasive effects. As such, it is essential to monitor how the introduced species behave in order to know at the earliest possible time when they may become a "harmful" one. This will would enable allow for the more effective management of urban biodiversity so as to reduce the potential risks of associated with invasive species (Mao et al., 2013).

The past four decades have witnessed a significant change in the distribution of rare or endangered plants and national key preserved plants. On the one hand, some distribution sites close to urban areas have disappeared because of urban construction, such as the distribution sites of *M. hangchowensis*. The reduction of water bodies and the pollution of water environments have also led to the loss of the distribution sites of some rare or endangered plants such as *I. sinensis* and *T. incisa*. In the Frankfurt/Mainz region of Germany, large-scale deforestation has also led to the loss of a huge number of species (Gregor et al., 2012). On the other hand, the renewed emphasis on biodiversity conservation has contributed to the discovery of some new distribution sites of rare or endangered plants. These new sites are now well protected and play a key role in local biodiversity conservation. This also provides the basis for the implementation of species conservation and plant resource management plans (Godefroid, 2001).

Ecological requirements of the urban flora

Socioeconomic factors influence the composition and structure of urban plants through urban management, the changing of urban infrastructure and hydrological conditions (Grimm et al., 2008). A comparison of EIVs shows that the flora in the research area have changed due to urban construction. The study of the changes of urban plants in Brussels also found that newly added plants preferred places with light (Chocholouskova and Pyšek, 2003). However, urban greening has also brought about more shade, leading to an increase in the number of shade plants, which is a similar finding to that of Salinitro et al. (2019). The present study found a larger number of sun plant species than shade-tolerant plants. This can be related to the decrease in forest and arable land and improvements in

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construction areas and open places, such as like roads and squares over the last 40 years. Among the newly added plant species, thermophiles accounted for the majority. Due to global warming, some foreign plants prefer urban areas with higher temperatures. Research has shown that the rapid urbanization in east China has led to a significant increase in urban temperatures. The temperature of the research area increased at a rate of 0.28-0.44℃ every ten years (Xie et al., 2007; Dai et al., 2011). Whilst the irrigation in urban green areas may disrupt the effect of natural rainfall on the distribution of plant ecotypes, the increase in the number of hydrophytes has barely any correlation with irrigation. Instead, the significant increase is related to changes in urban wetland landscapes because some aquatic ornamental plants have been introduced to urban water landscapes. The development of agriculturalization entails the heavy use of fertilizers, especially phosphorus-based fertilizers, which has led to the alkalization of some areas and created favorable conditions for the growth of alkaline plants. This finding is similar to the study of Sukopp, Blume, and Kunick (Sukopp et al., 1979) conducted in West Berlin. However, plants introduced for ornamental purposes tend to prefer acidic soil, resulting in a lower soil reaction value of acquired plants in this area. Plants that are tolerant of poor soil mainly appear in areas surrounding urban construction sites, the expansion of which also provides more survival space for such plants. Furthermore, urban nitrogen deposition will lead more fertilizer-loving plants to enter the urban habitat, thus increasing the soil fertility value of acquired plants.

Perennial herbs have seen a large increase in the number of species. This is especially so among ornamental plants which have increased by 65.38%. This is largely because Hangzhou, as a tourist destination, has introduced a large number of perennial ornamental herbs from home and abroad for urban greening and some plants better landscaping effects and adaptability to the local environment have been used for a long time.

Significance to urban planning

An analysis of the evolution of plant diversity not only provides information on the extent to which species numbers have changed but also offers possible reasons for such changes. Therefore, it forms the basis for landscape planning, and more importantly, decision making on species and biome conservation (Schaepe, 1990). There have increasingly been broader conservation efforts directed towards urban plant diversity of not only species, biota and ecology, but also landscapes, which has improved the living conditions of urban residents. Hence, green area planning and biodiversity conservation in urban planning essentially aim to improve the quality of urban environments. The improvement of urban environmental quality can broadly be achieved in three main ways: (a) expanding the total area of green spaces; (b) improving the quality of green spaces; (c) establishing an effective ecological network (Chocholouskova and Pyšek, 2003).

Changes in urban land use have reshaped current land patterns. Since the land used for urban construction has increased, urban planners now allocate part of this land for urban greening purposes to compensate for the reduction in arable land and forests. Studies have shown that the area set aside for urban greening is expanding at a rate larger than that of urban areas themselves (Fuller, 2009; Kabisch and Haase, 2013). As of 2020, the urban green area in Hangzhou accounts for 14.26% of the total built-up area (HBS, 2021). Urban green areas can provide more environments for plants to adapt thanks to their high habitat heterogeneity and operability in habitat construction and management. Some native plants that have disappeared can be artificially grown in urban green areas, so as to improve their plant diversity (Fan et al., 2016). However, the loss of native biodiversity

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_40074023

arising from the destruction of natural habitats can hardly be recovered by expanding urban green areas in the short term. Therefore, urban planning and land use should prioritize strengthening land conservation, including its species, biota, habitats and landscapes. Urban planners should not expect to compensate for the ecological losses due to land development through urban greening. The former is more beneficial to a stable urban ecological system and will more likely promote sustainable urban development. Cities or communities with higher levels of economic and social development will use more diverse and rare plant species to improve the quality of their green areas (Aronson et al., 2017), creating a "luxury effect". Some foreign plants are typically introduced to cities as ornamental plants. As Hangzhou welcomes more frequent trade and business exchanges with other cities, its urban biodiversity will accordingly be more susceptible to foreign species. Although the introduction of foreign species is inevitable, advocating the use of native plant species for ornamental purposes in urban planning should be highlighted.

Conclusions

In this study, Hangzhou city was taken as the research area. Based on the historical data of flora for the years 1981 and 2020, the dynamic changes in plant diversity in Hangzhou over the past four decades of rapid urbanization are analyzed, with the results revealing that:

1. An analysis of the change of urban biodiversity with existing literature may help with urban planning and biodiversity conservation. In this study, the plant diversity in Hangzhou city has changed in the past four decades due to rapid urbanization.

2. The loss of plant species in Hangzhou may relate to the way that land is used. The introduction of ornamental plants is not the main factor contributing to the increase in plant species and some of these have become invasive species in this area. As new distribution sites were found for rare and endangered plants, they are now well protected and play a key role in local biodiversity conservation.

3. A comparison of EIVs shows that the flora in the study area have changed due to urban construction. The number of species that are sun plants increased more than shade plants and there was a significant increase in the number thermophilous and hepophyte plant species.

4. Urban planning and land use should prioritize strengthening land conservation, including its species, biome, habitats and landscapes, which are more beneficial to a stable urban ecosystem and will more likely promote urban sustainable development. Although the introduction of foreign species is inevitable, advocating the use of native plant species for ornamental purposes in urban planning should be highlighted.

The species diversity of urban green space is of great significance for maintaining the ecological security and balance of urban system and improving the living environment. Urban green space with high plant diversity can improve ecosystem stability and provide sustainable ecosystem services for urban residents, such as providing heterogeneous habitats and alleviating urban heat island effect. However, there is little research connecting the urban plant diversity and its ecosystem services. The research on the relationship between the structure of urban plant diversity and ecosystem function is helpful to understand the impact of the spatial pattern of urban plant diversity on the stability of urban ecosystem and ecosystem service, which is an important direction of urban plant diversity study in the future. In addition, it should also pay attention to the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_40074023

ecological risks brought by the distribution of alien species and invasive species in cities and the potential health risks brought to urban residents, so as to reduce any harm to the sustainable development of the city and provide scientific suggestions for urban planning and construction.

Acknowledgements. This work was supported by Hangzhou Science and Technology Development Project (20201203B113).

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_40074023

COMPARISON OF EUROPEAN SARDINE (*SARDINA PILCHARDUS*, WALBAUM 1792) GREEK HAPLOTYPES WITH THOSE FOUND IN THE GLOBAL DISTRIBUTION OF THE SPECIES

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(Received 9th May 2021; accepted 12th Aug 2021)

Abstract. The identification of fish stocks is the first step in management and conservation processes. The European Commission has established among others the Protected Geographical Indication (PGI) protection label in order to identify an agricultural product, raw or processed, for which quality, reputation or other characteristics are linked to its geographical origin. In the present study, the Greek *Sardina pilchardus* haplotypes found with three mitochondrial segments (*COI*, *cytb*, *D-loop*), were compared with the ones previously submitted in the databases, to test the uniqueness of the discovered Greek haplotypes for a future PGI definition. For all the three mtDNA markers, the discovered Greek haplotypes were found to be common with species' haplotypes all over the world. These results reinforce the aspect that a single sardine individual cannot be classified as coming from a certain population (Mediterranean or Atlantic), as most of the data (present and former) support a single evolutionary unit for sardines.
Keywords: *product traceability, country of origin, phylogeography, Clupeidae, DNA barcoding*

Introduction

European sardine (*Sardina pilchardus*, Walbaum 1792) is a small pelagic clupeoid fish, inhabiting the Mediterranean Sea and part of the eastern Atlantic, from the North Sea to Senegal, with peripheral populations around the Azores, Madeira and the Canary Islands (Parrish et al., 1989). Adults usually swim close to the littoral zone, and display daily vertical movement capacity (Olivar et al., 2001). Spawning occurs in open waters and larvae remain in plankton for long periods of time (Olivar et al., 2001). Sardines show schooling and migratory behavior, as well as great dispersal capabilities both at the larval and adult stages. In the Mediterranean and Black Sea, landings between 2014 and 2016 are dominated by small pelagic fish (about 50%), mainly European anchovy and sardine (22% and 16%, respectively). Sardine (*S. pilchardus*) is the second main species landed (189,500 t on average between 2014 and 2016). In the same area, from the top twenty most important species by value, sardine is the fourth, accounting 9% of the total landing value (74,489,374 $) (FAO, 2018). In Greece, it is one of the two most

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_40254035

commercially important fish (after anchovy), with a total catch of 13,581.1 t in 2019, representing 16.6% of the total catch of marine species (Hellenic Statistical Authority, 2020).

The identification of fish stocks is the first step in management and biodiversity conservation processes (Waldman et al., 2005). Microsatellites are nuclear markers with higher mutation rates that have proved to be efficient and informative for detecting fine-scale population structure in marine pelagic fishes (Ruggeri et al., 2012, 2013). On the other hand, mitochondrial DNA is maternally inherited, lacks recombination, and shows relatively fast evolutionary rates, which make this molecular marker particularly suitable for inferring phylogeographic patterns (Avise, 2000). This marker is also appropriate for detecting historical vicariant or genetic bottleneck events, and has been very useful in describing present day phylogeography of taxa (Avise, 2000). In general, mtDNA genes show more population structure than nuclear genes do (Birky et al., 1989). This makes them particularly suitable indicators for the population genetic differentiation of marine organisms, which are generally high gene-flow species.

In 1992, according to regulation 2081/92, the European Union first adopted the system for the protection of geographical indications of agricultural products and foodstuffs, and according to the regulation 2082/92, the rules on the certificates of specific character for agricultural products and foodstuffs. In 2006, in order to improve the system, the above regulations were replaced by regulations (EC) 510/06 and (EC) 509/06, respectively, without changing their scope and feasibility. "Geographical indication" is a name which identifies a product, raw or processed: originating in a specific place, region or country, the quality, reputation or other characteristics of which are essentially attributable to its geographical origin and at least one of its production steps takes place in the defined geographical area. One hundred and twenty-five Greek products have been characterized as Protected Designation of Origin (PDO)/Protected Geographical Indication (PGI), while there is only one in the category "Fresh fish, molluscs, and crustaceans and products derived from them", Avgotaraho Messolonghiou (PDO - Council Regulations 1107/96 and 1263/96). Avgotaraho or botargo is the cured grey mullet roe. The mature roe pouches of the female grey mullet, *Mugil cephalus*, are processed as follow: removed from the fish, pressed, salted, sun-dried and dipped several times into bee's wax to preserve the product. It is produced mainly along the western coast of Greece; the most famous is produced outside Messolonghi.

In a previous study (Imsiridou et al., 2019), the PCR and further Sanger sequencing analysis of three mitochondrial segments (cytochrome oxidase subunit I - *COI*, cytochrome b - *cytb*, control region - D-*loop*) were applied for three Greek *S. pilchardus* populations, to assess the potential of using the population differentiation of the species as a tool for its future PGI definition. Three *S. pilchardus* populations were collected by professional fishermen from Amvrakikos Gulf (39° N, 20.8° W), the Ionian Sea (39.55° N, 20.1° W) (Western Greece) and Kalloni Bay in the Aegean Sea (39.15° N, 26.183333° W) (Northern Greece) during July 2017. In total, 641 bp at the 5′ end of the *COI* gene, 294 bp at the 5′ end of the *cytb* gene and 473 bp at the 5′ end of the *D-loop* region were sequenced for the majority of the individuals. For all the three segments, the statistical analysis revealed low levels of genetic structuring among populations. Additionally, most of the genetic variation was present within samples and the total Fst value was Fst=0.01232, which means that only 1.23% of genetic heterogeneity was apportioned among populations. Thirty-three haplotypes were detected for *COI* gene,

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DOI: http://dx.doi.org/10.15666/aeer/1905_40254035

twenty-five haplotypes were detected for *cytb* gene and fifty-three haplotypes were found for the *D-loop* region, in the three *S. pilchardus* populations. All haplotypes were deposited to GenBank under the accession numbers MH141137-MH141169 (*COI* haplotypes), MH127862-MH127879 (*cytb* haplotypes) and MH141170-MH141222 (*D-loop* haplotypes).

The different haplotypes inside a population represent the various nucleotide sequences its individuals have and consequently the uniqueness of its specimens. In the present study, the species' haplotypes found for each mitochondrial segment in the survey of Imsiridou et al. (2019), were compared with the ones previously submitted in the databases, to test the uniqueness of the discovered Greek haplotypes and consequently their geographic uniqueness, for further characterization of the Greek *S. pilchardus* populations as Protective Geographic Indication (PGI) products.

Materials and Methods

From the Nucleotide section of GenBank (http://www.ncbi.nlm.nih.gov/), all the registered *S. pilchardus* haplotypes for each mitochondrial segment (*COI, cytb, D-loop*) were retrieved. From the generated page of each haplotype, the relevant publication was recovered. Publications related to fish species identification, mislabeling of seafood samples and cooked products were excluded, because the actual *S. pilchardus* haplotypes should be included in the present study. Finally, the following haplotypes were chosen for each mitochondrial segment:

For COI gene: KJ768296, KJ768297 (Landi et al., 2014 - eighteen samples analyzed), KJ205156 - KJ205158 (Knebelsberger et al., 2014 - three samples analyzed), KC501213 - KC501232 (Keskin and Atar, 2013 - twenty samples analyzed), JQ775102 - JQ775107 (Costa et al., 2012 - six samples analyzed), JQ774896 - JQ774900 (Costa et al., 2012 - five samples analyzed), JQ774708 - JQ774712 (Costa et al., 2012 - five samples analyzed), KY176600 - KY176602 (unpublished), HQ340604 (Ardura et al., 2011), EF609451 (Ward and Holmes, 2007 - one sample analyzed), JQ623979 (unpublished), MG729573 - MG729589 (unpublished).

For cytb gene: JQ621900 (unpublished), JQ585750 - JQ585753 (unpublished), JQ237098 - JQ237114 (unpublished), AF291854 - AF291860 (Tinti et al., 2002 - three hundred and seven samples analyzed).

For the D-loop region: DQ139463 - DQ139723 (Atarhouch et al., 2006 - two hundred and sixty-one samples analyzed).

Then, the NCBI haplotypes from GenBank were converted to BioEdit v.7.0.5.3 files (Hall, 1999). Subsequently, the GenBank haplotypes were inserted, together with the discovered Greek haplotypes, into the same BioEdit files and were aligned with the CLUSTAL W algorithm (Thompson et al., 1997), as implemented in the software BioEdit v.7.0.5.3. As a result, three different BioEdit datasets were created, one for each segment. The three BioEdit datasets were processed with the DAMBE6 software package (Xia et al., 2017), which clusters the identical haplotypes. Then, from the "source" feature of GenBank, the locations of all the haplotypes were identified. Median networks for all the three groups of haplotypes of the total dataset were generated with the median joining algorithm (Bandelt et al., 1999), using the NETWORK 4.5.1.6 program.

The maximum likelihood approach was applied based on the Tamura-Nei model (Tamura and Nei, 1993), using MEGA7 software (Kumar et al., 2016), to depict the

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DOI: http://dx.doi.org/10.15666/aeer/1905_40254035

phylogenetic relationships among haplotypes. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 200.0000)). The rate variation model allowed for some sites to be evolutionarily invariable. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position.

Results and Discussion

For COI gene: The thirty-three Greek haplotypes for the *COI* gene were aligned with sixty-three *COI* haplotypes from GenBank, with the CLUSTAL W algorithm, and visually confirmed. The total dataset included ninety-six sequences and was analyzed with the DAMBE6 software package, in order to detect the common haplotypes. Eight Greek haplotypes (H1, H4, H7, H25, H27, H30, H31, H32) were found to be identical with haplotypes from different Mediterranean and Atlantic Ocean areas (Portugal, North Sea) (*Table 1, Figure 1, Figure 2*). *Figure 3A* depicts the phylogenetic relationships among *COI* haplotypes (data from *Table 1*).

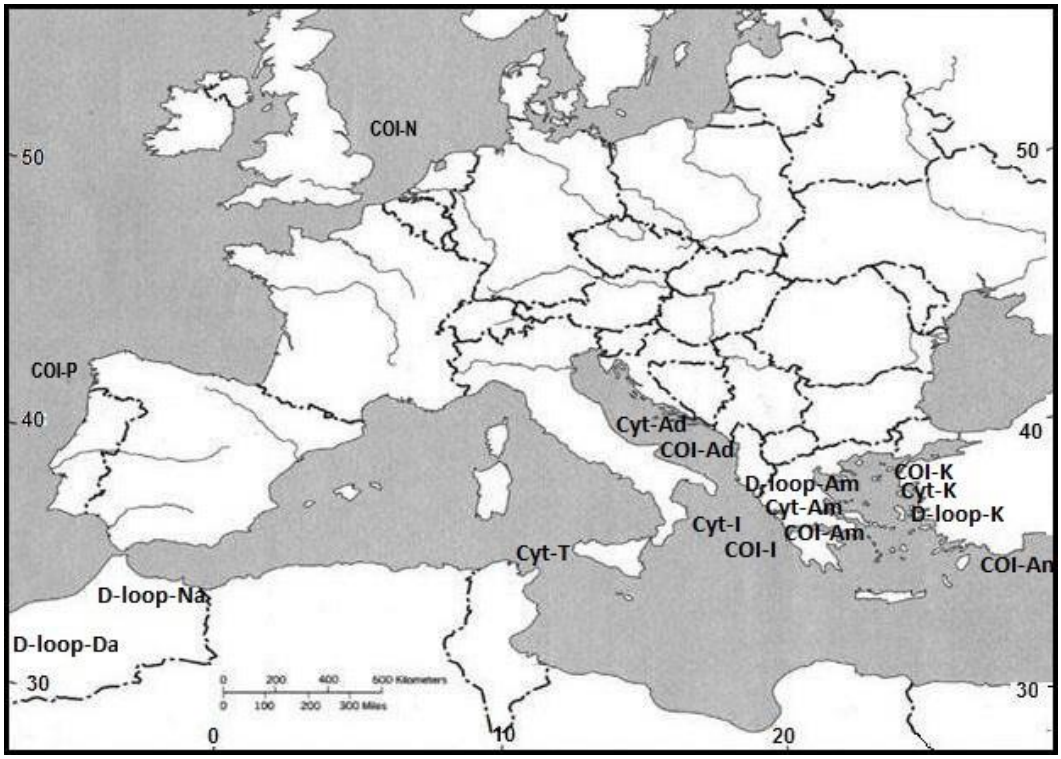

Figure 1. *Map showing the global distribution of the Greek haplotypes. COI-N: North Sea - Atlantic Ocean = H7; COI-P: Portugal - Atlantic Ocean = H1, H25, H30; COI-Ad: Croatia - Adriatic Sea = H1, H4, H25, H27, H31, H32; COI-An: Turkey, Antalya-Mediterranean Sea = H1; COI-Am: Amvrakikos Gulf, Greece = H1, H7; COI-K: Kalloni Bay, Greece = H1, H4, H25, H27, H30, H31, H32; COI-I: Ionian Sea, Greece = H1, H4; Cyt-T: Tunisian coast - Mediterranean Sea = H1, H10; Cyt-Ad: Adriatic Sea = H1, H6, H10; Cyt-Am: Amvrakikos Gulf, Greece = H1, H6; Cyt-K: Kalloni Bay, Greece = H1, H10; Cyt-I: Ionian Sea, Greece = H1; D-loop-Da: Dakhla-Atlantic coast of Morocco = H2; D-loop-Na: Nador-Mediterranean coast of Morocco = H2, H15; D-loop Am: Amvrakikos Gulf, Greece =H2, H15; D-loop-K: Kalloni Bay, Greece = H2*

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DOI: http://dx.doi.org/10.15666/aeer/1905_40254035

Table 1. *Distribution of Greek and GenBank haplotypes per gene, for species Sardina pilchardus*

COI gene								
Greek haplotypes	**H1**	**H4**	**H7**	**H25**	**H27**	**H30**	**H31**	**H32**
Location identified	Amvrakikos Gulf, Ionian Sea, Kalloni Bay	Ionian Sea, Kalloni Bay	Amvrakikos Gulf	Kalloni Bay	Kalloni Bay	Kalloni Bay	Kalloni Bay	Kalloni Bay
Common GenBank haplotypes	KJ768296 JQ775102 JQ775103 JQ775105 JQ775106 JQ774900 JQ774708 JQ774711 JQ774712 EF609451/ KY176602/ MG729576 MG729578 MG729584	MG729573	KJ205156	JQ775107/ MG729575	MG729580	JQ775104	MG729574	MG729579
Location identified	Portugal-Atlantic Ocean/ Turkey, Antalya-Mediterranean Sea/ Croatia-Adriatic Sea	Croatia, Adriatic Sea	North Sea - Atlantic Ocean	Portugal-Atlantic Ocean/ Croatia-Adriatic Sea	Croatia-Adriatic Sea	Portugal-Atlantic Ocean	Croatia-Adriatic Sea	Croatia-Adriatic Sea
Cyt b gene								
Greek haplotypes	**H1**	**H6**	**H10**					
Location identified	Amvrakikos Gulf, Ionian Sea, Kalloni Bay	Amvrakikos Gulf	Kalloni Bay					
Common GenBank haplotypes	AF291854/ JQ237101 JQ585750	AF291856	AF291855/ JQ585751					
Location identified	Adriatic Sea/ Tunisian coast-Mediterranean Sea	Adriatic Sea	Adriatic Sea/ Tunisian coast-Mediterranean Sea					
D-loop								
Greek haplotypes	**H2**	**H15**						
Location identified	Amvrakikos Gulf, Kalloni Bay	Amvrakikos Gulf						
Common GenBank haplotypes	DQ139479, DQ139589	DQ139597						
Location identified	Dakhla-Atlantic coast of Morocco/ Nador-Morocco	Nador-Mediterranean coast of Morocco						

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DOI: http://dx.doi.org/10.15666/aeer/1905_40254035

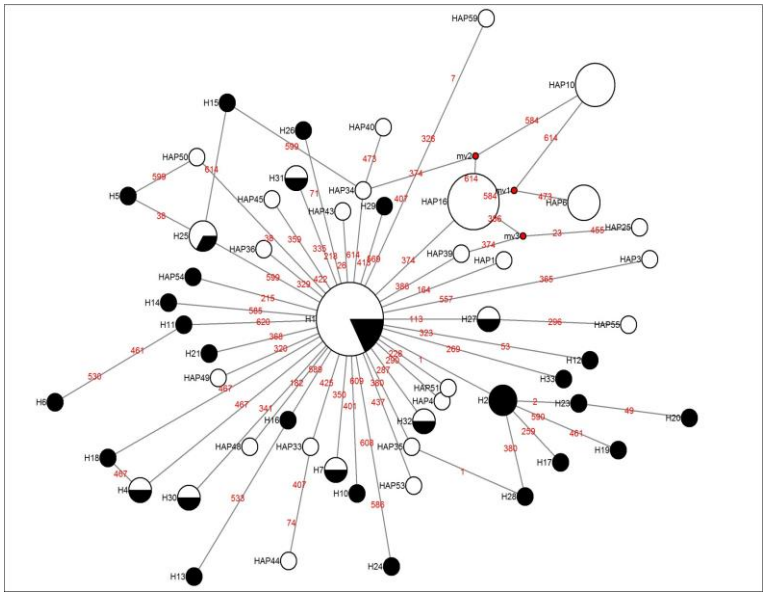

Figure 2. *Median-joining network of COI haplotypes of the total dataset (Greek haplotypes-black area, GenBank haplotypes-white area). The area of each circle is proportional to the number of individuals exhibiting that haplotype. Each line in the network represents one mutational step.*

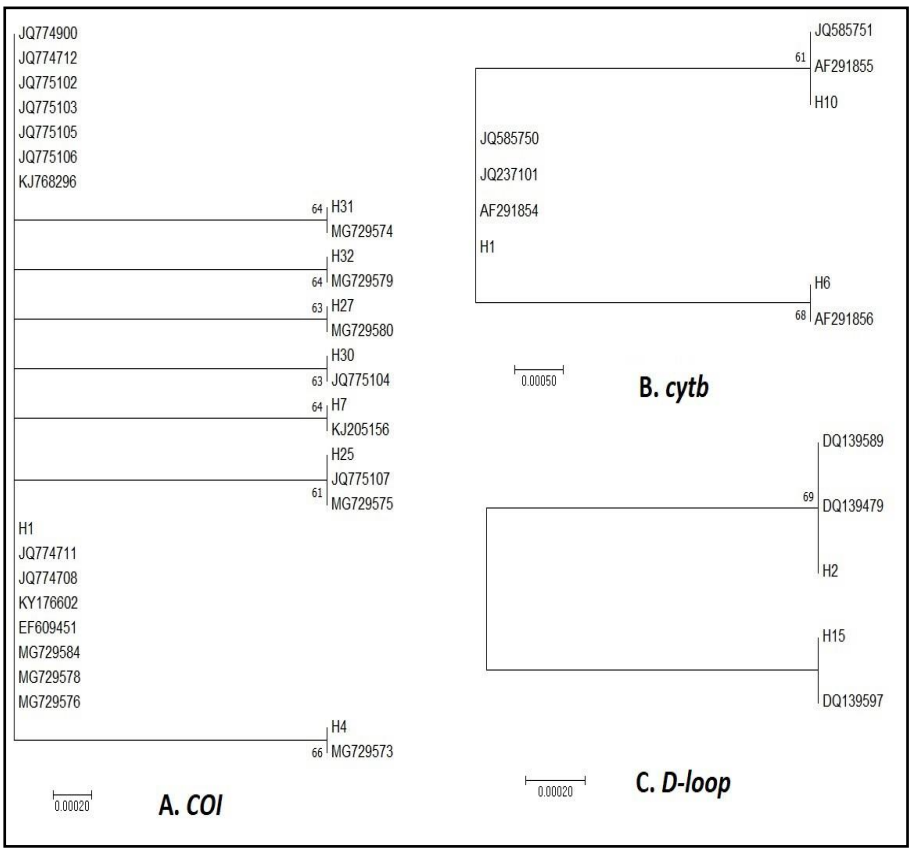

Figure 3. *Maximum likelihood trees, one per each mtDNA part (COI, cytb, D-loop), illustrating the genetic relationships among haplotypes. The model used was the Tamura-Nei model. Numbers above branches indicate bootstrap values among 1,000 replicates.*

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DOI: http://dx.doi.org/10.15666/aeer/1905_40254035

These findings are in agreement with previous microsatellite data (Gonzalez and Zardoya, 2007; Kasapidis et al., 2012) which do not support the existence of the two *S. pilchardus* subspecies. The degree of genetic differentiation between Atlantic and Mediterranean populations has been the subject of study for many marine organisms. In many cases, the Mediterranean and the adjacent Atlantic coasts support very similar faunas (Borsa et al., 1997; Bargelloni et al., 2003). For a proportion of species presently occurring in both the Atlantic and the Mediterranean, the Strait of Gibraltar represents a corridor sufficient for gene flow; thus, an ongoing exchange of individuals between the two seas is taking place (see reviews in Borsa et al., 1997). The fact that the Gibraltar Strait and the Almeria-Oran front may or not act as barrier to gene flow for different marine pelagic species, has been attributed to differences in life-history traits (e.g. dispersal capacity), and to the existence of distinct past demographical events (e.g. bottlenecks) (Gonzalez and Zardoya, 2007).

For cytb gene: The twenty-five *cytb* Greek haplotypes were compared with twenty-seven *cytb* haplotypes from GenBank, with the CLUSTAL W algorithm. The total dataset included fifty-two sequences. Three Greek haplotypes (H1, H6, H10) were found to be common with haplotypes from different regions of the Mediterranean Sea (*Table 1, Figure 1, Figure 4*). The relationships among *cytb* haplotypes are shown in *Figure 3B* (data from *Table 1*).

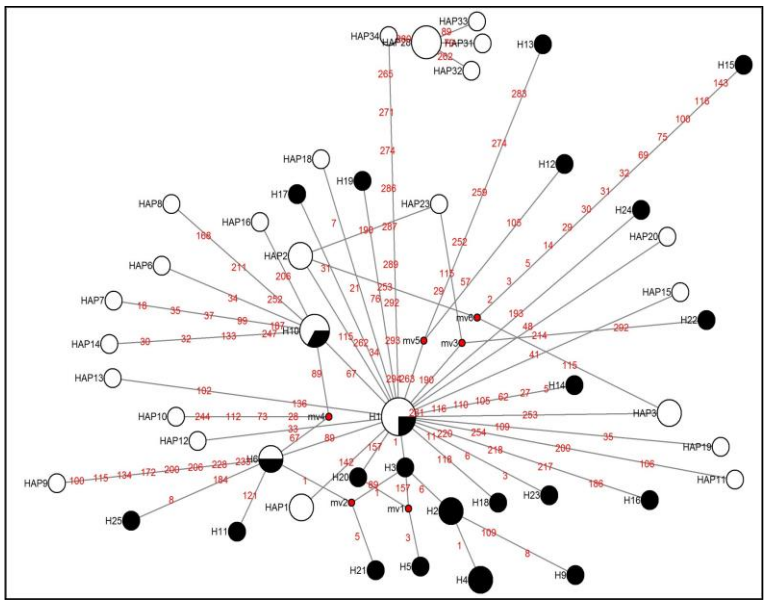

Figure 4. *Median-joining network of cytb haplotypes of the total dataset (Greek haplotypes-black area, GenBank haplotypes-white area). The area of each circle is proportional to the number of individuals exhibiting that haplotype. Each line in the network represents one mutational step*

A previous survey (Parrish et al., 1989) based on phenotypic variation mainly in gill raker counts and head length, has proposed the existence of two subspecies, that show a different distribution pattern with respect to the Atlantic Ocean-Mediterranean Sea transition: *Sardina pilchardus pilchardus* in the Eastern Atlantic from the North Sea to Southern Portugal, and *S. p. sardina* in the Mediterranean Sea and the Northwest African coast. In the present study, the Greek *cytb* haplotypes were found to be common

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DOI: http://dx.doi.org/10.15666/aeer/1905_40254035

with species haplotypes from the Mediterranean Sea (Adriatic Sea) and Northwest African coast (Tunisian coast). This result supports the existence of *S. p. sardina* subspecies in the Mediterranean Sea, as proposed, based on meristic (Parrish et al., 1989) and mtDNA studies (Atarhouch et al., 2006). Some mitochondrial data might be reflecting past isolation of sardine populations into two distinct groupings during Pleistocene (Atarhouch et al., 2006), whereas microsatellite data reveal the existence of present day gene flow among populations (Gonzalez and Zardoya, 2007; Kasapidis et al., 2012).

Moreover, the Greek Ionian Sea haplotypes were revealed to be common with GenBank Adriatic Sea haplotypes. This finding is consistent with a previous microsatellite analysis of European sardine from Adriatic and Ionian seas (Ruggeri et al., 2013), which indicated a lack of genetic differentiation between the two basins.

For the D-loop region: The fifty-three Greek haplotypes for the *D-loop* region were aligned with two hundred and sixty-one *D-loop* haplotypes from Genbank, with the CLUSTAL W algorithm. The total dataset included three hundred and fourteen sequences. Two Greek haplotypes (H2, H15) were found to be common with haplotypes from the Atlantic and the Mediterranean coast of Morocco (*Table 1, Figure 1, Figure 3C*). Median network for the control region haplotypes were generated with the median joining algorithm using the NETWORK 4.5.1.6 program (results not shown).

In the present survey, the Greek *COI* haplotypes were found to be common with haplotypes from different Mediterranean and Atlantic Ocean regions. Furthermore, the Greek *D-loop* haplotypes were revealed to be identical with Atlantic and Mediterranean sardine haplotypes. Previous studies also failed to find a barrier to gene flow for sardines in the Atlantic Ocean and the Mediterranean Sea, and to detect significant geographical structuring between sardine population samples in the two basins (Atarhouch et al., 2006; Gonzalez and Zardoya, 2007; Laurent et al., 2007; Chlaida et al., 2009; Baibai et al., 2012). Consequently, for sardine populations the Gibraltar Strait does not present a phylogeographic boundary, as there is no sign of Atlantic-Mediterranean division.

The study of population genetic variation of marine pelagic fish species has proven to be particularly challenging, because of the biological peculiarities of these fishes, including large effective population sizes and high dispersal capacities, as well as because of the apparent lack of physical barriers to gene flow in the marine realm (Avise, 1998; Graves, 1998; Hauser et al., 1998). In the marine environment, natural barriers are often absent and the seawater constitutes a potential means for dispersal, favoring intermixing of individuals over the species range. This phenomenon could commonly lead to high levels of current gene flow as a result of the species high dispersal abilities, both at larval and adult stages (Arnason and Palsson, 1996; Vis et al., 1997). Only a few migrants per generation are often sufficient to prevent detectable genetic differentiation between conspecific stocks (Hartl and Clark, 1997). For the three mtDNA markers used in the previous study (Imsiridou et al., 2019), the discovered Greek haplotypes were found to be common with species' haplotypes all over the world. This is evidence of the high gene flow among *S. pilchardus* populations and suggests high panmixia among them. All of these results reinforce the aspect that a single sardine individual cannot be classified as coming from a certain population (Mediterranean or Atlantic), as most of the data (present and former) support a single evolutionary unit for sardines.

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DOI: http://dx.doi.org/10.15666/aeer/1905_40254035

Conclusions

For the three mtDNA markers (*COI, cytb, D-loop*) used from a previous survey, the discovered Greek haplotypes were found to be common with species' haplotypes all over the world. This is evidence of the high gene flow among *S. pilchardus* populations. There have been many studies addressing the genetic structure of sardine in different parts of its global distribution, using different molecular markers. The results of these studies suggest low levels of genetic differentiation. Present and former results reinforce the aspect that a single sardine individual cannot be classified as coming from a certain population (Mediterranean or Atlantic), as most of the data support a single evolutionary unit for sardines. Conclusively, from the data so far, the Greek *S. pilchardus* populations could not be identified as Protective Geographic Indication products, provided that the Greek haplotypes are spread throughout the global distribution of the species. Nevertheless, these findings need to be enriched by more mtDNA or microsatellite data of additional sampling sites in the Ionian and Aegean basins, and further comparison of the new haplotypes with the existing ones, in order to strengthen or definitively reject the idea of characterizing the Greek sardine as a PGI product.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_40254035

EFFECTS OF VARIOUS WATER LEVELS AND LATERAL SPACING ON THE YIELD AND QUALITY OF DRIP-IRRIGATED COTTON (*GOSSYPIUM HIRSUTUM* L.) UNDER ARID CONDITIONS

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(Received 12th May 2021; accepted 12th Aug 2021)

Abstract. A field experiment involving cotton (*Gossypium hirsutum* L.) was conducted for two years to investigate the effects of deficit irrigation on seed cotton yield, fiber quality, and water productivity in Turkey. The main plots featured various drip line spacing, and the sub-plots included four irrigation levels based on class A pan evaporation in Turkey. Crop evapotranspirations were found to range from 585 to 1028 mm and from 601 to 1074 mm in consecutive years. Seed cotton yields ranged from 239.0 to 616.3 kg ha^{-1}, and the analysis of variance showed the presence of the statistically significant ($p \leq 0.01$) effects of irrigation levels on crop yield. The drip line spacing was effective for the yield only in the second year ($p \leq 0.05$). Employed limited irrigation affected ginning outturn significantly ($p \leq 0.01$) in both years. The results indicated that reduction in irrigation water did not have a significant effect on most fiber quality criteria. On the contrary, significant differences were observed for the leaf area index (LAI) traits of the treatments, and these reached the highest value approximately 100-110 days after planting. However, water productivity (WP) and irrigation water productivity (IWP) did not show significant differences among the limitations of irrigation water levels.
Keywords: *cotton, irrigation levels, water productivity, leaf area index, drip irrigation*

Introduction

The increasing urban and industrial demands for water, as well as the pollution of water resources have resulted in a decrease in the amount of water allocated to agriculture. In addition, global climate change, water constraint and drought cause serious problems for crop production all over the world (Katerji et al., 2008). Today, about 40% of the world's population suffers from water shortages (Steduto et al., 2012), but only 25% of agricultural land can be irrigated due to a lack of water resources. Turkey is one country that will face severe water shortages in the future.

Agricultural lands in Turkey are located in the arid and semi-arid climatic zones, so it is not possible to cultivate most summer crops without irrigation. Southeastern Anatolia (SEA) of Turkey is very hot and dry during the summer. Therefore, irrigation cannot be used for crop production. However, this region, where this study was carried out, is one of the most important agricultural zones in Turkey. Specifically, Harran Plain is the most important cotton-growing area, with cotton making up 85% of all plant patterns.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_40374053

Cotton is the main crop grown in arid regions of SEA under irrigation. The region is suitable for cotton cultivation due to its favorable climatic condition. In addition, cotton's economic return is higher than those of any other crops the region. Cotton seed can also be used for livestock feed and for edible cotton oil extraction in this area. All in all, cotton cultivation carries significantly higher return and an important crop in semi-arid regions, where it represents a significant source of income for both large- and small-scale farms (Papastylianou and Argyrokastritis, 2014). Mechanization is common in cotton agriculture, but a significant amount of manual labor is still employed. Along with the yield of cotton, its quality is of great importance. Genotype, weather conditions, farmer practices, fertilization, irrigation management, environment, and the interaction of genotypes by environments all influence cotton fiber quality parameters (Campbell and Jones, 2005; Bilalis et al., 2010).

The high irrigation water requirement of cotton (Cetin and Bilgel, 2002; Chapagain et al., 2006) depending on the climate conditions have resulted in considerably important irrigation and environmental problems in this region. Furrow irrigation is generally practiced in the plain; however, irrigation efficiency is very low due to a lack of land leveling coupled with farmers' habits. In addition, the charging of irrigation water is based on the irrigated area rather than on the amount of water used. This is another important reason for the lower water productivity in SEA. The lower level of water productivity in SEA has resulted in significant problems with the sharing and distribution of irrigation water on the plain. In addition, the overirrigation of cotton leads to excessive vegetative growth and causes the leaching of plant nutrients from the root zone. Furthermore, increased costs of chemical fertilizer and contamination of groundwater supplies (DeTar, 2008) are other major problems. In addition, there is irrigation water shortage in the downstream part of the plain and cotton yield decreases significantly due to insufficient irrigation.

As mentioned earlier, as agricultural producers face insufficient water supplies, they need to conserve and sustainably use inadequate water resources (Cai et al., 2002). The decrease in water supply for cotton growth has caused researchers to focus increase in water productivity (Dagdelen et al., 2009a). The use of modern irrigation systems is one key solution to increase water use efficiency and/or water productivity. Irrigation modernization requires the identification of the most appropriate solutions to increase yield and income, as well as increasing water productivity and water saving (Darouich et al., 2014; Cetin and Kara, 2019). For example, it is important to use modern irrigation technologies, such as drip irrigation (Cai et al., 2002). Many studies in different regions of the world have showed that drip irrigation has provided many advantages compared to surface irrigation for cotton (Cetin and Bilgel, 2002; Ibragimov et al., 2007; Kang et al., 2012; Fan et al., 2018). With this method, a low amount of irrigation water is delivered through drip irrigation, and it results in both a higher yield and water efficiency (Kang et al., 2012). Drip irrigation has been recommended as a means of supplying most types of crops with uniform and frequent applications of water. These applications are adaptable for different topographic and soil conditions (Cetin and Bilgel, 2002). With drip irrigation systems, it is possible to achieve high water application efficiency and to reduce evaporation losses in arid regions, such as the SEA region, where the relative humidity is low. However, when transitioning from surface irrigation methods to drip irrigation, the farmers were initially reluctant. One of the main reason obstacles to the common adoption of drip irrigation is its relatively higher costs of investment and operation. The cost of the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_40374053

drip irrigation system increase as long as increasing the system's uniformity (Wilde et al., 2009). However, at present, the decreased water allocation and the increased energy and labor costs have resulted in the rapid employment of drip irrigation systems.

In many parts of the world, drip irrigation has been used for long time and it is practiced increasing the crop yield and reducing soil-surface evaporation as well as increasing irrigation water use efficiency (WUE) (Sampathkumar et al., 2013). Studies have reported that drip irrigation provides a high yield, quality, and water productivity level in cotton while saving irrigation water. However, the values obtained differed according to the climatic conditions and agricultural techniques applied (Ibragimov et al., 2007; Rao et al., 2016; Zhang et al., 2016).

Due to insufficient water resources, limited water is sometimes applied in irrigation. However, the reduction of irrigation water reduces dry matter accumulation, the boll weight, boll production, the leaf area, the number of nodes and of cotton (Gerik et al., 1996; Pettigrew, 2004; Mert, 2005; Kang et al., 2012; Chen et al., 2020). Leaves are associated with evapotranspiration (ET) and photosynthesis, and the measurement of leaf growth is an application used in most agronomic and physiological studies, as well as plant growth studies (Guo and Sun, 2001). The leaf area index varies depending on the number of leaves and the leaf size of the plant, with stress and nutrient deficiency negatively affecting it (Longnecker, 1994). Deficite irrigation can be applied in various ways. These are: (a) not irrigating during periods when the plant is not sensitive to water, (b) extending the irrigation interval, (c) excluding areas with low yield potential, or (d) reducing the amount of water by certain amounts each time.

In this study, the drip irrigation method was applied to cotton plants grown in arid climatic conditions for the growing season applying different irrigation levels. The aims of the study were (1) to assess the effects of various irrigation water levels and drip line spacing on the seed cotton yield, and (2) to establish leaf area index, the lint quality and water productivity.

Materials and methods

Experimental site and climate

This study was carried out in the growing seasons of 2014 and 2015 in the experimental fields of Harran University (37°07' 38°48' and 467 m above sea level) of Sanliurfa, Turkey.

According to the Aydeniz climate classification, the region is included in the arid climate classification (Bölük, 2016). Summers are dry (very low relative humidity, 10-15%) and very hot and, and winters are relatively cold and wet. The long-term annual average rainfall in the area is about 360 mm, and the open-water surface evaporation is 1850 mm. Winter precipitation continues until early spring, however, the distributions differ significantly from year to year. June, July, August, and September are the hottest and driest months, whereas January and February are the coldest months. In July and August, the daily maximum temperatures often exceed 40 ° whereas the minimum temperatures seldom fall below 0 °C in the winter months.

The experimental soil is clay (USS 1954), and its infiltration rate is 9 mm h^{-1}. It is characterized as being salt free and slightly alkaline. Some analysis results of of the soil profile are given in *Table 1*. The lime is about 8.80%. In addition, the available water holding capacity of the 0- to 90-cm soil profile is 182 mm.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_40374053

Table 1. *Some physical and chemical properties of the soil in field trials*

Soil layers (cm)	Texture	Field capacity (g g^{-1})	Wilting point (g g^{-1})	Bulk density (g cm^{-3})	EC (dS m^{-1})	pH	Lime (%)
0-30	C	0.357	0.220	1.36	0.671	7.3	8.51
30-60	C	0.363	0.211	1.47	0.744	7.4	8.77
60-90	C	0.357	0.220	1.47	0.753	7.4	9.19

Agricultural applications

Cotton seeds were sown on 03 May 2014 and 01 May 2015 using a plot drill with 70-cm row spacing and 10-cm intrarow. The plant density was 142857 plant ha^{-1} in both years of the research. In the experiment, the cotton variety of Stoneville 468 was employed as experimental material. The plot size was 25.20 m^2 (6.00 × 4.20 m). However, during the harvest, the plot size was reduced to 14 m^2, thus eliminating the border effect. Hand harvesting was practiced twice during both years. The first harvest was practiced at the ball opening stage of 80-90%, and the second harvest was practiced after the remaining 10% of balls had been opened. Thus, the firsthand harvest was realized on 15 September 2014, and the secondhand harvest was realized on 16 September 2015. The second harvest was performed about three weeks after the first harvest was performed.

After the plant emergence, hoeing was done by machine and twice by hand. The plants in the plots were decreased, taking into consideration an intrarow spacing of 15-20 cm. For fertilizing, 80 kg N ha^{-1} and 80 kg P_2O_5 ha^{-1} were applied at planting. The fertilizer source was a compound fertilizer (20-20-0). Then, the remaining N was applied. It was split three times, with equal amounts of each being used via drip irrigation (Çetin and Akalp, 2019). In addition, total of 80 kg P_2O_5 ha^{-1} and 160 kg N ha^{-1} were applied as fertilizers. Some chemicals were applied against *Empoasca* sp. (active ingredients: %20 Acetamiprid 10 g da^{-1}) and thrips (active ingredients: 100 g l^{-1} Cyantraniliprole).

Experimental design and treatments

The experiment was conducted using split plots in randomized complete blocks with three replications. The main plots included various drip line spacings (L_1: 0.70 m and L_2: 1.40 m), and the subplots featured irrigation ratios based on different coefficients of Class A Pan evaporation (I_1, I_2, I_3, and I_4). Thus, eight different irrigation levels were applied during the field study. The treatments are given in *Table 2*.

The water used for irrigation was received from deep wells. The water from the deep wells had low sodium content and medium salt. p E and class of irrigation water is 7.87, 0.72 dS m^{-1}, and C_2S_1 respectively. In addition, the control unit for drip irrigation was formed using hydrocyclone, a sieve filter, pressure gauges, a fertilizer tank, and valves. The filtrated irrigation water was delivered by a 75-mm polyethylene pipe (PE) to the experimental site and was applied to the plots via 50-mm PE pipe lines. The diameter of drip lines was 16 mm. Also, the drip line spacing was 0.70 m in L_1 and 140 cm in L_2. Drippers in the drip line were placed 33 cm apart and had a flow rate of 4 L h^{-1}, and the selection of the drippers was based on the soil characteristics as described in Keller and Bliesner (1990). Furthermore, the operation pressure of the drip irrigation system was 1 atm. For the purpose of providing for the germination of the seeds, all of the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_40374053

experimental plots were irrigated two times with equal irrigation levels and with a small amount of irrigation using a sprinkler irrigation system. The drip irrigation system was installed following the second hoeing of the plots.

Table 2. *Experimental treatments*

Main plots (drip line spacings)	**Subplots (irrigation factors)**
L_1 (70 cm) L_2 (140 cm)	I_1 (K_p = 1.50) (full irrigation) I_2 (K_p = 1.25) I_3 (K_p = 1.00) I_4 (K_p = 0.75)

Irrigation water applied

The amount of irrigation water applied was calculated via multiplying the evaporation amount from the Class A Evaporation Pan based on various K_p coefficients. Thus, the total amount of evaporation for each of the seven days was multiplied by considering various coefficients and crop cover percentages. The crop cover percentage was scored by measuring the width of the plant crown prior to each irrigation. The first irrigation was practiced when 50% of the available water in the 0- to 60-cm soil profile was consumed. All experimental plots were irrigated until the field capacity was reached during the first irrigation, whereas the subsequent irrigations were based on the requirements of the experimental treatments. The amount of water given to the plots was measured using a water gauge, and the amount of rainfall was obtained from the meteorological station in the experimental area (Ünlü et al., 2011). The equation in calculation of the amount of irrigation water is given below:

$$IW = A \times E_{pan} \times K_p \times Pc \quad \text{(Eq.1)}$$

where IW: the amount of irrigation water applied (mm); E_{pan}: the cumulative evaporation from the Class A Pan at each irrigation interval (mm); K_p: the coefficient used to obtain a different irrigation level; and Pc: the plant canopy cover (it was estimated by measuring whole canopies of five plants selected randomly from each plot throughout the irrigation season). The evaporation was measured using a screened Class A Pan located at the meteorological station near the experimental field.

The soil moisture in all experimental plots was monitored at a soil depth of 0-120 cm through the gravimetric method. The actual evapotranspiration was computed using the equation of water balance (James, 1988):

$$ETc = I + P + D_p \pm R_{off} \pm \Delta S \quad \text{(Eq.2)}$$

where ETc: crop evapotranspiration (mm); I: the amount of irrigation water applied (mm); P: the rainfall (mm); D_p: the deep percolation (mm); R_{off}: the runoff (mm); and ΔS is the change in the moisture content at a root depth of 0-90 cm (mm).

Irrigation water productivity (IWP) and water productivity (WP) were calculated through the gauge effects of irrigation programs (Pereira et al., 2012). The equations are as follows:

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DOI: http://dx.doi.org/10.15666/aeer/1905_40374053

$$WP = Y/ET \quad \text{(Eq.3)}$$

$$IWP = Y/IW \quad \text{(Eq.4)}$$

where Y: the economical yield (kg da^{-1}); ET: the seasonal evapotranspiration (mm); and I: the amount of seasonal irrigation water (mm).

Analysis and statistical evaluation

Plant samples were taken to score the leaf area index (LAI) throughout the growing season at intervals of 10 days starting from the 31st day of sowing in the first year (DAS), and at intervals of 12 days starting from DAS 38 in the second year. A portable leaf area meter was used for leaf area measurements (LI-COR 3100). In this study, 50 bolls were randomly selected from each plot. The seed cotton weight per boll, seed cotton yield, ginning turnout (based on Worley et al., 1976), fiber length, fiber fineness (micronaire), and fiber strength were scored using the methods specified in Anonymous (1997).

The results of the study were evaluated using Minitab 16 statistics software. First, analysis of variance (ANOVA) was performed to evaluate the effects of various irrigation treatments on the yield and quality of the cotton. The Tukey test was used for the mean grouping of the treatments.

Result and discussions

Irrigation and crop evapotranspiration

The amount of irrigation water applied according to the treatments, coupled with the seasonal crop evapotranspiration (ETc) of the treatments are given in *Table 3*.

Table 3. *Amount of irrigation water and evapotranspiration for the treatments*

Treatments		2014				2015			
		Rainfall (mm)	**ΔS (mm)**	**IW (mm)**	**ETc (mm)**	**Rainfall (mm)**	**ΔS (mm)**	**IW (mm)**	**ETc (mm)**
L_1	I_1	56	11	983	1,028	11	-31	1,032	1,074
	I_2	56	0	824	880	11	-44	867	922
	I_3	56	-13	666	735	11	-51	702	764
	I_4	56	-22	507	585	11	-67	536	614
L_2	I_1	56	36	983	1,003	11	-6	1,032	1,049
	I_2	56	29	824	851	11	-23	867	901
	I_3	56	-16	666	738	11	-41	702	754
	I_4	56	-39	507	602	11	-54	536	601

ΔS: the change in the moisture content at a root depth of 0-90 cm, IW: Irrigation water, ETc: Seasonal crop evapotranspiration

Generally, almost the entire water requirement of the crop was met through irrigation, as no rainfall occurred during this period. However, excess spring precipitation in the late growing period delayed the initial irrigation, and thus, the irrigation water need decreased. The region's long-term annual precipitation average for

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DOI: http://dx.doi.org/10.15666/aeer/1905_40374053

the cotton growing period (May-September) was 30 mm. During the first year of the experiment, it was 56 mm, and during the second year, it was 11 mm. Therefore, the plant's water need was fully met through irrigation.

Laterals were placed 0.70 m apart during L_1 treatments. Meanwhile, irrigation was performed with the laterals spaced 1.40 m apart during L_2 treatments. Therefore, the irrigation times for L_2 treatments were twice as long as those of L_1 treatments. During the first year of the field trial, the amount of irrigation water applied to the treatments varied between 507 mm and 983 mm, and the amount was between 536 and 1032 mm during the second year. The water consumption associated with the trial treatments varied depending on the amount of irrigation water used. Treatments with less irrigation water benefited more from the moisture available in the soil at the time of sowing. Especially in the second year, all treatments utilized the moisture stored in the soil at different levels, whereas in the first year only, I_4 and I_3 treatments benefited from the moisture present at the time of planting. In addition, except for in the case of the L_1-I_2 treatments during the first year, it was observed that some of the irrigation water delivered during the treatments of I_1 and I_2 were stored in the soil.

Seed cotton yield and some yield parameters

ANOVA was performed for all scored characteristics and irrigation ratios, and the lateral spacing were found to be partially significant. The ANOVA table was not given here. However, the seed cotton yield and some of the yield factors during the experimental years are given in *Table 4*.

Table 4. *Means and statistical groups for the seed cotton yield, boll seed cotton weight, and 100 seed weight values of cotton for the treatments*

Treatments	Seed cotton yield (kg da^{-1})		Seed cotton weight (g boll^{-1})		100 seed weights (g)	
	2014	2015	2014	2015	2014	2015
L_1	437.3	388.0b	4.18b	3.96	8.49	8.29
L_2	468.5	432.8a	4.36a	4.02	8.45	8.36
P (Factor A)	ns	*	***	ns	ns	ns
I_1	596.7a	536.1a	4.59a	4.21a	8.64	8.58a
I_2	509.5ab	463.2a	4.40b	4.05ab	8.41	8.48ab
I_3	408.7b	371.8b	4.22c	3.87b	8.42	8.15b
I_4	296.7c	270.5c	3.87d	3.82b	8.41	8.11b
P (Factor B)	**	**	*	**	ns	**
L_1-I_1	577.0	522.3	4.50ab	4.13	8.64	8.44
L_1-I_2	491.0	444.3	4.33bcd	4.06	8.30	8.46
L_1-I_3	396.3	346.4	4.19de	3.84	8.68	8.13
L_1-I_4	284.7	239.0	3.70f*	3.81	8.34	8.15
L_2-I_1	616.3	549.8	4.68a	4.29	8.64	8.72
L_2-I_2	528.0	482.1	4.46bc	4.05	8.52	8.50
L_2-I_3	421.0	397.2	4.25cde	3.90	8.15	8.17
L_2-I_4	308.7	302.0	4.04e	3.83	8.49	8.06
P (A*B)	ns	ns	**	ns	ns	ns

**Significant at 1%, *significant at 5%, ns: not significant
[a]The treatments which have the same letter are not significantly different at the 5% level by Tukey's test

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DOI: http://dx.doi.org/10.15666/aeer/1905_40374053

In this study, decreasing the amount of irrigation water resulted in a decrease in the cotton seed yield, and a statistically significant decrease in the yield was found. In the first year of the experiment, the seed cotton yields depended on the first two groups (*Table 4*). Both the drip line spacing ($p \leq 0.05$) and the water levels ($p \leq 0.01$) significantly affected the cotton yields in the second year. However, the effect of the main treatment and sub-treatment interaction was found to be nonsignificant. The yields in L_2, where two plant rows were irrigated using a single drip line, was higher than that of L_1. Although the yield of L_2 was 31.2 kg more in the first year, it was statistically nonsignificant. However, in the second year, the yield of L_2 was 44.8 kg more than that of L_1, and this was found to be statistically significant.

In this study, seed cotton weights of 50 bolls that were randomly selected from each plot were averaged to score the seed cotton weights of the bolls used in the experimental treatments. The average boll cotton weights of the samples taken in the first year of the experiment varied between 3.70 g and 4.68 g depending on the treatment. It was found that the drip line spacing ($p \leq 0.01$), irrigation levels ($p \leq 0.01$), and interaction of both ($p \leq 0.01$) affected the boll cotton weight significantly. However, the irrigation of two plant rows from one lateral side had a positive effect on the boll seed cotton weight. Moreover, as the amount of irrigation water increased, the boll seed cotton weight increased. The highest seed cotton weight per boll was obtained from the treatment of L_2-I_1 with 4.68 g, and this treatment ranked in the first group in the Tukey grouping.

The drip line spacing had no significant effect on the boll seed cotton weight in the second year. However, the irrigation levels significantly affected the boll seed cotton weight ($p \leq 0.01$). In this year, as the amount of irrigation water increased, the boll seed cotton weight increased, and the highest seed cotton yield per boll was obtained from the highest irrigation level (I_1). The I_1 treatment ranked in the first group, and the I_4 and I_3 treatments were in the last group.

In 2014, the 100 seed weights of the treatments varied between 8.15 g and 8.64 g, but no significant difference arose from the factors. In the second year, although drip line spacing had no effect on the 100 seed weights, the effect of the amount of irrigation water used was significant ($p \leq 0.01$). As with other factors, irrigation had a positive effect on seed weight, and the highest of the 100 seed weights was obtained from the treatment with the highest water doses (8.58 g). This treatment alone ranked in the first Tukey grouping.

Fiber quality parameters

The effect of different lateral spacing and water amounts on the quality criteria of the cotton is given in *Table 5*.

One of the most important quality criteria in cotton is fiber fineness. It is desirable for the fiber to be thin. In this study, the amounts of various irrigation doses in the first year had no effect on fiber fineness. In other words, reducing the amount of irrigation water did not have a positive or negative effect on fiber fineness. However, different drip line spacing amounts affected fiber fineness ($p \leq 0.01$). The single lateral irrigation of two plant rows increased fiber thickness, but the interaction of drip line spacing and irrigation level had no significant effect on fiber fineness. When the results of the second year were evaluated, no statistically significant differences were found among irrigation treatments for fiber fineness. Even though it was statistically nonsignificant, it was found that the fiber fineness decreased with the increase of the drip line spacing. This situation showed that the results of the first year might be coincidental.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_40374053

Table 5. *Means and statistical groups for some fiber quality characteristics of treatments*

Treatments	Fiber fineness micronaire		Fiber length (mm)		Fiber strength (g tex^{-1})		Ginning outturn (%)	
	2014	2015	2014	2015	2014	2015	2014	2015
L_1	4.42b	4.72	27.6	26.8	28.4	27.4	42.4	41.2
L_2	4.69a	4.60	27.7	27.1	28.7	27.4	43.2	41.8
P (Factor A)	**	ns	ns	ns	ns	ns	ns	ns
I_1	4.65	4.70	27.8	26.9	28.1	27.7	48.2a	45.1a
I_2	4.50	4.57	27.5	27.0	28.5	26.8	42.7b	42.3ab
I_3	4.70	4.68	27.5	26.5	28.8	26.8	41.2b	40.2bc
I_4	4.38	4.69	27.6	27.3	28.8	28.1	39.0c	38.4c
P (Factor B)	ns	ns	ns	ns	ns	ns	**	**
L_1-I_1	4.42	4.72	27.7	27.0	28.4	27.4	47.8	44.7
L_1-I_2	4.45	4.68	27.3	27.2	28.7	27.2	42.1	41.9
L_1-I_3	4.64	4.80	27.7	26.1	28.4	26.7	41.0	40.3
L_1-I_4	4.18	4.68	27.6	26.9	28.2	28.3	38.6	37.9
L_2-I_1	4.88	4.67	27.9	26.9	27.8	28.0	48.6	45.5
L_2-I_2	4.54	4.47	27.7	26.9	28.4	26.5	43.3	42.8
L_2-I_3	4.76	4.56	27.4	26.8	29.2	27.0	41.4	40.1
L_2-I_4	4.58	4.71	27.6	27.7	29.4	28.0	39.4	38.9
P (A*B)	ns	ns	ns	ns	ns	ns	ns	ns

**Significant at 1%, *significant at 5%, ns: not significant
[a]The treatments which have the same letter are not significantly different at the 5% level by Tukey's test

Similar results were also found for fiber strength. Although the strength values varied between 27.8 and 29.4 g tex^{-1} in the first year and between 26.5 and 28.3 g tex^{-1} in the second year, these fluctuations between the treatments were not statistically significant.

The results obtained from this study revealed that the drip line spacing during both years of the field trial had no effect on the yield. Again, the interaction between the main plots and subplots did not have any significant effect on ginning efficiency. However, the irrigation levels significantly affected the yield in both years ($p \leq 0.01$). As the irrigation doses increased, the ginning efficiency also increased up to 48.2% in the first year and 45.1% in the second year for the highest amount of irrigation (I_1). However, in the I_4 treatments, these values remained at 39% and 38.4%, respectively. In previous studies, some conflicting results between irrigation water doses versus ginning efficiency have been revealed.

Leaf area index and water use efficiencies

The LAI was determined by taking plant samples at regular intervals during the plant growing season. In *Figure 1,* LAI variations for the consecutive years are given.

When the temporal variation of the LAI results of trial treatments was examined, a sigmoidal relationship was found between the LAIs and times in both years. The LAI started to increase when the plants began to emerge, and it continued until approximately 100-110 days after sowing (DAS), at which point it reached its maximum level. Although the LAIs of all treatments increased continuously during this period, the LAI values of the treatments started to differ with the initiation of irrigation

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at different doses. The negative effects of water stress on the LAI began to be observed with the start of the irrigation practices used in the treatments. Since DAS 60, the differentiation in the LAI values regarding the applications has occurred distinctly (*Fig. 1*). As the amount of irrigation water increased, the LAI increased more quickly, and the I_1 treatments reached the highest values in both years. The LAI indices decreased after DAS 110 until the harvest. The LAI values of the fully irrigated L_1-I_1 and L_2-I_1 treatments reached 4.83 and 5.16 in the first year, and 5.00 and 5.07 in the second year, respectively.

The highest LAI value was obtained from the full irrigation treatment. However, these studies showed variations in the amount of time required to reach the highest LAI. As mentioned above, the employed agricultural techniques, environment, and cotton varieties played an important role in this situation.

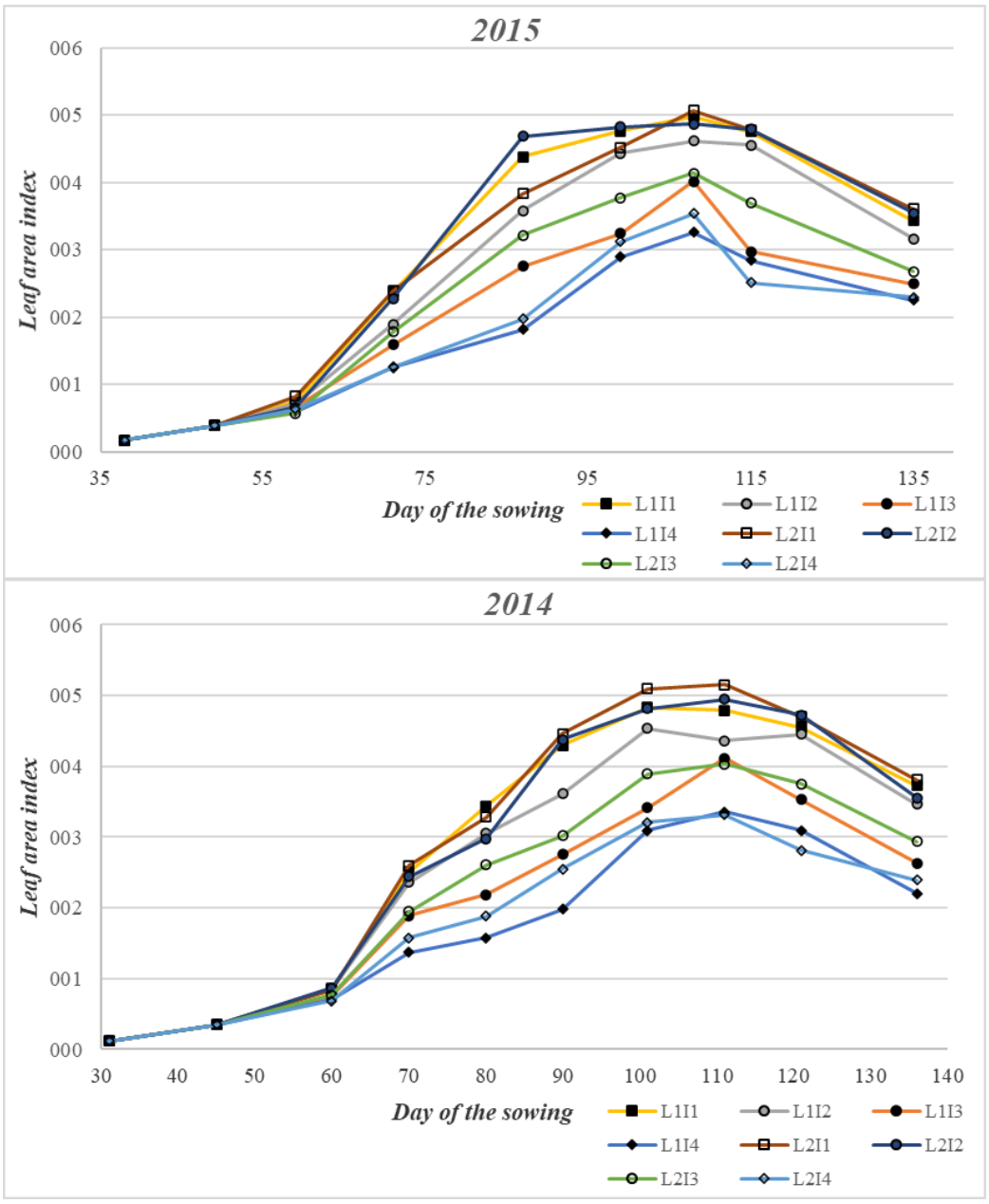

Figure 1. *Temporal changes in leaf area index*

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DOI: http://dx.doi.org/10.15666/aeer/1905_40374053

Water productivity is an important indicator in determining the most suitable irrigation program in restricted irrigation studies. The WP and IWP values were calculated for this study and are given in *Figure 2*.

Figure 2. *Water productivity and irrigation water production for the treatments*

The IWP values varied between 0.60 kg m^{-3} and 0.67 kg m^{-3} in the first year and between 0.48 kg m^{-3} and 0.61 kg m^{-3} in the second year.

In the first year, the IWP values did not differ significantly depending on the amount of irrigation water doses used, but the IWP values of the L_2 treatments were higher than those of the L_1 treatments in the same irrigation treatments. Although the same amount of irrigation water was applied, the more effective use of irrigation water in the 140-cm lateral range was due to the lower evaporation losses in this lateral range. Similar results were obtained in the second year, whereas higher WP_{irr} values were obtained from the L_2 treatments. However, no obvious differences were found due to the irrigation levels.

The WP values were lower than the IWP values were. These values varied between 0.48 kg m^{-3} and 0.61 kg m^{-3} in the first year and decreased to 0.39 for L_1-I_4, which was

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DOI: http://dx.doi.org/10.15666/aeer/1905_40374053

scored even lower in the second year. At the same time, the differences among the IWP values of the second year were more pronounced. In both years, the lowest WP values were obtained from the L_1-I_4 treatments. This was due to the proportionally higher amount of evaporation in the ET. When drip line spaces were evaluated, the WPs of the L_2 treatments were higher than those of the L_1 treatments. In this case, the wetted zone in the L_2 treatments consisted of a narrow yet deep profile. The low wetted soil surface caused the amount of water that entered the atmosphere through evaporation to be lower, considering the very hot and low relative humidity conditions of the region.

Discussion

In the study, the highest yield was found to be full irrigation treatment. During the cotton growing period, the region was very hot, and the relative humidity was also low, thus requiring intensive irrigation in cotton as pointed out by Chapagain et al. (2006) and Darouich et al. (2014). Some researchers also obtained similar results for semi-arid regions (Karam et al., 2006; DeTar, 2008; Onder et al., 2009; Singh et al., 2010; Ünlü et al., 2011). In addition, the factors of relative humidity, temperature, radiation and wind speed played an important role in plant water consumption (Rao et al., 2016). Therefore, in arid climates, the plant water consumption was higher than that of semi-arid climate. The amount of water consumption obtained from the study confirmed the research findings of Tüzel and Ul (2003) and Hunsaker et al. (2015).

The lateral spacing of 70 and 140 cm did not affect the ETc values significantly. The highest water consumption level occurred in L1-I1 factor in both years. Although no significant difference was found among the water consumption levels of the main treatments, the water consumption levels of the L1-I1 and L1-I2 treatments were relatively higher than those of the L2-I1 and L2-I2 treatments. This resulted in that the evaporation losses were high due to the double wetted surface area resulting from the lateral laying of each row in L1.

Although the same amount of irrigation water was given to plots, the main reason for the higher yield in L2 (140 cm) comparing L1 (70 cm) could be explained by the different amounts of evaporation loss among the main treatments. Although more evaporation was observed in L1 comparing in L2, the percentage of wetted area in L1 was higher than that of L2. This can be attributed totally the climatic condition in the experimental region (hot and featuring very low relative humidity). Similar results were also obtained in another study conducted earlier (Sarı, 2010).

The highest seed cotton yields were obtained by the factor without any water restrictions (I1). The yield obtained from this treatment was similar to that of Yang et al. (2015) and Zhang et al. (2016). However, the yields obtained by our study were higher than those of some other studies (Yavuz, 1993; Ertek and Kanber, 2001; Cetin and Bilgel, 2002; Onder et al., 2009; Çetin and Kara, 2019; Chen et al., 2020). These differences could be derived from the climate, soil characteristics, cotton variety, and cultivation techniques of the research sites. In terms of boll seed cotton yield, the findings on the boll seed cotton weight were similar to those of previous studies (Pettigrew, 2004; Rao et al., 2016). Moreover, in previous studies it was stated that the increasing amount of irrigation water also increased the weight of the 100 grains (Dagdelen et al., 2009a; Sari and Dagdelen, 2010)

Fiber quality also was affected by the factors implemented in our study. The fiber fineness values obtained from the experiment were higher than those of the conditions

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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obtained in similar climatic conditions (Thorp et al., 2020). This difference may be due to the characteristics of the cultivar. Lateral spacing or water limitation did not affect fiber fineness. Other studies on water restriction in cotton have also reported that water restriction had no effect on fiber fineness (Karam et al., 2006; Dagdelen et al., 2009b; Ünlü et al., 2011; Papastylianou and Argyrokastritis, 2014). Fiber length was another important quality criterion in cotton. Practicing various factors in both years of this study had no effect on fiber length. At the same time, irrigation factors with different lateral spacings did not affect fiber length either positively or negatively. Contrary to the results in this study, Thorp et al. (2020) reported that water restriction reduced the short fiber content. The research findings in this study were mostly confirmed by other researchers (Ünlü et al., 2011; Papastylianou and Argyrokastritis, 2014). The water shortages or limitations in the lateral range had also no effect on the fiber strength, which showed nonsignificant differences between the drip line spacing and irrigation doses. Our results were in agreement with those of Karam et al. (2006), Booker et al. (2006), Ünlü et al. (2011) and Papastylianou and Argyrokastritis (2014). Sari (2010) also confirmed our findings, whereas Dagdelen et al. (2009b) stated that irrigation water did not affect the ginning turnout. Onder et al. (2009) furthermore stated that the increase in irrigation water decreased the ginning efficiency. The most important reason for these variations was the characteristics of the varieties used in the experiments, as well as many other factors, such as climate and agricultural practices.

Although LAI varied due to applied agricultural techniques, the environment, and the cotton variety That of full irrigation were in accordance with the results of previous studies (Stone et al., 2001; Yazar et al., 2002; Karam et al., 2006; Kang et al., 2012; Sampathkumar et al., 2013).

WP and IWP values of irrigation increase in arid regions. In these regions, almost no rainfall occurs during the cotton growing period. Furthermore, the plant water consumption increases to a level higher than in other climatical conditions. In previous studies, some researchers stated that WP and IWP increased with water restriction (Ünlü et al., 2011; Yang et al., 2015; Fan et al., 2018; Garibay et al., 2019). However, some researchers reported confirming our finding that no significant effect occurred in WP and IWP due to water shortages (Chen et al., 2020).

Conclusion

The widespread adoption of limitations in irrigation water in arid regions throughout the entire growing season resulted in significant decreases in the cotton yield. Therefore, if limited irrigation operations are to be practiced, the water sensitivity of plant growth periods should be taken into account, and irrigation should be limited when cotton is insensitive to water. The reduction of irrigation water on quality did not have such a severe effect on the yield, and even some quality criteria were never affected by the amount of irrigation water.

In addition, in drip irrigation, irrigation of two rows instead single row from only one lateral resulted in higher yielding contrary to low yield expect. Decreasing the number of lateral spaces by half caused the wetted area to decrease at the same rate. This was derived from a decrease in evaporation losses from the soil. This situation also caused the IWP and WP values to be higher in the wide drip line spacing than in the narrow ones.

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The adoption of drip line spacing in areas where soil conditions are suitable for instance, soils with high water usage productivity will also reduce the lateral space related expense of the irrigation system by half. This is another important advantage of this system.

Acknowledgments. This work was financially supported by the Harran University Application and Research Center for Science and Technology, (Project number: 14051).

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_40374053

EXCESSIVE GROWTH OF FENNEL PONDWEED (*Stuckenia pectinata)* IN THE KIZILIRMAK RIVER, CAPPADOCIA, TURKEY

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(Received 12th May 2021; accepted 30th Aug 2021)

Abstract. An excessive growth of submerged vegetation has been observed in some regions of the Kızılırmak river. In this research, conducted below Yamula Dam, monthly changes in submerged macrophytes in the river course around Avanos (Nevsehir) were investigated. Submerged plant samples were taken from four stations and some water quality parameters were measured. The submerged plant which had increased excessively was identified as fennel pondweed, *Stuckenia pectinata* (L.) Börner. Molecular identification using a partial sequence of the ITS region has found that sampled *S. pectinata* specimens were not closer to sequences belonging to hybrid samples. Rather, nucleotide sequences of analysed fragment of ITS region have shown that the phytogeographic distribution of the haplotype, lies between eastern and western examples. The mean submerged plant biomass was estimated as 721 ± 288 g dry weight/m^2 in the research period. The excessive increase of *S. pectinata* in the Kızılırmak can be attributed to the regulated flow in the river, and its salt tolerance and preference for hard waters. Since it was the single dominant species of submerged macrophyte in the study area, macrophytes indices could not be applied. Mechanical/physical control was recommended for the control of submerged plant biomass in the area.
Keywords: *submerged plant, fennel pondweed, biomass, phylogenetics, impacts of dams*

Introduction

Rivers are much more variable ecosystems than standing waters. From their source to their outflow, they are affected by the soils of the regions they flow through and climatic conditions. Streams originate from different sources, gradually widening and gouging out a bed until they flow into a lake or the sea. In this way, they show variations in their physical, chemical, and biological characteristics, which greatly affects people. Flowing waters are mostly affected by pollution, agricultural activity, and hydromorphological modifications such as dams: the lake forming behind a dam changes the river's flow regime.

Aquatic plants are the primary producers of this environment: they provide nutrition and shelter, and form a place for fish and other organisms to lay their eggs, and they have great importance in the sustainable use, management and restoration of the environment. Macrophytes affect the distribution, particle size and composition in the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_40554067

sediments of fresh and marine waters, and so their presence in freshwater and marine ecosystems is fundamental to improving water quality and to preventing turbidity and sediment mixing (Madson et al., 2001; Cirik et al., 2007).

Aquatic macrophytes directly affect water quality and its composition of organisms. In both flowing water and lakes, aquatic macrophytes are used as indicators in the monitoring of environmental effects and eutrophication, and macrophyte monitoring programs have been developed. Aquatic macrophytes are an important indicator in monitoring ecological quality especially in shallow lakes, but they can also be used as a measure of ecological quality in rivers (Melzer, 1999; Birk et al., 2006; Kuhar et al., 2011; Gecheva et al., 2013; Ciecierska and Kolada, 2014). The construction of dams and canals cause changes in the ecological characteristics of the water by affecting the flow. The Kızılırmak – 'Red River' – got its name from the characteristic red colour of the clay particles that it carries (Akbulut et al., 2009). Large dams constructed on the Kızılırmak have regulated its turbulent flow pattern, and over the long term, there has been a reduction in its flow. It has been found that with the reduction in the amount of alluvium, the Kızılırmak delta has ceased expanding (Bahadır, 2011). After the construction of the Yamula Dam, the increase in the black fly population in the middle Kızılırmak basin between the provinces of Kayseri and Nevşehir reached disastrous numbers. The flies were identified as black flies of Simuliidae species. Several researches were performed on the taxonomy of black flies in Turkey (Kazancı and Clergue-Gazeau, 1990; Kazancı and Ertunç, 2008; Başören and Kazancı, 2016). This outbreak of flies had a negative effect on tourism and farming of this area of recreational importance (Yılmaz et al., 2007). This species of fly lays its eggs on several substrates such as underwater plants, and we think that excessive plant growth had an effect on increasing the fly population. The larvae and pupa of the insects were observed on the aquatic plants. It is reported that commercial preparations containing *Bacillus thuringiensis israelensis* (Bti) were used against the fly larvae in the area (Yılmaz et al., 2010). The treatments against the flies in the river are still going on. In the River Nile, it was reported that the construction of the Aswan Dam caused important changes in the aquatic ecosystem and especially in aquatic plant communities and that with the regularization in the flow regime of the river, a suitable habitat was formed for the growth of many underwater macrophytes such as *Ceratophyllum demersum* and *Stuckenia pectinata*. It was also found that the increased plant growth was a factor in an increase of disease-carrying black flies (El-Shinnawy et al., 2000). Similarly, when large dams were built on the River Ebro in Spain, a reduction in suspended solid matter caused an increase in the species *S. pectinata* associated to the black fly *Simulum erythrocephalum*, and negative effects were reported on local people and tourists (Ibanez et al., 2008). Changes in river ecosystems can have many negative ecological consequences. The plants have caused an increase in the black fly population and have created recreational problems.

This research aims to identify the aquatic macrophytes of the Kızılırmak in the region of the district of Avanos in Cappadocia, to perform phylogenetic characterization of the excessively growing plant, and to investigate monthly changes in plant biomass. In addition, plant control methods have been discussed to suggest which one is the most suitable for the region.

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DOI: http://dx.doi.org/10.15666/aeer/1905_40554067

Materials and methods

Study site and sampling

The Kızılırmak is Turkey's longest river, and stretches for 1355 km. It rises on the slopes of Kızıldağ in the north-east of Anatolia and runs through the provinces of Sivas, Kayseri, Nevşehir, Kırıkkale, Ankara, Çankırı, Çorum and Samsun, gathering water from many streams and rivers before meeting the Black Sea at Bafra. Kızılırmak is Turkey's second largest river basin, constituting approximately 10.9% of the total surface area of the country (Yıldız and Özkıran, 1991). Its main tributaries are the Delice, Devrez and Gökırmak rivers. Fed with rain and snowmelt, the river used to have an irregular flow. It has a low rate of flow from July to February, and then in March it begins to swell rapidly, reaching its highest levels in April. Its mean flow rate is 184 m^3/s, while over a 35-year observation period its minimum mean flow was 18.4 m^3/s and its maximum was 1673 m^3/s (DSI, 2009). There are 16 dams on the river, and there are four dams upstream of the sites where samples were taken. This study was conducted on the section of the Kızılırmak below the Yamula Dam, where it passes through the district of Avanos in Cappadocia.

This study was conducted on a monthly basis between March and December 2010. Samples were taken from four stations between 38°42'974"- 38°43'050" N and 34°51'526"- 34°50'878" E in the district of Avanos. Stations 3 and 4 were in the town of Avanos, and there was a distance of approximately 1470 m between stations 1 and 4 (*Fig. 1*). The width of river in the sampling stations changed between 55 and 125 m. In the field work, the river was crossed from bank to bank by boat along a transect, and the frequency of occurrence of the water plants was recorded. The abundance of the plants was measured according to Kohler (1978) on a five-degree scale, from 1 = very rare to 5 = abundant, predominant. After that, the plants were taken from the bottom using a long-handled rake in three repetitions at equal distances from the sampling points (Westlake, 1986; Demir et al., 2020). Also, water samples were taken from immediately below the surface. The plants collected were placed in plastic bags and labelled.

Because of adverse weather conditions, plant samples were not taken from station 1 in July. In September, the river water was cut off to collect water in the newly constructed Bayramhacılı dam upstream of Avanos and the river bed was dry, so that plant samples could not be collected from stations 1, 2 and 4, and it was not possible to measure water depth or Secchi depth. However, it was possible to collect water samples from the flowing water.

Analysis

The aquatic plant was identified using a hand lens and stereomicroscope according to relevant literatures (Davis, 1965-1985). DNA extraction was performed using CTAB chloroform: isoamylalcohol method (Štorchová et al., 2000). The 18S rRNA, ITS1, 5.8S rRNA, ITS2, and 26S rRNA regions of the samples were amplified using the ITSF and ITS4 primer pair (Kaplan and Fehrer, 2004). PCR were carried out in a final volume of 50 µl containing 8 µl of 5x FIREPol Master Mix Ready to Load (12.5mMMgCl2) (Solis BioDyne, Estonia), 1 µl of each primer (F, R), 2 µl of template DNA and 28 µl of ddH_2O. PCR amplifications were all set with a negative control sample. PCR amplifications were performed using a BioRad CFX96 thermal cycler using an initial denaturation step at 95°C for 7 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 48°C for 30 seconds, and extension at 72°C for 20 seconds

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and followed by a final extension at 72°C for 5 minutes (Keskin et al., 2016). PCR products of 945 base pairs were purified prior to sequencing. Bidirectional Sanger sequencing was performed using ABI SeqStudio Genetic Analyzer. Nucleotide sequences were aligned to reference sequences, and checked for possible reading errors, insertions and deletions. Analyses were performed based on a trimmed and quality checked 574 base pair fragment together with reference sequences obtained from NCBI GenBank. Nucleotide composition, nucleotide pair frequencies and codon usage were analysed for the nucleotide data and pairwise genetic distances were calculated using Jukes Cantor. The evolutionary relationship among species was analysed by constructing a Neigbor-Joining tree calculated using 1000 iteration bootstrap tests. All analyses were performed using MEGA X (Kumar et al., 2018). The biomass by unit area of aquatic plants was determined on the basis of dry weight. The plants were first sorted by species, and weighed. After the wet weight were measured, the samples of the plants were placed in weighed crucibles, dried in a desiccator at 105°C for 24 hours and cooled, after which dry weight was determined (Wetzel and Likens, 1991).

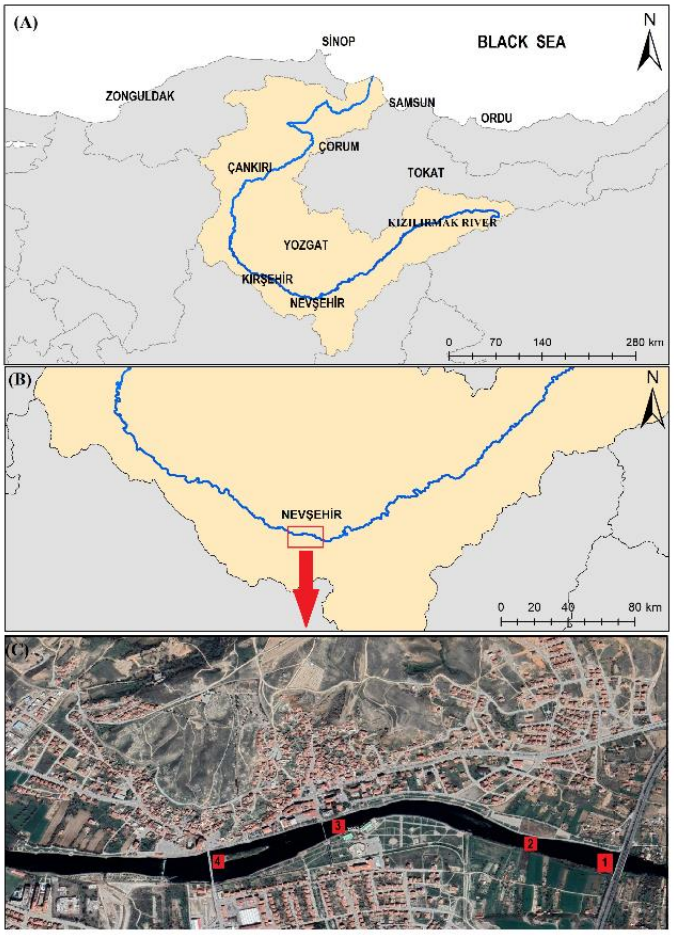

Figure 1. *A; The location of Kızılırmak Basin, B, C; Study site and sampling stations*

Measurements were made of water temperature, dissolved oxygen (with a YSI ProPlus Oxygenmeter), pH (with a Consort P901 Model pHmeter), electrical conductivity (with a Schott CG855 Model Conductivitymeter) and Secchi depth (with a

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20-cm diameter Secchi disk). Analyses of water samples for alkalinity, total hardness, nitrate, nitrite, ammonium, orthophosphate, total phosphorus were performed according to standard methods (APHA, 2005).

Statistical analysis

The variations of plant biomass between the sampling stations were evaluated by One-Way Analysis of Variance (ANOVA) and post-hoc Tukey test using SPSS 17 (Kesici and Kocabaş, 2007). For all parameters, logarithmic (Log X+1) data transformation was applied (except pH values). The Detrended Correspondence analysis (DCA) was used to select the appropriate model (linear or unimodal) for the ordination and it was determined by the Canoco v4.5 software (Ter Braak and Smilauer, 2002). According to the results, Principal Components Analysis (PCA) was applied by the covariance matrix at the XLSTAT 2014.5.03.

Results and Discussion

The species of aquatic plant showing an excessive increase in the sampling sites was determined as *Stuckenia pectinata* (L.) Börner (syn. *Potamogeton pectinatus* L.). Kaplan (2008), described the varieties and variation in *Stuckenia* species around the world, and separated them from the genus *Potamogeton*. Species grouped under the genus *Stuckenia* have long leaf sheaths, a characteristic leaf and a pendiculous anatomy, with a high ploidy level (hexaploidy) distinguishing them from the generally diploid or tetraploid *Potamogeton* species. However, hybridization with other *Potamogeton* species makes identification by morphological characteristics difficult.

Genetic characterization of the species *Stuckenia pectinata* was performed using the ITS region, and phylogenetic analyses were made comparing it with *Stuckenia pectinata, Stuckenia filiformis* as outgroup and *Stuckenia pectinata x Stuckenia filiformis* hybrid samples from various countries (*Table 1, Fig. 2*). Molecular methods have been used to distinguish *Stuckenia* and *Potamogeton* species. The nuclear ITS region has become the most used marker because it provides the possibility of copying both maternally and paternally derived DNA sequences. Results obtained show variation between species to be up to 1-1.3% level within species and hybrids and up to 4% among *S. pectinata* and *filiformis* specimens. In nucleotide composition analysis results, the mean G-C ratio was calculated to be 50.7% and the ratio of transitional pairs to transversional pairs was found to be R = 0.49. The mean genetic difference between the samples used in the analysis was calculated to be 0.02. Examining the tree constructed by the Neighbor-Joining method, it was seen that the specimen obtained from Turkey was identical with the reference sequence gathered from Czech Republic in terms of nucleotide composition. After examining the DNA sequences of the samples of *S. pectinata* taken in the section of the Kızılırmak and comparing them with DNA sequences in the literature and in international data banks, it was concluded that it was not a hybrid as it is clustered with *S. pectinata* specimen from Czech Republic, very close to other *S. pectinata* specimen from China and clearly separated from *S. pectinata* x *S. filiformis* hybrid specimen and *S. filiformis* specimens. These results strengthen the idea that the ITS region is a suitable gene for genetic characterization, including the phylogeographic analysis of the species *S. pectinata*. It is intended in future studies to analyse different populations of the species, thereby revealing the genetic structure of the populations of Turkey.

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Table 1. *Pairwise genetic distance matrix of S. pectinata haplotype sequenced in this study together with reference sequences from different countries gathered from NCBI GenBank*

		1	2	3	4	5	6
1	*Stuckenia pectinata* (This study-Turkey)		0.000	0.004	0.004	0.008	0.008
2	MH171027 *Stuckenia pectinata* (Czech Republic)	0.000		0.004	0.004	0.008	0.008
3	DQ840277 *Stuckenia pectinata* (China)	0.013	0.013		0.002	0.007	0.007
4	FJ956925 *Stuckenia pectinata* x *Stuckenia filiformis* (China)	0.010	0.010	0.003		0.007	0.007
5	KT175311 *Stuckenia filiformis* (China)	0.041	0.041	0.033	0.030		0.000
6	MH171032 *Stuckenia filiformis* (Czech Republic)	0.041	0.041	0.033	0.030	0.000	

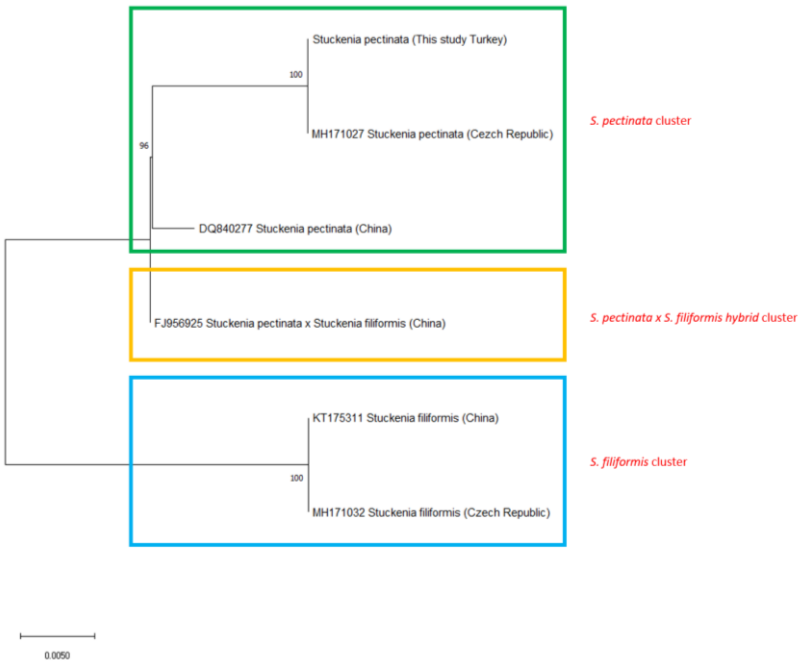

Figure 2. *Phylogenetic relationship (Neighbor-joining tree with Jukes Cantor estimation via 1000 bootstrap tests) among S. pectinata specimens sequenced in this study and specimens from other countries gathered from NCBI GenBank*

S. pectinata was not reported around Avanos before dam construction, but grew excessively when flow was regulated by dams. The Kızılırmak is faced with a great threat of pollution. In a research covering all Kızılırmak basin, 73% of stations are below good ecological status according to diatom index (EPI-D) and eutrophication/organic pollution are reported (Çetin et al., 2021). The name of the river – Kızılırmak means 'red river' – comes from the characteristic reddish colour of the clay particles which it carries (Akbulut et al., 2009). Dams built on the river have affected the flow regime, and it has been found that the growth of the Kızılırmak delta has ceased because of a reduction in the amount of alluvium (Bahadır, 2011). *S. pectinata* is a cosmopolitan species, lives in almost all waters and adapts to different environments. It was reported from the wetlands of the Kızılırmak basin, delta and

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Sultan Sazligi and widely distributed in Turkey in lakes and wetlands, from freshwaters to brackish lagoons and ponds (Seçmen and Leblebici, 1997; Demirezen and Aksoy, 2004). *S. pectinata* is a monocotyledonous angiosperm of the Potamogetonaceae family. It is a perennial macrophyte species, one of the most widespread aquatic plants, growing in fresh or brackish water, in stagnant water, in wet areas and in flowing water. It can grow excessively in irrigation canals and recreational areas. Its seeds are resistant to drying and high salinity, and are spread by water birds. It can also tolerate changes in water level. Among Potamogeton species, only *S. pectinata* tolerates high salinity, pH and alkalinity; it can be found in eutrophic waters and can even live in very polluted areas (Kantrud, 1990).

The mean depth of the sampling area was found as 2.25 ± 0.44 m, and Secchi depth was 0.90 ± 0.20 m. Water depth and Secchi depth showed monthly variation over the study period and monthly variation in Secchi depth was significant (F= 16.47, p= 0.00) (*Table 2*). The lowest Secchi depth were found to be lower than the values from the Yamula dam lake. In the Yamula dam, situated on the Kızılırmak in the upstream, the Secchi depth varied from 0.85 to 4.70 m (DSI, 2009). The dam lake causes an increase in the Secchi depth. One of the most important abiotic factors limiting macrophyte development is light penetration in the water. In clean water with high light intensity, the plant size, internode length and leaf length of *S. pectinata* are reduced, while in turbid waters with low light intensity, plant size, internode length and leaf length are increased (Santamaria, 2002). It has been reported that *S. pectinata* is found in running water habitats of less than 2.5 m depth (Kantrud, 1990). According to this, the depth of the sampling stations was suitable for plant growth. The height of the plants varied between 1.43 and 3.1 m in research period. It is thought that the excessive growth of *S. pectinata* in the Kızılırmak means that it has adapted to low light intensity and high salinity so that it has covered the whole river bed and grown up to the surface.

Water temperature at the sampling stations increased from March onwards, and the highest temperature, 14.9°C, was measured in July (*Table 2*). Dissolved oxygen concentrations at the sampling stations varied from 11.1 to 15.2 mg/l, pH from 6.9 to 8.3, and conductivity from 1000 to 1291 µS/cm. The variation in parameters between sampling stations was found not to be statistically significant, but the monthly variations were significant (F and P values of these parameters are; Water temperature F= 45.214, p= 0.00, Dissolved oxygen F= 47.76, p= 0.00, pH F= 63,70, p= 0.00, Conductivity F= 2.20, p= 0.049).

Mean alkalinity in the Kızılırmak was determined as 81.58 ± 18.89 mg $CaCO_3$/l, and the mean total hardness value was 39.10 ± 6.91°FS. the river flows through generally gypsum soils and it has high conductivity. The tolerance of macrophytes for salinity varies. Many freshwater plants are very sensitive to increasing salinity, but *S. pectinata* can grow in saline waters. It is reported that *S. pectinata* is found in waters with a pH of between 6.3 and 10.7, and an alkalinity of 0.018 to 34.7 g/L $CaCO_3$ (Kantrud, 1990). It was found that *S. pectinata* was widespread, and occurred at high alkalinity and pH values, and used bicarbonate effectively as a source of dissolved inorganic carbon (Vestergaard and Sand-Jensen, 2000). Kızılırmak are in the category of very hard waters in terms of total hardness. The chemical characteristics of the water have an important effect on the macrophyte community. The relationships between calcium concentration, hardness, dissolved inorganic carbon and pH affect macrophyte composition (Fox, 1992). It is thought that one of the reasons for the excessive increase in *S. pectinata* in the Kızılırmak are its salt tolerance and its preference for hard waters.

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Table 2. *Monthly changes of physical and chemical water quality parameters in in Kızılırmak river during research (mean ± standard deviation)*

WQ Parameters	March n=4	April n=4	May n=4	June n=4	July n=4	August n=4	Septem. n=4	October n=4	November n=4	December n=4	Mean±SD n=44*
Depth (cm)	125±31	246±11	227±33	250±14	205±17	220±8	-	268±30	248±13	235±24	226±44
Secchi D (cm)	98±17	69 ±8	91±6	88±9	108±12	110±8	-	50±0	96±11	103±9	90±20
Wat Temp (°C)	4.1±0.1	10.9±0.4	10.7±0.4	11.8±0.1	14.9±0.1	14.3±3.0	11.7±0.4	8.9±0.5	7.9±0.1	6.9±0.1	10.2±3.3
pH	7.15±0.19	8.09±0.02	7.93±0.10	7.96±0.10	8.32±0.02	8.25±0.02	8.03±0.05	8.26±0.04	8.21±0.02	7.96±0.11	8.01±0.33
EC (µS/cm)	1264±26	1132±59	1178±70	1113±84	1210±42	1137±60	1181±47	1167±47	1190±62	1209±71	1178±67
D. Oxygen (mgO_2/l)	12.7±0.3	11.9±0.2	12.2±0.2	11.8±0.4	13.1±0.1	11.5±0.4	11.6±0.3	14.8±0.4	12.9±0.3	11.9±0.2	12.4±0.9
NH_4-N (mg/l)	1.16±0.08	0.99±0.07	0.93±0.06	1.0±0.06	0.89±0.09	0.90±0.08	0.90±0.05	1.15±0.12	0.93±0.04	0.79±0.11	0.96±0.13
NO_2-N (mg/l)	0.08±0.01	0.03±0.01	0.02±0.01	0.01±0.0	0.01±0.0	0.01±0.0	0.02±0.0	0.13±0.01	0.04±0.01	0.04±0.0	0.04±0.02
NO_3-N (mg/l)	2.94±0.59	1.75±0.23	1.83±0.28	1.67±0.43	1.64±0.32	1.90±0.21	2.05±0.43	3.14±0.73	1.88±0.05	1.23±0.08	2.0±0.66
PO_4-P (mg/l)	0.02±0.003	0.01±0.0	0.011±0.0	0.01±0.0	0.01±0.0	0.02±0.0	0.02±0.01	0.03±0.01	0.02±0.01	0.01±0.0	0.02±0.01
TP (mg/l)	0.035±0.01	0.024±0.0	0.028±0.0	0.023±0.0	0.033±0.0	0.042±0.0	0.028±0.0	0.051±0.0	0.04±0.0	0.032±0.0	0.034±0.01
Hardness (°FS)	39.8±2.2	38.5±1.9	36.8±0.5	37.0±1.8	38.9±2.6	36.0±6.0	35.3±3.9	28.7±2.3	50.9±4.7	49.1±3.7	39.1±6.9
Alkalinity ($mgCaCO_3/l$)	80.0±16.3	85.0±10.0	72.5±18.9	77.5±5.0	85.0±19.2	75.0±10.0	87.5±9.6	117.5±17.1	70.0±20.0	65.0±10.0	81.5±18.9
Flow** (m^3/s)	62.6	174.7	95.3	58.9	56.2	75.5	77.8	75.3	97.6	94.1	83.7

*the number of repetitions, **Monthly values of outflow after Yamula Dam was taken from General Directorate of State Hydraulic Works

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PCA of plant biomass and environmental parameters showed that water temperature, pH, total phosphorus and alkalinity affected plant biomass positively. The first two axes of PCA accounted for 80% of the total variance (*Fig. 3*). But plant biomass was negatively affected with flow rate. It was found that in the River Wye, there was a negative correlation between the speed of flow and the macrophyte cover and biomass. Similarly, in Switzerland, it was found that when the flow of a river was below 0.3 m/s, the macrophyte cover increased, but that it decreased at higher rates of flow (Wade, 1994). Increases in flow rates cause reductions in macrophyte biomass. When flow rate increased, biomass and shoot density were observed to decrease (Chambers et al., 1991). Monthly mean values of outflows from the Yamula Dam in research period are given in *Table 2*. The mean outflow rate from the Yamula dam during the study period was 83.7 m^3/s, and the highest flow was in April at 174.7 m^3/s (DSI, 2009). According to measurements made in April on the 4th sampling station, the flow rate was 0.5 m/s. It has been reported that *S. pectinata* spreads in waters with a flow rate of less than 1 m/s (Kantrud, 1990). Water flow is one of the most important abiotic factors affecting the location and composition of plant communities in rivers.

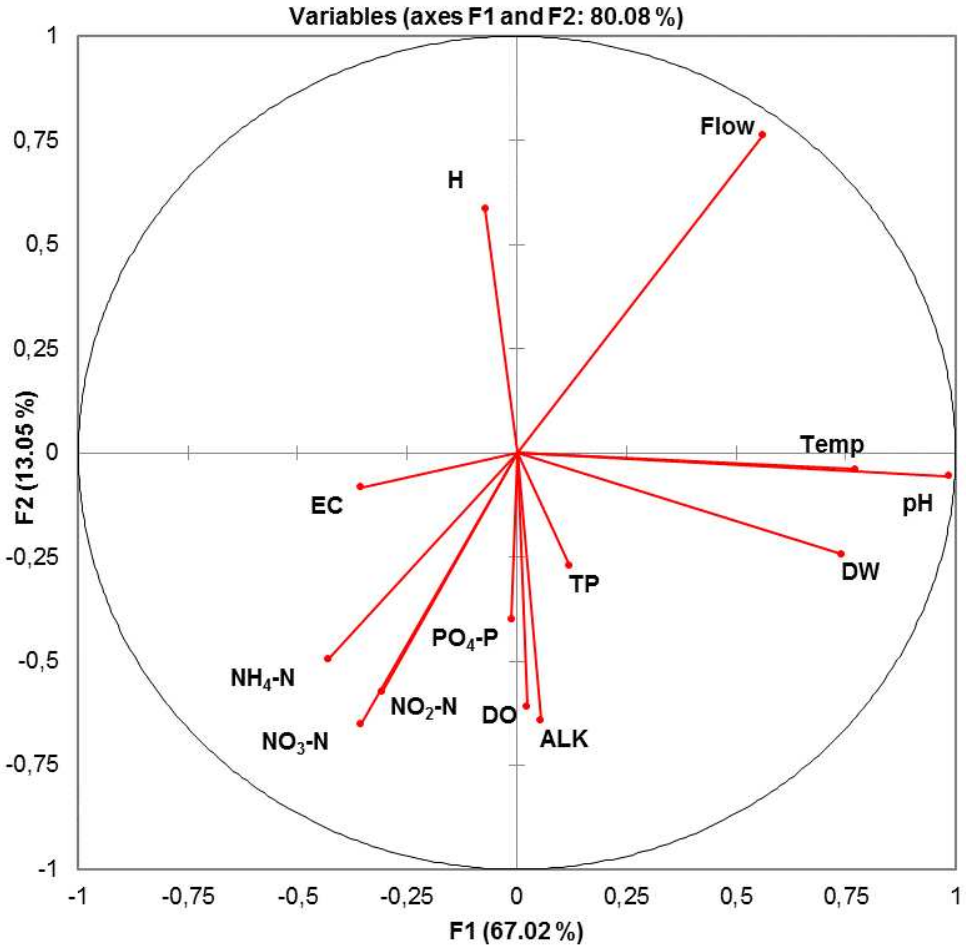

Figure 3. *PCA of macrophyte biomass and environmental parameters in Kızılırmak. DW: dry weight of plants, Temp: water temperature, EC: conductivity, DO: dissolved oxygen, ALK: alkalinity, H: hardness, TP: total phosphorus, PO_4-P: orthophosphate, NO_2-N: nitrite-nitrogen, NO_3-N: nitrate-nitrogen, NH_4-N ammonium-nitrogen*

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During the period of the study, the above-ground biomass of *S. pectinata* increased from March to August (*Fig. 4*). In September, the river bed was emptied to fill the Bayramhacılı dam, and so the amount of plant biomass declined. However, in October, November and December, it was found to be high again. Over the study period, the mean plant biomass varied from 226 to 1086 g dry weight/m^2 and the mean plant biomass was found to be 720.9 ± 288.0 g/m^2. *S. pectinata* biomass that it varies from >5 to 1988 g dry weight/m^2, and that plant growth is limited by turbidity, other macrophytes and competing species, water flow, wind, wave movement, changes in water depth, pollution and eutrophication, grazing by fish and water birds, excessive salinity, sediment, river bottom construction and toxic materials (Kantrud, 1990). The variation in *S. pectinata* biomass by sampling stations was found to be statistically insignificant, but the monthly variation was found to be significant (F= 21.52, p= 0.00) (*Fig. 4*). According to the Tukey test, the highest mean biomass was recorded in November (significanly higher than March, April, May, June, July and September, p<0.05). The variations between the plant biomass in June, July, August, September, October and December were not statistically significant.

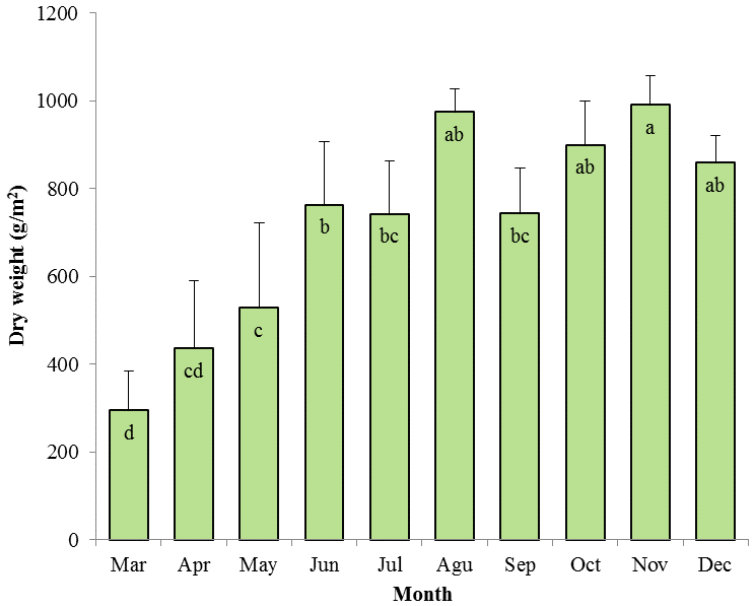

Figure 4. *Monthly mean values of plant biomass (g dry weight/m^2) and standard deviations (vertical lines on bars) (n=4) in Kızılırmak. Variations of the mean biomass with different letters (a, b, c) at each bar are statistically significant according to the post-hoc, Tukey test (p<0.05)*

The distribution of the aquatic plant *S. pectinata* in the Kızılırmak was found to be predominant (1 = very rare, 2 = rare, 3 = widespread, 4 = common, 5 = predominant). Because only one species was predominant in the Kızılırmak, macrophyte indices could not be used in determining ecological quality. The indices used for this purpose are applied when there are at least three, or two, species (Schneider and Melzer, 2003; Kuhar et al., 2011). Nevertheless, according to Melzer (1999), *S. pectinata* is in indicator group 4 which indicates heavy organic pollution in lakes. Kuhar et al. (2011) developed River Macrophyte Index to determine the ecological condition of rivers, and

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reported *S. pectinata* to be in group B, with ecologically medium level pollution load. The ecological status of Kızılırmak river in the same locations (R16 and R17) were also found as medium according to diatom indexes (Çetin et al., 2021). Kızılırmak and its tributaries were subjected to several impacts. *S. pectinata* is tolerant of eutrophication and salinity. It is thought that its excessive growth in the Kızılırmak along with other factors is an indicator of organic pollution.

Among the aquatic plant control methods, biological control and chemical control methods are not suggested for Kızılırmak river. Biological control methods possess the danger of spreading non-targeting organisms. The use of grass carp was not suggested because of the spreading non-native fish to river ecosystem. It was reported that herbicides can control *S. pectinata* in static or slow flowing rivers. But, the turions are unaffected and regrow after the herbicide degraded (CEH, 2004). Chemical methods such as the herbicide application is not suitable for Kızılırmak river because of high flow rate and the use of river water as drinking water in the river course. Pondweeds can be cut but regrowth is often rapid after early season cuts (CEH, 2004). The possible aquatic plant control method for the river may be physical/mechanical control by the means of harvesters or water level/flow rate control by the means of dams.

Conclusions

In conclusion, *S. pectinata* in the Kızılırmak constitutes a problem for the recreational use of the river. The plant was not previously found in the section of the river passing through the district of Avanos, but now forms a dense plant cover. It is thought that the main reason for the excessive growth of the *S. pectinata* is regulated flow by dams. Physical/mechanical methods are recommended for the control of aquatic plants in the Kızılırmak. However, the most suitable time and cutting periods, the design of machinery resistant for the high flow, and the uses for the cut plant material need further investigation. Future studies may focus on the location-specific applicability of the control methods based on field test.

Acknowledgements. This study was funded by The Scientific and Technological Research Council of Turkey (TUBITAK), Project No: 110Y210. We are grateful to Vet. Ahmet Demircioglu and Avanos Municipality for their help in the field studies.

Author contribution. Nilsun Demir and Eniz Ozge Balcı conceptualized and planned the study. Nilsun Demir, Eniz Ozge Balcı and Ozden Fakıoglu conducted the field studies and laboratory studies. Ozden Fakıoglu conducted nutrient analysis. Emre Keskin conducted phylogenetic analysis and Tolga Coşkun conducted statistical analysis and laboratory analysis. Nilsun Demir interpreted the results and wrote the text with contributions of all authors.

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DOI: http://dx.doi.org/10.15666/aeer/1905_40554067

SPATIAL AND TEMPORAL VARIATIONS IN THE GROWTH AND PHOTOSYNTHESIS OF SUBMERGED MACROPHYTES IN SONGKHLA LAGOON

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(Received 18th May 2021; accepted 12th Aug 2021)

Abstract. Increases in organic matter and temperature are major threats to benthic vegetation survival in the Songkhla Lagoon in Southern Thailand, leading to a loss of ecosystem functions and altered biodiversity. This project aimed to investigate the spatial and temporal variations in photosynthesis, growth, and carbon capture potential of submerged macrophytes in Songkhla Lagoon, including potential inhibitory effects of macrophytes on phytoplankton bloom. Percentage cover, photosynthetic performance, morphology and organic content of submerged macrophytes were investigated *in situ* bimonthly for a year. Environmental parameters such as organic matter content and carbon and nitrogen contents in sediment, nutrients, water temperature, and light were assessed. Free floating macrophytes, submerged macrophytes and macroalgae were observed from May 2017 to May 2018. CCA indicates that the main factors influencing submerged macrophytes were chlorophyll *a* concentration and dissolved oxygen concentration in the water, depth, organic carbon in sediment, and phosphate concentration in water. This project suggests that the relationship between environmental parameters and composition of macrophytes can be a helpful tool for predicting ecosystem processes under future climate change scenarios. This study further suggests that there is a potential role for macrophytes as bioindicators for the ecological status of Songkhla Lagoon that contributes to the development of a sustainable lake management plan.
Keywords: *bioindicator, organic content, CCA, lagoon management, anthropogenic change*

Introduction

During the past several centuries since the industrial revolution, human activities have caused strong changes in the structure and function of their environment (Smith et al., 1999). The growth of human population has increased demands on both aquatic and terrestrial ecosystems altering hydrological processes and geochemical cycles of carbon (C), nitrogen (N) and phosphorus (P) (Vitousek et al., 1997a; Smith et al., 1999). Large input of N and P into terrestrial systems from agriculture (from the use of fertilizers) and other human activities (e.g. use of detergents, sewage) have increased the nutrient supply in aquatic systems by: 1) accumulating in sediments, and 2) moving from land into surface waters and ground waters (Vitousek et al., 1997b; Smith et al., 1999) which can lead to eutrophication. Steffen et al. (2015) proposed P and N bounds for the earth system at a sustained flow of 11 Tg P y^{-1} and 62 Tg N y^{-1}, respectively; however, the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_40694101

current global rates of P and N release (22 Tg P y^{-1} and 62 Tg N y^{-1}) already exceed these limits, making eutrophication the major problem in aquatic systems globally (Steffen et al., 2015). Furthermore, the anthropogenic release of greenhouse gases e.g. carbon dioxide (CO_2) into the atmosphere has led to increased average global temperatures through the trapping of heat by the Greenhouse Effect. As a consequence, surface water temperature has increased and is predicted to increase in the future. Terrestrial, marine and freshwater ecosystems and their resident organisms are expected to be affected by climate change (IPCC, 2007). It is indicated that eutrophication problems will be worsened by the combination of climate change and anthropogenic activities in lakes and estuaries, as this is predicted to occur in many areas in the world (Moss et al., 2010).

Eutrophication is a condition of increased nutrient supply caused by human activities and natural processes as the lakes age and become filled with sediments (Smith et al., 1999). Eutrophication and sediment runoff have usually been the main problems in water quality management of lakes and reservoirs globally, including the Songkhla Lagoon in Southern Thailand (Sompongchaiyakul et al., 2004; Sompongchaiyakul and Sirinawin, 2007; Chesoh and Lim, 2008). Nutrient enrichment induces excessive growth of microalgae, macroalgae and aquatic plants and results in the disruption of ecosystem functions, by depleting the oxygen needed for fish to survive and reducing light penetration to the lake bottom (Smith et al., 1999). Increased temperature can also lead to a higher degree of eutrophication as it promotes growth of algae and aquatic plants and induces algal blooms (Yang et al., 2008). Eutrophication, accumulation of organic matter and increased temperature are also the major threats to benthic vegetation survival in coastal and freshwater environments, leading to a loss of ecosystem function and altered biodiversity (Soana, 2012).

Macrophytes play an important role as ecosystem engineers by acting as habitat structure and refugia for aquatic organisms, maintaining high physical and biological diversity, and ecosystem functions and providing nutrient cycling capacity, and (Sand-Jensen, 1997; Wigand et al., 2000; Cronk and Fennessy, 2001; Qiu et al., 2001). Due to their capacity for nutrient cycling and prevention of phytoplankton blooms, macrophytes can potentially be used as natural tools for sustainable water quality improvement in lakes and reservoirs (Guo-feng et al., 2014; Lone et al., 2014). The growth, photosynthesis and reproduction of submerged macrophytes can be affected by temperature (Barko et al., 1991; Chotikarn et al. 2021a), light (Korschgen et al., 1997; Chotikarn et al. 2021b), and high organic loads in sediments (Barko and Smart, 1983) due to several coupled biological, physical and chemical processes that modify benthic system (Raun et al., 2010). Jiang et al. (2018) showed that Maximum quantum yield (F_v/F_m) of six submerged macrophytes decreased in midday and this was induced by high light. High and low light intensity, which depend on depth, affected growth and biomass of submerged macrophytes by chlorophyll contents changes (Jin et al., 2020). Dissolved organic carbon (DOC) and humic substances (HS) limit macrophyte photosynthesis at greater depths and lead to lower macrophyte abundance and species diversity (Reitsema et al., 2018). Faster organic decomposition and higher oxygen consumption from microbial activity have been reported in organic enriched sediment, which can lead to anoxia in sediments, decreasing plant growth coupled with reduction in chlorophyll content (Atapaththu et al., 2018), greater formation of organic acids and phytotoxic compounds (Pezeshki, 2001; Colmer, 2003), and can induce physiological stresses such as shorter roots and reduced growth (Raun et al., 2010; Møller and Sand-

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Jensen, 2011; Atapaththu et al., 2018). In addition, sediment particle size distribution and physical properties in organic enriched sediments have significant effects on nutrient-holding capacity (Kuriata-Potasznik et al., 2018). Therefore, the different responses of each species of macrophytes to organic enriched sediment can cause a change in species composition in benthic vegetation.

Songkhla Lagoon is a tropical estuarine lagoon system located on the eastern side of the southern Thai Peninsula (Pongpiachan et al., 2019) that not only supports biodiversity and ecosystem functions but also a number of people whose livelihoods depend on that biodiversity via several ecosystem services such as fishery, aquaculture, and tourism (Hue, 2018). However, Songkhla Lagoon is currently experiencing serious water pollution and eutrophication due to human activities, such as release of pesticides and fertilizers from agricultural activities, and nutrient inputs from shrimp farming and livestock waste (Sompongchaiyakul et al., 2004; Pornpinatepong et al., 2010). This could lead to the loss of valuable ecosystem services and functions e.g., carbon sequestration, controlling algal blooms, and nutrient cycling. Several species of submerged macrophytes were observed in Songkhla Lagoon including *Ceratophyllum demersum*, *Cladophora* sp., *Najas malesiana*, *Najas marina*, *Najas graminea*, *Hydrilla verticillata*, and *Potamogeton malaianus* (Thongkao et al., 2001). Eutrophication is a complex process and is likely to affect each organism in each system differently due to interactions with other factors such as temperature, nutrient loading and light. Hence, it is essential to identify the effects of several environmental factors on these macrophytes in order to successfully manage the lagoon (Howarth et al., 2000).

Due to eutrophication in Songkhla lagoon, ecosystem management is needed to prevent eutrophication. However, there is lack of knowledge about spatial and temporal variations, relation and responses of macrophytes to environmental factors, as well as the roles of submerged macrophytes in nutrient cycling, controlling algal bloom, and carbon storage (Sompongchaiyakul et al., 2004; Sompongchaiyakul and Sirinawin, 2007; Chesoh and Lim, 2008). Thus, this study aims to investigate the spatial and temporal variations in ecophysiology of submerged macrophytes in Songkhla Lagoon, the effects of organic enrichment in sediment on photosynthesis of submerged macrophytes, and the roles of submerged macrophytes in nutrient cycling, controlling algal blooms, and carbon storage. This project could allow for identifying the main driver for growth and photosynthesis of submerged macrophytes and predicting their performance under changing environmental conditions and would allow for the development of sustainable lake management plan and eutrophication model.

Materials and methods

Experimental design

To investigate: 1) the spatial and temporal variations in photosynthesis of submerged macrophytes, 2) the effects of organic enrichment in sediment on photosynthesis of submerged macrophytes, 3) the carbon capture potential of submerged macrophytes, and 4) the inhibitory effects of macrophytes on phytoplankton blooms in 3 sites in Songkhla Lagoon, with sites 1, 2 and 3 at near, middle, and far from the lake shore, respectively. Photosynthetic performance (photosynthetic efficiency, leaf pigment; $n = 3$) and morphology (leaf length, leaf dry weight; $n = 10$) of submerged macrophytes from high and low organic sediment sites in Songkhla Lagoon were investigated *in situ* bimonthly for a year covering the rainy and summer seasons. High and low organic

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sediment sites in Songkhla Lagoon were selected for preliminary data on level of organic sediments in Songkla Lagoon (*Fig. 1*).

Three replicates of submerged macrophytes and sediments ($n = 3$) from each site and time of collection were collected for organic content and carbon (C) and nitrogen (N) content analyses. Sediments ($n = 3$) from each location and time of collection were collected for particle size analysis using a laser particle size analyser (LPSA) (LS230, Beckman Coulter, USA). Water (2 L) ($n = 3$) from each location and time of collection was collected for phytoplankton biomass and nutrient (phosphate and nitrate) analyses. Dissolved oxygen, light intensity, air and water temperature, salinity, pH, turbidity, and rainfall data were collected. Rainfall, maximum and minimum sea surface temperature (SST), and wind speed in Kukud, Songkhla Lagoon area were collected from State of the Ocean (2018).

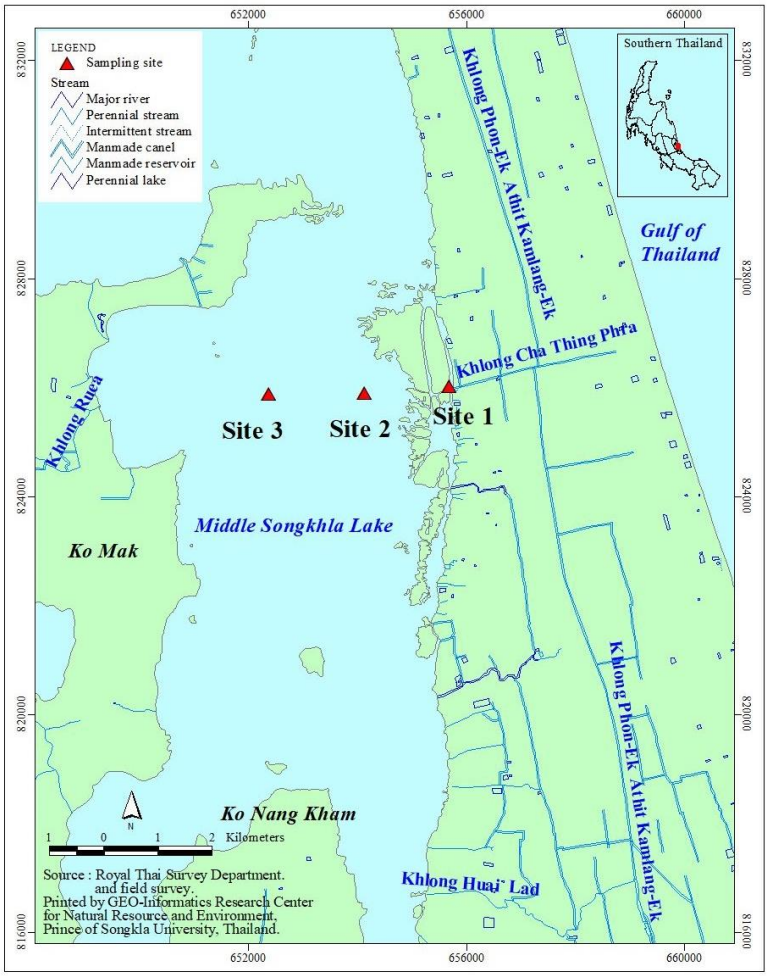

Figure 1. *Map showing Site 1 (7°28'18.2"N 100°24'37.8"E), Site 2 (7°28'16.7"N 100°23'46.1"E) and Site 3 (7°28'13.0"N 100°22'51.7"E) in Kukud, Songkhla Lagoon*

Percentage cover of submerged macrophytes

Percentage cover of submerged macrophytes from each time of collection was randomly estimated in 50 × 50 cm^2 quadrats ($n = 3$). Macrophytes along water body in quadrat were estimated in percentage by visual census, modified from Mellors (1991).

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Photosynthetic efficiency

The photosynthetic performances of each species of submerged macrophyte found at each site and time of sampling were investigated by determining Rapid Light Curves (RLCs) ($n = 3$) at the same time each day, using a 6-millimeter diameter fiberoptic probe connected to a pulse-amplitude modulated (PAM) fluorometer (MINI-PAM, Walz, Germany). RLCs with 9 increasing actinic light intensities (0, 149, 215, 383, 450, 806, 1074, 1658, 2576 μmol photons m^{-2} s^{-1}) were applied, with 0.8 s saturating pulses (>4500 μmol photons m^{-2} s^{-1}) between each actinic light intensity every 10 s. Effective quantum yield of PSII ($\Delta F/F_M'$; Schreiber, 2004), maximum relative electron transport rate ($rETR_{max}$), minimum saturating irradiance (I_k) and initial slope (α) of RLCs were calculated using the curve fitting protocols in Ralph and Gademann (2005).

Leaf pigments

Photosynthetic pigment concentration (chlorophyll (Chl) *a* and *b*) of submerged macrophytes were determined using the standard spectrophotometric method of Ritchie (2006). Chlorophyll *a* and *b* (μg g^{-1} fresh weight (fw)) were extracted by homogenizing samples in 4 ml of 90% acetone at 4 °C for 24 h. Samples ($n = 3$) were centrifuged at 1500 *g* for 10 min and the supernatant placed into a quartz cuvette in a spectrophotometer (SP8001, Metertech Inc., Taiwan), and the absorbance was measured at 647, 664 and 750 nm.

Organic content in macrophytes and sediments

Macrophyte samples ($n = 3$) were oven dried at 105 °C and ground to a particle size of less than 1 mm. 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). Sediment samples were oven dried at 105 °C and a 1.0 g dried sample was ashed at 550 °C for 4 h. Organic matter in macrophytes was determined using the data obtained from the ashed samples on mineral matter (MM) (*Eq. 1*), as the organic matter (OM) (*Eq. 2*) and organic carbon (OC) (*Eq. 3*) contents were computed using the following equations:

$$\%MM = (AW / DW) \times 100 \quad \text{(Eq.1)}$$

$$\%OM = 100 \times (DW - AW)/DW \quad \text{(Eq.2)}$$

$$\%OC = \%OM / 1.724 \quad \text{(Eq.3)}$$

where AW and DW are ash weight and dry weight of the sample, respectively.

Sediment composition

Sediment samples ($n = 3$) were collected using a 60 mm corer for grain size distribution and organic content analysis. Texture of each sample (grain size distribution) was investigated using a laser particle size analyzer (LS 230, Beckman Coulter, USA). Sediment < 2 mm in diameter was classified into eight size classes (clay, silt, fine sand, and coarse sand at < 3.9, 3.9-62.5, 62.5-500, and 500-2000 μm, respectively).

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Carbon and nitrogen contents in macrophytes and sediment

Carbon and nitrogen contents in sediments were analyzed with a standard method using a carbon nitrogen analyser (CN628, Leco, USA) at Office of Scientific Instruments and Testing (OSIT), Prince of Songkla University, Thailand.

Carbon capture potential

Macrophyte samples were dried at 60 °C for 96 h in an oven and weighted. All the samples were ground in a laboratory fine grinder and then passed through a 40-mesh screen before further analysis. Carbon content in each macrophyte species was analyzed by carbon nitrogen analyser (CN628, Leco, USA). Ash content was analyzed by heating at 600 °C in a muffle furnace (FHX, DAIHAN, China). Higher heating values (HHV_{ult}) were estimated from the carbon composition using the ultimate analysis formula (Sheng and Azevedo, 2005).

$$HHVult\ (MJ/kg) = 0.3259\ (Carbon\ content\ (\%)) + 3.4597 \quad (Eq.4)$$

In addition, higher heating values (HHV_{prox}) were also estimated from the carbon composition using the proximate analysis formula (Sheng and Azevedo, 2005).

$$HHVprox\ (MJ/kg) = 19.914 - 0.2324\ (Ash\ (\%)) \quad (Eq.5)$$

Carbon and ash contents ($n = 3$) for each macrophyte species were analyzed from the equal mixture of oven dried samples collected each time at each plot in each site.

Phytoplankton biomass

Phytoplankton biomass was determined as chlorophyll *a* concentration in water column. Water samples (150 ml) ($n = 3$) were filtered through 25-mm GF/F filters under dim light and pigments on filters were extracted with 100% acetone. The mixture was sonicated for 45 s on ice and the extracts were stored at 0 °C in the dark for 30 min. The extracts were then centrifuged at 2000 *g* for 5 min. Chlorophyll *a* in the supernatant was determined spectrophotometrically using equations of Jeffrey and Humphrey (1975) (Doblin et al., 1999).

Statistical analysis

Two-way ANOVA tests were used to test for significant differences among sites over time in chlorophyll fluorescence parameters ($\Delta F/F_M'$, $rETR_{max}$, I_k, α), leaf pigments, morphological properties, species composition, organic content, C and N contents, particle size distribution, nutrient, chlorophyll *a* in water column, and other physical parameters. All tests were performed with a significance level of 95%, and Tukey's honestly significant difference *post hoc* tests were used to identify the statistically distinct groups. If data did not meet the assumptions of normality (Kolmogorov-Smirnov test) and equal variance (Levene's test), the data were transformed using square root or $\log_{10}$. If transformed data did not meet the assumptions, non-parametric tests were used. Canonical Correspondence Analysis (CCA) was used to assess the relationship between biological and environmental variables among sites and times using MVSP (Kovach Computing Services).

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Results

Environmental parameters

Environmental data from State of the Ocean (2018)

Lowest rainfall was found in March 2018, while highest rainfall was observed in February 2018. Average SST in Kukud, Songkhla Lagoon from May 2017 to May 2018 ranged from 28 to 31 °C. Lowest average SST was found in February 2018 (28.318 ± 0.106 °C/24 h), while highest average temperature was observed in May 2018 (31.113 ± 0.072 °C/24 h) (*Table 1*). Rainfall and SST data indicate that March to July represented dry season, while October to February was the rainy season in this area. Wind speed ranged from 1.2 to 2.3 km hr^{-1}.

Water quality

Water depth and transparency showed spatial variation ($P < 0.001$) (*Fig. 2a, b*) in which water level was the shallowest in site 1 and the deepest in site 3 ($P < 0.001$); and the water level at all sites increased with the highest rainfall in February 2018 while transparency was highest at site 3 and lowest at site 1 ($P < 0.001$). Water temperature and SST showed similar trends (*Table 1*) at all sites (*Fig. 2c*) being highest at site 1 and lower at sites 2 and 3 ($P < 0.001$).

Table 1. *Mean ± SE of rainfall, maximum and minimum sea surface temperature (SST), and wind speed in Kukud, Songkhla Lagoon area from May 2017 to May 2018 (State of the Ocean, 2018) (mean ± SE)*

Times	Rainfall (mm/24 h)	Max SST (°C/24 h)	Min SST (°C/24 h)	Wind speed (km/h)
May: 2017	9.30 ± 1.98	30.85 ± 0.09	30.70 ± 0.09	2.06 ± 0.22
July: 2017	3.11 ± 0.57	30.69 ± 0.03	30.54 ± 0.03	1.61 ± 0.26
Oct: 2017	13.67 ± 2.07	30.37 ± 0.07	30.22 ± 0.07	1.97 ± 0.35
Feb: 2018	33.30 ± 9.99	28.47 ± 0.08	28.32 ± 0.11	2.32 ± 0.29
Mar: 2018	1.40 ± 0.00	29.93 ± 0.08	29.78 ± 0.08	1.29 ± 0.24
May: 2018	12.29 ± 2.54	31.11 ± 0.07	30.96 ± 0.07	1.19 ± 0.23

Temporal variation was seen in water temperature with the lowest water temperature found in March 2018 and the highest water temperature observed in May 2018 ($P < 0.001$). Total Dissolved Solid (TDS) showed temporal variation ($P < 0.001$) with the lowest value in February 2018 and the highest in May 2018 (*Fig. 2d*). Water salinity showed temporal variation ($P < 0.001$) with the lowest salinity in March 2018 and the highest in May 2018 (*Fig. 2e*). Water pH showed both spatial and temporal variations ($P < 0.001$) at sites 2 and 3 being higher from May to October 2017 and lower from February to May 2018 (*Fig. 2f*).

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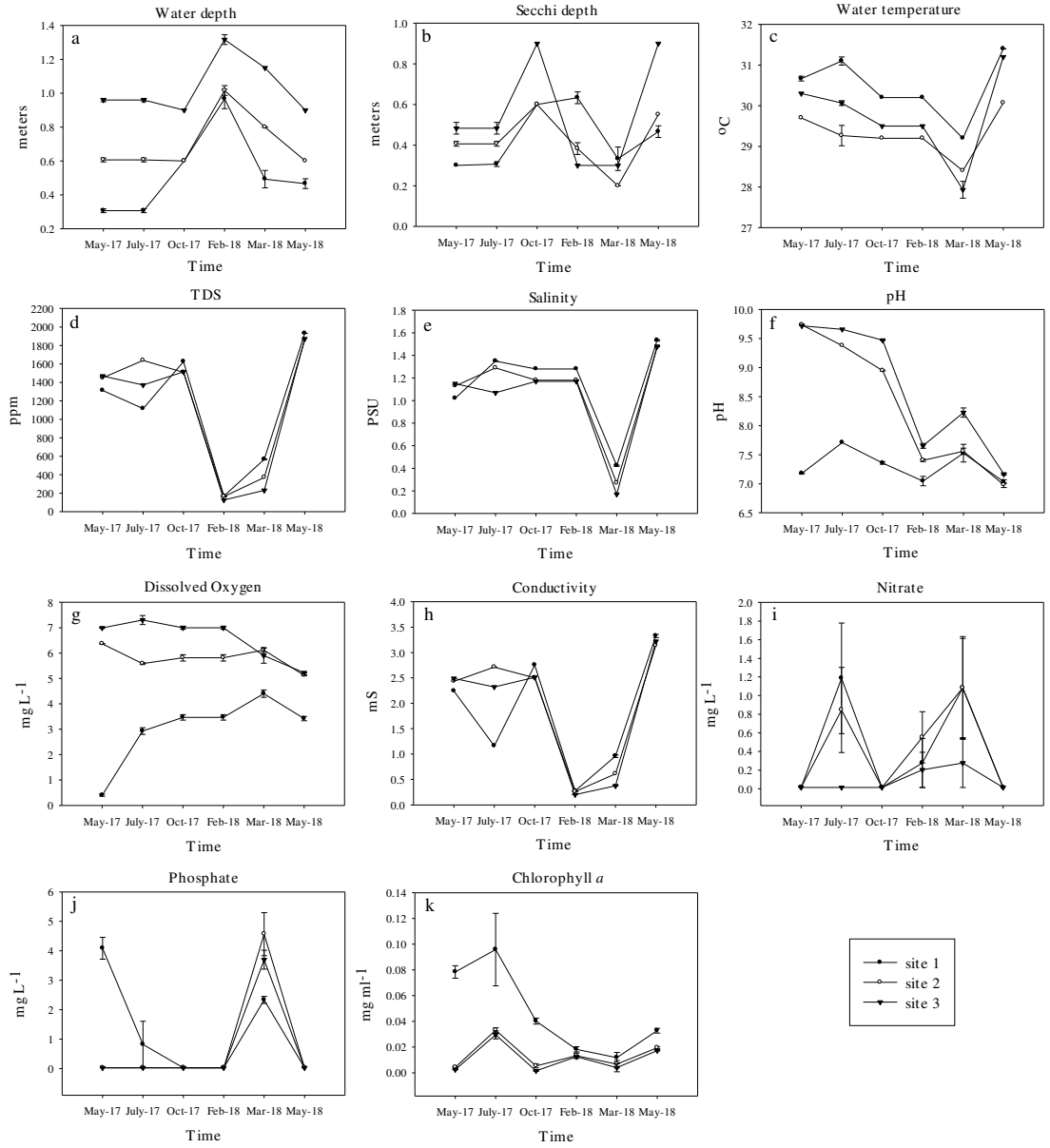

Figure 2. *Water depth (a), secchi depth (b), water temperature (c), TDS (d), salinity (e), pH (f), dissolved oxygen (g), conductivity (h), nitrate (i) and phosphate concentration (j) and chlorophyll a concentration (k) in Kukud, Songkhla Lagoon area from May 2017 to May 2018 (Data presented as mean ± SE)*

There were temporal variations in dissolved oxygen concentration (DO) ($P < 0.001$) at site 1, while sites 2 and 3 had higher DO from May 2017 to March 2018 and slightly decreased level in May 2018. DO at site 3 was significantly higher than at sites 1 and 2 ($P < 0.001$) (*Fig. 2g*). Water conductivity showed temporal variations ($P < 0.001$) at all sites and the highest conductivity was observed in May 2018, while the lowest conductivity was found in February 2018 ($P < 0.001$) (*Fig. 2h*). There was no significant difference in nitrate concentration among the sites (1, 2 and 3) at any time of collection ($P > 0.05$). The phosphate concentration was significantly higher at sites 1 and 2 ($P = 0.003$ and 0.028) (*Fig. 2i, j*). Chlorophyll *a* concentration in water was

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significantly higher at site 1 ($P < 0.001$) and in May and July 2017 ($P < 0.001$), which indicates elevated concentration of phytoplanktons and possible eutrophication during the dry season (*Fig. 2k*).

Sediment characteristics

Organic matter (OM) and organic carbon (OC) in sediment from site 1 were significantly higher than at sites 2 and 3 at all times of collection ($P < 0.001$). Site 2 also had significantly higher OM and OC than site 3 ($P = 0.014$). There was no significant difference in OM or OC among the times of collection ($P > 0.05$) except that in May 2017 these were significantly higher than at other times ($P < 0.001$) (*Fig. 3*).

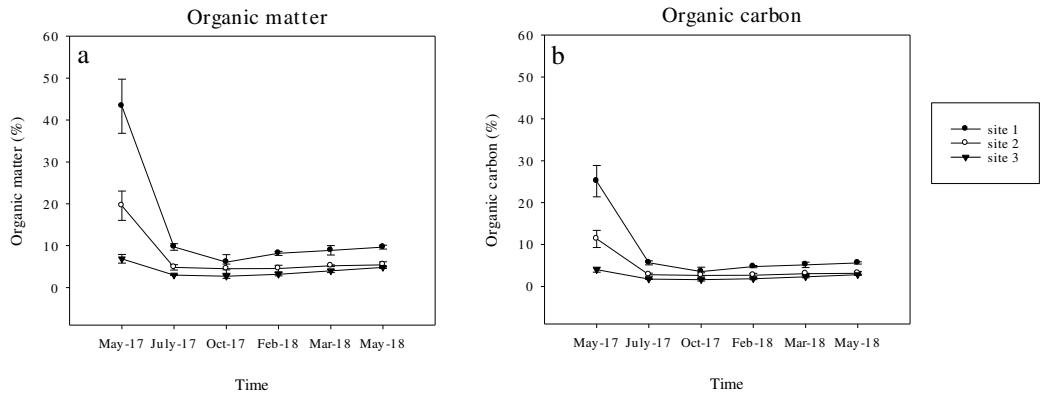

Figure 3. *Organic matter (a) and organic carbon (b) in sediment from sites 1 – 3 (Near, Mid, Far) in Kukud, Songkhla Lagoon area from May 2017 to May 2018 (data presented as mean ± SE)*

Carbon (C) and Nitrogen (N) contents in sediment from site 1 were significantly higher than at sites 2 and 3 for all times of collection ($P < 0.001$) except for October 2017 and February 2018 ($P > 0.05$). There was no significant difference in C or in N among the times of collection in sites 2 and 3 ($P > 0.05$) (*Fig. 4a, b*). C:N ratios at site 1 were significantly higher than at sites 2 and 3 for all times of collection ($P < 0.001$) (*Fig. 4c*). At site 1, C and N contents were significantly higher in May, July 2017 and March and May 2018 ($P < 0.001$), while C:N ratio was significantly higher in October 2017 and February 2018 ($P < 0.001$) (*Fig. 4c*) (*Table A2*).

Sediment in Kukud, Songkhla Lagoon was classified as silt. Large proportions of silt, clay, fine sand and coarse sand were observed. There were no significant differences in percentages of clay, silt, fine sand and coarse sand among sites and times of collection ($P > 0.05$) (*Fig. 5*).

Similarity of environmental parameters among sites and times of collection

The analysis of similarity of environmental parameters among sites and times of collection was done by using Cluster Analysis (CA). All environmental parameter data including wind speed, rainfall, sea surface temperature, water temperature, depth, secchi depth, salinity, dissolved oxygen concentration, pH, nitrate, phosphate, Chl *a* concentration, conductivity, TDS, organic matter and organic carbon, C and N contents, C:N ratios in sediments, and sediment particle size were used in the analyses. The

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results show two distinct clusters with 80.10% similarity. First cluster is T1Near, which had different environmental parameters from the other sites and times. Second cluster (Cluster 2) had 87.06% similarity and was separated into 2 clusters: Cluster 3 with 88.50% similarity (T1Mid, T1Far, T3Mid, T3Far, T2Mid, T2Far, T3Near, T6Near, T6Mid, T6Far, T4Near, T4Mid and T4Far) and Cluster 4 with 88.91% similarity (T2Near, T5Near, T5Mid and T5Far).

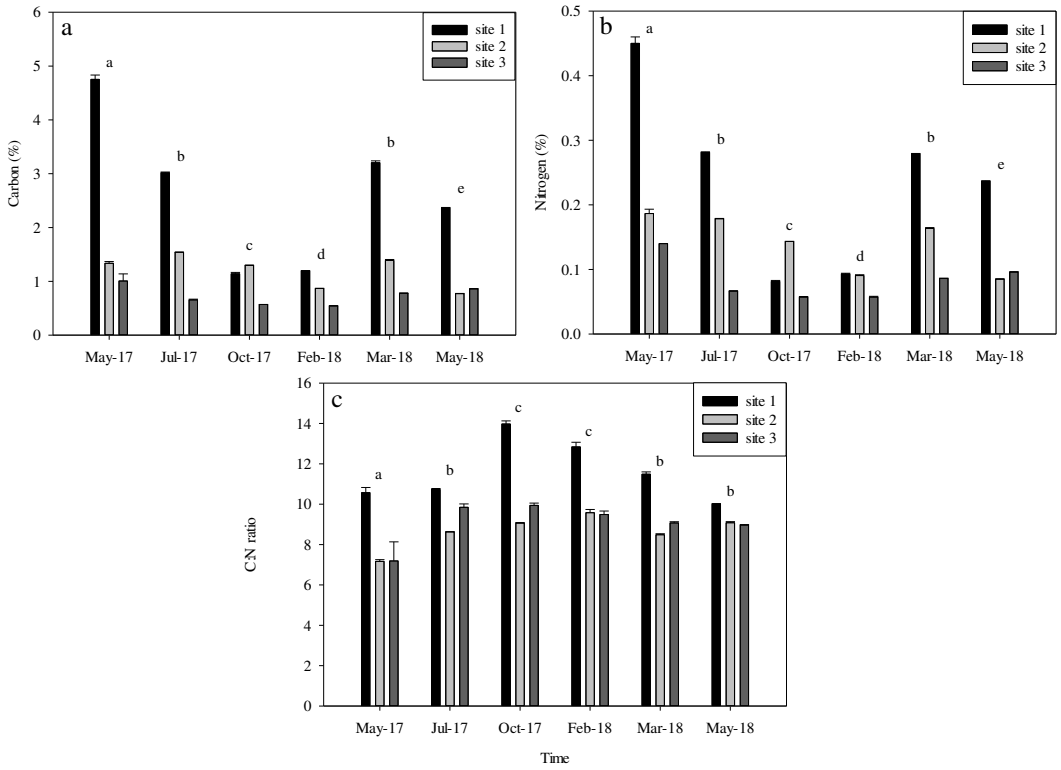

Figure 4. *Carbon (C; a) and nitrogen (N; b) contents and C:N ratios in sediments from sites 1 – 3 (Near, Mid, Far) in Kukud, Songkhla Lagoon area from May 2017 to May 2018 (data represent mean ± SE)*

When the similarity of environmental parameters among times was analyzed regardless of study site, we found that May 2017 was distinct from the other times of collection (Cluster 1 with 87.06% similarity). Cluster 2 (March 2018) had 89.67% similarity and cluster 3 (July and October 2017 and February and May 2018) had 90.48% similarity. Environmental parameters in July and October 2017 and May 2018 had 95% similarity. This indicates temporal variations in the environmental parameters among observation times.

When the similarity of environmental parameters among sites was analyzed regardless of time, the results showed two distinct clusters with 92.11% similarity where site 1 (Near) was different from the other sites. Site 2 (Mid) and 3 (Far) had 97.01% similarity.

Percentage cover of submerged macrophytes

Temporal and spatial variations in percentage cover and composition of free floating and submerged macrophytes and macroalgae were observed in this study (*Fig. 6*). Eight

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species were observed namely *Cladophora* sp., *Ceratophyllum demersum, Salvinia cucullata, Potamogeton malaianus, Najas marina, Najas graminea, Chara zeylanica* and *Elodea Canadensis* during May 2017 to May 2018. Highest macrophyte cover was found in site 3, while site 1 had a significantly lower percentage cover of submerged macrophytes than sites 2 and 3 ($P < 0.001$). From May to October 2017, submerged macrophytes cover was highest in sites 2 and 3, while no macrophytes were observed at site 1 in February to May 2018 and at site 2 in February to March 2018 (*Table A1*) which were in the rainy season.

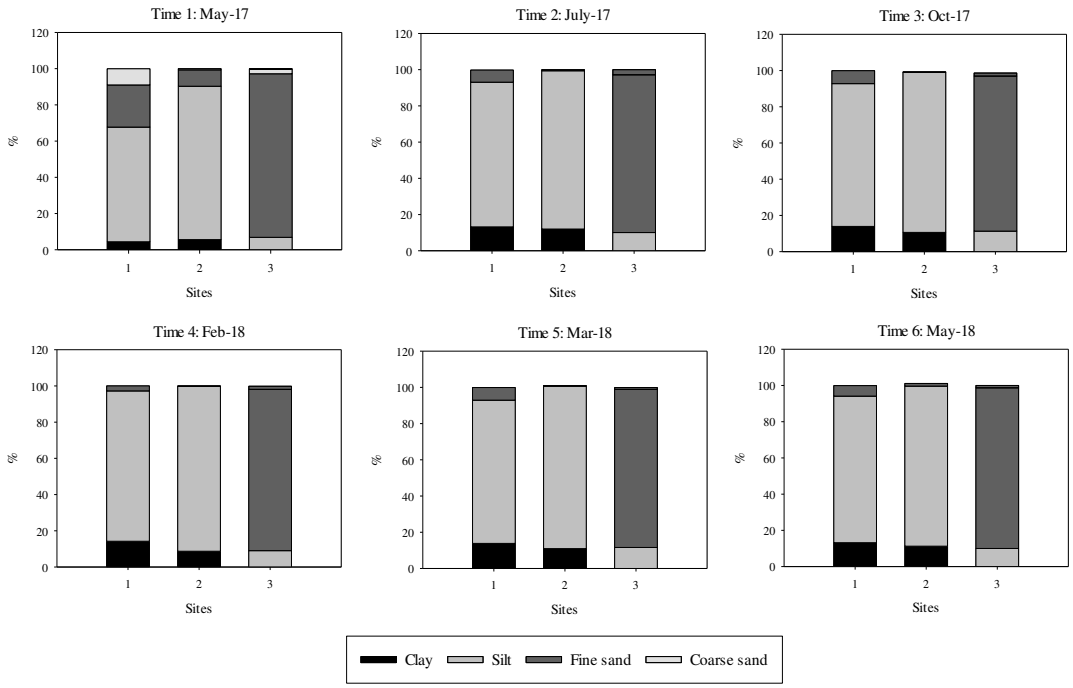

Figure 5. *Particle size proportions (clay, silt, fine sand, and coarse sand) of sediments from sites 1 – 3 (Near, Mid, Far) in Kukud, Songkhla Lagoon area from May 2017 to May 2018 (data represent mean ± SE)*

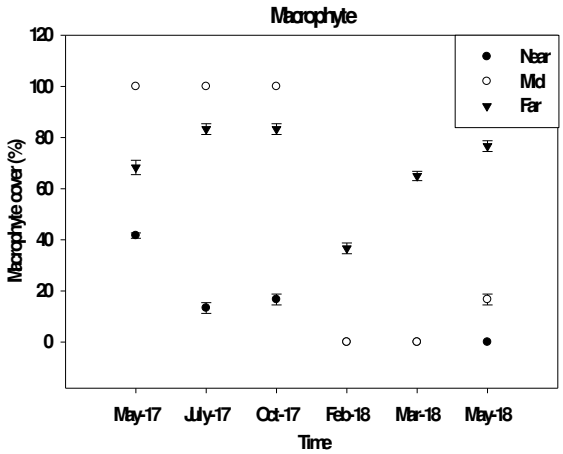

Figure 6. *Percentage cover of submerged macrophytes from three locations (sites 1 – 3 (Near, Mid, Far)) in Kukud, Songkhla Lagoon from May 2017 to May 2018 (data represent mean ± SE)*

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Cladophora sp. cover was significantly higher in May to October 2017 at site 2 ($P < 0.001$) (*Table A2*), while there were no *Cladophora* sp. at sites 1 and 3 at any time of collection except for May 2017 and October 2018, respectively. *C. demersum* was only observed in site 1 in May to October 2017 and site 2 in May to July 2017. *S. cucullata* was only observed in site 1 in May and July 2017. *P. malaianus* was only observed in sites 2 and 3 and high percentage cover was found at site 3 in October 2017, and March and May 2018. *N. marina* was observed at site 1 in May to October 2017 and May 2018 and at site 3 in May to July 2017. *N. graminea* was only found at site 2 from May to July 2017 and at site 3 from May to October 2017. High percentage cover of *C. zeylanica* was found at site 2 from May to October 2017. *E. canadensis* showed highest percentage cover at site 3 from July 2017 to May 2018.

Photosynthesis of submerged macrophytes

Effective quantum yield of PSII (Y(II)), which indicates photosynthetic efficiency of *C. Demersum,* was significantly higher at site 1 than at site 2 ($P < 0.001$) (*Fig. 7; Table A3*). There were no significant differences in Y(II) among sites and times of sampling observed in *P. malaianus*, *N. marina*, *N. graminea*, *C. zeylanica* and *E. canadensis* ($P > 0.05$). Saturating irradiance (I_k) showed no significant differences among sites and times of sampling observed in *C. demersum*, *P. malaianus*, *N. marina*, *N. graminea*, *C. zeylanica* and *E. canadensis* ($P > 0.05$) (*Fig. 7*). There were significant differences in maximum relative electron transport rate ($rETR_{max}$) among sites and times of sampling observed in *C. demersum, P. malaianus*, and *N. graminea*, ($P > 0.05$) (*Fig. 7*). $rETR_{max}$ of *N. marina*, *C. zeylanica* in October 2017 was significantly higher than that at the other times of sampling ($P = 0.006$ and $P = 0.047$, respectively). $rETR_{max}$ of *E. canadensis* was significantly lower in July 2017 ($P = 0.018$). There were no significant differences in Alpha among sites and times of sampling observed in *C. demersum*, *P. malaianus*, *N. marina*, *N. graminea*, *C. zeylanica* and *E. Canadensis* ($P > 0.05$) (*Fig. 7*).

Morphology (leaf length, leaf dry weight)

There were no significant differences in leaf length among sites and times of sampling observed in *C. demersum*, *P. malaianus*, *N. marina*, *N. graminea*, *C. zeylanica* and *E. canadensis* ($P > 0.05$) (*Fig. 8*).

There were no significant differences in leaf dry weight among sites and times of sampling observed in *P. malaianus*, *C. zeylanica* and *E. canadensis* ($P > 0.05$) (*Fig. 8*) (*Table A3*). Leaf dry weight of *C. demersum* was significantly higher at site 2 than at site 1 ($P = 0.024$). In *N. marina*, leaf dry weight was significantly higher at site 3 than at site 2 ($P = 0.001$), while this was reversed in *N. graminea* ($P < 0.001$).

Organic contents

Organic matter (OM) and organic carbon (OC) contents of *C. demersum* were significantly lower at site 1 in May 2017 compared to other sites and times of sampling ($P < 0.05$) (*Fig. 9*). There were significant differences in OM and OC among sites and times of sampling observed in *P. malaianus* and *C. demersum* ($P > 0.05$) (*Table A3*). Significantly higher OM and OC at site 2 compared to site 3 were observed in *N. marina, N. graminea* and *C. zeylanica* ($P < 0.001$, $P < 0.001$ and $P = 0.001$, respectively).

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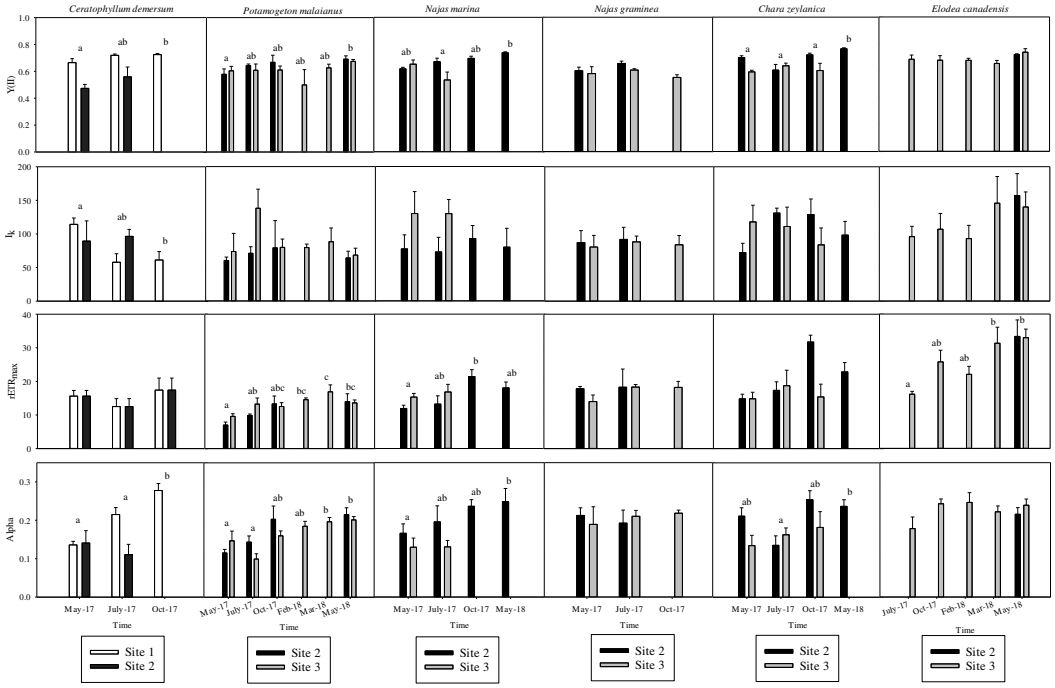

Figure 7. *Photosynthetic efficiency (Y(II)), saturating irradiance (I_k), maximum relative electron transport rate ($rETR_{max}$) and Alpha of Ceratophyllum demersum, Potamogeton malaianus, Najas marina, Najas graminea, Chara zeylanica and Elodea canadensis from three locations (sites 1-3 (Near, Mid and Far)) in Kukud, Songkhla Lagoon from May 2017 to May 2018 (data represent mean ± SE)*

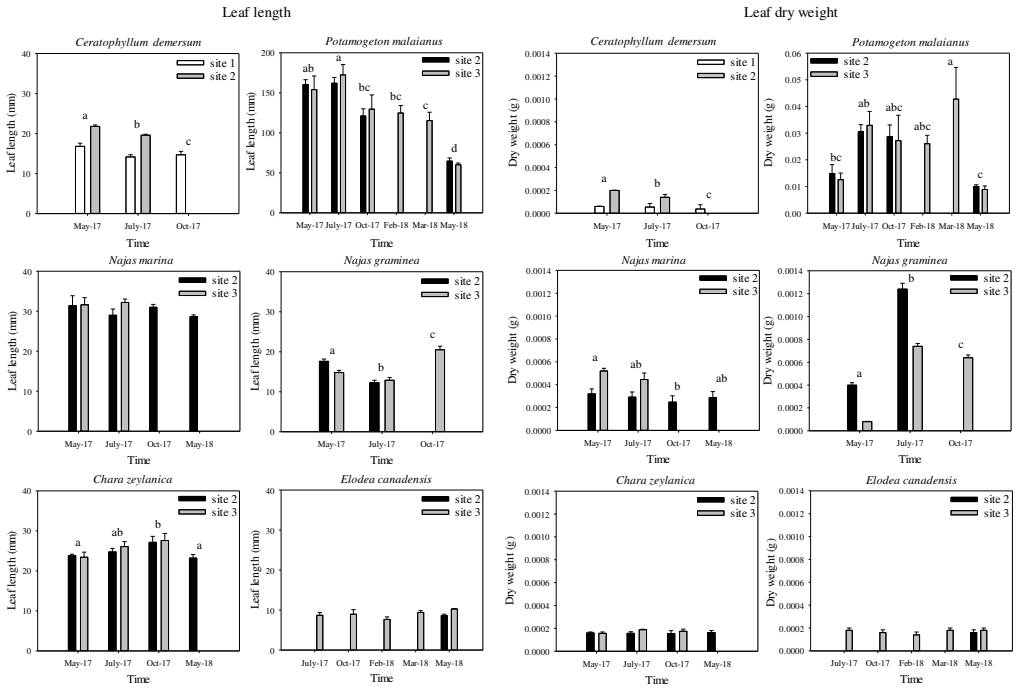

Figure 8. *Leaf length and Leaf dry weight of Ceratophyllum demersum, Potamogeton malaianus, Najas marina, Najas graminea, Chara zeylanica and Elodea canadensis from three locations (sites 1-3 (Near, Mid and Far)) in Kukud, Songkhla Lagoon from May 2017 to May 2018 (data represent mean ± SE)*

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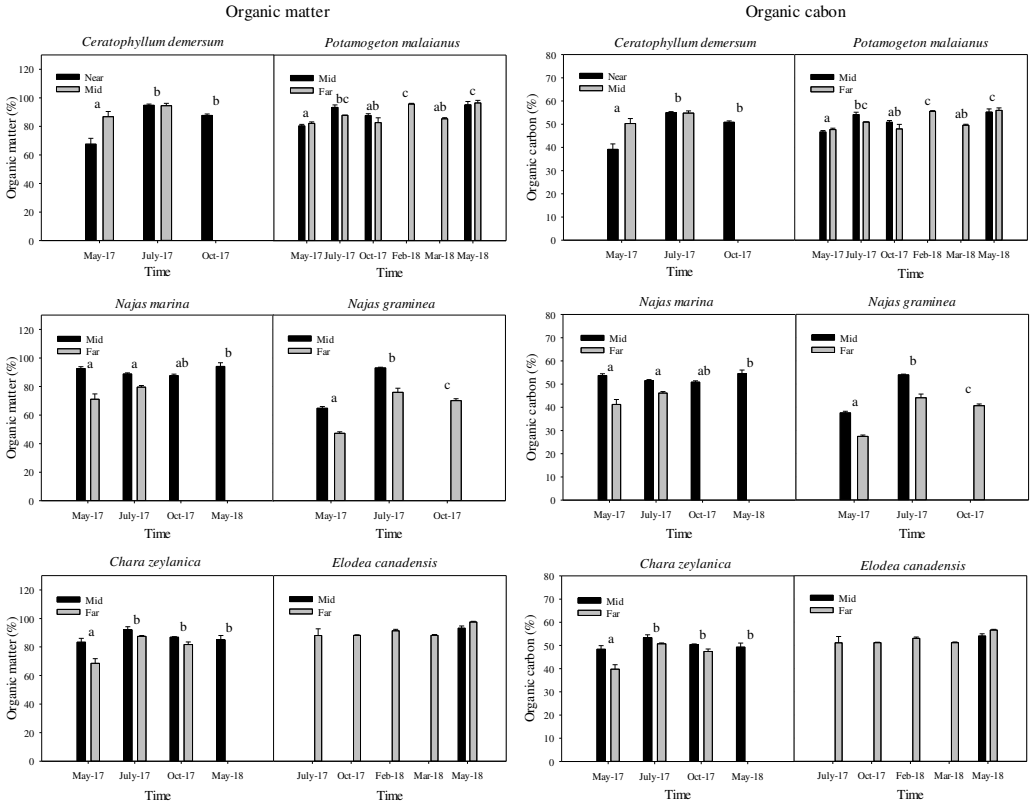

Figure 9. *Organic matter and organic carbon contents of Ceratophyllum demersum, Potamogeton malaianus, Najas marina, Najas graminea, Chara zeylanica and Elodea canadensis from three locations (sites 1-3 (Near, Mid and Far)) in Kukud, Songkhla Lagoon from May 2017 to May 2018 (data represent mean ± SE)*

CCA

CCA showed relationships among environmental parameters and biological parameters of macrophytes indicating that depth, wind, water quality, dissolved oxygen, nitrate and phosphate concentration in water, carbon and nitrogen contents, and silt concentration in sediment had significant impacts on biology of submerged macrophytes with 90.74% and Eigenvalue 0.03 (*Fig. A1*). Higher organic matter content in submerged macrophytes was related to higher depth, while EQY, $rETR_{max}$, I_k, Alpha, HHV_{ult} and HHV_{prox} were high at higher wind. Percentage cover of submerged macrophytes was found at higher silt concentration in sediment and high pH, while water depth, dissolved oxygen in water, and organic matter, organic carbon, C and N contents, and silt and fine sand contents in sediment had significant impact on percentage cover of submerged macrophytes with 83.94% and Eigenvalue 0.823 (*Fig. A2*). High percentage cover of macrophytes was observed with high dissolved oxygen. *C. demersum* and *S. cucullata* were found in area with high Chl *a* in water. High *N. marina* and *C. zeylanica* were found at high water pH, while *E. canadensis* and *P. malaianus* were high at increased depth and secchi depth.

CCA indicated that Chl *a* concentration in submerged macrophytes was influenced by water depth, TDS, nitrate concentration in water and organic carbon, organic contents, C, N and fine sand contents in sediment with 89.49% and Eigenvalue 0.491 (*Fig. A3*). Chl *a* concentration in submerged macrophytes was related to water depth.

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High Chl *a* content in *C. demersum* was found in area with high nitrate concentration. High Chl *a* content in *S. cucullata*, *N. marina*, *C. zeylanica* and *P. malaianus* were found in area with high organic matter and organic content in sediment but it was lowest at increased depth. High Chl *a* content *E. canadensis* was found at increased depth.

Then, CCA showed that effective quantum yield (EQY) and alpha of macrophytes were associated with chlorophyll *a* in water, water depth, dissolved oxygen, nitrate and phosphate in water and organic matter, organic carbon, C and N contents and silt and fine sand contents in sediment with 92.53% and 87.88% and Eigenvalues of 1.000 and 0.729, respectively (*Figs. A4* and *A5*). EQY and alpha of *Najas marina, Najas graminea, Chara zeylanica* were high in area with high dissolved oxygen, pH and conductivity and silt sediment, while EQY and alpha of *E. canadensis* and *P. malaianus* were high in area with higher depth and secchi depth, while $rETR_{max}$ and I_k of macrophytes were influenced by chlorophyll *a* in water, dissolved oxygen, water depth, nitrate and phosphate in water and organic matter, organic carbon, C and N contents, and silt contents in sediment with 83.33% and 89.50% and Eigenvalues of 0.484 and 0.644, respectively (*Figs. A6* and *A7*). $rETR_{max}$ and I_k of *C. demersum* were high in area with high phosphate, nitrate and Chl *a* in water, while $rETR_{max}$ and I_k of *Najas marina, Najas graminea, Chara zeylanica* were high in area with high dissolved oxygen, pH and silt sediment. $rETR_{max}$ and I_k of *E. canadensis* and *P. malaianus* were high in area with higher depth and secchi depth.

CCA indicated that organic matter of macrophytes was influenced by depth, water salinity, organic matter, organic carbon, C and N contents, and fine sand and coarse sand contents in sediment with 88.15% and Eigenvalue 0.474 (*Fig. A8*). Carbon in sediment has largest effect on organic matter of macrophytes. Organic matter of *S. cucullata, N. marina,* and *C. canadensis* was high in area with high salinity, while it was low in area with high rainfall and water depth. Organic matter of *E. canadensis* and *P. malaianus* was high in area with higher depth and clay sediment, while organic carbon of macrophytes was influenced by depth, pH, secchi depth, organic matter, organic carbon, C and N contents, and clay contents in sediment with 90.08% and Eigenvalue 0.647 (*Fig. A9*). Carbon in sediment has the largest effect on organic carbon of macrophytes. Organic carbon of *N. graminea, N. marina,* and *C. zeylanica* was high in area with elevated pH. Organic carbon of *E. canadensis* and *P. malaianus* was high in area with increased depth, secchi depth and clay sediment.

Carbon capture potential

HHV_{ult} and HHV_{prox} of submerged macrophytes collected from Kukud, Songkhla were between 12.05 – 22.07 MJ kg^{-1} and 7.23 – 19.80 MJ kg^{-1}, respectively (*Fig. 10*). HHV_{ult} in *C. demersum*, *N. gramine* and *Chara zeylanica* varied among sites and times of sampling ($P < 0.05$) (*Table A3*). There were no significant differences in HHV_{ult} and HHV_{prox} of *P. malaianus* and *E. Canadensis* among sites and times of sampling ($P > 0.05$). HHV_{ult} and HHV_{prox} in *N. marina*, *N. graminea* and *C. zeylanica* were significantly higher at sites 2 and 3 ($P < 0.05$).

CCA showed that chlorophyll *a* in water column, depth, secchi depth, dissolved oxygen, nitrate concentration in water, organic matter, organic carbon, and silt content in sediment had significant impacts on HHV_{ult} and HHV_{prox} of submerged macrophytes while silt content had the most significant effect (*Figs. A10* and *A11*). Higher HHV_{ult} and HHV_{prox} in *C. demersum* would be found in an area with high phosphate, nitrate and chlorophyll *a* concentration in water and high organic matter and organic carbon in

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sediment. On the other hand, HHV_{ult} and HHV_{prox} in *N. marina*, *N. graminea* and *C. zeylanica* would be high in an area with high dissolved oxygen and pH in water and silt sediment. *E. canadensis* and *P. malaianas* would have high HHV_{ult} and HHV_{prox} under higher depth and clay sediment.

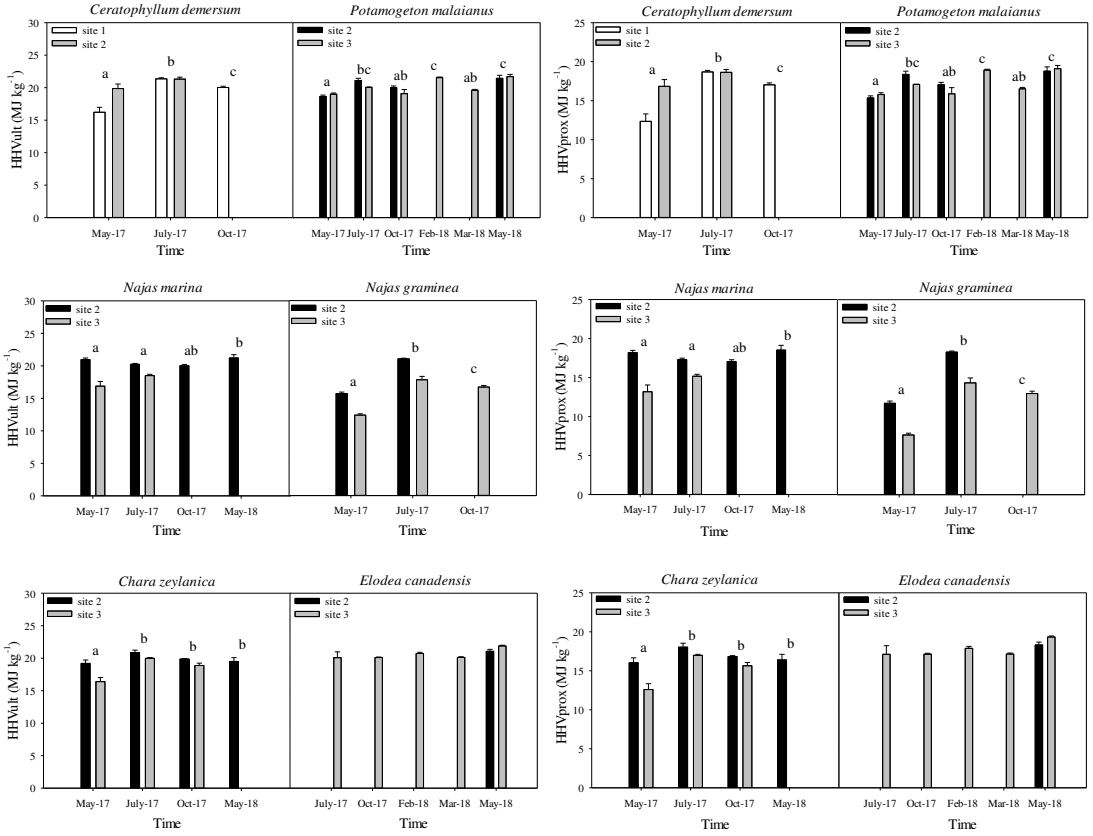

Figure 10. *HHV_{ult} and HHV_{prox} of submerged macrophytes in Kukud, Songkhla Lagoon area from May 2017 to May 2018*

Relationship between submerged macrophytes and phytoplankton bloom

This study used chlorophyll a concentration in water as an indicator of phytoplankton bloom. Linear regression showed a negative relationship between submerged macrophytes and phytoplankton bloom ($P < 0.001$, $R^2 = 0.057$) (*Fig. 11*). Multiple regression among submerged macrophytes, nitrate and phosphate concentration and chlorophyll a in water showed significant relationship among these parameters ($P < 0.001$, $R^2 = 0.381$).

Discussion

Spatial and temporal variations in photosynthesis of macrophytes

Six from twenty-one species of submerged macrophytes found in Songkhla Lagoon and one species of macroalgae were observed in the middle lake (Kukud) in this study. These include a floating plant (*Salvinia cucullata*), submerged plants (*Ceratophyllum demersum*, *Najas marina*, *Potamogeton malaianuis*, *Elodea canadensis, Chara*

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zeylanica and *Najas graminea*), and algae (*Cladophora* sp.). There are spatial and temporal variations in photosynthetic capacity, growth and species composition of submerged macrophytes.

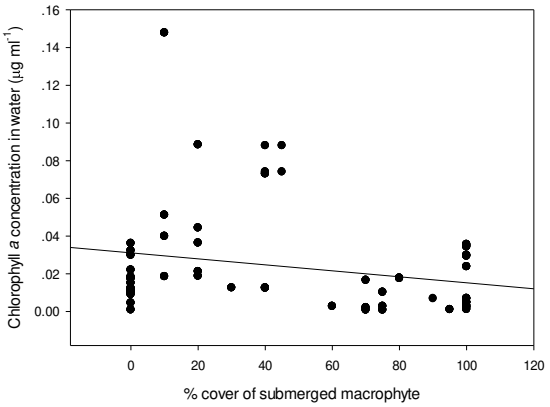

Figure 11. *Relationship between % cover of submerged macrophyte and chlorophyll a concentration in Kukud, Songkhla Lagoon area from May 2017 to May 2018*

Site 1 had significantly lower percentage cover of submerged macrophytes than sites 2 and 3 and presented only *C. demersum* and *S. cucullata,* which might be due to high nutrients and Chl *a* in water. It is suggested that trophic status is the major ecological determinant of the floristical composition (Sahu et al., 2020). *C. demersum* was found in site 1, which has high nutrients and high turbidity. This study confirms that *C. demersum* is an indicator of eutrophication in a lake. In lowland rivers with different habitat conditions, Bytyqi et al. (2020) found that the presence of macrophyte species varies by nutrient level, and macrophyte based indices represent the water quality. Further, macrophytes can serve as tools for biomonitoring heavy metals in river water (Yasar et al., 2019).

Macrophyte cover correlated with rainfall being low in high rainfall (February) and increasing with low rainfall (October). *Cladophora* sp. cover was significantly higher in May to October 2017 at site 2, while there were no *Cladophora* sp. at sites 1 or 3 at any time of collection except for May 2017 and October 2018, respectively. *N. graminea* was only found at site 2 from May to July 2017 and at site 3 from May to October 2017. High percentage cover of *C. zeylanica* was found at site 2 from May to October 2017. This might be due to fluctuations of several parameters, such as nutrients in water and sediment, turbidity, light availability, and water flow (Hilt et al., 2018; Schneider et al., 2018).

Songkhla lagoon water bodies are exposed to frequent water level fluctuations due to rainfall, freshwater run-off, and tides, which leads to a high instability of habitats and might affect macrophyte species composition and abundance (Schneider et al., 2018). For example, particle resuspension or turbidity could limit light availability for photosynthesis and growth (Reitsema et al., 2018). Water flow can directly reduce submerged macrophyte biomass due to the strong mechanical strain and the damage caused to plant tissues via breaking and uprooting (Zhang et al., 2014; Schneider et al., 2018). Changes of water depth could be catastrophic for submerged macrophytes (devoid of cuticle and supporting tissues) when exposed to air conditions or to very large floods (Sousa et al., 2010; Schneider et al., 2018). Our study showed *P. malaianus*

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and *E. canadensis* as abundant at site 3, which had more depth, higher light and lower turbidity. *P. malaianus* might have a high breaking strength and high phenotypic plasticity (Brewer and Parker 1990; Idestam-Almquist and Kautsky, 1995; Hilt et al., 2018) while *E. canadensis* has been found to be in deep water and possess a lower tensile strength (Brewer and Parker, 1990).

It has been suggested that light and water flow are important factors that influence submerged macrophytes (Zhang et al., 2012; Schneider et al., 2018). However, the macrophytes found in this study were rooted submerged macrophytes. Therefore, nutrient status in sediment could play a significant role in determining abundance of these submerged macrophytes. CCA showed that water depth, dissolved oxygen in water, and organic matter, organic carbon, C and N contents, and silt and fine sand contents in sediment had significant impact on percentage cover of submerged macrophytes. Therefore, a combination of several factors could play a significant role to these submerged macrophytes.

Effect of sediment enrichment on macrophytes photosynthesis

CCA showed that photosynthetic capacity (Effective quantum yield of PSII, alpha, saturating irradiance, and maximum relative electron transport rate) is influenced by several factors such as chlorophyll *a* in water, water depth, dissolved oxygen, nitrate and phosphate in water and organic matter, organic carbon, C and N contents, and silt and fine sand contents in sediment. Lower photosynthetic efficiency (effective quantum yield) was observed in area with more silt and clay sediment. This is consistent with findings in Ruan et al. (2010) which suggest that highly enriched sediment reduced leaf chlorophyll, leaf biomass and root length due to plant stress. Organic enrichment can enhance organic decomposition, oxygen consumption and acid formation, which lead to anoxia in sediment and roots and greater formation of organic acids, S^{-2}, Fe^{2+} and Mn^{2+}, which results in physiological stress. This stress can reduce root length, growth of plant and rood anchorage (Schutten et al., 2005; Ruan et al., 2010). The responses of macrophytes to organic enriched sediments are also species specific and the effects could be stronger on rooted macrophytes. For example, *Lobelia dortmanna* could have critically poor anchorage on highly organic sediments as the anoxic sediments impair root development (Sand-Jensen and Møller, 2014). As a result of the varied responses by species, this can cause changes to the species composition and reduce the variety of species found in benthic vegetation.

Ability of macrophytes to capture carbon and factors that affect carbon capture potential

One of the options to cope with the climate change issue is carbon capture and sequestration by natural ecosystems (Wang et al., 2011; Maqbool and Khan, 2013). An aquatic system (known as blue carbon habitat) can capture atmospheric CO_2 via photosynthesis by photosynthetic organisms such as macrophytes, mangrove, and seagrass, and convert it into a permanent form of fixed carbon stored in live tissues and sediments (McLeod et al., 2011; Trevathan-Tackett et al., 2015). It is found that blue carbon system is more efficient at long-term carbon sequestration than are terrestrial systems (Trevathan-Tackett et al., 2015). The inland lake waters, which cover only 2.1% of the global landscape, contribute 260 g C m^{-2} y^{-1} of net primary productivity, suggesting that the inland lakes can be sinks for carbon. With high growth rate, high

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productivity (545 mg C m^{-2} d^{-1}) and fragmentation of submerged macrophytes, they could make a significant contribution as carbon donors to blue carbon sediments. In addition, macrophyte biomass has potential for bioenergy production (Wang et al., 2011).

Organic carbon content of macrophytes was influenced by water depth, pH, secchi depth and sediment characteristics and varied by site and time of sampling. The mechanism of carbon sequestration through photosynthesis depends on several factors that influence growth of plants, like temperature, light, and nutrients. Therefore, summer season with higher temperature and more light could lead to higher carbon sequestration potential. However, too high levels of light and temperature could reduce photosynthesis and growth due to photosynthetic stresses, and lead to reduced carbon sequestration (Lolu et al., 2018). Moreover, higher nutrient status of lake leads to higher carbon sequestration (Kvet et al., 2008).

Highest organic carbon contents and HHV_{ult} and HHV_{prox} were found in *P. malaianus*, *E. canadensis* and *N. marina* and they are in the same range as submerged macrophytes reported in Wang et al. (2011). It is suggested that carbon contents were positively correlated with HHV_{ult} and HHV_{prox}, and negatively correlated with ash content.

Macrophytes as phytoplankton bloom inhibitors

Eutrophication is indicated by excessive growth of algae, cyanobacteria and macrophytes (Penning et al., 2008) due to relaxing one or more limitations of growth factors needed for photosynthesis, such as light, low CO_2, temperature, and nutrients such as phosphorus and nitrogen (Chislock et al., 2013). These nutrients are essential for algal growth (Scholten et al., 2005). Therefore, eutrophication results in an increase in algal and cyanobacteria productivity (bloom), which will subsequently reduce light availability for other photosynthetic species such as macrophytes and will result in poor water quality such that the water is anoxic and toxic (Scholten et al., 2005). The management and restoration of a lake suffering from eutrophication is challenging. It is suggested that nutrients (N, P) can be removed by biological processes, such as photosynthesis, respiration, fermentation, nitrification (N only), de-nitrification (N only), microbial activity; and by physical processes such as solute transport which was influenced by longitudinal dispersion. (Riis et al., 2020). It has been shown that submerged macrophytes can play an important role in controlling phytoplankton in enclosed shallow water bodies (Guo-feng et al., 2014). Macrophytes have a high capacity for absorption of nutrients (N and P) directly from the water, they store nutrients for long periods, and the accumulation of macrophyte biomass leads to N limitation for the phytoplankton (Guo-feng et al., 2014).

This study used chlorophyll *a* concentration in water as an indicator of phytoplankton bloom. Linear regression showed a negative relationship between submerged macrophytes and phytoplankton bloom and multiple regression among submerged macrophytes, nitrate and phosphate concentration and chlorophyll *a* in water showed significant relationship among these parameters. The results suggest that macrophytes could potentially be used for controlling phytoplankton blooms. However, the types and amounts of macrophytes used for nutrient removal should be considered as they differ in nutrient uptake, growth rate and physical structure (Lee et al., 2009). It has been shown that macrophytes also produce allelopathic substances, which can destroy the cell structure of algae and affect photosynthesis, respiration, and enzymatic

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activity of algal cells (Mohamed, 2017). This can lead to a competitive advantage over other macrophytes and phytoplankton (Mohamed, 2017). It has been found that allelochemicals of macrophyte *Myriophyllum spicatum* decreased the photosynthesis activity of *Microcystis aeruginosa* (Zhu et al., 2010) and macrophyte assemblages had greater allelopathic effect on cyanobacteria and diatoms than monocultured macrophytes (Rojo et al., 2013). Moreover, we found high percentage cover of macrophytes with high dissolved oxygen, which indicates that the macrophytes are a source of oxygen in the lake and play an important role in the functioning of aquatic ecosystem by changing water quality parameters, such as turbidity, total organic carbon, total nitrogen, and chemical oxygen demand (Souza et al., 2015; Lv et al., 2018). These changes can affect epiphytic algal community structure due to high transparency, low nutrient concentration and more habitats and spatial niches for epiphytic algae (Lv et al., 2018).

This study suggests that macrophytes can be used to improve water quality of the lake or lagoon by nutrient removal which will help manage an eutrophic lake. The suitable submerged macrophyte biomass for lake management could be determined. Excessive amount of submerged macrophytes can be problematic to human activities and affect ecosystem services of the lake. It is suggested that when submerged macrophyte biomass is more than 6000 g m^{-2}, dissolved oxygen concentration becomes too low for the survival of fish and invertebrates and a suitable submerged macrophyte biomass range is 3000-6000 g m^{-2} (Ishikawa et al., 2019). The actions to manage excessive macrophytes include reduction and elimination methods: mechanical (e.g., hand tools, mechanical devices), environmental (e.g., light, nutrients), chemical (e.g., herbicides, deoxygenation) and biological methods (e.g., grass carp) (Cabrera Walsh et al., 2017; Hussner et al., 2017; Ishikawa et al., 2019). Moreover, excessive macrophytes can be used as sources of feedstock for incineration or bioenergy production while unattended macrophyte biomass could become detritus organics and transform at the sediment layer into methane, which is a greenhouse gas, and nutrient uptake by the macrophytes can be partially returned to the water body (Wang et al., 2011).

This study confirms that water quality and environmental conditions of the lake related to both human activities and natural events are important for abundance, diversity, and distribution of submerged macrophytes in Songkhla Lagoon. Nutrients (nitrogen and phosphorus), light, and water flow are important factors. It is suggested that the relationship between environmental parameters and composition of macrophytes can be a helpful tool for predicting ecosystem processes under future climate change scenarios (Schneider et al., 2018). Reduction of nutrients is a priority for lake management in order to compensate for the increased eutrophication due to global warming (Stefanidis et al., 2019).

Conclusions

This study showed temporal and spatial variations in photosynthesis, growth, carbon sequestration potential and bioenergy potential of submerged macrophytes. It is suggested that trophic status might be a major ecological determinant of the floristical composition. As climate change is currently an issue, future research on the impacts of climate change on macrophytes and lagoonal ecosystems could be pursued. Interactive effects of elevated temperature, light, nutrient enrichment, and acidification on macrophyte species, community structure and lagoonal ecosystem functions could be

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DOI: http://dx.doi.org/10.15666/aeer/1905_40694101

determined and predicted. This project suggests that the relationship between environmental parameters and composition of macrophytes can be a helpful tool providing bioindicators of the ecological status; for predicting ecosystem processes of Songkhla Lagoon under future climate change scenarios; and can contribute to the development of a sustainable lake management plan.

Acknowledgements. We thank Pimchanok Buapet, Raymond Ritchie, Mikael Kim and Oramas Suttinun for their valuable support. We would also like to thank the Division of Biological Science, Faculty of Science, Prince of Songkla University (PSU), Faculty of Environmental Management, PSU, Marine and Coastal Resources Institute, PSU, and Coastal Oceanography and Climate Change Research Center, PSU for research facilities. This work was supported by the Thailand Research Fund and co-funded by The Commission of Higher Education [Grant number MRG6080129]; Prince of Songkla University [Grant number ENV59026N and Grant number COR59024N]. Authors would like to thank Assoc. Prof. Seppo Karrila and Research and Development Office, PSU for English language check.

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APPENDIX

Table A1. *Presence (x) of 8 species including free floating macrophytes (Salvinia cucullata), submerged macrophytes (Ceratophyllum demersum, Elodea canadensis, Najas marina, Potamogeton malaianus, Chara zeylanica and Najas graminea) and macroalgae (Cladophora sp.) for each site in Kukud, Songkhla Lagoon area from May 2017 to May 2018*

Species	Site 1						Site 2						Site 3					
	May-17	Jul-17	Oct-17	Feb-18	Mar-18	May-18	May-17	Jul-17	Oct-17	Feb-18	Mar-18	May-18	May-17	Jul-17	Oct-17	Feb-18	Mar-18	May-18
Cladophora sp.	x						x	x	x			x		x	x			
Ceratophyllum demersum	x	x	x				x	x										
Salvinia cucullata	x	x																
Potamogeton malaianus							x	x	x			x	x	x	x	x	x	x
Najas marina							x	x	x			x	x	x				
Najas graminea							x	x					x	x	x			
Chara zeylanica							x	x	x		x		x	x	x			
Elodea canadensis												x		x	x	x	x	x

Table A2. *Two-Way ANOVA showed significant differences (*) among time, site, and site*time in percentage cover of 8 species including free floating macrophytes (Salvinia cucullata), submerged macrophytes (Ceratophyllum demersum, Elodea Canadensis, Najas marina, Potamogeton malaianus, Chara zeylanica and Najas graminea) and macroalgae (Cladophora sp.), water quality, and sediment characteristics in Kukud, Songkhla Lagoon area from May 2017 to May 2018*

	Time			Site			Time*site		
	df	*F*	*sig*	*df*	*F*	*sig*	*df*	*F*	*sig*
Percentage cover									
Ceratophyllum demersum	5	200.066	<0.001*	2	547.783	<0.001*	10	172.047	<0.001*
Potamogeton malaianus	5	212.102	<0.001*	2	2720.078	<0.001*	10	190.661	<0.001*
Najas marina	5	87.162	<0.001*	2	122.119	<0.001*	10	83.320	<0.001*
Najas graminea	5	839.550	<0.001*	2	844.200	<0.001*	10	299.550	<0.001*
Chara zeylanica	5	791.210	<0.001*	2	3015.267	<0.001*	10	594.662	<0.001*
Elodea Canadensis	5	20.558	<0.001*	2	299.129	<0.001*	10	20.558	<0.001*
Salvinia cucullata	5	22.000	<0.001*	2	40.000	<0.001*	10	22.000	<0.001*
Cladophora sp.	5	1346.571	<0.001*	2	3375.536	<0.001*	10	749.893	<0.001*
Water quality									
Temperature	5	1981.010	<0.001*	2	880.026	<0.001*	10	49.287	<0.001*
Salinity	5	778315.700	<0.001*	2	12060.500	<0.001*	10	5375.900	<0.001*
DO	5	146.741	<0.001*	2	3864.699	<0.001*	10	257.338	<0.001*
Conductivity	5	134619.463	<0.001*	2	1220.529	<0.001*	10	4934.839	<0.001*
TDS	5	5218393.498	<0.001*	2	25363.744	<0.001*	10	75124.303	<0.001*
pH	5	2327.978	<0.001*	2	3530.389	<0.001*	10	430.549	<0.001*
Depth	5	597.082	<0.001*	2	2331.330	<0.001*	10	46.673	<0.001*
Secchi Depth	5	531.581	<0.001*	2	229.586	<0.001*	10	137.414	<0.001*
Nitrate	5	5.045	0.001*	2	2.907	0.068	10	0.993	0.467
Phosphate	5	74.949	<0.001*	2	6.969	0.003*	10	16.345	<0.001*
Chlorophyll *a*	5	15.600	<0.001*	2	46.456	<0.001*	10	6.217	<0.001*
Organic matter	5	43.852	<0.001*	2	45.714	<0.001*	10	12.012	<0.001*
Organic carbon	5	43.852	<0.001*	2	45.714	<0.001*	10	12.012	<0.001*
Sediment characteristics									
Carbon	5	608.693	<0.001*	2	3720.636	<0.001*	10	343.078	<0.001*
Nitrogen	5	1471.328	<0.001*	2	4150.365	<0.001*	10	521.708	<0.001*
Carbon:Nitrogen ratios	5	42.141	<0.001*	2	232.473	<0.001*	10	9.843	<0.001*

*Significant difference

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Table A3. *Two-Way ANOVA showed significant differences (*) among time, site, and site*time in leaf length, leaf dry weight, organic matter, organic carbon, HHV_{ult}, HHV_{prox}, EQY, ETR, I_k, and Alpha of 6 submerged macrophytes species in Kukud, Songkhla Lagoon area from May 2017 to May 2018*

	Ceratophyllum demersum			***Potamogeton malaianus***			***Najas marina***			***Najas graminea***			***Chara zeylanica***			***Elodea canadensis***		
	df	***F***	***p***	***df***	***F***	***p***	***df***	***F***	***p***	***df***	***F***	***p***	***df***	***F***	***p***	***df***	***F***	***p***
Time																		
Leaf length	2	8.214	0.002*	5	23.277	<0.001*	3	0.574	0.638	2	45.273	<0.001*	3	3.949	0.018*	4	2.175	0.102
Leaf dry weight	2	127.980	<0.001*	5	6.936	<0.001*	3	0.721	0.549	2	351.834	<0.001*	3	0.283	0.837	4	0.640	0.639
Organic matter	2	24.014	<0.001*	5	21.667	<0.001*	3	2.032	0.163	2	176.247	<0.001*	3	12.588	<0.001*	4	3.802	0.032*
Organic carbon	2	24.014	<0.001*	5	21.667	<0.001*	3	2.032	0.163	2	176.247	<0.001*	3	12.588	<0.001*	4	3.802	0.032*
HHV_{ult}	2	24.014	<0.001*	5	21.667	<0.001*	3	2.032	0.163	2	176.247	<0.001*	3	12.588	<0.001*	4	3.802	0.032*
HHV_{prox}	2	24.014	<0.001*	5	21.667	<0.001*	3	2.032	0.163	2	176.247	<0.001*	3	12.588	<0.001*	4	3.802	0.032*
EQY	2	1.973	0.174	5	1.743	0.155	3	2.532	0.090	2	1.688	0.218	3	3.141	0.047*	4	1.639	0.208
ETR	2	1.028	0.381	5	4.035	0.006*	3	5.727	0.006*	2	0.564	0.581	3	3.141	0.047*	4	3.944	0.018*
I_k	2	3.592	0.053	5	.865	0.516	3	0.240	0.867	2	0.159	0.854	3	1.134	0.358	4	1.721	0.189
Alpha	2	7.475	0.006*	5	7.306	<0.001*	3	1.764	0.190	2	0.147	0.864	3	2.933	0.057	4	1.886	0.157
Site																		
Leaf length	1	71.643	<0.001*	1	0.068	0.795	1	1.292	0.267	1	2.563	0.125	1	0.228	0.636	1	3.253	0.084
Leaf dry weight	1	5.960	0.024*	1	0.022	0.884	1	13.988	0.001*	1	197.881	<0.001*	1	1.492	0.232	1	0.400	0.533
Organic matter	1	12.969	0.005*	1	2.330	0.143	1	55.222	<0.001*	1	122.047	<0.001*	1	19.194	0.001*	1	2.167	0.167
Organic carbon	1	12.969	0.005*	1	2.330	0.143	1	55.222	<0.001*	1	122.047	<0.001*	1	19.194	0.001*	1	2.167	0.167
HHV_{ult}	1	12.969	0.005*	1	2.330	0.143	1	55.222	<0.001*	1	122.047	<0.001*	1	19.194	0.001*	1	2.167	0.167
HHV_{prox}	1	12.969	0.005*	1	2.330	0.143	1	55.222	<0.001*	1	122.047	<0.001*	1	19.194	0.001*	1	2.167	0.167
EQY	1	21.297	<0.001*	1	0.237	0.630	1	2.822	0.110	1	1.464	0.245	1	7.508	0.012*	1	0.272	0.608
ETR	1	1.850	0.194	1	1.419	0.243	1	3.541	0.076	1	0.470	0.504	1	4.398	0.048*	1	0.005	0.943
I_k	1	0.330	0.574	1	2.940	0.097	1	10.083	0.005*	1	0.221	0.645	1	0.273	0.607	1	0.406	0.532
Alpha	1	4.942	0.042*	1	0.726	0.401	1	3.321	0.085	1	0.010	0.922	1	3.657	0.070	1	0.654	0.429
Time*site																		
Leaf length	1	0.116	0.737	3	0.320	0.811	1	1.006	0.326	1	6.122	0.022*	2	0.260	0.773	0	-	-
Leaf dry weight	1	66.225	<0.001*	3	0.070	0.976	1	.236	0.631	1	9.535	0.006*	2	0.653	0.528	0	-	-
Organic matter	1	13.642	0.004*	3	2.751	0.070	1	8.846	0.012*	1	0.020	0.889	2	3.245	0.070	0	-	-
Organic carbon	1	13.642	0.004*	3	2.751	0.070	1	8.846	0.012*	1	0.020	0.889	2	3.245	0.070	0	-	-
HHV_{ult}	1	13.642	0.004*	3	2.751	0.070	1	8.846	0.012*	1	0.020	0.889	2	3.245	0.070	0	-	-
HHV_{prox}	1	13.642	0.004*	3	2.751	0.070	1	8.846	0.012*	1	0.020	0.889	2	3.245	0.070	0	-	-
EQY	1	0.169	0.687	3	0.106	0.956	1	7.979	0.011*	1	0.226	0.641	2	4.275	0.028*	0	-	-
ETR	1	0.190	0.669	3	0.809	0.499	1	0.004	0.952	1	0.498	0.491	2	5.734	0.010*	0	-	-
I_k	1	7.077	0.018*	3	0.975	0.418	1	0.018	0.896	1	0.020	0.890	2	4.697	0.021*	0	-	-
Alpha	1	5.940	0.028*	3	0.667	0.579	1	0.264	0.614	1	0.526	0.479	2	2.594	0.098	0	-	-

*Significant difference

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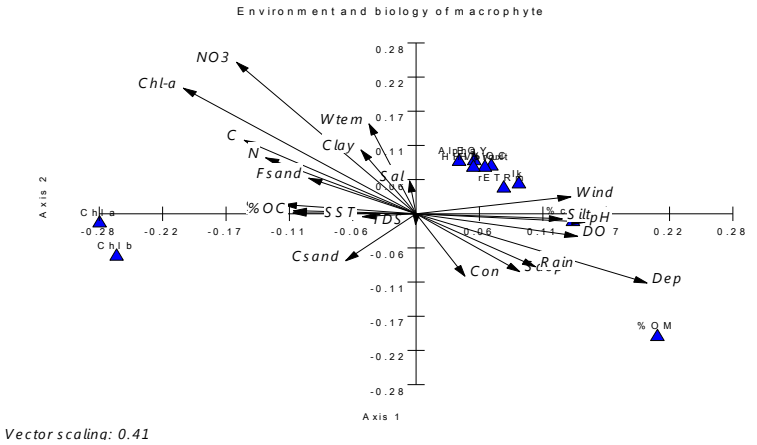
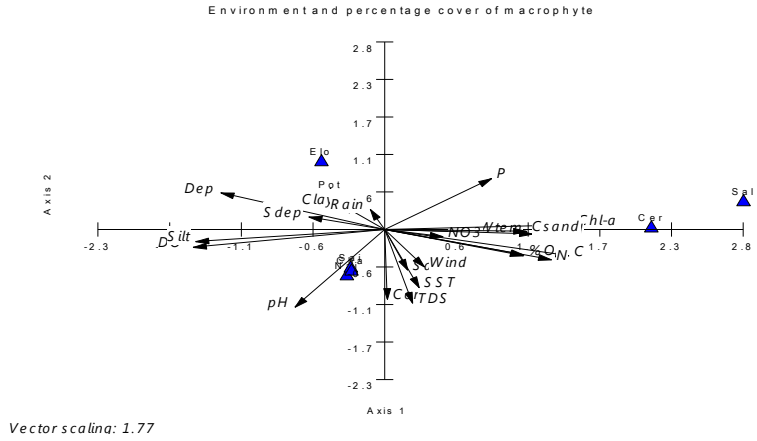


Eigenvalues		
	Axis 1	**Axis 2**
Eigenvalues	0.03	0.007
Percentage	72.728	18.017
Cum. Percentage	72.728	90.745
Cum. Constr. Percentage	72.728	90.745
Spec.-env. correlations	1.000	1.000

Biplot scores for env. variables		
	Axis 1	**Axis 2**
Depth	0.493	-0.273
DO	0.345	-0.088
NO_3	-0.384	0.601
P	-0.284	0.037
C	-0.368	0.292
N	-0.322	0.223
Silt	0.312	-0.02
Wind	0.33	0.068

Figure A1. *Relationships among environmental parameters and biology of submerged macrophytes in Kukud, Songkhla Lagoon area from May 2017 to May 2018*



Eigenvalues		
	Axis 1	**Axis 2**
Eigenvalues	0.823	0.392
Percentage	56.871	27.067
Cum. Percentage	56.871	83.939
Cum.Constr.Percentage	56.871	83.939
Spec.-env. correlations	1.000	1.000

Biplot scores for env. variables		
	Axis 1	**Axis 2**
Depth	-0.734	0.315
DO	-0.858	-0.151
%OM	0.619	-0.224
%OC	0.622	-0.217
C	0.823	-0.223
N	0.745	-0.257
Silt	-0.847	-0.103
Fsand	0.661	-0.037

Figure A2. *Relationship among environmental parameters and percentage cover of submerged macrophytes in Kukud, Songkhla Lake area from May 2017 to May 2018*

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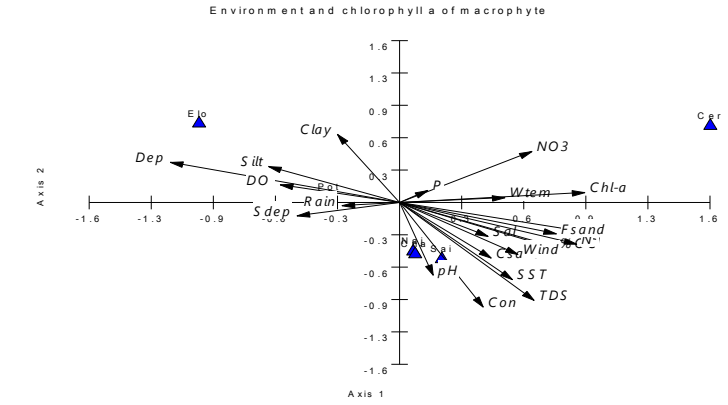
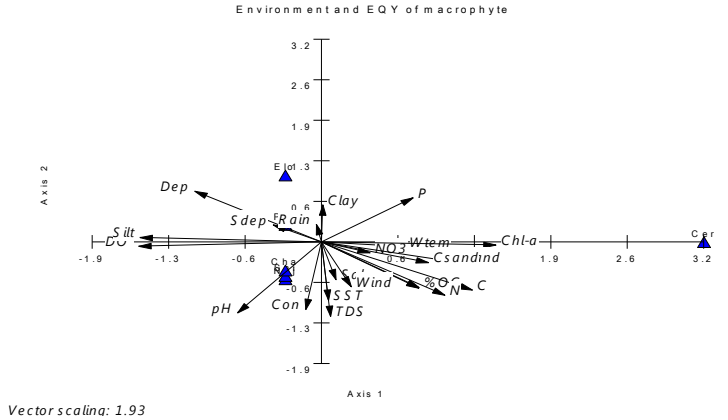


Eigenvalues		
	Axis 1	**Axis 2**
Eigenvalues	0.491	0.27
Percentage	57.755	31.73
Cum. Percentage	57.755	89.485
Cum.Constr.Percentage	57.755	89.485
Spec.-env. correlations	1.000	1.000

Biplot scores for env. variables		
	Axis 1	**Axis 2**
Depth	-0.666	0.224
TDS	0.389	-0.543
NO3	0.383	0.284
%OM	0.456	-0.251
%OC	0.451	-0.256
C	0.555	-0.169
N	0.513	-0.231
Fsand	0.454	-0.174

Figure A3. *Relationship among environmental parameters and chlorophyll a content of submerged macrophytes in Kukud, Songkhla Lake area from May 2017 to May 2018*



Eigenvalues		
	Axis 1	**Axis 2**
Eigenvalues	1.000	0.35
Percentage	68.557	23.978
Cum. Percentage	68.557	92.535
Cum.Constr.Percentage	68.557	92.535
Spec.-env. correlations	1.000	1.000

Biplot scores for env. variables		
	Axis 1	**Axis 2**
Depth	-0.56	0.423
NO3	0.213	-0.089
P	0.403	0.369
Chl-a	0.769	-0.025
%OM	0.428	-0.384
%OC	0.429	-0.378
C	0.664	-0.398
N	0.543	-0.442
Silt	-0.801	0.037

Figure A4. *Relationship among environmental parameters and photosynthetic efficiency (Effective quantum yield of PSII) of submerged macrophytes in Kukud, Songkhla Lake area from May 2017 to May 2018*

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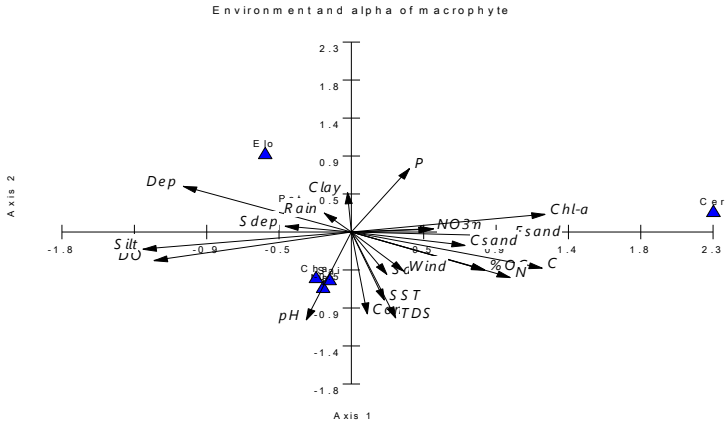
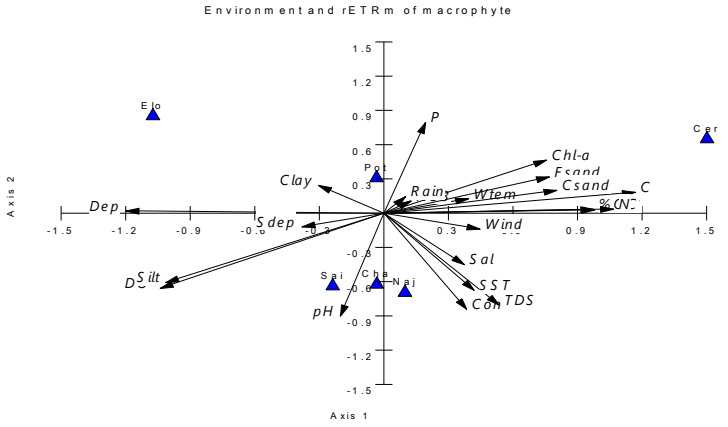


Eigenvalues		
	Axis 1	**Axis 2**
Eigenvalues	0.729	0.353
Percentage	59.18	28.697
Cum. Percentage	59.18	87.877
Cum.Constr.Percentage	59.18	87.877
Spec.-env. correlations	1.000	1.000

Biplot scores for env. variables		
	Axis 1	**Axis 2**
Depth	-0.644	0.334
NO3	0.307	0.02
P	0.222	0.463
Chl-a	0.739	0.13
%OM	0.509	-0.278
%OC	0.508	-0.274
C	0.729	-0.262
N	0.606	-0.33
Silt	-0.798	-0.124

Figure A5. *Relationship among environmental parameters and alpha of submerged macrophytes in Kukud, Songkhla Lake area from May 2017 to May 2018*



Eigenvalues		
	Axis 1	**Axis 2**
Eigenvalues	0.484	0.4
Percentage	45.61	37.718
Cum. Percentage	45.61	83.328
Cum.Constr.Percentage	45.61	83.328
Spec.-env. correlations	1.000	1.000

Biplot scores for env. variables		
	Axis 1	**Axis 2**
Depth	-0.84	0.015
DO	-0.728	-0.46
Chl-a	0.528	0.327
%OM	0.685	0.021
%OC	0.682	0.025
C	0.817	0.129
N	0.746	0.026
Silt	-0.71	-0.425

Figure A6. *Relationship among environmental parameters and maximum relative electron transport rate of submerged macrophytes in Kukud, Songkhla Lake area from May 2017 to May 2018*

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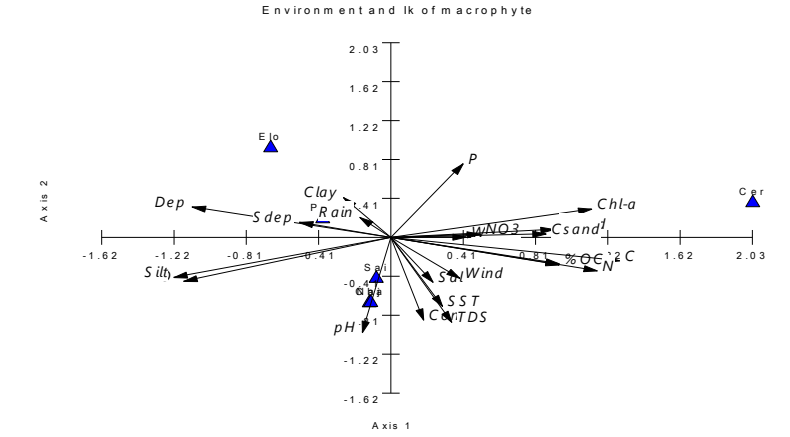
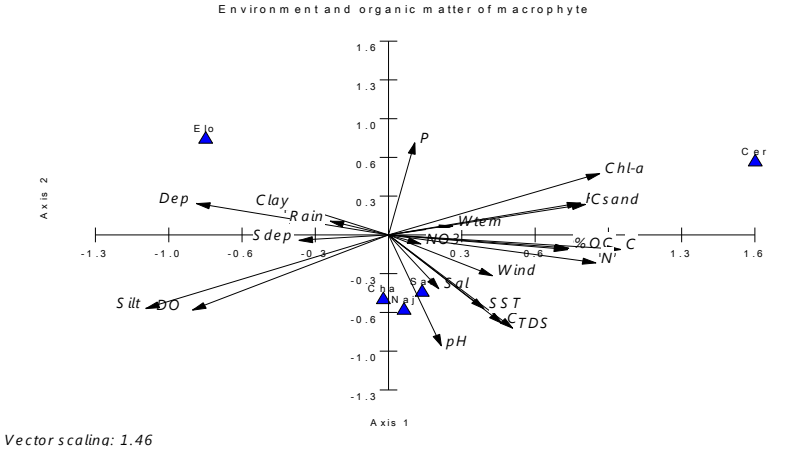


Eigenvalues		
	Axis 1	**Axis 2**
Eigenvalues	0.644	0.343
Percentage	58.418	31.085
Cum. Percentage	58.418	89.503
Cum.Constr.Percentage	58.418	89.503
Spec.-env. correlations	1.000	1.000

Biplot scores for env. variables		
	Axis 1	**Axis 2**
Depth	-0.714	0.204
DO	-0.744	-0.292
NO3	0.32	0.023
P	0.259	0.492
Chl-a	0.72	0.19
%OM	0.603	-0.182
%OC	0.603	-0.175
C	0.82	-0.155

Figure A7. *Relationship among environmental parameters and saturating irradiance of submerged macrophytes in Kukud, Songkhla Lake area from May 2017 to May 2018*



Eigenvalues		
	Axis 1	**Axis 2**
Eigenvalues	0.474	0.286
Percentage	55.009	33.142
Cum. Percentage	55.009	88.152
Cum.Constr.Percentage	55.009	88.152
Spec.-env. correlations	1.000	1.000

Biplot scores for env. variables		
	Axis 1	**Axis 2**
Depth	-0.576	0.179
Sal	0.15	-0.304
%OM	0.535	-0.082
%OC	0.539	-0.074
C	0.694	-0.081
N	0.621	-0.157
Fsand	0.575	0.181
Csand	0.589	0.174

Figure A8. *Relationship among environmental parameters and organic matter of submerged macrophytes in Kukud, Songkhla Lake area from May 2017 to May 2018*

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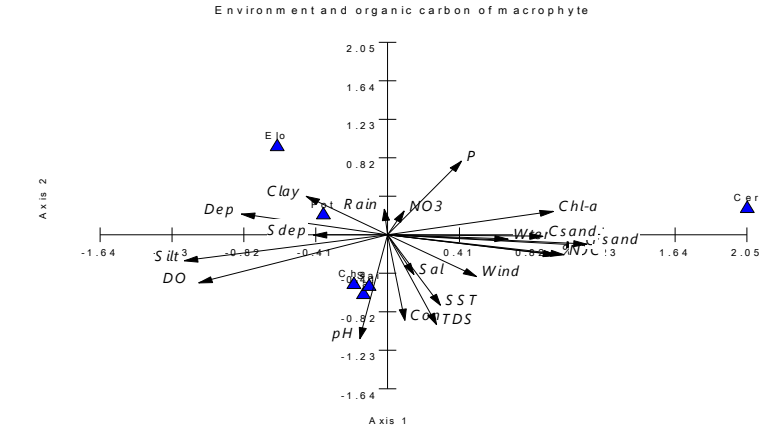
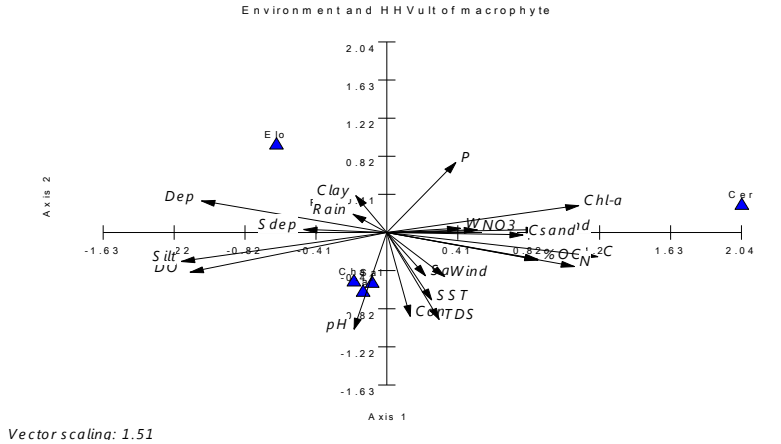


Eigenvalues		
	Axis 1	**Axis 2**
Eigenvalues	0.647	0.308
Percentage	61.023	29.054
Cum. Percentage	61.023	90.077
Cum.Constr.Percentage	61.023	90.077
Spec.-env. correlations	1.000	1.000

Biplot scores for env. variables		
	Axis 1	**Axis 2**
Depth	-0.524	0.14
Clay	-0.292	0.258
pH	-0.1	-0.693
Sdep	-0.266	0
%OM	0.604	-0.135
%OC	0.604	-0.133
C	0.688	-0.069
N	0.631	-0.132

Figure A9. *Relationship among environmental parameters and organic carbon of submerged macrophytes in Kukud, Songkhla Lake area from May 2017 to May 2018*



Eigenvalues		
	Axis 1	**Axis 2**
Eigenvalues	0.645	0.308
Percentage	60.988	29.109
Cum. Percentage	60.988	90.097
Cum.Constr.Percentage	60.988	90.097
Spec.-env. correlations	1.000	1.000

Biplot scores for env. variables		
	Axis 1	**Axis 2**
Depth	-0.705	0.224
Sdep	-0.317	0.023
Chl-a	0.728	0.191
DO	-0.749	-0.28
NO3	0.343	0.02
%OM	0.573	-0.193
%OC	0.573	-0.188
Silt	-0.782	-0.204

Figure A10. *Relationship among environmental parameters and HHVult of submerged macrophytes in Kukud, Songkhla Lake area from May 2017 to May 2018*

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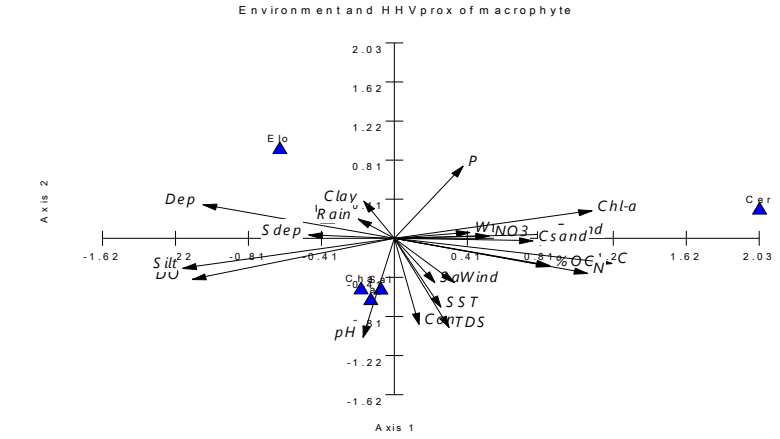
DOI: http://dx.doi.org/10.15666/aeer/1905_40694101



Eigenvalues		
	Axis 1	**Axis 2**
Eigenvalues	0.643	0.306
Percentage	61.051	29.053
Cum. Percentage	61.051	90.103
Cum.Constr.Percentage	61.051	90.103
Spec.-env. correlations	1.000	1.000

Biplot scores for env. variables		
	Axis 1	**Axis 2**
Depth	-0.705	0.232
Chl-a	0.726	0.191
Sdep	-0.316	0.024
DO	-0.745	-0.281
NO3	0.348	0.018
%OM	0.572	-0.192
%OC	0.571	-0.187
Silt	-0.78	-0.205

Figure A11. *Relationship among environmental parameters and HHVprox of submerged macrophytes in Kukud, Songkhla Lake area from May 2017 to May 2018*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_40694101

CHARACTERIZATION OF TEA (*CAMELLIA SINENSIS* L.) GENOTYPES GROWN IN TURKEY BY ISSR MARKERS

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(Received 25th May 2021; accepted 3rd Sep 2021)

Abstract. The aim of this study was to determine the genetic diversity of 18 tea (*Camellia sinensis*) genotypes and 6 varieties collected from different provinces in the Black Sea region of Turkey. DNA isolation was performed according to the CTAB method of tea samples. PCR was performed for all DNA samples with 59 ISSR primers. In order to determine the genetic relationship between tea genotypes among these primers, 15 polymorphic ISSR primers were used. At the end of the PCR procedures, the polymorphism rate was determined as 77% from the bands formed. As a result of the analysis of products obtained by PCR studies, 109 bands were obtained in ISSR primers. The obtained bands were scored as presence (1) and absence (0) and their files were created. A genetic relation dendrogram was formed according to the obtained band results. Genetic diversity of tea genotypes and varieties of the Black Sea region was determined by molecular markers.
Keywords: *Camellia sinensis, CTAB, endangered, Taq polymorphism, PCR*

Introduction

Turkey has a rich flora due to its geographical location, soil structure, and climate diversity. These characteristics allow Turkey to be one of the leading countries in the world in terms of plant diversity since it has many plant species growing naturally or is the gene center for many of them (Briggs and Knowles, 1967; Harlan, 1995; Kaya and Raynal, 2001; Ercisli, 2004; Altindal, 2019). Turkey has a diverse ecology allowing cultivation of a lot of horticultural crops of temperate, subtropical and semi tropical climates, in a narrow area in the south. Tea (*Camellia sinensis* L.) is a plant owes which its leaves and buds are used to produce beverages. The species belongs to the Theaceae family that grows in humid climates (Benzie and Szeto, 1999; Ferruzzi, 2010; Panda, 2011). Although it originates from is South and Southeast Asia, it is also grown in tropical and subtropical regions around the world (De Costa et al., 2007). The plants are small trees less than 2 m and are evergreen Tea is produced in 45 countries in an area of approximately 5 million 561 thousand hectares in the world. China is the leading country (36%) in world's dry tea production and Turkey ranks fifth (4%) (Anonymous 2016; Anonymous, 2018). Tea is cultivated in an area of provinces encompasses the Rize (65%), Trabzon (21%), Artvin (11%), Giresun (2%), and Ordu (1%) in 82.951 ha land in the Eastern Black Sea region, Turkey. Approximately, 22.331.000 tons of fresh tea leaves are harvested by 212.692 growers, and later transformed into 230,000 tons of dried leaves for brewing every year (Anonymous, 2018).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_41034114

The first tea cultivation in Turkey started in 1937 with the importation of 20 tons of tea seeds from Georgia. With these seeds, the first tea orchards were established in Rize and Artvin provinces. Over the years, new orchards and gardens in other provinces have continued to be established with the seeds obtained from these gardens. New varieties have been obtained by selecting individuals with superior characteristics, which differ in terms of morphological features, from these gardens, which were formed from seeds over time. Tea plants are mostly propagated by seeds in Turkey, although there are several clones, which creates wide genetic diversity resulting in variation in yield and quality. While this variation is not desirable for a standardized cultivation and high-quality leaf, it could provide new opportunities in development of new cultivars with superior characteristics from the established population by phenotypic selection. Therefore, it is important to determine the tea genetic sources. These differences also led to the emergence of close and distant inbreeding in the plant. Genetic differences were evaluated by researchers (Kafkas et al., 2009). In general, morphological characteristics are used in identifying superior genotypes, however, they are affected by cultivation techniques and ecological factors. Besides, selection breeding takes long time many years. Molecular marker technology has enabled the adoption of wide-ranging new applications to improve selection strategies in plant breeding (Upadhyaya et al., 2008; Bandyopadhyay, 2011; Abdel-Mawgood, 2012; Rajpal et al., 2016; Rahimi et al., 2018). Information obtained in recent years on plant molecular marker genetics can be reflected in plant breeding studies. For this reason, newly developed or modified plant breeding methods should be used based on information obtained from plant molecular biology studies. With the use of molecular markers in plant breeding, the efficiency of classical breeding is improved by providing advantages such as backcross breeding, creation of gene pyramids, selection of recessive genes, gene transfer from wild genetic resources, and early selection, thus accelerating the development of new varieties (Nybom et al., 2014). Although molecular marker applications alone cannot be used as a substitute for classical breeding, they are considered as a complementary technique that increases the success of classical breeding. In tea, several molecular markers have been used (Roy et al., 2009; Zang et al., 2018). Different markers have different advantages such as SSR and AFLP markers for polymorphism, RAPD and ISSR markers for cost efficiency, RFLP, SSR, SRAP, ISSR, and AFLP markers for repeatability. One of the widely used markers is ISSR markers (Lai et al., 2001; Yao et al., 2008; Ji et al., 2011; Rahimi et al., 2019).

Tea is grown naturally in the provinces of Rize, Artvin, Trabzon, Giresun, and Ordu in the eastern Black Sea region of Turkey. Of the total of 829.505 decares planted tea areas in these regions, 65% was in Rize, 21% in Trabzon, 11% in Artvin, 2% in Giresun, and 1% in Ordu. Every year, around 212.692 farmers harvest around 22.331 million tons of fresh tea leaves in the region. Approximately 220,000-230,000 tons of dry teas are produced from this amount of fresh leaves (Anonymous, 2018). Seeds are used as plant material in the creation of tea fields in our country. A large proportion of genetic variation occurs in gardens created from seeds. Therefore, it is important to determine the tea genetic sources in our country. In this study, by using the ISSR markers, it was aimed to make distinctions of tea genotypes and registered tea varieties selected as representatives of tea fields grown in Turkey.

The aim of the study was to molecular characterization of tea genotypes with seedling origin and registered tea clones grown in the Eastern Black Sea Region of Turkey by using the ISSR markers.

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DOI: http://dx.doi.org/10.15666/aeer/1905_41034114

Materials and methods

Plant material

A total of 24 *Camellia sinensis* L. genotypes representing all of the intensive tea growing provinces in the Eastern Black Sea region were used in the study. The genotypes were consisted of 18 seedlings selected from provinces (8 from Rize, 5 from Giresun, 2 from Artvin, 2 from Trabzon, and 1 from Ordu), and 6 clonally propagated standard varieties maintained in collection plots of Çaykur Atatürk Tea and Horticultural Research Institute in Rize (*Table 1; Fig. 1*). Leaf samples were collected from freshly growing shoots

DNA isolation

Genomic DNA isolation was performed by cetyltrimethylammonium bromide (CTAB) method (Saghai-Maroof et al., 1984) in the Genome and Stem Cell Research Laboratory at Erciyes University, Kayseri. DNA concentrations were determined and the purity (A260/A280) was checked by using BioSpec-nano Shimadzu Biotech spectrophotometer that DNA sufficient quality was used in ISSR-PCR studies. DNA concentration was diluted to 20 ng/µl, and the DNA was maintained at -20 °C until use.

Table 1. *Places where they are taken and codes of tea genotypes and varieties used in the study*

No	Province of origin	Country
1	Pazar1	RİZE
2	Pazar2	RİZE
3	Hopa	ARTVİN
4	Arhavi	ARTVİN
5	Fındıklı	RİZE
6	Ardeşen	RİZE
7	Hayrat1	TRABZON
8	Of	TRABZON
9	İyidere	RİZE
10	Derepazarı	RİZE
11	Güneysu	RİZE
12	Çayeli	RİZE
13	Fener3	RİZE and AÇBKAE
14	Fener	RİZE and AÇBKAE
15	Hayrat2	RİZE and AÇBKAE
16	Hamzabey	RİZE and AÇBKAE
17	Muradiye10	RİZE and AÇBKAE
18	Enstitü	RİZE and AÇBKAE
19	Tirebolu1	GİRESUN
20	Tirebolu2	GİRESUN
21	Tirebolu3	GİRESUN
22	Tirebolu4	GİRESUN
23	Tirebolu5	GİRESUN
24	Perşembe	ORDU

AÇBKAE: Atatürk Tea and Horticultural Research Institute

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DOI: http://dx.doi.org/10.15666/aeer/1905_41034114

Figure 1. *Distribution and locations of sampling points. ● Eighteen randomly collected C. sinensis genotypes. ▼ Six C. sinensis varieties provided by the Rize Ataturk Tea and Horticulture Research Institute*

For genetic diversity study

ISSR primers

Genomic DNAs were tested on 59 randomly selected ISSR primers. From these primers, 15 of them, which could be repeatable and polymorphic, were used in the PCR reactions (*Table 2*).

Polymerase chain reaction (PCR) protocols

PCR analysis was performed on a Bio-Rad T100 ™ device. For PCR reaction, a total of 15 µl solution, which included 1.2 µl dNTP (2.5 mM), 1.5 µl 10X Taq Buffer, 1.5 µl HQ Buffer, 1 pmol ISSR marker, 5 µl genomic DNA, 3.8 µl dH2O, 1 µl (2.5 units) DNA Taq polymerase, was prepared on 96 x 0.2 ml PCR plates. For–amplification, fragment replications were made in the form of 300 s at a final extension temperature of 72 °C after 40 cycles, after 1 cycle pre-denaturation at 95 °C for 180 s, 40 cycles at 95 °C for 45 s, 40 cycles at marker binding temperature of 55 °C for 45 s, and 40 cycles at 72 °C for 2 min. PCR products were separated by running them on 2% agarose gel. Separated DNA bands were placed on a UV transilluminator and DNA bands were displayed.

Determination of polymorphism rates of primers

The polymorphism ratios of ISSR primers were calculated by following formula:
Polymorphism rate (%) = (Number of polymorphic bands / Tptal number of bands) x 100

Statistical analysis

Of the 59 ISSR primers tested, 15 produced clear and polymorphic bands and were selected for further analysis. The bands on the agarose gel images were scored either "1" or "0" according to the presence or absence. Strong bands were evaluated in scoring and a similarity index was created.

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DOI: http://dx.doi.org/10.15666/aeer/1905_41034114

Dendrogram data, based on the similarity index, with the results obtained, were analyzed according to the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method (Rohlf, 1998).

Table 2. *ISSR primers used in the study*

Primer name	Primer sequence (5'-3')	Primer name	Primer sequence (5'-3')
UBC 808	(AG)8C	UBC 834	(AG)8YT
UBC 809	(GA)8T	UBC 836	(AG)8YA
UBC 816	(CA)8T	UBC 841	(GA)8YC
UBC 825	(AC)8T	UBC 842	(GA)8YG
UBC 826	(AC)8C	UBC 843	(CT)8RA*
UBC 827	(AC)8G*	UBC 845	(CT)8RG
UBC 840	(GA)8YT*	UBC 847	(CA)8RC
UBC 841	(GA)8YC*	UBC 848	(CA)8RG
UBC 844	(CT)8RC	UBC 851	(GT)8YG
UBC 850	(GT)8YC*	UBC 852	(CT)8RA*
UBC 855	(AC)8YT*	UBC 856	(AC)8YA
UBC 868	(GAA)6	UBC 857	(AC)8YG*
UBC 807	(AG)8T	UBC 858	(TG)8RT*
UBC 810	(GA)8T	UBC 859	(TG)8RC*
UBC 811	(GA)8C	UBC 860	(TG)8RA*
UBC 812	(GA)8A	UBC 866	(CT)8C
UBC 814	(CT)8A	UBC 887	DVD(TC)8
UBC 815	(CT)8G	UBC 888	BDB(CA)8
UBC 818	(CA)8G	UBC 889	DBD(AC)8
UBC 819	(GT)8A	UBC 890	VHV(GT)8
UBC 820	(GT)8T*	UBC 891	HVH(TG)7
UBC 821	(GT)8T	ISSR 7	(AG)8YC*
UBC 822	(TC)8A	ISSR 9	(CT)9RC
UBC 823	(TC)8C	ISSR 16	(TCC)5RY
UBC 824	(TC)8G	ISSR 21	(AG)8YT*
UBC 825	(AC)8T*	ISSR 28	A(GAAA)4G
UBC 826	(AC)8C	ISSR 43	(GT)8YA
UBC 828	(TG)8A	ISSR 47	(AG)8Y
UBC 830	(TG)8G		

*Polymorphic primers

Results and discussion

ISSR amplification results

In this study, molecular characterization of 18 seedling tea genotypes and 6 standard varieties grown in the Eastern Black Sea Region was performed by 59 ISSR primers. First 59 ISSR primers were tested on 8 genotypes, then 15 ISSR primers producing reproducible and polymorphic bands, were used in the molecular characterization of all samples (*Table 2*). Gel images and amplification products are shown in *Figure 2*. Today, it is important to perform genetic diversity analysis to have information about changes that may occur about the sustainable use and future status of plant genetic resources, to evaluate the evolutionary process of a population, and conservation of

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species (Pan et al., 2017; Yun-qinq et al., 2019). Many DNA molecular marker techniques based on PCR technology have been developed in the characterization of plant gene sources. The most known DNA marker techniques and used by many researchers are ISSR, RAPD, AFLP, SRAP. The ISSR DNA molecular marker technique, which is frequently used in the fields of genetic diversity, gene tagging, phylogenetic studies, genome mapping, and evolutionary biology, is among the most preferred, especially due to its ease of application and repeatability (Kafkas et al., 2006; Norozi et al., 2009; Nazafzadeh et al., 2014). Furthermore, the fact that ISSR markers are more efficient than other markers in genetic diversity assessment and their ability to produce more polymorphic bands increases the applicability of these markers (Kumar and Agrawal, 2019). The researches revealed that varieties that cannot be separated by RAPD markers can be separated by ISSR markers (Indu et al., 2020). This finding suggests that the evolution rate of ISSRs may be faster than the RAPDs in the tea samples studied. Therefore, the ISSR marker technique is suitable for use in the study of genetic diversity and the determination of genetic relationships of closely related tea varieties. Different studies have also indicated that ISSR markers show higher levels of polymorphism compared to RAPD markers (Yang et al., 1996; Nagaoka and Ogihara, 1997; Parsons et al., 1997; Esselman et al., 1999).

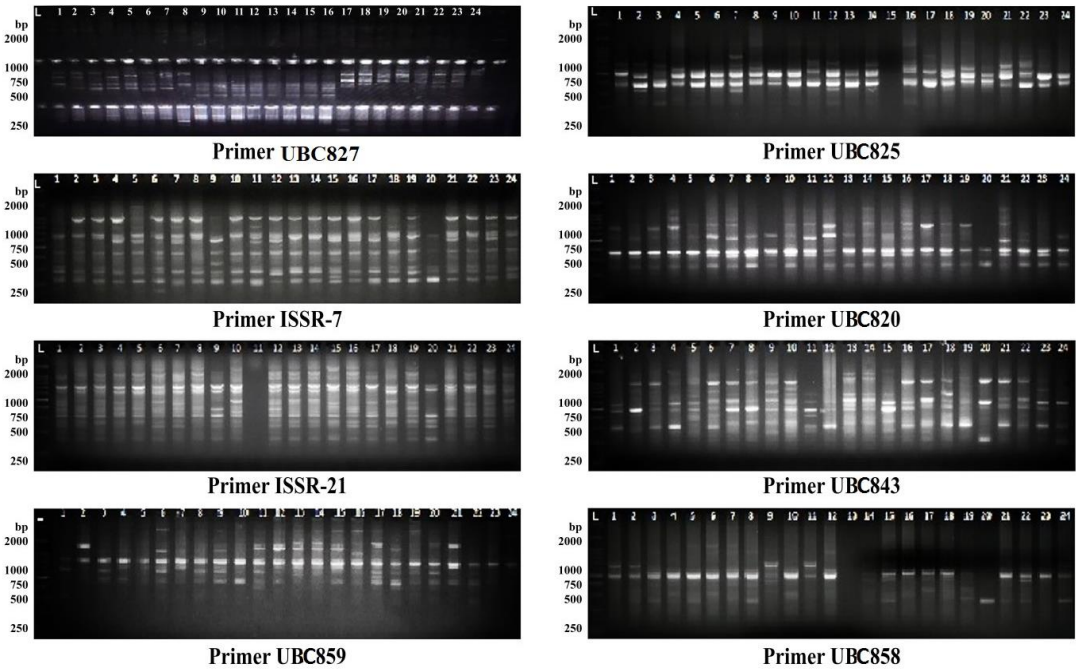

Figure 2. *Image of amplification products generated with the ISSR Primers, L: 5 µl DNA (ladder)*

In this study the total number of clearly visible bands was 109, and 84 of them were polymorphic. Band sizes ranged between 150 and 1500 base pairs (bp) and the polymorphism rate (PR) was 77%. Number of polymorphic ISSR bands ranged between 2 and 8 that minimum and maximum number of bands were given by UBC 858, and UBC 820 and UBC 843 primers, respectively. Polymorphism rates were observed in the range of 50% to 100% among the primers that ISSR 21 showed the lowest polymorphism, while UBC 843, UBC 850, UBC 860, and UBC 852 primers showed the highest polymorphism

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(*Table 3*). All available primers cannot be used in all plant species, so it is necessary to develop and select appropriate primers for each plant species and variety. Christopoulos (2010) used 95 ISSR primers in walnut, and 7 of them were polymorphic; Zafar-Pashanezhad et al. (2020) tried 30 ISSR primers in *Eruca sativa*, and 19 of them were polymorphic; Atalmış (2007) detected 15 ISSR primers as polymorphic out of 17 primers in melon; Demirel (2013) found that 14 primers as polymorphic among 54 ISSR primers used in wheat; Kaç (2013) found a total of 12 primers as polymorphic of 21 ISSR primers used in tea, and Çeker (2008) tried 65 ISSR primers in chickpeas and determined that 11 of them were polymorphic. These results are similar to the results of our study. Polymorphic primers will be the resource for future studies to be conducted on tea. Karadut and Kafkas (2013) used 137 ISSR primers in pistachio, walnut, apple, apricot, and cherry varieties, and they obtained 791 bands in pistachio, 613 bands in walnuts, 696 bands in apples, 666 bands in apricots, and 514 bands in cherries. In a study conducted with 91 chickpea genotypes of Turkish origin and 2 chickpea varieties, researchers managed to produce a total of 242 bands from 11 polymorphic ISSR primers (Çeker, 2008). A total of 391 bands were obtained from 11 polymorphic ISSR primers in the genus *Ankyropetalum* Fenzl found in Turkey (Atgüden, 2016). Although a smaller number of bands we obtained in tea samples, 84 bands were sufficient to distinguish tea genotypes and varieties from each other. In a study using 6 different ISSR primers on the endangered rare endemic species *Parrotia subaequalis*, the researchers determined that there were large genetic differences among 25 separate samples in the population with a polymorphism rate of 79.66% (Yun-xia et al., 2020). In another biodiversity study, on *Trichosanthes dioica*, using ISSR, SCoT, and SRAP markers, the researchers reported that 20 different ISSR primers gave a 95.96% polymorphism ratio and an average number of 15.25 bands. The researchers reported that ISSR markers out of these 3 different marker types used were more informative in the assessment of genetic diversity and produced a higher average number of polymorphic bands (Kumar and Agrawal, 2019). Although the highest polymorphism rates obtained in these studies on different species were lower than the polymorphism rates found in this study, the average number of bands obtained were higher compared to our study.

Similarity index and genetic relationship dendrogram

The numbering sequence created for the genotype and varieties of tea was presented in *Table 1*. Clustering analysis was performed by the UPGMA method (*Fig. 3*). The Dendrogram showed that two branches, A and B, were formed. It was detected that the group B contains 1 genotype, and the remaining 23 genotypes constitute the group A which was mainly divided into two as A1 and A2. The A1 contains 2 genotypes and the A2 contains 21 genotypes, and the A2 group is also divided into subgroups. The dendrogram revealed that the most distantly related genotypes to the others were Güneysu and Pazar, while the most similar genotypes to each other were İyidere and Derepazari. A2 group forms the main group with various genotypes consisting of 6 subgroups. The first subgroup consisted of Tirebolu1-Hamzabey-Çayeli. In the second subgroup, the Tirebolu3-Hayrat1-Ardeşen genotype and their varieties are intertwined. There are 8 genotypes in the third subgroup, and this of-Hayrat2-Fener3 - Fener-Tirebolu4-Tirebolu5-Iyidere-Derepazari genotype and its varieties are also intertwined. İyidere-Derepazarı genotypes were found genetically identical. The genotypes of Pazar2-Hopa-Muradiye-Institute in the fourth subgroup also formed a group among themselves. The last subgroup contains 3 genotypes and has been sampled from nearby

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provinces. Pazar-Arhavi- Fındıklı in this group show a close relationship among them. The similarity levels varied between 0.62 and 0.97 among the genotypes. In another study, the genetic similarity levels between 19 tea varieties taken from the Rize region were between 0.26 and 0.75 (Kaç, 2013). When the tea genotypes collected from the current tea growing regions were examined, a 62% similarity was detected among them. The ISSR molecular marker technique was used to determine the genetic relationships among 40 wild tea plants grown in Greece, and the genetic similarity rates were determined as 0.41, 0.37, and 0.55 (Liu et al., 2012). Yao et al. (2008) used the ISSR molecular marker technique to determine the genetic diversity and relationship among 48 tea varieties brought from China, Japan and Kenya, and they found that there was a greater amount of diversity among Chinese varieties than in Japan, and Kenya. The coefficient of gene differentiation (GST) was found as 0.202, indicating a high degree of genetic diversity in these populations. In another study conducted to reveal the possible genetic variation of *C. sinensis* caused by electromagnetic fields, the researchers used 10 ISSR and 8 SCoT primers, and they reported that ISSR primer sequences showed more variation compared to SCoT sequences (Azizi et al., 2020). Looking at the tea varieties in general, it is observed that genetic expansions have emerged. It is argued that this expansion is due to cross-pollination. The frequent preference of ISSR primers in *C. sinensis* genetic diversity studies in general by researchers supports our study. The first tea seeds to come to Turkey were taken from the same region. For many years, the seeds of plants grown from these seeds have been used in the establishment of new tea orchard. In this respect, since the population has been pollinated and fertilized within itself for many years, the genotypes and varieties formed from the seeds have shown a great deal of similarity. However, those that differ genetically should be examined and investigated morphologically.

Table 3. *Allele numbers and polymorphism rates of polymorphic ISSR primers used in tea genotypes and varieties*

Primer name	Total number of bands	Number of polymorphic bands	Percentage polymorphism (%)
UBC 820	10	8	80.0
UBC 825	8	6	75.0
UBC 827	6	4	66.6
UBC 840	6	4	66.6
UBC 841	8	6	75.0
UBC 843	8	8	100.0
UBC 850	7	7	100.0
UBC 852	7	7	100.0
UBC 855	6	5	83.3
UBC 858	3	2	66.6
UBC 857	6	5	83.3
UBC 859	5	4	80.0
UBC 860	6	6	100.0
ISSR 7	11	6	54.5
ISSR 21	12	6	50.0
Total	109	84	-
Mean	7.3	5.6	77

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DOI: http://dx.doi.org/10.15666/aeer/1905_41034114

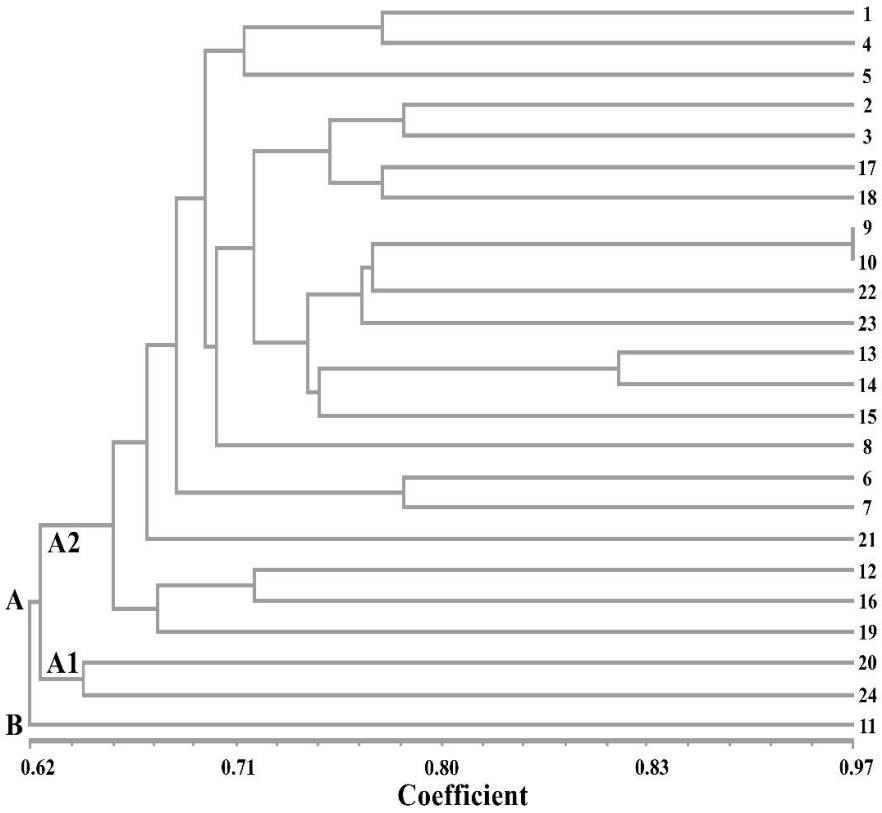

Figure 3. *Dendrogram based on the similarity index between C. sinensis genotypes and varieties using ISSR markers*

Conclusions

The study was conducted with 18 different genotypes and 6 different varieties of standard tea. Genotypes were selected as a representation from tea orchard of Artvin, Rize, Trabzon, Giresun, and Ordu provinces where tea is grown in Turkey. In the study, a total of 59 different ISSR primers were used and 15 of them were determined to be polymorphic for tea. It has been found that generally tea genotypes propagated by seed are genetically similar at a rate of 62%. For example, the genetic structures of the Iyidere-Derepazarı genotypes were found to be similar to each other (*Fig. 3*). A total of 38% of the tea population was determined to show a difference, which was found to occur due to genetic expansion. In the market, the idea that the tea of different companies or regions is of better quality is not entirely due to genotype, but also that the ecology in which the tea grows is effective in climate and soil characteristics, as well as processing technology. At the same time, this genetic difference can be used in breeding study.

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DOI: http://dx.doi.org/10.15666/aeer/1905_41034114

MODELING THE MAXIMAL ORGANIC CARBON SATURATION CAPACITY IN KARST FOREST SOILS OF CHINA: COMPARING THE EXISTING MODELS

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(Received 26th May 2021; accepted 4th Sep 2021)

Abstract. Karst forests are special and complex, featuring soil that typically exists as a thin layer, with a low C(Carbon) storage capacity but is rich in calcium and alkali. However, the maximum saturated capacity of the soil organic carbon (SOC) remains unclear. To address this crucial knowledge gap, a total of 10 typical karst forest (climax communities) topsoil samples of China were investigated using SOC fractionation methods, for which a maximum saturated capacity model of SOC was derived via least-squares linear regression (LSR) and boundary line analysis (BL). For our data, we found that the over-saturated proportion (the actual measured carbon content is greater than estimated by the model) of organic C data according to the Hassink model (y = 0.37x + 4.09), LSR (y = 0.35x + 6.83), and the Feng model (y = 1.07x) were 56.7%, 40.0%, and 16.7%, respectively, while that according to the BL model (y = 1.30x) was only 3.3%. This comparative analysis, based on validations with a robust sample size (N = 30), shows that the Hassink model, LSR, and Feng model all performed poorly at estimating the maximal organic C saturation capacity in karst forest, in contrast to the BL model (y = 1.30x), which is more suitable and accurate.

Keywords: *climax communities, boundary line analysis, least-square linear regression, soil organic carbon, soil fine particles*

Introduction

The soil carbon pool is critical for the global C cycle because it is the largest C pool within the surface (0–20 cm) of terrestrial ecosystems, of which forests are vital components (Trumbore et al., 2006; Banger et al., 2009). Globally, forest ecosystem soils

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_41154128

collectively store 40% of the organic C in the top 0-1 m soil layer, and its accumulation and decomposition processes directly impact the global C balance (Schlesinger, 1990; Ding et al., 2012; Zhu et al., 2017; Chen et al., 2019; Liu et al., 2020). Therefore, the size and dynamics of the forest SOC pool within the global C cycle and under ongoing climate change is particularly important (Dixon et al., 1994; Houghton et al., 2001; Pan et al., 2005).

Karst forest is a distinctive ecosystem type, especially those located in southwest China, which reside in a subtropical monsoon climate, where the soluble carbonate rocks result in slow soil formation, thereby generating soil rich in calcium and alkali that differs starkly from that in non-karst areas. Accordingly, the relationship between calcium carbonate in soil and karst SOC directly affects the maintenance of the karst forest ecosystems (Su et al., 2015; Hu et al., 2016), imparting with specific features regarding soil C cycling (Lu et al., 2014; Wang et al., 2015; Li et al., 2017). Therefore, it is of great significance to study how the SOC pool is balanced in karst forest.

Many studies find that SOC fractions show different response mechanisms under levels of long-term exogenous C input. For example, Stewart et al. (2008, 2009) showed that with an increased C input level, non-protected organic C and physically protected organic C exhibit a linear growth trend, while chemically protected organic C and biochemically protected organic C followed a "curve saturation" growth trend. In other works, by Six et al. (2002), West et al. (2007), Chung et al. (2008, 2010), and Luo et al. (2020), it was found that small aggregate organic C (< 20 μm) also showed a "curve" increase with more C inputs into soil. This phenomenon was defined by Stewart et al. (2008) as "soil C saturation"; that is, after putting a certain amount of exogenous C into the soil, the SOC will increase over time until it reaches a state of dynamic equilibrium, where the total level of soil C input equals that of C release. However, should new C sources be continually injected into the soil, this delicate C balance will likely become disrupted and this effect will intensify and persist through time, perhaps reaching a new stable state. However, if exogenous C is continually added yet the SOC remains unchanged, the type of balance is termed "soil C saturation," that is, the maximum C capacity of the soil has been attained (West et al., 2007; Rillig et al., 2019; Jing et al., 2020).

In an earlier work, Hassink (1997) compared the stable C content of natural grassland and cultivated soil, finding the former had a higher organic C content driven by >20 μm soil particles, since there was no difference in the organic C content of <20 μm particles between the two kinds of soil. Therefore, the protection capacity of fine particles for organic C was proposed, that is, fine particle organic C is stable organic C, and the capacity is limited. On this basis, by collecting data and focusing on fine particle C with relatively stable C sequestration in soil, many researchers generated empirical models and parameter indexes for predicting the saturated capacity of SOC, which could be used to estimate the turnover rate of SOC and the C saturation deficit in a specific region (Williams et al., 1974; Hassink, 1997; Six et al., 2002; Liang et al., 2009; Feng et al.,

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
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2013; Di et al., 2018). For example, Hassink (1997) used least-squares linear regression (LSR) to obtain a simple straightforward model for estimating the saturated capacity of fine particle organic C, namely y = 0.37x + 4.09, where x is the mass proportion of the < 20 μm fine particles. This empirical model has since been widely used (Angers et al., 2011; Zhang et al., 2014; Guo et al., 2016; Bastida et al., 2019). Later, Feng (2013) made a comparative study of grassland, cultivated land, and forest soils, uncovering some shortcomings of the Hassink model. The estimation of the maximum saturated capacity of SOC was improved by instead applying a boundary line (BL), thus providing a model specific for forests taking the form of y = 1.07x (where x is the mass ratio (proportion) of <20 μm fine particles).

In the karst forest region of China, we previously compared with the research objects of Hassink and Feng, the regional location, soil type and climatic conditions are different, so whether the model can be used to estimate the maximum saturated capacity of SOC in karst forest of China remains to be further verified. Therefore, the primary research questions addressed in the present study are: Is the Hassink model or Feng model suitable for estimating the maximum saturated capacity of SOC in the karst forests of China? If neither is suitable, how might we construct an improved model to predict the maximum saturated capacity of SOC?

Materials and methods

Field investigation and soil sample collection

Based on multiple field investigations and our working experience, 10 climax communities having the oldest forest stands and the least disturbance were selected for study in a typical karst area of Guizhou Province in China. The 10 sites were the Maolan national nature reserve (‘Maolan’), Daozhen dashahe nature reserve (‘Dashahe’), Shibing yuntai mountain nature reserve (‘Yuntai mountain’), Nayong gongtong nature reserve (‘Nayong’), Pogang karst vegetation nature reserve (‘Pogang’), Suiyang kuankuoshui nature reserve (Kuankuoshui), Puding huoyan mountain nature reserve (‘Huoyan mountain’), Jiangkou huanggu mountain nature reserve (‘Huanggu mountain’), Kaiyang zijiang rift valley (‘Zijiang rift valley’), and Wangmo bijia mountain (‘Bijia mountain’); all locations are shown in *Figure 1*.

In December 2019, a minimum sampling area of 900 m^2 (30 m × 30 m) was determined by drawing a "species–area curve". Three sampling plots were constructed in each climax community, for which the longitude and latitude, slope aspect, slope, soil type and altitude were recorded, in addition to determining the dominant plant species names as well as the abundance (counts), height, crown width, coverage, and DBH (diameter at breast height [1.3 m]) of individual trees (*Table 1*). In addition, from each site, one composite soil samples (one per plot) were taken from the surface layer (0-20 cm depth). These samples were then sieved through 2 mm and 0.25 mm mesh screens; any roots and stubble were removed, and 30 samples (There were 10 climax communities, each climax community

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_41154128

was set with 3 plots, and 1 soil samples were collected in each plot) were analyzed separately.

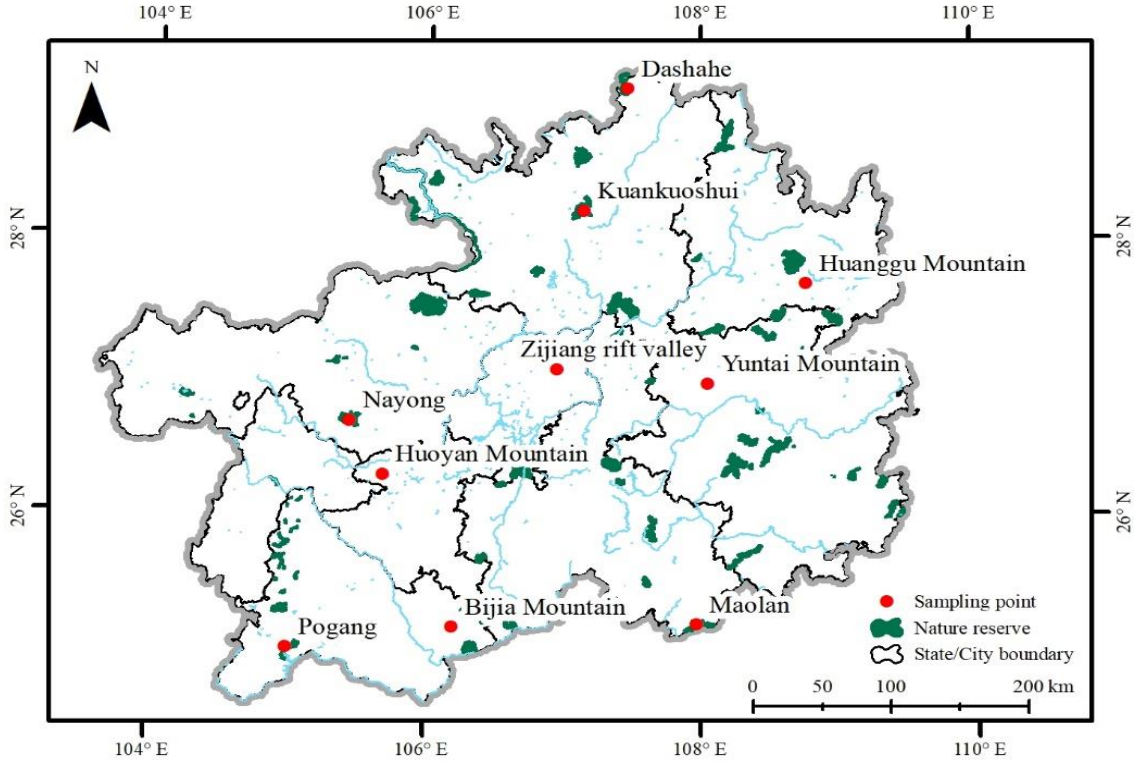

Figure 1. *Geographical map showing the locations in Guizhou Province (China) of the sampled karst forest sites*

Soil fractionation and C analyses

Six et al. (2002) divided SOC fractionation into three size fractions by wet sieving-isolation of microaggregates: >250 μm (coarse particles), 53-250 μm (microaggregate), and <53 μm (fine particles). Each soil subsample (20 g) was placed on a 250 μm sieve and carefully shaken with 30 glass beads on the top of the microaggregate isolator containing water filled to two-thirds of its volume. After 20 min, the fraction corresponding to the >250 μm particle size and that fraction whose particles had a size between 250 and 53 μm were collected in an aluminum specimen box. The remaining suspension harboring the <53 μm size and water was flocculated with 20 mL of $CaCl_2$ (0.25 M) and centrifuged at 127 × g for 7 min, to separate and isolate the fine particle fraction. All three fractions were dried at 60°C, weighed, and stored. Their respective SOC was determined using the potassium dichromate method. The fractionation and respective determinations of SOC were repeated three times for each soil sample.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_41154128

Table 1. *Basic information on the environmental background of different sampled sites for karst forest in China*

Sampling point site	Vegetational form	Location	Elevation, longitude & latitude	Slope aspect and position	Dominant tree species	Soil bedrock	Soil type	Total cover extent
Maolan	Climax vegetation	Baixian mountain	850 m, 107.99, 25.19	Southwest central	Carpinus pubescens, Swida wilsoniana, Pittosporum brevicalyx, Cyclobalanopsis multiervis, Acer wangchii	Dolomite limestone	Limestone soil	89.45%
Dashahe	Climax vegetation	Shahe	1304 m, 107.58 & 29.17	North central	Machilus pingii,Fagus longipetiolata, Pistacia chinensis Bunge, Lindera communis	Carbonate rock	Limestone soil	88.32%
Yuntai mountain	Climax vegetation	Yuntai mountain	875 m, 108.11 & 27.12	Northwest central	Machilus pingii, Pistacia chinensis Bunge, Acer cordatum Pax	Carbonate rock	Limestone soil	85.74%
Nayong	Climax vegetation	Jinzhulin village	1861, m 105.44 & 26.68	Northwest central	Davidia involucrata, Decaisnea insignis, Dipentodon sinicus, Cyclobalanopsis argyrotricha	Carbonate rock	Limestone soil	87.96%
Pogang	Climax vegetation	Minzu village	1280 m, 105.09 & 25.11	North central	Eucalyptus robusta, Platycarya strobilacea, Itoa orientalis Hemsl	Dolomite limestone	Limestone soil	86.63%
Kuankuoshui	Climax vegetation	Matixi	1450 m, 107.06 & 28.18	Southwest central	Fagus longipetiolata, Emmenopterys henryi, Tulip poplar	Carbonate rock	Limestone soil	91.34%
Huoyan mountain	Climax vegetation	Fenglin huoyan mountain	1680 m, 105.79 & 26.47	West central	Rhododendron stamineum, Birch, Oak	Carbonate rock	Limestone soil	90.17%
Huanggu mountain	Climax vegetation	Heitanggou	1020 m, 108.78 & 27.54	North central	Fagus longipetiolata, Buxus sinica, Davidia involucrata, Hemlock	Carbonate rock	Limestone soil	87.15%
Huanggu mountain	Climax vegetation	Heitanggou	1020 m, 108.78 & 27.54	North central	Fagus longipetiolata, Buxus sinica, Davidia involucrata, Hemlock	Carbonate rock	Limestone soil	87.15%
Zijiang rift valley	Climax vegetation	Liangchahe	720 m, 107.04 & 26.90	Southwest central	Betula luminifera, Cinnamomum camphora, Pistacia chinensis, Liquidenbar formosana	Carbonate rock	Limestone soil	81.25%
Bijia mountain	Climax vegetation	Liangfengao	1083 m, 106.14 & 25.12	Northeast central	Cyclobalanopsis oak, Carpinus pubescens, Celtis sinensis, Ormosia saxatilis	Carbonate rock	Limestone soil	90.18%

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Data analysis

Building on the original work of Hassink (1997), as well as more recent studies (Hassink, 1997; Six et al., 2000, 2002; Stewart et al., 2007, 2008, 2009, 2012; Feng et al., 2013), we applied several different methods to estimate the stabilization capacity of the fine particles, based on statistical analyses of the relationship between the C contents of the fine particles and mass proportions. Combined with the karst site characteristics, the two classical modeling approaches (LSR and BL) were compared and analyzed. Through model verification, a model for estimating the maximum saturated capacity of SOC in karst forest was constructed.

The Hassink (1997) method is based on the LSR of the mass ratio of fine particles (g fraction 100 g^{-1} soil) as a function of the SOC content of fine particles (mg C g^{-1} soil); it is described as $y = 0.37x + 4.09$, where y is the maximum C capacity that the soil can retain (mg C g^{-1} soil), and x is the fine particles mass ratio (g fraction 100 g^{-1} soil).

The BL method described by Feng (2013) was also applied herein to estimate the C stabilization capacity. The BL method is a statistical approach to define the upper limits of a dependent variable where there is evidence for a limiting response to an independent variable(s) along a defined boundary. Briefly, the method involves applying linear regression analysis to the upper-most (e.g., the top 10%) values of fine fraction C content across a wide range of fine particle masses. This process involved sorting the data into nine groups based on the mass proportions of the soil fine particles, at intervals of 10 g fraction 100 g^{-1} soil, ranging from < 10 to > 90 g fraction 100 g^{-1} soil. The upper 10% of soil C values in each group were identified and extracted along with the corresponding mass proportion data. These boundary line data were then subjected to a regression analysis where the y-intercept was forced through the origin, based on the assumptions outlined above. The forest model of Feng (2013) is $y = 1.07x$, where y is the maximum organic C capacity that the soil can retain (mg C g^{-1} soil), and x is the fine particles mass ratio (g fraction 100 g^{-1} soil).

Results

Changes to soil fine particles and total organic C

Table 2 reports our analysis of the mass proportion and C content of soil fine particles as well as the total organic C content in 10 karst forest climax communities. The mass proportions of soil fine particles in Maolan, Yuntai mountain, and Zijiang rift valley were the highest, at 31.36, 28.61 and 26.31 g fraction 100 g^{-1} soil, respectively. However, both the C content of soil fine particles and total organic C content were greatest in Nayong, at 23.56 mg C g^{-1} soil and 147.11 mg C g^{-1} soil, respectively. There was no significant difference in the ratio of soil fine particles to total organic C among the 10 climax community sites.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
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Table 2. *Mass proportion of soil fine particles and proportion of organic C content of the total organic C content in karst forest of the 10 nature reserves*

Sampling point site	Mass proportion of soil fine particles (g fraction 100 g^{-1} soil)	Organic C content of soil fine particles (mg C g^{-1} soil)	Total organic C content (mg C g^{-1} soil)	Proportion of organic C content (g fraction 100 g^{-1} soil)
Maolan	31.36 ± 3.13a	17.17 ± 0.73b	94.31b ± 3.60	18.28 ± 0.67
Dashahe	22.05 ± 1.27c	15.52 ± 1.46b	78.75cd ± 2.45	19.45 ± 1.36
Yuntai mountain	28.61 ± 2.05b	16.10 ± 2.48b	82.13c ± 5.01	19.60 ± 1.08
Nayong	18.17 ± 2.95d	23.56 ± 3.87a	147.11a ± 6.55	18.42 ± 0.52
Pogang	17.74 ± 1.13d	15.44 ± 1.43b	81.78c ± 8.89	18.88 ± 0.34
Kuankuoshui	22.99 ± 1.32c	11.45 ± 0.28c	59.50de ± 4.79	19.24 ± 1.17
Huoyan mountain	16.65 ± 2.69d	9.03 ± 1.73d	49.43e ± 0.63	21.09 ± 0.70
Huanggu mountain	22.26 ± 1.41c	15.62 ± 1.77b	75.79cd ± 1.39	20.61 ± 2.26
Zijiang rift valley	26.31 ± 1.72b	8.30 ± 0.78d	42.05f ± 1.85	19.74 ± 1.76
Bijia mountain	14.06 ± 0.77de	12.26 ± 1.49c	65.41d ± 6.03	18.82 ± 0.25

Different letters within a column indicate a significant difference ($P < 0.05$) between the nature reserve sites. The C content and proportion are reported as the mean ± SE

Regressions of organic C contents of soil fine particles against mass proportions

The relationship between organic C content of soil fine particles and mass proportion in karst forest was fitted by the LSR and BL methods (*Figure 2, Table 3*), whose respective best-fitting functions were y = 0.35x + 6.83 and y = 1.30x. The LSR had a slope similar to that of the Hassik model (y = 0.37x + 4.09) but with a larger intercept value, while the BL slope exceeded that of the Feng forest model (y = 1.07x).

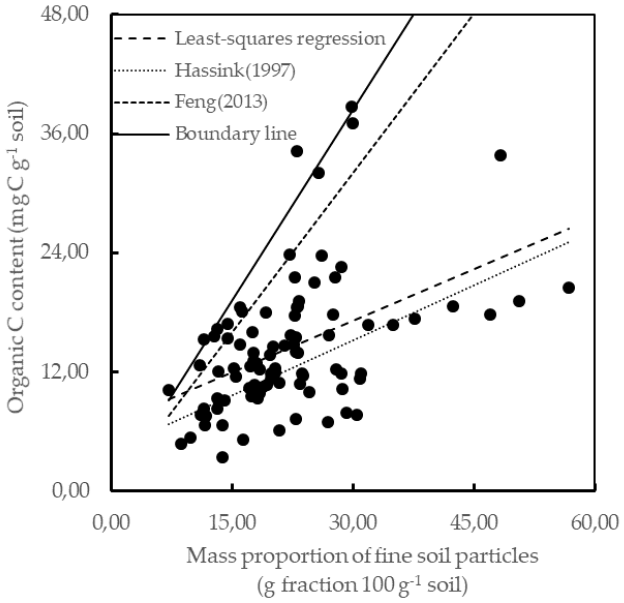

Figure 2. *Scatterplot of soil organic carbon in karst forest as a function of the amount of fine soil particles examined with four linear modeling approaches*

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DOI: http://dx.doi.org/10.15666/aeer/1905_41154128

Table 3. *Estimated maximal organic C stabilized by soil fine particles as determined by the slope of least-squares linear regression (LSR), the Hassink (1997) model, boundary line analysis (BL), and the Feng (2013) forest model*

Method	Slope[b]	Intercept[b]	R^2	P	N[a]
Hassink (1997)	0.37	4.09	0.89	0.001	40
LSR	0.35± 0.01	6.83 ± 0.38	0.32	< 0.01	30
Feng (2013)	1.07 ± 0.08	-	0.98	0.0058	3
BL	1.30 ± 0.01	-	0.96	< 0.01	3

[a] Only data points from the top tenth percentile of organic C content were included in the BL analysis, [b] Slopes and intercepts are reported as the mean ± SE

Modeling of maximal SOC saturation capacity

The maximum saturated capacity of the SOC for the karst forest sites was estimated using the Hassink and Feng models along with the LSR and BL models. Data on the organic C content and mass proportion of soil fine particles at 30 sampling point locations (across all 30 plots of the 10 sites) were verified and analyzed (*Table 4*). Overall, 17, 12, and 5 samples were over-saturated, corresponding to 56.7%, 40.0%, 16.7% of the data fitted by the Hassink model, LSR, and Feng model, respectively. In contrast, when fitted by the BL model, the proportion of soil samples over-saturated was only 3.3%.

Table 4. *Over-saturation of organic C content of soil fine particles by comparing the measured maximal organic C to predicted maximal organic C, estimated using least-squares linear regression (LSQ), the Hassink (1997) model, boundary line analysis (BL), and the Feng (2013) forest model*

Method	Land use	Total sample size	Over-saturated sample size	Over-saturated ratio (%)
Hassink (1997)	Forest	30	17	56.7
LSQ	Forest	30	12	40.0
Feng (2013)	Forest	30	5	16.7
BL	Forest	30	1	3.3

Discussion

There was no significant difference in the proportion of total organic C represented by fine particle C among the 10 sampled karst forest climax communities. This result indicates that the SOC tends to be stable, as expected. In this study, the maximum saturated capacity of SOC in karst forests in China was determined using the Hassink, Feng, LSR, and BL models. Of the four models, the BL model had the least over-saturated capacity, at 3.3%; this value was 5-15 times higher for the other three models. Hence, the BL model is suitable for estimating the maximum saturated capacity of SOC in karst forest, and the other models should not be used for this application in this region.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_41154128

There are several reasons for the above-mentioned interpretation. First, available field studies of soil types are generally inconsistent. Hassink (1997) and Feng (2013) respectively studied farmland and natural grassland. The level of human disturbance activity incurred by farmland soil is substantial, and the fine particle organic C in these systems is severely damage or even destroyed. By contrast, the natural grassland soil fluctuates greatly, as it is in an early stage of vegetation succession for which the SOC stability is poor. However, the current study takes the climax forest community with less man-made disturbance in karst nature reserves as the research object. In these protected areas, the soil fine particle organic C is less prone to degradation from anthropogenic disturbances; thus, the SOC tends to be more stable. Karst forest is a unique forest ecosystem, one that is distributed on karst landforms. Due to the influence of the bedrock, a karst forest ecosystem is formed in a habitat of neutral to slightly alkaline lime soils rich in calcium and magnesium ions, whose distribution is restricted by soil topography (Zhou, 1987; Yu, 1998). Compared with farmland and grassland ecosystems on non-karst terrain, this special and complex forest ecosystem differs tremendously in terms of its ecological environment and plant-soil-water dynamics (Lal et al., 2007; Wei et al., 2008; Luo et al., 2017; Lin et al., 2019). At the same time, because the soil layer in karst forests is thin, the gravel content is high, and the SOC storage is low. By contrast, the farmland and grassland soil in non-karst zones have thick surface soil layers capable of high C storage.

Much research has proven that about half of the SOC is combined with soil minerals (Elliott et al., 1996; Ge, 2008; Pan et al., 2009). Calcium and oxidizable organic C are mainly accumulated in the form of calcium-bonded organic C to form a stable calcium-bonded complex in the calcium-rich karst soil, which limits the contact between soil C and soil enzymes and degradable microorganisms. In this way, SOC is protected from microbial degradation and is conducive to the long-term accumulation of SOC. In calcium-rich calcareous soil environments, soil microbial activity is extremely active (Hu et al., 2011; Tian et al., 2016; Guan et al., 2019), fostering the rapid decomposition of organic matter into humus. Many calcium ions can easily combine with this humus in the soil to form highly condensed and stable humus calcium substrate, which improves the overall stability of karst SOC, a view that is consistent with our modeling results ($y = 1.30x$). Compared with the Hassink and Feng models, the slope of this model ($y = 1.30x$) is the highest, indicating that with each per-unit increase of the mass proportion of fine particles, the SOC increases more rapidly and tends to more quickly reach a stable state, a process arguably related to the soil calcium in the karst forest. Therefore, models that are suitable for predicting maximum saturated capacity of SOC in farmland and grassland in non-karst zones are inadequate for use in karst forest.

Second, the Hassink model has some disadvantages (West et al., 2007; Feng et al., 2013; McNeill et al., 2014). Notably, it assumes that the regression line represents the maximum saturation capacity, which means that all the sample data that generate the regression line must lie close to the maximum stability of SOC (Six et al., 2000, 2002; Stewart et al., 2007, 2008, 2009, 2012). If the C content of soil fine particles does not

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_41154128

reach the maximum value, then it is unreasonable to use this method to predict the maximum saturation capacity of SOC. In Hassink's study, although the uncultivated farmland and grassland soil were used as research objects, the degree of C saturation for each of the data building regression models was not mentioned (Carter et al., 2002; Chung et al., 2008, 2010). Further, the regression line of the Hassink model is forced through the middle of all the data points in the bivariate regression, representing the average, which tends to leave a considerable portion above the regression line over-saturated; this latter outcome is inconsistent with the actual expected estimation of the maximum saturation capacity of SOC (Virto et al., 2008, 2010; Feng et al., 2013; McNeill et al., 2014). As such, the Hassink model seriously underestimates SOC capacity. In this study, we used empirical data interspersed across 10 sites to analyze the Hassink model, finding that it yields the most over-saturated data, amounting to 56.7%.

In the Feng model, only the data of fine particle C lying above a 10%-threshold for the maximum stability of SOC were used to carry out regression analysis, and a maximum saturated capacity model of SOC was established (Elliott et al., 1996; Schuman et al., 2002; Tong et al., 2014). Compared with the LSR, the slope of this model is larger, because its intercept is zero (i.e., forced through the origin), thus leaving much data below the fitted regression line, which reduces the occurrence of over-saturation. In our verification analysis, the over-saturated data of the Feng model reached 16.7%, perhaps because of between-site habitat differences or pronounced within-site heterogeneity (Tian et al., 2016; Guan et al., 2019). With only 3.3% of the data over-saturated according to the BL model, we conclude the BL model is a relatively robust method for estimating the maximum saturated capacity of SOC. Nevertheless, estimations based on this model could also differ depending on the karst forest area and regional scales investigated (Zhang et al., 2010, 2012; Di, 2017).

Conclusions

Using comparative analysis and data verification of the Hassink model, Feng model, LSR, and BL model, we derived a maximum saturated capacity model of SOC for karst forest; the model exhibits a simple form: $y = 1.30x$. Although this model can be used to estimate the maximum saturated capacity of SOC in karst forest, we did not consider the effects of calcium carbonate and other exogenous substances on SOC in the studied karst areas. In future work, it is necessary to study the key soil characteristics and main mechanisms (calcium carbonate) that affect the stability of SOC, as well as any biochemical factors that could limit the stabilization rate of organic C, so as to improve our ability to estimate the maximum saturated capacity of SOC in karst forests in China.

Author contributions. L.Z. and Y.W. analyzed the data and wrote the manuscript. L.Z. and Y.W. performed the experiments. J.C. participated in data analysis. L.Y. provided helpful suggestions in design of the project. L.Y. conceived and designed the project. All authors read and approved the final manuscript.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_41154128

Acknowledgments. Funding was provided by the Project of National Key Research and Development Program of China (grant number 2016YFC0502604); the Application Foundation Major Project of Guizhou Province (Qian Ke He JZ [2014] 2002); the Construction Program of Biology First-class Discipline in Guizhou (GNYL [2017]009); and the Project of Promoted Innovation of Colleges and Universities of Guizhou Province (Qian Jiao He Collaborative Innovation [2014]01).

Conflict of Interests. The authors declare no conflict of interests.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_41154128

DOES SEED TRAIT VARIABILITY SUPPORT PRELIMINARY SEED TRANSFER ZONES FOR HUNGARY?

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(Received 29th May 2021; accepted 23rd Aug 2021)

Abstract. Maladaptation of populations can lead to a failed restoration when seeds are transferred to non-local target sites. Seed transfer zones (STZs) can guide transfer for restoration practices. In a previous study, we delineated STZs for Hungary based on biogeographic maps and potential natural vegetation models. The present aims were to compare the STZ with the pattern of seed trait variability of sand grasslands species (*Festuca vaginata*, *Stipa borysthenica, Dianthus serotinus* and *Centaurea arenaria*) from 34 localities. We assessed the effect of location as well as geographical, ecological, and environmental distances between locations on seed mass, germination, and emergence in a common garden. We detected that seed trait differentiation between the populations is a signal of local adaptation or phenotypic plasticity. Geographical distance did not affect the variability of seed traits, except on the emergence of *C. arenaria*. Ecological distance reflected in STZ delineation also did not explain seed trait variability except for the seed mass of *C. arenaria*. Aridity had a significant effect on the germination or emergence of the two forbs, but not on the grasses. In conclusion, we do not suggest restricting seed transfer among the populations over the sampled area. Further studies might complement these findings and lead to more general conclusions.
Keywords: *aridity, germination, ecological distance, local provenance, thousand seed weight*

Introduction

Grasslands are an important target of restoration due to their biodiversity and ecosystem services (Török and Dengler, 2018). In Europe, grasslands are jeopardized primarily by habitat fragmentation and degradation, climate change, invasive species, intensification of land use, and the abandonment of management at secondary grasslands (EC, 2008). Grassland restoration is used to mitigate the impact of degradation and fragmentation, restoring the structure, function and dynamics of the communities (Kiehl et al., 2014). Sowing seeds is a widely used method regarding species introduction for grassland restoration (Kiehl et al., 2010; Török et al., 2011; Kövendi-Jakó et al., 2019). Large seed quantities are needed for large-scale restoration projects (Merritt and Dixon, 2011; Lengyel et al., 2012) to respond to the spatial extent of degradation.

The use of seeds from local provenance is frequently suggested to avoid maladaptation (Broadhurst et al., 2008; Breed et al., 2018). A meta-analysis revealed that local species

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_41294149

with large distributions and population sizes perform better than foreign plants in 71% of studies (Leimu and Fischer, 2008). The concept of ”local” is rarely defined in papers; the best approach for the definition would be to rely on genetic information (McKay et al., 2005; Breed et al., 2018). However, genetic data is available only for very few species used in restoration (Bucharova et al., 2017). Therefore, the main constraint in selecting the most suitable provenance is the missing evidence of genetic differentiation for most native species.

In case of limited genetic information, the guidance of seed movement for restoration is suggested to be based on general seed transfer zones (STZs) that are geographically distinct areas within which seeds can be moved with little or no negative fitness consequences (Mijnsbrugge et al., 2010; Bower et al., 2014; Bucharova et al., 2019). STZs delimit areas with a particular set of environmental variables within which plant species are probably similarly adapted to the abiotic conditions (Bower et al., 2014). General STZs are usually recommended for multiple species (Durka et al., 2017). Studies that detect and test STZs usually select widespread generalist species that are not limited to a particular community (Bucharova et al., 2017; Durka et al., 2017). However, these species might demonstrate limited local adaptation (Reiker et al., 2015), and their use can miss the detection of zones.

The probability of gene flow among populations is assumed to decrease with geographical distance (Leimu and Fischer, 2008; Bucharova et al., 2019), as a result, genetic differentiation can occur with increasing distance (Baruch et al., 2004). However, it was also found that ecological distance (ecological differences between habitats) can better predict local adaptation than geographical distance (Herrera et al., 2002; Raabová et al., 2007). Phenotypic fitness traits (e.g. plant height, leaf length, inflorescence length) are significantly influenced by altitude, growing season, latitude, temperature and other environmental parameters and drivers of genetic differentiation (Miller et al., 2011; St. Clair et al., 2013).

Several studies have shown the relevance of eco-regions or biogeographical zones mirror species adaptive trait variabilities (Bower et al., 2014; Gibson and Nelson, 2017). Understanding the scale of local adaptation will help to define regions from which plants can be transported without detrimental effects on population fitness (Hufford and Mazer, 2003).

Experiments that compare species performance from different locations can substitute genetic analyses by sampling fitness traits, but these are also missing for most of the herbaceous species (Bucharova et al., 2017). The comparison of performance is generally done in common garden experiments, where different provenances are grown in a standard environment (Leger and Rice, 2007; Gibson et al., 2016; Germino et al., 2019). The climate of sites of population origin is generally a strong predictor of common garden performance of populations (Macel et al., 2007; Bischoff and Müller-Schärer, 2010).

The study of traits of early life stages at the seed and germination stages, may indicate adaptive differentiation (Postma and Ågren, 2016). Moreover, Raabová and colleagues (2007) found more evidence for local adaptation in seedlings than adults. Breen and Richards (2008) found that seed size was the most important factor in determining germination, survival and growth of a shrub. This connection was valid for a grass species, as well (Elgabra et al., 2019). Although seed mass is shown to demonstrate low plasticity (Hernández et al., 2019), some differences were observed in seed mass as a result of irrigation in experimental settings (Drenovsky and Richards, 2005; Breen and Richards, 2008). Germination is the earliest trait expressed by plants under strong natural

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selection before they can express other adaptive traits in later life stages, so it greatly contributes to local adaptation (Donohue et al., 2010; Cochrane et al., 2015).

Early life traits are also influenced by the parent environment during seed development, with aridity as an important driver (Elgabra et al., 2019). Drought tolerance can be locally adapted, therefore, non-adapted propagule introduction may induce reduced assimilation and resistance to pathogens, resulting in increased mortality (McDowell et al., 2008). Communities and species with higher drought tolerance might also gain importance in restoration in the future (Fry et al., 2018). Soil properties also have a great influence on the local adaptation of plants (Gibson et al., 2019). Especially the water holding capacity of soils and organic matter are related to trait variation (Johnson et al., 2010). Seed traits could thus be good indicators to identify provenances for seed transfer during restoration, but there are not enough published studies to conclude the differentiation of seed traits along abiotic gradients (Cochrane et al., 2015).

Pannonian sand grasslands are endangered habitat types of European Union conservation importance listed in the Habitats Directive (EC, 2013). Sand grasslands are edaphic communities adapted to drought that might gain further importance in restoration due to predicted future climate change with more frequent drought events. If the abandonment of cultivation occurs in sandy areas due to low profitability and frequent droughts, there is a high potential to turn these areas into native sand grasslands (Biró et al., 2013).

In our previous work, we analyzed the current situation in Hungary regarding seed transfer and delineated STZs based on the best available knowledge, combining biogeographic maps and potential natural vegetation models (Cevallos et al., 2020). In the present study, we assessed how the seed trait variability of two grass and two forb species from Pannonian sandy grasslands matches the delineated STZs, and geographical and environmental distance (based on site aridity) of their source location. For this reason, we collected seeds of the four species from altogether 34 locations in six STZ zones in Hungary and measured seed mass, germination in growth chamber and emergence in a common garden.

We conducted the research to respond to the following questions: a) Do seed traits differ according to provenance (location)? b) Do geographical and ecological (STZ) distances explain seed trait differences between locations? c) Do the site conditions defined as aridity, explain seed trait differences between locations?

Materials and Methods

Study region

The study was carried out within the Pannonian biogeographic region, in the extended sandy areas of Hungary (*Figure 1*). The annual mean temperature varies from 10.5 to 11.0 °C, the annual precipitation ranges from 500 to 600 mm (Bihari et al., 2018). The soil type of the largest sandy regions, e.g. Danube-Tisza Interfluve region and Nyírség, is characterized by mainly blown sand skeletal soil (arenosol) with low humus content (below 1%). Sandy soils in the Transdanubian region and the Northern foothills are composed of sandy loam with up to 2% of organic matter content (Pásztor et al., 2018).

Pannonic sand steppes are protected at the EU level (code 6260; EC, 2013). Most extensive sand steppes are distributed in the Danube-Tisza Interfluve and Nyírség regions, and in smaller patches of other parts of the Great Hungarian Plain, the foothills

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of the North Hungarian Mountains and the Transdanubian region (*Figure 1*; Bölöni et al., 2011).

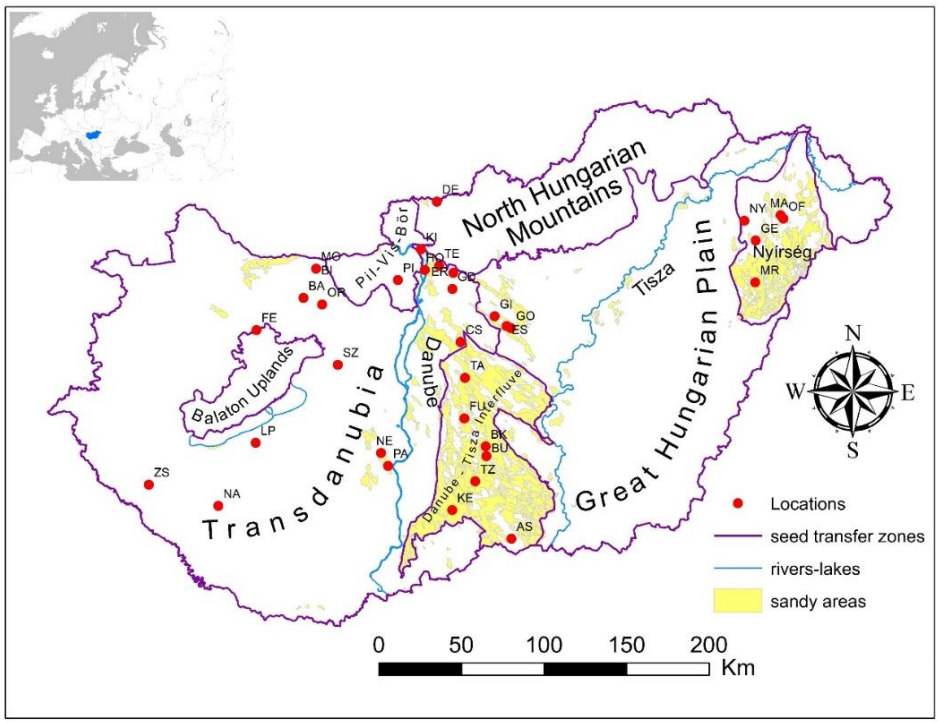

Figure 1. *Map of locations of seed collection of the four species and main sandy regions in Hungary (Pásztor et al. 2018) with the seed transfer zone borders (Cevallos et al. 2020). Location codes and coordinates can be found in Table S1. The Pilis-Visegrád-Börzsöny region is abbreviated as Pil-Vis-Bör.*

Field sampling

The selection of species of the open sand steppe community was based on sampling the main constituent grasses and subordinate forbs. The selected species are sufficiently frequent to provide the necessary number of populations. Four native species were selected for the study. Two dominant grasses: *Festuca vaginata, Stipa borysthenica,* and two dominant forbs: *Centaurea arenaria,* and *Dianthus serotinus*. The seeds of the studied species (*Figure 2*) were collected in the growing season of 2019 from a total of 34 different locations within the Pannonian biogeographic region (*Figure 1*; *Table S1*), with different timing according to the ripening season of the given species, as follows: *S. borysthenica* in May-July; *F. vaginata* in June-July; *C. arenaria* in July; *D. serotinus* in August-September. The 34 sampled locations can be found in six STZs, which are: Transdanubia, Pilis-Visegrád-Börzsöny Mountains, Danube-Tisza Interfluve, North Hungarian Mountains, Great Hungarian Plain, and Nyírség (Cevallos et al., 2020). Seeds were harvested by hand at each location to collect approximately 50 seeds from 15 individuals. Seed families were treated as one sample. In the case of *S. borysthenica* that has a lower average seed yield per individual, we considered the seeds collected from neighboring individuals as one family and worked with fewer seeds in all the analyses. The seeds were stored at dry room conditions (temperature: 25±3 °C, humidity: 38±4%) until further processing.

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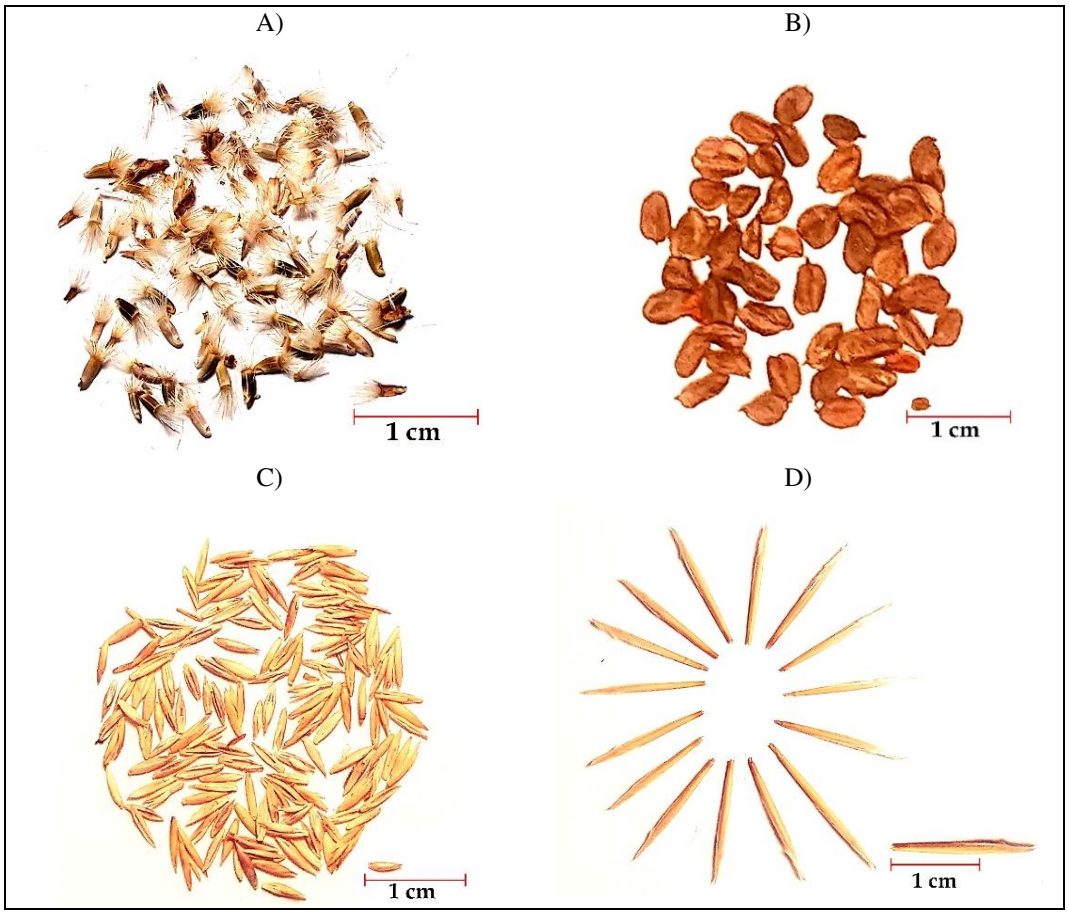

Figure 2. *Seed photographs of A) Centaurea arenaria, B) Dianthus serotinus, C) Festuca vaginata, D) Stipa borysthenica.*

Seed mass and germination

The dry weight of 50 seeds or 25 seeds (for *S. borysthenica*) from all locations (15 replicates each) was measured by an analytical balance with 0.0001 g accuracy. Seed mass was expressed as thousand seed weight in grams.

Two germination experiments were conducted: 1) under regulated conditions in a plant growth chamber and 2) in a common garden under outdoor conditions (irrigation was provided if necessary). Chamber germination tests were based on seed germination methods of Peti et al. (2017) and RBGK (2020). Thirty-five seeds (*C. arenaria*, *D. serotinus*, and *F. vaginata*) and ten seeds (*S. borysthenica*) of six samples per location per species were placed on wet filter paper in sterile Petri dishes and placed into an Aralab climatic chamber (FITOCLIMA D1200 PHL) with regulated temperature (20 °C), light (8 h light and 16 h dark) and humidity (50%) conditions for all the species. Experiments were carried out between October 2019 and March 2020. Seed coats were not sterilized. The seed samples of *S. borysthenica* were treated by mechanical scarification to break seed dormancy. Germination was defined as the emergence of a 2 mm long radicle. The germinated seeds were counted and removed from the Petri dishes weekly for three weeks for C. *arenaria, D. serotinus*, and *F. vaginata*, and for nine weeks for *S. borysthenica*. The viability of non-germinated seeds was not further tested.

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A common garden experiment was established at a roof garden at the National Botanical Garden, Vácrátót (47.708° N, 19.237° E). Average temperature: 10.75 °C, average relative humidity: 73.37% (data obtained by wireless weather station data loggers between 31 01 2020 and 30 03 2021 on site). Fifteen seeds from each sample were manually sown in a regular layout: 5 x 3 seeds within a pot of 13 x 13 x 13 cm. The seeds in a pot originated from one seed family (neighboring plants for *S. borysthenica*). Fifteen pots were seeded per location per species, resulting in 285 pots for *C. arenaria*, 240 for *D. serotinus*, 435 for *F. vaginata*, and 210 for *S. borysthenica*. Seeding was carried out in September 2019 for all species, emergence of the four studied species was monitored three times, in October 2019 and in March and April 2020. The cumulative emergence was used for the analyses.

Data analysis

The following three seed traits were selected for the analyses: seed mass expressed as thousand seed weight (TSW), the fraction of germinated seeds in a plant-growth chamber, and the cumulative seedling emergence in the outdoor common garden experiment. Average values of seed mass, germination and emergence, including standard errors are presented in *Table S2*.

The effect of the provenance (sampling location) on the three seed traits for each species was evaluated by applying linear models (LM) and conducting Tukey HSD post hoc tests between pairs of locations to reveal significant differences (*multcomp* package; Hothorn et al. 2020) with p values adjusted by the method of Holm-Bonferroni (Holm, 1979). We calculated the rate of significant differences between locations (SDL) for each species by dividing the number of significant pairs by the total number of location pairs.

To assess the impact of geographical distance, the absolute difference of the mean values for each seed trait (seed mass, germination, and emergence) was calculated for each location pair. The Euclidean distance between the sampling locations was calculated based on their GPS coordinates. LM was used to determine the relationship between the difference of three seed trait values and geographical distance. In these models, the three seed traits were treated as response variables and Euclidean distance as an explanatory variable. The effect of ecological distance (STZs) on the three seed traits was assessed using separate LME models where mean values of each trait for each location were used as a response variable, whereas STZ zone identity served as a fixed effect, and locations were treated as a random factor. For post-hoc pairwise comparisons, Tukey HSD post hoc tests were applied using the multicomp package (Hothorn et al., 2020) with p values adjusted by the method of Holm-Bonferroni (Holm, 1979).

Aridity effects on the three seed traits were tested using LME models. In these analyses, aridity was treated as a fixed effect, the seed traits as response variables and the sampling location as a random factor. We used the Global Aridity Index to characterize the aridity of collection locations (Trabucco and Zomer, 2019). This index is represented by the ratio between mean annual precipitation and potential evapotranspiration with a resolution of 30 arc seconds, therefore lower values indicate higher aridity.

Square, cube root and squaring transformations of the response variables were used to approximate assumptions of normality and homoscedasticity when it was necessary. All statistical analyses were performed using the R version 3.6.2 (R Core Team 2019). ArcGIS 10.2 was used to calculate the geographic distance.

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Results

The effect of provenance

The location of origin of the sampled populations significantly affected all seed traits (seed mass, germination, and emergence) for all studied species, except for the germination of *S. borysthenica* (*Table 1*). The rate of significant differences between locations (SDL) was variable among the species and the seed traits. *S. borysthenica* showed the highest rate of SDL for seed mass and emergence, while *C. arenaria* demonstrated the highest variability in germination. In the case of *D. serotinus*, the rate of significant differences was quite low (under 0.2) for all three traits. For *F. vaginata*, we found a low rate of SDL for seed mass and germination but a high rate for emergence.

Table 1. *Effect of locations on the response variables for the study species. Linear models were used. Used transformations are shown. The number of significant differences of response variables between location pairs (SDL) versus total number of location pairs and their % is given. Significant results are in bold.*

Species	Traits	Best conversion	F-value	df	p-value	SDL
C. arenaria	seed mass	no	**9.999**	**18**	**<0.05**	52/171 (0.3%)
	germination	cube root	**35.140**	**18**	**<0.05**	102/171 (0.63%)
	emergence	square root	**12.819**	**18**	**<0.05**	58/171 (0.34%)
D. serotinus	seed mass	no	**7.175**	**15**	**<0.05**	22/120 (0.18%)
	germination	squaring	**2.985**	**15**	**<0.05**	3/120 (0.03%)
	emergence	squaring	**5.989**	**15**	**<0.05**	21/120 (0.17%)
F. vaginata	seed mass	square root	**6.793**	**28**	**<0.05**	37/406 (0.09%)
	germination	no	**7.088**	**27**	**<0.05**	43/378 (0.11%)
	emergence	no	**15.375**	**28**	**<0.05**	146/406 (0.35%)
S. borysthenica	seed mass	squaring	**15.373**	**13**	**<0.05**	41/91 (0.45%)
	germination	square root	1.9036	13	0.192	0/91 (0%)
	emergence	squaring	**15.069**	**13**	**<0.05**	37/91 (0.4%)

The effect of geographical and ecological distance

There was no significant effect of the geographical distance on the measured seed traits (seed mass, germination, emergence) between populations from different locations, except for a significant effect for the emergence of *C. arenaria* (*Table 2, Figure 3*). The STZs had no effect on the response variables (seed mass, germination, emergence) for the studied species, except for the seed mass of *C. arenaria* (χ^2=8.47, df=3, p <0.05). In this case, the seed mass was significantly higher in the Great Hungarian Plain than in Transdanubia (z=-2.78, p=0.033; *Figure S1*).

The effect of aridity of the site

The emergence of *C. arenaria* was negatively influenced by site aridity (higher aridity index, better emergence; χ^2=5.244, df=1, p<0.05, *Figure 4a*). Site aridity had a positive effect (aridity index lower with higher trait values) both on germination and emergence of *D. serotinus* (germination: χ^2=5.159, df=1, p<0.05; emergence: χ^2=6.375, df=1, p<0.05; *Figure 4b,c*), when all sites considered (*Figure 4b,c*, line A). However, when sites with outlier high aridity index were skipped, no effect of aridity was found on *D. serotinus* seed traits (*Figure 4b,c*, line B). Site aridity had no effect on other species.

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Table 2. *Effect of ecological distance (STZ), geographical distance and aridity on the response variables for the study species. Used transformations and models are shown. For linear models (LM) results are shown with F values whereas in linear mixed models (LME) with chi-square (χ^2) values. Significant results are in bold.*

Species	Trait	Parameter	model	Best conversion	χ^2	F	df	p
C. arenaria	**Seed mass**	STZ	LME	no	8.470		3	**0.037**
		distance	LM	square root		3.208	1	0.075
		aridity	LME	no	0.023		1	0.879
	germination	STZ	LME	square root	3.555		3	0.314
		distance	LM	square root		0.185	1	0.668
		aridity	LME	square root	1.483		1	0.223
	emergence	STZ	LME	square root	4.084		3	0.253
		distance	LM	square root		7.160	1	**0.008**
		aridity	LME	square root	5.244		1	**0.022**
D. serotinus	**Seed mass**	STZ	LME	no	0.711		3	0.871
		distance	LM	square root		0.209	1	0.649
		aridity	LME	no	2.437		1	0.119
	germination	STZ	LME	squaring	4.014		3	0.260
		distance	LM	cube root		1.243	1	0.267
		aridity	LME	squaring	5.159		1	**0.023**
		aridity (nohigh)	LME	squaring	0.006		1	0.939
	emergence	STZ	LME	squaring	2.724		3	0.428
		distance	LM	cube root		2.798	1	0.097
		aridity	LME	squaring	6.215		1	**0.013**
		aridity (nohigh)	LME	squaring	0.791		1	0.374
F. vaginata	**Seed mass**	STZ	LME	square root	3.049		4	0.550
		distance	LM	square root		0.074	1	0.787
		aridity	LME	square root	0.725		1	0.395
	germination	STZ	LME	no	2.466		4	0.651
		distance	LM	square root		0.216	1	0.643
		aridity	LME	no	1.206		1	0.272
	emergence	STZ	LME	no	2.392		4	0.664
		distance	LM	square root		0.0005	1	0.982
		aridity	LME	no	0.001		1	0.974
S. borysthenica	**Seed mass**	STZ	LME	squaring	1.864		4	0.761
		distance	LM	square root		2.292	1	0.134
		aridity	LME	squaring	0.316		1	0.574
	germination	STZ	LME	square root	6.086		4	0.193
		distance	LM	square root		0.177	1	0.675
		aridity	LME	square root	1.736		1	0.188
	emergence	STZ	LME	squaring	1.748		4	0.782
		distance	LM	cube root		1.310	1	0.256
		aridity	LME	squaring	0.057		1	0.811

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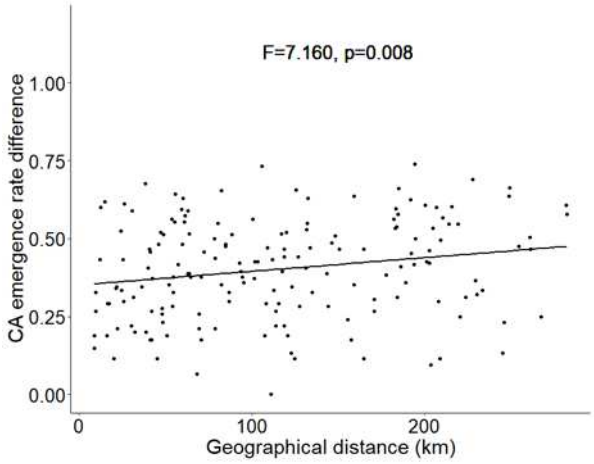

Figure 3. *Relationship between C. arenaria emergence rate difference and geographical distance for locality pairs.*

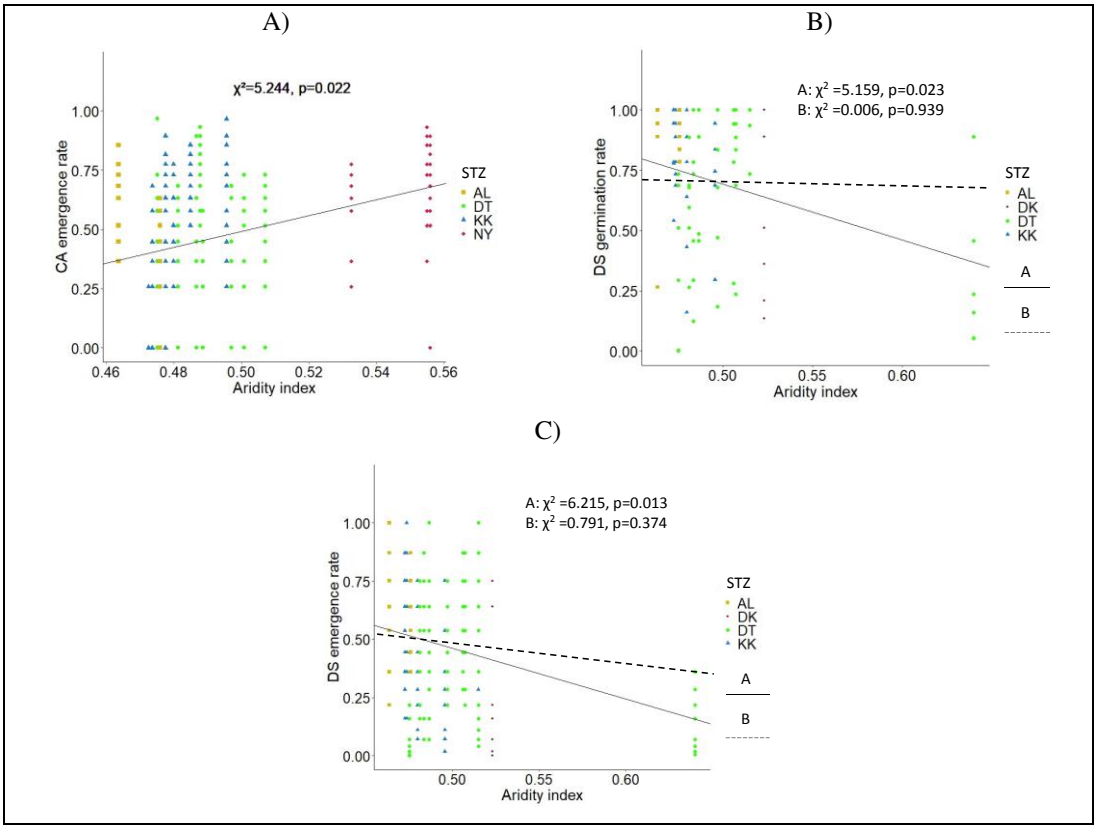

Figure 4. *Significant effect of aridity of collection locations on A) the emergence of C. arenaria (CA); B) the germination of D. serotinus (DS) and C) the emergence of D. serotinus (DS). For Figs B and C, line A represents all the values including locations with low aridity (high aridity index >0.6), whereas line B refers to the values excluding high aridity index locations. Note that higher value of the index refers to lower aridity. Locations coloured according to their seed transfer zones (STZ). Seed zones are represented with abbreviations: Great Hungarian Plain (AL, Alföld); Danube-Tisza Interfluve (KK, Kiskunság); Nyírség (NY); Pilis-Visegrád-Börzsöny region (DK); and the Transdanubia (DT, Dunántúl).*

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Discussion

Our main aim was to shed light on the pattern of the variability of species traits among populations that can reflect local adaptation to avoid failure during reintroductions due to maladaptation. We tested multiple locations distributed in six of the seven STZs developed for Hungary (Cevallos et al., 2020) over a large area (approx. 40.000 km^2); this level of sampling intensity is rather rare in studies, usually species from a few sites are investigated (Gibson et al., 2016; Bhatt et al., 2019).

We detected significant differences between provenance pairs, and the effect of location was significant for all four species characteristic to Pannonian sand grasslands considering every assessed seed trait. Seed mass is an important fitness trait (Cordazzo et al., 1995; Cochrane et al., 2015). The higher amount of resources in larger seeds provides an advantage for germination and emergence. It is however not clear, to what extent this advantage remains during emergence and development, and in later stages to support survival after introduction during restoration. In annual plants, the advantage of larger seeds can be detected even at the reproduction phase (Metz et al., 2010). Seed mass had high within-species variability in the present study among populations, as was reported in other studies as well (Cochrane et al., 2015; Bhatt et al., 2019; Elgabra et al., 2019). We found differences in the germination rate under laboratory conditions and the outdoor emergence for three species (*C. arenaria, D. serotinus,* and *F. vaginata*), similar to other research results (Clarke and Davidson, 2004; Kövendi-Jakó et al., 2017). The lack of this result for *S. borysthenica* can be explained by the fact that only very few seeds germinated under laboratory conditions, but emergence was variable among sites of origin, as for the other species. The mechanical scarification applied for breaking seed dormancy was ineffective, but the outdoor seeding with a prolonged incubation in the soil during the winter resulted in nearly 80% germination of *S. borysthenica* by spring. Seed dormancy breaking methods have to be further tested for this species, as it was done for *S. tenacissima* (Gasque and García-Fayos, 2003).

This within-species variability of seed traits is a signal of local adaptation of the different provenances, as it has been shown in numerous published common garden experiments for native species (Dorman et al., 2009; Bischoff et al., 2010), also aiming to test seed transfer zones (Bucharova et al., 2017; Durka et al., 2017; Gibson and Nelson, 2017). *C. arenaria* had the highest rate of significant differences between pairs of locations. This output could be the result of the high variability within this species as there are three subspecies in Hungary (Király, 2009).

The differences between locations in seed mass, germination and cumulative seedling emergence values were not explained by the geographical distance between populations unlike some other studies (Herrera et al., 2002; Baruch et al., 2004). However, it was also demonstrated in several cases that ecological distance (also expressed in STZs) is more important than geographical distance (Raabová et al., 2007; Bradburd et al., 2013; Durka et al., 2017). In our study, site aridity is represented by the precipitation-evapotranspiration ratio, thus, lower values represent higher aridity. This value had a significant effect on the seed traits of the two forbs (*C. arenaria* and *D. serotinus*), but not on the two grass species (*F. vaginata* and *S. borysthenica*). We found a significant negative effect of aridity on the emergence of *C. arenaria*. For *D. serotinus,* the effect of site aridity on the germination in the growth chamber was positive. However, in the latter case, if the three localities with aridity values above 0.6 were not considered, the positive effect diminished. Therefore, more studies on samples from locations with lower aridity would be needed in the future to confirm the reliability of this effect. The effect of aridity

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on the studied forbs suggests a higher sensitivity in early life traits regarding water availability compared to grasses. In contrast, Guo et al. (2017) found that both grasses and forbs, which were exposed to different aridity gradients showed an increase in leaf thickness with higher aridity. Aridity did not explain differences in seed mass in our studied species. Similar to our results, a study on seed traits also found the lack of environmental effect on the seed mass of *Helianthus annuus* (Hernández et al., 2019).

The differences in seed traits found do not correspond to the STZ delineation developed by using biogeographic maps and potential natural vegetation models (Cevallos et al., 2020). Raabová et al. (2007) found similarly no pattern in the local adaptation of a rare herb species, despite estimating genetic variation parallel to trait measurements. Only in the case of *C. arenaria*, we found a significant difference between two STZs (Great Hungarian Plain and Transdanubia) in seed weight. The most fragmented and remote populations in the Nyírség (Eastern part of the Great Hungarian Plain; *Fig. 1*) did not differ from the other zones for *C. arenaria* traits despite having the lowest aridity, among its sampled populations; however, the emergence of the same species was significantly affected by aridity.

The unfolding of the complex influences of the environment on the studied traits, and the importance of local adaptation compared to phenotypic plasticity needs other measurements and approaches. Further studies testing STZs can either focus on species at the genetic level (Jørgensen et al., 2016; Durka et al., 2017; Listl et al., 2018) or cover other traits and habitat types.

Conclusions

We found significant differences for all seed traits for the four species among the sampled locations. However, these differences did not correspond to STZ zones delineated based on biogeographic maps and potential natural vegetation models. We also found a limited effect of site aridity on the traits. Potentially the drivers of seed trait variability function at a finer scale. However, we can state that geographical, ecological (STZ), and environmental distance (aridity) had a stronger effect on the seed traits of the studied forbs than grasses. Based on our results, we suggest allowing seed transfer among the populations of the studied species over the sampled area. However, further studies are suggested to shed light on the relationship between other plant traits and seed transfer zones. Later life-history stages of the studied populations, the involvement of more populations, the implementation of reciprocal transplant experiments, the observation of phenotypic differences among populations in the further offspring to identify maternal effect are possible directions of research.

Acknowledgements. The study was financed by the Széchenyi 2020 Development Program (KEHOP-4.3.0-VEKOP-15-2016-00001) (KT). The study of DC was supported by the Stipendium Hungaricum program. AKJ was supported by the National Talent Program (NTP-NFTÖ-19-B-0159). MH was supported by NKFI FK127996 grant.

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DOI: http://dx.doi.org/10.15666/aeer/1905_41294149

APPENDIX

The R scripts and the basic data are available upon request: david.cevallos@ecolres.hu.

Table S1. *Locations with coordinates, STZ, aridity and fertility values of the seed collection from the studies species. STZ Codes: AL: Great Hungarian plain (Hungarian: Alföld), DK: Pilis-Visegrád-Börzsöny region, DT: Transdanubia (Dunántúl), EH: North Hungarian mountains (Északi-középhegység), KK: Danube-Tisza Interfluve (Kiskunság), NY: Nyírség. Aridity represents the ratio of mean annual temperature and evapotranspiration based on the (Global-ET0) Version 2 global dataset, with high resolution (30 arc-seconds). The timeframe of the weather data was from 1970 to 2000 (Trabucco and Zomer, 2019), and soil fertility values were obtained from the soil productivity map of Hungary in an ordinal scale (Pásztor et al., 2013).*

		Coordinates			Studied species			
Code	**Location name**	**Latitude**	**Longitude**	**STZ Code**	*Centaurea arenaria*	*Dianthus serotinus*	*Festuca vaginata*	*Stipa borysthenica*
AS	Ásotthalom	N46° 12' 50,63"	E19° 47' 18,77"	KK	✓	✓	✓	✓
BA	Bársonyos	N47° 31' 25,20"	E18° 08' 02,31"	DT		✓	✓	
BI	Billegpuszta	N47° 41' 01,96"	E18° 14' 00,98"	DT		✓		
BU	Bugac	N46° 39' 54,05"	E19° 35' 47,07"	KK	✓		✓	✓
BK	Bugac külső	N46° 43' 04,80"	E19° 35' 27,88"	KK			✓	✓
CS	Csévharaszt	N47° 17' 12,27"	E19° 23' 44,89"	DT	✓	✓	✓	✓
DE	Dejtár	N48° 03' 06,00"	E19° 12' 32,35"	EH				✓
ER	Erdőkertes	N47° 39' 46,79"	E19° 20' 26,11"	DT	✓	✓	✓	✓
ES	Erdőszőlő	N47° 22' 18,35"	E19° 45' 55,33"	AL				✓
FE	Fenyőfő	N47° 20' 42,06"	E17° 45' 36,96"	DT			✓	
FU	Fülöpháza	N46° 52' 14,59"	E19° 25' 24,31"	KK	✓	✓	✓	✓
GE	Geszteréd	N47° 48' 27,21"	E21° 47' 09,34"	NY	✓			
GI	Gicei-hegy	N47° 25' 32,27"	E19° 40' 14,72"	AL	✓	✓	✓	
GO	Göbölyjárás	N47° 21' 38,73"	E19° 47' 59,19"	AL	✓	✓	✓	
GD	Gödöllő	N47° 34' 33,59"	E19° 19' 55,19"	DT	✓		✓	
HO	Horány	N47° 40' 45,54"	E19° 06' 36,78"	DT	✓	✓	✓	✓
KE	Kéleshalom	N46° 22' 20,09"	E19° 19' 26,95"	KK	✓	✓	✓	✓
KI	Kisoroszi	N47° 47' 31,55"	E19° 04' 41,66"	DT	✓		✓	✓
LP	Látránypuszta	N46° 43' 52,73"	E17° 46' 06,89"	DT			✓	

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DOI: http://dx.doi.org/10.15666/aeer/1905_41294149

		Coordinates			Studied species			
Code	**Location name**	**Latitude**	**Longitude**	**STZ Code**	*Centaurea arenaria*	*Dianthus serotinus*	*Festuca vaginata*	*Stipa borysthenica*
MA	Magy	N47° 56' 25,90"	E21° 59' 40,95"	NY			✓	
MR	Martinka	N47° 34' 47,24"	E21° 46' 12,42"	NY	✓		✓	
MO	Mocsa	N47° 41' 01,88"	E18° 13' 59,35"	DT			✓	✓
NA	Nagybajom	N46° 23' 04,38"	E17° 29' 00,02"	DT		✓	✓	
NE	Németkér	N46° 41' 00,58"	E18° 45' 46,33"	DT	✓	✓	✓	
NY	Nyíregyházi lőtér	N47° 55' 01,71"	E21° 41' 58,68"	NY			✓	
OF	Ófehértó	N47° 55' 15,81"	E22° 00' 59,11"	NY	✓			
OR	Oroszlány	N47° 29' 18,94"	E18° 17' 05,89	DT		✓	✓	
PA	Paks	N46° 36' 45,96"	E18° 49' 05,06"	DT	✓	✓	✓	
PI	Pilisvörösvár	N47° 37' 27,61"	E18° 53' 35,13"	DK		✓	✓	✓
SZ	Székesfehérvár	N47° 09' 40,55"	E18° 24' 54,22"	DT			✓	
TA	Táborfalva	N47° 05' 30,31"	E19° 25' 48,54"	KK	✓	✓	✓	
TZ	Tázlár	N46° 31' 41,46"	E19° 30' 25,90"	KK	✓		✓	
TE	Tece (Vácrátót)	N47° 42' 07,74"	E19° 13' 32,13"	DT	✓		✓	✓
ZS	Zsigárdmajor	N46° 29' 26,30"	E16° 55' 55,49"	DT			✓	
			Total locations		19	16	29	14

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Table S2. *Average values ± standard error of seed traits for each species organized by location. (*** not enough seeds for germination). Seed mass is expressed in grams, gemination and emergence in ratio. The sample size is in italics and it is represented for the seed mass by the number of measurements per location, and for the germination and emergence by the total number of seeds used in the measurements. Location codes are in Table S1.*

Location	*Centaurea arenaria*			*Dianthus serotinus*			*Festuca vaginata*			*Stipa borysthenica*		
	seed mass	germination	emergence	seed mass	germination	emergence	seed mass	germination	emergence	seed mass	germination	emergence
AS	1.792 ±0.076 *15*	0.181 ±0.079 *210*	0.262 ±0.043 *225*	0.498 ±0.030 *15*	0.780 ±0.090 *210*	0.573 ±0.055 *225*	0.395 ±0.025 *15*	0.286 ±0.134 *210*	0.404 ±0.070 *225*	12.442 ±0.345 *14*	0.166 ±0.071 *60*	0.848 ±0.026 *210*
BA	-	-	-	0.635 ±0.019 *15*	0.970 ±0.023 *210*	0.608 ±0.064 *225*	0.490 ±0.017 *15*	0.532 ±0.070 *210*	0.591 ±0.076 *225*	-	-	-
BI	-	-	-	0.482 ±0.034 *15*	0.719 ±0.098 *210*	0.742 ±0.054 *225*	-	-	-	-	-	-
BU	2.239 ±0.110 *15*	0.530 ±0.064 *210*	0.386 ±0.057 *225*	-	-	-	0.436 ±0.017 *15*	0.759 ±0.044 *210*	0.586 ±0.043 *225*	13.528 ±0.281 *14*	0.116 ±0.040 *60*	0.909 ±0.021 *210*
BK	-	-	-	-	-	-	0.490 ±0.022 *15*	0.493 ±0.094 *210*	0.577 ±0.058 *225*	13.132 ±0.315 *15*	0.033 ±0.021 *60*	0.924 ±0.020 *225*
CS	2.113 ±0.126 *15*	0.233 ±0.092 *210*	0.244 ±0.065 *225*	0.269 ±0.046 *15*	0.500 ±0.155 *210*	0.383 ±0.073 *225*	0.390 ±0.024 *15*	0.057 ±0.025 *210*	0.128 ±0.043 *225*	14.442 ±0.330 *15*	0.120 ±0.066 *60*	0.906 ±0.026 *225*
DE	-	-	-	-	-	-	-	-	-	12.406 ±0.146 *15*	0.016 ±0.016 *60*	0.893 ±0.022 *225*
ER	2.221 ±0.087 *15*	0.553 ±0.087 *210*	0.275 ±0.041 *225*	0.502 ±0.012 *15*	0.856 ±0.078 *210*	0.760 ±0.038 *225*	0.534 ±0.030 *15*	0.564 ±0.109 *210*	0.715 ±0.043 *225*	15.462 ±0.216 *15*	0.100 ±0.044 *60*	0.937 ±0.016 *225*
ES	-	-	-	-	-	-	-	-	-	10.919 ±0.429 *14*	0.016 ±0.016 *60*	0.764 ±0.043 *210*

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_41294149

Location	*Centaurea arenaria*			*Dianthus serotinus*			*Festuca vaginata*			*Stipa borysthenica*		
	seed mass	germination	emergence	seed mass	germination	emergence	seed mass	germination	emergence	seed mass	germination	emergence
FE	-	-	-	-	-	-	0.493 ±0.020 *15*	0.776 ±0.049 *210*	0.675 ±0.055 *225*	-	-	-
FU	1.667 ±0.074 *15*	0.398 ±0.029 *210*	0.013 ±0.007 *225*	0.576 ±0.038 *15*	0.907 ±0.039 *210*	0.680 ±0.047 *225*	0.481 ±0.018 *15*	0.658 ±0.076 *210*	0.644 ±0.051 *225*	15.113 ±0.345 *15*	0.117 ±0.099 *60*	0.933 ±0.017 *225*
GE	1.904 ±0.077 *15*	0.400 ±0.118 *35*	0.373 ±0.048 *225*	-	-	-	-	-	-	-	-	-
GI	2.184 ±0.099 *15*	0.419 ±0.126 *210*	0.156 ±0.035 *225*	0.565 ±0.019 *15*	0.952 ±0.019 *210*	0.760 ±0.022 *225*	0.479 ±0.022 *15*	0.649 ±0.095 *210*	0.601 ±0.069 *225*	-	-	-
GO	2.374 ±0.104 *15*	0.628 ±0.066 *210*	0.342 ±0.047 *225*	0.479 ±0.031 *15*	0.900 ±0.078 *210*	0.764 ±0.042 *225*	0.286 ±0.013 *15*	0.814 ±0.056 *210*	0.707 ±0.039 *225*	-	-	-
GD	1.608 ±0.082 *15*	0.265 ±0.086 *210*	0.204 ±0.041 *225*	-	-	-	0.514 ±0.023 *15*	0.029 ±0.011 *210*	0.049 ±0.020 *225*	-	-	-
HO	1.561 ±0.057 *15*	0.274 ±0.044 *210*	0.191 ±0.034 *225*	0.644 ±0.018 *15*	0.766 ±0.059 *210*	0.627 ±0.038 *225*	0.456 ±0.022 *15*	0.483 ±0.052 *210*	0.529 ±0.053 *225*	8.450 ±0.965 *14*	0.067 ±0.067 *60*	0.400 ±0.090 *210*
KE	1.948 ±0.102 *15*	0.521 ±0.117 *210*	0.427 ±0.067 *225*	0.464 ±0.038 *15*	0.825 ±0.061 *210*	0.524 ±0.069 *225*	0.538 ±0.020 *15*	0.747 ±0.035 *210*	0.751 ±0.032 *225*	10.759 ±0.627 *14*	0.017 ±0.017 *60*	0.640 ±0.065 *210*
KI	1.766 ±0.085 *15*	0.584 ±0.024 *210*	0.551 ±0.048 *225*	-	-	-	0.472 ±0.036 *15*	0.433 ±0.121 *210*	0.462 ±0.054 *225*	9.478 ±1.016 *15*	0.017 ±0.096 *60*	0.516 ±0.096 *225*
LP	-	-	-	-	-	-	0.356 ±0.028 *15*	*** *0*	0.120 ±0.043 *225*	-	-	-
MA	-	-	-	-	-	-	0.518 ±0.029 *15*	0.795 ±0.095 *210*	0.724 ±0.048 *225*	-	-	-

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Location	*Centaurea arenaria*			*Dianthus serotinus*			*Festuca vaginata*			*Stipa borysthenica*		
	seed mass	germination	emergence	seed mass	germination	emergence	seed mass	germination	emergence	seed mass	germination	emergence
MO	-	-	-	-	-	-	0.483 ±0.016 *15*	0.781 ±0.063 *210*	0.693 ±0.037 *225*	12.589 ±0.257 *15*	0.033 ±0.021 *60*	0.848 ±0.023 *225*
MR	2.102 ±1.936 *15*	0.624 ±0.045 *210*	0.56 ±0.062 *225*	-	-	-	0.567 ±0.015 *15*	0.838 ±0.037 *210*	0.867 ±0.040 *225*	-	-	-
NA	-	-	-	0.342 ±0.040 *10*	0.495 ±0.113 *210*	0.347 ±0.055 *150*	-	-	-	-	-	-
NE	1.439 ±0.071 *15*	0.059 ±0.071 *210*	0.120 ±0.038 *225*	0.458 ±0.047 *15*	0.808 ±0.090 *210*	0.716 ±0.037 *225*	-	-	-	-	-	-
NY	-	-	-	-	-	-	0.445 ±0.027 *15*	0.238 ±0.1145 *210*	0.244 ±0.071 *225*	-	-	-
OF	1.839 ±0.098 *15*	0.405 ±0.084 *210*	0.489 ±0.057 *225*	-	-	-	-	-	-	-	-	-
OR	-	-	-	0.471 ±0.018 *15*	0.898 ±0.076 *210*	0.764 ±0.035 *225*	0.455 ±0.028 *15*	0.703 ±0.132 *210*	0.711 ±0.080 *225*	-	-	-
PA	1.610 ±0.088 *15*	0.110 ±0.075 *210*	0.156 ±0.055 *225*	0.604 ±0.047 *15*	0.87 ±0.058 *210*	0.707 ±0.059 *225*	0.359 ±0.017 *15*	0.210 ±0.109 *210*	0.244 ±0.060 *225*	-	-	-
PI	-	-	-	0.440 ±0.071 *15*	0.680 ±0.104 *210*	0.431 ±0.084 *225*	0.459 ±0.027 *15*	0.702 ±0.053 *210*	0.356 ±0.077 *225*	14.642 ±0.833 *14*	0 *60*	0.636 ±0.055 *210*
SZ	-	-	-	-	-	-	0.491 ±0.038 *15*	0.269 ±0.064 *210*	0.204 ±0.059 *225*	-	-	-
TA	1.719 ±0.052 *15*	0.211 ±0.070 *210*	0.124 ±0.036 *225*	0.585 ±0.022 *15*	0.914 ±0.028 *210*	0.813 ±0.042 *225*	0.442 ±0.020 *15*	0.500 ±0.101 *210*	0.307 ±0.059 *225*	-	-	-

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Location	*Centaurea arenaria*			*Dianthus serotinus*			*Festuca vaginata*			*Stipa borysthenica*		
	seed mass	germination	emergence	seed mass	germination	emergence	seed mass	germination	emergence	seed mass	germination	emergence
TZ	2.0771 ±0.090 *15*	0.562 ±0.071 *210*	0.471 ±0.038 *225*	-	-	-	0.503 ±0.018 *15*	0.600 ±0.121 *210*	0.573 ±0.049 *225*	-	-	-
TE	1.642 ±0.070 *15*	0.123 ±0.022 *210*	0.169 ±0.022 *225*	-	-	-	0.483 ±0.017 *15*	0.685 ±0.098 *210*	0.649 ±0.050 *225*	14.655 ±0.337 *15*	0.183 ±0.060 *60*	0.986 ±0.007 *225*
ZS	-	-	-	-	-	-	0.426 ±0.023 *15*	0.731 ±0.079 *210*	0.489 ±0.028 *225*	-	-	-

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_41294149

EFFECTS OF TEMPERATURE, FOOD CONCENTRATION, AND INITIAL ROTIFER DENSITY ON THE INTERSPECIFIC COMPETITION BETWEEN TWO ROTIFER SPECIES

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(Received 1st Jun 2021; accepted 3rd Sep 2021)

Abstract. There is often intense competition among species in an environment with limited resources and space, leading to the exclusion of inferior competitors, reduced species diversity, and changes in zooplankton communities. This study investigated the interspecific competition between two dominant rotifer species in the Pearl River Basin, *Brachionus calyciflorus* and *Keratella valga,* through laboratory experiments at different temperatures, food concentrations, and initial rotifer densities. In separate cultures, the population growth of both rotifer species was affected by changes in temperature, food density, and initial density. In mixed cultures, *B. calyciflorus* was inhibited by competition, leading to an overall decrease in its population growth rate and maximum population density. *K. valga* was also affected by competition, leading to an increase in its population growth rate and maximum population density at 20 ℃ or at an initial density of 1.0×10^6 cells/mL. *B. calyciflorus* was excluded by *K. valga* at low and medium food concentrations (0.5×10^6 and 1.0×10^6 cells/mL), whereas the opposite was found at high food concentration (3.0×10^6 cells/mL). Food concentration is obviously the main factor affecting outcome. Temperature, initial density, and interactions between the three factors also have an effect on it.
Keywords: *Brachionus calyciflorus, Keratella valga, species diversity, population growth rate, maximum population density*

Introduction

Zooplankton is an important component that plays a key connecting role in the aquatic food chain. Rotifers are one of the important groups of zooplankton widely distributed worldwide. They reproduce rapidly and have a short life cycle. They are not only important for the structure and function of the aquatic ecosystem, but are also meaningful to aquaculture feed science. They can also be used as a test organism for environmental monitoring and ecotoxicology research worldwide (Van Dijk and Van Zanten, 1995; Devetter, 1998; Andrzej and Jolanta, 2005).

Competition among rotifer species is influenced by various factors such as body size, feeding habits, food type and nutritional quality, temperature, salinity, food level, inoculation density and diapause (Fernández-Araiza et al., 2005; Montero-Pau and Serra, 2011; Divya et al., 2012; Li and Niu, 2015; Rebolledo et al., 2018; Zhang et al., 2019).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_41514168

The outcome of rotifer population competition is ultimately related to population growth and reproduction; whereas the outcome of interspecific competition is the change in the community structure of the rotifer population. The experimental results of Wang et al. (2014) showed that temperature significantly affected the population parameters of *Brachionus calyciflorus*. The most suitable temperature for the growth of *B. calyciflorus* is 28-32 ℃, and the highest temperature should not exceed 40 ℃ (Wang, 1995). Many studies have confirmed that the seasonal changes of rotifer population distribution and the spatiotemporal dynamics of its community structure are largely affected by temperature (Ruttner-Kolisko, 1974; May, 1983; Huang et al., 1985; Herzig, 1987; Arora, 2003; Wen et al., 2004). Zooplankton can quickly detect slight changes in temperature (Benincà et al., 2011). Different plankton species have different sensitivity to temperature. Therefore, the outcome of population competition is likely to vary with temperature (Feniova et al., 2011).

Rotifers feed on algae and protozoa for population growth and reproduction. Thus, there will inevitably be interspecific or intraspecific competition of rotifers when food is insufficient. Therefore, food has a significant impact on the population growth rate and density of rotifers (Zhang et al., 2005; Tian et al., 2009; Ma et al., 2004; Dong et al., 2004). The number and diversity of zooplankton populations and the outcome of interspecific competition are largely restricted by food factors (Sterner and Hessen, 1994; Elser et al., 2001; Ferrao-Filho et al., 2003; Persson et al., 2008; Espinosa-Rodríguez et al.,2012). The change in competition outcome will change the rotifer community structure and population density. In short, food resources affect the community structure and population changes of rotifers (Yoshida, 2003; Aoyagui and Bonecker, 2005; Lin et al., 2005). Interspecific competition of rotifers often leads to a decrease in the population growth rate of competitors, but the degree of decrease changes with food density. When food availability meets competition demands, the population growth rates of competitors play a huge role in the outcome of interspecific competition. Species with low population growth rates are usually at a disadvantage in the interspecific competition. However, the population growth rate does not fully represent the interspecific competitiveness of a competitor. Reproductive capacity under extremely low food density plays a more decisive role (Stemberger and Gilbert, 1985). Therefore, rotifers with higher population growth rates are likely to gain growth advantages when food is scarce (Stemberger and Gilbert, 1987).

Many studies have shown that the growth and reproduction of rotifers are largely affected by it (Xi et al., 2000a; Zhang et al., 2005; Tian et al., 2009). Therefore, the competitiveness of rotifers must be closely related to the initial density. When there is a very large difference in the competitive capacity of competitors, the competitor with a greater competitive capacity will inevitably gain an advantage in the competition. However, when the difference is small, the initial density of competitors will play a crucial role.

Food levels may modify the outcome of competition between two rotifer species which differ widely in their growth rates. When two species of rotifers compete for limited food, normally the one with higher population growth rate may be expected to outcompete the other with lower growth rate (Rothhaupt, 1990). However, this does not appear to hold true for many species (Sarma et al., 1996, 2007; Sarma and Nandini, 2002; Divya et al., 2012). Xi et al. (2019) studied the relationship between the results of competition and their population growth rate for *Brachionus angularis* and *B. calyciflorus* when they competed for limited food resources.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_41514168

B. calyciflorus and *Keratella valga* are the dominant rotifer species in the Pearl River Basin. However, there is no report on the interspecific competition between the two rotifer species. This study is the first to investigate the interspecific competition between *B. calyciflorus* and *K. valga* at different temperatures, food concentrations, and initial densities. It aims to better understand the competitive relationship between freshwater rotifers and reveal the effects of temperature, food, and initial density on the community structure of freshwater planktonic rotifers in the Pearl River Basin. The results have important theoretical implications for understanding the interspecific competition and community structure and function of zooplankton.

Materials and methods

Source and culture of the two rotifer species

B. calyciflorus was purchased from Qianjiangshui Water Biotechnology Co., Ltd. (Guangzhou, China). *K. valga* was collected from fish pond No. 9 (23°4'N, 113°13'E) of Pearl River Fisheries Research Institute, Chinese Academy of Fishery Sciences. The two rotifer species were incubated in EPA medium in a constant temperature incubator at 25 ± 1 ℃ under natural light and fed every 24 h with *S. obliquus* cultivated in BG11 medium and in exponential growth period (at a feeding density of 0.5×10^6, 1.0×10^6, or 3.0×10^6 cells/mL according to preculture conditions) for clonal culture. Before the start of the competition experiments, the two rotifer species were pre-cultured for more than 48 h by feeding with *S. obliquus* at 0.5×10^6, 1.0×10^6 and 3.0×10^6 cells/mL, respectively, in a constant temperature incubator under natural light at 20 ± 1, 25 ± 1, and 30 ± 1 ℃.

For the preparation of EPA medium, 0.096 g of sodium bicarbonate, 0.06 g of calcium sulfate, 0.06 g of magnesium sulfate, and 0.004 g of calcium chloride were weighed using an analytical balance and transferred to a 1000-mL beaker. Next, 1000 mL of deionized water was added with stirring using a glass rod to achieve full dissolution (U.S. EPA, 1985).

Equipment

The equipment used in the experiments are shown in *Table 1*.

Table 1. *Equipment*

Equipment	Model/specification	Manufacturer
Analytical balance	FB2004A	Jinghong Experimental Equipment Co., Ltd. (Shanghai, China)
Pen-type pH meter	PH-902	Sierte Electromechanical Equipment Co., Ltd. (Changzhou, China)
Plankton net	No. 25 trawl-type	Dengxun Instrument Equipment Co., Ltd. (Xiamen, China)
Intelligent light incubator	MGC-250BP-2	Yiheng Technology Co., Ltd. (Shanghai, China)
Biomicroscope	BX53	Olympus Corporation (Beijing, China)
High-speed centrifuge	10 - 1000 μL; 1000 - 5000 μL	Pingfan Instrument Co., Ltd. (Changsha, China)
Single-channel manual pipette	2 - 10 mL	Gaoxin Chemical Glass Instrument Co., Ltd. (Shanghai, China)
Pipette tip	1 mL, 5 mL, 10 mL	Labgic Technology Co., Ltd. (Beijing, China)
Beaker	25 mL, 1000 mL	Leigu Instrument Co., Ltd. (Shanghai, China)
Erlenmeyer flask	500 mL	Leigu Instrument Co., Ltd. (Shanghai, China)
Plankton counting chamber	1 mL	Purity Instrument Co., Ltd. (Beijing, China)

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_41514168

Reagents

The reagents used in the experiments are shown in *Table 2*.

Table 2. *Reagents*

Reagent	Chemical formula	Molecular weight	Grade	Source
Sodium bicarbonate	$NaHCO_3$	84.01	AR	Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China)
Calcium sulfate	$CaSO_4$	172.17	AR	Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China)
Magnesium sulfate	$MgSO_4$	120.37	AR	Aladdin Biochemical Technology Co., Ltd. (Shanghai, China)
Potassium Chloride	KCl	74.55	AR	Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China)
Sodium nitrate	$NaNO_3$	84.99	AR	Sigma-Aldrich Trading Co., Ltd. (Shanghai, China)
Dipotassium phosphate	K_2HPO_4	174.18	AR	Aladdin Biochemical Technology Co., Ltd. (Shanghai, China)
Magnesium sulfate heptahydrate	$MgSO_4·7H_2O$	246.47	AR	Aladdin Biochemical Technology Co., Ltd. (Shanghai, China)
Calcium chloride heptahydrate	$CaCl_2·7H_2O$	237.11	AR	Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China)
Citric acid	$C_6H_8O_7$	192.13	AR	HWRK Chem Co., Ltd. (Beijing, China)
Ferric ammonium citrate	$C_6H_8FeNO_7$	261.98	AR	Aladdin Biochemical Technology Co., Ltd. (Shanghai, China)
Ethylenediaminetetraacetic acid disodium salt dihydrate	$EDTANa_2$	336.21	AR	Aladdin Biochemical Technology Co., Ltd. (Shanghai, China)
Sodium carbonate	Na_2CO_3	105.99	AR	Aladdin Biochemical Technology Co., Ltd. (Shanghai, China)
Boric acid	H_3BO3	61.8	AR	Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China)
Manganese chloride tetrahydrate	$MnCl_2·4H_2O$	197.91	AR	Aladdin Biochemical Technology Co., Ltd. (Shanghai, China)
Zinc sulfate	$ZnSO_4$	161.45	AR	Aladdin Biochemical Technology Co., Ltd. (Shanghai, China)
Sodium manganate	Na_2MnO_4	164.92	AR	Aladdin Biochemical Technology Co., Ltd. (Shanghai, China)
Copper sulfate pentahydrate	$CuSO_4·5H_2O$	41.05	AR	Aladdin Biochemical Technology Co., Ltd. (Shanghai, China)
Cobalt nitrate hexahydrate	$Co(NO_3)_2·6H_2O$	291.03	AR	Aladdin Biochemical Technology Co., Ltd. (Shanghai, China)

Competition experiments

Experiments were performed to determine the outcome of the competition between *B. calyciflorus* and *K. valga* under the following conditions:

Experiment 1: at different temperatures and food concentrations at an initial rotifer density of 3 ind./mL.

Experiment 2: at different temperatures and initial rotifer densities (1, 3, and 5 ind./mL) at a food concentration of 0.5×10^6 cells/mL.

Experiment 3: at different food concentrations and initial rotifer densities (1, 3, and 5 ind./mL) at a temperature of 25 ℃.

In each experiment, separate cultures of *B. calyciflorus* and *K. valga* were used as the control group, and the mixed cultures of the two rotifer species were used as the competition group. Each experiment was repeated three times. Rotifers for inoculation

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_41514168

were randomly selected from the culture system in the exponential growth period under the corresponding culture conditions. The experiment was performed with a volume of 20 mL in a 25-mL beaker. After the start of the experiment, complete counting or three rounds of parallel counting by sampling of 1-3 mL (depending on the population density) of the separate and mixed rotifer cultures were performed every day. After counting, all surviving animals were transferred back to the original beakers. The competition experiments continued until all animals of one species in the mixed culture system died or until 30-35 days had elapsed.

The experimental culture is shown in *Figure 1*.

Figure 1. *Photo of the experimental culture*

Data collection and calculation of rotifer population density

Data were collected for each experimental group every 24 h after the start of the experiment. Every day at 9 a.m., after the beakers were shaken, 1 mL of rotifer culture medium was randomly transferred using a pipette to a 1-mL plankton counting chamber and counted under a biomicroscope to calculate the average population density of rotifers in each group.

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DOI: http://dx.doi.org/10.15666/aeer/1905_41514168

Parameter calculation and curve plotting

After the end of the experiments, the population growth curves were plotted for each rotifer species under each combination of experimental conditions based on the population densities recorded.

Four to 6 data points during the exponential growth period were selected to calculate the population growth rate for each repetition (Dumont et al., 1995).

The population growth rate was calculated from:

$$r = (\ln N_t - \ln N_0)/t \quad \text{(Eq.1)}$$

where N_0 and N_t are the population densities of rotifers at the beginning of the experiment and on Day t, respectively; and t is the number of days in the experiment.

The histograms of the maximum population densities under each combination of experimental conditions were plotted based on the highest point of the corresponding population growth curve.

Data analysis

The differences in the population growth rates and maximum population densities of *B. calyciflorus* and *K. valga* were analyzed by three-way analysis of variance and multiple comparisons using SPSS 25.0. Statistical significance was tested at $\alpha = 0.05$. T-test was used for mean comparison in this study.

Results and analysis

Outcome of competition between B. calyciflorus and K. valga at different temperatures and food concentrations

The population growth dynamics and outcome of competition between *B. calyciflorus* and *K. valga* at different temperatures and food concentrations are shown in *Figure 2*.

In general, regardless of competition, the population densities of both *B. calyciflorus* and *K. valga* increased and then decreased with increasing temperature. However, the population densities of both species remained at a relatively stable level of 2 ind./L at 20 °C and a food concentration of 0.5×10^6 cells/mL. In mixed cultures, interspecific competition inhibited the population density growth of both species, leading to lower population densities of both species compared to those in separate cultures. However, they promoted the population density growth of each other at the lowest temperature of 20 ℃ and food concentrations of 0.5×10^6 and 1.0×10^6 cells/mL, leading to higher population densities of both species in mixed cultures than in separate cultures.

Regardless of temperature, *B. calyciflorus* was competitively excluded by *K. valga* at lower food concentrations (0.5×10^6 and 1.0×10^6 cells/mL). At the highest food concentration (3.0×10^6 cells/mL), *K. valga* was competitively excluded by *B. calyciflorus*; and the duration of competitive exclusion decreased with increasing temperature and food concentration.

The results of three-way analysis of variance for temperature, food concentration, and competition affecting the population growth rates and maximum population densities of *B. calyciflorus* and *K. valga* are shown in *Table 3*. Food concentration had a

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_41514168

significant effect on the maximum population density of *B. calyciflorus* ($p < 0.05$), whereas other factors and interactions had no significant effect on the population growth rate or maximum population density of *B. calyciflorus* ($p > 0.05$). Temperature, food concentration, competition with *B. calyciflorus*, and their interactions had no significant effect on the population growth rate or maximum population density of *K. valga* ($p > 0.05$).

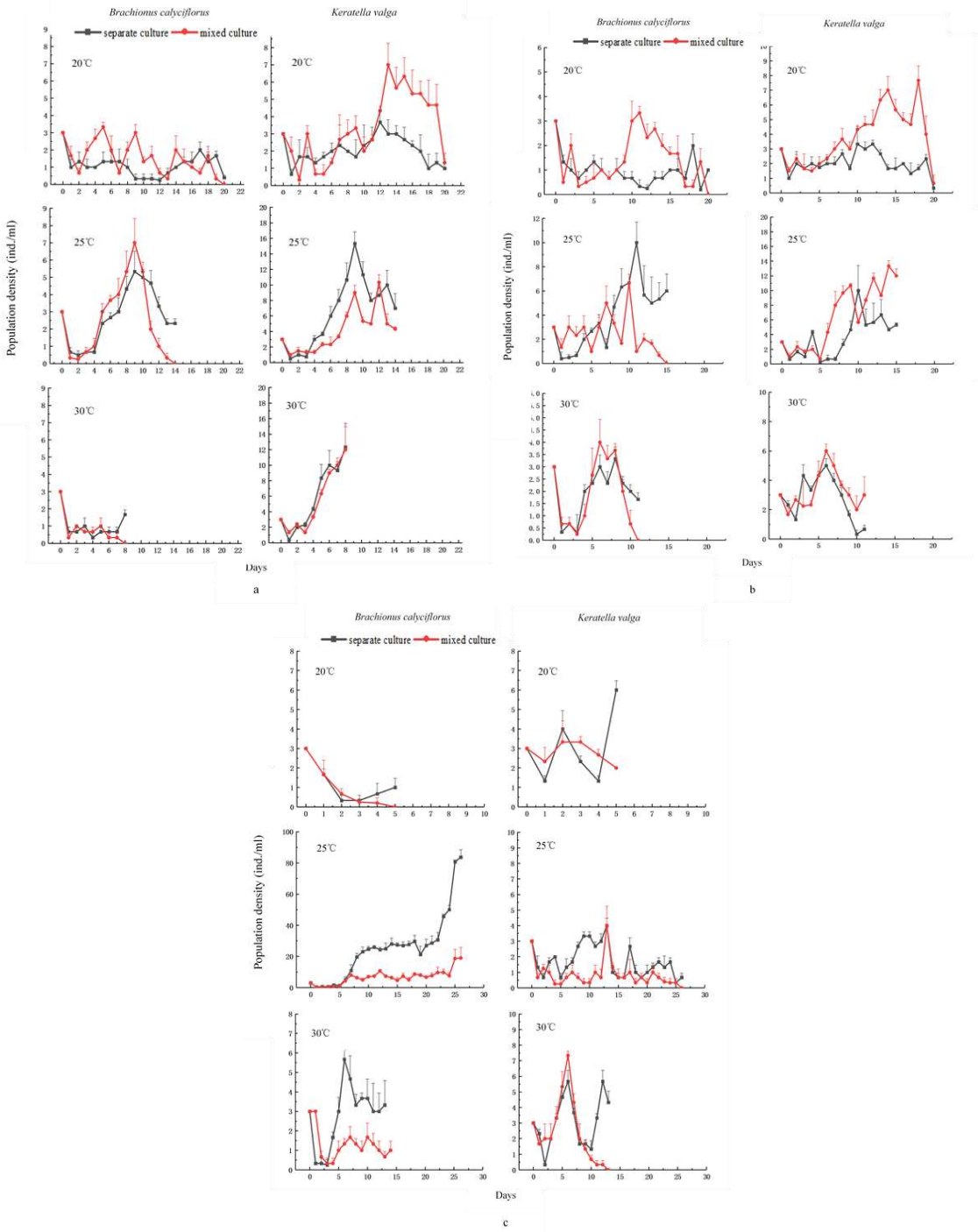

Figure 2. *Population growth of B. calyciflorus and K. valga in separate and mixed cultures at different temperatures (mean ± standard errors) (a. food concentration = 0.5 × 10^6 cells/mL; b. food concentration = 1.0 × 10^6 cells/mL; c. food concentration = 3.0 × 10^6 cells/mL)*

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Table 3. *Three-way analysis of variance of population growth rates and maximum population densities of B. calyciflorus and K. valga (p-value)*

Competitor	Parameter	T	F	C	T*F	T*C	F*C	T*F*C
B. calyciflorus	Population growth rate	0.106	0.361	0.544	0.553	0.586	0.976	0.229
	Maximum population density	0.139	**0.048**	0.530	0.143	0.976	0.752	0.796
K. valga	Population growth rate	0.475	0.316	0.914	0.675	0.526	0.919	0.534
	Maximum population density	0.970	0.663	0.734	0.683	0.919	0.861	0.871

T: temperature, F: food concentration, C: competition

Regardless of temperature and food concentration, the competition with *K. valga* inhibited the population growth of *B. calyciflorus*, leading to a decrease in the population growth rate and maximum population density of *B. calyciflorus*. On the other hand, the competition with *B. calyciflorus* promoted the population growth of *K. valga*, leading to a slight increase in the population growth rate and maximum population density of *K. valga* (*Table 3; Fig. 3*).

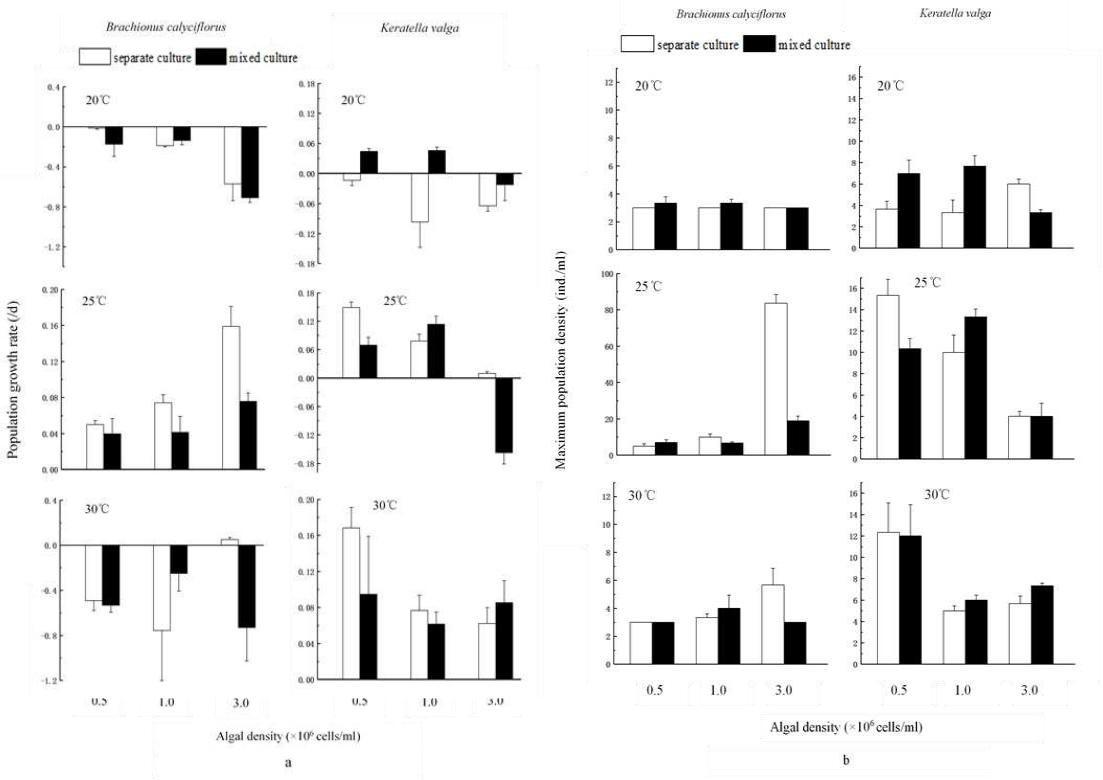

Figure 3. *Population growth rates (a) and maximum population densities (b) of B. calyciflorus and K. valga in separate and mixed cultures at different temperatures and food concentrations (mean ± standard errors)*

The population growth rates of *B. calyciflorus* and *K. valga* at different temperatures and food concentrations in separate and mixed cultures are shown in *Figure 3a*. At any food concentration, the growth rate of *K. valga* increased with increasing temperature. The population growth rate of *B. calyciflorus* was lower in mixed cultures than in separate cultures, and lower than that of *K. valga* in mixed cultures. Regardless of

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competition, the population growth rate of *B. calyciflorus* was positive at 25 °C only, and negative at the other two temperatures. At 20 °C, the population growth rate of *K. valga* was higher in mixed cultures than in separate cultures.

The maximum population densities of *B. calyciflorus* and *K. valga* at different temperatures and food concentrations in separate and mixed cultures are shown in *Figure 3b*. Regardless of competition, the maximum population density of *B. calyciflorus* in separate and mixed cultures increased with increasing temperature ($p < 0.05$). Regardless of temperature, the maximum population density of *K. valga* was higher than that of *B. calyciflorus* in mixed cultures.

Outcome of competition between B. calyciflorus and K. valga at different temperatures and initial densities

The population growth dynamics and outcome of competition between *B. calyciflorus* and *K. valga* at different temperatures and initial densities are shown in *Figure 4*.

In general, regardless of competition, the population densities of *B. calyciflorus* and *K. valga* increased with increasing initial density, but did not change significantly with increasing temperature. Regardless of initial density and temperature, the competition with *K. valga* inhibited the population growth of *B. calyciflorus*, leading to a decrease in the population density of *B. calyciflorus* in mixed cultures, and eventually competitively eliminating *B. calyciflorus.* Moreover, the duration of competitive exclusion decreased with increasing temperature, and with increasing initial density. On the contrary, the competition with *B. calyciflorus* promoted the population growth of *K. valga*, resulting in significantly higher population densities of *K. valga* in mixed cultures than in separate cultures, except at a temperature of 25 °C and an initial density of 3 ind./mL, at which the population density of *K. valga* was lower in mixed cultures than in separate cultures.

The results of three-way analysis of variance for temperature, food concentration, and competition affecting the population growth rates and maximum population densities of *B. calyciflorus* and *K. valga* are shown in *Table 4*. Temperature, initial density, competition with *K. valga*, and their interactions had no significant effect on the population growth rate or maximum population density of *B. calyciflorus* ($p > 0.05$). Similarly, temperature, initial density, competition with *B. calyciflorus*, and their interactions had no significant effect on the population growth rate or maximum population density of *K. valga* ($p > 0.05$).

Table 4. *Three-way analysis of variance of population growth rates and maximum population densities of B. calyciflorus and K. valga (p-value)*

Competitor	Parameter	T	I	C	T*I	T*C	I*C	T*I*C
B. calyciflorus	Population growth rate	0.114	0.224	0.573	0.317	0.786	0.883	0.974
	Maximum population density	0.338	0.071	0.518	0.301	0.806	0.660	0.812
K. valga	Population growth rate	0.864	0.454	0.816	0.671	0.689	0.925	0.969
	Maximum population density	1.000	0.626	0.929	0.572	0.728	0.899	0.962

T: temperature, I: initial density, C: competition

Regardless of temperature and initial density, the competition with *K. valga* inhibited the population growth of *B. calyciflorus*, leading to a significant decrease in the population growth rate and a small decrease in the maximum population density of *B. calyciflorus*. When competing *B. calyciflorus*, the population growth rate and maximum

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population density of *K. valga* increased significantly at 20 °C, and decreased significantly at 25 °C and 30 °C (*Table 4; Fig. 5*).

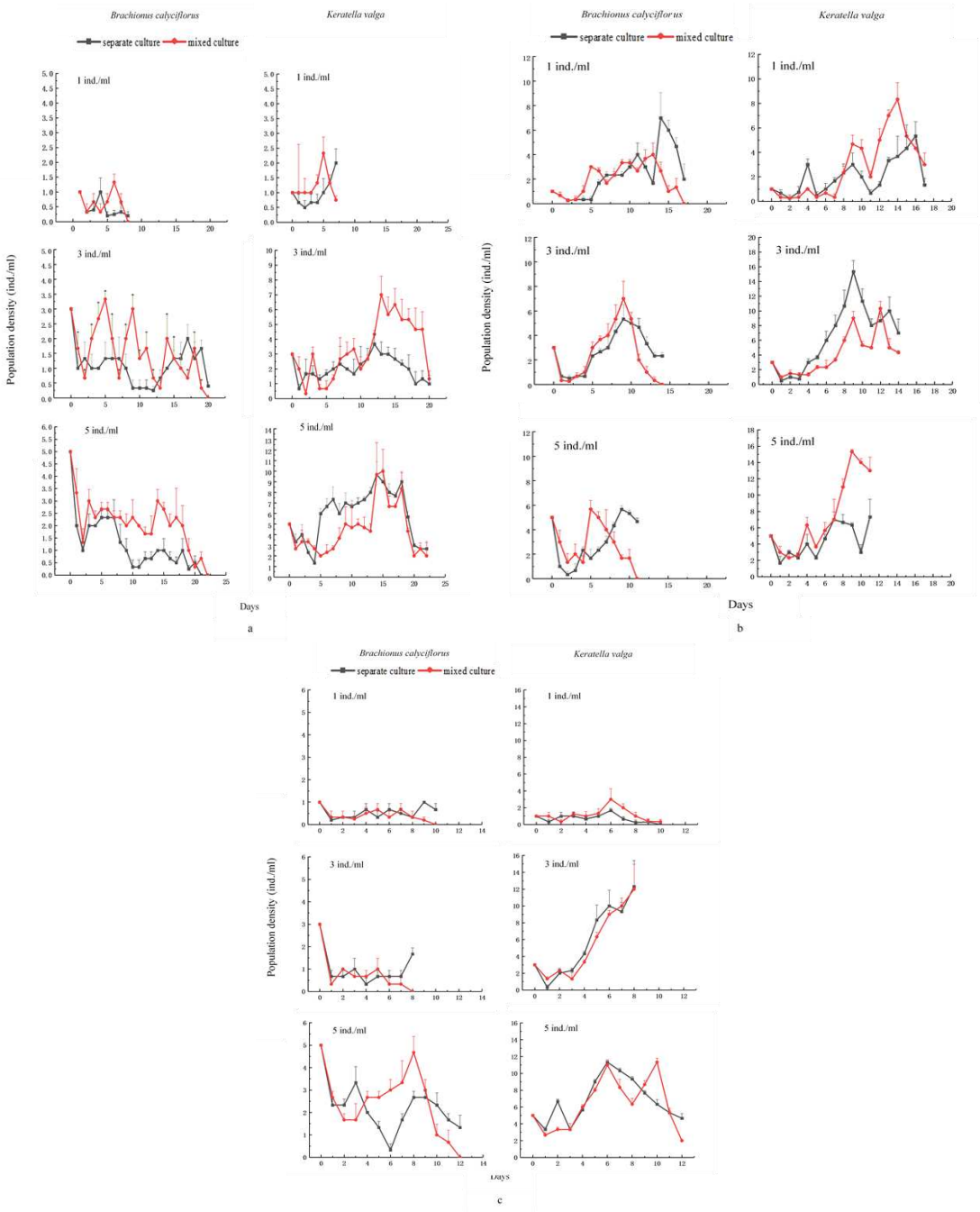

Figure 4. *Population growth of B. calyciflorus and K. valga in separate and mixed cultures at different initial densities (mean ± standard errors) (a. 20 ℃; b. 25 ℃; c. 30 ℃)*

The population growth rates of *B. calyciflorus* and *K. valga* at different temperatures and initial densities in separate and mixed cultures are shown in *Figure 5a*. At any temperature, the population growth rate of *K. valga* in mixed cultures decreased with increasing initial density. Regardless of initial density, *K. valga* suppressed the

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population growth rate of *B. calyciflorus* at 20 °C and 25 °C; and *B. calyciflorus* suppressed that of *K. valga* at 25 °C and 30 °C. Overall, competition had an inhibitory effect on the population growth rates of both rotifer species in mixed cultures at 25 °C.

The maximum population densities of *B. calyciflorus* and *K. valga* at different temperatures and food concentrations in separate and mixed cultures are shown in *Figure 5b*. Regardless of competition, the maximum population densities of both rotifer species increased with increasing initial density in separate and mixed cultures at 20 °C and 30 °C. Regardless of initial density, interspecific competition had no significant effect on the maximum population densities of both rotifer species in mixed cultures at 20 °C and 30 °C.

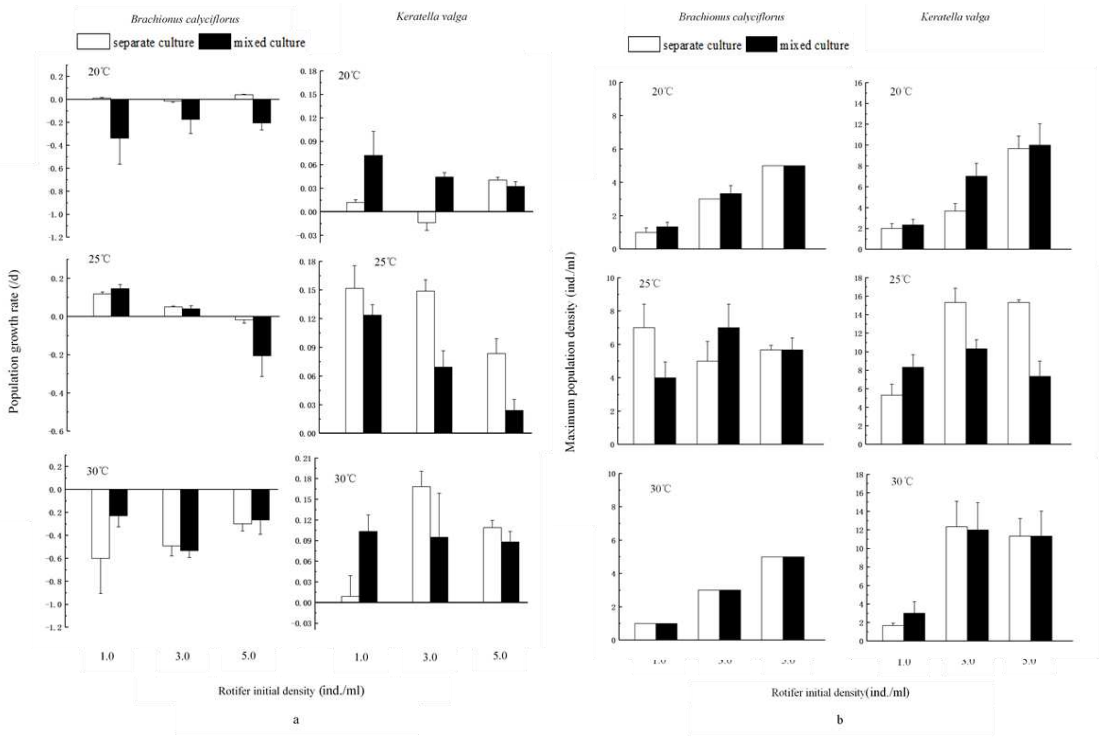

Figure 5. *Population growth rates (a) and maximum population densities (b) of B. calyciflorus and K. valga in separate and mixed cultures at different temperatures and food concentrations (mean ± standard errors)*

Outcome of competition between B. calyciflorus and K. valga at different food concentrations and initial densities

The population growth dynamics and outcome of competition between *B. calyciflorus* and *K. valga* at different food concentrations and initial densities are shown in *Figure 6*.

In general, regardless of competition, the population densities of both *B. calyciflorus* and *K. valga* increased with increasing food concentration, but did not change significantly with increasing initial density.

Interspecific competition inhibited the population density growth of both species, leading to lower population densities of both species in mixed cultures than in separate cultures. However, the population densities were higher in mixed cultures than in separate cultures at the lowest initial density. Regardless of initial density, *B. calyciflorus* was competitively excluded by *K. valga* at lower food concentrations (0.5 ×

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10^6 and 1.0×10^6 cells/mL). At the highest food concentration (3.0×10^6 cells/mL), *K. valga* was competitively excluded by *B. calyciflorus*; and the duration of competitive exclusion was not significantly related to initial density or food concentration.

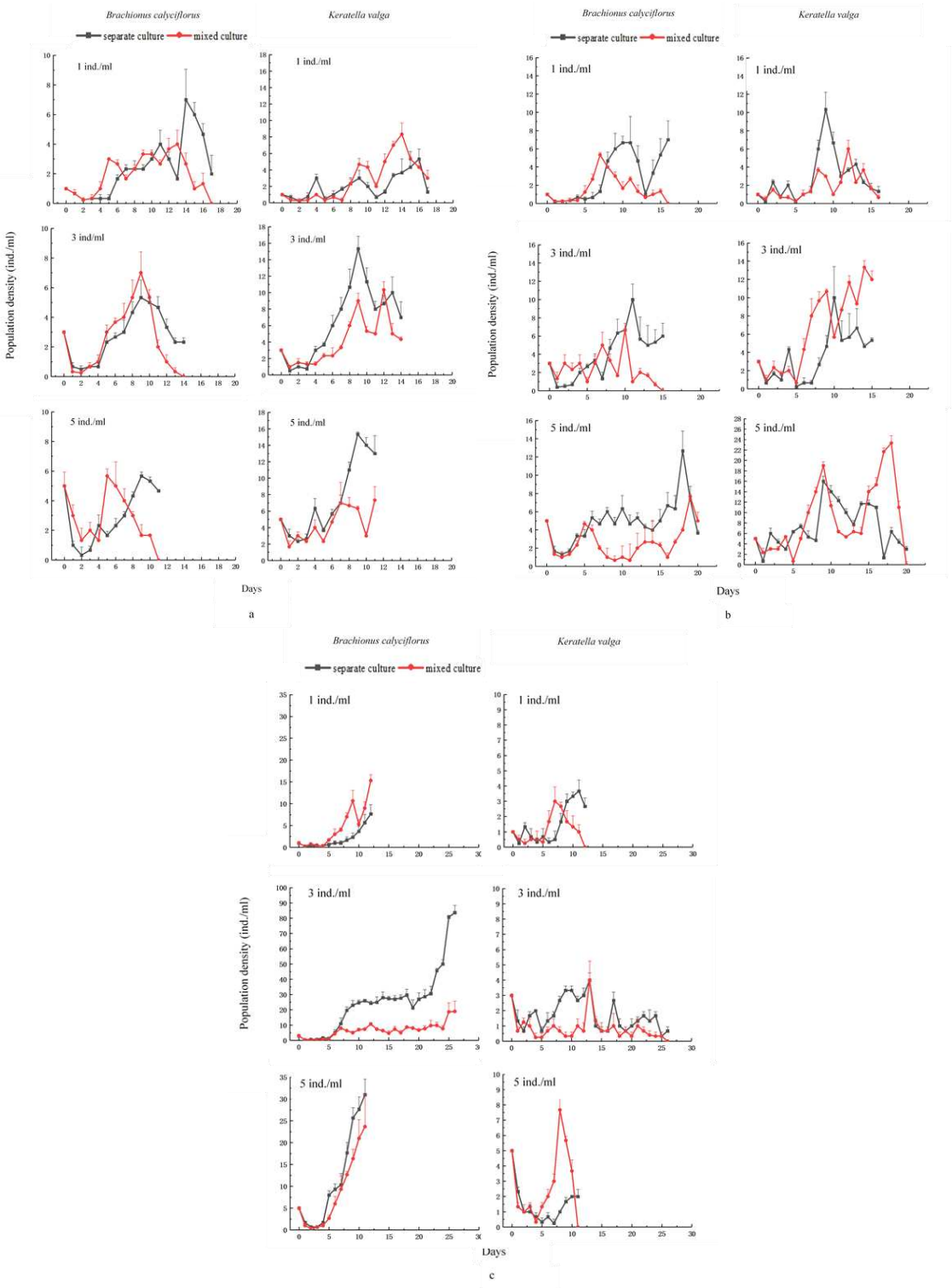

Figure 6. *Population growth of B. calyciflorus and K. valga in separate and mixed cultures at different initial densities (mean ± standard errors) (a. food concentration = 0.5 × 10^6 cells/mL; b. food concentration = 1.0 × 10^6 cells/mL; c. food concentration = 3.0 × 10^6 cells/mL)*

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The three-way analysis of variance showed that food concentration and initial density had a significant effect on the population growth rate of *B. calyciflorus*, and that food concentration, initial density, competition with *K. valga*, and their interactions had a significant effect on the maximum population density of *B. calyciflorus* ($p < 0.05$). Food concentration, initial density, competition with *B. calyciflorus*, and their interactions had a significant effect on the population growth rate of *K. valga* ($p < 0.05$). All the factors had no significant effect on the maximum population density of *K. valga* ($p < 0.05$) *(Table 5)*.

Table 5. *Three-way analysis of variance of population growth rates and maximum population densities of B. calyciflorus and K. valga (p-value)*

Competitor	Parameter	F	I	C	T*I	T*C	I*C	T*I*C
B. calyciflorus	Population growth rate	**0.045**	**0.026**	0.180	0.081	0.924	0.388	0.586
	Maximum population density	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.01$	**0.019**	$p < 0.01$
K. valga	Population growth rate	$p < 0.01$	$p < 0.01$	0.428	0.124	0.114	**0.047**	**0.030**
	Maximum population density	0.434	0.658	0.622	0.829	0.508	0.891	0.681

F: food concentration, I: initial density, C: competition, T: temperature

Regardless of food concentration and initial density, both *B. calyciflorus* and *K. valga* were inhibited by competition with each other, resulting in a significant decrease in their population growth rates. The maximum population density of *B. calyciflorus* also decreased, whereas that of *K. valga* slightly increased (*Table 5; Fig. 7*).

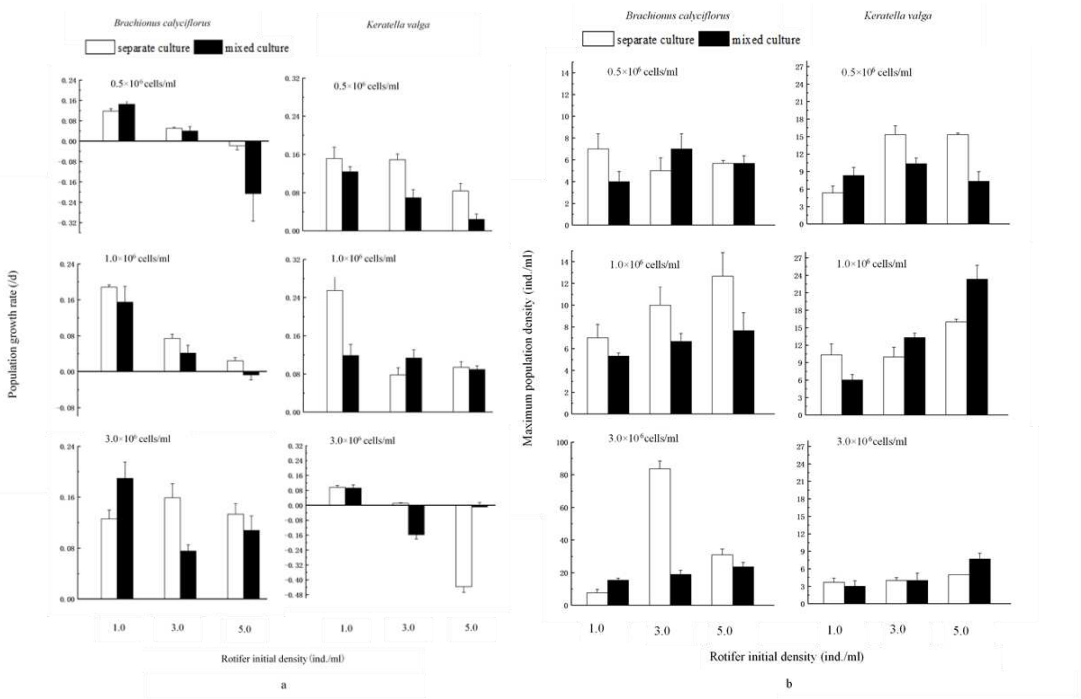

Figure 7. *Population growth rates (a) and maximum population densities (b) of B. calyciflorus and K. valga in separate and mixed cultures at different food concentrations and initial densities (mean ± standard errors)*

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The population growth rates of *B. calyciflorus* and *K. valga* at different temperatures and food concentrations in separate and mixed cultures are shown in *Figure 7a*. Regardless of competition, the population growth rate of *B. calyciflorus* increased with increasing food concentration ($p < 0.05$) and decreased with increasing initial density ($p < 0.05$), whereas that of *K. valga* decreased with both increasing food concentration and initial density ($p < 0.01$). At any initial density, competition with *K. valga* inhibited the population growth of *B. calyciflorus* at low and medium food concentrations (0.5×10^6 and 1.0×10^6 cells/mL), resulting in a significant decrease in the population growth rate of *B. calyciflorus*.

The maximum population densities of *B. calyciflorus* and *K. valga* at different temperatures and food concentrations in separate and mixed cultures are shown in *Figure 7b*. Regardless of competition, the maximum population density of *B. calyciflorus* significantly increased with both increasing food concentration and initial density ($p < 0.01$). Similarly, the maximum population density of *K. valga* slightly increased with both increasing food concentration and initial density. Regardless of food concentration and initial density, competition with *K. valga* inhibited the population growth of *B. calyciflorus*, leading to a decrease in the maximum population density of *B. calyciflorus*.

Discussion

The population structure and population dynamics of zooplankton in natural water are mainly caused by the influence of many environmental factors. The interspecific competition of rotifers is mainly chemical interference competition and resource utilization competition, without mechanical interference (Rothhaupt, 1988). Therefore, the competition between *B. calyciflorus* and *K. valga* is mainly chemical interference competition and resource utilization competition. Combined with the above analysis, it is easy to know that temperature and food concentration are the main factors affecting the interspecific competition between the two rotifers. Generally, the population growth of rotifers will increase with the increase of temperature and food concentration within the range of temperature and food concentration for rotifers to adapt to survival (Kauler and Enesco, 2011). In general, *B. calyciflorus* and *K. valga* in single culture or mixed culture conform to the law of population dynamics, and the experimental duration will shorten with the increase of temperature. However, because the intrinsic growth rate of *K. valga* is the largest at 25 ℃ and decreased at 20 ℃and 30 ℃, the most suitable water temperature is about 25 ℃ according to the life history parameters such as average life span, oviposition and net reproductive rate of *K. valga*. Therefore, with the increase of experimental temperature (20 ℃, 25 ℃ and 30 ℃), the population growth in the single culture and competition groups showed an increasing or decreasing trend. In addition to the factors of temperature and food concentration, the initial density of rotifer also had a great influence on the population growth dynamics of *B. calyciflorus*. In the early stage of the experiment, the population density of rotifer increased with the increase of initial density. With the extension of culture time, the difference caused by initial density changed irregularly. The results of this experiment are roughly the same (Xi and Huang, 2000).

The critical food density, individual size and hunger tolerance of the two rotifers also play an important role in the outcome of the competition. Among them, in terms of the impact of the relative size of competitors on the outcome of competition, *B. calyciflorus*

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is larger than *K. valga* under the comparison of individual size, and both are phytophagous zooplankton. Larger zooplankton consume less energy when swimming and are dominant in population competition, but the larger the individual is, the greater the energy required for its own metabolism. Therefore, *B. calyciflorus* may have an advantage in interspecific competition at high food concentration, which is opposite at low food concentration. In the mixed culture group, regardless of the initial density of rotifers, at low and medium food concentrations (0.5 and 1.0 $\times 10^6$ cells/ml), *K. valga* rejected rotifer *B. calyciflorus*, but the food concentration was high (3.0×10^6 cells/ml), *B. calyciflorus* rejected *K. valga*.

The effect of single factor on interspecific competition of rotifer is more regular, while the effect of the interaction of two or more factors on interspecific competition may be more complex. Some studies have shown that the interspecific competition results of *B. calyciflorus* (large size) and *Anuraeopsis fissa* (small size) are jointly determined by rotifer size, food concentration and rotifer inoculation density (Sarma et al., 1996), which shows that multiple factors and their interactions jointly affect the competition outcome. After the research of this experiment, it can be further discussed.

Under the same mixed culture condition at 25 ℃, regardless of the initial density of rotifers, at low and medium food concentrations (0.5 and 1.0×10^6 cells/ml competition group, most of *K. valga* rejected *B. calyciflorus*, while the food concentration was high (3.0×10^6 cells/ml, *B. calyciflorus* rejected *K. valga*, but at medium food concentration (1.0×10^6 cells/ml), when the initial density of rotifer increased to 5 ind./ml, *B. calyciflorus* rejected *K. valga* on the contrary. Regardless of the initial density of rotifer, the population growth rate of rotifer *B. calyciflorus* was mostly positive, while *K. valga* has positive growth at low and medium food concentrations (0.5 and 1.0×10^6 cells/ml) and has negative growth mostly at high food concentration (3.0×10^6 cells/ml).

This part of the experiment can also show that food concentration is the main factor affecting interspecific competition. We find that at the temperature of 25 ℃, the food concentration of 1.0×10^6 cells/ml and the initial density of 1, 3 ind./ml, *K. valga* repels *B. calyciflorus*. When the initial density increases to 5 ind./ml, the competition result is opposite, which also obtains the effect of the initial density on the interspecific competition of rotifer. Of course, this part of the experimental results also depends on temperature, food concentration, initial density and their interaction.

Conclusion

This study investigated the interspecific competition between *B. calyciflorus* and *K. valga* under pairwise combinations of temperature, food concentration, and initial density. The following conclusions have been reached:

First, in separate cultures, the population growth of both *B. calyciflorus* and *K. valga* was affected by temperature and food concentration. The population growth rates and maximum population densities of both rotifer species were higher at 30 °C than at 20 °C and 25 °C. The population growth rate and maximum population density of *B. calyciflorus* increased overall with increasing food concentration, whereas the opposite was found for *K. valga*. The initial density had no obvious effect on *B. calyciflorus*. The population growth rate of *K. valga* decreased with increasing initial density, leading to a slight increase in maximum population density.

Second, in mixed cultures, *B. calyciflorus* was significantly inhibited by competition with *K. valga,* leading to an overall decrease in the population growth rate and

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maximum population density of *B. calyciflorus*. Similarly, *K. valga* was also affected by *competition* with *B. calyciflorus*. However, the population growth rate and maximum population density of *K. valga* increased under some of the experimental conditions (at a temperature of 20 °C or an initial density of 1.0×10^6 cells/mL) and decreased under others.

Third, the outcome of the interspecific competition between *B. calyciflorus* and *K. valga* was mainly affected by food concentration. *B. calyciflorus* was excluded by *K. valga* at low and medium food concentrations (0.5×10^6 and 1.0×10^6 cells/mL), whereas the opposite was found at high food concentration (3.0×10^6 cells/mL). Moreover, the combination of 20 ℃, a food concentration of 3.0×10^6 cells/mL, and an initial density of 3 ind./mL and that of 25 ℃, 1.0×10^6 cells/mL, and 5 ind./mL yielded opposite outcomes of competition. Temperature, initial density, and interactions between the three factors also had non-negligible effects on the outcome of the competition between *B. calyciflorus* and *K. valga*.

Finally, although we have made some achievements in the research on the interspecific competition and mechanism of the two rotifers, there are still many areas that need to be improved, including increasing the types of competitive species, which is closer to the actual situation of interspecific competition of rotifers in natural water. It is necessary to increase the types of influencing factors, because the predation pressure in natural water and the stress of environmental pollutants also have a great impact on the interspecific competition of rotifers. By studying the interspecific competition of rotifers and analyzing the influencing factors, we can build a competition model to simulate, predict and analyze the interspecific competition outcome under various competition conditions.

Acknowledgements. This work was supported by Guangdong Basic and Applied Basic Research Foundation (No. 2018A030313848), National Key R&D Program of China on "Blue Granary Technology Innovation" (No. 2018YFD0900802), and Central Public-interest Scientific Institution Basal Research Fund of Chinese Academy of Fishery Sciences (No. 2021SJ-TD1).

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_41514168

THE EFFECTS OF SEEDING RATE AND ROW SPACING ON THE PHOTOSYNTHETIC ACTIVITY OF SOYBEAN (*GLYCINE MAX* (L.) MERR.)

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(Received 2nd Jun 2021; accepted 3rd Sep 2021)

Abstract. With an advent of early-maturing soybean varieties that have weaker ability to branch, there is a need to study the increase in seeding rates with different combinations of row spacing, in order to determine the effects of narrowing the area of plant nutrition on photosynthetic plant activity. This is the purpose of our research. The multifactorial experiment was performed by splitting sites in four replications. The research has demonstrated that the Baika variety sown with a row spacing of 15 cm and a seeding rate of 1.2 million pcs./ha - 40.5 ths. m^2/ha was close to the optimal leaf surface. The Annushka soybean plants were inferior to variety Baika in this regard, with the difference up to 6.0 ths. m^2/ha, due to differences in the leaf structure of the soybean varieties. The weather conditions have been found to play the dominant role; namely, a strong direct correlation was found between the amount of precipitation and the photosynthetic productivity of plants. For the Annushka variety, it was in the range of r = 0.714… 0.843, and for the Baika variety, r = 0.899… 0.947. Thus, using different combinations of seed sowing rate and row spacing, it is possible to adjust the photosynthetic productivity of soybean plants and, as a result, to provide better conditions for their development and higher yields.
Keywords: *soy bean (Glycine max (L.) Merr.), leaf areas, seeding rate, sowing method, correlation*

Introduction

The area of soybean (*Glycine max* (L.) Merr.) crops in Ukraine has already reached 1.84 million hectares, the top position in Europe, and the soybean production amounts to 3,688.3 thousand tons ranking the country the eighth producer worldwide (Drobitko, 2007; State Statistics Service of Ukraine, 2019). This progress is due to a number of factors including the development of new varieties, the improvement of cultivation technologies and the growing demand for crops in the market. However, the yield of 2.4 t/ha remains low and far from the highest observed for this crop, which is 11.5 t/ha (Yilmaz, 2003; Ogurtsov et al., 2016; Rozhkov et al., 2021).

Soybeans, similarly to most crops, provide a yield of 2–6 t/ha using only 0.5–1.5% of photosynthetically active radiation (PAR) (Nichiporovich et al., 1969; Purcell et al., 2002; Amelin et al., 2011). Meanwhile, by selecting the optimal sowing rates and sowing methods, it is possible to increase this value up to 4–5%, which will bring the yield to the highest possible of 10–15 t/ha (Amelin et al., 2011; Fontana et al., 2012).

Photosynthesis is the main factor in the formation of 90–95% of dry matter (Nichiporovich et al., 1969; Babych and Tkachuk, 2003; Baranov and Ugo Toro Korrea, 2006; Drobitko, 2007; Borovoj and Belik, 2009; Chuprina et al., 2021a). Up to 45% of

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_41694184

dry matter is composed of carbon, which is assimilated by the plant due to solar energy (Nichiporovich et al., 1969). The efficiency of photosynthesis under experimental conditions could reach 25%, and in the field it is 2.5% or lower (Sims et al., 1998). This is accounted for by a number of reasons, the majority of these being the insufficient supply of mineral nutrition and water for plants, inadequate selection of the optimal plant density, and the premature loss of leaf surface (Silva et al., 2013; Ribeiro et al., 2017). Therefore, the formation of high and sustainable productivity of soybean crops largely depends on the intensity of this process (Babych et al., 2003).

All legumes, including soybeans, are C_3–plants (Hikosaka and Terashima, 1995; Ogurtsov et al., 2016; Golovan et al., 2019). Plants of this type are characterized by slow leaf formation and low-rate growth for almost thirty days after seedling emergence. There is also increased abortion of flowers and beans, poor resistance to shading, the need for high concentrations of carbon dioxide along with optimal insolation and temperature, increased respiratory activity and, as a result, unproductive dry matter consumption (Leshhenko, 1962; Miyazawa et al., 1998; Chuprina et al., 2021b).

Scholars have found that the optimal conditions for photosynthesis were achieved when the leaf surface increased rapidly at the beginning of the vegetation period to its maximum size and was maintained for a prolonged period of time. Soybean plants have the maximum size of their leaves over the period of flowering and bean formation (BBCH 67–77) (Abaevet et al., 2009; Ribeiro et al., 2017). With the higher stocking density of soybean plants the leaf index of sowing is increasing, while the leaf area of one plant is decreasing (Babych et al., 2003; Procópio et al., 2014; Balbinot et al., 2015). However, the latter decreases at a slower rate than the increase in plant density (Gureeva and Xramoj, 2009).

Leaves also function as an organ of transpiration. The leaf surface area of 40.000 to 50.000 m^2/ha, in order to absorb up to 90% of light energy, should have easily accessible moisture of at least 20–50 m^3/ha per day. Therefore, increasing the area over 60.000 to 70.000 m^2/ha or higher is a negative factor that affects getting high crop yields considering the impeded light access to the crops and, accordingly, the reduced photosynthesis productivity (Nichiporovich et al., 1969; Sauer et al., 2007; Chuprina et al., 2020). The activity of the leaves at different tiers and over different periods of growth and development varies. At the initial stages of soybean plants growth their leaves direct the accumulated dry matter primarily to the roots and the growing parts of the stem (Leshhenko, 1962; Kokubun, 1988). New leaves receive products of photosynthesis from the old ones while the former have a small working surface. With the advent of fruit, the movement of dry matter is redirected to the beans where it is distributed between the leaves and seeds. The upper leaves remain underdeveloped; all the growth processes are slowed down, and then stop completely (Ogurtsov, 2008). During this period, the dry matter is not transported from one leaf to another in the lower, shaded tier; the leaves are starving, and this fact accounts for their premature falling off in the lower tier (Leshhenko, 1962; Ono et al., 2001). The typical feature of soybean plants is that the leaves provide nutrition only for those beans that are located in their axils (Kizilova, 1974; Calmeset et al., 1988). This is the reason of the bean loss after the leaf loss in the corresponding node, which is easily noticed in thickened crops (Leshhenko, 1962). However, the photosynthetic ability of the leaves located in different parts of the plant varies; thus, the nutrition of beans in the upper and lower tiers differs (Kizilova, 1974), and the quality of the seeds formed in the fruits located in different places varies considerably (Ogurtsov et al., 2016).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_41694184

The photosynthetic potential (PP) of soybeans depends significantly on the location of the leaves on the plant, the length of the day, the intensity of solar insolation, temperature and humidity, water supply, nutrients, etc. (Ogurtsov et al., 2016). The most active PAR absorption by leaves of plants occurs in the morning and afternoon hours. At noon, a pronounced decline in photosynthesis activity, at the average of 40%, is observed (Cunha and Volpe, 2010; Amelinet et al., 2011). The size and dynamics of PP throughout the growing season are dependent not only on varietal characteristics and the phase of plant development, but also on the soybean technology features (Zherebko et al., 2003; Ivebor, 2006). The highest values of PP were observed in years of high precipitation, and the lowest in those with dry seasons (Medvedeva and Babarykina, 2011).

Net productivity of photosynthesis (NPP) varies depending on the cultivating conditions; the dynamics during the growing season in soybean plants is sinusoidal (Dziubailo and Myhal, 2011; Myhal, 2011). It has the form of a two-vertex curve with peaks in the phases of branching and fruiting (BBCH 21–29 and BBCH 70–79) (Babych et al., 2003). Thus, soybeans have the highest indicators of NPF during the period from branching to the beginning of flowering (from BBCH 21–29 to BBCH 60–64) (Babych and Tkachuk, 2003).

In sparse crops, where conditions for the photosynthetic operation was better, the net productivity of photosynthesis was higher. Therefore, the maximum grain yield from each plant was observed. However, the actual yield of soybean grain per unit area at such a plant density was insignificant. On the other hand, in thickened crops, where the NPP was lower, the yield of soybeans from one plant decreased significantly (Babych et al., 2003).

Increased leaf surface area and photosynthetic potential results in a decrease in the productivity of photosynthesis (Babych and Tkachuk, 2003). The correlation between these indicators is negative ($r = –0.81$, $d = 0.66$) (Babych et al., 2003). This depends on the activity of the leaves of different tiers, and the uneven distribution of light to them. In the leaves of the lower tiers, due to shading, the intensity of photosynthesis is considerably reduced, which affects the supply of beans located there with the necessary substances. Therefore, they are defective or fall off. This is especially evident in thickened crops, in which the yield is reduced due to fewer beans and their lower weight (Andreyuk, 2010; Ribeiro et al., 2017).

Sowing rates and sowing methods are important or the formation of the leaf surface area of crops and the efficiency of their use. Ensuring their more uniform distribution and optimizing the feeding area of each plant enables to achieve maximum efficiency of their functioning and the assimilation of a larger share of photosynthetically active radiation (Mizerna and Nosulia, 2016; Ogurtsov et al., 2016).

Therefore, the research on the response of new modern soybean varieties to different plant density rates becomes apparent. The aim of this study is to evaluate the impact of the three sowing methods and five seeding rates on the photosynthetic productivity of plants in soybean varieties of different maturity groups.

Materials and methods

The research was conducted over the period of 2015–2018 in the field of the grain steam-row crop rotation of the Department of Plant Breeding of Kharkiv Dokuchaev NAU, Ukraine (latitude 49.893815°, longitude 36.449448°). The soil of the

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_41694184

experimental field is chernozem, a typical deep heavy loam on forest carbonate. The content of humus in the arable layer is 4.4–4.7%, mobile phosphorus - 13.8 mg, potassium - 10.3 mg/100 g of soil (Tikhonenko and Degtyarev, 2016).

The format of the three-factor field experiment applied was as follows. Factor A: soybean varieties (two variants) – Annushka (very early-ripening variety 0000) and - Baika (early-ripening variety 000); factor B: sowing methods (three options): 1 –a row having a 15 cm spacing; 2 and 3 - wide-row with a row spacing of 45 and 70 cm; factor C: seed sowing rate (five options): 0.8, 0.9, 1.0, 1.1 and 1.2 million pcs./ha. The experiment was based on the method of split sites, in four replications. Areas of the first order are varieties; the second order includes sowing methods; the third order - seeding rates (Rozhkov et al., 2016). The phases of development were determined visually according to the international classification BBCH (Biologische Bundesanstalt für Land- und Forstwirtschaft, Bundessortenamt und CHemische Industrie, in German) (Meier et al., 2011).

The evaluation of photosynthetic activity was performed based on the following indicators: the leaf surface area, photosynthetic potential, and the net productivity of photosynthesis. The area of the leaves was determined by the method of "cutting off": the leaves of the sample were weighed with the accuracy to the second decimal value; a spanner of a preset diameter was used to make cuts. With the mass and area of cuttings and the total weight of the leaf determined, the area of the leaf was calculated using *Equation 1:*

$$s = \frac{M * S_1 * k}{m}, \quad \text{(Eq.1)}$$

where: sis leaf area, cm^2; M - the total weight of the leaf, g; S_1 – the area of one cut-off, cm^2; k - the number of cuts, pcs; m - mass of cuts, g (Nichiporovich et al., 1969; Rozhkov et al., 2016).

The photosynthetic potential was determined by *Equation 2:*

$$PP = \frac{(S_1 + S_2) * T}{2 * 1000}, \quad \text{(Eq.2)}$$

where: PP – photosynthetic potential, million m^2/ha per day; S_1, S_2 - leaf area at the beginning and end of a certain period (development phase), ths. m^2/ha; T - duration of the period, days (Nichiporovich et al., 1969; Rozhkov et al., 2016).

The net productivity of photosynthesis was determined using *Equation 3:*

$$NPP = \frac{(M_2 - M_1) * T}{0.5 * (S_1 + S_n) * T}, \quad \text{(Eq.3)}$$

where: NPP - net productivity of photosynthesis, g/m^2 per day; M_1, M_2 - mass of plants per pcs area at the beginning and end of a certain period (development phase), g; S_1, S_2 - leaf area in the same periods (development phases) definition, cm^2; T - the duration of the period, days (Nichiporovich et al., 1969; Rozhkov et al., 2016).

The variability of photosynthetic productivity of soybean plants and individual phases of development was determined by the indicators of the arithmetic mean, standard deviation ($S_{\bar{x}}$, %), coefficient of variation (V, %), and confidence interval of averages. Statistical data processing (correlation of average air temperature and

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_41694184

precipitation with photosynthetic productivity of plants) was performed using correlation and regression analyses. Evaluation of the reliability of the obtained data in comparison with the average was checked using Student's test (t-criterion) using Excel (X16-45328-01) (Ermantraut et al., 2007).

Soil preparation and cultivation were typical for the region (Tishchenko et al., 2015). Maximum weed control, moisture accumulation and favorable conditions for the growth and development of soybean plants were envisaged. The predecessor of soybeans was spring wheat. After harvesting the predecessor, disking was carried out, then plowing to a depth of 25–27 cm. The sowing was carried out with a selection seeder SSFK–7, followed by rolling with ring-spur rollers. Two or three manual loosening operations between rows were performed during the cultivation season before closing the rows of plants. Soybeans were harvested in the phase of full ripeness of beans with a grain moisture content of 16–18% using Sampo harvester.

Results and discussion

Weather conditions during the soybean growing season over the years of research had certain features. Taking into account the average long-term observations, soybean sowing began in the first decade of May, and harvesting was in the second decade of September. Considering this, the focus of observing the hydrothermal conditions was on the period from May to September, which determined the features of the formation of soybean crop productivity.

The growing season of soybeans in 2015 was characterized by dry conditions. There were nine abnormally warm decades, the warmest were the first and the third decades of July, with a deviation of 3.2 and 2.7 °C. The second decade of July was abnormally cool, with a deviation from the norm of 2.7 °C (the night temperature dropped to 11.1 °C). The amount of precipitation was 215.9 mm, which is 74.1 mm less than the long-term norm. The average daily air temperature over the period was 19.6 °C (2.5 °C higher than normal). The total of the temperature values 10 °C during the vegetation season was 3082 °C, or 377 °C higher than normal.

The optimal conditions of the growing season developed in 2016. The amount of precipitation was 344.4 mm, which exceeded the normal by 54.4 mm. The average daily air temperature during the growing season was 19.6 °C; the sum of active temperatures was 3207.8 °C, which was 502.8 °C higher than the average long-term.

The growing season of soybeans in 2017 was characterized by dry conditions with eight abnormally warm decades, with the first and the second decades of April having been the warmest (deviations from the norm of 6.8 and 7.2 °C). The second decade of May was abnormally cool (deviation from the norm of 4.7 °C). The amount of precipitation during the growing season was 163.9 mm, which is 149.1 mm (52.4%) less than the long-term norm. The air temperature during the growing season was 18.2 °C (2.1 °C more than normal) the total of active temperatures was 3176 °C, or 471 °C higher than the average long-term (*Table 1*).

The growing season of soybeans in 2018 was less favorable; it was characterized by dry conditions with nineteen abnormally warm decades; the warmest were the first decades of May and September (deviations from the norm of 9.3 and 6.9 °C). The amount of precipitation during the growing season was 107.8 mm, which is 35.9% of the normal value. Rains were few in the first decades of April and June and in the third decade of July, only 14.0%. The air temperature during the growing season was

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_41694184

20.5 °C; the total of active temperatures (values above 10 °C) during the growing season was 3291.5 °C, which was 586.5 °C higher than normal.

Table 1. *Meteorological data for the soybean growing season in the years of research according to the meteorological station of Kharkiv Dokuchaev NAU (latitude 49.893815°, longitude 36.449448°)*

Months	Decades	Average air temperature, °C					The amount of precipitation, mm				
		2015	2016	2017	2018	For 50 years	2015	2016	2017	2018	For 50 years
V	1	14.1	19.5	16.7	23.2	13.9	31.7	65.6	1.8	0.0	15.0
	2	16.4	16.0	11.1	16.7	15.8	7.8	19.9	24.9	15.9	13.0
	3	21.2	19.5	18.3	19.9	16.4	7.0	5.9	8.9	0.0	21.0
		17.2	*18.3*	*15.4*	*19.9*	*15.4*	*46.5*	*91.4*	*35.6*	*15.9*	*49.0*
VI	1	22.2	17.2	19.2	17.9	18.7	13.6	0.5	1.2	2.2	15.0
	2	22.8	21.2	19.2	22.9	18.9	16.4	35.3	7.9	6.7	22.0
	3	21.7	25.6	22.9	24.1	19.9	74.5	7.5	9.5	34.6	22.0
		22.2	*21.3*	*20.4*	*21.6*	*19.2*	*104.5*	*43.3*	*18.6*	*43.5*	*59.0*
VII	1	23.4	22.3	19.3	22.0	20.2	0.3	4.7	1.1	6.7	17.0
	2	18.2	25.8	21.5	22.1	20.9	23.6	94.8	15.1	18.5	29.0
	3	23.2	21.9	24.3	25.0	20.5	18.7	6.9	15.4	3.5	25.0
		21.6	*23.3*	*21.7*	*23.0*	*20.5*	*42.6*	*106.4*	*31.6*	*28.7*	*71.0*
VIII	1	24.2	22.8	27.3	24.0	20.5	0.0	14.5	0.0	0.0	16.0
	2	21.0	20.9	27.3	25.1	20.1	0.0	36.1	0.0	0.0	21.0
	3	21.5	24.7	19.4	24.7	18.3	0.0	0.0	11.4	0.0	19.0
		22.2	*22.8*	*24.7*	*24.6*	*19.6*	*0.0*	*50.6*	*11.4*	*0.0*	*56.0*
IX	1	21.8	19.4	17.8	23.2	16.3	3.5	0.0	25.1	6.8	17.0
	2	17.2	14.7	21.3	20.1	13.7	3.3	1.1	0.6	3.9	13.0
		19.5	*17.1*	*19.6*	*21.7*	*15.0*	*6.8*	*1.1*	*25.7*	*10.7*	*30.0*

Over the period of research, the leaf areas have been found to vary, on the average, in the phase of BBCH 13. The value of this feature ranged from 5.6 ths. m^2/ha in plants of Annushka variety, for 70 cm row spacing sowing and a seeding rate of 0.8 million pcs./ha, to 10.3 ths. m^2/ha with the Baika variety with row sowing method and sowing rates of 1.2 million pcs./ha. In terms of the years, the smallest area of 5.1 ths. m^2/ha was formed under less favorable, arid conditions in 2018 (in plants of the Annushka variety) while the largest was under favorable conditions in 2016 amounting to11.5 ths. m^2/ha (in the Baika variety with a row spacing of 15 cm and seeding rates of 1.2 million pcs./ha) (*Table 2*).

During the growth and development of soybean plants, the leaf area increased, on the average over four years, reaching the maximum in the BBCH 77 phase. In this phase, the largest surface leaf area was actually 35.0 ths. m^2/ha, observed in soybean plants Baika with a row sowing method and the maximum seeding rate in the experiment (1.2 million pcs./ha); the lowest was 26.5 ths. m^2/ha in areas of Annushka soybean variety with a row spacing of 70 cm and the minimum seeding rate in the experiment (0.8 million pcs/ha). As for the years, the smallest area was formed in 2018 – 23.8 ths. m^2/ha (in plants of the Annushka variety for row spacing of 70 sowing and

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_41694184

seeding rate of 0.8 million pcs./ha) while the largest was observed in 2016, 40.5 ths. m^2/ha (in the Baika variety with 15 cm row spacing and seeding rates of 1.2 million pcs./ha).

Table 2. *Dynamics of growth of the leaf surface area of soybean plants depending on the research factors, ths m^2/ha (mean ± sx for 2015–2018)*

Variety (factor A)	Row spacing, cm (factor B)	Sowing rate, million pcs./ha (factor C)	Phases of development (classification BBCH)				
			BBCH 13	BBCH 61	BBCH 67	BBCH 77	BBCH 92
Baika	15	0.8	7.3 ± 1.09^{ns}	16.2 ± 1.75^{ns}	28.7 ± 3.26^{ns}	32.2 ± 3.71^{ns}	28.9 ± 3.49^{ns}
		0.9	8.2 ± 1.22^{ns}	17.0 ± 1.74^{ns}	29.5 ± 3.11^{ns}	32.9 ± 3.70^{ns}	29.7 ± 3.67^{ns}
		1.0	8.8 ± 1.19^{ns}	17.6 ± 1.64*	30.3 ± 2.90*	33.7 ± 3.31^{ns}	30.4 ± 3.24^{ns}
		1.1	9.5 ± 1.30*	18.3 ± 1.14*	31.1 ± 2.14*	34.2 ± 2.53*	31.0 ± 2.57^{ns}
		1.2	10.3 ± 1.32*	19.1 ± 1.13*	31.9 ± 1.99*	35.0 ± 2.49*	31.7 ± 2.46*
	45	0.8	6.9 ± 0.77*	15.5 ± 1.44^{ns}	27.8 ± 2.72^{ns}	31.4 ± 3.18^{ns}	28.0 ± 2.97^{ns}
		0.9	8.1 ± 0.97^{ns}	16.2 ± 1.30^{ns}	28.5 ± 2.39^{ns}	32.1 ± 2.94^{ns}	28.7 ± 2.85^{ns}
		1.0	8.6 ± 1.04^{ns}	17.0 ± 1.13*	29.2 ± 1.92^{ns}	32.7 ± 2.29^{ns}	29.5 ± 2.19^{ns}
		1.1	9.3 ± 0.91*	17.6 ± 0.99*	30.1 ± 1.84*	33.4 ± 2.17^{ns}	30.1 ± 2.18^{ns}
		1.2	10.1 ± 0.93*	18.5 ± 1.14*	31.0 ± 1.94*	34.0 ± 2.48*	30.9 ± 2.70^{ns}
	70	0.8	6.5 ± 0.73*	15.1 ± 1.42^{ns}	27.1 ± 2.67^{ns}	30.6 ± 3.06^{ns}	27.5 ± 2.99^{ns}
		0.9	7.2 ± 0.69*	15.8 ± 0.93^{ns}	27.8 ± 1.57^{ns}	31.3 ± 2.01^{ns}	28.1 ± 2.25^{ns}
		1.0	8.0 ± 0.66^{ns}	16.2 ± 0.91^{ns}	28.6 ± 1.55^{ns}	31.8 ± 2.08^{ns}	28.9 ± 1.96^{ns}
		1.1	8.7 ± 0.83^{ns}	16.8 ± 0.92*	29.4 ± 1.52*	32.7 ± 1.93^{ns}	29.6 ± 1.91^{ns}
		1.2	9.3 ± 0.89*	17.6 ± 0.92*	30.2 ± 1.34*	33,3 ± 1.77^{ns}	30.4 ± 1.99^{ns}
Annushka	15	0.8	6.7 ± 0.69*	14.2 ± 1.15*	24.9 ± 1.72*	28.9 ± 2.24^{ns}	26.5 ± 2.45^{ns}
		0.9	7.7 ± 0.89^{ns}	14.8 ± 1.19^{ns}	25.6 ± 1.66^{ns}	29.5 ± 2.27^{ns}	27.0 ± 2.51^{ns}
		1.0	8.5 ± 1.18^{ns}	15.5 ± 1.20^{ns}	26.4 ± 1.81^{ns}	30.4 ± 2.33^{ns}	27.8 ± 2.64^{ns}
		1.1	9.0 ± 1.14^{ns}	16.4 ± 0.99^{ns}	27.7 ± 1.66^{ns}	31.6 ± 2.37^{ns}	28.7 ± 2.79^{ns}
		1.2	9.9 ± 1.25*	17.3 ± 1.29*	28.7 ± 2.14^{ns}	32.6 ± 2.88^{ns}	29.5 ± 2.94^{ns}
	45	0.8	5.9 ± 0.42*	12.7 ± 0.60*	23.2 ± 1.28*	27.5 ± 1.95*	25.0 ± 1.98*
		0.9	6.8 ± 0.54*	13.3 ± 0.69*	23.8 ± 1.44*	28.2 ± 2.14*	25.6 ± 2.15*
		1.0	7.6 ± 0.71^{ns}	13.9 ± 0.77*	24.5 ± 1.45*	28.9 ± 2.21^{ns}	26.2 ± 2.29^{ns}
		1.1	8.5 ± 0.71^{ns}	14.8 ± 0.87^{ns}	25.7 ± 1.33*	29.9 ± 2.03^{ns}	27.2 ± 2.24^{ns}
		1.2	9.3 ± 0.78*	15.6 ± 1.08^{ns}	26.7 ± 1.42^{ns}	30.7 ± 2.04^{ns}	28.2 ± 2.21^{ns}
	70	0.8	5.6 ± 0.49*	11.9 ± 0.39*	22.1 ± 0.90*	26.5 ± 1.55*	24.1 ± 1.67*
		0.9	6.4 ± 0.46*	12.6 ± 0.38*	22.7 ± 0.78*	27.0 ± 1.47*	24.6 ± 1.59*
		1.0	7.1 ± 0.53*	13.1 ± 0.52*	23.4 ± 0.92*	27.8 ± 1.59*	25.3 ± 1.65*
		1.1	8.0 ± 0.72^{ns}	14.1 ± 0.48*	24.2 ± 0.94*	28.5 ± 1.42*	26.1 ± 1.57*
		1.2	8.7 ± 0.81^{ns}	14.9 ± 0.73*	25.3 ± 1.20*	29.3 ± 1.64^{ns}	26.8 ± 1.85^{ns}
Average			7.9	15.2	26.5	30.4	27.5
V, %			12.8	7.5	7.9	9.1	10.4
S_x, %			4.5	2.7	2.8	3.2	3.7

± – confidence interval; * – $t_{fact.} \geq t_{theor.}$ (essential for the level 0.05); ns – $t_{fact.} < t_{theor.}$ (insignificant for the level 0.05)

During the BBCH 92 phase, soybean plants began to lose their lower tier leaves and fall off. This has reduced the leaf surface. In this phase, the smallest leaf surface area - 24.1 ths m^2/ha - was in soybean plants of the variety Annushka with a row spacing of 70 cm and a seed sowing rate of 0.8 million pcs./ha. The largest was - 31.7 ths. m^2/ha - in the variety Baika with a row method of sowing and a seeding rate of 1.2 million pcs./ha. By years, the smallest area was formed in 2018 - 21.4 ths. m^2/ha (in varieties Annushka for sowing with a row spacing of 70 cm and a seeding rate of 0.8 million pcs./ha), and

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_41694184

the largest was in 2016 - 37.5 ths. m^2/ha (in the variety Baika with a row spacing of 15 cm and seeding rates of 1.2 million pcs./ha).

The formation of the leaf surface area is a prerequisite for obtaining maximum crop yields. Both in our observations and according to the results of many studies in the Forest Steppe of Ukraine, it was proved that the optimal leaf surface area for soybeans was 40–50 ths. m^2/ha (Babych et al., 2003; Dzhemesiuk et al., 2015; Nichiporovich et al., 1969; Rahman et al., 2011), and in the Polissya zone, depending on sowing dates and sowing rates, fluctuated within 44–60 ths. m^2/ha (Didora et al., 2013).

On the average over the period of four years of research, the variety-related difference has been observed. It included the following: in the BBCH 13 phase, a super early (0000) Annushka variety formed a leaf area by 0.7 ths. m^2/ha smaller than the early (000) Baika variety. The maximum difference between the varieties in leaf surface area, 4.4 ths m^2/ha, was observed in the period BBCH 61 - BBCH 67, which was confirmed by studies of other researchers (Mikheev, 2014; Ogurtsov, 2008). Later, the difference gradually reduced, namely, in the phase of BBCH 77 it was 3.6 ths. m^2/ha, and in the phase of BBCH – 92 - 3.0 ths. m^2/ha.

In row crops, the leaf surface area of soybean plants was larger than in broad-row crops, as shown also in other studies (Cox and Cherney, 2011; Mikheev, 2012; Shepilova and Petrenko, 2017). In the BBCH 13 phase, the difference was 0.5 and 1.0 ths. m^2/ha at row spacing of 45 and 70 cm, respectively. During the period BBCH 61 – BBCH 67 the difference increased to 1.4 and 2.4 ths. m^2/ha, as per the sowing methods. In the phase of BBCH 77, at the maximum leaf area, the difference was 1.2 and 2.2 ths. m^2/ha.

Increasing the seeding rate from 0.8 to 1.2 million pcs/ha contributed to an increase in leaf area. In the BBCH 13 phase, the difference amounted to 3.1 ths. m^2/ha, in the period BBCH 61 – BBCH 67, the difference increased to 3.4 ths. m^2/ha. Over the time, the difference gradually decreased, and in the phase of BBCH 92 seeds it was - 2.9 ths. m^2/ha, depending upon the seeding rates. The research results are consistent with those described by other authors (Ogurtsov, 2008). However, certain studies demonstrate that crop thickening caused no changes in leaf area (Kazachenko, 2010; Raniele et al., 2016), and in sometimes even resulted in its reduction (Shepilova and Petrenko, 2017).

Crop productivity was determined by photosynthetic potential (PP). It characterizes the dynamic changes in leaf area over the vegetation period and demonstrates the typical features of plant growth and development including the formation of the soybean leaf surface depending on the conditions that influence its progress (Baranov and Ugo Toro Korrea, 2006).

Our observation shows that, on the average over the years of research, the PP index values varied in the interphase period of BBCH 13–61. The values ranged from 0.045 million m^2/ha per day in plants of Annushka variety with 70 cm row spacing sowing and seeding rate of 0.8 million pcs./ha to 0.149 million m^2/ha per day in soybean plants of Baika variety, row sowing method, and sowing rates 1.2 million pcs./ha (*Table 3*). Across years, the lowest PP formed under less favorable, dry conditions in 2017, with 0.040 million m^2/ha per day (in plants of Annushka variety, 70 cm row spacing, seeding rate 0.8 million pcs./ha while the highest value, in more favorable conditions of 2016 with more precipitation, it was 0.195 million m^2/ha per day (in the Baika variety, 15 cm row spacing, seeding rates 1.0 million pcs./ha).

In the process of growth and development of soybean plants, PP increased, reaching the maximum values in the interphase period BBCH 77–92. The largest PP,

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_41694184

2.458 million m^2/ha per day, was observed in areas of Baika variety, row sowing method, seeding rate 0.9 million pcs./ha; the lowest was 1.510 million m^2/ha per day in areas of Annushka variety, 70 cm row spacing, seeding rate 0.8 million pcs./ha. In the observed years, the smallest area was formed in 2018, namely, 1.249 million m^2/ha per day (Annushka variety, row spacing 70 cm, seeding rate 0.9 million pcs./ha) while the highest, in 2016, was 3.239 million m^2/ha per day (Baika variety, 15 cm row spacing, seeding rates 0.9 million pcs./ha).

Table 3. *Dynamics of soybean plants photosynthetic potential (PP) growth depending on the factors studied, million m^2/ha per day (mean ± sx for 2015–2018)*

Variety (factor A)	Row spacing, cm (factor B)	Sowing rate, million pcs./ha (factor C)	Interphase periods of growth and development of soybean plants			
			BBCH 13–61	BBCH 61–67	BBCH 67–77	BBCH 77–92
Baika	15	0.8	0.142 ± 0.065^{ns}	1.102 ± 0.36^{ns}	2.030 ± 0.312^{ns}	2.414 ± 0.356*
		0.9	0.142 ± 0.059^{ns}	1.114 ± 0.32^{ns}	2.064 ± 0.302*	2.458 ± 0.346*
		1.0	0.146 ± 0.064^{ns}	1.134 ± 0.33^{ns}	2.072 ± 0.287*	2.439 ± 0.326*
		1.1	0.139 ± 0.050^{ns}	1.108 ± 0.25^{ns}	2.033 ± 0.236*	2.400 ± 0.274*
		1.2	0.149 ± 0.052*	1.155 ± 0.25*	2.080 ± 0.230*	2.430 ± 0.258*
	45	0.8	0.124 ± 0.054^{ns}	1.048 ± 0.30^{ns}	1.944 ± 0.277^{ns}	2.315 ± 0.316^{ns}
		0.9	0.134 ± 0.056^{ns}	1.058 ± 0.28^{ns}	1.943 ± 0.256^{ns}	2.320 ± 0.293^{ns}
		1.0	0.127 ± 0.053^{ns}	1.063 ± 0.25^{ns}	1.941 ± 0.229^{ns}	2.289 ± 0.263^{ns}
		1.1	0.132 ± 0.053^{ns}	1.068 ± 0.24^{ns}	1.942 ± 0.221^{ns}	2.297 ± 0.254^{ns}
		1.2	0.130 ± 0.048^{ns}	1.111 ± 0.25^{ns}	2.014 ± 0.236*	2.356 ± 0.261*
	70	0.8	0.120 ± 0.053^{ns}	1.006 ± 0.30^{ns}	1.856 ± 0.271^{ns}	2.221 ± 0.310^{ns}
		0.9	0.124 ± 0.047^{ns}	1.008 ± 0.22^{ns}	1.844 ± 0.208^{ns}	2.183 ± 0.236^{ns}
		1.0	0.121 ± 0.041^{ns}	1.001 ± 0.21^{ns}	1.823 ± 0.204^{ns}	2.162 ± 0.237^{ns}
		1.1	0.125 ± 0.050^{ns}	1.030 ± 0.22^{ns}	1.870 ± 0.208^{ns}	2.193 ± 0.234^{ns}
		1.2	0.121 ± 0.043^{ns}	1.033 ± 0.20^{ns}	1.861 ± 0.201^{ns}	2.190 ± 0.225^{ns}
Annushka	15	0.8	0.063 ± 0.017*	0.765 ± 0.15^{ns}	1.501 ± 0.137^{ns}	1.760 ± 0.196^{ns}
		0.9	0.060 ± 0.018*	0.765 ± 0.13*	1.492 ± 0.123^{ns}	1.727 ± 0.182^{ns}
		1.0	0.061 ± 0.019*	0.785 ± 0.16^{ns}	1.527 ± 0.142^{ns}	1.769 ± 0.204^{ns}
		1.1	0.064 ± 0.018*	0.811 ± 0.14^{ns}	1.571 ± 0.136^{ns}	1.819 ± 0.196^{ns}
		1.2	0.059 ± 0.023*	0.821 ± 0.17^{ns}	1.590 ± 0.147^{ns}	1.825 ± 0.208^{ns}
	45	0.8	0.056 ± 0.014*	0.688 ± 0.10*	1.384 ± 0.106*	1.604 ± 0.162*
		0.9	0.053 ± 0.019*	0.704 ± 0.11*	1.395 ± 0.105*	1.620 ± 0.165*
		1.0	0.057 ± 0.018*	0.724 ± 0.12*	1.424 ± 0.117*	1.654 ± 0.177*
		1.1	0.050 ± 0.021*	0.733 ± 0.11*	1.434 ± 0.104*	1.648 ± 0.158*
		1.2	0.054 ± 0.021*	0.760 ± 0.12*	1.482 ± 0.111*	1.694 ± 0.168*
	70	0.8	0.045 ± 0.015*	0.645 ± 0.08*	1.297 ± 0.086*	1.510 ± 0.140*
		0.9	0.048 ± 0.014*	0.660 ± 0.07*	1.314 ± 0.088*	1.527 ± 0.139*
		1.0	0.051 ± 0.015*	0.667 ± 0.09*	1.319 ± 0.090*	1.518 ± 0.138*
		1.1	0.047 ± 0.019*	0.686 ± 0.08*	1.350 ± 0.083*	1.546 ± 0.134*
		1.2	0.051 ± 0.020*	0.718 ± 0.11*	1.403 ± 0.109*	1.607 ± 0.165*
Average			0.093	0.899	1.693	1.983
V, %			16.9	12.7	16.1	17.3
$S_{\bar{x}}$, %			5.3	6.4	4.7	4.9

± – confidence interval; * – $t_{fact.} \geq t_{theor.}$ (essential for the level 0.05); ns – $t_{fact.} < t_{theor.}$ (insignificant for the level 0.05)

On the average over four years of research, the variety-related difference was observed. In the interphase period BBCH 13–61, the super early (0000) variety

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_41694184

Annushka formed PP by 0.077 million m^2/ha lower than the early (000) Baika variety. Over time, the difference gradually increased reaching the maximum over the period BBCH 77–92, namely, 0.656 millionm^2/ha per day.

In row crops, soybean plant PP was higher than in wide-row crops, observed also by other authors (Mikheev, 2014; Tolmachev and Sinegovskaja, 2009). In our studies, during the interphase period of BBCH 13–61, the difference was 0.011 and 0.017 million m^2/ha per day at rows of 45 and 70 cm, respectively. Over time, the difference gradually increased reaching the maximum over the period of BBCH 77–92, with the difference of 0.124 and 0.238 million m^2/ha per day, respectively, for rows 45 cm and 70 cm.

The increase in seed sowing rate from 0.8 to 1.2 million pcs./ha caused the increase in PP. Over the period BBCH 13–61 the difference reached 0.002 million m^2/ha per day, over the period of BBCH 61–67 the difference increased up to 0.057 million m^2/ha per day, and during the period of BBCH 67–77, it amounted to 0.069 million m^2/ha per day. After that, the difference gradually decreased, and during the period of BBCH 77–92 the difference was up to 0.046 million m^2/ha per day.

The relevant factor is not only the area of leaves, but also the period of its active operation. The crops are deemed to be of sufficient productive performance provided their PP is 2 million m^2/ha per day for every 100 days of vegetation (Nichiporovich et al., 1969; Tretjakov et al., 2003), which value was actually observed in our studies.

An important feature of the plants' potential for crop formation is the net productivity of photosynthesis (NPP) (Sidorovich, 2002). It reflects the productivity of the crop per 1 m^2 of leaf area during the day. In contrast to the overall productivity of photosynthesis, NPP does not contain the organic matter consumed by plants for respiration, only that which accumulates per day. Thus, the NPP reflects the actual opportunities of agrobiocenosis for the synthesis of organic matter in a more comprehensive way than the area of the leaves. It is one of the most important parameters, and the yield level correlates with it (Caulfield and Bunce, 1988). Direct yield relationship between the maximum values of NPP and seed yield is, however, not always observed (Ogurtsov, 2008). The net productivity of photosynthesis depends on both the biological characteristics of the plant and the environmental factors including solar radiation, air temperature, soil moisture, mineral nutrition, and others (Babych et al., 2003).

In contrast to the formation of the assimilation surface of the leaves, the dynamics of soybean NPP during the growing season develops differently. From BBCH 13 to BBCH 61 it increases, acquires an absolute maximum, and in the period BBCH 61–67 decreases; during the period of BBCH 67–77 it is growing again and reaches the second maximum, although compared to the first increase in NPP, the second is significantly lower. Next, the NPP is again reduced and the formation of NPP demonstrates a sinusoidal pattern.

On the average over the years of research, in the interphase period BBCH 13–61, NPP indexes were found to vary. The value of this indicator ranged from 9.8 g/m^2 per day in Annushka varieties for 70 cm row spacing sowing and a seeding rate of 0.8 million pcs./ha to 12.4 g/m^2 per day in soybean plants of Baika variety with row method sowing, sowing rates 1.0 million pcs./ha. Over the years, the lowest NPP was formed in 2018 under less favorable, dry conditions, which was 8.2 g/m^2 per day (in plants of the Annushka variety, 45 cm row spacing, seeding rate 0.8 million pcs./ha),

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_41694184

while the highest was in more favorable, wet conditions of 2016 – 17.3 g/m^2 per day (Baika variety, 15 cm row spacing, seeding rates 1.0 million pcs./ha) (*Table 4*).

During BBCH 61–67, the NPP decreased almost 1.5-fold, although the assimilation surface area almost doubled during this period. In the interphase period BBCH 61–67, the highest NPP of 7.8 g/m^2 per day was observed in areas of the Baika variety, row sowing method, seeding rate 1.0 million pcs./ha, while the lowest was 6.1 g/m^2 per day in areas of Annushka variety, 70 cm row spacing and a seeding rate 0.8 million pcs./ha.

The highest NPP of 8.1 g/m^2 per day was observed during the interphase period BBCH 67–77 in areas of the Baika variety, row sowing method, seeding rate 1.0 million pcs./ha while the lowest was 6.7 g/m^2 per day in areas of Annushka variety, 70 cm row spacing, seeding rate 0.8 million pcs./ha.

Table 4. *The dynamics of growth of net photosynthetic performance (NPP) of soybean plants depending on the factors studied, g/m2 per day (mean ± sx for 2015–2018)*

Variety (factor A)	Row spacing, cm (factor B)	Sowing rate, million pcs./ha (factor C)	Interphase periods of growth and development of soybean plants			
			BBCH 13–61	BBCH 61–67	BBCH 67–77	BBCH 77–92
Baika	15	0.8	12.1 ± 1.10^{ns}	7.4 ± 0.63^{ns}	7.6 ± 0.34^{ns}	3.9 ± 0.39^{ns}
		0.9	12.2 ± 1.11^{ns}	7.5 ± 0.67^{ns}	7.8 ± 0.39^{ns}	4.1 ± 0.41^{ns}
		1.0	12.4 ± 1.16^{ns}	7.8 ± 0.75^{ns}	8.1 ± 0.48*	4.3 ± 0.50*
		1.1	12.3 ± 1.07^{ns}	7.7 ± 0.62^{ns}	8.0 ± 0.32*	4.2 ± 0.36*
		1.2	12.3 ± 0.98^{ns}	7.6 ± 0.56^{ns}	7.9 ± 0.28*	4.2 ± 0.31*
	45	0.8	11.6 ± 1.17^{ns}	7.2 ± 0.67^{ns}	7.5 ± 0.36^{ns}	3.8 ± 0.38^{ns}
		0.9	11.8 ± 1.18^{ns}	7.3 ± 0.67^{ns}	7.7 ± 0.39^{ns}	4.0 ± 0.41^{ns}
		1.0	11.8 ± 1.16^{ns}	7.4 ± 0.63^{ns}	7.8 ± 0.35^{ns}	4.0 ± 0.38^{ns}
		1.1	11.8 ± 1.06^{ns}	7.5 ± 0.58^{ns}	7.8 ± 0.30*	4.0 ± 0.34^{ns}
		1.2	11.8 ± 1.06^{ns}	7.4 ± 0.58^{ns}	7.7 ± 0.30^{ns}	3.9 ± 0.32^{ns}
	70	0.8	11.1 ± 1.25^{ns}	6.8 ± 0.72^{ns}	7.1 ± 0.48^{ns}	3.5 ± 0.42^{ns}
		0.9	11.3 ± 1.18^{ns}	7.0 ± 0.65^{ns}	7.4 ± 0.38^{ns}	3.7 ± 0.37^{ns}
		1.0	11.4 ± 1.13^{ns}	7.0 ± 0.63^{ns}	7.3 ± 0.37^{ns}	3.7 ± 0.34^{ns}
		1.1	11.4 ± 1.07^{ns}	7.0 ± 0.61^{ns}	7.3 ± 0.38^{ns}	3.7 ± 0.33^{ns}
		1.2	11.1 ± 1.06^{ns}	6.8 ± 0.63^{ns}	7.1 ± 0.40^{ns}	3.5 ± 0.34^{ns}
Annushka	15	0.8	10.5 ± 0.48^{ns}	6.7 ± 0.35^{ns}	7.2 ± 0.17^{ns}	3.6 ± 0.22^{ns}
		0.9	10.7 ± 0.54^{ns}	6.8 ± 0.37^{ns}	7.4 ± 0.17^{ns}	3.7 ± 0.23^{ns}
		1.0	10.8 ± 0.50^{ns}	6.9 ± 0.37^{ns}	7.5 ± 0.17^{ns}	3.8 ± 0.23^{ns}
		1.1	11.2 ± 0.48^{ns}	7.1 ± 0.38^{ns}	7.6 ± 0.17^{ns}	3.9 ± 0.23^{ns}
		1.2	11.4 ± 0.42^{ns}	7.3 ± 0.37^{ns}	7.7 ± 0.17*	4.0 ± 0.23^{ns}
	45	0.8	10.3 ± 0.57^{ns}	6.6 ± 0.30^{ns}	7.1 ± 0.17*	3.4 ± 0.19*
		0.9	10.4 ± 0.47^{ns}	6.7 ± 0.32^{ns}	7.2 ± 0.17^{ns}	3.5 ± 0.20^{ns}
		1.0	10.7 ± 0.53^{ns}	6.9 ± 0.30^{ns}	7.3 ± 0.16^{ns}	3.6 ± 0.21^{ns}
		1.1	10.9 ± 0.58^{ns}	7.0 ± 0.32^{ns}	7.5 ± 0.19^{ns}	3.7 ± 0.22^{ns}
		1.2	10.8 ± 0.41^{ns}	7.0 ± 0.24^{ns}	7.5 ± 0.11^{ns}	3.7 ± 0.13^{ns}
	70	0.8	9.8 ± 0.34*	6.1 ± 0.22*	6.7 ± 0.17*	3.2 ± 0.17*
		0.9	10.0 ± 0.46*	6.2 ± 0.20*	6.8 ± 0.17*	3.3 ± 0.16*
		1.0	10.7 ± 0.48^{ns}	6.6 ± 0.20^{ns}	7.1 ± 0.13*	3.5 ± 0.19^{ns}
		1.1	10.3 ± 0.29*	6.5 ± 0.14*	6.9 ± 0.15*	3.4 ± 0.14*
		1.2	10.3 ± 0.29*	6.4 ± 0.11*	6.8 ± 0.13*	3.3 ± 0.12*
Mean			11.2	7.0	7.4	3.7
V, %			17.1	16.7	9.1	18.2
$S_{\bar{x}}$, %			3.2	3.2	1.7	3.7

± – confidence interval; * – $t_{fact.} \geq t_{theor.}$ (essential for the level 0.05); ns – $t_{fact.} < t_{theor.}$. (insignificant for the level 0.05)

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_41694184

In the course of further growth and development of soybean plants, NPP decreased, reaching its minimum in the interphase period BBCH 77–92. In particular, the highest NPP over this period of 4.3 g/m^2 per day was observed in areas of the variety Baika, row sowing method, seeding rate 1.0 million pcs./ha. The lowest one was 3.2 g/m^2 per day in areas of Annushka variety, row spacing 70 cm, seeding rate 0.8 million pcs./ha. As to years, the lowest, 2.5 g/m^2 per day, was observed in 2018, plants of Annushka variety, 70 cm row spacing sowing, seeding rate 0.8 million pcs./ha, while the highest of 6.5 g/m^2 per day was recorded in 2016, with the variety Baika, 15 cm row spacing, seeding rates of 1.0 million pcs./ha.

On the mean over four years of research, a variety-related difference was observed. These features included the following: in the interphase period BBCH 13–61, the super early (0000) Annushka variety formed a NPP of 1.187 g/m^2 per day less compared to the early (000) Baika variety. Over time, the difference gradually decreased, reaching its minimum in the period of BBCH 77–92, when it was 0.322 g/m^2 per day.

In row crops, the NPP of soybean plants was higher than in wide-row crops. In the interphase period of BBCH 13–61, the difference was 0.403 and 0.858 g/m^2 per day at 45 and 70 cm row spacing's, respectively. Over time, the difference gradually decreased, dropping down to its minimum in the period of BBCH 77–92, with the difference of 0.218 and 0.508 g/m^2 per day, at rows of 45 and 70 cm, respectively.

Increasing the seeding rate from 0.8 to 1.2 million pcs./ha contributed to an increase in NPP. Over the period of BBCH 13–61, the difference amounted to 0.433 g/m^2 per day; over the period of BBCH 61–67, the difference increased to 0.317 g/m^2 per day, and over the period of BBCH 67–77, the increase was up to 0.317 g/m^2 per day. After that, the difference gradually decreased, and in the period of BBCH 77–92 it was at 0.279 g/m^2 per day.

The results of the correlation analysis demonstrate that the photosynthetic productivity (S, PP and NPP) of soybean plants of the Annushka variety had a strong direct correlation with the amount of precipitation r = 0.738, 0.843 and 0.714, which was in the range of 51–71% of the sample (d = 0.509–0.710), respectively. The inverse correlation of medium strength was observed with the average air temperature r = –0.557; –0.657 and –0.503, which was within 25–41% (d = 0.253–0.432) of the sample (*Table 5*).

Table 5. *The results of correlation analysis of photosynthetic activity of soybean plants (S, PP, NPP) depending on the climate parameters (mean for 2015–2018)*

Climate indicators	Variety of soybean *Annushka*			Variety of soybean *Baika*		
	r	d	Regression equation	r	d	Regression equation
Leaf surface area (S) of soybean plants, ths. m^2/ha						
Precipitation, mm	0.738	0.545	y = 18.686x – 393.27	0.932	0.869	y = 17.158x – 390.44
Average air temperature, °C	–0.557	0.310	y = –0.2023x + 28.317	–0.604	0.365	y = – 0.2497x + 28.85
Photosynthetic potential (PP) of soybean plants, million m^2/ha per day						
Precipitation, mm	0.843	0.710	y = 253.46x – 268.36	0.899	0.809	y = 154.52x – 185.99
Average air temperature, °C	–0.657	0.432	y = –2.8352x + 27.116	–0.803	0.645	y = –3.0965x + 27.833
Net photosynthesis productivity (NPP) of soybean plants, g/m^2 per day						
Precipitation, mm	0.714	0.509	y = 108.99x – 236.29	0.947	0.897	y = 71.913x – 107.65
Average air temperature, °C	–0.503	0.253	y = –1.1019x + 26.341	–0.547	0.299	y = – 0.9791x + 24.472

r – correlation coefficient; d – coefficient of determination

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_41694184

A similar pattern has been observed with the soybean plants of Baika variety; however, it had a closer correlation, namely, a very strong direct relationship between precipitation r = 0.932; 0.899 and 0.947, which was in the range of 87–95% of the sample (d = 0.869–0.948), respectively. Also, the average feedback was observed with the average air temperature r = –0.604; –0.803 and –0.547, which was in the range of 30–65% of the sample (d = 0.299–0.645).

Conclusions

As a result of research, an intensive increase in the leaf surface of soybean plants before the BBCH 77 phase was found. After that, the death of the lower tier leaves was observed, which caused a decrease in the plants leaf area. Its highest value, close to the optimal one, characterized the Baika variety with 15 cm row spacing sowing and the seeding rate of 1.2 million pcs./ha - 40.5 ths. m^2/ha. The soybean plants of the Annushka variety were inferior to the Baika variety soybean plants in terms of leaf area. The difference amounted to 6.0 ths. m^2/ha, which was due to the variety-related differences in the structure of the leaf area.

On the average over four years, the highest value of photosynthetic potential (PP) of 2.311 million m^2/ha per day was formed by soybean plants of the Baika variety, which was 28.4% more than in the Annushka variety. Wide-row sowing methods with 45 and 70 cm row spacing reduced PP by 5.9% and 11.3% compared to row sowing. The sowing rate factor had the least impact on PP; increasing the rates to 1.1 and 1.2 million pcs/ha resulted in an in significant increase in PP (0.7% and 2.3%).

The highest value of NPP, 11.75 g/m^2 per day, or 1.187 g/m^2 per day more than in the Annushka variety, was formed by soybean plants of the Baika variety. Increasing the row spacing up to 45 and 70 cm caused a decrease in NPP compared to the row sowing method (width 15 cm), the difference was 2.3–3.5% and 7.4–8.5%. Seeding rates increased NPP; the difference was 11.32 g/m^2 per day.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_41694184

COMMUNITY ENGAGEMENT IN FOREST REHABILITATION WITHIN THE CONTEXT OF A TROPICAL ISLAND: INSIGHTS FROM PRASLIN, SEYCHELLES

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(Received 5th Jun 2021; accepted 3rd Sep 2021)

Abstract. Community involvement gained momentum after the establishment of the Terrestrial Restoration Action Society Seychelles (TRASS) in 2009 to address the concerns of forest rehabilitation on the fire ravaged lands on Praslin. This study assesses issues pertaining to community participation in forest rehabilitation and proposes strategies for improvement. Three shared-dialogue workshops and 180 household surveys conducted via a stratified random sampling was applied for the data collection. The collected data was analyzed using the Statistical Package for Social Sciences (SPSS) version 20. Results indicated that arson, accidents during agricultural practices, negligence during rubbish burning, cigarettes butts on trails and harvesting wild honey as well as the extended drought period during the dry season are the predominant causes of forest fires on Praslin. Membership in a community-based organization was the only determinant that influenced household participation in forest rehabilitation while other factors such as household size, age, gender and number of schooling years were not significant. Most benefits of forest rehabilitation were perceived in-kind rather than financial through the provisioning of ecosystem goods and services. Technical challenges manifesting in the form of inadequate knowledge and skills for tree planting on very steep slopes often covered with boulders and also exposed to soil erosion were of greater concern. This reinforces the point that forest rehabilitation needs to be adaptive to local conditions in order for it to prevail and it is a learning-by-doing process to develop the best practices that can ensure greater success. Lastly, some proposed strategies for improvement of rehabilitation programmes include awareness and education campaigns, additional manpower, the development of an efficient monitoring and evaluation system, conducting regular enrichment planting, and also greater involvement of community members at the planning stage but also during the execution of forest rehabilitation programmes.
Keywords: *forest landscape, participation, capacity building, tree knowledge, forest fire, TRASS*

Introduction

Forest degradation goes beyond a reduction of the forest area to a decrease in the quality of forest which in turn affects the ecosystem goods and services they provide (Yin et al., 2016). Despite the importance of forests as a source of income, food values, protection of watersheds, carbon and sinks among others, the conversion of grasslands, woodlands, and forests into croplands and pastures has risen dramatically

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_41854217

during the last two decades globally. For example, disturbances in tropical dry forests have resulted in the fragmentation, degradation, and in some cases disappearance of these forests (Mbow et al., 2013). The main drivers behind these changes are a combination of population growth (Stéphenne and Lambin, 2004), rising demand for agricultural products, dietary changes, agricultural trade and adjustment, dependence on wood energy, recurrent bush fires (Ouedraogo et al., 2015), infrastructural development (mainly road construction), expansion of settlements and agricultural fields (Makunga and Misana, 2017).

Madagascar, one of the 34 global biodiversity hotspots has a high prevalence for forest degradation. According to Yesuf et al. (2019), such degradation is mainly driven by weak governance structure and unclear land tenure that has necessitated the encroachment of large-scale commercial agriculture into forest areas. Furthermore, Sudan is considered a hotspot for forest degradation with dramatic land use change from 5.3% in 1973 to 22.2% in 2016 in the Erawashda forest driven by an increase in cultivated land through mechanized farming into forest areas (Sulieman, 2018). Corroborating the views of other studies, Vásquez-Grandón et al. (2018) concluded that over exploitation of forests products is the primary drivers of forest degradation, alongside overgrazing, wildfires, and the spread of invasive species or pests. Between the years 2001 to 2015, 27% of global forest loss was caused by permanent land use change for commodity production and 23% due to wild fire (Curtis et al., 2018).

In the case of Seychelles, deforestation and forest degradation (DD) can be traced to two main phases of historic forest loss – commercial logging of timber between 1770-c1820 and then between c1910-1970s for cinnamon extraction and distillation (Kueffer et al., 2013). Therefore, historical events such as unsustainable harvesting of timber and forest products also played a major role in removing vegetation and thereby exacerbating the impacts of climate change on the exposed top soil layer. After the first settlement of humans some 240 years ago there was widespread exploitation of hard wood from the forest for various uses including housing and boat construction in Seychelles (Etongo et al., 2019). In addition, DD is severe given the total landmass of 455 km^2 (GoS, 2020) spread across 115 islands, with intense land use competition from multiple uses as a consequence. More than 80% of the land area on Praslin and Curieuse Island has been affected by forest fires and consequent severe erosion. Dry weather conditions due to the extended period of drought during the dry season (Fleischmann et al., 2020) coupled with the availability of flammable materials in the forest create ideal conditions for forest fires to occur (Senterre, 2009). The exposed top soil with its little organic matter on the predominantly mountainous landscape in Seychelles (see *Fig. 1*) are easily eroded by runoff after torrential rainfall.

Although the causes of forest fires are human-induced, climate change manifestation through the extended period of drought during the dry season have created a favorable condition for fire to occur, persist and spread in the forest. Therefore, the predominant causes of fire on Praslin are anthropogenic but amplified by the impacts of climate change – a view supported by Senterre (2009). According to this same study, the main drivers of forest fires in the Seychelles are human ignition (proximity to trails), vegetation (post-fire colonizing species are more fire prone) and environment (dry spell, and topography). The amount of fuel available in the understory, e.g. typically palm forests with a relatively larger amount of fuel on the ground from the palm leaves are more vulnerable to fire outbreak. The climate change projections in Seychelles shows that rainfall, while increasing in overall terms, will become even more irregular (GoS,

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DOI: http://dx.doi.org/10.15666/aeer/1905_41854217

2020). Much of the precipitation is falling in sharp bursts, creating heavy flooding in the wet season, while imposing an extended period of drought during the dry season (Fleischmann et al., 2020).

Figure 1. *La Hauteur Mountain, overlooking the district of Baie Ste Anne, has been sporting bare red patches for almost thirty years following forest fires and soil erosion. Photo credit: Romano Laurence (Seychelles News Agency, 2015)*

Historically fires were set on purpose on both Praslin and Curieuse by the sailors/traders as a result of disputes. They collected the priceless Coco-de-mer nut and then set fires so that others could not get access to them but also to force the prices of the Coco-der-mer up on the international market. This was before the 1700. After colonization of the Seychelles in 1772 and much later on in the 1900, fires were mainly accidental during agricultural practices. Battles between pirates, around 1700, conflicts between groups of settlers around 1770, then war between French and English around 1800, etc., have resulted in unprecedented forest fires especially on the grounds of the Coco-de-mer, whereby fires were set on the Coco-de-mer forests to raise its value on the market (Senterre, 2009). The development of agriculture and timber extraction in the 19th century, and the development of the cinnamon industry, in the 20th century, have further deforested lands, increasing their flammability. During the heated political times of the 1960s to 1980s fires were set deliberately due to political frustrations but also because of conflicts between neighbours (arson fires as a protest tool). Forest fires during this period on average occurred twice a year and it destruction on the vegetation cover was relatively extensive, burning up to 20 ha which has diminished to 1-2 ha with some years without fire outbreak in the forest (Senterre, 2009). The other main cause of forest fire is negligence while burning rubbish, cigarette stump thrown on trails or harvesting wild honey, and also results from the non-awareness of importance of forests for the community (Senterre, 2009). Forest fires have become recurrent during the last

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_41854217

five decades with vast expanse of both private and public lands including the National Park on Praslin being ravaged by fires. This lead to the initial forest rehabilitation activities on Praslin and elsewhere in Seychelles pioneered by the government entities responsible for forestry and National Parks. However, community engagement was minimal given that rehabilitation activities were mostly implemented by contractors and government staffs in the environment and forestry divisions. The creation of a non-governmental organization - the Terrestrial Restoration Society of Seychelles (TRASS) in 2009 became a game changer and has witnessed community participation through volunteerism on the one hand (Seychelles News Agency, 2015), and on the other hand, building partnership through joint implementation of projects with government institutions such as the Ministry of Agriculture, Climate Change and Environment (MACCE) and Seychelles National Parks Authority (SNPA).

For example, during a one-day activity organized by TRASS in November 2020, a total of 1,000 seedlings of native tree species were planted at Pasquière, Praslin Island by a group of volunteers from Raffles Hotel, Seychelles Employee Transition Scheme (SETS), Seychelles Island Foundation (SIF), as well as students from Vijay International, Baie Ste. Anne and Grand Anse schools (Seychelles News Agency, 2020). Involving local community in forest rehabilitation activities from the planning, design, implementation and monitoring of such interventions has the potential for greater success and concomitantly to ensure sustainability when rehabilitation projects phase out. Community engagement in forest rehabilitation has multiple benefits including social learning through peer to peer support, development and transfer of new skills, increase awareness on the importance of forest in terms of livelihood values and environmental protection.

One of the main objectives of TRASS is to rehabilitate fire ravaged forests on Praslin and in order to achieve this objective, an estimated 15,000 endemic plants are cultured every year for this purpose at the TRASS nursery. Tree and mangrove planting activities are carried out on a voluntary basis witnessing the participation of community members of different age groups and even tourists in some cases. Continuous engagement with schools and local communities is an effective way to pass on relevant information and raise awareness on sensitive issues regarding management of forests (Senterre, 2015). Forest rehabilitation is a process and not an activity that could be limited solely to tree planting. It also contributes to Sustainable Development Goals 13 and 15 – Climate Action, and Life on Land. More importantly, forest rehabilitation is not limited to Praslin but occurring on other islands in the Seychelles. The total land area rehabilitated by TRASS is 23 ha during phase 1 and 2 of the Ecosystem Based Adaptation (EBA) project where 5 ha (2016–2017) and 18 ha (2019–2020) respectively. The phase 1 was mostly a learning-by-doing process in which the seedlings of native trees were planted on predominantly mountainous landscapes with steep slopes that have very little humus layer, from which lessons were learnt to ensure that best practices were implemented in phase 2. Therefore, insights from community involvement in forest rehabilitation and some lessons learnt especially within the context of a Small Island Developing State (SIDS) can provide baseline information to guide future rehabilitation projects on Praslin and elsewhere in the Seychelles. This study therefore addresses the following issues: (i) causes of forest fires on Praslin based on community perceptions, (ii) determinants and levels of household participation in forest rehabilitation, (iii) challenges and benefits of forest rehabilitation, and (iv) proposed strategies to improve forest rehabilitation.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Forest rehabilitation – conceptual perspective

Motivational drivers and types of participation in forest rehabilitation

Rehabilitation is often used interchangeably with ecological restoration with the most recent and comprehensive definition provide by the Society for Ecological Restoration (SEC) (Gann et al., 2019). Rehabilitation: Management actions that aim to reinstate a level of ecosystem functioning on degraded sites, where the goal is renewed and ongoing provision of ecosystem services rather than the biodiversity and integrity of a designated native reference ecosystem. Ecological restoration: The process of assisting the recovery of an ecosystem that has been degraded, damaged, or destroyed. (Ecosystem restoration is sometimes used interchangeably with ecological restoration, but ecological restoration always addresses biodiversity conservation and ecological integrity, whereas some approaches to ecosystem restoration may focus solely on the delivery of ecosystem services). While the latter is to restore a degraded forest to its original state – that is, to re-establish the presumed structure, productivity and species diversity of the forest originally present at a site, the former which is the focus of the current study, is to restore the capacity of degraded forest land to deliver forest products and services. The philosophy of participation is no longer new to forest rehabilitation (Evans et al., 2017). Viewed as a catalyst of social change, participation has not only been spread through technology transfer in development research, but also by challenging state control in the management of natural resources (Ballet et al., 2009). Participation involves three interconnected, but different processes: (i) the involvement of local communities in decision-making; (ii) the inclusion of local community's perspectives into programmes; and (iii) the assurance of community participation in benefit sharing from the process (United Nations, 1975).

The application of participatory methodologies in natural resource governance has been acknowledged by development practitioners as an effective mechanism for managing existing conflicts, while minimizing the tendency for future conflicts to occur (FAO, 2014). Despite this acknowledgment, participation has been described in some cases as a new form of tyranny (Cooke and Kothari, 2001), especially in cases where tokenism predominates, shielding the dominance of elites who end up capturing the entire forest rehabilitation process and the benefits thereof (Arnstein, 1969). In this regard, those who participate in the management of natural resources in general and forest rehabilitation in particular are hardly the true representatives of stakeholders who are directly affected by the decisions being made (Marshall and Jone, 2005). Agarwal's typology (Agarwal, 2001) presents a useful tool to appreciate different forms of participation in which he identified six levels of participatory behavior (*Table 1*). In principle, people's perceived benefits could improve their participation, placing them within the active and interactive participation strata. However, several factors (beyond perceived benefits) could also shape people's decision to participate in these arrangements.

Some of the scientific literatures have proven that the driving force behind participation in forest rehabilitation is contextual as people are motivated to participate due to their embedded socio-cultural, economic, and political benefits (Bagdi and Kurothe, 2014). Some of the widely documented drivers include cultural benefits, financial benefits, incentives, and prior established links with conservation agencies, among others (Raufirad et al., 2017). The literature on what motivates people to participate in forest rehabilitation is negligible, at least in the context of

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Seychelles. This study contributes to unmask the range of factors that (de)motivate community members' participation in forest rehabilitation in the context of Seychelles and Praslin Island in particular. Lessons learnt from ongoing rehabilitation activities are essential to inform future forest rehabilitation in the Seychelles given the increase in degraded forest areas amidst recurrent forest fires under the impacts of a changing climate.

Table 1. *Agarwal's typology of participation*

Form of participation	Characteristic features
Nominal participation	Membership in the group
Passive participation	Being informed of decisions ex post facto; or attending meetings and listening decision-making, without speaking up
Consultative participation	Being asked an opinion in specific matters without guarantee of influencing decisions
Activity-specific participation	Being asked to (or volunteering to) undertake specific tasks
Active participation	Expressing opinions, whether solicited, or taking initiatives of other sorts
Interactive (empowering) participation	Having voice and influence in the group decision-making

Source: Adopted from Agarwal (2001)

Guidelines for forest rehabilitation

Brown and Lugo (1994) characterized rehabilitation as a management strategy to reverse the negative impacts of deforestation and degradation. Achieving such an objective that has potential for environmental win-win will require adhering to certain guidelines that has proven to deliver a greater rate of success. Elliott et al. (2013) also suggested five actions that are important for forest rehabilitation as follows: (i) removing stressors such as forests fires, over grazing and biomass loss, (ii) to add animals and plants to the area depending on the level of degradation, (iii) experimental work by Schumacher indicated that additional nutrient conferred few benefits to endemic species and great benefits to non-native species – obviously this is a very different situation on fire degraded lands on Praslin that have lost humus compared to Mahe. Trials on Praslin indicated that adding humus and charcoal to plant increased plant growth by 2- 4 times. The addition of calcium carbonate through coral fill to improve soil pH also had beneficial advantages (Henriette et al., 2013; Senterre et al., 2012), (iv) regulating energy inputs in the system as a means of controlling ecosystem development, and (v) removing the most high impact stressors (e.g. poor land policy and overharvesting/exploitation of forest resources). The latter two is said to be the most difficult and time consuming (Brown and Lugo, 1994). Budiharta et al. (2014) stresses on the importance of identifying the characteristics of the degraded sites and level of degradation before choosing the rehabilitation techniques. For example, highly degraded forest lands may require intensive tree planting with a high cost compared to minimally degradation forest lands in which low-cost options such as enrichment planting (gap or strip planting) might be imperative. Additionally, the biodiversity of the area is an important consideration given that Key Biodiversity Areas (KBA) are hotspots that should be prioritized for rehabilitation/buffer zones so as to restore ecosystem goods and services.

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In order to ensure greater success rate in forest rehabilitation, Hahn et al. (2004) put forward the following guidelines as follows: (i) reduce the use of clear cutting, instead make use of productive and stable tree species, which secure soil fertility, (ii) use local variation and natural succession to secure constant supply of species and promote rare, native species, (iii) only native species should be used, except in circumstances where exotic species fulfill requirements such as site adaptation and biological integration, (iv) use species whose growth are not dependent on pesticides and or fertilizers, (v) promote and use natural regeneration techniques, (vi) apply an ecosystem based technique that will have minimal impact on the environment and on the local communities, (vii) securing biodiversity by creating forest reserves and promoting silviculture, and (viii) getting local community involved in the process and enhance their interactions with the forest through increasing aesthetic values and recreational opportunities and potential for economic activities is also an encouragement for participation.

Some considerations pertaining to forest rehabilitation in the case of Seychelles include climatic condition, nature of the topography, soil quality and vegetation types which have an impact on site selection and costs for rehabilitation. More importantly, the choice of site for rehabilitation is dependent on a number of factors such as its land use, proximity to high biodiversity areas, the human and financial resources available, and land ownership. According to Senterre et al. (2012), the predominant rehabilitation technique on degraded forest lands on Praslin is through strip clearing (only done in shrublands dominated by Chrysobalanus-Dicrapnopteris thickets) and tree planting mainly with native tree species while exotic species are used only in trials to compare with native species. The ecology of plant species should also be considered to achieve better success. Initial forest rehabilitation trials were conducted by TRASS from 2010 to 2014 with the aim of implementing the guidelines that reflects local realities. Despite some recorded success stories, ensuring the sustainability of forest rehabilitation activities should consider the availability of tree seedlings in nurseries, knowledge on planting techniques and preferred season for planting, nutrient supply for newly planted trees, continuous implementation of enrichment planting, proper maintenance of fire breaks, constant removal of invasive plant species and the active participation of community members at the planning, implementation and monitoring stages of forest rehabilitation.

Community-based forest rehabilitation in Seychelles

Involving local community in forest rehabilitation activities from the planning, implementation and monitoring are important to ensure sustainability of rehabilitation activities because skills and capacity are built across different levels. Community participation in forest rehabilitation is not new in the Seychelles. It can be traced to several projects and activities implemented during the last four decades by the Plant Conservation Action (PCA) group of Seychelles, SNPA and the MACCE (Kapisen, 2008). The momentum was rekindled by TRASS - a local NGO that has been in existence for 12 years with the mandate to restore the degraded forest areas on Praslin through the active engagement of local communities. Its effort has been to rehabilitate fire ravaged forest areas under private and public ownership.

A large area of the Praslin National Park is also being rehabilitated by TRASS. With support from mostly volunteers from both the public and private sector, TRASS has been able to achieve its target on planting trees and mangroves. For example, between 2015 to 2019, a total of 24,923 trees were planted on Praslin with the assistance of

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1,166 volunteers excluding TRASS own members. Most of the plantings occurred during the North West Monsoon between October to March, during which Seychelles experiences the most rainfall - an ideal situation for the growth of plants. The community engagement approach targets all individuals from Praslin and other nearby islands (Senterre et al., 2012). Community based rehabilitation has proven to be quite effective and is gaining more popularity as an effective approach to ensure sustainability of forest management while also promoting benefits for local livelihoods and the environment (Blay et al., 2008).

Materials and methods

Case study site

Praslin, the second largest island in Seychelles has a total land size of 38 km^2 with an annual average temperature of 30^0 C and a minimum of 25^0C (Etongo et al., 2020). Furthermore, annual average figures for rainfall maximum and minimum ranges between 292 mm and 140 mm. According to the Seychelles Meteorological Authority (SMA), the yearly precipitation for Praslin during the last five years from 2020 to 2016 was 2803 mm, 2947 mm, 1990 mm, 2146 mm, and 2080 mm. Praslin has a population of 7,533 people and comprises two administrative districts: Baie Sainte Anne and Grand Anse. The main settlements are the Baie Ste Anne, Anse Volbert and Grand Anse. It was named Isle de Palmes by explorer Lazare Picault in 1744. Seychelles in general and Praslin in particular suffers from low economic diversification and vulnerability to external shocks, given its dependence on tourism and fisheries and fishery-related manufacturing (OECD, 2013).

Two types of soils prevail in the Seychelles: (i) ferralitic soil, commonly known as "la Terre Rouge" or red soil and originating from the weathering of granitic rock, is widely extended over the slopes, hills and mountains of the granitic islands: (ii) calcareous sandy (Shoiya series) soil exists on the small plateaus on the coast of the granitic islands and on the coralline islands (FAO, 2005). The case study location is found within the National Park and is a predominantly mountainous area around the Fond B'offay Watershed (4.33807° S and 55.75368° E) on Praslin with active participation from TRASS to ensure the rehabilitation of the degraded forest areas (see *Fig. 2*).

The ferralitic soil type is the dominant soil that occurs at the Fond B'Offay Watershed area and also across other mountainous sites on Praslin Island (see *Fig. 3*). Despite the favourable climate conditions of rainfall and temperature that support tree growth, the forest landscape especially on mountainous areas dominated by boulders have not allowed sufficient accumulation of top soils. The soil humus layer is easily washed away given the dominance of steep slopes that are not properly covered by vegetation. In addition to the steep slopes, larger boulders are easily spotted on the mountainous landscape (see *Fig. 3*), and in some cases, they prevent the firm grip of tree roots into the soil. Forest fires date back to the last 70 years (Senterre (2009) documented a chronology of events) and it has become less frequent with the most recent on Praslin during the month of August 2020. TRASS have been involved in forest rehabilitation across the fire ravaged sites through domestic and internationally funded projects. The Fond B'Offay watershed is a Government of Seychelles (GOS) Adaptation Fund-UNDP Ecosystem based Adaptation (EBA) project site and the target indicator of forest rehabilitation within this project is 25 ha of degraded forest between 2014–2021 (UNDP, 2013).

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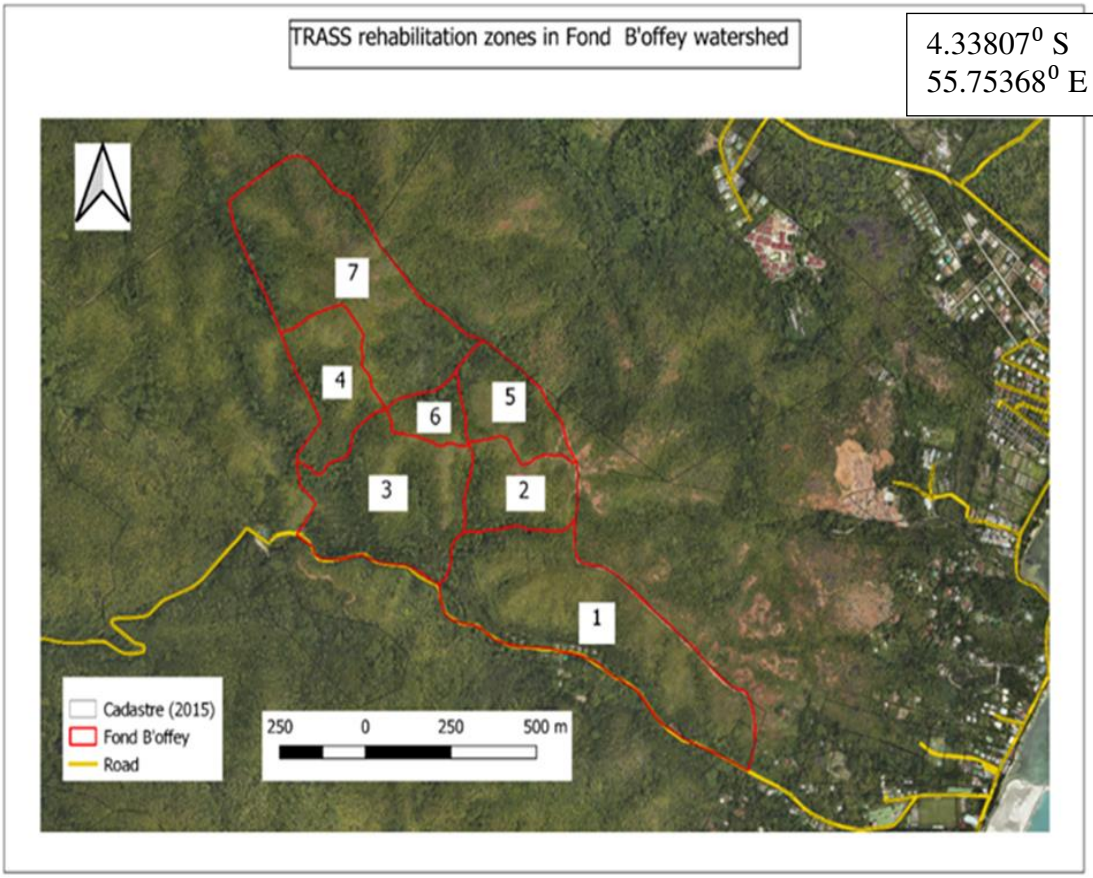

Figure 2. *Different forest rehabilitation zones managed by TRASS at the Fond B'Offay watershed*

Figure 3. *Ferralitic soil type on mountainous sites on Praslin that suffers from intense degradation. Photo credit: Daniel Etongo (Etongo et al., 2020)*

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Research design and data collection

Information from key informants and field observations

This study began with a shared dialogue workshop (SDW) with opinion leaders from different organizations that are involved in forest rehabilitation on Praslin. The opinion leaders were representatives from the MACCE, SNPA, TRASS and community members. A SDW is a forum that brings academics as well as technicians, practitioners, community leaders, and household members together at regular intervals to identify challenges and obstacles to, and opportunities for, the participation of households and community members in the management of a natural resource (Etongo et al., 2018) and in this case - forest rehabilitation. Taking these criteria into consideration, three SDWs were conducted between the months of September to November 2020. These workshops were conducted entirely in the local vernacular (Creole). Some of the issues discussed were a list of tree species used in rehabilitation, type of land management techniques implemented, challenges encountered in rehabilitation activities, and capacity needs for forest rehabilitation.

The database of TRASS which include data on types and number of trees planted, number of hectares rehabilitated, number of participants during tree planting and rehabilitation activities also provided additional information. For a comprehensive lists of indigenous, endemic and exotic tree species planted by TRASS, see *Appendix 1*. Field observation at two of the rehabilitation sites occurred in the month of December 2020. The main focus during the field visit included the types of trees planted, the nature of the terrain (steepness of slope and accessibility), the layer of humus in the soil was also assessed visually, vegetation cover and extent of damage caused by fire with some photographs taken to support findings from the survey. Field observations can provide unique information, because it does not rely on other's verbal interpretations of situations, but on observation made while in the field.

Sampling and data collection

To successfully achieve the aims of this research, qualitative as well as quantitative data was collected from 180 households (from a total of 2490 households) on Praslin using the stratified random sampling technique in order to understand people's perception on issues pertaining to forest rehabilitation. The sampling was carried out in close collaboration with the National Bureau of Statistics (NBS) Seychelles given that NBS have a comprehensive database of households across the Seychelles. The two administrative districts of Praslin - Baie Sainte Anne and Grand Anse were considered as two separate strata for the sampling of households. This was followed by the identification of all Enumeration areas (EA) in the two main districts that amounted to 68 EAs in total. To interview householders from all of these EAs could have been time consuming and costly. Therefore, the EAs were later grouped according to regions, nine (9) in total with a random selection of 200 households corresponding to 95 and 105 householders in Grand Anse district and Baie Ste Anne district respectively (see *Table 2*).

In some regions, less respondents were reached given that a household member was not available at the time of the survey while others were unwilling to participate due to lack of time and in some cases for political reasons that their political party just lost an election. That notwithstanding, a total of 180 surveys were completed across the nine regions in both districts. The survey questionnaire was clustered in categories from socio-demographic data to benefits of forest rehabilitation, factors influencing

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household participation in forest rehabilitation, among other. Some examples of qualitative questions included (i) what do you think could be the cause of forest fire on Praslin? (ii) What do you think could be done to improve on the rehabilitation of fire ravaged forest lands on Praslin? For more details, see the supplementary questionnaires.

Table 2. *Number of households surveyed across the nine regions in both districts on Praslin*

District	Region	Total sampled household	Respondents reached and interviewed
Grand Anse	Cherimont (Mont Plaisir	40	30
	Anse kerlan	30	33
	Amitie	25	21
Baie Sta Anne	Anse Boudin/Anse Lazio	20	20
	Zimbabwe	14	10
	Cote D'or	26	22
	Baie Ste Anne	30	33
	Moulignee Estate	15	11
Total		200	180

Data analysis

Descriptive and inferential statistics have been used to analyze the quantitative data. For the qualitative questions such as (i) perceived causes of forest fire on Praslin and proposed strategies to improve forest rehabilitation, these questions were analysed first by identifying main themes and assigning responses to their corresponding themes which was then coded for quantitative analysis. Regarding the determinants at the level of the households that influences participation in forest rehabilitation, the dependent variables are categorical – participants and non-participants in forest rehabilitation. Therefore, the logistic regression model was applied in order to understand the determinants at the household level that influence participation in forest rehabilitation activities. All analysis were performed using the Statistical Package for Social Sciences (SPSS) Version 20 and the results are presented in the form of tables and column graphs. A regression analysis is used to describe the relationship between a set of independent variables and a dependent variable. In this case, the dependent variable is participation in forest rehabilitation (with two groups – those that have participated, and those that have not) while the independent variables were gender of the household heads, age, schooling years, membership in a CBO and family size. For the multiple response questions, they were grouped using the multiple response function in SPSS and bar charts were produced to highlight the results in percentages. Lastly, Mean, counts and percentages were used to provide a summary statistics for the socio-demographic profile of the surveyed participants. The results are presented in bar charts and tables.

Results

Socio-demographic profile of householders and perceived causes of forest fires

The survey results showed that the mean age for householders at Grand Anse and Baie Ste Anne did not differ significantly. On the other hand, more females participated

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in the survey especially at Baie Ste Anne (35.0%) in which the figure almost double that of male participants (see *Table 3*). The category "other employments" was mentioned by a relatively greater number of households (20.6% for Grand Anse and 18.9% for Baie Ste Anne) and included those that are working with security agencies and other related activities within the tourism sector.

Table 3. *Socio-demographic information of householders that participated in the survey*

	Grand Anse	**Baie Ste Anne**	**TOTAL**
Age (mean)	44	43	
Gender (household head)			
Male	41 (*22.8*)	33 (*18.3*)	74 (*41.1*)
Female	43 (*23.9*)	63 (*35.0*)	106 (*58.9*)
Main occupation (household head)			
Farmer	4 (*2.2*)	7 (*3.9*)	11 (*6.1*)
Fisher	1 (*0.6*)	11 (*6.1*)	12 (*6.7*)
Business owner	18 (*10.0*)	25 (*13.9*)	43 (*23.9*)
Civil servant	24 (*13.3*)	19 (*10.6*)	43 (*23.9*)
Other employments	37 (*20.6*)	34 (*18.9*)	71 (*39.4*)
Education (household head)			
No formal education	2 (*1.1*)	0 (*0.0*)	2 (*1.1*)
Primary	20 (*11.1*)	7 (*3.9*)	27 (*15.0*)
Secondary	23 (*12.8*)	38 (*21.1*)	61 (*33.9*)
Post-secondary	31 (*17.2*)	47 (*26.1*)	78 (*43.3*)
University	8 (*4.4*)	4 (*2.2*)	12 (*6.7*)
Household dependency ratio			
Low	36 (*20.0*)	30 (*16.7*)	66 (*36.7*)
Medium	24 (*13.3*)	29 (*16.1*)	53 (*29.4*)
High	24 (*13.3*)	37 (*20.6*)	61 (*33.9*)
Knowledge of TRASS activities			
Yes	57 (*31.7*)	78 (*43.3*)	135 (*75.0*)
No	27 (*15*.0)	18 (*10*.0)	45 (*25.0*)

The figures in parentheses represent percentages (%)

In terms of educational level of the respondents, an estimated 84% have acquired secondary to university education while a miniscule 1.1% had no formal education and another 15.0% had completed just primary education in both districts. Information gathered from the resource persons during the SDWs indicated that education is free in the Seychelles up to post-secondary and scholarship opportunities are available for university studies locally and abroad. This further explains while majority of the respondents have acquired education at different levels. The household dependency ratio was spread across the three categories – low (36.7%), medium (29.4%) and 33.9% for high dependency ration (see *Table 3*). It is expected that householders with relatively higher dependency ratios are expected to be less active in forest rehabilitation activities given that the burden of the household is borne by the few adults of the working age group. Regarding knowledge on TRASS activities, 15.0% and 10.0% of

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respondents at Grand Anse and Baie Ste Anne mentioned that they were unaware of activities implemented by TRASS whereas 75.0% of respondents in both districts had knowledge on such activities (*Table 3*).

Causes of forest fires on Praslin were attributed mostly to ignition from cigarette butts as reported by 40.6% of the 180 respondents that participated in the survey (*Fig. 4*). This was followed by another 31.1% of respondent who were of the perception that the extended drought period during the dry season is the cause of forest fires on Praslin. Additionally, 13.3% of the respondents attributed the cause of forest fires to bee hunting for the harvesting of honey. The causes of forest fire based on information during the SDWs have not changed, but it is amplified by the extended drought period. However, the frequency of fire has changes from 1 to 2 occurrences during the last two decades compared to an average of 5 occurrences during the 60s and 70s.

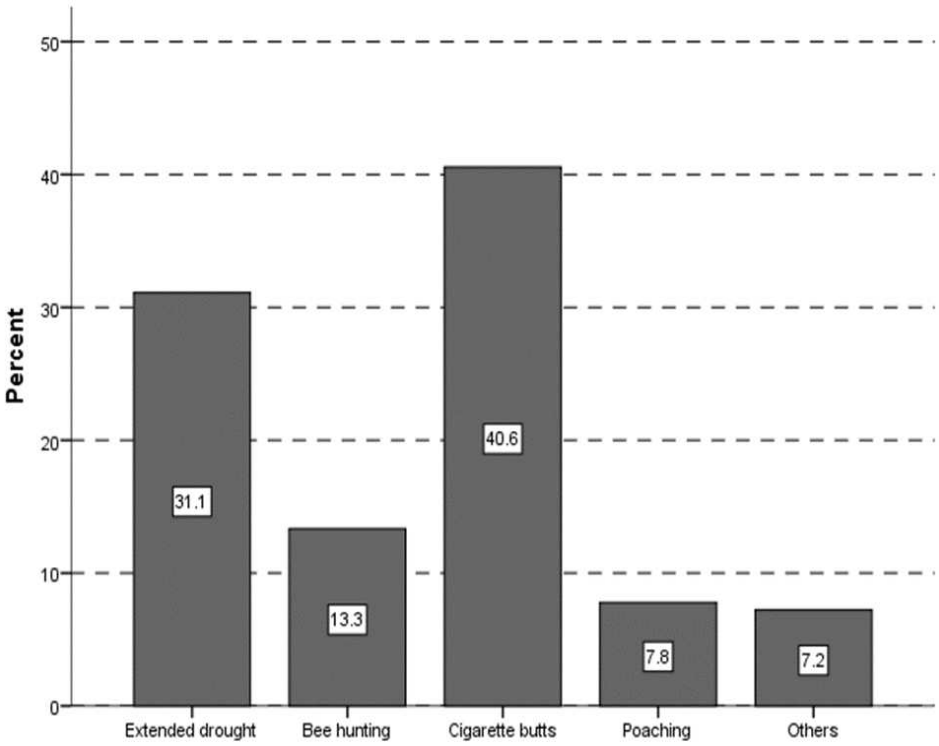

Figure 4. *Causes of forest fires on Praslin Island based on local perceptions (N = 180)*

Information gathered from opinion leaders indicated that than 40% of the land area on Praslin was affected by forest fires while Curieuse Island was almost entirely burnt. Furthermore, opinion leaders were of the views that forest fires have reduced over the last three to four decades, but that the vegetation remains flammable and risks of forest fires is still a major concern especially in the face of climate change and variability. Therefore, dry weather conditions coupled with steep slopes and mountainous landscapes that experiences relatively stronger wind speeds create ideal conditions for forest fires to occur. The exposed top soil with its little organic matter based on field observation at the affected sites, then erodes easily by runoff after torrential rainfall. After losing the vegetative cover, the top soil and organic matter, these affected soils will crust and bake in the sun, and become rock-hard preventing the establishment of vegetation.

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DOI: http://dx.doi.org/10.15666/aeer/1905_41854217

Determinants of household participation in forest rehabilitation

Family size, age and gender does not influence household participation in forest rehabilitation. However, membership in a community-based organization (CBO) was significant at the 5% level indicating that it did influence participation in forest rehabilitation (see *Table 4*). The number of schooling years of householders was not significant and that could be further explained that the level of education did not have an influence on the participation in forest rehabilitation. Participation in forest rehabilitation is not gender sensitive and this was supported by information gathered from the key informants. To further substantiate this claim, *Figure 5* highlights the fact that both male and female of different age groups actively participate in tree planting activities as part of forest rehabilitation on Praslin. Forest fires on Praslin has a long history and there is a nation-wide awareness given that these incidents are reported via several News Papers, Radio and Television Stations and different social media platforms with a greater reach to community member. Seychelles is a Small Island State and information easily circulate around the communities; however, all other factors did not affect participation in forest rehabilitation except for membership in a CBO.

Table 4. *Regression model of household determinants towards forest rehabilitation*

		B	**S.E.**	**Wald**	**df**	**Sig.**	**Exp(B)**	**95% C.I.for EXP(B)**	
								Lower	**Upper**
Step 1[a]	Gender	-.105	.311	.113	1	.736	.901	.489	1.658
	Age	.004	.012	.111	1	.739	1.004	.981	1.027
	SchoolYears	.079	.074	1.136	1	.287	1.082	.936	1.252
	CBO	1.185	.619	3.671	1	**.055****	3.271	.973	10.996
	FamSize	.035	.044	.640	1	.424	1.036	.950	1.130
	Constant	-1.305	1.296	1.014	1	.314	.271		

[a]Variable(s) entered on step 1: Gender, Age, SchoolYears, CBO, FamSize
*0.1 (10%), **0.05 (5%) and ***0.01 (1%) level of significance

Of the 96 respondents that are yet to participate in forest rehabilitation attributed their lack of participation to the following reasons (*Fig. 6*). Chief among these reasons was the lack of information on TRASS activity especially for tree planting as reported by 63.5% of the 96 respondents. This was followed by time constraints (61.5%), access to sites/the difficult nature of the terrain (45.8%), health related issues (37.5%) and a miniscule 13.5% reported distance to sites as the major factor that prevent their participation in forest rehabilitation activities being undertaken by TRASS (*Fig. 6*). Social media platforms, networking with other organizations and verbal communication are the medium used by TRASS to disseminate information pertaining to tree planting activities. Information gathered from TRASS showed that regular Facebook posts is the most used medium of communication. However, most of the community members that are yet to participate in forest rehabilitation were of the opinion that they are not on social media and hardly get timely information on planned tree planting activities. Therefore, alternative communication strategies that are relevant to the non-social media users will be important to improve engagement among this group of community member. Another reason mentioned by some of the community members who had prior

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access to activities organized by TRASS, also cited the timing of these activities occurring during the weekends as an impediment. While some of the respondents mentioned that they work even during weekends especially on Saturday mornings, for others, weekends were most preferred for buying groceries and performing other household chores.

Figure 5. *Both males and females of different age group participating in tree planting activity on Praslin organized by TRASS. Photo credit: Elvina Henriette (GEF, 2020)*

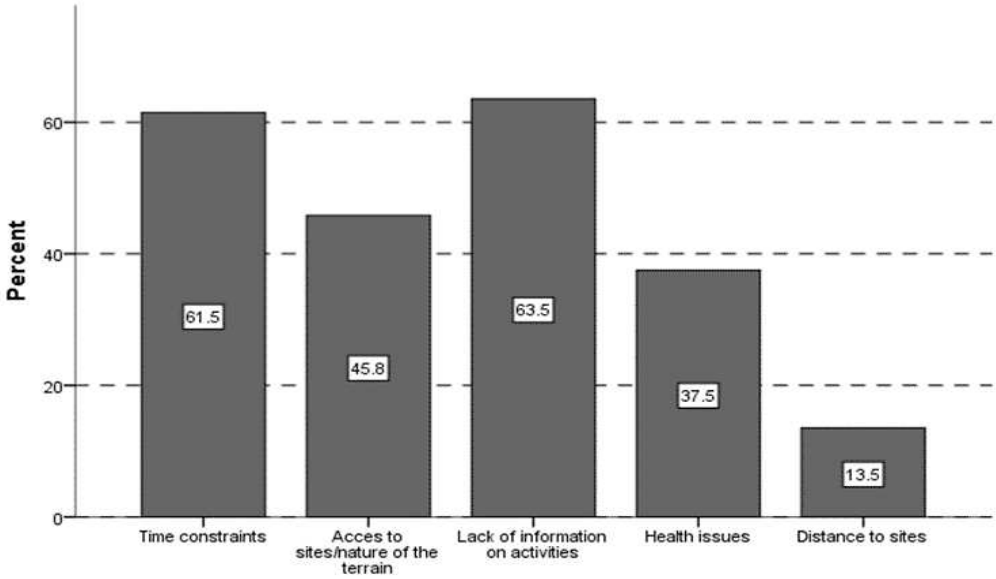

Figure 6. *Factors that prevent community member from participating in forest rehabilitation activities. Result is based on the 96 (53.3%) out of the 180 community members that have not participated in forest rehabilitation. Multiple responses with percentage > 100*

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Furthermore, another important reason mentioned by some of the community members and also observed during field visits is that the nature of the terrain is too steep to the extent that some form of support is needed to walk from one part of the forest landscape to another. To corroborate this view regarding the steep nature of the terrain making it difficult to access specific sites, a visit to one of the project sites at Fond B'Offay by the EBA project team further revealed the issue of accessibility. In some cases, a polyethylene rope is needed to access those sites with steep slopes (*Fig. 7*). Therefore, the nature of the terrain is the third most important factor mentioned by community members that prevent their participation in forest rehabilitation.

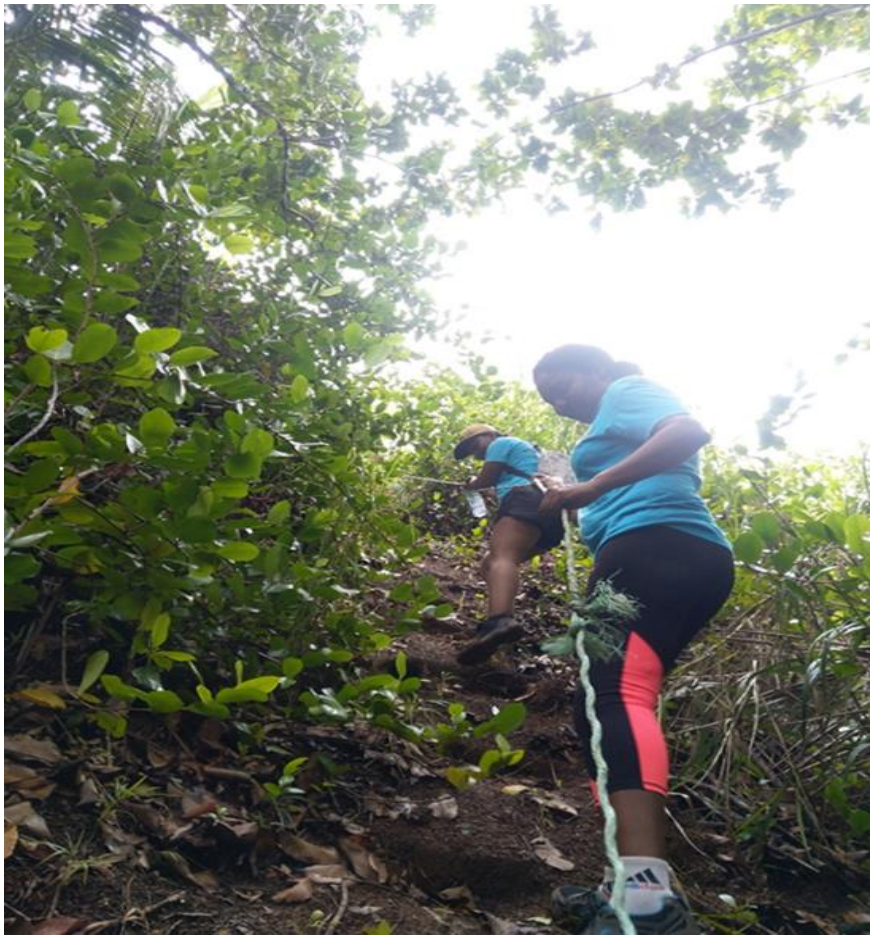

Figure 7. *A visit to the forest rehabilitation site by the EBA project team with the use of a polyethylene rope in order to access steep sites of the forest landscape. Photo credit: Vicky Stravens (TRASS; 2020)*

Community participation across the three stages of forest rehabilitation

The three stages of forest rehabilitation considered in the current study include the following: planning, implementation and monitoring stage. Data collected and summarized in *Appendix 2* shows that majority of the community members were involved in the implementation stage with activities such as transportation of tree seedlings and tree planting. Though TRASS benefit from the support of domestic and international donors, the planning and monitoring stages are still dominated by its

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members with a minuscule level of participation of the MACCE, SNPA, EBA Seychelles project, other NGOs and volunteers at these stages. Three interrelated activities in the implementation stages that recorded most responses are transportation of seedlings, tree planting and participation in training programs. A short training as was observed during field visit is usually provided prior to tree planting by TRASS in order to ensure a greater success rate of planted trees. Those participating in tree planting benefits from such trainings and they also transport the seedlings from the assembly point to the planting sites. The activity that received the least responses is the cutting of tracks which require a lot of physical labour (see *Appendix 2*). However, this activity according to key informants have been performed jointly at some of the rehabilitation sites by members of TRASS, SNPA and the Praslin Watershed Committee (WSC). Within the context of the EBA project for Seychelles, the WSC is a CBO given that its composition is entirely made up of community member with the mandate to led activities such as mangrove restoration, tree planting on degraded lands, protection of watersheds, and raising awareness across their communities on the need to protect and restore forest ecosystems and the multiple benefits it provides.

Perceived benefits and challenges encountered in forest rehabilitation

In general, community members perceive a lot of benefits that are linked to forest rehabilitation. The first four benefits based on the survey results include soil protection services from erosion, biodiversity conservation, landscape improvement (98.3%) and water quality and quantity (90%) as indicated in *Figure 8*. The multiple benefits mentioned by community members goes to show that they understand the importance of forest and the ecosystem goods and services it provides. Perceived benefits were viewed more in-kind rather than forest income. Forests is more than just trees and provide multiple benefits for local livelihoods in the form of food, energy, income, to environmental protection including carbon habitats that are important to climate change mitigation.

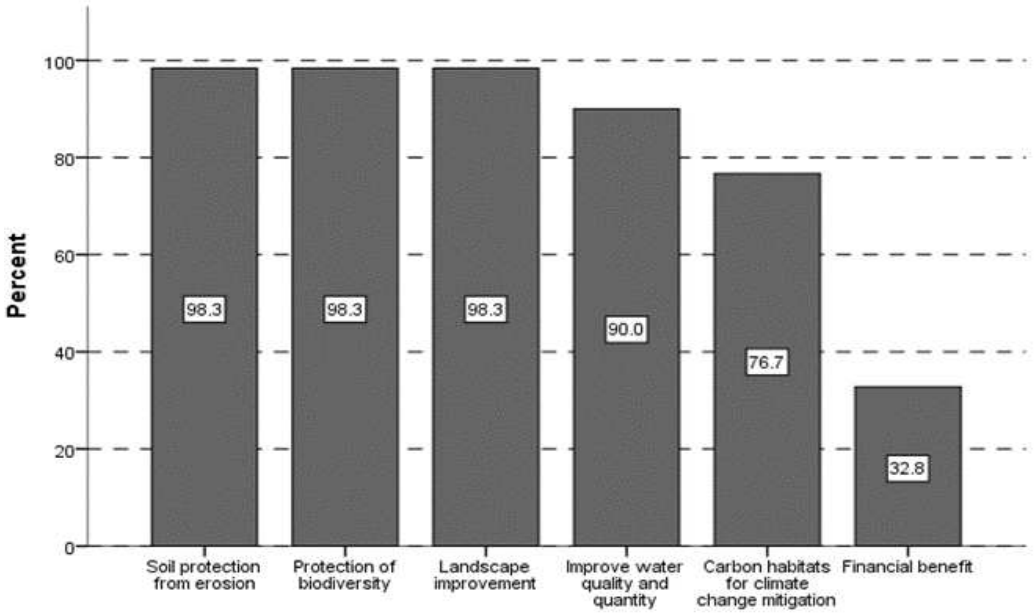

Figure 8. *Benefits of forest rehabilitation as perceived by community members. Multiple response question with overall percentage > 100*

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The challenges reported by respondents include technical and human capacity needs, availability of seedlings, impacts of climate change among others. Technical challenges that were prominent among those mentioned by community members are soil erosion reported by 64.1%, inadequate knowledge and skills (61.8%) preventing the effective rehabilitation of forest on Praslin, shortage of seedlings (61.2%), and 47.6% for those that mentioned recurrent forest fires (*Fig. 9*). Shortage of seedlings is a contrary view community members given that SNPA has two well-equipped nurseries at New Comb and Fond B'Offay with the active involvement of its staffs to grow stocks, harvest seeds and propagate them. TRASS on the other hand has a main nursery behind its office at Fond B'Offay which has an average of over 15,000 seedlings at any given time, most of the work is done by TRASS members and before the Covid-19 pandemic engaged volunteers or other community groups mainly during the weekends. It is through the involvement of volunteers almost every Saturday that TRASS managed to boost up its nursery stocks. However, TRASS and SNPA both agree that one of their main challenges is getting enough participants to cover all the area that needs rehabilitation on Praslin. SNPA further expresses concerns that their work force is not only limited but ageing and there is a need to employ more youths. Other challenges mentioned by community members pertain to governance issue that can be translated to land and tree tenure security and also the success rate of the planted trees given the steep nature of the terrain and the relatively small humus content of the top soil especially on mountainous slope with fewer trees.

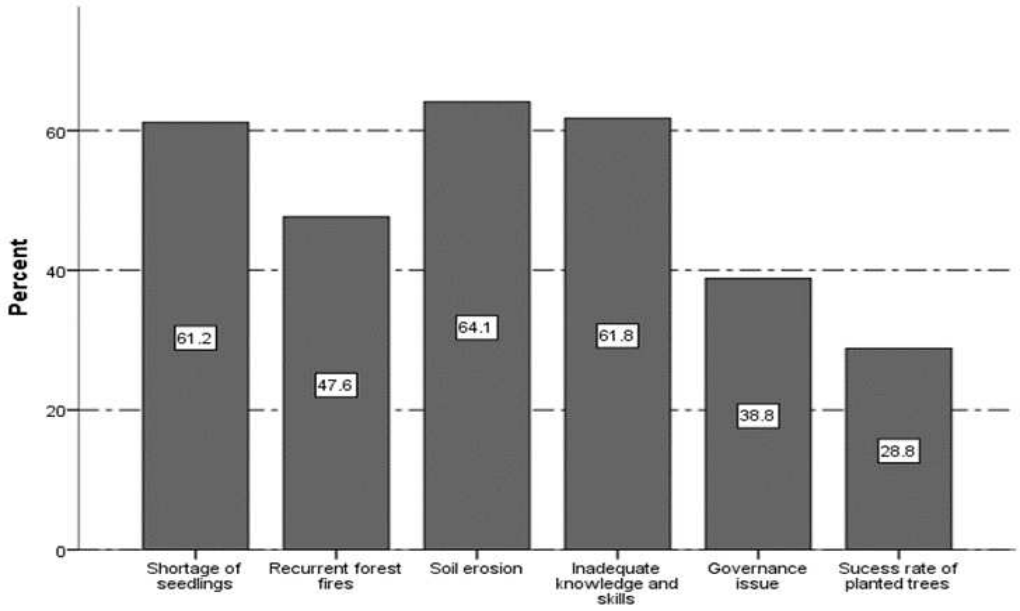

Figure 9. *Challenges encountered by community members in forest rehabilitation*

Multiple response question with overall percentage > 100

Proposed strategies to improve forest rehabilitation

A wide range of strategies were proposed by community members as options to improve forest rehabilitation activities on Praslin. Chief amongst them included the following: i) to improve on awareness and education campaigns (63.3%), ii) to produce

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more seedlings of native tree species for planting (63.3%), and iii) more manpower needed (60.6%) especially for cutting of tracks, weeding, transportation of seedlings, planting, among others (see *Fig. 10*). Two other strategies that were mentioned are improvement in community participation and also to develop an efficient monitoring and evaluation system as reported by 52.2% and 49.4% of the respondents respectively (*Fig. 10*). Failures of past tree planting efforts on degraded forest areas in Praslin has been associated with low fertility of soil, lack of technical skills as well as constraints imposed by the nature of the terrain and recurrent forest fires. Opinion leaders from TRASS proposed that some best practices already implemented should be up-scaled to other sites. These include (i) the application of 1 kg of humus to newly planted tree seedlings, (ii) the use to stones to construct an erosion control barrier for seedlings potted on steep slopes or at sites that could easily be affected by erosion to prevent seedlings from being washed away, and (iii) a short training provided prior to tree planting to ensure best practices are followed.

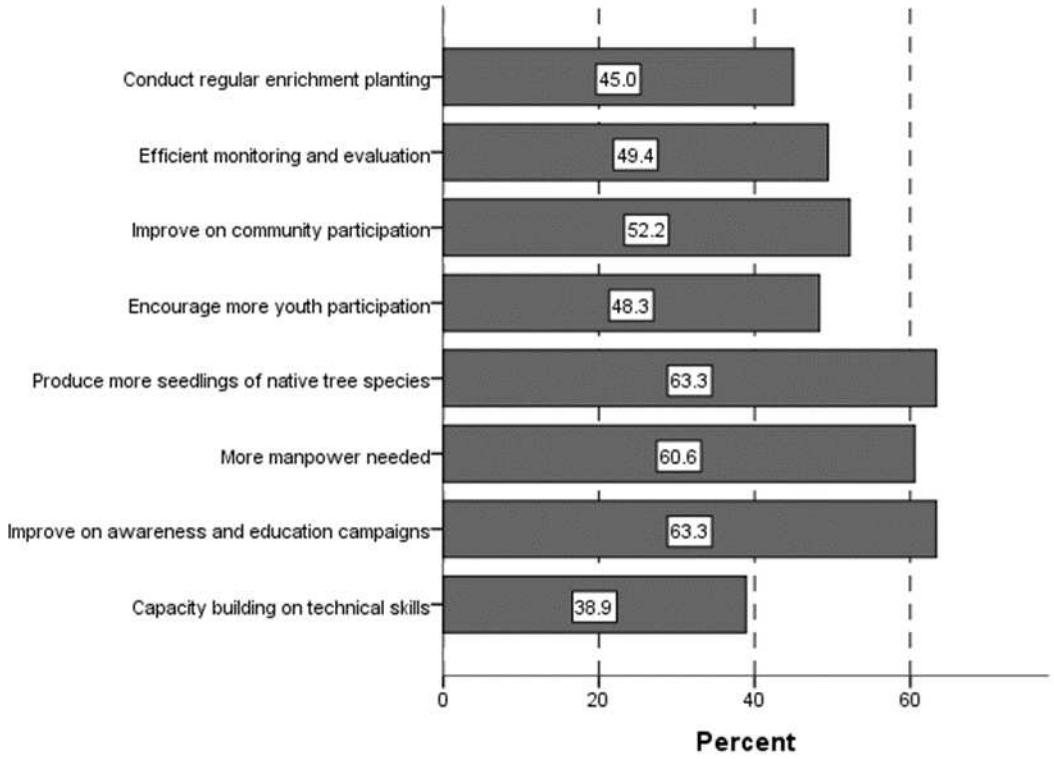

Figure 10. *Proposed strategies by community members to improve forest rehabilitation. Multiple response question with overall percentage > 100*

Discussion

According to a featured story by the Global Environment Facility (2020), the impacts of climate change and environmental degradation are increasingly evident on Praslin Island and the predominantly mountainous landscapes bear evidence of degradation due to anthropogenic and climate-induced forest fires intensified by the extended period of drought. The implication is that leaf litter become combustible due to the extended drought and create a favorable condition for fire to persist when such materials are ignited by cigarette butts or other drivers. Two studies in Seychelles (Senterre, 2009;

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Kapisen, 2008) corroborate the view that the most important factors that determine the impact of forest fire in Seychelles are the depth of the leaf litter layer, the amount of flammable plant species such as the fern *Dicrapnopteris linearis* and the intensity of the fire. According to this study, when there is not much leaf litter on the ground, the fire passes quickly and some of the native palms survive.

The Global Environment Facility (2020) further reported that bush fire is the main threat to the unique forest ecosystem on Praslin. Also, the long and harsh drought periods intensified by climate change increases the risk of their occurrence as well as intensity once alight. It should be noted that Praslin's La Hauteur watershed in the Midland region suffered from 12 recorded human-induced forest fires between 1996 and 2008. Furthermore, the year 2008 witnessed two remarkable forest fires based on the extent of damage caused - at Fond d'Albaretz on Praslin, which affected 4.2 ha of prime native vegetation, and the other at the summit of Ste Anne Island, where some 40 ha was burned, including large areas with endemic palms and shrubs (Kapisen, 2008). A complicating factor is that most burned, severely degraded land is privately owned and private land owners are required neither to rehabilitate degraded lands nor to provide government with access for land rehabilitation (MEECC and UNCCD, 2018).

Regarding the determinants that influenced household participation in forest rehabilitation activities is linked to membership in a CBO. This result can be attributed to TRASS approach of working in collaboration with CBOs in forest rehabilitation activity which is a common approach that yield better outcome as highlighted by another study in Nepal (Gurung et al., 2013). However, the results further revealed that community members registered greater participation in the implementation phase rather than the planning and monitoring phases of forest rehabilitation. A much greater engagement of different stakeholders in the implementation phase spanning from the private sector, the government, NGOs and CBOs in forest rehabilitation has been made possible by the TRASS that has enabled tree planting capacity to increase over the years. For example, tree planting occurred on 5 ha of land as part of the forest rehabilitation initiative between the year 2016-2017 and another 18 ha between 2019-2020 implemented by the TRASS within the framework of the Ecosystem Based Adaptation (EBA) Seychelles Project. Over 20 volunteer organizations and 1,300 community members have benefitted from both projects to date. The community has also increased its resilience and ability to adapt to climate change. A core group of 12 individuals, in addition to a peripheral group of 20, were trained to transfer knowledge and know-how to the wider community. Through training and upskilling initiatives, local community capacity has been enhanced to ensure that they continue towards the end goal of developing, implementing, and sustaining their own solutions to current problems (GEF, 2020). The importance of forest rehabilitation was reiterated by one of TRASS volunteers as shown below:

"Through the project, I not only learned plant propagation techniques, I was also able to improve my livelihood and household income by earning additional income from plant preparation."

Such expression goes to reaffirm that community engagement in forest rehabilitation enhances skills and also provide financial benefits even to non-members of the core organization driving the initiative – a view supported by Blay et al. (2008). Another study in the Seychelles also mentioned continuous engagement with schools and local communities is an effective way to pass on relevant information and raise awareness on sensitive issues regarding management of forests (Senterre, 2015).

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The EBA project provided funding for the rehabilitation of fire ravaged forest lands on Praslin's Fond B'Offay watershed which also led to the creation of the Watershed Committee. Given the nature of the terrain and soil types, forest rehabilitation was a learning-by-doing process and that explains the relatively small area that was rehabilitated during phase 1 of the EBA project. In addition to implementation of experimental trials which is time consuming, resource persons from TRASS also mentioned that getting the right contractors on-board was a major challenge because not all of them could work in the burnt, baked sun, dense shrubs and steep terrain. However, lessons learnt during phase 1 led to the development of best practices and coupled with greater community participation resulted to 18 ha of land rehabilitated during phase 2 of the project as opposed to 5 ha during phase 1. Community involvement through volunteerism is considered a priority for tree planting activities while members of the TRASS prepare the planting strips and grow nursery stocks. Therefore, good collaboration between stakeholders and communities provides greater success in forest rehabilitation on Praslin – a finding supported by another study from Zimbabwe (Matsvange et al., 2016).

In terms of benefits to community members, forests are known to boost fresh water storage through the root system of plants that allow for effective filtration of water (Thompson et al., 2011). However, an earlier study in Seychelles documented historical evidence supporting the importance of forest in providing livelihood benefits especially as a source of income for the country prior to independence. For example, 40% of the country's GDP was derived from the agricultural sector in 1970 through the export of copra and cinnamon. During this period, both the government and private companies engaged in exportation of forests products such as timber, Coco-de-mer, cinnamon and palm hearts which in turn created several local employment opportunities. With the fast-growing tourism industry in the 1990's, pressure was released from forestry-related activities (Emerton, 1997), enabling the transitioning from agriculture and forestry to tourism and fisheries dependent economy. Such a transition in the economy has meant that the forestry sector is generally perceived as marginal with its GDP contribution, including wood industries estimated at 0.4% (MEECC and UNCCD, 2018). That notwithstanding, the forest ecosystem continues to provide several indirect economic and environmental benefits on Praslin and elsewhere in the Seychelles. For example, the extremely vulnerable water supply is highly dependent on the vegetation cover provided by the forest (MEECC and UNCCD, 2018).

Despite the benefits provided by forest rehabilitation to local livelihood and the environment in terms of ecosystem goods and services, community members also mentioned some of the challenges encountered in the forest rehabilitation process. Technical challenges manifesting in the form of inadequate knowledge and skills for tree planting on steep slopes often covered with boulders and coupled with soil erosion were of greater concern. Therefore, specific technical skills and resources are needed for the rehabilitation of upland forests that in some cases has proven more difficult than the coastal and lowland zones – a finding that is supported by another study conducted in Seychelles (Kapisen, 2008). In addition, climate variability and change are another challenge that confront forest rehabilitation in Seychelles with too much rainfall and droughts during across different years. One of the respondents reported that "*this year 2020 we faced challenges with heavy rainfall down steep slopes uprooting and carrying away plants in some instances while in some cases, seedlings were deeply buried into the soil by eroded sediments. Hence, a lot of maintenance to remove sediments from*

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around the plants and also maintaining the stone barriers constantly that act as erosion control barriers. Another problem that we have notice is the proliferation of invasive native creepers which covers and strangles seedlings especially during the early stages of growth". Another manifestation of rainfall variability is drought which sometimes occur over an extended period than usual during the dry season. Dry spells also create conditions for fire to spread quickly and in some cases due to negligence especially during slash and burn agriculture, ignition from cigarette butts, bee farming, among others that cause fire which easily spreads in the forest (Etongo, 2021). According to the same study, landslide also pose a problem to the health of the forest. For example, landslides create gaps which are invaded by invasive alien species (IAS) such as Albizia and Clidemia which compete and overgrow native species.

Some suggestions were proposed by community members in order to improve forest rehabilitation activities on Praslin. More awareness and education campaigns, the production of more seedlings of native tree species and additional manpower were the three most reported strategies. The lack of monitoring associated with little manpower from the responsible ministry and other relevant stakeholders such as the Seychelles National Parks Authority (SNPA) has also contributed to a low positive outcome regarding previous rehabilitation activities (Seychelles News Agency, 2020). The role of contractors seems to have diminished over time especially after the creation of the TRASS eleven years ago. On the other hand, government institutions such as the SNPA and MACCE work closely with TRASS that already have a wider reach to community members. In general, local community members on Praslin are more involved in forest rehabilitation when compared to other actors such as hired contractors.

In addition, improvement towards community participation also featured prominently which could partly be linked to better communication on TRASS planned activities especially to people not using social media, on the one hand, and creating an enabling environment for ownership that will motivate participation at the planning, implementation and monitoring phases of forest rehabilitation. Information gathered during the SDWs from TRASS representatives stated that "*We also want to develop initiatives with economic benefits e.g. beekeeping on rehabilitated sites, agroforestry, trails for tour guides but funding still remains a major constraint*". Furthermore, the creation of the watershed committee was cited as a step in the right direction that will enhance community participation and also develop skills through peer-to-peer trainings. Wekesa (2017) also reaffirmed that community involvement in forest rehabilitation can develop skills and create employment opportunities for locals. However, it is inconclusive whether financial incentives will increase the level of participation of community members in forest rehabilitation and at what stage. As such, further study is needed given that forest rehabilitation offers a wide range of benefits, the majority of which provides indirect economic benefits.

Conclusions

This study demonstrated that the predominant causes of forest fires on Praslin based on the perceptions of community members was ignition from cigarette butts followed by an extended drought period during the dry season. In some instances, the fire ravaged forests after losing its vegetative cover and soil organic layer, will crust and bake under the scorching sun and become hard thereby presenting a challenge to forest rehabilitation. The influence of climate and the loss of plant biomass implies that forest

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rehabilitation activities are important to Sustainable Development Goals 13 and 15 – Climate Action, and Life on Land.

Forest rehabilitation requires the active participation of community member whose engagement in the process could be passive or active with the influence of several factors at play ranging from economic, socio-cultural, and institutional among others. Membership in a CBO was the only determinant at the household level that was significant regarding participation in forest rehabilitation while other factors such as household size, age, gender and number of schooling years were not. Therefore, communication channels that can provide access to information regarding planned activities of TRASS to non-social media users through different platforms such as News Papers, Schools, Radio and Television Stations should be considered as strategies to reach a wider proportion of community members. However, resource persons from TRASS did mentioned that these communication platforms have been used in the past but did not prove effective and hence not cost effective.

Both males and females of different age groups including teenagers participated in forest rehabilitation. Forest rehabilitation is a process and not an activity of which majority of the community members were mostly involved in the implementation phase participating in the transportation of tree seedlings and tree planting. Some community members do participate in plant propagation given that they have acquired trainings provided by TRASS from which additional income are derived. However, majority of the householder perceived in-kind benefits provided by forest rehabilitation through the provisioning of ecosystem goods and services such as soil protection services from erosion, biodiversity conservation especially for native tree species, landscape improvement and the protection of watersheds. Another important benefit cited was the creation of the Praslin Watershed Committee within the framework of the EBA project. Members of this committee have benefited from basic trainings to ensure the continual implementation of best practices in forest rehabilitation.

Furthermore, community members also mentioned the lack of specialized skills to enable the effective rehabilitation of specific sites that are predominantly mountainous with limited access due to very steep slopes. Therefore, technical challenges manifesting in the form of inadequate knowledge and skills for tree planting on very steep slopes were of greater concern especially those covered with boulders and also exposed to soil erosion. This reinforces the point that forest rehabilitation needs to be adaptive to prevailing local conditions and it is a learning-by-doing process through adaptive management in order to develop best practices that can ensure greater success. The phase 1 of the EBA project on Praslin clearly demonstrated this in which just 5 ha of forest was rehabilitated as opposed to 18 ha during phase after which some best practices were developed based on challenges encountered in phase 1. Currently, TRASS is conducting an erosion control experiment in order to determine soil loss under two scenarios – land with tree cover, and land without tree cover with plots established on same side of the slope and at the same inclination. Such an experiment will provide quantitative information and recommendations on soil loss from erosion on the one hand, and effective soil and water conservation techniques on the other hand, to be implemented on mountainous forest landscapes. Recommendations for future studies are needed in the following key areas as follows: (i) factors affecting the survival of tree seedlings on rehabilitated lands, (ii) impact of forest fire frequency on tree diversity and species regeneration, (iii) does tourism affects the frequency of forest fires, and (iv) a multi-criteria analysis of ecosystem services derived from rehabilitated forests.

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Lastly, some of the strategies for improvement as suggested by community members included awareness and education campaigns, more manpower needed especially during the cutting of strips in dense shrubland for planting, to develop an efficient monitoring and evaluation system, conduct regular enrichment planting, eradication of invasive species, and also greater involvement of community members at the planning stage of forest rehabilitation. Further strategies for improvement have been proposed by resource persons from TRASS who were part of the SDWs. One of the strategy that seems to have been tried in the past and has proven to increase motivation of community members to participate in forest rehabilitation is called the "treat system". According to TRASS, "*volunteers receive points whenever they participate in our activitieas and the volunteers with most points are favoured to benefit from 'treats' such as an island trip, a boat trip, international training etc. This works very well because it provides incentives to volunteers as they look forward to the 'treat' and compete amongst themselves for most points. But managing this is quite a challenge if there is not someone totally dedicated to it*". Another strategy proposed by TRASS is to continue to engage school children which is seen as a good way forward. One of the respondents stated that "*we have proof of kids who are now young adults working in the field of environment after growing up with TRASS. Kids also bring along their parents hence having more participation*". Finally, TRASS proposed another financial incentive mechanism through a different lens and mentioned that "another strategy TRASS would like to work on is one involving economic benefits like beekeeping, tour guiding, agrotourism and agroforestry by having a sort of cooperative where people who participate to rehabilitate degraded lands via the above-mentions approaches receives financial benefits from these activities.

Acknowledgements. We would like to thank the National Bureau of Statistics (NBS) Seychelles for guiding the sampling process based on available information from its comprehensive database of households across the Seychelles. The contributions of resource persons from the TRASS, MACCE, SNPA and all the community members that participated in survey, are greatly acknowledged. Lastly, we are thankful to the following students in the Environmental Science Program at the University of Seychelles who participated in the household interviews in the person of Maryssa Samedi; Bianca Marzocchi; Kelsy Gill; Alisha Belle; Kelly Etienne; Elgin Créa; Larrisha Serret; Louisette Hoareau.

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APPENDIX

Appendix 1. *A comprehensive lists of indigenous, endemic and exotic tree species planted bY TRASS on degraded forest areas on Praslin Island*

Local name	Scientific name	Status
Bwa dir	*Pyrostria bibracteata*	Indigenous
Bwa gayak	*Intsia bijuga*	Indigenous
Bwa kalou	*Memecylon elaeagni*	Endemic
Bwa kwiyer	*Tabernaemontana caffeoides*	Indigenous
Bwa mon per	*Pouteria obovata*	Indigenous
Bwa ponm	*Syzygium wrightii*	Endemic
Bwa rouz	*Dillenia feruginea*	Endemic
Bwa sandel	*Draceana reflexa*	Indigenous
Bwa siro	*Premna seratifolia*	Indigenous
Bwadnat	*Mimusops sechellarum*	Endemic
Kafe maron gran fey	*Paragenipa wrightii*	Endemic
Lafous gran fey	*Ficus lutea*	Indigenous
Lagati	*Adenantera pavivona*	Indigenous
Letiver	*Mapanea sp.*	Endemic
Prin maron	*Ludia mauritiana*	Endemic
Vetiver	*Chrysopogon zizanioides*	Exotic
Bwa bannann	*Polyscias crassa*	Endemic

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1905_41854217

Bwa koulev	*Psychotria pervillei*	Endemic
Bwadrenet	*Dodonaea viscosa*	Indigenous
Kafe maron pti fey	*Erythroxylum sechellarum*	Endemic
Koko maron	*Curculigo seychellensi*	Endemic
Latanyen fey	*Phoenicophorium borsigianum*	Endemic
Latanyen milpat	*Nephosperma vanhoutteanum*	Endemic
Palmis	*Deckenia nobilis*	Endemic
Vakwa parasol	*Martiledendron hornei*	Endemic
Bwa zoliker	*Pittosporum senacia subsp. wrightii*	Endemic
Kapisen	*Northea hornei*	Endemic
Lantannyen lat	*Verschaffeltia splendida*	Endemic
Lantannyen oban	*Roscheria melanochaetes*	Endemic
Lerb razwar	*Lophoschoenus hornei*	Endemic

Appendix 2. *Participation of community members at the planning, implementation and monitoring stages of forest rehabilitation on Praslin*

Location		**Location of householder**		**Total (*N*)**
		Grand Anse	**Baie St. Anne**	
Planning Stage	Attended meetings	9 (*20.9*)	10 (*23.3*)	19
	Suggested information	11 (*25.6*)	10 (*23.3*)	21
	Suggested any idea	10 (*23.3*)	10 (*23.3*)	20
	Motivated community members	9 (*20.9*)	14 (*32.6*)	23
	Shared information	10 (*23.3*)	13 (*30.2*)	23
	Total (*N*)	**49**	**57**	**106**
Implementation	Raise nursery	18 (*11.7*)	10 (*6.5*)	28
	Prepare sites to be restored	14 (*9.1*)	17 (*11.0*)	31
	Select nursery tree species	14 (*9.1*)	10 (*6.5*)	24
	Transportation of seedlings	54 (*35.1*)	53 (*34.4*)	107
	Planting of trees	64 (*41.6*)	53 (*34.4*)	117
	Attended training programs	56 (*36.4*)	60 (*32.5*)	116
	Total (*N*)	**220**	**203**	**423**
Monitoring	Participated in site visit	8 (*25.8*)	5 (*16.1*)	13
	Engaged in enrichment planting	17 (*54.8*)	5 (*16.1*)	22
	Motivate other community members	10 (*32.3*)	6 (*19.4*)	16
	Maintain fire breaks	7 (*22.6*)	2 (*6.5*)	9
	Collect data for TRASS	6 (*19.4*)	3 (*9.7*)	9
	Total (*N*)	**48**	**21**	**69**

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DOI: http://dx.doi.org/10.15666/aeer/1905_41854217

Appendix 3. *Questionnaire*

Assessing local communities' involvement in the rehabilitation of degraded forests: some lessons from Praslin Island

I am a final year student at the University of Seychelles currently doing my 3rd year thesis in Environmental science within the framework of the Ecosystem Based Adaptation to Climate Change Project Seychelles. My research aims to assess community involvement in forest rehabilitation on Praslin. The feedback provided from participants will shed light on the factors that influence community involvement and thus encourage relevant stakeholders including experts and scientists to better engage with communities. The findings of this study can also help decision makers to better plan and allocate funding for rehabilitation activities and adapt this strategy in other areas. Your participation is very important to the success of my thesis and the information provided will be treated with all confidentiality.

Section A – Socio demographic information

1. Gender: □ male, □ female
2. Age ……..
3. Family size (in numbers) ……...
4. How many household members are between the ages 16 and 60 years of age?.....
5. Of the household members that are between the ages of 16 – 60 years (Q. 4 above), how many of them are employed?.....
6. Gender of the household head. □ male, □ female
7. Address: _____

Section B – Socioeconomic information

8. Highest level of education
   □ Primary
   □ Secondary
   □ Post-Secondary
   □ University degree/higher

8. Occupation
   □ Farmer
   □ Fishermen
   □ Business owner
   □ Civil servant
   □ Others

9. Do you directly financially benefit from rehabilitation?
   □ yes , □ no , □ I don't know

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DOI: http://dx.doi.org/10.15666/aeer/1905_41854217

Section C

10. Are you a member of any community organization on Praslin?
□ yes , □ no , □ I don't know

11. What do you think could be the main cause of forest fire on Praslin?
_____

12. Do you think tree planting benefit fire ravaged sites through …

		Yes	No	I don't know
i)	Soil protection from erosion			
ii)	Protection of biodiversity			
iii)	Improving the landscape			
iv)	Improving water quality and quantity			
v)	Carbon capture for climate change mitigation			
vi)	Other: _____			

13. Do you think … is a problem for tree planting on fire ravaged sites?

		Yes	No	I don't know
i)	Shortage of seedlings			
ii)	Recurrent fire			
iii)	High tree mortality rate			
iv)	Inadequate knowledge/skills on restoration			
v)	Ownership of planted trees			
vi)	Other: _____			

14. Have you ever participated in Rehabilitation work on Praslin?
□ yes , □ no

15. (a) If, **yes** to the question above, with which of the following organizations?
□ SNPA , □ MEECC , □ TRASS , □ Others

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16. (b) Frequency of participation?
□ Once, □ Several times per week, □ Several times per month, □ Several times per year

(But if no to the question above, please move to Section E. Those who answer yes, should end in Section D)

Section D – Participation indicators at different stages of forest rehabilitation

Planning stage

17. Did you participate in any of the following activities?

	Yes	No	I don't know
Attended planning meetings for forest restoration?			
Suggested information on how the fire ravaged forest should be restored			
Suggested any idea during planning regarding techniques in the restoration of the fire damaged sites such as track cutting, weeding, preferred tree species to be planted, etc.			
Motivate your fellow community members to participate in planning regarding forest restoration on Praslin?			
Share information or experience about forest restoration after participating in planning meetings?			

Implementation stage

18. Did you participate in any of the following activities?

	Yes	No	I don't know
Raising the TRASS nursery either through financial contribution or labour			
Preparing the restored sites in activities such as track cutting, slashing, fire breaks, etc.			
Selecting the tree species raised in the nursery			
Transportation of seedlings from the nursery to the sites to be restore			
Tree planting at the fire damaged sites			
Training program on restoration on issues such as how to raise a nursery, types of trees to be planted, how to restore degraded sites, etc.			

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DOI: http://dx.doi.org/10.15666/aeer/1905_41854217

Monitoring stage

19. Do you participate in any of the following activities?

	Yes	No	I don't know
Site visit after trees are planted in the degraded site			
Replanting of trees that could not survive			
Motivate fellow community member to contribute labour or money for replanting, weeding, track cutting, etc.			
Maintaining fire breaks			
Providing/collecting data for TRASS database			

Section E (Non-Participants)

20. Do you know of the NGO TRASS? □ yes , □ no , □ I don't know

21. Do you know of the work that they do? □ yes , □ no , □ I don't know

22. Do you think the work they are doing is important? □ yes , □ no , □ I don't know

23. How?.....
.....

24. Do you wish to participate in rehabilitation works undertaken by TRASS?
□ yes , □ no , □ I don't know

25. If yes to the above question, what is currently preventing you from participating?
□ Time constraint
□ Access to site/too far and steep
□ Lack of information on activities
□ Health issues
□ Others (please specify)
……
……

26. There are different activities involve in the rehabilitation works undertaken by TRASS. Are any of the following activities of interest to you?
□ Nursery Maintenance
□ Track cutting
□ Tree planting
□ Site monitoring

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27. Which of the stages of forest restoration will you like to be involved in? (Multiple choice question)
    □ Planning stage
    □ Implementation stage
    □ Monitoring stage

28. What do you think could be done to improve on the restoration of the fire ravaged forests on Praslin?
    _____

29. Would you like to be contacted for future activities? □ yes , □ no , □ I don't know
    Contact details: _____

* * *

Estimated distance from home to rehabilitated forest (km) _____

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