ALLELOPATHIC EFFECTS OF AQUEOUS EXTRACTS FROM DIFFERENT PLANT PARTS OF CANADA GOLDENROD (*SOLIDAGO CANADENSIS* L.) ON SEED GERMINATION AND SEEDLING GROWTH OF KOREAN LAWNGRASS (*ZOYSIA JAPONICA* STEUD.)

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Abstract. The invasion mechanism *of Solidago canadensis* L., been extensively investigated as its one of the most destructive exotic invasive plants. The present study showed that allelopathy is beneficial to the successful invasion of invasive alien species (IAS). However, the main allelopathic part of *S. canadensis* L. is less known to the researchers. Therefore, we experimented with the allelopathic effects of extracts obtained from various parts of *S. canadensis* L. (root, litter, aboveground parts) at concentrations of 50 g L^{-1} and 150 g L^{-1} on seed germination and seedling growth of *Zoysia japonica* Steud. The results showed that the extract from the *S. canadensis* L. aboveground parts had prominent inhibition effects on Zoysiagrass seed germination rate, germination potential, vigour value, root length and plant height, and the suppression increased significantly at high concentrations. With the treatment of aqueous extract from the aboveground parts of *S. canadensis* L. (50 g L^{-1}), the content of malondialdehyde (MDA) concentration and peroxidase (POD) activity increased significantly. The root extract at the concentration of 50 g L^{-1} did not affect the parameters except POD activity, but the concentration of 150 g L^{-1} inhibited all the parameters significantly except catalase (CAT) activity. Litter aqueous extracts did not show any significant effect on any parameters of *Zoysia japonica* Steud.
Keywords: *allelochemicals, growth development, invasion, invasive species, litter extracts, enzyme activity*

Introduction

Allelopathy refers to the beneficial or harmful effects of one plant on another plant, in both crop and weed species, caused by release of biochemicals, known as allelochemicals, from plant parts by leaching, root exudation, volatilization, residue decomposition, and other processes in both natural and agricultural systems. It can also be defined as the phytotoxicity of a component or a series of components released from plant parts by root exudation, volatilization, leaching or residue decomposition of susceptible plants (Ferguson et al., 2013; Wang et al., 2019; Anžlovar and Anžlovar, 2019). The phenomenon of allelopathy has been used to explain how some invasive species harm to native ones or even produce specific biochemicals (Callaway, 2002; Hu and Zhang, 2013; Sun et al., 2017). Furthermore, the allelopathy has been involved in the success of certain widespread plant invaders. According to novel weapons hypothesis, invasive species exude unique biochemical constituents may contribute to their successful invasion (Callaway and Aschehoug, 2000; Zhang et al., 2017).

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Allelochemicals are a subset of secondary metabolites not required for metabolism (growth and development) of the allelopathic organism. Allelochemicals with negative allelopathic effects are an important part of plant defense against herbivory (i.e., animals eat plants as their primary food) (Lorenzo et al., 2011; Ullah et al., 2021). More specifically, it was explained in the novel weapons hypothesis that through the production of biochemicals some invasive plants regenerate successfully in the invaded range. Thus these biochemicals may have the potential to cause negative effects on native plants. Moreover, the novel biochemical effects widely used in competing plants, soil biota and generalist herbivores (Wang et al., 2017a; Zhang et al., 2017; Qi et al., 2020). The basic approach used in allelopathic research for agricultural crops has been to screen for both crops and natural vegetation for their capacity to suppress weeds. To demonstrate allelopathy, plant origin, production, and identification of allelochemicals must be established as well as persistence in the environment over time in concentrations sufficient to affect plant species (Lorenzo et al., 2011; Wang et al., 2017b, 2020c).

S. canadensis L. is a perennial rhizomatic plant and belongs to Compositae (Zhang and Wan, 2017; Anžlovar and Anžlovar, 2019). In the 1930s, it introduced into China as an ornamental plants from North America and originally cultivated in Shanghai and Nanjing area, and later spread to the wild. In the 1980s it began to spread to Jiangsu and Zhejiang area. The spread of *S. canadensis* L. causes serious damage to the ecological environment of China and considered as an invasive malignant weeds (Wang et al., 2020b). At present, there are many studies found about the allelopathy of *S. canadensis* L. (Yuan et al., 2013; Wang et al., 2019) found that the extracts of *S. canadensis* L. showed greater allelopathic effects on seedlings of native plants in the new location than in the original location, and the allelopathy enhance the competitive ability of *S. canadensis* L. in the invaded range (Yuan et al., 2013). Also, they reported that the secondary metabolites of *S. canadensis* L. may increase its competitiveness through enhancing the AMF symbionts (Abhilasha et al., 2008; Wang et al., 2020a) demonstrated that root exudates of *S. canadensis* L. significantly inhibited Arabidopsis growth and it was concentration-dependent. What's more, the addition of activated carbon can relieve the inhibitory of tested plants under more realistic conditions in soil (Wang et al., 2018). Sun et al. (2006a) investigated the effects of ethanolic and aqueous extracts from leaves, stems and rhizomes of *S. canadensis* L. to mulberry, morning glory, wheat and rape seed germination and seedling growth. They found higher concentration of this extracts can both significantly inhibit seed germination and seedling growth of the four species (Wu et al., 2019).

However, these studies mainly focus on the competitiveness between different plant species or the organic solvent of the whole plant or an organization tissue to evaluate the allelopathic effect, but whether these so-called allelochemical can release to the soil environment through the leaching and decomposition of litter and have a real ecological effect also deserves further research. Some researches already demonstrated the aqueous extracts allelopathy in different parts of invasive plants (Gao et al., 2009; Li and Jin, 2010; Wei et al., 2020), but rarely the allelopathy of *S. canadensis* L. has been noticed Therefore, our study use aqueous extracts of different parts of *S. canadensis* L. applied to *Zoysia japonica* Steud. (Zoysiagrass) seeds germination and seedling inhibition experiments to clarify its allelopathic inhibition mechanism and provide a basis to the assessment of actual ecological effect of allelochemical from *S. canadensis* L. to the soil environment.

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Materials and methods

Plant materials

There were two species used in this experiment i.e. *S. canadensis* L. and *Zoysia japonica* Steud. (Zoysiagrass). The *Zoysiagrass* seeds was used in the experiment bought from seeds market in Zhenjiang of Jiangsu Province, China. In December 2013, root, litter, aboveground parts of *S. canadensis* L. were collected from wild in Zhenjiang of Jiangsu Province, China (32.171058° N, 119.540535° E).

Preparation of S. canadensis L. extracts

The plant samples of *S. canadensis* L. were separated into root, litter and aboveground parts; each plant part was cut into 2-3cm pieces, which were soaked for 48 h with 1L distilled water and then filtered. The final concentration of the root, litter, and aboveground parts aqueous extracts of *S. canadensis* L. was 400 g L^{-1}, 150 g L^{-1}, 180 g L^{-1}, respectively. All the aqueous extracts stored at 4°C.

Effects of aqueous extracts on seed germination and seedling growth of Zoysiagrass

Thirty seeds of *Zoysiagrass* were sown in Petri dishes (9 cm in diameter) containing 10 mL aqueous extracts (the root, litter, aboveground parts aqueous extracts of *S. canadensis* L.) or 10 mL distilled water (control). Two concentrations were set for each aqueous extract i.e. 50 g L^{-1} and 150 g L^{-1} with five replicates per treatment. The Petri dishes were cultivated at room temperature (20°C) and the germination rate was measured daily for 5 days.

Lipid peroxidation and enzyme analyses

Growth parameters, content of malondialdehyde (MDA) as well as catalase (CAT) and peroxidase (POD) activity were assayed to evaluate the effects of allelochemicals and aqueous extracts from different parts of *S. canadensis* L. Lipid peroxidation was determined in 0.5 g fresh whole plants by measuring the content of MDA, a production of lipid peroxidation, by the thiobarbituric acid reaction (Gossett et al., 1994). 0.5 g whole plants were collected and ground with mortar with pestle on the ice immediately, suspended in 2 ml 50 mM phosphate buffer (PBS) at pH 7.8. Extracts were centrifuged at 12,000 × g for 20 min (4°C) and the supernatant was used for the determination of enzyme activity. CAT activity was assayed in a reaction solution (3 ml) containing 0.3% H_2O_2 and distilled water. The reaction was started by adding 100 µl crude extracts to the reaction solution and the activity was followed by monitoring the decrease in absorbance at 240 nm as a consequence of H_2O_2 consumption. POD activity was assayed in a reaction solution (3 ml) including 50 mM PBS at pH 7.0, 0.2% guaiacol, 0.3% H_2O_2 and 100 µl crude extracts. The increasing absorbance on account of oxidation of guaiacol was measured at 420 nm (Xu et al., 2015).

Determination of indexes and methods

In accordance with the rules of the international seed germination (We considered radicle length is half of the seed as germination). Determination of germination index formula is (Gao et al., 2009):

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$$Germination\ rate\ (\%) = (no.of\ germinated\ seeds \div total\ no.of\ seeds) \times 100 \quad \text{(Eq.1)}$$

$$Germination\ potential\ (\%) = (4th\ day\ no.of\ germinated\ seed \div total\ no.seeds) \times 100 \quad \text{(Eq.2)}$$

Daily germination number in the 4th day was the maximal (Jing, 2006).

$$Vigour\ value\ (V) = \left(\frac{a}{1} + \frac{b}{2} + \frac{c}{3} + \frac{d}{4} + \cdots \frac{x}{n}\right) \times \left[\frac{100}{S}\right] \quad \text{(Eq.3)}$$

where a, b, c… respectively, represent the number of seeds germinated after 1, 2, 3 days of inhibition, x is the number after (n) days and (S) is total number of germinated seeds (El-Soud et al., 2013).

In addition, selected 10 seedlings randomly from each Petri dishes in the 6th day and measured the root length and plant height. Aboveground parts extracts at the concentration of 150 g L^{-1} absolutely inhibited the growth of *Zoysiagrass* seedlings. Thus we cannot get the data of MDA, CAT and POD in *Zoysiagrass* seedlings.

Statistical analysis

Measurements were performed by using five replicates randomly. All measurements were examined statistically through SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) and Origin Pro 9.0. Variance analysis with two crossed fixed factors was applied to discriminate the effects of aqueous extracts of *S. canadensis* L. on the seed germination of *Zoysiagrass* varied with different types and concentrations of the extracts. Data were presented in means ± SE. All the parameters in the control and treatments were compared for each aqueous extract using an ANOVA and (Newman-Keuls or Student–Newman–Keuls) tests to determine the significant differences ($P < 0.05$). The graphs were produced in origin pro9.

Results

Effect of the extracts on the seed germination of Zoysiagrass

The effect of the aqueous extracts of *S. canadensis* L. on the seed germination of *Zoysiagrass* varied with different types and concentrations of the extracts. As compared with the control, the aqueous extract from the *S. canadensis* L. litter (50 g L^{-1} and 150 g L^{-1}) showed no significant influence to the germination rate, germination potential and vigour value of *Zoysiagrass* seeds ($P > 0.05$; *Figs. 1,2,3*). The 50 g L^{-1} extract from the root had no obvious effect to Zoysiagrass seeds germination rate, germination potential and vigour value ($P > 0.05$), but the effects of 150 g L^{-1} extract were significantly inhibitory ($P < 0.05$; *Figs. 1,2,3*). The extract from the aboveground parts of *S. canadensis* L. showed significant inhibitory effect ($P < 0.05$), and the suppression strengthened with the increasing of the extract concentration (*Figs. 1,2,3*). The allelopathic effects of aboveground parts extract on *Zoysiagrass* seeds were the strongest, followed by the root extract, and smallest for the litter extract. At the concentration of 50 g L^{-1}, only

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aboveground parts extract significantly decreased the germination rate, germination potential and vigour index of *Zoysiagrass* ($P < 0.05$). Root extract and aboveground parts extract inhibited germination rate, germination potential and vigour index obviously at 150 g L^{-1} ($P < 0.05$), and aboveground parts extract showed stronger inhibitory effect (*Table 1*).

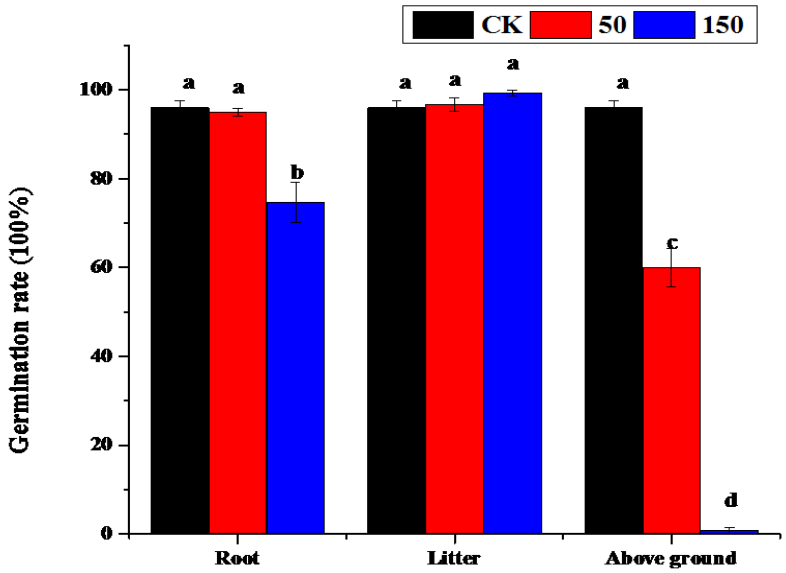

Figure 1. *Effects of the aqueous extracts of S. canadensis L. on the germination rate of Zoysiagrass seeds. Data with different superscript letters indicate a significant difference ($P < 0.05$). The concentrations of aqueous extract are given in g L^{-1}*

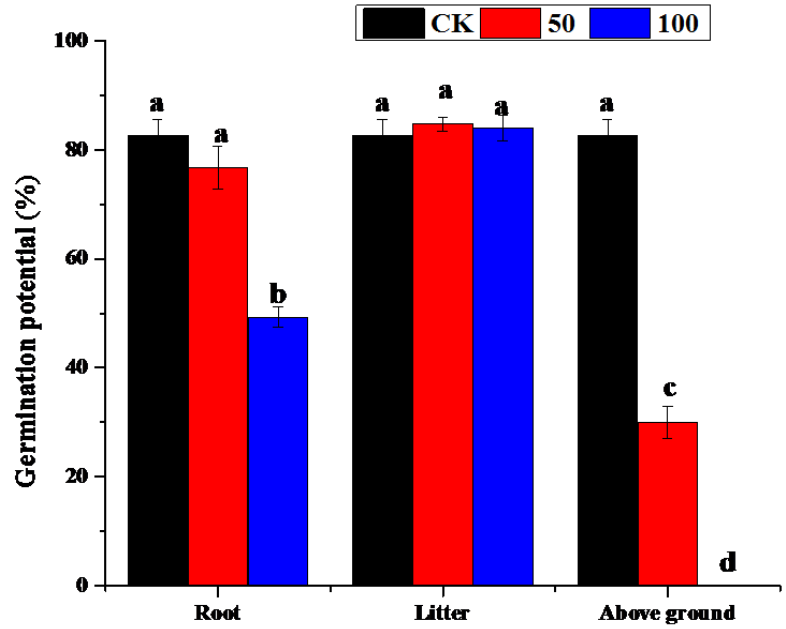

Figure 2. *Effects of the aqueous extracts of S. canadensis L. on the germination potential of Zoysiagrass seeds. Data with different superscript letters indicate a significant difference ($P < 0.05$). The concentrations of aqueous extract are given in g L^{-1}*

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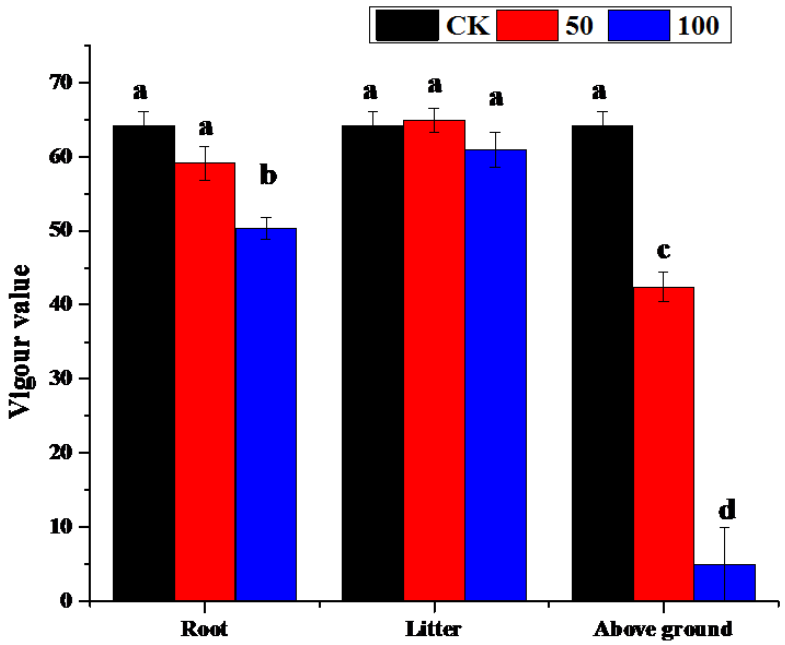

Figure 3. *Effects of the aqueous extracts of S. canadensis L. on the vigour value of Zoysiagrass seeds. Data with different superscript letters indicate a significant difference (P < 0.05). The concentrations of aqueous extract are given in g L^{-1}*

Table 1. *Summary of S-N-K tests of effect of treatment with different parts of S. canadensis L. indifferent concentrations*

	ck	Root		Litter		Aboveground part	
		50 g L^{-1}	150 g L^{-1}	50 g L^{-1}	150 g L^{-1}	50 g L^{-1}	150 g L^{-1}
GR	96.0±1.633a	95.3±0.816a	74.7±4.546b	96.7±1.491a	99.3±0.667a	60.0±4.346c	0.7±0.667d
GP	82.7±2.867a	76.7±3.944a	49.3±1.944b	84.7±1.333a	86.0±2.449a	30.0±2.981c	0d
VI	64.2±1.916a	59.1±2.270a	50.3±1.461b	64.9±1.652a	60.9±2.364a	42.4±2.019c	5.0±5.000d
PH	2.4±0.097a	2.4±0.116a	1.4±0.130b	2.3±0.113a	2.6±0.117a	0.8±0.100c	0.0±0.020d
RL	2.2±0.085a	2.2±0.075a	1.7±0.099b	2.1±0.076a	2.2±0.088a	0.4±0.064c	0.0±0.013d
MDA	2.0±0.113ab	1.4±0.085ab	1.7±0.177ab	2.1±0.131ab	1.9±0.294ab	2.7±0.304a	
CAT	9.2±0.467bc	8.0±0.686c	8.2±0.410c	10.3±0.787ab	11.0±0.338a	8.7±0.266bc	
POD	34.7±1.000b	24.6±1.060c	25.0±1.046c	32.2±2.439b	29.6±1.034bc	55.1±3.483a	

All the data present in mean ± SE, and data with different superscript letters behind indicate a significant difference (P < 0.05). Independent samples S-N-K tests were performed using SPSS version 17.0 (SPSS Inc., Chicaogo, IL). GR is germination rate, GP is germination potential, VI is vigour index, PH is plant height, RL is root length. The control treatment was conducted for each plant extracts

Effect of the extracts on the growth of the Zoysiagrass seedlings

Aqueous extract of *S. canadensis* L. litter (50 g L^{-1} and 150 g L^{-1}) and the 50 g L^{-1} extract from the root had no obvious impacts on the root length and plant height of *Zoysiagrass* seedlings (P > 0.05), but the aqueous extracts from root (150 g L^{-1}) and aboveground parts (50 g L^{-1} and 150 g L^{-1}) had significant inhibition on *Zoysiagrass* seedlings (P < 0.05), and the degree of suppression increased at a high concentration (150 g L^{-1}) (*Figs. 4,5*). Aboveground parts extract with the concentration of 50 g L^{-1} decreased the plant height and root length of *Zoysiagrass* while root extract and litter

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extract have no effects on them ($P < 0.05$). At the concentration of 150 g L^{-1}, root extract and aboveground parts extract significantly inhibited plant height and root length ($P < 0.05$), and aboveground parts extract had more negative effects (*Table 1*).

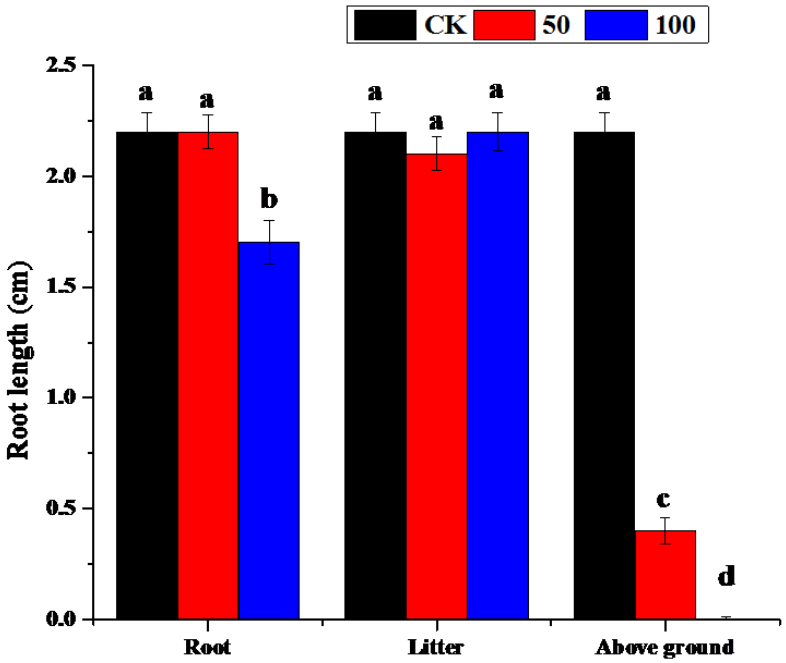

Figure 4. *Effects of the aqueous extracts of S. canadensis L. on the root length of Zoysiagrass seedlings. Data with different superscript letters indicate a significant difference ($P < 0.05$). The concentrations of aqueous extract are given in g L^{-1}*

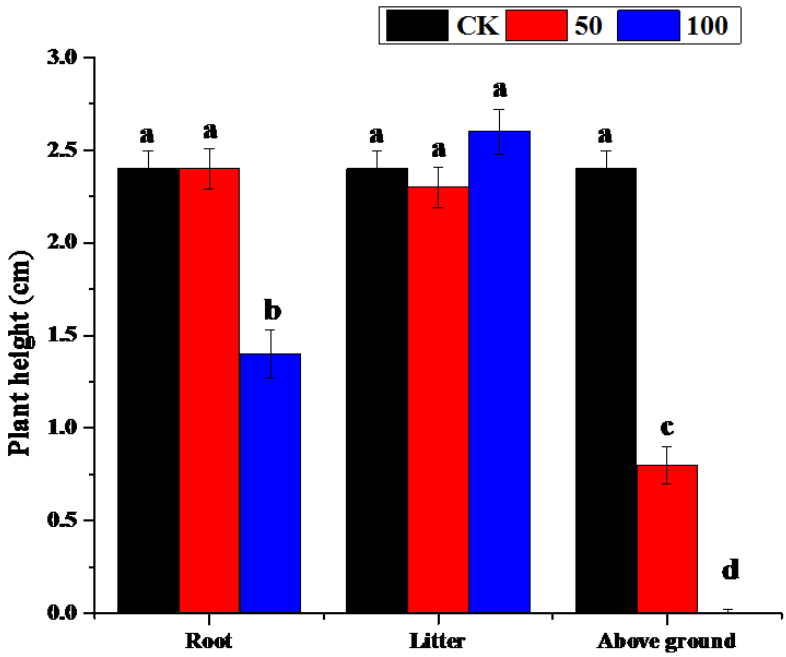

Figure 5. *Effects of the aqueous extracts of S. canadensis L. on the plant height of Zoysiagrass seedlings. Data with different superscript letters indicate a significant difference ($P < 0.05$). The concentrations of aqueous extract are given in g L^{-1}*

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Effect of the extracts on the MDA concentration of the Zoysiagrass seedlings

MDA content was a measurement of lipid peroxidation. The effect of the aqueous extracts of *S. canadensis* L. on the MDA concentration of the *Zoysiagrass* seedlings varied with different types of the extracts (*Fig. 6*). At 50 g L^{-1}, the root aqueous extract significantly decreased the MDA concentration of the *Zoysiagrass* seedlings ($P < 0.05$), but at 150 g L^{-1} the suppression was not significant compared with the control ($P > 0.05$). The aqueous extract of *S. canadensis* L. litters (50 g L^{-1} and 150 g L^{-1}) showed no obvious effects ($P > 0.05$). The 50 g L^{-1} aboveground extracts increased the MDA concentration obviously ($P < 0.05$).

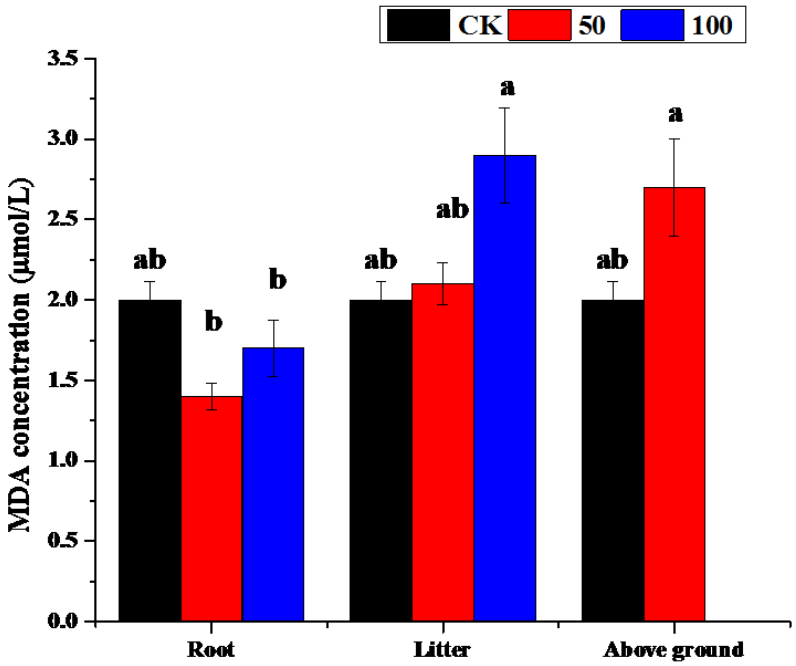

Figure 6. *Effects of the aqueous extracts of S. canadensis L. on the MDA concentration of Zoysiagrass seedlings. Data with different superscript letters indicate a significant difference ($P < 0.05$). The concentrations of aqueous extract are given in g L^{-1}*

Effect of the extracts on CAT and POD activity of the Zoysiagrass seedlings

The aqueous extracts of root, litter and aboveground parts from *S. canadensis* L. had no significant difference to the CAT activity of the *Zoysiagrass* seedlings ($P > 0.05$; *Fig. 7*). The extract of *S. canadensis* L. root (50 g L^{-1} and 150 g L^{-1}) significantly inhibited the activity of POD, while aboveground parts extract (50 g L^{-1}) obviously increased it (*Fig. 8*; $P < 0.05$). The litter extract of *S. canadensis* L. did not affect the POD activity ($P > 0.05$).

Relationship between different parameters of Zoysiagrass

Table 2 showed the pearson correlation analysis of all the parameters present in the experiment. MDA did not have any relationship with all the parameters, and POD had no obvious relationship with germination potential, plant height and as well as with root length. But beyond of this, the other parameters have significantly correlation with others.

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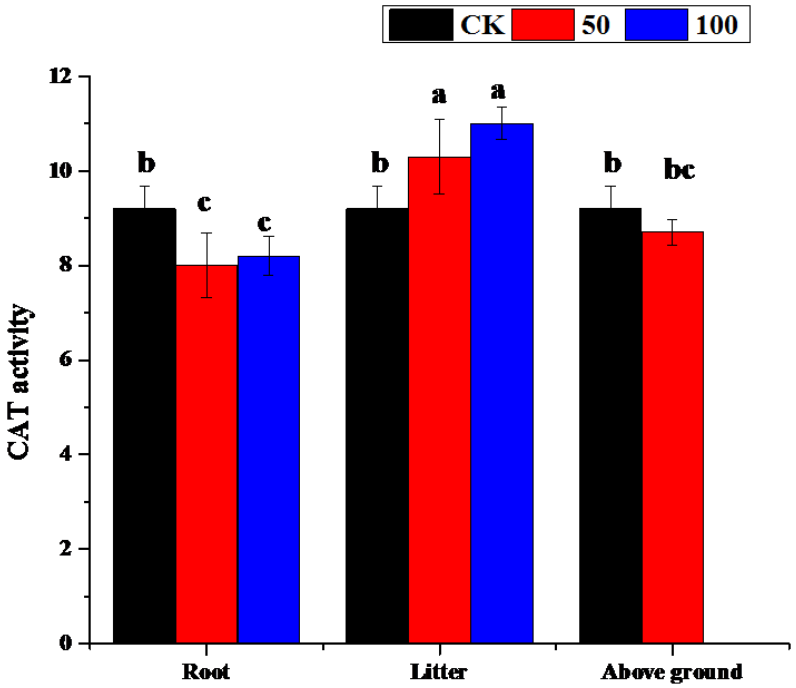

Figure 7. *Effects of the aqueous extracts of S. canadensis L. on the CAT activity of Zoysiagrass seedlings. Data with different superscript letters indicate a significant difference ($P < 0.05$). The concentrations of aqueous extract are given in g L^{-1}*

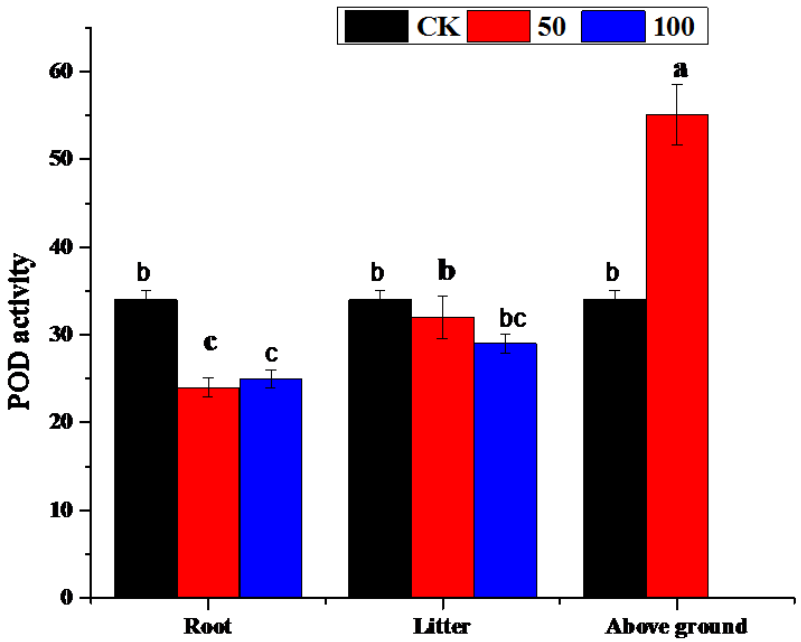

Figure 8. *Effects of the aqueous extracts of S. canadensis L. on the POD activity of Zoysiagrass seedlings. Data with different superscript letters indicate a significant difference ($P < 0.05$). The concentrations of aqueous extract is given in g L^{-1}*

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Table 2. *Pearson correlation analysis of all the parameters present in the experiment*

	GR	GP	VI	PH	RL	MDA	CAT	POD
GR	1	.946**	.945**	.931**	.897**	.267	.890**	.522**
GP		1	.925**	.973**	.931**	.298	.795**	.324
VI			1	.899**	.852**	.311	.858**	.518**
PH				1	.956**	.321	.745**	.279
RL					1	.036	.699**	.159
MDA						1	.265	.458**
CAT							1	.709**
POD								1

Behind each data, asterisks (*) show significant differences ($P < 0.05$) between each parameter. The analysis was performed using SPSS version 17.0 (SPSS Inc., Chicaogo, IL). GR is germination rate, GP is germination potential, VI is vigour index, PH is plant height, RL is root length

Discussion

The success of some of the worst plant invaders depend on the mechanism of allelopathy (Callaway and Aschehoug, 2000), including *Mikania micrantha* (Chen et al., 2009), *Ageratina adenophora* (Zhou et al., 2013) and *Fallopia japonica* (Dommanget et al., 2014). The significant inhibition that the aqueous extracts from the *S. canadensis* L. root and aboveground parts imposed on the germination rate, germination potential, vigour value, root length and plant height of *Zoysiagrass* seedlings indicated the existence of allelopathic water-soluble substances in the extracts. These results in accordance with the previous findings that aqueous extracts of *S. canadensis* L. suppress seed germination and seedling growth of some plants (Sun, 2006; Ledger et al., 2015). Moreover, some studies represented that the effects of different parts extracts of *S. canadensis* L were varied, and low concentrations of the extracts always showed positive effects on seed germination and seedling growth of native plants, and high concentration demonstrated negative effects on native seed germination and seedling growth (Sun et al., 2006b; Huang et al., 2009; Megenhardt, 2015). To some extent, our result in accordance with it, root extract and litter extract had no effect on Zoysiagrass seed germination, plant height and root length at low concentration (50 g L^{-1}) and inhibited these parameters at high concentration (150 g L^{-1}). Aboveground parts extract inhibited these parameters of *Zoysiagrass* at both 50 g L^{-1} and 150 g L^{-1}, and the inhibition strengthened with the increasing concentration (*Table 2*). In one of proceedings studies it was noted that growth rate reduced of target species in the presence of both aqueous and ethanolic extract of *S. canadensis* L. at different concentrations (Możdżeń et al., 2020). Similarly, in another study, leaf extracts with high concentration significantly decreased root length, leaf shape index, germination percentage, germination potential, germination index, germination vigor index, and germination rate index of lettuce (Wang et al., 2019).

Besides the reduction in growth of *Zoysiagrass* seedlings, the membranes were affected by *S. canadensis* L. aqueous extracts. Allelochemicals can impair the cell membranes through direct interaction with membrane constituents or due to impairment of some metabolic functions necessary to maintain the membrane functions (Bertin et al., 2003). Higher concentrations of aboveground part aqueous extracts of *S. canadensis* L. caused more serious lipid peroxidation damage, and the result was consistent with some other research (Han et al., 2012). But the root extract of *S. canadensis* L. caused obvious

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reduction of MDA, and it showed that the root extract can relieve the lipid peroxidation of the Zoysiagrass seedlings. Through the results, we speculated that there were some allelochemicals in aqueous extracts of root, litter and aboveground parts of *S. canadensis* L. These alllelochemicals could harm to cell membrances of the *Zoysiagrass* seedlings through direct interaction with a constituent of the menbrance or as a result of an impairment of some metabolic function necessary to the maintenance of membrane function.

As previous researches showed, CAT can be induced in plant species and CAT can produce and metabolise H_2O_2. What's more, CAT activity is directly regulated by the concentration of H_2O_2 and the accumulation of H_2O_2 can stimulate the CAT activity (Fornazier et al., 2002). Both the aqueous extracts of *S. canadensis* L. had no significant effects on CAT activity of *Zoysiagrass* seedlings, suggesting that the aqueous extracts could not induce and promote the metabolise of H_2O_2 in *Zoysiagrass* seedlings.

Aqueous extracts of *S. canadensis* L. strongly affects the activity of peroxidases in both root and aboveground parts. But it showed pronounced increase of POD activity in aboveground parts and decrease in root. Previous studies in Eucalyptus extracts and other allelochemicals on plant seedling illustrated that allelochemicals absorbed by plant cells should be antidotal and the antidotal action and other responses of plant cells caused the increase of POD activity. The decrease of POD activity of *S. canadensis* L. root extract, indicating the allelochemicals in the root injury the POD enzyme by toxicity. But some other researches gained the opposite results, higher concentration levels of *Pogostemon cablin* aqueous extracts might have exceeded the rate of detoxification which then result in inhibitory effect on plant growth and dramatically decreasing on POD activity (Xu et al., 2015; Meiners et al., 2017).

Conclusion

In conclusion, the aqueous extracts of *S. canadensis* L. could influence the *Zoysiagrass* seeds germination and seedlings growth through water-soluble allelopathic substances. And the regulation of the anti-oxidase activity of CAT and POD and the oxidization of the cell membranes may make a contribution to the effects. Our demonstration of suppression of Zoysiagrass by aqueous extracts of *S. canadensis* L. suggest that allelopathy plays a more important role than other mechanisms do in the out-competition of *S. canadensis* over other plants, and make it invasive in new habitats and offered an approach to explain the invasive of *S. canadensis* L. but the invasive mechanism still needs further study.

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IMPACT OF WHEAT (*TRITICUM AESTIVUM* L.) SEED CLEANING, HERBICIDES, AND THEIR INTERACTIONS ON WEED CONTROL, YIELD, AND YIELD COMPONENTS IN TWO DIFFERENT LOCATIONS

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Abstract. A factorial experiment (2 × 5) was applied according to Randomized Complete Block Design (RCBD) with four replications in two different locations, Qlyasan and Kani Panka in Sulaimani Governorate, Kurdistan Region of Iraq, during the winter season of 2018-2019. The objectives were to determine the effects of wheat (*Triticum aestivum* L.) seed cleaning (factor 1), weed control treatments (factor 2) and their interactions on weed control, wheat yield and yield components at two different locations. Seed cleaning decreased weed dry weight and increased number of spikes m^{-2}, number of grains $spike^{-1}$, weight of 1000 grains, grain yield m^{-2} and weed control efficiency significantly. Hand weeding and herbicides mixture decreased weed dry weight and increased grain yield.m^{-2} with some yield parameters, and weed control efficiency significantly. The interactions between locations and seed cleaning decreased weed dry weight, and increased grain yield and yield components and weed control efficiency significantly. Interactions between seed cleaning and weed control treatments decreased weed dry weight and increased grain yield m^{-2}, yield components, and weed control efficiency significantly. The triple interactions between locations, seed cleaning and herbicide mixture decreased weed dry weight and increased number of spikes m^{-2} 1000 grains weight, grain yield m^{-2} (6.36 $t.ha^{-1}$) and weed control efficiency significantly.
Keywords: *crop production, cleaning machinery, grain weight, clodinafop-propargyl, Granstar*

Introduction

Wheat (*Triticum aestivum* L.) is of prime importance in the kingdom of food crops standing second globally in terms of grain production and is the most widely cultivated food crop followed by rice, maize, sorghum and pearl millet. The total area of the world under wheat cultivation is around 220.5 million ha with a grain yield of 760.4 million tons, and an average wheat yield of 3.45 ton ha^{-1} (FAO, 2019).

Wheat production in Iraq is 5.1 million tons in over a total cultivated land of 2.12 million ha (FAO, 2014). In Kurdistan Region wheat fields occupy 567627 ha, which represents the largest acreage crop there, covering about 78% of rain-fed area (Al-Najafi, 1989), which has produced about 500000 tons during 2015, with an average wheat yield of about 0.88 ton ha^{-1}, this figure is very low comparing to the international average grain yield (only 25%), but there is potential opportunity to improve it significantly (Mazid, 2015). Weeds are a perennial problem for farmers; they are considered one of the important factors limiting crop production. Weeds are widely spread and reduce yield of crops considerably. Weeds also lower crop quality and may reduce the protein content of the grain (Monaco et al., 2002). Among the biotic factors weeds are one of the major constraints in wheat production as they reduce productivity due to competition,

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allelopathy and by providing proper habitats for pathogens as well as serving as alternate host for various insects, fungi and increase harvest cost (Ayana, 2019).

Farmers spend a lot of resources to reduce weeds impact, many a times quite unsuccessfully (ISWS, 2018). It is estimated that losses caused by weeds for wheat production ranges between 29 to 31% (Oerke, 2006; Gharde et al., 2018) while other studies mentioned that this figure may reach to 65% (Amare et al., 2014).

From the beginning of agriculture until the introduction of herbicides, weed management in agriculture depends largely on crop rotation, tillage, and seed cleaning. But the heavy reliance on chemical weed control is nowadays considered nonacceptable or not good enough (Das, 2008), this is mainly due to extensive use of chemicals with potential destructive side effects on food safety, public health, and the environment. Cropping systems focusing on using herbicides for weed control are becoming progressively at risk, as herbicide resistance are considerably creating situations where some weed species cannot be controlled by chemical methods (Kumar, 2014).

Weed control is becoming harder due to economic expenses of weed control; the elevating herbicidal prices; higher yield demands; economic and political factors. Unethical use of herbicides causes serious damage not only to the crop but even to the agro-environment. Misuse of herbicides increases herbicide resistance weed plants, soil and irrigation water contamination eventually causes killing of non-target organisms which might alter the natural balance of the area (Labrada et al., 1994).

Also frequent using of herbicides produce weed resistance to herbicides, therefore, to minimize this problem and for efficient weed management, by applying non-chemical weed management tactics or by reducing herbicide applications such as cleaned or weed-free cultivated seeds should be adopted in conjunction with chemicals (like herbicide mixture and rotation, optimum spray time, dose, and methods) (Chicouene, 2020; Norsworthy et al., 2012) or through minimizing herbicides amount. Some of the non-chemical agronomic strategies like tillage, sowing time and methods, competitive crop cultivars, higher crop density, crop rotation and sanitation practices (weed-free crop seeds and seed cleaning) can be adjusted and adopted in such a manner that they provide the competitive edge to the crop over weeds (Owen and Powles, 2020; Hossain, 2015; Michael et al., 2010). Several studies have found that cleaning the seeds reduces the return of weed seeds to the soil and increases wheat grain yield (Lollato et al., 2020; Burkov et al., 2018; Norsworthy et al., 2012; Walker, 1995).

The project's objectives are to determine the effect of seed cleaning on wheat crop yield and yield components, and to compare these results with the traditional method of weed control by herbicides or hand weeding to determine whether seed cleaning could replace or minimize the use of herbicides in wheat fields.

Materials and methods

Experimental design and land preparation

Field experiments were carried out at two locations; Qlyasan Research Station/College of Agricultural Engineering Sciences/University of Sulaimani (coordination 35°34'18"N, 45°22'01"E with altitude of 749 m asl) and Kani Panka Agricultural Research Station/Ministry of Agriculture/Kurdistan Regional Government (coordination 35°22'27.35"N, 45°43'02.48"E with altitude of 540 m asl), 40 Km south east of Sulaimani city, Kurdistan region/Iraq (*Fig. 1*).

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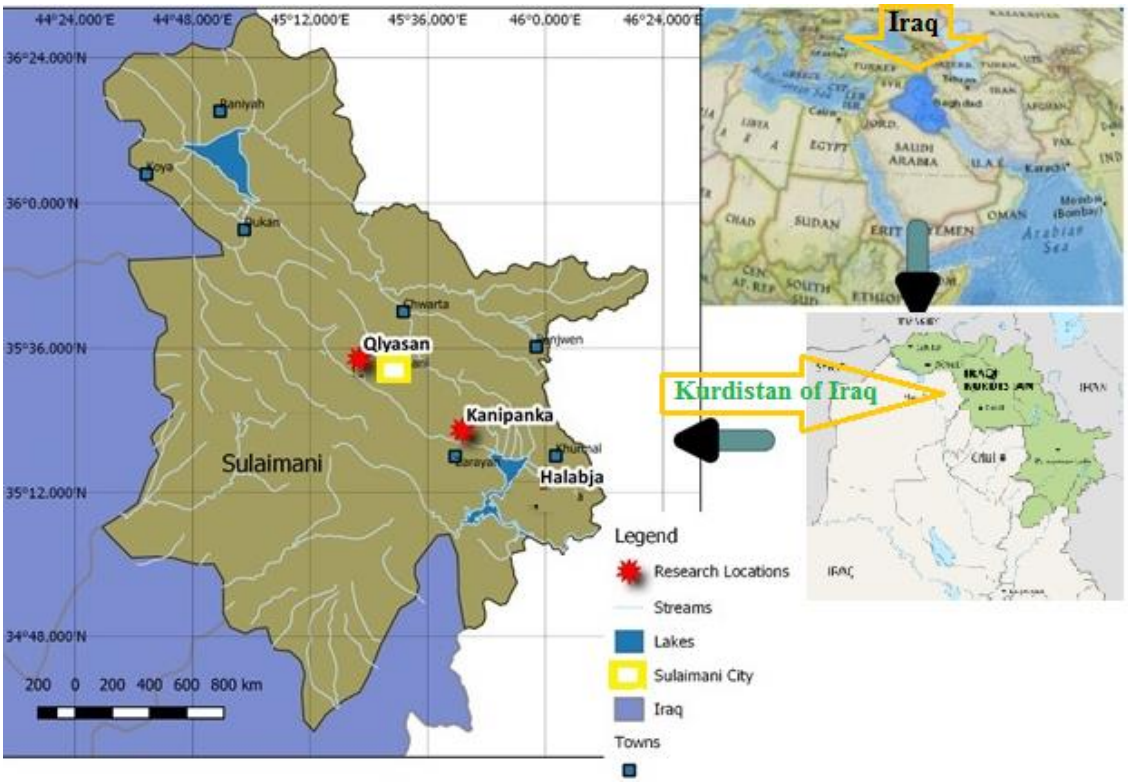

Figure 1. *Map of Sulaimanie governorate (Kurdistan region/Iraq) explaining field site locations*

A combined experiment was applied using Two ways-ANOVA randomized complete block design (RCBD) with four replications. Two factors were tested, the first factor was wheat seeds with two treatments: cleaned seeds, that were cleaned by seed cleaning machine after harvesting by the combine harvester to get rid of any weed seeds, infested seeds by insects or fungus, debris, dirt, dust, immature seeds, empty seeds, shrunken seeds, discolored seeds, peeled or broken seeds; and Non-clean seeds, which in this treatment wheat seeds left without cleaning after harvesting by the combine harvester. The second factor was herbicide application at five levels: Control: no herbicide and no methods of weed control was used; hand weeding for one time; narrow leaved herbicide (Topic); broad leaved herbicide (Granstar); and mixture of both herbicides (Topic + Granstar).

Qlyasan and Kani Panka lands were cultivated on 14/11/2018 and 15/11/2018 respectively, by moldboard plow and treated with disk harrows. Land area allocated in each site was 527 m^2, divided into four blocks, each block consisted of ten plots 2 × 3 m (6 m^2), 1 m was left between plots in all directions to avoid seepage between them, plots were divided into 15 rows by a handy tool, space between rows was 20 cm and rows length was 2 m. Rows were vertical on ground slope.

Cleaning and sowing wheat seeds

Wheat seeds (var. Adana) were collected from the fields of College of Agricultural Engineering Sciences, harvested by the combine harvester during June 2018. Those seeds were divided into two parts; the first part was used in the experiments of this

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study as clean seeds which was cleaned by the seed cleaning machine (Agrosaw mobile seed cleaning machine) to get rid of any weed seeds, infested wheat seeds by insects or fungus, debris, dirt, dust, immature seeds, empty seeds, shrunken seeds, discolored seeds and peeled or broken seeds. But the second part not cleaned seeds were harvested by the combine harvester without cleaning.

Wheat seeds (cleaned and non-cleaned) were sown at a rate of 120 kg ha^{-1} (which means 72 g. $plot^{-1}$ or 4.8 $g.row^{-1}$) in both Qlyasan and Kani Panka locations on 22/11/2018 and 29/11/2018 respectively. For the cleaned seeds number of seeds per row was 120 seed for each 4.8 gram, but for uncleaned seeds the weight was the measurement method due to containing many other bodies such as weed seeds, empty or broken seeds, debris and other materials that are similar in size to wheat seeds.

Cultural practices were conducted normally including fertilizing with di-ammonium phosphate (DAP 18-46) which was applied in all treatments with cultivation, in a dose of 174 kg ha^{-1}, while urea (46% N) was applied in a dose of 106 kg ha^{-1}, divided into two parts, the first part was applied in tillering stage and the second application was in booting stage for both locations.

Weed control treatments by herbicides application

Herbicides were applied using spray method. Knapsack sprayer was prepared to be calibrated after filling with water, sprayed on area of four treatment unites ($4 \times 6 = 24$ m^2) till complete wetting of all plants, the amount of water used was calculated to be 0.5 L, calculations of herbicide solution were made upon 200 L solution for 1 ha.

On 24 Jan 2019 and 30 Jan 2019 herbicide applications were done in Qlyasan and Kanipanka respectively in calm warm days (*Table 1*).

Table 1. *Monthly temperature and precipitation for Qlyasan and Kani Panka for the season 2018-2019*

Months		Qlyasan				Kani Panka			
		Min. temp. (°C)	**Max. temp. (°C)**	**Avg. temp. (°C)**	**Rainfall (mm)**	**Min. temp. (°C)**	**Max. temp. (°C)**	**Avg. temp. (°C)**	**Rainfall (mm)**
2018	October	10.8	36	23	41.5	9.7	34.3	22	27
	November	5	25.9	15.4	101	4.2	24.5	14.4	114.5
	December	2.3	18	10.1	324	3.1	17	10.5	328.4
2019	January	-2.5	15	6.5	152	-1.2	15.18	7	155.6
	February	1.5	17	9.2	135	2.4	13.3	7.8	153
	March	1.6	19.5	10.5	266	2	16	9	170
	April	5.5	26	15.7	177	7	23	15	120
	May	10.4	36	18.2	44.1	11	34	22.5	22
	June	21.6	42	32	4.6	19	38	29	2
Total rainfall					1245				1093

Water table level was measured for both locations, through nearest existing wells on the experiments land, using a sounder instrument electrical measuring tape, it was found

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that water table in Qlyasan is 22 m, while in Kani Panka it was 14 m from the earth surface. Plot controls were left without treating by any herbicides or weeding activities.

Hand weeding

Hand weeding treatment have been done for plots of cleaned and non-cleaned seeds for Qlyasan and Kanipanka on 4th and 7th March, 2019 respectively, in which all weed plants were cut above ground and classified in the laboratory to narrow and broad leaf weeds, fresh weights of weed plants was measured, then all weeds were kept in punched paper bags and dried in the oven on 70 °C, for 72 h, dry weight of narrow and broad leaf weeds was also calculated.

Topic 080 EC herbicide

Topic 080 EC herbicide (clodinafop-propargyl $C_{17}H_{13}ClFNO_4$) (FAO, 2010) was applied to control narrow leaved weeds in wheat fields. Topic 080 EC is an emulsion, that can be mixed with water and other herbicides, clodinafop-propergyl inhibits acetyl co-enzyme A carboxylase and belongs to the aryloxy phenoxy propionate family, Topik herbicides are taken up by foliage and are translocated via the phloem to areas of new growth accumulate in the tips (meristems), which is the site of action, as a results cell division and elongation are stopped , resulting weak and stunted plants of susceptible treated weeds (Ali et al., 2016; Cavanaugh et al., 1998). The recommended amount of Topic herbicide to control narrow leaved weeds in wheat fields is 0.8 L. ha^{-1}, which is also equal to 0.8 L/200 L of water for 1 ha.

Five liters of water was powered into the knapsack tank with 40 cc of Topic herbicide and the solution volume was completed to 10 L by tap water. Metal land marks for treatment units were fixed on the ground according to field map (in each replication two marks were used, one treatment unit for cleaned seeds and another unit for non-cleaned seeds), spray was done from 30 cm height ensuring all plants inside treatment unit were equally receiving herbicide solution.

Calculations for herbicide application were as follows:

$$\frac{0.8\ Liter\ herbicide}{one\ hectare\ of\ land} = \frac{0.8\ Liter\ herbicide}{200\ liter\ of\ water}$$

$$\frac{800\ cc\ herbicide}{200\ liter\ of\ water} = \frac{40\ cc\ herbicide}{10\ liter\ of\ water}$$

Granstar herbicide

Granstar (75% tribenuron methyl $C_{15}H_{17}N_5O_6S$ + 25% inert) is an herbicide used to control broad leaf weeds as post emergence in wheat fields (Mukherjee et al., 2015), Granstar is a member of sulfonyl urea family, a selective and translocated herbicide that is absorbed through the leaves to the meristematic tissues, which can move via the phloem to all parts of the plant, and inhibit amino acid synthesis namely, acetolactate synthase enzyme (ALS ase) to prevent the production of specific amino acids, the key building blocks for normal plant growth and development (Haghighi et al., 2019; Baghestani et al., 2007), the trade formula is granular dissolve in water , meanwhile capable to be mixed with other herbicides. Two grams of granular formula of Granstar herbicide was dissolved in 5 L of water in the Knapsack tank and the solution was completed with tap water to 10 L. The spray operation was done as in Topic herbicide application.

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Herbicide mixture (Topic + Granstar)

For the herbicide mixture treatments, the same amount of Topic and Granstar herbicides (as mentioned above) were poured into the knapsack tank containing 10 L of water, mixed well and sprayed.

Studied characters of wheat

On 1st and 3rd June 2019 Kanipanka and Qlyasan experiment fields were harvested respectively, 1 m^2 in the center of each treatment unit was harvested, all plants above ground were collected for each experiment unit and kept in labeled plastic bags. Bags were transferred immediately to the laboratory; wheat plants were separated from weed plants. *Tables 3* and *4* illustrate weed plants that still exist (as dry or fresh status) with wheat plants, during harvest period, in both fields of Qlyasan and Kanipanka.
The following parameters were registered for all harvested wheat plants:

Dry weight of weed plants

Weeds enclosed within 1 m^2 harvested from each plot were separated from the wheat plants, weeds were dried easily due to the high temperatures during harvest period in June, in addition to the low relative humidity. The dry weights obtained were expressed in $g.m^{-2}$.

Number of spikes m^{-2}

Spikes were counted in an area of 1 m^2 of the harvested wheat in each plot.

Number of kernels (grains) spike^{-1}

Ten spikes of wheat plants were taken randomly from 1 m^2 of the harvested plot, thrashed manually, grains were separated from straw and counted using electrical grain counter, then averaged to get the number of grains spike^{-1}.

Grain weight (1000 grains)

One thousand grains were counted from representative samples of each treatment drawn from winnowed and cleaned produce, and their weight in grams was determined.

Grain yield m^{-2}

All wheat spikes of 1 m^2 of the harvested plots were thrashed manually, grains were separated from straw and weighed.

Statistical analysis

Statistical analysis for all measured variables was performed using the XLSTAT software (XLSTAT, 2016). For direct comparison of treatments, least significant difference tests (LSD) at level of 0.05 was used, and the data were subjected to analysis of variance (ANOVA).

Preparation of soil samples and soil analysis

Soil samples of both experimental locations Qlyasan and Kanipanka were taken using an auger at a depth of 0-30 cm from the soil surface. Subsamples taken from

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Qlyasan location were mixed carefully, then a representative sample soil free from plant roots and other debris, was gently air dried, crushed, and sieved using a 2 mm stainless steel sieve, then the sample was taken for physical and chemical analysis. Particle size distribution for textural class assessing was carried out by international pipette method as described by Black et al. (1965).

Hydrogen ion concentration (pH) and electrical conductivity (EC) were measured in a suspension ratio of 1:10, soil to H_2O as determined by Gupta (2004), using pH model of WTW 330i, whereas for EC the model WTW 330i EC-meter was used.

Organic matter percentage (O.M.%) were determined by wet oxidation method according to Walkley-Black method (Black et al., 1965). O.M. % was calculated according to the following equation:

$$\text{O.M. \%} = \text{Organic carbon\%} \times 1.724 \text{ (factor)}$$

Calcium carbonate $CaCO_3$% (g kg^{-1}) was determined according to a 23C method of U.S. Salinity Laboratory Staff, 1954, as mentioned in Black et al. (1965) water table level was measured for each location through measuring the water level in the wells of each land using a geotech water level meter (*Table 2*).

Table 2. *Physical and chemical properties of the soil samples for experimental locations (Kanipanka and Qlyasan)*

Physicochemical properties		Locations	
		Kani Panka	**Qlyasan**
Particles size distribution (g kg^{-1})	Sand	36	107
	Silt	529	435
	Clay	435	458
	Texture	Silty Clay	Silty Clay
PH		7.70	7.59
Ece (micro Siemens cm^{-1}) or (µS cm^{-1})		218	490
O.M. (g kg^{-1})		22.4	14.8
$CaCO_3$ (g kg^{-1})		208.3	304.3
Water table (m) from ground level		14	22

Weed identification

Weed plants of both locations were collected and classified into narrow and broad leaved, and identified to their Scientific, English and Kurdish (local) names (*Tables 3* and *4*).

Results and discussion

Effects of seed cleaning, weed control treatments and their interaction on weed dry weight

Weight of weeds that accompany the wheat crop after harvest are a good indicator for the effectiveness of weed control or management process that is applied, and it is reflected directly on the crop yield and quality.

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It is found from *Table 5* that Qlyasan location registered the highest weeds dry weight (160.2 g.m^{-2}) which was significantly higher than Kani Panka (120.5 g.m^{-2}), this might be due to environmental diversity and water table level, or may be to the initial invading of weeds in Qlyasan because of high rain precipitation during the season 2018-2019 (*Table 1*), specially the rains during spring months in Qlyasan registered 1.5 to 2 times more rain fall comparing to Kani Panka, this gave chance to a group of weed seeds to grow during spring months.

Table 5 illustrates the significant effect of seed cleaning on weed dry weight, figures were 168.8 g.m-^{2} for non-cleaned seeds versus 112.02 g.m^{-2} for cleaned wheat seeds, in both locations cleaned seeds recorded significant effect on minimizing the weed dry weight, the reduction of weed dry weight reached 34% in both locations, these results are similar to what Norsworthy et al. (2012) reported that using of cleaned seeds is an effective method to control weeds in addition it minimizes introducing new weeds, mechanical cleaned seeds also enhance the establishment of weed-free fields and then keep fields as weed free as possible. Chauhan (2013) also emphasized that cleaning of seeds is one of the strategic methods to minimize effect of weeds. Results of this study are in line with what Hossain (2015) found that seed cleaning resulting in fewer weed seeds being sown with crop seed, also the results were in line with the previous studies by Owen and Powles (2020) which found that crop seed cleaning reduced weed seed contamination.

Table 3. *Scientific names, family, English and Kurdish names of weed plants in Qlyasan location*

Qlyasan narrow leaved weeds			
Scientific name	**Family**	**English name**	**Kurdish name**
Avena fatua L.	Poaceae	Wild oat	*Qalas, paraspelka*
Hordium balbosum	Poaceae	Bulbous barley	*Gezar geya*
Phalaris minor Retz.	Poaceae	Little seed canary grass	*Kapank, bashan, qaram*
Cyperus rotundus L.	Cyperaceae	Nut grass (nut sedge)	*Simel, sotka*
Lolium rigidum	Poaceae	Rigid ryegrass	*Giya ganem*
Lolium temulentum L.	Poaceae	Darnel ryegrass	*Ganema marana*
Sorghum halepense	Poaceae	Johnson grass	*Karoush*
Qlyasan broad leaved weeds			
Carthamus oxyacanthus	Asteraceae	Wild safflower	*Dirkazarda*
Centaurea Pallescens	Asteraceae	Knapweed	*Gawra gla*
Cichorium intybus L.	Asteraceae	Common chicory	*Chaqchaqoka*
Silybium marianum L.	Asteraceae	Milk thistle	*Chaoubaza, Qalughan*
Lactuca virosa L.	Asteraceae	Wild lettuce	*Talishk, Kahowakewi*
Sinapis nigra L.	Brassicaceae	Black mustard	*Khartala, aspand*
Sinapis arvensis L.	Brassicaceae	Wild mustard	*Khartala, garmazhen*
Lupinus albus L.	Fabaceae	Field lupine	*Pulka, wulara*
Galium tricorontum	Rubiaceae	Rough corn bedstraw	*Gertik, noosaka*
Vaccuria pyramidata	Caryophayllaceae	Cow herb	*Glenah*
Melilotus indicus L.	Fabaceae	Sweet clover	*Gochanbakhe*
Vicia calcarata	Fabaceae	Wild vetch	*Paqlamarana (paqloka)*
Convolvulus arvensis L.	Convolvulaceae	Field bindweed	*Laulawa*
Glycyrrhiza galabra	Fabaceae	Liquorice	*Bahlak*
Euphorbia peplus	Euphorbiaceae	Milk weed	*Khursheelk*

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Table 4. *Scientific names, family, English and Kurdish names of weed plants in Kani Panka location*

Kanipanka narrow leaved weeds			
Scientific name	**Family**	**English name**	**Kurdish name**
Avena fatua L.	Poaceae	Wild oat	*Qalas, paraspelka*
Hordium balbosum	Poaceae	Bulbous barley	*Gezar geya*
Phalaris minor Ritz.	Poaceae	Little seed canary grass	*Kapank, bashan, qaram*
Cyperus rotundus	Cyperaceae	Nut grass (nut sedge)	*Simel, sotka*
Lolium rigidum	Poaceae	Rigid ryegrass	*giya ganem*
Lolium temulentum	Poaceae	Darnel ryegrass	*Ganema marana*
Sorghum halepense	Poaceae	Johnson grass	*Karoush*
Kanipanka broad leaved weeds			
Glycyrrhiza galabra	Fabaceae	Liquorice	*Bahlak*
Galium tricorontum	Rubiaceae	Rough corn bedstraw	*Gertik, noosaka*
Sinapis nigra	Brassicaceae	Black mustard	*Khartala, aspand*
Sinapis arvensis	Brassicaceae	Wild mustard	*Khartala, garmazhen*
Silybium marianum L.	Asteraceae	Milk thistle	*Chaoubaza, Qalughan*
Xanthium strumarium	Asteraceae	Rough cocklebur	*Moosanak, Pizh*
Vicia calcarata	Fabaceae	Wild vetch	*Paqlamarana (paqloka)*
Lactuca virosa L.	Asteraceae	Wild lettuce	*Talishk, Kahowakewi*
Carthamus oxyacanthus	Asteraceae	Wild safflower	*Dirkazarda*
Centaurea Pallescens	Asteraceae	Knapweed	*Gauwragla*
Convolvulus arvensis L.	Convolvulaceae	Field bindweed	*Laulawa*

Table 5. *Effect of seed cleaning, weed control treatments and their interactions on weeds dry weight (g.m^{-2}) in two locations*

Locations (L)	**Seed cleaning (A)**	**Weed control (W)**					**Location * seed cleaning**
		Control	**Hand weeding**	**Narrow leafed herbicides**	**Broad leafed herbicides**	**Mixture**	
Qlyasan	Cleaned seeds	264.709 b	26.380 ijk	223.312 c	123.097 ef	26.410 IJK	132.781 c
	Not cleaned seeds	373.458 a	59.705 h	262.514 b	174.640 d	68.152 gh	187.694 a
Kani Panka	Cleaned seeds	228.790 c	11.520 k	153.037 de	48.897 hi	14.090 jk	91.2670 d
	Not cleaned seeds	342.335 a	45.325 hij	215.387 c	95.100 fg	51.452 hi	149.920 b
Qlyasan		319.083 a	43.0425 g	242.913 c	148.868 e	47.2812 g	160.237 a
Kani Panka		285.562 b	28.4225 g	184.212 d	71.9987 f	32.7712 g	120.593 b
Cleaned seeds		246.749 b	18.950 g	188.175 c	85.9975 e	20.250 g	112.024 b
Not cleaned seeds		357.896 a	52.515 f	238.950 b	134.870 d	59.8025 f	168.807 a
Weed control mean		302.323 a	35.7325 d	213.562 b	110.433 c	40.0262 d	

LSD 0.05 L = 12.9732, LSD 0.05 A = 10.4237, LSD 0.05 W = 16.4813, LSD 0.05 L * A = 14.7413, LSD 0.05 L * W = 23.3081, LSD 0.05 A * W = 23.3081, LSD 0.05 L * A * W = 32.9626. Different letters represent significant differences between the mean values according to LSD Test ($p \leq 0.05$)

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It is noticed from *Table 5* that cleaned wheat seeds had less weeds grown in both locations and that might not be because only less weed seeds accompanied the crop seeds, but also because cleaning of crop seeds will lead to select large crop seeds to be cultivated, this will raise the vigor of the crop seeds, and overbalance the competition to support the crop among weeds, this is in line with previous studies by Shi et al. (2017) and Kandasamy et al. (2020).

Impact of weed control on weed dry weight

Results in *Table 5* revealed that all weed control treatments were statistically significant in reducing weed dry weight, it is found that hand weeding and herbicides mixture recorded 88% and 86% reduction in weed dry weight respectively comparing to the control results, although both hand weeding and herbicides mixture had significant effect on reducing weed dry weight but there were no significant differences between those two treatments, these results are similar to what found by Hamouda et al. (2021) and Kareem et al. (2018). On the other hand, 63% and 29% reduction in weed dry weight was recorded in the case of broadleaved and narrow leaved herbicides, respectively, also these two treatments had significant differences between them and within all treatments. From the data shown in *Table 5* it is noticed that dry weight of weeds after application of narrow leaved herbicide was 213.56 $g.m^{-2}$ while dry weight of weeds after broadleaved herbicide application was only 110.43 $g.m^{-2}$, this shows that narrow leave weeds are more resistant to herbicides due to narrow selectivity between grassy weeds and wheat crop being both of grass in nature exhibits similar physiology and reaction to herbicides compared to broad-leaved weeds.

There was a significant effect of locations on weed dry weight, *Table 5* explained that Qlyasan record was higher than Kani Panka (160.237 versus 120.593 $g.m^{-2}$ respectively) and this might be due to extra number of weed species that existed in Qlyasan but were not found in Kani Panka (*Tables 3* and *4*).

Seed cleaning and locations interactions

Both *Table 5* and *Figure 2* elucidate significant effects of cleaned wheat seeds in different locations, it is noticed that the best combination for seed cleaning in both locations to minimize weed dry weight was in Kani Panka for cleaned seeds by recording only 91.26 $g.m^{-2}$,while the highest weed dry weight was found in Qlyasan for not cleaned seeds which registered 187.69 $g.m^{-2,}$ In the same trend clean seeds for Qlyasan and not cleaned seeds for Kani Panka registered 132.78 and 149.92 $g.m^{-2}$ respectively. It is obvious from the results that seed cleaning in both locations had significant effect on minimizing weed dry weight, these results were in agreement with those reported by Norsworthy et al. (2012), Qasem (2006), Worku (2010) and Cabardo (2003) meanwhile Qlyasan location recorded higher weed dry weight compared to Kani Panka and that might be due to existing of additional number of weeds in Qlyasan field such as (Common chicory *Cichorium intybus L.*, Field lupine *Lupinus albus L.*, Cow herb *Vaccuria pyramidata*, Sweet clover *Melilotus indicus L.*, Milk weed *Euphorbia peplus*) (*Tables 3* and *4*) which were not found in Kani Panka field. Moreover, rain fall in Qlyasan location was higher than Kani Panka by 13% (1245 mm and 1093 mm, respectively), the extra rainfall concentrated in spring months (march, April, May) when Qlyasan had 56% more rain comparing to Kani Panka.

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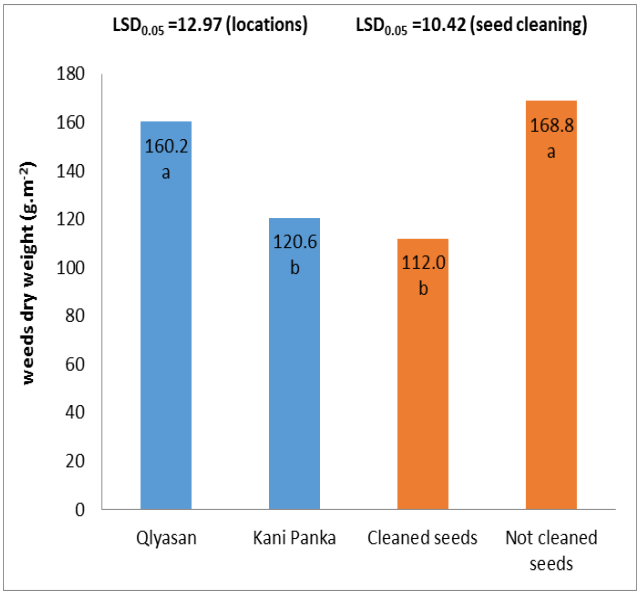

Figure 2. *Effect of locations and seed cleaning on weed dry weight (g.m^{-2})*

Influences of weed control and locations interactions on weed dry weight

As indicated in *Tables 5* and *A1* (in the *Appendix*) the application of herbicides was found to reduce weed dry weight significantly at both locations**.** The maximum and significant reduction in total weed dry weight realized by mixture of both Topic and Granstar herbicides treatment compared with the control treatment at both locations.

Data shown in *Table 5* exhibited the significant effect of weed control treatments on the weed dry weight in both locations, the best result in minimizing weed dry weight was 28.42 g.m^{-2} which obtained in Kani Panka * hand weeding, and this result have no significant differences with Kani Panka * herbicides mixture, Qlyasan * hand weeding and Qlyasan * herbicides mixture, which recorded significant reduction in weed dry weight (32.77, 43.03 and 47.28 g.m^{-2} respectively). Low weed dry weight in the treated pots specially those treated with herbicides mixture may be referred to the suppression of weed growth when herbicides are applied and ultimately reduced weed dry weight over the weedy check plots. From the previous results it is found that herbicide mixtures had reduced weed dry weight in Qlyasan and Kani Panka in a ratio of 85% and 88% respectively, while usage of broadleaved herbicide reduced weed dry weight by 53% in Qlyasan versus 75% in Kani Panka, on the other side the effect of using of narrow leaved herbicides reduced weed dry weight by 23% in Qlyasan versus 35% for the same treatment but in Kani Panka. From the above results it is noticed that weed dry weight reduction because of using herbicide mixtures have convergent results in both locations when compared to the control plots of each location, in addition to superior effect over using narrow leaved and broadleaved herbicides separately, which might be due to synergism effect of both herbicides when used as mixtures together, which means that herbicides treatment exhibited the same trend in reducing weed dry weight at both locations. These results are in line with what was found by Hamouda et al. (2021), Zand et al. (2007), Singh et al. (2008), and Kumar et al. (2018). Effect of the mixture might be due to its high selectivity to both narrow and broad-leaved weeds in the wheat crop.

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Effects of seed cleaning and weed control interactions on weed dry weight

Table 5 explained the significant effect of seed cleaning and weed control on minimizing weed dry weight, the lowest weed dry weight registered significant differences in treatment cleaned seeds with hand weeding and cleaned seeds with herbicides mixture recording (18.95 and 20.25 g.m-^{2}) respectively. On the other hand, the highest value for weed dry weight due to herbicide application was in the treatment of not cleaned seeds * no weed control recording 373.45 g.m^{-2} which shows that non-cleaned seeds and no herbicide usage have the highest infestation of weeds, meanwhile cleaning of wheat seeds with any herbicide treatment show significant differences. It is also seen in the same table that the effect of seed cleaning treatment with no herbicide had same records on weed dry weight (no significant difference) versus non-cleaned seeds with narrow weed herbicide (246.7 and 238.9 g.m^{-2} respectively), which shows that only cleaning of wheat seeds minimizing effect on weed dry weight is equal statistically to the effect of using narrow leaf herbicide (Topic), this result will be a good chance to select seed cleaning instead of using herbicides. From the same table it is illustrated that using of broad leaved herbicides (in both cleaned and non-cleaned seeds) registered less weed dry weight compared to using narrow leaved herbicides and this might be mainly due to the sensitivity of broadleaved weeds to herbicides which also is a result of the big area of broadleaved weeds in addition to the ability of broadleaved weeds in absorption of herbicide droplets solution, and also less cuticle coating leaves of broad weeds, on the other side leaves of narrow weeds are mostly hairy leaves that are coated with wax which minimize the penetration of herbicide solution, primary tissue of the weed leaves is epidermal, mesophyll which is a wax like material retards movement of herbicide solution in and out of leaves, also the angle of leaves for narrow leaved weeds is acute angle which minimize the exposure to herbicide solution while broadleaved weeds are mostly disposed parallel to the soil surface therefore are easier to hit with spray solutions if applied.

Seed cleaning, weed control and locations interactions on weed dry weight

Table 5 showed the significant interactions of all treatments in both Qlyasan and Kani Panka locations on weed dry weight, the lowest weed dry weight was 11.52 g.m^{-2} recorded in the treatment Kani Panka * cleaned seeds * hand weeding and this record did not differ significantly with (Kani Panka * cleaned seeds * herbicides mixture) or (Qlyasan * cleaned seeds * hand weeding) and Qlyasan * cleaned seeds * herbicides mixture), which emphasize that cleaned seeds with herbicides mixtures in both locations lowered weed dry weight significantly, these results of herbicide mixtures are similar with the results of Delchev (2018), Hamouda et al. (2021), Kareem et al. (2018), and Tityanov et al. (2015) when they reported that herbicides mixture of clodinafop-propargyl + tribenuron-methyl had a significant effect on weeds, in addition cleaned seeds also found to be significant on minimizing weed dry weight, and this was supported by results of Hossain (2015), Michael et al. (2010), and Owen and Powles (2020).

Effects of seed cleaning, weed control treatments and their interactions on number of spikes.m^{-2} in two locations

Number of spikes per unit area is considered as one of the important parameters that controls the grain yield in wheat.

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Effects of locations on number of spikes.m^{-2}

Results in *Table 6* illustrated that different locations had different significant effect on the number of spikes/area. Kani Panka location registered 413.47 spikes.m^{-2}, while in Qlyasan it was only 242.30 spikes.m^{-2}, this shows clearly that Kani Panka location was superior in increasing number of spikes per area, and this might be due to the environmental conditions in Kani Panka were better for the growth of wheat crop that season, among those factors humidity or water availability is one of the limiting factors, and as it was recorded the water table of Kani Panka is closer to the surface comparing to Qlyasan, 14 m vs 22 m respectively.

Table 6. *Effect of seed cleaning, weed control treatments and their interaction on number of spikes. m^{-2} in two locations*

Locations (L)	Seed cleaning (A)	Weed control (W)					
		Control	Hand weeding	Narrow leaved herbicides	Broad leaved herbicides	Herbicides mixture	Locations * seed cleaning
Qlyasan	Cleaned seeds	229.25 h	271.75 g	248.50 gh	236.50 gh	271.25 g	251.450 c
	Not cleaned seeds	219.75 h	240.25 gh	234.75 gh	233.25 gh	237.75 gh	233.150 c
Kanipanka	Cleaned seeds	391.25 cde	432.25 bc	375.00 ef	440.50 b	511.25 a	430.050 a
	Not cleaned seeds	346.00 f	420.50 bcd	381.75 def	390.25 de	446.00 b	396.900 b
Qlyasan		224.50 e	256.00 d	241.625 de	234.875 de	254.50 d	242.300 b
Kani Panka		368.625 c	426.375 b	378.375 c	415.375 b	478.625 a	413.475 a
Cleaned seeds		310.250cde	352.000 b	311.750 cde	338.500 bc	391.250 a	340.750 a
Not cleaned seeds		282.875 e	330.375 bcd	308.250 de	311.750 cde	341.875 b	315.025 b
Weed control mean		296.562 d	341.187 b	310.00 cd	325.125 bc	366.562 a	

LSD 0.05 L = 26.0923, LSD 0.05 A = 13.0851, LSD 0.05 W = 20.6893, LSD 0.05 L * A = 18.5051, LSD 0.05 L * W = 29.2592, LSD 0.05 A * W = 29.2592, LSD 0.05 L * A * W = 41.378 Different letters represent significant differences between the mean values according to LSD Test ($p \leq 0.05$)

Effects of seed cleaning on number of spikes.m^{-2}

Table 6 explains the effect of seed cleaning on the number of spikes.m^{-2} in both Qlyasan and Kani Panka locations, through recording 251.45 and 430.05 spikes.m^{-2} respectively. This might be as a result of cleaned seeds which lead to select big size and homogenized seeds for sowing (Tibola et al., 2016) and this was close to what Guillen-Portal et al. (2006) mentioned on wheat plants derived from large seed had a noticeable negative effect on weeds (wild oat) via a reduction in panicles.m^{-2} and seed weight, whereas wheat established from small seed wild oat competition reduced wheat spikes m^{-2}.

In addition, cleaned seeds lead to less weed seeds associated with wheat seeds resulting less weed plants grown and compete the crop plants, the cleaned wheat seeds also meant accurate number of healthy wheat plants per area as mentioned by Lollato (2016) and Elgersma (1990), these wheat plants produce a greater number of spikes (Ries and Everson, 1973; De Lucas Bueno and Froud Williams, 1996).

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Effects of weed control on number of spikes.m^{-2}

Results in *Table 6* illustrates that in Qlyasan area the differences were clearly significant, the highest number of spikes.m^{-2} was recorded in the treatment of hand weeding and using of herbicides mixture, recording 256 and 254.5 spikes.m^{-2} respectively, meanwhile control treatment in Qlyasan was 224.5 spikes.m^{-2}. On the other hand, broad leaved and narrow leaved herbicides individually also recorded significant effect but less than the herbicide mixture, these results are in line with what Al-Chalabi and Al-Agidi (2010) pointed out that using of herbicides to control weeds in wheat fields increased number of spikes.m^{-2}, they referred this result to the absence of weeds that allow the crop to grow with minimum competition on growth factors. Also, in Kani Panka location weed control treatments found to be significant comparing to the control treatment. These results were emphasized by Tawaha et al. (2002) in their study on barley, they indicated that herbicides application resulted in more spikes m^{-2}. Using of narrow leaved herbicides in both locations had significant influence on the number of spikes.m^{-2}, this was also reported by El-Metwally et al. (2015) that the highest value of the wheat spike (358 spikes m^{-2}) was obtained from clodinafop-propargyl spraying (Topic/narrow leaved herbicide).

Effects of seed cleaning and locations interactions on number of spikes.m^{-2}

Data in *Tables* 6 and *A1* for number of wheat spikes.m^{-2} exhibited significant differences among cleaned seeds versus non-cleaned at the locations, the highest records were for the cleaned seeds in Kani Panka, while the lowest were for non-cleaned seeds in Qlyasan recording 430.05 and 251.45 spikes.m^{-2} respectively, high number of spikes may be a result of cleaned seeds which means less weed seeds are competing with wheat seeds when sown, this will be reflected on the vigour of wheat seeds, strong wheat seedling and finally more spikes/area, similar results of clean seeds minimizing effect on weeds was gained by Verma et al. (2015), Cabardo (2003) and Worku (2010).

Effects of weed control and locations interactions on number of spikes.m^{-2}

Results illustrated in *Table 6* showed that weed control in different locations recorded significant influence on the number of spikes/area, the highest record was in the treatment herbicides mixture in Kani Panka and the lowest was in the control (un weeded) of Qlyasan location, 478.62 and 224.50 spikes.m^{-2} respectively. From the data shown in *Table 6* it is clear that herbicides mixture and hand weeding treatments had superiority among other treatments but it is also noted that location effect was significant (*Table A1*), the effect of locations is seen clearly in both control treatments (368.62 spikes.m^{-2} for Kani Panka versus 224.50 spikes.m^{-2} for Qlyasan) and this might be as a result of underground water near to the surface in Kani Panka compared to Qlyasan, which made more moisture to be available for wheat plants, that moisture is in the first order because it is one of the main factors limiting plant growth. It is found from the same table that weed control (either herbicides mixture or even one type of herbicide) showed significant effect on the increasing number of spikes (Tawaha et al., 2002; Turk et al., 2003) but it came in the second order compared to location effect.

Effects of seed cleaning and weed control interactions on number of spikes.m^{-2}

Results obtained from this study showed that seed cleaning with weed control treatments had a notable effect on the number of spikes.m^{-2} (*Table 6*), the highest values

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were in the cleaned seeds with herbicides mixture and cleaned seeds with hand weeding which recorded 391.25 and 352 spikes.m^{-2} respectively, while lower records were found in the uncleaned seeds with no weeding (control) treatment, which registered 282.87 spikes.m^{-2}. In a Comparison between cleaned seeds with herbicides mixture and not cleaned seeds with no weed control, it showed that the effect of using cleaned seeds with herbicides mixture increased the number of spikes.m^{-2} by 28%.

Effects of seed cleaning, weed control and locations interactions on number of spikes.m^{-2}

Wheat spikes number have been calculated under the combination of seed cleaning and weed control treatment of weed management as shown in *Tables 6* and *A1*. Significant difference in number of spikes m^{-2} as a result of seed cleaning and herbicide application compared with control treatments were recorded at both locations. The highest values of wheat spikes number (511.25 spikes.m^{-2}) were obtained from cleaned seeds with herbicides mixture in Kani Panka. Contrarily, the lowest values of the previous trait were obtained from un-weeded check at Qlyasan (219.75 spikes.m^{-2}). Such superior seed cleaning and weeded treatment minimize weed-crop competition and save more available environmental resources for crop plants that improved growth traits which positively reflected on spikes numbers of wheat. The positive effect of weed control practices on yield attributes of wheat crop have been confirmed by several authors. The reason may be mainly due to their effective control of weeds by reducing dry matter of weeds (*Table 5*) with recording a high Weed Control Efficiency % (WCE%) (appendixes *A2*). It is also confirmed by Zoheir Y. Ashrafi (2009) that line sowing treated with herbicides gave the maximum number of spikes m^{-2} (293 spikes.m^{-2}).

In Qlyasan location using of narrow leaved herbicide did not have significant effect compared with using broadleaved herbicides which might be due to the symmetrical effect of each type of weeds alone (Narrow or broadleaved) on the number of wheat spikes.m^{-2}. On the other hand, it is found in Kani Panka location that there are significant differences between the effect of narrow leaved weeds and broadleaved weeds, and this might be because of the proportional effect of different types of weeds between the locations.

Effects of seed cleaning, weed control treatments and their interaction on number of kernels (grains). spike $^{-1}$ in two locations

Number of grains. spkie^{-1} is one of yield components.

Effects of locations on number of kernels (grains) spike^{-1}

There were no significant differences among locations in the number of kernels. spike^{-1}, but Kani Panka location showed higher results 30.052 versus 29.588 kernel. spike^{-1} for Qlyasan location (*Table 7*). Meanwhile Qlyasan location showed significant differences in both cleaned seeds and weed control separately, the cleaned seeds resulted 32.101 and the highest records in weed control was for herbicides mixture was (32.014). The same trend for the weed control treatment effectiveness was observed in Kani Panka location, all treatments recorded significant differences with the control unit (no herbicide application), the highest records were in hand weeding and herbicides mixture (resulting 31.763 and 31.480 grain. spike^{-1} respectively).

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Effects of seed cleaning on number of kernels (grains) spike^{-1}

Results obtained from *Table 7* showed that seed cleaning recorded 31.087 grains.spike^{-1}, which was significantly higher than uncleaned seeds (28.554 grains.spike^{-1}), this might be related to minimizing of weed seeds that are taken out when wheat seeds were cleaned, in addition cleaning of wheat seeds gave a chance to select bigger wheat seeds to be cultivated which finally led to produce healthy plants that may produce better, these results were mentioned by Weimarck (1975) for the effect of cleaned seeds on selecting larges seeds, and the results of De Lucas Bueno and Froud Williams (1996) regarding of the vigour of large seeds, meanwhile Lollato (2016) mentioned that cleaned seeds are more similar in volume, shape and weight which is reflected on the accurate number of plants per area, that all leads to better crop production due to minimizing weeds (Khazaei et al., 2016).

Table 7. *Effect of seed cleaning, weed control treatments and their interactions on number of Kernels (grains). spike^{-1} in two locations*

Locations (L)	Seed cleaning (A)	Weed control (W)					Locations * seed cleaning
		Control	Hand weeding	Narrow leaf herbicides	Broad leaf herbicides	Herbicides mixture	
Qlyasan	Cleaned seeds	28.870 defg	34.400 a	33.417 ab	30.240 cdef	33.577 ab	32.101 a
	Not cleaned seeds	23.355 h	26.015 gh	28.555 efg	27.007 g	30.450 cdef	27.076 c
Kani Panka	Cleaned seeds	31.847 abc	32.272 abc	28.717 defg	27.170 g	30.355 cdef	30.072 b
	Not cleaned seeds	27.112 g	31.252 bcde	27.750 fg	31.437 bcd	32.605 abc	30.031 b
Qlyasan		26.112 e	30.207 abcd	30.986 abc	28.623 d	32.013 a	29.588 a
Kani Panka		29.480 bcd	31.762 a	28.233 d	29.303 cd	31.480 ab	30.052 a
Cleaned seeds		30.358 bcd	33.336 a	31.067 bc	28.705 de	31.966 ab	31.087 a
Not cleaned seeds		25.233 f	28.633 de	28.152 e	29.222 cde	31.527 ab	28.554 b
Weed control mean		27.796 d	30.985 ab	29.610 bc	28.963 cd	31.746 a	

LSD 0.05 L = 1.6225, LSD 0.05 A = 0.9052, LSD 0.05 W = 1.4313, LSD 0.05 L * A = 1.2802, LSD 0.05 L * W = 2.0242, LSD 0.05 A * W = 2.0242, LSD 0.05 L * A * W = 2.8627 Different letters represent significant differences between the mean values according to LSD Test ($p \leq 0.05$)

Effects of weed control on number of kernels (grains) spike^{-1}

Weed control have a significant effect on number of kernels (grains). spike^{-1}, the highest record was for the treatment of herbicides mixture and the lowest number was in the control treatment (no weed control method is applied), that recorded 31.746 and 27.796 grains. spike^{-1} respectively (*Tables 4-8*), also hand weeding showed a significant effect on the number of kernels (grains) of the spike (30.985).

Effects of seed cleaning and locations interactions on number of kernels (grains) spike^{-1}

Table 7 shows the significant differences between treatments on kernels number in spike, the highest record was for the cleaned seeds in Qlyasan location while the lowest was for not cleaned seeds in Qlyasan which registered 32.101 and 27.076 kernels.spike^{-1}

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respectively, while in Kani Panka location no significant differences were observed between cleaned and not cleaned seeds.

Effects of weed control and locations interactions on number of kernels (grains) spike^{-1}

Results obtained from *Table 7* illustrates significant differences between treatments, herbicides mixture in Qlyasan have the high number of kernels.spkie^{-1}, while the lowest result was in the control treatment for Qlyasan location,(32.013 and 26.112 kernels.spike^{-1} respectively), from the same table it is found that there is no significant differences between treatments Kani Panka * hand weeding, Kani Panka * herbicides mixture, Qlyasan * narrowleaf herbicides and Qlyasan * hand weeding (31.762, 31.480, 30.986 and 30.207 respectively), it is understood from these results that herbicides mixture and hand weeding treatments found to be effective on increasing number of kernels.spike^{-1} compared to the control treatment.

Effects of seed cleaning and weed control interactions on number of kernels (grains) spike^{-1}

Interactions of seed cleaning and weed control treatments have significantly influenced the number of wheat kernels.spike^{-1} (*Table A1*), the highest results were registered by cleaned seeds with hand weeding treatment, while the lowest records were in not cleaned seeds with no weed control, that registered 33.336 and 25.233 kernels.spike^{-1} respectively (*Table 7*), however herbicides mixture for both cleaned and not cleaned seeds have significant effect compared to the control, this result is in line with what Khan and Gul (2006) found that weed control affected the number of kernels.spike^{-1}. Using of narrow leaved herbicides with the cleaned seeds significantly and positively affected number of kernels.spike^{-1} (31.067), this agree to what noticed by Ali et al. (2016) that using of narrow leaved herbicides will increase grain production, adding the effect of seed cleaning to narrow herbicide in this treatment has additional positive effect on increasing the grain yield (Edwards and Krenzer Jr, 2006) and forage and total yield production as mentioned by Pinto et al. (2019).

Effects of seed cleaning, weed control and locations interactions on number of kernels (grains). spike $^{-1}$

Results illustrated in *Table 7* shows significant effect of the interaction between seed cleaning, weed control and locations treatments on number of kernels. spike^{-1}, the highest value was in the treatment Qlyasan * cleaned seeds * hand weeding, and the lowest value was Qlyasan * not cleaned seeds * no weed control (34.400 and 23.355 kernels.spike^{-1} respectively). This might be explained by the availability of moisture in Kani Panka due to ground water close to the surface compared to Qlyasan, and also because that cleaned seeds have provided big wheat seeds that had more vigour (Khazaei et al., 2016; Weimarck, 1975; De Lucas Bueno and Froud Williams, 1996), and similar in size which obtained accurate seeding rate (plants/area) (Pinto et al., 2019; Ries and Everson, 1973). In addition, cleaned wheat seeds minimize or even restrict any foreign seeds to accompany sowing wheat seeds that leads to less grown weeds in the plots (Hossain, 2015). On the other hand, using of herbicides mixture (Topic with Granstar) eliminated and controlled more weeds that have been grown from soil buried seeds (seed bank) which reflected on the availability of less weeds and more nutrients, moisture, space and even sun light to wheat crop plants, that led to grow powerful wheat

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plants (Abouziena et al., 2008; Kareem et al., 2018; Al-Wagaa and Mohammed, 2020), all these factors enhanced increasing total spikes weight.

Effects of seed cleaning, weed control treatments and their interaction on 1000 kernel (grain) weight in two locations

Effects of locations on 1000 kernel (grain) weigh

Results shown in *Table 8* indicated that all factors in Qlyasan location have significant effect on 1000 kernel weight, cleaned seeds versus not cleaned seeds have recorded 37.331 and 35.631 g for 1000 kernels weight respectively, also the weed control treatments in Qlyasan have positively affected 1000 kernels weight. The interacting effect of both factors cleaning seeds and weed control was significant comparing to the control treatment, the best combination was in the treatment cleaned seeds * herbicide mixture and cleaned seeds * hand weeding, which both recorded 40.1 g.1000^{-1} kernel weight, that shows as far as weeds existence are minimized through seed cleaning with either hand weeding or herbicides mixture enhanced the increase of 1000 grain weight. In Kani Panka location treatments had significant effect on the 1000 grain weight, results from (*Table 8*) shows that cleaned seeds increased 1000 grain weight of the wheat compared to non-cleaned seeds recording 39.52 and 37.39 g.1000^{-1} respectively, the same trend was found when applying weed control treatments in Kani Panka, hand weeding and herbicides mixture showed superiority compared to the control in increasing 1000 kernel weight, which recorded 40.840 and 40.291 g.1000^{-1} respectively while the control treatment was 35.299 g.1000^{-1} kernel.

Table 8. *Effect of seed cleaning, weed control treatments and their interaction on 1000 grain wheat weight (g) in two locations*

Locations (L)	Seed cleaning (A)	Weed control (W)					Locations * seed cleaning
		Control	Hand weeding	Narrow leafed herbicides	Broad leafed herbicides	Herbicides mixture	
Qlyasan	Cleaned seeds	34.655 ijk	40.120 b	35.672 ghi	36.032 fgh	40.175 b	37.331 b
	Not cleaned seeds	33.470 k	39.155 b	34.882 hij	34.642 ijk	36.005 fgh	35.631 c
Kanipanka	Cleaned seeds	36.320 efg	42.365 a	37.705 cd	39.582 b	41.670 a	39.528 a
	Not cleaned seeds	34.277 jk	39.315 b	37.022 def	37.452 de	38.912 bc	37.396 b
Qlyasan		34.063 f	39.638 b	35.277 e	35.338 e	38.090 cd	36.48 b
Kani Panka		35.298 e	40.840 a	37.364 d	38.518 c	40.291 ab	38.46 a
Cleaned seeds		35.487 f	41.243 a	36.688 de	37.808 c	40.923 a	38.43 a
Not cleaned seeds		33.874 g	39.235 b	35.953 ef	36.048 ef	37.458 cd	36.51 b
Weed control mean		34.68 d	40.24 a	36.32 c	36.93 c	39.19 b	

LSD 0.05 L = 0.6625, LSD 0.05 A = 0.4148, LSD 0.05 W = 0.6558, LSD 0.05 L * A = 0.5867, LSD 0.05 L * W = 0.9276, LSD 0.05 A * W = 0.9276, LSD 0.05 L * A * W = 1.3118 Different letters represent significant difference between the mean values according to LSD Test ($p \leq 0.05$)

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Effects of seed cleaning on 1000 kernel (grain) weight

Cleaned seeds had a significant effect on increasing 1000 kernel weight. Cleaning registered 38.43 versus 36.51 g. for uncleaned seeds (*Table 8*), this result agrees to what Hossain (2015) found that cleaning wheat seeds minimize weeds and to what Al-Chalabi and Al-Agidi (2010) emphasized that minimizing weeds will increase yield and its components.

Effects of weed control on 1000 seeds weight

Results obtained from this experiment revealed the significant effect of weed control treatments on 1000 kernel weight of wheat crop. Data shown in *Table 8* explains that all treatments have the same trend in increasing 1000 kernel weight, hand weeding and herbicides mixture recorded remarkable significant results (40.24 and 39.19 $g.1000^{-1}$ weight respectively), however broadleaved and narrow leaved herbicides treatment showed significant differences compared to the control treatment, 36.93 and 36.32 versus 34.68 $g.1000^{-1}$ weight respectively. This result was in agreement with the findings of Riaz et al. (2006) who reported that maximum 1000-kernel weight of wheat was produced with weed control by herbicides + hand weeding. Also, Hamouda et al. (2021) emphasized that using herbicides to control weeds in wheat fields increased 1000 kernel weight.

Effects of seed cleaning and locations interactions on 1000 kernel (grain) weight

The examination of the data presented in *Table 8* showed the effect of seed cleaning treatments and locations on 1000 kernel weight of the wheat crop, the highest results was in cleaned seeds in Kani Panka and the lowest was in not cleaned seeds in Qlyasan location, 39.528 and 35.631 g 1000^{-1}. kernel weight respectively. However, cleaned seeds in both locations registered higher results compared to uncleaned seeds, while Kani Panka showing superiority over Qlyasan might be due to environmental differences or different soil properties (*Tables 1* and *2*).

Effects of weed control and locations interactions on 1000 kernel (grain) weight

With respect to the wheat thousand kernel weight, the data presented in *Table 8* showed a significant effect of the weed control treatments and locations interactions, the highest record was in Kani Panka * hand weeding and the lowest found in Qlyasan * no weed control (40.840 and 34.063 $g.1000^{-1}$ kernel weight respectively). In Qlyasan location both narrow leaved and broadleaved herbicides have statistically the same result, while in Kani Panka there were significant differences between treatments of broad leafed and narrow leafed herbicides which recorded 38.518 and 37.364 respectively, they also have significant differences compared to the control treatment. This might be referred to soil moisture content due to close water table in Kani Panka compared to Qlyasan, also the diversity of weeds between both locations; as Qlyasan location found to have more broad leave weeds (*Tables 3* and *4*).

Effects of seed cleaning and weed control interactions on 1000 kernel (grain) weight

Cleaned seeds with hand weeding or herbicides mixture treatments recorded significant results (41.243 and 40.923 g 1000^{-1} kernel weight respectively) compared to the control (not cleaned seeds with no method of weed control 33.874 g) as

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explained in *Table 8,* these results explain how much seed cleaning with hand weeding or herbicides mixture was effective to increase 1000 kernel weight. These results reflect controlling and minimizing weeds in these treatments (*Tables 5* and appendix A-1).

Effects of seed cleaning, weed control and locations interactions on 1000 kernel (grain) weight

Results displayed in *Table 8* explained that interaction effect of seed cleaning, weed control and locations was significant on 1000 kernel weight, the highest value was in the treatment Kani Panka * cleaned seeds * hand weeding while the lowest record was for the treatment Qlyasan * not cleaned seeds * no weed control (42.365 and 33.470 $g.1000^{-1}$ kernel weight respectively). Meanwhile in Kani Panka the treatments cleaned seeds * hand weeding and cleaned seeds * herbicides mixture did not register any statistical differences, the same was observed in Qlyasan for the treatments cleaned seeds * hand weeding and cleaned seeds * herbicides mixture (40.120 versus 40.175 $g.1000^{-1}$ kernel weight respectively), these results show that the effect of hand weeding is equal to herbicides mixture.

Effects of seed cleaning, weed control treatments and their interaction on wheat yield (grain yield) in two locations

Grain yield is the aim of any production process and the most important parameter of any weed control program.

Effects of locations on wheat yield

Results collected from this study illustrates that research factors have significant effect on wheat grain yield, *Table 9* shows significant effect of the location on grain yield, it is found that Kani Panka was superior compared to Qlyasan (487.10 and 274.55 $g.m^{-2}$ respectively), which means 43.6% production difference between both locations, this might be referred mainly to the water availability in Kani Panka (ground water is found at the depth of 14 m in Kani Panka versus 22 m for Qlyasan) or soil property (*Table 2*), in addition this difference in the production might be the result of the weed diversity between both locations, Qlyasan was invaded by more weed species (*Tables 3* and *4*).

Effects of seed cleaning on wheat yield

A perusal of data indicate that seed cleaning had a significant effect on wheat yield. Results in *Table 9* showed that cleaned seeds recorded 405.06 $g.m^{-2}$ while wheat grain yield for uncleaned seeds was 356.60 $g.m^{-2}$, these results are combined with the minimizing of weed dry weight as explained in *Figure 2*. Cleaned seeds led to increase in grain yield, this result was emphasized by Edwards and Krenzer Jr (2006) who mentioned that cleaned seeds will increase grain yield.

Effects of weed control on wheat yield

Results in *Tables 9* and *A1* illustrate significant impact of weed control treatments on wheat grain yield, a close scanning of the data showed that among different herbicidal treatments, the highest significant value of wheat grain weight was obtained from

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herbicides mixture treatment (467.86 gm^{-2}) while the lowest value was (293.568 gm^{-2}) the result of unweeded (control) plots**.** The enhancement of wheat growth in the weeded plots might be attributed to the efficiency of herbicides in weed elimination (*Table 5*), and consequently reduced weed competitive ability against wheat crop, these results are in a good correlation with those of Al-Wagaa and Mohammed (2020) and El-Metwally et al. (2010) who reported that herbicides application to cereal crops during early stages of development have greatly decreased weed population over weedy check which ultimately increased grain yield by reducing competition among weeds and crop plants for light, nutrients, moisture and other growth requirements.

Table 9. *Effect of seed cleaning, weed control treatments and their interaction on wheat yield ($g.m^{-2}$) in two locations*

Locations (L)	Seed cleaning (A)	Weed control (W)					Locations * seed cleaning
		Control	Hand weeding	Narrow leafed herbicides	Broad leafed herbicides	Herbicides mixture	
Qlyasan	Cleaned seeds	231.53j	334.46g	299.00h	245.05ij	366.04f	295.219 c
	Not cleaned seeds	199.83k	249.93ij	267.21i	226.46jk	325.97gh	253.887 d
Kani Panka	Cleaned seeds	397.35e	573.74b	484.00d	483.14d	636.20a	514.891 a
	Not cleaned seeds	345.54fg	535.21c	408.11e	464.41d	543.21c	459.300 b
Qlyasan		215.68 i	292.20 g	283.11 g	235.76 h	346.01 f	274.55 b
Kani Panka		371.45 e	554.48 b	446.06 d	473.78 c	589.71 a	487.10 a
Cleaned seeds		314.443 f	454.10 b	391.50 c	364.10 d	501.12 a	405.06 a
Not cleaned seeds		272.693 g	392.57 c	337.665 e	345.44 de	434.59 b	356.60 b
Weed control mean		293.56 d	423.33 b	364.58 c	354.77 c	467.86 a	

LSD 0.05 L = 13.4528, LSD 0.05 a = 8.8165, LSD 0.05 w = 13.940, LSD 0.05 L * a = 12.468, LSD 0.05 L * w = 19.7144, LSD 0.05 a * w = 19.7144, LSD 0.05 L * a * w = 27.8804 Different letters represent significant differences between the mean values according to LSD Test ($p \leq 0.05$)

Effects of seed cleaning and locations interactions on wheat yield

Cleaned wheat seeds produce a high vigour seed that create more strong seedlings and assist to establish growing of healthy crop plants, which will produce more grain yield. Results obtained from this study showed that significant superiority in grain yield was in the treatment cleaned seeds * Kani Panka, while the lowest grain yield was found in not cleaned seeds * Qlyasan (514.891 and 253.887 g m^{-2} respectively), results shown in *Table 9* ensures what Keeble and Hale (1982), Shi et al. (2017) found, who mentioned that cleaned seeds will produce more healthy plants that gives higher grain yield (Edwards and Krenzer Jr, 2006; Lollato, 2016; Worku, 2010), cleaned wheat seeds minimized or eliminated weed seeds which is reflected on minimizing weed plants that germinate and grow in those plots leading to less competition with wheat crop plants, same trend is noticed in *Table 5*, the lowest weed

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dry weight (91.267 $g.m^{-2}$) led to register the highest wheat grain yield (514.891 $g.m^{-2}$) for the treatment Kani Panka * cleaned seeds (*Table 9*).

Effects of weed control and locations interactions on wheat yield

Results from *Table 9* obviously shows the significant effect of different weed control treatments on wheat grain yield, the highest grain yield registered in the treatment of herbicides mixture in Kani Panka location (589.71 gm^{-2}), while the lowest records were in the treatment control (no-weeding) in Qlyasan (215.68 $g.m^{-2}$), simultaneously weed control had notable effects on the grain yield in Qlyasan. Herbicides mixture and hand weeding had higher grain yield compared to the control treatment (346.01, 292.19 and 215.68 $g.m^{-2}$ respectively) the use of herbicides mixture minimized weed dry weight significantly (*Table 5*) through minimizing both narrow and broadleaved weeds, the highest record was found in herbicides mixture in Kani Panka treatment while the lowest spikes weight was also registered in the control (no-weeding) in Qlyasan treatment.

While all weed control treatments in Kani Panka registered significant effects on wheat grain yield compared to the control, among weed control treatments and due to using of herbicides the highest record was in the herbicides mixture treatment and the lowest was in the narrow leaved (Topic) herbicide treatment 589.710 and 446.056 $g.m^{-2}$ respectively (*Table 9*).

Effects of seed cleaning and weed control interactions on wheat yield

Data exhibited in *Table 9* showed a significant effect of the interactions between both factors, the highest records were in the cleaned seeds with herbicides mixture, while the lowest record was in the control treatment (uncleaned seeds with no weed control) which registered 501.126 and 272.693 $g.m^{-2}$ respectively, it is found from the same results that cleaned wheat seeds with hand weeding treatment have the same effect (no significant difference) of uncleaned seeds with herbicides mixture (454.10 and 434.59 $g.m^{-2}$ respectively), on the other hand cleaned seeds with hand weeding have similar effect (no significant difference) with cleaned seeds but with the use of only narrow leaved herbicide (392.57 and 391.5 $g.m^{-2}$ respectively).

Effects of seed cleaning, weed control and locations interactions on wheat yield

Results collected from this study clearly shows the significant effect of seed cleaning, weed control and locations interactions on wheat yield, it is found from *Table 9* that Kani Panka * cleaned seeds * herbicides mixture treatment have the highest grain yield while the treatment Qlyasan * not cleaned seeds * no weed control gained the lowest records (636.20 and 199.83 $g.m^{-2}$ respectively). The high records may be referred to different reasons, first of all it is clearly noticed that different locations have significant effect on wheat grain yield, the underlining reason might be linked to weed flora in wheat and their competitive abilities differ with changes in the environment and the availability of soil moisture. Water table closeness to soil surface in Kani Panka location prepared better conditions for growing wheat crops compared to Qlyasan location, in addition weed flora diversity (*Tables 2, 3* and *4*) which illustrated that more weed types were found in Qlyasan location that leads to more stress on wheat plants in Qlyasan, and as a result the wheat grain yield in Kani Panka was higher than Qlyasan. Furthermore, cleaned wheat seeds are more vigour and

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powerful to compete weed plans and this result was assured by De Lucas Bueno and Froud Williams (1996), Khazaei et al. (2016) and Al-Wagaa and Mohammed (2020). Less weed existence was observed when seeds were cleaned before sowing, this result was also emphasized by Hossain (2015), Verma et al. (2015), and Worku (2010) who mentioned that cleaning of crop seeds before sowing minimize weeds in the field. However, the cleaned seeds effect to increase grain yield through minimizing weeds was mentioned by several researchers (Chicouene, 2020; Butovchenko et al., 2018; Norsworthy et al., 2012).

The interaction combination of the treatment Kani Panka * cleaned seeds * herbicides mixture which had the higher grain yield (636.20 $g.m^{-2}$) may be referred to the effect of the use of herbicides mixture which minimized and controlled weed plants that have been grown with the wheat crop after sowing. Those weed seeds and propagules were a part of the soil seedbank, consist of narrow and broadleaved weeds, are controlled by Topic and Granstar herbicides mixture. Controlling of weeds that came from the soil by herbicides gave additional chances for wheat crop to use growth factors efficiently, which resulted to establish strong, tall plants, and productive wheat plants that produced remarkable grain yield. The positive effect of herbicides mixture to increase grain yield was supported by Al-Wagaa and Mohammed (2020).

On the other side the lowest yield was in the treatment of un-cleaned seeds with no weeding (199.93 $g.m^{-2}$), this treatment registered the highest weed dry weight (373.45 $g.m^{-2}$) as shown in *Table 5* and this reflects the general combined relation; high weed dry weight influences negatively and significantly the grain yield, this insures that high grain yield is associated with minimizing of weeds through seed cleaning or weed control after sowing.

Also from *Table 9* it is found that using cleaned seeds with narrow leaved herbicides in Qlyasan had significant difference compared to the treatment of cleaned seeds and broadleaved herbicides (299 and 245.05 $g.m^{-2}$ respectively), which showed the increase of yield by 23% and 14% respectively compared to control treatment, and this reflects the significant controlling effect of narrow leaved weeds, this result agrees to what Khan and Haq (2002) found in their study on wheat crop yield losses due to weeds when they mentioned that narrow leaved weeds are in charge of 30% of the wheat yield loss versus 24% losses caused by broadleaved weeds.

The same trend was found in the interactions of two factors in Kani Panka location, the highest record was in cleaned seeds with herbicides mixture while the lowest record was in the uncleaned seeds with no herbicide application (636.20 and 345.54 $g.m^{-2}$ respectively), this ensures that cleaned wheat seeds which contained no weed seeds show greater vigour due to bigger seeds selected and similarity in shape and size because of cleaning process can produce more healthy plants (Shi et al., 2017; Tibola et al., 2016; Ries and Everson, 1973) and as a result will produce more grain yield (Edwards and Krenzer Jr, 2006), in addition using of herbicides mixture enhance the weed control for those weed seeds that exist in the soil and grow with the crop plans and finally lead to higher grain yield, many papers have supported this approach (Kareem et al., 2018; Salim et al., 2017; Ali et al., 2016; Tityanov et al., 2015)

Conclusion

The results of this study indicated that herbicides mixture found to be effective in minimizing weed dry weight and in increasing of wheat yield, also the results assured

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that seed cleaning before sowing will enhance wheat yield and minimize weed dry weight, therefore in order to minimize using herbicides which cause environmental pollution it is recommended to apply seed cleaning process for wheat before sowing.

Recommendations

Seed cleaning process is effective in minimizing weeds; therefore, it is recommended to implement new studies on the comparison between, the cost and the environmental impact of using of herbicides as traditional method, compared to only using of seed cleaning.

As there are several seed cleaning factories and different mobile machines in Kurdistan region/Iraq, it is suggested to study and evaluate those machines in order to find out which type is most feasible and suitable to local conditions (weeds, crops, land and environment factors).

Seed cleaning machines work in different adjustments such as speeds and variable air discharge in addition to various sieve holes and shapes, additional research studies are needed to find which type of weeds are most vulnerable to seed cleaning method and adjustments, and the evaluation of the impact of captured weed seeds due to cleaning by machines on the crop production.

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APPENDIX

Table A1. *ANOVA table for the land experiment*

Source	DF	Means square (MS)					
		Weeds dry weight (g/m^2)	Number of spikes/m^2	Spikes weight (g/m^2)	Number of grains/spike	1000 grain weight	Grain yield (g/m^2)
Locations	1	31433.6**	5582 81**	1485562**	4.29201 ^{NS}	78.507**	3882588**
Replicates [location] and random	6	562.201 ^{NS}	2497.1**	2993.71*	8.79384 ^{NS}	1.46627 ^{NS}	735.72 ^{NS}
Seed cleaning	1	64485.2**	427.813 ^{NS}	34493.2**	128.296**	73.4403**	70589**
Locations * seed cleaning	1	69.9698 ^{NS}	10328.5**	4934.86*	124.176**	0.93528 ^{NS}	6535.94*

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Weed control	4	214000**	11379.7**	101707**	39.7716**	80.09**	103949**
Locations * weed control	4	3044.34**	3935.18**	14333.3**	21.0086**	2.6579 *	20035.9**
Seed cleaning * weed control	4	3890.09**	1902.5*	2929.16*	25.2626**	3.9114**	2987.07 ^{NS}
Locations * seed cleaning * weed control	4	134.203 ^{NS}	2584.33**	3486.9 *	7.75345 ^{NS}	1.67539 ^{NS}	5101.79**
Error	54	540.6	634.4	821.6	4.0777	0.8562	1205

Table A2. *Effect of seed cleaning, weed control treatments and their interactions on weed control efficiency % (WCE%) in two locations*

Locations (L)	**Seed cleaning (A)**	**Weed control (W)**					
		Control	**Hand weeding**	**Narrow leafed herbicides**	**Broad leafed herbicides**	**Herbicides mixture**	**Locations * seed cleaning**
Qlyasan	Cleaned seeds	--------	90%	15.6%	53.49%	90%	29.25%
	Not cleaned seeds	--------	84%	29.7%	53.23%	81.75%	---------
Kanipanka	Cleaned seeds	--------	94.96%	33.11%	78.62%	93.84%	39.12%
	Not cleaned seeds	---------	86.76%	37.08%	72.22%	84.97%	---------
Qlyasan		--------	86.51%	23.87%	53.33%	85.18%	---------
Kani Panka		-------	90.04%	35.49%	74.47%	88.52%	
Cleaned seeds		---------	92.32%	23.73%	65.14%	91.19%	---------
Not cleaned seeds		---------	85.32%	33.23%	62.31%	83.29%	
Weed control mean		**---------**	88.18%	29.35%	63.47%	86.76%	---------

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EFFECTS OF STRAW ENRICHMENT AND DEEP INCORPORATION ON HUMUS COMPOSITION AND HUMIC ACID STRUCTURE OF BLACK SOIL PROFILE IN NORTHEAST CHINA

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Abstract. Straw enrichment and deep incorporation (SEDI) is a new concept for soil restoration and conservation. It has been shown to improve soil organic carbon (SOC), humic C fractions, and enhance the structure of humic acid (HA). However, few studies have focused on the changes of humus components and structure of HA after SEDI at different depths of soil profile, especially under the subsurface soil. The purpose of study is to evaluate the effects of different soil profile (0-80 cm) on soil humus composition and structural characteristics of HA after SEDI in northeast China. Two different treatments, including CK (no straw) and SEDI (returning straw at a time with a total amount of 15385 $kg.hm^{-2}$, incorporating straw into 20-40 cm depth of black soil). Our results showed that SEDI significantly increased SOC contents, humus substances (HEC), humic acid carbon (HAC) and fulvic acid carbon (FAC). Among all the profiles, the increase most significant at the depth of 20-30 cm, the content of SOC, HAC, FAC and HMC was increased by 11.6%, 38.89%, 13.37% and 33.40%. The FTIR spectra and elemental results revealed that SEDI increased the aliphaticity of the HA structure. It can, therefore, be inferred that straw application will improve humus compositions, SOC content and enhance the stability of HA structure.
Keywords: *corn straw, deep incorporation, HA structure, soil profile, soil organic carbon*

Introduction

Over the past few years, the organic carbon storage of the black soil in Northeast China has significantly decreased (Han et al., 2018), due to excessive reclamation and long-term heavy application of chemical fertilizer, which resulted in shallow tillage layers and the degradation of black soil (Yi et al., 2020). According to Yin's study (Yin et al., 2018), crop straw is a good soil organic fertilizer. Consequently, straw return has become a routine practice in China, owing to its beneficial effects on crop productivity. Several studies (Wei et al., 2019; Chatterjee et al., 2013;Ingelmo et al., 2012; Hu et al., 2013; Li et al., 2018) have shown that the straw return is not only reflected in its effect on the change of soil organic carbon content, but can also improve the quality of SOM and crop productivity.

Scholars (Dou et al., 2011) usually divided the 0-40 cm soil layer into surface soil (0-20 cm) and subsurface soil (20-40 cm). However, most studies on straw return mainly focused on the surface soil. Shallow straw application significantly increases the content of organic carbon and humus components in soil surface according to a micro area experiment (Dong et al., 2017). Straw mulching improves the structure of surface soil and reduces soil compaction (Fan et al., 2018), but have little effect on the content of SOC in the subsurface soil, it affects seeding emergence and makes tillage difficult.

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However, Batjes showed that more than 50% of soil organic carbon is stored in the soil layer with a depth of 30-100 cm (Batjes et al., 1996). Thus, a new returning method is, hereby, invented, that is straw enrichment and deep incorporation (SEDI). Cong found that straw deep return improves the SOC content and humus C fractions in the 20-40 cm soil depth (Cong et al., 2019). Soil humic substances are important constituents of SOC (Brunetti et al., 2007). Zhang showed that straw deep return can also improve the quality and activity of soil humic substances (Zhang et al., 2012). However, Dou et al demonstrated that incorporating corn straw into 20-40 cm soil depth is more conducive to the accumulation of soil organic matter and humic C fractions (Dou et al., 2019), which enhances the aromatic C and aliphatic C groups, making HA structure more complicated (Cui et al., 2017; Zhang et al., 2016).

Previous studies mainly focus on the changing of soil organic matter after returning straw in the surface soil, but less talked about this kind of change in the subsurface soil. In this study, we conducted a year field trial, to (i) determine SOC and humic C fractions contents and (ii) to compare changes in HA characteristics under different depths.

Materials and methods

Site description

The soil was collected from the Enyu town of Changchun City, Jilin Province (N44°50′10.61″, E126°33′25.14″) at 0-80 cm soil depth. The region belongs to a temperate continental monsoon climate and receives an annual rainfall of 536.4 mm. According to the American soil classification system, the field is classified by black soil, belonging to Argiudolls. It belongs to the monsoon temperate semi humid climate in Northeast China. The temperature is mostly affected by the monsoon. It is dry and windy in spring, wet and rainy in summer, mild and cool in autumn and long and cold in winter. The basic properties of the tested soil are presented in *Table 1*. And the basic elemental composition of the straw is shown in *Table 2*.

Table 1. *Basic properties of the tested soil*

Depth/cm	Organic matter (g.kg^{-1})	2000-20 μm	20-2 μm	< 2 μm	pH
0-20	19.90	39.08	29.87	31.05	6.43
20-40	19.19	33.46	32.21	34.33	6.71

Table 2. *Basic properties of corn straw*

C (g.kg^{-1})	N (g.kg^{-1})	H (g.kg^{-1})	O + S (g.kg^{-1})	C/N
338.6	6.87	58.77	457.65	49.29

Experiment design

The experiment was initiated in the three week of June 2016. The straw was returned to the field under equal straw mass of 15385 kg/ha (equivalent to 100% straw return). Two treatments were randomly with three replications. The area of each plot is 18.2 m^2. The treatments included (i) CK (no straw applied), (ii) SEDI (rake the corn straw in the

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field together into rows at a ratio of 4:1 with a finger-plate rake, then crush the corn straw and bury it into subsoil, 20-40 cm deep along designated strips with a wind-driven input cylindrical plough (*Fig. 1*). Finally sow seeds into the strips with no corn straw buried in between the strips with corn straw buried in a normal way with a non-tillage seeder, to realize separation of the seeded strips (narrow rows) from the strips (wide rows) with straw buried in a wide and narrow row alternating cultivation mode). CK treatment was not disturbed.

Figure 1. *The wind-driven input cylinder plough*

Soil sampling and analysis

The soil was collected at the end of the growing season (October 2017), employing a 3-point sampling method, to make one composite mixture. Three random undisturbed soil samples were collected from each treatment at different profiles (0 to 10 cm, 10 to 20 cm, 20 to 30 cm, 30 to 40 cm, 40 to 50 cm, 50 to 60 cm, 60 to 70 cm, and 70 to 80 cm). After visible organic debris and stones were removed, the soil sample was air-dried and passed through a 2-mm sieve (*Fig. 2*).

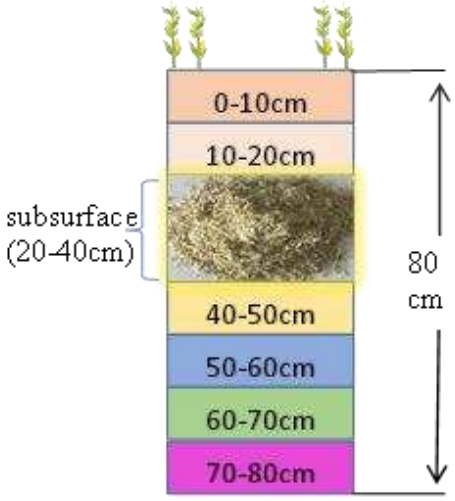

Figure 2. *Different soil layers in the same profile*

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Laboratory analysis

SOC was determined by potassium dichromate external heating method (Cambardella and Elliott, 1993), by treating 0.2 g soil sample into a 50 ml volumetric flask with 10 ml 0.4 N $K_2Cr_2O_7$-H_2SO_4 solution, and heating it at 180 ℃ until the first drop from the funnel. Then it was titrated with 0.2N $FeSO_4$.

Soil humus composition was analysed following the method by (Dou, 2010). Briefly, 5 g of soil sample was extracted with 30 ml of distilled water. After continuous shaking at 70 ℃ for 1 h in the water bath. The residue was extracted with 30 ml of a mixture of 0.1 N alkali solution (NaOH + $Na_4P_2O_7$). The C contents of HA (HA-C), FA (FA-C) and HM (HM-C) were determined.

HA isolation and purification were processed using the procedure described by International Humic Substances Society (Kuwatsuka, 1992). Briefly, 50 g of soil sample was treated with 1 N HCL in succession and shaking for 1 h. Then samples were extracted with 0.1 N NaOH solution to clean the precipitate and adjust pH to 13-14. The resulting liquid was low-speed centrifugation, high-speed centrifugation, electro-dialysed, rotary evaporation, freeze-drying and other processes.

HA element composition such as C, H, N and O were determined by a Vario EL III elemental analyzer (German).

The Fourier transform infrared spectrometer (Brucker TENSOR 27, German) was used to analyze the HAs, and the wave number was ranged from 4000 to 400 cm^{-1}, following the KBr tablet method.

Thermogravimetry (TG) and DTG analysis of solid HA samples were performed using STA 2500 Regulus thermogravimetric analyser (Netzsch, Germany). About 4–6 mg of solid HA sample was heated to 750 °C at a constant heating rate of 158 °C min^{-1}.

Data analysis

SPSS software was used for variance analysis to evaluate the effects of different depth on all measured soil parameters. Significance differences among treatment means were evaluated using the significant difference test with T test analysis at $p < 0.05$. All graphs and spectra were compiled using Origin 7.5 software. The DTA and TG charts were analyzed by Universal Analysis software. The humification index (PQ) was calculated as the formula:

$$PQ = \text{HA-C}/(\text{HA-C} + \text{FA-C}) \quad \text{(Eq.1)}$$

Results

Organic C contents and humus composition under different soil profile

After 16 months of field trial (*Fig. 3*), the contents of SOC ranged from 7.09 to 20.31 $g.kg^{-1}$, the SOC content decreased as the soil layer was deeper from the surface. Compared to CK, the SOC in SEDI treatment in 0-10 cm and 10-20 cm soil layers increased by 3.8% and 1.9%, respectively. The increase was most significant at the depth of 20-30 cm (11.59% higher than CK). The C content of humus components significantly increased after corn straw was returned, increasing in the order of SEDI > CK in the 0–40 cm depth (*Table 3*), but the soil layer changes in 40-80 cm are not significant. With the increasing of the depth of soil, the content of humus components decreased. Among all the profiles, HA-C, FA-C and HM-C contents had

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the most significant change in the 20-30 cm soil layer increased by 38.89%, 13.37% and 33.40%.

PQ value (*Eq. 1*) is the proportion of HA in humic substances, which can reflect the degree of humification of SOC. Compared with CK, the treatment of straw returning to the field increased the PQ value of the soil to varying degrees. And the SEDI treatment increased the PQ value of in the 20-30 cm soil the most, which increased from 62.07% to 66.72% compared with CK.

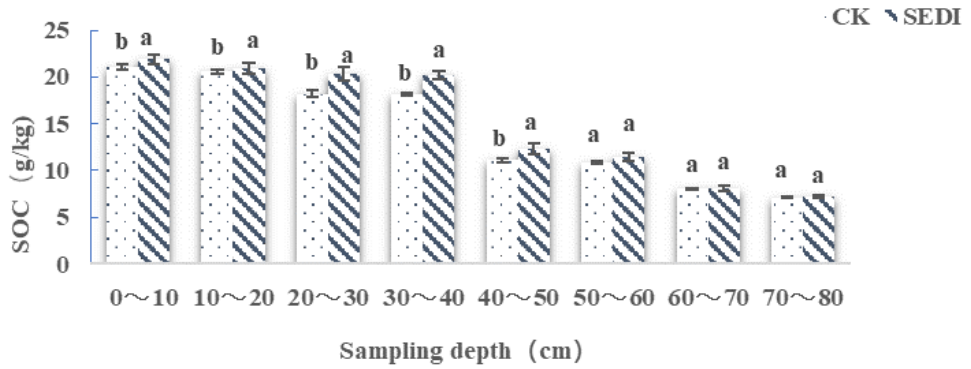

Figure 3. *Effects of straw enrichment and deep incorporation on soil organic carbon content. CK: no straw is applied in normal tillage; SEDI: straw enrichment and deep incorporation. Error bars represent the standard deviations of the mean (n = 3). Different lowercase letters indicate significant differences among treatments ($p < 0.05$)*

Table 3. *Effect of SEDI on the content of organic carbon in soil humus components*

Depth (cm)	Treatment	HA (g/kg)	FA (g/kg)	HM (g/kg)	PQ (%)
0~10	CK	3.51 ± 0.11^{b}	2.06 ± 0.26^{a}	5.26 ± 0.21^{b}	63.10
	SEDI	4.31 ± 0.14^{a}	2.14 ± 0.06^{a}	6.44 ± 0.21^{a}	66.82
10~20	CK	3.45 ± 0.11^{b}	2.04 ± 0.13^{a}	5.18 ± 0.24^{b}	62.82
	SEDI	4.26 ± 0.12^{a}	2.13 ± 0.17^{a}	6.42 ± 0.25^{a}	66.67
20~30	CK	3.06 ± 0.07^{b}	1.87 ± 0.05^{b}	4.67 ± 0.10^{b}	62.15
	SEDI	4.25 ± 0.07^{a}	2.12 ± 0.21^{a}	6.23 ± 0.17^{a}	66.72
30~40	CK	2.96 ± 0.09^{b}	1.83 ± 0.13^{b}	4.61 ± 0.23^{b}	61.80
	SEDI	4.03 ± 0.19^{a}	2.04 ± 0.26^{a}	6.04 ± 0.15^{a}	66.42
40~50	CK	1.76 ± 0.20^{b}	1.12 ± 0.26^{a}	3.17 ± 0.10^{a}	61.15
	SEDI	1.98 ± 0.06^{a}	1.17 ± 0.14^{a}	3.24 ± 0.17^{a}	62.79
50~60	CK	1.53 ± 0.21^{a}	0.96 ± 0.27^{a}	3.07 ± 0.17^{a}	61.50
	SEDI	1.57 ± 0.18^{a}	0.97 ± 0.21^{a}	3.13 ± 0.25^{a}	61.66
60~70	CK	1.31 ± 0.08^{b}	0.98 ± 0.24^{a}	2.05 ± 0.25^{a}	59.82
	SEDI	1.37 ± 0.16^{a}	0.91 ± 0.14^{a}	2.02 ± 0.20^{a}	60.05
70~80	CK	1.16 ± 0.13^{a}	0.79 ± 0.22^{a}	1.93 ± 0.26^{a}	59.63
	SEDI	1.23 ± 0.13^{a}	0.85 ± 0.24^{a}	1.97 ± 0.27^{a}	59.13

CK: no straw is applied in normal tillage; SEDI: straw enrichment and deep incorporation. The values represent the means ± standard error (SE). Means that do not share the same letter superscript within a column are significantly different ($p<0.05$). PQ is the humification index computed as PQ = HA-C/(HA-C + FA-C)

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Element composition of HA

Table 4 shows the elemental composition of soil HA across all treatments under different treatments, and the range of each attribute was as follows: C content was 478.5 to 525.8 g.kg^{-1}, N content was 20.00 to 29.85 g.kg^{-1}, H content was 35.70 to 41.90 g.kg^{-1}, and O content was 398.3 to 450.5 g.kg^{-1} (*Table 4*). Elemental C, H and N contents in HA were higher in SEDI treatment than in CK treatments. The O content showed a significant decrease in SEDI. These differences indicate that SEDI promotes the consumption of O element and the increases accumulation of C, N and H in soil HA. Among the all profiles, the H/C and O/C ratio decreased from the upper layer to lower layer of the soil. After SEDI, the H/C ratio of HA in surface layer and subsurface layer decreased. The O/C ratio decreased 9.75% compared with the CK group at the depth of 20-30 cm, which was the most significant decline in all profiles.

Table 4. *Effect of SEDI on HA element composition of black soil profile*

Depth (cm)	Treatment	C (g.kg^{-1})	H (g.kg^{-1})	N (g.kg^{-1})	O (g.kg^{-1})	The molar ratio	
						O/C	H/C
0~10	CK	510.4±0.4^{b}	40.40±0.30^{b}	25.18±0.19^{b}	420.1±0.6^{a}	0.617±0.001^{a}	0.950±0.007^{a}
	SEDI	525.8±0.4^{a}	41.90±0.35^{a}	29.85±0.08^{a}	415.2±0.5^{b}	0.592±0.001^{b}	0.956±0.007^{a}
10~20	CK	509.5±0.5^{b}	40.10±0.36^{b}	25.10±0.32^{b}	425.7±0.6^{a}	0.627±0.001^{a}	0.944±0.009^{b}
	SEDI	521.9±0.4^{a}	41.50±0.40^{a}	29.50±0.2^{a}	412.5±0.3^{b}	0.593±0.001^{b}	0.954±0.009^{a}
20~30	CK	498.2±0.6^{b}	38.70±0.46^{b}	22.70±0.26^{b}	436.2±0.6^{a}	0.657±0.001^{a}	0.932±0.011^{b}
	SEDI	520.3±0.6^{a}	40.90±0.44^{a}	29.00±0.47^{a}	411.1±0.5^{b}	0.593±0.002^{b}	0.943±0.009^{a}
30~40	CK	492.9±0.6^{a}	38.00±0.35^{a}	22.16±0.17^{b}	440.7±0.5^{a}	0.670±0.001^{a}	0.925±0.008^{a}
	SEDI	491.7±0.4^{a}	38.00±0.26^{a}	27.80±0.36^{a}	398.3±0.5^{b}	0.608±0.001^{b}	0.927±0.006a
40~50	CK	490.4±0.4^{a}	37.10±0.61^{a}	21.43±0.13^{b}	442.9±0.4^{a}	0.677±0.001a	0.908±0.015^{b}
	SEDI	491.0±0.3^{a}	37.80±0.36^{a}	22.79±0.17^{a}	438.9±0.6^{b}	0.670±0.002^{b}	0.924±0.008^{a}
50~60	CK	489.1±0.3^{a}	36.80±0.26^{b}	21.06±0.13^{a}	443.8±0.6^{a}	0.681±0.001^{a}	0.903±0.006^{a}
	SEDI	488.8±0.5^{a}	37.00±0.53^{b}	21.71±0.55^{a}	440.9±0.5^{b}	0.677±0.002^{b}	0.908±0.015^{a}
60~70	CK	486.3±0.5^{b}	36.30±0.44^{b}	20.81±0.12^{b}	445.7±0.3^{a}	0.687±0.001^{a}	0.896±0.011^{b}
	SEDI	488.7±0.4^{b}	36.70±0.36^{b}	21.10±0.23^{a}	441.0±0.3^{b}	0.676±0.001^{b}	0.901±0.008^{a}
70~80	CK	480.2±0.4^{a}	35.70±0.30^{a}	20.00±0.18^{b}	450.5±0.4^{a}	0.704±0.001^{a}	0.892±0.007^{b}
	SEDI	478.5±0.5^{b}	35.90±0.52^{a}	20.62±0.24^{b}	445.4±0.4^{b}	0.695±0.001^{b}	0.900±0.013^{a}

The values represent the means ± standard error (SE). Means that do not share the same letter superscript within a column are significantly different ($p<0.05$). CK: no straw is applied in normal tillage; SEDI: straw enrichment and deep incorporation

FTIR spectra of HA

The FTIR spectrum of soil HA for different treatments are shown in *Figure 4*, and the corresponding relative intensity of the main absorption peaks are listed in *Table 5*. The spectra bands across all treatments exhibited similar characteristics (*Fig.2*), differing only in their relative intensities of absorption (*Table 5*). Absorption peaks were assigned to organic functional groups following (Zhang et al.,2017). The SEDI treatment showed a relative abundance of aromatic peaks at 1620 cm^{-1} (*Table 5*).

The ratio of I_{2920}/I_{1720} and I_{2920}/I_{1620} which reflects the strength of the ratio of aliphatic C to carboxyl C and aromatic C in the HA structure, respectively, revealed that SEDI treatment increased the aliphatic/carboxyl C and aliphatic/aromatic C (*Table 5*). Compared with CK, the ratio of I_{2920}/I_{1720} and I_{2920}/I_{1620} of HA in 20-30 cm soil layer increased significantly than that of CK, with increase by 144.06% and 104.15%, respectively.

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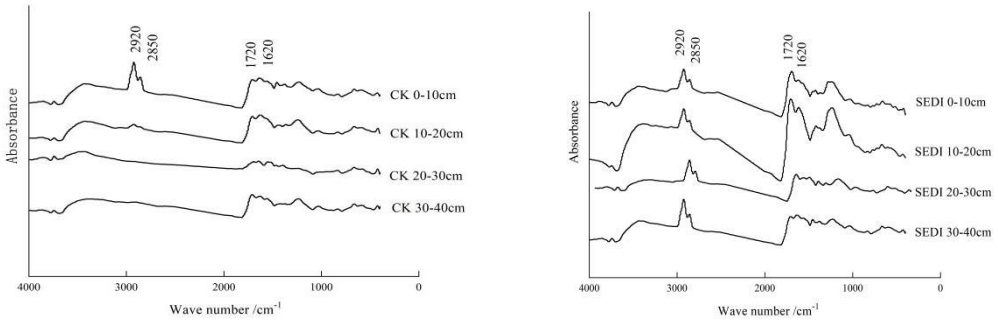

Figure 4. *FTIR spectrum of soil HA under different treatments. FTIR spectra of HA structure in soil layers 0~20 cm and 20~40 cm from left to right. CK: no straw is applied in normal tillage; SEDI: straw enrichment and deep incorporation*

Table 5. *Effect of SEDI on the relative absorption intensity of soil HA*

Depth (cm)	Treatment	Relative intensity (%)				Ratio	
		2920 cm^{-1}	2850 cm^{-1}	1720 cm^{-1}	1620 cm^{-1}	2920/1720	2920/1620
0~10	CK	3.78 ± 0.009^{b}	1.497 ± 0.007^{b}	7.563 ± 0.013^{a}	7.712 ± 0.010^{b}	0.473 ± 0.001^{b}	0.464 ± 0.001^{b}
	SEDI	5.547 ± 0.007^{a}	1.933 ± 0.013^{a}	7.315 ± 0.007^{b}	9.373 ± 0.014^{a}	0.758 ± 0.001^{a}	0.592 ± 0.001^{a}
10~20	CK	2.532 ± 0.015^{b}	1.217 ± 0.014^{b}	7.472 ± 0.006^{a}	7.531 ± 0.003^{b}	0.339 ± 0.002^{b}	0.336 ± 0.001^{b}
	SEDI	5.032 ± 0.019^{a}	1.872 ± 0.006^{a}	7.237 ± 0.091^{b}	9.019 ± 0.008^{a}	0.695 ± 0.007^{a}	0.558 ± 0.002^{a}
20~30	CK	2.019 ± 0.019^{b}	1.203 ± 0.007^{b}	7.381 ± 0.006^{a}	7.435 ± 0.007^{b}	0.274 ± 0.003^{b}	0.272 ± 0.002^{b}
	SEDI	4.752 ± 0.007^{a}	1.856 ± 0.010^{a}	7.118 ± 0.007^{b}	8.572 ± 0.007^{a}	0.668 ± 0.001^{a}	0.554 ± 0.001^{a}
30~40	CK	1.934 ± 0.010^{b}	1.119 ± 0.120^{b}	7.201 ± 0.107^{a}	7.353 ± 0.018^{b}	0.269 ± 0.002^{b}	0.263 ± 0.001^{b}
	SEDI	4.246 ± 0.010^{a}	1.801 ± 0.010^{a}	7.019 ± 0.005^{b}	8.351 ± 0.007^{a}	0.605 ± 0.001^{a}	0.508 ± 0.001^{a}
40~50	CK	1.851 ± 0.010^{b}	1.017 ± 0.056^{b}	6.985 ± 0.004^{a}	7.112 ± 0.005^{b}	0.265 ± 0.001^{b}	0.26 ± 0.002^{a}
	SEDI	2.038 ± 0.017^{a}	1.386 ± 0.066^{a}	6.873 ± 0.010^{b}	8.098 ± 0.015^{a}	0.297 ± 0.002^{a}	0.252 ± 0.002^{a}
50~60	CK	1.726 ± 0.010^{a}	0.996 ± 0.009^{b}	6.533 ± 0.007^{a}	6.688 ± 0.009^{b}	0.264 ± 0.002^{a}	0.258 ± 0.002^{a}
	SEDI	1.73 ± 0.012^{a}	1.217 ± 0.020^{a}	6.541 ± 0.008^{a}	7.579 ± 0.008^{a}	0.264 ± 0.002^{a}	0.228 ± 0.001^{b}
60~70	CK	1.558 ± 0.009^{b}	0.873 ± 0.008^{b}	5.897 ± 0.007^{b}	6.535 ± 0.007^{b}	0.264 ± 0.002^{a}	0.238 ± 0.001^{a}
	SEDI	1.603 ± 0.01^{a}	1.053 ± 0.013^{a}	6.003 ± 0.007^{a}	7.462 ± 0.011^{a}	0.267 ± 0.002^{a}	0.215 ± 0.001^{b}
70~0	CK	1.472 ± 0.010^{b}	0.862 ± 0.056^{b}	5.658 ± 0.007^{b}	6.432 ± 0.006^{b}	0.26 ± 0.002^{a}	0.229 ± 0.001^{a}
	SEDI	1.526 ± 0.006^{a}	1.012 ± 0.006^{a}	5.992 ± 0.092^{a}	7.361 ± 0.009^{a}	0.255 ± 0.003^{a}	0.207 ± 0.001^{b}

The values represent the means ± standard error (SE). Means that do not share the same letter superscript within a column are significantly different ($p<0.05$). I_{2920}/I_{1720}, absorption intensity ratio of 2920 cm^{-1} and 2850 cm^{-1} calculated as (2920 + 2850)/1720; I_{2920}/I_{1620}, absorption intensity ratio of 2920 cm^{-1} and 1620 cm^{-1} calculated as (2920 + 2850)/1620. CK: no straw is applied in normal tillage; SEDI: straw enrichment and deep incorporation

Thermal analysis of HA

TG (Thermogravimetric) and DTA (Differential Thermal Analysis) curves of soil HA under different treatments are shown in *Figure 5*. During the pyrolysis process, HA samples mainly showed moderate temperature exotherm (299–340°C) and high-temperature exotherm (467–501°C) (*Fig. 5*). The TG curves of all studied HA samples showed gradual weight loss with increasing temperatures, and then steep weight loss at temperatures greater than 400°C was observed. The semi-quantitative fundamental results presented in *Table 6*, revealed that SEDI treatment had a significant increase in exothermic heat and weight loss at moderate temperature, compared with CK, but decreased at the high/moderate heat ratio temperature and the high/moderate mass loss.

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It shows that SEDI can also increase the aliphatic compounds and aromatic compounds, and reduce the thermal stability.

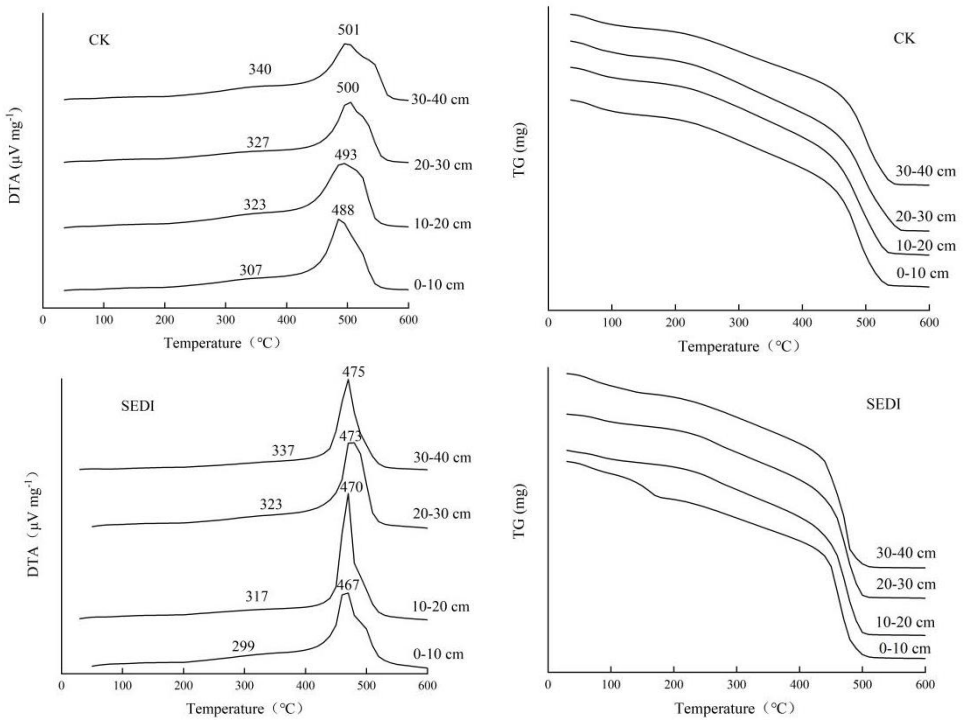

Figure 5. *Effect of different treatments on differential thermogravimetric analysis (DTA) curve of soil HA. From left to right are the heat DTA diagram of HA and the TG diagram of HA. CK: no straw is applied in normal tillage; SEDI: straw enrichment and deep incorporation*

Table 6. *Effects of SEDI on heat release and weight loss of HA*

Depth	Treatment	Exothermic heat ($kJ.g^{-1}$)		Exothermic heat ratio of moderate and high temperature	Mass loss ($mg.g^{-1}$)		Ratio of high and moderate mass loss
		Moderate temperature	High temperature		Moderate temperature	High temperature	
0~10	CK	0.108 ± 0.002^{b}	7.100 ± 0.122^{a}	65.946 ± 0.216^{a}	138.5 ± 0.458^{b}	576.8 ± 0.436^{b}	4.16 ± 0.110^{a}
	SEDI	0.125 ± 0.002^{a}	7.119 ± 0.045^{a}	57.107 ± 0.742^{b}	140.6 ± 0.520^{a}	583.1 ± 0.529^{a}	4.15 ± 0.017^{a}
10~20	CK	0.0902 ± 0.001^{b}	7.085 ± 0.007^{b}	78.550 ± 0.628^{a}	130.6 ± 0.520^{b}	558.2 ± 0.557^{b}	4.27 ± 0.013^{a}
	SEDI	0.141 ± 0.002^{a}	7.441 ± 0.027^{a}	52.903 ± 0.687^{b}	144.8 ± 0.520^{a}	599.5 ± 0.458^{a}	4.14 ± 0.017^{b}
20~30	CK	0.088 ± 0.001^{b}	7.077 ± 0.019^{b}	80.422 ± 0.707^{a}	128.4 ± 0.458^{b}	555.6 ± 0.436^{b}	4.33 ± 0.013^{a}
	SEDI	0.149 ± 0.002^{a}	7.715 ± 0.007^{a}	51.669 ± 0.555^{b}	170.1 ± 0.557^{a}	610.5 ± 0.400^{a}	3.59 ± 0.009^{b}
30~40	CK	0.084 ± 0.001^{b}	6.823 ± 0.068^{b}	81.229 ± 0.600^{a}	125.1 ± 0.458^{b}	541.3 ± 0.458^{b}	4.33 ± 0.013^{a}
	SEDI	0.165 ± 0.003^{a}	7.937 ± 0.007^{a}	48.208 ± 0.706^{b}	175.6 ± 0.794^{a}	630.3 ± 0.436^{a}	3.59 ± 0.015^{b}
40~50	CK	0.070 ± 0.002^{b}	5.788 ± 0.109^{b}	82.302 ± 0.253^{a}	115.2 ± 0.600^{b}	524.5 ± 0.360^{b}	4.55 ± 0.021^{a}
	SEDI	0.112 ± 0.002^{a}	7.079 ± 0.012^{a}	63.398 ± 0.780^{b}	138.6 ± 0.458^{a}	589.2 ± 0.721^{a}	4.25 ± 0.010^{b}
50~60	CK	0.063 ± 0.001^{b}	5.096 ± 0.046^{b}	83.713 ± 0.722^{a}	109.8 ± 0.624^{b}	513.7 ± 0.624^{a}	4.68 ± 0.024^{a}
	SEDI	0.083 ± 0.001^{a}	6.589 ± 0.013^{a}	79.396 ± 0.800^{b}	119.3 ± 0.458^{a}	503.7 ± 0.529^{b}	4.22 ± 0.013^{b}
60~70	CK	0.048 ± 0.001^{b}	4.463 ± 0.020^{b}	88.817 ± 0.679^{a}	95.6 ± 0.520^{a}	500.6 ± 0.557^{b}	5.24 ± 0.034^{b}
	SEDI	0.062 ± 0.002^{a}	5.349 ± 0.106^{a}	86.744 ± 0.787^{a}	96.7 ± 0.458^{a}	514.7 ± 0.458^{a}	5.32 ± 0.021^{a}
70~80	CK	0.053 ± 0.006^{a}	4.203 ± 0.012^{b}	89.006 ± 0.588^{b}	90.3 ± 0.557^{a}	500.1 ± 0.600^{b}	5.54 ± 0.028^{a}
	SEDI	0.054 ± 0.001^{a}	4.968 ± 0.011^{a}	92.571 ± 0.798^{a}	90.8 ± 0.557^{a}	504.7 ± 0.458^{a}	5.59 ± 0.030^{a}

The values represent the means ± standard error (SE). Means that do not share the same letter superscript within a column are significantly different ($p<0.05$). CK: no straw is applied in normal tillage; SEDI: straw enrichment and deep incorporation

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Discussion

Effects of SEDI on SOC and humus composition at different depths of the black soil profile

Our results showed that from the soil profile, the component of soil organic matter and soil humus decreased gradually from upper to lower layers in all the treatments (*Table 3*). This may be due to the fact that activated carbon tends to accumulate in the surface layer of soil (Liao et al., 2015). As the profile goes down, the source of organic carbon decreases, and the decomposition rate of straw decreases, so that the organic carbon content continues to decrease (Hao et al., 2017). In addition, the mineralization of microorganisms continuously consumes the original soluble organic matter, so that the increase of SOC is continuously reduced, showing a downward trend (Na et al., 2015). As the SOC content gradually decreases, the content of each component of humus also shows a downward trend. After straw enrichment and deep incorporation, as the depth of the profile increases, the content of soil organic matter and humic substances also decrease layer by layer, while they are all higher than CK on the same profile. Among them, the SEDI has the most significant effect on the accumulation of organic matter in the 20-30 cm soil layer, and its influence range is 0-50 cm. It may be because the straw is crushed and returned to the field, which reduces the volume of the straw and increases the contact area between the microorganisms and the straw. This leads to the decomposition rate of straw was accelerated, so that the organic matter and the nutrient can be fully released and enhance the organic matter content (Li et al., 2006; Kubar et al., 2018). And after SEDI, the soil was loosened, the soil microbial biomass and its activity were improved, which was conducive to the development of roots to the lower soil layer, and the content of organic matter in subsurface layer was higher than that of the surface layer.

Studies have shown that straw deep incorporation can significantly increase the organic matter content of subsurface layers (Cong, 2019). It also increases significantly the soil humus components and the PQ value (Cui et al., 2017). Our current study further confirmed that SEDI increased the carbon contents of humus components (HA, FA, and HM), with the greatest improvement in the 20-30 cm soil layer. The reason for a more significant increase of HA content may be that the stem, root or root exudates of crop straw provide a large amount of carbon source input to the soil (Ma et al., 2020), increase the content of soil organic carbon, improve the composition of humus, and then enhance the soil Hu Fu ratio (HA/FA), that is, increase the degree of soil humification (Cong, 2019). On the other hand, the enriched straw was buried in the subsurface of the soil, which disturbs the subsurface of the soil, increases the oxidizable carbon in the soil, and then increases the soil carbon source, stimulates the microbial activity (Lobe et al., 2011), and leads to the increase of microbial metabolic rate (Peng et al., 2013).

SEDI improves the structural characteristics of HA

Studies have shown that SEDI can enhance the HA aliphatic structure and make the HA molecular structure simpler and youthful (Cui et al., 2017). Our results show that H/C ratio, I_{2920}/I_{1720} and I_{2920}/I_{1720} ratio decrease from top to bottom with the increase of soil depth. This indicates that the degree of oxidation and aromaticity of HA in humus decreases as the soil layer increases. After straw enrichment and deep incorporation, the content of aliphatic chain hydrocarbon and aromatic carbon of HA increased, the

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content of Carboxylic Carbon decreased, the hydrophobicity of HA increased, and the structure of HA became simpler.

Research have shown that the hydrophobicity of aliphatic carbon was significant for maintaining the stability of soil organic carbon (Bai, 2017). Straw returning could increase the aliphatic carbon content of HA molecule (Zhang, 2020), and make the aliphatic carbon in HA molecule hydrophobic (Chen, 2020). As shown in *Table 3*, the (O + S)/C ratio of HA after straw enrichment and deep incorporation was lower than that of CK, indicating that straw application could increase the content of aliphatic compounds in soil and increase the hydrophobic C content of HA (Li et al., 2016). Among the different depth, the (O + S)/C ratio of 20-30 cm treatment in SEDI is the lowest, which indicates that HA molecules have less oxygen-containing functional groups, lower oxidation degree, and tend to simplify the structure (Vance, 1987). The H/C ratio of 20~30 cm treatment in SEDI was the highest, which indicated that the reduction trend of HA molecular condensation degree was the most obvious.

SEDI treatment increased the number of aliphatic chain hydrocarbon and aromatic hydrocarbon functional groups in FITR spectrum of HA and decreased the content of carboxyl group (*Table 4*). The results showed that straw returning could increase the content of aliphatic chain hydrocarbons and aromatic hydrocarbons in soil, which was consistent with Dong (Dong, 2017). For different treatments, the content of aliphatic chain hydrocarbon and aromatic hydrocarbon functional groups increased most obviously in 20-30 cm treatment of SEDI. This may be because that straw buried in the subsurface layer increases the carbon source of the soil, enhances the activity of microorganisms, and promotes the decomposition of HA (Kwiatkowska Malina, 2017). On the other hand, after the straw is enrichment and deep incorporation, the alkyl carbon and alkoxy carbon of HA will increase, which will increase the aliphaticity of HA and the carboxyl carbon will decrease, resulting in the reduction of the oxidation degree of soil HA (Wu, 2005). In elemental and differential thermal analysis, the (O + S)/C of the surface and sub-surface layers after deep incorporation are both higher than CK, and H/C ratio is lower than CK, the exothermic high/medium ratio and the mass loss high/medium ratio increased, indicating that the straw enrichment and deep incorporation reduces the degree of oxidation of HA molecules, the degree of condensation decrease and the thermal stability is reduced. This result is consistent with FTIR spectral analysis.

Conclusion

We used quantitative analysis of SOC and humus composition as well-qualitative analysis of humic acid (HA) and found that:

i. Addition of corn straw amendments in the soil significantly increased SOC content and humus composition. As the depth of the profile increases, the content of SOC and humic substances decreases layer by layer. Compared with CK, the SOC, HA-C, FA-C and HM-C of different depth soil layers in SEDI treatment increased in varying degrees, with the most increase in 20-30 cm soil layer.
ii. SEDI treatments increased the aliphaticity of the HA molecular structure, making it simpler.

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Therefore, in conventional planting, application of corn straw will improve SOC content, reduced the oxidation degree, condensation degree and thermal stability, and has the most significant effect on the 20-30 cm soil layer in the black soil of Northeast China. However, further research is needed to confirm the present conclusions by determining the long-term responses of humic substances and SOC to SEDI treatments.

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PHENOTYPIC VARIATION OF NATURAL *POPULUS PRUINOSA* SCHRENK POPULATIONS SHAPED BY GEOGRAPHICAL AND CLIMATIC FACTORS

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Abstract. *Populus pruinosa* Schrenk (*P. pruinosa*) is an important domestic forest species, only distributed in Xinjiang in China. To better protect the genetic germplasm resources of *P. pruinosa populations*, we study the phenotypic variations of leaf, seed, and fruit traits affected by geographical and climatic factors through investigation of natural *P. pruinosa* populations. Here, twenty-eight traits associated with leaf, seed and fruit characteristics were measured in the seventeen populations. The results of phenotypic variation show that leaf phenotypic traits directly exposed to the air undergo the greater morphological changes in order to adapt to changes in the external environment. The changes of phenotypic traits of different natural *P. pruinosa* populations can reveal different patterns of geographic variation. The phenotypic variation of *P. pruinosa* among populations (44.45%) was significant more than that within populations (18.14%), suggesting that the former was the main source of the total variation in phenotypic traits. The correlation analysis of phenotypic traits of natural *P. pruinosa* populations and environmental factors show that changes in annual average precipitation and evaporation have the most significant effects on the phenotypic traits. These results indicate that the phenotypic traits of natural *P. pruinosa* populations in the arid area are more sensitive to the damp environment over a long period of evolution.
Keywords: *natural forest, quantity trait, morphological change, population variation*

Abbreviations: CA, capsule area (cm^2); CI, capsule index; CL, capsule length (cm); CP, capsule perimeter (cm); CW, capsule width (cm); GI, germination index (%); GP, germination potential (%); GR, germination rate (%); LA, leaf area ($cm^{2)}$; LBA, leaf base angle; LL, leaf length (cm); LLW, left leaf width (cm); LP, leaf perimeter (cm); LSI, leaf shape index; LTA, leaf tip angle; LW, leaf width (cm); PEL, petiole length (cm); PL, podetium length (cm); SA, seed area (mm^2); SL, seed length (mm); SP, seed perimeter (mm); SSI, seed shape index; SW, seed width (mm); TSW, thousand seeds weight (g); VA, vein angle; VD, vein density (n); VN, vein number (n); WBD, wide base distance (cm)

Introduction

Global climate change have seriously affected the distribution and behaviour of most species, leading to a gradual increase in the number of endangered species, and it has also increased people's attention to biodiversity, ecosystem integration, and ecological health. As a result, conservation biology has naturally become a popular topic in academic

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research (Hoban and Schlarbaum, 2014; Trombulak et al., 2004; Flanagan et al., 2018). The genetic diversity of a species is not only the responded to environmental changes but also an important component of biodiversity (Flower et al., 2018). Plant phenotypic diversity is the result of interactions between genetic and environmental factors, and plays an important role in adaptive evolution (Wagner and Mitchell-Olds, 2018; Ghalambor et al., 2015; Hendry, 2015). Environmental changes can cause a plasticity effect on phenotypic traits of species, which can be produced by genetic differentiation at the population level or by individuals changing their phenotypes or choosing an environment to adapt to the new habitat (Sun et al., 2016; Edelaar et al., 2017; Hallsson and Björklund, 2012). Due to the plasticity of phenotypic traits, phenotypic diversity of species is ubiquitous in organisms (Holloway, 2002). Hence, Species can be genetically marked at the morphological, physiological and molecular levels, all of which give us a further knowledge of the genetic diversity of a particular species. Among them, phenotype determination at the morphological level is the most realistic and direct methods (Sreekanth et al., 2014; Xu et al., 2016). Studies on phenotypic traits could not only reveal phenotypic differences among and within populations, but also reflect the characterization of genetic variation (Brochmann et al., 1992). Up to now, stable plant phenotypic traits have been widely used by many scholars in research of cash crops (Rabara et al., 2014; Khadivi, 2018; Mashilo et al., 2017; Ngwepe et al., 2021), forest trees (Singh et al., 2015; Li et al., 2018), horticultural varieties (Zhang et al., 2017) and invasive species (Wang et al., 2018; Dong et al., 2015; Liu et al., 2014).

Leaf morphology is closely related to plant nutrition, physiology and ecological factors, so leaf phenotypic variation have a high research value (Chechowitz et al., 1990). For example, with the increase of altitudinal gradients, the plant populations show that leaves become smaller and thicker, along with the increase of nutrient content (Bresson et al., 2011). Under drought stress, the morphological and anatomical structure of leaves show the obvious characteristics of drought resistance, improve the photosynthetic capacity and the content of osmotic regulation substances (Zhai, 2020). Secondly, the phenotypic traits of fruit and seed can not only determine the mode and ability of species dispersal, but also affect seed germination and seedling settlement, and then affect the distribution pattern of populations (Li et al., 2013). For example, the variation pattern of seed characteristics on a large scale is usually related to climate and latitude gradient (García et al., 2010; Zhao et al., 2015). The zonal changes of seed morphological characters have become one of the contents of seed geography research (Fang and Yu, 2012). Therefore, it is imperative to measure the phenotypic traits of the *P. pruinosa* leaf, seed and fruit to understand the effects of environmental filtering on plant functional traits and the resulting biogeographic distribution pattern of species (Wright et al., 2007).

Populus pruinosa Schrenk and *Populus euphratica* Oliv. belong to the sect. Turanga in Populus of Salicaceae. *P. pruinosa* is only distributed in Xinjiang in China and mainly exists near the Hetian River and Ye Er-Qiang River and along the upstream coast of the Tarim Basin, where it often forms a large-scale mixed forest with *P. euphratica* (Yuan et al., 2020). Because of its excellent resistance to saline and alkali conditions, drought, heat and sand, *P. pruinosa* forests have become the main species used to maintain the balance of the ecosystem and the survival of the desert “green corridor” in the extremely arid area of the Tarim Basin. it played an irreplaceable role in maintaining the regional ecological balance and the safe development of the local society and economy. Although recent researches involved the anatomical structural indexes of heteromorphic leaves (Zhai et al., 2019) and the genetic structure investigated by SSR molecular markers (Zhang et al.,

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2012), studies on the genetic structure and environmental adaptability of natural *P. pruinosa* by analyzing the phenotypic variation of leaf, fruit and seed have not been reported. Changes in phenotypic traits were indispensable for describing species variation. Here, the phenotypic traits were measured and analysed in the *P. pruinosa* populations, and the genetic diversity of different natural *P. pruinosa* populations was studied as follows: (1) Analyse the phenotypic traits of different natural populations areas to study the geographical variation characteristics of morphological changes. (2) Explore the source of population variation and evaluate the impact of different environmental factors in phenotypic traits. The aim of our study was to advance our knowledge of the biological characteristics of different *P. pruinosa* populations, promote the collection of genetic germplasm resources and breeding of high-quality germplasm resources of the species.

Materials and methods

Study site

When investigating the distribution of *P. pruinosa* in the northwestern China, experimental materials were ultimately collected from 17 natural populations (*Fig. 1*). The date of sampling was collected from May to September in 2020. For each natural population, the tree age was calculated through the Logistic equation (Gu et al., 2013), 15 50-year-old trees displaying good growth that are free from pests are randomly selected as the samples, with the distance between the samples being greater than 100 m. For each tree, 3 replicates of matured broad ovate leaves, 15 replicates of fruit and 10 replicates of seeds are used. The longitude, latitude and altitude values are measured in the field by a GPS device, and other environmental factors were obtained by Wheat A software (http://www.wheata.cn) (*Table A1*). The general geographic distribution map is shown in *Figure 2*.

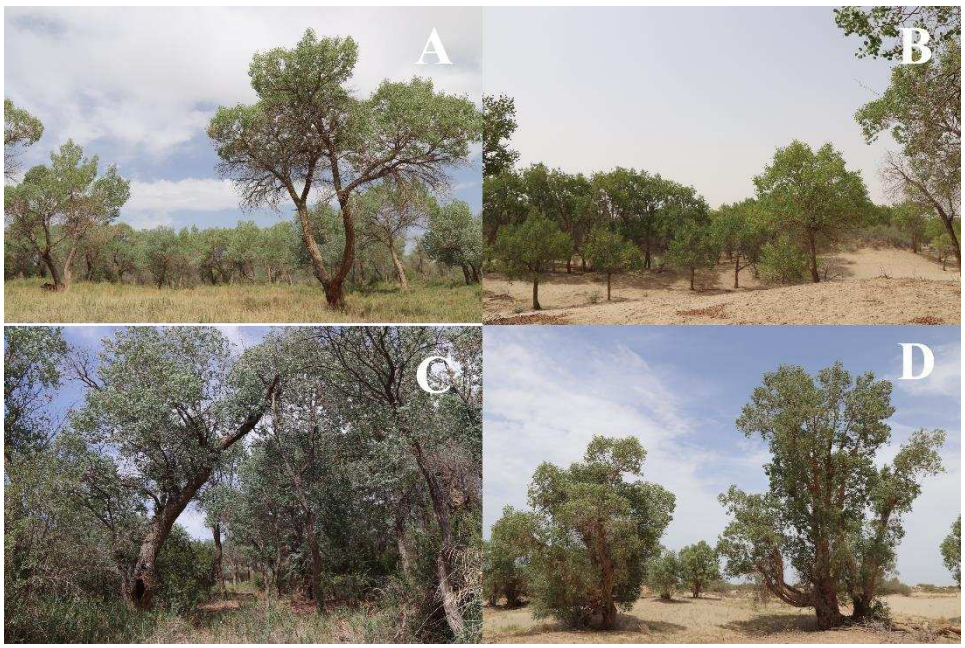

Figure 1. *Sampling sites of natural Populus pruinosa populations. (A) natural zpx population, (B) natural clx population, (C) natural ale population, (D) natural scx population*

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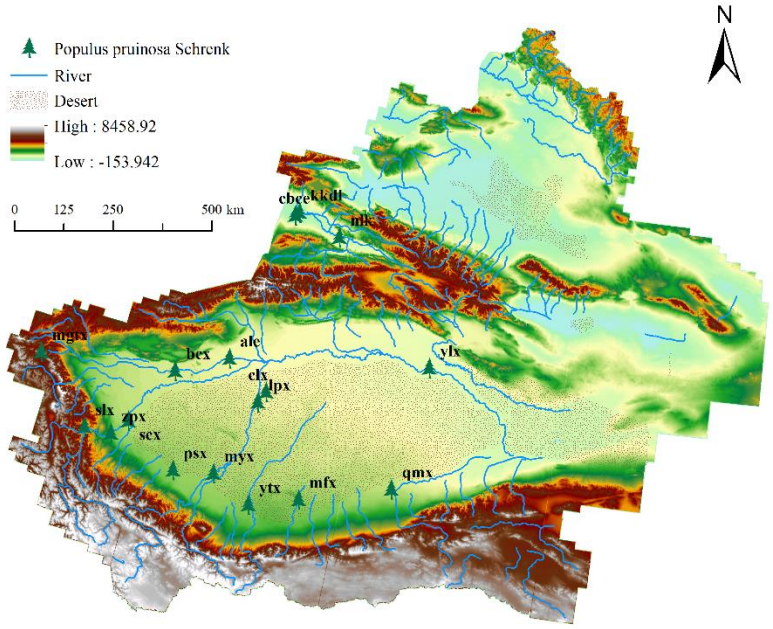

Figure 2. *Geographical distribution map of 17 natural Populus pruinosa populations*

Measurement of phenotypic traits

The leaves, fruits of natural *P. pruinosa* populations were photographed by using a digital camera, and its length, area, perimeter and angle of phenotypic traits were measured by *pruinosa* software (https://imagej.net/software/fiji/downloads). The sampling leaves were collected at the third nodes from the base of current branch. The leaf traits included leaf length/cm (LL), leaf width/cm (LW), wide base distance/cm (WBD), left leaf width/cm (LLW), petiole length/cm (PEL), leaf tip angle (LTA), leaf base angle (LBA), vein angle (VA), leaf area/cm^2 (LA), leaf perimeter/cm (LP), vein numbers/n (VN), leaf shape index (LSI) and vein density/n (VD). The fruit traits included capsule length/cm (CL), capsule width/cm (CW), capsule index (CI), capsule area/cm^2 (CA), capsule perimeter/cm (CP) and podetium length/cm (PL). The seed traits included seed length/mm (SL), seed width/mm (SW) seed area/mm^2 (SA), seed perimeter/mm (SP), seed shape index (SSI), germination rate (GR), germination potential (GP) and the germination index (GI), thousand seeds weight (TSW). The WBD is the distance from the maximum leaf width to the leaf base. The LLW refers to the distance from the left edge of the widest leaf to the centre. The LTA refers to the angle between the blade edge and the main vein at the blade tip. The VN refers to the number of primary veins on the left and right sides.

The computational formulas are as follows:

$$\text{Vein density} = \frac{\text{vein numbers}}{2\times\text{ength}}\times 100\% \quad \text{(Eq.1)}$$

$$\text{Leaf shape index} = \frac{\text{leaf length}}{\text{leaf width}}\times 100\% \quad \text{(Eq.2)}$$

The seed traits were measured by stereoscopic microscopy. The thousand seeds that was uniformly and randomly selected from 15 trees for each population was measured 4

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times by an electronic balance with an accuracy of 0.0001 g. Finally, the seeds from 5 randomly selected trees for each population were subjected to germination experiments with 3 replicates per tree and 100 seeds per replicate.

A solution of 1% NaClO was used to saturate seeds for an hour, after which the seeds were immediately washed with distilled water and blotted with absorbent paper. We placed the seeds evenly into Petri dishes lined with 2 layers of sterilized filter paper, added 8 mL distilled water to each Petri dish, weighed each dish, and then moved the Petri dishes into a light incubator for culturing. The experimental conditions were as follows: 08:00-24:00, light, temperature 30 °C; 0:00-08:00, dark, temperature 25 °C. Water was added to the dish at 14:00 every day to maintain moisture. The germination of seeds was recorded every day. all the germination experiments were ended at 7 days. The relevant formulas of seed germination are as follows:

$$\text{Seed germination rate (GR)} = \frac{\text{numbers of seed germination}}{\text{numbers of experimental seeds}} \times 100\% \quad \text{(Eq.3)}$$

$$\text{Germination potential (Gp)} = \frac{\text{max numbers of seed germination}}{\text{number of experimental seeds}} \times 100\% \quad \text{(Eq.4)}$$

$$\text{Germination index (GI)} = \sum \frac{\text{Numbers of seed germination per day (Gt)}}{\text{seed germination days (Dt)}} \times 100\% \quad \text{(Eq.5)}$$

Statistical analysis

The observed and recorded data were sorted and summarized in the Excel 2016 software. The coefficient of variation (CV) was calculated according to the average (X) and standard deviation (SD) of phenotypic traits of *P. pruinosa*. The significance test using Duncan multiple comparison method, Nested design of variance and Person related analyses of 28 phenotypic traits in the seventeen populations were performed with SPSS 19.0 software (IBM Corp., Armonk, NY, USA). the linear model of nested design of variance: $Y_{ijk} = u + S_i + T_{(i)j} + e_{(ij)k}$, where Y_{ijk} was the K^{th} observation of the J^{th} individual of the i^{th} population; μ was the overall value; S_i was the effected value in the i^{th} population; $T_{(i)j}$ was the effected value of the J^{th} individual in the i^{th} population; and $e_{(ij)k}$ was the random error. The formula of differentiation coefficients of phenotypic traits (V_{st}) was as follows (Ge et al., 1988).

$$\text{Seed germination rate (GR)} = \frac{\text{variance among populations } (\delta 2t/s)}{\text{variance among and within population } (\delta 2t/s+\delta 2s)} \times 100\% \quad \text{(Eq.5)}$$

Results

Phenotypic variation

The extreme value ratio of phenotypic traits could reflect the evolutionary and adaptive potential of traits in response to environmental changes (Benstock and Cegla, 2017). In terms of evolutionary potential (*Table 1*), these phenotypic traits ranged from high to low as follows: CA > CP > SA > CW > CI > LA > PL > CL > VD > PEL > TSW > WBD > VA > SL > LL > LTA > LP > SP > LBA > LW > LSI > LLW > SSI > SW > VN. The results showed that the capsule area, capsule perimeter and seed area had a relatively high evolutionary potential, but the seed shape index, seed width and vein numbers were low. The standard deviation can reflects the degree of dispersion of

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a dataset. The orders of standard deviations of different phenotypic traits were comprehensively analysed as follows: VD > LTA > LBA > VA > LA > LP > CP > VN > PEL > LW > LL > CI > LLW > CA > WBD > SP > SSI > CL > CW > SL > PL > SA > LSI > SW > TSW. The average levels of leaf traits (SD = 4.499) was more larger than fruit trait (SD = 0.379) and seed trait (SD = 0.119). The results show that the leaf phenotypic traits have undergone greater morphological changes than fruit and seed changes.

Table 1. *Phenotypic variation of natural Populus pruinosa populations*

Traits	Min	Max	Median	Extreme difference value	Average	Standard deviation	Coefficient of variation (%)
LL	3.085	4.938	4.234	1.852	4.218	0.536	12.709
LW	4.956	7.116	5.941	2.16	5.834	0.673	11.545
WBD	1.199	2.072	1.598	0.873	1.643	0.258	15.726
LLW	2.504	3.485	2.945	0.981	2.91	0.325	11.158
PEL	2.773	5.064	3.098	2.291	3.35	0.687	20.499
LTA	90.686	142.946	115.617	52.26	117.606	14.235	12.104
LBA	84.479	129.583	111.566	45.104	111.483	10.683	9.583
VA	23.31	40.115	34.965	16.805	34.47	4.303	12.483
LA	11.668	27.354	20.372	15.686	19.668	4.269	21.707
LP	13.423	20.784	17.827	7.36	17.177	2.202	12.82
VN	8.201	11	9.25	2.799	9.45	0.766	8.106
LSI	0.59	0.833	0.734	0.243	0.731	0.068	9.256
VD	91.215	166.754	118.201	75.538	117.494	19.479	16.579
Leaf average	26.007	43.234	34.334	17.227	34.31	4.499	13.406
CA	0.141	1.269	0.347	1.128	0.442	0.293	66.388
CP	1.602	5.66	3.619	4.058	3.638	0.988	27.154
PL	0.317	0.736	0.358	0.418	0.416	0.119	28.63
CL	0.583	1.348	0.942	0.765	0.964	0.186	19.289
CW	0.27	0.845	0.372	0.575	0.423	0.157	37.153
CI	1.313	3.232	2.598	1.919	2.432	0.533	21.912
Fruit average	0.704	2.182	1.373	1.477	1.386	0.379	33.421
SL	0.658	1.115	0.944	0.457	0.94	0.127	13.529
SW	0.313	0.424	0.384	0.111	0.377	0.027	7.075
SA	0.157	0.549	0.279	0.391	0.29	0.084	28.811
SP	1.708	2.642	2.337	0.934	2.288	0.255	11.137
SSI	2.12	2.897	2.495	0.778	2.492	0.21	8.424
TSW	0.045	0.082	0.057	0.037	0.058	0.01	17.606
Seed average	0.834	1.285	1.083	0.451	1.074	0.119	14.43
GR	0.075	1.049	0.258	0.973	0.343	0.259	75.503
GP	0.005	0.381	0.108	0.376	0.144	0.114	79.242
GI	1.076	14.982	3.686	13.906	4.898	3.698	75.505
Total average	9.182	15.567	12.263	6.385	12.257	1.666	24.701

Differentiation among populations

The analysis of variance (ANOVA) results for the 24 phenotypic traits of the leaves, fruits and seeds among and within *P. pruinosa* populations was showed (*Table 2*). The Left leaf width was highly significantly different among populations and not significantly different within populations. The capsule area, podetium length and

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capsule width showed no significant differences within and among populations. The capsule length was no significant differences within populations but highly significantly different among populations. The other traits were all highly significantly different within and among populations, which showed the phenotypic variation existed in the morphological characters.

Table 2. *Variance analysis of phenotypic traits of natural populus pruinosa populations*

Traits	Mean squared			F	
	Among population	Within population	Random errors	Among population	Within population
LL	13.37	1.73	0.37	7.74**	4.73**
LW	20.81	2.06	0.32	10.09**	6.49**
WBD	2.94	0.46	0.19	6.33**	2.46**
LLW	251.45	219.04	3.79	1.15	57.74**
PEL	20.07	1.56	0.36	12.89**	4.30**
LTA	54037.36	1554.85	905.05	34.75**	1.72**
LBA	10458.7	973.28	304.58	10.75**	3.20**
VA	1171.58	87.56	37.81	13.38**	2.32**
LA	903.92	94.5	14.67	9.57**	6.44**
LP	230.42	18.3	2.79	12.59**	6.56**
VN	41.77	5.87	2.79	7.11**	2.10**
LSI	0.22	0.04	0.01	5.94**	4.07**
VD	13850.51	1825.34	725.93	7.59**	2.51**
CA	1.79	1.56	4.84	1.15	0.32
CP	97.82	6.55	2.75	14.93**	2.38**
PL	0.52	1.55	4.82	0.33	0.32
CL	4.74	1.91	4.29	2.48**	0.45
CW	0.69	1.35	4.81	0.51	0.28
CI	25.39	3.16	3.45	8.05**	0.92
SL	1.27	0.15	0.01	8.28**	19.38**
SW	0.06	0.01	0.003	4.29**	4.97**
SA	0.32	0.04	0.003	7.44**	16.04**
SP	5.98	0.75	0.04	7.96**	18.85**
SSI	3.77	0.52	0.13	7.31**	4.05**

*P < 0.05; **P < 0.01, the same applies below

The variance component accounted for 18.14% and 44.45% among the intrapopulation and interpopulation of total variation respectively, which indicated that there existed some extent of variation. The differentiation coefficients of the phenotypic traits ranged from 2.92% to 98.71%, with an average of 71.02%. At the population level, the capsule index of different populations existed in the largest differentiated coefficients (98.71%), followed by capsule perimeter (96%) and leaf tip angle (94.17%). The trait of the least differentiated coefficients was left leaf width (2.92%) (*Table 3*). In In general, the variance of most phenotypic traits among population was larger than that within population, so the former was the main source of phenotypic variation.

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Table 3. *Variance components and differentiation coefficients of phenotypic traits of natural populus pruinosa populations*

Traits	Variance component			Percentage of variance component (%)			Differentiation coefficients of phenotypic traits (Vst) (%)
	Among population	Within population	Random errors	Among population	Within population	Random errors	
LL	0.78	0.45	0.37	48.65	28.46	22.88	63.09
LW	1.25	0.58	0.32	58.16	27.05	14.79	68.25
WBD	0.16	0.09	0.19	37.01	20.59	42.39	64.25
LLW	2.16	71.75	3.79	2.78	92.34	4.88	**2.92**
PEL	1.23	0.40	0.36	61.89	19.97	18.14	75.60
LTA	3498.83	216.60	905.05	75.72	4.69	19.59	94.17
LBA	632.36	222.90	304.58	54.52	19.22	26.26	73.94
VA	72.27	16.58	37.81	57.05	13.09	29.85	81.34
LA	53.96	26.61	14.67	56.66	27.94	15.41	66.97
LP	14.14	5.17	2.79	63.99	23.39	12.62	73.23
VN	2.39	1.03	2.79	38.53	16.53	44.94	69.98
LSI	0.01	0.01	0.01	39.88	30.45	29.68	56.71
VD	801.68	366.47	725.93	42.33	19.35	38.33	68.63
CA	0.02	0.22	4.84	0.30	4.31	95.39	6.57
CP	6.08	0.25	2.75	66.93	2.79	30.28	96.00
PL	0.07	0.22	4.82	1.35	4.27	94.38	23.95
CL	0.19	0.16	4.29	4.07	3.42	92.51	54.33
CW	0.04	0.23	4.81	0.86	4.54	94.60	15.93
CI	1.48	0.02	3.45	29.96	0.39	69.65	**98.71**
SL	0.07	0.01	0.01	76.84	15.02	8.15	83.65
SW	0.002	0.001	0.002	43.73	15.85	40.41	73.39
SA	0.02	0.004	0.003	73.24	15.98	10.79	82.09
SP	0.35	0.07	0.04	75.85	15.48	8.67	83.05
SSI	0.22	0.04	0.13	56.61	10.13	33.26	84.82
Mean	—	—	—	44.45	18.14	37.41	71.02

Variation in phenotypic traits in different populations

The 24 phenotypic traits of *P. pruinosa* leaf, fruit and seed traits among populations can be showed (*Table 4*). The qmx population had the largest leaf base angle, while wide base distance, vein numbers and leaf shape index were the smallest. The lpx population had the largest vein numbers and seed width. The mfx population had the smallest leaf tip angle, leaf base angle and vein angle. The psx population had the largest leaf tip angle, seed area and seed shape index, while its capsule area, capsule perimeter, podetium length, capsule length and capsule width were the smallest. The ytx population had the largest wide base distance, leaf shape index, seed length and seed perimeter. The vein density of the scx population was the smallest. The slx population had the largest leaf length, leaf width, left leaf width, petiole length, leaf area**,** leaf perimeter and capsule index. The vein angle of the ale population was the largest. The capsule length of the nlkx population was the largest. The vein density, capsule area, capsule perimeter, podetium length, and capsule width of the cbce population were the largest, while its leaf length, leaf area, capsule index, seed length, seed width, seed area, seed perimeter and seed shape index were the smallest. The leaf width, left leaf width, petiole length and leaf perimeter of the kkdl population were the smallest. The phenotypic traits of the ylx, clx, myx, bcx, mgtx and zpx populations were all moderate, revealing no special phenotypic characteristics. The phenotypic traits of *P. pruinosa* leaf, fruit and seed had the significant differences among populations, but the overall performance was continuous variation.

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Table 4. *Variation of phenotypic traits in different populations*

Populations	LL	LW	WBD	LLW	PEL	LTA	LBA	VA	LA	LP	VN	LSI
qmx	4.09±0.28cde	6.94±0.41a	1.20±0.24f	3.48±0.23a	4.07±0.34b	101.61±21.42fg	129.58±15.38a	35.90±2.38abcd	23.52±2.36ab	20.73±1.30a	8.20±0.91g	0.59±0.04f
ylx	3.76±0.25ef	5.53±0.42cdef	1.34±0.14ef	2.76±0.24cdef	3.14±0.31cde	113.68±12.30def	108.85±11.62de	34.47±3.46cd	17.00±2.23cde	16.06±1.11de	8.60±0.90fg	0.68±0.06de
clx	4.42±0.48abcd	6.26±0.90bc	1.57±0.19cde	3.14±0.47abc	3.68±0.40bc	121.59±17.82bcde	101.67±16.28e	29.99±4.70e	21.80±5.88b	17.94±2.45bcd	10.33±1.59abc	0.71±0.06cde
lpx	4.66±1.05abc	6.14±0.49bc	2.04±0.43a	3.07±0.21bc	3.06±0.49cde	138.26±17.07ab	116.84±14.28bcd	34.97±4.75cd	21.89±3.97b	18.08±1.63bcd	11.00±1.31a	0.76±0.14abcd
mfx	4.23±0.54bcde	5.24±0.77efg	1.95±0.26ab	2.61±0.41efg	3.49±0.66bcd	90.69±14.63g	84.48±11.79f	23.31±3.29f	16.40±4.72cdef	15.69±2.24ef	9.18±1.29defg	0.82±0.09ab
psx	4.11±0.63cde	5.72±0.83bcdef	1.63±0.46bcde	2.78±0.40cdef	3.00±0.34de	142.95±34.40a	116.62±22.75bcd	37.20±5.12abcd	18.88±3.98bcd	16.72±2.05cde	9.95±1.68bcd	0.73±0.14bcd
ytx	4.60±0.77abc	5.62±0.78cdef	2.07±0.44a	2.78±0.42cdef	2.79±0.81ef	133.22±33.01abc	106.95±16.38de	35.48±6.85bcd	19.59±4.07bcd	16.85±1.67cde	8.80±0.99efg	0.83±0.18a
myx	4.59±0.87abc	6.01±1.24bcd	1.88±0.45abc	2.94±0.65bcde	3.14±1.06cde	117.82±14.02cdef	111.57±12.48cde	34.72±4.31cd	22.41±8.43ab	18.05±4.00bcd	10.62±1.88ab	0.78±0.10abcd
bcx	4.17±0.76cde	5.94±0.98bcde	1.55±0.35cde	2.98±0.46bcde	3.58±0.71bcd	115.62±15.26cdef	123.78±15.57ab	30.09±6.25e	20.37±6.49bc	17.83±2.94bcd	9.49±1.32cdef	0.71±0.13cde
mgtx	4.41±0.79abcd	6.46±1.04ab	1.54±0.50cde	3.24±0.56ab	3.93±1.13b	113.22±16.04def	122.13±12.17abc	33.14±4.27de	23.32±7.87ab	19.28±3.11ab	9.04±0.95defg	0.69±0.08cde
scx	4.87±0.93a	5.95±0.88bcde	1.75±0.553abcd	3.00±0.42bcd	4.04±0.74b	129.44±18.45abcd	111.38±9.91cde	39.13±7.35ab	22.86±7.14ab	18.27±2.67bc	8.56±0.59fg	0.82±0.15ab
slx	4.94±0.67a	7.12±0.63a	1.83±0.41abc	3.48±0.32a	5.06±1.06a	108.69±21.68efg	121.49±11.33abc	38.12±3.90abc	27.35±4.88a	20.78±1.90a	9.07±0.92defg	0.70±0.08cde
zpx	3.71±0.84efg	5.42±0.93def	1.55±0.23cde	2.69±0.48defg	2.87±0.58ef	103.56±10.40fg	103.86±13.30e	30.61±4.45e	15.61±5.11def	15.24±2.53efg	9.71±1.26bcdef	0.69±0.11cde
ale	4.83±0.71ab	6.17±0.29bc	1.60±0.27cde	3.10±0.16bc	2.99±0.31de	132.32±22.57abcd	117.44±12.40bcd	40.12±4.69a	23.22±3.15ab	18.71±1.47abc	9.86±0.69bcde	0.78±0.10abc
nlkx	3.94±0.51de	5.16±0.40fg	1.76±0.39abcd	2.60±0.18efg	2.77±0.47ef	122.95±33.56bcde	102.99±8.60e	40.02±4.87a	15.98±2.63cdef	15.14±1.17efg	9.05±0.99defg	0.77±0.10abcd
cbce	3.16±0.45g	5.02±0.62fg	1.43±0.25def	2.51±0.33fg	2.80±0.56ef	110.36±19.33ef	103.59±10.44e	33.23±4.88de	12.18±3.02f	13.65±1.77fg	9.40±1.02cdef	0.63±0.04ef
kkdl	3.29±0.61fg	4.55±1.11g	1.32±0.31ef	2.32±0.57g	2.30±0.67f	106.04±20.06efg	111.87±10.75cde	36.26±3.20abcd	12.48±4.76ef	13.22±2.98g	9.25±1.09defg	0.75±0.13abcd

Table 4. *Continued*

Populations	VD	CA	CP	PL	CL	CW	CI	SL	SW	SA	SP	SSI
qmx	100.92±9.05efg	0.47±0.15cde	4.16±0.77b	0.43±0.05bcde	1.23±0.22ab	0.44±0.07cde	2.81±0.28bc	1.05±0.04ab	0.39±0.02bc	0.31±0.02bc	2.49±0.10ab	2.70±0.12bc
ylx	116.08±17.11cdef	0.52±0.12cd	4.09±0.65b	0.48±0.10bc	0.99±0.18cdef	0.48±0.09cd	2.09±0.22f	0.88±0.05def	0.37±0.03c	0.26±0.03bc	2.20±0.13de	2.38±0.14ef
clx	118.77±21.82bcde	0.31±0.09efg	3.28±0.48def	0.35±0.07de	0.99±0.19cdef	0.35±0.05efgh	2.85±0.34bc	0.91±0.11cde	0.37±0.04c	0.26±0.06bc	2.23±0.24cde	2.50±0.21de
lpx	123.25±26.91bcd	0.26±0.13fgh	3.13±0.61ef	0.33±0.06de	0.85±0.15efg	0.29±0.05gh	2.98±0.22b	1.08±0.11a	0.42±0.04a	0.35±0.06b	2.58±0.23a	2.56±0.19cd
mfx	109.84±13.71cdefg	0.24±0.04gh	3.03±0.28f	0.33±0.03de	0.80±0.11fg	0.31±0.04fgh	2.62±0.22cd	0.92±0.10cde	0.37±0.03c	0.27±0.05bc	2.23±0.21cde	2.48±0.18de
psx	125.86±33.28bc	0.14±0.04h	1.60±0.20g	0.32±0.10e	0.58±0.09h	0.27±0.04h	2.22±0.27ef	1.10±0.18a	0.38±0.05c	0.55±0.90a	2.60±0.38a	2.90±0.31a
ytx	98.13±11.92fg	0.25±0.07gh	2.21±0.33g	0.32±0.12e	0.85±0.14fg	0.34±0.07efgh	2.60±0.41cd	1.11±0.11a	0.39±0.03bc	0.34±0.05b	2.64±0.24a	2.84±0.22ab
myx	118.91±21.57bcde	0.35±0.14efg	3.72±0.73bcdef	0.36±0.05de	1.00±0.26cdef	0.35±0.06efgh	2.83±0.37bc	0.96±0.09cd	0.39±0.03bc	0.28±0.07bc	2.34±0.19bcde	2.48±0.23de
bcx	118.20±26.04bcde	0.32±0.05efg	3.31±0.27def	0.34±0.10de	0.87±0.07efg	0.37±0.04efgh	2.36±0.26def	0.99±0.11bc	0.39±0.03bc	0.30±0.05bc	2.40±0.22bc	2.58±0.24cd
mgtx	106.72±24.39cdefg	0.36±0.08efg	3.62±0.52bcdef	0.38±0.11cde	0.94±0.18defg	0.39±0.06def	2.41±0.39de	0.98±0.09bc	0.38±0.03bc	0.30±0.05bc	2.36±0.20bcd	2.56±0.22cd
scx	91.22±18.45g	0.30±0.07efgh	3.35±0.50cdef	0.34±0.06de	0.95±0.18defg	0.33±0.03fgh	2.85±0.44bc	1.04±0.05ab	0.41±0.01ab	0.33±0.03bc	2.47±0.11ab	2.55±0.11cde
slx	93.35±12.37g	0.31±0.05efg	3.82±0.50bcde	0.45±0.11bcd	1.06±0.09bcde	0.33±0.03fgh	3.23±0.21a	0.94±0.07cde	0.37±0.03c	0.27±0.04bc	2.27±0.15cde	2.59±0.16cd
zpx	136.11±28.03ab	0.35±0.11efg	3.34±0.56def	0.34±0.05de	0.89±0.12efg	0.39±0.07defg	2.24±0.11ef	0.87±0.16ef	0.38±0.05bc	0.27±0.08bc	2.16±0.36ef	2.26±0.22fg
ale	105.17±19.67defg	0.43±0.07def	3.96±0.38bcd	0.40±0.08cde	1.17±0.12abc	0.43±0.04cde	2.71±0.09bc	0.94±0.17cde	0.39±0.03bc	0.30±0.08bc	2.34±0.37bcde	2.41±0.31def
nlkx	120.30±23.43bcde	1.05±0.41b	5.51±1.45a	0.64±0.31a	1.35±0.42a	0.75±0.22b	1.78±0.14g	0.72±0.16g	0.33±0.06d	0.19±0.07bc	1.85±0.37gh	2.21±0.37g
yl-cbce	152.81±21.34a	1.27±0.68a	5.66±2.25a	0.74±0.40a	1.11±0.57bcd	0.85±0.42a	1.31±0.24h	0.66±0.08g	0.31±0.04d	0.16±0.04c	1.71±0.20h	2.12±0.23g
yl-kkdl	147.92±31.60a	0.59±0.25c	4.06±1.30bc	0.52±0.19b	0.76±0.33gh	0.52±0.21c	1.46±0.29h	0.81±0.09f	0.36±0.03c	0.21±0.05bc	2.02±0.22fg	2.26±0.34fg

Different lowercase letters in the same column indicated the significant differences ($p<0.05$)

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Correlations between phenotypic traits

As it was showed (*Table 5*), there were significant correlations among 28 phenotypic traits related to leaf, fruit and seed. For example, in the significant correlation associated with leaf traits, leaf length among 28 phenotypic traits (n = 6) had more positive correlation numbers than other traits, followed by leaf width (n = 5), left leaf width (n = 5), leaf base angle (n = 3). There were few negative correlations with leaf traits. The larger leaf length, leaf width and left leaf width had great influence on leaf morphology. In the significant correlation associated with fruit traits, capsule area (n = 4) had more positive correlation numbers than other traits, followed by capsule perimeter (n = 3), vein density (n = 3). The leaf length (n = 4) had more negative correlation numbers than other trait, followed by leaf area, leaf perimeter. The fruit correlation traits was more greatly affected by leaf length, capsule area, capsule perimeter and vein density. In the significant correlation associated with seed traits, seed length (n = 5) had more positive correlation numbers than other traits, followed by leaf length, leaf area, leaf perimeter, capsule index and seed width. The capsule area (n = 5), capsule perimeter (n = 5), podetium length (n = 5) and capsule width (n = 5) had more negative correlation numbers than other trait, followed by vein density. The results indicated seed morphological changes was more greatly affected by leaf traits, followed by fruit traits and seed traits.

Correlations of the phenotypic traits with environmental factors

The 28 phenotypic traits of *P. pruinosa* were not significantly correlated with longitude, annual average temperature, annual max temperature, soli temperature and soil moisture, which indicated that these phenotypic traits were relatively stable and not easily changed by these influences. The latitude was significantly positively correlated with vein density, capsule area, capsule perimeter, podetium length and capsule width. The altitude was significantly positively correlated with leaf length, leaf width, left leaf width, leaf area, leaf perimeter, capsule index, seed length, seed width, seed perimeter and seed shape index. The sunlight intensity was significantly positively correlated with leaf length, leaf width, left leaf width, leaf area, leaf perimeter, leaf shape index, seed length, seed width, seed perimeter and seed shape index. The relative humidity was significantly positively correlated with capsule area, podetium length and capsule width and so on (*Table 6*). In terms of the number of significant correlations with the phenotypic traits of *P. pruinosa*, the environmental factors were ranked as follows: count = 16 (annual average precipitation, evaporation) > count = 15 (altitude, latitude, sunlight intensity) > count = 9 (relative humidity) > count = 4 (annual min temperature) > count = 3 (topsoil organic carbon) > count = 1 (topsoil texture classification) > count = 0 (longitude, annual average temperature, annual max temperature, soil temperature, soil moisture).

Discussion

Variation in plant phenotypic traits result from genetic variation in “composition” and gene expression influenced by environmental factors (Burns and Strauss, 2012). The richer the phenotypic variation, the better the adaptability of plants to different environments (Verbeeck et al., 2014; Saenger and West, 2018). It has also been showed that phenotypic plasticity could respond to environmental changes, indicating that the

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same genotype expressed different phenotypes in different environments, or occurred through genetic variation in average phenotypes, but the evolutionary potential is not significant (Brunet and Larson-Rabin, 2012). Studies of *Styrax tonkinensis* populations show that the phenotypic variation within populations (69.60%) is greater than that among populations by using the molecular markers (Li et al., 2012), but the phenotypic variation among populations (59.08%) is greater than that within populations by using phenotypic traits (Liu et al., 2011). Similar to the results of *P. pruinosa* populations, the variation within population (88%) is the main source of phenotypic variation by using the molecular markers (Zhang et al., 2012). This research of *P. pruinosa* populations shows that the variation among population (71.02%) is greater than that within populations. Accordingly, the variation among population is the main source of the population variation by using phenotypic traits (*Table 3*). The possible reasons are as follows. Although the *P. pruinosa* has the biological characteristics of wind pollination, in fact, the natural populations are distributed in the extreme desert region and the effective distance of seed dispersal may be less than the actual distance, which increases the difference between different population. In addition, Xinjiang is located in arid and semi-arid regions. There are large conflicts between the supply and demand of water resources in different distribution areas, and the frequent climate changes (Zhang et al., 2020), which has caused obvious environmental heterogeneity among different populations. The uplift of the Tianshan Mountains directly blocks the gene flow between the north and south of Xinjiang (Zeng et al., 2018; Jia et al., 2020), and blocks the transport of water vapor from north to south of Xinjiang. The huge geographical and environmental differences are an important reason for the differences of natural *P. pruinosa* populations. Finally, *P. euphratica* has the multiple pedigrees and forms a parallel group with *P. pruinosa*. There is also a strong gene introgression between *P. pruinosa* and *P. euphratica* populations (Ma et al., 2018), which increases the genetic diversity of *P. pruinosa* population and promotes the phenotypic diversity of different populations. Therefore, there are significant differences in the phenotypic traits of different natural *P. pruinosa* populations (*Table 4*).

Plant trait correlation can indirectly reflect the phenotype of another trait through the phenotype of one trait, improve the selection efficiency and accelerate the breeding process (Rui et al., 2018). For example, leaves are vegetative organs that provide energy to plants. Changes in leaf shape result from responses to the ecological environment and evolutionary history (Nicotra et al., 2011). Leaf shape is correlated with other phenotypic traits. For example, Mclellan finds significant correlations between leaf shape and the presence, number and size of trichomes in plants (Mclellan, 2005). As is showed (*Table 5*), the correlations between most phenotypic traits of natural *P. pruinosa* populations have a significant level, indicating that these phenotypic traits show a strong linkage effects to adapt the environmental changes.

An important content of plant ecology research is to determine and quantify the dominant dimensions of the ecological variables of each species and try to explain its mechanism (Wright et al., 2007). By analyzing the phenotypic variation based at the population level for specific plant groups on a larger scale, studying the relationship between phenotypic traits and geography-climate factors could often reveal the pattern of plant variation and its ecological response strategies more accurately (Yang et al., 2016; Meng et al., 2017). For example, different environmental factors may affect the distribution range of plants in response to climate change (Mccauley et al., 2014; Galván-Hernández et al., 2015).

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Table 5. *Correlations between phenotypic traits*

Traits	LL	LW	WBD	LLW	PEL	LTA	LBA	VA	LA	LP	VN	LSI	VD	CA	CP	PL	CL	CW	CI	SL	SW	SA	SP	SSI	TSW	GR	GP
LL																											
LW	0.71**																										
WBD	0.61**	0.06																									
LLW	0.70**	0.99**	0.02																								
PEL	0.57*	0.82**	0.04	0.82**																							
LTA	0.42	0.11	0.37	0.09**	-0.22																						
LBA	0.26	0.63**	-0.33	0.64**	0.37	0.26																					
VA	0.23	0.184	-0.06	0.19	-0.01	0.57*	0.52*																				
LA	0.88**	0.94**	0.26	0.94**	0.77**	0.26	0.58*	0.27																			
LP	0.79**	0.98**	0.12	0.98**	0.80**	0.15	0.64**	0.21	0.97**																		
VN	0.17	-0.01	0.37	-0.04	-0.28	0.39	-0.09	-0.14	0.06	-0.05																	
LSI	0.48	-0.28	0.75**	-0.29	-0.24	0.41	-0.42	0.08	0.03	-0.13	0.19																
VD	-0.83**	-0.70**	-0.35	-0.70**	-0.7**	-0.145	-0.27	-0.21	-0.80**	-0.79**	0.38	-0.30															
CA	-0.63**	-0.46	-0.36	-0.43	-0.35	-0.21	-0.19	0.21	-0.57*	-0.52*	-0.20	-0.35	0.52*														
CP	-0.43	-0.19	-0.40	-0.15	-0.08	-0.38	-0.05	0.21	-0.30	-0.25	-0.23	-0.40	0.31	0.89**													
PL	-0.63**	-0.40	-0.40	-0.38	-0.25	-0.25	-0.12	0.27	-0.52*	-0.48	-0.27	-0.40	0.48*	0.97**	0.88**												
CL	0.05	0.27	-0.21	0.31	0.20	-0.17	0.12	0.36	0.18	0.24	-0.27	-0.30	-0.201	0.60*	0.79**	0.55*											
CW	-0.67**	-0.48*	-0.41	-0.45	-0.39	-0.22	-0.18	0.20	-0.60*	-0.55*	-0.25	-0.36	0.53*	0.993**	0.88**	0.95**	0.58*										
CI	0.90**	0.81**	0.48	0.80**	0.68**	0.18	0.26	0.002	0.89**	0.85**	0.18	0.23	-0.80**	-0.73**	-0.47	-0.71**	0.02	-0.77**									
SL	0.67**	0.55*	0.36	0.53*	0.32	0.45	0.43	0.06	0.64**	0.63**	0.07	0.28	-0.61**	-0.86**	-0.83**	-0.85**	-0.45	-0.86**	0.70**								
SW	0.63**	0.45	0.31	0.45	0.21	0.35	0.38	0.02	0.57*	0.52*	0.21	0.33	-0.49*	-0.83**	-0.68**	-0.854**	-0.39	-0.83**	0.69**	0.87**							
SA	0.41	0.31	0.23	0.26	0.10	0.60*	0.33	0.15	0.37	0.355	0.19	0.21	-0.30	-0.70**	-0.84**	-0.68**	-0.59*	-0.69**	0.39	0.82**	0.60*						
SP	0.68**	0.55*	0.36	0.53*	0.29	0.47	0.44	0.08	0.64**	0.63**	0.09	0.288	-0.61**	-0.85**	-0.80**	-0.85**	-0.43	-0.85**	0.71**	0.99**	0.88**	0.82**					
SSI	0.57*	0.54*	0.31	0.50*	0.37	0.42	0.40	0.07	0.59*	0.60*	-0.05	0.18	-0.60*	-0.74**	-0.81**	-0.70**	-0.43	-0.74**	0.59*	0.92**	0.60*	0.84**	0.91**				
TSW	0.26	0.30	-0.04	0.33	0.10	0.12	0.40	0.24	0.31	0.37	-0.14	0.01	-0.33	-0.26	-0.12	-0.33	0.14	-0.27	0.40	0.49*	0.64**	0.20	0.50*	0.27			
GR	0.11	-0.16	0.26	-0.149	-0.17	0.18	-0.32	0.03	-0.09	-0.11	0.13	0.33	-0.06	0.16	0.10	0.05	0.28	0.17	-0.06	-0.19	-0.23	0.03	-0.17	-0.15	-0.25		
GP	0.20	0.07	0.26	0.03	-0.09	0.03	-0.01	0.03	0.17	0.11	0.43	0.2	0.02	-0.09	0.02	-0.13	0.07	-0.12	0.20	0.04	0.10	-0.01	0.05*	-0.01	0.11	-0.10	
GI	0.18	-0.07	0.30	-0.07	-0.06	0.16	-0.29	0.07	-0.01	-0.04	0.11	0.31	-0.12	0.14	0.11	0.06	0.31	0.15	0.01	-0.20	-0.25	0.02	-0.18	-0.13	-0.29	0.98**	-0.11

* $p < 0.05$, ** $p < 0.01$, the same below

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Table 6. *Correlations of the phenotypic traits with environmental factors*

Traits	Longitude	Latitude	Altitude	AAVT	AMAT	AMIT	AAP	SI	RH	EV	ST	SM	TTC	TOC
LL	-0.43	-0.67**	0.74**	0.05	-0.07	0.38	-0.58*	0.62**	-0.43	-0.61**	-0.06	0.17	0.36	-0.32
LW	-0.12	-0.58*	0.62**	0.19	0.14	0.42	-0.65**	0.56*	-0.51*	-0.64**	0.12	0.30	0.14	-0.12
WBD	-0.33	-0.38	0.37	0.09	-0.01	0.24	-0.22	0.35	-0.28	-0.24	0.06	-0.23	0.20	-0.25
LLW	-0.11	-0.55*	0.61**	0.15	0.12	0.37	-0.62*	0.53*	-0.47	-0.61**	0.08	0.32	0.16	-0.13
PEL	-0.15	-0.45	0.45	0.03	0.01	0.20	-0.46	0.47	-0.28	-0.47	-0.10	0.17	0.01	0.13
LTA	-0.31	0.01	0.11	0.01	-0.10	0.08	-0.01	-0.13	0.06	-0.06	0.12	0.23	0.34	-0.33
LBA	-0.07	-0.17	0.08	0.18	0.14	0.28	-0.23	0.15	-0.18	-0.21	0.16	0.44	-0.09	0.10
VA	-0.29	0.29	-0.27	-0.25	-0.27	-0.18	0.35	-0.34	0.41	0.31	-0.32	0.30	-0.21	0.23
LA	-0.28	-0.63**	0.68**	0.15	0.06	0.44	-0.64**	0.60*	-0.47	-0.64**	0.03	0.29	0.22	-0.18
LP	-0.14	-0.64**	0.66**	0.17	0.12	0.42	-0.67**	0.63**	-0.52*	-0.66**	0.08	0.31	0.22	-0.20
VN	-0.15	-0.10	0.22	0.22	0.12	0.24	-0.14	-0.01	-0.23	-0.15	0.37	-0.09	0.15	-0.21
LSI	-0.39	-0.22	0.24	-0.13	-0.22	0.02	-0.01	0.18	0.02	-0.05	-0.23	-0.17	0.31	-0.27
VD	0.19	0.63**	-0.64**	0.01	0.05	-0.28	0.56*	-0.65**	0.38	0.57*	0.15	-0.18	-0.34	0.30
CA	0.24	0.76**	-0.75**	-0.18	-0.05	-0.51*	0.80**	-0.70**	0.58*	0.81**	-0.22	-0.25	-0.44	0.43
CP	0.24	0.59*	-0.58*	-0.22	-0.09	-0.46	0.63**	-0.52*	0.47	0.64**	-0.30	-0.18	-0.43	0.44
PL	0.27	0.81**	-0.82**	-0.10	0.02	-0.45	0.80**	-0.73**	0.57*	0.82**	-0.19	-0.23	-0.60*	0.58*
CL	0.17	0.16	-0.07	-0.21	-0.11	-0.28	0.25	-0.11	0.16	0.23	-0.29	-0.12	-0.06	0.08
CW	0.25	0.76**	-0.75**	-0.19	-0.07	-0.51*	0.81**	-0.71**	0.59*	0.82**	-0.23	-0.22	-0.42	0.40
CI	-0.24	-0.79**	0.83**	0.10	0.02	0.41	-0.78**	0.76**	-0.59*	-0.81**	0.06	0.12	0.39	-0.30
SL	-0.17	-0.69**	0.64**	0.21	0.13	0.47	-0.74**	0.66**	-0.55*	-0.74**	0.24	0.30	0.45	-0.44
SW	-0.27	-0.68**	0.62**	-0.01	-0.11	0.32	-0.69**	0.60*	-0.42	-0.73**	0.07	0.41	0.46	-0.44
SA	-0.17	-0.40	0.39	0.16	0.07	0.34	-0.49*	0.34	-0.32	-0.50*	0.31	0.38	0.37	-0.43
SP	-0.16	-0.70**	0.65**	0.22	0.13	0.48*	-0.75**	0.66**	-0.57*	-0.76**	0.26	0.31	0.48	-0.48
SSI	-0.05	-0.57*	0.54*	0.37	0.31	0.52*	-0.65**	0.59*	-0.57*	-0.63**	0.34	0.16	0.36	-0.36
TSW	-0.01	-0.41	0.27	-0.30	-0.26	-0.14	-0.33	0.40	-0.11	-0.42	-0.19	0.10	0.39	-0.14
GR	-0.05	0.01	0.16	-0.27	-0.24	-0.29	0.22	-0.06	0.10	0.19	-0.10	-0.10	0.39	-0.50*
GP	-0.01	-0.23	0.15	0.21	0.15	0.31	-0.17	0.29	-0.23	-0.15	0.08	-0.24	0.37	-0.50*
GI	-0.09	-0.02	0.17	-0.25	-0.23	-0.24	0.20	-0.03	0.07	0.17	-0.10	-0.12	0.31	-0.41
Count	0	15	15	0	0	4	16	15	9	16	0	0	1	3

AAVT = annual average temperature; AMAT = annual max temperature; AMIT = annual min temperature; AAP = annual average precipitation; SI = sunlight intensity; HR = relative humidity; EV = evaporation; ST = soil temperature; SM = soil moisture; TTC = topsoil texture classification; TOC = topsoil organic carbon

The different geographical factors could affect plant phenotypic traits and seed germination rate and quality (Yan et al., 2014; Butnor, 2019). Plants could adapt to the warmth of their surroundings by adjusting their phenotypic morphology (Supratya et al., 2020; Bahuguna and Jagadish, 2015). As is showed (*Table 6*), most phenotypic traits and environmental factors in the study are significantly correlated, indicating that changes in habitat conditions have a great influence on the phenotypic traits of *P. pruinosa*. For example, annual average precipitation and evaporation are significantly correlated with most of the phenotypic traits, while longitude, annual average temperature and annual maximum temperature have no significant correlations with the phenotypic traits. The results indicate that the annual average precipitation and evaporation are the main factors driving the variation of phenotypic traits of *P. pruinosa*, and the tropism evolution of phenotypic traits is to meet the growth needs of extreme drought environments in different desert regions.

The evaluation of phenotypic traits is an important step in the identification and protection of germplasm resources and plays a vital role in the comprehensive potential of agronomic traits and breeding (Scarano et al., 2014). *P. pruinosa* is one of the few

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species that only exists in extreme desert environments, which requires that the plant has a regulatory mechanisms allowing them to adapt to changes in harsh conditions. it means that the germplasm resources of natural *P. pruinosa* populations have high economic value and social benefits. Currently, the survival rate of asexual reproduction from natural *P. pruinosa* populations have been very low. So seed reproduction is an extremely important method for population reproduction of *P. pruinosa*. Moreover, the population habitat fragmentation caused by human factors and the increasingly poor ecological environment lead to a dramatically sharp increase in the decline rate of *P. pruinosa* populations; thus, in order to better protect the existing genetic resources and achieve sustainable growth of natural *P. pruinosa* populations, we suggest the following: (1) Implement in situ and off-site protection mechanisms to maximize the ecological value and economic benefits of genetic resources. (2) In the future, explore excellently genetic stress-resistance resources in different populations that are adapted to environmental factors such as salt and alkali, drought and heat at the molecular level to provide a basis for breeding protection. (3) Variation among populations is the result of adaptation to the environment and evolution, and conservation and utilization should be strengthened. Screening excellent germplasm resources will be beneficial to the conservation of existing *P. pruinosa*. (4) Superior natural populations selected from different provenances will be pollinated by seed reproduction to build a parent forest or seed orchard and obtain capsule tree species with high ecological value.

Conclusions

The phenotypic diversity associated the leaf, fruit and seed traits of seventeen natural *P. pruinosa* populations show that there are abundant variations in phenotypic traits. In addition, there are significant differences in phenotypic traits between populations and within populations, and the variation among populations is greater than that within populations. The different geographical and climatic factors have significant influence on the variation of phenotypic traits, The results indicate that the differences of adaptation mechanisms and the sensitivity in response to environmental factors of plant phenotypic traits may lead to the different patterns of geographic variation in morphological changes during the growth and development *P. pruinosa* populations. It is imperative that all of natural *P. pruinosa* populations in China is studied by molecular markers in the future to fully reveal the variation law and genetic diversity level of different groups. Finally, using resistance genes associated with molecular markers, such as salt-tolerance gene and water-stressed gene, is to distinguish the differences among populations, construct core collections of populations from the level of genetic diversity, and consider the populations of phenotypic diversity to achieve the vital protection of natural *P. pruinosa* populations.

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APPENDIX

Table A1. *General information of seventeen natural Populus pruinosa populations in Xinjiang*

Region	Site	Code	Longitude (°E)	Latitude (°N)	Altitude (m)	AAVT (℃)	AMAT (℃)	AMIT (℃)	AAP (mm)	SI (J/m²/d)	RH (%)	EV (mm)	ST (℃)	SM (m^3/m^3)	TTC	TOC (%)
Bayingol Mongolian Autonomous Prefecture, Southern Xinjiang	Qiemo County	qmx	85°23′32″	38°18′13.00″	1212.5	11.21	39.72	-20.36	35.55	253.71	28.43	39.65	7.78	0.17	9	0.99
Bayingol Mongolian Autonomous Prefecture, Southern Xinjiang	Yuli County	ylx	85°48′27.9″	41°08′24.5″	899	13.16	39.34	-17.3	64.28	234.46	33.53	65.68	15.83	0.18	9	0.42
Hotan, Southern Xinjiang	Qira County	clx	81°06′43.48″	36°49′35.29″	1454	12.06	38.4	-19.74	62.21	237.47	31.27	57.61	9.54	0.09	13	0.43
Hotan, Southern Xinjiang	Lop County	lpx	80°56′46.32″	39°33′32.69″	1123.2	12.24	38.38	-19.01	64.9	236.92	30.08	57.69	15.34	0.2	9	0.42
Hotan, Southern Xinjiang	Minfeng County	mfx	82°47′38.27″	37°38′37.05″	1308	12.46	39.59	-18.51	46.78	257.63	27.02	54.67	9.31	0.08	9	0.42
Hotan, Southern Xinjiang	Karakax County	myx	80°11′15.33″	37°46′14.09″	1248.2	13.91	39.48	-14.43	51.26	254.82	28.25	59.38	10.22	0.09		
Hotan, Southern Xinjiang	Pishan County	psx	79°0′28.37″	37°36′44.54″	1299.7	13.81	39.31	-14.48	56.32	253.23	27.88	69.25	10.3	0.1	10	0.43
Hotan, Southern Xinjiang	Yutian County	ytx	81°23′33.44″	37°16′37.04″	1288.8	13.47	39.26	-16.18	52.25	255.25	26.97	54.7	16.53	0.08	13	0.43
Kashgar, Southern Xinjiang	Marabishi County	bcx	78°18′29.00″	39°46′22.78″	1126.7	12.68	38.43	-16.13	84.93	237.6	34.31	125.35	6.93	0.32	5	0.49
Kashgar, Southern Xinjiang	Makit County	mgtx	74°17′30.13″	39°19′31.14″	1143.2	2.22	26.96	-26.43	126.08	238.79	48	104.05	-9.8	0.23	11	1.41
Kashgar, Southern Xinjiang	Yarkant County	scx	77°21′28.48″	38°24′43.48″	1207.5	12.79	37.87	-15.14	66.47	247.8	30.83	71.11	8.25	0.14	2	2.94
Kashgar, Southern Xinjiang	Shule County	slx	76°10′25.00″	38°0′17.37″	1270.8	4.21	28.38	-22.11	87.08	241.82	39.14	81.14	2.61	0.22	9	0.58
Kashgar, Southern Xinjiang	Zepu County	zpx	76°58′04.51″	38°02′06.45″	1407	10.6	35.23	-16.56	71.4	245.29	32.38	74.96	5.96	0.21	9	0.41
Aksu, Southern Xinjiang	Alaer County	ale	79°47′40.46″	40°21′31.06″	1049.3	12.84	38.57	-17.28	88.28	234.01	34.36	95.99	15.46	0.22	9	0.42
Ili Kazak Autonomous Prefecture, Southern Xinjiang	Nilka County	nlk	82°12′32.30	43°35′53.58″	754.8	7.16	34.29	-26.43	255.77	217.19	44.95	273.71	2.48	0.1	9	0.46
Ili Kazak Autonomous Prefecture, Southern Xinjiang	Qapqal Xibe Autonomous County	cbce	80°40′48.36″	43°50′34.87″	538.5	11.14	37.82	-22.11	219.21	215.61	43.69	244.66	7.11	0.11	2	2.94
Ili Kazak Autonomous Prefecture, Southern Xinjiang	Cocodala city	kkdl	80°47′20.93″	43°55′47.64″	553.3	11.14	37.82	-22.11	219.21	215.61	43.69	244.66	3.64	0.13	2	2.94

AAVT = annual average temperature; AMAT = annual max temperature; AMIT = annual min temperature; AAP = annual average precipitation; SI = sunlight intensity; HR = relative humidity; EV = evaporation; ST = soil temperature; SM = soil moisture; TTC = topsoil texture classification; TOC = topsoil organic carbon

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ESTIMATION OF ATMOSPHERIC NITROGEN DEPOSITION TO TAIHU LAKE FROM TAIHU WATERSHED, CHINA

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Abstract. In this study, the catchment area of Taihu Lake, China, was divided into four parts: water area, farmland area, construction land and vegetation area, and the total amount of atmospheric nitrogen deposition from the catchment area into the Taihu Lake was estimated. The results showed that the total nitrogen deposition from the water area, farmland, urban construction land and vegetation area into the water were 38667.5 t, 124596.1 t, 52653.2 t and 11107.8 t, respectively. The atmospheric nitrogen deposition entering into water was 227024.6 t eventually, accounting for 80.5% of total atmospheric nitrogen deposition, which has exceeded the nitrogen content of agricultural non-point source pollution, industrial sewage, domestic sewage and other exogenous pollution. Nitrogen deposition in farmland area is the main source of entering Taihu Lake. Atmospheric nitrogen deposition may be the main pollution source into the Taihu basin. Atmospheric nitrogen deposition plays an important role in the input of exogenous nitrogen in Taihu Lake, which is an important source of nitrogen and plays an important role in eutrophication. It is recommended to take necessary measures to control atmospheric nitrogen deposition, for example, reduce energy-intensive industries, build buffer strips, ecological ditches, and three- dimensional greening.
Keywords: *water area, farmland, vegetation, agricultural production, buffer strips, three-dimensional greening*

Introduction

Reactive nitrogen (Nr) has significantly increased over the past 150 years because of emissions from fossil fuels burning, industry and fertilizer and transportation development, at the rate of about 10 times higher than a century ago (Meunier et al., 2016). Excessive atmospheric nitrogen (N) deposition resulted in the negative impacts on the function and biodiversity of terrestrial ecosystems (Porter et al., 2013; Stevens et al., 2018). Atmospheric N deposition is also an important reason for water eutrophication (Xu et al., 2018), especially in some plateau and mountain lakes (Hundey et al., 2015). Compared with other pollution sources, atmospheric N deposition also affected food production, environmental quality and climate change from the regional to global scales, and further threat to human health especially in highly populated regions (Yu et al., 2019). However, it is difficult to observe and quantify atmospheric N deposition, especially dry deposition (Ti et al., 2018).

In the temperate terrestrial ecosystems, constant anthropogenic N input accelerated plant growth, but N over-enrichment would result in N loss eventually, which has changed the species composition and caused ecosystem degradation (Gilliam et al.,

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2016). Anthropogenic N emissions lead to acid rain and soil acidification, which damages plants through leaves and soil, and leads to nutrient deficiency and forest decline (Abrahamsen et al., 2012; Zheng et al., 2018). In addition, soil acidification increased the activity of soil aluminum ion and elements in soil, which was toxic to plants and humans (Fujii et al., 2018; Meng et al., 2019).

Atmospheric N deposition is an important source of lakes pollution (Xie et al., 2007; Ellis et al., 2105; Chen et al., 2018). It has resulted in acidification and eutrophication of freshwater ecosystems (Brittain and Strecker, 2018). It changed abundance and composition of phytoplankton in lakes (Lepori and Keck, 2012; Benavides-Ordillo et al., 2019). It also affected biogeochemical cycle, trophic dynamics and biological diversity (Meunier et al., 2015). Excess N deposition resulted in acidification of water and soil, eutrophication of water in lakes and rivers, depleted or wiped out the fish stocks, which also affected the aquatic animals and plants, and the structure and function of aquatic ecosystem (Gao et al., 2014; Geng et al., 2021; Mcdonnell et al., 2021).

In China, N deposition amount was 13.2 kg N ha^{-1} in 1980s, and reached 21.1 kg N ha^{-1} in the 2000s (Liu et al., 2013). Taihu Lake is the third largest freshwater lake in China. Taihu Lake basin is one of the most densely populated and urbanized areas in China. Industry, agriculture and transportation are well developed in this area. Atmospheric N deposition is also an important N input source of the Taihu Lake, which would affect the water quality of the lake and the growth of plankton (Di et al., 2015). Taihu basin has been a hotspot for atmospheric N deposition. In 1980s, the total amount of N deposition was less than 3000 t yr^{-1} in the Taihu basin because of less industrial development, and less N emissions during industrial and agricultural production (Xu et al., 2019). N emissions and deposition have also increased with the development of industrial and agricultural production since the 1990s. Total N deposition entering into the lake was 9981 t yr^{-1} from 2002 to 2003, which represents about 48.8% of total annual N inputs via inflow rivers (Yang et al., 2007). In the Taihu basin, the total N deposition was 20978 t yr^{-1} in 2011 (Liu et al., 2012). The N wet deposition from 2003 to 2004 was 7329.5 t yr^{-1} (Wang et al., 2009), from 2009 to 2010 was up to 10868 t yr^{-1} (Yu et al., 2011) and was up to 12062 t yr^{-1} in 2012 (Li et al., 2015). N deposition was 50-60 kg N ha^{-1} yr^{-1} in 2013, but the same latitude of Sichuan was only 15-21 kg N ha^{-1} yr^{-1}, the Tibetan Plateau without industrial pollution only 10-14 kg N ha^{-1} yr^{-1} (Zhu et al., 2015), the N deposition in Taihu Lake would reach 18447.5-22137.0 t yr^{-1} in 2013 according to this result. It is evident that the total amount of atmospheric N deposition in Taihu Lake was increasing, and significantly aggravated lake eutrophication (Luo et al., 2007). Previous studies have shown that the N load transmitted from the atmosphere to water body may be close to the load of rivers and other point sources in Taihu Lake. N deposition in contaminated regions contributed by precipitation may be elevated by 1-2 orders of magnitude compared to uncontaminated regions (Wang et al., 2017).

In the above-mentioned estimation process, the total amount of atmospheric N deposition from the water surface of Taihu Lake was estimated, but the amount of N deposition entering into the lake from the whole watershed was not estimated. The total area of Taihu lake catchment is 36895 km^2, but the water area of Taihu is only 2338 km^2, accounting for 6.3% of the catchment area. Therefore, the main objective of this study is to analyze the contribution of atmospheric N deposition entering into the Taihu lake from the whole watershed, analyze the total amount of N uptake and leach by different land use, and find out the main source of N deposition entering into the Taihu Lake, and to provide scientific suggestions for controlling lake eutrophication.

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So, the watershed catchment can be divided into different areas depending upon the variability of absorption of N deposition, which fully considers the impact of atmospheric N deposition on water body in the whole watershed, and plays an important role in analyzing the impact of atmospheric N deposition on water body, especially the external sources of N.

Materials and methods

Study area

Taihu Lake is located in the Yangtze River delta, the most developed area of China in industry and agriculture (*Fig. 1*). A large amount of N is released in the process of industrial, agricultural production and transportation. The water quality of Taihu Lake is deteriorating gradually. In recent decades, with the deterioration of water quality, the algal bloom outbreaks are frequent. Taihu Lake has brought a great impact on economic and social development within the Taihu basin. Since the 1980s, the amount of pollutants discharged into the Taihu Lake has increased dramatically, which resulted in deterioration of the lake ecosystem and appearance of harmful algal blooms. Many studies have been taken to restore the lake ecosystem since the 1990s. However, the lake water quality has not shown any significant improvement.

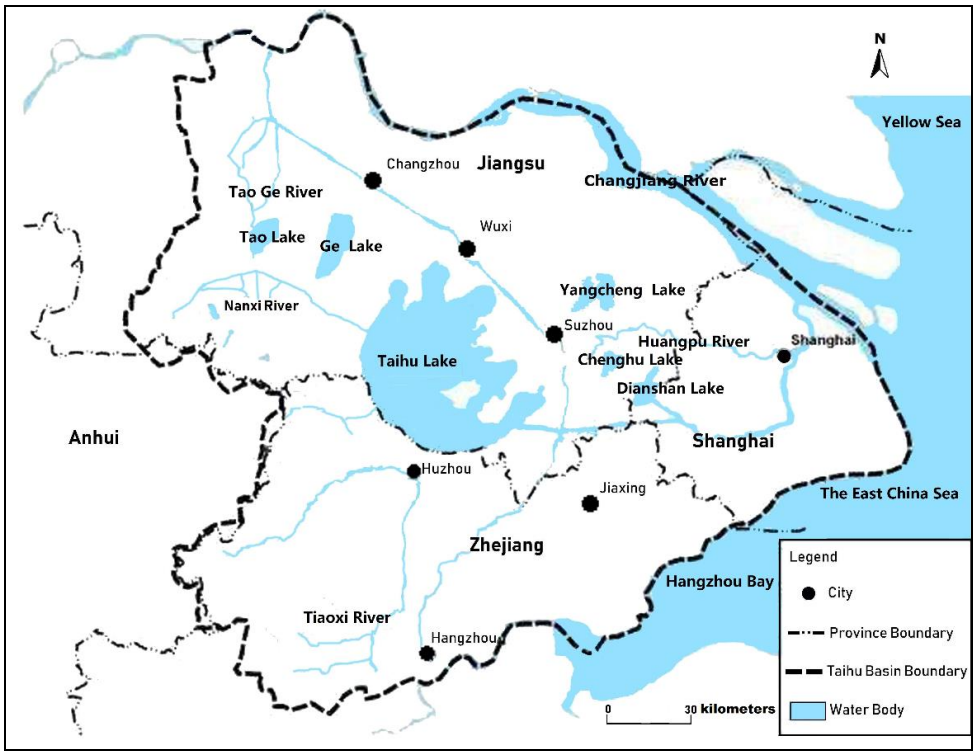

Figure 1. *Lake and city distribution in Taihu basin*

Data sources

Some important cities and water areas in the Taihu basin are shown in *Figure 1*. Precipitation N ion content was obtained from environmental monitoring stations in

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Suzhou, Wuxi and Changzhou in 2014, and the value of the dry deposition rate in 2014 is referenced by Ti et al. (2018). N ion concentration of precipitation (from Aug. 2013 to Aug. 2014) in Hangzhou, Jiaxing and Huzhou was acquired from the research of Wang et al. (2015). N ion of precipitation (from Sep. 2014 to Sep. 2015) in Shanghai was obtained from a study of Deng et al. (2016). Precipitation data came from Statistical Yearbooks of the cities in 2014 (Suzhou, Wuxi, Changzhou, Hangzhou, Jiaxing, Huzhou, Shanghai (excluding Chongming)). Data on vegetation and land use in Taihu basin was taken from land use map at the scale of 1:100,000 (2015) (*Fig. 2*). The data of the agricultural output, poultry and livestock output, chemical fertilizer, population were obtained from Statistical Yearbooks of Suzhou, Wuxi, Changzhou, Jiaxing, Huzhou, Hangzhou and Shanghai (excluding Chongming) respectively.

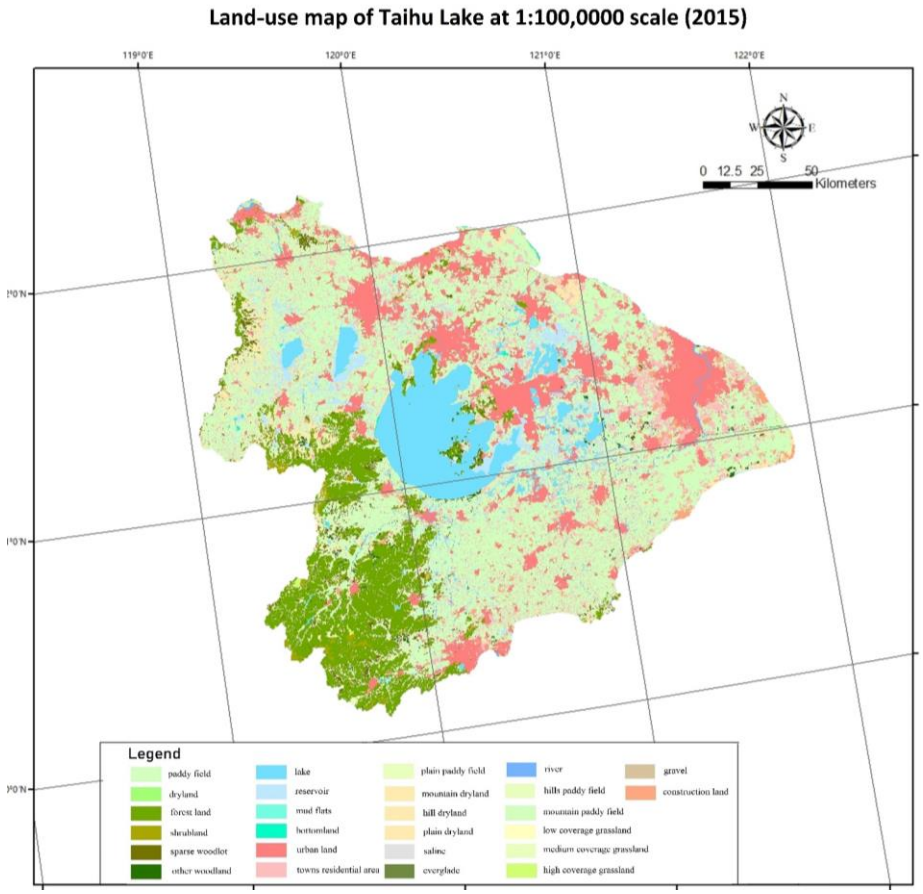

Figure 2. *Land-use map of Taihu Lake basin at 1:100,000 scale (2015)*

Estimation method of N deposition

Estimation method of N deposition from catchment into Taihu Lake

According to the landuse vectorgraph in Taihu basin at the scale of 1:100,000 (2015) (*Fig. 2*), Taihu Lake basin was divided into four sections in accordance with the land-use classification, those are water area (lake, river, reservoir and everglade), farmland (paddy field, plain paddy field, hills paddy field, mountain paddy field, dryland, mountain dryland, hill dryland, plain dryland), vegetation area (forest land, shrubland, sparse woodlot, low-coverage grassland, medium-coverage grassland, high-coverage

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grassland), urban construction land (UCL) (urban land and towns residential area), respectively. Some gravel and saline were not included in the statistics, because their area was less than 0.003%. There are great differences in atmospheric N deposition in different land-use patterns. The atmospheric N can be directly accepted by the water area, while the atmospheric N deposition will be absorbed by crops in the farmland and then discharged into the Taihu Lake. In forest and grassland, N deposition is absorbed by vegetation. If N cannot be absorbed completely by the vegetation, the excessive N will be discharged into the Taihu Lake, if N is absorbed completely, the total amount of N deposition entering into the Taihu Lake will be zero. When the atmospheric N deposition is only absorbed by the urban green space in the urban area, the remaining part will afflux into the Taihu Lake.

Therefore, N deposition from catchment entering into Taihu Lake can be expressed as:

$$N_{input} = N_{wat} + N_{far} + N_{veg} + N_{urb} \quad \text{(Eq.1)}$$

where N_{wat} represents N deposition from water area, N_{far} represents N deposition from farmland into the lake, N_{veg} represents N deposition from vegetation area, and N_{urb} represents N deposition from UCL into the lake.

Estimation method of each component

(1) Estimation of total N deposition (N_{total})

The estimation method of wet deposition is the precipitation collection method. The total amount of wet deposition is calculated using N deposition in each precipitation, so the wet N deposition is expressed as:

$$F_w = \sum_{i=1}^{n} Ci \times Pi \quad \text{(Eq.2)}$$

where F_w represents the total amount of wet N deposition (kg N·hm^{-2}); C_i represents the average concentration of N deposition (mg N·L^{-1}) in every area; P_i represents per precipitation (mm).

Dry N deposition is calculated according to particulate ammonium and nitrate concentrations in the aerosols. It can be expressed as:

$$F_{dry} = F_d \times A = C_a \times V_d \times A \quad \text{(Eq.3)}$$

where F_d is the dry deposition rate of N (kg N km^{-2}·d^{-1}), C_a is the concentration of N in aerosol particles, V_d is the dry deposition rate of aerosol particles. A represents the basin area. So the total N deposition is equal to the sum of dry deposition and wet deposition.

$$N_{total} = F_w + F_{dry} \quad \text{(Eq.4)}$$

(2) Estimation method of N deposition in water area (N_{wat})

There are many water areas in Taihu watershed, including Taihu Lake, Ge Lake, Yangcheng Lake etc., they are scattered in the Taihu basin. The dry N deposition and wet N deposition can be deposited into water portion.

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N deposition in water area is expressed as:

$$N_{wat} = (R_{wat} / R_{tot}) \times N_{total} \quad \text{(Eq.5)}$$

where R_{wat} is the water area, R_{tot} is the total basin area.

(3) Estimation method of N deposition from farmland entering into the lake (N_{far})

The N absorption rate is used to estimate the N deposition from farmland into Taihu Lake. There are four main ways of N input in farmland in the Taihu Lake basin, i.e. chemical fertilizer (N_{fert}), N input from human and livestock excreta (N_{exc}), biological N fixation (N_{fix}) and atmospheric N deposition (N_{dep}) in farmland. In this study, it is assumed that all forms of N input are equally absorbed by the crops. Therefore, the rest of the atmospheric N deposition is considered to enter into the lake after absorption by all plants.

① The chemical fertilizer is the most important N input in the process of agricultural production in Taihu basin. The final N input amount of chemical fertilizer (N_{fert}) is calculated as follows:

$$N_{fert} = \sum_{i=1}^{m} N_{fert_i} \quad \text{(Eq.6)}$$

where N_{fert} represents the total N amount of chemical fertilizer to farmland, N_{ferti} represents the i kind N chemical fertilizer to farmland (pure N).

② N input from human and livestock excreta (N_{exc}): The N input of excreta mainly comes from the agricultural population, livestock and poultry in the Taihu basin. The data of agricultural population, the amount of livestock and poultry in the Taihu basin is obtained by the Statistical Yearbooks of all regions. The calculation equation of livestock and poultry quantity is as follows (Yan et al., 2010):

$$Lpp = Bs + S \times 0.542 \quad \text{(Eq.7)}$$

where Lpp represents livestock and poultry population, Bs represents breeding stock, S represents slaughter capacity.

The N input is calculated as follows:

$$N_{exctotal} = \sum_{i=1}^{m} N_{exci} \quad \text{(Eq.8)}$$

where N_{exci} is N input from all types of human or livestock waste, $N_{exctotal}$ is the total amount of N in excreta.

Because the rate of excrements as a fertilizer to fields is low in Taihu basin, therefore, the N input from excrement does not return to the fields completely, the amount of N to fields is calculated as the following:

$$N_{exc} = r_{exc} \times N_{exctotal} \quad \text{(Eq.9)}$$

where r_{exc} is the coefficient (r_{exc}) from excrement to fields.

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③ N fixation by crop (N_{fix}): The N fixation rate of crops is quite different between various crops. The yield of main crops is obtained from the local Statistical Yearbooks, and the total N fixation amount of crops is calculated according to the N fixation coefficient of various crops. Therefore, the N fixation of crops can be calculated by the following equation:

$$N_{fix} = \sum_{i=1}^{n} A_i \times Ci \quad \text{(Eq.10)}$$

where N_{fix} represents the N fixation amount of the crops, A_i represents the plant area of certain crops, C_i represents the N fixation coefficient of a certain crop.

④ Atmospheric N deposition of farmland (N_{dep}): The amount of N deposition in farmland can be calculated by the following equation:

$$N_{dep} = R_2 \times N_{total} \quad \text{(Eq.11)}$$

where R_2 represents the ratio of the farmland area to the Taihu basin area.

⑤N assimilation in crops (N_{ass}): The N content of the main crops in the Taihu Lake area is calculated according to the N absorption rate of the main crops. The N uptake (N_{ass}) can be calculated by the following equation:

$$N_{ass} = \sum_{i=1}^{m} O_i \times Ri \quad \text{(Eq.12)}$$

where N_{ass} represents the N uptake amount of the crops, O_i represents the output of certain crops, R_i represents the absorption coefficients of N of certain crop.

If the crops absorb all forms of N uniformly, the absorption coefficients of crops to various forms of N can be obtained by the following equation:

$$r = N_{ass} / (N_{fert} + N_{exc} + N_{fix} + N_{dep}) \quad \text{(Eq.13)}$$

Therefore, the calculation equation of N entering into the Taihu basin through atmospheric N deposition is as follows:

$$N_{farm} = (1 - r \times N_{dep} / (N_{fert} + N_{exc} + N_{fix} + N_{dep})) \times N_{dep} \quad \text{(Eq.14)}$$

(4) Estimation method of N deposition from UCL entering into the lake （N_{urb}）

The afforestation rate of UCL is rather higher in the Taihu basin, so atmospheric N deposition in UCL does not all enter into the water body. Some of N deposition is absorbed by the urban green spaces, while the rest of N deposition can be regarded as entering into the lake. So the calculation equation is the following:

$$N_{urb} = R_3 \times N_{total} - \sum_{i=1}^{n} A_i \times f_{gb} \quad \text{(Eq.15)}$$

where R_3 represents the ratio of UCL in Taihu basin. A_i represents the urban green space , f_{gb} represents N absorption rate of green space ($kg \cdot hm^{-2}$).

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(5) Estimation method of N deposition from vegetation area entering into the lake (N_{veg})

In the vegetation area, when the atmospheric N deposition is absorbed and saturated by vegetation and soil, the excess N will be discharged to the lake. Therefore, it is necessary to consider the critical point of different soil types when estimating the N deposition entering into water bodies. Therefore, the important step is to estimate whether the regional atmospheric N deposition exceeded the N critical loads. If it exceeds the N critical loads, the excess part is regarded as entering into the water; if it does not exceed the loads, it is regarded as being absorbed and utilized by vegetation (Song et al., 2014).

At present, the simple mass balance method (SMB) is widely used to estimate the critical load of atmospheric N deposition. The basic equation is as follows:

$$CL_N = N_i + N_{up} + N_{de} + N_{le,crit} \quad \text{(Eq.16)}$$

where CL_N represents the critical loads of N deposition; N_i represents the mineralization rate of soil N; N_{up} represents the rate of N uptake by vegetation; N_{de} represents the denitrification rate of N in soil; $N_{le,erit}$ represents the critical point of soil N leaching rate (Posch et al., 2015).

N_{de} is the denitrification rate of N, the denitrification rate is linear with the net N input, Therefore, the calculation equation is as follows (Posch et al., 2015):

$$N_{de} = \begin{cases} f_{de}(N_d - N_i - N_{up}) & N_d > N_i + N_{up} \\ 0 & N_d \le N_i + N_{up} \end{cases} \quad \text{(Eq.17)}$$

where N_d represents N deposition in the vegetation area of Taihu basin. According to the calculation of total N deposition in the region, the calculation equation is:

$$N_d = R_4 \times N_{total} \quad \text{(Eq.18)}$$

where R_4 is the percentage of vegetation area in the watershed.

N_{de} can be ignored in well-drained vegetation cover areas. According to the above calculation, *Equation 16* can be simplified as follows:

$$CL_N = N_i + N_{up} + \frac{N_{le,crit}}{1 - f_{de}} \quad \text{(Eq.19)}$$

where f_{de} represents the denitrification rate, $N_{le,erit}$ represents the critical leaching rate.

The calculation equation of the critical leaching rate ($N_{le,erit}$) of N is as follows:

$$N_{le,erit} = Q \times [N]_{crit} \quad \text{(Eq.20)}$$

where Q represents the runoff, $[N]_{crit}$ represents the critical leaching concentration of N. The calculation equation of N_{veg} is as follows:

$$N_{veg} = \begin{cases} N_d - CL_N \times A_f & N_d > CL_N \times A_f \\ 0 & N_d \le CL_N \times A_f \end{cases} \quad \text{(Eq.21)}$$

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where A_f represents the vegetation area. When the N deposition (N_d) is larger than the critical loads in natural vegetation area, the excess part of N deposition can be regarded as entering into the basin. When N_d in vegetation area is less than the critical load, it can be regarded as 0.

Results

Watershed division of the Taihu Lake basin

According to the land-use vectorgraph in Taihu basin at the scale of 1:100,000 (2015) (*Fig. 2*), the water area accounts for 13.70%, farmland 46.71%, vegetation area 13.91% (forest 13.44%, grassland 0.47%) and UCL 25.67%.

Total N deposition (N_{total})

The concentration of N deposition, the rate of wet N deposition and dry N deposition rate and total amount of wet N deposition and dry N deposition are listed in *Table 1*.

Table 1. *Wet N deposition and dry N deposition rate and the total amount in every city*

City	Concentration of N deposition (mg N •L^{-1})	Wet N deposition (Kg/km^2 yr)	Rainfall (mm)	Total amount of wet N deposition (t)	Dry deposition rate of N (Kg/km^2 yr)	Total amount of dry N deposition (t)
Suzhou	3.82	4840.5	1265.7	41088.6	2693.9	20582.8
Wuxi	4.45	5885.7	1322.5	27235.7	2858.2	41238.0
Changzhou	4.19	5504.1	1313.0	24135.5	2712.3	11893.4
Hangzhou	2.48	2561.6	1359.9	6045.3	2474.3	5839.3
Jiaxing	2.72	3170.3	1580.8	12411.5	2415.6	9457.0
Huzhou	2.69	3506.7	1443.6	20401.6	1444.1	8401.7
Shanghai	3.84	4972.4	1295.3	25747.1	5560.4	27714.2
Total				157065.2		125126.5

In this study, the total amount of wet N deposition (*Fw*) is 157065.2 t according to *Equation 2*, the total dry N deposition is 125126.5 t according to *Equation 3*, the total N deposition (N_{total}) 282191.8 t according to *Equation 4*.

N deposition in water area (N_{wat})

The ratio of R_{wat}/R_{tot} is 0.1370 in this study, so the amount of N deposition (N_{wat}) directly from the Taihu basin into the lake is 38667.5 t according to *Equation 5*.

Total N deposition from farmland entering into the lake (N_{far})

① N_{fert}: The N fertilizer (N_{fert}) in the Taihu basin in 2014 is listed in *Table 2* according to the Statistical Yearbooks of Suzhou, Wuxi, Changzhou, Jiaxing, Huzhou, Hangzhou and Shanghai (excluding Chongming). So N_{fert} is about 213263.6 t by *Equation 6*.

Table 2. *N amount of chemical fertilizer in Taihu basin (pure N)*

City	Suzhou	Wuxi	Changzhou	Hangzhou	Jiaxing	Huzhou	Shanghai	Summation
N amount of chemical fertilizer (t)	3977	3160	22739	46800	29200	78100	29287	213263

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② N_{exc}: The number of agricultural population, the amount of livestock and poultry in the Taihu basin is obtained from the Statistical Yearbooks of the Taihu basin, and as shown in *Table 3*. The livestock and poultry population is calculated according to *Equation 7*, the N coefficient of people and animal excreta is referenced by the study from Zhang in 2014 (Zhang et al., 2014), the results are listed in *Table 3*. The total amount of N in excreta (N_{exc}) according to *Equation 8* is 342506.7 t according to *Equation 8*. The value of r_{exc} is 0.1 in Taihu basin according to the study from Zhang in 2014 (Zhang et al., 2014), so the actual amount of N through excrement into farmland is 34250.7 t.

Table 3. *N excretion rates and total content by human and domestic animals*

Parameters	People (N kg/person·year)	Pig (N kg/each·year)	Cattle (N kg/each·year)	Sheep (N kg/each·year)	Rabbit (N kg/each·year)	Poultry (N kg/each·year)	Summation (t)
N discharge coefficient*	4.0	4.5	61.1)	2.28	1.0	0.275	
Amount (10000)	1863.5	1452.9	215.0	268.7	189.0	22925.2	
N discharge amount (t)	74539.2	65524.6	131382.2	6126.3	1890.1	63044.3	342506.7

*N discharge coefficient refers to the research results of Zhang et al. (2014)

③ N_{fix}: The planting area and the N fixation rate of the main crops in Taihu Lake Basin are shown in *Table 4*. The N fixation amount of the crops (N_{fix}) is 36350.3 t according to *Equation 10*.

Table 4. *N fixation coefficient and the quantity of N fixation of staple crops*

Parameters	Wheat	Rice	Maize	Soybean	Potatoes	Oil-bearing	Cotton	Vegetable and fruits	Other crops	Total
Acreage (×1000 hm^2)	263.9	447.7	28.7	39.6	18.7	75.2	2.2	367.0	113.4	
Fixed N coefficient (N kg/hm^2)*	15.0	45.0	15.0	80.0	15.0	15.0	15.0	15.0	15.0	
Amount of fixed N (Nt)	3958.7	20148.3	431.0	3165.6	279.8	1128.0	33.5	5505.0	1700.6	36350.3

*Fixed N coefficient refers to the research results of Zhang et al. (2014)

④ N_{dep}: In this study, R_2 is 0.4671, N_{total} is 282191.8. Therefore, the calculation result of N_{dep} is 131811.1 t according to *Equation 11*.

⑤ N_{ass}: The main agricultural output and their N absorption coefficient are shown in *Table 5,* the absorption coefficients of N can be referenced from some researches (Xu et al., 2006; Zhang et al., 2014; Gao et al., 2014). Ultimately, the N uptake (N_{ass}) of the crop in the Taihu Lake basin can be calculated through *Equation 12*. The total N uptake of crops is calculated as 217634.6 t.

According to the above calculation, N_{ass} is 217634.6 t, N_{fert} is 213263.0 t, N_{exc} is 342506.7 t, N_{fix} is 36350.3 t, N_{dep} is 131811.1 t, and r is equal to 0.2981 by *Equation 13*.

According to the calculation, the N deposition entering into the Taihu Lake from farmland is 124596.1 t by *Equation 14*, it accounts for 94.5% of N deposition in the farmland area, only 5.70% is absorbed by crops in Taihu basin, which is only 7215.0 t.

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Table 5. *Absorption coefficient and absorptive amount of staple crops*

Parameters	Rice	Wheat	Maize	Soybean	Potato	Oil-bearing	Cotton	Vegetables	Total
N absorption coefficient (kg/t)*	19	28	14	45	25	35	30	3.5	
Output (10^4t)	4615141	1300441	176803	1268147	115441	222467	3057	6636749	
N uptake amount (t)	87687.7	36412.3	2475.2	57066.6	2886.0	7786.3	91.7	23228.6	217634.6

*N absorption coefficient refers to the research results of Xu et al. (2006), Zhang et al. (2014) and Gao et al. (2014)

Total N deposition from UCL entering into the lake (N_{urb})

UCL of every region is shown in *Table 6, R_3* is equal to 0.2567 in this study, so the product of R_3 and N_{total} is 72446.3 t. The value of f_{gb} in this study is 0.116 t N·hm^{-2} (Zhang et al., 2019). The amount absorbed N is 19793.1 t. N_{urb} is 52653.2 t by *Equation 15*.

Table 6. *The area of UCL in Taihu basin*

UCL	Suzhou	Wuxi	Changzhou	Hangzhou	Jiaxing	Huzhou	Shanghai	Total
Area/hm^2	34909	18543	8813	18386	5659	4792	80041	160692

Total N deposition from vegetation area entering into the lake (N_{veg})

① It is difficult to obtain the average annual mineralization rate of the soil (N_i), the value of N_i in the Taihu basin is 0.25 keq·hm^{-2}·a^{-1} according to Duan and Liu's researches (Duan et al., 2015; Liu et al., 2020). So N_i is 0.25 keq·hm^{-2}·a^{-1}.

② The rate of N uptake by forest vegetation types (N_{up}) is 2.06 keq·hm^{-2}·a^{-1} in the Taihu basin according to Sun and Duan's researches (Sun and Xie, 2014; Duan et al., 2015).

③ R_4 is 0.1391. N_{total} is 282191.8 t, the final calculation of N_d is 39252.9 t by *Equation 18*.

④ The average runoff depth in Taihu basin is 553 mm in 2014 according to the Taihu Basin and Southeast Rivers Water Resources Bulletin, so Q = 553 mm. Based on considerations to protect groundwater, a lower limit of $[N]_{crit}$ = 20.0 mg/L is suggested as the critical limit (Vries et al., 2015). The value of $N_{le,erit}$ can be calculated by *Equation 20*. The value of f_{de} is 0.5 according to Duan's research in 2015 (Duan et al., 2015) in the calculation *Equation 17*. Finally, CL_N is 5.446 gN·m^{-2}·a^{-1} by *Equation 19*.

A_f is 5168.04 km^2 in this study, the maximum N load in vegetation area is calculated as 28145.1 t according to this critical load (CL_N), and less than the actual N deposition (N_d, 39252.9 t). So, the amount of N deposition entering into Taihu Lake through the vegetation area is 11107.8 t by *Equation 21*.

Total N deposition from the entire Basin entering into the lake (N_{input})

The N deposition in each region and the total N deposition entering into Taihu Lake is shown in *Table 7*. The final contribution of atmospheric N deposition entering into the water body is calculated by *Equation 1*. The N amount directly into the water (N_{wat}) is 38667.5 t. The final amount of N into the water after the crops absorption (N_{far}) is

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124596.1 t. The total amount of N into the water after the absorption of urban green plants (N_{urb}) is 52653.2 t. The amount of N after the absorption of vegetation area (N_{veg}) is 11107.8 t. The final N deposition entering into the basin (N_{input}) is 227024.6 t.

Table 7. *The total amount of N deposition entering the lake from the different regions*

Parameter	N_{wat}	N_{far}	N_{urb}	N_{veg}	N_{input}
N deposition/t	38667.5	124596.1	52653.2	11107.8	227024.6
Percentage/%	17.0	54.9	23.2	4.9	100.0

Discussion

According to the above calculation, the total N deposition in the catchment area of Taihu Lake was 282191.8 t. After absorption by vegetation, soil and crops and so on, the 227024.6 t of N will eventually enter into the lake, accounted for 80.5% of the total deposition. The least N deposition was absorbed by farmland, most of N entered into Taihu Lake. It has become the main source of entering into Taihu Lake, accounted for 54.9%. The most N deposition was absorbed by vegetation, and thus few N into the water from the vegetation areas, only accounted for 4.9%. UCL reduced the N deposition entering into the water body because of the absorption of urban green space.

The improper or excessive fertilization is becoming more and more serious in Taihu Lake, and causes the environmental pollution from the non-point pollution of farmland, not only less N deposition is absorbed by it, but also becomes an important source of N emission. The agricultural production was the most contributor (62–69%) in China, especially in the developed and coastal regions that have high population densities (Xian et al., 2018). The livestock excrement is also an important source of atmospheric N emissions from the livestock production (Ti et al., 2019). There are not enough buffer strips around the farmland to absorb N deposition, which may eventually result in large amounts of N entering the water (Li et al., 2019). Therefore, the least of N deposition was absorbed by crops and most entered into the water.

Taihu basin covers only 0.38 percent of the country, but the population accounts for 10.2% of China's population, and GDP accounts for 9.9% which is 2.2 times the national average. Industry, agriculture and transportation are very developed in the Taihu Lake area in China and the number of automobiles is very high. In 2012, the proportion of urbanization in Taihu Lake has reached 77.6%. The coal burning, power production, and car ownership will all be the source of N emissions. Some models predict that China's NOx emissions decreased by 9.11% on average during 2019–2030 because of the decline in the proportion of industry (Yu and Liu, 2020). The coal consumption, electricity production, electricity production and car ownership are the source of N emissions (Wei et al., 2018), they will directly affect the level of N deposition. Continuing urbanization will reduce the vegetation area in Taihu basin, UCL is up to 24.95% in Taihu Basin, and there is little absorption of N deposition by natural vegetation in this area. Only 27.3% of the N deposition is absorbed by the urban green space, therefore, it is necessary to increase urban green space.

There are many water bodies in Taihu Basin, for example, Taihu Lake, Ge Lake, Yangcheng Lake, Dingshan Lake, etc., they can directly accept N deposition. Although the area of these lakes is only 2,838 km^2, accounting for 7.7% of the entire basin, and the area of other ponds and rivers only accounts for 13.7% of the entire basin. Since this

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water area is not covered by any vegetation or other objects, there will be no conversion and interception of N deposition. N from the atmosphere can enter the water body through atmospheric (rain, snow) and occult (dew, frost) precipitation, etc. (Kurzyca and Frankowski, 2019), or directly enter the water body through dry deposition. Therefore, a large amount of N can get into the water directly, the amount of N deposition in water area has reached 17.0%. Wang et al. (2017) also reported that the annual flux loads of TN from surrounding rivers, atmospheric deposition, and sediment suspension was 29600 t in 2014, and 23.5% of the TN load comes from atmospheric deposition directly entering into the lake. This result is also in agreement with our research.

N deposition can be absorbed by many plants (Gong et al., 2019), but the N deposition is absorbed to a certain extent, and more than the critical loads, N will not be absorbed (Kosonen et al., 2019), and a small amount of excess N is leached out and discharged into the water. Therefore, although the vegetation area only accounts for 13.7% of the Taihu Lake Basin, but it was found that 71.7% of the N deposition in the area was absorbed.

N deposition in the catchment area of Taihu Lake was 282191.8 t in this study, and the 227024.6 t of N deposition entered the lake. The total N emission into the Taihu Lake only reaches 141150.5 t in 2014 according to Wang's research (Wang et al., 2019), which is only 50.0% of the atmospheric N deposition, and it is difficult to reach the impact caused by atmospheric N deposition. According to the Overall Plan for Integrated Water Environment Management in the Taihu basin (revised in 2013), by the year 2015, the maximum allowance total N emissions will be 100,818 t, but the atmospheric N deposition has exceeded this threshold. Therefore, the atmospheric N deposition may be an important source of N in the Taihu basin, which will seriously affect the development of aquatic ecosystems. The influence degree of atmospheric N deposition may exceed the total of industry, agriculture, domestic sewage.

Conclusions and prospects

The catchment area of Taihu Lake was divided into four areas (water area, farmland area, UCL and vegetation area) to calculate the atmospheric N deposition. The amount of N deposition entering into the water from the four areas was 38667.5 t, 124596.1 t, 52653.2 t and 11107.8 t, respectively. The total N deposition entering into the lake is 227024.6 t, which contributed to 80.5% of the atmospheric N deposition in the basin. The total N into Taihu Lake through atmospheric N deposition is more than agricultural non-point source pollution, domestic sewage and industrial wastewater. Atmospheric N deposition may be the largest pollution source in Taihu Lake.

It is very important to control the atmospheric N deposition in terms of the external N input in Taihu Basin. After the calculation of agricultural non-point source pollution, domestic sewage, industrial wastewater, internal release and other control measures have achieved results, it is very important to control the atmospheric N emission. But the air pollution from thermal power plants, automobile transportation, petrochemical industry, large-scale application of chemical fertilizers and pesticide problems is becoming more and more serious. The atmospheric reactive N mainly comes from N fertilizer synthesis, fossil fuel combustion, N fertilizer applied in farmland and a large amount of volatilization of human and animal waste, as well as a small number of biological combustions, lightning process. Therefore, it is very important to control N emissions.

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It is a recommended way to popularize control-released fertilizer and optimized N management in the agricultural production, which could effectively improve N utilization efficiency and reduce N release in the course of agricultural production. Some strategies can be used in the livestock excrement treatment, for instance, manure acidification and deep manure placement, urease inhibitor, and so on. It will be effective to reduce high energy consumption industries in the process of industrial production, so the fossil fuel consumption can be decreased. It is necessary to develop clean energy vigorously, and increase the recovery and treatment of N in the combustion process of fossil fuels. In the meantime, control the number and travel of motor vehicles, develop public transport, these measures will effectively reduce N emissions.

In addition, increasing N absorption can significantly reduce the total amount of N deposition entering into the lake. Buffer strips and ecological channels can be build around the farmland, the buffer strips may intercept and retain nutrients from surface runoff, the ecological channels can absorb and remove nutrients such as N and phosphorus, and reduce the concentration of nutrient into the lake. The urbanization of the Taihu Lake Basin is at a relatively high level in China, and the land for urban green space is very limited. Therefore, three-dimensional greening is a very good choice, and can be vigorously developed.

How to accurately determine the dry deposition and more accurately estimate the contribution of the N and phosphorus deposition in the river basin to the water body, and the ability of the above methods to reduce N emissions and increase N absorption are the focus of future research.

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CORRELATIONS BETWEEN SOIL PROPERTIES AND ANT POPULATION IN AGRICULTURAL HABITAT AT AURANGABAD MAHARASHTRA, INDIA

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Abstract. Ants have a profound impact on the edaphic factors of the agricultural ecosystem as they increase the soil nutrient and plant growth. Hence in agricultural ecosystem farmers promote ant populations in their farm. Ants help to investigated soil properties included Nitrogen, Phosphorus, Potassium and organic carbon as well as Water holding capacity, pH etc. on agriculture field. The Ants were collected by using handpick, pitfall trap, scented trap methods. The soil properties Some of the environmental variables like rainfall, temperature, and relative humidity were analyzed from June 2015 to May 2016 at an agricultural field at Aurangabad city. The correlation coefficient of ant's population with soil properties and climatic factors were studied. In that nitrogen, organic carbon, relative humidity, rainy season shows significant positive correlation with all ant species $r = 0.507$, $r = 0.649$ at $p = 0.05$ level, and $r = 0.825$, $r = 0.793$ at $p = 0.01$ level of significant, respectively. The present study revealed that ants had the ability to modify chemical properties of soils and showed the correlation between ant diversity and soil.
Keywords: *agricultural field, ants, correlation coefficient, soil disturbance*

Introduction

There are various studies that reveal the role of ants in the maintenance of the ecosystem in which they live. Ants positively impact agricultural systems by rapidly consuming large numbers of pest insects, disturbing pests during feeding and oviposition, and increasing soil quality and nutrients. Naturally occurring ant species in milpas, mango, citrus, coconut, cashews, and cotton control many pest insects (Choate and Drummond, 2011). In food chains ants are considered as an integral part because of their capability to affect the flow of water, energy and nutrients through many terrestrial ecosystems (Jurgensen et al., 2008). Species of ants differentially affects the soil environment and it is of significant interest to understand how the distribution and composition of ant species are affected by environmental factors. Soil heterogeneity is increased by the activity of ants and reflects their long term engineering activities (Jouquet et al., 2006). In turn while searching a spot for building their nest, ants select microhabitats with specific physico-chemical conditions according to the species preferences (Johnson, 2000). In soil ants build several types of nest some are completely or partially present in the soil that consist of corridors and chambers can be covered by dead wood, stones and some other natural structure on the soil surface. In nest ants accumulates food in large amounts and deposits some food in the nest, which may alter the nutrient status of the soil and increase drainage and soil aeration, increasing the porosity of soil (Pokarzhevskij, 1981). How ants affect soil properties is determined by many factors such as nesting strategy, colony age, mound size, feeding behavior, and nutritional demand (Dauber and Wolters, 2000). Several biological and physical factors such as solar radiation, water, temperature, affect abundance and species richness of community of ant inhibiting particular environments because insects ants are small

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sized with high surface to volume ratio that make them prone to desiccation (Edney, 1977). Ants are very important in below ground processes by altering chemical and physical environment and their effects on soil organisms, microorganisms, plants (Folgarait, 1998). In the colony of ants, there are many workers, this leads to intensive mixing of soil from different soil depths and changes soil textural properties (Cammeraat and Risch, 2008). Ants are not considered as soil biota but in relation to their contact with soil, soil biota categorized into three groups such as endogeic (those living in soil), anecic (those which transfer materials between soil and litter habitats) and epigeic (those which process organic matter on or near the soil surface). In these ants could be placed in either the epigeic or anecic groups. In the present study twenty-three ant species collected in that *Dorylus labiatus* are endogeic species. Ant species *Tetramorium walshi, Meranoplus bicolor, Leptogenys chinensis, Solenopsis geminata* are epigeic species. In anecic group *Myrmicaria brunnea, Camponotus compressus*, *Camponotus sericeus* are present. These suggestions should be tested on the level of population because the significant evidence of the habitation is not present on these species. Soil biota categorized according to size and ants are considered to be microfauna. It is seen that ants' role in soil functioning are similar to earthworms, termites (Bouche, 1977). Ants act as an indicator because ants respond quickly to the surrounding environment. The process of construction of the nest, foraging activity increases the fertility of the soil. The contribution of active ants mounds to the surrounding soil ecosystems involves long-term nutrient release into the system (Chate and Chavan, 2021). Ants are abundant and diverse part of soil fauna to increase crop yield and restore soil quality in agroecosystems (Jesovnik et al., 2019). Hence the present study was undertaken to study the correlation coefficient (r) of ant population in agriculture fields of periurban site, with soil parameters like nitrogen, phosphorus, potassium, organic carbon, water holding capacity, pH and climatic parameters such as rainfall, temperature, and relative humidity.

Materials and Methods

Study Site

Aurangabad city is located mainly in Godavari Basin on the bank of its tributary Khamriver. The city is surrounded by hills in all directions, latitude $19^0$53’ and longitude $75^0$23’ of Aurangabad. Aurangabad city experiences three distinct seasons: the rainy season from June to September, winter season from October to January and summer season from February to May. The average rainfall of Aurangabad district is 734 mm and minimum temperature is 5.6^0 C and maximum temperature is 45.9^0 C (www.aurangabad.gov.in.). The study area was agriculture field is located within 15 km of the city center near about 6 ha or more in size planted with *Zea mays* (Maize), *Saccharum officinarum* (Sugarcane), *Gossypium* (cotton), vegetables such as *Spinacea oleracea* (Spinach), *Rhaphanus sativus* (Radish) and major trees were *Mangifera indica*, *Tamarindus indica* etc. (*Fig. 1*). This historic Aurangabad city is facing civilization and urbanization during a couple of decades with modification of natural and artificial habitats for different purposes. In the present study ant sampling was carried by three methods as handpick, pitfall trap, scented trap, all out search method was in morning 6 to 8 am once in a month. In agriculture field in each cultivated plants as Zea mays (Maize), Saccharum officinarum (Sugarcane), Gossypium (cotton) six ant colony were observed and for soil analysis soil sample was collected from near the

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colony of ants once in the month. Blocks of soil 15 cm long, 6 cm wide and 16 cm deep were excavated from each of six ants mounds the section of cut soil sample 0-5 cm deep were taken 20 m x 20 m in rectangular area of one-hectare plot of surroundings ants mounds in agriculture field. Collected ants specimens and soil samples were brought to the laboratory. Soil samples were air dried overnight and next day sieved soil through a 2 mm screen. Ants were preserved in 70% alcohol. Identification is made with the help of stereoscope trinocular microscope based on standard taxonomic keys suggested by Bolton (1994), Holldobler and Wilson (1990), Mathew and Tiwari (2000) and Sheela (2008) etc. Physico-chemical parameters such as nitrogen, phosphorus, potassium, organic carbon, water holding capacity, and pH were analyzed in soil testing Marathwada Institute of Technology laboratory Aurangabad as per standard protocol described by Somawanshi et al. (2012), and Trivedy and Goel (1987). Meteorological data such as rainfall, temperature, and relative humidity were obtained from the Government of Maharashtra Water Resource Department, Aurangabad. The study was done in the agriculture field in Aurangabad city from June 2015 to May 2016. The study evaluate how nest of ants affects different characteristic on soil nutrient, soil cation. The article aimed to investigate the way ant populations influence physical and chemical soil properties of agricultural fields near Aurangabad city, India, focusing on the correlation between ant populations, soil properties and climatic factors.

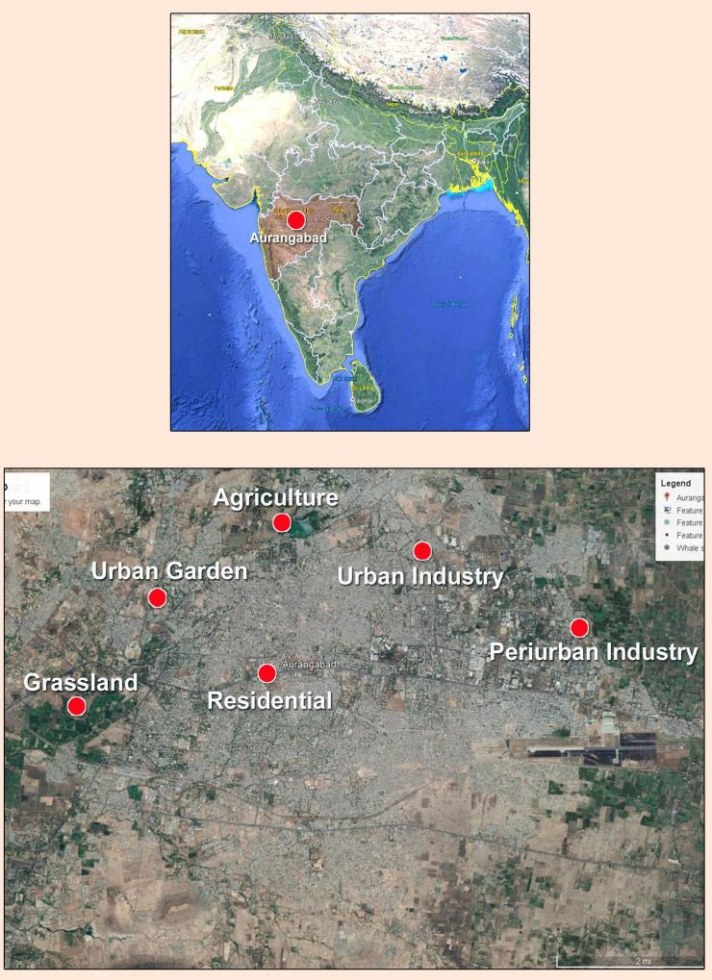

Figure 1. *Map of Agriculture site from Aurangabad*

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Site of agriculture field shows in *Fig. 2A* and in *Fig. 2B* shows Pitfall trap.

(A) (B)

Figure 2. *(A) Agriculture field, (B) Pitfall trap*

Result and Discussion

In the present study total twenty-three species of ants belonging to seventeen genera and six subfamilies were recorded. Month wise climatic parameters in the agriculture field represent in *Table 1*. Month wise soil parameters such as nitrogen, phosphorus, potassium, organic carbon, water holding capacity, pH are shown in *Table 2*.

Table 1. *Month wise climatic parameter in Agriculture Field. (Government of Maharashtra, Water Resource Department, Aurangabad)*

Month	Rainfall (mm)	RH (%)	Temperature (^{0}C)
June	243	83.0	30
July	32	57.1	33.3
August	82	78.7	32
September	(Max.245.8)	(Max.84.2)	30.1
October	33.2	64.0	34.8
November	0	45.2	35.5
December	0	51.5	29.8
January	0	47.1	(Min.28.6)
February	0	(Min.44.1)	34.0
March	13.3	49	38.2
April	0	55	40.5
May	(Min.2)	46.4	(Max.42.5)

Correlation coefficient (r) of ant species diversity are shown in *Table 3*, with soil properties and climatic factors in the agriculture field. Nitrogen shows significant positive correlation with subfamily Dolichoderinae (r = 0.567), subfamily Pseudomyrmecinae (r = 0.566) and all ants species (r = 0.507) at p = 0.05 level and negative correlation with phosphorus (r = -0.587). Potassium showed significant

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positive correlation with p^H (r = 0.804) at p = 0.01 level and negative correlation with temperature (r = - 0.53). Organic carbon showed significant positive correlation with all ants species (r = 0.649) at p = 0.05. Water holding capacity showed negative correlation with subfamily Dolichoderinae (r = - 0.723) at p = 0.01 level. Relative humidity showed significant positive correlation with subfamily Myrmicinae (r = 0.62), Ponerinae (r = 0.669), Pseudomyrmecinae (r = 0.74), all ants species (r = 0.825) at p = 0.01 and subfamily Formicinae (r = 0.535) at p = 0.05 level. Rain showed significant positive correlation with relative humidity (r = 0.897) at p = 0.01, subfamily Myrmicinae (r = 0.558), Formicinae (r = 0.574), Dolichoderinae (r = 0.583), Ponerinae (r = 0.56), Pseudomyrmecinae (r = 0.596) at p = 0.05, respectively and all ants species (r = 0.793) at p = 0.01. Temperature showed negative correlation with subfamily Dolichoderinae (r = -519), Pseudomyrmecinae (r = - 0.568) and all ants species (r = - 0.61) at p = 0.05 level (*Table 3*). In the agriculture field, climatic factors such as relative humidity and rainfall were positively correlated with ant communities. In this study 23 species of ants were sampled, which were distributed amongst 17 genera and six subfamilies (*Table 4*). Myrmicinae was most speciose subfamily belongs to eight species followed by subfamily Formicinae belongs to four species followed by subfamily Ponerinae and subfamily Pseudomyrmecinae belongs to three species and respectively subfamily Dolichoderinae and Dorylinae belongs to one species respectively. The species of the *Solenopsis geminate are* distinguished for their aggressiveness in the use of litter and soil found in agroecosystems. Ants of *Solenopsis* genera are able to withstands long periods of food scarcity and have effective strategies for mass recruitment. The *Camponotus* genus distributed wide consisting of terrestrial habit, dominant organism and omnivorous species. They are commonly known as Carpenter ants because of their ability to build nest in the dead or decaying logs at the base of trees. *Meranoplus bicolor* nest of these species present on ground surrounding with a small crater they feed on harvest grass seed and floral nectar. Species *Leptogenys Processionalis* are predatory and nomadic. These ants are ground dwelling, nest of these species seen in loose soil.

Table 2. *Month wise soil parameter in Agriculture Field during June 2015 - May 16. (Government of Maharashtra, Water Resource Department, Aurangabad)*

Month	pH (1 :2.5)	OC (%)	N (kg ha^{-1})	P (kg ha^{-1})	K (kg ha^{-1})	WHC %
June	7.66	2.17	(Max.815)	(Min.3.99)	450	69.3
July	7.98	1.16	250	16.4	637	(Min.11.5)
August	8.00	(Min.0.99)	(Max.815)	(Min.3.99)	450	72.6
September	8.16	1.66	250	45.6	655	50.1
October	7.48	2.92	388	27.9	453	78.1
November	8.13	3.88	551	66.1	585	94.9
December	(Max.8.28)	1.56	(Min.150)	(Max.70.4)	608	68.3
January	8.13	1.61	288	48.8	(Max.663)	46.3
February	7.30	(Max.4.25)	401	41.2	473	49.8
March	7.84	1.22	401	57.5	651	(Max.95.5)
April	(Min.6.97)	1.67	338	69.1	(Min.346)	65.9
May	7.53	2.16	338	28.1	393	82.13

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Table 3. *Correlation coefficient (r) of ant population with soil properties and climatic factors in Agriculture Field site during June 2015 to May 2016*

	N	**P**	**K**	**OC**	**WHC**	**pH**	**RAIN**	**RH**	**TEMP**	**MYRME**	**FORMI**	**DOLICH**	**PONER**	**PSEUDO**	**DORYL**	**Total Ant**
N	0.000	-0.587	-0.401	0.1	0.36	-0.095	0.385	0.476	-0.084	0.295	0.246	**0.567**	0.088	**0.566**	-0.246	**0.507**
P		0.000	0.271	0.168	0.266	0.044	-0.463	-0.577	0.193	-0.28	0.097	-0.359	-0.295	-0.247	-0.309	-0.29
K			0.000	-0.228	-0.275	**0.804**	0.058	-0.092	-0.53	-0.104	-0.085	0.046	0.2	0.031	0.294	-0.01
OC				0.000	0.224	-0.304	-0.186	-0.355	0.127	-0.304	0.013	0.05	-0.179	-0.246	-0.281	**0.649**
WHC					0.000	-0.091	-0.129	-0.119	0.422	0.093	0.317	0.187	-0.253	0.025	-0.723	0.148
pH						0.000	0.18	0.139	-0.631	0.119	0.135	0.216	0.327	0.387	0.149	0.301
RAIN							0.000	**0.897**	-0.464	**0.558**	**0.574**	**0.583**	0.56	**0.596**	-0.076	**0.793**
RH								0.000	-0.443	0.62	0.535	0.449	**0.669**	0.74	-0.034	**0.825**
TEMP									0.000	-0.377	-0.38	-0.519	-0.338	-0.568	-0.057	-0.61
MYRME										0.000	0.319	**0.676**	-0.013	0.393	-0.078	**0.771**
FORMI											0.000	0.425	0.319	**0.641**	-0.740	**0.762**
DOLICH												0.000	-0.181	0.392	-0.236	**0.739**
PONER													0.000	**0.565**	0.085	0.344
PSEUDO														0.000	-0.201	**0.809**
DORYL															0.000	-0.47
Total Ant																0.000

N – Nitrogen, P – Phosphorus, K – Potassium, OC – Organic Carbon, WHC – Water holding capacity, RH – Relative humidity, Temp – Temperature, MYRME – Myrmicinae, FORMI – Formicinae, DOLICH – Dolichoderinae, PONER - Ponerinae PSEUDO - Pseudomyrmecinae, DORYL – Dorylinae

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Table 4. *Ant distribution in agriculture field from Aurangabad city*

Subfamily	*Genus*	*Species*
Dorylinae	*Dorylus*	*Dorylus labiatus*
Myrmicinae	*Crematogaster*	*Crematogaster subnuda* *Crematogaster brunnea contemta*
	Solenopsis	*Solenopsis geminata*
	Pheidole	*Pheidole spathifera*
	Tetramorium	*Tetramorium walshi*
	Monomorium	*Monomorium indicum*
	Trichomyrmex	*Trichomyrmex destructor*
	Myrmicaria	*Myrmicaria brunnea*
	Meranoplus	*Meranoplus bicolor*
Formicinae	*Camponotus*	*Camponotus compressus* *Camponotus anguisticolis* *Camponotus sericeus*
	Oecophylla	*Oecophylla smaragdina*
	Paratrechina	*Paratrechina longicornis*
	Polyrhachis	*Polyrhachis dives*
Ponerinae	*Leptogenys*	*Leptogenys chinensis* *Leptogenys processonalis*
	Anochetus	*Anochetus graeffei*
Dolichoderinae	*Tapinoma*	*Tapinoma melanocephalum*
Pseudomyrmecinae	*Tetraponera*	*Tetraponera allaborans* *Tetraponera nigra* *Tetraponera rufonigra*

Ants act as ecosystem engineers and also respond quickly to change in soil chemical and physical properties by contributing in agriculture practices as increasing nutrient quantity, drainage and aeration (Folgarait, 1998). Studies on ant diversity in agriculture ecosystem help to identify species of ants with potential for biological control in different types of crops (Fernandes et al., 1994). During the present study the agricultural habitat was under investigation to study the correlation between nest soil parameters and 23 species of ants. Silva et al. (2017) studied the correlations between 34 species of ants with other arthropods. In the present study, temperature showed a significant negative correlation with ants species. This is in agreement with the result obtained by Watanasit et al. (2000) and Sudd and Franks (1987). It is observed that there is significant positive correlation of nitrogen, organic matter in agriculture ecosystem, this is due to ants burrowing activity, construction of ant nest this is in agreement with Wang (2017). Soil organic matter, total nitrogen, available Phosphorous, available potassium and total potassium increased in ant mound soil due

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to excavation activities by ants, accumulation of organic matter and other nutrient at the time of building of mound construction. Chemical properties C, P, Na, K, pH, Ca, Organic matter were quantified in the nest of ant species *Myrmica ruginodis* and surrounding soil in the forest. All properties except Phosphorous were higher in the nest of ant species *Myrmica ruginodis* than in surrounding soil investigated by Vele et al. (2010). Cammeraat and Risch (2008) studied the effect of soil dwelling ants on properties of soil aggregate stability which were improved by ant activity. Nest of ants showed higher values for pH phosphorus, potassium, and nitrogen content, which help in soil turnover process in young spruce forest stands of steppe rangelands of Iran noticed by Ghobadi et al. (2016).

Conclusion

The present study concluded the correlation between diversity of ants in agriculture field periurban site from Aurangabad city, with soil properties that included the parameters as nitrogen ($r = 0.507$) at $p = 0.05$ level, potassium, phosphorus, organic matter ($r = 0.649$) at $p = 0.05$ level, water holding capacity and climatic factors such as humidity ($r = 0.825$) at $p = 0.01$ level, temperature, rainy season ($r = 0.793$) at $p = 0.01$ level. Except potassium, phosphorus, water holding capacity and temperature, all other parameters showed significant positive correlation with ants, this was due to bioturbation, nesting habitat and foraging activity of ants in the agriculture field. Such activities caused by ants will help to improve the properties of soil of the area.

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SPATIOTEMPORAL CHANGES OF EXTREME TEMPERATURES IN THE PAST 49 YEARS AND THEIR RELATIONSHIP WITH THE ENSO IN SOUTHWESTERN CHINA

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Abstract. In the present research, spatiotemporal changes of extreme temperatures in Southwestern China as well as their relationship with the El Niño–Southern Oscillation (ENSO) events were determined for the 1969 to 2017 period using the Mann-Kendall test, the Sen's slope estimator, and the empirical orthogonal function (EOF) analysis. For this purpose, daily temperature data of 93 weather stations and 17 extreme temperature indices (ETIs) were analyzed. Our results suggested that: (1) during the past 49 years, ETIs showed a warming trend in Southwestern China, and differences in warming trends were observed in different geographical regions. (2) In the 1990s and early 21st century (2001-2011), abrupt extreme temperature changes were observed. (3) Results of the EOF analysis also indicated abrupt changes in extreme temperature indices. The regions susceptible to changes in the number of ice days (ID) were the Hengduan Mountains and part of the Yunnan-Guizhou Plateau. The high-value midpoints of summer days (SU) were found in the Zoige Plateau, Sichuan Basin, and part of the Yunnan-Guizhou plateau. (4) The ETIs were closely related to the ENSO events. The extreme cold indices were more significantly influenced by La Niña, and the extreme warm indices were more susceptible to El Niño events.
Keywords: *extreme temperature indices, mutation test, Sen's slope estimator, EOF analysis, multiple variation analysis, SOI, ONI*

Introduction

Since the First World Climate Conference (FWCC) held in Geneva in 1979, global warming has been an important topic worldwide. In 2014, the Fifth Assessment Report of the Intergovernmental Panel on Climate Change (IPCC) stated that in the past 130 years, the global temperature increased by 0.65~1.06 ℃. In addition, as the warming trend continues, extreme temperatures occur more frequently and for longer periods of time. Different studies have shown that climate warming has changed the spatiotemporal distributions of water resources, leading to more severe and frequent occurrences of related natural disasters, such as droughts and floods. Both, experimental observations and modeling results, have indicated that the ENSO events are also influenced by global warming, with a significant increase in frequency and intensity. Abnormal climate phenomena such as floods in the south and droughts in the north and warmer winters and cooler summers occur as a result of ENSO (IPCC, 2013; Gao et al., 2017; Qiu, 2013). Extreme climate events have given rise to disastrous environmental and socio-economic consequences in many regions, drawing the attention of the public, government, and academics (Gao and Xie, 2016; An et al., 2019).

In the early 21st century, global warming has caused a dramatic increase in both, the frequency and intensity of extreme weather and climate events. Extreme temperature events

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are considered the main signs of global warming, and their effects have been observed all over the world. Alexander et al. (2006), Ding et al. (2018) and Zhao et al. (2016, 2017) indicated that global warming has significantly decreased the number of cold nights and increased the number of warm nights in most parts of the world. In the past decades, the frequency of global heat and cold wave events has increased by 2.7 and 6.4 times, respectively (Karl et al., 1991; Cui et al., 2018). According to Diffenbaugh et al. (2017) during historical records, over 80% of the observed regions worldwide displayed a significant increase in the annual number of hottest months and the annual number of hottest days. In addition, model results have also indicated that, for a given area, historical climate forcing increased the probability of observing the driest year and wettest 5-d period at 57% and 41%, respectively. Grotjahn et al. (2016) applied the peaks-over-threshold (POT) method to indirectly determine the generalized extreme value (GEV) in North America. The results showed that the broadest region of warming occurred in the cold tail of the minimum temperature distribution. In some regions of North America, an increasing warming trend was observed (90% confidence interval). Similar results have been reported for China (Zhao et al., 2016, 2017), where intensities were comparable. However, opposite trends for cold night index and warm night index were observed in most parts of China, which indicated a good symmetry. The diurnal temperature change displayed considerable asymmetry, with small diurnal temperature range (DTR). This phenomenon affects the variation of extreme temperatures and accelerates water circulation. In consequence, water resources distribution is also affected (Yang et al., 2010; Chen and Min, 2017; Li et al., 2019). In this context, Pi et al. (2020) identified an abrupt climate change in the arid regions of Northwestern China, with increasing temperatures. The number of summer days (SU25), warm days (TX90p), warm nights (TN90p), and warm spell duration index (WSDI) significantly increased. The increasing rate was 2.27, 1.49, 3, and 2.28 d/10a, respectively. On the other hand, the number of frost days (FD), cold days (TX10p), cold nights (TN10p), and cold spell duration index (CSDI) significantly decreased at a rate of -3.71, -0.86, -1.77, and -0.76 d/10a, respectively. Tong et al. (2020) determined that the maximum and minimum temperatures increased either within the whole Beijing-Tianjin-Hebei Region (BTHR) or locally. The frequency of extreme heat also increased, while the frequency of cold weather decreased.

The warming trend of atmosphere and ocean significantly influences the air-sea interaction (L'Heureux et al., 2017). In this context, ENSO events are considered abnormal phenomena and strong signals of the interaction between the tropical oceans and atmosphere, and they exert a significant impact on temperature in many regions of the world. They also vary in intensity and stability, which results in notable regional features (Yang et al., 2013). For example, in 1982 and 1997 two extreme El Niño events occurred. They are known as the strongest ones of this type ever recorded in history. In each event, the ocean surface temperature in the eastern equatorial Pacific exceeded 28 ℃. These two El Niño events caused an economic loss between 20 and 35-45 billion US dollars each worldwide, and claimed the lives of over 20 thousand people (Shi, 2017; Glantz et al., 1998; Sponberg, 1990). Therefore, the relationship between the ENSO events and the increased intensity and frequency of extreme temperature events has also become one of the major research interests in this area of knowledge (An et al., 2019; Vincent and Mekis, 2006; Choi et al., 2009). In this context, Wang et al. (2017) found out that, from 1961 to 2012, the ENSO events affected winter temperatures in Northern China by modulating the atmospheric circulation over the North Atlantic area. This feature changed the convective transport in zonal temperatures in the mid and high latitudes of Eurasia and the intensity of

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Siberian High. The El Niño events caused a decrease in the daily mean temperature of Northeastern China and an increase in the frequency of extreme low temperature events. Lim and Schubert (2011) showed that, as compared with El Niño, La Niña phenomenon played a more critical role in causing more frequent extreme climate events. ENSO offered a reasonable explanation for the upper temperature limit, but not for the lower temperature limit.

Southwestern China has a subtropical monsoon climate, where the annual mean temperature is 14~24 ℃ (Zhao, 1997). In the past 53 years (1960-2012), major natural disasters in Southwestern China have included frequent droughts (Wang et al., 2014a). Data indicated that Southwestern China was hit by extreme droughts in the periods 2006-2007 and 2009-2010. In addition, Sichuan and part of Chongqing were affected by extreme droughts from 2006 to 2007. The cropping area slightly affected by droughts in Chongqing was 1.327 million ha, which represented a direct economic loss of 9.07 billion RMB. In addition, the cropping area moderately affected by droughts in Sichuan was 1.166 million ha. The direct economic loss produced by summer droughts was 12.57 billion RMB (China Meteorological Disaster Yearbook, 2007). A rare extreme drought hit Southwestern China from 2009 to 2010 affecting a population of 51 million, and a cropping area of 6.25 million ha (Qiu, 2013). It has been also reported that, in 2006, the southern oscillation index (SOI) reached a value of -0.28. In addition, in the year of 2007, the oceanic Nino index (ONI) was smaller than -0.5 for six months consecutively. Thus, it was concluded that El Niño phenomenon occurred during the period 2005-2006. In 2009, the ONI was above 0.5 for six months consecutively, while 2010, it increased up to 0.95. This indicated a strong La Niña event. Most studies have concluded that ENSO events are the important external forcing factors causing the two above-mentioned droughts. Later, from October 2012 to the first ten days of March 2013, Yunnan, Sichuan, Chongqing and western Guizhou were also hit by severe droughts, followed by a continuous drought that lasted from autumn to winter and then to spring of the next year. The severe local droughts that occurred from 2015 to 2019 in several stages, caused important economic losses in the provinces of Southwestern China, posing a threat to people's properties and lives (Wu et al., 2014). In particular, the event that occurred in the Sichuan Basin in the summer of 2017 with prolonged high-temperatures and drought, with the temperature as high as 44 ℃, has been known as the worst since the local meteorological facility initiated operations in 1951. Similarly, the big drought event that occurred in 2017 in Chongqing, caused the longest known summer drought event registered in the meteorological record.

Regarding ETIs, several studies have been conducted in Southwestern China. For example, Yuan et al. (2015) showed that, from 1962 to 2012, FD, TN10p and TX10p significantly decreased at a rate of 2.7, 4.6, and 3.5 d/10a, respectively. It was also determined that TX90p, TN90p, annual minimum value of daily minimum temperature (TNn), and annual minimum value of daily maximum temperature (Txx) increased, at a rate of 3.6, 4.9, 0.4, and 0.1 ℃/10a, respectively. The numbers of extreme high- and low-temperature events in Southwestern China increased and decreased, respectively. Yang et al. (2010) and He et al. (2011) found out that DTR decreased in Southwestern China. In addition, the amplitude increase of extreme low temperatures was considerably larger than that of extreme high temperatures. Furthermore, there was a significant difference in the spatial distribution of extreme temperature events, which highlighted the complexity and distinctiveness of temperature changes in Southwestern China (Yuan et al., 2015; Zhu et al., 2018; Liu and Xu, 2014). Based on existing studies, we selected 17 ETIs and analyzed daily temperatures in the long-term series. Several methods were applied to investigate

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spatiotemporal changes and potential trends of extreme temperatures during the past 49 years in Southwestern China. In addition, we identified mutation time of extreme temperature changes, as well as the period of the event and potential influencing factors. We also analyzed the relationship between ETIs and ENSO events in Southwestern China. Our research findings will provide new insights on the development of disaster prevention and relief strategies in events caused by extreme temperatures in Southwestern China.

Study area and data sources

Study area

Southwestern China (21°08' ~ 34°19' N, 97°21' ~ 110°11' E) borders with South Central China to the east, Vietnam, Laos and Burma to the south, Tibet Plateau to the west, and Northwestern China to the north. It displays a downwards slopping terrain from the northwest to the southeast. Southwestern China extends over the first and second steps of China's ladder topography, resulting in a diversity of climate features. According to China's meteorological and geographical division, the Southwestern Chinese region studied in this paper consists of four provinces and cities: (a) Chongqing city; (b) Sichuan Province; (c) Guizhou Province; and (d) Yunnan Province. In the present research, Southwestern China was divided into the four landform units based on climate and hydrologic conditions, geographical location, as well as altitude from north to south: (a) Zoige Plateau; (b) Hengduan Mountains; (c) Sichuan Basin; and (d) Yunnan-Guizhou Plateau. The Zoige Plateau covers part of Northern Sichuan; Hengduan Mountains cover Western and Southern Sichuan and Northern Yunnan; the Sichuan Basin covers most of Chongqing City and eastern and central Sichuan; and the Yunnan-Guizhou Plateau covers most of Guizhou as well as part of Southern Yunnan.

Due to the special geographical location and complex topographic structure in Southwestern China, the development of urban and social economy in this region is restricted. 1978 was the first year of the implementation of China's reform and opening up policy. Sichuan (Chongqing was originally under the jurisdiction of Sichuan Province and became a municipality in 1997), Guizhou and Yunnan Province had a total urban population of 14.84 million people, a GDP (Gross Domestic Product) of 30.03 billion yuan, and an urbanization rate of 11.8%. In the latest 20 years, due to the implementation of the western development strategy and the new urbanization road construction, by the end of 2017, the total urban population of the four provinces and cities in Southwestern China was 100.76 million people, the GDP was 8,632.21 billion yuan, and the urbanization rate was 51.89%. Compared with 1978, the urbanization rate increased by 40.09%. In recent years, the urbanization growth rate of the four provinces and cities has exceeded the national urbanization level. By the end of 2017, the total construction land and agricultural land of the four provinces and cities in Southwestern China were 4,388 thousand ha and 96,843.8 thousand ha, respectively, accounting for 3% and 85% of the total land area of the four provinces and cities (China Statistical Yearbook, 2018).

Data sources

Data quality control was performed for the input of daily maximum and minimum temperatures as a previous step for ETIs (Zhang and Yang, 2004). Daily temperature data were obtained from the Meteorological Data Service Center of China (MDSCC) (http://data.cma.cn) for the period 1969 to 2017. Ninety-three weather stations located

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in Southwestern China were chosen for the study. Data were processed considering the principles of continuity, homogenisation and longest possible duration. Three quality control methods were used for the treatment of the data: (a) internal consistency check; (b) climate limit check; and (c) station extremum check. Interpolation was performed to fill the data gaps in adjacent stations by using the linear regression method, which ensured that the corrected meteorological data involved high accuracy, homogenisation and continuity (Gao et al., 2017; Zhu et al., 2018). *Figure 1* displays the geographical locations of the 93 studied weather stations in Southwestern China and the digital elevation model (DEM) of the four geographical divisions. *Table 1* shows the distributions of the 93 weather stations in the four geographical divisions. According to *Table 1,* four stations are located in the Zoige Plateau, 22 in the Hengduan Mountains, 23 in Sichuan Basin, and 44 in the Yunnan-Guizhou Plateau. DEM data was obtained from the SRTM data (http://www.cgiar-csi.org/) published by the National Imagery and Mapping Agency. In order to determine the relationship between ETIs and ENSO events, we selected the SOI data from the Climatic Research Unit of the University of East Anglia (http://www.cru.uea.ac.uk/cru/data/soi/) (Wang et al., 2014a). In addition, the ONI data were obtained from the oceanic El Niño index data provided by the NOAA Center for Weather and Climate Prediction and updated once every five years (http://www.cpc.ncep.noaa.gov) (Jiang et al., 2017).

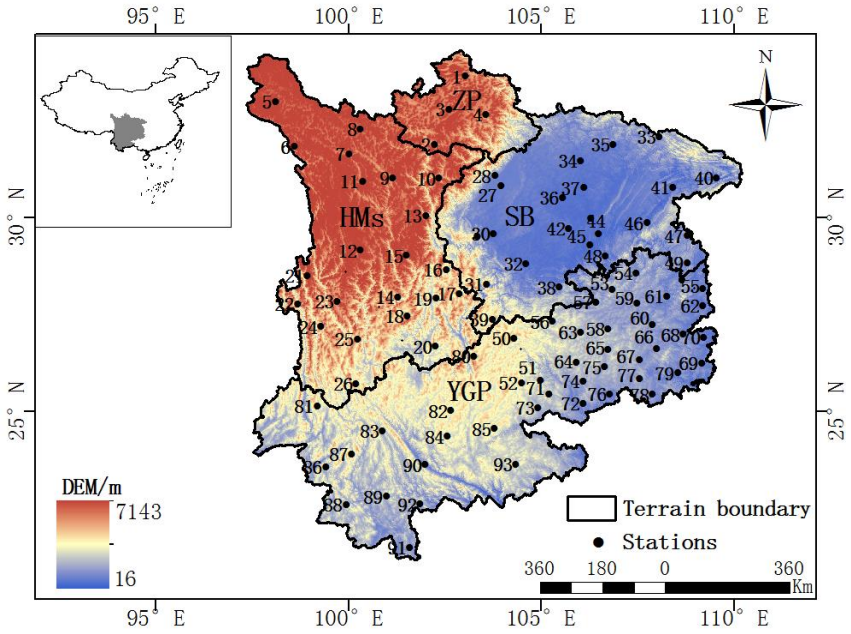

Figure 1. *Topographical division and spatial distribution of weather stations in Southwestern China*

Extreme temperature indices and main research methods

Extreme temperature indices

Numerous studies, both on the global and local scales, have used averages of long- and medium-term low-resolution data to analyze climate change (Croitoru et al., 2013). However, data on changes in ETIs may be more appropriate to use than averages when monitoring, testing, and analyzing the influence of climate change (Peterson et al.,

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2008). Herein, 17 ETIs were selected to analyze the features of extreme temperature events in four provinces and cities of Southwestern China (*Table 2*). This was performed in accordance with the Expert Team on Climate Change Detection and Indices (ETCCDI) recommended by the Commission for Climatology (CCI) of World Meteorological Organization (WMO) and Climate Variability and Predictability Program (CLIVAR) (Liu and Xu, 2014). The ETIs were calculated by using the RClimDex (1.0) software developed by Xuebin Zhang and Feng Yang (http://etccdi.pacificclimate.org) (Zhang and Yang, 2004).

Table 1. *Distribution in four terrain zones of 93 weather stations studied in the present research*

Topographical division	Numbers and names of weather stations
Zoige Plateau (ZP)	1 Zoige, 2 MaErKang, 3 HongYuan, 4 SongPan
Hengduan Mountains (HMs)	5 ShiQu, 6 DeGe, 7 GanZi, 8 SeDa, 9 DaoFu, 10 XiaoJin, 11 XinLong, 12 DaoCheng, 13 KangDing, 14 MuLi, 15 JiuLong, 16 YueXi, 17 ZhaoJue, 18 YanYuan, 19 XiChang, 20 HuiLi, 21 DeQin, 22 GongShan, 23 Shangri-La, 24 WeiXi, 25 LiJiang, 26 DaLi
Sichuan Basin (SB)	27 WenJiang, 28 DuJiangYan, 29 EMeiShan, 30 LeShan, 31 LeiBo, 32 YiBin, 33 WanYuan, 34 LangZhong, 35 BaZhong, 36 SuiNing, 37 GaoPing, 38 XuYong, 39 ZhaoTong, 40 FengJie, 41 WanZhou, 42 DaZu, 43 HeChuan, 44 ShaPingBa, 45 JiangJing, 46 FengDu, 47 QianJiang, 48 QiJiang, 49 YouYang
Yunnan-Guizhou Plateau (YGP)	50 WeiNing, 51 PuAn, 52 PanXian, 53 TongZi, 54 ZhengAn, 55 SongTao, 56 BiJie, 57 HuaiRen, 58 XiFeng, 59 MeiTan, 60 YuQing, 61 SiNan, 62 TongRen, 63 QianXi, 64 AnShun, 65 GuiYang, 66 KaiLi, 67 DuYun, 68 SanSui, 69 LiPing, 70 TianZhu, 71 XingRen, 72 WangMo, 73 XingYi, 74 ZiYun, 75 HuiShui, 76 LuoDian, 77 DuShan, 78 LiBo, 79 RongJiang, 80 HuiZe, 81 BaoShan, 82 KunMing, 83 JingDong, 84 YuXi, 85 LuXi, 86 GengMa, 87 LinCang, 88 LanCang, 89 SiMang, 90 YuanJiang, 91 MengLa, 92 JiangCheng, 93 YanShan

Table 2. *List of the 17 selected extreme temperature indices*

Categories	No.	Descriptive names/units	Indices	Definitions
Extreme cold indices	1	Cold spell duration indicator/d	CSDI	Annual count of days with at least 6 consecutive days when TN < 10th percentile
	2	Frost days/d	FD	Annual count when Tn (daily minimum) < 0 ℃
	3	Ice days/d	ID	Annual count when Tx (daily maximum) < 0 ℃
	4	Cool nights/d	TN10p	Number of days when Tn < 10th percentile
	5	Cool days/d	TX10p	Number of days when Tx (daily maximum) < 10th percentile
	6	Average daily minimum temperature/℃	TMINmean	Average Tn
	7	Min Tmin/℃	TNn	Annual minimum value of daily minimum temperature
	8	Min Tmax/℃	TXn	Annual minimum value of daily maximum temperature
Extreme warm indices	9	Warm spell duration indicator/d	WSDI	Annual count of days with at least 6 consecutive days when TX > 90th percentile
	10	Tropical nights/d	TR	Annual count when Tn (daily minimum) > 20 ℃
	11	Summer days/d	SU	Annual count when Tx > 25 ℃
	12	Warm nights/d	TN90p	Number of days when Tn > 90th percentile
	13	Warm days/d	TX90p	Number of days when Tx > 90th percentile
	14	Average daily maximum temperature/℃	TMAXmean	Average Tx
	15	Max Tmin/℃	TNx	Annual maximum value of daily minimum temperature
	16	Max Tmax/℃	TXx	Annual maximum value of daily maximum temperature
	17	Diurnal temperature range/℃	DTR	Monthly mean difference between TX and TN

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Main research methods

First, in order to obtain the temporal variation trend of ETIs in the past 49 years, a linear regression analysis was performed using the 17 ETIs. For this purpose, the SPSS (Statistical Program for Social Sciences) was used. Also, a FORTRAN code was written to obtain the univariate linear regression. The linear trend estimation of ETIs at 93 weather stations in Southwestern China was performed by loop computation. The spatial map showing the extreme temperature index spatial trend for the past 49 years in Southwestern China was plotted using ArcGIS. Then, in order to understand the stages of ETIs change, the ETIs trend and year of climate alteration were calculated using the M-K test and the Sen's slope estimator on MATLAB. Later, ETIs were subjected to EOF analysis to determine the spatial ETIs changes. Finally, the relationship between ETIs and ENSO was analyzed using the SOI and ONI. A description of the methods employed in the present study is presented below:

(1) Mann-Kendall test (M-K test): the M-K test is a nonparametric statistical tool that is used to analyze the variation trend of climate and hydrological series over time. This method is also used to identify trends of precipitation and drought frequencies caused by the influence of climate change (Cai, 2016). Samples used for the M-K test may or may not fit a normal distribution. In addition, the samples are not influenced by few outliers. Therefore, the M-K test outperforms other mutation tests for detecting abrupt climate changes (Ma et al., 2018).

For a given time series x, containing n samples, a rank series Sk is constructed as:

$$S_k = \sum_{i=1}^{k} r_i = \begin{cases} 1, x_i > x_j \\ 0, x_i \le x_j \end{cases}, \quad j = 1, 2, \ldots, i \qquad \text{(Eq.1)}$$

where k represents the sample size; r_i is used to determine whether the value at the time i is larger than the value at the time j. According to this, the rank series S_k is the cumulative number of the values, which are larger at the time j than those at the time i.

The statistics is defined as:

$$UF_k = \frac{S_k - E(S_k)}{\sqrt{Var(S_k)}}, k = 1, 2, \ldots, n \qquad \text{(Eq.2)}$$

where:

the mean: $$E(S_k) = \frac{n(n+1)}{4} \qquad \text{(Eq.3)}$$

the standard deviation: $$Var(S_k) = \frac{n(n-1)(2n+5)}{72} \qquad \text{(Eq.4)}$$

$UF_1 = 0$, $E(S_k)$ and $Var(S_k)$ are the mean and standard deviation of the rank series S_k, respectively; UF_k is the standard normal distribution. The described process is repeated according to the reverse order of the time series x, and $UB_k = -UF_k$. Subsequently, the plotted curves are analyzed. If $UF > 0$, the variable shows an increasing trend. On the other hand, if $UF < 0$, the variable displays a decreasing

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trend. In addition, an intersection between the statistic of UF and UB, which lies between the critical lines, corresponds to the start time of climate mutation (i.e., the year of climate mutation).

(2) Sen's slope estimator: Theil–Sen estimator, also known as Sen's slope estimator or Kendall robust line-fit method, is used to robustly fit the line to the sampling points distributed on a plane. This method uses the median of all lines' slopes, which are connected to the paired points. This estimator has proven to be an effective method. For skewed and heteroscedastic data, the Sen's slope estimator achieves a more clear and accurate result as compared to the non-robust simple linear regression. In terms of statistical power, the Sen's slope estimator outperforms the non-robust least square method even for the data obeying a normal distribution. Therefore, the Sen's slope estimator is known as the most popular nonparametric technique for estimating a linear trend. The Sen's slope estimator is represented by

$$\beta = Median\left(\frac{X_j - X_i}{j - i}\right) \quad j > i = 1, 2, \ldots, n \qquad \text{(Eq.5)}$$

where X_j and X_i are elements of the j and i moments, respectively, in the series to be analyzed. The moment i precedes moment j; $\frac{X_j - X_i}{j-i}$ is the slope between the moments j and i; Median is the median function. When $\beta > 0$, the time series displays an upward trend; when $\beta < 0$, the time series displays a downward trend (Croitoru et al., 2013).

(3) Multiple variation analysis and the determination of years of temperature anomalies: ETIs multiple variation analysis between ETIs and ENSO events provided evidence of the influence of global temperature changes on extreme temperature events in Southwestern China. The composite rate of ETIs was defined as the ratio of mean ETIs value in the years when ENSO occurred to long-term ETIs mean values. Two indices, SOI and ONI, were chosen for comparison and mutual corroboration to determine whether ENSO events occurred. SOI corresponds to the standard deviation of the atmospheric pressure observed in Darwin Island and Tahiti Island. Generally speaking, negative SOI indicates an El Niño event, and positive SOI a La Niña event. ONI is an indicator of the occurrence of an El Niño event based on sea surface temperature anomalies in 1, 2, 3, and 4 regions. Thus, ONI > + 0.5 °C corresponds to an El Niño event. On the other hand, ONI < -0.5 °C indicates a La Niña event.

(4) EOF analysis: EOF, also known as eigenvector analysis (EA) or principal component analysis (PCA), is used to determine structural features of the matrix data by extracting the principal eigenvalues. No established function is used in EOF. In addition, there is no need for a specific function as the basis function in order to carry out the decomposition. EOF analysis decomposes the signals from irregularly distributed stations within a finite region. This method performs a rapid convergence, easily decomposing the information of the variable field into several modes. The spatial structure analyzed by the EOF analysis provides physical information. Because of these benefits, the EOF analysis has become a major tool for analyzing the variable field features in climate change studies. The calculations are performed as follows:

① The anomaly is processed in order to normalize the original data matrix X. Later, the covariance matrix is calculated as S = XX', where S corresponds to the m×m real symmetric matrix, and m is the number of space points in the variable field.

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② The eigenvalue ∧ and eigenvector V of the matrix S are obtained by the eigenvalue and eigenvector method of real symmetric matrix.

③ The matrix ∧ is the diagonal matrix, and the diagonal element is the eigenvalue of XX', $\lambda = (\lambda_1, \lambda_2, \ldots, \lambda_m)$. The eigenvalues are ranked in descending order: $\lambda_1 \geq \lambda_2 \geq \ldots \geq \lambda_m \geq 0$.

④ The time coefficient matrix T is obtained using T = V'X.

⑤ The variance contribution R_k of each eigenvector and the cumulative variance contribution G of the top p eigenvectors are calculated according to:

$$R_k = \lambda_k \Big/ \sum_{i=1}^{m} \lambda_i \quad k = 1, 2, \ldots, p(p < m) \tag{Eq.6}$$

$$G = \sum_{i=1}^{P} \lambda_i \Big/ \sum_{i=1}^{m} \lambda_i \quad p < m \tag{Eq.7}$$

Results

ETIs variability

Interannual and interdecadal variability

Figure 2 displays the interannual and interdecadal changes of ETIs in Southwestern China from 1969 to 2017. According to the regression analysis performed with data of 93 weather stations, the linear trend rates of the 8 extreme cold indices among the 17 extreme temperature indices indicated an upward temperature trend and a downward trend for the number of cold days. Thus, a warming trend was consistently identified. The interannual change trend rates were -1.1 d/10a (CSDI), -2.34 d/10a (FD), -0.41 d/10a (ID), -3.52 d/10a (TN10p), -1.34 d/10a (TX10p), 0.28 ℃/10a (TMINmean), 0.43 ℃/10a (TNn), and 0.31 ℃/10a (TXn), where $P \leq 0.01$. In addition, the coefficient of determination (R2) for the regression line between TN10p and TMINmean was 0.7, which indicated a good fit. The 8 extreme warm indices and 8 extreme cold indices were validated with each other, all of them indicating a warming trend. The interannual change trend rates were 1.64 d/10a (WSDI), 2.44 d/10a (TR), 0.81 d/10a (SU), 1.87 d/10a (TN90p), 3.09 d/10a (TX90p), 0.22 ℃/10a (TMAXmean), 0.20 ℃/10a (TNx), and 0.27 ℃/10a (TXx). All of them passed the significance test at the 95% confidence interval (CI). A large difference was observed in the amplitude variations between different types of ETIs. Specifically, the difference in amplitude between TN10p and TX90p was higher than that observed between other ETIs. In addition, DTR decreased, indicating a gradual reduction of minimum and maximum temperatures differences. However, the fitting results failed to pass the statistical test, indicating a poor fit. Also, amplitude variations of extreme cold and warm indices were similar, indicating symmetry of the temporal changes of the two types of indices.

Data also indicated that the degree to which warming occurred varied between regions. *Table 3* displays specific change rates for different indices. Data indicated that the mean change rates of the top five extreme cold indices (CSDI, FD, ID, TN10p, and TX10p) in the Zoige Plateau, the Hengduan Mountains, Sichuan Basin, and Yunnan-Guizhou Plateau were -2.68, -2.49, -1.37, and -1.52 d/10a, respectively. The mean change rates of the top five extreme warm indices (WSDI, TR, SU, TN90p, and TX90p)

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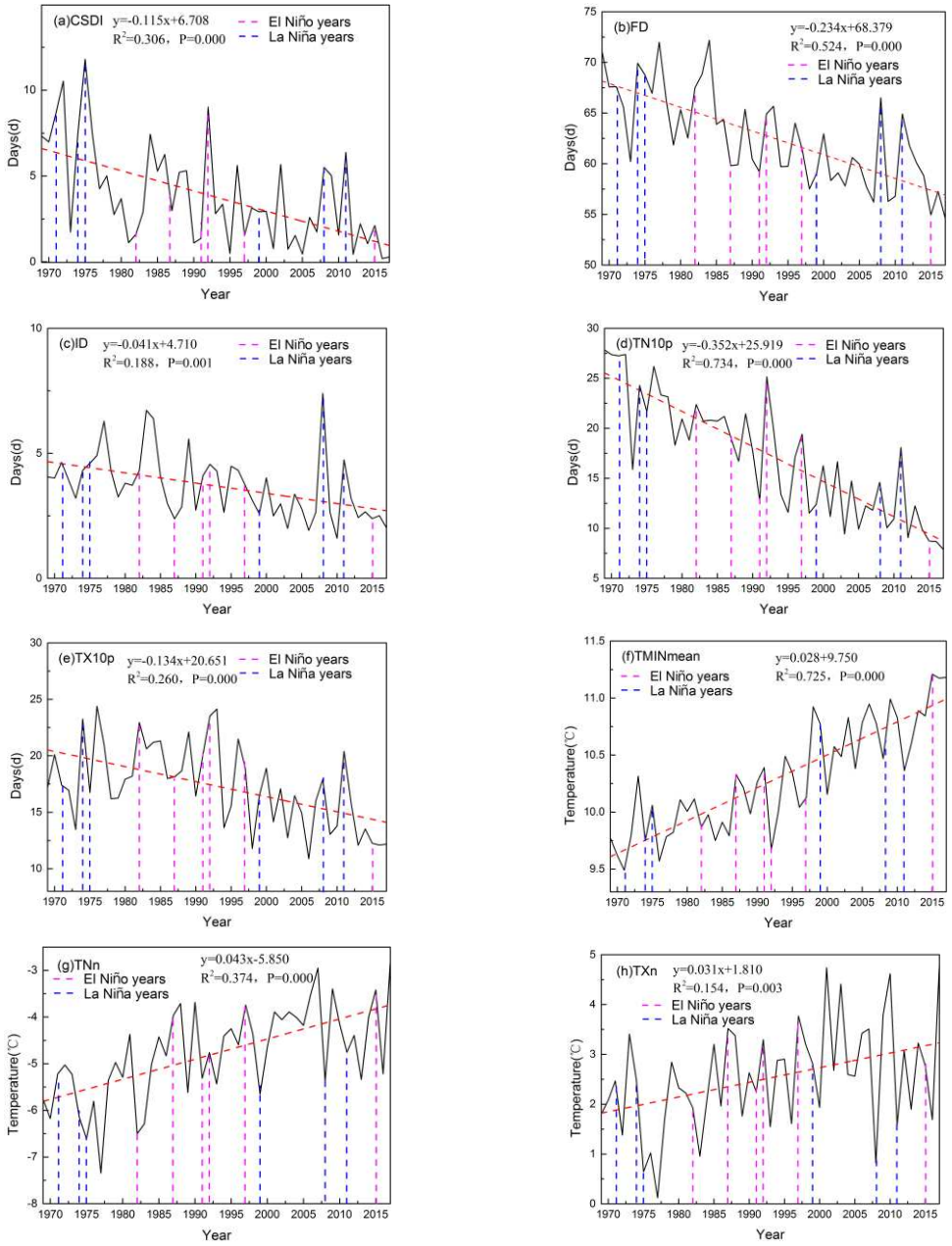
in the Zoige Plateau, the Hengduan Mountains, Sichuan Basin, and Yunnan-Guizhou Plateau were 1.66, 2.03, 2.58, and 1.57 d/10a respectively. It was also determined that the mean change rates of the last three extreme cold indices (TMINmean, TNn, and TNx) were 0.43, 0.35, 0.30, and 0.34 ℃/10a, which corresponded to the Zoige Plateau, the Hengduan Mountains, Sichuan Basin, and Yunnan-Guizhou Plateau, respectively. Furthermore, the mean change rates of the last three extreme warm indices (TMAXmean, TNx, and TXx), were 0.32, 0.27, 0.28, and 0.18 ℃/10a in the Zoige Plateau, the Hengduan Mountains, Sichuan Basin, and Yunnan-Guizhou Plateau, respectively. Thus, according to the results, the Zoige Plateau was the region were extreme cold indices decreased the most and temperature showed the highest increase. In addition, the indices of extreme warm temperature mostly increased in the Sichuan Basin. As previously stated, the climate change pattern varied across the geographical regions, showing a warming trend.



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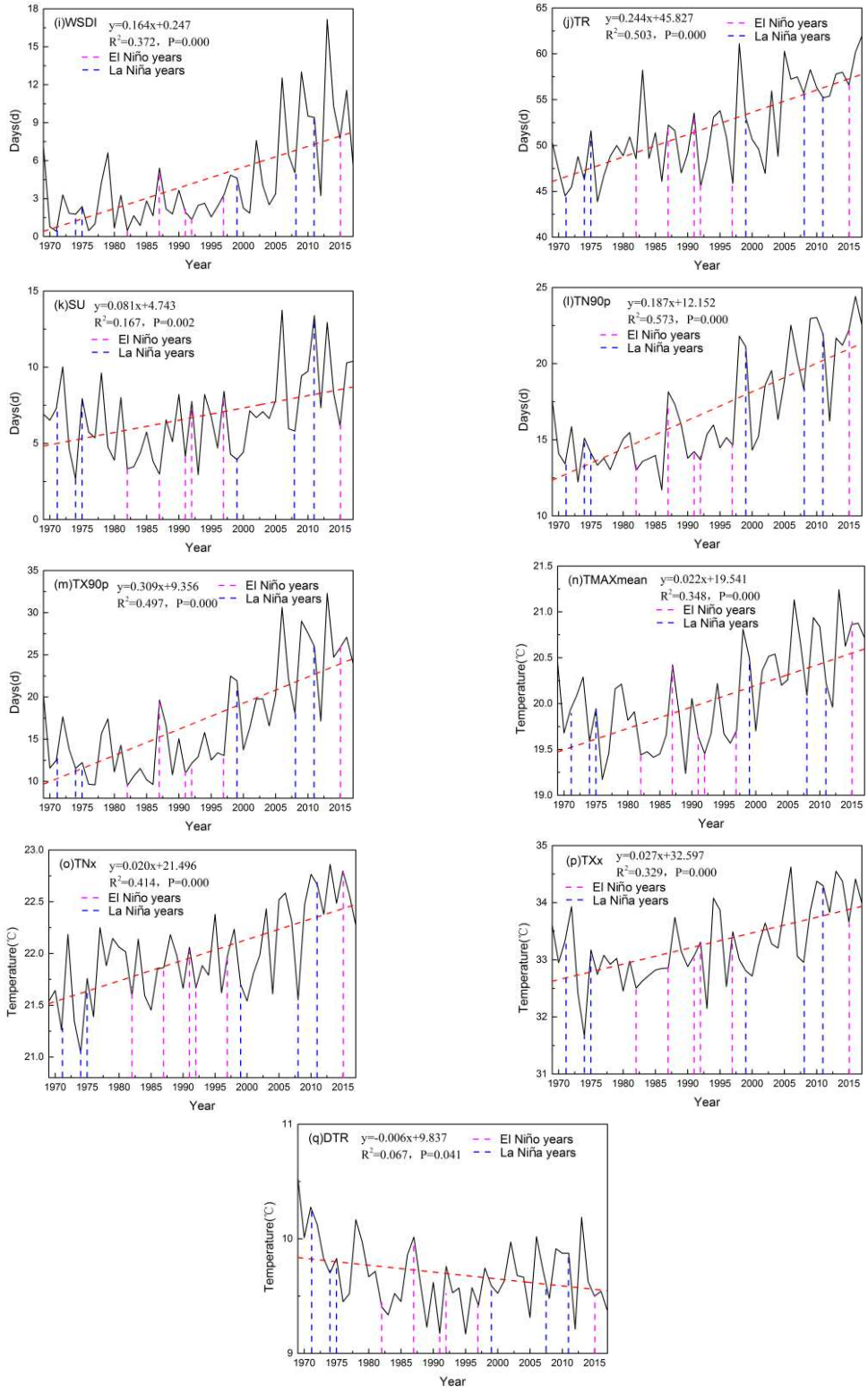

Figure 2. *Time trend of 17 ETIs (referring to Table 2) in Southwestern China from 1969 to 2017*

Results of the comparison between SOI and ONI indicated that the years of strong ENSO events were 1982, 1987, 1991, 1992, 1997, and 2015, while those of strong La Niña events were 1971, 1974, 1975, 1999, 2008, and 2011. Combining this information

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with that in *Figure 2,* it was observed that, in some years, ETIs displayed a correspondence to the ENSO events. Thus, in the El Niño years, the value of the extreme cold indices CSDI, FD, ID, TN10p, and TX10p decreased. However, a peak was observed in TMINmean, TNn, and TNx values, indicating an upward trend. The opposite was observed in the La Niña years. In addition, in the El Niño years, a peak occurred in the extreme warm indices, which indicated a warming trend. Thus, the warming trend in Southwestern China presented a certain connection with the ENSO events.

Table 3. *Change rates of 17 ETIs (referring to Table 2) in four different zones*

Division	CSDI (d/10a)	FD (d/10a)	ID (d/10a)	TN10p (d/10a)	TX10p (d/10a)	TMINmean (℃/10a)	TNn (℃/10a)	TXn (℃/10a)	
RP	-0.79	-4.89	-1.65	-4.15	-1.94	0.39	0.55	0.36	
HMs	-1.26	-4.29	-0.89	-4.18	-1.82	0.33	0.42	0.29	
SB	-0.82	-1.51	-0.27	-2.78	-1.47	0.23	0.33	0.35	
YGP	-1.18	-1.85	-0.13	-3.44	-0.98	0.27	0.46	0.28	
Division	**WSDI (d/10a)**	**TR (d/10a)**	**SU (d/10a)**	**TN90p (d/10a)**	**TX90p (d/10a)**	**TMAXmean (℃/10a)**	**TNx (℃/10a)**	**TXx (℃/10a)**	**DTR (d/10a)**
RP	1.75	0	0.02	3.59	2.95	0.3	0.29	0.38	-0.08
HMs	1.95	0.36	0.14	3.95	3.75	0.29	0.24	0.28	-0.05
SB	2.09	2.41	1.68	3.04	3.69	0.26	0.23	0.36	-0.05
YGP	1.29	3.26	0.75	-0.01	2.54	0.16	0.15	0.22	-0.1

Spatial variations characteristics

Figure 3 presents the spatial distribution of the regression coefficients of ETIs obtained at 93 weather stations in Southwestern China for the past 49 years. The spatial change rates and the results of the significance tests are shown in *Table 4*. Results indicated differences in the ETIs change trends as regions varied. Specifically, the top five extreme cold indices showed a downward trend from east to west. Change rates for CSDI, FD, ID, TN10p, and TX10p were -1.15, -2.47, -0.41, -3.52, and -1.34 d/10a, respectively. In the Hengduan Mountains, these five indices dramatically decreased to values of -1.26 d/10a (CSDI), -4.29 d/10a (FD), -0.89 d/10a (ID), -4.18 d/10a (TN10p), and -1.82 d/10a (TX10p). The increase in value and amplitude between these extreme cold indices in the Hengduan Mountains exceeded those observed in other regions. In other words, the warming trend was more severe in high-altitude areas. TMINmean (0.28 ℃/10a), TNn (0.42 ℃/10a), and TXn (0.31 ℃/10a) all showed an upward trend. Moreover, TNn displayed the highest increase in amplitude. Weather stations with a dramatic increase in TNn corresponded to the Hengduan Mountains and the eastern Yunnan-Guizhou Plateau. The highest TNn value, which was 1.30 ℃/10a, was observed at the Shangri-La Station in Yunnan. The maximum positive TXn change rate was observed in Fengjie, Chongqing, with a value of 1.30 ℃/10a. The maximum TMINmean increase was observed in Muli, Sichuan, with a value of 0.9 ℃/10a. Every extreme warm index showed an increasing trend (1.66 d/10a for WSDI, 2.22 d/10a for TR, 0.81 d/10a for SU, 1.83 d/10a for TN90p, 3.13 d/10a for TX90p, 0.22 ℃/10a for TMAXmean, 0.20 ℃/10a for TNx, and 0.28 ℃/10a for TXx). Also, it was determined that the lowest statistical significances were obtained for the upward trend of TNx, and

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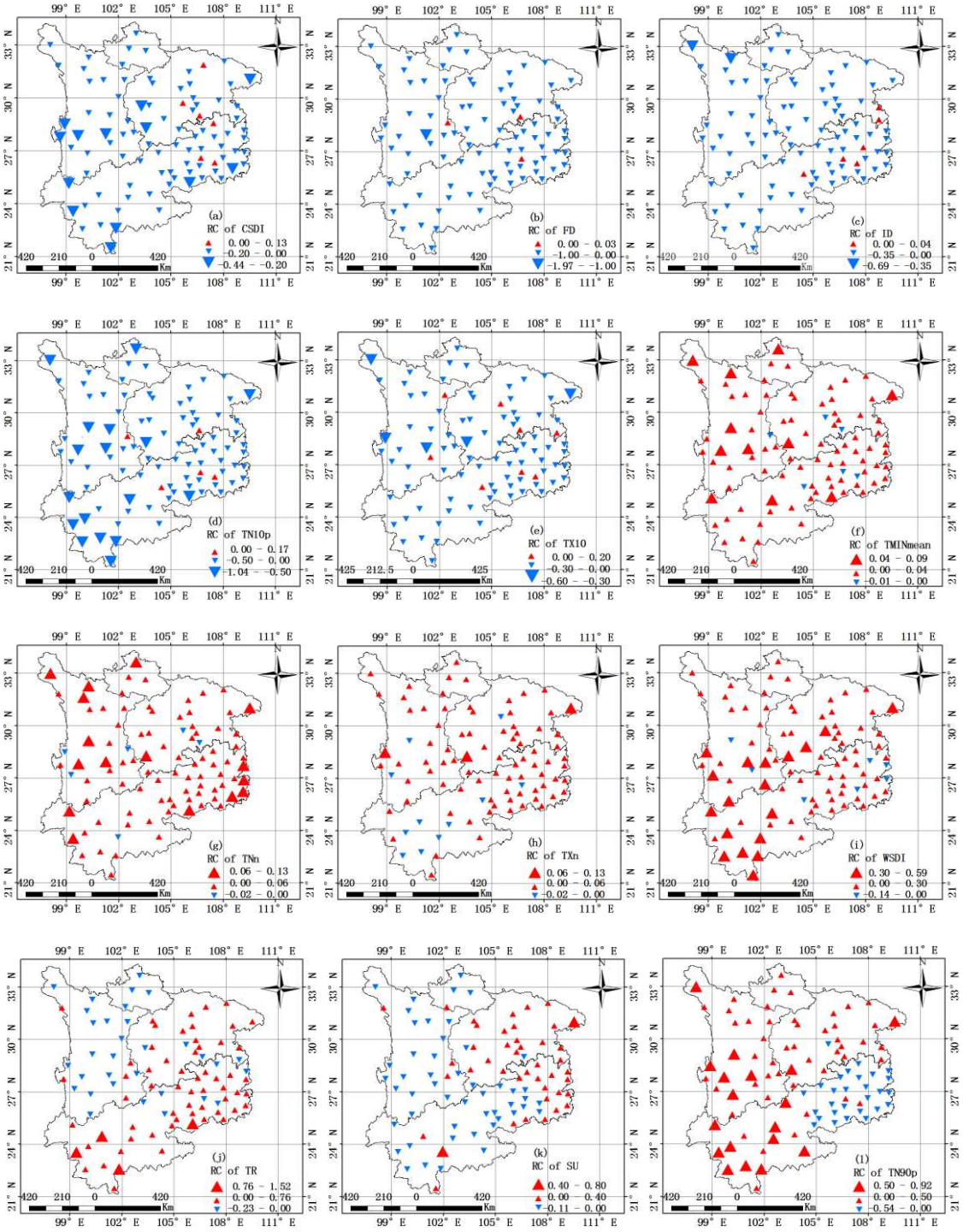
such a trend was only observed in the Sichuan Basin (0.23 ℃/10a) and northeastern Yunnan-Guizhou Plateau (0.15 ℃/10a). When all the warm extreme indices were considered, Sichuan Basin displayed the most severe warming trend. The temperature extreme occurred more frequently in Fengjie, Chongqing (5.93 d/10a for WSDI, 8.91 d/10a for TX90p, and 0.85 ℃/10a for TMAXmean, all passing the significance test at the 0.01 level), and the weather station in Fengjie displayed the most severe warming trend. In addition, DTR significantly decreased (-0.05 d/10a), indicating a decreasing difference between daytime and nighttime temperatures.



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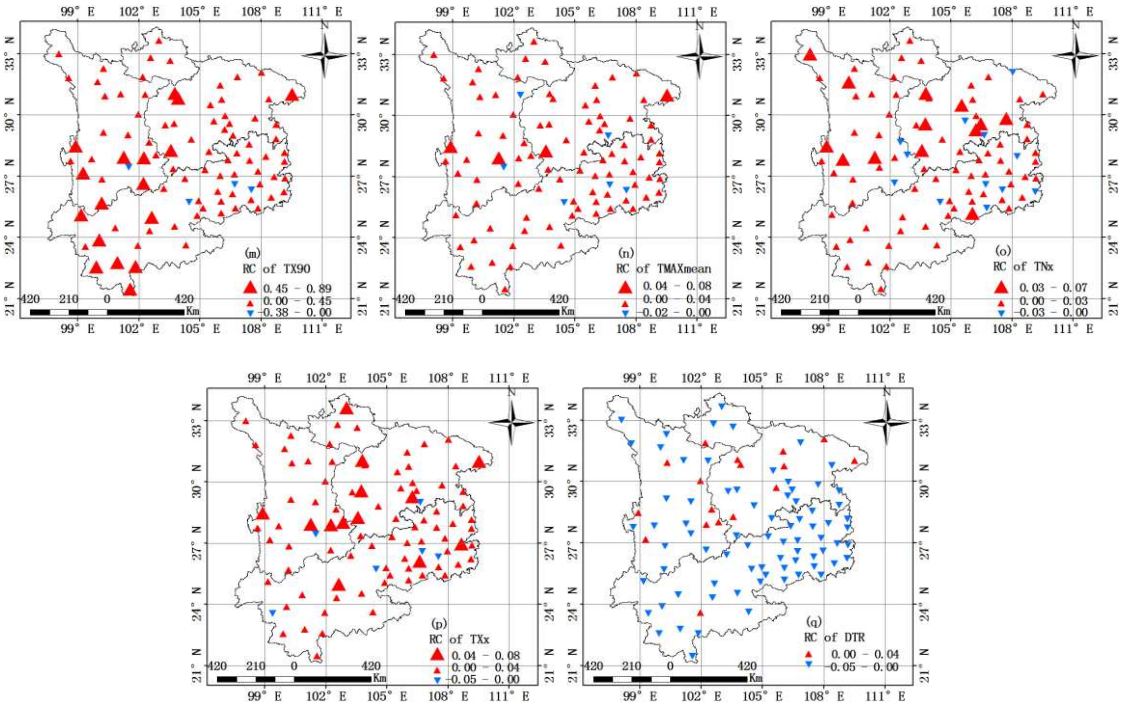

Figure 3. *Spatial change trends of 17 ETIs (referring to Table 2) in Southwestern China from 1969 to 2017*

Table 4. *The spatial change rates and the results of the significance test level in 17 ETIs (referring to Table 2)*

Indices	**CSDI**	**FD**	**ID**	**TN10p**	**TX10p**	**TMINmean**	**TNn**	**TXn**	
Change rates	-0.115	-0.247	-0.041	-0.352	-0.134	0.028	0.043	0.031	
P ≤ 0.01	30.11%	61.29%	50.54%	88.17%	45.16%	90.32%	53.76%	23.66%	
P ≤ 0.05	47.31%	75.27%	51.61%	91.40%	72.04%	91.40%	69.89%	48.39%	
P ≤ 0. 1	58.06%	83.87%	55.91%	92.47%	80.65%	92.47%	82.80%	55.91%	
Indices	**WSDI**	**TR**	**SU**	**TN90p**	**TX90p**	**TMAXmean**	**TNx**	**TXx**	**DTR**
Change rates	0.164	0.244	0.081	0.187	0.309	0.022	0.020	0.027	-0.006
P ≤ 0.01	50.54%	68.82%	58.06%	86.02%	80.65%	72.04%	59.14%	39.78%	39.78%
P ≤ 0.05	55.91%	75.27%	66.67%	90.32%	91.40%	84.95%	65.59%	61.29%	49.46%
P ≤ 0.1	65.59%	82.80%	70.97%	91.40%	92.47%	90.32%	70.97%	69.89%	56.99%

Mutation characteristics

Climate mutation manifests as abrupt changes in the statistical spatiotemporal variability of the climate and is usually indicated by the mutation points (Fu and Wang, 1992). The change trend and mutation points of ETIs were determined by using the M-K test and the Sen's slope estimator to ensure the scientificity and accuracy of the research. These tests were performed using MATLAB. *Table 5* shows the results of the M-K test and Sen's slope estimator of ETIs for the period 1969 to 2017. According to the data shown in *Table 5,* the mutation years of extreme cold indices were identified between the 1990s and early 21st century (2005-2011), while the mutation years of extreme warm indices occurred in the early 21st century (2001-2008). It was also determined that the

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mutation years of extreme warm indices fell behind those of the extreme cold indices. Combining this information with that in *Figure 2,* it was observed that the number of days of extreme cold indices suddenly decreased in the 1990s, which was accompanied by a significant increase in temperature. Data also indicated that, in the early 21st century, an abrupt change in the extreme warm indices, with a higher number of warm days and temperature increase occurred. The results from the Sen's slope estimator were in accordance with those obtained applying the regression coefficients of interannual and interdecadal ETIs variations. In this case, the change trend was exactly the same. The change trend of the Sen's slope on the UF curve was the same as the trend estimated by the regression coefficient of the interannual and interdecadal changes; however, due to an insignificant change in DTR, the results were the opposite.

Table 5. *M-K test and Sen's slope estimation analysis results of 17 ETIs (referring to Table 2)*

Categories	Indices	M-K test	Sen's slope estimation (time series)	Trend	Sen's slope estimation (UF)	Trend
Extreme cold indices	CSDI	1989, 1992	-0.102	Decline	-0.085	Decline
	FD	1991, 1992, 1993	-0.238	Decline	-0.123	Decline
	ID	1998	-0.043	Decline	-0.100	Decline
	TN10p	1991, 1993	-0.366	Decline	-0.127	Decline
	TX10p	2005, 2007, 2008, 2011	-0.131	Decline	-0.069	Decline
	TMINmean	1996	0.028	Rise	0.154	Rise
	TNn	1986	0.043	Rise	0.113	Rise
	TXn	1986-1987, 1989	0.031	Rise	0.081	Rise
Extreme warm indices	WSDI	2001-2002	0.128	Rise	0.113	Rise
	TR	1998, 1999, 2002	0.231	Rise	0.107	Rise
	SU	2006, 2008	0.077	Rise	0.066	Rise
	TN90p	2002	0.179	Rise	0.157	Rise
	TX90p	2002	0.291	Rise	0.161	Rise
	TMAXmean	2002-2003	0.022	Rise	0.110	Rise
	TNx	2002	0.021	Rise	0.073	Rise
	TXx	2004	0.028	Rise	0.113	Rise
	DTR	1971, 2009-2011, 2012-2013, 2014-2015	-0.005	Decline	0.006	Rise

Figure 4 displays the M-K test curves for 17 ETIs. According to the results, the upper outlier limits of the top five extreme cold indices and DTR passed the significance test at the 95% CI. In addition, the lower outlier limits of other extreme cold indices and most extreme warm indices passed the significance test at the 95% CI. Numerous outliers were observed for CSDI and TNx on the UF curve (*Fig. 4a*), and most of them were located in the tail (on the side of larger values). Since the medians were larger than the means, the UF curve was skewed to the left. The UB curve of TNx (*Fig. 4b*) was skewed to the right, and most outliers displayed small values. Moreover, CSDI, FD, ID and TN10p showed a descending trend, while TX10p only decreased slightly. All upper outlier bounds passed the significance test at the 95% CI. In addition, TMINmean, TNn and TXn showed an upward trend, and their lower outlier limits also passed the significance test. According to *Table 5,* the mutation points occurred in the 1990s. That is, the extreme cold events atypically decreased during this period. Eight extreme warm indices, WSDI, TR, SU, TN90p, TX90p, TMAXmean, TNx, and TXx, showed an increasing trend and their lower outlier limits passed the significance test. The mutation points occurred in the early 21st century. That is, the extreme warm events increased abnormally during this period, and they were accompanied by a very high temperature rise. DTR showed a downward trend,

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and the upper limit of the outliers on the UF curve passed the significance test. Since the mutation points occurred in the early 21st century (2009-2015), it was concluded that DTR decreased suddenly at this point in time, indicating smaller differences between daily maximum and minimum temperatures.

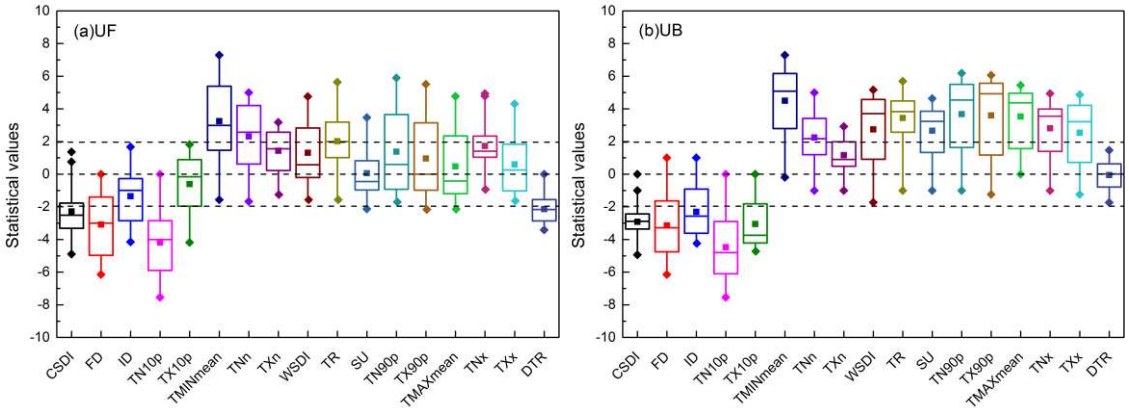

Figure 4. *M-K inspection box diagrams of 17 ETIs (referring to Table 2)*

EOF analysis

ETIs were extended by using the EOF analysis, and North criterion was used to test the significance. The cumulative variance percent value of the top five loading vectors of ID and SU was up to 90%, which indicated a proper representation of the original vector field. Among the 17 ETIs, the smallest number of loading vectors with the highest cumulative variance contribution rates were those corresponding to ID and SU. Therefore, ID (extreme cold index) and SU (extreme warm index), were chosen for the subsequent EOF analysis. *Table 6* presents the eigenvalues, cumulative variance contribution rates, and the variance contribution rates of the first five modes for ID and SU indices. In both cases, the cumulative variance contribution rates of the two top loading vectors were 75%, approximately. Hence, the top two loading vectors indicated the spatiotemporal changes of ETIs in Southwestern China. In the present research, only the two top loading vectors of the ID and SU indices were analyzed. *Figures 5–6* show the time coefficient series and the spatial distribution modes of the two top loading vectors of ID and SU after EOF analysis, respectively.

Table 6. *Eigenvalues and variance contribution rates of the first five modes for ID and SU indices*

Variable	Modal	Eigenvalue	Cumulative variance contribution rate	Variance contribution rate
ID	1	35656.14	52.17%	52.17%
	2	14817.06	73.85%	21.68%
	3	6054.771	82.71%	8.86%
	4	3566.85	87.93%	5.22%
	5	1958.087	90.80%	2.87%
SU	1	102025	60.11%	60.11%
	2	26453.18	75.70%	15.59%
	3	12814.79	83.25%	7.55%
	4	5383.14	86.42%	3.17%
	5	3684.315	88.59%	2.171

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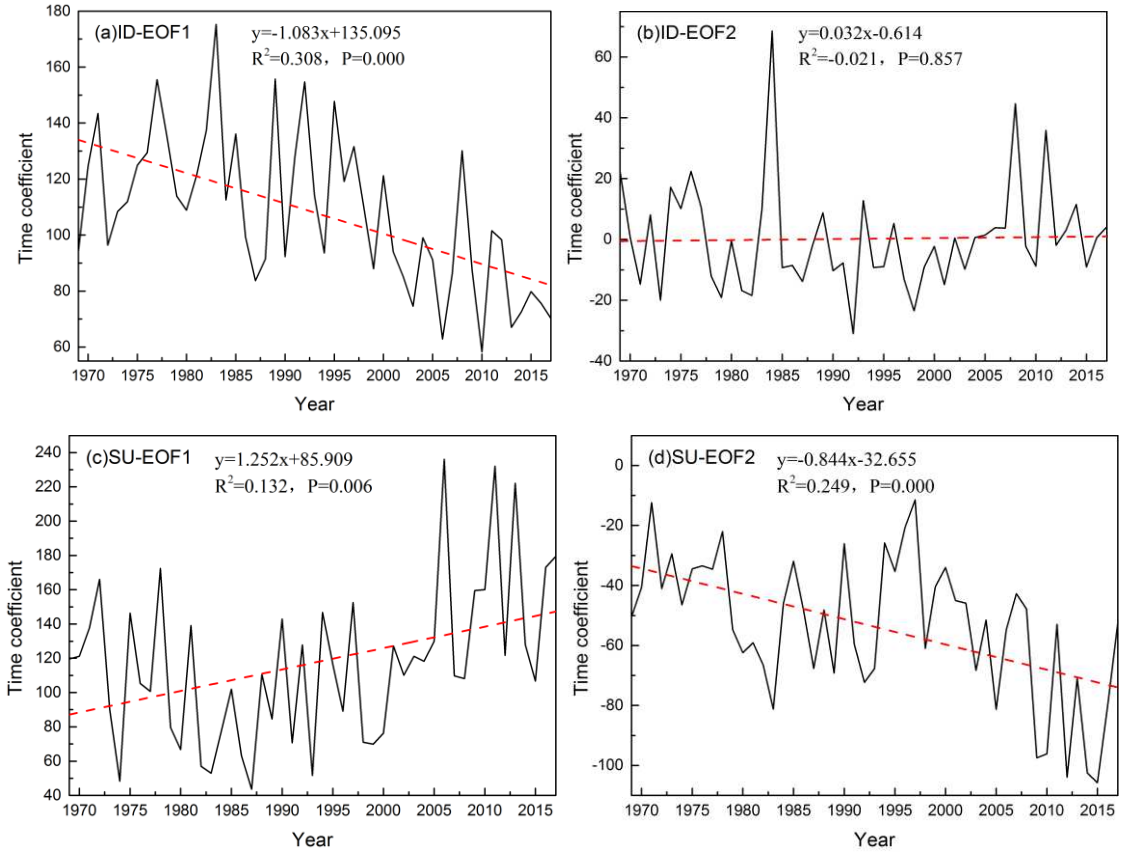

Figure 5. *Temporal EOF variation for ID and SU*

Figure 5 shows that the top one loading vectors of ID and SU were positive values, indicating a positive correlation for both ID and SU in Southwestern China. The change trends for both indices were highly consistent. Results indicated that, for the past 49 years in Southwestern China, ID decreased and SU increased. In addition, around 1990, ID significantly decreased. Moreover, SU suddenly increased in the early 21st century, which indicated the appearance of the mutation point. According to the data presented in *Figure 6,* the first ID mode showed a positive response. Furthermore, a small area in Western Hengduan Mountains and most of the Western Yunnan-Guizhou Plateau presented high loading values. It was also determined that the loading components reached or exceeded values of 0.5. These areas were susceptible to ID changes and the susceptibility decreased from southwest to northeast. Moreover, according to the data displayed in *Figure 5,* ID decreased from southwest to northeast. The percentage of variance of the second ID mode was 21.68% and the loading component was negative in the southwest. This is, the high-value region of the first mode became the low-value region of the second mode. Also, the high-value region of the second mode was positive in the east and northeast. As indicated by the time series data, a downward trend in the western Yunnan-Guizhou Plateau and an upward trend in most of the eastern Yunnan-Guizhou Plateau were observed. The amplitude decreased from west to east; however, no significant difference was observed. The first SU mode displayed a positive response, with a central high-value located in the eastern Zoige Plateau, part of Sichuan Basin, and the central Yunnan-Guizhou Plateau. *Figure 5* also shows that SU significantly increased in these regions, indicating a temperature

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increase. The high loading component of the second SU mode corresponded to the central Yunnan-Guizhou Plateau, and the low loading component to the northern Sichuan Basin. Also, as shown by the downward trend in the time series, SU generally decreased from south to north.

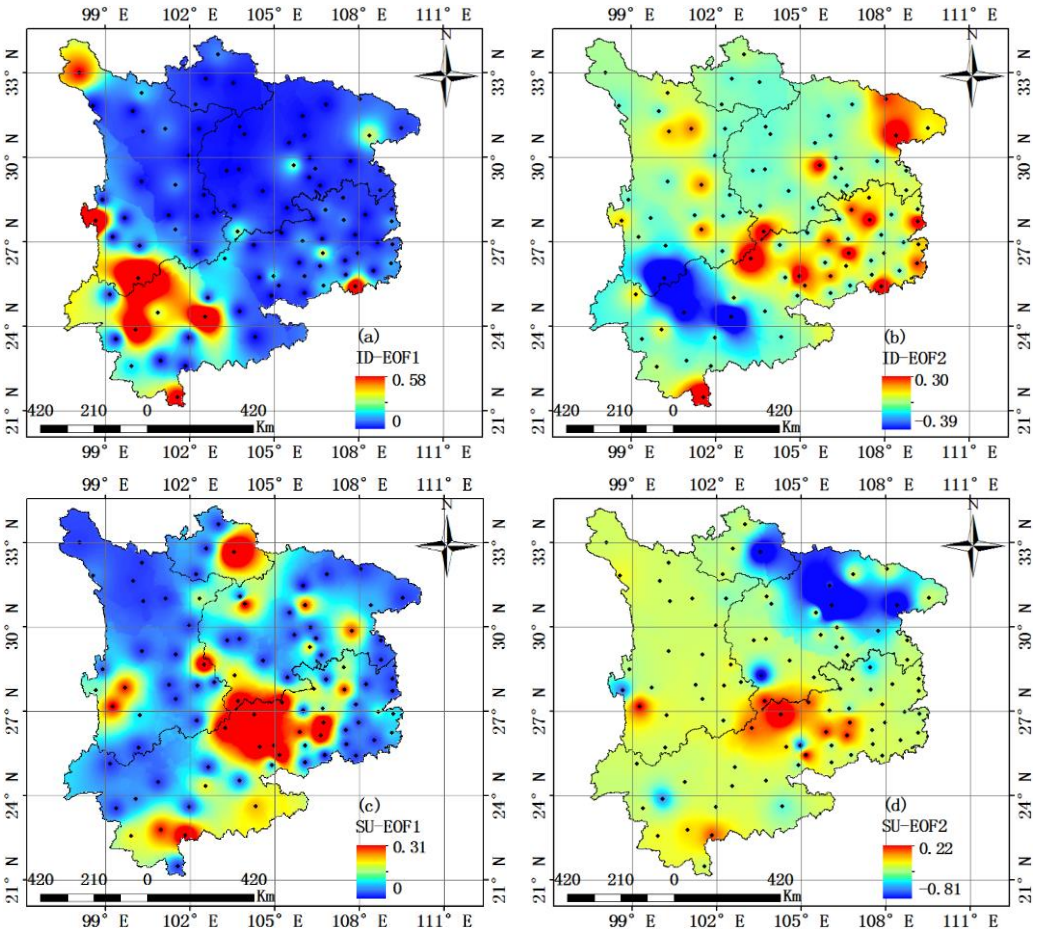

Figure 6. *Spatial EOF variation for ID and SU*

Analysis of the relationship between ETIs and ENSO events

Two indices, SOI and ONI, were chosen for mutual validation in order to prove the occurrence of ENSO events. Further comparisons showed that strong El Niño phenomena (SOI << 0 and ONI >> + 0.5 °C) occurred in the years of 1982, 1987, 1991, 1992, 1997, and 2015. On the other hand, strong La Niña phenomena (SOI >> 0 and ONI << + 0.5 °C) occurred in the years of 1971, 1974, 1975, 1999, 2008, and 2011. Once the composite ETIs rate was calculated, the influence of ENSO events on ETIs was determined, and results are shown in *Table 7*. Data indicated that CSDI was the most affected index as a consequence of ENSO events. The change rate in the El Niño year was -16.86%. That is, the cold spell duration decreased. Furthermore, the change rate increased to 86.68% during La Niña year. The number of cold days during the abnormal years was about twice that of the long-term average number of days. The cold spell duration was considerably extended. FD, ID, TNn, and CSDI values were similar. A descending trend was observed for these indices during El Niño years, while an

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ascendant trend was observed in the La Niña years. In other words, warmer temperatures were observed in the El Niño years and colder temperatures in the La Niña years. The amplitude increase in the number of cold days in the La Niña years was significantly higher than the amplitude decrease in the number of cold days during the El Niño years. Both, TN10p and TX10p, showed an increasing trend in the ENSO years, while TMINmean decreased, indicating a temperature reduction. The change in TXn value indicated a warming trend in the El Niño years, and a cooling trend in La Niña years. WSDI, TR, and TMAXmean showed a downward trend in the ENSO years. It was also observed that this reduction was higher in El Niño years than in La Niña years. SU, TN90p, and TX90p decreased in El Niño years; however, they increased in La Niña years. With respect to these indices, the amplitude reduction was much higher than the amplitude increase. In relation to TNx, this index did not show variations in El Niño years. However, it displayed decreasing values during La Niña years. DTR decreased in El Niño years, which indicated that smaller temperature differences occurred during the same day. However, DTR increased in La Niña years, indicating that larger temperature differences were observed during the same day.

Table 7. *Correlation between 17 EPIs (referring to Table 2) and ENSO events in Southwestern China*

Categories	Indices	El Nino compound ratio	Change rates (%)	La Niña compound ratio	Change rates (%)
Extreme cold indices	CSDI	0.83	-16.86%	1.87	86.68%
	FD	0.98	-1.99%	1.06	5.75%
	ID	0.97	-3.00%	1.28	27.87%
	TN10p	1.05	4.83%	1.15	15.44%
	TX10p	1.12	11.81%	1.07	7.44%
	TMINmean	1.00	-0.24%	0.99	-1.44%
	TNn	0.97	-3.09%	1.18	17.66%
	TXn	1.15	15.40%	0.71	-28.94%
Extreme warm indices	WSDI	0.76	-23.59%	0.92	-7.69%
	TR	0.97	-2.98%	0.98	-1.61%
	SU	0.81	-19.48%	1.01	1.24%
	TN90p	0.95	-4.74%	1.03	2.81%
	TX90p	0.89	-11.27%	1.00	0.28%
	TMAXmean	0.99	-0.89%	1.00	-0.23%
	TNx	1.00	0.00%	0.98	-1.52%
	TXx	1.00	-0.41%	0.99	-0.72%
	DTR	0.99	-1.47%	1.01	1.18%

As previously mentioned, ETIs were closely related to ENSO events. In the El Niño years, the number of cold days decreased (-1.04%), while temperature increased (4.03%), as indicated by the extreme cold indices. On the other hand, during La Niña years, the number of cold days increased (28.64%), and temperature decreased (-4.24%). According to values of the extreme warm indices obtained in the present research, the number of warm days during El Niño years decreased (-12.41%), and temperature value was reduced (-0.43%). Moreover, in La Niña years, the number of

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warm days decreased (-0.99%), while temperature was reduced (-0.82%). According to these results, the extreme cold indices were more susceptible to the influence of the ENSO events, and the change in amplitude was significantly larger than that observed for extreme warm indices. Thus, La Niña had a greater impact on extreme cold indices, while El Niño displayed a more significant impact on extreme warm indices.

Discussion

(1) According to the interannual ETIs variation (*Fig. 2*), the results on extreme cold indices indicated a warming trend. Similarly, the extreme warm indices showed an increasing trend. Our results agreed with other investigations that determined overall changes in extreme temperatures (a) on a global scale (Alexander et al., 2006); (b) in China (Yang et al., 2010); and Southwestern China (Wang et al., 2014a; Luo et al., 2015; Luo et al., 2016). Most ETIs change trends obtained in the present research were similar to those published by Yuan et al. (2015). However, the amplitude of the change varied slightly depending on the dataset or time scale used. Yuan et al. (2015) reported a significant difference in the amplitude change between different types of ETIs. Specifically, the amplitude change for TN10p and TX90p was greater than that for other ETIs, which was also consistent with our findings. In relation to the spatial trend, the extreme cold indices decreased from west to east. The warming trend was more pronounced in the extreme cold indices in the Hengduan Mountains than in other regions. In addition, the Sichuan Basin and southwestern Yunnan-Guizhou Plateau displayed the extreme warm events with the highest frequency and intensity. According to our results, DTR showed a decreasing trend, indicating a gradual reduction of the daily temperature differences. Similarly to other indices, the results published by Yang et al. (2010) were alike. Thus, even when there are slight differences in climate variability across different geographical divisions, the warming trend seems definite. This means that Southwestern China has been experiencing a warming trend for the past 49 years.

(2) As indicated by the Sen's slope estimator (*Table 5*), the number of cold days in Southwestern China suddenly decreased in the 1990s, and temperature increased. Later, in the early 21st century, an abrupt change in extreme warm indices with a higher number of warm days and higher temperatures occurred. The results of the Sen's slope estimator corroborated the regression coefficients of the interannual and interdecadal ETIs changes, as well as the UF statistics calculated by SPSS. Thus, in Southwestern China, an upward temperature trend has been observed for the past 49 years. In addition, an abrupt change in temperature occurred in the 1990s and the early 21st century (2001-2011). This conclusion was consistent with the data published by Zhu et al. (2018) the region of Guizhou. However, according to He et al. (2011), the amplitude increase in extreme low temperature was significantly higher than that of the extreme high temperature, which was consistent with our findings. Moreover, He et al. (2011) suggested that the abrupt change in the extreme droughts occurred during the monsoon season of 2003 in Southwestern China. Also, the abrupt change in the non-monsoon season occurred in 1989. Even when the methods used by He et al. (2011) differ from ours, the findings were similar. Jia et al. (2018) showed that droughts hit Southwestern China at a higher intensity in the years of 1970 and 2000. Since extreme temperature events usually occur concomitantly with droughts, their results also support our findings.

(3) The results of the EOF analysis also indicated a decrease in the extreme cold indices and an abrupt change in the 1990s. Extreme warm indices increased, and their

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abrupt change generally occurred in the early 21st century. Furthermore, regions susceptible to the changes in ID were identified in a small area of the western Hengduan Mountains and in a large area of the western Yunnan-Guizhou Plateau. The high-value centers of SU were found in the eastern Zoige Plateau, part of Sichuan Basin, and the central Yunnan-Guizhou Plateau. These findings also proved the following spatiotemporal characteristics of ETIs in Southwestern China: (a) the warming trend in the extreme cold indices was the highest in the Hengduan Mountains; (b) extreme warm events were more frequent in the Sichuan Basin and southwestern Yunnan-Guizhou Plateau than in the rest of the regions. Wang et al. (2014a, b), who studied the drought events in Southwestern China, found out that droughts were more likely to occur in the Yunnan-Guizhou Plateau and part of the Sichuan Basin. The frequency of droughts was higher in the Zoige Plateau and southwestern Sichuan Basin than in other regions. It was also determined that the Southwestern Yunnan-Guizhou Plateau was susceptible to droughts. These findings support the conclusion that extreme warm events occurred more frequently in the Sichuan Basin and southwestern Yunnan-Guizhou Province, where the susceptibility to droughts was also higher with respect to other regions.

(4) Shi (2017) suggested that ENSO were more intense between 1970s and 1990s. The ENSO geographical range of influence was wider in the north-south direction. The midpoint of maximum anomaly in sea surface temperature was identified in the east. This finding confirmed that the ENSO years selected in the present study were appropriate, and also indicated an upward temperature trend in the ENSO years. Lim and Schubert (2011), Shi et al. (2018) and Moon et al. (2011) all concluded that extreme climate events were associated with the circulation index. These researchers proved that the correlation between ETIs and ENSO events was significant. Wang et al. (2014a) analyzed the correlation between drought events and ENSO events in Southwestern China and identified a correlation between the ENSO index and drought index in a long time series. However, the correlation between these two indices was lower, and the relationship more complex than originally hypothesized. Also, comparing different regions, Sichuan Basin and Zoige Plateau were susceptible to droughts in the El Niño years, while these events were less frequent in La Niña years. However, for the Yunnan-Guizhou Plateau, droughts were more likely to occur in La Niña years. Moreover, the probability of droughts was higher in El Niño years and lower in the La Niña years. The present conclusion corroborated the reliability of our findings. That is, ETIs were correlated to ENSO events. In addition, upward and downward temperature trends were identified in the El Niño and the La Niña years, respectively. Wang et al. (2019) determined that the southeast monsoon was weakened when SOI entered the negative phase and the El Niño phenomenon occurred. They also suggested that large-scale droughts in Southwestern China were related to the El Niño phenomenon and Yunnan-Guizhou Plateau was more vulnerable to the influence of SOI. Existing studies have shown that extreme temperature events are also remotely related to the circulation indices, including the AO (Arctic Oscillation) (Lim et al., 2011), AMO (Atlantic Multidecadal Oscillation), DMI (Dipole Mode Index) (Shi et al., 2018), MJO (Madden–Julian Oscillation) (Moon et al., 2011), and NAO (North Atlantic Oscillation) (Grotjahn, 2011). In addition, in recent years, due to the implementation of the western development strategy and the construction of new urbanization roads in China, the built-up areas of the four provinces and cities in Southwestern China have been expanding and the urbanization process has been accelerated. Cities as areas of concentration of human activities, urban heat island phenomenon will appear in different degrees due to

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the change of underlying surface properties and the emission of anthropogenic heat. Many studies also showed that the impact of urbanization on extreme temperature events cannot be ignored (Jiao et al., 2020; Lin et al., 2020).

Conclusions

Extreme weather events refer to a type of rare events that occur at specific sites and at a specific time. The occurrence of extreme weather events can be determined based on the values of extreme indices. In this study, data on daily mean temperatures as well as daily maximum and minimum temperatures were obtained in 93 weather stations in Southwestern China for the period 1969 to 2017. Seventeen ETIs were selected according to the climate indices recommended by the ETCCDMI. These index data were analyzed using linear regression, M-K trend test, Sen's slope estimator, and EOF analysis. In addition, extreme spatiotemporal climate changes for the past 49 years were analyzed. The results of this analysis were used to determine the relationships between: SOI and ONI with ETIs, as well as the correlation between ETIs and ENSO events in Southwestern China. The following conclusions were reached:

(1) during the past 49 years, ETIs showed a warming trend in Southwestern China, which responded positively to global warming. Also, differences in warming trends were observed in different geographical regions. For example, increasing temperatures were more evident in the Hengduan Mountains. In addition, in the Sichuan Basin and southwestern Yunnan-Guizhou Plateau, the extreme warm events occurred more frequently and at a higher intensity as compared to other regions. The diurnal temperature range (DTR) decreased, indicating increasingly small daily temperature differences.

(2) In the 1990s and early 21st century (2001-2011), abrupt temperature changes were observed. While in the 1990s the number of days of extreme cold indices suddenly decreased, in the early 21st century (2001-2008) the extreme warm indices suddenly increased.

(3) Results of the EOF analysis also indicated abrupt changes in temperature indices. The regions susceptible to changes in the number of ice days (ID) were the Hengduan Mountains and part of the Yunnan-Guizhou Plateau. The high-value midpoints of summer days (SU) were found in the Zoige Plateau, Sichuan Basin, and part of the Yunnan-Guizhou plateau.

(4) ETIs were closely related to the ENSO events. The extreme cold indices were more influenced by La Niña, and the extreme warm indices were more susceptible to El Niño events. The amplitude change of the extreme cold indices was more significant than that of the extreme warm indices. The number of days with cold indices consistently decreased, and the temperature increased during the El Niño years. On the other hand, in La Niña years, the number of days with warm indices increased, and temperature decreased. The extreme warm indices showed a decreasing trend in all the ENSO years. However, the amplitude change of some of the indices was negligible. DTR indicated a reduced difference between the daily maximum and minimum temperatures in the El Niño years, and an increased difference in the La Niña years.

Extreme temperature events have important implications for agricultural and livestock production, resource protection and socio-economic development in Southwestern China. However, due to the specificity and complexity of extreme temperature events themselves, their influencing factors involve not only natural factors

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but also human factors caused by human activities. The research in this paper only analyzed the characteristics of extreme temperature index changes from the perspective of climate statistics, and explored the causes of influence less, which has certain limitations. In the future, in order to clarify the influencing factors and physical mechanisms of its spatiotemporal change trends, a more in-depth and comprehensive discussion can be conducted from various aspects and perspectives, such as atmospheric circulation anomalies, water vapor transport, solar activity, urbanization process, underlying surface land use changes, and greenhouse gas emissions. At the same time, in order to cope with the future extreme climate risks caused by continuous global warming, an early warning system for extreme temperature events can be established and improved, and CMIP6 model data can be applied to project extreme temperature events in Southwestern China for different scenarios and different time scales, which will be further analyzed and discussed in depth in the future.

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RESPONSE OF METHANE EMISSION AND GROWTH PERFORMANCE OF YEARLING MALE BOER GOATS TO AN INCLUSION OF *ACACIA KARROO* (SWEET THORN) IN *AVENA SATIVA* (COMMON OAT) HAY BASED DIET

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Abstract. This study determined the response of methane emission and growth performance of yearling male Boer goats fed a basal diet of *Avena sativa* hay supplemented with Acacia karroo (sweet thorn*)* leaf meal. Twelve yearling male Boer goats with initial mean live weights of 23 ± 2 kg were used in a 21-day experiment. The goats were randomly assigned to four dietary treatments, each containing sweet thorn leaf meal at 10, 15, 20 and 30% with *Avena sativa* hay as a basal diet. The data collected was subjected to analysis of covariance and analysis of variance in a completely randomized design using Statistical Analysis Software. Differences were separated at 5% level of probability. Sweet thorn leaf meal inclusion level had no effect ($p > 0.05$) on diet intake, methane emission, live weight changes and digestibility. Feed conversion ratio improved linearly with increased Sweet thorn leaf meal inclusion level. The low tannin contents in sweet thorn leaves indicate that these leaves can be safe to use as a source of protein in animal nutrition if used sparingly. Although, sweet thorn leaf meal reduced methane emission, the optimal dose was not determined. Further validation is required to determine sweet thorn inclusion levels for optimal methane production and emission by goats.
Keywords: *feed conversion ratio, optimal dose, live weight, leaves, tannins*

Introduction

Africa's livestock accounts for one-third of the global livestock population (AU-IBAR, 2015) and about 40% of agricultural GDP in Africa, ranging from 10% to 80% in individual countries. Livestock will be increasingly important in the future in sub-Saharan Africa (SSA) because the demand for animal-source food is projected to increase due to population growth, increased incomes, and urbanization. In South Africa, goats play multiple roles in the livelihoods of the populace (Ng'ambi et al., 2013). The total national goat population in the commercial sector of South Africa is approximately 3.2 million (DALRRD, 2020). Although the exact population of goats in the South African communal sector cannot be readily established, the Eastern Cape and Limpopo provinces are the largest communal goat-producing provinces. However, methane produced by goats from enteric fermentation contributes to the emission of greenhouse gases (GHG) into the atmosphere. According to Peters et al. (2018), methane along with other GHG arising from anthropogenic activities have a greater contribution to global warming. Furthermore, Canul-Solis et al. (2020) stated that ruminant production systems contribute between18% and 33% of total methane emissions. Gerber et al. (2013) reported that African livestock have significant impacts on the environment. Tubiello et al. (2014) ascertained that more than 70% of agricultural GHG emission in Africa comes from the livestock sector dominated by

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enteric methane (CH_4) emission. Production of methane translates into lost energy which can negatively affect ruminant productivity (Ramin and Huhtanen, 2013). Therefore, in Africa, sustainable livestock production must address food security and climate change concerns simultaneously in addition to social and economic aspects. Currently, most countries in Africa rely on the Intergovernmental Panel on Climate Change (IPCC) tier 1 methodology to estimate their livestock-based emissions.

Furthermore, there is a general lack of detailed, precise activity data and accurate estimates of natural resource use and environmental impact by livestock to inform the adoption of climate-smart livestock interventions (Gaitán et al., 2016. This situation calls for more research into the potential use of tropical foliage trees, pods, and secondary metabolites to reduce methane emissions from ruminant supply chains. Optimal livestock production that couples the use of environmentally friendly practices within the tropical regions is attainable when the relevant stakeholders have a better knowledge of the available strategies for efficient foliage use (Ku-Vera, 2020). Most tropical forages contain high tannin levels and some studies have reported beneficial effects of tannins in livestock production and more recent studies have revealed beneficial effects of tannins in domestic ruminants. For example, Anantasook et al. (2014), Gunun et al. (2016) and Canul-Solis et al. (2020) reported that tannins containing plants contribute to inhibiting the enteric methane emission. Thus, the mitigation of rumen methane production with the foliage and metabolites of tropical trees represents an interesting challenge in the field of ruminant nutrition. Research on the utilization of tropical forages is particularly important in goat production. Goat mouth structure allows them to select the high-quality parts of the woody plants and they can effectively detoxify secondary compounds such as tannins and terpens. These observations have led to a growing interest in finding feed alternatives, which may help to mitigate methane production in the goat rumen. It was observed by Canul-Solis et al. (2020) that the presence of a vast range of secondary metabolites in tropical trees (coumarins, phenols, tannins, and saponins, among others) may be a valuable alternative to manipulate rumen fermentation and partially defaunate the rumen, and thus reduce enteric methane production.

This study explored sweet thorn as an alternative for sustainable livestock production in South Africa. Sweet thorn is one of the tropical trees that have been reported to have a potential to mitigate methane emissions from Boer goats. Aboagye and Beauchemin (2019) pointed out that CH4 mitigation options that are inexpensive would allow wide implementation to reduce enteric CH4 emissions associated with ruminant production. The use of tannins may offer such a possibility because they are naturally occurring in numerous plants, and hence widely available to ruminant producers. There is neither extensive nor conclusive data on tanniniferous sweet thorn leaf meal inclusion levels for optimal reduction in methane production and emission in the ruminant animals. Additionally, extensive knowledge on inclusion levels of sweet thorn leaf meal for optimal goat productivity is also minimal.

Materials and methods

This study was conducted at the University of Limpopo Experimental farm, Limpopo province, South Africa. The farm is situated 10 km north-west of main campus of the University of Limpopo. The University of Limpopo lies at latitude 27.55°S and longitude 24.77°E. The ambient temperatures around this area are above 32 °C during summer and

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between 5 and 25 °C during winter seasons. The mean annual rainfall ranges between 446.8 and 468.4 mm. The dry season occurs between April and October and the rainy season occurs between November and March (Kutu and Asiwe, 2010). Vegetation structure is a Savanna type (bushveld) that is characterised by trees, shrubs and grass undercover. Browsing animals, traditionally, keep a balance between trees and grass.

The experimental diets consisted of *Avena sativa* hay as a basal diet mixed with sweet thorn leaves. Sweet thorn leaves were hand-harvested at the University of Limpopo Experimental farm. The leaves were then shade-dried and stored indoor for 14 days prior to chopping. The samples were chopped, using a 2 mm screen, and then stored in airtight bags until needed for analysis and feeding. *Avena sativa* hay was bought from a local supplier. The hay was harvested before summer rains while still green, soft and less stalky. It was shade-dried before being chopped and stored in bags under a shade until needed for analysis and feeding.

The first part of the study determined tannin levels of sweet thorn leaf meals and *Avena sativa* hay. The dietary means were compared using Fisher's least significant difference at the 5% level of probability. The second part of the study determined the effect of sweet thorn leaf meal inclusion level on diet intake and digestibility, methane emission and growth response of yearling male Boer goats. Twelve yearling male Boer goats with initial mean live weights of 23 ± 2 kg were used in a 21-day experiment. The goats were randomly assigned to four dietary treatments, each containing sweet thorn leaf meal inclusion levels at 10, 15, 20 and 30% with *Avena sativa* hay as a basal diet. The experimental diets were as indicated in *Table 1*. These inclusions include high and low tannin-levels as indicated in the literature (Makkar, 2003). The pens and surrounding environment were cleaned thoroughly and disinfected. Each goat was housed in well-ventilated individual metabolic pen. The diets were replicated three times. The data collected were subjected to analysis of covariance and analysis of variance in a completely randomized design using SAS. Differences were separated at 5% level of probability.

The feeding trial lasted for 21 days, with the last 7 days being for data collection. The goats were fed once at 8:00am on daily basis. The feed was offered *ad libitum*. The goats were weighed at the start of the experiment, on the 14th day when data collection commenced and on the last day of the experiment. Methane emissions were measured at the start of the experiment and again on the last 5 consecutive days of the experiment together with intake and digestibility. Dry matter digestibility was calculated according to Khan et al (2003). Methane emissions were measured using a hand-held portable laser methane detector (LMD) manufactured by Growcon according to Chagunda et al. (2009).

Table 1. *Dietary treatments for sweet thorn and Avena sativa hay diet*

Diet code	Diet description
$H_{As90}AK_{10}$	Yearling male Boer goats fed a mixed diet of 90% *Avena sativa* hay and 10% *sweet thorn*
$H_{As85}AK_{15}$	Yearling male Boer goats fed a mixed diet of 85% *Avena sativa* hay and 15% *sweet thorn*
$H_{As80}AK_{20}$	Yearling male Boer goats fed a mixed diet of 80% *Avena sativa* hay and 20% *sweet thorn*
$H_{As70}AK_{30}$	Yearling male Boer goats fed a mixed diet of 70% *Avena sativa* hay and 30% *sweet thorn*

All the diets and faecal output were analysed for dry matter, organic matter, ash, crude protein, fat, energy and minerals according to AOAC (2002). Neutral and acid

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detergent fibre contents of feeds and faeces were determined according to Van Soest et al (1991). Tannin contents were determined using the methods of Waterman and Mole (1994). The total phenolic and tannin contents were measured using Folin-Ciocalteu method according to Makkar (2000). Hydrolysable tannins were measured using a modified method of Hartzfeld et al. (2002).

The nutrient and tannin contents of sweet thorn leaves and *Avena sativa* hay diets were subjected to analysis of variance (ANOVA) using SAS (2008). Intake, digestibility, live weight, weight gain and feed conversion ratio (FCR) were adjusted for final weight, FCR, dry matter intake, FCR and weight gain, respectively, by covariance analysis and were presented as adjusted least-square means. In the case of methane, data was scrutinised by ANCOVA using methane baseline values as the covariates to control statistically for differences in baseline values. Where the covariates showed no significant effect, the data were analysed with ANOVA in a completely randomized design at 5% ($p < 0.05$) level of probability with diet as a fixed factor (SAS, 2008). Where significant treatment effects were detected, means were separated by Fisher's least significant difference (LSD) using SAS (2008).

Results

Table 2 shows the tannin contents of sweet thorn leaves. Total tannins and condensed tannins were found in sweet thorn leaves. However, no hydrolysable tannins were detected. Sweet thorn leaf meal inclusion level had no effect on diet dry matter (DM) intake. Increasing sweet thorn leaf meal inclusion level in the diet increased total phenolics, total tannins and condensed tannins (CT). Sweet thorn leaf meal inclusion level had no effect on diet intake, methane emission, live weight and weight gain of goats (*Table 3*). Similarly, sweet thorn leaf meal inclusion level had no effect on diet DM digestibility by goats. Feed conversion ratio improved with increased sweet thorn leaf meal inclusion level. Boer goats on a diet containing a 30% sweet thorn leaf meal inclusion level had a higher ($p < 0.05$) FCR value than those on diets having 10, 15 or 20% sweet thorn leaf meal inclusion levels. Similarly, goats on a diet with a 15% sweet thorn leaf meal inclusion level had a higher ($p < 0.05$) FCR value than goats on a diet having a 10% sweet thorn leaf meal inclusion level. However, goats on diets having 10 or 20% sweet thorn leaf meal inclusion levels had similar ($p > 0.05$) FCR values. Similarly, goats on diets containing 15 or 20% sweet thorn leaf meal inclusion levels had similar ($p > 0.05$) FCR values.

Table 2. *Composition of tannins in the experimental sweet thorn diets*

Nutrient	Diet[#]			
	$H_{As90}AK_{10}$	$H_{As85}AK_{15}$	$H_{As80}AK_{20}$	$H_{As70}AK_{30}$
Total phenolics*	$0.28^{d} \pm 0.002$	$0.41^{c} \pm 0.002$	$0.55^{b} \pm 0.003$	$0.83^{a} \pm 0.005$
Total tannins*	$0.21^{d} \pm 0.001$	$0.32^{c} \pm 0.002$	$0.43^{b} \pm 0.003$	$0.64^{a} \pm 0.004$
Condensed tannins**	$0.16^{d} \pm 0.001$	$0.24^{c} \pm 0.001$	$0.32^{b} \pm 0.002$	$0.48^{a} \pm 0.003$
Hydrolysable tannins (mg/g)	0.00 ± 0.000	0.00 ± 0.000	0.00 ± 0.000	0.00 ± 0.000

[a, b, c, d]Means in the same row not sharing a common superscript are different ($p < 0.05$)
*Percentage DM tannic acid equivalent
**Percentage DM leucocyanidin equivalent
[#]Diet codes are explained in *Table 1*

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Table 3. *Effect of sweet thorn leaf meal inclusion level on diet intake, digestibility, methane emission, live weight change and feed conversion ratio of yearling male Boer goats fed an Avena sativa hay-based diet*

Variable	Diet[#]			
	$H_{As90}AK_{10}$	$H_{As85}AK_{15}$	$H_{As80}AK_{20}$	$H_{As70}AK_{30}$
Intake (g/goat/day)				
DM	264 ± 40.223	337 ± 40.451	370 ± 40.602	288 ± 40.247
Digestibility (decimal)				
DM	0.55 ± 0.147	0.55 ± 0.063	0.63 ± 0.078	0.42 ± 0.277
Methane emission (ppm-m)	13.80 ± 1.668	13.07 ± 0.668	13.33 ± 0.668	12.40 ± 0.698
Initial live weight (kg)	23.15 ± 3.715	22.52 ± 2.639	23.97 ± 2.965	22.85 ± 2.707
Final live weight (kg)	23.38 ± 3.534	22.88 ± 2.511	24.33 ± 2.821	23.27 ± 2.576
Weight gain (g/goat/day)	46.00 ± 200.621	73.33 ± 86.252	73.33 ± 106.608	83.33 ± 379.350
Feed conversion ratio	5.74[a] ± 0.241	4.60[b] ± 0.238	5.04[ab] ± 0.238	3.45[c] ± 0.242

[a, b, c]Means in the same row not sharing a common superscript are different ($p < 0.05$)
[#]Diet codes are explained in *Table 1*

Discussion

Some studies have attempted to provide dose-response information on the effect of including tannin based diets on methane emission in goats. Naumann et al. (2017) showed that total condensed tannins vs. methane production gave a correlation of $R^2 = 0.44$, suggesting that only 44% of the reduction in methane production is explained by total CT. Many studies have demonstrated that forage plants with diverse natural products as well as tannin rich legumes such as *Mimosa* spp. and *Acacia mearnsii* can decrease gas production by ruminants, including methane emissions (Bhatta et al., 2013; Durmic et al., 2014). Generally, condensed tannins (CT) and hydrolysable tannin (HT) supplementation have no deleterious effects (dose dependent) on animal performance (Krueger et al., 2010). Several studies reported that some tannins based forage are beneficial and some are deleterious (Dey and De, 2014). Tannins in higher concentrations reduce feed intake, carbohydrates metabolism, protein digestibility and ultimately animal production performance (Shewangzaw, 2016.

Sweet thorn leaf meal inclusion levels of 10 - 30% in the present study had no effect on nutrient intake per goat. The intakes, irrespective of dietary treatments, are consistent with the previous reports (Bwire et al., 2004; Pathak et al., 2014; Dey and De, 2014). These authors reported that moderate levels of 1 - 4% of CT in the diet from various plant sources exerted no significant effect on diet intake. Values of CT of 0.16 - 0.48% in sweet thorn leaf meal diets in the current study fell below the moderate levels of CT reported. The non-significant effect of sweet thorn leaf meal inclusion on intake by goats may be attributed to low concentration of CT in the diets. Pathak et al. (2013), also, reported non-significant differences in total intake of DM in lambs fed on diets with or without tannin leaf meal mixtures. It has been reported that diets containing up to 5% tannins are utilised efficiently by the animals without any harmful effect on intake (Barry and McNabb, 1999). This differs from the findings of Gwanzura et al. (2011) who reported a high level of voluntary intake by goats, which were fed tannin-rich diets. Ramírez and Ledezma-Torres (1997) reported that inclusion of *Acacia rigidula* and *Acacia farnesiana* did not adversely affect forage intake of goats. Nunez-

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Hernandez et al. (1989) observed similar DM intakes for Angora goats on a diet containing a high tannin shrub (*Juniperus monosperma*) compared with goats fed an alfalfa hay diet. Other studies have indicated that plant secondary metabolites (PSM) such as tannins may reduce dry matter intake of forage legumes by decreasing palatability (Estell, 2010). Brown et al. (2016) observed that tannin-rich plant leaves when fed in a mixed diet can influence preference and intake by goats. Tannins have been associated with low palatability values, however, medium to low levels may be tolerated by ruminant animals (Lamy et al., 2011). Reduced dry matter intake associated with tannin supplementation have been reported in some studies (Fernández, 2012). In a study by Aboagye et al. (2018), dry matter intake of beef steers was not affected by hydrolysable or a combination of hydrolysable and condensed tannin, at the inclusion level of 15 g/kg DM. The influence of tannin on nutrient intake may vary widely and depend on several factors such as the concentration of tannin in the diet, animal factor, biological characteristics of the tannin compound, effect of prolonged adaptation, rumen fermentation or diet characteristics (Dschaak, 2011; Archimède, 2016). The methane-reducing potential of Acacia tannin and other tannin additives has been noted in previous studies (Carulla, 2005; Grainger, 2009). The methane suppressing effects of tannins relate to a combination of direct toxicity on the methanogenic archaea or reduced fibre degradation (Patra and Saxena, 2011; Beauchemin, 2007). Feed conversion ratio (FCR) measures the amount of feed an animal requires to gain per unit body weight. In the present study, feed conversion ratios significantly improved as Sweet thorn inclusion level increased.

Ngambu et al. (2013) reported a higher growth performance in Xhosa lop-eared goats that were supplemented with sweet thorn and attributed this to the high nutritive value of the foarge (Ngongoni et al., 2007; Mapiye et al., 2009; Marume et al., 2012). The significance of tannins in ruminant diets lies in their effect on protein digestion. It has been observed that tannins can reduce the amount of protein that is degraded in the rumen and increase the protein flow for digestion in the small intestine (Mueller-Harvey, 2006). This tannin-protein interaction leads to increased protein metabolism into the muscle, leading to higher slaughter weights and heavier carcasses (Gleghorn et al., 2004). In a study by Mapiliyao et al. (2012), goats, which were allowed access Sweet thorn encroached areas, had higher growth responses as compared to those in open grassland.

Brown et al. (2018) reported improved body weight gains in Pedi goats on diets higher in sweet thorn inclusion levels and attributed this to an improved protein utilization in line with the findings of Solaiman et al. (2010). Similarly, Gwanzura (2011) reported higher body weight gains in Pedi goats fed taninniferous Mucuna hay. The variation in the feed conversion ratio among goats in the present study as compared to previous studies could be due to other factors which were not controlled which also concurs with previous studies that compared performance of sheep (Mukasa-Mugerwa et al., 2000; Mapiliyao et al., 2012), goats (Rumosa-Gwaze et al., 2009) and cattle (Mapiye et al., 2009). Results of Njidda and Ikhimioya (2010) with tannin-rich plants revealed that some rumen microorganisms are able to metabolize tannins or remain active in a high tannin environment and overcome their detrimental effects, which in turn improves animal performance. A study by Ash and Norton (1987) observed higher average daily gains in goats consuming a diet with a 50% sweet thorn leaf meal inclusion level and attributed this to increase in diet digestibility with an increase in sweet thorn inclusion level. However, Tanner et al. (1990) observed that higher

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concentrations of total polyphenols in a diet tended to reduce nutrient availability to the animals, thus adversely affecting their body weight gains. In the present study, the highest inclusion level for sweet thorn was 30% and this could have been lower to yield significantly higher weight gains.

Dry matter digestibility was affected by sweet thorn leaf meal inclusion level even though the differences across the treatments were not significantly different. Digestibility was optimised at 13.50 and 16.75% sweet thorn leaf meal inclusion levels, respectively. Boer goats on diets having 30% sweet thorn leaf meal inclusion level had the lowest digestibility values. The reduction in digestibility could be due to the negative effect of tannins and phenolics through formation of indigestible complexes with proteins and other nutrients (Dubey et al., 2012). This is also supported by the findings of Salem et al. (2006). The authors reported that ingestion of tannin-containing feed by ruminants may reduce nutrient digestibility. In other studies, digestibility in sheep was decreased by adding CT of *A. mearnsii* to grass-based diets (Carulla et al., 2005). Thus, effects of CT on dry matter digestion, in the present experiment, were expected.

Daily gain indicates production performance of animals which is determined by the feeding value of the feed consumed (Waghorn, 2008). In the present study, the goats attained desired live weight gains and remained in good health condition throughout the experiment. Initial and final live weights of Boer goats were similar irrespective of sweet thorn leaf meal inclusion levels. This accounted for comparable daily weight gains of the goats. These comparable results are in conformity with the findings of several workers (Anbarasu et al., 2001). The authors observed similar body condition in animals on various tannin-rich forage meals. However, Waghorn (2008) reported improved live weight gain in sheep fed temperate forages with CT. The author suggested that increased protein due to higher levels of sweet thorn leaf meal inclusion might also have contributed to improvements in live weight gains.

In the present study, inclusion of tanniferous sweet thorn in the diet showed a potential to reduce methane emission. However, the differences in the amount of methane emitted across the treatments were not significant. Earlier studies suggested that feeding forages that contain tannins for ruminants generally effectively inhibit CH_4 produced during enteric fermentation (Puchala et al., 2005; Animut et al., 2008). In this regard, tannins of lower molecular weights were found to be more effective against methanogens than their monomeric precursors or tannins of higher molecular weight (Tavendale et al., 2005). However, there is huge diversity in tannin structure and concentration, and other chemical constituents among the browses, which may affect variably, the observed response in rumen fermentation, digestibility of nutrients and methanogen activity (Mueller-Harvey, 2006**).** This indicates that tannin from different plants might show a different response in gas production, true digestibility and methane production. Tan et al. (2011) reported that with different graded levels of CT inclusion, total gas production, CH_4 production and total VFA concentration decreased at a decreasing rate with increasing levels of CT and so were the in vitro DM degradation and N disappearance.

Estimates of rumen methanogenic archaea and protozoa populations later showed linear reductions in total methanogens and total protozoa with increasing levels of CT (Tan et al., 2011) and this may justify the observed outcome. In another study, reduced emissions of CH_4 were observed at all levels with the greatest reduction observed at 50%. The values of methane (CH_4) emissions for goats are still being estimated (IPCC,

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2006; Moeletsi, 2017). There are reports indicating a decrease in CH_4 emission with inclusion of CT-containing forage (Carulla et al., 2005; Puchala et al., 2005). In the current study, variations in sweet thorn leaf meal inclusion level had different effects on the enteric CH_4 emission by the goats. Different effects of sweet thorn leaf meal inclusion levels on CH_4 emission may indicate that inclusion level of CT (0.16-0.48%) in this study were below the required effective level.

Inclusion of sweet thorn leaf meal in the diets of male Boer goats did not cause a significant reduction in CH_4 emission. This indicates that tannins in sweet thorn leaf meal at the inclusion levels used in the present study were not enough to inhibit methanogens. This differs from Woodward et al. (2001) who reported lower (decreased by 24 - 29%) CH_4 production relative to digestible dry matter intake (DMI) in sheep when CT-containing forage, *Lotus pedunculatus*, was fed compared with ryegrass or alfalfa. The same authors observed a similar decrease of 23% in CH_4 emission relative to DMI when cows were fed *Lotus corniculatus* silage compared with ryegrass silage. This is because CH_4 emission is less in animals fed legumes than grasses (Benchaar et al., 2001; Van Dorland et al., 2007). Grobler et al. (2014), also, did a study comparing CH_4 production in different cattle breeds and reported that Bonsmara, Jersey and Nguni cattle produced less CH_4 on forage sorghum compared to natural pasture. The same authors further concluded that less CH_4 production in forage sorghum is possibly due to the tannin content found in forage sorghum, which inhibits methanogens. Carulla et al. (2005) reported that inhibition of methanogens by CT was primarily the result of suppressed fibre degradation that limits hydrogen gas (H_2) derived from synthesis of acetate. Results of the present study revealed that tannin contents in sweet thorn leaf meal inclusions were not adequate for suppressing CH_4 production and emission by Boer goats.

Conclusions and recommendations

It is concluded that nutrient composition of sweet thorn leaves in this study appeared adequate for ruminant growth. The species contained more than 12% of crude protein. This is quite high and ideal for protein supplementation in animal nutrition. In general, it is advisable to use sweet thorn leaves as a source of protein. Additionally, the low tannin contents in sweet thorn leaves indicate that sweet thorn can be safe to use in animal nutrition if used sparingly. With regard to methane emission, sweet thorn leaf meal inclusion levels used in the present study did not have a significant reduction on methane emission. However, there is need to determine levels of inclusion for optimal methane emission reduction in goats.

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INVASIVE PLANT SPECIES AFFECT SODOM APPLE (*CALOTROPIS PROCERA* (AITON) W. T. AITON) AND ASSOCIATED PLANTS BY ALTERING SOIL PHYSIOCHEMICAL CHARACTERISTICS IN NORTHWEST PAKISTAN

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Abstract. Invasive plant species have deliberately affected and reduced the populations of native species, thereby increasing their risk of extinction. The objective of this research was to compare the effects of the two most aggressive invasive alien species currently invading habitats of *Calotropis procera* "an ecologically and medicinally important native plant" in northwest Pakistan, most probably by altering vegetation and soil physical and chemical characteristics. We sampled invaded and non-invaded vegetation of exotic invasive and native species in diverse habitats with corresponding environmental and soil parameters for multiple comparisons like species richness, diversity, and total plant coverage to assess the invasion effects. Our results exposed that soil physiochemical characteristics significantly differed among the native and exotic invaded-derived soils. Principal component analysis revealed that *C. procera* dominated vegetation was strongly affected by the invasion of *X. strumarium* and *P. hysterophorus* as a result of altered soil characteristics, thereby offering strong evidence that invasion of both exotic invasive species alter soil chemistry and ecology, which may create conditions favourable for invasive plants over native plants. Likewise, results showed that invasive species have completely dominated invaded sites (IV ≥45%) whereas in native vegetation both species are overlapping (IV ≥15%) and might be the strong competitors affecting community structure in the future.
Keywords: *plant invasion, native plants, alpha diversity, soil characteristics, importance values*

Introduction

Invasive plant species are considered the second highest global threat to ecosystems worldwide, decreasing biodiversity, increasing net primary productivity, and modifying the invaded environment (Vila et al., 2011; Pysek et al., 2012; Chen et al., 2015; Seifu et al., 2017). Biological invasion poses severe threats to agriculture and other natural resources, thereby affecting native communities, making them endangered, and causing extinction worldwide (Joshi, 2001; Kathiresan et al., 2005; Pysek et al., 2012). Nowadays, ecologists pay special attention to exotic species due to their adverse impacts on biodiversity loss, ecosystem invasibility, and especially disturbance of native flora (Grotkopp et al., 2002; Lake and Leishman, 2004). These exotic species cause economic loss worldwide; therefore, ecologists focus on the factors that enable their successful invasion (Schmidt and Drake, 2011). In this sense, soil characteristics play a fundamental role in the growth of plants by providing essential elements and nutrients for reproduction and development in specific habitats (Sperry et al., 2006). Soil nutrient availability and physicochemical properties play a crucial role in the establishment of exotic species, as invasive plants may alter various biotic and abiotic soil characteristics such as pH, nutrient pool, and moisture to promote invasion (Elgersma and Ehrenfeld, 2011; Osunkoya and Perrett, 2011; Lazzaro et al., 2014; Majewska et al., 2015). Many exotic

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plant invaders are known to interfere with native plants by changing soil physical and chemical properties such as moisture, temperature (Zavaleta, 2000; Belnap and Phillips, 2001; Hawkes et al., 2005; Sperry et al., 2006), soil pH (Kourtev et al., 1998; Timsina et al., 2011), amount of soil organic matter, aggregation (Windham and Ehrenfeld, 2003; Koutika et al., 2007), nitrogen, and phosphorus (Ehrenfeld, 2003; Chapuis-Lardy et al., 2006; Koutika et al., 2007; Dassonville et al., 2008). Likewise, soil microbial, micro-fauna behaviors (Belnap and Phillips, 2001; Evans et al., 2001; Chacon et al., 2009) and allelopathy intervention (Murrell et al., 2011) may also be key drivers in promoting successful invasion of many tropical plants.

Many studies emphasize that invasive species-dominated communities severely affect native plant species in biotic and abiotic factors (Allen et al., 2003; Yu et al., 2005; Stinson et al., 2006). Because of the change in phosphorus, nitrogen, and other element balances, the plants in disturbed soil have a significant impact on the local plant populations, leading to invasive species biomass expansion, that is greater than native vegetation (Dassonville et al., 2008; Liao et al., 2008; Castro-Díez et al., 2014). Pakistan has a long history of invasion and so far, about 700 invasive species have been reported (Nasim and Shabbir, 2012), of which *Xanthium strumarium* L., and *Parthenium hysterophorus* L., of the Asteraceae family, pose severe threats to native flora (Hussain and Zarif, 2003; Nasim and Shabbir, 2012). *P. hysterophorus* is an annual exotic ephemeral herb that is commonly known as white-top weed, which originated in the neo-tropical region but has expanded its populations now in the pan-tropical region as well (Navie et al., 1996; Mahadevappa, 1997; Jayasuriya, 2021). *P. hysterophorus* is spreading in rocky crevices, aggressively colonizing wastelands, along roadsides and water channels, as well as in agricultural lands (Shabbir, 2002). This plant has got international level importance because of its rapid propagation and invasion (Ali et al., 2018) due to its severe negative impacts on human health, crop production, and biodiversity (Chippendale and Panetta, 1994; McFadyen, 1995; Shabbir, 2002). Similarly, the genus *Xanthium* comprised of more than sixteen species (Dekker, 2011), in which *X. strumarium* (cocklebur) a monoecious annual herb, appeared to be one of the most noxious weeds in Pakistan and had been introduced due to the Russian-Afghan dispute in 1980s, thereby posing negative impacts on plant biodiversity, livestock and the economy (Rezene and Taye, 2014). This species was first described in Europe and later originated in North America (Stesevic and Petrovic, 2010; Dekker, 2011). Since then, a few sparse studies on both the species have been conducted with special emphasis on their invasive successes and ecological determinants in Pakistan (e.g., Khan et al., 2020; Ullah et al., 2021).

According to Shinwari and Qaiser (2011), about 7.8% species are endemic, and several are endangered in Pakistan. Among the native flora, *C. procera* (family Ascelpiadaceae) is one of the most important and critical native species distributed in the arid and semiarid regions of Pakistan (Abbas et al., 1992; Hassan et al., 2015). This Giant Milkweed is well-known for its allelochemicals or secondary metabolites like cardiac glycosides, sterols, flavonoids and triterpenes (Heneidak et al., 2006). It has been studied due to its photo-pathogenic properties (Kareem et al., 2008) and bio-controlling agents for plants and animals (Ahmad, 2005; Iqbal et al., 2005). Besides containing three toxic glycosides in its milky latex, it also has steroidal heart poisons (Zeng et al., 2008) and is also widely recognized for its adverse effects on the germination inhibition of several plants (Al-Zahrani and Al-Robai, 2007; Yasin et al., 2012). This evergreen shrub is native to Pakistan and distributed in diverse habitats (i.e., riverside, forests, roadsides, and areas

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containing ruderal species) at an elevation ranging from 200 to 1500 m (Hussain, 2020). Despite its adverse effects, this plant provides various ecosystem services, benefiting different stakeholders and hosting many beneficial insects (Bidak et al., 2015; Hassan et al., 2015). However, in the last few decades, this species has been severely exploited and therefore, under severe threat and at the risk of becoming endangered, primarily due to anthropogenic activities like explicit harvesting for medicinal and burning purposes by the local inhabitants. Secondly, the most severe and unnoticed threat to this vital plant and its associated vegetation is possibly due to the rapid invasion of two aggressive noxious invasive species *i.e. X. strumarium* and *P. hysterophorus,* which deliberately change the vegetation structure and soil composition, making the environment suitable for their invasion (Khan et al., 2020; Ullah et al., 2021). Here we hypothesized that *C. procera* native populations faced severe threats due to the rapid invasion of these invasive species through the interference of soil physical and chemical characteristics, thereby setting the objectives for this study 1) to compare the selected biotic and abiotic factors between *X. strumarium* and *P. hysterophorus* (invaded) and *C. procera* dominated vegetation (non-invaded sites), 2) to investigate the effects of abiotic variables on community structure and, 3) to assessing the factors that have a significant impact on the *C. procera* populations in their native range.

Materials and methods

Study site

The study was conducted in the selected administrative divisions (i.e., Malakand, Peshawar, and Hazara) of Khyber Pakhtunkhwa, Pakistan (34.11 to 34.59 N and 71.44 to 73.14 E) located in the northwest of the country (*Fig 1*). The climate is varied, with annual average rainfall ranging from 347 to 600 mm and an average annual temperature of 27°C, respectively. The province's topography ranges from rocky outcroppings in the south to forests and green plains in the north (Marwat et al., 2010). The province's elevation ranges from 327 to 7708 meters above sea level, whereas the sampling was conducted between 350 to 1456 meters across the current distribution ranges of the species. This province is situated at the crossroads of the Iranian plateau and the Eurasian land plate, where the Hindukush Mountains and the Eurasian land plate meet (Shad et al., 2011). It has a wide range of climatic conditions, with warm summers and bitterly cold winters in the mountainous north. On the other hand, the southern regions have very different climatic patterns, with topography ranging from dry, rocky areas in the south to woodlands and fertile plains in the north (Marwat et al., 2010).

Sampling design

A total of sixty-nine study sites (twenty-three each for *Xanthium*, *Parthenium*, and *Calotropis*) were randomly selected during the summer of 2019 in Khyber Pakhtunkhwa, Pakistan, for vegetation analysis and collection of soil samples after extensive field survey. All the sites were moderately or purely dominated by the investigated species where sampling was performed by following standard procedure (Vanderhoeven et al., 2005). At each study site, ten plots of 3×3 m were established along a 100 m transect and data was recorded in 345 plots, including two invasive (i.e., *X. strumarium* and *P. hysterophorus*) and one native *C. procera* dominated vegetation. The invaded and native plots were chosen as close as possible to elaborate the effect of invasion on

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vegetation populations, and at least 10m from the edge of a patch to minimize any effects of contrasting vegetation such as shading or litter-fall. The phytosociological variables recorded in each plot include the frequency, density, and cover of each plant species to calculate the importance value index (IVI) following Mueller-Dombois and Ellenberg (1974) and Brower et al. (1998). All the plants were identified following the Flora of Pakistan (Nasir and Ali, 1995) and also confirmed through various pictorial guides. Likewise, three biodiversity indices were calculated to compare vegetation between invading and non-invaded sites, i) species richness (*S*), referring to the number of species per plot in each vegetation group, ii) Shannon diversity index *H'* was computed using the formula H' = -$\sum pi$ ln *pi*, where pi = frequency of (*i*th) species per plot. Furthermore, the Evenness index (J') was calculated to estimate the relative abundance of different species in a particular community type using the formula given:

$$\text{Evenness index } (J') = \frac{H'}{\ln S} \qquad \text{(Eq.1)}$$

where *H'* is Shannon-Wiener index and *S* is the total number of species in each site (Mahdavi et al., 2013; Zhang et al., 2017).

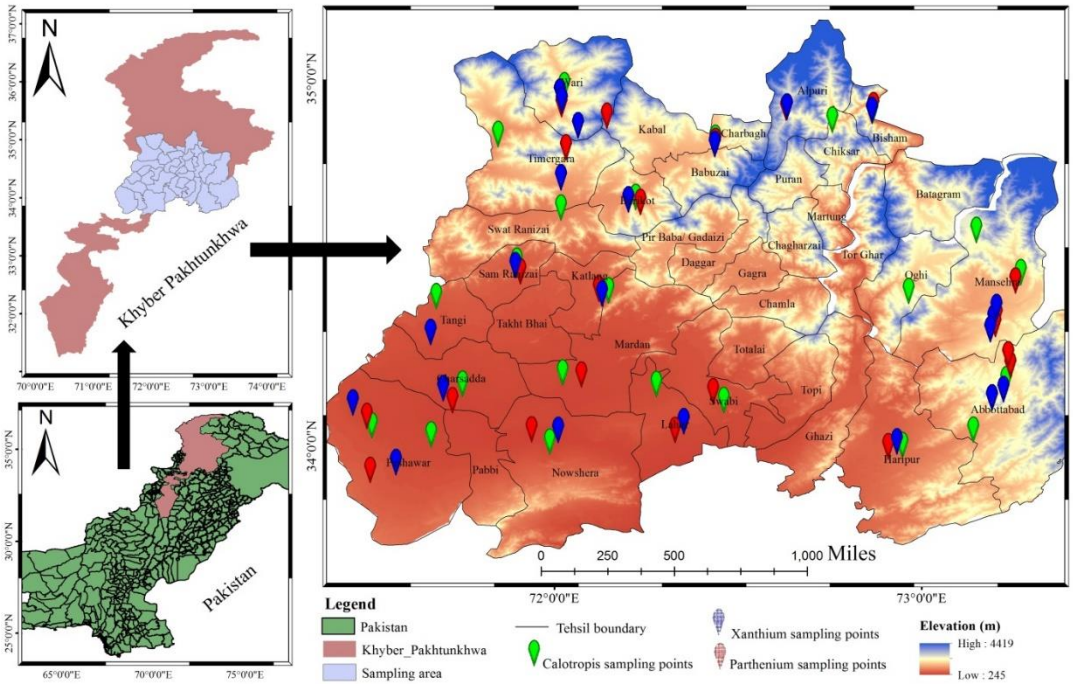

Figure 1. *Location of the sampling points of the investigated species in Khyber Pakhtunkhwa, Pakistan showing invaded and non-invaded sites*

Spatial parameters of the sites were recorded using a Geographic Positioning System (GPS), altimeter, and clinometer. Likewise, soil bulked samples (3 replicates per stand) were collected during the peak season at a depth of 20cm following a completely randomized design. All samples were air-dried, and visible fine roots were removed before physiochemical analysis in the laboratory. All the collected samples of native and invasive species were brought to the Agriculture Research Institute Swat (ARIS) for further analysis.

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Soil physiochemical analysis

We measured fourteen soil physical and chemical properties, namely soil texture (sand, silt and clay), pH, organic matter, nitrogen (N^{3-}), phosphorus (P^{3-}), potassium (K^{1+}), total carbon (TC) and soil hydraulic properties (i.e., EC µS/cm, wilting point (mm/m), field capacity (mm/m), saturation point (%) and available water (%). Soil samples were sieved (2-mm mesh) by mechanical sieve shakers before determining dry weight and moisture content after drying treatment at 105 °C overnight. We followed the hygrometer procedure proposed by Bouyoucos (1936) for soil texture properties. Although, this is a simpler and quicker analytical method, it is considered to be less accurate than other methods, like Pipette and Bouyoucos_M-T methods (Beretta et al., 2014; de Oliveira Morais et al., 2019). Soil pH was measured within 15 minutes by preparing 1:5 soil-water suspensions using a digital pH meter (McLean, 1983). Soil organic matter (SOM %) was determined by the loss-on-ignition method following Salehi et al. (2011), whereas nitrogen, phosphorus, and potassium (NPK) were determined using an optical transducer method recently introduced by Masrie et al. (2017). Likewise, we estimated different soil hydraulic properties (SHP) that included electrical conductivity (EC), wilting point (WP), field capacity (FC), saturation point (SP) and available water (AW) following the methods outlined by Marandi et al. (2013), Assi et al. (2019) and Mbah (2012), respectively.

Statistical analysis

Descriptive statistics of the data coupled with inferential parameters were used to describe and interpret the information obtained from the data. Analysis of variance (ANOVA) was performed on log-transformed (for data uniformity and better results) data, followed by a post-hoc Tukey HSD test for inter-group differences in environmental and soil parameters. All soil and vegetation variables were subjected to Principal component analysis (PCA) to distinguish relationships between them and produce a limited number of principal components (PCs) that represented the key sources of heterogeneity in the soil dataset. To achieve a better understanding of invasion effects on soil (some PCs can be difficult to read if loadings on all variables within a PC are not very high), we performed split-plot ANOVA to use all PCs and variables with the highest loading on each PC as dependent variables. The split-plot ANOVA was used in order to investigate the impact of site types (3-site types, with *C. procera*, *X. strumarium* or *P. hysterophorus*, including both native and invasive plots; whole plot factor), locations (2-locations –dominated by native and invasive species; whole-plot factor) and invasion (2-plot types within sites– invaded vs. native; split-plot factor), and their interaction on soil physiochemical characteristics, density (D. ha^{-1}) and cover per hectare (C. ha^{-1}) respectively (Stefanowicz et al., 2017). In addition, contrast analyses were also performed for the comparison of invaded and non-invaded sites separately to measure the effects of invasion at each level. All the statistical analyses interpreted in this research paper were performed using the software's PAST (ver. 2.17) and SPSS (ver. 22).

Results

The floristic diversity revealed a total of 105 species belonging to 43 families at invaded and non-invaded study sites. Species richness was found higher in invaded sites comprising of 52 different species as compared to native vegetation (40 species). Species

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distribution was uneven among the families e.g., Asteraceae (17 species) followed by Poaceae (11 species), Fabaceae (7 species), Euphorbaceae and Amaranthaceae each with 6 species were the families of high species richness. Brassicaceae and Solanaceae were represented by 5 species, Polygonaceae and Lamiaceae with 4 species and Rhamnaceae and Moraceae with 3 species each. However, the remaining families contributed only two or one species. The invaded communities were dominated by *P. hysterophorus* and *X. strumarium* with a mean importance value of 47.49 ± 3.52% and 46.36 ± 1.5% respectively. In contrast, *C. procera* occurred with very low importance values (0.16 ± 0.15% and 0.59 ± 0.43%) in these invaded sites. The non-invaded communities in all the studied sites were dominated by *C. procera* with a mean importance value of 22.71 ± 3.81%, where the invasive species were found in all sites almost competing with 17.23 ± 1.56% and 15.38 ± 1.78% of importance values. The invasive *Xanthium* and *Parthenium* were overlapping with the native species in all the non-invaded sampling sites (*Table 1*). The vegetation study also revealed that the selected invasive species were completely dominating in the invaded area (IVI ≥45%). In contrast, in the native sites, these are the strong competitors of the native species and were found overlapping in the vegetation affecting the whole structure of the communities.

Similarly, we also observed clear differences in soil physiochemical characteristics between native and invaded sites (*Table 2*). Among the environmental variables, elevation and aspect angle did not differ significantly between the invaded and non-invaded sites. Concerning soil texture properties, the invaded sites represent the highest percent of sand (31.4 ± 3.3 and 41.1 ± 2.6 for *X. strumarium* and *P. hysterophorus* respectively) and silt (42.8 ± 4.1 and 35.1 ± 2.1 for *X. strumarium* and *P. hysterophorus* respectively) compared to clay content (25.7 ± 2.4 and 27.9 ± 2.4 for *X. strumarium* and *P. hysterophorus*, respectively) which shows a significant variation ($p < 0.05$) between invaded and non-invaded sites. Invaded sites exhibited higher levels of phosphorus (P), organic matter (OM %), total carbon (TC), and electrical conductivity (EC) than native sites showing significant variations ($p < 0.05$), suggesting that invaded areas are nutrient-rich and may better sustain plant development. In contrast, some soil nutrients i.e. Potassium (K) and Nitrogen (N) show less variation among the invaded and non-invaded sites. In soil hydraulic properties saturation point (SP) and available water show significant differences among the invaded and non-invaded sites ($p < 0.05$) as revealed in figure (2).

Plant species composition differed significantly between the invaded and non-invaded sites i.e. species richness (S) having $F = 3.838$, and $p = 0.0287$ indicating a smaller number of species in native sites compared to invaded site. Similarly, Shannon index (H') and evenness index (J') also show the same significant pattern of distribution (F = 20.099, P = 1.597E-7: F = 34.649, P = 2.15E-18) between the invaded and non-invaded sites (*Fig. 2*). Moreover *X. strumarium* and *P. hysterophorus* (invaded) characterized by high density and cover per hectare when compared to the *C. procera* (non-invaded) dominated communities indicating abundance of species in the invaded sites. Three Principal Components (PCs) were selected as a representation of soil properties based on a screen plot and the strength of correlations between the PCs and original variables. The PCs explained 76.6% of the total variance in the soil data (*Table 3*). PC 1 correlated strongly with Clay, Wilting point, Field capacity, sand, and organic matter. PC 2 correlated mainly with species richness, sand, Shannon index, and available water, whereas, PC 3 correlated with available water, silts, and percent sand content.

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Table 1. *Comparison of species importance values between the non-invaded and invaded communities*

Species name	A	S	Capr (Mean±SE)	Xast (Mean±SE)	Pahy (Mean±SE)
Calotropis procera (Aiton) W.T.Aiton	Capr	N	22.81±0.72	0.59±0.43	0.16±0.15
Xanthium strumarium L.	Xast	I	15.38±1.78	46.36±1.5	2.61±0.56
Parthenium hysterophorus L.	Pahy	I	17.23±1.56	8.33±1.3	47.49±3.52
Acacia nilotica L.	Acni	Nt	0.33±0.24	-*	0.13±0.09
Aloe barbadensis Mill	Alba	C	0.11±0.11	-*	-*
Amaranthus viridis L.	Amvi	I	2.09±1.19	3.76±0.85	2.31±0.84
Artemisia absinthium L.	Arab	N	2.01±1.38	-*	-*
Asparagus gracilis Royle	Asar	N	1.38±0.94	-*	-*
Avena sativa L.	Avsa	I	3.21±1.73	-*	-*
Cannabis sativa L.	Casa	I	3.17±1.52	11.23±1.5	6.01±1.2
Carthamus oxycantha M. Bieb.	Caox	Nt	4.06±1.53	0.70±0.70	0.39±0.39
Chenopodium album L.	Chal	I	1.44±1.01	3.63±0.88	0.81±0.76
Conyza bonariensis L.	Cobo	Cs	1.78±1.26	-*	2.23±0.73
Cynodon dactylon L.	Cyda	N	6.99±1.69	2.62±0.77	17.31±2.21
Dodonaea viscosa (L.) Jacq.	Dove	I	1.14±0.81	-*	0.32±0.22
Erigeron canadensis (L.) Cronq.	Erca	I	1.83±1.19	0.48±0.36	0.36±0.35
Eucalyptus camaldulensis L.	Euca	I	1.03±0.57	-*	0.36±0.35
Euphorbia helioscopia L.	Euhe	I	2.31±1.20	-*	0.10±0.09
Ficus carica L.	Fica	N	1.03±0.52	-*	0.11±0.07
Ipomoea purpurea (L.) Roth	Ippu	I	1.004±0.72	-*	-*
Jasminum officinale L.	Jaof	I	0.61±0.61	-*	-*
Lamium amplexicaule L.	Laam	N	0.30±0.30	-*	-*
Malva sylvestris L.	Masy	I	0.56±0.56	-*	-*
Medicago denticulata L.	Mede	I	4.52±1.79	-*	-*
Melia azedarach L.	Meaz	Cu	2.63±1.15	-*	0.31±0.15
Mentha longifolia (L.) Huds	Melo	Cu	1.61±1.12	0.77±0.58	1.19±0.74
Morus alba L.	Moal	Nt	0.11±0.11	-*	1.04±1.03
Ricinus communis L.	Rico	I	1.95±1.07	-*	-*
Rumex dentatus L.	Rude	N	2.28±1.27	-*	1.15±0.49
Silybum marianum (L.) Geartn	Sima	I	1.43±0.82	2.42±0.80	-*
Solanum nigrum L.	Soni	I	1.99±1.18	0.16±0.15	0.59±0.30
Sonchus asper (L.) Hill	Soas	N	2.04±1.40	0.57±0.33	0.06±0.06
Trianthema portulacastrum L.	Trpo	I	2.52±1.14	-*	-*
Trifolium repens L.	Trre	I	1.49±1.04	2.07±0.89	0.56±0.35
Triticum aestivum L.	Trae	Cu	2.48±1.37	-*	-*
Urtica dioica L.	Urdi	N	2.39±1.35	1.57±0.33	-*
Verbascum Thapsus L.	Veth	I	1.93±0.80	0.72±0.5	0.19±0.11
Zanthoxylum armatum DC. Prodr	Zaar	N	1.31±0.91	-*	-*
Ziziphus mauritiana L.	Zima	N	1.09±0.76	-*	1.11±0.87
Alternanthera pungens Kunth	Alpu	I	-*	0.84±0.34	-*
Amaranthus spinosus L.	Amsp	I	-*	0.19±0.19	-*
Brassica campestris L.	Brca	N	-*	0.84±0.44	0.53±0.39
Capsella bursa pestoris (L.) Medik	Cabp	I	-*	1.19±0.5	0.18±0.13
Cenchrus ciliaris L.	Ceci	N	-*	0.35±0.34	-*
Calendula arvensis L.	Caar	I	-*	1.81±0.75	-*
Cassia occidentalis L.	Caoc	I	-*	0.70±0.70	-*
Cyprus rotundus L.	Cyro	N	-*	0.41±0.23	1.61±1.05
Chrozophora tinctoria (L.) Raf.	Chti	N	-*	0.21±0.20	-*

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Species name	A	S	Capr (Mean±SE)	Xast (Mean±SE)	Pahy (Mean±SE)
Datura metel L.	Dame	I	-*	3.11±1.3	0.060±0.02
Eclipta alba L.	Ecal	N	-*	0.58±0.41	-*
Helianthus annuus L.	Hean	I	-*	0.13±0.12	-*
Heliotropium curassavicum L.	Hecu	N	-*	0.11±0.10	-*
Justicia adhatoda L.	Juad	N	-*	0.91±0.62	-*
Lepidium sativum L.	Lisa	N	-*	0.88±0.36	-*
Mirabilis jalapa L.	Mija	N	-*	0.77±0.58	0.09±0.08
Oxalis corniculata L.	Oxca	Nt	-*	0.14±0.14	2.33±0.7
Polygonum aviculare L.	Poav	I	-*	0.16±0.16	0.11±0.11
Physalis minima L.	Phmi	N	-*	2.42±0.80	-*
Tagetes erecta L.	Taer	I	-*	1.42±1.08	-*
Tagetes minuta L.	Tami	Nt	-*	1.04±1.03	0.56±0.35
Tribulus terrestris L.	Trte	N	-*	0.10±0.09	0.05±0.04
Zea mays L.	Zema	Cu	-*	0.17±0.17	1.11±0.87
Achyranthes aspera L.	Acas	N	-*	-*	0.06±0.05
Ailanthus altissima L.	Alal	I	-*	-*	2.31±0.84
Broussonetia papyrifera (L.) L'Herit. ex Vent	Brpa	I	-*	-*	0.22±0.20
Centaurea cyanus L.	Cecy	N	-*	-*	0.53±0.39
Cirsium arvense (L.) Scop.	Ciar	I	-*	-*	0.81±0.76
Convolvulus arvensis L.	Coar	N	-*	-*	0.41±0.32
Cucurbita pepo L.	Cupe	Cu	-*	-*	0.18±0.13
Desmostachya bipinnata L.	Debi	I	-*	-*	0.044±0.02
Dichanthium annulatum (Forssk).	Dian	I	-*	-*	1.61±1.05
Dysphania ambrosioides	Dyam	I	-*	-*	0.52±0.37
Eichhornia crassipes (Mart.) Solma in DC	Eicr	I	-*	-*	0.33±0.14
Eragrostis minor L.	Ermi	N	-*	-*	0.060±0.02
Eryngium coeruleum M.Bieb.	Erco	N	-*	-*	0.36±0.35
Euphorbia hirta L.	Euhi	N	-*	-*	0.076±0.07
Euphorbia prostrate L.	Eupr	I	-*	-*	1.03±0.73
Fragaria indica L.	Frin	N	-*	-*	0.24±0.16
Hackelia virginiana L.	Havi	I	-*	-*	0.13±0.13
Hibiscus mutabilis L.	Himu	I	-*	-*	0.69±0.34
Indigofera gerardiana L.	Inge	N	-*	-*	0.08±0.06
Mangifera indica L.	Main	N	-*	-*	0.06±0.01
Mallotus Philippines L.	Meph	I	-*	-*	0.09±0.08
Mentha spicata L.	Mesp	I	-*	-*	0.07±0.06
Narcissus tazetta L.	Nata	I	-*	-*	0.14±0.11
Nasturtium officinale W.T. Aiton	Naof	Nt	-*	-*	0.15±0.11
Persicaria maculosa S.F.Gray	Pema	N	-*	-*	0.15±0.07
Phragmites karka (Retz). Trin. Ex Saud	Phka	I	-*	-*	2.33±0.77
Pinus roxburghii L.	Piro	N	-*	-*	0.11±0.11
Poa annua L.	Poan	N	-*	-*	0.21±0.13
Populus nigra L.	Poni	I	-*	-*	0.06±0.06
Prosopis juliflora (Sw.) DC.	Prju	I	-*	-*	0.21±0.16
Robinea pseudoacacia L.	Rops	I	-*	-*	0.20±0.11
Rubus fruticosus L.	Rufr	N	-*	-*	1.15±0.49
Rumex hestatus L.	Ruhe	I	-*	-*	0.04±0.02
Salvia moorcroftiana Wall. Ex. Benth	Samo	N	-*	-*	0.16±0.11

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Species name	A	S	Capr (Mean±SE)	Xast (Mean±SE)	Pahy (Mean±SE)
Sisymbrium officinale (L.) *Scop.*	Siof	I	-*	-*	0.76±0.64
Solanum melongena L.	Some	N	-*	-*	0.15±0.12
Solanum xanthocarpum (SX). Schard and Wendl.	Soxa	N	-*	-*	0.042±0.01
Sonchus oleraceus L.	Sool	N	-*	-*	0.59±0.30
Taraxicum officinale Weber	Taof	I	-*	-*	0.06±0.06
Trianthema portulacastrum L.	Trpo	I	-*	-*	0.065±0.06
Triticum aestivum L.	Trae	N	-*	-*	0.05±0.04
Ziziphus nummularia (Burm.f.). Wight and Arn.	Zinu	I	-*	-*	0.047±0.01
Zizipus oxyphyla L.	Ziox	I	-*	-*	0.047±0.02

Note: A (Acronyms): S (Status): N(Native): Nt (Naturalized): I(Invasive): Cu (Cultivated): values are presented as mean and standard error: -* (absence of species)

Table 2. *Comparison of environmental drivers and diversity indices in the plant communities of invaded and non-invaded sites*

Factors	*Capr*	*Xast*	*Pahy*	F-value	P-value
	Mean+SE	Mean+SE	Mean±SE		
Lat	34.33±0.06^{a}	34.20±0.45^{a}	34.708±0.03^{a}	1.684	0.194
Long	72.382±0.22^{a}	71.81±0.51^{a}	72.100±0.36^{a}	0.502	0.684
Elev	841±42^{a}	822±50^{a}	802±37^{a}	1.376	0.3011
AA	160±20^{a}	159±20^{a}	190±24^{a}	0.98	0.3806
Cl%	24.7±2.6^{a}	25.7±2.4^{a}	27.9±2.4^{a}	1.998	0.14
Si%	32.5±2.4^{ab}	42.8±4.1^{a}	35.1±2.1^{b}	3.1395	**0.051***
Sa%	36.56±2.4^{b}	31.4±3.3^{b}	41.1±2.6^{a}	3.01	**0.0484***
pH	6.7±0.1^{a}	6.71±0.08^{a}	6.93±0.11^{a}	1.029	0.3631
OM%	0.91±0.1^{c}	1.08±0.1^{b}	2.29±0.23^{a}	18.912	**3.27E-7*****
N	0.12±0.02^{ab}	0.04±0.09^{b}	0.127±0.1^{a}	4.006	0.0911
P	4.48±0.2^{a}	4.5±0.23^{a}	3.30±0.51^{b}	3.8413	**0.0327***
K	90.5±7.9^{b}	108±8.4^{a}	104.7±8.0^{a}	1.607	0.2083
EC	281±14^{c}	294±20^{b}	365±1.8^{a}	11.1	**6.94E-05*****
TC	1.49±0.07^{b}	1.88±12^{a}	0.985±0.12^{c}	18.028	**4.64E-01*****
WP	0.17±0.07^{a}	0.15±0.01^{a}	0.15±0.02^{a}	2.9981	0.3741
FC	0.30±0.08^{a}	0.31±0.01^{a}	0.28±0.01^{a}	1.5971	0.2744
SP	0.49±0.01^{b}	0.48±0.05^{a}	0.47±0.07^{a}	2.9981	**0.051***
AW	0.13±0.04^{b}	0.14±0.06^{a}	0.039±0.03^{b}	3.398	**0.038***
S'	5.603±0.22^{c}	7.52±0.56^{b}	9.12±0.88^{a}	3.838	**0.0287****
H	1.769±0.03^{a}	1.39±0.05^{b}	1.18±0.08^{c}	20.099	**1.597E-7*****
J	0.94±0.05^{a}	0.71±0.09^{b}	0.56±0.03^{c}	34.649	**2.15E-18*****
D/ha	3067±807^{b}	11697±55^{c}	8193±230^{a}	8.522	**0.0029****
C/ha	3866±785^{c}	12318±68^{a}	7282±128^{b}	11.113	**2.41E-02*****

Note: Native plots were compared with invasive plots of *Xanthium strumarium* and *Parthenium hysterophorus* using One-way ANOVA followed by Tukey's HSD test. A significant difference was shown by different letters. SD (standard deviation), Lat (latitude), Long (longitude), Ele (elevation), AA (aspect degree), OM (organic matter), N (nitrogen), P (phosphorous), K (potassium), EC (electrical conductivity), TC (total carbon), WP (welting point), SP (saturation point), AW (available water), S' (species richness), H' (Shannon index), J' (evenness index), D/ha (Density ha^{-1}), C/ha (Cover ha^{-1})

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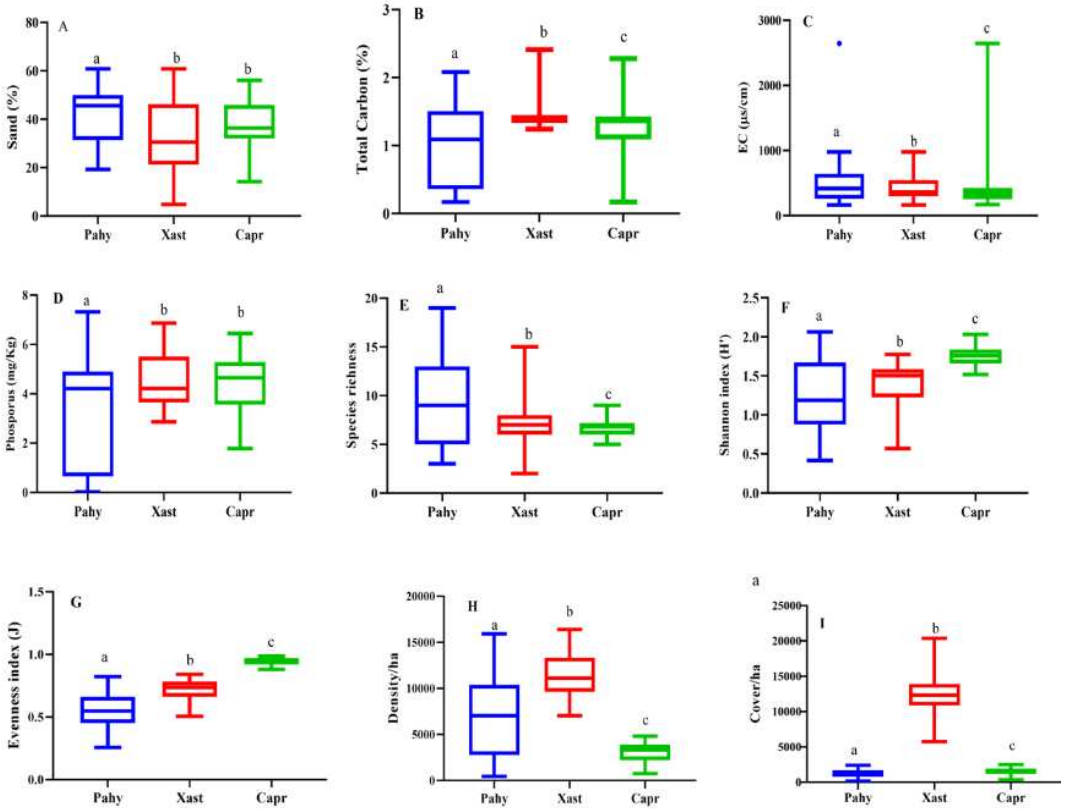

Figure 2. *Box-plot diagrams for comparison of significant values among the native and invasive plots. Different letters indicate significant difference between the native and invasive species (Calculated through post-hoc Tukey HSD test at p<0.05). A. % of Sand: B. % of Total Carbon: C. Electrical conductivity (µs/cm): D. Phosphorus (mg/Kg): E. Species Richness: F. Shannon index (H'): G. Evenness index (J): H. Density ha^{-1}: I. Cover ha^{-1}*

Table 3. *Three major axis of principal components with its explained variance and loading value of associated environmental variables*

Factors	% variance explained	Variables loading (with value >0.6)
PC-1	37.0	Clay (0.979), WP (0.965), FC (0.864), SP (0.932), Sand (-0.796), EC (-0.829), OM (-0.733)
PC-2	15.7	S (0.897), Sand (0.858), H' (0.832), Ele (0.723), AW (-0.788), Si (-0.721), J' (0.785), D ha^{-1} (0.727), OM (0.635), N (0.666), C ha^{-1} (-0.788)
PC-3	23.5	AW (0.965), Sand (-0.920), Silt (0.899)

Cumulative variance percentages along the PCA axis are presented in *Table 4*, showing that 22 axes cumulatively show 100% variance of the entire axis and can be considered for variation. The 1st four axes showed the highest cumulative variance (44.5%) having 9.2-13.3% of variance for a single axis. Plant invasions strongly reduced the number of native plant species and their cover while total plant cover remained unaffected (*Tables 3 and 4*). As far as soil properties are concerned, most variables were unaffected by invasion or by its interaction with site type or location (*Tables 5*). PCA diagram showed that *C. procera* vegetation was strongly affected by *X. strumarium* and

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P. hysterophorus community invasions along with the soil characteristics (*Fig. 3*). Plant invasion effects on PC2 and available water depended on invasive species as indicated by significant invasion × site type interactions (*Table 5*). Contrast analysis revealed that PC2 changed due to the invasion compared to control. Still, the direction of the changes differed between species (a decrease in the former species and an increase in the latter). The only significant invasion × location interaction was observed in PC2 (*Table 5*), but contrast analysis did not confirm that the invasion impact differs between locations.

Table 4. *Percentage cumulative variance and the axes of Principal Component (PC) of different environmental variables associated with invaded and non-invaded communities*

Axis	Eigen-values	% variance explained	Cumulative variance	Broken-stick Eigenvalues
X_1	151.8	13.3	13.3	141.1
X_2	137.6	12.1	25.4	106.6
X_3	112.8	9.9	35.3	89.3
X_4	104.5	9.2	44.5	77.8
X_5	94.6	8.3	52.8	69.2
X_6	83.9	7.4	60.2	62.3
X_7	74.3	6.5	66.7	56.5
X_8	65.3	5.7	72.4	51.6
X_9	57.9	5.1	77.5	47.3
X_{10}	45.2	4.0	81.5	43.5
X_{11}	43.7	3.8	85.4	40.0
X_{12}	32.4	2.8	88.2	36.9
X_{13}	29.3	2.6	90.8	34.0
X_{14}	24.2	2.1	92.9	31.3
X_{15}	22.3	2.0	94.8	28.9
X_{16}	18.8	1.7	96.5	26.6
X_{17}	11.5	1.0	97.5	24.4
X^{18}	9.9	0.9	98.4	22.4
X_{19}	8.9	0.8	99.2	20.5
X_{20}	4.5	0.4	99.6	18.7
X_{21}	3.5	0.3	99.9	16.9
X_{22}	1.6	0.1	100.0	15.3

Table 5. *The effects of site type, location, invasion, and their interactions portrayed by the first three principal components (PCs), soil and vegetation characteristics using split-plot analysis of variance (ANOVA)*

Variables	Site type		Location		Invasion		Site type ×Location		Invasion ×Site type		Invasion ×Location		Invasion ×Site type ×Location	
	F	P	F	P	F	P	F	P	F	P	F	P	F	P
PC 1	**34.00**	**<0.00**	1.50	0.31	0.16	0.24	6.30	**0.00**	0.94	0.64	0.85	1.74	1.92	0.3
PC 2	1.35	0.106	**6.03**	**0.00**	**2.30**	**0.01**	1.43	0.45	**7.23**	**0.00**	0.44	0.391	1.85	0.26
PC 3	0.293	0.232	0.4	0.51	0.30	0.36	**5.30**	**0.00**	0.22	1.43	0.80	0.421	1.90	0.31
Clay	**5.87**	**0.004**	0.54	0.4	1.34	0.26	**8.96**	**0.00**	16.8	3.08	**6.70**	**0.008**	1.31	0.54
S	0.487	0.044	1.48	0.09	**11.3**	**<.01**	1.33	0.11	0.38	1.06	1.47	0.810	**15.1**	**<.01**
Aw	1.97	0.24	0.93	0.57	0.49	0.14	1.08	0.40	**5.87**	**0.00**	0.97	0.567	**10.5**	**0.00**
D h^{a-1}	**12.54**	**<0.00**	0.74	0.24	**30.4**	**<.00**	1.99	0.64	2.49	1.91	**2.87**	**0.010**	0.42	0.82
C ha^{-1}	**4.65**	**0.015**	1.65	0.19	0.40	0.70	1.76	0.97	0.56	0.46	0.93	0.579	0.47	0.88

Note: Soil physiochemical variables were chosen based on the principal component analysis. Significant values (p<0.05) are shown in bold

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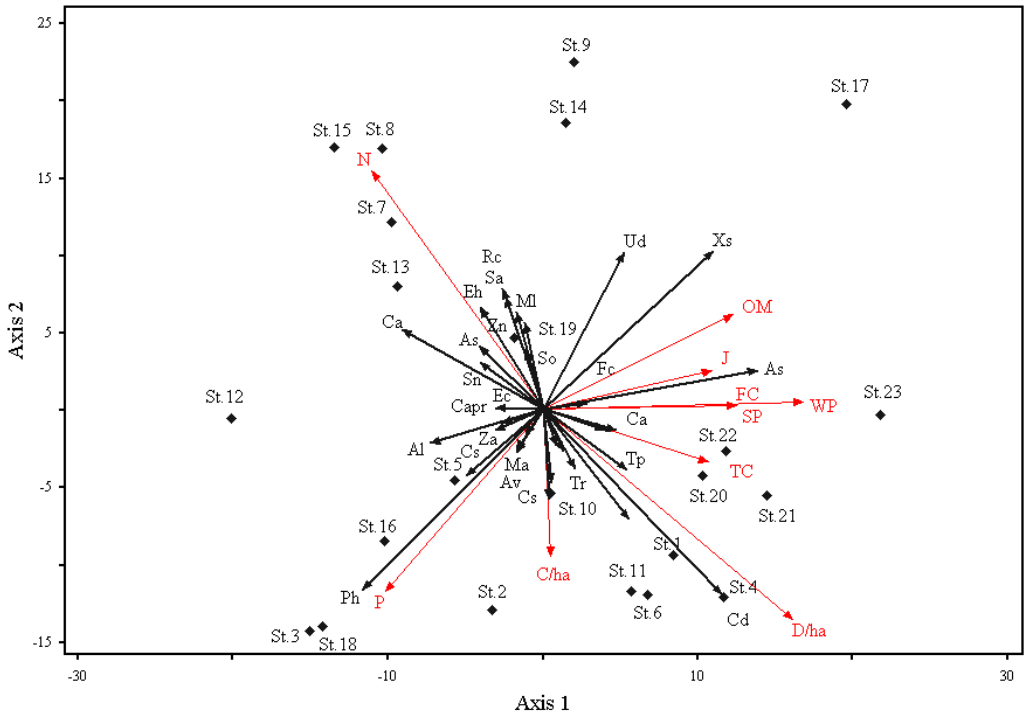

Figure 3. *PCA-ordination showing the effect of environmental variables on plant communities*

Discussion

Invasion of plant species causes significant changes in soil physio-chemistry and vegetation characteristics, and invasive species are frequently found to disrupt native vegetation quickly and intensely (Ullah et al., 2021) The invaded sites demonstrated low IVI of *C. procera* and species diversity revealing that invasive species have negatively affected the native plant communities. These results were in compliance with the findings reported by Grice (2006); Mc Fadyen (1992); Sakai et al. (2001) for Australia, India, and Ethiopia, where the native and associated species were found to be strongly influenced by the invasion of invasive species. Invaded sites have a more diversified floristic composition (70 plant species) compared to non-invaded areas (45 plant species) presumably because invasion improves soil nutrient retention, which supports various heterogeneous populations. Physiochemical soil characteristics supported these impacts, showing that invaded areas had greater nutrient levels than non-invaded sites. Similarly, diversity indices are linked and reflect the theory proposed by Bimova et al. (2004), Dunbar and Facelli (1999), Kohli et al. (2004), and Pysek and Pysek (1995) in their findings. Our target invasive species was discovered near other invasive species such as *Silybum marianum* and *Amaranthus caudatus*, complementing Timsina et al. (2011), that disturbed communities' overlap, increasing the threat to native plant species. Changes in the physiochemical properties of soils caused by invading species are also considered feedback mechanisms with various eco-physiological manifestations, increasing the chances of invasive species establishment and propagation, and eventually posing a threat to native species (Stefanowicz et al., 2017). These changes illustrate how introduced plants will expand, disturb the natural ecology, and promote their role in causing human health and economic disruption (Charles and Dukes, 2008). In contrast, some authors are of the view that invasive species may be economically and ecologically beneficial in

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proportion to their negative consequences (Mack et al., 2000). Therefore, it is difficult to assume that the modification by invasive plants of the physiochemical characteristics of soil will always be lethal, harmful, or just beneficial, depending on the circumstances (Pimentel et al., 2000). The rise of nutrients in the soil area would also encourage many more plant species to thrive and spread with invasive plants that increase their diversity.

The findings clearly show significant disparities in soil properties between invaded and non-invaded locations, indicating that invasive species alter soil qualities in order to establish themselves in a new region, disrupting native plant populations and allowing invaders to expand rapidly. Similarly, Soti and Jayachandran (2016) reported that *lygodium microphyllum,* an invasive species in Florida, has changed and increased soil nutrients in areas of three different locations. Plant growth and productivity require organic matter in the soil (Lehmann and Kleber, 2015). Invaded sites with *X. strumarium* and *P. hysterophorus* had more organic matter than non-invaded sites compared to the native species *C. procera*, which might be due to quicker decomposition of soil litter in the root region caused by invasive plants, as advocated by Duda et al. (2003). Due to the differences in eco-physiological processes between invasive and non-invasive plants, invasive sites exhibit greater levels of nitrification and nitrogen mineralization than non-invaded sites (Hibbard et al., 2001; Ehrenfeld et al., 2001; Windham and Ehrenfeld, 2003). Our findings showed that invaded sites had higher nitrogen concentrations than non-invaded sites, owing to a faster decomposition rate and the process of nitrification may possibly enhance the process in the root region which might possibly cause increased soil fertility (Borken and Matzner, 2009). Changes in carbon and nitrogen concentrations would have a major influence on recycling, which may be as a consequence of invasive plant species' invasion and establishment in new habitats (Horgan et al., 2014). Hughes and Uowolo (2006) record a higher rate of invasion and a different mode of plant growth in an environment invaded by invasive plant species compared to the non-invaded area, which is in compliance with the findings of our results.

The pH differences between invaded and non-invaded sites were found to be inconsistent. According to Osunkonya and Perret (2011), invaded sites have a higher pH than non-invaded sites, which is consistent with our findings in urban and rural areas. Higher pH was found to increase cation concentration, allowing nutrient absorption for faster plant growth and production (James et al., 2005). The shift in pH of roadside soil, on the other hand, is in the opposite direction, confirming Kuotika et al. (2011). Many scholars, including Dassonville et al. (2007) for *Fallopia japonica*, Herr et al. (2007), Scharfy et al. (2009), and Quist et al. (2014) for *Solidago gigantean* and Maurel et al. (2010) for *Reynoutria japonica*, have documented this inconsistency of pH changes. Gordon (1988) reported that invasive species have greatly altered soil organic matter and texture, which ultimately have an effect on soil pH that can be easily detected.

Conclusion and recommendation

The study revealed higher species diversity in invaded areas than non-invaded sites and a higher concentration of nutrient and physiochemical characteristics. Thus, the increase in nutrients leads to an increase in the density and population of invasive species, thereby decreasing the native population of *Calotropis procera*, which is progressively be reduced in the region and may be endangered shortly. Many other factors may also contribute to threatening the native plant species, which may be the environmental conditions that may be better utilized by the invasive species and the plant's internal

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genetics, which is essential irrespective of the environmental conditions. The study recommends that the local and federal governments conduct extensive monitoring and preventive measures to eradicate and control these invasive species, which will not only flourish native species but also regulate the other ecosystem services. Here we recommend further detailed studies on the invasive behaviors of *X. strumarium* and *P. hysterophorus* and their impacts on soil micro-biota, nutrient pools in plant biomass and the content of secondary metabolites in both homogenous (in control environment) and heterogeneous environments. Likewise, we suggest investigating the seasonal changes in the influence of plant invasion on soil, which might be helpful, especially on the range of impacts of these invasive taxa and the overall magnitude of their increasing threats.

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INVASIVE PLANT ALLIGATOR WEED (*ALTERNANTHERA PHILOXEROIDES* (MART.) GRISEB.) PERFORMS BETTER TO SALINITY, DROUGHT AND ABSCISIC ACID STRESSES THAN NATIVE PLANT SESSILE JOY WEED (*ALTERNANTHERA SESSILIS* (L.))

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Abstract. Invasive plant species have been reported to have advantages over native species in growth- and physiological traits; however, such characteristics have not been discussed under saline, water-scarce conditions. Therefore, the present study was undertaken to investigate the effect of salinity and drought stress and abscisic acid (ABA) application on the morphological and physiological traits of invasive and native weed species. We established three experiments as (i) salinity stress, control with no salt application, 100 and 150 mM NaCl; (ii) drought stress, control-no drought, 75 and 100 gL^{-1} PEG-6000; and (iii) ABA application, control-no application, 25 and 50 ulL^{-1}. Two weed species, the invasive *Alternanthera philoxeroides* and native *Alternanthera sessilis*, were used in all experiments. The study was laid out in a completely randomized design (CRD) with factorial arrangements and twelve replications. Results revealed that both invasive *A. philoxeroides* and native *A. sessilis* species survived under high salt and drought stress conditions. *A. philoxeroides* species generally showed higher morphological and physiological growth under high salt and drought levels, showing higher tolerance to stress conditions than *A. sessilis.* Moreover, the morphological and physiological traits of *A. philoxeroides* were elevated more under ABA application. Our results showed higher values of morpho-physiological traits might partly explain the success of *A. philoxeroides* under saline and drought conditions.

Keywords: *invaders, abiotic stresses, growth, water deficit stress, polyethylene glycol, physiological attributes, comparative analysis*

Introduction

Invasive weeds are among the major problems for crop production, and also exerts a harmful impact on the environment (Pyšek and Richardson, 2008; Balachandar et al., 2021). The typical approach used to study these plants is to compare them with their native counterparts (Kettenring and Adams, 2011). However, only limited studies have discussed the performance of invasive and native species under different environmental conditions (Yu and He, 2021). It is commonly known that invasive species can perform better under abiotic stress stimuli, in terms of higher growth rate, effective resource use efficiency, and reproducibility capacity, compared with their native species (Funk, 2013). In a comparative study of native and invasive species, Godoy et al. (2011)

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reported that invasive species had performed better even under limited resource availabilities than their native ones. Also, in a meta-analytic study, invasive weeds have been reported as more dominant in morphological and physiological traits than native species (Palacio-López and Gianoli, 2011). However, in some studies, authors have reported higher growth rate and resource use efficiencies in terms of greater instantaneous photosynthetic energy-use efficiency and photosynthetic nitrogen-use efficiency in native species than invasive plants (Heberling and Fridley, 2013). Among invasive weeds, *Alternanthera philoxeroides* is an amphibious stoloniferous perennial herb native to South America, and firstly was introduced to China and Japan as a forage crop (Myanmar et al., 2016). It is characterized as a higher growth rate and vegetative reproduction than their corresponding native species (Yang et al., 2019). Also, new plants are produced through the splitting from roots and stems (Amsellem et al., 2000). Now a days, it is found across the globe, both in aquatic and terrestrial environments. *A. philoxeroides* had dominant characteristics and diversified habits in term of better plasticity to cope with different abiotic stresses (Chen et al., 2013). Additionally, it can re-grow aggressively under extreme conditions and has dominant biological control through their morphological traits (Zuo et al., 2012). *A. philoxeroides* have the ability to grow rapidly under different abiotic stresses, including long submerged conditions (Fan et al., 2015).

Furthermore, *Alternanthera sessilis* is an amphibious dicotyledon, as invasive weed can grow abundantly in disturbed areas and in moist and dry soils (Abbasi et al., 2019). It is a pioneer species typically growing on marginal lands, ranging from poor sandy/alkaline soils to loam soils, and it can be found in invading floodplain wetlands, margins of rivers, streams, canals, and damp forest. It is regarded as fast-growing species in dry regions as well as in seasonally-waterlogged areas and is defined as a weed in fields with different crops (Holm et al., 1997; Gupta, 2014). Consequently, this species has been reported as invasive in different countries and as a noxious weed in the United States (USDA-NRCS, 2014). It becomes particularly harmful for paddy and other extensive irrigated crops due to its allelopathic effects (Kumar et al., 2020). Both *A. sessilis* and *A. philoxeroides* are dominant in nature and grow well even under abiotic stress conditions. While adapting to different environmental stresses, these species pass through various changes at morphological, physiological, and molecular levels (Mallick et al., 2019). By reducing the leaf water potential and slow down the growth and development, these species adapt well under stress conditions (Parkash and Singh, 2020). Drought is a significant abiotic stress, can influence the various physiological and biochemical functions, including reduced photosynthesis and chlorophyll synthesis (Dong et al., 2016; Wang et al., 2018). In addition to drought stress, high salt concentrations in rhizosphere also restrict the plant growth and development (Vurukonda et al., 2016); however, the inhibitory effects of salt stress vary with salt types, plant growth stage, and environmental conditions (Shahverdi et al., 2018). Variable growth responses of different weed species have been documented under drought stress (Webster and Grey, 2008; Chauhan, 2013). Drought stress markedly reduced the photosynthetic rates, mainly through the closure of stomata, reduced mesophyll conductance, and supply of carbon dioxide (CO_2) (Sagardoy et al., 2010).

Moreover, it is commonly known that abscisic acid (ABA) is a crucial regulator during plant responses to stresses, and torrent weeds can accumulate ABA faster than that of sanative weeds (Radhakrishnan et al., 2016). Abscisic acid also reported to play

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an essential role in maintaining the plant growth under different stress conditions (Vishal and Kumar, 2018). As discussed above, in recent years, many studies have conducted on the comparative performance of *A. sessile* and *A. philoxeroides*. However, little is known regarding the comparative performance of these species under abiotic stress conditions. Therefore, for this work, it was hypothesized that *A. philoxeroides* showed better performance under drought and salt stress than *A. sessile*. For this study, our specific objectives were (a) to investigate the performance, in terms of morphological and physiological traits, of native and invasive weeds under drought and salt stresses, (b) and to evaluate the effectiveness of these weed species to exogenous ABA application.

Materials and methods

Experimentation and treatments implementation

The experiment was performed under greenhouse conditions at Jiangsu University, Jiangsu (32.20°N, 119.45°E), China. Ramets of *A. sessilis* and *A. philoxeroides* were collected outside of the greenhouse, where they were grown for experimental studies. To culture the plant materials, the rooted seedlings were cut into ramets of equal lengths. Ramets were prepared in seedling trays with sand as a culture medium. These trays were placed in a greenhouse, where the temperature of 25 ± 5 °C during the day, 18 ± 2 °C during the night, and 70% relative humidity were maintained throughout the experimental period. All these seedlings were subjected to normal daily watering. When these ramets had two fully expanded leaves, they were sampled and re-planted into the plastic pots (having height of 10 cm, outer- and inner diameter of 13 and 6 cm, respectively) for salinity and ABA experiments and to hydroponic culture for drought experiment. After one week of seedling transfer, the treatments were imposed. In experiments I and III (salinity and ABA application), there were total of 72 plastic pots for each experiment and filled with well-sieved sandy soil. The physical and chemical properties of the soil used for pot filling are given in *Table 1*. In experiment II, for imposition of drought stress, plants of invasive and native species with two fully expanded leaves were cultivated in hydroponic culture with Hoagland nutrient solution (Hoagland and Arnon, 1950). After seven days of transplanting, drought treatments were imposed as control-no drought, 75 g L^{-1} PEG 6000, and 100 g L^{-1} PEG 6000. The nutrient solution was continuously aerated throughout the experimentation. In experiment I, salinity treatments included on: control-no salt application, 100 mM NaCl and 150 mM NaCl. In experiment III, abscisic acid was applied as control, (no ABA application), 25 ul L^{-1} and 50 ul L^{-1} (*Table 2*). The study was laid out in a completely randomized design (CRD) with factorial arrangements, and there were total twelve replications.

Table 1. *Physical and chemical characteristics of sandy soil used for pot filling*

Soil parameter	Sandy soil
pH	6.60
Organic matter %	0.34
Total nutrient %	0.98
Water content %	15
Electrical conductivity Ds m^{-1}	1.2

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Table 2. *Treatment plan for different experiments*

Experiments	Treatments		
	T1	**T2**	**T3**
Experiment-I (salt stress)	Control	100 mM NaCl	150 mM NaCl
Experiment-II (drought stress)	Control	75 g L^{-1} PEG-6000	100 g L^{-1} PEG-6000
Experiment-III (abscisic acid application)	Control	25 ul L^{-1}	50 ul L^{-1}

Data recording

Morphological and chlorophyll data

Fifteen days after treatments application, plants were harvested for the measurement of growth and physiological traits. From each treatment, six plants were selected for the measurement of growth and physiological parameters. Plant height was measured using a ruler and fresh and dry weights of seedlings by using a weighing balance. Each plant was separated into roots and leaves, dried at 60 °C for 48 h, and weighed. A plant chlorophyll meter (Oakoch OK-Y104, China) was used to measure the stomatal conductance, net photosynthesis rate, water use efficiency, and intercellular CO_2. Young leaves were preferably selected for evaluating the photosynthetic attributes. All data were recorded during full sunshine at 9:30–11:30 a.m. The following settings were noted on chlorophyll meter during data collection: photosynthetic active radiation (PAR) of 800 μmol m^{-2} s^{-1}, the temperature of 28 °C and CO_2 concentration of 500 μmol mol^{-1}.

Statistical analysis

Data analysis was performed statistically with SPSS 17 software (SPSS, IL, USA). Before further analysis, assumptions of parametric statistics were tested to verify the normality and homogeneity of variance using the Shapiro–Wilk normality test and Levene's test. A one-way analysis of variance (ANOVA) technique was applied to examine the impact of different levels of salinity, drought, and abscisic acid on the growth and physiological traits of *A. sessile* and *A. philoxeroides*. The least significant difference test at 5% probability level was applied to determine the differences between means (n = 12).

Results

Experiment 1: impact of salt stress on the performance of native and invasive weed species

Growth measurements

The growth traits (fresh and dry root weight, fresh and dry leaf weight, plant height, and dry weight biomass) of both *A. philoxeroides* and *A. sessilis* remained unaffected by salt stress treatments (*Table 3*; *Fig. 1*). Nonetheless, all growth measures differed significantly for both *A. philoxeroides* and *A. sessilis* species (*Fig. 1*). Regardless of salt treatments, *A. philoxeroides* recorded an increase in fresh root weight by 45.91%, dry root weight by 172.63%, fresh leaf weight by 50.78%, dry leaf weight by 66.40%, plant height by 46.56%, and dry weight biomass by 21.75%, respectively, compared to *A. sessilis* on

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average. Moreover, there was a non-significant salt treatment × species interaction for all growth traits except for plant height and total dry weight biomass (*Table 3*; *Fig. 1*).

Physiological attributes

Salt stress did not have same effect on the physiological attributes of both species. For instance, the intracellular CO_2 and net photosynthesis rate in both *A. philoxeroides* and *A. sessilis* species did not differ under salt stress (*Fig. 3*). However, salt treatments significantly affected the stomatal conductance and water use efficiency in both species (*Table 3*; *Fig. 2*). In general, there was a significant difference for all physiological traits, including stomatal conductance, net photosynthesis rate, water use efficiency and intercellular CO_2 of both species. Compared to *A. sessilis*, an increase in intracellular CO_2 by 8.89%, stomatal conductance by 20.35%, and water use efficiency by 52.46% were recorded for *A. philoxeroides* on average. Moreover, there was a non-significant salt treatment × species interaction for all physiological traits (*Table 3*).

Experiment 2: Impact of drought stress on the performance of native and invasive weed species

Growth measurements

In this study, drought stress did not influence all growth measures of both *A. philoxeroides* and *A. sessilis* (*Table 3*; *Fig. 3*). Among native and invasive species, there was a significant difference for all growth traits. Regardless of drought treatments, *A. philoxeroides* reported an increase in fresh root weight by 71.66%, dry root weight by 139.09%, fresh leaf weight by 27.47%, dry leaf weight by 57.72%, plant height by 19.45%, and dry weight biomass by 21.83% compared to *A. sessilis* on average. Moreover, the only significant drought treatment × species interaction was for plant height (*Table 3*).

Table 3. *Influence of salt and drought stresses and ABA application on morphological and physiological traits of A. sessilis and A. philoxeroides*

Variables	Salt stress			Drought stress			Abscisic acid application		
	Treatment	Species	T × S	Treatment	Species	T × S	Treatment	Species	T × S
Morphological traits									
FRW	2.11^{ns}	44.65**	0.75^{ns}	0.43^{ns}	199.45**	0.03^{ns}	0.08^{ns}	84.30**	1.51^{ns}
DRW	2.91^{ns}	684.99**	1.55^{ns}	0.58^{ns}	446.44**	2.46^{ns}	1.96^{ns}	463.00**	1.14^{ns}
FLW	0.83^{ns}	131.32**	0.00^{ns}	1.64^{ns}	39.30**	0.05^{ns}	57.58^{ns}	1.76**	0.85^{ns}
DLW	0.24^{ns}	54.58**	0.21^{ns}	0.31^{ns}	63.45**	1.00^{ns}	0.13^{ns}	103.29**	0.31^{ns}
PH	6.43^{ns}	216.34**	0.44**	1.62^{ns}	29.20**	1.10*	0.43^{ns}	50.88**	0.11^{ns}
DWB	0.44^{ns}	24.98**	1.15*	0.68^{ns}	38.17**	0.64^{ns}	1.40^{ns}	20.78**	0.29^{ns}
Physiological traits									
iCO_2	0.81^{ns}	6.46*	0.34^{ns}	0.94^{ns}	0.60^{ns}	0.14^{ns}	0.11^{ns}	4.72*	0.11^{ns}
SC	5.81**	9.20*	0.61^{ns}	5.08*	7.34**	0.78^{ns}	3.88*	41.13**	0.12^{ns}
NPR	1.44^{ns}	21.21**	0.68^{ns}	2.49^{ns}	33.91**	0.32^{ns}	4.43*	12.24*	1.03^{ns}
WUE	6.30**	61.42**	0.30^{ns}	3.05^{ns}	4.31^{ns}	0.91^{ns}	11.70**	10.06**	3.17^{ns}

FRW, fresh root weight; DRW, dry root weight; FLW; fresh leaf weight; DLW, Dry leaf weight; PH, plant height; DWM, dry weight biomass; iCO_2 intracellular CO_2; SC, stomatal conductance; NPR, net photosynthesis rate; WUE, water use efficiency

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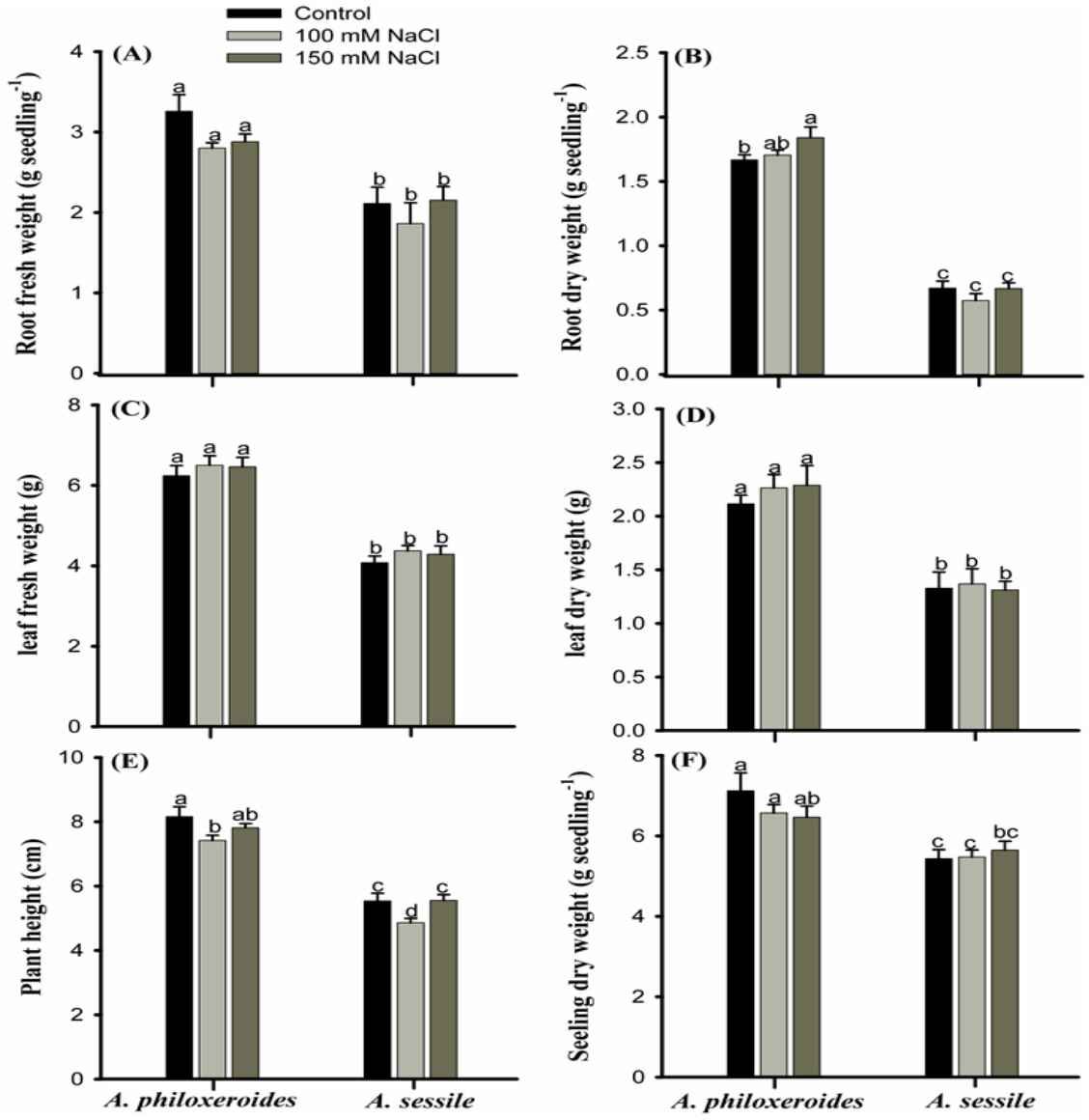

Figure 1. *Fresh root weight (A), dry root weight (B), fresh leaves weight (C), dry leaf weight (D), plant height (E), and dry weight biomass (F) of A. philoxeroides and native A. sessilis plants exposed to different levels of salt stress. The data are presented as the means ± S.E., n = 12. Different letters above bars indicate significant differences among treatments at (P < 0.05)*

Physiological attributes

Physiological measures in both species did not differ significantly between drought treatments, except for stomatal conductance of both species (*Fig. 4*). However, there was a significant difference in all physiological traits between *A. philoxeroides* and *A. sessilis*, except for intracellular CO_2 and water use efficiency (*Fig. 4*). The stomatal conductance and net photosynthesis rate of *A. philoxeroides* were increased by 14.74 and 61.51%, respectively, compared to *A. sessilis* on average (*Fig. 4*). In addition, there was a non-significant drought treatment × species interaction for all physiological traits (*Table 3*).

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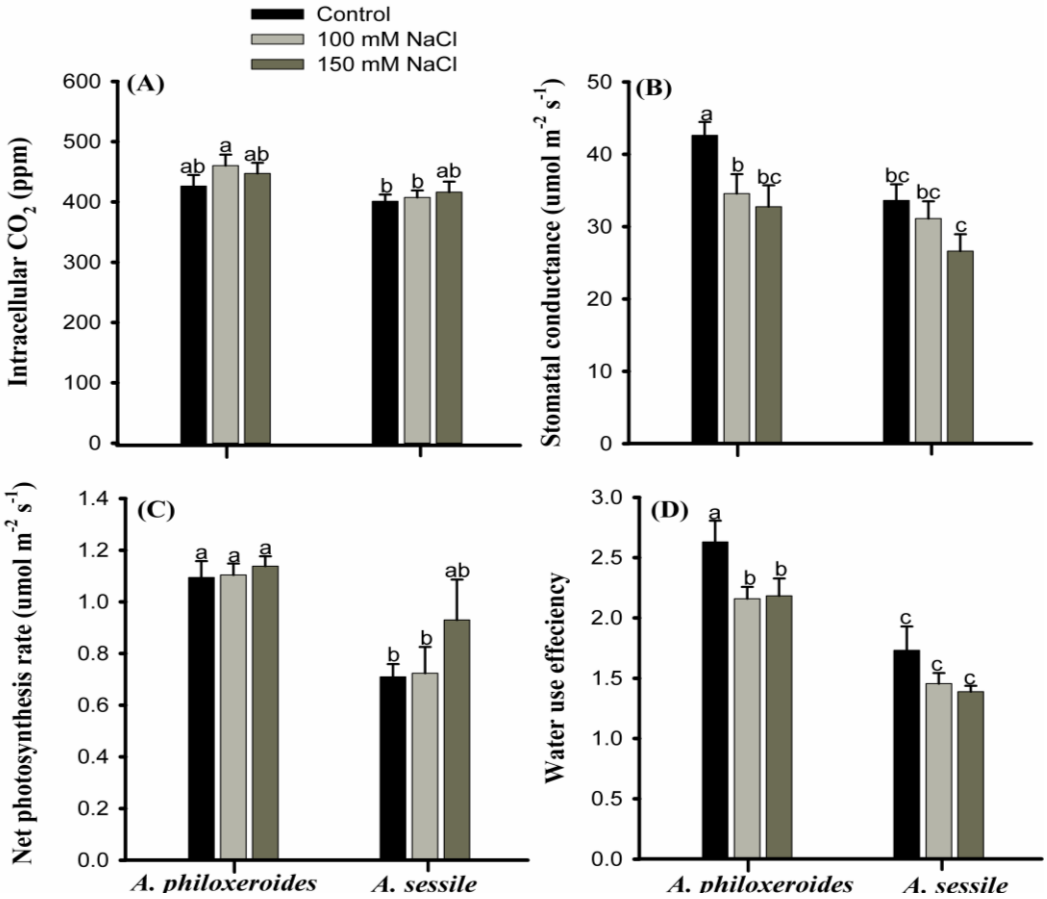

Figure 2. *Intracellular CO_2 (A), stomatal conductance (B), net photosynthesis rate (C), and water use efficiency (D) in A. philoxeroides and native A. sessilis plants exposed to salt treatments. The data are presented as the means ± SE, n = 12. Different letters above bars indicate significant differences among treatments at ($P < 0.05$)*

Experiment III: Comparative performance of native and invasive weed species under ABA application

Growth measurements

Abscisic acid (ABA) application non-significantly affected the growth measures in both species (*Fig. 5*). Nonetheless, all growth measures were differed significantly for both *A. philoxeroides* and *A. sessilis* species. Overall, for the individual effect of species, *A. philoxeroides* showed greater values of growth measures than *A. sessilis.* An increase in fresh root weight by 90.74%, dry root weight by 145.96%, fresh leaf weight by 48.35%, dry leaf weight by 56.10%, plant height by 26.12%, and dry weight biomass by 19.78% was noted for *A. philoxeroides*, as compared to *A. sessilis* on average. Moreover, there was a non-significant ABA treatment × species interaction for all growth parameters (*Table 3*).

Physiological attributes

Data regarding the physiological activities (i.e., intracellular CO_2, stomatal conductance, net photosynthesis rate and water use efficiency) of both *A. philoxeroides* and *A. sessilis* species under ABA application are presented in (*Fig. 6*). ABA

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application significantly affected both species' stomatal conductance, net photosynthesis rate, and water use efficiency. However, there was a non-significant difference for intracellular CO_2 under ABA application. In general, there was a significant difference for all these traits of both *A. philoxeroides* and *A. sessilis* species (*Fig. 6*). Compared to *A. sessilis*, an increase in intracellular CO_2 by 8.40%, stomatal conductance by 37.66%, net photosynthesis rate by 39.54%, and water use efficiency by 31.55% was recorded for *A. philoxeroides* on average. Moreover, there was a non-significant ABA treatment × species interaction for all physiological traits (*Table 3*).

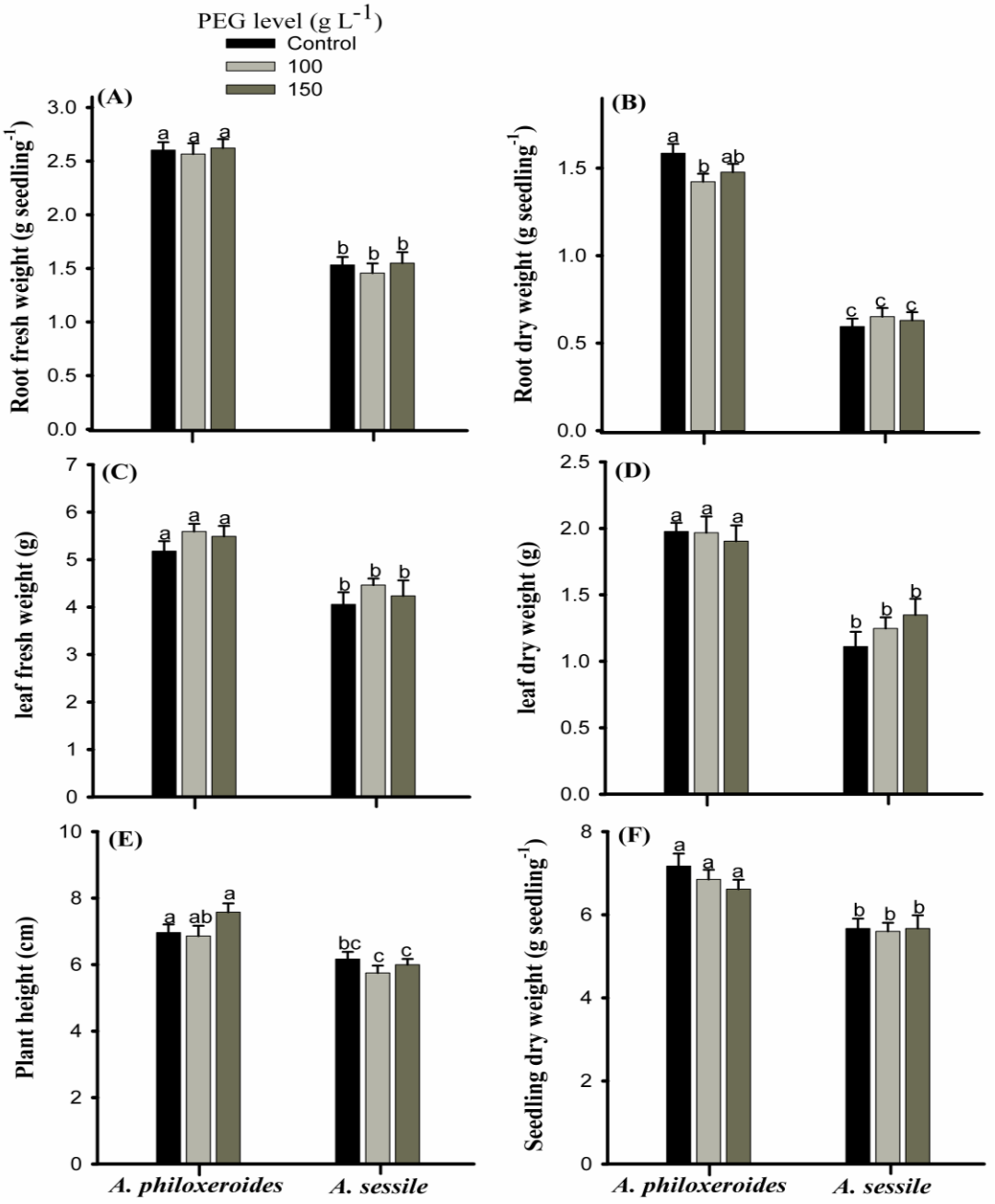

Figure 3. *Fresh root weight (A), dry root weight (B), fresh leaves weight (C), dry leaves weight (D), plant height (E), and dry weight biomass (F) of A. philoxeroides and native A. sessilis plants exposed to drought stress. The data are presented as the means ± SE, n = 12. Different letters above bars indicate significant differences among treatments at (P < 0.05)*

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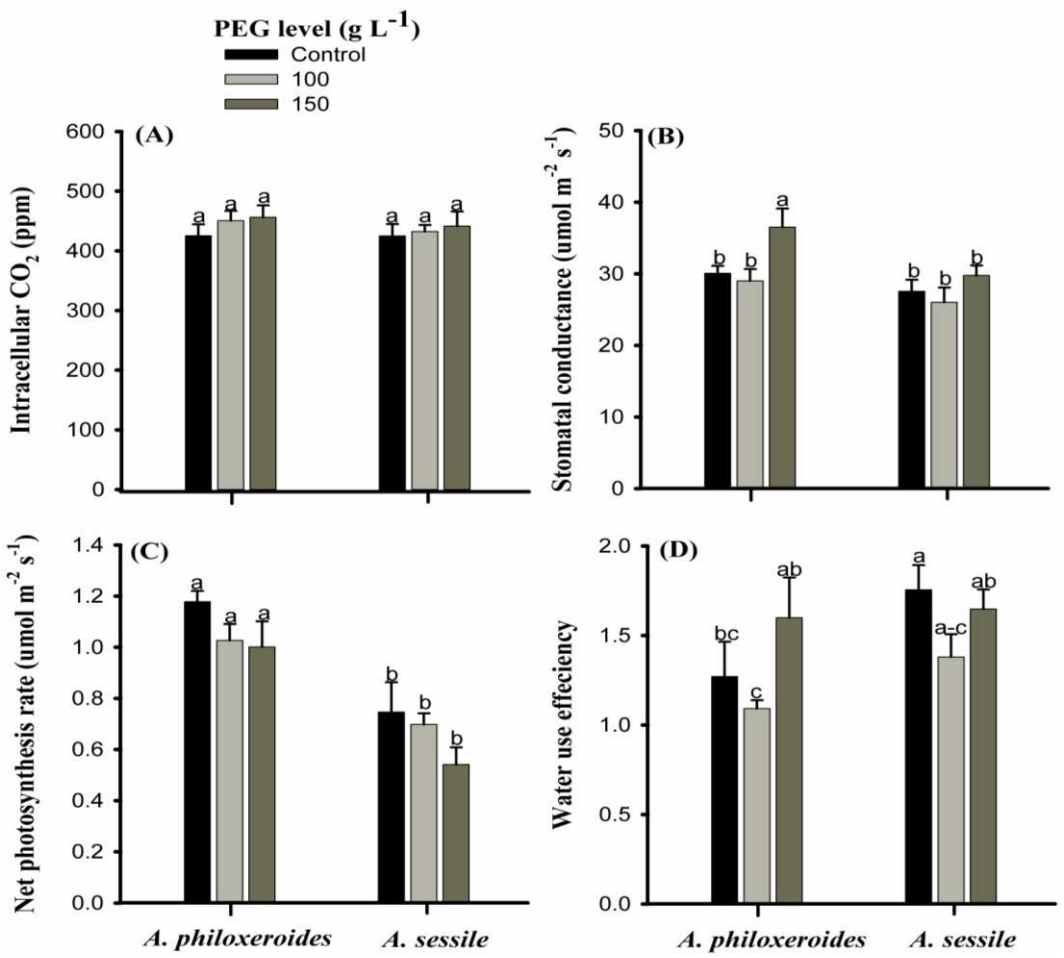

Figure 4. *Intracellular CO_2 (A), stomatal conductance (B), net photosynthesis rate (C), and water use efficiency (D) in A. philoxeroides and native A. sessilis plants exposed to drought stress. The data are presented as the means ± SE, n = 12. Different letters above bars indicate significant differences among treatments at ($P < 0.05$)*

Discussion

Among abiotic stresses, salinity and drought are the major limiting factors for crop plant growth and productivity (Fita et al., 2015). Although major studies discuss the impact of abiotic stresses on field-grown crops (James et al., 2018), only a few studies have examined the comparative performance of native and invasive weed species under environmental cues (Gioria and Pyšek, 2017). In this experiment, salinity and drought stress did not affect the growth of both invasive *A. philoxeroides* and native *A. sessilis* species, indicating their tolerance abilities to salinity and drought stress (*Figs. 1, 3*). These findings are inconsistent with the results of (Chen et al., 2013), who reported that stress (waterlogged) conditions decreased the growth of both native and invasive species. Similarly, according to (Madawala et al., 2014), high soil salinity influences both native and invasive species.

Performance of invasive and native species under salt stress

Invasive *A. philoxeroides* and native *A. sessilis* species differed in growth and physiological traits under a salt environment (*Fig. 1-2*). In general, *A. philoxeroides*

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recorded significantly more values of growth traits than *A. sessilis* (*Fig. 1*). In agreement with these findings, Monaco et al. (2016) reported that invasive *Bromus diandrus* accumulated significantly higher total biomass than the native *Bromus carinatus* under salt stress. Invasive species grow under salt stress mainly due to their higher growth rate. Similar findings were also reported for waterlogging stress (Chen et al., 2013). The authors reported that *A. philoxeroides*, an invasive species, showed more reduction in growth traits under waterlogged conditions than *A. sessilis*. Compared to non-invasive species, higher growth rates of invasive species have been well documented in previous studies (Van Kleunen et al., 2010). The physiological traits under salinity also differed between invasive and native species (*Fig. 1*). Compared to *A. sessilis*, an increase in intracellular CO_2, stomatal conductance, and water use efficiency was recorded for *A. philoxeroides*. Our results consisted of previous studies, which showed that *A. philoxeroides* showed greater physiological traits under heat and drought stress (Geng et al., 2006; Sun et al., 2010). Higher root to shoot ratio and chlorophyll concentrations of *A. philoxeroides* under stress conditions might reduce the respiratory losses and improve the light interception, availability of oxygen, and carbohydrate status of plants (Luo et al., 2011; Zhang et al., 2020).

On the other hand, in *A. philoxeroides*, leaves necrosis under stress conditions (Chen et al., 2013) might also reduce respiratory failure (Colmer and Voesenek, 2009). It is generally believed that by producing more carbon-rich compounds, the invaders (invasive) avoid excessive irradiance (Godoy et al., 2011) and disposes of enough carbon to reduce the carbon trade-off between growth and tissue protection (Villar et al., 2006), thereby reducing the risk of photo-inhibition causing damage to chlorophyll, which in turn can increase the carbon gain and crop growth (Naciri et al., 2021). Compared to *A. sessilis*, a less decline in growth traits and higher values of physiological parameters under salinity might contribute to higher tolerance of *A. philoxeroides* (*Figs. 1-2*). A relatively more substantial decline in growth traits of *A. sessilis* suggesting more sensitivity to environmental stresses (Javed et al., 2019).

Performance of invasive and native species under drought stress

Both *A. philoxeroides* and *A. sessilis* did not differ regarding growth traits (dry root and plant biomass, and fresh leaf weight) under drought stress (*Fig. 3*). However, *A. philoxeroides* had increased the dry root weight, fresh leaf weight, and dry weight biomass compared to *A. sessilis* (*Fig. 3 A, C*). These findings are consistent with previous reports that invasive species showed higher growth rate than native species (Van Kleunen et al., 2010). However, these results are inconsistent with earlier findings of Molina-Montenegro et al. (2011), where the authors have reported lower biomass and leaf size in invasive species than in native type during water scares conditions. In this study, under drought stress, there was a significant difference in physiological traits for both species (*Fig. 4*). Stomatal conductance, net photosynthesis rate, and water use efficiency were increased significantly in *A. sessilis* than in *A. philoxeroides*. These findings do not match previous studies suggesting that invasive showed higher values of physiological traits (photosynthesis and transpiration rates, and water use efficiency) than native species (Van Kleunen et al., 2010; Godoy et al., 2011). Similarly, in contrast to our findings, Chen et al. (2013) have reported that invasive *A. philoxeroides* showed the ability to maintain the photosynthetic capacity even under stress conditions.

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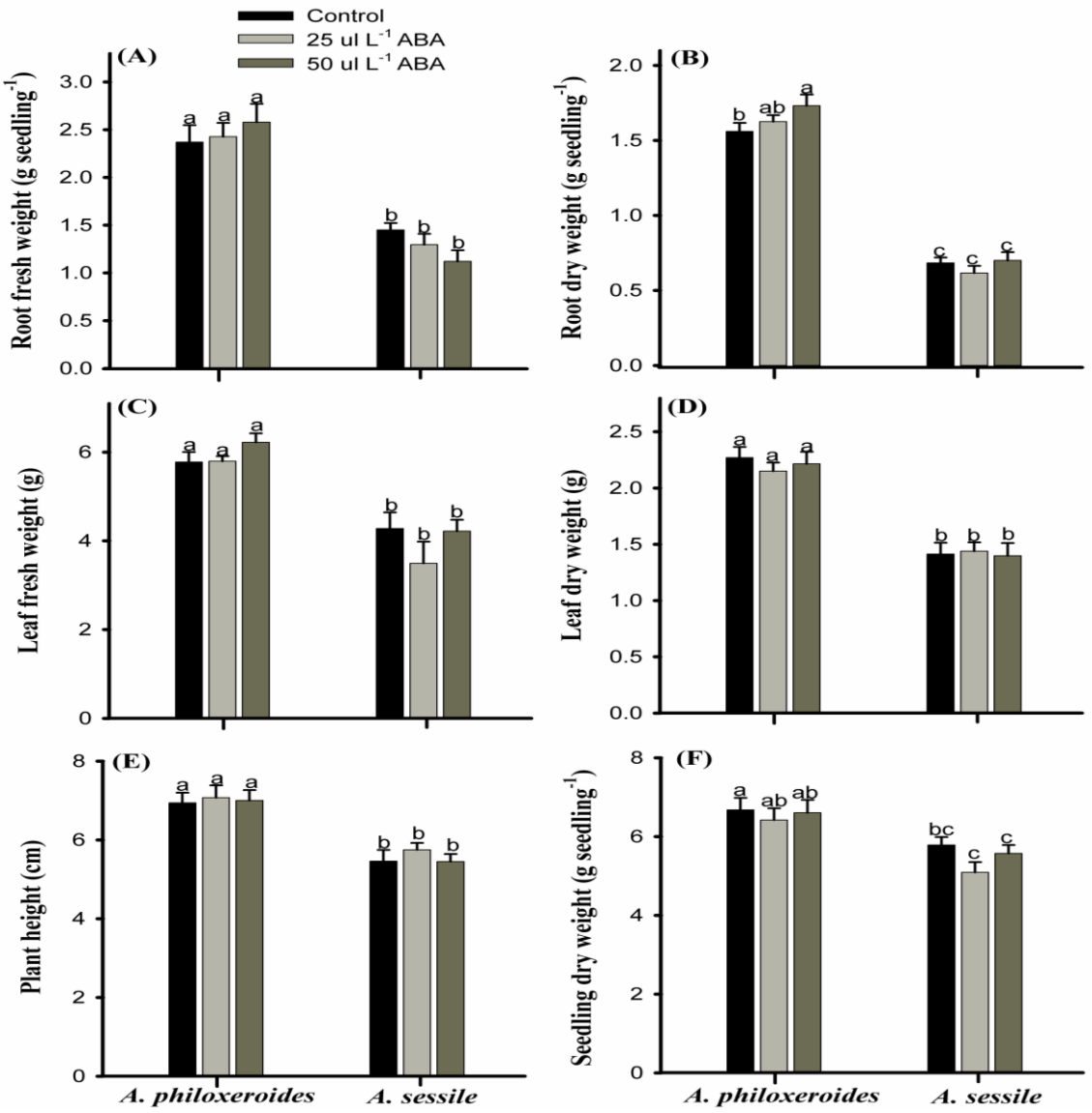

Figure 5. *Influence of abscisic acid (ABA) application on Fresh root weight (A), dry root weight (B), fresh leaves weight (C), dry leaves weight (D), plant height (E), and dry weight biomass (F) of A. philoxeroides and native A. sessilis plants. The data are presented as the means ± SE, n = 12. Different letters above bars indicate significant differences among treatments at (P < 0.05)*

Response of invasive and native species to abscisic application

For the first time, our results have reported the performance of invasive and native species under ABA application. In this study, *A. philoxeroides* recorded significantly higher values of growth measures and physiological traits than *A. sessilis* under ABA application (*Fig. 6*). These findings are inconsistent with previously published reports that suggest that seedling growth in most of the invasive species significantly inhibited under ABA application (Liu et al., 2015). In *A. philoxeroides,* higher values of growth traits may be due to improved water relations of the plants under ABA application. Abscisic acid acts as an endogenous messenger in regulating plant-water status (Rai et al., 2011). The enhanced plant growth under ABA application has been

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well documented in various studies (Wei et al., 2015, 2017). In addition, ABA application may also result in the closure of stomata and reduced transpirational water loss (*Fig. 6*).

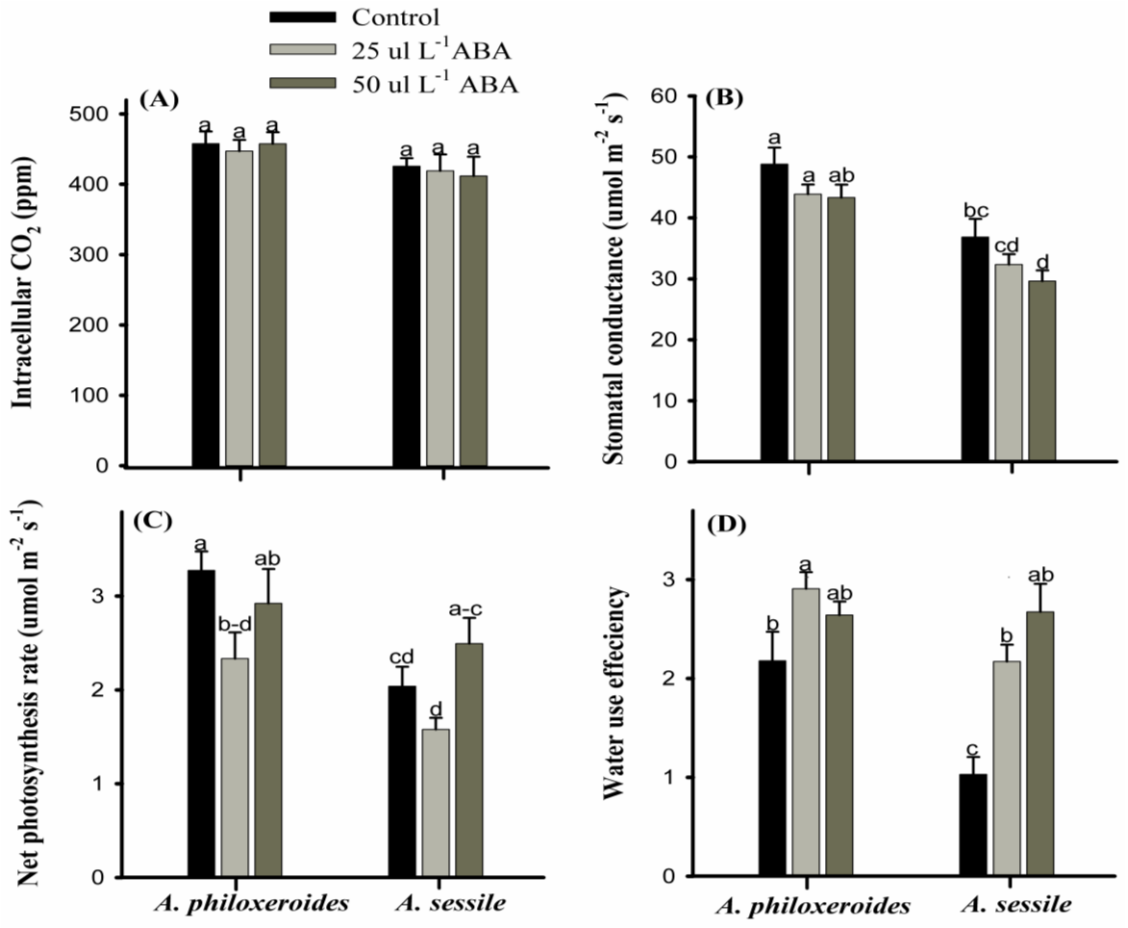

Figure 6. *Influence of abscisic acid (ABA) application on intracellular CO_2 (A), stomatal conductance (B), net photosynthesis rate (C), and water use efficiency (D) in A. philoxeroides and native A. sessilis weeds. The data are presented as the means ± SE, n = 12. Different letters above bars indicate significant differences among treatments at ($P < 0.05$)*

Conclusion

In sum, the results of this study showed that both invasive *A. philoxeroides* and native *Alternanthera sessilis* showed great tolerance to salt and drought stress. In general, under stress conditions, two species differed in growth and physiological traits: *A. philoxeroides* displayed higher values of morphological and physiological characteristics. In addition, under ABA application, *A. philoxeroides* showed less sensitivity as compared to *A. sessilis*. Higher tolerance of salt and drought stress may partly explain the ability of *A. philoxeroides* to invade saline and water-scarce areas. We predict that *A. philoxeroides,* which is dominant invasive species, might have the potential to become a serious problem in saline soils, including coastal areas, and in dry areas in the future. More attention should focus on monitoring the occurrence of *A. philoxeroides* on saline soils and in dry areas, timely prevention should be implemented to control new invaders.

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

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ASSOCIATION BETWEEN PHTHALATE EXPOSURE AND INSULIN RESISTANCE: A REVIEW

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Abstract. Insulin resistance refers to a state in which the target tissues have reduced biological sensitivity to insulin. Insulin resistance is considered the common pathophysiological basis of many chronic non-communicable diseases, including type 2 diabetes, non-alcoholic fatty liver, atherosclerosis and coronary heart disease. These diseases have become the major public health problems that seriously endanger human health and economic development nationwide, and the prevention and control of the occurrence of insulin resistance is a crucial issue. The occurrence of insulin resistance is a complex process influenced by genetics and environment. Genetic susceptibility, higher body mass index and less physical exercise are the main risk factors for insulin resistance. Besides, environmental endocrine disruptors such as phthalates may increase the incidence of metabolic disorders and explain a part of its increase. Therefore, this study reviews the epidemiological studies on the association between phthalate exposure and insulin resistance, and discusses the potential biological mechanisms. In conclusion, the majority of studies supported the hypothesis that phthalate exposure was significantly associated with the increased risk of insulin resistance. High oxidative stress, abnormal systemic inflammation and lipid metabolism were underlying mechanisms of insulin resistance induced by phthalates.

Keywords: *environmental endocrine disruptors, cardiovascular disease, oxidative stress, chronic inflammation, obesity*

Abbreviations

Phthalic acid diester		Phthalate metabolite	
Abbreviation	**Whole name**	**Abbreviation**	**Whole name**
DMP	Dimethyl phthalate	MMP	Monomethyl phthalate
DEP	Diethyl phthalate	MEP	Monoethyl phthalate
DiBP	Di-iso-butyl phthalate	MiBP	Mono-iso-butyl phthalate
BBzP	Butyl benzyl phthalate	MBzP	Monobenzyl phthalate
DEHP	Di(2-ethylhexyl) phthalate	MEHP	Mono(2-ethylhexyl) phthalate
		MEOHP	Mono(2-ethyl-5-oxyhexyl) phthalate
		MEHHP	Mono(2-ethyl-5-carboxyhexyl) phthalate
DiNP	Di-iso-nonyl phthalate	MiNP	Mono- iso-nonyl phthalate

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Introduction

Phthalates are a family of artificially synthesized chemicals since 1920s. They have been widely used as plasticizers in the production of polyvinyl chloride since 1950s (Kimber et al., 2010). For instance, high molecular weight phthalates including butyl benzyl phthalate (BBzP) and di(2-ethylhexyl) phthalate (DEHP), are commonly used in building materials, food packages, child toys, medical equipments, etc. Low molecular weight phthalates including diethyl phthalate (DEP) and dibutyl phthalate (DBP) are often used in personal care products. However, phthalates are not bonded to polymers with chemical bond. They are easily released into the surrounding environment, especially under high temperature or high fat conditions. Therefore, human is exposure to phthalates through digest, inhalation, dermal contact and medical treatment. A variety of phthalate metabolites can be detected in human urine, serum and other biological samples, and the detection frequency is almost 100%. It suggests that people have been generally exposed to phthalates. Therefore, it is very important to study the effects of phthalates on human health. In 2009, Grün and Blumberg reported that environmental endocrine disruptors are "obesogens" that can disrupt homeostasis and reward mechanisms, and increase individual sensitivity (change in weight set point) (Grün and Blumberg, 2009). In 2015, the Parma Consensus stated that the range of “obesogens” that increase obesity and metabolic syndrome susceptibility should be expanded and referred to as environmental “metabolic disruptors” (Heindel et al., 2015). At present, a large number of studies have conducted intense discussions on the obesity-causing effects of phthalates exposure. The increase in obesity is closely related to the development of insulin resistance, but insulin resistance can also occur independently of obesity.

The increasing prevalence of obesity and metabolic syndrome is a huge challenge in the field of public health. Insulin resistance is an important pathological feature of obesity and metabolic syndrome (Samuel and Shulman, 2012). Therefore, improving insulin sensitivity is an important strategy to prevent, delay or treat type 2 diabetes and metabolic syndrome. Although genetic susceptibility and lifestyle (such as diet, exercise, smoking, etc.) are considered as important causes of type 2 diabetes and metabolic syndrome, these reasons cannot fully explain the sharp increase in incidence of metabolic syndrome in recent decades. Studies have pointed out that exposure to environmental chemicals may be another important cause.

Therefore, this study aimed to review contemporary epidemiological literatures on the association between phthalate exposure and insulin resistance as well as its underlying mechanisms.

Overview of epidemiological studies on the association between phthalate exposure and insulin resistance

A systematic literature search was used for MEDLINE database on March 17, 2021. The search strategy was TS= (phthalate OR “phthalic acid ester” OR “endocrine disruptor” OR “endocrine disrupting chemical”) AND TS= (“insulin resistance” OR “insulin sensitivity” OR “pre-diabetic state” OR “hyperinsulinemia” OR “glucose intolerance” OR “diabetes” OR “metabolic syndrome”). Fifteen cross-sectional studies were found to have analyzed the association between phthalate exposure and insulin resistance (*Table 1*). The minimum age of the research subjects was 6 years old, but most of participants were teenagers, adults and the elderly people. The research

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publication year ranged from 2007 to 2020. The research sites included Asia [China (N= 4) and South Korea (N= 3)], America [the United States (N= 4) and Canada (N= 1)], and Europe [Sweden (N= 1), Belgium (N= 1) and Serbia (N= 1)], etc. The basic characteristics of each study were shown in *Table 1*.

Table 1. *The basic characteristics of studies analyzed the association between phthalate exposure and insulin resistance*

Reference	Publication year	Country	Study design	Sample size	Age of participants (year)
Stahlhut et al.	2007	United States	cross-sectional	1451	＞18
Lind et al.	2012	Sweden	cross-sectional	1016	70
Trasande et al.	2013	United States	cross-sectional	766	12～19
Kim et al.	2013	South Korea	cross-sectional	560	＞60
Dirinck et al.	2015	Belgium	cross-sectional	123	41±12.5
Attina &Tresande	2015	United States	cross-sectional	356	12～19
Lin et al.	2016	China	cross-sectional	793	12～30
Chen et al.	2017	China	cross-sectional	786	12～30
Dales et al.	2018	Canada	cross-sectional	4437	12～79
Dong et al.	2018	China	cross-sectional	300	＞50
Kim et al.	2018	South Korea	cross-sectional	137	6～13
Ko et al.	2019	China	cross-sectional	435	32.16±6.43
Li et al.	2019a	United States	cross-sectional	1605	12～85
Lee et al.	2019a	South Korea	cross-sectional	459	20～48
Milošević et al.	2020	Serbia	cross-sectional	305	18～50

Relationship between phthalate exposure and insulin resistance in children

Study only included girls aged 6～13 years as participants. It found that the percent of mono(2-ethyl-5-carboxyhexyl) phthalate (MEHHP%) of overweight girls was significantly higher than that of the control group. MEHHP% was positively correlated with girls' body mass index (BMI) percentile, body fat percentage, waist circumference and homeostasis model assessment-insulin resistance (HOMA-IR) (Kim et al., 2018).

Relationship between phthalate exposure and insulin resistance in teenagers

Among adolescents and young people, Lin et al. (2016) observed that there was a significant positive relationship of the urinary concentration of mono(2-ethylhexyl) phthalate (MEHP) with HOMA-IR and the particle level of vascular injury markers in circulation. Chen et al. (2017) performed more detailed analyses and obtained similar results in the total population and the youth population, but this phenomenon was not observed in the adolescent population. Trasande et al. (2013) found that for one logarithmic unit increase of concentrations of DEHP metabolites, HOMA-IR increased by 0.27 (0.14 to 0.40); Compared with the lowest quartile group of DEHP concentration (the incidence of insulin resistance, defined as HOMA-IR > two-fold standard deviations, was 14.5%), the incidence of insulin resistance was 21.6% in the third quartile concentration group (Trasande et al., 2013). To further verify the relationship of DEHP and its substitute di-iso-nonyl phthalate (DiNP) exposure with insulin resistance, another study found that for one logarithmic unit increase of DiNP, HOMA-IR

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increased by 0.08 (p value= 0.001); Compared with the lowest quartile group of DiNP and DEHP concentrations (the prevalence of insulin resistance was 23.4% and 20.5%, respectively), prevalence of the third quartile concentration groups was 34.4% and 37.7%, respectively (Attina and Trasande, 2015).

Relationship between phthalate exposure and insulin resistance in adults

Based on the data analyses of the National Health and Nutrition Survey of the United States in different years, Stahlhut et al. (2007) found that exposure to monoethyl phthalate (MEP) and monobenzyl phthalate (MBzP) was positively associated with the increase of waist circumference and insulin resistance in adult. In Canada, one study showed that DEHP metabolites were positively correlated with the increase of fasting blood glucose, fasting insulin, HOMA-IR and HOMA-β (the indicator of the islet β cell function). For one interquartile range increase of DEHP concentration, HOMA-IR increased by 0.15 (0.04～0.26), HOMA-β increased by 10.24% (3.71%～16.77%) (Dales et al., 2018). In 123 Belgium obese subjects without type 2 diabetes, phthalates exposure was associated with increased insulin resistance, decreased insulin sensitivity, and impaired pancreatic β-cell function (Dirinck et al., 2015). A total of 305 Serbians (18～50 years old) were divided into obesity group, type 2 diabetes group and healthy control group. Only in type 2 diabetes patients, serum insulin levels and HOMA-IR showed significantly difference in different MEP exposure groups (Milošević et al., 2020). To analyze the associations of phthalates exposure with the risks of insulin resistance and metabolic syndrome, the results revealed that dimethyl phthalate (DMP) exposure was associated with high HOMA-IR [odd ratio (OR) = 1.686, 95% confidence interval (CI) = 1.079～2.634) and risk of metabolic syndrome (OR = 2.329, 95%CI = 1.263～4.295). In addition, the role of HOMA-IR in mediating the effect of dimethyl phthalate exposure on metabolic syndrome was 43% (Ko et al., 2019). In the population of adult women, whether it was a single substance or a multi-pollutant model, several chemicals, including monomethyl phthalate (MMP), mono-iso-butyl phthalate (MiBP) and bisphenol S, had always been shown significantly positive associations with the concentrations of adipokines or the risk of insulin resistance (Lee et al., 2019a). Li et al. (2019a) found that serum antioxidant β-carotene had a protective effect on insulin resistance induced by DEHP.

Relationship between phthalate exposure and insulin resistance in elderly

In the elderly population (>50 years old), Dong et al. (2018) observed that the concentrations of most phthalate metabolites were positively correlated with HOMA-IR and the concentrations of 8-hydroxy-2'-deoxyguanosine (8-OHdG) and malondialdehyde, suggesting that phthalate exposure was related to insulin resistance and oxidative stress. A study in South Korea reported that the urinary concentrations of the secondary metabolites of DEHP, MEHHP and mono(2-ethyl-5-oxyhexyl) phthalate (MEOHP), were related to the risks of insulin resistance and diabetes (Kim et al., 2013). Besides, a Swedish study reported that high levels of MMP, MEP and MiBP were related to the increased prevalence of diabetes; MiBP was related to insufficient insulin secretion, and MMP and MEP were related to insulin resistance (Lind et al., 2012). The oxidative stress marker malondialdehyde was related to both DEHP and insulin resistance, suggesting that oxidative stress might play a role in mediating the effect of DEHP exposure on insulin resistance (Kim et al., 2013).

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Potential biological mechanisms

Most studies supported a positive correlation between phthalate exposure and risk of insulin resistance. It indicated that phthalates exposure might be related to glucose metabolic disorders, not only in obese and diabetic patients, but also in normal-weight non-diabetic individuals. The biological mechanisms of the relationship between phthalates and insulin resistance is still unclear. However, several biological mechanisms of insulin resistance occurring have been reported, including defects in the insulin signaling pathway, ectopic lipid accumulation, systemic inflammation, mitochondrial dysfunction, oxidative stress and endoplasmic reticulum stress.

Exposure to phthalates and oxidative stress

Studies have found that nutrient overload, insufficient physical activity, hypoxia, excessive psychological stress, and exposure to environmental pollutants could induce a series of responses including cellular stress, stress response and stress response disorders. These responses jointly inhibit the insulin signals in target cells, such as endothelial cells, liver cells, skeletal muscle cells, hypothalamic neurons, fat cells, etc. Therefore, the insulin resistance occurs (Onyango, 2018). Cellular stresses that induce insulin resistance include oxidative stress, nitrosative stress, carbonyl/electrophilic stress, genotoxic stress and endoplasmic reticulum stress. A large number of epidemiological studies have observed that phthalates exposure increases the level of oxidative stress in the body. Holland et al. (2016) reported that DEHP and the sum of high molecular weight phthalate metabolites were statistically associated with the level of isoprostaglandin, one of the oxidative stress markers, and this association exists throughout the whole period of pregnancy. Using urinary 8-isoprostaglandin F2α as a biomarker of oxidative stress, one study had found that the concentration of most phthalate metabolites were related to the increase of the concentration of 8-isoprostaglandin F2α (Van et al., 2019). In the population of children, some phthalate metabolites also showed positive relationships with malondialdehyde and 8-OHdG (Lee et al., 2019b). A male mouse experiment observed that DEHP inhibited miR-17 to disrupt the Keap1-Nrf2 redox system and activate the oxidative stress response Txnip in skeletal muscle; Oxidative stress upregulated miR-200a, which directly targeted the 3' untranslated sequence of Insr and Irs1, leading to obstruction of insulin signaling and impaired insulin-dependent glucose uptake in skeletal muscle, which ultimately promotes the development of insulin resistance (Wei et al., 2020). The role of oxidative stress in mediating DEHP and insulin resistance had also been confirmed in some cross-sectional studies (Kim et al., 2013; Dong et al., 2018), and serum antioxidant *β*-carotene was revealed a protective effect on insulin resistance induced by phthalates exposure (Li et al., 2019a). Li et al. (2019b) reported that the association between phthalates exposure and the risk of type 2 diabetes was mediated partlyby the oxidative stress induced by phthalates, especially by the biomarker of 8-OHdG.

Exposure to phthalates and systemic inflammation

A large number of animal experiments and epidemiological evidence demonstrate that phthalates exposure can cause an inflammatory cascade. An epidemiological study observed that higher concentrations of phthalates in urine were correlated with higher levels of high sensitive C reaction protein (hs-CRP), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), and were positively associated with the risk of

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cardiovascular disease, type 2 diabetes and hypertension (Bai et al., 2017). An animal experiment found that DEHP exposure induced the infiltration of macrophages in rat adipose tissue, promoted the secretion of interleukin-1β (IL-1β), TNF-α and the formation of inflammation, and interfered with normal lipid metabolism to result in the lipid metabolism disorders (Zhou et al., 2019). MEHP can regulated Sirtuinsgene expression in macrophages to further activate inflammasomes, leading to increased inflammation (Park et al., 2019). Nuclear factor kappa-B (NF-κB) is the most common signaling pathway related to inflammation. In vitro experiments had revealed that exposure to dibutyl phthalate during pregnancy could induce inflammation in the offspring's testicular cells, possibly by activating the lipid receptor CD68 and leading to intracellular P65 phosphorylation and transfermation to the nucleus, NF-κB was activated and then the cells synthesized TNF-α, IL-6, monocytes chemotactic protein-1 (MCP-1) and chemokine CXCL-10, NOD-like receptor protein 3 (NLRP3) inflammasomes accumulated. Pellino-dependent NLRP3 recruited Caspase precursors through the Caspase recruitment domain to release mature Caspase-1, and further recruited the precursor of IL-1 to release mature IL-1. Finally, the NF-κB/NLRP3 cascade signal resulted in the typical features of inflammation (Zhou et al, 2020). IL-1 directly inhibited the insulin signaling pathway and induced the occurrence of insulin resistance by reducing the phosphorylation of tyrosine of the insulin receptor substrate-1 and negatively regulating the gene expression of this substrate (Jager et al., 2007).

Exposure to phthalates and obesity and lipid metabolic disorder

Obesity is closely related to insulin resistance, especially the increase in lipid deposition and abnormal distribution patterns in insulin target organs. As mentioned above, phthalates have been classified as environmental "obesogens" and "metabolic disruptors", probably by increasing the number of fat cells, increasing the volume of fat cells, changing the basal metabolic rate, and regulating appetite and satiety, etc. (Muscogiuri et al., 2017). In vitro studies had shown that phthalates could bind to estrogen receptor alpha or androgen receptor, thus exhibited weak estrogenic activity and strong antiandrogenic activity (Gray et al., 2000; Takeuchi et al., 2005). The level of sex hormones in the body would affect the amount and distribution of fat. Phthalates were known as ligands to peroxisome proliferator-activated receptors (PPARs) (Hurst and Waxman, 2003; Bility et al., 2004). PPARs are a family of transcription factors with a key role in adipogenesis and lipid metabolism (Grun, 2009). PPARs mainly regulate adipocyte development, and promote adipocyte differentiation and maturation after activation (Evans et al., 2004). In addition, phthalates are known to interfere with thyroid function and may reduce the level of thyroxine in the circulatory system (Yao et al., 2016; Gao et al., 2017). Thyroid function plays a key role in maintaining basal metabolism and the regulation of energy balance. However, these biological mechanisms are complicated and potential interaction. For instance, in vitro experiment had observed that MEHP induced lipid accumulation by inhibiting the JAK2/STAT5 pathway, and damaged the liver parenchyma by aggravating oxidative stress and leaded to the occurrence of non-alcoholic fatty liver (Zhang et al., 2019).

Conclusions

Human exposure to phthalates is related to the risk of insulin resistance, which is consistent with our recent findings through an update of a systematic review and meta-

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analysis (Gao et al., 2021). These may be mediated by lipid metabolism disorders induced by chronic inflammation and oxidative stress. It is impossible to draw a causal relationship between phthalate and insulin resistance, because of lack of prospective cohort studies to date. Only one study population was girls aged 6 to 13, and the other study populations were adolescents and adults (including the elderly) aged over 12 years old. For young children, it is not clear whether phthalates exposure in early life is related to risk of insulin resistance. This is very important for whether moving or not the insulin resistance prevention window to early life. For the short biological half-life chemicals, phthalate metabolites were measured by one-time urine sample to evaluate exposure in the majority of studies. This might introduce exposure misclassification bias. Only three studies used Bayesian nuclear machine learning or hazard index models to evaluate the effect of co-exposure to multiple phthalate metabolites on insulin resistance. As phthalates are a class of chemicals with similar chemical structures and health effects, it is necessary to take the cumulative risk assessment of multiple phthalates combined exposure into consideration.

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Conflicts of interests. The authors declare no conflict of interests.

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COTTON PINK BOLLWORM (*PECTINOPHORA GOSSYPIELLA*) MANAGMENT WITH THE GOAL OF ERADICATION FROM THE COTTON PRODUCING COUNTRIES OF THE WORLD

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Abstract. Bt-toxin is not effective anymore in controlling cotton pink bollworm in the major cotton producing countries of South Asia. The cultivation of Bt-cotton without the use of 5% non-Bt plants as a refuge crop has made pink bollworm resistant to this toxin. On the other hand this pest has been successfully eradicated from the main cotton growing areas of the United States. The wise use of 5% non-Bt cotton as refugee has provided successful control over this pest in Australia and China. The genetically engineered cotton encoding the Cry1Ac, Cry2Ab, or Cry1F protein does not have a stable insecticidal efficacy. There is fluctuation in its expression during different cotton plant growth stages. Significant reduction in Bt-toxin expression and resistance for pink bollworm has been reported especially near crop maturation. The early crop termination through early maturing varieties, harvest aid chemicals and use of growth regulators can be a key to disrupt pink bollworm diapause phase. Varieties with Bt-toxin along with several morphological and anatomical features that provide natural shelter against bollworm attack should be cultivated in the affected regions. The collective use of integrated pest management approach, sterile PBW moth release, pheromone treatments, crop management for host free period, use of early maturing varieties, natural insect pest resistant varieties, adaptation toward the shortening of crop season, use of 5% refuge crop and transgenic Bacillus thuringiensis (Bt) cotton have great impact on the management of this pest of cotton and can change the current suboptimal worse condition of the effected cotton growing areas of the world.

Keywords: *cotton chewing insects, Bt-toxin resistance, pink bollworm damage, integrated pest management, pink bollworm invasion*

Abbreviations: Bt: Bacillus thuringiensis; Cry1Ac, Cry2Ab: crystal insecticidal proteins produced by a bacterium (Bacillus thuringiensis); Cry1F: crystaline entomocidal protoxin; CDFA: The California Department of Food and Agriculture; USDA: The United States Department of Agriculture

Introduction

With the introduction of Bt-cotton in South Asia the farmers of the region did not pay attention to the value of refuge crops. Pink bollworm (*Pectinophora gossypiella*) the destructive pest of cotton has developed resistance against Bt-toxin and these nations are facing huge economic losses. This article is all about the background of how this destructive pest developed resistance against Bt-toxin, how developed nations have worked wonder by eliminating this destructive pest from their fields and what measures the currently suffering nations can carry out to eradicate it (Tabashnik et al., 2021). After almost a century of struggle United States cotton is free from the devastating pink bollworm (USDA**,** 2018). More concerning is the fact that the worm is happily chewing up Bollgard-II and is causing severe cotton production losses of worth millions of

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dollars in south Asia (Kranthi, 2011; Ingole et al., 2019). According to an estimate one million bales of cotton is destroyed due to pink bollworm every year in Pakistan (Sayyed et al., 2008). On one side insect resistance to transgenic cotton (Tabashnik et al., 2009; Carrière et al., 2010; Bagla, 2010; Downes et al., 2010; Zhang et al., 2011; Wan et al., 2012; Tabashnik and Carrière, 2010) and pesticides (Schmutter, 1985; Labbe et al., 2005; Levot and Sales, 2008; Sayyed et al., 2008) is proving Darwin's theory of "survival of the fittest" (Tabashnik and Carrière, 2019). On the other hand, world top cotton producing nations either eradicating it like in America, declining its resistance against Bt-cotton like in China or have full control like in Australia (Tabashnik and Carrière, 2019). Invasive life forms when survive and widespread in a new habitat become not only difficult to eradicate but also emerge as a global threat (Pyšek et al., 2020; Martinez et al., 2020 and Simberloff et al., 2020).

The pink bollworm is causing serious economic losses to the cotton crop throughout the world. According to an estimate the destructive pest is causing huge losses with an average range of 3 to 50 per cent in seed cotton yield and oil contents (Anjum et al., 2019). American states of Arizona and California have witnessed a great deal of cotton crop damage after invasion of pink bollworm in 1978. In just a single decade the total cotton crop area dropped from 79,942 ha to 37,130 ha. The total value of cotton dropped approximately $174,009,389 and the pesticides costs raised to $264.39/ha. Meanwhile the whitefly populations in the cotton fields also rise to an exponential level resulting in appearance of several disease symptoms and yield losses (Johnson et al., 1982). The year prior to Bt cotton introduction (1995), at a cost of $373 million and nearly two thirds of cotton crop cultivated land in the United States was treated with insecticides to control cotton pink bollworms (Williams, 1996). Pink bollworm was causing cotton yield damage worth of above 0.25 billons dollar even after the introduction of Bt-cotton in USA (Frisvold and Tronstad, 2003). Each year in America pink bollworm control and yield losses costs cotton producers more than US$32 million (McGaughran et al., 2017). Before cultivation of Bt-cotton in China it reduced cotton yield by 10% (Cai et al., 1985). In the major cotton growing area of China (Wuhan) the fibre yield losses were estimated about 17-26% (Luo et al., 1986). In the top cotton producing country of the world including Brazil and Egypt it still causes 20% or higher losses in cotton crop (Ismail, 2019). If 1% infestation of pink bollworm in cotton would increase then almost 2.5-6% of cotton yields would decrease in Turkey (Unlu and Bilgic, 2004). In Sudan, the potential yield losses due to pink boll worm infestation could reach up to 10.7% (Darling, 1951). Different surveys results revealed a 20 and 30% yield losses due to widespread infestation of pink bollworm on Bt cotton across the different sites in a range of 40-95% in India (Fand et al., 2019). During 2017-18 there was epidemic level pink bollworm infestation in major cotton growing states of India. The infestation ranged between 8-92% with corresponding yield losses of 10-30% (Anonymous, 2019). It is estimated that in Pakistan, farmers spend US$300 million on pesticides annually, of which more than 80% is used on cotton, especially for bollworms (Rao, 2007). After 1911, the year of pink bollworm invasion into North America, the cotton growers used different efforts to control its population in the cotton fields. These practices included the use of conventional pesticides, cultivation of modern Bt-cotton varieties, different cultural managements like shredding of cotton residues during winter to kill pink bollworm larvae. The government also planned disrupted mating technique by sterile male dispersal and using sex pheromones etc. Although these efforts resulted in the suppression of pink bollworm population but the

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cost for these efforts accounted for a worth of $32 million per year. This leads to the idea of pink bollworm complete eradication by using all these control methods together by the National Cotton Council (Grefenstette et al., 2009; Marjorie, 2019). The article aimed to review literature discussing methods meant to control cotton pink bollworm, with the aim to investigate the idea of global eradication. Here we identify the problem areas that resulted in pink bollworm resistant to Bt-cotton and the consequences of this negligence. Further, we focused the priority areas helping in its control and eradication. Here we summarize the invasion history and global spread of PBW. Moreover, the impact of collaborative efforts between the governments and different farmer organizations are summarized. Further, the results of different integrated pest management methods in combination with Bt-cotton are discussed.

Pink bollworm invasion history

It is believed that the centre of origin of pink bollworm is Australia or its neighbouring islands (*Fig. 1*), on a malvaceous family plant other than cotton. The positional relationship of Indonesia with the origin is very important in investigating the history of spreader of pink bollworm. As Indonesia is not a cotton producing country therefore it is ignored in the investigations but the earliest record of pink bollworm on Java dates from 1903 (Kalshoven et al., 1981). More recently, the presence of pink bollworm on Bali and its adjacent islands suggested that it is widespread in Indonesia (Burrows et al., 1982). The trade and Hindu expansion is a hypothetical scenario that explains the movement of pink bollworm into the India. From here it reached to the African continent by traders. With the import of American cotton into India, this insect preferred it and quickly shifted its hosts. Seed transport may have resulted in its movement to Egypt another historical cotton growing country, "the land of Nile". The movement of this destructive pest to South American continent with famous cotton growing areas of Brazil and Mexico occurred through the transport of infested seed originating in Egypt. On the other hand India is considered the centre of spreader to Malaysia, China and Hawaii through the transport of infested seed. Even its spreader is reported to USA during 19 from Indian sub-continent (Brown, 2002; Noble, 1969). Pink bollworm has been described from India in 1843 and subsequently recovered in Hawaii (1901), East Africa (1904), Malaysia and Burma (1906), Egypt (1907), Australia (1911), Mexico (1911), Brazil (1913), Texas (1917), China (1918) and California (1965) (Burrows et al., 1982).

Mechanism of pink bollworm resistance

Transgenic cotton has reduced the need for chemical pesticides. Millions of growers worldwide are cultivating this transgenic cotton. This genetically engineered cotton produces caterpillar-killing proteins from a bacterium source (Bacillus thuringiensis*)* (Bt) (Tabashnik and Carrière, 2019). These pests have developed resistance against this killer protein and reduced its efficacy (*Table 1*). Initially Bt cotton showed perfect results against pink bollworm but with the passage of time the pest undergoes mutations and developed resistance against Bt toxin (Qiu, 2010; Wan et al., 2012; Ojha Sree et al., 2014). In India during 2010-2017 pink bollworm resistance to transgenic cotton (Cry1Ac) and (Cry1Ac + Cry2Ab) have been located in ten major cotton growing states. This study provides the evidence of field evolved resistance of these pests to Bt-I and Bt-II cotton (Naik et al., 2018). These mutations were rare before the commercialization of this

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transgenic cotton. However, when two resistant pink bollworm moths get a chance to mate, they produce resistant off-springs. If their population remains unchecked, this increases the proportion of resistant pests in every generation. In a study in central Indian states, pink bollworm resistant samples have been collected from Bt and non-Bt cotton fields. They have observed reduction in labelled Cry1Ac protein binding activity to the receptors membrane of pink bollworm larva showing very high tolerance to Cry1Ac (Lu et al., 2013). They pooled Cry1Ac-resistant strains and feed them on Cry1Ac diet. This pooled sample of resistance pink bollworm again showed significant reduction in protein binding activity. The reduced binding of Cry1Ac to receptors could be anticipated to pink bollworm getting resistant against Bt-toxin and Bollgard hybrids (Ojha et al., 2014). The knowledge of level of dominance for Bt protein resistance in a population is very important because it affects the success of the refuge strategy. This approach of refuge crop is most widely adopted strategy to delay pest resistance development to Bt cotton throughout the world (Zhang et al., 2012).

Figure 1. *The history of spreader of pink bollworm. (A) Pink bollworm spreader to the whole world starts from the centre of origin 'Australia' or its neighbouring islands. From here through the way of Indonesia (Java) it reached to India through trade. (B) From India (centre of spreader) it reached to the African continent (Egypt), USA, China and Malaysian islands by traders. (C) From Africa (Egypt) it reached Brazil and Mexico through the transport of infested seed*

Refuges can delay resistance

One strategy to maintain progeny of Bt-toxin susceptible moths within the insect population in the cotton field is the use of non-Bt cotton as refuse crop. The normal moth mate with resistance or normal moth the progeny will remain Bt-toxin susceptible. And probability of mating two resistant moths will remain low. Now most of the countries either use refuge crop to maintain Bt-toxin susceptible insect progeny (Wan et al., 2012) or cultivating doubled toxins Bollgard hybrids that would require pink

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bollworm to mutate twice in order to survive (Tabashnik et al., 2008). Here we must relate the story of "pink bollworm versus Bt cotton" in the three different areas of the world (Tabashnik and Carrière, 2019). In United States when Bt-cotton was first introduced and planted for cultivation Environmental Protection Agency of the country made it a mandatory requirement to plant 5% of their cotton acreage as non-Bt cotton state-wide. They also dropped billions of sterile pink boll worm moths from airplanes into cotton fields during 2006 (Tabashnik et al., 2010). This was to reduce the probability of Bt-toxin resistance moth to get a little chance of mating with another resistant moth. This would lead to mate a sterile moth to stop reproduction of the fertile offspring. The cultivation of Bt-cotton with the use of 5 per cent non-Bt cotton as refuge, sterile moths, along with other control methods (Lu et al., 2012), eventually led to the eradication of pink bollworm from commercial cotton-growing areas (USDA**,** 2018). In China the situation was a bit different as pink boll worm was not the major chewing worm in the cotton fields. Instead army worm (*Helicoverpa armigera*) was the main destructive pest which already has the option of other crops to feed unlike the pink bollworm that feed exclusively on cotton. These other crops naturally serve as refuges for army worm (*Helicoverpa armigera*). Meanwhile the pink boll worm population resistant to Bt-toxin begin to increase in the Bt-cotton field in the absent of refuges (Wan et al., 2012). Luckily the cotton seed companies begin to sell second generation Bt-cotton seed mixed with 5% non-Bt-cotton seed and growers were not aware of this refuge mixer. They preferred hybrid seed with second generation of Bt-toxin to boost yield and reduce their production cost. This reverse the situation and pink bollworm resistance to Bt-toxin began to reduce because of increase in refuge (Tabashnik et al., 1990). In India the Bt-cotton seed was sold with a separate packing of non-Bt-cotton seed as refuge. The cotton growers were not properly educated with the importance of refuges and within a time span of less than a decade pink bollworm got resistance against first generation of Bt-toxin. Meanwhile most of the growers switched toward cultivation of second generation of Bt-cotton. Here pink bollworm was already resistant to the first toxin, and resistance to the second toxin evolved rapidly. Now the situation in India is worse as Bollgard hybrids with two Bt-toxins provides little or no control over the devastating pest. The possible way out seems adopting toward shortening the crop season (Tabashnik and Carrière, 2019). The evolution of resistance is real and the only way to coop with it is to slow down this resistance process. And a general principal that can work for any transgenic crop is "refuges can delay resistance" (Shen et al., 2010; Tabashnik and Carrière, 2019; Kranthi et al., 2005).

Fluctuation in Bt-protein expression and varied effectiveness

The Cry toxin crystals get soluble in the insect midgut and bind to the receptors. This damages the mid-gut apical membrane and ultimately death of the insect occurs (Kranthi et al., 2005; Qiong et al.; 2013; Jurat-Fuentes and Crickmore, 2017). The genetically engineered Bt-cotton, encoding the Cry1Ac, Cry2Ab, or Cry1F protein, effectively guard against the bollworm damage (Shen et al., 2010 and Levine et al., 2016) however, the insecticidal efficacy is not stable (Liu et al., 2019). The production and maintaining of Bt-toxin expression throughout the Bt-cotton growing season in an adequate and consistent amount in the susceptible plant organs is very important to prevent the harm of targeted pests. However, many studies have reported its fluctuation in expression during different cotton plant growth stages, resulting in varied insecticidal

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efficacy (Wan et al., 2005; Chen et al., 2017 and Bravo et al., 2018). Significant reduction in Bt-toxin expression and resistance for Helicoverpa spp. was reported especially after flowering (Wu, 2007; Knight and Rogers, 2013; Chen et al., 2017). Near crop maturation significant reduction in Cry1Ac protein expression in the plant leaves was also reported (Wu et al., 2003 and Chen et al., 2004). The Bt-toxin proteins level in second generation Bt-cotton carrying both Cry1Ac and Cry1Ab genes, was reported to be higher during the early plant stages and from anthesis onwards decrease significantly. The Bt-toxin protein resistance against targeted pests dropped below the lethal level of 1.9 μg g-1 and maintained only for 110 days. After this time period pink bollworm can easily harm the cotton crop (Guo et al., 2001; Kranthi et al., 2005).

Table 1. *The different aspects of the pink bollworm resistance and their possible solution*

Sr. no.	Different aspects of the pink bollworm resistance	References	Possible solutions
1.	The cultivation of Bt-cotton without the use of 5% non-Bt plants	Tabashnik and Carriere, 2019; Wan et al., 2012; Lu et al., 2012; Tabashnik et al., 2010, 2008	Refuge crop can delay PBW resistance development against Bt-cotton
2.	Fluctuation in Bt-protein expression and varied effectiveness	Bravo et al., 2018; Chen et al., 2017; Kranthi et al., 2005; Wan et al., 2005; Guo et al., 2001	Development of new Bt-cotton varieties with standard Bt-protein expression
3.	Bt-cotton varieties with prolong stand in the field (sowing timing early Feb and late maturing) favouring PBW life cycle	Asif et al., 2017; Sarwar, 2017; Reddy et al., 2015; Jatoi et al., 2012; Costa et al., 2011; Qureshi et al., 2009; Yu and Huang, 1990; Michel and Gomez, 1992; Singh et al., 1988	Cultivation of short duration and early maturing varieties
4.	Lack of integrated pest management practices	Tabashnik et al., 2021; Maruti et al., 2020; Marjorie, 2019; Sarwar, 2017; Grefenstette et al., 2009; Arif et al., 2007	Integrated pest management practices especially pheromone application for mating disruption and sterile pink bollworm moth releases
5.	Lack of coordination between government and farming community	Tabashnik et al., 2021; Marjorie, 2019; CDFA, 2018; Grefenstette et al., 2009; Henneberry and Naranjo, 1998	Following example of USDA Area-wide PBW Eradication Program

Mode of action throughout growing season

Depending on environmental conditions, pink bollworms can live and produce up to 1-4 summer generations. The cotton bolls are the favourite and most desirable egg-laying and feeding site for the development of larvae (Saunders, 1843; Beasley and Adams, 1995). The pink bollworm larvae bore into the squares and bolls after hatching from eggs laid by pink bollworm moths and cause rosette blooms. In the cotton bolls, the growing pink bollworm larvae feed and destroy 1-5 seeds before completing development and dropping to the soil for pupation. The lint is also damage by it by cutting it through its mouthparts (Peter et al., 1987). The pink bollworm spends the winter in the larval stage in diapause (Singh et al., 1988; Michel and Gomez, 1992) which is largely photoperiod induced. The larval entrance into the diapause increases from mid of September to the mid of October with an approximate percentage of 5-

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90%. The early crop termination can be a key to disrupt this diapause phase and ultimate control of this destructive pest in cotton. Harvest aid chemicals and use of growth regulators (Costa et al., 2011) can also be utilized for the effective destruction of unwanted squares and small bolls which can serve as cite of overwintering feeding and shelter for the pink bollworm population. Cutting off irrigation to eliminate the food supply and production of green bolls by the early September can be a good strategy. Immediately after crop termination and harvest, shredding the cotton plants increases the chances of larvae destruction. Unharvested bolls are rapidly dried by shredding the plants and high temperature further destroys larvae. Plough to a depth of at least 6 inches can be very helpful. 50 to 70% flooding in December can reduce populations of overwintering pink bollworms more effective than flooding in November or January (Anonymous, 2019). The cotton crop should be timely sprayed with insecticides until the phase of 3 weeks old last harvestable boll (Henneberry and Naranjo, 1998).

Breeding strategies

The technology behind the eradication of the pink bollworm has four primary components: 1) extensive survey; 2) transgenic Bt cotton; 3) pheromone application for mating disruption; and, 4) sterile pink bollworm moth releases (Tabashnik et al., 2021). The latest breeding strategy to confer resistance in cotton plant against pink bollworm is the genetic engineering for the production of Bt-toxin (Tabashnik and Carrière, 2019; (Kranthi et al., 2005; Qiong et al.; 2013; Jurat-Fuentes and Crickmore, 2017). Furthermore, before the introduction of genetically engineered Bt-cotton different morphological and physiological characters of cotton plant in combination of insect behaviour were considered for the development of resistance against this devastating pest of cotton. The feeding preferences of insects are dependent on several morphological and anatomical features of a host plant. The different plant characters like leaf and stem color, Shape of bracts (frego), leaf shape (okera), presence of hairs and spines on leaves, nectaries with deposition of waxes, plant cell wall lignifications, sturdiness of tissue etc., define the fate of insect attack either alone or in combination (Ponti, 1977; Painter, 1951; Din et al., 2016; Silva et al., 2008; Rahman et al., 2013). Different varieties have different resistance level against insect pest attack (Goussain et al., 2005; Pathan, et al., 2007; Inbar and Gerling, 2008; Nawab et al., 2014). Pink bollworm (*Pectinophora gossypiella*) had minimum infestation on certain genotypes with Okra leaves frego bract, red leaf color. These traits alone or in combination are found effective to minimize insect pest attack. These traits should be considered during any breeding program especially for commercial cultivars development (Indrayiani and Sumaitini, 2007). **Okra leaf:** Okra leaf type genotypes have less surface area and provide better light penetration and air circulation so are resistant to pink bollworm (Wilson and George, 1982; Wilson, 1986). **Frego Bracts:** In narrow and small bract genotypes the boll are naked and therefore are less damaged as compared to broad and normal bract genotypes. These define the pink bollworm moth preference for oviposition (Baloch et al., 2001; Rahman et al., 2008; Jones et al., 1989; Brazzel and Martin, 1959). **Glabrous and nectariless:** The genotypes with glabrous (hairs and spines on leaves) and nectariless traits show lower bollworm damage due to less preference of moth for oviposition (Jones et al., 1989; Brazzel and Martin, 1959). **Red leaf color:** Pink bollworm had minimum infestation on certain genotypes with, red leaf color and is found effective to restrict insect pest population (Indrayiani and Sumaitini,

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2007). **Early maturity:** Controlling the number of overwintering pink bollworms by reduced their food supply later in the year through the use of early maturing varieties and plant growth regulators (Jatoi et al., 2012; Kairon and Singh, 1996; Beasley and Adams, 1995).

In certain studies backcross breeding techniques is utilized to transfer these morphological traits to the next generations to develop resistant plant material. The genetic analysis verified these traits under additive genetic control with significantly high narrow sense heritability estimates and genotype × environment interactions were not significant for these traits. Therefore, it should not be difficult to transfer these resistance traits into desirable backgrounds (Wilson and George, 1983). Although Bt-cotton provide resistance against chewing insects but the evolution of resistance against this toxin in the insect pest is an alarming situation. In china another breeding technique gave promising results in eleven year field study which involves crossing Bt-cotton with conventional non-Bt cotton and then sowing the second generation. This strategy yields a random mixture within fields of three-quarters of plants that produce Bt protein and one-quarter that does not. This outcome illustrates that non-Bt plants in a seed mixture can boost survival of susceptible insects and delay resistance (Wan et al., 2017). Another breeding strategy to increase the insect resistance of cotton plants would be to combine the Bt-toxin trait with other plant morphological traits resistance against insects and pest traits such as nectariless, okra leaf, and early maturity, known to reduce pink bollworm and other pest insects (Douglas et al., 1992). Early maturing cotton varieties are desirable for a number of the reasons as they require relatively less inputs like fertilizer and irrigation and also uses less labour. Thus, early maturing varieties provide comparatively increased economic returns on account of reduced cost of inputs and crop management. Besides, early maturing cottons are exposed to unfavourable environmental conditions for a relatively shorter period, hence provides an escape to late season pest attack, especially boll worm pest complex. Short season cottons are also of immense importance in the countries where sequence of other crops succeed cotton crop, thus fitting very well in cropping pattern of wheat followed by cotton crop for sustainable agriculture (Jatoi et al., 2012). A number of indicators are proposed that determine earliness in cotton are reduction in monopodia, profuse flowering, higher boll setting and opening at earlier stages of crop growth, less and smaller leaves, short internode length, semi-determinate types, lower sympodial node number, short sympodial branches, cluster fruit bearing types, medium boll size, sub-okra types and dwarf stature plants (Kairon and Singh, 1996; Baloch and Baloch, 2004). Short-season upland cotton (*Gossypium hirsutum* L.), which is also called early-maturing cotton, generally exhibits a dwarf, compact phenotype with fewer leaves, shorter internodes and fruiting branches, and shorter growth period than middle-late-maturing cotton (Yu and Huang, 1990). Making use of the markers linked closely to QTL for early-maturing traits for MAS is an effective method for the simultaneous improvement of early maturity and other properties in cotton (Chengqi et al., 2013). The majority of yield in cotton plant is produced from the first and second fruiting sites on main-stem nodes 9 through 14. As much as 80% of the yield originates at these sites (Costa et al., 2011) Because of these growth characteristics, ways to modify and control the flowering/fruiting of the cotton plants are often desirable. This encourages the management practices to terminate crop at this stage to have maximum economic yield benefits along with suppressing overwintering insect population. The other option is the use of plant growth regulators (PGRs).

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A success story

The managerial activities have successfully prevented incipient infestations of PBW from becoming established in the cotton growing areas of California. A cooperative integrated pest control program by the cotton growers of the California and the United States Department of Agriculture (USDA) has been in continual operation since 1967. The major funding contributors are the cotton growers themselves and USDA share is nearly about 8% (CDFA, 2018). The success of this program is because of the collective use of the previous researches all together like integrated pest management approach, sterile PBW moth release (Tabashnik et al., 2021; Staten and Walters, 2020; Leonard et al., 2020) host free period including crop destruction and plant back restrictions, pheromone treatments and 100% transgenic Bacillus thuringiensis (Bt) cotton in the region to keep pink bollworm infestations below economic impact levels. In this program no pesticides were applied instead sterile PBW moths were utilized. They were produced at the CDFA/USDA pink bollworm rearing facilities in Phoenix, Arizona. The pheromone-baited insect traps were used to inspect weekly from April through October in order to know the proper time and stage to release sterile moths. Different areas were targeted and the sterile pink bollworm moths were released by aircrafts on daily bases. After successful reduction of pink bollworm the release of sterile moths ended in 2012. This helped in reduction of millions of pounds of pesticides which cost by $100- $150 per acre (CDFA, 2018). This program also used pheromones to disrupt the moths' mating activities. With a collaborative efforts of the local agriculture departments the cultural controls activities were applied by managing a "host-free" period through identification favourable of planting and crop destruction periods. The identification of favourable planting and crop destruction periods was monitored. These were carried out through collaboration between the local agriculture departments and farming community. Complete shredding of cotton stalks and the dislodging of cotton plant roots to prevent regrowth were encouraged. These all activities resulted in successful completion of this integrated pest control program. In addition to these activities the 95% cotton growing area is under Bt-cotton cultivation which is in compliance with the USDA Area-wide PBW Eradication Program (Henneberry and Naranjo, 1998). Although area-wide application of these methods resulted in its suppression but it accounted for at least the costs of $32 million per year. As a result, the National Cotton Council proposed its eradication by using all of these control methods together (Grefenstette et al., 2009; Marjorie, 2019).

Conclusions

The reason behind pink boll worm resistance to Bt-toxin in the main cotton growing areas of South Asia can be significant reduction in its expression near the crop maturation. The early crop termination through early maturing varieties, harvest aid chemicals and use of growth regulators can be a key to disrupt pink bollworm diapause phase. The cultivation of Bt-cotton with the strict use of 5% non-Bt as refuge and timely utilization of different integrated pest management practices is essential for management of pink bollworm in the cotton crop. A collaborative international revolution against this pest is needed as a pest in one part of the world can be in any part of the world.

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CHARACTERISTICS OF BACTERIAL COMMUNITIES IN THE RHIZOSPHERE SOIL OF *ARALIA CONTINENTALIS* KITAG. BASED ON HIGH THROUGHPUT SEQUENCING

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Abstract. By analyzing the structure of communities and distribution law of soil bacterial communities in different years, this paper provides a reference for cultivation management aiming to improve growth, development, and yield. The changes of soil bacterial community structure were analyzed by Illumina Miseq high-throughput sequencing techniques using growing period soils that were unplanted (A1), planted for 1 year (A2), 4 years (A3), or 10 years (A4). The V3-V4 hypervariable regions of 16 S rRNA genes were PCR amplified by genomic extraction, after sequencing Illumina HiSeq 2500 a high-throughput sequencing platform, bioinformatics were used to analyze the abundance, diversity index and community structure of soil bacteria in different planting years. The soil bacteria belonged to 25 Phylums, 76 Classes, 175 Orders, 255 Families, 395 Genuses, 423 Species. Among them, the dominant bacteria are Proteus, Acinetobacter, Actinomycetes, etc. There are significant differences in soil bacterial communities in different places. The diversity of bacterial communities increased with planting years the community structure, the abundance of beneficial microorganisms such as Proteus, Acinetobacter, Actinomycetes, Bacteroides and Chlorophyta decreased, which suggests that planting years are the main factors affecting soil microbial activity. The results have deepened the understanding of the microbial community structure and diversity in the rhizosphere of Aralia continentalis Kitag.
Keywords: *continuous cropping obstacles, Aralia continentalis Kitag., bacterial community, high throughput sequencing*

Introduction

Aralia continentalis Kitag. is a species of the Aralia genus distributed in the northeastern region (Wang, 2017). Aralia is similar to ginseng. The root and root bark of *Aralia continentalis* Kitag. have a wide range of medicinal properties. The total saponin content of *Aralia continentalis* Kitag. root bark is three times that of ginseng, and the curative effect of some diseases is similar to that of ginseng (Fan et al., 2010). Its buds that grow in early spring are rich in protein, carbohydrates, fat, cellulose, calcium and amino acids, and have high nutritional value (Duan et al., 2019). Its potential medicinal and edible values have gradually attracted people's attention.

In recent years, artificially domesticated *Aralia continentalis* Kitag. has been widely planted, and some achievements have been made in the introduction and domestication of *Aralia continentalis* Kitag. at home and abroad (Han et al., 2003; Fan et al., 2013). There are few reports on the research on the soil bacterial community of *Aralia continentalis* Kitag. Soil bacteria play a key role in the ecosystem, participating in energy conversion and material circulation (Jiang et al., 2018). The diversity of soil bacteria composition has a key impact on crop growth and production (Han et al., 2020). Existing studies have shown that soil bacteria and plants have formed a mutually beneficial relationship. On one hand, plants affect the changes

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in soil physical and chemical properties through rhizosphere exudates or litter, thereby affecting the growth of bacteria, thereby affecting the composition of the community, species richness and diversity, etc. (Yin et al., 2020); on the other hand, some plants show more vigorous growth in the presence of the entire bacterial community, and some bacteria can also inhibit plant diseases or related pathogenic bacteria and promote improved disease resistance and stress resistance (Qiu et al., 2019). This study uses high-throughput sequencing to analyze the status of soil bacterial communities in *Aralia continentalis* Kitag. at different ages, which can provide some references for exploring the diversity of soil bacteria in *Aralia continentalis* Kitag., and can also be used to study more effective soil management methods and changes in *Aralia continentalis* Kitag.

Materials and methods

Sample collection and analysis

This study was carried out with *Aralia continentalis* Kitag. experimental base of Changchun University in China, and 4 experiment fields with consistent field management were set up (*Fig. 1*). The row of *Aralia continentalis* Kitag. is 15-20 cm soft and 7-8 cm wide, covering 0.5-0.8 cm of soil. No other plants were planted in the experimental base during this period. Soil sampling was carried out in July 2019, and the soil in the nursery area without *Aralia continentalis* Kitag. (A1) and the soil with *Aralia continentalis* Kitag. was planted for 1 year (A2), 4 years (A3), and 10 years (A4) were taken. Six plots of 2 m × 2 m were set up on each plot, each plot was distributed, and rhizosphere soil was sampled by "shaking root method ", collected soil samples were numbered and loaded into sterile polyethylene sealing bags for DNA extraction and high-throughput sequencing.

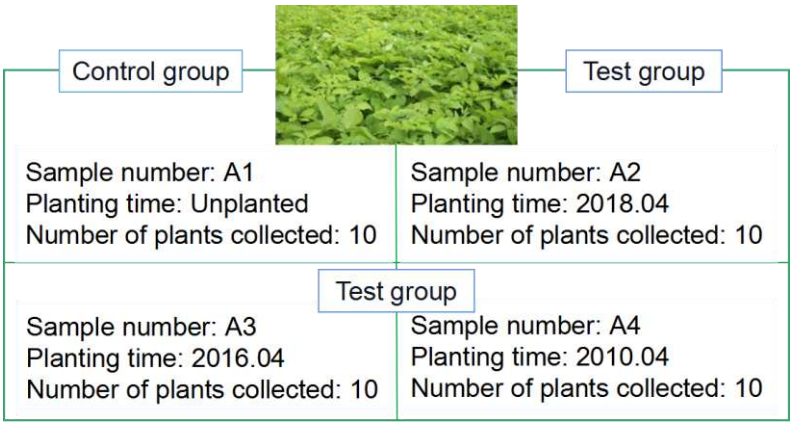

Figure 1. *Test plot information*

Test process

After extracting the total DNA of the sample, primers are designed according to the conserved regions, and sequencing adapters are added to the ends of the primers, PCR amplification is performed, and the products are purified, quantified and homogenized to form a sequencing library. The built library is first subjected to

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library quality inspection. The qualified library was sequenced with Illumina HiSeq 2500 (*Table 1*).

Table 1. *Primer list*

Name	Sequenced region	Primer name	Primer sequence
Bacteria	16s: V3 + V4	338F 806R	5'ACTCCTACGGGAGGCAGCA-3' 5'-GGACTACHVGGGTWTCTAAT-3'

Data analysis

The original sequencing data were filtered and merged to obtain the optimized sequences. The optimized sequences were clustered to get OTU, then the species classification was obtained according to the sequence composition of OTU; Based on OTU analysis results, taxonomic analysis was performed on samples at various taxonomic levels to obtain the community structure map, species cluster heat map, genus level phylogenetic tree and taxonomic tree of each sample at the taxonomic level of phylum, class, order, family, genus, and species. Alpha diversity analysis was used to study the species diversity within a single sample. Ace, Chao1, Shannon and Simpson indices of each sample were statistically calculated at 97% similarity level to draw sample dilution curve and rank abundance curve; Beta diversity analysis was used to compare the differences of species diversity between different samples.

According to overlap relation between PE reads, the paired-ends sequence data obtained from Hiseq sequencing were merged into tags of one sequence, then quality control and filtering was performed for reads quality and Merge effect. (1) PE reads merge: FLASH v1.2.7 software was used to merge reads through overlap, the obtained merged sequences were Raw Tags; (2) Tags filtering: Trimmomatic v0.33 software was used to filter merged Raw Tags to get high quality Clean Tags; (3) Chimera removal: UCHIME v4.2 software was used to identify and remove chimera sequences to get Effective Tags.

Results and analysis

OTU analysis and species classification of soil bacteria in Aralia continentalis Kitag. with different planting years

It can be seen from *Table 2* that there is a big difference in the number of soil samples from different planting years. A total of 689980 pairs of reads were obtained by sequencing. After splicing and quality control filtering, 654659 optimized sequences were obtained. Among them, the number of reads of A1 (65367 pairs) is the least, and that of A4 (292452 pairs) is the most, which is more than four times of A1. There was no significant difference in avglen (BP), GC (%), Q20 (%), q30 (%) and effective tags (%) among A1, A2, A3 and A4 samples. It can be seen from *Figure 2* that, at the 97% similarity level, the number of OTUs in A4 is the most, while the number of OTUs in A1 is the least (1526 in A1, 1673 in A2, 1694 in A3 and 1703 in A4). The total number of OTUs is 1493, accounting for 86.90% of the total OTUs of soil bacteria in *Aralia continentalis* Kitag. The number of OTUs unique to A3 and A4 is 6, It accounted for 0.35% of the total OTU of soil bacteria in *Aralia continentalis* Kitag., and the number of OTU unique to A1 and A2 was 0.

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Table 2. *Statistics of sequencing data processing*

Sample ID	PE reads	Raw tags	Clean tags	Effective tags	AvgLen (bp)	GC (%)	Q20 (%)	Q30 (%)	Effective (%)
A1	65367	62245	62157	61008	419	56.43	97.08	91.93	93.33
A2	108213	102223	102062	98315	420	56.06	96.81	91.3	90.85
A3	223948	213012	212724	207687	420	55.87	96.96	91.65	92.74
A4	292452	278087	277716	270767	420	56.5	96.99	91.81	92.59

PE Reads is the paired-ends reads count obtained from sequencing; Raw Tags is the count of original sequences merged from paired-ends reads; Clean Tags is the count of optimized sequences after filtering of raw tags; Effective Tags is the count of effective sequences after filtering chimera from Clean Tags; AvgLen (bp) is the average sequence length of sample; GC(%) is the GC content of sample, i.e. the percentage of G and C bases in all bases; Q20(%) is the percentage of bases which quality value is no less than 20 in all bases; Q30(%) is the percentage of bases which quality value is no less than 30 in all bases; Effective (%) is the percentage of Effective Tags in PE Reads

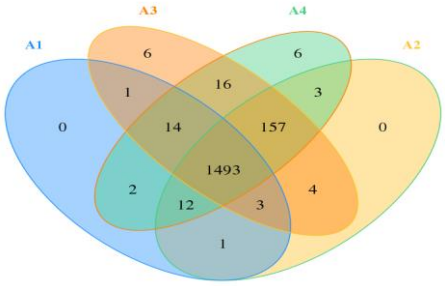

Figure 2. *OTU Venn. The number of overlapping parts between multiple color patterns is the total number of OTUs among multiple samples, and the number of non-overlapping parts is the unique number of OTUs of each sample*

Analysis of bacterial community structure and abundance in soil of Aralia continentalis Kitag. with different planting years

The OTU representative sequences of each sample were annotated at each level to obtain the statistical information of species at each level of each sample (*Table 3*). The average number of species of A1, A2, A3 and A4 at the phylum, class and order level were 23, 74 and 170 respectively, and the relevant values of each sample were very close to the average value. There were significant differences in the number of species at the family, genus, and species level, and A1 was in the family, genus, and order level The number of species was the least at the level of 240, 371 and 394, respectively, and A4 was the most at the level of 253, 391 and 419, respectively.

It can be seen from *Figure 3A* that *Aralia continentalis* Kitag. growing for 10 years has the largest number of soil bacteria (25 phyla), and A1 has the least number of soil bacteria (22 phyla). The species abundance information of each sample is shown in *Figure 3A* (in order to make the histogram more intuitive and clearer in reflecting the species composition, only the top 10 species with higher abundance are shown, and other species are classified as others, unclassified represents the species without taxonomic annotation on the pair.) The relative abundances of *Proteobacteria, acidobacteria, Actinobacteria, Bacteroidetes* and *Chloroflex I* were 35.6%, 18.3%, 9.6%, 8.7% and 8.3%, respectively. In the four soil samples, the sum of the relative

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abundances of the five phyla accounted for 80.5% of the total number of soil bacteria. However, the content of *Proteus* in A2, A3 and A4 was higher than that in A1, *acidobacteria* and *Curvularia* in A1, A3 and A4 The content of actinomycetes in A3 and A4 was lower than that in A1 and A2, which indicated that different planting years had different effects on the growth of different bacteria. According to the similarity of species or richness, the samples were clustered, and the species richness clustering heat maps of different classification levels were obtained (*Fig. 3B*). The bacteria in the four samples were roughly divided into four clusters.

Table 3. *Statistics of species of samples by grade*

Sample	Kingdom	Phylum	Class	Order	Family	Genus	Species
A1	1	22	72	168	240	371	394
A2	1	23	73	170	246	384	411
A3	1	23	74	171	249	388	416
A4	1	25	75	174	253	391	419
Total	1	25	76	175	255	395	423

Analysis results of bacterial community abundance at phylum level

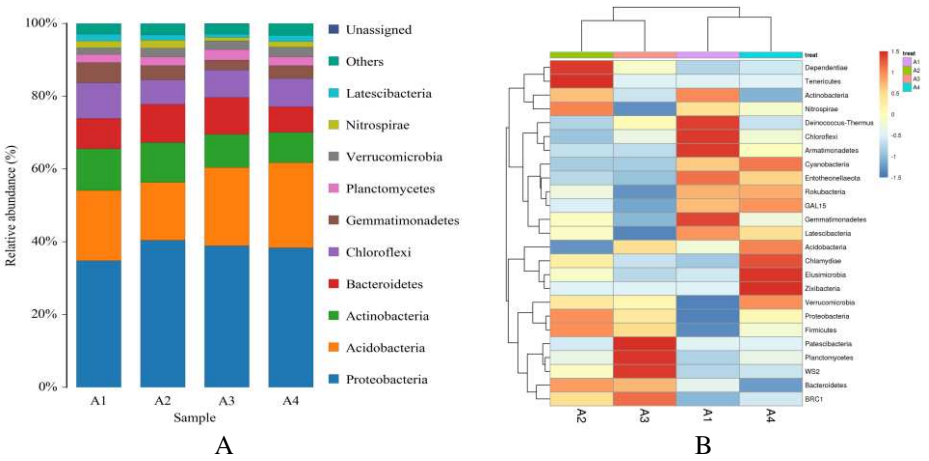

Figure 3. *The relative abundance histogram (A) and the relative abundance cluster diagram (B) of bacteria community on the phylum level*

Analysis results of bacterial community abundance at class level

According to *Figure 4A*, the main groups of soil bacterial community of *Aralia continentalis* Kitag. are gamma *Proteobacteria, Alpha Proteobacteria, subgroup_6, Bacteroidia, Blastocatellia _ Subgroup_*, and their relative species abundance averaged were 4%, 12.3%, 5.6%, 4.9% and 3.7% respectively. *Gammaproteobacteria* in the rhizosphere soil of *Aralia continentalis* Kitag. is the dominant class. It can be seen from *Figure 4B* that among the four samples, *bacteria* are roughly divided into four clusters, 15 classes such as *Dehalococcoidia* and *Chlamydiae* are closely related to each other, 17 classes such as *Deltaproteobacteria* and *Actinobacteria* are the second cluster, 22 classes such as *Gemmatimonadetes* and *Anaerolineae* are the third cluster, and 22 classes such as *Parcubacteria* and *Rubrobacteria* are the fourth cluster.

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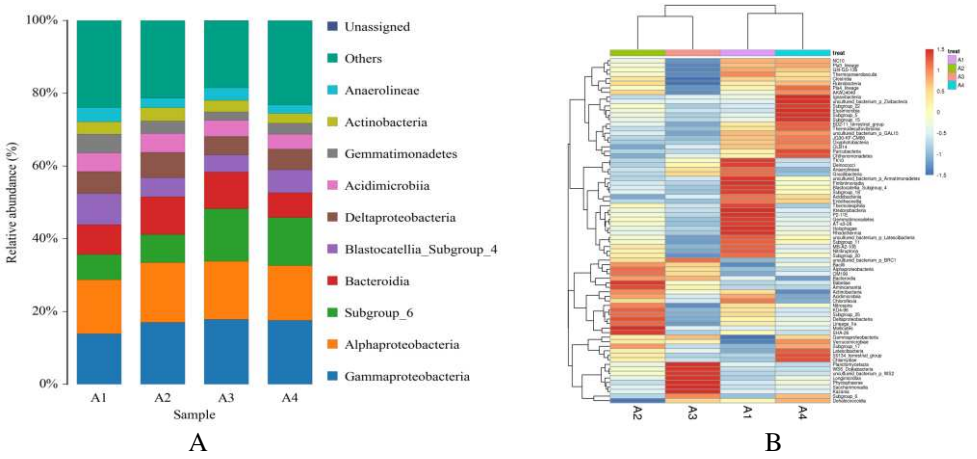

Figure 4. *The relative abundance histogram (A) and the relative abundance cluster diagram (B) of bacteria community on the class level*

Analysis results of bacterial community abundance at order level

It can be seen from *Figure 5A* that the main bacterial communities in the four samples are betaproteobacteriales, uncultured _ bacterium _ Subgroup _ 6, Rhizobiales, Chitinophagales, Pyromonadales, Sphingomonadales, Gemmatimonadales, Myxococcales, Microtichales, Cytophagales, and their relative species abundance averaged were 5.5%, 10.2%, 5.1%, 4.8%, 3.9%, 3.2%, 2.9%, 3.1%, 2.8% and 2.3%, respectively. The dominant strains were Betaproteobacteriales and Uncultured _ bacterium _ Subgroup _ 6. It can be seen from *Figure 5B* that in the four samples, bacteria are roughly divided into four clusters, 26 orders such as Caulobacterales and Anolineales are the first cluster, 13 orders such as Mbnt15, Bacteroidales and Pseudomonas are the second cluster, 28 orders such as S085, Enterobacteria and Chthoniobacteria are the third cluster, and 33 orders such as Babeliales, Micromonosporales and Babeliales are the fourth clusters. At order level, the bacterial composition of the four samples was significantly different. The main bacteria in sample A2 are the second cluster, the main bacteria in sample A3 are the fourth cluster, the main bacteria in sample A1 are the first cluster, and the main bacteria in sample A4 are the third cluster.

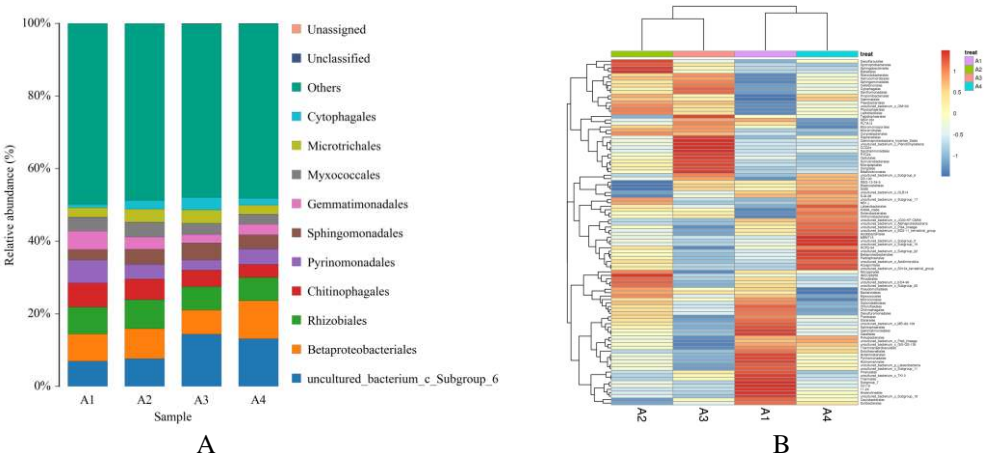

Figure 5. *The relative abundance histogram (A) and the relative abundance cluster diagram (B) of bacteria community on the order level*

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Analysis results of bacterial community abundance at family level

The main bacterial communities in the four samples were uncultured _ bacterium _ c_Subgroup_6, Pyinomonadaccae, Chidinophagaccae, Nitrosomonadacae, Sphingomonadaceae, Gemmatimonadaceae, Xanthobacteraceae, Microscillaceae, Burkholderiaceae, and their relative species abundance averages were 8.9%, 3.9%, 3.7%, 3.5%, 3.2%, 2.8%, 2.5%, 2.0%, 1.8%, respectively. It can be seen from *Figure 6B* that the composition of soil bacterial community of Aralia continentalis Kitag. planted for one year, four years and ten years is similar, while the composition of soil bacterial community of Aralia continentalis Kitag. planted for less than one year is larger than that of the three. In the four samples, soil bacteria are roughly divided into three clusters, and 33 families, such as Anaeroline Aceae, Scolimonadaceae and Micrococcaceae, are closely related to each other, 28 families, such as Methylophilaceae and Pedosbhaeraceae, are clustered in the second cluster, while 39 families, such as Devosiaceae and Polyangiaceae, are clustered in the third cluster (*Fig. 6*).

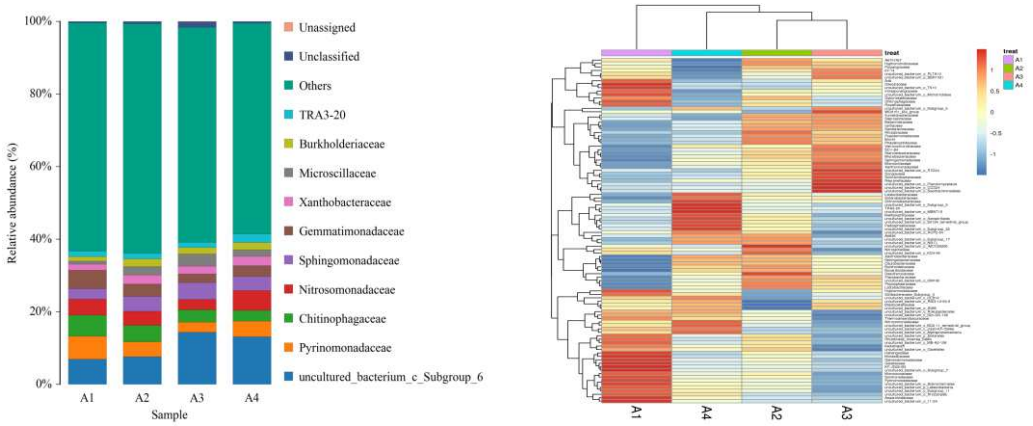

Figure 6. *The relative abundance histogram (A) and the relative abundance cluster diagram (B) of bacteria community on the family level*

Analysis results of bacterial community abundance at genus level

Figure 7A shows that the main bacteria in the soil of the four samples were uncultured _ bacterium _ c _ Subgroup _ 6, RB41, uncultured _ bacterium _ f _ Gemmatimonadaceae, Sphingomonas (sphingosine monomonas), uncultured _ bacterium _ f _ Chitinophagaceae, MND1, uncultured _ bacterium _ f _ Microscillaceae, uncultured _ bacterium _ f _ TRA3-20, Nitrospira, uncultured _ bacterium _ o _ PLTA13 relative species abundance averages were 6.5%, 7.5%, 4.9%, 3.5%, 3.5%, 3.5%, 2.6%, 2.3%, 1.8%, respectively. *Figure 7B* shows that the bacterial composition of the soil with less than one year of cultivation is similar to that of the long white log with 1 year and 10 years. The difference is large after four years of cultivation compared to other years.

Phylogenetic analysis of dominant bacteria in soil of Aralia continentalis Kitag. with different planting years

The representative sequence data of genera with relatively high abundance were selected, by QIME software, and multiple sequence alignment was carried out. Then the

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phylogenetic tree was built, and the graph was drawn (*Fig. 8*). Data on the occurrence of representative sequence systems correspond to the first 13 genera and bacteria with high abundance in the soil samples. The results showed that there were 13 main genera in the rhizosphere soil samples of *Acidobacteria, Patescibacteria, Proteobacteria, Nitrospirae, Rokubacteria, Actinobacteria, Planctomycetes, Chloroflexi, Bacteroidtes, Firmicutes, Latescibacteria, Verrucomicrobia, Gemmatimonadetes*. Among them, *Proteobacteria* accounted for the largest proportion.

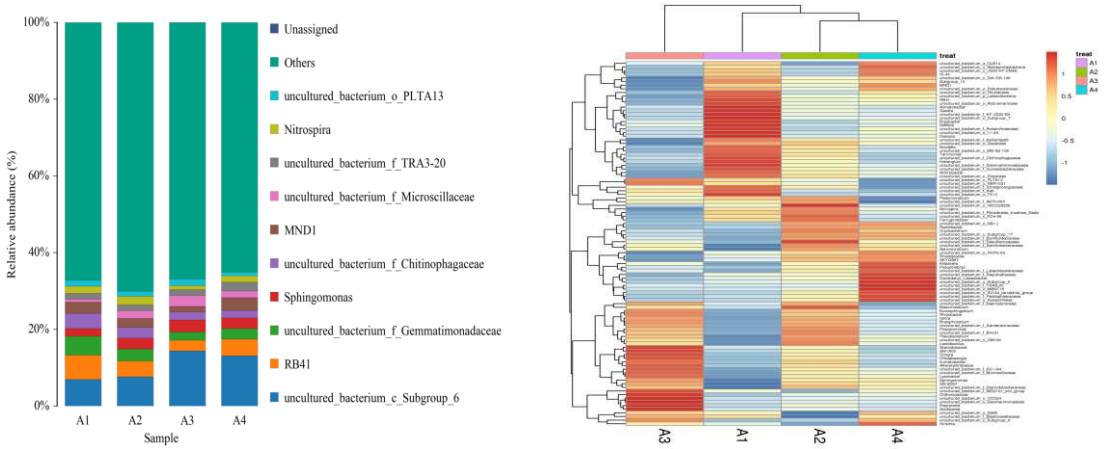

Figure 7. *The relative abundance histogram (A) and the relative abundance cluster diagram (B) of bacteria community on the genus level*

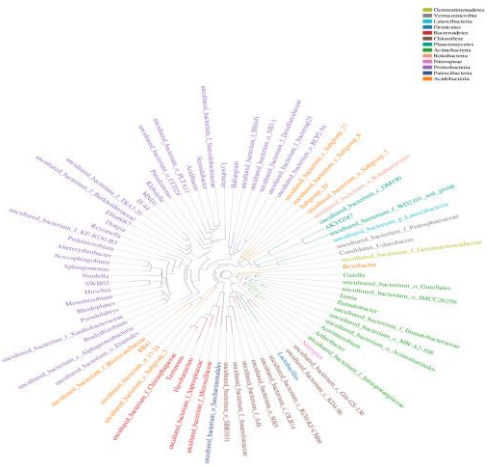

Figure 8. *Species phylogenetic tree*

Analysis of soil bacterial diversity index of Aralia continentalis Kitag. in different planting years

The diversity index of soil alpha in different planting years is shown in *Table 4*. The OTU coverage rates of A1, A2, A3 and A4 were all above 99%, indicating that the sequencing results reflected the real situation of soil bacteria in the samples. The difference between ACE index and Chao1 index of each sample was obvious, which indicated that the species abundance of each sample was significant. The ace and Chao1

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indices of sample A4 were the highest, 1705.1625 and 1, respectively. The results showed that the abundance of bacteria in the soil of *Aralia continentalis* Kitag. planted for 10 years was the highest, while the ace and Chao1 indices in sample A1 were the lowest, indicating that the abundance of bacteria in the soil of *Aralia continentalis* Kitag. planted for less than 1 year was the lowest; the Shannon index and Simpson index of sample A4 and A2 were the highest, indicating that the bacterial diversity in the soil of *Aralia continentalis* Kitag. planted for 4 and 10 years was the highest. According to the alpha diversity index of each sample, the abundance and diversity of soil bacteria of newly planted *Aralia continentalis* Kitag. are relatively low, and with the increase of planting years, the abundance and diversity of soil bacteria are also increasing, among which, the increase range is larger in the two years after planting, and the increase range is smaller and smaller with the increase of planting years. The results showed that *Aralia continentalis* Kitag. interacted with soil bacteria in the process of growth, and soil bacteria would adapt to the environment with the change of environment.

Table 4. *Alpha diversity index statistics*

Sample ID	OTU	ACE	Chao1	Simpson	Shannon	Coverage
A1	1,526	1,572.04	1,580.79	0.0057	6.1821	0.9972
A2	1,673	1,683.11	1,686.89	0.0041	6.3762	0.9993
A3	1,694	1,699.83	1,701.67	0.0042	6.3267	0.9998
A4	1,703	1,705.16	1,706.46	0.0041	6.376	0.9999

OTU presents the number of OTU; Chao1, Ace, Shannon and Simpson represent each index respectively. Coverage represents the Coverage of the sample library

With the continuous random sampling of effective sequences, the number of OTU is increasing, but after the OTU reaches a certain value, the curve tends to balance and no longer changes with the increase of sequence number. It shows that the sequencing data can reflect species diversity and species richness in the sample. *Figure 9* shows that at the level of 97% sequence similarity, the OTU number shows the following trend: A1 < A2 < A4 < A3, sparse curve of soil bacteria in different planting years tend to be flat, and the OTU coverage of the samples has reached saturation. It can reflect the composition of bacterial community in the soil of L. The curve of A3 and A4 is flatter than that of A1 and A2, which indicates that the uniformity of soil bacteria planted with long years is higher than that of short years (*Fig. 10*).

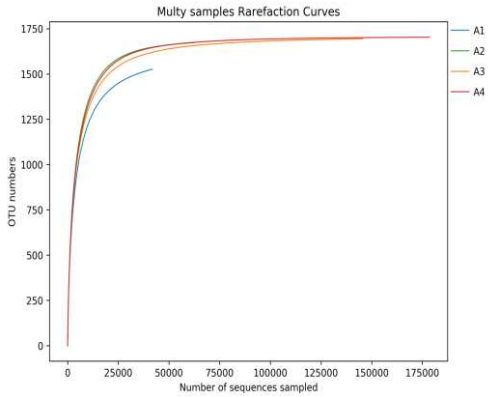

Figure 9. *Dilution curve of soil samples at 97% level*

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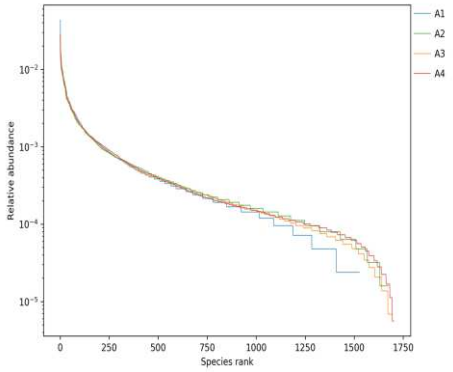

Figure 10. *Rank-abundance curve of soil samples*

Based on the distance Matrix between samples obtained by the distance algorithms (binary, Bray, weighted, unweighted) , the heat map of soil bacterial community diversity is drawn by the R language tool. In *Figure 11A* and *B*, the cross between sample A1 and A3 is the deepest red. The results showed that the greatest difference was measured between the bacteria in the soil of the unplanted and cultivated land. The cross between sample A3 and A4 is deepest blue. It shows that the similarity of soil bacteria between the newly planted 4 years and the cultivated 10 years is the greatest.

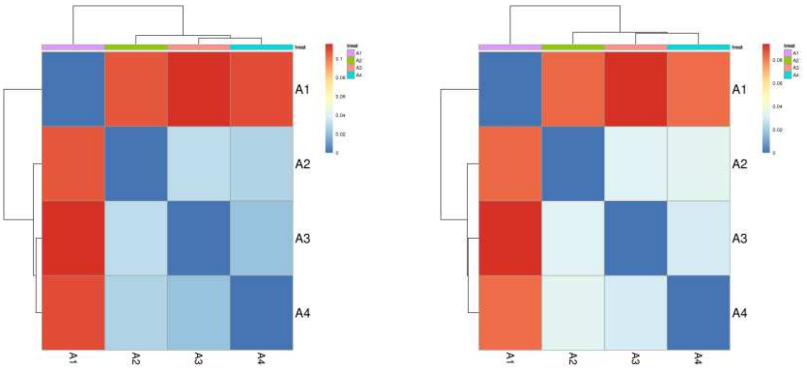

Figure 11. *Heat map of soil bacterial community diversity in different samples*

Correlation analysis of soil bacterial species of Aralia continentalis Kitag. in different planting years

According to the abundance and change information of species in various varieties, Spearman rank correlation analysis and screening data were used to construct the genus level correlation network (*Fig. 12*). The average abundance of RB41 (acidobacteria) was the highest, followed by iamia, but they had little correlation with other species, Novosphingobium and subgroup_ The average abundances of Novosphingobium and Devosia are small, but they are highly correlated with many other species_10 was negatively correlated with Devosia. The results showed that the species composition and diversity of bacterial community were influenced not only by the interaction between different bacterial species, but also by other factors such as environmental factors.

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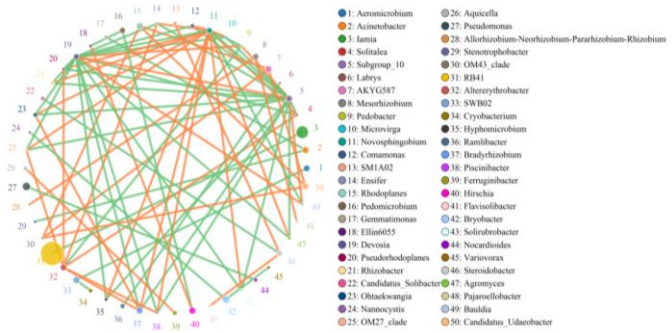

Figure 12. *Species-related networks of soil sample bacteria at the genus level*

Discussion

Interannual variation characteristics of soil bacterial community structure of Aralia continentalis Kitag.

There are many kinds of bacteria in soil. In different soil environments, the bacterial composition is similar and different, and the plants growing or planted in soil also have a certain degree of influence on the composition and structure of soil bacterial community, species richness, bacterial distribution uniformity and diversity (Dong et al., 2018; Zhu et al., 2019). At present, high-throughput sequencing technology is widely used to study plant rhizosphere soil microbial community, and the results obtained by high-throughput sequencing are more comprehensive and accurate than traditional sequencing technology (Wang et al., 2018). In this study, high-throughput sequencing technology was used to analyze the soil samples of *Aralia continentalis* Kitag. in different planting years. There were 25 phyla, 76 classes, 175 orders, 255 families and 423 species of soil bacteria in four different planting years. The dominant bacteria at phyla level were Proteus, Acidobacteria, Actinomycetes, Bacteroides and Curvularia, among which Proteus accounted for the largest proportion. This is similar to the related research results of selenium sand melon, cotton, ramie and wolfberry (Qiao et al., 2018; Zhu et al., 2018; Xiao et al., 2018; Yue et al., 2020). Proteus and Actinomycetes can participate in the carbon and nitrogen cycle of various organic matter. Nitrogen fixing bacteria play a certain role when crops absorb nitrogen from soil (Guo, 2019). Acidobacteria can degrade plant residues, carry out photosynthesis, participate in carbon metabolism, etc., and play a very important role in soil material cycle and ecological environment construction (Wang et al., 2016). In this study, the abundance of soil degenerative bacteria, actinomycetes and acidobacteria in different planting years of *Aralia continentalis* Kitag. showed the following trend: A4 < A3 < A2, indicating that continuous cropping may reduce the accumulation of soil beneficial bacteria and hinder soil material circulation and fertility renewal. Curvularia viridis is a kind of microbe that can produce energy through photosynthesis and CO_2 as carbon source. It also has strong competitiveness in soil with low organic matter and other nutrients (Kou et al., 2020). In this study, the dominant bacteria in soil of *Aralia continentalis* Kitag. were nitrifying Spirochetes, Sphingomonas, Proteus, Acidobacter and other beneficial bacteria, which had important value in the control of plant pathogens (Lalloo et al., 2010). With the extension of planting years, the abundance of flora increased, which indicated that the effective utilization of photosynthetic products in the process of planting *Aralia continentalis* Kitag. would be conducive to the growth of *Aralia continentalis* Kitag. (Zhu et al., 2016).

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Interannual characteristics of soil bacterial community diversity of Aralia continentalis Kitag.

Soil bacterial nutrients mainly come from plant root exudates. The types and quantities of organic acids, inorganic ions, and other substances in plant root exudates at different growth stages are different, which affect the growth, distribution, abundance and diversity of soil bacteria, and soil bacteria also play a crucial role in soil physical and chemical reactions (Ding et al., 2018). In this study, we found that the diversity and evenness of soil bacterial community of *Aralia continentalis* Kitag. planted in different years were different. In terms of soil bacterial abundance, diversity and evenness, the soil bacteria of *Aralia continentalis* Kitag. planted for 1 year, 4 years and 10 years were significantly higher than those of *Aralia continentalis* Kitag. planted for less than 1 year, and with the increase of years, the abundance and diversity of bacterial species increased, and the increasing extent increased with the increase of years. Therefore, root exudates increased the abundance and diversity of soil bacterial community (Luo et al., 2019). In this study, we also found that there were 6 unique OTUs in soil bacteria of *Aralia continentalis* Kitag. planted for 4 years and 10 years, and 0 in soil bacteria of *Aralia continentalis* Kitag. planted within one year. Through the analysis of the effective sequence length distribution map and OTU number distribution map of each sample, it indicated that there were abundant soil bacteria species in *Aralia continentalis* Kitag. Most of the soil bacteria species planted in different years had great similarity with their distribution, but there were significant differences in the composition and distribution of a few soil bacteria. With the growth of *Aralia continentalis* Kitag. growing years increasing, the soil bacteria species of *Aralia continentalis* Kitag. growing in different years had great similarity with their distribution. The number of different kinds of soil bacteria showed the same or opposite changes, and some unique strains could also be produced, which may be affected by the physiological metabolism of *Aralia continentalis* Kitag. in different periods and the artificial cultivation and management measures in different periods (Zhou et al., 2018). There are many factors affecting soil bacterial diversity. To maintain soil bacterial diversity and richness, we should strengthen soil nutrient management and avoid soil acidification, so as to increase the quality and yield of *Aralia continentalis* Kitag. (Duan et al., 2012).

Conclusion

The soil bacterial community of *Aralia continentalis* Kitag. planted was significantly different from that of *Aralia continentalis* Kitag. unplanted. With the increase of planting years, the diversity of bacterial communities increases. In terms of community structure, the abundance of beneficial microorganisms such as *Proteobacteria, Acidobacteria, Actinomycetes, Bacteroides* and *Chloroflexus,* etc. decreases. This study has deepened the understanding of the microbial community structure and diversity in the rhizosphere of *Aralia continentalis* Kitag., combined with the environmental requirements of *Aralia continentalis* Kitag. growth and the factors of the physiological and metabolic activities of plant roots, it is speculated that there are other influencing factors and pathways in microbial regulation, these issues need to be further studied.

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SPECIES COMPOSITION AND ABUNDANCE OF EGG AND LARVAL FISH IN THE LOWER REACHES OF GANJIANG RIVER UNDER CASCADED DEVELOPMENT

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Abstract. Egg and larval fish investigation can be deduced the distribution and reproductive characteristics of adult fish. Ganjiang River is the longest river flowing into the Poyang Lake, and one of the important tributaries of the Yangtze River. Due to the construction of cascade water conservancy projects on the mainstream of the river, the fish resources have been affected. In order to correctly design protection measures of fish resources, egg and larval fish species composition and abundance at Fengcheng section in lower reaches of the river were investigated between May and June 2019. The results indicated that: (a) of the 29 species of egg and larval fish collected, and the individuals of *Hemiculter bleekeri*, *Rhinogobius giurinus*, *Parabramis pekinensis*, *Squalidus argentatus* and *Xenocypris argentea* were most abundant; (b) egg abundance was higher through May while larval abundance was higher during June; (c) the egg abundance of the four carps further declined and two new spawning grounds were found. Therefore, we suggested that the ecological regulation of the Ganjiang River should be managed to create flood peaks and make the daily increment of water level greater than 0.55 m when water temperature was between 18-24 °C during May to July.
Keywords: *Ganjiang River, hydraulic complex, early-stage fish recourse, diurnal variation, ecological regulation*

Introduction

The resources and spawning grounds of most fish have been negatively affected by some factors, such as water pollution, hydraulic engineering construction, overfishing, and so on (Sun et al., 2015; Xu et al., 2015). The dynamics of egg and larval fish resources are sensitive indicators of environmental changes (Gogola et al., 2013). In addition, early life history stages play an important role in fish population dynamics (Kamler, 1992). Meanwhile, knowledge about the mechanisms underlying recruitment success during the early life history stages provides essential information to explain population dynamics (Trippel and Chambers, 1997). The abundance, spawning grounds, and reproductive traits of adult fish can be inferred from information on the distribution patterns of egg and larval fish (Doyle et al., 1993; Nonaka et al., 2000; Bialetzki et al., 2001; Cao et al., 2007). Therefore, knowledge of egg and larval fish assemblages provides fundamental information that is valuable for conservation and management.

The black carp (*Mylopharyngodon piceus*), grass carp (*Ctenopharyngodon idella*), silver carp (*Hypophthalmichthys molitrix*) and bighead carp (*Hypophthalmichthys*

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nobilis), referred to as the "four major Chinese carps", are all commercially important freshwater fish species in China. Ganjiang River is the largest river flowing into the Poyang Lake, and one of the important tributaries (the 7th largest) of the Yangtze River (Xiong, 2007). Historically, a total of 118 fish species were recorded in the Ganjiang River (Tian, 1989). And in 1960s, there were 12 spawning grounds for the four major Chinese carps in the river (Tian, 1989). Due to the separation of dams in the Ganjiang River, the water environment has changed, and the fishery resources have been affected (Tang et al., 1993). Meanwhile, the function of these spawning grounds has begun to decrease, and the 12 spawning grounds in the Ganjiang River had been investigated and found that only three spawning grounds were well preserved and other spawning grounds had degenerated (Liu et al., 2009; Hu et al., 2015; Guo et al., 2020). At present, six hydro-junctions on the Ganjiang River has been gradually completed, which has blocked the connection between the upstream and downstream of the Ganjiang River. After the cascade hub construction on the Ganjiang River have been completed and put into operation, it is expected that the larval fish abundance will be further reduced. In this case, a pressing matter of the moment is to explore the ecological impact of the larval fish, and to correctly design and implement protection measures.

In order to further understand the existing fishery resources in the Ganjiang River, the larval fish abundance was investigated through the fixed-point collection in the lower reaches of Ganjiang River. The present study was to evaluate: 1) species composition of egg and larval fish, 2) diurnal pattern of egg and larval abundance, 3) spawning grounds and scale of the four major Chinese carps, and 4) relationships of larval assemblages to environmental variables. The results of the research will benefit to fill in the blank of the early fish resources information after the construction of water conservancy project in the Ganjiang River, and clarify the ecological functions of the fish in this region.

Materials and methods

Study area

Studies on early-stage fish resources have been conducted in China since the early 1950s (Cao et al., 2007). Subsequently, a number of researchers investigated the early-stage fish resources of important rivers such as the Yangtze River, especially the four major carps in China, and identified many important spawning grounds by extrapolating the developmental periods of eggs and larvae and water flow rates (Cao et al., 2007). By the 1990s, due to the completion of the Three Gorges Water Conservancy Project, researchers conducted long-term and continuous surveys of Yangtze River eggs and larvae. The Ganjiang River, however, is one of the eight major rivers of the Yangtze River and is an important river for replenishing fish resources in the Yangtze River (Guo, 2018).

Ganjiang River, the longest river in Jiangxi Province, is more than 823 km long and has a drainage basin area of 82809 km^2 (Xiong, 2007). The riverhead is located on the Shiliaodong (in Yangdi Town) with a geographical coordinate of 116°22′ E and 25°57′ N, and the estuary is located on the Wangjiangting (in Wucheng Town) with a geographical coordinate of 116°01′ E and 29°11′ N (Xiong, 2007). The lower reaches of the river are below Xingan and have a length of about 208 km, and from Ganzhou to Xingan is the middle reaches with a length of about 303 km, and the upper reaches are above Ganzhou and have a length of about 312 km (Xiong, 2007; *Fig. 1*). The basin presents a mid-subtropical humid climate, and the mean annual precipitation and river runoff are about 1580.8 mm and 2125 m^3/s, respectively (Xiong, 2007).

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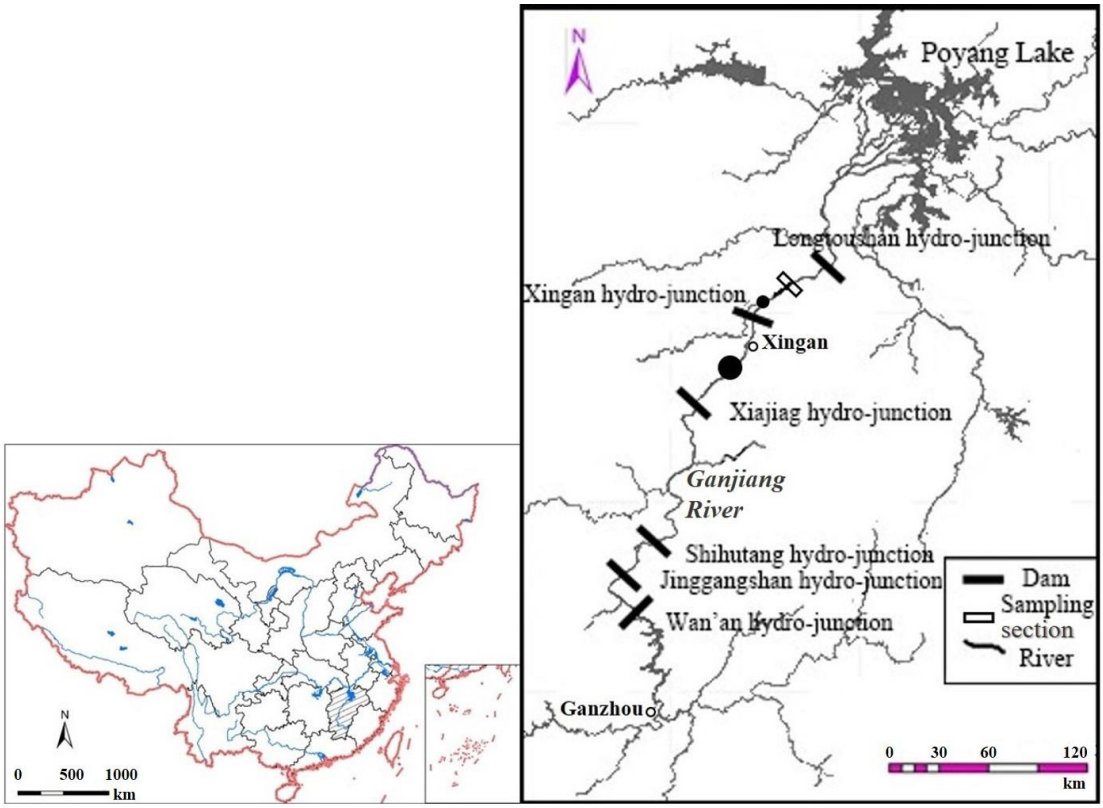

Figure 1. *Locations of sampling section and spawning grounds (●) of the four major Chinese carps in the Ganjiang River*

Samples collection

Based on the results of the survey of eggs and larvae in the Ganjiang River during the historical period (Liu et al., 2009; Hu et al., 2015; Guo et al., 2020), as well as the findings of some other rivers (Hu et al., 2019; Wang et al., 2020), it was concluded that the peak period of eggs and larvae occur mostly from May to July, so the eggs and larvae were collected day by day at the Fengcheng section (28°12′4″N, 115°46′47″E) during May 3 to June 23, 2019 (*Fig. 1*). Diurnal sampling was carried out twice between 7: 00 and 8: 00, and between 17: 00 and 18: 00 (Yi et al., 1988; Li et al., 2016). Each collection lasted about 30 min. Sampling was conducted with a conical net (1.0 m length, 0.5 m in diameter, with 0.3 mm in mesh size) to collect eggs and larvae just below the water surface (water depth, 0.5 m) (Yi et al., 1988, 2016; Guo et al., 2020). A mechanical flow meter was attached to the mouth of the net to calculate the water volume filtered during sampling. Sampling was conducted near the shore because of the high current velocity in the middle of the river (Li et al., 2016; Guo et al., 2020).

Eggs and larval fish were separated and counted immediately after sampling. The developmental stages and developmental times of each stage were defined according to Yi et al. (1988) and Cao et al. (2007). Eggs were incubated in a tank (0.18 m high, 0.2 m in diameter) and identified to species level. Each aquarium was stocked with 5–10 eggs, water temperature was maintained at 22–24 °C with air conditioning and the water was aerated (Li et al., 2016). The larvae were preserved in 5% sodium phosphate-buffered formalin (Ren et al., 2016). Species were identified according to Yi et al. (1988) and Cao et al. (2007).

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Water temperature, pH and transparency were obtained at sample site with a YSI recorder. Daily water level and water runoff were acquired from the Jiangxi Hydrology Information Network (http://www.jxssw.gov.cn/) measured at Fengcheng section.

Data analysis

The runoff of eggs and larvae in the sampling section was calculated according to the method of Yi et al. (1988, 2016) and Guo et al. (2020):

$$d = m / (v \times a \times t) \quad \text{(Eq.1)}$$

$$D = \sum_{i=1}^{n} di/n \quad \text{(Eq.2)}$$

$$M = \left(\frac{Q}{q}\right) \times D \quad \text{(Eq.3)}$$

where ***d*** is the density of eggs and larvae collected at the sampling site (ind./m^3), ***m*** is the number of eggs and larvae obtained in a single sampling, ***v*** is the velocity of river water passing through the mouth of conical net (m/s), ***a*** is the area of cone net mouth (m^2), and ***t*** is the duration of single sampling (s); ***di*** is the density of eggs and larvae at the *i* sampling point (ind./m^3), ***D*** is the average density of cross-section eggs and seedlings (ind./m^3); ***q*** is the river water flow in the network (m^3/s), ***Q*** is the average water flow of sampling site (m^3/s), ***M*** is the runoff of eggs and larvae in a single sampling (ind./m^3).

The runoff of eggs and larvae flowing through the section is calculated by interpolation method in the free time between the two samples, and the calculation is as follows: $M' = t' / 2 (M_1/t_1 + M_2/t_2)$. Where ***t'*** is the interval time between the two acquisitions (s), t_1 and t_2 represent the duration of the two acquisitions, M_1 and M_2 are the number of eggs and larvae collected before and after two times.

The runoff (N_m) of fish eggs and larvae passing through the survey section per day and night is the sum of the runoff of eggs and larvae collected regularly within 24 h and the sum of the runoff of eggs and larvae collected in non-collection time between the two samplings, and the calculation is as follows: $N_m = \sum M + \sum M'$.

According to the development period of fish eggs and larvae, combined with the calculation of river water velocity, the location of spawning ground is inferred, and the calculation is as follows: $L = V \times T$. Where ***L*** is the drifting distance of eggs from spawning ground (m); ***V*** is the water flow velocity of sampling site (m/s), ***T*** is the time that fish eggs have drifted in the water from spawning to sampling site (s).

The daily recorded hydrological data such as water temperature, pH, flow, water level and transparency are input and calculated by SPSS 20.0, and the graph is drawn by using GraphPad. The relationship between spawning quantity and environmental factors was compared by variance analysis, and the relationship between environmental factors and larval density was calculated by redundancy analysis.

Results

Environmental factors

Water level ranged from 23.1 m to 31.5 m with a mean of 25.5 ± 2.1 m (mean ± SD) during May to June in 2019. And it had a peak on 11 June. Water flow, ranging from

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3260 to 17279 m^3/s with a mean of 6212 ± 3436 m^3/s, showed a similar pattern to that of water level. Water temperature ranged from 21.5 to 27.8 °C with a mean of 24.8 ± 1.7 °C. It showed an increased slowly through May to June in 2019. Transparency ranged from 0.17 to 0.54 m and showed a clear daily variation with higher values during May and lower values during June. There was no clear diurnal pattern for pH value (*Fig. 2*).

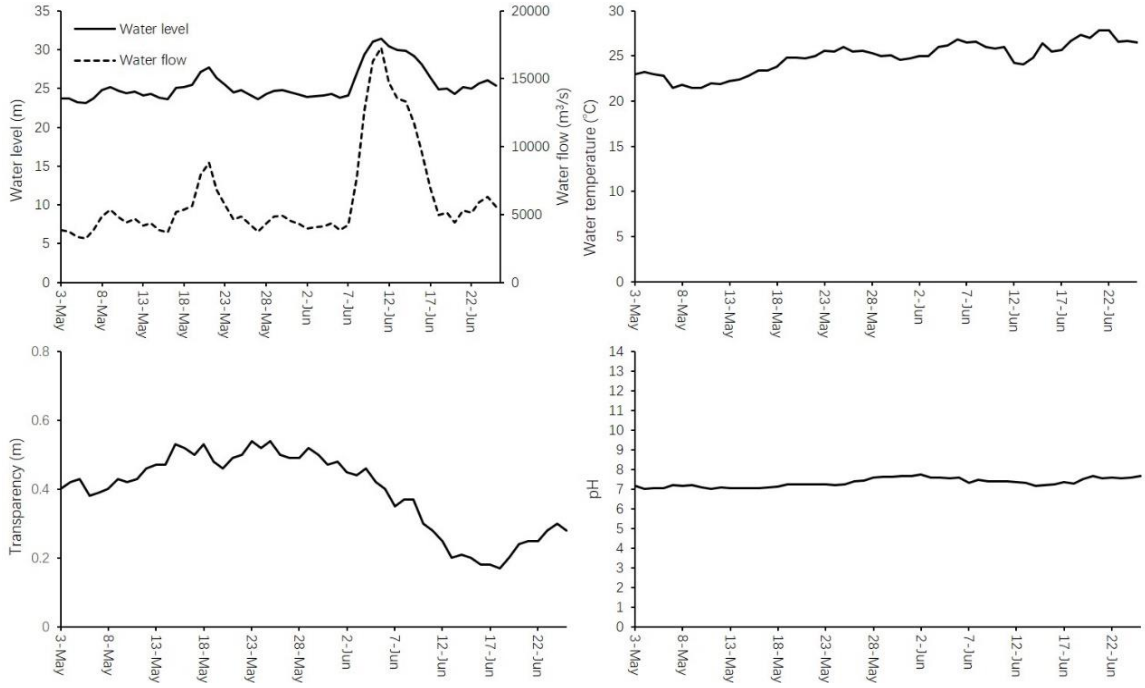

Figure 2. *Environmental variables measured at Fengcheng section in the Ganjiang River during May to June 2019*

Species composition of egg and larval fish

A total of 100 eggs and 3816 larvae, belonging to 7 families and 29 species, were collected during the study period (*Table 1*).

Squalidus argentatus was the most egg abundant species, comprising 33% of the total number of fish eggs, followed by *Parabramis pekinensis* (25%) and *Xenocypris argentea* (18%). And egg abundance of the four major Chinese carps accounted for 10% (*Table 1*).

In addition, the larval abundance of *Hemiculter bleekeri* was over half of the total number larval fish, followed by *Rhinogobius giurinus* (28.69%) and *Parabramis pekinensis* (6.18%). And larval abundance of the four major Chinese carps accounted for 2.86% (*Table 1*).

Diurnal pattern of egg and larval abundance

The mean densities of eggs and larval fishes during May to June in 2019 were 4.27 ind./1000 m^3 and 66.09 ind./1000 m^3, respectively.

The diurnal trend of egg and larval density during May to June in 2019 was different (*Fig. 3*). Egg abundance was higher in May 2019 and peaked on 11 May (10.78 ind./1000 m^3). But larval abundance was higher during June than that in May,

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with three peaks on 24 May (131.79 ind./1000 m^3), 7 June (176.03 ind./1000 m^3), and 21 June (191.70 ind./1000 m^3), respectively.

Egg abundance and spawning ground of four major Chinese carps

The egg abundance of four major Chinese carps was 6.1 million during May to June in 2019. Moreover, grass carp, silver carp and bighead carp were accounted for 41.0%, 50.8% and 8.2%, respectively.

Table 1. *Number of eggs and larvae of fishes collected in the Fengcheng section of Ganjiang River from May to June 2019*

Family and species	No. of egg	Ratio	No. of larvae	Percentage (%)
Salangidae				
Neosalanx taihuensis			16	0.42
Cyprinidae				
Ctenopharyngodon idellus	3	0.03	46	1.21
Squaliobarbus curriculus	2	0.02	30	0.79
Opsariichthys bidens			2	0.05
Parabramis pekinensis	25	0.25	236	6.18
Megalobrama amblycephala			6	0.06
Zacco platypus			3	0.08
Hemiculter bleekeri	6	0.06	1987	52.07
Pseudolaubuca sinensis			74	1.94
Pseudolaubuca engraulis	3	0.03	117	3.07
Culter alburnus	1	0.01	8	0.21
Culter mongolicus			7	0.18
Cultrichthys erythropterus			7	0.18
Hypophthalmichthys molitrix	3	0.03	59	1.55
Aristichthy nobilis	4	0.04	4	0.10
Squalidus argentatus	33	0.33	14	0.37
Xenocypris argentea	18	0.18	15	0.39
Xenocypris davidi			11	0.29
Xenocypris microlepis			6	0.16
Pseudorasbora parva			4	0.10
Spinibarbus sinensis			36	0.94
Acheilognathus tonkinensis			3	0.08
Saurogobio dabryi			3	0.08
Gobiobotia filifer	2	0.02	5	0.13
Cobitidae				
Leptobotia elongate			2	0.05
Serranidae				
Siniperca kneri			15	0.39
Odontobutidae				
Odtontobutis obscura			2	0.05
Gobiidae				
Rhinogobius giurinus			1095	28.69
Adrianichthyidae				
Orizias latipes			3	0.08

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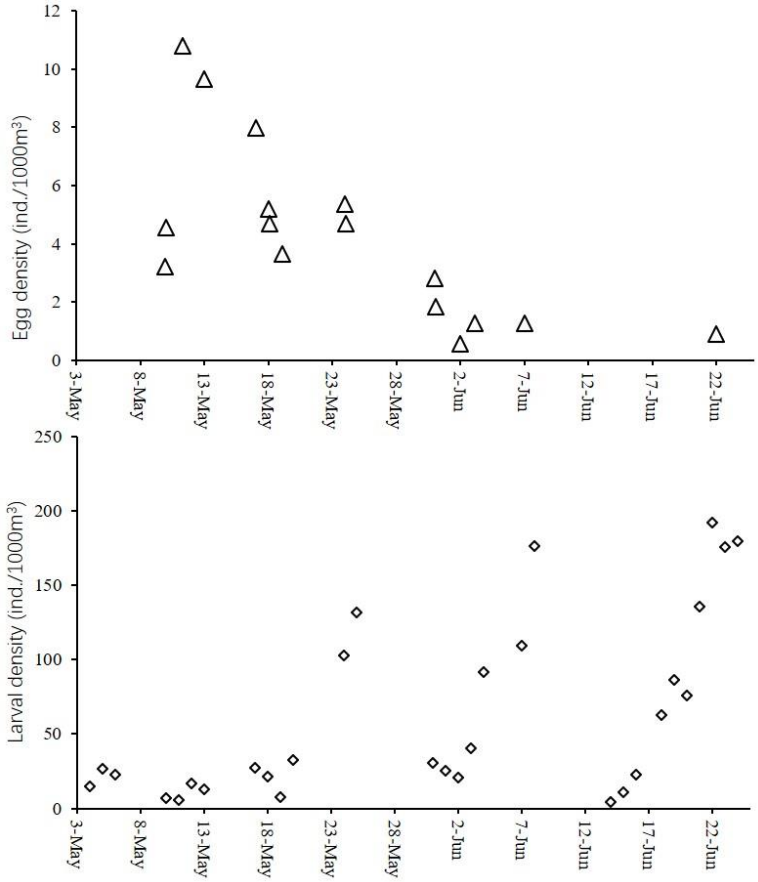

Figure 3. *Diurnal trend of egg and larval density in the Fengcheng section of the Ganjiang River during May to June 2019*

Among the samples, the egg developmental stages were divided into neurula stage, closure of blastopore stage, tail bud stage and rudiment of heart stage. Approximately 86% of the eggs were in the tail bud and rudiment of heart stages, which last 19-29 h after fertilization (Yi et al., 1988). Mean current velocity at the sampling site was 1.01 ± 0.23 m/s. Based on the egg developmental stages and mean river velocity, two main spawning grounds were identified upstream the Fengcheng section in the Ganjiang River during 2019. One was about a 4 km stretch between Zhoushang to Yongtai, and the other was about an 18 km stretch from Kengdong to Renhe (*Fig. 1*).

Relationships of egg and larval assemblages to environmental variables

The egg density had a significant negative correlation with water temperature and pH ($P < 0.01$). But larval density had a significantly positive relationship with water temperature and pH ($P < 0.01$), and a negative relationship to transparency ($P < 0.05$).

Five environmental factors (i.e., water temperature, transparency, pH, water level and flow) were contributed significantly to the explanation of the larval fish assemblages. The correlation efficiencies of the first and second RDA axes were 0.94 and 0.87 of the species-environment relationships, respectively. The first axis was highly correlated with the water temperature; the second axis was highly correlated with the water level (*Fig. 4*). The ordination diagram showed that *Hypophthalmichthys*

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molitrix scattered near the origin, which indicated average values in relation to the environmental variables for the species; *Parabramis pekinensis*, *Pseudolaubuca engraulis*, *Hemiculter bleekeri* and *Rhinogobius giurinus* were along the first axis, showing mainly a positive relationship with water temperature; *Pseudolaubuca sinensis* was mainly along the second axis, showing a negative correlation with the water level (*Fig. 4*).

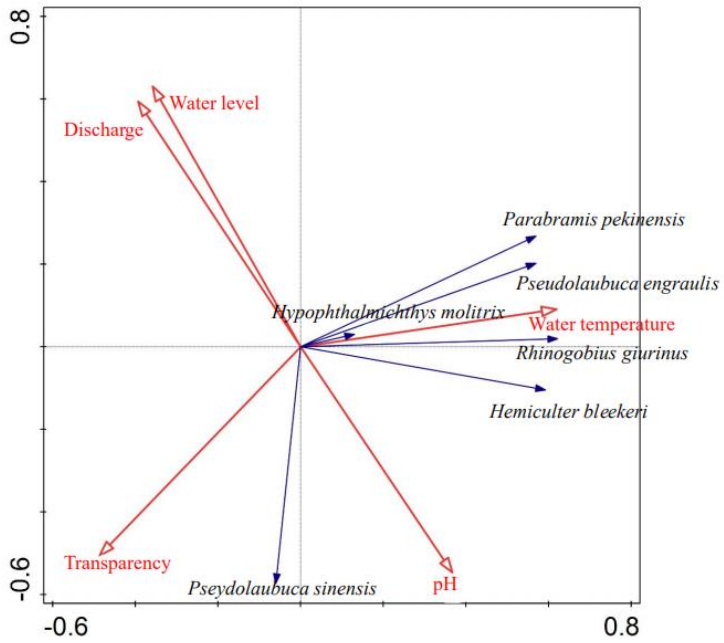

Figure 4. *Biplots of the first two axes of the redundancy analysis (RDA) ordination of larval fish assemblages in the Fengcheng section of the Ganjiang River*

Discussion

A total of 29 species of egg and larval fish were identified at Fengcheng section in the lower reaches of Ganjiang River from May to June during 2019. And *Hemiculter bleekeri*, *Rhinogobius giurinus*, *Squalidus argentatus*, *Xenocypris argentea* and *Parabramis pekinensis* were dominant species. Meanwhile, the mean densities of eggs and larval fishes were 4.27 ind./1000 m^3 and 66.09 ind./1000 m^3, respectively. The species richness and mean densities of eggs and larval fishes in the present study were less than that recorded in the recent research (Guo et al., 2020), while dominant species of egg and larval fish at the section of the river were similar. Compared to the egg and larval fish assemblage in the Yangtze River, the number of species collected in the present study was much less than those recorded in some recent research, e.g., 51 species recorded in Li et al. (2011), 52 species recorded in Ren et al. (2016), 44 species recorded in Guo et al. (2017), 34 species recorded in Hu et al. (2019), 37 species recorded in Chen et al. (2020) and 49 species recorded in Chang et al. (2021). In addition, the mean densities of eggs and larval fishes that occurred in the upper (Tang et al., 2010), middle (Li et al., 2011; Guo et al., 2017; Hu et al., 2019), or lower (Ren et al., 2016) reaches of the Yangtze River were much higher than that presented in this research. Egg and larval fish collection are proposed as a more efficient approach to investigate fish diversity and to assess the resource status of fish populations for the

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higher abundance and usually lower labor investment compared to traditional methods targeting adults (Cao et al., 2007; Ren et al., 2016). The sampling in this research was conducted using a conical net by placing it facing the water flow and thus mainly targeting the drafting egg and larval fish. Thus, further research should be designed with sedimentary larvae included.

The diurnal patterns of egg and larval fish assemblages at Fengcheng section in the lower reaches of Ganjiang River were clearly observed. Briefly, larval fish were highly abundant through June, while the emergence of a high abundance of eggs occurred during May. This temporal patterns of egg and larval fish occurrence were also observed in the recent research (Guo et al., 2020). The timing of fish reproduction has evolved through time to optimize recruitment success by matching the emergence of larvae with appropriate water temperature and physical habitat conditions (Lowe-McConnel, 1987; Munro et al., 1990). In temperate regions, optimal conditions for reproduction of most riverine fishes are predicted to occur when flooding coincides with the appropriate temperature (Bayley, 1991). The spawning of most fish in the middle and lower reaches of the Yangtze River occurs when the water temperature is above 18 °C (Cao et al., 2007). Water temperature at Fengcheng section in the lower reaches of Ganjiang River rose to this temperature and remained increased through May to June (*Fig. 2*); meanwhile, the water flow and water level increased sharply and maintained a high-level during May to June (*Fig. 2*). The results suggested that spawning of most fish occurred at a high-water level and water flow and when water temperature was appropriate. Thus, the results demonstrated that egg and larval fish assemblages in the lower reaches of the Ganjiang River were mainly determined by water temperature, water flow, and water level.

The four major Chinese carps (i.e., black carp, grass carp, silver carp, and bighead carp) lay their drifting eggs in rivers, the fertilized eggs hatch and the larvae develop during downstream drift, the larvae and juveniles then enter floodplain lakes to grow and mature (Yi et al., 1988; Cao et al., 2007). So far, reproduction of these carps has declined since the construction of dams due to an altered water flow and thermal regime in key river systems in China (Hu et al., 2015). Historically, 12 spawning grounds for the four carps were found in the mainstream of the Ganjiang River, located from Ganzhou to Xingan within a range of 303 km (Tian, 1989). After construction of the Wan'an Dam (completed in 1990), Shihutang Dam (closed in 2012) and Xiajiang Dam (completed in 2017), nine spawning grounds were inundated by these reservoirs (Guo et al., 2020). This caused that the egg abundance of the four domestic fishes had decreased from 2.5 billion in the 1960s, 1.9 billion in the 1970s, 1.3 billion in the 1980s, 0.5 billion in the 1990s, to just 20 million in 2000 (Liu et al., 2009; Hu et al., 2015). By 2017, their egg production had dropped further to 11 million (Guo et al., 2020). In present study, the egg abundance of the four carps in the Fengcheng section of Ganjiang River was only 6.1 million during May to June in 2019; and based on the egg developmental stages and mean river velocity, two main spawning grounds were identified upstream the Fengcheng section in the Ganjiang River during 2019 (*Fig. 1*). But along with the Xingan Dam (completed on November 2019), Jinggangshan and Longtoushan dams (under construction) have been gradually completed (*Fig. 1*), all spawning grounds of the four major Chinese carps in the Ganjiang River will be flooded. Some studies indicated that the density of drifting eggs of the four carps was mainly influenced by the diurnal variation of water level, the diurnal variation of water discharge, water temperature, humidity, and air pressure (Duan et al., 2009; Li et al.,

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2013). When water temperature was between 18 °C and 24 °C, especially in association with the diurnal increase of water level greater than 0.55 m/d, spawning activities was always favored (Li et al., 2013). Thus, in order to ensure the successful spawning of the four carps during May to July, we suggested that the ecological regulation of the Ganjiang River should be managed to create flood peaks and make the diurnal increase of water level greater than 0.55 m/d when water temperature was between 18-24 °C.

Conclusion

Ganjiang River is the longest river flowing into the Poyang Lake, and one of the important tributaries of the Yangtze River. Historically, 12 spawning grounds for the four major Chinese carps were recorded in the river. Due to the construction of dams (such as Wan'an Dam, Shihutang Dam and Xiajiang Dam) in mainstream of the river, the egg abundance of the four carps had been declined. Although two new spawning grounds for the four carps were found in present study, but all spawning grounds will be flooded along with the cascade water conservancy projects have been gradually built. In order to ensure the successful spawning of the four carps, the ecological regulation of the Ganjiang River should be managed to create flood peaks and make the diurnal increase of water level greater than 0.55 m/d when water temperature was between 18-24 °C during May to July. Meanwhile, in order to better protect the fish resources of Ganjiang River, it is recommended that: (1) scientific scheduling of water conservancy hubs at all levels of Ganjiang River according to fish breeding rhythms; (2) regular monitoring of fish resources; and (3) ecological supplementation of important species, such as the stocking of sexually mature individuals or fry, to increase the abundance of fish populations in Ganjiang River water bodies.

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

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DETERMINING THE OPTIMUM RATIO OF POLYCULTURED SHELLFISH AND SEAWEED: A MICROCOSM STUDY

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Abstract. The polyculture of shellfish and seaweed together is an effective way to solve the current bottleneck in developing the shellfish culture industry, and determining the optimal ratio is an important prerequisite for its application. Large-scale oyster monocultures in the Beibu Gulf (South China) have led to environmental degradation and exceeded the aquaculture carrying capacity. In order to cope with the problem, this study designed a microcosm of polyculture with shellfish and seaweed, trying to explore the optimal ratio with both environmental protection and economic benefits. Different densities of *Gracilaria tenuistipitata* (0.33–4.17 kg/m^3) combined with a fixed density (83.3 ind/m^3) of *Crassostrea hongkongensis* were investigated to measure the growth rate and nutrient removal effects. The oyster growth rates in the polyculture groups were 6–14-fold that of the oysters in the monoculture group. The highest oyster and seaweed growth rates were in the medium-density seaweed group (0.83 kg/m^3). Compared with the low-density seaweed groups, a high density of seaweed effectively removed the nutrients released by the oysters. A response surface analysis showed that a seaweed density of 1.567 kg/m^3 was the optimal proportion with 83.3 ind/m^3 (10 individuals) of *C. hongkongensis*, with a predicted growth rate of 0.110%/day for the oyster and 0.564%/day for the seaweed. This research indicates that combining *G. tenuistipitata* in a polyculture system could increase oyster production and provide the exciting prospect of improving seawater quality in the Beibu Gulf.
Keywords: *marine ecological restoration, sustainable development, seawater quality, Gracilaria tenuistipitata, Crassostrea hongkongensis, Beibu Gulf*

Introduction

Mariculture wastewater increases marine pollution and aggravates eutrophication in the Beibu Gulf. This decline in water quality affects public health in coastal areas and directly threatens the development of the mariculture industry. In addition, the increasing monoculture has resulted in decreased yield and frequent disease outbreaks (Neori, 2007), and the culture capacity is approaching its limit (Nunes et al., 2003).

The solution to the problem of monoculture and environmental pollution is to develop a polyculture system (McVey et al., 2002; Neori et al., 2004; Wartenberg et al., 2017). Macroalgae are important species for mixed cultivation and the environmental

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restoration of bays (Neori et al., 2004; McGlathery et al., 2007; Yang et al., 2015; Buschmann et al., 2017). The seaweed uses the nutrients in aquaculture wastewater to form new tissues (Qian et al., 1996; Sandoval-Gil et al., 2016), increases dissolved oxygen for the animals via photosynthesis (Yang et al., 2015), and improves the culture environment. Macroalgae have been widely used as a biofilter in treating aquacultural wastewater from shellfish farming (Mao et al., 2009; Sandoval-Gil et al., 2016), shrimp ponds (Jones et al., 2001; Mai et al., 2010), and fish ponds (Hayashi et al., 2008), as well as for the ecological restoration of bays and estuaries (Yang et al., 2006; McGlathery et al., 2007). Several studies have shown that seaweed and animals in mixed cultures have higher growth rates than those in monoculture (Qian et al., 1996; Mao et al., 2009). In addition, many kinds of seaweeds have economic value and can be used as raw materials for such things as agar and edible and medicinal products (Neori et al., 2004; Yang et al., 2015).

Determining the optimal ratio of seaweed and animals is the key premise of polyculture, as a proper ratio is an important condition for the balance and stability of a polyculture system. Excessive seaweed could reduce the seaweed and animal growth rates (Yang et al., 2006), while too little seaweed may be ineffective at improving the system environment and promoting growth (Mao et al., 2009). The response surface methodology (RSM) is an effective tool for studying optimal ratios under the influence of multiple factors. Li et al. (2015) used RSM to study the optimal ratios for *Gafrarium tumidum* (tropical clam) and *Eucheuma gelatinae* (macroalga) and selected two optimal ratios for field validation tests. The results showed that both combinations could significantly decrease the microalga density and dissolved nutrients in a sea mesocosm.

The Beibu Gulf is a primary oyster culture center in southern China as well as an important natural oyster seedling center. Studies have shown that as marine suspended filter-feeders, oysters can reduce suspended matters (e.g., phytoplankton, inorganic matter, and particulate organic matter) through water filtration, which purifies the seawater (Schröder et al., 2014; Petersen et al., 2016) and reduces the risk of red tide. However, oysters also release dissolved nutrients through metabolism (Newell et al., 2005; Hoellein and Zarnoch, 2014), especially ammonia (NH_4^+), which can increase seawater eutrophication. The increasing scale of oyster cultivation in recent years has aggravated eutrophication in the Gulf, especially increasing the concentration of inorganic nitrogen (Wang et al., 2016). Moreover, it has resulted in negative impacts on oyster growth such as a decrease in the individual weight of commercial oysters and frequent mass deaths in the spring (Yu et al., 2016). These negative phenomena indicate there may be a bottleneck in the oyster carrying capacity of the Beibu Gulf, making it necessary to adjust the cultivation patterns to achieve sustainable economic growth and environmental protection.

To examine how to deal with this oyster cultivation bottleneck and mitigate eutrophication in the Beibu Gulf, this study attempted to construct a microcosm of polyculture system with *Gracilaria tenuistipitata* (edible red seaweed) and *Crassostrea hongkongensis* (Hong Kong oyster). The growth performance and nutrient removal effects of *G. tenuistipitata* and *C. hongkongensis* were measured to determine their optimal ratio, which would be conducive for the further implementation of a shellfish and seaweed polyculture program in the Gulf and improve seawater quality. *Gracilaria tenuistipitata* is the dominant species of macroalgae in the mangrove conservation area of the Gulf, and it has great economic value as a raw material for agar. Our hypothesis was that *G. tenuistipitata* could promote the growth of *C. hongkongensis*.

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Materials and Methods

Experimental design

The experiment was designed with 7 groups, which included a seaweed-monoculture, an oyster-monoculture, 4 groups with different levels of seaweed and oysters mixed, and one group as a blank control. In addition to the control group, each of the other groups had three replicates. To determine the optimal amount of *G. tenuistipitata* biomass in combination with one string of *C. hongkongensis*, 10 oysters of equal size were used in the oyster-monoculture and mixed groups, while 200 g (wet weight) of *G. tenuistipitata* was used in the seaweed-monoculture group. The 4 mixed groups included 40 g (0.33 kg/m^3), 100 g (0.83 kg/m^3), 200 g (1.67 kg/m^3), and 500 g (4.17 kg/m^3) of *G. tenuistipitata*, respectively. Details of the experimental design are shown in *Table 1*. The experiment lasted 7 days, between 21–28 March 2017. To study the growth rates of the oyster and seaweed, the shell lengths of *C. hongkongensis* and wet weights of *G. tenuistipitata* were measured before and after the experiment. The concentrations of ammonia (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-), and phosphate (PO_4^{3-}) were determined daily during the experiment to observe the effect of *G. tenuistipitata* on removing dissolved nutrients.

Table 1. *Experimental polyculture design with Crassostrea hongkongensis and Gracilaria tenuistipitata*

Group	*G. tenuistipitata* (kg/m^3)	*C. hongkongensis* (ind/m^3)
Control	–	–
Monoculture		
Oyster-monoculture	–	83.3
Seaweed-monoculture	1.67	–
Polyculture		
Low-seaweed	0.33	83.3
Medium-seaweed	0.83	83.3
High-seaweed	1.67	83.3
Ultra-seaweed	4.17	83.3

Experimental materials and environmental conditions

Crassostrea hongkongensis was obtained from the oyster raft culture area at Longmen Port (21°40'41" N, 108°36'46" E) in Qinzhou, China. The experimental oysters were all 2-year-old individuals with an average shell length of 98.47 ± 4.61 mm and a soft body dry weight of 1.63 ± 0.27 g, which acclimated for one week before the experiment. *Gracilaria tenuistipitata* was obtained from the Golden Bay Mangrove area near Beihai (21°25'15" N, 109°12'02" E) in Guangxi province, which provided one week of training using Provasoli enriched seawater culture medium before the experiment. Several high-density polyethylene cylindrical tanks (44 cm diameter, 80 cm height, 120 L capacity) were used as the containers for this experiment. The filtered experimental seawater (water temperature 21–23 °C, salinity 26–28 ppt, dissolved oxygen 7.49–7.54 mg/L) was obtained from the Zhulin area of Tieshan Port in Beihai (Beibu Gulf). The experiment was performed outdoors under a semi-transparent sunshade in the Shellfish Comprehensive Experimental Station of the Guangxi Academy of Fishery Sciences in Beihai with an illumination intensity of 3300–6000 lux (light:dark was 14 hr:10 hr). The oysters were placed evenly at the bottom of the tanks,

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and the seaweeds were hung 20 cm under the water surface with a nylon rope below a float pipe. During the experiment, 600 mL water samples were siphoned from 30 cm under the water surface at 10:00 am every day to determine the concentration of dissolved nutrients, and a 300 mL algae culture solution of *Chlorella vulgaris* (about 5×10^8 cells/L) was fed to the oysters. Each tank was equipped with two air stones, which provided continuous aeration to ensure sufficient dissolved oxygen.

Sample determination and data analysis

To determine the dissolved nutrient concentrations, the water samples were filtered through 0.7 μm GF/F filters (Whatman, England), and the filtrates were stored at −20 °C until the analysis (within one month). The NH_4^+, NO_3^-, NO_2^-, and PO_4^{3-} concentrations were determined using an AutoAnalyzer 3 (SEAL, Germany).

The growth rates for *C. hongkongensis* and *G. tenuistipitata* were determined by the following equation 1 and 2, respectively:

$$LGR\ (\%/\text{day}) = (ln\ L_t - ln\ L_0) / t \times 100 \quad \text{(Eq.1)}$$

$$WGR\ (\%/\text{day}) = (ln\ W_t - ln\ W_0) / t \times 100 \quad \text{(Eq.2)}$$

where *LGR* is the shell length growth rate of the oysters, L_t is the shell length of the oysters at the end of the experiment, and L_0 is the shell length of the oysters at the beginning of the experiment; *WGR* is the biomass growth rate of the seaweed, W_t is the wet weight of the seaweed at the end of the experiment, and W_0 is the wet weight of the seaweed at the start of the experiment; t is the days of culture.

The response surface analysis was performed using Design Expert 12 to estimate the seaweed density at the organics' maximum growth and nutrient removal rates. A one-way analysis of variance (ANOVA) was performed using SPSS 19.0 to test the significance between different treatment groups with the significance level set at 0.05. Sigma 14.0 and Origin 9.0 were used for visualizations.

Results

Growth of the oyster and seaweed under monoculture and polyculture conditions

During the experiment, *C. hongkongensis* grew well in all groups, with a 100% survival rate. The growth status of *G. tenuistipitata* in the polyculture groups was good, appearing glossy and a healthy brown color. However, in the seaweed-monoculture group, some of the leaf tips were whitening by Day 6. *Figure 1* shows the growth performance of the oyster and seaweed. The results show that the growth rates of the oysters (LGR) in the polyculture were higher ($p < 0.05$) than those in the monoculture group. The LGRs were ranked as follows: medium-seaweed group (0.11%/day) > high-seaweed group (0.10%/day) > ultra-seaweed group (0.08%/day) > low-seaweed group (0.05%/day) > oyster-monoculture group (0.01%/day). With the exception of the algae in the ultra-seaweed group, the growth rates of *G. tenuistipitata* (WGR) in polyculture were significantly higher than that in the seaweed-monoculture group ($p < 0.05$). The WGR ranking for the seaweed was medium-seaweed group (0.81%/day) > high-seaweed group (0.48%/day) = low-seaweed group (0.48%/day) > seaweed-monoculture group (0.28%/day) > ultra-seaweed group (0.09%/day).

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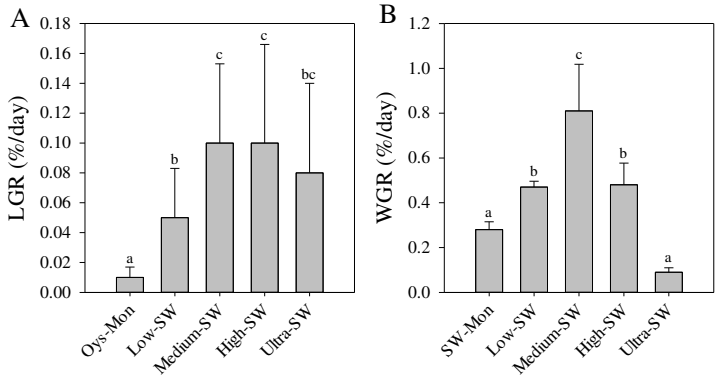

Figure 1. *The length growth rate (LGR) of Crassostrea hongkongensis (n = 10) and biomass growth rate (WGR) of Gracilaria tenuistipitata (n = 3). Different letters (a–c) indicate a significant difference (p < 0.05). Oys, oyster; SW, seaweed; Mon, monoculture*

Effect on nutrients of the oyster and seaweed in monoculture and polyculture

The effect of the seaweed on the dissolved nutrient concentrations is shown in *Figure 2*. First, dissolved nutrients were continuously released in the monoculture oyster group during the experiment, with the final NH_4^+, NO_3^-, NO_2^-, and PO_4^{3-} concentrations at 4.2-, 1.6-, 13.3-, and 8.0-fold the initial concentrations, respectively. Second, the monoculture seaweed group greatly affected nutrient removal (except for the NO_2^- index for the high-seaweed group and the PO_4^{3-} index for the ultra-seaweed group). Third, compared with the control group, the dissolved nutrient concentrations for the high- and ultra-seaweed groups were significantly lower from the third day forward ($p < 0.05$), indicating these seaweed densities could effectively remove nutrients. The highest NH_4^+, NO_3^-, NO_2^-, and PO_4^{3-} removal rates were 89.24%, 76.89%, 93.89%, and 90.97%, respectively. The NH_4^+ and PO_4^{3-} concentrations in the medium- and low-seaweed groups were higher than those in the control group after the second day, indicating the seaweed density of these two groups was insufficient to remove nutrients.

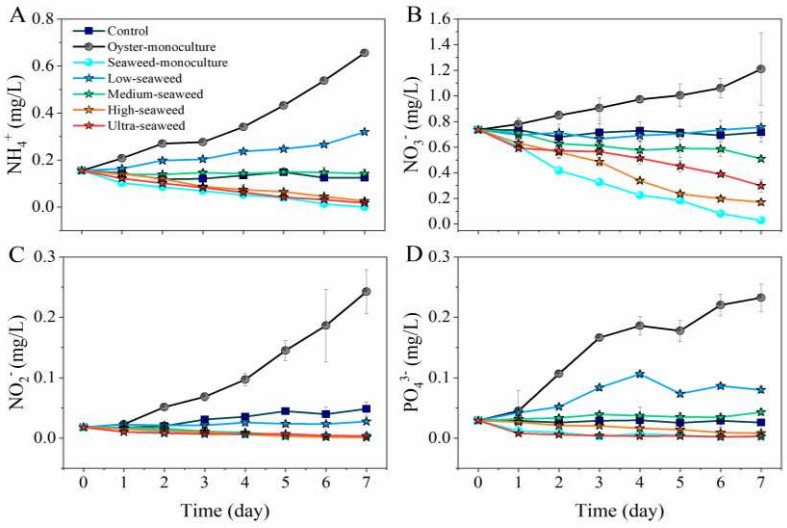

Figure 2. *Variation in dissolved-nutrient concentrations changed with time under the different treatments. All values are the mean ± SD (n = 3)*

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Optimal proportion of oysters and seaweed in a polyculture system

To simulate the optimal proportion of oysters and seaweed for the highest growth and nutrient removal rates in a polyculture system, the constraints in the response surface analysis were set to "maximize" for the growth rates of the oyster (LGR) and seaweed (WGR), and to "minimize" for NH_4^+, NO_3^-, NO_2^-, and PO_4^{3-}. The variance analysis (ANOVA) showed that the confidence levels for LGR, WGR, NH_4^+, NO_3^-, NO_2^-, and PO_4^{3-} in the response surface model were high ($p < 0.01$). The simulation results showed that when the seaweed density was 1.567 kg/m^3, the growth and nutrient removal rates reached their highest expectation (0.824). At this level, the LGR of the oyster was 0.11%/day, the WGR of the seaweed was 0.564%/day, and the concentrations of NH_4^+, NO_3^-, NO_2^-, and PO_4^{3-} were 0.030 mg/L, 0.207 mg/L, 0.001 mg/L, and 0.011 mg/L, respectively (*Figure 3*).

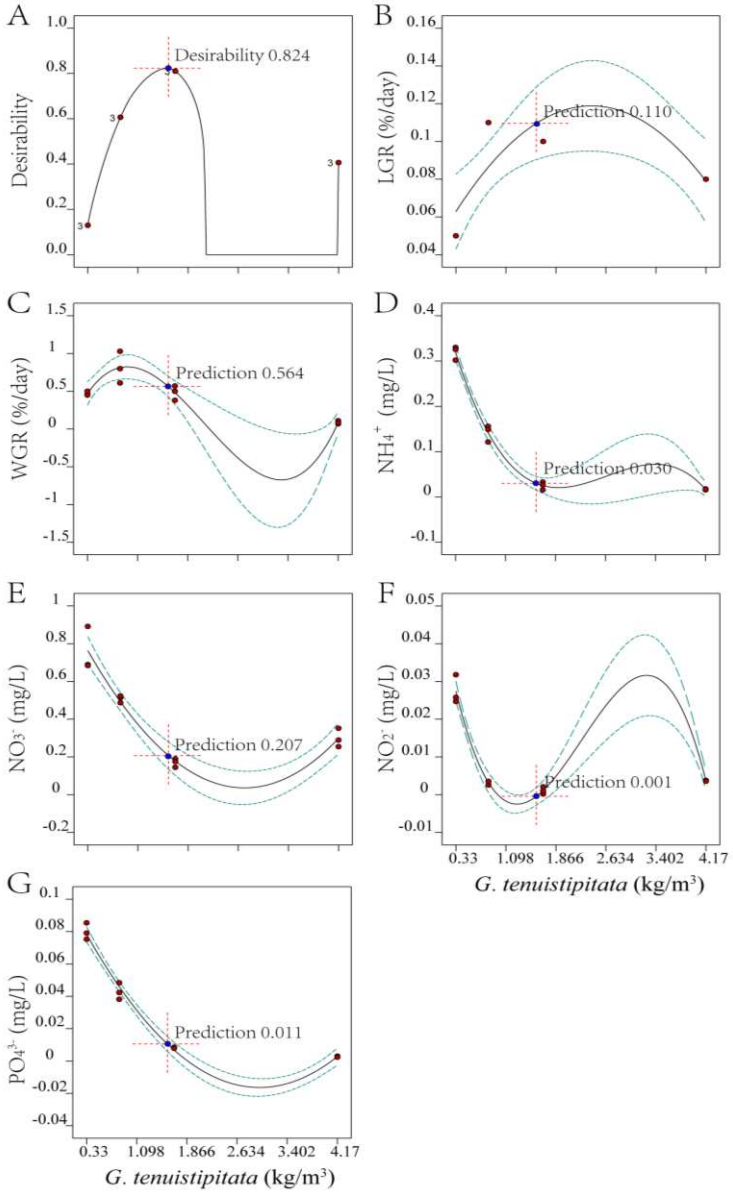

Figure 3. *Optimal solution for Crassostrea hongkongensis and Gracilaria tenuistipitata in polyculture as calculated by the response surface analysis*

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Discussion

As a biofilter, the red seaweed *G. tenuistipitata* can significantly reduce the inorganic nutrients in an integrated culture system. Studies have reported the use of *G. tenuistipitata* in a variety of polyculture systems such as those for shrimp (Anh et al., 2019), tilapia (An and Anh, 2020), and rainbow trout (Haglund and Pedersén, 1993). The NH_4^+, NO_3^-, NO_2^-, and PO_4^{3-} concentrations were effectively reduced in these polyculture systems, while NH_4^+ and PO_4^{3-} concentrations were reduced by 95.71% and 95.74%, respectively, under the polyculture conditions used with 3.5 g/L of the shrimp *Penaeus vannamei* (Sarkar et al., 2019). In the mixed culture system, *G. tenuistipitata* synthesizes its own tissue, obtaining nutrients from the aquatic animals. The growth rate of *G. tenuistipitata* can reach 4%/day under the optimal conditions of the three summer months (Haglund and Pedersén, 1993) and reach 2.4%/day under natural pond conditions over a year (Wu et al., 1994). In this study, the daily growth rate of *G. tenuistipitata* was less than 1%, which might be related to the short culture period (one week), when it may still be in the environmental adaptation stage. However, *G. tenuistipitata* significantly reduced the amount of nutrients (76.89–93.89%) in polyculture with the oyster, indicating that it could play a role in improving water quality in a mixed-culture system.

In this study, both the oyster and seaweed grew better in the polyculture system than in monoculture. Other studies have reported similar results. Qian et al. (1996) found higher growth rates for the red algae *Kappaphycus alvarezii* and pearl oyster *Pinctada martensi* in a field co-culture. The mechanisms operating during the polyculture of shellfish and seaweed can be explained by the following aspects. On the one hand, the great filtration skill of the oysters increases the transparency of seawater (McVey et al., 2002; Schröder et al., 2014; Petersen et al., 2016), which may improve the photosynthetic efficiency of the seaweed (Newell et al., 2005). In addition, the rich dissolved nutrients released by the oyster (Newell et al., 2005; Hoellein and Zarnoch, 2014), especially NH_4^+, could benefit the seaweed's growth (Sandoval-Gil et al., 2016). On the other hand, seaweed photosynthesis releases oxygen, providing more dissolved oxygen for the oyster, which would be conducive to its growth (Yang et al., 2015). The seaweed also removes NH_4^+ excreted by the oyster, which reduces ammonia toxicity for the oysters, helping them maintain healthy growth. In this polyculture system, the oyster and seaweed each obtain what they need, forming a mutually beneficial and stable symbiotic system.

In the polyculture system, a moderate seaweed density is the key to promoting the growth of the organisms and improving the culture environment. Studies have shown that at a medium density, seaweed greatly effects nitrogen and phosphorus removal in a mixed-culture system (Huang et al., 2010). In this study, the appropriate seaweed density promoted the growth rate of both oyster and seaweed, and the nutrient removal rate was high. However, the growth and nutrient removal rates were low under the low-density and high-density seaweed conditions. For instance, in the medium-density seaweed group, the growth rates of the oysters and seaweed were roughly 14 and 3 times higher than that in their respective monoculture groups. Under the high-density seaweed conditions, the nutrient removal rates reached 77–94%. Therefore, while a medium seaweed density can promote growth, high seaweed density is conducive to improving water quality. To balance the economic benefits and environmental protection, it is necessary to find the optimal oyster to seaweed ratio.

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Polyculture is defined as putting two or more species with different ecological niches into a culture environment that combines their nutritional resources and living space, improves nutrient utilization, minimizes cost and maximizes economic value, and benefits the environment. To maximize the economic and environmental benefits, the optimum proportion of the different species in the polyculture need to be studied. Huang et al. (2010) found that better nitrogen and phosphorus removal was achieved when 6 kg/m^3 of the red alga *Kappaphycus striatum* was combined with 60 ind/m^3 of the bivalve *Paphia exarata*, while the effect was poor when the densities were too high or too low. Qian et al. (1996) found that 200 g of *K. alvarezii* could effectively absorb the total amount of nitrogen released by 80 *P. martensi* individuals within 6 hr. In our study, the optimal densities for *G. tenuistipitata* and *C. hongkongensis* were 1.567 kg/m^3 and 83.3 ind/m^3 (10 individuals), respectively, which resulted in a balance of the biological growth rate and the nutrient removal rate.

Conclusions

Both *C. hongkongensis* and *G. tenuistipitata* grew better in a polyculture system than in a monoculture system. The appropriate seaweed density removed significant amounts of the dissolved nutrients released by the oysters. Considering both the economic benefits and environmental protection, the best combination was 10 oysters (83.3 ind/m^3) with 1.567 kg/m^3 seaweed. Our results indicate that seaweed in co-culture could effectively reduce dissolved nutrients, improve water quality, and increase oyster production. *Gracilaria tenuistipitata* could be used in the ecological restoration of the bay to mitigate eutrophication and improve seawater quality, especially in the Beibu Gulf, where the oyster aquaculture center is in South China. In recent years, eutrophication has intensified, the risk of red tide has increased, aquaculture diseases have frequently appeared, and the size of commercial oysters has decreased. In this case, combining seaweed with the oyster culture system would be expected to extend the carrying capacity barrier and achieve a balance of economic growth and environmental protection. The optimal ratio in this study was obtained under semi-outdoors condition. Future studies will verify the actual effect of this ratio in the field, as well as explore the optimal ratio of other seaweeds and the oysters.

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ZOOPLANKTON COMMUNITY STRUCTURE IN THE HAMIZ LAKE AND ITS RELATIONSHIPS WITH ENVIRONMENTAL FACTORS

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Abstract. The present study reports ten identified species of zooplankton in an Algerian lake. Seven stations were chosen according to different criteria and eleven physicochemical parameters were estimated to describe the water quality of each station. Qualitative and quantitative samples were collected from the lower horizon to the surface monthly, in October 2006 and from January to March 2007. In January, the highest species richness (S=9) was recorded in the upper lake corresponding to greater diversity (H'=1.41). The maximum density (28101 ind./L) was observed at the most vegetated site. The crustacean community consisted of 5 Cladocerans, 2 Cyclopidae and 1 Calanoida, however, rotifers were scarce. The CCA analysis reveals that most cladocerans seem to be associated with water hardness contrary to rotifers and copepods. The zooplankton structure was dominated in the north by copepods. However, cladocerans showed an inverse pattern from north to south. Taken together, our data indicate that community structure changes over time during our sampling surveys and in the lake, three zooplanktocenoses were identified. Therefore, it seems that environmental factors linked to the Mediterranean climate play a fundamental role in shaping the architecture of the food webs and the distribution of zooplanktocenoses of the Hamiz reservoir.
Keywords: *zooplanktocenoses, density index, bioindicators, water quality, sub-humid region*

Introduction

Hamiz lake, is one of the first dams of the Algerian hydrotechnical infrastructure (Remini, 2017), with a capacity of more than 15 million m^3 (Cheikhlounis et al., 2011), it supplies the population of Algiers with drinking water through the Keddara reservoir. This aquatic ecosystem has been the subject of various researches like parasitology (Ould Rouis et al., 2016) ecology of ichtyofauna (Ould Rouis, 2016) and reproductive biology (Ould Rouis et al., 2012). However, data on diversity and dynamics of zooplankton is still inadequate.

More than 94 water reservoirs were built in Algeria (National Agency of Dams and Transfers) to supply the urban population with drinking water, irrigate agricultural land and develop aquaculture in some dam lakes (Utomo et al., 2019) in order to enrich people's diet with proteins (Lynch et al., 2016). However, fish constitute good indicators of water quality but most fry feed on the three main groups of zooplankton: rotifers, cladocerans and copepods thus, considerably modifying the specific composition of zooplankton. Simultaneously, temperature and oxygen are the most important factors in the regulation of the zooplankton communities, as well as the spatial and temporal scale of lake ecosystems (Itigilova, 2019).

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Zooplankton species are crucial components of aquatic food webs (Yang et al., 2018) and knowledge of these communities can provide useful information on water quality. Indeed, the study of plankton is an integrative measure of water quality (Suthers and Rissik, 2009). Zooplankton inhabit lakes and ponds that functionally are "aquatic islands" in the landscape and both community composition and richness depend on the one hand, on their ability to disperse and their post-dispersal colonization abilities (Hessen et al., 2019) and to use the available resources (nutrient content and concentration) on the other hand (Walz, 1995). This ability to disperse makes them interesting tools to test the hypothesis of dispersion (Battuello et al., 2016). They are important for understanding ecosystem functioning and stability (Msiteli-Shumba et al., 2017). These species are commonly used as bioindicators (Gökçe and Özhan Turhan, 2014) with their short life cycles rapidly responding to changes in environmental conditions (Suikkanen et al., 2007; Suthers and Rissik, 2009) also used as model systems to maintain biological diversity (Aránguiz-Acuña et al., 2018). Moreover, the composition, abundance and dynamics of zooplankton are themselves shaped by the environment and biotic interaction (Wetzel, 2001; Fernández-Rosado and Lucena, 2001) and for this, it seems especially in reservoirs, difficult to isolate the primary causes explaining the variation in abundance (Velho et al., 2001).

In the dam lake Hamiz we investigate, for the first time, the spatial heterogeneity and the distribution of zooplanktonic species in relation to environmental parameters. Our objectives were to identify the most common freshwater zooplankton occurring presently in Hamiz reservoir (hereafter Hamiz) and then determine: (1) whether physicochemical properties influence the occurrence of species and thus characterize zooplankton assemblages in relation to environmental variables (2) zooplankton structure based on the variation in species density over time between groups of sampled stations and (3) the allocation of zooplanktonic communities and define the proportion of space and how consistently one or another species occupies the water area of the reservoir. Based on these results and knowledge of the ecology of these species, we highlight the zooplanktocenoses living in the Hamiz, discuss possible reasons of their organization in response to the temporal dynamics of environmental indicators, taking into account their interrelationships and their roles in the evolution of these species.

Finally, knowledge of the distribution, abundance and dynamics of this important link in the food web will allow us to both understand and contribute to the restoration and protection of our aquatic ecosystems.

Materials and Methods

Sampling area

The study took place in Algeria, from 2006 to 2007 (in October 2006 and from January to March 2007). Hamiz lake is situated under a Mediterranean climate in the sub-humid region where, winters are relatively cool and wet. The drought lasts five months (late May to October) while the rainy period covers the remaining months of the year. The dam reservoir is located in *Mitidja* (36°35′59″N, 3°20′50″E), 158 m above sea level and 35 km east of Algiers.

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Sampling sites

Seven stations (from A to G) were chosen on the lake (*Fig. 1*), according to bottom topography (depths), North/ South exposure, the place where the tributaries/ small rivers flow in as well as vegetation distribution. Station A (exceeding 20 m), is located a few meters from the dike, northeast side. Station B (not exceeding 16 m) is near northwest coast of water reservoir. Going from Ghar Tabarante (north bank), approximately towards the center is station C (up to 20 m). Station D is situated rather towards the southwest where, Oued Arbatache (a little river) throws into the reservoir. On the south shore, stations E and F are located respectively opposite Chabet Djnane M'Sada and Chabet El M'Ghassel. The latter three stations are characterized by a strong presence of emergent plants. Opposite station A is station G, which receives water from a small tributary that crosses Ghabet Tararesse and flows into the lake.

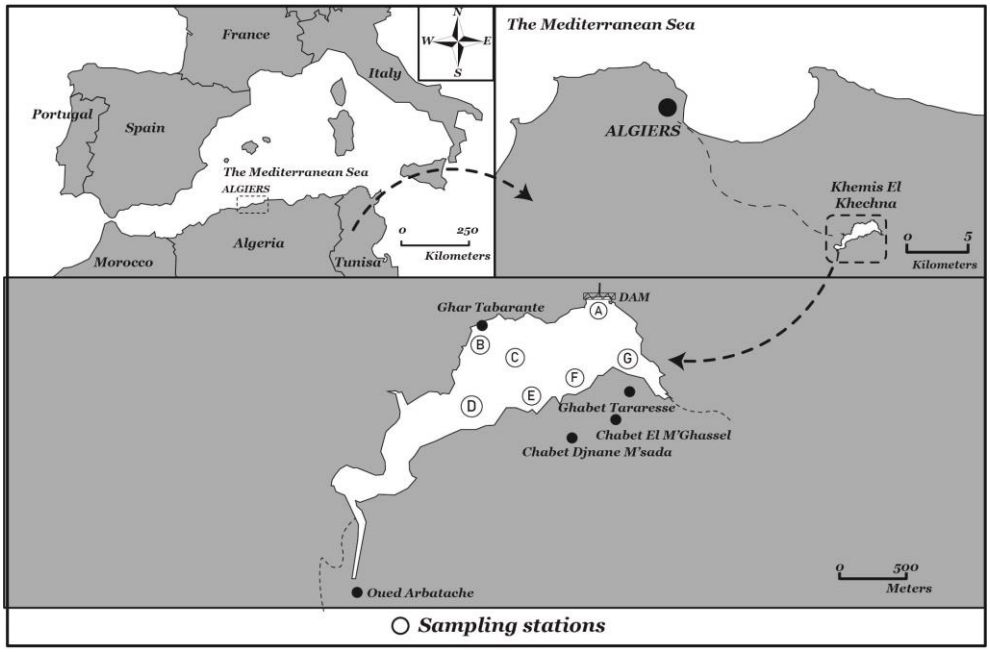

Figure 1. *Sampled stations on the Hamiz (from A to G)*

Water quality estimation

To characterize the quality of the water column at each station, eleven parameters, both physical and chemical, were estimated and measured together with zooplankton sampling. Water temperature, pH and dissolved oxygen (O_2) were assessed by means of the multi-parameter (Multi 340i/ SET WTW) while, transparency was determined using a black and white Secchi disk 20 cm in diameter. The remaining chemical parameters studied (Ca^{2+} - calcium, Mg^{2+} - magnesium ion, PO_4^- - orthophosphate phosphorus, NO_3^- - nitrate nitrogen, NO_2^- - nitrite nitrogen, Cl^- - chloride ion, H_2CO_3 - carbonic acid) were analyzed according to Rodier et al. (2009).

Zooplankton determination

Qualitative zooplankton sampling was determined by a hand net (50 µm mesh size) fired over a distance of about 10 m (horizontal hauls) at the surface (level 0 m).

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However, quantitative sampling was collected via a Ruttner's bathometer (1-liter capacity). We throw it at different depth (vertical hauls) from the lower horizon to surface; at the deep stations A, B and C samples were selected in three points of vertical water column: from surface (0.0 m-1.0 m), in the middle layers (1.5 m-4.5 m) and in the lower layers of water (5.0 m-9.0 m). At shallow-water, E and F stations were sampled from two horizons and the rest stations (D and G) only from surface. On the lake, stations A and B are considered to be located along the northern bank. We assume that station C is in the center and stations D, E, F and G are situated on the south shore (on the opposite side). The selected samples of water were filtered through the planktonic net during sampling then, we use the zooplankton collected by the pill container for calculation and identification of organisms. A total of 60 quantitative and 28 qualitative samples were collected and processed. All these samples were preserved in 90% ethanol. Zooplankton individuals were identified to the species level using a compound microscope (Zeiss) at high power magnifications. Identification keys of Einsle (1993, 1996), Dussart (1967, 1969) and Stella (1982) were used for copepods, while cladocerans were identified following Margaritora (1983), Ould Rouis (1995) for Algerian species and Alonso (1996). For Rotifera, we used Pourriot and Francez (1986).

Quantitative zooplankton abundance is expressed as number of organisms per liter. Each sample was counted on at least half of the total volume. Therefore, to characterize the structure of zooplankton communities and describe diversity, the Shannon-Wiener index (H) (Wolda, 1983) and the species Evenness (E) were calculated according to Pielou (1966).

Finally, to determine which particular species is constantly present at a given station (Bayanov, 1997), we used the density index according to Pidgajko (1978), which specifies the spatial volume and the period when the species are present in the water reservoir, which are then presented in the form of column diagrams. The square root of the biomass of the whole zooplankton population at a specified date was theoretically considered as the maximum possible density. This latter is divided by 4 for the calculation of four intervals of the density. The particular verbal characteristic corresponding to a species with density values within a particular interval: the species in the upper interval were called dominant species, in the next interval sub-dominant species, in the third typical and in the last minor. The communities were therefore called according to the names of the dominant species or, if there were no apparent dominants, after the sub-dominants and typical species.

Data analysis

A hierarchical clustering using Euclidean distances between species was performed to check whether the sampled stations and the collected species are close together. To estimate similarity between stations, Steinhaus coefficient (Legendre and Legendre, 2012) was carried out then, a Chi-square test of homogeneity was conducted to compare zooplankton structure between northern and southern station groups. The variation of species abundance among the station groups was highlighted by using box-whisker plots model. The relationship between water quality and zooplankton species was determined by canonical correspondence analysis (CCA) using XLSTAT software (version 2018). Statistical analysis was performed with the Statistica 7.0 software.

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Results

Environmental factors

During the study period, the dam waters were alkaline and well oxygenated with dissolved oxygen levels averaging between 4.0 mg/L and 6.0 mg/L, with a highest mean value recorded at station A. The average water temperature is approximately the same at all stations. Phosphate values are less than 0.1 mg/L. The hardness of the water is medium, well represented by the values of calcium and magnesium ions. It appears that the calcium contents are higher than those of the magnesium ions.

The maximum average value for chlorides is less than 40.0 mg/L. The levels of bicarbonates evolve almost similarly in the waters of Hamiz, minimum and maximum values were recorded respectively at stations C and D (*Table 1*).

Table 1. *Physicochemical parameters of water at each station*

		Stations						
Parameters	**Variables**	**A**	**B**	**C**	**D**	**E**	**F**	**G**
Depth (m)		9.1-14.5	2.4-8.0	4.0-9.0	0.8-2.0	1.8-6.5	2.5-5.0	1.0-3.0
Water Temperature (°C)	Mean	17.0	17.8	17.3	17.6	18.2	17.8	17.6
	SD	4.0	4.8	4.8	5.2	5.1	5.0	4.5
Transparency (m)	Mean	1.1	1.2	1.3	0.9	1.2	1.3	0.7
	SD	0.7	1.0	1.0	0.7	1.0	1.0	0.2
pH	Mean	8.1	8.0	8.2	8.1	8.0	8.1	7.9
	SD	0.1	0.1	0.1	0.2	0.2	0.3	0.3
O_2 (mg/L)	Mean	6.0	4.7	4.2	4.0	4.3	4.4	4.4
	SD	1.1	2.1	2.0	2.1	1.7	1.9	1.9
Ca^{++} (mg/L)	Mean	65.9	66.9	65.5	64.7	64.4	64.6	64.2
	SD	31.2	30.8	30.6	29.9	29.5	29.7	29.8
Mg^{++} (mg/L)	Mean	35.6	35.3	35.9	44.2	39.8	38.9	42.1
	SD	13.8	14.0	14.7	18.7	16.8	17.0	17.6
PO_4^- (mg/L)	Mean	0.02	0.05	0.07	0.08	0.08	0.09	0.09
	SD	0.03	0.05	0.04	0.00	0.00	0.01	0.01
NO_3^- (mg/L)	Mean	2.0	2.1	2.2	2.4	2.3	2.4	2.5
	SD	0.9	1.0	0.5	0.3	0.3	0.4	0.4
NO_2^- (mg/L)	Mean	0.02	0.03	0.03	0.05	0.04	0.04	0.06
	SD	0.03	0.03	0.02	0.02	0.02	0.02	0.04
Cl^- (mg/L)	Mean	23.6	23.4	28.0	38.8	39.9	39.1	38.9
	SD	10.6	10.2	12.7	1.8	2.1	2.2	1.9
H_2CO_3 (mg/L)	Mean	227.2	226.3	224.7	261.2	256.6	245.4	257.7
	SD	11.4	13.7	19.5	32.5	35.5	17.8	19.2

Zooplankton community structure and diversity

Ten species (10) of zooplanktonic fauna were collected from the lake (*Table 2*). These species belong to three main groups (cladocera, copepoda and rotifera).

Shannon-Wiener diversity index during the sampling period varied monthly from 0.43 to 1.41 (*Table 3*). The highest value of species richness (S) was recorded in January (9) corresponding to the highest diversity (H=1.41) in the dam. February and March have the same richness (S=7). The Pielou's evenness was not so different between months however, the minimum value (0.18) was observed in October.

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Table 2. *Zooplankton species occurring in Hamiz*

Zooplankton groups/ species	Code
Cladocera	
Ceriodaphnia reticulata (Jurine, 1820)	Cla1
Daphnia magna (Straus, 1820)	Cla2
Daphnia longispina (O.F. Müller, 1785)	Cla3
Daphnia similis (Claus, 1876)	Cla4
Daphnia hyalina (Leydig, 1860)	Cla5
Copepoda	
Copidodiaptomus numidicus (Gurney, 1909)	Cop1
Cyclops abyssorum (O.G. Sars, 1863)	Cop2
Acanthocyclops americanus (Marsh, 1893)	Cop3
Rotifera	
Keratella quadrata (O.F. Müller, 1786)	Rot1
Filinia longiseta (Ehrenberg, 1834)	Rot2

Table 3. *Species diversity and evenness of zooplankton in Hamiz*

Indices	October 06	January 07	February 07	March 07
Total number of species (S)	5	9	7	7
Total number of Individuals	2400	5512	90631	15315
Shannon-Wiener Index (H)	0.43	1.41	1.28	1.14
Pielou's Evenness Index (E)	0.18	0.44	0.46	0.41

The zooplankton community fluctuates from a station to another; in fact, it appears that station D has a higher total density (28101 ind./l) than all other stations: A (15477), B (14834), C (3658), E (1292), F (26820) and G (23672). In addition, we noticed that the zooplankton species were more developed in the vegetated sites than in unvegetated areas in the reservoir.

Zooplankton assemblages in relation to environmental factors

On the lake, we noted that zooplankton assemblages are strongly related to the physicochemical nature of water (*Fig. 2*). Indeed, environmental factors such as water hardness (calcium and magnesium), dissolved oxygen and nitrates are strongly associated with the first two axes. CCA analysis reveals that the first two axes provide 83.91% of the total information on zooplankton species assemblages with environmental parameters. The first axis accounts for 64.41%. It discriminates between zooplankton species associated with hard and well oxygenate waters on the right of the axis and on the left; rotifers seem to be insensitive to the physicochemical nature of the lake waters. The second canonical axis, explaining 19.50% of the variance, shows mainly species that prefer nutrient-rich waters. The species-environment correlations were 0.849 for axis 1 and 0.871 for axis 2. Thus, the zooplankton distribution seems to be highly related to water quality. In fact, some species such as Daphnids (*Ceriodaphnia reticulata*, *Daphnia similis* and *Daphnia longispina*) and the Copepod (*Copidodiaptomus numidicus*) were all strongly and positively influenced by calcium, magnesium, bicarbonates and dissolved oxygen in water. Others such as, *Cyclops abyssorum* and *Daphnia magna* are sensitive to phosphates, chlorine and nitrates ions but do not appear to be influenced by water hardness. Furthermore, most of cladocerans seem to be associated to water's hardness contrary to rotifers and copepods.

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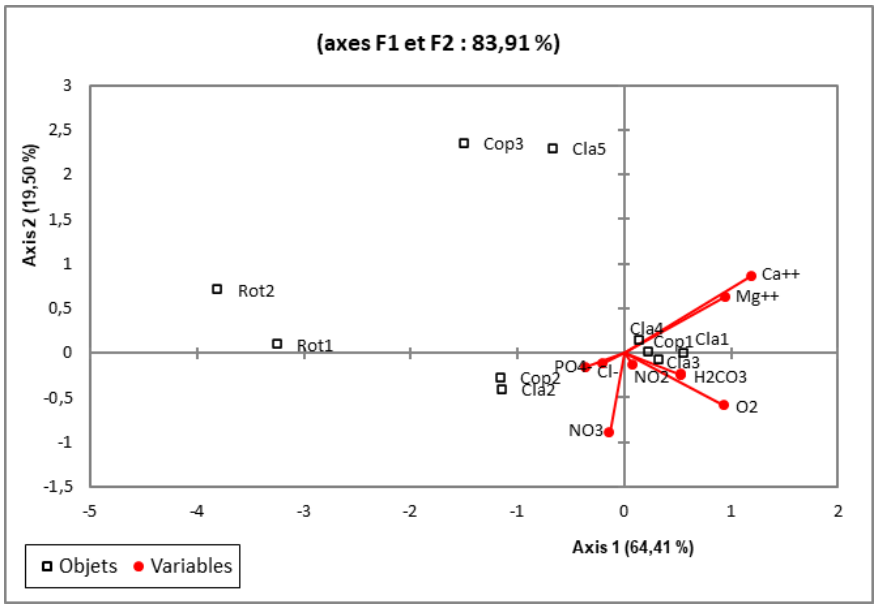

Figure 2. *CCA according to zooplankton assemblages (10 species, codes as in Table1) and environmental parameters (8) on the lake during the study period*

Similarity between stations

Based on the abundance of zooplankton species, the CLUSTER analysis divides the sampling stations into three groups (*Fig. 3*). At the first cutoff value 4000: two clusters each contains two sites A-B; C-E (northern stations; the center station and the one closest to it) and the third one formed squarely by the southern stations (G-F-D). This is in accordance with the locations of the stations. Taking into account the presence/absence of species, two main groups of stations reappear (*Table 4*); similarity between C-E stations is greater than 50% however between the other stations, similarity exceeds 56% and can exceed 90% as it is the case for stations F and D.

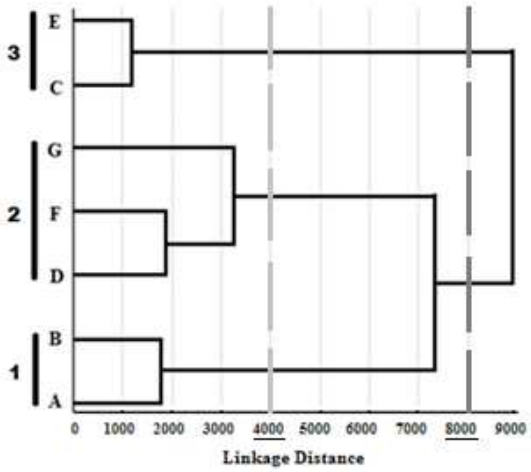

Figure 3. *Dendrogram showing the clustering of the sampling stations based on the abundance of zooplankton species*

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Table 4. *Similarity matrix of sampling stations based on Steinhaus coefficient*

E							
C	**0.522**						
G	0.104	0.232					
F	0.092	0.203	**0.842**				
D	0.088	0.228	**0.872**	**0.926**			
B	0.158	0.292	**0.715**	**0.565**	**0.595**		
A	0.153	0.371	**0.765**	**0.601**	**0.663**	**0.890**	
	E	C	G	F	D	B	A

In addition, the Chi-square test of homogeneity showed a significant difference ($p < 0.05$) among groups of stations taken in pairs and this difference is highly significant (*p-value* < 0.00001) between northern group and southern one. The probable reason that could explain this difference is the variation in species abundance (*Fig. 4*). Quantitatively, the southern stations enjoy maximum abundance and qualitatively, they host an important diversity (H=1.33; S=10 and E=0.4). In the Hamiz, copepods were the dominant group in determining total zooplankton abundance and contributed with 91.74% in the north, 86.48% in the center and 80.78% in the south. However, cladocerans show increasing proportions from north to south (8.19%, 12.83% and 19.06%) and rotifers are extremely rare in the lake.

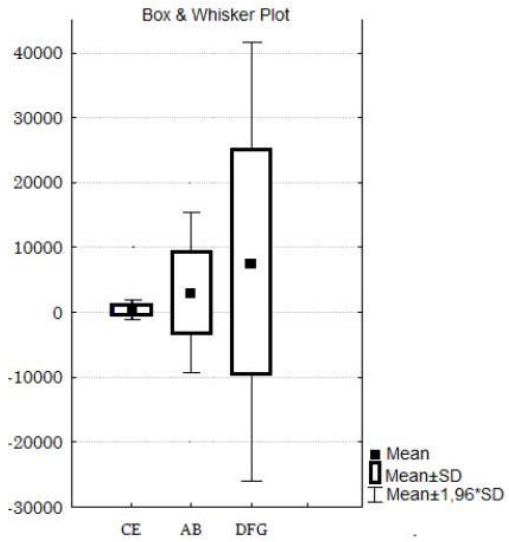

Figure 4. *Box and Whisker Plot of the abundance species variation in the station groups*

Dynamics of the Hamiz zooplanktocenoses

Horizontal structure (Table 5; Fig. 5)

From October to January, the zooplankton population was represented by the dominant species *Cyclops abyssorum*. The lowest abundance was found in October (5 species only), then it increased significantly in January up to 9 species. It should be noted that the highest abundance of species could be observed in the Hamiz especially

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in January. In February *C. abyssorum* loses its leading position, transferring to typical species category and making place for the thermophilic *Copidodiaptomus numidicus*. In this period, the typical species includes also *Daphnia longispina*. Neither *Filinia longiseta* nor *Acanthocyclops americanus* were detected during February. In March *C. numidicus* community was retained in the whole water reservoir. In addition, *D. longispina* was included in the typical species. *C. abyssorum* was excluded from the typical species, replaced with *Acanthocyclops americanus* and *Filinia longiseta* was not detected at all.

Table 5. *Dynamics of zooplankton communities in Hamiz (2006-2007)*

Months / Depth (m)		October	January	February	March
Horizontal structure (0.0)		*Cyclops abyssorum*		*Copidodiaptomus numidicus*	
Vertical structure	0.0-1.0	*Cyclops abyssorum*		*Copidodiaptomus numidicus*	
	1.5-4.5	*Cyclops abyssorum*	*C. abyssorum – C. numidicus*		
	5.0-9.0	*C. numidicus – C. abyssorum*	*C. abyssorum*	*C. numidicus*	*C. abyssorum - C. numidicus*

Vertical structure (Table 5; Fig. 5)

In October, *C. abyssorum* was distributed along the whole water column. If in the upper horizons *C. abyssorum* apparently dominated in the community then in the depths (5.0-9.0 m), this species was equally represented along with *C. numidicus* (taking into account that the common density of the organisms is less by an order than the density in the upper horizons and lessening the abundance of species from five to two species). In January, the zooplankton organisms were distributed in the water thickness more evenly and with rather high density. The abundance of the species was as usual noticeably lower in the near bottom horizons where only *C. abyssorum*, *C. numidicus* and *Ceriodaphnia reticulata* were available. Here *C. abyssorum* is very strong with its dominant position. In the surface layer of the water, this species also outnumbers the rest of the species and the cenosis of middle horizons can be determined as *C. abyssorum - C. numidicus* communities, as the second species is considered as subdominant. The significant restructuring (change of the dominants) took place from January till February. Indeed, *C. numidicus* community occupies all the horizons in February. The ex-dominant *C. abyssorum* is transferred to the typical species, where *D. longispina* makes appearance as well as *C. reticulata* in the lower layers of the water. In March *C. numidicus* retained its dominant position in the whole water column. *C. abyssorum*, was subdominant in the lower horizon only, and it could not be detected in the middle layers of the water and sometimes could be detected near the surface.

Temporal structure

When considering variations in community structure over time, the density index gives us the possibility to define the zooplankton communities typical for each station (*Fig. 6*) and we can therefore, identify four zooplankton communities in the Hamiz (*Table 6*).

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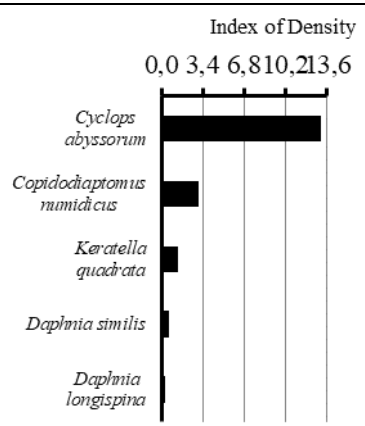
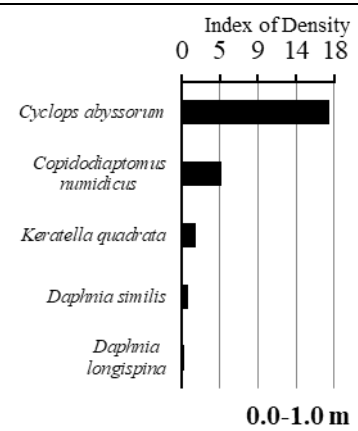
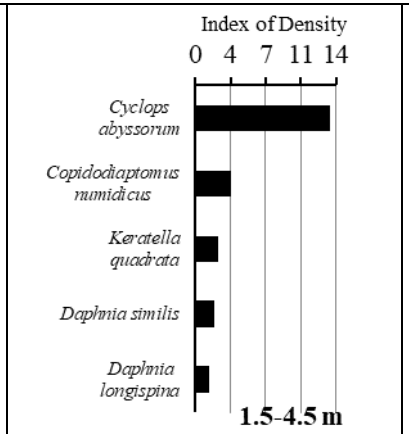
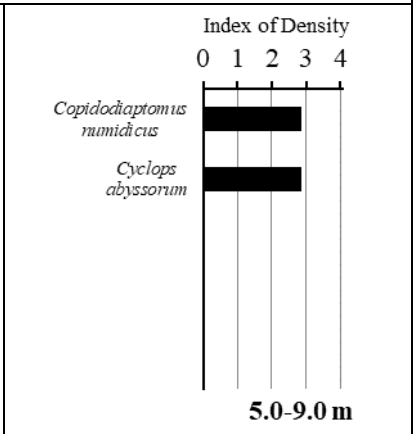
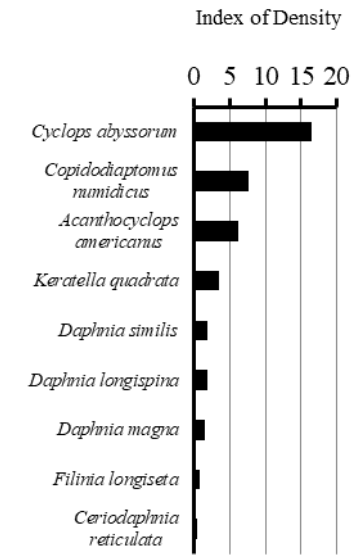
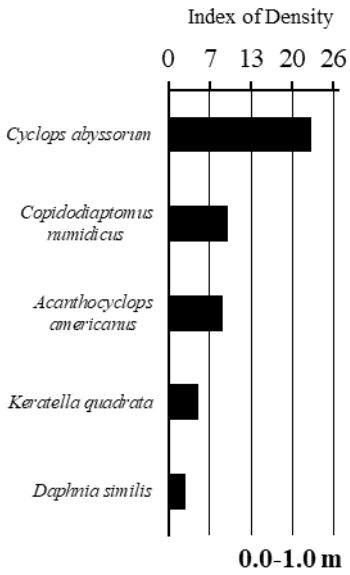
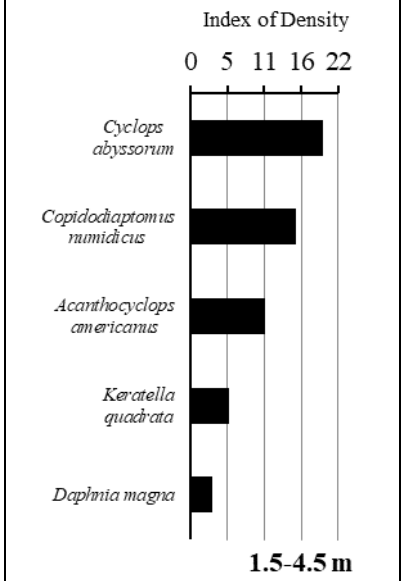
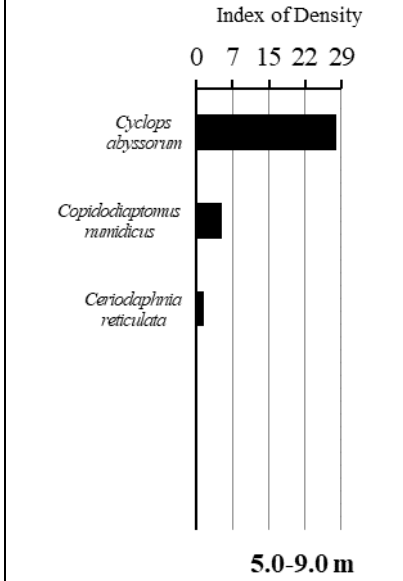
Months	Horizontal structure (0 m)	Vertical structure (0.0-9.0 m)		
October				
January				

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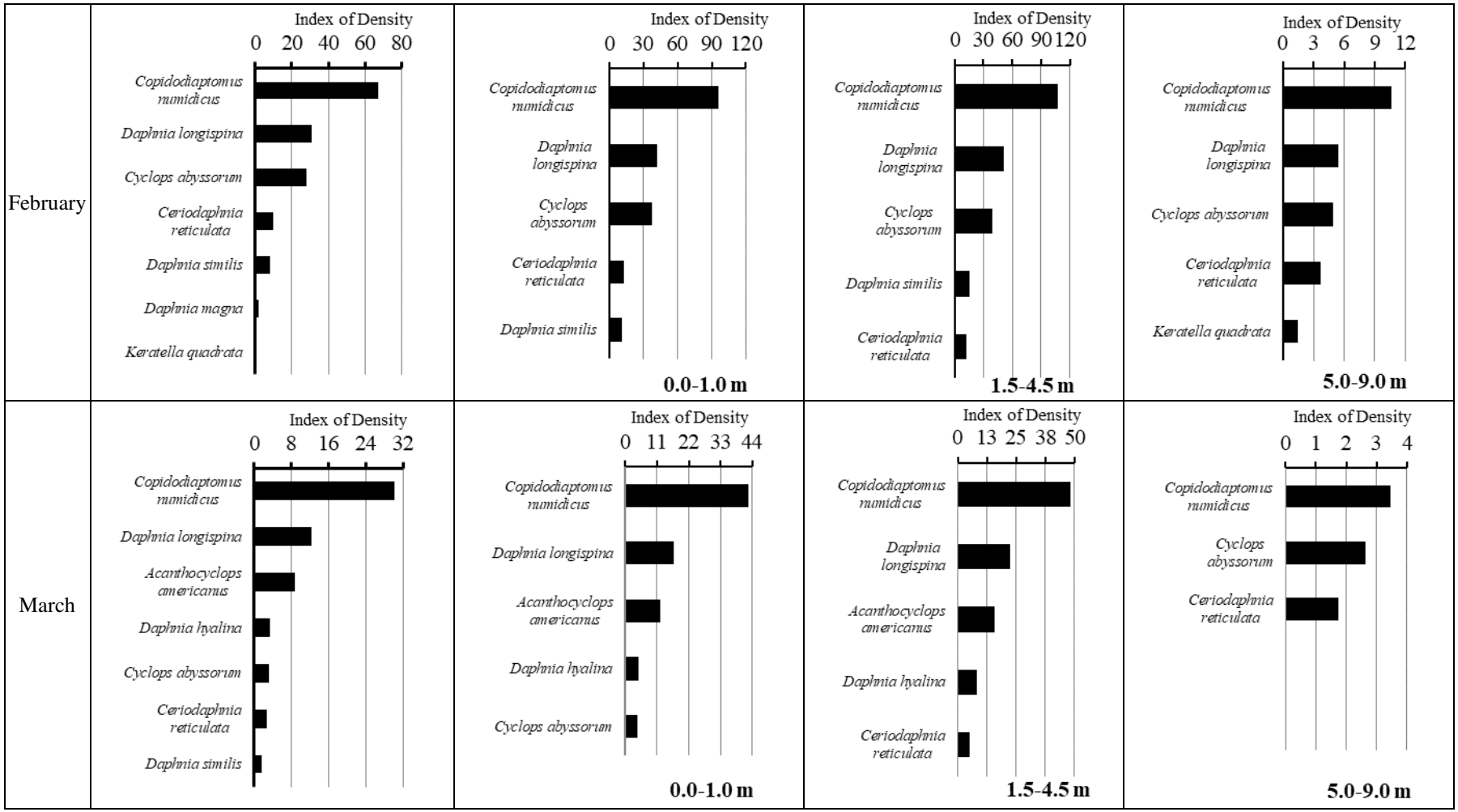

Figure 5. *Zooplanktocenoses at different depths on Hamiz*

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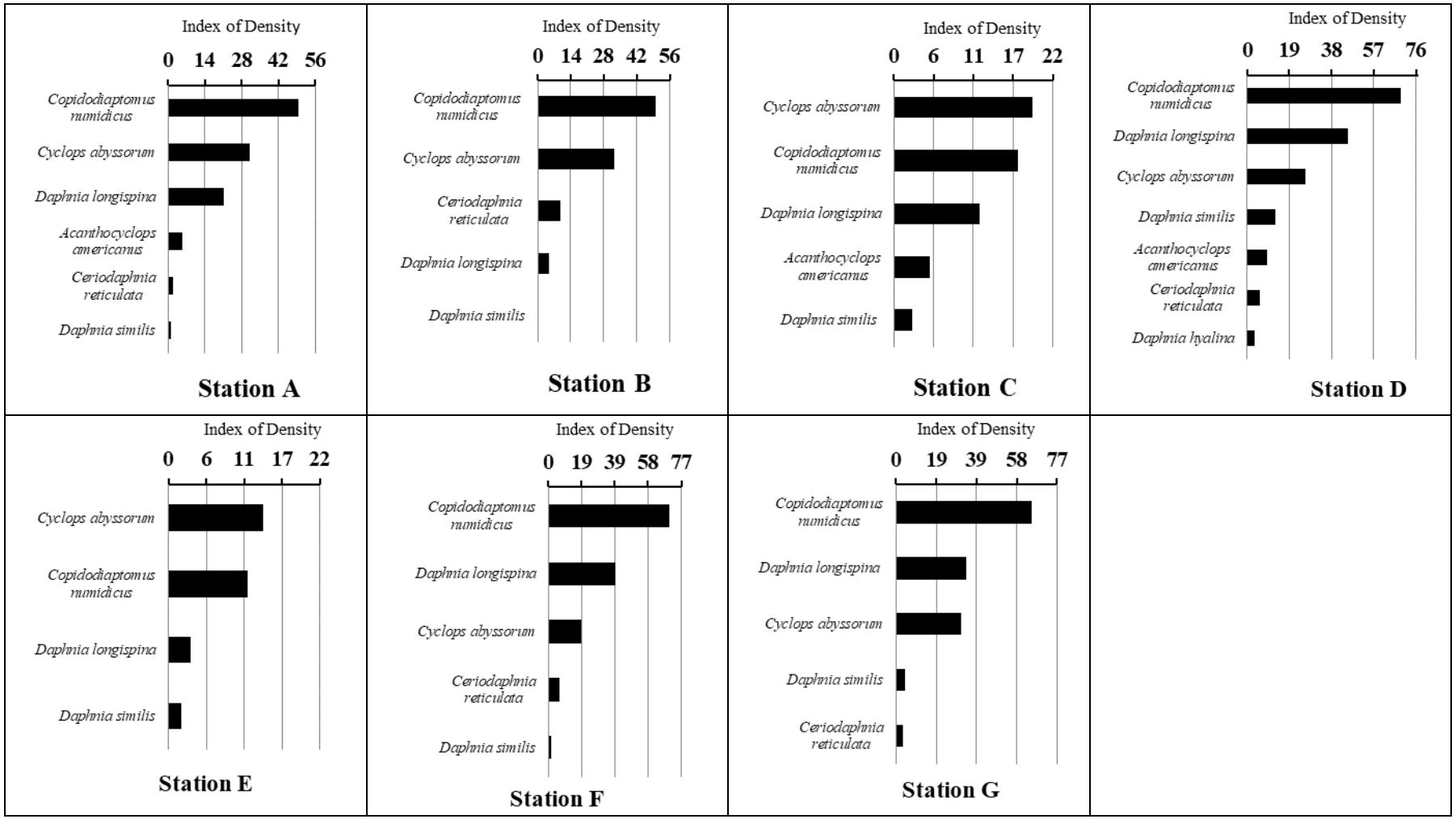

Figure 6. *Zooplanktocenoses at each station*

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Table 6. *Zooplankton communities in Hamiz (2006-2007)*

Zooplankton communities at different stations			
C. numidicus – C. abyssorum	*C. abyssorum – C. numidicus*	*C. numidicus – D. longispina*	*C. numidicus*
A-B	C-E	D	F-G

1. The zooplankton community where the only species *C. numidicus*, dominates for most of the year. It is the typical community of stations F and G, located along the south-west bank of the water reservoir.

2. The very close to this community is the zooplankton community *C. numidicus* - *C. abyssorum*, which is typical of stations A and B, which are located near the dam (North-West) and near the north-east bank of the water reservoir.

3. The community *C. numidicus* - *D. longispina*, which is typical of station D. This community is also related to the first two communities.

4. The *C. abyssorum* - *C. numidicus* community is typical of stations C and E, which are located next to each other.

Discussion

The Hamiz ecosystem is not only a real biotope that hosts different links in the food webs, especially zooplankton, but also continues to play a vital role by supplying the population with drinking water despite its structure, which was built more than a hundred years ago. Our survey on this lake confirms the presence of ten species belonging mostly to cladocerans and copepods while rotifers, were represented by only two species. Copepods were the major component of zooplankton throughout the reservoir followed by cladocerans. Rotifers are ubiquitous, occurring in almost all types of freshwater habitats (Tasevska et al., 2019) and they constitute with the two last groups most of the zooplankton. In Hamiz, they are very rare mainly represented by *K. quadrata*, which is found occasionally but not recorded near the dam wall where, planktivorous fish are abundant (Ould Rouis et al., 2016) thus modifying the zooplanktonic community structure. These latter are formed mainly by Copepoda and Cladocera since the Rotifera group does not appear well in the lake while their total abundance is a more sensitive indicator of the trophic state of the lake than the composition of the species (Liang et al., 2019). During our study period, the water temperature was almost the same between stations. This ecological factor is very important for several biological compartments, including zooplankton. Indeed, when temperature increases, zooplankton metabolic activity rates change and they can also swim faster (Simoncelli et al., 2019). The oxygenation of the water is sufficient and the pH values recorded characterize alkaline waters, which are common in most of the freshwater ecosystems including lakes, ponds, rivers and streams (Ishaq and Khan, 2013). Moreover, the geology (Cooper et al., 2004) and soil type (Shirazi et al., 2001) of the mountainous massif bordering the Mitidja plain, where the Hamiz basin is located presents schisto-sandstone and marno-calcareous terrains, (Durozoy, 1952; Cheikhlounis et al., 2011). Therefore, the waters that cross these soils have a high concentration of carbonate and a high pH (Angelier, 2000). Phosphate contents are low may be, because of the assimilation of mineral phosphorus dissolved by aquatic plants

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and then rapidly recycled (Simoneau et al., 2004). In addition to the above given factors, calcium and magnesium ions describing the hardness of the water may arise from the subsurface ground waters having higher hardness (Sharma et al., 2016). In Hamiz, variations in environmental parameters are mainly related to precipitation and temperature as well as the geological nature of the soil. Runoff from the watershed and agricultural activity around the lake are also major contributors to changes in nutrient levels, which also provide a picture of water quality (Rossetti et al., 2004). The CCA analysis shows significantly, strong correlations between species influenced par calcium-magnesium ions and others that prefer nutrient-rich waters. Cladocerans were the richest group represented by a single family where, *D. longispina*, *D. similis* and *Ceriodaphnia reticulata* were the most abundant species. It seems that the environmental conditions of the lake suit them. Indeed, the water quality of the Hamiz is generally acceptable. *D. longispina* prefers waters with low mineralization and is very sensitive to magnesium (Gonçalves et al., 2007), while *D. similis* tolerates a wide range of fluctuations of physicochemical parameters of the water. Therefore, the zooplankton community and its distribution in the water column are strongly linked to water quality. Copepods are predominant in the continental waters of Algeria, but some species are regional (Hamaidi et al., 2010). Furthermore, the specific composition of an ecosystem at a regional scale is more likely to be due to local environmental conditions (García-Chicote et al., 2019). However, *C. numidicus* is widely distributed in the western Mediterranean regions (Samraoui et al., 1998), is endemic to North Africa (Mouelhi et al., 2000) and in the Hamiz, this copepod colonized the reservoir from February and now, it becomes the most abundant species. In addition, the distribution of this Calanoida is probably associated with the supply of nutrients, in late winter and spring, by the wadi Arbatache that feeds this lake. Knowing that *C. numidicus* is herbivorous, it was considered to be a potential competitor of cladocerans species (Geraldes and Boavida, 2004). Moreover, the presence of this invasive species, associated with the trophic status of the lake, could be the reason for the rarity of rotifers, because biotic factors are important in shaping the structure of this group (Dumont, 1977). During our survey, the only species recorded are thus *Keratella quadrata* and *Filinia longiseta* without forgetting that the whole genus Keratella is considered eurytopic and cosmopolitan (Segers and De Smet, 2008). The waters of the Arbatache wadi and the small tributary which cross the "Tararesse forest" and then flow into the lake, enrich the latter with nutrient inputs from human activities in the watershed, providing favorable conditions for the development of aquatic plants. In these shallow sites, macrophytes offer food supplies as well as refuge for zooplankton species from pelagial predators (Špoljar et al., 2012). Indeed, it seems that certain areas of the lake usually host the same species, which suggests that the configuration of the food webs in Hamiz is shaped by the orientation of the basin from the northeast to the southwest on the one hand and the depth, which increases as one moves northwards, on the other hand. To these factors are added the macrophytes that are developing well on the southern bank. These differences in both physical and chemical conditions and in the characteristics of the northern and southern shores of the reservoir can lead to different zooplanktocenoses, whose existence and evolution in a given space and time can be an indicator of the fertility of the water and reflect the environmental conditions prevailing in the lake.

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Conclusion

Heterogeneity of physico-chemistry of the water and topography of the basin's reservoir are the main factors that draw and regulate the distribution of the zooplankton communities of the Hamiz. Our results demonstrated, for the first time, that this oligo-mesotrophic lake, has a low zooplankton diversity compensated by a high abundance of species especially copepods, which form the majority of the zooplankton population. The information provided by our findings on the distribution of zoobiocenoses in relation to abiotic factors, may be essential for monitoring and understanding the functioning of lake ecosystems. Additionally, they lead us to predict that the morphology and the exposure of the lake play a fundamental role in shaping the architecture of the food webs and the distribution of these communities not only in the superficial layers but also in the deep layers of the lake. Finally, we recommend to do more investigations considering the characteristics of the studied water mass, the abundance of zooplankton populations while completing the obtained results by a thorough knowledge of phytoplankton species, first link of the food chain.

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CHANGES IN LIGHT INTENSITY AFFECT LEAF GAS EXCHANGE, CHLOROPHYLL FLUORESCENCE, AND NON-STRUCTURAL CARBOHYDRATES OF MA BAMBOO (*DENDROCALAMUS LATIFLORUS* MUNRO)

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Abstract. Many physiological traits are thought to play an important role in the low-light adaptation of plants. Therefore, we tested light intensity regulation of chlorophyll pigments, gas exchange, chlorophyll fluorescence, and non-structural carbohydrates in Ma bamboo (*Dendrocalamus latiflorus* Munro) leaves under five varying light intensities; such as L0 (100%), L1 (40%), L2 (30%), L3 (20%), and L4 (10%). We found that Ma bamboo grown under low light conditions synthesized more chlorophyll [total chlorophyll (Chls) and carotenoids (Car)], whereas net photosynthetic rate (P_n) under 10-40% light treatments was lower than that of those established under full sunlight. The decrease in light intensities increased PSII actual photochemical efficiency (Φ_{PSII}), electron transport rate (*ETR*), and PSII photochemistry (F_v/F_m), while low radiation led to a reduction in photochemical quenching coefficient (*qP*) and an increase in non-photochemical quenching coefficient (*NPQ*) of the leaves. Low light intensity was more conducive to accumulating non-structural carbohydrates with higher soluble sugar and starch. These parameters analyzed by principal component analysis (PCA) could be considered critical physiological traits to evaluate Ma bamboo's adaptation to low light. Our findings implied that Ma bamboo had better adaptive strategies in low light conditions, which had substantial implications for the management and cultivation of Ma bamboo's plantation.

Keywords: *low-light conditions, bamboo seedlings, shade tolerant, photosynthetic efficiency, photosynthetic products*

Abbreviations: Chls - total chlorophylls, Car - carotenoid, Chl a/b - chlorophyll a/b, Car/Chls - carotenoid/total chlorophylls, P_n - net photosynthetic rate, g_s - stomatal conductance, C_i - intercellular CO_2 concentration, T_r - transpiration rate, Φ_{PSII} - PSII actual photochemical efficiency, ETR - apparent photosynthetic electron transport rate, F_v/F_m - PSII photochemistry, qP - photochemical quenching coefficient, NPQ - non-photochemical quenching coefficient, SS/starch – soluble sugar/starch, and NSCs - non-structural carbohydrates

Introduction

Light is an essential abiotic factor affecting plant growth and productivity. Light is absorbed by photosynthetic pigments and converted into biochemical energy (ATP and NADP), which are used for carbon dioxide (CO_2) fixation to produce carbohydrates or plant biomass (Matsudo et al., 2012; Minagawa, 2013). Average plant growth requires optimal light irradiance, however excessive or low irradiance might impact plant photosynthesis, which is critical for plant productivity and severely restricts plant growth (Ma et al., 2015). In forests, plants growing in the understory are subjected to some degree of shade during their life span due to varying

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light gradients from plant canopies (Valladares and ülo Niinemets, 2008; Lu et al., 2018). As a result, plants progressively evolve various traits to adapt to low-light conditions during their life cycle, especially in the early stages, such as morphological and physiological plasticity, biochemistry, and metabolic regulation (Nicotra et al., 2010; Liu et al., 2020).

Plants grown under low-light conditions can strengthen their adaptation by changing their photosynthetic traits. Generally, plants increase their light-use efficiency by prioritizing light capture over photosynthetic capacity to adapt to low light conditions (Var, 2017; Tang et al., 2020). In addition, plants grown under low-light conditions are known to optimize their light absorption efficiency by increasing pigment production (Lichtenthaler et al., 2007a; Tang et al., 2015; Shafiq et al., 2020). Chlorophyll fluorescence focuses on photosynthesis regulation and plant response to the environment due to its sensitivity, convenience, and non-destructive characteristics (Dai et al., 2009; Tang et al., 2015). In a light-induced environment, plants tend to improve photosystem II (PSII) efficiency, which protects the photosynthetic machinery from irreversible damage caused by excessive light energy (Cai and Xu, 2002). As a result, in the current research, we attempt to gain insight into the ability of Ma bamboo (*Dendrocalamus latiflorus* Munro) grown under varying light conditions, to confirm the significance of their photosynthetic attributes.

Furthermore, as a source of plant energy storage, leaf non-structural carbohydrates (NSCs) (including soluble sugars and starch), can be mobilized to support growth or other plant physiological functions and can be used to resist external adverse environments (Poorter and Kitajima, 2007; Richardson et al., 2015; Hartmann and Trumbore, 2016). The NSCs storage can enhance the ability of plants to adapt to low light (Sala et al., 2012). Some studies have shown that plants have higher NSC contents under low light conditions by reducing their growth rate and increasing their chances of survival during periods of carbon budget imbalance (Meyer et al., 2006; Jing et al., 2009; Athar et al., 2016; Lin et al., 2018). Starch and soluble sugar can also help plants to resist external interference, such as temperature stress (Jain et al., 2007; Lu et al., 2014; Yang et al., 2020), water deficit (Meier et al., 1990; O'Brien et al., 2014), salt stress (Boriboonkaset et al., 2013), etc., and play an important role in the recovery after interference. Consequently, we attempt to explore the response mechanism of non-structural carbohydrates to different light intensities, especially low light.

Ma bamboo is a wide-distribution clump bamboo species in China. The south area of the Yangtze River in China is the main production area of Ma bamboo shoots, with abundant resources to meet the needs of bamboo growth. However, in the early stage of bamboo shoots, the main production area has continuous rainfall and Ma bamboo has a large leaf area, which would exacerbate the lack of sunlight in the forest understory. Ma bamboo bushes, composed of different quantities of individual bamboo, have different light gap sizes from the canopy. In contrast, the mosaic of diffuse light and direct light from the gaps may affect early shoot development to promote understory renewal. However, its physiological aspects, such as the potential photosynthetic or carbohydrate storage to widely varying light conditions, are unknown.

Therefore, we hypothesized whether Ma bamboo has good adaptability to low-light conditions or varying light intensities will influence their photosynthetic activity and NSCs storage. To test this hypothesis, we used various light intensities; from full sunlight (100%) to a minimum (10%) to determine whether Ma bamboo would reach its growth potential in light levels approximately equivalent to the forest-edge gradient.

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The objectives of this study were: (i) to examine the variation of leaf photosynthetic characteristics in Ma bamboo, (ii) to examine responses in NSCs to different levels of light intensities in Ma bamboo. The study will provide valuable insights into optimum light conditions for the growth of Ma bamboo.

Materials and methods

Study site

The present study was conducted out at Fujian Agriculture and Forestry University, Fujian Province, China. Geographically it is situated 26°05'29.88" North at latitude, 119°14'47.37" East at longitude (*Fig. 1*). Climatically the study area falls in the subtropical oceanic monsoon regions of south China, where the climate is warm and humid. The mean annual sunshine is 1700-1980 h with the mean annual frost-free period of 326 days and the mean annual precipitation of 900-2100 mm. The mean annual temperature is 16-20 ℃ with minimum and maximum temperatures of 1.2 ℃ in January or February and 42.3 ℃ in July or August, respectively.

Description of plant material and soil

Three-year-old Ma bamboo seedlings were selected as materials, which were purchased from Yunnan Zhenzhu Agricultural Technology Co., Ltd. The mean height of seedlings was 105.5 cm, the mean ground diameter was 4.66 mm, and the crown width (north-south × east-west) was 71.1 cm × 69.5 cm. The seedlings were transplanted in non-woven bags in October 2018, with a size of 35 cm × 33 cm × 30 cm (upper diameter × lower diameter × height) (*Fig. 1*).

Potted substrate soil was yellow soil and peat soil (volume ratio 3:1). The mean weight of one potted substrate was 15.48 kg with a pH value of 5.77 and organic carbon content of 13.67 $g \cdot kg^{-1}$. The total nitrogen, total phosphorus, and total potassium content were 0.35, 0.50, and 50.01 $g \cdot kg^{-1}$, respectively.

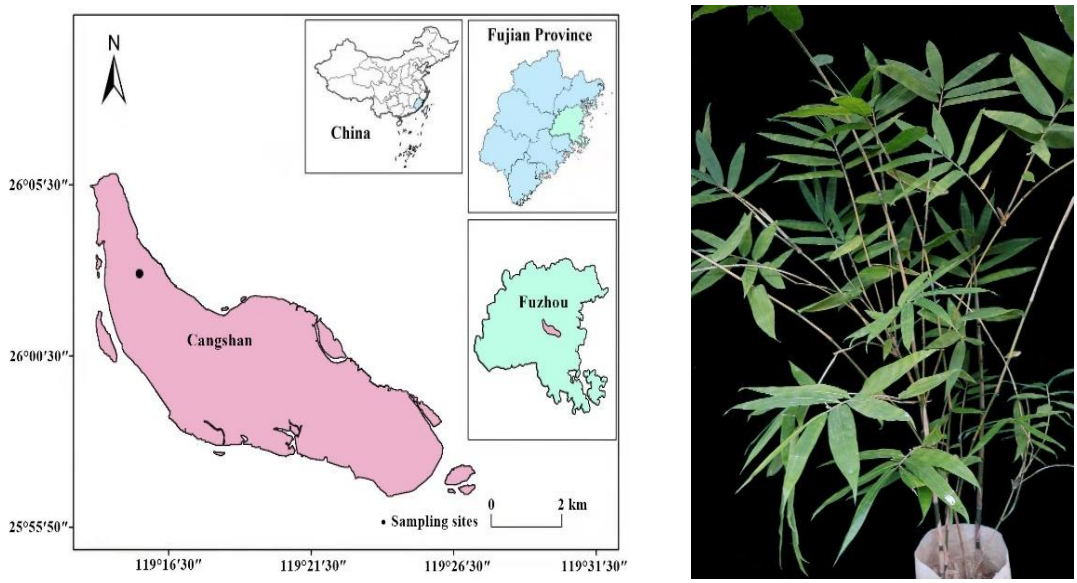

Figure 1. *Geographical location of Fujian Agriculture and Forestry University (left) and Ma bamboo seedlings (right)*

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Experimental design and growing conditions

After transplanting, Ma bamboo seedlings were grown in the greenhouse of the Forestry College of Fujian Agriculture and Forestry University. The shading treatment was carried out on April 10, 2019. The experiment included five light intensity treatments: 100% (L0 as control), 40% (L1), 30% (L2), 20% (L3), and 10% (L4) of natural light. All the pots were arranged in a completely randomized design with four replicates for each treatment.

A black plastic shade net was used to build a shade chamber (height = 3 m, width = 1.2 m, length = 3 m), and different light intensities were managed by covering plastic shade nets with different needles and layers. Each shade chamber was oriented east-west to allow full exposure of light.

The light intensity gradients were chosen based on the daily variable spectrum of light intensity under natural management conditions of the Ma bamboo forest, forest gap, and forest edge. The Taiwan Hipoint handheld spectrometer (HP350) measured the light intensity under different shaded chambers (*Table 1*). The shaded chambers were separated by 1.2 m to avoid mutual interference between treatments. Six months after the experiment, gas exchange attributes were measured and the leaf tissues were sampled for analyzing photosynthetic pigments and leaf non-structural carbohydrate contents. During the experiment, the field water holding capacity was maintained at more than 60%. Inorganic fertilizers (N: P: K-15: 15: 15) of 10 gram was applied to all treatments at one time. The maximum temperature in the shaded chamber was controlled (below 35 ℃), and the relative air humidity was kept above 85%. Furthermore, carbendazim was sprayed every 15 days for disinfection, and weeds were artificially eradicated.

Table 1. *Light intensity of different shading treatments*

Treatments	Light transmittance (%)	Illuminance (lx)	PPFD (μmol·m^{-2}·s^{-1})
L0	100	10343.67 ± 233.59a	185.66 ± 4.37a
L1	40	4062.00 ± 154.02b	73.252 ± 2.97b
L2	30	3116.67 ± 240.25c	55.96 ± 4.35c
L3	20	2066.00 ± 172.39cd	49.44 ± 4.27cd
L4	10	1054.00 ± 101.25d	39.25 ± 2.04d

L0, L1, L2, L3, and L4 refer to 100%, 40%, 30%, 20%, and 10% of natural light, respectively. Different letters indicate significant differences ($P < 0.05$) of means and ± denotes the standard errors of the means (SE) (n = 4)

Determination of chlorophyll pigments

The chlorophyll pigment contents were estimated using the same samples used to monitor gas exchange attributes. After collection of leaf samples, these were washed with distilled water, dried on filter paper, cut along midribs, and mixed to weigh 0.1 g. After that, the chlorophyll pigments were directly extracted from 25 ml mixed solution (acetone: absolute ethanol: distilled water = 4.5: 4.5: 1) for 48-72 h in darkness until their color changed completely to white (Gao, 2006). The absorbance of the extracted solution was measured at 645 nm, 663 nm, and 470 nm, respectively. Later the chlorophyll concentrations [chlorophyll a (Chl a), chlorophyll b (Chl b), total

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chlorophylls (Chls), and carotenoid (Car)] were calculated using the equations of Lichtenthaler (1987). Besides, the ratio of Chl a and Chl b (Chl a/b) and the ratio of Car and Chls (Car/Chls) were also calculated.

Determination of leaf gas exchange attributes

The upper-middle and healthy leaves were selected from each replicate (five readings) to monitor the gas exchange attributes which include net photosynthetic rate (P_n), stomatal conductance (g_s), intercellular CO_2 concentration (C_i), and transpiration rate (T_r). All the measurements were recorded between 08:30-11:30 am. A standard leaf chamber (2 cm × 3 cm) with red/blue light sources and the portable photosynthesis instrument (LI-6400 XT, LI-COR Biosciences, Lincoln, NE, USA) were used to test photosynthetic characteristics at a saturated light intensity of 1600 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetic photon flux density (PPFD), with a measurement period of 120-180 s. The CO_2 was supplied by an external CO_2 small steel bottle, and the concentration was adjusted to 400 $mmol \cdot mol^{-1}$. Prior to the measurements, the testing leaves were illuminated under a light intensity of 1600 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (PPFD) for 20-30 min.

Determination of chlorophyll fluorescence

The upper-middle and healthy leaves from each replicate were chosen for measuring chlorophyll fluorescence using the OS5p portable chlorophyll fluorescence instrument (OPTI-sciences, USA). After dark-adaptation of samples for 30 min, the maximum quantum efficiency of PSII photochemistry (F_v/F_m) was measured after 5-7 min of ambient light. In addition, other fluorescent parameters were determined under ambient light, including PSII actual photochemical efficiency (Φ_{PSII}), apparent photosynthetic electron transport rate (*ETR*), photochemical quenching coefficient (*qP*), non-photochemical quenching coefficient (*NPQ*).

Determination of the non-structural carbohydrate contents

From each replicate, the fresh leaves were cut into pieces and mixed to weigh 0.1 g. The soluble sugar and starch concentrations were estimated using the chemical kits (Suzhou Keming Biotechnology Co., Ltd.) based on anthrone colorimetry method at an absorbance of 620 nm by UV spectrophotometer (TU-1901, Beijing, China) and the ratio was also calculated. The non-structural carbohydrate contents (NSCs) were calculated as the sum of the soluble sugar and starch contents.

Statistical analysis

The statistical analysis was done using SPSS program (version 20.0, IBM Corp., Armonk, NY, USA). The analysis of variance (ANOVA) was applied to all the results. However, if there were significant differences between various treatments, the means were differentiated ($\alpha = 0.05$) using the Tukey HSD (honestly significant difference). Besides, principal component analysis (PCA) was performed using correlation matrix data for comprehensive analysis of the test data under varying light intensities and reflecting the relationship between different parameters. The data was presented as means ± standard errors (SE). Prism v. 8.0.1 (GraphPad, San Diego, CA, USA), Origin 9.5 (OriginLab OriginPro, 2019), and Microsoft Excel-2016 were used for graphical illustrations and tables, respectively.

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Results

Changes in the concentrations of photosynthetic pigments under different light intensities

We found that the plants established under L2 and L3 treatments (30% and 20% light intensities, respectively), their leaves' Chls increased significantly ($P < 0.05$) compared to L0 (*Fig. 2A*). Similarly, the leaf's Car contents also increased significantly ($P < 0.05$) under L2 and L3 treatments compared to all other treatment combinations (*Fig. 2B*). Compared to L0, no significant differences were noticed for Chl a/b concentrations under low light treatments (*Fig. 2C*). However, Car/Chls decreased significantly ($P < 0.05$) to 4.75% and 6.10% under L2 and L3 treatments, respectively compared to L0 (*Fig. 2D*).

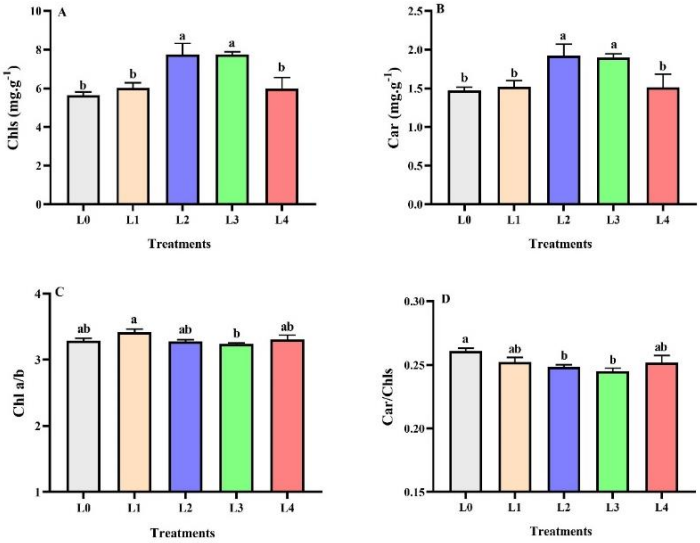

Figure 2. *Changes in photosynthetic pigments of Ma bamboo under different light treatments. (A) Chls - total chlorophylls, (B) Car - carotenoid, (C) Chl a/b - chlorophyll a/b, and (D) Car/Chls - carotenoid/total chlorophylls, respectively. L0, L1, L2, L3, and L4 refer to 100%, 40%, 30%, 20%, and 10% of natural light, respectively. Values are the means ± SE of four replicates per treatment. Different lowercase letters above the bars represent significant differences ($P < 0.05$) between treatments*

Changes in gas exchange attributes under different light intensities

Compared to L0, P_n decreased significantly ($P < 0.05$) as the light intensity was reduced (*Fig. 3A*). Similarly, g_s showed parallel changes with P_n under light treatments (*Fig. 3B*). Compared to other shade treatments, the values of P_n (8.65 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and g_s (0.12 $mmol \cdot m^{-2} \cdot s^{-1}$) were higher under L2 treatment (*Fig. 3A* and *B*). Additionally, no significant differences were observed for C_i under low light treatments compared to L0 (*Fig. 3C*). Compared to L0, T_r increased significantly ($P < 0.05$) up to 29.76% under L2 treatment (*Fig. 3D*).

Changes in chlorophyll fluorescence parameters under different light intensities

Compared to L0, Φ_{PSII} and *ETR* increased significantly ($P < 0.05$) under all light treatment combinations as shown in *Figures 4A* and *B*. In comparison to L0, seedlings established under L4 treatment significantly ($P < 0.05$) improved their Φ_{PSII} by 47.67%

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and *ETR* by 47.78%. F_v/F_m increased as the light intensity decreased, while no significant impact of varying light intensities was noticed for the values (*Fig. 4C*). The values of *NPQ* were higher than that of *qP*, which decreased significantly ($P < 0.05$) in shading treatments compared to L0 (*Fig. 4D* and *E*).

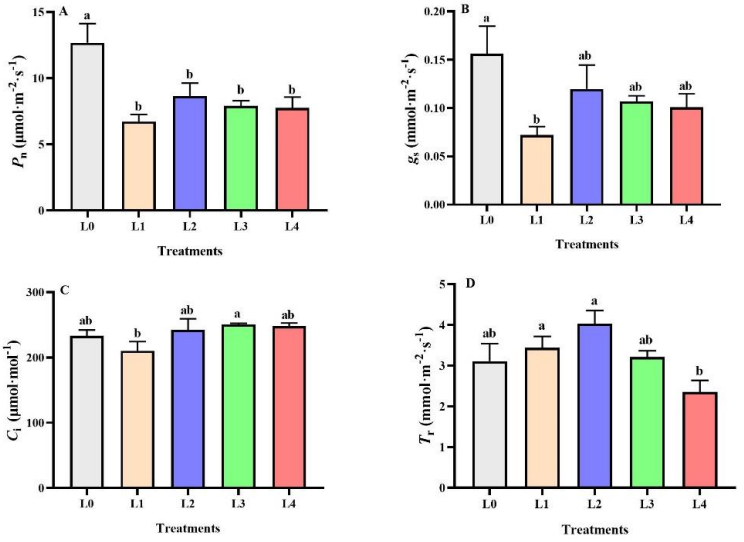

Figure 3. *Changes in gas exchange attributes of Ma bamboo under different light treatments. (A) P_n - net photosynthetic rate, (B) g_s - stomatal conductance, (C) C_i - intercellular CO_2 concentration, and (D) T_r - transpiration rate. L0 refers to full light as the control level. L1, L2, L3, and L4 refer to 40%, 30%, 20%, and 10% of natural light, respectively. Values are the means ± SE of four replicates per treatment. Different lowercase letters above the bars represent significant differences ($P < 0.05$) between treatments*

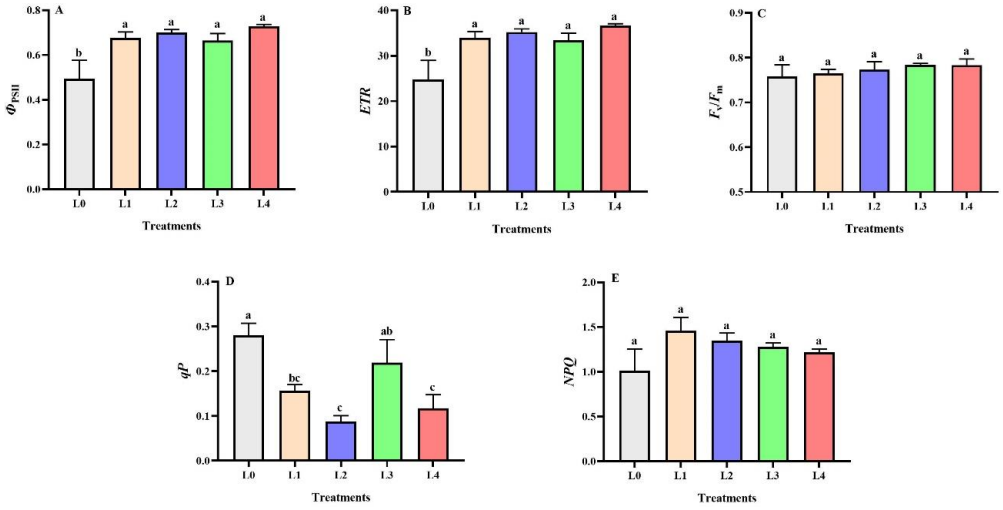

Figure 4. *Changes in chlorophyll fluorescence of Ma bamboo under different light treatments. (A) Φ_{PSII} - PS Ⅱ actual photochemical efficiency, (B) ETR - apparent photosynthetic electron transport rate, (C) F_v/F_m - PSII photochemistry, (D) qP - photochemical quenching coefficient, and (E) NPQ - non-photochemical quenching coefficient. L0 refers to full light as the control level. L1, L2, L3, and L4 refer to 40%, 30%, 20%, and 10% of natural light, respectively. Values are the means ± SE of four replicates per treatment. Different lowercase letters above the bars represent significant differences ($P < 0.05$) between treatments*

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Changes in light intensity affect non-structural carbohydrate contents

Compared to L0, a certain degree of reduction in light intensity increased the leaves soluble sugar up to 13.78% under L2 treatment, while a minimum reduction of 0.07% was found under L4 treatment (*Fig. 5A*). Overall, the seedlings established under various shading treatments exhibited greater starch and NSCs over L0 treatment. Compared to L0, L2 treatment resulted in significant ($P < 0.05$) increases in starch by 11.30% and NSCs by 11.91% (*Fig. 5B* and *C*). The soluble sugar/starch did not show any differences under various light treatments (*Fig. 5D*).

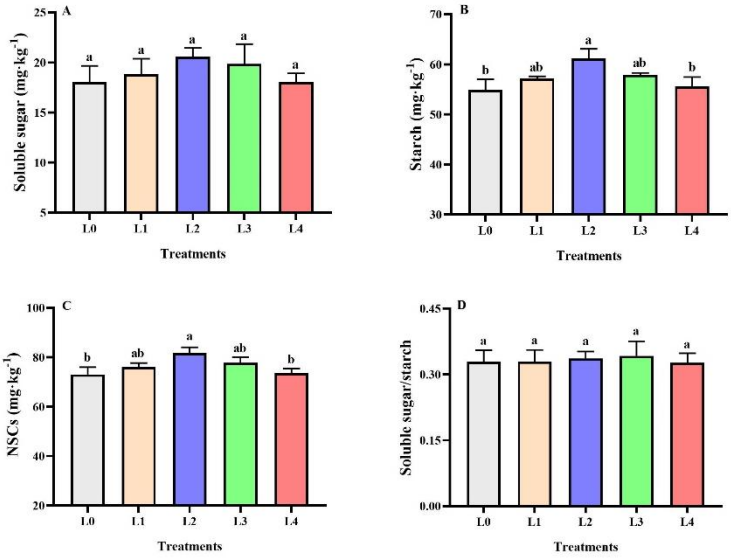

Figure 5. *Changes in leaf non-structural carbohydrates of Ma bamboo under different light treatments. (A) soluble sugar, (B) starch, (C) NSCs - non-structural carbohydrates, and (D) soluble sugar/starch. L0 refers to full light as the control level. L1, L2, L3, and L4 refer to 40%, 30%, 20%, and 10% of natural light, respectively. Values are the means ± SE of four replicates per treatment. Different lowercase letters above the bars represent significant differences ($P < 0.05$) between treatments*

Principle component analysis (PCA) on all parameters under different light intensities

The results of the PCA revealed that the first two components accounted for 78.22% of the total variation, and the first and second PCA accounted for 52.08% and 26.14% of the total variability, respectively as depicted in *Figure 6*. The photosynthetic pigments (Chls and Car) grouped with non-structural carbohydrate parameters (soluble sugar, starch, and NSCs) presented with PC1 were favored under L2 and L3 treatments. The gas exchange (P_n and g_s) and chlorophyll fluorescence (qP) parameters had significant positive correlations with PC2 under L0 treatment.

Discussion

Changes in light intensity affect the contents of photosynthetic pigments

Plants' absorption of light energy and the mechanism of photosynthesis are regulated by photosynthetic pigments, and their quality and ratio are essential measures of how well

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plants respond to their environment (Jin et al., 2016; Wu et al., 2020). The leaves acclimated to low light require more chlorophyll synthesis to improve the photosynthetic efficiency (Sharma and Sharma, 2001). We found that when the seedlings were exposed to low light intensity, leaf Chls and Car increased, which was conducive to improving light utilization and enhancing the light-harvesting capacity of leaves in a low light environment, which has been confirmed by the previous research (Stewart et al., 2015). Under 20-30% light, the growth of Ma bamboo exhibited lower Chl a/b and higher Chls, indicating that the seedlings tend to enhance the absorption of blue-violet light and effectively capture more available light energy (Bell et al., 2000; Wittmann et al., 2001). In comparison, the seedlings grown in full light with a strong reduction in Chls and Car and a higher Car/Chls might dissipate excess light energy by Car to protect the photosynthetic mechanism from severe light damage (Yao et al., 2017).

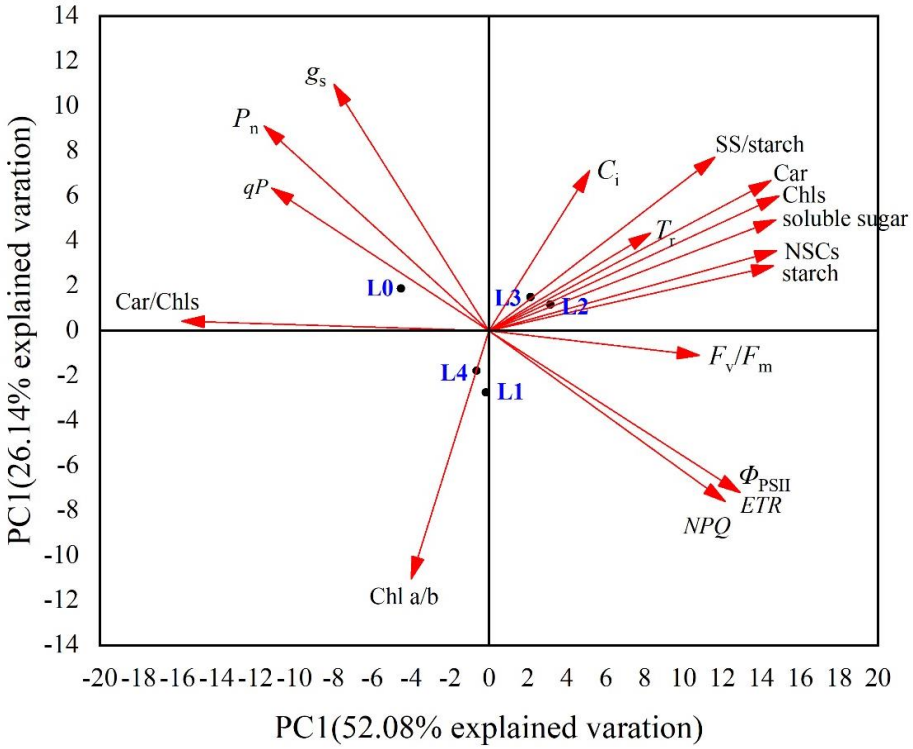

Figure 6. *Biplot of principal component analysis of the first two principal components of all parameters and light intensities. Chls - total chlorophylls, Car - carotenoid, Chl a/b - chlorophyll a/b, Car/Chls - carotenoid/total chlorophylls, P_n - net photosynthetic rate, g_s - stomatal conductance, C_i - intercellular CO_2 concentration, T_r - transpiration rate, Φ_{PSII} - PS Ⅱ actual photochemical efficiency, ETR - apparent photosynthetic electron transport rate, F_v/F_m - PSII photochemistry, qP - photochemical quenching coefficient, NPQ - non-photochemical quenching coefficient, SS/Starch - soluble sugar/starch, and NSCs - non-structural carbohydrates. L0 refers to full light as the control level. L1, L2, L3, and L4 refer to 40%, 30%, 20%, and 10% of natural light, respectively*

Changes in light intensity affect the characteristics of gas exchange attributes

Leaf photosynthetic characteristics exhibit remarkable plasticity in response to variations in the light intensities (Katahata et al., 2007). A reduction in light intensity

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due to shading has detrimental impacts on leaf gas exchange parameters (Lakshmanakumar et al., 2015). In our research, shading significantly reduced the PPFD value of the canopy of Ma bamboo seedlings. Plants at high irradiance increase P_n to fix large amounts of carbon for photosynthesis (Tang et al., 2015; Var, 2017). Similarly, our findings are in the line with previous research where the plants exposed to full light intensities exhibited significantly greater P_n over shaded plants (Shafiq et al., 2020). Therefore, we reported that L2 was advantageous to other shade treatments in terms of increasing P_n to enhance the carbon sequestration ability of Ma bamboo. At saturating PPFD, the decrease in g_s was attributed to the decrease in P_n, indicating that low g_s led to weakening leaf photosynthetic biochemical pathways and the accumulation of photosynthetic products (Lichtenthaler et al., 2007a, b). However, low g_s can reduce or prevent transpiration and related water loss compared to full light (Tarin et al., 2020). Compared with L0, the maximum T_r value under L2 treatment showed a higher water transport capacity and was able to maintain a better leaf water state during the day to avoid the restriction of noon stomata (Zhang et al., 2013). These traits enhanced the ability of Ma bamboo seedlings to tolerate under low light conditions.

Changes in light intensity affect chlorophyll fluorescence

Φ_{PSII} is an indicator that reflects the actual photosynthetic efficiency of plants and the relative rate of electron transfer, whereas *ETR* determines the efficiency of external photosynthetic electron transfer in plant leaves under actual light intensity (Szabó et al., 2014; Wang et al., 2021). Under 10-40% light, the differences in Φ_{PSII} and *ETR* of Ma bamboo showed parallel changes, which were significantly ($P < 0.05$) higher than that of full light. The weak light can improve the photochemical efficiency of Ma bamboo, speed up the electron transmission efficiency, and enhance the ability to adapt to low light conditions. On the contrary, under 100% light, Φ_{PSII} and *ETR* decreased, where there was obvious light suppression. The increase of F_v/F_m with the decrease of light intensities is an obvious feature of plants adapting to low-light environments (Hussain et al., 2019). Our research confirmed that Ma bamboo showed strong resistance to negative under 10-20% light intensity with the increased F_v/F_m. In a non-stress environment, plants can maintain a stable range of F_v/F_m (0.75-0.85) (Rascher et al., 2010). The normal values of F_v/F_m in our study suggested that Ma bamboo performed well in low light. *qP* indicates the strength of the electron transfer activity of plant PSII (Wang, 2014). *NPQ* indicates the ability of plants to convert excess light energy into heat dissipation (Müller et al., 2001). At a low light-induced period, plants are not able to utilize the absorbed energy, whereas under these conditions an increase in non-radiative dissipation can be observed as non-photochemical quenching of *NPQ* (Busch et al., 2008). In the current research, the proportion used for photochemical reactions decreased, while the proportion of conversion into heat dissipation increased, which was considered a protective mechanism formed by the adaptation of Ma bamboo to low light.

Changes in light intensity affect the accumulation of NSCs

Generally, plants cannot maximize growth under low light conditions, but transfer a large number of photosynthetic products for storage, so that plants can tolerate periods of low light close to or below the entire plant's light compensation point (Montgomery and Chazdon, 2002). In the previous research, Ma bamboo had good light energy

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utilization under 20-30% light intensity (*Table A1*), which demonstrated strong resistance to shade. Many studies have shown that shade-tolerant tree species can accumulate higher carbohydrate content under low light conditions (Myers and Kitajima, 2007; Poorter and Kitajima, 2007; Liu et al., 2020). Our research showed that soluble sugar, starch, and NSCs contents of the seedlings grown under L2 treatment were all higher than those under L0 treatment, indicating that 30% light was more conducive to the conversion and accumulation of non-structural carbon. However, as the degree of light intensities decreased, the contents of soluble sugar, starch, and NSCs all decreased, indicating that moderate shading can promote the accumulation of photosynthetic products in the leaves of seedlings. The carbon assimilation of Ma bamboo leaves was reduced under L1 treatment, which may result in sugar consumption with the increased respiration rate to maintain growth.

In our research, the reduction in the accumulation of carbohydrates in leaves was possibly related to photosynthetic capacity. Ma bamboo had higher light-harvesting chlorophylls under low light, which allows plants to capture light energy under low light, which is conducive to plant carbon accumulation (Cave et al., 1981; Meyer et al., 2006). In addition, the photosynthetic rate is sensitive to the demand for carbon assimilation (Chantuma et al., 2009). Since the light intensities reduced, Ma bamboo tended to increase the distribution of photosynthetic products and carbohydrates accumulation in leaves which negatively regulated the photosynthetic rate (Evans and Poorter, 2001; Moreau et al., 2012). However, Ma bamboo seedlings maintained high P_n in full light, more likely allocated more carbon to meet the needs of other metabolisms (Wu et al., 2019) and therefore NSCs decreased. The ratio of soluble sugar and starch in leaves reflects the distribution of NSCs (Xie et al., 2018). We noticed that Ma bamboo exposed to 20-30% light had higher soluble sugar/starch with higher NSCs, indicating that the seedlings had a strong ability to produce soluble sugar converted from starch to promote the metabolic activities for new bamboo growth consumption, which has already been confirmed in the previous research (Xie et al., 2018; Liu et al., 2020).

Evaluation of all parameters under different light intensities by PCA

PCA can fully explain the interrelationships between test indicators (Lu et al., 2021). The PCA analyzed with strong correlations may be screened out and can be used to assess plant adaptation to varying light intensities (El-Hendawy et al., 2017). The photosynthetic pigments were grouped with non-structural carbohydrates, which exhibited strong correlations with PC1, indicating that Ma bamboo can strengthen their tolerance to low light conditions by accumulating more chlorophylls and NSCs. Ma bamboo under 20-30% light can alleviate the low light stress by these mechanisms. The negative correlations between P_n and g_s, with photosynthetic pigments and non-structural carbohydrates, revealed that Ma bamboo was accumulating more photosynthetic products as a consequence of a higher photosynthetic rate. As a result, all photosynthetic parameters and carbohydrates could be considered as key physiological traits to evaluate the low light tolerance of Ma bamboo.

Conclusion

Differences in light intensity affected leaf photosynthetic characteristics and non-structural carbohydrates of Ma bamboo. We found that Ma bamboo grown in low light conditions increased light utilization efficiency by preferentially synthesizing more

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chlorophyll (Chls and Car) to tolerate low light at the expense of photosynthesis capacity. The decrease in light intensities increased Φ_{PSII}, *ETR*, and F_v/F_m, which was conducive to enhancing shade-tolerate ability. However, low radiation can improve the heat dissipation capacity of Ma bamboo and weaken the phenomenon of light inhibition. The increase of NSCs was the adaptive strategies of shade-tolerant Ma bamboo to poor light environments. As a result, Ma bamboo may undergo physiological plasticity changes in response to low light. These results have implications for the management of Ma bamboo: (i) moderate shading may provide sufficient light to the growth of young seedlings; (ii) the number or structure of bamboo clumps can be adjusted by artificial pruning, to create a higher understory lighting environment for shoot regeneration and a further increase in productivity.

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

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APPENDIX

Table A1. *Various light response parameters of Ma bamboo under shading treatments*

Treatments	α ($\mu mol \cdot \mu mol^{-1}$)	*LSP* ($\mu mol \cdot m^{-2} \cdot s^{-1}$)	*LCP* ($\mu mol \cdot m^{-2} \cdot s^{-1}$)	R_d ($\mu mol \cdot m^{-2} \cdot s^{-1}$)
L0	0.052	1532.245	23.195	0.999
L1	0.043	2113.669	11.573	0.453
L2	0.061	2647.497	11.129	0.616
L3	0.072	2466.379	10.698	0.713
L4	0.084	1054.247	6.695	0.537

α - initial quantum efficiency, *LSP* - light saturation point, *LCP* - light compensation point, and R_d - dark respiration rate respectively. L0 refers to full light as the control level. L1, L2, L3, and L4 refer to 40%, 30%, 20%, and 10% of natural light, respectively

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SUMMER AND WINTER OF PROKARYOTIC COMMUNITY STRUCTURE AND THEIR RELATIONSHIP WITH ENVIRONMENTAL FACTORS IN XIANGXI RIVER AND SHENNONG CREEK IN CHINA

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Abstract. Few studies have reported about the summer and winter seasonal changes of prokaryotic community structure and their relationship with environmental factors in Xiangxi River and Shennong Creek in China. In this study, the 16S ribosome RNA (rRNA) gene cloning library was used to test the diversity of the aquatic prokaryotes along the Three Gorges Reservoir of the Yangtze River in China over two seasons in 2013. We analyzed the effects of summer and winter seasonal variation of the prokaryotic community composition and environmental factors. Diversity index analysis revealed that Shannon index, Chao1 index, Observed species index, and PD whole tree index of Xiangxi River were the highest in winter, and the four indices of Shennong Creek were the lowest in summer. Spatial analysis reported that the species diversity of Xiangxi River was significantly higher than that of Shennong Creek. Temporal analysis reported that the species diversity of Shennong Creek and Xiangxi River were higher in winter than in summer. The community classification analysis indicated that: at the phylum level, *Proteobacteria*, *Actinobacteria* and *Bacteroidetes* were the dominant bacteria, and their relative abundance accounted for 65.05% of the total community. At the class level, *Acidimicrobiia* had the highest relative abundance, accounting for 13.72% of all sequences, followed by *Betaproteobacteria* (13.12%) and *Alphaproteobacteria* (11.28%). The change of temperature was the main factor causing the summer and winter seasonal differences of prokaryotic biodiversity and community structure.
Keywords: *16S rRNA, Illumina MiSeq sequencing, biodiversity, Three Gorges Reservoir, spatiotemporal variation*

Introduction

Prokaryotes are single-celled organisms that have no real morphological nuclei or mitochondria and play important role in the microbial community, mainly archaea and bacteria (Tanaka, 2009). It has become evident that, although prokaryotes generally lack membrane-bound organelles, they have a sophisticated intracellular organization (Rudner and Losick, 2010; Govindarajan and Amster-Choder, 2016; Surovtsev and Jacobs-Wagner, 2018). The ocean is the birthplace of prokaryotes, and its area covers about 70% of the world (Sun et al., 2010), because of the prokaryotic adaptability to a wide range of habitats and their high variability, which makes them spread all over the world. In aquatic ecosystems, prokaryotes play important roles in some processes, such as the circulation of biomass elements (Sorokin et al., 2014; Li and Qin, 2015), organic decomposition

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(Gomez-Consarnau et al., 2012) and pollutant purification (Celik et al., 2008; Das and Chandran, 2011). In addition, prokaryotic plankton communities have complex effects and rapid response to changes in environmental conditions, hence the changes of their aggregate structure composition can serve as a good indicator of the environmental status of the water bodies (Massana and Logares, 2013). Prokaryotes play an important role in water quality monitoring, especially in lakes and reservoirs (De Wever et al., 2005). At present, great progress has been made in studies on the distribution of prokaryotes community in freshwater bodies (Li et al., 2017). However, there are few studies on the diversity of prokaryotes in reservoir ecosystems.

The Three Gorges Dam on the Yangtze River is the world's largest hydropower station. Reservoirs perform invaluable functions such as fresh water supply, flood control, and hydropower generation (Ouyang et al., 2021). After the first impoundment of the Three Gorges Reservoir in 2003, the hydrodynamic conditions and hydrological environment had changed remarkably (Yang et al., 2015), the eutrophication and algal blooms of some tributaries have attracted extensive attention at home and abroad (Ye and Cai, 2011; Zhang et al., 2016a). Xiangxi River and Shennong Creek are the first and second-largest tributaries of the Three Gorges Reservoir area, the ecological environment was directly related to the water quality of the Three Gorges Reservoir. After the storage of the Three Gorges Reservoir, Dinoflagellate and Diatom blooms have been a frequent occurrence in the Xiangxi River every spring (Fang et al., 2013). At different stages of operation, Shennong Creek has different forms of trunk flow backward, and often accompanied by cyanobacterial blooms (Wang et al., 2011). Large quantities of researches have certified that algal blooms in freshwater have led to serious environmental and societal problems (Rose et al., 2019), with long-term negative effects on water quality. Therefore, it is particularly important to study the structure of prokaryotic plankton communities in the Xiangxi River and Shennong Creek.

With the emergence of new molecular technologies, the field of microbial ecology has been promoted and developed, which makes the study of microorganisms in the freshwater environment more mature (Uyaguari-Diaz et al., 2016). In recent years, high-throughput sequencing technology (Lee et al., 2017) has developed rapidly. Based on the PCR amplification of the 16S rRNA gene of prokaryotes and high-throughput sequencing, their rapid and sensitive advantages have greatly promoted the depth and breadth of environmental microbial diversity research (van Vliet, 2010). In this study, Xiangxi River and Shennong Creek were selected as the research regions. The prokaryotic biodiversity and community composition were studied by 16S rRNA Gene-Illumina Miseq high-throughput sequencing and further biological information analysis. In addition, the correlation of environmental factors is of vital imporance to reveal Xiangxi River and Shennong Creek prokaryotic plankton community structure and temporal and spatial evolution law of summer and winter, it provides a reference and scientific basis for the Three Gorges Reservoir area environmental quality monitoring.

Materials and methods

Sampling and determination of environment variables

We selected representative sampling of Xiangxi River and Shennong Creek, six sampling points were set up, each tributary three sampling points (*Fig. 1*). Water samples were collected from the Three Gorges Reservoir of the Yangtze River of China in August (summer) and December (winter) of 2013 (*Fig. 1*). At each sampling point, 3 bottles of

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surface water (900 mL/bottle) are collected and then mixed. And Water samples were collected at a depth of 0.5 m beneath the water surface using a 5-L plexiglass sampler and were pre-filtered through 5 μm pore-size polycarbonate filters (Millipore) followed by filtration through GF/F filters (0.45 μm, Whatman) for molecular analyses. The samples were immediately frozen in liquid nitrogen and stored at -80°C until analysis.

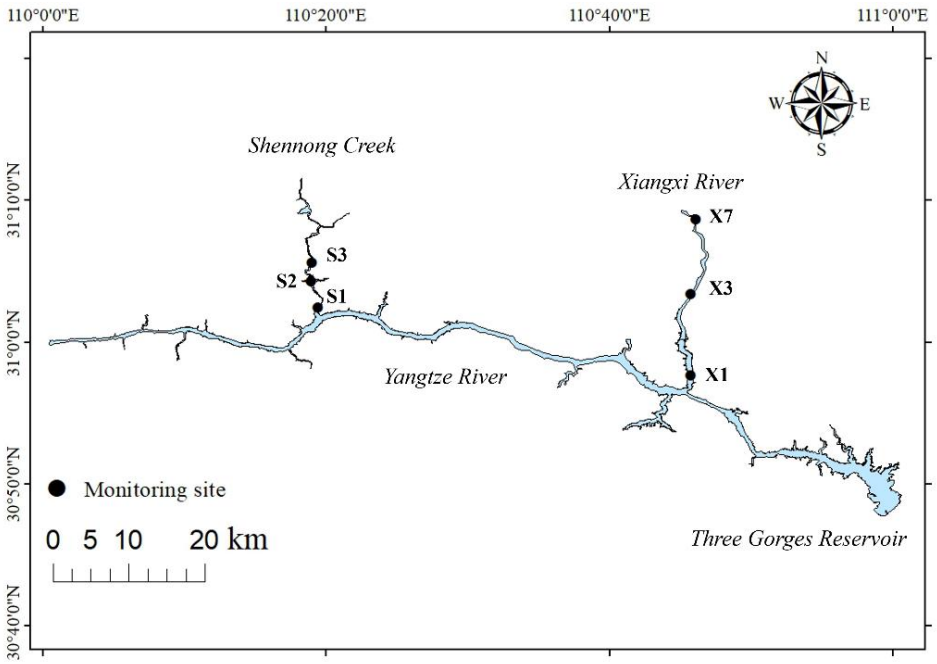

Figure 1. *Locations of the monitoring sites in the Xiangxi River and Shennong Creek in Three Gorges Reservoir*

The main physical and chemical indicators of water environment were tested through field test records and laboratory tests. The physical and chemical indexes of field tests include water temperature (WT), Turbidity (NTU), dissolved oxygen (DO), transparency (SD), pH, conductivity (SPC). The indicators of indoor water chemistry are total nitrogen (TN), total phosphorus (TP), orthophosphate (P-PO_4), nitrate (N-NO_3), ammonium (N-NH_4), chlorophyll a (Chl *a*) and chemical oxygen demand (COD). The physical and chemical data of the field were measured mainly through YSI (EXO3, USA), transparency disk (Secchi, Shanghai ShuoGuang Electronic Science and Technology Ltd. Co), and the determination method of water chemical indexes was mainly measured by the national standard method (Administration, 2002).

Ribonucleic acid (RNA) extraction, polymerase chain reaction (PCR) amplification and sequencing of 16S rDNA

Total community RNA was extracted from each core from each site. The extraction method used was a modification of that described by (Irastortza-Olaziregi and Amster-Choder, 2020; Dell'Orso et al., 2021). Amplification of the V4 region of the 16S rDNA from the samples, the following prokaryotes specific primers were used: 515F (5'-GTGCCAGCMGCCGCGG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT- 3') (Bates et al., 2011; Liao et al., 2015). PCR amplification and purification were based on

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our laboratory's own methods. PCR was carried out under the following conditions: 35 cycles (denaturation at 94 °C for 30 s, annealing at 55 °C for 45 s, extension at 72 °C for 2 min) proceeded by 3 min denaturation at 94 °C and finally, an extension at 72 °C for 8 min. PCR products were quickly checked using 1% agarose gel electrophoresis and purified using the Gel Extraction Kit (Tiangen biotech China).

The purified amplicons were quantified with TBS-380 (Turner BioSystems Inc, USA). Paired-end (PE) sequencing of purified amplicons was carried out on an Illumina MiSeq PE250 platform (Majorbio Bio-Pharm Technology Ltd, Shanghai, China) (Hou et al., 2020) to perform batch analysis.

Statistical analyses

The raw data obtained by Illumina MiSeq sequencing platform were divided, primers were removed, Paired-end (PE) Reads were spliced, Tags' quality and length were filtered and intercepted and chimeras were removed to obtain the final effective tags. Then, Operational Taxonomic Units (OTUs) were formed by clustering all effective tags sequences (the default selected identity is 97%). Based on the homogenization OTU abundances table, use the QIIME software package of alpha_diversity. Py (http://qiime.org/scripts/alpha_diversity.html) script for four kinds of diversity indexes (Shannon, Chao1, Observed species, PD whole tree) calculation (Wang et al., 2012). Using the Bray-Curtis distance matrix as the method of clustering analysis, community structure was compared with Non-Metric Multi-Dimensional Scaling (NMDS). Analysis of similarities (ANOSIM) was based on Bray-Curtis distance measurements, which measured differences between sample groups in permutations. Both NMDS and ANOSIM were carried out using R package "vegan" (Warton et al., 2012). R (https://www.processon.com/diagraming/5fd8629cf346fb07100fe483) version 3.0.1 was used to draw relative abundance heat map of class level and cluster analysis between samples and species was also performed. First, detrended correspondence analysis (DCA) was used to detect the length of the environmental gradient. For the result of detrending correspondence analysis (DCA) was more than or equal to 4, Canonical correspondence analysis (CCA) was adopted (Lepš and Šmilauer, 2003). When conducting CCA analysis, the selected environmental factors were examined by the Monte Carlo permutation test (999 permutations) to ensure that the environmental factors had a good explanation for the microbe. When the results presented significant differences (P<0.05), the relationship of environmental factors and prokaryotes community was analyzed using CCA in CANOCO 5 software (Borcard and Legendre, 2011).

Results

Spatiotemporal variation of environmental parameters

Eleven physical and chemical factors were listed in this study (*Table 1*). These factors didn't change much among all sampling sites, but they varied greatly in different seasons of summer and winter. The variation patterns of each factor between the Xiangxi River and Shennong Creek were basically similar. COD, P-PO_4, DO, pH, turbidity and N-NH_4 were highest in summer, and ORP and N-NO_3 were highest in winter and increased rapidly between summer and winter. However, the turbidity peaked in summer and droped sharply in winter.

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Table 1. *Spatiotemporal variation of environment variables*

	Summer						Winter					
	XX01	**XX03**	**XX07**	**SNX01**	**SNX02**	**SNX03**	**XXO1**	**XX03**	**XX07**	**SNX01**	**SNX02**	**SNX03**
TP (mg/L)	0.08	0.06	0.26	0.04	0.04	0.02	0.12	0.14	0.10	0.10	0.080	0.10
TN (mg/L)	1.60	1.51	1.34	1.18	1.75	1.41	1.49	1.61	1.54	1.52	1.56	1.50
COD (mg/L)	2.59	2.39	3.69	2.40	2.72	2.25	1.34	1.30	1.50	1.34	1.34	1.46
P-PO_4 (mg/L)	0.10	0.06	0.05	0.16	0.16	0.01	0.07	0.09	0.08	0.05	0.04	0.030
N-NH_4 (mg/L)	0.02	0.04	0.07	0.08	0.04	0.05	0.00	0.00	0.04	0.02	0.02	0.02
N-NO_3 (mg/L)	1.00	1.05	0.12	0.67	0.88	0.75	1.38	1.39	1.48	1.33	1.33	1.30
WT (°C)	29.60	29.60	29.50	30.90	30.00	30.90	18.10	18.10	17.70	17.50	18.20	17.60
DO (mg/L)	12.53	11.01	15.55	14.51	10.46	10.06	7.33	6.94	7.65	6.03	6.95	7.13
ORP (mV)	61.20	56.10	48.10	68.90	62.90	64.70	129.20	157.80	133.70	146.60	167.80	152.20
pH	9.31	9.23	9.75	9.49	9.34	9.43	8.56	8.71	8.58	8.63	8.60	8.67
Turbidity (NTU)	8.20	6.10	17.80	7.60	9.10	7.30	2.70	2.60	2.70	1.70	1.40	1.30

Analysis of alpha diversities

For the analyzed 12 samples in this study, a total of 602175 sequences (an average of 256 bp) were obtained after denoising and chimera detection, Then, a cut-off value of 97% sequence identity was performed and a total of 1691 OTUs were generated.

Alpha diversities of prokaryotes were high and varied greatly among the 12 samples, with Shannon index of 6.45 to 7.54, Chao 1 index of 595.60 to 824.80, Observed species index of 476 to 778 and PD whole tree index of 37.54 to 60.84 (*Table 2*). In the two different tributaries of Xiangxi River and Shennong Creek, the species diversity in winter was higher than that in summer. At the different stations of Xiangxi River and Shennong Creek, the differences were not significant in the same season ($P > 0.05$); but at the same site, the differences of most species diversity indices between different seasons were significant ($P < 0.05$). The community assemblages of diversity indices of all samples were congregated comparing α-diversity indices of prokaryotic communities among different groups. In the intergroup distribution map of the α-diversity indices, Shannon index (*Fig. 2A*), Chao1 index (*Fig. 2B*), Observed species index (*Fig. 2C*) and PD whole tree index (*Fig. 2D*) of XXwin were the highest, indicated that the abundance and evenness of species composition in Xiangxi River were highest in winter, and corresponding species indices had the highest group diversity. The four diversity indices of Shennong Creek in summer were the lowest, indicated that Shennong Creek had the lowest species diversity in summer. Differences in most species diversity indices were significant ($P < 0.05$). On the whole, the changes of the four indices were mostly similar. In space, the species diversity of Xiangxi River was significantly higher than that of Shennong Creek. In season, the species diversity of Shennong Creek and Xiangxi River was higher in winter than in summer.

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Table 2. *Indices of the alpha diversity of sampling*

Seasons	Shannon	Chao 1	Observed species	PD whole tree
XX01sum	6.54	752.62	625	46.67
XX03sum	6.63	595.60	560	43.32
XX07sum	6.50	677.87	598	44.63
XX01win	7.45	824.89	742	57.23
XX03win	7.15	763.11	711	54.39
XX07win	6.57	749.56	664	51.87
SNX01sum	6.51	562.80	513	39.23
SNX02sum	6.91	712.44	636	47.23
SNX03sum	6.45	513.14	476	37.54
SNX01win	7.08	819.92	778	60.85
SNX02win	6.69	657.32	645	52.26
SNX03win	7.00	794.42	687	52.46

Note: XX01sum, XX03sum, XX07sum represent Xiangxi Creek 01, 03, 07 sites in summer, respectively; XX01win, XX03win, XX07win represent Xiangxi Creek 01, 03, 07 sites in winner, respectively; SNX01sum, SNX02sum, SNX03sum represent Shennong Creek 01, 02, 03 sites in summer, respectively; SNX01win, SNX02win, SNX03win represent Shennong Creek 01, 02, 03 sites in winner, respectively

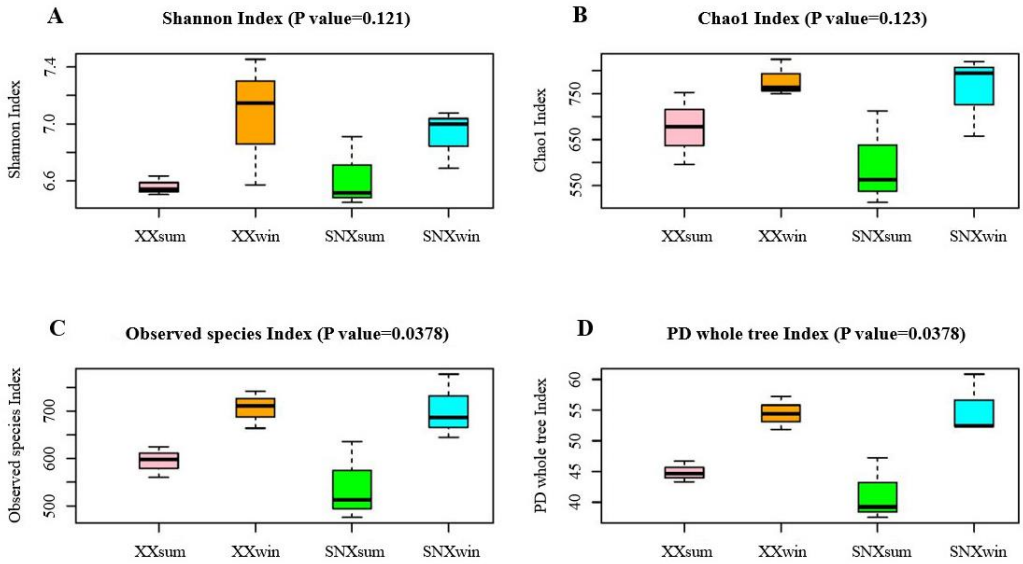

Figure 2. *Alpha diversities of prokaryotes. XXsum represent Xiangxi Creek in summer; XXwin represent Xiangxi Creek in winner; SNXsum represent Shennong Creek in summer; SNXwin, represent Shennong Creek in winter*

Distribution of prokaryotic communities

Based on the phylogenetic analyses, 34 taxa at the level of phylum were identified. These 34 phyla contain 71 classes, 111 orders, 206 families and 311 genera.

At the phylum level, we divided the community into two parts according to the season: summer (*Fig. 3A*) and winner (*Fig. 3B*). The results revealed that the prokaryotic communities of Xiangxi River and Shennong Creek in the Yangtze River basin were composed of 34 species. *Proteobacteria*, *Actinobacteria* and *Bacteroidetes* were dominant species, accounting for more than 65% of total communities. *Planctomycetes*,

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Verrucomicrobia, *Firmicutes*, Cyanobacteria, *Chloroflexi*, *Acidobacteria*, and Saccharibacteri were the subdominant populations of prokaryotes in the two major water areas, while the relative abundance of other phyla were relatively low. *Figure 3* provides an overview of the community composition of prokaryotes in two seasons. The dominant bacteria in summer were *Proteobacteria* (26.63%), *Actinobacteria* (24.23%), *Bacteroidetes* (19.37%) and Cyanobacteria (13.48%). In winter, *Proteobacteria* (39.25%) were dominant, followed by *Actinobacteria* (25.61%) and *Bacteroidetes* (9.30%), *Planctomycetes* (6.21%) and *Acidobacteria* (5.92%). Comparing the dominant phyla of the two seasons, it was found that the composition of prokaryotes in Xiangxi River and Shennongxi Creek in the Yangtze River basin was significantly related to the seasons, among which the number of *Proteobacteria* and *Bacteroidetes* varied greatly from summer to winner.

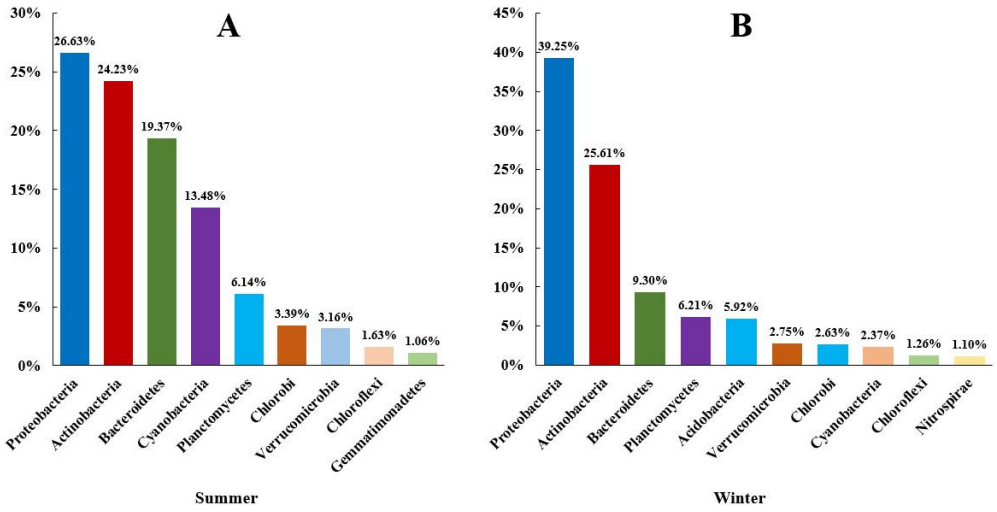

Figure 3. *Prokaryotic communities of Xiangxi River and Shennong Creek were divided into two parts (3A and 3B) according to the season of summer and winter*

Heat map of prokaryotes with a level relative abundance greater than 0.1% was selected. Throughout the whole profile, the prokaryotic communities of Xiangxi River and Shennong Creek presented a unique distribution pattern (*Fig. 4*). The clustering results based on the horizontal similarity of the OTUs community revealed that the prokaryotic communities exhibited obvious seasonal differences and station differences. The relative abundance heat map based on class level revealed that *Acidimicrobiia* had the highest relative abundance, accounting for 13.72% of all sequences, followed by *Betaproteabacteria* and *Alphaproteobacteria*, accounting for 13.12% and 11.28% of the total sequences respectively. *Armatimonadia* had the lowest relative abundance, accounting for only 0.11%. Further analysis founded that *Spartobacteria* and *Chloroflexia* tended to change with the seasons, and their relative abundance was higher in summer than in winter. In contrast, *Holophagae*, *Nitrospira* and *Jg30-KF-CM66* were higher in winter than in summer. Sequences assigned to Cyanobacteria accounted for 2.5% of the total number, which dominated in Xiangxi River in summer. The comparison between Xiangxi River and Shennong Creek revealed that the relative abundance of Shennong Creek was smaller than that of the Xiangxi River. The species composition of Xiangxi River was richer and the community structures were more complex.

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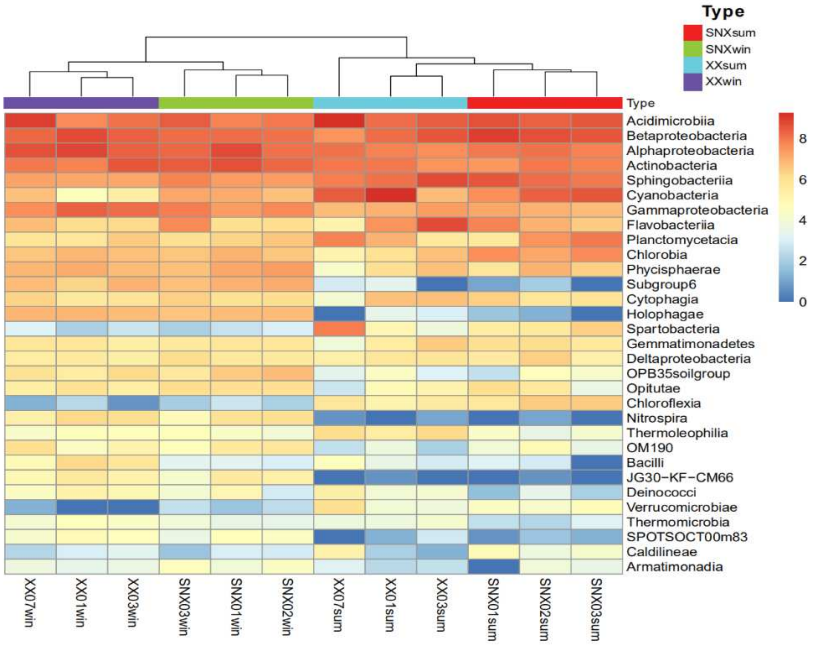

Figure 4. *The community distribution and relative abundance of Prokaryotes. The operational taxonomic units (OTUs) were defined at 97% sequence similarity threshold. Tree chart of 12 samples from two large bodies of water in winter and summer, based on the similarity of Bray-curtis. The color-coding at the end of the branch is based on four groups of six sampling sites. The heat map shows the relative abundance of each sample at the class level. The class with a relative abundance greater than 0.5% is selected and combined with the phylogenetic data to achieve the sample clustering (longitudinal clustering) at the level of the class. The sequence data is standardized by scaling the range of each sample (0-8)*

Variations of community structure and composition

A total of 1961 OTUs were generated and the shared OTUs of the different groups were further identified by the Venn diagram (*Fig. 5*). 345 OTUs were shared among the two seasons of Xiangxi River and Shennong Creek. Among them, Shennong Creek has the highest OTUs value in winter, and has the lowest OTUs value in summer. The shared OTUs of Shennong Creek and Xiangxi River in summer were 621, and 735 in winter. OTUs were higher in winter than in summer.

Based on the calculation of the Bray-Curtis distance matrix, the Non-metric multidimensional scaling (NMDS) analysis method was used to study the differences of prokaryotic community structures in Xiangxi River and Shennong Creek in summer and winter (*Fig. 6*). NMDS analysis revealed that the difference between XX03 summer and SNX01 summer is the largest, while that between SNX03 summer and SNX02 summer was the smallest. Basically, there were significant differences (P<0.05) in the community structures of prokaryotes between Shennong Creek and Xiangxi River in summer and winter. The species similarity of Xiangxi River in winter is similar to that of Shennong River in winter. And Xiangxi River in summer is similar to that of Shennong River in summer. The species similarity in winter is higher than in summer.

Analysis of similarities (ANOSIM) revealed that there were significant differences (P=0.001) between groups (*Fig. 7*). It indicted that differences between groups were greater than differences within groups. Among the groups, the similarities from small to

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large are as follows: XXwin (Xiangxi River in winner) - SNXwin (Shennong River in winter) - SNXsum (Shennong River in summer) - XXsum (Xiangxi River in summer).

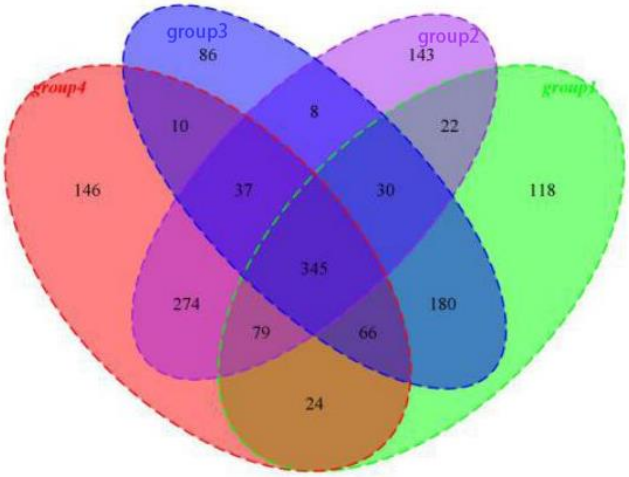

Figure 5. *Unique and shared OTUs of prokaryotes in Xiangxi River and Shennong Creek. Group1 represents XXsum; Group2 represents XXwin; Group3 represents SNXsum; Group4 represent SNXwin*

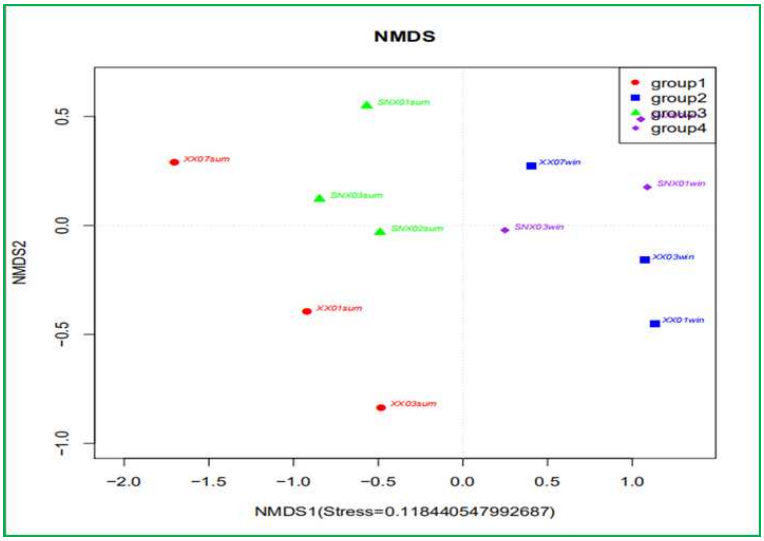

Figure 6. *Non-metric multidimensional scaling (NMDS) ordination of the prokaryotic community based on Bray-Curtis distance. The points in the figure represent the samples, and the distance between the point and the point indicates the degree of difference. When Stress is less than 0.2, it indicates that NMDS analysis has certain reliability*

Influences of species and physicochemical factors

Prokaryotes grow in water and were affected and restricted by a variety of physical and chemical factors in water, such as WT, ORP, N-NH_4, P-PO_4, DO and pH, which may affect the prokaryotic biodiversity. From the 11 environmental physicochemical factors, 4 influential factors (P<0.05) were selected for CCA analysis. Based on CCA (*Fig. 8*), the bacterial composition was significantly correlated with temperature (P=0.06), pH

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(P=0.08), ORP (P=0.018) and N-NH_4 (P=0.05). The relationships between the 8 dominant bacteria groups (relative abundance greater than 2%) and environmental factors were as follows: *Bacteroidetes* were significantly positively correlated with WT; *Chloroflexi* was significantly positively correlated with pH, N-NH_4. Cyanobacteria were positively correlated with pH, N-NH_4, WT, and negatively correlated with ORP; *Firmicutes*, *Actinobacteria*, *Proteobacteria* and Planctomycetes all showed a positive correlation with ORP.

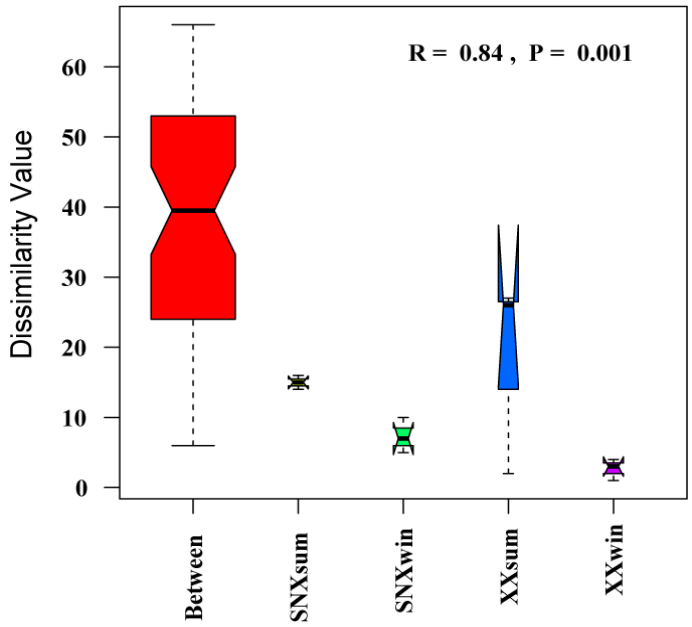

Figure 7. *Analysis of similarities (ANOSIM) of between and within groups in Xiangxi River and Shennong Creek in summer and winter*

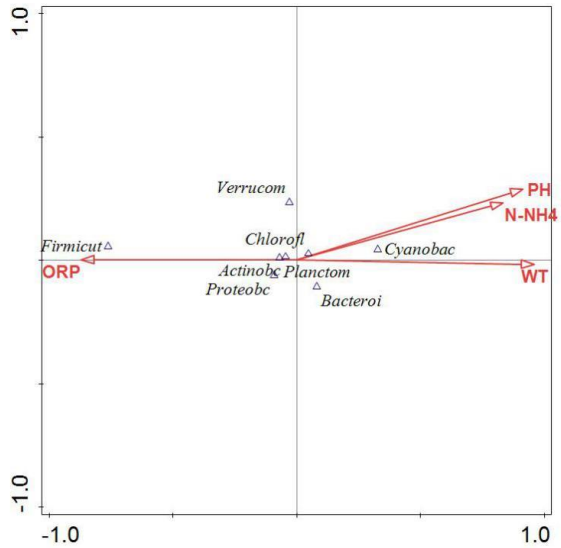

Figure 8. *Canonical correspondence analysis (CCA) of the prokaryotic community-environmental factors relationships. Firmicut:Firmicutes; Verrucom: Verrucomicrobia; Chlorofl: Chloroflexi; Actinobc: Actinobacteria; Planctom: Planctomycetes; Proteobc: Proteobacteria; Bacteroi: Bacteroidetes; Cyanobac: Cyanobacteria*

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Discussion

Seasonal variation of dominant bacteria and corresponding ecology in summer and winter

Prokaryotic diversity and community composition of Xiangxi River and Shennong Creek in summer and winter were analyzed by using the 16S rRNA gene cloning library. Although this method could not reflect all prokaryotic groups in the sample, it can reflect the main dominant bacterial groups in the sample. At the phylum level, *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, Cyanobacteria, Planctomycetes and *Acidobacteria* were common bacteria in summer and winter, it indicates that the two-bay has formed a stable prokaryotic community. The composition is similar to that of other dominant groups of freshwater habitats (Simonato et al., 2010). The study has found that Cyanobacteria, *Actinobacteria*, *Verrucomicrobia* and *Proteobacteria* dominated in two shallow and small natural lakes (Ávila et al., 2016). The previous study has reported on the diversity of bacterial communities in Taihu Lake, the main bacterial communities in this lake included *Actinobacteria*, *Proteobacteria*, *Bacteroidetes*, *Chloroflexi*, Planck oomycetes, *Verrucomicrobia* and *Chlorobi* (Tang et al., 2017). All these studies have confirmed that *Proteobacteria* and *Actinobacteria* were typical freshwater phyla and have relatively high abundance distributions in different freshwater habitats. In this survey, the dominant groups in the Xiangxi River and Shennong Creek were *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, and Cyanobacteria. The distribution of dominant bacteria in two seasons was different, among them, the dominant bacteria were *Bacteroidetes* and Cyanobacteria in summer and *Proteobacteria* in winter. Significant seasonal changes of the dominant bacteria may be attributed to the high summer temperature, which is conducive to the growth of *Bacteroidetes* and Cyanobacteria. In addition, the seasonal variation of bacteria may be related to the content of nutrients in the water, which will lead to the growth of some bacteria being promoted or inhibited (Dai et al., 2016). Our study found that the number of *Proteobacteria* increased gradually as the season transited from summer to winter and the *Bacteroidetes* decreased. Proteobacteria is usually an important indicator of water eutrophication, to some extent, the water quality of Xiangxi River and Shennong Creek has changed. Recently, several studies have been carried out on different marine ecosystems, these studies have reported the main flora of the bacterioplankton community in the South China Sea or its sediments. In the Southern Ocean, Antarctica, the main flora of the Bacterioplankton community were *Alphaproteobacteria*, *Gammaproteobacteria*, *Bacteroidetes*, and *Firmicutes* (Singh et al., 2015). In the northern region of the South China Sea, *Proteobacteria*, *Firmicutes, Planctomycetes*, *Actinobacteria* and *Chloroflexi* were most diverse (Zhu et al., 2013). Thus, it implies that the composition of prokaryotes in marine and freshwater environments is a little similar, but the difference between dominant bacteria in each group is more significant ($P < 0.05$).

Effects of summer and winter seasonal variation on prokaryotic biodiversity and community structure

Alpha diversities are an important indicator of species diversity. The analysis results of 16S rDNA sequencing on prokaryotic communities in Xiangxi River and Shennong Creek showed that the diversity index was different in summer and winter, and the diversity of prokaryotic communities was higher in winter than in summer. Our findings may be related to the optimum growth temperature for most prokaryotes. A study on the seasonal variation of bacterioplankton community structure in Mochou Lake and Zixia

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Lake in Nanjing, and found that the season is an important factor affecting the phytoplankton community structure, and the seasonal changes lead to changes in the water temperature, pH and other environmental factors (Cao et al., 2016). The water temperature of Xiangxi River and Shennong Creek in winter is about 18℃, which is favorable for the growth of prokaryotes. However, the water temperature in summer is 29 - 31°C, which may limit the growth of prokaryotes. Some studies on the gulf and lakes showed that the main driving force of the change of bacterial community structure was the evolution of environmental factors such as temperature caused by seasonal changes (Wu et al., 2007; Bucci et al., 2014). Therefore, the water temperature is an important factor influencing the seasonal difference of prokaryotic biodiversity.

Clustering results at the phyla level indicated that the composition of prokaryotic communities in Xiangxi River and Shennong Creek was related to the summer and winter different seasons ($P < 0.05$), but had little correlation with the sites ($P > 0.05$). This was similar to the research that the time change of the microbial community in Taihu Lake was significantly greater than that in space (Li et al., 2015). Other studies have found that seasonal changes have a more significant impact on the community structure (Tsuchiya et al., 2011; Chen et al., 2013), which may be the common characteristics of eutrophic lakes. These results provide important information for the study of bacterial community composition and spatiotemporal changes in other freshwater lakes. At the same time, the study of seasonal changes of phytoplankton community structure in lakes is of great significance for understanding the relationship between prokaryotic community and environmental factors.

Correlation between the community structure of prokaryotes and environmental factors

Previous studies suggested that the bacterial community was significantly impacted by environmental factors, the contribution of different environment variables to the change of planktonic bacteria community was different (Song et al., 2012; Zhang et al., 2016b). In the present study, the CCA analysis showed that bacterial communities were significantly correlated with temperature, followed by pH, ORP and the concentrations of $N\text{-}NH_4$. These factors were important environmental factors influencing the community composition of prokaryotes in Xiangxi River and Shennong Creek. Some studies have reported that the variation of bacterial community structure was related to water temperature in lakes and streams (Sakami, 2008; Adams et al., 2010). As a result, it was found that the water bodies of the Xiangxi River and Shennong Creek were alkaline in summer and winter. The average pH value of 6 stations was 8.6 in winter and 9.4 in summer, while the proportion of Cyanobacteria amount was 2.3679% and 13.4813%, respectively. The quantity of Cyanobacteria was more abundant in summer. However, other research found that Cyanobacteria was more abundant in the lake bodies with lower alkalinity (Ballot, 2004). The difference may be related to physical environmental factors. Water characteristics, especially water temperature, might play a significant impact on the abundance of Cyanobacteria (Wang et al., 2008). In our study, the water temperature was significantly higher in summer than in winter, which might result in the Cyanobacteria population increasing.

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Conclusions

Prokaryotic communities in Xiangxi River and Shennong Creek were related to the summer and winter different seasons, but had little correlation with the sites. The number of *Proteobacteria* increased gradually as the season transited from summer to winter and the *Bacteroidetes* decreased. Our research found that the composition of prokaryotes in marine and freshwater environments is a little similar, but the difference between dominant bacteria in each group is more significant ($P < 0.05$). This may be related to the optimum growth temperature for the growth of prokaryotes.

In future research, we can combine more molecular biology techniques such as qPCR, single molecule real-time sequencing with HPLC-CHEMTAX technology to study prokaryotic communities in Xiangxi River and Shennong Creek. And future research we can combine four seasons and interannual variability to study succession law of prokaryotic plankton community structure in Xiangxi River and Shennong Creek.

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EVALUATION OF A BOTANICAL INSECTICIDE, LAVENDER (*LAVANDULA ANGUSTIFOLIA* (M.)) ESSENTIAL OIL AS TOXICANT, REPELLENT AND ANTIFEEDANT AGAINST LESSER GRAIN BORER *(RHYZOPERTHA DOMINICA* (F.))

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Abstract. In this study, the chemical composition of *Lavandula angustifolia* (Miller.1768) have been determined by gas chromatography-mass spectrometry. Then, we have evaluated the fumigant toxicity, the repellent and antifeedant properties, and also the effects on some biomarkers of essential oil extracted from *L. angustifolia* on adult of *Rhyzopertha dominica* (F. 1792) (Coleoptera: Bostrichidae). GC-MS analysis showed that this oil contains 56 compounds with linalool (20.42%) and linalyl acetate (13.24%) as the major components. This essential oil was found to exhibit insecticidal activity depending on the concentration and exposure period. In addition, the obtained results revealed an increase in the percent repellency. The enzymatic measurements showed a neurotoxic activity as evidenced by an inhibition of acetylcholinesterase (AChE). In addition, we observe a stimulation of the detoxification system as showed by an increase in glutathione-S-transferase (GST) activity and a decrease in gluthatione (GSH) rate. Lastly, essential oil was investigated on nutritional indices. Results showed a decrease in the relative growth rate, relative consumption rate, efficiency of conversion of ingested food, and an increase in feeding deterrent index, accompanied by a decrease in digestive enzymes tested, α-amylase and protease in treated series when compared with control.
Keywords: *biopesticide, fumigant toxicity, repellency, nutritional indices, biomarkers, digestive enzymes*

Introduction

Stored grains are destroyed by insects, fungi, and vertebrates, but insect pests assume the greatest significance due to the favorable environmental conditions that promote their development (Aref and Valizadegan, 2015). According to the Food and Agriculture Organization (FAO), about 10-25% of the world's harvested food is destroyed by rodents and insect pests (Goergen et al., 2005). They induce qualitative and quantitative losses, such as grain weight loss, decrease in nutritional value and germination capacity of seeds, and product devaluation (Scheepens et al., 2011).

Producers rely on chemical insecticides to avoid losses (Ebadollahi and Sendi, 2015). Intensive use of synthetic insecticides, including phosphine, and sulfuryl fluoride, induce negative effects, such as residue threats, toxicity to non-target organisms, and outbreaks of secondary pests (Ngassoum et al., 2007). In Algeria, aluminium phosphide (Phostoxin^R) is commonly used to control infestations (Soltani-Mazouni et al., 2012). However, these insecticides possess strong secondary effects on the environment. In this context, there is an urgent need to develop eco-friendly materials and methods that only have slightly adverse effects on the consumers and the environment at large (Ebadollahi and Sendi, 2015).

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Botanical insecticides are often effective alternatives to organophosphates or other neurotoxins for pest control (Gökçe et al., 2010). Plant materials, especially essential oils, which affects both behavioral and physiological aspects, have received elicited a great deal of attention as pest control substances (Isman et al., 2011). Many plant extracts can be used for stored product pest control. They act as bioactive chemicals, are selective, and have little or no harmful effects on the environment and non-target organisms (Regnault-Roger et al., 2012). They have been successfully exploited as insecticides (Tang et al., 2007), insect repellents (Islam et al., 2009) or insect anti-feedants (Gonzalez-Coloma et al., 2006) and may affect some biological parameters such as growth rate (Nathan et al., 2008), life span and reproduction (Isikber et al., 2006).

Fumigants from plant origins are said to have a greater potential in the future based on their efficacy, economic value, and use in large-scale storage (Lee et al., 2004). Among botanical insecticides, essential oils are natural products and can negatively affect the food consumption of insects; they are known as feeding deterrents or antifeedants (Wawrzyniak, 1996). *Lavandula angustifolia* Miller or true lavender is a perennial shrub of the family of Lamiaceae (Lis-Balchin, 2002), recognized for their antimicrobial, antioxidant, antifungal, and insecticidal activities (Yazdani et al., 2013; Costa et al., 2014; Duda et al., 2015; Badreddine et al., 2015). The oil profile of different sources of lavender might be different, so it is necessary to analyze the chemical composition of this oil by GC-MS.

Rhyzopertha dominica (F.), lesser grain borer, is regarded as a major stored product pest (Phillips et al., 2010), due to its high potential and wide host range of products. Both larvae and adults consume the germ and endosperm of wheat and rice during their development in grain, causing extensive damage (Dowdy and Mc Gaughey, 1992). A recent literature review was carried out to provide information on several subjects like biology, ecology, and control of *R. dominica* (Edde, 2012). The adults include sturdy fliers, which fly from warehouse to warehouse, causing severe infestation and convert the stored grains to mere frass (Negbenebor and Nura, 2020).

Chintala and Virani (2018) found that the total developmental period on the wheat variety under laboratory conditions at an average temperature of 30 ± 10 °C, and 70 ± 5% relative humidity was varied, with an average of 52.20 ± 5.66 days for males, whereas it was 95.85 ± 9.19 days for females.

In the present study, we determined the chemical composition of *L. angustifolia* EO and found that this oil is toxic to adults of *R. dominica*. In addition, we investigated the effects of this plant on *R. dominica* adults such as fumigant toxicity, repellent activity, feeding by measurement of some indices (relative growth rate, relative consumption rate, the efficacy of conversion of ingested food, and feeding deterrence index), and digestive enzymes. Finally, biomarkers of neurotoxicity and detoxification were also examined to give additional information on its mode of action. Our results indicate that the EO tested has the potential for controlling lesser grain borer.

Materials and methods

Insect rearing

The insect species used in this study i.e. lesser grain beetle borer, *R. dominica* was procured from a farmer (Youkous-Hammamet –Tebessa-Algeria; Latitude: 36° 23′59″ North, Longitude: 10° 37′00″ East, Altitude above sea level: 13 m). In cube containers, 1 kg wheat was used for insect rearing. For ventilation purposes, the containers (volume

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2 L) were covered by mesh cloth. The incubators (SNIJDERS SCIENTIFIC-France) with 27 ± 1 °C and relative humidity of 65 ± 5% were used for insect rearing and experiments, as described by Aref and Valizadegan (2015). Experiments were carried out between May and July 2016, and in all experiments 7- to 14- old adult insects were used.

Plant material and essential oil extraction

L. angustifolia (Miller.1768) were harvested during May-July 2016 from the Tebessa area (Northeast Algeria) and identified with the help of plant taxonomist Dr. Hioune Soraya (Department of Biology, Larbi Tebessi University, Tébessa). Then, the plant parts were washed with tap water, to eliminate soil and other surface contaminants. After the dryness, at laboratory temperature and obscurity, the plant material was cut into small pieces. Subsequently, 50 g of the air-dried aerial parts of the species were subjected to hydro distillation for 3 hours to 500 ml distilled water using a Clevenger-type apparatus according to the method recommended in the British Pharmacopoeia (1988). The oil obtained was collected and dried over anhydrous sodium sulfate and then stored in screw-capped glass vials in a refrigerator at 4 °C before the analysis. Thereafter, the yield was calculated based on the dried weight of the samples (Costa et al., 2014).

Gas chromatography-mass spectrometry analysis

The essential oil of *L. angutifolia* was subjected to GC and GC-MS analysis. Gas chromatography-Mass spectrometry (GC-MS) analysis was performed with an HP Agilent 6890 plus gas chromatograph (GC) equipped with an HP-5MS column (a length of 30 m × internal diameter of 0.25 mm, and 0.25 mm film thickness). The column oven temperature was set at 60 °C for 8 min and then increased to 250 °C at the rate of 2 °C/min. The injector and detector temperatures were kept at 250 and 270 °C, respectively. The carrier gas was helium, the flow through the column was 0.5 ml/min, and the split ratio was set to 50:1 with an injection of 0.2 µl of oil sample. The GC/MS analysis was performed with a Quadruple mass spectrometer that operated at 70eV. Constituent's identification was based on a comparison of retention times with those of corresponding reference standards using the NIST and WILEY libraries (Adams, 2001). Percentage compositions of essential oils were calculated in accordance with the area of the chromatographic peaks.

Fumigant toxicity assay

The fumigant toxicity of essential oil on *R. dominica* was tested in 60 ml glass vials. In each of them, 20 adults (both sexes, male or female, 7-14 days old) were released. No.2 Whatman filter paper disks were cut to 2.5 cm in diameter and attached to the undersurface of glass vial screw caps. Filter papers were impregnated with series of pure concentrations of essential oil: 25, 50, 100, 150, and 200 µl/l air. The choice of applied concentrations was established after a preliminary screening using a range of concentrations. Control insects were kept under the same conditions without essential oil. Each dose was replicated six times. After 24, 48, and 72 hours, from the beginning of exposure, numbers of dead and alive insects were counted. In these experiments, those insects incapable of moving their heads, antennae, and body were considered dead. This was followed by a correction in the mortality percentage. Sublethal and lethal concentrations (LC_{10}, LC_{25}, and LC_{50}) and 95% confidence limits (95% FL) were determined.

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Repellent bioassay

The repellent effect of the essential oil against adults of *R. dominica* was evaluated using the method of the preferred area on filter papers as described by Mc Donald et al. (1970). Thus, the filter paper discs of 9 cm in diameter used for this purpose have been cut into two equal parts. Four concentrations were prepared (1, 2, 4, and 8 µl/ml) and diluted with acetone. Then, 0.5 ml of each solution prepared was spread evenly over one half of the disc. After 15 min, the time required for completing evaporation of the solvent dilution, the two halves of the discs were glued together using adhesive tape. The filter paper disc was restored and placed in a box before kneading a batch of 10 adult insects was placed in the center of each disk. Each treatment was replicated six times and the percentages of insects present on treated **(G)** and control **(P)** areas were recorded after 30 min, 3 h, 6 h, and 24 h. The percentage of repulsion (RP) was calculated using the following formula (Mc Donald et al., 1970):

$$PR = [(P-G) / (P+G)] \times 100 \quad \text{(Eq.1)}$$

The average values were then categorized in accordance with the following scale: [Class 0 (RP < 0.1%), class I (RP = 0.1%–20.0%), class II (RP = 20.1%–40.0%), class III (RP = 40.1%–60.0%), class IV (RP = 60.1%–80.0%) and class V (RP = 80.1%–100%)].

Determination of nutritional indices

To examine the impact of EO on feeding efficiency, some nutritional indices were determined. The assay was conducted as previously described by Bahrami et al. (2016). The experiment was carried out in Petri dishes (diameter 9 cm). The adults of *R. dominica* were starved for 3 h before the experiment to exude gut contents. The solutions were prepared from the EO by dilution in ethanol to produce two concentrations (1 and 4%, W/W) applied with a micropipette in 5 g of wheat, as elucidated by Gökçe et al. (2010). After evaporation for 15 min at room temperature, 10 individuals were introduced into each box. Wheat, to which only the solvent had been applied, was used as the control. The adults were incubated for 72 h at 25 ± 2 ºC and on a 16:8 (L: D). The weight of the adult and wheat before and after each experiment was also determined. Weight loss of diet caused by water evaporation was quantified by establishing two positive control treatments of 5 g of diet treated with a plant extract or solvent. The nutritional indices were calculated for adults according to the formula specified by Huang et al. (2000):

$$\text{Relative Growth Rate (RGR)} = (A - B)/(B \times \text{day}) \quad \text{(Eq.2)}$$

where,
A: weight of live insect after the experiment (mg to each insect)
B: weight of insect before the experiment (mg to each insect)

$$\text{Relative Consumption Rate (RCR)} = D/(B \times \text{day}) \quad \text{(Eq.3)}$$

where,
D: dried weight of food consumed by insects (mg)

$$\text{Efficacy of Conversion of Ingested Food (ECI)} = RGR/RCR \times 100\% \quad \text{(Eq.4)}$$

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$$\text{Feeding Deterrence Index (FDI)} = [(C - T)/C] \times 100\% \quad \text{(Eq.5)}$$

where,
C: food consumed in control (mg)
T: food consumed in treatment (mg).

Digestive enzyme assay

Enzyme extracts adults were sampled from controls and treated series (LC_{25} and LC_{50}) by fumigation. The whole body was homogenized in 1 ml of universal buffer (pH 7) and centrifuged (13,000 g for 15 min) as previously described by Valizadeh et al. (2013). The supernatant was used as the enzyme source. Of the 10 insects, three replicates each were used for each dose. The protein concentrations in each sample were determined in parallel according to Bradford (1976) and used to calculate the specific activity. The α-amylase activity was measured in line with what was described (Kilani-Morakchi et al., 2017) using dinitrosalicylic acid (DNS) as the reagent and 1% soluble starch as substrate. 20 µl of the enzyme were incubated for 30 min at 35 °C with 100 µl universal buffer (pH 7) and 40 µl soluble starch. To stop the reaction, 100 µl dinitrosalicylic acid (DNS) was added and heated in boiling water for 10 min. Thereafter, absorbance was read at 540 nm after cooling. One unit of α-amylase activity was defined as the amount of enzyme required to produce 1 mg maltose in 30 min at 35 °C.

Protease activity was assayed using casein (1%) as substrate following the procedure of Garcia-Carreno and Haard (1993). Briefly, 200 µl of 1% casein solution was added to 100 µl enzyme and 100 µl universal buffer (pH 7), and the mixture was incubated at 37 °C for 60 min. Proteolysis was stopped by the addition of 800 µl of 5% trichloroacetic acid (TCA). The mixture was centrifuged at 8000 g for 15 min and the absorbance was red at 280 nm. The activity was calculated from a curve using tyrosine (Sigma, Italy) as a standard. Six replicates were used for each concentration. Data were expressed in µmol/min/mg protein.

Biomarker assay

The lethal concentrations (LC_{25} and LC_{50}) were applied on *R. dominica* adult. Their effects were examined on AChE and GST activities at various times (24, 48, and 72 hours) following treatment. The AChE activity was determined using acetylthiocholine as a substrate according to the method of Ellman et al. (1961) as previously described (Dris et al., 2017). Succinctly put, adult heads were homogenized in the detergent solution D [38.03 mg EGTA (ethylene glycol tetra-acetic acid, 1 ml Triton X-100, 5.845 g NaCl, and 80 ml Tris bufer (10 mM, pH 7)]. The AChE activity was determined from the absorbance changes at 412 nm for 20 min. The activity was expressed as nM/ min/mg proteins.

The assay of GST was carried out according to Habig et al. (1974) with the use of GSH (5 mM). The adult decapitated body was homogenized in 1ml phosphate buffer (0.1 M, pH 6). The homogenate was centrifuged (14000 rpm for 30 min). 200 µl of the resulting supernatant was added to 1.2 ml of the mixture GSH-CDNB in phosphate buffer (0.1, pH 7). Changes in absorbance were measured at 340 nm every minute for 5 min. The activity was expressed as nM/min/ mg proteins.

The rate of GSH was then determined following the method of Weckberker and Cory (1988). Adult bodies were homogenized in 1 ml of EDTA (0.02 M, pH 6). The homogenate was subjected to a deproteinisation with sulfosalysilic acid (SSA, 0.25%)

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(W/V). The optical density was measured at 412 nm. The amount was expressed as nM/mg proteins.

Statistical analysis

The number of individuals tested in each series is given with the results. Data are presented as the mean ± standard errors (SE). Data of corrected mortality and the significance between different series were subjected to one-way analysis of variance (ANOVA) followed by a post-hoc HSD Turkey test. All statistical analyses were performed using Prism 7 (GraphPad Software Inc., www.graphpad with a significant level $p < 0.05$).

Results

Extraction yield and chemical analysis

The results of the steam distillation show that the yield of extraction of *L. angustifolia* essential oil was 3.2 ± 0.15% (dry matter of the plant).

Gas chromatography-mass spectrometer analysis of *L. angustifolia* essential oil led to the identification of 56 components. The percentages and the retention times of the identified compounds of the essential oil of *L. angustifolia* are listed in *Table 1*. The oil profile is characterized by linalool (20.48%), camphor (13.15%), linalyl acetate (13.24%), 1,8 Cineole (12.96%), Borcenol (10%), α- Cadinol (4.25%) and α- Terpineol (4.07%) (*Fig. 1*).

Fumigant toxicity assay

Figure 2 shows the percent mortality of *R. dominica* after exposure to different concentrations of the tested essential oils. The highest percentage of mortality was seen at 200 µl/liter air concentrations of *L. angustifolia*. Since 100% mortality was achieved at 72 h after exposure at the highest concentration (200 µl L^{-1} air) of the tested oils, we calculated LC_{10}, LC_{25}, and LC_{50} values of the essential oil along with their fudicial limits (*Table 2*).

Repellency bioassay

In this study, this test was applied to *R. dominica* adult. The percent repellency of *R. dominica* adult against 1, 2, 4, and 8 µl ml^{-1} concentrations of *L. angustifolia* essential oil at different periods after treatment are presented in *Table 3*. The obtained results showed an increased repellency percentage depending on the exposure period and concentration. The maximum repellency rate of 86.96% wax recorded with a dose of 8 µl ml^{-1} at 24 hours.

Effects on biomarkers

To obtain information on the mode of action of *L. angustifolia* EO, activities of AChE and GST and GSH amounts were determined following the treatment of *R. dominica* adult. The results are shown in *Table 4*. A significant decrease ($p < 0.001$) of AChE activities was observed when essential oil was used at their LC_{25} and LC_{50} at 24, 48, and 72 h.

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Table 1. *Chemical composition of L. angustifolia essential oil: retention time (RT) and concentration (%) of different constituents*

N°	RT	Compound	Area
1	7.921	α- Pinene	0.51
2	8.693	Camphene	0.62
3	10.264	β-Pinene	0.62
4	11.030	3-Octanone	0.26
5	11.276	ß-Myrcene	0.73
6	12.293	Δ. 3-Carene	0.11
7	12.813	Acetic acide, hexyl ester	0.35
8	14.004	**1,8 Cineole (Eucalyptol)**	**12.96**
9	14.418	Cis-Ocimene	0.44
10	15.073	ß-Ocimene	0.51
11	15.652	γ-Terpinene	0.14
12	16.707	Linalool oxide Cis	**0.75**
13	17.657	α- Terpinolene	0.28
14	17.825	Furfuryl Alcohol	0.48
15	**19.638**	**Linalool**	**20.48**
16	19.975	Octen-1-ol, acetate	0.46
17	20.597	Trimethyl cyclo pentadiene	0.09
18	22.124	**Camphor**	**13.15**
19	22.322	Propanoic acid	0.29
20	22.635	Neroloxide	0.21
21	22.987	Pinocarvone	0.08
22	**23.936**	**Borcenol**	**10**
23	24.380	Terpinene-4-ol	1.00
24	24.804	Cryptone	0.22
25	**25.590**	**α- Terpineol**	**4.07**
26	25.773	2- Pinen-10-ol	0.10
27	26.322	Berbenone	0.24
28	26.968	2,6- Dimethyl-3,5,7-Octatriene-2-ol,E	0.20
29	27.460	Borneol	0.36
30	28.168	Cis-Geraniol	0.84
31	28.399	Butyl 2-Methyl butanonoate	0.32
32	28.746	Carvone	0.36
33	**30.115**	**Linalyl Acetate**	**13.24**
34	30.804	ß- Citral	0.16
35	31.537	1 α- Bornyl acetate	0.31
36	32.144	Lavandulyl acetate	0.77
37	33.797	Cuminol	0.11
38	33.797	Thymol	0.11
39	34.679	Hexyl-Tiglate	0.31
40	36.959	Neryl acetate	1.39
41	38.246	Geranyl acetate	1.65
42	39.918	Caryophyllene	0.62
43	41.041	α- Bergamotene	0.08
44	42.516	Trans-ß Farnesene	0.11
45	44.636	Bicyclogermacrene	0.08
46	45.561	ß-Bisabolene	0.09
47	45.764	Naphtalene	0.36
48	46.241	L-Calamenene	0.12
49	47.913	Caryophylene oxide	0.12
50	49.798	1-Methylene-2-vinylcyclopentane	1.66
51	51.663	Carotol	0.31
52	**53.451**	**α- Cadinol**	**4.25**
53	53.957	Bisabolol oxide	0.38
54	55.012	Azunol	0.31
55	55.798	α- Bisabolol	2.14
56	58.675	Naphthalenone	0.15

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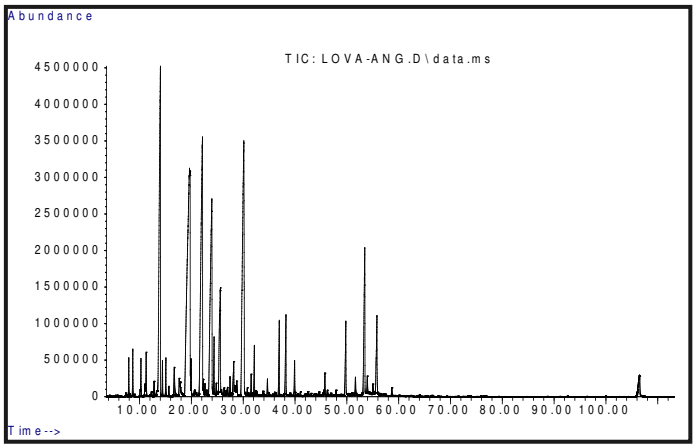

Figure 1. *GC-MS chromatogram for essential oil obtained from L. angustifolia*

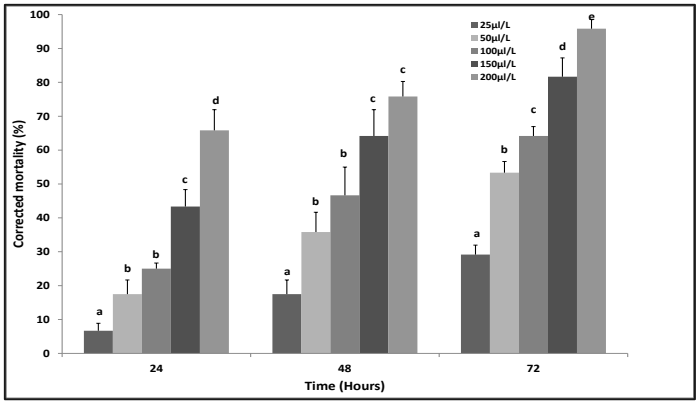

Figure 2. *Efficacy of essential oil of L. angustifolia applied on adult of R. dominica: corrected mortality (mean ± SE, n = 6 replicates each containing 20 adults)*

Table 2. *Lethal concentrations (µl/liter air, FL= 95%) of L. angustifolia essential oil against adult of R. dominica*

Time (Hours)	Hill Slope	R^2	Concentrations (µl/liter air)		
			LC_{10} (95% FL)	LC_{25} (95% FL)	LC_{50} (95% FL)
24	**1.77**	**0.94**	**46.23** (20.23-105.68)	**85.90** (55.46-133.04)	**159.22** (118.30-214.28)
48	**1.20**	**0.97**	**14.75** (6.71-32.35)	**36.72** (23.28-57.94)	**91.62** (71.12-118.03)
72	**1.36**	**0.94**	**9.79** (3.07-31.18)	**21.92** (10.54-45.49)	**48.97** (32.21-74.47)

Concerning GST activities, the treatment caused a significant induction at 24 h ($p < 0.01$; $p < 0.01$) and at 48 h ($p < 0.05$; $p < 0.01$) with LC_{25} and LC_{50}, respectively, as well as at 72 h only with the highest concentration ($p < 0.001$). The amounts of GSH showed a significant decrease in the treated series at 24 h ($p < 0.01$) with LC_{50} at 48 h ($p < 0.05$; $p < 0.01$) and at 72 h ($p < 0.01$; $p < 0.001$) with the two tested concentrations LC_{25} and LC_{50}, respectively.

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Table 3. *Repellent activity (%) of essential oil of L. angustifolia against R. dominica adults at different exposure times*

Concentrations	Time after treatment	RP (%)	Class
1 µl/ml	**30min**	26.66	**II**
	3hours	40.00	**II**
	6hours	43.33	**III**
	24hours	50.00	**III**
2 µl/ml	**30min**	32.66	**II**
	3hours	50.00	**III**
	6hours	53.33	**III**
	24hours	60.00	**III**
4 µl/ml	**30min**	50.00	**III**
	3hours	60.00	**III**
	6hours	63.33	**IV**
	24hours	73.33	**IV**
8µl/ml	**30min**	65.00	**IV**
	3hours	73.33	**IV**
	6hours	83.33	**V**
	24hours	86.96	**V**

Table 4. *Effect of L. angustifolia essential oil applied at two concentrations (LC_{25} and LC_{50}) on the AChE, and GST activity (nM/min/mg of proteins) and GSH rate (nM/mg of proteins) in R. dominica adults (mean ± SE, n= 3 pools each containing 20 individuals)*

Time (hours)		Control	LC_{25}	LC_{50}
24	**AChE**	23.77 ± 0.50 **a**	13.03 ± 0.09 **b**	10.31 ± 0.48 **c**
	GST	27.64 ± 0.98 **a**	42.78 ± 0.32 **b**	43.25 ± 4.68 **b**
	GSH	14.78 ± 0.43 **a**	12.84 ± 1.10 **a**	9.63 ± 0.76 **b**
48	**AChE**	22.84 ± 0.45 **a**	14.36 ± 0.13 **b**	10.24 ± 0.75 **b**
	GST	28.99 ± 1.24 **a**	38.24 ± 0.35 **b**	42.15 ± 3.60 **b**
	GSH	17.94 ± 0.74 **a**	13.28 ± 1.30 **b**	13.03 ± 0.83 **b**
72	**AChE**	22.96 ± 0.29 **a**	10.24 ± 0.75 **b**	8.84 ± 0.54 **b**
	GST	27.73 ± 0.32 **a**	35.00 ± 2.48 **a**	46.36 ± 2.99 **b**
	GSH	18.51 ± 0.31 **a**	14.83 ± 0.76 **b**	11.65 ± 0.38 **c**

For the same biomarker, the different letters indicate significant differences based on Tukey's HSD test ($p < 0.05$)

Effects on nutritional indices

Nutritional analyses revealed that the EO influenced all nutritional indices used when ingested by *R. dominica* adults (*Table 5*). When fed with a diet containing 1 and 4% (w/v) of EO, an increase in FDI ($p < 0.01$) of adults was observed for the two tested concentrations. However, a decrease in ECI ($p < 0.05$) was recorded for the higher concentration (4%). In addition, the RGR index was inversely proportional to the EO concentrations and it was significantly reduced ($p<0.001$) with increased concentrations of EO (*Table 5*). This has shown that the highest concentration forces *R. dominica* to use less food and to have reduced growth. It was found that the essential oil of *L. angustifolia* has no effect ($p > 0.05$) on the RCR index.

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Table 5. *Effects of essential oil (% (w/v) of L. angustifolia on nutritional indices of R. dominica adults (mean ± SE, n= 3 pools each containing 10 individuals)*

Concentrations	RGR	RCR	ECI	FDI
	(mg/mg/h)		(%)	
0 %	0.17 ± 0.01^{a}	2.23 ± 0.46^{a}	8.39 ± 2.09^{a}	-
1%	0.07 ± 0.01^{b}	1.73 ± 031^{a}	4.51 ± 1.41^{a}	34.11 ± 10.08^{b}
4%	0.02 ± 0.00^{c}	1.60 ± 0.05^{a}	1.55 ± 0.14^{b}	54.39 ± 5.80^{a}
P	0.0002	0.4120	0.0438	0.0035
F	45.79	1.032	5.513	16.76
df	2	2	2	1

RGR: relative growth rate; **RCR:** relative consumption rate; **ECI:** efficiency of conversion of ingested food; **FDI:** Feeding Deterrence Index; Different letters in the same column indicate significant differences ($P \leq 0.05$) between treatments according to ANOVA and Tukey's Multiple Range Test

Effects on digestive enzyme activities

α-amylase is an enzyme hydrolyzing starch to maltose and glycogen to glucose. Adults EO-treated with LC_{50} showed a significantly lower α-amylase activity in comparison to controls ($F_{2,6}$ = 11.88; $p < 0.01$) (*Fig. 3*). The mean values recorded were 5.63 ± 0.68 µmol/min/mg proteins for controls, 4.56 ± 0.39 µmol/min/mg proteins for the LC_{25}, and 3.17 ± 0.16 µmol/min/mg proteins for the LC_{50}. Statistical analysis revealed a significant difference between the control series and the highest tested concentration series ($p < 0.01$).

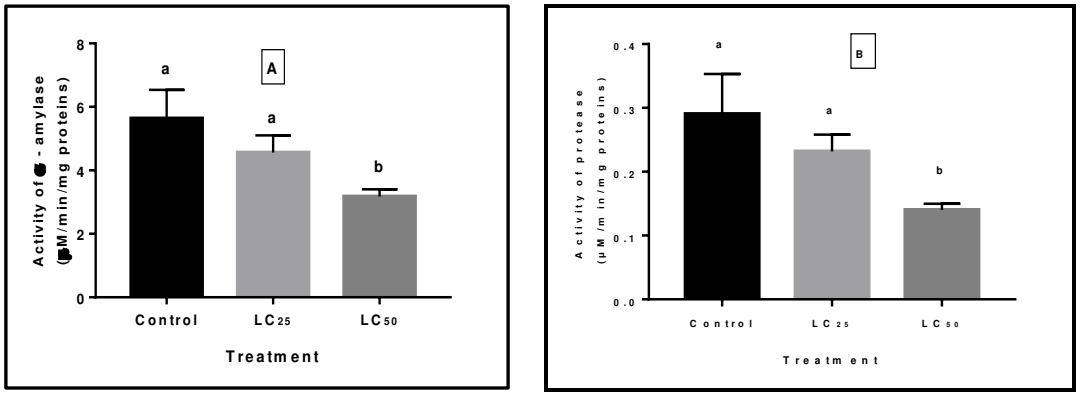

Figure 3. *Effect of L. angustifolia EO, applied on adults of R. dominica on activity of α-amylase* ***(A)*** *and protease* ***(B)****, (m ± SE; n= 3 replicates each of 10 insects). Different small letters indicate a significant difference between control and treated series (p<0.01)*

General protease activity was lower in EO-treated adults with LC_{50} as compared to the controls ($F_{2,6}$ = 10.96; $p < 0.01$) (*Fig. 3*). In control series, the mean values recorded were 0.29 ± 0.004 µmol/min/mg. In treated series, the mean values recorded were 0.23 ± 0.01 µmol/min/mg proteins for the lowest concentration (LC_{25}) and 0.14 ± 0.00 µmol/min/mg proteins for the highest concentration (LC_{50}). According to statistical analysis, there was a significant difference between the control series and the highest tested concentration series ($p < 0.01$).

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Discussion

Essential oil yield and composition

The qualitative and quantitative composition of lavender essential oil depends on genotype, growing location, climatic conditions, and morphological features (Prusinowska and Śmigielski, 2014). The quality of this oil depends on the high content of these major compounds and their mutual proportions (higher than 1) (Prusinowska and Śmigielski, 2014). Lavender oil primarily consists of linalyl acetate, linalool, lavandulol, 1,8-cineole, lavandulyl acetate, and camphor (Prashar et al., 2004). However, the relative level of each of these constituents varies in different species (Cavanagh and Wilkinson, 2002; Woronuk et al., 2011). The various lavenders have similar ethnobotanical properties and major chemical constituents (Cavanagh and Wilkinson, 2002).

In this study, the major components of *Lavandula angustifolia* (Lamiaceae) oil were linalool (20.48%) and linalyl acetate (13.24%) with a total yield of 3.2% relative to the dry matter. The EO of *L. angustifolia* from Iran contains 1,8-cineole (65.40%), borneol (11.50%), and camphor (9.50%) as the most abundant compounds (Hajhashemi et al., 2003). Whereas, 1,8-cineole is the primary compound (38.40%), followed by cis-verbenol (4.30%) and cymene-8-ol (3.80%) in the EO of *L. angustifolia* from the Cherchell region (North Algeria) (Dob et al., 2005). These variations could either be attributed to differences in elevation, the genetic makeup of the plant, or due to an adaptive process to particular ecological conditions (Verma et al., 2010). Moreover, compositional variations can be observed in oils from different organs of the same species (Salleem et al., 2012). Distillation may also influence the composition of the oil, because isomerization, saponification, and other reaction may occur under distillation conditions (Zheljazkov et al., 2013). The results obtained by Zheljazkov et al. (2013) revealed the influence of the duration of distillation on the lavender oil yield and its composition. The maximum efficiency of the distillation process (2%) is achieved after 2 h and the minimal oil yield (1%) is obtained after 40 min of distillation (Wesołowska et al., 2010).

Insecticidal activity

EOs are lipophilic in nature and interfere with basic metabolic, biochemical, physiological, and behavioral functions of insects (Brattsten, 1983). These physical properties, such as high boiling point, high molecular weight, and low vapour pressure, are barriers for application in large-scale fumigation (Daglish, 2006).

In Africa, EOs have traditionally been used by small farmers to protect stored grains from insect pests. Inspired by the traditional practices in Guinea, extracts of four West African plant species, *Tagetes minuta* (Asteraceae), *Hyptis suaveolens* (Lamiaceae), *Ocimum canum* (Lamiaceae), and *Ocimum basilicum* (Lamiaceae), were assayed against adults of the bruchid *Callosobruchus maculatus* (Chrysomelidae) as protectants for stored cowpeas (Keita et al., 2000). In Algeria, powders from dry leaves of four plant species: *Ficus carica* (Moraceae), *Eucalyptus globulus* (Myrtaceae), *Olea europaea* (Oleacea), and *Citrus limon* (Rutaceae), were evaluated under controlled conditions against *C. maculatus* (Kellouche and Soltani, 2004). Meanwhile, in developing countries, aromatic plants were widely used for stored-product insects in traditional agricultural systems. Currently, there is a move to replace these plants with steam-distilled EOs (Regnault-Roger et al., 2012). Botanical insecticides affect various insects in different ways depending on the physiological characteristics of the insect species and the type of the insecticidal plant (Hikal et al., 2017). The components of various botanical

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insecticidal can be classified into six groups: repellents (Isman, 2006), feeding deterrents/antifeedants, toxicants (Tripathi et al., 2001), growth retardants (Papchristos and Stamopoulos, 2002), and attractants on stored product insects (Rajashekar et al., 2012).

In our study, the essential oil of *L. angustifolia* applied on *Rhyzopertha dominica* by fumigation was evaluated. Mortality was found to increase with the applied concentration and the exposure time. Fumigation studies showed that the essential oil had a "knockdown effect" on the test insect.

Essential oils usually extracted from various parts of the plant are traditionally used through fumigant or contact action to protect grains against storage pests, in some Asian and African countries (Shaaya et al., 1991). The insecticidal activity of some essential oils from Lamiaceae and other plants has been evaluated against several stored product insects (Negahban et al., 2007; Ayvaz et al., 2009). Rozman et al. (2007) revealed significant toxicity of *L. angustifolia*, *Rosmarinus officinalis* (Lamiaceae), and *Thymus vulgaris* (Lamiaceae) against *T. castaneum* (Tenebrionidae), *S. oryzae* (Dryophthoridae), and *R. dominica* (Bostrichidae). Shaaya et al. (1997) found that oils extracted from a Lamiaceae species have fumigant toxicity against four major stored-grain insects, namely *R. dominica*. As per the findings, an increase in concentration led to an increase in mortality rates. In the study of Shokri-Habashi et al. (2011), the essential oil of *Carum copticum* L. (Apiaceae). against *R. dominica* was evaluated and the LC_{50} values were equal to 19.01 and 15.12 µl/l air after 24 and 48 h exposure times, respectively.

A monoterpenoid, linalool, has been demonstrated to act on the nervous system, affecting ion transport. The release of acetylcholine (Barocci et al., 2000) and can inhibit acetylcholinesterase (Mazzonetto, 2002) which at least in part accounts for the insecticidal effects of lavender EO. The insecticidal activity varied with insect species, oil concentrations, and exposure time. It can be concluded that essential oil products are generally broad-spectrum, due to the presence of several active ingredients that operate via several modes of action (Chiasson et al., 2004). Toxicity of pure and mixed compounds depends on their physicochemical properties and is the final result of the different toxicokinetic and toxicodynamic steps (penetration, distribution, metabolism, and interaction with the site of action) (Mc Donald et al., 1970).

Ho et al. (1995) concluded that adult mortality might be attributed to the contact toxicity or to the abrasive effect on the pest cuticle (Mathur et al., 1985), which might also interfere with the respiratory mechanism of insects (Agarwal et al., 1988).

Repellency activity

The repellent activity is a physiological phenomenon that occurs in insects as a defense mechanism against toxins secreted by plants. A botanical pesticide has a repellent property, which keeps insect pests away and protects the crops (Isman, 2006) with minimal impact on the ecosystem, as they remove insect pests from the treated materials by stimulating olfactory or other receptors (Talukder, 2006). The effectiveness and duration of repellency of chemicals depend on the type of repellent (active ingredient and formulation), the mode of application, and the local condition (temperature, humidity, and wind) (Barnard, 2000).

The present study revealed the effective repellent activity of *L. angustifolia* EO against *R. dominica*. The results showed that all tested concentrations induced a repellent activity with concentration and period-response relationship. According to Mc Donald et al. (1970), this plant belongs to the repellent class V. The repellent activity is related to major

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active compounds and other chemical constituents (Abdelaziz et al., 2014). Hori (2004) revealed that, as one of the principal components of shiso oil, linalool displays a repellent activity against *L. serricone (*Anobiidae) (Mauchline et al., 2008). We found that *L. angustifolia* EO contained high amounts of linalool, as other researchers have reported (Danh et al., 2013). Similar observations have been made after application of Citrus limonum EO which have revealed significant repellent effects on *Sitophylus granarius* (Guettal et al., 2020). Zhang et al. (2017) reported the repellent activities of six *Zanthoxylum* (Rutaceae) species essential oils against two storage pests including *T. castaneum* (Tenebrionidae) and *L. serricorne (*Anobiidae) adults. Ghavani et al. (2017) found that *Ziziphora tenuiore* (Lamiaceae)*, Myrtus communis* (Myrtaceae)*, Achillea wilhelmsii* (Compositae) and *M. piperita* (Lamiaceae) essential oils have repellent activities against human fleas, *Pulex irritans* (Pulicidae). Rahdari and Hamzei (2017) demonstrated the efficacy of *M. piperita* (Lamiaceae)*, R. officinalis* (Lamiaceae)*,* and *Coriandrum sativum* (Apiaceae) oils for applying in organic food protection due to the repellent activity of essential oils on *T. confusum* (Tenebrionidae). The repellent activity depends on the anti-insect mechanism and non-persistent volatility of essential oil sample (Hikal et al., 2017). However, the effectiveness of the repellents is might be attributed to multiple factors including the type of active ingredients, formulation, mode of application, environmental factors (temperature, humidity, and wind), the sensitivity of the insects to repellents, and the biting density (Hikal et al., 2017).

Effects on nutritional indices

In a bioassay of no-choice tests, the parameters used to evaluate antifeedant activity were relative growth rate, relative consumption rate, efficiency on the conversion of ingested food, and feeding and deterrence indices.

The discovery of novel toxins and/or antifeedants from plant extracts has been recently emphasized as a potential method for the development of ecologically safe pesticides (Wheeler et al., 2001). A high antifeedant index normally indicates a decreased rate of feeding, thereby resulting in the starvation of an insect. According to Isman (2002), the concept of using insect antifeedant for crop protectants/insect control is intuitively attractive; he listed many potent insect antifeedants that are extracted from the plant along with their chemical composition. Botanical pesticides inhibit feeding or disrupt insect feeding by rendering the treated materials unattractive or unpalatable (Talukder, 2006; Rajashekar et al., 2012). The insects remain on the treated material indefinitely and eventually starve to death (Hikal et al., 2017). In this regard, Liao et al. (2017) demonstrated that the oil of *M. alternifolia* (Myrtaceae) and their constituents possessed obvious antifeedant activities against *Helicoverpa armigera* (Noetuidae). However, Rama Rao et al. (2005) reported antifeedant and growth inhibitory effects of seed extracts of custard apple in hexane, ethyl acetate, and methanol against *Trogoderma granarium* (Dermestidae). The various bioassay showed that crude seed extracts of *A. squamosa* (Annonaceae) have both toxic as well as antifeedant properties as reported by Leatemia and Isman (2002). *Piper nigrum* (Piperaceae) and *Jatropha curcas* (Euphorbiaceae) extracts showed an antifeedant action against *C. cephalonica* (Pyralidae*)* larvae which increased with increasing extract concentrations (Khani et al., 2012); these results are in line with the present findings where concentration-dependent antifeedant and insecticidal potency was observed against the adult of *R. dominica*.

Reduction of RCR, ECI, and ECD led to a delay in adult growth and formation of smaller adults which have a direct relationship with the fecundity and longevity of the

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insect (Sogbesan and Ugwumba, 2008). The observed decrease in ECI indicates that more food is being metabolized for energy and less is being concerted to body substance (growth); ingested EO also exhibited some chronic toxicity. Similarly, this result is in consonance with previous findings' reporting on the antifeedant activity of *M. azedarach* such as those of Coria et al. (2008) for *Aedes aegypti* (Culicidae), Bullangpoti et al. (2012) for *S. frugiperda* (Noctuidae), and Aouadia et al. (2012) for *Drosophila* melanogaster (Drosophilidae). ECI is an overall measure of an insect's ability to utilize the food ingested for growth and development (Koul et al., 2004). The relative consumption rate is used for measurement exploitation of food by insects. This index shows the rate of feed connected weight in insects at a certain point in time. The RGR and RCR reduction may be an indication of damages caused by allelochemicals present in the essential oil to the peritrophic membrane or cell surfaces in the midgut (Nasr et al., 2017). The rate of feeding in insects depends on the water and physicochemical properties of food (Srinivasan and Uthamasamy, 2005). The aqueous and ethanolic extracts of *Melia azedarach* exhibited an antifeedant activity and reduced the food consumption of *S. littoralis* (Noctuidae) larvae according to the applied concentrations (Akacha et al., 2017). In the study carried out by Gökçe et al. (2010), *Humulus lupulus* (Cannabaceae) and *Arctium lappa* (Asteraceae) exhibited antifeedant activity in *Choristoneura rosaceana* (Tortricidae) larvae, in addition to contact toxicity, while *Bifora radians* (Apiaceae) was an antifeedant and exhibited toxic effects when ingested. These plants are also known to deter the feeding of *Leptinotarsa decemlineata* (Chrysomelideae) larvae (Gökçe et al., 2006). Correspondingly, the experiment of Taghizadeh et al. (2014) revealed a decrease of nutritional indexes, RGR, RCR, and ECI of *L. decemlineata* (Chrysomelidae) with increased concentrations of EOs of six tested plants. In fact, due to the tendency of insects to consume food, growth rate and food consumption decreased. Also, FDI increased with increased concentrations of all essential oils.

This indicated that the active compounds present in the plant inhibit the larval feeding behavior while others disrupt hormonal balance or make the food unpalatable. These active substances may directly act on the chemosensilla of the larvae resulting in feeding deterrence (Hikal et al., 2017).

Effect on digestive enzyme activities

Digestion refers to the process wherein ingested macromolecules by insects break down to smaller ones to be absorbable via epithelial cells of the midgut. Several enzymes based on food materials play critical roles in this process. Any disruption in their activity disables insects to provide their nutrients for biological requirements (Zibaee, 2011). Digestive enzymes, such as amylases, lipases, and proteases, play an important role in the body of insects by converting complex food materials into smaller molecules necessary in order to provide energy and metabolites (Teimouri et al., 2015).

Our results showed a clear disruption of digestive enzyme's activities responsible for the broken down of dietary components before its absorption by the intestinal epithelium. Many of the natural plant compounds used in the control of insect pests are known to affect digestive enzymes.

α-amylase is a midgut and salivary enzyme involved in starch and other carbohydrate metabolism and its activity level depends on feeding diet (Shekari et al., 2008). This enzyme was reduced in the series treated with LC_{25} and LC_{50} compared to the control series. The reduction of this enzyme activity could be caused by a cytotoxic effect of

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different extracts on epithelial cells of the midgut that synthesize α-amylase (Jbilou et al., 2008).

Shekari et al. (2008) demonstrated that α-amylase activity level in *Xanthogaleruca luteola (*Chrysomelidae) treated by *A. annua* (Asteraceae) extract decreased after 24 h and sharply increased after 48 h. Zibaee and Bandani (2010a) showed that *Artemisia annua* (Asteraceae) extract caused a reduction of α-amylase activity as the function (as concentrations) of plant extract in *Eurygaster integriceps* (Scutelleridae). Merkx-Jacques and Bede (2005) also demonstrated that increased activity of amylase in *Spodoptera exigua* (Noctuidae) larvae fed on artificial diets in comparison with the larvae fed on legume, *Medicago truncatula* L. (Fabaceae). Azadirachtin was reported to disrupt insect physiology and its ability to digest food (Senthil-Nathan, 2013; Shannag et al., 2015). It significantly reduced the activity of α-amylase of adults of *D. melanogaster* (Drosophilidae) surviving to azadirachtin-treated third instar larvae (LD_{25}, LD_{50}) (Kilani-Morakchi et al., 2017).

Proteases are a group of enzymes that hydrolyze peptide bonds in proteins and convert them into their respective amino acids. Proteases have a crucial role in the food digestion of insects. Three subclasses of proteinases are involved in digestion classified according to their active site group: serine, cysteine, and aspartic proteinases. The oligopeptides resulting from proteinase action are attacked from the N-terminal end by aminopeptidases and from the C-terminal end by carboxypeptidases (Zibaee, 2011). Studies by Johnson et al. (1990) and Senthil-Nathan et al. (2006) inferred those botanical insecticides may interfere with the production of certain types of proteases and disable them to digest ingested proteins. In the present study, compared with the controls, protease activity was significantly reduced in *R. dominica* after the treatment. The reduction of protease activity under botanical insecticide treatment was reported in several insects' species (Paranagama et al., 2001). Increased activity of proteases in *Ectomyelois ceratoniae* (Pyralidae) is probably caused by the need of insects for protein (Teimouri et al., 2015). Hemati et al. (2012) found significant differences in proteolytic activities in *Helicoverpa armigera* (Noctuidae) larvae reared on different host plants.

These results may reflect interference of *Lavandula* essential oil with the regulation of feeding and metabolism, which clearly supports the secondary antifeedant action of this oil that included a reduction in food consumption and digestive efficiency, thus reducing the access of nutrients for biological requirements. Perturbations of digestive enzymes cause a reduction in energy and metabolites and consequently affect normal growth.

Effects on biomarkers

Previous studies have shown that compounds extracted from diverse plants exhibited anti-insect properties by disturbing neuro-endocrine and growth regulatory processes (Xiao et al., 2012). Four types of detoxifying enzymes have been found to react against botanical insecticides including general esterases (EST), glutathione S-transferase (GST), and phosphatases (Zibaee, 2011).

Acetylcholinesterase is a key enzyme that terminates nerve impulses by catalyzing the hydrolysis of the neuro-transmitter acetylcholine in the nervous system (Wang et al., 2010) and is an important target for insecticides (Van Leeuwen et al., 2005). Huignard et al. (2008) observed that the EO of *O. basilicum* (Lamiaceae) inhibited neuronal electrical activity by decreasing the amplitude of action potentials and reducing both the post-hyperpolarization phase as well as the firing frequency of action potentials. Several monoterpenes contained in EOs are neurotoxic to insects (Regnault-Roger et al., 2012).

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Zibaee and Bandani (2010b) showed that *Artemisia annua* (Asteraceae) extract induced inhibition of the AChE activity in higher doses in *Eurygaster integriceps* (Scutelleridae), which agreed with the findings of studies about the effect of botanical insecticides on AChE inhibition. The alteration of AChE was observed in the cockroach, *Periplaneta americana* (Blattidae) (Shafeek et al., 2004) and *Blatta orientalis* (Blattidae) treated with AZA (Tine et al., 2016). A number of monoterpenes also act on acetylcholinesterase. Terpinen-4-ol and 1,8-Cineole, found in EOs of *Eucalyptus globulus* (Myrtaceae), *Laurus nobilis* (Lauraceae), and *Origanum majorana* (Lamiaceae) (Regnault-Roger et al., 1993), inhibit acetylcholinesterase (Mills et al., 2004). Our results are similar to those obtained by Al-Sarar et al. (2014) showing that EOs of *Mentha longifolia* (Lamiaceae) and *Lavandula dentata* (Lamiaceae) inhibited the AChE activity in *C. maculatus* (Chrysomelidae) adults. The AChE was also inhibited in the treated mosquito larvae by *O. basilicum* (Lamiaceae) (Dris et al., 2017). The results of Acheuk et al. (2017) revealed that the exposure of *T. castaneum* (Tenebrionidae) adults to *Limoniastrum guyonianum* (Plumbaginaceae) extract significantly reduced AChE activity. This acetylcholinesterase inhibition could be a possible mechanism of action of *L. guyonianum*. Kabir et al. (2013) indicate that the extract of *Seseli diffusum* (Apieae) exhibited a potent larvicidal activity and induced strong neurobehavioral toxicity against the 4th instar larvae of *A. aegypti* (Culicidae). Inhibition of AChE causes accumulation of acethylcholine at the synapses, which will lead to paralysis and eventually, death of the insect. In a recent study, Gade et al. (2017) noted that stigmasterol and 1-hexacosanol, biological compounds from *Chromolaena odorata* (Asteraceae) were responsible for larvicidal activity against *Cx. quinquefasciatus* (Culicidae) via their neurotoxicity. At a molecular level, these compounds were found able to inhibit the acetylcholinesterase activity in *C. quinquefasciatus* and *A. aegypti* (Culicidae).

On the other hand, glutathione S-transferases (GST) are the mainly cytosolic enzymes that catalyze the conjugation of reduced glutathione (GSH) with a wide range of lipophilic toxicants bearing electrophilic sites (Habig et al., 1974). GSTs play an important role in insecticide resistance (Zibaee et al., 2009) and participate in the primary detoxification, in phytophagous insects, of plant allelochemicals (Yu, 1987). Some plants defend allelochemicals against the GST activity induced by phytophagous insects (Yu, 1982; Vanhaelen et al., 2001). Vanhaelen et al. (2001) showed that Brassicacea secondary metabolites induced GST activity in *Myzus persicae* (Aphididae) and several Lepidopteran species such as *Heliothis virescens* (Noctuidae*)*, *Trichoplusia ni (*Noctuidae and *Anticarsia gemmatalis* (Erebidae). The influence of plant allelochemicals on GST activity is not limited to the herbivores and was observed in several predators (Francis et al., 2000). By applying *A. annua* (Erebidae) extracts on *E. integriceps* (Scutelleridae*)* adults, Zibaee and Bandani (2010b) and Zibaee (2011) reported that activity level of GST increased significantly at 24 h post-treatment. The induction of GST was observed in *B. orientalis* treated with AZA (Tine et al., 2016) and in *Cx. pipiens* (Culicidae) treated with *O. basilicum* (Lamiaceae) (Dris et al., 2017).

For GSH, this tripeptide is known as an antioxidant, preventing damage to important cellular components caused by reactive oxygen species such as free radicals and peroxides. It has been confirmed as a good indicator for oxidative stress; the amounts of GSH within cells are often used as a measure of cellular toxicity. In our experiments, it was evident that the increase of GST activity was also correlated with a decrease in GSH amounts after treatment with EO of *L. angustifolia*. Indeed, GSH is known as a non-enzymatic oxidative stress parameter; GSTs conjugate xenobiotics with the use of

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reduced GSH. The reduction of GSH was observed in *Z. variegatus* (Pyrgomorphidae) exposed to pyrethroids (PYRs) and *O. gratissimum* (Lamiaceae) leaf extract, in *B. orientalis* (Blattidae) treated with AZA (Tine et al., 2016) and in *Cx. pipiens* (Culicidae) treated with *Thymus vulgaris* (Lamiaceae) (Bouguerra et al., 2018).

Conclusion

In recent years, the use of synthetic insecticides in the fight against agricultural pests has inflicted unintended damages on both human life and the environment. Plant materials with insecticidal properties have been traditionally used for generations in some parts of the world. These studies provide an interesting opportunity to develop bioinsecticides, repellents, and antifeedant formulations based on the extracts from plants. For all these reasons, we can infer that the essential oils of *L. angustifolia* could be considered a natural alternative in the control of stored grains insects such as *R. dominica.*

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INFLUENCE OF ENVIRONMENTAL FACTORS ON THE DEVELOPMENT OF POTATO BLACKLEG DISEASE

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Abstract. Climatic conditions play a crucial part regarding the development of blackleg caused by *Pectobacterium atrocepticum (Pa)*, which is one of the major threats the potato industry faced. These factors affect the stage of growth, host susceptibility, succulence, vigor, survival, rate of multiplication, direction of pathogen dispersion, rate of spore penetration and germination. In the present experiment, effect of these environmental variables like maximum and minimum temperature (max and min T), relative humidity (RH), rainfall (RF), and wind speed (WS) on disease development were studied. Significant positive correlation was observed for all twenty-five varieties and a multiple regression model ($Y = + 24.382 + 0.3592X_1 + .0970X_2\text{-}0.2551X_3 + 1.982X_{4)}$) based on the two-year study was developed to find out the relationship between environmental parameters and disease projection. Goodness of model was indicated by coefficient determination value (97.5%). Correlation of these environmental factors on the development of disease on twenty-five potato varieties during 2018, 2019 was done. Significant positive correlation was observed on all twenty-five varieties. In conclusion, it was established that environmental factors, including max. T (28 °C), min. T (15 °C), RH 70%, and WS 3.5 Km/h were conducive for the development of blackleg. Current study would be helpful for researchers in designing better disease management strategies under changing climatic conditions in the future.
Keywords: *Pectobacterium atrocepticum, blackleg, environmental variables, regression model, correlation*

Introduction

Potato (*Solanum tuberosum*) belongs to the family of *Solanaceae*. It is a perishable food crop. It is a tuber bearing plant, that is cultivated worldwide and ranked 4th after maize (*Zea mays*), wheat (*Triticum aestivum*) and rice (*Oryza sativa*). In temperate regions, it is a major field crop (Kroschel et al., 2020). Potato tubers are not only used as a seed but also used as a food source for market and processed products. In developing countries, potato crop is a major source of employment and income (Horten et al., 2019). It has become the staple food of Irish people (De Jong, 2016). It contains water (70-80%) and organic matter (20%) (Nyawade et al., 2018). It has dietary materials (22%), food energy (325 kcal), protein (7.6 g), fat (0.04 g), carbohydrates (72.8 g), calcium (42 mg), phosphorus (213 mg), iron (2.7 mg), vitamin A (70 IU), riboflavin (0.15 mg), niacin (4.4 mg), ascorbic acid (64 mg) and also rich in antioxidants (Tunio et al., 2020). Potato crop is facing several biotic and abiotic constraints, in which blackleg caused by *Pectobacterium atrosepticum (Pa)* is a major threat among all (Rivedal et al., 2021). In Pakistan it was firstly reported in 1984 from swat valley. In India, losses due to this disease recorded up to 45% (Raza et al., 2021). When disease incidence is above 5-10%, Pakistan economy damaged due to yield reduction and potato degradation (Sharma et al., 2017). Characteristic symptom of this

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disease is a slimy, wet, black rot lesion appearing on tuber and spreading from infected tuber up to stem (Elhalag et al., 2020). Maceration occurs due to enzymes (pectinase, protease and cellulose) released by bacteria. Then the stem turns into inky black color and becomes soft. Above ground parts of plant drop down because plant becomes infected from the soil. Shoots become stunted and stem turns into black color at the bottom and hence it is called blackleg (Aboshama et al., 2019) (*Fig. 1*).

Figure 1. *Characteristic symptoms of black leg disease of potato*

Epidemiology of any disease is necessary to be studied to gain knowledge about all environmental factors which directly or indirectly affect the development of epidemic. Environmental factors have a critical role in blackleg disease development (Skelsey et al., 2018). Temperature is a key factor in this disease, and cause rotting of tubers. *Pa* is more vigorous at temperature lower than <25 °C (Kaczynska et al., 2019). In rotting of mother tubers, if more than one pathogen species is present, it will depend on temperature that which pathogen will predominate. A species gains maximum growth rate at its ideal temperature. Moisture is also an important factor for tuber rotting.

There is dire need to study these epidemiological factors and understand the characterization of these factors for disease management. Present study will be designed to assess the epidemiological factors like max and min temperature, rainfall, wind speed, relative humidity etc.) and their impact on disease development. By following this information, researchers develop disease predictive model to minimize the losses and to guide the farmers that how they can tackle these situations and get maximum yield.

Material and methods

Data collection

A comprehensive study was designed for two years to collect disease incidence data from the experimental area of Department of Plant Pathology, University of Agriculture Faisalabad. For pathogenicity, three weeks after stem emergence, 100 uL of bacterial inoculum at 10^8 CFU/mL was injected in the collar region of the stem. Five genotypes (Harmony, Lady rosetta, Faisalabad white, Accent and Desiree) were tested for the assessment of different environmental factors playing role in disease development. All

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were susceptible against the black leg disease. Environmental factors data including max and min temperatures (°C), wind speed (Km/h), rainfall (mm) and relative humidity (%) was obtained from meteorological station located at the Agronomy research area, UAF for the whole duration of crop (from sowing to harvesting) and their conduciveness with the disease development was checked by regression and correlation analysis and a predictive model was developed based upon the results.

Regression analysis

For determining the relationship b/w the climatic/environmental factors and disease development/incidence, regression analysis was used. There are actually two types of regression models that were used, first one is a simple regression model, and the other is multiple regression model (*Eqs. 1* and *2*). Regarding the development of simple linear regression models, Y act as response variable in case the of disease while X will be worked as explanatory variable which represent the environmental/climatic factors. The equation for simple linear regression is:

$$Y = \beta 0 + \beta 1X \quad \text{(Eq.1)}$$

where β0 denotes as intercept and β1 is the slope.

In the case of multiple linear regression models, more than one explanatory or predictor variables (X) are included as compared to the simple linear regression analysis. The equation of multiple regression model with relationship of variables is described by:

$$Y = \beta 0 + \beta 1x1 + \beta 2x2 + \cdots + \beta i\ xi + \in \quad \text{(Eq.2)}$$

Here, x characterizes as the compilation of predictors x1, x2... xi in the model, and β1, β2...βi act for the corresponding regression coefficients and ∈ is the random error or interruption in the experiment.

Characterization of environmental factors conducive for black leg of potato

All environmental data as well as disease incidence (%), and alterations in the environmental factors and disease incidence observed on potato cultivars, were checked by (LSD at $P < 0.05$) least significance difference test. The effect of environmental factors on blackleg disease was studied by correlation. Mean square error (MSE) Mallows Cp and R2 were criteria used for selecting the best models (Gottwald and Irey, 2007).

Goodness of model

Correlation was used for determining goodness of the fit model (Steel and Torrie, 1997) and varieties/cultivars in which, more than 50% of the environmental variables exert significant effects which were plotted and the most conducive environmental factors for development of blackleg disease was determined with the help of regression analysis. The manipulation of these factors on the establishment of blackleg was tested by drawing a comparison in between the recorded and predicted values of disease incidence with the help of multiple regression models. Furthermore, disease predictive model depending on environmental conditions was developed which has significant influence on blackleg disease development.

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Results

Development and evaluation of blackleg predictive model based on two years data

Regression analysis expressed that relative humidity exhibited statistically non-significant response. So, it is removed from the model. The multiple regression model based on data of two years: $Y = 24.382 + 0.3592X_1 + .0970X_2\text{-}0.2551X_3 + 1.982X_4$ (X_1 = Max.T, X_2 = Mini.T, X_3 = Rainfall X_4 = Wind speed) explained 97% variability. The normal probability plot showed (*Fig. 2*) that most points were present on the reference line at different points. In the residual verses fitted value, all the points were distributed within range -1 to +1. Only four points were present along the reference line.

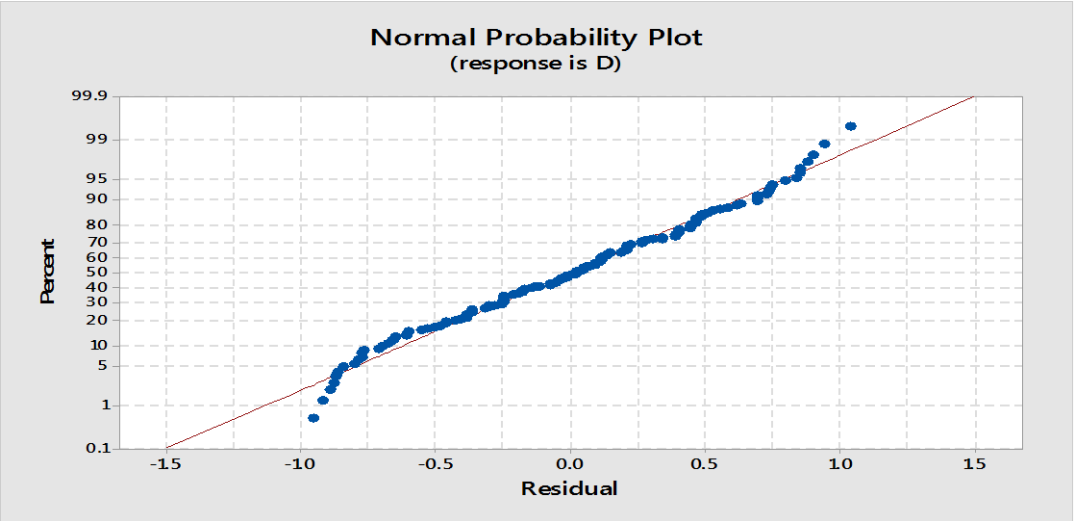

Figure 2. *Normal probability plot for the model of blackleg disease of potato based on data of two years*

Assessment of disease predictive model by comparing the dependent variables with regression coefficient through physical theory

Disease predictive model expressed $R^2 = 0.97$ coefficient of determination which showed that the model is good for disease prediction with low standard error. Temperature (Max. and min.) rainfall (mm) and wind speed (km/h) have significant contribution in the development of blackleg disease of potato (*Table 1*).

Table 1. *Regression model's coefficients of variables, standard error, t stat and P-value for blackleg*

Parameters	Coefficients	Standard error	t Stat	*P*-value
Intercept	24.382	0.456	53.41	0.000*
Max.T (°C)	0.3592	0.0255	14.06	0.000*
Mini. Temp.	0.0970	0.0461	2.10	0.037*
Rainfall	0.2551	0.0652	-3.92	0.000*
Wind speed	1.982	0.115	17.30	0.000*

*Significant at $P < 0.05$

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Estimation of model on the basis of predicted and observed values

For assessing the reliability of model value differences of observed and predicted data points were estimated. Among the observed values fourteen data-points were beyond reference line (standard error = 0.485174) and created an error in the experiment. According to the graphs, maximum prediction values having differences (less than 5) were consolidated between 95% interval of confidence (C.I) and 95% predictive interval (P.I) showed that there was a good fit in between predictive and observed values (*Fig. 3*).

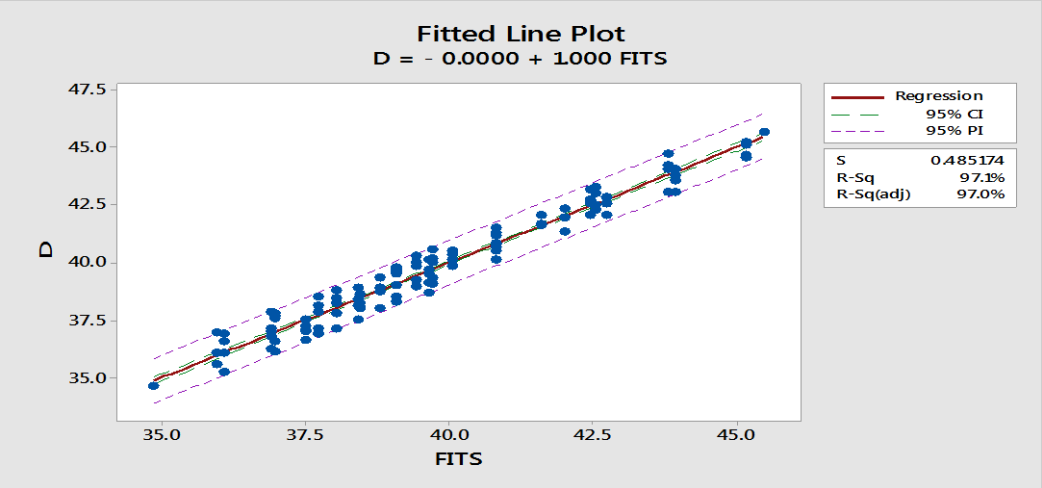

Figure 3. *A fitted line plot for blackleg with observed and predicted data points at 95% confidence and predictive interval*

Correlation of environmental factors for the development of blackleg on various varieties during 2018 and 2019

The environmental dependent variables i.e. maximum temperature (°C), minimum temperature (°C), relative humidity (R.H %), rain fall (mm) and wind speed (Km/h) expressed significant positive correlations with all varieties Cardinal, Vales Everest, Paramount, Atlantic, Orla, Melody, Patrones, Harmony, Multa, SH70, Desiree, Hermes, Diamant, Raja symphonia, Ajax, Fontane, Lal-e-Faisal, Ultimas, FSD white, FSD red, Sante, Lady Rosetta, Agria, Accent and Prima during 2018 (*Table 2*) and similar results were obtained during the year of 2019 (*Table 3*).

Discussion

Numerous environmental factors influenced the successful growth of plants (Li et al., 2020). Environmental factors viz. maximum air temperature, minimum air temperature (°C), moisture (%), relative humidity (%), rainfall (mm) and wind speed (km/h) are the imperative sources that predispose the plants for the development of infection (Saharan et al., 2016). Abrupt changes in the climatic conditions can impose an effect on plant diseases (Serdeczny et al., 2017). Environmental factors play an influential role in the resistance and susceptibility of the host plant. They can also alter rate of pathogen growth, reproduction, dissemination, infection, survival, and host pathogen interaction. So, in present study impact of environmental factors on the development of disease, was observed.

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Table 2. *Correlation of environmental factors with blackleg disease on different varieties of potato for 2018*

Sr#	Variety name	Max. T (°C)	Min. T (°C)	RH (%)	RF (mm)	WS (km/h)
1	Cardinal	0.960 0.000**	0.849 0.000**	0.894 0.000**	0.602 0.023*	0.960 0.000**
2	Vales Everest	0.966 0.000**	0.913 0.000**	0.922 0.000**	0.703 0.005*	0.983 0.000**
3	Paramount	0.967 0.000**	0.887 0.000**	0.913 0.000**	0.656 0.011*	0.986 0.000**
4	Atlantic	0.956 0.000**	0.827 0.000**	0.918 0.000**	0.555 0.039*	0.955 0.000**
5	Orla	0.970 0.000**	0.889 0.000**	0.923 0.000**	0.663 0.010*	0.980 0.000**
6	Melody	0.964 0.000**	0.820 0.000**	0.944 0.000**	0.535 0.048*	0.951 0.000**
7	Patrones	0.980 0.000**	0.933 0.000**	0.930 0.000**	0.737 0.003*	0.993 0.000**
8	Harmony	0.987 0.000**	0.899 0.000**	0.918 0.000**	0.671 0.009*	0.985 0.000**
9	Multa	0.951 0.000**	0.859 0.000**	0.897 0.000**	0.609 0.021*	0.967 0.000**
10	SH70	0.965 0.000**	0.865 0.000**	0.939 0.000**	0.628 0.016*	0.978 0.000**
11	Desiree	0.955 0.000**	0.795 0.001*	0.925 0.000**	0.548 0.042*	0.940 0.000**
12	Hermes	0.942 0.000**	0.842 0.000**	0.886 0.000**	0.586 0.028*	0.952 0.000**
13	Diamant	0.977 0.000**	0.912 0.000**	0.912 0.000**	0.693 0.006*	0.989 0.000**
14	Raja symphonia	0.966 0.000**	0.804 0.001*	0.931 0.000**	0.527 0.053*	0.950 0.000**
15	Ajax	0.968 0.000**	0.848 0.000**	0.934 0.000**	0.614 0.019*	0.961 0.000**
16	Fontane	0.961 0.000**	0.825 0.000**	0.920 0.000**	0.561 0.037*	0.561 0.037*
17	Lal-e-faisal	0.960 0.000**	0.843 0.000**	0.902 0.000**	0.587 0.027*	0.970 0.000**
18	Ultimas	0.960 0.000**	0.849 0.000**	0.894 0.000**	0.602 0.023*	0.960 0.000**
19	FSD white	0.966 0.000**	0.913 0.000**	0.922 0.000**	0.703 0.005*	0.983 0.000**
20	FSD red	0.967 0.000**	0.887 0.000**	0.913 0.000**	0.656 0.011*	0.986 0.000**
21	Sante	0.958 0.000**	0.868 0.000**	0.902 0.000**	0.652 0.02*	0.962 0.000**
22	Lady Rosetta	0.955 0.000**	0.895 0.000**	0.897 0.000**	0.672 0.009*	0.973 0.000**
23	Agria	0.991 0.000**	0.906 0.000**	0.938 0.000**	0.678 0.008*	0.990 0.000**
24	Accent	0.982 0.000**	0.860 0.000**	0.932 0.000**	0.608 0.021*	0.980 0.000**
25	Prima	0.970 0.000**	0.872 0.000**	0.908 0.000**	0.638 0.014*	0.983 0.000**

Upper values indicated Pearson's correlation coefficient. Lower values indicated level of significance at 5% probability. Ns = non-significant; *Significant ($P < 0.05$); **Highly significant

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Table 3. *Correlation of environmental factors with blackleg disease on different varieties of potato for 2019*

Sr#	Variety name	Max. T (°C)	Min. T (°C)	RH (%)	RF (mm)	WS (Km/h)
1	Cardinal	0.837 0.000**	0.965 0.000**	0.951 0.000**	0.688 0.007	0.954 0.000**
2	Vales Everest	0.911 0.000**	0.963 0.000**	0.980 0.000**	0.780 0.001	0.990 0.000**
3	Paramount	0.885 0.000**	0.961 0.000**	0.983 0.000**	0.764 0.001*	0.989 0.000**
4	Atlantic	0.820 0.000**	0.952 0.000**	0.948 0.000**	0.655 0.011	0.954 0.000**
5	Orla	0.878 0.000**	0.977 0.000**	0.967 0.000**	0.745 0.002	0.000 0.979
6	Melody	0.891 0.000**	0.977 0.000**	0.964 0.000**	0.765 0.001	0.970 0.000**
7	Patrones	0.866 0.000**	0.969 0.000**	0.970 0.000**	0.705 0.005	0.964 0.000**
8	Harmony	0.870 0.000**	0.975 0.000**	0.972 0.000**	0.706 0.005	0.972 0.000**
9	Multa	0.836 0.000**	0.944 0.000**	0.955 0.000**	0.674 0.008	0.958 0.000**
10	SH70	0.931 0.000**	0.955 0.000**	0.993 0.000**	0.784 0.001	0.986 0.000**
11	Desiree	0.875 0.000**	0.982 0.000**	0.975 0.000**	0.733 0.003	0.974 0.000**
12	Hermes	0.900 0.000**	0.966 0.000**	0.972 0.000**	0.786 0.001	0.970 0.000**
13	Diamant	0.879 0.000**	0.959 0.000**	0.973 0.000**	0.752 0.002	0.982 0.000**
14	Raja symphonia	0.889 0.000**	0.978 0.000**	0.982 0.000**	0.744 0.002	0.981 0.000**
15	Ajax	0.842 0.000**	0.974 0.000**	0.966 0.000**	0.699 0.005	0.961 0.000**
16	Fontane	0.856 0.000**	0.982 0.000**	0.947 0.000**	0.711 0.004	0.950 0.000**
17	Lal-e-Faisal	0.900 0.000**	0.963 0.000**	0.978 0.000**	0.776 0.001	0.982 0.000**
18	Ultimas	0.933 0.000**	0.964 0.000**	0.981 0.000**	0.807 0.000**	0.991 0.000**
19	FSD white	0.898 0.000**	0.990 0.000**	0.972 0.000**	0.748 0.002	0.974 0.000**
20	FSD red	0.874 0.000**	0.970 0.000**	0.981 0.000**	0.728 0.003	0.980 0.000**
21	Sante	0.854 0.000**	0.956 0.000**	0.956 0.000**	0.723 0.004	0.955 0.000**
22	Lady Rosetta	0.829 0.000**	0.970 0.000**	0.953 0.000**	0.679 0.008	0.946 0.000**
23	Agria	0.820 0.000**	0.952 0.000**	0.948 0.000**	0.655 0.011	0.954 0.000**
24	Accent	0.878 0.000**	0.977 0.000**	0.967 0.000**	0.745 0.002	0.979 0.000**
25	Prima	0.909 0.000**	0.971 0.000**	0.983 0.000**	0.740 0.002	0.972 0.000**

Upper values indicated Pearson's correlation coefficient. Lower values indicated level of significance at 5% probability. Ns = Non-significant; *Significant ($P < 0.05$); **Highly significant

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Present study was designed to check the collective effect of environmental factors including temperature, rain fall, relative humidity and wind speed which showed positive correlation with the blackleg disease incidence. The results of present study are hand in line with the findings of Agrios (2005) and it was concluded that various environmental factors have great impact on blackleg disease development, maximum and minimum temperature is the most serious factor in the disease development. Favorable conditions for blackleg development included cool and wet conditions (Charkowsky et al., 2020). In this current study, maximum number of cultivars showed positive correlation with the blackleg disease development and collectively all environmental factors including maximum, minimum temperature, wind speed, rain fall and relative humidity. The highest disease incidence was observed at 9-15 °C minimum temperature, 20-28 °C maximum temperature, 65-70% relative humidity, 3.5 km h^{-1} wind speed. The results of this study are supported by the findings of Quiroz et al. (2018) who concluded that temperature within 15-22 °C with high air moisture are the most crucial for blackleg disease development. Dispersal of disease significantly increased by warm and wet climatic conditions. In the favorable atmospheric conditions, the entire potato crop may be destroyed within short time periods. Ideal conditions for disease spread are high relative humidity of more than 75% and temperature above 10 °C. Rainfall had great impact in infecting young tubers. The potato crop is significantly affected in warm temperature (15-20 °C) and high humidity (80-100%) (Rykaczewska, 2017).

Conclusion

All the environmental factors (Max. T, Min. T, RH, WS and RF) have significant positive correlation on all varieties of potato for two years. Due to sudden fluctuations in the climatic conditions, continuous monitoring of environmental variables is necessary for accurate prediction of blackleg and its management. Installation of weather stations in the major potato growing areas would be helpful in risk assessment and forecasting systems in a specific area. On the basis of data collected from different areas, a Decian Support System can be developed for precise management of disease.

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GENETIC TRANSFORMATION OF THE EPSPS HERBICIDE RESISTANCE GENE IN AGROBACTERIUM MEDIATED PEANUT (*ARACHIS HYPOGAEA* L.) AND EFFECTIVE REVIVAL OF TRANSGENIC PLANTS

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Abstract. The current study was based on Agrobacterium-mediated peanut genetic transformation and effective remediation of transgenic plants. The effect of Agrobacterium tumefaciens strain LBA4404 with a binary vector of *PxCSG m-YFP* 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene has been evaluated on two commercial varieties and on two sources of explants (Embryo and cotyledon), each containing a CaMV 35S promoter. The comparative regeneration and transformation efficiency analyses revealed that the embryo is a better target tissue than cotyledon, and early-ripe peanut genotypes with relatively small seeds (such as Bard-479) have proven to be comparatively responsive to transformation. Explant culture in media containing 3 mg L 1 indole-3-butyric acid and 0.1 mg L 1 napthalene acetic acid resulted in strong roots of virtually all transgenic plants. More than 87% of the transplanted plants were able to produce morphologically normal blooms and pods containing viable seeds. The phenotypic and genotypic monitoring of the EPSPS gene inheritance over two generations exhibited the desired 3:1 inheritance. Our findings suggest that Agrobacterium-mediated transformation is a feasible and valuable tool for peanut breeding and functional genomics research.
Keywords: *binary vector, EPSPS gene, transformation efficiency, tissue culture, regeneration performance, peanut breeding*

Introduction

Agrobacterium-mediated transformation is a common and effective method for transferring foreign DNA into a broad range of plant species. Low transgene copy number, capacity to produce lines devoid of selectable flag genes, and stable integration of lengthy strains of DNA with specified ends are among its benefits over other transformation techniques (Travella et al., 2005). Long term fertility building requires a combined methodology instead of short range approach and targeted way out instead of conventional agriculture approaches, therefore combined efforts of agriculture scientists (of diverse discipline) specially plant breeders and geneticist is required to overcome loss inflicted by lethal disease both by biotic and abiotic sources i.e. by incorporation of resistance/require alleles in various high yielding crops through genetic transformation

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breeding programs (Khattak et al., 2020; Waqar et al., 2018). As a result, development of these modern biotechnological approaches and exploitation of transgenic plants has become increasingly important for better and sustainable production of different cash crops (Bangash et al., 2013).

One of the best ways is *Agrobacterium-mediated* DNA transfer that has become a significant tool in functional genomics because it is the most reliable method to produce gain-of-function or loss-of-function mutants to evaluate gene activity (Curtis and Grossniklaus, 2003). Peanut, an economically significant oil and protein-rich crop, can adapt to a broad range of climatic conditions and thrives in a variety of habitats between latitudes 408N and 408S (Sharma et al., 2006). However, a number of limitations to peanut production result in significant yearly economic losses (Sharma and Anjaiah, 2000; Sharma and Ortiz, 2000). Genetic transformation is quickly becoming a crucial part in the process of varietal improvement, as it may offer a strong tool for identifying genes that regulate essential agronomical characteristics related to disease resistance and/or abiotic stressors (Graham-Rowe et al., 2014).

Although *A. mediated* transformation has been used to transfer many genes into the peanut genome (Iqbal et al., 2012), only a few peanut cultivars exhibit significant transformation efficiency. *A. mediated* transformation may be limited by factors that influence the interaction of host cells with bacteria (Gelvin, 2003). High-throughput sequencing techniques, such as genome or transcriptome sequencing are confronting fundamental new problems in plant biology. For example, they make it easier to determine the function of anticipated genes and to introduce new characteristics into existing genotypes via genetic engineering. In the case of peanuts, the pedigree selection procedure may take many years. In the face of an unforeseen danger, only plant genetic change might provide an acceptable response (Iqbal et al., 2012). Another issue is the absence of effective procedures for regenerating whole plants (Geng et al., 2011). This necessitates the regeneration of adventitious shoot buds from changed tissues in vitro as well as the development of roots from transformed shoots. Apart from peanuts, poor rooting in vitro hinders the efficient creation of transgenic lines of dicotyledonous crops (Jin et al., 2006; Dutt and Grosser, 2010; Belide et al., 2011). Roots that have been induced in vitro are often weak and do not survive the transition from tissue culture medium to soil (Dutt and Grosser, 2010; Tiwari and Tuli, 2012). The transplanted plants' subsequent establishment in the greenhouse is equally critical. As a result, in addition to an effective transformation method, an effective regeneration mechanism is also required to generate transgenic peanut lines for crop improvement and successful gene function research. The objectives of this study were to compare the effects of different peanut genotypes, explant sources, and inoculation treatments on transformation efficiency and the regeneration of adventitious shoot buds, (ii) determine the culture conditions required for efficient shoot proliferation and in vitro root formation, and (iii) determine the conditions required for efficient transplantation.

Materials and methods

Plant materials

Mature Dehusked seeds of peanut genotypes Bard-479 and Potohar (obtained from the Bari Research Institute in Chakwal, Pakistan) were utilized. Dehusked seeds were sterilized by immersing them in 70% (v/v) ethanol for 5 min and then in 50% Clorox for 30 min. They were then rinsed with five to six changes of sterilized water before being

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immersed in double-distilled water for 3 h. The embryo and the cotyledon were utilized as explants for transformation. Overnight, the explants were pre-cultivated on MS media.

Agrobacterium strain and binary vector

The DH5-alpha plasmid which contains the *EPSPS* gene in T-DNA, is an expression vector. Using the freeze-thaw technique, the construct was deployed into *Agrobacterium* host strain *LBA4404* (Hoekema et al., 1983). The PAT gene sequence was utilised as a selective marker for transgenic plant tissue (under the control of a CaMV35S promoter). Bacteria were grown on Luria broth agar plates with 50 mg L 1 ampicillin and 50 mg L 1 rifampicin (Sambrook et al., 1989).

Inoculation and co-cultivation

A single bacterial colony was inoculated into 20 mL of liquid YEB medium (Tiwari and Tuli, 2012) containing 50 mg L1 Ampicillin and incubated at 280 °C and 160 rpm overnight. The medium used for bacterial culture was then transferred into 100-200 mL of liquid YEB medium and cultivated for 4-5 h until the culture reached an absorbance (A600) of 0.6. Bacteria were collected by centrifugation for 10 min at 8000 rpm, and resuspended in the same volume of MS liquid medium (30 g/L sucrose) (Murashige and Skoog, 1962) containing 100 µM acetosyringone (As). Explants were dipped into this suspension, and then immersed in the suspension to inoculation. Inoculation conditions were modified to establish efficient conditions for transformation and regeneration. The infected calli were dried on cleaned filter paper. Three separate co-cultivation media, (100 µM, 200 µM and 300 µM), were examined and co-cultivated at 25 ± 2 °C under light (under a photoperiod of 16/8 h) conditions. After co-cultivation, surplus *Agrobacterium* was washed away from calli using antibiotic 250 mg/l cefotaxime (bacteriostatic). First purified distilled water was used for washing the calli 3-4 times for 20 min then MS liquid with cefotaxime (250 mg/l) was used for washing for 15 min the calli were desiccated out on filter paper for 10 min. Excess bacterial suspension was removed from the explants by placing them on sterile filter paper, and the *Agrobacterium* infected embryo and cotyledons were then cultured in darkness for 3 days on MS medium supplemented with 0.4 mg L_1 6-benzylaminopurine (BAP) and 100 µM As and 0.1 mg _L NAA.

Statistical analysis was conducted to find out the best concentration for transformation in both the cultivars. Complete randomized design (CRD) was used followed by Least significant difference (LSD). Graphically representation was also done to illustrate the transformation efficiency difference for each of the concentration used.

Control of agrobacterium regrowth, and regeneration and selection of transformed tissue

The developing explants were moved to callus proliferation and regeneration medium (CPM), which consisted of MS media supplemented with 0.4 mg/L BAP, 0.1 mg/L NAA, 100 µM Phosphothricine acetyl transferase *PAT*, and 250 mg/L cefotaxime after three days in darkness. Explants were grown for 12 days at 25 ± 2 °C in a light (16/8 h photoperiod) condition. PAT Selection medium (PATSM), which consisted of CPM supplemented with 100 µM PAT, was used to cultivate the

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developing calli for 17 days. After 7 days on PATSM, the number of regenerated calli was counted. For a second round of selection, Calli that developed shoot-like structures were transplanted to new PATSM for a further 17 days. The number of calli that generated green shoots, as well as the quantity of shoots produced, were both counted.

Vigorous shoot culture and rooting of transgenic plants

Green shoots were carefully separated and grown in fresh strong shoot culture medium (SSCM), which consisted of MS media supplemented with 250 mg/L cefotaxime and 100 µM PAT, for 20 days in jam jars. To improve the effectiveness of transgenic calli shoot proliferation and growth, media added with various combinations and doses of phytohormones (*Table 1*) were tested. The green shoots were then placed to fresh SSCM for another 20 days. Transgenic shoots (2.6 - 5 cm) were counted and then moved to root induction medium (RIM), which consisted of half-strength MS media (15 g/L sucrose) supplemented with IBA and/or napthaleneacetic acid (NAA). Different concentrations of IBA and NAA were compared in order to improve rooting efficiency and achieve vigorous root growth. Plants were grown in RIM for 35 days before being transplanted to pots, as stated below.

Hardening and transplantation of rooted transformants

Plantlets with excellent rooted systems were moved to the culture chamber for hardening at 25 ± 2 °C for 10 days under light (16/8 h photoperiod) conditions with an ambient humidity of 80%. The jar lids were gradually opened throughout this time: first, the vent covers were opened for three days, and then the jar covers were gently opened (25% open for 2 days, then 50% open for an additional 2 days, and then fully open for 3 days). The plantlets were carefully removed from the jam jars, and leftover medium-covered roots were rinsed with sterile water before being cultured for 20 min in liquid MS media supplemented with 0.3 mg/L IBA and 0.1 mg/L NAA. After 30 s in 0.2% potassium permanganate, the plantlets were rinsed three times with sterile water. The plantlets were then moved to pots with an autoclaved soil combination (soil/sand/vermiculite/7:2:1) and placed in a greenhouse with the same light and humidity conditions as the hardening culture chamber. The plantlets were fertilized with 100 ml of half strength MS saline solution seven days after transplantation. For the first two weeks following transplanting to the soil mixture, the young plants were covered with clear polythene bags to maintain high humidity and prevent nutrient deficiencies. The plants were then cultivated in a greenhouse with a 16-h photoperiod and enhanced light intensity (50 mmol m/2 s/1). Every fourth day, the plants were irrigated with 10 - 25 cm3 of water per plant.

PCR analysis of transgenic plants

To validate transgene incorporation by PCR analysis, genomic DNA was extracted from the control and the putatively transgenic plants using the cetyl-dimethyl-ammonium-bromide technique (Doyle and Doyle, 1987). Leaf DNA extracted from 25 putative T0 and T1 plants (one plant chosen at random each transformation event), as well as non-transformed controls was examined using PCR. Two sets of primers were developed.

The 293-bp EPSPS (PAT-1) gene fragment was amplified using the primers
EPSPS-F = 5'CACCGCTTTCCCACTTGTTG3' and
EPSPS-R = 5'GCATAACGGTGGTTCCCTCA3'

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The primers used to amplify the 245-bp EPSPS (PAT-2) gene fragment were EPSPS F = 5'GCTTGCTGGAGGAGAGGATG3' PPT-R = 5'CTCCCTCGGTGCAATCAACT3'. The amplified products were examined by electrophoresis using 1.5% agarose gels.

Table 1. *Different types of culture media used for regeneration and transformation of two genotypes of peanut*

Culture media	Composition
Germination media (GM)	MS basal medium (Murashige and Skoog, 1962), 30 g/l sucrose, 2.8 g/l gellen gum, pH 5.8
YEP medium	10 g/l yeast extract, 10 g/l bacto-peptone, 5 g/l Nacl, pH 7.0 g/l, 8.0 g/l Phytagel (for solid and liquid medium) (Sabir et al., 2014)
LB medium	5 g/l yeast extract, 10 g/l bacto-tryptone, 10 g/l Nacl, pH:7.0 g/l, 8.0 g/l Phytagel (for solid and liquid medium) (Sabir et al., 2014)
Inoculation medium (IM)	MS basal medium, 30 g/l sucrose, 100 uMacetosyringone, 2.8 g/l gellen gum, pH 5.8
Pre-culture medium (PCM)	MS basal medium (Murashige and Skoog, 1962), 30 g/l sucrose, BAP 0.4 mg/l, NAA 0.1 mg/l, 2.8 g/l gellen gum, pH 5.8
Co-cultivation medium (CCM)	MS basal medium (Murashige and Skoog, 1962), 30 g/l sucrose, BAP 0.4 mg/l, NAA 0.1 mg/l, 100 µM acetosyringone, 2.8 g/l gellen gum, pH 5.8
Pre-selection medium (PSM)	MS basal medium (Murashige and Skoog, 1962), 30 g/l sucrose, BAP 0.4 mg/l, NAA 0.1 mg/l, 250 mg/l cefotaxime, 2.8 g/l gellen gum, pH 5.8
Selection medium (SM)	MS basal medium (Murashige and Skoog, 1962), 30 g/l sucrose, BAP 0.4 mg/l, NAA 0.1 mg/l, 100 µM *PAT*, 2.8 g/l gellen gum, pH 5.8
Shoot induction medium	MS basal medium (Murashige and Skoog, 1962), 30 g/l sucrose, BAP 0.4 mg/l, NAA 0.1 mg/l, 100 µM acetosyringone, 250 mg/l cefotaxime, 100 µM *PAT*, 2.8 g/l gellen gum, pH 5.8
Root induction medium	MS basal medium (Murashige and Skoog, 1962), 30 g/l sucrose, IBA 3.0 mg/l, NAA 0.1 mg/l, 250 mg/l cefo, 100 µM *PAT*, 2.8 g/l gellen gum, pH 5.8

Results

Shoot regeneration from Agrobacterium-infected explants

The viability of fast and effective shoot regeneration from callus acquired from both Embryo and Cotyledon generated from two distinct types of peanut was shown in this research. Before being transferred to CPM, the infected explants were first cultured in darkness on MS medium supplemented with 0.4 mg/L BAP and 0.1 mg/L NAA and 100 µM Acetosyringone. The morphological characteristics of callus development were apparent after 7-10 days, and globular formations formed on the callus' top surface on the CPM (*Fig. 1A*). On PATSM, these formations grew into dark-green shoot-like structures (*Fig. 1B*) and ultimately became full shoots. After 10-12 days of growth on PATSM, several shoot-like structures started to emerge from the *Agrobacterium*-infected callus. The tiny protuberances that arose from the epidermal cell layer at the base of each callus piece ultimately evolved into shoot clusters. Green shoots were carefully separated from one another and put to SSCM in jam jars to allow for continued development and elongation (*Fig. 1C*). Root formation in transgenic, shoots and transplantation of rooted plants are two important aspects of plant breeding. The

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shoots were transplanted to RIM enriched with phytohormones when they reached 5-6 cm in length (*Fig. 1D*). The RIM needed both IBA and NAA for strong roots. Prior cultivation of shoots on the SSCM improved rooting effectiveness in all genotypes studied, with rooting efficiency reaching 100% in some. The roots of shoots that grew where clumps of callus were broken at their natural point of weakness developed better than those of shoots that grew where the callus was mechanically disrupted. Roots were completely differentiated and were developing rapidly after 4-5 weeks of being transferred to RIM. The rooted plantlets were initially moved to a culture chamber to harden, where they thrived (*Fig. 1E*). During this period, it was essential to maintain high humidity (80%) and low irradiance (30 mmol m 2/s) (*Fig. 1F*).

The healthy plantlets were then placed in plastic pots filled with an autoclaved combination of soil, sand, and vermiculite and placed in a greenhouse. The same parameters (temperature, humidity, light intensity, and photoperiod) were utilized during the first stage of transplanting as they were during the hardening stage. More than 95% of the transplanted plantlets generated new shoots within 2 weeks. The light intensity was then raised to 50 mmol m 2/s and the plantlets were maintained in the greenhouse to continue growing (*Fig. 1G-L*). The transplanted plants developed properly, and 87% of them produced morphologically normal flowers and pods with viable seeds (*Fig. 1K-L*).

Influence of genotype and explant source on shoot regeneration and transformation efficiency

Two distinct peanut varieties and two distinct explants of embryo and cotyledonary tissues were evaluated under our transformation settings to determine their impacts on shoot regeneration and transformation efficiency (*Table 2*). The experiment was repeated twice, with 55-69 explants in each batch within each duplicate. The data were statistically examined, and the confidence index was computed according to Ribas et al. (2011) Bard-479 early maturity cultivar with tiny seeds and Potohar early-maturing cultivar with long seeds. In our study, Bard-479 provided the best results as compared to Potohar.

ANOVA clearly depicted significant variations for both the cultivars on Aceto and Cefotaxime concentration. In control no chemical was injected and transformation efficiency was the least. Similarly for Bard-479 and Potohar concentration of 100 µM of acetosyringone showed the best results with highest LSD value “a”. LSD was constructed to find out the best concentration statistically. Similarly the Cefotaxime concentration of 250 mg/L showed the best results (*Table 2; Fig. 2A, B*).

The greatest proportion of shoot regeneration, with 87% from embryo-derived explants and 75% from cotyledon-derived explants, respectively. Potohar, an early ripening variety with comparatively big seeds than bard-479, had a lower regeneration percentage, with levels of 75% and 59% from embryo-derived explants, respectively, compared to the other varieties tested in other studies. The variety Bard-479, which develops very early and produces tiny seeds as compare to Potohar, had the greatest rate of shoot regeneration, with levels of 87% from embryo-derived explants and 87% from seedlings, respectively, in laboratory experiments.

It was also discovered that the transformation efficiency of various kinds and explants exhibited the same trend as that found for shoot regeneration. The greatest transformation efficiency for Bard-479 was achieved using embryonic and cotyledon-derived explants, respectively (87% and 75%, respectively).

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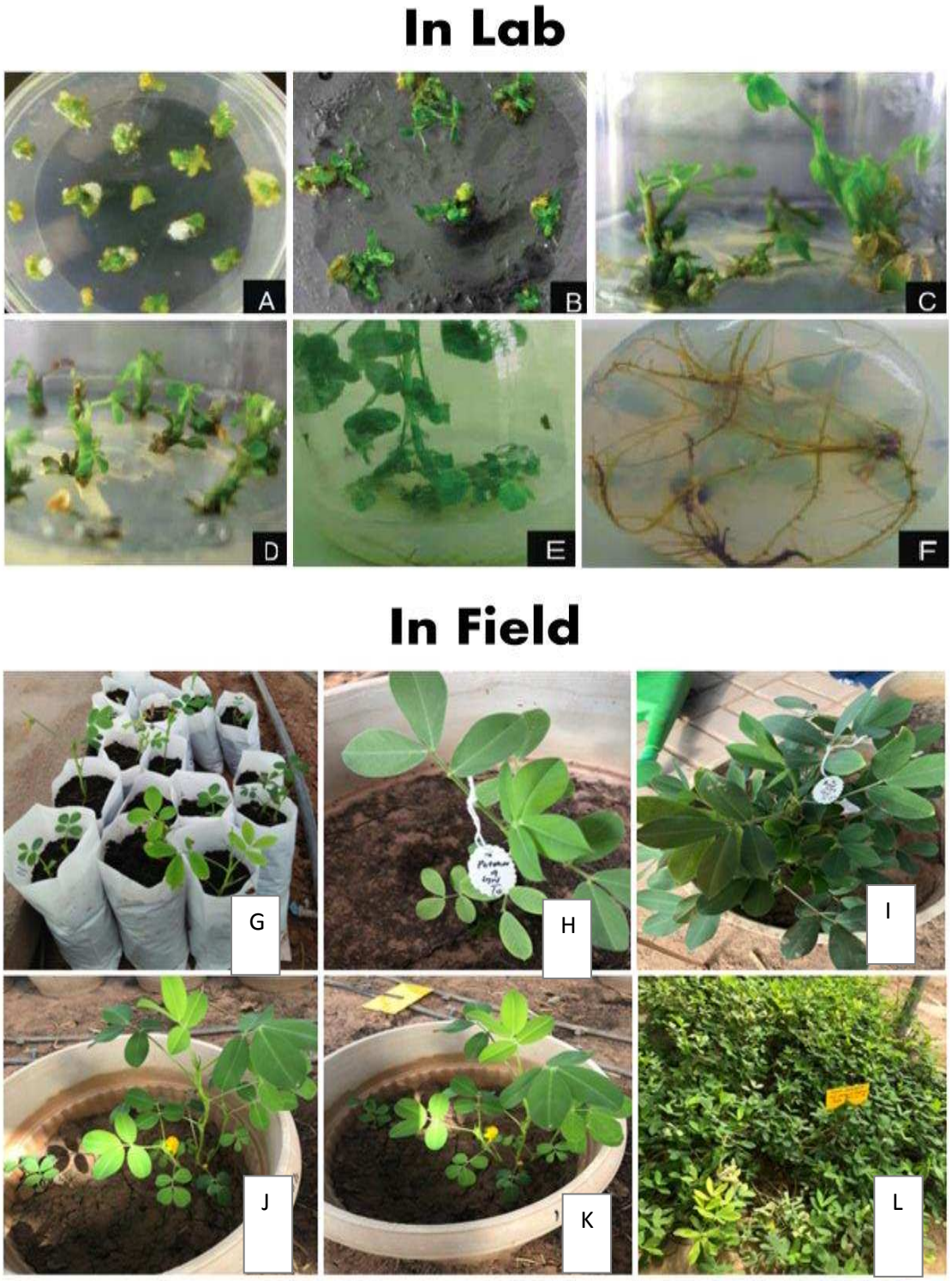

Figure 1. *(A). In Lab. Shoot regeneration from Agrobacterium-infected calli on the PAT selection medium. (B) Shoot clump formation on the strong sprout culture medium (C). Elongated shoots growing on PAT selection medium in jam bottle (D-E). Complete plantlet with vigorous roots on root induction medium in jam bottle (F). In field, (G) Hardening and acclimatization of peanut plants (H). Growing of complete transgenic peanut plant 2 weeks after transplanting Bard-479 and Potohar (I-J). Growing of flowering in transgenic peanut plant 2 weeks after transplanting Bard-479 and Potohar (K). The complete growth and development of transgenic peanut plant in the greenhouse (L)*

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Comparative studies of all genotypes revealed that the percentage of shoots regenerated from cotyledon 75% was somewhat lower than the percentage of shoots regenerated from embryo-derived explants (87%). The efficiency of transformation followed a similar pattern. As demonstrated in (*Table 2*), much greater transformation efficiency (87%) was achieved in Bard-479 using embryos as compared to Potohar from cotyledon-derived explants, indicating that embryos are more efficient at transformation.

Table 2. *Shoot regeneration and transformation efficiency of both the cultivars on application of Acetosyringone and Cefotaxime concentration*

Variety	Aceto-conc. µM	Efficiency*	Cefotaxime conc. mg/L	Efficiency*
Bard-479	0	8.3 ± 2.1^{b}	100	14.0 ± 5.6^{c}
	40	12.3 ± 3.8^{b}	150	30.0 ± 13.2^{bc}
	60	21.7 ± 1.2^{b}	200	47.3 ± 14.6^{b}
	80	55.0 ± 6.0^{a}	250	$^{**}100.0 \pm 0.0^{a}$
	100	$^{**}73.7 \pm 13.0^{a}$	300	7.0 ± 2.6^{c}
Potohar	0	12.7 ± 6.0^{c}	100	16.7 ± 5.5^{cd}
	40	11.7 ± 1.2^{c}	150	31.0 ± 14.0^{c}
	60	23.0 ± 3.6^{c}	200	52.3 ± 3.5^{b}
	80	$^{**}53.0 \pm 2.6^{b}$	250	$^{**}100.0 \pm 0.0^{a}$
	100	71.7 ± 9.5^{a}	300	8.0 ± 3.6^{d}

*Small letters indicated the LSD (least significant difference) values
**Letter "a" indicates the highest while letter "d" indicates the least difference

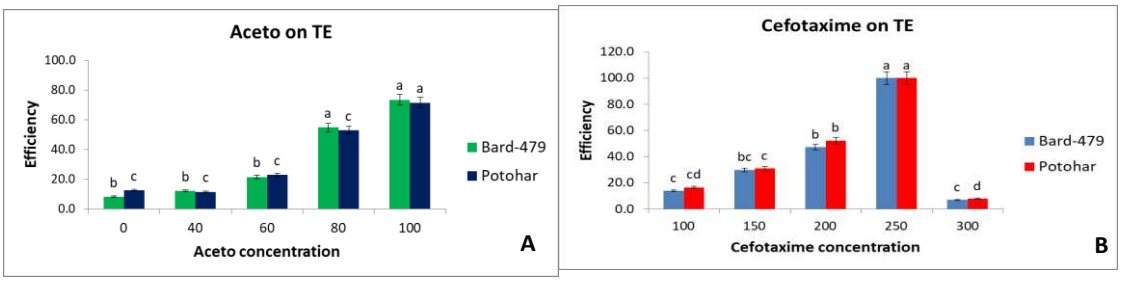

Figure 2. *Effect of Aceto and Cefotaxime concentration on transformation efficiency of both cultivars*

Effect of inoculation treatment on shoot regeneration and transformation efficiency

Between the co-cultivation and selection stages, the pre-selection period is the most significant step for transformation efficiency since it improves the 100% recovery of transgenic plants (Husaini, 2010). To prevent infected cells being directly exposed to selecting agents, a delay period (pre-selection) is needed for transgenic recovery from infection, allowing more time for stable integration of the desired gene as well as production of the selection marker gene (Zhao et al., 2004).

ANOVA clearly depicted significant variations for both the cultivars on *PAT* and Co-cultivation days' concentration. For Bard-479 and Potohar a concentration of 100 µM of

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Phosphinothricin-acetyl-transferase (*PAT*) showed the best results with highest LSD value "a". While best Co-cultivation days 5 showed best results (*Table 3; Fig. 3A, B*).

For embryo-derived explants, a comparison of two inoculation days revealed that the plant regeneration percentage and transformation efficiency were somewhat greater for 2 days of inoculation than for 5 days of inoculation. The percentages of plant regeneration were 87% and 75% for 2 and 5 days, respectively, while the corresponding transformation efficiencies were 80.3% and 67%.

Inoculating the explants in bacterial solution for 10 min was 80.3 ± 6.1^{a} marginally more efficient in Bard-479 than submerging them for 15 min, and in Potohar was 67.0 ± 7.2^{a} for ten min according to statistical analysis. When the two inoculation techniques were compared, it was discovered that combined immersion was superior to immersion alone. There were no significant variations in the percentage of shoot regeneration between the immersion technique and the optimum method when the two immersion periods were compared.

Table 3. *Effect of PAT and co-cultivation days on transformation efficiency of both the cultivars*

Variety	PAT	Efficiency*	Co-cultivation days	Efficiency*
Bard-479	50	8.3 ± 2.1^{c}	2	$^{**}80.3 \pm 6.1^{a}$
	100	$^{**}73.7 \pm 13.0^{a}$	5	66.3 ± 13.8^{a}
	150	48.0 ± 10.6^{b}	7	24.7 ± 2.1^{b}
	200	34.0 ± 5.3^{b}	15	13.3 ± 2.1^{b}
Potohar	50	10.3 ± 2.9^{c}	2	$^{**}67.0 \pm 7.2^{a}$
	100	$^{**}65.7 \pm 8.1^{a}$	5	55.0 ± 7.9^{a}
	150	53.7 ± 4.0^{ab}	7	22.7 ± 4.7^{b}
	200	33.3 ± 16.4^{bc}	15	10.3 ± 2.1^{b}

*Small letters indicated the LSD (least significant difference) values
**Letter "a" indicates the highest while letter "c" indicates the least difference

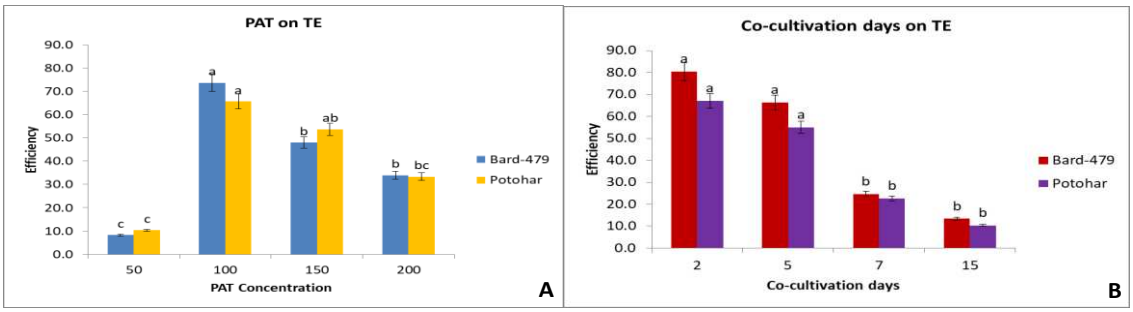

Figure 3. *Effect of PAT concentration and Co-cultivation days on transformation efficiency of both cultivars*

Effect of phytohormones on shoot proliferation and rooting

The kinds and quantities of phytohormones supplied to the SSCM before to root formation was critical for effective calli shoot growth and therefore transformation success. To investigate the effects of various concentrations and ratios of the cytokinin BAP and the auxin NAA on shoot proliferation, we selected the cytokinin BAP and the

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auxin NAA (*Fig. 2*). The proliferation ratio was highest on SSCM supplemented with 0.4 mg/l BAP and 0.1 mg/l NAA, and the respective proliferation ratios were lowest on SSCM supplemented with 0.4 mg/L BAP and 0.2 mg/L NAA as 0.875, and the respective efficient proliferation ratio with BAP 0.4 mg/L and 0.1 mg/L NAA was 17.25. The combination of BAP and NAA improved proliferation efficiency. The proliferation ratio was highest (20.25) on medium supplemented with both 0.4 mg/L 1 BAP and 0.1 mg/L NAA through embryo-derived explants and was comparably high (16.0) on medium supplemented with both 0.4 mg/L BAP and 0.1 mg/L NAA through cotyledon-derived explants, according to ANOVA analyses of the six combinations tested. On the six combination media, the efficient proliferation ratios were 20.25 and 16.0, respectively. The two other combinations examined (0.2 mg/L BAP coupled with 0.1 mg/L NAA and 0.2 mg/L BAP mixed with 0.5 mg/L NAA) had reduced shoot proliferation. These findings suggest that a 4:1 BAP/NAA ratio is acceptable, and that a combination of 0.4 mg L 1 BAP and 0.1 mg L 1 NAA is effective for shoot proliferation. The rooting media was changed to provide optimal conditions for root development and growth. Six media were compared, each supplemented with various amounts and ratios of IBA and NAA (*Fig. 3*). On medium enriched with 0.2 mg/L IBA and 0.3 mg/L NAA, the rooting percentage was greatest (almost 95%). The medium supplemented with 0.2 mg/L IBA and 0.1 mg/L NAA had the lowest rooting percentage. The three other media examined, which included 0.3 mg/L IBA alone, 0.2 mg/L NAA solely, or a combination of 0.3 mg/L IBA and 0.1 mg/L NAA, had no significant variations in rooting percentage (90.00-91.78%). These findings suggest that the phytohormones ratio had a significant role in transgenic shoot roots. Each experiment was repeated three times, the data was statistically evaluated, and standard error values for each duplicated set were produced.

PCR analyses of transgenic peanut plants

Independent transformed plants produced from the same *Agrobacterium*-infected explant were numbered and kept separate for DNA analysis and future generation propagation. Each transformants seeds were dried and kept at 4 °C until needed. The EPSPS gene of the presumably altered plants was detected via PCR analysis. The existence of the gene in all of the plants examined (*Fig. 4A, B, C*) was confirmed by DNA fragments of the expected sizes, indicating that PAT selection is very effective. Neither fragment could be amplified from DNA taken from an untransformed plant or a blank negative control. The inheritance of the EPSPS gene in the T1 generations was also used to determine transgenic transmission in future progenies. In the T1 generation, PCR results revealed a 3:1 segregation pattern.

Discussion

Transformed peanut plants were successfully recovered after *Agrobacterium-mediated* transformation of derived explants from two different peanut varieties. On an average, 105-130 days were needed between the start of explant transformation and the transfer of rooted plants to the greenhouse. It was shorter than the 120 to 150 days reported by Sharma and Anjaiah (2000), Sharma and Ortiz (2000). Although *Agrobacterium mediated* transformation of peanut was reported previously, these studies revealed a marked disparity between the conditions considered to enable optimal efficiency of transformation (Iqbal et al., 2012).

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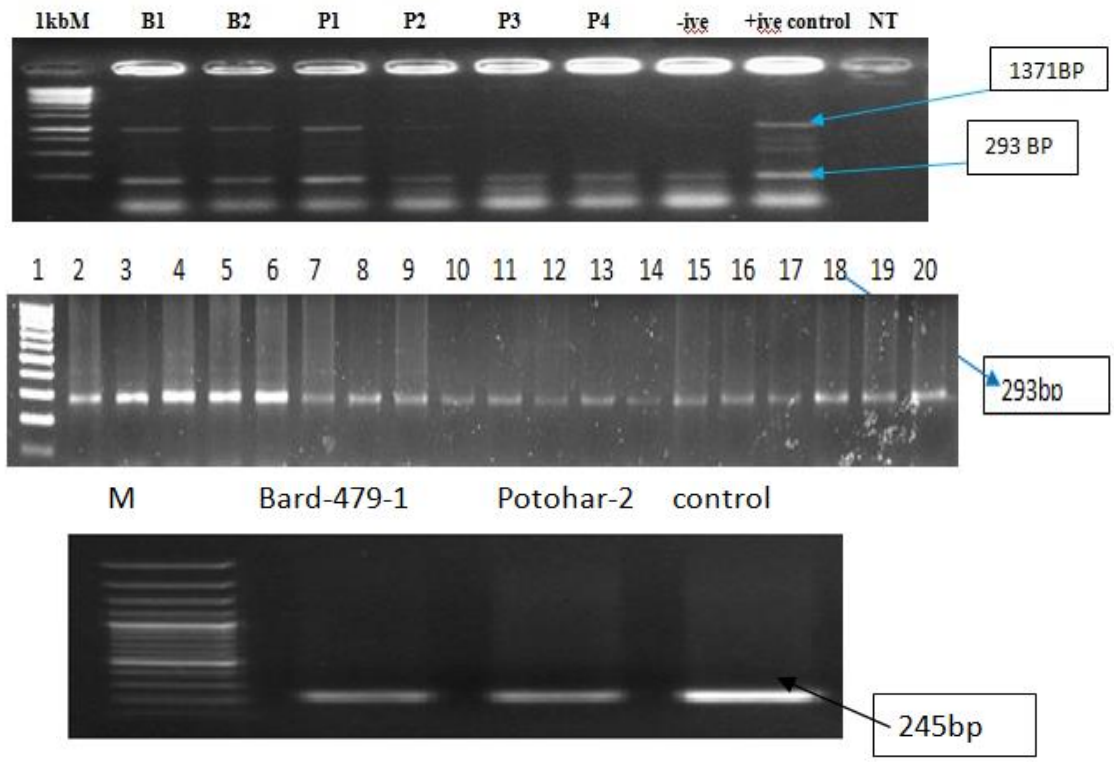

Figure 4. *(A) RT- PCR screening for EPSPS AND PAT genes in putative Peanut transgenic plants Lanes M 1 kb Ladder (Fermentas), Lane P(9) Positive control (Plasmid), Lane N (10) Negative control (non transgenic Peanut), Lane B1 and B2 for bard-479 transgenic plants and Lane (P1-P4) transgenic plants of Potohar. (B) Segregation analysis of Peanut lines produced by self-fertilized T1 progeny by Mendelian inheritance ratio (3:1). (I) Segregating analysis of Bard-479 transgenic line (II) Segregating analysis of Potohar Transgenic line. (C) Segregation analyses of Peanut lines produced by self-fertilized T1 progeny by Mendelian inheritance ratio (3:1). (I) Segregating analysis of Bard-479 transgenic line (II) Segregating analysis of Potohar Transgenic line*

The current study demonstrated that factors such as genotype selection, explant source, *Agrobacterium* interaction efficiency with target tissue cells, and a reproducible complete plant regeneration protocol are critical for successful peanut transformation. The auxin combination used to induce rooting from transgenic shoots, as well as the transplantation procedures, play critical roles in ensuring successful *Agrobacterium-mediated* transformation. Given the recalcitrance of legumes to transformation, there has been limited progress in their improvement using transgenic approaches, and this is especially evident in the case of peanut.

A major limitation of the available in vitro regeneration protocols for legumes is that they are highly genotype-specific, with only a few genotypes amenable to regeneration after *Agrobacterium-mediated* transformation (Matand and Prakash, 2007; Bhattacharjee et al., 2010). We developed two phenotypes of peanuts separately for this study: one (Bard-479) that matures early and has relatively tiny fruit, and Potohar that matures early and has very big fruit. Explants for transformation were obtained from two target tissues: embryo and cotyledon. It was discovered that Bard-479 had greater regeneration percentage and transformation efficiency than Potohar.

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The regeneration percentage of shoots from cotyledons was somewhat lower than that of shoots from embryos, but transformation efficiency was much greater with embryo-derived explants than with cotyledon-derived explants. The majority of earlier research on *Agrobacterium-mediated* peanut transformation utilized leaf discs, cotyledons, cotyledonary nodes, and embryo axes as target tissues (Anuradha et al., 2006; Iqbal et al., 2012). Explants produced from cotyledons are ideal for the transformation and regeneration of fertile plants, including agricultural species such as chickpea, rapeseed, and peanut (Bhattacharjee et al., 2010; Iqbal et al., 2012). However, our comparative studies using cotyledon- and embryo-derived explants revealed that embryo was superior to cotyledon as a vehicle for *Agrobacterium-mediated* peanut transformation.

Embryos incubated for 2 days and cotyledon explants cultured for 5 days on pre-selection media were shown to be suitable for explant rehabilitation after transformation therapy in the current research. *PAT*-selected explants producing shoots had better survival rates after 2 days of pre-selection in embryo-derived explants and 5 days in cotyledon-derived explants. With increasing pre-selection time, the survival percentage of chosen explants dropped.

Sun et al. (2011) investigated the genetic transformation and regeneration of pear leaf disc segments using pre-selection medium containing 250 mg/l cefotaxime and 100 µM *PAT* for two days in the dark at 250 °C and found that the explant shifted to pre-selection medium containing 250 mg/l cefotaxime and 100 µMm *PAT* yielded the highest transformation efficiency of 87%. The impact of various factors and inoculation times on encouraging regeneration and transformation were compared in this research.

Furthermore, peanut genotypes with early maturation and tiny fruit (Bard-479) seem to be more vulnerable to transformation than Potohar examined. The interaction of *Agrobacterium* with target tissue cells was also shown to be a significant restriction for effective transformation. The *Agrobacterium* strain *LBA4404* containing the binary vector *PxCSG m-YFP* was grown to the mid-log phase of the growth cycle (A600 0.6) for this research. *LBA4404* has been shown to be more successful than other strains in the transformation of legumes such as chickpeas (Senthil et al., 2004). However, the efficacy of other techniques of inoculation does not seem to have been studied, with the immersion approach being virtually exclusively utilized (Jones et al., 2005; Bhattacharjee et al., 2010).

The current research looked at the consequences of changing the immersion technique. The inoculation technique, in which the explants were immersed in bacterial solution, was a significant advancement in peanut research. Our findings indicate that combining immersion allowed for more efficient transformation than immersion alone. Comparative studies of the factors revealed that immersion duration had an effect on both the plant regeneration percentage and the transformation efficiency. Given that this seems to be the first time the impacts of these variables on *Agrobacterium* infection have been studied, the findings may be applicable to other plant species than peanut. Other techniques for promoting root development, in addition to RIM culture, include hydroponics culture and in vitro grafting using scions from pre-existing seedlings (Dutt and Grosser, 2010). However, such technologies are technique-specific, time-consuming, and sometimes have success rates that differ across labs. Previous studies found that rooting media enriched with IBA or NAA alone was effective for root development of transgenic peanut and other legumes (Anuradha et al., 2006; Iqbal et al., 2012). The current research compared rooting medium added with IBA or NAA alone

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to rooting media supplied with IBA and NAA in various ratios. The findings showed that rooting was effective on every medium, but the efficiency of rooting was influenced by the various kinds and ratios of phytohormones. The combination of 0.3 mg L 1 IBA and 0.1 mg L 1 NAA allowed nearly all transgenic peanut shoots to root. Root development should be aided by vigorous branch growth prior to rooting. Roots produced in vitro are often extremely weak and do not survive the transition from tissue culture medium to soil (Dutt and Grosser, 2010; Geng et al., 2011). The root systems we acquired, on the other hand, were robust and active. This was evident in their high rates of survival following transplanting, which was most likely due to the vigor and vitality of the plantlets produced through robust shoot culture. More than 95% of the transplanted plantlets developed new shoots within 2 weeks, and 87% of the transplanted plants produced morphologically normal flowers and pods with viable seeds. The survival rate was considerably greater than the stated figure of 58-75% in many investigations (Anuradha et al., 2006; Iqbal et al., 2012). Furthermore, the composition and concentration of phytohormones contained in the SSCM played a significant function in encouraging efficient shoot growth and therefore allowing transformation success. When compared to other phytohormones compositions, the efficient proliferation ratio of shoots was greatest on the medium supplemented with 0.4 mg L 1 BAP and 0.1 mg L 1 NAA (87% and 75%, respectively). The purpose of this research was to look into the impact of *Agrobacterium-mediated* transformation on peanut shoot growth. Another important factor for effective *Agrobacterium-mediated* transformation is high survival efficiency following transplanting from tissue culture medium to soil. However, there have been no studies on optimizing the conditions for transferring converted peanut plantlets from culture to soil. In this research, regenerated plantlets with strong root systems were moved to a culture chamber for hardening before being transferred to soil and grown in a greenhouse. Moisture, temperature, light intensity, and nutrition were all regulated throughout the process. The transplanted plantlets developed normally as a result of these treatments, with more than 87% of them generating new shoots in two weeks. More than 87% of the transplanted plantlets generate normal-looking blooms and pods with viable seeds.

Conclusion

The protocol for *Agrobacterium-mediated* peanut transformation reported here enables highly efficient regeneration of adventitious shoots from explants derived from either cotyledons or embryo from two peanut genotypes. For all two genotypes, *Agrobacterium mediated* transformation of embryo enabled more superior regeneration and transformation efficiencies than cotyledon. Additionally, transgenosis is viable in peanut genotypes that mature early and have relatively small fruit (Bard-479) when using the transformation and regeneration protocols defined here. Submersion of *Agrobacterium* solutions in which target tissues were submerged increased the efficiency of transformation relative to submersion alone.

The current study also identified an efficient method for vigorous rooting and transplantation of the transgenic plantlets. Analyses by PCR indicated that the PAT selection used to identify transformants was highly efficient. The availability of an efficient and reliable transformation procedure in peanut will soon be indispensable for the vast increase in functional genomics studies enabled by the availability of the complete genome sequence of peanut. The article aimed to investigate the genetic

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transformation of the EPSPs herbicide resistance gene of peanut mediated by an *Agrobacterium tumefaciens* strain, as well as to determine the this method is useful in peanut breeding and functional genomics research.

Furthermore protocol must be optimized for each variety due to varietal effect and result may differ for different varieties. Varieties that have high transformation efficiency should be used in order for successful transformation. Further conventional approaches may take lot of time and in present day scenario time is not luxury, and non-conventional techniques like tissue culturing, transformation, Crisper, etc. must be adopted. Similarly successful transformation is totally different from hardening of plant in screen house so proper agronomic practices must be followed even if the variety performs best in term of transformation.

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EFFECTS OF LAND USE CHANGE ON THE RIPARIAN ZONES' QUALITY ALONG THE ZAT RIVER AND ITS TRIBUTARIES: HIGH ATLAS OF MOROCCO

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Abstract. This study was conducted to assess the quality of riparian zones of Zat basin of Morocco by using the QBR index, and to analyze land use changes over 30 years along the Zat River and its tributaries by using remote sensing and Geographic Information System. Five land use/land cover classes were identified: Forest, building area, water, bare soil, and agricultural land using Landsat images. Also, the QBR index was evaluated in 14 localities distributed along the Zat River and its tributaries from 2018-2019. For instance, the total forest area was reduced from 959.07 ha (15.35%) in 1990 to 890.41 ha (14.25%) and 713.25 ha (11.41%) in 2005 and 2020, respectively. Whereas agricultural land and building area classes increased with an estimated rate of change of 24.99% and 33.81% respectively over the last 30 years. Furthermore, our results indicate that 35.7% of the banks in the riparian zone are of very poor quality, 14.3% of poor quality, 28.6% of average quality and 21.4% of good quality. The finding demonstrates that the low QBR score obtained by some sampling localities especially downstream is the result of multiple anthropogenic interventions in riparian ecosystems, including forest plantations, land use for agriculture, and road infrastructures.
Keywords: *QBR index, LULC changes, riparian vegetation, remote sensing, Zat basin, Morocco*

Introduction

The riparian forest also called "riparian buffer" corresponds to the transition zone between the aquatic and terrestrial environments. It is composed of a group of trees, shrubs, and herbaceous plants and its main role is to maintain water quality (Naiman and Decamps, 1997; Bertoldi et al., 2011). Due to its filtration capacity, the riparian buffer strip performs many functions: it contributes to the reduction of non-point pollution of surface water by reducing the nutrient and sediment load of runoff from agricultural land, it serves as a refuge for biodiversity (fauna and flora), and it also provides protection against erosion and limits the rate of evaporation (Pusey and Arthington, 2003; Stevaux et al., 2012). In addition to its ecological, remediation, and protection functions, it also plays a very essential role in maintaining the integrity and aesthetics of the landscape (Naiman and Decamps, 1997). One of the characteristics of riparian strips is the edge vegetation (Ripisylves) which, in addition to its role in structuring landscapes, through its root system, serves to stabilize the banks and provide refuge areas for wildlife (Ater et al., 2008; Naiman and Decamps, 1997).

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However, changes in the use of land surrounding water bodies and pollution have had a deep impact on the functionality of riparian ecosystems (Jetz et al., 2007). These changes lead to modification in species richness, species composition, and species relative abundance (Pereira et al., 2012), and may also lead to the introduction of exotic plant species (Hood and Naiman, 2000; Richardson et al., 2007) which can cause the extinction of local plant species, which have severely degraded their structure and ecological function (Cushing et al., 1995; Nilsson and Berggren, 2000; Hughes and Rood, 2003).

Since it plays an essential role in maintaining the integrity of a hydro system, there is undoubtedly a need for methods to guide managers in maintaining and restoring these complex systems. The development of methods to quickly and effectively assess the ecological status of riparian ecosystems has received a great deal of attention in recent times, and it is on this basis that sampling techniques and methods have been developed by several researchers to assess the biological and ecological quality of riparian strips (Stella et al., 2013; Valero et al., 2014).

The QBR ("riparian forest quality") index, proposed by Munné et al. (2003), is currently the most widely used measure for riverbank analysis; it is used to describe the quality of in situ vegetation. The QBR index is useful for assessing riparian forest quality and the degree of bank alteration in four distinct components; three of these are based on the characteristics of riparian vegetation (cover, structure, and nature), while the fourth refers to changes in the river channel (Munné et al., 2003).

The QBR index has already been tested in Mediterranean streams (Gonzalez del Tanago and Anton, 1998; Prat et al., 1999; Carrascosa and Munné, 2000; Suarez and Vidal Abarca, 2000; Martinez and Lozano, 2004; Gonzalez del Tanago et al., 2006; Gonzalez del Tanago et al., 2010; Stella et al., 2013) with good results. In Morocco, the studies on riparian zones are those carried out by Ater et al. (2008), which focused on the structure and diversity of riparian zones and how they constitute a refuge for the avifauna in the Laou River. Another example is Ennabili (1999) who studied the ecology and context of hygrophilous vegetation and its role in wastewater treatment. Similarly, Ater et al. (2008) and Khamlichi et al. (2008) studied riparian or riverine vegetation in the Laou and Tahddart basins respectively to highlight the structure and diversity of riparian vegetation.

Like other Moroccan river systems, the Zat basin has undergone several changes in time and space, including deforestation, the elimination of natural vegetation in favor of cultivated areas, overgrazing, and the occurrence of natural disasters; As a result, the water resources of the rivers of Zat and its tributaries are being depleted day after day (Mostakim et al., 2021; AHT Group, 2016; Ait Mlouk et al., 2015). The Zat basin has been the subject of several sectoral and/or localized studies (Mostakim et al., 2021; Mostakim et al., 2020; Ait Mlouk et al., 2015), however, little is known about the chronological trend of Land Use Land Cover (LULC) in the Zat basin and assessment of riparian zone quality using QBR index. Based on the landscape of this area, the LULC assessment is carried out on the following five categories, namely: Building (all land covered by infrastructure), Water Body (all surface water areas), Vegetation (area covered by deciduous or coniferous woody vegetation), Agricultural Land (land used for the production of agricultural products) and Bare Soil (non-forested, non-agricultural land). This study considered 1990 as the base year and examined LULC from 1990 to 2020 to assess the trend of change over the past 30 years.

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This paper attempts to improve thematic knowledge on changes in land use and landscape structures and to describe the ecological status of riparian zones in Zat basin, using the QBR index and to analyze how the quality of riparian vegetation and the species composition is related with altitude, species richness, and proportion of exotic species of the study area.

Materials and methods

Study area

The study was carried out in the Zat basin in High Atlas of Morocco (*Fig. 1*), it is part of the hydraulic system of the Tensift watershed, between 31° 17' and 31° 32' North and 07° 29' and 07° 34' West. It is drained by the Zat River with a length of 89 km, which has its source at the foot of Taska n'Zat (3905 m) on the right bank and Tougroudaden (3736 m) on the left bank and drains a catchment area of about 525 km². The surface area of the riparian zones is approximately 62.47 km² (*Fig. 1*).

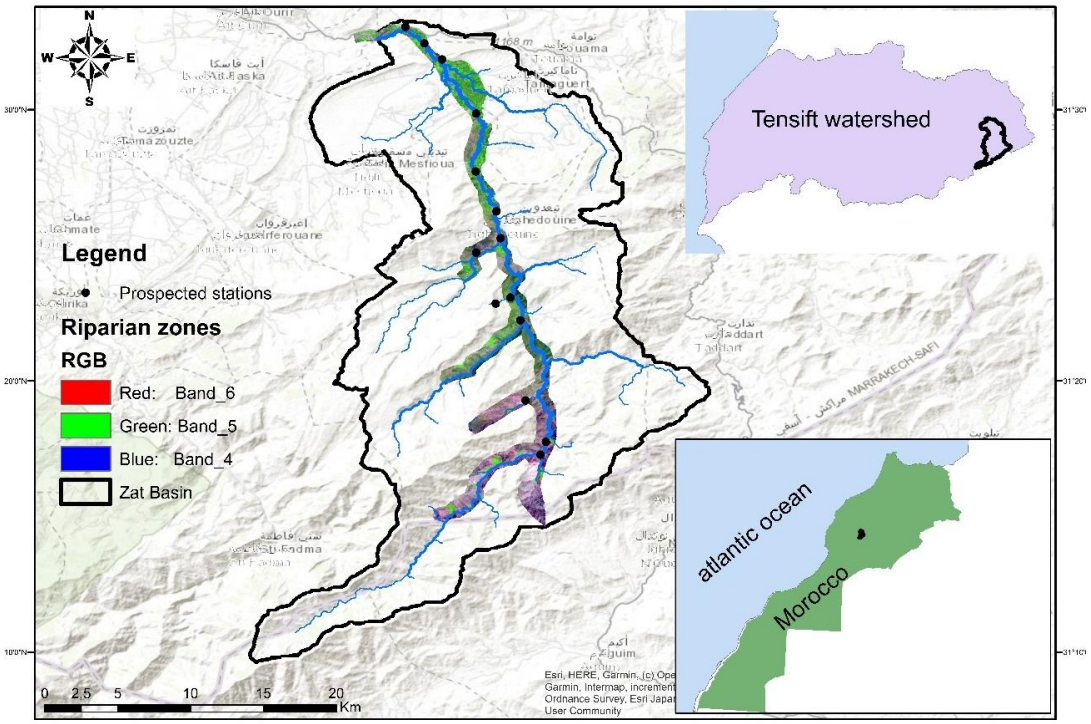

Figure 1. *Geographic location of sampling localities of riparian zones of Zat basin*

The Zat basin is bounded to the east by the Ghdat sub-Basin, to the south by the High Atlas Mountains, to the north by the Tensift watershed, and to the west by the Ourika sub-Basin (*Fig. 1*). It is characterized by an arid to semi-arid climate downstream and sub-humid in the high mountains (Ait Mlouk et al., 2015). The type of climate in this region is Mediterranean with a cold and rainy winter (average annual rainfall of 382 mm) from October to April and a hot and dry summer (5.2-37.1 °C) from May to September (Lovich et al., 2010; AHT Group, 2016). It is a perennial mountain stream, largely fed by snowmelt, with a pebble substrate. Clearwater supports dense accumulations of filamentous algae on the bedrock substrate (Lovich et al., 2010).

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Sampling

Data acquisition and image processing

The data used for the Zat basin consist of Landsat satellite images and GPS data collected during fieldwork, these Landsat images are an important tool in land use mapping and resource planning and management, they are descriptive and provide spatial and spectral information, much more important than other sources of information (Uddin and Gurung, 2010). Multi-date Landsat images from 1990, 2005 and 2020 (*Table 1*) captured by satellite and downloaded from the USGS (United States Geological Survey; (http://earthexplorer.usgs.gov/) website by selecting those with the least disturbance, including cloud cover. The period of time when the images were taken was also taken into account in order to be able to make an effective comparison between the different dates. Since the site is often swampy and flooded during the rainy season and dry in summer, priority was given to satellite images taken during the summer season (July and August) to visualize the different land uses. Image data were used to generate the LULC for the study period.

Table 1. *Satellite data specification*

Satellite	Sensor	Path/Row	Year of acquisition	Spectral bands	Resolution
Landsat-1	MSS	146/44	15/4/1990	G, R, NIR, 4,5,6,7	60 + m
Landsat-5	ETM +	136/44	26/4/2005	G, R, NIR, 2,3,4,	30 m
Landsat-8	OLI	136/44	02/5/2020	G, R, NIR, Panchromatic, 3,4,5,8	30 m (For Panchromatic 15)

Image processing and classification

For image processing and supervised classification of satellite images, we have performed radiometric calibration of the sensor and atmospheric corrections of our images. These operations were carried out by combining them in a single step to preserve radiometric integrity (Maimouni et al., 2011). Sensor calibration allows the conversion of the digital number (DN) to visible luminance to correct sensor-specific anomalies and to obtain accurate, reliable, and precise information (Uddin and Gurung, 2010). The calibration coefficients published in the metadata file were used for each image. This apparent luminance was then transformed into apparent reflectance by introducing the solar illuminance, the angle of incidence, and the "Sun-Earth" distance. In the latter case, we convert the apparent reflectance into ground reflectance using the parameters of the acquisition date, solar zenith angle, atmospheric model, aerosol model, and ground visibility (Uddin and Gurung, 2010; Chowdhury, 2018). Next, all satellite data were studied by assigning pixel signatures in ArcGis 10.2.2. The entire area of the Zat basin was differentiated into five classes: building area, forest, agriculture land, water body, and bare soil, which are described in *Table 2*.

Table 2*. Classes delineated based on supervised classification of Landsat image.*

Class name	Description
Building area	Areas designated as residential, commercial, industrial, scattered rural settlements with forests, roads and transportation
Water	Rivers, lakes, ponds and reservoirs, as well as wetlands in the wet season and dry areas in the dry season, perennial wetlands and riparian vegetation
Forest	Areas covered with natural and planted trees
Agriculture land	Cultivated fields and fallow land
Bare soil	Exposed soil and barren area

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Land use/cover changes

After classifying the images, the geographic extent in terms of hectares for the land use and land cover class was calculated for each period mentioned and the magnitude of the change in land use type during and between periods was compared. The change in the different land use and land cover classes was carried out using ArcGIS 10.2.2 software and finally, the following calculation was used to find out the rate of change in hectares/year and the percentage share of each class in the periods studied.

$$\Delta A(\%) = \frac{At2-At1}{At1} \times 100 \quad \text{(Eq.1)}$$

where: ΔA(%) = percentage change in the area of land use and land cover class type between initial time At1 and period At2 At1 = area of land use and land cover type at initial time At 2 = area of LULC type at final time As stated by Abate (2011) the rate of change of LULC type was calculated by the following formula:

$$R\Delta(ha/year) = (Z - X)/W \quad \text{(Eq.2)}$$

where: RΔ = rate of change Z = recent area of LULC type in ha X = previous area of LULC type in ha W = time between Z and X in years.

QBR index

The QBR index (Munné et al., 1998a, b, 2003) was applied, without modification, to assess the condition and the quality of riparian vegetation in both streams of 14 localities distributed along the Zat River and its tributaries from 2018-2019. This index focuses on four fundamental aspects of riparian systems: (1) the degree of vegetation cover, (2) vegetation structure, (3) vegetation cover quality, and (4) the degree of naturalness of the river channel. Vegetation cover assesses the connectivity between the riparian zone and adjacent terrestrial ecosystems by considering the percentage of tree, shrub and helophyte cover, in conjunction with the connection to the adjacent terrestrial community. Vegetation structure assesses the structural complexity of the riparian ecosystem, considering that environmental heterogeneity can increase its biodiversity. To evaluate it, the percentage of presence of each functional group and the presence of plantations and isolated plots in the riparian zone were taken into account. The quality of the vegetation allows us to determine the naturalness of the plant formations present, which is assessed based on the number of native species in the sampling area and depends on the geomorphological type of the riparian zone. The determination of plant composition was carried out according to the European standard for the study of macrophytes in rivers (Känel et al., 2017).

The degree of naturalness of the river channel has mainly taken into account anthropogenic modifications to the bed, those that modify, alter and disturb the riparian habitat. Each section was assessed independently at each sampling locality, with a score from 0 to 25, and the sum of the scores of the four sections fluctuated between 0 and 100, each aspect is initially scored with one of four values: 0, 5, 10 or 25; intermediate values cannot be scored.

The resulting quality values are divided into five ranges: very good quality, natural condition (QBR ≥ 90); good quality, slightly disturbed vegetation (QBR 75-90); intermediate quality, significant alteration (QBR 55-70); poor quality, significant

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alteration (QBR 30-50); and poor quality, extreme degradation (QBR ≤ 25). To calculate the QBR index, a 100 m long section (with an average separation of 1 km) was selected at each sampling station, and the full potential width of the forest and/or riparian vegetation was taken into account. It is the sum of four scores, based on four aspects of riparian quality (Munné et al., 2003).

Statistical analysis

All the sampled localities were ranked using the value of QBR index, considered as an indicator of the conservation status of riparian forests. We evaluated whether riparian forests with less exotic species, higher species richness, and higher altitudes had a more suitable conservation status. For this purpose, we performed Pearson correlations between QBR and plant species richness, percentage of exotic species, and altitude. This statistical analysis is to evaluate whether any of them have more influence or better explains the variations in the quality of the banks.

Results

Land use/cover change detection from the year 1990-2005

The dynamics of land use in the Zat basin between 1990 and 2005 show a strong anthropization of natural ecosystems. *Table 3* shows that the area of forest ecosystems has considerably decreased by -7.16%, from more than 959.07 ha in 1990 to less than 890.41 ha in 2005. An important part of these plant formations has been transformed into agricultural fields and building areas which respectively occupy an area of 1340.63 ha and 1898.56 ha of the riparian zones in 2005. On the other hand, the water class experienced a negative change with a rate of change of -44.76% and an average annual change of -27.35 ha/year (*Fig. 2)*.

Table 3. *Land use change assessment of Zat basin of Morocco over the last 30 years*

Classes of land use/land cover	1990		2005		2020	
	Area (ha)	Coverage rate (%)	Area (ha)	Coverage rate (%)	Superficie (ha)	Coverage rate (%)
Forest	959.07	15.3509	890.41	14.2520	713.25	11.4163
Agriculture lands	1030.96	16.5016	1340.63	21.4582	1561.63	24.9956
Build up area	1728.13	27.6606	1898.56	30.3885	2112.22	33.8083
Bare soil	1612.74	25.8136	1611.56	25.7947	1342.01	21.4803
Water	916.73	14.6732	506.36	8.1048	517.63	8.2852
Riparian zones	6247.63	100	6247.63	100	6247.63	100

Land use/cover change detection from the year 2005-2020

From 2005 to 2020, there is a significant decline in forest class with a rate of -19.90% (*Table 3*). The average annual evolution of the forest class is -11.81 ha/year, it has been mainly replaced by agriculture. During this period, the share of bare soil also decreased significantly, with an average annual change of -17.96 ha/year, i.e. a rate of change of -16.72% compared to the previous period, and was mainly converted to agriculture and urban construction, the latter two experiencing respectively an average

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annual change of 14.73 ha/year and 14.29 ha/year, i.e. a rate of change of 16.48% and 11.29%. The category that was mainly changed in 2005 is that of the forest. There was a decrease in the share of forest ecosystems from -7.16% to -19.90%, with an average of -11.81 ha/year (*Fig. 2*).

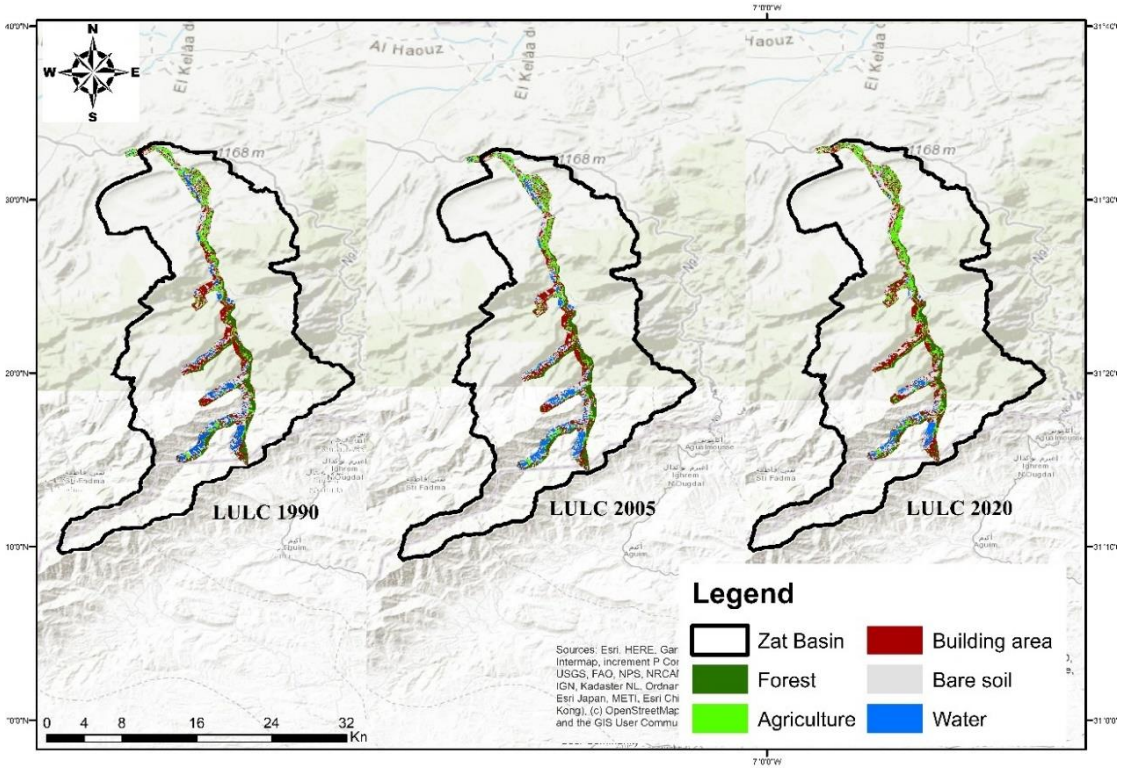

Figure 2. *Relative changes in Land use/Land cover of riparian zones in Zat basin of Morocco between 1990 and 2020*

Land use/cover change detection from the year 1990-2020

An overall comparison of each LULC class from 1990 and 2020 shows that there has been a considerable change over the past 30 years. Over this period, the forest and bare soil class in the riparian zones have decreased by -25.63% and -16.78% respectively (*Fig. 2*), with an average annual change of -8.19 ha and -9.02 ha. While the agricultural and urban land class have increased significantly over the last 30 years with an estimated rate of change of 51.47% and 22.26% respectively, equivalent to an average annual change of 17.69 ha and 12.82 ha (*Fig. 3*).

QBR index

The results obtained indicate that the QBR value of the Zat River and its tributaries ranged from 15 to 85 (*Table 4*). Approximately 35.7% (5 localities) of the all the sampled localities are with extreme degradation and worst Quality (including QBR index ≤ 25), 14.3% (2 localities) with strong alteration, and poor quality. However, 28.6% (4) of the study areas are of "Fair Quality" and almost 21.4% (3 localities) stations are of "Good Quality" ($75 <$ QBR Index ≤ 90) (*Fig. 4*). It should be noted that there is no locality with natural habitat characteristics, i.e., with a QBR index ≥ 95.

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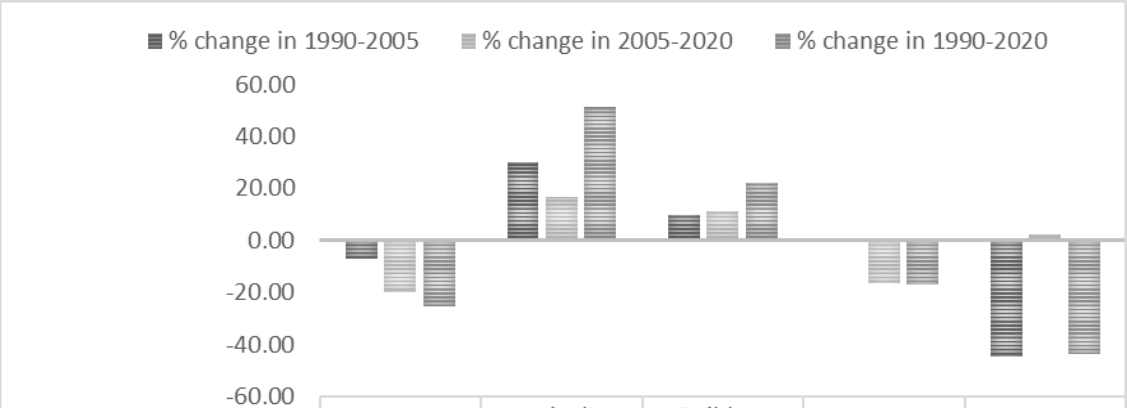

	Forest	Agricultu re lands	Build up area	Bare soil	Water
% change in 1990-2005	-7.19	30.04	9.86	-0.07	-44.76
% change in 2005-2020	-19.90	16.48	11.29	-16.72	2.23
% change in 1990-2020	-25.63	51.47	22.26	-16.78	-43.53

Figure 3. *Relative changes in Land use/Land cover of Za basin of Morocco over the last 30 years between 1990 and 2020*

Table 4. *Coordinate system, Elevation, percentage of native and exotic species collected, and QBR index score of each sampled localities of Zat basin of Morocco*

N° of station	Name	Coordinate system (X-Y)		Elevation (m)	% of native species	% of exotic species	Specific richness	QBR score
		Latitude (Y)	Longitude (X)					
1	Tafriat	31°32'38.81"N	7°35'53.51"W	733	20	80	11	15
2	Talbanine	31°32'54.18"N	7°35'1.20"W	746	24	76	13	20
3	Imin tghrist	31°31'51.2" N	7°33'43.8" W	819	60	40	18	35
4	Timzilite	31°29'51.68"N	7°32'27.98"W	850	40	60	12	20
5	Tassourte	31°27'43.33"N	7°32'28.73"W	965	35	65	13	20
6	Igherm Melloulne	31°26'14.9" N	7°31'42.6" W	1043	86	14	23	35
7	Tighadouine	31°25'15.7" N	7°31'33.5" W	1038	73	27	25	20
8	Tamal	31°24'43.7" N	7°32'27.9" W	1149	100	0	19	60
9	Ait slimane	31°22'13.98"N	7°30'49.80"W	1245	89	11	17	55
10	Yagour 1	31°23'4.54"N	7°31'11.90"W	1953	90	10	15	75
11	Yagour 2	31°22'51.01"N	7°31'44.52"W	2120	100	0	18	85
12	Ikkis	31°19'7.07"N	7°30'49.14"O	1456	96	4	23	80
13	Tizart	31°17'44.97"N	7°29'53.08"W	1520	80	20	19	55
14	Wansa	31°17'16.7"N	7°30'5.00" W	1430	78	22	17	55

Stations of "very poor quality" (QBR < 25) (1, 2, 4, 5 and 7) are mostly characterized by low vegetation cover on both the right and left sides of the shoreline, and poor connectivity between the riparian zone and adjacent terrestrial ecosystems, these localities also showed the poor quality of structure and cover, indeed these localities were marked by the non-negligible presence of exotic plant species such as *Nicotiana glauca* L. *Ricinus communis* L. *Fraxinus angustifolia* L. and *Dittrichia viscosa* L.).

Also, the localities with poor QBR quality (3 and 6) are marked by low tree cover on both banks of the creek with slight connectivity between the different strata, and poor

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quality of cover structure due to the presence of human and agricultural activities, as well as the localities with the lowest quality, we evaluated in these two stations the presence of exotic plants such as *Fraxinus angustifolia* and *Phragmite australis*, which affect the quality of the creek cover.

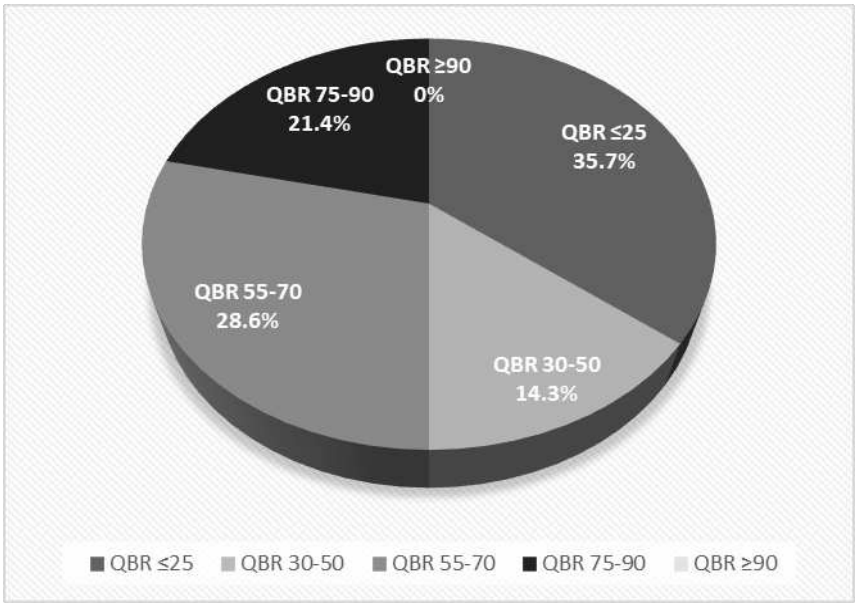

Figure 4. *Percentage of sampling localities over the total number sampled in each quality class according to the QBR index*

Then, the localities (8,9,13, and 14) with average QBR quality, (50 < QBR-index < 70) are characterized by an average vegetation cover and show alterations in the river (and its surroundings) such as the presence of roads, retaining walls for flood protection, and are marked by the presence of arboriculture and planted crops (*Ficus carica* L. and *Juglans regia* L.) in the riverside vegetation as well as dwellings on the river terraces.

Finally, the localities with a QBR value > 75 (10, 11, and 12), are the best in terms of the quality index (good quality) and have good vegetation cover in terms of quality, structure, and unmodified river channels on the left and right sides of the bank. They are located upstream of the river (higher altitudes). This area is an important reservoir of native riparian vegetation, acting as a buffer against increasing anthropogenic threats.

Significant alterations were identified in the quality of riparian vegetation using the QBR index, species richness, and the rate of exotic plants. Our results indicated that there is a significant correlation between the QBR index and altitude ($r = 0.72$, $N = 14$, $p = 0.01$) (*Table 5*), and the percentage of exotic plants ($r = -0.71$, $N = 14$, $p = 0.001$). We also obtained a non-significant correlation between the QBR index and the species richness of aquatic plants ($r = 0.40$, $N = 14$, $p = 0.01$).

Discussion

The results of this study investigate the effect of land use/land cover changes on the quality of riparian zones, particularly in the Zat basin in Morocco. This change has a negative impact on water resources throughout the watershed. The surrounding land

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cover change had a significant impact on both suspended solid and nitrate nitrogen loadings (Chu et al., 2013; Ding et al., 2015). Furthermore, Schilling et al. (2010) and Adusumilli et al. (2011) showed that agricultural activities have an important impact on the quality of the water more than climate change. So, the increasing of agricultural activities and settlements within Zat basin is a matter of great concern.

Table 5. *Pearson correlation coefficients matrix between the QBR index and variables "Elevation; % of native and exotic species, and specific richness" of study area*

	Elevation (m)	**% of native species**	**% of exotic species**	**Specific richness**	**QBR score**
Elevation (m)	1				
% of native species	0.71549	1			
% of exotic species	-0.71549	-1	1		
Specific richness	0.20028	0.66771	-0.66771	1	
QBR score	0.88206	0.81404	-0.81404	0.25068	1

Moreover, our results indicated that there is a significant correlation between the QBR index and altitude, and the percentage of exotic plants. This result is in agreement with other studies (Carrascosa Gómez and Munné, 2000; Suárez and Vidal-Abarca, 2000; Sirombra and Mesa, 2012) showing the increase in bank quality in high altitude sites due to the greater distance from urban areas and the inaccessibility of these places. In addition, this relationship was not as strong as we had expected, and this was related to the lower QBR value at higher altitude sites in Zat basin such as Tizart and wansa. The distance of these sites from urban areas explains the lower quality of the riparian strips at these stations.

In addition, the low QBR score obtained by some sampling localities located downstream of the Zat basin is the result of multiple anthropogenic interventions in riparian ecosystems, including land use for agriculture, road infrastructure and the abundance of invasive alien species such as (e.g. *Nicotiana glauca* L., *Ricinus communis* L., *Fraxinus angustiolia* and *Dittrichia viscosa* L.). The results published by Tüzün and Albaryrak (2005) and Valero et al. (2014) indicate that the areas classified as lower QBR correspond to those closest to human settlements and road infrastructure, which is consistent with the situation observed in this study, since the proximity of riparian ecosystems to population, productive land uses and corresponding road infrastructure leads to the modification and fragmentation of riparian habitats, resulting in low QBR scores.

During the study, agriculture was found to be the predominant activity observed in the riparian zone of the surveyed localities. According to the latest censuses by the Department of Agriculture of Morocco (2013) (AHT Group, 2016), the surface area of the Zat basin (512 km²), covers approximately 186 km² of agricultural area, which represents 35% of the total surface area. In addition, the riparian cover in the study area was subject of high degradation due to different varieties of crops that have been cultivated on both banks of the rivers. This leads to the introduction of exotic plants, which displace native species, altering their capacity for regeneration and dispersal. We have identified in this study, 54 species, represented by 51 genera and 31 families. 75.93% of the identified species were native (*Table A1* in the *Appendix*), while 24.07% of the species were non-native (Taleb et Bouhache, 2006). As well as influencing the wildlife living in these ecological corridors. The same finding was made for Chandni

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Nalla by Chaurasia et al. (2015) and the Bhahner River by Bahsir et al. (2015). The predominance of agricultural practices on both banks of Chandni Nalla was also reported by War et al. (2014). It is responsible of soil erosion and the ecological degradation of the river.

Conclusion and recommendation

The study was carried out on the quality of the riparian zones of the Zat River and its tributaries using the QBR index in order to know the current state of the riparian vegetation and to identify the different areas that are most affected and that deserve special attention. Although the study was not carried out on all sections of the sub-Basin, due to the limited accessibility in some sections of the rivers, in these cases we suggested that the QBR index be complemented by spatial analysis tools, namely geographic information systems (GIS) and remote sensing, which make it possible to deduce the ecological characteristics of riparian areas. The results of this study showed that at some stations where the QBR index was low, there was a high abundance of infrastructure and crop fields, rock removal for building construction, and an abundance of exotic plants. Therefore, proper attention should be given to the restoration and management of the riparian area of the studied river. However, these assessment tools should be used in conjunction with biological and physico-chemical assessments to ensure a clear understanding of the status of riparian ecosystems. The development of a multi-metric index capable of assessing the entire ecosystem in a single value would be of great interest for future research.

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APPENDIX

Table A1. *List of identified taxa, indicating origin of Arboreal, shrub and herbaceous species present in the riparian zones of Zat River and its tributaries*

Taxa	Origin (Taleb and Bouhache 2006)	Taxa	Origin (Taleb and Bouhache 2006)
Pistacia lentiscus L.	Native	*Cistus salviifolius* L.	Native
Nerium oleander L.	Native	*Euphorbia hirsuta* L.	Native
Ricinus communis L.	Non-Native	*Astragalus caprinus* L.	Native
Retama monosperma L.	Native	*Mentha longifolia* (L.) Hudson.	Native
Quercus ilex L.	Native	*Mentha retondifolia* L.	Native
Juglans regia L.	Native	*Mentha pulegium* L.	Native
Ficus carica L.	Native	*Lavendula multifida* L.	Native
Fraxinus angustifolia Vahl.	Non-Native	*Marrubium vulgare* L.	Non-Native
Olea europea. L.	Native	*Scrophulaira auriculata* L.	Native
Ziziphus lotus L.	Native	*Hyoscyamus albus* L.	Non-Native
Rubus ulmifolius Schott.	Native	*Ononis repens* L.	Native
Rosa sempervirens L.	Non-Native	*Convolvulus althaeoides* L.	Native
Populus alba L.	Native	*Frankenia laevis* L.	Native
Populus nigra L.	Native	*Potentilla reptans* L.	Native
Salix purpurea L.	Non-Native	*Juniperus oxycedrus* L.	Native
Salix pedicellata L.	Native	*Oppentium ficus-indica* L.	Non Native
Tamarix africana L.	Native	*Juncus acutus* L.	Native
Nicotiana glauca L.	Non-Native	*Phragmites australis* L.	Non-Native
Phoenicum vulgare L.	Native	*Typha angustifolia* L.	Native
Asphodilus microcarpus L.	Native	*Launea arborescens* L.	Native
Asparagus horridus L.	Native	*Phoenicum vulgare* L.	Native
Dittrichia viscosa L.	Non-Native	*Verbascum sinuatum* L.	Non-Native
Atriplex halimus L.	Native	*Astragalus caprinus* L.	Native
Onopordum arenarium	Non-Native	*Arundo donax* L.	Native
Cistus monosplensis L.	Native	*Polygala balansa* Cosson	Native
Euphorbia hursita L.	Native	*Scolymus hispanicus* L.	Non-Native
Ononis natrix L.	Native	*Thymus satureioides* Cosson	Native

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Figure A1. *Riparian zone in Downstream of Zat River (S4) showed the implantation of buildup and agriculture land*

Figure A2. *Talbanine station (S2) located in downstream of Zat River showed the presence de agriculture land*

Figure A3. *Tafriat station (S1) located in downstream of Zat basin*

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Figure A4. *Tighadouine station (S7) present the implantation of building area in riparian zone*

Figure A5. *Igherm melloulne station (S6) located in upstream of Zat basin*

Figure A6. *Ait Slimane station (S9) located in upstream of Zat basin*

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Figure A7. *Tizart station (S13) located in upstream of Zat basin*

Figure A8. *Ikkis station (S12) located in upstream of Zat basin*

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EFFECT OF SILICON FOLIAR APPLICATION ON THE ASSIMILATION AREA AND PHOTOSYNTHETIC PIGMENT CONTENTS OF POTATO (*SOLANUM TUBEROSUM* L.)

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Abstract. The effect of silicon-based biostimulant Optysil (200 g SiO_2 and 24 g Fe in 1 dm^3) on the assimilation area and photosynthetic pigment contents of potato was investigated. Optysil was applied once at the leaf development stage (BBCH 14-16) or at the tuber initiation stage (BBCH 40-41) and twice at the leaf development and tuber initiation stages, at the dose of 0.25 dm^3 ha^{-1} or 0.50 dm^3 ha^{-1} in each treatment. Optysil resulted in an enlargement of assimilation area and an increase in leaf area index (LAI), leaf dry weight, chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and carotenoids (Car) contents, and decreased the Chl *a*/Chl *b* ratio under water deficit conditions, but had no effect on the specific leaf area (SLA) and Chl (*a* + *b*)/Car ratio. Under periodical water deficits during potato growth, the plants produced the largest assimilation area with two Optysil applications at 0.50 dm^3 ha^{-1}, whereas the biosynthesis of photosynthetic pigments was most stimulated by Optysil at 0.25 dm^3 ha^{-1} applied at the tuber initiation stage. Under drought conditions, leaf growth was most stimulated by Optysil application at 0.50 dm^3 ha^{-1} at the initial potato growth period, whereas the biosynthesis of photosynthetic pigments by the double Optysil application at 0.50 dm^3 ha^{-1}.
Keywords: *sodium silicate, leaf area index (LAI), specific leaf area (SLA), chlorophyll a, chlorophyll b, carotenoids*

Introduction

Potato (*Solanum tuberosum* L.) growth is influenced by many biotic and abiotic stresses. Among abiotic stresses, drought is one of the most serious regarding potato growth and productivity. The most sensitive periods for water shortage are the vegetative and tuberization stage (Wagg et al., 2021). Periods of high temperature and drought are becoming more frequent in Central Europe, South-Central Asia, southeastern South America and the southeastern United States (Carrão et al., 2016). Under climate change conditions, biostimulants play an important role in mitigating abiotic stresses in plants. These products contain organic (seaweed extracts, humic substances, hydrolyzed proteins, amino acids containing products, microorganisms) or inorganic (Ti, Se, Si) compounds which enhance nutrient use efficiency, abiotic stress tolerance and/or crop quality traits, regardless of its nutrient content (du Jardin, 2015; Van Oosten et al., 2017; Bulgari et al., 2019; Drobek et al., 2019). In recent years, the use of silicon (Si) as a biostimulant has been increasing. Silicon plays an important role in plant growth by regulating both physiological and biochemical processes and

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mitigating biotic and abiotic stresses. Silicon stimulates plant growth in a species-specific manner. It can influence water relations and nutrient uptake, increase the content of photosynthetic pigments and regulate the activities of certain photosynthetic enzymes, as well as increase photosynthesis rate and decrease oxidative stress (Balakhnina and Borkowska, 2013; Zhu and Gong, 2014; Cao et al., 2015; Savvas and Ntatsi, 2015; Cooke and Leishman, 2016; Yavaş and Ünay, 2017; Zargar et al., 2019). Foliar application of silicon increases chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and carotenoids (Car) content in sweet pepper, onion, wheat and canola leaves (Lobato et al., 2009; Maghsoudi et al., 2016; Rangwala et al., 2019; Abdelaal et al., 2020; Bukhari et al., 2020) and Chl *a* and Chl *b* content in tomato and cucumber leaves (Abd El-Aziz, 2020; Gou et al., 2020). Since silicon plays an important role in mitigating biotic and abiotic stresses, silicon application is recommended for the improvement of vegetable crop production (Kaushik and Saini, 2019).

To date, few studies have focused on the effect of silicon foliar application on assimilation area and photosynthetic pigment contents in potato plants. A greenhouse pot experiment showed that both soil (soluble $SiO_2 \cdot HO_2$) and foliar (soluble concentrate stabilized silicic acid) application of silicon caused enlargement of leaf area and increased specific leaf area (SLA), as well as Chl *a* and Car contents in potato leaves, but had no effect on Chl *b* content. Silicon applied only to the soil increased dry weight of leaves. Silicon application alleviated water-deficit stress in potato. Chl *a* and Car concentrations, Chl *a*/Chl *b* ratio and tuber yield of water-deficit plants treated with silicon were similar to well-watered plants (Pilon et al., 2013, 2014). Another a greenhouse experiment showed that silicon ($NaSiO_3$) added to a nutrient solution caused enlargement of leaf area, and increased the leaf number and biomass of potato grown in a hydroponic system (Dorneles et al., 2018). Knowledge is scarce on the effect of silicon on potato growth under field conditions. A one-year field experiment carried out in Iran showed that foliar application of silicon (silica SiO_2 or sodium silicate nanoparticles Nano-$NaSiO_3$) enhanced the quantum yield of photosystem II (PS II) and Car content in potato leaves under salinity stress, but had no effect on Chl *a* and Chl *b* content. Under non-stress conditions, foliar application of silicon (sodium silicate nanoparticles Nano-$NaSiO_3$) increased Chl *a* and Chl *b* content, but had no effect on Chl *a*/Chl *b* ratio and Car content (Kafi et al., 2019). Silicon stimulated potato growth only at low doses. A higher concentrations of silicon in a nutrient solution induced reduction in potato growth parameters, mainly in leaf area (Dorneles et al., 2018). The excess of silicon on leaf surface may reduce transpiration so that it reduces photosynthesis (Hodson et al., 2005; Farooq and Dietz, 2015). The excess of silicon may also lead to the immobilization of essential elements for plant growth (Mg, Zn, Fe, Mo) in the apoplast (Moussa, 2006; Farooq and Dietz, 2015). Foliar application of silicon is effective only at very low doses and when started early in the vegetative stage (Laane, 2017). In the current study, it was hypothesized that silicon foliar application could contribute to enhancing assimilation area and photosynthetic pigment contents in potato under abiotic stress. The assumption was also made that potato response to silicon depends on the dose and time of application. The current study aimed to determine the effect of the dose and time of silicon foliar application on the assimilation area and photosynthetic pigment contents in potato leaves.

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Materials and methods

Experimental site and season

A field experiment was carried out in central-eastern Poland (52°03´N, 22°33´E), over three growing seasons (2016-2018). The experiment was located on soil classified as Haplic Luvisol (LV-ha) with a sandy loam texture (WRB FAO, 2015), with an acid-to-slightly-acid reaction (pH_{KCl} 5.2–5.6). The content of available phosphorus in the soil ranged from 97 to 114 mg P kg^{-1}, potassium from 93 to 124 mg K kg^{-1} and magnesium from 23 to 42 mg Mg kg^{-1} of soil.

Over the three years of the study, the most favorable hydrothermal conditions for the early crop potato culture were in the warm and moderately wet growing season of 2017 (*Table 1*). The year 2016 was warm with drought periods during potato growth, whereas 2018 was warm and very dry. In 2016 total precipitation in May was similar and in June over 40% lower than long-term average. In 2018 total precipitation in May and June was 2 times lower than the long-term average.

Table 1. *Mean air temperature and precipitation total in potato growing period recorded at the meteorological station of the Siedlce University of Natural Sciences and Humanities*

Years	Temperature; °C			Rainfalls; mm		
	April	May	June	April	May	June
2016	9.1	15.1	18.4	28.7	54.8	36.9
2017	6.9	13.9	17.8	59.6	49.5	57.9
2018	13.1	17.0	18.3	34.5	27.3	31.5
Many-year mean (1981-2010)	8.3	12.2	16.8	41.2	53.0	63.8

Plant material and experimental design

In this experiment, the silicon (Si) source was the liquid biostimulant Optysil (Intermag Ltd. Olkusz, Poland), which activates natural plant defense mechanisms against stress and stimulates their growth and development. Optysil contains 200 g SiO_2 (16.5 m/m) and 24 g Fe (2 m/m) in 1 dm^3, in the form of sodium metasilicate (Na_2SiO_3) and iron chelate (Fe-EDTA). The recommended dosage of the Optysil for potato harvested when fully ripe is 0.5 dm^3 ha^{-1} at the beginning of tuber formation stage and a repeat the treatment when tubers reach 70-80% of the final mass, and the optional 0.50 dm^3 ha^{-1} at the beginning of plant growth.

The effect of dose and time of Optysil application on the assimilation area and photosynthetic pigment contents in potato leaves were determined. The field experiment was established as a split-plot design with a control object without Optysil with three replications. The main plots were Optysil dose: 0.25 dm^3 ha^{-1} and 0.50 dm^3 ha^{-1}, and the sub plots time of Optysil application: the leaf development stage – according to the Biologische Bundesanstalt, Bundessortenamt and Chemical Industry (BBCH) the 14-16 stage, tuber initiation stage – BBCH 40-41, at both the leaf development stage and tuber initiation stage – BBCH 14-16 and BBCH 40-41 (Meier, 2018). Potato plants sprayed with water were used as a control. A one plot-size control was located between main plots containing three sub plots.

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The drought-sensitive very early potato cultivar Catania (Europlant Pflanzenzucht GmbH, Germany) registered on the Common Catalogue of Varieties of Agricultural Plant Species (CCV) was grown. It is one of the most widely grown very early potato cultivars in central-eastern Poland.

At the tuber formation stage (BBCH 46-48), the assimilation leaf area, leaf dry weight, leaf area index (LAI) and specific leaf area (SLA) and photosynthetic pigment (Chl and Car) contents were determined. The measurements were made on four successive randomized plants per plot. The assimilation leaf area was measured by the weight method based on the weight of pieces with a known diameter and total weight of leaves per plant (Wadas and Kalinowski, 2017). LAI and SLA were calculated as the ratio of the assimilation leaf area/ground area and the ratio of assimilation leaf area/dry weight of leaves, respectively (Pietkiewicz, 1985). The relationship between the tuber weight per plant and the assimilation area and photosynthetic pigment contents were also determined. The tuber weight per plant were determined on ten successive plants per plot.

Agrotechnics and plant protection

In each year of the study, spring triticale was grown as a potato forecrop. Potato cultivation was carried out according to common agronomical practices. Farmyard manure was applied in autumn at the rate of 25 t ha^{-1}, and mineral fertilizers were applied at rates of 80 kg N (ammonium nitrate), 35 kg P (superphosphate), and 100 kg K (potassium sulphate) per hectare in spring.

Six-week pre-sprouted seed potatoes were planted on 6 April 2016, 10 April 2017 and 9 April 2018, with a row spacing of 25 cm and 67.5 cm between rows (96 plants per plot). Potatoes were harvested 75 days after planting (the end of June).

Colorado potato beetle (*Leptinotarsa decemlineata*) was controlled using thiamethoxam (Actara 25 WG; Syngenta Crop Protection AG Basel, Switzerland).

Determination of photosynthetic pigment content

The photosynthetic pigment (Chl *a*, Chl *b*, Car) contents were determined in the youngest fully expanded leaves, i.e. the fourth and fifth leaves from the top, by the Arnon method (Arnon, 1949). The photosynthetic pigments were extracted by 80% acetone in 2 g fresh leaves and absorbance was measured at the 663 nm, 645 nm and 470 nm wavelengths, respectively, for Chl *a*, Chl *b* and Car using a UV-1800 spectrophotometer (Rayleigh, UK). Chl *a*, Chl *b*, Chl ($a + b$) and Car contents were calculated by using the following formulas (*Eqs. 1-4*):

$$Chl\ a = \frac{(12.7\,A663 - 2.7A645)V}{1000W} \quad \text{(Eq.1)}$$

$$Chl\ b = \frac{(22.9A645 - 4.7A663)V}{1000W} \quad \text{(Eq.2)}$$

$$Chl\ (a+b) = \frac{(20.2A645 + 8.02A663)V}{1000W} \quad \text{(Eq.3)}$$

$$Car = 1000A470 - 2.27Chl\ a - \frac{81.4Chl\ b}{227} \quad \text{(Eq.4)}$$

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where: A – absorbance at specific wavelength; V– volume of the extract (cm^3); W – weight of the leaves used for extraction (g).

The contents of photosynthetic pigments were expressed as milligrams per gram of the fresh weight of potato leaves. The ratios of Chl *a*/Chl *b* and Chl (*a* + *b*)/Car were also calculated.

Statistical analysis

The results of the three-year study were analyzed statistically using a two-factor analysis of variance (ANOVA) for the split-plot design (Optysil dose × time of Optysil application × years) with a control object. The analysis of the results of the study was conducted using the orthogonal contrast to compare the control without Optysil with the test objects with Optysil. Fisher-Snedecor *F* test was used to check the significance of sources of variability, and Tukey's test was used to check the significance of differences between compared averages at $p \leq 0.05$. Calculations were performed using Statistica 12 PL software (StatSoft, Tulsa, OK, USA). Results are shown as mean ± standard deviation (SD). The linear correlation was used to determine relationship between the tuber weight and, assimilation area and photosynthetic pigment contents in potato leaves ($n = 21$).

Results

Assimilation area and dry weight of leaves

Silicon-based biostimulant Optysil caused enlargement of the assimilation area and increased dry weight of leaves but had no effect on the SLA (*Table 2*). Optysil effect depended on the weather conditions during potato growth. Optysil had a significant effect on the assimilation area and dry weight of leaves under water deficit conditions in the years 2016 and 2018. In the warm growing season of 2016 with periodical water deficits, Optysil caused enlargement of assimilation area on average by 578 cm^2 (14%) and increased leaf dry weight by 4.79 g (23%) compared with the control plants. As a result, the LAI for the treated plants was higher on average by 0.34 than for control plants. In the warmer and very dry growing season of 2018, following application of Optysil, the assimilation area was larger on average by 327 cm^2 (9%), LAI value was higher by 0.19 and leaf dry weight by 1.84 g (11%) compared with the control plants.

The dose and time of Optysil application had a significant effect on the assimilation area but had no effect on the leaf dry weight (*Tables 3* and *4*). The study demonstrated the significant effect of the interaction of the years and the dose of Optysil and the interaction of the years and the time of Optysil application on the assimilation area.

The Optysil dose had a significant effect on the assimilation area only in the warm and very dry growing season of 2018 (*Table 3*). In that year, after the application of 0.50 $dm^3\ ha^{-1}$ of Optysil, the assimilation area was larger on average by 534 cm^2 (12%) and LAI was higher by 0.32 as compared with the values at the dose of 0.25 $dm^3\ ha^{-1}$. The time of Optysil application had a significant effect on the assimilation area in 2016 with the lower water deficit than in 2018 (*Table 4*). Regardless of Optysil dose, in 2016, the assimilation area was the largest with two Optysil applications, at the leaf development stage and with a repeated treatment at the tuber initiation stage (BBCH 14-16 and BBCH 40-41).

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Table 2. *Effect of Optysil on assimilation area and dry weight of leaves*

Treatment	Years			Mean
	2016	2017	2018	
Assimilation area; cm^2				
Without Optysil	4240 ± 305 b	6006 ± 102 a	3571 ± 210 b	4605 ± 1091 b
With Optysil	4818 ± 568 a	5784 ± 894 a	3898 ± 380 a	4833 ± 1005 a
Leaf area index (LAI); cm^2 cm^2				
Without Optysil	2.51 ± 0.02 b	3.56 ± 0.06 a	2.12 ± 0.02 b	2.73 ± 0.65 b
With Optysil	2.85 ± 0.34 a	3.43 ± 0.53 a	2.31 ± 0.23 a	2.86 ± 0.59 a
Leaf dry weight; g				
Without Optysil	20.97 ± 2.52 b	22.93 ± 3.01 a	16.57 ± 1.53 b	20.16 ± 3.20 b
With Optysil	25.76 ± 3.10 a	23.37 ± 3.26 a	18.41 ± 2.02 a	22.51 ± 4.17 a
Specific leaf area (SLA); cm^2 g^{-1}				
Without Optysil	202.2 ± 14.6 a	264.4 ± 14.3 a	216.8 ± 15.0 a	227.8 ± 30.03 a
With Optysil	187.7 ± 9.9 a	246.9 ± 19.4 a	211.5 ± 10.7 a	215.4 ± 28.12 a

Means within columns for each data followed by the same letters do not differ significantly at $p \leq 0.05$

Table 3. *Effect of Optysil dose on assimilation area and dry weight of leaves*

Optysil dose	Years			Mean
	2016	2017	2018	
Assimilation area; cm^2				
0.25 dm^3 ha^{-1}	4718 ± 440 a	5865 ± 904 a	3631 ± 228 b	4738 ± 1091 b
0.50 dm^3 ha^{-1}	4918 ± 685 a	5704 ± 931 a	4165 ± 306 a	4929 ± 922 a
Leaf area index (LAI); cm^2 cm^2				
0.25 dm^3 ha^{-1}	2.79 ± 0.26 a	3.47 ± 0.53 a	2.15 ± 0.16 b	2.81 ± 0.64 b
0.50 dm^3 ha^{-1}	2.91 ± 0.40 a	3.38 ± 0.55 a	2.47 ± 0.18 a	2.92 ± 0.54 a
Leaf dry weight; g				
0.25 dm^3 ha^{-1}	25.32 ± 3.38 a	23.14 ± 3.54 a	17.46 ± 1.68 a	21.97 ± 4.43 a
0.50 dm^3 ha^{-1}	26.21 ± 2.91 a	23.59 ± 3.16 a	19.37 ± 1.96 a	23.06 ± 3.89 a

Means within columns for each data followed by the same letters do not differ significantly at $p \leq 0.05$

The study demonstrated the significant effect of the interaction of the years, the dose and the time of Optysil application on the assimilation area (*Table 5*). In 2016 with drought periods during potato growth, the plants produced the largest assimilation area with two Optysil applications at 0.50 dm^3 ha^{-1}, at the leaf development stage and with a repeated treatment at the tuber initiation stage (BBCH 14-16 and BBCH 40-41). In the very dry growing season of 2018, the assimilation area was largest after the application of 0.50 dm^3 ha^{-1} of Optysil at the leaf development stage (BBCH 14-16).

Photosynthetic pigment contents

Silicon-based biostimulant Optysil had a significant effect on photosynthetic pigment contents in potato leaves under water deficit conditions in the years 2016 and 2018 (*Table 6*). In the warm growing season of 2016 with periodical water deficits, Optysil

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increased leaf Chl *a* content on average by 0.055 mg g^{-1} (11%) compared with the control plants but had no effect on the Chl *b* or Car contents. As a result, the Chl *a*/Chl *b* ratio was higher than in the control plants, whereas the Chl (*a* + *b*)/Car ratio did not differ significantly. In the warmer and very dry growing season of 2018, following the application of Optysil, the leaf Chl *a* content was higher on average by 0.167 mg g^{-1} (35.5%), Chl *b* by 0.108 mg g^{-1} (54%) and Car by 0.007 mg g^{-1} (43.8%) compared with the control plants. In that year, Optysil caused a decrease in the Chl *a*/Chl *b* ratio but had no effect on the Chl (*a* + *b*)/Car ratio.

Table 4. *Effect of time of Optysil application on assimilation area and dry weight of leaves*

Time of Optysil application	Years			Mean
	2016	2017	2018	
Assimilation area; cm^2				
Leaf development stage	4776 ± 494 b	5825 ± 833 a	4103 ± 436 a	4901 ± 929 ab
Tuber initiation stage	4325 ± 393 b	5764 ± 414 a	3801 ± 181 a	4630 ± 914 b
Leaf development and tuber initiation stages	5352 ± 250 a	5763 ± 679 a	3791 ± 439 a	4969 ± 1176 a
Leaf area index (LAI); cm^2 cm^2				
Leaf development stage	2.83 ± 0.29 b	3.34 ± 0.50 a	2.44 ± 0.26 a	2.90 ± 0.55 a
Tuber initiation stage	2.56 ± 0.26 b	3.42 ± 0.25 a	2.26 ± 0.11 a	2.74 ± 0.54 b
Leaf development and tuber initiation stages	3.17 ± 0.16 a	3.42 ± 0.80 a	2.25 ± 0.26 a	2.94 ± 0.69 a
Leaf dry weight; g				
Leaf development stage	24.66 ± 2.73 a	23.22 ± 3.99 a	18.80 ± 2.22 a	22.23 ± 4.04 a
Tuber initiation stage	23.86 ± 2.01 a	23.57 ± 1.87 a	18.12 ± 2.41 a	21.85 ± 7.49 a
Leaf development and tuber initiation stages	28.74 ± 2.15 a	23.35 ± 4.09 a	18.32 ± 1.70 a	23.46 ± 4.52 a

Means within columns for each data followed by the same letters do not differ significantly at $p \leq 0.05$

Table 5. *Interaction effect of years, dose and time of Optysil application on assimilation area*

Time of Optysil application	Years and Optysil dose					
	2016		2017		2018	
	0.25 dm^3 ha^{-1}	0.50 dm^3 ha^{-1}	0.25 dm^3 ha^{-1}	0.50 dm^3 ha^{-1}	0.25 dm^3 ha^{-1}	0.50 dm^3 ha^{-1}
Assimilation area; cm^2						
Leaf development stage	4473 ± 352 b	5119 ± 366 a	5977 ± 381 a	5673 ± 242 a	3813 ± 247 ab	4393 ± 401 a
Tuber initiation stage	4509 ± 289 b	4141 ± 448 b	5666 ± 217 a	5862 ± 489 a	3678 ± 200 ab	3923 ± 164 ab
Leaf development and tuber initiation stages	5210 ± 236 a	5493 ± 280 a	5952 ± 553 a	5576 ± 290 a	3403 ± 184 b	4180 ± 247 a
Leaf area index (LAI) cm^2 cm^2						
Leaf development stage	2.62 ± 0.21 b	3.03 ± 0.22 a	3.54 ± 0.22 a	3.36 ± 0.15 a	2.28 ± 0.15 ab	2.61 ± 0.24 a
Tuber initiation stage	2.67 ± 0.17 b	2.45 ± 0.26 b	3.36 ± 0.13 a	3.47 ± 0.29 a	2.18 ± 0.12 ab	2.33 ± 0.10 ab
Leaf development and tuber initiation stages	3.09 ± 0.14 a	3.25 ± 0.16 a	3.53 ± 0.32 a	3.30 ± 0.17 a	2.06 ± 0.11 b	2.48 ± 0.15 a

Means within columns for each data followed by the same letters do not differ significantly at $p \leq 0.05$

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Table 6. *Effect of Optysil on photosynthetic pigment contents*

Treatment	Years			Mean
	2016	2017	2018	
Chlorophyll *a* (Chl *a*); mg g^{-1}				
Without Optysil	0.490 ± 0.010 b	0.716 ± 0.094 a	0.470 ± 0.024 b	0.558 ± 0.128 a
With Optysil	0.545 ± 0.079 a	0.661 ± 0.064 a	0.637 ± 0.105 a	0.614 ± 0.096 a
Chlorophyll *b* (Chl *b*); mg g^{-1}				
Without Optysil	0.209 ± 0.008 a	0.447 ± 0.092 a	0.200 ± 0.010 b	0.285 ± 0.130 a
With Optysil	0.216 ± 0.042 a	0.410 ± 0.069 a	0.308 ± 0.069 a	0.311 ± 0.100 a
Chl (*a* + *b*); mg g^{-1}				
Without Optysil	0.700 ± 0.028 b	1.164 ± 0.180 a	0.671 ± 0.026 b	0.845 ± 0.256 a
With Optysil	0.761 ± 0.118 a	1.072 ± 0.101 a	0.946 ± 0.166 a	0.926 ± 0.182 a
Carotenoids (Car); mg g^{-1}				
Without Optysil	0.016 ± 0.001 a	0.030 ± 0.007 a	0.016 ± 0.001 b	0.021 ± 0.008 a
With Optysil	0.018 ± 0.002 a	0.032 ± 0.003 a	0.023 ± 0.004 a	0.024 ± 0.007 a
Chl *a*/Chl *b*				
Without Optysil	2.349 ± 0.116 b	1.620 ± 0.184 a	2.342 ± 0.108 a	2.104 ± 0.383 a
With Optysil	2.546 ± 0.177 a	1.648 ± 0.253 a	2.105 ± 0.252 b	2.100 ± 0.435 a
Chl (*a* + b)/Car				
Without Optysil	43.44 ± 0.85 a	38.50 ± 2.47 a	42.75 ± 1.75 a	41.56 ± 2.78 a
With Optysil	41.80 ± 2.24 a	33.12 ± 2.50 a	42.09 ± 3.73 a	39.01 ± 5.08 a

Means within columns for each data followed by the same letters do not differ significantly at $p \leq 0.05$

The study demonstrated the significant effect of the interaction of the years and the dose of Optysil and the interaction of the years and the time of Optysil application on the photosynthetic pigment contents (*Tables 7* and *8*).

Table 7. *Effect of Optysil dose on photosynthetic pigment contents*

Optysil dose	Years			Mean
	2016	2017	2018	
Chlorophyll *a* (Chl *a*); mg g^{-1}				
0.25 dm^3 ha^{-1}	0.568 ± 0.076 a	0.679 ± 0.058 a	0.609 ± 0.067 b	0.619 ± 0.080 a
0.50 dm^3 ha^{-1}	0.522 ± 0.079 a	0.644 ± 0.064 a	0.665 ± 0.131 a	0.610 ± 0.111 a
Chlorophyll *b* (Chl *b*); mg g^{-1}				
0.25 dm^3 ha^{-1}	0.228 ± 0.051 a	0.396 ± 0.064 a	0.278 ± 0.043 b	0.300 ± 0.088 a
0.50 dm^3 ha^{-1}	0.204 ± 0.031 a	0.424 ± 0.076 a	0.338 ± 0.079 a	0.322 ± 0.112 a
Chl (*a* + *b*); mg g^{-1}				
0.25 dm^3 ha^{-1}	0.796 ± 0.124 a	1.076 ± 0.108 a	0.888 ± 0.104 b	0.920 ± 0.161 a
0.50 dm^3 ha^{-1}	0.736 ± 0.108 a	1.068 ± 0.099 a	1.004 ± 0.201 a	0.933 ± 0.505 a
Carotenoids (Car); mg g^{-1}				
0.25 dm^3 ha^{-1}	0.018 ± 0.002 a	0.030 ± 0.004 a	0.021 ± 0.002 b	0.024 ± 0.007 a
0.50 dm^3 ha^{-1}	0.018 ± 0.003 a	0.032 ± 0.002 a	0.024 ± 0.005 a	0.025 ± 0.007 a
Chl *a*/Chl *b*				
0.25 dm^3 ha^{-1}	2.532 ± 0.214 a	1.774 ± 0.236 a	2.213 ± 0.240 a	2.163 ± 0.391 a
0.50 dm^3 ha^{-1}	2.560 ± 0.141 a	1.552 ± 0.243 a	1.997 ± 0.260 a	2.037 ± 0.471 a

Means within columns for each data followed by the same letters do not differ significantly at $p \leq 0.05$

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Table 8. *Effect of time of Optysil application on photosynthetic pigment contents*

Time of Optysil application	Years			Mean
	2016	2017	2018	
Chlorophyll *a* (Chl *a*); mg g^{-1}				
Leaf development stage	0.540 ± 0.048 ab	0.658 ± 0.070 a	0.582 ± 0.098 b	0.593 ± 0.086 a
Tuber initiation stage	0.606 ± 0.079 a	0.637 ± 0.036 a	0.630 ± 0.045 ab	0.624 ± 0.055 a
Leaf development and tuber initiation stages	0.488 ± 0.066 b	0.688 ± 0.083 a	0.698 ± 0.133 a	0.625 ± 0.114 a
Chlorophyll *b* (Chl b); mg g^{-1}				
Leaf development stage	0.205 ± 0.020 a	0.397 ± 0.107 a	0.272 ± 0.044 b	0.294 ± 0.101 a
Tuber initiation stage	0.224 ± 0.057 a	0.415 ± 0.040 a	0.320 ± 0.082 a	0.327 ± 0.092 a
Leaf development and tuber initiation stages	0.205 ± 0.023 a	0.418 ± 0.054 a	0.331 ± 0.073 a	0.313 ± 0.110 a
Chl (*a* + *b*); mg g^{-1}				
Leaf development stage	0.754 ± 0.064 ab	1.576 ± 0.142 a	0.855 ± 0.138 b	0.889 ± 0.258 a
Tuber initiation stage	0.852 ± 0.132 a	1.053 ± 0.048 a	0.951 ± 0.124 ab	0.952 ± 0.132 a
Leaf development and tuber initiation stages	0.678 ± 0.089 b	1.108 ± 0.099 a	1.031 ± 0.203 a	0.939 ± 0.233 a
Carotenoids (Car); mg g^{-1}				
Leaf development stage	0.018 ± 0.002 a	0.031 ± 0.003 a	0.021 ± 0.003 a	0.023 ± 0.006 a
Tuber initiation stage	0.020 ± 0.002 a	0.032 ± 0.001 a	0.022 ± 0.001 a	0.025 ± 0.009 a
Leaf development and tuber initiation stages	0.016 ± 0.002 a	0.034 ± 0.003 a	0.024 ± 0.006 a	0.025 ± 0.007 a
Chl *a*/Chl *b*				
Leaf development stage	2.540 ± 0.126 a	1.736 ± 0.383 a	2.145 ± 0.204 a	2.140 ± 0.417 a
Tuber initiation stage	2.516 ± 0.276 a	1.550 ± 0.182 a	2.048 ± 0.382 a	2.038 ± 0.716 a
Leaf development and tuber initiation stages	2.584 ± ± 0.105 a	1.659 ± 0.126 a	2.122 ± 0.151 a	2.122 ± 0.407 a

Means within columns for each data followed by the same letters do not differ significantly at $p \leq 0.05$

The Optysil dose had a significant effect on the leaf photosynthetic pigment contents only in the warm and very dry growing season of 2018 (*Table 7*). In that year, after the application of 0.50 dm^3 ha^{-1} of Optysil, the Chl *a* content was higher on average by 0.056 mg g^{-1} (9%), Chl *b* by 0.060 mg g^{-1} (21.6%) and Car by 0.003 mg g^{-1} (14%) as compared with the values at the dose of 0.25 dm^3 ha^{-1}. The Optysil dose had no effect on the Chl *a*/Chl *b* ratio. Time of Optysil application had a significant effect on the leaf photosynthetic pigment contents under water deficit conditions in the years 2016 and 2018 (*Table 8*). In 2016 with the lower water deficit, the Chl *a* and Chl *b* contents were the highest with a single application of Optysil at the tuber initiation stage (BBCH 40-41) whereas during the very dry growing season of 2018, the contents of Chl *a* and Chl *b* were the highest with two Optysil applications, at the leaf development stage and with a repeated treatment at the tuber initiation stage (BBCH 14-16 and BBCH 40-41). The time of Optysil application had no effect on the Chl *a*/Chl *b* ratio.

The study demonstrated the significant effect of the interaction of the years, the dose and the time of Optysil application on the leaf photosynthetic pigment contents (*Table 9*). In 2016 with drought periods during potato growth, the Chl *a*, Chl *b* and Car

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contents were the highest after the application of 0.25 dm^3 ha^{-1} of Optysil at the tuber initiation stage (BBCH 40-41). In the very dry growing season of 2018, the content of these pigments was highest after the application of 0.50 dm^3 ha^{-1} of Optysil at the leaf development stage (BBCH 14-16) and with a repeated treatment at the tuber initiation stage (BBCH 40-41) with the same dose of Optysil.

Table 9. *Interaction effect of years, dose and time of Optysil application on photosynthetic pigment contents*

Time of Optysil application	Years and Optysil dose					
	2016		2017		2018	
	0.25 dm^3 ha^{-1}	0.50 dm^3 ha^{-1}	0.25 dm^3 ha^{-1}	0.50 dm^3 ha^{-1}	0.25 dm^3 ha^{-1}	0.50 dm^3 ha^{-1}
Chlorophyll *a* (Chl *a*); mg g^{-1}						
Leaf development stage	0.506 ± 0.023 b	0.575 ± 0.040 ab	0.676 ± 0.086 a	0.640 ± 0.062 a	0.621 ± 0.095 b	0.543 ± 0.103 b
Tuber initiation stage	0.655 ± 0.068 a	0.557 ± 0.061 ab	0.640 ± 0.036 a	0.634 ± 0.043 a	0.607 ± 0.016 b	0.653 ± 0.059 ab
Leaf development and tuber initiation stages	0.543 ± 0.016 ab	0.433 ± 0.042 b	0.720 ± 0.014 a	0.657 ± 0.104 a	0.599 ± 0.093 b	0.798 ± 0.074 a
Chlorophyll *b* (Chl *b*); mg g^{-1}						
Leaf development stage	0.198 ± 0.008 a	0.228 ± 0.014 a	0.344 ± 0.063 a	0.450 ± 0.082 a	0.288 ± 0.048 ab	0.256 ± 0.042 b
Tuber initiation stage	0.278 ± 0.065 a	0.213 ± 0.027 a	0.396 ± 0.023 a	0.433 ± 0.049 a	0.256 ± 0.015 b	0.384 ± 0.067 a
Leaf development and tuber initiation stages	0.208 ± 0.011 a	0.171 ± 0.014 a	0.447 ± 0.062 a	0.388 ± 0.032 a	0.288 ± 0.062 ab	0.374 ± 0.061 a
Chll (*a* + *b*); mg g^{-1}						
Leaf development stage	0.704 ± 0.031 ab	0.804 ± 0.045 ab	1.022 ± 0.146 a	1.091 ± 0.160 a	0.910 ± 0.136 b	0.800 ± 0.141 b
Tuber initiation stage	0.934 ± 0.126 a	0.771 ± 0.087 ab	1.037 ± 0.026 a	1.068 ± 0.066 a	0.864 ± 0.015 b	1.038 ± 0.125 ab
Leaf development and tuber initiation stages	0.750 ± 0.027 ab	0.605 ± 0.055 b	1.169 ± 0.074 a	1.046 ± 0.089 a	0.888 ± 0.154 b	1.174 ± 0.135 a
Carotenoids (Car); mg g^{-1}						
Leaf development stage	0.016 ± 0.001 ab	0.019 ± 0.002 ab	0.031 ± 0.004 a	0.031 ± 0.001 a	0.021 ± 0.003 b	0.020 ± 0.004 b
Tuber initiation stage	0.022 ± 0.002 a	0.019 ± 0.002 ab	0.032 ± 0.001 a	0.031 ± 0.002 a	0.022 ± 0.001 b	0.023 ± 0.002 b
Leaf development and tuber initiation stages	0.018 ± 0.001 ab	0.015 ± 0.001 b	0.036 ± 0.002 a	0.032 ± 0.002 a	0.019 ± 0.002 b	0.030 ± 0.004 a

Means within columns for each data followed by the same letters do not differ significantly at $p \leq 0.05$

Relationship between tuber weight and, assimilation area and photosynthetic pigment contents in potato leaves

The yield-forming effect of Optysil depended on water conditions during the potato growth period (*Table 10*). In the 2016 with a drought periods during potato growth, with the use of Optysil the average tuber weight per plant was higher by 83.0 g (23%) compared with the cultivation without biostimulant. In very dry growing season of 2018, following the application of Optysil the average tuber weight per plant was higher by 22.3 g (13%). A significant positive correlation was found between the tuber weight per plant and assimilation area and dry weight of leaves (*Table 11*). The tuber weight was not significantly correlated with photosynthetic pigment contents.

Table 10. *Effect of Optysil on tuber weight per plant; g*

Treatment	Years			Mean
	2016	2017	2018	
Without Optysil	361.3 ± 17.5 b	407.3 ± 14.1 a	170.7 ± 13.2 b	313.1 ± 109.1 b
With Optysil	444.6 ± 39.2 a	424.4 ± 30.3 a	193.0 ± 29.4 a	354.0 ± 119.7 a

Means within columns for each data followed by the same letters do not differ significantly at $p \leq 0.05$

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Table 11. *Linear correlation coefficients (n = 21) between tuber weight and assimilation area, leaf dry weight and photosynthetic pigment contents*

Specification	Correlation coefficient
Assimilation area	0.6719*
Leaf dry weight	0.7973*
Leaf area index (LAI)	0.6695*
Specific leaf area (SLA)	0.0986
Chlorophyll *a* (Chl *a*)	-0.0773
Chlorophyll *b* (Chl *b*)	0.1092
Chl (*a* + *b*)	0.0507
Carotenoids (Car)	0.2290

*Significant at $p \leq 0.05$

Discussion

Assimilation area and photosynthetic pigment contents are important parameters of assessment plant growth. Previously, a greenhouse pot experiment showed that three times (10, 20 and 30 days after emergence) silicon foliar application (1.425 mM Si water solution of soluble concentrate stabilized silicic acid) caused enlargement of the leaf area and increased SLA of very early potato cultivar Agata (resistant to a short-term drought) on Typic Acrortox soil, but had no effect on the dry weight of leaves (Pilon et al., 2013). In the present study, the silicon-based (Na_2SiO_3) biostimulant Optysil caused enlargement of the assimilation area and increased leaf dry weight of drought-sensitive very early potato cultivar Catania under water deficit on Haplic Luvisol soil, but had no effect on the SLA. The enlargement of assimilation area does not always results in an increase in the tuber yield. The relationship between the rate of tuber yield and LAI value is rather complex, because potato responds very sensitively to weather changes during vegetation that may cause the falling or new growth of leaves (Zrůst et al., 1999). According to Howlander and Hoque (2018), LAI increases progressively over time, reaching a peak at 60 days after potato planting and thereafter declining. The LAI describes the growth in lowland fields, whereas the growth of individual plants is characterized by the SLA (Van Delden et al., 2000). The SLA for potato depends on the growth stage and temperature (Howlander and Hoque, 2018; Van Delden et al., 2000).

Silicon stimulated potato growth only at low doses. A higher concentration of silicon in a nutrient solution induced reduction in potato leaf area (Dorneles et al., 2018). In the present study, the effect of dose and time of Optysil application on assimilation area depended on a water deficit during potato growth. Under conditions of periodical water deficits during potato growth, the plants produced a largest assimilation area with two Optysil applications at 0.50 dm^3 ha^{-1}, at the leaf development stage and with a repeated treatment at the tuber initiation stage (BBCH14-16 and BBCH 40-41). Under drought conditions, growth of leaves was most stimilated by Optysil application at 0.50 dm^3 ha^{-1} only at the initial potato growth period (BBCH14-16).

There is scarce knowledge of the effect of silicon foliar application on the biosynthesis of photosynthetic pigments by potato plants. Photosynthetic pigment contents and their ratios may be used as an indicator of plant response to environmental conditions (Strzałka et al., 2003; Zhou et al., 2019). Chlorophyll concentration in leaves is an indicator of potato tuber yield under water-shortage conditions (Ramírez et al.,

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2014). A greenhouse pot experiment showed that five-times (10, 20, 30, 40 and 50 days after emergence) foliar application of silicon (1.425 mM Si water solution of soluble concentrate stabilized silicic acid) increased Chl *a* and Car content in leaves of very early potato cultivar Agata (resistant to a short-term drought) under water deficit conditions on Typic Acrortox soil, but had no effect on Chl *b* content (Pilon et al., 2014). Previously, a one-year field experiment carried out in Iran showed that two-time (40 and 50 days after potato planting) foliar application of silicon (1000 ppm silica SiO_2 or 400 ppm sodium silicate nanoparticles Nano-$NaSiO_3$) increased Chl *a* and Chl *b* content in leaves of medium late potato cultivar Agria (drought-resistant) on Silty Loam soil (Kafi et al., 2019), which is confirmed in the present study. Optysil increased Chl *a*, Chl *b* and Car contents in leaves of drought-sensitive very early potato cultivar Catania under water deficit on Haplic Luvisol soil. An increase in the Chl *a* and Chl *b* content following Optysil application resulted in a decrease in the Chl *a*/Chl *b* ratio compared with the control plants. Optysil had no significant effect on the Chl (*a* + *b*)/Car ratio in potato leaves. A decrease in the Chl *a*/Chl *b* ratio is the plants' response to abiotic stress (Sumanta et al., 2014). Silicon application is beneficial for plants under water deficit conditions (Bukhari et al., 2020), which is confirmed in the present study. A decrease in chlorophyll content under drought stress is associated with both a decrease in chlorophyll synthesis and an increase in the degradation of chlorophyll by chlorophyllase enzyme (Zahedi et al., 2019; Bukhari et al., 2020). Silicon mitigates the adverse effect of drought by inducing synthesis of new chlorophyll and protecting existing chlorophyll from oxidative stress induced by drought (Cao et al., 2015; Bukhari et al., 2020). The positive responses of photosynthetic pigments to silicon application under drought stress may be associated with a protective role of silicon on chloroplast ultrastructure (Cao et al., 2015; Savvas and Ntatsi, 2015) and increase activities of some antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (CAT), that are located in the chloroplast. Catalase (CAT) is a principal enzyme that prevents chlorophyll degradation (Balakhnina and Borkowska, 2013; Pilon et al., 2014; Zhu and Gong, 2014; Savvas and Ntatsi, 2015; Bukhari et al., 2020).

There is a scarce knowledge of the effect of different doses and time of silicon foliar application on the biosynthesis of photosynthetic pigments by potato plants. Previous, a one-year field experiment carried out in Iran showed that two-times (40 and 50 days after potato planting) application of 400 ppm sodium silicate nanoparticles Nano-$NaSiO_3$ stimulated the biosynthesis of Chl *a*, Chl *b* and Car more strongly than 1000 ppm silica SiO_2 (Kafi et al., 2019). In the present study, the effect of dose and time of Optysil application on leaf photosynthetic pigment contents depended on a water deficit during potato growth. In a year with periodical water deficits during potato growth, an Optysil dose of 0.25 dm^3 ha^{-1} application at the tuber initiation stage (BBCH 40-41) stimulated the biosynthesis of Chl *a,* Chl *b* and Car in potato leaves more strongly than the dose of 0.50 dm^3 ha^{-1}. In a very dry year, the biosynthesis of photosynthetic pigments was most stimulated by two Optysil applications at 0.50 dm^3 ha^{-1}, at the initial plant growth stage (BBCH 14-16) and with a repeated treatment in the tuber initiation stage (BBCH 40-41).

The silicon-based biostimulant Optysil reduced drought stress and improved the yield of field crops (Ciecierski and Kardasz, 2014; Artyszak, 2018), which is confirmed in the present study. In the present study, following Optysil application, the tuber weight per plant of drought sensitive very early potato cultivar Catania 75 days after planting was higher, on average by 23% under periodical water deficits during potato

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growth, and by 13% under drought conditions. A significant positive correlation was found between the tuber weight and assimilation area, and leaf dry weight and LAI. A positive correlation between leaf area and tuber yield suggests thet the enlargement of leaf area could enhance the export of photosynthetic products and cause an increase in tuber yield (Li et al., 2013). According to Ascione et al. (2013), the tuber growth rate is only slightly corelated with LAI, and still less so with SLA, which is confirmed in the present study. No correlation was found between tuber weight and SLA, and between the tuber weight and photosynthetic pigment contents.

Conclusions

This study demonstrated possibility of silicon use to promote potato growth and biosynthesis of photosynthetic pigments under water deficit. Silicon-based biostimulant Optysil caused enlargement of assimilation area and increased the leaf dry weight, Chl *a*, Chl *b* and Car contents, and decreased the Chl *a*/Chl *b* ratio, but had no effect on the SLA and Chl $(a + b)$/Car ratio. Optysil effect depended on the dose and time of application. The dose and time of Optysil application had a greater effect on the assimilation area than on the leaf dry weight. Optysil at 0.50 dm^3 ha^{-1} stimulated biosynthesis of photosynthetic pigments more strongly than at 0.25 dm^3 ha^{-1}. The time of Optysil application had a greater effect on the Chl content than on the Car content.

Results of this study increased current knowledge to potato response on silicon foliar application under field conditions and provides data for future recommendations for silicon application in early crop potato culture. However, future studies are necessary to evaluate responses of various potato cultivars on silicon, and optimize dose and time of silicon application for environmental conditions to achieve the expected outputs.

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COMPARISON OF CALCIUM ACCUMULATION, DISTRIBUTION, AND USE EFFICIENCY AMONG CHINESE FIR (*CUNNINGHAMIA LANCEOLATA* (Lamb.) Hook) CLONES

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Abstract. Screening Chinese fir (*Cunninghamia lanceolata)* clones with efficient calcium utilization is an effective strategy to maintain soil fertility and improve their productivity and adaptability to nutrient stress habitats. The calcium content of 17-year-old Chinese fir progenies was measured, and calcium accumulation, distribution, and utilization efficiency were compared to select clones that had high calcium utilization efficiency (CaUE). Significant differences in calcium content and accumulation were observed in different vegetative organs and clones. The highest average calcium content and accumulation were in needles, and the lowest averages were in stems and dead branches. Clone M43 had the highest total calcium accumulation, whereas clone M37 had the lowest. Among the tested clones, the CaUE of the whole tree for M23, M37, and M25 was significantly high, whereas that for M43 was the lowest. The CaUE of stems was the highest in M23 and lowest in M17. Thus, calcium content and accumulation in the vegetative organs are important factors affecting the CaUE of whole trees and stems of different Chinese fir clones. Considering the comprehensive CaUE and bioaccumulation in whole trees and stems, M23, M25, and M10 were clones with high CaUE that should be used in future studies on high-yield plantations.
Keywords: *Chinese fir, plantation, productivity, nutrient stress adaptation, nutrient accumulation, utilization efficiency*

Abbreviations: NUE, nutrient utilization efficiency; CaUE, calcium utilization efficiency

Introduction

Calcium is an important nutrient essential for plant growth and development. It participates in cell growth, the formation of cell walls, plasma membranes and wood, as well as in the regulation of physiological processes in plants. Calcium ions have evolved over countless mega-anna and formed a relatively rigorous and complex signal regulation mechanism (Hepler, 2005; Lautner et al., 2007; Kudla et al., 2010). The amount of calcium in the soil depends on many factors, such as the parent material, degree of weathering, leaching intensity, and application of lime. Generally, the calcium content in the soil can meet the regular growth needs of plants; however, plants show calcium deficiency owing to the influence of environmental factors, such as high soil acidity or alkalinity, and other causal factors (White and Broadley, 2003; Guo et al., 2022). For example, soil calcium content is low in red soil areas of subtropical regions because of strong soil weathering and leaching. Damp and rainy conditions can easily cause leaching

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and reduce calcium content and oxygen concentrations in the soil, which affects the absorption of calcium and causes calcium deficiency in plants (Huang, 2004). Therefore, these factors gradually restrict the growth of crops and forest trees. Research has shown that improving the nutrient utilization efficiency (NUE), which enables the production of new biomass with minimum nutrient uptake, is a major strategy for adapting to poor environments. Although some scholars in China and abroad have attached great importance to the study of plant NUE, most research has focused on carbon, nitrogen, phosphorus, potassium, and other nutrient elements. Most studies on efficient calcium utilization by plants focus on crops, and only a few have been conducted on forests.

Nutrient utilization is an important factor when studying forest ecosystem productivity and nutrient cycles. Studies have shown that nutrients play an important role in forest productivity as forest nutrients are mainly used in material production (Wu and Ma, 2009). During the growth process, plants are often in a nutrient-stressed environment for various reasons. In the long-term evolution process, they gradually adapt to the varying nutrient resources in their habitats, forming a selectivity to different nutrient levels and nutrient forms. This selectivity is mainly reflected in a series of physiological adaptations, such as nutrient absorption, assimilation, and photosynthesis, as well as the requirements for nutrient content, morphological adaptation, and productivity accumulation. These different adaptation mechanisms are not only observed among different plant species but also among different strains of the same plant. Studies have shown that different plants or different strains of the same plant have significant differences in the absorption and utilization efficiency of soil nutrients (Nourgholipour et al., 2018). Forest site conditions, especially the supply level of soil nutrient elements, are crucial for a genetic response or adaptation mechanism to nutrient deficiency in plants. Therefore, screening forest genotypes with high NUE is an important way to improve forest productivity and the theory and methods of forest nutritional genetics.

The Chinese fir (*Cunninghamia lanceolata* (Lamb.) Hook) is an important tree species in subtropical regions that is fast-growing, has a high yield per unit area, and has good material quality. For a long time, many researchers have been committed to investigating the selection and breeding of Chinese fir and have screened out many excellent genotypes that have greatly improved the productivity of Chinese fir plantations. In recent years, based on the characteristics of fast growth, fertilizer tolerance, and drought resistance of Chinese fir clones, high-yield varieties that do not require copious amounts of fertilizer and have adapted to different site conditions with high water and NUE have been studied. For example, it is important to screen clones with high utilization of nutrients, such as nitrogen and phosphorus in Chinese fir (Ma et al., 2002; Wu et al., 2011; Zou et al., 2018), and cultivate excellent seed sources of different aluminum-resistant and drought-resistant types of Chinese fir (Li et al., 2020; Xu et al., 2020). Furthermore, reducing the growth pressure of fast-growing tree species is important to forest fertility and alleviating the decline in productivity due to the continuous planting of Chinese fir plantations. Previous studies have shown the importance of screening genotypes with high calcium utilization to enhance plant resistance and improve plant fruit quality. Xing et al. (2020) reported that the accumulation rate of calcium in plant tissues increased concurrently with the calcium supply level. An appropriate amount of calcium can promote the absorption and utilization of nitrogen by improving plant photosynthesis, nitrogen metabolism, and enzyme activity. Nourgholipour et al. (2018) reported there are differences in the absorption of phosphorus and calcium among different varieties of spring oilseed rape under low phosphorus conditions. High calcium absorption can increase the

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bioavailability of calcium-bound phosphate in the soil and promote the absorption and utilization of phosphorus in spring rape seeds. Hippler et al. (2018) identified that a reasonable calcium supply could maintain photosynthesis by directly enhancing the antioxidant system and protecting related antioxidant enzyme activities to minimize the stress damage of copper to plant roots and needles. Calcium application can also promote the nutritional growth and reproductive growth of peanuts, significantly increasing peanut yield and promoting the absorption, utilization, and accumulation of phosphorus in peanut plants (Suo et al., 2021). However, most of these studies focused on crops such as fruits and vegetables, and only a few studies on genotypes of high calcium utilization in trees. Current research shows that because of factors such as acid rain, a large amount of soil calcium is lost, and an appropriate amount of lime or other calcium fertilizer applied to the forest land in acidic soils can alleviate the decline in productivity of *Eucalyptus*, *Pinus*, and other plantations by increasing the effectiveness of calcium (Rochaa et al., 2019). However, adding an appropriate amount of calcium in barren soil can promote the growth of Chinese fir (Rashid et al., 2020) and cypress (Zhang et al., 2020) seedlings, which provides a scientific basis for screening clones with high calcium utilization in forest trees. Therefore, it is very important to screen Chinese fir clones with high calcium acquisition and utilization efficiency. It is not only an effective way to maintain the productivity of Chinese fir plantations in subtropical red soil forest areas and improve their productivity, but also an important strategy to enhance the adaptation of Chinese fir to nutrient stress habitats.

Therefore, based on previous research, we aimed to screen Chinese fir clones with high calcium utilization efficiency (CaUE) from 15 Chinese fir clones (17-year-old) by investigating the calcium nutrient concentration in various organs, the calcium accumulation levels in different Chinese fir stands, and the CaUE of whole trees and stems of different Chinese fir clones, to elucidate the efficiency of Chinese fir to soil calcium and provide a reference for the sustainable management practice of Chinese fir plantations.

Materials and methods

Study site

The study site was located in the Wuyi state-owned forest farm of Zhangping, Fujian Province (117°35'E, 25°15'N), China, which belongs to the general production area of Chinese fir. The annual average temperature is 20.3 °C, the average temperature in the coldest month (January) is 9.7 °C, the average temperature in July is 28.1 °C, the frost-free period is 300 days, the accumulated temperature ≥10 °C is 4956.5 °C, the annual sunshine hours are over 1878 h, and the annual average precipitation is 1508.8 mm (Hu, 2008). In 1991, an experiment on Chinese fir clone determination and afforestation was conducted in the Shibankeng work area of this study site (Wuyi state-owned forest farm). The terrain of the Shibankeng work area is concave, with a southwest slope direction and an altitude of 300 m. The soil is categorized as deep and fertile mountain red soil. The site type belongs to class II, and the previous stubble was pine and miscellaneous forest cuttings.

We used excellent Chinese fir clones screened by the Fujian Chinese Fir Research Center and Fujian Chinese Fir Progeny Determination Cooperation Group for this study. Spike strips were collected from September to October and were cut in the nursery. Intensive management was conducted from January to March of the following year for

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transplanting. Watering was conducted in the afternoon, once per day for the first 30 days, once per two days after 30 days, and weeding was performed once per month. In the spring of the third year, strong symmetrical seedlings with well-developed roots and a uniform size were selected for afforestation. A randomized block design was adopted for afforestation: two block groups with four Chinese fir trees in each group and six replicates, arranged longitudinally along the mountain in two columns. During afforestation, a random arrangement within and between groups was achieved.

The afforestation of forest land involved grass cutting, mountain refining, and strip land preparation. The belt spacing was 2.0 m, the plant spacing was 1.5 m, an open hole (60 cm × 40 cm × 30 cm) was excavated. Comprehensive weeding and tending were performed 1–2 times a year in the first 3 years after afforestation. One to two rows of *Michelia macclurei* were planted around the trees for protection. In this study, 15 clones (M1, M3, M8, M9, M10, M17, M19, M23, M24, M25, M34, M37, M41, M43, and M45) were selected for analysis. The tested Chinese fir clones were 17 years old, and the growth conditions and treatment methods of the experimental forest were consistent.

Investigation method

The characteristics of the 17-year-old progeny of different Chinese fir clones (15 clones) were measured using a wood gauge. Tree height, average diameter at breast height (DBH), and average tree height of different clones were calculated (*Table 1*). Based on the average wood height and DBH of all trees at the sample site, one standard tree was selected from each sample site and dug out from the roots. The fresh mass of stems, barks, branches, and needles in each section was determined by dividing the stem into 2 m segments. The fresh weight of the root was also determined. All samples were taken back to the laboratory, where they were dried and measured for dry matter weight. After the plant samples were dried, crushed, and sieved, the calcium content of different organs was determined using atomic absorption spectrophotometry (Thermo Scientific iCE 3000), and the calcium accumulation and CaUE of whole trees and stems were calculated using equations 1, 2, and 3, with three replications:

$$\text{Calcium accumulation=Total dry biomass in different organs of Chinese fir} \times \text{Calcium content in different organs} \quad \text{(Eq.1)}$$

$$\text{CaUE of the whole tree} = \frac{\text{Total dry biomass of the whole tree}}{\text{Calcium accumulation of the whole tree}} \quad \text{(Eq.2)}$$

$$\text{CaUE of stem} = \frac{\text{Total dry biomass of stem}}{\text{Calcium accumulation of the whole tree}} \quad \text{(Eq.3)}$$

Statistical analyses

All measurements are reported as mean ± standard deviation. Microsoft Excel 2016 was used to calculate the data, and IBM SPSS Statistics 19 software was used to analyze the data. At a significance level of 5%, the Tukey test was used to compare the average values of variables showing significant differences, and Origin 2021b (OriginLab) was used for Pearson correlation analysis and mapping.

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Table 1. *Comparison of growth among different testing clones of 17-year-old Chinese fir plantations (Tao et al. 2021)*

Clone	diameter at breast height (DBH)/cm	Height/m
M1	22.13 ± 2.84	16.13 ± 1.35
M3	23.99 ± 2.29	16.06 ± 0.56
M8	22.46 ± 3.42	16.47 ± 1.15
M9	19.70 ± 4.78	14.64 ± 3.16
M10	20.58 ± 4.53	15.70 ± 1.80
M17	24.37 ± 2.55	17.15 ± 1.35
M19	21.79 ± 4.24	16.02 ± 1.99
M23	21.40 ± 3.16	16.06 ± 1.67
M24	22.06 ± 2.95	16.00 ± 1.42
M25	19.47 ± 3.42	16.14 ± 1.28
M34	22.32 ± 4.50	15.64 ± 2.93
M37	21.47 ± 3.14	15.31 ± 2.31
M41	20.07 ± 5.39	15.19 ± 3.69
M43	24.17 ± 2.37	17.76 ± 1.36
M45	23.60 ± 2.58	15.58 ± 0.43

Results

Calcium content in vegetative organs of different Chinese fir clones

The calcium content in different vegetative organs of different Chinese fir clones varied greatly. Among the 15 tested clones, the average calcium content in needles was the highest and differed significantly from in the other organs ($P < 0.05$); The average calcium content of bark was the second, but was only 38.98% of that of needles; The average calcium content of living branches and barks was similar, and the difference was only 0.03 g kg^{-1}; The average calcium content of the roots and stems was low, with only 0.04 and 0.01 g kg^{-1}. The calcium content of the stem and root organs of the multiple clones was zero. Among all tested clones, M17 had the highest calcium content, which differed significantly from the other clones ($P < 0.05$), followed by M43, M41, and M24, but there was no significant difference ($P > 0.05$). M23 had the lowest calcium content, which was only 33.90% of the average value. The calcium content of living branches M43, M19, M10, and M41 was higher, and the difference was not significant ($P > 0.05$); The calcium content of M1 living branches was the lowest, with only 9.52% of its average value (*Table 2*).

Calcium accumulation in different Chinese fir clones

Calcium accumulation in different vegetative organs of Chinese fir clones also varied greatly. The average calcium accumulation in the needles was the highest, which differed significantly from that in the other organs ($P < 0.05$). The average calcium accumulation in the cortex was 46.01% of that in the needles; the average calcium accumulation of living branches, stems, roots, and dead branches decreased successively, with dead branches showing the lowest, at only 7.06% of that in the needles. The average calcium

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accumulation in the roots and stems was 11.16% and 12.83%, respectively, and many clones showed no calcium accumulation in the stem and roots. The calcium accumulation pattern of the needles, living branches, and bark of vegetative organs were similar in all clones, with high calcium accumulation in M17, M41, and M43 and the lowest in M45, M1, and M23. Calcium accumulation in the 15 tested clones was significantly different among the different Chinese fir clones. Clone M43 had the highest calcium accumulation in the whole plant, which differed significantly from that in the other clones ($P < 0.05$) and was 1.95 times the average of all tested clones. The calcium accumulation of M17 and M41 was 5.98% and 17.45% lower than M43, respectively. M37 had the lowest calcium accumulation in the whole plant, with only 24.76% of that of M43 (*Table 3*).

Table 2. *Comparison of calcium content among different organs in different Chinese fir clones (g kg^{-1})*

Clone	Needle	Living branch	Dead branch	Bark	Stem	Root
M1	1.44 ±0.02Abcd	0.04 ± 0.06CDc	0.41 ± 0.10Bab	0.29 ± 0.05BCcde	0.00 ± 0.00Da	0.09 ± 0.11CDab
M3	1.13 ±0.01Acde	0.39 ± 0.07Bbc	0.13 ± 0.02CDc	0.24 ± 0.21BCde	0.00 ± 0.00Da	0.00 ± 0.00Db
M8	0.99±0.01Acdef	0.54 ± 0.03Bab	0.21 ± 0.01CDbc	0.34 ± 0.15Cbcde	0.01 ± 0.01Ea	0.03 ± 0.04DEab
M9	1.17 ±0.39Acde	0.52 ± 0.06BCabc	0.19 ± 0.01CDbc	0.83 ± 0.04Aba	0.00 ± 0.00Da	0.04 ± 0.06Dab
M10	1.43 ±0.21Abcd	0.61 ± 0.15Bab	0.25 ± 0.12CDbc	0.42 ±0.09BCabcde	0.02 ± 0.02Da	0.06 ± 0.06Dab
M17	2.21 ± 0.18Aa	0.48 ± 0.09Babc	0.60 ± 0.14Ba	0.56 ± 0.18Babcd	0.00 ± 0.00Da	0.03 ± 0.03Dab
M19	0.86 ±0.04Adef	0.61 ± 0.14Bab	0.20 ± 0.02Dbc	0.41 ± 0.04Cabcde	0.05 ± 0.05Da	0.02 ± 0.04Dab
M23	0.40 ± 0.01Af	0.16 ± 0.09Bbc	0.20 ± 0.04Bbc	0.06 ± 0.05Be	0.05 ± 0.06Ba	0.03 ± 0.05Bab
M24	1.54 ± 0.04Abc	0.29 ± 0.05Cbc	0.32 ± 0.01Cbc	0.58 ± 0.12Babcd	0.02 ± 0.03Da	0.00 ± 0.00Db
M25	0.77 ± 0.18Aef	0.24 ± 0.09Cbc	0.29 ± 0.01BCbc	0.46 ± 0.05Babcde	0.00 ± 0.00Da	0.00 ± 0.00Db
M34	0.87 ±0.06Adef	0.32 ± 0.02Bbc	0.40 ± 0.06Babc	0.41 ± 0.20Babcde	0.06 ± 0.01Da	0.01 ± 0.02Db
M37	0.62 ± 0.20Aef	0.37 ± 0.16ABbc	0.30 ± 0.03Bbc	0.20 ± 0.04BCde	0.00 ± 0.00Ca	0.00 ± 0.00Cb
M41	1.57 ± 0.04Abc	0.60 ± 0.06Bab	0.28 ± 0.07BCbc	0.60 ± 0.12Babcd	0.00 ± 0.00Ca	0.13 ± 0.12Cab
M43	1.96 ± 0.17Aab	0.86 ± 0.08Ba	0.29 ± 0.11CDbc	0.72 ± 0.11BCabc	0.00 ± 0.00Da	0.17 ± 0.18Da
M45	0.78 ± 0.12Aef	0.24 ± 0.30Bbc	0.29 ± 0.01Bbc	0.77 ± 0.01Aab	0.00 ± 0.00Ba	0.06 ± 0.09Bab
Means	1.18 ± 0.51	0.42 ± 0.23	0.29 ± 0.12	0.46 ± 0.23	0.01 ± 0.03	0.04 ± 0.08

Different lowercase letters in the same column indicate significant differences between different clones ($P < 0.05$); different uppercase letters in the same row indicate significant differences between different organs ($P < 0.05$)

CaUE of different Chinese fir clones

The calcium utilization efficiency of whole tree and stem varied greatly among different Chinese fir clones, with an average of 7011.95 and 4140.60 g g^{-1}, respectively. The utilization efficiency of the whole tree and stem of M23, M37, and M25 were 1.79, 1.66, and 1.39 times and 1.86, 1.57, and 1.38 times their average values, respectively, which were significantly higher than the other tested clones ($P < 0.05$). The CaUE of the whole tree and stem of clone M43 was 51.79% and 52.33% of their average values, respectively, which were the lowest among the tested clones, and significantly different from that of the other clones ($P < 0.05$) (*Fig. 1*).

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Table 3. *Comparison of calcium accumulation among different organs in different Chinese fir clones (g per plant)*

Clone	Needle	Living branch	Dead branch	Bark	Stem	Root	Whole tree
M1	13.11 ± 0.33Aef	0.47 ± 0.02Eg	1.15 ± 0.03Dbc	4.31 ± 0.04Bi	0.00 ± 0.00Fg	2.17 ± 0.09Cc	21.21 ± 0.44de
M3	16.76 ± 0.90Ad	5.82 ± 0.32Bd	0.32 ± 0.01Dj	2.19 ± 0.08Cj	0.00 ± 0.00Dg	0.00 ± 0.00Dg	25.10 ± 1.30d
M8	14.52 ± 0.44Ae	8.34 ± 0.33Bc	0.72 ± 0.02Dfg	4.73 ± 0.12Ci	0.88 ± 0.02Df	1.18 ± 0.08De	30.36 ± 0.96c
M9	8.55 ± 0.45Ai	4.82 ± 0.04Be	0.45 ± 0.02CDi	8.41 ± 0.43Ac	0.00 ± 0.00Dg	1.06 ± 0.09Cef	23.29 ± 1.03d
M10	8.99 ± 0.12Ahi	4.20 ± 0.15Ce	0.68 ± 0.02Fg	4.94 ± 0.10Bhi	2.23 ± 0.04Dd	1.78 ± 0.07Ed	22.81 ± 0.48d
M17	31.17 ± 2.07Aa	9.64 ± 0.47Bb	2.45 ± 0.06Ca	9.23 ± 0.30Bb	0.00 ± 0.00Dg	1.00 ± 0.07CDef	53.50 ± 2.96a
M19	11.64 ± 0.52Afg	8.38 ± 0.11Bc	0.65 ± 0.02Dg	5.57 ± 0.18Cgh	6.08 ± 0.10Cc	0.75 ± 0.01Df	33.07 ± 0.49c
M23	5.61 ± 0.10Bj	2.49 ± 0.08Cf	0.78 ± 0.01Df	0.75 ± 0.02Dk	6.70 ± 0.58Ab	1.21 ± 0.07De	17.54 ± 0.86ef
M24	11.83 ± 0.58Afg	2.87 ± 0.14Cf	0.93 ± 0.05De	7.69 ± 0.51Bcd	1.61 ± 0.02De	0.00 ± 0.00Eg	24.93 ± 1.29d
M25	7.83 ± 0.23Ai	3.10 ± 0.11Cf	1.19 ± 0.05Db	6.14 ± 0.16Bfg	0.11 ± 0.00Eg	0.00 ± 0.00Eg	18.38 ± 0.54e
M34	10.68 ± 0.12Agh	4.56 ± 0.17De	1.11 ± 0.02Ebcd	6.78 ± 0.23Cef	7.68 ± 0.37Ba	0.20 ± 0.02Fg	31.00 ± 0.92c
M37	5.42 ± 0.09Aj	5.03 ± 0.09Bde	1.04 ± 0.03Dd	2.59 ± 0.09Cj	0.00 ± 0.00Eg	0.00 ±.00Eg	14.09 ± 0.30f
M41	21.23 ± 0.82Ac	10.01 ± 0.76Bb	0.54 ± 0.01Dh	10.38 ± 0.44Ba	0.00 ± 0.00Dg	4.80 ± 0.20Cb	46.97 ± 2.21b
M43	24.87 ± 0.63Ab	15.20 ± 0.49Ba	0.88 ± 0.03Ee	9.68 ± 0.31Cab	0.00 ± 0.00Eg	6.27 ± 0.42Da	56.90 ± 1.87a
M45	5.36 ± 0.38Bj	2.20 ± 0.07Cf	1.09 ± 0.03Dcd	7.42 ± 0.31Ade	0.00 ± 0.00Eg	1.63 ± 0.08Dd	17.70 ± 0.80ef
Means	13.17 ± 7.39	5.81 ± 3.77	0.93 ± 0.49	6.06 ± 2.80	1.69 ± 2.70	1.47 ± 1.78	29.12 ± 13.05

Different lowercase letters in the same column indicate significant differences between different clones ($P < 0.05$); different uppercase letters in the same row indicate significant differences between different organs ($P < 0.05$)

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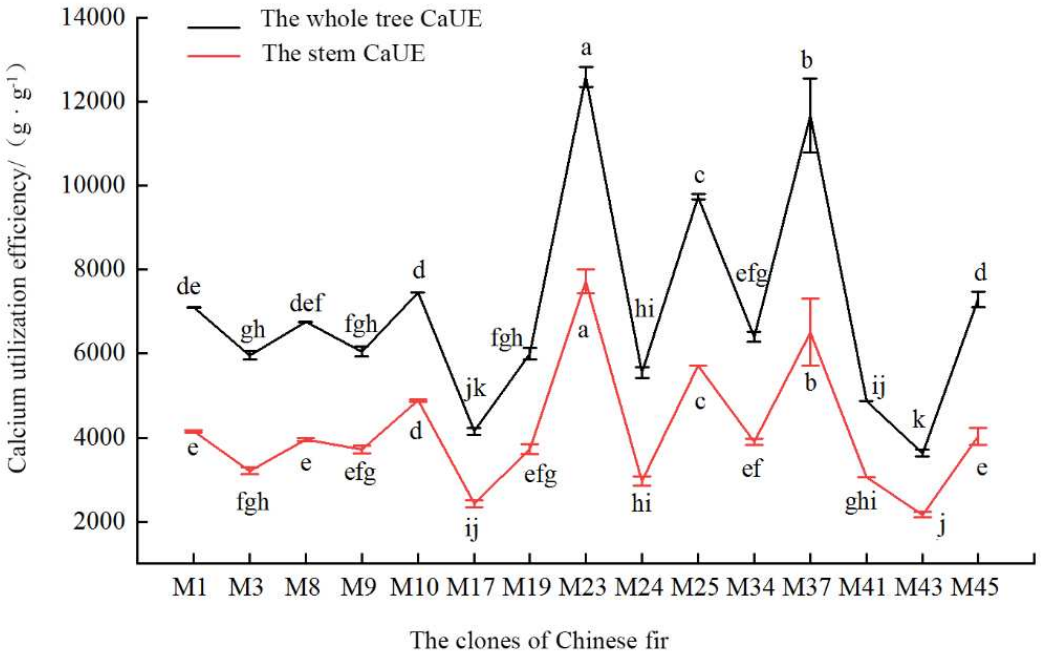

Figure 1. *Comparison of calcium use efficiency among different clonal test plantations of Chinese fir. Different lowercase letters indicate significant differences between different clones ($P < 0.05$)*

Correlation between calcium accumulation and CaUE among different Chinese fir clones

The CaUE of different Chinese fir clones showed different correlations with calcium content (*Fig. 2*) and calcium accumulation (*Fig. 3*) in different vegetative organs. In the $P = 0.05$ level test, there was a significant positive correlation between CaUE of whole tree and stem. There was a significant negative correlation between CaUE and calcium content and calcium accumulation in needles, living branches, and barks of vegetative organs. The CaUE of the stem was significantly negatively correlated with the calcium content of needles and barks and the calcium accumulation of needles, living branches, and bark. The CaUE of the whole tree and stem was positively correlated with the calcium content and calcium accumulation of stems and negatively correlated with the calcium content of needles, living branches, dead branches, barks, and roots. The CaUE of whole tree and stem was positively correlated with the calcium content and calcium accumulation in the stem and negatively correlated with the calcium content of needles, living branches, dead branches, barks, and roots. Except for the significant positive correlation between the calcium content of needles and dead branches, the correlation between the calcium content of different vegetative organs did not reach a significant level (*Fig. 2*). In contrast, there was a significant positive correlation between the calcium accumulation of needles and living branches and barks, and between roots and living branches and barks. In addition, the correlation between calcium accumulation in other vegetative organs did not reach a significant level (*Fig. 3*).

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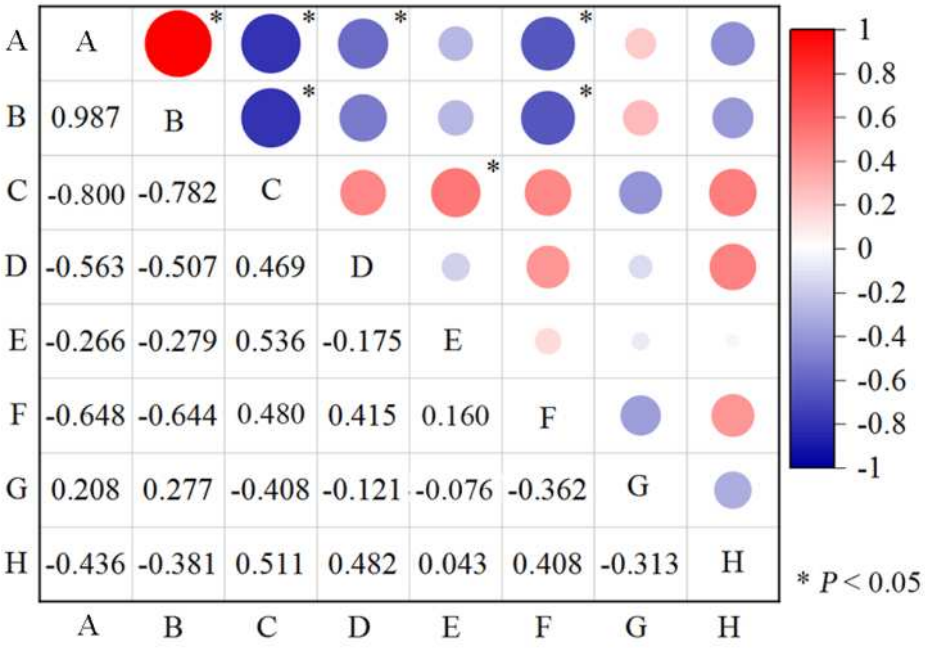

Figure 2. *Correlation between CaUE and calcium content in different organs. A: The whole tree CaUE; B: The stem CaUE; C, D, E, F, G, and H are Ca contents of needles, living branches, dead branches, barks, stems and roots, respectively*

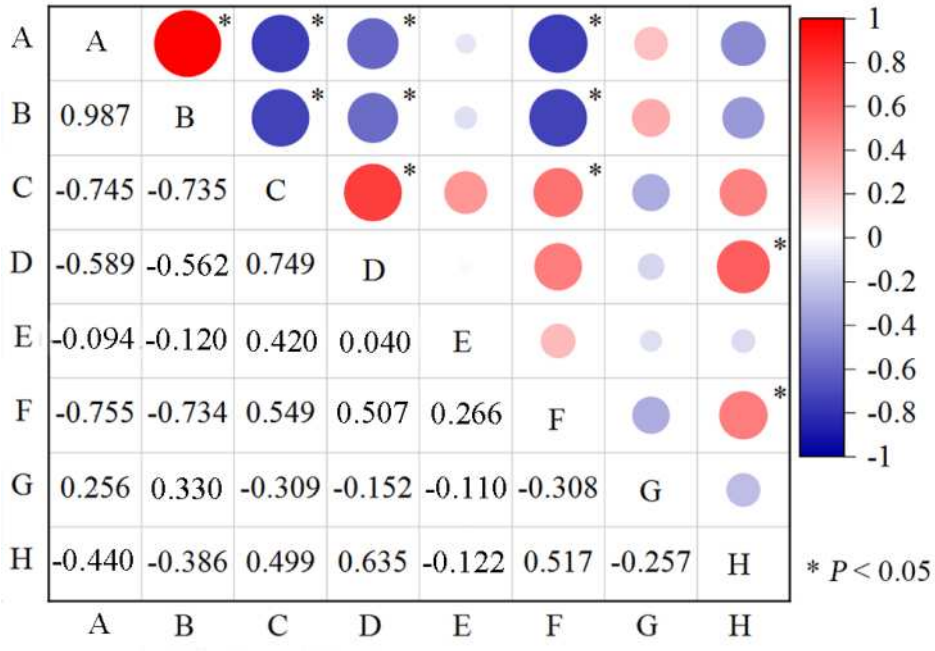

Figure 3. *Correlation between CaUE and calcium accumulation in different organs. A: The whole tree CaUE; B: The stem CaUE; C, D, E, F, G, and H are Ca accumulation in needles, living branches, dead branches, barks, stems and roots, respectively*

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Discussion

The nutrient element content in plants reflects the ability of plants to absorb and accumulate mineral nutrients from soil under certain habitat conditions. Because of the different components of organic matter in different organs, their physiological functions are different. Furthermore, the functions of different nutrient elements in plants are also different, resulting in differential distributions of nutrient elements in the same organ and the same nutrient element in different organs. For example, the content of nutrient elements in living tissues is higher than that in aging tissues, usually in needles, branchlets, and other organs, which have the highest nutrient input, whereas only low nutrient content is found in dry organs, which indicates an effective utilization of nutrients by plants (Zhao et al., 2016; Zhang et al., 2018). According to our results, the calcium content in the needles of the tested Chinese fir clones was the highest, and the stems calcium content was the lowest. This is mainly because the needles are assimilation organs, which are important for photosynthesis and organic matter synthesis; their growth cycle is short, and their metabolic activities are the most vigorous, so their nutritional content is generally the highest. The stem mainly comprises wood, supports the plant body and has the weakest physiological function. Most nutrients contained are utilized or transferred, and thus their nutrient element content is the lowest. In addition, the function, transport, and distribution of calcium in plants are also important reasons for this phenomenon. Generally, the calcium content in the apical meristem is high because this is where vigorous plant metabolism occurs. This is also because long-distance transportation of calcium in the plant mainly depends on the xylem, and the transportation power of the xylem is in transpiration. Calcium first reaches the vigorous growth parts, such as treetops and needles, through the xylem due to transpiration. After calcium reaches these tissues and organs, it generally becomes relatively stable because there is no re transportation and distribution, there is poor mobility of calcium in the phloem, and transportation volume is small. This characteristic also indicates that more calcium is present in the aboveground organs and less in the underground roots.

Plant nutrient accumulation is an indispensable link in the material cycle of ecosystems and the basis for studying the logistics and energy flow of forest ecosystems. It is important to maintain the structure and function of forest ecosystems. The accumulation of plant nutrient elements depends on the size of the biomass and the content of nutrient elements. Zhou et al. (2019) showed that the calcium accumulation in a 16-year-old Chinese fir forest was 376.64 $kg \cdot hm^{-2}$. However, the calcium accumulation of Chinese fir clones in this study was not high (between 46.91 and 189.47 $kg \cdot hm^{-2}$), which may have been caused by many factors, such as low wood density, slow growth, and low calcium concentration in tissues (mainly roots and stems). The accumulation and distribution of biomass in different organs of Chinese fir clones were different. Wu et al. (2012) reported that the proportion of stem biomass in stand biomass was the greatest, ranging from 53.37% to 65.48%, and the average biomass value of each organ was stem > root > living branch > bark > needle > dead branch. Chen et al. (2019) identified that the characteristics of Chinese fir as a timber (stem utilization) species, indicating that its stem biomass is the main component of stand biomass. In this study, the average calcium content of roots and stems was low, and the difference between the average values was only 0.03 g kg^{-1}. Therefore, although the nutrient content of the stem was the lowest among all organs, its nutrient accumulation was higher than the roots. Zhou et al. (2019) studied nutrient accumulation and distribution characteristics in the tree layer of Chinese fir forests of different forest ages. The results showed that in the same Chinese fir forest, the contents

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of five nutrient elements (N, P, K, Ca, and Mg) in each organ were needle > branch > bark > root > stem, and the distribution law of nutrient accumulation in each organ was needle > branch > bark > stem > root, which differs slightly from the results, but the accumulation in the needles was the highest. It also shows that stand biomass and its distribution in different organs and nutrient content in Chinese fir are the main factors affecting nutrient accumulation and distribution in Chinese fir forests. Research has shown that plant tissues or organs maintain the relative balance of nutrient absorption and transfer during growth (Guyonnet et al., 2018). Therefore, the growth of Chinese fir and its nutrient demand at different growth stages also affect nutrient accumulation and distribution.

Under specific nutrient supply conditions, nutrient efficiency can be divided into nutrient absorption efficiency and NUE. The factors affecting plant NUE include soil nutrient content and effectiveness (such as soil quality and nutrient management) and differences in plant strains. Efficient nutrient absorption and utilization are two important characteristics of nutrient-efficient strains. Nutrient efficiency is not only related to plant root morphology and configuration, rhizosphere processes, and nutrient transmembrane transport; it is also closely related to the behavior of nutrients in plants, especially how they affect the synthesis, transport, distribution, transformation, and transport of photosynthetic substances and their storage in yield organs (Mi, 2017). Our results indicated that the CaUE of different Chinese fir clones was significantly different, and the CaUE was significantly related to the calcium content and calcium accumulation in needles, living branches, and barks organs of Chinese fir. Clones with high CaUE can carry out normal growth and metabolism with low nutrient concentrations in their bodies and produce a certain biomass. Considering the CaUE and biological accumulation of whole trees and stems of different Chinese fir clones, M23, M25, and M10 were preliminarily selected as clones with high CaUE. Plant lines with high nutrient utilization have ideal root morphology and reasonable root distribution, strong adaptive response in the rhizosphere under nutrient stress, high specific absorption rate for low concentration nutrients, strong ability for nutrient transportation and reuse in plants, and high nutrient utilization rate or low metabolic demand (Wu and Ma, 2009; Wu et al., 2011; Zou et al., 2018). In addition, Hawkesford et al. (2014) pointed out that the difference in plant genetic characteristics significantly affects the NUE, which can be improved through improved seed cultivation. The functions of different nutrients in plants vary because of the differences in physical and chemical properties between different soil nutrients and the complex interactions between them. For example, the phosphorus content in soil affects the absorption and utilization of calcium by plants (Ding et al., 2018; Suo et al., 2021). Soil nutrient conditions are also an important factor affecting the utilization efficiency of plant nutrients. Therefore, nutrient absorption and utilization efficiency reflect the relationship between nutrient processes and plant growth processes and directly reflects nutrient acquisition and utilization of different plant lines.

The purpose of studying plant nutrient efficiency is to improve the genetic characteristics of plant nutrition and improve the yield and quality of crops. Notably, the NUE in plants is a complex dynamic process involving multiple physiological processes such as transportation, distribution, utilization, and reuse of nutrients in plants, which needs to be analyzed from the whole growth period of plants. Calcium is a nutrient element not easy to move in plants, and calcium ions have formed a more rigorous and complex signal regulation mechanism after hundreds of millions of years of evolution. At present, research regarding calcium signaling has entered the molecular level, and

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calcium signal elements in some plant cells have been identified and analyzed. However, the operation mechanism of calcium signaling in plants is not very clear, and still many aspects need to be further studied. Therefore, the selected calcium-efficient Chinese fir clones should be monitored, and their nutrient efficiency during the entire growth period should be dynamically tracked. Furthermore, emerging technologies such as bioinformatics, genomics, proteomics, and metabolomics should be applied to study the physiological processes of calcium transport, distribution, utilization, and reuse in Chinese fir through pot control experiments. In addition, the calcium metabolism, transmission, perception response, and expression of stress signals, especially the response mechanism of calcium messengers in Chinese fir toward stressors, should be explored. Such extensive studies can help us utilize the potential of Chinese fir for calcium absorption and can provide a reference for maximizing the yield from Chinese fir plantations.

Conclusion

Among the vegetative organs of different Chinese fir clones, the calcium content and accumulation were the highest in the needles and the lowest in the stem and dead branches. There were significant differences in calcium content and accumulation among different Chinese fir clones, and there were also significant differences in the CaUE of whole tree and stem. There was a significant negative correlation among CaUE, calcium content, and calcium accumulation in Chinese fir needles, living branches, and barks. Clones with high CaUE can normally grow and metabolize with low nutrient concentrations in their bodies and produce a certain biomass. The calcium content and accumulation in the vegetative organs of Chinese fir are important factors affecting the CaUE of whole tree and stem among different Chinese fir clones. Considering the CaUE and biological accumulation of whole tree, M23, M25, and M10 were preliminarily selected as clones with high CaUE. It is an effective way to maintain the productivity of Chinese fir plantations in subtropical red soil forest areas to improve their productivity. Furthermore, it is an important strategy to enhance the adaptation of Chinese fir to nutrient-stressed habitats. This experiment only studied 17-year-old Chinese fir, which could not reveal the CaUE of Chinese fir forests at different growth stages and its impact on productivity. In the future, we should strengthen the research on the production, calcium utilization, and utilization efficiency of Chinese fir forests at different growth stages.

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CHARACTERIZATION OF METAL POLLUTION IN SURFACE SEDIMENTS ALONG THE NORTHEASTERN COAST OF LIBYA

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Abstract. The present investigation aimed to assess metal pollution status (Fe, Al, Cu, Zn, Mn, Cd and Pb) in sediments of four cities along the northeastern coast of Libya. The mean metal concentrations in sediment samples from highest to lowest showed the following order Al > Fe > Mn > Zn > Cu > Pb > Cd. All metal concentrations were lower than their background values except for Cd at sites 1, 2 and 3. Sediment quality guidelines showed that biological hazardous effects are related to 25% of Cd samples while other studied metals are biologically safe. The overall evaluation indicates moderate to severe pollution status with Cd along sites 1 and 3 while other metals showed marginal or safe limits. Metal pollution status along the coastline of major cities proved indisputably to be due to anthropogenic activities. The geoaccumulation index (I_{geo}) showed efficient indication of individual metals while the potential ecological risk index (RI) could clearly discriminate the risks posed by metal mixtures.
Keywords: *Mediterranean Sea, sediment quality, anthropogenic activities, ecological risks*

Introduction

The Mediterranean coastline of Libya extends for about 1950 km starting from the western borders with Tunisia to the eastern borders with Egypt. The Libyan coast and its lagoons play an important role in the biodiversity and productivity of the Mediterranean marine life as it is characterized by gigantic beds of marine plants serving as important feeding and nursery grounds for different marine species. Thus it is considered one of the most productive commercial fisheries along North Africa (Haddoud and Rawag, 2007). Rapid industrialization and uncontrolled urbanization around many Libyan cities and coastal areas have brought alarming levels of metal contamination to the aquatic environment as metals have high enrichment factors and slow removal rates (Soliman et al., 2015). Metals constitute a core group of aquatic pollutants and have a particular significance in ecotoxicology due to their high persistence as well as non-biodegradable and bio-accumulative properties (Paudel et al., 2016). The elevated metal levels in aquatic ecosystems raises worldwide environmental and health concerns (Rakib et al., 2021).

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Pollutants related to anthropogenic activities have various sources such as mining, industry, urbanization, agriculture and many other every-day activities. Metals released from mining and industrial processes are among the major contaminants of concern in marine environments, where they accumulate in the water, sediments and marine organisms. Sources of pollution in aquatic ecosystems are mostly land-based but in some cases, the pollutants originate or remobilize in the aquatic environment itself (Tornero and Hanke, 2016). Integrated management strategies to protect aquatic ecosystems include evaluating metal pollution of sediments as well as differentiating the influence of natural and/or anthropogenic sources (Yu et al., 2008).

Procedures of sediment pollution assessment using calculated indices such as contamination factor (CF), enrichment factor (EF), modified contamination degree (mC_d), pollution load index (PLI) and geoaccumulation index (I_{geo}) are widely applied to evaluate metal pollution status and enrichment in sediments (Wang et al., 2014; Omar et al., 2016; Saleh, 2021). These indices are considered as sensitive indicators of the influence of different anthropogenic activities on aquatic ecosystems (Omar et al., 2016).

The present work aims to characterize metal pollutants in sediments of four coastal sites with different levels of anthropogenic activities along the northeastern coast of Libya.

Materials and Methods

The study sites

The Mediterranean Sea coast of Libya is closely associated with the major geological features of the African continent. The hydrological conditions of the region are dominated by three layers of water masses, which are the surface layer, the intermediate layer and the deep layer also it is characterized by lack of natural freshwater inputs (Al-Hassan, 1999). The Libyan coastline has four important lagoons, which are Farwa (at the northwestern coast), Ain Zayana (Benghazi city), Ain Gazala (Tobruk city) and Al-Burdy lagoons (Kerambrun, 1986). Three of these lagoons are located in the northeastern coast of Libya and were investigated in the present work.

Site 1: located at Ain Zayana lagoon, Benghazi city, with GPS coordinates of 32° 14' 59.98" N, 20° 4' 47.72" E. Benghazi is the second largest Libyan city after Tripoli. The city is characterized by its port on the Mediterranean Sea with various urban and industrial activities.

Site 2: located at Susah city with GPS coordinates of 32° 55' 6.49" N, 21° 57' 26.23" E. The area is characterized by its shallow waters suitable for fishing activities which are uncontrolled or illegal in most cases.

Site 3: located at Ain Ghazala lagoon, Tobruk city, with GPS coordinates of 32° 3' 51.37" N, 24° 1' 14.00" E. The city is well known by the shipping and shipment activities associated with oil pollution.

Site 4 (Reference site): located at Al-Burdy lagoon, Al-Burdy city, with GPS coordinates of 31° 45' 38.32" N, 25° 6' 11.20" E. The city is characterized by minor urban activities which are mostly eco-friendly, thus this site is considered as a reference site in the present study.

The four sites cover a distance of about 500 km along the Libyan Mediterranean coast (*Fig. 1*).

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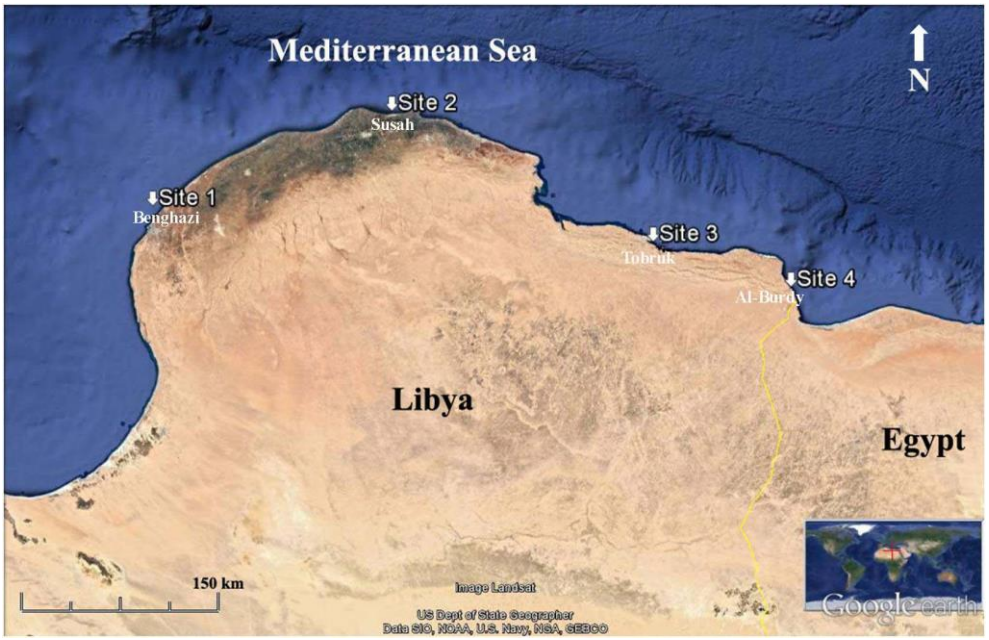

Figure 1. *Map of the study sites*

Sediment sampling

Duplicates of eight surface sediment samples, up to 10 cm in depth, were collected using sampling dredge from four localities in a radius of 1 km^2 around the reported GPS coordinates of the studied during summer season 2019. The samples were immediately stored at 4ºC for further analyses (Cabrera et al., 1992).

Metal concentrations in sediment samples

Sediment samples were dried at 105ºC for 12 hours then burned in a muffle furnace at 550ºC for 16 hours. Samples were then acid digested and diluted with de-ionized water to known volume using the dry ashing procedure proposed by Issac and Kerber (1971) and Hseu (2004). Metal concentrations (Fe, Al, Cu, Zn, Mn, Cd and Pb) were determined using flame atomic absorption spectrophotometer (Thermo Scientific ICE 3300, UK) provided with double beam and deuterium background corrector according to APHA (2005).

Quality assurance and quality control (QA/QC) procedures

Analytical blanks were run in the same way as the samples, and metal concentrations were determined using standard solutions prepared in the same acid matrix. All used reagents were of analytical grade (Merck Co.). Standards for instrument calibration were prepared on the basis of mono-element certified reference solution (Merck Co.). Standard reference material (SRM 2702) was used to validate extraction procedures. All analyses were carried out in duplicates and the metal recoveries ranged from 92 to 107% for all measured samples.

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Risk assessment of sediment pollution

Sediment quality guidelines (SQGs)

The detected metal concentrations were compared to the corresponding quality guidelines. Two sets of SQGs proposed by the U.S. National Oceanic and Atmospheric Administration (NOAA) and Canadian guidelines developed for marine sediment (MacDonald et al., 2000) were applied in the present study: (1) the threshold effect level (TEL)/probable effect level (PEL) values; and (2) the effect range low (ERL)/effect range-median (ERM) (Long et al., 1995; MacDonald et al., 2000).

Contamination factor (CF)

It was calculated using the following equation (*Eq. 1*) (Hakanson, 1980).

$$CF_i = Cm_i / Cb_i \quad \text{(Eq.1)}$$

where Cm_i is the mean concentration of metal i in sediment sample and Cb_i is the background value, also called shale value or crustal value, of that metal. The average background values of the studied metals as indicated by Turekian and Wedepohl (1961) are 46000, 80000, 45, 95, 850, 0.3 and 20 mg/kg dry wt. for Fe, Al, Cu, Zn, Mn, Cd and Pb, respectively.

Enrichment factor (EF)

Calculation of EF involves normalization of the detected metal with respect to a reference element such as Al, Fe and Mn (Karbassi et al., 2008). Al was used as a normalizer as it exists in sediments at a respective high level and it is rich in the Earth's crust assuming that it is free from anthropogenic impact. EF values for each metal in sediment were calculated as follows (*Eq. 2*) (Buat-Menard and Chesselet, 1979).

$$EF = (Cm_i/C_{Al})_{sample} \; / \; (Cm_i/C_{Al})_{background} \quad \text{(Eq.2)}$$

where $(Cm_i/C_{Al})_{sample}$ is the ratio of concentration of metal i to that of Al in each sampling site and $(Cm_i/C_{Al})_{background}$ is the same ratio of the background concentrations reported by Turekian and Wedepohl (1961).

Modified contamination degree (mC_d)

It was applied to estimate the overall contamination degree at a particular site based on the following equation (*Eq. 3*) (Abrahim and Parker, 2008).

$$mC_d = \sum_{i=1}^{n} CF_i \; / \; n \quad \text{(Eq.3)}$$

where n is the number of the studied metals.

Pollution load index (PLI)

The PLI was calculated using the following equation (*Eq. 4*) (Tomlinson et al., 1980).

$$PLI = (\prod_{i=1}^{n} CF_i)^{1/n} \quad \text{(Eq.4)}$$

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PLI < 1 indicates safe limits, whereas PLI ≥ 1 indicates progressive pollution (Tomlinson et al., 1980).

Mean effect range-median quotient (M-ERM-Q)

The mean ERM quotient (M-ERM-Q) was calculated according to the following equation (*Eq. 5*) (Long et al., 2000).

$$\text{M-ERM-Q} = \frac{\sum_{i=1}^{n} Cmi \,/\, ERM}{n} \quad \text{(Eq.5)}$$

where *ERM* is the effect range median of the metal. The *ERM* values of Cu, Zn, Cd and Pb are 270, 410, 9.6 and 218, respectively (Long et al., 1995).

Potential ecological risk

The potential ecological risk factor (Er_i) and the potential ecological risk index (RI) were calculated according to Hakanson (1980) as (*Eq. 6* and *Eq. 7*, respectively)

$$Er_i = Tr_i \times CF_i \quad \text{(Eq.6)}$$

$$\text{RI} = \sum_{i=1}^{n} Er_i \quad \text{(Eq.7)}$$

where Tr_i is the toxic response factor of metal *i*. The toxic response factors of Cu, Zn, Cd and Pb are 5, 1, 30 and 5, respectively (Hakanson, 1980) and that of Mn is 1 (Xu et al., 2008).

Geoaccumulation index (I_{geo})

A common approach to estimate the enrichment of metal concentrations above the background or baseline concentrations is to calculate the geoaccumulation index (I_{geo}). The I_{geo} values were calculated for the studied metals as indicated by Müller (1969) as (*Eq. 8*):

$$I_{geo} = Log_2 \left[Cm_i / (1.5 \times Cb_i)\right] \quad \text{(Eq.8)}$$

where, the 1.5 factor is the background matrix correction factor. Lu et al. (2009) defined the background matrix correction factor as a constant introduced to minimize the effect of possible variations in the background values, which may be attributed to lithologic variations in sediments.

Quality criteria of SQGs and all studied ecological risk assessment indices are illustrated in *Table 1*.

Statistical analyses

The results are expressed as mean ±S.E. Data were statistically analyzed using one-way analysis of variance (ANOVA) and Duncan's multiple range test to determine difference in means as indicated by different case letters in the descending order A, B, C and D at $P<0.05$ using SAS (Statistical Analysis System) version 9.1 (SAS, 2006).

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Table 1. *Quality criteria of SQGs and ecological risk assessment indices*

Index	Classification	Description	Reference
TEL and PEL guidelines	< TEL	Not associated with hazardous biological effects	MacDonald et al. (2000)
	≥ TEL < PEL	May occasionally be associated with hazardous biological effects	
	≥ PEL	Frequently associated with hazardous biological effects	
ERL and ERM guidelines	< ERL	Minimal effects range	Long et al. (1995)
	≥ ERL < ERM	Effects would occasionally occur	
	≥ ERM	Effects would frequently occur	
Contamination factor (CF)	< 1	Low contamination	Hakanson (1980)
	1 - 3	Moderate contamination	
	3 - 6	Considerable contamination	
	> 6	Very high contamination	
Enrichment factor (EF)	< 1	No enrichment	Birch and Olmos (2008)
	1-3	Minor enrichment	
	3-5	Moderate enrichment	
	5-10	Moderately severe enrichment	
	10-25	Severe enrichment	
	≥ 25	Extremely severe enrichment	
Modified contamination degree (mC_d)	< 1.5	Nil to very low degree of contamination	Abrahim and Parker (2008)
	1.5 - 2	Low degree of contamination	
	2 - 4	Moderate degree of contamination	
	4 - 8	High degree of contamination	
	8 - 16	Very high degree of contamination	
	16 - 32	Extremely high degree of contamination	
	> 32	Ultra-high degree of contamination	
Mean effect range-median quotient (M-ERM-Q)	< 0.1	9% probability of toxicity	Long et al. (2000)
	0.11 - 0.5	21% probability of toxicity	
	0.51 - 1.5	49% probability of toxicity	
	> 1.5	76% probability of toxicity	
Potential ecological risk factor (*Eri*)	< 40	Low risk	Hakanson (1980)
	40 - 80	Moderate risk	
	80 - 160	Considerable risk	
	160 - 320	High risk	
	> 320	Very high risk	
Potential ecological risk index (RI)	< 50	Low risk	Hakanson (1980)
	50 - 100	Moderate risk	
	100 - 200	Considerable risk	
	> 200	Very high risk	
Geoaccumulation index (I_{geo})	< 0	Unpolluted	Müller (1969)
	0 - 1	Unpolluted to moderately polluted	
	1 - 2	Moderately polluted	
	2 - 3	Moderately to heavily polluted	
	3 - 4	Heavily polluted	
	4 - 5	Heavily to extremely polluted	
	> 5	Extremely polluted	

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Results and Discussion

Metal concentrations

Metal concentrations along the studied sites showed the order Al > Fe > Mn > Zn > Cu > Pb > Cd (*Table 2*). All detected mean values were less than their corresponding background values except for Cd in sites 1, 2 and 3. The highest values of Cd and Zn were recorded significantly in site 1 followed by site 3. Meanwhile the highest values of Cu and Pb were recorded significantly in both sites 1 and 3. The lowest values of Cd, Zn, Cu and Pb were recorded in site 4 (the reference site). Omar et al. (2016) reported that Cd, Zn, Cu and Pb are the main metal pollutants in sediments arising from anthropogenic inputs. Meanwhile manganese, iron and aluminium are common natural components of the Earth's continental crust (WHO, 2017), thus have no significance as sediment pollutants unless their current concentrations exceed their corresponding background concentrations. However, their nanoparticles occurring in anthropogenic effluents, such as iron based nanoparticles, have high efficiencies to adsorb other metals due to their large surface areas and the linked particles mostly settle on surface sediments (Alhadhrami et al., 2019). Yu et al. (2008) mentioned that marine sediments act as the ultimate destination of metal pollutants into the aquatic ecosystems.

Table 2. *Metal concentrations (mg/kg dry wt.; mean ±SE) in sediment samples and sediment quality guidelines (SQGs)*

		Fe	Al	Cu	Zn	Mn	Cd	Pb
Sampling sites	Site 1	424.80 ±8.85^{AB}	365.0 ±20.18^{C}	4.32 ±0.14^{A}	7.87 ±0.32^{A}	34.14 ±1.99^{C}	0.83 ±0.11^{A}	1.10 ±0.19^{A}
	Site 2	413.60 ±95.40^{B}	560.90 ±13.78^{A}	3.48 ±0.12^{B}	4.90 ±1.33^{C}	63.23 ±1.65^{B}	0.43 ±0.02^{C}	0.61 ±0.13^{B}
	Site 3	481.70 ±38.01^{A}	448.80 ±24.98^{B}	4.20 ±0.54^{A}	6.29 ±0.36^{B}	32.56 ±1.44^{C}	0.66 ±0.03^{B}	1.14 ±0.48^{A}
	Site 4	477.0 ±54.86^{A}	545.20 ±25.36^{A}	3.43 ±0.13^{B}	4.03 ±0.14^{D}	94.18 ±2.28^{A}	0.27 ±0.06^{D}	0.16 ±0.01^{C}
TEL*				19	124		0.7	30
PEL*				108	271		24	112
ERL**				34	150		1.2	46.7
ERM**				270	410		9.6	218
Percentage of TEL and PEL guidelines								
< TEL				100	100		75	100
≥ TEL < PEL				0	0		25	0
≥ PEL				0	0		0	0
Percentage of ERL and ERM guidelines								
< ERL				100	100		100	100
≥ ERL < ERM				0	0		0	0
≥ ERM				0	0		0	0

Statistical significant differences (P<0.05) are shown with different capital letters along the studied sites for each metal. TEL: Threshold effect level; PEL: Probable effect level; ERL: Effect range-low; ERM: Effect range-median, * MacDonald et al. (2000), ** Long et al. (1995)

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The detected elevated levels of Cu, Zn, Cd, and Pb in site 1 and site 3, are directly associated with different anthropogenic activities including illegal fishing prevailing at these areas and untreated sewage discharge. Metwally and Fouad (2008) illustrated that the concentrations of Cu, Pb, Zn, Cd and Hg along Khomse coast, Libya, significantly increased in comparison to their concentrations 17 years ago, except for Cd. They also indicated that untreated domestic wastewater as well as agricultural and industrial wastes are discharged directly through many drainages and outfalls along this coastal area.

The detected high concentrations of Al in all studied sites is in agreement with Abdel-Ghani (2015) who detected elevated levels of Al among other studied metals in samples collected from Marsa Matrouh City at the northwestern Mediterranean coast of Egypt. Sadauskas-Henrique et al. (2011) stated that sewage effluents contain high levels of Fe, Cd, Cu, Zn, Mn and Pb.

Consequently, assessment of metal pollutants in marine sediments of sites nearby populated areas is useful to investigate anthropogenic impacts on ecosystems and to indicate risks posed by waste discharges (Yi et al., 2011; Saleh, 2021). In addition, these persistent pollutants may remain for a long time in the ecosystem even after cutting-off direct inputs (Ali and Abdel-Satar, 2005).

Risk assessment of sediment pollution

SQGs indicate the possibility that a sediment associated toxicant may cause hazardous biological effects as well as impairment of aquatic organisms and the entire aquatic ecosystem (MacDonald et al., 2000). TEL and PEL guidelines showed lower concentrations of Cu, Zn and Pb than their TEL values indicating no association with hazardous biological effects. Meanwhile, 75% of Cd samples were lower than its TEL value while 25% of samples lied between its TEL and PEL values and may occasionally be associated with hazardous biological effects. 100% of Cu, Zn, Cd and Pb samples were lower than their ERL values illustrating a minimal effects range (*Tables 1 and 2*). Several risk indices including SQGs, CF, EF, Er_i and I_{geo} are useful in assessing adverse effects of individual metals but do not consider the cumulative effect of several metals in mixture as usually encountered in most environmental conditions. The cumulative effect of a metal mixture is assessed by mC_d, PLI, M-ERM-Q and RI (Saleh, 2021).

Values of CF, EF, mC_d, PLI and M-ERM-Q of metals are illustrated in *Table 3*.

Table 3. *Contamination factor (CF), enrichment factor (EF), modified contamination degree (mC_d), pollution load index (PLI) and mean effect range-median quotient (M-ERM-Q) of metals in sediment samples*

	Site 1		**Site 2**		**Site 3**		**Site 4**	
	CF	**EF**	**CF**	**EF**	**CF**	**EF**	**CF**	**EF**
Fe	0.009	1.98	0.009	1.28	0.010	1.86	0.010	1.53
Al	0.005	-	0.007	-	0.006	-	0.007	-
Cu	0.096	19.70	0.077	10.30	0.093	15.60	0.076	10.50
Zn	0.083	21.50	0.052	8.70	0.066	14.0	0.042	7.40
Mn	0.040	9.40	0.074	11.30	0.038	7.30	0.111	17.30
Cd	2.760	566.40	1.440	192.80	2.210	369.10	0.890	122.40
Pb	0.053	9.60	0.030	3.60	0.057	8.40	0.008	1.0
mC_d	0.43		0.24		0.35		0.16	
PLI	0.06		0.05		0.06		0.04	
M-ERM-Q	0.031		0.026		0.027		0.027	

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The calculated CF values (*Table 3*) and their related guidelines (Hakanson, 1980) showed that all studied sites had low metal contamination levels (CF < 1) except for cadmium in sites 1, 2 and 3 that showed moderate contamination levels (1 ≤ CF < 3).

Based on EF values (*Table 3*) and their quality criteria (Birch and Olmos, 2008), Fe showed minor enrichment along all sites (1 ≤ EF < 3). Cu showed severe enrichment (10 ≤ EF < 25) along all sites. Zn showed severe enrichment (10 ≤ EF < 25) in site 1 and site 3 while it showed moderate severe enrichment (5 ≤ EF < 10) in site 2 and site 4. Mn showed severe enrichment (10 ≤ EF < 25) in site 2 and site 4 while it showed moderate severe enrichment (5 ≤ EF < 10) in site 1 and site 3. Cd showed extreme severe enrichment (EF ≥ 25) along all sites. Pb showed moderate severe enrichment in site 1 and site 3, moderate enrichment in site 2 and minor enrichment in site 4. EF calculations for Al were not conducted due to its usage as a normalizer (reference element) in EF calculations of other metals.

According to Ergin et al. (1991), EF value < 1.5 indicate a natural source of metal. Meanwhile, EF value ≥ 1.5 indicate anthropogenic sources. All metals' EF values of the present study correspond to anthropogenic activities except for Fe in site 2 and Pb in site 4.

All detected mC_d values (*Table 3*) are less than 1.5 indicating nil to very low contamination degree (Abrahim and Parker, 2008). Also PLI values (*Table 3*) were lower than 1 indicating that sediment samples collected from all sites were in safe limits (Tomlinson et al., 1980). The mC_d and PLI were applied to determine the overall metal accumulation at the studied sites. Meanwhile, the M-ERM-Q was used to evaluate the potential biological effect of metals in mixture. All detected M-ERM-Q values (*Table 3*) were lower than 0.1 showing 9% probability of toxicity (Long et al., 2000).

The Er_i and RI are illustrated in *Table 4*. Values of Er_i of Cd in site 2 and site 3 showed moderate risk (40 ≤ Er_i < 80) while in site 1 it showed considerable risk (80 ≤ Er_i < 160). All other Er_i of metals along the studied sites showed low risk (Er_i < 40) (Hakanson, 1980). Meanwhile, RI values showed low risk in site 2 and site 4 (RI < 50) while it showed moderate risk in site 1 and site 3 (50 ≤ RI < 100) (Hakanson, 1980).

Table 4. *The potential ecological risk factor (Eri) and the potential ecological risk index (RI) of metals in sediment samples*

	Er_i					RI
	Cu	**Zn**	**Mn**	**Cd**	**Pb**	
Site 1	0.48	0.08	0.04	82.8	0.27	83.67
Site 2	0.39	0.05	0.07	43.2	0.15	43.86
Site 3	0.47	0.07	0.04	66.3	0.29	67.17
Site 4	0.38	0.04	0.11	26.7	0.04	27.27

The I_{geo} values of Cd confirm the present findings where site 1 and site 3 were unpolluted to moderately polluted with Cd (0 ≤ I_{geo} < 1) while site 2 and site 4 were unpolluted with Cd (I_{geo} < 0). Sediment samples of all other sites were unpolluted with Fe, Al, Cu, Zn, Mn and Pb (I_{geo} < 0) (*Fig. 2 and Table 1*). The I_{geo} indicates metal enrichment in sediments in relation to background concentrations (Zhang et al., 2009). However, it is not analogous to other pollution indices because its calculation involves a logarithmic function and a correction factor (Zahra et al., 2014).

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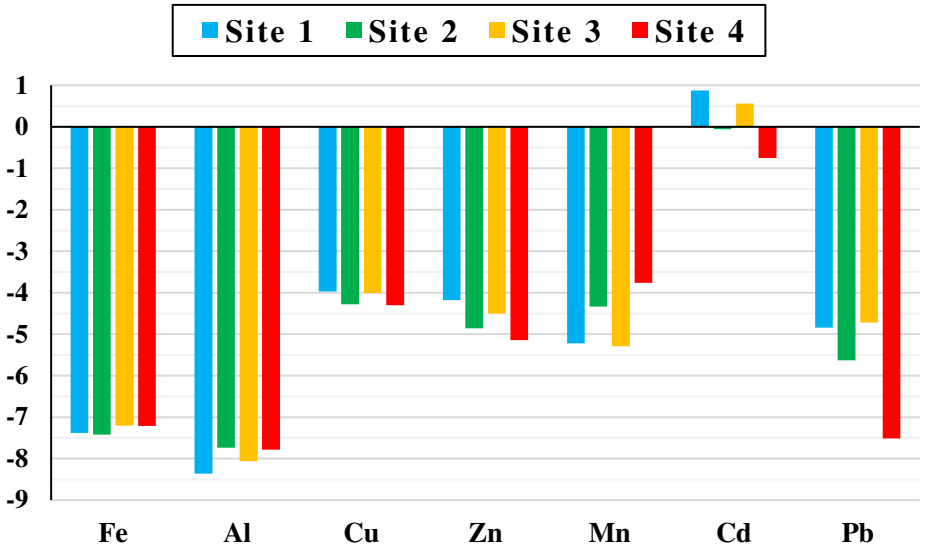

Figure 2. *Geoaccumulation index (I_{geo}) of metals in sediment samples*

The formerly discussed risk assessment indices have been widely used to evaluate the contamination degree and sediment enrichment due to different anthropogenic inputs (Wang et al., 2014; Omar et al., 2016; Saleh, 2021). The background values used to interpret geochemical data have a specific importance. The continental crustal values are usually used as reference baselines. Alternatively, the obtained data are compared to previously reported local or regional data (Birch, 2017). Due to lack or variations of reference data of metal concentrations along the study area, the background values reported by Turekian and Wedepohl (1961) were utilized.

The present findings confirm cadmium pollution condition in site 1 and site 3. These results coincide with Hamouda and Wilson (1989) who confirmed elevated levels of metals (Cu, Ni, Mn, Zn and Cd) in surface sediments of Benghazi Bay, Libya, especially Cd and Cu indicating hazardous anthropogenic activities. Moreover, Hasan and Ul Islam (2010) reported significant increase in metal concentrations (Fe, Mn, Zn, Cu, Cd, Co, Pb, Ni and Cr) in sites polluted with domestic, agricultural and industrial effluents along Al-Gabal Al-Akhdar coast, Libya. According to Soliman et al. (2015), Cd showed low concentrations among individual metals (Fe, Pb, Mn, Ni, Zn, Cr, Cu, Co and Cd) in 14 sites along the Libyan coastline but it was the only metal to show very high contamination levels in regard to CF guideline values. Soliman et al. (2015) also found evidences that Mn, Cu, Cd, Zn, Co and Ni have similar sources and/or the same geochemical processes that control their levels in sediments.

The elevated metal levels in samples of site 1 followed by site 3, in comparison to site 2 and site 4, may be attributed to the high load of untreated pollutants that are released directly at these lagoons and the general increase of anthropogenic activities as well. Several studies confirmed the anthropogenic origin of Cd, Pb, Cu and Zn such as household wastes, exhaust emissions, municipal effluents, shipping activities, antifouling paints, phosphate fertilizers and pesticides, and many others. In fact, Cd and Pb specifically originate mainly from anthropogenic activities and their natural sources of emission are rare (Ungureanu et al., 2016; Guan et al., 2018).

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The present work confirms the potential ecological risks posed by Cd among other studied metals due to various anthropogenic activities along major Libyan cities such as Benghazi and Tobruk. Similar results were reported in the Mediterranean coast of Sfax City, Tunisia (Houda et al., 2011), in Manzala Lake, Egypt (Zahran et al., 2015), in the Red Sea coast of Hodeida, Yemen Republic (Omar et al., 2016) and in 9 sites along Yemen's Red Sea coast (Saleh, 2021).

Conclusion

The continued uncontrolled discharge of domestic, industrial and agricultural effluents to the aquatic ecosystems in large cities such as Benghazi and Tobruk proved to be of critical concern and may disrupt natural attenuation mechanisms. The utilization of CF, EF, mC_d, PLI and I_{geo} and other sediment pollution indices proved to be efficient in surveying and monitoring the pollution conditions of marine ecosystems with variable and complex pollution gradients. Comprehensive metal risk assessment measures act as early warning of any expected potential risks at densely populated areas, thus continuous and periodic contaminant assessment surveys are recommended.

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Declaration of competing interests. The authors declare no conflict of interests.

Authors contributions. Wael A. Omar performed the sample processing, measurements also conducted the writing and editing of the manuscript. Mariam A. Busaadia conducted the practical work, sample collection, sample preparation and participated in writing and editing the manuscript. Yousef S. Saleh and Abdulraheem S.A. Almalki conducted the data processing, statistical analysis as well as preparation of tables and figures. Mohamed-Assem S. Marie reviewed and edited the final version of the manuscript. The present work was performed as a continuation of the survey conducted along the northeastern coast of Libya as partial fulfillment of the requirements for the Ph.D. Degree of Science awarded by Cairo University to Mariam A. Busaadia under supervision of Wael A. Omar and Mohamed-Assem S. Marie.

Ethical approval. All procedures performed in the present study did not involve human or animal participants.

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AGRONOMIC PERFORMANCE AND GENETIC PARAMETERS OF SOYBEAN (*GLYCINE MAX* (L.) MERR.) LINES IN TIDAL SWAMP LAND

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Abstract. This study aimed to find out the agronomic performance and genetic parameters of soybean lines in tidal swampland. A total of 100 soybean lines were planted in the tidal swampland using a randomized complete block design. The results showed that the broad genetic diversity was reflected in plant height (HGT), number of branches (BRC), number of productive nodes (NOD). Number of filled pods (POF), number of unfilled pods (POU), 100-seed weight (SEW), and seed yield (YLD). Those seven characteristics also had high heritability, except for YLD. Phenotypic correlations were found in all characters except for SEW with POF, POU, and YLD, while genotypic correlations were only found between NOD with BRC and POF, and YLD with POF. The moderate heritability of YLD results in measurement bias when used as the single selection criterion. Therefore, it is necessary to have other characters with significant genetic correlation with YLD as supporting characters in the selection of soybean lines for high YLD. POF as a character that had a significant correlation with YLD can be used to select soybean lines for high YLD.
Keywords: *genetic variability, phenotypic variability, genotypic correlation, phenotypic correlation, heritability*

Introduction

Plants need water for growth and development, but excess water can reduce productivity and affect their survival. Flash floods or high tide cause plants to completely submerge. Plants that grow in tidal areas experience multiple stresses, especially when they sink due to the high tides. These stresses include low oxygen levels, low light intensity, nutritional deficiencies, and a high risk of disease infection. Then as the floodwaters recede, submerged plants are suddenly exposed to higher oxygen concentrations and greater light intensity, which can lead to post-drowning injuries caused by oxidative stress, high light, and dehydration (Tamang and Fukao, 2015).

Improvements in the characteristics of high productivity soybean and tidal swampland adaptive are still to be done. The study on genetic variability, phenotypic variability, and interactions between genotype and environment will assist the selection process to obtain adaptive soybean varieties in tidal swamplands. Agronomic characters are influenced by variances of genetics, the environment, and interactions between the

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genotype and the environment (Kuswantoro et al., 2020). The wide genetic and phenotypic variabilities help plant breeders to identify parents for the hybridization program and to obtain the desired offspring with a higher degree of heterosis (Kumar et al., 2018).

The selection of genetically diverse parents is a prerequisite for a plant trait improvement program because it increases the chances of a better selection of offspring for various characters. Selection criteria are very important for determining the selection of adaptive lines for tidal fields. Physiological, morphological, and agronomic characters can be used to identify resistance mechanisms that are owned by the selected genotype. According to Kuswantoro (2010), the selection criteria for adaptive tidal land genotypes that can be used practically in the field are chlorosis, necrosis, and leaf defoliation.

Study on genetic diversity, phenotypic diversity, and heritability has been carried out in soybean plants (Aditya et al., 2011; Akram et al., 2016; Baraskar et al., 2014; Dilnesaw et al., 2013; Ghodrati, 2013; Reni et al., 2013; Malek et al., 2014). Liu et al. (2017) revealed that genetic diversity in soybean germplasm can be determined by several methods including pedigree information, phenotypic observation, and low-density polymorphism markers. Meanwhile, biochemical methods, plant morphology, and plant agronomic characters can be used to determine the phenotypic diversity between soybean cultivars from China and North America. The heritability value can be used as a predictive value for the phenotype, whether the trait displayed is caused by genetic factors or environmental factors. The high heritability value for agronomic characters such as 50% flowering age, plant height, number of branches per plant, number of pods per plant, pod length, the 100-seed weight, harvest index, and seed weight per plant can indicate that genetic factors have a greater influence than the environmental factors. These characters can be used for initial selection (Reni and Rao, 2013). According to research by Baraskar et al. (2014), genetic factors are more influential than environmental factors on the character of protein content and oil content in addition to other agronomic characters. Malek et al. (2014) identified and separated the diversity of 27 mutant soybean genotypes and 4 parent genotypes into 5 clusters based on the diversity of morphological characters and it was found that the environmental effect was lower than the genetic effect on all observed morphological characters.

Materials and methods

Plant materials and experimental design

A total of 100 soybean lines were planted in Barito Kuala Regency, South Kalimantan, Indonesia, with coordinates 1°11’50”, 140°4’9”, 17 m above sea level. Planting was carried out on May 8, 2019. The experimental design used was a randomized block design, repeated twice. Each line was planted on a plot measuring 1.6 m × 3 m, spacing 40 cm × 15 cm, two plants per hill. Soil analysis was carried out on soil samples taken before planting. The soil properties are presented in *Table 1*.

Cultural practice

Fertilization was done using 125 kg of urea, 250 kg SP36, and 150 kg KCl. Fertilization was carried out in stages, namely at the time of planting as much as 40% Urea + 100% SP36 + 100% KCl/ha, while the rest (60% Urea) was given when the

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plants approaching flowering. Soil tillage was carried out optimally to obtain an ideal soil structure for the growth of soybean plants. Making drainage channels was done before planting and herbicides were applied.

Table 1. *Soil properties*

Soil properties	Value
pH	4.8^{A}
N (%)	0.14^{L}
P_2O_5 (ppm)	58.5^{H}
C-Org (%)	1.8^{L}
SO_4 (ppm)	58.1^{L}
Cu (ppm)	0.86^{VH}
Mn (ppm)	6.39^{VH}
Fe (ppm)	341.41^{VH}
Zn (ppm)	2.36^{H}
K ($Cmol^+/kg$)	0.19^{VL}
Ca ($Cmol^+/kg$)	0.09^{VL}
Mg ($Cmol^+/kg$)	1.05^{VL}
CEC ($Cmol^+/kg$)	8.46^{L}
Al_{ex} ($Cmol^+/kg$)	0.42
H_{ex} ($Cmol^+/kg$)	1.99

A = acid, L = low, VL = very low, H = high, VH = very high

Observation

Before the plants were harvested, a sample of 10 plants was taken. Observations were done on plant height (HGT), number of branches (BRC), number of filled pods (POF), number of empty pods (POU), number of productive nodes (NOD), 100-seed weight (SEW), seed yield (YLD).

Data analysis

The expected mean square was used for calculating phenotypic and genotypic variability coefficient and broad-sense heritability (Singh and Chaudary, 1985). The significance of genotypic variability was determined using the genetic standard deviation. Phenotypic and genotypic correlations were calculated according to Singh and Chaudary (1985). A t-test was performed to determine the phenotypic and genotypic correlation significance (Dabi et al., 2016). Analysis of variance was performed using PKBT STAT 3.1. Phenontypic and genotypic correlation were calculated using MS Excel.

Results and discussion

Tidal land is acid soil with the main problems of micronutrients poisoning and the lack of macronutrients. In this study, macronutrients such as N, P, and K were classified as low, high, and very low, respectively. The other macronutrients, Ca and Mg, were

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also very low. All micronutrients were at high levels, where Zn was classified as high, while Cu, Mn and Fe were very high (*Table 1*).

All the agronomic characters observed were significantly different between the tested lines (*Table 2*). This shows that all tested lines had differences in the observed characters. Character differences between the lines tested can also be seen in *Figures 1-7* describing the large ranges.

Table 2. *Mean square of agronomic characters*

	Replication	Genotype	Error
HGT	4.91	60.63 **	1.61
BRC	2.69 **	0.65 **	0.05
NOD	3.26 *	15.82 **	0.59
POF	74.57 **	81.97 **	1.84
POU	23.33 **	0.94 **	0.14
SEW	68.86 **	7.39 **	0.09
YLD	11.51 **	0.64 **	0.27

HGT = plant height (cm), BRC = number of branches per plant, NOD = number of productive nodes per plant, POF = number of filled pods per plant, POU = number of unfilled pods per plant, SEW = 100 seeds weight (g), YLD = seed yield per plot (t/ha), * = significant at 0.05, ** = significant at 0.01

The 100 lines tested had an average plant height (HGT) of 33.21 cm. The largest distribution of lines had HGT around 30-35 cm. The lines that had HGT above 55 cm were less than 5 lines. According to Felici et al. (2019), the soybean genotype is strongly influenced by the environment. Knowing the interactions between the genotype and the environment will help identify an adaptive and stable genotype. Peter et al. (2019) reported that plants with high plant posture, a large number of filled pods on the main branch, and each pod containing 3 soybeans had high yields. Soybean plants that had an HGT below 48 cm may be caused by off-season planting or they are grown in suboptimal conditions that affect the growth process.

The number of branches per plant (BRC) ranged from 1.2 to 4.2 branches per plant with an average BRC of 2.8 branches per plant. Based on *Figure 2,* the largest distribution of BRC was 2.6-3.0 branches per plant. Less than 50% of the genotypes had BRC above 3 branches per plant. The lines with BRC 4.2 branches per plant were less than 5 genotypes. According to Kareem et al. (2015), soybean yields had a positive correlation with leaf number, seed weight, and had a strong correlation with BRC.

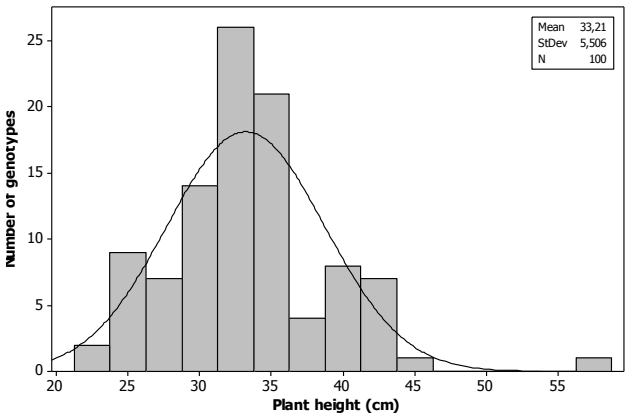

Figure 1. *Plant height of soybean lines*

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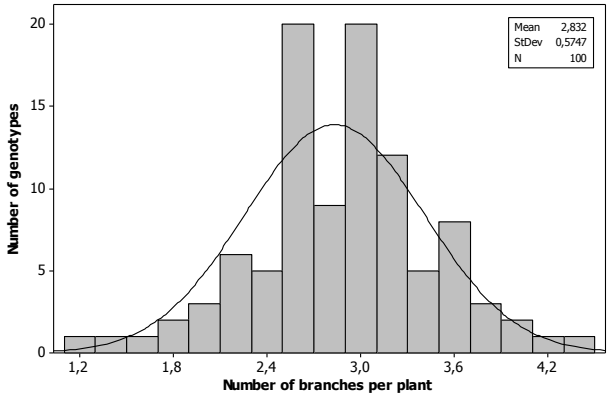

Figure 2. *Number of branches per plant of soybean lines*

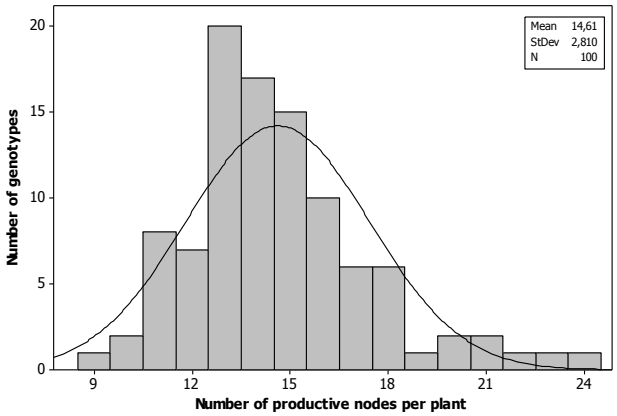

Figure 3. *Number of productive nodes per plant of soybean lines*

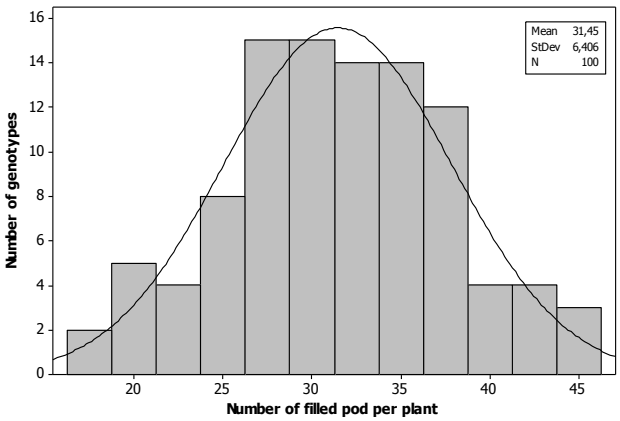

Figure 4. *Number of filled pods per plant of soybean lines*

The number of productive nodes per plant (NOD) of the 100 lines tested ranged from 9-24 nodes with an average of 14.6 nodes. Of the 100 soybean lines tested, the most number of lines had 13 NOD. Soybean lines that had more than 20 NOD were less than 10 lines. According to Peter et al. (2019) that HGT can affect NOD formation.

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The average number of filled pods (POF) of the 100 soybean lines tested was 31.45 POF. The highest distribution of lines was in 27.5-30 filled pods, followed by 32.5-35 filled pods. Soybean lines that had filled pods above 25 pods per plant were more than lines that had POF below 25 pods per plant. Soybean lines that had POF above 40 were less than 15 lines. There were about two soybean lines that had POF below 20 pods. Malek et al. (2014) also reported that the number of pods and number of seeds was the main characters that affected yield. High yields of soybeans are obtained with pods containing 2-3 seeds per pod. Hakim et al. (2014) reported that NOD can be used to identify the soybean genotype with high seed yield.

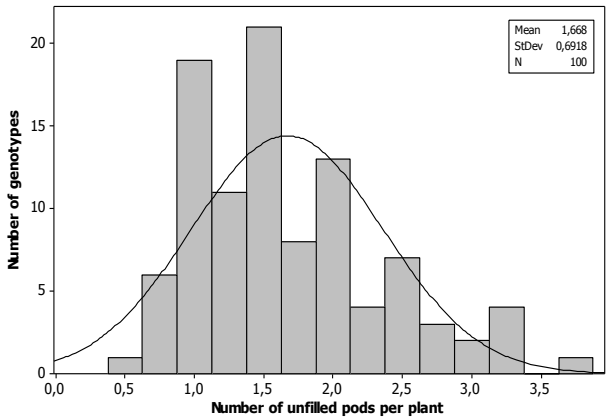

Figure 5. *Number of unfilled pods per plant of soybean lines*

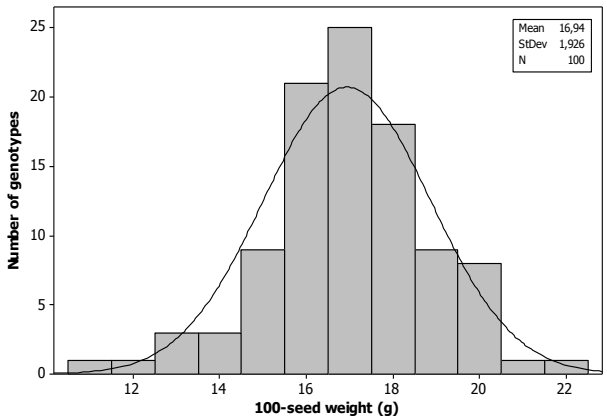

Figure 6. *100-seed weight of soybean lines*

The average number of unfilled pods per plant (POU) was 1.6 pods. The highest POU was around four pods. There were less than five soybean lines that had more than 3.5 POU. Of the 100 tested soybean lines, the most number of lines had 1.5 POU reached 20 soybean lines.

The average 100-seed weight (SEW) was 16.94 g/100 seeds. Based on the SEW results, all tested lines had medium to large seed sizes. *Figure 6* describes that more soybean lines had SEW higher than 14 g/100 seeds. Less than 15 lines had SEW larger than 20 g/100 seeds. Most soybean lines had SEW of 17 g/100 seeds, which was about

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25 soybean lines. Jin et al. (2010) stated that an increase in soybean yields was correlated with an increase in the number of pods, while the size of seeds and the number of seeds per pod were not very influential at any time. Hakim et al. (2014) and Aditya et al. (2011) stated that seed size characters had high heritability values on plant height (HGT), maturity, and the number of pods per plant.

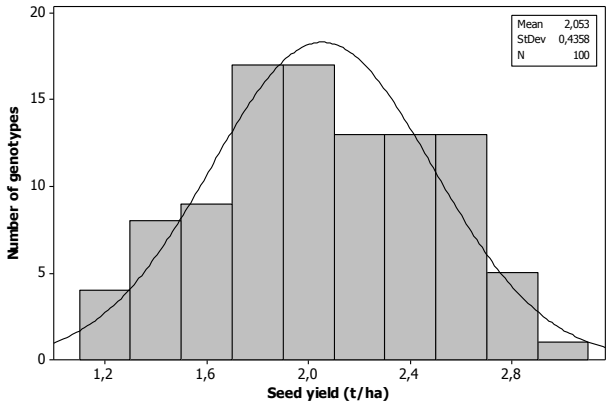

Figure 7. *Seed yield of soybean lines*

The average seed yield (YLD) was 2 t/ha with the range of 1.00-3.57 t/ha. Seed yield positively correlated with plant population, HGT, number of leaves per plant, stover weight, number of pods per plant, number of seeds per pod, number of seeds per plant, and negatively correlated with SEW and harvest index. The characters that can be used to select superior soybean genotypes include the number of seeds per pod, number of pods per plant, and weight of stover (Ali et al., 2013). In line with Jin et al. (2019) YLD correlates with the number of pods per plant. Akram et al. (2016) stated that YLD had a positive correlation with BRC, number of pods per plant, number of seeds per plant and SEW. Mahbub et al. (2015) stated that the characters of the number of seeds per pod, SEW, pod length, maturity, and HGT showed a direct positive effect on YLD.

The genetic variance of all observed characters was lower than the phenotypic variance because the phenotypic variance was the result of both genetic variability and environmental variability. In general, the observed characters had relatively small differences in PCV-GCV, except for POU and YLD. However, all character's genetic diversity observed were broad (*Table 3*). It is confirmed that the difference of PCV-GCV does not affect the genetic diversity determined by GSD. In other words, genetic diversity is not determined by PCV, but by GCV and GSD.

Broad genetic diversity is very useful in the development of superior varieties because it allows for high genetic advances. With the broad genetic diversity of all observed characters, selection can be made based on each of these characters. Broad genetic diversity was also reported by Kuswantoro (2017 a, b) in BRC, NOD, SEW, and YLD, but narrow genetic diversity in HGT.

Heritability describes the proportion of genetic variance to phenotypic variance. Similar to PCV and GCV, the genetic variance is lower than phenotypic variance because phenotypic variance is the sum of genetic variance and environmental variance. All of the seven characters observed were genetic variance in the high category except YLD (*Table 4*). This means that the appearance of the six characters is more influenced

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by genetic factors than environmental factors. Therefore, selection based on these six characters can avoid errors due to environmental influences. Chandrawat et al. (2017) reported that the seven characters observed in this study also had high heritability except for NOD which was not observed. Getnet (2018) also reported that YLD also had high heritability. This difference occurs because the plant materials are different. Differences can also occur due to differences in an environment so that the tested lines do not show their genetic potential optimally.

Table 3. *Phenotypic and genotypic coefficient variation of agronomic characters*

	PCV	GCV	GSD	Category
HGT	16.81	16.37	4.27	Broad
BRC	21.04	19.48	0.05	Broad
NOD	19.63	18.91	1.11	Broad
POF	20.60	20.14	5.77	Broad
POU	44.69	38.47	0.07	Broad
SEW	11.42	11.28	0.52	Broad
YLD	26.04	15.04	0.03	Broad

HGT = plant height (cm), BRC = number of branches per plant, NOD = number of productive nodes per plant, POF = number of filled pods per plant, POU = number of unfilled pods per plant, SEW = 100 seeds weight (g), YLD = seed yield per plot (t/ha), PCV = phenotypic coefficient variation, GCV = genotypic coefficient variation, GSD = genetic standard deviation

Table 4. *Heritability of agronomic characters*

	Vp	Vg	Ve	H bs	Category
HGT	31.12	29.51	1.61	0.95	High
BRC	0.35	0.30	0.05	0.86	High
NOD	8.21	7.62	0.59	0.93	High
POF	41.91	40.07	1.84	0.96	High
POU	0.54	0.40	0.14	0.74	High
SEW	3.74	3.65	0.09	0.98	High
YLD	0.29	0.10	0.19	0.33	Medium

HGT = plant height (cm), BRC = number of branches per plant, NOD = number of productive nodes per plant, POF = number of filled pods per plant, POU = number of unfilled pods per plant, SEW = 100 seeds weight (g), YLD = seed yield per plot (t/ha), Vp = phenotypic variance, Vg = genotypic variance, Ve = environment variance, Hbs = broad sense heritability

YLD is a character that is controlled by many genes so that it is influenced by environmental factors. Thus, selection based on YLD will be more difficult because the genotype obtained does not necessarily have the genetic structure desired. Therefore, the other characters are necessary to support the selection of a line with high YLD so that it is not biased by environmental factors. The phenotypic correlations on the agronomic characters were all significant, except between SEW with POF, POU, and YLD (*Table 5*). The significant YLD relationship with BRC was also reported by Kumar et al. (2018) but the relationship between YLD with POD and HGT was not significant. Although almost all agronomic characters had significant phenotypic correlations, while genotypic correlations were only found in the correlations between NOD with BRC and POF, and POF with YLD. Genotypic correlation is very important because environmental factors that cause bias in the phenotypic correlation have been

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eliminated. Kumar et al. (2018) also did not find any genotypic correlation between agronomic characters.

The selection of YLDs with moderate heritability needs to be supported by other characters that had significant genotypic correlation. Although there was a significant phenotypic correlation between YLD with HGT, BRC, NOD, POF, and POU, this correlation still involves environmental factors that can provide a bias in the assessment of lines. In this study, POF had a significant genotypic correlation to YLD. Thus, selection for high-yielding lines can be done based on POF. A high POF will result in a high YLD as well.

Table 5. *Phenotypic correlation and genotypic correlation of agronomic characters*

	BRC	**NOD**	**POF**	**POU**	**SEW**	**YLD**
HGT	0.395**	0.449**	0.435**	0.265**	-0.328**	0.269**
	0.430	0.480	0.455	0.314	-0.341	0.483
BRC		0.795**	0.722**	0.296**	-0.205*	0.361**
		0.814*	0.756	0.363	-0.222	0.669
NOD			0.855**	0.458**	-0.209*	0.399**
			0.874**	0.506	-0.219	0.707
POF				0.470**	-0.124	0.500**
				0.541	-0.134	0.887*
POU					0.033	0.372**
					0.032	0.811
SEW						-0.080
						-0.165

Upper = phenotypic correlation, Lower = genotypic correlation, HGT = plant height (cm), BRC = number of branches per plant, NOD = number of productive nodes per plant, POF = number of filled pods per plant, POU = number of unfilled pods per plant, SEW = 100 seeds weight (g), YLD = seed yield per plot (t/ha), * = significant at 0.05, ** = significant at 0.01

Conclusion

The genetic diversity of the agronomic characters of the soybean lines tested was broad, allowing for the selection of these lines easily. YLD as a character that is influenced by other characters as a component of the yield had moderate heritability which can provide bias during selection, so it is necessary to have other characters associated with YLD. POF was a character that had a genetic correlation with YLD, so POF can be used as a selection criterion to obtain soybean lines with high YLD. Counting filled pods are more laborious than a yield measurement, so the proposed approach could be applied at an early phase of breeding.

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METAGENOMIC ANALYSIS OF THE GUT BACTERIOME OF USHERHOPPER, *POEKILOCERUS BUFONIUS* (KLUG) FROM HADDA, SAUDI ARABIA

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Abstract. Microbial communities that colonize insect guts contribute positively to the absorption of nutrients, immunity, and the overall health of the host. Recent studies have been tapered towards only economically important arthropods, particularly honeybees. On the other hand, arthropods such as grasshoppers are considered pests because they create havoc leading to economic losses. Grasshoppers are considered phytophagous pests that have a large appetite for plant fibers, and digestion depends largely on the bacterial communities in their guts. This study characterizes the gut microbiome in Usherhopper, *Poekilocerus bufonius* using metagenomics methods through next generation sequencing (NGS). A total of 59,072,222 bacterial reads were recorded which were classified at the phylum and genus levels. *Proteobacteria* were the most common at the phylum level whereas *Wolbachia* was the most dominant genus based on the total reads. The host-microbiome interactions and their perceived influence on the ecosystem are yet to be fully explained, therefore a detailed study is pivotal in order to enforce effective conservation and pest management.
Keywords: *gut microbiome, NGS, 16s rRNA, bacteria, archaea*

Abbreviations: RNA, ribonucleic acid; DNA, deoxyribonucleic acid; OUT, operational taxonomic unit

Introduction

Insects are considered as the largest class of the Arthropoda phylum, which is the largest in the animal phyla in diversity, ecological adaptations and biomass (Muratore et al., 2020). Their diversity and evolutionary success are partly attributed to their interaction with the beneficial bacteria that colonize their digestive tracts (Engel and Moran, 2013; Jing et al., 2020). They assist host digestion and protect them from parasites and pathogenic bacteria by producing antimicrobial peptides (Chen et al., 2016) and influence host behaviors like aggregation into large groups (Dillon and Charnley, 2002). Interactions between insect hosts and their microbiomes show

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comprehensive effects on the host, and by extension, the ecosystem (Lü et al., 2019). The contribution of gut bacteria to insects' function is highly important from a medicinal, agricultural and ecological perspective. In the order Orthoptera, which includes grasshoppers, katydids, and crickets although relatively abundant in the ecosystem, their microbiomes are yet to be extensively characterized unlike other more charismatic insect species such as butterflies, moths, caterpillars, and Hymenopterans like bees (Muratore et al., 2020; Ojha and Zhang, 2019).

Grasshoppers are an important herbivore in grassland ecosystems that provide crucial ecosystem services like nutrient cycling. In contrast, they are considered as pests that require effective management and control strategies (Wang et al., 2020). Studies have generalized that the gut microbiome of most insect herbivores is comprised predominantly of *Proteobacteria, Firmicutes, and Actinobacteria* (Wielkopolan and Obrępalska-Stęplowska, 2016). Whether this generalization holds true across all insect herbivores has not been fully investigated. Compared with termites and cockroaches, grasshoppers have a very sparse microbiome (Dillon and Dillon, 2004).

Research had shown that any alteration in the gut microflora constitution influences the survival rate of grasshoppers (Tan et al., 2020). The polyphagous Usherhopper *Poekilocerus bufonius* (Orthoptera: *Pyrgomorphidae*) has been typically recorded in different regions in Saudi Arabia with a particular higher presence in the western region (Alghamdi et al., 2017; Noor et al., 2020; Sayyed and Patel, 2011). This is where the *Rhazya stricta* plant is widely distributed in the rangeland of Hadda, Saudi Arabia. This plant is well known for its allelopathic effects and has been explored in traditional medicine (Baeshen et al., 2014; Noor et al., 2020). Thus, the present study aimed to determine the microbiota bacterial communities of *P. bufonius* that habituates the western region of Saudi Arabia and harvest R. *stricta* plant with all its life cycle forms, which inspired us to investigate the microbiome of this insect that lives on this toxic shrub which is avoided by many herbivores (Baeshen et al., 2015).

Metagenomics based molecular techniques based on 16S rRNA sequencing have proven efficient in the characterization of insect's gut microbiome (Ahn et al., 2012; Prabhakar et al., 2013). The characterization of grasshopper gut microbiome communities in conjunction with information on host-associated variation in bacteria composition is obligatory for a total perception of insect ecology and the improvement of pest management action plan.

The aim of the study is to point out the bacterial and archaeal communities living inside the gut of the usherhooper that consumes toxic plants and lives in harsh environmental conditions.

Materials and methods

Sample collection

Usherhoppers were collected from the leaves of *R. stricta* plants, from Hadda (Coordinates: Lat: 21.444271 – Lon: 39.5316938), Saudi Arabia, in August 2014 at 11:00 am while the temperature was 40 °C and transported to the laboratory according to standard protocol (Mancini et al., 2018; Muratore et al., 2020).

Sample treatment

Usherhoppers were dissected in aseptic conditions. The entire intestinal tract was removed. Gut parts were separated, stored at -20 °C till further use.

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Extraction of genomic DNA, metagenomics and bioinformatics analysis

The stored gut parts were crushed and powdered in liquid nitrogen. Total genomic DNA was extracted using the Wizard® Genomic DNA Purification kit (www.promega.com) and shipped to Beijing Genomics Institute, China for next generation sequencing.

Based on the PCR results, a high-quality original library was prepared by Illumina kit by removing short fragments. Illumina (USA) HiSeq/MiSeq 2000 encoded 16S rRNA gene amplicons were used to observe the gut microbiome diversity (Moussa et al., 2017). The quality of the raw data sequences was checked using *FastQC* v0.11.9. Filtration of the raw data was done to obtain clean reads, and tags were clustered at 97% sequence identity to an operational taxonomic unit (OTU), which assigned its taxonomy.

Results

Sequence analysis

Sequence analysis was performed by EDGE bioinformatics (https://edgebioinformatics.org/). A total of 6,000,838 paired-end reads were obtained by sequencing and 5,907,222 clean tags were generated after splicing and filtering the paired-end reads with a covered percentage of 98.44%, and the unpaired reads were 83,418 with a percentage of 1.41% (*Table 1*).

Table 1. *Assembly's result of the P. bufonius sample*

Sample name	Before trimming		After trimming		Unpaired reads	Paired reads	GC (%)
	Total bases	Read count	Total bases	Read count			
Poekilocerus bufonius gut bacteriome	600083800	6000838	582766134 (97.11%)	5907222 (98.44%)	83418 (1.41%)	5823804 (98.59%)	58.93%

The results of the bacterial communities in the samples at phylum-level taxonomic distribution show that they belong to eight phyla based on the total reads. *Proteobacteria* were the most shared at the phylum-level and followed by; *Actinobacteria, Firmicutes, Nitrospirae, Bacteroidetes, Tenericutes, Cyanobacteria,* and *Acidobacteria* (*Fig. 1*).

At the genus-level, the taxonomic distribution showed that they belong to eighty-nine (89) genera. *Wolbachia* were the most abounds at the genus-level and followed by, *Acinetobacter, Pseudomonas, Azospira, Enterobacter, Shewanella, Vibrio, Photobacterium, Serratia, Acidovorax, Sphingobium, Nitrospira, Sphingomonas, Rhodoferax, Stenotrophomonas,* and *Bacillus* (*Fig. 2*).

At the species-level, the taxonomic distribution showed that they belong to one hundred eighty-two (182) species. Wolbachia sp. wRi, Wolbachia endosymbiont of Drosophila simulans, Acinetobacter oleivorans, Acinetobacter pittii, Azospira oryzae, Pseudomonas putida, Pseudomonas fluorescens, Enterobacter lignolyticus, Pseudomonas sp. UW4, Pseudomonas stutzeri, Pseudomonas brassicacearum, Pseudomonas savastanoi, Pseudomonas syringae, Photobacterium profundum were the most abundant based on the total reads (*Fig. 3*). A heatmap was generated at the genus

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and species level to explain the diversity in the composition of the taxonomy based on all the total reads (*Fig. 4*) while the bacterial communities that are present in the sample are attached (see *Appendix*).

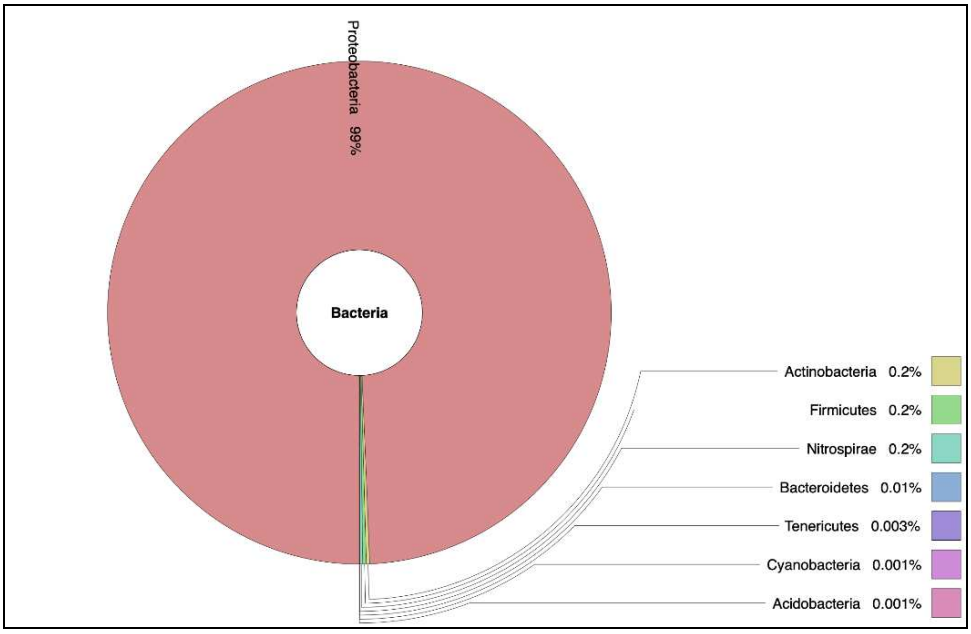

Figure 1. *The bacterial communities at the phylum-level*

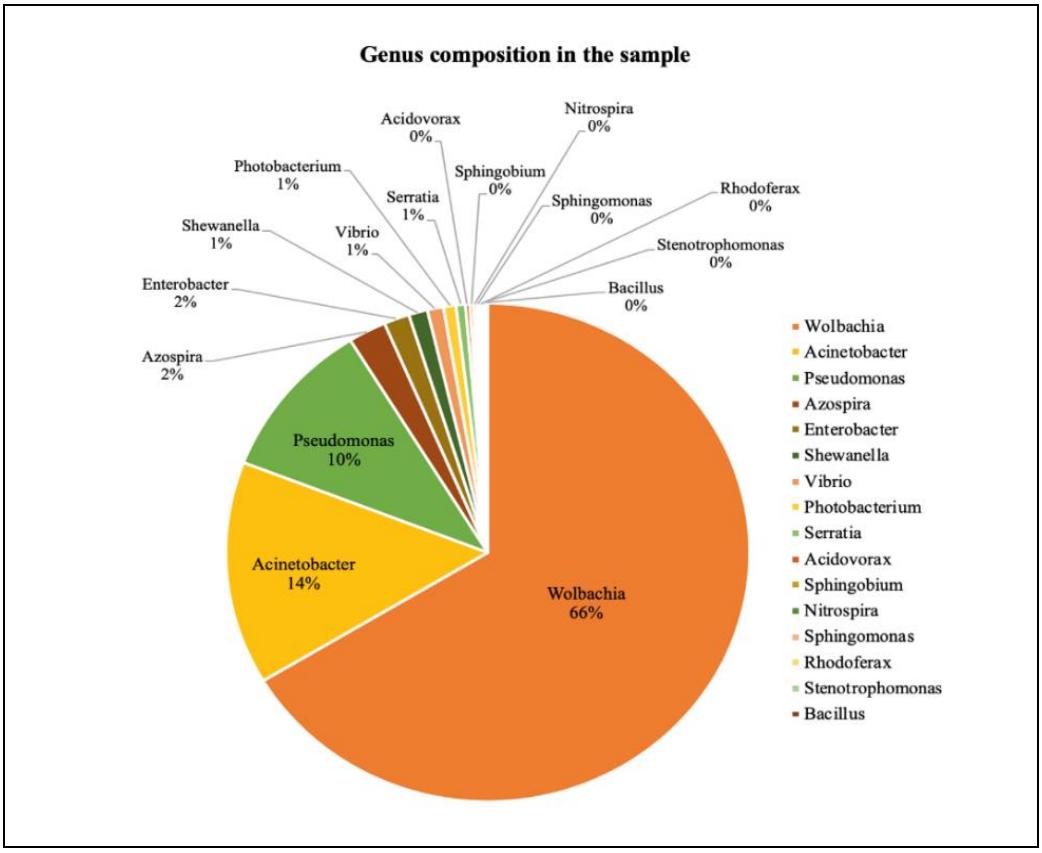

Figure 2. *The bacterial communities at the genus level*

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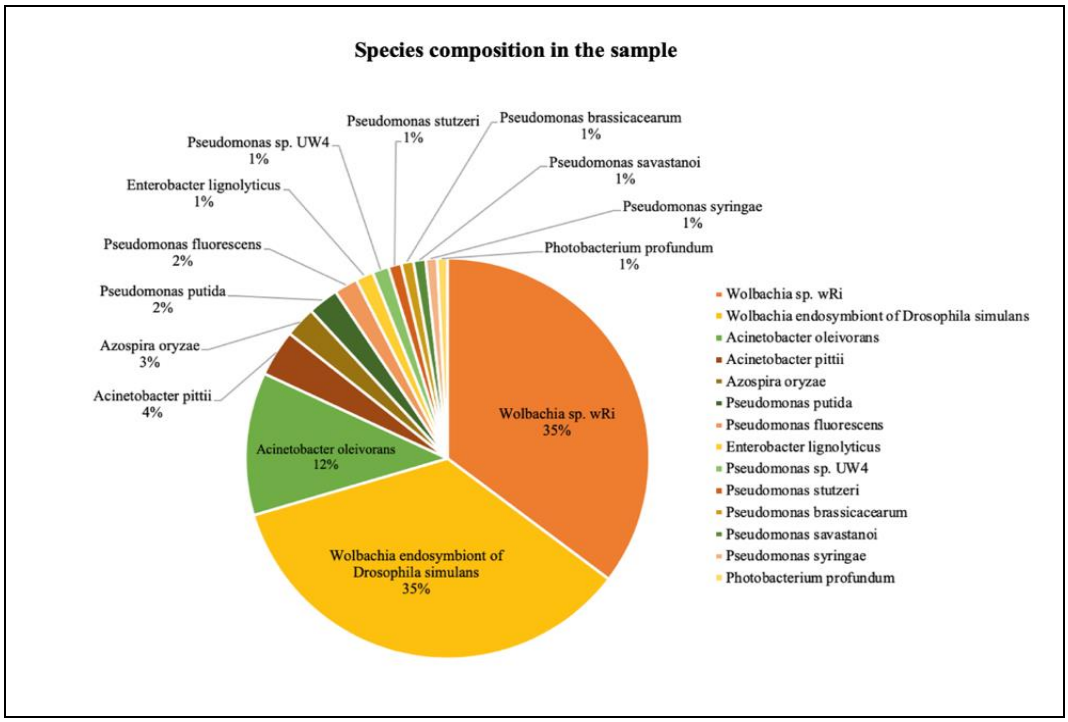

Figure 3. *The bacterial communities at the species level*

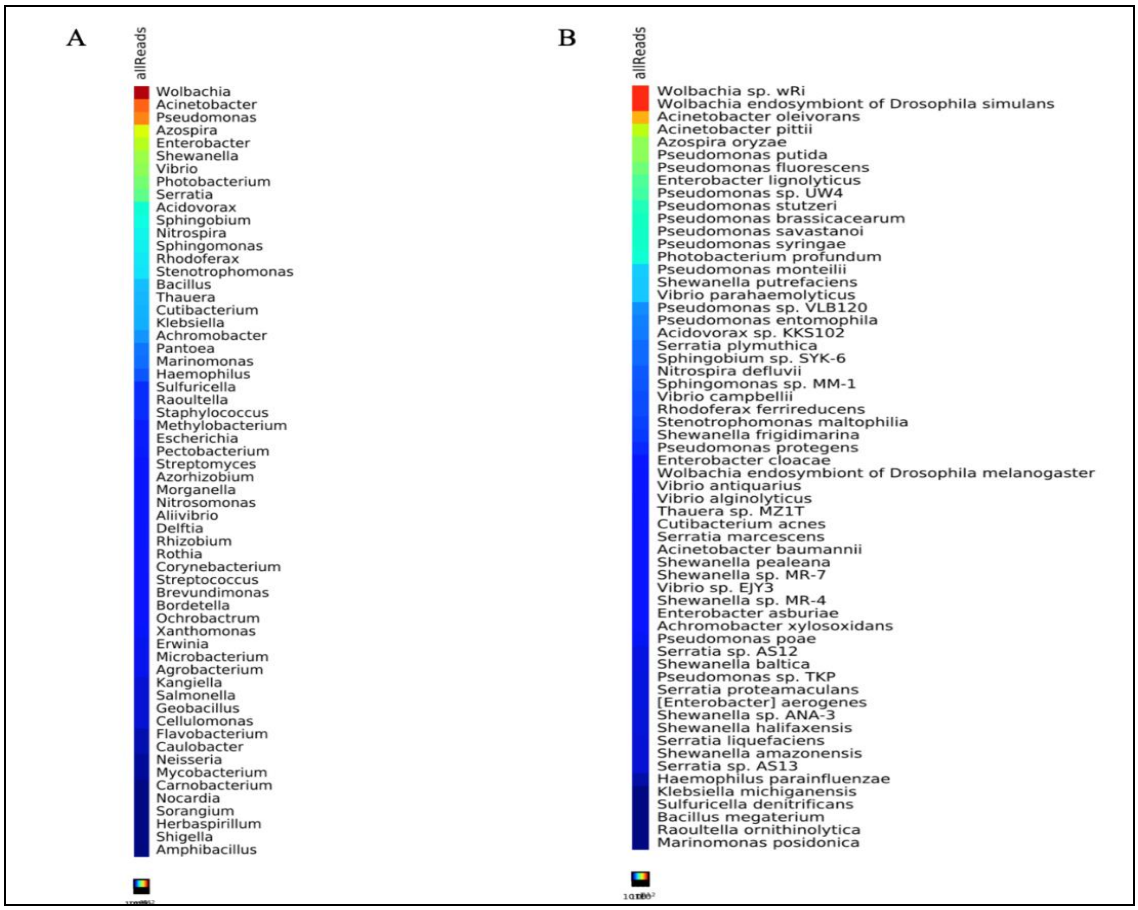

Figure 4. *The heatmap of the bacterial community at; (A) genus level, (B) species level*

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Discussion

The microbiomes present in the guts of insects serve as major contributors to nutrient absorption, enhancing immunity and ecological fitness (Morimoto et al., 2019; Muratore et al., 2020). The evolutionary powers of *P. bufonius* in terms of their high tolerance to varying environmental conditions are evidenced by the bacterial community presented in their guts (Griffith et al., 2018). Metagenomic analysis used to study the bacterial diversity in the gut of *P. bufonius* resulted in 59072222 sequences which were classified into phylum and genus levels. A total of 182 bacterial taxa at the species level were present with *Wolbachia sp.* were having high relative abundance. Meanwhile, results of sequencing showed that the taxonomic distribution of the bacterial communities at the phylum-level indicated eight phyla, indicating that *Proteobacteria* were the most shared at the phylum-level and followed by: *Actinobacteria, Firmicutes, Nitrospirae, Bacteroidetes, Tenericutes, Cyanobacteria,* and *Acidobacteria.* Several studies have shown that *Proteobacteria, Firmicutes,* and *Actinobacteria* have been found in the intestinal bacteria of locusts, orthopterans and the well-characterized microbiota of honeybees (Anjum et al., 2018; Engel et al., 2012; Stoops et al., 2016).

On the other hand, Cyanobacteria appeared remarkably in the sample because it is part of the diet of some insects such as mosquito *Larvae* (Berry, 2014), In other studies, (Meng et al., 2019) high abundance of cyanobacteria family was found present in dessert soils of Israel, while (Wynn-Williams, 2000) observed that *Cyanobacteria* constitute a large population of microalgae in hot desert rocks as a result of their ability to withstand high temperatures.

At the genus-level, the taxonomic distribution showed eighty-nine (89) genera. This diversity is attributed to a combination of factors such as nutrition, environmental conditions, and the gut environment (Brune and Dietrich, 2015; Wang et al., 2020; Yun et al., 2014). *Wolbachia* were the most abounds at the genus-level and followed by, *Acinetobacter, Pseudomonas, Azospira, Enterobacter, Shewanella, Vibrio, Photobacterium, Serratia, Acidovorax, Sphingobium, Nitrospira, Sphingomonas, Rhodoferax, Stenotrophomonas,* and *Bacillus* based on the total reads. Many studies demonstrated that *Wolbachia* has a role in controlling the reproductive characteristics of host insects (Hancock et al., 2011; Saridaki and Bourtzis, 2010). In addition, grasshopper gut bacteria, such as Acinetobacter, Pseudomonas and Klebsiella, have the ability to produce siderophore (Sonawane et al., 2018). The bacteria that produce siderophore have an effective role in promoting plant growth and are considered effective in insecticides, which are environmentally friendly and can be a valuable alternative solution to chemical insecticides (Kramer et al., 2020; Rungin et al., 2012; Sepehri and Khatabi, 2021). Microorganisms colonizing the gut of *P. bufonius* are capable of degrading cellulose, which is the functional role of *Bacillus sp* and *Pseudomonas sp* in the gut (Wang et al., 2020). The study of the gut bacteria in *P. bufonius* carried out by (Alghamdi et al., 2017) was done through traditional methods by isolation. Their work showed four bacteria: *Bacillus subtilis, Staphylococcus aureus, Klebsiella sp.* and *Streptococcus sp*. However, the results of this study revealed ninety-two species that describe the whole bacterial community using metagenomic methods. Metagenomics is considered as a powerful technique and future feature that can open the space to understand the diversities and the functions of the bacterial community in their environment (Baeshen et al., 2020).

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Conclusion

In this study, we demonstrated the conservation of one dominant phylum, *Proteobacteria* present in *P. bufonius*. Differences in the bacterial community compositions are likely influenced by their dietary preferences including *R. stricta* plant as compared to other studies on grasshoppers. The host-microbiome interactions and their subsequent impact on the ecosystem are yet to be fully established, therefore a detailed study is needed to ensure effective conservation, pest management, and other biotechnological applications in industry and environment.

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ELECTRONIC APPENDIX

This manuscript has an electronic appendix.

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MODELLING ENERGY CONSUMPTION AND CARBON DIOXIDE EMISSIONS OF FOSSIL FUELS AND NUCLEAR ENERGY USING LOTKA-VOLTERRA EQUATIONS

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Abstract. This study applied the Lotka-Volterra model to energy consumption and carbon dioxide (CO_2) emissions from 1965 to 2017 in the United States to explore the feasibility of replacing fossil fuels with nuclear energy. Parameter estimations suggested that the consumption of nuclear energy increases the consumption of fossil fuels, thereby increasing CO_2 emissions. Consistent with the arguments in Sovacool (2008) and Lenzen (2008), our results suggested that the mining, milling, conversion, enrichment, fuel fabrication, construction of nuclear plants, nuclear waste treatment, and decommissioning increase the consumption of fossil fuels and hence CO_2 emissions. Utilization of nuclear energy fails to reduce fossil fuel consumption and CO_2 emissions to achieve the environmental protection goals in the United States. By applying the Lyapunov functions to conduct equilibrium analysis, our investigation verified that the consumption of fossil fuels will ultimately be ten-fold the consumption of nuclear energy in the long term. Furthermore, the results of forecast accuracy show that the predictive ability of our proposed Lotka-Volterra model is superior to that of Bass model both in forecasting energy consumption and CO_2 emissions because the Lotka-Volterra model considers how nuclear energy affects the consumption and CO_2 emissions of fossil fuels, while the Bass model does not.
Keywords: *decommissioning, Lyapunov functions, equilibrium analysis, forecast accuracy, CO_2*

Introduction

This study applied the Lotka-Volterra model to explore how the utilization of nuclear energy affects fossil fuel consumption and CO_2 emissions in the United States (US) since the US is the country with the most nuclear power plants. CO_2 emissions generated from burning fossil fuels cause global warming and extreme weather. Monahan et al. (2016) used data from the past 112 years and found that 76% of American national parks have an early arrival of spring. Abatzogloua et al. (2016) demonstrated that rising temperatures have doubled the range of fires in the western US in the past 30 years. Rietbroek et al. (2016) showed that sea warming is one of the most important causes of global sea-level rise. Because global warming affects human life globally, scholars are making efforts to explore the factors that affect CO_2 emissions. Al-Harbi et al. (2020) investigate higher consumption of the greenhouse gas emissions for cooling purposes and traffic activities in four major urban cities in summer compared to winter and higher CO_2 emission accelerate climate change. García-Martosa et al. (2013) stated the continued use of fossil fuels cause increased CO_2 emissions. Besides, Zhang et al. (2019) indicate industrial development led to high carbon emissions in China and Cui et al. (2019) elucidated that fossil fuel combustion, cement and non-fossil singular usage during Chinese industrial plural caused more than quarter of the world total CO_2 to be emitted from China. It is critical to control CO_2 emissions to avoid extreme global warming. Gustavssonab et al. (2011) recovered, refined and transported logging residues to replace fossil fuels and obtain the benefits of CO_2

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reductions. The US has the most nuclear power plants in the world, including 98 operating commercial nuclear reactors at 60 nuclear power plants in 30 states, at the end of 2018, according to the statistics of United States Energy Information Administration. For the purpose of environmental protection, the US was motivated to use nuclear energy to replace fossil fuels. Electricity generation by nuclear power plants does not require fossil fuels, so no CO_2 is emitted during power generation. Consequently, the CO_2 emission of nuclear power is regarded as zero, so more and more countries follow US to adopt nuclear energy to generate electricity. Although the nuclear energy generates steady, reliable, and controllable electricity with much lower carbon emissions than emitted by fossil fuel generation, the true greenhouse gas emissions from the radioactive waste management and nuclear plant constructions are controversial. The United States Energy Information Administration (2012) also emphasized that although nuclear power plants do not consume fossil fuels during their operation, nuclear energy consumes fossil fuels during the entire life cycle of nuclear power plants, from exploration, enrichment, transformation, production and transport of uranium raw material, to the treatment, solidification, transport, and burial of radioactive wastes, as well as decommissioning or undertaking long-term management. The US has accumulated approximately 70,000 metric tonnes of high-level nuclear waste by 2019. The spent high-level nuclear fuel is stored at reactor sites in wet pools to cool off, so that its radiation can be shielded. The absence of a long-term repository for high-level waste and the complicated waste treatment also requires the use of electricity (Alley et al., 2012).

Previous research has estimated how much fossil fuel is needed during the life cycle of nuclear plants. Lenzen (2008) and Sovacool (2008) have estimated the amount of greenhouse gas emissions arising from the consumption of fossil fuels during the entire life cycle of nuclear energy production. Lenzen (2008) estimated that greenhouse gas emissions per kilowatt-hour (kWh) of nuclear energy are equivalent to 65 grams of CO_2. Although nuclear power yields zero CO_2 emissions during heat- and electricity-generation, the extensive system of upstream supply stages requires fossil fuels and nuclear energy production indirectly involves the emission of greenhouse gases. Sovacool (2008) indicated that throughout the lifetime of nuclear energy production, from the construction of a nuclear plant, the implementation of the plant, uranium-mining and concentrating process, through to decommissioning, relies on facilities driven by the energy provided by fossil fuels. The average value of emissions over the lifetime of a nuclear reactor was estimated to be 66 g CO_2/kWh. In addition, Torfs et al. (1998) estimated that the nuclear material, enriched uranium (3.8% U-235), in its extraction, concentration, conversion, manufacturing, and transport steps, directly consumes approximately 10,500 Joules of fossil fuel per milligram of enriched uranium. Nuclear Energy Agency (2012) indicated the emissions stem from the mining and milling of uranium, clean-up of the mine site, the construction, operation and decommissioning of the nuclear power plant and the treatment of low and high-grade ore waste, respectively. The emissions of nuclear power ranges from 3 to 40 g CO_2/kWh. These studies have shown that a large amount of reinforced concrete must be used in the construction of nuclear power plants, explaining why the whole life cycle of nuclear energy consumes large amounts of fossil fuels.

These aforementioned studies demonstrate that nuclear energy production increases the volume growth of fossil fuel consumption when we take the whole life cycle of nuclear power into account. However, the US has tended to utilize nuclear power to

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substitute fossil fuels and lessen CO_2 emissions for more than 50 years, since the Atomic Energy Act of 1954. Because the US consumes the great amount of fossil fuels and emits large volume of CO_2 globally, it is critical to provide tangible evidence of the consumption and the CO_2 emission relationship between fossil fuels and nuclear energy in the US. For this reason, the purpose of this study was to examine whether nuclear energy can take the place of fossil fuels, thereby decreasing CO_2 emissions in the US. This work concentrates on CO_2 emissions and energy consumption in the US as the research sample and empirically investigated whether nuclear energy can substitute fossil fuels. Tsai et al. (2016) have used Lotka-Volterra model to analyze the relationships between the low-carbon energy and fossil fuels in the United States and found that low-carbon energy increases the consumption of fossil fuels. The low-carbon energy includes renewable energy and nuclear energy. However, Tsai et al. (2016) emphasize that mining, milling, conversion, enrichment, waste treatment and decommission of low-carbon energy needs electricity generated by the fossil fuels, but it did not indicate which kind of low-carbon energy increases the fossil fuel consumption. Only nuclear energy deals with the mining, milling, conversion, enrichment, fuel fabrication and decommission, while renewable energy does not. In addition, Lenzen (2008) indicated that the greenhouse gas emissions caused by upstream and downstream processing stages of nuclear life cycle from nuclear plants could not be neglected. Greenhouse gas emissions are exactly higher for the wind turbines and hydroelectricity, but slightly lower for the solar photovoltaic. The conclusion also implies that we may not simply categorize renewable energy into one class while analyzing. Therefore, this research will focus on the analysis of fossil fuels and nuclear energy to simplify our analysis.

Researchers in many fields have applied an S-curve model to explore diffusion of an individual ethnic group (Tsai, 2013a; Tsai, 2013b; Sodero et al., 2017; Lee et al., 2017). However, the S-curve model does not take into account interactions among different groups. As an analogy, the traditional S-curve model also cannot be used to examine the substitution of nuclear energy for fossil fuels. To overcome the drawbacks, many previous studies applied Lotka-Volterra's mathematical model to effectively illustrate the phenomenon of competitive behavior among different groups (Lukas et al., 2017; Kamimura et al., 2006; Tsai et al., 2016; Tsai, 2017; Pac et al., 2018). Tsai and Chen (2020) used the binary Lotka-Volterra model to show that CO_2 emissions in Taiwan and China, where there is a strong dependence on industry and trade, have a significant interactive effect. In this study, for the first time, the forward-looking Lotka-Volterra model was used to quantify the interactive relationship between nuclear energy and fossil fuels in the US to explain the correlation of CO_2 emissions and energy consumption. Furthermore, previous studies ignored the interactive effects of competitive or cooperative subjects and applied a Bass diffusion model to determine the diffusion of products and technology (Fan et al., 2017). Thus, this work also utilizes the Bass model to predict energy consumption and CO_2 emissions of each energy source and compares the prediction ability between the Bass model and the Lotka-Volterra model. We compared the forecast accuracy of the Lotka-Volterra and Bass models and determined whether the Lotka-Volterra model, which considers the substitutive or complementary relations among various energy resources, has a better predictive ability.

This study has three research objectives. The first objective of this study was the determination of whether nuclear energy in the US increased the consumption of fossil fuels, instead of curtailing it. We explored whether it is infeasible for the US

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environmental protection to utilize the nuclear energy instead of fossil fuels and reduce to CO_2 emissions. Second, equilibrium analysis was also conducted to ascertain whether the consumption of fossil fuels and nuclear energy will reach a stable constant level in the long term. Third, this work was motivated to explore the forecast accuracy of our proposed Lotka-Volterra model. The results of this study find that nuclear power plants fail to reduce CO_2 emissions in the US, suggesting that the construction of nuclear power plants, nuclear waste treatment and decommissioning of nuclear power plants consume a large amount of fossil fuels and increase CO_2 emissions. Also, this study for the first time uses equilibrium analysis to find that the fossil fuel consumption in the US is ultimately ten-fold nuclear energy consumption under the incumbent technology. Fossil fuels are still the main sources of power generation and CO_2 emissions. Therefore, reducing CO_2 emissions in the US requires new technologies to improve the efficiency of fossil power generation.

Materials and methods

Lotka-Volterra model of energy consumption

The Lotka-Volterra model combines logic equations and the interaction variables representing other energy sources (Lin, 2013). The consumption relationship between fossil fuels and nuclear energy was analyzed in this work. The following two differential equations represent the interaction between two energy sources:

$$\frac{dX_1}{dt} = (a_1 - b_1X_1 - c_1X_2)X_1 = a_1X_1 - b_1X_1^2 - c_1X_1X_2 \quad \text{(Eq.1)}$$

and

$$\frac{dX_2}{dt} = (a_2 - b_2X_2 - c_2X_1)X_2 = a_2X_2 - b_2X_2^2 - c_2X_2X_1 \quad \text{(Eq.2)}$$

where $X_1 \geq 0$ and $X_2 \geq 0$. The terms of X_1 and X_2 represent the consumption of fossil fuels and nuclear energy in every year, respectively; X_1^2 and X_2^2 refer to the self-influence of the same energy source; X_1X_2 and X_2X_1 represent the different energy interactions; $\frac{dX_1}{dt}$ and $\frac{dX_2}{dt}$ denote the annual growth rate of fossil fuels and nuclear energy, respectively.

Since the Lotka-Volterra equation is a continuous model that cannot fit our specific samples at different times, these equations have to be converted to a discrete-time version (Leslie, 1958). The discrete-time format of *Equations 1* and *2* are expressed as follows:

$$X_1(t+1) = \frac{\alpha_1 X_1(t)}{1+\beta_1 X_1(t)+\gamma_1 X_2(t)} \quad \text{(Eq.3)}$$

and

$$X_2(t+1) = \frac{\alpha_2 X_2(t)}{1+\beta_2 X_2(t)+\gamma_2 X_1(t)} \quad \text{(Eq.4)}$$

where α_i and β_i represent the logistic parameters of individual energy consumption, and γ_i denotes the magnitude of the impact of one energy source on the growth rate of

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consumption of another energy source. The coefficients in the continuous form of Lotka-Volterra model have relationships with those in the converted discrete-time format (*Equations 3* and *4*). The parameters of Lotka-Volterra model a_i, b_i, c_i can be expressed in *Equations 5-7*.

$$a_i = \ln \alpha_i \quad \text{(Eq.5)}$$

$$b_i = \frac{\beta_i \alpha_i}{\alpha_i - 1} = \frac{\beta_i \ln \alpha_i}{\alpha_i - 1} \quad \text{(Eq.6)}$$

$$c_i = \gamma_i \frac{b_i}{\beta_i} = \frac{\gamma_i}{\beta_i} \frac{\beta_i \ln \alpha_i}{\alpha_i - 1} = \frac{\gamma_i \ln \alpha_i}{\alpha_i - 1} \quad \text{(Eq.7)}$$

In Equation *7*, γ_i has the same sign as c_i because $\frac{\ln \alpha_i}{\alpha_i - 1}$ is always positive when α_i is not equal to one. This means that the sign of γ_i can determine the inter-relationship of two energy sources. Similar to *Equation 7*, coefficients β_i and b_i in *Equation 6* also have the same sign, which represents the self-influence effect. A positive γ_i means that an increase in energy consumption will reduce consumption of a second energy source. Conversely, negative γ_i indicates that the consumption of one kind of energy increases the consumption of another kind of energy. How nuclear energy and fossil fuel consumption affect each other can be explored by determining the sign of γ_i in conjunction with statistical significance.

Lotka-Volterra model of CO_2 emissions

The CO_2 emissions from fossil fuels and CO_2 emission savings from nuclear energy are also analyzed using Lotka-Volterra models in this study after analyzing and investigating energy consumption. We have to define the CO_2 emissions from fossil fuels and CO_2 emission savings from nuclear energy before the model constructions. A zero-sum choice is made by policymakers when adopting either nuclear energy or fossil fuels. The condition is determined by either selecting nuclear energy by abandoning fossil fuels, or *vice versa*. Policymakers choose either nuclear energy, thus giving up fossil fuels, or fossil fuel, thus giving up nuclear energy. The overall CO_2 emissions will be reduced if policymakers use nuclear energy, instead of fossil fuels. Using nuclear energy reduces the same consumption amount of fossil fuels, leading to a net reduction of CO_2 emissions. In this work, we developed a CO_2 emission savings for nuclear energy, which expresses how nuclear energy can reduce CO_2 emissions when nuclear energy is used instead of fossil fuels. The indicator of CO_2 emission savings is defined in *Equation 8*:

$$ES^{Nuclear} = UE^{Fossil\_fuel} \times Consumption^{Nuclear} \quad \text{(Eq.8)}$$

where $ES^{Nuclear}$ represents the CO_2 emissions saving from nuclear energy, which is the reduced emissions arising from the use of nuclear energy to replace fossil fuels. UE^{Fossil_fuels} refers to the CO_2 emissions per unit fossil fuels (Tonnes of carbon/Tonnes of oil equivalent). Because nuclear energy generates electric power without any CO_2 emissions, the CO_2 emission saving from using nuclear energy to replace fossil fuels $ES^{Nuclear}$ is equal to nuclear consumption $Consumption^{Nuclear}$ multiplied by CO_2 generated per unit fossil fuels UE^{Fossil_fuel}. Since $ES^{Nuclear}$ illustrates how nuclear

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energy produces clean-up benefits, policymakers can thus regard $ES^{Nuclear}$ as an indicator of the degree of environmental protection issues at the expense of economic development.

On the other hand, policymakers can also choose to use a certain amount of fossil fuels instead of using nuclear energy. Because fossil fuels emit more CO_2 emissions than nuclear energy, choosing a certain amount of fossil fuels to replace the same amount of nuclear energy results in a net increase in CO_2 emissions. The amount of CO_2 emission due to the use of fossil fuels implies that if policymakers choose fossil fuels to obtain economic benefits, it will eventually lead to enhanced pollution; this can be calculated by *Equation 9*:

$$EC^{Fossil\_fuels} = UE^{Fossil\_fuels} \times Consumption^{Fossil\_fuel} \quad \text{(Eq.9)}$$

where EC^{Fossil_fuels} represents the CO_2 emissions due to fossil fuel use, which increases owing to the replacement of nuclear energy; and UE^{Fossil_fuels} refers to the quantity of CO_2 emissions per unit fossil fuels (Tonnes of carbon/Tonnes of oil equivalent). $Consumption^{Fossil\_fuels}$ denotes the quantity of fossil fuels consumed. As fossil fuels generate electric power with emitting a large amount of CO_2, EC^{Fossil_fuels} is equal to the total fossil fuel consumption $Consumption^{Fossil\_fuels}$ multiplied by the CO_2 generated per unit fossil fuels UE^{Fossil_fuels}. This CO_2 emissions indicator of fossil fuels EC^{Fossil_fuels} explain how fossil fuels produce more CO_2 pollution for economic advantage; therefore, policymakers can thus regard EC^{Fossil_fuels} as an indicator of the degree of economic benefit issues at the expense of environmental protections. After we define the CO_2 emissions from fossil fuels and CO_2 emission savings from nuclear energy. We utilize Lotka-Volterra models to analyze the CO_2 emissions from fossil fuels and CO_2 emission savings from nuclear energy as *Equations10* and *11*:

$$\frac{dY_1}{dt} = (p_1 - q_1Y_1 - r_1Y_2)Y_1 = p_1Y_1 - q_1Y_1^2 - r_1Y_1Y_2 \quad \text{(Eq.10)}$$

and

$$\frac{dY_2}{dt} = (p_2 - q_2Y_2 - r_2Y_1)Y_2 = p_2Y_2 - q_2Y_2^2 - r_2Y_2Y_1 \quad \text{(Eq.11)}$$

where $Y_1 \geq 0$ and $Y_2 \geq 0$. Y_1 and Y_2 denote the annual CO_2 emissions of fossil fuel and annual CO_2 emission savings of nuclear energy, respectively; Y_1^2 and Y_2^2 refer to the self-influence of the same energy source; Y_1Y_2 and Y_2Y_1 represent the interactions between both energy sources; $\frac{dY_1}{dt}$ and $\frac{dY_2}{dt}$ denote the annual growth rate of CO_2 emissions from fossil fuel and CO_2 emission savings from nuclear energy.

Equations 10 and *11* have to be converted to a discrete-time version because these equations are in a continuous format, which cannot fit our samples at different times. The discrete-time format of *Equations10* and *11* are expressed as *Equations 12* and *13*:

$$Y_1(t+1) = \frac{\delta_1 Y_1(t)}{1+\lambda_1 Y_1(t)+\theta_1 Y_2(t)} \quad \text{(Eq.12)}$$

and

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$$Y_2(t+1) = \frac{\delta_2 Y_2(t)}{1+\lambda_2 Y(t)+\theta_2 Y_1(t)} \quad \text{(Eq.13)}$$

where δ_i and λ_i represent the logistic parameters of individual energy sources relative to CO_2 emission indicators, and θ_i denotes the magnitude of the influence of one energy on the CO_2 emissions indicator growth rate of another energy source. The relationships of the coefficients of the Lotka-Volterra model and the ones in the converted discrete-time format (*Equations12* and *13*). The parameters of Lotka-Volterra model p_i, q_i, r_i can be expressed in *Equations 14-16*.

$$p_i = \ln \delta_i \quad \text{(Eq.14)}$$

$$q_i = \frac{\lambda_i \delta_i}{\delta_i - 1} = \frac{\lambda_i \ln \delta_i}{\delta_i - 1} \quad \text{(Eq.15)}$$

$$r_i = \theta_i \frac{q_i}{\lambda_i} = \frac{\theta_i}{\lambda_i} \frac{\lambda_i \ln \delta_i}{\delta_i - 1} = \frac{\theta_i \ln \delta_i}{\delta_i - 1} \quad \text{(Eq.16)}$$

In *Equation 16*, θ_i has the same sign as r_i because $\frac{\ln \delta_i}{\delta_i - 1}$ is always positive when δ_i is not equal to one. This means that the sign of θ_i or r_i reflects the competitive relationship between the two energy sources; the coefficients λ_i and q_i can determine the self-influential effect. Through the determination of the sign and the statistical significance of θ_i, this work examined how the CO_2 emission savings of nuclear energy and CO_2 emissions of fossil fuels, affect each other. In *Equations12* and *13*, Y_1 and Y_2 represent annual CO_2 emissions of fossil fuel and annual CO_2 emission savings of nuclear energy, respectively. A positive θ_1 indicates a competitive relationship and implies that as more nuclear energy is used, less CO_2 emissions are produced by fossil fuels in the US. A positive θ_1 can be an indicator of governmental concern for environmental protection efforts; the value usually represents how policymakers evaluate the cleansing effect of nuclear energy. In contrast, a negative θ_1 indicates that when more nuclear energy is used, more CO_2 emissions are produced by fossil fuels in the US. In this instance, nuclear energy does not result in increased environmental protection.

Equilibrium analysis

When equilibrium is reached, the variables or trajectories do not change over time. In the Lotka-Volterra model, when *Equations 1* and *2* are equal to zero, it represents equilibrium stability. Thus,

$$\frac{dX_1}{dt} = 0 \text{ ,and } \frac{dX_2}{dt} = 0 \quad \text{(Eq.17)}$$

Solving *Equation 17* yields:

$$X_1 = \frac{a_1 - c_1 X_2}{b_1}, and\ X_2 = \frac{a_2 - c_2 X_1}{b_2} \quad \text{(Eq.18)}$$

The intersection of the two lines, $\frac{dX_1}{dt} = 0\ and\ \frac{dX_2}{dt} = 0$, implies an equilibrium point, where the consumption of the two energy sources reaches equilibrium and does not change

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over time. If the equilibrium is unstable, the consumption of nuclear energy or fossil fuels will increase infinitely. The intersection of lines, $\frac{dY_1}{dt} = 0$ and $\frac{dY_2}{dt} = 0$, also implies an equilibrium. The stable values of Y_1 and Y_2 can be solved by *Equations 19* and *20*:

$$\frac{dY_1}{dt} = 0, \text{and } \frac{dY_2}{dt} = 0 \quad \text{(Eq.19)}$$

Solving *Equation 19* yields:

$$Y_1 = \frac{p_1 - r_1 Y_2}{q_1}, and\ Y_2 = \frac{p_2 - r_2 Y_1}{q_2} \quad \text{(Eq.20)}$$

The trajectories of CO_2 emission savings from nuclear energy and CO_2 emissions from fossil fuels moving to equilibrium point demonstrates that the annual emissions of CO_2 generated by fossil fuels will saturate and stop increasing. If the equilibrium is stable, fossil fuels may not increase to generate CO_2 emissions infinitely. This study further utilizes the estimated parameter values in the proposed Lotka-Volterra equation to test the equilibrium point's stability through two criteria, Jacobian matrix eigenvalues at the equilibrium point and the Lyapunov function. We can verify whether the final equilibrium point of energy consumption and CO_2 emission indicators between nuclear energy and fossil fuels is stable by calculating the eigenvalues of Jacobian matrix *A* at the equilibrium point with the method of Tsai and Chen (2020). Hritonenko and Yatsenko (1999) denote the condition that both eigenvalues' real parts are negative will let equilibrium point stable. This investigation examines whether the real parts of the Jacobian matrix's eigenvalues are negative at the equilibrium points to ascertain that the energy consumption and CO_2 emissions trajectories stably reach an equilibrium point. In addition, we applied the Lyapunov function to inspect the nonlinear differential equations' stability. If the value of Lyapunov function is positive and the first order differential of Lyapunov function is negative, the trajectories satisfy the conditions in which energy consumption and CO_2 emissions stably reach an equilibrium point.

Analyses of forecast accuracy

We constructed the Bass model for further comparison with the Lotka-Volterra model to demonstrate the accuracy of forecasts. Energy consumption in the Bass (1969) model are expressed as *Equations 21* and *22*, respectively:

$$\frac{dX_1}{dt} = (g_{X1} + h_{X1}X_1)(m_{X1} - X_1) \quad \text{(Eq.21)}$$

$$\frac{dX_2}{dt} = (g_{X2} + h_{X2}X_2)(m_{X2} - X_2) \quad \text{(Eq.22)}$$

where X_1 and X_2 denote the consumption of fossil fuels and nuclear energy, respectively. By following Bass (1969), we estimated the parameters g_{X1}, h_{X1}, m_{X1}, g_{X2}, h_{X2}, and m_{X2}. Parameters m_{X1} and m_{X2} are defined as the maximum consumption of fossil fuels and nuclear energy, respectively.

Also, we constructed the Bass model for further comparison with the Lotka-Volterra model to demonstrate the accuracy of forecasts. Energy CO_2 indicators in the Bass (1969) model are expressed as *Equations 23* and *24*, respectively.

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$$\frac{dY_1}{dt} = (g_{Y1} + h_{Y1}Y_1)(m_{Y1} - Y_1) \quad \text{(Eq.23)}$$

$$\frac{dY_2}{dt} = (g_{Y2} + h_{Y2}Y_2)(m_{Y2} - Y_2) \quad \text{(Eq.24)}$$

where Y_1 and Y_2 denote the CO_2 emissions of fossil fuels and CO_2 emission savings of nuclear energy, respectively. By following Bass (1969), we estimated the parameters g_{Y1}, h_{Y1}, m_{Y1}, g_{Y2}, h_{Y2}, and m_{Y2}. Parameters m_{Y1} and m_{Y2} are defined as the maximum CO_2 emission of fossil fuels and maximum CO_2 emission savings of nuclear energy, respectively.

By using the training samples from 1965 to 2010, we estimated all the parameters g_{X1}, h_{X1}, m_{X1}, g_{X2}, h_{X2}, m_{X2}, g_{Y1}, h_{Y1}, m_{Y1}, g_{Y2}, h_{Y2}, and m_{Y2} in the Bass and then derived the forecast values of the test period, from 2011 to 2017. The prediction accuracy of our proposed Lotka-Volterra model was compared with the Bass model. This work applies mean absolute percentage error (MAPE) to measure the predictive ability of the Lotka-Volterra model, calculated by:

$$MAPE = \frac{1}{n}\sum_{t=1}^{n}\frac{|Z_t - \hat{Z}_t|}{Z_t} \quad \text{(Eq.25)}$$

where Z_t and $\hat{Z}_t$ are the actual and predicted values, respectively, of energy consumption or CO_2 emission indicators. We followed the criteria proposed by Martin and Witt (1989) to evaluate the predictive ability of our Lotka-Volterra Model and the Bass model into four levels. The forecasting accuracy of a model is "excellent" when MAPE is smaller than 10%; it is "good" when MAPE is between 10% and 20%; and "reasonable" when MAPE is between 20% and 50%. This work compares the forecast accuracy of the Lotka-Volterra and Bass models and determines whether the Lotka-Volterra model, which considers the substitutive or complementary relations among various energy resources, has a better predictive ability.

Materials

This study focused on energy consumption and CO_2 emissions in the US. In 2011, nuclear energy accounted for 47% of low-carbon energy in the US, implying that nuclear energy plays an important role in carbon management. This is why we chose nuclear energy in our investigation, to examine whether nuclear energy reduces CO_2 emissions. The data on energy consumption, from 1965 to 2017, were collected from the 2018 Statistical Review of World Energy, widely recognized as a key source of data on energy markets, published on the BP Public Limited Company website. The consumption of fossil fuel in our study is equal to the sum of gas consumption, coal consumption and oil consumption on the BP Public Limited Company website. According to the methodology definition of BP Public Limited Company, the gas consumption includes derivatives of coal and those consumed in gas-to-liquids transformation. Coal consumption includes only solid fuels, whereas oil consumption includes international aviation, marine bunkers, refinery fuel and inland demand. Data on CO_2 emissions of fossil fuels from 1965 to 2017 were collected from the ICOS Carbon Portal, a research infrastructure to quantify the greenhouse gas balance. The sample period was divided into two parts: the training period from 1965 to 2010, and

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the test period, from 2011 to 2017. The model was constructed by using data from the training period. The integrity of the model can be analyzed by examining the parameters obtained by the model. In addition, accuracy can be tested by comparing predicted and observed values in the test period.

Results

Parameter estimation results

Relationship between consumption of fossil fuels and nuclear energy

To understand interactions between fossil fuels and nuclear energy consumption, we explored whether nuclear energy affects the consumption of fossil fuels. The Lotka-Volterra model estimation results of the interaction between nuclear energy and fossil fuels are shown in *Table 1*. The parameter results in *Table 1* show that γ_1 was -3.32×10^{-4}, which is significantly negative at 10% significance level. Thus, the coefficient of the nuclear impact on fossil fuel c_1 is statistically significant since c_1 is a function of γ_1, $c_1 = \gamma_1 \frac{ln\alpha_1}{\alpha_1 - 1}$. It implies nuclear energy X_2 substantially increases the growth of fossil fuel $\frac{dX_1}{dt}$ from the calculation of *Equation 1*. On the other hand, γ_2 was insignificant, according to the *t*-statistics. It implies that fossil fuel X_1 does not obviously affect the growth of nuclear energy $\frac{dX_2}{dt}$. Therefore, these results signify that nuclear energy consumption can enhance the growth of consumption of fossil fuels, but fossil fuel consumption does not have an obvious impact on the growth of consumption of nuclear energy. The relationship between nuclear energy and fossil fuels is one of commensalism. These results are possibly caused by the fact that the frontend mining, the construction of large-scale nuclear power plants, operation, backend waste treatment and decommissioning phases in nuclear life cycle need a substantial amount of electricity generation, the fabrication of which consumes fossil fuels. The US is the country with the most nuclear power plants to replace fossil fuels with nuclear energy. In contrast to the US expectations, our findings provide the evidence that nuclear power plants fail to reduce CO_2 emissions in the US.

Table 1. *The Lotka-Volterra model's parameter estimation results of energy consumption*

	Coefficient	**t-statistics**		**Calculation value**
Fossil fuels				
α1	1.2463	12.9911 ***	*ai*	0.2202
β1	1.56×10^{-4}	2.3824 **	*bi*	1.39×10^{-4}
γ1	-3.32×10^{-4}	-1.7350 *	*ci*	2.97×10^{-4}
Nuclear energy				
α2	1.006	7.7466 ***	*ai*	0.0959
β2	0.0011	3.3000 **	*bi*	9.6×10^{-4}
γ2	-4.7×10-5	-0.5191	*ci*	-4.50×10^{-5}

*p < 0.1, **p < 0.05, ***p < 0.01

In addition, the processes of enriching uranium, treating radioactive waste, and the decommissioning and management of a nuclear plant, consume a considerable amount of fossil fuels. Consistent with the viewpoints in previous studies (Lenzen, 2008;

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Sovacool, 2008), our results emphasize that nuclear energy will increase the growth of fossil fuel consumption when we take the whole life cycle of nuclear power into account, even though the electricity generation of nuclear power plants does not require fossil fuels. Nuclear energy is not the suitable resource to reduce fossil fuel consumption for the environmental protection purpose of the US. These results explain why large amounts of fossil fuels are consumed during the whole life cycle of nuclear energy. In addition, the self-influence coefficient, β_i, was significantly positive, which means that there was a negative force within fossil fuels, or nuclear energy, to suppress its own growth. The growth rate of fossil fuels will be slowed by their own power of suppression.

Relationships between CO_2 emissions of fossil fuels and nuclear energy

The Lotka-Volterra model estimation results of the relationship between the CO_2 emission savings of nuclear energy and CO_2 emissions of fossil fuels is shown in *Table* 2. The parameter results in *Table* 2 demonstrate that θ_1 was -0.0005 and was significantly negative according to the *t*-statistics. The signs of θi and ci are the same, so this negative θ_1 suggests that greater CO_2 emission savings of nuclear energy lead to more CO_2 emissions from fossil fuels in the US. However, θ_2 was insignificant, according to the *t*-statistics. CO_2 emissions of fossil fuel do not have significant impact on the CO_2 emissions from nuclear energy in the US. In summary, nuclear energy can increase the growth of CO_2 emissions of fossil fuels, but fossil fuels did not have an obvious impact on the growth of nuclear energy. Therefore, these results signify that the CO_2 emissions relationship between nuclear energy and fossil fuels is one of commensalism. The results suggest that CO_2 emissions savings from the usage of nuclear energy enhance the CO_2 emissions growth from fossil fuels.

Table 2. *The Lotka-Volterra model's parameter estimation results of CO_2 emissions*

	Coefficient	**t-statistics**		**Calculation value**
Fossil fuels				
δ_1	1.2319	13.1874 ***	p_i	0.2086
λ_1	1.9×10^{-4}	2.2698 **	q_i	1.7×10^{-4}
θ_1	-0.0005	-1.7091 *	r_i	-4.4×10^{-4}
Nuclear energy				
δ_2	1.1104	8.0010 ***	p_i	0.1047
λ_2	0.0013	2.9714 ***	q_i	0.0012
θ_2	-5.9×10^{-5}	-0.5017	r_i	-5.5×10^{-5}

*$p < 0.1$, **$p < 0.05$, ***$p < 0.01$

Combining the results of *Tables 1* and *2,* nuclear energy contributes to the consumption of fossil fuels in *Equation 1* throughout its product life cycle, leading to an increase in the CO_2 emissions of fossil fuels in *Equation 10*. Both consumption and CO_2 emissions indicators exhibited a similar relationship, whereby nuclear energy enhanced fossil fuel growth and fossil fuels exerted no effect on nuclear energy. Because it is not feasible for nuclear energy to replace fossil fuels to generate electric power, the "carbon-free" nuclear energy is not an effective energy resource to reduce CO_2 emissions in the US. Lenzen (2008) indicated that the most of the greenhouse gas

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emissions are caused in processing stages upstream and downstream in nuclear life cycle from nuclear plants. Sovacool (2008) illustrates that the nuclear generate greenhouse gas emissions from frontend, construction, operation, backend and decommissioning phases and greenhouse emission intensity in the frontend and decommissioning phases is larger than those in the other phases of the nuclear life cycle. The viewpoints of Lenzen (2008) and Sovacool (2008) concurred with the empirical results of parameter estimations in our proposed Lotka-Volterra model.

Equilibrium analysis results

Jacobian matrix at the equilibrium point of energy consumption

The X-axis and Y-axis in *Figure 1* represent nuclear energy and fossil fuel consumption, respectively. The values of the trajectory in 1965 start from area IV: ($X_1 < \frac{a_1 - c_1 X_2}{b_1}$ and $X_2 < \frac{a_2 - c_2 X_1}{b_2}$) and transit to area I: ($X_1 < \frac{a_1 - c_1 X_2}{b_1}$ and $X_2 > \frac{a_2 - c_2 X_1}{b_2}$). From 1979 to 1983 fossil fuel consumption decreased continually: $\frac{dX_1}{dt} < 0$, during the initial phase of using of nuclear energy. However, fossil fuel consumption increased substantially and moved into area I in 2007. This implies that nuclear energy reduced fossil fuel consumption only during the early stages of nuclear plant operation, but enhanced fossil fuel consumption during the middle and late stages because waste treatment, management, and decommissioning of nuclear plants require a lot of electricity, which was generated from fossil fuels.

The real parts of the eigenvalues of the Jacobian matrix $\boldsymbol{A}$ at the equilibrium point were -0.3087 and -0.1423. The negative real parts of eigenvalues indicate that the orbits computed using our Lotka–Volterra equations satisfy the Hritonenko and Yatsenko (1999) stability conditions. By applying the eigenvalues of the Jacobian matrix at the equilibrium point, our investigation verified that the annual consumption of fossil fuels finally reaches a long-term equilibrium point 1990.00 million tonnes oil equivalent, approximately ten times the equilibrium point of nuclear consumption 193.18 million tonnes oil equivalent. Fossil fuels of 1,945.88 million tonnes oil equivalent were consumed in 2010, which will increase by 2.27% by approximately 2075.

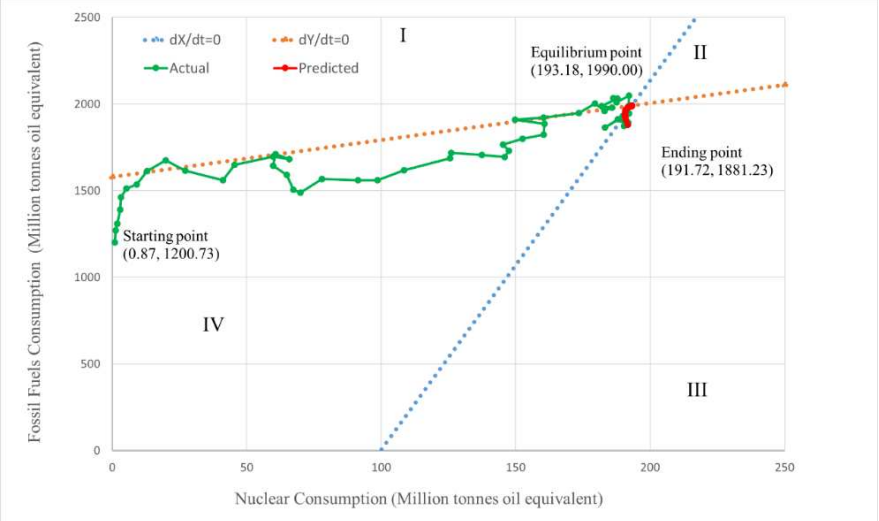

Figure 1. *Phase diagram for the consumption of annual fossil fuel and nuclear energy*

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Lyapunov analysis of energy consumption

The Lyapunov stability equilibrium analysis established that $V(x') = 1.0229 \times 10^7 > 0$, $\dot{V}(x') = -3.9973 \times 10^7 < 0$, demonstrating that the trajectory satisfied the conditions for a stable equilibrium point at approximately 2071, with fossil fuel consumption ultimately being ten-fold the consumption of nuclear energy. *Figure* 2 depicts actual and forecasted consumption of fossil fuels and nuclear energy from 1965 to 2017 and the consumption forecast of fossil fuels and nuclear energy subsequent to 2018. Fossil fuel consumption decreased with the increased use of nuclear energy from 1979 to 1983. From 1983 to 2007, the use of nuclear energy accelerated the consumption of fossil fuels because the construction, management and decommissioning of nuclear plants require a lot of electricity generated from fossil fuels. From 2007 to 2009, the decreasing consumption of nuclear energy from 192 to 190 million tonnes oil equivalent led to a reduction of fossil fuel consumption from 2,049 to 1,873 million tonnes of oil equivalent. The trajectory of fossil fuel consumption was positively related to nuclear energy consumption; there is no obvious evidence that nuclear energy can replace fossil fuels.

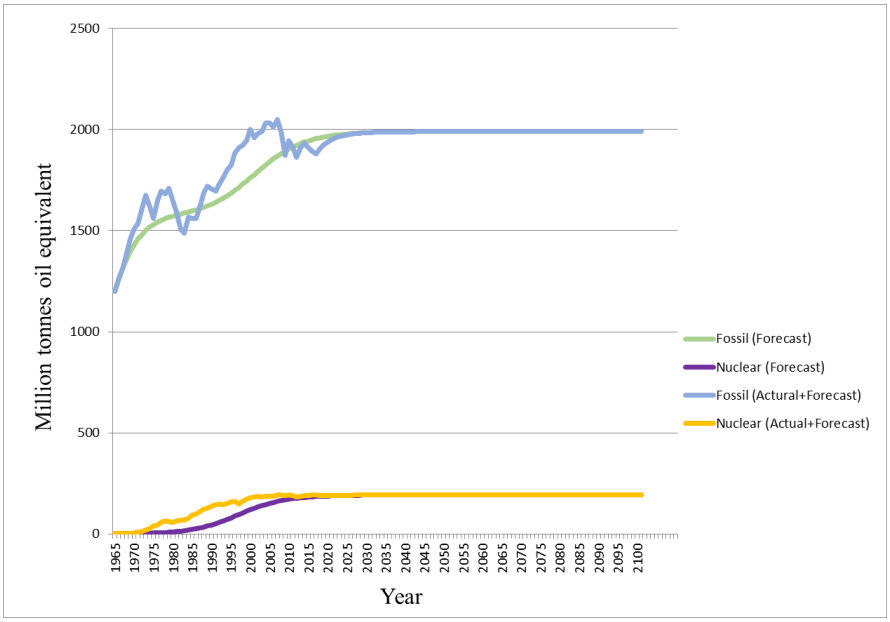

Figure 2. *Time-series consumption of fossil fuels and nuclear energy*

Jacobian matrix at the equilibrium point of CO_2 emissions

The X-axis and Y-axis in *Figure* 3 represent the CO_2 emission saving of nuclear energy and the CO_2 emissions of fossil fuels, respectively. The trajectory starts in 1965 from area IV ($Y_1 < \frac{a_1 - c_1 Y_2}{b_1}$ and $Y_2 < \frac{a_2 - c_2 Y_1}{b_2}$) and change to area I ($Y_1 < \frac{a_1 - c_1 Y_2}{b_1}$ and $Y_2 > \frac{a_2 - c_2 Y_1}{b_2}$). From 1979 to 1983, CO_2 emissions of fossil fuel decreased continually: $\frac{dY_1}{dt} < 0$, during the initial stage of using of nuclear energy. However, fossil fuel CO_2 increased substantially and changed to area I in 2007. The use of nuclear energy accelerated the CO_2 emissions of fossil fuels. Our investigation verified that CO_2 emission of fossil fuels finally reaches a long-term equilibrium point 1,623.56 million

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tonnes of carbon, approximately ten times the equilibrium point of nuclear CO_2 emission savings 156.52 million tonnes of carbon.

The real parts of eigenvalues of the Jacobian matrix $\boldsymbol{A}$ at the equilibrium point were -0.4322 and -0.2074. The negative real parts of eigenvalues indicate that the trajectories of our Lotka–Volterra equations satisfy the Hritonenko and Yatsenko (1999) stability conditions. Although the CO_2 emissions of fossil fuels and CO_2 emissions-savings of nuclear energy were 1,438.19 and 147.53 million tonnes of carbon in 2017, respectively, the CO_2 emissions of fossil fuels and CO_2 emission savings of nuclear energy would finally converge to their equilibrium point in the future, at 1,623.56 and 156.52 million tonnes of carbon, respectively.

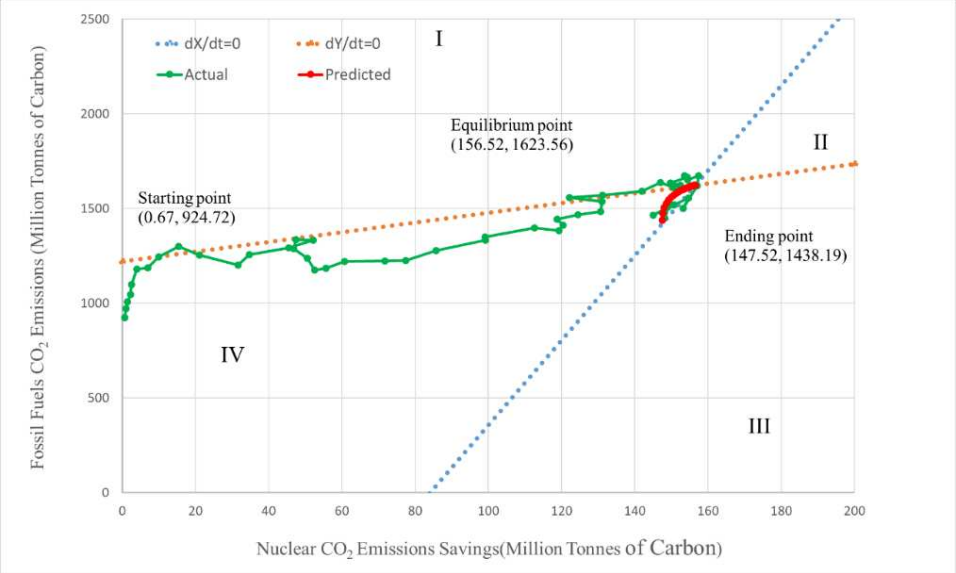

Figure 3. *Phase diagram for the fossil fuel CO_2 emission and the nuclear CO_2 emission savings*

Lyapunov analysis results of CO_2 emissions

The Lyapunov stability equilibrium analysis established that $V(x') = 4.8424 \times 10^6 > 0$, $\dot{V}(x') = -2.6585 \times 10^7 < 0$, demonstrating that the trajectory satisfied the stability conditions of its equilibrium by approximately 2075. The fossil fuel CO_2 emissions will ultimately be ten-fold the CO_2 emission savings of nuclear energy. *Figure* 4 depicts actual and forecasted CO_2 emissions of fossil fuels and CO_2 emission savings of nuclear energy from 1965 to 2017 and the consumption forecast of fossil fuels and nuclear energy subsequent to 2018. Fossil fuel CO_2 emissions decreased with the increasing use of nuclear energy from 1979 to 1983. From 1983 to 2007, the use of nuclear energy accelerated the CO_2 emission of fossil fuels because the construction, waste management, and decommissioning of nuclear plants require a lot of electricity generated from fossil fuels. Combining the results of fossil fuel CO_2 emissions with nuclear consumption, decreasing the consumption of nuclear energy from 192 to 190 million tonnes oil equivalent led to the reduction of CO_2 emissions from 1,673 to 1,500 million tonnes of carbon from 2007 to 2009. This supports the conclusion that in the long run, using nuclear energy will increase fossil fuel consumption and thus increase CO_2 emissions, even though the use of nuclear energy in the short term can reduce CO_2 emissions.

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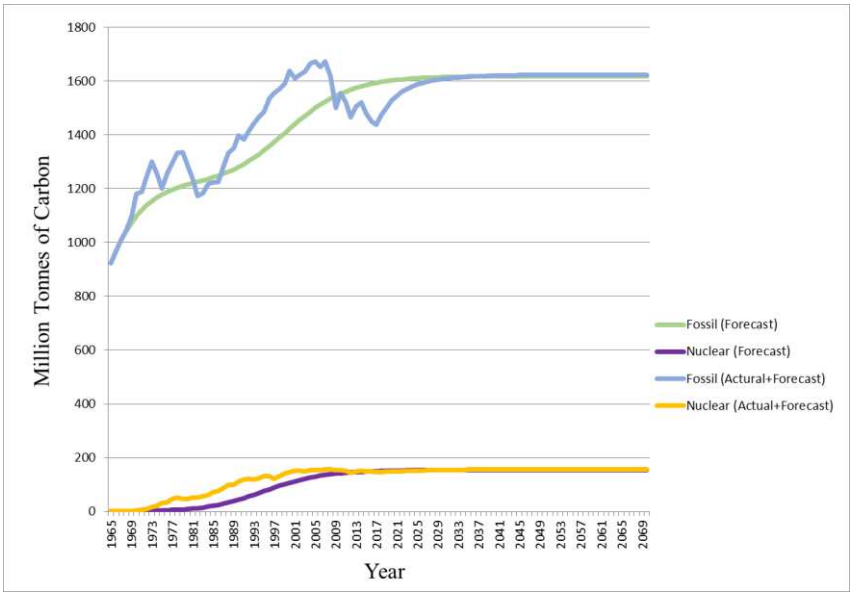

Figure 4. *Time-series CO_2 emissions of fossil fuels and CO_2 emission savings of nuclear energy*

Results of forecast accuracy analysis

We then applied the parameter values to our Lotka-Volterra model to predict the consumption of fossil fuels and nuclear energy in the test period, using the Lotka-Volterra and Bass models (*Fig. 5a* and *b*). Predicted consumption of the Lotka-Volterra model were extremely close to the actual consumption of fossil fuels and nuclear energy. In contrast, the predicted consumption of the Bass model was far from the actual consumption of fossil fuels and nuclear energy. Because our proposed Lotka-Volterra model sets the negative signs before interaction items in *Equation 2*, negative interaction parameter c_i in *Table 1* denotes that the consumption of fossil fuel increased the consumption of nuclear energy. However, the Bass model does not consider the enhance of fossil fuels on nuclear energy consumption, so the Bass model underestimated the nuclear clear consumption in test period. This can explain why the forecasted error of our proposed Lotka-Volterra model is much smaller than the Bass model in predicting nuclear energy consumption.

Figure 6a depicts the actual and predicted values using the Lotka-Volterra and Bass model for the CO_2 emissions of fossil fuels and nuclear energy, respectively, during the test period. *Figure 6b* shows that the forecast CO_2 emission savings predicted by the Lotka-Volterra model is closer to the actual CO_2 emission savings of nuclear energy than the values predicted by the Bass model. Because the Bass model does not consider the enhance of fossil fuels CO_2 emission on nuclear energy CO_2 emission savings, the Bass model underestimated the nuclear CO_2 emission savings in test period.

Table 3 summarizes the forecast accuracy of fossil fuel and nuclear energy consumption and CO_2 emissions indicators during the test period. Graphs stagnation of the Lotka-Volterra predicted value caused by the fact that the error rate of the prediction value of Lotka-Volterra model is lower than 8% as shown in *Table 3*. It implies the prediction is already very close to the actual value. However, the difference between actual value and predicted value is relatively small, leading a smooth prediction curve and no corresponding trend can be seen. Particularly, *Figures 5* and *6* also exhibits the

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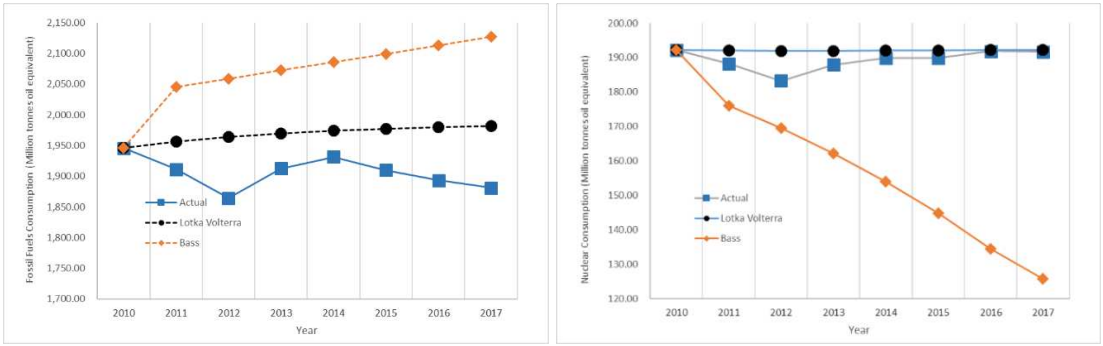
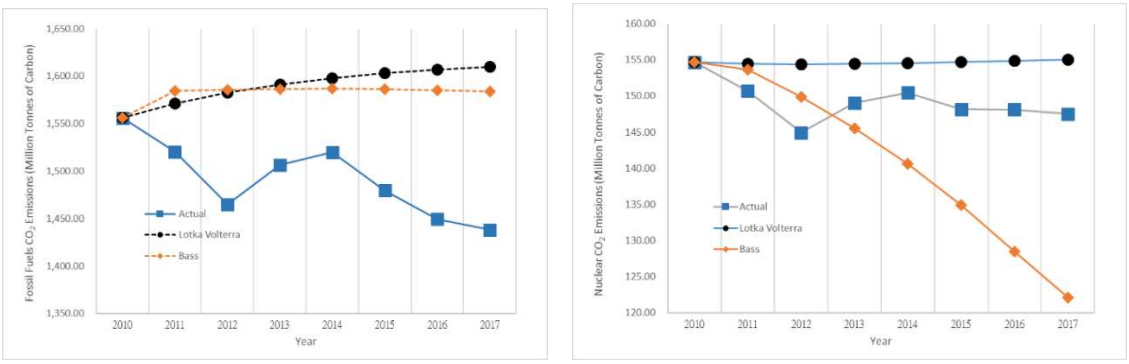
prediction of the Bass model. Due to the large error rate of Bass value, the predicted value of Lotka-Volterra model in *Figures 5* and *6* looks more rigid at a certain level.



(a) Fossil fuel (b) Nuclear energy

Figure 5. *The consumption trajectory for fossil fuel (a) and nuclear energy (b)*



(a) Fossil fuel (b) Nuclear energy

Figure 6. *The trajectory of fossil fuel CO_2 emission (a) and nuclear CO_2 emission savings (b)*

Table 3. *Forecast accuracy of fossil fuel and nuclear energy*

Consumption	**Lotka-Volterra**	**Bass**
Fossil fuel	3.77%	9.79%
Nuclear energy	1.66%	19.22%
CO_2 emission indicators		
CO_2 emissions of fossil fuel	7.62%	6.98%
CO_2 emission savings of nuclear energy	4.21%	7.66%

In the course of the test period, the MAPEs of the predicted consumption of the Lotka-Volterra model for fossil fuels were 3.77%, whereas the result was 1.66% for nuclear energy. All MAPEs of the predicted values, derived from the Lotka-Volterra model, were lower than 10%, indicative of an excellent predictive accuracy (Martin and Witt, 1989). The results demonstrate the reliability of our Lotka-Volterra model in predicting energy consumption. However, the MAPEs of the predicted consumption of the Bass model were 9.79% for fossil fuels and 19.22% for nuclear energy within the test periods. The MAPEs of the predicted consumption forecast by our Lotka-Volterra

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model were lower than those forecast by the Bass model during the test period. Because the Bass model does not consider the enhance of fossil fuels on nuclear energy consumption, the Bass model underestimated the nuclear clear consumption in test period. This explains why our proposed Lotka-Volterra model outperforms the Bass model in predicting the nuclear consumption.

Table 3 also denote that the MAPEs of the predicted CO_2 emissions from fossil fuels and the savings of CO_2 emission with nuclear energy, derived from our Lotka-Volterra model, were 7.62% and 4.21%, respectively, over the test period. All of the MAPEs of the values predicted by the Lotka-Volterra model were lower than 10%, indicative of an excellent predictive accuracy. The analytical results demonstrated the reliability of our prediction with Lotka-Volterra model in CO_2 emissions. In contrast, the MAPEs of the predicted CO_2 emissions of the Bass model were 6.98% and 7.66%, from fossil fuels and nuclear energy, respectively, throughout the test period. The MAPE difference of the predicted CO_2 emissions generated from fossil fuels between Lotka-Volterra and Bass models were no more than 1%, but the MAPE of the predicted CO_2 emissions savings of nuclear energy forecasted by the Lotka-Volterra model was lower than that of the CO_2 emissions savings forecasted by the Bass model by more than 3%.

The predictions of the CO_2 emissions and consumption of fossil fuels and nuclear energy with Lotka-Volterra model, were excellent, according to the criteria of Martin and Witt (1989). The analytical results demonstrated the trustworthiness of energy consumption prediction with the Lotka-Volterra model, and the MAPE of the Bass model was larger than 10%, indicative of a good, but not excellent, predictive ability. Except for the CO_2 emissions due to fossil fuels, the analytical results demonstrated that the forecast accuracy of the Lotka-Volterra model was superior to that of the Bass model. This work proved the dynamic interactions between fossil fuel and nuclear energy, thereby explaining the CO_2 emissions and predicting future energy consumption in the US.

Discussion

Regarding the long-term CO_2 emission calculation for the two energy production ways, nuclear power generation relies on nuclear fission without greenhouse gas emissions, while fossil fuel power generation relies on fuel combustion with greenhouse gas emissions. The greenhouse gas emissions are mainly CO_2 emissions. In order to compare the two energy under the consistent basis, we deducted the total emissions of fossil fuel power generation from fuel combustion. Our comparison of fossil fuels and nuclear energy are based on the long-term greenhouse gas emissions from the frontend mining, construction, operation, backend waste treatment to decommissioning phases.

For fossil fuels, Hondo (2005) reveals life cycle greenhouse gas emission of coal-fired, oil-fired, liquefied natural gas-fired and liquefied natural gas combined cycle power generation. There are 3.6, 32, and 52.9 gCO_2e/kWh in the construction, operation and methane leakage phases, respectively for coal-fired power generation. There are 2.3, 35.2, and 0.3 gCO_2e/kWh in the construction, operation and methane leakage phases, respectively for oil-fired power generation. There are 2.9, 117.7, and 9.1 gCO_2e/kWh in the construction, operation and methane leakage phases, respectively for liquefied natural gas-fired power generation. There are 2.7, 100.9, and 7.7 gCO_2e/kWh in the construction, operation and methane leakage phases, respectively for liquefied natural gas combined cycle power generation. Since fossil fuels are

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composed by coal-fired, oil-fired, liquefied natural gas and liquefied natural gas combined cycle, there are in average 2.875, 71.45, and 17.5 gCO_2e/kWh in the construction, operation and methane leakage phases, respectively for fossil fuels.

For nuclear energy, Sovacool (2008) reveals nuclear fuel cycle, and indicates the quantity CO_2 footprint per kWh at every stage. There are at least 0.58 gCO_2e/kWh, at most 118 gCO_2e/kWh, in average 25.09 gCO_2e/kWh in the frontend phase, including mining, milling, conversion, enrichment, fuel fabrication and transportation. Then, there are at least 0.27 gCO_2e/kWh, at most 35 gCO_2e/kWh, in average 8.2 gCO_2e/kWh in the construction phase, including all materials and energy input for building the facility. Additionally, there are at least 0.1 gCO_2e/kWh, at most 40 gCO_2e/kWh, in average 11.58 gCO_2e/kWh in the operating phase, including maintenance, cooling and fuel cycles, backup generators during outages and shutdowns. Moreover, there are at least 0.4 gCO_2e/kWh, at most 40.75 gCO_2e/kWh, in average 9.2 gCO_2e/kWh in the backend phase, including fuel processing, conditioning, reprocessing, interim and permanent storage. Lastly, there are at least 0.01 gCO_2e/kWh, at most 54.5 gCO_2e/kWh, in average 12.01 gCO_2e/kWh in the decommissioning phase, including deconstruction of facility and land reclamation. From Sovacool (2008), we could observe that the CO_2 emission intensity in the frontend and decommissioning phases is larger than those in the other phases. Nuclear energy totally causes long-term CO_2 emissions at least 1.36, gCO_2e/kWh in average 66.08 gCO_2e/kWh, and at most 288.25 gCO_2e/kWh. CO_2 emissions of the nuclear energy seem no less than fossil fuels CO_2 emissions 91.8 gCO_2e/kWh if fossil fuels and nuclear energy are compared based on the long-term greenhouse gas emissions from the frontend mining, construction, operation, backend waste treatment to decommissioning phases. Under the technology in our sample period from 1965 to 2017, our findings indicate nuclear life cycle requires electricity generated from fossil fuels so as to emit incremental CO_2.

Conclusion

This study assessed energy consumption and CO_2 emissions in the US and examined the relationship between fossil fuels and nuclear energy. We determined whether nuclear energy can replace fossil fuels, thereby reducing CO_2 emissions in the US. Our results show that nuclear energy increased the consumption of fossil fuels, rather than deterring the use of fossil fuels. Uranium mining, concentration, conversion, manufacturing, transportation, and the disposal of radioactive waste, including curing, transportation, landfill, the construction and management of treatment plants, and decommissioning and long-term management of nuclear power plants, all consume a large amount of traditional fossil fuels. Therefore, the use of nuclear energy inevitably contributes to a growth in the consumption of fossil fuels and concomitant CO_2 emissions. Nuclear energy is not the suitable resource to reduce fossil fuel usage and CO_2 emissions for the environmental protection and public health purpose of the US. Our results regarding the relationship between nuclear energy and fossil fuels are in agreement with those of previous studies. While the use of nuclear energy may not emit CO_2 during electricity generation, the use of nuclear energy increases fossil fuel consumption throughout the entire life cycle of nuclear power, from the construction of the plant until its decommissioning, and thereby further increases CO_2 emissions. This result verifies that from the construction of a nuclear plant until its decommissioning,

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nuclear power plants increase fossil fuels consumption, which explains the difficulty of the CO_2 emission reductions in the US.

In addition, we find that nuclear energy consumption decreased fossil fuels in a short run from 1979 to 1983, but nuclear energy consumption positively increased fossil fuel consumption and CO_2 emission in the long run. The results of equilibrium analysis show the consumption of fossil fuels and nuclear energy will reach a stable and constant level in the long term. The consumption of fossil fuels will ultimately be ten-fold the consumption of nuclear energy. This implies that nuclear energy reduced fossil fuel consumption only during the early stages of nuclear plant operation, but enhanced fossil fuel consumption during the middle and late stages because waste treatment, management, and decommissioning of nuclear plants require a lot of electricity, which was generated from fossil fuels. Fossil fuels are still the main sources of power generation and CO_2 emissions. Finally, this work explored the forecast accuracy of our Lotka-Volterra model and compared the forecast accuracy of the Lotka-Volterra model with the Bass model in predicting the amount of energy consumed and CO_2 emission indicators of these energy sources. Our estimations of forecast accuracy showed that the Lotka-Volterra model, which considers the substitutive or complementary relations among various energy sources, generally performed better than the Bass model in predicting the consumption and CO_2 emission indicators of fossil fuels and nuclear energy. To sum up, reducing CO_2 emissions in the US requires new technologies to improve the efficiency of fossil power generation. Nowadays, the importance of renewable energy production needs to be emphasized as well. The power demand of nuclear waste management could be supplied from renewable sources and this can modify the whole CO_2 emission. However, this paper dose not investigate the feasibility of renewable energy to replace fossil fuels to generate electricity. Future studies can further explore the consumption relationships among fossil fuels, renewable energy and nuclear energy to examine the feasibility of renewable energy to replace fossil fuels to reduce CO_2 emissions.

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THE IMPACT OF URBAN POPULATION CHANGE ON FORESTS, IN-FOREST RECREATION AREAS AND URBAN FORESTS IN TURKEY

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Abstract. This study aimed to investigate the effects of rapid population growth and urbanization on forests, in-forest recreation areas and urban forests in Turkey. Therefore, through the consideration of the urban population changes in Turkey between 1973-2019, "Forestry Statistics" related to the changes in the forest existence, in-forest recreational areas and urban forests were evaluated. Within the scope of "Forestry Statistics", both forestry data and population data were considered separate variables, and 36 variables were created in this context. According to this, while the total forest area in Turkey was 20,199,296 ha in 1973, it increased by 2,541,001 ha (12.6%) and reached 22,740,297 ha by 2019. According to the up-to-date data, there have been increases in growing stock and annual current increment, as well as forest areas in Turkey. It is a fact that there is a transformation in favor of forests in rural areas of the country. However, it is not possible to talk about a similar situation in urban areas. Although there is a positive correlation between the urban population and forest presence across the country, it is not at the same rate. The urban population increases several times faster than the extent of forest areas and growing stock.
Keywords: *urbanization, rural changes, forestry statistics, growing stock, annual current increment, Turkey*

Introduction

Along with the Industrial Revolution, the increase in population, production and consumption, development of technology, and increasing economic activities brought along rapid urbanization worldwide. Rapid urbanization, which started much earlier in developed countries, has caused intense migration from rural areas to urban areas all over the world since the 1900s; consequently; the urban population has continuously increased, while the rural population has been constantly decreasing.

The rapid increase of the population in urban areas and migration caused unplanned and distorted urbanization, especially in underdeveloped countries, which brought many multi-dimensional problems. Urbanization is, therefore, a phenomenon that has demographic, economic, political, social, technological and environmental dimensions, and also changes and affects human attitudes and behaviors. Today, while rapid urbanization has caused changes in the amount and structure of forest areas in the world and in Turkey, it has also caused a change in the habits of urban people in terms of benefitting from forest areas and spending time in these areas. In the last decade, global attention has begun to focus more on ecological and environmental issues (United Nations Environment Program (UNEP, 2020; Pata et al., 2020; Nathaniel et al., 2020; Zambrano-Monserrate et al., 2020; Guo et al., 2020; Zhou et al., 2022). Because with the change in living standards in the last decade, people have become more interested in outdoor activities (Lin and Liu, 2021).

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This situation causes people to need natural environments where they can spend their leisure time socially, culturally and physiologically for various purposes. In order to meet these needs, in-forest recreation areas and urban forests, which are relatively natural and unspoilt today, are of great importance. In this sense, recreation is an intangible ecosystem service that is affected by the preferences and needs of individuals, and the development of recreation should be based on certain criteria and indicators that will be realized with the participation of the public (Scholte et al., 2018; Nigussie et al., 2021). In addition to its personal benefits, recreation has various and multidimensional advantages such as leadership development, participation in the community, ethnic and cultural interaction, strong family formation, preventive health services, productive workforce, reduction in crime and violence, tourism, environmental health, wildlife protection, and rehabilitation (Broadhurst, 2001; Özgüç, 2017).

Human beings have depended upon forests to survive since prehistoric times (Misbahuzzaman and Smith-Hall, 2015). The demand for forest resources is closely related to economic development and population growth (Wang et al., 2015). According to Department for International Development (DFID) (2002) and Yeşildal (2020), today about 840 million people living on an income of less than 1 USD $ a day live in rural areas with high dependence on natural resources such as forests. While the world's forests covered an area encompassing 4.128 billion ha in 1990, it decreased by 129 million ha to 3.999 billion ha in 2015. The proportion of forests covering 31.8% of the land surface has decreased to 30.8% and the forest area per capita has decreased from 0.8 to 0.6 ha in the world between 1990 and 2015 (Food and Agriculture Organization of the United Nations (FAO), 2015). Although the change in forest areas decreased from 30.6% to 26.8% in underdeveloped countries, it increased from 30.9% to 31.3% in OECD (Organization for Economic Development and Cooperation) countries and from 35% to 38% in European Union countries (World Bank (WB), 2017). Therefore, developed countries increase their forest areas and growing stock by preserving their existing forests and establishing new forests with afforestation, while forest areas in underdeveloped and poor countries decrease.

When the reasons for the changes in forest areas are considered for all countries, population changes at the national level stand out (Ryan et al., 2017). In addition, many human-induced negative impacts, such as opening forest areas for agricultural land, differences in socioeconomic structure at global, national and local levels (Lambin and Meyfroidt, 2010; Shi et al., 2017), illegal use of forest areas (Kuemmerle et al., 2009), legal regulations based on short-term populist policies that do not take into account the sustainable management of forests (Min-Venditti et al., 2017), and removing forest cover for illegal settlements, play an important role in this change.

Since 99.9% of the forest areas in Turkey are under state ownership, Turkish forestry is based on public administration and property. These areas are managed the General Directorate of Forestry (GDF) and the General Directorate of Nature Conservation and National Parks (GDNCNP), which are affiliated to the Ministry of Agriculture and Forestry.

While there is a decrease in forest areas in the world in general, Turkey is among the countries that increased the extent of its forests such as China. According to the current data, there have been increases in growing stocks and current annual increment, as well as forest areas in Turkey. On the other hand, with the rapid urbanization process, the number and amount of in-forest recreation areas and urban forests have also increased, as the demand of the people living in metropolitan areas for recreation has increased. This study

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was conducted in order to investigate the effects of rapid population growth and urbanization on Turkish forestry, forest areas, growing stock, annual current increments, in-forest recreation areas and urban forests. For this purpose, in this study, in which the relevant data between 1973-2019 were evaluated, the changes in forest areas, growing stock, current annual increment, in-forest recreation areas, and urban forests were analyzed and their transformations were examined through the consideration of the total population, urban population and rural population changes in Turkey.

Material and method

An important part of the data used in the study was taken from "Forestry Statistics" which has been regularly published every year since 2007 by the General Directorate of Forestry (GDF) (GDF, 2021a). In Turkey, since 1963, data collection and inventory techniques have been used throughout the country on forest areas, and the first comprehensive information based on spatial measurement was published by GDF in 1973. However, during the 26 years from 1973 to 1999, the forest inventory was not published. Then, in 2004, 2012 and 2015, 2018 and most recently in 2019, the statistics including data about the forest areas distribution, growing stock, current annual increment, urban forests, recreation areas, silviculture and afforestation services, etc. were shared with the public. Therefore, since the first data on forestry based on measurement and research started to be shared GDF in 1973, the year 1973 was taken as the starting year in this study. Turkey's forest existence and its location on the world are given in *Figure 1*.

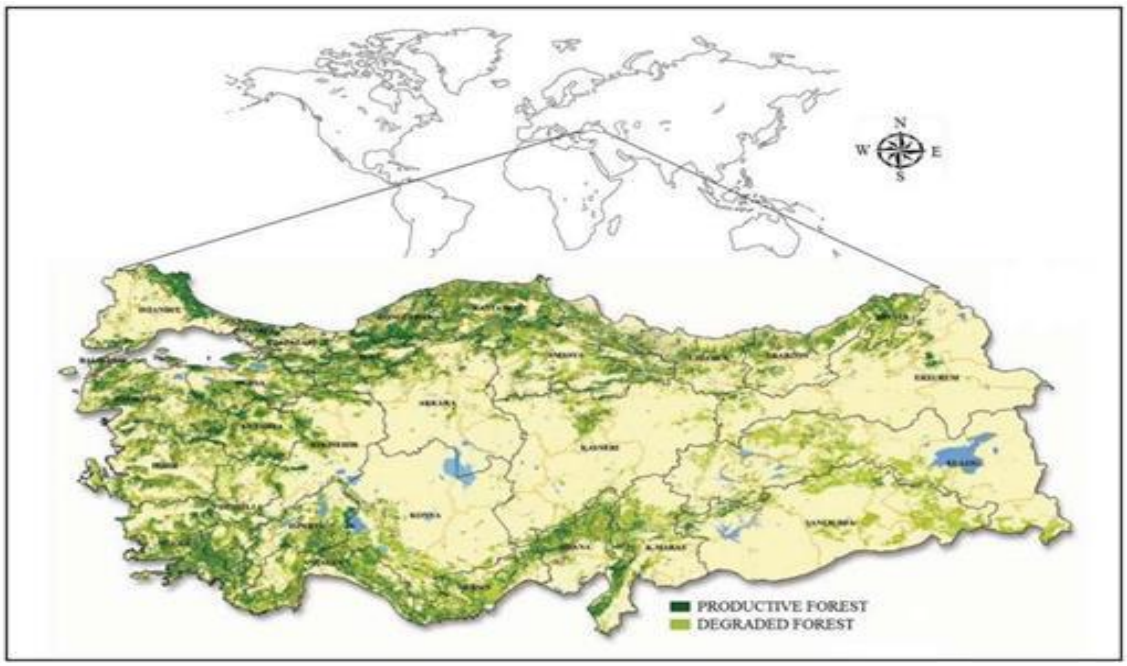

Figure 1. *Turkey's forest existence and its location on the world (GDF, 2021b)*

Therefore, in this study, through the consideration of the total population, urban population and rural population changes in Turkey between 1973-2019, "Forestry Statistics" related to the changes in the forest areas, growing stock, current annual

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increment, in-forest recreation areas and urban forests were evaluated. Within the scope of "Forestry Statistics", each data related to forestry were accepted as a separate variable, and years, the general population of the country, the population living in urban areas and the population living in rural areas were also defined as variables (GDF, 2021a; Turkish Statistical Institute (TUIK), 2020). As a result, a total of 36 variables were created within the scope of this study (*Table 1*).

Table 1. *Variables used in the research*

No	Variable name	Code	Type	No	Variable name	Code	Type
1	Elapsed time	ET	Year	19	Annual current increment	ACI	m^3/year
2	Rural population	RP	Person	20	Average increment	AI	m^3/ha
3	Urban population	UP	Person	21	Growing stock per person	GSPP	m^3/person
4	Total population	TP	Person	22	Number of in-forest recreation areas of type A	NFRA	Number
5	The share of the urban population in the total population	UP/TP	%	23	Area of in-forest recreation areas of type A	AFRA	ha
6	The share of the rural population in the total population	RP/TP	%	24	Number of in-forest recreation areas of type B	NFRB	Number
7	Productive forest area	PFA	ha	25	Area of in-forest recreation areas of type B	AFRB	ha
8	The ratio of productive forest area to total forest area	PF/TF	%	26	Number of in-forest recreation areas of type C	NFRC	Number
9	Hollowed closed forest area	HCF	ha	27	Area of in-forest recreation areas of type C	AFRC	ha
10	The ratio of hollowed closed forest area to total forest area	HCF/TF	%	28	Total number of in-forest recreational areas	TNFR	Number
11	Total forest area	TFA	ha	29	Total area of in-forest recreation areas	TAFR	ha
12	Forest area per person	FAPP	ha	30	In-forest recreation area per person	FRPP	m^2/person
13	Productive forest growing stock	PFGS	m^3	31	Number of urban forests	NUF	Number
14	The ratio of productive forest growing stock to total growing stock	PFGS/TGS	%	32	Urban forest area	UFA	ha
15	Hollowed closed forest growing stock	HCGS	m^3	33	Urban forest area per person	UFPP	m2/person
16	The ratio of hollowed closed forest growing stock to total growing stock	HCGS/TGS	%	34	Total number	TN	Number
17	Total growing stock	TGS	m^3	35	Total area	TA	ha
18	Average growing stock	AGS	m^3/ha	36	Total area per person	TAPP	m^2/person

The criteria used in the definitions of rural and urban population differ among researchers. Some researchers take the population into account and some consider the functions of the settlement, whereas some take the population density into account. Since the available statistics can only be obtained from the Turkish Statistical Institute in this study, the criteria and data of the Turkish Statistical Institute are taken as basis. On the other hand, the Turkish Statistical Institute evaluates the population living in provincial and district centers as urban population, and the population of other settlements as rural population.

The frequency distributions and rates of change of the variables were calculated, evaluated and interpreted. In addition, the significance, strength and direction of the relationships between these variables were tested with correlation analysis. Correlation analysis is a statistical method which reveals the direction, degree and importance of the relationship between variables. In order to evaluate the research data and perform statistical analysis, Microsoft Excel and Statistical Package for Social Science 23.0 programs were used.

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Results and discussion

Urban population and change of forest areas

The increase in the urban population emerges as a phenomenon experienced in the development process in Turkey as well as in the world. The rural-urban migration movement, which started with the Industrial Revolution in Western Europe, started after 1950 in Turkey (Tümertekin, 1973; Doğanay, 1997; Keleş, 2012). The share of the urban population in the total population increased continuously in the 1950-1980 period, but the rural population continued to increase in quantity during this period (Yılmaz, 2015).

According to *Table 2,* the urban population has a high-level positive significant relationship (r = 0.97) with the total population, and a high-level negative significant relationship with the rural population (r = -0.92). Similarly, the elapsed time (year) has a high level negative significant relationship with the rural population (r = -0.83), and a high-level positive significant relationship with the urban population (r = 0.98) and the total population (r = 0.99). As a matter of fact, while the share of the rural population in the total population was 15,702,851 (75.0%) in 1950, it was 25,091,950 (56.1%) in 1980 (TUIK, 2020). However, the share of the rural population in the total population started to decrease in amount for the first time after 1980 (Yılmaz, 2015). The issue to be considered here is that while the general population in Turkey continues to increase continuously, the rural population started to decrease in quantity for the first time in 1980. In this context, according to *Table 3,* when a general assessment of the population change in Turkey between 1973-2019 is made, it is seen that the total population was 38,450,702 in 1973 and 83,154,997 in 2019, with an increase of approximately 116.3%. While the share of the urban population in the total population (UP/TP) was 15,597,881 (40.6%) in 1973, this figure has increased continuously and reached 77,151,280 (92.8%) in 2019. The share of the rural population (RP/TP) in the total population has steadily decreased to 22,852,821 (59.4%) in 1973 and to 6,003,717 (7.2%) in 2019. In Turkey, where the population census is carried out every five years, the share of the rural population in the total population was 56.1% in 1980 and 46.9% in 1985 (TUIK, 2020). The urban population and the rural population were equalized once between 1980 and 1985, and in the following years, the gap between them gradually widened in favor of the urban population (*Fig. 2*).

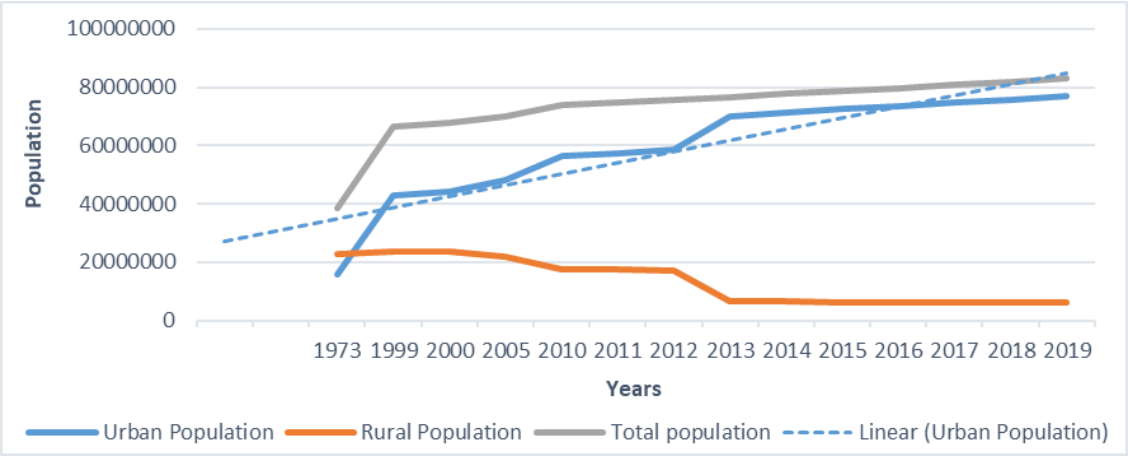

Figure 2. *Population change in Turkey by years*

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Table 2. *Correlation analysis matrix*

T. population	U. population	R. population	Elapsed time	
			1.00	Elapsed time
		1.00	-0.83	R. population
	1.00	-0.92	0.98	U. population
1.00	0.97	-0.80	0.99	T. population
0.93	0.98	-0.94	0.95	Pr. For. area
-0.94	-0.99	0.94	-0.95	H. c. forest area
0.92	0.97	-0.94	0.94	T. forest area
-0.95	-0.88	0.64	-0.94	Forest area p. per.
0.96	0.99	-0.92	0.97	Pr. for. growing stock
-0.83	-0.91	0.93	-0.86	H. c. for. growing stock
0.96	0.99	-0.92	0.97	T. growing stock
0.97	0.99	-0.90	0.98	Av. growing stock
0.94	0.99	-0.93	0.96	An. current increment
0.95	0.98	-0.91	0.96	Av. increment
-0.56	-0.38	0.03	-0.51	Growing stock p. per.
0.77	0.80	-0.74	0.77	Num. of type A
0.74	0.74	-0.64	0.74	Area of type A
0.76	0.75	-0.65	0.75	Num. of type B
0.75	0.64	-0.37	0.73	Area of type B
0.81	0.80	-0.73	0.84	Num. of type C
0.11	-0.00	0.14	0.59	Area of type C
0.88	0.91	-0.84	0.91	T. num. of rec. areas
0.25	0.16	0.02	0.27	T. area of rec. areas
0.20	0.09	0.09	0.22	Rec. area p. per.
0.87	0.87	-0.83	0.90	Num. of u. forests
0.46	0.43	-0.39	0.53	U. forest area
-0.15	-0.24	0.28	-0.09	U. for. area p. per.
0.88	0.91	-0.85	0.91	Total number
0.33	0.24	-0.06	0.35	Total area
0.26	0.16	0.02	0.28	T. area p. per.

As can be understood from the data obtained above, for various reasons, especially after the 1980s, the urban population in Turkey started to increase rapidly and the rural population started to decrease. It is a fact accepted by many researchers that rural-to-urban migration is the most important reason for the population increase in urban areas in Turkey (Gümüş, 2004; İlter and Ok, 2004; Şen and Toksoy, 2006; Türker et al., 2017); Günşen and Atmiş, 2019; Köse, 2020). With the continuous increase of the population since 1927 in Turkey, the agricultural lands passed through inheritance have been divided into smaller units. In addition, many factors, such as the increase in mechanization in agriculture; the higher employment, education and health opportunities in big cities compared to rural areas; and the transformation of big cities into attraction centers in the eyes of the public in recent years, can be counted as the main reasons for migration from rural to urban centers.

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Table 3. *Distribution of the population and forest areas by years (GDF, 2021a; TUIK, 2020)*

Year	Urban population*	UP/TP (%)	Rural population*	RP/TP (%)	Total population*	Productive forest area** (ha)	PF/TF (%)	Hollowed closed forest area** (ha)	HCF/TF (%)	Total forest area** (ha)	Forest area per person (ha/person)
1973	15,597,881	40.6	22,852,821	59.4	38,450,702	8,856,457	43.8	11,342,839	56.2	20,199,296	053
1999	42,938,282	64.4	23,732,556	35.6	66,670,838	10,027,568	48.3	10,735,680	51.7	20,763,248	0.31
2000	44,006,274	64.9	23,797,653	35.1	67,803,927	10,126,510	48.6	10,707,654	51.4	20,834,165	0.31
2005	48,107,406	68.9	21,683,899	31.1	69,791,305	10,621,221	50.1	10,567,526	49.9	21,188,747	0.30
2009	54,807,219	75.5	17,754,093	24.5	72,561,312	10,972,509	51.3	10,417,274	48.7	21,389,783	0.29
2010	56,222,356	76.3	17,500,632	23.7	73,722,988	11,202,837	52.0	10,334,254	48.0	21,537,091	0.29
2011	57,385,706	76.8	17,338,563	23.2	74,724,269	11,380,753	52.7	10,226,860	47.3	21,607,613	0.29
2012	58,448,431	77.3	17,178,953	22.7	75,627,384	11,558,668	53.3	10,119,466	46.7	21,678,134	0.29
2013	70,034,413	91.3	6,633,451	8.7	76,667,864	11,940,495	54.5	9,959,240	45.5	21,899,734	0.29
2014	71,286,182	91.8	6,409,722	8.2	77,695,904	12,322,321	55.7	9,799,013	44.3	22,121,335	0.28
2015	72,523,134	92.1	6,217,919	7.9	78,741,053	12,704,148	56.9	9,638,787	43.1	22,342,935	0.28
2016	73,671,748	92.3	6,143,123	7.7	79,814,871	12,797,148	57.0	9,638,787	43.0	22,435,935	0.28
2017	74,761,132	92.5	6,049,393	7.5	80,810,525	12,890,148	57.2	9,638,787	42.8	22,528,935	0.28
2018	75,666,497	92.3	6,337,385	7.7	82,003,882	12,983,148	57.4	9,638,787	42.6	22,621,935	0.28
2019	77,151,280	92.8	6,003,717	7.2	83,154,997	13,083,510	57.5	9,656,787	42.5	22,740,297	0.27

*Prepared by using the data of the Turkish Statistical Institute (TUIK, 2020)
**Values between 1999-2005, 2005-2009, 2009-2012, 2012-2015, 2015-2018 were calculated by interpolation from data for 1973, 1999, 2005, 2009, 2010, 2012, 2015, 2018, 2019 (GDF, 2021a)

There is a hihg-level significant positive correlation between the elapsed time (year) and the amount of total forest area (r = 0.94) and productive forest area (r = 0.95), whereas there is a hihg-level significant negative relationship between the elapsed time (year) and the amount of hollowed closed (degraded) forest area (r = -0.95) (*Table 2*). In this sense, when the situation is evaluated according to *Table 3,* in the period between 1973, when the first inventory was published, until 2019, when the last inventory was published, the total and productive forest areas of Turkey increased continuously and the hollowed closed forest area decreased. Accordingly, in 1973, Turkey's total forest area was determined to be 20,199,296 ha. Of this, 8,856,457 ha (43.8%) (PF/TF) is productive, whereas11,342,839 ha (56.2) (HCF/TF) is hollowed closed forest. The concept expressed as productive forest here is the forest areas where the canopy, which is the degree of the trees covering the soil surface, is more than 10%, where wood raw material is produced. The forest areas where the canopy is 10% or less and where wood raw material is not produced are considered as hollowed closed forest. The concept of "hollowed closed forest" has been used by GDF since 2015. Previously, the concept of "degraded forest" was used instead of this concept.

Likewise, according to *Table 3,* although the rate of hollowed closed forest area in Turkey decreased to 42.5% (9,656,787 ha) in a 46-year period between 1973 and 2019, the proportion of productive forest areas increased to 57.5% (13,083,510 ha). On the other hand, the total forest area increased by 2,541,001 ha (12.6%) in the same period and reached 22,740,297 ha in 2019. The surface area of Turkey is 78,356,200 ha (URL-1), and the ratio of forest areas to country surface area increased from 25.8% in 1973 to 29.0% in 2019. When we look at the ratio of total surface measurements of the forest areas of some countries in the world, this rate is 73.7% in Finland, 68.7% in Sweden,

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49.8% in the Russian Federation, 42.7% in Bosnia-Herzegovina, 40.6% in Georgia, 37.2% in Spain, 35.9% in Bulgaria, 32.7% in Germany, 32.5% in Italy, 31.5% in France and 22.7% in Hungary (URL-2). When the situation of Turkey is compared to countries such as Bosnia-Herzegovina, Bulgaria, Italy, France and Georgia with similar ecological conditions, it is seen that Turkey is in the last ranks.

However, in Turkey, especially the afforestation works that started from the 1960s and continued increasingly until today, the rehabilitation works of degraded areas and their reflection on the records have caused the rate of productive forest areas to increase continuously. In addition, the migration from rural areas to urbans, which started in the 1950s and accelerated after the 1980s (Yılmaz, 2015), caused a significant decrease in the population of forest villages and a change in the socio-economic and demographic structure. This situation has resulted in the elimination or significant reduction of many negative human-induced factors such as grazing pressure, illegal utilization of forests, opening forest areas for agriculture and settlement. This is another situation which causes an increase in both total and productive forest areas.

In fact, the difficulties experienced still continue due to the incompatibility between the definition of forest in the 1st article of the Forest Law No. 6831 in Turkey and the definition of forest by the United Nations Food and Agriculture Organization (FAO). By FAO, *areas larger than 0.5 hectares with trees that have more than five meters of height and more than 10 percent top closure or that can reach these threshold values in their natural area are accepted as forest* (FAO, 2010a). In addition, in order for an area to be considered as a forest, the forest use of this area must be the dominant use. The areas where the canopy is less than 10% are called other wooded areas (Global Observations of Forest Cover and Land-use Dynamics (GOFC–GOLD), 2009).

In Turkey, *"tree and shrub communities grown with labor are considered as forests together with their locations"* (URL-3). Here, as seen in the last version of the Forest Law No. 6831 in force, it is stated that not only the vegetation but also the land itself is considered forest. However, all wooded places are not classified as forest, there are some exceptions in the definition of forest such as "reed areas, all kinds of thorny areas, parks, places covered with all kinds of trees, and shrubs on the owned land whose surface area does not exceed three hectares" (GDF, 1956).

In this context, there is no limit value for the size of the area and the canopy regarding the acceptance of a place as a forest in Turkey. However, in owned lands, the area should be a minimum of 3 ha. According to the 2010 data, Turkey's forest existence is 21,537,091 ha, of which 11,202,837 ha are productive forest (GDF, 2021a). FAO, on the other hand, specified Turkey's forest area as 11,334,000 ha in the same year (FAO, 2010b). Almost half of the figure calculated by GDF is recognized as Turkey's forest area by FAO. This difference is due to the use of forest definitions accepted by Turkey and FAO. As a matter of fact, international institutions such as FAO give only productive forest areas as Turkey's forest area (Foresters' Association of Turkey (FAT), 2019).

However, forest definitions accepted in many countries are different from the forest definition made by FAO. For example, the canopy criterion is taken as 30% in some countries such as Brazil, Peru, Mexico, Malaysia, Thailand; as 25% in Malaysia, Paraguay and Morocco; as 15% in India and Ghana (Sasaki and Puntz, 2009); and as 20% in China (Shi et al., 2011). The differences in forest definitions of different countries are not only due to the canopy criteria; the minimum area (0.5 ha) and minimum tree height (5 m) criteria given by FAO also vary from country to country.

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According to the United Nations Framework Convention on Climate Change (UNFCCC), the forest is an area of minimum 0.05-1.0 ha, consisting of trees that can reach a height of at least 2-5 m, with 10-30% hill cover (UNFCC, 2002). Therefore, in many countries, including Turkey, some areas that FAO does not accept as forest are considered as forests, and some areas that FAO accepts as forest are not considered as forest. According to GOFC-GOLD (2009), the Designated National Authority (DNA) in each country is responsible for the forest definition.

There is a high-level significant negative relationship (r = -0.88) between the amount of forest area per capita across the country and the urban population, whereas there is a high-level significant negative relationship (r = -0.95) between the amount of forest area per capita across the country and the total population (*Table 2*). Accordingly, as the total population and urban population in the country increase, the total and productive forest areas per capita decrease. As seen in *Table 3,* while the total forest area in Turkey increased by 12.6% between 1973-2019, the total population increased by 116% in the same period. Due to this rapid increase in the total population, forest area per capita continued to decrease continuously. Forest area per capita was calculated as 0.53 ha/person in 1973 and 0.27 ha/person in 2019. As can be seen, in the 46-year period between 1973 and 2019, the total amount of forest area per person including degraded forest areas decreased by almost half. Productive forest area per person is less, and it was found as 0.23 ha/person and 0.16 ha/person in 1973 and 2019, respectively.

However, in order to evaluate the adequacy of the productive forest area per capita in Turkey, it is necessary to compare it with the forest area per capita in other countries. When evaluated in this sense, it is estimated that the total forest area of the world was just over 4 billion ha in 2010, which corresponds to an average of 0.6 ha of forest per person (FAO, 2010b). According to FAO (2015), in Russia, one of the richest countries in the world in terms of forest, the amount of forest area per capita is 5.75 ha, whereas it is 9.74 ha in Canada and 2.23 ha in Brazil. On the other hand, forest areas per capita in some countries in the Mediterranean climate zone, including Turkey, with similar ecological conditions to Turkey, are as follows: 1.33 ha/person in Montenegro, 0.63 ha/person in Georgia, 0.55 ha/person in Bosnia-Herzegovina, 0.53 ha/person in Bulgaria, 0.45 ha/person in Croatia, 0.39 ha/person in Spain, 0.38 ha/person in Serbia, 0.35 ha/person in Greece, 0.30 ha/person in Portugal, 0.28 ha/person in Albania, 0.26 ha/person in France and 0.16 ha/person in Italy (Forest Europe, 2018; Foresters' Association of Turkey (FAT), 2019). Looking at these figures, Turkey ranks last, along with Italy. If Turkey makes the degraded forest areas productive, it will be able to improve its rank among other countries.

Growing stock and increment changes in forests

In the 46-year period between 1973-2019, *Table 4* shows the changes of the growing stock, which express the total wood amount of forests in Turkey, and the increment values, which express the increases in growing stock.

There is a hihg-level significant positive relationship (r = 0.97) between the elapsed time (year) and total growing stock, and there is similarly a hihg-level significant positive relationship (r = 0.97) between the elapsed time (year) and productive forest growing stock; however, there is a hihg-level significant negative relationship (r = -0.86) between the elapsed time (year) and the hollowed closed forest growing stock (degraded) (*Table 2*). In this direction, when the data in *Table 4* are evaluated, between the years of 1973-2019, the growing stock in all forests in Turkey increased, regardless

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of the distinction between productive and degraded. While all forests had a growing stock of 935,512,150 m^3 in 1973, this value increased by 79.5% (743,844,060 m^3) in 2019 and reached the level of 1,679,356,210 m^3. According to this, it is understood that the growing stock of Turkey's forests has increased by an average of 16,170,523 m^3 each year over the course of 46 years.

Table 4. *Distribution of the population, growing stock and annual increment values by years (General Directorate of Forestry, 2020; Turkish Statistical Institute (TUIK), 2020)*

Year	Productive Forest Growing Stock* (m^3)	PFGS/TGS (%)	Hollowed Closed Forest Growing Stock* (m^3)	HCGS/TGS (%)	Total Growing Stock* (m^3)	Average Growing Stock (m^3/ha)	Current Annual Increment* (m^3/year)	Average Increment (m^3/ha)	Growing Stock Per Person (m^3/person)
1973	847,033,015	90.5	88,479,135	9.5	935,512,150	46.3	28,063,205	1.4	24.3
1999	1,113,612,229	92.7	87,179,408	7.3	1,200,791,637	57.8	34,269,650	1.7	18.0
2000	1,127,849,222	92.8	87,497,938	7.2	1,215,347,160	58.3	34,605,090	1.7	17.9
2005	1,199,034,187	93.1	89,090,585	6.9	1,288,124,772	60.8	36,282,291	1.7	18.5
2009	1,290,450,115	93.9	83,790,811	6.1	1,374,240,926	64.2	38,454,916	1.8	18.9
2010	1,347,453,572	94.3	81,051,145	5.7	1,428,504,717	66.3	40,061,594	1.9	19.4
2011	1,373,751,906	94.8	76,069,420	5.2	1,449,821,326	67.1	40,543,474	1.9	19.4
2012	1,400,050,239	95.2	71,087,695	4.8	1,471,137,934	67.9	41,025,353	1.9	19.5
2013	1,446,641,335	95.3	71,375,352	4.7	1,518,016,687	69.3	42,651,596	1.9	19.8
2014	1,493,232,432	95.4	71,663,008	4.6	1,564,895,440	70.7	44,277,840	2.0	20.1
2015	1,539,823,528	95.5	71,950,665	4.5	1,611,774,193	72.1	45,904,083	2.1	20.5
2016	1,555,964,749	95.6	71,258,046	4.4	1,627,222,795	72.5	46,269,389	2.1	20.4
2017	1,572,105,971	95.7	70,565,427	4.3	1,642,671,398	72.9	46,634,694	2.1	20.3
2018	1,588,247,192	95.8	69,872,808	4.2	1,658,120,000	73.3	47,000,000	2.1	20.2
2019	1,609,841,860	95.9	69,514,350	4.1	1,679,356,210	73.8	47,200,000	2.1	20.2

*Values between 1999-2005, 2005-2009, 2009-2012, 2012-2015, 2015-2018 were calculated by interpolation from data for 1973, 1999, 2005, 2009, 2010, 2012, 2015, 2018, 2019 (GDF, 2021a)

At the same time, there is a hihg-level significant positive relationship (r = 0.98) between elapsed time and average growing stock (*Table 2*). As a matter of fact, while the average growing stock per hectare was 46.3 m^3/ha in 1973, this value increased by 59.5% to 73.8 m^3/ha in 2019. It seems that the increase in total growing stock generally occurs in productive forests. In terms of growing stock per unit area, Turkey is seen to be in the last ranks when compared to countries with similar ecological conditions. For example, the growing stock per unit area is 216 m^3/ha in Croatia, 164 m^3/ha in Bosnia-Herzegovina, 183 m^3/ha in Bulgaria, 173 m^3/ha in France, 161 m^3/ha in Georgia, 154 m^3/ha in Serbia, 149 m^3/ha in Italy, 147 m^3/ha in Montenegro, 99 m^3/ha in Albania, 66 m^3/ha in Spain and 48 m^3/ha in Greece (FAO, 2015). Growing stock per unit area in Turkey is lower than in countries with similar ecological conditions, which can be related to the fact that the proportion of degraded forest areas in the total forest area is high and that forest areas generally have poor habitat characteristics.

When evaluating the condition of the forests, it is necessary to consider the annual current increment amounts in addition to the growing stock. According to the statistical analysis (*Table 2*), there is a positive significant relationship between the annual current increment amount and the urban population (r = 0.99) and the elapsed time (r = 0.96).

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Accordingly, when the data in *Table 4* are evaluated, the annual current increment amount of all forests in Turkey was 28,063,205 m^3 in 1973, and this figure increased by 68.2% in 2019 and reached 47,200,000 m^3. The annual current increment amount per unit area (ha) of the country's forests is parallel to this situation. In other words, while the annual current increment amount per unit area in all forests was 1.4 m^3/ha in 1973, this figure increased to 2.1 m^3/ha in 2015.

According to the 2015 data (FAO, 2015), when we look at the situation in countries with the richest forest existence in the world in terms of the annual current increment per unit area, it is seen that it is 1.3 m^3/ha in the Russian Federation, 3.6 m^3/ha in China and 2.9 m^3/ha in the United States. In countries with similar ecological conditions to Turkey, the annual current increment amounts are 4.9 m^3/ha in France, 4.2 m^3/ha in Croatia, 3.8 m^3/ha in Bulgaria, 3.5 m^3/ha in Italy, 2.4 m^3/ha in Montenegro, 1.9 m^3/ha in Spain and 0.3 m^3/ha in Albania (Forest Europe, 2018; Foresters' Association of Turkey (FAT), 2019). Considering the annual current increment per unit area in terms of fertile forests, it can be said that Turkey has an average value among the countries with similar ecological conditions.

While forest areas increased in 46 years between 1973 and 2019 in Turkey, there were also increases in growing stock and annual current increments. It can be said that these increases are caused by the afforestation, maintenance and rehabilitation works carried out by the forestry organization for many years. On the other hand, especially since the 1980s, with the increase of migration from rural to urban areas, human-induced pressures (illegal cutting, illegal opening and settlement in forest areas, animal grazing, etc.) have disappeared or decreased significantly. This situation enabled the forest areas to increase and degraded forest areas to become productive. For example, in a study conducted by Turkish Environmental Foundation (TEF) (2001), it was stated that the village population in the Black Sea Region decreased by up to 70% and that the empty agricultural areas turned into forest ecosystems.

In addition to these, with an administrative decision put into effect in 2006, the works carried out within the scope of transforming forests that are operated as coppice forest throughout the country as a high forest are an important factor in the increase in growing stock and annual current increments. As a matter of fact, according to GDF (2021a), the forest area operated as coppice forest in Turkey in 1973 constituted 45.6% (9,264,689 ha) of the whole forest area. This value decreased to 22.6% in 2010 and to 5.0% in 2019. Thus, the forests in the areas transformed from coppice forests to high forests were not cut down every 20 years, and annual increment has thus been ensured for many years.

There is a medium-level significant negative relationship ($r = -0.51$) between growing stock per capita and the elapsed time (*Table 2*). In this sense, although the growing stock and the current annual increment in Turkey have been constantly increasing, the growing stock per capita has continuously decreased due to the faster increase in the total population in the country. As a matter of fact, while the growing stock per capita was 24.3 m^3/person in 1973, this figure was calculated as 20.2 m^3/person in 2019. According to FAO (2010 b), the total growing stock in the world's forests is 527 billion m^3 or 131 m^3/ha. However, the growing stock per hectare in the world's forests is generally increasing, except for North America and the Russian Federation. On the other hand, when we look at the situation in countries with similar ecological characteristics with Turkey, the growing stock per capita is as follows: 195.4 m^3/person in Montenegro, 102.4 m^3/person in Bosnia-Herzegovina,

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101.5 m^3/person in Georgia, 97.6 m^3/person in Croatia, 96.2 m^3/person in Bulgaria, 58.4 m^3/person in Serbia, 43.3 m^3/person in France, 26.0 m^3/person in Spain, 23.1 m^3/person in Italy, 18.8 m^3/person in Albania, and 16.8 m^3/person in Greece (Forest Europe, 2018; Foresters' Association of Turkey (FAT), 2019). Therefore, in terms of growing stock per capita, Turkey falls behind many countries with similar conditions except for Albania and Greece.

In-forest recreation areas and urban forests

The concept of urban forest was first expressed in Canada in 1965 by Jorgenson (Johnston, 1996; Konijnendijk, 2003; Randrup et al., 2005). However, according to Raundrup et al. (2005), the beginning of the urban forest concept in Europe was in the 1980s, with studies in England and the Netherlands. In Turkey, the concept of urban forest has been brought to the agenda after the 1980s (Serin and Gul, 2006; Köse, 2020).

When the development of urban forestry is examined, landscape development and mental and physical health come to the fore as the starting point, while issues such as air and sound pollution as well as microclimate changes have started to take priority due to environmental problems caused by urbanization (Carter, 1995; Nowak et al., 2006; Vos et al., 2013). The concepts of "urban forest" and "urban forestry" are defined differently in many countries. For example, according to Konijnendijk (2003), urban forest and urban forestry definitions are as follows: In Finland, "it is a forest area in or around the urban area, whose main purpose and function is recreation." In the Netherlands, the term "urban green" is used instead of the concept of urban forest, and it covers all of the urban green areas. In the United States (USA), "it is the whole of vegetation and green areas that benefit the enrichment of the quality of life of the society." In countries with low levels of urbanization (Finland, Sweden, etc.), traditional forestry works generally come to the fore, whereas in countries with more urbanization such as England and the Netherlands, works on urban forest/forestry are increasing (Ottisch, 1999). As it can be understood from here, the concepts of "urban forest" and "urban forestry" vary according to the state of the countries in terms of forest and their expectations from urban forests.

GDF, which has the ownership of forests on behalf of the Treasury in Turkey, has all kinds of rights such as management, planning, operation, etc. on forests. The expectations of the urban people from forests were met from the green areas in the urban centers, such as parks, gardens, etc., which were previously under the administration of municipalities. However, GDF has started to establish in-forest recreation areas (A, B and C type) and urban forests (D type) in forest areas in order to meet the recreational needs of the urban people for more than 40 years.

The main purpose of in-forest recreation areas and urban forests is to produce recreational services. According to the National Parks Regulation (URL-4, 1986), in-forest recreation areas are defined as "forest area with recreational and aesthetic resource values". On the other hand, according to the Regulation on Recreation Areas issued by GDF in 2013 (URL-5, 2013), the definition of recreation areas is as follows: "They are forest areas that meet the various rest, entertainment and sports needs of the society, provide daily or overnight accommodation needs of the people, and have recreational and aesthetic resource values." In addition, this regulation defined three different in-forest recreation areas as A, B and C type and urban forests as type D. A type A in-forest recreation area is a recreation area with overnight accommodation,

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whereas a type B in-forest recreation area is a recreation area where overnight accommodation is not available. A type C in forest recreation area, on the other hand, refers to the places that are used daily and that allow the local people to sell their local products.

Establishment of urban forests and in-forest recreation area in Turkey is subject to the permission of the Ministry of Agriculture and Forestry. According to the legislation in force in Turkey (URL-5, 2013), the term urban forest, which is considered in the D-type recreation area category, is a concept that is still discussed in developing countries. Since it is not yet fully a system settled and fit for purpose in Turkey, the differences between urban forest, in-forest recreation area (type A, B, C) and green areas in and around urban areas other than other forest regimes have not been clearly revealed. However, considering the Regulation on Recreation Areas, Application Communiqué on Recreation Areas and the situations in practice, the differences between urban forests and in-forest recreation areas can be explained as follows.

Accordingly, the basic criteria that distinguish urban forests from in-forest recreation areas and the functions that should be found in urban forests are as follows: (1) Offering the social functions of forests such as health, sports, aesthetics and culture to the service of the public, apart from the traditional picnic concept; (2) introducing the technical forestry activities and local flora and fauna; (3) giving the young generations the love and awareness of the forest; (4) enabling activities for tourism purposes; (5) reflecting the historical and cultural characteristics of the region. In addition, according to the application instruction of the law published by GDF (URL-6, 2014), except in compulsory cases, a minimum of 2 ha and a maximum of 50 ha of area should be allocated for A and B type in-forest recreation areas, whereas a minimum of 1 ha and a maximum of 20 ha of area should be allocated for C type in-forest recreation areas and a minimum of 5 ha and a maximum of 300 ha of area should be allocated for urban forests. In addition, in this application instruction, the number (54 different structures and facilities for A-type, 46 different structures and facilities for B-type, 30 different structures and facilities for C-type and 42 different structures and facilities for D-type urban forests were proposed) and characteristics of buildings and facilities which could be built in each type of in-forest recreation areas and urban forests were specified. However, in practice, it is seen that the rule regarding area sizes is not followed. For example, according to GDF (2021a), the smallest urban forest is 1.5 ha (Aydın/Karacasu Urban Forest), and the largest urban forest is 847.5 ha (Kanuni Sultan Süleyman Urban Forest). In terms of in-forest recreation area, the smallest is 0.02 ha (Karabük/Yenice İncedere C-type), while the largest is 768.0 ha (Adana/Karaisalı Topalak Dörtler C-type).

According to *Table 5,* a total of 40 (A-type: 17, B-type: 23) in-forest recreation areas were established in 1973, and their total area is 1,127 ha. The number of in-forest recreation areas established in 2019 reached 1,387 (A-type: 164, B-type: 342 and C-type: 881) on 17,009 ha of land. The duty of operating in-forest recreation areas and urban forests is largely transferred by GDF to municipals and village legal entities in return for rent. The General Directorate of Forestry has concentrated on the establishment of in-forest recreational areas in forest villages outside the city centers in order to contribute to rural development, especially since 2003. Accordingly, between 2005 and 2011, there has been a significant increase in the number and size of in-forest recreation areas, whereas there has been a sudden decrease in this sense since 2012 (*Table 5*). This situation was caused by the closure of some of the in-forest recreation

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areas, as the forest villagers could not earn enough income from the in-forest recreation areas and had difficulty in paying the annual rental fees they had to pay to the General Directorate of Forestry.

Table 5. *Distribution of the number and area changes of urban forests and in-forest recreation areas in Turkey by years (GDF, 2021a)*

Year	Type A		Type B		Type C		Total (A + B + C)		Urban forest (Type D)		Urban forest per person (m^2/person)	Total		Total area per person (m^2/person)
	Number	Area (Ha)	Number	Area (Ha)	Number	Area (Ha)	Number	Area (Ha)	Number	Area (Ha)		Number	Area (Ha)	
1973	17	614	23	513			40	1,127				40	1,127	0.72
1980	25	981	51	1,103			76	2,084				76	2,084	1.06
1985	31	1,122	65	1,553	1	32	97	2,707				97	2,707	1.01
1990	4	1,851	94	2,464	10	102	108	4,417				108	4,417	1.33
1995	56	2,242	120	3,578	19	123	195	5,943				195	5,943	1.54
2000	59	2,311	132	4,119	45	499	236	6,929				236	6,929	1.57
2005	102	3,976	178	5,625	213	33,402	493	43,003	33	4,840	1.01	526	47,843	9.95
2010	106	4,257	191	6,043	1,039	144,664	1,336	154,964	85	9,662	1.72	1,421	164,626	29.28
2011	46	1,539	108	3,384	1,295	149,388	1,449	154,311	110	11,230	1.96	1,559	165,541	28.85
2012	46	1,539	105	3,295	1,286	10,528	1,437	15,362	122	12,720	2.18	1,559	28,082	4.80
2013	53	1,678	105	2,774	1,239	9,924	1,397	14,376	126	11,867	1.69	1,523	26,243	3.75
2014	77	2,739	114	3,038	1,123	9,060	1,314	14,837	127	9,946	1.40	1,441	24,783	3.48
2015	122	4,190	173	3,515	1,016	8,165	1,311	15,870	133	10,315	1.42	1,444	26,185	3.61
2016	124	4,254	205	3,979	975	8,033	1,304	16,266	145	10,550	1.43	1,449	26,816	3.64
2017	130	4,066	234	4,302	953	7,890	1,317	16,258	142	10,444	1.40	1,459	26,702	3.57
2018	149	4,498	290	4,694	936	7,497	1,375	16,689	137	10,361	1.37	1,512	27,050	3.57
2019	164	4,914	342	5,088	881	7,007	1,387	17,009	134	10,199	1.32	1,521	27,208	3.53

There is a medium-level significant positive relationship ($r = 0.53$) between the amount of urban forest area and the elapsed time, and a medium-level significant positive relationship ($r = 0.43$) between the amount of urban forest area and urbanization (*Table 2*). Urban centers, which offer better social and economic conditions for people in Turkey, as in the whole world, are transformed into a built ecosystem where all of their time is spent, and there is therefore an increase in the demand and need for green space in urbans (Köse, 2020). As a result of these expectations, 134 urban forests were established in a total area of 10,199 ha between 2003 and 2019, in line with the "urban forest project" that was put into practice in Turkey in 2003. As seen in *Table 5,* the number of urban forests in Turkey has increased (145) until 2016, and has decreased after this date. It is considered that this situation is caused by the closure of a part of the urban forests that were opened to operation, since sufficient resources were not allocated for the operation of the urban forests.

According to *Table 5,* the total urban forest area in 2019 covers approximately 0.04% of Turkey's total forest area, 0.08% of the productive forest area, and 0.01% of the total

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land area. In 2019, the total number of both in-forest recreation areas and urban forests was 1,521, and the area covered by them was 27,208 ha. In 2019, the total area of urban forests and in-forest recreation areas covers approximately 0.1% of Turkey's total forest area, 0.2% of the productive forest area, and 0.03% of the total land area. In this sense, the amount of green area (urban forest + in-forest recreation area) per person in Turkey is 3.53 m^2. The World Health Organization (WHO) states that the green area per person in cities should be at least 9 m^2, and 10 to 15 m^2 is ideal (Götze et al., 2008; Pogue, 2010). According to the data of 2013, the green area per person in developed countries is around 20 m^2 on average, while it varies between 1-9 m^2 in the urban centers of Turkey (URL-7, 2013). For example, the amount of green space per person in some European cities is 23.6 m2 in Berlin, 29.2 m2 in Brussels, 26.1 m2 in Milan, 32.9 m^2 in Liverpool, 18.0 m2 in Barcelona, and 124.7 m2 in Vienna (Anonymous, 2000).

According to the provisions of the "Regulation on Principles for Plan Making" (URL-8, 2001), which is still in force in Turkey, the amount of green space per person in urban areas has been determined as at least 10 m^2. In this sense, many scientific studies were conducted in order to determine the amount of green areas per person in urban centers, and it was aimed to consider the deficiencies observed on the urban green space presence. For example, it was determined that there was 3.1 m^2 green area per person in Antalya (Ortaçeşme et al., 2000), 1.9 m^2 in Istanbul (Aksoy, 2001), 3.0 m^2 in Isparta (Gül and Küçük, 2001), 5.4 m^2 in Kayseri (Öztürk, 2004), 1.4 m^2 in Kahramanmaraş (Doygun and İlter, 2007), and 4.0 m^2 in Burdur (Yenice, 2012). Therefore, it is clear that the current urban green space amount per capita in Turkey is well behind both the green space standards specified within the scope of the Zoning Legislation for Turkish cities and the available green space amount in many European cities.

Conclusions and recommendations

Since people, who would like to get rid of the tiring and depressing life of metropolises, whose population continues to increase rapidly, turn to forests and urban centers expand to forest borders, an increasing number of urban people in Turkey and the world have become interested in forests and natural areas.

Since 1927, the total population and urban population have continuously increased in Turkey (TUIK, 2020). In addition, while the share of the rural population in the total population decreased with the migration process, the share of the urban population increased significantly. The decrease in the rural population has reduced the negative effects of the rural population on forests. In other words, because the number of people living in the countryside has been decreasing, the demand for firewood and round timber raw materials from forests has been decreasing, grazing pressure on forests and pastures has been easing as the number of animals has been decreasing, and new areas have not been opened from forests for illegal settlements and agriculture. In addition, forest areas, which were previously opened as agricultural land, have been turning into forests again as they have been abandoned due to migration. Therefore, it is a fact that there is a transformation in favor of forests in the rural areas of Turkey.

However, it is not possible to talk about a similar situation in urban areas. According to the statistical analysis, although there is a positive relationship between urban population and forest area, and growing stock and annual increment, it is not at the same rate. The urban population increases several times more than the forest area, growing

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stock and annual increment. This increase creates significant pressures on forests. As cities grow, they approach the forest boundary. This situation brings about illegal settlements in forested areas due to the populist policies of the political powers. These illegal settlements have been paved the way with the changes made in the Constitution and laws in different times from past to present. On the other hand, allocations given from forest areas for education, health, mining, industrialization, transportation, recreation and tourism, etc. create significant threats on forests. While giving these allocations, the concept of public benefit is taken into account in practice. However, since the criteria and conditions of the concept of public benefit are not revealed in a concrete way, they are often interpreted differently by different people. Therefore, the criteria and conditions of the concept of public benefit should be clearly and comprehensively presented in a way which would not harm forests.

In addition, despite the increase in total forest area and growing stock in Turkey, the amount of forest area and growing stock per total population and urban population is gradually decreasing. This situation should be taken into account in the development of forward-looking forestry policies. At the same time, any regulations in the Constitution and laws, which would be against forests (implementations of taking out of forest boundaries with regulations 2B, etc.), should be avoided.

There is no significant difference between forests, which are separated as urban forests with a separate definition and sign, and other in-forest recreation areas in the forest in terms of purpose, application and public utilization demands, and it is indeed quite possible to state that all types of in-forest recreation areas in the forest serve urban forestry (Sağlam and Elvan, 2017). In fact, considering the examples in the world in urban forestry practices as mentioned in this study, it can be said that a large part of the green open areas outside the forest regime created in the urban also serve urban forestry, which is contrary to the understanding in Turkey.

Urban forests in Turkey are on average 8.4 years old (=134/16). 134 urban forests have been established in 16 years, mostly for populist and political purposes, which do not comply with the understanding of urban forestry in developed countries. Such an application, which is incompatible with urban forest planning and management principles, brought many problems and caused some of them to be closed later. This has led to a waste of labor and capital. In urban forests and other in-forest recreation areas, buildings and facilities that are not suitable for the natural structure of forests are being built by using the recreation demands of the people as an excuse. In fact, it is against the Constitution and the Laws to make structuring in forest areas restricted by the Forest Law (Law No. 6831), and even to issue regulations such as guidelines and communiqués to legitimize what has been done. The construction of these structures and facilities causes the forests and natural structure (ecological balance, wildlife, etc.) to deteriorate and not to return to their former state for many years. At the same time, this situation is contrary to the understanding of urban forestry of developed countries in the world. Municipalities are the biggest operators of urban forests and recreation areas in Turkey. Nevertheless, municipalities and special administrations do not take sufficient initiative in the establishment, planning and management of urban forests. In this sense, municipalities and special administrations should act together with the forestry organization.

Therefore; especially in urban settlements, in order to prevent the activities of the urban people against the forests such as deforestation, settlement, illegal logging, those working in practice should be more careful, political authorities should abandon

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populist policies, and researchers and academicians should make more scientific publications to attract the attention of the public on this issue. It is important that this subject is scientifically supported by forestry research institutions in order to reveal the criteria of the concept of "public benefit" in detail regarding the areas subject to permission and easement in forests. Due to the fact that there is no significant difference between urban forests and other recreation areas in terms of application and utilization demands of the people of the city. The definition of all forest recreation areas, city parks and wooded green areas in and around the urban should be re-evaluated within the scope of urban forest according to the current legislation. In this context, the definition of "urban forest" in the current legislation should be rearranged.

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STUDIES ON INFLUENCING FACTORS OF PHYTOPLANKTON FUNCTIONAL GROUPS COMPOSITION AND ECOLOGICAL STATUS OF THE DONGTA SPAWNING GROUNDS IN THE PEARL RIVER, CHINA

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Abstract. To explore the spatial and temporal distribution characteristics of phytoplankton functional groups (PFGs) of the Dongta spawning grounds in the Pearl River, China, quarterly surveys of this area were carried out during March, 2016 to December, 2018, and the ecological status was analyzed and evaluated according to PFGs assemblage index (*Q* index). 26 PFGs were identified, and the dominant PFGs were C, D, J, K, L_0, MP, N, P, S1, S2, T, W1, W2, X2, Y in this area. Phytoplankton *Q* indexes varied from 1.83 to 3.73, with the mean value 2.91, which showed that the ecological status of the Dongta spawning grounds was "medium" and "good", but parts occasionally were "tolerable". Temporally, the general ecological status was good in March and June, but it was medium in September and December. Basing on our study, the results showed that: (1) factors including water velocity (V), DO, Chla, phosphate, oxidation reduction potential, total phosphorus, electrical conductivity, total dissolved solid and salinity were significantly correlated with the dominant PFGs; (2) the phytoplankton *Q* index was significantly correlated with the V. It was suggested that after the construction and operation of Datengxia Water Dam and its upper reaches cascade dams, attention should be paid to regulate the water flow and velocity in the lower reaches of the river by means of ecological operation, so as to ensure the functions of the spawning grounds and protect the fishery conservation zones in the mainstream of the Pearl River.
Keywords: *Dongta spawning grounds, phytoplankton functional groups, assemblage index, water quality, relationship analysis*

Introduction

Phytoplankton is the main primary producer in water areas, which releases oxygen through photosynthesis and forms the basis of material circulation and energy transfer in aquatic ecosystems (Reynolds, 1984). In addition, phytoplankton is an important indicator for water environments because of its small size, rapid growth, short life

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cycle, highly unified living space and water environment, and rapid response to environmental changes (Huisman et al., 2004; Istvanovics et al., 2010). Its community composition and population changes can directly and quickly reflect the living environment (Reynolds, 2006; Istvanovics et al., 2010; Wang et al., 2017). Traditional phytoplankton ecology is studied mainly by identifying algae species based on original Linnaean classification system, followed by the analysis of the variation of phytoplankton species composition, density, biomass and dominant species, to reflect and evaluate the environmental quality of specific waters (Reynolds, 2006). However, the process was time-consuming and laborious. Besides, dominant species, diversity index and other indicators could only reflect one or several aspects of the environmental status, the results were often having large difference, the interpretation of the results and conclusions was usually arbitrary and subjective, and could not reflect the habitat conditions (Reynolds et al., 2002). In order to accurately reflect the real functions of phytoplankton in the aquatic ecosystem, Reynolds et al. (2002) and Padisák et al. (2006) referenced the research methods on land plants, after gradually expanding and improving these approaches, and finally formed a relatively complete theory of phytoplankton functional groups (PFGs, coda) methods. These methods combined phytoplankton habitat characteristics with community ecological process, simplified the complexity of traditional classification system, and provided a powerful tool for explaining the selection mechanism of phytoplankton community and predicting community succession results more rationally (Padisák et al., 2009). In the world, PFGs classification methods had become one of the main techniques to study the ecological structure and function of rivers, reservoirs and lakes (Crossetti et al., 2008; Xiao et al., 2011; Abonyi et al., 2012; Devercelli et al., 2013; Cupertino et al., 2019; Wang et al., 2020b). At the same time, a new ecological status estimation method for waters basing on PFGs assemblage index (Q index), was proposed to assess ecological status of different types of water (Padisák et al., 2006). Q index combines the weight of functional groups relative to the total biomass, with a factor number for each assemblage related to the type of water body. The Q index method had been successfully applied in ecological status assessment for extensive regional waters (Wang et al., 2011; Zhu et al., 2013; Santana et al., 2017).

Dongta spawning grounds is located in the upstream of the Xunjiang River, from the junction of Qianjiang and Yujiang Rivers to Dongta Village, Xun Wang Town, Guiping County, with a length of about 7 km, which lies in the area of E 110.0956°, N 23.4054° and E 110.0967°, N 23.4003° to E 110.1387°, N 23.4560° and E 110.1443°, N 23.4550°. It is known as the second largest spawning grounds of the four Chinese farmed carps, i.e. black carp, grass carp, silver carp and bighead carp, and the most abundant fish biodiversity in the main stream of the Pearl River, China (Shuai et al., 2016). The Dongta spawning grounds had important economic value and aquatic biological resources protection value for aquatic living resources, especially the Chinese carp fish (Li et al., 2009, 2021). At present, the Datengxia Water Control Project, upstream of Dongta spawning grounds, is under construction. It is only about 15 kilometers away from the downstream boundary of the Dongta spawning grounds. Previous studies showed that dam construction would have many important impacts on water velocity, sediment and biological resources in the upstream and downstream of the river, and affected the distribution of phytoplankton (Zeng et al., 2006; Zhou et al., 2011; Zuo et al., 2019; Maavara et al., 2020). Therefore, the influence of Datengxia hydroelectric power plant during the construction and water storage period

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on the changes of water environment and biological resources in Dongta spawning grounds should be attracted early attention. In the past, the division of fish spawning grounds was mainly based on the status of fishery resources, and the location and boundary area of spawning grounds were determined through investigation or detection of fish resources in various sections of rivers (Tan et al., 2011a, b; Li et al., 2021). It was difficult to scientifically explain the formation and change mechanism of fish spawning grounds, and to formulate corresponding protection and restoration measures.

Good water environment in the Dongta spawning grounds is the basically conditions to ensure fish spawning and breeding, which is also beneficial to realize its protection function (Fellman et al., 2015; Deng et al., 2019; Li et al., 2021). Since the PFGs could explain and predict the water condition, farther more, it had far-reaching impact on fishery resources distribution, the present study selected the habitat of the Dongta spawning grounds in the Pearl River, through monitoring and analysis its environmental factors and PFGs, as well as the relationship between the environment variables and PFGs, to analyze the distribution characteristics of functional groups and its main influencing factors, and to understand the ecological status of the Dongta spawning grounds. Comprehensive conclusions would be discussed and proposed that how to ensure the functions of the spawning grounds and protect the fishery conservation zones in mainstream of the Pearl River after the construction and operation of Datengxia Water Dam and its upper reaches cascade dams.

Materials and methods

Study site and sampling

Surface water samples were collected in March, June, September and December of the year 2016, 2017 and 2018 in the Dongta spawning grounds of the Pearl River, China (*Fig. 1*), and the investigation was launched on the last ten days of the sampling month. The sampling stations of the Dongta spawning grounds were Bailan (BL, downstream and near the Datengxia Dam, E 110.0352°, N 23.4675°), Guiping (GP, under the Qianjiang bridge of the Guiping County, E 110.0807°, N 23.4103°), Yujiang (YJ, near the river mouth of the Yujiang connection to the mainstream of the Pearl River, E 110.1021°, N 23.3961°), Dongta (DT, Dongta Village, Xun Wang Town, Guiping County, E 110.1261°, N 23.4188°) and Shizui (SZ, the Shizui Town, Guiping County, E 110.1773°, N 23.4690°).

Determination of water physical and chemical factors

Water velocity was measured using water current meter (Global water, USA). Water transparency (SD) was measured using a Secchi disk according to a standard method (Zhang and Huang, 1991). Water temperature (WT, °C), pH, dissolved oxygen (DO, mg/L), oxidation–reduction potential (ORP, mV), conductivity (Cond, μS/cm) and total dissolved solids (TDS, g/L) were measured using a smart portable multi-parameter water quality analyzer (YSI, USA). Approximately 500 ml water was filtered using a Whatman GF/C filter membrane and was then used to measure the chlorophyll-a content (Chla, μg/L) according to a previously described method (The State Environmental Protection Administration, 2002). The concentrations of ammonium (NH_4-N), nitrate (NO_3-N), NO_2-N, total nitrogen (TN), total phosphorus (TP) and

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Silicate (SiO_3-Si) were determined using a flow injection water quality analyzer (Skalar, Netherlands) by standard procedures. The concentration of un-ionized ammonia (NH_3, mg/L) was calculated using NH_4-N, pH and WT according to previously studying method (Liu et al., 2021).

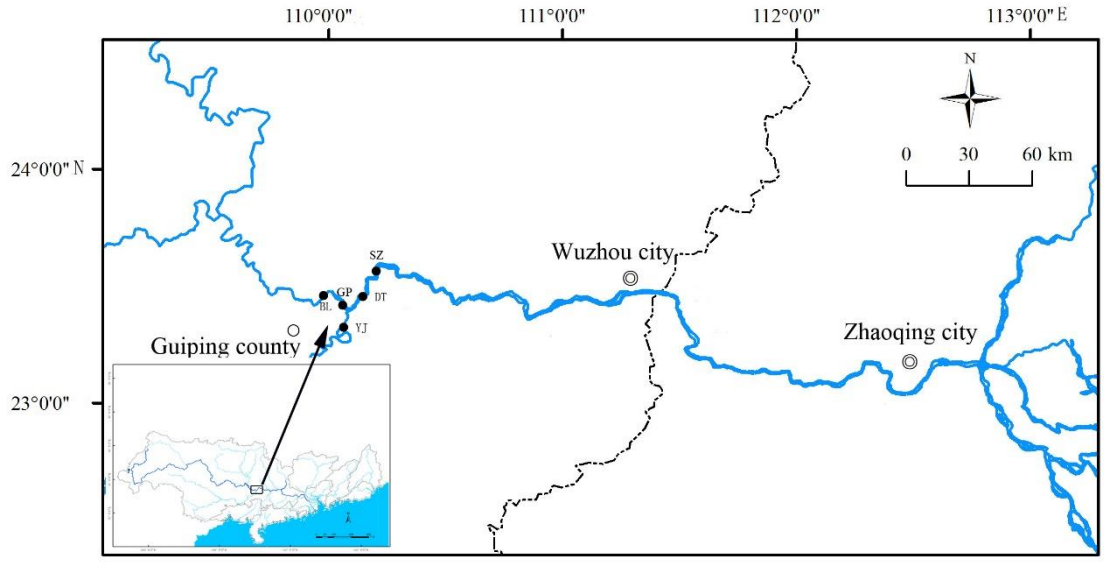

Figure 1. *The sampling stations of the Dongta spawning grounds in the Pearl River, China. In the figure, BL, GP, YJ, DT, SZ standing for Bailan, Guiping, Yujiang, Dongta and Shizui, respectively*

Phytoplankton sample collection and analysis

For each phytoplankton sample, 1 L of water was collected 0.5 m below the surface using a 5-L HQM-1 sampler, put into a polyethylene bottle, and fixed immediately with neutral Lugol's solution (5%). The phytoplankton samples were taken to laboratory, and concentrated to 30 mL using the settling technique. Phytoplankton was quantified using a light microscope (Olympus CX21, Japan) at 400× magnification. The units (cells, colonies and filaments) were enumerated in random fields, and at least 100 fields or 200 individuals of the most frequent species were counted (Zhang and Huang, 1991), and phytoplankton species were identified according to Hu and Wei (2006). Phytoplankton biomass was calculated from the biovolume of each species, assuming unit specific gravity, by geometrical approximation according to Hillebrand et al. (1999).

Phytoplankton taxa were classified into characteristic PFGs according to Reynolds et al. (2002) and Padisák et al. (2009). PFGs contributing more than 10% to the total biomass of each sample were categorized as dominant PFGs; meanwhile, some algal species usually contributed more than 10% to the density of the sample, and the PFGs the algae belonging to were also considered as dominant functional groups.

Evaluation of the water ecological status

The functional group approach had been applied in estuary, lake and reservoir ecosystems (Crossetti and de M. Bicudo, 2008; Wang et al., 2011; Santana et al., 2017; Cao et al., 2018), and can potentially be developed to assess the water quality more

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consistently. According to phytoplankton functional classification and typology of lakes, Padisák et al. (2006) developed an assemblage index, *Q* index, to assess ecological status of different lake types established by the Water Framework Directive (WFD). *Q* index combines the weight of functional groups relative to the total biomass, with a factor number for each assemblage related to the type of water body. The calculation of *Q* is the following:

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

Where $p_i = n_i/N$, n*i* is the biomass of the *i*th group, while *N* is the total biomass; F_i represents the assignment value of the *i*th PFG in the water body, and *i* represents the water quality index to range between 0 (the worst) and 5 (the best). Ecological status based on *Q* index can be classified into 5 grades: 0–1: bad; 1–2: tolerable; 2–3: medium; 3–4: good; and 4–5: excellent. The *Q* index method had a sufficiently solid theoretical basis, and it had been successfully applied in ecological status assessment for several reservoirs recently (Crossetti and de M. Bicudo, 2008; Santana et al., 2017).

Data treatment

Statistical analysis was carried out using the SPSS 18.0 package (one-way ANOVA). Variables of phytoplankton and chemical data were visualized using the EXCEL 2010, Origin (8.0) and R (R Studio) program with vegan package. To analyze the influence of these environmental factors on PFGs, Redundancy Analysis (RDA) was performed to investigate the relationship between the main environmental parameters and the dominant coda, using the R package Vegan. During the analysis, the PFGs biomass data were Hellinger transformed, as well as the environmental parameters were standardized, to reduce the effects of extreme values. A forward selection of environmental factors was applied to avoid using collinear environmental factors in the same constrained ordination model (Wang et al., 2020b). Only those parameters contributing significantly ($P < 0.05$) to PFGs dynamics were considered as the main influencing variables.

Results

Physical and chemical variables

The results of water environmental variables in the Dongta spawning grounds were shown in *Table 1*. The mean water velocity in the survey area was 0.9 m/s, with the lowest velocity in December (mean value 0.5 m/s) and the highest velocity in June (mean value 1.4 m/s). Water pH values were most in a range of 7.43 ~ 8.64, and the pH value in June was slightly lower than other period. The change of water temperature varied obviously seasonally. The water salinity varied in the range of 0.08‰ to 0.17‰. The mean value of water TDS content in three years was roughly the same, but it was obviously higher in wet season than in other periods. ORP of the study area had a widely variation range, with the minimum value of 15.50 mV and maximum value of 730.50 mV. The water Cond showed an obvious changes with hydrological period. Water DO content varied in the range of 5.33~9.43 mg/L, meeting the oxygen conditions needed for fish living. Affected by rainfall in the sampling periods, the water transparency varied in a large range, with the mean values were about 92 cm, 30 cm,

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74 cm and 98 cm in the four quarters, respectively. TN content was higher than 1.0 mg/L in most periods with its maximum value 2.74 mg/L, and the quarterly average value was 2.11 mg/L, 1.89 mg/L, 1.94 mg/L and 2.02 mg/L, respectively, which had an obviously increase in wet season. The variation trend of NO_3-N, NH_4-N and SIN (soluble inorganic nitrogen, NO_3-N + NO_2-N + NH_4-N + NH_3) was similar to that of TN. Silicate concentration showed an obvious change in the hydrological period, and the concentration in wet season (June and September) was significantly higher than that in level season and dry season (March and December). The contents of NO_2-N and NH_3 were at a low level, but peaked in some periods. The ratio of nitrogen to phosphorus was significantly different, with the minimum value of 4.31 and maximum value of 131.54.

Phytoplankton community composition

Identification of phytoplankton samples collected from the Dongta spawning grounds showed that a total of 176 phytoplankton species, including a few varieties and forma, were distributed among the following 7 major taxonomic categories: Bacillariophyceae (69 taxa), Chrysophyceae (59 taxa), Euglenophyceae (23 taxa), Cyanophyceae (14 taxa), Dinophyceae (4 taxa), Cryptophyceae (4 taxa) and Xanthophyceae (3 taxa). The Percentages of the 7 categories to the total species were 39.20%, 33.52%, 13.07%, 7.95%, 2.27%, 2.27% and 1.70%, orderly.

The quarterly phytoplankton community composition during March 2016 to December 2018 were showed in *Figure 2A*. In March, a total of 117 phytoplankton species, including a few varieties and forma, were distributed among the following 7 major taxonomic categories: Bacillariophyceae (46 taxa), Chlorophyceae (39 taxa), Euglenophyceae (15 taxa), Cyanophyceae (7 taxa), Dinophyceae (3 taxa), Cryptophyceae (4 taxa) and Xanthophyceae (3 taxa). In June, a total of 130 phytoplankton species, were distributed among the 7 major taxonomic categories: Bacillariophyceae (53 taxa), Chlorophyceae (39 taxa), Euglenophyceae (18 taxa), Cyanophyceae (12 taxa), Dinophyceae (2 taxa), Cryptophyceae (4 taxa) and Xanthophyceae (2 taxa). In September, a total of 118 phytoplankton species, were distributed among 7 major taxonomic categories: Bacillariophyceae (42 taxa), Chlorophyceae (43 taxa), Euglenophyceae (16 taxa), Cyanophyceae (8 taxa), Dinophyceae (2 taxa), Cryptophyceae (4 taxa) and Xanthophyceae (2 taxa). In December, a total of 98 phytoplankton species, were distributed among 7 major taxonomic categories: Bacillariophyceae (32 taxa), Chlorophyceae (38 taxa), Euglenophyceae (13 taxa), Cyanophyceae (7 taxa), Dinophyceae (2 taxa), Cryptophyceae (4 taxa) and Xanthophyceae (2 taxa). And the phytoplankton species composition in each sampling station were showed in *Figure 2B-F*.

Phytoplankton abundance

The phytoplankton cell density in the sampling area ranged from 8.40×10^4 cell/L to 4.08×10^6 cell/L. The average density was 5.01×10^5 cell/L. The maximum density appeared at the GP station in December 2018, and the minimum value appeared at the SZ site in June 2018 (*Fig. 3A*). The variation range of phytoplankton biomass was 0.14~2.55 mg/L, with an average of 0.94 mg/L. The maximum biomass appeared at the BL station in September 2016, and the minimum biomass appeared at the DT station in December 2017 (*Fig. 3B*).

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Table 1. *Means and ranges of environmental variables in the Dongta spawning grounds*

Environmental factors*	Mean and variation ranges			
	March	**June**	**September**	**December**
V (m/s)	0.86 ± 0.10 (0.30~1.50)^{a**}	1.42 ± 0.11 (0.70~2.20) ^{b}	0.81 ± 0.07 (0.50~1.30) ^{a}	0.53 ± 0.06 (0.30~0.90) ^{c}
pH	8.00 ± 0.07 (7.67~8.64)	7.88 ± 0.08 (7.45~8.48)	8.02 ± 0.04 (7.60~8.23)	8.03 ± 0.10 (7.43~8.64)
WT (℃)	18.48 ± 0.25 (17.48~20.55)^{a}	27.14 ± 0.38 (23.98~29.43)^{b}	28.29 ± 0.23 (26.95~29.68)^{c}	20.84 ± 0.43 (19.09~24.61)^{d}
Sal (‰)	0.13 ± 0.01 (0.08~0.15)^{ab}	0.12 ± 0.01 (0.08~0.15)^{b}	0.14 ± 0.003 (0.11~0.16)^{ac}	0.15 ± 0.004 (0.11~0.17)^{c}
TDS (g/L)	0.18 ± 0.01 (0.13~0.21)^{a}	0.18 ± 0.01 (0.15~0.22)^{a}	0.19 ± 0.005 (0.14~0.22)^{ab}	0.20 ± 0.005 (0.15~0.23)^{bc}
ORP (mV)	82.31 ± 13.29 (30.40~151.90)^{a}	93.19 ± 13.05 (15.50~167.70)^{a}	146.85 ± 32.86 (37.70~348.70)^{ab}	198.93 ± 52.95 (25.30~730.50)^{b}
DO (mg/L)	7.92 ± 0.20 (6.64~8.93)^{a}	7.34 ± 0.24 (5.33~8.31)^{ab}	7.07 ± 0.20 (5.75~8.31)^{b}	7.81 ± 0.24 (6.47~9.43)^{a}
Cond (μS/cm)	265.60 ± 9.95 (177.00~312.90)^{a}	272.04 ± 10.09 (176.00~337.89)^{ab}	314.35 ± 7.33 (238.00~356.19)^{c}	291.76 ± 6.46 (236.00~325.49)^{bc}
SD (cm)	92.00 ± 5.87 (70.00~150.00)^{a}	29.67 ± 3.53 (10.00~50.00)^{b}	74.00 ± 12.71 (30.00~190.00)^{a}	97.67 ± 6.53 (60.00~140.00)^{ac}
TP (mg/L)	0.08 ± 0.02 (0.02~0.25)^{a}	0.10 ± 0.01 (0.03~0.20)^{a}	0.18 ± 0.02 (0.02~0.31)^{b}	0.25 ± 0.01 (0.14~0.32)^{c}
PO_4-P (mg/L)	0.05 ± 0.01 (0~0.17)^{a}	0.06 ± 0.02 (0.01~0.18)^{a}	0.12 ± 0.02 (0.01~0.26)^{b}	0.12 ± 0.02 (0.01~0.28)^{b}
TN (mg/L)	2.11 ± 0.06 (1.75~2.51)	1.89 ± 0.08 (1.01~2.40)	1.94 ± 0.12 (0.64~2.65)	2.02 ± 0.08 (1.55~2.74)
NO_3-N (mg/L)	1.71 ± 0.07 (1.25~2.08)^{a}	1.25 ± 0.10 (0.15~1.79)^{b}	1.35 ± 0.13 (0.29~2.06)^{bc}	1.60 ± 0.09 (1.04~2.06)^{ac}
NO_2-N (mg/L)	0.04 ± 0.02 (0.00~0.21)	0.04 ± 0.02 (0.00~0.32)	0.03 ± 0.01 (0.00~0.09)	0.06 ± 0.01 (0.00~0.18)
NH_4-N (mg/L)	0.21 ± 0.05 (0.01~0.50)^{ab}	0.33 ± 0.04 (0.04~0.55)^{b}	0.31 ± 0.05 (0.11~0.69)^{b}	0.14 ± 0.03 (0.00~0.33)^{a}
NH_3(mg/L)	0.01 ± 0.003 (0.00~0.03)^{a}	0.03 ± 0.01 (0.00~0.07)^{b}	0.02 ± 0.003 (0.00~0.05)^{b}	0.005 ± 0.001 (0.00~0.02)^{a}
Si (mg/L)	6.89 ± 0.80 (2.42~10.16)^{ab}	7.01 ± 0.98 (2.78~12.27)^{ab}	9.41 ± 1.15 (3.54~16.07)^{a}	6.11 ± 0.79 (1.70~11.76)^{b}
SIN (mg/L)	1.97 ± 0.06 (1.35~2.40)^{a}	1.65 ± 0.12 (0.63~2.26)^{b}	1.71 ± 0.13 (0.43~2.35)^{ab}	1.80 ± 0.08 (1.17~2.18)^{ab}
N/P	40.72 ± 8.69 (9.44~131.54)^{a}	26.10 ± 3.99 (9.16~56.54)^{ab}	19.42 ± 6.66 (4.31~105.63)^{b}	8.78 ± 0.78 (5.06~14.43)^{b}
Chla (ug/L)	3.59 ± 0.24 (1.89~4.88)^{a}	2.34 ± 0.21 (1.05~3.81)^{b}	2.81 ± 0.26 (1.33~5.00)^{b}	1.54 ± 0.10 (0.70~2.28)^{c}

*V, water velocity; WT, water temperature; Sal, salinity; TDS, total dissolvable solid; ORP, oxidation-reduction potential; DO, dissolved oxygen; Cond, conductivity; SD, water transparency; TP, total phosphorus; PO_4-P, phosphate; TN, total nitrogen; NO_3-N, nitrogen nitrate; NO_2-N, nitrite nitrogen; NH_3, un-ionized ammonia; Si, Silicate, SiO_3-Si; NH_4-N, ammonium nitrogen, SIN, soluble inorganic nitrogen, SIN; N/P, ratio of nitrogen to phosphorus; Chla, chlorophyll-a

**Different letters indicated significantly different, $P < 0.05$

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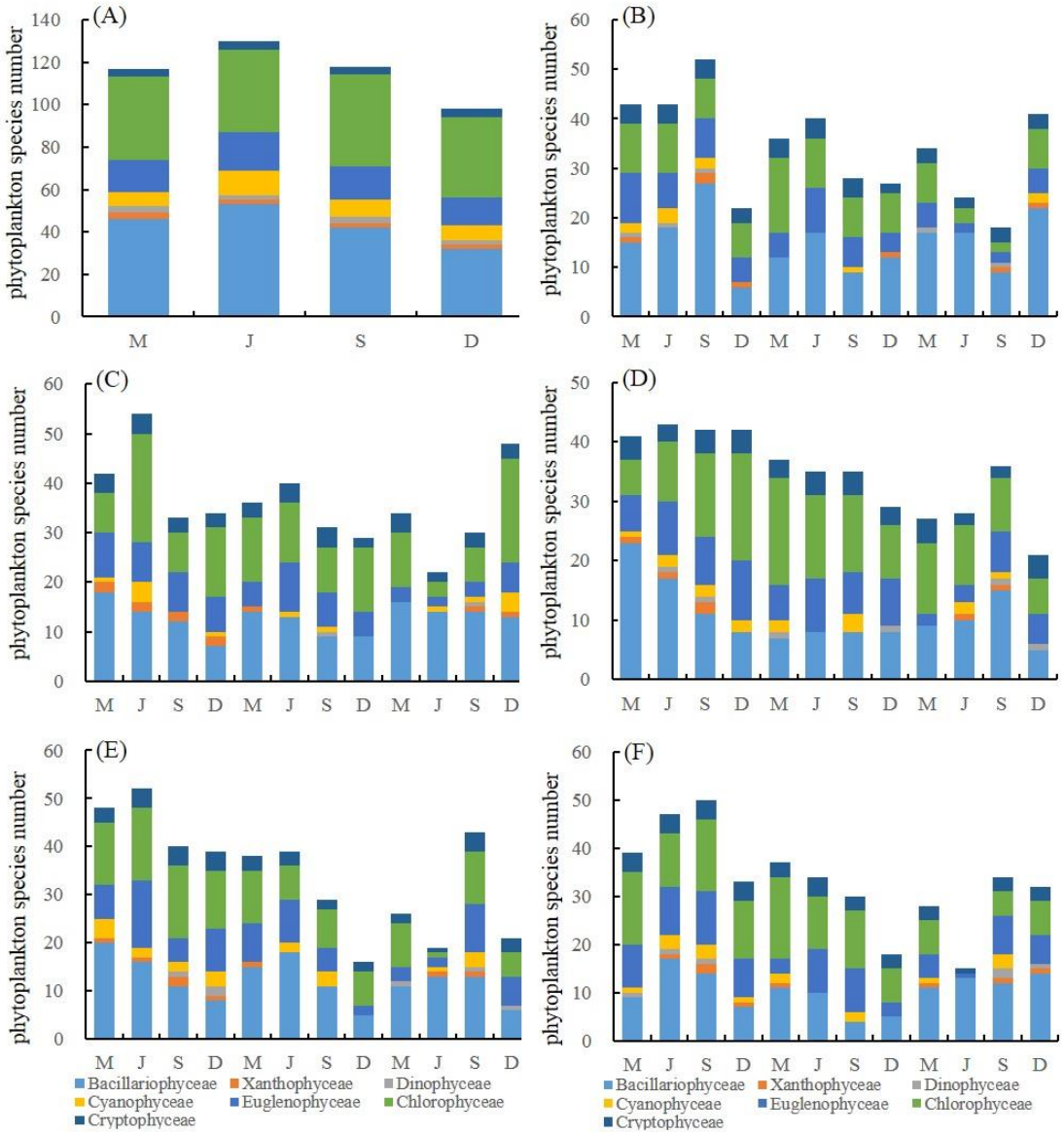

Figure 2. *Phytoplankton species composition in the Dongta spawning grounds. (A) The quarterly phytoplankton species composition during March 2016 to December 2018. (B) Phytoplankton species composition of Bailan sampling station during March 2016 to December 2018. (C) Phytoplankton species composition of Guiping sampling station during March 2016 to December 2018. (D) Phytoplankton species composition of Yujiang sampling station during March 2016 to December 2018. (E) Phytoplankton species composition of Dongta sampling station during March 2016 to December 2018. (F) Phytoplankton species composition of Shizui sampling station during March 2016 to December 2018. In the figure, M, J, S, D standing for March, June, September and December, respectively*

Phytoplankton functional groups

According to the PFGs methods, the planktonic algae in the Dongta spawning grounds could be divided into 26 functional groups, including A, B, C, D, F, G, H1, J, K, L_1, L_M, L_0, M, MP, N, P, S1, S2, T, W1, W2, W_S, X1, X2, X_{Ph} and Y, and the dominant phytoplankton functional groups were 15 coda, including C, D, J, K, L_0, MP,

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N, P, S1, S2, T, W1, W2, X2 and Y. The frequency analysis showed that 13 functional groups, including W1, MP, P, Y, K, X2, J, C, D, F, W2, T and G, were common functional groups in this area, with their frequencies of occurrence ranging from 50 to 100%; The occurrence frequency of functional groups L_0, B, S1 and X_{Ph} ranged from 25 to 50%, belonging to the sub-common functional groups in this area; The frequency of functional groups L_1, H, M, LM, Ws, N and X1 was between 5 and 25%, belonging to the uncommon functional groups; The frequency of functional groups S2 and A was less than 5%, which belonged to the rare or occasional functional groups in this area (*Fig. 4A*). The biomass of each functional group was shown in *Figure 4B*. It can be seen that W1, P, Y, D, MP, W2, C, J, K and F coda had large average biomass values, which were the most important PFGs in the Dongta spawning ground.

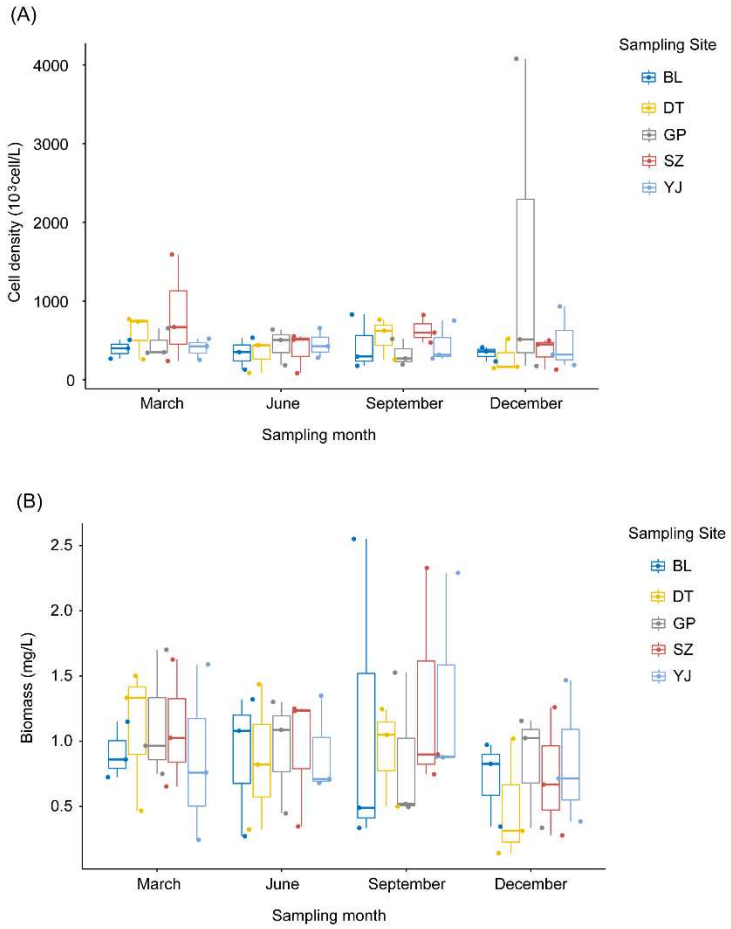

Figure 3. *Phytoplankton density and biomass in each sample stations of the Dongta spawning grounds. In the figure, BL, GP, YJ, DT, SZ standing for Bailan, Guiping, Yujiang, Dongta and Shizui, respectively*

The distribution of dominant PFGs in the Dongta spawning grounds was shown in *Table 2*. Based on the three years' investigation, the temporal characteristics of dominant PFGs had some difference. In March, there were 11 dominant functional groups including C, D, K, MP, N, P, S1, W1, W2, X2 and Y; the dominant functional groups in In June were D, MP, P, T, W1, W2 and Y; the dominant functional groups in September were D, L_0, MP, P, S2, W1, W2 and Y; and D, J, K, L_0, MP, P, S1, W1, W2,

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X2, Y coda were dominated in December. Spatially, six dominant PFGs were dominated BL station, which were D, L_0, MP, P, W1, Y; nine functional groups including D, K, MP, P, S1, W1, W2, X2 and Y, were dominated in GP station; ten dominant functional groups including D, K, MP, N, P, T, W1, W2, X2 and Y, were dominantly found in YJ station; eleven dominant functional groups (C, D, J, K, L0, MP, P, W1, W2, X2, Y) were dominantly found in DT station. Similarly, eight dominant functional groups, including D, MP, P, S1, S2, W1, W2 and Y were found at SZ station. The quarterly biomass of dominant phytoplankton functional groups in each sampling station of the Dongta spawning grounds were showed in *Figure 5*.

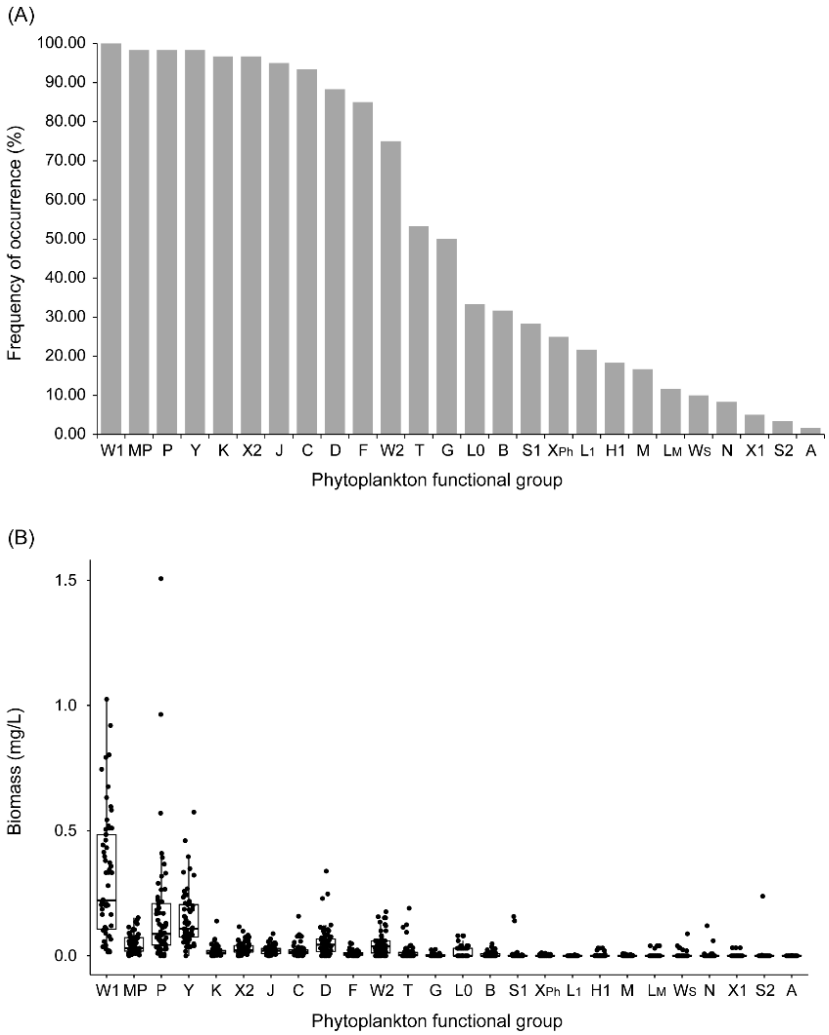

Figure 4. *Occurrence frequency analysis of phytoplankton functional groups and their biomass in the Dongta Spawning Grounds. (A) Occurrence frequency of phytoplankton functional groups; (B) Biomass of phytoplankton functional groups*

Phytoplankton functional groups assemblage index and water ecological status

Through habitat analysis, representative species, taxonomic status and *F* factor assignment of each PFG identified in the Dongta spawning grounds were listed in *Table 3*. The phytoplankton *Q* index was calculated according to Padisák et al. (2006) and Zhu et al. (2013).

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Table 2. *Spatial and temporal distribution of dominant phytoplankton functional groups in the Dongta spawning grounds*

	C	D	J	K	L_0	MP	N	P	S1	S2	T	W1	W2	X2	Y
March	+ *	+		+		+	+	+	+			+	+	+	+
June		+				+		+			+	+	+		+
September		+			+	+		+		+		+	+		+
December		+	+	+	+	+		+	+			+	+	+	+
BL		+			+	+		+	+			+			+
GP		+		+		+		+				+	+	+	+
YJ		+		+		+	+	+			+	+	+	+	+
DT	+	+	+	+	+	+		+				+	+	+	+
SZ		+				+		+	+	+		+	+		+

*"+ " representing the appearance of this phytoplankton functional group

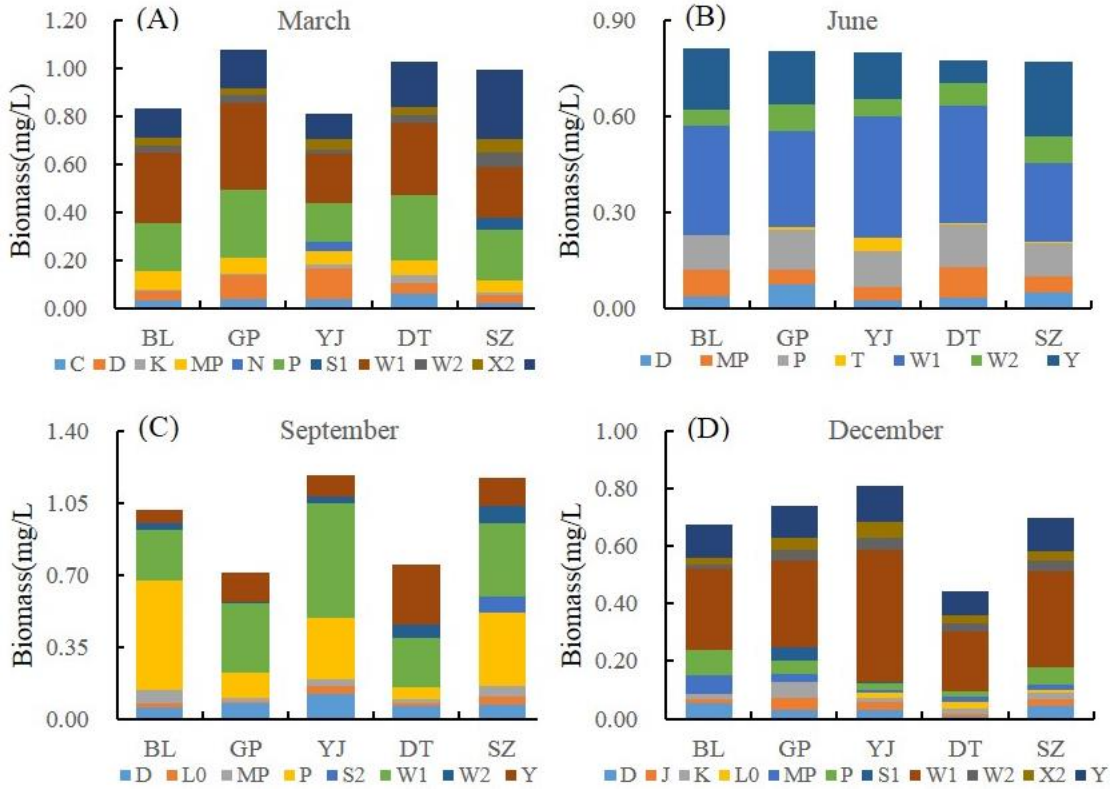

Figure 5. *The quarterly biomass of dominant phytoplankton functional groups in the Dongta spawning grounds. In the figure, BL, GP, YJ, DT, SZ standing for Bailan, Guiping, Yujiang, Dongta and Shizui, respectively*

The *Q* index of PFGs at each sampling station in Dongta spawning grounds ranged from 1.83 to 3.73, with an average of 2.91 and the median of 2.86 (*Fig. 6*). In March, the *Q* index varied from 2.77 to 3.46, with mean value of 3.05. The lowest *Q* index was observed in March 2017, and the highest was observed in March 2018. In June, *Q* index varied from 2.55 to 3.73, with a median value of 2.87 and mean value of 3.05. YJ station had the lowest value in June 2017, and SZ site had the highest value in June

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2018. In September, *Q* index varied from 1.83 to 3.43, with a median value of 2.84 and mean value of 2.82. The SZ station in September 2017 was the lowest, and the BL station was the highest in September 2016. In December, *Q* index varied from 2.31 to 3.12, with a median value of 2.73 and average value of 2.72. YJ station had the lowest value in December 2016, and SZ site had the highest value in December 2018. To the sample stations, the *Q* index variation range of BL station was 2.53~3.64, with mean value of 2.99. The *Q* index of Guiping samples ranged from 2.45 to 3.49 with mean value of 2.91. The *Q* index of YJ ranged from 2.31 to 3.23, with an average of 2.83. The *Q* index of DT area ranged from 2.65 to 3.67, with an average of 2.92. The *Q* index of SZ site ranged from 1.83 to 3.73, with an average value of 2.90. It can be seen that the *Q* index difference between March and December was small (less than 1), but the difference between September was larger (over 1.5), and June' *Q* index difference was in the middle range.

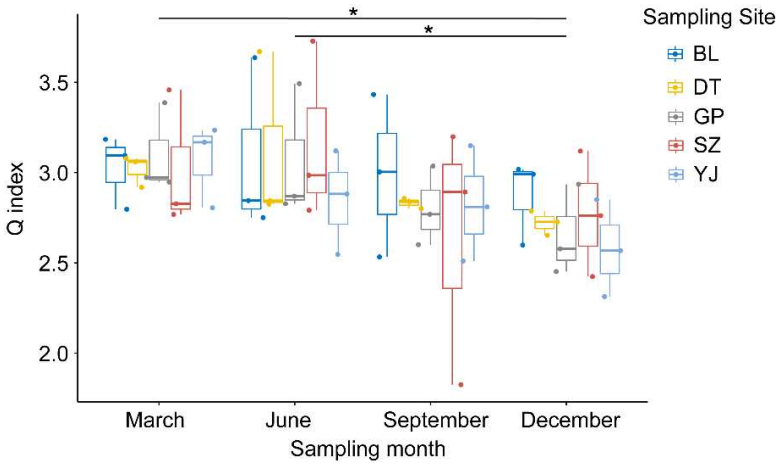

Figure 6. *Phytoplankton functional groups assemblage index (Q index) at each sampling station in the Dongta spawning ground. In the figure, BL, GP, YJ, DT, SZ standing for Bailan, Guiping, Yujiang, Dongta and Shizui, respectively (*P < 0.05)*

According to the phytoplankton *Q* index, it can be seen that the overall ecological status of the waters in the Dongta spawning grounds was in the range of "tolerable" to "good". Most of investigation periods, the ecological status of the Dongta spawning grounds was "medium" or "good", but it was "tolerable" occasionally with some areas' *Q* index less than 2. The ecological status in March and June was in the range of "medium" to "good", and the ecological status in these two periods was similar without significant difference ($P > 0.05$). The average *Q* indexes were 3.05 in these two sampling periods, which presented ecological status was "good" as a whole. In September, with an average *Q* index of 2.82, but there was a great difference in ecological status. A few sampling sites were "tolerant", while most of the sites were at the "medium" to "good", which was significantly different from March and June ($P < 0.05$). In December, the mean value of *Q* index was 2.72, and the water body was at the level of "medium" to "good", but the significance was lower than that of March, June and September ($P < 0.05$). The average annual *Q* index of the five sampling sites were 2.99, 2.91, 2.83, 2.92 and 2.90, respectively. There was no significant difference among the five stations ($P > 0.05$), and their quarterly average values *Q* index closing to 3.0, and their ecological status were in the critical state of "medium" to "good" (*Table 4*).

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Table 3. *The phytoplankton functional groups, representative species, taxonomic group and their F factor of the Dongta spawning grounds*

Functional group	The main phytoplankton representative species	Taxonomic group	*F* factor
Y	*Cryptomonas* sp.	Cryptophyceae	3
MP	*Nitzschia* sp.	Bacillariophyceae	4
MP	*Cymatoplcura* sp.	Bacillariophyceae	4
MP	*Stauroneis* sp.	Bacillariophyceae	4
MP	*Cymbella* sp.	Bacillariophyceae	4
MP	*Gomphonem*a sp.	Bacillariophyceae	4
MP	*Navicul*a sp.	Bacillariophyceae	4
L_0	*Peridiniosis nie*i	Dinophyceae	2
L_0	*Peridinium* sp.	Dinophyceae	2
L_0	*Ceratium hirundinella*	Dinophyceae	2
B	*Cyclotella* sp.	Bacillariophyceae	4
B	*Aulacoseira* sp.	Bacillariophyceae	4
C	*Cyclotella meneghiniana*	Bacillariophyceae	3
C	*Asterionella formosa*	Bacillariophyceae	3
D	*Synedra* sp.	Bacillariophyceae	3
X2	*Chroomonas* sp.	Cryptophyceae	3
X2	*Chlamydomonas* sp.	Chlorophyceae	3
J	*Pediastrum* sp.	Chlorophyceae	2
J	*Scenedesmus* sp.	Chlorophyceae	2
P	*Fragilaria* sp.	Bacillariophyceae	4
P	*Aulacoseira granulata*	Bacillariophyceae	4
K	*Aphanocapsa* sp	Cyanophyceae	2
G	*Eudorina* sp.	Chlorophyceae	2
G	*Pandorina* sp.	Chlorophyceae	2
F	*Oocystis* spp.	Chlorophyceae	5
F	*Kirchneriella* sp.	Chlorophyceae	5
H1	*Anabaena flos-aquae*	Cyanophyceae	0
M	*Microcystis* sp.	Cyanophyceae	0
N	*Cosmarium* sp.	Zygnemaphyceae	3
S1	*Pseudanabaena* sp.	Cyanophyceae	0
W1	*Euglena* sp.	Euglenophyceae	2
A	*Rhizosolenia* sp	Bacillariophyceae	5
L_1	*Geitlerinema* sp	Cyanophyceae	3
L_M	*Ceratium* sp	Dinophyceae	2
S2	*Spirulina* sp	Cyanophyceae	0
T	*Tabellaria* sp	Bacillariophyceae	2
W2	Trachelomonas	Euglenophyceae	4
W_S	*Strombomonas* sp	Euglenophyceae	3
X1	*Chlorella* sp	Zygnemaphyceae	2
X_{Ph}	*Phacotus* sp	Zygnemaphyceae	4

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Table 4. *Quarterly average values of phytoplankton functional groups assemblage index and the ecological status at each sampling station of the Dongta spawning grounds*

	March		June		September		December		Annual	
	Q**	E-S***	Q	E-S	Q	E-S	Q	E-S	Q	E-S
BL*	3.03	Good	3.08	Good	2.99	Medium	2.87	Medium	2.99	Medium
GP	3.10	Good	3.06	Good	2.80	Medium	2.65	Medium	2.91	Medium
YJ	3.07	Good	2.85	Medium	2.82	Medium	2.58	Medium	2.83	Medium
DT	3.02	Good	3.11	Good	2.83	Medium	2.72	Medium	2.92	Medium
SZ	3.02	Good	3.17	Good	2.64	Medium	2.77	Medium	2.90	Medium

*BL, GP, YJ, DT, SZ standing for Bailan, Guiping, Yujiang, Dongta and Shizui, respectively
** Q, phytoplankton functional groups assemblage index (Q index); *** E-S, ecological status

Correlation analysis of environmental factors

Results of correlation analysis of environmental factors showed that TP and PO_4-P were significantly correlated with Sal, TDS, ORP, Cond, DO, V, pH, SD environmental factors ($P < 0.05$). In addition, WT, SD, TP and NH_3 were significantly correlated with water velocity (*Table 5*).

Relationship between phytoplankton functional groups and environmental factors

Redundancy analysis (RDA) were done between environmental factors and the dominant PFGs to find the main influence factors, and the results were shown in *Figure 7*. In the biplot diagrams for redundancy analysis, the interpretation degree of the measured environmental indicators for the first two axes of PFGs reached 50.72%. The results of showed that: factors including DO, Chla, V, PO_4-P, ORP, TP, Cond, TDS and Sal were significantly correlated with the dominant PFGs (Monte Carlo test, $P < 0.05$; *Fig. 7A*); V was reversely related with most of the dominant PFGs, meanwhile, it had a reverse relationship with most environmental factors except Chla and DO in the biplot diagrams; V had a significant positive correlation with codon MP ($P < 0.05$), and was significant negatively correlated with codon J ($P < 0.01$, *Fig. 7B*).

Relationship between phytoplankton Q index and water velocity

Correlation analysis results showed that phytoplankton *Q* index was positively correlated with water velocity, with a correlation coefficient of 0.263 ($P < 0.01$). Further, linear fitting between *Q* index and V was done which showed a good correlation (*Fig. 8*).

Discussion

Distribution characteristics of PFGs in the Dongta spawning grounds

PFGs corresponded to the characteristics of water habitats. The dynamic sequence of phytoplankton was mainly the result of the interaction of environmental factors such as thermal stability, hydrodynamic characteristics, nutrient status, physiological adaptation characteristics, light conditions, zooplankton and fish grazing pressure, and hydrological dynamics (Reynolds, 2006; Abonyi et al., 2012). These environmental factors induced the phytoplankton appear or disappear in a particular habitat selection

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mechanism, and also impacted on PFGs. Generally, the more PFGs in the water, the higher diversity of habitats in this water environment (Padisák et al., 2006; Wang et al., 2011a; Santana et al., 2017; Zuo et al., 2019;). With the change of water habitat conditions, the composition of PFGs had obvious characteristics of temporal and spatial succession (Zhu et al., 2013; Wang et al., 2020b). In the present study, after three years of sampling investigation and microscopic analysis in different quarters, the planktonic algae of the Dongta spawning grounds were classified to 26 PFGs, including A, B, C, D, F, G, H1, J, K, L1, LM, L_0, M, MP, N, P, S1, S2, T, W1, W2, W_S, X1, X2, X_{Ph}, Y, and the dominant PFGs were C, D, J, K, L_0, MP, N, P, S1, S2, T, W1, W2, X2, Y. Compared with other global water bodies (Zhu et al., 2013; Santana et al., 2017; Zuo et al., 2019), the waters of the Dongta spawning grounds had more numerous of PFGs and dominant functional groups, which showed that the water quality in this area was relatively good. In addition, the current of this spawning grounds was fast flowing with many reefs in river channel, which formed a continuous turbulent environment, and become a good spawning ground for cyprinid fish, principally the four Chinese farmed carps, i.e. black carp, grass carp, silver carp and bighead carp, green grass silver carp and bighead carp (Tan et al., 2011a).

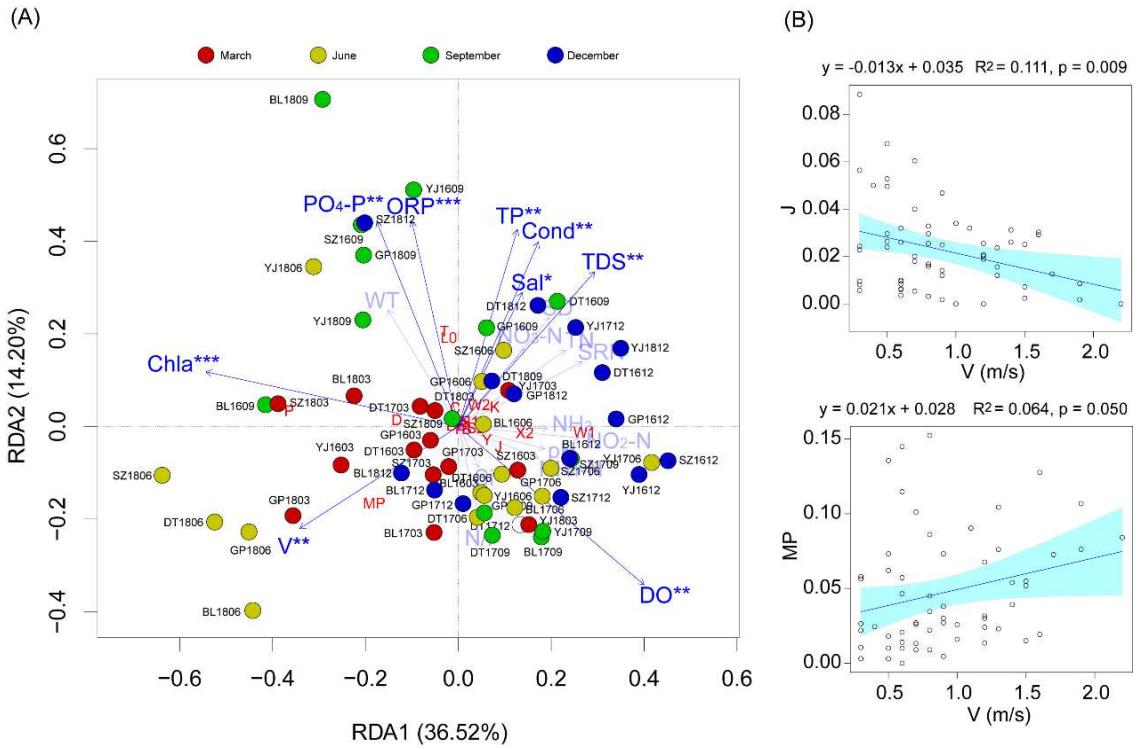

Figure 7. *Relationship between dominant phytoplankton functional groups and environmental factors at each sampling point of Dongta spawning Ground. (A) Biplot diagrams for redundancy analysis between environmental factors and the dominant PFGs; (B) fitting relationship between water velocity and dominant phytoplankton functional group J and MP. WT, water temperature; DO, dissolved oxygen; Cond, conductivity; TDS, total dissolvable solid; SD, water transparency; ORP, oxidation-reduction potential; TP, total phosphorus; PO_4-P, phosphate; TN, total nitrogen; Si, Silicate, SiO_3-Si; Chla, chlorophyll-a. NH_4-N, ammonium nitrogen, NO_3-N, nitrogen nitrate; NO_2-N, nitrite nitrogen; V, water velocity; Sal, salinity; NH_3, un-ionized ammonia; SIN, soluble inorganic nitrogen, SIN; N/P, ratio of nitrogen to phosphorus. * P < 0.05; **, P < 0.01; *** P < 0.001*

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Table 5. *Correlation between environmental factors of the Dongta Spawning founds*

Factors***	WT	Sal	TDS	ORP	DO	Cond	SD	PO_4-P	TP	TN	NO_3-N	NO_2-N	NH_4-N	NH_3	Si	SIN	N/P	Chla	V
pH	-0.103	0.299*	0.394**	-0.166	0.409**	0.120	0.049	-0.337**	-0.179	0.175	0.202	-0.360**	-0.025	0.352**	0.240	0.158	0.238	-0.221	-0.157
WT		-0.022	0.062	0.095	-0.437**	0.360**	-0.464**	0.242	0.079	-0.234	-0.402**	-0.108	0.394**	0.495**	0.256*	-0.241	-0.186	-0.073	0.371**
Sal			0.681**	0.174	0.086	0.775**	0.208	0.346**	0.475**	0.304*	0.306*	-0.009	-0.044	0.009	0.300*	0.296*	-0.316*	-0.116	-0.208
TDS				0.202	0.181	0.618**	0.066	0.273*	0.407**	0.217	0.182	0.048	-0.030	0.151	0.150	0.188	-0.224	-0.224	-0.202
ORP					-0.415**	0.179	-0.065	0.677**	0.551**	-0.246	-0.251	-0.098	-0.100	-0.124	-0.134	-0.321*	-0.228	-0.113	-0.145
DO						-0.058	0.184	-0.598**	-0.398**	0.087	0.181	-0.068	0.024	0.151	-0.078	0.192	0.292*	-0.322*	-0.171
Cond							0.069	0.350**	0.396**	0.175	0.023	0.015	0.309*	0.236	0.334**	0.168	-0.318*	-0.089	-0.044
SD								-0.003	0.158	-0.044	0.198	0.138	-0.376**	-0.378**	-0.418**	0.048	0.011	0.140	-0.499**
PO_4-P									0.833**	-0.052	-0.049	0.182	-0.054	-0.134	0.038	-0.052	-0.537**	-0.006	-0.097
TP										0.105	0.136	0.291*	-0.226	-0.248	0.072	0.077	-0.704**	-0.210	-0.286*
TN											0.782**	0.202	0.047	0.033	0.347**	0.857**	0.054	0.015	-0.100
NO_3-N												-0.004	-0.281*	-0.198	0.140	0.901**	-0.004	0.107	-0.155
NO_2-N													-0.198	-0.244	-0.057	0.050	-0.240	-0.016	-0.087
NH_4-N														0.755**	0.418**	0.140	0.166	-0.245	0.205
NH_3															0.365**	0.124	0.195	-0.267*	0.310*
Si																0.330*	-0.131	-0.085	0.214
SIN																	0.040	-0.008	-0.072
N/P																		0.093	0.023
Chla																			0.173

*Significantly correlated at 0.05 level. **Significantly correlated at 0.01 level

***V, water velocity; WT, water temperature; Sal, salinity; TDS, total dissolvable solid; ORP, oxidation-reduction potential; DO, dissolved oxygen; Cond, conductivity; SD, water transparency; TP, total phosphorus; PO_4-P, phosphate; TN, total nitrogen; NO_3-N, nitrogen nitrate; NO_2-N, nitrite nitrogen; NH_3, un-ionized ammonia; Si, Silicate, SiO_3-Si; NH_4-N, ammonium nitrogen, SIN, soluble inorganic nitrogen, SIN; N/P, ratio of nitrogen to phosphorus; Chla, chlorophyll-a

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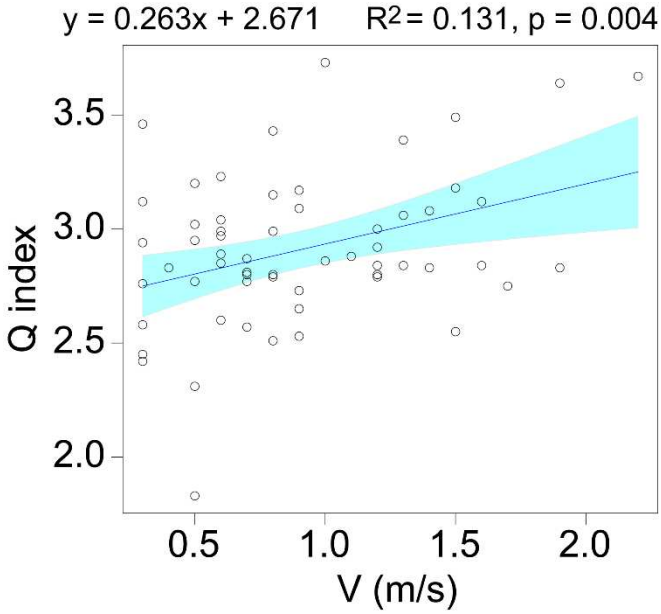

Figure 8. *Relationship between phytoplankton functional groups assemblage index (Q index) and water velocity in the Dongta spawning grounds. V, water velocity*

In the Pearl River Basin, May to September was the main rainy season, with rainfall accounting for 70% of the annual rainfall (Li et al., 2021). The main dry season was from December to March of the following year. During dry period, rainfall was scarce and the river bed was exposed in part of the river. In this study, there were 11 dominant PFGs in the Dongta spawning grounds' waters in March and December, and 7 and 8 dominant PFGs in June and September, respectively. It shows that the number of dominant PFGs in the dry season was obviously more than that in the wet season. The reason may mainly due to the increasing rainfall, turbid water, and increased river runoff in the rainy season, which was not conducive to the growth and reproduction of most algae, resulting in fewer functional groups during this period. Similarly, a study by Salmaso and Zignin (2010) found a negative correlation between phytoplankton biomass and runoff. Six dominant PFGs existed throughout the year in the Dongta spawning grounds, which were D, MP, P, W1, W2 and Y coda. Among them, codon D is tolerant to scouring action, and the representative algae include *Stylus acuminata*, *Nitzschia*, etc. Codon P is tolerant to moderately low light, and its main habitat is continuous or semi-continuous mixed water layer. The representative algae mainly include *Fragillaria*, etc. Codon MP is tolerant to mixed agitation, the main habitat is frequent agitation, turbid water; Codon Y is also tolerant to low light; and coda W1 and W2 represent the habitat conditions of "water bodies that obtain organic matter from farmland or sewage" and "medium or eutrophic water bodies", respectively. From the perspective of the composition of functional groups, these PFGs that exist throughout the year constitute the basic PFGs of the waters of Dongta spawning grounds, and also represented the habitat conditions and pollution factors (Reynolds, 2002; Padisák et al., 2009). Geographically, the Dongta spawning grounds undertake runoff from a large area of the upper reaches of the Pearl River, including the main rivers such as Nanpan River, Beipan River, Hongshui River and Liujiang River. Because the river bank from Wuxuan to Bailan is the high mountain and gorge area, the river width downstream of

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Bailan increases greatly, and a large amount of rainwater runoff begin to burst, forming a faster water velocity and violent scouring force, which led to higher water turbidity and weaker underwater light. Therefore, D, MP, P, Y groups suitable for this habitat (Padisák et al., 2009), became the dominant PFGs. As the geographical conditions from Bailan to Shizui is flat, which is adjacent to the Guiping County urban area and several towns. The concentrated residential, developed field farming, established multiple factories and enterprises, produce domestic sewage, agricultural fertilizers, and factory sewage, which would also be carried into the river by runoff, causing the concentration of nutrients and organic matter in the water body to increase (Abonyi et al., 2012; Guo et al., 2016). Thus, the dominance coda (W1 and W2) adapted to this environment increasing gradually.

Temporally, there was no significant difference between PFGs in March and December, with the difference rate of 18.18%. Similarly, there was no significant difference between PFGs in wet season, with the difference rate of 25%, indicating that the ecological environment in this region was relatively stable in dry season or wet season. Codon C was the unique functional group of March's PFGs, the algae in this group mainly include *Asterionella formosa* and *Cyclotella meneghiniana*, etc. The habitat condition indicated by these coda was medium or small nutrient-rich waters beginning to stratify (Padisák et al., 2009). It was corresponded to the river's state. Since March was the deep dry periods of the Pearl River basin, as the decreasing of rainfall and runoff, and the downstream impoundment of Changzhou Hydraulic Complex (Tan et al., 2011a, b), the Dongta spawning grounds water velocity reduced, most river area in static or half static state, water may form a weak layer in this period. In June, codon T was dominant. The environmental characteristics indicated by T group were continuous mixing layer and light as the limiting factor (Padisák et al., 2009). It was consistent with the characteristics of water body in this period, either. June was the most important rainy season in the Pearl River Basin, and the inflow of heavy rainfall and surface runoff introduced the water velocity and sediment content in this period reach the peak of the whole year (Wang et al., 2017, 2020a), thus forming the complete mixing of water body and weak underwater light. In September, codon S2 was the unique functional group, and one of the important habitat characteristics indicated by S2 was "water body warmth" (Padisák et al., 2009). The actual monitoring also showed that the water body temperature was the highest in September. It was consistent with the regional environment and climate characteristics of this period. In September, the rainfall in the Pearl River Basin gradually decreased, and the sediment content decreased, but this period was still a high temperature period in southern China. Besides, the sunny and warm weather was dominant in this period, so the water body had good light transmission, sufficient light and warm water, leading the adaptive PFGs proliferating.

Comparing with the 5 sampling positions, the number of dominant PFGs in BL station were the least. According to the above information, the BL sample site was located at the junction of canyon and open river section, where the water velocity was higher than other areas all the year round, and the PFGs suitable for this environment were limited. The DT station had the most PFGs, which may be caused by: the sampling site was located in the core area of the Dongta spawning grounds, which area had the widest river width, with the rock forest of gully region (with high water velocity) and the broad waters of aquatic plants growing in the region (with low water velocity). The multifarious habitat provided the important place of producing sticky egg and fish

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breeding, and the varied topography also provided different algae with their appropriate habitat conditions (Abonyi et al., 2012; Nagy-László et al., 2020). For example, apart from the coda MP, P and W1, algae suitable for slow water velocity, such as *Chlorella meniensis* (codon C), *Chlorella convolvula* and *Chlorella sclerotii* (codon G), were also noticeably appeared in this area. Therefore, the number of PFGs was higher than other regions. This study also showed that the YJ station contained some PFGs, such as coda N and T, the main representative species being *Euastrum* spp and *Tribonema*. spp, which were significantly different from other sites. Unlike the other stations, YJ sampling site located in the river mouth of Yujiang River tributaries into the Pearl River. The Yujiang River flowing through the large or medium cities, such as Nanning city and Guigang city of Guangxi Zhuang Autonomous Region, China. Densely populated residents, many ports and factories along the river banks, which possesses many nutrients sources into water, but there was no water exchange with the Pearl River main stream. Therefore, the YJ area had an independent water environment. *Cosmarium* belonging to codon N and *Tribonema* belonging to codon T were large algae, which were more suitable for living in static water environment (Padisák et al., 2009). Since the runoff of Yujiang River was lower than the main stream of Pearl River, and the estuary area was relatively wide, so the water velocity was low, which provides conditions for the growth and reproduction of these groups.

The relationships between phytoplankton functional groups and environmental factors in the Dongta spawning grounds

The RDA analysis results showed that environmental factors such as DO, Chla, V, PO_4-P, ORP, TP, Cond, TDS and Sal were significantly correlated with the dominant PFGs. Both Rodrigues et al. (2018) and Qu et al. (2019) pointed out that phytoplankton communities were more impacted by the hydrological index than other factors. In our RDA ordination diagram, V was reversely correlated with many environmental factors other than Chla and DO, as well as the dominant PFGs other than MP, D and P. Large number of hydroecological studies had shown that the water velocity was an important impact factor to change the biological and non-biological factors of the water environment (Zeng et al., 2006; Devercelli, 2013). The speed of water flow affects the input and output of nutrients in rivers, such as sediment content, transparency, suspended particulate matter and dissolved oxygen content. In our study, V was significantly positively correlated with water temperature, and significantly negatively correlated with SD and TP, while water temperature, transparency and TP played an important role in the growth of phytoplankton (Reynolds, 2006; Santana et al., 2017). In addition, previous studies showed that phosphorus was the limiting nutrient relative to nitrogen in the middle and upper reaches of the Pearl River (Liu et al., 2019). In other regions of China, such as the Three Gorges Basin of the Yangtze River, the source and flow areas of tributaries were rich in phosphate minerals and phosphorus processing enterprises, resulting in phosphorus heavy load in rivers and becoming major pollution factors. After the impoundment of the Three Gorges Dam, with the slowing down of water velocity (Zeng et al., 2006; Zhu et al., 2013), a large amount of nutrients accumulates in the reservoir bays, and finally algal blooms appeared in several tributaries (Zhou et al., 2011). However, the Pearl River basin was different from the Yangtze River basin in that there were few phosphate ore and phosphorus products processing enterprises in its basin. Due to the low input of nutrients, the accumulation of nutrients was relatively slow after the construction of the Dam. Therefore, the

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changes of algal structure in the water, especially the appearance of algal blooms, may not be as obvious as after the construction of the Three Gorges Dam. But water dams changed the river hydrological dynamics, and the aquatic organisms' structure transformation would be inevitable (Tan et al., 2011; Jung et al., 2014; Steinschneider et al., 2014; El-Karim, 2015; Li et al., 2021). Such as after the completion of the Danjiangkou Reservoir since 1958, its water environment had changed in these 60 years: (1) A tendency to gradually increase the reservoir eutrophication degree (Zhu et al., 2008); (2) The whole reservoir phytoplankton density increased 16 times; (3) Phytoplankton composition changed from Bacillariophyta to Bacillariophyta-Cyanophyta-Chlorophyta type, and then was gradually replaced by Bacillariophyta-Pyrrophyta-Cryptophyta-Cyanophyta (Shen et al., 2011). Similarly, in other rivers, a series of environmental problems had also been reported due to the changes in hydrology and water regime of the upstream and downstream rivers after damming (Zhou et al., 2011; Leslie et al., 2014; Steinschneider et al., 2014; Jung et al., 2014; Deng et al., 2019). Basing on the present study, analysis and lots of relevant literature, we concluded that water velocity was a very important factor influencing phytoplankton functional groups composition and river ecological status. However, the dominant influence of flow velocity should be fatherly studied in the future.

Water ecological status of Dongta spawning grounds based on phytoplankton functional groups assemblage index

PFGs assemblage index (*Q* indexes) reflected the ecological status of water from the perspective of composition and the habitat conditions of phytoplankton species. In this study, the *Q* indexes in Dongta spawning grounds were obtained by investigating the BL, GP, YJ, DT and SZ sampling stations to evaluate the water ecological status. On the whole, the ecological status of the Dongta spawning grounds was fair to middling among the reported study areas, showing a "medium" to "good" ecological status and occasionally "tolerant" status in some areas (Padisák et al., 2006). There was no significant difference in *Q* index among the five sampling stations, meaning exciting smaller habitats in the main stream of the Pearl River.

The sampling sites of BL, GP, DT and SZ were located in the main stream of the Pearl River. The spatial distance between them was small, and the climate, human geography and other factors were very close. Moreover, due to the high water velocity in this region, the whole region had good connectivity between upper and lower levels, and the ecological status changes had no much difference. Previous studies indicated that the greater the spatial distance, the greater the difference in regional ecological environment (Thomaz et al., 2007; Graco-Roza et al., 2020; Wang et al., 2020b). For the YJ station, due to the low water velocity, the proportion of green algae in the algal community structure increased and the *Q* index was slightly lower than other sites, but the geographical proximity did not make it significantly different from other sample sites. The results of this study showed that the ecological conditions of the SZ station had changed in larger ranges, and "tolerable" condition appeared in some periods. The actual investigation found that there were some sand digging and transport ships in this area, and a large number of fishery boats rested and the fishermen lived near the bank. As a result, some mechanical interference, domestic sewage and garbage would be generated. Once it encountered a period of high temperature and low rainoff, the output could not be timely, and the environment would become worse. Temporally, there were significant differences in *Q* index in different periods. March and June were grouped

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together, and their *Q* indexes were significantly higher than other periods; while September and December's *Q* indexes were grouped respectively. The analysis indicated that the main reason was that the water temperature in March was low, biological activities were not active, because February was generally during the Chinese Lunar New Year holiday, human production activities had been drastically reduced, and pollutants discharged accordingly had been reduced (Guo et al., 2016). March was the deep dry period, though water velocity dropped drastically in this period, but after a long period of normal and dry periods, most of the nutrients and pollutants in the water were used or degraded, so the water ecology showed the "good" condition. June was the main rainy period, when the inflow of rainwater runoff allowed the river to be replenished with fresh water and facilitated the output of nutrients. So the ecological state of this period was also in "good" condition. The same results mentioned by Okogwu and Ugwumba (2012) basing on their studies in the Cross River showed that a sudden increase in runoff resulted in the dilution effect on nutrient contents of the river, and water quality improved. In our study, September and December were in the transitional period, and they were also the most prosperous period of people's production and living in this area. As water supply gradually decreased and the influx of pollutants related to people's production increasing, the ecological status would be reduced (Abonyi et al., 2012), which displayed as "medium" conditions in these two periods. As other studies and proposals (Leslie et al., 2014; Steinschneider et al., 2014; Chen and Li, 2015), it was recommended that after the completion and operation of the Datengxia Dam and the upstream cascade dams, attention should be paid to the regulation of the water volume and water flow velocity of the downstream reaches in the way of ecological operation, to ensure the function of the spawning grounds and fishery conservation zones in the Pearl River.

Conclusion

There were 26 PFGs identified in the Dongta spawning grounds, and the dominant PFGs were C, D, J, K, L_0, MP, N, P, S1, S2, T, W1, W2, X2, Y. Phytoplankton *Q* indexes were varied from 1.83 to 3.73, with the mean value 2.91, the corresponding result showed that most of the time, the ecological status of the Dongta spawning grounds was "medium" and "good", but parts occasionally was "tolerable". Spatially, the mean values of *Q* index in Bailan, Guiping, Yujiang, Dongta and Shizui sampling stations were 2.99, 2.91, 2.83, 2.92 and 2.90, respectively, which were in the critical state of "medium" to "good", but without statistically significant difference among the 5 sampling sites. Temporally, the *Q* index average values in March and June were the same for 3.05 showing the "good" ecological status, and they were significantly higher than September and December. Factors including DO, Chla, V, PO_4-P, ORP, TP, Cond, TDS and Sal were significantly correlated with the dominant PFGs; especially, V was reversely related with most of the dominant PFGs and most environmental factors except Chla and DO; besides, V had a significant positive correlation with codon MP, and was significant negatively correlated with codon J. Basing on the present study and analysis, we conclude that water velocity was an very important factor influencing phytoplankton functional groups composition and river ecological status, but the dominant influence of flow velocity should be fatherly studied in the future. Nevertheless, it is also suggested that after the construction and operation of Datengxia Water Dam and its upper reaches cascade dams, attention should be paid to regulating

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the water flow and velocity in the lower reaches of the river by means of ecological operation, so as to ensure the functions of the spawning grounds and protect the fishery conservation zones in mainstream of the Pearl River. This study provided important guidance for drawing up protection measures for the ecological status of the Dongta spawning grounds of its downstream after the completion of Datengxia Water Control Project.

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MICROBIAL SIGNATURES IN THE RHIZOSPHERE AND SURROUNDING BULK SOILS AND DIFFERENTIAL ABUNDANCE DUE TO WATERING FOR SWEET INDIAN MALLOW (*ABUTILON FRUTICOSUM*)

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Abstract. The present study aims to decipher microbiota signatures of rhizosphere soil of the medicinal plant *A. fruticosum* and surrounding bulk soil. The study also investigates differential response of microbes potentially serving to promote drought tolerance in the plant. Microbiomes of rhizosphere and bulk soils were collected after 0, 24 and 48 h of watering, deep sequenced and annotated to the different taxonomic ranks. The results strongly indicated higher relative abundance in rhizosphere soil microbiomes compared to those in the bulk soil of phyla Proteobacteria, Actinobacteria, Bacteroidetes, Firmicutes and Acidobacteria. Of these, growth of Acidobacteria and Firmicutes, in addition to Gemmatimonadetes, Melainabacteria, Elusimicrobia and Armatimonadetes, responded positively to watering across microbiome source. While Cyanobacteria was the only abundant phylum in bulk soil and showed lower abundance in rhizosphere soil due to watering. At the genus level, *Bacillus*, *Microvirga*, *Adhaeribacter*, *Sphingomonas*, *Arthrobacter* and *Pontibacter* are the most abundant in rhizosphere soil, while growth of genera *Ramlibacter*, *Haliangium*, *Gemmatimonas* and unidentified genera of taxon Acidobacteria significantly increased 24 h after watering. Results of the present study warrant comprehensive research to dissect factors influencing differential stress responses and plant-bacterial relationships in order to provide feasible soil management programs in the future.
Keywords: *drought, PGPB, OTU, alpha and beta indices, phylogenetic tree*

Introduction

Sweet Indian mallow (*Abutilon fruticosum*) is a drought tolerant perennial herb that belongs to the family Malvaceae (also known as Mallow family). This family is among the largest families of angiosperm that have high medicinal and economic potentials as many parts of this plant generate biological activities useful in the treatment of several human diseases, like piles, inflammation of the bladder, ulcers and rheumatism (Husain and Baquar, 1974; Patel and Rajput, 2013). Therefore, it is of crucial importance to monitor conservation processes of this important family and investigate ways to promote tolerance to drought stress in their native habitat. As plant species of genus *Abutilon* are not toxic to biological systems, they are eaten by wild and domesticated ungulates and birds. This medicinal plant is native to USA, Mexico, Africa and southwestern Asia including Saudi Arabia (Fryxell, 2002).

Desert plants harbor several metabolic strategies to tolerate drought stress, but very little is known about the possible contribution of the surrounding microflora in promoting abiotic stress tolerance in plants. Recent studies demonstrated that the microbiome in the surrounding root-soil interface, e.g., rhizosphere, plays a role in augmenting the plant's ability to withstand drought by changing microbiome structure (Xu et al., 2018). Rhizosphere is described as the hotspot of plant-microbe interaction

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within their soil environment. This spot is occupied by a diversity of microbial communities that are mutually influenced by intact plant and soil types (Philippot et al., 2013). For example, plant root exudes a variety of chemicals and nutritional compounds into the rhizosphere to attract a variety of microorganisms including bacteria and archaea (Mendes et al., 2013; Geng et al., 2018). Such attracted microbes harbor diverse metabolic potentialities that, in turn, promote plant growth and overall performance especially under abiotic stresses (Dennis et al., 2010; Dai et al., 2019). The latter interaction refers to the symbiotic relationship between plant and its intact microbes.

Some of the recent studies indicated that shifts in structure of microbiomes within the root endosphere and in the rhizosphere are in favor of the Actinobacteria and other Gram-positive taxa (Naylor et al., 2017; Timm et al., 2018; Xu et al., 2018, 2021; Xu and Coleman-Derr, 2019). Interestingly, these shifts are proportional to the strength and duration of drought and can rapidly diminish upon alleviation of the stress (Xu et al., 2018). Recent reports implicate the root external environment (rhizosphere) is involved in a wide range of stress responses in plant (Dai et al., 2019).

The present study aims at the detection of microbiota signature of rhizosphere soil of the medicinal plant *A. fruticosum* and its surrounding bulk soil, and differential response of microbes that can potentially serve in promoting plant growth under drought stress.

Materials and methods

Watering regime and collection of bulk and rhizosphere soils

The experiment was conducted 10 km away from Jeddah, Saudi Arabia in June (2021) in a spot in Southern Jeddah province that received no rainfall for at least three months prior experiment. We have assigned plots (1 m^2 each) of three single-grown plants of *Abutilon fruticosum* perennial herb with similar size, in addition to three neighboring bulk soil plots for the experiment. Distance between selected plots was ~10 m. Microbiomes of the two types of soils were harvested (between 10-30 cm depth) as previously described (Geng et al., 2018; Dai et al., 2019). Rhizosphere and bulk soil samples were collected at dawn after 0 (prior watering), 24 and 48 h of watering (25 l dH_2O/plot). The amount of water is based on the average amount received by rainfall in summer season. Harvested samples were immediately put in liquid nitrogen and transferred to the laboratory for storage until further DNA extraction.

Genomic DNA extraction and 16S rRNA sequencing

Total genomic DNAs of soil microbiome samples were extracted using CTAB/SDS method. DNA concentration and purity was monitored by electrophoresis on 1% agarose gels and concentration was adjusted to 1 ng/μL using sterile double-distilled water. Then, metagenomic DNA samples were shipped to Novogene (HK) Co., Ltd. (Singapore) for deep sequencing and bioinformatics analyses. The 16S rRNA gene at region V3-V4 was amplified using primers 515F/806R and PCR was carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). Amplicons were run on 2% agarose gel to detect quality and quantity and those with 400-450 bp length were chosen for further analysis. Then, amplicons were purified with Qiagen Gel Extraction kit (Qiagen, Germany) and libraries were generated with NEBNext® Ultra™ DNA Library Prep kit for Illumina platform. Deep sequencing generated ~250 bp paired-end (PE) raw reads that were quantified via Qubit and Q-PCR.

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Data processing, OTU analysis and taxonomic annotation

Nomenclature and grouping of raw read datasets were made for microbiome samples of surrounding bulk (S) and rhizosphere (R) soils of *Abutilon fruticosum* that were collected in three replicates at dawn after 0 (S11-S13 [group A] and R11-R13 [group D], respectively), 24 (S21-S23 [group B] and R21-R23 [group E], respectively) and 48 h (S31-S33 [group C] and R31-R33 [group F], respectively) of watering. The grouping type ABCDEF represent interaction between source of microbiome, e.g., rhizosphere and bulk soils, and time after watering, e.g., 0, 24 and 48 h. Raw data was also grouped based on source of microbiome regardless of time after watering, e.g., bulk (group J) and rhizosphere (group K) soils comprising grouping type JK, and on time after watering regardless of source of microbiome, e.g., 0 (group G), 24 (group H) and 48 h (group I) comprising grouping type GHI.

Raw data was then merged using FLASH (V1.2.7 (Magoč and Salzberg, 2011)) and raw tags were subjected to quality filtering to obtain high-quality clean tags (Bokulich et al., 2013) consulting QIIME software V1.7.0 (Caporaso et al., 2010). The tags were compared with the reference database (SILVA database, http://www.arb-silva.de/) in order to detect chimeric sequences for removal using UCHIME algorithm (Edgar et al., 2011). Then, effective tags were analyzed using Uparse software V7.0.1090 (Edgar, 2013), where sequences with $\geq 97\%$ similarity were assigned to the same OTU. Representative sequences of different OTUs were screened for annotation. Then, QIIME software V1.7.0 (Altschul et al., 1990) in Mothur method was performed against SSUrRNA dataset of SILVA database to annotate taxa with threshold of 0.8-1 at taxonomic levels including kingdom, phylum, genus and species and to obtain taxa-based abundance distribution (Quast et al., 2012).

Alpha diversity analysis

Statistical indices of alpha diversity (e.g., Shannon and Simpson) (at p value < 0.05) were generated with QIIME software V1.7.0 and displayed with R software V2.15.3 to detect richness and evenness of microbial communities of individual samples, respectively (Core Team, 2013; Li et al., 2013). Boxplots were formed to analyze significant differences in the two alpha diversity indices between (e.g., grouping type JK) and among (e.g., grouping types ABCDEF and GHI) groups using t-test and Tukey, respectively. Biodiversity rarefaction curves were also created by randomly selecting certain amount of sequencing data from each sample, then counting the number of the represented species (i.e., the number of OTUs). The sequencing data volume was detected for rationality of the overall analysis as steep curve indicates that many species remain to be discovered, while flat curve indicates that data volume is saturated and all species, except scarce species, were detected (Lundberg et al., 2013). Then, species accumulation boxplot or curve (Specaccum) was generated where gradual increase of the curve occurs with the increase of number of sequenced samples until the curve is flattened. This test determines the possibility of collected microbiomes to satisfy all microbes in the soil metagenomes.

Beta diversity analysis

Analysis of similarity (anosim) was performed by R software (Vegan package: anosim function) as a comparative tool of microbial communities at the groups level. Anosim boxplots were generated, where Y-axis represents the distance rank between

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samples, and X-axis represents the results between groups. R value generally stands between 0 and 1, where value close to 0 with $P \leq 0.05$ indicates no significant inter- or intra-group differences, while value close to 1 indicates that inter-group differences are greater than intra-group differences.

Beta diversity indices to assess the differences or distances between or among microbial communities were estimated using weighted and unweighted unifrac matrices of QIIME software V1.7.0. The data matrices have generated heatmaps of weighted and unweighted unifrac beta diversity and also used in measuring unweighted pair-group method with arithmetic means (UPGMA) as a hierarchical clustering method and in measuring principal coordinate analysis (PCoA) that ensures incorporation of ecological distance. UPGMA method is used to construct phylogenetic trees with relative abundance of each sample by phylum (Lozupone and Knight, 2005; Lozupone et al., 2007, 2011). PCoA is displayed by WGCNA package, stat packages and ggplot2 package in R software V2.15.3.

Evolutionary phylogenetic trees of representative sequences for individual samples and their grouping types at genus level (the top 100 genera in abundance) were also constructed using MUSCLE V3.8.31 (Edgar, 2004) and relative abundance of each genus in each tree was displayed. Finally, taxa of each sample or group at the taxonomic ranks phylum, genus and species were selected based on statistical significance at levels of sample (S11-S33 and R11-R33) and different grouping type (e.g., ABCDEF, JK and GHI) in order to detect the taxa with differential abundance and their proportions.

Results

In this study, deep sequencing of 16S rRNA partial-length gene was performed for microflora of rhizosphere soil of the medicinal plant *Abutilon fruticosum* and its surrounding bulk soil in order to explore microbe signatures in the two soil types and microbe's differential abundance due to watering. We have ensured that water is maintained and its amount (25 liters dH_2O/m^2) is enough to keep the soil moist for at least 48 h. This amount was based on the fact that maximum rainfall amount in the selected spot is ~25 cm, therefore, the amount of water to be received by individual plant in 1 m^2 plot equals 25000 cm^3. We speculated that microflora will immediately respond to watering by changing diversity, an approach to adapt to the new environmental condition. Therefore, we thought we will have the chance to detect differential growth patterns and responses of microbes after watering and elevating the stress. We also compared the influence of the plants interacting with their intact microbes with the microbes of surrounding bulk soil in terms of alpha and beta diversities.

Statistics of 16S rRNA sequencing data

Illumina MiSeq was used in analyzing microbiomes of rhizosphere soil of *Abutilon fruticosum* and surrounding bulk soil in three replicates after 0, 24 and 48 h of watering and raw statistical data is shown in *Table 1*. Nomenclature of microbiome grouping types was decided based on the interaction between source of microbiome and time after watering (e.g., grouping type ABCDEF and derivatives), regardless of time after watering (e.g., grouping type JK), and regardless of microbiome source (e.g., grouping type GHI and derivatives). The sequence length per read ranges

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between 409-416 bp with average raw, clean and effective tag numbers of 191474, 187706 and 142646, respectively. Average percentages of Q20 and Q30 with sequencing error rates of < 1 and 0.1% are 97.64 and 92.85%, respectively, while average percentage of effective tags in raw data is 68.58% (*Table 1*). The data in *Figure 1* indicates that average number of OTUs per sample is as high as 2563, while numbers of total, taxon and unique tags are 142646, 133598 and 9046, respectively. As expected, average percentage of archaeal sequences was far lower (0.43%) than that of bacterial sequences (99.57%) (*Fig. A1* in the *Appendix*). The data in *Figure A2* indicates that number of taxa taged per sample at the genus level was, expectedly, much higher than that at the species level. Sequences taged with unidentified species of a given genus refer to new species.

Table 1. *Statistics of raw sequencing data of microbiomes collected from surrounding bulk (S) and rhizosphere (R) soils of Abutilon fruticosum in three replicates after 0 (S11-S13 [group A] and R11-R13 [group D], respectively), 24 (S21-S23 [group B] and R21-R23 [group E], respectively) and 48 h (S31-S33 [group C] and R31-R33 [group F], respectively) of watering. Samples data were also grouped based on source of microbiome regardless of time after watering, e.g., bulk (group J) and rhizosphere (group K) soils and on time after watering regardless of source of microbiome, e.g., 0 (group G), 24 (group H) and 48 h (group I)*

Group			Sample Name	Raw PE (no.)	Raw tags (no.)	Clean tags (no.)	Effective tags (no.)	Base (nt no.)	Avg. len. (nt)	Q20 (%)	Q30 (%)	GC %	Effectivity %
A	J	G	S11	202,329	186,930	183,790	154,094	63,095,793	409	97.74	93.11	55.32	76.16
			S12	206,537	190,785	187,527	148,590	60,878,051	410	97.69	92.92	55.36	71.94
			S13	218,208	201,086	197,323	155,552	63,785,697	410	97.72	93.12	55.65	71.29
B		H	S21	197,737	183,095	179,734	139,042	57,092,281	411	97.69	92.96	55.76	70.32
			S22	209,771	194,101	190,670	154,235	63,478,777	412	97.66	92.86	55.36	73.53
			S23	208,498	192,432	188,865	146,495	60,307,978	412	97.74	93.10	55.4	70.26
C		I	S31	204,937	187,333	183,688	150,196	61,669,902	411	97.62	92.81	55.28	73.29
			S32	185,214	171,658	168,277	125,520	51,521,913	410	97.72	92.91	55.23	67.77
			S33	209,161	192,144	188,426	145,921	59,991,150	411	97.68	92.98	55.41	69.76
D	K	G	R11	208,662	191,660	187,621	144,935	60,221,366	416	97.62	92.83	55.85	69.46
			R12	206,340	189,234	185,407	137,133	56,884,636	415	97.63	92.87	56.20	66.46
			R13	217,927	200,569	196,680	149,840	62,190,891	415	97.63	92.84	56.17	68.76
E		H	R21	216,328	197,344	193,135	145,040	60,218,141	415	97.50	92.51	55.91	67.05
			R22	204,765	187,535	183,319	127,405	52,939,215	416	97.53	92.60	56.21	62.22
			R23	207,956	191,328	187,303	133,975	55,594,046	415	97.58	92.71	56.27	64.42
F		I	R31	204,624	189,285	185,563	131,411	54,479,082	415	97.72	92.92	55.92	64.22
			R32	219,603	199,398	194,673	139,841	57,776,395	413	97.48	92.50	56.67	63.68
			R33	216,950	200,607	196,714	138,410	57,370,972	415	97.65	92.77	56.31	63.80

Raw PE no. represents number of original paired-end (PE) reads after sequencing. Raw tags no. represents number of tags merged from PE reads. Clean tags no. represents number of tags after filtering. Effective tags no. represents number of tags after filtering chimera and can be finally used for subsequent analysis. Base nt no. is the number of bases of the Effective Tags. Avg. len. (nt) represents average length of Effective Tags. Q20 and Q30 are the percentages of bases whose quality value in Effective tags is greater than 20 (sequencing error rate is less than 1%) and 30 (sequencing error rate is less than 0.1%). GC (%) represents GC content in Effective Tags. Effectivity (%) represents the percentage of Effective Tags in Raw PE

Description of the raw sequencing data along with the recovered OTUs at different bacterial taxonomic levels (kingdom, class, order, family, genus and species) is shown in *Table S1*. The total number of OTUs across samples is 4562 of which number of archaeal OTUs is 29. Number of OTUs with average number of sequencing reads over

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1000 is 12. Of which, the largest average number of reads was assigned to OUT1 (~26343), followed by OTU4 (~4825), OTU7 (~3671) and OTU2 (2514). These OTUs refer to unidentified genus of taxon Cyanobacteria, unidentified species of genus *Microvirga*, *Bacillus niacini* and unidentified species of genus *Adhaeribacter*, respectively (*Table S1*).

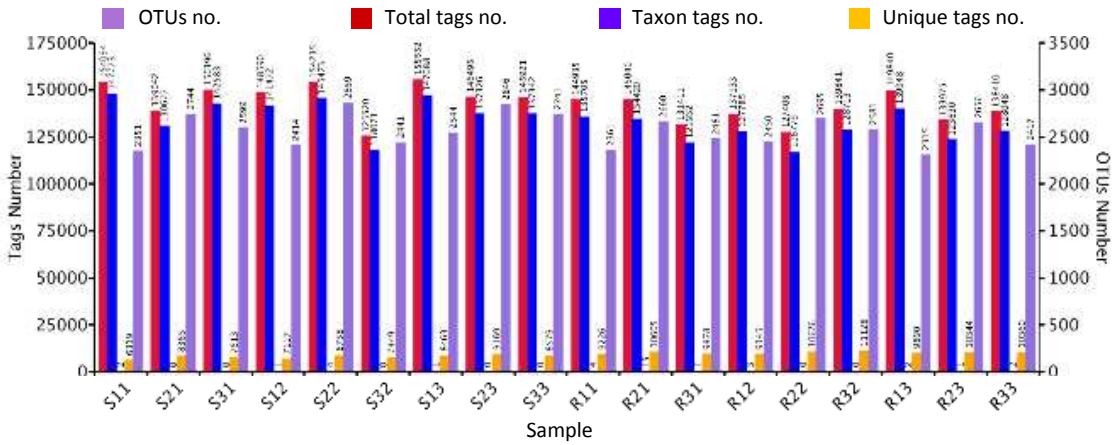

Figure 1. *Description of Tag and OTU information of microbiomes collected from surrounding bulk (S) and rhizosphere (R) soils of Abutilon fruticosum three replicates after 0 (S11-S13 and R11-R13, respectively), 24 (S21-S23 and R21-R23, respectively) and 48 h (S31-S33 and R31-R33, respectively) of watering. OTUs no. (Purple bars) refers to the number of OTUs to identify the numbers of OTUs in different samples. Total tags no. (Red bars) refer to the number of effective tags; Taxon Tags no. (Blue bars) refer to the number of annotated tags; Unique tags no. (Orange bars) refers to the number of tags with a frequency of 1 and only occurs in one sample*

Alpha diversity analysis

The results for the number of observed species (S_{obs}) at the individual level were not influenced by the source of the microbiome or time after watering, while those for Shannon and Simpson indices significantly indicated higher index values for individual bulk soil microbiomes compared with those of rhizosphere soil (*Fig. A3*). Similar results were reached at the grouping type level, where Shannon and Simpson values were significantly higher in grouping type DEF (or group K) than in grouping type ABC (or group J) (*Fig. 2*). This indicates that taxa richness and evenness are significantly higher in the rhizosphere soil than in the bulk soil.

In terms of microbiomes collected at different times after watering across microbiome source (e.g., grouping type GHI), there were no distinctive differences in both Shannon and Simpson indices (*Fig. 2*).

Rarefaction curves indicated that the maximum depth permitted to retain all samples in the dataset is ~120,000 sequence reads (*Fig. A4*). The curves were about to be flattened indicating that microbial data volume is almost saturated. This conclusion was confirmed via the species accumulation curve or Specaccum (*Fig. A5*), where the increase of number of individual sequenced samples resulted in the gradual increase of the curve until it is flattened indicating that number of samples (18) in this experiment is comprehensive.

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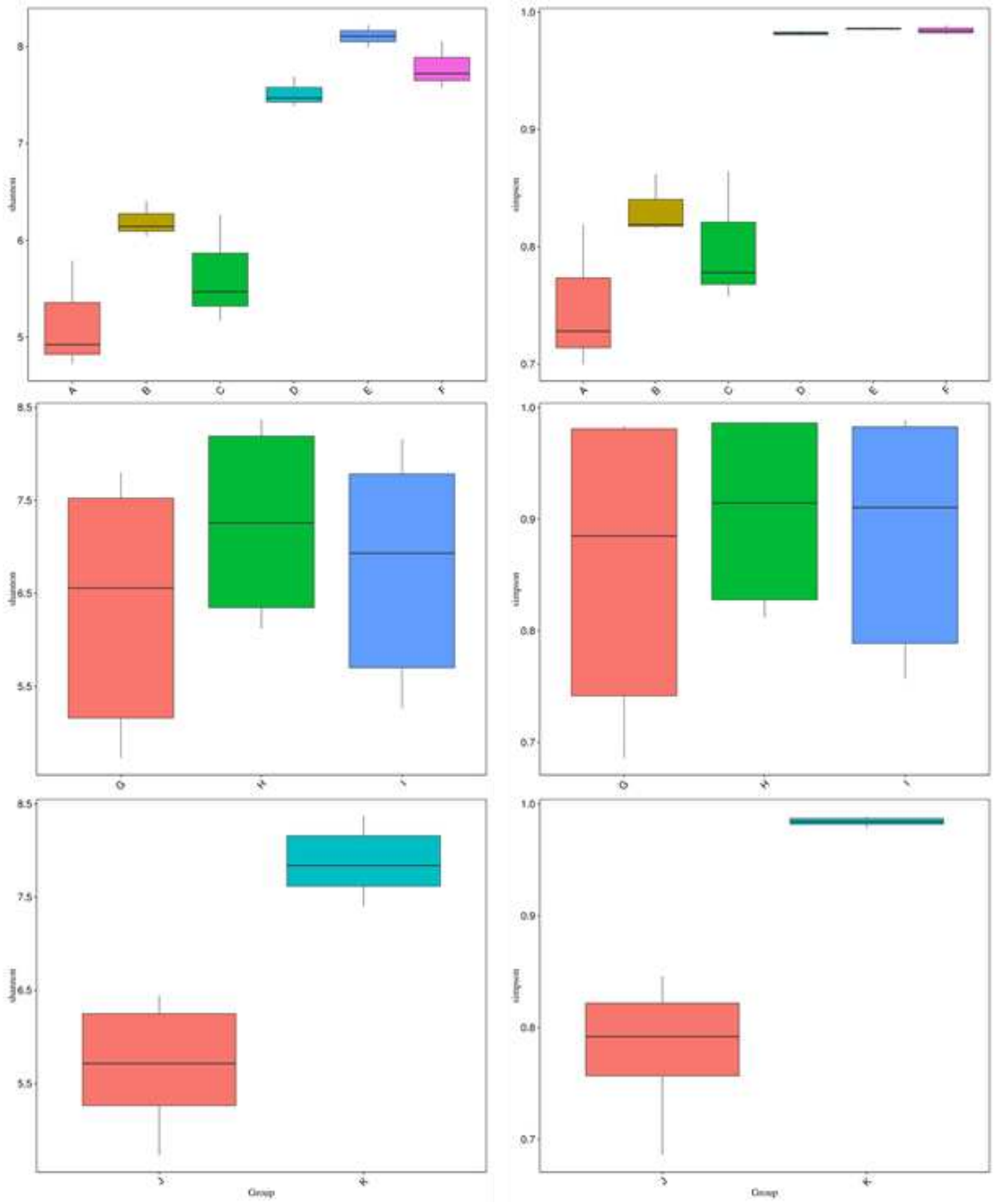

Figure 2. *Boxplots of Shannon and Simpson alpha diversity measures referring to taxa richness and evenness, respectively, of microbiomes collected from surrounding bulk (grouping type ABC) and rhizosphere (grouping type DEF) soils of Abutilon fruticosum after 0 (grouping type AD), 24 (grouping type BE) and 48 h (grouping type CF) of watering. Samples data were also grouped based on source of microbiome regardless of time after watering, e.g., bulk (group J) and rhizosphere (group K) soils and on time after watering regardless of source of microbiome, e.g., 0 (G), 24 (group H) and 48 h (group I)*

Beta diversity analysis

Anosim results at the level of grouping type and derivatives of JK, and grouping type GHI (e.g., GH, GI and HI) are shown in *Figure A6*. Anosim is a nonparametric test

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displayed as boxplots to be used in justifying the reason of dividing groups and evaluating whether variation among groups is significantly higher or lower than variation within groups. The results of anosim boxplots indicate that R values between groups G and H (R = 0.15, P = 0.081), between G and I (R = 0.046, P = 0.519) and between H and I (R = 0.46, P = 0.245) refer to insignificant inter and intra-group differences in global microbiome structure. On the other hand, anosim boxplot between groups J and K showed an R value of 1 (P = 0.001) indicating that inter-group differences are significantly greater than intra-group differences. This indicate that grouping of bulk soil (grouping type ABC or group J) and rhizosphere soil (grouping type CDE or group K) has high rationality (*Fig. A6*), while grouping of microbiomes regardless of microbiome source at the three time points 0 (grouping type AD or group G), 24 (grouping type BE or group H) and 48 h (grouping type CF or group I) after watering has low rationality. Therefore, we expected to detect very few taxa with significant abundance due to watering regardless of microbial soil source.

Heat maps of weighted and unweighted unifrac beta diversity measures at the individual (*Fig. A7*) and group (*Fig. 3*) levels as well as dendrogram trees or hierarchical clustering by phylum (*Fig. A8*) were generated.

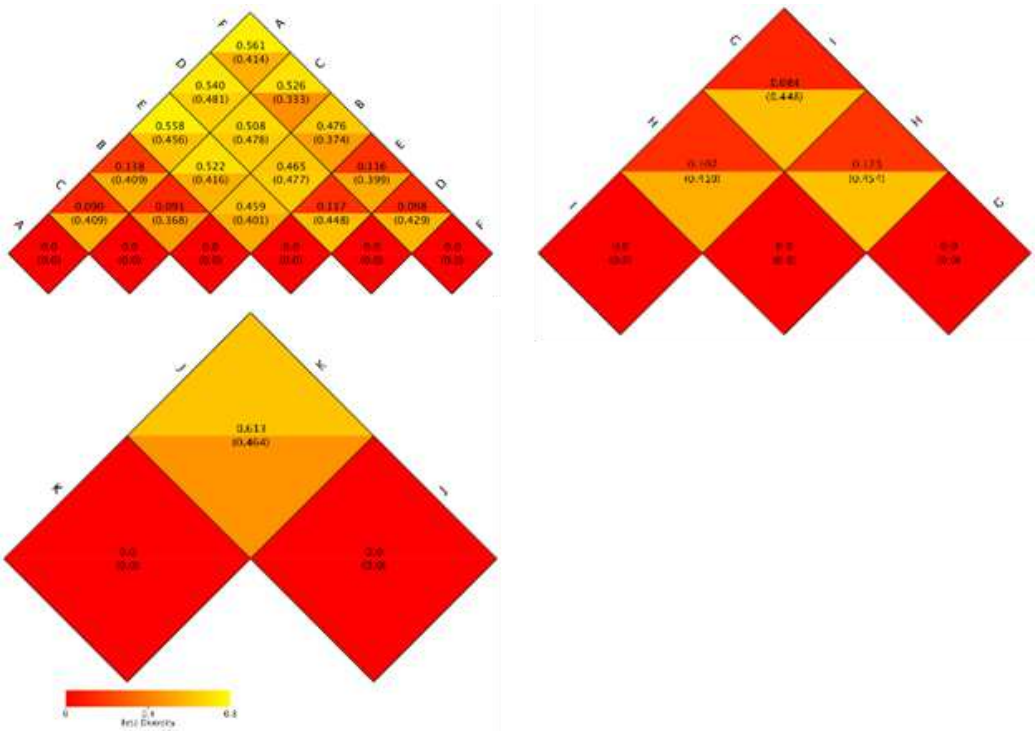

Figure 3. *Heatmaps of weighted (top record) and unweighted unifrac beta diversity measures of microbiomes collected from surrounding bulk (grouping type ABC) and rhizosphere (grouping type DEF) soils of Abutilon fruticosum after 0 (grouping type AD), 24 (grouping type BE) and 48 h (grouping type CF) of watering. Samples data were also grouped based on source of microbiome regardless of time after watering, e.g., bulk (group J) and rhizosphere (group K) soils and on time after watering regardless of source of microbiome, e.g., 0 (G), 24 (group H) and 48 h (group I)*

Weighted uniFrac refers to the relative abundance of sequences, while unweighted uniFrac refers to the unique species in the environmental samples (Lozupone et al.,

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2007, 2011). The results of weighted unifrac heat map and hierarchical cluster at the individual level indicated distinctive separation between microbiomes of bulk soil (S11-S33) and rhizosphere soil (R11-R33) samples. While, unweighted unifrac results indicated no distinctive distances based on the unique microbes in either source of soil microbiome samples (*Fig. A8*). The weighted and unweighted unifrac heatmaps in *Figure 3* for grouping types ABCDEF and JK align with those at the individual level, while those for grouping type GHI indicated no distinctive differences either with weighted or unweighted unifrac matrices. The latter results support our claim of focusing more on the interactive influence of time after watering and microbiome source in addition to the influence of microbiome source regardless of time after watering.

When uniFrac is measured solely, it refers to the phylogenetic information of environmental samples, while when coupled with standard multivariate statistical techniques, it refers to principal coordinates analysis (PCoA) to map distances among microbial communities. When similarity among individual samples or groups is high, then they are closely located. The results at the grouping types ABCDEF and JK in *Figure 4* indicate complete separation between microbiomes of bulk and rhizosphere soils as the latter (grouping type DEF or group K) are located in the positive direction of PCoA 1 (PC1), while bulk soil microbiomes (grouping type ABC or group J) are located in the negative direction of PCoA 1 (PC1). Interestingly, at the PCoA 2 (PC2) level, grouping type BE (or group H) is located in the positive direction, while grouping types AD (or group G) and CF (or group I) are located in the negative direction (*Fig. 4*). The latter results indicated the high tendency of gathering microbiomes collected after 24 h, e.g., grouping type BE (or group H), compared with microbiomes collected after 0 or 48 h of watering, e.g., grouping type AD (or group G) or CF (or group I), respectively. The latter conclusion is further supported by the existence of microbiome samples S11-S13 (or group A) and S31-S33 (or group C), on one hand, and existence of microbiome samples R11-R13 (or group D) and R31-R33 (or group F), on the other hand, in close vicinity. While, microbiome samples S21-S23 (or group B), on one hand, and R21-R23 (or group E), on the other hand, tend to be located far from their respective source (S or R) groups (*Fig. 4*).

Evolutionary phylogenetic trees of representative sequences by genus (the top 100 genera in abundance) for individual samples (*Fig. A9*) and their grouping types, e.g., ABCDEF (*Fig. A10*), JK (*Fig. A11*) and GHI (*Fig. A12*) were constructed. They indicated high abundance of the unidentified genera of *Cyanobacteria* and *Rickettsiales* families in bulk soil microbiomes, while genera *Bacillus*, *Ammoniphilus*, *Adhaeribacter*, *Pontibacter*, *Arthrobacter* and *Microvirga* in rhizosphere soil microbiomes (*Fig. A9-A11*). For grouping type GHI, only genus *Massilia* showed lower abundance in H group of microbiomes collected 24 h after watering compared with the other two groups G and I of microbiomes collected 0 and 48 h after watering, respectively (*Fig. A12*).

Results of relative abundance of individual microbiome samples as well as microbiome groups and grouping types in the bulk and rhizosphere soils of *A. fruticosum* at phylum level is shown in *Figures 5, A13, A18, A21* and *A24*. Abundance (of the phylogenetic trees) and relative abundance at the genus level is shown in *Figures 6, A9-A12, A14, A15, A19, A22* and *A25,* while relative abundance at the species level is shown in *Figures 7, A16, A17, A20, A23 and A26.*

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Relative abundance of individual microbiome samples was detected for selected taxa at the taxonomic ranks phylum, genus and species (*Figs. A13, A14* and *A16,* respectively). Taxa selection was based on statistical significance of relative abundance at the three taxonomic ranks for grouping types ABCDEF (*Figs. A18-A20,* respectively), JK (*Figs. A21-A23,* respectively) and GHI (*Figs. A24-A26,* respectively). Genera with differential abundance across grouping types belong to seven phyla as shown in the heat map of *Figure A15*. It is clear that the largest number of taxa with significantly different relative abundance at the three taxonomic levels was detected for grouping type JK. It is worth noting that species *Bacillus niacini* showed differences within replicates of rhizosphere soil microbiomes after 0 (e.g., samples S11-S13 or group D) and 48 h (e.g., samples S31-S33 or group F) of watering (*Fig. A17*), although this species showed significant high abundance in group K (*Figs. 7, A16* and *A23*).

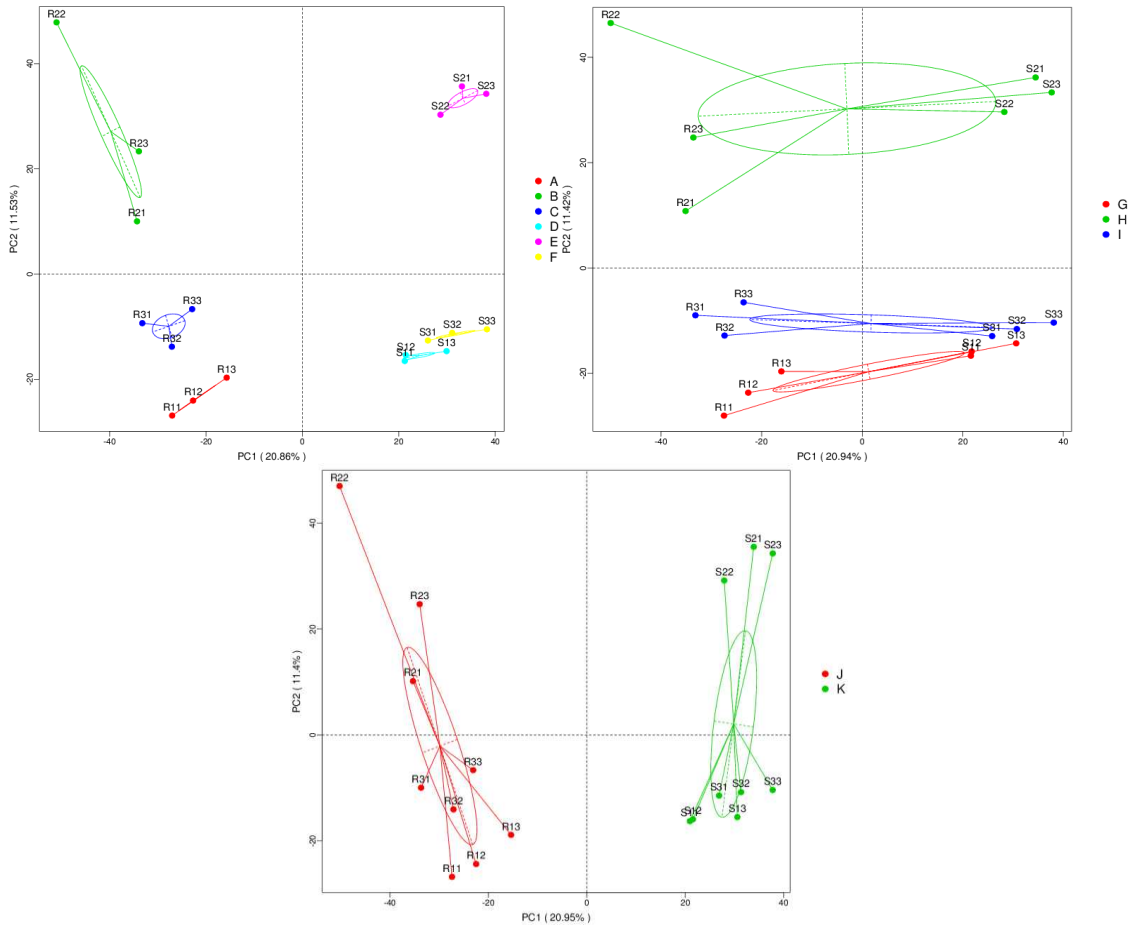

Figure 4. *Principle coordinate analysis (PCoA) based on OTU abundance of microbiomes collected from surrounding bulk (grouping type ABC) and rhizosphere (grouping type DEF) soils of Abutilon fruticosum after 0 (grouping type AD), 24 (grouping type BE) and 48 h (grouping type CF) of watering. Samples data were also grouped based on source of microbiome regardless of time after watering, e.g., bulk (group J) and rhizosphere (group K) soils and on time after watering regardless of source of microbiome, e.g., 0 (group G), 24 (group H) and 48 h (group I). A colored dot represents a given sample in one group, and similar colored dots refer to samples of the same group. X-axis is the first principal coordinate and Y-axis is the second. Number in brackets represents contributions of PCoA to differences among samples*

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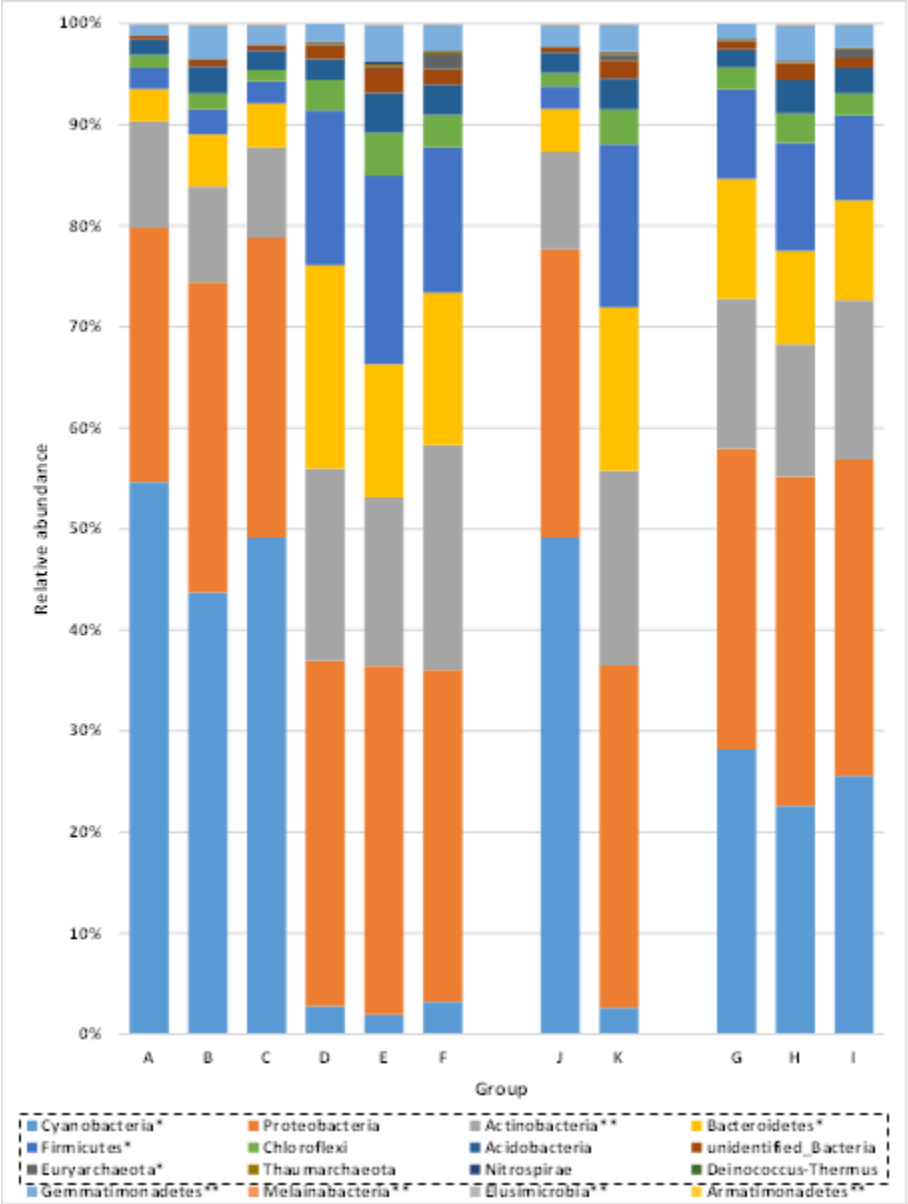

Figure 5. *Relative abundance at the phylum level within different grouping types of taxa that showed significant differences in microbiomes collected from surrounding bulk (grouping type ABC) and rhizosphere (grouping type DEF) soils of Abutilon fruticosum after 0 (grouping type AD), 24 (grouping type BE) and 48 h (grouping type CF) of watering. Samples data were also grouped based on based on source of microbiome regardless of time after watering, e.g., bulk soil (group J) and rhizosphere soil (group K), and time after watering regardless of source of microbiome, e.g., 0 (group G), 24 (group H) and 48 h (group I). Taxa with (*) refer to occurrence of significant differences within grouping type ABCDEF, taxa with (**) refer to occurrence of significant differences within grouping type GHI, while first 12 taxa (inside the dotted black box) refer to occurrence of significant differences between grouping type JK. Statistical analysis is shown in Metastat boxplots of Figures A18-A26*

For grouping type analysis, relative abundance of microbiomes was studied for taxa at the three taxonomic levels, e.g., phylum, genus and species (*Figs. 5-7,* respectively). The results of grouping type ABCDEF significantly indicated higher relative abundance

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in rhizosphere soil microbiomes compared with those in bulk soil of two bacterial phyla, e.g., Bacteroidetes (e.g., groups D vs. A) and Firmicutes (e.g., groups E vs. B) and an archaeal phylum, e.g., Euryarchaeota (e.g., group F vs. grouping type ABCDE) (*Figs. 5* and *A18*). Opposite results were reached for phylum Cyanobacteria that significantly showed higher relative abundance in bulk soil microbiomes compared with their respective ones in rhizosphere soil (e.g., groups B vs. E and groups C vs. F). The results of the above-mentioned four phyla of grouping type ABCDEF almost align with theirs at the grouping type JK (*Figs. 5* and *A21*). Grouping type JK for the other eight phyla (seven bacterial and one archaeal) significantly showed higher relative abundance in rhizosphere soil microbiomes (group K) compared with those in bulk soil (group J). These phyla include Proteobacteria, Actinobacteria, Chloroflexi, Acidobacteria and Deinococcus-Thermus and a new unidentified bacteria as well as the archaeal phylum Thaumarchaeota (*Fig. A21*).

At the genus level, *Ramlibacter* of grouping type ABCDEF showed significantly higher relative abundance in microbiomes of group C than that in group A (*Figs. 6* and *A19*). Other genera include *Planococcus*, *Procabacter* and *Raineyella.* Relative abundance of genus *Planococcus* in rhizosphere soil microbiomes of group E was significantly higher than that in bulk soil microbiomes of group B. Relative abundance of genus *Procabacter* in rhizosphere soil microbiomes of group D was significantly higher than that of group F, while that of genus *Raineyella* showed unexplainable significant differences (*Fig. A19*). The results of grouping type JK indicated that relative abundance in 10, out of 12, phyla was significantly higher in rhizosphere soil microbiomes (group K) than those in bulk soil (group J). These genera are *Bacillus*, *Microvirga*, *Adhaeribacter*, *Pontibacter*, *Nocardioides*, *Arthrobacter*, *Sphingomonas*, *Ammoniphilus*, *Rubellimicrobium* and unidentified bacteria. While, opposite results were reached for unidentified genera of taxa Cyanobacteria and Rickettsiales (*Figs. 6* and *A22*).

At the species level, relative abundance of *Acidobacteria* bacterium WWH111 and *Bdellovibrio* sp. oral clone CA006 was significantly higher in microbiomes of group A than that of group D, while in group D than that group F of bacterium YC-ZSS-LKJ66 (*Figs. 7* and *A20*). In terms of the significant differences in relative abundance in grouping type JK at the species level, eight taxa showed higher significance values in rhizosphere soil microbiomes (group K) compared with those of bulk soil (group J). These taxa are *Bacillus niacini*, *Arthrobacter subterraneus*, *Blastococcus aggregatus*, soil bacterium WF55, *Bacillus selenatarsenatis*, *Sphingomonas wittichii*, *Bacillus funiculus* and *Rhodocista* sp. SCSIO 13435 (*Figs. 7* and *A23*). Opposite results were reached for *Amycolatopsis ruanii*, *Massilia albidiflava*, *Streptomyces mutabilis* and *Aquabacterium citratiphilum*, where relative abundance was significantly higher in bulk soil microbiomes (group J) compared with those in rhizosphere soil (group K). Interestingly, genus *Massilia* showed lower abundance regardless of soil microbiome sources 24 h after watering compared with microbiome sources collected 0 and 48 h after watering (*Fig. A11*).

The results of grouping type GHI indicated significant differences of relative abundance for five phyla, e.g., Acidobacteria, Gemmatimonadetes, Melainabacteria, Elusimicrobia and Armatimonadetes (*Figs. 5* and *A24*). Interestingly, relative abundance of these phyla significantly increased after 24 h of watering regardless of microbiome source (group H), while original abundance was significantly restored 48 h after watering (group I) for the two phyla Elusimicrobia and Gemmatimonadetes. Phylum Elusimicrobia is known as a rare bacterial phylum in the matagenomic data (Wei et al., 2017). Similar results were reached at the genus and species levels for other

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taxa, where relative abundance of the four genera *Ramlibacter*, *Haliangium*, *Gemmatimonas* and unidentified genera of taxon Acidobacteria (*Figs. 6* and *A25*) as well as the two species of *Acidobacteria* bacterium LP6 and SCN69-37 (*Figs. 7* and *A26*) significantly increased after 24 h of watering (group H).

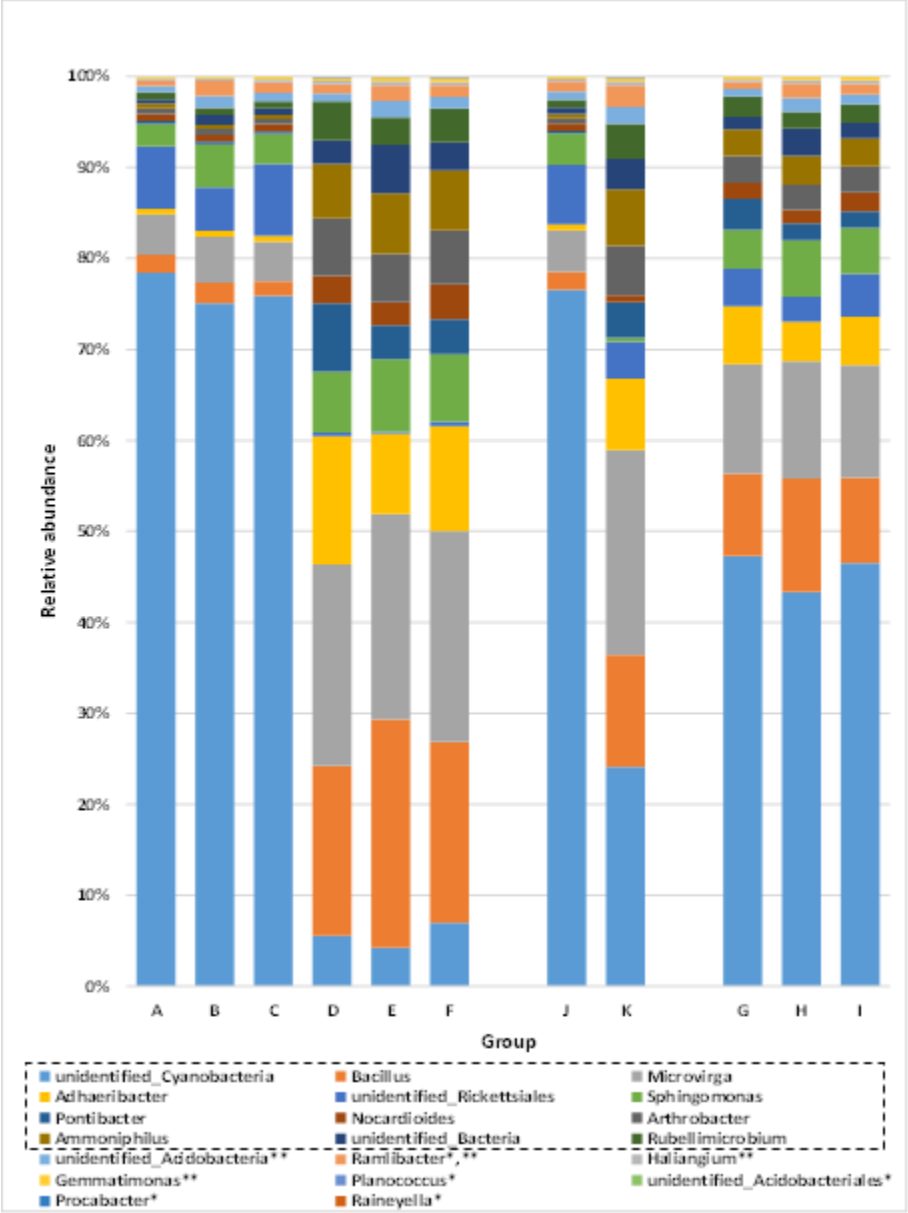

Figure 6. *Relative abundance at the genus level within different grouping types of taxa that showed significant differences in microbiomes collected from surrounding bulk (grouping type ABC) and rhizosphere (grouping type DEF) soils of Abutilon fruticosum after 0 (grouping type AD), 24 (grouping type BE) and 48 h (grouping type CF) of watering. Samples data were also grouped based on based on source of microbiome regardless of time after watering, e.g., bulk soil (group J) and rhizosphere soil (group K), and time after watering regardless of source of microbiome, e.g., 0 (group G), 24 (group H) and 48 h (group I). Taxa with (*) refer to occurrence of significant differences within grouping type ABCDEF, taxa with (**) refer to occurrence of significant differences within grouping type GHI, while first 12 taxa (inside the dotted black box) refer to occurrence of significant differences between grouping type JK. Statistical analysis is shown in Metastat boxplots of Figures A18-A26*

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Discussion

Differential abundance due to microbiome source

(a) Phylum level

Rhizosphere soil is a composite of root surface soil and soil around roots, while bulk soil refers to the area near the selected plants where the closest growing flora is at least 10 m^2 away. In the present study, relative abundance of phylum Cyanobacteria (*Figs 5* and *A21*) or its descendent unidentified genus of family Cyanobacteria (*Fig. 6* and *A22*) is significantly higher in bulk soil compared with that in rhizosphere soil. Cyanobacteria is an above-ground phylum and genera of Gram-negative bacteria representing a major component of the surface soil biota that has internal membranes of flattened sacs, named thylakoids, to obtain energy via photosynthesis (Liesack et al., 2000; Sinha and Häder, 2008; Liberton et al., 2013; Ranjan et al., 2016). Then, this microbe can exist independently in bulk soil as its favorite option, but it can also initiate symbiotic associations in rhizosphere soil with a diverse range of plant kingdom members as the alternative option. The symbiotic association represents a phenomenon similar to legume-rhizobium symbiosis (Nilsson et al., 2005) as microbes of this phylum can act as a cyanobiont receiving its main carbon source from the host, while providing, in return, nitrogen-rich products (citrulline and glutamate) to the host (Lindblad et al., 1991; Bergman et al., 2007; Cuddy et al., 2012). The microbe also has a positive role in plant growth by promoting substances such as IAA and provides organic matter, amino acids, vitamins and oxygen to plant rhizosphere. The microbe can also ameliorate salinity and solubilize phosphates to be available to the plant roots (Prasanna et al., 2009). We speculate that this association will not be mandatory after watering and plant will be able to survive independently until water diminishes again or, otherwise, the plant might associate with other microflora that have alternative mechanisms to promote its growth under normal or drought condition.

Prior phylogenetic studies of Cyanobacteria revealed the natural occurrence of 30 OTUs in the soil. In the present study, a number of 32 OTUs were generated of which OTU1 comprises the most abundant taxon across bulk and rhizosphere soils (*Table S1*). Interestingly, none of the OTUs of this phylum was identified in the present study down the phylum level indicating that new versions of this microbe exist in the rhizosphere soil of *A. fruticosum* and surrounding bulk soil. A scientific evidence indicates that each individual plant root might host a unique taxon of cyanobionts (Costa et al., 1999; Gehringer et al., 2010). This observation might, at least, explain the high variation in the cyanobiont diversity in rhizosphere soil of *A. fruticosum*.

Phyla Bacteroidetes, Firmicutes, Actinobacteria, Acidobacteria, Chloroflexi and Proteobacteria were proven to mutually dominate rhizospheres of several plants possibly as a result of plant's specific root exudates (Lundberg et al., 2012; Dai et al., 2019; Zhang et al., 2019). In the present study, these bacterial phyla were proven to favor rhizosphere soil for growth (*Fig. 5* and *A21*) indicating that root exudates of *A. fruticosum* represents a main factor influencing proper growth of these bacteria. Many reports indicated that microbial abundance in rhizosphere soil can be as high as 10-1000 times that in bulk soil (Miransari, 2011; Zuo et al., 2021). In the present study, microbial abundance in the bulk soil is far less than that of rhizosphere soil of *A. fruticosum*, especially for the most abundant taxa like Cyanobacteria (*Table 1; Figs. 5-7*).

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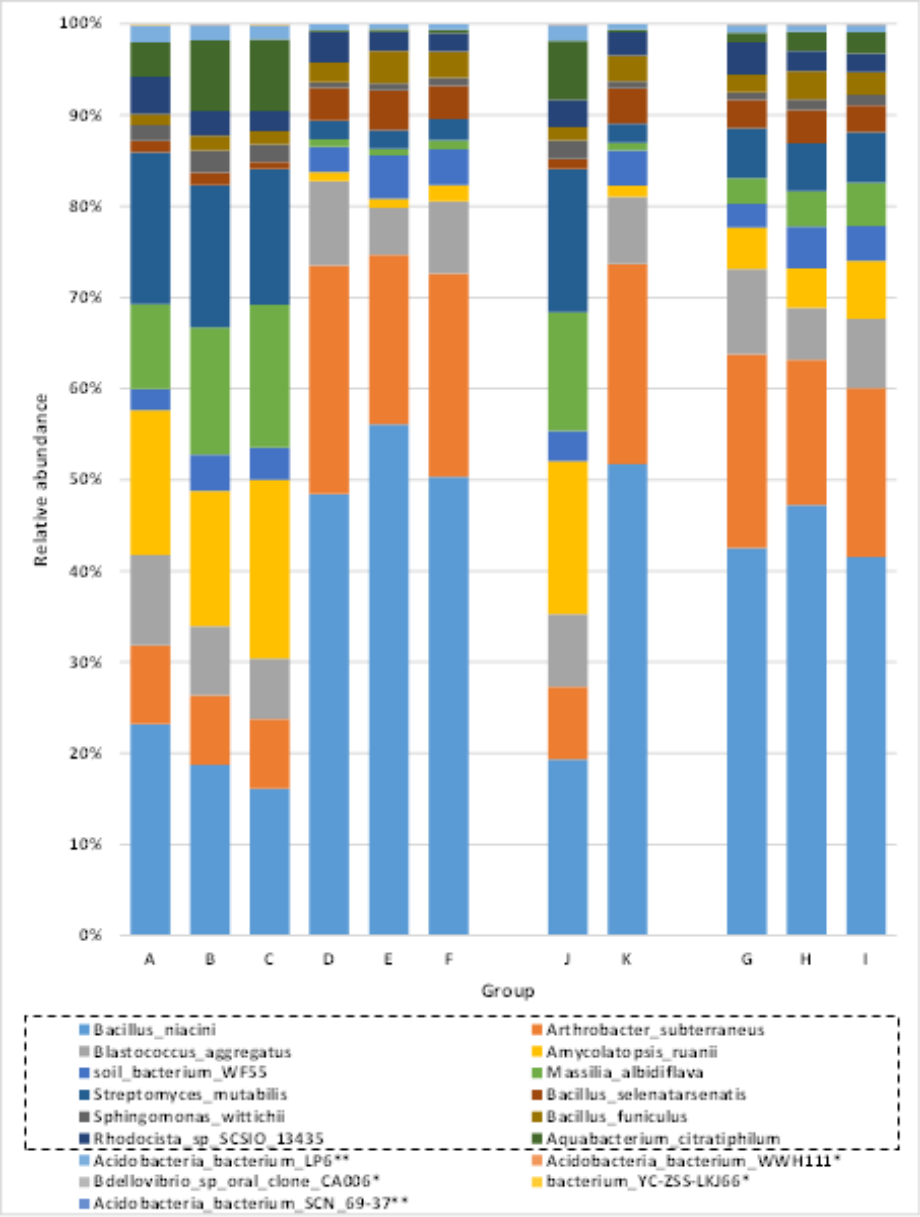

Figure 7. *Relative abundance at the species level within different grouping types of taxa that showed significant differences in microbiomes collected from surrounding bulk (grouping type ABC) and rhizosphere (grouping type DEF) soils of Abutilon fruticosum after 0 (grouping type AD), 24 (grouping type BE) and 48 h (grouping type CF) of watering. Samples data were also grouped based on based on source of microbiome regardless of time after watering, e.g., bulk soil (group J) and rhizosphere soil (group K), and time after watering regardless of source of microbiome, e.g., 0 (group G), 24 (group H) and 48 h (group I). Taxa with (*) refer to occurrence of significant differences within grouping type ABCDEF, taxa with (**) refer to occurrence of significant differences within grouping type GHI, while first 12 taxa (inside the dotted black box) refer to occurrence of significant differences between grouping type JK. Statistical analysis is shown in Metastat boxplots of Figures A18-A26*

Among other factors influencing high abundance of phyla in rhizosphere soil of *A. fruticosum*, Lopes et al. (2021) indicated that range of soil pH implies shaping of soil microbiomes. In terms of the differential abundance of bacteria in rhizosphere and bulk

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soils, it was proven that microbes that favor high soil pH grow better in rhizosphere soil, while those favoring low pH (≤5) grow better in bulk soil (Chodak et al., 2015). As soil pH in the western region of Saudi Arabia was estimated to be > 5, this might explain the high abundance of members of these phyla in rhizosphere soil. In addition, symbiotic association of microbes of these phyla might be a favorite option to the plant under watering condition. Other factors may also refer to the consequence of natural co-existence of the two highly abundant phyla Acidobacteria and Proteobacteria that are responsible for sustainability of the other phyla in the rhizosphere whose abundance after watering (Wei et al., 2017). Thus, these phyla will perform parallel, in terms of growth rate, to those of the phyla Acidobacteria and Proteobacteria. Highly abundant Phyla Firmicutes and Bacteroidetes also co-exist in the soil in order to imply minimal competition for resources through cooperation and/or specialization (Wei et al., 2017), where Firmicutes, for example, favors lipid nutrients in biogas reaction, whereas Bacteroidetes favors starch nutrients (Kampmann et al., 2012).

Phylum Acidobacteria was also reported to have genes for colonization in the rhizosphere to establish symbiotic relationships with plants (Kalam et al., 2020). Other genes encode enzymes for carbon metabolism, like the degradation of complex carbohydrate polymers, metabolic intermediates in the soil (Belova et al., 2018), enzymes for inorganic and organic sources of nitrogen metabolism (Eichorst et al., 2018), sulfur metabolism (Tank and Bryant, 2015) as well as enzymes for acid tolerance to help it survive in highly acidic conditions (Sun et al., 2012). The above-mentioned characteristics of the microbe indicate why Acidobacteria favors growth in rhizosphere soil in the present study (*Fig. 5* and *A21*).

Nonetheless, Lazcano et al. (2021) claimed in strawberry that archaeal phylum Thaumarchaeota, and bacterial phyla Chloroflexi and Firmicutes and Acidobacteria exhibited higher abundance in bulk soil relative to rhizosphere soil, while Proteobacteria, Bacteroidetes and Actinobacteria showed opposite results. Previous reports on several other plants indicated that phylum Acidobacteria showed higher abundance in the bulk soil (Walker et al., 2010), while phyla Actinobacteria (Deiglmayr et al., 2006), Bacteroidetes (Schmidt et al., 2008) and Proteobacteria (Mukhtar et al., 2021) showed higher abundance in rhizosphere soil. Interestingly, Na et al. (2019) indicated that phyla Acidobacteria, Chloroflexi, and Cyanobacteria are enriched in rhizosphere of broomcorn millet at the jointing stage, while decreased as the plants continue growing. We refer these contradicting results to the natural growth dynamics of microbiota responding differently to the surrounding plants and environmental conditions.

(b) Genus and species levels

Descendent genera of phyla Bacteroidetes (e.g., *Adhaeribacter* and *Pontibacter*), Firmicutes (e.g., *Bacillus* and *Ammoniphilus*), Actinobacteria (e.g., *Nocardioides* and *Arthrobacter*), and Proteobacteria (e.g., *Microvirga*, *Sphingomonas* and *Rubellimicrobium*), as well as *Bacillus niacini*, *B. funiculus*, *B. selenatarsenatis*, *Arthrobacter subterraneus*, *Blastococcus aggregatus*, soil bacterium WF55, *Sphingomonas wittichii* and *Rhodocista* sp. SCSIO 13435 showed significant higher microbial abundance in rhizosphere soil compared with that of bulk soil (*Figs. 6, 7, A22* and *A23*). While, opposite results were reached for descendent of phylum Proteobacteria, e.g., unidentified genus of family Rickettsiales, *Acidobacteria* bacterium

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WWH111, *Bdellovibrio* sp. oral clone CA006, *Amycolatopsis ruanii*, *Massilia albidiflava*, *Streptomyces mutabilis* and *Aquabacterium citratiphilum*.

Genus *Adhaeribacter* (Bacteroidetes) was proposed to predominantly exist in rhizosphere of Broomcorn Millet (Na et al., 2019) aligning with the results of the present study (*Table 1; Figs. 6* and *A22*). Genus *Pontibacter* (Bacteroidetes) was reported to possess menaquinone-7 (MK-7) as predominant respiratory quinone that is essential for the production of human vitamin K-2 (Joshi et al., 2012), while this genus was recently reported to contribute to the hormetic responses of soil alkaline phosphatase (a potential endpoint) when low levels of cadmium (stressor) presented in soils (Fan et al., 2018).

Other reports indicated that abundance of genus *Bacillus* (Firmicutes) and members of Actinobacteria in non-cultivated bulk soil is higher than that of members of Proteobacteria, while these taxa showed similar abundance in rhizosphere soil of *Capsicum annum* plant (Marasco et al., 2013). Nonetheless, abundance of genera *Streptomyces* (Actinobacteria) and *Microvirga* (Proteobacteria) was high in *Calotropis procera* rhizosphere, while that of genera *Bacillus* and *Ammoniphilus* (Firmicutes) decreased. Interestingly, the latter three genera act as plant growth promoting bacteria (PGPB) (Wang et al., 2017). In phylum Actinobacteria, genus *Nocardioides* showed higher abundance in maize rhizosphere soil (Piutti et al., 2003), while genus *Arthrobacter* was proven to be an important fraction of the microflora that promotes plant growth especially under drought stress. Members of genus *Arthrobacter* are resistant to desiccation and can survive for a long time under starvation condition (Chukwuneme et al., 2020). In the present study, the genus *Arthrobacter* showed no differential response to watering.

Aligning with the results of the present study, genus *Sphingomonas* (Proteobacteria) is more abundant in rhizosphere and is known to promote growth of *Arabidopsis thaliana*. However, it can survive in bulk soil by taking energy from degrading organic pollutants (Luo et al., 2019). Genus *Sphingomonas* is also known for its ability to tolerate drought stress and promote tolerance in the neighboring roots (Luo et al., 2019). However, this genus showed no differential response to watering in the present study. Information regarding genus *Rubellimicrobium* (Proteobacteria) is rare except that it was shown to be highly enriched in oil-contaminated and non-contaminated rhizosphere soil of barley and alfalfa compared with its level in sandy clean soil (Kumar et al., 2018). Family Rickettsiales was proven to be highly abundant in bulk soil (*Figs. 6* and *A22*), however, there is no information in the literature to support its role in the soil.

At species level, no solid information regarding the roles of most highly abundant bacteria in rhizosphere soil (*Figs. 7* and *A23*), except that *Sphingomonas wittichii* is among bacteria that make bioremediation and has the ability to degrade xenobiotic compounds dibenzofuran and dibenzo-p-dioxin (Coronado et al., 2014), while *Bacillus niacini* and *Streptomyces mutabilis* were reported to be a potential plant growth promoting (PGP) rhizobacteria (Suralta et al., 2018; Bhattacharyya et al., 2020) with the ability, as a potential bio-fertilizers, to fix nitrogen and solubilize phosphorous (Latif et al., 2020).

Contradiction of the results at the genus level can be due to the fact that microflora react differentially with the root system of the different interacting plant or otherwise abundance of members of the same genus in different soil types differs at species level. Wang et al. (2017) indicated that plant genotype and growth stage, plant disease

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incidence, soil nutrient contents, soil enzyme activities and pH are main factors affecting abundance of microbes in different soil types.

Differential abundance due to watering

Generally speaking, Gram-positive (diderm) bacteria are known to be more drought tolerant than Gram-negative (monoderm) bacteria as the first has stronger cell wall and drought avoidance strategies (ex., spore formation) (Potts, 1994). Although drought-induced microbial enrichment refers to the boundaries between monoderm and diderm lineages, Xu et al. (2018) indicated that the discriminating factors can to the structure and properties of cell wall, rather than the presence or absence of outer membrane. Accordingly, the latter authors confirmed that root microbiomes of different sources under normal watering conditions prefer to be colonized by diderm bacteria, rather than by monoderm bacteria.

Watering of drought-stressed plants was speculated to rapidly disturb natural progression of microbiome development, which can be eventually restored after water is diminished. Xu et al. (2018) speculated that a maximum of one week following watering is a maximum time to restore microbiome pattern of drought-treated plant roots. In the present study, two days after watering were enough to recover the natural growth pattern of some microbes at phylum and genus levels and to restore their previous or natural growth pattern under drought condition (*Figs. A24* and *A25*), while it seems that this short period is not enough for the majority of microflora to do both actions, e.g., induction of disturbed growth pattern, and restoration of natural growth pattern of drought-stressed microbes. This supports our claim that growth dynamics of microbiota respond differently to the genotypes of interacting plant and environmental conditions.

The results of the present study for phylum Cyanobacteria showed significant increase of relative abundance in bulk soil groups B and C due to watering compared with those of rhizosphere soil groups E and F, respectively (*Figs. 5* and *A18*). This indicates that members of this phylum responded positively to watering in the present study possibly due to the fact that roots of *A. fruticosum* reduced carbon exudation rate to complement drought stress pressure (Reid and Mexal, 1977). Besides, the microbe itself might have strong alterative mechanisms for independent survival when water becomes available.

Relative abundance of phylum Bacteroidetes increased before watering in rhizosphere soil microbiome group D compared with that of bulk soil microbiome group A (*Figs. 5* and *A18*). This might indicate that watering abolished differential microbial abundance between the two types of soil, although the overall growth across time after watering was in favor to the rhizosphere soil (*Figs. 5* and *A21*). While, relative abundance of phylum Firmicutes and its descendent genus *Planococcus* showed significant increase in microbiome abundance of rhizosphere soil group E after 24 h of watering compared with that of the respective bulk soil group B (*Figs. 5, 6, A18* and *A19*). In addition, relative abundance of descendent unidentified taxon of taxon Acidobacteria (*Figs. 6* and *A25*) and descendent genus of phylum Proteobacteria, e.g., *Haliangium* (*Figs. 6* and *A25*), as well as the two species of genus Acidobacteria, e.g., LP6 and SCN69-37 (*Figs. 7* and *A26*), significantly increased 24 h after watering regardless of microbiome source. This might refer to the instant response of some microbes to watering regardless of soil type. Although not statistically proven, the results for the other descendent genus of phylum Proteobacteria, e.g., *Massilia* indicated

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lower abundance level (*Fig. A11*). Interestingly, the other descendent genera of phylum Proteobacteria, e.g., *Ramlibacter* and *Procabacter*, indicated significant increase in microbiome abundance of groups C and D compared with that in groups A and F, respectively (*Figs. 6* and *A19*). This indicates that *Ramlibacter* benefitted from watering in the rhizosphere soil, while abundance of *Procabacter* was declined due to watering. Regardless of microbiome source, abundance of *Ramlibacter* increased significantly 24 h after watering supporting the latter results in *Figure A19* that this genus benefits from watering. Results of archaeal phylum Euryarchaeota, which has no distinctive differentially abundant descendent genera at any grouping type, indicated that relative abundance was significantly increased in rhizosphere soil 48 h after watering (*Figs. 5* and *A18*). This indicates that this phylum also benefited from watering but responded later than bacterial phylum Firmicutes (*Figs. 5* and *A18*).

Previous studies indicated that drought significantly affects microbial composition in the rhizosphere and mitigates the adverse effect of drought stress on plant growth, which can be used as an emerging strategy for improving drought stress tolerance in plants (Xu et al., 2018). Genus *Planococcus* was reported to exist in rhizosphere and root endosphere of halophytes indicating that this genus is salt tolerant (Mukhtar et al., 2021) and can promote growth of plants when exists in their rhizosphere soil (Rajput et al., 2013). In addition, *Bacillus*-generated biofertilizers are highly tolerant to diverse environmental stresses due to their ability to form spores, which are resistant to desiccation (Barnard et al., 2013). Bacterial responses to drought and rewetting stress are based on genetic makeup of the bacteria, the surrounding environment, e.g., contents of soil N and organic C and heavy metal, and host-microbe interaction (Chodak et al., 2015). Stress tolerant bacteria, in turn, induce physiological response in the plant root vicinity in order to help them alleviate adverse effects of drought stress (Bokhari et al., 2019). In maize seedlings, responses include stimulation of ribosomal synthesis and cell proliferation (Vardharajula et al., 2011). Relative abundance of Actinobacteria under drought was declared to be higher within the rhizosphere than that in the surrounding bulk soil (Naylor et al., 2017) in alignment with the results of the present study. It was reported that Actinomycetes has the ability to survive in unfavorable environments (Passari et al., 2015), thus, it is likely that relative abundance in bulk soil is quite high as well. The phylum is also known to produce bioactive secondary metabolites acting as plant growth promoting (PGP) and nitrogenous compounds in non-legumes and to make P solubilization via production of various organic acid including citric acid, gluconic acid, lactic acid, malic acid, oxalic acid, propionic acid, and succinic acid which aid in promoting plant growth (Sathya et al., 2017). Several other phyla, Bacteroidetes, Cyanobacteria, Firmicutes, and Proteobacteria were also reported to act as plant growth-promoting bacteria (Yadav et al., 2018). Jang et al. (2020) indicated that phyla affected by drought stress and the presence of plants include Armatimonadetes, Firmicutes and Proteobacteria. The authors indicated that relative abundance of phylum Armatimonadetes and Proteobacteria in bulk soil was lower under drought condition than that under watered conditions and vice versa in the rhizosphere soil of rice. Thus, the three phyla Armatimonadetes, Firmicutes and Proteobacteria are considered in several reports to have potential significance in assisting plants to withstand drought stress (Ma et al., 2020). These results are consistent with those of many other reports on various plants (Chodak et al., 2015; Bu et al., 2018; Gumiere et al., 2019). More recently, attention was given to the promising phylum Armatimonadetes as it is proposed to have insurance mechanisms against fungal invasions and drought stress in

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avocado rhizosphere (Bejarano-Bolívar et al., 2021). However, bacterial members of Armatimonadetes are difficult to isolate or purely (Hu et al., 2014). Then, it is highly recommended to consult culturomics approaches in order to explore proper culture conditions and recover pure cultures of members of this phylum for further beneficial use in improving plant tolerance against drought stress.

Drought and rewetting stress significantly alter structure of soil metagenomes (Chodak et al., 2015). The authors indicated that abundance of Gram-positive bacterial phyla, like Firmicutes and Actinobacteria, increases after the stress, whereas Gram-negative bacteria, like Proteobacteria, Bacteroidetes, Chloroflexi, Gammaproteobacteria, decreases. It seems that plant growth stage affects relative abundance of rhizosphere microbes under drought stress where abundance of Actinobacteria and Acidobacteria significantly increased at early stages in peanut, while that of Cyanobacteria and Gemmatimonadetes increased at flowering stage (Dai et al., 2019).

Finally, isolation of selected plant growth-promoting bacteria (PGPB) in this study using culturomics approach is highly recommended for soil management program to improve the tolerance of cultivated plant crops to drought stress.

Conclusion

In conclusion, the present study was able to detect the differential microbiota signatures in the rhizosphere of the wild plant *Abutilon fruticosum* and surrounding bulk soil. After watering plots of drought-stressed plants, some soil microbes responded positively and two days were enough for these microbes to restore their natural growth pattern. Such dynamics of microbial growth patterns act in restoring disturbed abundances due to the changes in the environmental condition. We also tried to raise the possible roles of Gram staining properties under drought stress and after watering, but it seems that this factor cannot be considered universally in justifying differential enrichment where phylum Proteobacteria, for example, is Gram-negative, yet members of this phylum (e.g., genera *Ramlibacter* and *Procabacter*) responded differently to watering. Therefore, the present study warrants comprehensive research to dissect other factors that can influence differential stress responses as well as tolerance of soil microbiota, and to shape accurate plant-bacterial relationships in order to help making more proper soil management decisions.

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APPENDIX

This manuscript has an electronic appendix with taxonomic data (OTUs).

Figure A1. *Abundance at the kingdom level (bacteria and archaea) of microbiomes collected from surrounding bulk (S) and rhizosphere (R) soils of Abutilon fruticosum in three replicates after 0 (S11-S13 and R11-R13, respectively), 24 (S21-S23 and R21-R23, respectively) and 48 h (S31-S33 and R31-R33, respectively) of watering. Samples data were also grouped based on source of microbiome regardless of time after watering, e.g., bulk soil (group J) and rhizosphere soil (group K)*

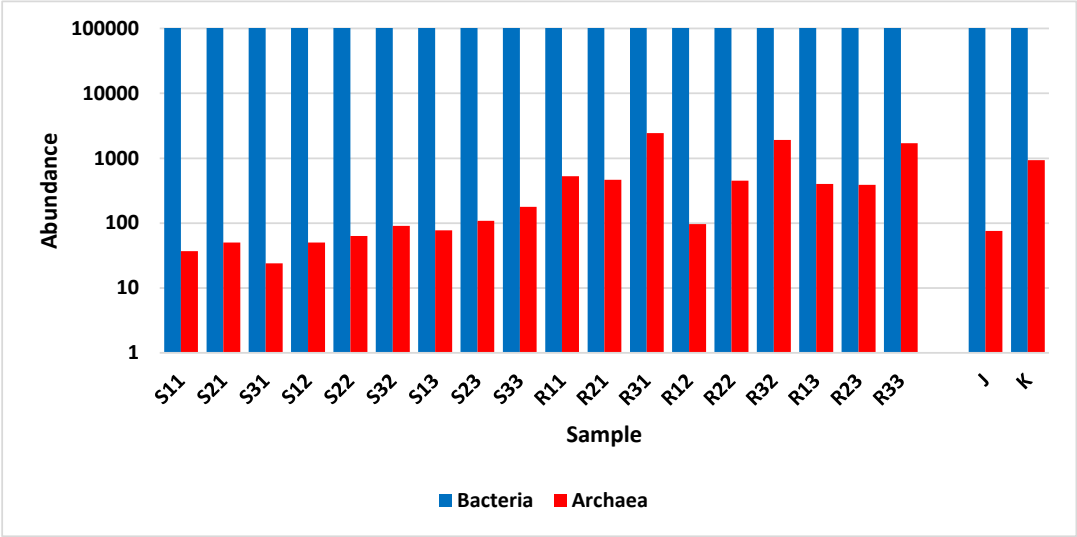

Figure A2. *Number of sequences of taxa taged at different taxonomic levels (kingdom, phylum, class, order, family, genus and species) of microbiomes collected from surrounding bulk (S) and rhizosphere (R) soils of Abutilon fruticosum in three replicates after 0 (S11-S13 and R11-R13, respectively), 24 (S21-S23 and R21-R23, respectively) and 48 h (S31-S33 and R31-R33, respectively) of watering*

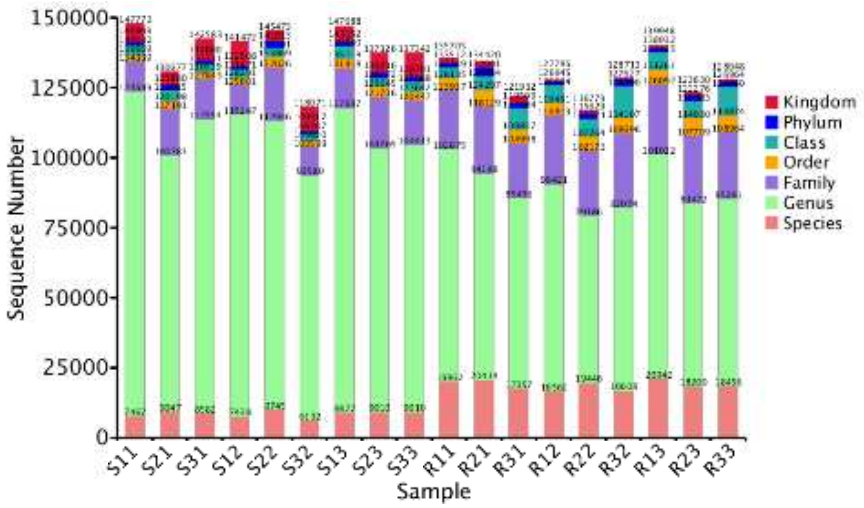

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Figure A3. *Records of observed species (S_{obs}), Shannon and Simpson alpha diversity indices of microbiomes collected from surrounding bulk (S) and rhizosphere (R) soils of Abutilon fruticosum in three replicates after (S11-S13 and R11-R13, respectively), 24 (S21-S23 and R21-R23, respectively) and 48 h (S31-S33 and R31-R33, respectively) of watering*

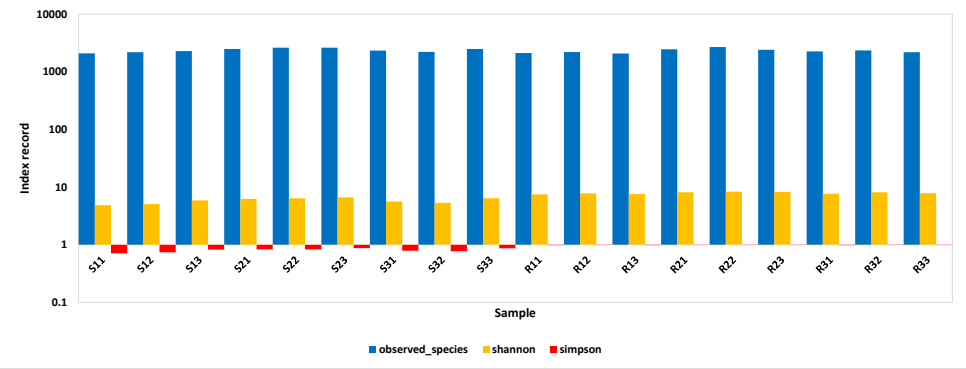

Figure A4. *Number of observed species as a rarefaction measure to describe the maximum depth permitted to retain all samples in the dataset (~120,000 sequence reads) for studying taxonomic relative abundance of microbial community as a beta diversity measure*

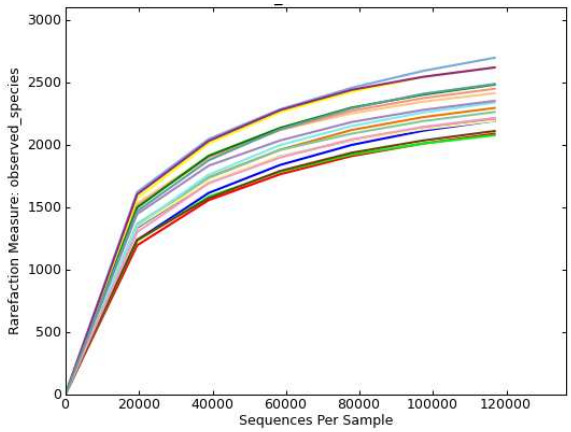

Figure A5. *Specaccum or species accumulation curve of 18 microbiome samples collected from Abutilon fruticosum rhizosphere soil and surrounding bulk soil*

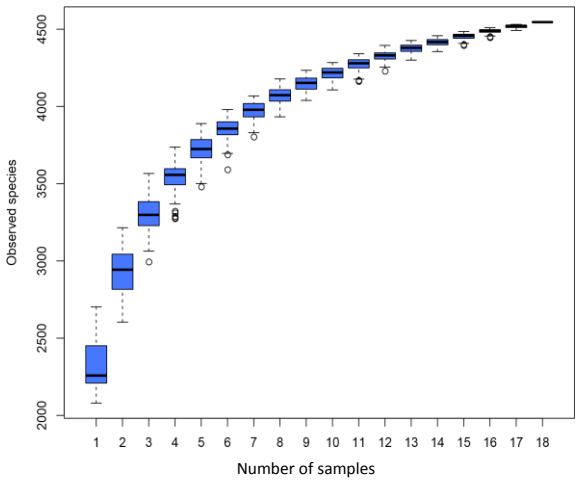

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Figure A6. *Anosim plots of microbiomes collected from surrounding bulk and rhizosphere soils of Abutilon fruticosum regardless of time after watering, e.g., bulk soil (group J) and rhizosphere soil (group K). Samples data were also grouped based on time after watering regardless of microbiome source, e.g., 0 (group G), 24 (group H) and 48 h (group I)*

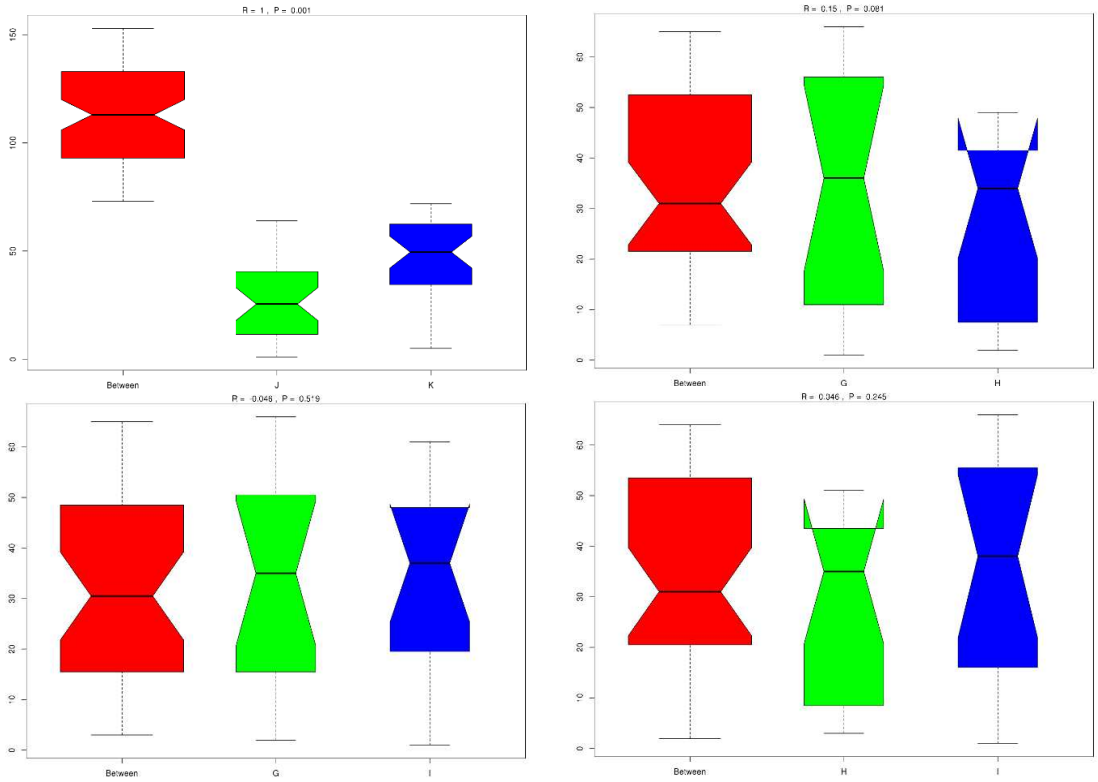

Figure A7. *Heatmap of weighted (top records) and unweighted (bottom records) unifrac beta diversity measures of microbiomes collected from surrounding bulk (S) and rhizosphere (R) soils of Abutilon fruticosum in three replicates after 0 (S11-S13 and R11-R13, respectively), 24 (S21-S23 and R21-R23, respectively) and 48 h (S31-S33 and R31-R33, respectively) of watering*

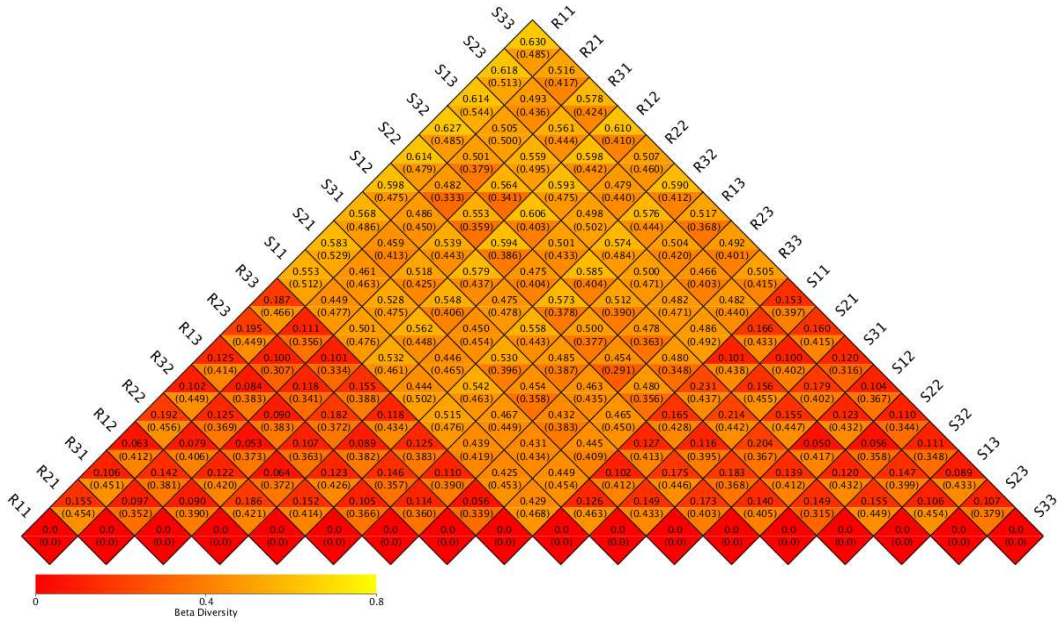

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Figure A8. *Dendrogram trees of unweighted pair-group method with arithmetic mean (UPGMA) cluster analysis describing the calculated weighted (a) and unweighted (b) unifrac beta diversity distances of microbiomes collected from surrounding bulk (S) and rhizosphere (R) soils of Abutilon fruticosum in three replicates after 0 (S11-S13 and R11-R13, respectively), 24 (S21-S23 and R21-R23, respectively) and 48 h (S31-S33 and R31-R33, respectively) of watering*

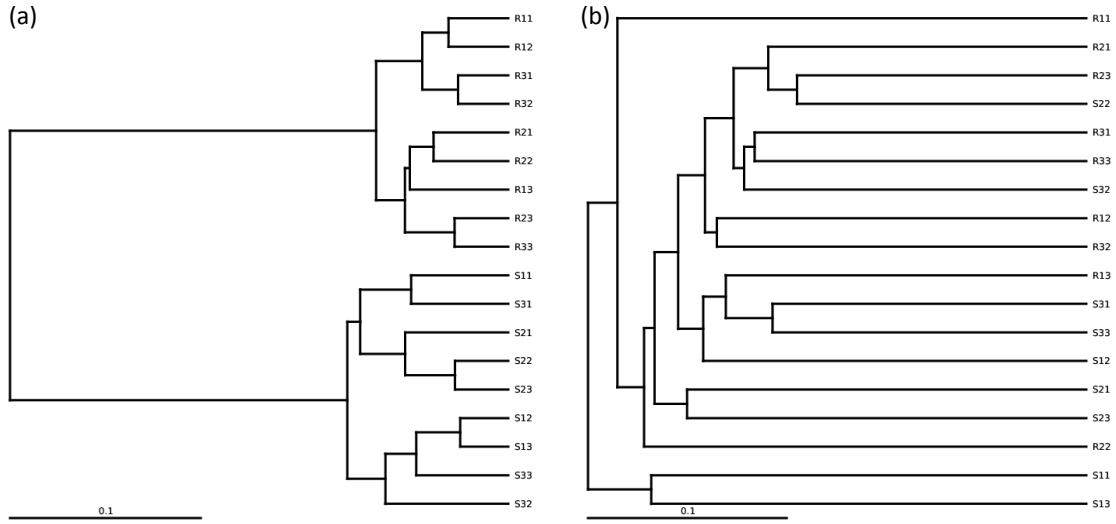

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Figure A9. *Genus evolutionary tree describing abundances of microbiome samples collected from surrounding bulk (S) and rhizosphere (R) soils of Abutilon fruticosum in three replicates after 0 (S11-S13 and R11-R13, respectively), 24 (S21-S23 and R21-R23, respectively) and 48 h (S31-S33 and R31-R33, respectively) of watering*

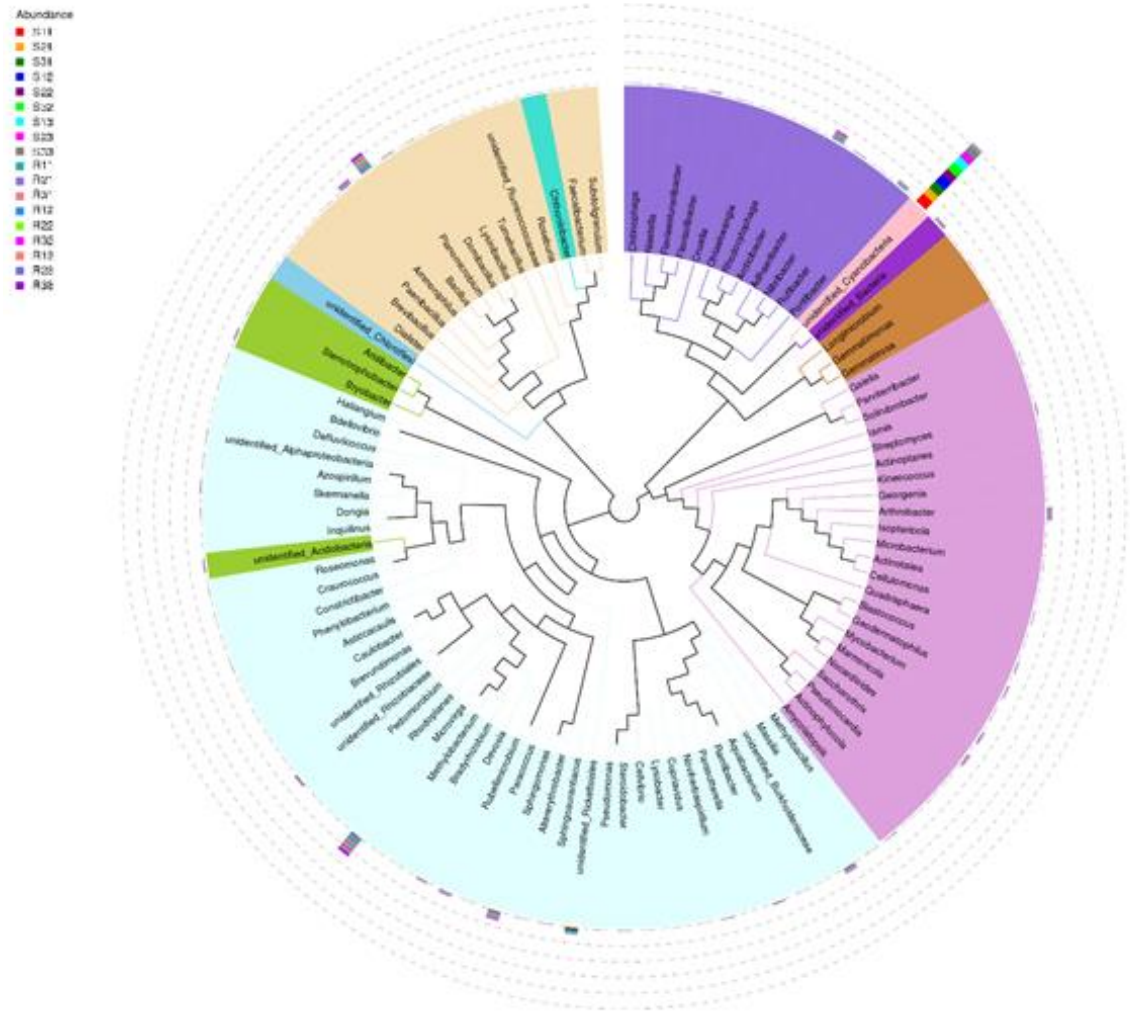

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Figure A10. *Genus evolutionary tree describing abundances of microbiomes collected from surrounding bulk and rhizosphere soils of Abutilon fruticosum after 0 (groups A and D, respectively), 24 (groups B and E, respectively) and 48 h (groups C and F, respectively) of watering*

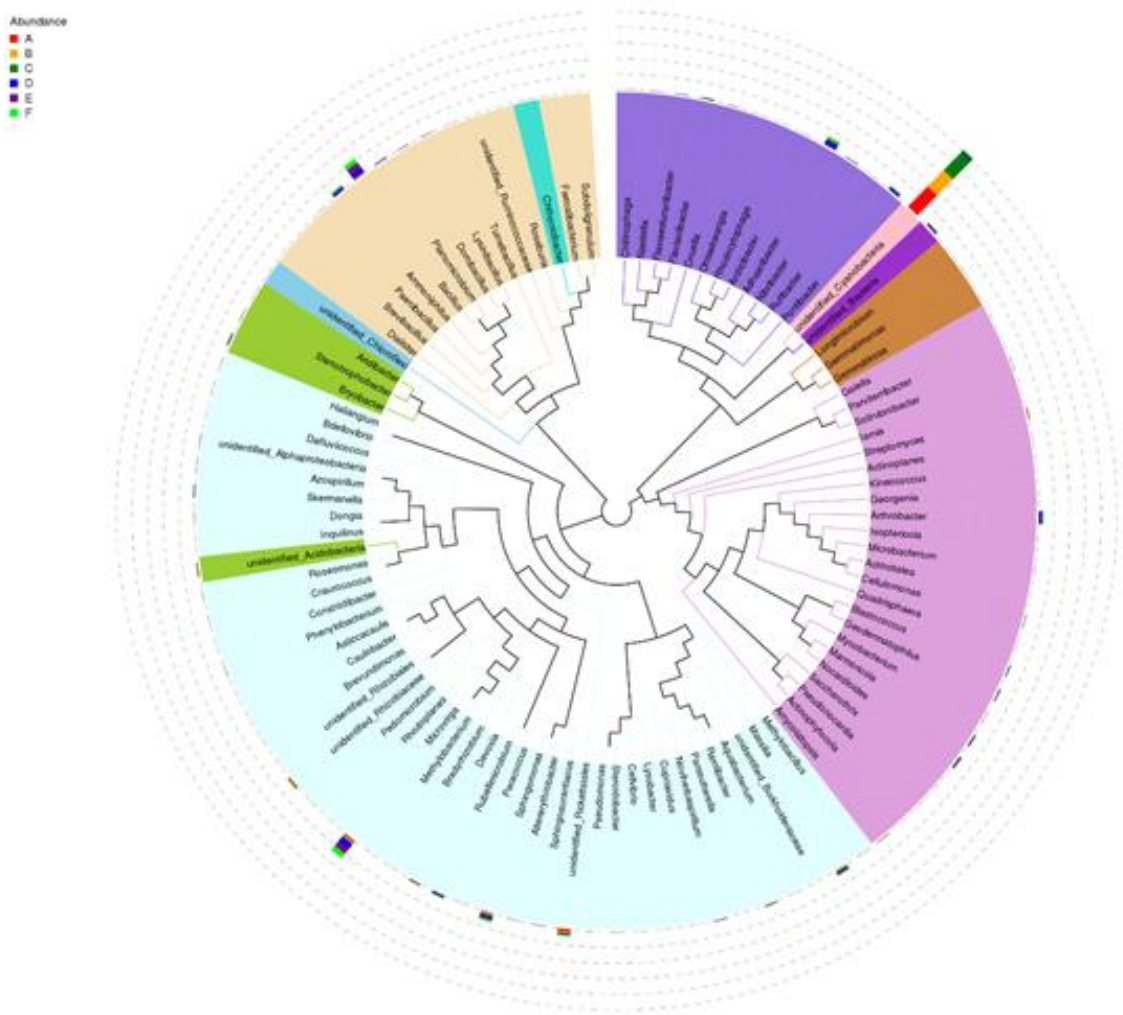

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Figure A11. *Genus evolutionary tree describing abundances of microbiomes collected from surrounding bulk (group J) and rhizosphere (K) soils of Abutilon fruticosum regardless of time after watering*

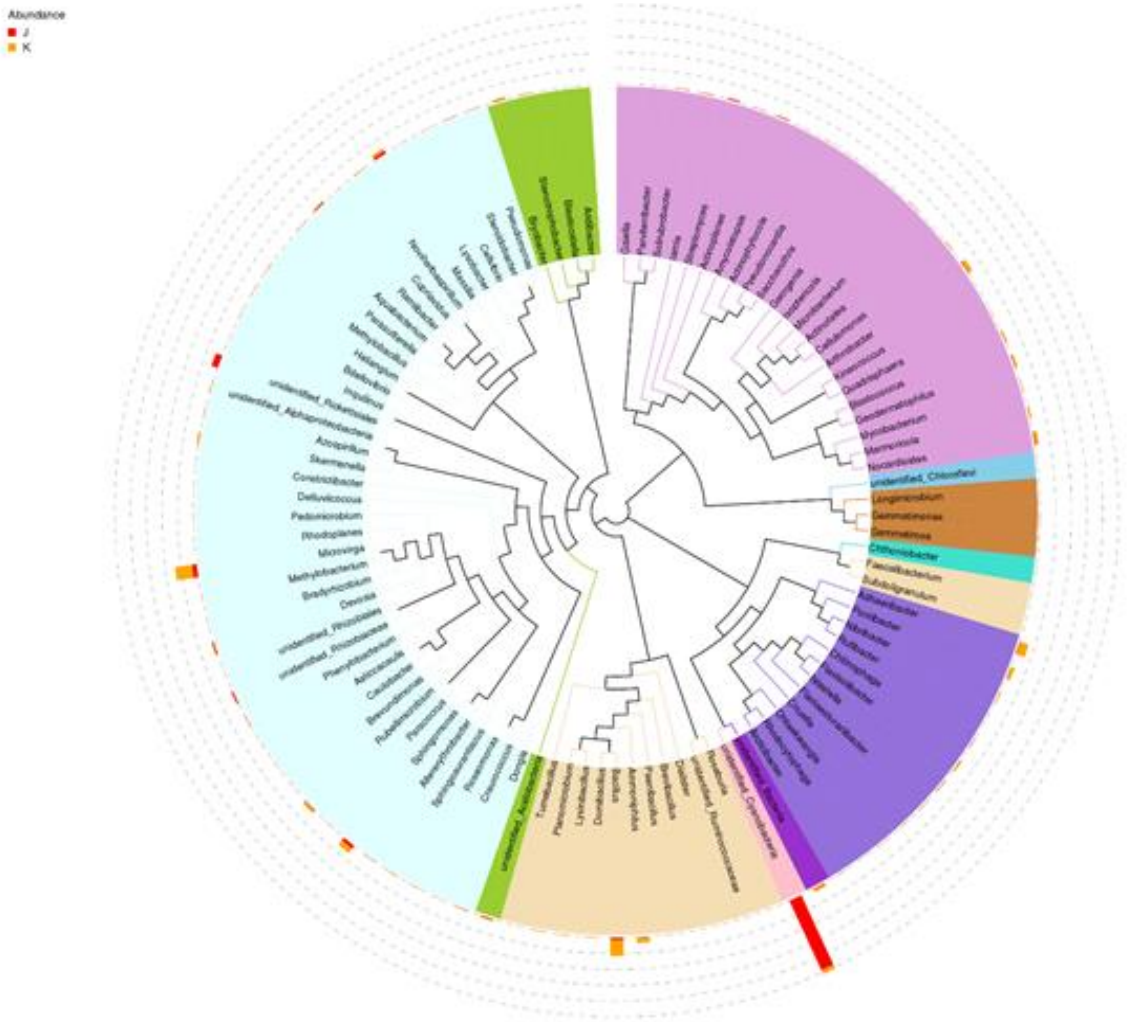

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Figure A12. *Genus evolutionary tree describing abundances of microbiomes collected from surrounding bulk and rhizosphere soils of Abutilon fruticosum after 0 (grouping type AD or group G), 24 (grouping type BE or group H) and 48 h (grouping type CF or group I) of watering regardless of source of microbiome*

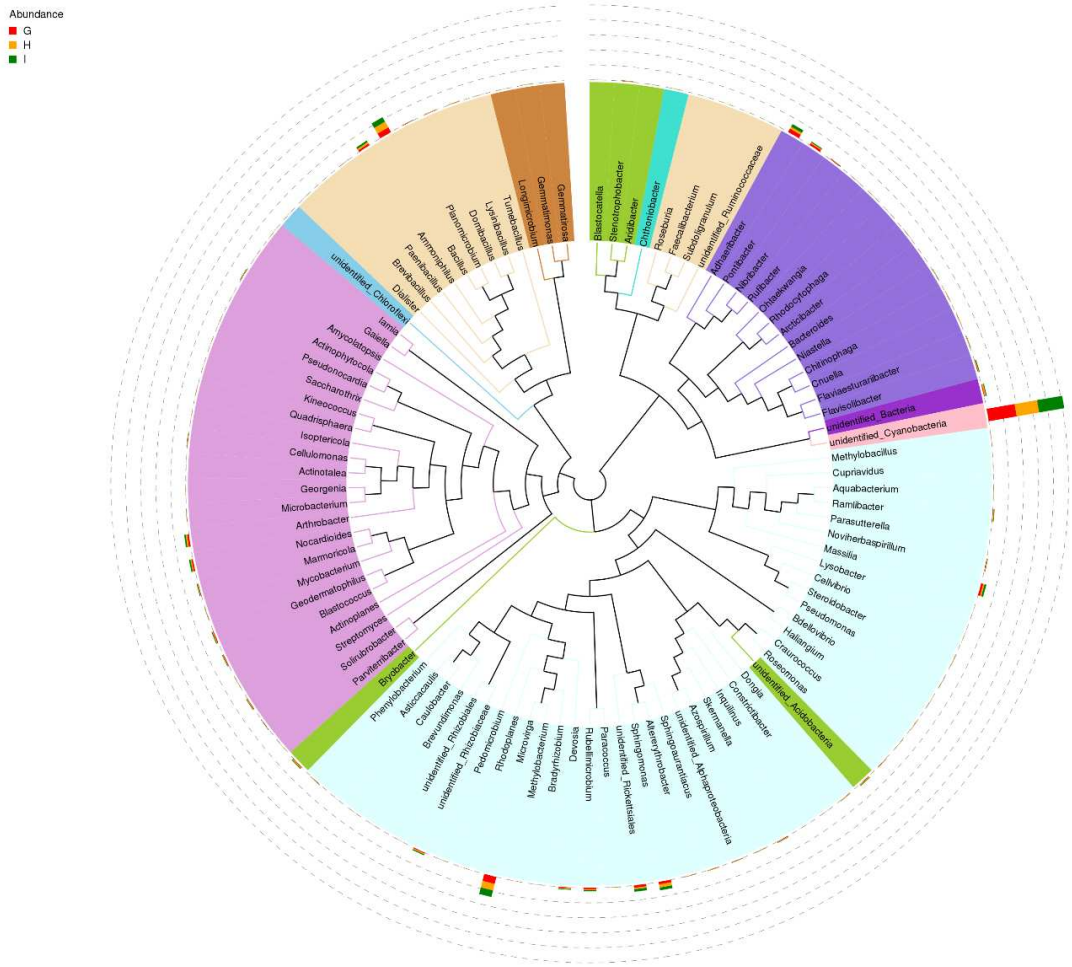

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Figure A13. *Relative abundance of individual samples at the phylum level emphasizing taxa that showed significant differences within different types of grouping of microbiomes collected from surrounding bulk (S) and rhizosphere (R) soils of Abutilon fruticosum in three replicates after 0 (S11-S13 [A] and R11-R13 [D], respectively), 24 (S21-S23 [B] and R21-R23 [E], respectively) and 48 h (S31-S33 [C] and R31-R33 [F], respectively) of watering. Samples data were also grouped based on based on source of microbiome regardless of time after watering, e.g., bulk soil (group J) and rhizosphere soil (group K), and time after watering regardless of source of microbiome, e.g., 0 (group G), 24 (group H) and 48 h (group I). Taxa with (*) refer to occurrence of significant differences within grouping type ABCDEF, taxa with (**) refer to occurrence of significant differences within grouping type GHI, while first 12 taxa (inside the dotted black box) refer to occurrence of significant differences within grouping type JK. Statistical analysis is shown in Metastat boxplots of Figures S18-S26*

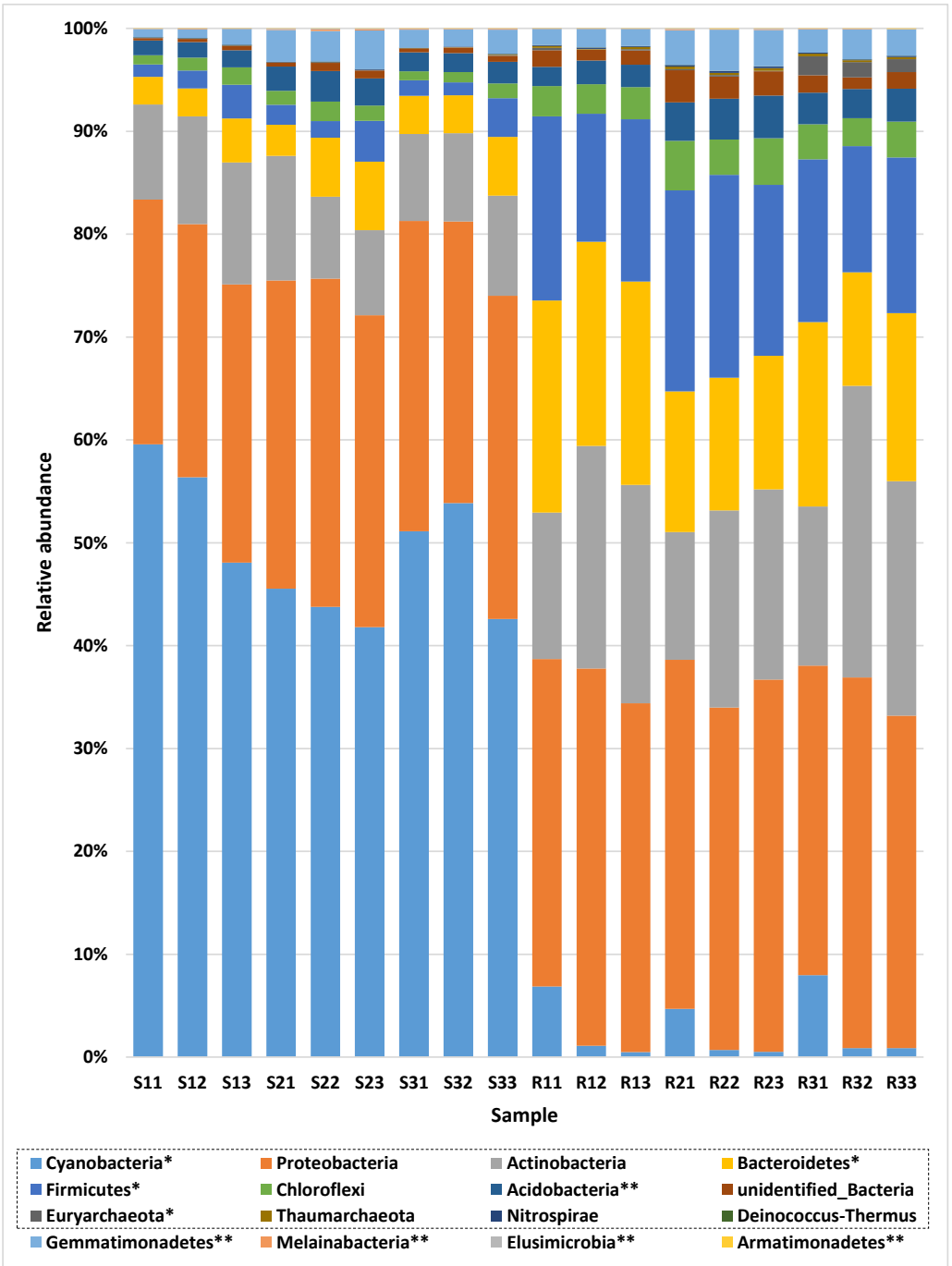

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Figure A14. *Relative abundance of individual samples at the genus level emphasizing taxa that showed significant differences within different types of grouping of microbiomes collected from surrounding bulk (S) and rhizosphere (R) soils of Abutilon fruticosum in three replicates after 0 (S11-S13 [A] and R11-R13 [D], respectively), 24 (S21-S23 [B] and R21-R23 [E], respectively) and 48 h (S31-S33 [C] and R31-R33 [F], respectively) of watering. Samples data were also grouped based on based on source of microbiome regardless of time after watering, e.g., bulk soil (group J) and rhizosphere soil (group K), and time after watering regardless of source of microbiome, e.g., 0 (group G), 24 (group H) and 48 h (group I). Taxa with (*) refer to occurrence of significant differences within grouping type ABCDEF, taxa with (**) refer to occurrence of significant differences within grouping type GHI, while first 12 taxa (inside the dotted black box) refer to occurrence of significant differences within grouping type JK. Statistical analysis is shown in Metastat boxplots of Figures S18-S26*

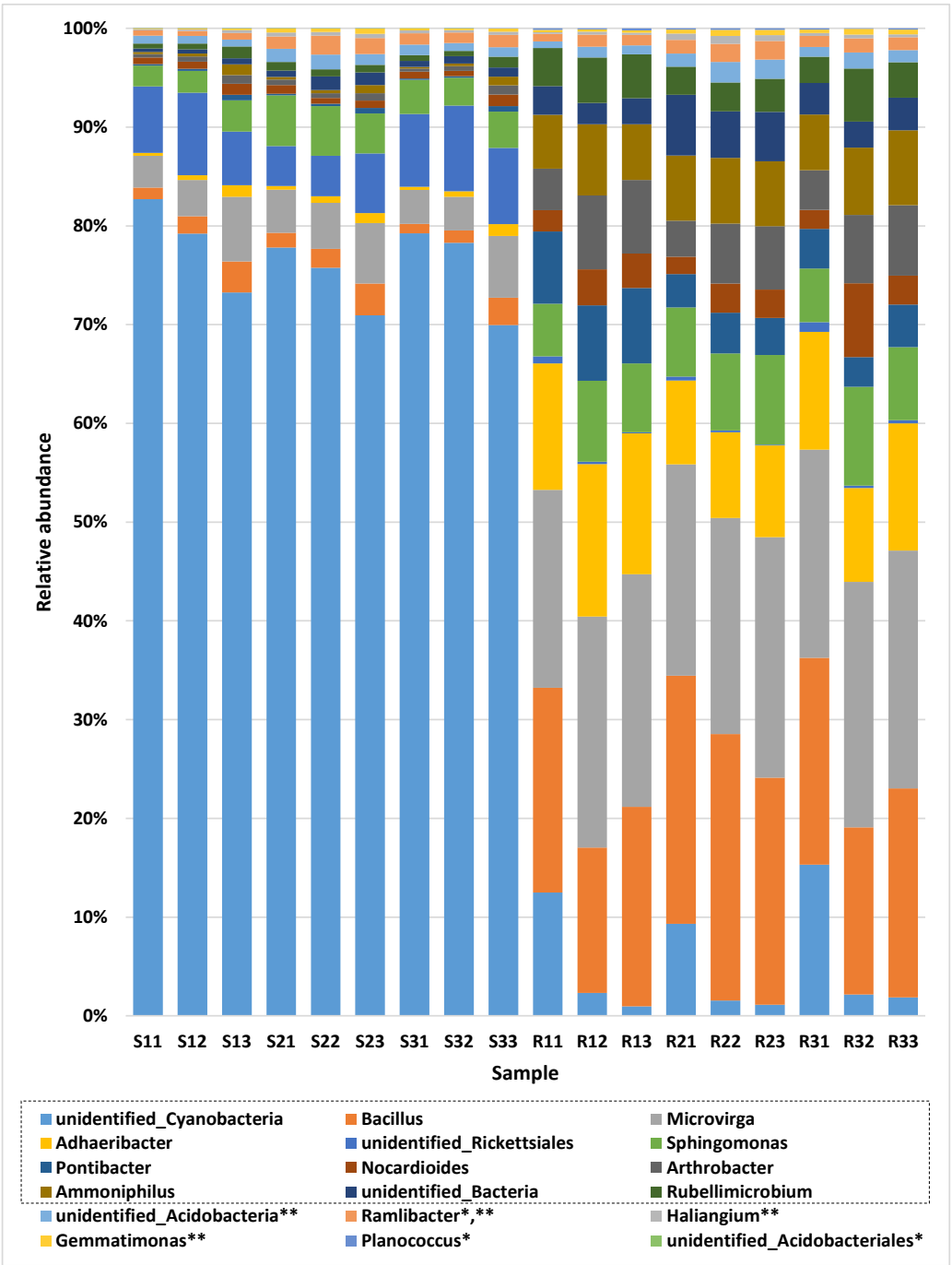

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Figure A15. *Heat map referring to the most abundant 35 genera and their phylum of individual samples microbiomes collected from collected from surrounding bulk (S) and rhizosphere (R) soils of Abutilon fruticosum in three replicates after 0 (S11-S13 and R11-R13, respectively), 24 (S21-S23 and R21-R23, respectively) and 48 h (S31-S33 and R31-R33, respectively) of watering*

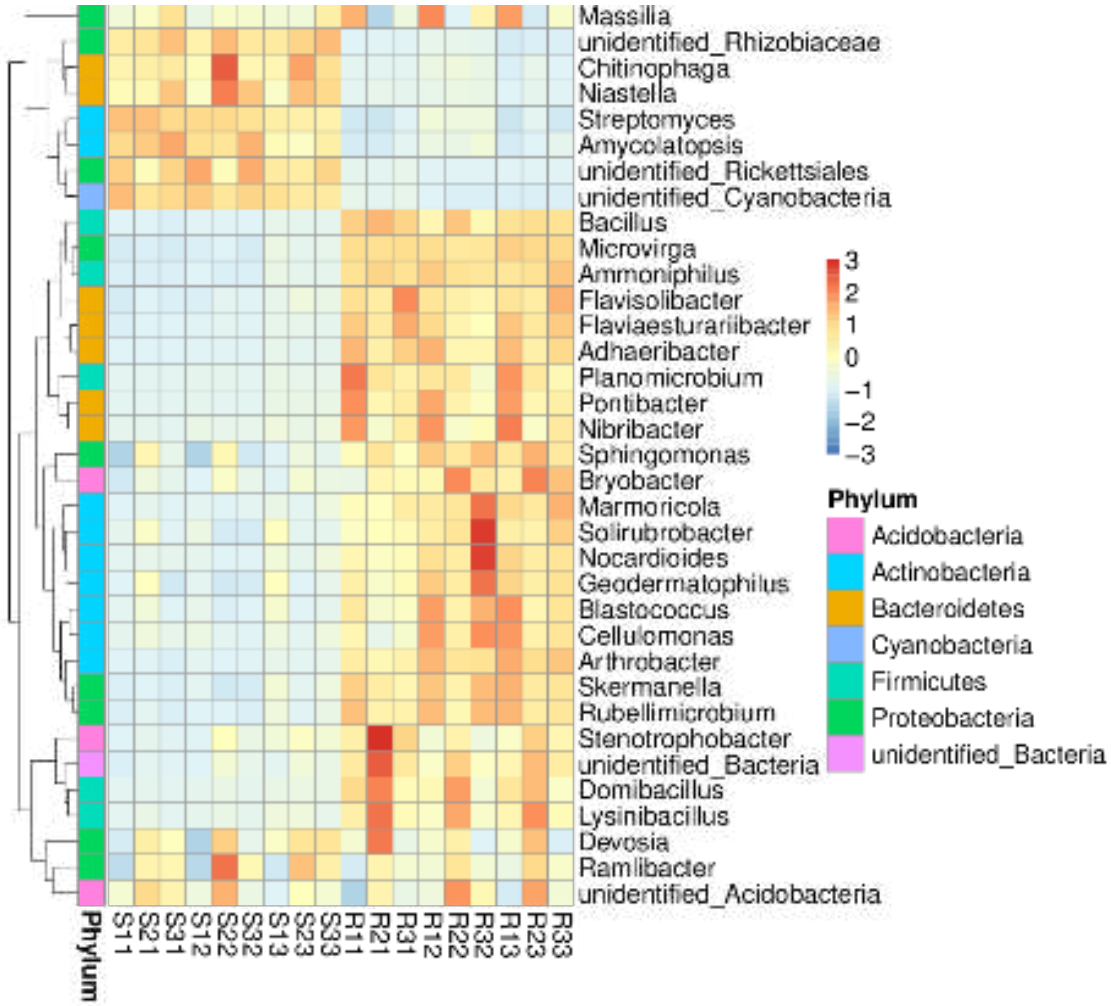

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Figure A16. *Relative abundance of individual samples at the species level emphasizing taxa that showed significant differences within different types of grouping of microbiomes collected from surrounding bulk (S) and rhizosphere (R) soils of Abutilon fruticosum in three replicates after 0 (S11-S13 [A] and R11-R13 [D], respectively), 24 (S21-S23 [B] and R21-R23 [E], respectively) and 48 h (S31-S33 [C] and R31-R33 [F], respectively) of watering. Samples data were also grouped based on based on source of microbiome regardless of time after watering, e.g., bulk soil (group J) and rhizosphere soil (group K), and time after watering regardless of source of microbiome, e.g., 0 (group G), 24 (group H) and 48 h (group I). Taxa with (*) refer to occurrence of significant differences within grouping type ABCDEF, taxa with (**) refer to occurrence of significant differences within grouping type GHI, while first 12 taxa (inside the dotted black box) refer to occurrence of significant differences within grouping type JK. Statistical analysis is shown in Metastat boxplots of Figures S18-S26*

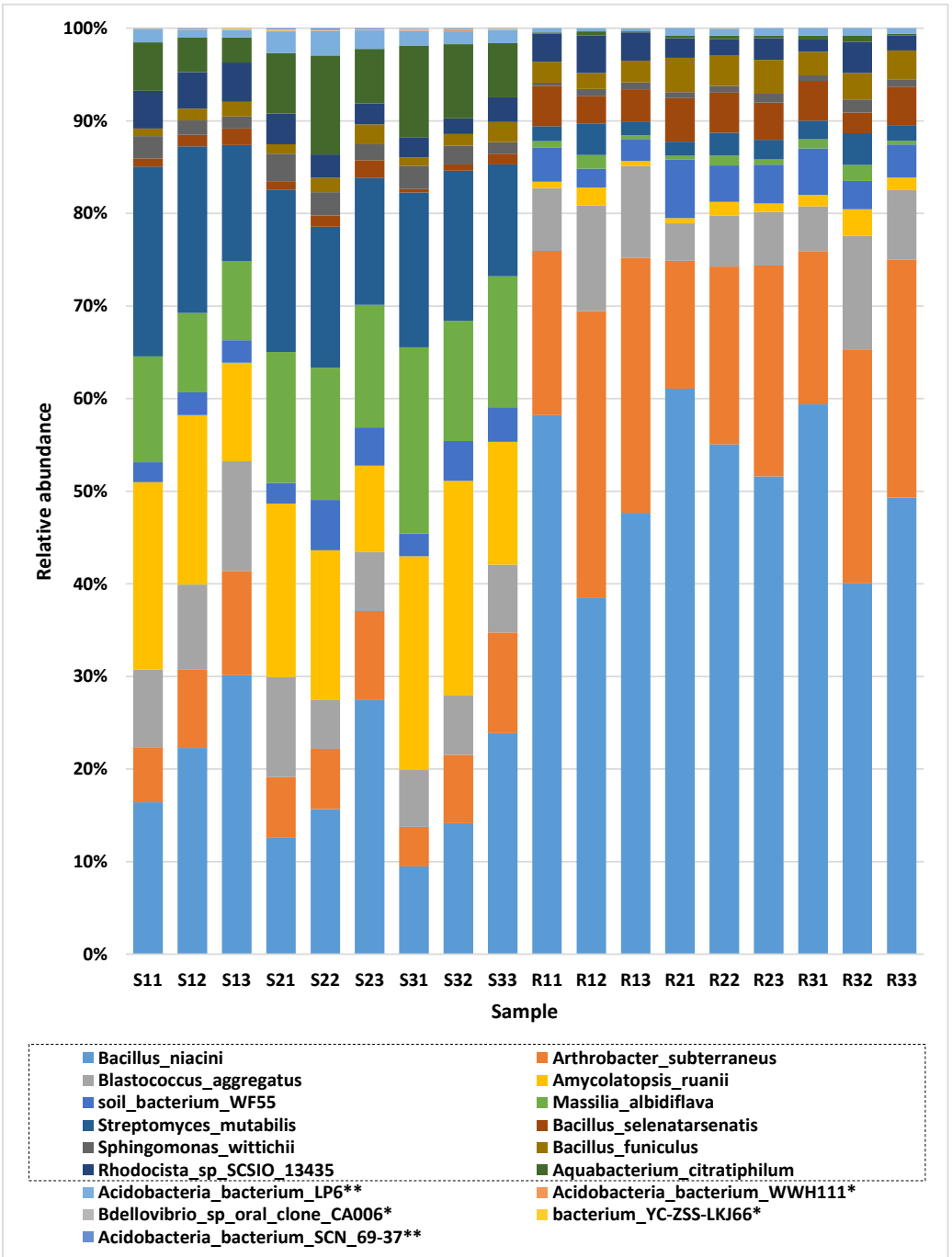

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Figure A17. *Differential abundance of Bacillus niacini of individual samples collected from surrounding bulk (S) and rhizosphere (R) soils of Abutilon fruticosum in three replicates after 0 (S11-S13 [A] and R11-R13 [D], respectively), 24 (S21-S23 [B] and R21-R23 [E], respectively) and 48 h (S31-R33 [C] and S31-S33 [F], respectively) of watering*

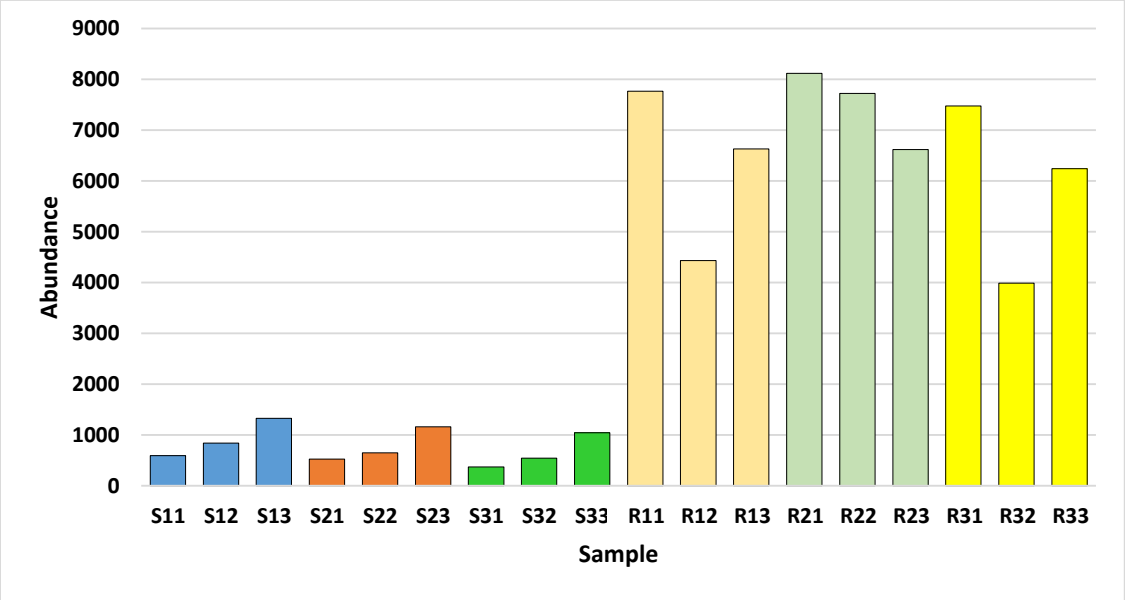

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Figure A18. *Metastat boxplots at the phylum level for the taxa that showed significant differences within grouping type ABCDEF of microbiomes collected from surrounding bulk (grouping type ABC) and rhizosphere (grouping type DEF) soils of Abutilon fruticosum after 0 (grouping type AD), 24 (grouping type BE) and 48 h (grouping type CF) of watering*

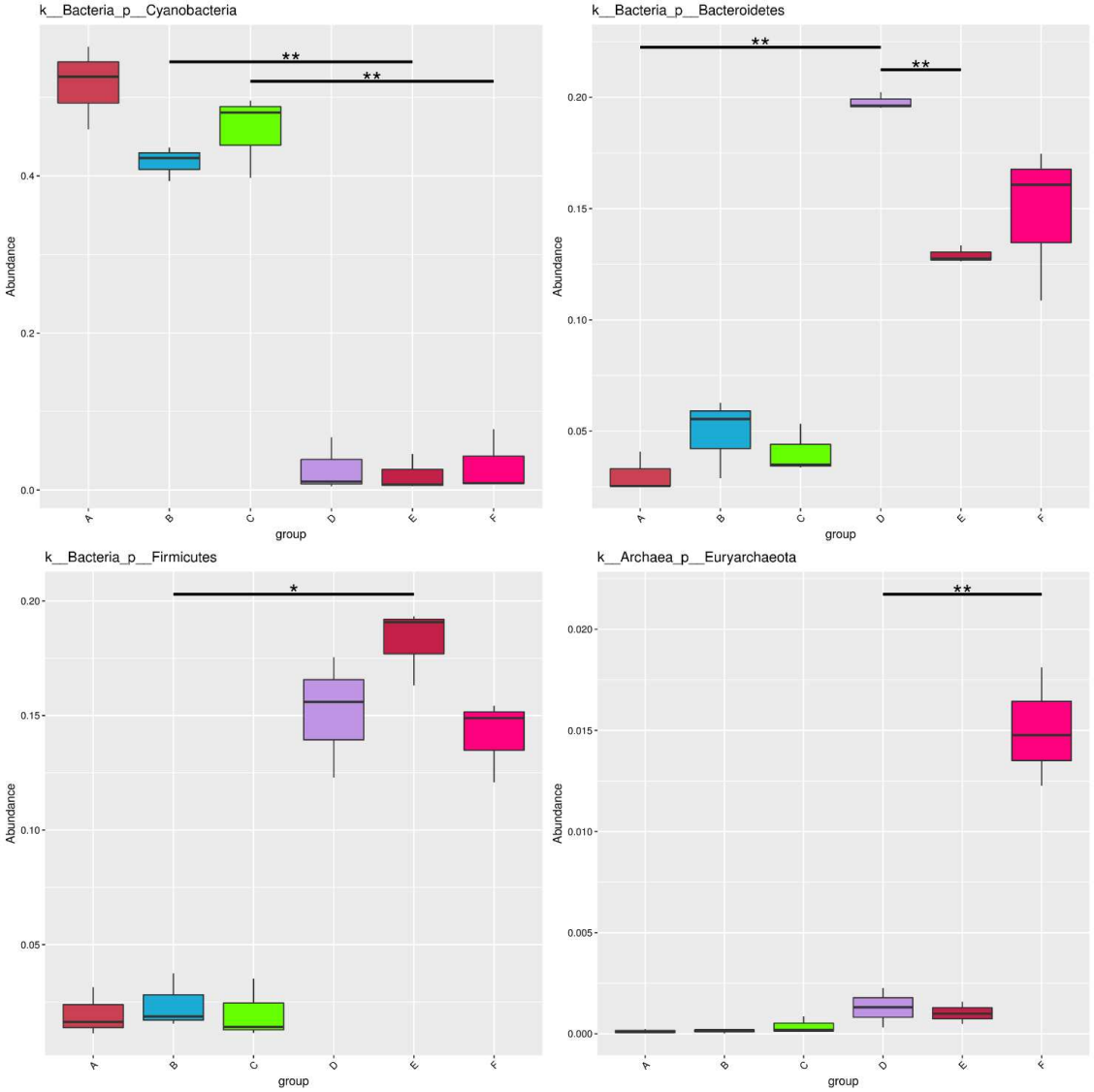

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Figure A19. *Metastat boxplots at the genus level for the taxa that showed significant differences within grouping type ABCDEF of microbiomes collected from surrounding bulk (grouping type ABC) and rhizosphere (grouping type DEF) soils of Abutilon fruticosum after 0 (grouping type AD), 24 (grouping type BE) and 48 h (grouping type CF) of watering*

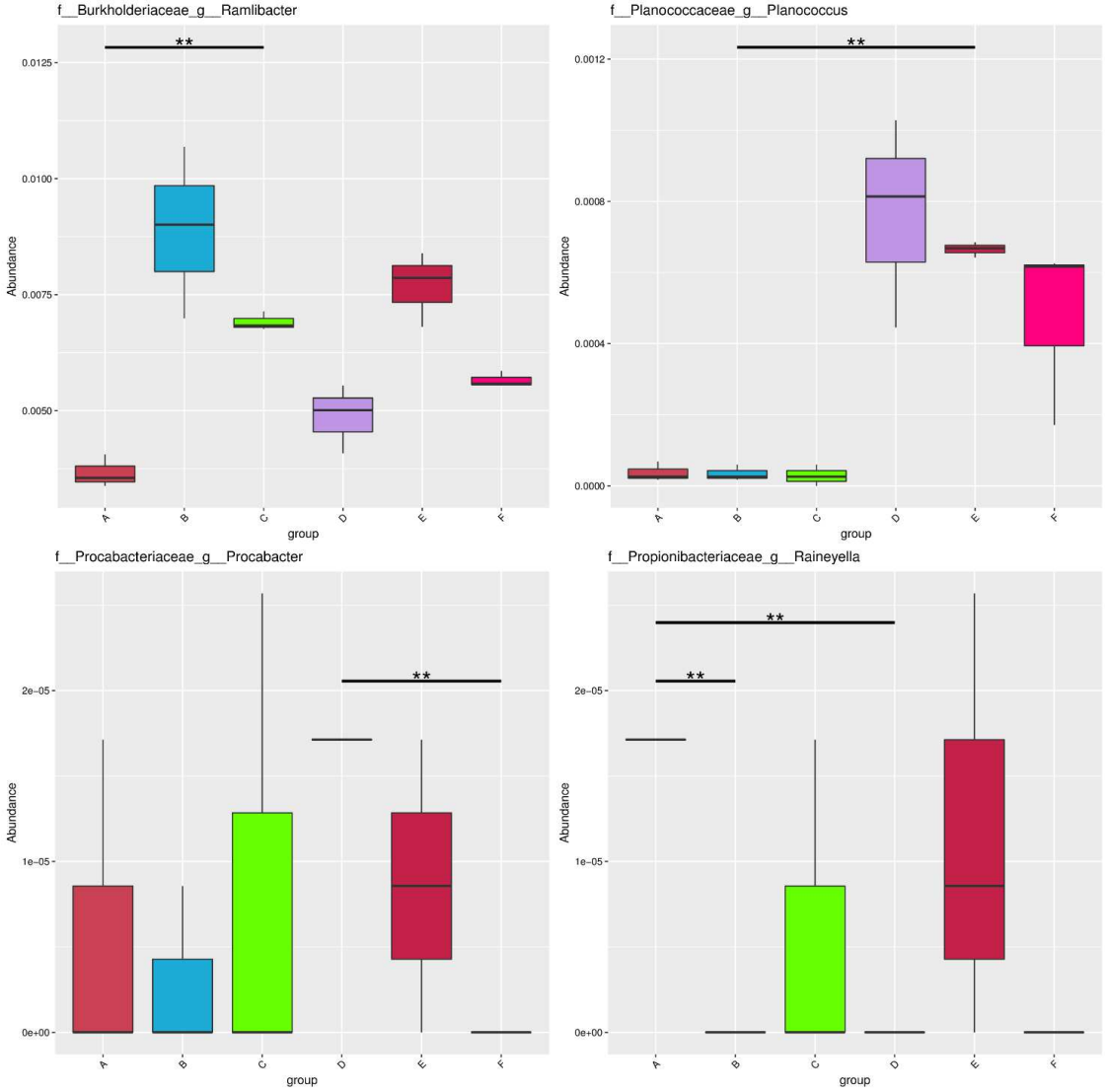

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Figure A20. *Metastat boxplots at the species level for the taxa that showed significant differences within grouping type ABCDEF of microbiomes collected from surrounding bulk (grouping type ABC) and rhizosphere (grouping type DEF) soils of Abutilon fruticosum after 0 (grouping type AD), 24 (grouping type BE) and 48 h (grouping type CF) of watering*

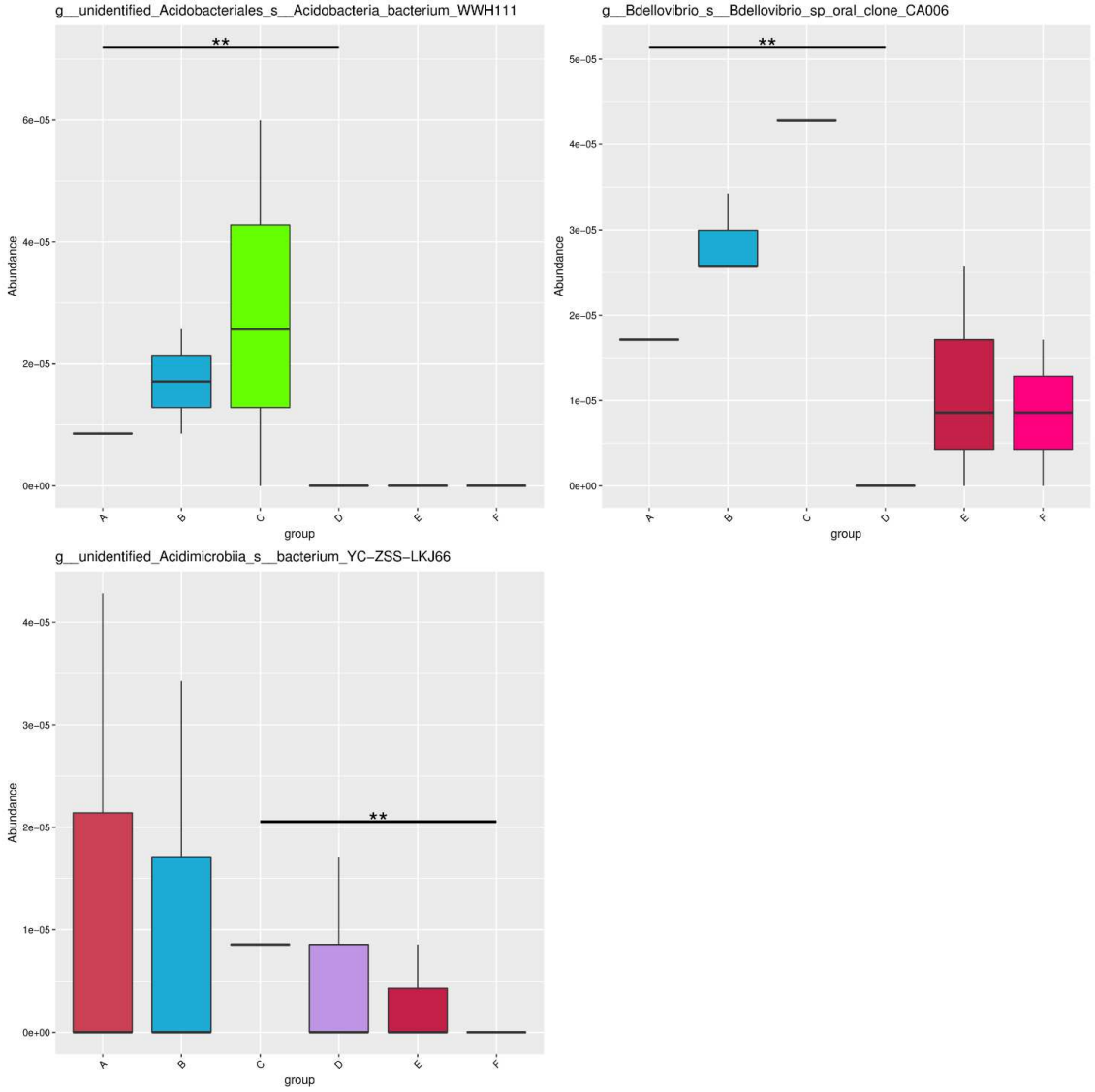

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Figure A21. *Metastat boxplots at the phylum level for the top 12 taxa that showed significant differences between groups of microbiomes collected from surrounding bulk (group J) and rhizosphere (group K) soils of Abutilon fruticosum regardless of time after watering*

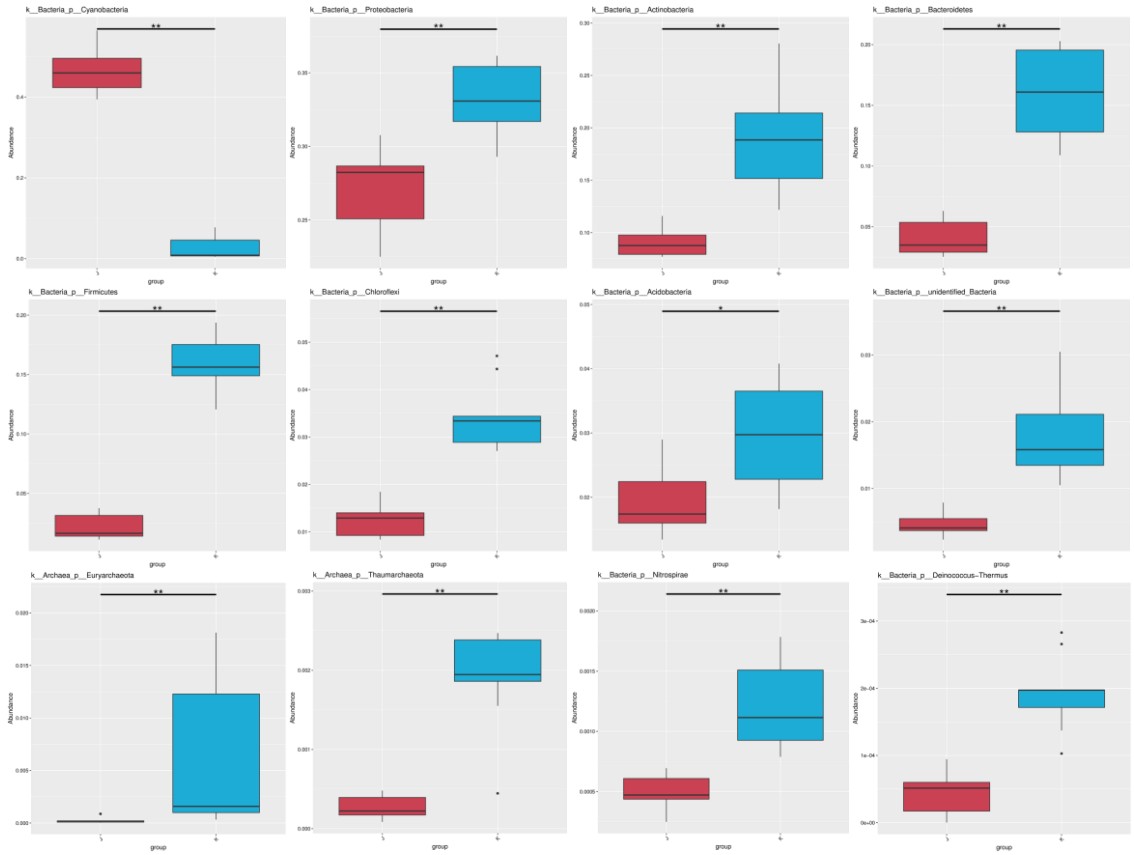

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Figure A22. *Metastat boxplots at the genus level for the top 12 taxa that showed significant differences between groups of microbiomes collected from surrounding bulk (group J) and rhizosphere (group K) soils of Abutilon fruticosum regardless of time after watering*

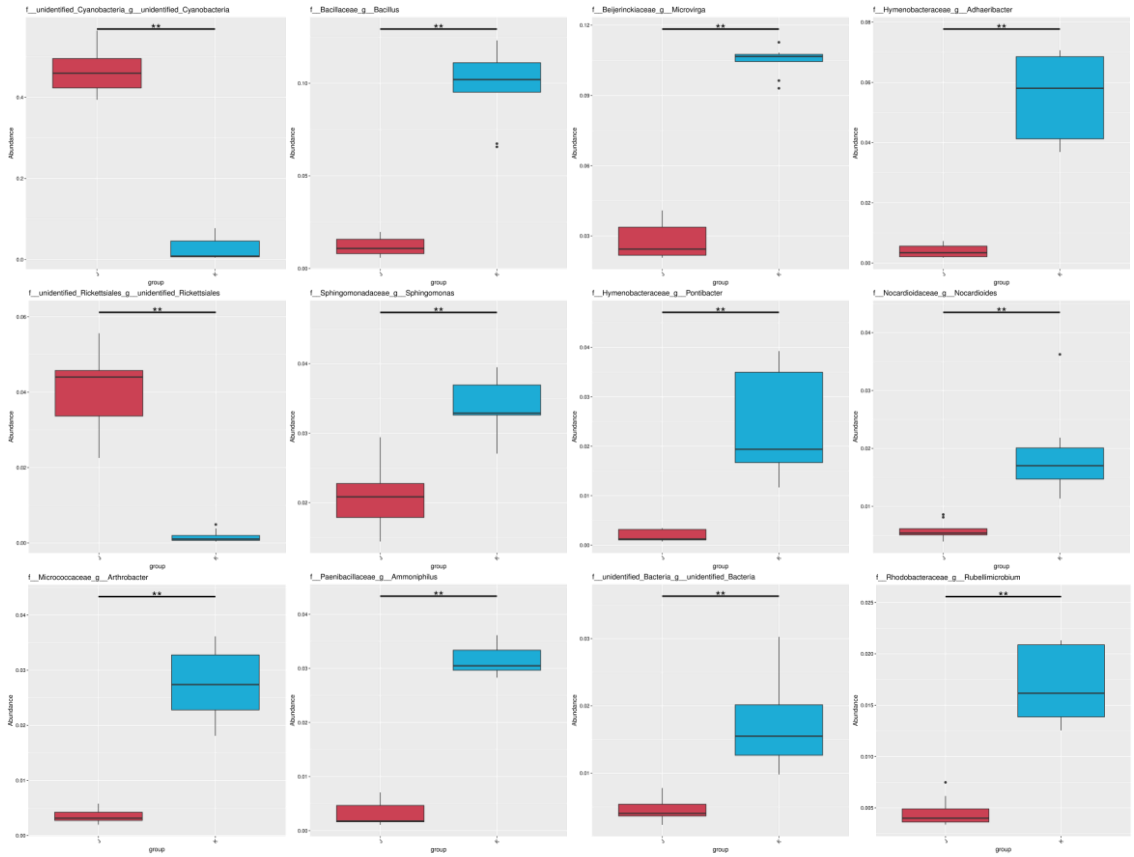

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Figure A23. *Metastat boxplots at the species level for the top 12 taxa that showed significant differences between groups of microbiomes collected from surrounding bulk (group J) and rhizosphere (group K) soils of Abutilon fruticosum regardless of time after watering*

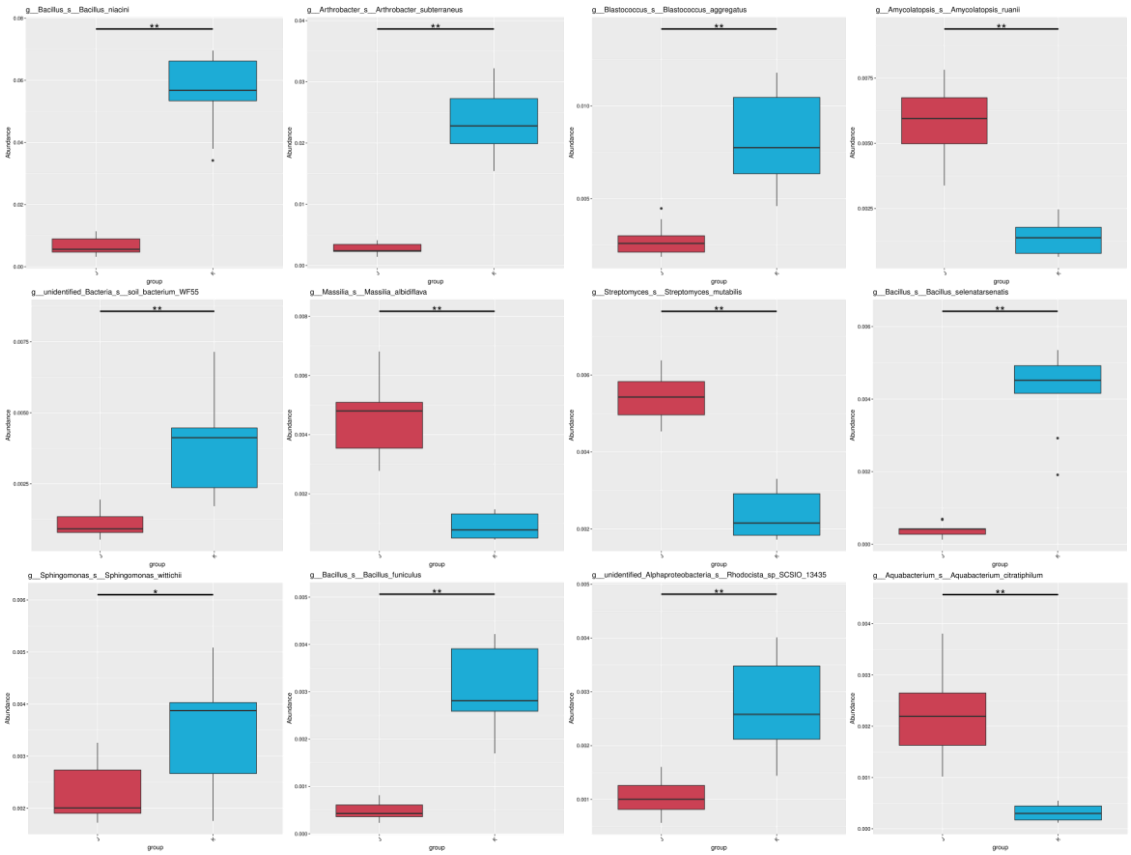

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Figure A24. *Metastat boxplots at the phylum level for the taxa that showed significant differences within grouping type GHI of microbiomes collected from surrounding bulk and rhizosphere soils of Abutilon fruticosum after 0 (group G), 24 (group H) and 48 h (group I) of watering regardless of source of microbiome*

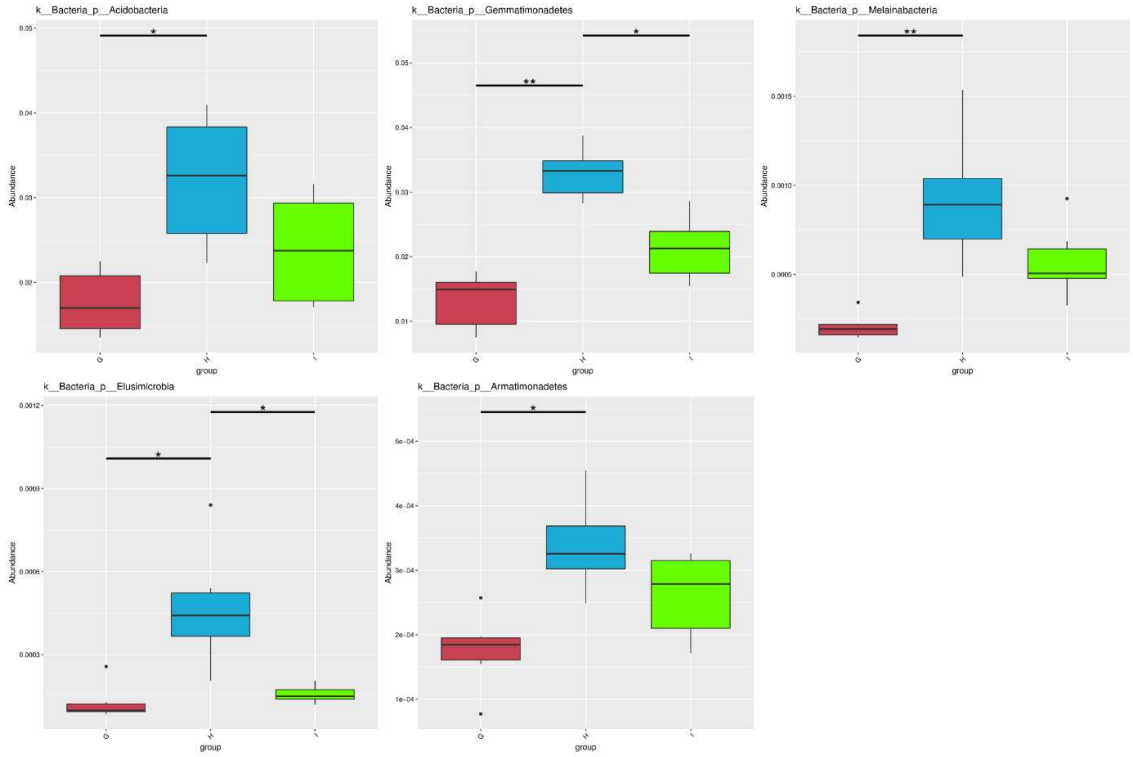

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Figure A25. *Metastat boxplots at the genus level for the taxa that showed significant differences within grouping type GHI of microbiomes collected from surrounding bulk and rhizosphere soils of Abutilon fruticosum after 0 (group G), 24 (group H) and 48 h (group I) of watering regardless of source of microbiome*

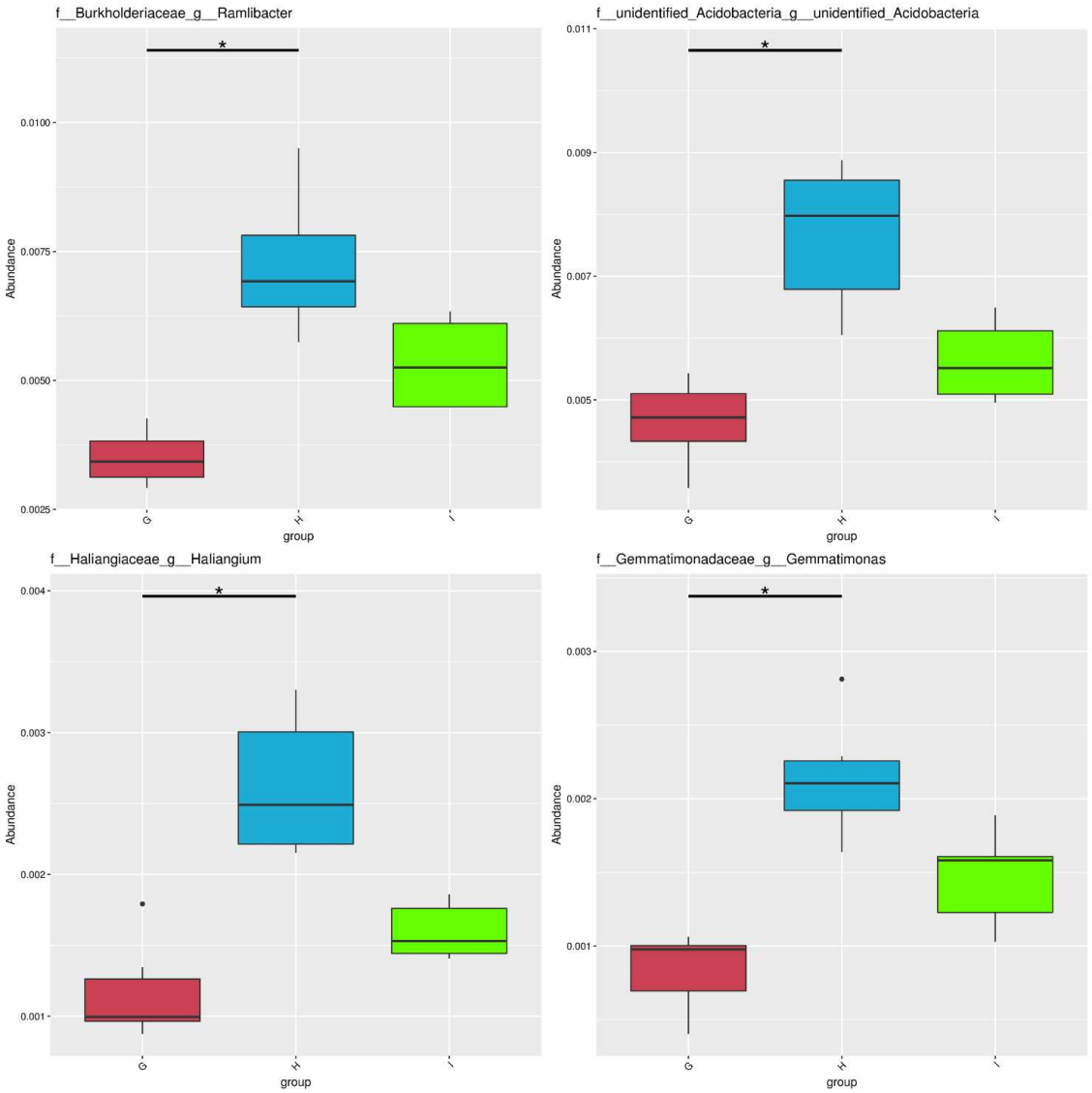

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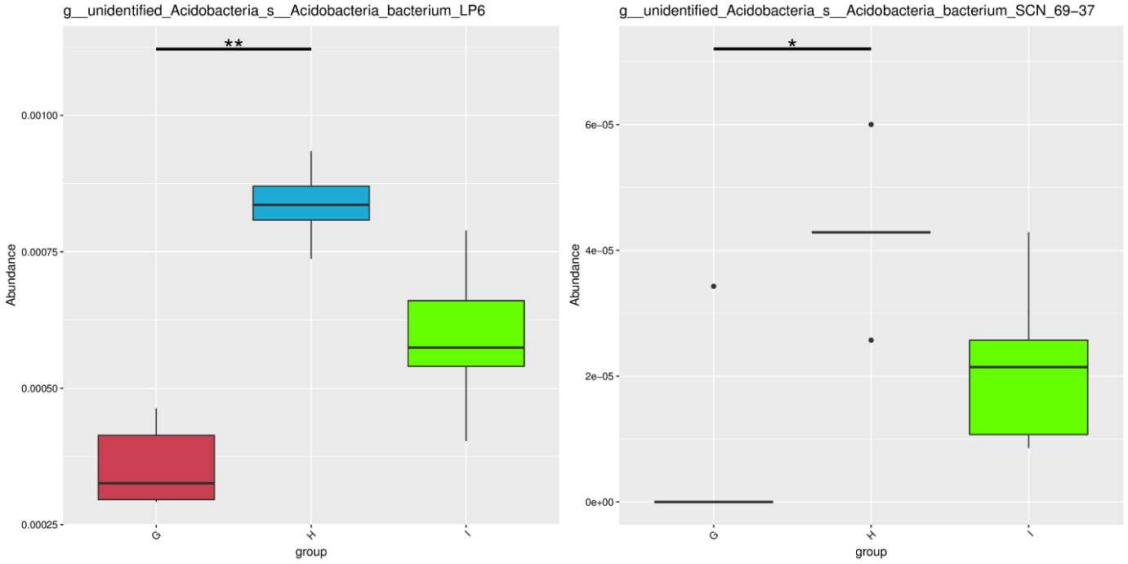
Figure A26. *Metastat boxplots at the species level for the taxa that showed significant differences within grouping type GHI of microbiomes collected from surrounding bulk and rhizosphere soils of Abutilon fruticosum after 0 (group G), 24 (group H) and 48 h (group I) of watering regardless of source of microbiome*



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DIFFERENCES IN DENITRIFYING COMMUNITY STRUCTURE OF THE NITROUS OXIDE REDUCTASE (*nosZ* I) TYPE GENE IN A MULTI-STAGE SURFACE CONSTRUCTED WETLAND

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Abstract. Nitrous oxide (N_2O)-reducing microorganisms with the capacity to reduce the greenhouse gas N_2O to harmless dinitrogen gas are affected by environmental conditions. We applied pyrosequencing of the *nosZ* clade I gene to investigate denitrifying communities in a restoring constructed wetland ecosystem in China. We collected sediments from an ecological retention pond (0) and a five-stage (I, II, III, IV, and V) surface-flow constructed wetland. Content of ammonium-nitrogen (N), nitrate-N, available phosphorus, total N, organic matter, and available potassium as well as pH value in the sediment decreased in the last stage (i.e., V). Richness and diversity of *nosZ* clade I communities in stage V were slightly higher than those of other stages. The *nosZ* clade I communities in stage IV and V samples were clearly distinguished from the other four samples. The proportion of Actinobacteria and Chloroflexi was lower in wetlands than in the ecological retention pond. *Bradyrhizobium* was significantly lower in all five stages (17.9–37.8%) than in the ecological retention pond (44.7%). Of 32 abundant operational taxonomic units, 22 were significantly correlated with more than at least one of the measured physicochemical properties. Our results are essential for understanding the interrelationship between N-related microbial distribution and the pollution status of wetlands for the evaluation of constructed wetlands.
Keywords: *N_2O-reducing microorganisms, nosZ I abundance, Artificial Wetlands, ecological retention, water quality*

Introduction

As the decomposers of ecosystems, including the biosphere, microbial composition and function are closely interrelated with the surrounding environment (Cao et al., 2017), including in wetland sediments (Overbeek et al., 2019). The reducing conditions in wetland sediments favor microbial respiratory processes that use alternate electron acceptors, such as denitrification, which contribute to nitrogen (N) removal in a variety of environments (Lansdown et al., 2016; Cheng et al., 2020). In the absence of oxygen in wetlands, denitrifiers can sequentially respire nitrate (NO_3^-) to nitrite (NO_2^-), nitric oxide (NO), nitrous oxide (N_2O), and ultimately to dinitrogen gas (N_2) (Zhang et al., 2019). Novel N2O-reducing microorganisms with the capacity to reduce the third most important anthropogenic greenhouse gas N2O (Gao et al., 2021) to harmless N_2 gas are receiving increased attention due to rising N_2O emissions (Hallin et al., 2018; Gao et al., 2021). The *nosZ* gene, encodes N_2O reductase, which is related to the reduction of N_2O to N_2, and is a suitable marker for investigating the diversity and composition of the N_2O-reducer community (Li et al., 2020). A recent important discovery showed that *nosZ* phylogeny has two major clades, I and II (Conthe et al., 2018). Denitrifiers are present in both clades, but organisms in clade I, which is dominated by Proteobacteria

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under all conditions (Miguel et al., 2020), were more likely to be complete denitrifiers, while a large fraction of organisms with clade II *nosZ* lacked the genetic capacity to produce N_2O (Juhanson et al., 2017). Community composition of denitrifiers within clade I could influence in situ net N_2O fluxes (Hallin et al., 2018), because these denitrifiers reduce N_2O at different rates (Wang et al., 2020).

Nansi Lake, with a total surface area of 1.2×10^3 km^2, is one of the largest shallow freshwater lakes (Zhang et al., 2011), and an important storage reservoir and regulation works for the South-to-North Water Transfer Project of China (Tian et al., 2013). It is becoming the main freshwater fishery production base in Shandong due to its fertile water and large lake surface (Wang et al., 2014; Zhu et al., 2015). However, this lake has been polluted by inefficiently treated effluent from industrial and municipal activities, runoff from mining sites and agriculture land and deposition of air pollutants since the 1970s (Wang et al., 2012a, 2014). Accordingly, its water quality is poor. In 2002, water quality was inferior to Class V, which refers to the categories of "polluted" and "dirty" (Kondrateva et al., 2009), according to the "China surface water quality standard (GB3838–2002)" and U.S. Environmental Protection Agency, and the chemical oxygen demand reached thousands of milligrams per liter (Wang et al., 2016).

When the eastern route of the South-To-North Water Diversion Project was completed, the government started to take measures to improve water quality, one of which was constructed wetlands (CWs). The CWs are widely used, cost-effective, and energy-efficient solutions for purifying water, and enhance a watershed's environmental capacity to self-defend from pollution (Wang et al., 2016; Rajan et al., 2018). The "Artificial Wetlands Demonstration Project" has been widely applied in the whole basin and in an area of 120 km^2 in the estuaries of the Xinxue River from 2005, and has been shown to be effective in improving the water quality of rivers flowing into the lake and increasing the environmental capacity of the entire lake (Cao et al., 2015). However, the effects of pH on denitrifier community richness and diversity in sediment remain unclear. Our previous research confirmed that there was abundant bacterial population diversity in the sediments of Nansi Lake, and the bacterial population diversity is positively correlated with the total phosphorus (P) content in the environment. Among these, relative abundance of the genus *Devosia* in the class α-Proteobacteria is reduced. Most microorganisms in this group are N-fixing, and are functional in the N cycle (Lu, 2013). However, the shift in the N_2O-reducer community after ecological restoration remains unclear.

The wetland effluent is collected by the tail water collection channel and flows into Nansi Lake. This CW is a flow-through system, and the water flowing into the wetland originates from the adjacent Xinxue River. According to the landform characteristics of Xinxue River Estuary, the process scheme of the demonstration project is determined as follows: ecological detention pond (0) + multistage (I, II, III, IV, and V) surface flow CW system. The water plants in the wetland are mainly *Arundo donax* and *Phragmites australis*, the floating leaf plants are *Nymphoides peltatum*, and the submerged plants are *Potamogeton crispus*. The CW has achieved good results, and its pollutant removal effect is obvious. The standard rate of wetland effluent is 80%, and water quality is class III, which refers to the category of "moderately polluted" (Zhang et al., 2014). In this study, we collected sediments from the Xinxue Estuary CW water purification project, including 0 + five-stages CW, to investigate the changes in denitrification communities. The plan layout of Xinxue River CW is shown in *Figure A1* in the *Appendix*. We hypothesized that composition of denitrifiers would be affected by CW; such a shift may directly or indirectly derive from a shift in pH.

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Materials and methods

Site description

The Xinxue River wetland (34°45′N, 117° 09′E) was built in 2005 (Zhang et al., 2008) and is located in the flood discharge area on the west side of the embankment at the entrance of Xinxue River into Nansi Lake, Shandong Province, China (*Fig. A1*). The Xinxue River is intercepted and stored in a rubber-lined dam, enters the diversion channel through the metering system, and successively enters the ecological detention pond and surface flow CW through the water distribution channel. The dam is 170 m long and the dam crest elevation is 33.55 m. The water level of the river is raised by the rubber dam to form a river storage pond. The river water is pretreated here to remove some suspended substances through physical sedimentation and then passes through to the river. The photosynthesis of aquatic plants in the channel pre-oxygenates the river water to reduce some pollutants. The river water raised by the rubber dam enters the ecological detention pond (0) through the diversion channel. The diversion channel has the form of a trapezoidal artificial open earth channel, with a length of about 250 m and a slope of 0.02%. At 300 m downstream of the rubber dam, the ecological detention pond was constructed using the low-lying beach and pond inside the embankment, with an area of about 333,350 m^2, a length of 1400 m, a width of 120–360 m, and a water level elevation of 32.75 m.

Totally, six surface sediment layers of the ecological retention pond (0) and five stages (I–V) of sampling stations were selected in September in 2018. Each station had three sampling sites at intervals of 20 m. Thus, a total of 18 surface sediment samples were collected in an icebox (4 °C) and transported back to the laboratory. One part of each sample was immediately put in a refrigerator at −80 °C for later microbial investigation, and the other part was for nutrient determination.

Physicochemical properties

Sediment samples were air-dried for 1 week at room temperature and sieved through a 1-mm screen. The sediment monitoring items were pH, NO_3^-, ammonium (NH_4^+), total N (TN), available P (AP), available potassium (AK), and organic matter (OM), measured by the Analysis and Testing Center in the Chinese Academy of Agricultural Sciences (IEDA, CAAS, Beijing, China) according to Chinese national standard assay methods (pH: GB 7859-1987; N: GB 7173-1987, AP: GB 7853-1987, AK: GB 7856-1987; OM: GB 9834-1988).

Pyrosequencing of nosZ clade I gene

The process of DNA extraction from 1 g of sediment (Enwall et al., 2010) was performed using a Fast DNA SPIN Kit (MP Biomedicals, Solon, OH, USA) and primers *nos*Z1840-F/*nos*Z2090-R (Henry et al., 2006) were used to amplify the *nosZ* clade I gene by touchdown PCR using TransGene high-fidelity enzyme. The PCR process follows: 95 °C predenaturation for 5 min, 94 °C for 30 s, annealing at 55 °C for 35 s, 72 °C for 30 s, with a total of five cycles; 94 °C for 30 s, annealing at 55–51 °C for 35 s, extending at 72 °C for 30 s, with a total of 25 cycles. The amplified PCR products were sequenced on an Illumina MiSeq PE 2 x 250 platform at BeiJing Fixgene Co., Ltd (Beijing, China). The obtained raw sequences were submitted to the NCBI Sequence Read Archive (SRA) under accession PRJNA734135.

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Bioinformatic analysis

Adaptor, barcode, and primer sequences were filtered from the raw data. The pair-end reads were then joined according to their overlapping sequences using FLASH (v1.2.7) with the properties set to a shortest length of 10 bp (Peng et al., 2019) and a maximum mismatch ratio of 0.3. Then, the remaining sequences were converted to amino acid sequences using the FunGene Pipeline of the Ribosomal Database Project according to Penton et al. (2015). The sequences encoding proteins that contained termination codons or that did not match the *nosZ* clade I protein sequence were removed (Zhou et al., 2021). High-quality sequences obtained through the above processing were rarefied and subsampled based on the lowest number of reads in a sample for subsequent alpha and beta analysis (Zhou et al., 2021). Operational taxonomic units (OTUs) were classified with a similarity of 97%. The classification system from the FunGene pipeline database was used (Penton et al., 2015), and the blastn method was used for annotation (Hou et al., 2012).

Statistical analysis

The least significant difference test was used to identify the differences in sediment physicochemical properties, absolute *nosZ* I gene abundance, and alpha diversity among the six sediments at $P < 0.05$ (Liang et al., 2020), using IBM Statistic SPSS 21. A non-metric multidimensional scaling (NMDS) and hierarchical clustering based on pairwise Bray–Curtis dissimilarities was performed to determine the relationship between individual sediment samples using PAST (version 3.01, folk.uio.no/ohammer/past/). Permutational MANOVA (PERMANOVA) using Bray–Curtis dissimilarity was used to compare differences in *nosZ* clade I community composition among samples.

We analyzed *nosZ* clade I taxa at the genus level to identify, which were different among the six sediment samples. We identified genera whose proportions significantly differed ($P < 0.05$). We use the term 'X-enriched taxon' to refer to a genus found in significantly higher proportion in that group compared to the other group (Zhou and Fong, 2021). A phylogenetic tree was inferred for these enriched genera using the neighbor-joining method in MEGA and displayed using the iTOL (Interactive Tree of Life, https://itol.embl.de/) (Wei et al., 2020; Zhou and Fong, 2021), together with data on genera average relative abundance. We identified all observed genera whose proportions significantly differed (FDR-corrected, $P < 0.05$, Wilcoxon rank-sum test) for 0 and V. Additionally, Pearson's correlation between 'X-enriched taxon' and physicochemical properties was determined using IBM Statistic SPSS 21 and visualized with Origin Pro, 2019b. Redundancy analysis (RDA) was used to explain the relative effects of soil properties on the microbial community (at OTU level) using CANOCO 5.0 (Zhou, 2016).

Results

Sequencing results

In analysis of the 13 sediment samples, we obtained a total of 2,571,816 raw *nosZ* clade I sequences (average 142,878 per sample, *Table A1* in the *Appendix*). The Good's coverage values at 97% cutoff similarity were 98.6–99.6% (*Table A1*), indicating that the current numbers of sequence reads were sufficient to capture the *nosZ* clade I diversity in these samples. High-quality sequences were rarefied and subsampled to 24,000 reads per sample, leaving 6450 OTUs for alpha and beta diversity analysis.

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Alpha diversity of nosZ clade I communities in relation to physicochemical properties

Physicochemical properties in sediments significantly differed (*Table 1*). Sediment NH_4^+-N, NO_3^--N, AP, TN, OM, and AK contents, and pH value in the last stage (V) decreased significantly ($P < 0.05$) compared to the ecological retention pond. These properties decreased from stage I to V. Richness (Chao and ACE indices, *Fig. 1B* and *A*, respectively) and diversity (Shannon index, *Fig. 1D*) of *nosZ* clade I communities in stage V were slightly, but non-significantly, higher than in the ecological retention pond and other stages. The pH value and NO_3^- content were negatively and significantly correlated with ACE and Chao ($P < 0.05$, *Table 2*).

Table 1. *Physicochemical properties of sediments in Nansi Lake*

Stage	pH	NO_3^- (mg/kg)	NH_4^+ (mg/kg)	AP (mg/kg)	AK (mg/kg)	TN (%)	OM (%)
0	7.7 ± 0.4cd	6.7 ± 0.1c	20 ± 0.1c	19.7 ± 0.1d	625.6 ± 0.4e	0.5 ± 0.02e	10.8 ± 0.4d
I	7.7 ± 0.3d	5.9 ± 0.1bc	13.3 ± 1.1b	13.6 ± 0.3c	327.9 ± 1.7d	0.3 ± 0.01d	6.4 ± 0.1c
II	7.6 ± 0.2bc	5.1 ± 0.1b	12.5 ± 1.5b	11.1 ± 0.2b	201 ± 1.3a	0.2 ± 0.01c	4.7 ± 0.2b
III	7.5 ± 0.1b	4.9 ± 0.1b	11.7 ± 0.5a	10.7 ± 0.5b	282.6 ± 2.8c	0.2 ± 0.03b	2.5 ± 0.3a
IV	7.5 ± 0.2b	4.5 ± 0.1b	9.4 ± 0.4a	7.5 ± 0.3a	273.1 ± 1.6b	0.2 ± 0.01ab	2.4 ± 0.1a
V	7.2 ± 0.1a	2.3 ± 0.4a	7.7 ± 1.3a	6.7 ± 1.0a	201 ± 1.3a	0.2 ± 0.01ab	2.2 ± 0.1a

Values are the mean ± standard deviation (n = 3). Values within the same column followed by a different letter indicate a significant difference ($P < 0.05$)
AP, available phosphorus; AK, available potassium; TN, total nitrogen; OM, organic matter

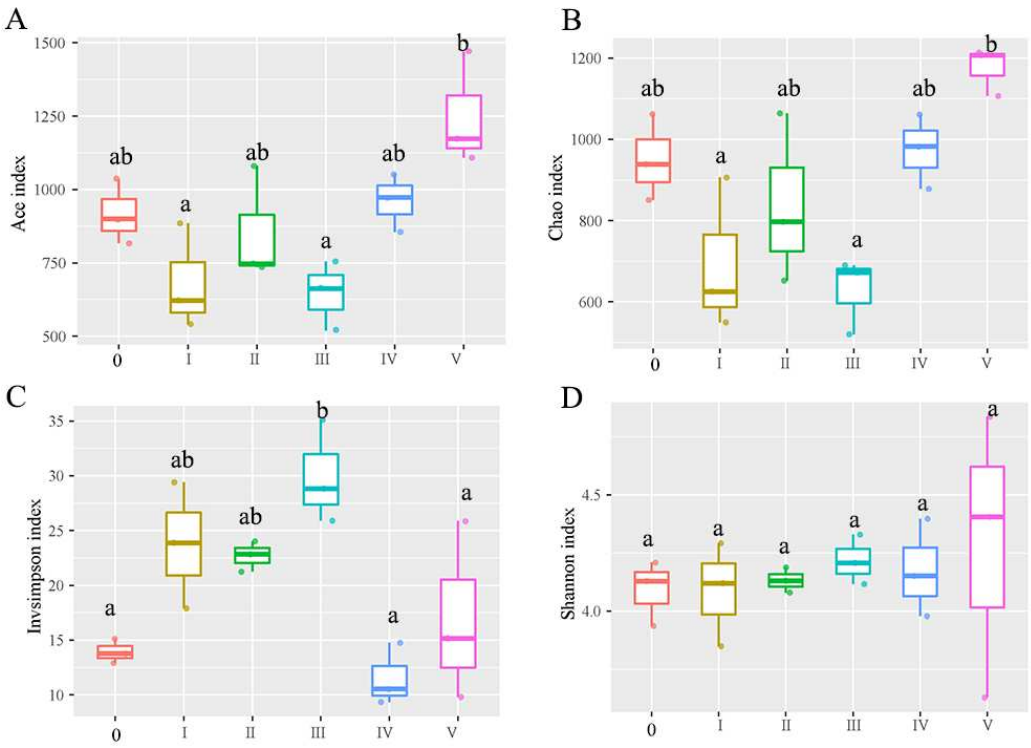

Figure 1. *Alpha diversity indices of nosZ clade I communities*

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Table 2. *Pearson correlation between physicochemical properties and alpha diversity of nosZ clade I communities*

	pH	NO_3^-	NH_4^+	AP[1]	AK[2]	TN[3]	OM[4]
Ace	-0.675**	-0.742**	-0.143	-0.327	-0.127	-0.124	-0.165
Invsimpson	0.239	0.358	-0.231	-0.028	-0.276	-0.209	-0.188
Shannon	-0.098	-0.137	-0.176	-0.180	-0.174	-0.191	-0.238
Chao	-0.563*	-0.708**	-0.018	-0.227	-0.027	-0.030	-0.077

* $P < 0.05$; ** $P < 0.01$
AP, available phosphorus; AK, available potassium; TN, total nitrogen; OM, organic matter

Beta diversity

Hierarchical clustering (at OTU level) revealed that *nosZ* clade I communities in IV and V samples were clearly distinguished from the other four samples, which were divided into two groups: I and III; and 0 and II (PERMANOVA, $P = 0.001$; *Fig. 2A*). Similarly, based on NMDS results, all 18 samples were separated into three groups: IV and V (red ellipse); 0 and II (green ellipse); and I and III (brown ellipse) ($P < 0.05$, *Fig. 2B*).

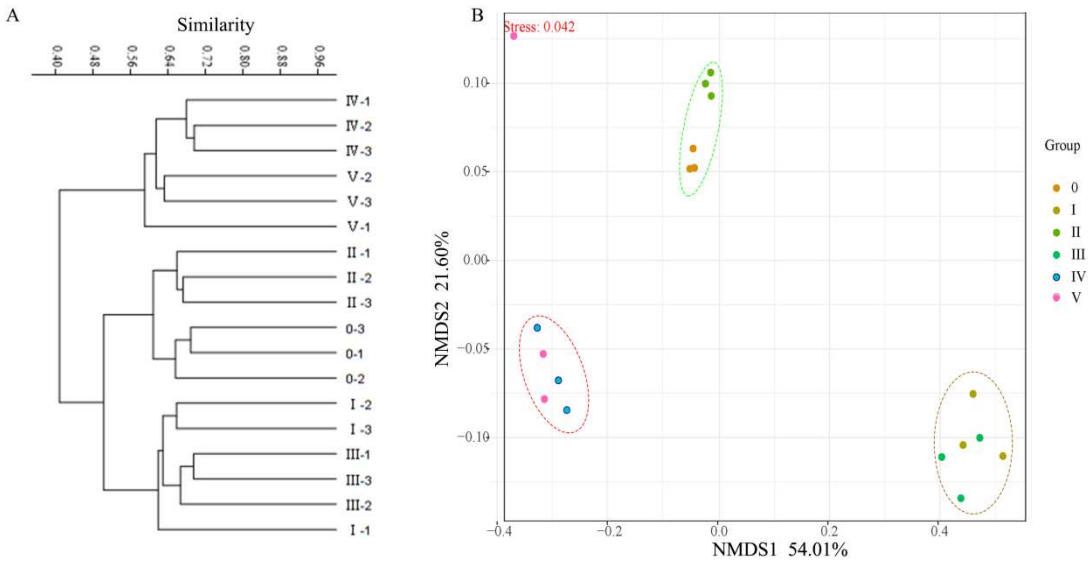

Figure 2. *Hierarchical clustering and NMDS of nosZ clade I communities based on all samples*

Taxa significantly different among samples

The sequence affiliated with the phylum Proteobacteria occupied the highest proportions (81.3–92.7%) of the *nosZ* sequences in wetland sediments, followed by Terrabacteria (0.02–0.5%) (*Table A2*). At the class level, different wetland stages had significantly different relative abundances of classes Alphaproteobacteria, Deltaproteobacteria, Actinobacteria, and Chloroflexi rather than Betaproteobacteria and Gammaproteobacteria. Notably, the proportion of Actinobacteria was lower in IV and V than in 0, I, II, and III. Similarly, the proportion of Chloroflexi was lower in III, IV, and V than in 0, I, and II (*Table A2*).

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At the family level, Bradyrhizobiaceae, which was the most abundant group, was lower in all five stages than in the ecological retention pond (*Fig. 3A*). However, the percentages of Rhizobiaceae, Burkholderiaceae, Gemmatimonadales, Brucellaceae, and Cytophagia were higher in all stages than in the ecological retention pond (*Fig. 3B–F,* respectively).

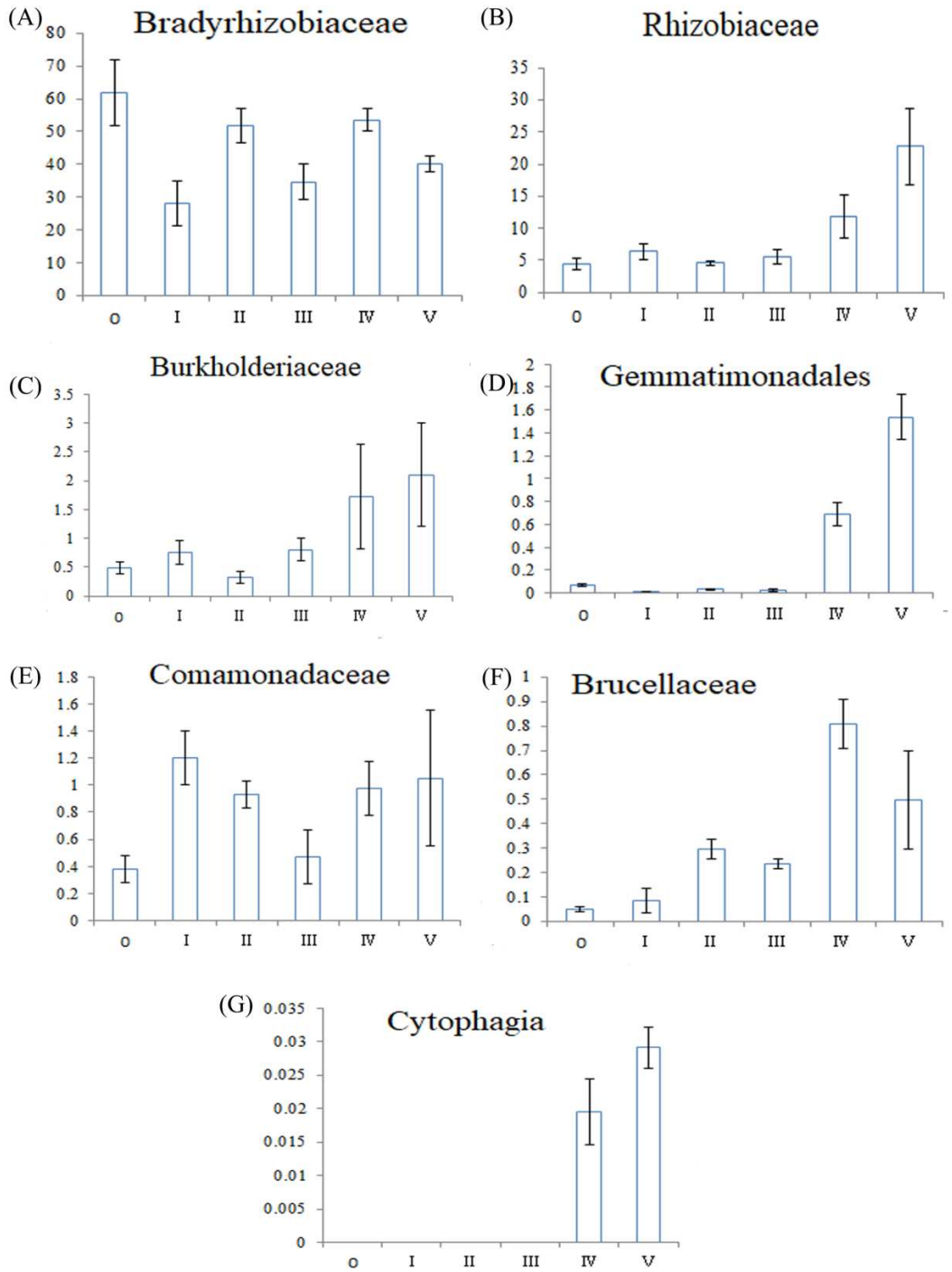

Figure 3. *Relative average abundances of the six most abundant families for different wetland stages. Error bars indicate the standard deviation of relative abundance between three replicate samples. The numbers on the ordinate are relative average abundances (%) of microbial families*

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A total of 32 OTUs significantly differed among the six sediment samples (*Fig. 4; Table A2*, $P < 0.05$). Among them, 30 OTUs belong to Proteobacteria, with 19 classified as Alphaproteobacteria, eight as Betaproteobacteria, and four as Gammaproteobacteria. Only OTU80 was affiliated with Actinobacteria, and OTU200 with Gemmatimonadetes (*Fig. 4*). The top two most abundant *nosZ* clade I genera were *Bradyrhizobium* and *Mesorhizobium*, with proportion ranges of 17.94–44.74% and 1.72–17.30%, respectively (*Table A3*).

For the most abundant genus *Bradyrhizobium*, abundance was significantly ($P < 0.05$) lower in all five stages (17.9–37.8%) than in the ecological retention pond (44.7%) (*Table A3*). *Nitrospirillum*, *Paracoccus*, Nocardioidaceae, *Oligotropha*, and *Ralstonia* showed the same trend as *Bradyrhizobium.* However, we observed significantly ($P < 0.05$) higher proportions of genera *Rhizobium* and *Polyangium* in all five stages than in the ecological retention pond (*Table A4*).

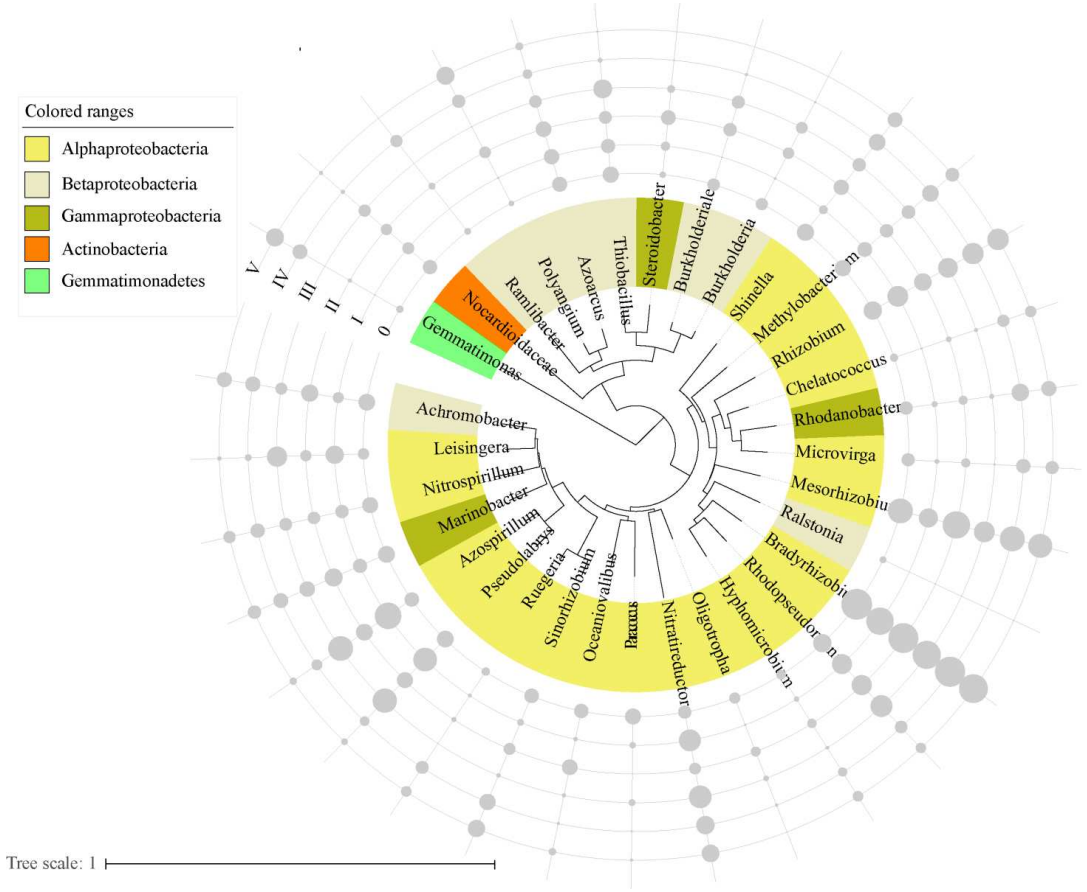

Figure 4. *Top OTU members of the nosZ I community enriched in wetland stages and the ecological retention pond. Gray circles represent the relative abundance of each OTU. Taxonomic dendrogram shows the inferred evolutionary relationship of the enriched microbiota of each sample. Total relative abundances of all OTUs and significant effects across soil compartments are listed in Table A3*

Correlation between physicochemical properties and nosZ I community

Of the 32 abundant OTUs, 22 were significantly correlated with more than at least one of the physicochemical properties (*Fig. 5*). *Ralstonia*, *Paracoccus*, *Oligotropha*,

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Nocardioidaceae, and *Nitrospirillum* were positively and significantly related with sediment contents of NH_4^+, AP, AK, TN, and OM. Of these 22 taxa, 10 had significant correlations with sediment content of NO_3^-, in which *Azospirillum*, *Rhodopseudomonas*, *Pseudolabrys*, *Nitratireductor*, and *Leisingera* were positively correlated and *Hyphomicrobium*, Polyangium, Gemmatimonadaceae, and Sinorhizobium_Ensifer were negatively correlated (*Fig. 5; Table A5*).

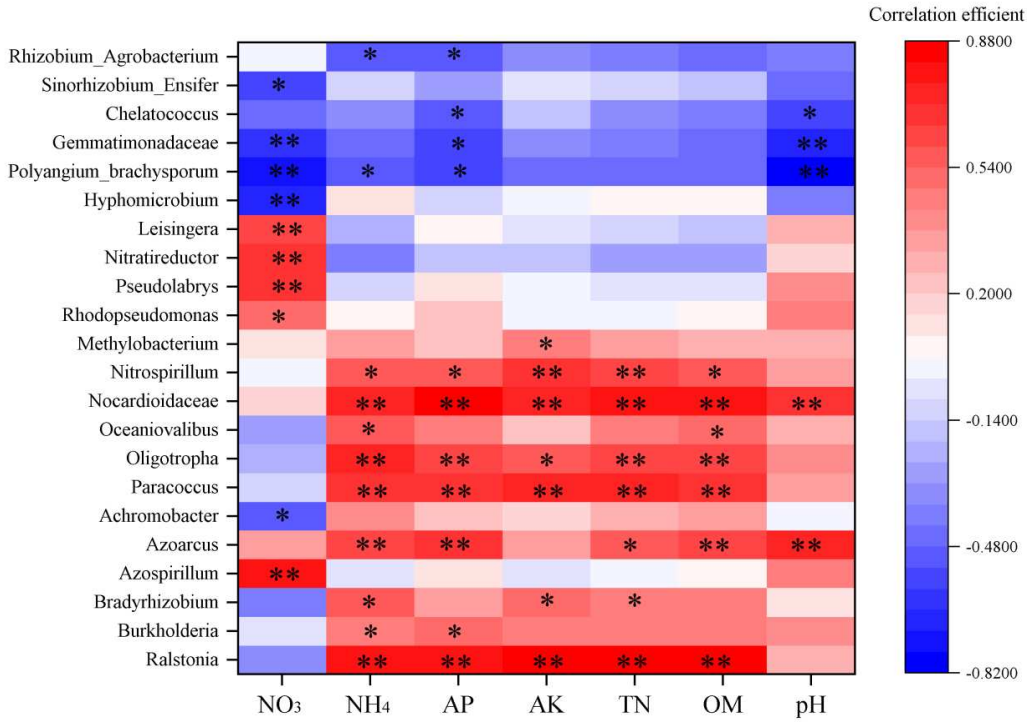

Figure 5. *Pearson correlations between 'X-enriched taxon' and physicochemical properties. *P < 0.05, **P < 0.01*

The RDA of physicochemical properties is shown in *Figure 6*. Seven properties explained 67.5% of the total variation of *nosZ* I communities. Sediment content of NO_3^- (explaining 24.2%, $P = 0.002$) was the most important factor shaping *nosZ* I communities, followed by pH (explaining 13.2%, $P = 0.004$) and NH_4^+ (explaining 9.6%, $P = 0.004$) (*Table A5*). All *nosZ* I communities in 18 samples were separated on the first axis into two groups: 0, I, II, and III (blue ellipse); and IV and V (green ellipse) (*Fig. 6*).

Discussion

Sediment physicochemical properties and adsorption

The obvious decrease in sediment content of NH_4^+, NO_3^-, AP, TN, and OM can be attributed to the absorption of these substances as nutrients by the four plants in CWs. *Arundo donax*, which favors high absorption and a high level of macronutrient translocation, has the ability to remove a greater quantity of nutrients per unit area in the pilot system (Leto et al., 2013). *Phragmites australis* was reported to remove NH_4^+-N, NO_2^--N, TN, and total P (Tomoko et al., 2002). *Nymphoides peltatum* and *Potamogeton crispus* (Li et al., 2008) have the potential to absorb NH_4^+-N and total P, respectively.

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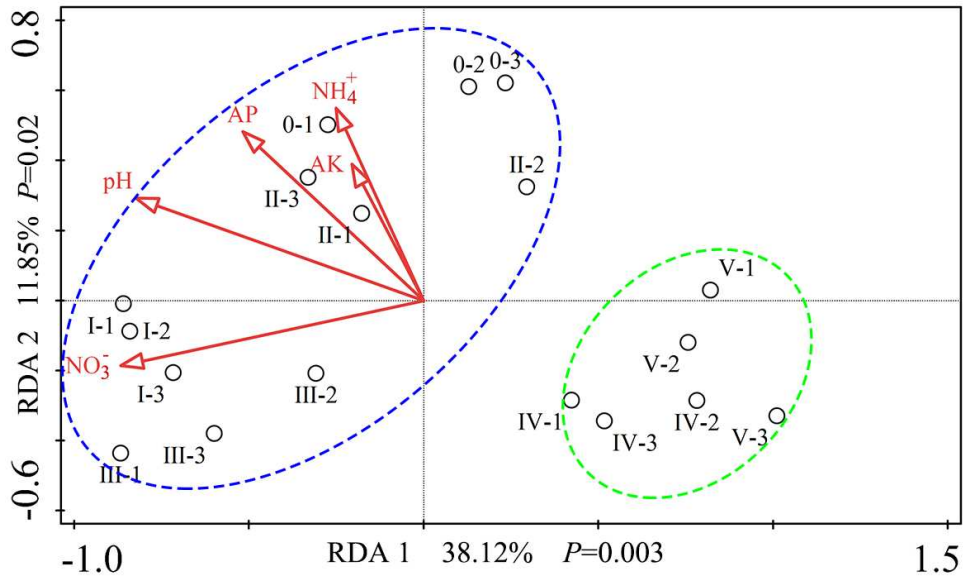

Figure 6. *RDA results between physicochemical properties and nosZ I community at OTU level*

The limited decline in pH value in wetland systems has been speculated to be due to the production of CO_2 resulting from decomposition of carbohydrates (i.e., plant residues), removal of various components of the wastewater retained in the plant root area (Leto et al., 2013), and nitrification of ammonia (Levinik-Hfferle et al., 2012). Studies showed that most horizontal subsurface-flow CW systems buffer wastewater, which is slightly acid or basic at the inflow and bring the pH values back to near neutral in an experimental site (Leto et al., 2013) and a pilot plant (Mitsch, 1997).

Richness and diversity of nosZ-denitrifier community

The slight but non-significant increase in alpha diversity indices of the *nosZ*-denitrifier community in the last stage of multi-stage CWs indicated linkage among the microbial community, treatment performance, and design of the CWs. Chen et al. (2020) stated that there was a negative relationship between the richness of denitrifying bacteria and the contents of NH_4^+-N and TN, but no such relation was detected in our study. We observed that richness indices were significantly and negatively related to sediment pH, consistent with results of Szukics et al. (2010). In contrast, Li et al. (2020) showed that pH was positively correlated with richness of *nos*Z-encoding denitrifier in mangrove wetlands.

Jha (2015) reported that bacterial denitrifier gene abundance was correlated with NO_3^-content in soils. We found a negative correlation between *nosZ*-denitrifier richness and NO_3^-, and soil NO_3^- was the most important single predictor of denitrification potential (Xiong et al., 2017). The above three results showed that NO_3^- has a very important effect on denitrifying processes.

nosZ-denitrifier community composition

The hierarchical clustering showed that the *nos*Z-denitrifying flora in sediments of stages IV and V significantly differed from the other four.

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Proteobacteria was the predominant phylum in all sediment samples. The abundances of Deltaproteobacteria – which are mainly involved in sulfate reduction (Wang et al., 2012b), can fix atmospheric nitrogen, and many denitrify (Lower and Bazylinski, 2013) in anaerobic conditions – were higher in stages IV and V. The most abundant genus *Bradyrhizobium* (within order Rhizobiales and class Alphaproteobacteria), which can act as sinks or sources for N_2O (Tian et al., 2020), has been reported to denitrify. Many *Bradyrhizobium* strains have truncated denitrification pathways lacking one or more of the steps, and all N_2O-reducing *Bradyrhizobium* strains prefer N_2O to NO_3^- as an electron acceptor (Gao et al., 2021). The proportion of *Bradyrhizobium* decreased in sediments of CWs compared to those in the ecological retention pond (*Fig. 4; Table A3*), indicating that the lower levels of N (NO_3^-, NH_4^+, and TN) to be metabolized in the sediment reduced the demand for *Bradyrhizobium* after purification of the CAWs.

Brucellaceae are Gram-negative bacteria that cause brucellosis, one of the most distributed zoonoses worldwide, and are transmitted to humans by contact with either infected animals or their products (Casabuono et al., 2017). Rhizobiaceae strains are reported to utilize a wide range of organic substrates, such as aromatic constituents present in polyphenols and lignin (Rich, 2003). However, Brucellaceae and Rhizobiaceae in Rhizobiales showed increased relative abundance in CAWs.

The obvious decrease in relative abundance of Nocardioidaceae (in phylum Actinobacteria; *Fig. 4*) in stages IV and V, which are all active in the degradation of recalcitrant chemicals, may indicate a reduction in the content of chemical pollutants (Zhang et al., 2020) in the water and sediment after the depuration by CWs. Whether this assumption is correct requires further verification.

Linking soil physical and chemical properties to nosZ- denitrifier communities

Soil pH has been shown to be important in the denitrifying bacterial community in pristine forest (Szukics et al., 2010) and wetland (Bowen et al., 2020) soils. The sediment pH was also found to be closely correlated with *nosZ*-denitrifiers in CW in this study in the following four ways. First, we found that water in the last stage (i.e., V) was purified in this experiment, resulting in a lower pH in sediment than for the other five groups. This may be the reason for the obvious reduction of alkalinity in stage V that led to the non-significant increase in diversity of denitrifying bacteria (*Fig. 2D*). Second, the negative correlation between pH and richness indices (ACE and Chao; *Table 2*) also confirmed that the decrease of pH increased the abundance index of denitrifying bacteria. Third, pH was significantly related to Nocardioidaceae, *Azoarcus*, *Chelatococcus*, Gemmatimonadaceae, and Polyangium_brachysporum (*Fig. 5*). Fourth, pH was the second most important factor ($P = 0.004$; *Table A5*) shaping *nosZ* community structure.

Nitrate pollution is responsible for algal blooms and eutrophication, which also pose public health risks (Beman et al., 2005) and are recognized as vital environmental threats (Tilman et al., 2002) by researchers and agricultural policymakers (Kramer et al., 2006). Kramer et al. (2006) showed the important link between reduced NO_3^- leaching and enhanced denitrifier activity and efficiency. In this study, we also found that NO_3^- content was negatively related to *nosZ*-denitrifier communities including richness indices (ACE and Chao, *Table 2*) and community structure ($P = 0.002$, *Table A5*). Additionally, 10 out of 22 OTUs were correlated with NO_3^- content (*Fig. 5*). However, Li et al. (2015) concluded that NO_3^- positively affected the *nirK* and *nirS*

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denitrifier community distribution in sediments of a *Myriophyllum elatinoides* purification system for treating swine wastewater.

Chen et al. (2020) confirmed that N salts (NH_4^+, NO_2^-, NO_3^-, and TN) are important factors affecting the composition and distribution of *nosZ*-denitrifiers in samples. This study is consistent with our results, confirming that the reduced N content in water changed the composition of denitrifying bacteria, i.e., the adaptation mechanism of bacteria in different habitats was associated with different environmental factors.

Conclusion

In this study, we investigated the denitrifying community structure of the *nosZ* clade I in a multi-stage surface-flow CAWs to obtain a holistic view of how they respond to constructed wetland. The physical and chemical properties, diversity of denitrifying microorganisms, and community structure of the sediments were changed. The CAWs can remove suspended solids in river water by physical precipitation, and remove N, P, and some OM by the biological action of algae, microorganisms, and aquatic plants. It is necessary to study denitrification rate and denitrification process at RNA level in the future. Our study provides microbial community data supporting the idea that CAWs have positive effects on the environment and promote more sustainable wetland ecosystems.

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Availability of data and material. The data of Sequence Read Archive (SRA) that support the findings of this study are available in GenBank of NCBI at [https://www.ncbi.nlm.nih.gov] under Accession no. PRJNA734135.

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APPENDIX

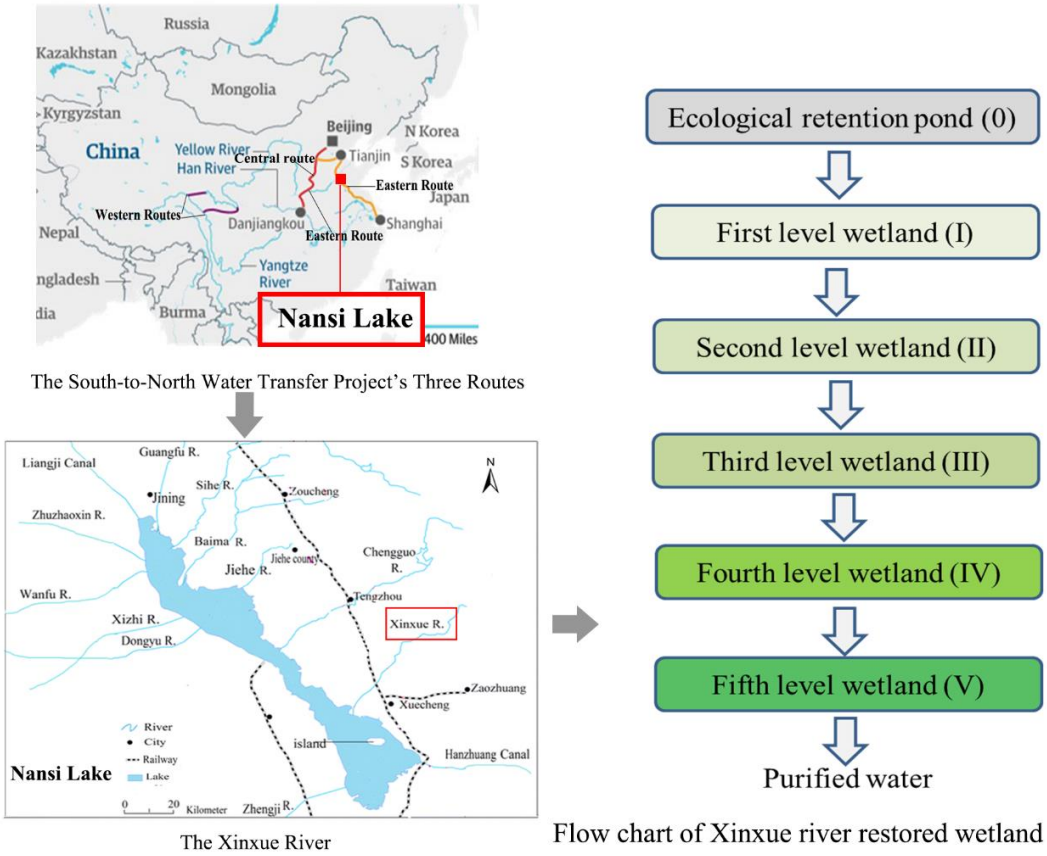

Figure A1. *Study site and experimental design*

Table A1. *Sequence information of nosZ clade I gene*

Sample	Rowdata	Final_clean	Percentm (%)	Goods coverage
0-1	164655	158355	96.17	99.2%
0-2	151354	146110	96.54	99.2%
0-3	220842	213293	96.58	99.0%
I-1	325573	307331	94.40	99.1%
I-2	28841	27879	96.66	99.6%
I-3	72431	68925	95.16	99.5%
II-1	149185	143253	96.02	99.4%
II-2	162141	154504	95.29	99.1%
II-3	240835	231836	96.26	99.3%
III-1	212982	206577	96.99	99.6%
III-2	69409	64853	93.44	99.4%
III-3	146448	140495	95.94	99.4%
IV-1	153800	145515	94.61	99.2%
IV-2	134196	126770	94.47	99.1%
IV-3	185788	176768	95.15	99.0%
V-1	54537	48365	88.68	99.1%
V-2	50561	47567	94.08	98.8%
V-3	48238	45685	94.71	98.6%

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Table A2. *The relative abundance (%) of different taxa in sediment groups*

Phylum	Class	0	Ⅰ	Ⅱ	Ⅲ	Ⅳ	Ⅴ	*P* value
Proteobacteria	Alphaproteobacteria	83.19 ± 1.2c	69.24 ± 5.26a	82.78 ± 0.93b	74.55 ± 1.65a	84.77 ± 2.23c	78.06 ± 2.27b	0.02
	Betaproteobacteria	5.42 ± 0.29a	7.78 ± 3.08a	5.12 ± 0.69a	7.9 ± 1.31a	4.78 ± 1.02a	6.82 ± 1.03a	0.06
	Gammaproteobacteria	4.02 ± 0.65a	4.21 ± 0.75a	5.06 ± 0.58a	5.25 ± 3.39a	2.81 ± 0.26a	5.93 ± 3.7a	0.07
	Deltaproteobacteria	0.02 ± 0.01a	0 ± 0a	0.01 ± 0.01a	0 ± 0a	0.1 ± 0.03b	0.1 ± 0.02b	0.008
Terrabacteria	Actinobacteria	0.49 ± 0.15c	0.24 ± 0.1ab	0.22 ± 0.02ab	0.26 ± 0.08b	0.02 ± 0.01a	0.02 ± 0.01a	0.005
	Chloroflexi	0.03 ± 0.01ab	0.1 ± 0.1b	0.03 ± 0.03ab	0 ± 0a	0 ± 0a	0.01 ± 0a	0.005
Unclassified_Bacteria	Unclassified_Bacteria	6.7 ± 0.6a	18.35 ± 6.2c	6.69 ± 1.0a	11.79 ± 5.6b	6.74 ± 0.9a	7.33 ± 0.9a	0.001

Table A3. *The proportion of enriched nosZ clade I taxa in each group*

	0	Ⅰ	Ⅱ	Ⅲ	Ⅳ	Ⅴ	Phylum	Class	Order	Family	Genus
Otu1	0.447395	0.179402	0.346458	0.21754	0.378721	0.263073	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Bradyrhizobium
Otu6	0.086991	0.01719	0.17302	0.027799	0.090421	0.068269	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	Mesorhizobium
Otu26	0.014236	0.027837	0.0114	0.020881	0.032925	0.033827	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Rhizobium_Agrobacterium_group
Otu16	0.014043	0.02252	0.023828	0.039114	0.002841	0.00151	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Rhodopseudomonas
Otu64	0.009985	0.000941	0.006041	0.003525	0.008886	0.005445	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Shinella
Otu45	0.009188	0.004384	0.012819	0.006407	0.005389	0.003643	Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	Marinobacter
Otu30	0.009073	0.001811	0.01048	0.002369	0.005876	0.005597	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Achromobacter
Otu138	0.009017	0.063023	0.007391	0.074398	0.001198	0.000471	Proteobacteria	Alphaproteobacteria	Rhizobiales	Xanthobacteraceae	Pseudolabrys
Otu74	0.007795	0.004501	0.003076	0.016509	0.000251	6.93E-05	Proteobacteria	Betaproteobacteria	Nitrosomonadales	Thiobacillaceae	Thiobacillus
Otu235	0.007754	0.00534	0.003702	0.003887	0.00879	0.003893	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	Methylobacterium
Otu153	0.007467	0.000427	2.78E-05	0.000488	6.97E-05	0.000111	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Paracoccus
Otu105	0.00745	0.003516	0.00277	0.003023	0.00461	0.002563	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	Nitrospirillum
Otu107	0.00572	0.006158	0.006806	0.002801	0.001156	0.000693	Proteobacteria	Betaproteobacteria	Rhodocyclales	Zoogloeaceae	Azoarcus
Otu286	0.005037	0.000472	0.002339	0.000209	0.007965	0.010442	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Sinorhizobium_Ensifer_group
Otu10	0.004858	0.139632	0.009159	0.085686	0.001769	0.000596	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	Azospirillum
Otu67	0.004692	0.000222	0.00682	0.000181	0.000111	0.000623	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Oceaniovalibus
Otu80	0.004636	0.002335	0.001976	0.002563	0.000195	0.000194	Actinobacteria	Actinobacteria	Propionibacteriales	Nocardioidaceae	Nocardioidaceae
Otu292	0.003466	0.037677	0.002158	0.047468	0.014356	0.011857	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	Nitratireductor
Otu103	0.003453	0.000376	0.000988	0.000265	0.011073	0.005872	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Rhodanobacteraceae	Rhodanobacter
Otu85	0.00334	0.00025	0.001044	6.97E-05	0.004582	0.003297	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	Microvirga
Otu87	0.002771	5.55E-05	0.003549	0.003273	0.000125	1.39E-05	Proteobacteria	Betaproteobacteria	Burkholderiales	Unclassified_Burkholderiales	Burkholderiales_Genera_incertae_sedis

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Otu32383	0.002521	9.6E-05	0.002074	0.002188	6.96E-05	0	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Burkholderia
Otu71	0.002005	0.014114	0.001921	0.024204	0.000488	0.000208	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Leisingera
Otu657	0.001852	6.92E-05	0.001308	0.000321	0.000125	2.77E-05	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Oligotropha
Otu91	0.001182	0.000292	0.0011	0.000153	0.001031	0.001593	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Hyphomicrobium
Otu165	0.000807	0.000166	0.000278	4.18E-05	0.003147	0.00162	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Ruegeria
Otu157	0.000752	6.94E-05	0.000334	0.000181	0.002563	0.002022	Proteobacteria	Alphaproteobacteria	Rhizobiales	Chelatococcaceae	Chelatococcus
Otu200	0.000571	5.57E-05	0.000195	2.78E-05	0.005403	0.012992	Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae	Gemmatimonas
Otu710	0.000501	0	5.57E-05	0	0	5.54E-05	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Ralstonia
Otu245	0.000473	0	0.000501	0	0.002548	0	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Ramlibacter
Otu120	0.000445	0.001054	0.005164	0.000683	5.57E-05	2.77E-05	Proteobacteria	Gammaproteobacteria	Nevskiales	Sinobacteraceae	Steroidobacter
Otu63	0.000306	6.92E-05	0.002186	4.18E-05	0.003442	0.012898	Proteobacteria	Betaproteobacteria	Burkholderiales	Unclassified_Burkholderiales	Polyangium_brachysporum

Table A4. *The Pearson correlation between 'X-enriched taxon' and physicochemical parameters*

	NO_3^-	**NH_4^+**	**AP**	**AK**	**TN**	**OM**	**pH**
Sinorhizobium_Ensifer_group	-0.549*	-0.129	-0.282	-0.076	-0.131	-0.183	-0.438
Rhodopseudomonas	0.494*	0.036	0.227	0.000	0.029	0.058	0.454
Rhizobium_Agrobacterium_group	0.040	-0.481*	-0.488*	-0.316	-0.416	-0.462	-0.368
Ralstonia	-0.331	0.790**	0.784**	0.858**	0.877**	0.827**	0.292
Pseudolabrys	0.707**	-0.138	0.115	-0.011	-0.055	-0.040	0.390
Polyangium_brachysporum	-0.728**	-0.484*	-0.574*	-0.440	-0.426	-0.456	-0.819**
Paracoccus	-0.139	0.680**	0.667**	0.758**	0.731**	0.669**	0.360
Oligotropha	-0.204	0.740**	0.639**	0.560*	0.648**	0.650**	0.382
Oceaniovalibus	-0.302	0.559*	0.463	0.235	0.435	0.491*	0.292
Nocardioidaceae	0.197	0.749**	0.867**	0.763**	0.811**	0.794**	0.686**
Nitrospirillum	0.014	0.581*	0.547*	0.678**	0.609**	0.557*	0.367
Nitratireductor	0.657**	-0.386	-0.149	-0.171	-0.285	-0.301	0.188
Methylobacterium	0.141	0.348	0.246	0.472*	0.334	0.293	0.293
Leisingera	0.614**	-0.221	0.045	-0.049	-0.124	-0.143	0.308
Hyphomicrobium	-0.698**	0.101	-0.091	-0.080	0.054	0.037	-0.396
Gemmatimonadaceae	-0.604**	-0.436	-0.540*	-0.347	-0.395	-0.444	-0.70**
Chelatococcus	-0.424	-0.328	-0.498*	-0.184	-0.324	-0.383	-0.548*
Burkholderia	-0.059	0.481*	0.523*	0.434	0.455	0.427	0.418
Bradyrhizobium	-0.387	0.555*	0.369	0.502*	0.471*	0.432	0.115
Azospirillum	0.790**	-0.054	0.142	-0.031	-0.017	0.045	0.450
Azoarcus	0.327	0.616**	0.665**	0.335	0.553*	0.646**	0.732**
Achromobacter	-0.485*	0.375	0.247	0.173	0.301	0.314	-0.018

*$P < 0.05$, **$P < 0.01$

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Table A5. *Forward selection results of RDA*

Name	Explains (%)	Contribution (%)	pseudo-F	*P* value
NO_3^-	24.2	35.6	5.1	0.002
pH	13.2	19.4	3.2	0.004
NH_4^+	9.6	14.2	3	0.004
AP	9.3	13.6	2.5	0.014
AK	5.5	8	1.3	0.202
TN	3.2	4.1	0.5	0.341
OM	2.5	1.6	0.7	0.732

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MULTI-SPECIES *BACILLUS* INOCULANT AND ITS EFFECT ON POTATO GROWTH AND CONTROL OF POTATO (*SOLANUM TUBEROSUM* L.) BLACKLEG DISEASE

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Abstract. Potato blackleg disease (PBD) is a common bacterial disease of potato, causing serious losses in potato (*Solanum tuberosum* L.) production. Using a probiotic biofertilizer that improves the structure of rhizosphere microbiota may be an effective way to prevent the disease. To develop a mixed *Bacillus*-based inoculant that would control PBD, active strains resistant to PBD were screened by the plates, and the fertilizer effect of the multi-species *Bacillus* inoculant on potato was tested in field experiment; the abundance of rhizosphere bacteria, actinomycetes, ammonifying bacteria, and nitrogen-fixing bacteria, and enzyme activities in potato leaves and rhizosphere were analyzed in the pot experiment. Our results showed that *Bacillus licheniformis* exhibited the most antagonistic effect on the PBD pathogen, followed by *Bacillus pumilus* and *Bacillus megaterium*. The highest yield of potato tubers was 2.05 kg/plant with the application of the multi-species *Bacillus* alternated with Gexi Tianzhuang fertilizer. The amendment also effectively improved the activity of defense enzymes in potato rhizosphere and leaves, and significantly changed the composition of potato rhizosphere microbiota. These results showed the multi-species *Bacillus* inoculant could effectively improve potato yield and had the potential to resist PBD.
Keywords: *compound agent, probiotic bio-fertilizer, microbial fertilizer, rhizosphere microbiota, soil*

Introduction

Potato (*Solanum tuberosum* L.) is one of the main crops worldwide (Bera et al., 2015). Soil-borne bacterial diseases (SBD) cause serious reductions in the yield of potato (Mao et al., 2019,2020). Potato blackleg disease (PBD) caused by *Pectobacterium atroseptica* is one of the most common SBD; it is transmitted mainly through the diseased potato seeds and soil. PBD occurs in all growth stages of potato. The infected plants grow slowly, the leaves turn yellow, wither, and fall off, and the stem base is brown to black soft rot, and extends upward (Cheng, 2020), which eventually leads to plant lodging, seeding shortage and loss, and in serious cases, the yield is reduced by 30 - 50% (Czajkowski et al., 2011; Mao et al., 2019). It propagates rapidly and is difficult to control, which seriously affects potato production (Mao et al., 2020). Although potato varieties resistant to PBD have been cultured, and thiazolidone, streptomycin sulfate, and other chemical agents were used to control PBD, the effect is not ideal.

Microbial fertilizers can improve the utilization of chemical fertilizers, reduce the occurrence of crop diseases, and decrease the use of pesticides, improving the agricultural ecology (Gharib et al., 2008; Ortíz-Castro et al., 2008; Mahdi et al., 2010; Haneef et al., 2014; Fan, 2017). *Bacillus* species are widely used in microbial fertilizers, biological control and environmental protection due to their advantages of high stress resistance and production of a variety of beneficial metabolites (Ongena et al., 2004; Liu, 2015). *Bacillus* can antagonize many common plant pathogens and effectively inhibit their growth (Ongena et al., 2004; Chen et al., 2007; Hu et al., 2019). Additionally, the high stress resistance of

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Bacillus makes it advantageous in the production and transport of microbial fertilizers, and survival in the harsh farmland environments (Ongena et al., 2004; Li et al., 2008).

To develop a multi-species *Bacillus* fertilizer to effectively prevent PBD and enhance growth, the activities and yields of defense enzymes and the dynamics of microbial communities in potato rhizosphere were characterized by pot and field experiments in this study. This study provides an important technical guidance for the ecological control of PBD.

Materials and Methods

Source of bacterial strains

Bacillus subtilis, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus pumilus*, *Bacillus mucilaginosus*, and *Bacillus amyloliquefaciens*, were isolated from the rhizosphere of different plants in the nursery and vegetable fields of Huizhou University, China. Pathogenic *Pectobacterium atroseptica* MB001 was isolated from Jiuhua Potato Base located in Tieyong Town, Huizhou City, China. The strains were grown on nutrient agar (NA) medium. *B. mucilaginosus* was inoculated into a silicate liquid medium (Huankai Bio-Science, China). Other strains were inoculated into the lysogeny broth (LB) liquid medium (Huankai Bio-Science, China), and were cultured at 28 °C with 120 r/min for 18 h.

In vitro experiments

Antagonism of Bacillus strains to the PBD pathogen

The bacterial plates of *P. atroseptica* MB001 were prepared by gradient dilution. The pathogenic strain was cultured at 28 °C for 24 h, and the optical density (OD) of culture suspensions reached 1.0. After 100 μL of the suspension was evenly spread on LB solid culture medium, filter paper pieces soaked with 5 μL of *Bacillus* suspensions were placed on the medium. The antagonistic effect of *Bacillus* strains to the pathogenic strain was judged based on the size of the bacteriostatic halo zone. Each treatment contained three replicates.

Determination of nitrogen fixation, phosphorus release and potassium release by Bacillus strains

Each *Bacillus* strain was cultured in organic phosphorus medium, inorganic phosphorus medium, silicate bacterial fermentation medium, and nitrogen-free medium in three replicates. The effect of phosphorus and potassium release and nitrogen fixation was judged from the growth of each *Bacillus* strain on the plates (Song, 2015).

Field experiments

Fertilizer effect of multi-species Bacillus strains

The multi-species *Bacillus* (FH) inoculant was a liquid microbial preparation composed of equal proportions of the six *Bacillus* species, with a live bacterial content of 1×10^8 CFU/mL. The tested potato variety was Holland No. 7. The fertilizer effect of the multi-species *Bacillus*, Gexi Tianzhuang fertilizer (GT; Tanghua Shiye, China), and LvShuo No. 1 fertilizer (LS; LvShuo Bio-Tech, China) was characterized in the field experiment by comparing the average yield per potato plant. The GT was a water-soluble fertilizer. The

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ratio of nitrogen (N), phosphorus (P) and potassium (K) in the GT was 1:1:1. N, P_2O_5 and K_2O contents were greater than or equal to 50% w/w; and the total content of cuprum (Cu), ferrum (Fe), manganese (Mn), zinc (Zn), boron (B), and molybdenum (Mo) was 0.3%-3.0% w/w. The LS was an organic fertilizer with spores of various microbes, and contained fulvic acid, alginic acid, mixed microbes (*Bacillus belesii*, *Bacillus subtilis*, and *Bacillus licheniformis*), soybean powder, and trace elements. The effective viable bacteria content is 5×10^8 CFU/mL.

The field experiment was conducted in the Experimental Base of Huizhou University (114.692 E, 23.067 N; altitude is 30 m). It is located in subtropical monsoon climate. The experimental field area was approximately 300 m^3. The experiment adopted a randomized grouping design, with 5 treatment groups and 1 control group, and each group had 3 repetitions. The area of each repetition was approximately 50.0 m^2. Potatoes were planted on ridges, with a length of 15 m, a width of 1.2 m and a plant spacing of 0.3 m. Approximately 100 plants were planted in each repetition. The plant density was 2 plants/m^2. The potatoes were sowed on November 23, 2017. When sowing, the seed potatoes were cut longitudinally, and each potato block had bud eyes. Then the potato blocks were sowed to ridges with 5 cm depth. After sowing, the ridges were covered by 5 cm of straw and soil, and then covered with black plastic film to keep warm, moisturize, and prevent weeds. On or about January 3, 2018, potato seedlings emerged successively. After emergence, the seedlings were pulled out of the plastic film manually, and the potato field was irrigated for 3 times. When the potato plants grown to 6 - 8 leaves (on January 13, 2018), apply corresponding fertilizers to each group according to the fertilization scheme shown in *Table 1*. The FH fertilizer was applied at 20 mL to each plant. Other fertilizers were applied according to the manufacturer's instructions. The rhizosphere soil pH was 5.81 ± 0.09. Content of organic matter, alkali-hydrolysable nitrogen, available phosphorus, and available potassium in the soil were 118.98 ± 1.50 g/kg, 130.22 ± 7.02 mg/kg, 31.02 ± 1.41 mg/kg, and 66.99 ± 10.07 mg/kg, respectively. These soil chemical indices were determined as described previously (Mao et al., 2020). Pathogenic *P. atroseptica* MB001 culture medium (2×10^7 CFU/mL) was sprayed on potato plants with a watering can for three times on January 29, February 1, and February 4, 2018, with an average of about 3 mL per plant each time. Routine field management and observation of potato plants were carried out after February 4, 2018 (Vashisht et al., 2015). The potatoes were harvested on March 15, 2018 (*Fig. 1*), and the average yield was determined by weighing tubers of 6 potato plants in each of three replicates. Moreover, the infection rate of each group was counted.

Table 1. *The experiment design of fertilizer effect of multi-species Bacillus inoculant*

Treatment	First day	Third day	Fifth day	Seventh day	Nineth day
GT	GT	GT	GT	GT	GT
GL	GT	LS	GT	LS	LS
FH	CW	MSB	CW	MSB	MSB
GF	GT	MSB	GT	MSB	MSB
LS	CW	LS	CW	LS	LS
BC	CW	CW	CW	CW	CW

GT fertilizer was diluted to 300 times with clear water to irrigate the root of potato; LS fertilizer was diluted to 100 times with clear water to irrigate the root of potato; The compound Bacillus was diluted to 200 times with clear water to irrigate the root of potato. MSB, multi-species Bacillus; CW, clean water; GT, GT fertilizer; GL, GT fertilizer and LvShuo No. 1 fertilizer; FH, multi-species Bacillus inoculant; GF, GT fertilizer and multi-species Bacillus inoculant; LS, LvShuo No. 1 fertilizer; BC, Blank control

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Figure 1. *Photos of field experiment. (A) photo during fertilization; (B) pre-harvest photo*

Determination of enzyme activities in potato rhizosphere soil and leaves

After the last fertilization of potato at the seeding stage, three plants were randomly selected from each group to measure the urease and saccharase activities in the rhizosphere soil of potato at the third day, sixth day, nineth day, and 12th day after fertilization. The activities of peroxidase (POD), superoxide dismutase (SOD), catalase (CAT), polyphenol oxidase (PPO), and phenylalanine ammonialyase (PAL) in potato leaves were measured at the sixth day after fertilization according to the previous description (Lin et al., 2010; Yang, 2014).

Rhizosphere microbiome composition

The total DNA of rhizosphere microbiota was extracted from three parallel soil samples before (PecQ) and after (PecH) infection with *P. atroseptica* MB001 as well as from the non-infected control using the improved CTAB method (Ni et al., 2017) and was purified as our previously described (Mao et al., 2019). The 16S rDNA V4 - V5 hypervariable region was amplified using the 515F and 909R primers according to the published reports (Huang et al., 2018). Polymerase chain reaction products were purified and pooled together at equal molar amounts from each sample as previously described (Ni et al., 2019), and then sequenced using a MiSeq system (Illumina, USA) at Guangdong Meilikang Bioscience, Ltd., China.

The raw reads were merged using FLASH 1.2.8 software to get merged sequences (Magoc and Salzberg, 2011), and the low-quality merged sequences and chimeric sequences were filtered out as previously descripted before further analysis (Ni et al., 2019). The high-quality sequences were clustered into operational taxonomic units (OTUs) at 97% identity using UPARSE (Edgar, 2013). The phylogenetic taxon of each OTU was assigned using the Ribosomal Database Project classifier (Wang et al., 2007) with greengene gg_13_8_otus dataset.

Data analysis

The data were showed as the mean ± standard error. One-way analysis of variance (ANOVA) with Tukey-Kramer post-hoc test was conducted using R 3.5.1 (Dixon, 2003). Correspondence analysis (CA), principal component analysis (PCA) and non-parametric multivariate analysis of variance (PERMANOVA) (Anderson, 2001) were conducted using the vegan package of R 3.5.1 (Dixon, 2003). The Kruskal-Wallis test was conducted using

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the Statistical Analysis of Metagenomic Profiles software. Box plots were drawn using the ggpubr R package. $P < 0.05$ was considered significantly different.

Results and Discussion

In vitro experiments

Antagonistic effect of Bacillus against P. atroseptica

Previous studies showed that *Bacillus* inhibited the growth of pathogenic bacteria and fungi in soil to prevent soil borne diseases of plants (Cai et al., 2012; Patel et al., 2015; Gond et al., 2015). We found in present study that the antagonistic effects of various *Bacillus* species to *P. atroseptica* MB001 were *B. licheniformis* > *B. pumilus* > *B. megaterium* > *B. subtilis*, with the diameters of their bacteriostatic halos being 14.32 ± 0.53 mm, 13.72 ± 0.12 mm, 11.60 ± 0.23 mm, and 2.28 ± 0.10 mm, respectively (One-way ANOVA, $p < 0.001$; *Fig. 2*). *B. amyloliquefaciens* and *B. mucilaginosus* were not antagonistic to *P. atroseptica* MB001 (*Fig. 2*).

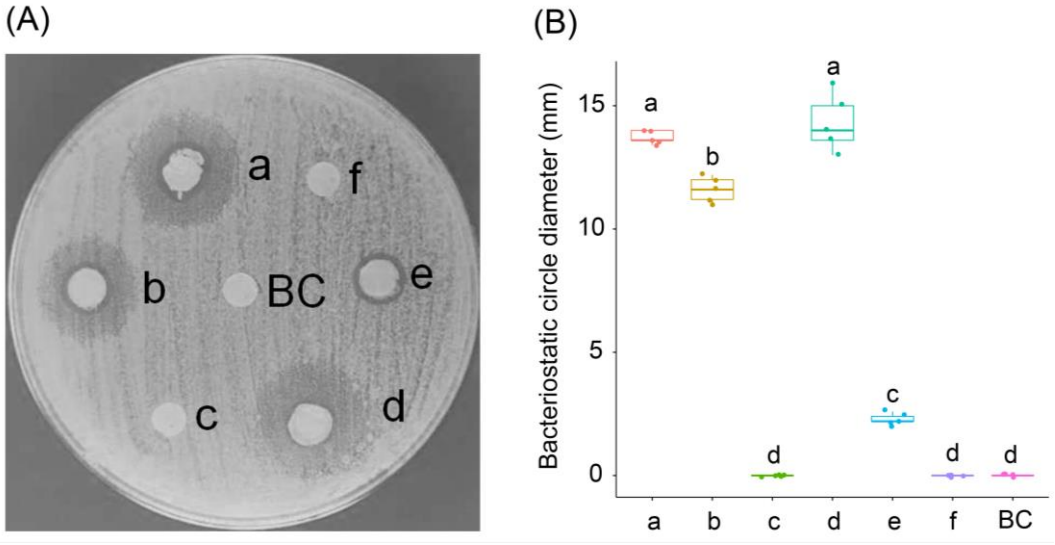

Figure 2. *Inhibition of Pectobacterium atroseptica by Bacillus pumilus (a), Bacillus megaterium (b), Bacillus amyloliquefaciens (c), Bacillus licheniformis (d), Bacillus subtilis (e), Bacillus mucilaginosus (f), and blank control (BC). Different letters above the boxplots indicate significant differences*

Nitrogen fixation and phosphorus and potassium release by Bacillus strains

Probiotics are used extensively in crop pest control and for improving yield because of their safety and environmental friendliness (Berendsen et al., 2012; Patel et al., 2015; Marcano et al., 2016). *Bacillus* is used widely in developing biological fertilizers because it can fix nitrogen, and solubilize unavailable forms of phosphorus and potassium to make them available (Glick, 2012; Patel et al., 2015; Fan, 2017; Prabhukarthikeyan et al., 2018). Our results showed that *B. licheniformis* and *B. mucilaginosus* could grow on the four types of selective media, which showed that they had the capacity to fix nitrogen and dissolve phosphorus and dissolve potassium. *B. licheniformis* and *B. mucilaginosus* were the only species with the capacity to dissolve potassium. *B. subtilis* could not use organic

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phosphorus, fix nitrogen or dissolve potassium, but had a strong capacity to use inorganic phosphorus. These results implied that the multi-species *Bacillus* amendment could not only prevent and control PBD, but also promoted the growth of potato (*Table 2*).

Table 2. *Growth of different Bacillus strains on nitrogen free medium, inorganic phosphorus medium, organophosphorus medium, and silicate bacterial fermentation medium*

Bacillus strains	Nitrogen free medium	Inorganic phosphorus medium	Organophosphorus medium	Silicate bacterial fermentation medium
B. lincheniformis	+	+	+	+
B. subtilis	-	+	-	-
B. pumilus	+	+	+	-
B. megaterium	+	+	+	-
B. mucilaginosus	+	+	+	+
B. amyloliquefaciens	+	+	+	-

"+" indicates the strain could grow on the medium; "-" indicates the strain could not grow on the medium

Field experiments

Effects of multi-species Bacillus inoculant on potato yield, rhizosphere soil and leaf enzyme activities

The potato yields of the treatment groups were significantly higher (one-way ANOVA, $F = 112420$, $p < 0.001$; *Fig. 3A*). The GF treatment had the highest yield, followed by the GL treatment (*Fig. 3A*). The infection rate of the GF treatment was the lowest, followed by the FH and GL treatments. The infection rate of BC was the highest (one-way ANOVA, $p < 0.001$; *Fig. 3B*).

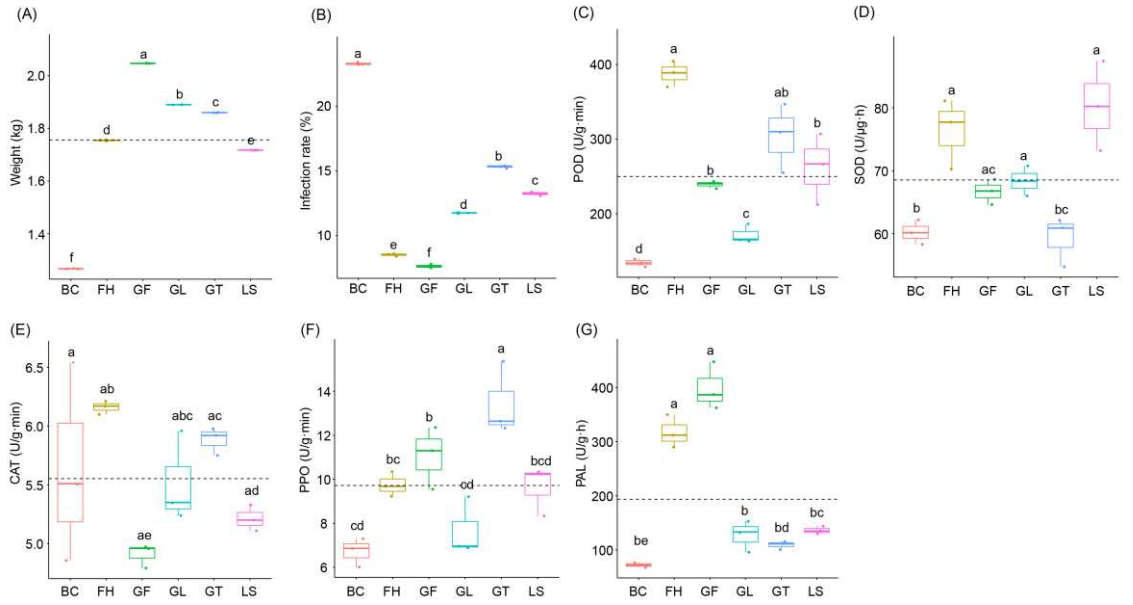

Figure 3. *Average yield per plant (A) and activities of peroxidase (B), superoxide dismutase (C), catalase (D), polyphenol oxidase (E), and phenylalanine ammonialyase (F) in potato leaves. GT, GT fertilizer; GL, GT fertilizer and LvShuo No. 1 fertilizer; FH, Compound Bacillus; GF, GT fertilizer and Compound Bacillus; LS, LvShuo No. 1 fertilizer; BC, Blank control. Different lower-case letters above the box diagrams show the significant difference among the treatments*

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B. amyloliquefaciens was reported to induce defense gene expression, including pathogenesis related protein 1 and pathogenesis related protein 4, which act against fungal pathogens (Gond et al., 2015). Thuricin 17 and bacthuricin F4 purified from *Bacillus* strains induced defense-related enzymes in soybean leaves (Jung et al., 2011). The activities of POD, SOD, PPO, and PAL in potato leaves in the FH and LS treatments were significantly increased compared with the control group in present study. The potato leaf activities of POD and SOD in the GL treatment, and those of POD, PPO and PAL in the GT treatment, were also increased significantly (*Fig. 3C-3G*). According to the results of enzyme activities of potato leaves, the FH treatment improved them to a greater extent than the other treatments.

Sucrase can hydrolyze sucrose into glucose and fructose that are easy to be absorbed and utilized in soil, and can increase soluble nutrients in soil, which is one of the important indicators characterizing the biochemical activity in the rhizosphere soil (Li et al., 2012). Soil urease is the key enzyme of nitrogen transformation in soil, and urease activity is often used to characterize the nitrogen status of soil and evaluate soil fertility (Kang et al., 2017). In this study, urease and saccharase activities of potato rhizosphere soil in each treatment were significantly higher, except for urease activity in the FH treatment on day 12. However, the changes in the urease and saccharase activities differed among the treatments (*Fig. 4*). In the GL treatment, the urease activity reached the peak on the sixth day after fertilization, those in the GF and GT treatments reached the peak on the ninth day after fertilization, whereas those in the LS and FH continued to decrease from the third day after fertilization (*Fig. 4A*). The saccharase activity in the GL treatment reached the peak value on the 6th day after fertilization, that in the GT reached the peak value on the 9th day after fertilization, and other treatments maintained relatively stable saccharase activity (*Fig. 4B*).

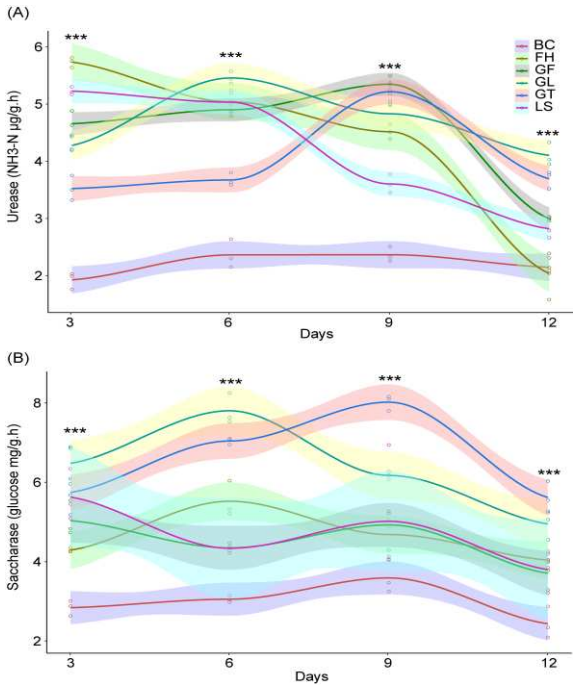

Figure 4. *Changes in urease (A) and saccharase (B) activities in the potato rhizosphere soil. GT, GT fertilizer; GL, GT fertilizer and LvShuo No. 1 fertilizer; FH, Compound Bacillus; GF, GT fertilizer and Compound Bacillus; LS, LvShuo No. 1 fertilizer; BC, Blank control. ***, $p < 0.001$*

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Changes in bacterial abundance and composition in the rhizosphere soil

Rhizosphere microbiota is vital in plant disease resistance (Mao et al., 2019, 2020). However, no significant difference was detected in bacteria, actinomycetes, ammonifying bacteria, and nitrogen-fixing bacteria abundances in the rhizosphere soil during the experiment (one-way ANOVA, $p > 0.05$; *Fig. 5*).

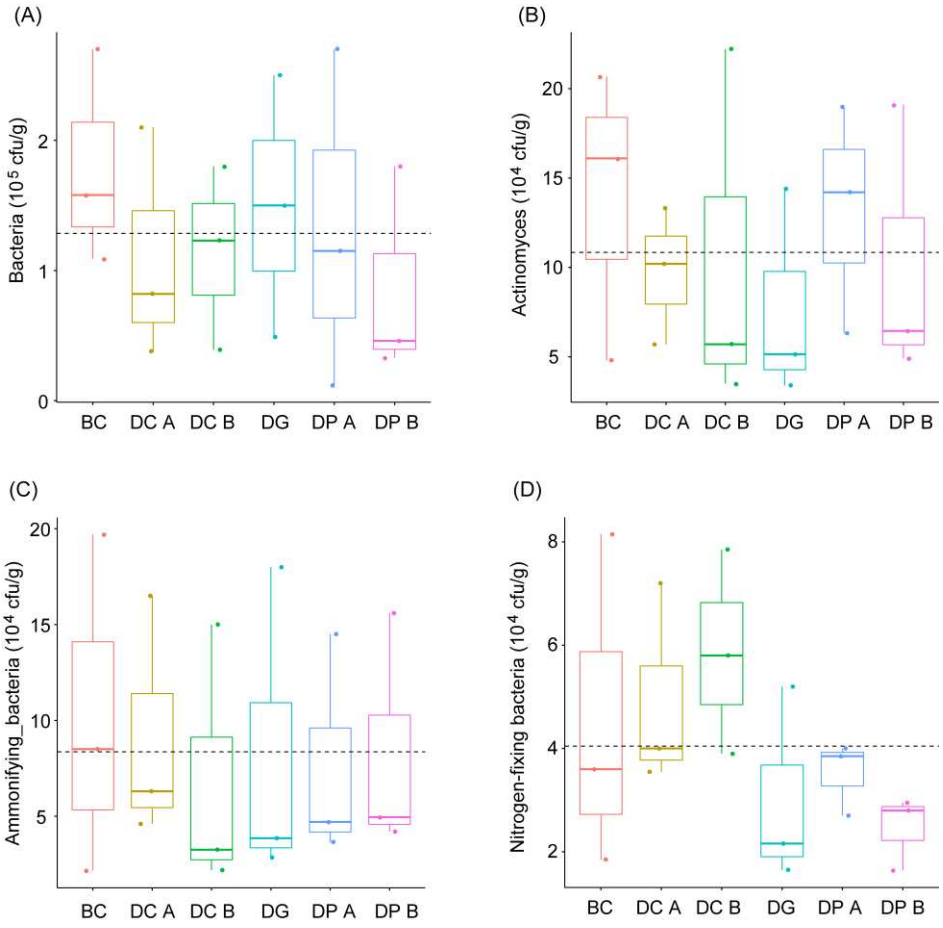

Figure 5. *Amount changes of microorganisms in potato rhizosphere. DC, disease cure group; DG, disease group; DP, disease prevention group; BC, blank control*

Total of 402,392 (44,710 ± 2,713) high-quality sequences were obtained from the nine rhizosphere samples. Finally, 33,004 sequences were randomly resampled from each sample for subsequent analyses. These sequences were classified into 15,619 OTUs. The Shannon index (one-way ANOVA, $F = 32.63$, $p < 0.001$; *Fig. 6A*) and Chao1 coverage (one-way ANOVA, $F = 8.025$, $p = 0.02$; *Fig. 6B*) of rhizosphere microbiota were significantly higher in the treatments. The bacteria were classified into 56 phyla. However, only 11 phyla dominated the microbiomes (*Fig. 6C*). Proteobacteria was significantly reduced in the control than PecQ and PecH, whereas Actinobacteria in BC was significantly enhanced (*Fig. 6D*).

CA and PERMANOVA based on the compositions of all genera and dominant genera in the rhizosphere microbiome showed that there were significant differences in the rhizosphere soil microbiota between the treatments and the control (PERMANOVA,

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F = 3.52, p = 0.015 for all genera, and F = 4.73, p = 0.025 for dominant genera; *Fig. 7A* and *7B*). The LefSe and heatmap results showed that *Carnobacterium* was significantly enriched in the multi-species *Bacillus*-amended rhizosphere soil microbiome before infection with *P. atroseptica* MB001, whereas *Mesorhizobium*, *Aquicella* and *A17* were significantly enriched after the infection (*Fig. 7C* and *7D*). The bacteria with altered abundances were related to plant diseases (Young et al., 2014; Town et al., 2016; Wang et al., 2018). However, the effects of the change in the rhizosphere microbiome on the occurrence of PBD needs further experimental verification.

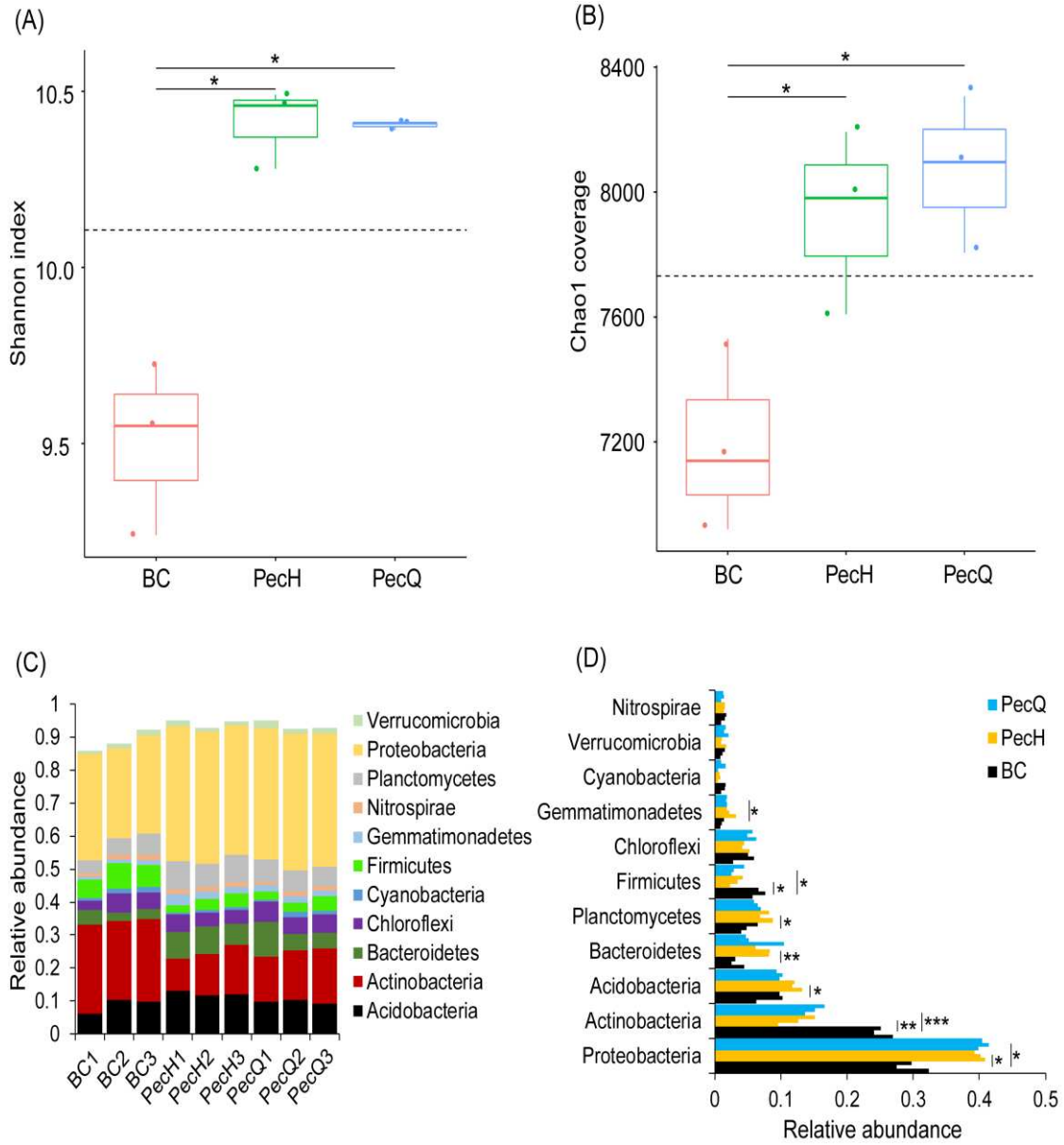

Figure 6. *The Shannon index (A), Chao1 coverage (B), dominant phylum compositions (C), and dominant phylum differences in the rhizosphere microbiome among different treatments (D). BC, blank control; PecH, the samples before infection with Pectobacterium sp. MB001; PecQ, the samples after infection with Pectobacterium sp. MB001. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$*

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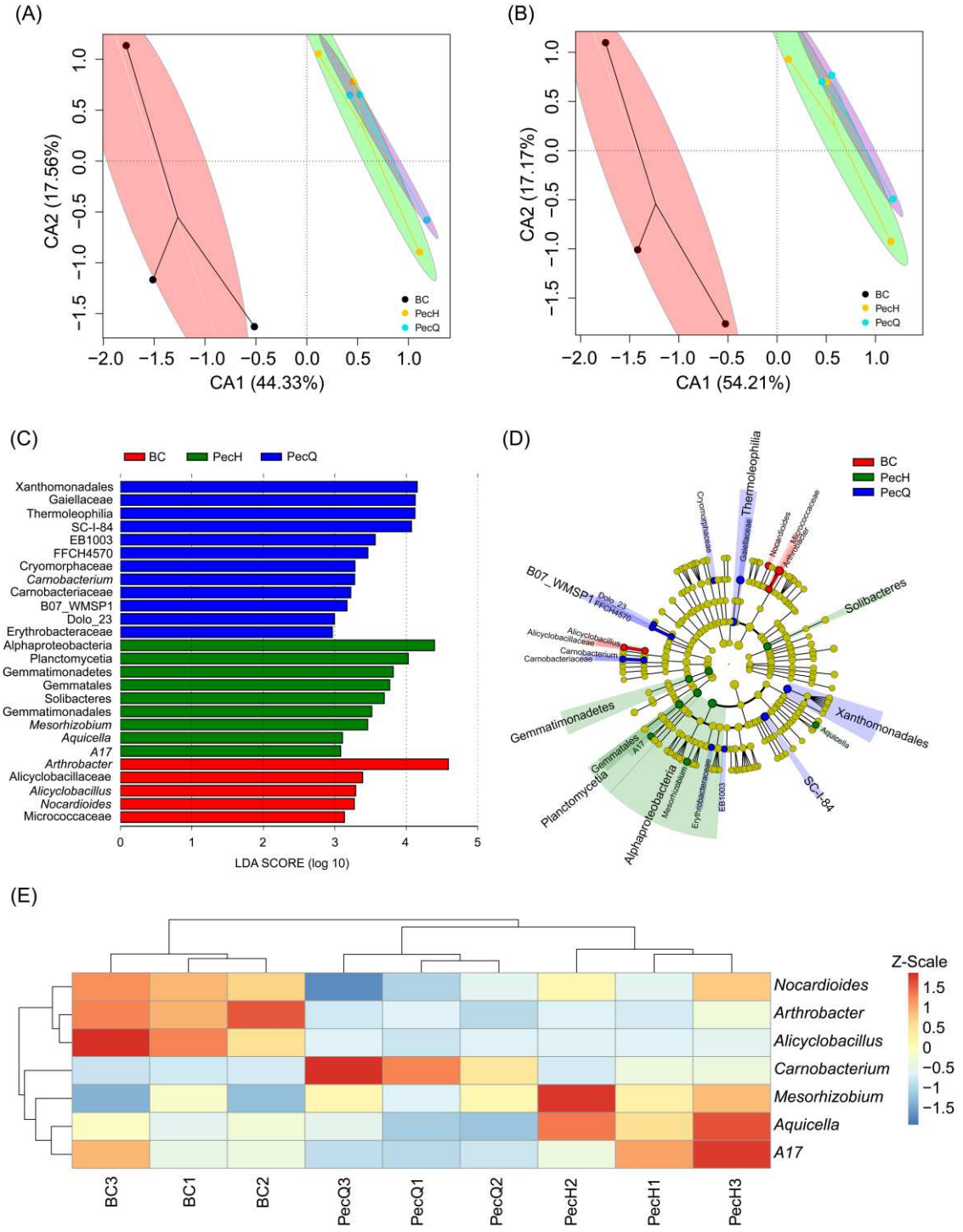

Figure 7. *Changes in microbial composition in the potato rhizosphere. (A), CA profile for all the genera in the potato rhizosphere microbiome; (B), CA profile for the dominant genera in the potato rhizosphere microbiome; (C), LEfSe results; (D), Cladogram profile of LEfSe results; (E), Heatmap of the significantly different genera among different treatments. BC, blank control; PecH, the samples before infection with Pectobacterium sp. MB001; PecQ, the samples after infection with Pectobacterium sp*

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Conclusion

We constructed a multi-species *Bacillus* inoculant and showed that it increased potato yield and the potential PBD resistance, and also changed the composition of the potato rhizosphere microbiome. A larger area of field experiment, the impact of large-scale production and storage, and the molecular mechanism of preventing PBD of the multi-species *Bacillus* inoculant on its function need to be further studied in the future.

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IN VITRO ANTIBACTERIAL, ANTIDERMATOPHYTE AND ANTICANDIDA POTENTIALS OF DIFFERENT ORGANIC EXTRACTS AND TOTAL ALCALOIDS OF INDIAN THORN APPLE (*DATURA METEL* L.) FROM TUNISIA

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Abstract. Leaf and seed extracts of *Datura metel*, a species known as a folkloric medicinal plant in Tunisia, were prepared with different solvents: PE, CH_2Cl_2, ACOEt and MeOH. Both the leaves and seed samples contained alkaloids, tannins, saponins, Iridoids, and flavonoids. Extracts were tested in vitro for antimicrobial activity against 9 fungi and 11 bacteria. Results have shown that different extracts have various levels of inhibition against all tested bacterial species (with MIC varied from 0.625 to 10 mg/mL). Differences in antibacterial activities between seed and leaf extracts were also observed. The highest inhibition potentials were shown for *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus subtilis* strains. Most fungi are quite resistant to PE and CH_2Cl_2 extracts at concentrations lower than 500 µg/mL, while the ACOEt and MeOH extracts demonstrated the greatest antifungal potential. The MIC against *Aspergillus fumigatus,* was found to be the lowest (62.5 µg/mL). The most promising results were shown against *Candida albicans,* which was totally inhibited by 250 µg/mL of both ACOEt and MeOH extracts. Findings from this study highlight not only the rich antibacterial potentials of *D. metel*, but also its very effective antidermatophytic and anticandidose activities, thus recommending the use of this plant against certain skin diseases.
Keywords: *antimicrobials, antifungal activity, organic extracts, phytochemical screening, organ variability*

Introduction

The emergence of newer multidrug resistant pathogens is threatening the clinical efficacy of existing antimicrobials. A lot of interest is given nowadays to new active substances with potent antimicrobial activity in all over the word. Polyphenols derived from plants and spices have been shown to have antimicrobial properties that could be used as a replacement for antibiotics (Luis et al., 2014; Khameneh et al., 2019). *Datura metel*, also known as Indian Thorn apple in Europe, is a member of the Solanaceae family, which includes over 100 genera and 3000 plant species. It is a worldwide shrub that can

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be cultivated for its chemical and ornamental properties. In traditional medicine, the plant is used widely. In Nigeria for example, *D. metel* is administered in case of cough, asthma convulsion and insanity. In other countries, it was used to treat skin diseases and burns, animal bites, cerebral as well as herpetic problems and diarrhoea. Mixed with other plants it is used as cataplasm against eczema (Priya et al., 2002; Dabur et al., 2004; Alam et al., 2021). Traditional Chinese medicine has used the plant for centuries to treat asthma, pain, convulsions, rheumatism, and antibronchitis (Xia et al., 2019). In Tunisia, *D. metel* is a spontaneous herb. Le Floch (1983) reported that the Arab physicians use this species for various uses: against insomnia, hair loss and injury oedema. The leaves are a source of scopolamine, used as nerve sedative, antiparkinsonian and in combination with morphine as a pre-anesthetic. However, according to many Pharmacopeia, it is extremely recommended that the plant should not be administered as ingestion form (Spadari et al., 2008).

Keeping in view the traditional beliefs, several research studies have investigated the antimicrobial activities of different aerial parts of *D. metel* and have documented promising antimicrobial effects (Sakthi et al., 2011; Rinez et al., 2013; Lim et al., 2020; Bawazeer and Rauf, 2021).

To our knowledge, none of the previous work however has considered tests on the seed extracts of the plant. In the same way, surprisingly, the antibacterial and antidermatophyte properties of Tunisian *D. metel* extracts have not been studied. As a result, this is the first attempt to determine the in vitro antimicrobial activity of different organic extracts from seeds, leaves, and total alkaloids from Tunisian *D. metel* roots against a variety of strains of bacteria, dermatophytes, hyphamycets, and one opportunist pathogenic yeast.

Material and methods

Plant material collection

D. metel was found in the Monastir region (35°46'N, 10°50'E) of Tunisia's central-east. Separately, the leaves, seeds, and roots were washed with tap water and dried in the shade at room temperature. They were powdered after drying and stored separately in a sterile and airtight container until further use. Pr. Fathia Skhiri, a Botanist at the Laboratoire de Génétique Biodiversité et Valorisation des Bio-ressources, Institut Supérieur de Biotechnologie de Monastir (ISBM), Université de Monastir, identified the plant material.

Plant extract preparation

Extracts from dried powder (100 g) were obtained by soaking it in increasing polarity organic solvents such as petroleum ether, dichloromethane, ethyl acetate, and methanol for one week. The resulting mixture was filtered through membrane filters with a diameter of 1 mm. The filtrates were vacuum evaporated in a rotary evaporator (BUCHI, Germany), and the dried extracts were stored at 4°C in the dark until further processing. Because the biosynthesis of alkaloids in *Datura* occurs primarily in the roots (Kohnen-Johannsen et al., 2019), total alkaloids were extracted and isolated from this organ using a liquid-liquid extraction based on the difference in solubility of the alkaloids in acidic and alkaline conditions. An electric grinder was used to finely crush 300 mg of roots. Sulfuric acid (3%) was added, followed by a 3-hour incubation at room temperature.

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Following filtration, the filtrate was basified with ammonia solution NH_4OH, which allowed alkaloids to transition from salt to organic form. A rotary evaporator is used to evaporate the collected extract under vacuum. The total alkaloids are represented by the dry residue.

Phytochemical screening of extracts

The leaves and seeds of *D. metel* were subjected to preliminary Qualitative phytochemical screening in order to detect major groups such as tannins, alkaloids, iridoids, saponins, and flavonoids by following the standard procedures described by Harbone (1998).

Tannins. After boiling 200 mg of plant material in 10 mL distilled water and adding a few drops of $FeCl_3$ to the filtrate, a blue-black precipitate indicated the presence of Tannins.

Alkaloids. 200 mg plant material was boiled and filtered in 10 mL methanol. 1% hydrochloric acid was added, followed by six drops of Dragendorff reagent, and the brownish-red precipitate was used to confirm the presence of alkaloids.

Iridoids. Trim & Hill color reagent was used to detect iridoid glycosides in the drug: 1 g of powered drug was placed in a test tube with 5 ml of 1 percent aqueous hydrochloric acid, and after 3–6 hours 0. 1 ml of the macerate is decanted into another tube containing 1 ml of the Trim–Hill reagent. After being heated, a dark color indicates the presence of iridoids.

Saponins (Frothing test). To 200 mg plant material, 5 mL distilled water was added. The filtrate was diluted to a volume of 5 mL with distilled water and vigorously shaken for 2 minutes. The presence of saponins is indicated by the formation of stable foam.

Flavonoids. 5 mL dilute ammonia solution was added to the aqueous filtrate, followed by concentrated H_2SO_4. The presence of flavonoids was indicated by a yellow coloration.

Antibacterial activity

Test microorganisms

For the antimicrobial tests, eight Gram-negative and three Gram-positive bacteria strains were used. The American Type Culture Collection (ATCC) provided the reference strains (USA). Stock cultures were stored at 80°C in the laboratory LR 99 ES 27, Monastir, Tunisia, as glycerol stock (20%). Before any antimicrobial testing, all strains were subcultured three times.

Method of disc diffusion

Different extracts of *D. metel* leaves, seeds, and total alkaloids were tested for antibacterial activity in Muller Hinton agar using the standard disc diffusion method. The bacterial inoculum was set at 0.5 Mcfarland (Bel Hadj Salah et al., 2006). Each extract (10 mg/ml) was inoculated onto the surface of the media using 6 mm sterilized Whatman No. 3 paper discs. As a positive control, a standard antibiotic disc (gentamycin, 30 µg /disc) was used, and a disc impregnated with ethanol 99 percent was used as a solvent control. The incubation of the plates is at 37°C for 24 hours. The diameter of the inhibition zone in mm, was used to calculate the antibacterial activity.

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Minimum inhibitory and minimum bactericidal concentrations (MIC and MBC)

The Minimum Inhibitory Concentration (MIC) of *D. metel* organic extracts was determined using the serial dilution technique (Hajlaoui et al., 2009). In brief, 96-well plates were filled with 95 µl of nutrient broth, 5 µl of bacterial inoculum (10^6 cfu/ml), and 100 µl of plant extract (the initial concentration was 10 mg/ml). A total of 100 µl from their serial dilutions was transmitted into six consecutive wells. Column 12 was set aside for solvent control (ethanol), while column 1 was set aside for extract control. Columns 10 and 11 served as controls for bacterial culture and nutrient broth medium, respectively. Under the same conditions, the antibiotic gentamycin was manipulated. The test microplates were then incubated at 37°C for 24 hours, and the presence of turbidity and a pellet on the well bottom was used to assess bacterial growth. The minimum inhibitory concentration (MIC) was defined as the concentration that totally inhibited visible cell growth after a 24-hour incubation period at 37°C. 10 µl of each well medium with no visible growth was subcultured in MH plates to determine the minimum bactericidal concentration (MBC) results. The number of surviving pathogens was determined after 24 hours of incubation at 37°C. MBC was defined as the lowest concentration at which 99% of the bacteria were killed (Sahin et al., 2004). All experiments were done in triplicate.

Antifungal activity

Fungal strains

The fungal strains were acquired from the microbiology laboratory at the University of Franche-Comté in Besançon and the Pasteur Institute (France). Every three months, the strains were subcultured on Sabouraud Dextrose Agar (SDA) medium and incubated for seven days.

Agar incorporation method

The antifungal activity was measured using the method explained by Bel Hadj Salah et al. (2006). SDA medium containing 0.05% chloramphenicol was prepared. Appropriate amounts of leaf and seed extracts were mixed in the medium, kept cool to 45 to 50°C to obtain concentrations of 500, 250, 125, and 62 µg/ml. The medium was thoroughly mixed with the plant extracts. In sterile Petri dishes (33 mm), 3 ml of each concentration were poured. Following solidification, 5 mm diameter mycelial plugs were collected with a pre-sterilized cork borer from a 5 to 7 day old culture of test fungus and put in each Petri plate. On the SDA medium, a conidial suspension of *C. albicans* was placed. Incubation was 24 h at 37°C for *Candida* and *Aspergillus* and 7 days at 24°C for Scopulariopsis and dermatophytes. Each treatment was replicated thrice for every concentration and microorganism.

"Percentage growth inhibition of the fungal colonies (Eq1) was calculated by applying the following formula":

$$\text{Growth/inhibition (\%)} = (dC-dE)/dC \times 100 \text{ (Singh et al., 1993)} \qquad \text{(Eq.1)}$$

where dC: "*the diameters of the colonies in the control plate*, dE: *diameters of colonies in the treated plate"*.

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The minimal inhibitory concentration (MIC), for each species was also calculated. This is defined as the lowest concentration that suppresses observable growth of fungus throughout the incubation period.

Statistical analysis

Three replicates were analyzed using one-way analysis of variance (ANOVA) in the SPSS program Ver. 20. The mean of the replicates is used to calculate the standard deviation (SD). All tests were performed in triplicates (n = 3) and the error bars represent the SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ to compare the treatment, the differences were declared low, high and very high statistically significant, respectively.

Results

Phytochemical analysis

Organic extracts of *D. metel* were found to have a variety of chemical components. *Table 1* highlights the phytochemical content of leaves and seeds of *D. metel* extracts. All the extracts contained flavonoids, tannins, Iridoids, alkaloids and saponins.

Table 1. *Phytochemical Screening of Datura metel L leaves and seeds*

Phytochemicals	Seeds	leaves
Alkaloids	+	+
Flavonoids	+	+
Saponins	+	+
Iridoids	+	+
Tannins	+	+

Antibacterial activity

The diameters of the inhibition zones of various organic extracts were measured in mm. The antibacterial activities of leaf and seed extracts were then compared (*Fig. 1A, B, C and D*), as well as those of total alkaloids extract and the reference antibiotic Gentamicin (*Fig. 1E*). All of the organic extracts were efficacious against *Bacillus subtilis*, having inhibition zones ranged from 7 to 16 mm, whereas no inhibitory effects of the same extracts were observed against *Vibrio parahaemolyticus*. In most cases, methanolic extracts appeared to be more active over other extracts with zones of inhibition varying from 7 to 15 mm. Results also showed that the *D. metel* seed extracts possess in general significantly higher antibacterial activities over leaves extracts ($p < 0.01$, *Fig. 1*).

The total alkaloids extract, on the other hand, has lower activity than the control Gentamicin, as well as other leaf and seed extracts. It was efficient against *B. subtilis* (14 mm) (*Fig. 1E*).

We used a second antibacterial technique, the serial dilution in 96 plates, to confirm the results obtained from the agar diffusion method. If we consider the problems of miscibility of organic extracts in an aqueous medium, it is said to be more efficient.

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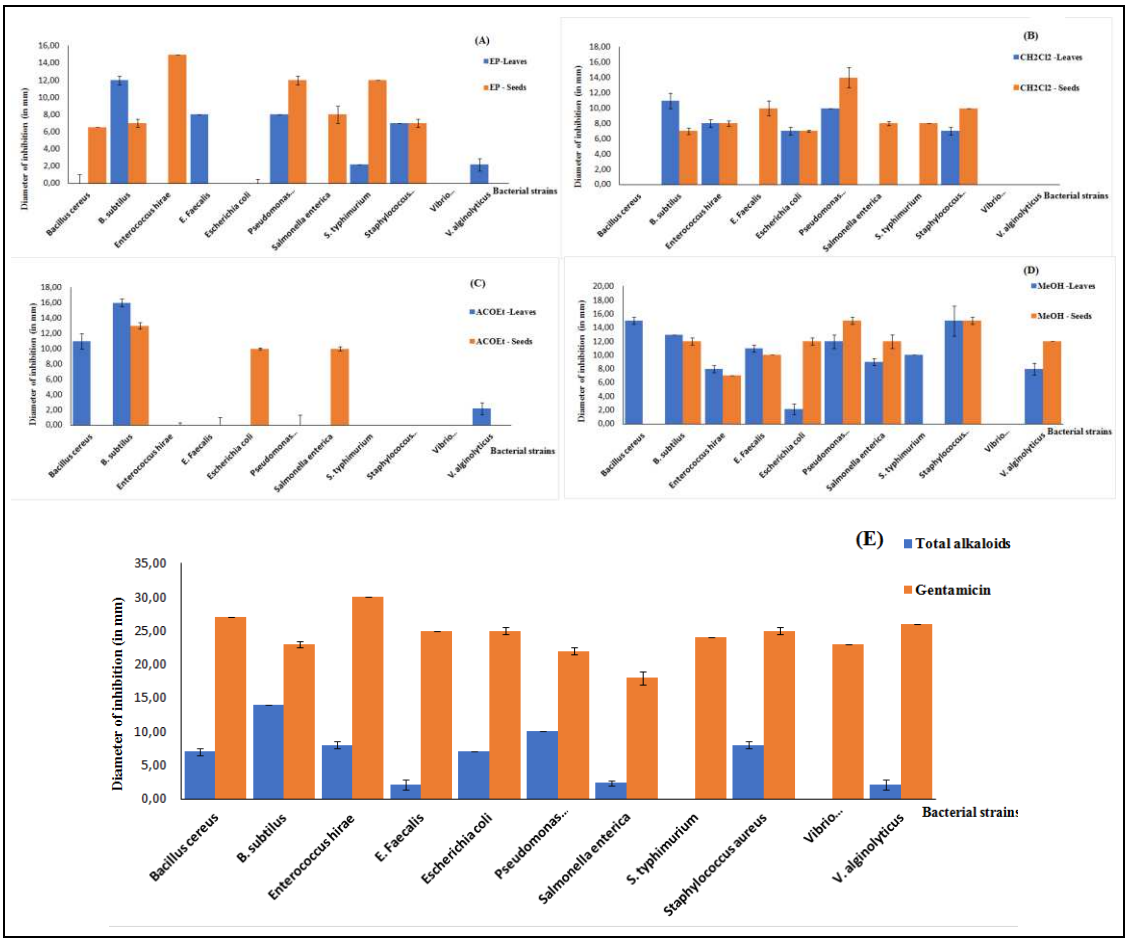

Figure 1. *Comparison of the Diameters of inhibition (in mm) of D. metel L. leave and seed extracts (1mg/mL): (A) Petroleum Ether (PE); (B) Dichloromethane (CH_2Cl_2); (C) Ethyl acetate (ACOEt); (D)Methanol (MeOH);(E): total alkaloids (1mg/mL) and the reference antibiotic Gentamicin (30 µg /disc); bacterial strains are in the same order as in the Figure : Bacillus cereus(ATCC10987); Bacillus subtilus (ATCC6633); Enterococcus hirae (ATCC10541); Enterococcus faecalis (ATCC29212); Escherichia coli (ATCC25922); Pseudomonas aeruginosa (ATCC9027); Salmonella enterica (ATCC10708); Salmonella typhimurium (ATCC1408); Staphylococcus aureus (ATCC25923); Vibrio parahaemolyticus (ATCC17802) and Vibrio alginolyticus (ATTC17749). All tests were performed in triplicates (n = 3) and the vertical bars represent the standard deviations. All Treatments are highly significant at $p < 0.01$*

Fig. 2 represents the minimum inhibitory concentrations (MICs) of different *D. metel* extracts against the tested bacterial strains. *B. subtilis* (MIC from 1.25 to 2.5 mg/ml except the CH_2Cl_2 seed extract) and *P. aeruginosa* (MIC from 0.625 to 10 mg/ml) were the most vulnerable pathogens, whereas *V. parahaemolyticus, V. alginolyticus, Staphylococcus typhimurium, Escherichia coli* and *Enterococcus faecalis* were among the most resistant ones (MIC: 10 mg/ml for leave extracts). The lowest CMI/CMB (0.078/0.156) was observed with the EP seed extract against *Enterococcus hirae*. Differences in activities are very high significant ($p < 0.001$) (*Fig. 2 and Table 2*). These results correlate with those recorded with the disc diffusion method.

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Figure 2. *Comparison of MIC values (mg/mL) of D. metel L. leave and seed extracts (10mg/mL) Petroleum Ether (PE): A; Dichloromethane (CH_2Cl_2): B; Ethyl acetate (ACOEt): C; Methanol (MeOH): D; bacterial strains in the same order as in the Figure: Bacillus cereus (ATCC10987); Bacillus subtilus (ATCC6633); Enterococcus hirae (ATCC10541); Enterococcus faecalis (ATCC29212); Escherichia coli (ATCC25922); Pseudomonas aeruginosa (ATCC9027); Salmonella enterica (ATCC10708); Salmonella typhimurium (ATCC1408); Staphylococcus aureus (ATCC25923); Vibrio parahaemolyticus (ATCC17802) and Vibrio alginolyticus (ATTC17749). All tests were performed in triplicates (n = 3) and the vertical bars represent the standard deviations. All Treatments are very highly significant at $p < 0.001$ except Total alkaloid and Gentamicin are not Significant*

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Table 2. *Minimal bactericide concentrations values (MBC mg/mL) of D. metel organic extracts against bacterial pathogens*

Bacterial strains	Leave extracts				Seeds extracts				MBC	
	PE	CH_2Cl_2	ACOEt	MeOH	PE	CH_2Cl_2	ACOEt	MeOH	T. alkaloids	Gentamicin
B. cereus (ATCC10987)	*	*	1.25	1.25	*	*	*	2.5	*	5
B. subtilus (ATCC6633)	2.5	2.5	5	2.5	5	*	2.5	2.5	2.5	5
E. hirae (ATCC10541)	5	*	*	*	0.156	2.5	*	*	*	*
E. Faecalis (ATCC29212)	*	*	*	5	*	*	*	*	*	5
E.coli (ATCC25922)	*	*	*	*	*	*	5	2.5	*	5
P. aeruginosa (ATCC9027)	*	5	*	2.5	5	2.5	*	1.25	5	*
S. enterica (ATCC10708)	*	*	*	*	*	*	*	5	*	5
S. typhimurium (ATCC1408)	*	*	*	5	5	*	*	2.5	*	5
S. aureus (ATCC25923)	*	*	*	1.25	*	5	*	0.62	*	5
V.parahaemolyticus (TCC17802)	*	*	*	*	*	*	*	*	*	5
V.alginolyticus (ATTC17749)	*	*	*	*	*	*	*	*	*	5

*: MBC more than 10 mg/mL

The seed extracts are slightly more active over the leaves, and the methanolic extracts (0.625 to 10 mg/ml) seem to be more active over the organic extracts. All treatments are highly significant at $p < 0.001$ except Total alkaloid and Gentamicin which are not Significant. For many of the bacterial strains tested, *Datura* leaves and seeds extracts seem to have less MICs than the reference antibiotic Gentamicin (*Fig. 2*). The minimum bactericidal concentrations MBCs of *D. metel* organic extracts were noted to be close to the MIC values (*Table 2*).

Antifungal activity

The effects of different tested extracts on fungal growth are presented in *Fig. 3* and *Fig. 4*.

The comparison of the growth inhibition percentages (%) of different organic Leave & Seed extracts from *D. metel L.*, at a concentration of 500 µg/ml are represented on *Fig. 4*. The percent inhibition values vary from 0 to 100%.

The MeOH and ACOEt extracts showed the strongest inhibition effects with percent inhibition values ranging from 56.5 to 100% and from 31 to 100% respectively with a very high significance ($p < 0.001$). The most inhibited fungi were *A. fumigatus* (90.9%), *A. niger* (82.7%), *S. brevicaulis* (83.3%), *T. mentagrophytes* (86.6%) and *C. albicans* (100%) with the methanolic leave and seed extracts. They were more effective against the majority of fungal strains (*Fig. 4*). MIC values ranged from 62.5 to 250 g/ml as shown on *Table 3*. No revealing difference was observed between the leaves and seed extracts, only with ACOEt extracts (*Table 3*).

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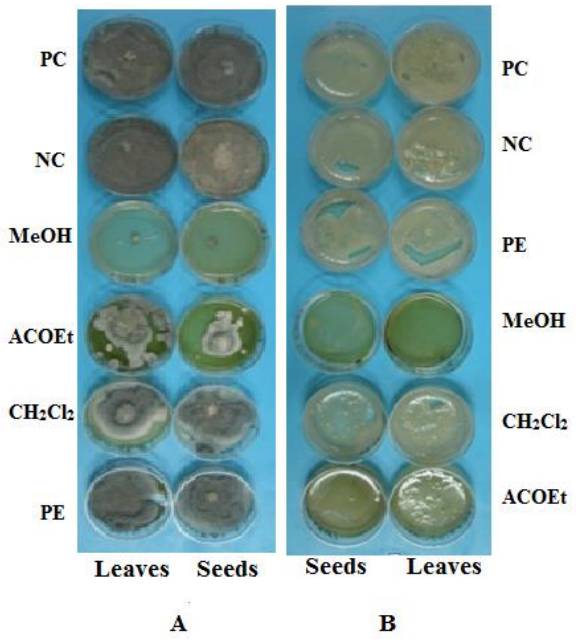

Figure 3. *The antifungal activity of D.metel Leaves and seeds extracts 500µg/ml; (A) Aspergillus fumigatus; (B) Candida albicans; (PC) positive control; (NC) negative control: alcohol 99%; (PE) petroleum ether; (CH_2Cl_2) dichloromethane; (ACOEt) ethyl acetate; (MeOH) methanol*

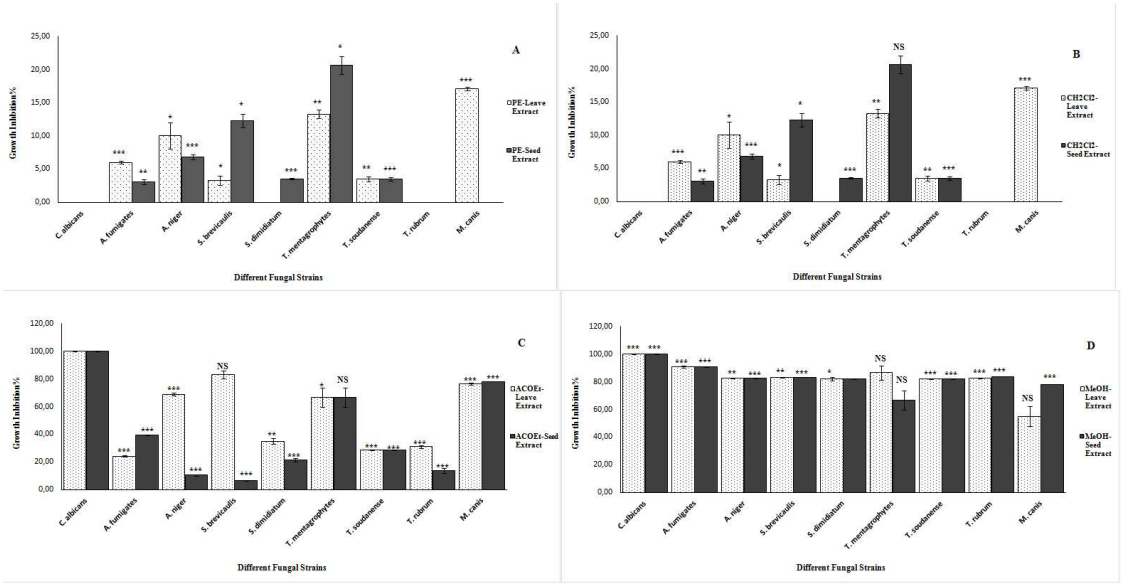

Figure 4. *Comparaison of the Growth Inhibition percentage (%) of. different organic Leave & Seed extracts from D. metel L. (500µg/ml): Petroleum Ether (PE): A; Dichloromethane (CH_2Cl_2): B; Ethyl acetate (ACOEt): C; Methanol (MeOH): D; fungal strains strains in the same order as in the Figure: Candida albicans; Aspergillus fumigates; Aspergillus niger; Scopulariopsis brevicaulis; Scytalidium dimidiatum; Trichophyton mentagrophytes; Trichophyton soudanense; Trichophyton rubrum and Microsporum canis. Inhibitory power was interpreted as follows: 0–25%, no or little inhibition; 26–50%, average inhibition; 51–100%, strong inhibition. All tests were performed in triplicates (n = 3) and the error bars represent the SD. Asterisks denotes significance level: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$*

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Table 3. *The MIC (µg/mL) of Datura metel organic extracts against fungal strains*

Fungal strains	MIC (µg/mL)							
	Leaves extracts				Seeds extracts			
	PE	CH_2Cl_2	ACOEt	MeOH	PE	CH_2Cl_2	ACOEt	MeOH
Candida albicans (B)	*	*	250	250	*	*	250	250
Aspergillus fumigates (B)	*	*	*	62.5	*	*	*	62.5
Aspergillus niger (B)	*	*	*	250	*	*	*	250
Scopulariopsis brevicaulis (B)	*	*	250	250	*	*	*	250
Scytalidium dimidiatum (IP)	*	*	*	250	*	*	*	250
Trichophyton mentagrophytes (B)	*	*	*	250	*	*	*	*
Trichophyton soudanense (B)	*	*	*	250	*	*	*	250
Trichophyton rubrum (B)	*	*	*	250	*	*	*	250
Microsporum canis (IP)	*	*	250	*	*	*	125	250

*: minimum inhibitory concentration (MIC): more than 500 µg /mL; (B): Microbiological Laboratory, Besancon; (IP): Institut Pasteur, Paris; Petroleum Ether (PE); Dichloromethane (CH_2Cl_2); Ethyl acetate (ACOEt)

Discussion

Due to their wealth of active components, plant extracts are widely assessed for their biological properties. To our knowledge, this is one of the few studies that have investigated the antibacterial, antidermatophyte, and anticandida properties of Tunisian *D. metel* organic extracts from seeds, leaves, and total alkaloids from roots.

The qualitative preliminary phytochemical analysis of *D. metel* extracts from seeds and leaves revealed the presence of saponins, alkaloids, tannins, Iridoids and flavonoids. Our findings in this context are in line to several other reports (Nandakumar et al., 2017; Partap et al., 2019; Alam et al., 2021; Sharma et al., 2021). On the other hand, tannins were not identified in the extracts of *D. metel* in some other literature but are present in seed and leave extracts considered in this study (Irudayaraj et al., 2010; Muthusamy et al., 2014).

Phytochemical substances are involved in a wide range of biological activities, including antimicrobial properties. Numerous among them, have shown significant antibacterial action (Cowan, 1999; Mahizan et al., 2019). In our study, *D. metel* extracts inhibited all of the microorganisms examined, except for the genus *Vibrio*. This discovery supports the use of this herb in traditional medicine to cure cough, diarrhea, and certain skin disorders (Dabur et al., 2004; Al-Snafi, 2017; Alam et al., 2021). Furthermore, our findings are consistent with earlier studies that found antibacterial properties of *D. metel* extracts from Oman, Pakistan and India, but with variable potency (Al-Jafari and Hossain, 2015; Krishnan et al., 2017; Bawazeer and Rauf, 2021).

Moreover, the comparison of the bacteriostatic and bactericid action of extracts with different polarities in the present study is as follow: MeOH > Gentamicin > ACOEt >

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CH2Cl2 > total alkaloids > EP. Several earlier studies have documented the antibacterial potentials from alkaloid component (Al-Jafari and Hossain, 2015; Pervaiz et al., 2019; Sharma et al., 2021). In our study, total alkaloids extracted from the roots of *D. metel* were active against only two bacteria among a total of eleven strains tested. This difference in activity among different extracts could be explained by the synergistic action between phytochemicals found in every crude extract. We call synergism, the fact that the therapeutic effect of two or more compounds is higher than if each one is used alone. Thus, toxicity can be decreased since fewer concentrations can be used from both compounds (Chung, 2011).

In the same context, *E. coli*, was not only inhibited but completely killed by 2.5 mg/ml of seed methanolic extract from Tunisian *D. metel*, while it was not inhibited by "5`, 7 `, dimethyl 6 ` hydroxyl 3 amine β-yne sitosterol", isolated from the leaves of Nigerian *D. metel* (Donatus et al., 2009). In another study, the methanolic extract of *D. metel* was effective against *E. coli* and *P. aeruginosa* (Bachheti, 2018). Furthermore, for the same solvent, seed extracts were significantly more active than leaf extracts. Previous antibacterial research has also deduced differences in activity between extracts and organs of *D. metel* (Al-Snafi, 2017; Sharma et al., 2021). According to Carrubba et al. (2021), the variability in activity for the same species may be related to the existence of bioactive secondary metabolites, which are known to cause damage to cell membranes and vary from organ to another, as well as climatic circumstances, according to the place of collection (geographical origin) and physiological growth period of the plant (Rouis et al., 2013).

Continuing with the antimicrobial activities, *D. metel* leaf and seed extracts were tested for antifungal activity. Our findings indicated a high significant inhibition of many harmful fungi (*T. mentagrophytes, T. soudanense, T. rubrum, C. albicans and M. canis*). As shown in our experiments, the methanol extract of *D. metel* was the most efficacious, with the highest percentage of inhibition seen against *S. brevicaulis* radial growth (83.3%). Similarly, Bawazeer and Rauf (2021) discovered that the chloroform and methanol fractions of *D. metel* exhibited a promising antifungal activity against *Aspergillus flavus, Fusarium solani,* and *Microsporum canis,* but *Candida albicans*, on the other hand, was resistant to all extracts tested.

Furthermore, our data support the use of *D. metel* in traditional medicine to treat dermatitis of the hair, nails, and skin. Although *C. albicans* frequently demonstrated resistance to natural extracts in our previous researches (Bel Hadj Salah et al., 2006; Ben Othman, 2017), the current findings revealed an interesting and a high significant anti-Candida activity for ACOEt and MeOH extracts of *D. metel* (MIC 250 µg/ml). The presence of flavonoids, saponins and alkaloids might explain Thornapple′s intriguing antifungal effectiveness. According to Trdá et al. (2019), saponins have been reported to have antifungal potential (Tagousop et al., 2018). Moreover, flavonoids were revealed to have inhibition effects against the germination of plant fungi (Al Aboody et al., 2020).

Tropane alkaloids scopolamine and atropine characterise *D. Metel* in many reports and indole alkaloids were identified in seeds of *D. metel* (Yang et al., 2010; Kohnen-Johannsen et al., 2019; Pervaiz et al., 2019; Alam et al., 2021; Sharma et al., 2021).

Moreover, Atropine has a strong antiviral impact against HSV-1 DNA viruses PI-3 as well as HIV-1 RNA viruses and COVID-19 (Devkar et al., 2021).

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Conclusion

Overall, the present study suggests that extracts from Tunisian *D metel* have efficient and a high significant antimicrobial potential, supporting its folkloric use. This research also points to the promising value of *D. metel* as anticandida and antidermatophyte source. Further analysis, dealing with bioactive molecules from the organic extracts and their eventual cytotoxicity, are required to confirm the possible therapeutic and/or industrial valorization of this plant.

Conflict of interests. The authors state that they do not have any conflict of interests.

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COMBINED NITROGEN AND PHOSPHORUS APPLICATION SYNERGISTICALLY REGULATE GRAIN YIELD AND PROTEIN CONTENT IN WINTER WHEAT (*TRITICUM AESTIVUM* L.)

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Abstract. To provide technical support for better quality and to get high-yield of winter wheat in Shanxi Province, four winter wheat genotypes are investigated in the field experiments to reveal the synergistic change regularity of grain protein content and yield, determine the protein threshold of winter wheat, and obtain the optimum fertilization application of nitrogen (N) and phosphorus (P). The study depicts P has a significant effect on the protein content and yield of winter wheat. Increasing the application rate of P can significantly increase the yield, while its influence on protein content varies within different varieties. Similarly, application rate of N has a prominent effect on both yield and protein content as both of which are enhanced with the increase of N application. We also record a synergistic increase in yield and protein content between certain intervals; the yield and protein content increase first, and then decrease with increasing applications of N fertilizer, which can be characterized with a parabola. Applying the appropriate amount of N and P can cause a synergistic increase for protein content and yield, and the most ideal threshold intervals of protein corresponding to the optimal P and N application rate are 150 kg/ha-200 kg/ha and 180 kg/ha-255 kg/ha, respectively.
Keywords: *threshold intervals of protein, nitrogen fertilizer, phosphorus fertilizer, genotypes of winter wheat, grain protein, grain yield*

Introduction

Winter wheat (*Triticum aestivum* L.) is an important grain crop in China and also the second top grain crop in Shanxi Province. However, medium and low-yield winter wheat with 3721.15 kg/hm^2 which is lower than the average yields in China with 5495.8 kg/hm^2 accounts for more than 50% of total wheat fields in Shanxi Province. Increasing the yield per unit area could improve self-sufficiency rate of wheat in Shanxi Province (Yang et al., 2010). Meanwhile, with the economic development and the improvement of people's living standard, purchasing the production of high yield, high quality and high efficiency has become an important goal of wheat production nowadays. Fertilizer application is one of the important factors that influence output and quality of wheat and an appropriate application amount of fertilizer to enhance the output and yield of wheat simultaneously is an important way to enhance production of wheat.

Both water and fertilizer conditions have a great effect on wheat yield and quality (Huang et al., 2015; Mostafa et al., 2018; Wang et al., 2019), with N fertilizer as the largest contributing factor (Bouacha and Nouaigui, 2014; Nan et al., 2015; Zhen et al., 2016). It is generally believed that within certain range of N application, the yield of wheat grain will be improved with the increasing application rates. But when N application amount exceeds a certain level, it can be non-significant and even decrease yield. Likewise, the grain protein content of wheat also shows a similar trend with the increasing application amount of N (Fan et al., 2021; Wang et al., 2021; Zhang et al., 2021). Appropriately increasing N application level can improve wheat yield and grain

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protein content, which can synchronously increase grain yield and protein content. Too high N were negatively correlated with grain protein and yield (Wang et al., 2003; Hawkesford, 2014; Zörb et al., 2018). Researchers also demonstrated similar findings indicating a "threshold value of protein content" (Lin et al., 2004). The effect of increasing yield reaches its maximum value when the N application amount remains 284 kg/hm^2 and the effect of increasing grain protein is the most significant when it is 384 kg/hm^2 (Deng and Jiao, 2021). Similarly grain yield of wheat and protein content increased with the increasing application amount of N at 270 kg/hm^2 but when N application amount is raised to 360 kg/hm^2, grain yield and protein content reduced (Xu et al., 2012).

Same is the case for P fertilizers which play a good role in promoting tiller and root growth of wheat, but its effect on grain yield and protein content remains ambiguous. Most of researches believed that the yield increased differentially in response to P fertilizer (Xing et al., 2015; Bekalu and Mano, 2016; Abbas et al., 2016). For example, Yu et al. (2013) reported that the yield of winter wheat had maximum values when P application amount was 172.5 kg/hm^2, but Xing et al. (2015) reported 240 kg/hm^2 P-fertilizer as optimum dose while wheat yield decreased at 480 kg/hm^2. Similar reports have been reported earlier by many researchers (Sun et al., 2006; Wang et al., 2006, 2009; Xing et al., 2015; Abbas et al., 2016; El-Sobky, 2017; Dambeniece-Migliniece et al., 2018).

It is of great importance to study the rational application amount of N and P to promote wheat production. Both N and P fertilizer application amount exhibit a strong relation to the variation of grain yield and protein content (Zhao and Yu, 2006; Zhao et al., 2010; Xu et al., 2012; Xing et al., 2015). Most interestingly, within this range there is a possibility that the grain yield and protein content may synergistically change (Bouacha and Nouaigui, 2014; Wei et al., 2021). Meanwhile, there are protein content threshold intervals with relatively high protein content and wheat yield (Lin et al., 2004). Previous studies have attached importance on the effects of N and P fertilizer on wheat yield and protein content, but there have been relatively few studies which focus on the effect of NP treatment on the relation and variation regularity between wheat yield and protein contents. The determination of protein threshold intervals is of therefore greater significance and could be important for production of high quality wheat. This study investigated the synergistic effects of N-P application rates on change regularity of winter wheat yield and the grain protein contents.

Materials and methods

Experiment details

Experiment was conducted at farming station of Shanxi Agricultural University in Taigu County, China from October 2013 to June 2015 with four varieties viz. V1, Jintai182; V2, Jintai170; V3, Jingdong12; V4, Lumai14.

Field experiment

The soil texture of experimental field at farming station is Calcaric Cambisols which developed from the loess parent material and has medium-level soil fertility. The content of organic matter, total nitrogen, available nitrogen, total phosphorus, available phosphorus and available potassium were 21.7 g/kg, 0.184%, 54.7 mg/kg, 0.12%, 17.5 mg/kg and 239.8 g/kg, respectively. Four different genotypes of winter wheat cultivars which were

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extensively used and strongly suggested by the local government were selected to conduct fertilizer experiment and the randomized block design were adopted for three repetitions. The details of grain quality for all winter wheat varieties were showed in *Table 1*.

Table 1. *The grain quality details of four selected winter wheat*

Varieties	Protein content (%)	Wet gluten (%)	Stable time (min)	Sedimentation value (ml)	Absorption (%)
Jintai 182 (V1)	16.17	32.8	3.6	29.5	59.2
Jintai 170 (V2)	16.49	35.1	21.2	60.6	61.8
Jingdong 12 (V3)	17.40/18.20	41.00/37.90	4.20/3.40	33.5/30.9	61.10/62.80
Lumai 14 (V4)	11.76	25.38	-	-	-

Each cultivar was treated by 3 P fertilizer levels and 4 N fertilizer levels (*Table 2*). With the addition of a control group P0N0, there were 13 treatments in total. The area of each block was 16 m² and there were guarding row among each block. Meanwhile, the row distance and plant density are 20 cm and 4.5×106 plant·hm^{-2}, respectively. The varieties of nitrogen and phosphorus fertilizers used in the experiment were urea and calcium superphosphate, and the base fertilizer was applied before sowing. The P fertilizer was applied at one time, N fertilizer used method of topdressing at the returning green stage, and the ratio of basal fertilizer and top-dressing was 1:1.

Table 2. *Combined application of N and P under different experiments of wheat varieties*

Treatments No.	Treatments	Amount of fertilizer application (kg/hm^2)		N/P
		N	P_2O_5	
1	N0P0	0	0	-
2	N1P1	105	100	1.05:1
3	N1P2	105	150	1:1
4	N1P3	105	200	0.525:1
5	N2P1	180	100	1.8:1
6	N2P2	180	150	1.2:1
7	N2P3	180	200	0.9:1
8	N3P1	255	100	2.55:1
9	N3P2	255	150	1.7:1
10	N3P3	255	200	1.275:1
11	N4P1	330	100	3.3:1
12	N4P2	330	150	2.2:1
13	N4P3	330	200	1.65:1

Determination of yield traits and grain protein contents

The yield attributes of plants were recorded from 5 plants per treatments and the proteins contents were quantified via Kjeldahl method.

Data analysis and processing method

The range transformation was used to standardize the values of wheat yield and the content of grain protein (eliminating dimension of quantity).

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$$y_i' = \frac{y_i - y_{min}}{y_{max} - y_{min}} \quad \text{(Eq.1)}$$

where, y_i' refers to yield (protein content) standard value, $y_i$refers to actually measured yield (protein content), y_{min} refers to the measured minimum output (protein content) and y_{max} refers to the maximum output (protein content). The figures were made by using the DPS, Excel 2013 and Origin softwares.

Results

Effect of different ratios of N and P on the yield of winter wheat

The effects of different combinations of *N and P applications* on the grain yields was analyzed (*Table 3*), and it showed that the grain yield of the four varieties were 4978.4 kg/hm², 5011.6 kg/hm², 5143.3 kg/hm² and 5146.2 kg/hm², respectively, under the condition of P0N0 in the control group. The yield of each variety had a trend of increasing initially and then decreasing with different nitrogen application ratio under the constant phosphorus application rate of each variety, and the variation range existed. For V1, there are significant differences under P1N2, P2N3, and P3N4 conditions; V2 under P1N3, P2N3, P3N2, P3N3, and P3N4 conditions, respectively; and V3 under P1N2, P1N3, P2N3, P2N4, and P3N3 conditions, respectively. There were significant differences in V4 under the conditions of P1N2, P1N3, P1N4, P2N2, P2N3, P3N2, and P3N3, respectively. Moreover, under different nitrogen fertilization treatments, the yield of each cultivar increased with the increase of phosphorus application, V1 was significantly different under N1, N3 and N4 conditions, and P3N1, P2N3, P3N3, P2N4 and P3N4 conditions; V2 wheat yield increased with phosphorus application, and increased with the increasing of the amount, and the difference was significant under N2, N3 and N4 conditions. The wheat yield of cultivar V3 increased with the increasing of phosphorus application under N1 and N4 conditions, and the difference was significant under P2N1, P3N1 and P3N4 conditions. Variety V4 increased significantly with the increase of phosphorus application under N2 and N3 conditions.

Table 3. *The effects of different N and P applications on grain yield of four varieties*

Variety	Treatment	N1	N2	N3	N4
V1	P1	5614.9±22.77 de	7174.7±25.25 c	6152.0±27.12 d	5156.3±21.21 e
	P2	5650.0±23.11 de	5927.6±22.67 d	6871.6±21.89 c	5890.4±24.23 d
	P3	7198.3±25.71 c	7335.4±24.98 bc	7342.4±24.37 bc	6071.3±22.91 d
V2	P1	5697.2±23.68 de	6160.7±24.25 d	7329.2±25.14 bc	5566.7±22.43 de
	P2	6368.1±22.97 cd	7415.7±25.09 bc	8840.0±29.05 a	6549.2±24.12 cd
	P3	6656.5±23.33 cd	7823.8±24.73 b	8913.8±26.35 a	7824.0±25.14 b
V3	P1	5268.7±22.26 e	6767.7±23.27 c	7867.4±25.11 b	5538.1±23.26 de
	P2	6099.4±24.15 d	7890.7±27.24 b	7120.8±28.16 c	6193.4±24.74 d
	P3	6657.4±23.78 cd	7322.5±29.03 bc	8372.6±28.33 b	6975.7±22.17 c
V4	P1	6102.4±23.36 d	7437.3±25.27 bc	8088.1±29.22 b	7880.0±27.81 b
	P2	6732.3±22.29 cd	9701.7±29.54 a	8848.5±28.25 a	6044.5±22.33 d
	P3	8261.9±26.74 b	9656.7±28.31 a	8925.3±27.71 a	7901.5±26.09 b

To clearly show the effect of different applications of N, P and its interactive combinations on the grain yield of four varieties, the radar maps (*Fig. 1*) which could

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represent the optimal combinations of N and P application for achieving the highest grain yields and also visually indicate the interval distribution effect between the different N levels, and different P levels.

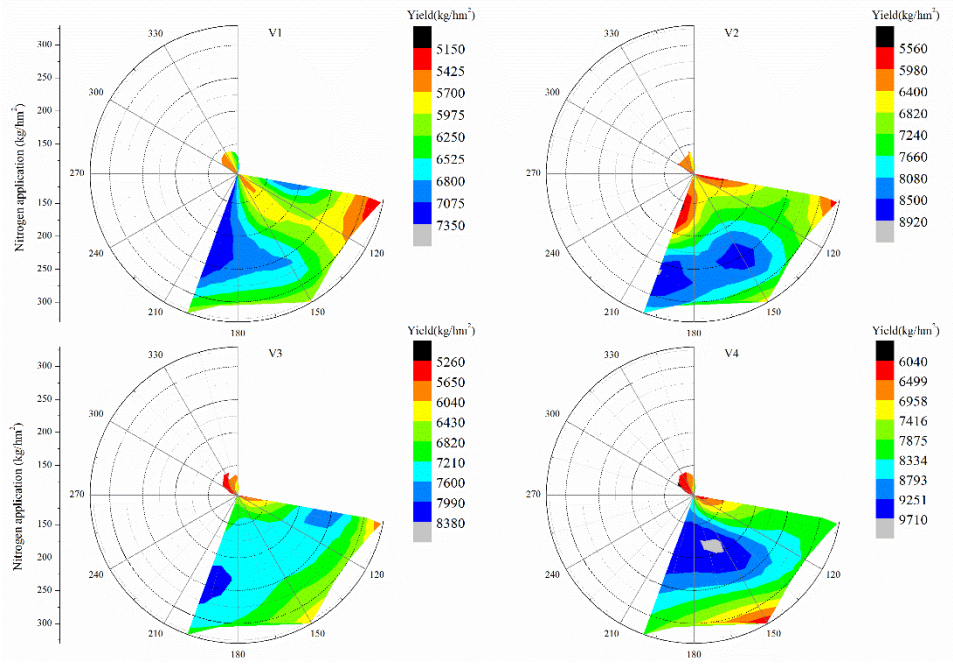

Figure 1. *The grain yield contour map of wheat varieties with different ration of N and P*

It shows that the relation of different wheat cultivars towards different N and P fertilizer doses is presented. When P application amount remained constant, the yield of winter wheat showed a trend of increasing firstly and then decreasing with the increasing N application. When N application amount remained constant, the yield of winter wheat increased with the increasing P application. Under the condition of low and high P application, V1, V3 and V4 had different degrees of insensitivity to N fertilizer respectively. Especially, when P application was 200kg/ hm², V1 exhibited the highest insensitivity towards N fertilizer and acquired higher yield when N application amount kept within the range of 0-275 kg/hm^2. The cultivars V1 and V3 exhibited higher yield under low and high P application, while V2 and V4 showed remarkably increasing trends with the increased P application. Above all, the yield of four winter wheat cultivars increased first and then decreased with the increased N application, and almost all reached the highest levels at N2 or N3 treatments respectively. By contrast, too low or high N applications resulted in lower yield of different cultivars in response to different P treatments.

Effect of different N and P application rates on protein content of four varieties

The effects of different combinations of N and P applications on the grain yields was further analyzed (*Table 4*), and it showed that under the condition of P0N0 in the control group, the protein contents of the four varieties were 8.61%, 7.54%, 8.93% and 6.07%, respectively. There were significant differentially-expressed in the highest protein content under different nitrogen and phosphorus combined application ratios. Under low P treatment, V1 and V2, V4 had significant differences in the highest protein content, and

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V3 had significant differences with V2, V4 in the highest protein content; under medium P treatment, V2 and V1 and V3 had significant differences in protein content, and V4 had significant differences with V1 and V3. Under high P treatment, V1 had significant differences with V2 and V4 protein content, and V3 had significant difference with V2 and V4 protein content in their highest values. Overall, under the condition of a certain amount of phosphorus fertilizer, the four varieties of winter wheat showed higher protein content under the medium nitrogen treatment, and most of the treatments were significantly different.

Table 4. *The effects of different N and P applications on protein content of four varieties*

Variety	Treatment	N1	N2	N3	N4
V1	P1	9.42±0.37 d	13.26±0.51 b	16.69±0.39 a	10.80±0.42 cd
	P2	9.52±0.43 d	11.22±0.28 c	13.39±0.25 b	10.13±0.53 cd
	P3	9.77±0.26 d	14.27±0.32 ab	13.02±0.35 b	11.84±0.44 c
V2	P1	8.48±0.22 de	13.22±0.18 b	15.48±0.24 cd	9.10±0.35 d
	P2	8.67±0.16 de	10.86±0.21 cd	15.27±0.37 cd	10.92±0.19 c
	P3	10.06±0.27 cd	13.48±0.31 b	15.26±0.36 c	10.46±0.47 cd
V3	P1	9.11±0.21 d	11.11±0.29 c	15.96±0.33 a	12.97±0.41 b
	P2	9.43±0.29 d	12.93±0.36 b	17.22±0.43 a	9.89±0.37 d
	P3	10.21±0.25 cd	13.20±0.29 b	17.87±0.32 a	13.75±0.21 b
V4	P1	10.76±0.49 cd	11.16±0.14 c	12.56±0.58 bc	9.76±0.28 d
	P2	6.42±0.11 e	8.62±0.19 de	10.97±0.21 c	6.82±0.15 e
	P3	8.31±0.16 de	9.00±0.22 d	7.86±0.34 e	6.24±0.18 e

The radar maps (*Fig. 2*) was also made to clearly showed the optimal combinations of N and P application for achieving the highest protein content and also visually indicate the interval distribution effect between the different N levels, and different P levels.

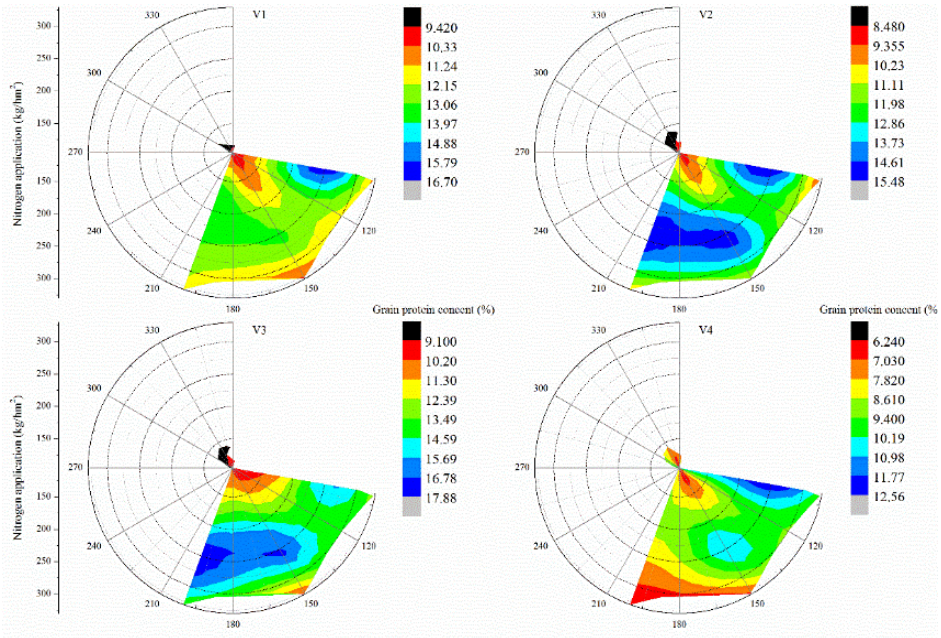

Figure 2. *The grain protein contour map of wheat varieties with different ration of N and P*

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The *Fig. 2* indicates that the grain protein content of four winter wheat cultivars exhibited differential trends of variation with the increase of P application. The cultivar V1 exhibited decrease initially and then slowly increased with the increasing application of P and reached peak values when treated with low P treatment. The grain protein content of V2 and V3 exhibited the same trends as that of V1 with the increasing application of P, and reduced to the lowest value within the range of low to medium P application. The grain protein content of V4 reached the highest level when given high P application, and then gradually decreased with the increasing application of P. Overall, the grain protein content of V1 and V4 reached the highest levels when given lower P treatment, while the grain protein content of V2 and V3 increased highest levels when given medium and high P treatment.

When P fertilizer amount remained constant, the grain protein content of four winter wheat cultivars increased initially, and decreased with the increasing application of N reaching peak values in the range of N2 to N3. Under the condition of low P treatment, peak values for grain protein content of all four cultivars were evident in N3 treatment; under the condition of medium P treatment, higher values of four cultivars when given N3 treatment, of which V2, V3 exhibited higher peak values. The cultivar V4 and V1 had least values under the condition of high P treatment, grain protein content of V1, V4 were highest (remaining relatively low) when given N2 treatment. While, the grain protein content of V2 and V3 reached to the highest values (remaining relatively high) under the N3 treatment.

Threshold intervals of grain yield and protein

To find out synergistic increase intervals of yield and protein content and the corresponding N application amount under different P fertilizer treatments, the research adopted Range Transformation to evaluate standard value, and standard value variation curve of yield and protein were drawn according to the variation of N application amount in *Fig. 3*.

The *Fig. 3* indicated that V1 and V4 showed poor synergistic effects under low P application. However, the V2 showed the optimum synergistic effect in terms of protein content and yield when the P application amount was within the range of medium and high levels and the N application amount was close to N3. The V3 exhibited a mediate synergistic effect when low or high P fertilizer was applied.

All figures *(Figs. 1 to 3) also* indicated that the standardized yield and protein content of the four cultivars can be shown with convex parabolas. With the increase of fertilizer application, the yield and protein content of each cultivar simultaneously increase to the maximum intervals with the increase of fertilizer application, which is the optimum intervals for high yield and high quality, also called the protein threshold intervals.

For V1, the protein threshold interval was (14.8% and 16.6%) under low P treatment and the corresponding optimal amount of N application was 217.5-255 kg/hm^2; its protein threshold interval was (11.6% and 13.39%) under medium P treatment and the corresponding optimal amount of N application was 217.5-292.5 kg/hm^2; the protein interval was (13.02% and 15.48%) under high P treatment and the corresponding optimal amount of N application was 180-255 kg/hm^2. For V2, the protein threshold interval was (12.29% and 14.35%) under low P treatment and the N application amount was 217.5-292.5 kg/hm^2; the protein threshold interval was (13.07% and 15.27%) under medium P treatment and the N application amount was 217.5-292.5 kg/hm^2; the protein threshold interval was (12.77% and 15.26%) under high P treatment, and the N

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application amount was 217.5-292.5 kg/hm^2. For V3, the protein threshold interval under low P treatment was (13.54% and 17.96%) and the corresponding optimal amount of N application was 217.5-292.5 kg/hm^2; the protein threshold interval under medium P treatment was (12.93% and 17.22%) and the N application amount was 180-255 kg/hm^2; the protein threshold interval under high P treatment was (15.54% and 17.87%) and the N application amount was 217.5-292.5 kg/hm^2. For V4, the protein threshold interval under low P treatment was (11.86% and 12.56%) and the N application amount was 217.5-255 kg/hm^2; the protein threshold interval was (8.90% and 10.97%) under medium P treatment, the N application amount was 217.5-255 kg/hm^2; the protein threshold interval under high P treatment was (8.43% and 9.00%), and the N application amount was 142.5-217.5 kg/hm^2.

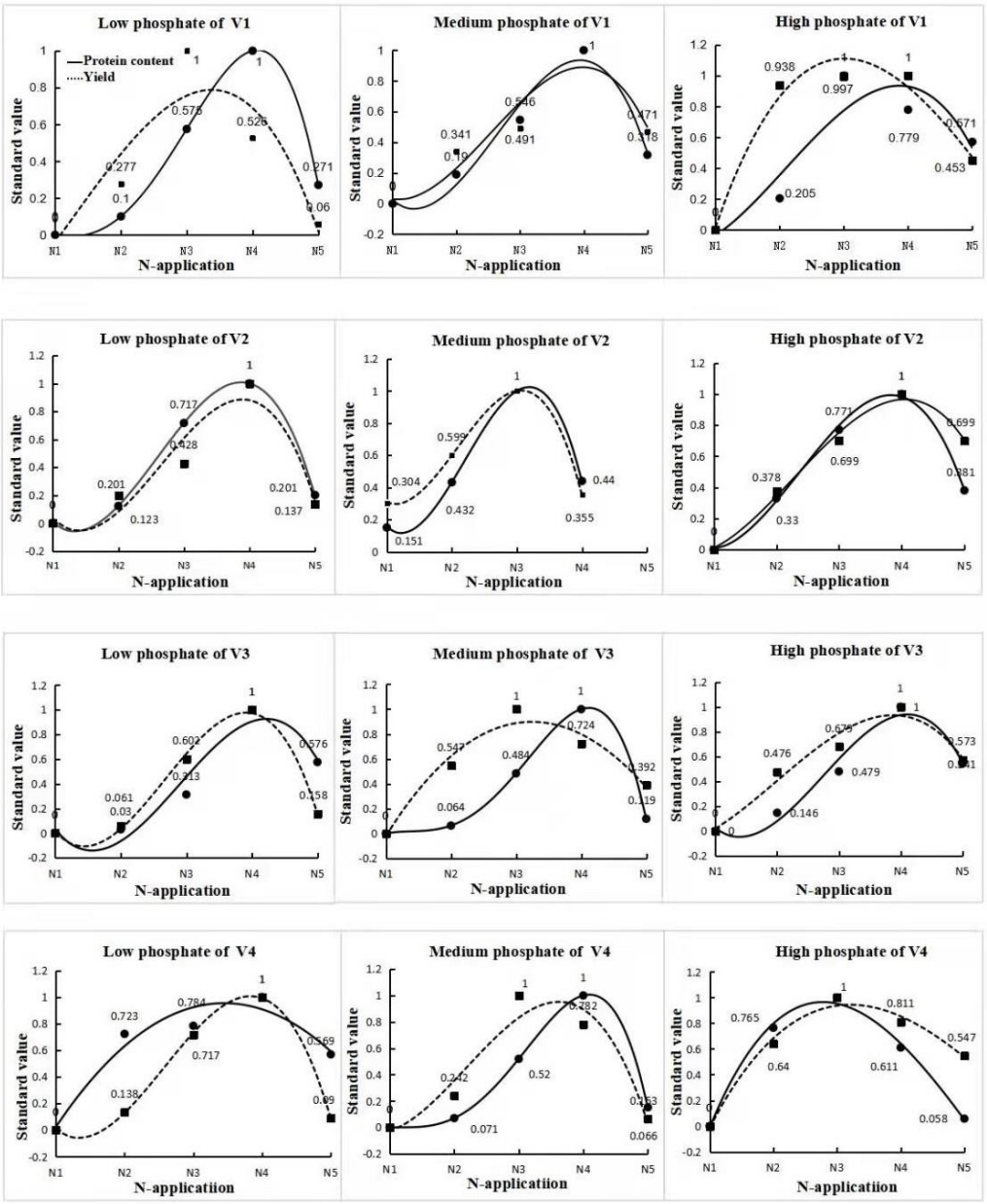

Figure 3. *Dynamics of yield and protein content in different P-application of the 4 varieties*

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The fitted equation of the grain protein content and yield of winter wheat

Under the condition of applying a certain amount of P fertilizer, a polynomial regression was employed to establish the fitted equation of grain protein content and yield of winter wheat. The cubic equation with one variable can be used to demonstrate the changing regularity of protein content and yield with the increase of N fertilizer application (*Fig. 3*). As *Table 5* shows, the R^2 of the model after fitting is above 0.82 and the regression effects of the equations were good.

Table 5. *Fitted equation of N utilization efficiency on yield and protein under different P-application*

Varieties	Treatments	Fitted equation $y=a_0+a_1x+a_2x^2+a_3x^3$				
		a_0	a_1	a_2	a_3	R^2
V1	P1	-0.4664	0.3254	0.1369	-0.0365	0.8252
		1.2432^p	-2.1308^p	1.0248^p	-0.1274^p	0.9900
	P2	0.4756	-0.9138	0.5366	-0.0706	0.8955
		0.9768^p	-1.7106^p	0.8589^p	-0.1085^p	0.9762
	P3	-1.5864	2.0407	-0.4629	0.0274	0.9684
		-0.1170^p	-0.1737^p	0.3012^p	-0.0481^p	0.8518
V2	P1	1.1852	-2.0019	0.9684	-0.1218	0.8983
		1.1212^p	-2.0028^p	1.0108^p	-0.1294^p	1
	P2	0.5856	-1.1308	0.6498	-0.0864	0.9589
		1.0796^p	-1.8147^p	0.8624^p	-0.1048^p	0.9396
	P3	0.0232	-0.2793	0.3103	-0.0454	0.9905
		0.3554^p	-0.8385^p	0.5685^p	-0.0799^p	0.9977
V3	P1	1.4212	-2.4219	1.1508	0.1433	0.9959
		1.4048^p	-2.2543^p	0.9870^p	-0.1137^p	0.9429
	P2	-1.0524	1.2367	-0.2061	0.0032	0.9563
		1.5384^p	-2.5613^p	1.1866^p	0.1461^p	0.9688
	P3	-0.1344	-0.0437	0.2357	-0.0396	0.9572
		0.9752^p	-1.6635^p	0.8023^p	-0.0972^p	0.9677
V4	P1	1.1680	-2.0842	1.0546	-0.1362	0.9995
		-0.9068^p	1.0937^p	-0.1650^p	0.0012^p	0.9342
	P2	0.1900	-0.6876	0.5539	-0.0845	0.9308
		1.4628^p	-2.4561^p	1.1498^p	-0.1421^p	0.9789
	P3	-1.2454	1.5398	-0.3221	0.0171	0.9861
		-1.6442^p	2.1131^p	-0.5074^p	0.0305^p	0.9944

R^2 refers to determination coefficient. a_0, a_1, a_2, a_3 refer to the coefficients of yield equation. a_0^p, a_1^p, a_2^p, a_3^p refer to the coefficients of protein content equation

Discussion

Through fertilization experiments on four cultivars of winter wheat, our study verified that protein content and yield can change synergistically under certain fertilization condition, and there are protein threshold intervals, within which the grain protein content and yield can reach up to higher levels. Under the same conditions of ecological circumstance, soil fertility, and fertilizer application, the maximum protein content of each cultivar is different, which may be related to the differences in the nitrogen assimilation ability, nutrient redistribution ability, as well as genotypic differences of

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plants' total nitrogen content at flowering stage and nitrogen transferring (Sun et al., 2006; Wang et al., 2006, 2009).

A previous study suggested that the yield of wheat decreases with the increase of P application after it has reached a certain amount, showing a trend of increasing firstly then decreasing with the increase of P application (Xing et al., 2015; Abbas et al., 2016; Zhang et al., 2018). Our experiment indicated that the yield of four cultivars under high P application > the yield under medium P application > the yield under low P application. In contrast to our research findings, Yu et al. (2013) reported that wheat yield reaches to the maximum level when the applied amount of P fertilizer is 72.5 kg/hm^2, which is because the effective phosphorus content of the soil is 38 mg/kg. Xing identified that the yield of winter wheat will reach to the highest level when P application amount is 240 kg/hm^2 and that the yield will decrease when P application amount is 400 kg/hm^2. The effective phosphorus content of the soil in Xing's experiment is 18.59 mg/kg (Xing et al., 2015), which is basically the same as that of our experiment. In our experiment, the total phosphorus content of the soil accounts for 0.12%, and the effective phosphorus content is 17.58 mg/kg, suggesting the phosphorus content of the soil is at the medium level. The optimal amount of P application in Xing's study is basically consistent with the results of our experiment. Therefore, the inconsistency of various studies in the optimal amounts of P application may be result from the differences of soil fertility.

Under the condition that N application amount is certain, the P fertilizer had different influences on the protein content of the four cultivars. The V3 had higher protein content under medium or high P application than low P application, which is probably because the nitrogen use efficiency is greatly improved under the treatment of medium or high P fertilizer (El-Sobky, 2017). The V4 had higher protein content under the treatment of low P fertilizer than medium or high P fertilizer, which is consistent with the published research results (Fu et al., 2008; Wang et al., 2009). It is important to mention that the quality of medium and weak gluten wheat decreases with the increase of P fertilizer. In our experiment, the protein content of V2 initially decreased and then increases with the increase of P fertilizer application and minimum at under medium and low P application. At the same time, according to the change in the yield of this cultivar, a conclusion can be drawn that the application of medium and high P fertilizer ensures both high yield and high protein content. Therefore, in practice, we should take into consideration that wheat varieties have influences on the effects of fertilization.

The changing regularities of protein content and yield with the increase of P fertilizer application can be shown with convex parabolas. N application amounts corresponding to the maximum yield and protein content were not exactly the same. Under some treatments, the highest critical values of yield and protein content corresponded to the same N application amount. Under some treatments, the protein content reaches to the critical values earlier than the yield with the increase of N fertilizer. While under other treatments, the yield reached to the critical values earlier than protein content when N application increases. But in the three cases, when the yield and protein content reach the critical values, the corresponding N application amount ranges from N2 to N3, which is probably because excessive application of N fertilizer reduces net photosynthetic rate of wheat. When nitrogen use efficiency decreases, the flat grains will increase and the yield will decrease. When the critical values of yield and protein content correspond to the same N application amount, the yield and the protein content were always positively correlated to each other with the increase of N application. However, when the two critical values corresponded to different N application amount, the yield had a negative correlation with

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protein content within a certain extent. Therefore, some previous studies have concluded that yield and protein content are negatively correlated (Wang et al., 2003), while other studies have suggested that yield and protein content can increase simultaneously under suitable conditions (Cooper et al., 2001; Danid and Triboy, 2002), which is because N application amounts corresponding to the critical values of yield and protein content are diverse under different experimental conditions. At the same time, the study has figured out the synergistic intervals of yield and protein content with the increase of N application under different amounts P application, according to fitted equation of N utilization efficiency on yield and protein under different P application (*Table 5*).

Conclusion

In this study, four winter wheat cultivars were treated with combined N and P fertilizers to study the synergistic change regulation of grain protein content and yield for the purpose of obtaining the protein threshold intervals of winter wheat. The results clearly showed that grain yield and protein content could synergistically increase under the medium and high P fertilizer treatment and under the condition of N fertilizer treatment within the range from N2 to N3. At the same time, the protein threshold intervals can be calculated by the fitted equation, however, the difference among the winter wheat genotypes need to be further explored as their varied genetic characteristics which will ultimately affect the yield components and grain quality even though at the same fertilizer treatments. Moreover, the combined effect between the water and nitrogen, phosphorous need also revealed as the absorption of nitrogen, phosphorous by crops is affected by the soil water which was hard to be controlled in our study. Thus, finding out the interactive effect among these factors would be beneficial for understanding the relationship between the grain yield and the protein content.

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STUDY ON THE P FACTOR OF TILLAGE PRACTICES IN THE TYPICAL BLACK SOIL AREAS OF NORTHEAST CHINA UNDER EXTREME RAINFALL CONDITIONS

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Abstract. The support practice factor (P) is an essential component of the common Universal Soil Loss Equation (USLE) model. Although concerns about soil loss in the typical black soil areas of Northeast China are increasing, little research exists on the P factor of extreme rainfall in soil loss. Therefore, we conducted an orthogonal simulation experiment of artificial rainfall employing different rainfall intensities, slopes, and tillage practices. The aim of the design was to establish the values and calculation methods for the P factor of different tillage practices under various slope and rainfall intensity conditions. Our results showed that for No tillage (NT) and longitudinal wide ridge (LWR), a significant logarithmic decreasing relationship was found between the P factor and the product of rainfall erosivity (R) and slope factor (S). When *RS* was < 9.18 MJ·mm/(hm^2·h), contour ridge tillage was the optimal practice to decrease soil loss. The P factor of contour narrow ridge (CNR) was higher than those of other tillage practices after contour ridge failure occurred. When maximum rainfall intensity was > 75 mm/h, NT was superior in reducing soil loss. The results could provide a basis for predicting soil loss under extreme rainfall conditions.

Keywords: *USLE, simulation experiment, rainfall erosivity, slope factor, hydraulic characteristic, ridge system*

Abbreviations: A: soil loss modulus, A_F: soil loss modulus for contour ridge failure, CNR: contour narrow ridge, CWR: contour wide ridge, D: runoff depth, Fr: Froude number, LNR: longitudinal narrow ridge, LWR: longitudinal wide ridge, n: roughness, NT: no tillage, Qr: runoff volume measured by the flowmeter at the outlet of the soil tank, R: rainfall erosivity, S: slope factors, V_F: volume for soil loss for contour ridge failure, W_F: width for soil loss for contour ridge failure

Introduction

Soil loss is a crucial factor in the loss and degradation of land resources that humans depend on for survival. To provide suggestions on reducing the loss of soil, a tool able to diagnose the "problem" of soil loss is required to provide the scientific basis for preventing and controlling the occurrence and aggravation of such a loss. This tool is the soil loss model (Lane et al., 1992; Kinnell, 2010; Di Stefano et al., 2019). In regard to soil modelling mean, soil loss models can be categorised as either empirical or mechanistic (Aksoy and Kavvas, 2005; Batista et al., 2019). Empirical models are a series of

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mathematical equations based on the statistical analysis of a large amount of experimental observation data (Foster et al., 2001; Khosravi et al., 2012). Mechanistic models are primarily based on physical causes and quantitatively describe and depict the soil loss process by simulating various effects (Nearing et al., 1989; Licznar and Nearing, 2003; Nouwakpo et al., 2018). These effects include rainfall infiltration, runoff formation, raindrop splashing, and runoff scouring on a series of main processes, such as soil separation, sediment transport and deposition, plant growth, and stubble decomposition. Empirical soil loss models are applied widely because of their low input requirements for parameters and variables (Kinnell and Risse, 1998; Ibearugbulem et al., 2018). However, mechanistic models are still in the research stage in most countries and regions and have not been applied widely because of the difficulty to obtain model parameters, operate the models (Shoemaker et al., 2005; Lobo and Bonilla, 2019), and the cost is high.

Among the empirical models, the Universal Soil Loss Equation (USLE) has a long application history and wide application scope (Wischmeier and Smith, 1958; Ahamed et al., 2000; Tyner et al., 2011). Its first official version was launched through Agricultural Manual No. 282 in 1965 (Wischmeier and Smith, 1965) and the second version was launched through Agricultural Manual No. 537 in 1978 (Wischmeier and Smith, 1978). In 1997, the Revised Universal Soil Loss Equation (RUSLE) was released officially (Renard et al., 1997). The USLE includes the main factors that affect soil loss on a sloping surface and uses a wide range of data; hence, it has been applied widely in rainfall soil loss prediction in many countries to conduct soil loss investigations (Yu et al., 1999; Van der Kinff et al., 2000; Mausbach and Dedrick, 2004; Brazier, 2016). In 1975, the Soil Health Institute in the United States developed the MUSLE (Modified Universal Soil Loss Equation) model based on USLE, which can simulate soil loss at the watershed scale with a single rainfall event as the simulation step (Williams, 1975). Since then, MUSLE has been adopted by the crop productivity model (Williams et al., 1983), watershed non-point source pollution model (Gassman et al., 2007), and others (Shoemaker and Dai, 2005), and has played an important role in the simulation of the surface ecological process. The factors that affect the soil loss used in this model, such as soil erodibility, slope length, coverage, management measures, and soil conservation measures, have the same meanings as those in the USLE.

According to the definition of the Universal Soil Loss Equation, the P factor for soil conservation practices refers to the soil loss ratio with the implementation of conservation measures when directly tilling up and down along a sloping farmland. Such soil conservation measures can be divided into two categories, namely engineering measures and tillage practices (Liu et al., 2002; Xin et al., 2019). Engineering measures reduce soil loss by changing a certain scope of the micro-topography (e.g., terraces) to retain surface runoff and increase rainfall infiltration. Tillage practices involve improving the anti-soil-loss performance of the soil, preserving water and soil and preventing soil loss through farming, specifically by using ploughs, hoes, harrows, and other farming tools or machinery to increase surface coverage, increase soil infiltration, and the like. The difference between tillage practices and engineering measures is that the former does not change the micro-topography and is implemented only on agricultural land. The establishment of a soil loss model is primarily based on a large quantity of observations and experimental data. Long-term, high-quality experimental and monitoring data comprise the basis for establishing this model and are an important and reliable means to obtain the P factor (Chen et al., 2017). When calculating the P factor, with regard to the kind of underlying surface and tilling methods that should be

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considered as the objects for reference and contrast, USLE and RUSLE stipulate up-and-down slope culture (Wischmeier and Smith, 1978; Renard et al., 1997). However, under the same longitudinal tillage method, the soil loss amount could differ substantially because of different tillage systems with different ridge spaces, widths and heights (Xu et al., 2019). Therefore, the objects for reference and contrast should be further concretised.

Soil loss has become increasingly concerning in the typical black soil areas of Northeast China (Lal et al., 2001; Liu et al., 2008), which comprise approximately 17.27 million hm^2 of cropland, accounting for 14% of total croplands in China. Longitudinal ridge tillage has been a common practice for a long time in this area; typical sampling survey in Heilongjiang Province showed that 3/4 of the farmland has adopted longitudinal or quasi-longitudinal ridge tillage practices (Zhao et al., 2012). In recent years, with the promotion of black soil land protection, soil loss prevention measures have been popularised and implemented gradually. Black soil areas are an important commodity grain base, and are the main producers of crops such as sugar beet, flax, and sunflower (Cui et al., 2007; Wang et al., 2015). To ensure farmland quantity and grain output, implementing vegetation measures such as returning farmland into forest or grassland is not suitable. At present, in the distribution zones of black soil and chernozem in these typical black soil areas, nearly half of the black soil layers in the profiles have a thickness of < 40 cm, whereas in the chernozem distribution areas, the thickness of black soil layers in more than half of the profiles is < 20 cm (Wang et al., 2009). If soil conservation engineering measures were implemented, the excavation depth in the thin soil layer would exceed the tillage layer, leading to the destruction of surface soil resources. Therefore, soil conservation tillage practices currently comprise the main method to prevent and control soil loss in these areas.

Recommendations to improve soil and water conservation include adjusting longitudinal ridge tillage to contour ridge tillage or no tillage (NT). Research has been conducted on the effects of such tillage practices on soil and water loss and sediment reduction, and the conservation tillage practices in these areas could increase gradually in the future (Xu et al., 2018; Zhang et al., 2012). However, few studies have been conducted on the P factor values of different tillage practices in this area and, particularly, no studies could be located on calculating the P value under extreme rainfall conditions.

A high-intensity rainstorm of short duration on a slope under the contour ridge tillage system usually causes concentrated stream soil loss and intensified gully-cutting soil loss. Usually, these affects can be ascribed to the infiltration-excess runoff in the furrows gradually gathering and then breaking out from the ridges in fragile or low-lying places of the contour ridges (Meng et al., 2009). In recent years, climate change and its significant global effects on water resources, environment, ecology, and the like, have become pressing environmental problems. The fifth assessment report of the United Nations Intergovernmental Panel on Climate Change (IPCC) showed that global warming has changed the global water cycle process (Ohmura and Wild, 2002), resulting in changes in the temporal and spatial distribution of precipitation and the frequent occurrence of extreme rainfall events (Stocker et al., 2013). Research has indicated an increase in the frequency and intensity of extreme rainfall worldwide in recent decades, and this trend could continue (Singh and Kumar, 1997; Arnell, 1999; Bartholy and Pongrácz., 2007). The influence of extreme rainfall events on the effects of conservation tillage practices on soil and water requires further investigation.

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In this study, we employed an indoor artificial rainfall simulation experiment to determine the reference objects for calculating the P factor; furthermore, we propose calculation methods for the P factor value under different slope and rain intensities with the purpose of predicting soil loss.

Materials and methods

Materials

The soil used in the experiment was obtained from a long, gentle sloping farmland from the Soil and Water Conservation Experimental Station in Keshan County, Heilongjiang Province, where longitudinal ridge tillage is adopted in the field (*Fig. A1* in the *Appendix*). The slope of the plot is primarily between 3° and 5°, and the soil type is black soil. The geographical coordinates of the soil collection site are 48°03′17″N and 125°49′23″E. The location has a temperate continental monsoon climate, with an annual average temperature of 3.0 ℃, and the annual average precipitation concentrated from June to September is 514.9 mm. The land plot is located at the southern foot of the Lesser Khingan Mountains in the transition zone next to the Songnen Plain. Soybean is the main crop, and mechanisation is adopted mainly in field operations.

The test soil derives from typical cultivated black soil within 40 cm from the surface of the sloping farmland. Soil particles comprise clay (<2 μm), silt (2-50 μm), and sand (50-2000 μm), accounting for 23.7%, 72.5%, and 3.8% of the soil particle composition, respectively. The soil organic matter content is 29.67 g/kg, and the pH value is 6.81.

Our artificial rainfall simulation experiment was conducted in the soil conservation hall of the Yanqing Experimental Base of the China Institute of Water Resources and Hydropower Research. The rainfall equipment comprised a side-spray artificial rainfall device, which uses computer software to control rainfall intensity in the range of 10 to 200 mm/h. The rainfall height was 13.8 m, and rainfall uniformity was more than 85%.

The dimensions of the test soil tank (fixed hydraulic lifting and descending steel tank) were 8 m × 3 m × 1 m (length × width × height) (*Fig. 1*). Tipping-bucket flowmeters were used at the runoff collector of the tank to perform measurements in the runoff yield process at a resolution of 3 L. Tipping-bucket self-recording rain gauges were used to measure the simulated rainfall at a resolution of 0.2 mm.

Design of the artificial rainfall simulation experiment

We adopted an orthogonal design for the artificial rainfall simulation experiment. The primary factors we considered were rain intensity, surface slope, and tillage practices. Forty experimental sessions (4 × 2 × 5) were conducted, with two replicates for each session.

The rain intensity design of our artificial rainfall experiment was based on the intensities of the rainstorms in different recurrence periods in the soil source area. The rainfall intensities were analysed and determined, considering the reports on erosive rainfall intensities in the typical black soil areas in Northeast China. The 1 h rainstorm intensities in the 20%, 10%, 5%, 2%, and 1% recurrence periods of Keshan, where the test soil is located, are 35.7, 43.6, 49.3, 60.5, and 68.2 mm/h (Ye et al., 2014), respectively. According to Lu et al. (2016), in the heavy rainfall soil loss events with above-moderate soil loss intensities, the instantaneous rainfall intensities range from 42.6 to 103.2 mm/h. On this basis, we designed four typical rainfall intensities for this study, namely 30, 50, 75, and 100 mm/h, with the simulated rainfall events all lasting 1 h.

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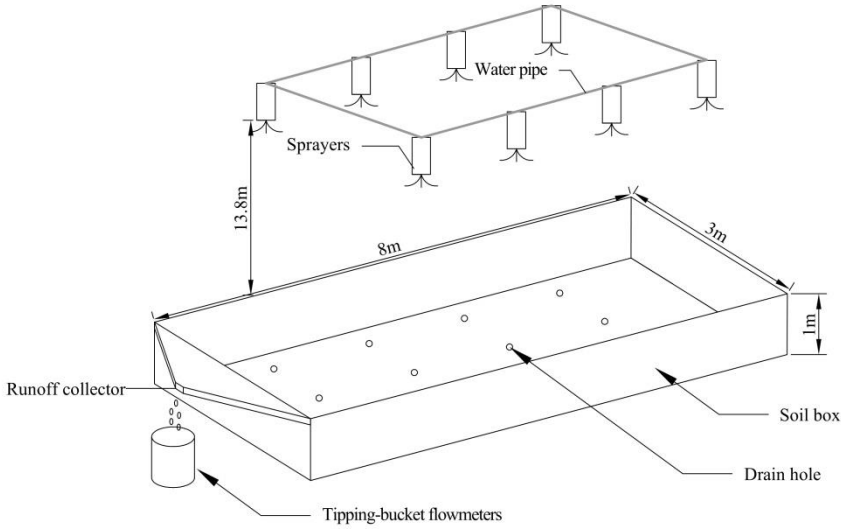

Figure 1. *Schematic diagram of test soil tank and artificial rainfall system*

Typically, black soil areas in Northeast China have gentle and long slopes. The slope of the ground of the soil collection site was mainly between 2° and 6°, and the slope length mainly between 100 and 1000 m. Therefore, two common angles of slope were designed for the experiment, with angles of 3° and 5°.

Tillage practices adopted in the sloping farmland in the source area include mainly longitudinal ridging, contour ridging, and no tillage (Chen et al., 2008; Shen et al., 2020). For ridged sloping farmland, there were two types of ridges, namely wide and narrow ridges (Xu et al., 2018). For wide ridges, the distance was 100–120 cm, width was 65–75 cm, furrow width was 35–45 cm, and height was 7–12 cm. For narrow ridges, the distance was 55–70 cm, width was 30–35 cm, furrow width was 25–35 cm, and height was 12–20 cm. Therefore, we adopted five tillage practices, namely no tillage (NT), longitudinal wide ridge (LWR), longitudinal narrow ridge (LNR), contour wide ridge (CWR), and contour narrow ridge (CNR) (*Fig. 2*). For wide ridges, the distance was 110 cm, ridge width was 70 cm, furrow width was 40 cm, and average furrow depth was 10 cm. For narrow ridges, the distance was 60 cm, ridge width was 30 cm, furrow width was 30 cm, and average furrow depth was 15 cm (*Table 1*).

Table 1. *Comparison between actual plot conditions and experimental simulation conditions*

Primary factor	Actual conditions	Simulation conditions
Rain intensity (mm/h)	1 h rainstorm intensity in 20% recurrence period is 35.7 mm/h; range of heavy rainfall intensity is between 42.6 and 103.2 mm/h	30, 50, 75, and 100
Slope (°)	2–6	3 and 5
Slope length (m)	100–1000 m	8
Ridge furrow width (cm)	Wide ridge 35–45, narrow ridge 25–35	Wide ridge 40, narrow ridge 30
Ridge width (cm)	Wide ridge 65–75, narrow ridge 30–35	Wide ridge 70, narrow ridge 30
Ridge height (cm)	Wide ridge 7–12, narrow ridge 12–20	Wide ridge 10, narrow ridge 15

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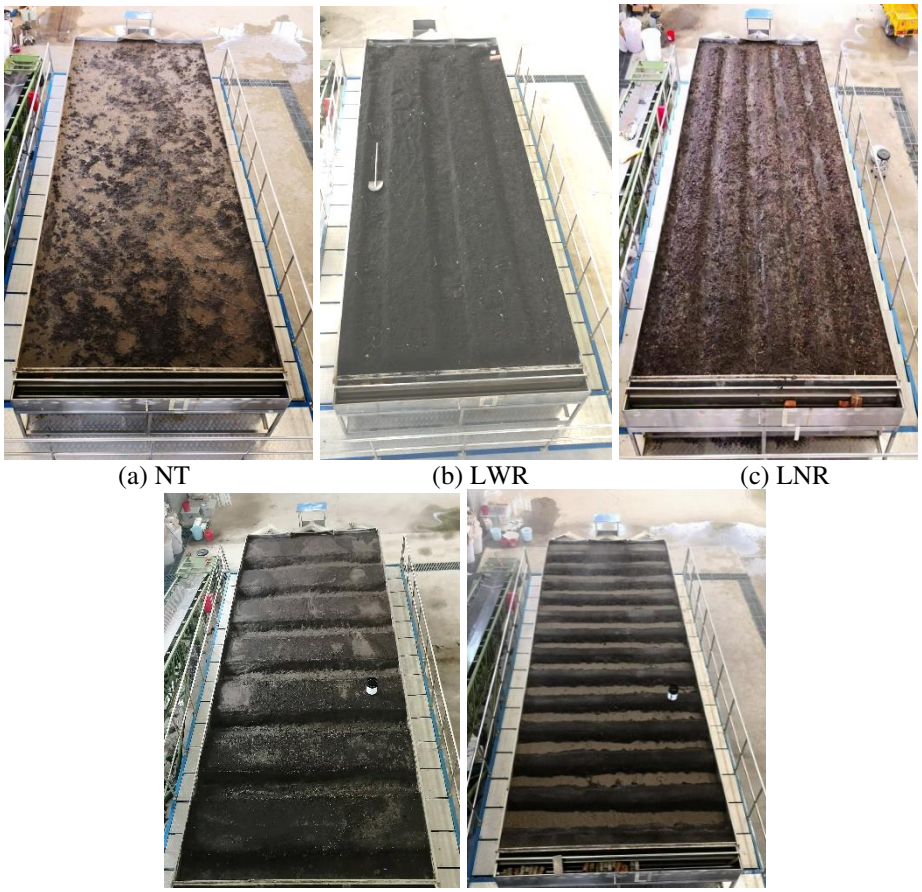

(a) NT (b) LWR (c) LNR

(d) CWR (e) CNR

Figure 2. *Photos of tillage practices adopted in the indoor simulation experiment*

Steps in the artificial rainfall simulation experiment

(1) Before loading the soil, evenly spaced drainage holes were drilled at the bottom of the test soil tank; these drainage holes were first filled with gauze, and then with fine sand up to 20 cm in thickness to form a permeable layer and ensure adequate water permeability. The test soil tanks were filled according to the soil bulk densities of the plough pan, tilth, and ridge layers measured in the field. Above the sand layer, the plough pan layer was filled to a thickness of 30 cm with a soil bulk density of 1.35 g/cm^3, then the tilth layer was filled to a thickness of 30 cm with a soil bulk density of 1.20 g/cm^3. The layered filling method was adopted; with one layer every 5 cm. Additionally, when filling the soil, the peripheral boundary of the test soil tank was compacted to reduce the influence of the boundary effect. Ridges were built on the tilth layer with a height of 15 cm, spacing of 65 cm, and soil bulk density of 1.20 g/cm^3.

(2) On the day before the experiment, the test soil tank was covered with gauze, and pre-rainfall was conducted at a rainfall intensity of 30 mm/h until runoff yield on the slope surface, which ensured the consistency of soil conditions in the early stage of the experiment. After pre-rainfall, the test soil tank was covered with plastic cloth to prevent the evaporation of soil moisture and slow down the formation of a crust. The rainfall for the experiment began after a resting period of 12 h.

(3) After the start of the formal rainfall experiment, the runoff and soil loss on the slope were carefully observed. When runoff occurred, the first sample was collected at

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the outlet of runoff collector, followed by sample-collection every 3 min. If the contour ridge tillage becomes damaged because of runoff, samples should be taken every 1 min thereafter. After rainfall, the supernatant of the runoff sample was removed, and placed in an oven at a constant temperature of 105 ℃. The sediment quality was determined after drying.

Calculation of P factor and analysis of main influencing factors

P factor calculation

The equation form of the USLE (Wischmeier and Smith, 1978; Renard et al., 1997) is:

$$A = RKLSCP \quad \text{(Eq.1)}$$

Soil loss modulus A ($t \cdot hm^{-2}$) is the soil loss at a given period. R is the rainfall erosivity factor ($MJ \cdot mm \cdot hm^{-2} \cdot h^{-1}$) for any given period and is obtained by summing for each rainstorm, the product of total storm energy (E) and the maximum 30-min intensity (I_{30}). K is the soil erodibility factor ($t \cdot h \cdot MJ^{-1} \cdot mm^{-1}$), which is defined as the rate of soil loss per unit of R as measured on a unit plot. L and S (dimensionless) are the slope and slope length factors respectively, and account for the effect of the topography on soil erosion. C is the coverage-management factor, which is defined as the ratio of soil loss from land with a specific vegetation to the corresponding soil loss from continuous fallow. P is the support practice factor (*Eq. 1*), which is defined as the ratio of soil loss with a specific support practice to the corresponding loss with up-and-down slope culture.

In *Equations 2* and *3,* the S factor can be calculated according to the slope θ (°):

$$S = 10.8\sin\theta + 0.03, \theta < 5° \quad \text{(Eq.2)}$$

$$S = 16.8\sin\theta - 0.5, \theta = 5° \quad \text{(Eq.3)}$$

In calculating the P factor, the amount of soil loss caused by up-and-down slope culture should be determined; this refers to the amount of soil loss caused by longitudinal ridge tillage. Both LWR and LNR are longitudinal ridge tillage practices, with differences only in the ridge and furrow sizes. To obtain unified contrast objects in calculating the P factor, the tillage practice with larger overall soil loss in LWR and LNR should be considered as the contrast objects. The other four tillage practices can be considered as soil and water conservation effects. The P factor value in the single rainfall event was determined by the ratio of soil loss amounts observed in the artificial simulated rainfall experiments.

Main influencing factors affecting P factor

We analysed the main factors that could influence variation in the P factor, including rainfall, slope, and hydraulic characteristics, to obtain the equation for calculating the P factor.

The effects of rainfall and surface slope are characterised by R and S, respectively. The hydraulic characteristics of surface runoff are reflected mainly by roughness n and the Froude number Fr (RöMkens et al., 2002; Cremers et al., 2010; Omidvar et al., 2019):

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$$n = R_h^{2/3} \cdot J^{1/2} \cdot V_{OV}^{-1} \quad \text{(Eq.4)}$$

$$Fr = V_{OV}^{-1} \cdot (gR_h)^{-0.5} \quad \text{(Eq.5)}$$

where n is a parameter comprehensively reflecting the influence of the roughness of the ridge furrow surface on water flow (*Eq. 4*); Fr is used to judge the state of water flow (*Eq. 5*); R_h is the hydraulic radius (m), which is the ratio of the cross-sectional area of the flow and the wetted perimeter, and the wetted perimeter refers to the perimeter of the runoff in contact with the ridge furrow profile; J is the hydraulic slope (m/m), i.e., the soil tank slope; g is the Gravitational acceleration; V_{OV} is the flow velocity (m/s) of runoff in the ridge furrow, and is obtained by dividing the runoff discharge in the ridge furrow by the cross-sectional flow area. In this study, the discharge of each ridge furrow was considered equal under the condition of longitudinal ridge tillage, and the V_{OV} was calculated based on the outlet runoff volume of the soil tank (*Eq. 6*).

$$V_{OV} = Q_r \cdot (Nr \cdot R_r)^{-1} \quad \text{(Eq.6)}$$

where Q_r is the runoff volume (m^3) measured by the flowmeter at the outlet of the soil tank, Nr is the number of furrows in the soil tank, and R_r is the cross-sectional flow area in the furrows.

Analysis of suitable conditions for the application of tillage practices

We suggested appropriate conditions for the implementation of the tillage practices, considering soil loss reduction by these various tillage practices under the combined conditions of different rainfall erosivities and slopes. With regard to contour ridge tillage, if the ridge is penetrated by runoff, the contour ridge is considered damaged. In such a case, concentrated surface runoff could be formed in a short time, resulting in a significant increase in the soil loss amount. The threshold of hydrological conditions for contour ridge failure were analysed in this study.

Results

Runoff and soil loss under different tillage practices

The runoff depth and soil loss modulus of the five tillage practices, namely NT, LWR, LNR, CWR, and CNR under extreme rainfall conditions are shown in *Figure 3*. Generally, the order of the runoff depth (D) of the various tillage practices was LNR > LWR > NT > CNR > CWR. The order of the soil loss modulus (A) was CNR > LNR > CWR > LWR > NT. Under extreme rainfall conditions, the soil loss amount of the contour ridge tillage was higher than that of the longitudinal ridge tillage, which was the opposite of the observation data of the runoff plot in a short time series (Xin et al., 2019). Therefore, to analyse the effect of water and soil loss reduction by soil conservation tillage practices, it was necessary to consider extreme rainfall conditions.

If the product (RS) of rainfall erosivity R and slope factor S is used to characterise the interaction of rainfall and slope on the soil loss process, the runoff depth (D) of various tillage practices has a significant linear function relationship with RS (*Fig. 4a*). The soil loss modulus (A) of the tillage practice has a significant exponential increasing

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relationship with *RS* (*Fig. 4b*). Generally, the increasing rate of *D* with the increase in *RS* is relatively stable, and the increasing rate of the contour ridge tillage is slightly higher than is that of the longitudinal ridge tillage. However, when *RS* was ≥ 15 MJ·mm/(hm^2·h), the increasing rate of *A* of all tillage practices increased with *RS*. Among these, the increasing rate of the soil loss modulus in CNR was the largest, even a little higher than it was in the LNR. During continuous heavy rainfall, a large amount of runoff collected gradually in the furrows in contour ridges. If the contour ridge was damaged under the effects of continuous runoff scouring, a large amount of runoff can be formed in a short time to scour the surface, resulting in substantial soil loss. Compared with other tillage practices, with NT it was not easy to form a concentrated confluence path on the surface that weakens the scouring and erosion capacity of per unit runoff.

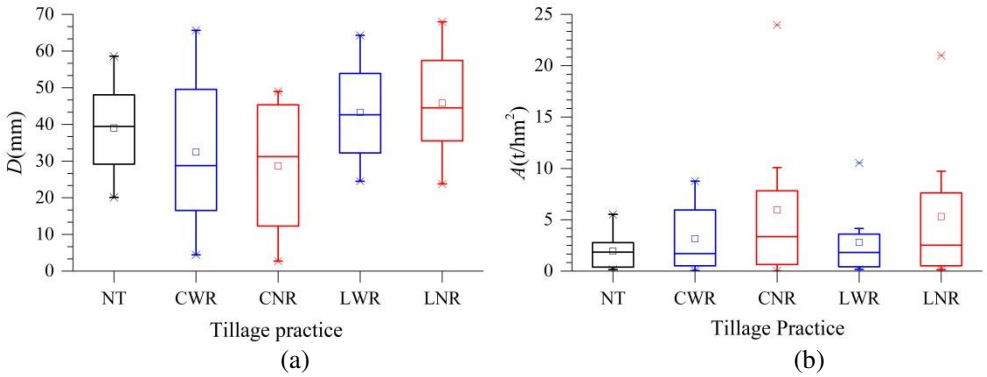

Figure 3. *Relationship between runoff depth (a) and soil loss modulus (b) of various tillage practices*

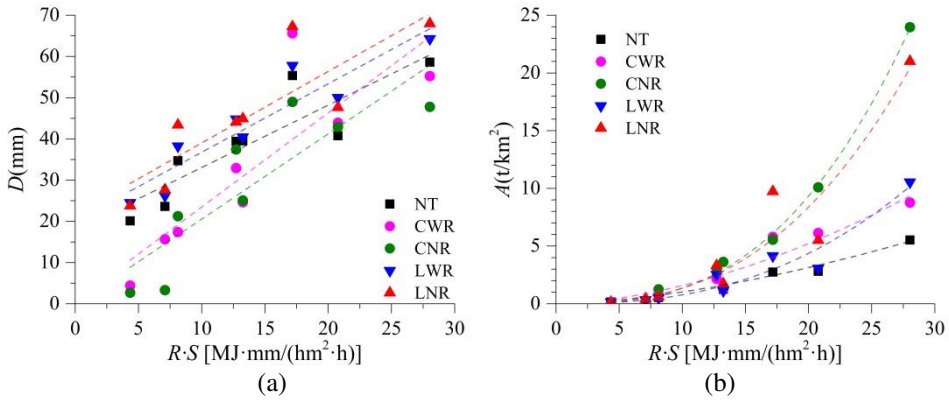

Figure 4. *Relationship of RS with runoff depth (a) and soil loss modulus (b)*

P factor under different tillage practices

Under extreme rainfall conditions, the soil loss modulus of LWR varied from 0.17 to 10.54 t·hm^2, with an average of 2.80 t·hm^2. The soil loss modulus of LNR varied from 0.14 to 21.01 t·hm^2, with an average of 5.31 t·hm^2, i.e., the latter was generally higher. Therefore, LNR was considered the contrast object when calculating the P factor, LWR, CWR, CNR and NT could all be regarded as soil conservation tillage practices. See

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Figure 5 for calculated P factor values. Among these, the P factor of LWR varied from 0.426 to 1.223, with an average of 0.712; the P factor of NT varied from 0.263 to 1.094, with an average of 0.634; the P factor of CWR varied from 0.302 to 1.375, with an average of 0.706; and the P factor of CNR varied from 0.051 to 2.090, with an average of 1.097. Clearly, the P factor value of NT was the lowest and, generally, there was little difference between LWR and CWR, whereas the P factor value of CNR was significantly higher than those of other tillage practices.

Regarding CWR, if the contour ridge was not damaged during the runoff yield, the average P factor value was 0.384. In case of damage, the average P factor value was 0.813. As regards CNR, the average P factor value was only 0.067 if the contour ridge was not damaged during the runoff yield. In case of damage, the average P factor value was 1.440. When other conditions were the same, if the contour ridge was damaged, the soil loss amount of the slope surface was close to or higher than that of longitudinal ridge tillage. Further, a concentrated runoff path could be formed in a short time, leading to a large amount of concentrated runoff scouring and resulting in more substantial soil loss.

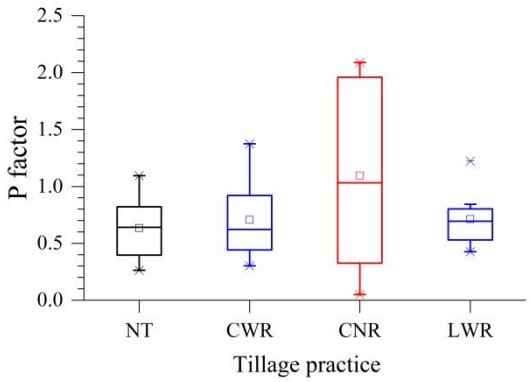

Figure 5. *P factor values under various tillage practices*

Main factors affecting the P factor

Combined influence of rainfall intensity and slope

For NT and LWR, there was a significant logarithmic decreasing relationship between the P factor value of a single runoff event and *RS* (*Fig. 6*):

$$\text{NT: } P_{NT} = -0.392\text{Ln}(RS) + 1.607, (r^2 = 0.69, p < 0.05) \quad \text{(Eq.7)}$$

$$\text{LWR: } P_{LWR} = -0.364\text{Ln}(RS) + 1.616, (r^2 = 0.80, p < 0.01) \quad \text{(Eq.8)}$$

Along with the increase in rain intensity and slope, the relative soil loss reduction effect of NT and LWR was more significant. For LNR, the average height difference between the adjacent lowest point of the ridge furrow and the highest point of the contour ridge was 15 cm, and the average horizontal distance between them was 30 cm, with the ratio being 0.50. For LWR, the average height difference of the highest points of the two was reduced to 10 cm, and the average horizontal distance was expanded to 55 cm, with the ratio being 0.18, i.e., significantly lower than was that of the former. The amount of soil loss in the process of extreme rainfall was relatively low on the

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surface of sloping farmland with micro-topographic relief. Regarding farmlands with a long, gentle slope, the amount of soil loss could be reduced in the process of runoff scouring by appropriately reducing the degree of micro-topographic relief.

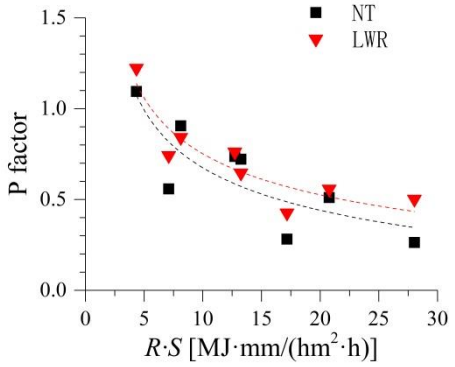

Figure 6. *Relationship of P factor value with rainfall erosivity (R) and slope factor (S) under extreme rainfall conditions*

Hydraulic characteristics of longitudinal ridge tillage

Roughness (*n*) and Froude number (*Fr*) are important parameters that affect the soil loss process of the slope surface under longitudinal ridge tillage. For LWR, *n* varied from 0.124 to 0.227, with an average of 0.191, and for LNR, *n* varied from 0.061 to 0.144, with an average of 0.100. Research has suggested that *n* of bare ridge tillage farmland is 0.06 to 0.12, and an average value of 0.09 is adopted usually when no actual measurement is conducted (Neitsch et al., 2002). The variation range of *n* under LNR was slightly higher than the suggested range, whereas the *n* value under LWR was generally higher than this range. In our study, the Manning roughness coefficient *n* reflected the blocking effect of the roughness of furrows on water flow. The typical black soil areas in Northeast China have a gentle slope, and the depth of runoff in furrows is shallow, mostly less than 2 cm, which leads to an increase in *n*. Compared with LNR, LWR had greater width at the bottom of furrows and shallower depth of water flow; therefore, it had a greater blocking effect on runoff from the slope surface. This effect could reduce the flow velocity on the slope surface, thereby reducing the soil loss and scouring from the runoff.

As regards the flow pattern in the furrows, the *Fr* under LWR and LNR varied from 0.147 to 0.619, with an average of 0.297. The *Fr* values were all greater than 0.10, which showed that the flow pattern in the furrows was slow. Generally, the *Fr* value under LNR was slightly higher. There is a significant quadratic relationship in the *n* and *Fr* values of LWR with *RS* (*Fig. 7*):

$$n = -0.0004\ (RS)^2 + 0.0154RS + 0.0776 \quad \text{(Eq.9)}$$

$$Fr = 0.0005(RS)^2 - 0.0173RS + 0.322 \quad \text{(Eq.10)}$$

With an increase in *RS*, the *n* value of furrows under LWR gradually increased, but the growth rate of the *n* value decreased significantly from $RS \geq 15$MJ·mm/(hm^2·h) (*Fig. 7a*). This indicated that with the increase in runoff, the blocking effect of the

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surface on the slope runoff became limited gradually, leading to the soil loss modulus increasing significantly with an increase in *RS* (*Fig. 4b*) and the decline rate of the P factor value gradually slowing down (*Fig. 6*). There was no significant correlation of the *n* and *Fr* values of furrows under LNR with *RS* (*Fig. 7b*).

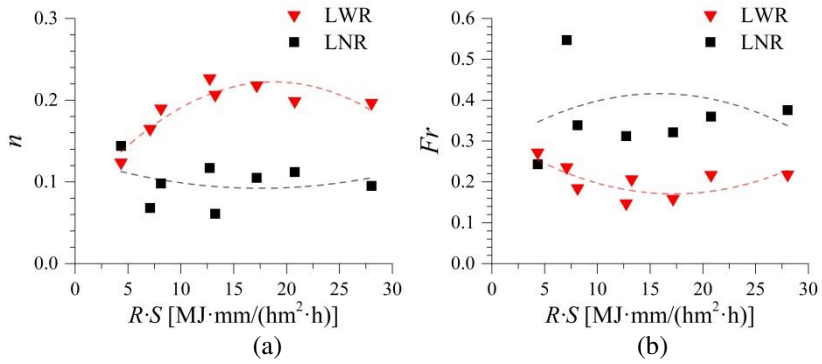

Figure 7. *Relationship of RS with roughness (a) and Froude number (b)*

Suitable conditions for application of main tillage practices

Figure 8 shows the changes in soil loss rate with time under different experimental rainfall intensities. With a rainfall intensity of 30 mm/h, the soil loss rate under CNR was significantly lower than under NT or LWR. Although the soil loss rate under CWR increased significantly after 45 min, and was between that of NT and LWR (*Fig. 8a*), the average soil loss rate during the runoff yield process was still lower than under NT or LWR. With a rainfall intensity of 50 mm/h, the soil loss rates under CWR and CNR rapidly increased at 30 and 42 min, respectively, because the contour ridges had been damaged during the runoff yield; however, the average soil loss rates of the entire runoff yield process did not differ much from those under NT and LWR. With rain intensities of 75 and 100 mm/h, the time points of sudden increase in the soil loss rate under CWR and CNR advanced further, which were 21 and 33 min under 75 mm/h, and 19 and 33 min under 100 mm/h, respectively. The average soil loss rates under both tillage practices were significantly higher than those under NT and LWR. Compared with CWR, the soil loss rate under CNR increased faster after breaking the ridges. With 100 mm/h as an example, the soil loss rate under CNR increased by 173.0 times and that of CWR by 12.5 times within 6 min after breaking the ridge. The soil loss rates under NT and LWR showed little difference under conditions of 50 and 75 mm/h, and the soil loss rate under NT was lower than under LWR with conditions of 30 and 100 mm/h.

With extreme rainfall, soil loss caused by contour ridge failure is a significant source of soil loss and sediment yield on the slope surface under contour ridge tillage. Considering the test slope as a whole, the soil loss modulus *Arw* and *Arn* corresponding to contour ridge failure under CWR and CNR increased significantly linearly with *RS* (*Fig. 9*):

$$\text{CWR: } Arw = 1.65RS\text{-}15.85, (R^2 = 0.73, p < 0.1) \quad \text{(Eq.11)}$$

$$\text{CNR: } Arn = 5.09RS\text{-}46.74, (R^2 = 0.96, p < 0.01) \quad \text{(Eq.12)}$$

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Although the slopes and intercepts of the above two linear equations were quite different, when contour ridge failure under CWR and CNR started to occur, the *RS* values were 9.61 and 9.18 MJ·mm/(hm^2·h), respectively, i.e., considerably close to each other. These values could be used as the threshold of hydrological conditions for contour ridge failure.

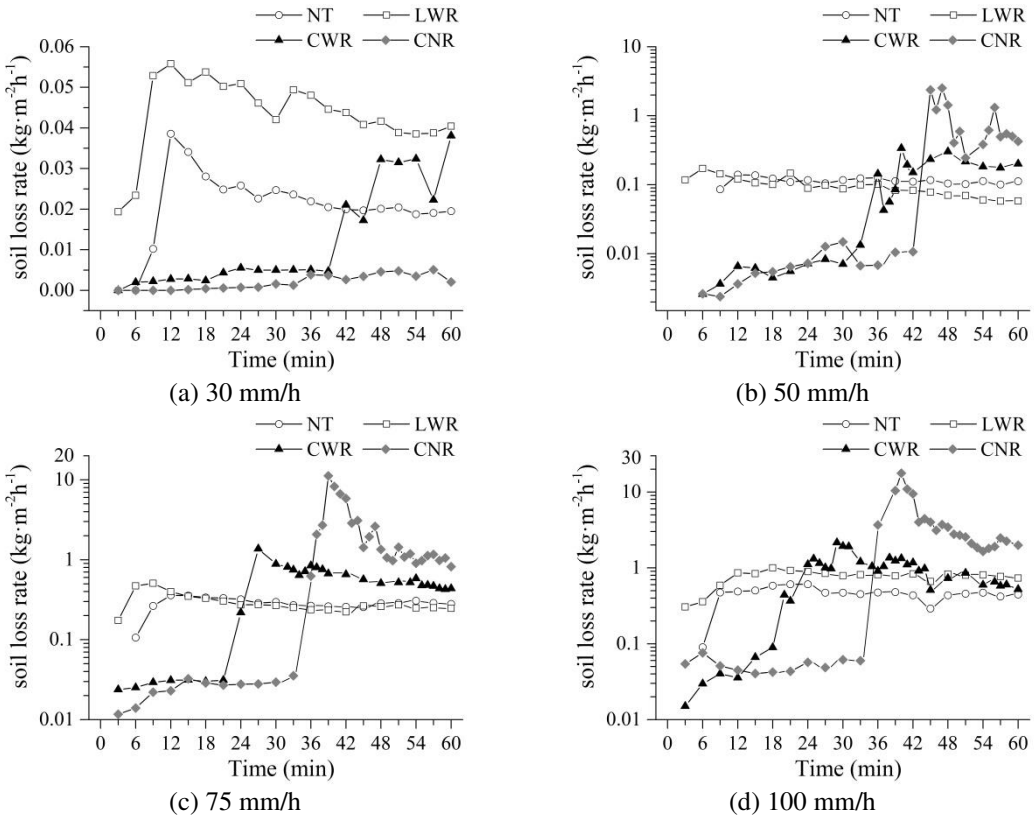

Figure 8. *Changes in soil loss rate under various tillage practices under different rainfall intensities. Note: the average soil loss rate is the average value of soil loss rates under 3° and 5° under this tillage practice*

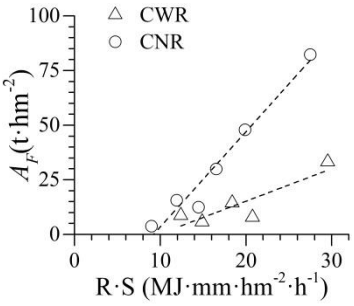

Figure 9. *Relationship of soil loss modulus for contour ridge failure with rainfall erosivity and slope factor*

According to the above analysis, when *RS* was < 9.18 MJ·mm/(hm^2·h), contour ridge tillage was the optimal tillage practice to reduce soil loss. In a unit area, the impoundment

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runoff of furrows under CNR was 2.08 times that of CWR, i.e., the effect of reducing water and sediment was more significant. When *RS* was ≥ 9.18 MJ·mm/(hm^2·h) and the maximum rainfall intensity was < 75 mm/h, there was no significant difference between CWR and NT in respect of the soil loss reduction effect. When the maximum rainfall intensity was > 75 mm/h, NT had the optimal effect in reducing soil loss.

Discussion

Comparison with annual average field observation values

When the P factor value of this study was compared with the multi-year average P factor values observed in the runoff plots (*Table 2*), the former was substantially higher. The rainfall intensity of the artificial rainfall experiment was primarily designed based on the highest local 1 h rainfall intensity over 5–100 years. The observation of soil loss in this area began relatively late, and the continuous observation data of many field runoff plots cover no more than 5 years; consequently, the soil loss under extreme rainfall is not reflected fully. However, the soil loss caused by extreme rainfall accounted for a significant proportion of the total soil loss, and this fact should not be ignored. In the extreme rainfall events, soil loss of the LWR and LNR systems were both severe, and the P value increased. For most of the erosion event in the field runoff plot, the soil loss rates were relatively low without ridge failure, and the P factors were relatively low. While in the extreme rainfall events, after the ridge failure, the increasing trends for soil loss rates were significant, especially for higher rainfall intensities, and the P factors increased significantly. Accordingly, the amount of soil loss for CWR and CNR systems could be underestimated if the annual average P observed from the runoff plot was used in the calculations.

Table 2. *Comparison between P factor value of this study and observation value of field runoff plot*

Tillage practice	Observation time	Observation site	Slope (°)	Average P factor	Literature source
LWR	1 year	Keshan	5	0.33	Wang et al., 2019
	Extreme rainfall	Indoor (artificial rainfall)	3	0.81	This study
	Extreme rainfall	Indoor (artificial rainfall)	5	0.61	This study
CWR	6 years	Jiusan	5	0.08	Xin et al., 2019
	3 years	Binxian	6	0.12	Xin et al., 2019
	3 years	Zhalantun	7	0.05	Xin et al., 2019
	Extreme rainfall	Indoor (artificial rainfall)	3	0.73	This study
	Extreme rainfall	Indoor (artificial rainfall)	5	0.68	This study
CNR	3 years	Meihekou	7	0.39	Xin et al., 2019
	3 years	Fuxin	12	0.71	Xin et al., 2019
	Extreme rainfall	Indoor (artificial rainfall)	3	0.91	This study
	Extreme rainfall	Indoor (artificial rainfall)	5	1.29	This study

Differences in soil loss forms

Indoor artificial rainfall observation showed that for NT, LWR, and LNR, the main forms of soil loss were sheet and inter-rill erosion, and no ephemeral gully erosion

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occurred under any rainfall intensity. For CWR and CNR, if no contour ridge failure occurred, the soil loss form was mainly sheet and inter-rill erosion, with ephemeral gully erosion being rare. However, if contour ridge failure occurred, it led to the occurrence and aggravation of ephemeral gully erosion. After extreme rainfall, the width at contour ridge failure sites under CWR varied from 9 to 68 cm, with an average of 30.6 cm, and the maximum width at 92.3% of contour ridge failure sites exceeded 20 cm. The width at contour ridge failure sites under CNR varied from 7 to 138 cm, with an average of 33.1 cm, and the maximum width at 86.0% of contour ridge failure sites exceeded 20 cm. When the width of a rill on the slope surface exceeded 20 cm, it could easily develop into ephemeral gully erosion (Zhao et al., 2012). Therefore, generally, if contour ridge failure occurred because of rainfall runoff, it could easily induce ephemeral gully erosion.

With an increase in slope surface runoff, the width and volume of contour ridge failure soil loss enlarged correspondingly, resulting in the gradual occurrence and aggravation of ephemeral gully erosion (Douglas-Mankin et al., 2020). Using a single contour ridge as the analysis object, runoff is an important factor in the degree of contour ridge failure. There was a significant exponential increasing relationship between the failure volume V_F of a single contour ridge and the runoff Q in the corresponding catchment area of this contour ridge (*Fig. 10a*). With an increase in Q, the rate of increase of contour ridge failure in 5° narrow ridges was the fastest, the increase rate in contour ridge failure in 3° narrow ridges was the slowest, and the increase rate of contour ridge failure in wide ridges was between the two.

As shown in *Figure 10b*, there is a significant exponential increasing relationship between failure width W_F and Q of a single contour ridge in contour ridge tillage. Specifically (*Eqs.13–15*):

$$5° \text{ CNR: } WF5 = 8.89\exp(2.42Q) , (R2 = 0.81, p < 0.01) \quad \text{(Eq.13)}$$

$$3° \text{ CNR: } WF3 = 11.96\exp(1.42Q) , (R2 = 0.44, p < 0.01) \quad \text{(Eq.14)}$$

$$\text{CWR: } WFW = 15.71\exp(0.99Q) , (R^2 = 0.59, p < 0.01) \quad \text{(Eq.15)}$$

If $W_F = 20$ cm is considered the critical value for the beginning of ephemeral gully erosion on the slope surface, and this value is substituted into *Equations 13–15,* it can be inferred that the threshold values of runoff in the upstream catchment for the contour ridge failure are 0.335, 0.362, and 0.244 m^3, for substantial contour ridge failure in the 5° narrow ridge, 3° narrow ridge, and the wide ridge, respectively. The typical black soil areas in Northeast China have a gentle topography, with slopes mostly <5° and a surface slope length mostly between 300 and 1500 m. The catchment area in the upper reaches of the farmland plot is large, and a large amount of surface runoff can be collected during the rainstorm process, which scours and damages the contour ridges and forms an ephemeral gully erosion path. The catchment area of a single plot could be reduced significantly by building a reasonable drainage or runoff diversion path, thereby realising the purposes of arranging water flow and reducing soil loss.

Sediment deposition on slope surface under contour ridge tillage

With regard to a slope surface with ephemeral gully erosion, the soil loss modulus A_F corresponding to the contour ridge failure during extreme rainfall was compared with

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the slope soil loss modulus A. If A_F is considered the independent variable and A the dependent variable, the slope of the zero-crossing linear equation fitted by the two was only 0.267 (*Fig. 11*), which showed that an average of at least approximately 73% of the soil loss sediment was deposited in the slope surface confluence. The deposition sites were mainly the furrows. When contour ridge failure occurred, if the contour ridge located at its downstream was not damaged immediately, the soil loss sediment generated by the contour ridge failure could be easily deposited in the gullies. In some furrows, the deposited sediment blocked the horizontal movement of water in the furrow, forming a significant ephemeral gully erosion path (*Fig. 12*).

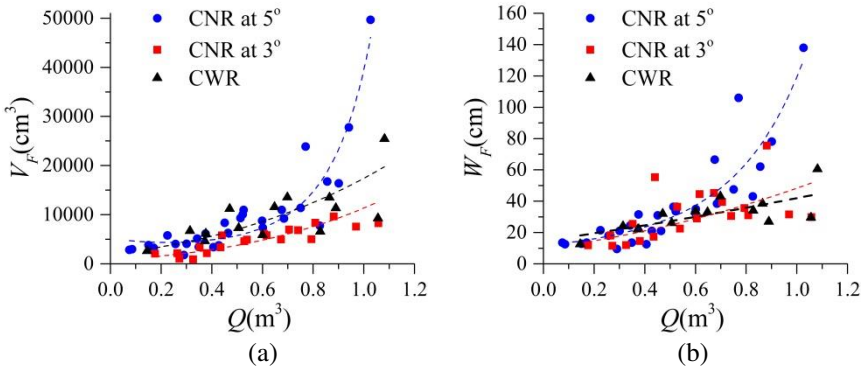

Figure 10. *Relationship of failure volume (a) and failure width (b) of a single contour ridge with runoff in its upstream catchment*

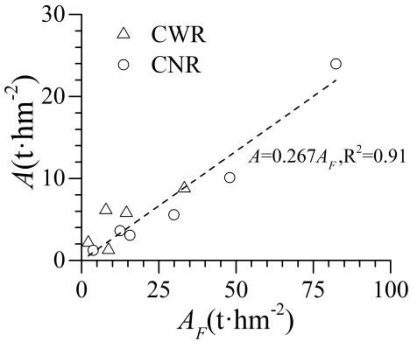

Figure 11. *Relationship between slope soil loss modulus and sediment yield modulus in ephemeral gully erosion process*

In their study, Gao et al. (2016) selected a small watershed with an area of 2.1 km^2, with the proportion of farmland amounting to 95% in the typical black soil areas of Northeast China. Based on the observation data of soil loss and sediment yield, the average sediment delivery ratio in this watershed was calculated as 0.38. The analysis showed that most of the sediments from soil loss were deposited before entering the river channel, caused mainly by the topographic characteristics of gentle and long slopes. Because of the slow confluence speed, runoff could infiltrate easily and, coupled with the high summer temperature, evaporation was relatively strong. When surface runoff infiltrates and evaporates, the sediments carried by it would also deposit at the foot of a slope, meadow, and other places with gentle terrain or high surface resistance.

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The accumulated water in the low-lying areas of the earth's surface contributes to soil loss materials being deposited easily. It should be recognised that furrows could serve as the location where a large amount of soil loss sediment is deposited (*Fig. 12*), which increases the complexity of sediment deposition in such areas.

Figure 12. *Sediment deposition in furrows*

Conclusion

(1) In longitudinal ridge tillage practices, LNR, with a higher soil loss amount was considered the contrast object when calculating the P factor, and LWR, CWR, CNR, and NT were all regarded as soil conservation tillage practices. Under extreme rainfall conditions, the P factor of LWR varied from 0.426 to 1.223, with an average of 0.712; the P factor of NT varied from 0.263 to 1.094, with an average of 0.634; the P factor of CWR varied from 0.302 to 1.375, with an average of 0.706; and the P factor of CNR varied from 0.051 to 2.090, with an average of 1.097. The P factor value of NT was the lowest and, generally, there was not significant difference between LWR and CWR; the P factor value of CNR was significantly higher than those of other tillage practices.

(2) The P factor value is influenced by the combined action of rainfall erosivity(R) and the slope factor(S). For NT and LWR, there was a significant logarithmic decreasing relationship between the P factor value and RS. Under NT, $P_{NT} = -0.392\text{Ln}(RS) + 1.607$, and under LWR, $P_{LW} = -0.364\text{Ln}(RS) + 1.616$. On the surface of sloping farmland with relatively small topographic relief, the amount of soil loss in the process of extreme rainfall was relatively low. With an increase in RS, the n of furrows under LWR gradually increased; however, when RS was ≥ 15 MJ·mm/(hm^2·h), the growth rate of the n value decreased significantly. Further, the blocking effect of surface on the slope runoff gradually became limited, and the soil loss modulus increased significantly with the increase in RS.

(3) When RS was < 9.18 MJ·mm/(hm^2·h), contour ridge tillage was the best tillage practice to reduce soil loss. In a unit area, the impoundment runoff of furrows under CNR was 2.08 times that of CWR, i.e., its effect of reducing water and sediment was more significant. When RS was ≥ 9.18 MJ·mm/(hm^2·h) and the maximum rainfall intensity was ≤ 75 mm/h, there was no significant difference between CWR and NT in their soil loss reduction effect. If the maximum rainfall intensity was higher than 75 mm/h, NT showed the optimal effect in reducing soil loss.

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(4) For CWR and CNR, if no contour ridge failure occurred, the soil loss form was primarily surface soil loss, with ephemeral gully erosion being rare. However, when contour ridge failure occurred, the damage failure and width increased exponentially with the runoff in the upstream catchment, which could easily lead to the development of ephemeral gully erosion. At least approximately 73% of the soil loss sediment was deposited in the slope confluence during the soil loss process, and the deposited sites were mainly furrows. Deposited sediment blocked the lateral communication of water flow between some furrows, forming a significant ephemeral gully erosion path.

Changing the microtopography and thus regulating the confluence path to shorten the slope length and construct a drainage path, is a potential conservation practice for reducing soil loss in contour ridge systems in the typical black soil region of Northeast China, especially in extreme rainfall events. Study for the potential conservation practices is needed in future.

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APPENDIX

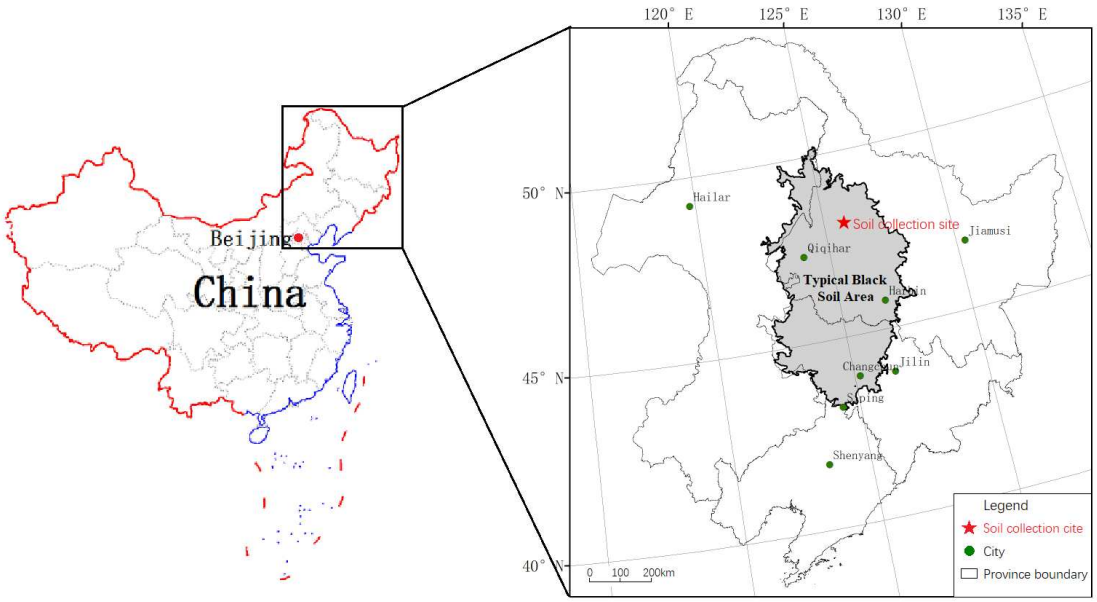

Figure A1. *Location of the soil collection site*

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EFFECT OF AMENDMENTS ON SOIL MOISTURE, SALINITY, Cl^- CONTENT, SAR, AND K^+/Na^+ RATIO

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Abstract. Application amendments to saline-alkaline soils is considered to be a good practice for soil remediation. The effects of different amendments (e.g., biochar soil amendment, microorganism agents, polyacrylamide) on saline soils were investigated under a wheat-maize rotation system from 2017 to 2018 in Binzhou, Shandong Province, China. The field experiment was conducted with four soil treatments: control (CK), biochar soil amendment (BSA), polyacrylamide (PAM) and microorganism agents (MA). Surface soil (0-20 cm) moisture increased consistently under various amendment treatments compared with CK during crops growing season, especially under BSA treatment. The effect of amendments on subsoil (20-60 cm) moisture was not significant. Simultaneously, soil amendment remarkably reduced soil salinity at the 0-60 cm depth during wheat growing season especially under BSA treatment. During maize growing season, surface soil (0-20 cm) salinity decreased. Surface soil (0-20 cm) salt content was the lowest under PAM treatment, but the salt content in deeper soil (20-60 cm) changed little. Furthermore, amendments significantly reduced Cl^- content and sodium adsorption ratio (SAR), and increased K^+/Na^+ ratio. Soil moisture content was higher and salinity content was lower during maize growth season compared with those during wheat growth season. As a result, application of BSA, among the three amendments, produced the best effects. Therefore, it can be recommended for wide application in similar saline-alkali soil conditions.
Keywords: *soil moisture, salinity, amendment, soil deep profile*

Introduction

Saline-alkali land is an important reserve resource for the development of regional economies. Rational exploitation and improvement of land can relieve the pressure for arable land resources. In China, there are about 3 600×10^4 ha salt-affected soils (Yang, 2008), which is distributed in Northeast China Plain, Central-north area, Northwest inland, North China and coastal areas (Liu et al., 2007). However, the physical and chemical properties of saline-alkaline soil are poor due to the high content of salt, low natural desalination rate, heavy salinization. It has been one of the major environmental problems threatening agricultural productivity for a long time (Rengasamy, 2006). It is important to improve saline-alkaline soils especially mildly saline soils for ensuring the quantity of cultivated land, maintaining food security and promoting the sustainable development of ecological environment.

Shandong province is located in the northern coastal areas of China, which has distinctive physicochemical properties with about 592673 ha coastal saline-soil which accounts for more than 3.78% of the province area (Zheng et al., 2017). It is a typical distribution area of coastal saline-alkali soil in China. The coastal areas at the junction of sea and land, has high groundwater level, tidal erosion, frequent supply of seawater and

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evaporation-concentration, thereby a large expanse of saline-soil is formed (Rengasamy, 2006; Li et al., 2014). This coastal area has specific characteristics, primarily including harsh environmental conditions such as high salinity, shallow groundwater, a high evaporation-precipitation ratio, poor drainage and secondary salinization (Xia et al., 2019). Salt toxicity has negative effects on soil properties, including high pH, high level of sodium adsorption ratio (SAR) and exchangeable sodium percentage (ESP), poor soil structure and also low water permeability (Singh et al., 2012). Shandong province is one of the main bases of facility agriculture production in China, thereby it is essential to improve coastal saline-soil for Shandong's ecological environment and development of economy. Therefore, various methods have been investigated for the effectiveness in saline soil remediation.

In recent years, a growing number of literatures demonstrated that amendments application effectively improves soil quality in saline-alkali soils. Application amendments such as farm manure, poultry manure, compost and flue gas desulfurization (FGD) gypsum could be effective in improving plant growth through their beneficial impacts on physical, chemical, nutritional and biological properties of saline-alkali soil (Li et al., 2014; Cacini et al., 2020; Iqbal et al., 2020). These findings of amendments on soil improvement were mainly attributed to soil amendment type, soil type (soils varying in organic matter, texture and mineralogy), amendment application amount, application method and so on (Liang et al., 2019; Yu et al., 2019; Urra et al., 2020).

Biochar is considered as a potential amendment in accelerating salt-leaching process and improving saline-alkali soil quality (Chaganti et al., 2015a; Elshaikh et al., 2018; He et al., 2020; Quan et al., 2020; Zhang et al., 2020; Zhao et al., 2020) due to its unique physicochemical properties (i.e. high porosity and large specific surface area, etc.). Recently, numerous studies have been conducted to investigate the effects of biochar on improving soil quality (O'Laughlin et al., 2009). Biochar can improve soil structure by, such as reducing soil bulk density, promoting the formation and stabilization of soil aggregate, and improving hydrodynamic parameters (Wang et al., 2017; Fei et al., 2019; Zhang et al., 2020). Simultaneously, it can rapidly increase the content of soil organic matter, nitrogen, phosphorus and potassium, which is conducive to water conservation, salt leaching and salt suppression of saline soil (Yue et al., 2016). Biochar application to soil is widely recommended for a variety of reasons related to utilizability and sustainability. The application of biochar in salt-affected soil not only increases organic carbon content and nutrients, especially cationic ones (e.g. K^+, Ca^{2+}, Mg^{2+}, Zn^{2+}, Mn^{2+}), but also improves soil quality (Rajkovich et al., 2012; Usman et al., 2016; Yue et al., 2016; Zheng et al., 2018). Lashari et al. (2015) found that combined crop straw biochar with manure compost and pyroligneous solution could ameliorate salinity stress to maize and improve productivity in saline croplands in arid/semi-arid regions threatened increasingly by global climate change. Zhang et al. (2013) observed that with the increased amount of biochar, Na^+ content in saline-alkali soils has decreased by 20 times, and K^+, Ca^{2+}, Mg^{2+} and other ion contents have increased. Some scholars demonstrated that biochar has significantly contribute divalent cations (Ca^{2+} and Mg^{2+}) and facilitated the exchange of sodium from soil exchange sites. Use of biochar reduced soil salinity and alkalinity as much as or more than control treatment (Chaganti et al., 2015a). Among these applications, biochar has attracted a particularly increasing attention as an effective means in the remediation of salinity-affected soils (Ali et al., 2017; Saifullah et al., 2018).

Polyacrylamide (PAM) is a water-soluble polymer with strong cohesiveness and a strong water absorption capacity, it is used in numerous applications such as food

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industry, well drilling, and wastewater treatment as a flocculation agent (Albalasmeh et al., 2021). PAM has been widely used to modify soil structure (Lentz et al., 2015). Previous study found that addition of PAM can effectively improve soil aggregate structure, soil bulk density, soil permeability, and decrease surface and nutrient runoff (Green et al., 2004). In addition, PAM also affect saline-alkali soil structure and hydraulic properties (Fei et al., 2019). A study found that soil salinity could affect the effects of PAM on soil physical properties and water evaporation (Zhang et al., 2012). Large numbers of studies show that under brackish water infiltration, PAM application could reduce soil infiltration rate and increase soil water retention performance (Wang et al., 2014). Some studies have found that PAM also has positive effects on saline-alkali soil physical properties. PAM could increase the content of aggregates in saline alkali soil, improve soil porosity, and thus enhance the permeability coefficient of soil (Zhang et al., 2012; Zhang et al., 2017). Understanding the mechanism is also needed to keep knowledge of the contradictory effects of PAM on soil salinity. However, soil improvement by PAM is mostly used in laboratory experiments, studies carried out on field experiments are limited.

Microbial agents (MA) effectively improve soil quality, soil health, soil microbial activities and nutrient cycling (Talaat et al., 2019; Liu et al., 2020). MA was sufficient to significantly improve the number of potassium bacteria and bacillus subtilis, soil organic matter content, and available N, P, K content in salinized soil (Pang et al., 2011). Bossuyt et al. (2001) reported that microorganisms could promote the formation of soil aggregates, loosen soil after fungal repair, reduce bulk density, cut off capillary pores in the soil, increase non-capillary pores, accelerate salt leaching, and alleviate salt accumulation. Application MA in saline soil could reduce pH, EC and ESP of soil and increase soil fertility (Wang et al., 2015). In addition, MA can produce a variety of enzymes through their own life activities in saline soil (Song et al., 2007). However, the MA effects on soil salt ions, are still not clear and the improvement effect of MA on saline-alkali soils needs further research.

Amendments application to saline soils is one viable solution for improving soil quality. Therefore, in accordance with the characteristics of coastal saline soils in Shandong, three amendments were selected: Biochar soil amendment (BSA), Microbial agents (MA), Polyacrylamide (PAM). Therefore, the objectives of this study were to (1) investigate the effects of the three amendments on soil moisture and soil salt profile during 2017 to 2018 wheat-maize rotation growing season, (2) evaluate the influences of different amendments addition on soil salt content and salt ions. (3) determine the optimal amendment applied to obtain maximum soil moisture and minimum salt content in salinized Chao soil of Binzhou, Shandong Province, China.

Materials and methods

Details of experimental site

A winter wheat (*Triticum aestivum* L.) and summer maize (*Zea mays* L.) rotation field experiment was carried out in 2017 and 2018 at an experimental research station (37° 29′ N, 118° 03′ E) for ecological and sustainability research in Binzhou, Shandong Province, China. This region has a semi-humid climate with an average annual rainfall of 560 mm, with a majority (70%) of the precipitation occurring from June through August. The mean annual air temperature was 12.7°C (Shi et al., 2019). The precipitation and average temperature from October 2017 to October 2018 are shown in *Fig. 1*. The

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precipitation during the test period was 787 mm, which was more than the multi-year average precipitation. The highest temperature is above 26℃ in July 2018, the lowest temperature was lower than 0℃ in January 2017. The groundwater table in experimental area was shallow, mostly between 0.8 and 2.4 m. The chemical properties of the topsoil (0–20 cm) in the experimental field are briefly described in *Table 1*.

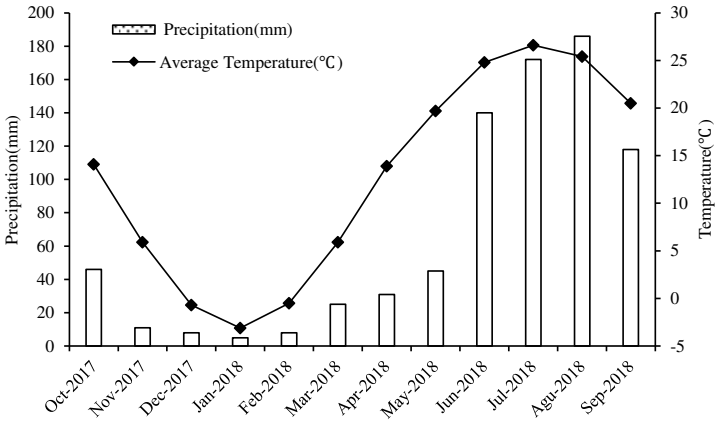

Figure 1. *Monthly climate data from October 2017 to October 2018 at the pilot site*

Table 1. *The initial salt content of topsoil (0-20 cm)*

Soil	EC ($mS\ cm^{-1}$)	pH	Salt ions concentration ($cmol\ kg^{-1}$)							
			CO_3^{2-}	HCO_3^-	Cl^-	SO_4^{2-}	Ca^{2+}	Mg^{2+}	K^+	Na^+
Salinized chao soil	0.83	7.93	*	0.53	2.08	0.18	0.52	1.94	0.04	20.07

Soil amendments

Three types of amendments were used in this experiment. Three amendments were biochar soil amendment (BSA), microbial agents (MA) and anionic polyacrylamide (PAM), respectively.

Biochar soil amendment (BSA), used in this study was a black particle with size of 2 mm, produced by Shanghai Shike biotechnology LTD (China), it was made from bamboo and the average pyrolysis temperature was 650°C. Microbial agents (MA) used in this study was black particle with size of 2-4 mm produced by Beijing Hengyuan Jiada technology LTD (China). It is mainly composed of nitrogen-fixing bacteria, phosphate-dissolving bacteria, potassium-resolving bacteria, bacillus, etc. MA's carrier is organic matter. Anionic polyacrylamide (PAM) used in this study was a white powder with a molecular weight of 8 million Daltons and was produced by Chengdu YiSheng Environmental Engineering Technology Company (China).

Experimental design

The experiment was carried from Oct. 2017 to Oct. 2018, in a winter wheat (*Triticum aestivum* L.) and summer maize (*Zea mays* L.) rotation systems. The winter wheat (*Triticum aestivum* L.) cultivar "Kaimai 18" was used in this study. Its seed were sowed

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at a density of 187.5 kg hm^{-2} on October 7th, 2017 and harvested on June 9th, 2018. The maize (*Zea mays* L.) variety "Denghai 605" was tested in the experiment. Its planting densities was 90,000 plants hm^{-2} on 15th June, 2018 and harvested on 18th October, 2018. Four treatments with three replications were established: (1) no amendment (CK), a local traditional field management regime; (2) Biochar soil amendment (BSA) treatment with 1500 kg ha^{-1}; (3) Microbial agents (MA) treatment with 1500 kg ha^{-1}; and (4) Polyacrylamide (PAM) treatment with 11.5 kg ha^{-1}. We chose these dosages according to the research results of the research group in the early stage. The field experiment was laid out in a randomized block design and each plot had an area of 7 m × 6 m = 42 m^2, and a protected zone with the width of 1 m was arranged between adjacent plots to eliminate the impact of the lateral movement of soil water and salinity. Before the wheat and maize sowing the same amount was used each time, all amendments (BSA, MA, PAM) were manually spread in each plot, and then evenly mixed into the soil with a spade to a depth of about 20 cm.

In each plot winter wheat was planted after maize harvest. No general irrigation practices were adopted, and the crop growth depended on natural precipitation. The other management practices, including weeding, and insect control were carried out based on local farmer's experience.

Soil sampling and analyses

This experiment followed a completely randomized block design with 4 treatments and 3 replicates. Soil samples were taken by soil drilling, and total soil sampling depth was 0-60 cm, with 10 cm increment. Samples were collected at different growth stages of wheat and maize including seedling (BBCH00), tiller (BBCH21), wintering (BBCH25), revival (BBCH30), heading (BBCH51), maturity (BBCH99) stage of winter wheat, jointing (BBCH30), tasseling (BBCH51) and maturity (BBCH99) of maize. (BBCH73 according to Meier (2019)).

The collected soil samples were stored in sealed plastic bags and shipped to the laboratory. All soil samples were divided into two parts, one part was oven dried (DHG-9003, Shanzhi, Shanghai, China) at 105°C for 8-10 h to determinate moisture content. The other part was air-dried and passed through a 2 mm sieve and stored at room temperature for the determination of chemical properties. After that, 10 g of each soil sample was removed, sifted (through a 1 mm sieve) and then placed in a 150 mL Erlenmeyer flask with 50 mL of distilled water to analyze salt properties. The Erlenmeyer flask (water: soil mass ratio=5:1) was stirred by an oscillator (Shanghai, China) for 10 min. After standing for 15 min, electrical conductivity (EC) of the water extract was measured using a DDS-307 conductivity meter (INESA, Shanghai, China). Soil was analyzed for soluble ions (K^+, Na^+, Ca^{2+}, Mg^{2+}, Cl^-) following the methods described by Bao (2000). Soluble sodium and potassium were measured with a flame photometer (Jenway PEP-7) having Na^+ and K^+ filters, whereas Ca^{2+}, $Mg^{2+}$and Cl^- were determined by titration. Ca^{2+} and Mg^{2+} by titration with EDTA, Cl^- with $AgNO_3$ using K_2CrO_4 indicator.

Data analysis

A higher soil sodium adsorption ratio (SAR) poses a greater risk to soil. SAR are traditionally used as indices for assessing soil structural stability upon interaction with water (Bennett et al., 2016). The SAR was calculated according to *Eq.1* (McGeorge, 1954):

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$$SAR = \frac{\left[Na^+\right]}{\sqrt{\frac{1}{2}\left(\left[Ca^{2+}\right]+\left[Mg^{2+}\right]\right)}} \quad \text{(Eq.1)}$$

where $[Na^+]$, $[Ca^{2+}]$ and $[Mg^{2+}]$ are expressed in cmol L^{-1}.

However, the chemical components of clay structural integrity are primarily a function of ionic valence and hydrated radius, meaning that not only Na^+ but also K^+ expected to affect clay structure on this basis (Bennett et al., 2016). The ratio of exchangeable K^+ to exchangeable Na^+ is given by the following *Eq.2*:

$$K^+ / Na^+ \text{ Ratio} = \frac{\left[K^+\right]}{\left[Na^+\right]} \quad \text{(Eq.2)}$$

where $[Na^+]$, $[K^+]$ are expressed in cmol L^{-1}.

All data were showed as the average value of three replicates. SPSS Statistics 19.0 software was used to conduct the Analysis of Variance (ANOVA). Least significant difference (LSD) tests at $\alpha = 0.05$ level was used to determine significant differences between all treatments.

Results

Dynamics of soil moisture

Fig. 2 showed soil water dynamic change in soil layers of different treatments. During both crops growing season, the soil moisture varied with the applied type of amendments and soil depth.

The soil moisture during crops growing season in 0–30 cm soil layer showed strong temporal variability because the depth of infiltration under precipitation was within the range of the 0–30 cm soil layer. However, in the topsoil layer (0–10 cm), the soil moisture was greater in BSA treatment than that of the CK treatment ($P<0.05$). The difference of soil moisture in all treatments was significant ($P<0.05$). Application of amendments mainly caused a difference of soil moisture in the 0–30 cm soil layer. However, in the deep soil layers (30–60 cm depth), the difference disappeared (*Fig. 2d, e, f*).

Dynamics of soil salinity

During crops growing season, the dynamics of soil salinity within 0-60 cm depth were shown in *Figure 3*. Under CK and BSA treatments, soil salinity in surface layer (0-10 cm) increased from winter seeding to wintering and then gradually decreased. Under MA and PAM treatments, soil salinity in surface layer had a peak during winter wheat revival stage. Amendments affect infiltration of rainfall and loss of soil water from the 0-20 cm due to evaporation, which primarily affects the salt distribution in the soil profile (0-60 cm). There was almost no difference in salt concentration in all soil layer between the MA and PAM treatment during late growth stage of winter wheat and maize growing season. However, the salt content under BSA treatment were significantly lower than under CK treatment, the average salt content for the CK, BSA, MA and PAM in the 0-60 cm soil layer were 2.68 mS cm^{-1}, 1.83 mS cm^{-1}, 2.58 mS cm^{-1} and 2.09 mS cm^{-1},

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respectively. There was intensive rainfall during maize growing season, which decreased salt content in the upper soil layer and increased them in the deeper soil layer under all treatments. Compared with the growing season of wheat, average soil salt content during maize growing season decreased 47.9%, 35.6%, 56.4% and 39.7%, respectively. Soil salinity of all amendment treatments were also lower than that of CK treatment in 0-60 cm and 20-40 cm during the winter wheat seeding season. Furthermore, from maize tasseling to harvest, no significant differences in salinity were observed comparing BSA, MA and PAM treatments in these layers.

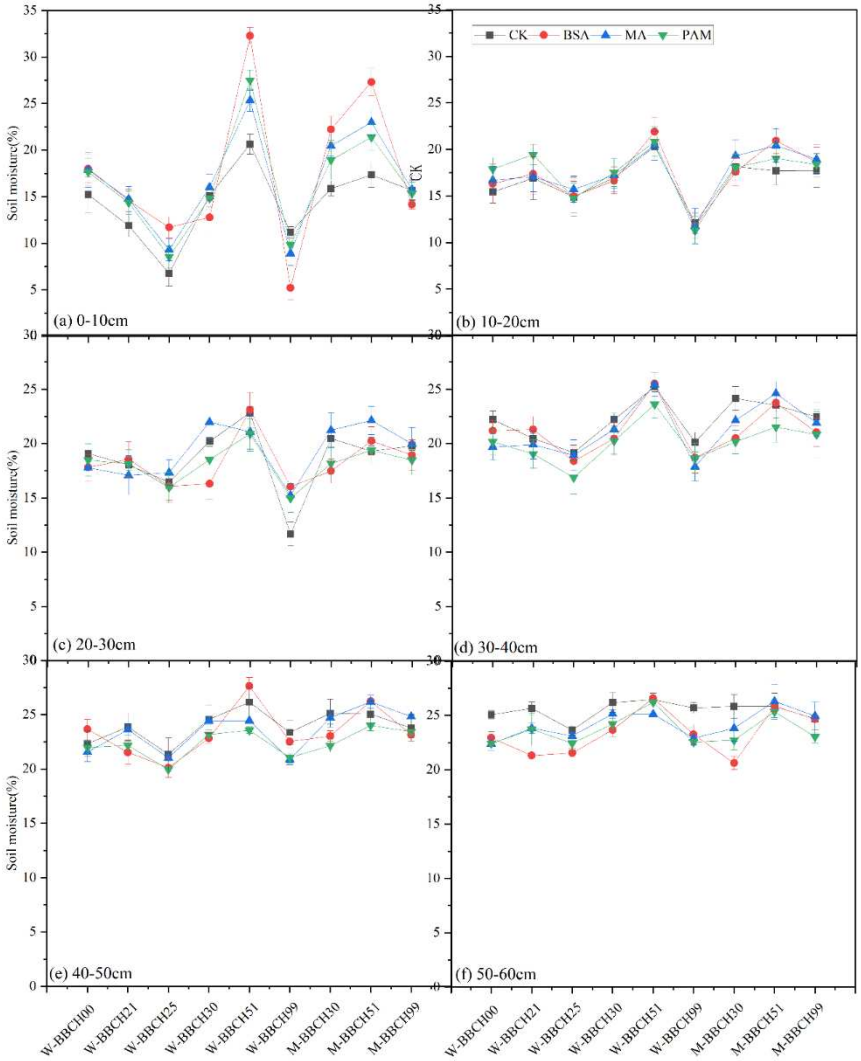

Figure 2. *Dynamics of soil moisture in the soil layers of 0-10 cm, 10-20 cm, 20-30 cm, 30-40cm, 40-50 cm and 50-60 cm under CK, BSA, MA, and PAM treatments during crops growing season. Error bar refers to the $LSD_{5\%}$ error value. W, winter wheat; M, summer maize. Seedling(BBCH00), tiller(BBCH21), wintering(BBCH25), revival(BBCH30), heading(BBCH51), maturity(BBCH99) stage of winter wheat. Jointing(BBCH30), tasseling(BBCH51) and maturity(BBCH99) of maize*

Spatial characteristics of soil Cl^- content

Fig. 4 showed the soil Cl^- content under different treatments. During winter wheat growing season, BSA decreased the soil Cl^- content. However, PAM caused a higher soil

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Cl^- content during winter wheat tillering stage. At wheat maturity, the soil Cl^- content of MA treatment was significantly greater higher than CK in 30-60 cm soil layer.

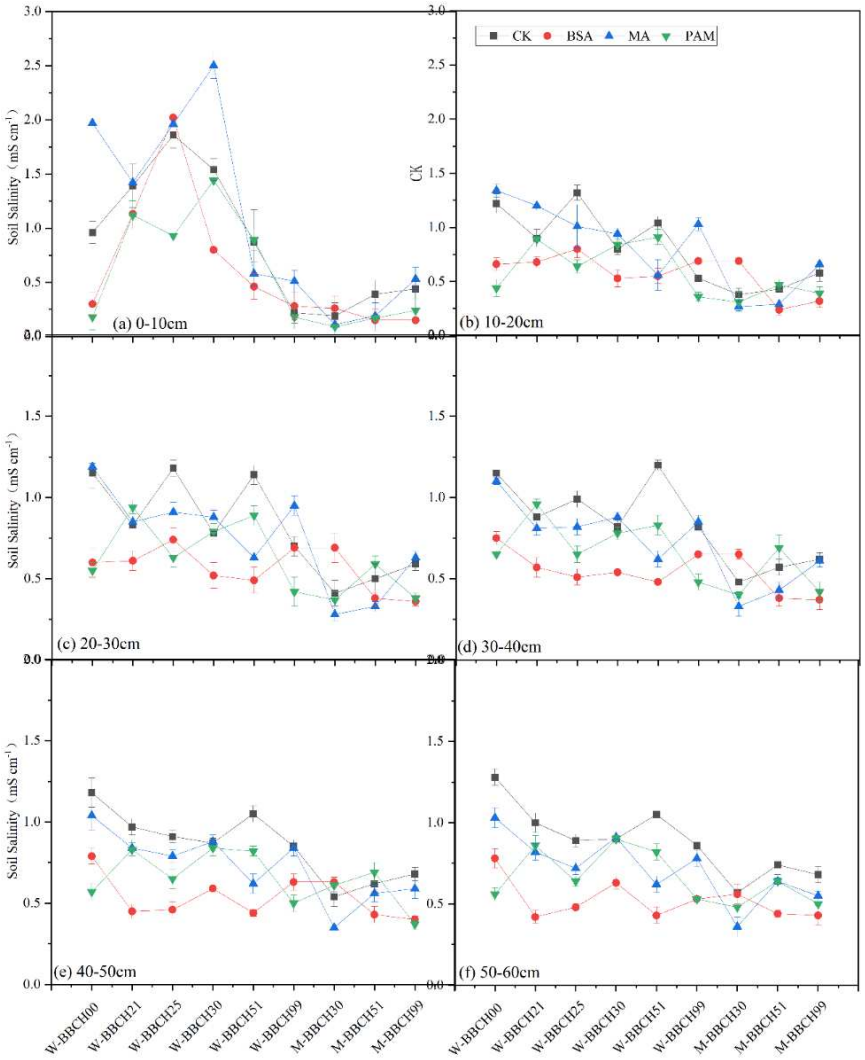

Figure 3. *Dynamics of soil salinity in the soil layers of 0-10 cm, 10-20 cm, 20-30 cm, 30-40 cm, 40-50 cm and 50-60 cm under CK, BSA, MA and PAM treatments during crops growing season. Error bar refers to the $LSD_{5\%}$ error value; W, winter wheat; M, summer maize. Seedling(BBCH00), tiller(BBCH21), wintering(BBCH25), revival(BBCH30), heading(BBCH51), maturity(BBCH99) stage of winter wheat. Jointing(BBCH30), tasseling(BBCH51) and maturity(BBCH99) of maize*

From winter wheat tillering to revival period, all treated surface soil (0-10 cm) Cl^- content increased and tended to migrate upward, and soil Cl^- content showed a surface accumulation pattern. From winter wheat revival to harvest, soil Cl^- content increased with increasing soil depth and tended to migrate downward, soil Cl^- content showed a bottom accumulation pattern. Throughout winter wheat growing season, at a depth of 0-20 cm, Cl^- content in all treatments greatly changed. The Cl^- content in the 30-60 cm soil layer decreased slightly(P<0.05). At winter wheat seedling, Cl^- distribution under BSA and CK treatment profiles showed similar trends. The Cl^- content of BSA treatment was

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0.46-1.33 cmol kg^{-1}, and Cl- content in CK treatment was 2.35-2.91 cmol kg^{-1}. During this period, Cl^- content was the lowest under BSA treatment, followed by PAM treatment. During winter wheat tillering, soils under BSA, MA and PAM treatment had more Cl^- content compared with CK treatment. From winter wheat revival to maturity, the mean soil Cl^- content was lower than 1.8 cmol kg^{-1} under BSA treatment. During the winter wheat revival stage, in vertical direction, soil Cl^- content under MA and PAM treatment compared with CK treatment showed no significant difference.

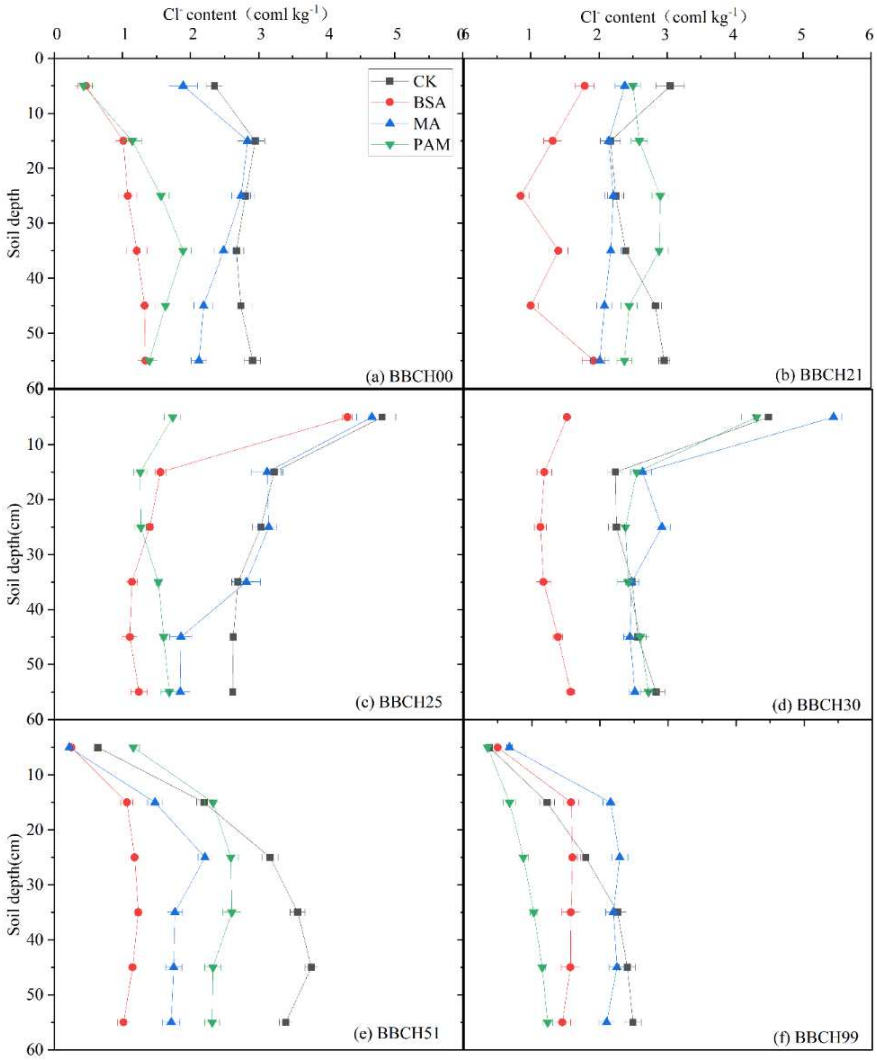

Figure 4. *Spatial characteristics of soil Cl^- content in winter wheat growth season. Error bar refers to the $LSD_{5\%}$ error value; Seedling(BBCH00), tiller(BBCH21), wintering(BBCH25), revival(BBCH30), heading(BBCH51), maturity(BBCH99) stage of winter wheat*

Soil Cl^- content under different treatments during maize growing season were shown in *Figure 5*. With increasing soil depth, soil mean Cl^- content showed the following vertical distribution characteristic: 40-60 cm > 20-40 cm > 10-20 cm> 0-10 cm, thus revealing a bottom accumulation tendency. During maize growing season, soil Cl^- content under PAM treatment was higher than CK treatment in most soil layer at jointing (BBCH30) and maturity stage (BBCH99). During tasseling stage (BBCH51), all

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treatments soil Cl^- content was lower, and there was no significant difference in all soil layers. The results indicated that BSA treatment had lower soil Cl^- content, compared to other treatments. What's more, during the whole growing season of maize, the soil Cl^- also showed the bottom accumulation tendency. In addition, soil Cl^- content at the growing season of maize was lower than that during wheat season.

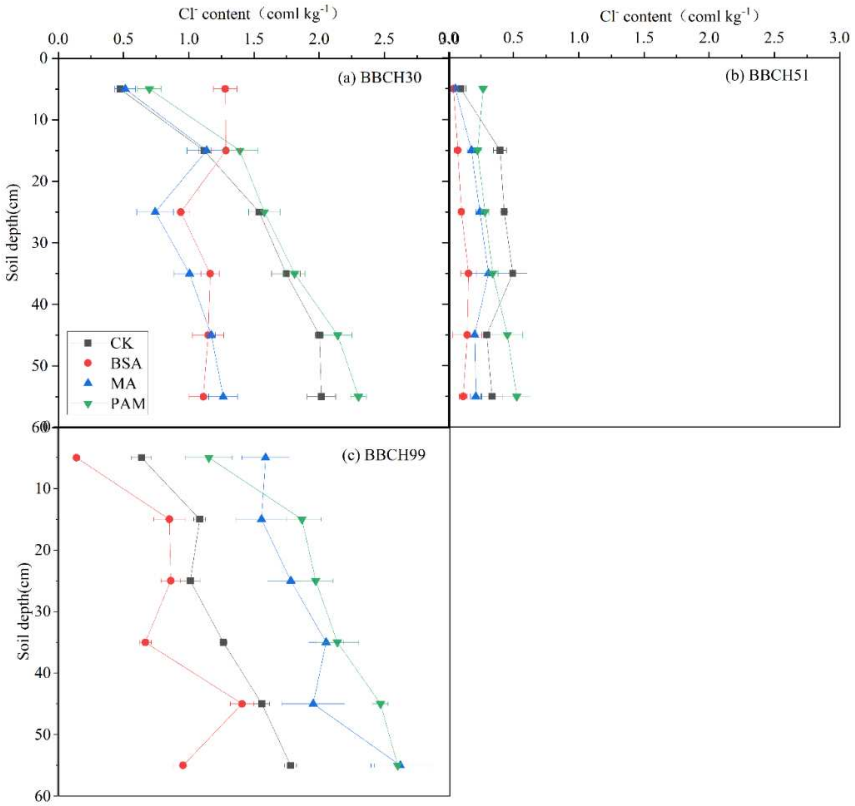

Figure 5. *Spatial characteristics of soil Cl^- content during summer maize growth season. Error bar refers to the $LSD_{5\%}$ error value; Jointing(BBCH30), tasseling(BBCH51) and maturity(BBCH99) of maize*

Dynamics of soil SAR

SAR in soil profile varied greatly among experimental year (*Tables 2,3*). During winter wheat growing season, the soil SAR increased first but then decreased. At wheat revival stage soil SAR was the highest, and maize tasseling stage soil SAR was the lowest. Soil SAR in maize growing season was lower than that in winter wheat growing season. Among these treatments, surface soil (0-10 cm) SAR under BSA, PAM and MA treatments were consistently lower than that under CK treatment throughout an-experimental-year. Compared with CK, soil SAR average decreased of BSA, MA and PAM by 48.1%, 13.5%, 18.9%, respectively ($P < 0.05$). In addition, soil SAR under BSA treatment were also significantly lower than that under CK treatment in all soil layer during crops growing season. There was a significant difference between 0-20 cm soil SAR under MA treatment and CK treatment (except during wheat wintering period). However, at depth of 20-60 cm soil layer, SAR under MA treatment showed significantly lower than that under CK treatment, whereas the difference comparing MA and CK treatment was not significant. In general, (*Table 3*) soil SAR under PAM treatment

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decreased gradually during maize growing season. From wheat tillering to maturity, the average of each soil layer SAR from PAM treatment decreased by 20-30.5% compared with CK, whereas no significant difference in ANOVA. Furthermore, no significant differences in SAR were observed between PAM and MA treatment in 20-60 cm layers during crops growing season.

Table 2. *Dynamics of soil SAR in soil layers of 0-10 cm, 10-20 cm, 20-30 cm, 30-40 cm, 40-50 cm and 50-60 cm under CK, BSA, MA, and PAM treatments during winter wheat growing season. Different letters in the same column indicates significant differences between different treatments at $P < 0.05$*

Soil depth (cm)	Treatment	Winter wheat growing season					
		Jointing (BBCH30)	Tasseling (BBCH51)	Maturing (BBCH99)	Revival (BBCH30)	Heading (BBCH51)	Maturity (BBCH99)
0-10	CK	8.92±1.4b	29.15±1.3a	25.93±3.2a	33.91±4.2a	8.4±2.1b	5.26±1.9ab
	BSA	4.71±1.5c	9.36±1.7c	9.84±2.4c	10.38±1.3b	4.55±1.9c	8.25±2.1ab
	MA	13.66±1.9a	16.12±1.4b	15.36±3.6b	27.33±3.6a	1.02±0.9c	13.9±2ab
	PAM	5.03±2.1c	26.27±1.5a	15.84±2.1b	23.75±2.5a	36.38±3.4a	2.21±0.5b
10-20	CK	29.88±2.9a	19.88±2.2a	19.9±2.3a	27.93±2.9a	29.46±4.5a	17.3±2.1ab
	BSA	11.95±2.1b	11.16±2.3b	10.66±1.3b	18.93±1.7b	15.57±2.6b	22.46±1.9b
	MA	24.94±1.2a	17.37±2.4ab	14.45±1.1b	25.2±1.8a	14.51±2.7b	25.05±3.1b
	PAM	13.93±1.1b	26.92±2.4a	11.43±1.1b	28.11±1.5a	27.29±2.4a	11.66±2.1a
20-30	CK	29.02±1.3a	22.64±2.5a	23.13±2.4a	28.25±2.6a	26.63±3.4a	19.57±1.9a
	BSA	12.44±1.2b	10.66±4.5a	11.42±2.9b	17.89±1.5b	13.19±3.1b	21.27±2.4a
	MA	26.29±1.4a	21.92±2.4b	16.33±1.3ab	29.74±2.9a	21.91±2.6a	26.53±2.5a
	PAM	18.2±2.4a	22.51±3.1a	14.23±2.4b	29.96±3.1a	26.63±3.8b	16.14±2.1a
30-40	CK	28.25±2.3a	22.43±2.9a	23.98±2.1ab	33.14±2.5a	29.35±2.9a	21.16±1.9a
	BSA	13.09±1.4b	11.9±1.3a	11.92±1.4b	18.09±1.6b	13.87±2.6b	18.27±1.6a
	MA	29.56±2.3a	31.62±3.4b	29.02±1.5a	39.18±4.7a	25.74±2.4a	29.24±1.6a
	PAM	20.75±1.4ab	23.47±2.4a	19.78±1.7ab	26.17±2.4a	24.36±2.5a	18.65±1.5a
40-50	CK	30.51±1.1a	26.59±2.4a	26.18±1.4a	29.2±2.7a	29.47±3.4a	22.11±2.5a
	BSA	14.25±1b	10.99±2.7b	12.82±1.5b	19±2.3b	14.34±2.3b	18.16±2.9a
	MA	24.97±1.5ab	21.87±2.6a	20.67±2.4ab	23.99±2.4ab	16.22±2.5b	21.1±2.7a
	PAM	16.45±1.3b	22.04±3.1a	19.65±2.1ab	27.46±2.5a	24.05±1.4a	17.85±2.5a
50-60	CK	30.02±2.9a	25±1.3a	26.29±1.9a	31.52±3.5a	32.35±2.5a	22.97±2.3a
	BSA	12.96±1.3b	10.33±1.7b	13.27±1.7b	18.83±1.4b	12.6±1.9b	17.46±2.7a
	MA	24.21±1.3a	28.32±1.5a	20.79±1.2a	30.13±3.6a	17.17±2.6ab	14.25±1.8a
	PAM	15.66±1.2b	22.07±1.8a	20.15±1.4a	27.98±2.8a	27.32±2.3a	19.49±1.9a

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Table 3. *Dynamics of soil SAR in soil layers of 0-10 cm, 10-20 cm, 20-30 cm, 30-40 cm, 40-50 cm and 50-60 cm under CK, BSA, MA, and PAM treatments during summer maize growing season. Different letters in the same column indicates significant differences between different treatments at $P < 0.05$*

Soil depth (cm)	Treatment	Maize growing season		
		Jointing (BBCH30)	Tasseling (BBCH51)	Maturing (BBCH99)
0-10	CK	10.89±2.3a	11.54±2.3a	20.73±3.1a
	BSA	1.93±0.9b	0.46±0.2c	2.26±0.3c
	MA	4.49±0.7b	0.83±0.3c	14.27±1.4b
	PAM	2.99±0.9b	6.25±0.9b	4.89±1.3c
10-20	CK	21.32±2.3a	5.97±0.9a	19.6±2.1a
	BSA	10.34±1.9b	1.26±0.6b	8.45±0.9b
	MA	13.58±2.5b	4.3±1.1ab	20.57±2.3a
	PAM	16.48±2.9ab	8.26±2.1a	12.96±1.3ab
20-30	CK	23±3.4a	5.42±1.3b	18.68±1.5a
	BSA	11.51±3.1b	1.91±0.3b	9.23±1.4b
	MA	8.81±2.1b	10.33±1.8a	22.25±2.5a
	PAM	19.15±2.7a	8.49±1.6a	13.95±2.1b
30-40	CK	27.07±3.7a	4.47±1.3b	19.52±1.3b
	BSA	14.64±2.9c	2.59±0.8b	11.06±1.1c
	MA	18.41±1.4b	8.87±2.1a	28.8±2.3a
	PAM	19.8±2.6b	7.46±1.6a	14.96±1.2c
40-50	CK	29.28±3.2a	2.56±0.4a	21.67±3.1a
	BSA	15.06±2.5b	2.17±0.5a	11.99±1.2b
	MA	15.6±2.5b	2.7±0.6a	17.26±2.3a
	PAM	30.12±3.6a	4.7±2.1a	12.38±2.1b
50-60cm	CK	30.67±4.2a	2.95±0.4a	21.27±2.2a
	BSA	13.83±2.1b	1.31±0.3a	12.26±1.2b
	MA	18.63±1.6a	2.42±1.1a	18.63±1.3ab
	PAM	16.34±1.3b	4.46±0.8a	13.78±1.4b

Dynamics of soil K^+/Na^+ Ratio

As shown in *Table 4* and *Table 5*, different amendments affected soil K^+/Na^+ ratio in all soil layers during crops growing season. The K^+/Na^+ ratio of the topsoil layer (0-10 cm) could be increased significantly after application amendments. Relative to CK, an increase of BSA, MA and PAM were average 2.5, 2.9 and 4.8 times. However, in the subsoil layers (20–30 cm depth), the difference decreased. What's more, at the depth of 30-60 cm soil layer, there was no significant difference in soil K^+/Na^+ ratio comparing all

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treatments. In addition, the average of soil K^+/Na^+ ratio comparing all treatments was in the order BSA>MA>PAM>CK during experimental year.

Table 4. *Dynamics of soil K^+/Na^+ ratio in the soil layers of 0-10 cm, 10-20 cm, 20-30 cm, 30-40 cm, 40-50 cm and 50-60 cm under CK, BSA, MA, and PAM treatments during winter wheat growing season. Different letters in the same column indicates significant differences between different treatments at $P < 0.05$*

Soil depth (cm)	Treatment	Winter wheat growing season					
		Jointing (BBCH30)	Tasseling (BBCH51)	Maturing (BBCH99)	Revival (BBCH30)	Heading (BBCH51)	Maturity (BBCH99)
0-10	CK	0.238±0.01c	0.083±0.01b	0.091±0.03b	0.064±0.03b	0.221±0.02b	0.201±0.02b
	BSA	0.380±0.01b	0.257±0.02a	0.310±0.02a	0.316±0.02a	0.259±0.03b	0.160±0.01b
	MA	0.177±0.02c	0.273±0.01a	0.278±0.06a	0.165±0.03ab	1.577±0.04a	0.151±0.03b
	PAM	0.757±0.04a	0.245±0.03a	0.392±0.06a	0.243±0.01a	0.12±0.01b	2.249±0.01a
10-20	CK	0.039±0.01b	0.050±0.04b	0.061±0.03b	0.050±0.04b	0.041±0.01a	0.035±0.02b
	BSA	0.105±0.03a	0.136±0.01a	0.152±0.04a	0.144±0.02a	0.092±0.02a	0.071±0.02b
	MA	0.058±0.02b	0.112±0.08a	0.131±0.02a	0.058±0.03a	0.096±0.01a	0.052±0.01b
	PAM	0.106±0.04a	0.072±0.01b	0.122±0.04a	0.048±0.02a	0.078±0.01a	0.114±0.03a
20-30	CK	0.034±0.01a	0.037±0.01a	0.042±0.04b	0.034±0.01a	0.030±0.03a	0.030±0.02a
	BSA	0.085±0.03a	0.090±0.02a	0.129±0.01a	0.059±0.01a	0.063±0.02a	0.065±0.01a
	MA	0.044±0.02a	0.059±0.03a	0.064±0.02a	0.028±0.04a	0.036±0.02a	0.041±0.02a
	PAM	0.071±0.03a	0.070±0.02a	0.095±0.05ab	0.034±0.01a	0.089±0.01a	0.082±0.03a
30-40	CK	0.031±0.01a	0.026±0.03a	0.029±0.05a	0.022±0.04a	0.023±0.04a	0.025±0.01a
	BSA	0.066±0.03a	0.061±0.01a	0.079±0.03a	0.051±0.05a	0.051±0.04a	0.045±0.01a
	MA	0.041±0.02a	0.031±0.03a	0.042±0.01a	0.021±0.02a	0.046±0.02a	0.033±0.02a
	PAM	0.047±0.01a	0.054±0.04a	0.038±0.02a	0.024±0.04a	0.070±0.04a	0.054±0.02a
40-50	CK	0.029±0.02a	0.018±0.03a	0.019±0.03a	0.041±0.01a	0.019±0.03a	0.021±0.01a
	BSA	0.058±0.03a	0.049±0.03b	0.062±0.01a	0.030±0.04a	0.041±0.04a	0.032±0.02a
	MA	0.037±0.01a	0.022±0.01a	0.030±0.02a	0.020±0.02a	0.054±0.03a	0.027±0.01a
	PAM	0.050±0.03a	0.055±0.04a	0.025±0.03a	0.019±0.03a	0.045±0.04a	0.041±0.03a
50-60	CK	0.028±0.02a	0.017±0.03a	0.019±0.01a	0.018±0.04a	0.021±0.02a	0.020±0.01a
	BSA	0.053±0.03a	0.041±0.02a	0.059±0.04a	0.029±0.05a	0.046±0.02a	0.031±0.01a
	MA	0.036±0.01a	0.020±0.04a	0.025±0.02a	0.025±0.02a	0.054±0.03a	0.033±0.02a
	PAM	0.049±0.03a	0.034±0.01a	0.026±0.04a	0.020±0.04a	0.054±0.02a	0.037±0.04a

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Table 5. *Dynamics of soil K^+/Na^+ ratio in soil layers of 0-10 cm, 10-20 cm, 20-30 cm, 30-40 cm, 40-50 cm and 50-60 cm under CK, BSA, MA, and PAM treatments during maize growing season. Different letters in the same column indicates significant differences between different treatments at $P < 0.05$*

Soil depth (cm)	Treatment	Maize growing season		
		Jointing (BBCH30)	Tasseling (BBCH51)	Maturing (BBCH99)
0-10	CK	0.123±0.03b	0.079±0.02b	0.062±0.02b
	BSA	0.667±0.02a	0.991±0.01a	0.814±0.02a
	MA	1.016±0.01a	0.567±0.01a	0.379±0.03a
	PAM	1.507±0.01a	0.257±0.01a	0.996±0.01a
10-20	CK	0.043±0.03b	0.083±0.01b	0.043±0.03b
	BSA	0.108±0.03a	0.159±0.01a	0.137±0.02a
	MA	0.111±0.02a	0.138±0.01a	0.073±0.03b
	PAM	0.104±0.02a	0.070±0.01b	0.120±0.01a
20-30	CK	0.027±0.01b	0.078±0.01a	0.029±0.01b
	BSA	0.069±0.02b	0.097±0.02a	0.008±0.01a
	MA	0.108±0.01a	0.077±0.01a	0.041±0.02a
	PAM	0.083±0.03b	0.049±0.03a	0.105±0.04a
30-40	CK	0.023±0.03a	0.059±0.02a	0.028±0.01a
	BSA	0.049±0.02a	0.103±0.03a	0.059±0.03a
	MA	0.045±0.03a	0.063±0.02a	0.025±0.03a
	PAM	0.057±0.02a	0.041±0.02a	0.071±0.02a
40-50	CK	0.020±0.03a	0.042±0.02a	0.025±0.02a
	BSA	0.031±0.04a	0.065±0.02a	0.035±0.03a
	MA	0.032±0.02a	0.048±0.03a	0.028±0.02a
	PAM	0.036±0.02a	0.041±0.02a	0.085±0.03a
50-60	CK	0.019±0.03a	0.030±0.02a	0.025±0.01a
	BSA	0.039±0.03a	0.057±0.03a	0.039±0.01a
	MA	0.027±0.02a	0.043±0.03a	0.025±0.02a
	PAM	0.040±0.01a	0.041±0.02a	0.053±0.01a

Discussion

Effect of amendment on soil moisture

Coastal saline-alkali soil is characterized by low porosity, easy compaction, and poor water and air permeability. The key to improve coastal saline soil is to improve soil moisture (Jia et al., 2017). Soil moisture is critical to plant growth and soil salinity leaching and forms one of the important physical properties used to evaluate the quality of saline-alkali soil. In present study, the applied field management efficiently increased retention of soil moisture, and reduced soil salinity levels. It is widely reported that

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application of MA have beneficial effects on soil properties by reducing soil bulk density (Gill et al., 2009; Clark et al., 2007). In this study, at the depth of 0-20 cm soil layer, moisture was higher under MA treatment compared with CK treatment. PAM is commonly adsorbed by soil through cationic bridges between soil and polymer anionic groups, and multivalent cations in the soil solution would bridge the negatively charged soil particles and polymers together (Laird, 1997; Chen et al., 2016). The effect of PAM on improving soil water holding capacity has been confirmed in many literatures (Tadayonnejad et al., 2017; Albalasmeh et al., 2021). In our experiment, PAM treatment consistently increased 0-20 cm layer soil moisture compared with CK during crops growing season. However, at the depth of 20-60 cm soil layer, there was no significant difference in soil moisture content between PAM and CK treatments. We used dry granular PAM, surface application dry granular PAM without a source of electrolytes was not effective in maintaining a good infiltration rate, 20-60 cm soil moisture content did not increase significantly (Abrol et al., 2013).

Effect of amendment on soil salinity

Biochar can hasten salt leaching and thus decrease the time required for reducing salt concentration to a level suitable for growing plants (Zhao et al., 2020; Zhang et al., 2020; He et al., 2020). In our study, in surface layer (0-20 cm), soil salinity content under BSA treatment was lower than that under CK treatment during crops growing season. But in deep soil layer (20-60 cm), the difference gradually decreases. This might be due to the that the application of BSA can improve salt-leaching efficiency of surface salts as explained by Yue et al. (2016).

Applying MA have beneficial effects on decreasing salt concentration, improving soil fertility and enhancing soil microbial activity (Clark et al., 2007; Gill et al., 2009). At winter wheat maturation stage, only 0-10 cm soil layer was desalination area, the scope of the desalination area was small. At maize matured, soil desalination rate increased from 17% to 30%. MA were rich in organic matter, addition of MA to soil could increase soil colloid concentration and EC. Moreover, the influence mechanism of MA on soil salinity needs further studies.

Application of PAM can effectively improve soil physical structure. After contacting with water, hydration occurs through these groups and osmotic pressure can absorb more water, improve the soil water retention capacity, and then reduce the salt content of the soil colloid (Zhang et al., 2012). In this study, at the depth of 0-60 cm soil layer, soil salinity under PAM treatment was lower than that in CK. Previous studies have found that PAM could improve the capillary porosity of saline-alkali soils, thereby increasing salt-leaching efficiency.

Effect of amendment on soil salt ions

Chloride ion (Cl^-) is often used to indicate the salinization degree of saline soil as the main reference index for the classification and improvement of saline soil (Bao, 2000). Cl^- is the anion with the highest content of salinized Chao soil. In this study, we found that Cl^- content in 0-10 cm layer soil is contradictory to soil moisture content, which also confirms the characteristics of effective Cl^- leaching. Compared with CK, Cl^- content in surface soil layer (0-20 cm) decreased after applying amendments. Soil profile Cl^- content under BSA treatment was lower than that under CK treatment during crops growing season. It is demonstrated that biochar has improved the infiltration characteristics of soil

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moisture, which in turn promoted the efficiency of water leaching of Cl^- (Zhang et al., 2019).

Soil SAR depend on the relative proportion of Na^+ and Ca^{2+} in soil solution. Soil porosity is expressed in terms of SAR, with high SAR values having the potential for deterioration of soil structure, low infiltration rate, specific-ion effect, and deficiencies of several micro and macro nutrients (Murtaza et al., 2006). In this study, we found that the change of soil SAR was consistent with soil salt content. In short, the soil SAR appears in order of BSA> MA> PAM> CK. Compared with CK, BSA can significantly reduce soil SAR during crops growing season. Many studies have confirmed the beneficial impact of BSA on decreasing SAR of saline soils (Lashari et al., 2013; Chaganti et al., 2015b). BSA could decrease SAR directly by providing exchangeable Ca^{2+} to replace Na^+ on the soil colloids, similar results were observed by Lashari et al. (2013) and Chaganti et al. (2015b). At the same time, BSA may increase partial pressure of CO_2 in the rhizosphere to mobilize Ca^{2+} from calcareous soils for replacing Na^+ from the soil colloids (Jalali and Ranjbar, 2009). PAM has no significant effect on soil SAR during the growth season of winter wheat, but it can significantly reduce soil SAR during maize growing season. In this study, we used anionic-PAM. According to Gungor's (2001) research, when the application amount of PAM is too large, the presence of exchangeable Na^+ will reduce the viscosity of the PAM aqueous solution, thereby increasing the rate of soil moisture infiltration. Since there was more precipitation in the maize growing season in field experiment area, application of PAM could improve the salt leaching efficiency and improve the effect of salt leaching. Therefore, during the growth season of maize, the SAR of soil was low. No matter what crops were grown, MA had no obvious effect on soil SAR. From the aspect of soil SAR, MA had no significance effect on improving salinized soil. In short, the interaction between microbial agents and soil salt ions needs further research.

Exchangeable K^+ can create similar effects as Na^+ on soil structure, but it has received less attention than Na^+ because K^+ amounts are typically low in salt-affected soils (Rengasamy, 2011). In other words, it is believed that K^+/Na^+ affect clay structure.

In present study, we investigated K^+/Na^+ in the soil during crops growing season to analyze the effect of amendments on soil salt ions. The K^+/Na^+ ratio of the topsoil (0-10 cm) could be increased significantly after application amendments. A conclusion can be drawn that the three amendments had beneficial effects on soil quality.

MA could improve the soil porosity and aggregate structure, increase soil permeability, and promote plant root growth; on the other hand, it relies on the beneficial microbial flora, organic and other substances it contains to increase the amount of soil microorganisms, and participate in humus formation (Pang, 2011). In this study, MA was rich in organic materials and a variety of microorganisms. Previous studies have demonstrated that application of organic materials to saline soils has been shown to accelerate Na^+ leaching and reduce exchangeable sodium percentage (ESP) and electrical conductivity (EC) (Tejada, 2006; Lopez-Valdez el al., 2010). In this study, SAR of the surface soil (0-10 cm) significantly decreased, especially during maize growing season.

Earlier studies demonstrated that the presence of Na^+ in soil could decrease the viscosity of PAM aqueous solution, and improve water infiltration and promote salt leaching (Silvestri, 2005; Tho, 2008; Liu et al., 2015a,b). PAM was an anionic polyamide, which may possess ability to absorb soil cations. In present experiment, at the depth of 0-20 cm, K^+/Na^+ value of the soil after applying PAM was significantly increased. In this study, surface soil moisture gradually decreased while soil salinity gradually increased

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under all treatments, and both showed a strong temporal variability, which was related to the seasonal precipitation and evaporation patterns (*Fig. 3*). Previous studies have demonstrated that soil salinity in coastal saline area has obvious seasonal variability, which is generally characterized by strong accumulation in winter and spring, and desalination in summer and autumn (He et al., 2015; Feng et al., 2018). This is mainly because the summer and autumn in the study area were rainy seasons, and the abundant precipitation makes the soil salt gradually leaching to the deeper layer. While the winter and spring were dry seasons, with less rainfall and strong evaporation, makes the soil moisture evaporates violently, and the salinity accumulates to the surface rapidly (Wu et al., 2009; Feng et al., 2018). In our experiment, winter wheat was sown in October 2017 and harvested in June 2018, with low rainfall and high evaporation. Summer maize was sown in June 2018 and matured in October. The whole growing season was in the rainy season with more rainfall. Therefore, during maize growing season, soil had higher water content and lower salt content than that during wheat growing season. At the same time, we observed soil salinity gradually increased and accumulated to the surface soil during early growing stages of wheat (from seedling to revival), which might attribute to the rapid rise of temperature and increase of evaporation in spring, making soil salinity accumulate to the surface (*Figs. 1 and 3*). Soil moisture and salinity also varied with different treatments in surface soil. In this experiment, we used three different properties of amendments. A consistently lower salt content was observed in the 0-40 cm soil layer treated with BSA than that of CK treatment (*Table 2*). However, soil moisture of BSA treatment decreased in the later growth stage, whereas the soil salinity increased rapidly (*Figs. 2 and 3*). This might be owing to the combined influence of transpiration and evaporation as explained by Huo et al. (2017). It should be noted that the water supply from deep soil layer (40-60 cm) was sufficient as no amendments were applied under CK treatment, and consequently a relative stable soil moisture and significantly higher soil salinity were observed for CK treatment during crops growing season.

Conclusions

The soil moisture and salt spatial distribution characteristics during wheat-maize rotation growing season were investigated in saline soil region. A conclusion can be demonstrated that all applied amendment treatments had significant effects on dynamics of soil moisture, soil salinity, Cl^-, SAR and K^+/Na^+ ratio. During the growing season, all amendment treatments increased the surface (0-20 cm) soil moisture compared with CK. Simultaneously, amendments decreased soil salinity at 0-20 cm depth. Moreover, soil amendment treatment apparently promoted salt-leaching, reduced Cl- and SAR, increased K^+/Na^+ ratio. During crops growth season, the maximum water retention of soil and salt leaching were obtained in 1500 kg $ha^{-1}$for BSA treatment. Therefore, when chemical measures were used to improve saline alkali land in Binzhou, Shandong, it is recommended to prioritize the application of biochar soil amendment (BSA), under the dosage of 1500 kg ha^{-1}, to improve soil quality and prevent secondary salinization. Our results provide insights into the effects of four different amendments added to saline-alkaline soils under a wheat-maize rotation system. Further investigations are suggested to find the best mixture of amendments to improve saline-alkali soil and crops production.

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ANALYZING THE IMPACT OF BURNING ON VEGETATION IN HIMALAYAN CHIR PINE (*PINUS ROXBURGHII* SARG.) FORESTS, INDIA

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Abstract. The study was conducted in three Forest Divisions i.e. Solan, Nahan and Rajgarh of Himachal Pradesh, India to determine the impact of controlled burning on the diversity of shrubs and herbs in chir pine forests. A total 2.00 ha was selected for study on each site. The controlled burning was conducted in 1.5 ha (B_1) during January 2017 and 1.0 ha (B_2) in January 2018. A 0.50 ha was designated for control treatments on each site. The field observations of phytosociology were recorded during November, 2018. A total number of 49 species was found at all three sites. The number of species varied from 27 at Lawasachowki (B_2) to 35 at Mangotimor (C). The diversity of shrubs ranged from 2.17 at Bagpashog (B_2) to 2.66 at Mangotimor (C), whereas for herbs it ranged from 2.82 at Mangotimor (C) and Lwasachowki (C) to 3.25 at Bagpashog (B_2). The density of shrubs and herbs of control (C) were significantly different from burning treatments (B_1 and B_2). The density and diversity of shrubs were found higher in control as compared to burning treatments on each sites and vice versa for herbs.
Keywords: *controlled burning, shrubs, herbs, density, diversity*

Introduction

Himalaya is one of the hotspots of the biodiversity (Myers et al., 2000; Singh et al., 2009) and provides ecological niches for plants and human beings (Sharma et al., 2018). Himalaya provides various ecosystem services, sustain biodiversity, regulate hydrological integrity and maintain slope stability (Chawla et al., 2008; Sharma et al., 2009). The plant diversity and communities in Himalaya changes with environmental gradient and also due to variation in geological conditions, topography, river systems, rainfall, altitude and climate (Sharma et al., 2014). The climate change, human and cattle population pressure, over grazing, habitat loss (Sharma et al., 2014) and development of infrastructures like roads, are the major threats to Himalayan ecosystems leading to increased rate of ecological degradation and disturbance to the equilibrium of Himalayan mountain ecosystem (Ahmad et al., 1990). Forest fire is one of the major challenges and requires proper planning of land use management in the region (Avesani et al., 2000).

The forest fire impacts the ecosystems through variety of ways as biogeochemical cycling, atmospheric chemistry and ecosystem structures (Joseph et al., 2009) and also modifies the atmosphere by mixing the greenhouse gases (Simmonds et al., 2005; Chiriaco et al., 2013) and contribute in acceleration of climate change (Stocks et al., 1998; Kiem et al., 2006; Gavin et al., 2007). The forest fires destroy the biomass (Chiriaco et al., 2013; Jharya et al., 2014) and impacts nutrients availability in the soil (Verma et al., 2019) resulting into the accelerating the soil erosion on the slopes in the

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Himalaya. The forest fire contributes for altering the plant communities (Whelan, 1995; Arevalo, 2014) changes the dynamics and structure of vegetation of landscape (Baeza, 2007; Arevalo, 2014). The woody and non woody vegetation are prone to forest fire which lead to decrease in the moisture content on plants (West, 1965; Gandiwa, 2011) leading to chnages into the functioning of the plants. The forest fire can be prevented by improving awareness among the people about damages caused by the fire and also by employing the legal enforcement and application of suitable silviculture practices such as fire lines and controlled burning (Bhardwaj and Narkhede, 1997).

Chir pine (*Pinus roxburghii* Sarg.) forests in Western Himalayas are prone to forest fire with recurrent fire incidences (Negi et al., 2016). Fire in chir pine forest influence plant community structure and also causing huge economic and environmental losses (Negi et al., 2016; Konsam et al., 2017; Sannigrahi et al., 2020). The fire affected area in the chir pine forest constitutes 2.36% of forest area of India (FSI, 2019). The incidences of the forest fire is decreased by the old age practice of controlled burning during winter when surface temperature is low (Anderson, 1983; Rego et al., 1991; Brose et al., 1998; Blake et al., 2000; Allain and Grace, 2001; Hutchinson et al., 2005; Baeza et al., 2007; Knapp et al., 2007; Gairola et al., 2008; Brose, 2010; Gandiwa, 2011; Balch et al., 2013; Arevalo et al., 2014; Edgar and Griscom, 2017; Kumar, 2019; Kumar et al., 2020; Alba et al., 2021; Jaffe et al., 2021; Jang et al., 2021; Perles et al., 2021). However, succinct analysis and authenticated database on impact of controlled burning on woody and non woody vegetation in Chir pine forests is not available inspite of the fact that the controlled burning can assist to decrease the chances of forest fire in chir pine forest (Kumar et al., 2020).

The lack of information about the impacts and control of forest fire undermine the efforts of the forest department to control the recurrent fire. Moreover, forest fire leads huge losses to exchequer leading to loss of properties and lives (Negi et al., 2016). The impact of controlled burning on understorey of forests is poorly known, therefore, controlled burning evaluations are a requirement for the effective management of vegetation in the forest ecosystems. Moreover, under the current global changes, the evaluations of impacts of forest fire would guide for fire control mechanism and also led to refrain the losses of properties and lives.

With this in view, the study hypothesizes that the controlled burning affects the density and diversity of the forest fire fuel specially the shrubs and herbs of the forest ecosystems. Therefore, objective of the present study was to analyze the impact of controlled burning conducted twice on phytosociology of understory vegetation in chir pine forests. The result of the study would supplement to the current understanding about the forest fire control mechanism along with generation of specific knowledge about forest fire management.

Material and methods

Selection of sites

The study focuses the Indian Himalayan forests of the Nahan and Solan Forest Circles of Himachal Pradesh (HP), India. The focus of the study was higher altitude zone (>1200 m above mean sea level) in Upper Himalayan Chir pine Forests (Group 9/C1b as per Champion and Seth, 1968 classification of Forests of India). Total three sites i.e., Mangotimor, Bagpashog and Lawasachowki were selected randomly for investigations in the forests (*Table 1, Figs. 1 and 2*) in Parwanoo, Sarahan and Jamta

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Forest Ranges, respectively. The monthly weather details of the areas are reported in *Figs. 3 and 4*. The dominant species at study sites were *Pinus roxburghii* with associate species as *Quercus leucotrichophora*, *Myrica esculenta* and *Pyrus pashia*. The climate of the study sites was transition between sub-temperate and sub-tropical. The winter, summer and rainy seasons are found in the area and the precipitation in the region is received in the form of the rains. The calcareous shales, carbonaceous shales, dolomite limestone are found on the ground of chir pine forests (Gupta et al., 2009). The depth of the soil in the study sites varied from 40 cm to 70 cm. The slope of the sites ranged from 10° to 25°.

Table 1. *Site details of the selected sites in Nahan and Solan Circles of Himachal Pradesh Forest Department*

Name of the site	Name of Forest Division	Geo-coordinate		Elevation (amsl)	Aspect
		Latitude (N)	Longitude (E)		
Mangotimor	Solan	30^0 53′ 49.3″	077^0 00′ 06.5″	1550m	Sothern Eastern
Bagpashog	Rajgarh	30^0 45′ 41.5″	077^0 09′ 52.6″	1522m	North West
Lawasachowki	Nahan	N30^0 40′ 32.8″	E077^0 11′ 45.7″	1344m	Sothern Eastern

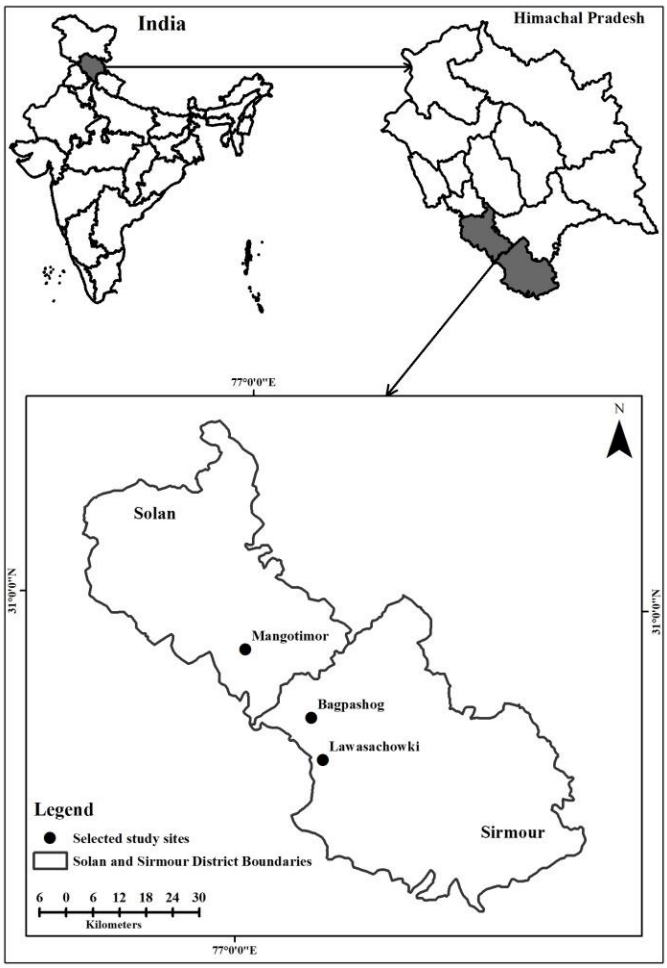

Figure 1. *Map of selected study sites in Solan and Sirmaur Districts in Himachal Pradesh*

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a) b) c)

Figure 2. *Photographs of the study sites, a. Mangotimor b. Bagpashog and c. Lwasachowki*

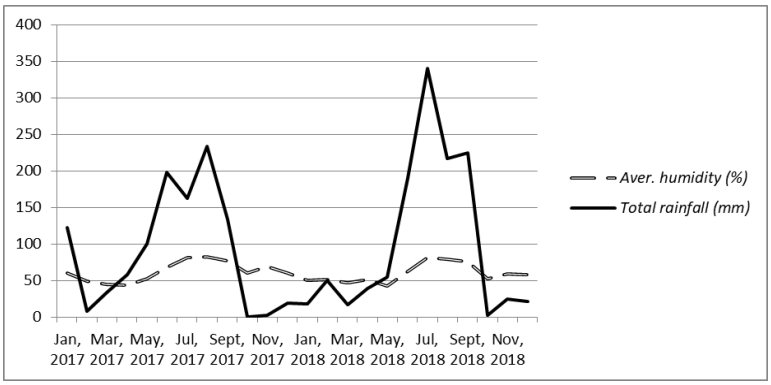

Figure 3. *Monthly average humidity (%) and total rainfall (mm) from January 2017 to December, 2018*

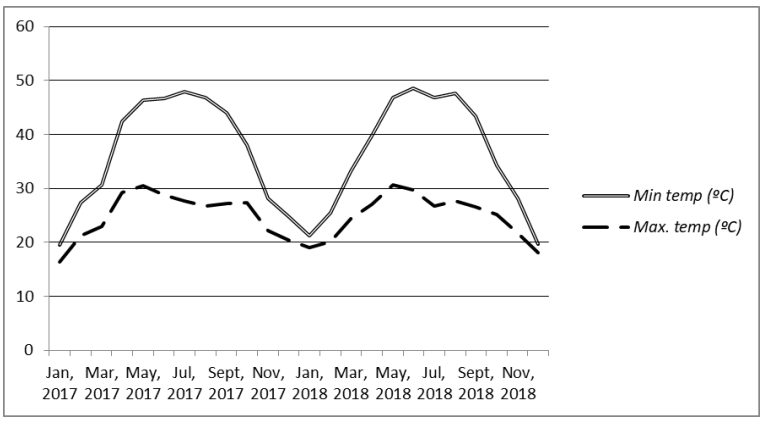

Figure 4. *Monthly max. temp (ºC) and min. temp (ºC) from January 2017 to December, 2018*

Fire management treatment

The forest fire can be managed by using controlled burning. Controlled burning is controlled firing of forest fuel either in their natural or modified state under specific environmental condition to fulfill the objective of plan resource management (Bhardwaj and Narkhede, 1997). The three treatments for forest fire evaluations were control (C), once burnt (B_1) and twice burnt (B_2). A total area of 2.00 ha homogenous patch of forests on each site was selected for the study and out of which the controlled burning (B_1) was conducted once in 1.50 ha on each site with 0.50 ha designated as control (C)

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during January, 2017. In January 2018, controlled burning was again conducted only in 1.00 ha out of the burnt area in 2017 on each site and considered as twice burnt (B_2), and remaining 0.50 ha of the burnt area in 2017 remains intact and treated as B_1. The designated control site of 0.50 of 2017 ha was control (C) on each site i.e. controlled burning was not undertaken on each sites in the area.

Collection of data

Total 20 quadrates for shrubs and 40 quadrates for herbs on each site were laid out randomly in control (C), once burnt (B_1) and twice burnt (B_2) areas. The size of the quadrates was 5 m x 5 m for shrubs and 1mx1m for herbs (Mishra, 1968). The number of individual and diameter of shrubs, saplings of trees and herbs including natural regeneration of trees for phyto-sociological analysis were recorded in 0.50 ha, 0.50 ha and 1.00 ha for C, B_1 and B_2, respectively during November, 2018.

Analysis of data

The density (D), frequency (F %), abundance (A) and importance value index (IVI) were determined as per Curtis and McIntosh, 1950. The sum total of relative value of density, basal area, and frequency was used for calculating IVI. Abundance to frequency ratio (A/F) was calculated and the distribution pattern (A/F) was used to categories as contiguous (>0.050), random (0.025-0.050) and regular (<0.050) (Curtis and Cottam, 1956). The Shannon-Wiener Diversity Index (H) was calculated for determining the plant diversity (*Eq. 1*) (Shannon-Wiener, 1963).

$$H = -\sum_{i=1}^{S} (ni/N)\, ln\, (ni/N) \qquad \text{(Eq.1)}$$

Concentration of dominance (Cd) was measured by Simpson's Index (*Eq. 2*) (Simpson, 1949).

$$Cd = \sum_{i=1}^{S} (ni/N)^2 \qquad \text{(Eq.2)}$$

where ni = Importance value of species i and N= total importance value of all the species in both the indices.

Statistical analysis

The effect of controlled burning on number of individuals of shrubs and herbs were evaluated as per the analysis of variance (ANOVA) with 3 treatments i.e. control (C), once burnt (B_1) and twice burnt (B_2). The mean of the four quadrates selected randomly from each patch i.e. for the three treatments was considered as one replication for herbs, whereas mean of two quadrates was treated as replication for shrubs. Total replications were ten for each treatment i.e., control (C), once burnt (B_1) and twice burnt (B_2) for shrubs and herbs on each site. The Least Significant Difference (LSD) test was applied for evaluation of critical difefrences between two treatment means.

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Results

The phytosociological analysis was conducetd for collected field data. The differences in the impacts of the treatments were compared through ANOVA. The details of the analysis have been described in subsequent paragraphs for each site for shrubs and herbs.

A total of 49 species was found across the three sites which belong to 49 genera and 28 families. The number of species was 45 at C, 49 at B_1 and 45 at B_2. A total number of species in control was 28 at Lawasachowki and 32 at Mangotimor. The total number of species in once burnt (B_1) varied from 28 (Lawasachowki) to 35 (Mangotimor), whereas in twice burnt (B_2), the number of speices ranged from 27 (Lawasachowki) to 33 (Mangotimor).

Carissa carandas, Myrsine africana, Rubus ellipticus and saplings of *Pinus roxburghii* were present across three sites at all three treatments. *Buddleja asiatica* was present only in B_2 at Mangotimor (*Table 2*). The saplings of *Myrica esculenta* was present only in C at Bagpashog. The saplings of *Pistacia integerimma* and *Rosa moschata* were present in C and B_1 at Mangotimor. The saplings of *Punica granatum* at Mangotimor were present in B_1 respectively. The saplings of *Sapium insigne* were present only in C and B_2 at Lwasachowki (*Table 2*).

Table 2. *Density/25 m^2 of shrubs under control (C) and burnt (B_1 and B_2) sites in Himalayan Chir Pine Forests*

S.No.	Name of species	Mangotimor			Bagpashog			Lawasachowki		
		C	B_1	B_2	C	B_1	B_2	C	B_1	B_2
1	*Berberis lycium*	1.05	0.95	0.5	1.5	1	0.75			0.75
2	*Buddleja asiatica*			0.4						
3	*Carissa carandas*	3.7	1.3	0.75	0.95	0.75	0.4	1.75	1.25	0.65
4	*Colebrookea oppositifolia*	0.9		0.4	1	0.8	0.65	0.9	1.2	0.5
5	*Ficus palmata**	0.5	0.55	0.5						
6	*Flacourtia indica**	0.6						0.95	0.95	0.25
7	*Lantana camara*	1.25	0.6	0.85						
8	*Maesa indica*							1.45	1.45	0.95
9	*Mallotus philippensis**					0.35	0.2	0.7	0.6	0.3
10	*Murraya koenigii*				0.7	1.45	0.45	1.5	1.25	0.95
11	*Myrica esculenta**				0.55					
12	*Myrsine africana*	2.4	2.85	0.65	1.5	1	0.95	1.25	1.1	0.6
13	*Pinus roxburghii**	0.45	0.3	0.5	0.4	0.3	0.2	0.6	0.4	0.25
14	*Pistacia integerimma**	0.25	0.5							
15	*Prinsepia utilis*	1.15	1.45	0.4						
16	*Punica granatum**		0.3							
17	*Pyrus pashia**	0.5	0.65	0.4	0.55	0.4	0.25	1	0.6	
18	*Rosa moschata*	0.35	0.65							
19	*Rubus ellipticus*	1.4	1.05	0.5	1	0.6	0.4	1.95	1.1	0.4
20	*Sapium insigne**							0.9		0.2
21	*Toona ciliata**	0.35		0.25	0.35			0.4	0.5	
22	*Woodfordia fruticose*	0.45	0.5	0.6				0.6	0.45	
23	*Zanthoxylum armatum*	0.75	0.95	0.35	0.9					
TOTAL		16.05	12.6	7.05	9.4	6.65	4.25	13.95	10.85	5.8

* Saplings of trees

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The data on groundflora (herbs, seedlings/natural regeneration of trees and shrubs) of burnt and unburnt treatments revealed that *Adiantum lunulatum, Anaphalis triplinervis, Cheilanthes farinosa, Chrysopogon montanus, Cirsium wallichii, Cissampelos pareira, Dicliptera bupleuroides, Fragaria vesca, Heteropogon contortus, Oxalis corniculata, Viola serpens* along with seelings of *Pinus roxburghii* were present in all the treatments and sites (*Table 3*). The seedlings of *Ficus palmata* was present in B_1 and B_2 only at Bagpashog and Lwasachowki. The seedlings of *Flacourtia indica* were present in B_1 and B_2 at Lwasachowki. The seedlings of *Lantana camara* and *Sapium insigne* were present in B_1 and B_2 at Mangotimor. *Thymus linearis, Pteris cretica* and seedlings of *Myrica esculenta* and *Prinsepia utilis* were present in B_1 and B_2 at Bagpashog (*Table 3*).

Density of shrubs and herbs

A. Study site: Mangotimor

Density (Ind./25 m^2) of shrubs

The highest density (3.70) at C was recorded for *Carisssa carandas* followed by *Myrsine africana* (2.40) and lowest (0.25) for *Pistacia integerrima* (*Table 2*). The maximum density (2.85) at once burnt (B_1) was found for *Myrsine africana* followed by *Prinsepia utilis* (1.45) and minimum (0.30) was recorded for *Pinus roxburghii* and *Punica granatum*. On twice burnt (B_2) site, maximum density (0.85) was recorded for *Lantana camara* followed by *Carissa carandas* (0.75) and minimum (0.25) for *Toona ciliata* (*Table 2*). Significance difference in total number of individuals/25 m^2 of all shrubs were found between C (16.05), B_1 (12.60) and B_2 (7.05) (*Table 4*).

Density (Ind./m^2) of herbs

The highest density (3.03) at C was recorded for *Carex meiogyna,* followed by *Chrysopogon montanus* (2.55) and lowest (0.08) was recorded for *Solanum xanthocarpum* and *Parthenium hysterophorus* (*Table 3*). At once burnt (B_1), highest density (5.88) was recorded for *Heteropogon contortus*, followed by *Chrysopogan montanus* (5.85) and lowest (0.18) was recorded for *Parthenium hysterophorus* at Mangotimor (B_1). The highest density (3.90) at twice burnt (B_2) was recorded for *Heteropogon contortus* followed by *Chrysopogan montanus* (3.83) and lowest (0.15) for *Sapium insigne* and *Anaphalis triplinervis* (*Table 3*). Significance difference in total number of individuals of all herbs/m^2 was observed between C (12.98), B_1 (36.23) and B_2 (22.93) (*Table 4*).

B. Study site: Bagpashog

Density (Ind./25 m^2) of shrubs

The highest density (1.50) at C was recorded for *Myrsine africana* and *Berberis lycium* followed by *Colebrookea oppositifolia* (1.00), *Rubus ellipticus* (1.00) and lowest (0.35) for *Toona ciliata* (*Table 2*). The highest density (1.45) at once burnt (B_1) was recorded for *Murraya koenigii*, followed by *Berberis lycium* (1.00), *Myrsine africana* (1.00) and least dominant (0.30) was *Pinus roxburghii*. The maximum density (0.95) at twice burnt (B_2) was recorded for *Myrsine africana* followed by *Berberis lycium* (0.75) and least dominant (0.20) was *Mallotus philippensis* and *Pinus roxburghii* (*Table 2*). Significance difference in number of individual/25 m^2 of all shrubs was found between C (9.40), B_1 (6.65) and B_2 (4.25) (*Table 4*).

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Table 3. *Density /m^2 of herbs under control (C) and burnt (B_1 and B_2) sites in Himalayan Chir Pine Forests*

S.No.	Name of species	Mangotimor			Bagpashog			Lawasachowki		
		C	B_1	B_2	C	B_1	B_2	C	B_1	B_2
1	*Adiantum lunulatum*	1.03	1.65	2.03	0.45	0.53	0.73	0.45	0.53	0.73
2	*Ageratum conyzoides*		3.28	1.13				0.3	0.53	0.65
3	*Ajuga bracteosa*				0.3	0.53	0.65			
4	*Anaphalis triplinervis*	0.33	0.43	0.15	0.38	0.65	0.75	0.38	0.65	0.75
5	*Artemisia vulgaris*				0.23	0.5	0.63			
6	*Berberis lycium***		0.7	0.45	0.23	0.73	0.83			
7	*Carex meiogyna*	3.03	3.98	2.25						
8	*Carissa carandas*	0.2	0.43	0.3				0.23	0.5	0.63
9	*Cheilanthes farinosa*	0.2	1.73	0.85	0.28	0.55	0.3	0.25	0.73	0.83
10	*Chrysopogon montanus*	2.55	5.85	3.83	2.3	5.38	4.73	1.78	5.88	4.73
11	*Cirsium wallichii*	0.2	0.35	0.2	0.23	0.63	0.85	0.23	0.63	0.85
12	*Cissampelos pareira*	0.15	0.35	0.23	0.13	0.4	0.4	0.23	0.55	0.3
13	*Dicliptera bupleuroides*	0.23	1.48	0.7	0.4	0.35	0.5	0.38	0.35	0.5
14	*Ficus palmata**					0.53	0.55		0.43	0.4
15	*Flacourtia indica***								0.53	0.55
16	*Fragaria vesca*	0.38	0.83	0.63	0.65	0.48	0.58	0.4	0.45	0.58
17	*Galium aparine*		0.58	0.43	0.38	0.58	0.43			
18	*Geranium wallichianum*		0.3	0.2	0.18	0.45	0.58	0.08	0.58	0.43
19	*Heteropogon contortus*	2.28	5.88	3.9	2.3	5.88	5.33	2.3	5.38	5.33
20	*Hydrocotyle asiatica*	0.4	0.75	0.35						
21	*Lantana camara***		4.98	0.65						
22	*Leucas lanata*		0.45	0.6	0.25	0.73	0.65	0.38	0.73	0.65
23	*Mallotus philippensis**							0.18	0.35	0.5
24	*Murraya koenigii***							0.38	0.78	0.65
25	*Myrica esculenta***					0.33	0.5			
26	*Myrsine africana***				0.3	0.58	0.5	0.3	0.45	0.5
27	*Oxalis corniculata*	1.13	0.28	2.23	0.28	0.35	0.5	0.3	0.7	0.63
28	*Parthenium hysterophorus*	0.08	0.18	0.45						
29	*Pinus roxburghii**	0.13	0.23	0.18	0.2	0.43	0.45	0.23	0.3	0.45
30	*Prinsepia utilis***					0.63	0.48			
31	*Pteris cretica*					0.78	0.63			
32	*Pyrus pashia**	0.1	0.53	0.23	0.18	0.45	0.55			
33	*Rubia cordifolia*				0.18	0.63	0.5			
34	*Rubus ellipticus***	0.15			0.25	0.35	0.65	0.2	0.38	0.48
35	*Sapium insigne*		0.23	0.15						
36	*Solanum xanthocarpum*	0.08			0.3	0.78	0.5			
37	*Sonchus asper*				0.28	0.45	0.63	0.23	0.53	0.55
38	*Thalictrum foliolosum*	0.15	0.43	0.25						
39	*Thymus linearis*					0.7	0.53			
40	*Viola serpens*	0.13	0.43	0.6	0.28	0.38	0.63	0.2	0.83	0.8
41	*Zanthoxylum armatum***	0.1			0.23	0.53	0.45			
TOTAL		13.03	36.31	22.97	11.17	26.27	25.99	9.41	22.77	22.47

*Natural regeneration of trees and ** Natural regeneration of the shrubs

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Table 4. *Mean and result of ANOVA for density of shrubs and herbs under control (C) and burnt (B_1 and B_2) traetment in the selected sites in Himalayan Chir Pine Forests*

Name of site	Treatment (Mean)				F value		$LSD_{(0.05)}$
	C	B_1	B_2	SE	Calculated	Critical value	
Shrubs							
Mangotimor	16.05	12.6	7.05	1.222	13.151	3.554	2.568
Bag Pashog	9.40	6.65	4.25	0.73	24.894	3.554	1.53
LawasaChowki	13.95	10.85	5.80	0.75	60.860	3.554	1.57
Herbs							
MangotiMor	12.98	36.23	22.93	2.791	34.919	3.554	5.865
Bag Pashog	11.10	26.175	25.92	2.861	18.201	3.554	6.011
LawasaChowki	9.35	22.70	22.43	1.899	32.269	3.554	3.990

Density (Ind./m^2) of herbs

The highest density (2.30) at C was recorded for *Heteropogon contortus* and *Chrysopogon montanus* followed by *Fragaria vesca* (0.65) and least dominant (0.13) was *Cissampelos pareira* (*Table 3*). The maximum density (5.88) at once burnt (B_1) was recorded for *Heteropogon contortus*, followed by *Chrysopogon montanus* (5.38) and minimum (0.33) for *Myrica esculenta*. The maximum density (5.33) at twice burnt (B_2) was recorded for *Heteropogon contortus* followed by *Chrysopogon montanus* (4.73) and minimum (0.30) for *Cheilanthes farinosa* (*Table 3*). The number of individuals of all herbs/m^2 was significantly different between C (11.10), B_1 (26.17) and B_2 (25.92). No significant difference was found between B_1 (26.17) and B_2 (25.92) (*Table 4*).

C. Study site: Lawasachowki

Density (Ind./25 m^2) of shrubs

The highest density (1.95) at C was shown by *Rubus ellipticus* followed by *Carissa carandas* (1.75) and lowest (0.40) for *Toona ciliata* (*Table 2*). The maximum density (1.45) at once burnt (B_1) was recorded for *Maesa indica* followed by *Carissa carandas* (1.25) and minimum (0.40) for *Pinus roxburghii*. *Maesa indica* (0.95) and *Murraya koenigii* (0.95) at twice burnt (B_2) showed highest value for density (Ind/25 m^2) followed by *Berberis lycium* (0.75) and least dominant (0.20) was *Sapium insigne* (*Table 2*). Significant difference in number of individuals/25 m^2 of all shrubs was found between C (13.95), B_1 (10.85) and B_2 (5.80) (*Table 4*).

Density (Ind./m^2) of herbs

The maximum density (2.30) at C was recorded for *Heteropogon contortus* followed by *Chrysopogon montanus* (1.78) and minimum (0.08) for *Geranium wallichianum* (*Table 3*). The highest density (5.88) at once burnt (B_1) was recorded for *Chrysopogon montanus* followed by *Heteropogon contortus* (5.38) and lowest (0.30) for *Pinus roxburghii*. Maximum value of density at twice burnt (B_2) was observed for *Heteropogon contortus* (5.33), followed *Chrysopogon montanus* (4.73) and lowest (0.30) for *Cissampelos pareira* (*Table 3*). The number of individuals of all herbs/m^2 between C (9.35) was significantly different from B_1 (22.70) and B_2 (22.43), whereas no significant difference was found between B_1 (22.70) and B_2 (22.43) (*Table 4*).

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Dominance of shrubs and herbs the basis of importance value index (IVI)

A. Dominance of shrubs

Carissa carandas (44.04), *Myrsine africana* (38.56) and *Carissa carandas* (30.84) were dominant species at C, B_1 and B_2, respectively at Mangotimor. *Pinus roxburghii* had the least IVI (10.70) at C and 10.69 at B_1 at Mangotimor. The least dominant was *Toona ciliata* (11.11) at B_2 at Mangotimor (*Table 5*).

Table 5. *Importance value index (IVI) of shrubs under control (C) and burnt (B_1 and B_2) sites in Himalayan Chir Pine Forests*

S.No.	Name of species	Mangotimor			Bagpashog			Lawasachowki		
		C	B_1	B_2	C	B_1	B_2	C	B_1	B_2
1	*Berberis lycium*	18.26	19.04	17.75	41.64	32.88	43.07			32.72
2	*Buddleja asiatica*			16.28						
3	*Carissa carandas*	44.04	23.81	30.84	28.91	32.43	32.23	31.06	25.81	29.6
4	*Colebrookea oppositifolia*	13.06		20.15	30.4	31.2	38.99	15.82	27.56	26.49
5	*Ficus palmata**	12.09	14.78	20.4						
6	*Flacourtia indica**	13.96						22.82	25.5	21.4
7	*Lantana camara*	16.24	13.46	25.28						
8	*Maesa indica*							31.23	34.89	34.53
9	*Mallotus philippensis**					23.54	22.98	16.84	18.94	25.74
10	*Murraya koenigii*				23.37	44.5	36.98	27.98	32.76	33.16
11	*Myrica esculenta**				20.87					
12	*Myrsine africana*	34.89	38.56	27.3	38.12	35.68	45.74	25.6	30.52	31.81
13	*Pinus roxburghii**	10.7	10.69	23.67	14.4	32.26	22.61	21.39	18.85	21.32
14	*Pistacia integerimma**	15.55	18.02							
15	*Prinsepia utilis*	21.13	32.64	18.69						
16	*Punica granatum**		30.23							
17	*Pyrus pashia**	17.79	21.92	21.12	22.86	31.65	26.24	24.5	22.81	
18	*Rosa moschata*	11.16	17.57							
19	*Rubus ellipticus*	22.54	25.19	21.57	30.26	35.87	31.17	26.92	24.2	24.84
20	*Sapium insigne**							26.23		18.38
21	*Toona ciliata**	12.41		11.11	20.15			15.34	19.02	
22	*Woodfordia fruticosa*	10.76	15.47	21.52				14.28	19.13	
23	*Zanthoxylum armatum*	25.43	18.63	24.33	29.03					
TOTAL		300	300	300	300	300	300	300	300	300

*Saplings of trees

Berberis lycium (41.64*), Murraya koenigii* (44.5) and *Myrsine africana* (45.74) was dominant species at C, B_1 and B_2, respectively at Bagpashog. The least dominant at Bagpashog was *Pinus roxburghii* (14.40), *Mallotus philippensis* (23.54) at B_1 and *Pinus roxburghii* (22.61) at B_2 (*Table 5*).

Maesa indica was dominant species in treatments i.e., C (31.23) and B_1 (34.89) at Lawasachowki, whereas *Maesa indica* (34.53) showed highest value of importance value index (IVI) for dominant species at B_2. The least dominant was *Woodfordia fruitcosa* (14.28) at C, *Pinus roxburghii* (18.85) at B_1 and *Sapium insigne* (18.38) at B_2 at Lawasachowki (*Table 5*).

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B. Dominance of herbs

Heteropogon contortus showed the highest IVI value (42.87) at Mangotimor (C) and 44.82 at Lawasachowki (B_1) (*Table 6*). The least dominant at Mangotimor was *Cissampelos pareira* (4.04) at C, *Leucas lanata* (4.53) at B_1, *Circium wallichii* (3.48) at B_2. At Lawasachowki, the least dominant was *Geranium wallichianium* (3.46) at C, *Myrisine africana* (6.75) at B_1 and *Geranium wallichianum* (5.32) at B_2 (*Table 6*).

Table 6. *Importance value index (IVI) of herbs under control (C) and burnt (B_1 and B_2) sites in Himalayan Chir Pine forests*

S.No.	Name of species	Mangotimor			Bagpashog			Lawasachowki		
		C	B_1	B_2	C	B_1	B_2	C	B_1	B_2
1	*Adiantum lunulatum*	17.58	16.44	20.08	9.57	12.35	9.87	9.88	14.19	12.51
2	*Ageratum conyzoides*		14.97	13.24				7.67	7.12	9.67
3	*Ajuga bracteosa*				7.52	5.98	7.74			
4	*Anaphalis triplinervis*	6.03	10.23	6.58	17.03	10.01	10.5	36.71	11.61	13.27
5	*Artemisia vulgaris*				13.99	8.59	11.73			
6	*Berberis lycium***		9.27	10.58	5.5	6.68	6.27			
7	*Carex meiogyna*	39.61	22.12	21.34						
8	*Carissa carandas*	15.53	12.96	8.45				13.05	10.01	15.1
9	*Cheilanthes farinosa*	10.24	11.85	9.25	8.03	14.07	7.21	5.96	7.94	7.67
10	*Chrysopogon montanus*	35.91	26.88	27.59	34.65	37.99	30.9	36.95	42.58	36.86
11	*Cirsium wallichii*	10.22	7.97	3.48	7.08	7.95	7.96	5.95	9.33	9.76
12	*Cissampelos pareira*	4.04	9.11	7.49	9.04	5.77	8.62	11.58	16.1	9.35
13	*Dicliptera bupleuroides*	9.11	12.26	12.49	14.69	7.85	6.11	8.38	9.01	7.57
14	*Ficus palmata**					8.22	9.69		9.81	11.06
15	*Flacourtia indica***								9.62	12.33
16	*Fragaria vesca*	11.43	9.99	7.19	12.09	7.18	7.26	14.23	8.55	9.08
17	*Galium aparine*		12.19	5.82	20.05	8.97	4.31			
18	*Geranium wallichianum*		6.82	4.3	11.57	7.34	7.26	3.46	10.45	5.32
19	*Heteropogon contortus*	42.87	29.35	29.35	33.55	35.89	36.03	41.9	44.82	43.11
20	*Hydrocotyle asiatica*	11.16	8.39	4.7						
21	*Lantana camara***		20.53	9.35						
22	*Leucas lanata*		4.53	8.58	11.11	6.87	7.22	18.77	8.15	8.9
23	*Mallotus philippensis**							10.78	9.42	7.54
24	*Murraya koenigii***							12.01	8.93	12.17
25	*Myrica esculenta***					6.87	6.08			
26	*Myrsine africana***				7.96	8.97	6.08	8.05	6.75	11.56
27	*Oxalis corniculata*	15.17	4.9	21.03	7.71	8.15	9.05	8.03	9.49	12.17
28	*Parthenium hysterophorus*	7.09	5.8	11.17						
29	*Pinus roxburghii**	13.08	6.54	8.19	9.77	7.25	11.06	15.87	7.87	14.32
30	*Prinsepia utilis***					7.95	10.51			
31	*Pteris cretica*					7.49	9.57			
32	*Pyrus pashia**	8.04	9	14.9	5.62	7.75	6.2			
33	*Rubia cordifolia*				11.57	7.95	9.01			
34	*Rubus ellipticus***	6.8			11.11	8.15	9.58	9.28	8.87	13.56
35	*Sapium insigne*		13.74	18.71						
36	*Solanum xanthocarpum*	10.81			7.96	7.49	9.05			
37	*Sonchus asper*				7.71	5.65	9.57	6.1	10.35	7.67
38	*Thalictrum foliolosum*	6.15	6.76	7.6						
39	*Thymus linearis*					8.01	9.63			
40	*Viola serpens*	7.49	7.42	8.54	8.03	7.72	9.57	15.39	19.03	9.44
41	*Zanthoxylum armatum***	11.64			7.08	8.89	6.35			
TOTAL		300	300	300	300	300	300	300	300	300

*Natural regeneration of trees and ** Natural regeneration of the shrubs

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At Bagpashog, *Chrysopogon montanus* showed highest value of IVI (34.65) at C and 37.99 at B_1, whereas *Heteropogon contortus* (36.03) was dominant species at B_2. The least dominant was *Berberis lycium* (5.5) at C, *Sonchus asper* (5.65) at B_1, *Galium aparine* (4.31) at B_2 at Bagpashog (*Table 6*).

Distribution pattern

The distribution pattern was contiguous (A/F >0.05) for all the shrubs, saplings and herbs at all three sites.

Concentration of dominance (Cd)

The highest value of Cd for shrubs (*Table 7*) was 0.117 at Bagpashog (B_2) followed by 0.114 at Bagpashog (B_1) site and lowest (0.075) at Mangotimor (B_2). The highest value of Cd for herbs (*Table 8*) was 0.075 at Mangotimor (C) preceded by 0.074 at Lwasachowki (C) site and lowest (0.048) at Bagpashog (B_2).

Table 7. *Concentration of dominance (C) and diversity index (H) for shrubs under control (C) and burnt (B_1 and B_2) sites in Himalayan Chir Pine Forests*

Name of site	Control (C)		Burnt (B_1)		Burnt (B_2)	
	Cd	H	Cd	H	Cd	H
Mangotimor	0.079	2.66	0.080	2.58	0.075	2.61
Bagpashog	0.098	2.36	0.114	2.19	0.117	2.17
Lwasachowki	0.082	2.53	0.087	2.46	0.094	2.38

Table 8. *Concentration of dominance (C) and diversity index (H) for herbs under control (C) and burnt (B_1 and B_2) sites in Himalayan Chir Pine Forests*

Name of site	Control (C)		Burnt (B_1)		Burnt (B_2)	
	Cd	H	Cd	H	Cd	H
Mangotimor	0.075	2.82	0.052	3.09	0.054	3.06
Bagpashog	0.055	3.07	0.052	3.22	0.048	3.25
Lwasachowki	0.074	2.82	0.068	2.94	0.063	2.97

Shannon-Wiener index (H)

The highest value of Shannon-Wiener index (H) for shrubs (*Table 7*) was 2.66 at Mangotimor (C) followed by 2.61 at Mangotimor (B_2) and lowest was 2.17 at Bagpashog (B_2). The highest value of Shannon-Wiener Index (H) for herbs was 3.25 at Bagpashog (B_2) followed by 3.22 at Bagpashog (B_1) and lowest was 2.82 at Mangotimor (C) and Lwasachowki (C) (*Table 8*).

Discussion

The floristic composition and structure of communities are the main attributes of the forest ecosystems and depend upon environment and anthropogenic activities (Gairola et al., 2008; Shaheen et al., 2012; Bisht and Bhatt, 2013; Dar and Sundarapandian, 2016) like burning. The control burning in forests is a mechanism applied for

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management of forest fire (Whelan, 1995; Arevalo et al., 2014) and has been significantly affected by the number of individuals of shrubs and herbs of chir pine forests and depend on time, intensity and way of controlled burning undertaken. The intensity of controlled burning also decides the floristic composition as the low intensity fire substantially changes the understory composition and density (Brown, 1960; Swan, 1970; Nyland et al., 1982; Reich et al., 1990; Blake and Schuette, 2000). The change in composition of woody plants after burning is observed by Blake and Schuette (2000). In the study, controlled burning did not completely burn all available fuel loads on the ground rather total number of shrubs varied from 9 at Bagpashog (B_1 and B_2) to 16 at Mangotimor (C). The probable cause may be the associated factors which restrict the complete loss of fuel loads on the site. Moreover, the intensity of burning depends on temperature, wind speed, humidity, aspect, slope, plant type and fuel load (Maclean, 1992; Pyne and Bryant, 1994; Pyne, 1997; Blake and Schuette, 2000).

The result indicates that the controlled burning has varied impacts on woody vegetation of understory and herbs on the ground floor in chir pine forests. However, literature on the aspects for the chir pine forests is lacking. The consistent pattern for IVI was not observed for shrubs and herbs in all the three treatments and similar as Kumar et al. (2020). Density and diversity of shrubs were higher in control as compared to fire occurrence for both the burnt treatments and similar to Allain and Grace (2001). The new shoots of shrubs and natural regeneration of trees which emerge after controlled burning are easily palatable and grazed by cattle, however, these new shoots attract the herbivores in the burnt sites. The burning with intensity of the grazing affects the natural regeneration of trees and shrubs and ultimately affecting the diversity of woody vegetation of understory. The controlled burning also destroys the woody plants and has also been reported by various workers (Ensline et al., 2000; Gandiwa and Kativu, 2009; Gandiwa, 2011). The re-sprouting of woody vegetation has also been reported for woody plants after burning (Marrinan et al., 2005; Gandiwa, 2011). The higher temperature during burning decreases physiological functions, growth and increases the mortality of seedlings (Smith et al., 2017; Sharma et al., 2020). These are probable reasons of low density and diversity of shrubs and saplings of trees. The establishment of natural regeneration after burning was poor due to grazing pressure on the sites. The density and diversity after burning can be increased by following rotational grazing.

Total number of herbs varied from 21 at Lawasachowki (C) and Mangotimor (C) to 30 at Bagpashog (B_1 and B_2). Total density and diversity of herbs were higher in burnt sites (B_1 and B_2) as compared to C and is supported by various researchers (Elliot et al., 1999; Royo et al., 2010; Kumar et al., 2020). Density of the herbs was increased after controlled burning and may be due to decrease in competition among different species for soil nutrient and increase in solar insolation. The frequent occurrence of forest fire in chir pine forests (Kumar and Thakur, 2008; Singh et al., 2016; Attri et al., 2020) led to adaptation to forest fire. The various researchers have also reported the higher stems per plant in burnt sites as compared to unburnt sites (San Jose and Farinas, 1983; Scholes and Walker, 1993; Ensline et al., 2000). The higher density of herbs also changes or improves the microclimate of burnt sites by reducing soil erosion. The present result reveals that controlled burning significantly affect the number of the individuals of shrubs and herbs. The contiguous distribution pattern was observed for shrubs and herbs and has also been reported by as a common phenomenon in forest (Kershaw, 1973; Singh and Yadava, 1974; Kunhikannan et al., 1998; Gairola et al., 2011; Dar and

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Sundarpandian, 2016; Kumar et al., 2020). The variation in distribution pattern depends upon microenvironment and biotic factors (Joshi and Tiwari, 1990; Dar and Sundarpandian, 2016). The various factors like slope, soil type and soil moisture affect abundance and distribution pattern of the species (Nigh et al., 1985; Pallardy et al., 1988; Taft et al., 1995; Blake and Schuette, 2000). The higher value of concentration of dominance for herbs in control sites and shrubs in burnt sites revealed the homogeneous nature of community (Kohli et al., 2004; Kumar et al., 2020). The effect of the burning is mainly determined by intrinsic nature of understory vegetation which tends to regain its original structure (Fernandes and Botelho, 2003; Marino et al., 2010).

Conclusions

The diversity and density of shrubs were higher in control as compared to burnt sites. The diversity and density of herbs were higher in burnt sites as compared to control. The study concludes that controlled burning may be beneficial for forest management as burning may modify the diversity of the species while maintaining the density for the chir pine forests of the Indian Himalayan region as frequent forest fire has been noted in the recent years in the region. The significance of the study is many folds as the comparative analysis reveals the importance of burning for the forest management alongwith generating the information about the controlled burning impact analysis. The study may be applicable at local level however for larger spatial domain needs further evaluation on actual ground level basis. Interestingly, the forest fire occurs in small patch of land but in absence of proper management practices it converts into a large fire incidences leading to huge loss, however checking the fire at local level by controlled burning may reduce the chances of big fire in forests.

The study conculdes that the burning has immediate impacts on the diversity and density of the herbs and shurbs of the forest. The recording of the observations on same phytosociological parameter after long duration on these sites may be made to observe the responses and for specific recommendation. Therefore, forest mangement should be reorient for immediate silvicultural treatment for strenghtening and managing the understorey.

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EFFECTS OF LAND USE CHANGE ON THE STRUCTURE AND FUNCTION OF SOIL FUNGAL COMMUNITY IN SANJIANG PLAIN WETLAND

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Abstract. This study analyzed the impact of land use changes on the structure and function of soil fungal communities in the Sanjiang Plain wetland. Soil samples were collected from natural wetlands, paddy fields, and restored wetlands in the Sanjiang Plain, after which the internal transcribed spacer (ITS) of soil fungi was sequenced by Miseq technology. Our results demonstrated that the reclamation of wetlands into paddy fields can significantly reduce the α diversity index of soil fungi (P <0.05), whereas the restoration of wetlands can significantly increase the soil fungus Chao1 index (P <0.05). A total of 16 phyla were identified in three types of land use soil, among which Ascomycota, Basidiomycota, Mortierellomycota, and Rozellomycota were the most dominant (relative abundance > 1%). Mycorrhizal fungi and parasitic fungi are the main functional groups of fungi in natural wetlands and restored wetlands, whereas saprophytes were the main functional groups of paddy fields. Soil pH, total carbon, organic carbon, total nitrogen, and total phosphorus are the main factors affecting the diversity of soil fungal communities. Therefore, our findings indicated that resource development in natural wetlands increases potential ecological risks and reduces ecosystem stability.
Keywords: *soil pH, soil microbial structure, diversity, Miseq*

Introduction

Wetlands are an important natural resource and are among the three most important ecosystems on Earth. Specifically, wetlands play irreplaceable ecological roles such as soil conservation, water purification, and hydrological adjustment (Sui et al., 2021). In recent years, the ecological impacts of wetland land use changes have garnered increasing attention from the scientific community worldwide. Many studies have demonstrated that land use changes can alter the environmental conditions of wetlands and have an important impact on the quality and stability of the ecosystem (Huang et al., 2013; Wang et al., 2017). Overexploitation and excessive utilization of resources and environmental pollution are the main reasons for the loss of biodiversity and functional degradation of wetlands along rivers (Kang et al., 2018). Therefore, coordinating the relationship between wetland resource development and ecological protection has become a key measure to ensure the healthy development of ecosystems and sustainable resource utilization (Hou et al., 2021). Fungi are among the main groups of microorganisms in soil. These microorganisms not only break down recalcitrant substances such as lignin and cellulose derived from plant decomposition but also form tight symbiotic relationships with plants to promote plant metabolism and nutrient absorption, in addition to playing an important role in the accumulation, transformation, and material cycle of soil nutrients (Tedersoo et al., 2014; Yong et al., 2017). At the same time, the spatial distribution

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pattern of soil fungi has significant habitat-dependent characteristics, and changes in environmental conditions can thus affect the structure and function of soil microbial communities (Tedersoo et al., 2014). Therefore, the diversity of soil fungal communities is considered to be one of the most important indicators for evaluating the health and stability of ecosystems and is often used to predict changes in soil nutrient conditions and environmental quality (Wei et al., 2017; Rodriguez et al., 2019; Adamo et al., 2021).

The Sanjiang Plain encompasses several wetlands and is an important food production base in China. Therefore, this region is not only ecologically relevant but is also instrumental in ensuring national food security (Wang et al., 2013). Human activities such as reclamation, river sand excavation, and dam construction have changed the types of land use, thus leading to black soil degradation, wetland shrinkage, and biodiversity reduction, and seriously threatening the health and safety of wetland ecosystems (Dang et al., 2020). Therefore, improving habitat quality, biodiversity, and sustainability are key strategies to restore degraded wetland ecosystems (Zhang et al., 2021). Soil fungi are a crucial component of underground biodiversity and thus have an important role in shaping the overall biodiversity and functions of ecosystems. Higher levels of soil fungal community diversity are generally linked to more stable ecosystems (van der Wal et al., 2006; Sun et al., 2019). At the same time, the diversity of soil fungi is affected by natural or anthropogenic factors such as pH value, soil physical and chemical properties, as well as vegetation and land use patterns, and can interact with plants and soil to affect the ecological processes of wetland soil (Puangsombat et al., 2010; Deng et al., 2019). Therefore, characterizing the soil fungal community diversity and its influencing factors in different land use types in the Sanjiang Plain wetland would be conducive to the protection and restoration of this area. However, most studies on the impact of land use on wetlands have focused on the soil nutrients and the temporal and spatial distribution of physical and chemical properties, whereas the structure and function of soil fungal communities in inland river wetlands in northern China have remained largely uncharacterized.

Additionally, very few studies have assessed the status of black soil degradation in the region. Due to the unique geographical characteristics and cold climate of the study location, the soil microbial richness is low and the humus content is high. Further, the soil fungal community structure and its influencing factors are highly complex in this region due to the co-existence of both natural and anthropogenic disturbances (Dai et al., 2020). Therefore, this study sought to elucidate the impact of different land use types on the soil environment of the Sanjiang Plain wetland and evaluate the natural restoration potential of the degraded wetland ecosystem. To this end, the soil fungal community in the Sanjiang Plain wetland was examined using high-throughput sequencing technology and biological information to explore the impact of land use changes on the composition and function of the soil fungus community in the Sanjiang Plain wetland. Additionally, this study analyzed the interaction between the soil fungal community diversity and environmental factors to serve as a scientific basis for the improvement of wetland resource management strategies in the Sanjiang Plain.

Materials and Methods

Research area

This study was conducted in a region of the Heilongjiang Honghe National Nature Reserve (N46°57' 55”–47°14’7”, E130°24’51”–130°57’38”) located northeast of the

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Sanjiang Plain (*Figure A1*). The area north of the Lahong River and south of the Nongjiang River has a temperate continental monsoon climate, with an altitude of 65–81 m, annual rainfall of 548 mm, and annual evaporation of 1,155 mm. From mid-October through the freezing and thawing season and until mid-May of the following year, the average temperature for 5 months is below 0 °C, the annual average temperature is 2.1 °C, and the frost-free period lasts approximately 130 days. The main vegetation types include wet shrubs and herbaceous plants (e.g., *Deyeuxia angustifolia*, *Carex lehmannii*, and *Phragmites communis*) (Wang et al., 2013), with small areas of farmland and island-like plantations distributed throughout the study region.

Soil collection

In October 2017, three land use types [natural wetland (NW), restoration wetland (RW), and paddy field (PF)] were selected for research in the Honghe Nature Reserve, accounting for a total of nine plots (*Figure A2*). The latitudes and longitudes of three land use types were 47°44’42’’N and133°35’02’’E for natural wetland, 48°15’27’’N and 138°30’08’’E for restoration wetland and 49°50’32’’N and 128°25’07’’E for paddy field. Among them, the main vegetation types of the natural wetlands were *D. angustifolia* and *Carex sphaerocephala* (*C. lehmannii*). Further, the restoration of wetlands consisted of leveling abandoned farmlands and artificially transplanting *Carex angustifolia* and *Carex sphaerocarpa*. The paddy fields had been in operation since the beginning of wasteland cultivation in 2010. All plots exhibited small slopes that is between 0.5°～2° and consistent slope on north directions.

Soil samples were collected from each plot in October 2017. Three 10 m × 10 m plots were randomly established in each plot, and soil drills were used to collect surface soil (0–20 cm) samples. The soil samples taken from five points in the same plot were fully mixed, after which the soil samples from each spot were passed through a 2 mm soil sieve and divided into two equal parts (approximately 500 g each), then stored in a cooler with dry ice. Half of each sample was used to extract the genomic DNA of the soil fungi, whereas the other half was used to test the physical and chemical properties of the soil.

Determination of soil physical and chemical indices

The gravimetric method was used to determine soil moisture content (MC); a pH analyzer (HQ30d; Hach Company, United States) was used to determine the pH value of soil; an automatic carbon and nitrogen analyzer (ElementarVarioMaxCN, German Elementar) was used to detect the total soil nitrogen (TN) and organic carbon; and total phosphorus (TP) in the soil was determined via alkali fusion-molybdenum antimony spectrophotometry.

Extraction of soil fungal DNA and PCR amplification

A DNA extraction kit (power soil 12888 Extraction Kit) was used to extract the total soil DNA, and a universal primer pair (ITS1F/ITS2) was used to amplify the internal transcribed spacer (ITS) rRNA gene of soil fungi via PCR. Each reaction was conducted using TransStart Fastpfu DNA polymerase in 20 μL reaction volumes: 10 μL of PCR mix buffer, 1.0 μL of 5 μmol L^{-1} upstream primer (ITS1), 1.0 μL of 5 μmol L^{-1} downstream primer, 15 ng of DNA template, and enough double distilled water to reach a 20 μL volume. The following were the PCR reaction parameters: 30 cycles of 95 ℃ for 5 min; 95 ℃ for 60 s, 55 ℃ for 60 s, 72 ℃ for 60 s; 72 ℃ for 10 min. The PCR products were

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recovered using the AxyPrep DNA gel recovery kit, and the Illumina MiSeq sequencing platform was used to construct a library and conduct high-throughput sequencing.

Bioinformatics and statistical analyses

The QIIME software (version 1.17; http://qiime.org) (Caporaso et al., 2010) was used to analyze the sequencing data and divide different operational taxonomic units (OTU) according to a 97% similarity threshold, the USEARCH software (version 7. 1) was used to remove chimeric sequences (Edgar, 2010). The RDP (Ribosomal Database Project) Bayesian classification algorithm was used to perform taxonomic analysis on the OTU representative sequences according to a confidence threshold of 0.7 and the resulting sequences were compared with those in the Unite 8. 0 its_fungi database. Soil alpha-diversities diversity indices (Ace, Chao1, Shannon, Simpson) were performed by R software (version 3.0, vegan package) based on OTU level. Principal coordinate analysis (PCoA) and was performed by R software (vegan package) based on Bray-Curtis. Permutational multivariate analysis of variance (PERMANOVA) was used to identify soil fungal community characteristics with significant differences and performed by R software (version 3.0, vegan package) based on genus level. The Cladogram of soil fungal communities were performed based on LAD score = 2.5 by R software (version 3.0, vegan package). The Redundancy analysis (RDA) of the soil fungal community and environmental factors were performed by R software (version 3.0, vegan package) based on OTU level. The Funguild micro-ecological tools were then used to predict and analyze soil fungal functions according to nutritional methods. Pearson correlation between soil physicochemical properties and soil fungal α-diversity indexes used IBM SPSS Statistics 17.0 software at 0.05 and 0.01 levels. Significant differences between groups were identified via one-way analysis of variance (ANOVA) coupled with Duncan's multiple comparison and permutation test using the IBM SPSS Statistics 17.0 software.

Results

Differences in soil physical and chemical properties in different land use types

Changes in land use types have had a significant impact on the physical and chemical properties of wetlands in the Sanjiang Plain (*Table 1*). Our findings indicated that the soil pH of the restored wetland and paddy field was significantly higher than that of the natural wetland ($P<0.05$). The soil pH of the restored wetland was the highest (6.81), whereas that of the natural wetland was the lowest (5.57).

Table 1. *Comparison of soil physicochemical properties in different land use types of the Sanjiang wetland*

Land use types	pH	MC (%)	SOC (g/kg)	TN (g/kg)	TP (mg/kg)
NW	5.57±0.05c	40.20±3.15b	45.83±8.12a	6.20±0.51a	31.56±3.20a
RW	6.81±0.04a	25.35±2.46c	10.45±2.31c	1.51±0.12c	18.42±2.82c
PF	5.82±0.03b	52.45±3.20a	25.38±5.64b	3.25±0.20b	24.68±1.65b

Note: natural wetland (NW); restoration wetland (RW); and paddy field (PF). MC: moisture content; SOC: soil organic carbon; TN: total nitrogen; TP: total phosphors

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The soil water content of the paddy fields was the highest (52.45%), followed by natural wetlands (40.20%), and finally the restored wetlands (25.35%). From a soil nutrient content perspective, the natural wetlands had the highest soil organic carbon, total nitrogen, and total phosphorus content, followed by paddy fields, and finally the restored wetlands. Among these different soil conditions, organic carbon ranged from 10.45 to 45.83 g/kg, total nitrogen ranged from 1.51 to 6.20 g/kg, and total phosphorus ranged from 18.42 to 31.56 g/kg (*Table 1*).

DNA sequencing analysis of soil fungi in each plot

A total of 537,138 pruning sequences were detected in nine soil samples, with an average sequence number of 50,214. The number of soil fungi OTUs in all plots was 1,349. The number of soil fungi OTUs in each type of plot exhibited the following descending order: PF > NW > RW (*Figure 1*). Among them, the number of soil fungi OTUs unique to natural wetlands was 326, accounting for 24.1% of the total OTU number. The number of unique soil fungi OTUs in the restored wetland was the lowest, accounting for only 16.4% of the total OTU number. In contrast, paddy soil exhibited the highest number of unique soil fungus OTUs, accounting for 44.8% of the total OTU data. The number of OTUs shared by all types of plots was 36, accounting for approximately 2.6% of the total OTUs.

Figure 1. *Comparison of the number of OTUs in different land use types. Note: natural wetland (NW); restoration wetland (RW); and paddy field (PF)*

Comparison of soil fungal communities in different types of plots and analysis of indicator species

Taxonomic analysis of OTU at the phylum level (*Figure 2*) indicated that the soil fungi of all soil samples belonged to 16 known fungi phyla. Among them, Basidiomycota, Ascomycota, Mortierellomycota, and Glomeromycota were the main fungal phyla (relative abundance > 1%).

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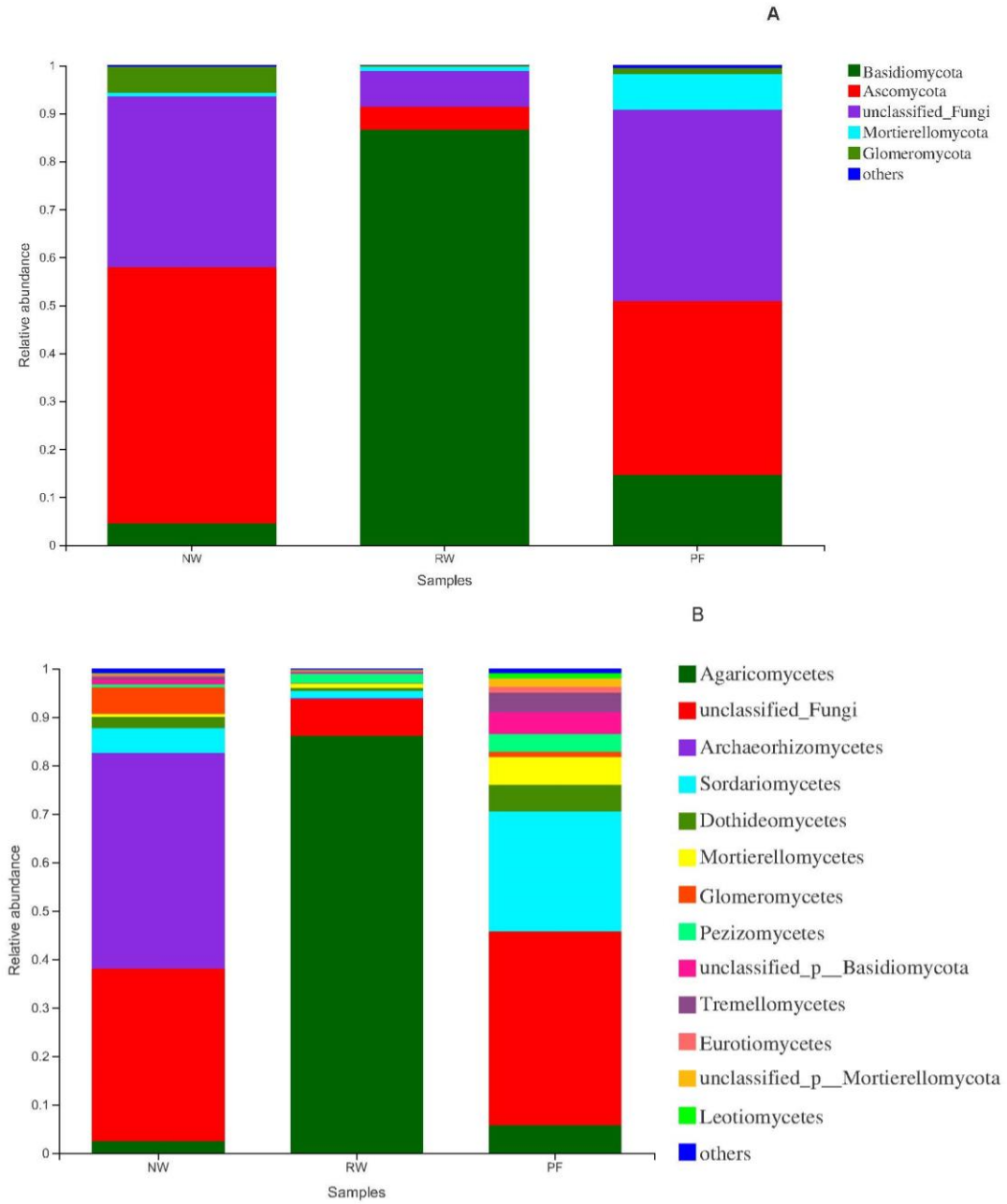

Figure 2. *Composition of the horizontal community structure of soil fungus phyla and classes under different land use types. Note: natural wetland (NW); restoration wetland (RW); and paddy field (PF)*

The results of the principal coordinate analysis of the beta diversity distance matrix (*Figure 3*) indicated that the differences in soil fungi under different land use conditions were mainly attributed to the different land use methods, whereas the internal differences were not significant. The differences between groups determined using PERMANOVA (*Figure 2*) indicated that there were significant differences in the soil fungal community structure in natural wetlands, restored wetlands, and paddy fields (P <0.05).

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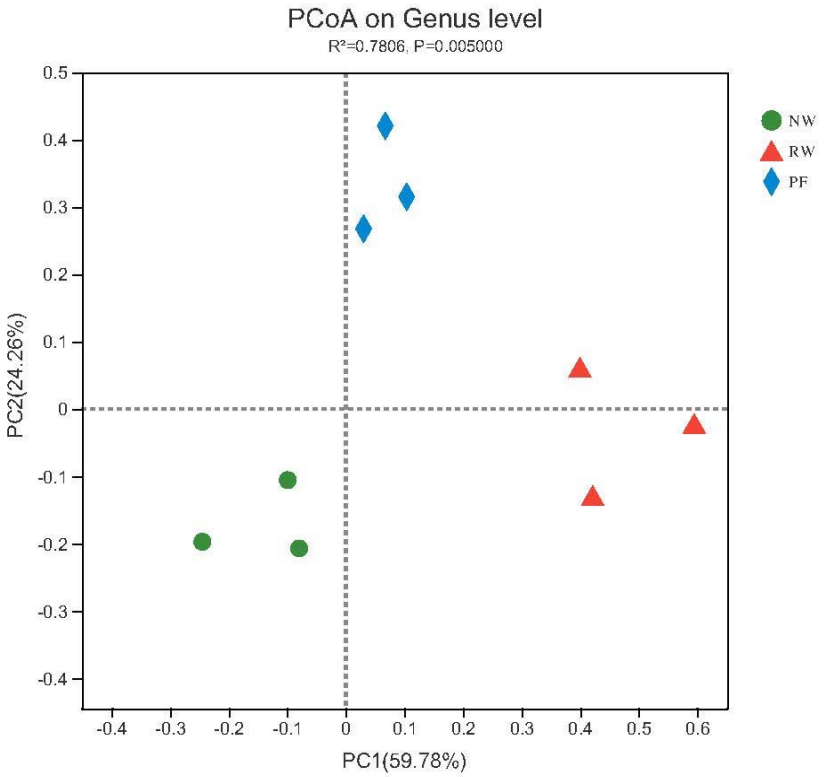

Figure 3. *Principal coordinates analysis (PCoA) of soil fungal communities in different land use types. Note: natural wetland (NW); restoration wetland (RW); and paddy field (PF)*

The analysis of the different characteristics of soil fungi of different land use types at each classification level (*Figure 4*) indicated that the dominant soil fungus groups in natural wetlands were Lentitheciaceae, Epicoccum, and Archaeorhizomycetes; the dominant soil fungus groups in rice fields were Ascobolaceae, Pseudombrophila, Dothideomycetes, Serophoma, Ophiosphaerella, Ascobolus, Trichophaeopsis, Mycosphaerellaceae, Setophoma, and Didymella; the dominant soil fungus groups in the restored wetland were Agaricales, Cortinariaceae, Cortinarius, Hebeloma, Inocybaceae, and Inocybe.

Analysis of the relationship between soil physical and chemical properties and soil fungal diversity

By comparing the α diversity of fungal communities in soil samples from different land use types (*Table 2*), the Ace index, Chao1 index, Shannon-Wiener index, and Sobs index of natural wetlands, restored wetlands, and paddy fields were significantly different ($P< 0.05$). Among them, the natural wetland soil fungus Chao1 index was the highest (405.2), whereas the paddy soil fungus Chao1 index was the lowest (165.2); the natural wetland soil fungus Ace index was the highest (425.2) and the paddy soil fungus Chao1 index was the lowest (170.3); the natural wetland soil fungus Shannon-Wiener index was the highest (4.15) and the paddy soil fungus Shannon-Wiener index was the lowest (2.29); the natural wetland soil fungus Sobs index was the highest (359.7), whereas the paddy soil fungus Sobs index was the lowest (151.3).

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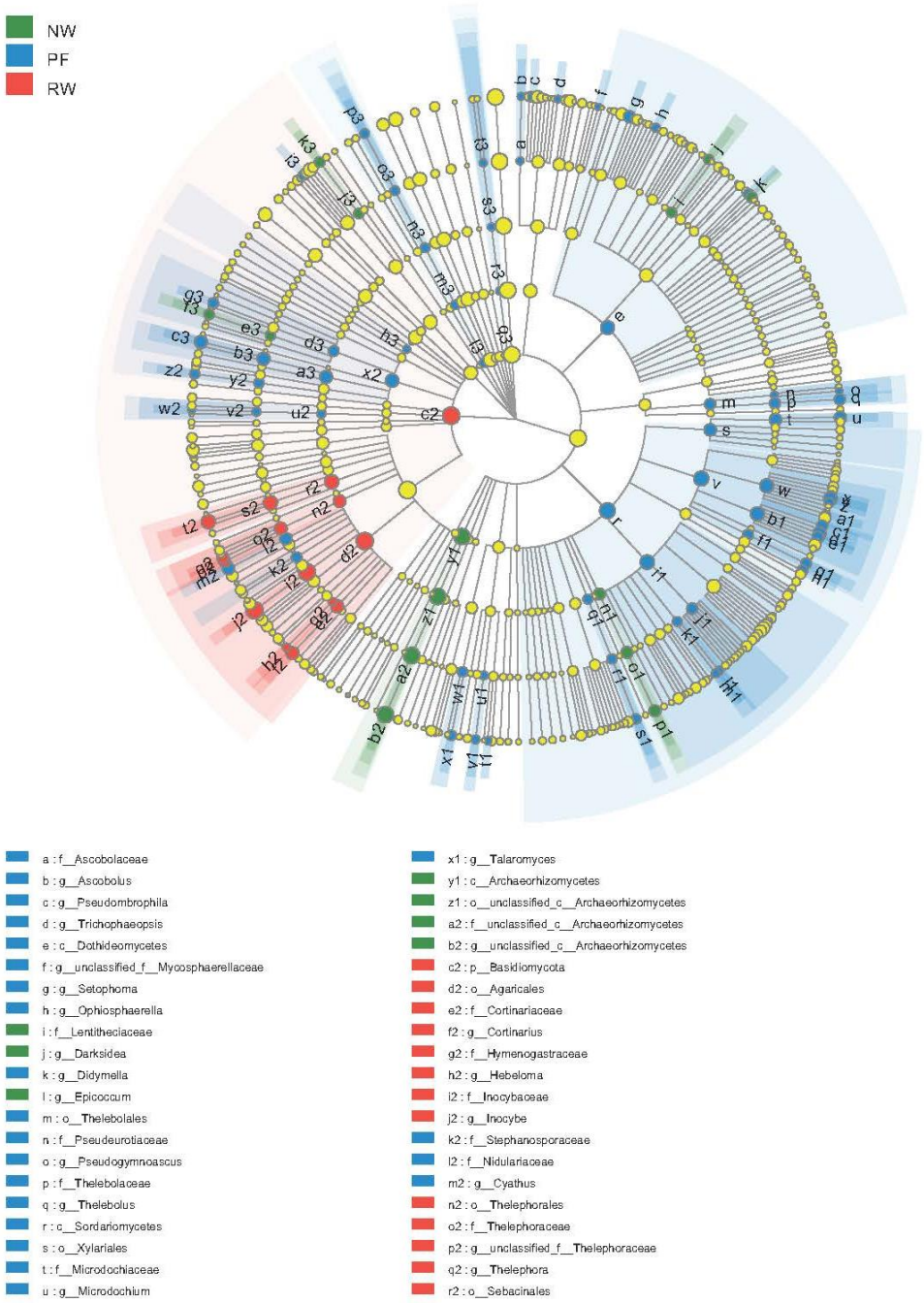

Figure 4. *Cladogram of soil fungal communities for the different land use types (LAD score = 2.5). Note: natural wetland (NW); restoration wetland (RW); and paddy field (PF)*

Table 2. *Comparison of α diversity index of soil fungi in different land use types*

Land use types	Chao1	Ace	Shannon-Wiener	Sobs
NW	405.2±102.79a	425.2±51.72a	4.15±0.44a	359.7±74.01a
RW	221.1±27.70b	220.9±27.26b	3.45±0.76a	202.7±23.02b
PF	165.2±55.79b	170.3±55.27b	2.29±0.40b	151.3±52.54b

Mean±SD (n=3); different lowercase letters indicate significant differences between different land use types (P<0.05). Note: natural wetland (NW); restoration wetland (RW); and paddy field (PF)

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The analysis of the correlation between soil physical and chemical properties and soil fungus α diversity (*Table 3*) indicated that the Ace, Chao1, and Sobs indices were significantly and negatively correlated with soil carbon to nitrogen ratio (P<0.05). Further, there was a significant negative correlation between the Shannon-Wiener index and soil pH (P<0.05), as well as a significant positive correlation between organic carbon, total nitrogen, and the Shannon-Wiener index (P<0.01).

Table 3. *Correlation coefficients between the soil physicochemical properties and soil fungal α-diversity indexes*

α-diversity	pH	SOC	TN	C/N
Sobs	-0.372	-0.009	0.281	-0.828**
Ace	-0.337	-0.037	0.247	-0.819**
Chao1	-0.352	-0.022	0.262	-0.815**
Shannon-Wiener	-0.650*	0.849**	0.884**	-0.587

*P <0.05; **P <0.01. Note: natural wetland (NW); restoration wetland (RW); and paddy field (PF); MC: moisture content; SOC: soil organic carbon; TN: total nitrogen; TP: total phosphors; C/N: soil organic carbon/total nitrogen

Prediction of soil fungus functions in different land use type plots

Using the FUNGuild fungus prediction tool, soil fungus functions were predicted based on the fungal community and assuming similar environmental resources (Li et al., 2018). As shown in *Figure 5*, the detected soil fungi were classified as symbiotrophs based on the way these microorganisms absorb and utilize environmental resources. Among the three types of soil, 12 pathotroph- and saprotroph-specific functions were identified. Concretely, the restored wetland soils exhibited a higher incidence of arbuscular mycorrhizal, plant pathogen, endophyte-litter, animal pathogen-saprotroph, parasite-plant, ectomycorrhizal, wood saprotroph, and arbuscular mycorrhizal functions, whereas plant pathogen functions were more common in natural wetlands, and wood saprotroph and dung saprotroph had significant advantages in rice fields.

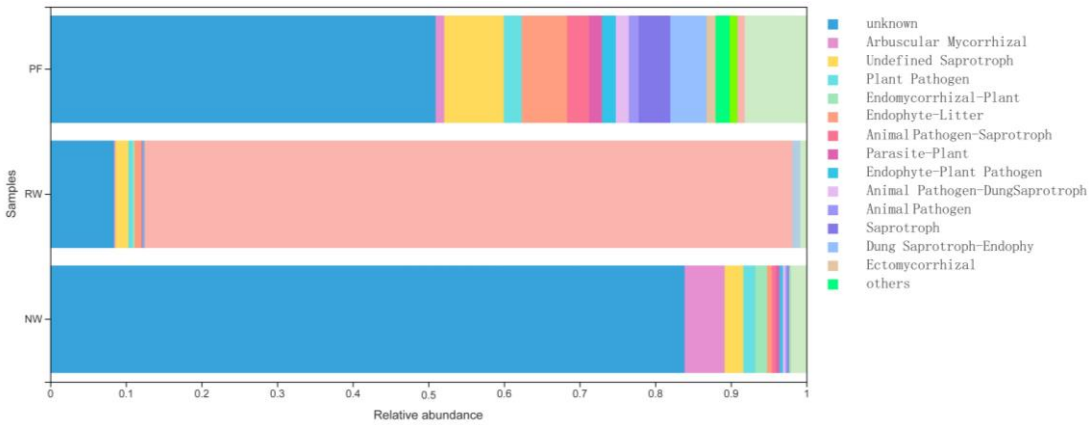

Figure 5. *Variations in the composition of the soil fungal functional communities based on the OTU level in different land use types. Note: natural wetland (NW); restoration wetland (RW); and paddy field (PF)*

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To analyze the key influencing factors that caused the changes in the soil fungus community structure of the Sanjiang Wetland, redundancy analysis (RDA) was carried out on the structure of the soil fungus community and the physical and chemical properties of the soil (*Figure 6*). The results indicated that the cumulative variation of the two axes reached 66.16%, which can explain nearly 70% of the soil fungal community change characteristics and their influencing factors. The results of the replacement test indicated that the soil MC (r^2 = 0.875, P = 0.03), total nitrogen (TN, r^2 = 0.682, P = 0.02), organic carbon (SOC, r^2 = 0.542, P = 0.05), C/N (r^2 = 0.758, P = 0.03), and pH (r^2 = 0.542, P = 0.01) were key environmental factors that shaped the structure of the fungal communities.

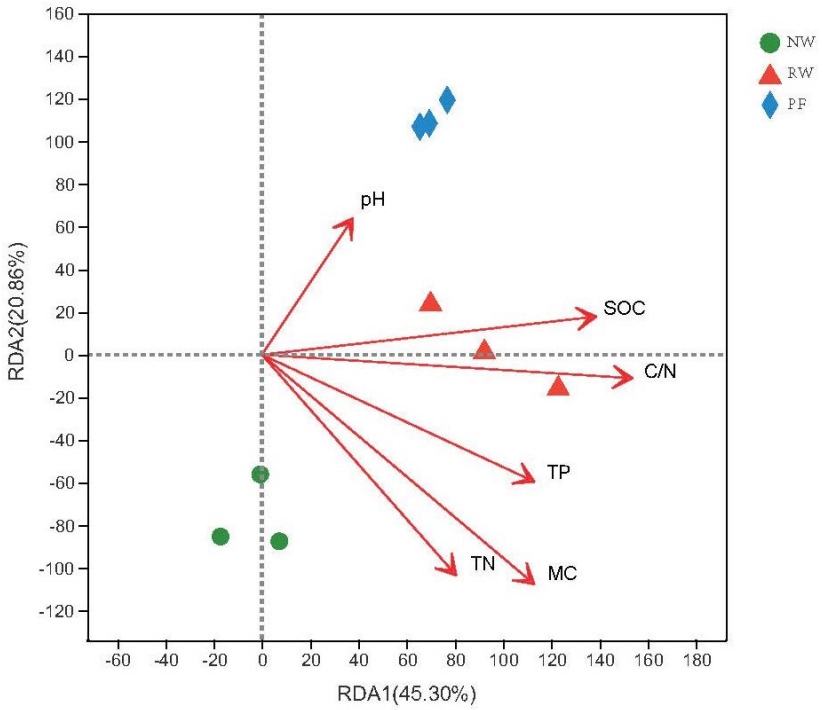

Figure 6. *RDA of the soil fungal community and environmental factors in the Sanjiang plain wetland. Note: natural wetland (NW); restoration wetland (RW); and paddy field (PF)*

Discussion

Comparison of soil fungal communities in wetlands of different utilization types

Environmental filtration, interspecies competition, and random diffusion mechanisms are among the most important factors to maintain the stability of soil fungal community structures (Li et al., 2018). In this study, we found that the soil fungal structure and diversity of natural wetland, restoration wetland and paddy field were significant changes in the Sanjiang Plain, particularly α diversity index of soil fungi decreasing from natural wetland to paddy field (*Table 2*). Our findings are consistent with the literature, indicating that land use types changed soil α diversity (Bossio et al., 2005; Sui et al., 2019;), because the condition of the soil environment in the paddy field was influenced by disturbances such as agricultural tillage, which resulted in a higher soil nutrient content than the wetland soils. Moreover, because of above-ground vegetation change, the abundance of litter inputs, the diversity of soil fungi in natural wetland was

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also higher than that in the paddy field (Sheik et al., 2012). Wetland restoration did not significantly change the soil fungal community, as short-term wetland restoration did not change the composition of the original fungal community in the soil, and therefore the original fungal community remained in the restored wetland soil. Reclamation of wetlands into paddy fields caused significant changes in the soil fungal community structure, presumably because part of the original soil fungal community structure gradually changed under the influence of chemical fertilizers and pesticides, and therefore the relative abundance of the Basidiomycota in the paddy field was often significantly high.

The α diversity index is an important indicator for evaluating the diversity of soil fungus communities. The diversity of soil fungus communities in wetlands can serve as an indicator of ecosystem health (Sui et al., 2016). Our findings demonstrated that the diversity and richness of the soil fungus communities in restored wetlands and paddy fields were significantly lower than those in natural wetlands. As expected, the diversity of soil fungi in undisturbed natural wetlands is higher than that of converted farmlands at different succession stages. This may be because rice crop cultivation practices severely inhibited the diversity of soil fungi, after which the spread of exogenous fungi was insufficient. Wetland restoration greatly increased the Ace and Chao1 indices of the soil fungal community diversity, whereas the Shannon index did not increase significantly. This may be ascribed to the restoration of soil vegetation after a short-term restoration of the wetland. Further, the restoration of above-ground vegetation might have led to habitat improvements, thus promoting bacterial proliferation. The rapid increase in the number was due to the increase in the Chao1 index; however, the Shannon index did not increase significantly due to the heterogeneous and steady restoration of the wetland ecosystem.

Determining the influencing factors that drive soil fungal community diversity is essential to promote the balance and health of wetland ecosystems. In this study, different land use types changed the physical and chemical properties of surface vegetation and soil, as well as the diversity of soil fungal communities. Further, changes in land use affected material circulation and energy flow in the soil ecosystem (Xu et al., 2017). This study also demonstrated that the diversity of soil fungal communities is closely related to changes in soil pH, total nitrogen, organic carbon, and total phosphorus, which is consistent with the factors affecting soil fungal community diversity reported in a previous study (Anderson et al., 2010). The high soil water content in the Sanjiang Plain wetlands is a prerequisite for a high diversity of soil fungi, and soil pH and nutrient conditions are the key limiting factors for the development of healthy soil fungi communities in wetlands. Most soil fungi in the environment prefer a moist and weakly acidic soil environment, and the community diversity increases with lower soil pH. However, when the pH reaches a certain limit, the soil fungus community diversity begins to decrease. Good soil nutrient conditions can provide the material and energy requirements for fungal survival. However, reclamation in natural wetlands can significantly reduce the content of soil total carbon, total nitrogen, organic carbon, and total phosphorus. The reduction of soil nutrient content also hinders the growth of soil fungi. Further, the survival of the community reduces the diversity of soil fungal communities in wetlands. Additionally, the existing form and content of soil nutrients in the natural environment may affect the use of carbon and nitrogen by soil fungi, which also explains why different forms of carbon and nitrogen are the key limiting factors for the diversity of wetland soil fungi (Anderson et al., 2010).

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Function analysis of wetland soil fungi of different utilization types

Higher abundances of soil fungi commonly translate to an increase in functional species, which can play a greater role in maintaining the stability of the structure and function of the ecosystem (Puangsombat et al., 2010). This study demonstrated that natural wetlands exhibited the highest diversity of fungal communities. Functional fungi such as ectomycorrhizal, moss parasitic fungi, lichen parasitic fungi, endophytic fungi, litter saprophytic fungi, and soil saprophytic fungi occupied a large proportion of the overall population, which is consistent with the fungal composition of natural wetlands. Further, the ecosystem exhibited stable structural and functional characteristics. Ectomycorrhizas mostly exist in forests and are aerobic fungi. The relatively high abundance of ectomycorrhizal fungi in natural wetlands may be due to drought conditions in the northern regions of China. The annual flooding duration in the Sanjiang Plain wetlands is very short, which provides several benefits. For example, these conditions promote the growth of oxygen fungi, and the relative abundance of animal and plant pathogenic fungi in natural wetlands is extremely low. This may be due to the elimination of lesser resistant species through natural selection, and therefore the remaining wetland vegetation has high resistance to animal and plant pathogenic fungi. Additionally, a stable interaction is formed between wetland plants and soil microorganisms, which can secrete metabolites and effectively inhibit pathogen invasion (Casper, 2006). The relative abundance of dung rot fungi in the restored wetlands was significantly higher than that of the other soil samples. This may be because wild animals often choose bare beaches as foraging grounds. Constructed wetlands often contain sand as a substrate, which slowly transforms into silt similar to natural wetlands. This results in a habitat type that is different from natural wetlands, thus serving as a large habitat and spawning ground for birds, frogs, and other animals. Fecal rot fungi are introduced by animal activities (Zhu et al., 2009). The relative abundance of animal and plant pathogenic fungi in rice fields was significantly higher than that of other habitats. This may be due to the imbalance of the soil-plant-microbial system interaction caused by human cultivation, fertilization, and pesticide use, as well as the loss of some fungal community components. These processes may introduce pathogenic microorganisms into rice paddy fields, which may be directly related to the occurrence of rice blast disease (Lumini et al., 2011). This study used Funguild microecological tools to classify fungal communities using similar environmental resources (Schmidt et al., 2019). As a result, many repeated classifications were identified, which is one of the shortcomings of this functional classification method.

Conclusions

Changes in wetland land use in the Sanjiang Plain can lead to a significant decrease in soil nutrient content and a gradual increase in soil pH, resulting in a continuous decrease in the diversity of soil fungal communities. As the habitat conditions continue to deteriorate, soil fungal communities tend to become less diverse, which increases the relative abundance of a few dominant fungal communities. The Sanjiang Plain wetland is naturally resilient, and therefore the diversity of fungal species can be quickly recovered. The relative abundance of plant pathogenic fungi in relatively closed farmland habitats was significantly higher than that in natural wetlands and restored wetland habitats, whereas the relative abundances of ectomycorrhizal fungi and parasitic fungi in natural wetlands were significantly higher than those in farmland and

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restored wetlands. The underlying mechanisms related to soil fungal composition and function deterministic factors are multifaceted and provide a fruitful area for further investigation.

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APPENDIX

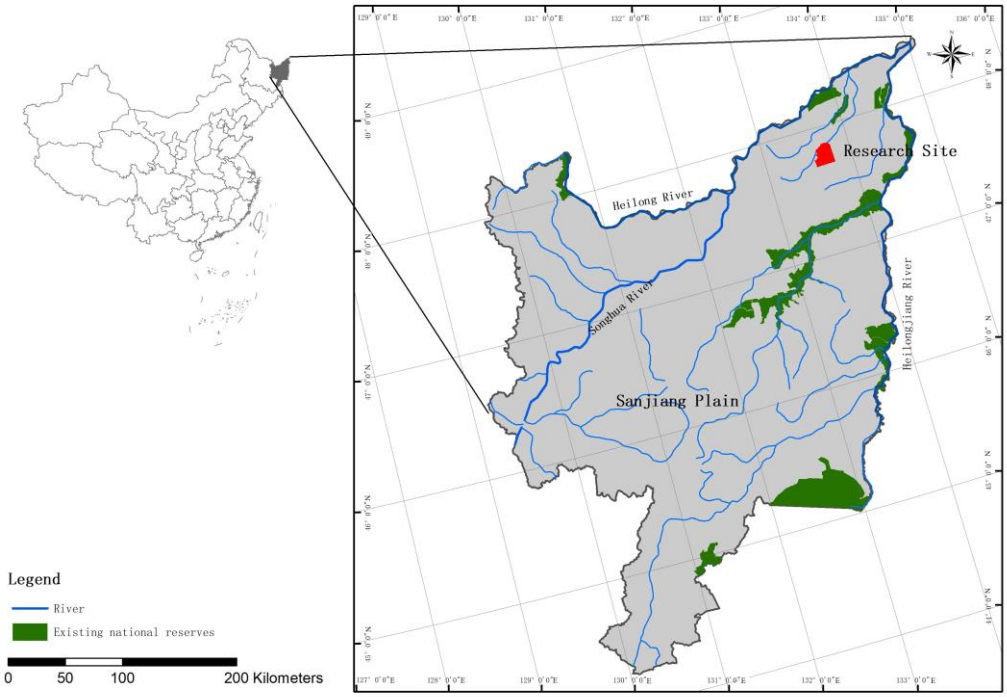

Figure A1. *Location of sampling sites in the Sanjiang Plain*

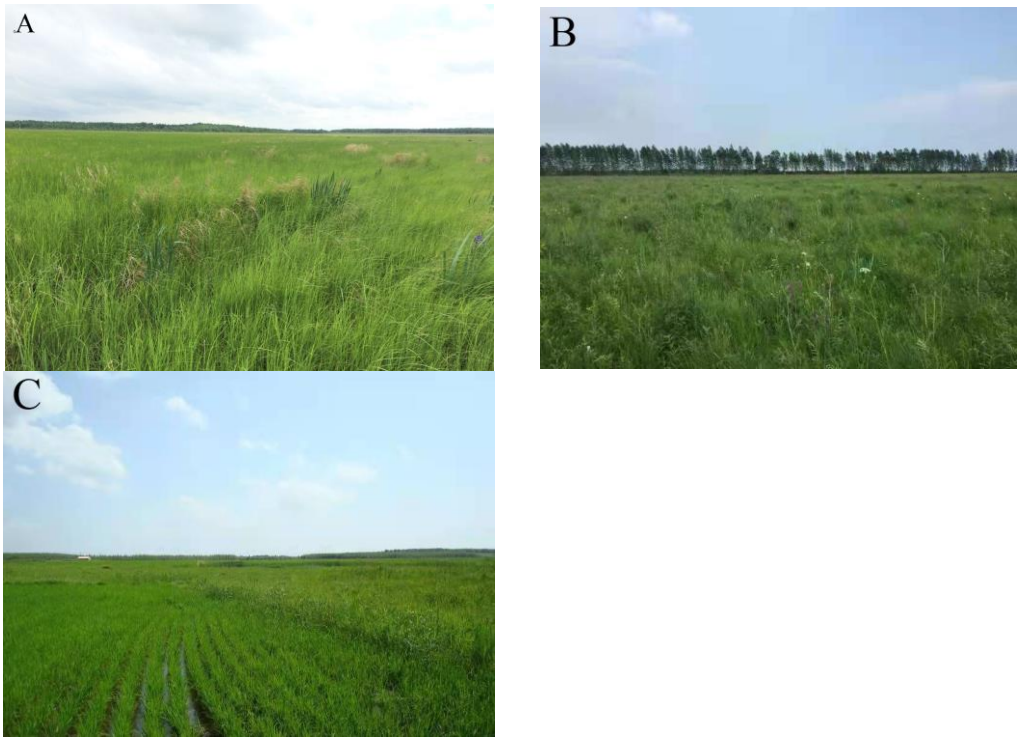

Figure A2. *Habitats of three land use types: natural wetland (A), restoration wetland (B) and paddy field (C) in the Sanjiang Plain*

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BIOREMEDIATION OF HEAVY METALS IN AGRICULTURAL SOILS IRRIGATED WITH TREATED WASTEWATER

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Abstract. The current study investigated the bioremediation abilities of bacteria on non-essential heavy metals in treated wastewater irrigated fields. Two fields, namely, cultivated field and fallowed field, each being 4 ha, were divided into 40 equal grids, for soil sample collection. Samples were analysed for five non-essential heavy metals namely arsenic, aluminium, cadmium, chromium, and lead. The isolated bacteria were identified as *Providencia rettgeri*, *Enterobacter cloacae, Bacillus cereus* and *Arthrobacter aurescens.* The bacteria were cultured and inoculated into heavy metal-contaminated soils and incubated for 12 weeks. Results showed that gram positive bacteria reduced concentrations of non-essential heavy metals separately and combined, especially in fallowed field. Cadmium and lead were significantly reduced by the combination of gram-positive bacteria by 95% and 83% respectively. Among the selected non-essential heavy metals chromium was the one which was most efficiently bioremediated with a 100% removal by *Providencia rettgeri* in cultivated field. No reduction was observed for cadmium by *Arthrobacter aurescens* in fallowed field. This study proved that bioremediation coupled with fallowing could be considered a solution in ameliorating heavy metal toxicity while naturally improving the quality of the soil.
Keywords: *water re-use, heavy metals toxicity, soil regeneration, soil health*

Introduction

Industries are rapidly expanding and improving and while that happens, great amounts of toxic wastes such as heavy metals get released and spread into the environment and water sources (USDA, 2016). These heavy metals are then transferred into wastewater through runoffs, whereby the wastewater is collected, purified and used for irrigation in agricultural fields. The treated wastewater containing large amounts of heavy metals results in soil heavy metal pollution (Hussain et al., 2019). There are several techniques that have been used to remove these heavy metals from water and soil and which includes chemical precipitation, oxidation or reduction, filtration, ion-exchange, reverse osmosis, membrane technology, evaporation and electrochemical inoculum (Selvi et al., 2019). Most of these techniques become unsuccessful when the concentrations of heavy metals are less than 100 mg/l (Masindi and Muedi, 2018). These cleaning methods are expensive, time consuming and still release some other toxic wastes after removal of heavy metals (Kanamarlapudi et al., 2018). Most heavy metal salts are water-soluble and get dissolved in wastewater, which means they cannot be separated by physical separation methods (Khulbe and Matsuura, 2018).

The use of microorganisms such as bacteria and fungi for remediation purposes is thus a more sustainable solution for heavy metal pollution since it includes maintainable remediation technologies to rectify and restore the natural condition of soil without giving off any more toxic substances (Dixit et al., 2015). The use of microorganisms

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will not only remove the toxic heavy metals in the soil but will lead to improved soil health; for example, more bacterial species in the soil will encourage relations with other organisms such as fungi. More fungi in the soil will help build stable aggregates with their hyphae or with the use of glomalin which is a carbon rich compound that they release (Wu et al., 2014). The build-up of carbons and presence of more stable aggregates in the soil will improve soil structure and improve water holding capacity of the soil.

Bioremediation refers to the use of microorganisms such as bacteria and fungi to detoxify heavy metals by either absorbing them or converting them into carbon oxide, energy or methane (Garima and Singh, 2014). Bioremediation relies on microbes that live naturally in soil and groundwater, and these microbes pose no threat to people at the site or in the community (EPA, 2016). This is further assured using indigenous microbes, which guarantees that the organisms can tolerate and exist in that particular ecosystem. Microbes that participate in bioremediation are referred to as bioremediators, and common examples are bacteria (*Bacillus subtilis, Pseudomonus putida, Enterobacter cloacae*) and fungi (*Penicillium, Aspergillus, Rhizopus);* they are potential microbial agents for the removal of heavy metals from aqueous solutions or in contaminated soils (Bahafi et al., 2013). Studies further reported that fungi were more tolerant to heavy metals as a group than bacteria (Igiri et al., 2018) However, diversity of bioremediators in agricultural soils remain necessary. Therefore, the objective of this study was to investigate the bioremediation abilities of indigenous gram-positive and gram-negative bacteria on non-essential heavy metals in treated wastewater irrigated agricultural fields. The research aimed at identifying specific microbes that were indigenous to the soils cultivated and fallowed soils contaminated with heavy metals following irrigation with treated wastewater, then used as inoculants for bioremediation.

Materials and methods

Description of the study site

The experiment was carried out in the Soil Science laboratory of University of Limpopo (28° 0’ 59.76” E; 25° 36’ 54” S), South Africa. Soil samples for the experiment were collected from a cultivated field (CF) in its third of onion cultivation and irrigated with treated water; and fallowed field (FF) which has been fallowed for 5 years following 3 years of cultivation and irrigation with treated wastewater. The 2 fields were adjacent to each other at University of Limpopo Experimental Farm (UL Farm) (-23°50’42.86” E; 29°42’44” S) (*Fig. 1*).

Research design

The experiment was a 2 × 7 factorial study in completely randomised design. The first factor was the 2 fields which were CF and FF and the second factor was the microorganism inoculants. The second factor was made up of a control with no inoculant (this sample was sterilised of microorganisms) (0I); two Gram-negative microorganisms (*Providencia rettgeri* (A) and *Enterobacter cloacae* (B)); the combination of the 2 Gram-negative microorganisms (AB); 2 Gram-positive microorganisms (*Bacillus cereus* (C) and *Arthrobacter aurescens* (D)) and the combination of two Gram-positive microorganisms (CD). Samples were collected at a depth of 0-20 cm and mixed to get a composite sample.

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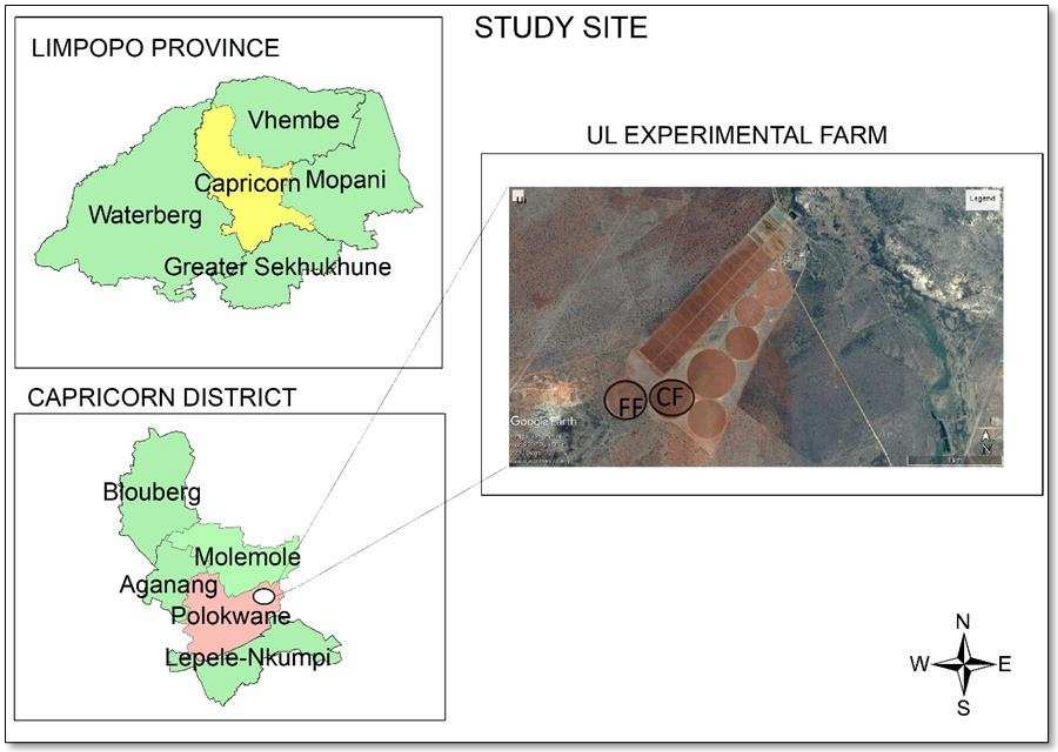

Figure 1. *Study area*

Data collection

Soil pH and heavy metal analysis

Soil pH (H_2O) and pH (KCl) were analysed using an electrode soil pH meter Mettler Toledo before commencing with the experiment (Reeuwijk, 2002). Non-essential heavy metals (As, Al, Cd, Cr and Pb) were extracted from the soil samples through the use of the nitric acid digestion method (Lu et al., 2017), whereby 1 g of soil was mixed together with 5 ml of 65% concentrated nitric acid in a centrifuge tube, heated in water bath at 95 °C temperature, cooled and then filtered and analysed through an ICP-OES Spectrophotometer machine from Ultima Expert (Habte et al., 2016).

Identification of isolated pure cultures microorganisms

Microorganisms were identified using a Maldi Biotyper through formic acid extraction method (Singhal, 2015), at the Biotechnology Unit, University of Limpopo. 14 Eppendorf tubes were sterilised and labelled according to the inoculums. 300 µl of deionized water was pipetted and transferred into each of the Eppendorf tubes. A quantity of pure culture biological material grown on agar plates (*Fig. 2*) (between one colony and 5-10 mg) was transferred into the tubes in accordance with the labels and the respective samples and mixed thoroughly by vortexing. 900 µl of alcohol was added into the tubes and mixed thoroughly. The samples were then centrifuged at maximum speed (15 000 rpm) for 2 min. The supernatant was decanted, and the samples were centrifuged again until the remaining alcohol was removed without disturbing the pellet. The alcohol-pellets were allowed to dry at room temperature for 2-3 min. 10 ml of 70% formic acid was then added to the pellets and then mixed thoroughly by vortexing.

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10 ml of acetonitrile was then added to the samples, and the samples were mixed thoroughly by vortexing. Samples were then centrifuged at 15 000 rpm for 2 min. 1 µl of the supernatant was transferred onto a Maldi target plate and allowed to dry at room temperature. The samples on the Maldi targets were then overlayed with 1 µl of α-Cyano-4-hydroxycinnamic acid (HCCA) solution within 1 h and allowed to dry at room temperature before being placed into the Maldi-tof for identification (Singhal, 2015).

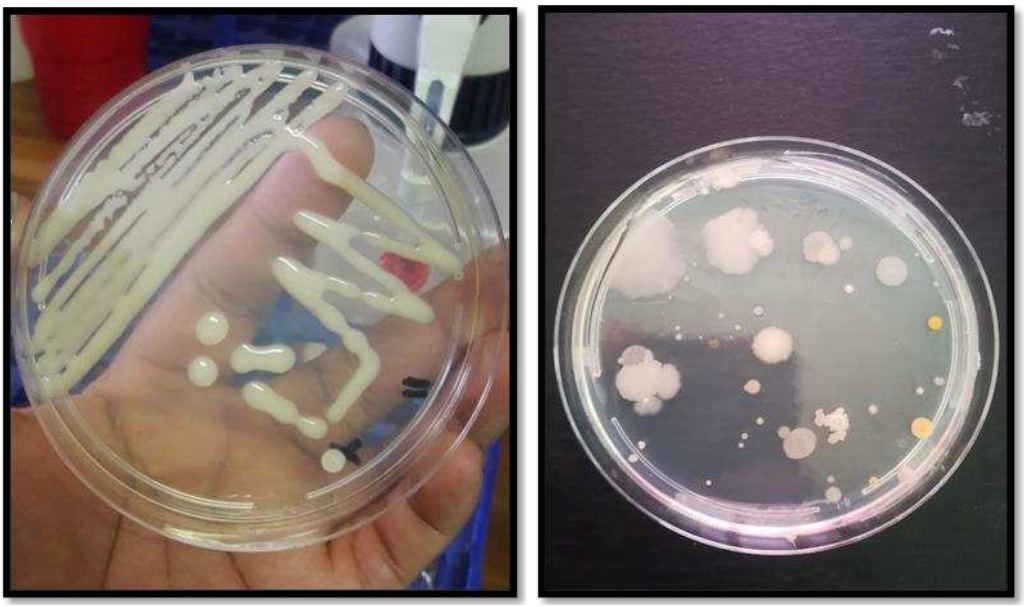

Figure 2. *Bacteria isolates sample used for bioremediation*

Morphological, microscopical and gram staining characteristics of the microorganisms used for the process of bioremediation

The morphological, microscopical and gram staining characteristics of the microorganisms used for the process of bioremediation are presented in *Table 1*. The first bacterium was identified as *P. rettgeri* which developed as a cream colony with medium rods, and it tested negative on gram stain. The second Gram-negative bacterium was identified as *E. cloacae* which grew as cream white colony with medium sized rods. The 2 Gram-positive bacteria were *B. cereus* which grew as small rods, light brown colony and the other one was *A. aurescens* grew as pink colony with big rounds (*Table 1*).

Microbial culturing of the identified microorganisms

Prior to inoculation, 4 microorganisms were cultured (Kim et al., 2021). Briefly, 4 Erlenmeyer flasks (500 ml) were sterilized and labelled as per organism. Cultures of the microorganisms were transferred into the flasks filled with 250 ml nutrient broth. The flasks were incubated on a shaker at 150 rpm at 25^0C for 72 h. After 72 h the samples were transferred into sterile centrifuge tubes and centrifuged at maximum speed in order to obtain single pellets. The supernatant was then discarded without disturbing the pellets. In reference to a paper by Kim et al. (2021), 5 ml of deionized water was added into the centrifuge tubes and mixed thoroughly. The samples were then ready to be used as inoculants (*Fig. 3a*) (Kim et al., 2021).

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Table 1. *Identified microorganisms that were used for bioremediation non-essential heavy metals on cultivated and fallowed fields*

Sample names	Morphological characteristics	Shapes under microscope	Gram staining results
Providencia rettgeri	Cream colony	Medium rods	Negative
Enterobacter cloacae	Cream-white colony	Medium rods	Negative
Bacillus cereus	Light-brown colony	Small rods	Positive
Arthrobacter aurescens	Pink colony	Big rounds	Positive

a **b**

Figure 3. *Inoculated samples (a) ready for incubation, and (b) incubated at 37 ^{0}C*

Bioremediation process

Composite samples were made from all the samples collected from 0-20 cm with respect to each field (CF and FF). Eight 50 g of soil were weighed from composite samples of CF and FF and transferred into 16 (100 ml) sterile glass beakers. Each of the glass beakers were replicated 3 times and labelled according to the inoculums of the microorganisms. Each glass beaker was inoculated with the prepared samples in the centrifuge tubes except for the control. The samples were incubated at 37 °C for 12 weeks as it was tested and proven to be a duration that will yield the most effective results (Fawole, 2017) and to maintain adequate moisture, the samples were irrigated two times a week with 20 ml of distilled water. After 12 weeks the soils were analysed again for bioavailable heavy metals (*Fig. 3b*) (Fawole, 2017). The incubators were well aerated and monitored for excess moisture.

Data analysis

All data were subjected to analysis of variance (ANOVA) through Statistix 10.0 version. Mean separation was done for significant means using Tukey's multiple range test at $P \leq 0.05$. Heavy metal reductions and additions are presented as relative impact (RI%), which was computed as follows:

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$$R.I(\%) = [(Inoculum\ treatment/Before\ Inoculum) - 1] \times 100. \quad \text{(Eq.1)}$$

A negative RI% indicates a reduction in non-essential heavy metals, while a positive RI% indicates addition. Before inoculum samples were used as a reference point.

Results

Description summary of soil pH for cultivated and fallowed field

Soil pH (H_20) and pH (KCl) values of CF ranged between minimum of 4.27 and 4.10 with maximum values of 8.94 and 6.605, respectively. Soil pH (H_2O) and pH (KCl) had average values of 7.67 ± 0.6381 and 5.54 ± 0.43 (*Table 2*).

Table 2. *Descriptive summary of soil pH for cultivated (CP) and fallowed (FF) fields*

Soil properties	Cultivated field (CF)				Fallowed field (FF)			
	Min	Max	Mean	St Dev	Min	Max	Mean	St Dev
pH (H_20)	4.27	8.94	7.67	0.63	5.58	7.58	5.35	0.46
pH (KCl)	4.10	6.60	5.54	0.49	4.55	6.58	5.35	0.44

Non-essential heavy metal distribution following bioremediation

The field × inoculation effects were significant on Cd, Cr and Pb but were not significant on Al and As. Factor A (field) was not significant for all the selected non-essential heavy metals. Factor B (Inoculum) was highly significant on Al, As, Cd, Cr and Pb. Since the field × inoculation and Factor A (field) effects were not significant on Al and As, RI% was only computed to report on effects of Factor B (Inoculum) for the two metals.

Distribution of cadmium

Relative to the reference sample in CF, OI reduced Cd by 67% whereas, AB, CD, A, B, C, and D reduced Cd concentration by 77%, 95%, 73%, 80%, 58% and 90%, respectively. Relative to the reference sample in FF, OI reduced Cd by 54% whereas, AB, CD, B, C, and D reduced Cd concentration by 3%, 26%, 60%, 14% and 0%, respectively. A increased Cd concentration by 8%, respectively (*Fig. 4*).

Distribution of chromium

Relative to the reference sample in CF, OI reduced Cr by 89% whereas, AB, CD, A, B, C, and D reduced Cr concentration by 84%, 93%, 100%, 76%, 98% and 63%. Relative to the reference sample in FF, OI reduced Cr by 64%, whereas AB, CD, B, C, *D* and A reduced Cd concentration by 64%, 98%, 46%, 65%, 73% and 83%, respectively (*Fig. 5*).

Distribution of lead

Relative to the reference sample in CF, OI reduced Pb by 80% whereas, AB, CD, A, B, C, and D reduced Pb concentration by 77%, 80%, 80%, 83%, 83% and 82%, respectively. Relative to the reference sample in FF, OI reduced Cr by 79% whereas, AB, CD, B, C, D and A reduced Pb concentration by 79%, 80%, 83%, 83%, 83% and 82%, respectively (*Fig. 6*).

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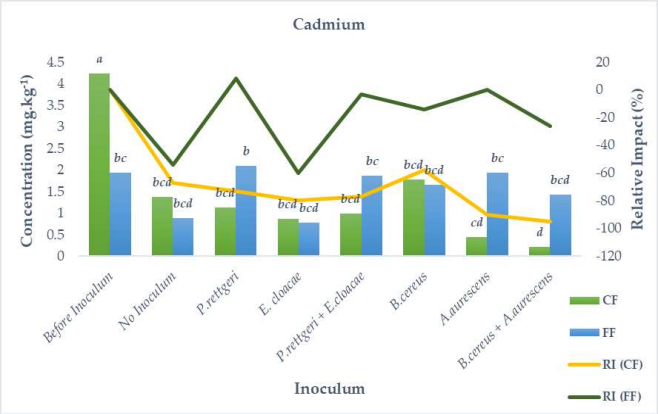

Figure 4. *Response of cadmium (Cd) in cultivated field (CF) and fallowed field (FF) following bioremediation with selected bacteria. (Different letters means statistical difference in the mean values according to the Turkey's multiple range test at the probability level $p < 0.05$)*

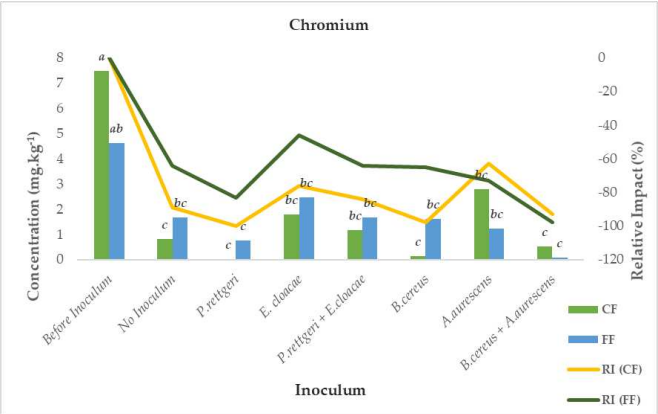

Figure 5. *Response of chromium (Cr) in cultivated field (CF) and fallowed field (FF) following bioremediation with selected bacteria. (Different letters means statistical difference in the mean values according to the Turkey's multiple range test at the probability level $p < 0.05$)*

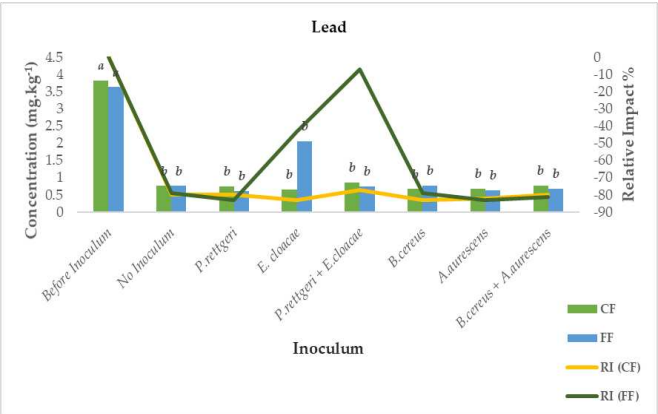

Figure 6. *Response of lead (Pb) in cultivated field (CF) and fallowed field following bioremediation with selected bacteria. (Different letters means statistical difference in the mean values according to the Turkey's multiple range test at the probability level $p < 0.05$)*

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Distribution of aluminium

Relative to the reference sample, OI reduced Al by 61% whereas, AB, CD, A, B, C, and D reduced Al concentration by 46%, 62%, 63%, 60%, 61% and 59%, respectively (*Fig. 7*).

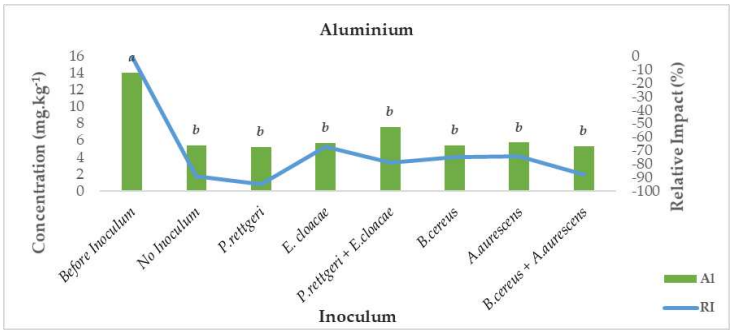

Figure 7. *Response of aluminium (Al) following bioremediation with selected bacteria. (Different letters means statistical difference in the mean values according to the Turkey's multiple range test at the probability level $p < 0.05$)*

Distribution of arsenic

Relative to the reference sample, OI reduced As by 89% whereas, AB, CD, A, B, C, and D reduced As concentration by %, 80%, 95%, 67%, 75% and 74%, respectively (*Fig. 8*).

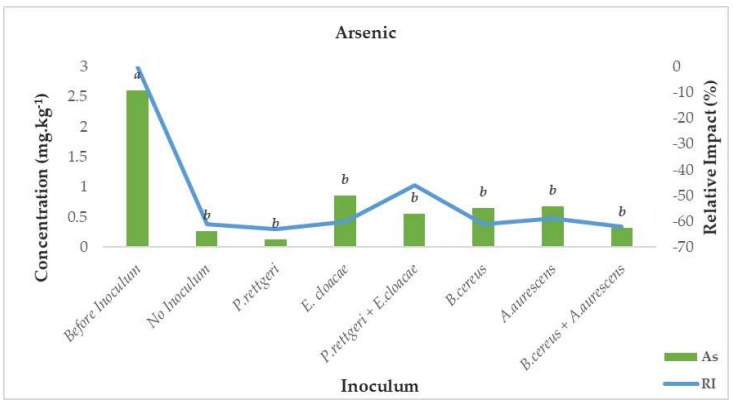

Figure 8. *Response of arsenic (As) following bioremediation with selected bacteria. (Different letters means statistical difference in the mean values according to the Turkey's multiple range test at the probability level $p < 0.05$)*

Discussion

Soil pH is a very crucial chemical indicator of organism habitat, which to an extent determines soil microbial diversities. Different organisms grow differently in varying pH ranges, with reports indicating that most bacteria prefer neutral pH, as acidity may impact microbial growth and functions (Sullivan et al., 2017). Soil pH means for the current study was found to be neutral in both CF and slightly acidic in FF, respectively. This observation could have influenced the performance of the isolated bacteria,

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however, this study was designed to show potential of indigenous bacteria as bioremediators, where there was contamination, which promotes resilience even when other conditions are not conducive.

Inoculum A (Providencia rettgeri)

Providencia rettgeri is a Gram-negative bacterium that is commonly found in both water and land environments (Triverdi et al., 2015). A strain of *P. rettgeri* was isolated from wastewater and solid water compost in Tunisia, and it showed tolerance to chromium, copper and other heavy metals (Das and Osborne, 2018). Likewise, it could be obtained in polluted effluents (Foti et al., 2009). Since it is an ubiquitous microorganism, it could have been in the soil naturally, brought by run off or even brought by the treated wastewater during irrigation at the study site. The trend of heavy metals was Cr > As > Pb > Cd > Al at CF and As > Pb > Cr > Al > Cd in order of the most reduced heavy metals to the least reduced by *P. rettgeri.* It was able to reduce 83% of Cd at its highest bioremediation compared to the other microorganisms. *Providencia rettgeri* could grow and reduce chromate to 100% at a concentration ranging from 100–300 mg/l and 99.31% at a concentration of 400 mg/l, pH 7 and temperature 37 °C (Thacker et al., 2006). The latter finding was better than the current research results. This could be due to the fact that the pH at both CF and FF was not kept constant at 7 like that of the latter report (Thacker et al., 2006). A different study reported that among other microorganisms, *P. rettgeri* was highly resistant to high concentrations of cadmium, copper and cobalt in polluted activated sludge (Bestawy et al., 2013).

Inoculum B (Enterobacter cloacae)

Enterobacter cloacae is a rod-shaped Gram-negative bacterium that live in mesophilic environments with an optimal temperature of 37 °C (Devin-Regli, 2015). It is aerobic and facultative anaerobic. *Enterobacter cloacae* is a human pathogen that can cause infections but can also act as a bioremediator. Under anaerobic conditions, it is able to convert toxic selenite in water sources that come from fossil fuel combustion to elemental, insoluble and non-toxic selenium (Devin-Regli, 2015). The trend of the bioremediated heavy metals was Pb > Cd > Cr > Al > As in CF and in FF was As > Al > Cd > Cr > Pb in order of the most reduced heavy metal to the least reduced heavy metal. Its lowest reduction in FF was Pb but it was its highest remediated heavy metal in CF. On average it performed best in CF. In a different study, maximum resistance was tested against *E. cloacae* with increasing concentrations of silver (Ag), Pb, and Cd. The maximum biosorption capacities of *E. cloacae* to the heavy metals were reported to be 65% at 200 mg/kg, 54.28% at 100 mg/kg and 74.46% at 300 mg/kg (Banerjee et al., 2009). In a polluted soil bioremediation study conducted in 2015 in India, *E. cloacae* bioaccumulated 95.25% of Pb, followed by 64.17% of Cd then by 36.77% Ni after 72 h of inoculation (Banerjee et al., 2009). The results of this research were more comparable to the latter, and this proves the ability and resilience of the organism.

Inoculum C (Bacillus cereus)

Bacillus cereus is said to be aerobic and facultatively anaerobic. This means that it makes adenosine try-phosphate (ATP) by aerobic respiration if oxygen is present but can switch to fermentation or anaerobic if oxygen is absent (Rohini and Jayalakshmi, 2015). It is also motile and commonly found in soil and food (Olaniran et al., 2013).

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Bacillus cereus is widely reported as a soil bacterium and occurs in the rhizosphere of some plants (Xiao et al., 2017), and some strains of *B. cereus* produce antibiotics able to suppress fungal diseases of the rhizosphere (Thacker et al., 2006). *Bacillus cereus* and *Bacillus thuringiensis* have been reported to increase extraction of Cd and Zn from soil and soil polluted with effluent from metal industry (Chibuike and Obiora, 2014).

A 70% decrease of chromium from the soil by two strains of *B. cereus* was reported (Ghalib et al., 2009). From the results of this study, it was observed that *B. cereus* was able to remediate Cr > Pb > As > Al > Cd in CF and in FF the trend was Pb > As > Cr > Al > Cd in order of the most reduced heavy metal to the least reduced. Based on the results obtained from this research, *B. cereus* was able to reduce 100% of the bioavailable Cr in CF, which was a huge improvement from the previous study. *Bacillus cereus* bioremediates heavy metals by bio-absorbing them from the soil solution, assisted by its cell wall characteristic (Thacker et al., 2006). This is due to affinity of hydroxylated and carboxylic functional group molecules on bacterial surfaces for heavy metals leading to their effective adsorption and precipitation and hence it shows resilience in bioremediating rather the most toxic heavy metals such as Pb (Thacker et al., 2006). Its least performance was on Cd, whereby, it increased it by 8% in FF. One study indicated that *B. cereus* was tolerant to a minimum level of 100 ppm to the metals, Cd and Co (Garima and Singh, 2014; Selvi et al., 2019) and this was in contrast with the current study as it could not bioremediate Cd efficiently.

Inoculum D (Arthrobacter aurescens)

Arthrobacter aurescens are basic soil bacteria that can fix nitrogen in the soil (Mongodin et al., 2006; Zhang et al., 2011) and perform several important functions of removal of toxic chemicals (Singhal et al., 2015; Wu et al., 2014). It has been reported that *A. aurescens* can reduce hexavalent chromium, which can cause severe irritations to humans, and they are also known to degrade agricultural pesticides in the soil (Fu et al., 2014). Hexavalent chromium is 100 times more toxic than trivalent chromium because of its oxidation state, and is also much more soluble in water, allowing it to seep into groundwater very easily (Fu et al., 2014). This research revealed that *A. aurescens* was able to reduce heavy metals in CF at a trend of Cd > Pb > As > Cr > Al in CF and in FF, it was Pb > As > Cr > Al > Cd in order of the most reduced heavy metal to the least reduced. Which is a very interesting finding because it was able to reduce more of Cd in CF but the lowest in FF.

Combination of Gram-positive and Gram-negative bacteria

From the results obtained, the combination of the Gram-positive bacteria (*B. cereus* + *A. aurescens*) always had the highest reduction of heavy metals than the combination of Gram-negative bacteria (*P. rettgeri* + *E. cloacae*). To add, the reduction was always highest at FF than at CF, meaning that bioremediation in this case was highly favourable at conditions of FF than that of CF. Although *B. cereus* + *A. aurescens* generally performed the best in comparison with *P. rettgeri* + *E. cloacae,* it was observed to have the lowest reduction of Cd in FF. Even when the individual microbes were used, they still had a poor performance. This could be because the Gram-positive bacteria used are not resistant to high levels of Cd. The trend of the non-essential heavy metals in CF was Cr > As > Cd > Pb > Al for *P. rettgeri* + *E. cloacae* and Cd > Cr > As > Pb > Al in for *B. cereus* + *A. aurescens* in the order of the most reduced

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heavy metal to the least. In FF the trends were As > Pb > Cr > Al > Cd for *P. rettgeri* + *E. cloacae* and Cr > As > Pb > Al > Cd for *B. cereus* + *A. aurescens* in the order of the most reduced to the least reduced heavy metal.

Both Gram-negative and Gram-positive bacteria have their cell wall charged with a negative charge. This is due to carboxyl, hydroxyl and phosphyl groups, thus in the presence of positive heavy metal cations, these groups are very important in cation sorption (Bahafi et al., 2013). Metals and metalloids get attached to these ligands on cell surfaces, which displace essential metals from their normal binding sites (Ayangbenro and Babalola, 2017). Once the metal and metalloid are bound, microbial cells can transform them from one oxidation state to another, thus reducing their toxicity (Gupta et al., 2015; Lesmana et al., 2009). By so saying, this defines the act of bioremediation observed in the study whereby the concentrations of non-essential heavy metals reduced in the soil.

Conclusion

Gram-positive bacteria performed the best individually and as a combination in bioremediation of the bioavailable non-essential heavy metals. Generally, this was mostly observed at FF than at CF. This means that fallowing of soils helps in bioremediation process, and this could be because the soil conditions are not constantly changed through the irrigation with treated wastewater. All the identified microbes were able to reduce the heavy metal concentration in the soil at different conditions of CF and FF, but worrying observations were seen with low reduction of concentrations of Cd at FF such that bacteria *A* increased it by 8% and D could not even reduce it at all. Another observation was that there wasn't any significant difference between the effectiveness of the inoculums of Pb, Al and As, further investigation needs to be done to see what the cause of this is. For further studies, these microorganisms must be screened for Cd resistance in soils of FF to understand the negative performance observed. More research must also be done on bioremediation of these non-essential heavy metals on both treated wastewater and polluted soils, especially with varying bacteria strains. Furthermore, a follow-up study that will investigate the mechanism with which this remediation occurs must be conducted, as well as a study that will implement the recommended findings in agricultural fields. It can be concluded that there are other soil factors that contributes to the effectiveness of this process and also that must be investigated. In conclusion, bioremediation using bacteria coupled with fallowing has shown to have great potential in the removal of non-essential heavy metals in soils. Therefore, it could be recommended for adoption by farmers who experience heavy metal pollution in their fields and as well as those who rely on treated wastewater for irrigation purposes.

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APPENDIX

Table A1. *Analysis of variance for Cd at cultivated and fallowed fields following bioremediation with seven (7) Gram-negative and positive bacterial species*

Source	DF	SS	MS	F	P
Rep	2	0.41	0.21		
Field	1	0.46	0.46	1.61	0.21
Trt	7	22.31	3.19	11.18	0.00
Field #Trt	7	15.98	2.28	8.01	0.00
Error	30	8.55	0.29		
Total	47	47.71			

Table A2. *Analysis of variance for Cr at cultivated and fallowed fields following bioremediation with seven (7) Gram-negative and positive bacterial species*

Source	DF	SS	MS	F	P
Rep	2	4.86	2.43		
Field	1	0.07	0.07	0.05	0.82
Trt	7	142.60	20.37	14.98	0.00
Field #Trt	7	22.32	3.19	2.34	0.04
Error	30	40.81	1.36		
Total	47	210.65			

Table A3. *Analysis of variance for Al at cultivated and fallowed fields following bioremediation with seven (7) Gram-negative and positive bacterial species*

Source	DF	SS	MS	F	P
Rep	2	10.08	5.04		
Field	1	0.12	0.12	0.02	0.90
Trt	7	385.49	55.07	7.82	0.00
Field #Trt	7	34.88	4.98	0.71	0.67
Error	30	211.17	7.04		
Total	47	641.74			

Table A4. *Analysis of variance for As at cultivated and fallowed fields following bioremediation with seven (7) Gram-negative and positive bacterial species*

Source	DF	SS	MS	F	P
Rep	2	1.89	0.94		
Field	1	1.20	1.20	1.69	0.20
Trt	7	25.75	3.68	5.17	0.00
Field #Trt	7	1.51	0.22	0.30	0.95
Error	30	21.37	0.71		
Total	47	51.71			

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RELATIONSHIP BETWEEN LANDSCAPE PATTERN AND ECOSYSTEM SERVICES VALUE IN THE KARST AREA OF SOUTHWEST CHINA

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Abstract. Ecological problems such as environmental degradation are urgent problems to be solved in karst areas. It is of great theoretical and practical significance to explore the landscape pattern and the relationship between landscape pattern and ecosystem service value (ESV) in karst areas. Based on the remote sensing data of Guiyang City, China, this paper uses landscape index method to evaluate landscape pattern, uses value equivalent factor method to estimate ESV, and uses gray correlation analysis to analyze the relationship between landscape pattern and ESV from patch type level and landscape level. The results showed that: (1) the landscape fragmentation slowed down, the complexity decreased, and the stability increased. (2) The total value of ESV shows an upward-downward-upward trend. Spatially, it showed a law of geographical center as the core, low in the middle and high in the surroundings. (3) ESV value is closely related to landscape aggregation and shape complexity.
Keywords: *grey correlation analysis, rocky desertification, landscape index, remote sensing, Fragstats 4*

Introduction

Ecosystem services and products that humans obtain directly or indirectly from the ecosystem, are closely related to human production and life (Constanza et al., 2014; Kubiszewski et al., 2017). In the 1990s, the evaluation of global ecosystem service value conducted by Costanza et al. (1997) greatly promoted the quantitative study of ecosystem service value. Since then, many scholars have accounted for different regions and different types, and improved the estimation methods (Luo and Yan, 2018; Wang and Li, 2020; Zhu et al., 2020; Zan et al., 2020; Hao et al., 2020; Wang et al., 2020; Xie et al., 2008). At present, the evaluation methods of ecosystem service value have not yet formed a unified framework system (Kubiszewski et al., 2017; Yu and Bi, 2011). Chinese researchers mainly quote the original or revised equivalent based on the value of China's ecosystem services calculated by Xie et al. (2013, 2015b).

Landscape pattern refers to the spatial structure characteristics of landscape, which is one of the core issues of landscape ecology research (Wu, 2007). Changes in landscape pattern will cause changes in the types and areas of ecosystems (He et al., 2018; Lu et al., 2019). At the same time, the flow of various materials, energy and information in the landscape will also affect ecosystem services to a significant extent, and reflect the impact of human activities to a certain degree (Zhu, 2012; Gardner, 1987). The quantification of landscape pattern changes is a substantial reflection of ESV changes (Wang et al., 2017). Some scholars in China have conducted research on the relationship between landscape pattern and ecosystem service value. For example, Wang et al. (2017), Song et al. (2018) and Zhu et al. (2018) analyzed the relationship between landscape pattern and ecosystem service value from a qualitative perspective. Yu et al. (2021), Tong et al. (2020), Zhang et al. (2010), Su and Fu (2012) tried to

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explore the quantitative response relationship between regional landscape pattern and ecosystem service value. Quantitative analysis methods mainly include correlation coefficient method, regression analysis method and grey correlation analysis method. Among them, grey correlation method has more advantages in exploring the correlation degree. The existing results have laid a good foundation for quantitative research on the response relationship between landscape patterns and ecosystem services, but they still only stay at simple analysis showing the quantitative correlation, and do not dig into its ecological significance (Su and Fu, 2012).

The karst area is nearly 2,200 km^2, accounting for about 15% of the global land area, which seriously affects the production and life of about 1 billion people (Wang, 2003). The karst areas have serious ecological degradation due to the high vulnerability and sensitivity of the geological environment and the multiple pressures of population, economy and society (He et al., 1996). Fragile ecological environment, rocky desertification, prominent contradiction between man and land, environmental degradation, and social and economic backwardness are the main problems that need to be solved urgently in karst areas (Cai, 1996). At present, there are relatively few studies on the landscape pattern and the value of ecosystem services in the southwest karst area. The exploration of the landscape pattern in the southwest karst area, especially the rocky desertification landscape pattern, and the relationship with ESV has important theoretical and practical guidance in the karst area. Few studies have taken rocky desertification landscapes as an independent type of landscapes. As a result, there is a lack of theoretical basis for rational planning and configuration of landscapes in karst areas (Tang et al., 2019). Guizhou Province is one of the experimental areas of ecological civilization in China. There exists a high-grade and large-scale rocky desertification landscape, which is very typical in karst areas (Li et al., 2017). Therefore, Guiyang City was selected as a case study. In this paper, the quantitative relationship between landscape pattern and ecosystem services were discussed both at the landscape level and patch type level using remote sensing data from 1998 to 2018 and revised ecological service value coefficients. It aims to reveal the relationship between landscape pattern and ecosystem services value in the karst area of southwest China, and provide a theoretical basis and reference significance for related research and development planning in karst areas in China and even in the world.

Materials

Study area

Guiyang City (106°~108°E, 26°~28°N) is located in southwest China, with a total area of 8043 km^2 (*Fig. 1*). It is the economic, cultural and political center of Guizhou Province, as well as an important eco-tourism city in China. It is also an Ecological Civilization Demonstration Zone, an important transportation hub and the economic growth pole of southwest China. The annual average temperature of Guiyang is 15.3 ℃, the annual average relative humidity is 77%, the annual average total precipitation is 1129.5 mm, the annual average sunshine hours are 1148.3 h, and the annual snowfall days are few, with an average of only 11.3 days. It has a topography dominated by hilly basins and a large area of karst landscape. It has varied soil types such as yellow soil and fluvo-aquic soil, but the soil erosion is relatively serious and the soil conditions are relatively poor. Artificial vegetation dominated by Masson pine accounts for a great proportion of vegetation cover.

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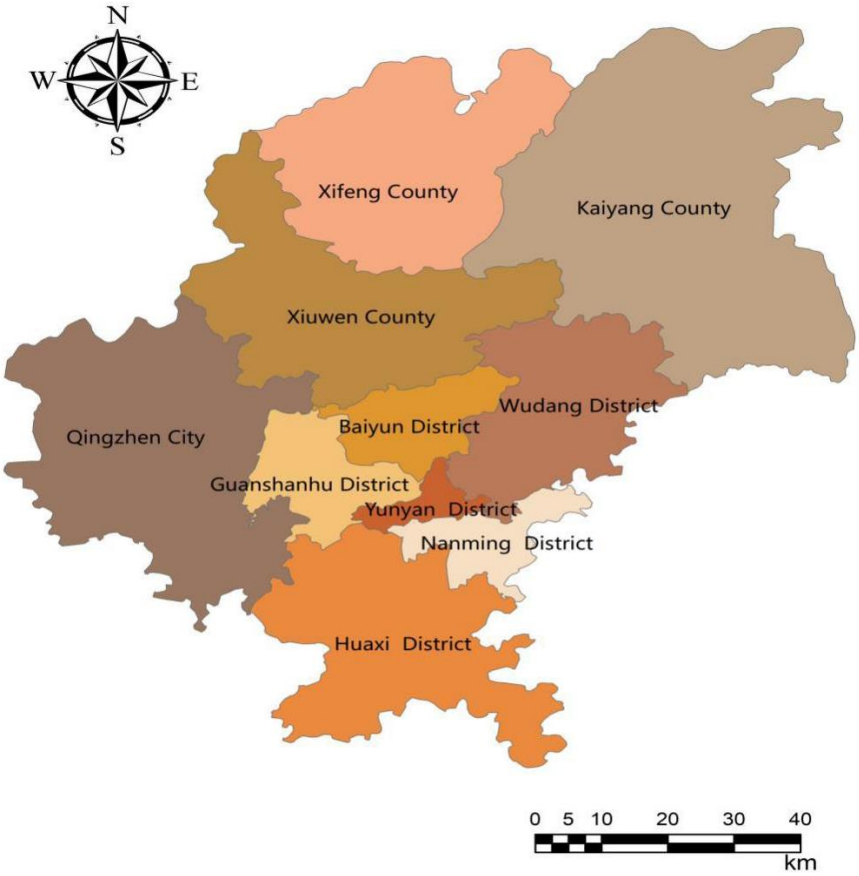

Figure 1. *Administrative map of Guiyang City*

Data sources

This paper selects Landsat 5-tm remote sensing satellite images taken on April 13, 1998, April 11, 2003 and April 8, 2008, and landsat-8 oli remote sensing satellite images taken on September 25, 2013 and March 19, 2018, with a resolution of 30 m. All these remote sensing images come from the geospatial data cloud website (http://www.gscloud.cn). The economic and social data comes from the "Guiyang City Statistical Yearbook" and the "Guiyang City National Economic and Social Development Statistical Bulletin". ENVI5.3 software was used to process the images including map mosaic, geometric correction and atmospheric correction. With reference to "Classification of Land Use Status", combined with the current conditions of Guiyang City, landscape types are divided into forestry landscape, agricultural landscape, construction landscape, rocky desert landscape, and water landscape using Support Vector Machine (SVM) supervision classification method.

Methods

Analysis of landscape pattern

Landscape index method

The prerequisite for studying the relationship between landscape pattern and ecological process is to conduct a quantitative analysis of the landscape pattern and to explore its

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dynamic changes (Wang and Bao, 1999). Landscape index is a general quantitative index to reflect the composition structure and spatial configuration of landscape, which is the concentration of landscape pattern information (Xie et al., 2003). It is often used to study the fragmentation, diversity and other landscape heterogeneity characteristics of the entire landscape or a fixed landscape element in the study area (Chen, 2011). Many landscape indexes express similar landscape meanings and have strong correlation (Bu et al., 2005; He and Zhang, 2009; Zhang et al., 2008). Repeated selection of landscape indexes will lead to more redundancy and less accuracy. Therefore, the indicators with less correlation were selected in this paper (*Table 1*), and the calculation formula and meaning are detailed in the literature (Wu, 2007).

Table 1. *Landscape index*

Index type	Landscape level	Patch level
Patches, density index	Number of Patches (NP) Patch Density (PD) Largest Patch Index (LPI)	Number of Patches (NP) Patch Density (PD) Largest Patch Index (LPI)
Shape Index	Landscape Shape Index (LSI) Mean Fractal Dimension Index (FRAC_MN)	Landscape Shape Index (LSI) Mean Fractal Dimension Index (FRAC_MN)
Contagion Index	Aggregation Index (AI) Interspersion and Juxtaposition Index (IJI) Contagion Index (CONTAG)	Aggregation Index (AI) Interspersion and Juxtaposition Index (IJI)
Diversity Index	Shannon's Diversity Index (SHDI) Shannon's Evenness Index (SHDI)	

Number of patches (NP), reflecting the spatial pattern of the landscape, is often used to describe the heterogeneity of the whole landscape. NP has an impact on many ecological processes, such as determining the spatial distribution characteristics of various species and secondary species in the landscape, and changing the stability of interaction and synergistic symbiosis among species. NP is the total number of patches in the landscape. Value range: NP ≥ 1, no upper limit. NP is equal to the total number of patches of a block type in the landscape at the type level. At the landscape level, it is equal to the total number of patches in the landscape.

$$NP = N \quad \text{(Eq.1)}$$

Patch density (PD) is the number of patches per square kilometer, reflecting the heterogeneity and fragmentation of the landscape as a whole and the fragmentation degree of a certain type, and reflecting the heterogeneity of the landscape per unit area. Value range: PD > 0, no upper limit.

$$PD = N/A \quad \text{Eq.2)}$$

The largest patch index (LPI) is equal to the proportion of the largest patch in a patch type occupying the whole landscape area. It is helpful to determine the model land or advantage type of the landscape. Its value determines the abundance of dominant species and internal species in the landscape. The change of its value can change the intensity and frequency of interference and reflect the direction and strength of human activities.

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$$LPI = \frac{Max(a_1,\ldots,a_n)}{A}(100) \quad \text{(Eq.3)}$$

The landscape shape index (LSI) helps to determine the model land or advantage type of the landscape. Its value determines the ecological characteristics such as the abundance of dominant species and internal species in the landscape. The change of its value can change the intensity and frequency of interference and reflect the direction and strength of human activities. The total length (m) of all patch boundaries in the landscape divided by the square root of the total area (m^2) in the landscape and multiplied by the square correction constant. Value range: LSI ≥ 1, when the patch shape in the landscape is irregular or deviates from the square, the LSI value increases.

$$LSI = \frac{0.25E}{\sqrt{A}} \quad \text{(Eq.4)}$$

Mean fractal dimension index (FRAC_MN), which can be intuitively understood as the non-integer dimension of irregular geometry, mainly describes the complex characteristics of patch shape in the landscape, and can reflect the changes of landscape shape to a certain extent. The shape of patch affects many ecological processes and produces edge effects on natural patches. In the formula, K is the proportional constant, and different values are taken according to the different definition of p_{ij} and the shape of the grid: if expressed by the length of one side of the patch, k = 1, and if expressed by actual perimeter, k = 4 (grid is square), or K = 1.26 (grid is circular), or K = 3.72 (grid is hexagonal).

$$FRAC\_MN = \frac{\sum_{i=1}^{m}\sum_{j=1}^{n}\left(\frac{2\ln(0.25P_{ij})}{\ln(a_{ij})}\right)}{N} \quad \text{(Eq.5)}$$

Aggregation index (AI) investigated the connectivity between patches of each landscape type. The smaller the value, the more discrete the landscape is. Where g_{ii} is the number of similar adjacent patches of corresponding landscape type, AI is calculated based on the common boundary length between pixels of the same type of patch. When there is no common boundary between all pixels in a type, the aggregation degree of this type is the lowest; When the common boundary between all pixels in the type reaches the maximum, it has the maximum aggregation index. Value range: AI∈ (0,100].

$$AI = \left[\frac{g_{ii}}{max \rightarrow g_{ii}}\right](100) \quad \text{(Eq.6)}$$

Interspersion and juxtaposition index (IJI), IJI is one of the most important indicators to describe landscape spatial pattern. IJI reflects the distribution characteristics of ecosystems seriously restricted by certain natural conditions. For example, various ecosystems in mountain area are seriously affected by vertical zonality, and their distribution is mostly circular, and the value of IJI is generally low. Where e_{ij} is the total edge length (m) adjacent to patch type i and k (non-adjacent variable). Value range: 0 < IJI ≤ 100.

$$IJI = \left[\frac{-\sum_{k=1}^{m}\left[\frac{e_{ik}}{\sum_{k=1}^{m} e_{ik}}\ln\left(\frac{e_{ik}}{\sum_{k=1}^{m} e_{ik}}\right)\right]}{\ln(m-1)}\right](100) \quad \text{(Eq.7)}$$

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Contagion index (CONTAG), where Pi *is* area percentage of type i patch;g_{ik} is the number of adjacent patches of type i and type k, m is the total number of patch types in the landscape. If the CONTAG value is large, indicating that the dominant patch types in the landscape form a good link. On the contrary, it shows that the landscape is a spread pattern with multiple factors. CONTAG was negatively correlated with edge density and highly correlated with dominance and diversity index. Value range is 0 < contact ≤ 100.

$$CONTAG = \left[1 + \frac{\sum_{i=1}^{m}\sum_{k=1}^{m}\left[(Pi)\left(\frac{g_{ik}}{\sum_{k=1}^{m} g_{ik}}\right)\right]\left[\ln(Pi)\frac{g_{ik}}{\sum_{k=1}^{m} g_{ik}}\right]}{2\ln(m)}\right] \quad \text{(Eq.8)}$$

Shannon's diversity index (SHDI), a measurement index based on information theory, is widely used in ecology. This index can reflect landscape heterogeneity, especially sensitive to the unbalanced distribution of each block type in the landscape, that is, it emphasizes the contribution of rare block types to information, which is also different from other diversity indexes. The proportion of each patch type in the total landscape area multiplied by its logarithm, and then summed to take a negative value. Value range: SHDI ≥ 0, no upper limit. When there is only one patch type in the landscape, SHDI = 0. When the patch type increases or the area proportion of each type of patch tends to be similar, the value of SHDI increases accordingly.

$$SHDI = -\sum_{i=1}^{m}(P_i \ln(Pi)) \quad \text{(Eq.9)}$$

Shannon's evenness index (SHEI) is equal to Shannon's diversity index divided by the maximum possible diversity under a given landscape abundance (each block type is equally distributed). SHEI = 0 indicates that the landscape is only composed of one kind of block without diversity's = 1 indicates that all block types are evenly distributed and have the greatest diversity. Like SHDI index is also a powerful means for us to compare the diversity changes of different landscapes or the same landscape in different periods. When the value of SHEI is small, the dominance is generally high, which can reflect that the landscape is dominated by one or a few dominant block types. When SHEI approaches 1, the dominance is low, indicating that there is no obvious dominant type in the landscape, and each block type is evenly distributed in the landscape.

$$SHEI = \frac{-\sum_{i=1}^{m}(P_i \ln(Pi))}{\ln(m)} \quad \text{(Eq.10)}$$

Landscape dynamics

The dynamic calculation formula is as below:

$$K = \frac{|U_b - U_a|}{U_a} \times \frac{1}{T} \times 100\% \quad \text{(Eq.11)}$$

K is the dynamics of the landscape in a certain research period, U_a is the initial landscape area of the research period; U_b is the landscape area at the end of the research period, and T is the length of the research period.

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ESV estimation

Coefficient correction

Existing studies indicate that the economic value of an ecosystem value in China was equivalent to 535.16 dollars/hm^2 in 2010 (Xie et al., 2015a, b; Zhang et al., 2017; Xu et al., 2019; Lei et al., 2020). The average grain output per unit area was 4,947 kg/hm^2 in China in 2010, and Guiyang's grain output was 5,301 kg/hm^2 in the same year. Consequently, the ecosystem service value for 1 standard equivalent factor in Guiyang is 570.48 dollars/hm^2 using a correction coefficient of 1.066, and the ecosystem service value coefficient per unit area in Guiyang is determined as in *Table 2*.

Table 2. *Ecosystem service value coefficient of unit area in Guiyang (dollars/hm^2)*

Service function	Cultivated land	Woodland	Grassland	Water area	Construction land	Unused land
Gas regulation	285.21	1996.59	456.37	0.00	0.00	0.00
Climate regulation	507.69	1540.22	513.43	262.39	0.00	0.00
Water conservation	342.26	1825.43	456.37	11625.73	0.00	17.08
Soil conservation	832.87	2224.74	1112.40	5.67	0.00	11.41
Waste disposal	935.57	747.32	747.32	10370.79	0.00	5.67
Biological protection	404.99	1859.66	621.80	1420.43	0.00	193.92
Food production	570.48	57.05	171.16	57.05	0.00	5.67
Raw materials	57.05	1483.16	28.49	5.67	0.00	0.00
Entertainment culture	5.67	730.17	22.82	2475.72	0.00	5.67
Total	3941.80	12464.34	4130.17	26223.47	0.00	239.44

ESV estimation

Based on the ecosystem service value proposed by Costanza et al. (1997), combined with the revised ecosystem service value coefficient per unit area, the ecosystem service values in study area were estimated using the formula as follows:

$$ESV = \sum_{1}^{n} A_k \times V_k \quad \text{(Eq.12)}$$

ESV is the total value of all ecosystem services in the study area; K is the number of land types in the study area; A_k is the k-th type of land use area in the study area, and V_k is the ecosystem service per unit area of the k-th land type value.

Grey correlation analysis

Grey correlation method is a research method to calculate the degree of correlation between two variables. In this paper, NP, LPI, LSI, FRAC_MN, CONTAG, IJI, SHDI, SHEI and AI were selected as the environmental variable groups at the landscape level, and NP, LPI, LSI, FRAC_MN, IJI, and AI were selected as the environmental variable groups at the patch type level, and the grey correlation analysis was carried out to access the relationship between those landscape indexes and ecosystem service value.

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First, the values of various ecosystem services in the study area were chosen as the reference sequence, and the landscape pattern indexes at patch type level and landscape level were chosen as the comparison sequence. Second, all these numbers were nondimensionalized using the range-standardization method. Finally, the correlation coefficients and correlation degree between the landscape pattern index and ecosystem services were calculated. The detailed calculation formula refers to the literature (Sun, 2010).

Results

Landscape pattern

Dynamics of landscape area

According to the area calculation results (*Table 3*), the forestry landscape area accounts for 54% in 2018. The forestry landscape has increased by 1,014.72 km^2 from 1998 to 2018. The growth trend of the construction landscape is also obvious, increasing from 359.95 km^2 in 1998 to 935.84 km^2 in 2018. Both agricultural landscape and rocky desertification landscape show a downward trend, of which agricultural landscape has decreased the most obvious (*Fig. 2*), and the area has decreased by 1,324.84 km^2 in 20 years.

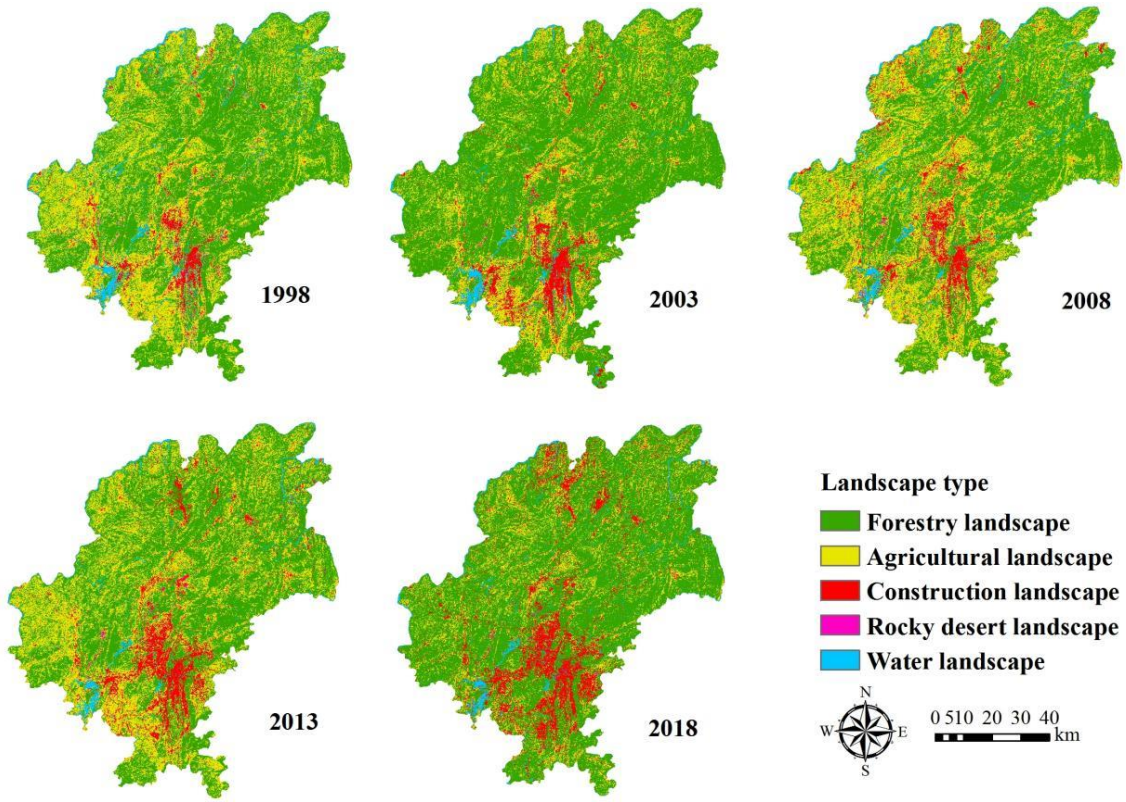

Figure 2. *1998-2018 land use distribution*

According to the calculation results of the dynamic degree (*Table 4*), the construction landscape has the highest dynamic degree of 8.00% from 1998 to 2018, followed by the

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rocky desert landscape at 3.53%, and the forestry landscape has the least dynamic degree of 1.14%. The dynamic degree of different landscape types varies over different time periods. The dynamic degree of forestry landscape is only 0.60% over the period from 2008 to 2013, and it is basically maintained at 4.0%-5.0% over the period from 2013 to 2018. For agricultural landscape, the dynamic degree of is the largest over the period from 2003 to 2008, which reaches 10.14%, and it is the smallest over the period from 2008 to 2013, which is only 1.06%. For construction landscape, the dynamic degree shows a trend of first declining and then rising, it is all above 5.0% except the period from 2003 to 2008. For rocky desertification landscape, the dynamic degree varies greatly, which is 35.28% over the period from 2003 to 2008, and only 6.29% over the period from 2008 to 2013. For water landscape, the dynamic degree shows a continuous downward trend, but the decline rate is gradually decreasing.

Table 3. *Dynamic change of landscape type area in Guiyang from 1998 to 2018 (km^2)*

Year	Forestry landscape	Agricultural landscape	Construction landscape	Rocky desert landscape	Water landscape	Total area
1998	4,450.28	2,729.69	359.95	56.93	444.30	8,041.15
2003	5,370.96	1,910.58	523.51	19.83	216.27	8,041.15
2008	4,262.47	2,879.70	536.48	54.82	307.68	8,041.15
2013	4,391.16	2,727.19	692.58	37.59	192.63	8,041.15
2018	5,465.01	1,404.85	935.84	16.76	218.69	8,041.15
Changes from 1998 to 2018	1,014.72	-1,324.84	575.89	-40.17	-225.61	

Table 4. *Dynamic degree of different landscape types in Guiyang from 1998 to 2018 (%)*

Landscape type	1998-2003	2003-2008	2008-2013	2013-2018	1998-2018
Forestry landscape	4.14	4.13	0.60	4.89	1.14
Agricultural landscape	6.00	10.14	1.06	9.70	2.43
Construction landscape	9.09	0.50	5.82	7.03	8.00
Rocky desert landscape	13.03	35.28	6.29	11.08	3.53
Water landscape	10.27	8.46	7.48	2.71	2.54

According to the transition matrix of landscape types (*Table 5; Fig. 3*), forestry landscapes are mainly transferred into agricultural landscapes and construction landscapes, with the transferred areas being 220.53 km^2 and 198.75 km^2 respectively. Agricultural landscapes are mainly converted to forestry landscapes and construction landscapes, with an area of 1,221.57 km^2 and 436.53 km^2 respectively. Rocky desertification landscapes are mainly converted to construction landscapes, agricultural landscapes, and forestry landscapes, with a transfer area of 19.57 km^2, 18.59 km^2, and 12.06 km^2 respectively. Water landscapes are mainly converted to forestry landscapes with an area of 215.43 km^2 and a small amount of them are converted to construction landscapes. In general, the main landscape type which is transferred into is construction landscape, of which the area transferred from forestry landscape and agricultural landscape is the largest.

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Table 5. *Transfer matrix of different landscape types in Guiyang from 1998 to 2018 (km^2)*

Landscape type	Forestry landscape	Agricultural landscape	Construction landscape	Rocky desert landscape	Water landscape
Forestry landscape	3,995.93	220.53	198.75	5.22	51.58
Agricultural landscape	1,221.57	1,056.91	436.53	7.08	10.44
Construction landscape	64.40	62.47	218.57	1.15	7.21
Rocky desert landscape	12.06	18.59	19.57	2.41	0.41
Water landscape	215.43	19.47	55.26	0.29	140.14

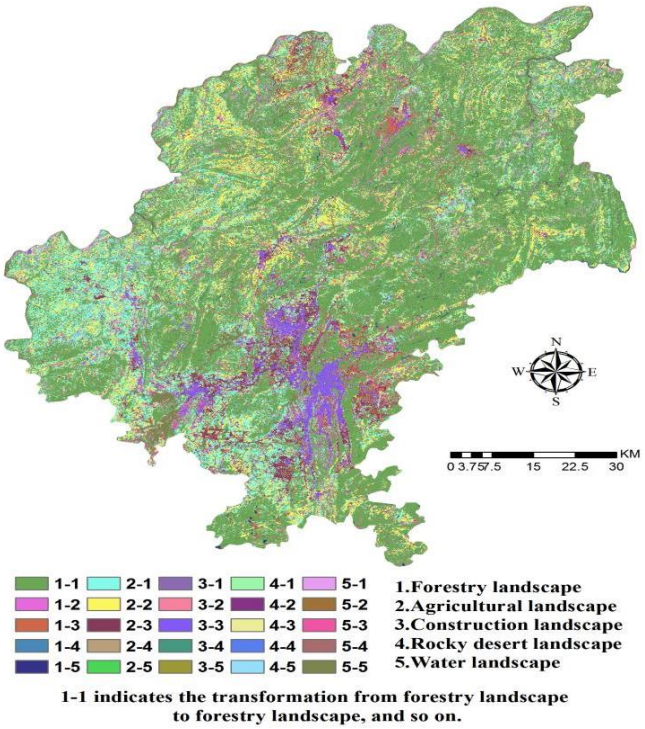

Figure 3. *land use transfer from 1998 to 2018*

Landscape index

Landscape index at landscape level

At the landscape level (*Table 6*), the number of patches and patch density show a clear descending trend from 1998 to 2013, and then slightly increased from 2013 to 2018. The number of patches decreased by 11,418 in total, and the patch density dropped from 21.477 to 20.057, and both decreased by 6.6%. The largest number of patches shows a fluctuating growth trend, from 36.339 in 1998 to 50.904 in 2018, indicating that the process of landscape fragmentation in the study area has slowed down in the past 20 years. The average fractal dimension remains almost unchanged from 1998 to 2018, and the patch shape index shows a decreasing-increasing-decreasing trend, reaching a maximum of 237.945 in 1998. It shows that the complexity of the landscape has decreased, human interference has tended to be less, the boundary of the landscape has become more regular, and the stability has increased. Both the spreading index and the aggregation degree show an increasing-decreasing-increasing trend, and

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the maximum value appeared in 2003. The scatter and juxtaposition index, the Shannon diversity index and the Shannon uniformity index all show a decreasing trend, indicating the landscape diversity and uniformity have decreased. Although a certain dominant landscape still has good connectivity, the probability of interpenetration among landscape patches has decreased, hindering the flow of material and energy, and reducing biological safety.

Table 6. *Landscape index changes at landscape level from 1998 to 2018*

Year	NP	PD	LPI	LSI	FRAC_MN	CONTAG	IJI	SHDI	SHEI	AI
1998	172,745.00	21.477	36.339	237.945	1.046	51.351	58.269	1.029	0.639	84.210
2003	147,796.00	18.375	51.821	213.032	1.044	57.472	46.113	0.901	0.560	85.876
2008	146,365.00	18.197	31.253	233.908	1.046	51.180	54.118	1.044	0.649	84.481
2013	125,845.00	15.646	33.546	226.726	1.047	52.625	45.556	1.023	0.636	84.962
2018	161,327.00	20.057	50.904	222.823	1.048	55.539	55.336	0.929	0.577	85.220

Landscape index at patch-type level

In terms of the patch and density index (*Fig. 4*), the agricultural patches have the largest patch number and patch density, and the rocky desertification patches have the smallest patch number and patch density. The patch number and patch density of agricultural patches and construction patches show an upward trend, while the rocky desertification patches, forestry patches and water body patches show a downward trend. The maximum patch index of forestry patches is significantly higher than that of other patch types, and it shows a trend of fluctuating increasing. The maximum patch index of other patch types fluctuated slightly within a certain range, remained almost the same or slightly increased. It shows that the fragmentation of agricultural patches is the largest, and that of rocky desertification patches is the least. The fragmentation of agricultural patches and construction patches is intensified, and that of other patch types tends to be gentle.

In terms of the shape index (*Fig. 4*), the patch shape index and average fractal dimension of agricultural patches are at the highest level and those of rocky desertification patches are at the lowest level. The patch shape index and average fractal dimension of agricultural and construction patch types show an upward trend, and the other patch types show a fluctuated downward trend. This shows that the shape of agricultural patches is the most complex and irregular, and the shape of rocky desert patches is relatively simple and regular. In addition, the shape of agricultural and construction patches has become more complex, tending to develop in an irregular direction, and are more and more seriously affected by the interference of human activities. The situation of forestry and water patches is just the opposite.

In terms of the index of spreading degree (*Fig. 4*), the forestry patches are the largest agglomeration, and the rocky desert patches are the smallest. The degree of agglomeration of forestry patches remained basically unchanged. The degree of agglomeration of agricultural patches declined, and those of the other patch types increased in fluctuation. The scatter and parallel index are more complicated. On the whole, only the forestry patch shows an increasing trend, and the rest show a significant decreasing trend. It shows that the distribution of forest patch types is the most concentrated, and the distribution of rocky desert patch types is more scattered. Agricultural patches tend to be in a scattered distribution, and the tendency of interpenetrating with other types of landscape patches is increasing.

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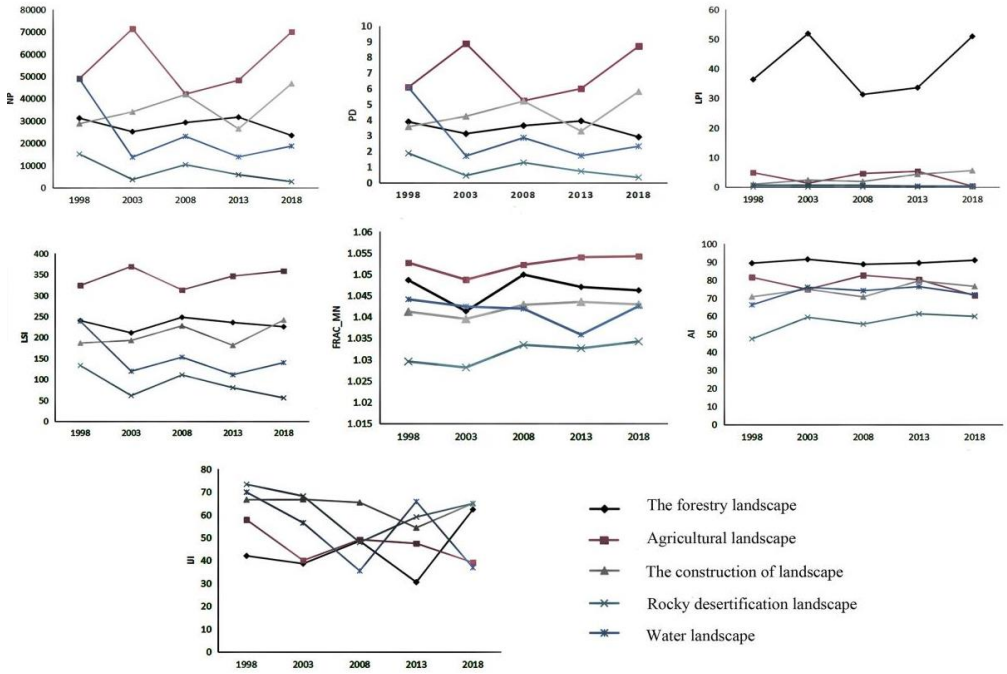

Figure 4. *Changes in the landscape index of patch types from 1998 to 2018*

Ecosystem services value

The total value of ESV shows a trend of first increasing, then decreasing, and increasing again. It increased from 1998 to 2003, decreased continuously from 2003 to 2013, and began to rise again from 2013 to 2018, and reached the maximum value of 8.0152 billion dollars in 2003. The total value of ESV increased 0.1499 billion dollars from 1998 to 2018. Regulating services contribute the most to the total value of ESV in Guiyang, followed by supply services and cultural services. In terms of the secondary service types, the value of all service types has increased except the food production and waste treatment functions in the past 20 years, indicating that the climate, soil and water sources of Guiyang have improved to a certain extent in recent years (*Table 7*).

Table 7. *Distribution of ESV in Guiyang city (billion dollars)*

Year	ESV total value	supply service		Adjustment service						Cultural service
		Food production	Raw materials	Gas regulation	Climate regulation	Water conservation	Soil conservation	Waste treatment	Biological protection	Entertainment and culture
1998	7.7895	0.1836	0.6758	0.9663	0.8356	1.4224	1.2177	1.0488	1.0023	0.4366
2003	8.0152	0.1409	0.8077	1.1269	0.9299	1.2973	1.3542	0.8044	1.1072	0.4468
2008	7.2561	0.1904	0.6488	0.9332	0.8108	1.2345	1.1883	0.9071	0.9541	0.3891
2013	7.0544	0.1818	0.6669	0.9545	0.8199	1.1189	1.2042	0.7831	0.9552	0.3698
2018	7.9394	0.1126	0.8186	1.1313	0.9187	1.3000	1.3330	0.7666	1.1046	0.4540

Counted by counties (*Table 8*), the total value of ESV of Huaxi district, Kaiyang county, Xiuwen county and Qingzhen city, which are outside the geographic center of Guizhou city, all increased to a varying extent from 1998 to 2018. The largest increase occurred in Qingzhen city, which is located in the west of Guizhou city, with the increase of 0.17 billion dollars. Xifeng county, which is also on the periphery, showed a decreasing trend, as well as the six regions near the geographic center, of which Xifeng

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county showed the largest decrease of 54.36 million dollars. Obviously, it shows that the landscape pattern of Qingzhen city and Xifeng county has changed drastically. Yunyan cistrict, Nanming cistrict, Baiyun district and Guanshan Lake district have the smallest proportion of ESV total value, while Kaiyang dounty and Qingzhen city have the largest proportion. In general, the ESV in Guiyang city has a spatial pattern with the geographic center as the core, and the greater the value toward the periphery (*Fig. 5*).

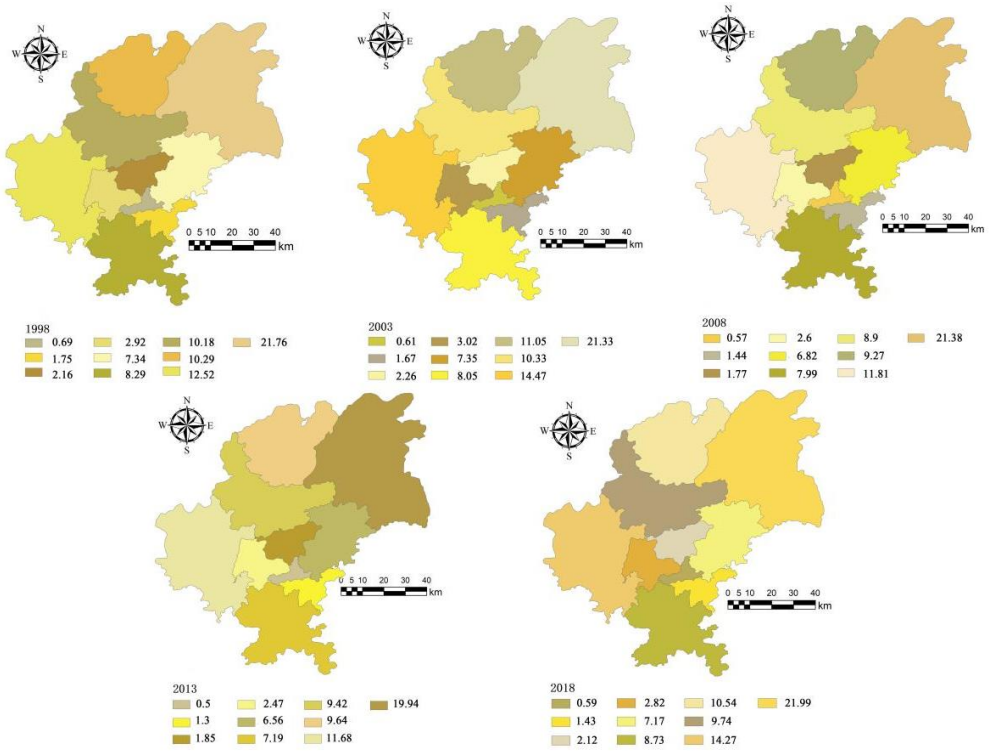

Figure 5. *ESV spatial distribution indifferent years (unit: 100 million dollars)*

The relationship between landscape pattern and ESV

Landscape level

At the landscape level (*Table 9*), the correlation between the landscape index and the total value of ESV in descending order is AI > FRAC_MN > CONTAG > LPI > IJI > LSI > SHDI > SHEI > NP, all above 0.663, and the AI with the highest correlation degree is 0.863, indicating that the total value of ESV is closely related to the degree of landscape aggregation, landscape shape complexity, and fragmentation degree. NP, IJI and LSI have the greatest correlation with water conservation. LPI has the greatest correlation with gas regulation. FRAC_MN, AI, CONTAG have the greatest correlation with soil conservation, and SHDI and SHEI have the greatest correlation with food production, which indicates that the number of landscape patches, scattered and juxtaposed conditions, and the complexity of patch shapes are the main factors affecting the water conservation function. The proportion of the largest patch number is closely related to the gas regulation function. The degree of landscape agglomeration and spread profoundly affect the soil conservation function. The diversity and uniformity of the landscape greatly affect the function of food production.

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Table 8. *Distribution of ESV in districts and counties of Guiyang city*

Administrative district	1998		2003		2008		2013		2018	
	Total value /100 million dollars	Percentage/ %	Total value /100 million dollars	Percentage /%	Total value /100 million dollars	Percentage /%	Total value /100 million dollars	Percentage /%	Total value /100 million dollars	Percent-age /%
Nanming District	1.75	2.24	1.67	2.09	1.44	1.99	1.30	1.84	1.43	1.80
Yunyan District	0.69	0.89	0.61	0.76	0.57	0.79	0.50	0.71	0.59	0.74
Huaxi District	8.29	10.64	8.05	10.04	7.99	11.01	7.19	10.20	8.73	10.99
Wudang District	7.34	9.43	7.35	9.17	6.82	9.40	6.56	9.30	7.17	9.03
Baiyun District	2.16	2.77	2.26	2.82	1.77	2.44	1.85	2.62	2.12	2.67
Guanshan Lake	2.92	3.75	3.02	3.77	2.60	3.58	2.47	3.50	2.82	3.55
Kaiyang County	21.76	27.94	21.33	26.62	21.38	29.46	19.94	28.27	21.99	27.70
Xifeng County	10.29	13.21	10.33	12.89	9.27	12.78	9.64	13.66	9.74	12.27
Xiuwen County	10.18	13.06	11.05	13.78	8.90	12.26	9.42	13.35	10.54	13.28
Qingzhen City	12.52	16.08	14.47	18.06	11.81	16.28	11.68	16.56	14.27	17.97

Table 9. *Correlation between landscape level index and ecosystem service value*

Landscape index	ESV total value	Waste treatment	Climate regulation	Gas regulation	Biological protection	Food production	Water conservation	Soil conservation	Entertainment culture
NP	0.663	0.784	0.577	0.551	0.591	0.574	0.856	0.575	0.700
LPI	0.762	0.685	0.755	0.776	0.772	0.651	0.721	0.750	0.756
LSI	0.748	0.603	0.741	0.702	0.754	0.692	0.772	0.742	0.682
FRAC_MN	0.838	0.580	0.814	0.772	0.782	0.715	0.691	0.828	0.784
CONTA-G	0.791	0.579	0.929	0.861	0.893	0.691	0.651	0.941	0.764
IJI	0.762	0.797	0.658	0.634	0.680	0.682	0.844	0.654	0.751
AI	0.863	0.576	0.823	0.776	0.793	0.709	0.678	0.836	0.810
SHDI	0.678	0.597	0.711	0.691	0.674	0.760	0.741	0.726	0.628
SHEI	0.677	0.596	0.709	0.689	0.672	0.759	0.741	0.724	0.627

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Patch-type level

At the patch type level (*Table 10*), the total value of ESV has the greatest correlation with the FRAC_MN for water patches, agricultural patches, construction patches, and rocky desertification patches, and the total value of ESV has the greatest correlation with AI for forestry patches. Generally speaking, in terms of the characteristics of fragmentation, the degree of fragmentation of water patches is closely related to waste treatment.

Table 10. *Correlation between horizontal landscape index of each patch type and ecosystem service value*

Landscape type	Landscape index	ESV total value	Waste treatment	Climate regulation	Gas regulation	Biological protection	Food production	Water conservation	Soil conservation	Entertainment	Culture raw materials
Water landscape	NP	0.544	0.617	0.524	0.516	0.528	0.598	0.579	0.523	0.552	0.513
	LPI	0.656	0.729	0.591	0.568	0.601	0.628	0.703	0.590	0.676	0.559
	LSI	0.565	0.686	0.536	0.526	0.542	0.672	0.619	0.535	0.578	0.522
	FRAC_MN	0.840	0.581	0.821	0.779	0.787	0.722	0.693	0.835	0.785	0.756
	IJI	0.674	0.729	0.645	0.629	0.662	0.792	0.682	0.641	0.654	0.623
	AI	0.699	0.527	0.802	0.771	0.782	0.610	0.592	0.810	0.689	0.734
Forestry landscape	NP	0.725	0.800	0.715	0.706	0.712	0.817	0.708	0.718	0.674	0.702
	LPI	0.762	0.685	0.755	0.776	0.772	0.651	0.721	0.750	0.756	0.793
	LSI	0.698	0.587	0.727	0.689	0.687	0.773	0.729	0.724	0.646	0.675
	FRAC_MN	0.830	0.579	0.810	0.769	0.777	0.719	0.693	0.824	0.776	0.746
	IJI	0.730	0.732	0.711	0.708	0.714	0.697	0.780	0.710	0.741	0.708
	AI	0.884	0.582	0.843	0.795	0.812	0.709	0.681	0.857	0.827	0.769
Agricultural landscape	NP	0.734	0.671	0.774	0.796	0.766	0.678	0.704	0.771	0.734	0.811
	LPI	0.709	0.701	0.712	0.709	0.712	0.752	0.683	0.712	0.685	0.708
	LSI	0.781	0.587	0.919	0.889	0.895	0.654	0.652	0.914	0.752	0.849
	FRAC_MN	0.836	0.580	0.813	0.771	0.781	0.715	0.692	0.827	0.782	0.748
	IJI	0.681	0.867	0.618	0.605	0.634	0.758	0.780	0.614	0.729	0.601
	AI	0.698	0.601	0.734	0.705	0.694	0.753	0.756	0.737	0.649	0.690
Rocky desert landscape	NP	0.590	0.662	0.568	0.562	0.574	0.619	0.630	0.567	0.603	0.560
	LPI	0.662	0.618	0.679	0.691	0.678	0.620	0.641	0.679	0.662	0.698
	LSI	0.635	0.755	0.600	0.592	0.609	0.680	0.702	0.597	0.658	0.589
	FRAC_MN	0.838	0.579	0.812	0.769	0.780	0.716	0.689	0.825	0.784	0.745
	IJI	0.662	0.667	0.577	0.549	0.590	0.559	0.834	0.575	0.698	0.538
	AI	0.639	0.533	0.698	0.739	0.688	0.591	0.576	0.699	0.628	0.766
Construction landscape	NP	0.737	0.623	0.754	0.776	0.759	0.661	0.664	0.751	0.714	0.784
	LPI	0.625	0.611	0.631	0.634	0.630	0.620	0.617	0.631	0.623	0.635
	LSI	0.784	0.606	0.799	0.795	0.795	0.698	0.670	0.795	0.758	0.801
	FRAC_MN	0.837	0.578	0.811	0.769	0.780	0.715	0.689	0.825	0.783	0.745
	IJI	0.825	0.639	0.735	0.687	0.731	0.609	0.778	0.741	0.830	0.665
	AI	0.793	0.573	0.850	0.783	0.833	0.655	0.649	0.861	0.772	0.752

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The degree of fragmentation of forestry patches, agricultural patches and construction patches is highly related to raw materials. The degree of fragmentation of rocky desertification patches also greatly affects waste treatment and water conservation functions. In terms of the complexity of landscape shapes, the shape complexity of water patches is mainly closely related to food production. The shape complexity of forestry patches and construction patches are closely related to waste treatment. The complexity of agricultural landscapes and rocky desertification landscapes are closely related to soil conservation and entertainment culture. In terms of landscape aggregation, the degree of agglomeration of water bodies and construction patches mainly affects the value of soil conservation. The degree of agglomeration of forestry patches mainly affects entertainment culture. The agglomeration of rocky desertification patches and agricultural patches play an important role in the function of water conservation.

Discussion

Landscape pattern

In this study, the agricultural landscape mainly includes arable land, tea gardens and orchards, and the forestry landscape mainly includes woodland and shrubland. In terms of landscape area, the water body landscapes fluctuate greatly during study period due to the variations in annual precipitation, of which the annual precipitation of Guiyang City in 1998 and 2008 was significantly higher than that in 2003, 2013 and 2018. The landscape matrix of Guiyang City is forestry landscape. During the study period, the construction landscape expanded rapidly, and the forestry and agricultural landscapes were inevitably invaded during the process of urbanization. Meanwhile, certain area of construction land was changed into the forestry and agricultural landscapes due to the measures of construction land reclamation and reforestation. Guiyang City has implemented a series of measures such as returning farmland to forests for rocky desertification control, which has led to a continuous decline in the area of rocky desertification landscapes, some of which have been transformed into agricultural landscapes, and a considerable part of agricultural landscapes have also been transformed into forestry landscapes. This shows that the phenomenon of rocky desertification has improved and the control measures have achieved a certain degree of effectiveness.

Ecosystem services

The ESV evaluation results of this study are similar to that of Wei et al. (2015) and Han et al. (2020) in Guiyang. Although the total value of ESV is not exactly the same, the change trend of ESV is consistent, showing a fluctuating upward trend with the development of urbanization. Existing studies have shown that under normal circumstances, economic and social development and the increase in the area of construction land landscapes will lead to a decline in ESV. For example, Mobeen et al. (2020) evaluated the ecosystem service value of land use/cover change and found that the simultaneous conversion of vegetation and unused land into construction land resulted in a net ESV reduction of $7.96 million in Lahore city, Pakistan. Meng took a case study in Ningbo City and found that economic urbanization and land urbanization had a significant negative impact on ESV (Meng, 2016). Qu and Guo took a case study

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in Nanjing City to quantitatively study the coupling between urbanization level and ESV, and found that the development of urbanization will inevitably cause damage to the ecological environment (Qu and Guo, 2016). In this study, the ESV of Guiyang city shows a spatial pattern with the geographic center as the core, low in the middle and high around it. The main reason is that the nearby area of geographic center that has the most developed economy and construction landscape is poor in ecological environment, while the regional economic development outside the geographic center is relatively backward, and the degree of land use development is low. However, since 2013, with the rapid expansion of the construction landscape, the ESV has also increased in Guiyang city. This is mainly due to the policy of returning farmland to forests and some measures such as rocky desertification control and natural forests protection projects. Although the area of water landscapes and agricultural landscapes has decreased, the area of forestry landscapes with a higher coefficient with ESV per unit area has shown an increasing trend, and rocky desertification landscapes have also decreased. This is similar to the conclusions of the study conducted by Bai et al. (2020) on the coupling relationship between urbanization and ESV in Guiyang city.

The relationship between landscape pattern and ecosystem services

The correlation relationships between landscape patterns and ESV are roughly the same at the landscape level and at patch type level (*Tables 9* and *10*). At the landscape level, the highest correlation with food production is the Shannon Diversity Index and the Shannon Uniformity Index, which is a little different with the correlation at the patch type level. The main reason is that these two indices are not available at the patch type level. Exclude these factors, the correlation degree of the patch type level is basically consistent with the landscape level. There are still individual differences in the correlation degree between landscape type level and the patch type level. Therefore, when exploring the relationship between the landscape pattern and ESV in the southwest karst area, analysis at the patch type level can get more accurate and detailed results.

Highlights and limitations

Highlights

(1) Dividing the rocky desertification landscape into an independent category

At present, few studies have divided rocky desertification landscapes into an independent landscape type in the researches on ecosystem services of karst areas (Wei et al., 2015; Xiao et al., 2013; Ivajnsic and Kaligari, 2014; Luo et al., 2021). Rocky desertification landscapes in karst areas account for a large proportion, and it is the most serious ecological problem in southwestern China, which severely restricts the sustainable development of the local economy. Therefore, it is of great significance to classify rocky desertification landscapes into one category in karst areas. It is possible to further analyze and study the relationship between rocky desertification landscapes and ecosystem service values, to lay the foundation for subsequent further research and to a certain extent quantify and reflect the control effects of various rocky desertification ecological projects. In addition, it can also provide theoretical support for the scientific management of rocky desertification from the perspective of landscape ecology.

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(2) Using the grey-level correlation analysis method

Most of the existing studies on the relationship between landscape pattern and ecosystem services use correlation and regression methods, which often require a large sample size and obvious distribution characteristics. Correlation analysis is essentially a linear relationship analysis between two variables (Wang et al., 2017; Song et al., 2018; Zhang et al., 2010; Yohannes et al., 2021). However, existing studies have also shown that the landscape pattern and the value of ecosystem services are not simple linear relationship (Zhong, 2019). The gray-level correlation analysis method can more accurately measure the correlation between two variables based on its advantages in comprehensively analyzing the multi-factor interaction of the system (Tan and Deng, 1995). This study attempts to use the gray-level correlation analysis method to analyze the relationship between landscape pattern and ecosystem services, and obtain more reasonable research results, which provides a new idea for the study of the impact relationship between landscape pattern and ecosystem services.

Limitations

This research is a case study, taking Guiyang, the capital of Guizhou Province, as the study area, using local data for research and analysis, so as to draw some specific conclusions. The study area is located in the center of East Asia, one of the three karst concentrated distribution areas in the world. Many problems of karst area in the world need to be studied and solved here (Yu, 2008). The complexity of Guizhou ecosystem, the vulnerability of karst environment and the emergence of rocky desertification are very representative in China and even the world. According to the principle of comparability of climate background and karst characteristics, Li selected several karst areas worldwide to compare with karst in southern China, and found that they are similar to karst in southern China (Li, 2014). Therefore, although this is a local study, the results can be generalized to other karst areas in the world, which can provide theoretical basis and reference significance for the rational allocation of landscape pattern and the implementation of ecological restoration technology in other karst areas in the world, and further enrich relevant research theories. In addition, the ideas and research methods of this paper can also be used for reference to the research on the relationship between other landforms and ecosystems, so as to provide new ideas and methods.

Another limitation is that our research only focuses on the relationship between landscape pattern and ESV, whereas it might be important to include the mechanism of the relationship as well. In fact, the inclusion of the mechanism in the future study would enable us to improve our understanding of the interaction between landscape pattern and ESV.

Conclusions

Landscape pattern

The landscape matrix of Guiyang City is forestry landscape. During the 20 years of the study period, the water body landscape remained basically unchanged. The agricultural landscape and rocky desertification landscape decreased, and the forestry landscape and construction landscape increased. Both the construction landscape and the rocky desertification landscape maintain a high degree of dynamics. The rocky desertification landscape is mainly converted to the construction landscape, but the

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construction landscape is mainly transferred from the agricultural landscape and forestry landscape. The phenomenon of rocky desertification in Guiyang City has improved, and the corresponding control measures have achieved results. The fragmentation of the landscape slows down, the complexity decreases, and the stability increases. However, the diversity and uniformity of the landscape are showing a downward trend, the material flow, information flow, and energy flow between patches are reduced, and the biological safety is reduced. The agricultural patch type has the largest degree of fragmentation, the patch shape is the most complex and the most irregular, and the rocky desert patch type is just the opposite. Agricultural patches and construction patches have become more fragmented, more complex in shape, and tend to develop in irregular directions, and are more and more seriously affected by interference from human activities. Forestry and water patches have the opposite situation. The distribution of forest patch types is the most concentrated, while the distribution of rocky desertification patch types is more scattered. The trend of mutual penetration between agricultural patches and other types of landscape patches is increasing, while the situation of other patch types is just the opposite.

Ecosystem services

The total value of ESV shows a trend of first increasing, then decreasing and then increasing. The most important contribution to the total value of ESV is the regulation service. Among the secondary service types, except for the reduction of food production and waste treatment functions, the rest have increased. The climate, soil and water resources of Guiyang City have been improved to a certain extent. In terms of spatial distribution, the west has the most value-added, and the north has decreased significantly. ESV presents a law of geographic center as the core, low in the middle and high around it.

The relationship between landscape pattern and ecosystem services

The total value of ESV is closely related to the degree of landscape aggregation, the degree of complexity of landscape shape and the degree of fragmentation. The number of landscape patches, their dispersal and juxtaposition, and the complexity of patch shapes all mainly affect the water conservation function. The proportion of the largest patch number is closely related to the gas regulation function. The degree of agglomeration and spread of the landscape has a profound impact on the soil conservation function, while the diversity and uniformity of the landscape greatly affects the food production function. At the patch type level, the total ESV value has the greatest correlation with the average fractal dimension of water landscapes, agricultural landscapes, construction landscapes and rocky desertification landscapes, and the degree of aggregation of forestry landscapes. On the whole, the degree of association between each landscape type and the secondary service type is different in terms of fragmentation characteristics, complex shapes, and aggregation and dispersion.

In the future, we will conduct the study on the interaction direction between karst area, landscape pattern and ecosystem service value, so as to further study the specific correlation mechanism between them. The evaluation method of ecosystem service value in karst area can be innovated to be more in line with the actual situation of karst area. In addition, we should strengthen the implementation of measures such as returning farmland to forest and rocky desertification control to help improve ESV.

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A STUDY OF THE IMPACT OF THE CHANGES IN WINTER PRECIPITATION PATTERN ON THE WINTER CROPS YIELD IN SOUTH CENTRAL REGION OF BULGARIA

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Abstract. The paper discusses the impact of winter seasons climate change on crop yields in the South Central Region of Bulgaria. Twenty-two years long field observations show a progressive increase in air temperature and a decrease in snow cover in the winter seasons. The reduction of snow and the air temperature rise are supposed to lead to reduction of water availability in the spring for the winter crops and respectively to lowering their yield. By compiling observed climatic data and those obtained through a spatially distributed climatic model for the region area analysed is the relationship between these climatic parameters and the respective harvest of the winter crops for the last 5 winter seasons. The observed in the analysed period winter crops yield does not show distinctly the supposed dependence on the decrease of the snowfall amount and snow cover residence time. The yields of the last winter season 2019/2020 with the least snow cover and 2°C higher air temperature exceeded those of all other seasons. This effect is explained with the favourable combination of the high air temperature with the amount and monthly distribution of the precipitation in the season.
Keywords: *climate change, snow cover, winter crop yield, hydrological simulations, snow to precipitation ratio*

Introduction

Wheat, barley and oats are one of the most important agricultural crops in Bulgaria - they occupy great part of the country land and provided 62% of the production of cereals in 2019 (MAFF, 2021).

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A study conducted by Bonchev (2020) on a test field in the town of Sadovo (South Central Region) for 6 sorts of wheat in years with different climatic conditions (2016/2017 and 2017/2018) showed that for the formation of the yield of different sorts the influence of the genotype is not so significant, as the hydro-climatic conditions are. Shrestha et al. (1999) also consider that climate change will have significant impact on water availability by changes in the temperature and precipitation patterns, thus affecting the crop yield (Fodor and Pasztor, 2010). The temperature increase leads to diminishing or lack of snow cover in winter, which creates favourable conditions for drought in spring (Brázdil et al., 2015).

The snow cover has a regulating role on the hydrological cycle, recharges groundwater and plays a very important role in solving irrigation problems (Petkova, 2014). Snow to precipitation ratio controls the catchment water storage and the summer runoff (Meriö et al., 2019). The snow cover maintains water balance and creates proper conditions for overwintering of field crops as it protects them against low temperatures (Wimmerová et al., 2017). According to Watson (2020) snow is more beneficial than rain for the wheat crop in several ways. Moisture from snow does not evaporate immediately because of the cold weather. So it moves down into the soil and remains there keeping the soil warmer during winter as it takes much longer for wet soils to get cold than dry soils. Also, moisture from snow helps the roots to continue growing even when the top growth of crops is dormant during the cold periods. Third, snow cover keeps the soil from blowing, thus preventing wind erosion. Snow cover insulates the soil from the low air temperatures and protects the crown of the wheat plant from cold injury.

Climate change decreases the fraction of precipitation falling as snow and the timing of snowmelt as well. This may have negative effects on food production in basins with agriculture depending heavily on snowmelt runoff (Watson, 2020). Therefore, having information about snow cover formation processes is important in order to know the expected water availability and thermal regime in the region agriculture, respectively the winter crops yield. The soil and climatic conditions in Bulgaria are favourable for growing winter crops. Studying the changes in snow hydrology parameters during the latest years are of great importance for development of resilient agricultural practices.

The main objective of the present study is to assess the impact of climate change, which has already been identified in recent decades on the winter crops. This in particular relates to the change of the amount of the total precipitation, snow-rain ratio and air temperature. The study was done at a regional level in Bulgaria.

To achieve this goal, an analysis of the available information on winter precipitation, air temperature, snow cover and thickness, related to the yield of selected crops, was performed. The above noted hydrological data was obtained from field observations or by use of on-going spatial climatic databases. Due to the lack of sufficient field measurements, the area distributed snow data as snow part of the total precipitation were estimated using hydrological model.

Incomplete data concerning the above discussed information is available for the study area of the South Central Region of Bulgaria at a few hydro-meteorology observation stations. This however does not enable a correct enough assessment of the changes in agro-climatic conditions for the whole territory of the region for implementation of the above targeted studies.

That is why here a physically based Community Land Model (CLM) (Oleson et al., 2004) to study and monitor the processes of snow hydrology has been applied. The

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model is calibrated for the soil and climatic conditions of Bulgaria. As source of its input information - climate, land cover and soil data used is the developed gridded NCEP / NCAR and IGBP database (Kalney et al., 1996). The output of the CLM model is also represented in a gridded format, which facilitates the preparing averaged regional hydrological assessments during the winter season.

By employment of the model, maps of the monthly values (from November until March) of rain and snow, snow depth, snow cover and air temperature for 5 winter seasons have been prepared. They show an increase in the air temperatures, varying snow part of the total precipitation and decrease of the snow cover area and residence time.

Materials and methods

Study area and winter crops characteristics

The South-Central Planning Region of Bulgaria was selected as the study area. The territory includes a large part of the mountain chain of Central Balkan, part of the Rila mountain and the entire massif of the Rhodopes. The north border follows the ridge of Central Balkan - Sredna Stara Planina (*Fig. 1*). The southern boundary of the region reaches the frontier with the Republic of Turkey and the Hellenic Republic. The region's area is 22 400 square kilometres, which represents 20.1% of the country's territory. The relief consists of valleys, lowlands, hills and mountains. It contains the most fertile part of Bulgarian land – the Thracian valley.

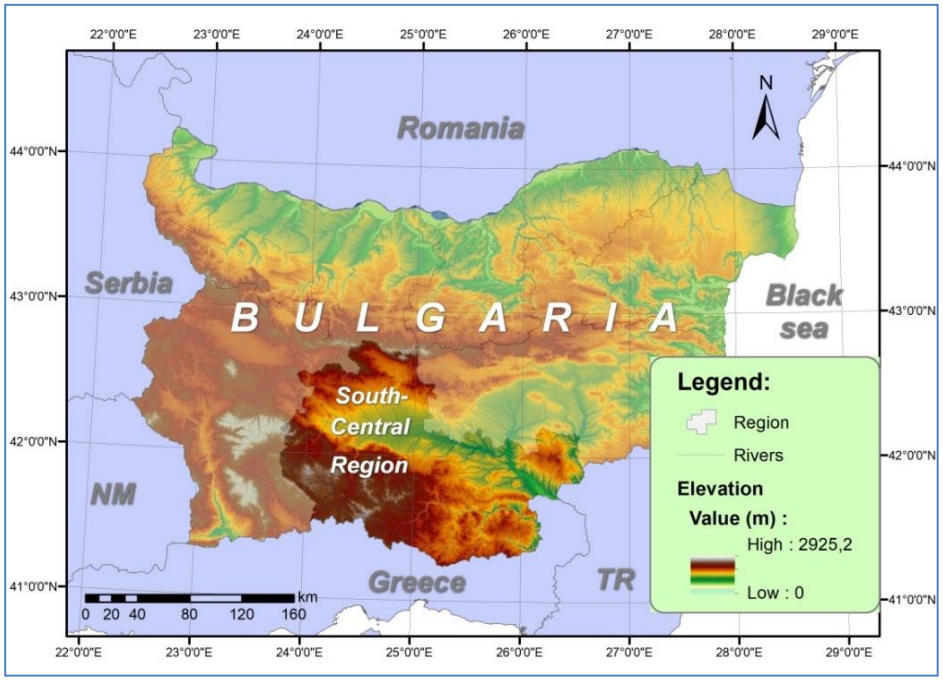

Figure 1. *Map of Bulgaria and South Central Region of Bulgaria*

The climate is predominantly temperate-continental with influence from the Mediterranean Sea in the south-eastern part of the region. It is characterized by long frost-free periods, relatively warm winter and summer and high total temperatures. 1/5

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of the country's surface water resources and about 1/4 of the country's groundwater are concentrated in the region. The main river is Maritsa with about 100 tributaries, more significant of which in the region are Arda and Vycha. The Maritsa River springs from Rila Mountain at 2378 m above sea level The mountains in the South Central Region provide water for downstream agriculture activities along the Maritsa and Arda rivers. In the flat part the precipitation is between 450 and 500 mm/a, while in the mountain and hilly parts it is between 1000 – 1200 mm/a (Santourdjian, 2000).

The most important soil resources are Chromic Luvisols, Chromic Cambisols, Planosols, Vertisoils, Fluvisols; there are also sandy light soils on deforested slopes. The region includes five Bulgarian administrative districts – Pazardzhik, Plovdiv, Smolyan, Haskovo, and Kardzhali with Plovdiv being the regional capital (*Fig. 2*). As of 2016 farmland covers 840 650 hectares, of which the utilized agricultural area is 650 190 hectares and arable land covers 358 860 hectares (Eurostat, 2021).

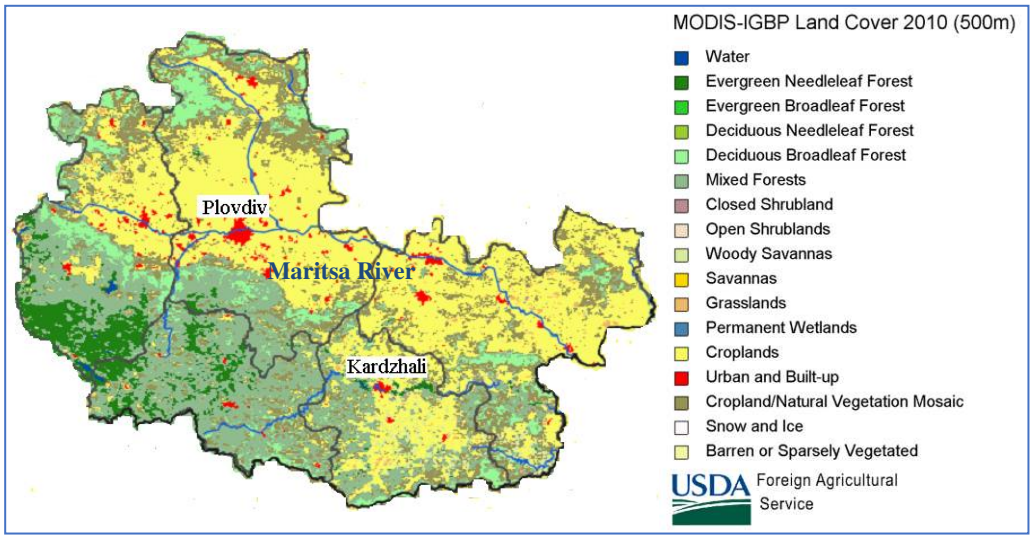

Figure 2. *Land Cover map of the South-Central Planning Region of Bulgaria*

Wheat and barley are sown mainly in late September and early October. Thus, they use autumn moisture in the soil to germinate and develop in their initial stages (Savova, 1994). These phases are essential for their overwintering. The main phase is tillering, during which up to 3 tillers develop on one root. The number of tillers is basic to the high yields of these crops. Decreased tillering and spring tillering lead to a strong decrease in yields (Yanchev and Tahsin, 2000; Kolev et al., 2004). Soil moisture in May-June, combined with high temperatures (above 25^{o}C) during grain maturing (June) are also favorable for good yields.

Of the three crops, oats yield is the lowest because oats are most susceptible to frost. That is why it is grown mainly as a spring crop. It is sown usually in March. This leads to much lower yields because of possible dry periods in the end of May and in June. In such a case the autumn development of the crop is lost and there is an accelerated development in the spring in not always favorable temperature and humidity conditions (Savova, 1994; Georgieva, 1995). With global warming, oats will be able to be sown mainly as a winter crop in September and October and this would increase its yields (Forsberg and Reeves, 1992; Savova, 1994).

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Data and methods

Necessary data about the atmospheric forcing for the research purposes and the modeling of the hydrology processes on the territory of the South-Central region of Bulgaria for the period 2013-2020 are available. Available are also published data about the yields of the winter crops - wheat, barley and oats (MAFF, 2021), as well as data about the number of days with snow cover for the period 2013-2020 on the area of Plovdiv and Kardzhali, and long data series (1999-2021) with measured temperatures, precipitation and the snow cover thickness for the region of Kardzhali. For assessments of the whole South-Central region hydrology applied is the Community Land Model version 3 (CLM3).

CLM is a physically-based hydrologic model developed in the US National Center for Atmospheric Research (Oleson et al., 2004, 2008). The energy and water fluxes are estimated through vegetation processes, energy budget calculations, and water balance calculations based on the theory of mass conservation and Fick‘s law with specified initial and boundary conditions.

The water balance for a SnowpackSlab is presented by *Eq.1* (Arsenault, 2010):

$$Qsnow = Qsnowsubl + Qsnowmelt + \Delta Qsnow(t) \quad \text{(Eq.1)}$$

where Q_{snow} is the snowfall (kg m^{-2}) (when air temperature is <0°C), $Q_{snowsubl}$ is the sublimation from snow surface (kg m^{-2}), $Q_{snowmelt}$ is the snowmelt (kg m^{-2}), $\Delta Q_{snow(t)}$ is the change in snow amount (kg m^{-2}), t is time step length.

Snow depth formation is calculated by *Eq.2*:

$$Zsnow = \frac{Qsnow}{\rho snow}.t \quad \text{(Eq.2)}$$

where Z_{snow} is the snow depth (m), ρ_{snow} is the bulk density of newly fallen snow (kg m^{-3}), for dry new snow ρ_{snow} =50-70 (kg m^{-3}), for damp new snow ρ_{snow} =100-200 (kg m^{-3}).

The snow depth decreases along the time due to sublimation, melting and compaction.

CLM3 uses a one-dimensional vertical, multilayer snow model based on the parameterizations developed by Anderson (1976), Jordan (1991), and Dai and Zeng (1997).

The model input uses gridded climate variables such as solar radiation (W/m^2), precipitation (mm/s), air temperature (K), wind speed (m/s), atmospheric pressure (Pa), and specific humidity (kg/kg), with spatial resolution 180 x 180 km and time resolution 6 hours. Soil and plant data are presented by the information for soil texture (% of sand and % of clay), soil color and vegetation parameters (monthly leaf LAI and stem SAI indices), where the spatial resolution is 5 x 5 km. Database with the listed parameters is publicly available. These are aggregated ground-based and satellite observations from different countries and organizations, which are prepared in a uniform grid that is suitable for input of regional climate and hydrological models.

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Results and Discussion

As discussed above, the main factors responsible for the growth and development of winter crops are the amount and monthly distribution of total precipitation, snow cover thickness and residence time, respectively the average snow depth and air temperature during the winter season. As such considered is the period from November to March. Globally, one of the hitherto undisputed effects of climate change is the trend of rising air temperatures and reduction in the share of snow from total precipitation.

Confirmation of this trend for the studied area is seen in the available field data about the precipitation, air temperature and snowdepth during the last 22 winter seasons, measured near to the town of Kardzhali. The town is located in the southeast part of the regarded region at about 280 m above sea level (*Fig. 2*). Graphs of the time data series, averaged for the winter seasons (from November to March) during the period 1999-2021 are shown on *Figure 3*. Plotted are the values of the precipitation (snow plus rain), air temperature and snowdepth, together with the trendlines of their change.

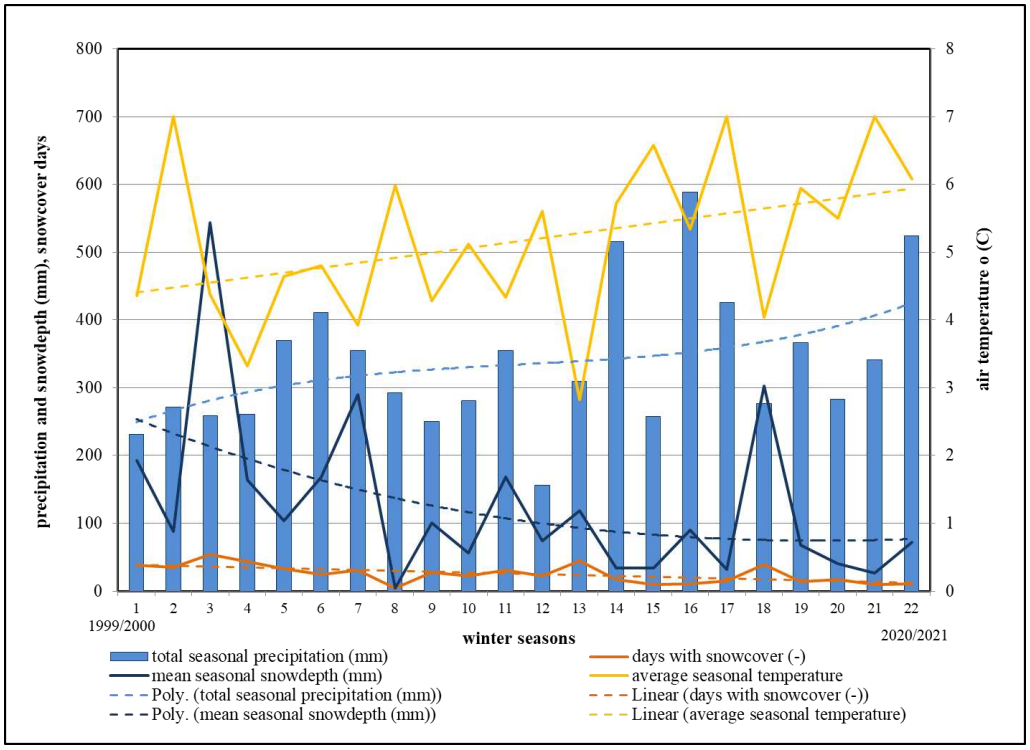

Figure 3. *Trends of the seasonal total precipitation, air temperature and snowdepth at Kardjali station from 1999/2000 to 2020/2021, (for months Nov, Dec, Jan, Feb and March)*

This was prepared using the public databases STRINGMETEO (2021) and National Institute of Meteorology and Hydrology (NIMH) monthly bulletins (2021). However, they don't provide information about the part of the snow from the total precipitation. From the measured average monthly snowdepth it is very difficult to recover the amount of precipitation in the form of snow.

The time distribution of winter precipitation and temperatures presented on *Figure 3* shows an increasing trend for the period 1999-2021, while the snowdepth measured in the field decreases during the same period. Temperature rise leads to accelerating of the

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snowpack melting. This shortens the time of water availability due to winter precipitation. The decrease of snow cover normally is supposed to worsen winter agro-climatic conditions.

Checking the degree of validity of these considerations on the importance of the amount of snow and the time of its retention for the development and the yield of winter crops, a comparative assessment of their values during the winter seasons of 5 years with rather different climatic characteristics has been done. For the purpose selected were the last 4 winter seasons – the period 2016-2020, including the months from November to March. The winter season 2013/2014 was additionally added to them as being with a similar scarce amount of rain and snow as the season 2019/2020.

The observed climatic data at the Kardzhali station, as well as the similar at the stations at Plovdiv have local character and do not allow accurate enough hydrological and climatic estimates for the whole area of the region. They don't show the snowfall part from the total winter precipitation either. An estimate covering the entire area is necessary for a well based and reasonable assessment of the considered climatic parameters value and their influence on the region winter crops yield.

This was achieved by employment of the hydrological model CLM to study and supplement the hydrological description of the winter conditions for analysis and comparisons on the considered area, half of which is agricultural land. Mathematical simulations have been made for evaluation of the snow part of the total precipitation and the average monthly snow depth during the five selected years.

For demonstration purposes the model computed monthly averaged snowdepth spatial distribution in December 2018 is presented on *Figure 4*. The highest average monthly values of 5 cm are calculated for the mountainous and hilly parts of the studied region, the lowest values of 1-2 cm are along the Maritsa River, where the agricultural lands are situated.

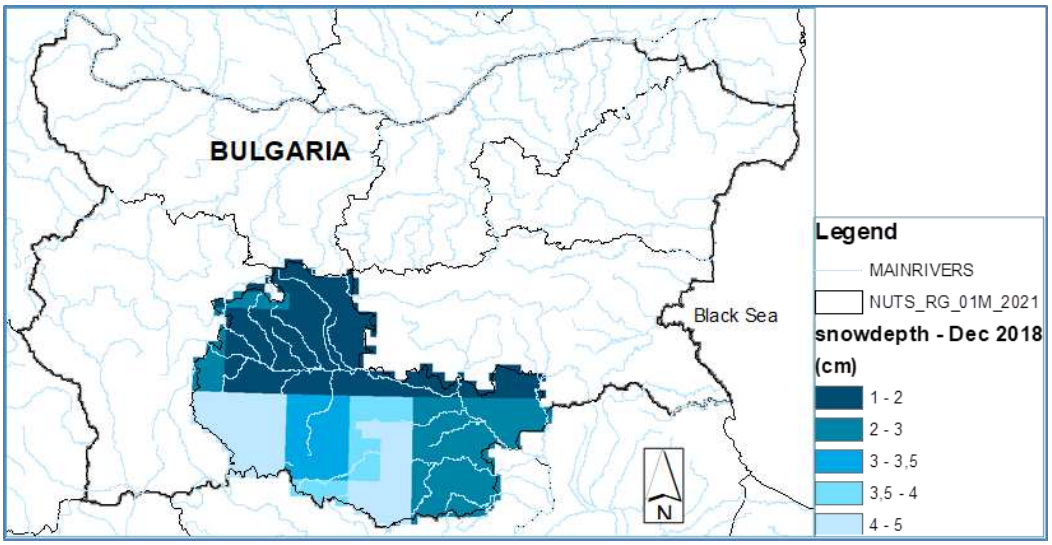

Figure 4. *Map of CLM model calculated average snowdepth on the Bulgarian South Central Region in December 2018*

The calculated for the South-Central Region mean monthly values of rainfall, snowfall, air temperature and snow depth are shown on *Figure 5*. There is a tendency to

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progressive reduction of the snow depth and increase of the air temperature at the end of the period. The average monthly snowdepth is quite small in the last two winter seasons, being at the critical minimum in 2019/2020. Its highest value of 18 cm is in January 2017, when the air temperature is the lowest.

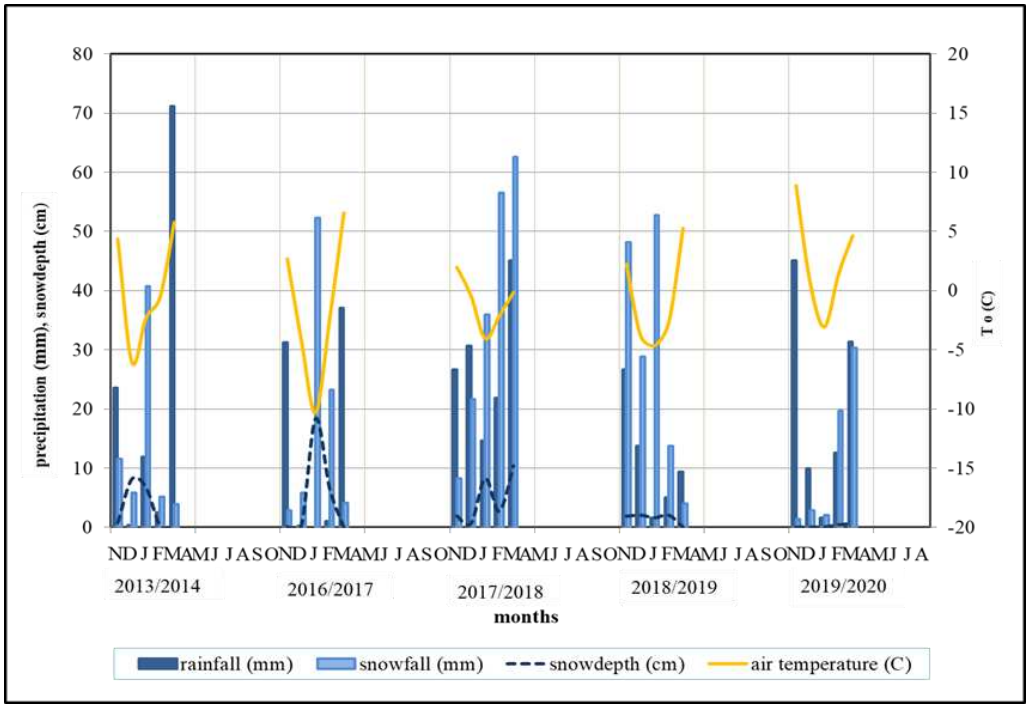

Figure 5. *Model calculated monthly values of rainfall, snowfall, air temperature and snowdepth during winter seasons*

The credibility of the results comes from comparing the trend of change of the modeled snowdepth with the trend of the number of days in the months with snow cover measured in the field and published by NIMH (NIMH, 2013-2020). The distribution of the days in the month with snow cover is in line with the calculated snowfall and snowdepth. The reliability of model results from simulation of winter conditions was tested in another study on the mountain catchment in the upper reaches of the Struma River (Nitcheva et al., 2021). The modeling of the physical and climatic processes in the South Central Region also shows good enough agreement of the calculated data for snow cover with measured one. The comparisons are made with summarized data for the whole region. The estimated degree of realism of the snow characteristics calculated according to the model is sufficient for confidence in the unambiguousness of the survey results.

To facilitate the analysis, the modeled values are averaged over the 5 season months and presented graphically on *Figure 6*. On the same figure shown are the yields of the winter crops – wheat, barley and oats. These data together with the snowfall / precipitation ratio and the annual yields of wheat, barley and oats, average estimates for the whole South Central region, are shown on *Table 1*.

Table 1 and *Figure 6* show the seasonally summarized values of the hydrological and thermal effects in the considered 5-year period, influencing the soil thermal and humidity regime during the growth of the winter crops. The total precipitation except

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for the seasons 2017/2018 is close to the normal for that part of the year but the snowfall/precipitation ratio changes significantly. For the seasons with the least snowfall 2013/2014 and 2019/2020 it is around 38-36%. The air temperature in the last season is rather positive making the snow cover depth and residence time negligible. At the same time the seasons 2016/2017 and 2017/2018 enjoy nearly normal winter climatic conditions with more snow than rain, negative air temperature and satisfactory for the current stage of climate change snow cover. Within this small span of time, these changes can be assumed as significant and they follow the typical pattern accepted as inherent to the ongoing climate conditions.

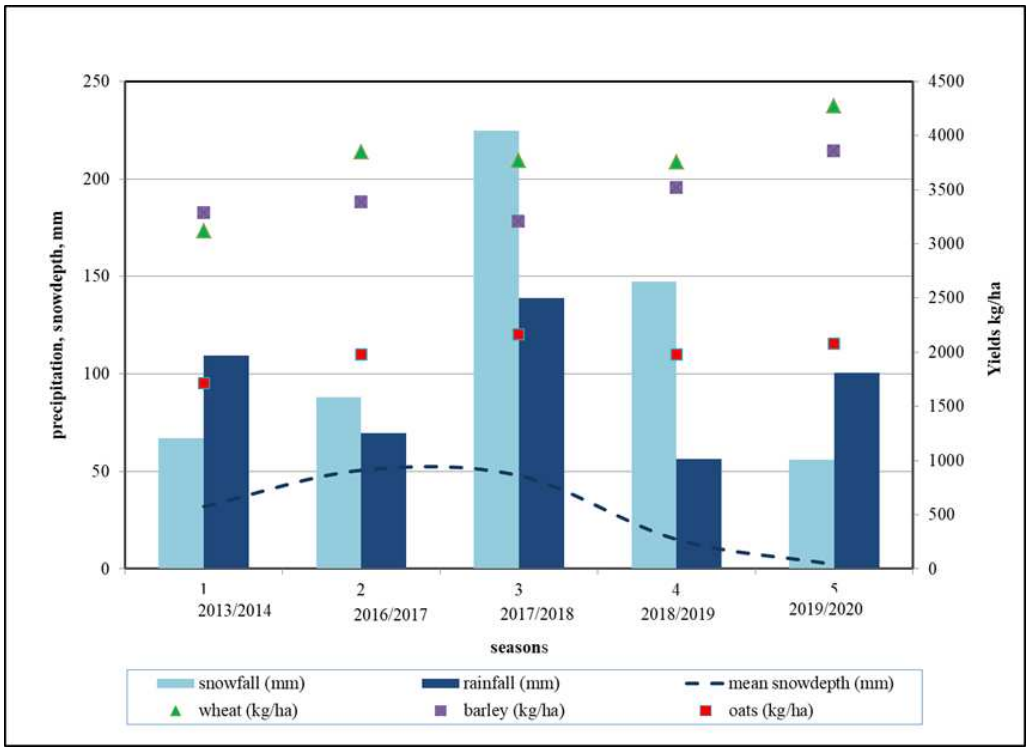

Figure 6. *Model calculated seasonal values of the total snowfall, total rainfall and average snowdepth with the crops yield*

Table 1. *Seasonal precipitation components in mm and air temperature in °C calculated by the model CLM and observed winter crops yield in kg/ha for the South Central Region of Bulgaria*

Winter seasons	2013-2014	2016-2017	2017-2018	2018-2019	2019-2020
Total rainfall (mm)	110	70	139	57	101
Total snowfall (mm)	67	89	225	148	57
Total precipitation (mm)	177	159	364	205	158
Snowfall/precipitation (%)	38	56	62	72	36
Air temperature (°C)	0,3	-1,5	-1,0	-0,7	2,5
Wheat yield (kg/ha)	3479	3849	3768	3754	4272
Barley yield (kg/ha)	3283	3386	3207	3517	3858
Oats yield (kg/ha)	1716	1978	2163	1976	2078

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The yield of the winter crops, which as a rule in Bulgaria are not irrigated, depends on the thermal and moisture regime of the soil during the autumn-winter season. However, the exposed on the *Table 1* yields of wheat, barley and oats in the analyzed 5 winter seasons don't show proportional response to the considered as negative reduction of the snowfall part from the total precipitation amount. Judging from the data on the *Table 1* they are not so sensitive even to the total amount of the precipitation.

Actually the yields of the season 2013/2014 with snowfall much less than the ones in the winter seasons of 2016, 2017 and 2018 are around 10% lower than the yields in these 3 years. Although less pronounced, this result is in line with the logics of the adverse effects of snow reduction on the winter crops growth. On the other hand, noteworthy is the yield of the season 2019/2020, which exceeds the yield of all other considered winter seasons at the same time being with the least snowfall. Most probable explanation for that fact may be the significant rainfall in November (*Fig. 5*) and the positive seasonal mean air temperature, stimulating the early growth and development of the crops.

These conclusions are confirmed by MARS JRC research (bulletin March 2020), based on field and remote satellite observations and model simulations. The CLM model simulations in the present study is in line with those of the European Commission research team (Baruth et al., 2020).

Conclusions

Carried is out an overview study of the impact of the occurring changes in the winter season precipitation pattern and air temperature on the yields of the winter crops in the South Central Region of Bulgaria. The analysis is based on field measurements of climatic data such as total precipitation and air temperature and through a hydrological model estimated snow characteristics for the last 5 winters, covering the whole area.

The general understanding is that the reduction of snowfall and snow cover residence time should lead to a reduction in winter crop yields. The results of the study show that this influence is not strictly observed for the last 5 winter seasons in this region. If it is to some extent confirmed for the season 2013/2014 with little snow, then for the last winter season 2019/2020 with even less snow cover, but with 2^{o}C higher average temperature of air, the yields exceed those of all other seasons. One of the reasons for this phenomenon is supposed to be the high mean air temperature of the season and the monthly distribution of the precipitation with significant rainfall in November.

The main conclusion from the study is that the changes in the main parameters of the climate such as the decrease in the snow part of the precipitation and the increase in air temperature, as well as their distribution in the winter months have divergent effects on winter crops. Their yield depends on the favorable combination of air temperature with the amount, type and monthly distribution of precipitation, which can reduce the role of snow as a determining factor for good yields. The conclusion is in accordance with the agricultural logic. It shows the importance of the reliability of long-term weather forecasts facilitating adequate solutions in the cultivation of winter crops.

Studies with similar purpose, using information from ground observations for precipitation and air temperature and obtained by hydrological models snow amount and residence time, should be performed for other agricultural regions of the country. They will serve for a detailed analysis of the amount and nature of changes in these basic hydro-climatic parameters and in the soil moisture and infiltration as well. As a

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result, their influence on the cultivation and harvesting of winter crops will be clarified. These assessments will be useful for improving the current agricultural practice in the country and for its adaptation to the ongoing agro-climatic changes.

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META-ANALYSIS OF THE POSITIVE EFFECT OF GINGER FEED ADDITIVE ON HEALTH AND PRODUCTION INDICES OF LAYING HENS

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Abstract. Meta-analysis of the effects of ginger (*Zingiber officinale*) supplementation on outcome measures (health and production indices) in laying hens was investigated. A database of eighteen studies on the subject was systematically compiled based on set inclusion criteria and were used for the analysis. Outcome measures were pooled using a random-effects model and quantified using standardised mean difference (SMD) at 95% confidence interval (CI). The pooled results indicate that ginger supplementation improved blood antioxidant markers in laying hens. The results also showed that layers on ginger supplementation had significantly better plasma cholesterol concentrations (SMD = -1.140 mg/dl, 95% CI: -1.622 to -0.658) and hen day egg production (SMD = 0.217%, 95% CI: 0.080 to 0.354) compared to the controls. In addition, layers on ginger supplementation had significantly higher egg mass (SMD = 0.245 g egg/hen/day, 95% CI: 0.017 to 0.473) and egg weight (SMD = 0.096 g, 95% CI: 0.029 to 0.163) when compared to the controls, taking cognizance of heterogeneity. Meta-regression found that studied moderators explained most of the sources of variation. This meta-analysis suggests that ginger could improve health indices, hen day egg production, egg mass and egg weight in laying hens.
Keywords: *layers, blood characteristics, egg production, egg quality, data synthesis, meta-regression*

Introduction

The global demand for animal proteins is on the increase due to the rapid growth in human population. To meet up the increasing demand for high-quality animal products, the modern egg production industry needs to develop strategies to improve laying performance at a reduced feed to egg ratio. Feed to egg ratio, calculated as the quantity of feed consumed per unit of eggs is a vital production trait as feed contributes about 60 – 70% of the overall production costs in the egg production industry. Hence, optimizing feed efficiency is the key to a sustainable and profitable egg production enterprise. One of the methods to achieve this is the use of phytogenics. Phytogenics are products derived from plants and included in animal feed to enhance growth rate and productivity. Ginger (*Zingiber officinale*), is a flowering plant widely cultivated in the tropics and is belong to the family Zingiberaceae. It is used as a spice in human food preparation; and is rich in beneficial bioactive compounds and essential oil (Ahn et al., 2002; Akoachere et al., 2002; Dragland et al., 2003). Studies have shown that ginger has antioxidant activity and increases blood flow and nutrient uptake in chickens (Incharoen and Yamauchi, 2009; Kothari et al., 2019). Though the positive effects of ginger supplementation in laying hens have been reported (Incharoen and Yamauchi, 2009; Akbarian et al., 2011), there is consensus that many of these studies lack consistency, as

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results differ greatly from one research station to another (Windisch et al., 2008; Okoro, 2016; Gurbuz and Salih, 2018; Kumar et al., 2019; Ogbuewu and Mbajiorgu, 2020).

Resolving this conflict requires pooling results of individual studies on the effects of ginger on health and productive indices in laying hens using a meta-analysis approach. Meta-analysis is a statistical means of resolving conflicting research results using a quantitative approach. It aggregates data and determine treatment effects that ordinarily may not be detected by the individual study (Van Houwelingen et al., 2002). However, there is scanty information on the effect of ginger feed additive on health and productivity of laying hens using meta-analysis in the literature, hence this research. This meta-analysis is targeted at furnishing the egg production industry and poultry nutritionists of the needed information on the effects of ginger feed additive and explanatory variables (ginger presentation form, supplementation level and duration, chicken age, and breed/strain/lines) on health and productive indices of laying hens.

Materials and methods

Search strategy and inclusion criteria

A literature search of peer-reviewed original articles on the effect of ginger intervention on health and productive markers of laying hens was performed in Scopus, Google Scholar and AGORA using different search queries (OR, AND, $, and *) and keywords (ginger, "laying hens, egg production, antioxidant and blood markers). The reference lists of relevant papers identified during the search were manually screened for eligible studies. We conducted a manual search of papers published in offline journals. The included papers met the following criteria: (i) the title was on the effect of ginger on all, or any of the outcome measures: feed intake (FI), feed to egg ratio (F:E), blood makers (glutathione peroxidase, total antioxidant capacity, malondialdehyde or superoxide dismutase, packed cell volume, red blood cell, cholesterol, total protein, calcium or phosphorus), egg production and egg quality in laying hens. (ii) studies were peer-reviewed and published in English, (iii) included studies that have both control and experimental treatments, and administered ginger as extract, oil, or powder, (iv) diet is free of growth promoters and (v) trial reported the mean, number of animals used, and a measure of dispersion. Identified studies were independently assessed for eligibility and disparity was resolved by consensus. The Preferred Reporting Items for Systematic Review and Meta-analysis (PRISMA) flow chart as presented in *Fig. 1* summarizes the search details.

Exclusion criteria

100 papers identified were excluded and five were excluded for being duplicates (*Fig. 1*). Fifty-five studies were also removed from the study for the title not being on any of the measured outcomes and study not published in English, mixed ginger with other plant materials and reported in animal other laying hens. In addition, 22 papers were removed from the remaining 40 studies for lack of randomization and being a review articles.

Data extraction and synthesis

Data presented in graphs were extracted using a ruler while studies that provided standard error (SE) instead of the standard deviation (SD), the SD values were computed

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from the SE using a standard formula (Koricheva et al., 2013). Data on the number of hens included in the control and treatment group, first author's surname, publication year and study location were extracted from each study that met the set inclusion criteria. Information on blood and antioxidant markers such as glutathione peroxidase (GSH-Px), total antioxidant capacity (TAOC), malondialdehyde (MDA) and superoxide dismutase (SOD), performance [feed intake (FI), feed to egg ration (F:E), hen day egg production (HDEP) and egg quality indices] and blood parameters [packed cell volume (PCV), red blood cell (RBC), cholesterol, total protein (TP), calcium (Ca) and phosphorus (P)] of laying hens in control and treatment group were also extracted. In addition, data on covariates (breeds, age of the birds at the beginning of the experiment, presentation form of ginger, duration of ginger supplementation and ginger supplementation levels) of hens in the control and treatment group were also extracted. We contacted 14 authors to supply missing information on housing conditions (natural or controlled environment), essential amino acids (lysine and methionine), cage dimensions, number of hens per cage, ambient temperature and rearing type (deep litter or battery cage), building type (closed or open-sided) however, no response was received from any of the authors and therefore, the influence of these predictor variables on performance of laying hens on ginger intervention was not analysis.

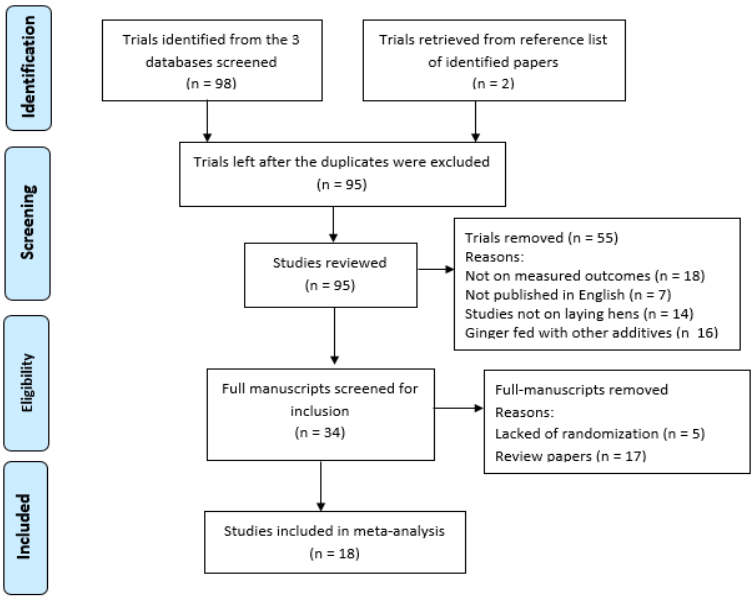

Figure 1. *Flow diagram of the article selection steps*

Statistical analysis

Forest plots and funnel graphs were built from dataset extracted from the 18 studies that met the set inclusion criteria using OpenMEE software (Wallace et al., 2016). Map was generated in Tableau software (version 2020.2). Bar graphs and pie charts were generated in SPSS 20.0 (SPSS, 2019). Publication bas as determined using funnel graphs and Rosenberg's fail-safe number (Nfs). Meta-analysis results were considered robust in the presence of publication bias when Nfs > 5 (n = number of layers in each treatment group) + 10 (Jennions et al., 2013; Ogbuewu et al., 2020). Publication bias was not performed on outcomes with less than 10 studies (Egger et al., 1997). Heterogeneity

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across trials was computed using the Cochran Q test and I^2 – index (Higgins et al., 2003; Higgins and Deeks, 2011). Subgroup analysis, meta-regression and funnel graph were not conducted in measured outcomes with less than 10 studies because of the report that test power from such analysis is usually low (Borenstein et al., 2009; Higgins and Green, 2009). We performed meta-regression in studies with non-significant heterogeneity test because non-significant test for heterogeneity does not guarantee homogeneity across studies used in meta-analysis (Thompson and Higgins, 2002). The diamond at the bottom of the forest plot represents the overall mean estimation (SMD = 0) and is said to be significant when the CI did not include zero (Koricheva et al., 2013). The points to the left of the line of no effect (i.e. where SMD = 0) denote a reduction in the measured outcome and the opposite is the case when the points are to the right of the line of no effect.

Results

Study characteristics

The summary of the 18 studies with 3600 hens that met the set inclusion criteria is presented in *Tables 1-5*. The studies included in this meta-analysis were conducted in 10 locations drawn from four continents: North America (n = 1), Europe (n = 2), Asia (n = 11) and Africa (n = 4) as shown in *Fig. 2* and *Fig. 3*. Out of the 18 articles used for the meta-analysis, 3 studies (16.67%) each were conducted in Nigeria, Iran and China, whereas 2 studies (11.11%) each were performed in Sudan and Japan as described in *Fig. 2*. The articles used for the analysis were published between 2009 and 2020, and span 12 years (*Fig. 4*).

Table 1. *Number of studies and hens included in the analysis*

Outcomes	T^n	Datasets	T^a	T^b	T^c
Antioxidant status					
Glutathione peroxidase (× 10^2U/mL)	2	9	435	840	1275
Malondialdehyde (nmol/mL)	3	11	570	1110	1680
Superoxide dismutase (U/mL)	3	10	570	975	1545
Total antioxidant capacity (U/mL)	3	11	570	1110	1680
Blood indices					
Packed cell volume (%)	2	4	32	72	104
RBC count	2	4	32	72	104
Cholesterol (mg/dl)	7	14	271	537	808
Phosphorus (mg/dl)	2	5	38	96	134
Calcium (mg/dl)	2	5	38	96	134
Total protein (g/dl)	3	5	350	390	740
Performance					
Feed intake (g/hen/day)	14	29	663	1444	2107
Feed to egg ratios (g/g)	12	26	589	1308	1897
Egg Traits					
Hen day egg production (%)	15	30	921	1660	2581
Egg mass (g egg/hen/day)	10	20	527	1144	1671
Egg weight (g)	13	28	949	1713	2662
Egg quality traits					
Haugh unit	10	21	490	934	1424
Egg yolk weight (g)	8	19	327	628	955
Egg yolk cholesterol (mg/g yolk)	3	8	64	168	232
Egg shell thickness (mm)	10	21	490	934	1424
Egg shell weight (g)	5	12	106	238	344

T^n – number of studies included in the meta-analysis; T^a – Number of hens used in the control groups; T^b – Number of hens used in the treatment groups; T^c – Total of number of hens used for the analysis

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Table 2. *Overview of articles used to assess antioxidant ability of ginger in laying hens*

References	Location	Covariates					Outcome
		Breed	Hen's age (week)	PF	DS (day)	SL (%)	
An et al. (2019)	China	Hyline	25	Extract	49	0, 0.1	1, 2, 3, 4
Zhao et al. (2011)	Canada	Hyline	27	Powder	35	0, 0.5	1, 2, 3, 4
Zhao et al. (2011)	Canada	Hyline	27	Powder	35	0, 1	1, 2, 3, 4
Zhao et al. (2011)	Canada	Hyline	27	Powder	35	0, 1.5	1, 2, 3, 4
Zhao et al. (2011)	Canada	Hyline	27	Powder	35	0, 2	1, 2, 3, 4
Zhao et al. (2011)	Canada	Hyline	27	Powder	70	0, 0.5	1, 2, 3, 4
Zhao et al. (2011)	Canada	Hyline	27	Powder	70	0, 1	1, 2, 3, 4
Zhao et al. (2011)	Canada	Hyline	27	Powder	70	0, 1.5	1, 2, 3, 4
Zhao et al. (2011)	Canada	Hyline	27	Powder	70	0, 2	1, 2, 3, 4
Yang et al. (2017)	China	Hyline	40	Powder	28	0, 1	2, 4
Yang et al. (2017)	China	Hyline	40	Powder	56	0, 1	2, 3, 4

1- GSH-Px; 2 - MDA; 3 - SOD; 4 – TAC; PF – presentation form; DS – duration of supplementation; SL – supplementation level

Table 3. *Summary of studies used to assess the effect of ginger on feed intake and FE ratios in laying hens*

References	Location	Covariates					Outcome
		Breed	Hen's age (week)	PF	DS (day)	SL (%)	
Akbarian et al. (2011)	Iran	Hyline	30	Powder	56	0, 0.25	1, 2
Akbarian et al. (2011)	Iran	Hyline	30	Powder	56	0, 5	1, 2
Akbarian et al. (2011)	Iran	Hyline	30	Powder	56	0, 0.75	1, 2
Akanbi et al. (2020)	Nigeria	Isa Brown	24	Powder	56	0, 3	1
Gurbuz and Salih (2018)	Turkey	Atak-S	25	Powder	56	0, 1	1, 2
Gurbuz and Salih (2018)	Turkey	Atak-S	25	Powder	56	0, 2	1, 2
Gurbuz and Salih (2018)	Turkey	Atak-S	25	Powder	56	0, 3	1, 2
Incharoen and Yamauchi (2009)	Japan	Leghorn	24	Powder	140	0, 1	1, 2
Incharoen and Yamauchi (2009)	Japan	Leghorn	24	Powder	140	0, 5	1, 2
Kumar et al. (2019)	India	Leghorn	28	Powder	84	0., 0.5	1, 2
Kumar et al. (2019)	India	Leghorn	28	Powder	84	0, 1	1, 2
Malekizadeh et al. (2012)	Iran	Leghorn	103	Powder	63	0, 1	1, 2
Malekizadeh et al. (2012)	Iran	Leghorn	103	Powder	63	0, 3	1, 2
Nasiroleslami and Torki (2010)	Iran	Lohmann		Oil	42	0, 0.03	1, 2
Kumar et al. (2019)	India	Leghorn	28	Powder	84	0., 0.5	1, 2
Okoro (2016)	Nigeria	nr	73	Powder	49	0, 0.5	1, 2
Okoro (2016)	Nigeria	nr	73	Powder	49	0, 1	1, 2
Wen et al. (2019)	China	Hyline	40	Extract	56	0, 0.01	1, 2
Zhao et al. (2011)	Canada	Hyline	27	Powder	70	0, 0.5	1, 2
Zhao et al. (2011)	Canada	Hyline	27	Powder	70	0, 1	1, 2
Zhao et al. (2011)	Canada	Hyline	27	Powder	70	0, 1.5	1, 2
Zhao et al. (2011)	Canada	Hyline	27	Powder	70	0, 2	1, 2
Zomrawi et al. (2014b)	Sudan	Hisex	27	Powder	nr	0, 0.5	1, 2
Zomrawi et al. (2014b)	Sudan	Hisex	27	Powder	nr	0, 1	1, 2
Zomrawi et al. (2014b)	Sudan	Hisex	27	Powder	nr	0, 1.5	1, 2
Yang et al. (2017)	China	Hyline	40	Powder	28	0, 1	1, 2
Yang et al. (2017)	China	Hyline	40	Powder	56	0, 1	1, 2
Sittiya et al. (2017)	Japan	Sonia	53	Powder	56	0, 0.005	1
Sittiya et al. (2017)	Japan	Sonia	53	Powder	63	0, 0.005	1
Soliman and Kamel (2020)	SA	Hisex	35	Powder	70	0, 1	1, 2

SA-Saudi Arabia; nr - not reported; 1 – Feed Intake; 2 - Feed to egg ratio

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Table 4. *Summary of studies included to assess the impact of ginger on blood markers in laying hens*

References	Location	Covariates					Outcome
		Breed	Hen's age (week)	PF	DS (day)	SL (%)	
Akanbi et al. (2020)	Nigeria	Isa Brown	24	Powder	56	0, 3	1, 2, 3, 7
Zomrawi et al. (2014b)	Sudan	Hisex	27	Powder	nr	0, 0.5	1, 2, 3, 4, 5, 6, 7
Zomrawi et al. (2014b)	Sudan	Hisex	27	Powder	nr	0, 1	1, 2, 3, 4, 5, 6, 7
Zomrawi et al. (2014b)	Sudan	Hisex	27	Powder	nr	0, 1.5	1, 2, 3, 4, 5, 6, 7
Malekizadeh et al. (2012)	Iran	Leghorn	103	Powder	63	0, 1	4, 5, 6
Malekizadeh et al. (2012)	Iran	Leghorn	103	Powder	63	0, 3	4, 5, 6
Akbarian et al. (2011)	Iran	Hyline	30	Powder	56	0, 0.25	6
Akbarian et al. (2011)	Iran	Hyline	30	Powder	56	0, 5	6
Akbarian et al. (2011)	Iran	Hyline	30	Powder	56	0, 0.75	6
Nasiroleslami and Torki (2010)	Iran	Lohmann		Oil	42	0, 300	6, 7
Okoro (2016)	Nigeria	nr	73	Powder	49	0, 0.5	6
Okoro (2016)	Nigeria	nr	73	Powder	49	0, 1	6
Wen et al. (2019)	China	Hyline	40	Extract	28	0, 0.01	6
Wen et al. (2019)	China	Hyline	40	Extract	56	0, 0.01	6
Soliman and Kamel (2020)	SA	Hisex	35	Powder	70	0, 1	6

1- Hb, 2- PCV; 3- RBC; 4- Calcium; 5- Phosphorus; 6- Cholesterol; 7- Total protein

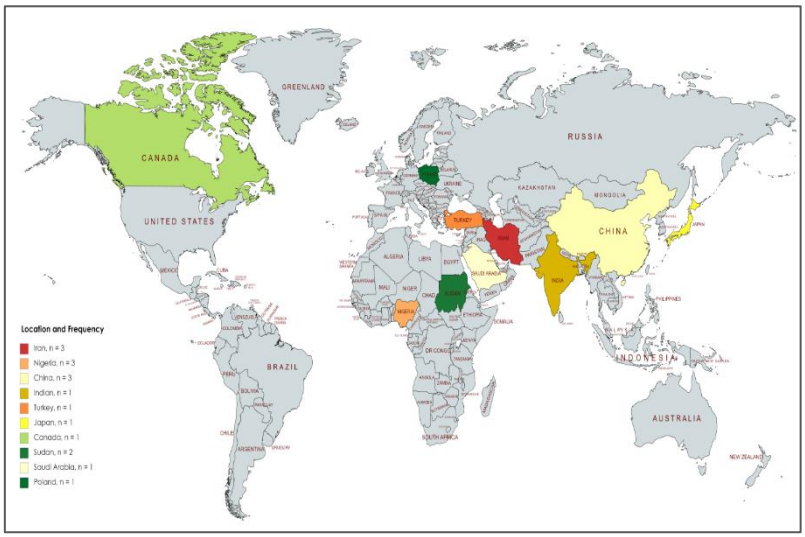

Figure 2. *Study location of papers used for the analysis*

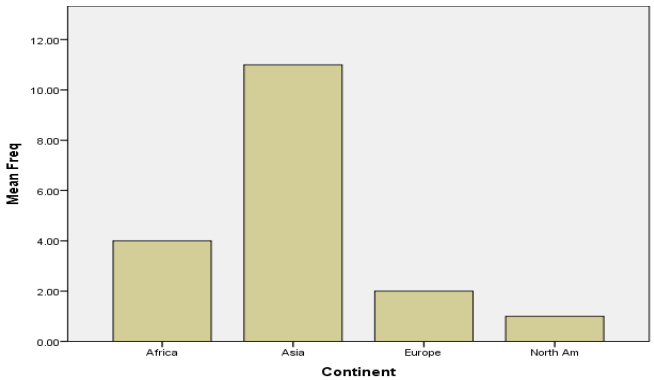

Figure 3. *Bar graph for study continent used for analysis*

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Table 5. *Overview of studies used to explore the impact of ginger on laying performance in hens*

References	Location	Covariates					Outcome
		Breed	Hen's age (week)	PF	DS (d)	SL (%)	
Akbarian et al. (2011)	Iran	Hyline	30	Powder	56	0, 0.25	1, 2, 7, 8
Akbarian et al. (2011)	Iran	Hyline	30	Powder	56	0, 0..5	1, 2, 7, 8
Akbarian et al. (2011)	Iran	Hyline	30	Powder	56	0, 0.75	1, 2, 7, 8
Akanbi et al. (2020)	Nigeria	Isa-brown	24	Powder	56	0, 3	1
An et al. (2019)	China	Hyline	25	Extract	49	0, 0.1	1
Gurbuz and Salih (2018)	Turkey	Atak-S	25	Powder	56	0, 1	1, 2, 3, 4, 5, 6, 8
Gurbuz and Salih (2018)	Turkey	Atak-S	25	Powder	56	0, 2	1, 2, 3, 4, 5, 6, 8
Gurbuz and Salih (2018)	Turkey	Atak-S	25	Powder	56	0, 3	1, 2, 3, 4, 5, 6, 8
Incharoen and Yamauchi (2009)	Japan	Leghorn	24	Powder	84	0, 1	1, 3, 4, 5
Incharoen and Yamauchi (2009)	Japan	Leghorn	24	Powder	84	0, 5	1, 3, 4, 5
Kumar et al. (2019)	India	Leghorn	28	Powder	49	0, 0.5	1, 2, 3
Kumar et al. (2019)	India	Leghorn	28	Powder	49	0, 1	1, 2, 3
Malekizadeh et al. (2012)	Iran	Leghorn	103	Powder	63	0, 1	1, 2, 3
Malekizadeh et al. (2012)	Iran	Leghorn	103	Powder	63	0, 3	1,2, 3
Nasiroleslami and Torki (2010)	Iran	Lohmann	nr	Oil	42	0, 0.03	1, 3, 4, 5, 6
Okoro (2016)	Nigeria	nr	73	Powder	49	0, 0.5	1, 2, 4, 5, 7, 8
Okoro (2016)	Nigeria	nr	73	Powder	49	0, 1	1, 2, 4, 5, 7, 8
Wen et al. (2019)	China	Hyline	40	Extract	28	0, 0.01	1, 3, 4, 5
Wen et al. (2019)	China	Hyline	40	Extract	28	0, 0.01	4, 5
Zhao et al. (2011)	Canada	Hyline	27	Powder	70	0, 0.5	1, 2, 3
Zhao et al. (2011)	Canada	Hyline	27	Powder	70	0, 1	1, 2, 3
Zhao et al. (2011)	Canada	Hyline	27	Powder	70	0, 1.5	1, 2, 3
Zhao et al. (2011)	Canada	Hyline	27	Powder	70	0, 2	1, 2, 3
Zomrawi et al. (2014b)	Sudan	Hisex	27	Powder	nr	0, 0.5	1, 2, 4, 5, 6, 7, 8
Zomrawi et al. (2014b)	Sudan	Hisex	27	Powder	nr	0, 1	1, 2, 4, 5, 6, 7, 8
Zomrawi et al. (2014b)	Sudan	Hisex	27	Powder	nr	0, 1.5	1, 2, 4, 5, 6, 7, 8
Yang et al. (2017)	China	Hyline	40	Powder	28	0, 1	1, 2, 3, 4, 5, 8
Yang et al. (2017)	China	Hyline	40	Powder	56	0, 1	1, 2, 3, 4, 5, 8
Sittiya et al. (2017)	Japan	Sonia	53	Powder	56	0, 0.005	1, 3, 4, 5, 6, 8
Sittiya et al. (2017)	Japan	Sonia	53	Powder	63	0, 0.005	1, 3, 4, 5, 6, 8
Soliman and Kamel (2020)	SA	Hisex	35	Powder	70	0, 1	1, 2, 3
Damaziak et al. (2018)	Poland	Isa brown	16	Extract	112	0, 0.04	2, 4, 5, 8
Udeh et al. (2018)	Nigeria	Isa brown	35	Powder	30	0, 2.5	2, 4, 6, 8
Udeh et al. (2018)	Nigeria	Isa brown	35	Powder	30	0, 2.5	2, 4, 6, 8
Udeh et al. (2018)	Nigeria	Isa brown	35	Powder	30	0, 2.5	2, 4, 6, 8

1- HDEP; 2- EW; 3- EM; 4- HU; 5- EST; 6- ESW; 7- EYC; 8- EYW

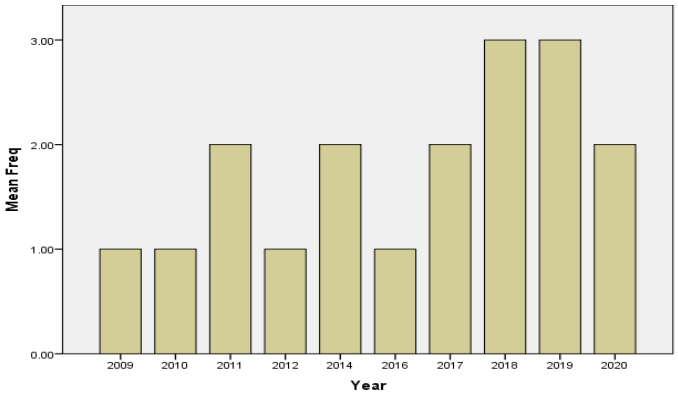

Figure 4. *Bar graph for the years the studies included in the analysis were published*

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Antioxidant capacity

Pooled estimate of the effect of ginger on blood antioxidant markers as presented in *Table 6* suggests that ginger intervention significantly increased GSH-Px (SMD = 9.485 U/mL; 95% CI: 9.148 to 9.822), TAOC (SMD = 10.03 U/mL; 95% CI: 9.466 to 10.593), SOD (SMD = 9.642 U/mL; 95% CI: 9.388 to 9.895) and reduced MDA (SMD = -2.092 nmol/mL; 95% CI: -2.863 to -1.320) in laying hens compared to controls. There was significant heterogeneity amongst the articles used to determine the impact of ginger intervention on the concentrations of MDA (I^2 = 98.62; p < 0.001) and TAOC (I^2 = 79.99; p < 0.001) in the plasma of laying hens. Studies used to evaluate the impact of ginger intervention on blood antioxidant markers were less than 10 studies (*Table 1*), as a result, we could not proceed to determine the relationships between blood antioxidant outcomes and our chosen covariates using stratified subgroup and meta-regression analysis.

Table 6. *Blood GSH-Px, TAOC, MDA and SOD in laying birds administered ginger*

Outcome	SMD	95% CI		SE	P-value	Q	df	I^2 (%)	*P-value
		Lower	Upper						
GSH-Px, U/mL	9.485	9.148	9.822	0.172	0.001	12.96	8	38.28	0.113
MDA, nmol/mL	-2.092	-2.863	-1.320	0.394	<0 .001	276.34	10	98.62	< 0.001
SOD, U/mL	9.642	9.388	9.895	0.129	<0 .001	4.475	9	0.00	0.877
TAOC, U/mL	10.03	9.466	10.593	0.288	< 0.001	49.98	10	79.99	< 0.001

*P-val – Probability value for heterogeneity; SE – Standard error; I^2 – Heterogeneity; GSH-Px - Glutathione Peroxidase; TAOC - Total Antioxidant Capacity; MDA – Malondialdehyde; SOD - Superoxide Dismutase

Blood markers

Table 7 indicated significant effect of ginger intervention on RBC (SMD = 1.573 × 10^{12}/L; 95% CI: 0.078 to 3.067) and plasma cholesterol level (SMD = -1.140 mg/dl; 95% CI: -1.622 to -0.658) of laying hens in treatment and control groups. Results indicate that ginger supplementation had similar PCV (SMD = 0.130%; 95% CI: -0.426 to 0.691), calcium (SMD = 0.690 mg/dl; 95% CI -0.051 to 1.143), phosphorus (SMD = 0.480 mg/dl; 95% CI: -0.460 to 1.420) and total protein (SMD = 0.498 mg/dl; 95% CI: -0.151 to 1.147) with the controls.

Table 7. *Aspects of blood markers in laying birds administered ginger*

Outcome	SMD	95% CI		SE	P - val	Q	df	I^2 (%)	*P-val
		Lower	Upper						
Packed cell volume (%)	0.13	-0.426	0.691	0.285	0.642	8.27	3	63.74	0.041
RBC (× 10^{12}/L)	1.573	0.078	3.067	0.763	0.039	43.80	3	93.15	< .001
Calcium (mg/dl)	0.690	-0.051	1.431	0.378	0.068	24.37	4	83.59	< .001
Phosphorus (mg/dl)	0.480	-0.460	1.420	0.480	0.317	38.75	4	89.68	< .001
Cholesterol (mg/dl)	-1.140	-1.622	-0.658	0.246	< .001	153.25	13	91.52	< .001
Total protein (g/dl)	0.498	-0.151	1.147	0.331	0.133	37.23	4	89.26	< .001

P-val – Probability value for heterogeneity; SE – Standard error; SMD – Standardised Mean Difference; I^2 – Heterogeneity; RBC- Red blood cell

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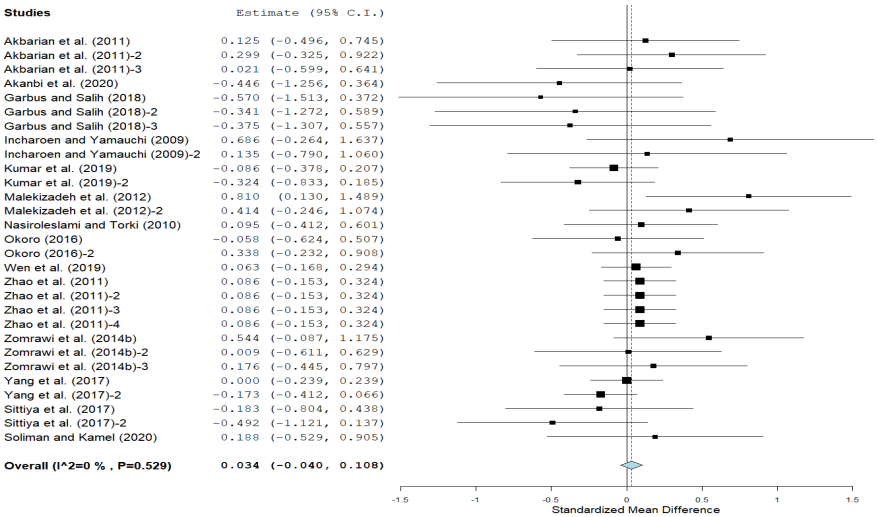
Feed intake and the ratio of feed to egg

The meta-analysis of the influence of ginger intervention on feed intake as presented in *Table 1* was computed with 14 publications that comprised 29 datasets and 2107 layers (663 hens for the control and 1444 hens for the treatment group). Twelve (12) publications with 26 datasets having 1897 layers that comprised 589 hens for the control group and 1308 hens for the treatment groups were used to assess the impact of ginger intervention on feed to egg ratio. The overall pooled result indicated that ginger intervention had no significant influence on feed intake (SMD = 0.034 g/hen/day; 95% CI: -0.040 to 0.108; *Fig. 5a*) and feed to egg ratio (SMD = -0.147; 95% CI: -0.307 to 0.013 *Fig. 5b*). There is evidence of significant heterogeneity (I^2 = 69.26%, p < 0.001; *Fig. 5b*) among the articles used to examine the influence of ginger on feed to egg ratios in laying hens. Also, meta-regression analysis results (*Table 8*) indicated that hen's age, duration of supplementation and breed were significant predictors of treatment effect and accounted for the majority of the sources of variations.



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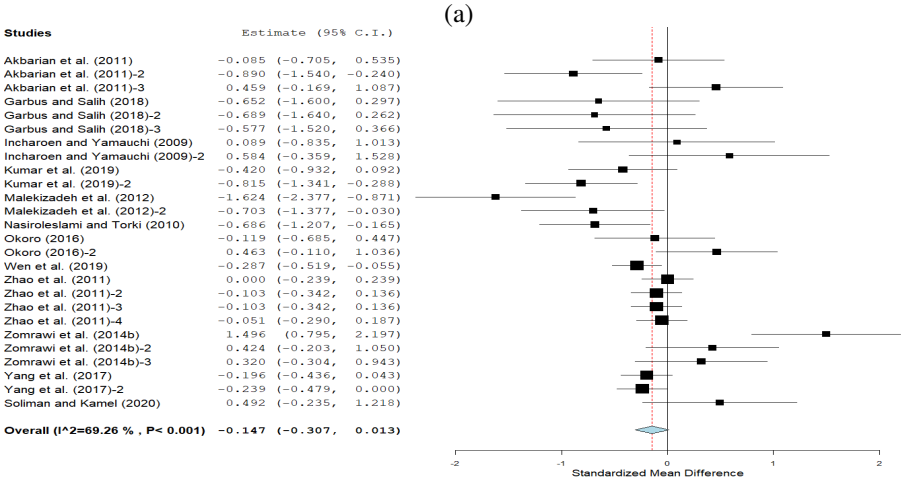

(b)

Figure 5. *Forest plot of the effect of ginger on: (a) feed intake and (b) feed to egg ratio. The thick vertical line is the line of no effect where the mean effect size is equal to zero. The dotted vertical line depicts the point estimate with the diamond at the bottom representing the 95% CI for the overall estimate. The point estimate is considered significant when the CI did not include zero*

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Table 8. *The relationships between the study moderators and ginger treatment*

Parameters	Moderators	Q_B	df	P-val	R^2 (%)
F:E ratio	Supplementation duration (day)	53.80	8	< .001	100.00
	Hen's age (week)	22.90	8	0 .004	55.44
	Supplementation level (%)	5.78	10	0.833	0.00
	Breed	49.00	5	< .001	99.96
HDEP (%)	Supplementation duration (day)	40.00	7	< .001	100.00
	Hen's age (week)	46.69	9	< .001	100.00
	Supplementation level (%)	6.52	11	0.836	0.00
	Breed	56.19	7	< .001	100.00
	Presentation form	0.001	1	0.981	0.00
Egg mass (g egg/hen/day)	Supplementation duration (day)	19.66	6	0.003	55.55
	Hen's age (week)	56.55	7	< .001	91.72
	Supplementation level (%)	3.50	8	0.900	0.00
	Breed	19.46	6	0.003	52.93
	Presentation form	0.046	2	0.977	0.00
Egg weight (g)	Supplementation duration (day)	9.79	8	0.280	99.99
	Hen's age (week)	11.74	8	0.163	100.00
	Supplementation level (%)	19.62	13	0.105	100.00
	Breed	5.02	4	0.285	0.00
	Presentation form	8.11	1	0.004	100.00
Haugh unit	Supplementation duration (day)	8.09	8	0.425	5.29
	Hen's age (week)	13.24	7	0.066	33.46
	Supplementation level (%)	22.10	11	0.024	57.74
	Breed	10.79	6	0.095	28.70
	Presentation form	15.47	2	< .001	62.33
Shell thickness (mm)	Supplementation duration (day)	36.50	8	< .001	74.66
	Hen's age (week)	57.79	7	< .001	91.08
	Supplementation level (%)	13.68	11	0.251	5.99
	Breed	56.18	6	< .001	90.94
	Presentation form	1.18	2	0.556	0.00

Q_B - Coefficient of covariates; df - Degree of freedom; p-val - Probability value; R^2 - Percentage of heterogeneity explained by the studied covariates

Egg production outcomes

A meta-analysis of 15 studies (*Table 1*) with 30 datasets having 2581 layers (921 and 1660 for control and ginger treatment, respectively) indicate that ginger significantly increased HDEP when compared with the controls (SMD = 0.217%; 95% CI: 0.080 to 0.354; *Fig. 6a*) with evidence of significant heterogeneity across studies included in the analysis (I^2 = 65.47%, p < 0.001; *Fig. 6a*). Meta-regression analysis (*Table 8*) results showed that hen's age, breed and duration of ginger supplementation had significant predictors of the treatment effect. The R^2 index which is used to quantify the proportion of variance explained by covariates explained majority of the heterogeneity. Furthermore, the point estimate of 10 studies, 20 datasets (*Table 1*), with 1671 layers having 527 hens for control and 1144 hens for the treatment group indicate that ginger significantly increased EM in comparison with controls (SMD = 0.245 g egg/hen/day, 95% CI: 0.017 to 0.473; *Fig. 6b*) with evidence of significant heterogeneity (I^2 = 83.64%, p < 0.001; *Fig. 6b*). *Table 8* indicated that chicken breed, hen's age and duration of ginger

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supplementation were predictors of the treatment effect and accounted for most of the heterogeneity. On the other hand, meta-analysis of 13 publications with 28 datasets (*Table 1*) indicate that ginger intervention increased EW (SMD = 0.096 g, 95% CI: 0.029 to 0.163; *Fig. 6c*) compared to the control, with proof of substantial heterogeneity (I^2 = 0%, p = 0.499; *Fig. 6c*). We also found significant relationships between ginger treatment and ginger presentation form for EW as presented in *Table 8*.

Egg quality characteristics

Overall pooled estimate of 10 articles having 21 datasets with 1424 laying chickens (i.e. 490 and 934 hens for control and treatment group, respectively) as described in *Table 1* showed that birds ginger supplementation had comparable HU (SMD = 0.123; 95% CI: -0.079 to 0.326; *Fig. 7a*) with the controls with evidence of significant heterogeneity (I^2 = 74.08%, p < 0.001; *Fig. 7a*). Meta-regression analysis of the explanatory moderator variable found that dosage (supplementation level) and ginger presentation form were predictors of the impact of ginger on HU (*Table 8*). Similarly, eight articles with 19 datasets comprising 955 layers (i.e. 327 hens for the control and 628 hens for treatment group) revealed that ginger intervention had no effect on egg yolk weight compared with the control, SMD = 0.063 g; 95% CI: -0.104 to 0.229; *Fig. 7b*) without evidence of significant heterogeneity across studies (I^2 = 47.13%, p = 0.012; *Fig. 7b*). Pooled effect estimate of 3 studies having 8 datasets, with 232 laying hens having 64 and 168 birds for control and ginger group, respectively (*Fig. 7c*) showed that ginger supplementation did not affect egg yolk cholesterol level when compared with the control (SMD = -0.049 mg/g yolk; 95% CI: -0.610 to 0.512) with evidence of significant heterogeneity (I^2 = 84.37%, p < 0.001; *Fig. 7c*). Ten publications having 21 datasets with 1424 layers (i.e. 490 for the control group and 934 for the treatment group) were used analyze the impact of ginger treatment on eggshell thickness (*Table 1*). Results revealed that birds on ginger supplement had similar eggshell thickness with control group (SMD = -0.029 mm; 95% CI: - 0.266 to 0.209; *Fig. 8a*) with evidence of substantial heterogeneity (I^2 = 81.28%; p < 0.001; *Fig. 8a*). *Table 8* found that breed, duration of ginger supplementation and hen's age were significant predictors of the treatment effect and explained the majority of the between-study variance. Also, the mean effect estimate of 5 trials comprising 12 datasets with 344 layers (106 for the control group and 238 for the treatment group) showed that layers on ginger had comparable ESW with the controls (SMD = 0.213 g; 95% CI: -0.051 to 0.477; *Fig. 8b*) without evidence of significant heterogeneity (I^2 = 50.14%, p = 0.024; *Fig. 8b*) across the 5 studies used for the meta-analysis.

Analysis of publication bias

Publication bias is a common issue in a meta-analysis, as it may alter the summary effect estimate of an intervention. Funnel plots asymmetry appeared to be higher in meta-analyses that used few publications. In the present study, analysis of bias was not conducted in studies that assessed the impact of ginger intervention on antioxidant and blood outcomes in laying hens as the number of studies included for their computation was less than ten. Publication bias was assessed for FI. F:E, HDEP, EM, EW, HU and EST using funnel graphs (*Figs. 9a,b and 10a-e*). The funnel graphs obtained in the current study were near asymmetry suggesting the possibility of minimal publication biases, and this could be attributed to a small-study effect, variations in test procedures, study design,

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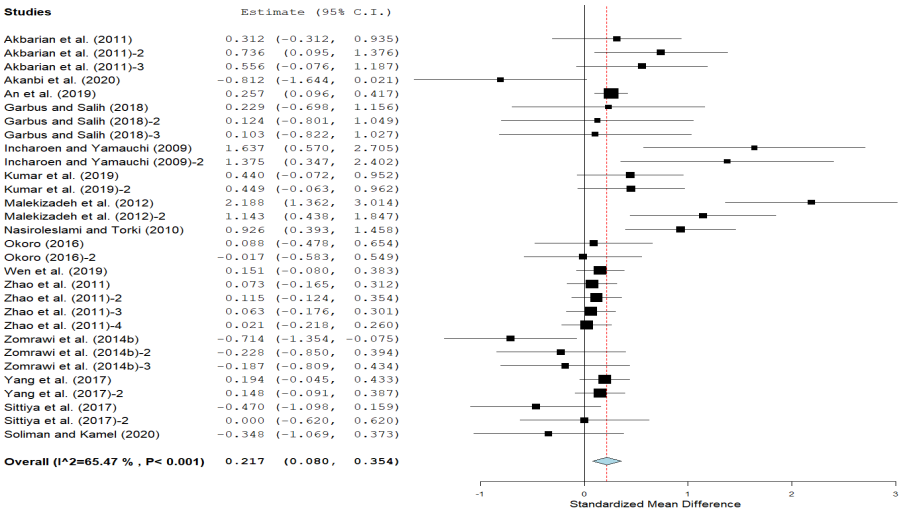
environmental factors and chance. This may also be due to language bias, as the current meta-analysis employed only studies published in English.



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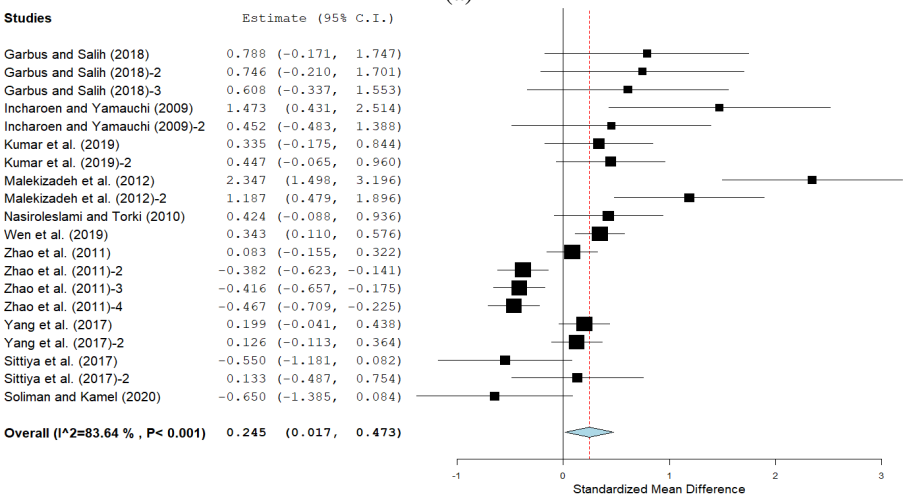

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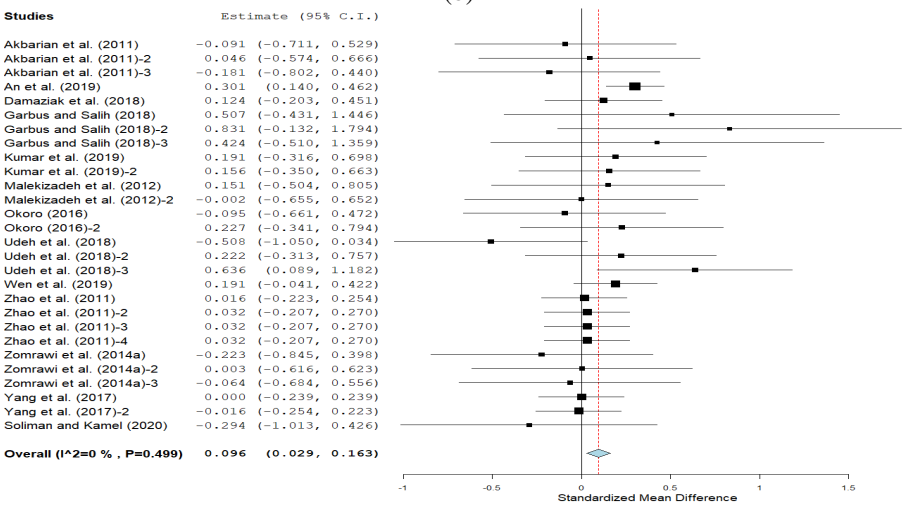

(c)

Figure 6. *Forest plot of the effect of ginger on: (a) HDEP (b) egg mass and (c) egg weight*

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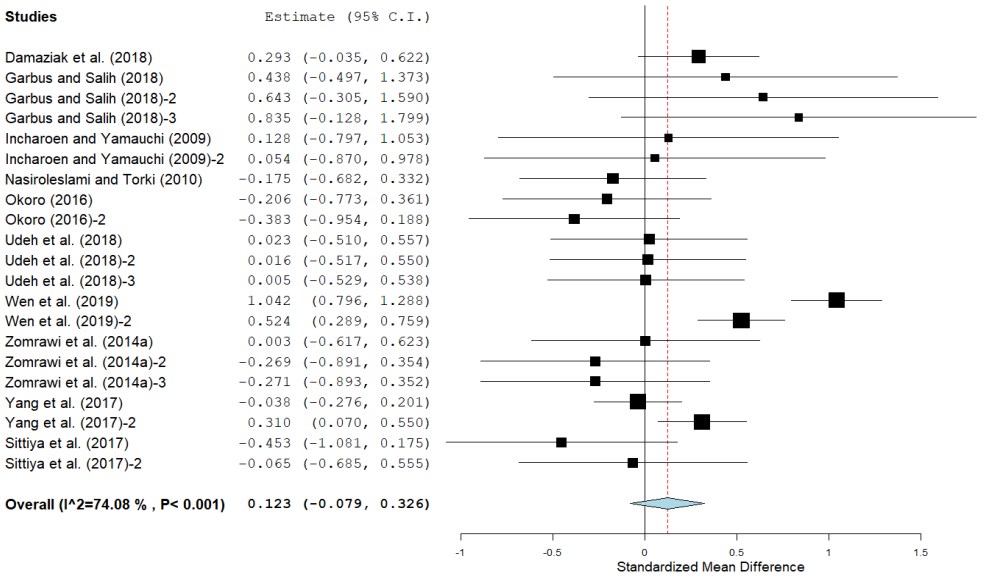

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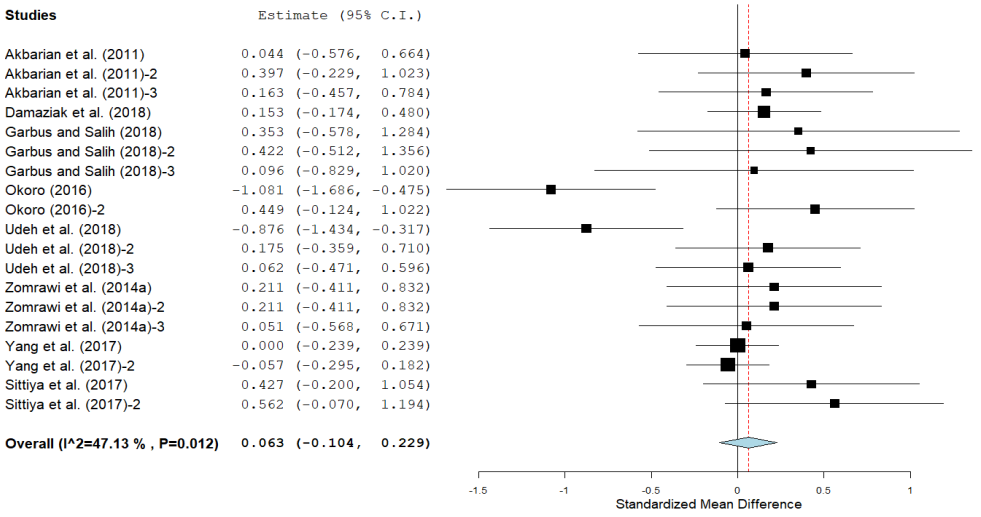

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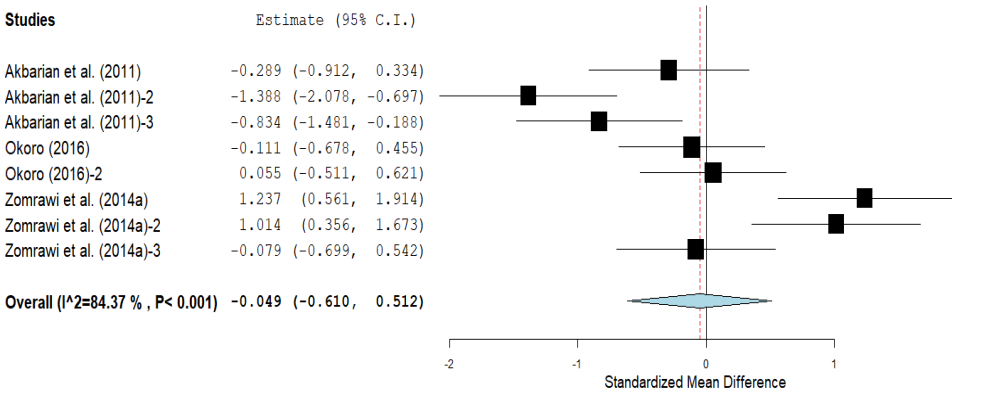

(c)

Figure 7. *Forest plot of the effect of ginger on: (a) Haugh unit; (b) egg yolk weight and (c) egg yolk cholesterol*

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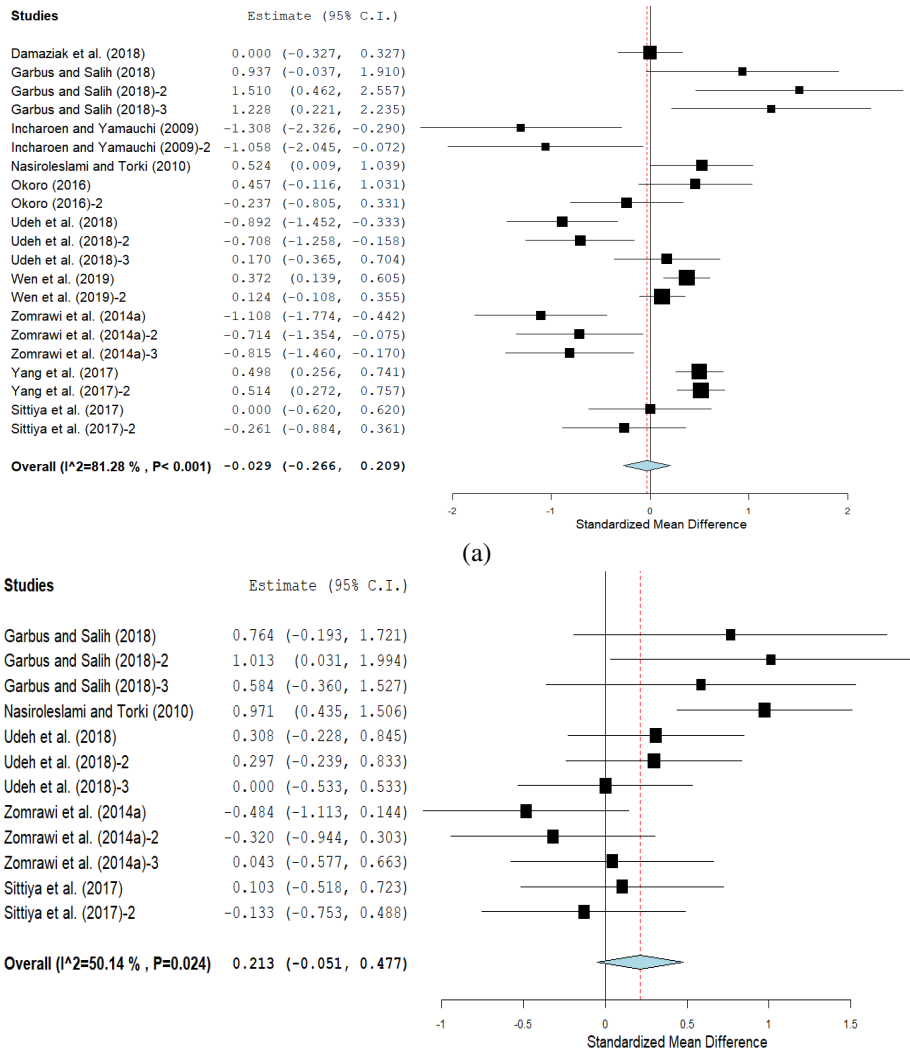

Figure 8. *Forest plot of the effect of ginger on: (a) egg shell thickness and (b) egg shell weight*

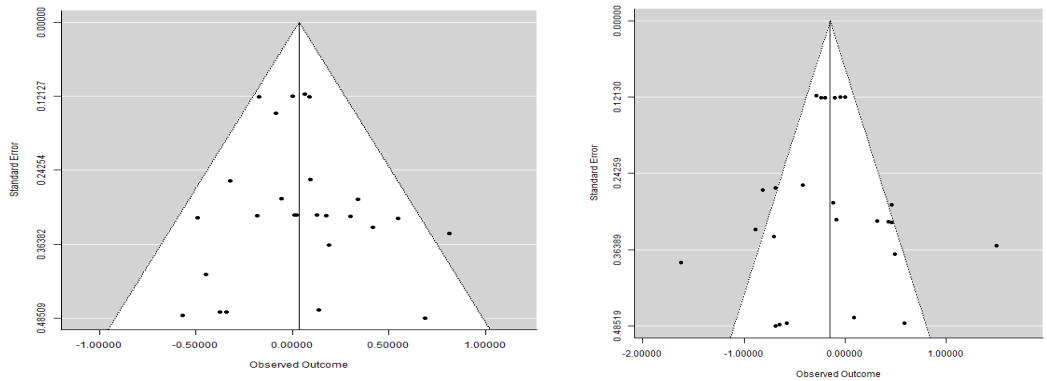

Figure 9. *Funnel graphs of studies assessing the impact of ginger on: (A) feed intake and (B) feed to egg ratios in laying hens*

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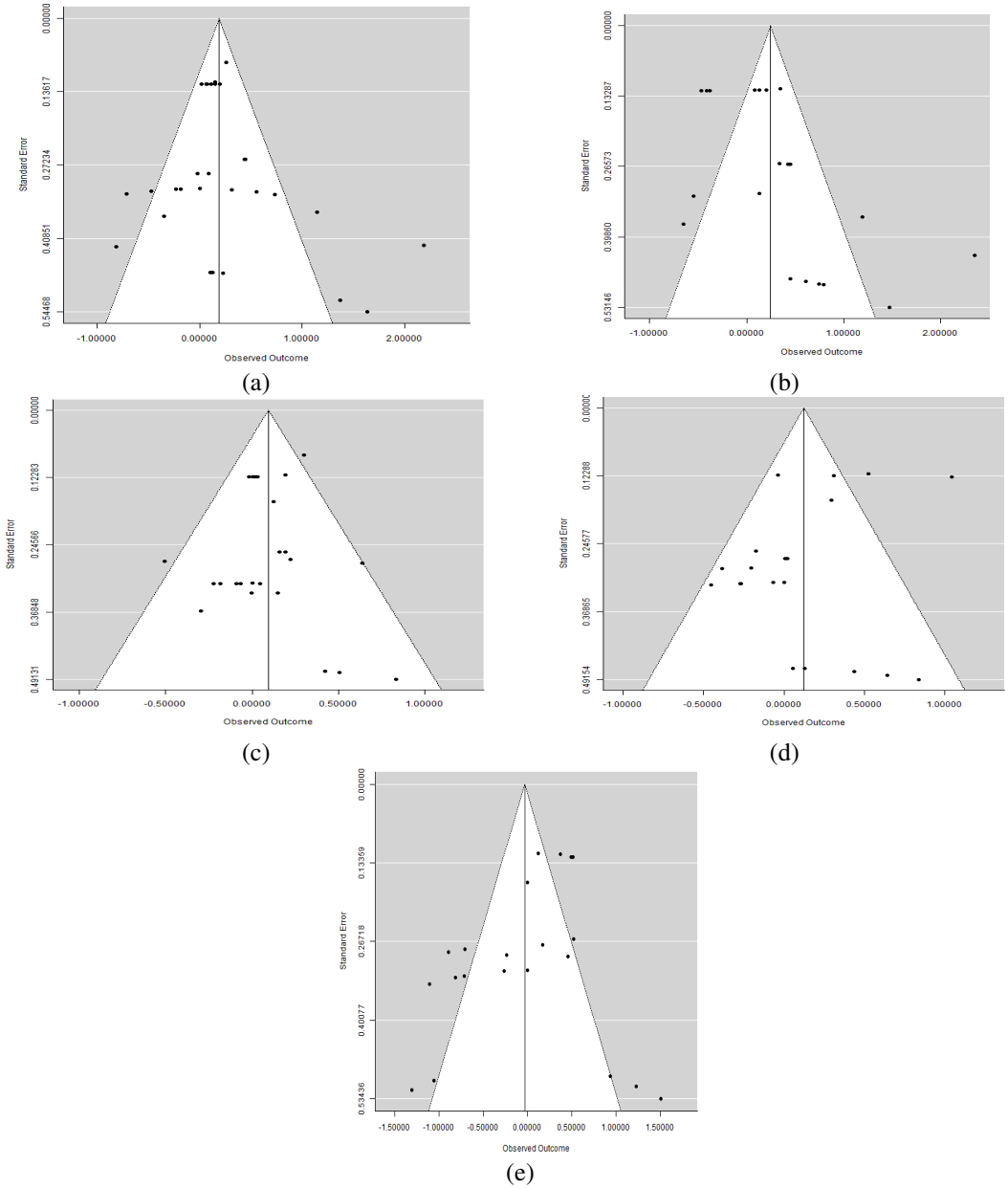

Figure 10. *Funnel graphs of studies assessing the impact of ginger on: (a) HDEP; (b) egg mass; (c) egg weight; (d) Haugh unit; and (e) egg shell thickness in laying hens*

Discussions

Ginger is known to possess several pharmacological actions including anti-inflammatory, anti-microbial and antioxidant properties (Ahn et al., 2002; Akoachere et al., 2002; Dragland et al., 2003). Ginger has been reported to enhance blood flow, feed intake and taste of feed, nutrient uptake and increase the secretion of digestive enzymes in chickens (Incharoen and Yamauchi, 2009; Kothari et al., 2019). Also, ginger has been shown to stimulate the flow of digestive enzymes in small laboratory animal models (Platel and Srinivasan, 1996; Platel and Srinivasan, 2000). Besides the pharmacological actions, ginger is also endowed with beneficial bioactive compounds whose composition and concentration are influenced by the season the rhizome was harvested, storage

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conditions and processing methods (Windisch et al., 2008). Our results indicate that ginger supplement had no effect on feed intake and feed to egg ratios in layers. Similar results have been reported in broiler chickens (Ogbuewu and Mbajiorgu, 2020) and layers (Incharoen and Yamauchi, 2009). Ogbuewu and Mbajiorgu (2020) have found ginger improves feed conversion ratio (FCR) in broiler chickens. However, the observed improved FCR was at variance with the results of this mea-analysis, and the observed disparity may be attributed to the different breeds used. There is presence of substantial heterogeneity amongst the 12 papers used to evaluate the effect of ginger supplement on feed to egg ratio, and meta-regression analysis suggested that hen's age, breed and duration of supplementation explained most of the variations.

The use of tropical medicinal plants with antimicrobial and antioxidant potentials in animal production is on the rise due to the restriction on the use of antibiotics in animal feed (Ahn et al., 2002; Dragland et al., 2003). Antioxidant function is routinely used to assess the physiological status of animals, and MDA, SOD, GSH-Px, and TAOC are the four common markers that reflect the antioxidant ability of chickens. The results of this meta-analysis revealed that ginger products increased the concentrations of GSH-Px, TAOC and SOD and reduced the concentrations of MDA in the plasma of laying hens. The significantly improved GSH-Px, TAOC, MDA and SOD concentration in laying hens on ginger intervention implied that ginger products can improve the antioxidant enzyme system of laying chickens, which is good for the health and productivity of layers. It is well known that cells under anaerobic environments are continuously faced with the dilemma of oxygen paradox. Cells need oxygen to work, but cell metabolites such as reactive oxygen species (ROS) may alter cell functions as well as threaten its survival. To avoid the adverse impacts of excessive generation of ROS, cells possess both enzymatic and non-enzymatic antioxidant defense systems that work together to prevent the harmful influences of ROS from aerobic mechanisms (Ogbuewu et al., 2010). The significantly increased plasma levels of GSH-Px and SOD in layers on ginger products over the controls indicate better cell functions through enhanced ability to mitigate the adverse effect of oxygen free radicals and the significantly reduced MDA implied lower oxidative degradation of lipids also called lipid peroxidation. On the other hand, the elevated blood TAOC that is composed of both enzymatic and non-enzymatic antioxidant defense systems suggests that ginger increased the blood antioxidant capacity of laying hens. Research has also shown that ginger is rich in bioactive substances such as gingerol and its derivatives (Fuhrman et al., 2000; Kota et al., 2008) known to have potent antioxidant ability (Ahn et al., 2002). The better antioxidant defense system of layers on ginger products as indicated by increased concentrations of GSH-Px, TAOC and SOD and reduced MDA in this meta-analysis could suggest the ability of ginger bioactive compounds to stimulate the secretion of both enzymatic and non-enzymatic antioxidant defense systems in laying hens (Fuhrman et al., 2000; Kota et al., 2008). The improved antioxidant markers as observed in layers on ginger treatment over the control layers also suggest the potential of ginger to enhance cell function by reducing the harmful effect of free radical attack through the stimulation of enzymatic and non-enzymatic antioxidant defense systems as corroborated by the increased concentrations of plasma TAOC.

Meta-analysis results of aspects of blood parameters of laying hens on ginger supplementation revealed that ginger significantly increased RBC count in laying hens. The significantly high RBC levels reported in layers on ginger supplementation over the controls suggest the high ability of the ginger treatment to support RBC production in the bone marrow leading to improved health indices and laying performance. In contrast,

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ginger had no significant influence on PCV, plasma calcium, phosphorus and total protein concentration in laying hens. Research has demonstrated the influence of medicinal plant-based diets on hepatic and plasma cholesterol concentration (Yalcin et al., 2006). Cholesterol biosynthesis and secretion in avian species occur principally in the liver (Ryś et al., 1996) and our pooled results showed significantly decreased plasma cholesterol value in laying hens on ginger intervention when compared with the controls. The mechanism of action behind the hypocholesterolemic property of ginger in laying birds is less clear. However, the observed reduction in plasma cholesterol concentration may be ascribed in part to increased uptake of cholesterol, one of the principal egg yolk precursors from the liver, the site of biosynthesis to the ovary where it is utilized for yolk synthesis under the influence of estrogen (Johnson, 2015). Besides the influence of ginger on blood variables as assessed in this study, it is important to note that blood parameters in chickens are strongly associated with the physiological state, genotype, age, environmental factors and sex (Ogbuewu et al., 2015). These factors were not evaluated in this meta-analysis because the requisite data needed to determine their influence on blood profiles of layers were not reported in most of the studies used for the analysis.

Results showed that birds on ginger intervention had higher HDEP with similar feed intake to the control. From an economic viewpoint, the success of the egg production enterprise lies in the total number of eggs laid (Sinha et al., 2018). Although the mechanism of action of ginger on egg production in chickens is not well understood, the observed higher HDEP in layers on ginger supplementation could be attributed to the activities of phytochemicals contained in ginger to enhance the secretion of gastric juices and digestive enzymes as well as increase blood circulation and nutrient uptake from the gut (Platel and Srinivasan, 2000; Ali et al., 2008; Incharoen and Yamauchi, 2009). Another likely explanation for the increased HDEP is the action of ginger to increase the synthesis and secretion of egg precursors such as egg yolk proteins (vitellogenins and apolipoprotein), triglycerides, phospholipids and cholesterol in the liver under the influence of estrogen (Johnson, 2015) leading to improved follicle development and release. Similarly, egg mass and weight were increased in layers on ginger treatment over the control, and this indicates the superiority of ginger over the control. The significantly increased egg mass and egg weight in layers on ginger over the controls may be due to the ability of ginger to balance intestinal microbiota and reduce the production of proinflammatory cytokines in the intestine owing to their antimicrobial properties, which in turn relieve intestinal challenge and immune stress, thus enhancing nutrient uptake of nutrient from the gastrointestinal tract. Even though our analysis found that ginger improved HDEP, egg mass and weight, the influence of other variables like housing temperature, lighting schedule and air velocity known to affect laying performance that are not analysed in this study may not be ruled out completely as these variables affect laying performance (Devi and Reddy, 2005; Parmar et al., 2006; Sinha et al., 2018). Haugh unit used as an index of albumen quality was not significantly influenced by ginger treatment likewise the egg yolk weight and egg yolk cholesterol. There are claims that ginger has positive effects on egg quality attributes in laying hens (Zhao et al., 2011; Okoro, 2016). The lack of significant effects of ginger on egg yolk weight and egg yolk cholesterol could be due to differences in preparation form (i.e. powder, extract and oil), inclusion level, hen's age or breed of chickens used.

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Limitations and strengths of the analysis

This meta-analysis was limited to laying hen studies alone and may not apply to other animal species. The authors encountered few articles on the impact of ginger on antioxidant capacity indices, blood parameters and aspects of egg quality attributes, and therefore it was difficult to run subgroup and meta-regression analysis based on these few studies. The differences in analytical methods used by the individual studies may pose a constraint. Despite these constraints, the strength of this meta-analysis includes a systematic characterization of uncharacterized studies by aggregating data from different studies to resolve uncertainty, identify knowledge gaps, and create new insights on the effect of ginger intervention on health and production outcomes in laying hens.

Conclusion

The results of this study provided vital scientific insight into the positive effect of ginger intervention on health and production markers in laying hens. The results suggest that ginger significantly increased hen day egg production, egg weight and mass in laying hens when compared with the controls. The results also indicate that ginger did not affect feed intake, feed to egg ratio, eggshell thickness, eggshell weight, Haugh unit, egg yolk weight and yolk cholesterol content. Layers on ginger intervention experienced reduced plasma cholesterol content and enhanced red blood cell and blood antioxidant markers in comparison with the control. However, few studies were used for the blood and antioxidant marker assessment, thus these results need to be validated using a large number of studies. The studied covariates influenced the findings of this meta-analysis. Although laying performance in avian species were strongly related to lighting program and ambient temperatures, the evidence-based information on increased egg production and aspects of egg quality in laying hens on ginger intervention over the control may help poultry farmers, animal nutritionists and policymakers in making informed management decision on the use of ginger as feed additive in laying hens.

Implications for further research

Firstly, more research on the long-term effect of ginger intervention on health and laying performance are therefore needed, since the majority of the articles included in this analysis used layers at their first production cycle as this may increase the chance of the adoption of these findings in the commercial egg production enterprise. Secondly, the majority of the trials included in the analysis had missing data on housing type, lighting schedule, housing temperature, cage dimensions and number of layers included in each cell and rearing type (deep litter and battery cage). Thus, it has become vital for authors to report these variables known to influence egg production in laying chickens in their studies. Thirdly, none of the studies used for the analysis reported mortality or its absence. Future research should address this aspect. Fourthly, insufficient data prevented us from determining the effect of ginger presentation form (i.e. powder, extract and essential oil) on laying performance in hens in this meta-analysis. Thus, the current meta-analysis strengthens the call for more studies in this area.

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SPECIES DIVERSITY OF DRAGONFLIES ON THE SANGIHE ISLANDS, NORTH SULAWESI, INDONESIA

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Abstract. The diversity of dragonflies on an island is strongly influenced by habitat degradation, as well as the size and distance from the mainland. Therefore, this study aims to analyze the species diversity of dragonflies in the Sangihe Islands, North Sulawesi, Indonesia. It was carried out in five types of habitats namely forests, waterfalls, dams, agricultural land, and settlements. In each habitat, four-line transects with a length of 100 m each were made and placed around the river. The results showed 6 families which included 32 species and 3020 individuals. Libellulidae is the family with the highest number of species. Furthermore, the dominant species in the suborders Anisoptera and Zygoptera were *Orthetrum pruinosum* and *Rhinocypha frontalis*, respectively. Forests, waterfalls, and dams tend to have the highest species richness and diversity index compared to other habitats. Based on the results, the diversity of dragonflies at the observation site is strongly influenced by the complexity of the vegetation and environmental factors, including temperature and humidity. The presence of the families Chlorocyphidae, Calopterygidae, and Platycnemididae in each of the studied habitats indicates that the water quality is still very good and supports the life of dragonflies.
Keywords: *dams, forests, Libellulidae, waterfalls, Rhinocypha frontalis*

Introduction

Sangihe is the outermost island in North Sulawesi with an area of approximately 11,863.58 km^2 consisting of 736.98 km^2 landmass (6.2%) and 11,126.61 km^2 ocean (93.8%). Geographically, the Sangihe Islands regency is located at 4° 4’ 13” and 4° 44’ 22” north latitude - 125° 9’ 28” and 125° 56’ 57” east longitude between the Sulawesi and Mindanau (Philippines) islands. It consists of 105 large and small islands of which 26 are inhabited and 79 are uninhabited (Setiawan et al., 2016). These Islands have several types of ecosystems including forests, plantations, rivers, beaches, seas, and others, each ecosystem is inhabited by various flora and fauna such as dragonflies.

Dragonflies, an Odonata species, occupy forests, agricultural land, lakes, rivers, and other aquatic habitats (Kanaujia et al., 2015). The number of Odonata species including damselflies and dragonflies found in the world is approximately 5900 (Van Tol, 2000; Potapov et al., 2020). In Indonesia, approximately 700 species of dragonflies have been discovered and some are endemic to North Sulawesi (Murwitaningsih et al., 2019), which reportedly has over 143 (Van Tol, 2000). These insects tend to colonize a wide array of extreme environments such as hot springs, sulfidic, acidic, alkaline, and hypoxic water bodies, as well size ephemeral wetlands, pools, salt-water wetlands, and mangroves (Potapov et al., 2020).

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Furthermore, dragonflies are an important component in maintaining the balance of ecosystems and biodiversity (Monteiro-Júnior et al., 2014; Saha and Gaikwad, 2014; Das et al., 2012; Harabiš and Dolný, 2012; Willigalla and Fartmann, 2012; Siregar and Bakti, 2016). The nymphs produced by these insects function as predators in aquatic ecosystems, while the adult prey on pests of agricultural crops, hence, they are used as biological control agents (Willigalla and Fartmann, 2012; Agus et al., 2017). Adult dragonflies often lay eggs in clean water, hence, they need a natural habitat to support daily living. These insects are also used as bioindicators of forest and aquatic environmental quality (Dolný et al., 2011; Ilhamdi et al., 2020; Das et al., 2012).

Information on the distribution and diversity of flora and fauna in different habitat types at the local and regional levels is key to biodiversity conservation. Several studies on the diversity of dragonflies have been carried out, particularly in relation to ecosystems and as environmental indicators (Dolný et al., 2011; Mapi-ot et al., 2013; Kannagi et al., 2016).

In Indonesia, studies on dragonfly diversity have been conducted in various areas such as in Central Kalimantan Province (Hendriks, 2020), Genus Orthetrum in the Mount Prau Nature Reserve, Central Java Province (Setyawati et al., 2017), Asmat and Mappi Papua Regencies (Kaize and Kalkman, 2011), as well as in the Cibodas Botanical Gardens, West Java Province (Murwitaningsih et al., 2019). Moreover, the community structure and diversity of Odonata were investigated at the West Lombok Nature Tourism Park, West Nusa Tenggara Province (Ilhamdi et al., 2020), while the variety and composition of the dragonflies community were analyzed in the Tangkoko Nature Reserve, North Sulawesi Province (Koneri et al., 2017). A similar study was also conducted in Bogani Nani Wartabone National Park North Sulawesi (Nangoy and Koneri, 2017), as well as in the Tunan waterfall area, North Minahasa, North Sulawesi Province (Koneri et al., 2020), and in Bawean Islands Nature Reserve, East Java Province (Rohman et al., 2020).

Meanwhile, studies on dragonfly diversity in North Sulawesi Province were mostly carried out on the mainland and only a few were conducted on the islands. Furthermore, there are no studies on Odonata diversity in the outer islands of this province including Sangihe. The islands are experiencing problems due to forest destruction, conversion of forest habitats into agricultural areas, as well as a decrease in water quantity and quality (Lee et al., 2001). These conditions tend to reduce dragonflies diversity, therefore, this study aims to analyze the diversity of dragonflies species in the Sangihe Islands, North Sulawesi, Indonesia. The results are expected to become basic data for the conservation of dragonflies diversity and species in the Sangihe Islands.

Material and methods

Study area and land-use types

Sampling was carried out on adult dragonflies from May to August 2021 in the Sangihe Islands, North Sulawesi Province, Indonesia (*Table 1; Fig. 1*). It was conducted along the river in 5 types of habitats, consisting of forests, waterfalls, dams, plantations, and settlements. Forest is a natural habitat with a complex vegetation structure dominated by various types of trees such as *Ficus sp* (Moraceae), *Alstonia macrophylla* (Rubiaceae), *Litsea* sp. (Lauraceae), and *Garcinia* sp. (Fagaceae). Furthermore, waterfalls are habitats with vegetation dominated by *Ficus* sp. (Moraceae), ferns, and grasses *(Poaceae),* while dam is a river flow for hydroelectric power generation and is

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dominated by *Ficus* sp. (Moraceae), *Litsea* (Lauraceae), bush vegetation (Astreaceae), and grasses *(Poaceae),* as well as teki (*Cyperaceae* sp.). Plantations is managed by the community and are planted with various crops such as clove (*Eugenia aromatica*), coconut (*Cocos nucifera*), banana (*Musa* sp.), and nutmeg (*Myristica fragrans*). Meanwhile, settlement habitats are rivers flowing around residential areas and vegetated with grass (*Poaceae*) (*Fig. 2*).

Table 1. *The sampling habitats and coordinates on the Sangihe Island*

Habitats	Transect	Latitude (N)	Longitude E	Habitats	Transect	Latitude (N)	Longitude E
Dam	DM1	03°30'09.14"	125°31'45.87"	Plantation	PT3	03°26'30.31"	125°36'02.34"
Dam	DM2	03°30'06.30"	125°31' 44.10"	Plantation	PT4	03°26' 27.50'	125°36'01.40"
Dam	DM3	03°30'03.45"	125°31'42.44"	Settlement	ST1	03°27'33.20"	125°30' 35.20"
Dam	DM4	03°30'01.41"	125°31'41.87"	Settlement	ST2	03°27' 37.50"	125°30'33.90"
Forest	FR1	03°30'11.21"	125°31'45.64"	Settlement	ST3	03°26'09.62"	125°36'00.85"
Forest	FR2	03°27'08.96"	125°34'47.17"	Settlement	ST4	03°26'05.20"	125°36'05.20"
Forest	FR3	03°27'05.33"	125°34'48.92"	Waterfall	WF1	03°26'59.52"	125°34'53.74"
Forest	FR4	03°26'33.12"	125°35'56.10"	Waterfall	WF2	03°27'01.55"	125°34'49.66"
Plantation	PT1	03°29'51.22"	125°31'44.42"	Waterfall	WF3	03°26'40.89"	125°35'55.47"
Plantation	PT2	03°27'13.38"	125°34'48.50"	Waterfall	WF4	03°26'36.93"	125°35'55.13"

Sampling

The purposive random sampling method was used on forest habitats, dams, waterfalls, plantations, and settlements of the Sangihe Islands. For each of these habitat types, four line transects with a length of 100 m each were made along the river body. The width of the transect was 2 m: 1 m on the edge and 1 m on the water body from the edge in line (Sugiman et al., 2020).

Dragonflies collection was carried out in sunny weather from 8:00 am to 4:00 pm when most of the insects were still active (Renner et al., 2015; Khan, 2017). The sampling of dragonflies was conducted monthly for four months. This was performed along the transect line using insect nets (40 cm, 65 cm deep) with an aluminum handle 90 cm long (Mapi-ot et al., 2013). The observation was carried out directly or using binoculars and cameras while the samples were identified through field guidance. Samples that were not directly identified were caught using insect nets for further identification. The captured dragonflies were placed into kill bottles containing some tissue paper and filled with ether. After death, they were immediately removed from the bottle, dried in the sun, and then stored in triangular paper envelopes measuring 30 x 20 cm with the wings folded above the body (Koneri et al., 2020).

The identification process was carried out based on external morphological characteristics using dragonflies identification books (Watson and O'farrell, 1991; Miller, 1995; Wilson, 1995; Orr and Hämäläinen, 2003; Kalkman and Orr, 2013; Orr and Kalkman, 2015). Subsequently during sampling, environmental parameters such as air temperature, humidity, wind speed, and light intensity were observed. The air temperature and humidity were measured using a thermo-hygrometer (Deko 637 Thermo-hygrometer), while wind speed and light intensity were calculated with the Anemometer and Lux meter, respectively (Lutron LM8010 Type K). Additionally, the coordinates and elevation of the study location were simultaneously recorded using the Global Positional System (Garmin GPSMAP 78s).

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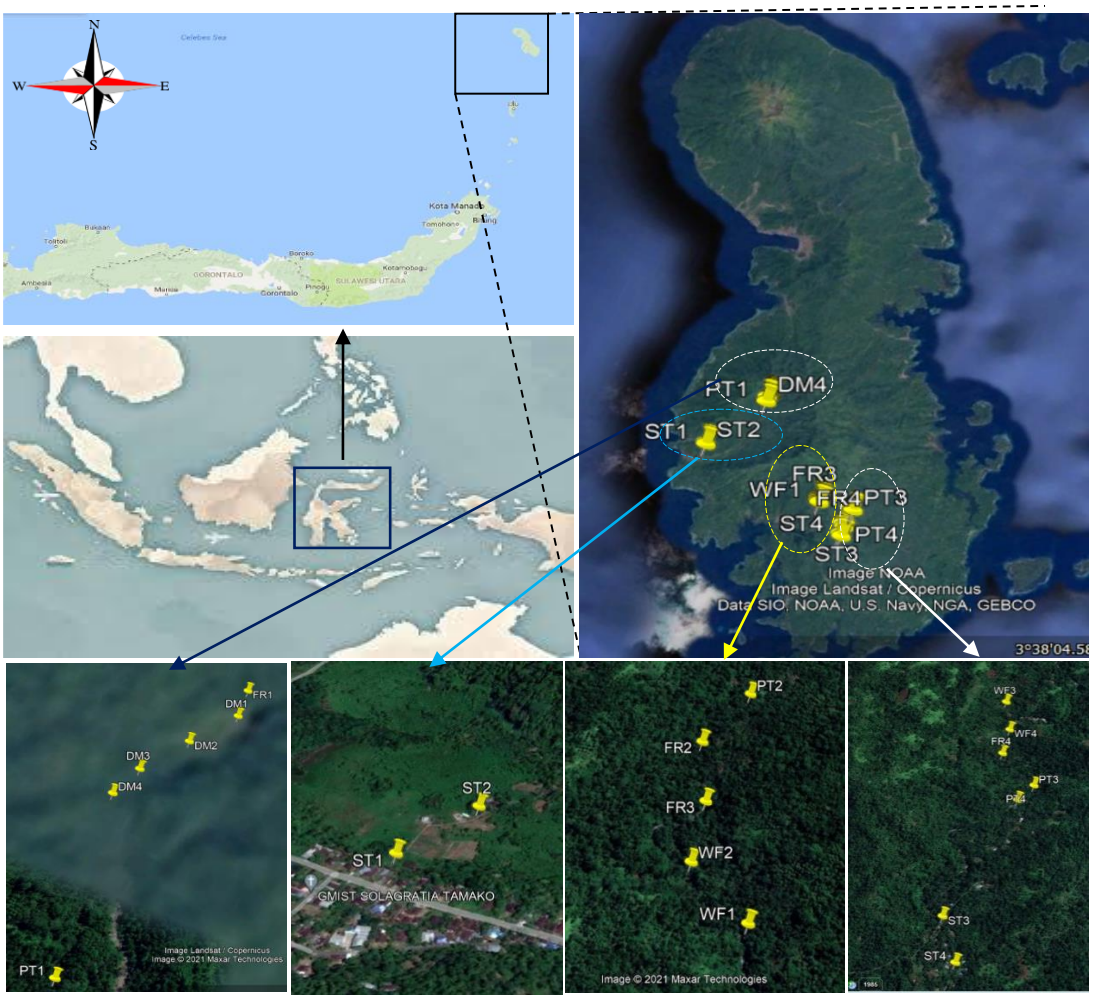

Figure 1. *Map of the study area in Sangihe Islands, North Sulawesi. (FR: forest; DM; dam; WF: waterfall; PT: plantation; ST: settlement)*

Figure 2. *Some photos of sampling site: forest (1); dam (2); waterfall (3); plantation (4); settlement (5)*

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Data analysis

The dragonflies abundance and richness data were tabulated for each habitat. The Rank-abundance curve was analyzed by an Excel program based on the percentage of total individuals, while species richness (Chao1) was used to estimate the number of species in each habitat. Meanwhile, interpolation-extrapolation rarefaction curves of species were created using the software an R package iNEXT (iNterpolation/EXTrapolation) (Hsieh et al., 2016).

Species diversity was calculated using the richness, diversity, and evenness indices. The species richness index was calculated using the formula from Margalef (R1) as follows:

$$RI = \frac{S-1}{LogN} \quad \text{(Eq.1)}$$

Description: R: Richness Index; S: Number of species (n1, n2, n3, ….) and N: total individuals in sampling (Magurran, 1988). Furthermore, the species diversity index was calculated using the Shannon-Wiener index (Omayio et al., 2019) as follows:

$$H' = -\sum_{i=1}^{s}(P_i \ln P_i) \quad \text{(Eq.2)}$$

Description: Pi = ni/N; H': Shannon-Wiener diversity index, ni: number of individuals for each species, N: number of individuals for all species (Magurran, 1988). Meanwhile, the species evenness index was calculated using the Shannon (E) evenness index (Magurran, 2004) as follows:

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

Description: E: Evenness index, H': Shannon-Wiener diversity index, S: number of species (n1, n2, n3, ….).

To test the significant differences in individual abundance, species richness, Shannon diversity, and evenness indices of dragonflies between the five habitats, the one-way ANOVA statistical analysis and Tukey's test at 95% confidence level with Statistica version 6 software were used (Bashir et al., 2019; Ajerrar et al., 2020).

The differences in the sample composition for each habitat were analyzed using analysis of similarity (ANOSIM), while differences between the habitat types were assessed with non-metric dimensional scaling (NMDS). ANOSIM and NMDS were analyzed based on the Bray-Curtis inequality index, while the principal component analysis (PCA) was conducted to determine the relationship between the sampling location and the measured environmental factors. ANOSIM, NMDS and PCA were analyzed using Paleontological Statistics software (PAST software 3.10) (Cuartas-Hernández and Gómez-Murillo, 2015; Wakhid et al., 2021).

Results

Dragonflies composition

Dragonflies found in five habitats on the Sangihe Islands were 2 suborders namely Anisoptera and Zygoptera consisting of 6 families, 32 species, and 3020 individuals. 2

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families were found for Anisoptera including Libellulidae with 16 species and Macromiidae with 1 species, while Zygoptera consisted of 4 families including Platycnemididae with 6, Chlorocyphidae with 4, Coenagrionidae with 4, and Platystictidae with 1 species (*Table 2*).

Table 2. *The list of species of dragonflies in all habitats*

Sub Ordo/Family/Species	Dam	Forest	Plantation	Settlement	Waterfall	Total	%
Anisoptera							
Libellulidae							
Diplacina militaris	1	0	2	1	9	13	0.43
Diplacina sanguinolenta	65	36	52	4	19	176	5.83
Diplacodes trivialis	3	4	0	0	10	17	0.56
Lathrecista asiatica	0	0	0	3	0	3	0.10
Nannophya pygmaea	13	0	1	9	59	82	2.72
Neurothemis manadensi	12	0	2	14	11	39	1.29
Neurothemis ramburii	39	4	13	33	22	111	3.68
Neurothemis stigmatizans	11	0	2	12	24	49	1.62
Neurothemis terminata	2	0	2	4	16	24	0.79
Orthetrum glaucum	17	0	5	0	7	29	0.96
Orthetrum pruinosum	77	9	42	154	47	329	10.89
Orthetrum sabina	1	3	2	21	0	27	0.89
Pantala flavescens	5	0	0	6	5	16	0.53
Tetrathemis irregularis	0	1	0	0	4	5	0.17
Tetrathemis leptoptera	0	3	0	0	2	5	0.17
Tetrathemis platyptera	0	0	0	6	1	7	0.23
Macromiidae							
Macromia melpomene	0	0	0	0	2	2	0.07
Zygoptera							
Chlorocyphidae							
Celebargiolestes cinctus	2	7	0	0	3	12	0.40
Celebargiolestes orri	0	2	0	0	0	2	0.07
Libellago daviesi	59	124	132	59	112	486	16.09
Rhinocypha frontalis	72	124	156	78	151	581	19.24
Coenagrionidae							
Agriocnemis femina	22	1	7	53	4	87	2.88
Pseudagrion crocops	1	11	0	0	0	12	0.40
Pseudagrion pilidorsum	53	51	26	0	62	192	6.36
Pseudagrion ustum	7	1	0	0	0	8	0.26
Platycnemididae							
Nososticta emphyla	3	16	9	0	3	31	1.03
Nososticta flavipennis	37	85	85	54	131	392	12.98
Prodasineura autumnalis	0	7	0	0	0	7	0.23
Teinobasis laidlawi	5	41	0	0	6	52	1.72
Teinobasis rufithorax	3	1	0	0	21	25	0.83
Teinobasis sp	25	40	86	3	28	182	6.03
Platystictidae							
Proposticta simplicinervis	0	9	2	0	6	17	0.56
Total	**535**	**580**	**626**	**514**	**765**	**3020**	**100**

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Zygoptera had the highest abundance in all habitat types namely 60.07%, especially in waterfalls with 25.30%, while Anisoptera only consisted of 1 family of Libellulidae with 11 species. Furthermore, Zygoptera consisted of 6 families including Coenagrionidae with 5 species, Argiolestidae, Calopterygidae, Chlorocypidae, Platycnemididae, and Lestidae with 1 species, respectively.

The percentage composition of the dragonflies family based on the abundance of individuals in each habitat was different. Chlorocyphidae of the sub-order Zygoptera had the highest abundance in forests with 44.3%, followed by plantations 46.0%, and waterfalls 34.8%. Meanwhile, Libellulidae of the suborder Anisoptera was mostly found in dams with 46.0% and settlements 51.9%. Similarly, Macromiidae were the family with the least abundance of individuals namely 0.3% and were only found in waterfalls (*Fig. 3*).

Species from the suborder Zygoptera with the highest abundance were *Rhinocypha frontalis* with 19.24% and *Libellago daviesi* 16.09%. Both species belong to the family Chlorocyphidae, while the species from Zygoptera with the lowest abundance was *Prodasineura autumnalis* (0.23%) (*Fig. 4*).

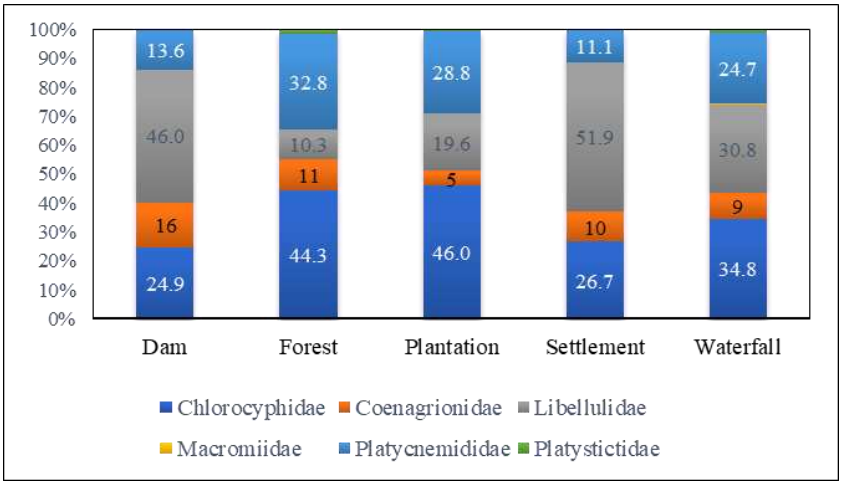

Figure 3. *The relative abundance of dragonflies based on the family in five habitats*

Figure 4. *Several species of dragonflies suborder Zygoptera in five habitat types in the Sangihe Islands. (1-2: Chlorocyphidae: Libellago daviesi (1); Rhinocypha frontalis (2); 3-4: Coenagrionidae: Pseudagrion pilidorsum (3); Agriocnemis femina (4); 5-7: Platycnemididae: Nosoticta flavipennis (5); Teinobasis sp (6); Nosoticta emphyla (7); Platystictidae; Proposticta simplicinervis (8)*

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Furthermore, the species with the highest abundance from the sub-order Anisoptera was *Orthetrum pruinosum* with 10.89%, followed by *Diplacina sanguinolenta* with 5.83%, both species belong to the family Libellulidae (*Fig. 5*). Meanwhile, *Macromia melpomene* was the species with the lowest abundance of 0.07% (*Table 2*).

Based on the results, 8 species were widely distributed in all habitat types, 8 were also found in 4 habitats, while 7 and 5 species were found in 3 and 2 respectively. Moreover, 4 species were only found in one habitat, namely *Lathrecista asiatica* (Anisoptera: Libellulidae) in settlements, *Celebargiolestes orri* (Zygoptera: Chlorocyphidae) and *Prodasineura autumnalis* (Zygoptera: Platycnemididae) in forests, as well as *Macromia melpomene* (Anisoptera: Macromiidae) in waterfalls (*Table 2*).

Figure 5. *Several species of dragonflies sub order Anisoptera in five habitat types in the Sangihe Islands (Libellulidae: Orthetrum pruinosum (1); Diplacina sanguinolenta (2); Neurothemis stigmatizans (3); Nannophya pygmaea (4); Neurothemis ramburii (5); Orthetrum glaucum (6); Orthetrum sabina (7); Diplacodes trivialis (8).*

Species ranking

Among the 32 species of dragonflies found, *Rhinocypha frontalis, Libellago davesi, Nososticta flavipennis,* and *Orthetrum pruinosum* were ranked 1, 2, 3, and 4 with relative abundances of 19.24%, 16.09%, 12.98%, and 10.89, respectively. The next three species between ranks 5-7 had a relative abundance range of 6.36%-5.83%. The low steepness of the curve indicates a high evenness of species (*Fig. 6*).

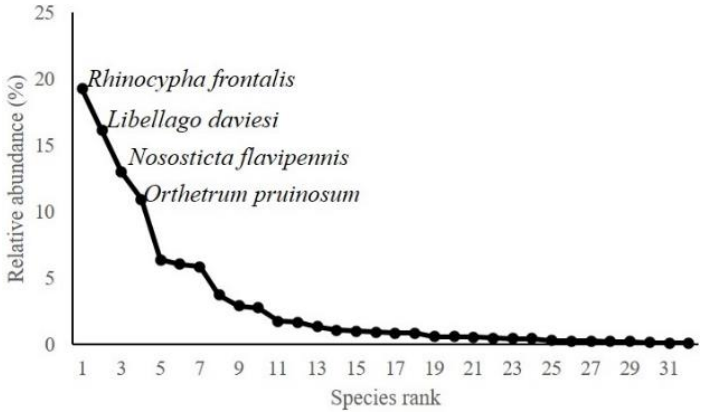

Figure 6. *The rank-abundance curve of dragonflies in all habitats*

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Dragonflies species richness and diversity

The interpolation-extrapolation rarefaction curve for each habitat showed a rapid increase at the beginning of the sampling and then approached the asymptote point (*Fig. 7*). The rarefaction curve interval of dragonflies species in agricultural land and settlements was separated compared to the other three habitats indicating that species richness in agricultural land and settlements is lower. Based on the extrapolated rarefaction curve, it is assumed that the number of dragonflies species found in the five habitats is raised by increasing the number of samples.

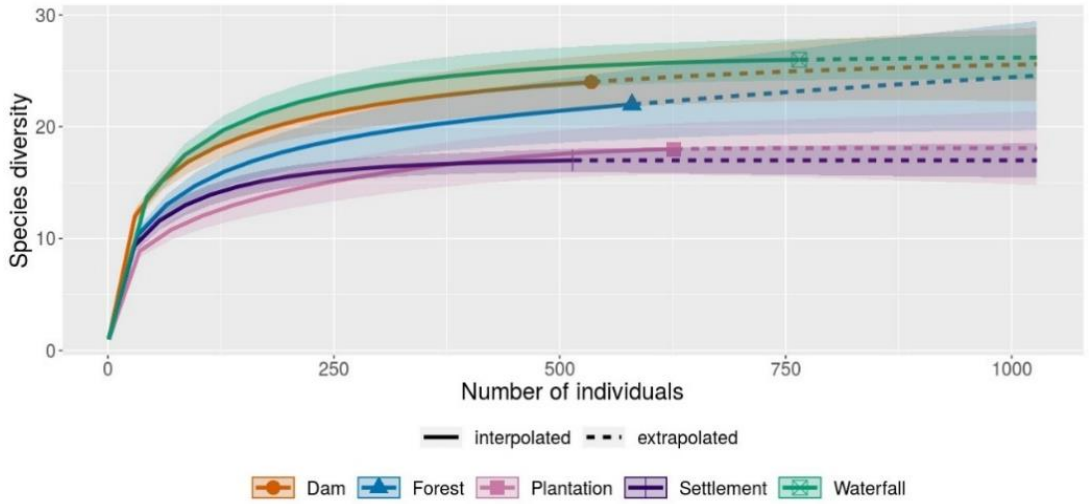

Figure 7. *The individual rarefaction curve of dragonflies for five habitats*

Among the five observed habitats, the highest average abundance and species diversity index were found in waterfalls with abundance = 191.3 individuals; H = 2.12, while the lowest was found in settlements with an abundance of 128.5, as well as individual and a diversity index of 1.60. Furthermore, dams and settlements were the habitats with the highest (2.48) and lowest (1.60) species richness indices, respectively. Meanwhile, the highest and lowest species evenness indices were found in dams (0.64) and forests (0.58), respectively (*Fig. 8a-d*). The statistical analysis showed that the average individual abundance with ANOVA: F4, 19 = 0.478; P = 0.752, Margalef species richness index ANOVA: F4, 19 = 1.743; P = 0.193, Shannon diversity index ANOVA: F4, 19 = 2.732; P = 0.069, and Pielou evenness index with ANOVA: F4, 19 = 0.471; P = 0.756 did not differ between the habitats (*Fig. 8a-d*).

Composition of dragonflies species between habitats

Based on the Analysis of Similarity (ANOSIM) results, the composition of dragonflies in five habitats did not show a significant difference with R = 0.00083; P = 0.4597. This was also observed in the NMDS ordinance results which show that the ordinance points in each habitat were close together and overlap (*Fig. 9*).

Effect of environmental factors

The average temperature showed a small variation between the three habitats with the lowest in forests namely 28.92 °C and the highest in settlements with 31.61 °C.

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The highest and lowest light intensities were found in settlements with 9215.0 Lux and forests 6695.9 Lux. Furthermore, settlements were the habitats with the highest wind speed namely 0.62 m/s, while the lowest was found in forests and waterfalls with 0.00 m/s. The mean relative humidity showed little variation between the five habitats with the lowest in settlements of 72.47% and the highest in waterfalls 84.31% (*Table 3*).

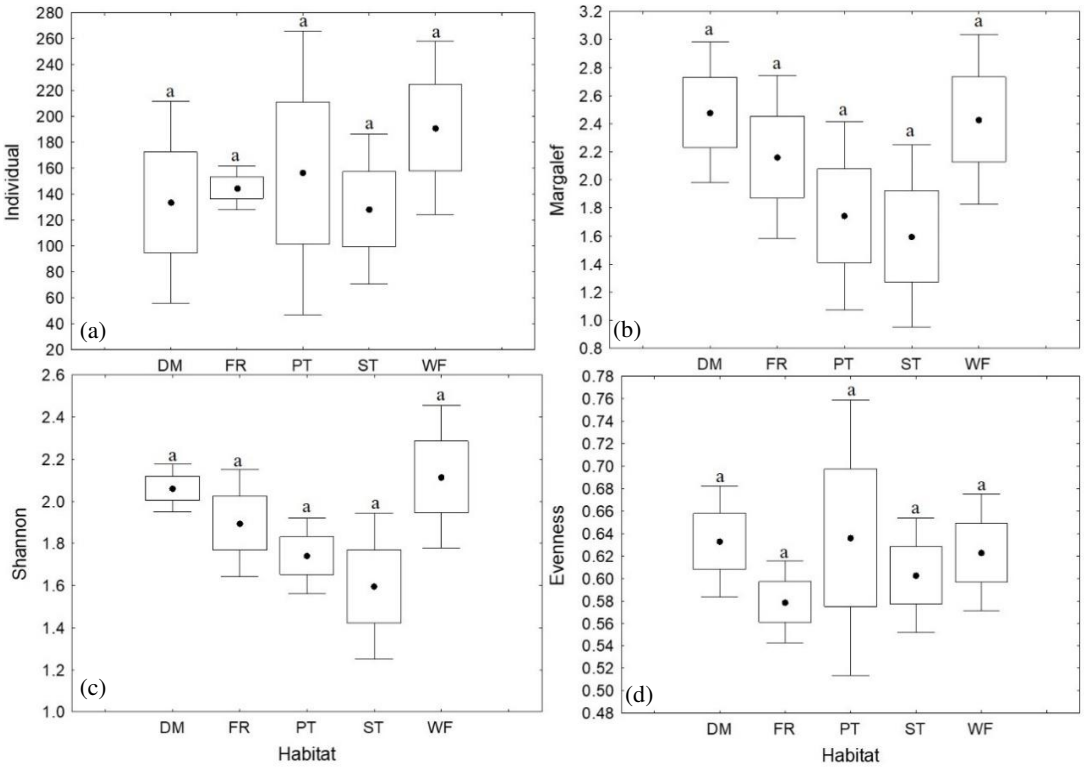

Figure 8. *The community structure of dragonflies in five habitats ((a): abundance, (b) richness, (c) diversity and (d) evenness species indexes; DM: Dam; FR: Forest; PT: Plantation; ST: Settlement; WF: Waterfall; (●): Mean, (□): ± SE, (⊥): ± SD. The same letter in the same picture does not differ significantly according to Tukey's test 95% confidence level*

Table 3. *Environmental factor of five habitats*

Environmental factors	Dam		Forest		Plantation		Settlement		Waterfall	
	Mean	**SE**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**
Temperature (°C)	28.93	0.29	28.92	0.33	31.20	0.27	31.61	0.21	29.09	0.19
Light (Lux)	8784.2	1122.5	6695.9	3306.6	8602.5	1403.8	9215.0	1425.8	6750.3	2125.5
Wind (m/s)	0.48	0.14	0.00	0.00	0.08	0.06	0.62	0.21	0.00	0.00
Humidity (%)	81.42	0.81	82.23	0.36	79.05	1.42	72.47	1.48	84.31	0.90

The PCA ordinances show a clear variation in the spatial pattern of environmental factors from the five observed habitats (*Table 4*). The plot obtained shows two distinct habitat groups, the first consists of adjacent and overlapping forests, waterfalls, and dams, while the second consists of settlements and plantations. The adjacent and

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overlapping ordinances between habitats are influenced by the high similarity of environmental characteristics between habitats. Furthermore, the PCA results showed that waterfalls and forests are characterized by high relative humidity and low air temperature, while settlements have high air temperature with low relative humidity (*Fig. 10*).

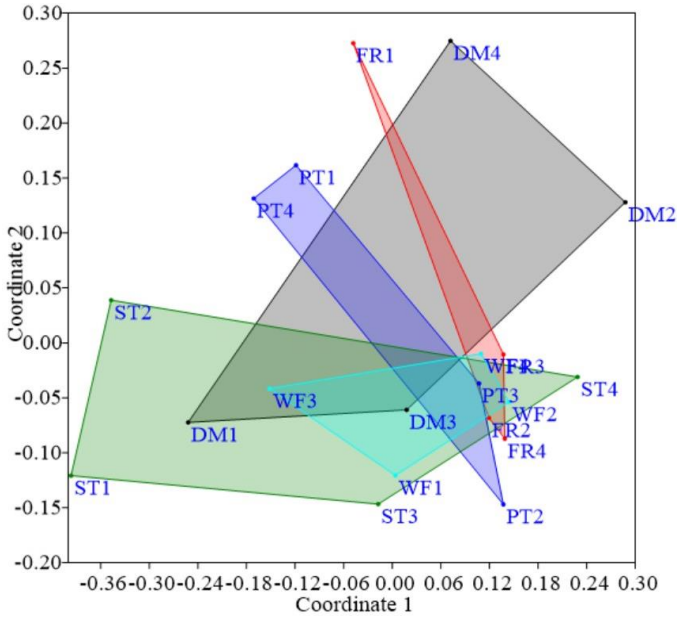

Figure 9. *Non-metric dimensional scaling (NMDS) of dragonflies composition in five habitats (stress value: 0.15); ANOSIM (R = 0.00083; P = 0.4597). (DM: dam; FR: forest; PT: plantation; ST: settlement; WF: waterfall)*

Table 4. *Environmental factor and PCA scores of five habitats*

Habitats	Environmental factors					PCA scores			
	Transect	Temperature (°C)	Light (Lux)	Wind (m/s)	Humidity (%)	Axis 1	Axis 2	Axis 3	Axis 4
Dam	DM1	28.70	11495.00	0.77	83.87	-0.17707	15.334	-12.181	0.3609
Dam	DM2	28.03	7325.33	0.77	79.67	-0.15989	0.63368	-18.433	-0.3776
Dam	DM3	79.67	10891.67	0.00	79.67	-0.17173	0.35597	0.6501	-0.4288
Dam	DM4	29.20	5425.00	0.40	82.47	-0.59201	-0.02225	-0.7923	0.29446
Forest	FR1	27.97	400.33	0.00	83.57	-20.228	-0.89074	-0.8187	-0.2274
Forest	FR2	28.70	3858.33	0.00	81.97	-13.156	-0.5291	-0.2884	-0.2802
Forest	FR3	28.97	3858.33	0.00	81.97	-12.032	-0.58375	-0.1910	-0.1627
Forest	FR4	30.03	19466.67	0.00	81.43	0.20544	1.96	14.635	-0.3989
Plantation	PT1	32.23	3390.67	0.00	73.77	11.737	-18.907	0.8644	0.07843
Plantation	PT2	30.80	11233.33	0.00	79.77	0.26396	0.21555	10.507	0.02026
Plantation	PT3	31.00	10893.00	0.00	82.17	0.02128	0.279	11.191	0.47066
Plantation	PT4	30.77	8893.00	0.33	80.50	0.42707	0.09444	0.1811	0.49217
Settlement	ST1	32.00	12391.67	1.07	68.37	35.936	0.18105	-0.723	-0.23902
Settlement	ST2	31.87	12391.67	1.07	72.00	30.762	0.45673	-0.7334	0.23364
Settlement	ST3	30.80	6420.67	0.00	77.60	0.26392	-0.79545	0.63505	-0.1053
Settlement	ST4	31.77	5656.00	0.33	71.90	17.548	-12.785	0.19067	-0.1983
Waterfall	WF1	29.33	3582.67	0.00	83.07	-12.096	-0.63042	-0.0726	0.16511
Waterfall	WF2	29.67	2863.33	0.00	81.67	-0.93076	-0.92424	-0.0229	0.13742
Waterfall	WF3	28.80	14768.00	0.00	86.37	-12.076	17.068	0.6847	-0.0270
Waterfall	WF4	28.57	5787.33	0.00	86.13	-17.897	0.12848	0.13568	0.19324

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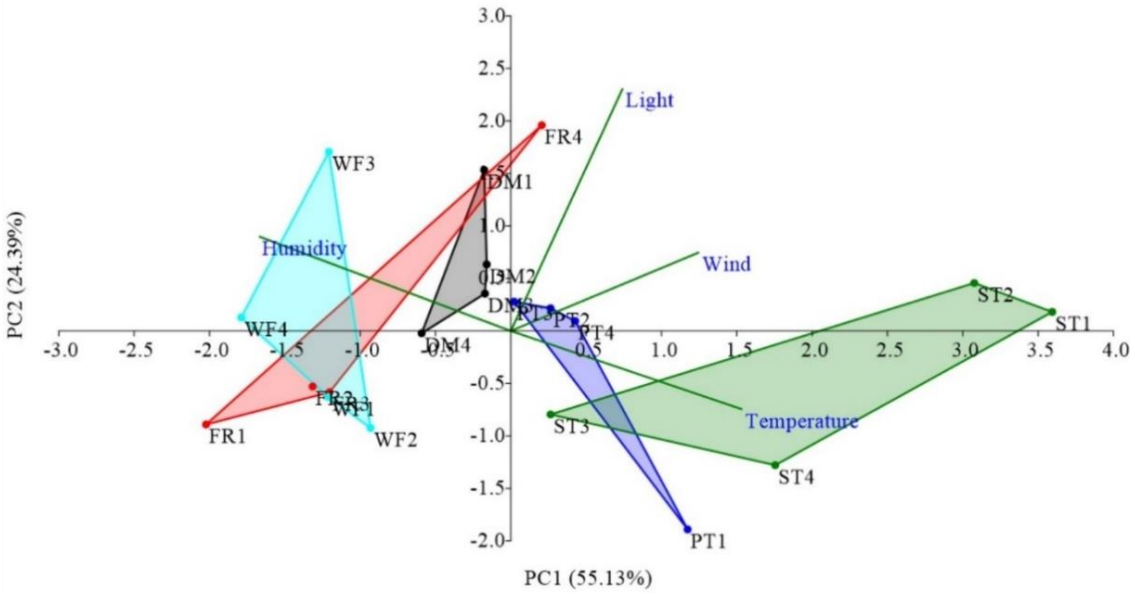

Figure 10. *PCA ordinations of five habitats. (DM: dam; FR: forest; PT: plantation; ST: settlement; WF: waterfall)*

Discussion

Dragonflies species found in the Sangihe Islands was only 0.54% of the 5,900 species in the world (Van Tol, 2000; Potapov et al., 2020), 3.56% of the 900 in Indonesia, and 21.92% of the 146 in Sulawesi (Lupiyaningdyah, 2020). The number of species found is smaller than that of the mainlands with a total of 146, this difference is due to the variation in the area of the islands. The Sangihe Islands have a land area of about 736.98 km^2 (Mokodompis, 2020), while the mainland of Sulawesi is approximately 174,600 km^2 (Decentralization Support Facility, 2011).

The number of species on an island reportedly depends on the total area. Previous studies showed that the island area has a positive correlation with the number and diversity of species. It directly affects the species diversity in two ways, first, large islands allow the species to spread widely, second, it supports more flora and fauna as the habitats and ecosystems are more diverse than small islands (Farizawati et al., 2014).

The sub-order Anisoptera has more species than Zygoptera due to its wide distribution and adaptability to various types of habitats. Meanwhile, Zygoptera are very sensitive to environmental conditions, have limited ability to spread, and prefer to live in vegetated and slightly shaded habitats (Pujiastuti et al., 2017). Several studies reported that Anisoptera are more common than Zygoptera (Dolný et al., 2011; Kaize and Kalkman, 2011; Narender et al., 2016; Seidu et al., 2017; Nilamsari et al., 2021).

Zygoptera are dragonflies with the highest abundance in waterfall and forest as these habitats have vegetation and shade with undisturbed environmental conditions. In tropical rain forests, they have limited distribution and competition outside the preferred habitats and are highly sensitive to changes in riparian vegetation. According to (Rahadi et al., 2013), Zygoptera are found around clean and flowing river waters with moderate intensity of sunlight or under tree shades. Moreover, (Narender et al., 2016) stated that the canopy cover and water vegetation in rivers were favored by Zygoptera compared to Anisoptera.

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Libellulidae has the highest number of species compared to other dragonflies families. This is because it is the largest family in the sub-order Anisoptera with a distribution pattern and high adaptability. This family is one of the common dragonflies and is often found in stagnant and flowing as well as fresh or slightly brackish water. Previous studies stated that Libellulidae are more common than other dragonflies families (Das et al., 2012; Narender et al., 2016; Acharjee and Karzee, 2016; Siregar and Bakti, 2016).

The most common habitat for Libellulidae is settlements characterized by little vegetation and less tree canopy cover. (Kalkman and Orr, 2013) reported that Libellulidae were found in almost all habitat types and were dominant in stagnant as well as flowing water. This family has the greatest migratory ability, including a distribution that spans more than one area and is also found on isolated islands (Van Tol, 2008). Another factor that causes this family to have the highest number of species is due to a shorter life cycle and tolerance to various habitats (Arulprakash and Gunathilagaraj, 2010). Libellulidae prey on all types of aquatic organisms as well as pests found in food crops and plantations, hence, they are used as biological control agents. Additionally, Rohman and Faradisa (2020) reported that Libellulidae are aggressive predators that feed on almost all insects.

Rhinocypha frontalis (Chlorocyphidae) and *Orthetrum pruinosum* (Libellulidae) are the dominant dragonflies found from the Suborders Zygoptera, and Anisoptera, respectively. *R. frontalis* was found mostly in waterfalls, forests, and agricultural land, while the lowest abundance was in settlements. During the observation, many of these species were found perched on the leaves and twigs of trees on the riverbank and rocks in the river. According to (Rahadi et al., 2013) *R. frontalis* is generally found in clean waters with moderate intensity of sunlight. This type perches on branches under the tree canopy and are commonly found in various clean springs (Pamungkas and Ridwan, 2015).

O. pruinosum species were dominantly found in settlements and rarely found in forest habitats. These species are generally found flying around rivers and occasionally perching on plants found in settlements. In sunny weather, these dragonflies fly low around the water, before finally landing on aquatic plants to reduce the body temperature. The adult dragonflies of *O. pruinosum* are flying insects, hence, they move from one place to another. Corbet (1999), stated that several species from the sub-order *Anisoptera* are flying insects which migrate long distances thereby affecting the distribution. These dragonflies travel at a maximum speed of 36 km/h (Amir and Kahano, 2003).

Based on the results, the abundance, as well as richness, diversity, and evenness indices were not significantly different between the habitats. This is caused by the microhabitats were not segregated from the surrounding habitats. This could be the reason for the detected similarities. However, waterfalls, dams, and forests tend to have higher species richness and diversity indices compared to other habitats. The three habitats have the same type of vegetation characteristics and do not receive significant human disturbance. Waterfalls and forest habitats have varied types such as rocky rapids and complex vegetation structures. Meanwhile, the habitat type is a factor that supports the diversity of dragonflies species. Herlambang et al. (2016) stated that the main factors that cause diversity differences in a habitat include food resources, habitat type, light intensity, temperature, humidity, and vegetation structure. Habitat variation also leads to the availability of various food types for dragonflies prey, hence, species

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diversity becomes high. Moreover, Hendriks (2020) stated that habitat is directly proportional to the physical condition of the environment, meaning that each type of habitat has its physical condition, which is also influenced by several factors such as vegetation density, canopy cover, and altitude. Therefore, these limits affect the presence and distribution of dragonflies species.

The vegetation in a habitat influences the diversity of dragonflies, forests with very dense vegetation as well as no disturbance and conversion of functions tend to have a high diversity (Dolný et al., 2011). Several previous studies reported that an increase in vegetation cover and plant biodiversity elevates the species richness and diversity. The presence of vegetation around rivers greatly influences the behavior of adult dragonflies such as sunbathing, foraging, roosting, and sheltering (Buchwald, 1992; da Silva Monteiro Júnior et al., 2013; Kolozsvári et al., 2015; Hendriks, 2020).

Based on the environmental factors measured, forest habitats, waterfalls, and dams form one group, indicating that there were high similarities between these habitats. Meanwhile, agricultural land and settlements form another group. Furthermore, the PCA results showed that waterfalls, dams, and forests are characterized by high relative humidity and low air temperature, while settlements habitats are characterized by high air temperatures and low relative humidity. The low temperature and high humidity in the forest and waterfalls due to the slightly denser canopy conditions and the presence of many trees on the riverbanks caused the inhibition of the air temperature rate and the intensity of sunlight. Differences in environmental factors between habitats affect the diversity of dragonflies species. Feriwibisono et al. (2016), reported that the diversity in a habitat is strongly influenced by environmental quality factors, such as pH, temperature, humidity, chemical conditions, and food availability.

Humidity and temperature affect plants and animals, especially small insects which are the main food of dragonflies. Also, temperature determines the various activities in finding a place to rest, as well as flight and breeding time. Furthermore, light intensity is an abiotic factor that affects several activities including sunbathing and foraging for food. At the optimal level, dragonflies bask and forage for food, but when it is extremely low or high, they spend more time resting in the habitat (Susanto dan Zulaikha, 2021).

Celebargiolestes orri (Zygoptera: Chlorocyphidae) and *Prodasineura autumnalis* (Zygoptera: Platycnemididae) are dragonflies species used as bioindicators of environmental health. Both species are only found in forest habitats where the water environment is not polluted. Female adult dragonflies in oviposition choose clear and clean water habitats, as the nymphs are sensitive to polluted water quality (John, 2001; Villalobos-Jimenez et al., 2016). In general, the water condition at the study site namely Sangihe Islands is categorized as clean and unpolluted. This is due to the presence of three families namely Chlorocyphidae, Calopterygidae, and Platycnemididae in each of the studied habitats. According to Hasanah et al. (2021) the presence of dragonflies from these families describes the condition of clean waters.

Conclusions

This study describes the diversity of dragonflies species in different habitats of the Sangihe Islands. Based on the results, there are 6 families which include 32 species and 3020 individuals. The diversity in the five habitat types showed no significant differences, but the richness and species diversity indices tend to be higher in the forest,

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dam, and waterfall habitats. Based on the environmental factors, forests and waterfalls have the same characteristics. Furthermore, the presence of the families *Chlorocyphidae, Calopterygidae,* and *Platycnemididae* in each studied habitat indicate that the water quality is generally good and supports the life of dragonflies. Therefore, the local government is recommended to preserve the river and not changing the forest function into agricultural land and housing to support dragonfly conservation as most part of the life cycle is highly dependent on water quality. Recommendations for further research are the identification of dragonflies in the Sangihe Islands through molecular phylogenetic analysis. The results of this follow-up study are expected to produce molecular-based basic data on dragonfly diversity and storage of dragonfly molecular data on GenBank and BOLD.

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PREDICTION OF PRODUCTION INDICES OF BOSCHVELD CHICKENS ON DIETARY PROBIOTIC-YEAST SUPPLEMENTATION LEVELS USING QUADRATIC OPTIMISATION MODEL

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Abstract. The dose response effect of yeast on productive indices of indigenous Boschveld chickens was investigated. 600 unsexed Boschveld chickens were divided into 6 groups of 100 and each group replicated five times. Chickens received starter mash (1 to 49 days) and grower mash (50 - 91 days) supplemented with probiotic-yeast at 0, 2.5, 5.0, 7.5, 10.0 and 12.5 g/kg feed. Data were collected on productive indices and analysed using a one-way analysis of variance, and significant means were compared using Duncan's test for multiple comparisons. A quadratic model was used to determine probiotic-yeast levels for optimum production parameters which differed at $p < 0.05$. Dietary yeast levels that supported the optimum feed conversion efficiency (FCE), average daily gain (ADG) and nitrogen retention for Boschveld chickens aged 1 - 49 days were higher than the levels that optimised the same production variables at 50 - 91 days and 1 - 91 days, except for FCE that was higher at 1 – 91 days. This has implications in diet formulation for Boschveld chickens. It is concluded that probiotic-yeast at 7.4 - 9.0, 7.1-7.3 and 7.1 - 9.0 g/kg supported optimum productivity in Boschveld chickens reared from 1 - 49, 50 - 91 and 1- 91 days of age, respectively.
Keywords: *local chicken, Saccharomyces cerevisiae, dose response, regression analysis, performance*

Introduction

Indigenous chickens play a vital part in enhancing food security and improving the nutritional status (meat and eggs) and income of several smallholder farmers in many developing countries. In South Africa like in other developing countries, the cost of feeding chickens for optimal productivity is on the increase because of the direct competition for grains between chickens, humans, and the industry. This trend has led to a decrease in the contribution of indigenous chickens in reducing food insecurity in developing nations because of their productivity, and the continuous increase in the cost of eggs and meat from commercially produced exotic poultry breeds has exacerbated these problems. Imported chicken breeds are highly prolific, resulting in a quick return on investment. This improvement in the productivity of imported chicken breeds was achieved through improved genetics and enhanced intensive management strategies. The nutrient requirements of indigenous chickens differ from that of imported breeds, with the imported breeds requiring grains such as maize and soybean which are directly consumed by humans. As a result, resource-poor rural farmers find the rearing of imported breeds such as broilers to be unsustainable and prefer to rear native chicken strains that are adapted to free-range rearing systems. These chicken strains are known to

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be products of their environment and can be produced at a low cost. However, research to improve the productivity of indigenous chickens under confinement is still limited. Thus, to contribute effectively to food security, it is important to improve and promote the production of indigenous chicken breeds such as Boschveld chickens. Boschveld chicken is a hybrid of three South Africa native breeds (Venda, Ovambo, and Matabele chickens) and is the only locally developed indigenous breed in Africa (Bosch, 2018). Earlier studies in our research station found significant improvement in gut health, blood characteristics, and carcass quality of Boschveld chickens fed yeast (*Saccharomyces cerevisiae*) supplemented diets (Maoba et al., 2021a,b). A quadratic optimization model is increasingly being used in the animal science discipline to determine the inclusion levels of feedstuffs for optimal productivity (Mbajiorgu et al., 2011; Ogbuewu and Mbajiorgu, 2020; Anyanwu et al., 2021). The knowledge will help in the formulation of a diet to optimise growth and productivity in livestock and chickens. Therefore, knowing yeast requirements for optimal productivity will help in the formulation of diets to optimize the productivity of the indigenous Boschveld chickens. This study was therefore designed to determine the probiotic-yeast supplementation levels that supported optimum productivity of Boschveld chickens reared from aged 1-91 days of age using a regression optimisation model.

Materials and methods

Study site and source of test probiotics

The study was done in Portion 22 of the farm Elandsfontein 334 IQ (26.3898 S 27.9235 E) in Gauteng Province, South Africa during January to April 2019 in strict adherence to the guidelines of the Animal Ethics Committee, University of South Africa's (approval number: 2018/CAES/101). Baker's yeast used for this study was procured from Anchor Yeast 22 Bunsen Street, Industria, Johannesburg, South Africa. Six hundred (600) unsexed day-old Boschveld chicks (27.8±1.07 g/bird) were purchased at Boschveld Ranching Limited in Bela Bela, Limpopo Province, South Africa.

Experimental procedures

The poultry house is partitioned into 30 pens with equal surface area (2 square metres per pen) and divided into three rows of 10 pens. The floor of the pens was covered with wood shavings to a depth of 7 cm. The pens were preheated for 12 hours with infra-red lamps before the arrival of the chicks, in the first week of age, brooding temperature (33°C) was sustained and moderately reduced by 2°C per week to around 20°C on completion of the trial. 600 unsexed Boschveld chickens were randomly divided into 6 treatment groups of 100 chickens with five replicates, each replicate having 20 chickens. Chickens were fed starter mash (1 to 49 days) and grower mash (50 - 91 days) supplemented with probiotic-yeast at 0, 2.5, 5.0, 7.5, 10.0 and 12.5 g/kg feed for 91 days. Birds were fed starter mash (1 - 49 days) and grower mash (50 - 91 days) as shown in *Table 1*. Birds were offered diets and water *ad libitum* throughout the feeding trial. Twenty-four hours of light was available per day throughout the study. The birds were vaccinated following the Boschveld chicken management guide (Bosch, 2018). We used unsexed chickens in the present study to reflect the current production practices of smallholder farmers, who are the primary target audience.

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Table 1. *Proximate composition of the experimental diets*

Nutrients (g/kg)	Starter feed (1 – 49 days)	Grower feed (50 - 91 days)
Crude protein*	200.00	180.00
Lysine*	13.30	10.50
Methionine*	4.70	4.40
Moisture*	120.00	120.00
Crude fat*	25.00	25.00
Crude fibre*	50.00	60.00
Calcium*	10.50	9.50
Phosphorus*	6.00	4.52
Determined analysis		
Dry matter	914.63	912.53
Moisture	85.37	87.47
Crude protein	229.96	193.19
Ash	58.96	47.79
Nitrogen free extract	55.06	58.65
Gross energy MJ/kg	17.74	17.17
Metabolisable energy, Kcal/kg	3009.98	3001.38

* As illustrated in the feed label, Kcal – kilocalorie

Data collection

The weight of the chicks was recorded at the beginning of the feeding trial and thereafter on a weekly interval per pen. The average voluntary feed consumption per chicken was recorded daily using an electronic weighing scale for the duration of the trial. The mean live weight per chicken was determined weekly per pen by dividing the total live weight by the total number of chickens in each pen. ADG was calculated as weight gain divided by the duration of the feeding trial, whereas FCE was calculated as feed intake divided by weight gain. Digestibility assay was done between days 42 - 49 days and 84 - 91 of feeding trial. On days 42 and 84, two birds per replicate were transferred to a metabolic cage equipped with a drinker and feeder. A 3-day stabilization period was allowed before a daily faecal collection period of 3 days commenced. Faeces voided by each bird were collected at 0900 h and weighed daily. Care was taken to avoid contamination from feathers, feed, scales and other debris. Faecal samples were analysed for apparent metabolisable energy (AME) and nitrogen retention contents using a standard method (AOAC, 2008). Proximate composition of experimental diets was analysed for dry matter, ash, ether extract (EE), crude fibre (CF), crude protein (CP) in triplicates following the procedures of AOAC (2008). Metabolisable energy (ME) was calculated using the prediction equation of Pauzenga (1985) as follows: ME = 37 × CP % + 81.8 × EE % + 35.5 × nitrogen-free nitrogen (NFE) %. The gross energy (GE) of the diets was determined (AOAC, 2008) using bomb calorimeter.

Data analysis

The responses in final live weight (FLW), ADG, FCE and nitrogen retention to dietary probiotic yeast supplementation levels were modeled using the following quadratic optimisation equation (SAS, 2010): $Y = a + b_1x + b_2x^2$. Where, Y is the dependent variable (FLW, ADG, FCE and nitrogen retention); a is the intercept on Y-axis; b_1 and b_2 are coefficients of the quadratic function; x is the independent variable (yeast inclusion level) and $-b_1/2b_2$ is the x value for optimum response. The quadratic model was used because of the adequacy of fit compared to other regression types. The quadratic model was determined to

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be the best-fit model of the data based on the coefficient of determination (r^2) and thus optimum levels for data were based on this model.

Results

The effect of probiotic yeast on the productive traits of Boschveld chickens is shown in *Table 2*. Results indicate differences among means for various traits under consideration in Boschveld chickens following probiotic-yeast supplemental level. However, probiotic-yeast supplementation levels had a quadratic effect on the productive traits of Boschveld chickens (*Table 3*). The effect of probiotic-yeast level on optimum production variables of indigenous Boschveld chickens 1 to 49 days of age are presented in *Figures 1-4*. Results of the quadratic analyses demonstrated that different dietary yeast levels of 7.7 and 7.4 g/kg feed supported optimum FLW (*Figure 1*) and FCE (*Figure 2*), while yeast levels of 7.7 and 9.0 g/kg feed optimised ADG (*Figure 3*) and nitrogen retention (*Figure 4*), respectively. There is little or no information in the literature on ideal dietary yeast levels for optimal production responses in Boschveld chickens. Results of the effect of yeast level on optimum production variables of Boschveld chickens aged 50 - 91 days are presented in *Figures 5-7*. Dietary yeast had a quadratic effect ($p<0.05$) on ADG (*Figure 5*), FCE (*Figure 6*) and nitrogen retention (*Figure 7*), and were optimised at yeast levels of 7.1, 7.3 and 7.3 g/kg feed, respectively. Curve estimation of the influence of yeast level on optimum production parameters of Boschveld chickens aged day-old up to 91 days is presented in *Figures 8-11*. As the dietary yeast level increased, FLW, FCE, N-retention and ADG of Boschveld chicken aged day-old up to 91 days also increased until they were optimized at different dietary yeast levels. The yeast level of 7.3 g/kg feed supported optimum FLW and ADG as shown in *Table 3*.

Table 2. *Effect of probiotic yeast on production indices of Boschveld chickens reared from 1 to 91 days*

Parameters	Probiotic-yeast supplementation levels (g/kg)						SEM
	SC0	SC2.5	SC5.0	SC7.5	SC10.0	SC12.5	
d 1 - 49							
Initial live weight (g/bird)	28.4	26.4	27.7	27.0	29.4	27.5	0.44
Final live weight (g/bird)	540.5^{c}	548.3^{c}	633.3^{ab}	646.9^{a}	634.6^{ab}	583.7^{b}	19.1
Feed intake (g/bird/d)	38.1	36.8	34.8	37.2	36.4	37.3	0.45
FCE	3.7^{a}	3.5^{a}	2.8^{c}	2.9^{c}	3.0^{bc}	3.3^{ab}	0.14
ADG (g/bird/d)	10.5^{c}	10.7^{c}	12.4^{ab}	12.7^{a}	12.4^{ab}	11.4^{bc}	0.40
AME (MJ ME/kg)	11.3	10.7	11.9	11.7	11.6	10.8	0.20
N-retention (g/bird/d)	1.84^{c}	1.90^{c}	2.26^{b}	2.54^{a}	2.42^{ab}	2.26^{b}	0.11
d 50 - 91							
Final live weight (g/bird)	1313.8^{d}	1388.6^{c}	1587.8^{b}	1634.6^{ab}	1685.6^{a}	1380.4^{c}	63.67
Feed intake (g/bird/d)	76.0	75.7	74.4	75.3	74.4	72.6	0.50
FCE	4.1^{a}	3.9^{a}	3.3^{b}	3.2^{b}	3.0^{b}	3.9^{a}	1.10
ADG (g/bird/d)	18.4^{b}	20.0^{b}	22.7^{a}	23.5^{a}	25.0^{a}	19.0^{b}	0.19
AME (MJ ME/kg)	10.7	11.0	10.8	11.4	11.9	10.8	0.19
N-retention (g/bird/d)	1.8^{c}	2.2^{b}	2.4^{a}	2.4^{a}	2.5^{a}	2.1^{b}	0.10
d 1 - 91							
Final live weight (g/bird)	1313.8^{d}	1388.6^{c}	1587.8^{b}	1634.6^{ab}	1685.6^{a}	1380.4^{c}	63.67
Feed intake (g/bird/d)	57.1	56.2	54.6	56.2	55.4	54.9	0.38
FCE	4.0^{a}	3.7^{ab}	3.1^{c}	3.1^{c}	3.0^{c}	3.6^{ab}	0.16
ADG (g/bird/d)	14.4^{c}	15.3^{c}	17.6^{b}	18.1^{ab}	18.7^{a}	15.2^{c}	0.73
AME (MJ ME/kg)	10.96	10.84	11.35	11.56	11.71	10.80	0.16
N-retention (g/bird/d)	1.84^{d}	2.03^{c}	2.34^{ab}	2.48^{a}	2.46^{a}	2.20^{bc}	0.10

^{a,b,c,d} Means in the same row not sharing a common superscript are significantly different ($p < 0.05$). FCE: feed conversion efficiency, ADG: average daily gain, AME: apparent metabolisable energy, SD: standard deviation, CV: coefficient of variation, SEM: standard error of the mean

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Table 3. *Probiotic-yeast levels for optimal productivity of Boschveld chickens aged 1 to 91 days*

Variable	Formula	Optimal X-value	Optimal Y-value	r^2	p-value
d 1 - 49					
FLW (g/bird)	$Y = 522.35 + 29.969x - 1.951x^2$	7.7	637.5	0.82	0.001
FCE	$Y= 3.7821 - 0.2424x + 0.0163x^2$	7.4	2.9	0.86	0.001
ADG (g/bird/d)	$Y = 10.143 + 0.6131x - 0.04x^2$	7.7	12.4	0.83	0.001
N-retention(g/bird/d)	$Y =1.7214 + 0.1551x - 0.0086x^2$	9.0	2.4	0.90	0.001
d 50 - 91					
ADG (g/bird/d)	$y = 17.543 + 1.7434x - 0.1223x^2$	7.1	23.7	0.75	0.001
FCE	$y = 4.2607 – 0.297x + 0.0203x^2$	7.3	3.2	0.78	0.005
N-retention(g/bird/d)	$y = 1.8 + 0.1846x – 0.0126x^2$	7.3	2.5	0.94	0.001
d 1 - 91					
FLW (g/bird),	$Y = 1259.3 + 103.55x - 7.1223x^2$	7.3	1635.7	0.80	<.001
FCE	$Y = 4.0964 - 0.279x + 0.0186x^2$	7.5	3.0	0.90	<.001
ADG (g/bird/d)	$Y = 13.786 + 1.1966x - 0.0823x^2$	7.3	18.1	0.80	<.001
N-retention (g/bird/d)	$Y = 1.7818 + 0.1644x - 0.0102x^2$	8.1	2.4	0.94	<.001

FLW: final live weight; ADG: average daily gain; FCE: feed conversion efficiency; N: nitrogen; r^2: coefficient of determination

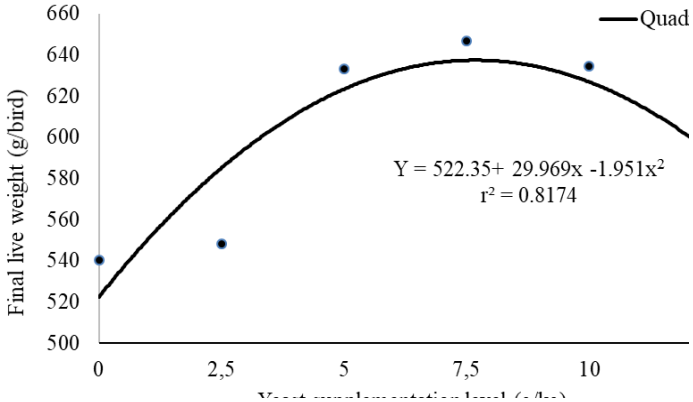

Figure 1. *Effect of probiotic-yeast on FLW in Boschveld chickens aged 1 to 49 days*

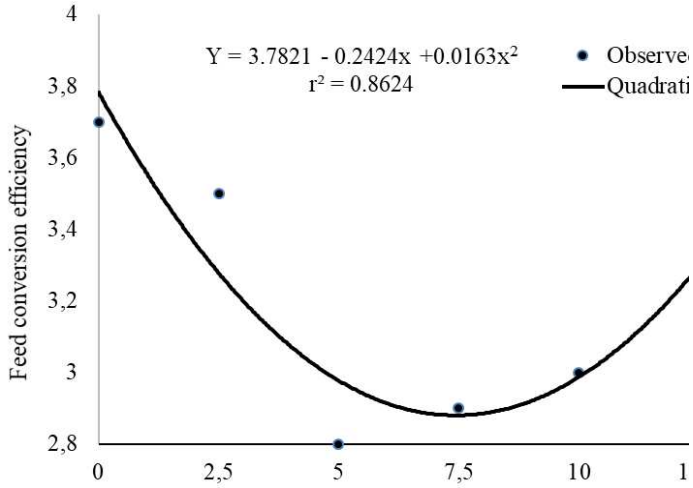

Figure 2. *Effect of probiotic-yeast on FCE in Boschveld chickens aged 1 to 49 days*

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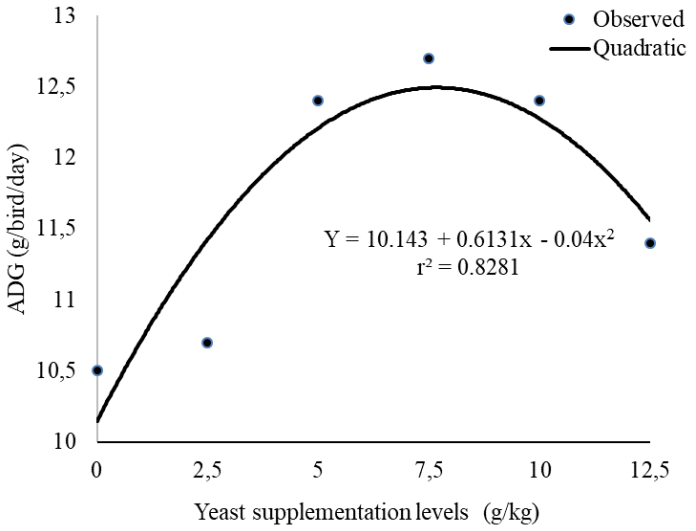

Figure 3. *Effect of probiotic-yeast on ADG in Boschveld chickens aged 1 to 49 days*

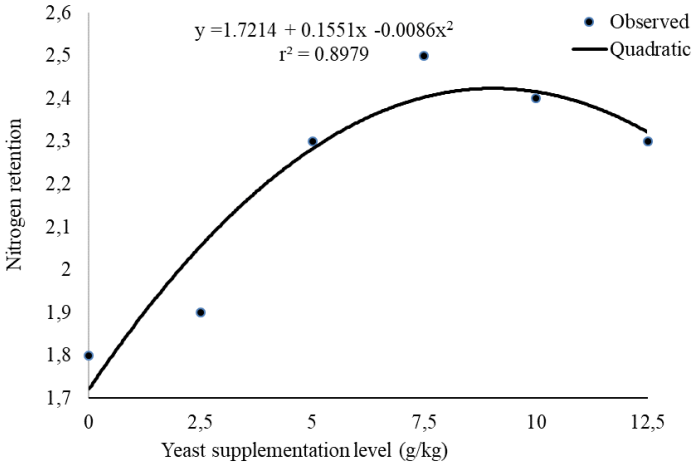

Figure 4. *Effect of probiotic-yeast on nitrogen retention in Boschveld chickens aged 1 to 49 days*

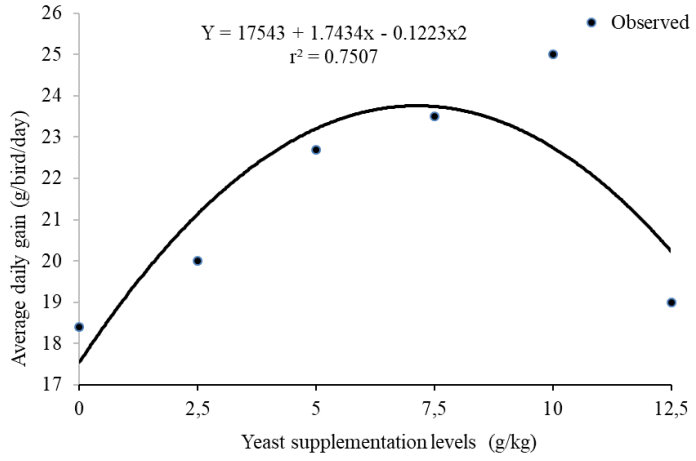

Figure 5. *Effect of yeast on ADG of Boschveld chickens from one 50 to 91 days of age*

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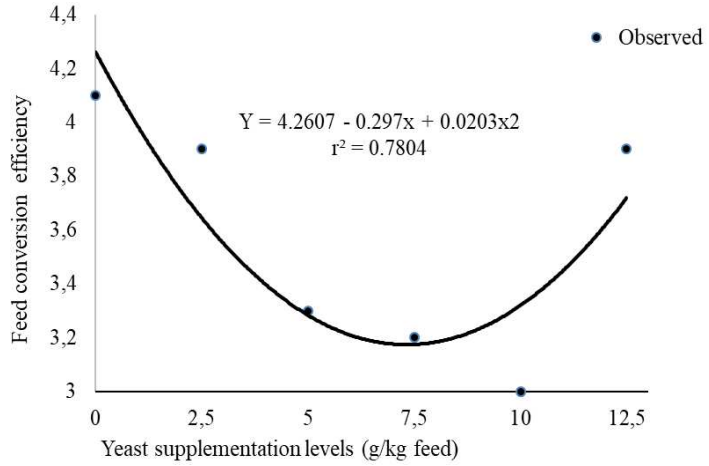

Figure 6. *Effect of yeast on FCE of Boschveld chickens from one 50 to 91 days of age*

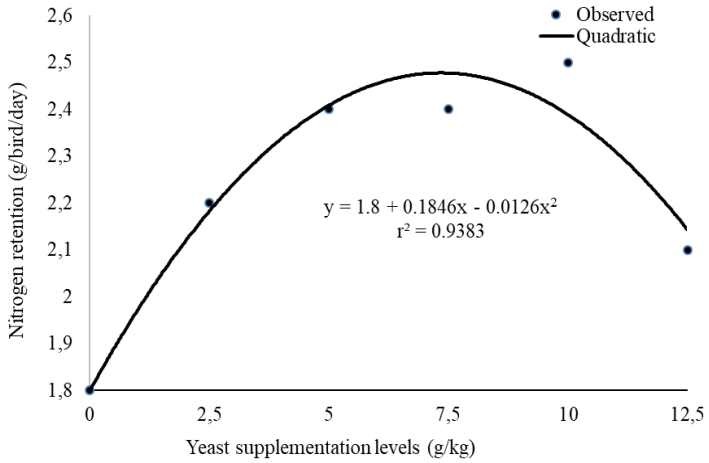

Figure 7. *Effect of yeast on nitrogen retention of Boschveld chickens from 50 to 91 days of age*

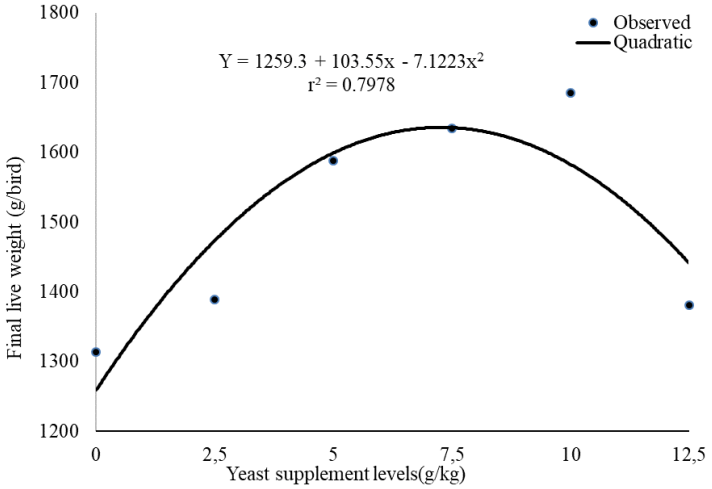

Figure 8. *Effect of yeast on live weight of Boschveld chickens aged day-old up to 91 days*

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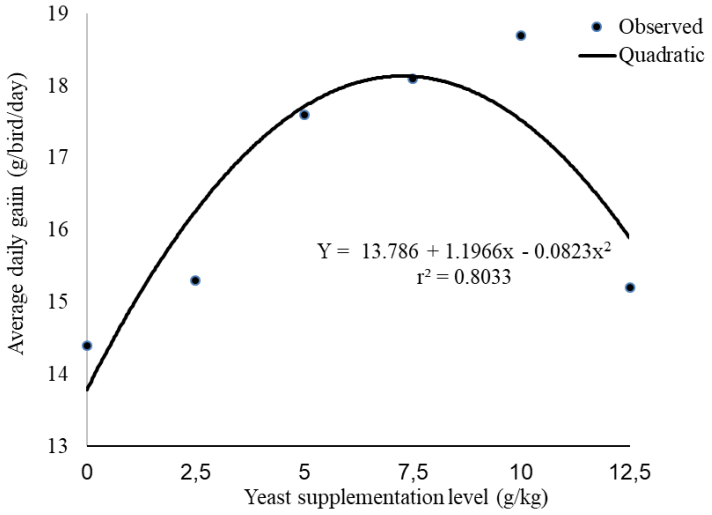

Figure 9. *Effect of yeast on ADG of Boschveld chickens aged day-old up to 91 days*

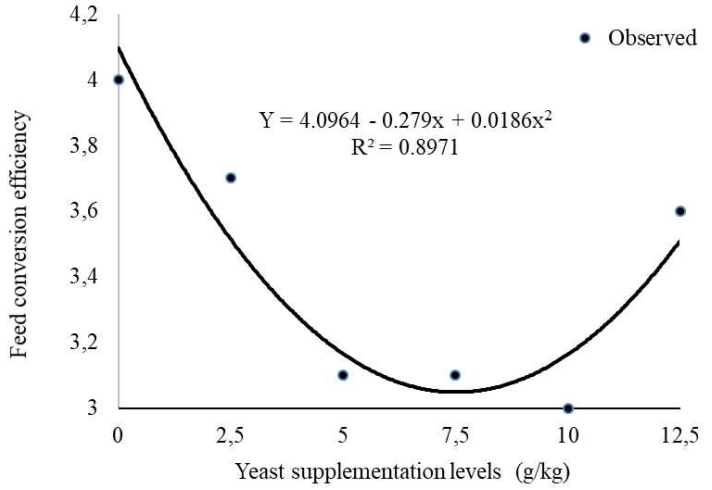

Figure 10. *Effect of yeast on FCE of Boschveld chickens aged day-old up to 91 days*

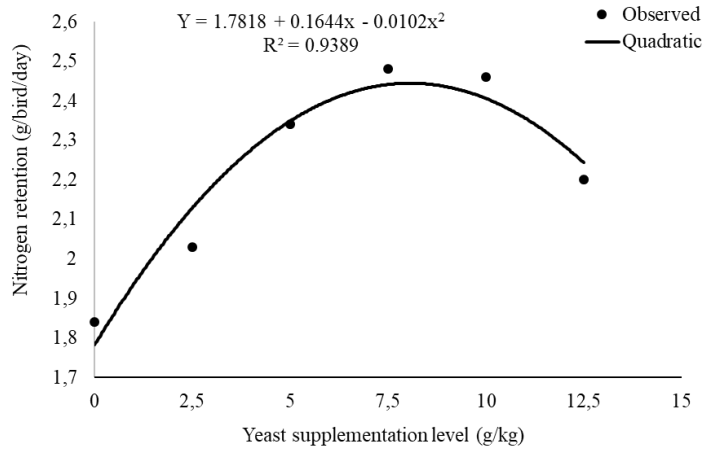

Figure 11. *Effect of yeast on nitrogen retention of Boschveld chickens aged 1 to 91 days*

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Discussion

There is little or no information in the literature on ideal dietary yeast levels for optimal production responses in Boschveld chickens. Probiotic-yeast supplementation levels had a quadratic effect on productive traits of Boschveld chickens aged 1 - 49 days. These findings supported Mbajiorgu et al. (2011) who reported that the amount of nutrients needed to optimise different production parameters in indigenous chickens is dynamic. The single yeast level of 7.7 g/kg feed that optimised FLW and ADG in Boschveld chickens aged 1-49 days in the present study is expected given that the two parameters are positively correlated (Mbajiorgu et al., 2011). The yeast levels of 7.4 and 7.7 g/kg feed that supported optimum FCE (2.9) and ADG (12.4 g/bird/d), respectively were higher than the levels of 2.5 and 4.5 g/kg feed calculated from the data of Ahmed et al. (2015) that optimised the same parameters in broiler chickens aged 1 - 21 days. This result is envisaged as indigenous Boschveld chickens are slow-growing chicken breed when compared to broiler chickens which are selected for high growth efficiency. The r^2 values for Boschveld chickens aged 1-49 days were ranged from 82 to 90% and were considered high. That implied that there is a very high strength of determination of the various production indices using the quadratic equation and that FLW, FCE, ADG and nitrogen retention could be predicted based on a given amount of yeast added to the diet.

Dietary yeast had a quadratic effect on ADG, FCE and nitrogen retention in Boschveld chickens aged 50-91 days and was optimised at yeast levels of 7.1, 7.3 and 7.3 g/kg feed, respectively. Quadratic effect was observed for ADG and nitrogen retention in response to yeast levels up to 7.5 g/kg feed and thereafter it started to decline. However, the reverse pattern was the case for FCE. The ADG and FCE were optimised at different yeast supplementation levels in Boschveld chickens aged 50-91 days, indicating that yeast level for optimal productivity in Boschveld chickens is dynamic and dependent on the production variable under consideration. Data estimated from the results of Gao et al. (2008) found that yeast levels of 2.3 and 2.5 g/kg feed supported optimum ADG and FCE, respectively in Arbor Acres broiler chickens aged 1 - 42 days of age fed yeast culture at the levels of 0, 2.5, 5.0, and 7.5 g/kg as similarly observed by Mulatu et al. (2019). The genetics of a chicken influences its digestibility; feed efficiency and growth rate in poultry (Rondelli et al., 2003). However, the yeast levels for optimum ADG and FCE in this study were higher than the levels of 2.3 and 2.5 g/kg feed estimated from the data of Gao et al. (2008) for optimal value (ADG and FCE, respectively) in broiler chickens. This indicates that yeast inclusion for optimal productivity in Boschveld chickens aged 50 – 91 days is higher than that of broiler chickens aged 1 - 42 days and this response could be ascribed to variation in genetics. The optimal yeast value of 1.2 g/kg feed (FCE) and 2.3 g/kg feed (ADG) estimated from the data of Ding et al. (2019) in Chinese native chickens fed yeast derivatives at levels between 0.5 and 2.0 g/kg feed for 42 days was lower than the values recorded in the current study. The observed discrepancies in animal response could be attributed to variations in yeast type, diet composition as well as the age of chickens in studies under consideration.

The results of the present study showed that a single yeast level of 7.3 g/kg feed optimised both FCE and nitrogen retention in Boschveld chickens aged 50 to 91 days. The yeast level of 7.3 g/kg feed recorded in the present was higher than the estimated value of 1.9 g/kg feed from the data of Oyedeji et al. (2008) that optimised nitrogen retention in broiler chickens. Results also support the view that Boschveld chicken is a slow-growing chicken strain when compared to broiler chickens. In contrast, the value of 7.3 g/kg feed that optimised nitrogen retention in Boschveld chickens aged 50 to 91 days

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was 19% lower than the value of 9.0 g/kg recorded in Boschveld chickens aged 1 to 49 days old. The observed difference is expected since the starter diet was high in protein (20.0%) than the grower diet (18.0%), thus the birds on the starter phase will tend to consume less diet to meet their daily protein requirements when fed diets supplemented with yeast that is high in crude protein (45%) as stated by Reed and Naodawithana (1999). The r^2 values of 75 to 94% recorded for production parameters in Boschveld chickens aged 50 to 91 days were high, meaning that there is a very high strength of determination of the response parameters using a quadratic model.

Our results indicated that as the dietary yeast level was increased, FLW, FCE, N-retention and ADG of Boschveld chicken aged 1 to 91 days also increased until they were optimized at different dietary yeast levels. A single yeast level of 7.3 g/kg feed supported optimum FLW and ADG. This level is higher than the single yeast level of 2.2 g/kg feed reported for FLW and ADG by Manal (2012) in broiler chickens aged 1 to 42 days. Indigenous chicken breeds are slow-growing chickens and, thus, may require higher yeast levels as their feed conversion efficiency are usually higher than that of broiler chickens (Mbajiorgu et al., 2011). This has implications in diet formulations for indigenous chickens. Our quadratic results also showed that yeast level of 7.5 g/kg feed supported optimum FCE, which was higher than the value of 0.23 g/kg feed (Manal, 2012) and 2.5 g/kg feed (Gao et al., 2008) recorded in broiler chickens aged 1 - 42 days. This is also higher than the level of 0.77 g/kg feed recorded for broiler chickens aged 1 - 42 days by Ahmed et al. (2015). This suggests that Boschveld chickens have poor feed conversion efficiency; as a result, broiler chickens were expected to outperform locally developed Boschveld chicken breeds (Padhi, 2016). Daily yeast supplementation level of 8.1 g/kg feed supported optimum nitrogen retention in Boschveld chickens which were lower than the levels of 9.0 g/kg that optimised the same parameter in Boschveld chickens aged 1 to 49 days. The yeast level of 8.1 g/kg feed that optimised nitrogen retention in the current study was higher than the value of 1.9 g/kg feed reported in fast-growing chickens (Oyedeji et al., 2008). The lower value estimated for broiler chickens from the data of Oyedeji et al. (2008) may be a genotypic factor achieved via selection and breeding for higher performance in broiler chickens. The r^2 values for FLW, FCE, ADG and nitrogen retention in the present study were regarded as being high (r^2 = 80 - 94%). This means that there is a very high strength of determination of these production variables using a quadratic equation. The r^2 indicated that yeast supplementation had a 94% influence on nitrogen retention of indigenous Boschveld chickens reared in a confined environment from day-old up to 91 days of age. This means that yeast had a greater influence on nitrogen retention and lesser influence on FLW, FCE and ADG of indigenous Boschveld chickens reared from day-old up to 91 days of age.

Conclusion

Daily supplementation with 7.7, 7.4, 7.7 and 9.0 g/kg feed probiotic-yeast level, respectively, supported optimum live weight, feed conversion efficiency, average daily gain and nitrogen retention in Boschveld chickens reared from 1 to 49 days of age. In addition, daily probiotic-yeast supplementation levels of 7.3, 7.1 and 7.3 g/kg feed optimised feed conversion efficiency, average daily gain and nitrogen retention, respectively in Boschveld chickens aged 50 to 91 days. Daily yeast levels of 7.3, 7.5, 7.3 and 8.1 g/kg feed, respectively optimised live weight, feed conversion efficiency, average daily gain and nitrogen retention in Boschveld chickens from 1 – 91 days of age.

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However, a single yeast value of 7.7 and 7.3 g/kg feed optimised live weight and average daily gain, respectively in Boschveld chickens aged 1 to 49 days and 1 to 91 days, while a single value of 7.3 g/kg feed optimised feed conversion efficiency and nitrogen retention in Boschveld chickens from 50 to 91 days of age. The results of this study also suggest that the optimum probiotic-yeast level necessary to maximize performance in terms of average daily gain and nitrogen retention decreases as the bird ages. It is, therefore, recommended that effect of dietary probiotic-yeast supplement on productive indices of male and female Boschveld chickens reared from 1 to 91 days of age be determined.

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INTERACTIVE EFFECTS OF MULCH, IRRIGATION AND NITROGEN ON MAIZE (*ZEA MAYS* L.) PRODUCTIVITY

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Abstract. Combined effects of mulch, irrigation and nitrogen application at optimum level played an important role in influencing crop and water use efficiency. A field experiment was conducted during main maize growing season i.e. June to October for two consecutive years i.e. 2020 and 2021 with three N levels i.e. N_1 (100 kg N ha^{-1}), N_2 (125 kg N ha^{-1}) and N_3 (150 kg N ha^{-1}) in main plots, two mulch treatments including without mulch (M1) and with mulch application at the rate of 6 t ha^{-1} (M2) in sub plots and two irrigation regimes {IW/PAN-E ratio 0.6 (I_1) and 0.9 (I_2)} in sub-sub plots. The pooled data of two years indicated that the N31I2 treatment under mulch application resulted in significant increase of plant height, hundred grain weight, crop biomass, grain yield and water use efficiency of maize by 23.1%, 14.3%, 41.3%, 46.9 %, and 31.3%, respectively, over N1I1 un-mulched treatment. The root mass density increased with increase in nitrogen level and more so under mulch application. However, the interactive effects of mulch, irrigation and nitrogen were found to be not significant for most of the plant parameters. The average soil moisture content recorded at the time of maize harvesting was also observed to be higher under mulch application by 26.6% over un-mulched plots.
Keywords: *crop residue, moisture content, biomass, maize growth, root distribution, crop productivity, water use efficiency*

Introduction

In India, maize is the third most important cereal crop after wheat and rice. In Punjab, maize occupied 144.6 thousand ha, with a production of 410.5 thousand tons and mean yield of 3582 kg ha^{-1} during 2019-20 (Anonymous, 2021). The diversification of rice with maize is being emphasized especially in areas with over-exploitation of ground water resources. In the recent past, due to lowering of the water table, increasing cost of pumping in rice and evolution of high yielding cultivars of maize, the area under maize-wheat rotation has shown increasing trends in north-western India. Mulch application along with irrigation and fertilization played an important role in improving land and crop productivity. Straw mulching can improve soil organic matter, reduces evaporation at soil surface, decreases weed growth and enhances crop and water productivity. Mulching reduces water losses through evaporation, mulch forms a blanket over the soil which breaks raindrop impact and reduces crusting and soil erosion losses (Gholami et al., 2013). The application of organic mulches improves soil and water conservation along with increasing yield by up to 50% compared to un-mulched plots (Ranjan et al., 2017). Mulch increases the water storage efficiency of soils. Mulch usage proved to be a significant factor in reducing the evapotranspiration rate throughout the year leading to overall water saving while simultaneously maintaining the same yield level (Sun et al., 2012). Meena et al. (2018), and Arora et al. (2011) concluded that straw mulching improved water productivity. The improvement in the yield was noted to be around 20%. The use of residue mulch also impacts plant canopy and results in higher water status and affects several plant growth parameters such as root proliferation, leaf area index and overall yield. Rani et al. (2019) reported that the

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use of residue mulch at the rate of 5 t ha^{-1} increased soil moisture storage by a degree of 7.7% along with a reduction in evaporative flux by 23.9%, in low rainfall years. Qamar et al. (2015) studied the effect of mulching on crop productivity using different types of mulches (straw, plastic, natural mulch) and concluded that the use of mulch improves yield and yield parameters, the highest yield was seen in plastic mulch plot. Singh and Singh (2018) reported that mulched plots showed higher soil moisture content, soil moisture storage from period of mulch application till harvest, lower maximum soil temperature, weed growth was nearly reduced by half, higher plant height, significantly higher water use efficiency and better yield. Thus, mulch application gave profound results in terms of overall plant growth, alteration of soil temperature and moisture content. Mbagatuzinde (2020) studied the effect of mulching on weed management and maize productivity and observed that using mulch increased maize yield by 27% and 24% in the year 2018 and 2019, respectively. Effect of mulch in combination with sub-surface drip irrigation produced 48% higher grain yield than conventional irrigated and un-mulched control plots.

Nitrogen (N) is a key nutrient in the production of cereal crops. It is a component in many biological compounds playing a noteworthy part in photosynthetic activity and crop yield. Its inadequacy constitutes one of the real yield constraining factors in cereal crops. Irrigation is another most essential agricultural input to achieve the optimum crop yield. Irrigation is a very important agricultural practice in achieving optimum productivity. Water deficit induces a reduction in water potential, leaf elongation, leaf photosynthesis and N metabolism and hence leads to significant influence on plant height, leaf area index (LAI), relative leaf water content (RLWC), chlorophyll content as well as nutrient uptake and hence indirectly affects the crop yield significantly. Motazedian et al. (2019) studied the effect of irrigation on sweet corn growth and grain yield as influenced by irrigation and suggested that when maize was irrigated at 70% (moderate stress) of the water requirement the yield was highest with improved water productivity as compared to 50 (severe stress) and 100% normal irrigation levels, the protein content and sugar content also show similar results. There is a lack of information regarding the interactive effects of N, irrigation regimes and mulching on maize productivity under Punjab conditions. Keeping the above facts in view, a study was conducted to investigate the interactive effect of N, irrigation and mulch on water use efficiency and maize productivity.

Materials and methods

Experimental site and treatment details

The study was conducted during main maize growing season locally named as kharif season i.e. from June to October during two consecutive years i.e. 2020 and 2021 in a sandy loam soil (basic properties mentioned in *Table 1*) at an experimental station (30° 56’ N and 75° 52’ E, latitude and longitudes and 247 meter above the mean sea level) of Punjab Agricultural University, Ludhiana, India. The climate of Ludhiana is semi-arid sub-tropical, with hot-dry summers from April to June, with humid and hot monsoon from July to September, and cold winters from November to January. The average summer and winter temperature undergoes a drastic change. Temperature varied from 40 °C in summer and as low as 0.5 °C during winters and thus, crops suffers from a hot dry spell during summers and frost during winters. The average annual rainfall received in the region is about 759 mm (Kaur et al., 2021). The climatic

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data of crop growing period for both the year is presented in *Table 2*. The research experiment design was a split-split design with three treatments (2 mulch, 2 irrigation and 3 N levels) and three replications, thus forming a total of 36 plots. In main plot treatments: three N levels (N_1:100 kg N ha^{-1}; N_2:120 kg N ha^{-1}; N_3:150 kg N ha^{-1}); in sub plot treatments: two mulch levels i.e. without mulch (M1) and with mulch at the rate of 6 t ha^{-1} (M2) and in sub-sub plot treatments: two irrigation levels based on irrigation water to open pan evaporation ratio (I_1: IW/PAN-E ratio 0.6; I_2: IW/PAN-E ratio 0.9) were studied. The maize was sown on June 10, 2020 and June 7, 2021, while the crop was harvested on September 29, 2020 and September 25, 2021, respectively, for two years. The row to row spacing was maintained at 60 cm, while the plant to plant spacing was maintained at 20 cm. The plot size was 32 m^2. Whole of the phosphorus was applied as basal dose and N was applied as per the treatment. The N was applied in three splits (i.e. at sowing, knee high stage and at pre-tasseling stage). Gravimetric method was used to determine the surface soil moisture content. These samples were oven dried (at 105 °C) till constant weight was obtained and water content was calculated on mass basis. The difference between wet and dry weights of the soil sample is expressed as soil water content on dry weight basis. The volumetric water content can be calculated by multiplying the mass water content with bulk density.

Table 1. *Soil characteristics of experimental site*

Depth (cm)	pH	EC (dS m^{-1})	OC (%)	Sand (%)	Silt (%)	Clay (%)	FC (%)	PWP (%)
0-15	7.6	0.25	0.42	62.3	21.5	16.1	21.7	11.3
15-30	7.3	0.26	0.36	62.7	18.3	19.0	18.4	10.4
30-45	7.5	0.21	0.23	60.4	19.4	20.2	19.7	12.8
45-60	7.4	0.22	0.18	58.6	20.1	21.3	20.6	13.5
60-75	7.4	0.20	0.08	58.0	19.3	22.7	21.5	14.3
75-90	7.1	0.18	0.06	56.4	19.6	24.0	22.9	13.8

Table 2. *Climatic data of the crop growing period*

Month	2020				2021				Normal value*			
	Max temp (°C)	Min temp. (°C)	Evaporation (mm)	Rain-Fall (mm)	Max. temp. (°C)	Min. temp. (°C)	Evaporation (mm)	Rain-Fall (mm)	Max temp. (°C)	Min temp. (°C)	Evapo ration (mm)	Rain-Fall (mm)
May	36.9	22.3	213.0	49.6	36.3	22.6	235.0	37.3	38.7	22.7	310.4	23.2
Jun	37.6	26.5	220.4	9.6	36.3	25.3	208.4	84.8	38.1	25.9	289.7	84.2
July	34.4	26.9	157.6	232.0	34.3	27.8	158.6	271.2	36.3	25.3	208.4	84.8
Aug	33.5	26.8	135.9	145.6	33.7	27.2	135.6	107.6	33.7	27.2	135.6	107.6
Sep	34.5	24.9	123.4	136.0	31.7	25.3	86.7	295.8	31.7	25.3	86.7	295.8

*Normal values are average of 40 years

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Agronomic data and observations

For plant height measurements, ten plants per plot were randomly selected and their height was measured from ground surface to the tip of the plant at 80 days after sowing (DAS). The soil plant analysis development (SPAD value) /chlorophyll content of the leaves were measured at 60 DAS with the SPAD meter. Chlorophyll content in leaves of the intact plants was measured with Minolta- SPAD 502 chlorophyll Meter. The SPAD 502 Chlorophyll Meter is a handheld, a convenient and non-destructive lightweight device developed by the Minolta Camera Co., Japan for calculating the amount of chlorophyll present in the leaves. For each observation third fully opened leaf from apex was selected from ten plants for each treatment by taking the precaution that midrib should not come under the sample area/sensor of the instrument. The mean value of 10 readings was recorded as SPAD value.

The relative leaf water content (RLWC) was measured using the method by Barrs and Weatherley (1962), modified later by Esparza-Rivera et al. (2006) at 70 DAS in maize. Three randomly selected plants were sampled per plot to calculate RLWC. The determination of RLWC was accomplished by cutting two discs leaf^{-1}, from the lower, medium and uppermost leaves, making 6 discs plant^{-1} and 18 discs plot^{-1}. The leaf discs were put in plastic vials and immediately weighed, providing the value of fresh weight (FW). Then the discs were made to soak in de-ionized water for four hours and weighed to achieve the turgid weight (TW). At last, the discs were oven dried at 60 °C until a constant weight is reached (dry weight i.e. DW). The RLWC was determined using following equation:

$$\text{Relative leaf water content (\%)} = \frac{\text{FW-DW}}{\text{TW-DW}} \text{ x100} \qquad \text{(Eq.1)}$$

The root sampling was done at 90 DAS from 0-15, 15-30, 30-45 and 45-60 cm depths by core sampler with 5 cm diameter. Sampling was done amid the plant rows, nearly 5 cm away from the plant base. The soil-root mass in the core was then collected in plastic nets and washed under running water, carefully separating the roots from soil. The washed roots were retained on the sieves and were cleaned more to eliminate any remaining organic debris, weed roots etc. with the help of forceps. After the roots were thoroughly cleaned, they were gently pressed between filter papers to soak the excess moisture. The root mass density (RMD) was calculated by weighing the oven dried roots on a precision balance.

One hundred grains per plot were manually counted and weighed precisely and presented in grams. The crop biomass was determined at harvesting and the above ground parts of the plants were dried and weighed from net area per plot. Grain yield was recorded in kg from 9 m^2 area per plot and presented in t ha^{-1}. The total water use (TWU) was calculated by adding the irrigation water applied (IW), amount of rainfall (RF) and profile water use (PWU) as given in *Equation 2*. The water use efficiency (kg ha^{-1}mm^{-1}) was measured by dividing the grain yield by the total water use for each treatment as mentioned in *Equation 3*.

$$\text{TWU} = \text{IW} + \text{RF} + \text{PWU} \qquad \text{(Eq.2)}$$

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where, TWU = total water use (mm), IW = total irrigation water applied (mm), RF = total rainfall received (mm), PWU = profile water use (mm).

$$\text{Water use efficiency} = \frac{\text{Grain yield (t ha-1)}}{\text{Total water use (mm)}} \quad \text{(Eq.3)}$$

The data was statistically analyzed using CPCS-I software according to Cochran and Cox (1957) and adapted by Cheema and Singh (1991) and it was compared at significance level of 5%.

Results and Discussion

Plant height

The data pertaining to the impact of mulch, N levels and irrigation regimes on maize plant height is presented in *Table 3*. The maximum plant height (m) in maize was observed in N3I2 (2.92 and 2.95) under mulched condition while the lowest was recorded in N1I1 (2.21 and 2.32) under un-mulched conditions during 2020 and 2021, respectively. Irrespective of nitrogen and irrigation regimes, the mean plant height (m) was observed to be 2.47 and 2.53 at M1 and 2.53 and 2.66 at M2 during 2020 and 2021, respectively. Higher plant height under mulch in comparison to un-mulched condition might be due to greater moisture and nutrient availability in mulched than un-mulched treatment. Mulching improved plant height at all stages in maize, this is because mulch improved the soil ecological environment, decreased soil temperature and increased moisture contents, promoted the growth of maize. Irrespective of N and irrigation regimes, 26.6% more soil moisture was recorded under mulching than control treatment as depicted in *Figure 1*. Maize plant height (m) was significantly increased with N addition and value at N3 (2.76 and 2.74) was found to be significantly higher than that at N1 (2.30 and 2.44) during 2020 and 2021, respectively. However, the effect of N at N3 and N2 levels were at par during both the years. Higher N applications increase the cell division, cell elongation, number and length of internodes, maintains higher auxin and protein level in plants and therefore encourages the shoot growth. Also, higher N levels increased the chlorophyll content which increased the photosynthetic rate and plant height. Irrigation regimes had no significant effect on maize plant height. Irrespective of N level and mulch conditions, maize plant height (m) at I2 (2.61 and 2.71) was higher than under I1 (2.50 and 2.57) during 2020 and 2021, respectively.

SPAD value

The data on SPAD value at 60 days after sowing (DAS) is presented in *Table 3*. The irrigation alone and the interactive effects of mulch, irrigation and nitrogen were found to be non-significant for both the years. However, the maximum SPAD was observed in N3I2 (54.0 and 55.4) under mulched condition which was significantly higher than that of N1I1 (43.2 and 41.7) under un-mulched conditions during 2020 and 2021, respectively. Irrespective of nitrogen and irrigation regimes, the mean SPAD value was observed to be 47.3 and 46.6 at M1 and 50.5 and 52.0 at M2 during 2020 and 2021, respectively. Higher SPAD value under mulch in comparison to un-mulched condition might be due to better utilization of applied N under mulched than un-mulched conditions.

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Table 3. *Effect of mulch, N levels and irrigation regimes on plant height and SPAD value*

Mulch rate	N and Irrigation levels																	
	N1			N2			N3			N1			N2			N3		
	I1	I2	Mean	I1	I2	Mean	I1	I2	Mean	I1	I2	Mean	I1	I2	Mean	I1	I2	Mean
	2020 (Plant height, m)									2020 (SPAD value)								
M1	2.21	2.25	2.23	2.46	2.57	2.51	2.60	2.75	2.68	43.2	44.8	44.0	45.9	49.1	47.5	49.4	51.6	50.5
M2	2.32	2.41	2.37	2.65	2.76	2.71	2.75	2.92	2.83	46.1	48.3	47.2	49.9	52.3	51.1	52.3	54.0	53.2
Mean	2.27	2.33		2.55	2.67		2.67	2.84		44.7	46.6		47.9	50.7		50.9	52.8	
Treatment means	M1= 2.47; M2= 2.53; N1 = 2.30; N2 = 2.61; N3 = 2.76; I1 = 2.50; I2 = 2.61									M1= 47.3; M2= 50.5; N1 = 45.7; N2 = 49.3; N3 = 51.9; I1 = 47.8; I2 = 50.0								
LSD (0.05)	M = 0.12; I = NS; N = 0.24; M×I×N = NS									M = 3.1; I = NS; N = 3.1; M×I×N = NS								
	2021 (Plant height, m)									2021 (SPAD value)								
M1	2.32	2.44	2.38	2.53	2.67	2.55	2.63	2.83	2.65	41.7	44.6	43.2	45.6	47.5	46.6	48.8	52.8	50.0
M2	2.46	2.54	2.50	2.72	2.82	2.71	2.75	2.95	2.77	47.4	50.3	48.9	51.0	53.9	52.5	53.7	55.4	54.6
Mean	2.39	2.49		2.63	2.74		2.68	2.89		44.6	47.5		48.3	50.7		51.3	54.1	
Treatment means	M1= 2.53; M2= 2.66; N1 = 2.44; N2 = 2.68; N3 = 2.74; I1 = 2.57; I2 = 2.71									M1= 46.6; M2= 52.0; N1 = 46.1; N2 = 49.5; N3 = 52.7; I1 = 48.1; I2 = 50.8								
LSD (0.05)	M = 0.15; I = NS; N = 0.16; M×I×N = NS									M = 3.7; I = NS; N = 4.2; M×I×N = NS								

M1 = Un-mulched; M2 = Mulched; I1 = IW/PAN-E = 0.6; I2 = IW/PAN-E = 0.9; N_1 = 100 kg N ha^{-1} N_2 = 125 kg N ha^{-1}; N_3 (150 kg N ha^{-1})

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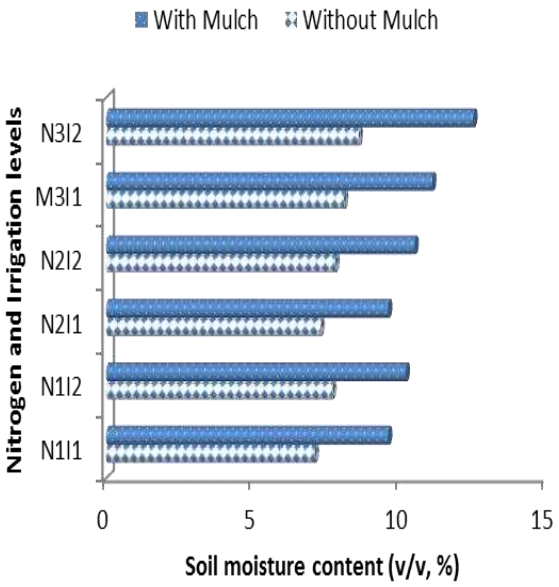

Figure 1. *Soil moisture variation at 0-15 cm depth as affected by nitrogen and irrigation regimes under mulched and un-mulch conditions*

SPAD value was found to be significantly increased with N addition and value at N3 (51.9 and 52.7) was found to be significantly higher than that at N1 (45.7 and 46.1) during 2020 and 2021, respectively. However, the effect of N at N3 and N2 levels were at par during both the years. Higher N levels increased the chlorophyll content which increased the photosynthetic rate. Irrigation regimes had no significant effect on SPAD value. Irrespective of N level and mulch conditions, maize SPAD value at I2 (50.0 and 50.8) was not significantly higher than under I1 (47.8 and 48.1) during 2020 and 2021, respectively. The higher dose of N might have produced greater values of leaf SPAD as N is considered an essential constituent of chlorophyll as reported by Schlemmer et al. (2005), Singh (2010) and Parija (2011) in maize. Munne-Bosch and Alegre (2000) observed that the SPAD value decreased with decreasing irrigation level and this decrease was correlated with relative water content in leaves. Chlorophyll decline is a negative outcome of water stress.

Relative leaf water content (RLWC)

The data regarding the effect of mulch practice and irrigation regimes on relative leaf water content (RLWC) is presented in *Table 4*. Maize RLWC (%) was found to be statistically at par under mulched (86.5 and 85.6) and un-mulched (84.4 and 83.1) treatments during 2020 and 2021, respectively. Irrespective of irrigation and mulch conditions, the RLWC increases with increase in N level and found to be 87.9% and 86.0% at N3 which was significantly higher than that under N1 82.3% and 82.2% during 2020 and 2021, respectively. The interaction effects of mulch, N and irrigation regimes were found to be non-significant. Increase in RLWC under mulching may be due to greater water extraction and uptake by plants owing to deeper and extensive rooting. Relatively more RLWC was reported under mulching may be due to greater moisture availability (Martorano et al., 2009).

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Table 4. *Effect of mulch, N levels and irrigation regimes on relative leaf water content (RLWC) and hundred grain weight*

Mulch rate	N and Irrigation levels																	
	N1			N2			N3			N1			N2			N3		
	I1	I2	Mean	I1	I2	Mean	I1	I2	Mean	I1	I2	Mean	I1	I2	Mean	I1	I2	Mean
	2020 (RLWC, %)									2020 (Hundred grain weight, g)								
M1	79.3	82.4	81.4	84.6	85.3	85.0	86.5	87.1	86.8	37.0	37.8	37.4	39.5	40.2	39.8	40.2	41.1	40.7
M2	82.6	84.7	83.9	85.7	87.9	86.8	88.2	89.6	88.9	37.6	38.1	37.8	40.5	41.1	41.0	41.6	43.1	42.4
Mean	80.9	83.6		85.2	86.6		87.4	88.4		37.3	37.9		40.0	40.9		40.9	42.1	
Treatment means	M1= 84.4; M2= 86.5; N1 = 82.3; N2 = 85.9; N3 = 87.9; I1 = 84.5; I2 = 86.2									M1=39.3; M2= 40.4; N1 = 37.6; N2 = 40.5; N3 = 41.5; I1 = 39.4; I2 = 40.3								
LSD (0.05)	M = 2.4; I = NS; N = 2.7; M×I×N = NS									M = 0.7; I = NS; N = 1.8; M×I×N = NS								
	2021 (RLWC, %)									2021 (Hundred grain weight, g)								
M1	80.7	81.9	81.3	82.5	83.8	83.2	84.1	85.6	84.9	37.5	39.1	38.2	39.1	42.0	40.5	39.5	42.9	41.2
M2	82.6	83.5	83.1	85.6	87.4	86.5	86.7	87.4	87.1	38.9	40.5	39.7	41.3	42.9	42.1	43.1	43.8	43.4
Mean	81.7	82.7		84.1	85.6		85.4	86.5		38.2	39.8		40.2	42.4		41.3	43.3	
Treatment means	M1= 83.1; M2= 85.6; N1 = 82.2; N2 = 84.9; N3 = 86.0; I1 = 83.7; I2 = 84.9									M1= 40.0; M2= 41.7; N1 = 39.0; N2 = 41.3; N3 = 42.3; I1 = 39.9; I2 = 41.8								
LSD (0.05)	M = 2.1; I = NS; N = 2.9; M×I×N = NS									M = 1.4; I = NS; N = 1.7; M×I×N = NS								

M1 = Un-mulched; M2 = Mulched; I1 = IW/PAN-E = 0.6; I2 = IW/PAN-E = 0.9; N_1 = 100 kg N ha^{-1}; N_2 = 125 kg N ha^{-1}; N_3 (150 kg N ha^{-1})

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With increase in irrigation level RLWC increased. Irrespective of N level and mulch conditions, the RLWC (%) at I2 (86.2 and 84.9) was not significantly higher as compared to I1 (84.5 and 83.7) during 2020 and 2021, respectively. Jayasankar and Ramakrishnyya (1993) in rice and Kumar (2005) in maize also reported increased RLWC with increase in irrigation, owing to higher uptake of water.

Hundred grain weight (HGW)

The data presented in *Table 4* represents the effect of mulch practice, N levels and irrigation regimes on maize HGW. The HGW increased with increase in N levels. The HGW (g) at N3 (41.5 and 42.3) was found to be significantly higher than that at N1 (37.6 and 39.0) during 2020 and 2021, respectively. This might be attributed to proper physiological functioning of N in tissue development, cell division, enhanced plant growth and thereby increased HGW. Low N supply decreases maize HGW due to decreased supply of carbohydrates and amino compounds to the grain. The mulching practice significantly affected HGW. Irrespective of irrigation and nitrogen levels, the HGW (g) under un-mulch treatment was 39.3 and 40.0, while the same was 40.4 and 41.7 under mulch during 2020 and 2021, respectively. Irrigation regimes did not significantly affect HGW, however, higher HGW (g) was observed in I2 (40.3 and 41.8) than in I1 (39.4 and 39.9) during 2020 and 2021, respectively. Increased translocation of photosynthates to the grain under I2 might be responsible for increasing the grain mass.

Root mass density (RMD)

The *Figure 2* depicts the effect of mulch, irrigation and N on RMD of maize. The RMD sharply declined with increase in the soil depth. Mulching increased root proliferation as well as the depth to which roots penetrated and also increased the biomass of deeper root. Higher RMD may also be because of reduction in soil strength and soil bulk density under mulching and also due to improved soil moisture storage. The pooled data of two years showed that RMD (μg cm^{-3}) at 0-15, 15-30, 30-45 and 45-60 cm depths was 1.38, 0.43 0.20 and 0.07 under un mulched treatment and 1.52, 0.55, 0.32 and 0.09 under mulched conditions. Martinez et al. (2008) reported higher RMD under mulch as compared to un-mulched conditions because of improved soil structure, greater availability of water and increased soil organic carbon content due to continuous crop resid1ues addition. The *Figure 2* presents data on effect of N levels on RMD in maize. Irrespective of much, irrigation and depth, higher value of RMD (μg cm^{-3}) was found under N3 (0.71), followed by N2 (0.55) and least under N1 (0.46). Increase in maize RMD with increase in N levels might be the result of favourable effect of N on plant biomass that also encouraged root growth (Singh, 2010).

Crop biomass

The data pertaining to the effect of mulching, N levels and irrigation regimes on maize biomass is presented in *Table 5*. Mulching showed significant impact on maize biomass yield. Irrespective of N and irrigation regimes, the increase in maize biomass yield was 7.5% and 11.4% during 2020 and 2021, respectively over un-mulched plots. The reason for higher value of plant biomass under mulching could be improved soil physical environment and reduced soil mechanical resistance to root penetration, because above ground biomass of plants is enhanced by a well-developed root system. More biomass under mulching might also be ascribed to taller plants and greater dry

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matter production. The greater values of plant biomass were also reported by Imran et al. (2015) in maize because of efficient utilization of the water and nutrients by plants under mulching. Similarly, the N level showed significant impact on biomass yield with increase in yield up to 29.5% and 28.6% in N3 over N1 during 2020 and 2021, respectively. Irrespective of mulch treatment, the maximum maize biomass yield (t ha^{-1}) was recorded under N3I2 (13.5) while the minimum was recorded under N1I1 i.e. 9.1 for pooled data of two years. Irrespective of irrigation treatments, the maize biomass (t ha^{-1}) was significantly higher under N3 (13.3) than N1 (9.4) but at par with N2 (11.8) for pooled data of two years. The increment in N levels lead to greater biomass accumulation, owing to greater photosynthesis, contributed by higher leaf area and greater N uptake resulting in the accumulation of significantly higher dry matter. However, irrigation regimes did not show significant influence on biomass yield of maize. Over pooled data of two years, there was only 4.2% increase in maize biomass yield under I2 as compared to I1 irrigation regime. In general, increase in irrigation level increased the biomass yield (Saren et al., 2004).

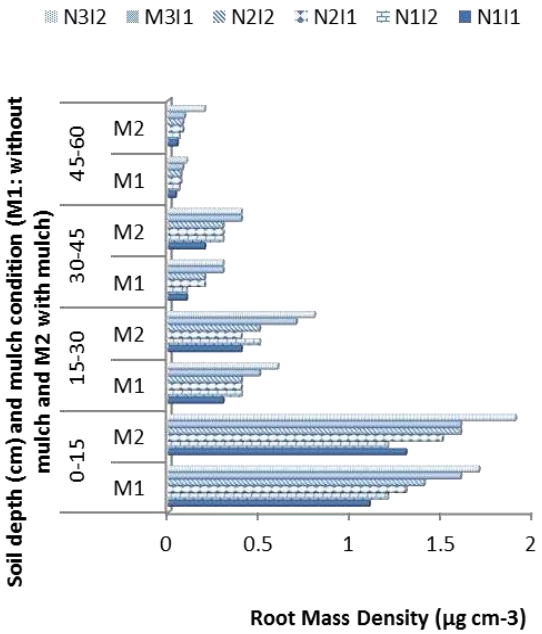

Figure 2. *Root mass density (µg cm^{-3}) as affected by nitrogen and irrigation regimes under mulched (M1) and un-mulched (M2) conditions*

Grain yield and water productivity

The *Table 5* illustrates the effect of N levels, mulch practice and irrigation regimes on maize grain yield. Significantly higher maize grain yield (t ha^{-1}) was observed under mulching in N3I2 (6.4 and 6.6) as compared to un-mulched N1I1 (3.4 and 3.5) treatment during 2020 and 2021, respectively. The reason behind higher yield under mulch was improved root proliferation in deeper layers and greater water and nutrient uptake. Irrigation regimes did not show significant effect on maize grain yield. However, higher grain yield (t ha^{-1}) was observed in I2 (5.4 and 5.6) and lower value under I1 (5.0 and 5.1) during 2020 and 2021, respectively.

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Table 5. *Effect of mulch, N levels and irrigation regimes on biomass yield and maize grain yield*

Mulch rate	N and Irrigation levels																	
	N1			N2			N3			N1			N2			N3		
	I1	I2	Mean	I1	I2	Mean	I1	I2	Mean	I1	I2	Mean	I1	I2	Mean	I1	I2	Mean
	2020 (Biomass yield, t/ha)									2020 (Grain yield, t/ha)								
M1	8.5	8.8	8.7	11.6	12.2	11.9	12.5	12.9	12.7	3.4	3.9	3.7	5.0	5.1	5.1	5.5	5.8	5.7
M2	9.4	10.0	9.7	12.0	12.9	12.5	13.2	14.1	13.7	4.0	4.6	4.4	5.6	5.8	5.7	6.1	6.4	6.3
Mean	9.0	9.5		11.8	12.4		12.9	13.5		3.7	4.3		5.3	5.4		5.8	6.1	
Treatment means	M1= 11.1; M2= 12.0; N1 = 9.3; N2 = 12.1; N3 = 13.2; I1 = 11.2; I2 = 11.8									M1= 4.8; M2= 5.5; N1 = 4.0; N2 = 5.3; N3 = 6.0; I1 = 5.0; I2 = 5.4								
LSD (0.05)	M = 0.7; I = NS; N = 2.3; M×I×N = NS									M = 0.3; I = NS; N = 0.8; M×I×N = 0.07								
	2021 (Biomass yield, t/ha)									2021 (Grain yield, t/ha)								
M1	8.4	8.9	9.7	10.4	10.6	10.5	12.2	12.5	12.4	3.5	4.2	3.7	5.2	5.6	5.3	5.7	6.1	5.9
M2	9.9	10.7	10.3	11.7	13.2	12.5	13.8	14.6	14.2	4.3	5.0	4.5	5.6	6.0	5.7	6.2	6.6	6.4
Mean	9.2	9.8		11.1	11.9		13.0	13.6		3.9	4.6		5.4	5.8		6.1	6.4	
Treatment means	M1= 10.9; M2= 12.3; N1 = 9.5; N2 = 11.5; N3 = 13.3; I1 = 11.4; I2 = 11.8									M1= 5.0; M2= 5.5; N1 = 4.2; N2 = 5.6; N3 = 6.2; I1 = 5.1; I2 = 5..6								
LSD (0.05)	M = 0.6; I = NS; N = 2.1; M×I×N = NS									M = 0.3; I = NS; N = 0.6; M×I×N = NS								

M1 = Un-mulched; M2= Mulched; I1= IW/PAN-E =0.6; I2= IW/PAN-E =0.9; N_1 = 100 kg N ha^{-1}; N_2 =125 kg N ha^{-1}; N_3 (150 kg N ha^{-1})

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The grain yield significantly increased with increase in N levels. Maize grain yield (t ha^{-1}) at N3 (6.0 and 6.2) was significantly higher in comparison to N1 (4.0 and 4.2) during 2020 and 2021, respectively. Higher yield with increase in N doses was due to higher dry matter accumulation in N3. There was a strong synergistic effect of irrigation and N on grain yields. Better root growth and better moisture extraction under mulch helped the crop to first develop adequate source (as reflected by high above-ground biomass accumulation) and then development of better sink size and capacity (higher grain mass as compared to un-mulched conditions). The effect of N and irrigation levels under mulch conditions on maize water use efficiency (WUE) is depicted in *Figure 3*. The N levels had a significant effect on maize WUE and it increased with increase in N levels, due to increased grain yield at higher N level. In case of mulching practice N3I2 had the maximum WUE and least under N1I1 un-mulched conditions. The WUE (t/ha-mm) was 10.5 under I2 irrigation regime, while it was 9.8 under I1 regime. Irrespective of N and irrigation levels the WUE (t/ ha-mm) varied significantly with mulching and was 9.8 under un-mulched condition and 10.6 under mulch application. Similarly, N showed significant effect as the pooled data for two years indicate WUE (t/ha-mm) of 11.4, 9.9 and 9.2 under N3, N2 and N1 levels, respectively. Halvorson et al. (2006) also reported that the WUE can be increased by N application.

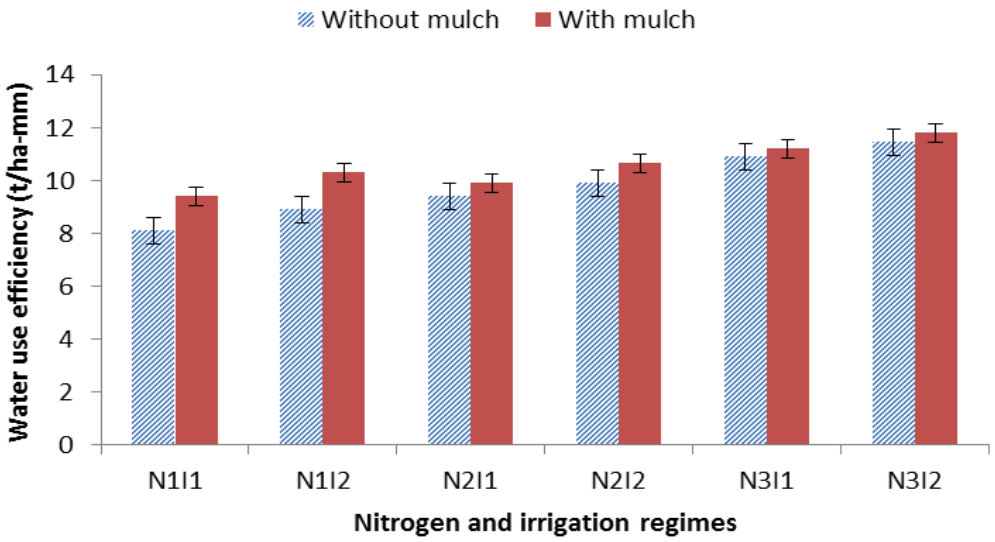

Figure 3. *Water use efficiency of maize (pooled data of 2 year) as affected by nitrogen and irrigation regimes under mulch and un-mulched conditions. (Bars showed standard error values)*

Conclusion

It is thus, concluded from the study that under mulch conditions even with lesser application of N i.e. @ 125 kg N ha^{-1} equal yield could be achieved to that with higher application of N @ 150 kg N ha^{-1} under un-mulched condition. Mulching thus, helped in saving nearly 20% of the nitrogen. All other plant parameters including height, root mass density, hundred grain weight and crop biomass also responded significantly more under mulching. For attaining maximum maize productivity of maize, the mulching practice along with nitrogen level of 125 kg N ha^{-1} and irrigation regime of IW/CPE

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ratio 0.9 needs to be practised in northwest India. However, there is need to conduct future studies on interactive effects of mulch, irrigation and nitrogen application under different soil textures and surface and sub-surface fertigation systems.

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YIELD, YIELD COMPONENTS AND OIL RATIOS OF IRRIGATED AND RAINFED SAFFLOWER CULTIVARS (*CARTHAMUS TINCTORIUS* L.) UNDER SEMI-ARID CLIMATE CONDITIONS

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Abstract. The field experiment was conducted in the experimental area of the Faculty of Agriculture in Harran University during of 2019-20, 2020-21 growing seasons to determine the yield and yield components of some safflower cultivars under irrigated and rainfed conditions. The experimental layout was divided into plots in randomized block desing with three replicates. Five safflower varieties (Göktürk, Koç, Dinçer, Safir and Balcı) were used as the plant material. Plant height (cm), number of lateral branches (number plant^{-1}), number of capitula (capitula plant^{-1}), number of seeds per capitulum (number capitulum^{-1}), 1000-seed weight (g), seed yield (kg ha^{-1}), crown yield (g), crude oil content (%) and crude oil yield (kg ha^{-1}) were investigated. Yields of irrigated cultivars (2425.8 and 2308.9 kg ha^{-1}) in both years were higher than those grown under rainfed conditions (857.3 and 578.1 kg ha^{-1}). The best performance under rainfed conditions was obtained from Dinçer cultivar, and the performance of Dinçer and Balci cultivars under irrigated conditions were better than of other three cultivars. In addition, oil content of varieties grown under irrigation were higher than varieties grown under rainfed conditions. Balcı variety had the highest oil content and best performance under rainfed and irrigated conditions for oil content.
Keywords: *safflower, yield components, irrigation, oil rate, economic analysis*

Introduction and literature review

Safflower (*Carthamus tinctorius* L.) an industrial plant has many different uses with its stem, leaves, seeds as well as flowers. The safflower is an ancient cultivar that was cultivated in the Middle East 3000 years ago (Knowles, 1982), and whole plant is used for the treatment of different diseases in India and Pakistan (Nimbkar, 2002). The flowers of safflower plants are used in food, cosmetics and paint-pharmaceutical industries (Abd El-Mohsen and Mahmoud, 2013). Safflower is an important alternative crop that can be cultivated in rainfed agricultural lands with its high tolerance to cold and heat, and in irrigated agricultural lands due to its tolerance to salinity and weeds (Kaya et al., 2003; Hussain et al., 2015; Emongor et al., 2015). Safflower (*Carthamus tinctorius* L.), which can be grown in winter, is an important annual oil plant (Eslam, 2010; Gürsoy et al., 2018). The safflower grows in wide range of environments and is more resistant to drought and low temperatures that can be used in fallow areas unlike other oil crops (Jhonson et al., 1993; Hussain, 2015; Emongor et al., 2015; Yeilaghi et al., 2015). Aforementioned characteristics reveal that safflower can be grown in rotation with wheat, barley, singular, etc. under rainfed conditions and can be considered an important oil seed crop for vegetable oil deficit of the country (Eryiğit et al., 2015; Köse, 2017).

Safflower seeds contain 13-46% oil (Doğan, 2021; Beyyavas and Haliloglu, 2021), of which approximately 90% consists of unsaturated fatty acids (oleic and linoleic acid)

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(Jhonson et al., 1993; Belgin et al., 2007). Safflower oil, which contains an average of 75% linoleic acid, also contains tocopherols with antioxidant effect and high vitamin E value (Weiss, 2000). Safflower is an important oilseed plant that grows optimally in rainfed conditions. However, lack of adequate moisture due to the severe drought conditions during phenological periods such as germination, stem elongation and branching and flowering negatively affect crop growth (Dajue and Mündel, 1996; Ekin, 2005).

Although safflower is a resistant crop to drought, the yield increases when irrigated during critical periods. The most critical periods for safflower seed yield are stem elongation and pre-blooming periods. Additional irrigation during these critical periods, when there is severe drought and insufficient moisture in the soil, will increase the yield of safflower (Omidi, 2012; Mohammedi et al., 2018; Santos et al., 2018; Koç, 2019; Doğan, 2021). This study was carried out to determine the effects of drought and yield of safflower cultivars, and suitable varieties by economic analyzes on the performance of five safflower cultivars grown under rainfed and irrigated conditions.

Materials and methods

Materials

The experiment was conducted in the Agricultural Research and Experimental Fields of Agriculture Faculty in Harran University during 2019-20 and 2020-21 growing seasons. Göktürk and Koç varieties obtained from Konya Bahri Dağdaş International Agricultural Research Institute, Balcı and Dinçer varieties from Eskişehir Transition Zone Agricultural Research Institute and Safir variety from Şanlıurfa GAP Agricultural Research Institute were used as plant safflower varieties.

Soil and climate characteristics

Soil samples were collected from 0-30 cm depth of the experimental site, and some of soil chemical characteristics of experimental site are given in *Table 1*.

Table 1. *Some of chemical properties of soils in experimental field*

Depth (cm)	pH	Organic matter (%)	Lime (%)	Macro and micro nutrients (mg kg^{-1})						
				P	K	Mg	Fe	Zn	Cu	Mn
0.30	7.92	1.12	29.6	4.70	180	303	3.46	0.72	4.64	0.44

Soil organic matter content (1.12%) of experimental site was low, and soil was slightly alkaline, very calcareous, slightly saline, clay-loam textured, low phosphorus and moderate potassium content (Ramazanoglu, 2019).

Şanlıurfa province is located in the Southeastern Anatolia Region of Turkey, between 36° 40’ and 38° 02’ north latitudes and 37° 50’ and 40° 12’ east longitudes. The continental transition climate prevails in the area, with hot and dry summers and cold winters. Average temperature and precipitation data of 2019-2020 and 2020-2021 growing periods and long-term (1929-2020) for Sanlıurfa province are given in *Table 2*. (Anonymous, 2021).

The average temperature values of the first and second years of the experiment were 14.99 and 15.97 °C. The average temperature value of long-term was 13.65 °C. Total

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precipitation in the first and second years of the experiment was 584 and 249 mm, and long-term average precipitation was 425.8 mm (*Table 2*). The second growing period was quite dry and the drought had a significant negative impact on yield and yield components.

Table 2. *Some climate data of Şanlıurfa province for 2019-2020, 2020-21 and long-term (between 1929-2020)*

Months	2019-2020		2020-2021		Long-term average 1929-2020	
	Avg. temp. (°C)	Total prep. (mm)	Avg. temp. (°C)	Total prep. (mm)	Avg. temp. (°C)	Total prep. (mm)
November	14.8	6.7	13.5	60.9	12.18	44.9
December	9.0	277.7	9.4	61.5	7.5	80.1
January	6.6	76.9	8.1	76.4	5.5	87.6
February	7.0	24.1	10.4	13.4	7.0	69.5
March	13.3	90.8	11.7	33.7	10.8	62.8
April	17.1	68.3	19.1	0.4	16.1	49.8
May	23.2	39.1	26.6	2.7	22.1	26.7
June	28.9	0.4	29.0	0.0	28.0	4.4
Total	-	584	-	249	-	425.8
Average	14.99		15.97		13.65	

Experimental design and treatment

The research was carried out in the experimental field of Field Crops Department, in Agricultural Faculty of Harran University (*Fig. 1*). Experimental layout was divided plots in randomized blocks. The treatments (irrigation and rainfed, IR) were placed in the main plots, and the cultivars were placed in the sub plots.

The plots were 6 m long and consisted of 5 rows, and the interrow and intra row distance was 30 and 10 cm, respectively. The experimental field was tilled with a plow and an herbicide (with *Trifluarin* active substance) was applied before planting using a hand pulverizer. Afterwards, the field was tilled with a cultivator at shallow depth and made ready for planting by pulling the tap. The sowing was carried using an experiment seeder on 5 November 2019 in the first year and on 8 November 2020 in the second year. After emergence, the plants were thinned in the 3-4 leaf period. Fertilizers were applied manually in a band on one side of the plant rows. Diammonium phosphate (80 kg P ha^{-1}; 30 kg N ha) was applied for the P requirement of the plant. Seven kg of urea (46% pure N) in the form of N was applied in February as top fertilizer. The rainfed plots were not irrigated and the plants were grown under natural precipitation conditions. Irrigation was carried out in the irrigation treatment plots, considering the climatic conditions. In the first, irrigation was applied 3 times (225 mm in total) during the flowering and seed setting periods, and in the second year, 4 times (300 mm) during the stem elongation, flowering and seed setting periods. Irrigation was applied homogeneously to the plots using a sprinkler system. On April 22 2020, 20 g L^{-1} acetamiprid active ingredient powder pesticide, effective against leafworm (*Spodoptera littoralis*) and aphid (*Aphidoidea*) pests, was applied to the experimental field.

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Figure 1. *Location of experiment area in Harran University*

Observations

When the plants reached the harvest maturity, one row from each side of plots and 1 m from the lower and upper parts of plots were cut with a sickle as a side effect, and the remaining 2 rows (0.6 m × 4.0 m = 2.4 m^2) were harvested. Plant height (cm), number of lateral branches (number $plant^{-1}$), number of capitula (piece $plant^{-1}$), number of seeds (number $capitulum^{-1}$), thousand seed weight (g), seed yield (kg ha) and crown yield (g) from 10 randomly selected plants from each plot were determined and averaged (Esendal,1992). To determine the oil content (%), 10 g of seeds from each variety were grounded, and dried in an oven at 70 °C for 72 h. Five g dried samples were taken and boiled for 6 h using n-hexane in the Soxhlet device and oil ratios (%) were determined (Bilsborrow et al., 1993). The results of percent oil content were multiplied by the seed yield per hectare and the results were converted to kg^{-1} ha by dividing 100 (*Fig. 2*).

Figure 2. *Observations in the experimental field*

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Data analysis

The data were analyzed using JMP 11 (SAS Institute, 2013) according to the experimental design of divided plots in randomized blocks. Means were grouped by the Tukey test (0.05).

Results and discussion

Plant height (cm)

As a result of the combined years analysis (ANOVA), there was a statistically significant difference between the years in terms of characteristics examined, and the data of each year were analyzed separately.

The rainfed-irrigation and variety treatments and rainfed-irrigation × variety interaction had a significant effect ($P < 0.01$) on plant height in both growing seasons (*Table 8*). The highest plant height value under rainfed-irrigation treatment in the first year was recorded in Dinçer variety (130.0 cm). This difference can be partially explained by the fact that the varieties grown under rainfed and irrigated conditions were not irrigated until May and the plants continued to grow with the precipitation. In the second year of the experiment, mean plant height measured under rainfed (60.88 cm) and irrigated (101.47 cm) varieties was significantly different from each other. The highest plant height was recorded in Koç variety (104.56 cm) (*Table 3*). Adverse climatic conditions and severe drought in the 2020-2021 growing season caused shortening the plant height of varieties grown under rainfed conditions. The results reported in the previous studies investigating the effects of rainfed and irrigated cultivation on plant height were in agreement with our findings. Öztürk et al. (2009) reported that plant height under rainfed and irrigated conditions was 86.9 and 108.2 cm, respectively, Jabbari et al. (2010) stated that irrigation levels had an important effect on plant height of safflower genotypes, Singh et al. (2016) reported that irrigation levels affected plant height, and Santos et al. (2018) found that the plant height in different irrigation periods was higher than that measured in the control plots.

Table 3. *Mean plant height (cm) and number of lateral branches (pieces/plant) of some safflower (Carthamus tinctorius L.) cultivars grown under rainfed and irrigated conditions*

Variety	Plant height (cm)				Number of lateral branches (piece/plant)			
	2019-2020		2020-2021		2019-2020		2020-2021	
	Rainfed	Irrigated	Rainfed	Irrigated	Rainfed	Irrigated	Rainfed	Irrigated
Safir	122.33bc	120.00cd	70.06c	100.10ab	5.47ab	5.30ab	4.96ab	5.63a
Koç	120.67c	120.67c	53.30d	104.56a	4.83b	5.27ab	4.03bc	4.63ab
Göktürk	124.67b	120.00cd	55.00d	102.90ab	6.00ab	5.30ab	3.36c	5.50a
Balcı	119.67cd	117.00d	56.03d	98.66b	6.27a	5.93ab	4.03bc	5.70a
Dinçer	130.00a	125.00b	70.00c	99.50ab	5.33ab	6.03ab	3.93bc	5.66a
Mean	123.46a	120.53b	60.88b	101.47a	5.58a	5.56a	4.06b	5.42a

Means that do not share a letter are significantly different. *$p \leq 0.05$, **$p \leq 0.01$

Number of lateral branches (piece plant^{-1})

The effects of rainfed-irrigation factor and rainfed-irrigation × variety interaction on the number of lateral branches were not statistically significant in 2019-2020 growing

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season. In contrast, the effects of rainfed-irrigation and variety factors and rainfed-irrigation × variety interaction had significant effect on the number of lateral branches in 2020-2021 growing season (*Table 8*). In the first year, the number of lateral branches counted under rainfed (5.58 pieces plant^{-1}) and irrigation (5.56 pieces plant^{-1}) conditions were similar. The highest number of lateral branches under rainfed conditions was obtained in Balcı variety (6.27 pieces plant-1) under rainfed-irrigation treatment. The similarity in the number lateral branches between rainfed and irrigation conditions can be attributed to the lack of water during vegetative period, the same amount of precipitation (no irrigation till May) and cultivation under the same growing conditions. In contrast, the difference in the number of lateral branches between rainfed (4.06 pieces plant^{-1}) and irrigation conditions (5.42 pieces plants^{-1}). The highest number of lateral branches (5.72 pieces plant^{-1}) among the varieties was counted in Balcı variety (*Table 3*). The irrigation during vegetation period increased the number of lateral branches. Similarly, Muhammedi et al. (2018) indicated that water stress decreased the number of lateral branches in plants.

Number of capitula (piece plant^{-1})

The effects of rainfed-irrigation and variety treatments and rainfed-irrigation interaction on the number of capitula were statistically significant ($P < 0.01$) in both years of the experiment (*Table 8*). The number of capitula in both years of the experiment was higher for irrigated varieties (9.16 and 8.04 piece plant^{-1}) compared to rainfed varieties (7.44 and 5.35 piece plant^{-1}). The highest number of capitula under irrigated and rainfed conditions was recorded in Dinçer variety (*Table 4*). Similar to the number of capitula recorded in this study, Santos et al. (2018) found higher number of capitula in different irrigation periods compared to the control.

Table 4. *Mean number of capitula (piece/plant) and number of seeds (piece/capitula) of some safflower (Carthamus tinctorius L.) cultivars grown under rainfed and irrigated conditions*

Variety	Number of capitula (piece/plant)				Number of seeds (piece/capitula)			
	2019-2020		2020-2021		2019-2020		2020-2021	
	Rainfed	Irrigated	Rainfed	Irrigated	Rainfed	Irrigated	Rainfed	Irrigated
Safir	7.40c	8.23c	6.83bc	7.10b	14.30ef	29.40bc	14.56c	18.35b
Koç	5.03d	8.70bc	4.20e	5.90cd	11.42f	27.52c	13.38c	18.87b
Göktürk	8.90bc	7.70c	5.30d	8.50a	19.27d	31.28ab	13.84c	18.53b
Balcı	7.87c	10.10ab	5.10de	9.40a	17.22de	32.51a	16.32bc	22.81a
Dinçer	8.00c	11.06a	5.33d	9.33a	19.22d	27.27c	18.95b	24.63a
Mean	7.44b	9.16a	5.35b	8.04a	16.28b	29.59a	15.41b	20.64a

Means that do not share a letter are significantly different. *p ≤ 0.05, **p ≤ 0.01, ns: non-significant

Number of seeds (piece capitulum^{-1})

The number of seeds per capitulum in both years was significantly different under rainfed-irrigated treatments. The effects of variety had also a significant effect ($P < 0.01$) on the number of seeds per capitulum in both years. The effect of rainfed × irrigation interaction on the number of seeds per capitulum was significant ($P < 0.01$) in 2019-20 growing season, while it was not significant in 2020-2021 growing season

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(*Table 8*). The number of seeds per capitulum for all varieties under irrigated conditions (29.59 and 20.46 piece plant^{-1}) was significantly ($P < 0.01$) higher in both years of the experiment than that obtained under rainfed conditions (16.28 and 15.41 piece plant^{-1}). The highest number of seeds under rainfed and irrigated conditions was obtained in Dincer and Balcı variety (*Table 4*). Öztürk et al. (2008) reported that the number of seeds per capitulum under irrigated condition was higher than that of the rainfed conditions, and the number of seeds per capitulum has significantly changed with genotypes and years. Similarly, Jabbari et al. (2010) indicated that irrigation has significant effect on the number of seeds per capitulum, the highest number of seeds per capitulum was 26.56 seed capitula^{-1} with irrigation during flowering and capitula formation periods. Similar to the findings reported by Jabbari et al. (2010) and Zarghami et al. (2011) stated that drought particularly during flowering and capitula formation has significant effect on safflower seed yield.

Thousand seed weight (g)

The variance analysis revealed that the treatments had a significant effect ($P < 0.01$) on 1000-weight in the first year, while the effect of rainfed-irrigation × variety interaction was not significant (*Table 8*). The one thousand seed weight under irrigated conditions (32.82 g) was significantly higher than that obtained under rainfed conditions (26.15 g). In the second year, the effect of rainfed-irrigation treatment on 1000-seed weight was not significant. The highest 1000- seed weight in the first and second year of the experiment was obtained Balcı (34.05 g) and Dinçer (40.38 g) variety (*Table 5*). Similarly, Omidi et al. (2012) and Muhammedi et al. (2018) found that cultivars and irrigation issues had a significant af1000- seed weight, and Santos et al. (2018) found that 1000-seed weight in different irrigation periods was higher than that obtained in the control plot.

Table 5. *Thousand seed weight (g) and yield (kg ha^{-1}) of some safflower (Carthamus tinctorius L.) cultivars grown under rainfed and irrigated conditions*

Variety	Thousand seed weight (g)				Yield (kg ha^{-1})			
	2019-2020		2020-2021		2019-2020		2020-2021	
	Rainfed	Irrigated	Rainfed	Irrigated	Rainfed	Irrigated	Rainfed	Irrigated
Safir	28.08c	33.11ab	36.42bc	37.28bc	866.5d	2339.8b	451.4fg	2250.5c
Koç	20.97e	31.59b	36.55bc	36.02c	420.9e	2180.8b	351.9g	1968.5d
Göktürk	28.71c	32.75ab	35.48c	35.71c	1003.1cd	221.98b	643.2ef	2295.1bc
Balcı	25.19d	34.05a	37.85abc	36.30bc	856.5d	2724.4a	655.9e	2464.9ab
Dinçer	27.83c	32.63ab	39.06ab	40.38a	1139.7c	2664.3a	787.8e	2565.7a
Mean	26.15b	32.82a	37.07a	37.14a	857.3b	2425.8a	578.1b	2308.9a

Means that do not share a letter are significantly different. *p ≤ 0.05, **p ≤ 0.01, ns: non-significant

Seed yield (kg ha^{-1})

The effect of all treatments on seed yield in 2019-2020 growing period was significant ($P < 0.01$). The effects of rainfed-irrigation and variety factors were also significant ($P < 0.01$) in 2020-21, while the effect of rainfed × irrigation interaction on seed yield was not significant (*Table 8*). The seed yields obtained under irrigated

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conditions (2425.8 and 2308.9 kg ha^{-1}) were higher in both years of the experiment compared to those obtained under rainfed conditions (857.3 and 578.1 kg ha^{-1}). Low yield of the varieties grown under rainfed conditions in the second year of the experiment may be associated with the negative climatic conditions, the absence of precipitation during the periods desired by safflower and severe drought throughout the year (*Table 5*). The best performance under rainfed conditions was obtained in Dinçer variety, and Dinçer and Balcı varieties under irrigated conditions (*Table 5*). The findings reported by other researchers were in agreement with our findings. Omidi et al. (2012) showed that the decrease in available water content causes a 10 to 38% decrease in yield. Nacar et al. (2016) stated that the yield increased with irrigation and Santos et al. (2018). determined that the yield was higher in irrigation compared to the control plots. Muhammedi et al. (2018) found that water stress reduced seed yield, and Koç (2019) revealed that precipitation in April, May and June has direct effect on seed yield.

Petal yield (g $plant^{-1}$)

The effect of all treatments on petal yield in 2019-2020 growing season was not significant. In contrast, the effects of rainfed-irrigation and variety factors on petal yield was statistically significant at $P < 0.01$ level in 2020-2021 growing period, and the effect of rainfed-irrigation × variety interaction was significant at $P < 0.05$ level (*Table 8*). The petal yield of cultivars grown under irrigation condition in the 2020-2021 growing season was higher than the petal yield of cultivars grown in rainfed conditions. Safir and Göktürk varieties had the best performance. Özel et al. (2004) reported that the petal yield varied between 0.46-1.60 g plant-1 depending on the planting time and the interrow distance, and Doğan (2021) indicated that the crown yield was between 0.38-0.67 g in irrigated and rainfed conditions.

Oil ratio (%)

The effect of all treatments in both growing seasons on oil ratio was significant ($P < 0.01$) (*Table 8*). The oil ratio of varieties under irrigated conditions was higher than the oil ratio record under rainfed conditions (*Table 6*). The highest oil ratio under rainfed and irrigation conditions was recorded in Balcı variety. The oil ratio under rainfed and irrigation conditions was reported between 40.3-44.2% by Bergman et al. (2001). Öztürk et al. (2009) indicated that mean crude oil ratio under rainfed and irrigated conditions was 28.3 and 32.7%, respectively. Eslam et al. (2010) reported that water deficit during seed filling period caused a decrease in oil ratio and yield. Similarly, Santos et al. (2018) showed that oil ratios of safflower varieties decreased with increasing drought, and Koç (2019) showed that irrigation had a positive effect on oil ratio.

Oil yield (kg ha^{-1})

The rainfed and irrigated treatments had a significant effect on oil rate in both growing seasons (*Table 8*). The cultivars grown under irrigation had higher oil content than cultivars grown under rainfed conditions (*Table 7*). In both years, Balcı variety had the best performance under rainfed and irrigated conditions and had the highest oil yield (705.1 and 911.6 kg ha^{-1}). Oil yields found in previous studies are consistent with the oil rates determined in this study. The oil yield reported by Öztürk et al. (2009) was 624 kg ha^{-1} and 264 kg ha^{-1} under irrigated and rainfed conditions; crude oil yield found

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by Sirel (2011) was between 180.6 and 392.3 kg ha^{-1}, and Yurteri (2016) indicated that the crude oil yield in summer and winter sowing was between 244.4 and 505.5 kg ha^{-1}, respectively. Similar to our findings, Santos et al. (2018) stated that safflower oil yield increased with irrigation; Mohammadi et al. (2018) indicated that lack of water reduces seed and safflower oil yield and safflower varieties showed different water stress responses; Ebrahimian et al. (2019) revealed that irrigation increased oil yield.

Table 6. *Mean petal yield (g plant^{-1}) and oil ratio (%) of some safflower (Carthamus tinctorius L.) cultivars grown under rained and irrigated conditions*

Variety	Petal yield (g plant $^{-1}$)				Oil ratio (%)			
	2019-2020		2020-2021		2019-2020		2020-2021	
	Rainfed	Irrigated	Rainfed	Irrigated	Rainfed	Irrigated	Rainfed	Irrigated
Safir	0.43^{ns}	0.54 ^{ns}	0.35c	0.48a	27.09bc	28.76ab	30.64b	34.91a
Koç	0.38	0.50	0.23d	0.38bc	25.38cd	25.91cd	34.93a	37.52a
Göktürk	0.55	0.44	0.25d	0.48a	28.89ab	28.09b	34.66a	37.50a
Balcı	0.44	0.53	0.24d	0.47ab	30.41a	26.02cd	35.53a	37.37a
Dinçer	0.67	0.64	0.23d	0.46ab	24.29d	20.51e	28.91b	29.03b
Mean	0.49	0.53	0.25b	0.46a	25.85b	27.21a	32.93b	35.26a

Means that do not share a letter are significantly different. *p ≤ 0.05, **p ≤ 0.01, ns: non-significant

Table 7. *Mean oil yield (kg ha^{-1}) of some safflower (Carthamus tinctorius L.) cultivars grown under rained and irrigated conditions*

Variety	Oil yield (kg ha^{-1})			
	2019-2020		2020-2021	
	Rainfed	Irrigated	Rainfed	Irrigated
Safir	234.7e	672.8ab	144.7de	785.9bc
Koç	106.8f	564.6cd	123.6e	738.5c
Göktürk	289.6e	623.7bc	223.2de	860.7ab
Balcı	260.4e	705.1a	244.6d	921.6a
Dinçer	276.9e	546.5d	227.7de	744.7c
Mean	233.6b	622.5a	192.8b	810.3a

Means that do not share a letter are significantly different. *p ≤ 0.05, **p ≤ 0.01, ns: non-significant

Economic analysis

Economic analysis were performed by collecting input and output data, and benefit cost ration was determined by dividing net return on total cost as described (Ali et al., 2019). Incomes from safflower varieties grown under rainfed and irrigated conditions in the 2019-2020 and 2020-2021 growing seasons were calculated (*Table 9*). The input cost (fertilizer, irrigation and spraying costs), total income and net income received by the farmer were calculated. Price of one kg of safflower seed for 2020 and 2021 was 0.45 $ kg^{-1} and 0.54 $ kg^{-1} respectively. Crop incentive by the government was 0.07/$ kg^{-1}. Net income of safflower cultivars grown under irrigated conditions was higher than those grown under rainfed conditions. The highest net income under irrigated conditions was obtained by Balcı and Dinçer cultivars (*Fig. 3*).

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Table 8. *F value for some safflower (Carthamus tinctorius L.) cultivars in rainfed and irrigated conditions, the degree of importance and % CV values*

2019-2020	F value								
	Plant height (cm)	**Number of lateral branches (pieces plant^{-1})**	**Number of capitula (piece plant^{-1})**	**Number of seed (piece capitula^{-1})**	**Thousand seed weight (g)**	**Yield (kg ha^{-1})**	**Petal yield (g plant^{-1})**	**Oil ratio (%)**	**Oil yield (kg ha^{-1})**
Rainfed-irrigated	60.50**	ns	81.45**	1239.47**	599.69**	4398.18**	ns	36.64**	2794.27**
Variety	65.08**	5.19**	23.55**	31.44**	36.68**	74.54**	ns	105.78**	49.04**
Rainfed-irrigated*variety	5.73**	ns	20.90**	15.52**	22.56**	23.33**	ns	28.00**	25.44**
% CV	1	8	6	5	3	4	23	2	5
2020-2021									
Irrigated-rainfed	3130.26**	83.11**	419.85**	147.69**	ns	5349.28**	255.21**	37.93**	2253.24**
Variety	20.28**	5.32**	40.95**	28.14**	15.04**	56.27**	9.86**	59.09**	17.49**
Rainfed-irrigated*variety	39.14**	4.25**	33.05**	ns	ns	ns	3.56*	ns	ns
% CV	2.43	8.61	5.38	6.53	2.67	4.48	9.63	3.03	7.10

Means that do not share a letter are significantly different. *$p \leq 0.05$, **$p \leq 0.01$, ns: non-significant. CV: coefficient of variations

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Table 9. *Economic analysis for safflower (Carthamus tinctorius L.) cultivars under rainfed and irrigated conditions*

Period	Safflower varieties	Yield (kg ha^{-1})	Total income ($ ha^{-1})	Total cost ($ ha^{-1})	Crop incentive ($ ha^{-1})	Net income ($ ha^{-1})	Benefit cost ratio
	Rainfed						
2019-2020	Safir	866.50	351.28	132.43	0.07	218.93	2.65
	Koç	420.90	170.64	132.43	0.07	38.28	1.29
	Göktürk	1003.70	406.66	132.43	0.07	274.30	3.07
	Balcı	856.50	347.23	132.43	0.07	214.87	2.62
	Dinçer	1193.70	462.34	132.43	0.07	329.68	3.49
	Irrigated						
	Safir	2339.80	948.57	259.46	0.07	689.18	3.66
	Koç	2180.80	884.11	259.46	0.07	624.72	3.41
	Göktürk	2219.80	899.92	259.46	0.07	640.53	3.47
	Balcı	2724.40	1104.49	259.46	0.07	845.10	4.26
	Dinçer	2664.30	1080.12	259.46	0.07	820.74	4.16
2020-2021	**Rainfed**						
	Safir	451.40	213.68	128.99	0.07	84.75	1.66
	Koç	351.90	166.58	128.99	0.07	37.65	1.29
	Göktürk	643.20	304.47	128.99	0.07	175.54	2.36
	Balcı	655.90	310.49	128.99	0.07	181.56	2.41
	Dinçer	787.80	372.92	128.99	0.07	243.99	2.89
	Irrigated						
	Safir	2250.50	1065.33	252.07	0.07	813.32	4.23
	Koç	1968.50	931.83	252.07	0.07	679.83	3.70
	Göktürk	2295.10	1086.44	252.07	0.07	834.43	4.31
	Balcı	2464.90	1166.82	252.07	0.07	914.81	4.63
	Dinçer	2565.70	1214.53	252.07	0.07	962.53	4.82

August 2020 1 TL (Turkish Lira): $7.40; August 2021 1 TL: $8.40
2020 crop income kg^{-1}: 0.45$; 2021 crop income kg^{-1}: 0.54$

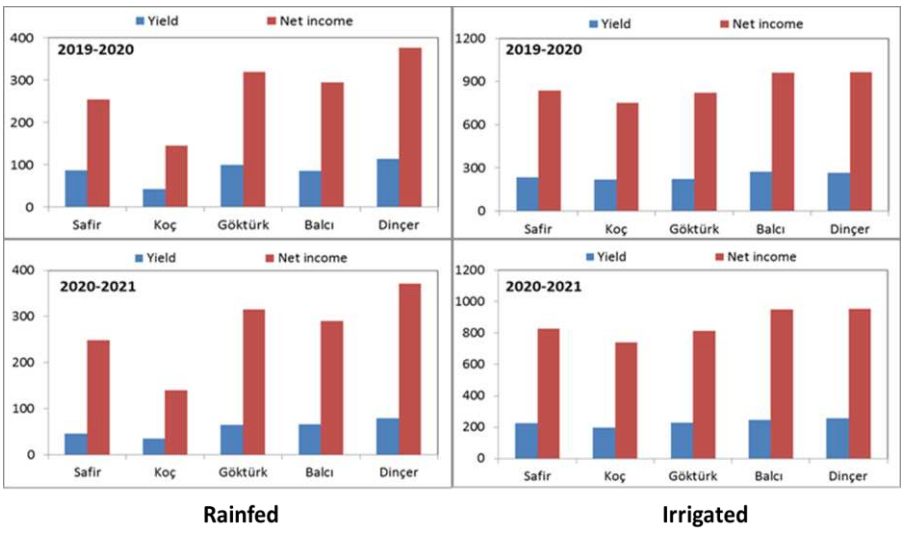

Figure 3. *Comparison of yield and net income for safflower (Carthamus tinctorius L.) cultivars under rainfed and irrigated conditions*

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Conclusion and recommendations

Seed yield of safflower cultivars grown under rainfed and irrigated conditions was significantly different. Safflower is not recommended and not economical due to low yield in arid conditions. The safflower plant, sown in winter, has to be irrigated to compete with wheat-barley plants, and to meet the water needed when plants need water. The possibility of irrigation should be considered especially if the sunflower is recommended to be grown in winter. In the second growing period of the experiment, the decrease in yield is more obvious seen due to insufficient rainfall. Dinçer variety had the best performance under rainfed conditions, and Dinçer and Balcı varieties under irrigated conditions. In addition, varieties grown by irrigation have higher oil content than varieties grown in rainfed conditions. The results revealed that Balcı had a high oil content, and variety showed the best performance under rainfed and irrigated conditions.

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EFFECTS OF DIFFERENT IMPROVEMENT MEASURES ON HYDROTHERMAL CARBON AND COTTON (*GOSSYPIUM HIRSUTUM* L.) YIELD IN SALINE-ALKALI SOIL

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Abstract. In order to explore the effects of different improvement measures on hydrothermal carbon and cotton (*Gossypium hirsutum* L.) yield of saline-alkali soil in Xinjiang, China, four treatments were applied: biochar (B, 20 t·ha^{-1}), desulfurization gypsum (D, 35 t·ha^{-1}), straw mulching (S, 16 t·ha^{-1}) and control (CK). The results showed that the soil moisture content in 0-20 cm and 20-40 cm deep soil layers under the three improved treatments was significantly higher than that of the control throughout the whole growth period. The spatial distribution pattern of soil water in each treatment profile showed the characteristics of lower wet and upper dry. Compared with CK, the improved treatments showed good warming and heat preservation effects, both showed warming effect at low temperature and cooling effect at high temperature. The three improvement measures could increase the content and density of soil organic carbon, among which the application of biochar was the most significant, and the density of organic carbon was 17.46% higher than that of the control. Compared with the control, the yield increase rate of cotton in treatment B was the highest, up to 32.28%. In conclusion, the application of 20 t·ha^{-1} biochar is suitable for soil improvement in the process of cotton planting in saline farmland in Xinjiang, China.
Keywords: *salinized soil, hydrothermal condition, organic carbon, yield, improved measures*

Introduction

Cotton (*Gossypium hirsutum* L.) planting in Xinjiang plays an important role in China's cotton production (Hou et al., 2021). In 2013, the cotton planting area in Xinjiang was 1.80×10^6 ha, accounting for 36.7% of the national cotton planting area, with a total output of 3.52×10^6 t, accounting for 55.9% of the total cotton output in China (Zong et al., 2021). The region has a dry climate, sparse rainfall, strong evaporation, and serious soil salinization (He et al., 2018). In 2005, the area of salinized cultivated land in Xinjiang reached 1.62×10^4 ha, accounting for 32.1% of the total cultivated land area (Wang et al., 2020). Soil salinization leads to the continuous reduction of farmland soil water and fertilizer conservation capacity and agricultural productivity, which seriously restricts the sustainable development of agriculture in Xinjiang, China (Tan et al., 2018). Therefore, it is urgent to apply appropriate soil improvement products to conserve water and fertilizer.

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In recent years, the research on the effect of different improvement products on saline-alkali soil has attracted extensive attention (Zhai et al., 2016; Zhao et al., 2018; Zhu et al., 2020; Zhou et al., 2021). For example, biochar contains organic carbon and has porous structure and large surface area (Liang et al., 2021). After being applied to the soil, it can effectively increase the content of soil organic carbon and improve its nutrient absorption and water holding capacities of the soil (Cui et al., 2021). Therefore, it is widely used in soil remediation and agricultural production. He et al. (2020) and Zhao et al. (2020) showed that the application of biochar had a good effect on improving soil water permeability and permeability and improving soil fertility. Desulfurized gypsum, as coal-fired desulfurization waste, is an economic and environment friendly soil conditioner, which has been proved to be an effective saline alkali soil improvement product (Zhang et al., 2021). Hammerschmitt et al. (2021) showed that desulfurization gypsum could improve soil physical and chemical properties, increase salinized soil organic matter and crop yield. Straw mulching can not only reduce environmental pollution and increase soil carbon pool, but also improve the water, fertilizer, gas and heat status of soil (Wang et al., 2018). A large number of studies have shown that straw mulching can improve soil properties, inhibit water evaporation, improve soil water holding capacity (Wang et al., 2021), effectively stabilize the change of ground temperature and increase the content of soil organic carbon (Ma et al., 2019).

At present, there are many studies on the improvement effect of single measure on saline alkali soil, while there are few reports on the comprehensive analysis and comparison of hydrothermal carbon and cotton yield of saline-alkali soil by selecting different improvement measures at the same time. In view of this, this paper carried out a field experiment in Shaya County, Aksu Prefecture, Xinjiang, China, to explore the effects of three common improvement measures of biochar (B), desulfurization gypsum (D) and straw mulching (S) on the hydrothermal carbon environment of saline-alkali soil and cotton yield, in order to determine more suitable soil improvement measures for improving the hydrothermal conditions of saline-alkali soil in arid areas and provide scientific basis and technical support for improving soil fertility and cotton yield.

Materials and methods

Overview of test area

The experiment was conducted in Shaya County (41°25'N, 84°47'E) in Aksu Prefecture, Xinjiang, China. This region is located in the south of the middle section of Tianshan Mountain, the north edge of Taklimakan Desert and the middle reaches of Tarim River, with an altitude of 946-1050 m. Moreover, the region is far from the sea, the East, South and West are surrounded by deserts, and the ecological environment is relatively fragile. In addition, the region is a typical continental warm temperate arid climate, with annual precipitation of 57.44 mm, evaporation of 2756 mm, average temperature of 11.32℃, annual sunshine number of 2965 h, wind speed of 2.54 $m \cdot s^{-1}$, and frost-free period of 148 days (Liang et al., 2020). Before the experiment, the physical and chemical properties of the initial soil were determined. The soil in the study area (0-40 cm) was sandy loam (63.32% sand, 34.23% silt, 2.45% clay) saturated water content was 0.396 $cm^3 \cdot cm^{-3}$, wilting water content was 0.048 $cm^3 \cdot cm^{-3}$, field water capacity was 0.203 $cm^3 \cdot cm^{-3}$ (Chen et al., 2018; Liang et al., 2019).

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Test materials

The biochar selected in the test was prepared by pyrolysis of corn straw used by Xinjiang Carbon Biotechnology Co., Ltd., China, the results for many years showed that the best application rate in the field was 20 $t \cdot ha^{-1}$ (Liang and Shi, 2021). The desulfurized gypsum was selected from the South Thermal Power Plant of Xinjiang Tianfu Energy Co., Ltd., China, and the optimal application rate was 35 $t \cdot ha^{-1}$ according to the principle of ion exchange reaction (Wang et al., 2017). The locally harvested corn straw cut into small sections of about 5 cm by hay cutter was selected, and the application rate was 16 $t \cdot ha^{-1}$ (Tan et al., 2017).

Experimental design

The test was conducted in April to October 2019 and 2020. Various modifiers were applied to the soil surface in April 2019 and evenly mixed with the topsoil soil with a rotary cultivator. The main sampling date was April to October 2020. A single factor completely randomized block design was adopted. Using the field test method, 4 treatments were set as follows: CK (blank control without any soil improvement measures), B (biochar, 20 $t \cdot ha^{-1}$), D (desulfurized gypsum, 35 $t \cdot ha^{-1}$) and S (straw mulching, 16 $t \cdot ha^{-1}$). Each treatment area was 30 m^2 (6 m long and 5 m wide), with 3 replicates. Cotton was planted by drip irrigation under the film. The cotton was cultivated by drip irrigation under film, and the cultivation mode was 1 film, 2 tubes and 4 rows, which the plant and row distance were 0.10 m and 0.20 m, respectively. The field management level in the later stage of treatment was the same. Conventional irrigation was adopted in the whole growth period of cotton, and the irrigation amount was consistent with the actual production. The irrigation quota of cotton growth period was 5250 $m^3 \cdot ha^{-1}$, the irrigation period was 7-10 days, and the total irrigation time was 10 times. The soil conditions of the test site before the test were shown in *Table 1*.

Table 1. *The condition of soil in the study area*

pH	Electric conductibility ($dS \cdot m^{-1}$)	Bulk density ($g \cdot cm^{-3}$)	Total salt content ($g \cdot kg^{-1}$)	Available phosphorus ($mg \cdot kg^{-1}$)	Available potassium ($mg \cdot kg^{-1}$)	Alkali-hydrolyzed nitrogen ($mg \cdot kg^{-1}$)	Organic matter ($g \cdot kg^{-1}$)
8.32	1.58	1.64	5.15	8.75	218.45	50.74	14.32

Investigate items and methods

Soil moisture content

At stages 13, 55, 61, 79 and 89 in BBCH scale for cotton, soil drill was used for multi-point collection of soil samples in different treatments. The sampling depth was 0-100 cm, and every 20 cm was a layer. After each layer of soil was mixed evenly, it was brought back to the laboratory, drying for 8 h to dry state under the constant temperature of 105℃, and then the soil mass moisture content was calculated.

Soil temperature

The soil temperature was measured by a WH55-405330 curved tube geothermometer (Dongfang Chemical Glass (Beijing) Technology Co., Ltd, China) for 3 consecutive days in each growth period. The soil temperature at the depth of 5, 15, 25 and 35 cm of the soil

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layer was read every 2 hours from 08:00 to 20:00 every day, and finally the average value was obtained.

Soil organic carbon content

At stages 13, 55, 61, 79 and 89 in BBCH scale for cotton, different treatments were sampled in 0-20 cm and 20-40 cm soil layers according to the multi-point method. The soil samples were crushed and mixed, dried and ground naturally, and the content of soil organic carbon was determined by potassium dichromate volumetric method. The organic carbon density of the *i* soil layer and the total organic carbon density of soil profile were calculated as *Eq.1* and *Eq.2*, respectively (Patton et al., 2019).

$$D_{SOCi} = C_i D_i E_i / 100 \quad \text{(Eq.1)}$$

$$D_{SOCt} = \sum_{i=1}^{n} D_{SOCi} = \sum_{i=1}^{n} C_i D_i E_i / 100 \quad \text{(Eq.2)}$$

where, D_{SOCi} represents the organic carbon density of the *i* soil layer, $kg \cdot m^{-2}$. D_{SOCt} represents the total organic carbon density of the soil profile, $kg \cdot m^{-2}$. C_i represents the content of soil organic carbon of the *i* soil layer, $g \cdot kg^{-1}$. D_i represents the soil bulk density of the *i* soil layer, $g \cdot cm^{-3}$. E_i represents the thickness of the *i* soil layer, cm. *n* represents the number of soil layers.

Cotton yield measurement

On September 18, 2020, the cotton yield under each treatment in the experimental plot of the study area was measured. 3 sample points were taken from each community. 11 lines were taken from each sample point to measure the line spacing and calculate the average line spacing. 21 plants in one row were randomly selected from each sample point to measure the plant spacing and calculate the average plant spacing. 3 rows were randomly selected from each sample point, 10 plants in each row, a total of 30 plants, and the number of bolls was investigated. 100 bolls were collected randomly at each sample point to weigh them after drying and calculate the average single boll weight. Cotton yield was calculated by the average line spacing, plant spacing, number of bolls per plant and average single boll weight.

Data processing and analysis

Data processing were carried out with Microsoft Excel 2016. The analysis of variance was performed by SPSS 20.0 statistical software. One-way ANOVA and LSD were used to test the significance of multiple comparison differences (α=0.05).

Results

Dynamic change characteristics of soil moisture content during cotton growth period

The dynamic changes of soil moisture content in 0-20 cm and 20-40 cm soil layers during the cotton growth period of each treatment were shown in *Fig. 1*. At stage 13 in BBCH scale for cotton (S1), plants were short and water consumption was low. The soil moisture content of CK treatment at 0-20 cm and 20-40 cm was lower than that of B and

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S treatments, while there was no significant difference between CK and D (p> 0.05) in 20-40 cm soil layer. At stage 55 in BBCH scale for cotton (S2), the growth indexes of cotton gradually increased, and the soil moisture content decreased due to water consumption. There was significant difference between the three improved treatments and the control (p< 0.05), while there was no significant difference between B and D treatments (p> 0.05), indicating that each improved measure had a good moisture conservation effect. The budding stage was the key period of cotton growth, and the appropriate water was more conducive to its growth and development. At stage 61 in BBCH scale for cotton (S3), the increase of soil moisture content in 0-20 cm soil layer B was the largest, up to 24.39%, and there was no significant difference between the three improved treatments and the control in 20-40 cm soil layer (p> 0.05). At stage 79 in BBCH scale for cotton (S4), the soil moisture content of each treatment in each soil layer was significantly different from that of the control (p< 0.05). At stage 89 in BBCH scale for cotton (S5), crop water consumption decreased in the mature stage, resulting in little change in soil moisture content. In 0-20 cm soil layer, B and S treatment were significantly higher than CK by 17.56% and 15.85%, respectively (p< 0.05). There was no significant difference between D treatment and CK (p> 0.05). In 20-40 cm soil layer, B treatment was significantly higher than CK (p< 0.05), with an increase of 16.00%, while there was no significant difference between D and S treatments (p> 0.05).

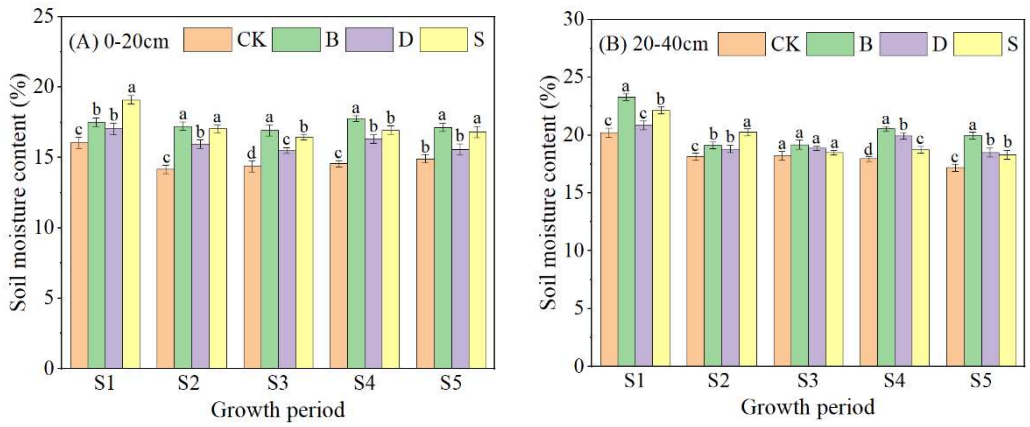

Figure 1. *Dynamic changes of soil moisture content in different treatments during whole growth period. CK represents blank control without any soil improvement measures, B represents biochar 20 t·ha^{-1}, D represents desulfurized gypsum 35 t·ha^{-1}, and S represents straw mulching 16 t·ha^{-1}. S1, S2, S3, S4 and S5 represent stages 13, 55, 61,79 and 89 in BBCH scale for cotton, respectively. The error lines represent the standard variance, and different lowercase letters represent significant differences among treatments in the same soil layer (p< 0.05)*

Profile distribution characteristics of soil moisture content

The spatial distribution characteristics of soil moisture in 0-100 cm soil layer under different treatments within 110 days of cotton growth period were analyzed (*Fig. 2*). It could be seen that the spatial distribution characteristics of soil moisture in 0-40 cm soil layer of was obvious (p< 0.05), and the soil moisture content of each improved treatment was higher than that of CK. In the 40-100 cm soil layer, the soil moisture content fluctuated among the treatments, and there was no obvious law between treatments

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(p> 0.05). At stage 13 in BBCH scale for cotton, the soil moisture content of 0-100 cm soil layer of each treatment was higher, and the soil moisture content of 0-100 cm soil layer of CK treatment was lower than that of B, D and S treatment. At stage 55 in BBCH scale for cotton, the soil moisture content of 0-40 cm soil layer of each improvement treatment was generally higher than that of the control, and the effect of 10-20 cm soil layer was the most obvious (p< 0.05). The order of soil moisture content from large to small was B > S > D > CK. At stage 61 in BBCH scale for cotton, the soil moisture content of each treatment was higher than that of CK. At stage 79 in BBCH scale for cotton, the moisture content contour of 10-30 cm soil layer of B treatment was dense, which reflected that the soil moisture content gradient was large and changed violently in space. The soil moisture content was significantly higher than that of CK treatment, with a maximum increase of 80.47%. In addition, the spatial distribution pattern of soil moisture in each treatment profile showed the characteristics of lower wet and upper dry, but the spatial distribution position and soil moisture content of dry and wet soil layer between different treatments were less different (p> 0.05).

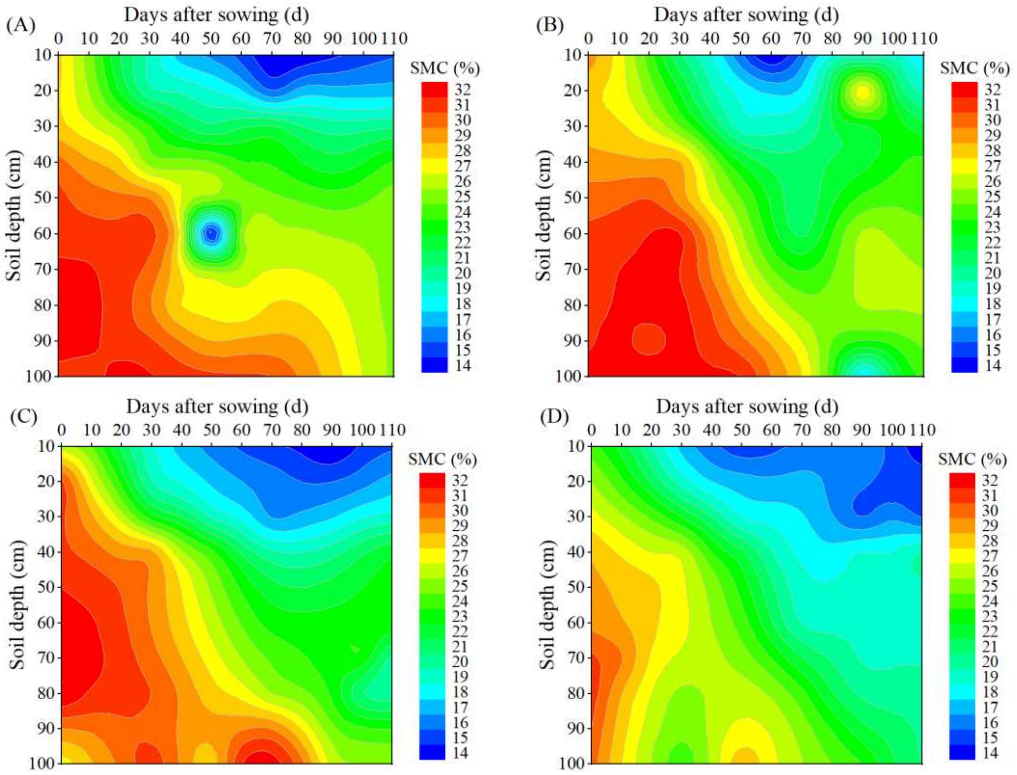

Figure 2. *Spatial distributions of soil moisture under different treatments. A represents the spatial distributions of soil moisture under biochar 20 t·ha^{-1}, B represents the spatial distributions of soil moisture under desulfurized gypsum 35 t·ha^{-1}, C represents the spatial distributions of soil moisture under straw mulching 16 t·ha^{-1} and D represents the spatial distributions of soil moisture under blank control without any soil improvement measures. SMC represents soil moisture cotton*

Effects of different improvement measures on soil temperature

The changes of soil multi-day average temperature in different growth stages of different treatments were shown in *Table 2*. At stage 13 in BBCH scale for cotton (S1),

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the heat preservation effects of treatment B and treatment D were the same. The depth temperature of each soil layer had no significant difference (p> 0.05), while they were higher than that of CK. There was a significant difference between treatment S and CK at the depth of 25 cm (p< 0.05). At stage 55 in BBCH scale for cotton (S2), D and S treatment had the same heat preservation effect, and the soil temperature at 15 cm was significantly higher than CK, increased by 2.5℃ and 2.44℃, respectively (p< 0.05). Compared with the control, B, D and S treatments could effectively improve the soil temperature in the early stage of cotton growth, make cotton emerge in advance and provide good soil temperature conditions for early growth. At stage 61 in BBCH scale for cotton (S3), the soil temperature of treatment B, D and S at the depth of 5-25 cm was 0.02-1.3℃ lower than that of CK, and the external temperature corresponding to this growth stage reached the peak value of the whole year. At stage 79 in BBCH scale for cotton (S4), due to the decrease of temperature at this time and the influence of physiological activities such as transpiration during plant growth, the relative humidity near the ground increased, resulting in the decrease of soil temperature. The thermal insulation effect of treatment B and D was equivalent, and the difference between treatment S and CK in 5-15 cm soil layer was significant (p< 0.05). At stage 79 in BBCH scale for cotton (S5), treatment B, D and S were significantly different from CK at 25 cm soil layer (p< 0.05), with an increase of 1.85℃, 1.74℃ and 1.89℃, respectively.

Table 2. *Comparison of soil temperature in different growth stages of cotton under different treatments*

Treatment	Soil depth (cm)	Soil temperature in different growth stages of cotton				
		S1(℃)	S2 (℃)	S3 (℃)	S4 (℃)	S5 (℃)
CK	5	21.32b	22.90a	26.23a	18.40b	15.11a
	15	20.90a	21.20b	25.11a	17.90b	14.11b
	25	19.31b	20.34a	23.60a	17.02a	13.22b
	35	19.21a	20.05a	21.06a	16.23a	13.22a
B	5	23.35ab	24.21a	25.13a	20.34ab	16.41a
	15	22.57a	22.79ab	23.85a	18.51ab	15.32ab
	25	21.90a	22.23a	23.01a	17.38a	15.07a
	35	20.86a	21.47a	21.04a	16.41a	14.25a
D	5	23.12ab	23.97a	26.20a	20.21ab	15.25a
	15	22.36a	23.70a	24.16a	18.14ab	15.07ab
	25	22.01a	22.12a	23.58a	18.08a	14.96a
	35	20.57a	21.04a	21.53a	17.18a	13.59a
S	5	23.80b	24.36a	26.01a	21.31a	16.21a
	15	22.41a	23.64a	24.02a	20.26a	15.73a
	25	21.80a	22.30a	22.90a	18.13a	15.11a
	35	21.03a	22.08a	21.46a	17.62a	14.27a

CK represents blank control without any soil improvement measures, B represents biochar 20 $t \cdot ha^{-1}$, D represents desulfurized gypsum 35 $t \cdot ha^{-1}$, and S represents straw mulching 16 $t \cdot ha^{-1}$. S1, S2, S3, S4 and S5 represent stages 13, 55, 61,79 and 89 in BBCH scale for cotton, respectively. The different lowercase letters represent significant differences among treatments in the same soil layer (p< 0.05)

Effects of different improvement measures on soil organic carbon content

The variation characteristics of soil organic carbon content in 0-20 cm and 20-40 cm soil layers of each treatment in the whole growth period were shown in *Fig. 3*. In 0-20 cm soil layer, the content of soil organic carbon in treatment B and D was significantly higher than CK at stage 13 in BBCH scale for cotton (S1) (p< 0.05), with an increase of 52.58%

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and 24.96%, respectively. There was no significant difference between treatment S and CK (p> 0.05). In 20-40 cm soil layer, only treatment B was significantly higher than CK (p< 0.05), with an increase of 17.34%. At stages 55 (S2) and 61 (S3) in BBCH scale for cotton, the content of soil organic carbon in 0-20 cm soil layers treated with B, D and S increased by 52.22%, 27.12%, 15.72% and 36.90%, 16.85% and 20% respectively compared with CK. In 20-40 cm soil layer, the maximum increases of treatment B, D and S compared with CK were 29.42%, 18.18% and 19.37%, respectively. At stage 79 in BBCH scale for cotton (S4), the soil organic carbon content of each treatment was significantly different from that of CK in 0-20 cm and 20-40 cm soil layers, with an increase of 12.95- 47.12% (p< 0.05). At stage 89 in BBCH scale for cotton (S5), the soil organic carbon content of B and S treatment increased by 15.92% and 16.90% respectively in 0-20 cm soil layer, while the increase of D treatment was not significant (p> 0.05). In 20-40 cm soil layer, the soil organic carbon content of B, D and S treatment increased by 26.22%, 15.18% and 10.05%, respectively.

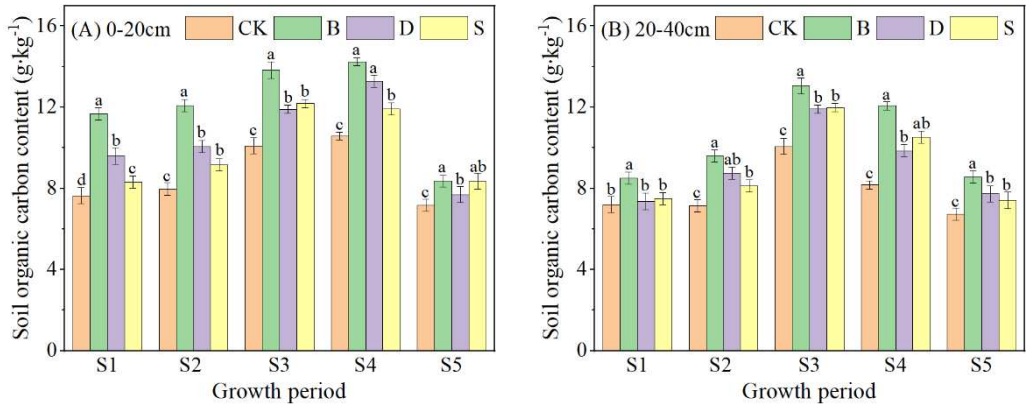

Figure 3. *Dynamic changes of soil organic carbon content under different treatments. CK represents blank control without any soil improvement measures, B represents biochar 20 t·ha^{-1}, D represents desulfurized gypsum 35 t·ha^{-1}, and S represents straw mulching 16 t·ha^{-1}. S1, S2, S3, S4 and S5 represent stages 13, 55, 61,79 and 89 in BBCH scale for cotton, respectively. The error lines represent the standard variance, and different lowercase letters represent significant differences among treatments in the same soil layer (p< 0.05)*

Changes of soil organic carbon density under different improvement measures

The total density of soil organic carbon in different soil layers of each treatment was higher than that of CK (p< 0.05, *Table 3*). The total density of soil organic carbon in 0-40 cm soil layer of CK, B, D and S treatments were 3.77, 4.43, 4.12 and 4.00 kg·m^{-2} respectively. Among them, B treatment was significantly higher than CK treatment (p< 0.05), with an increase of 17.46%. There was no significant difference among B, D and S treatments (p> 0.05). In 0-20 cm soil layer, the fluctuation range of soil organic carbon density among treatments was 1.93-2.19 kg·m^{-2}, in which treatment B and D were significantly higher than CK (p< 0.05), with an increase of 10.80% and 13.46% respectively. In the 20-40 cm soil layer, the fluctuation range of soil organic carbon density among treatments was 1.84-2.24 kg·m^{-2}. Among them, treatment B was significantly higher than other treatments (p< 0.05), 21.64%, 12.99% and 13.86% higher than CK, D and S, respectively. The soil organic carbon density between treatments D and S remained at the same level. The content of soil organic carbon in 0-20 cm soil layer

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was higher than that in 20-40 cm soil layer, indicating that the improvement measures were the main reason for the change of soil organic carbon density.

Table 3. *Soil organic carbon density under different treatments (kg·m^{-2})*

Treatment	Soil depth (cm)		Total
	0-20	20-40	
CK	1.93b	1.84b	3.77b
B	2.02ab	1.97b	4.00a
D	2.19a	2.24a	4.43a
S	2.14a	1.98b	4.12a

CK represents blank control without any soil improvement measures, B represents biochar 20 t·ha^{-1}, D represents desulfurized gypsum 35 t·ha^{-1}, and S represents straw mulching 16 t·ha^{-1}. The different lowercase letters represent significant differences among treatments in the same soil layer ($p< 0.05$)

Effects of different improvement measures on cotton yield

The cotton yield under different improvement measures was shown in *Table 4*. Compared with CK, different improvement measures could promote the cotton yield and single boll weight, and the increase of each treatment was significantly different from that of the control ($p< 0.05$). Under different treatments, the single boll weight of cotton remained between 5.93-8.07 g. The single boll weight and yield of B, D and S treatments were significantly higher than CK ($p< 0.05$), with an increase of 36.10%, 29.67%, 25.11% and 32.28%, 30.68% and 21.94%, respectively, and there was no significant difference among the three treatments ($p> 0.05$). The heat preservation effect of each treatment at the seedling stage was significantly better than that of the blank treatment, which could lead to early emergence. The soil moisture content and organic carbon content effectively increased after the application of modifier, which promoted the growth and development of cotton. Therefore, the yield of each improved treatment was better than that of the blank treatment.

Table 4. *Effects of different improvement measures on cotton yield*

Treatment	Single boll weight (g)	Yield (kg·ha^{-1})	Yield increase rate (%)
CK	5.93b	4212b	
B	7.42a	5136a	21.94
D	8.07a	5572a	32.28
S	7.69a	5504a	30.68

CK represents blank control without any soil improvement measures, B represents biochar 20 t·ha^{-1}, D represents desulfurized gypsum 35 t·ha^{-1}, and S represents straw mulching 16 t·ha^{-1}. The different lowercase letters represent significant differences among treatments in the same soil layer ($p< 0.05$)

Discussion

Effects of different improvement measures on soil moisture content and soil temperature

Water and heat conditions are the key factors affecting crop emergence rate, growth and development and improving crop yield (Yang et al., 2018a), while saline-alkali soil has hardened structure and poor ability of soil moisture and fertilizer conservation (Yan

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et al., 2021). Our results showed that the application of biochar, desulfurization gypsum and straw mulching could improve the soil moisture (*Fig. 1*) and temperature (*Table 2*) in varying degrees, and play the role of moisture conservation and heat preservation. In general, biochar treatment has better water retention and water holding capacity. Especially in the seedling stage, cotton plants are short, roots are shallow and transpiration is small. Evaporation between soil particles have become the main reason for soil water loss. At this time, biochar treatment can significantly increase soil water content, which mainly because biochar can increase soil porosity and improve soil water holding capacity. This is consistent with the research results of He et al. (2020) and Wu et al. (2019a). Desulfurized gypsum can also improve soil moisture content to a certain extent, mainly because it contains high valence ions, which can enhance soil ion adsorption capacity, improve soil aggregate structure and water holding capacity (Li and Wang, 2018). Straw mulching treatment can significantly increase soil moisture content in the early growth stage, and the increasing effect slightly weakened in the later stage. The main reason is that in the later stage, the cotton leaves grow luxuriantly, shading the soil, and plant transpiration is the major factor for the decrease of soil moisture content. In the later stage, the temperature gradually increases, the straw mulching enhances the ventilation capacity of the soil, and the plant water demand increases, which gradually reduces the moisture of the straw itself. Under the condition of drip irrigation, the amount of irrigation is not enough to offset the soil evaporation and crop water absorption. It leads to the decrease of soil moisture content in straw mulching treatment, which is basically consistent with the research results of (Xiao et al., 2019). Through the contour map of soil moisture content (*Fig. 2*), it could be intuitively found that this water retention phenomenon was more significant in biochar treatment. Most of the reasons are due to poor soil water holding capacity and leaching loss of soil organic matter (Bohara et al., 2019). Therefore, the application of biochar in saline-alkali soil can effectively alleviate this contradiction.

Different improvement measures could significantly regulate soil temperature (*Table 2*). Considering the whole growth period, the three improvement measures could improve the soil temperature to a certain extent. The main reason is that the biochar itself is black and the desulfurization gypsum is light gray (Nifong et al., 2019). After adding the improvers to the soil, the heat absorption capacity of the soil could be enhanced, so as to improve the soil temperature. This is consistent with the research results of Yang et al. (2018b). On the other hand, the porous structure of biochar may provide a favorable place for the survival of microorganisms (Shi et al., 2022). Microorganisms will release a lot of heat during their activities, thus increasing the soil temperature (Pagnossa et al., 2020). In addition, our results found that the improvement measures had the effect of increasing temperature and heat preservation in the early growth stage, which could promote the emergence, growth and development of cotton, and had a certain cooling effect in the highest temperature stage, so as to avoid the harm of high temperature and better regulate the soil temperature. The temperature regulating effect of each treatment was mainly in the soil layer of 5-25 cm and weakened at 35 cm. The reason may be that the soil surface temperature is greatly affected by solar radiation and added improvement measures, and with the increase of soil depth, the impact on soil temperature gradually decreases (Tian et al., 2019).

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Effects of different improvement measures on soil organic carbon

Farmland soil organic carbon pool is an active component in soil and the basis of high and stable yield of crops (Guo et al., 2020). Our results showed that the application of different improvement measures could improve the content of soil organic carbon (*Fig. 3*) and the total density of organic carbon (*Table 3*), among which the effect of biochar was the best. Due to the good effect of moisture conservation and heat preservation, biochar improves the soil structure, and the negative charge could increase the soil cation exchange capacity and reduce the soil nutrient loss (Razzaghi et al., 2020). Yuan et al. (2019) showed that the application of biochar could increase the content of soil total organic carbon and was conducive to the fixation of soil carbon, which was consistent with the results of our study. Desulfurized gypsum uses Ca^{2+} to replace Na^{+}, improves soil aggregate structure and contains a large number of trace elements, which can increase soil organic carbon content by changing soil physical and chemical properties (Liu et al., 2021). In addition, the soil organic carbon under desulfurization gypsum treatment was higher than that of straw mulching treatment. The reason may be that desulfurization gypsum reduces the degree of saline-alkali stress by reducing the soil salt content, which increases the effect of fertilizing salinized soil (Yonggan et al., 2020). It was also found that the content of soil organic carbon in the surface soil was higher, and the content of soil organic carbon decreased with the increase of soil depth. The reason may be that the change of soil organic carbon content in 0-20 cm plough layer is mainly affected by improvement measures, which further shows that improvement measures are very helpful to improve soil organic carbon.

Effects of different improvement measures on cotton yield

Compared with the control, the three improvement measures can significantly improve the yield and single boll weight of cotton (*Table 4*), and the yield increase rate of biochar is the highest, up to 32.28%, which is mainly due to the best water and fertilizer retention ability of biochar to soil in the whole growth period, so as to significantly improve the yield of cotton. This is similar to the research results of Wu et al. (2019b). Cao et al. (2019) showed that desulfurization gypsum has a significant effect on improving soil properties and increasing crop yield. Ma et al. (2019) found that straw mulching can significantly improve crop yield. Our results showed that the yield of desulfurized gypsum and straw mulching to the field increased by 30.68% and 21.94%, respectively, compared with the control, which was similar to the above results.

Biochar and straw are important measures to promote farmland sustainable development and resource recycling, and have broad research prospects (Xiu et al., 2019). As a waste of coal desulfurization, desulfurized gypsum is considered to be one of the soil improvement measures with economic, environmental protection and fast remediation rate (Zhang et al., 2021). Some studies have also shown that desulfurization gypsum should be combined with organic fertilizer and farming measures, and the effect is more remarkable (Febrisiantosa et al., 2018). This experiment mainly applied three different improvement measures in in Shaya County, Aksu Prefecture, Xinjiang, China, to study the effects of saline-alkali soil hydrothermal carbon and cotton growth. The results show that biochar treatment can better create good hydrothermal conditions, increase soil organic carbon and greatly improve cotton yield.

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Conclusions

1) The three improved treatments had good water storage effect. The application of biochar increased the most, up to 24.39%, desulfurization gypsum could stably increase the soil moisture content in the whole growth period, while straw mulching could significantly increase the soil moisture content in the early growth period.

2) In the whole growth period, the three improvement measures had a good stabilizing effect on the soil temperature of 5-25 cm soil layer, showing warming effect at low temperature and cooling effect at high temperature.

3) The three improvement measures could increase soil organic carbon content and organic carbon density, and the effect of applying biochar was the most significant, and the increase of straw mulching treatment was the smallest.

4) The three improvement measures could improve the yield and single boll weight of cotton. The application of biochar treatment had the highest yield, followed by desulfurization gypsum treatment, and the straw mulching treatment may have the lowest yield due to the short application life and the straw has not been fully decomposed. Therefore, the long-term effects of the improvement measures on saline-alkali soil and cotton growth need pay attention to and study in the future.

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A META-ANALYSIS OF DIETARY TURMERIC (*CURCUMA LONGA* L.) SUPPLEMENTATION ON THE ENHANCEMENT OF BROILER CHICKEN PRODUCTIVITY

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Abstract. The increased awareness of consumers on the health benefits of organically produced animals has triggered interest on the use of phytogenics to boost broiler chicken productivity. Therefore, this meta-analysis aimed to determine the effect of turmeric (*Curcuma* longa L.) supplementation on feed intake, feed conversion efficiency (FCE) and body weight gain (BWG) in broiler chickens. Data from 31 studies that met the selection criteria for the meta-analysis were pooled using a random-effects model and expressed as a natural log of the response ratio (In*R*) at 95% confidence interval (CI) for each investigation. Results indicated that broiler chickens fed treatment diets had better FCE and BWG than controls. Furthermore, broiler chickens fed diets supplemented with turmeric at 5–10 g/kg consumed less feed and exhibit better FCE (In*R* = -0.06 g/bird, 95% CI: -0.07 to -0.04) and BWG (In*R* = 0.05 g/bird, 95% CI: 0.03 to 0.08) in comparison to the controls. Meta-regression results showed that studied moderators (broiler strain, dosage and supplementation duration) had an impact on the results of the meta-analysis. It was concluded that turmeric supplementation increased broiler chicken performance, and could be used as a performance enhancing agent in the broiler chicken industry.
Keywords: *phytogenics, poultry, feed intake, feed conversion efficiency, body weight gain, data synthesis*

Introduction

Broiler chickens play a vital role in improving food security, especially in developing countries. However, because of the direct competition for grains between animals and humans, the cost of rearing broiler chickens for optimal productivity has escalated. As a result, the contribution of broiler chickens to food security in developing nations is on the decline. This is traced to the exorbitant price of broiler meat, which can be propelled by the high cost of chicken feed, and this has prompted animal nutritionists to use natural growth-promoting agents to mitigate this challenge. A large body of evidence has demonstrated the ability of some tropical medicinal plants to enhance animal productivity (Amad et al., 2011; Ogbuewu et al., 2020). The anti-stress, antioxidant, anti-inflammatory and antimicrobial properties of turmeric (*Curcuma* longa L), one of such medicinal plants have been reported (Gul and Bakht, 2015; Panahi et al., 2015). Turmeric is low in nutrients but high in essential oils (Ikpeama et al., 2014; Youssef et al., 2014) and its addition in broiler diets have been claimed to boost chicken performance and nutrient utilization (Gobiraju et al., 2017; Guil-Guerrero et al., 2017). However, the effect of turmeric on broiler chickens is inconsistent and therefore will not allow for a generalized conclusion on the effect of turmeric in broiler chicken performance. Several attempts made to ascertain the factors that were responsible for

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the inconsistent results among studies that investigated the impact of dietary turmeric supplementation in broiler chicken performance were not successful (Tajodini et al., 2015; Gobiraju et al., 2017; Guil-Guerrero et al., 2017). This may be due to fact that these studies used a qualitative method to pool and synthesize evidence across several published primary studies, which usually is subjective and easily biased. Meta-analysis is one of the methods used to resolve inconsistent results studies addressing the research objective. It uses a quantitative approach to pool and synthesizes evidence across several individual studies with conflicting findings. It also increases the chances of detecting the true effect of an intervention that ordinarily cannot be detected by the individual studies. Therefore, the objective of this study was to determine the meta-analysis effect of turmeric supplementation on the productivity of broiler chickens.

Materials and methods

Search protocols

The search on Scopus, Google Scholar, Google engine and AGORA databases was focused on the effect of diets with or without turmeric supplementation on the growth performance of broiler chickens. The search was done using Boolean operators ("and" / "or") and combinations of search words such as turmeric, growth performance, average daily gain, weight gain, body weight gain, feed conversion ratio, feed conversion efficiency, feed efficiency, feed consumption, feed intake and broiler chickens. The reference lists of retrieved studies were manually searched for other related studies. The search was not restricted by publication year as to our knowledge this is the first meta-analysis that studied the effect of turmeric supplementation in broiler chicken performance. One hundred and forty studies were identified at the end of the systematic search.

Inclusion and exclusion criteria

Thirty-one out of the 140 studies identified were included in the meta-analysis as illustrated in our study selection flow chart (*Fig. 1*). To be included in the meta-analysis, (1) the study randomly assigned animals to treatment and controlled groups; (2) study was published in English; (3) experimental diets must contain turmeric additive with no other added growth promoters; (4) study reported at least one of the response variables (FI, FCE or BWG) with their measures of variance; and (5) study provide sufficient data to determine effect size. On the other hand, studies were excluded if (1) experiment was not in broiler chickens; (2) study lacked sufficient data to compute effect size; (3) trial was not published in English; (4) study published only the abstract; (5) study that mixed turmeric with other feed additives; (6) study appeared in more than one of the selected databases and (7) studies were in heat-stressed broiler chickens.

Data extraction

Information on response variables (FI, FCE, and BWG), covariates (supplementation duration, dosage and broiler strain), surname of the first author, publication year, study country and continents were extracted from each study that met the inclusion criteria as shown in *Table 1*. In addition, information on the mean and the number of broiler chickens included in the treatment and control groups were also extracted.

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Moderator and subgroup analysis

Several moderators may influence the response of broiler chickens to turmeric supplementation. The moderators used in the present study conformed with some of the variables we presumed to influence the responses of broiler chickens to turmeric supplementation. The moderators tested for were broiler strain (Cobb, Ross, Anak, Arbor acres and Hubbard), Dosage (<5, 5–10 and > 10 g/kg feed) and supplementation duration (1–21, 1–28, 1–35, 1–42 and 1– > 42 d). They were taking from each article that passed the eligibility criteria. The same moderators were also used for subgroup analysis in the present meta-analysis. Heterogeneity was determined using Cochran's Q statistic and I^2 statistics.

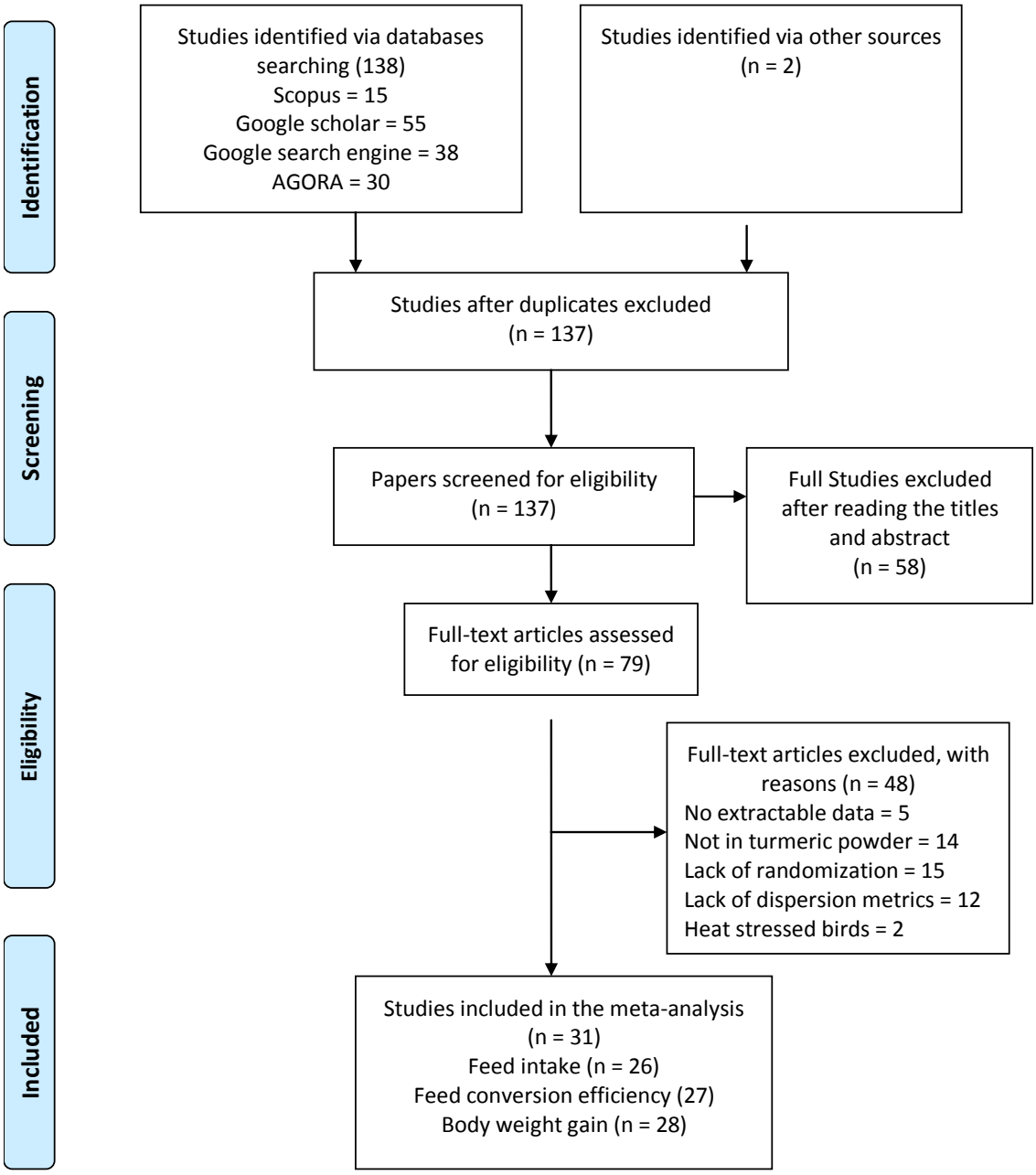

Figure 1. *Preferred reporting items for systematic review and meta-analysis flow diagram of study selection methods used in this meta-analysis*

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Table 1. *Studies used to determine the effect of turmeric supplementation in broiler chicken productivity*

References	Continent	Country	Dosage (g/kg)	Explanatory variables		Outcomes
				Duration (d)	Strain	
AL-Sultan (2003)	Asia	Saudi Arabia	0, 2.5, 5, 10	1–35	Ross	FI, FCR, BWG
Samarasinghe et al. (2003)	Asia	Sri Lanka	0, 1, 2, 3	1–28	Anak	FI, FCR, BWG
Durrani et al. (2006)	Asia	Pakistan	0, 2.5, 5, 10	1–35	Ross	FI, FCR, BWG
Emadi and Kermanshahi (2006)	Asia	Iran	0, 2.5, 5. 7.5	1–49	Ross	FI, FCR, BWG
Mehala and Moorthy (2008)	Asia	India	0, 1, 2	1–42	Cobb	FI, FCR, BWG
Nouzarian et al. (2011)	Asia	Iran	0, 3.3, 6.6, 10	1–42	Ross	FI, FCR, BWG
Sadeghi et al. (2012)	Asia	Iran	0, 5	1–42	Ross	BWG
Tirupati et. al. (2012)	Asia	India	0, 2.5	1–42	Cobb	FI, FCR, BWG
Raghdad (2012)	Asia	Iraq	0, 2.5, 10, 15	1–42	Ross	FI, FCR, BWG
Hussein (2013)	Asia	Iraq	0, 5, 7, 9	1–42	Ross	FI, FCR, BWG
Nayaka et al. (2013)	Asia	India	0, 2	1–42	Raja II	FCR
Abou-Elkhair et al. (2014)	Africa	Egypt	0, 5	1–35	Cobb	FI, FCR, BWG
Naderi et al. (2014)	Asia	Iran	0, 2.5, 7.5	1–21	Ross	FI, FCR, BWG
Anjali (2015)	Asia	India	0, 5	1–42	Cobb	FI, FCR, BWG
Mondal et al. (2015)	Asia	Bangladesh	0, 5, 10, 15	1–28	Ross	FI, BWG
Alagawany et al. (2015)	Africa	Egypt	0, 5, 10	1–35	Hubbard	FI, FCR, BWG
Fallah and Mirzaei (2016)	Asia	Iran	0, 5	1–42	Ross	FI
Kassu et al. (2016)	Africa	Ethiopia	1, 2	1–49	Cobb	FCR, BWG
Maha et al. (2016)	Africa	Egypt	0, 2.5	1–35	Ross	FI, FCR, BWG
Ukoha and Onunkwo (2016)	Africa	Nigeria	0, 10, 20, 30	1–56	Anak	FI, FCR, BWG
Arslan et al. (2016)	Asia	Pakistan	0, 5, 10, 15	1–35	Hubbard	FI, FCR, BWG
Kamdev et al. (2016)	Asia	India	0, 5, 10	1–42	Cobb	BWG
Łukasiewicz et al. (2017)	Europe	Poland	0, 7.5	1–42	Ross	FCR
Urusan and Bolukbasi (2017)	Europe	Turkey	0, 2, 4, 6, 8, 10	1–42	Ross	FI, FCR, BWG
Kafi et al. (2017)	Asia	Bangladesh	0, 5, 7.5	1–35	Cobb	FI, FCR, BWG
Esonu et al. (2017)	Africa	Nigeria	0, 10, 15, 20	1–21	Cobb	FI, FCR, BWG
Attia et al. (2017)	Africa	Egypt	0, 5, 10, 20	1–35	Hubbard	FI, FCR, BWG
Adegoke et al. (2018)	Africa	Nigeria	0, 2, 4	1–35	AA	FI, FCR, BWG
Ratika et al. (2018)	Asia	India	0, 5	1–21	Ross	FI, FCR, BWG
Choudhury et al. (2018)	Asia	India	0, 2.5, 5, 7.5	1–21	AA	FI, FCR, BWG
Shohe et al. (2019)	Asia	India	0, 2.5, 5, 7.5	1–21/1–42	Cobb	FI, FCR, BWG

d – day, AA – Arbor acres

Statistical analysis

We considered the random-effects model (REM) more appropriate in this analysis than the fixed-effects model (FEM) since the studies included in the meta-analysis were performed under different experimental conditions. Furthermore, researchers consider the REM approach more natural choice than FEM in the decision-making process (Ades and Higgins, 2005). The point estimate was expressed as InR with 95% confidence intervals. Forest and funnel plots were created in OpenMEE software (Wallace et al., 2016). Rosenberg's fail-safe number (Nfs) was used to determine the presence of publication bias which was assessed through a funnel graph. Symmetrically funnel graphs denote an absence of publication bias. Meta-analysis results were deemed robust despite publication bias when $Nfs > 5(n) + 10$, where n is the number of studies (Ogbuewu et al., 2020; Jennions et al., 2013). The diamond at the bottom of the plot represents the overall mean estimation (InR) and is said to be significant when CI did not include zero (Koricheva et al., 2013). The points to the left of the line of no effect (i.e. when $\text{In}R = 0$) connotes a

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reduction in the response variable; whereas the points to the right of the line of no effect indicate an increase. The relationships between the In*R* and the studied moderators were evaluated using the mixed-effects meta-regression analysis.

Results

The features of the 31 papers included in the meta-analysis are presented in *Table 1*. The articles used for the meta-analysis spanned 16 years, with the earliest study published in 2003 and the most recent being published in 2019. The studies used for the meta-analysis were conducted in 12 countries drawn from three regions around the world: Europe (n = 2), Asia (n = 21) and Africa (n = 8). Six studies were published in 2016 followed by seven studies published in 2017, whereas one study each was published in 2008, 2011 and 2019. The effect of turmeric supplementation on growth performance traits in broiler chickens is presented in *Figures 2–4*. The meta-analysis of 26 peer-reviewed journal papers, 79 comparisons, with 4392 broiler chickens (1128 and 3264 for the control and treatment groups respectively) indicated that turmeric supplementation significantly reduced feed intake when compared with the controls (In*R* = -0.02 g/bird, 95% CI: -0.03 to -0.01; I^2 = 69%; *Fig. 2*). The meta-analysis of 28 studies, 86 comparisons, with 4755 broiler chickens (1184 and 3572 each for control and treatment groups respectively) showed better FCE (computed as the quotient of average daily feed intake and the average in chickens) in treatment group when compared with the controls (In*R* = -0.06, 95% CI: -0.07 to -0.05, I^2 = 89%; *Fig. 3*). The meta-analysis of 28 peer-reviewed journal articles, 87 comparisons, with 4779 broiler chickens (1231 and 3548 each for control and treatment groups respectively) suggested that broiler chickens on turmeric supplementation had significantly higher BWG (calculated as final live weights - initial live weights divided by the duration of study) in comparison to the controls (In*R* = 0.05 g/bird, 95% CI: 0.03 to 0.07, *Fig. 4*), taking cognizance of significant heterogeneity ($p < 0.001$; I^2 = 92%).

Sub-analysis of the effects of moderators on the productivity of broiler chickens fed turmeric-supplemented diets is presented in *Table 2*. Broiler chickens fed turmeric supplemented diets at 5–10 and > 10 g/kg feed had significantly reduced FI in comparison with the controls. In contrast, broiler chickens fed turmeric supplemented diets at < 5 g/kg feed had similar FI with the controls. Broiler chickens offered turmeric supplemented diets at < 5, 5–10 and > 10 g/kg feed had significantly better FCE and BWG when compared with the controls, except for the birds fed turmeric supplemented diets at > 10 g/kg feed that had similar BWG with the controls. On the other hand, chickens from studies that fed turmeric supplemented diets for 1–42 d had significantly better FCE and gained weight at a reduced FI. Cobb, Ross, Hubbard, Arbor acres and Anak strains fed turmeric supplemented diets had significantly better FCE than the controls. Ross and Cobb broiler chickens gained weight at reduced FI in comparison with the controls. Conversely, Arbor acres and Hubbard broiler chickens gained weight at a similar FI with the controls.

Heterogeneity analysis is crucial in meta-analysis because it helps to determine the sources of variation between studies. A significant I^2-statistic indicates that the difference between study outcomes is more than anticipated naturally by chance. Significant heterogeneity was found among the studies included in the analysis as indicated by I^2-statistic and ranged from 69–92% as shown in *Figures 2–4*. The observed significant heterogeneity in treatment response as found in the present meta-

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analysis could perhaps be attributed to our chosen moderators. Meta-regression analysis as shown in *Table 3* showed that dosage is a significant predictor of the effect of turmeric on FI and accounted for about 26% of the source of heterogeneity. In contrast, duration and broiler strain had no significant influence on FI in broiler chickens. Furthermore, meta-regression analysis found significant relationships between dosage and FCE, which explained about 7% of the sources of heterogeneity. Similarly, we found significant associations between duration and strain and BWG in broiler chickens. However, the studied moderators and their interactions explained only 23% of the heterogeneity among the studies that evaluated the effect of turmeric on BWG.

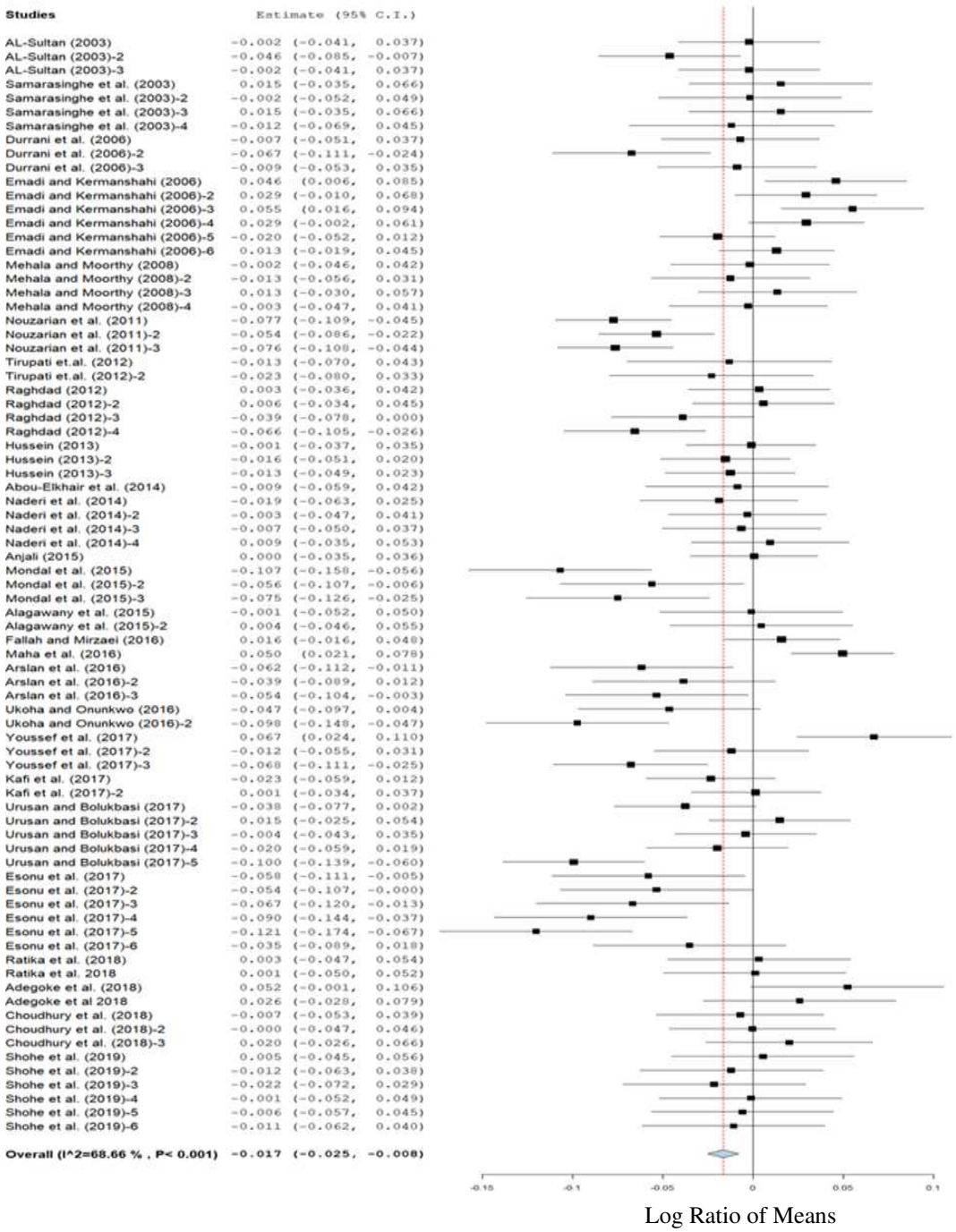

Figure 2. *Effect of turmeric supplementation on FI in broiler chickens*

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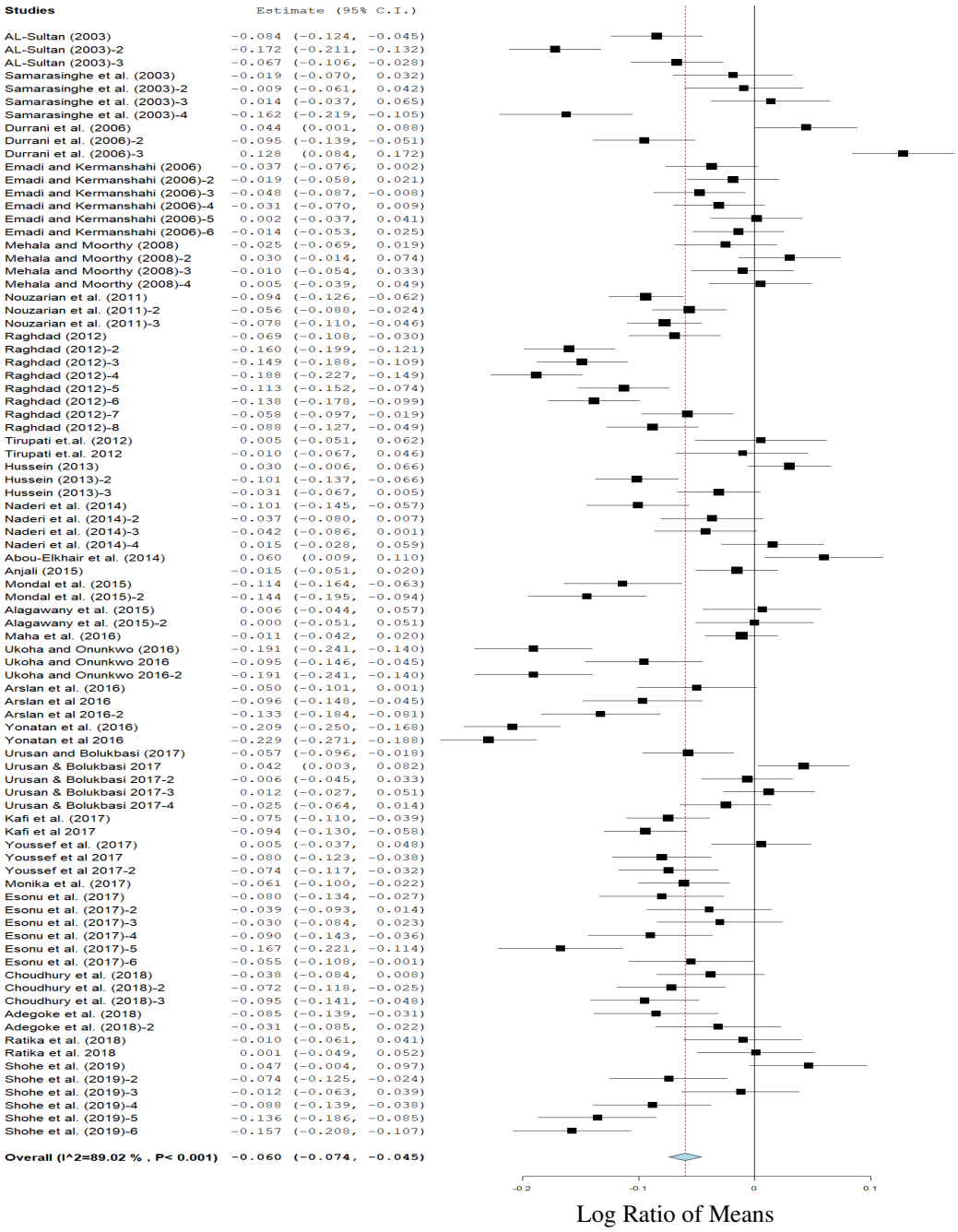

Figure 3. *Impact of turmeric supplementation on FCE in broiler chickens*

The funnel plots for all the production indices as shown in *Figure 5a–c* were asymmetrical. The Nfs of the dataset for FI, FCE and BWG were 709, 13662 and 7618, respectively, which is about 2, 32 and 17 times higher than the threshold of 405 (5 × 79 + 10), 430 (5 × 86 + 10) and 445 (5 × 87 + 10) needed to declare the mean effect sizes robust.

Discussion

India is the location with the highest number of studies in the present analysis followed by Iran, and this is expected since turmeric is native to Asia (Anantkawlas,

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2014). However, Europe (Turkey and Poland) contributed the least to turmeric research in broiler chickens in the current study, which could be attributed to the fact that turmeric is not widely available in this region. The observed yearly increase in turmeric research apart from the year 2019 may be due to the global campaign on the use of natural products in animal feed to curtail the rising incidence of antibiotic resistance and the appearance of antibiotic residues in animal products and the environment (FAO, 2003; Forgetta et al., 2012). However, the lower research figure put for the year 2019 may not reflect the true picture on the ground, as most of the works done in 2019 may be undergoing peer-review processes as at the time the literature search used for the analysis was conducted.

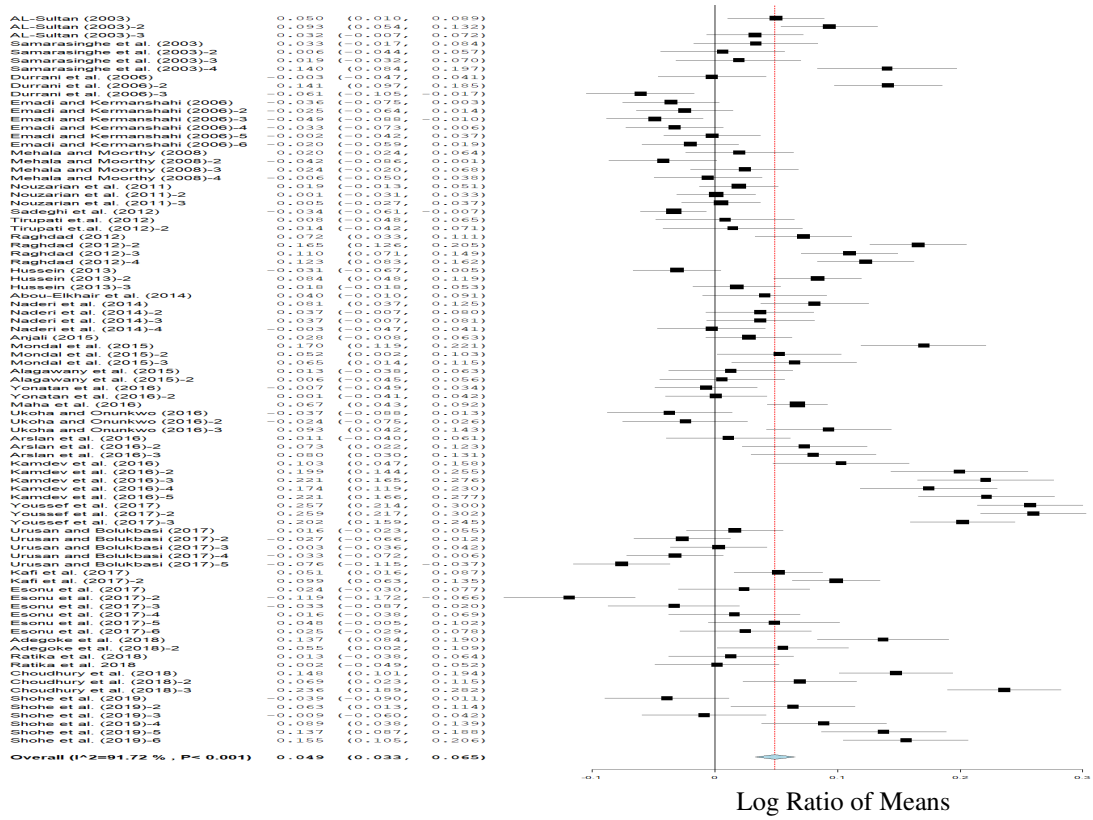

Figure 4. *Effect of turmeric supplementation on BWG in broiler chickens*

Findings of this meta-analysis revealed that chickens fed turmeric supplemented diets had improved FCE and BWG at reduced feed intake when compared with the controls and this is a welcome development. These findings agree with others (Halle et al., 2004; Windisch et al., 2008; Amad et al., 2011; Ogbuewu et al., 2020) who recorded improved FCE and BWG at lower feed consumption in broiler chickens fed diets supplemented with plant-based feed additives. For the digestive system to function optimally there must be equilibrium between the diet, the mucosa and the microbes. The significantly better FCE and BWG observed in broiler chickens fed turmeric supplemented diets over those fed control diets might be attributed to the inert ability of turmeric essential oils to favor the growth and multiplication of beneficial microbes in the digestive tract of chickens (Mohammad et al., 2010; Gul and Bakht, 2015). Furthermore, Trachoo and Boudreaux (2006) indicated that factors such as stress and

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diets may compromise the gut microbial ecosystem of chickens. Although the mechanisms of turmeric in increasing broiler chicken productivity is not clear, there is a hypothesis that bioactive compounds contained in turmeric may improve chicken productivity by alteration of gut metabolic processes in favor of production and secretion of endogenous digestive enzymes as well as the activation of gut defense systems (Ogbuewu et al., 2019).

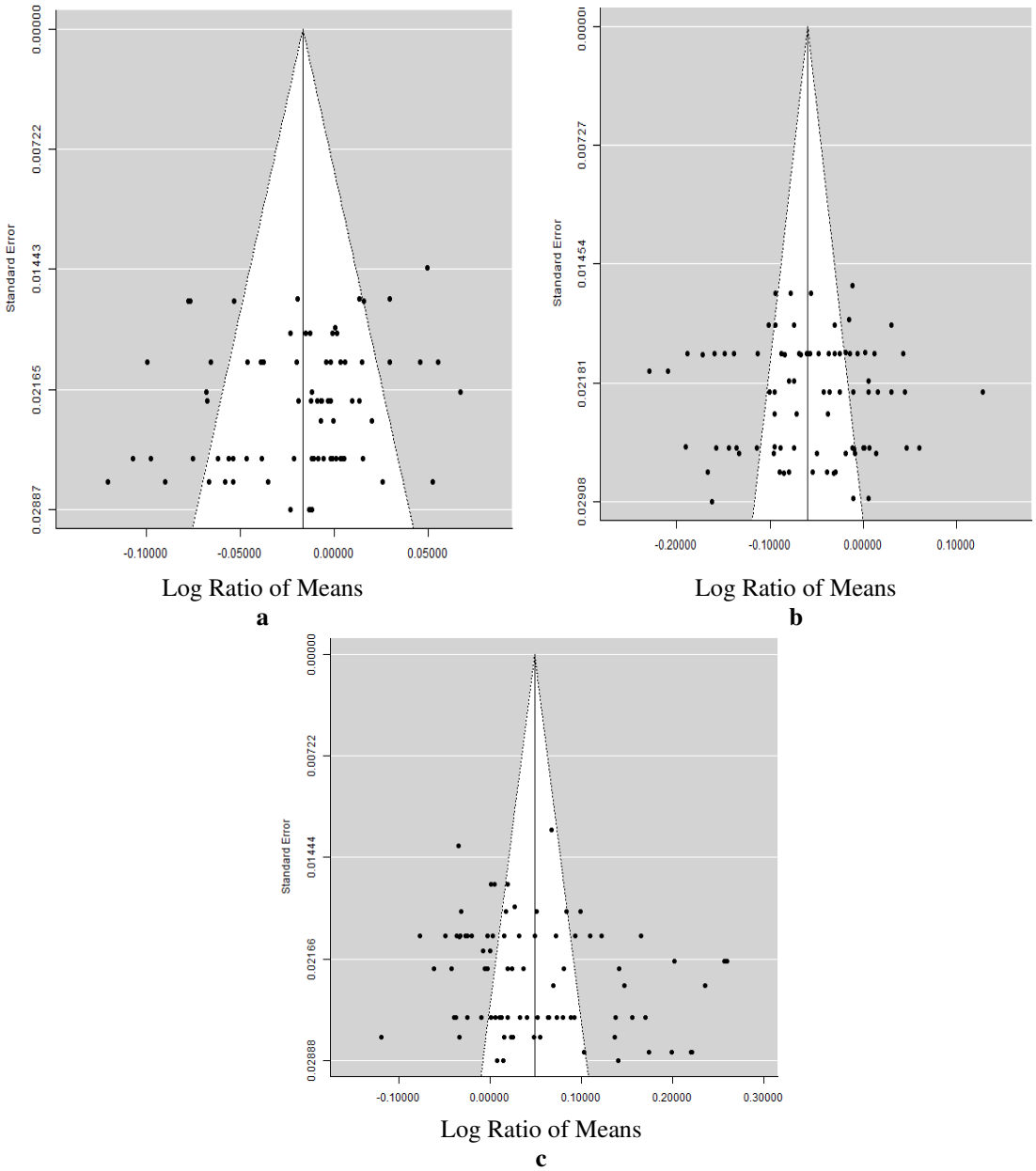

Figure 5. *Funnel graphs of the log ratios of means (InR) for dataset which compared production outcomes: FI (a), FCE (b) and BWG (c) between broiler chickens on diets with and without turmeric. X-axis is the InR, and Y-axis is the standard error*

In this meta-analysis, the supplementation dose was a significant predictor of feed intake and FCE, but not BWG. Results revealed that broiler chickens from studies that

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fed turmeric at < 5 g/kg feed had similar feed intake with the controls, nonetheless, chickens from studies that fed turmeric at the levels of 5–10 and > 10 g/kg feed consumed less feed than the controls. Knowledge of the process that controls feed intake in broiler chickens fed turmeric supplemented diets is still not clear. However, it is presumed that feed intake is regulated in neuroanatomical sites by well-organized neural and endocrine signaling networks (Richards and Proszkowiec-Weglarz, 2007). The observed difference in feed intake in broiler chickens fed diets supplemented with turmeric at < 5 g/kg feed and those fed diets supplemented with turmeric at 5–10 and > 10 g/kg feed indicate that < 5 g/kg feed may be below the threshold needed for the bioactive ingredients in turmeric to activate specific circuit in the neuroanatomical sites and its signaling molecules to modulate appetite in broiler chickens. The results of this restricted subgroup analysis showed that broiler chickens from studies that supplemented turmeric at the levels of < 5, 5–10 and > 10 g/kg feed had better FCE than the controls, suggesting the ability of turmeric to enhance feed digestion and nutrient uptake. The results of this meta-analysis show significantly heavier BWG due to turmeric supplementation in studies that included turmeric at levels of < 5 and 5–10 g/kg feed. In contrast, broiler chickens from studies that fed turmeric at > 10 g/kg feed had comparable BWG with the controls. This could mean that the optimal supplementation dose of turmeric expected to maximize body weight gain in broiler chickens falls between 5 and 10 g/kg feed. However, more studies are needed to determine the optimal turmeric supplementation dose that maximizes body weight gain in broiler chickens.

Our results indicate that supplementation duration was a significant predictor of BWG. Supplementation duration had a reduction effect on feed intake only in experiments that fed turmeric to broiler chickens from 1–42 d experiments. FCE was enhanced in 1–21, 1–28, 1–35, 1–42 and 1– > 42 d recorded better FCE. Broilers from studies that fed turmeric-supplemented diets from 1–28 d, 1–35 d and 1–43 d had significantly heavier BWG than the controls, suggesting that turmeric supplementation should not exceed 1–42 d for optimal BWG in broiler chickens. Probiotics have been reported to exert a higher beneficial impact on chicken gut during the early stage of life when a stable gut microflora has not been established, and therefore broiler chicks are more vulnerable to pathogens (Gaggia et al., 2010). However, we did not observe this pattern in the current study, as broiler chickens fed diets supplemented with turmeric from 1–21 d had similar BWG with the controls, suggesting that turmeric and probiotics may differ in their mechanism of action. Nevertheless, the poor weight gain seen in broilers fed turmeric-supplemented diets for 1–21 d suggests the low ability of broiler chicks to utilize turmeric-supplemented diets since their digestive tracts may have not been fully developed to handle the bioactive compounds contained in turmeric. The significantly lower weight gain obtained in chickens fed turmeric for more than 1–>42 d, indicates that 1–42 d may be the optimal supplementation duration for turmeric in the broiler chicken diet. This study, therefore, reaffirms the need for further research in this regard.

Strain is a significant predictor of BWG in the current meta-analysis. Ross and Cobb had better FCE and BWG at a reduced feed intake compared with the controls. In contrast, Hubbard Arbor acres gained weight at comparable feed intake with controls. This suggests that Cobb and Ross broilers may have a better ability to regulate turmeric intake than Anak, Hubbard and Arbor acres. Similarly, Korver et al. (2004) and Gonzales et al. (1998) reported the effect of strains on broiler chicken performance.

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These findings demonstrated that different broiler strains may exhibit different growth potentials. This finding may assist farmers in making informed decisions on which broiler strain (s) to rear especially when using turmeric supplements.

Table 2. *Sub-analysis of the effect of moderators on FI, FCE and BWG in broiler chickens fed turmeric*

Moderators	FI	FCE	BWG
	In*R* (95% CI)	**In*R* (95% CI)**	**In*R* (95% CI)**
Dosage (g/kg)			
< 5	0.02 (-0.01 to 0.02)	-0.05 (-0.07 to -0.03)	0.05 (0.02 to 0.08)
5–10	-0.02 (-0.03 to -0.01)	-0.06 (-0.07 to -0.04)	0.05 (0.03 to 0.08)
> 10	-0.08 (-0.11 to -0.05)	-0.11 (-0.15 to -0.08)	0.03 (-0.02 to 0.08)
Duration (d)			
1–21	-0.02 (-0.04 to 0.03)	-0.06 (-0.08 to -0.03)	0.01 (-0.02 to 0.02)
1–28	-0.03 (-0.07 to 0.004)	-0.07 (-0.13 to -0.01)	0.07 (0.02 to 0.12)
1–35	-0.01 (-0.03 to 0.01)	-0.05 (-0.08 to -0.02)	0.08 (0.05 to 0.12)
1–42	-0.02 (-0.03 to -0.01)	-0.05 (-0.08 to -0.03)	0.06 (0.03 to 0.08)
1– > 42	-0.02 (-0.06 to 0.02)	-0.12 (-0.19 to -0.05)	0.04 (-0.02 to 0.09)
Broiler strains			
Ross	-0.02 (-0.03 to -0.01)	-0.05 (-0.07 to -0.04)	0.03 (0.01 to 0.05)
Cobb	-0.02 (-0.04 to -0.01)	-0.06 (-0.09 to -0.03)	0.05 (0.02 to 0.08)
Hubbard	-0.02 (-0.05 to 0.01)	-0.05 (-0.09 to -0.02)	0.11 (0.04 to 0.19)
Anak	-0.02 (-0.06 to 0.01)	-0.09 (-0.16 to -0.03)	0.03 (-0.01 to 0.08)
Arbor acres	-0.02 (-0.01 to 0.04)	-0.07 (-0.09 to -0.04)	0.13 (0.07 to 0.19)

Table 3. *Meta-regression of the effect of moderators on performance of broiler chickens fed turmeric*

Outcome	Moderators	Q_M	df	P-value	R^2 (%)
FI	Duration	1.63	4	0.803	0
	Dosage	20.50	2	< 0.001	26
	Strain	4.22	4	0.377	0
FCE	Duration	7.78	4	0.099	4
	Dosage	7.65	2	0.022	7
	Strain	1.98	4	0.739	0
BWG	Duration	11.90	4	0.018	9
	Dosage	0.66	2	0.719	0
	Strain	15.70	4	0.003	14

R^2 = Percentage of heterogeneity explained by moderators

In the current meta-analysis, we included a large number of studies that could permit us to conclude that turmeric supplementation improves growth performance in broiler chickens. Our results were robust despite the presence of publication bias as suggested by Rosenberg Nfs and funnel graphs (Ogbuewu et al., 2020; Jennions et al., 2013; Higgins et al., 2003). Rosenberg Nfs indicated that a large number of unpublished

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studies would be needed to change the significant effects of turmeric supplements on FI, FCE and BWG as observed in the current meta-analysis. There is moderate to substantial heterogeneity in the present meta-analysis as supported by the I^2-statistic. Studied moderators were significant predictors of the study effect and explained most of the sources of heterogeneity. Other factors such as air velocity, ambient temperature, housing type, age, dietary composition and nutrient consistency may have accounted for the remaining heterogeneity. This meta-analysis, therefore, reinforces the need for another study in this area.

Conclusion

The results of the present meta-analysis indicate that turmeric supplementation improves growth performance indices in broiler chickens and may be used to increase the productivity of broiler chickens. Restricted subgroup results showed that dietary turmeric supplementation at the range of 5–10 g/kg feed significantly improved broiler chicken performance at a reduced feed intake, suggesting that turmeric can be included at this range in broiler chicken feed to enhance performance indices. However, more studies are needed to ascertain the exact amount of turmeric that optimizes growth performance indices in broiler chickens using a regression model. Meta-regression found significant relationships between the studied moderators and the variables of interest and explained most of the sources of heterogeneity. Nevertheless, the impact of other factors (ambient temperature, relative humidity, air velocity, housing type, dietary compositions/nutrient consistency and stocking density) that affect chicken performance that were not examined in this meta-analysis needs to be considered since such factors may explain the difference not accounted for by the studied moderators. More research is needed to determine the economics of production of broiler chickens fed diets supplemented with different levels of turmeric as such information is lacking in the literature.

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GLOBAL TRENDS IN ZOOPLANKTON RESEARCH OF FRESHWATER ECOSYSTEMS DURING 1991-2020: A BIBLIOMETRIC ANALYSIS

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Abstract. Zooplanktons play an important role in the whole freshwater ecosystem, which are the main quality food for almost all fish in their juvenile stage and are the main basic component of the high trophic levels in the aquatic food web. In the context of global environmental change, freshwater zooplankton is experiencing rapid loss of biodiversity and ecosystem integrity. However, our understanding of the geographical distribution pattern of freshwater zooplankton and the diversity-affected ecological factors is still very limited. In order to investigate the research status and future development trend of zooplankton in global freshwater, 26176 publications about freshwater zooplankton/zooplankter indexed by Web of Science from 1991 to 2020 were analyzed with a bibliometric approach in our study. The research contents mainly included the output trend of publications related to the freshwater zooplankton/zooplankter filed over time, the journals with greatest number of zooplankton publications, the countries from which the authors came from, the co-authors and cooperation across countries, the associated keywords in the research areas and so on. This study was searched on a scientific website for scientific study that have limited scientific relevance for the future researches on the freshwater zooplankton.
Keywords: *research hotspot, bibliometrics, aquatic food web, citations, scientific research, impact*

Introduction

Zooplanktons are small, microscopic and free-swimming animals, which are the good food sources for many aquatic animals (Rathod and Patil, 2019). The main nutrition sources of zooplankton in water are phytoplankton, bacteria and organic matter (Donk et al., 2011). Despite small individuals, zooplankton has a large number of species, which can adapt to and exist in various types of water such as oceans, lakes, reservoirs, freshwaters and so on (Krylov, 2013; Abe et al., 2020). Because the zooplanktons are in the middle of the aquatic ecosystem food chain, their community structure changes directly or indirectly affect the community structure changes of other aquatic organisms

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(Kar et al., 2016). The metabolism and secretion of zooplanktons can promote the decomposition and circulation of organic matter, which are also highly sensitive to the changes of the water environment. In general, the different types of zooplankton are distributed in various types of water bodies, so the dominant species and other information on zooplankton can reflect the pollution degree of water environment (Kang et al., 2020). And some surveys have shown that the amount, type and diversity of freshwater zooplankton have a significant impact on the entire aquatic food web (Pickhardt et al., 2005; Smyntek et al., 2010; Allard et al., 2011). The researches on the freshwater zooplankton have become an important topic worldwide in recent years because of the research significance of zooplankton (Lovern, 1935; Milstein et al., 2006; Zargar et al., 2007; Hannes et al., 2008; Balvay et al., 2009; Warren et al., 2016; Hitchcock et al., 2016; Elias-Gutierrez et al., 2018; Choi et al., 2019; Li et al., 2019; Tremblay et al., 2019; Kalcheva et al., 2020; Qin et al., 2020). However, it is difficult to find the research focus, research background and specific research direction in a large number of publications. Many problems have not been completely solved including the output trend of publications related to the freshwater zooplankton/zooplankter filed over time, the journals with greatest number of zooplankton publications, the countries from which the authors came from, the co-authors and cooperation across countries, the associated keywords in the research areas and so on. So it is rather necessary to find a new method to overcome these problems (Wang et al., 2015).

Currently, bibliometric analysis has been widely applied to quantitative and qualitative analysis of research results and trends in many disciplines (Keiser et al., 2005; Li et al., 2009; Zhang et al., 2013; Chen et al., 2020). In particular, this method can be applied to detect metal ions in freshwater environment (Irfan et al., 2021), detect the environmental health risks (Andrade et al., 2017), detect the carbon cycling (Zhi et al., 2015) and so on. However, with this method, the scientific research and development trend in the field of freshwater zooplankton are not clear. Web of Science is the citation index database in the ISI database, containing more than 8,000 of the world's most influential, peer-reviewed, high-quality journals (Sevinc, 2004; Zhang et al., 2016), which can search for the popular published articles in related fields according to the search keywords, author's name, title, publication year, affiliations (journals, countries, and regions) and other detail information (Mosicheva et al., 2018). In view of the importance of freshwater zooplankton in aquatic ecosystem function and the lack of bibliometric analysis in this field, this study uses the bibliometric analysis method to analyze the development status of freshwater zooplankton during 1991–2020. The research objectives of this study are as follows: (1) Which countries and journals are the dominant publishers in the field of freshwater zooplankton? (2) Which are the research focus in the field of freshwater zooplankton during the period 1991-2020? (3) Which kind of methods can be applied to accurately locate the gradient relationship between freshwater plankton and freshwater environmental parameters in the future?

Materials and methods

Establishment of database

The methods we used in the present study are cited from the previous literature (Rumin et al., 2020) with minor revisions. The establishment of database was built through a literature search. The use of "Web of Science" database was compulsory to obtain a format compatible with the bibliometric analysis using the analysis software. The

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keywords "zooplankton or zooplankter" and "freshwater" were used to list all relevant publications in the worldwide for the period 1991 to 2020. The relevant publications were from the European countries, American countries, Asian countries and Oceania countries. Furthermore, both “zooplankton” and “zooplankter” were selected as keywords due to the fact that the publications related to both were very similar.

Data sources

The data sources of this study mainly included the published articles related to the keywords "zooplankton or zooplankter" and "freshwater" from 1991 to 2020, all of which were inquired from all citation indices in the Web of Science. And then XML files can be obtained, which contained the title, keyword, abstract, publication year, published journal, authors’ name, authors’ affiliation, citation times and other related information. The search query was constructed as below: (TS = (zooplankton* OR zooplankter*) AND TS = (freshwater*)).

Extraction of country names

All the names of the countries were taken from the authors’ affiliations. The affiliations from Peoples R China, Hong Kong, Taiwan were treated as from China. The affiliations from England, Scotland and Wales were treated as from the United Kingdom. The names of some former countries were updated, containing “Ussr” (Russia) and so on. The author's relationship with the states/provinces does not take into account the country names. Publications with only one country name are considered to be co-published domestically, while publications with multiple country names are considered to be co-published internationally.

Co-word analysis of keywords and cooperation among countries

Co-word analysis is an analytical means to discover disciplinary structure of the field of science by analyzing the forms of items (words or pairs of noun phrases) that occur together in the same text body (Cheng et al., 2014; Ravikumar et al., 2015). The classical co-word analysis methods for the countries were mainly for the following steps: Firstly, extract the names of countries. Secondly, the preparation of the country name library. Thirdly, the construction of a co-occurrence matrix and the specific processes are as follows:

(1) a term-document-matrix was generated for the selected country names library; (2) the term-document-matrix was converted into a co-occurrence one. Finally, the ideas for cooperation between countries: a graph was generated where the countries were represented as nodes and their associations were represented as lines between each node. The line width and node width were trimmed for readers.

The procedures of the keywords co-word analysis were as follows: Firstly, the keywords of publication in the Web of Science were summarized. And the spellings of keywords were simplified as much as possible and changed to lowercase, such as “freshwater-zooplankton/zooplankter” to “freshwater zooplankton/zooplankter”; Secondly, the constriction of the top 20 popular keywords database. Thirdly, the construction of a co-occurrence matrix and the specific processes were as follows: (1) term-document-matrix was established for the popular topic keywords; (2) the term-document-matrix was converted into a co-occurrence matrix. Finally, the keywords were further visualized by co-word analysis.

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Furthermore, the number and percentage of the single or cooperation articles among the countries or institutions were determined according to the *Equations (1-4)* respectively as follows:

$$CA^1 = TA^1 - SA^1 \quad \text{(Eq.1)}$$

$$PCA = 1 - SA^1/TA^1 \times 100\% \quad \text{(Eq.2)}$$

$$SA^2 = TA^2 - CA^2 \quad \text{(Eq.3)}$$

$$S = SA^2/TA^2 \times 100\% \quad \text{(Eq.4)}$$

where CA^1 is the number of cooperation articles among the countries, TA^1 is the total number of country articles, SA^1 is the number of single country articles, PCA is the percentage of cooperation articles among the countries. SA^2 is the number of single institution articles, TA^2 is the total number of institution articles, CA^2 is the number of cooperation articles among the institutions, S is the percentage of single institution articles.

The trend analysis of keywords

The presence percentage of every keyword per year was calculated by dividing the annual frequency by the total frequency, and then adjusted the total annual frequency to compensate for the overall raise in the total frequency. Each keyword was checked for trends by MK (Mann-Kendal trend test, MK). In the case of p values less than 0.05, the trend was considered to be significant, otherwise neither increase nor decrease.

Statistical analysis

In this study, all the data handing, block generation and statistical analysis were carried out using the self-written R code. In addition, the MK trend test was used to examine the increase or decrease trend of keyword frequency and statistical quantity/percentage of the relevant publications on freshwater zooplankton during the study years. The formation and analysis of partial figures in the study were performed using OriginPro 8.5.1 data analysis and mapping software (OriginLab Co., MA, USA) and Excel data analysis and mapping software.

Results

The total number and rising tendency of total publications

The total number of publications in the area of freshwater zooplankton/zooplankter in the Web of Science from 1991 to 2020 is 26176. As shown in *Figure 1,* the publication output increased slowly before 2000. And then the total number of publication output increased rapidly after 2000 and reached the maximum in 2020. The total number of publications in the field of zooplankton/zooplankter in the Web of Science for each year from 2000 to 2020 was as follows: 2000 (77 publications), 2001 (845 publications), 2002 (848 publications), 2003 (1028 publications), 2004 (993 publications), 2005 (1094 publications), 2006 (1090 publications), 2007 (1104 publications), 2008 (1246 publications), 2009 (1197 publications), 2010 (1304 publications), 2011 (1342

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publications), 2012 (1401 publications), 2013 (1443 publications), 2014 (1446 publications), 2015 (1412 publications), 2016 (1434 publications), 2017 (1517 publications), 2018 (1537 publications), 2019 (1722 publications) and 2020 (1798 publications). The main reasons for the rapid increase in the number of publications after 2000 were the rapid development of the related information technologies and the substantial increase in the internet availability.

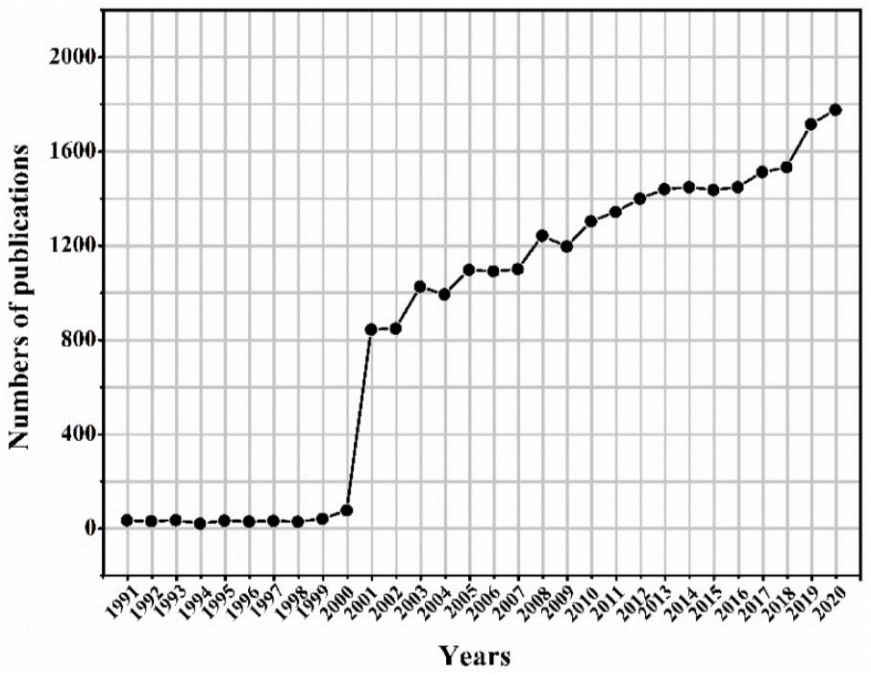

Figure 1. *Temporal trends of publications output in the field of freshwater zooplankton between the 1991 and 2020 in the Web of Science*

The details of top 10 journals of the total publications

The top 10 journals of the total publications are listed in *Table 1*. As depicted in *Table 1*, the journal "*Hydrobiologia*" topped the list with 1146 publications. However, the total citation of the journal "*Hydrobiologia*" was much lower than the journal "*Limnology and Oceanography*" and journal "*Plos One*" because of the low impact factor. As the second ranked in the number of publications, the journal "*Journal of Plankton Research*" also had a low impact factor and the number of total citations was the lowest in the top 10 journals, the impact factor and total citation number are 2.149 and 6746, respectively. Although the journal "*Progress in Oceanography*" had the highest impact factor (4.06), the number of total citations was much lower than the other journals excepted the journal "*Journal of Plankton Research*". Simultaneously, the journal "*Plos One*" had the highest total citations, but the number of the publications and the impact factor were relatively low.

The details of top 20 countries of the total publications and their cooperation

Figure 2 shows the schematic diagram of the top 20 countries published number in the Web of Science in freshwater zooplankton research area and their cooperative networks. And the detail information of the top 10 countries has also listed in *Table 2*. In terms of

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the total of publications, the USA contributed the most publications (26.3%) and played an important role in international cooperation networks. And the number of total publications was much higher than the Canada (Rank two in *Table 2*). Because the total of publications in the United States were much higher than those in other countries, all other metrics such as cooperation article and single country article were also ranked first. Although China ranked fourth for the number of total publications, it showed a higher percentage of single country article than other countries except USA. As for China, the number of single country article was 1211. Among the top 20 countries with the highest numbers of publication on the Web of Science between 1991 and 2020, there are thirteen countries in Europe, four countries in the Americas, two countries in Asia and one country in Oceania. Among the thirteen countries in Europe, six countries (Germany, UK, France, Spain, Norway and Russia) contributed more than 50 % to international cooperation.

Table 1. *The top 10 journals with the number of total publications and the category of journals related to the field of freshwater zooplankton between the 1991 and 2020 in the Web of Science*

Journal	TA (%)	IF	TC
Hydrobiology	1146 (4.4)	2.385	25221
Journal of Plankton Research	961 (3.7)	2.149	6746
Marine Ecology Progress Series	945 (3.6)	2.326	37574
Limnology and Oceanography	660 (2.5)	3.778	29383
Freshwater Biology	574 (2.2)	3.835	15145
Deep Sea Research Part II Topical Studies in Oceanography	514 (2.0)	2.697	11188
Progress in Oceanography	407 (1.6)	4.06	9511
Plos One	385 (1.5)	2.74	688786
Ices Journal of Marine Science	336 (1.3)	3.188	11699
Marine Biology	334 (1.3)	2.05	17198

TA: total article, IF: impact factor (2019), TC: total citations (2019)

Table 2. *Top 10 most productive countries in the publications related to the field of freshwater zooplankton between the 1991 and 2020 in the Web of Science*

Country	TA[1]	TA[1] R(%)	CA[1] (CR)	SA[1] (CR)	PCA (%)
USA	6986	1 (26.7)	2702 (1)	4284 (1)	38.7
CANADA	2400	2 (9.2)	1264 (4)	1136 (3)	52.7
GERMANY	2143	3 (8.2)	1380 (3)	763 (5)	64.4
P. R. CHINA	1819	4 (6.9)	608 (8)	1211 (2)	33.4
UK	1527	5 (5.8)	1502 (2)	25 (10)	98.4
FRANCE	1427	6 (5.5)	1047 (5)	380 (9)	73.4
SPAIN	1331	7 (5.1)	793 (7)	538 (7)	59.6
NORWAY	1271	8 (4.9)	835 (6)	436 (8)	65.7
RUSSIA	1225	9 (4.7)	395 (10)	830 (4)	32.2
JAPAN	1207	10 (4.6)	513 (9)	694 (6)	42.5

TA[1]: the total number of country articles, R: rank, CA[1]: the number of cooperation articles among the countries, SA[1]: the number of single country articles, PCA: the percentage of cooperation articles among the countries

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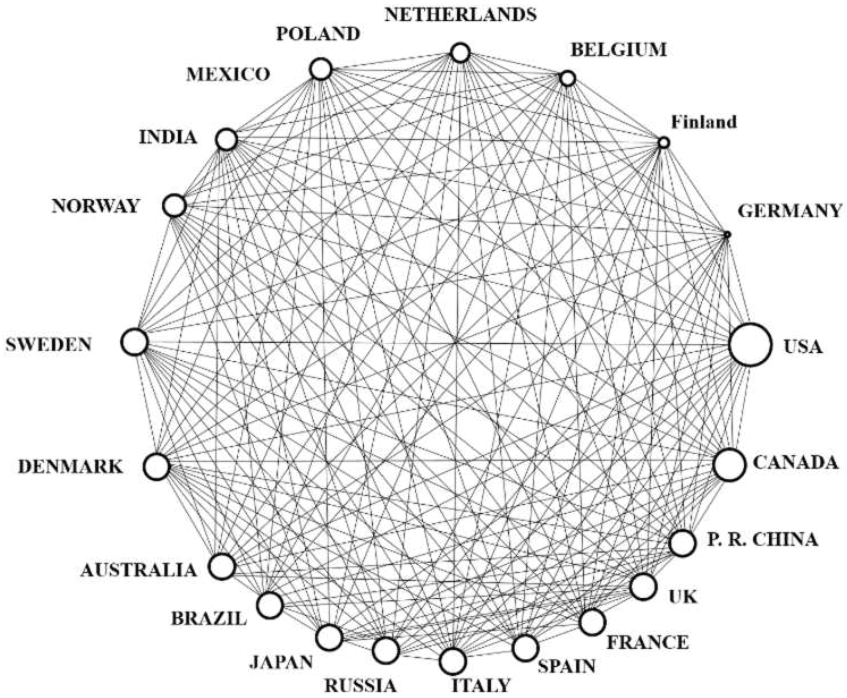

Figure 2. *Sequence and correlations of top 20 countries in the publications related to the field of freshwater zooplankton between the 1991 and 2020 in the Web of Science (The size of the circle in the figure shows a positive correlation with the number of publications)*

The details of top 10 productive institutions and their cooperation

The top 10 most productive organizations are listed in *Table 3*, three of which are from the USA, two from China, three from the European and two from Canada. The Centre national de la recherche scientifique (CNRS, France) topped the list with 961 publications and 960 cooperation articles. And the number of total publications of Chinese Academy of Sciences (China) was 696, ranking sixth in the list. Furthermore, as depicted in *Table 3*, most of the institutions were mainly dependent on the international cooperation, only the Fisheries Oceans Canada, Canada and NOAA, USA on a single publication with about ten percentages.

The sequence and correlations of top 20 keywords from freshwater zooplankton publications

The correlations of top 20 keywords from freshwater zooplankton publications are shown in *Figure 3*. As depicted in the *Figure 3*, the keyword "zooplankton" was strongly correlated with the "responses", "phytoplankton", "food web", "biodiversity" and "distribution". The keyword "biodiversity" was strongly correlated with the "species", "phytoplankton" and "zooplankton". The keyword "food web" was strongly correlated with the "phytoplankton", "species" and "zooplankton".

The uptrend and other trends of top 50 keywords from freshwater zooplankton publications

Figure 4 shows the top 50 keywords for uptrend and other trends, which can be classified into the following four types: (1) Research region, including "freshwater",

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"river" and "surface waters". (2) Research contents, including "zooplankton", "rotifera", "water quality", "food web", "organic carbon, "growth", "species", "distribution", "phytoplankton", "trophic structure", "community", "equilibrium", "distribution", "biomass", "stationary distribution" and so on. (3) Environments, including "ecological quality", "nutrients", "water quality", "pollutions", "eutrophication", "heavy metal", "water quality", "pollutions", "climate change", "aquatic environment", "environmental fluctuations", "nutrient limit", "river connectivity" and so on. (4) Research methods, including "sampling", "model", "monitor", "dynamical analysis" and biological evalution".

Table 3. *Top 10 most productive institutions in the publications related to the field of freshwater zooplankton between the 1991 and 2020 in the Web of Science*

Institutions	TA^2	TA^2 R	CA^2 (CR)	SA^2 (CR)	S (%)
CNRS, France	961	1	960 (1)	1 (5)	0.1
National Oceanic Atmospheric Admin, USA	901	2	890 (2)	11 (4)	1.2
Russian Academy of Sciences, Russian	899	3	886 (3)	13 (3)	1.4
Helmholtz Association, Germany	832	4	832 (4)	0 (6)	0
University of California System, USA	795	5	787 (5)	8 (5)	1.0
Chinese Academy of Sciences, China	696	6	695 (6)	1 (5)	0.4
Fisheries Oceans Canada, Canada	582	7	527 (8)	55 (2)	9.5
NOAA, USA	530	8	470 (10)	60 (1)	11.3
Chinese Academy SCI, China	529	9	529 (7)	0 (6)	0
CSIC, Canada	513	10	512 (9)	1 (5)	0.2

TA^2: the total number of institution articles, R: rank, CA^2: the number of cooperation articles among the institutions, SA^2: the number of single institution articles, S: percentage of single institution articles

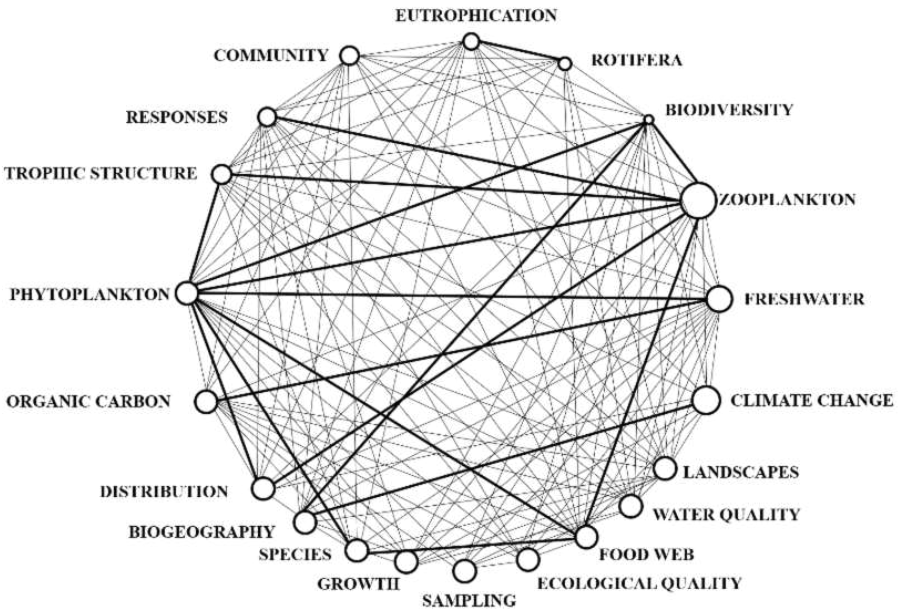

Figure 3. *Sequence and correlations of top 20 keywords in the publications related to the field of freshwater zooplankton between the 1991 and 2020 in the Web of Science (The size of the circle in the figure shows a positive correlation with the frequency of keywords)*

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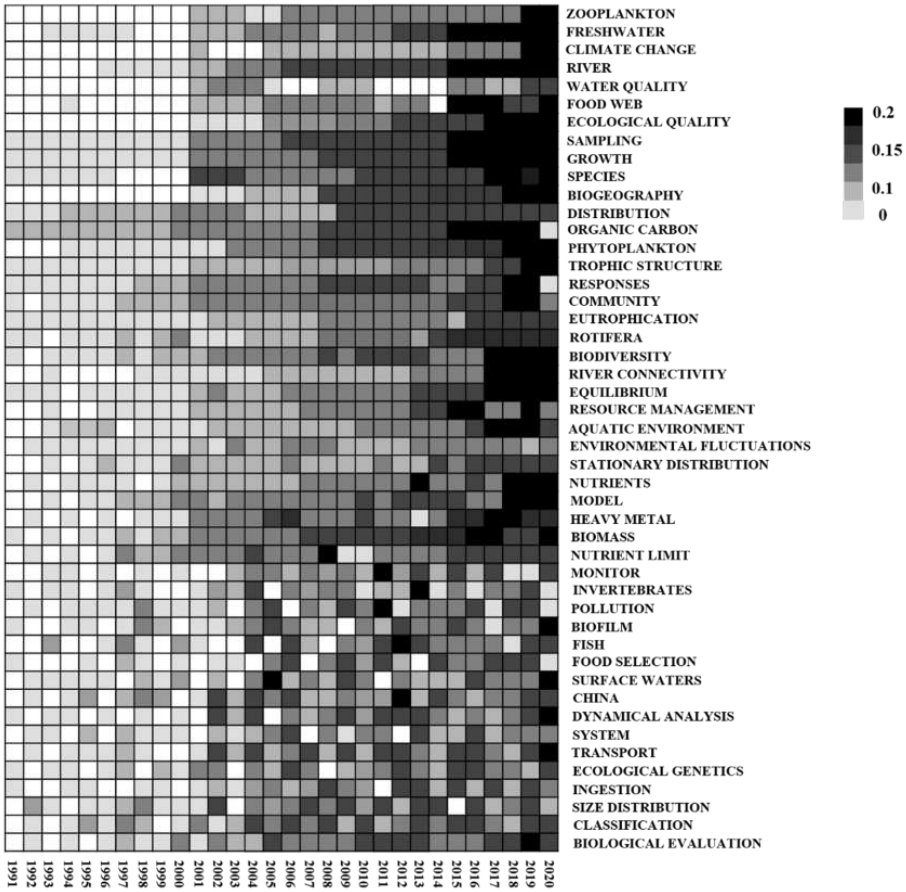

Figure 4. *Top 50 keywords with ascending trend in the publications related to the field of freshwater zooplankton between the 1991 and 2020 in the Web of Science. (Full spelling of all keywords): zooplankton, freshwater, climate change, river, water quality, food web, ecological quality, sampling, growth, species, biogeography, distribution, organic carbon, phytoplankton, trophic structure, responses, community, eutrophication, rotifera, biodiversity, river connectivity, equilibrium, resource management, aquatic environment, environmental fluctuations, stationary distribution, nutrients, model, heavy metal, biomass, nutrient limit, monitor, invertebrates, pollutions, biofilm, fish, food selection, surface waters, China, dynamical analysis, system, transport, ecological genetics, ingestion, size distribution, classification, biological evaluation)*

Discussion

Output trend of publications

As the above data depicted, the number of publications on the Web of Science showed a gradual upward trend between 1991 and 2020, which was consist with the growth trend in other research areas (Zhang et al., 2010; Yi and Jie, 2011; Liao and Huang, 2014; Beskaravainaya and Kharybina, 2018; Seftel, 2019). Particularly since 2001, the number of publications have been an extremely rapid growth trend, possibly due to the environmental pollution problems and shortages of traditional energy sources, and many countries had invested significant funds and implemented significant supportive policies for the sustainability of the development of natural water resources. For example, the EU Water Framework Directive (2000−2015a) provided a framework for the sustainable

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development of natural waters. Although the guidelines came into effect during different periods among the countries, their impact on the natural waters of the future was enormous.

Influencing factors of the total publications of journals

The impact factors (IFs) are put forward by E. Garfield in 1972, and have become the universal evaluation index of journals in the world (Lee et al., 2009), which are not only an index to measure the practicability and demonstration of journals, but also an important index to measure the academic level of journals and the quality of publications. The greater the impact factor, the greater the academic influence and function of the journal (Bence and Oppenheim, 2004). Furthermore, the citation frequency of articles is also an important index to evaluate the quality and influence of publications. The quality and influence of highly cited papers are usually very great (Pasterkamp et al., 2007). However, based on the research results in our study, it can be found that the citation frequency of papers published in top journals with high impact factors is not always high, which proves that there is no necessary correlation between the citation frequency and impact factor of publications (Wang et al., 2018).

The effect of international cooperation on the number of publications

Under the background of economic and technological globalization, international cooperation has played an increasingly important role in promoting various research fields. Many researchers have regarded the international cooperation as one of the most effective ways to publish articles and evaluate scientific and technological cooperations between countries (Leeuwen et al., 2009; Chen et al., 2016; Duan et al., 2018). At the same time, there are many factors that affect international cooperation, which mainly includes geographical location, political relations, cultural differences and many other factors. Based on the statistics of cooperation and publication between countries in different continents, it can be seen that among the top 20 countries with the highest numbers of publication on the Web of Science between 1991 and 2020, there are thirteen countries in Europe, four countries in the Americas, two countries in Asia and one country in Oceania. Among the thirteen European countries, six countries (Germany, UK, France, Spain, Norway and Russia) contributed more than 50% to international cooperation. In comparison with the Asia and America countries, the European countries have a significant advantage in the number of cooperation and jointly published articles, mainly because of the issue of the Water Framework Directive in Europe, which provides an excellent opportunity for cooperative researches and publications among European countries and provides a good case for an international range of collaborative researches (Barth and Fawell, 2001; Borja et al., 2006; Leeuwen et al., 2009; Chen et al., 2016). The application of similar directives to the Americas and Asia, and even the world at large, could further strengthen linkages and cooperation between neighboring countries and lead to the development of a harmonized directive on water resources assessment and management strategies on a global scale in the future.

The importance and development trend of the research topic

Through the publications in the field of freshwater zooplankton on the Web of Science, we can find that the researches on freshwater environment have been a popular research topic in recent years. Freshwater environment usually refers to the inland freshwater

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resources, including rivers, lakes, canals and underground water, which is vital to the entire ecosystem (Nnadozie and Odume, 2019; Choi et al., 2019; Cuassolo et al., 2020). Freshwater is the indispensable basis for the survival of human beings and the vast majority of organisms, and it has a function to protect biodiversity. Freshwater is an essential resource for irrigation in agricultural production, which determines the expansion and development of irrigation agriculture (Robin et al., 2006). Furthermore, the abundance of freshwater determines the development prospect of industry and the freshwater ecosystem plays an irreplaceable role and influence on pollution purification and climate regulation (Ruesink, 2005). However, the situation of global freshwater resources is not optimistic because of the impact of human production and living. The problems with serious water shortage and water quality deterioration have caused serious and irreversible impact on the natural hydrological conditions of freshwater and changed the quality of freshwater habitat, biological dynamics and ecological stability (Itahashi et al., 2019). In addition, due to the correlation between different aquatic ecosystems, the change of freshwater environment has also affected other aquatic ecosystems, mainly manifested by eutrophication of water environment, heavy metal ion pollution, high organic carbon content, poor stability of zooplankton community and other problems (Atamna et al., 2008; Raunio et al., 2011; Holland et al., 2011; Liu et al., 2020). In the recent years, many researchers have used many methods to solve the above problems. Firstly, the model method has been used to observe and predict the dynamics of zooplankton, and then feedback relevant information such as water quality, pollution level and eutrophication degree (Usha et al., 2006). Sampling method has been used to measure the stability and organic carbon of zooplankton community in real freshwater environment (Stéphane et al., 2004). The monitoring method has been used to collect real-time data and timely analyze the correlation coefficients of the freshwater environment so as to ensure the accuracy and timeliness of the experimental results (Ofukany et al., 2014). The dynamic method was used to analyze the changes and stability of the zooplankton community in the dynamic freshwater environment (Raquel et al., 2013).

The development trends in the field of freshwater zooplankton were reflected by analyzing the frequency of keywords. The results showed that the keywords with high frequency in recent years were as follows: climate change, water quality, food web, responses and so on. The climate change and the quality of freshwater ecosystem have become major research focuses because the quality of freshwater ecosystem has been in serious decline since the beginning of the 20th century due to frequent human activities. The zooplankton was used as a reliable environmental indicator in freshwater, including the streams and rivers, because of the short life cycle, high sensitivity to environmental change, easy sampling and wide distribution of zooplankton. Based on zooplankton to evaluate the degree of environmental pollution, the methods mainly include biomass, biological density, dominant species, zooplankton and other biological indexes and physical and chemical indexes. Recently, many researches have been conducted on the biological monitoring and biodiversity conservation in freshwater ecosystem according to freshwater indices and traits, in which the qualitative and quantitative way were widely applied to tackle a complex mixture of stressors. For example, the Prokopkin et al. developed a one-dimensional ecological model of the meromictic brackish Lake Shira (Russia, Khakasia). The kinetic fitting of oxygen and hydrogen sulfide and the simulated positions of the chemical incline and thermocline in the model are in good agreement with the data of zooplankton abundance, density and dominant species. This model opens the way for future investigations to examine various assumptions about the functioning

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of the Lake Sheila ecosystem and to analyse management options for this economically important lake (Prokopkin et al., 2010). Furthermore, the zooplankton formed the base of the food web in many aquatic ecosystems, the abundance and composition of which determined the quantity and quality of food available to other aquatic life (Choi et al., 2012). The food webs were very complex in common so the stable isotope analyses were applied to trace the origin and transfer of organic matter in aquatic food webs (Careddu et al., 2015), which can provide insight and build the trophic relationships among organisms. In recent years of researches, the eutrophication and humification impacted nutrient cycles and the efficiency of carbon transfer in the planktonic food webs (Karpowicz et al., 2020a). Zooplankton are an important link between trophic levels in aquatic ecosystems, and their response to organic carbon is likely to have broad implications for lake food webs (Bowszys et al., 2020). A fundamental question regarding energy flow in rivers and streams is whether the basal component of the food web is predominantly driven by allochthonous or autochthonous sources (Guo et al., 2016). Although many researches have been conducted and proved the terrestrial sources were mainly fuel stream food webs, the widespread use of biochemical tracers in the recent years has challenged the view of dominant terrestrial sources, and highlighted the importance of zooplankton food sources in freshwater food webs (Pitt et al., 2009). Based on studies using a number of biochemical tracers, the nutrients of zooplankton have been demonstrated to be the main basis of freshwater food webs (Ryan et al., 2013). Zooplankton based food chains are generally considered as an efficient method to transfer energy and carbon to higher trophic levels (Metillo et al., 2019).

The zooplankton can respond to changes in the water environment in a variety of ways. For example, the ecological response of zooplankton to reduced light is a measure of pollution in the aquatic environment (Williamson et al., 2020). The response of zooplankton communities with different population densities to different pesticides and insecticides (Chang et al., 2005). The response of zooplankton to nutrient enrichment and fish in shallow lakes (Vakkilainen et al., 2004). The zooplankton community has fast responses to oxygen stress in the aquatic environment (Karpowicz et al., 2020b). In a word, the response behavior of zooplankton to the aquatic environment can provide insights into the degree of pollution in the aquatic environment and other species diversity.

The present qualitative and quantitative methods in zooplankton

The production and life of human beings are closely related to climate change and the decline of water quality in freshwater ecosystem (Bollens et al., 2014). Since the 1990s, zooplankton research and comprehensive evaluation of freshwater ecosystem have been two important issues in zooplankton research. It is very necessary to study zooplankton systematically. Firstly, zooplankton is an important bait for fish and other economic animals in the upper and middle waters, which is of great significance to the development of fisheries. Secondly, because the distribution of many zooplankton is related to climate, zooplankton can be used as a sign of warm or cold current changes (Keister et al., 2012). Thirdly, due to eutrophication, many dominant populations of zooplankton in freshwater can be used as indicators of water pollution (Wei et al., 2017). Because of the above reasons, many new identification methods of zooplankton in freshwater environment have been developed and applied all over the world (Medellin and Escribano, 2013; Uusitalo et al., 2016; Berges et al., 2020). A part of them are quantitative research methods, which mainly studies the biomass and diversity index of zooplankton in

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freshwater environment (Tsuboko and Burton, 2018). The other part of them are the qualitative research methods, which are based on the characteristics, composition, spatial distribution and dominant species of zooplankton community (Ramdani et al., 2009). However, the results obtained by these two methods alone are often not satisfactory because they often ignore the impact of complex pressure factors on freshwater zooplankton. And the combination of qualitative method and quantitative methods can better deal with the complex mixed source pressure (Wang et al., 2017). In addition, compared with the traditional classification analysis method, the combination of qualitative and quantitative analysis can better transform the different classification components of geographical regions into similar complementary features. According to the results of this study, the common qualitative and quantitative research methods used in the field of freshwater zooplankton in the Web of Science from 1991 to 2020 are as follows: (1) Sampling; (2) Model; (3) Monitor (4) Dynamical analysis, which can provide a reference for the development and utilization of freshwater plankton germplasm resources in the future (Ringelberg et al., 2003; Morley et al., 2012; Woods et al., 2015; Li et al., 2017).

Application significance of integrated technology

Zooplankton refers to the small aquatic animals suspended in the water, which can't produce organic matter by themselves in the name of heterotrophic invertebrates and chordate larvae, and together with phytoplankton constitute plankton. Zooplankton which can participate in the decomposition and circulation of organic matter in aquatic ecosystem through their own excretion and secretion, not only feed on phytoplankton, bacteria and debris in water, but also be preyed on by fish and other aquatic animals (Day et al., 1990; Lochmann et al., 2007). Because many zooplankton species are very sensitive to pollutants, they can be used as indicators to monitor and evaluate water quality (Hallanger et al., 2011; Nizzetto et al., 2012; Peng et al., 2018). Zooplankton can bio-accumulate and transfer pollutants, playing an important role in ecotoxicology and water environmental protection (Fisk et al., 2001).

In addition to the traditional fixed-point sampling method, with the advance of science and technology, many new freshwater zooplankton detection technologies have realized the organic combination of micro detection and macro detection and promoted the development of freshwater zooplankton research. Among them, the most common micro-techniques include PFU artificial matrix method, which is easy to operate and suspend under water, not affected by water depth, water quality and water flow velocity (Xu et al., 2005; Jiang et al., 2007). It can avoid the influence of macro-zooplankton and other invertebrates on zooplankton measurement and ensure the authenticity of test results. Furthermore, the freshwater zooplankton can affect all aspects of freshwater, seawater and terrestrial ecosystems to a great extent because of the freshwater zooplankton plays an important part in the food web (Pereira et al., 2007). In order to better trace and analyze the specific situation of aquatic ecological environment, stable isotope analysis of carbon and nitrogen has been successfully applied to trace the source and transfer of organic matter in aquatic food webs, providing a comprehensive view of time and space for the nutritional relationship between organisms (Hoeinghaus et al., 2011). By tracing the transfer and transformation process of isotopes in the food chain, we can accurately analyze and locate the correlation coefficient of freshwater zooplankton, so as to investigate the aquatic environmental indicators such as water quality, eutrophication degree, environmental fluctuation, nutrient structure and nutrient limit (Logan et al.,

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2008). And the stable carbon and nitrogen isotope analysis method can not only trace the transfer and transformation process of freshwater zooplankton, but also challenge the view of the main terrestrial sources, and highlight the importance of zooplankton in the stream food web. The stable carbon and nitrogen isotope analysis showed that freshwater zooplankton is an important component of food web in aquatic ecosystem (Robson et al., 2016). In addition, the dominant community species and structure characteristics of freshwater zooplankton are very important to study the ecological structure, stability and pollution degree of freshwater environment. It is very necessary to find out the gradient relationship between freshwater zooplankton and freshwater environmental parameters, providing suggestions for the management and development of freshwater resources and environment in the future.

Conclusion

The number of publications on Web of Science showed a gradually increasing trend from 1991 to 2020, which was closely related to the strong support and mutual cooperation of the government. For example, the EU Water Framework Directive provides excellent opportunities for collaborative researches and publications among European countries, and a good case for collaborative research on an international scale. The adoption of similar directives among neighboring countries throughout the world could further strengthen ties and cooperation among neighboring countries, with the aim of gradually developing a unified directive on water resource assessment and management strategies on a global scale.

The impact factor (IF) of journals is related to a certain extent to the number of publications and their cited frequencies. And the freshwater zooplankton is the main food source of many aquatic animals as indicators to monitor and evaluate water quality, which plays an important role in ecotoxicology and water environmental protection. With the progress of science and technology, there are many new freshwater zooplankton detection technologies. Among them, the most representative technologies are PFU artificial matrix method and stable isotope analysis of carbon and nitrogen, which can not only combine qualitative and quantitative methods to deal with complex pressure source mixture, but also realize the organic combination of micro and macro with highly accurate results. By tracing the transfer and transformation process of isotopes in the food chain, we can accurately analyze and locate the correlation coefficient of freshwater zooplankton, so as to investigate the aquatic environmental indicators. And the stable carbon and nitrogen isotope analysis method also shows the freshwater zooplankton is an important component of food web in aquatic ecosystem. And the dominant community species and structure characteristics of freshwater zooplankton are very important for studying the ecological structure, stability and pollution degree of freshwater environment. It is very necessary to find out the gradient relationship between freshwater plankton and freshwater environmental parameters, providing suggestions about the management and development of freshwater resources and environment in the future.

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EFFECTS OF ARTIFICIALLY ALTERED SOIL STRUCTURE ON ^{15}N ABSORPTION AND UTILIZATION FOR MAIZE (*ZEA MAYS* L.) AT THE SEEDLING STAGE

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Abstract. Soil structure is an important parameter which governs soil and plant nitrogen (N) dynamics. N is essential in the development and growth of maize (*Zea mays* L.). In this field pot study carried out for four months, we investigated the effects of artificial soil structure alteration on N use efficiency (NUE) and N uptake by maize. Soil structure was artificially altered by (i) retaining soil with a blocky structure in a 2 mm sieve (CKU), and (ii) flooding the soil with 600 mL of distilled water (CKW). Unaltered soil was established as the control treatment (CK). Using a ^{15}N tracer method, soil ^{15}N retention, and ^{15}N utilization and absorption rate of maize during the seedling stage was studied. The results showed that plant height, dry matter weight, root status, chlorophyll contents, and ^{15}N utilization rate were significantly decreased by CKU and CKW. The ^{15}N utilization rates were 23.09%, 20.64% and 13.76%, under CK, CKU, and CKW, respectively. This indicated that after the structure of black soil was destroyed, maize growth declined, and ^{15}N absorption and utilization rates decreased at the seedling stage.
Keywords: *artificial altered, soil structure, maize seedling stage, ^{15}N utilization, ^{15}N residual*

Introduction

Soil structure controls many processes in the soil. Good soil structure plays an important role in agricultural production (Munkholm et al., 2003). Well-structured soils are high in organic matter and provide a continuous supply of nutrients to promote crop growth (Bimuller et al., 2016), improve soil fertility and increase crop yields (Liang et al., 2011). Contrarily, the soil with compact soil structure leads to deterioration of soil water and aeration conditions, which limits the growth of plant roots, thus hindering the effective use of nutrients and water by plants (Walia and Dick, 2018). It also reduces crop yield and causes serious nutrient leaching (Kavdır and Smucker, 2005). Panakoulia using principal component analysis identified clay content, bulk density, climatic conditions (precipitation and evapotranspiration), organic matter (OM) and its decomposition rates as the most important factors that controlled soil structure development (Panakoulia et al., 2017). However, most studies on soil structure have focused on the impact of a single influencing factor, in this study we focused on several.

Nitrogen (N) is one of the major limiting nutrients in agricultural production, and crop productivity depends largely on the high level of N applied to the soil (Alam et al., 2006). However, excessive application of N fertilizer can lead to serious ecological and environmental problems, as well as increase leaching of nitrate, resulting in eutrophication of surface water and pollution of underground water (Islam et al., 2007). The N in the soil is absorbed by crops mainly in inorganic forms (Pang, 2019), and the soil structure affects the transformation and residue of N (Bataung et al., 2012). Thusly, soil structure has a certain influence on the absorption and utilization of crop N.

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Maize seedling stage (code 19 according to the BBCH scale (Lancashire et al., 1991)) is a vegetative growth period with long roots as the center, rapid root growth and slow above-ground growth (Chen, 2007). From the perspective of the whole growth period of maize, seedling stage is the critical period for nutrient demand, and its growth and development status can be used as an important indicator to judge whether the location of nutrient application is good or bad (Yu et al., 2017). At the same time, seedling growth is the basis of maize growth and yield in the future.

In recent years, there have been many studies on soil structure and, N absorption and utilization by crops, but most of them focus on a certain influencing factor of soil structure. Little research has been done on N absorption and utilization after soil structure is destroyed. Li showed that damaged soil structure significantly reduces the amount of organic N mineralization, and affects the cumulative porosity (Li, 2018). The uptake and utilization of crop N in destroyed soil structure has not been sufficiently studied. The ^{15}N tracer method can distinguish whether the N absorbed by the crop comes from the fertilizer or the soil, so as to determine the main N source of the crop (Zuo, 2012). In this paper, the ^{15}N tracer method was employed to study the use of N fertilizer in artificially damaged soil structure during maize seedling. This will provide a basis for the use and scientific application of N fertilizer in soil with damaged structure in the future.

Materials and methods

Site description

The soil selected in this study are from a maize (*Zea may* L) continuous monocropping field of Jilin Agricultural University, located in Jingyue district, Jilin province of China (N43°48'43.57", E125°23'38.50"). Maize monocropping is the main cropping system in the region. The area has an average temperature of 4.8°C, with an average annual sunshine duration of 2 688 h, and mean annual precipitation of 617 mm. About 65% of annual precipitation is concentrated in July and August (summer). The field is characterized by black soil, classified as Mollisol according to the World reference base classification system, with a loamy clay texture. The soil was amended with corn straw and constant fertilizer for three consecutive years from October 2014 to October 2017.

In June 4, 2018, undisturbed soil samples were randomly collected from different locations in the 0–20 cm soil depth using a stainless-steel soil sampler (5 cm in diameter). Collected soil samples were carefully mixed to form a composite, placed in a plastic box, and then immediately transported to the laboratory. Upon arriving in the laboratory, the soil was gently broken along natural break points and allowed to pass through a 10 mm sieve to remove stones and other impurities. Visible plant residues were removed from the sieved soil with forceps and the soil was then air-dried for 72 hours. The initial basic soil properties are shown in *Table 1*.

Table 1. *Initial basic soil properties*

pH	Water content (%)	WSA (%)	BD (g cm^{-3})	SOM (g kg^{-1})	AH–N (mg kg^{-1})	P (mg kg^{-1})	K (mg kg^{-1})	Yield (kg ha^{-1})
6.55	26.78	76.85	1.08	19.61	106.1	24.3	116.1	11492

WSA, water stable aggregate-size fractions (sum of > 2 mm, 2 – 0.25 mm, 0.25 – 0.053 mm, and < 0.053 mm aggregates); BD, bulk density; SOM, soil organic matter; AH–N, alkaline hydrolysable nitrogen; P and K, available phosphorus and potassium, respectively; Yield, maize yield

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Experimental design

The experiment was initiated in June 2018, designed as pot experiment, and included three treatments which were replicated three times and arranged in a completely randomized design. The pot (high-type polyethylene plastic bucket) had the bottom and barrel diameter of 24 cm, and height of 22 cm. The treatments were prepared by: (i) adding exactly 3 kg of soil (< 10 mm) into a plastic pot container, and designated as a control (CK), (ii) passing soil through a 2 mm sieve to retain the soil with blocky structure in the sieve, and 3 kg of the soil retained by the sieve was added in a plastic pot container (CKU), and (iii) another, 3 kg of soil (< 10 mm sieve) was added into a plastic pot container and then flooded with 600 mL of distilled water, henceforth referred as CKW (before planting, the soil remained submerged under water for 24 hours, until the water slowly evaporated, resulting to soil crusting on the surface after day 10). CKW represent waterlogging-prone soil. Each pot was planted with 2 identical corn seeds (xiang yu 998) in June 6, 2018 and one plant was left after seeding. Prior to planting, ^{15}N labeled urea (isotope content 5.15%) obtained from Shanghai institute of chemical engineering - Ministry of chemical industry was applied. Since, corn seedling (stage 19 in BBCH scale) was of interest in our study, only 0.22 g of ^{15}N labeled urea, 0.08 g of potassium dihydrogen phosphate and 0.08 g of potassium sulfate were incorporated into each pot (i.e., one third of the recommended fertilizer for the entire growth cycle). The pots were randomly placed in an open cart (100 cm high), and watered by 200 mL on weekly bases. In August 5, 2018, maize plant including roots, and 30 g of soil was collected from each pot, and designated as the first sowing data set (S1). The soil was air-dried and passed through a 2-mm sieve.

The experiment was repeated using the same pot soils used in S1 (i.e., CK, CKU and CKW treatments), but without fertilizer addition and altering soil structure further. Maize seed was planted in each pot in August 6, 2018, and watered on weekly basis. Maize plant including the roots were harvested in September 25, 2018 (at stage 19 according to the BBCH scale) from each pot. In the same day, 30 g of soil was collected from each pot. Collected maize plant and soil samples were designated as second sowing data set (S2).

Laboratory analysis

Plant analysis

The number of roots was determined by lying flat the roots systems of maize and counting them. Root volume and root dry quality were determined by drainage method and drying method, respectively. Maize plant height was obtained by measuring the maximum length from the soil surface to its natural extension at stage 19 of BBCH (Lancashire et al., 1991).

Weight dry matter was measured by oven-dry method, by oven drying above-ground maize parts at 105°C for 30 min, thereafter maize plant parts were dried at constant temperature (80°C) for 12 h. The oven-dried maize sample was allowed to cool to room temperature, and the weight change was recorded by a sensitive electric balance. Maize plant N, phosphorus (P) and potassium (K) contents were measured according to the method described Lu (1999). In short, 0.2 g of crushed and sieved (0.25 mm) above-ground maize plant was treated with concentrated H_2SO_4-H_2O_2 solution. The N and P were analyzed by a two-channel automatic flow analyzer. While, maize plant K content was determined by FP640 ASTM flame photometer (Lu, 1999). The chlorophyll content in maize plant leaves was determined following extraction with acetone and measured by

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visible spectrophotometer (Model 722 spectrophotometer, Huanghua Faithful Instrument, China) according to the method describe in Lu (1999).

The $\delta^{15}N$ of corn plant sample was measured by the Isoprime100 Mass Spectrometer (Elementar Analysensysteme GmbH Inc. Germany), after corn plants were defoliated at 105°C for 30 min, and dried at 75°C for 48 h, thereafter weighed and grounded into powder.

Soil analysis: Physical and chemical properties

The soil from each data set (S1 and S2) was air-dried and allowed to pass through a 2–mm sieve, then analyzed for soil pH, moisture content, alkali-hydrolyzed N (AH–N), available P and K by referring to soil agricultural chemical analysis methods (Lu, 1999). Nitrate (NO_3^-–N) and ammonium (NH_4^+ –N) were extracted by 2 M KCl in a 1:5 soil-solution ratio and the extractant was determined by continuous flow analyzer (AA3 HR continuous flow analyzer). Soil organic carbon (SOC) was determined by potassium dichromate method (Nelson and Sommers, 1982), by treating 0.2 g of soil with $K_2Cr_2O_7$-H_2SO_4 solution and heating at 180°C for ~5 min. After cooling to room temperature, excess $K_2Cr_2O_7$ was titrated with 0.2 N $FeSO_4$ until the end point was reached.

Soil water stable aggregate-size (WSA) fractions were measured with a wet-sieving procedure (Cambardella and Elliot, 1993). This wet-sieving procedure resulted into four aggregate-size fractions: (i) large macro-aggregates (> 2 mm), (ii) small macro-aggregates (2-0.25 mm), (iii) micro-aggregates (0.25–0.053 mm) and (iv) silt/clay fraction (< 0.053 mm). All fractions were dried at 60°C. The $\delta^{15}N$ of air-dried and ground soil samples (100 mesh sieves) was measured with an isotope ratio mass spectrometer (IsoPrime100 Mass Spectrometer, Germany).

Statistical analysis and calculations

The data were analyzed by one-way analysis of variance using SPSS 22.0 (IBM Corporation, New York, USA). Significant differences among treatment means were evaluated using the least significant difference test at a $P < 0.05$. All figures were compiled using Origin 8.5 software.

The ^{15}N utilization rate (^{15}NUR, %) was calculated as follows:

$$\begin{aligned} {}^{15}NUR &= Npf/Nf \times 100 \\ Npf &= NP \times Npff \\ Npff &= Npp/Nppf \times 100 \end{aligned} \quad \text{(Eq.1)}$$

where: N_{pf} is the amount of N absorbed by the plant in ^{15}N fertilizer (g pot^{-1}), N_f is the amount of applied ^{15}N fertilizer (g pot^{-1}), N_P is the amount of total N absorbed by plants (g pot^{-1}), N_{pff} is the percentage of total plant N from fertilizer (%), N_{pp} is the atomic percentage of ^{15}N fertilizer in plants exceeds, N_{ppf} is the atomic percentage of ^{15}N-labled fertilizer.

The ^{15}N retention rate (^{15}NRR, %) was calculated as follows:

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$$^{15}NUR = Nsf/Nf \times 100$$
$$Nsf = Ns \times Nsff \quad \text{(Eq.2)}$$
$$Nsff = Nss/Nssf \times 100$$

where: N_{sf} is the amount of N in the soil from ^{15}N fertilizer (g pot^{-1}), N_f is the amount of applied ^{15}N fertilizer (g pot^{-1}), N_s is the amount of soil total N (g pot^{-1}), N_{sff} is the percentage of total soil N from fertilizer (%), N_{ss} is the ^{15}N atoms of total N in the soil, N_{ssf} is the ^{15}N-labeled fertilizer atomic excess percentage.

The ^{15}N loss rate (^{15}NLR, %) was calculated as follows:

$$^{15}NLR = 100 - {}^{15}NUR - {}^{15}NUR \quad \text{(Eq.3)}$$

Results and analysis

Changes in soil properties

Changes in soil aggregate-size distribution

The aggregate-size distribution of macro-aggregates (> 0.25 mm) across all treatments and sowing periods accounted for more than 60% of total soil weight. Of all aggregate-size fractions, the 2 – 0.25 mm aggregates were higher than that of other aggregate-size fractions in both data sets (*Table 2*). CKU and CKW treatments reduced 2 – 0.25 mm aggregate-size distribution by 11.67% and 17.84% compared to CK. But these treatments increased micro-aggregates (0.25 – 0.053 mm) and silt/clay fraction (< 0.053 mm). Compared with S1 results, the 2 – 0.25 mm and 0.25 – 0.053 mm soil aggregate-size fractions slightly increased in S2 across all treatments, whereas > 2 mm aggregates decreased slightly (*Table 2*).

Table 2. *Soil aggregate-size fractions (%) in soils' structure artificially altered*

Time	Treatment	>2 mm	2–0.25 mm	0.25–0.053 mm	<0.053 mm
S1	CK	21.25±0.08c	44.51±0.34a	25.77±0.12b	7.28±0.21c
	CKU	22.79±0.63b	39.86±0.48b	27.53±0.97a	8.21±0.29b
	CKW	23.97±0.62a	37.77±0.97c	25.47±0.66b	10.54±0.16a
S2	CK	20.78±0.50a	45.67±0.35a	26.48±0.71b	5.63±0.23c
	CKU	21.12±0.77a	41.38±0.51b	28.73±0.93a	6.22±0.21b
	CKW	21.78±0.57a	39.23±0.88c	27.11±0.64b	9.37±0.34a

Mean values ± SE. Means followed by the same letter in a column of a given sowing time across all treatments are not significantly different at $p < 0.05$. CK represents well-structured normal soil treated with corn straw return and constant fertilizer application for 3 consecutive years from October 2014 to October 2017. CKU is blocky soil structure retained by a 2 mm sieve; CKW represents a well-structed soil flooded with 600 mL of distilled water in CK. S1 represents the first sowing period; S2 represents the second sowing period

Soil organic carbon content changes

SOC content ranged from 11.42 to 14.02 g kg^{-1} across all treatments and data sets (*Fig. 1*). In both data sets, CK showed significantly ($P < 0.05$) high SOC contents than CKU and CKW. In fact, CKU decreased SOC by 8.23% in S1 and by 8.05 in S2 compared with CK. While, CKW reduced soil organic carbon by 15.22% and 15.79% in the S1 and

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S2, respectively, compared with CK (*Fig. 1*). These results indicate that altering soil structure significantly encourages SOC losses.

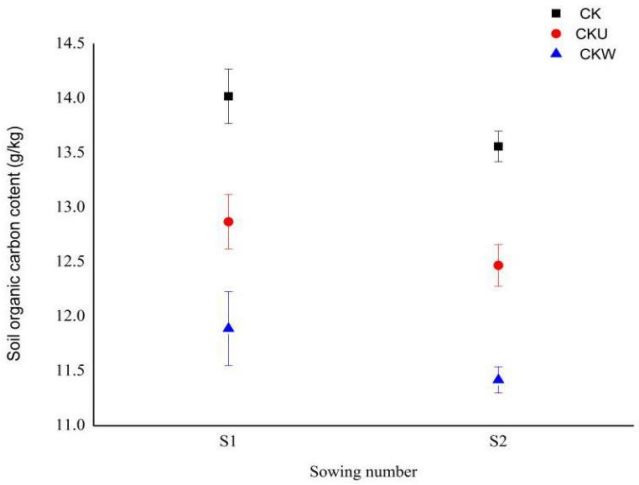

Figure 1. *Changes in organic carbon content after artificial soil destruction. Bars are standard error, and bars followed by the same letter for a given sowing time are not significantly different at $p < 0.05$. CK represents well-structured normal soil treated with corn straw return and constant fertilizer application for 3 consecutive years from October 2014 to October 2017. CKU is blocky soil structure retained by a 2 mm sieve; CKW represents a well-structed soil flooded with 600 mL of distilled water in CK. S1 represents the first sowing period; S2 represents the second sowing period*

Changes in soil NH_4^+ –N and NO_3^- –N contents

The NH_4^+ –N ranged from 3.03 to 4.06 mg kg^{-1} and NO_3^-–N ranged from 22.71 to 30.69 mg kg^{-1} for both data sets. Soil NH_4^+ –N and NO_3^-–N contents from both sowing times showed a similar trend, which increased in this order CK > CKU > CKW (*Fig. 2*). Compared with CK, the CKU and CKW decreased NH_4^+ –N content by 13.87–14.98% and 7.98–8.47%, respectively, and decreased NO_3^-–N content by 24.48–25.51% and 18.75–19.17%, respectively. This indicated that soil NH_4^+ –N and NO_3^-–N contents changes with soil structure.

Soil ^{15}N total residual, total loss rate

Total soil ^{15}N residue rate appeared to follow this trend CK > CKU > CKW, while total ^{15}N loss rate followed this order CK < CKU < CKW (*Table 3*). The CKU and CKW treatments decreased total soil ^{15}N residual rate of 7.67% and 8.85% compared with CK, respectively. While, the total ^{15}N loss rate of CKU and CKW increased by 21.53% and 47.56% compared with CK. These results showed that altering soil structure decrease total ^{15}N residual rate and increases ^{15}N loss rate.

Plant growth

Root system in maize seedling stage

The number of roots, root volume and dry root matter of maize at seedling stage significantly decrease after CKU and CKW (*Table 4*). Compared with CK, the dry root of maize decreased by 26.83% under CKU and by 34.15% under CKW, in S1. In the S2,

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CKW and CKU decreased the number of roots and dry root mass of maize at seedling stage (*Table 4*), suggesting that poor soil structure might impede root development.

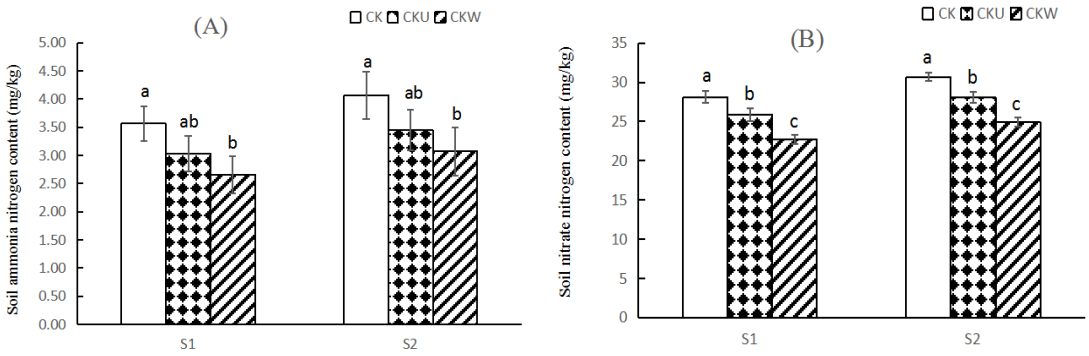

Figure 2. *Changes in soil (A) ammonium nitrogen, and (B) nitrate nitrogen contents after the soil was artificially damaged. Bars are standard error, and bars followed by the same letter for a given sowing time are not significantly different at $p < 0.05$. CK represents well-structured normal soil treated with corn straw return and constant fertilizer application for 3 consecutive years from October 2014 to October 2017. CKU is blocky soil structure retained by a 2 mm sieve; CKW represents a well-structed soil flooded with 600 mL of distilled water in CK. S1 represents the first sowing period; S2 represents the second sowing period*

Table 3. *^{15}N total residual, and total ^{15}N loss rate after artificial soil destruction*

Treatment	Total ^{15}N residual rate (%)	Total ^{15}N loss rate (%)
CK	48.31±1.97a	28.60±1.62c
CKU	44.60±1.80b	34.76±1.03b
CKW	44.03±1.74b	42.21±2.21a

Mean values ± SE. Means followed by the same letter in a column are not significantly different at $p < 0.05$. CK represents well-structured normal soil treated with corn straw return and constant fertilizer application for 3 consecutive years from October 2014 to October 2017. CKU is blocky soil structure retained by a 2 mm sieve; CKW represents a well-structed soil flooded with 600 mL of distilled water in CK. S1 represents the first sowing period; S2 represents the second sowing period

Table 4. *Root growth of maize at seedling stage after soil structure was artificially disturbed*

Treatment	S1			S2		
	Root number (piece)	Root volume (cm^3)	Dry root mass (g)	Root number (piece)	Root volume (cm^3)	Dry root mass (g)
CK	8.33±0.58a	2.90±0.20a	0.27±0.02b	7.67±0.58a	2.63±0.15a	0.25±0.02a
CKU	6.33±0.58b	2.22±0.11b	0.20±0.01b	6.00±1.00b	1.94±0.06b	0.18±0.01b
CKW	5.67±0.58b	1.98±0.08b	0.18±0.01b	4.67±0.58b	1.81±0.06b	0.16±0.01c

Mean values ± SE. Means followed by the same letter in a column across all treatments are not significantly different at $p < 0.05$. CK represents well-structured normal soil treated with corn straw return and constant fertilizer application for 3 consecutive years from October 2014 to October 2017. CKU is blocky soil structure retained by a 2mm sieve; CKW represents a well-structed soil flooded with 600 mL of distilled water in CK. S1 represents the first sowing period; S2 represents the second sowing period

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Growth of maize plant at seedling stage

In both data sets, the CKU and CKW appeared to significantly hinder plant growth, shown by a decrease in maize plant height and dry weight (*Table 5*). Both maize height and dry weight in S1 and S2 data sets were significantly higher under CK compared with CKU and CKW, and these parameters appeared to decreased in this order CKW < CKU < CK (*Table* 5).

Table 5. *Growth of maize at seedling stage after artificial soil structure destruction*

Treatment	S1		S2	
	Height (cm)	Weight (g)	Height (cm)	Weight (g)
CK	80.50±0.87a	7.37±0.11a	50.00±2.00a	3.06±0.17a
CKU	73.77±0.71b	6.38±0.05b	44.50±1.95b	2.32±0.18b
CKW	70.03±0.95c	6.16±0.06c	42.53±1.86b	2.14±0.12b

Mean values ± SE. Means followed by the same letter in a column across all treatments are not significantly different at $p < 0.05$. CK represents well-structured normal soil treated with corn straw return and constant fertilizer application for 3 consecutive years from October 2014 to October 2017. CKU is blocky soil structure retained by a 2 mm sieve; CKW represents a well-structed soil flooded with 600 mL of distilled water in CK. S1 represents the first sowing period; S2 represents the second sowing period

Chlorophyll content in maize leaves at seedling stage

The content of chlorophyll a ranged from 1.21–1.61 mg g^{-1}, and chlorophyll b ranged from 0.18–0.35 mg g^{-1} in S1 and S2 (*Table 6*). Only chlorophyll a appeared to be affected by soil structure, because it significantly decreased upon alteration in soil structure (*Table 6*). Whereas, chlorophyll b was not significantly affected by changes in soil structure in both sowing times (*Table 6*).

Table 6. *Chlorophyll content in maize leaves at seedling stage after artificial destruction*

Treatment	S1			S2		
	Chlorophyll a	Chlorophyll b	Total chlorophyll	Chlorophyll a	Chlorophyll b	Total chlorophyll
	mg g^{-1}					
CK	1.61±0.03a	0.35±0.04a	1.96±0.02a	1.44±0.06a	0.30±0.06a	1.74±0.04a
CKU	1.46±0.05b	0.30±0.05a	1.76±0.04b	1.27±0.04b	0.19±0.07a	1.46±0.04b
CKW	1.41±0.04b	0.29±0.05a	1.70±0.07b	1.21±0.02b	0.18±0.04a	1.39±0.03c

Mean values ± SE. Means followed by the same letter in a column across all treatments are not significantly different at $p < 0.05$. CK represents well-structured normal soil treated with corn straw return and constant fertilizer application for 3 consecutive years from October 2014 to October 2017. CKU is blocky soil structure retained by a 2 mm sieve; CKW represents a well-structed soil flooded with 600 mL of distilled water in CK. S1 represents the first sowing period; S2 represents the second sowing period

Nutrient content in maize seedling stage

Maize N, P, and K contents were significantly affected by changes in soil structure and ranged from 8.31 to 11.15 mg kg^{-1}, 3.32 to 4.90 mg kg^{-1}, and 2.49 to 4.69 mg kg^{-1}, respectively (*Table 7*). The contents of the same attributes were significantly ($P < 0.05$) higher under CK, followed by CKU, and then CKW. The CKW treatment showed the highest decrease in N, P, and K contents compared with CK, decreasing N by 20.71%, P

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by 20.13%, and K by 39.05% in S1 data set. In the S2 data set, N, P and K contents of CKU decreased by 12.27%, 19.76%, and 32.44%, respectively, compared with CK, which means N, P and K contents in maize plant decreases after the soil structure is artificially changed.

Table 7. *Nutrient content of maize at seedling stage after artificial destruction*

Treatment	S1			S2		
	N	P	K	N	P	K
	mg kg^{-1}					
CK	11.15±0.70a	4.98±0.04a	4.69±0.10a	10.08±0.32a	4.62±0.05a	4.19±0.10a
CKU	9.66±0.70ab	4.37±0.12b	3.17±0.10b	8.84±0.27b	3.70±0.10b	2.83±0.21b
CKW	8.84±0.88b	3.98±0.05c	2.86±0.20c	8.31±0.21b	3.32±0.17c	2.49±0.21b

Mean values ± SE. Means followed by the same letter in a column are not significantly different at $p < 0.05$. N, P, and K represents nitrogen, phosphorus, and potassium, respectively. CK represents well-structured normal soil treated with corn straw return and constant fertilizer application for 3 consecutive years from October 2014 to October 2017. CKU is blocky soil structure retained by a 2mm sieve; CKW represents a well-structed soil flooded with 600 mL of distilled water in CK. S1 represents the first sowing period; S2 represents the second sowing period

Change of utilization rate of ^{15}N in maize seedling stage

As shown from *Fig. 3*, the ^{15}N utilization rate exhibited similar trend in both S1 and S2 data sets, increasing in this order CK > CKU > CKW. The ^{15}N utilization rate of S1 was slightly higher than that in S2 across all treatments. Compared with CK, the ^{15}N utilization rate in CKU decreased by 11.51% and that in CKW decreased by 32.69%, in under S1. In S2 data set, the CKU and CKW decreased ^{15}N utilization rate by 9.56% and 49.91%, respectively *(Fig. 3)*. This indicate that soil altered by flooding (i.e., CKW treatment) significantly reduce the rate by maize utilizes ^{15}N during the seedling stage.

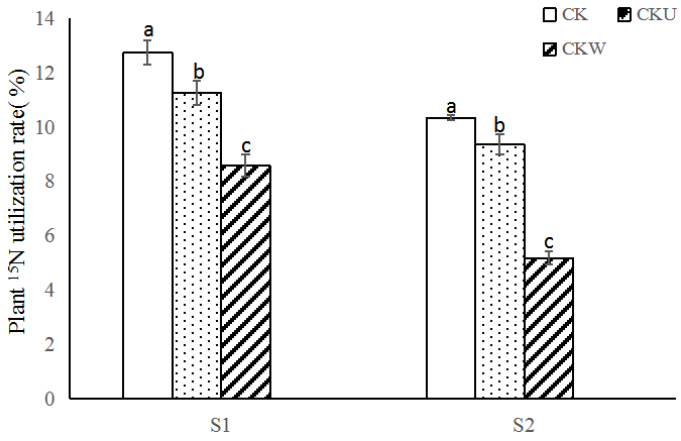

Figure 3. *The ^{15}N utilization rate after artificial soil structure destruction. Bars are standard error, and bars followed by the same letter for a given sowing time are not significantly different at $p < 0.05$. CK represents well-structured normal soil treated with corn straw return and constant fertilizer application for 3 consecutive years from October 2014 to October 2017. CKU is blocky soil structure retained by a 2mm sieve; CKW represents a well-structed soil flooded with 600 mL of distilled water in CK. S1 represents the first sowing period; S2 represents the second sowing period*

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Discussion

Effects of artificially altered soil structure on soil properties

The present study demonstrated that soil flooding (i.e., CKW treatment) and altering soil structure (i.e., CKU treatment) significantly decreases 2 – 0.25 mm aggregate-size fractions (*Table 2*), SOC content (*Fig. 1*) and soil mineral N (*Fig. 2*). These results indicate that altering soil structure and flooding affects soil mineral N, SOC content and soil physical properties, and is consistent with the results obtained by Harris (Harris et al., 2016) which showed that soils prone to flooding have low NO_3^- - N content and higher bulk density compared with not flooded soil. This may be because flooding lower N mineralization rates, and encourages volatilization of ammonia and denitrification losses (Bacon et al., 1986; Zhang and Wienhold, 2002). Whereas, structure disturbance experienced under CKU treatment may have increased accessibility of soil organic N and organic matter to soil micro-organisms for mineralization. Kristensen et al. (2000) also reported a decrease in soil mineral N and SOC contents when soil structure is disturbed.

Nitrogen fertilizer applied to the soil is converted into inorganic NO_3^-–N and NH_4^+–N, organic nitrogen (Caiyan et al., 2010). According to Mkhabela et al. (2008), NO_3^-–N and NH_4^+–N are the forms of inorganic N most greatly absorbed by the roots of maize. Therefore, the higher the availability of inorganic NO_3^-–N and NH_4^+–N in the soil and rhizosphere, and the utilization ability of the crop, the higher the N absorption by crops (Asibi et al., 2019). In the present study both CKW and CKU reduced soil NO_3^-–N and NH_4^+ –N (*Fig. 2*), resulting to losses of soil total ^{15}N and low soil total ^{15}N residual rate (*Table 3*). These results indicate that applied synthetic fertilizer N was not stabilized within the soil matrix, probably because flooding led to elevated nitrous (N_2O) production. Whereas, CKU may have influenced mineralization-immobilization turnover by increasing availability of protected N to microbes, and some of N stored in < 2 mm aggregates were lost due to sieving. Harris observed that waterlogging-prone soil can lead to elevated N_2O production, which reduces soil N (Harris et al., 2016). These results suggest that soils that had their structure disrupted have lower capacity for N retention and nutrient. Contrary to this, a study by Chilundo et al. (2018) showed that proper soil management practices reduce potential leaching losses of NO_3-N and NH_4-N, which in turn improves N retention. This is attested by results presented by our study which showed that non-destroyed soil structure (CK treatment) was more conducive in increasing NO_3-N and NH_4-N (*Fig. 2*), soil total ^{15}N residual rate, and significantly reduced soil total ^{15}N loss (*Table 3*).

Effects of artificial soil structure changes on plant characteristics

Root growth (*Table 3*), height and dry matter weight (*Table 4*) of maize at seedling stage appeared to cease after flooding and soil structural disturbance. These maize growth parameters (*Tables 3 and* 4) did not recover over the durations evaluated in this experiment. This could mean, when soil is flooded or the structure is disturbed it might take a relatively long time (> 6 months in the case of our study) to restore its inherent properties to support maize production. The decrease in roots system in flooded soil might be due to the scarcity or absence of oxygen at the root tip (Malik et al., 2002). Low chlorophyll content measured in flooded soil (CKW treatment) (*Table 5*), is attributable to chlorophyll destruction mediated by superoxide radicals formed under flooding stress (Wang et al., 2012). These results suggest that when the structure of the soil is altered, maize development is impaired, which could potentially affect crop yield.

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Effects of artificial soil structure alteration on plant nutrients and N utilization efficiency

Compared with CK, the CKW and CKU treatments showed significantly lower leaf N, P and K content in both data sets (*Table 6*), and is consistent with the study by Ren, which reported a decrease in leaf N in waterlogged compared with non-waterlogged soil (Ren et al., 2017). This could be due to leaching of nutrients below the rooting depth of maize. Whereas, soil structure disturbance accelerates N mineralization of previously protected soil microbial biomass N (Kristensen et al., 2000), thus reducing available N for plant uptake.

The results of our study showed that, compared with CK maize ^{15}N absorption and utilization rate decreased significantly after soil structure was artificially altered (*Fig. 3*). This is because artificially altering soil structure significantly reduced soil aggregate-size distribution (*Table 2*) and SOC content (*Fig. 1*), soil factors which control the rates and products of N mineralization (Seyfried and Rao, 1988). Getahun showed that soil with high bulk density and lower porosity, deteriorates soil structure, thus affecting transport and absorption of nutrients (Getahun et al., 2018).

Kong et al. (2007) observed that soil N was reduced by changes in macroaggregates (> 0.25 mm). Therefore, a decline in > 0.25 mm aggregate-size fractions after CKW and CKU treatments in our study (*Table 2*), could have also contributed to lower ^{15}N utilization in maize (*Fig. 3*), because majority of protected soil organic N was lost when > 0.25 mm aggregates turned over (Kong et al., 2007). Xu et al. (2015) and Zhou et al. (2019) reported that increase in > 0.25 mm aggregate-size distribution could promote nutrient circulation, increase nutrient supply and absorption of maize roots.

Lal et al. (2007) showed that increasing SOC storage improves soil stability and porosity, and reduce soil erosion and nutrient leaching, which in turn encourages nutrient absorption by crops. A decrease in SOC content under CKW and CKU treatments compared with CK in our study (*Fig. 1*), might have contributed to a decrease in maize ^{15}N absorption and utilization rate (*Fig. 3*). Conversely, greater soil organic carbon content under CK, enhanced soil structure and fertility, as a result maize ^{15}N absorption and utilization rates was increased accordingly.

These results imply that ^{15}N absorption and utilization rate is dependent on several soil properties, which are exacerbated by poor soil structure. Therefore, poor soil structure might limit nutrient absorption/adsorption which will ultimately affects crop productivity.

Conclusion

After the soil structure was artificially altered, the growth and development of maize was hindered. Artificial altering soil structure decreased soil N and P content, and increased soil N losses. The ^{15}N utilization rate and uptake by maize at seedling stage was reduced in altered soil structure under both sowing times. The total ^{15}N absorption rate by maize under CK was 23.14%, the CKU and CKW were down to 20.63% and 13.77%, respectively, after soil structure is altered. Therefore, altering soil structure have adverse effects on soil nutrients and maize development. Through the study of this paper, further research on soil structure improvement measures to improve nitrogen use efficiency and increase crop yield will play an important role.

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ETHNOBOTANICAL KNOWLEDGE ON THE PLANTS USED BY PEOPLE ON THE DATÇA PENINSULA (MUĞLA, TURKEY)

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Abstract. This study aims to document the traditional uses of plants in the district of Datça peninsula in Turkey. Ethnobotanical data on 85 plants from a total of 67 informants were collected between February 2018 and June 2019. Demographic characteristics of the informants, vernacular names of the plants, their used parts, and preparation methods were investigated and recorded. The data was analysed using quantitative indices of use-value (UV), information consent factor (ICF), and fidelity level (FL). The plants were used for different purposes, mainly for medicinal, food, and handicraft. *Origanum onites* L. (FL: 100, UV: 0.50), *Salvia fruticosa* Mill. (FL: 100, UV: 0.50), and *Sideritis leptoclada* O.Schwarz & P.H.Davis (FL: 100, UV: 0.50) have been determined as the taxon most commonly used for medicinal purposes. If *Olea europaea* L. var. *europaea* (FL: 96, UV: 0.50) has been determined as the taxon most used for handicrafts. The highest ICF was cited for rheumatism (0.80), followed by respiratory diseases (0.79) and diabetes (0.74). Additionally, different uses and purposes of some plants were observed in the study, some medicinal uses of *Drimia maritima* (L.) Stearn, *Rumex amanus* Rech.f., and *Opopanax hispidus* (Friv.) Griseb. were recorded for the first time.
Keywords: *ethnobotany, folk remedies, medicinal plant, traditional uses*

Introduction

Plants like humans migrate and adapt to various habitats of the world in a variety of ways. People also have a significant role in this process. Traditional societies all over the world have enriched their social and cultural values accumulated through prolonged interaction with the world. One of these accumulated values is traditional botanical knowledge. For generations, biologists and anthropologists have studied various aspects of human knowledge on plants. They put forward vast data on how plants were used, managed, and perceived by different societies (Cotton, 1996). Thus, ethnobotany, which is an interdisciplinary field that studies the relationships between plants and humans, was born. Its purpose is to compile all local information on plants utilized in various ways. Approximately 5000 years ago, with the emergence of writing, information on cultivated plants and medicinal plants began to be recorded. People needed to record the information and recipes of these plants and transfer them to the next generations (Ertug, 2014). Ethnobotanical studies reflect very precious knowledge that has been learned through experience and inherited from generation to generation.

Anatolian territories, where both biological diversity and cultural richness are present, are a prominent center of ethnobotany (Sadıkoğlu, 1998; Güner et al., 2000; Akbulut and Özkan, 2014; Erşen-Bak and Çifci, 2020). Also, geographic position, climate diversity,

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natural resources, the possibility of hosting agriculture and livestock, and its location at the intersection of trade routes can be listed as other factors (Uyanık and Yenigün, 2016). For this reason, Anatolia has become a center of attraction for many ethnobotanists and anthropologists, and much research has been done on the traditional and medicinal uses of herbs to date (Lyle-Kalças, 1974; Bottema and Woldring, 1990; Ertuğ, 2000). The oldest book on medicinal plants in Anatolia is the work named "Materia Medica" written by Dioscorides in the middle of the 1st century (Baytop, 1999).

In this context, Datça, which is a research area, has become an important center for an ethnobotanical study due to its 861 plant taxa (2.9% of which are endemic) (Tuzlacı, 2002) and its history dating back to the 2000s BC (Sertkaya-Doğan, 2008).

Materials and Methods

Study area

Datça Peninsula is situated in the South-west of Turkey. Datça, which is within the borders of Muğla province, is a narrow peninsula extending 70 km in the east-west direction. Its total area is approximately 418 km^2 and located between 36°42′26″ north latitude and 27°33′07″ east longitude. Datça Peninsula is hot in summers and has a relatively mild climate in other seasons, with an annual average temperature of 19 °C and an annual rainfall of 836 mm (Özalp, 1993). Datça Peninsula belongs to the Mediterranean flora region and falls within the southeast part of the C1 grid square according to the grid system (Davis, 1965).

Pinus brutia Ten. and *Cupressus sempervirens* L. are the dominant forest tree species in the region. The important vegetation type in Datça is maquis. The maquis community has been destroyed in the region and major species are *Laurus nobilis* L., *Quercus coccifera* L., *Q. ilex* L., *Arbutus andrachne* L., *Juniperus oxycedrus* L, *Nerium oleander* L., *Erica arborea* L., *Myrtus communis* L., *Ceratonia siliqua* L., *Olea europaea* L., *Pistacia lentiscus* L. Another type of vegetation seen in the region is garrigue. The main species of this vegetation type formed because of the destruction of maquis vegetation are *Cistus creticus* L., *Lavandula stoechas* L., *Euphorbia acanthothamnos* Heldr. & Condition. ex Boiss., and *Spartium junceum* L. *Medicago marina* L., *Alkanna tinctoria* (L.) Tausch, *Eryngium maritimum* L. These are the dominant species in the dunes on the southern coast of the peninsula (Özalp, 1993; Taşlıgil, 2008).

Data collection

Ethnobotanical data were obtained from February 2018 to June 2019. A total of 67 informants were interviewed in the study area. Informants were selected using the snowball method from people living in 10 villages in the Datça Peninsula (*Table 1, Figure 1*). Traditional knowledge is hardly known to the younger generation. The Snowball method focuses on finding people who know about the subject. This sampling method includes a primary data source that nominates other potential data sources that can participate in research studies. The sample continues entirely based on references. In this way, it is possible to reach the sources that have information on the subject (Wegner, 2007). While choosing the informants, care was taken to have local expert-witness on the history and culture of the region. The ages of them were middle and older and were ranged from 36 to 87. Especially crowded places such as bazaars and tea houses were selected for the survey interviews (*Figure 2*).

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Table 1. *Number of questionnaire and study locations*

Study area	Locations	Number of participants
Datça Peninsula (Muğla province)	Yazıköy	8
	Cumalı	3
	Yaka	8
	Sındı	11
	Mesudiye	5
	Karaköy	6
	Hızırşah	6
	Reşadiye	3
	Kızlan	3
	Emecik	14

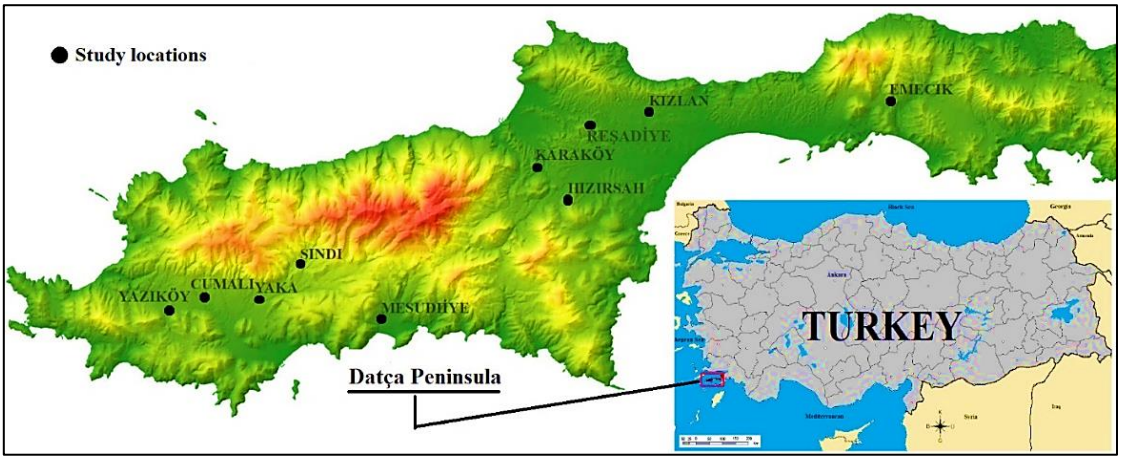

Figure 1. *The geographical location of Datça Peninsula*

Figure 2. *Informant interviews*

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In the villages visited, people were interviewed face to face, and a questionnaire was applied. Prior Informed Consent (PIC) was taken orally recently beginning each questionnaire. Ethical rules were taken from the Code of International Society for Ethnobiology (International Society of Ethnobiology, 2008). Informants were asked questions about their demographic characteristics, the plants they use, the purpose of use, and the way of using the plants (*Appendix*). The Informants were asked to show the plants they used, and samples were collected from these plants. Also, each plant was photographed. During this time 85 plant taxa used by local people were collected and defined. All voucher specimens were collected from their natural habitat. No samples were taken from cultural areas and gardens. Voucher specimens and photographs were identified and confirmed according to the Flora of Turkey by the authors (Davis 1965-1985; Davis et al., 1988). Identified plants were made of herbarium materials, and they were deposited in the Karadeniz Technical University Forest Faculty Herbarium (KATO) in Turkey. In the study, the taxonomic order was given alphabetically.

Data analysis

Data analyses were made based on ethnobotanical information provided by the informants and used various statistical methods. Use-value (UV), informant consensus factor (ICF), fidelity level (FL) of plants in the study area were calculated and their importance to the community was evaluated.

UV is a method used to determine how often local people actively use plants in their daily lives. In calculating the UV, the *equation (Eq.) 1* was (Trotter and Logan, 1986):

$$\mathrm{UV} = \frac{\mathrm{U}}{\mathrm{N}} \qquad \text{(Eq.1)}$$

where "U is the number of use citations by informants for any species, N is the number of informants".

ICF is a method used to determine the level of homogeneity between a specific disease and the plants used in its treatment and the effectiveness of the plants. In calculating the ICF, the *Eq. 2* was (Trotter and Logan, 1986; Heinrich et al., 1998):

$$\mathrm{ICF} = \frac{\mathrm{Nur}-\mathrm{Nt}}{\mathrm{Nur}-1} \qquad \text{(Eq.2)}$$

where "Nur is the number of citations in each category and Nt is the number of species used".

FL refers to the specificity of the plant species of choice for the diseases most frequently reported by informants. In calculating the FL, the *Eq. 3* was (Friedman et al., 1986):

$$\mathrm{FL}(\%) = \frac{\mathrm{Ip}}{\mathrm{Iu}} \times 100 \qquad \text{(Eq.3)}$$

where "Ip is the number of informants that suggested the use of a plant for a specific ailment and Iu is the total number of informants who mentioned that a species is used to treat any ailment".

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Results and discussion

Demographic characteristics of the informants

The distribution of informants by gender, educational level, age groups, and occupation were given in *Table 2*.

Table 2. *Demographic profile of informants*

Indicator		Number of Informants	Percentage (%)
Gender	Male	26	39
	Female	41	61
Educational level	Illiterate	1	1
	Primary school	63	94
	High school	3	5
Age groups	31-50	18	27
	51-70	32	48
	>70	17	25
Occupation	Farmer	24	36
	Retired	2	3
	Housewife	41	61

According to *Table 2*, a total of the 67 informants consists of 41 females, 26 males. Their age ranged from 36 to 87 years old, and the average age was 60. All female informants were housewives. Out of the 26 male informants, 24 were farmers and 2 were retired. Most informants (94%) have primary education.

Ethnobotanical knowledge and uses of plants

Data on the ethnobotanical use of a total of 85 plants belonging to 41 families were given in *Table 3*. Data on the traditional use was compared with primarily ethnobotanical studies in Turkey's Mediterranean and Aegean. Mediterranean countries were also considered in the comparison. It has been determined that plants were mostly used for medicinal (45) and food (44) purposes. These were followed respectively by handicrafts (11), fodder plants (8), building materials (7), cosmetics (5), spices (4), dyestuffs (3), and other purposes (toothpick, equipment, adhesive, superstition, silkworm care, cleaning materials, ornament, insect damage prevention) (18) (*Figure 3*).

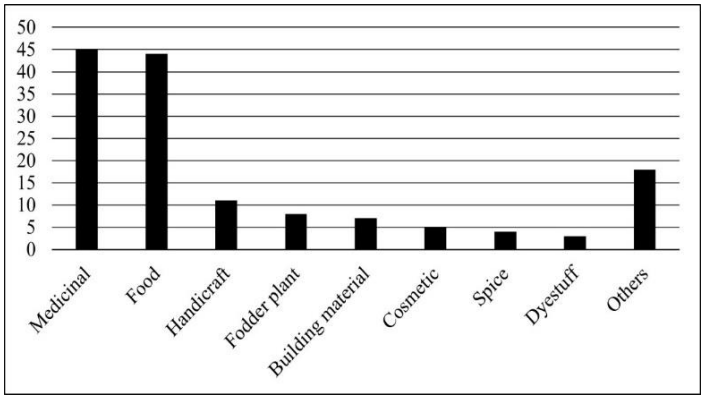

Figure 3. *Use purposes of the plants*

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Table 3. *Ethnobotanical uses of plants in Daçta (Muğla, Turkey)*

Plant species (voucher specimen)	Vernacular names	Parts used[a]	Purposes	Preparations	Traditional uses	UV
Amaranthaceae						
Amaranthus viridis L. (KATO 19670)	Sirken	L	food	cooking	It is eaten after roasting and added to pastries and omelets.	0.44
Amaryllidaceae						
Allium ampeloprasum L. (KATO 19671)	Çayır Soğanı, Körmen, Kördem	Ap	food	fresh, cooking	It is added to herbs roasting or pastries and is eaten raw with soups.	0.47
Anacardiaceae						
Pistacia lentiscus L. (KATO 19672)	Sakızlık, İlki, İlkicik	Br, St, Tw	handicrafts, building materials, food	boiling, drying	A garlic salad is made after boiling thin fresh twig. Branches are used to make arbors, baskets, and brooms. Since its wood is hard, it is used in making various hand tools.	0.15
Apiaceae						
Berula erecta (Huds.) Coville (KATO 19673)	Su Kazayağı, Karabaldır Otu	Ap	food	boiling	It is eaten after roasting and added to pastries.	0.23
Crithmum maritimum L. (KATO 19674)	Genevir, Kaya Koruğu, Kereviz Otu	Ap	food	boiling, pickle	It is boiled and consumed with hot oil and cheese, or with garlic yogurt. It is pickled.	0.38
Daucus carota L. (KATO 19675)	Diş Otu, Engin Otu	Fl, P	medicinal, toothpick	decoction	Its inhalation is used to treat inflammation in the mouth. Dried pedicels are used as a toothpick.	0.02
Ferula communis L. (KATO 19676)	Şavşır, Gabuş, Gamış, Kamış Otu	Ap	fodder plants, handicrafts, building materials	fresh, drying	Fresh is fed to goats for twin calving. When it is dried and ground, it is used as a plaster in fractures. Children make a toy out of the dried stem.	0.10
Foeniculum vulgare Mill. (KATO 19677)	Isıra, Arapsaçı, Rakı Otu, Rezene, Anason Otu	Ap	food, medicinal	cooking	It is eaten after roasting and added to pastries. When consumed as food, it is appetizing, strengthening, expectorant, digestive, curing diarrhea, and anthelminthic. It is used externally in the treatment of wounds and boils.	0.19
Opopanax hispidus (Friv.) Griseb. (KATO 19678)	Sarıot	Ap	food, medicinal	cooking	It is consumed as food to remove intestinal parasites from the body.	0.46

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Plant species (voucher specimen)	Vernacular names	Parts used[a]	Purposes	Preparations	Traditional uses	UV
Apocynaceae						
Nerium oleander L. (KATO 19679)	Zakkum, Ağcı Çiçeği	L	medicinal, equipment	drying, crushed	In the past, dried branches were used to string tobacco. The sap obtained from the oleander leaf is mixed with lime and aerolite. The mixture is used externally to treat scabies. (The mixture is kept in a container for 2 days)	0.14
Asparagaceae						
Asparagus acutifolius L. (KATO 19680)	Tilkicik, Tilki, Dilkicek, Tilkişen, Tilkimen	Tw	food, medicinal	cooking	Fresh shoots are roasted and then added to the omelet. It is also used externally for the treatment of scalp wounds.	0.47
Drimia maritima (L.) Stearn (KATO 19681)	Natariş Avusu, Pampampiyak, Tatayış Avusu	Bu, L	medicinal, adhesive, superstition	fresh	Bulbs are used externally for rheumatism pain. It is applied to the body against colds (caustic). Glue is made from the bulb. Used as an amulet. Olive oil is rubbed on the heated leaves and wrapped in the ear to treat mumps.	0.21
Asteraceae						
Achillea cretica L. (KATO 19682)	Civan Perçemi	Tw, Fl	medicinal	infusion	Infusion of flowers and twigs is used for the treatment of hemorrhoids and colds.	0.04
Anthemis altissima L. (KATO 19683)	Beyaz Papatya, Şifalı Papatya, Mayıs Papatyası	Fl	medicinal, cosmetics	infusion, decoction	Infusion of flowers is used to relieve the gas pains of babies and the treatment of stomach disorders. It is also used for eye drops in eye diseases. Decoction of flowers is used to nourish the hair and lighten the hair color.	0.23
Artemisia arborescens (Vaill.) L. (KATO 19684)	Pelin	L, Fl	medicinal	infusion	Infusion of flowers and leaves is used for blood pressure-lowering, appetizer, and diabetes treatment.	0.01
Calendula arvensis (Vaill.) L. (KATO 19685)	Aynısafa	Ap	medicinal	fresh	It is used externally for wound treatment.	0.07
Carlina gummifera (L.) Less. (KATO 19686)	Sakız Dikeni, Sakız Keyganası	R	medicinal, adhesive	latex	Gum of root is used for the treatment of teeth and gum diseases.	0.10
Chrysanthemum coronarium L. (KATO 19687)	Dallama	St, Fl	food	cooking	It has an appetizing feature. A salad is made with garlic and lemon.	0.36

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Plant species (voucher specimen)	Vernacular names	Parts used[a]	Purposes	Preparations	Traditional uses	UV
Cynara cardunculus L. (KATO 19688)	Peynir Otu, Mayaotu, Dikenli Enginar	Fl	food	drying	Used as rennet.	0.13
Glebionis segetum (L.) Fourr. (KATO 19689)	Sarı Papatya, Alimeç	St	food	fresh	It is eaten raw with meals.	0.30
Helichrysum stoechas subsp. *barrelieri* (Ten.) Nyman (KATO 19690)	Ayna Çiçeği, Sarı Çiçek	Fl	medicinal, cosmetics, superstition	infusion, fresh	Infusion of flowers is used to reduce kidney stones and relieve urinary tract disorders. It is added to bathwater to nourish hair and add vitality. Fresh flowers are hung on the door of houses in bunches to ward off snakes.	0.25
Notobasis syriaca (L.) Cass. (KATO 19691)	Yaban Kengeri	St	food	fresh	The stem is eaten fresh after the peel.	0.30
Scolymus hispanicus subsp. *hispanicus* (KATO 19692)	Kenger, Künger, Dikenkökü, Şevketibostan, Könger	Ap	food	fresh, cooking	The stem is eaten fresh or cooked after the peel. It is also eaten fresh when it is young.	0.36
Sonchus asper subsp. *glaucescens* (Jord.) Ball (KATO 19693)	Düdüklen	St	food	cooking	It is cooked after roasting in a pan.	0.10
Tragopogon dubius Scop. (KATO 19694)	Teke Sakalı	Ap	food	cooking	It is roasted by mixing with various herbs.	0.13
Brassicaceae						
Raphanus raphanistrum L. (KATO 19695)	Kızıl Turp, Turp Otu	L	food	cooking	It is roasted by mixing with various herbs.	0.38
Sinapis arvensis L. (KATO 19696)	Hardal Turpu, Turp Otu	L	food	fresh	Lemon and olive oil are added to the leaves and consumed as a salad.	0.38
Cactaceae						
Opuntia ficus-indica (L.) Mill. (KATO 19697)	Diken İnciri, Frenk İnciri, Fri İnciri, Mısır İnciri	Fr, L	food, medicinal	fresh, boiling	Fruits are eaten fresh. Boiled leaves are used externally for wound healing and rheumatism pain.	0.35
Capparaceae						
Capparis spinosa L. (KATO 19698)	Gebere, Kapari, Kebere, Sülük Dikeni	B, St, R	food, medicinal, superstition	pickle, fresh, decoction	Pickles are made from buds. In the past, the stem was used to extract leeches from animals' mouths. Decoction of roots is used for the treatment of the prostate.	0.42

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Plant species (voucher specimen)	Vernacular names	Parts used[a]	Purposes	Preparations	Traditional uses	UV
Caryophyllaceae						
Paronychia argentea Lam. (KATO 19699)	Dirfil	Ap	fodder plants	drying	It is used as livestock fodder in winter.	0.10
Silene vulgaris (Moench) Garcke (KATO 19700)	Kışyak, Gışıyak, Gavşık Otu	L	food	cooking, fresh	It is eaten by adding to the omelet or by adding garlic yogurt after cooking. Fresh leaves are also used in salads.	0.40
Cistaceae						
Cistus creticus L. (KATO 19701)	Pamuklan	L	medicinal	crushed	Crushed leaves are used externally in the treatment of tomies and wounds.	0.29
Cistus salviifolius L. (KATO 19702)	Pamuklan	L	medicinal	crushed	Crushed leaves are used externally in the treatment of tomies and wounds.	0.29
Cucurbitaceae						
Ecballium elaterium (L.) A.Rich. (KATO 19703)	Acı Kavun	Fr	medicinal	fruit juice	The fruit juice is mixed with olive oil and used in the treatment of sinusitis. It is also used externally in the treatment of jaundice.	0.31
Ericaceae						
Erica manipuliflora Salisb. (KATO 19704)	Piren, Püren	Fl, Ap	medicinal, dyestuffs, silkworm care	infusion, drying	Infusion of dried flowers is used as a sedative. The yellow paint obtained from the aerial parts is used in carpet and rug dyeing. It is also used in preparing a natural environment for silkworms to make cocoons.	0.10
Euphorbiaceae						
Euphorbia acanthothamnos Heldr. & Sart. ex Boiss. (KATO 19705)	Gavur Kefeni	Ap	Silkworm care	drying	It is used in preparing a natural environment for silkworms to make cocoons.	0.26
Fabaceae						
Anagyris foetida L. (KATO 19706)	Keçi Gevişi	Fl, Fr, Se	food, medicinal, superstition	fresh, drying	Seeds are divided into four and consumed internally by diabetics. When consumed orally, flowers leave a sweetish taste. Dried fruits are strung on a rope and hung as amulets on the shoulders of the kids.	0.13
Ceratonia siliqua L. (KATO 19707)	Keçi Boynuzu, Harnup	Fr, Br	food, medicinal, fodder plants,	fresh, drying, crushed	Fruits are eaten fresh or dried. A kind of dessert called "Gölemez" is made by adding crushed fruit and sugar to milk. This dessert	0.45

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Plant species (voucher specimen)	Vernacular names	Parts used[a]	Purposes	Preparations	Traditional uses	UV
			building materials, cleaning materials		is used to cure diarrhea and antitussive. Fruits are also used as livestock fodder. Branches are used in arbor construction. Leaves and branches are used in dishwashing.	
Lotus edulis L. (KATO 19708)	Konçalak	Fr	food	fresh	When consumed orally, fruits leave a sweetish taste.	0.24
Spartium junceum L. (KATO 19709)	Çalgılık, Katır Tırnağı, Katırkuyruğu	Ap	handicrafts, building materials	drying	It is used in making a fence, arbor, broom, and basket.	0.10
Trifolium nigrescens Viv. (KATO 19710)	Dirfil	Ap	fodder plants	drying	It is used as livestock fodder in winter.	0.10
Vicia faba L. (KATO 19711)	Bakla	Ap, Fr	food, fodder plants	cooking, drying	Various dishes with olive oil are made from fresh and dried fruits. Aerial parts are used as livestock fodder in winter.	0.16
Fagaceae						
Quercus coccifera L. (KATO 19712)	Kara Pınar	L	fodder plants	fresh	It is used as livestock fodder.	0.33
Geraniaceae						
Erodium cicutarium (L.) L Hér. (KATO 19713)	Beyaz Çiçekli İğnelik	L	food	cooking	It is eaten after roasting and added to pastries.	0.41
Erodium moschatum (L.) L Hér. (KATO 19714)	Pembe Çiçekli İğnelik	L	food	cooking	It is eaten after roasting and added to pastries.	0.41
Pelargonium quercetorum Agnew (KATO 19715)	Mis Çiçeği, Itır	L, Fl	food, medicinal	infusion, drying	Infusion of leaves is used to diabetes. It is used to flavor milk desserts. Dried flowers are used in making the dessert called Pelize. It is also used as a sweetener in making sherbet.	0.10
Hypericaceae						
Hypericum triquetrifolium Tura (KATO 19716)	Kızılcırık, Kızılcaerik, Zihircirik, Kılıçotu,	L, Br	medicinal, handicrafts	essential oil, decoction, drying	The essential oil of leaves is used for the treatment of wounds, burns, and tomies. Decoction of branches is used for the treatment of stomach disorders. It was used as a threshing broom in the past.	0.46
Iridaceae						

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Plant species (voucher specimen)	Vernacular names	Parts used[a]	Purposes	Preparations	Traditional uses	UV
Gladiolus illyricus W.D.J.Koch (KATO 19717)	Delikanlı Çiçeği	Ap	Ornament	fresh	It is used in ceremonial decorations and as an ornamental plant in homes. Youngsters used to present this plant to their loved ones as an expression of their feelings.	0.26
Juncaceae						
Juncus acutus L. (KATO 19718)	Gova, Govan, Süpürgelik	Ap	handicrafts, building materials	fresh, drying	It is used for local equipment such as the arbor, broom, basket, and rope production.	0.13
Lamiaceae						
Lavandula stoechas subsp. *stoechas* (KATO 19720)	Karabaş Otu, Karağan, Garanı, Karan	Fl	food, medicinal, insect damage prevention	infusion, drying	Jam is made from flowers. Infusion of flowers is used for the treatment of cardiovascular diseases, stomach pain, shortness of breath, hemorrhoids, and lowering blood fats. Incense is used for asthma treatment. It is put between woolen clothes against moth.	0.42
Mentha pulegium L. (KATO 19721)	Narpuz, Narpız	L	food, medicinal	infusion, fresh	Infusion of leaves is used for the treatment of colds, flu, and stomach pain. Fresh leaves are added to the salad.	0.24
Micromeria myrtifolia Boiss. & Hohen. (KATO 19722)	Kırkboğum Otu	L	medicinal	infusion	Infusion of leaves is used for the treatment of sore throat and stomach pain.	0.16
Origanum majorana L. (KATO 19723)	Mercanköşk, Sept Suyu	Fl, L	medicinal	infusion	Infusion of flowers and leaves is used for the treatment of stomach pain, menstrual pain, kidney and urinary tract diseases, and cough.	0.10
Origanum onites L. (KATO 19724)	İncir Kekiği, Bilyalı Kekik, Peynir Kekiği	Fl, L	medicinal, spice	infusion	The dried herb is used as a spice. Infusion of flowers and leaves is used for the treatment of colds, flu, and cough. It is also used as an antiflatulent.	0.50
Salvia fruticosa Mill. (KATO 19725)	Adaçayı, Ekmek Elması, Elmacık, Almecik	L	medicinal	infusion	Infusion of leaves is used in stomach disorders, shortness of breath, and as a diuretic. It is used as a mouthwash for gum and tonsil problems.	0.50
Satureja thymbra L. (KATO 19719)	Oğul Kekiği, Kara Kekik	L, Fl	medicinal, spice	infusion, drying	Dried flowers and leaves are used as a spice in meat dishes. Infusion of flowers and leaves	0.10

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Plant species (voucher specimen)	Vernacular names	Parts used[a]	Purposes	Preparations	Traditional uses	UV
					is used for the treatment of stomach disorders.	
Sideritis leptoclada O.Schwarz & P.H.Davis (KATO 19726)	Dağ Çayı, Kırkboğum Otu, Kızlan Çayı	Ap	medicinal	infusion	Infusion of aerial parts is used for the treatment of cold and stomach pain.	0.50
Teucrium polium L. (KATO 19727)	Tavşan Ütmeği, Pir Yavşağı,	Ap	medicinal, cosmetics	decoction	Decoction of aerial parts is used for the treatment of stomach pain, malaria, diabetes, anorexia, blood pressure, and as an anthelminthic in children. It is added to bathwater to nourish hair and add vitality.	0.33
Thymbra capitata (L.) Cav. (KATO 19728)	Eşek Kekiği	Fl, L	spice, medicinal	infusion, drying	Dried flowers and leaves are used as a spice in meat dishes. Infusion of flowers and leaves is used for the treatment of a cold.	0.27
Lauraceae						
Laurus nobilis L. (KATO 19729)	Defne, Çıbıklık	L	spice, cosmetics, insect damage prevention	drying	Leaves are used as a spice in fish and meat dishes, and as a fragrance in bathwater. It is placed among dried figs to prevent bug infestation.	0.24
Liliaceae						
Asphodelus aestivus Brot. (KATO 19730)	Çirgiş, Kirgiş, Ganlık	R, St	medicinal, handicrafts, building materials	crushed, drying	The crushed roots are used externally in the treatment of bee stings and wounds after the addition of olive oil. The liquid obtained from the crushed roots is used for the treatment of stomach pain, and as an adhesive in shoemaking. Toys and weathercock are made from their stems.	0.20
Lilium candidum L. (KATO 19731)	Dağ Zambağı	Fl	Ornament	fresh	It is used in ambient decorations.	0.25
Smilax aspera L. (KATO 19732)	Silcan	Tw	food	cooking	Fresh twigs are roasted and then added to the omelet.	0.10
Malvaceae						
Alcea heldreichii (Boiss.) Boiss. (KATO 19733)	Gül Hatmi, Deve Gülü, Gülhatmi, Gül Fatma	Fl	medicinal	infusion	Infusion of flowers is used for the treatment of asthma and as an antitussive.	0.10

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Plant species (voucher specimen)	Vernacular names	Parts used[a]	Purposes	Preparations	Traditional uses	UV
Malva sylvestris L. (KATO 19734)	Ebecik, Ebe Gümeci	Ap	food, medicinal	cooking, decoction	Local dishes are made from fresh twigs and leaves. Decoction of aerial parts is used to pass kidney stones, prevent itching on the skin, and cough.	0.33
Myrtaceae						
Myrtus communis subsp. *communis* (KATO 19735)	Yaban Mersini	Fr, L, Br	food, medicinal, handicrafts, superstition	fresh, drying, infusion, powder	Fruits are eaten. Infusion of leaves is used for the treatment of diabetes. Leaves are put in between food stored for winter and inside olive bins. The powdered leaves are used for baby rash. Branches are used in making baskets and arbors, and to decorate coffins in funerals. Branches are added to the bathwater so that newborn children don't smell.	0.21
Oleaceae						
Olea europaea L. var. *europaea* (KATO 19736)	Zeytin	Fr, L	medicinal, handicrafts, cosmetics, dyestuffs, building materials	fresh, infusion, oil	Fresh leaves are chewed to treat aphtha and sore throats. The compress is applied to the place where the nail sinks. Infusion of the leaf is used for the treatment of diabetes and blood pressure. Used for skin, and hair care. Used to produce green carpet dye. It is used to temper with whitewash. The broom is made from leafy branches. Oil for lamps is made from its fruits. This oil is used to extract sea urchin thistle.	0.50
Orchidaceae						
Serapias vomeracea (Burm.f.) Briq. (KATO 19737)	Köpek Kulağı	Tu	food	fresh, drying	It is eaten raw while its tubers are fresh. When dried, is made salep.	0.04
Oxalis pes-caprae L. (KATO 19738)	Ekşicek, Ekşikulak	Ap	food, dyestuffs, fodder plants	fresh	Used in salads, and to make henna. It is used as animal fodder. Especially for increasing egg production in fowl. In the past, used to shine shoes.	0.29
Papaveraceae						

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Plant species (voucher specimen)	Vernacular names	Parts used[a]	Purposes	Preparations	Traditional uses	UV
Papaver rhoeas L. (KATO 19739)	Zemperlik, Gelincik	L, Fl	food, medicinal	cooking, infusion	Leaves are eaten after roasting and added to pastries and omelets. Infusion of flowers is used for the treatment of cough.	0.36
Plantaginaceae						
Plantago lanceolata L. (KATO 19740)	Sinir Otu	Ap	medicinal	crushed	Crushed leaves are used externally on body aches and wounds.	0.21
Plumbaginaceae						
Limonium sinuatum (L.) Mill. (KATO 19741)	Deniz Otu, Mor Çiçek, Dilicek, Deniz Lavantası	Ap	food, superstition	fresh, boiling	Fresh or boiled aerial parts are added to garlic yogurt and pastries. Flowers are used in home decorations.	0.19
Poaceae						
Arundo donax L. (KATO 19742)	Çığ, Kargı	Ap	handicrafts, fodder plants	drying, fresh	The dried aerial parts are used in making baskets, arbor, end-blown flute, and locally for spinning wool and cotton. Fresh is also used as livestock fodder.	0.13
Polygonaceae						
Emex spinosa (L.) Campd. (KATO 19744)	Ispanak, İlaboda	L	food	cooking	Leaves are used in local dishes.	0.42
Rumex amanus Rech.f. (KATO 19745)	Labada, İlaboda	L, Se	food, medicinal	cooking, fresh	Leaves are used in local dishes. Fresh seeds are used to cure diarrhea.	0.15
Rumex bucephalophorus L. (KATO 19746)	Kuzukulağı	L	food	fresh	Fresh leaves are used as a garnish or in salads.	0.27
Portulacaceae						
Portulaca oleracea L. (KATO 19747)	Semiz Otu	Ap	food	fresh, cooking	Fresh leaves are used in salads and tzatziki. Also, an olive oil dish is made.	0.33
Rafflesiaceae						
Cytinus ruber (Fourr.) Fritsch (KATO 19748)	Gürlencik, Yer Narı, İnek Memesi, Kızılcık, Saya	Ap	food	fresh	Leaves are consumed fresh.	0.10
Rosaceae						
Rubus sanctus Schreb. (KATO 19749)	Böğürtlen, Orman Üzümü	L, Fr, Tw	food, medicinal	fresh, boiling, cooking	Fresh fruits are used to cure diarrhea, throat infection, and aphonia. Jam is also made from its fruit. Infusion of leaves is used for the treatment of diabetes. A salad with oil	0.33

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Plant species (voucher specimen)	Vernacular names	Parts used[a]	Purposes	Preparations	Traditional uses	UV
					and lemon is made after boiling thin fresh twigs. Fresh twigs are roasted and then added to the omelet.	
Santalaceae						
Osyris alba L. (KATO 19750)	Süpürge Çalısı	Ap	handicrafts	drying	Broom is made from leafy branches.	0.30
Solanaceae						
Datura innoxia Mill. (KATO 19751)	Zemberek, Boruçiçeği, Patlıcan Çiçeği, Sarhoş Çiçeği	Fl	medicinal	drying	Dried flowers are smoked as a cigarette in the treatment of asthma.	0.11
Mandragora autumnalis Bertol. (KATO 19752)	Adam Otu	Fr	superstition	fresh	It is believed to encourage chickens to brood. If the fruits are eaten, it causes short-term hallucinations.	0.10
Urticaceae						
Urtica pilulifera L. (KATO 19753)	Erkek Isırgan, Cızgan, Isırgan, Dalaygan, Dalayan	Ap	food, medicinal	cooking, fresh, decoction	It is eaten roasted and added to the pastries. It is used externally in the treatment of rheumatism. Decoction of aerial parts is used for the treatment of urinary tract diseases.	0.30
Urtica urens L. (KATO 19754)	Dişi Isırgan, Cızgan, Isırgan, Dalaygan, Dalayan	Ap	food, medicinal	cooking, fresh, decoction	It is eaten roasted and added to the pastries. It is used externally in the treatment of rheumatism. Decoction of aerial parts is used for the treatment of urinary tract diseases.	0.30
Verbenaceae						
Vitex agnus-castus L. (KATO 19755)	Hayıt	L, Se, Br	medicinal, handicrafts, other	infusion, decoction, crushed, drying	Infusion of leaves is used to prevent itching. Crushed leaves are used to treat fungus on the fingers. Crushed seed is used against snake and scorpion bites, and rheumatism pain. Decoction of seeds is used for infertility in women. Seeds are put into shoes against bad odors. Branches are used to make baskets.	0.29

[a] Parts used: Ap-Aerial parts, B-Bud, Br-Branch, Bu-Bulb, Fl-Flower, Fr-Fruit, L-Leaf, P-Pedicel, R-root, Se-Seed, St-Stem, Tu-Tuber, Tw-Twig

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The most common traditional preparation method of plants material was fresh (34 taxa), followed by drying (26 taxa), cooking (23 taxa), infusion (20 taxa), decoction (9 taxa), crushing (7 taxa), boiling (6 taxa), oil (2 taxa), pickle (2 taxa), powder (1 taxon), latex (1 taxon), fruit juice (1 taxon) (*Figure 4*). The infusion method is mostly preferred for the preparation of plants used for medicinal purposes, followed by decoction.

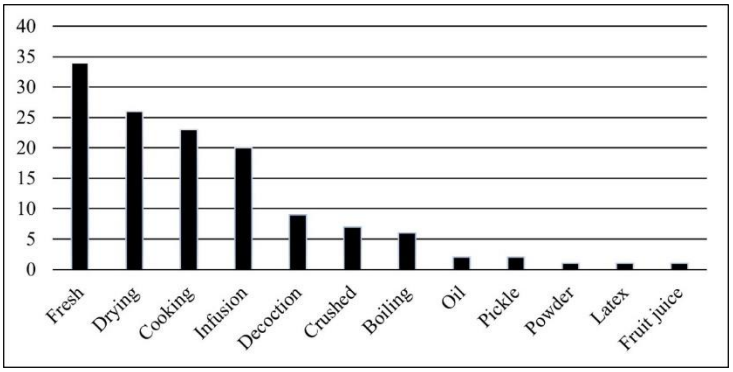

Figure 4. *Traditional preparation methods*

The most used families were Asteraceae (13 taxa), Lamiaceae (10 taxa), Apiaceae (6 taxa), and Fabaceae (6 taxa) (*Figure 5*). Similar results were obtained in studies in districts close to the research area (Bruni et al., 1997; Agelet and Vallès, 2003; Ertuğ, 2004; Ugulu et al., 2009; Bulut and Tuzlaci, 2013; Gürdal and Kültür, 2013; Fakir et al., 2016; Kawarty et al., 2020). While Lamiaceae ranked first in Greece, Asteraceae ranked first in Spain and Italy, which were among other Mediterranean countries (Guarrera and Lucia, 2007; Cornara et al., 2009; Benítez et al., 2010; Axiotis et al., 2018; Tsioutsiou et al., 2019).

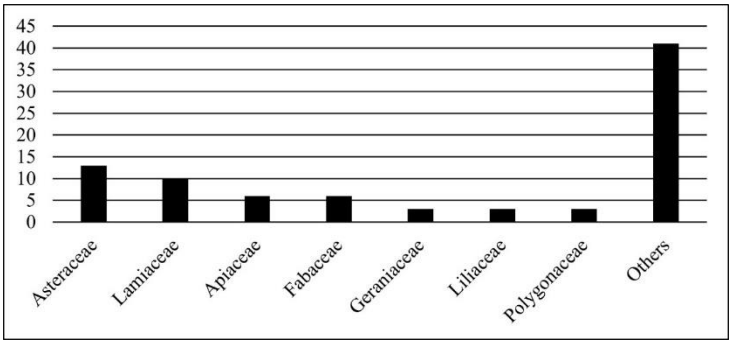

Figure 5. *Most preferred families*

Commonly used parts of plants for different purposes were leaves (31 taxa), aerial parts (29 taxa), flowers (19 taxa), fruits (10 taxa), and other parts such as seeds, branches, stems, tubers, bulbs, buds, branches, roots, pedicels (*Figure 6*). In many studies, the leaves and aerial parts of the plants were ranked in the 1st and 2nd ranks. The main reason for this is that they are easily collected and can be stored for a long time without deterioration (Sargin, 2015; Rehman et al., 2017; Chaachouay et al., 2019a; Polat, 2019).

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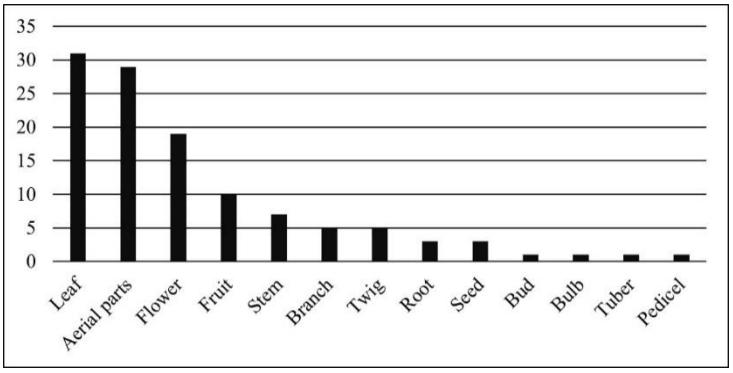

Figure 6. *Plant parts used*

Plants used as food mostly belonged to the Asteraceae family (7 taxa). The same result was obtained in a similar study conducted in Anatolia (Ertuğ, 2000; Pieroni, 2000; Rivera et al., 2005; Dogan, 2012; Saraç, 2013; Yeşil and İnal, 2019; Yeşil et al., 2019; Demir, 2020). Of the other plants used in food, 4 belonged to Apiaceae, 4 to Fabaceae, and 3 to Geraniaceae.

As in Anatolia, most of the plants used in handicrafts are used to make items such as baskets, brooms, arbors (10 taxa). In general, the stem or branches of these plants used in handicrafts are used. *Olea europaea* var. *europaea* was determined as the most used plant. *Pistacia lentiscus*, *Spartium junceum*, *Juncus acutus*, *Myrtus communis* subsp. *communis*, *Arundo donax*, and *Vitex agnus-castus* were also other plants that were frequently used in handicrafts such as baskets, broom, and arbor making.

Eight different plant taxa were used as animal fodder. Some forage crops such as *Vicia faba*, *Oxalis pes-caprae*, which are cut and dried during summer and fall and used as winter fodder, are also consumed by local people as fresh.

There were more than 100 plants that were used as dyes in handicrafts in Turkey (Doğan et al., 2003; Özgökçe and Yılmaz, 2003). Among these plants, *Erica manipuliflora* and *Olea europaea* var. *europaea* were used for carpet and rug dyeing in Datça.

Spice plants in the region (*Origanum onites*, *Satureja thymbra*, *Thymbra capitata*, and *Laurus nobilis*) were like the Mediterranean countries and other regions of Turkey (Ertuğ, 2004; Özgökçe and Özçelik, 2004; Kendir and Güvenç, 2010; Gürdal and Kültür, 2013; Yeşilyurt et al., 2017; Kizilarslan-Hançer et al., 2020). However, local people widely use these spice plants in meat dishes.

The use of *Drimia maritima* leaves in the treatment of mumps has been recorded for the first time. Bulbs were also used for medical purposes such as a diuretic, cardioactive, headache, expectorant, and rheumatic pain relief in Turkey (Baytop, 1999; Comlekcioglu and Karaman, 2008; Eşen, 2008; Akan et al., 2018).

Aerial parts of *Opopanax hispidus* were consumed as food to remove intestinal parasites from the body. Similar usage has not been found in the literature. In the literature, it was found that *O. hispidus* was used for different purposes such as antiseptic (Amiri and Joharchi, 2016), infertility in women (Tuzlacı and Erol, 1999), hemorrhoids (Gürhan and Ezer, 2004), and epilepsy (children) (Sahranavard et al., 2014).

Two endemic plant species have been identified in the study area. One of those was *Sideritis leptoclada* and the other was *Rumex amanus*. The use of *R. amanus* seeds to cure diarrhea has not been mentioned in the literature.

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Data analysis

In the study, it was determined that 45 plants were used for medicinal purposes. According to the information obtained from informants, the ailments were classified into 8 categories and the ICF values of each were indicated in *Table 4*.

Table 4. *Informant consensus factor (ICF) for each ailment*

Ailment categories	Number of citations (Nur)	Number of taxa (Nt)	ICF[b]
Cardiovascular disease	57	13	0.79
Diabetes	24	7	0.74
Gastrointestinal disease	87	27	0.70
Kidney disease	23	9	0.64
Respiratory disease	113	26	0.78
Rheumatism	21	5	0.80
Skin disease	102	33	0.68
Urinary tract diseases	17	8	0.56

[b]Informant consensus factor has been shown as ICF or FIC in previous studies (Polat and Satıl, 2012; Madani et al., 2017; Hossain and Rahman, 2018; Chaachouay et al., 2019a; Karaköse et al., 2019)

Local people mentioned medicinal plants most commonly for the treatment of respiratory diseases, followed by skin diseases, gastrointestinal diseases, cardiovascular diseases, diabetes, kidney diseases, rheumatism, and urinary tract diseases respectively. Rheumatism has the highest ICF value (0.80). Plants reported to be used by informants for the treatment of this disease are *Drimia maritima*, *Opuntia ficus-indica*, *Urtica pilulifera, Urtica urens*, and *Vitex agnus-castus.* Cardiovascular diseases have the 2nd highest ICF value (0.79), respiratory diseases have the 3rd highest ICF value (0.78), followed by diabetes 0.74 ICF, gastrointestinal diseases 0.70 ICF, skin diseases 0.68 ICF, kidney diseases 0.64 ICF. The lowest ICF value with 0.56 belongs to urinary tract diseases.

When the studies from west of Turkey in which the informant consensus factor (ICF) was calculated were examined, it was seen that in the study in Balıkesir by Polat and Satıl (2012), anorexia and hypertension stabilizer have the highest 0.87 ICF, followed by hemorrhoids 0.80 ICF. In the study in Turgutlu (Manisa) by Bulut and Tuzlaci (2013), the digestive system has the highest 0.73 ICF, followed by the skin and subcutaneous tissues 0.70 ICF and the respiratory system have 0.64 ICF. In the study in Marmaris (Muğla) by Gürdal and Kültür (2013), rheumatism has the highest 0.73 ICF, followed by diabetes 0.57 ICF and urinary disease have 0.56 ICF. In the study in İzmir by Ugulu et al. (2009), cold and flu have the highest 0.82 ICF, followed by respiratory tract diseases 0.73 ICF and stomach disorders have 0.68 ICF.

When looking at ICF values in neighboring Mediterranean countries; Tsioutsiou et al. (2019), the highest ICF value in Greece was found to be 0.85 for respiratory diseases. Chaachouay et al. (2019b), the highest ICF value in Morocco was found to be 0.98 for rheumatism. These results were like the current study. The high ICF values, that was, close to one, suggest that the medicinal plants used in the treatment of certain diseases were more effective.

The fidelity level (FL) of the 14 most important plant taxa was between 61 to 100% (*Table 5*). The high FL of a plant indicates the prevalence of a specific disease in a country

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and the utilization of plants by the local people to treat it (Srithi et al., 2009; Bibi et al., 2014; Umair et al., 2017.

Table 5. *Medicinal species for the most frequently reported ailment categories based on the fidelity level (FL) index*

Taxa	Uses	FL (%)
Origanum onites *Salvia fruticosa* *Sideritis leptoclada*	Respiratory disease	100
Olea europaea var. *europaea*	Diabetes	96
Asparagus acutifolius	Skin disease	90
Opopanax hispidus	Intestinal parasites	88
Hypericum triquetrifolium	Wounds, burns, and tomies	84
Ceratonia siliqua	Antitussive	84
Capparis spinosa	Prostate	82
Lavandula stoechas subsp. *stoechas*	Asthma	79
Malva sylvestris	Kidney stones	67
Ecballium elaterium	Jaundice	63
Urtica pilulifera *Urtica urens*	Rheumatism	61

The plants in the study area with a high FL were *Origanum onites*, *Salvia fruticosa*, *Sideritis leptoclada* for respiratory disease (100), *Olea europaea* var. *europaea* for rheumatism (96), *Asparagus acutifolius* for skin disease (90), *Opopanax hispidus* for intestinal parasites (88), *Hypericum triquetrifolium* for wounds, burns, and tomies (84), *Ceratonia siliqua* for antitussive (84), *Capparis spinosa* for prostate (82), *Lavandula stoechas* subsp. *stoechas* for asthma (79), *Malva sylvestris* for kidney stones (67), *Ecballium elaterium* for jaundice (63), and *Urtica* spp. for rheumatism (61) (*Table 5*). Another *Origanum* species in northeastern Turkey (*Origanum vulgare* FL:90) has been reported to be used for similar purposes (Eminağaoğlu et al., 2017). In another study, *Origanum onites* with the highest FL value was reported to be used against gastralgia disorders (Dogan and Ugulu, 2013).

Origanum onites (0.50), *Salvia fruticosa* (0.50), *Sideritis leptoclada* (0.50), *Olea europaea* var. *europaea* (0.50), *Allium ampeloprasum* (0.47), *Asparagus acutifolius* (0.47), *Opopanax hispidus* (0.46), *Hypericum triquetrifolium* (0.46), *Ceratonia siliqua* (0.45), *Amaranthus viridis* (0.44), *Capparis spinosa* (0.42), *Lavandula stoechas* subsp. *stoechas* (0.42) had the highest UVs (*Table 3*). The results showed that the medicinal plants in the region were widely used by local people to treat various ailments and were the first species that come to mind by local people in the treatment of a particular disease. It was seen that the plants with the highest UV values were medicinal plants and those used for food purposes. The lowest UV of *Daucus carota* (0.02), *Achillea cretica* (0.04), and *Serapias vomeracea* (0.04) maybe because they were less common in the region and therefore, they low ethnobotanical use. UV results of the study were comparable with those previously reported from Turkey. It has been reported that *Origanum onites* was used in the treatment of abdominal ache, toothache, headache, diabetes, high cholesterol, and itching (Polat and Satıl, 2012; Gürdal and Kültür, 2013; Akbulut et al., 2019). *Salvia fruticosa*, another type with high FL value, has been reported to exhibit hair tonic, anti-dandruff, weight loss, memory improvement, colic rates (Abdelhalim et al., 2017).

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Another medicinal plant, *Olea europaea* var. *europaea* has been studied against diabetes (Bulut and Tuzlaci, 2013), hypotensive, ripe pimples, constipation, small burns, astringent (Passalacqua et al., 2007), cholesterol, cardiovascular problems, and dental care (Mechchate et al., 2020). When the results of the study were evaluated, it was understood that plants with high FL values and plants with high UV values overlap. These results confirmed the high FL and UV of these species in the study area.

Conclusion

Research on traditional botanical knowledge provides first-hand information about the use and management of natural resources of cultures. Recording of most unwritten traditional information is essential for the continuity of cultural heritage. Datça is one of the transit ports from the Mediterranean to Anatolia and even to Asia. Therefore, it is a favorable resource area for ethnobotanical research.

A total of 85 plant taxa traditionally used in the peninsula have been identified and recorded. The medicinal plant category, together with the edible plant category, constitutes the highest percentage among useful plants. The fact that Datça peninsula is a more virgin area compared to its surroundings encouraged the local people to use plants for food purposes.

When the traditional uses of plants are considered one by one; the prominent taxa are *Origanum onites*, *Salvia fruticosa*, *Sideritis leptoclada* for medicinal purposes, *Asparagus acutifolius, Chrysanthemum coronarium, Crithmum maritimum* for food purposes, *Olea europaea* var. *europaea* for handicrafts, *Erica manipuliflora* for coloring, and *Teucrium polium* for cosmetic purposes. If *Olea europaea* var. *europaea* draws attention as the taxon that local people benefit the most with different uses such as medical, food, handicraft, building material.

The medicinal uses of *Drimia maritima*, *Rumex amanus*, and *Opopanax hispidus*, which were recorded for the first time in the current study, should be subject to new research. Local people should be informed about endemic plants (*Sideritis leptoclada* and *Rumex amanus*) in the region. Cultivation of these species for consumption and use should be encouraged.

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APPENDIX

Interview Questions

1. Name, surname, age, gender, occupation, educational level, permanent address.
2. What is the vernacular name of the plants?
3. For which purposes do you use the plants? (medicinal, food, construction material, equipment, etc.)
4. What plant do you prefer most for a particular disease?
5. How often do you use the plants?
6. What plants do you use for customs?
7. What plants do you use for livestock and fowl?
8. Which parts of the plant do you use? (aerial parts, leaf, fruit, flower, root, etc.)
9. How do you prepare the plants? (infusion, decoction, crushed, powder, fresh, drying, boiling, cooking, etc.)

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SPATIAL DISTRIBUTION AND ASSOCIATION PATTERNS OF *HOPEA PIERREI* HANCE AND OTHER TREE SPECIES IN THE PHU QUOC ISLAND EVERGREEN BROADLEAVED FOREST OF VIETNAM

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Abstract. The present study was conducted to better understand the ecology, spatial patterns and associations of *Hopea pierrei* – a vulnerable plant in the Vietnam Red Book and on the IUCN's Red List – in the medium natural evergreen broadleaved forest of Phu Quoc National Park, Kien Giang Province, Vietnam. Data was collected from all woody tree individuals with a diameter at breast height (dbh) ≥ 2.5 cm in three 1-ha plots (100 m × 100 m). The spatial point-pattern analysis method was used to analyze the data with Programita Noviembre 2018 and R 4.1.1 software. Our findings suggested that *H. pierrei* was aggregated and randomly distributed at various scales in the area where it appeared. The spatial patterns of *H. pierrei* were influenced by environmental heterogeneity. The intraspecific associations of *H. pierrei* were mainly attraction associations, while its interspecific associations with sixteen dominant species were mainly independent. Processes such as seed dispersal and self-thinning are the main mechanisms underlying the spatial patterns and associations of *H. pierrei*. Based on the present findings, it is possible to regulate density and choose an appropriate planting-hole distance when restoring forests or planting new forests with *H. pierrei* and dominant species with which it grows.
Keywords: *threatened species, point-pattern, interspecific association, ecological characteristics, conservation planning*

Introduction

The spatial patterns of species and interspecies interactions at different spatial scales have been considered evidence reflecting the dynamics of stands over time (Hai and Bang, 2020). Over the past 50 years, ecologists around the world have proposed many hypotheses, such as Neutral theory, Niche theory, and the Janzen-Connell hypothesis, to explain the spatial pattern and association of forest tree species (Quy et al., 2021a). Among the proposed hypotheses, Neutral theory holds that all individuals in a community are strictly equivalent regarding their prospects of reproduction and death (Hubbell, 2001; Chave, 2004). Niche theory emphasizes that different species are best suited to different habitats in which they are completely dominant and more abundant than in less suitable habitats (Hubbell, 2005). Janzen (1970) and Connell (1971) hypothesized that host-specific pests reduce recruitment near conspecific adults, thus

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freeing up space for other plant species. Although different theories can explain the mechanism of species coexistence on a certain spatial and temporal scales, no theory can explain the coexistence of species in different communities on a global scale (Yao et al., 2018; Liza et al., 2014). Therefore, studying the mechanisms of plant species coexistence is still very challenging and requires further in-depth research.

Dien and Hai (2016) suggested that the spatial distribution pattern of a forest tree population can be determined as aggregated, random or regular based on the coordinates of tree individuals in the population. According to Tilman (2004), the spatial patterns of forest trees are explained by the influence of environmental heterogeneity and species equilibrium status depending on dispersal limitation and plant–plant interactions, such as competition or facilitation. In a tropical rainforest, intra- and interspecific interactions are more complicated than in other forest types because this forest type has a very diverse tree species composition but a low density of each species (Dien and Hai, 2016; Quy et al., 2021a). Spatial point-pattern analysis (SPPA) in ecological research uses forest tree individuals with coordinate pairs to represent points on a two-dimensional spatial plane from which we can learn the arrangement or spatial patterns and associations among them in a space bandwidth (Franklin et al., 2010; Ben-Said, 2021). However, the application of SPPA to explain the role of theoretical ecological processes that affect the spatial distribution and association patterns of forest tree species is still limited, especially in Vietnam as well as many other tropical countries in Southeast Asia (Ben-Said, 2021; Quy et al., 2021a).

The Dipterocarpaceae family has 16 genera with 515 species; there are 13 genera with 470 species in Asia (Ashton, 1982; Bawa, 1998). The tree species of the Dipterocarpaceae family play important ecological and economic roles in Southeast Asian tropical rainforests (Trung, 1999; Turner, 2001). In Vietnam, Dipterocarpaceae species are mainly distributed in the South and Central Highlands regions (Con, 1991; Linh, 1996). The studies of Trung (1985) and Them (1992) showed that a region of South Vietnam has an evergreen forest type belonging to the flora of Malaysia - Indonesia with tree species of the Dipterocarpaceae family dominating; these tree species are mainly large timber trees, often used in construction, house building, handicraft production, and household appliances as well as for export. Dipterocarpaceae species are usually distributed at altitudes below 1,000 m a.s.l (Bunyavejchevin, 1983; Loc and Hiep, 1987). Forty-two species of the Dipterocarpaceae family have been recorded in Vietnam, many of which are of high conservation value and are included in the Vietnam Red Book (2007) and the IUCN's Red List (Nghia, 2003; Long et al., 2018).

Hopea pierrei Hance belongs to the genus *Hopea* of the Dipterocarpaceae family (Hop, 2002; Huy et al., 2012). This species is naturally distributed in Southeast Asian countries, including Cambodia, Laos, Malaysia, Indonesia, Thailand, and Vietnam. *H. pierrei* grows and develops well on red–yellow Feralit soil, but its ability to adapt to harsh environmental conditions is poor. *H. pierrei* is very sensitive to toxic chemicals (Appanah et al., 1998); therefore, *H. pierrei* forests in South Vietnam, Laos, and Cambodia were devastated during the Vietnam War (Ho, 1999; Ban et al., 2007). Adult trees of *H. pierrei* produce many fruits, the ability to regenerate by seeds is good, and juvenile trees are shade tolerant, whereas adult trees are light demanding (Ho, 1999; Hop, 2002). *H. pierrei* wood is hard, fine-grained, very durable in the air, not subject to termites, often used in construction and shipbuilding, and very valuable in the domestic consumption and export markets (Hop and Quynh, 2003). *H. pierrei* is a native species

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of Vietnam that is naturally distributed in some provinces, such as Thua Thien Hue, Dak Lak, Kien Giang, and Ho Chi Minh provinces (Lan et al., 2006). The number of *H. pierrei* individuals in the wild is low; the distribution area is narrow due to the influence of previous wars and illegal logging (Ban et al., 2007). *H. pierrei* is one of the twenty-eight species of plants prioritized for conservation in Vietnam and is listed in Vietnam Red Book (2007) and on the IUCN's Red List (2017) as "Vulnerable" due to many threats (Ban et al., 2007). Research on the ecological characteristics of *H. pierrei* in Vietnam and around the world is relatively limited, and most previous studies focused on its geographical distribution and description for species identification. There have been no studies using SPPA method to assess the ecological characteristics of this species in Southeast Asian tropical rainforests in general and in Vietnam in particular. Therefore, this study was carried out to provide in-depth scientific information for the conservation of the species *H. pierrei*.

We used SPPA method to explore the spatial patterns and associations of *H. pierrei* in the medium natural evergreen broadleaved forest belonging to Phu Quoc National Park, Phu Quoc Island, Kien Giang Province, Vietnam. The present study was focused on four (4) research questions: (i) In Phu Quoc Island evergreen broadleaved forests, which species often grow with *H. pierrei*, and what are their ecological roles? (ii) Are the spatial patterns of *H. pierrei* affected by environmental conditions? (iii) What is the difference between the intra- and interspecific associations of *H. pierrei*? (iv) Which underlying ecological mechanisms explain the spatial patterns and associations of *H. pierrei* and other tree species?

We will elucidate the ecological processes that help *H. pierrei* dominate in the area where it is distributed, and the findings will also provide information about the ecological characteristics of *H. pierrei* to serve as a basis for developing silvicultural measures to restore forests and expand the distribution area of this species.

Materials and methods

Study area

The study was conducted from March 2021 to July 2021 with three field surveys in Phu Quoc National Park, Phu Quoc Island, Phu Quoc District, Kien Giang Province, Vietnam (10°12'7" to 10°27'2" North latitude, 103°50'4" to 104°04'40" East longitude) (*Fig. 1)*.

The total forest land area under management by Phu Quoc National Park is 29,135.9 ha. Phu Quoc Island is located in a tropical monsoon climate zone with an equatorial nature but is strongly influenced by ocean dynamics, with two distinct seasons: the rainy season (from May to October) and the dry season (from November to April of the next year). The average temperature is 27.1 °C, while the average rainfall is 3,037 mm. The average wind speed is 3.9 m/s, with two main wind directions during the year: the prevailing East-North monsoon in the dry season (wind speed 2.8-4.0 m/s) and the West-South wind prevailing in the rainy season (wind speed 3.0-5.1 m/s). Strong winds on the island often occur in June-August, and the highest wind speed is up to 31.7 m/s. The elevation of the terrain decreases from north to south and west to east, ranging from 20-603 m a.s.l., with slopes of 5-45° (Quan et al., 2014).

The study plots are located in the restricted area of Phu Quoc National Park, which is less affected by human activities than other areas. Coordinates of the plots were as follows (*Fig. 2*): Plot 1 (Plot 1 - P1), 10°26'12.50" N latitude, 103°59'42.52" E

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longitude; Plot 2 (Plot 2 - P2), 10°23'2.80" N latitude, 104° 0'47.03" E longitude; and Plot 3 (Plot 3 - P3), 10°20'50.43" N latitude, 104° 3'17.40" E longitude. The dominant plant species in the study area include *Hopea pierrei, Diospyros venosa, Syzygium cuminii, Memecylon ligustrinum, Garcinia delpyana, Olea dioica, Garcinia vilersiana, Syzygium cuminii, Syzygium zeylanicum,* and *Diospyros sylvatica* (Quan et al., 2014).

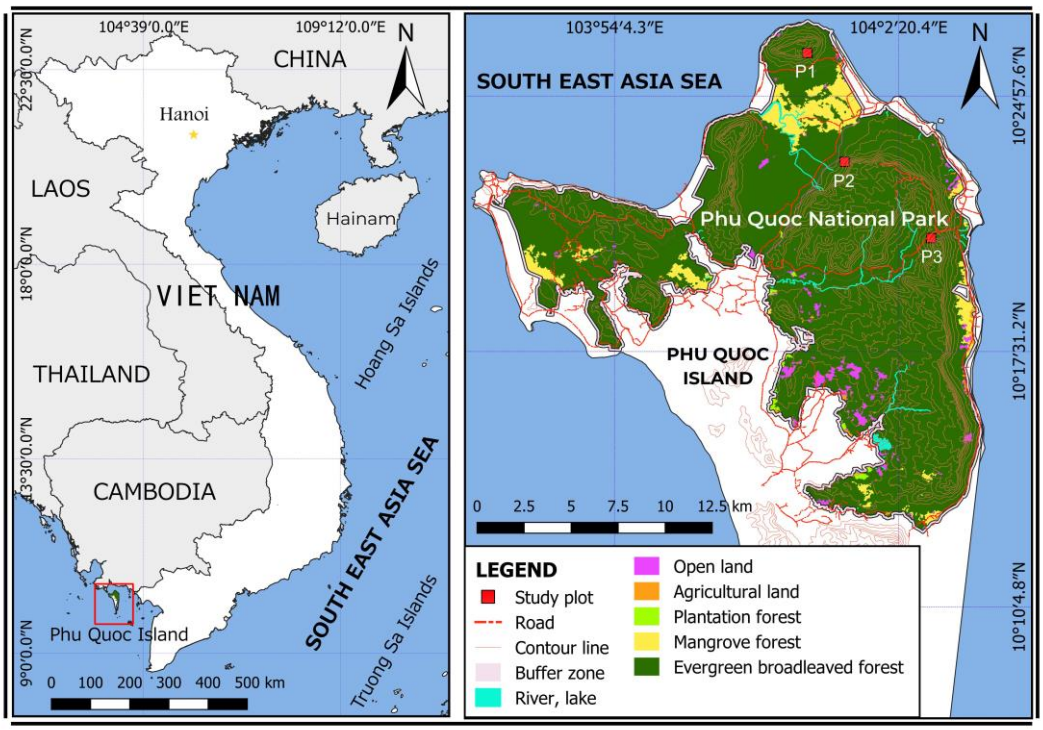

Figure 1. *Map of the study area. Maps of Vietnam (left) and Phu Quoc Island (right)*

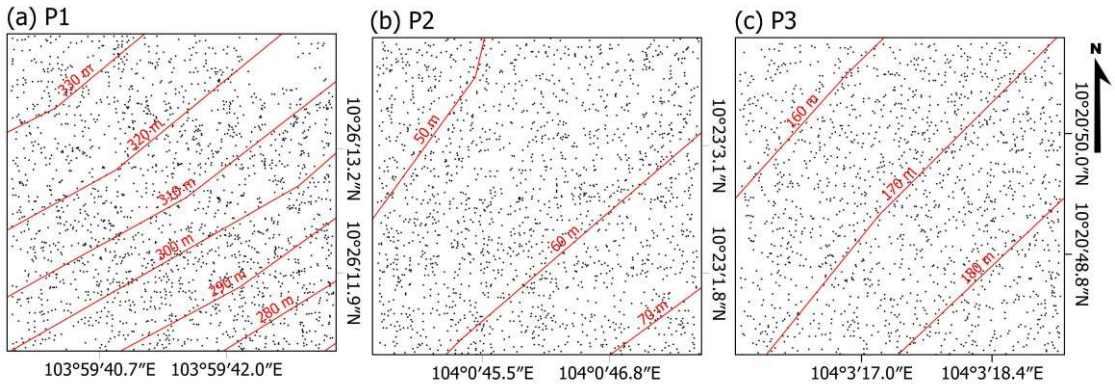

Figure 2. *Distribution maps of investigated trees in the three 1-ha study plots with 10 m contour lines of altitude (red lines)*

Data collection

In the study area, we selected stands where *H. pierrei* trees were concentrated and dominant to establish three 1-ha plots (100 m × 100 m). Using the square grid method, each plot was divided into 25 subplots; each subplot had an area of 400 m^2 (20 m ×

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20 m) to facilitate the survey and data collection and avoid missing investigated trees. In the subplot, information was collected for all tree individuals with a diameter at breast height (diameter at breast height - dbh) ≥ 2.5 cm, including the name of the tree species; dbh was measured by a diameter caliper, canopy diameter was measured with a measuring tape in two directions (east-west and south-north), and the overall height of trees (overall height of trees - H) was measured by a Blume-Leiss meter. The intersection point between the two edges of the plot in the north and south directions was taken as the origin according to the reference system, and the relative coordinates of each tree in the plot were determined by a laser distance meter (Leica Disto - D2) and compass.

Identification of tree species

The tree species were identified by the comparative morphology method, and the references used included Plants of Vietnam (Ho, 1999) and Vietnam Forest Trees (Hop, 2002). The scientific names of the species were corrected according to Kew Royal Botanic Gardens (http://www.plantsoftheworldonline.org), the World Flora Online (http://104.198.148.243).

All individual trees were assigned to one of three basic life-history stages, namely, juvenile (dbh < 5 cm), subadult (5 cm ≤ dbh < 10 cm), or adult (dbh ≥ 10 cm). The identification of trees was performed in the field during the plot inventory. When a tree could not be identified to species in the field, voucher specimens were collected, prepared, and subsequently identified by taxonomic specialists after the fieldwork.

Data analysis

Different parameters were calculated as below:

$$\mathbf{d = \frac{n_i}{S}} \quad \text{(Eq.1)}$$

where d is the stem density of species i (the number of individual trees ha^{-1}), n_i is the total number of individual trees calculated for species i (the number of individual trees) and S is the study plot area (in ha) (Brower et al., 1997).

$$\mathbf{BA = \pi \times r^2 = \frac{3.142 \times dbh^2}{200^2}} \quad \text{(Eq.2)}$$

where BA is the tree basal area (m^2), r is the radius and dbh is the diameter at breast height (cm) (Brower et al., 1997).

$$\mathbf{V = H \times BA \times F} \quad \text{(Eq.3)}$$

where V is the tree volume (m^3), H is the overall height of the tree (m), and F is the form factor.

Dominance was calculated by the important value index (IVI%) of the species through the number of individual trees and tree basal area. IVI% was calculated for all tree species in the stand with a dbh ≥ 2.5 cm according to the following formula:

$$\mathbf{IVI\% = \frac{RD + RBA}{2}} \quad \text{(Eq.4)}$$

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where RD is the relative density and RBA is the relative basal area. The relative basal area is defined as the total basal area of species i as a percent of the total basal area of all species. The ecological value of a species in a forest stand is obtained by determining its importance value index (Curtis and Macintosh, 1951).

According to Marmillod (1982), only tree species with an IVI% > 5% are truly ecologically significant in the stand. On the other hand, according to Trung (1978), a species group with a total value of IVI% ≥ 50% for the total number of species can be considered the dominant tree species group in the stand.

The evergreen broadleaved forest types of the study area were determined based on the volumes of the stand and Circular No. 33 issued in 2018, Circular of Vietnam Ministry of Agriculture and Rural Development: Regulations on survey and inventory and monitoring forest developments (Vietnam Ministry of Agriculture and Rural Development, 2018). In this classification, the rich forest type has a volume of > 200 $m^3 ha^{-1}$, the medium forest type has a volume of 100-200 $m^3 ha^{-1}$, and the poor forest type has a volume less than 100 $m^3 ha^{-1}$.

Analysis of spatial pattern and association

The data was analyzed by using Programita Noviembre 2018 (http://programita.org/) and R version 4.1.1 software (R Development Core Team, 2021). SPPA was performed using Programita software (Wiegand and Moloney, 2004). The package 'spatstat' implemented in R software was used to analyze spatial patterns and intra- and interspecific associations (Baddeley et al., 2015). We used the spatstat package to make a distribution map of all species in the study area, the pair-correlation function, Ripley's K functions, and the L-function, as explained by many researchers worldwide (Dixon, 2002; Sotirios and Alan, 2005; Getzin et al., 2006; Bolibok, 2008; Nguyen, 2017; Wédjangon et al., 2020; Quy et al., 2021a, c).

The environmental homogeneity of the study plots was assessed based on the spatial pattern of all individual trees with a dbh ≥ 15 cm by comparing the results of the two functions $g_{11}(r)$ and $L_{11}(r)$ (Dien and Hai, 2016). Individual trees with a dbh ≥ 15 cm were selected because they are capable of living in all possible areas and have undergone natural selection. Heterogeneous environmental conditions will be reflected in the distribution heterogeneity of adult trees (Getzin et al., 2008; Hai et al., 2014). We assumed no interaction between the points in the patterns.

Based on the coordinates of all individuals of *H. pierrei* and other dominant species in the study plots, the univariate pair-correlation function $g_{11}(r)$ was used to analyze the spatial pattern of species. The bivariate pair-correlation function $g_{12}(r)$ was used to analyze the intraspecies associations of *H. pierrei* at the different life-history stages as well as the interspecies associations between *H. pierrei* and the other dominant species in the study plots. The pair correlation function g(r) is the derivative of Ripley's K function given by $g(r) = K'(r)/(2\pi r)$, which shows the expected density of points at a distance r from any point (Ripley, 1976). According to the tree-tree distances, the function $g_{11}(r)$ (univariate pair-correlation function) describes the spatial distribution of trees at radius r using a standardized density. Consequently, when this parameter equals 1, it indicates complete spatial randomness, > 1 indicates aggregation, and < 1 indicates regularity at distance r among trees with the pattern. The function $g_{12}(r)$ (bivariate pair-correlation function) was used to describe the spatial association between two types of points. The function $g_{12}(r)$ is the expected density of points of type 2 at a distance r beginning from a randomly chosen point of type 1. Similar to the univariate version, =1

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indicates independence, <1 indicates repulsion, and > 1 indicates attraction between two tree species at distance r (Nguyen et al., 2014).

To eliminate errors in the judgment of spatial distribution, when conducting the analysis, it is necessary to pay attention to the selection of the null model (the theoretical model used to test the homogeneity of the environmental conditions) (Tuan et al., 2018). The null models used in this study included the following: (1) The null model of complete spatial randomness (complete spatial randomness - CSR) was used for the univariate pair-correlation function $g_{11}(r)$ and $L_{11}(r)$ function of all individual trees with a dbh ≥ 15 cm in the plots. (2) The null model of inhomogeneous Poisson process (inhomogeneous Poisson process - IPP) was used to analyze the spatial patterns of species when the environment of the plots was heterogeneous; conversely, if the environment was homogeneous, then the null model of CSR was used. (3) The null model of independence was used to test for intra- and interspecific associations of *H. pierrei* at the different life-history stages and between *H. pierrei* and the dominant species in the stand by immobilizing the position of the type 1 points (species 1) and randomly moving the positions of all type 2 points (species 2) around type 1 points. The null model of independence was used in the analysis of the bivariate pair-correlation function $g_{12}(r)$; we assumed that the two point patterns were created by two independent processes.

Epanechnikov kernel estimation was used for the intensity function with a bandwidth radius R = 30 m and a ring width (bins) of 1 m to analyze the spatial distribution and association patterns of forest tree species (Dien and Hai, 2016). All spatial models were performed in Programita 2018 software with 999 Monte Carlo simulations, using the 5th lowest and highest values of the 999 simulations to build approximately 95% confidence intervals. The distribution map of forest tree species was created with the 'spatstat' (https://cran.r-project.org/web/packages/spatstat/) and 'ggplot2' packages (https://cran.r-project.org/web/packages/ggplot2/) in R 4.1.1 software. All the R scripts used in this study can be found on GitHub (https://github.com/quyforest/hopea-pierrei).

Results

Composition of tree species growing with H. pierrei and their ecological roles

A total of seventy-six species were identified in the three 1-ha study plots of the medium natural forest type in Phu Quoc National Park. The number of tree species did not differ significantly among the study plots, but the density and volume of the stands were significantly different. The forty-eight species recorded in P1 had the highest density but the lowest volume among the three study plots (2,049 individuals ha^{-1} and 144.1 m^3 ha^{-1}); the numbers of species in P2 and P3 were forty-five (1,884 individuals ha^{-1} with 176.3 m^3 ha^{-1}) and fifty-one (1,671 individuals ha^{-1}, 185.1 m^3 ha^{-1}), respectively. The species composition according to the IVI% value is shown in *Table 1*.

According to the species composition of the study plots expressed by IVI% values, *H. pierrei* along with eleven other species, namely, *A. quocense, S. cuminii, S. roxburghii, S. cinereum, O. dioica, C. parthenoxylum, D. venosa, M. ligustrinum, S. superba, C. dryobalanoides,* and *D. sylvatica*, was an ecologically significant species (IVI% value > 5%). *H. pierrei* had the highest IVI% value in P2 (19.5%), and it combined with nine other dominant species (*S. cuminii, C. parthenoxylum, D. venosa, M. ligustrinum, S. roxburghii, O. dioica, G. delpyana, G. vilersiana,* and *D. sylvatica*) to form the dominant tree species group (total IVI% of the ten species > 50%). The IVI%

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value of *H. pierrei* was lower than that of the other dominant species in P1 and P3, where it ranked 3rd (P1) and 2nd (P3); Additionally, in these two plots (P1 and P2), *H. pierrei* combined with twelve other dominant species to form another dominant species group. Based on the IVI% values of the dominant species, P3 had the fewest species in the three plots (forty-six species), but *H. pierrei* had the highest IVI% value, which indicates that its ability to assert spatial dominance was better than that of other dominant species and even better than its ability to do so in P1 and P2.

The values of N, dbh, H, BA, V, and IVI% for dominant species with ≥ 50 individual trees are shown in *Table A1* in the *Appendix*.

Table 1. *Composition of tree species in the stands*

Plot	S	N	V	Composition of species by IVI% value
P1	48	2,049	144.1	20.7 *Archidendron quocense* + 12.5 *Syzygium cinereum* + 8.0 *Hopea pierrei* + 6.7 *Olea dioica* + 5.2 *Shorea roxburghii* + 4.1 *Garcinia delpyana* + 4.1 *Syzygium zeylanicum* + 3.8 *Calophyllum dryobalanoides* + 3.8 *Diospyros sylvatica* + 3.6 *Memecylon ligustrinum* + 3.3 *Syzygium cuminii* + 3.2 *Gironniera cuspidata* + 3.1 *Diospyros venosa* + 34.3 Other species
P2	46	1,884	176.3	19.5 *Hopea pierrei* + 14.5 *Syzygium cuminii* + 5.7 *Cinnamomum parthenoxylum* + 5.1 *Diospyros venosa* + 5.1 *Memecylon ligustrinum* + 4.3 *Shorea roxburghii* + 3.9 *Olea dioica* + 3.6 *Garcinia delpyana* + 2.8 *Garcinia vilersiana* + 2.5 *Diospyros sylvatica* + 32.9 Other species
P3	51	1,671	185.1	13.7 *Archidendron quocense* + 11.9 *Hopea pierrei* + 10.3 *Schima superba* + 7.8 *Shorea roxburghii* + 7.6 *Calophyllum dryobalanoides* + 6.7 *Syzygium cuminii* + 5.5 *Diospyros sylvatica* + 4.7 *Olea dioica* + 3.9 *Diospyros venosa* + 3.7 *Vatica odorata* + 2.7 *Garcinia delpyana* + 2.5 *Memecylon ligustrinum* + 2.1 *Garcinia vilersiana* + 35.6 Other species

S - Number of species; N - number of individuals; IVI - importance value index expressed as a percentage; V – stand volume (m^3 ha^{-1})

Environmental heterogeneity effects

The results of testing the null model of CSR with the univariate function $L_{11}(r)$ showed that the cumulative density of all trees with a dbh ≥ 15 cm was similar in P2 and P3; these plots had no patterns of aggregation at scales of 5-30 m, and their spatial patterns were random (*Fig. 3f, i*); for P1, the opposite was true, with the adult trees showing an aggregated pattern at scales of 8-22 m (*Fig. 3c*). In addition, the univariate pair-correlation function $g_{11}(r)$ also showed that the adult trees in two plots (P2 and P3) followed a random pattern at scales of 0-30 m (*Fig. 3e, h*), but the adult trees in P1 showed all three patterns (aggregation, random, and regular) at scales of 0-30 m (*Fig. 3b*). In addition, the distribution map of the adult trees in P2 and P3 showed that the adult trees were distributed in most of the locations in these two study plots (*Fig. 3d, g*), whereas the adult trees were not distributed in many locations of P1 (*Fig. 3a*). According to the test results of the two functions $g_{11}(r)$ and $L_{11}(r)$, the hypothesis about environmental homogeneity could be rejected for P1 and accepted for P2 and P3. Therefore, the null models that were selected to perform the spatial pattern and association analysis of the species were the null model of IPP (P1) and the null model of CSR (P2 and P3).

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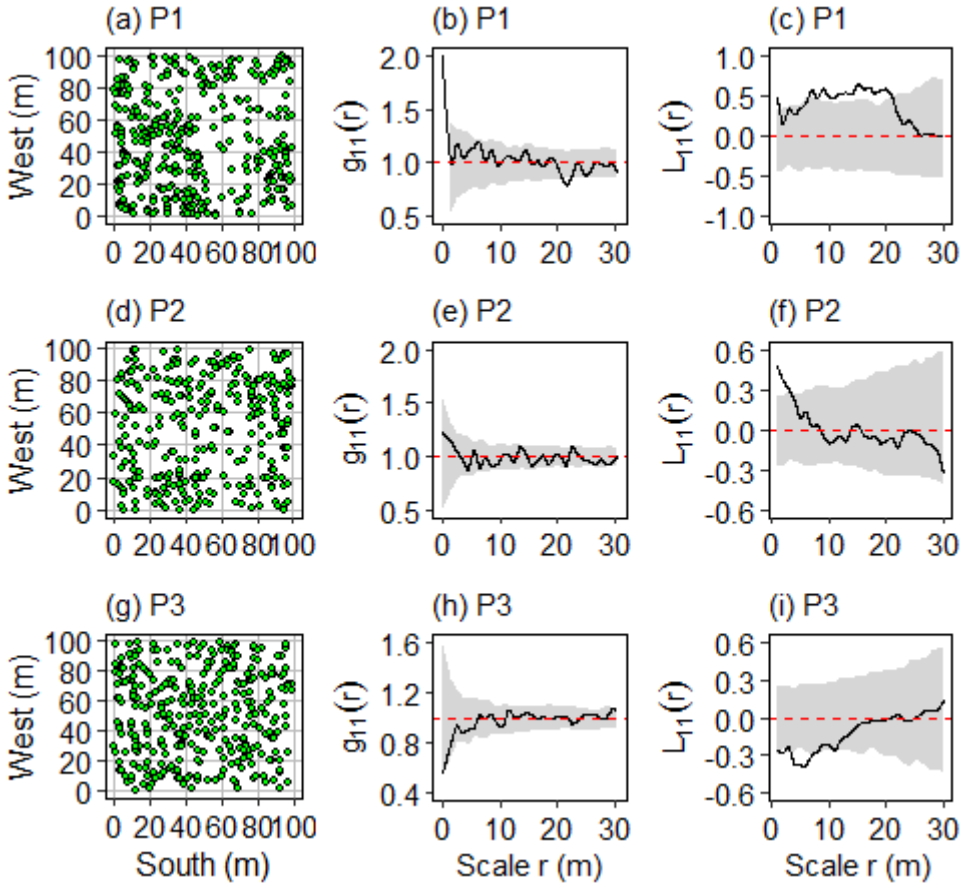

Figure 3. *Distribution map and spatial pattern of all individual trees with a dbh ≥ 15 cm in the study plots (they were analyzed by functions $g_{11}(r)$ and $L_{11}(r)$ under the null model of CSR. The dark line lying beyond the confidence envelope region (gray area) indicates a significant departure from the null model of CSR. The gray envelope region is the p = 0.05 confidence intervals from 999 Monte Carlo simulations (values < 1 indicate regularity; values > 1 indicate aggregation; values = 1 indicate randomness). The red dashed line is the expectation for spatial randomness between individual trees*

Spatial patterns of H. pierrei

The results of spatial pattern analysis of *H. pierrei* showed that all individual trees of this species showed only an aggregated pattern at scales of 0-30 m in a heterogeneous environment (*Fig. 4b*). However, individual trees of *H. pierrei* showed both aggregated and random patterns when the environment was homogeneous (*Fig. 4d, f*), and aggregation was found at scales of < 20 m. On the other hand, the distribution map of all individuals of *H. pierrei* showed that this species was highly concentrated in the eastern part of the study plot, where the elevation was lower than that in other locations in the plot (*Fig. 4*). This result showed that the same species, in this case *H. pierrei,* can exhibit a spatial pattern that differs among plots in a study area and it proved that heterogeneous environmental conditions significantly affected the spatial pattern of the study species.

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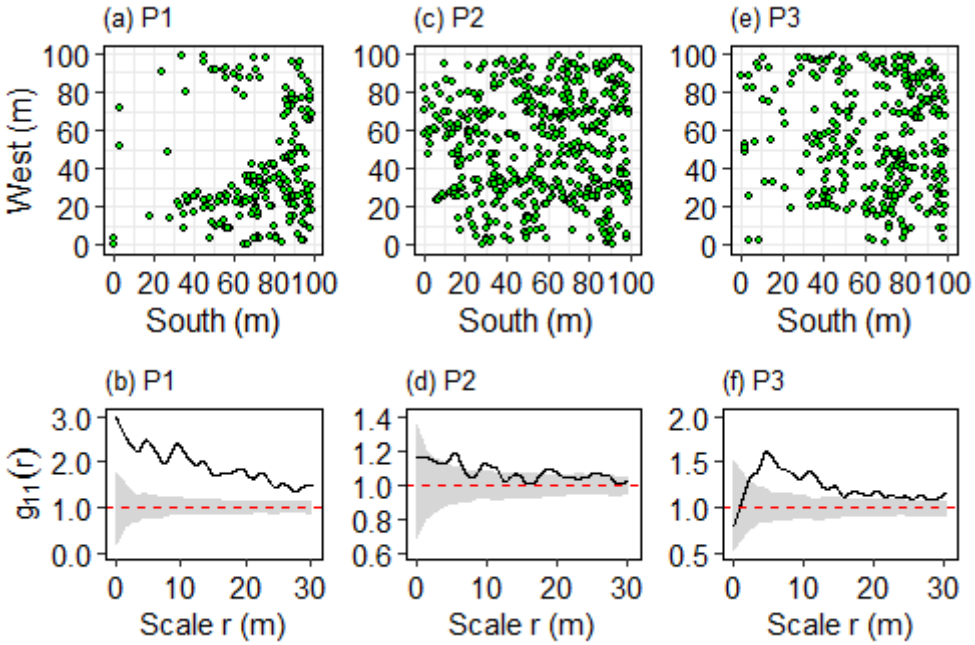

Figure 4. *Distribution map and spatial pattern of all individual trees of H. pierrei in the plots. The dark line lying beyond the confidence envelope region (gray area) indicates a significant departure from the null model. The gray envelope region is the p = 0.05 confidence intervals from 999 Monte Carlo simulations (values < 1 indicate regularity; values > 1 indicate aggregation; values = 1 indicate randomness). The red dashed line is the expectation for spatial randomness between individual trees of H. pierrei species*

The spatial patterns of *H. pierrei* at the different life-history stages are shown in *Figure 5,* in which *Figure 5a-c* show the spatial distribution patterns of the individual trees of this species in a heterogeneous environment under the null model of IPP (P1) and *Figure 5d-i* show the case of environmental homogeneity under the null model of CSR (P2 and P3). Just as the spatial pattern of all the individual trees of *H. pierrei* was considered above (*Fig. 4*), the spatial patterns of this tree species also showed a very clear difference among life history-stages when the environmental conditions in the study plot were variable. For the case of environmental homogeneity, the spatial pattern of *H. pierrei* tended to change from aggregation at the juvenile stage (*Fig. 5d, g*) to random at the subadult stage (*Fig. 5e, h*) and regular at the adult stage (*Fig. 5f, i*). In the case of environmental heterogeneity, *H. pierrei* showed only an aggregated pattern at the juvenile tree stage but an aggregated and random pattern at the subadult and adult stages, respectively (*Fig. 5a, b, c*).

Intraspecific associations of H. pierrei

The intraspecific associations of *H. pierrei* were analyzed using the bivariate pair-correlation function $g_{12}(r)$ (*Fig. 6*). The results showed that attraction associations accounted for a high proportion of 88.89% (eight of nine patterns) of the total associations. The repulsion association accounted for 11.11% (one of nine patterns) of the total associations. There was no repulsion in the intraspecific associations of *H. pierrei* with environmental homogeneity (*Fig. 6d-i*). Conversely, under environmental heterogeneity (*Fig. 6a-c*), there was repulsion in the intraspecific associations of *H.*

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pierrei, which was found between subadult and juvenile trees (*Fig. 6c*). If environmental heterogeneity was ignored, there were only attraction associations among the intraspecific associations of *H. pierrei* at all life-history stages (*Fig. 6d-i*).

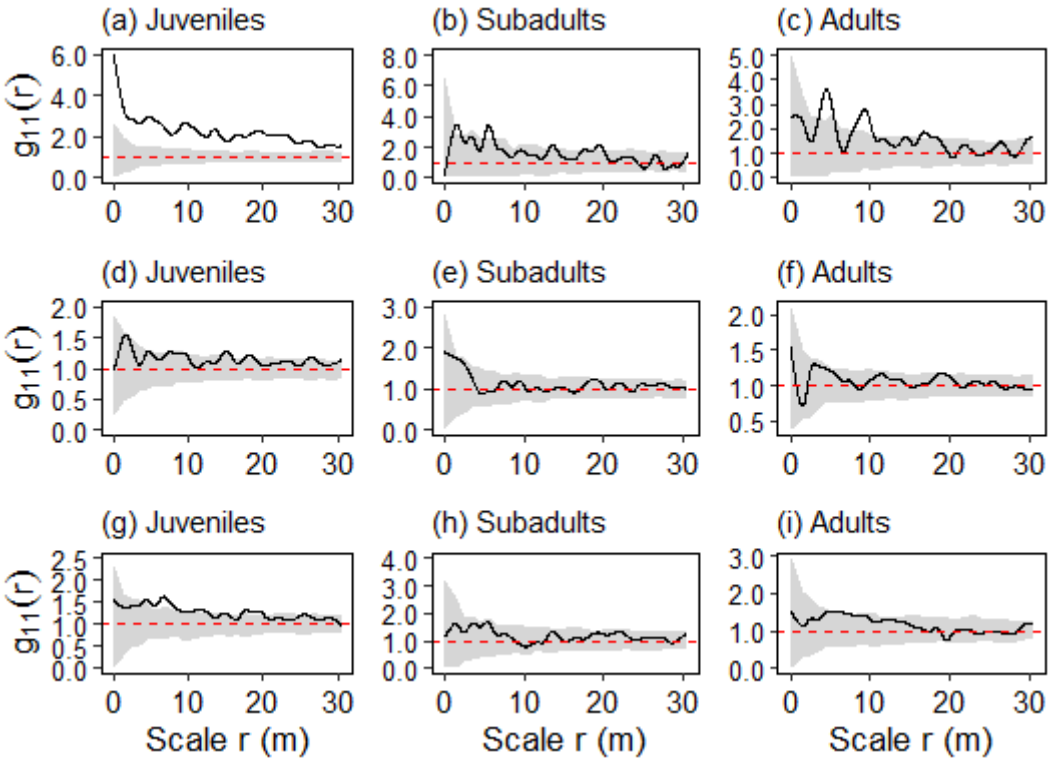

Figure 5. *Spatial distribution patterns of H. pierrei at different life-history stages in P1 (a-c), P2 (d-f), and P3 (g-i). The dark line lying beyond the confidence envelope region (gray area) indicates a significant departure from the null model. The gray envelope region is the p = 0.05 confidence intervals from 999 Monte Carlo simulations (values < 1 indicate regularity; values > 1 indicate aggregation; values = 1 indicate randomness). The red dashed line is the expectation for spatial randomness between individual trees of H. pierrei species at each life-history stage*

Interspecific associations of H. pierrei

The results of the spatial associations between *H. pierrei* and the species of the dominant species group in the three study plots (*Fig. 7*) showed that the majority of dominant species had an independent association with *H. pierrei* at scales of 0-30 m. An attraction association was found mainly at scales > 15 m. In contrast, a repulsion association was found at scales < 15 m. There was a difference in the interspecific associations of *H. pierrei* when the environmental conditions of the study plot changed. Under conditions of environmental heterogeneity, the number of species that had an independent association with *H. pierrei* tended to decrease as the scale increased; conversely, under conditions of environmental homogeneity, the number of species that had an independent association with *H. pierrei* tended to increase with increasing scale. Among the sixteen dominant species tested for associations with *H. pierrei* (*Figs. A1, A2, A3* in the *Appendix*), seven showed repulsion associations at all scales within 0-30 m, including *A. quocense, O. dioica, D. sylvatica, D. venosa, M. ligustrinum, S.*

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roxburghii, and *V. odorata*. Three species showed attraction associations at scales < 20 m, including *S. cinereum, G. delpyana,* and *S. superba*.

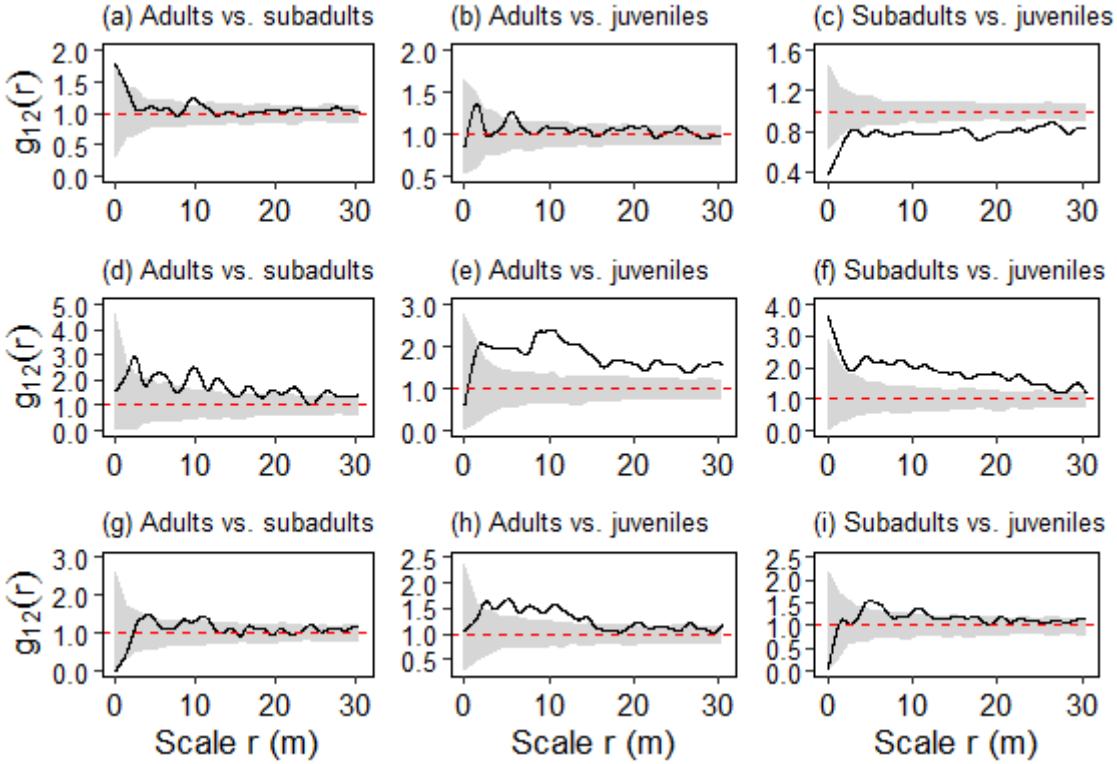

Figure 6. *Intraspecific associations of H. pierrei of P1 (a-c), P2 (d-f), and P3 (g-i) as analyzed by the bivariate pair-correlation function $g_{12}(r)$. The dark line lying beyond the confidence envelope region (gray area) indicates a significant departure from the null model of independence. The gray envelope region is the p = 0.05 confidence intervals from Monte Carlo simulations (values < 1 indicate repulsion; values > 1 indicate attraction; values = 1 indicate independence between two life-history stages). The red dashed line is the expectation for spatial independence between individual trees at two different life-history stages of H. pierrei*

Discussion

Tree species often growing with H. pierrei

In tropical forests, the tree species composition is very complex. To reflect the true status of the stand, it is necessary to identify the dominant tree species for management purposes (Quy et al., 2021b). The dominant species is the species that can significantly control forest plant community structure. The dominance of a tree species is expressed as the number of individuals of the species in the stand and the relative spatial relationship. The traditional methods of determining the predominance of tree species mainly use several ecological criteria of the tree species, such as the relative density, relative coverage, and relative total basal area of each species (Them, 2004; Hop et al., 2021). In addition, some authors believe that species composition may also reflect the dominant conditions of tree species in the stand (Quy et al., 2021b). Dominant tree species are the foundation of the entire forest plant community, and they greatly affect the stability of the community due to their high adaptability to the environment. The

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dominant species also determine the composition, morphology, and structure of the community, as well as the main features of the environment within the plant community (Thin, 2004).

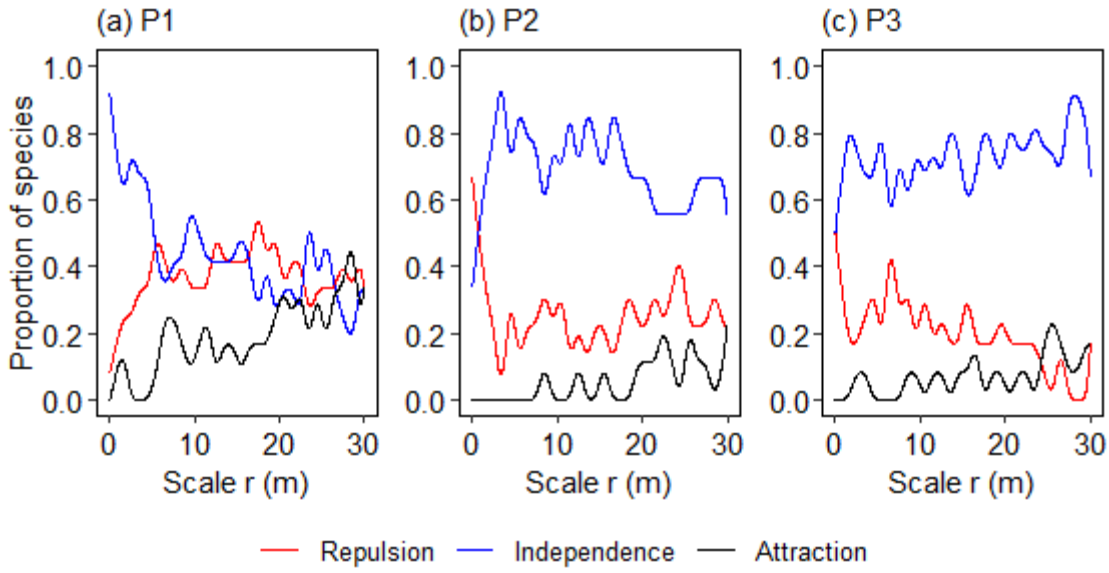

Figure 7. *Interspecific association patterns of H. pierrei and the species of the dominant species group as analyzed by the bivariate pair-correlation function $g_{12}(r)$ under the null model of independence*

Each type of vegetation was formed in different environments, so there were different dominant tree species (Lan et al., 2006). The results of the dominance analysis of tree species in the three study plots showed that *H. pierrei* often grew with sixteen other species, namely, *A. quocense, S. cinereum, O. dioica, S. roxburghii, G. delpyana, S. zeylanicum, D. sylvatica, C. dryobalanoides, M. ligustrinum, S. cuminii, G. cuspidata, D. venosa, C. parthenoxylum, G. vilersiana, S. superba,* and *V. odorata*. *H. pierrei* combined with these sixteen species to form a typical forest ecosystem on Phu Quoc Island, Vietnam.

Environmental heterogeneity effects

The spatial pattern of plant populations can be affected by environmental heterogeneity, such as variation in exposed rocks, slope, canopy cover, and soil nutrients, and in such cases, populations will exhibit spatial patterns that are not the same in different environments, such as aggregated, random or regular patterns (Hu et al., 2019). Getzin et al. (2008) suggested that if forest trees were aggregated at scales of > 10 m, the aggregation could be explained by environmental heterogeneity. Environmental heterogeneity in the same study plot is a very common phenomenon in tropical rainforests, with the cumulative density of adult individuals tending to change from a random pattern to an aggregated pattern at scales greater than 20 m (Wiegand et al., 2007). Wu et al. (2018) also suggested that environmental heterogeneity played an important role in species spatial aggregation in a study on the spatial patterns and associations of the main tree species in the evergreen broadleaved forest of Zhejiang

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Province, China. Similarly, Tuan et al. (2018) found that environmental heterogeneity in the study plots was the main reason for the large variation in the structural characteristics of the stand at different locations in the plot, thus creating spatial structural diversity of the research object. In our study, two *H. pierrei* populations (P2 and P3) showed both aggregated and random patterns when the null model of CSR was used as a theoretical model under environmental homogeneity. However, the spatial patterns of the *H. pierrei* population were only aggregation patterns at scales of 0-30 m when the null model of IPP was used as the theoretical model. In the results of environmental heterogeneity effects and the spatial patterns of *H. pierrei* in this study, we also found that environmental heterogeneity had a great influence on the spatial patterns of the studied species.

Spatial patterns of H. pierrei

The difference in spatial distribution patterns of plant species is often the result of the combined effects of many factors, such as the biological characteristics of the species, environmental factors, and especially the dispersal mechanism (Condit et al., 2000). Wu et al. (2018) found that *Cyclobalanopsis glauca* produces an acorn-like ovoid fruit, which combined with the steep slope of the study area, leads to the fruit and seeds of this species falling to the ground after a period and being driven to the foot of the mountain or places with shallower slopes and forming a clustered distribution at small scales. This phenomenon is very common in the Zhejiang evergreen broadleaved forest of China where the authors conducted the study. Hu et al. (2019) found that *Cunninghamia lanceolata* has a random distribution in all study plots at scales of 0-30 m because its seeds show low germination and the seedling survival rate is low, which causes the number of individuals of the species in the stand to be very low in the secondary forests of Hunan Province, China. Nguyen et al. (2014) also suggested that the dispersal limits of *Streblus macrophyllus* (fleshy fruit) regulated the spatial distribution patterns of this plant in the Cuc Phuong evergreen broadleaved forest of Vietnam. In our study, the spatial patterns of *H. pierrei* showed that this species was spatially aggregated at scales up to 20 m under environmental heterogeneity (*Fig. 2d, f*). On the other hand, based on the distribution map of *H. pierrei* and natural conditions in the study area, the spatial distribution patterns of *H. pierrei* were consistent with its ecological characteristics. *H. pierrei* produces a winged fruit (Ho, 1999; Ban et al., 2007); thus, *H. pierrei* has the ability to disperse seeds very well, and seedlings are often distributed far away from the mother tree and form a clumped distribution at large scales. On the other hand, the main wind direction on Phu Quoc Island is west-south, and this wind direction prevails in the rainy season (May-October); this is also the fruit ripening season of *H. pierrei*. Therefore, the number of *H. pierrei* individuals was more concentrated in the eastern area of the study plots than in the western area. The difference in the spatial distribution patterns of *H. pierrei* in the plots could be because P1 is located closer to the west coast of the island and P2 and P3 are located far inland and farther away from the west coast than P1.

The clumped distribution of *H. pierrei* at small scales is beneficial for juvenile trees at the early stage because this species is shade tolerant at this stage but will experience strong intraspecific competition for nutrients between individuals at later time points in the juvenile stage. Therefore, the opportunity for juvenile and subadult trees overtop the canopy of the mother plant is limited. For this reason, it is very important to develop appropriate silvicultural measures for increasing the number of *H. pierrei* adults in areas

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where juvenile and subadult trees can be placed in more suitable locations in the stand (under the canopy or near the mother tree) or in suitable parts of the ecological restoration area on Phu Quoc Island.

From the analysis results of the spatial distribution patterns of *H. pierrei*, it could be concluded that in addition to the influence of environmental heterogeneity, seed dispersal is a main determinant of the spatial distribution patterns of this species in the evergreen broadleaved forest in Phu Quoc National Park. In addition, the ecological characteristics of *H. pierrei* (a shade-tolerant plant at the juvenile stage) also showed that gap regeneration is not the mechanism explaining the spatial distribution pattern of *H. pierrei*. The spatial distribution of *H. pierrei* at different life-history stages showed a shift from a clustered distribution at the juvenile stage to a random distribution at the subadult stage and that there was strong competition between the adult individuals. The adult trees of *H. pierrei* were regularly distributed at scales of 2-3 m (*Fig. 5f*) and 18-19 m (*Fig. 5i*), which is evidence that *H. pierrei* has a self-thinning ability. Thus, self-thinning is also a mechanism underlying the spatial distribution pattern of *H. pierrei*.

Intra- and interspecific associations of H. pierrei

The spatial association patterns of forest tree species may reflect the biology of the population (Veblen et al., 1979). Hubbell (2001) suggested that direct tree-tree interactions occur at a distance of less than 30 m in the spatial association of forest tree species. The intraspecific association patterns of *H. pierrei* were mainly attraction patterns, accounting for 88.89%, while intraspecific competition accounted for 11.11% and occurred only when there was environmental heterogeneity. In our study, the association patterns between *H. pierrei* and the dominant species were mainly independent at scales of 0-30 m. Competition between *H. pierrei* and other dominant species was found at scales < 15 m. The results of our study are consistent with those of some previously published studies. Yang et al. (2014) found that the interspecies competitive relationship among plant species was mainly driven by competition for nutrients at small scales (<15 m) when studying the spatial association patterns of major tree species in the evergreen broadleaved secondary forest of Tai Bai Shan, China. Dien and Hai (2016) also found a competitive relationship between tree species in the evergreen broadleaved forest of A Luoi of Thua Thien - Hue Province, mainly at scales below 15 m. Hu et al. (2019) also suggested that competition or attraction relationships between species often appear at small scales; as the scale increases, the number of individuals in the large diameter class decreases, and the distance between them increases. Thus, most species are usually independent when considered at a large scale.

Conclusion

This study was conducted in the medium evergreen broadleaved forests of Phu Quoc National Park in Vietnam to understand the ecological mechanisms that regulate the spatial distribution and association patterns of *H. pierrei*. This Dipterocarpaceae species is a threatened species in South Asia, classified as “Vulnerable” in the Vietnam Red Book and on the IUCN’s Red List.

In the medium evergreen broadleaved forests of Phu Quoc National Park, *H. pierrei* often grows with sixteen dominant tree species, namely, *A. quocense, S. cinereum, O. dioica, S. roxburghii, G. delpyana, S. zeylanicum, D. sylvatica, C. dryobalanoides, M. ligustrinum, S. cuminii, G. cuspidata, D. venosa, C. parthenoxylum, G. vilersiana, S.*

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superba, and *V. odorata*. The ability of *H. pierrei* to occupy space was better than that of other dominant tree species when it was not affected by environmental factors. *H. pierrei* had both spatial distribution patterns (clustered and random distributions) where it was distributed. The spatial distribution patterns of *H. pierrei* were strongly influenced by heterogeneous environmental conditions, and when the environment was heterogeneous, this species showed only a clustered distribution at scales of 0-30 m. The spatial distribution patterns of *H. pierrei* at different life-history stages showed a shift from a clustered distribution at the juvenile stage to a random distribution at the subadult stage and a regular distribution at the adult tree stage. Among the types of intraspecific associations, repulsion associations of *H. pierrei* at different life-history stages were very infrequent (11.11%) and only occurred when the environment in the study plot was not homogeneous; attraction associations accounted for a high proportion (88.89%). Regarding interspecific associations at a distance of 0-30 m, *H. pierrei* mainly had independent associations with dominant species. In addition, seven of the sixteen dominant species had repulsion associations with *H. pierrei* at all scales of 0-30 m, namely, *A. quocense, O. dioica, D. sylvatica, D. venosa, M. ligustrinum, S. roxburghii,* and *V. odorata*, and three of the sixteen dominant species had attraction associations with *H. pierrei* within $r < 20$ m, namely, *S. cinereum, G. delpyana*, and *S. superba*. Seed dispersal and self-thinning are the ecological mechanisms driving the spatial distribution and association patterns of *H. pierrei*, leading to the formation of a clustered distribution at scales > 20 m and individuals distributed more in the western part of the study plots.

When restoring or planting new forests, imitating the laws of the natural world is always a way to avoid risks and obtain good results early. The results of this study have great significance for adjusting the density of *H. pierrei* and its neighboring species. The adjustment should be in the direction of increasing the density of species that have an attraction association and reducing the density of the species that have a repulsion association with *H. pierrei*. In addition, the interspecific associations of *H. pierrei* should also be considered in order to build a plant list and select an appropriate planting hole-spacing when restoring a forest or planting a new forest to expand the distribution area of *H. pierrei* in areas similar to the study area.

Regarding future research on *H. pierrei*, we recommend focusing on the influence of environmental factors such as soil nutrients, soil moisture, and temperature on the spatial distribution of *H. pierrei*, which will allow data collected on the ecological characteristics of this tree species to be more systematic, complete and detailed.

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APPENDIX

Table A1. *Characteristics of dominant species in three study plots*

Plot	Tree species	N	dbh	H	BA	V	IVI
P1	*Archidendron quocense*	267	14.2 ± 7.7	13.2 ± 2.4	5.45	37.41	20.7
	Syzygium cinereum	300	10.3 ± 3.8	11.5 ± 1.7	2.84	15.8	12.5
	Hopea pierrei	199	9.9 ± 3.7	11.9 ± 1.7	1.73	9.98	8
	Olea dioica	152	10.8 ± 3.5	12.0 ± 1.7	1.53	8.84	6.7
	Shorea roxburghii	129	9.7 ± 4.2	11.4 ± 1.7	1.14	6.52	5.2
	Garcinia delpyana	125	8.9 ± 1.9	10.9 ± 1.3	0.82	4.12	4.1
	Syzygium zeylanicum	57	14.2 ± 6.9	12.4 ± 2.2	1.11	7.02	4.1
	Diospyros sylvatica	111	8.9 ± 3.0	11.0 ± 1.6	0.77	4.07	3.8
	Calophyllum dryobalanoides	85	10.2 ± 5.1	11.5 ± 2.4	0.87	5.33	3.8
	Memecylon ligustrinum	102	9.4 ± 3.4	9.8 ± 1.8	0.79	3.71	3.6
	Syzygium cuminii	65	11.6 ± 5.0	12.2 ± 2.1	0.82	4.8	3.3
	Gironniera cuspidata	79	10.0 ± 3.4	11.6 ± 2.0	0.69	3.9	3.2
	Diospyros venosa	94	8.6 ± 2.8	10.7 ± 1.6	0.6	3.07	3.1
	Thirteen dominant species	1765	10.7 ± 4.9	11.7 ± 2.1	19.15	114.57	82.2
	Thirty-five other species	284	12.4 ± 6.8	12.2 ± 2.4	4.47	29.53	17.8
	All (forty-eight species)	2049	10.9 ± 5.3	11.8 ± 2.1	23.62	144.1	100
P2	*Hopea pierrei*	464	10.3 ± 4.7	10.7 ± 2.5	4.7	27.75	19.5
	Syzygium cuminii	168	15.3 ± 9.6	12.1 ± 3.7	4.3	31.75	14.5
	Cinnamomum parthenoxylum	72	14.0 ± 9.1	12.3 ± 4.3	1.57	12.51	5.7
	Diospyros venosa	173	8.3 ± 1.9	9.2 ± 1.6	0.98	4.29	5.1
	Memecylon ligustrinum	155	9.2 ± 2.6	9.1 ± 1.9	1.11	4.85	5.1
	Shorea roxburghii	115	9.8 ± 3.6	10.0 ± 2.1	0.99	5.01	4.3
	Olea dioica	104	10.0 ± 3.1	10.2 ± 1.7	0.9	4.48	3.9
	Garcinia delpyana	107	9.4 ± 2.6	9.9 ± 1.3	0.8	3.75	3.6
	Garcinia vilersiana	84	9.2 ± 2.3	9.6 ± 1.7	0.6	2.7	2.8
	Diospyros sylvatica	69	9.3 ± 4.4	9.7 ± 2.0	0.57	2.92	2.5
	Ten dominant species	1511	10.5 ± 5.4	10.3 ± 2.6	16.51	100.02	67.1
	Thirty-six other species	373	14.1 ± 10.8	11.9 ± 4.3	9.2	76.28	32.9
	All (forty-six species)	1884	11.2 ± 7.0	10.6 ± 3.1	25.71	176.3	100
P3	Archidendron quocense	105	18.3 ± 11.1	16.6 ± 4.5	3.78	34.66	13.7
	Hopea pierrei	297	9.0 ± 4.3	12.1 ± 2.8	2.32	14.91	11.9
	Schima superba	63	21.5 ± 11.4	16.8 ± 4.9	2.92	27.43	10.3
	Shorea roxburghii	109	13.4 ± 7.4	13.9 ± 3.9	2	15.46	7.8
	Calophyllum dryobalanoides	73	15.6 ± 10.3	15.6 ± 5.3	1.98	18.45	7.6
	Syzygium cuminii	129	10.1 ± 6.6	12.5 ± 3.3	1.48	11.01	6.7
	Diospyros sylvatica	78	13.0 ± 8.3	12.9 ± 3.7	1.45	10.57	5.5
	Olea dioica	91	9.7 ± 6.6	12.6 ± 3.1	0.97	8.17	4.7
	Diospyros venosa	117	7.9 ± 2.7	11.0 ± 2.0	0.64	3.41	3.9
	Vatica odorata	63	11.6 ± 6.3	14.1 ± 3.9	0.86	6.58	3.7
	Garcinia delpyana	73	8.9 ± 3.1	11.6 ± 1.9	0.5	2.82	2.7
	Memecylon ligustrinum	62	9.4 ± 4.2	11.2 ± 2.4	0.51	2.98	2.5
	Garcinia vilersiana	71	6.9 ± 1.8	10.1 ± 1.3	0.28	1.32	2.1
	Thirteen dominant species	1331	11.4 ± 7.7	12.9 ± 3.8	19.68	157.76	64.4
	Thirty-eight other species	340	10.1 ± 6.3	12.1 ± 3.2	3.79	27.34	35.6
	All (fifty-one species)	1671	11.1 ± 7.5	12.8 ± 3.7	23.47	185.1	100

N - number of individuals; dbh - diameter at breast height (mean ± Standard deviation) (cm); H - overall height of tree (mean ± Standard deviation) (m); BA - Tree basal area (m^2); V – stand volume (m^3 ha^{-1}); IVI - importance value index expressed as a percentage

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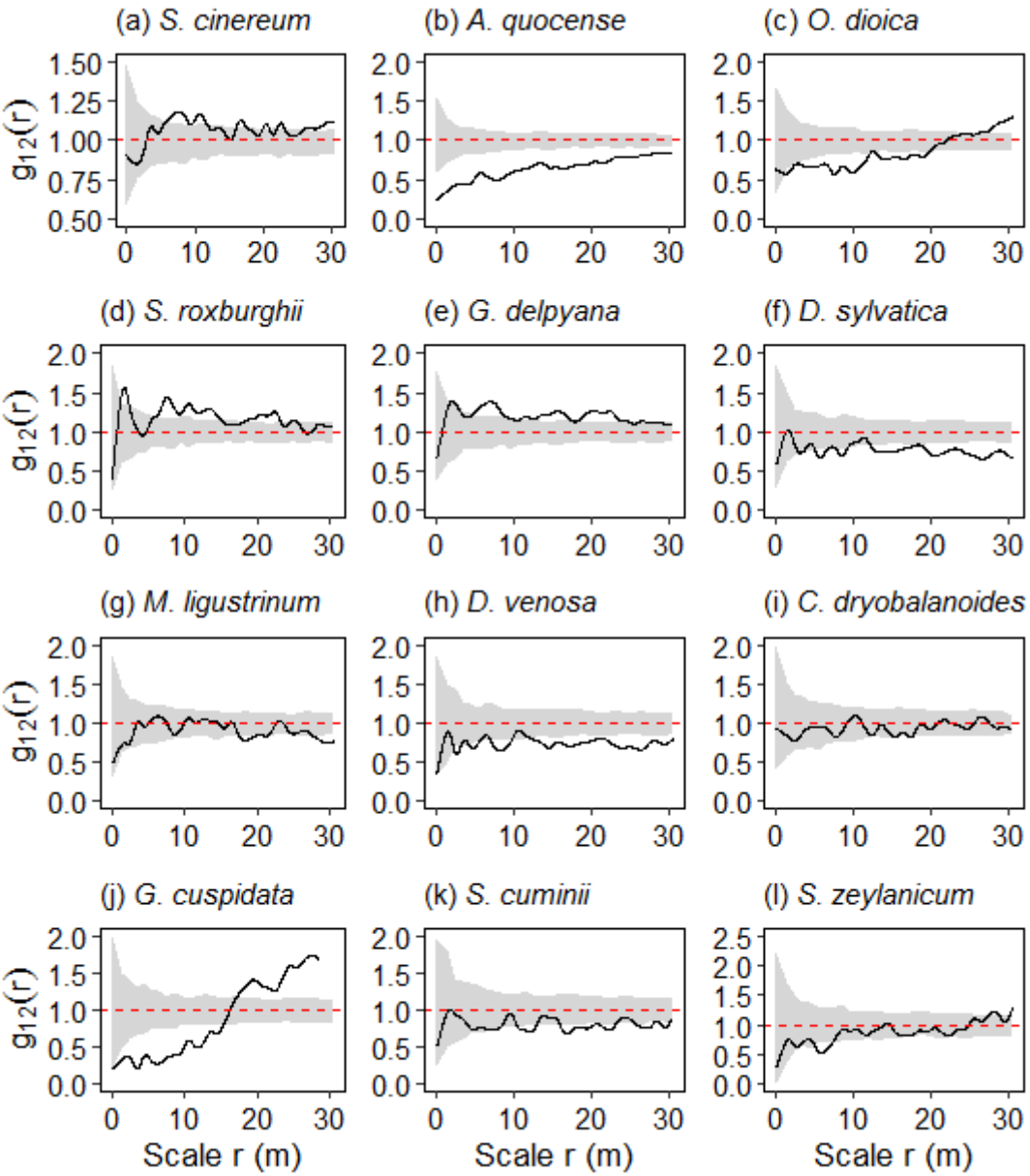

Figure A1. *Interspecific association patterns of H. pierrei and the species of the dominant species group as analyzed by the bivariate pair-correlation function $g_{12}(r)$ in P1. The dark line lying beyond the confidence envelope region (gray area) indicates a significant departure from the null model of independence. The gray envelope region is the p = 0.05 confidence intervals from 999 Monte Carlo simulations (values < 1 indicate repulsion; values > 1 indicate attraction; values = 1 indicate independence between two tree species). The red dashed line is the expectation for spatial independence between individuals of H. pierrei and the dominant species*

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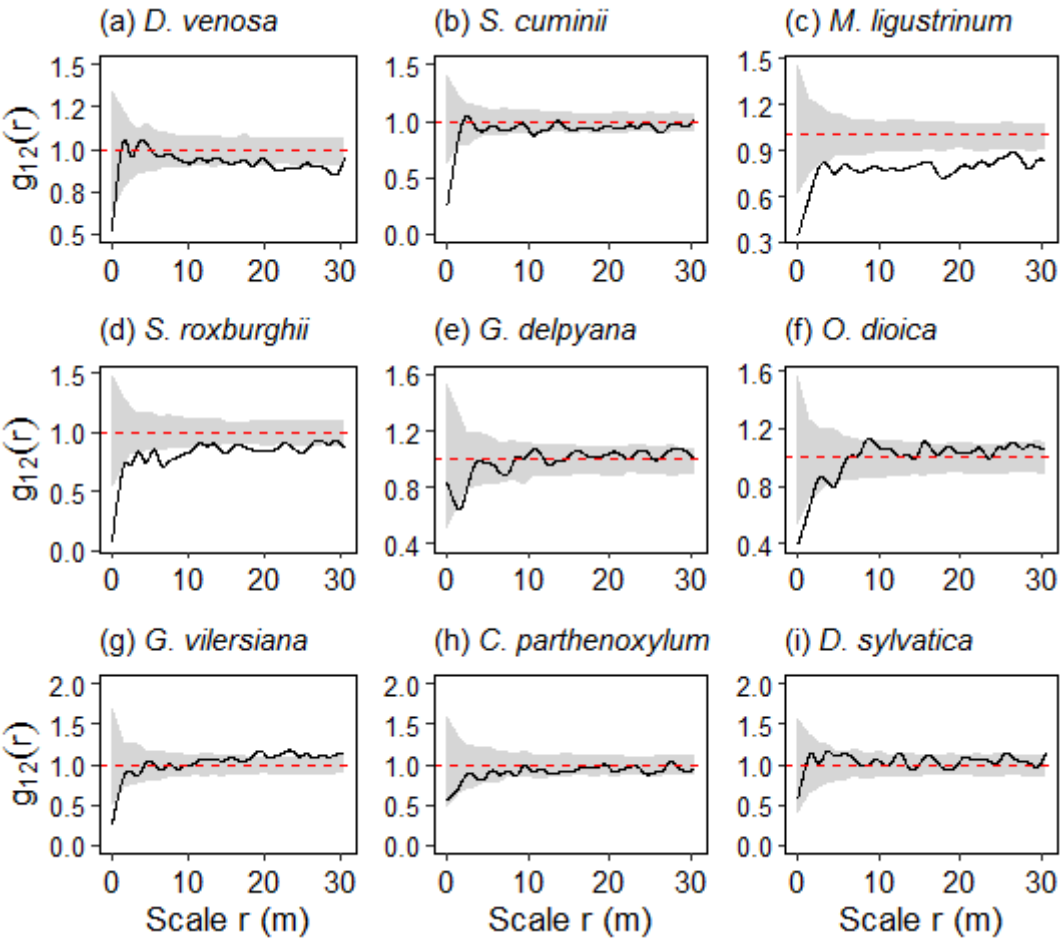

Figure A2. *Interspecific association patterns of H. pierrei and the species of the dominant species group as analyzed by the bivariate pair-correlation function $g_{12}(r)$ in P2. The dark line lying beyond the confidence envelope region (gray area) indicates a significant departure from the null model of independence. The gray envelope region is the p = 0.05 confidence intervals from 999 Monte Carlo simulations (values < 1 indicate repulsion; values > 1 indicate attraction; values = 1 indicate independence between two tree species). The red dashed line is the expectation for spatial independence between individuals of H. pierrei and the dominant species*

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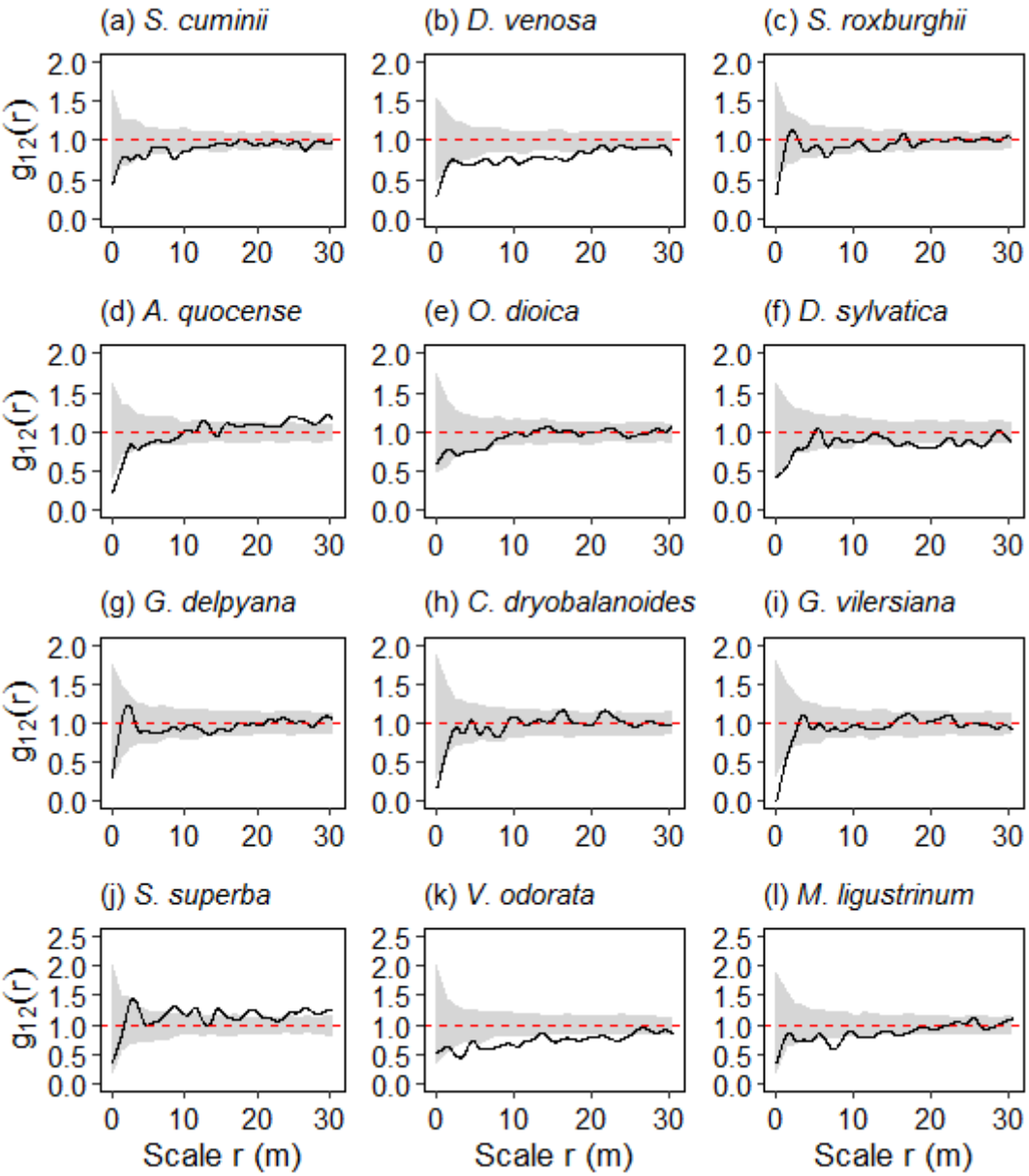

Figure A3. *Interspecific association patterns of H. pierrei and the species of the dominant species group as analyzed by the bivariate pair-correlation function $g_{12}(r)$ in P3. The dark line lying beyond the confidence envelope region (gray area) indicates a significant departure from the null model of independence. The gray envelope region is the p = 0.05 confidence intervals from 999 Monte Carlo simulations (values < 1 indicate repulsion; values > 1 indicate attraction; values = 1 indicate independence between two tree species). The red dashed line is the expectation for spatial independence between individuals of H. pierrei and the dominant species*

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EFFECTS AND MECHANISMS OF ORGANIC ACID INPUT ON THE PRIMING EFFECT OF DARK BROWN SOIL

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Abstract. In this study, malonic acid was used to simulate the organic acids in the root exudates of *Fraxinus mandshurica* to study their impact of organic acids in root exudates on the priming effect mechanism of organic carbon. Through incubation experiments, ^{13}C-labeled malonic acid was added to soil samples to study the effects of organic acids on the priming effect, physical and chemical properties, and bacterial diversity in dark brown soil. The results showed that after the addition of malonic acid, the cumulative soil respiration increased significantly, and soil organic matter generated a strong positive priming effect. There were no significant changes in the soil bacteria and microorganism species after adding malonic acid, but significant differences in species abundance were observed. Actinobacteria were dominant at the phylum level, followed by Firmicutes, Proteobacteria and Verrucomicrobia. At genus level, Bacillus, Micromonospora and Streptomyces were dominant during the whole incubation period. The addition of malonic acid demonstrated a strong priming effect on organic carbon present in this typical dark brown soil, showed relation with the soil physical and chemical properties, the typical microbial species, and the changes in the composition of the soil microbial community after the addition of fresh organic matter.
Keywords: *malonic acid, ^{13}C-labeled, soil respiration, soil organic carbon, microbial diversity, priming mechanism*

Introduction

The soil organic carbon pools play an important role in soil biology, with their size dynamically changing constantly, controlled by fixation and mineralization of plant organic carbon (Dai, 2018). Addition of exogenous substances to soil promotes the mineralization of organic carbon or organic nitrogen that is already present in the soil, and this is termed as the "priming effect" (Löhnis, 1926; Bingemann, 1953). It is necessary to properly understand the priming effect mechanism and its impact on mineralization, fixation, and equilibrium relationships in storing the forest soil organic matter, and the means to influence it to resolve agricultural production and global climatic problems.

The main exogenous substances in forest soils include forest floor litter and root exudates of forest plants, and the priming effect in forest soils is closely related to these. The root exudates are regarded as important carriers of material exchange and information transmission between plants and soil. They are the key factors in the micro-ecological rhizosphere characteristics of plants, and have important functions in biogeochemical cycles and in the regulation of soil micro-ecosystem structure and function (Woldendrop, 1963; Haichar, 2014), especially in the priming effect of soil organic carbon, regulation of complex interactions between soil and microorganisms,

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and in the priming effect of soil organic carbon (Ezékiel et al., 2003). Therefore, many studies have been conducted on the priming effect of root exudates on soil organic carbon. There are studies that observed the positive effect played by the root exudates on priming effect, while few other studies have observed a negative role. For example, Helal and Sauerbeck (1984), Sauerbech and Sauerbeck (1986), Fu et al. (2002) have found that the root exudates of corn, sunflower and soybean had positive priming effects on soil organic carbon and promoted decomposition of soil organic matter; while Reid and Goss (2010) and Sparling et al. (2010) have found that the root exudates of wheat, barley and sorghum had a negative priming effect on soil organic carbon and inhibited the decomposition of soil organic matter. Moreover, some studies have reported that the effect of root exudates on soil priming effects depends on the specific component of exudates to be input. Among them, Dalenberg and Jager et al. (1989) have found that aspartic acid generated a positive priming effect, while glucose produced a negative one. Also, Landi et al. (2006) have found that oxalic acid produced a positive priming effect on soil organic carbon, while glucose showed no obvious effect. In contrast, Shen and Bartha (1996) have found that benzoic acid had no effect on soil organic carbon, but glucose enhanced it.

The results of these studies with regard to the priming effect of root exudates on soil are different, and the mechanism of priming process still remained unclear. It has been suggested that priming might be related to the type and quantity of root exudates and soil properties (Wu et al., 1993; Luna-Guido et al., 2003; Xie et al., 2005). Furthermore, it also might be related to the interactions between root exudates and soil microorganisms. According to Landi et al. (2006) addition of artificial root exudates has significantly affected the soil bacterial community. Fountaine et al. (2004) have proposed that microbial populations can be divided into R-strategy species and K-strategy species, and that competition between R- and K-strategists affects the strength of the priming effect. There are currently two explanations for the priming effect mechanism: the co-metabolic mechanism and the N mineralization mechanism. When the nutrient elements in the soil are sufficient, then the soil microbial population is dominated by r-strategy species, thus generating a positive priming effect through co-metabolic mechanisms. When nutrient elements in the soil are scarce, then the soil microbial population is dominated by K strategy species, thus generating the positive priming effect through N mining mechanism. However, it is unclear as to which of these mechanisms are triggered by root exudates. In addition, as root exudates are mixed, it is difficult to analyze the priming effect, and is impossible to determine as to which substance plays a major role. Therefore, when studying the soil priming effect of root exudates, a single organic compound should be applied to study the resulting mechanism. In addition, most of the studies use carbohydrate compounds for analyzing the effect of root exudates on soil priming mechanism, while the study of organic acids on soil priming are still lacking. What impact does the addition of organic acids would bring to soil organic carbon, and what specific changes would microbial community have the impact is still unknown.

Hence, in this study, the typical soil from the mid-temperate region of China was incubated, and used malonic acid to simulate organic acids. The interactions between organic acids in root exudates, and the mechanisms by which they affected the priming of soil organic carbon, and the effect of root exudates on soil bacterial diversity were examined. The main objectives of this study were: (1) to determine the effect of organic

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acid on soil priming effect; and (2) to investigate how organic acid input change the soil properties and soil bacteria communities.

Materials and methods

Test soil

The soil test samples were collected from the Maoershan Experimental Forest Farm of MAPERSHAN, Harbin, China (45°20'N,127°30'E). The samples of dark brown soil were collected from the shallow layer at 0–10 cm depth using the snake shaped point sampling method. The soil samples were then taken to the laboratory for mixing and air drying, and the plant residues and other solid matter were removed as far as possible by screening with a 2 mm sieve. The basic physical and chemical properties of the soil were as follows: pH 6.7 ± 0.05; organic carbon content 11.12 ± 11.95 g/kg; total N content 1.42 g/kg; NH_4-N content 396.19 ± 3.43 mg/kg; NO_3-N content 90.70 ± 3.03; NO2-N content 0.27 ± 0.01 mg/kg; and available P content 40.02 ± 1.01 mg/kg.

^{13}C tracer experimental incubation method

In this study, ^{13}C-labeled malonic acid was used to simulate the organic acids. This was determined as the greatest amount of organic acids in the root exudates of *Fraxinus mandshurica* in our previous study, and is regarded as the most important economic tree species in mid-temperate region of China (Li, 2019). A fresh organic matter (FOM) treatment group and a control group were established, with 3 repetitions in each group. Three FOM treatment subgroups of Mal-1, Mal-2 and Mal-3 were created, and a base of malonic acid with 3% soil organic carbon and ^{13}C marker were added into each, followed by 1%, 2% and 3% additional soil organic carbon, respectively, with 3 repetitions each. Next, 50 g of soil was weighed for each repetition and put into a 500 mL incubation flask with 20 mL of deionized water, the flask was sealed, and then was pre-incubated at 25 °C for 7 d. The whole process was performed in the dark. After pre-incubation, the water content in the incubation bottles was adjusted to 60 ± 0.5% distilled water and mixed evenly. At the same time, the same amount of distilled water was added to the control group and mixed evenly. A 25 mL beaker containing 15 mL 0.5 mol/L NaOH solution was carefully placed into each incubation flask, and the flasks were resealed and incubated at a constant temperature of 25 ± 1 °C to stimulate the unplanted soil in natural conditions. The beakers were then removed on days 1, 3, 7, 14, 21, 2, 42, and 56, and the NaOH solution in the beaker was collected and placed into a centrifuge tube. Fresh NaOH solution was added to the beaker, and incubated further (Wang et al., 2013; Qin et al., 2016). The levels of CO_2-C and $\delta^{13}C$ isotope (products of soil respiration) were determined for each of the NaOH solution samples. The CO_2-C was determined as total organic content (TOC), and $\delta^{13}C$ was determined by using an isotope mass spectrometer (Flash, 2000 HT, Thermo Scientific, Inc., Waltham, Massachusetts, U.S.). The results were used to analyze the effects of soil CO_2 emission and the resulting soil priming effect.

Calculation of soil CO_2-C emission and the priming effect

1. Soil cumulative CO_2-C (Ctot) emissions: the sum total of the cumulative CO_2-C emissions of each sample.

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2. Contribution of malonic acid and soil organic matter (SOM) to CO_2 emissions.

The total CO_2 (C_{SOC}) from SOM plus the total CO_2 (C_{mal}) from FOM were calculated according to the *Equations 1* and *2* (Qiu et al., 2019).

$$C_{SOC} = (\delta_{mal} - \delta_T)/(\delta_{mal} - \delta_C) \times C_T \quad \text{(Eq.1)}$$

$$C_{mal} = C_T - C_{SOC} \quad \text{(Eq.2)}$$

where C_T represents the total C emission after adding FOM; C_{SOC} represents the C emission from the soil organic carbon (SOC) source after adding FOM; C_{mal} represents the C emission from the FOM source after adding FOM; δ_T represents the $\delta^{13}C$ of CO_2 after adding FOM; δ_C represents the $\delta^{13}C$ of CO_2 from control; and δ_{mal} represents the $\delta^{13}C$ of FOM.

Calculation of the cumulative priming effect on the soil

Based on the determination of soil CO_2-C release and $CO_2\delta^{13}C$, the cumulative priming effect (PE) was calculated using *Equation 3* (Hamer and Marschner, 2005).

$$PE = C_{SOC} - C_C \quad \text{(Eq.3)}$$

where C_{SOC} represents C emission from SOC source after adding FOM and C_C represents C emission from SOC in control.

Calculation of the daily cumulative priming effect on the soil

The calculation of the daily priming effect on soil and daily mineralization rate of FOM was based on *Equations 4* and *5*.

$$PEt_{1\text{-daily}} = (PEt_2 - PEt_1)/(t2 - t1) \quad \text{(Eq.4)}$$

$$FOMt_{1\text{-daily}} = (FOMt_2 - FOMt_1)/t2 - t1 \quad \text{(Eq.5)}$$

where: t1 is the date of the sampling day; t2 is the date of the next sampling; PEt1 and PEt2 are the priming doses calculated during the sampling of t1 and t2, respectively; and $PEt_{1\text{-daily}}$ is the priming rate of t1. FOM_{t1} and FOM_{t2} are calculated as malonic acid mineralization amounts on days t1 and t2, respectively. $FOM_{t1\text{-daily}}$ is the malonic acid mineralization rate on day t1.

Incubation method for the destructive sampling experiment

The group without incubation and without the addition of malonic marked was regarded as blank group. The analytically pure malonic acid was not added under consistent incubation conditions, and destructive samples were taken on days 3, 14, and 56, respectively (the three groups were marked as ck-1, ckl-2 and ck-3, respectively). The analytically pure malonic acid was added under consistent incubation conditions consistent using the ^{13}C tracer method. Destructive samples were taken on days 3, 14, and 56, respectively (the treatment groups are marked as mal-1, mal-2 and mal-3, respectively), and stored in a refrigerator at -80 °C for determining soil physical and chemical properties, and microbial diversity.

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Determination of soil physical and chemical indexes

The pH of the soil was determined using potentiometer (pH Meter Basic 20, Crison Instruments, Alella, Spain). The total C and total N contents of the soil were determined using an element analyzer (Vario EL, Analysensysteme GmbH, Hanau, Germany). The mineral N, which was determined as NH_4-N, NO_3-N and NO_2-N, were extracted using the KCl solution extraction method followed by determination with a flow analytical system (Smartchem 200, Zeal Quest Equipments, France) (Feng, 2019). The effective P in the soil was extracted using the double acid extraction method and a flow analytical system (Xu et al., 2014).

Determination of the soil bacteria and microorganism community structure and diversity

(1) Total DNA extraction and PCR amplification

The American FastDNA® Spin Kit was used to extract the total DNA in the soil samples. The concentration and purity of the extracted DNA were measured using a NanoDrop2000 Microvolume UV-Vis Spectrophotometer. The quality of the DNA was measured using 1% agarose gel electrophoresis. The V3-V4 variable region was amplified by PCR through 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') primers. The amplification procedure was as follows: pre-denaturation at 95 °C for 4 min, 27 cycles; denaturation at 95 °C for 30 s; annealing at 55 °C; extension at 72°C for 30 s; and finally, extension at 72 °C for 10 min (using a PCR analyser: ABI GeneAmp® 9700). The amplification system was 20 uL, 4 uL 5 * FastPfu buffer, 2 uL 2.5 mm dNTPs, 0.8 uL primer (5 μm), 0.4 μL FastPfu polymerase, and 10 ng DNA template (Wijaya et al., 2019).

(2) Identification, purification and quantification of polymerase chain reaction (PCR) products

Each sample was replicated with three PCR repeats and mixed with three repeated PCR products. Two percent agarose gel electrophoresis was used to detect the PCR products. The PCR products were then purified using an AxyPrep DNA Gel Extraction Kit. The PCR products were used for quantitative detection by using a Quantus™ Fluorometer. The corresponding proportions were mixed according to the sequencing quantity requirements of each sample (Belgrader, 1999).

(3) Construction of a priming effect (PE) library and Illumina sequencing

A NEXTFLEX® Rapid DNA-Seq Kit was used to build the library and connect the adaptors. Magnetic beads were used to screen and remove the self-linked segments of the adaptors. PCR amplification was used to enrich the library template. Magnetic beads were used to recover the PCR products to obtain the final library. The sequence was carried out using a Miseq PE300 platform (Illumina company, Shanghai Major Biomedical Technology Co., Ltd.).

(4) Analysis method

Trimmatotic software was used for quality control of the original sequencing, and Flash software was used for fragment assembly. UPARSE software (version 7.1 http://drive5.com/uparse/) was used to obtain operational taxonomic units (OTUs) by

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clustering the sequences to a 97% similarity. A ribosomal database project (RDP) classifier (http://rdp.cme.msu.edu/) was used for species classification and annotation of each sequence. The Silva database (SSU128) was used for alignment by setting the alignment threshold to 70%. In addition, OTU analysis, Alpha diversity analysis, dilution curve analysis, Venn diagram analysis, bar diagrams and heat map analysis of colony compositions, and principal co-ordinates analysis (PcoA) analyses were performed. Species difference analysis of multi-group comparisons between groups and phylogenetic tree analyses were also performed.

Statistical treatment

The experiment was conducted three times parallelly. SAS software was used for one-way ANOVA and Duncan analysis, and SigmaPlot 12.5 was used to prepare the figures and diagrams.

Results and analysis

Effects of malonic acid addition on soil respiration, and priming effects

After addition of malonic acid, the soil cumulative respiration has reached to 3831.52 mg C/kg at the end of incubation. However, the respiration of control soil has reached only to 204.63 mg C/kg instead (*Fig. 1*). The priming effect induced by mal was obvious, and the cumulative priming during the whole incubation period was 2874.69 mg/kg. As shown in *Figure 2,* the priming effect of soil caused by malonic acid was decreased with increasing incubation time. The priming effect remained the strongest on day 1 of incubation, then decreased rapidly in intensity, reaching the lowest level after 21 days, and then showed a slight upward trend. The C released in soil respiration of malonic acid mineralization was 546.23 mg/kg, in which only 4.55% that of the added amount. As shown in *Figure 3,* the mineralization rate of malonic acid was decreased with increasing incubation time. It remained the strongest on day 2 of incubation and then its intensity was decreased rapidly. After 7 days of incubation, the mineralization effect was weak. The accumulated mineralization effect due to malonic acid accounted for 95.65% of the total mineralization during the first 7 days of incubation.

Effects of malonic acid addition on soil physical and chemical properties

As shown in *Table 1,* the pH levels showed no significant differences between the treatments, and so no effect of added malonic acid was observed on soil pH. As shown by the TOC measurements in the three treatment groups, blank, the control group (ck1) and the experimental group Mal-1, no significant differences were observed in TOC between blank and ck1, while both were significantly lower than that of Mal-1 treatment after malonic acid addition. The soil TOC values were gradually decreased with increasing incubation time, and the treatments differed significantly ($p < 0.05$). Soil organic C was rapidly decomposed during the early stage of incubation, and the decomposition rate was slowed down during the middle and late stages of incubation. The NH_4-N, NO_3-N, and mineral N results showed that all the three were increased with increasing incubation time, and showed significant changes. In addition, NH_4-N and NO_3-N content in the groups without adding malonic acid were significantly higher than in the corresponding groups. The results showed no significant differences in the available phosphorous (AP) among the treatment groups.

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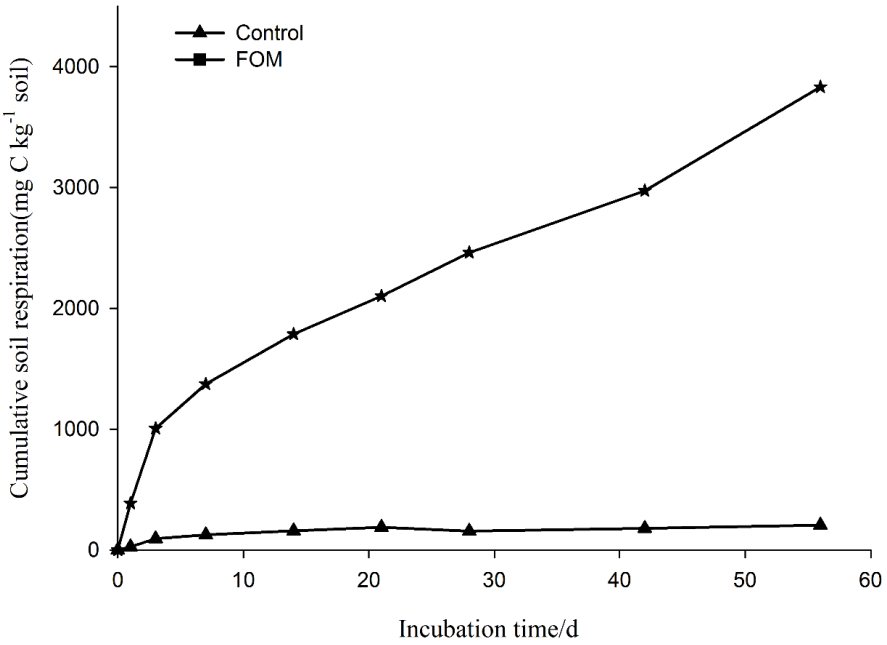

Figure 1. *The dynamics of cumulative CO_2-C emission derived from different carbon sources (n = 3). FOM: malonic acid addition was 3%. Control: no added malonic acid*

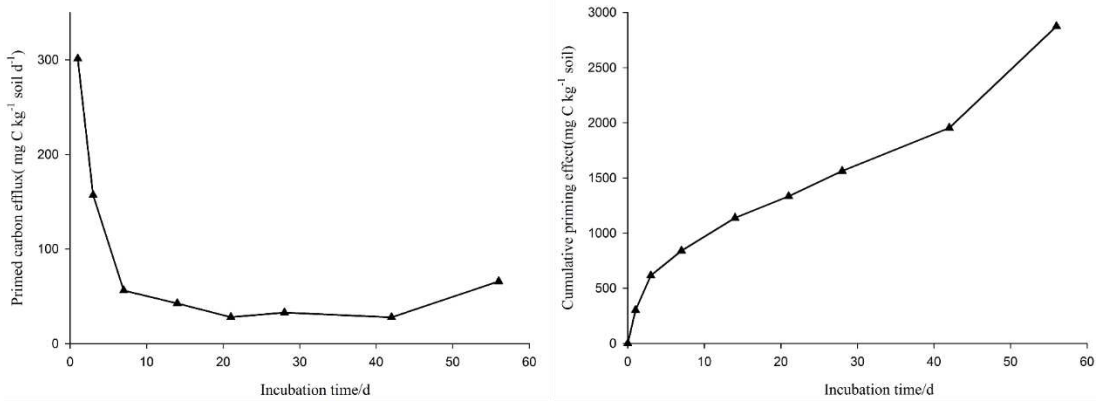

Figure 2. *The priming effects and cumulative priming effects of the soil (n = 3)*

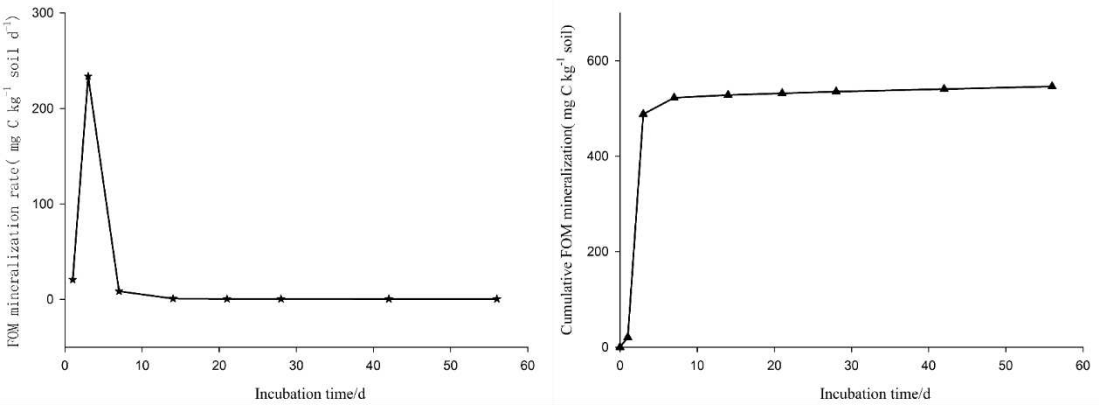

Figure 3. *The mineralization effects and the cumulative mineralization effects of the soil (n = 3)*

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Table 1. *the influence of malonic acid addition on soil physicochemical properties (n = 3)*

No.	pH	TOC mg/kg	NH_4-N mg/kg	NO_3-N mg/kg	Total inorganic N g/kg	AP mg/kg
blank	6.70 ± 0.05 [a]	832.48 ± 11.95[b]	396.19 ± 3.43[f]	90.70 ± 3.03[f]	486.89 ± 3.36[f]	40.02 ± 1.01[a]
ck1	6.68 ± 0.02[a]	866.97 ± 40.17[b]	539.25 ± 6.10[d]	115.54 ± 2.12[cd]	654.79 ± 4.27[d]	42.82 ± 1.21[a]
ck2	6.68 ± 0.02[a]	588.69 ± 38.56[d]	662.11 ± 26.82[b]	125.95 ± 4.89[c]	788.06 ± 16.75[b]	43.90 ± 0.78[a]
ck3	6.67 ± 0.02[a]	538.14 ± 15.64[e]	756.70 ± 25.00[a]	150.56 ± 1.70[a]	907.26 ± 18.34[a]	41.77 ± 1.69[a]
Mal-1	6.67 ± 0.03[a]	942.39 ± 46.79[a]	476.60 ± 12.32[e]	106.22 ± 4.82[e]	582.82 ± 9.58[e]	39.67 ± 1.05[a]
Mal-2	6.65 ± 0.01[a]	673.67 ± 24.26[c]	581.16 ± 11.22[c]	116.57 ± 5.42[c]	697.73 ± 8.31[c]	41.54 ± 1.03[a]
Mal-3	6.67 ± 0.02[a]	598.35 ± 12.91[d]	739.63 ± 12.62[a]	143.32 ± 5.29[b]	882.95 ± 10.57[a]	42.24 ± 1.13[a]

a-d show intra-group differences ($p < 0.05$). TOC: total organic carbon; AP: available phosphorous; Blank: The group without incubation and without the addition of malonic acid; ck-1: the group culture for 3 days without malonic acid; ck-2: the group culture for 14 days without malonic acid; ck-3: the group culture for 56 days with malonic acid. Mal-1: the group culture for 3 days with malonic acid; Mal-2: the group culture for 14 days with Malonic acid; Mal-3: the group culture for 56 days with malonic acid

Effects of malonic acid addition on soil bacterial diversity

Analyzing the OTU dilution curve and species accumulation curve at 97% similarity level revealed that both curves were relatively flat in all the treatment groups, showing that the amount of sequencing data used was adequate and that the depth of sample measurement was reliable. The sample size and library capacity were clearly large enough to represent that the majority of the bacteria in the experimental soil flora, and the species richness and library diversity have reached saturation point.

Diversity analysis of Venn graphs and PCoA

Venn graphs were generated to count the number of common and unique species in the samples, and to assess the similarity and overlap between the species composition in different samples. As shown in *Figure 4,* 1385, 1475 and 1449 OTUs were detected in Mal-1, Mal-2 and Mal-3 groups, respectively. Of the total 964 OTUs, Mal-1 and Mal-2 shared 137 OTUs; Mal-1 and Mal-3 shared 124 OTUs; and Mal-2 and Mal-3 shared 201 OTUs. Mal-1, Mal-2, and Mal-3 had 160, 173, and 160 unique OTUs, respectively, which accounted for 11.55%, 11.43%, and 11.04% of the total OTUs. The results showed that the number of bacterial species were increased initially and then were decreased with increasing incubation time, and showed significant differences in the composition of soil bacterial communities during different incubation periods. Principle component analysis (PCA) was carried out on the abundance of OTUs in each sample using 97% similarity level as the principle variable. The principal component factors were extracted from 16S rRNA sequences of each sample, and a PCA map was drawn using R language tools. As shown in *Figure 5,* the first two principal components, PC1 and PC2, accounted for 60.68% and 16.48% variation, respectively. Their cumulative contribution was 77.16%, and most of the variation was explained. *Figure 5* shows that the distribution distances between the experimental groups and the blank group was large, and significant. It can also be seen that the distribution distances of the experimental groups with different incubation times were relatively large, and significantly different, indicating that the structure of the bacterial flora of each

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experimental group had significant differences. The distance between Mal-2 and Mal-3 was relatively close, and the distance between Mal-2 and Mal-3 was relatively far from ck and Mal-1 in the control group. This indicated that the bacterial flora of Mal-2 and Mal-3 samples was relatively similar, and that the difference between ck and Mal-1 samples showed more significance. However, the distance between ck and Mal-1 was relatively close, indicating that the bacterial flora of ck and Mal-1 were relatively similar. Overall, the results showed that the structure of the soil bacterial communities were significantly different at different incubation times. The structure of the bacterial communities were similar during the middle and end of the incubation period, while there were large differences between them at the end of incubation.

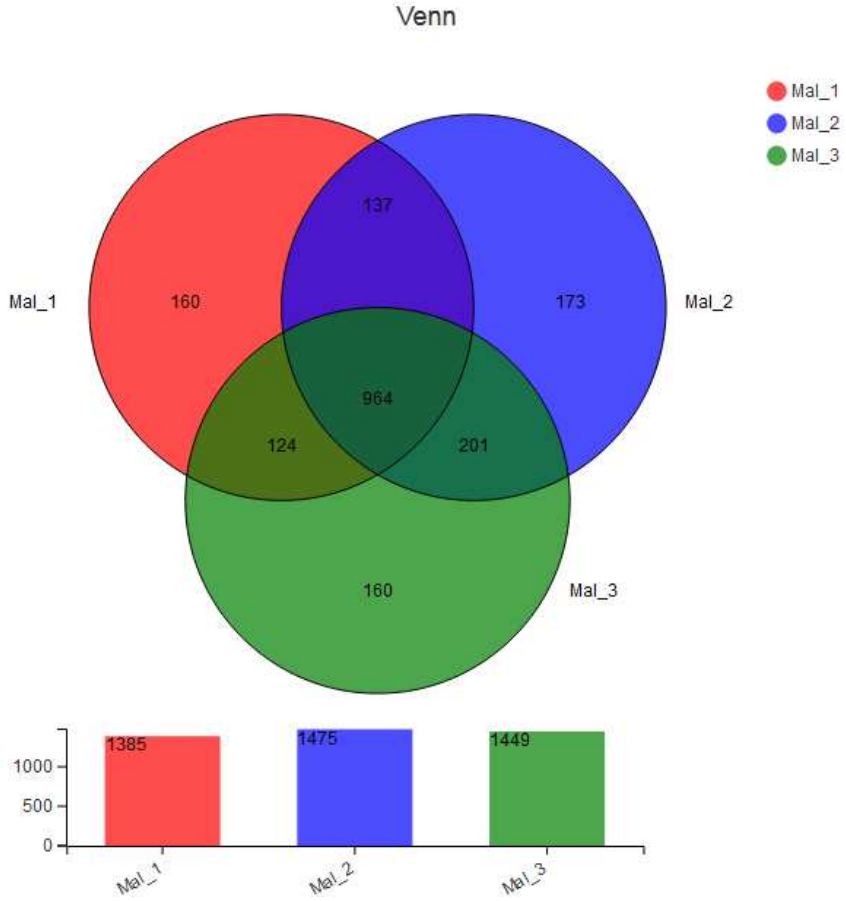

Figure 4. *A Venn diagram analysis of OTUs (97% sequence similarity). Mal-1: this group was incubated for 3 days with malonic acid; Mal-2: this group was incubated for 14 days with malonic acid; and Mal-3: this group was incubated for 56 days with malonic acid*

Soil bacterial community composition analysis of the samples

(1) Community diversity of soil bacteria at the phylum level

The dominant bacteria of each group were as follows (*Fig. 6a; Table 2*): Actinobacteria were shown to be dominant, followed by Firmicutes and Proteobacteria. The structure within each group was similar, but the proportions of the three groups differed. Actinomycetes were increased significantly during the early stage of incubation, Proteobacteria were increased significantly during the middle stage of

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incubation, and Actinomycetes were increased significantly again in the later stage of incubation when some bacteria in other phyla also showed an increasing trend.

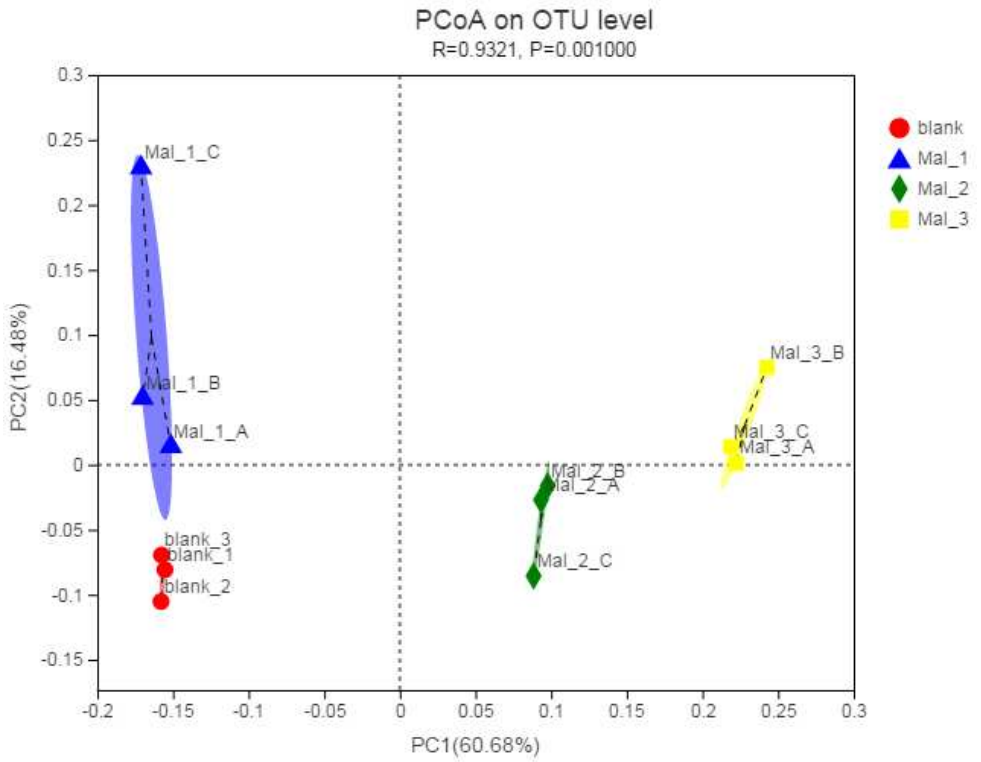

Figure 5. *The principal coordinates analysis (PCoA) analysis of OTUs (97% sequence similarity). PC1 and PC2: principal component1 and principal component2; Blank: The group without incubation and without the addition of malonic acid; Mal-1: this group was incubated for 3 days with malonic acid; Mal-2: this group was incubated for 14 days with malonic acid; Mal-3: this group was incubated for 56 days with malonic acid; A, B and C: parallel sample*

Table 2. *The bacterial composition at phylum level*

Proportion of phylum (%)	Group			
	Blank	Mal-1	Mal-2	Mal-3
Actinobacteria	44.70%	57.37%	39.82%	47.92%
Firmicutes	23.76%	23.72%	24.11%	13.56%
Proteobacteria	14.53%	10.13%	23.75%	24.45%
Verracomicrobia	8.38%	4.02%	4.12%	4.44%
Chloroflexi	3.78%	2.22%	2.12%	2.20%
Acidobacteria	2.51%	1.21%	1.41%	1.26%
Bacteroidetes	0.60%	0.22%	2.58%	2.16%
Gemmatimonadetes	0.7%	0.46%	0.83%	2.22%
Planctomycetes	0.37%	0.14%	0.68%	1.41%
Others	0.67%	0.51%	0.58%	0.38%
Total	100%	100%	100%	100%

Blank: The group without incubation and without the addition of malonic acid; Mal-1: the group culture for 3 days with Malonic acid; Mal-2: the group culture for 14 days with malonic acid; Mal-3: the group culture for 56 days with malonic acid

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(2) Community diversity of soil bacteria at the genus level

The *Bacillus* species were dominated (*Fig. 6b; Table 3*), followed by the *Micromonospora* and *Streptomyces* species. The species structure of each group was similar, but the proportions of bacterial genera differed. The number of *Micromonospora* spp. was increased significantly during the early stage of incubation, while that of *Bacillus* spp. was increased significantly during the middle stage of incubation. In addition, the similarity between the bacterial species during the middle and end stages of incubation was higher than that between the control group and the initial stage of incubation, which was consistent with the results of the PCoA analysis.

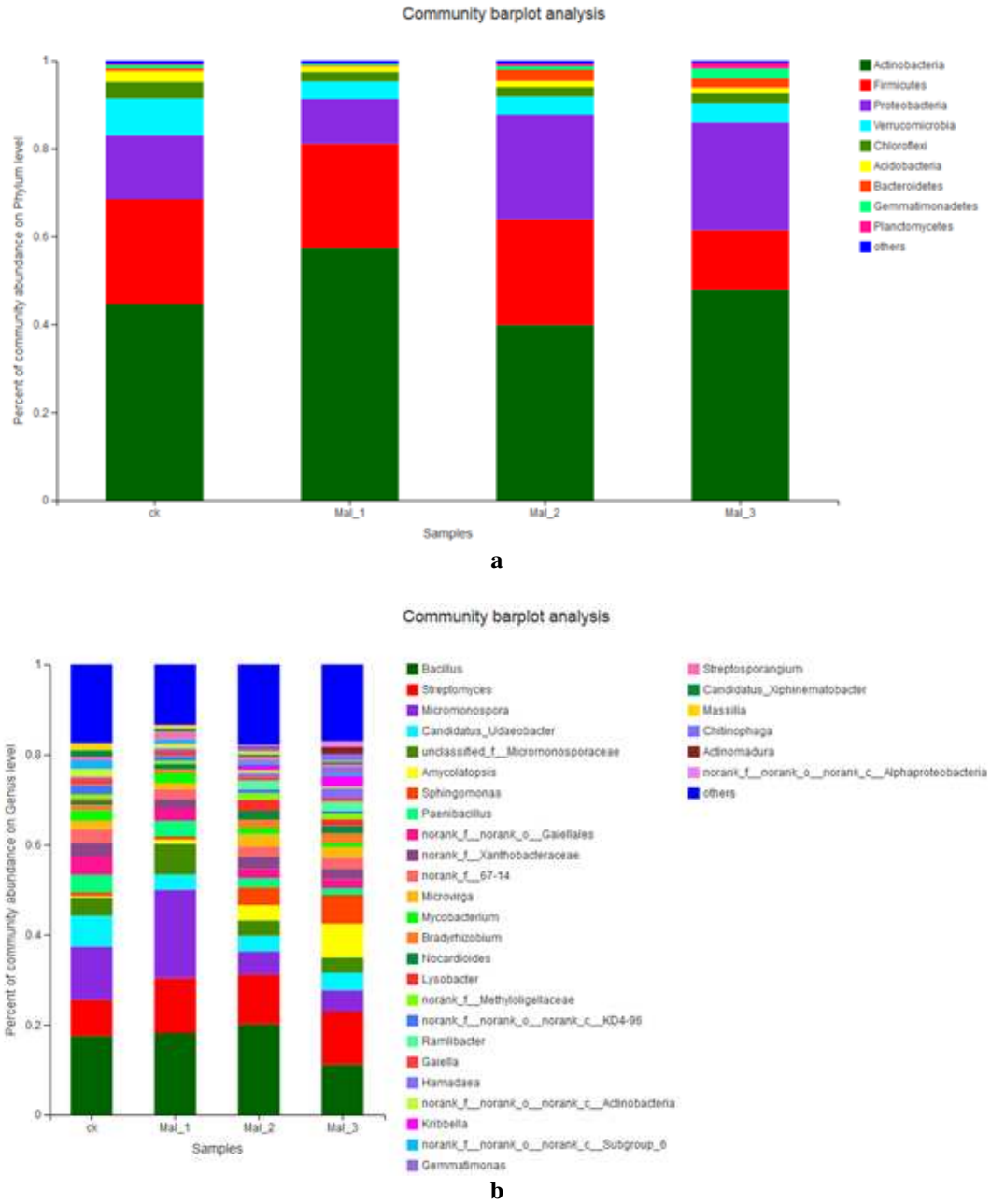

Figure 6. *Bar community composition analysis at (a) phylum and (b) genus levels. Mal-1: this group was incubated for 3 days with malonic acid; Mal-2: this group was incubated for 14 days with malonic acid; Mal-3: this group was incubated for 56 days with malonic acid*

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Table 3. *The bacterial composition at genus level*

Proportion of genus (%)	Group			
	Blank	**Mal-1**	**Mal-2**	**Mal-3**
Bacillus	17.49%	18.18%	20.11%	11.15%
Micromonospora	11.83%	19.61%	5.10%	4.69%
Streptomyces	8.01%	12.18%	11.05%	11.83%
Unclassified_f_Micromonosporaceae	4.06%	6.92%	3.40%	3.41%
Candidatus_Udaeobacter	6.91%	3.36%	3.49%	3.85%
paenibacillus	3.87%	3.49%	2.11%	--
norank_f_norank_o_Gaiellales	4.08%	2.77%	2.06%	--
norank_f_67-14	3.10%	2.17%	2.14%	2.48%
norank_f_xanthobacteraceae	2.92%	2.02%	2.72%	2.37%
Microvirga	1.90%	1.46%	2.82%	2.30%
Mycobacterium	2.24%	2.05%	--	--
Gaiella	1.60%	--	--	--
norank_f_norank_o_ norank_c_KD4-96	1.97%	1.12%	--	--
norank_f_norank_o_orank_c_Actinbaterria	1.72%			
Streptosporangium	--	1.55%	--	--
Bradyrhizobium	--	--	--	2.12%
Nocardioides	--	1.15%	--	--
Hamadaea	--	--	--	2.12%
Sphingomonas	--	--	3.89%	6.40%
Amycolatopsis	--	--	3.42%	7.5%
Lyspbacter	--	--	2.60%	--
Ramlibacter	--	--	1.93%	--
Kribbella	--	--	--	2.28%
Others	28.3%	21.97%	33.16%	40.91%
Total	100%	100%	100%	100%

Blank: The group without incubation and without the addition of malonic acid; Mal-1: the group culture for 3 days with malonic acid; Mal-2: the group culture for 14 days with malonic acid; Mal-3: the group culture for 56 days with malonic acid

Sample species difference analysis

Different significance tests and analyses between species groups were carried out for samples at different incubation times. At the phylum level, the abundance of Actinobacteria, Proteobacteria, Bacteroidetes, Nitrospirae, Gemmatimonadetes, Planctomycetes, and Entotheonellaeota showed significant differences among the four groups (one-way ANOVA, $p < 0.05$) (*Fig. 7a*). At the genus level, Micromonospora spp., Micromonosporaceae spp., Amycolatopsis spp., Sphingomonas spp., Paenibacillus spp., unidentified_Order_Gaiellales, unidentified_Family_Xanthobacteraceae, unidentified_Family_67-14, Microvirga spp., Mycobacterium spp., Bradyrhizobium spp., Nocardioides spp., Lysobacter spp., unidentified_Family_Methyloligellaceae, unidentified_Family_O, and unidentified_C_KD4-96 showed significant or extremely significant differences among the four treatment groups (one-way ANOVA, $p < 0.05$) (*Fig. 7b*).

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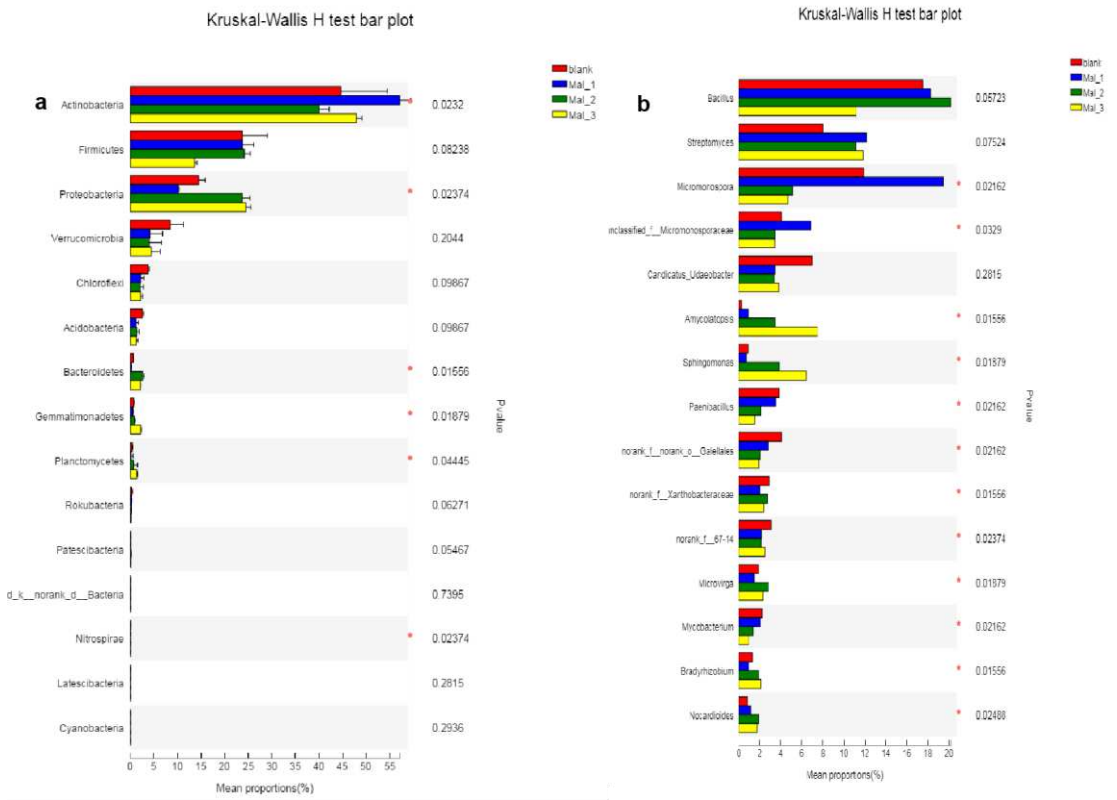

Figure 7. *The analysis of differences between species composition at a, phylum and b, genus levels. Mal-1: this group was incubated for 3 days with malonic acid; Mal-2: this group was incubated for 14 days with malonic acid; Mal-3: this group was incubated for 56 days with malonic acid*

Discussion

Effects of malonic acid addition on soil carbon mineralization and the priming effects

Following the addition of malonic acid, the cumulative respiration of the soil has been increased due to malonic acid mineralization by soil microorganisms, causing SOM to generate a strong positive priming effect. Previous studies have also shown that addition of organic acids into soil can promote mineralization of the original SOM, resulting in positive priming effect (Lonardo et al., 2017). A strong positive priming effect was found after adding oxalic acid to soil (Dutton et al., 1996), and this is possibly because oxalic acid can help fungal basidiomycetes to depolymerize cellulose and lignocellulose. In this study, the priming effect caused by the addition of malonic acid was shown to be positive during the whole incubation period, and remained the highest during the early stage of incubation. This might be due to the fact that during the early stage of incubation, just after the application of FOM to the soil, the dissolved organic carbon (DOC) content remained high and had sufficient energy available, and this led microorganisms to generate strong positive priming effects through co-metabolism while mineralizing the organic acids (Blagodatskaya et al., 2014). As incubation time increases, the DOC content in the soil is gradually decreased, resulting in decreased priming effect (Ma et al., 2018). However, the mineralization peak of malonic acid also occurred during the early stage of incubation, on day 3, before decreasing rapidly. The mineralization rate showed no significant fluctuation thereafter

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until the end of the incubation period. The accumulated malonic acid mineralization was only 4.55% that of the amount added initially, which accounted for relatively low values reported in other studies (Yuan et al., 2015; Wang et al., 2016). This might be because the FOM applied in this study was low molecular weight organic matter, and the substrate utilization efficiency of low molecular weight matter remained very high because microorganisms required no increase in extra-cellular enzyme metabolism during the process of decomposition and did not required not many enzymatic reactions. This in turn reduced the respiratory metabolism of microorganisms, so that their substrate utilization efficiency was low (Bosatta, 1999; Blagodatskaya and Kuzyakov, 2008; Öquist et al., 2017). At the same time, the availability of nutrient elements in the soil also affected their substrate availability. When the nutrient content was high, then the organic matter utilization capacity of microorganisms and the substrate availability were increased (Ågren et al., 2001; Manzoni et al., 2012). Additionally, the total N and P contents of the soil used in this study were very high, in which the N content was about 40 times higher and the P content was about 30 times higher than in other studies (Yuan et al., 2015; Wang et al., 2016). The mineralization rate of malonic acid in this study was therefore low.

Effects of malonic acid addition on the physical and chemical properties of the soil

Experiments on root soil have revealed that the application of organic acids can affect soil pH, thus increasing the availability of nutrients in the soil (Neumann and Römheld, 1999; Jones et al., 2010). In particular, it can affect the P content of the soil and increase the AP levels (Gardner et al., 1983; Marschner et al., 1986). However, in this study there were no significant changes in soil pH and AP after adding malonic acid. But the mineral N content in the soil was increased significantly with increasing incubation period after adding malonic acid, which is possibly due to the release of the mineral N by soil microorganisms while mineralizing SOM (Fontaine et al., 2004). Moreover, the mineral N content of the soil after malonic acid addition was less than that in the control group throughout the incubation period, and this is possibly due to the fact that soil microorganisms consume large amounts of existing mineral N when decomposing additional SOM and malonic acid. At the same time, the DOC content of the soil was increased significantly at the beginning of incubation period after adding malonic acid, and then decreased with increasing incubation time. The addition of FOM has quickly increased the DOC content of the soil, however, the mineralization of available substrate in the soil was increased with increasing incubation time, resulting in the decrease of available substrate in the soil and decrease of DOC content (Mary et al.,1992; Jans-Hammermeister, 1998).

Effects of added malonic acid on soil bacterial community structure

After addition of malonic acid, Actinobacteria were the dominant population, followed by Firmicutes, Proteobacteria and Verrucomicrobia. Actinobacteria were the most abundant during the early stage of incubation, but were decreased significantly during the middle stage of incubation, before rising to a certain extent during the later stage. After Actinobacteria, the population of *Micromonospora* spp. was significantly increased during the early stage of incubation, while *Streptomyces* spp. showed no significant differences in their abundance. Previous studies have also shown that Actinobacteria are dominant during the early stage of incubation (Padmanabhan et al.,

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2003), and this is perhaps because they tend to decompose organic matter more easily and can decompose cellulose at the same time. Firmicutes was the second most abundant microbial population during the early stage of incubation, but showed no significant differences with that during the middle and late stages of incubation. *Bacillus* spp. (Firmicutes) were the most abundant of the entire microflora throughout the entire incubation period, but did not differ significantly in abundance between the early, middle and late stages of incubation. As typical r-strategists, Firmicutes genera form the first breeding pioneer populations after the addition of FOM into the soil. Studies have shown that extracellular enzymes secreted by Firmicutes can decompose FOM and SOM at the same time (Fierer et al., 2007; Pascault et al., 2013). In addition, Chloroflexi and Verrucomicrobia are also r-strategists (Razanamalala, 2018), but no significant difference were observed in their abundance throughout the entire incubation period. We speculate that the priming effect observed might be caused by the co-metabolism of Actinobacteria dominated by *Micromonospora* spp. and bacteria dominated by *Bacillus* spp.

Proteobacteria are a well-known r-strategy group (Padmanabhan et al., 2003; Fierer et al., 2007), and ranked third in abundance throughout the entire incubation process, although their abundance during the early stage of incubation was significantly lower than that during the middle and late stages of incubation. This indicated that this r-strategy group was dominant during the middle and late stages of incubation, and that the positive priming effects were caused by co-metabolism. A study found that the priming effects during the later stage of incubation were related to Acidobacteria and Planctomycetes spp. (Razanamalala, 2018). In this study, the highest abundance of Acidobacteria spp. occurred during the early stage of incubation, while the population abundance of Planctomycetes and Gemmatimonadetes spp. increased significantly with increased incubation time. Previous studies have shown that these three phyla are K-strategists (Fierer et al., 2007; DeBruyn et al., 2011; Pascault et al., 2013). Marilley and Aragno (1999) have found that the abundance of Acidobacteria in rhizosphere soil is lower than that in non-rhizosphere soil (Marilley and Aragno, 1999). In addition, some studies showed that the abundance of Acidobacteria is lower in soil with more available energy (McCaig et al., 1999; Axelrood et al., 2002). However, in our study the highest abundance of Acidobacteria occurred at the end of the pre-incubation period, and was decreased after addition of malonic acid. This showed that when the available energy in the soil increases, the r-strategists in the soil increase and propagate vigorously, resulting in the loss of competitive advantage of K-strategists and their population decline (Héry et al., 2005). However, some studies have shown that Gemmatimonadetes can mineralize the mineral N by using some FOM or easily decomposed SOM in soil as an energy source (Razanamalala,). Therefore, in this study, we speculated that during the later stage of incubation, when energy and nutrient reserves in the soil became gradually consumed, the competitive advantage of K-strategists (Planctomycetes and Gemmatimonadetes) in the soil was relatively enhanced (Fontaine et al., 2004). This caused a priming effect through N mining mechanism, wherein the mineral N content during the later stage of incubation was significantly higher than during the early stage of incubation. Therefore, we speculate that the later priming effects were caused by the co-metabolism of r-strategy species, which are dominated by Proteobacteria, while the positive stimulation of K-strategy species, which are dominated by Gemmatimonadetes and Planctomycetes, resulted due to increased N availability.

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Conclusion

The addition of malonic acid significantly increased CO_2 respiration in dark brown soil. The SOM had a positive priming effect, but the cumulative mineralization of malonic acid remained low. The addition of malonic acid did not affect the soil pH and effective P, but increased the mineral N content of the soil. The addition of malonic acid had no significant effect on various species of soil bacteria and microorganisms present, but the relative abundance of each species was significantly affected with incubation time. In conclusion, addition of malonic acid had a strong priming effect on the original organic C content of dark brown soil, which was not only related to the physical and chemical composition of the soil, but also to the microbial populations in the soil and the changes in soil microbial community composition. Future studies should focus on the effect of malonic acid addition on the diversity of soil fungi, and the interactions between bacteria and fungi in order to further reveal the mechanisms behind the priming effects of organic acids in root exudates on soil organic C.

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THE COASTAL DISTANCES AND TENDING OPERATIONS INFLUENCE THE LEAF TRAITS AND LEAF ALLOMETRY OF BAMBOO *DENDROCALAMUS MINOR* VAR. *AMOENUS*

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Abstract. Exploring the variation of functional traits and leaf allometry under different tending operations and distances to seashore would aid in understanding the plant's response and adaptation to the environment as well as provide a suggestion for windbreak construction. As a result, communities of *Dendrocalamus minor* var. *amoenus* were selected in Chishan forestry farm, Dongshan Island, China, to determine their leaf traits and leaf allometry under different tending operations, at various distances to the seashore. The results showed that leaf area, length, width, calorific value, C and N contents in the plot under tending operations were significantly higher than that in the plot near to seashore without tending operations and the plot far away to seashore without tending operations (P<0.05). Tending operations and distance to seashore had effects on the leaf traits and leaf allometry. There was a trade-off between leaf morphological traits and leaf dry weight due to the changes in the allocation of resources and energy, caused by tending operations and distances to the seashore. We conclude that tending operations and distances to the seashore have a high impact on leaf morphological, functional traits by balancing the relationship between energy and growth.
Keywords: *functional traits, distance to seashores, tending operations, allometric growth, bamboo, sandy coast*

Introduction

China has a length of ca. 5948 km, of which the length of the sandy coastline is about 312 km (Department of Natural Resources, 2018). Plants growing on sandy coasts have to tolerate coastal stresses, such as salt spray, poor soil nutrients, and sand burial (Griffiths and Orians, 2004; Lin et al., 2017). Blowing wind and salt spray in the coastal area are prone to affect the morphological and physiological traits of plants (Griffiths and Orians, 2004; Pompeiano et al., 2016). Wind speed, salinity, and salt spray show a decreasing trend with increasing distance to the seashore (Lin et al., 2017; Huang et al., 2020). Different distances from the seashore have a significant effect on plant functional traits; For instance, plants near the coast are shorter (Todo et al., 2019) and have higher branching wood density (Lin et al., 2017). Environmental gradients have resulted in a large amount of intraspecific trait variation in species (Kumordzi et al., 2019).

Plant functional traits refer to traits that can easily respond to the environment and indicate plant growth, development, and reproduction (Cornelissen et al., 2003). Plant evolution and the environment have jointly shaped its growth and development (Chitwood and Sinha, 2016). The leaf is a representative organ that is quite sensitive to environmental changes and can generate phenotypic plasticity in response to abiotic stresses (Xu et al., 2009). Leaf functional traits can explain plants' response to past and future climate change (Heilmeier, 2019). Therefore, the leaves have been selected as a study object in most studies. The intensity of human disturbance affects the functional

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traits (Carreño-Rocabado et al., 2012; Yamashina et al., 2021). The response of communities to disturbances (direct human disturbances, biological pressure, environmental changes) can be predicted and quantified through leaf functional traits (Mouillot et al., 2013). The leaf construction cost is used to evaluate plant energy allocation, which indicates the adaptation strategy of the plant to the environment (Penning et al., 1974; Villar and Merino, 2001). Besides, morphological indexes and functional traits would change with the gradient environment (Guo et al., 2018). Therefore, functional traits, morphological indexes, and construction cost would be selected as indicators that evaluate the adaption of plants in previous studies.

Some scholars believe that appropriate forest management practices or cultural operations can mitigate the effects of coastal stress on plants, such as watering (Boch et al., 2020). In the recent past, the effects of altitudes, topography, climatic regions and distance from the seashore on the leaf functional traits have been reported (Guo et al., 2018; Pardos et al., 2021; Zhang et al., 2021; Zheng et al., 2021b), but there have been relatively few studies on the effects of various distances from the seashore and tending operations on the leaf functional traits.

The genetic and environmental factors influence the relationships between leaf traits (Granier and Vile, 2014). Evidence suggests that allometric growth relationships among traits can reveal plant adaptive strategies to the environment and forecast future climate responses (Zhang et al., 2021). The altitudinal patterns of leaf characteristics indicate an allometric growth relationship, but a trade-off between branch diameter and length by adjusting the allometric growth relationship to match the gradient distance to the seashore needs further investigation.

Bamboo species with highly ornamental value have high phenotypic plasticity and can improve soil quality compared to bare land (Zheng et al., 2020, 2021a). Bamboo plants have complex branching characteristics and have a good potential to adapt to coastal environments (Bamboo Phylogeny Group, 2012). More than 20 years ago, a large number of bamboo species were introduced to the sandy coast of Dongshan Island in Fujian Province, China for windbreak and landscape greening. Among them, *Dendrocalamus minor* var. *amoenus* is a typical bamboo species suitable for the sandy coast. This species grows well and is widely planted with strong adaptive ability. In this study, we evaluated the leaf traits and allometric growth relationship of *Dendrocalamus minor* var. *amoenus* under the various distance to the seashore and tending operations conditions, revealing the response of *Dendrocalamus minor* var. *amoenus* to diverse conditions, and providing a scientific basis for windbreak construction and tending operations.

Material and Methods

Study area

The study site is located at 23°40'N, 118°18'E, Dongshan Island, Fujian Province, China. The climate of the study site is a subtropical maritime monsoon climate. The mean annual precipitation and evaporation are 1113.9 mm and 2013.2 mm, respectively. The annual temperature ranges from 3.8 °C to 36.6 °C (Mean value is 20.8 °C) (Kong, 1999). The age of bamboo stands is similar, which is planted in 2016~2018. Three sample plots were selected with various distance and tending operations (*Table 1*). The DMY plot (near to seashore with tending operations) was established with NPK compound fertilizer (0.2 $kg \cdot m^{-2}$), and usual practices like weeding, cleaning, and

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irrigation were also performed. However, the HB plot (near to seashore without tending operations) and LM plot (far away to seashore without tending operations) plots were not nurturing. Each sample plot was divided into three sub-sample plots (20 m×20 m) where the morphological traits (base diameter, height, density) of *Dendrocalamus minor* var. *amoenus* and distance from the seashore were determined (*Table 1*).

Table 1. *The situations of Dendrocalamus minor var. amoenus*

Sample plot	Base diameter/(cm)	Height/(m)	Density/(Int/cluster)	Tending operations	Distance to seashore/(m)
DMY	3.24±0.43	7.43±0.73	19.80±6.16	Yes	50~80
HB	2.92±0.49	9.31±2.05	8.38±3.29	No	80~100
LM	3.26±0.89	10.31±1.33	39.25±45.42	No	500~800

Determination of functional traits

The 5~10 plants were randomly selected from each sub-sample plot (20 m×20 m) from July to August 2019. The healthy, fully expanded, unshaded leaves were collected from different directions and positions of the crown layer to determine their fresh weight and thickness. The area, length, and width of leaves were measured using Image J. The leaves were immersed in distilled water for 12 h under dark conditions and their saturated fresh weight was determined. The leaves were dried in an oven at 105 ℃ for 0.5 h and then placed in an oven at 80 ℃ for about 72 h until constant weight. The equations used to assess the specific leaf area (SLA, $cm^2 \cdot g^{-1}$) and Leaf dry-matter content (LDMC, $g \cdot g^{-1}$) have been described in Pérez-Harguindeguy et al. (2013).

The 1.0 g leaf powder was ashed at 700 ℃ for 6 h. The mass of ash was measured after cooling. The caloric value per gram was determined using a PC bomb calorimeter (HWR-15E, China). The ash-free caloric value(AFCV), CC_{mass} (Leaf construction cost per unit mass), and CC_{area} (Leaf construction cost per unit area) were calculated according to Williams et al. (1987).

$$\text{Ash } content\,(\%) = Ash_{mass} / Sample_{mass} \times 100\% \quad \text{(Eq.1)}$$

$$\text{AFCV}\,(\text{kJ} \cdot \text{g}^{-1}) = \text{CV}/(1 - \text{AC}) \quad \text{(Eq.2)}$$

$$CC_{\text{mass}} = [(0.06968AFCV - 0.065)(1 - AC)] + 7.5 \times (kN_{mass} / 14.0067)] / EG \quad \text{(Eq.3)}$$

$$CC_{\text{area}} = CC_{\text{mass}} / SLA \quad \text{(Eq.4)}$$

where AC: Ash content; AFCV: Ash-free caloric value; EG: The deviation of growth efficiency. The value of EG was estimated to be 0.87 (Penning et al., 1974); N_{mass}: Nitrogen content per unit mass; k: the oxidation state of the nitrogen content. The value of k was estimated to be 5 for the oxidation.

Allometric growth analysis

The allometric growth analysis was performed to test the relationship between the allocation of functional traits under the different distances and tending operations. Before allometric growth analysis, the log-transformation of all data values was

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conducted. The log-transformation is useful for testing the proportional relationships in allometric scaling. The standardized major axis (SMA) was performed to calculate the slope and elevation of the relationship between leaf traits.

$$\log Y = \log \beta + \alpha \log X \quad \text{(Eq.5)}$$

where Y refers to the dependent variable, X refers to the independent variable; β represents the elevation and α is the slope of the allometric scaling. If the slope of these relationships were not significantly different from |1.00|, they would be isometric. If the slope of these relationships were significantly different from |1.00|, they would be allometric.

Data analyses

The data presented in the current study is Mean ± SD. One-way ANOVA and pairwise comparison tests (Tukey's method) were performed to test the difference of leaf morphology, functional traits and construction cost among these sample plots. All the statistical analyses were performed using R v3.6.1 and allometric growth analysis was conducted using the Smatr package (Warton et al., 2012). The graphical illustration was done by using Origin 2013 (Origin Lab, USA).

Results

Leaf morphological traits under different conditions

Leaf area, length, and width were significantly higher in the DMY plot than that of the HB plot and LM plot (P<0.05, *Fig. 1*). There was no significant difference in leaf area, leaf length, leaf width, and L: W between HB and LM plot (P>0.05). Leaf thickness in the DMY plot was significantly higher than that in the HB plot (P< 0.05).

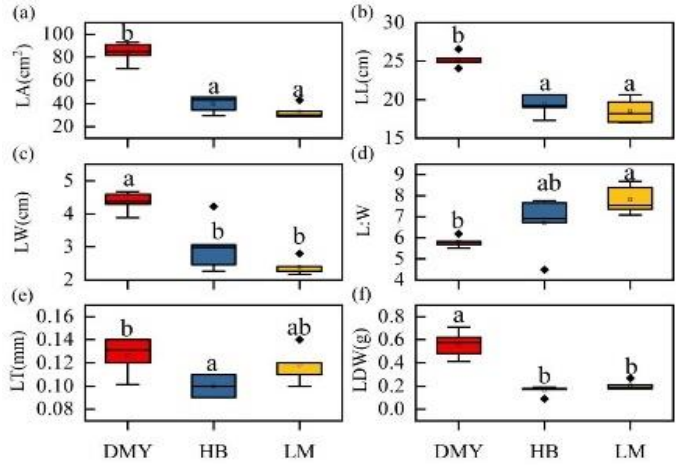

Figure 1. *The morphological traits under different conditions. Where DMY represents the plot near to seashore with tending operations; HM represents the plot near to seashore without tending operations; LM represents the plot far away to seashore without tending operations. (a) LA: leaf area; (b) LL: leaf length; (c) LW: leaf width; (d) L:W represents leaf length: leaf width; (e) LT: leaf thickness; (f) LDW: leaf dry weight. Different letters indicate the significant differences between different conditions (P>0.05)*

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Functional traits under different conditions

In terms of functional traits, no significant differences in leaf dry matter contents (LDMC) were observed among the three sample plots (P>0.05). However, SLA and ash contents in the HB plot were significantly (P<0.05) higher (253.25 $cm^2 \cdot g^{-1}$ and 7.57%, respectively) relative to other sample plots. Besides, the leaf calorific value in the DMY plot was significantly higher (18.62 $kJ \cdot g^{-1}$) than that of the HB and LM plot (P<0.05, *Fig. 2*).

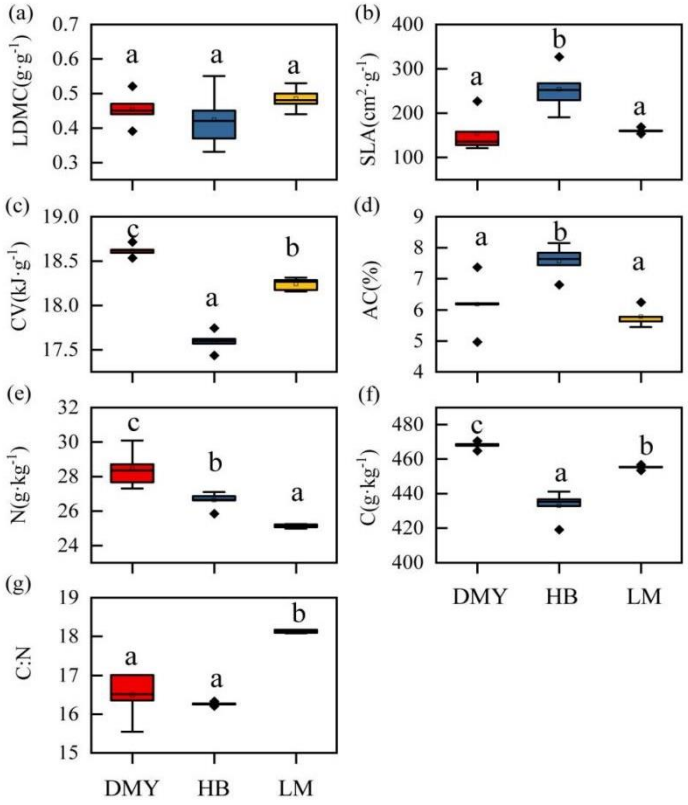

Figure 2. *Leaf C and N contents and C: N under different conditions. Where DMY represents the plot near to seashore with tending operations; HM represents the plot near to seashore without tending operations; LM represents the plot far away to seashore without tending operations. (a) LDMC: leaf dry matter content; (b) SLA: specific leaf area; (c) CV: calorific value; (d) AC: ash content; (e) N: nitrogen content; (f) C: carbon content; (g) C:N: carbon content: nitrogen content. Different letters indicate the significant differences between different conditions (P>0.05)*

Leaf C and N contents in the DMY plot were significantly higher than that of the HB and LM plot (P<0.05, *Fig. 3*). Leaf C contents in HB plot reduced significantly compared to LM plot (P<0.05), whereas leaf N contents in HB plot were significantly higher than that of LM plot (P<0.05). In addition, the C: N in the LM plot was the highest (18.13).

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The construction cost under different conditions

The CC_{mass} in the DMY plot was the highest, reaching up to 10.20 g glucose·g^{-1}, while CC_{mass} in the LM plot was the lowest (9.15 g glucose·g^{-1}, *Figure 3*). Furthermore, the CC_{area} in the HB plot was the lowest (389.68 g glucose·m^{-2}).

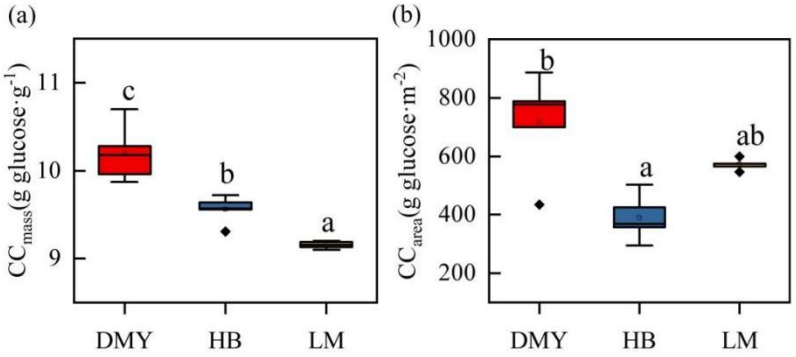

Figure 3. *The leaf construction cost under different conditions. Where DMY represents the plot near to seashore with tending operations; HM represents the plot near to seashore without tending operations; LM represents the plot far away to seashore without tending operations. (a) CC_{mass}: Leaf construction cost per unit mass; (b) CC_{area}: Leaf construction cost per unit area*

The allometric growth relationship

There was a significant allometric growth relationship between leaf area and leaf width in the three sample plots (P<0.05, $P_{1.0}$<0.05, see *Table 2*). Leaf area was significantly related to length, width, and L: W in HB and LM plot with allometric growth (P<0.05, $P_{1.0}$<0.05). Leaf area was not correlated with leaf thickness (P<0.05). The relationship between leaf area and thickness was isometric growth ($P_{1.0}$>0.05), which suggests the relationship will not change with different conditions.

Table 2. *The allometric relationship between leaf area and other leaf functional traits at different conditions*

Y	X	Conditions	*df*	R^2	Slope	5% CI	95% CI	Elevation	$P_{1.0}$-value	*P*-value
Leaf area	Length	DMY	62	0.16	1.19	0.95	1.50	0.24	0.133	0.001
		HB	101	0.72	1.99	1.80	2.21	-0.98	0.000	0.000
		LM	151	0.66	1.94	1.77	2.14	-0.96	0.000	0.000
	Width	DMY	62	0.89	1.80	1.66	1.96	0.76	0.000	0.000
		HB	101	0.80	1.59	1.45	1.73	0.89	0.000	0.000
		LM	151	0.72	1.57	1.44	1.71	0.91	0.000	0.000
	L:W	DMY	62	0.05	-1.16	-1.48	-0.90	2.77	0.245	0.078
		HB	101	0.07	-1.89	-2.28	-1.56	3.18	0.000	0.009
		LM	151	0.04	-1.57	-1.84	-1.34	2.89	0.000	0.015
	Leaf thickness	DMY	62	0.01	2.77	2.15	3.56	4.34	0.000	0.576
		HB	101	0.00	-1.93	-2.35	-1.58	-0.38	0.000	0.816
		LM	151	0.00	-3.21	-3.76	-2.73	-1.59	0.000	0.857

Where DMY represents the plot near to seashore with tending operations; HM represents the plot near to seashore without tending operations; LM represents the plot far away to seashore without tending operations. *df* represents degrees of freedom; R^2 represents determination coefficient; Slope represents scaling slope. 5% CI represent 5% confidence intervals; 95% CI represent 95% confidence intervals. $P_{1.0}$- value means the significance of the SMA slope value being different from |1.00|

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There was a significant allometric growth relationship between leaf dry weight and leaf length at DMY plot (P<0.05, $P_{1.0}$<0.05); leaf dry weight and leaf width, leaf dry weight, and leaf area in HB plot (P<0.05, $P_{1.0}$<0.05, see *Table 3*). Leaf dry weight has a significant allometric growth relationship with leaf thickness in all plots (P<0.05, $P_{1.0}$<0.05).

Table 3. *The allometric relationship between leaf dry biomass and other leaf functional traits at different conditions*

Y	X	Conditions	*df*	R^2	Slope	5% CI	95% CI	Elevation	$P_{1.0}$-value	*P*-value
Leaf dry weight	Length	DMY	62	0.07	1.59	1.24	2.02	-2.41	0.000	0.040
		HB	101	0.01	2.45	2.02	2.98	-3.85	0.000	0.221
		LM	151	0.00	-2.20	-2.58	-1.87	2.07	0.000	0.565
	Width	DMY	62	0.00	-2.40	-3.08	-1.86	1.30	0.000	0.959
		HB	101	0.04	1.95	1.61	2.37	-1.55	0.000	0.049
		LM	151	0.00	-1.77	-2.08	-1.51	-0.05	0.000	0.977
	L:W	DMY	62	0.06	1.54	1.21	1.96	-1.38	0.001	0.043
		HB	101	0.01	-2.32	-2.82	-1.91	1.26	0.000	0.245
		LM	151	0.00	-1.78	-2.09	-1.52	0.89	0.000	0.662
	Leaf thickness	DMY	62	0.25	3.68	2.96	4.58	3.04	0.000	0.000
		HB	101	0.04	2.37	1.96	2.88	1.69	0.000	0.046
		LM	151	0.05	3.63	3.10	4.24	2.78	0.000	0.007
	Leaf area	DMY	62	0.00	1.33	1.03	1.71	-2.74	0.026	0.886
		HB	101	0.04	1.23	1.02	1.49	-2.65	0.034	0.043
		LM	151	0.00	-1.13	-1.33	-0.96	0.98	0.132	0.674

Where DMY represents the plot near to seashore with tending operations; HM represents the plot near to seashore without tending operations; LM represents the plot far away to seashore without tending operations. *df* represents degrees of freedom; R^2 represents determination coefficient; Slope represents scaling slope. 5% CI represent 5% confidence intervals; 95% CI represent 95% confidence intervals. $_{P1.0}$- value means the significance of the SMA slope value being different from |1.00|

Discussion

Effect of tending operations on leaf morphology, functional traits, and construction cost

The current research was designed to assess the impacts of various distances and tending operations on the plant communities around the coastal line. We hypothesized that the tending operations would affect leaf morphology, functional traits, and construction cost. In this study, various traits such as leaf area, length, width, leaf thickness, leaf dry weight, leaf C and N content, calorific value, CC_{mass}, and CC_{area} in DMY plot were significantly higher than that observed in the HB plot (P<0.05), which indicates that tending operations have prompted larger leaves with higher carbon fixation capacity, calorific value and nutrient content. The thicker and higher N contents under tending operations facilitate resistance to physical damage and high water use efficiency (Wright et al., 1993). On the contrary, previous studies have reported that the thicker leaves reduce the N content (Pérez-Harguindeguy et al., 2013). Tending operations alters the correlation of traits by coupling coordination or combination (Baraloto et al., 2010; Freschet et al., 2010), It has been evident in previous reports that tending operations affect the functional traits of the vegetation (Carreño-Rocabado et al., 2012; Yamashina et al., 2021), and the soil organic matter has been the main factor

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influencing the variation in functional traits under different tending operations (Chen et al., 2018). Consequently, tending operations would supply nutrients to the soil, resulting in C and N content in leaves increased, photosynthesis, and carbon fixation increased.

SLA reflects the ability of plants to absorb resources from the environment, and it is positively correlated with the potential reproduction rate (Pérez-Harguindeguy et al., 2013). SLA and ash content in the DMY plot were significantly lower than that observed in the HB plot (P<0.05). The thicker and lower SLA would reduce water evaporation from the leaves, alleviate the damage caused by high temperatures, and drought stress in this research in this research in this research (Comstock and Mencuccini, 1998). It's the ecological strategy of *Dendrocalamus minor* var. *amoenus* with thicker leaf and lower SLA under tending operations in the DMY plot. SLA was negatively correlated with leaf longevity (Pérez-Harguindeguy et al., 2013). The high SLA in the HB plot had relatively shorter leaf longevity and lower CC_{mass} than that in the DMY plot. Leaf construction cost is used to evaluate plant energy allocation, which indicates the adaptation strategy of the plant to the environment (Penning et al., 1974; Villar and Merino, 2001). *Dendrocalamus minor* var. *amoenus* under tending operations had thicker leaves, higher CC_{mass}, and lower SLA, which suggest with the tending operations, *Dendrocalamus minor* var. *amoenus* have higher nutrient contents and water use efficiency, while relatively slow potential reproduction rate enhances resistance to physical damage (e.g., wind stress).

Effect of distances to the seashore on leaf morphology, functional traits, and construction cost

The distance to the seashore would affect the plant morphology (Todo et al., 2019). Previous research has shown that different distances to the seashore with gradient coastal stress influence branching functional characteristics (Lin et al., 2017; Zheng et al., 2021b). For instance, leaf C contents of *Pinus thunbergii* decreased with distance to seashore increased, however, C: N would not change with various distances (Sun et al., 2019). On the contrary, the leaf C content, and C: N of *Dendrocalamus minor* var. *amoenus* increased as the distance from the seashore increased. The inequalities in findings may be attributed to the various soil types and conditions. The plant absorbs the C content from the soil and in the current study, the C contents in sandy soil increased as the distance to the seashore increased. It is also possible that plant communities located far from the seashore would attract a large number of seabirds, and owing to their droppings the leaf litter would increase the C content in the soil (Huang et al., 2020).

The N contents in leaves are used primarily to construct proteins, which has been highly correlated with energy investment and construction cost (Griffin et al., 1996; Zhang et al., 2019). The leaf N contents and CC_{mass} decreased with increasing distance from the seashore. However, the relationship between leaf construction costs and the environment is controversial. Some scholars suggest that leaf construction costs increase when plants are subjected to environmental stress (Villar and Merino, 2001; Fortunel et al., 2012; Matías et al., 2012), and others suggest that plants increase their adaptability to environmental stress by reducing leaf construction costs (Suárez, 2003; Falcão et al., 2017). In this research, we found that when plants were subjected to environmental stress, the cost of leaf construction increased. The distance to the seashore is negative with coastal stress (Lin et al., 2017). The greater the distance from the seashore, the richer the environmental resources, and therefore the lower

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construction cost of *Dendrocalamus minor* var. *amoenus*, which needs less energy to develop plant tissues. However, the plant established closer to the seashore, requires more energy to develop tissues and resist environmental stress.

Tending operations and distances to the seashore on the allometric growth of leaf morphology

The mechanism of leaf size and adaptive strategy is generally considered to be highly related to the balance in leaf energy (Niinemets et al., 2006). The study of the allometric growth relationships of leaf morphology under different environmental conditions can help to understand the mechanism of how plants adapt to environmental stress. Plants adapt to environmental patterns by changing the allometric growth relationships of morphological traits (Guo et al., 2018). In this research, there was no correlation between leaf area and leaf thickness even though the environment was altered. Previous studies have suggested the correlations of some traits did not change with the environment, indicating strong covariance relationships of the traits (Chave et al., 2014; Zhang et al., 2015). The changes in leaf morphology indicate the adaptation strategies of plants to environmental resources; the longer and broader leaves are beneficial to resource acquisition and conservation (Xu et al., 2009). To adapt to the poor environment, leaf morphological traits in HB and LM plots adjust allometric growth relationships. The growth relationship between leaf length, width, and L: W with leaf area is significantly allometric in HB and LM plot (P<0.05, $P_{1.0}$<0.05), and leaf area was increased by adjusting the leaf morphological traits to increase photosynthetic efficiency to adapt to coastal pressure.

The leaf dry weight and leaf thickness exhibited the allometric growth relationships among different conditions. The slopes in the HB plot were low, and the slopes in the DMY plot and LM plot were relatively higher and similar, which suggests that tending operations and distance to seashore have a similar effect on allometric growth relationships between leaf dry weight and leaf thickness. Previously, studies have shown that the allometric growth relationships exist between leaf dry weight and leaf morphology in *Pleioblastus amarus* at different elevation gradients (Guo et al., 2018). Various distances to the seashore cause diversified environments, resulting in significant allometric growth relationships among traits (Zheng et al., 2021b). There were significant positive allometric growth relationships between leaf dry weight and leaf length, L: W, and leaf thickness in the DMY plot (P<0.05, $P_{1.0}$<0.05), and there were significant positive allometric growth relationships between leaf dry weight and leaf thickness, leaf width, and leaf area in HB plot (P<0.05, $P_{1.0}$<0.05). There was a significant positive allometric growth relationship between leaf dry weight and leaf thickness in the LM plot (P<0.05, $P_{1.0}$<0.05). The soil nutrient and water contents can be improved under tending operations, and various distances to the seashore are highly correlated with environmental resources (Lin et al., 2017; Huang et al., 2020). There was a trade-off allocation of resources and energy between leaf morphological traits and leaf dry weight in *Dendrocalamus minor* var. *amoenus*, which would change the allometric growth relationship between traits. Larger leaves will be more efficient for photosynthesis and might fix more carbon assimilation products, which helps the plant to resist abiotic stresses.

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Conclusion

Tending operations changed the correlation between traits, supplied more nutrients to the plant, and also increased the plant's resistance to coastal stress. The plant grows in places far away from the seashore with lower coastal pressure and construction costs. Tending operations and distances to the seashore have a high impact on leaf morphological, functional traits of *Dendrocalamus minor* var. *amoenus* by balancing the relationship between energy and growth. Future studies can focus on revealing the determining environmental factors of the functional traits and allometric growth relationships among various distance and tending operations. These findings would provide a reference for windbreak construction, and it also reveals the ecological meaning of plants adapting to the coastal environment.

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ANTIBACTERIAL ACTIVITY AND MOLECULAR CHARACTERISTICS OF INDIAN OLIBANUM (*BOSWELLIA SERRATA)* PHYTOCHEMICALS: AN IN SILICO APPROACH

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Abstract. An unhealthy community is a reservoir for the global threat of Nosocomial infection. Rapidly escalating drug resistance and nosocomial infections resulting from the search for a novel drug is a tough challenge to upcoming researchers. Indian olibanum extract is held in high regard in the field of traditional medicines. To evaluate the antibacterial properties of *B. serrata* aqueous and hydroethanolic extract was taken with an in silico approach using Autodock vena tool. The nosocomial pathogens were detected from wound and skin samples in the Hospitals and from around Namakkal. Among the gram-negative nosocomial pathogen *Klebsiella oxytoca* and gram-positive *Staphylococcus aureus* were branded by microscopic and biochemical profiling. In addition, the susceptibility and resistance to antibiotics were explored for *K. oxytoca* and *S. aureus* in the case of tetracycline (21 mm and 28 mm), chloramphenicol (23 mm and 16 mm), and gentamycin (14 mm and 21 mm) respectively. The seven bioactive compounds were attained by GCMS. The inhibition zone of 18 mm at 60 µg deliberation for both the isolates viz *K. oxytoca* and *S. aureus.* In silico approach of phyto-bioactives with Beta-lactamase (AmpC) was investigated by Autodock vena tool. In silico Molecular docking approach examined the binding energy as -7.6 (kcal/mol) for Cholan-24-oic acid, 3,12-bis(acetyloxy) followed by Pyrene, hexadecahydro- -7.5 (kcal/mol), 5(1H)-Azulenone, 2,4,6,7,8,8a-hexahydro-3,8-dimethyl-4-(1-methylethylidene)-, (8S-cis)- -6.7 (kcal/mol). The conclusion of the finding is that phyto-bioactives of *B. serrata* also promote natural healing and antibacterial potential.
Keywords: *bioactives, phyto-extracts, natural healing, nosocomial, molecular docking*

Introduction

Hospital-acquired nosocomial infection and drug resistance is a saddle in the health, medical field and this is a global health crisis (Patil and Rathod, 2020; Vakayil et al., 2021b). The antibiotic drugs are currently regained by the bioactive compounds of plants (Ansari et al., 2019) in this approach, the *B. serrata* achieves its significant place and exploring its usefulness towards antibacterial activity. The ancient (pre-historical) times used Boswellic acid contain infinite bioactives that are pharmacologically used for therapeutics (Sharma et al., 2016). *B. serrata* was well known in herbal treatment hundred years ago. Boswellic acid consists of two components α and β boswellic acid originating from resin, which is the primary component of *B. serrata*. Resin from the

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bark of the tree is rich in antioxidants. B. serrata is commonly known as Indian frankincense or Indian olibanum because the dried exudates from the bark of the tree are an ole-gum-resin. Dried gum looks like lumps or tears and is ivory (yellow and white) in colour. 'Pure incense" is another name for B. serrata derived from the French language. Olibanum means white or cream derived from Arabic languages so it is named as "olibanum". The bioactive compounds offer their effectiveness towards the ailments. In the antique era, the therapeutic value of natural medicine was unknown at global level. In modern days the numerous researcher studies explored the economical and therapeutic significance of this species (Niphadkar et al., 2017). The pharma of plants probed the importance of the species implicit, enormously cultivated medicinal properties wide-ranging of bioactive compounds are obtaining best prognosis (Fan et al., 2021; Sun et al., 2021). According to the agriculture sector, the production of B.serrata is not adequate, but somehow it reduces the virulence of the organism causing disease. Whaterver ailments occur to our body boswellic acid is the right choice (Niphadkar and Rathod, 2018). Thus, the salai gaugal heals inflammation, breathlessness, blood disorders, painful joints, heart, inability, malignants, and stomach upsets. In silico approach such as auto dock vena tool also helps us to briefly clarify the drug molecule and the receptor interactions by three-dimensional structures and its efficacy are exposed in the form of binding energies thus the healing process of an apparent wound (Chou et al., 2017) is preciously and powerfully treated and attained an expectational result by this phytotherapy which was conjoint with metal microparticles.

Methodology

Resin collection

Purchased wrinkled, golden amber-colored *B. serrata* resins from Kolli Hills, Namakkal DT, Tamil Nadu, India. After surface sterilization, it was dried in the shadow and then made into powder for extraction (Vakayil et al., 2021a).

Extraction of B. serrata

10 g of powdered resin was weighed and mixed with 100 ml of hydroethanolic and aqueous solvent in a 250 ml flask. Through the microwave method, it was extracted by placing the beaker on the circular plate. Maintaining the different parameters such as temperature and time the aqueous phase was obtained after filtering via Whatman filter paper no1 (Kumar et al., 2016). After that, the crude was placed in an icebox, and the container was sealed for future analysis.

GC-MS

Samples (solid and liquid) in GC–MS were prepared by dissolving inappropriate solvents (polar and nonpolar). International union of pure and applied chemistry (IUPAC) of the compound, Morphology, Molecular weight (MW), length, and internal diameter all are analyzed using the mass spectral library (NIST software) (Bhutada et al., 2017).

Collection of strains

Sufficient exudates of the wound and skin specimens using sterile needles, containers, and tubes, were procured from chronic wound beds without cross-

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contamination (Ohemu et al., 2018). Nearly twenty samples were collected from different laboratories in Namakkal DT, Tamil Nadu, India. Then sealed aseptically and transported to the laboratory in time (Kabeerdass et al., 2021).

Inoculation and screening

Isolation of the sample is done by inoculating it in their selective media such as MSA, and EMB and incubated at 37 °C for 24 h (Asante-Kwatia et al., 2020). Smear was prepared for gram staining to observe the morphology of the organism. Crystal violet, mordant, alcohol, and safranin are sprinkled one after the other for a minute, dried, and observed under the microscope to note whether it was a gram +ve or gram –ve. To confirm its physiological state some biochemical tests like Methylred, Voges Proskauer, indole, citrate oxidase, urease coagulase, and catalase were done (Vakayil et al., 2019).

Antibiotic sensitivity test

In the MHA plate, both gram-positive (*S. aureus*) and gram-negative (*K. oxytoca*) were streaked in a zig-zag manner on the plate. A circular antibiotic disc (6 mm) was impregnated on the plate (Vahabi et al., 2019*).* The plate was spread with *K. oxytoca* and *S. aureus* (nosocomial pathogens) against a spectrum of drugs such as Meropenem, vancomycin, and tetracycline (Abirami and Maghimaa, 2019).

Antibacterial activity

A diverse concentration of resin crude (20 µg, 40 µg, and 60 µg) was added to each well which was made by the cork Borell of 6 mm, then incubated for 24 h and the MIC values were observed and tabulated (Maghimaa and Alharbi, 2020; Arora et al., 2020; Kabeerdass et al., 2021; Vakayi et al., 2021f).

Ligand preparation

The structural (Schmiech et al., 2019) geometry in GC–MS were observed through the database and some chemical parameters like bond length, ionization energy, radius, efficient charges, strengths were ruled out (Khan et al., 2016). The efficacy of the antibiotics used and the three-dimensional structures of the compounds were identified in PDF format via molecular docking.

Molecular docking

Docking was used to calculate the protein-ligand chemistry (Taghizadeh et al*.,* 2018), torsion of the atom, van der Waals forces, its positions, the conjoining of the hydrogen atom (Du et al., 2015) between the un polar structure through the in silico mol soft tool Autodock vena (Bairwa and Jachak, 2016; Bolbolian et al., 2018; Vakayil et al., 2021d; Gaillard, 2018; Baburam et al., 2021).

Results

GC–MS analysis

A stream of seven plant-derived compounds in the hydroethanolic extract from the crude of *B. serrata* via GC-MS is shown in *Table 1*.

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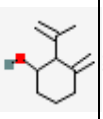
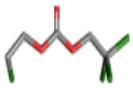
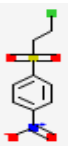
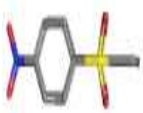
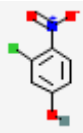
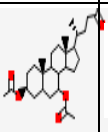
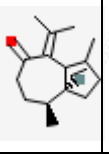
Table 1. *Phyto bioactive Compounds identified through GC–MS analysis of B. serrata*

S. No	Name of the compound	Retention time	Molecular formula	Molecular weight	Peak area	PUB CHEM ID	2D structure	3D structure
1.	o-Mentha-1(7),8-dien-3-ol	21.352	$C_{10}H_{16}O$	152.23 g/mol	2.67	564552		
2.	Carbonic acid, 2-chloroethyl 2,2,2-trichloroethyl ester	22.063	$C_5H_6Cl_4O_3$	255.9 g/mol	1.26	87646995		
3.	Benzene, 1-[(2-chloroethyl)sulfonyl 4-nitro	22.130	$C_8H_8ClNO_4S$	249.67 g/mol	1.07	80935		
4.	3-chloro-4-nitrophenol	26.318	$C_6H_4ClNO_3$	173.55 g/mol	1.62	10283		
5.	Cholan-24-oic acid, 3,12-bis(acetyloxy)	26.729	$C_{29}H_{46}O_6$	490.7 g/mol	8.88	21140628		
6.	Pyrene, hexadecahydro-	26.840	$C_{16}H_{26}$	218.38 g/mol	12.13	75524		
7.	5(1H)-Azulenone, 2,4,6,7,8,8a-hexahydro-3,8-dimethyl-4-(1-methylethylidene)-, (8S-cis)-	27.229	$C_{15}H_{22}O$	218.33 g/mol	45.36	91735354		

Observed bioactive compounds in the extract

Bioactive compounds examined were o-Mentha-1(7),8-dien-3-ol, Carbonic acid, 2-chloroethyl 2,2,2-trichloroethyl ester, Benzene, 1-[(2-chloroethyl)sulfonyl 4-nitro, 3-chloro-4-nitrophenol, Cholan-24-oic acid, 3,12-bis(acetyloxy), Pyrene, hexadecahydro-, and 5(1H)-Azulenone, 2,4,6,7,8,8a-hexahydro-3,8-dimethyl-4-(1-methylethylidene)-, (8S-cis)-s, the results are briefly given in *Table 1*. The docking score with the minimal binding energy of Cholan-24-oic acid, 3,12-bis(acetyloxy) shows -7.6 of BF (kcal/mol) with AmpC from *K. oxytoca* is explained in *Table 5*.

Isolation and identification

Dark pink shiny mucoid lactose fermenting colonies were isolated on McConkey agar plate which differ from other commensals. This is an encapsulated gram-negative bacillus that shows positive sign for Indole, Voges Proskauer, urease, citrate, catalase,

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and for gram-positive cocci positive results were obtained to VP, Citrate, Urease, catalase, coagulase methyl red, and oxidase.

Antibiotic sensitivity

An extensive spectrum of antibiotics such as tetracycline (30 µg), Chloramphenicol (30 µg)**,** and Gentamycin (10 µg) were effectively used against gram-negative bacilli *K. oxytoca* and also *S. aureus* which is a gram-positive coccus. In this *K. oxytoca* showed a high zone of inhibition (21 mm, and 23 mm) against tetracycline than chloramphenicol when compared with gram-positive bacilli *S. aureus* with a zone of inhibition (28 mm, and 16 mm) the results are presented in *Table 2*.

Table 2. *Antibiotic sensitivity test against the nosocomial pathogens (ZOI in mm)*

Isolates	Tetracycline (30 µg)		Chloramphenicol (30 µg)		Gentamycin (10 µg)	
	ZOI	Inf	ZOI	Inf	ZOI	Inf
Staphylococcus aureus	28	S	16	R	21	S
Klebsiella oxytoca	21	S	23	S	14	R

R – Resistance, I – Intermediate, S – Sensitivity, ZOI – Zone of Inhibition, Inf – Inference Te; Tetracycline: C; Chloramphenicol; Gm: Gentamycin

Antibacterial activity

In the case of resin crude, the aqueous and hydroethanolic extract of the nosocomial pathogen *K. oxytoca* and *S. aureus* shows the highest zone of inhibition of 18 mm at 60 µg concentration. This indicates the susceptibility of pathogens towards the extracts, their MIC values are shown in *Table 3*.

Table 3. *B. serrata shows susceptibility against the nosocomial pathogens*

S. no	Isolates	*B. serrata* resin extracts (ZOI in mm)					
		Aqueous extract (in mcg)			Hydroethanol extract (in mcg)		
		20	40	60	20	40	60
1	*Staphylococcus aureus*	15 ± 1.041	17 ± 0.763	18 ± 0.763	17 ± 0.763	18 ± 0.763	18 ± 0.763
2	*Klebsiella oxytoca*	13 ± 0.557	16 ± 0.771	18 ± 0.763	14 ± 1.041	15 ± 1.04	18 ± 0.763

Data are mean ± SE (n = 3)

Protein-protein interaction

Antibiotic Reference Drugs used for docking are shown in *Table 5*. Nonbonding and bonding interaction of the residues in the active site is shown via docking (*Table 4*). The AmpC from *K. oxytoca* shows a binding affinity at -7.6 (less binding energy) with Cholan-24-oic acid, 3,12-bis(acetyloxy), followed by Pyrene, hexadecahydro- -7.5 (kcal/mol) and 5(1H)-Azulenone, 2,4,6,7,8,8a-hexahydro-3,8-dimethyl-4-(1-methylethylidene)-, (8S-cis)- -6.7 (kcal/mol). Among the Phyto compounds, Cholan-24-oic acid, 3,12-bis(acetyloxy), show a binding affinity -7.6 (kcal/mol), and the reference drug tetracycline also has the same binding affinity -7.6 (kcal/mol) are shown by highlighting in *Table 5*. The binding score and 3D graphical structure are all shown

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below with their CID 21140628 > CID 75524 > CID 91735354 > CID 54675776 in which the color variation shows the binding affinity of the amino acids in *Figures 1-4*.

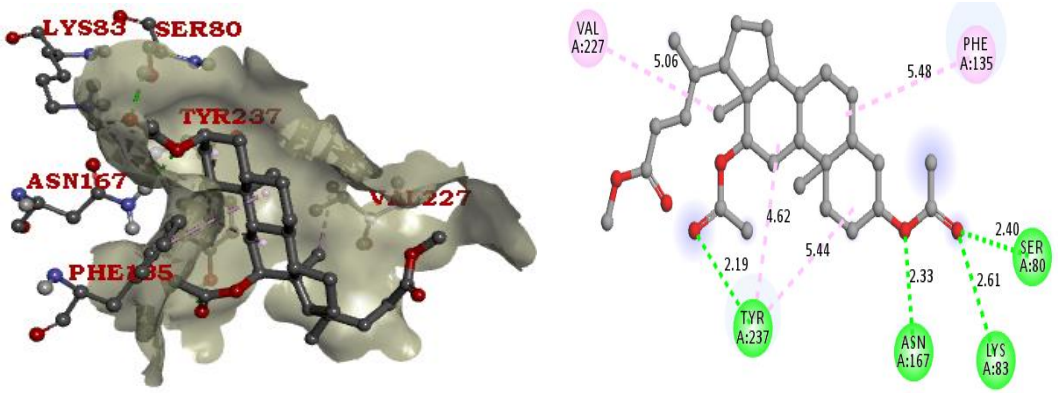

Figure 1. *AmpC K. oxytoco - CID 21140628 docking pose & interaction plot (-7.6 Kcal/mol)*

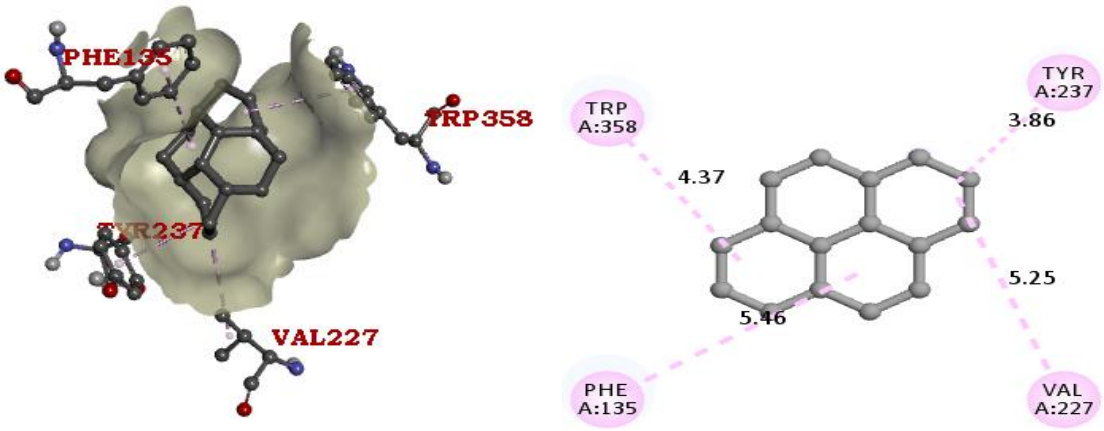

Figure 2. *AmpC K. oxytoco - CID 75524 docking pose & interaction plot (-7.5 Kcal/mol)*

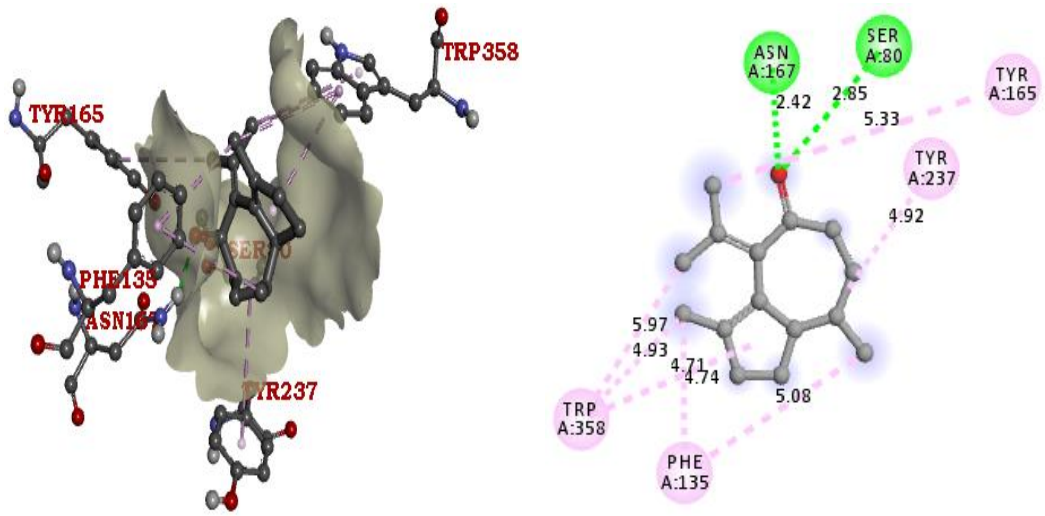

Figure 3. *AmpC K. oxytoco - CID 91735354 docking pose & interaction plot (-6.7 Kcal/mol)*

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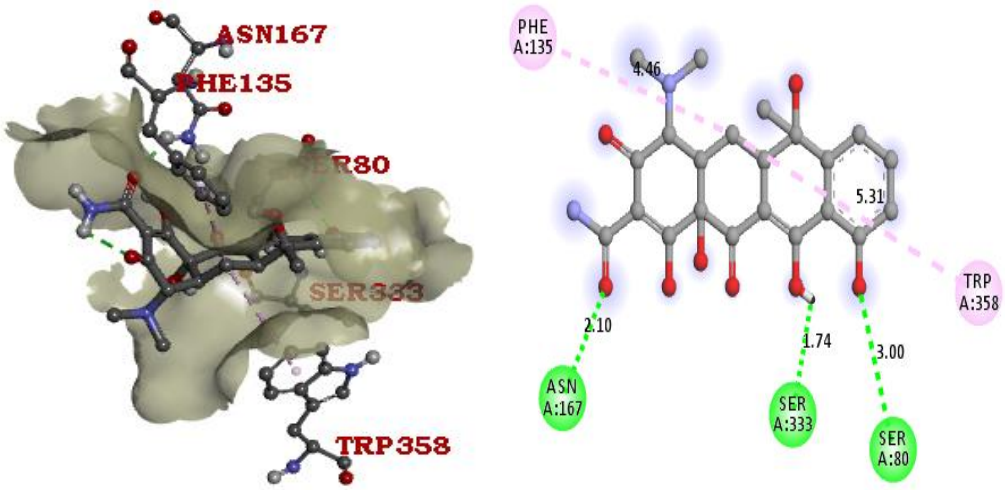

Figure 4. *AmpC K. oxytoco - CID 54675776 docking pose & interaction plot (-7.6 Kcal/mol)*

Table 4. *Docking interaction table for AmpC K. oxytoco ligand complexes*

S. No	Complex name	Bonded interactions	Non bonded interactions	Docking score (-K.Cal/mol)
1.	CID 564552	-	Met306,Leu134,Phe135,Tyr165	-5.4
2.	CID 87646995	Ser80,Ser333	Ser333	-4.5
3.	CID 80935	Asn167,Thr331	Gly305,Ser80,Phe135	-5.8
4.	CID 10283	Ala234,Phe185,Asp233	Leu232,Glu235	-5.2
5.	CID 21140628	Ser80,Lys83,Asn167,Tyr237	Phe135,Val227,Tyr237	-7.6
6.	CID 75524	-	Phe135,Val227,Tyr237,Trp358	-7.5
7.	CID 91735354	Ser80,Asn167	Phe135,Ytr165,Tyr237,Trp358	-6.7
8.	CID 5959	-	Ser333,Tyr237	-6.8
9.	CID 37569	Ser333,Ser80,Val136	Asn167	-6.6
10.	CID 54675776	Ser80,Asn167,Ser333	Phe135,Trp358	-7.6

Table 5. *Docking score of phytocompounds and antibiotic reference drug against AmpC*

Compounds	Binding affinities (kcal/mol) with AmpC from *K. oxytoca*
o-Mentha-1(7),8-dien-3-ol	-5.4
Carbonic acid, 2-chloroethyl 2,2,2-trichloroethyl ester	-4.5
Benzene, 1-[(2-chloroethyl)sulfonyl 4-nitro	-5.8
3-chloro-4-nitrophenol	-5.2
Cholan-24-oic acid, 3,12-bis(acetyloxy)	-7.6
Pyrene, hexadecahydro-	-7.5
5(1H)-Azulenone, 2,4,6,7,8,8a-hexahydro-3,8-dimethyl-4-(1-methylethylidene)-, (8S-cis)-	-6.7
Chloramphenicol	-6.8
Gentamycin	-6.6
Tetracyclin	-7.6

Discussion

Nosocomial drug resistance developed a rigorous crisis in health and hospital care, intensifying morbidity, fatality, extent the reside and raise the costs of healthcare (Exner et al., 2017). The nosocomial drug resistance exhibited variations among hospitals

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around the globe. The majority of the infections caused by bacteria are resistant to multiple drugs (Asres et al., 2017). This study explored the drug resistance of *S. aureus* and *K. oxytoca* from nosocomial infections. Similar findings were explored by other studies (Vakayil et al., 2020). This determined that drug resistance to nosocomial pathogens is a major health issue. A lofty number of patients, deprived infection management practices, self-therapeutic medication, maltreatment, improper overprescription, extended hospitalization, and deviation in aseptic dealings may be the causes. The crude of the *B. serrata* resin shows its inhibitory action against the nosocomial pathogen *K. oxytoca* (Jamwal and Sharma, 2018). *B. serrata* consists of immense therapeutic bioactive that diverge by the extraction process and compound nature. The small organic chemical compound obtained by microwave extraction shows its effective role in antibacterial, antifungal, ulcer healing, and control of nosocomial infection (Beghelli et al., 2017). Through GC-MS analysis the effective bioactive compound cholan-24-oic acid, 3,12-bis(acetyloxy) (Vakayil et al., 2021c; Perleberg et al., 2018) was extracted and proved its susceptibility character against tetracycline, chloramphenicol, and gentamycin via in silico tool (Siemoneit et al., 2017).

Conclusion

The phytochemicals in *B. serrata* are efficient in repairing the wound. Morphology and biochemical changes occur in the wound which leads to scar formation after exposure to the *B. serrata* extract. Protein-protein interaction of the nosocomial pathogen (*K. oxytoca*) against tetracycline, chloramphenicol, and gentamycin showed less effectiveness compared with the action of *B. serrata* extract. This proves that *B. serrata* is a cheap, eco-friendly, and safe remedy due to the rich source of antioxidant properties. In the future, it might be produced on a large scale and act as a medication for curing chronic ulcers.

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Conflict of interests. The authors declare no conflict of interests with this research.

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BIOPESTICIDE EFFICACY AGAINST MAIN PESTS, DISEASES, AND NATURAL ENEMIES OF MUNGBEAN (*VIGNA RADIATA* L.)

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Abstract. Synthetic pesticides are not effectively controlling pests and diseases of mungbean in Indonesia. The objective of this study was to examine the inundation of various types of biopesticides in controlling the main pests and diseases, and their impact on natural enemies in mungbean. The study consisted of five treatments namely P1 = preventive biopesticide (*Trichoderma harzianum*, neem seed powder (*Azadirachta indica*), *Spodoptera litura* Nuclear Polyhedrosis Virus, *Beauveria bassiana*, and galangal rhizome extract) inundation, P2 = biopesticide application based on control threshold (CT), P3 = scheduled synthetic pesticide application, P4 = synthetic pesticide application based on CT, and P5 = without control. The results showed that P1 inundation reduced the population of 11 types of mungbean pests. The P1 treatment had the same efficacy as the P3 treatment. Scheduled and inundated application of biopesticides maintained the survival of natural enemies *Oxyopes* sp., *Paederus* sp., *Coccinella* sp., *Andrallus* sp., *Sycanus* sp. and the parasitoids *Trichogramma* sp., *Telenomus* sp., and *Apantheles* sp. Synthetic pesticides on schedule or based on control threshold killed the entire population of natural enemies. Preventive application of biopesticides could be recommended for controlling the main pests and diseases of mungbean as well as an alternative to synthetic pesticides.
Keywords: *synthetic pesticides, application, control, impact, preventive*

Introduction

Mungbean (*Vigna radiata* L.) production in Indonesia is still classified as low, only around 1-1.1 t/ha, meanwhile the opportunity for increasing productivity is quite large because the yield potential of several superior varieties that have been released can reach a productivity of 2 t/ha. One of the obstacles in increasing mungbean productivity is the occurrence of organisms harmful to plants which attack in them from early growth stages until just before harvest, even in storage areas. The main pests that attack at the beginning of growth are bean flies and shoot borer. Pests that attack in the vegetative phase are armyworms, leaf rollers, looper cutworm, whitefly, and leaf suckers. Meanwhile, pests that attack in the generative phase include pod sucking bug, pod borer, and pod feeder (Brier, 2010).

The types of diseases found on mungbean plants at the beginning of plant growth are soil borne pathogens which can cause yield losses ranging from 30-100% (Hall, 2005). Meanwhile, diseases in the vegetative phase are leaf spot and rust disease except viral disease caused by whitefly (Karthikeyan et al., 2014). Mungbean pest control technology applied by farmers is the application of synthetic pesticides because the results of this technology can be immediately determined. In addition, farmers do not have sufficient knowledge about other control technologies that can suppress pest populations and yields loss. However, it is not realized that the application of synthetic pesticides which are not in the right type, dose, time or method of application can lead

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to resistance and resurgence due to the killing of all natural enemies, both predators and parasitoids (Panizzi, 2013).

One of several ways to reduce resistance and resurgence is by suppressing the use of synthetic pesticides and developing the roles of various types of biological agents that can be used as biopesticides in pest control. Various advantages of biopesticides for controlling plant pests due to the fact that biopesticides: (1) have more specific host, (2) are easily degraded in nature so that they do not leave any residue, (3) the products obtained are organic so that improves the health of consumers, although the selling price of crop products become more expensive (which is good for the farmers) and, (4) are easy to breed in an easy way, (5) are not toxic to livestock, domestic animals and humans, as well as (6) do not pollute water sources or the environment (Gupta and Dikshit, 2010). The results of a recent study reported by Pacheco et al. (2018) that the antagonist fungus *T. harzianum* and several other species are very effective in suppressing the development of soil-borne diseases caused by the fungus *S. rolfsii*, even exceeding the efficacy of synthetic fungicides.

Biopesticide Virgra from *Spodoptera litura* Nuclear Polyhedrosis Virus (*Sl*NPV) is quite effective in controlling several types of pests, including toxic in killing armyworm *S. litura* larvae (Bedjo, 2004, 2012). *Sl*NPV is also able to kill leaf-folding caterpillars (*Chrysodeixis chalcites*) and leaf-rolling caterpillars (*Lamprosema indicata*) (Indiati and Bedjo, 2017). Biopesticides derived from botanical pesticides, neem seed powder (NSP), have been reported to be effective in refusing to eat the targeted insect by *O. phaseoli* bean flies, *S. litura* armyworms and several other pests (Indiati et al., 2013). Furthermore, BeBas biopesticide from the entomopathogenic fungus *Beauveria bassiana* can kill all pod sucking pests (*R. linearis, N. viridula, P. hybneri*) (Prayogo, 2013; Prayogo and Tantawizal, 2015).

Trichol 8 biopesticide from the antagonistic fungi, *T. harzianum,* was reported to be quite effective in suppressing the development of soil borne diseases of *S. rolfsii* and *R. solani* (Redda et al., 2018). Soil borne diseases reported by Handayani and Purwoko (2008) could also be controlled with extracts of galangal rhizome (*Alpinia galanga*) because they contain essential compounds that function against fungi and bacteria. Therefore, this study aims to test the inundation efficacy of various types of biopesticides to control mungbean pests and diseases.

Methodology

Place and time of study

The research was conducted at the Ngale Agricultural Technology Research and Assessment Installation (ATRAI), Ngawi, East Java, Indonesia with an altitude of 50 meters above sea level at the coordinates of 7° 24′ 32.4″ and 111° 22′ 22.8″ East Longitude. The experiment was conducted during Dry Season (June-August) 2017. The field soil is classified as clay vertisol with pH 6.8, C-organik 1.2%, N Total 0.14, and climate type is C3 base on Oldeman system.

Research design

The study was arranged in a randomized block design (RBD) with five replications. The treatments tested were: P1 = preventive biopesticide inundation, P2 = biopesticide inundation based on control threshold (CT), P3 = scheduled synthetic pesticide

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application (weekly), P4 = synthetic pesticide application based on CT, and P5 = without control.

The types of biopesticides used were: (1) Trichol 8, a biofungicide containing the antagonistic fungi *T. harzianum;* (2) NSP, a botanical pesticide made from neem seed powder (NSP) (*Azadirachta indica*) containing azadirahtin compounds; (3) Virgra, an entomopathogenic virus containing particles of *S. litura* Nucear Polyhedrosis Virus (*Sl*NPV); (4) BeBas, a biopesticide containing the entomopathogenic fungal conidia *B. bassiana*; and (5) GE, a botanical pesticide made from galangal rhizome extract (*Alpinia galanga*).

Preventively inundation of biopesticides is the application in abundance amount with the expectation of reducing the pest population in a short time. Scheduled synthetic pesticide control was an application that was carried out every week regardless of the type or population of the pest. Control based on the control threshold (CT) value, namely the applications of both biopesticides and synthetic pesticide were adjusted with the control threshold value for each type of pest (*Table 1*).

Table 1. *Control threshold (CT) for each type of pest in mungbean and time of control*

No.	Type of pest	Control threshold for each type of pest in mung beans and time of control		
		Control threshold (CT)	**Biopesticide**	**Synthetic pesticide**
	Pests			
1.	*O. phaseoli*	1 adult/50 cluster, plant age of 5 DAP*	NSP** (7, 14, 21, 28 DAP)	Monocrotophos (7, 14, 21, 28 DAP)
2.	*S. litura* *L. indicata* *C. chalcites*	10 insects of instar 3/10 clump, plant flowering age; 13 insects /10 clump, pod filling stage	Virgra (28, 35, 42, 49 DAP)	Cyhalothrin (28, 35, 42, 49 DAP)
2.	*B. tabaci*	1 pair of adult/ 20 clump, plant age of 21 DAP	BeBas (21, 28, 35, 42 DAP)	Thiamethoxam (21, 28, 35, 42 DAP)
3.	*R. linearis* *N. viridula*	1 pair of adult/20 clump, plant age of 35 DAP	BeBas (35, 42, 49, 53 DAP)	Cypermethhrin (35, 42, 49, 53 DAP)
4.	*M. testulalis*	2 insects/20 clump, plant age of 42 DAP	Virgra (42, 49, 53 DAP)	Cypermethrin (42, 49, 53 DAP)
	Diseases			
1.	*S. rolfsii* *R. solani*	IP 10% at plant age of 7-35 DAP	Trichol 8 (seed maintenance, 7, 14, 21, 28, 35 DAP)	Captan (seed maintenance, 7, 14, 21, 28, 35 DAP)
2.	*E. polygoni* *C. canescens* *Uromyces* sp.	IP 35% at plant age of 35 DAP	GE (35, 42, 49, 53 DAP)	Benomyl (35, 42, 49, 53 DAP)

Land preparation

The soil was cultivated by plowing twice then adding organic fertilizer as much as 4 t/ha mixed with soil, then the plots were made with the size of 4 m × 5 m per plot as treatment units. The mungbean variety Vima 1 was planted in each plot with a plant spacing of 40 cm × 15 cm, 2 plants per hole. Inorganic fertilizers as much as 37.5 Kg N/ha, 73.5 Kg P_2O_5/ha, and 37.5 Kg K_2O/ha were all given at planting time by being distributed beside the planting hole. Weeding was done mechanically two times at the age of 14 and 28 days after planting (DAP).

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Biopesticides preparation

Trichol 8 biofungicide was obtained from the IETRI Mycology Laboratory, then cultured on potato dextrose agar (PDA) growing media in a petri dish. At the age of 21 days after inoculation (DAI), each fungal culture in the cup was added with 10 ml of water/cup, then the fungal colony was scraped off using a soft brush to knock out the formed conidia. The conidia suspension was put into a beaker glass, then 2 ml/L of Tween 80 was added and shaken using a shaker until it was homogeneous for 60 min. Conidia suspension was calculated using a hemocytometer to obtain a conidia density of 2.5×10^7/ml, then the conidia suspension was mixed with the seeds as a seed treatment before planting. Trichol 8 biofungicide, also applied to the vegetative phase to suppress airborne diseases.

BeBas biopesticide from the entomopathogenic fungus *B. bassiana* was obtained from the ILETRI Biopesticide laboratory collection, then the fungus was cultured in a petri dish using potatoes dextrose agar (PDA) and chitin as growth media. At the age of 21 DAI, 10 ml of water/cup was added to the fungal culture in a petri dish, then the fungi colony was scraped off using a soft brush until all conidia formed were fall out. The conidia suspension was then put into a beaker glass and 2 ml/L of Tween 80 was added and shaken using a shaker for 60 min. Conidia suspension was calculated using a hemocytometer to obtain a conidia density of 10^7/ml before application in the field. The application of BeBas biopesticide was sprayed right on the integument of target insect for optimal control.

Virgra biopesticide containing *S. litura* Nuclear Polyhedrosis Virus (*Sl*NPV) was a JTM97C isolate obtained from the ILETRI Entomology Laboratory. *Sl*NPV isolates were reproduced in the larvae of *S. litura* as a result of rearing in the laboratory. The larvae of *S. litura* were fed with soy leaves every day, after the IV instar was formed. The larvae were fed with soybean leaves that have been sprayed with the *Sl*NPV suspension, then the *S. litura* larvae were maintained until they die. Every 20 dead *S. litura* larvae were crushed using a mortar or blender until smooth, then added with 1000 ml of water. Viral suspension was calculated using a hemocytometer to obtain a density of 10^{11} PIB/ml, then added with Tween 80 as much as 2 ml/L and shaken before being applied in the field. The application of biopesticides derived from entomopathogenic viruses were attached to all plant organs, especially the leaves and pods eaten by insect pests.

NSP biopesticides containing azadirachtin compounds from neem seed powder (*A. indica*) were obtained from the Indonesian Sweetener and Fiber Crops Research Institute (ISFCRI). Every 50 g/L of NSP formulation was first boiled to a boiling point with the aim that all azadirachtin compounds were decomposed and then deposited one night before application in the field. The SBM solution was mixed with 2 ml/L adhesive and then applied in the afternoon to the plant organs according to the predetermined schedule.

Botanical pesticides of galangal rhizome (*A. galangal*) obtained from the Gadang market (Malang) were cut into pieces and then dried. The application dose of galangal extract (GE) was 50 g/L. First GE was boiled to a boil point in order to break down the essential compounds and alicin. Furthermore, biopesticide was applied in the evening to avoid ultraviolet rays and winds that affect the efficacy of biopesticides.

Data observed

The observed variables were: (1) types and populations of pests through direct observation of 10 sample plants or using sweep nets on each treatment plot, (2) types of diseases that appear were visually observed in 10 sample plants in each treatment plot,

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(3) leaf pest attack intensity, (4) pod pest attack intensity, (5) disease intensity observed in 10 sample plants by calculating the level of plant damage, (6) species and population of natural enemies were directly observed and harvested using insect nets in each treatment plot, and (7) seeds weight of determined 20 m^2 plot size.

Soil-borne disease intensity was observed based on disease incidence which referred to the van-Schoonhaven and Pastor-Carrales (1991) method with the following formula:

$$KjP = (A/B) \times 100\% \quad \text{(Eq.1)}$$

where KjP = incidence of disease (%), A = number of disease infected plants, and B = number of plants observed.

Leaf disease intensity was observed based on disease severity using the method developed by Paplomatas et al. (1992) with the following formula:

$$KrP = \sum \frac{(nxv)}{N\,x\,V}\, x\ 100\% \quad \text{(Eq.2)}$$

where KrP = disease severity (%), n = number of leaves from each attack category, v = score value for each attack category, N = number of all leaves observed, and V = the highest score of disease score. The type and population of arthropods were observed from an area of 20 m^2 by catching them using a sweep net and yellow trap, as done by Holcomb et al. (2011). Each catch obtained was taken to the laboratory and identified based on the key of determination, referring to the method developed by Gibb and Oseto (2020).

Data analysis

All observed data were analyzed using the Minitab 14 program. If there was a significant difference between treatments, it was followed by a multiple distance test (Duncan's Multiple Range Test) at the real level $\alpha = 0.05$.

Results and discussion

Soilborne disease

Soil-borne disease with wilting symptoms at the beginning of growth was caused by the fungus *S. rolfsii*. The results showed that the average incidence of wilt disease was 3-12% (*Fig. 1*). The lowest wilt disease intensity occurred in the inundation treatment of Trichol 8 (P1) by coating the seed before planting and continued application at the age of 14, 21, 28 and 35 DAP. The efficacy of Trichol 8 in suppressing soil borne diseases occured because the fungus of *T. harzianum* was antagonistic in addition to endophytes and decomposer so that it was able to remodel all litter into a source of nutrients that were more quickly available to plants. This resulted in the plants became more fertile and were able to increase plant resistance to pests and pathogenic infections.

The highest incidence of wilt disease was found in the treatment without control (P5) with an intensity of 12.60% and this intensity of the disease was not significantly different from the application of biopesticides or synthetic pesticides based on CT (P2 and P4 treatments). The results of this study indicated that Trichol 8 inundation and the application of chemical fungicides based on CT were not effective in suppressing the development of soil borne diseases. It was suspected that the *S. rolfsii* in the research location was tolerant to synthetic fungicides containing the captan active ingredient.

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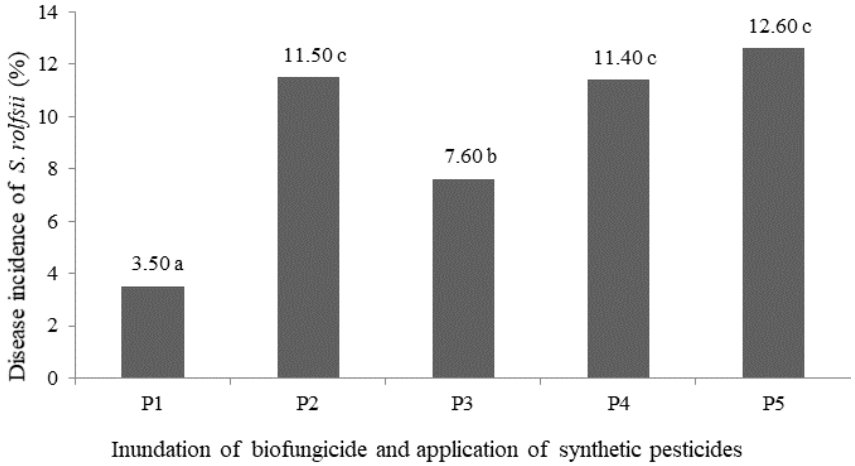

Figure 1. *Inundation of Trichol 8 biofungicide and synthetic pesticides against the incidence of S. rolfsii disease. The treatments: P1 = preventive biopesticide inundation, P2 = biopesticide inundation based on control threshold (CT), P3 = scheduled synthetic pesticide application (weekly), P4 = synthetic pesticide application based on CT, and P5 = without control*

Powdery mildew

The observation results showed that the average powdery mildew disease intensity ranged from 15-37% (*Fig. 2*). The lowest powdery mildew disease intensity occurred in the treatment of botanical pesticide application of galangal GE by inundation which was only 15.50% and the synthetic fungicide scheduled basis, namely 16.75%.

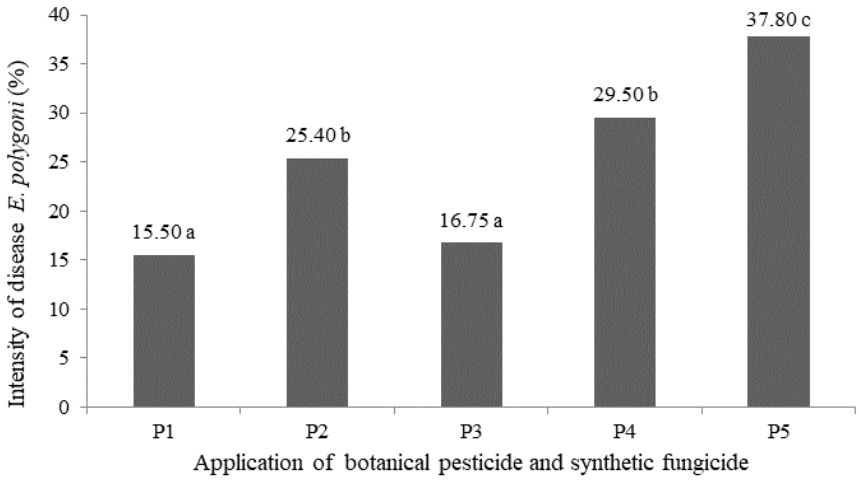

Figure 2. *Efficacy of galangal extract (GE) botanical pesticides and synthetic fungicides in suppressing the development of E. polygoni. The treatments: P1 = preventive biopesticide inundation, P2 = biopesticide inundation based on control threshold (CT), P3 = scheduled synthetic pesticide application (weekly), P4 = synthetic pesticide application based on CT, and P5 = without control*

The results of observations on the application of botanical pesticides from GE (P2) and synthetic fungicides based on CT (P4) were not able to reduce the progression rate of powdery mildew showed by the disease intensities of the two P2 and P4 treatments

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were 25.40% and 29.50%, respectively. This condition occurred because the rate of disease progression was very fast which was supported by the dry season and the severe wind so that the spores of *E. polygoni* could easily spread and infect other plant organs. Therefore, mung bean leaves that were heavily infected by *E. polygoni* on the entire leaf surface appeared white, covered by mycelium and fungal spores. Leaves heavily infected by powdery mildew turned slightly dark in color and eventually the leaves dried out and fell prematurely (*Fig. 3*).

Figure 3. *Mungbean leaves infected by E. polygoni (P1) which was applied preventive galangal extract (GE) (a) and P5 without control (b)*

Leaf spot and rust disease

Other leaf diseases found were leaf spot, namely *C. canescens* and *Uromyces* sp. with a disease intensity of less than 11% (*Fig. 4*). The intensity of rust disease appeared to be higher than the intensity of leaf spot disease. Inundation of GE preventively (P1) and scheduled synthetic fungicide application (P3) were able to suppress the development of spotting and leaf rust, which were showed by the intensity of the disease appeared to be lower when compared to control based on CT on P2 and P4 treatments. The intensity of leaf spot disease in P1 and P3 was only below 3%. This condition was due to the efficacy of GE and synthetic fungicides that were applied early, which could make pathogens difficult to develop.

Pests population

The results observation obtained 11 types of pests, namely; bean flies (*O. phaseoli*), armyworms (*S. litura*), *Empoasca* sp., *Megalurothrips usitatus*, leafminer (*Liriomyza* sp.), whitefly (*B. tabaci*), brown stink bug (*R. linearis*), green stink bug (*N. viridula*), pale green stink bug (*P. hybneri*), pod borer (*M. testulalis*) and pod feeder (*H. armigera*) (*Table 2*). The population of *S. litura* in the P1 and P3 treatments were 5.9 and 17.5 insect bodies, respectively was lower when compared to P4 (19 insect bodies) and P5 (26.5 insect bodies). The low population in the P1 was due to the Virgra containing *Sl*NPV being able to kill *S. litura* larvae.

Table 2 shows that the populations of *B. tabaci* and *M. usitatus* pests was higher in all treatments except for P3, even if the populations of the two types of pests were uncontrolled, they reached 42.10 and 92.30 insect bodies, respectively. The preventive application of biopesticide (P1) was still found 19 nymphs and adult of *B. tabaci*, while

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34.60 insect bodies of *B. tabaci* were found in P2. Scheduled application of synthetic insecticide thiamethoxam (P3) was found in the nymphs and adult *B. tabaci* with a higher population of 46 insect bodies and an increase in population to 66.90 insect bodies in P4. The results of this study as reported by Fang et al. (2018) which informed that the application of synthetic insecticides that were not in the right dose and target could trigger the explosion of the *B. tabaci* population, apart being caused by all existing natural enemies were killed due to the efficacy of synthetic insecticides in P4 treatment.

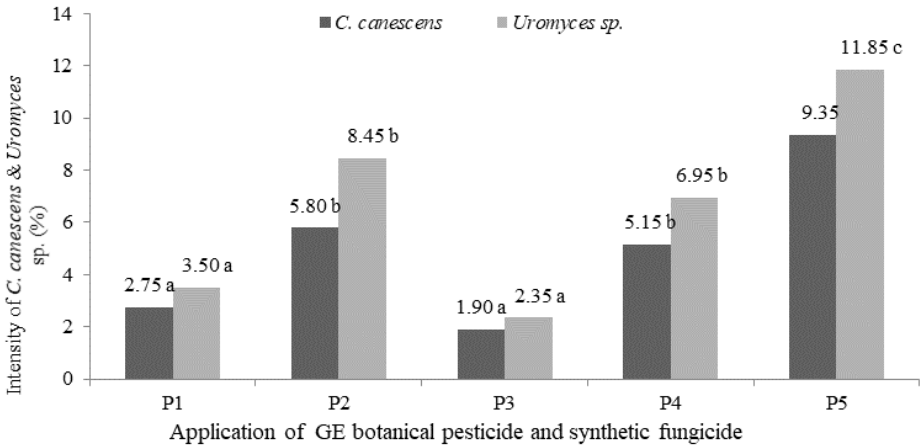

Figure 4. *Efficacy of botanical pesticides GE and synthetic fungicides against mung bean leaf spot and leaf rust. The treatments: P1 = preventive biopesticide inundation, P2 = biopesticide inundation based on control threshold (CT), P3 = scheduled synthetic pesticide application (weekly), P4 = synthetic pesticide application based on CT, and P5 = without control*

Table 2. *Pest populations per plot land of mung bean applied using biopesticides and synthetic pesticides*

No.	Type of arthropod	Pest populations (insect bodies) per plot at 49 DAP *				
		P1	**P2**	**P3**	**P4**	**P5**
1.	*O. phaseoli*	2.7 bc	4.1 b	2.6 bc	6.4 a	7.5 a
2.	*S. litura*	5.9 cd	0.9 c	17.5 b	19.0 b	24.5 a
3.	*Empoasca* sp.	7.1 d	23.1 b	12.4 d	21.4 bc	57.3 a
4.	*Liriomyza* sp.	3.5 a	2.4 ab	1.5 b	2.5 ab	3.1 a
5.	*M. usitatus*.	11.7 d	23.1 bc	4.5 e	29.0 b	42.1 a
6.	*B. tabaci*	19.0 e	34.6 cd	46.8 c	66.9 b	92.3 a
7.	*R. linearis*	2.4 d	9.7 bc	0.5 d	11.0 b	22.5 a
8.	*N. viridula*	1.7 d	6.1 bc	1.0 d	10.9 b	21.0 a
9.	*P. hybneri*	2.4 cd	3.7 c	2.0 cd	11.7 b	20.9 a
10.	*M. testulalis*	9.9 bc	15.1 ab	4.1 d	12.5 b	20.0 a
11.	*H. armigera*	2.5 b	7.4 a	2.4 b	6.5 a	6.9 a

*The numbers in the row followed by the same notation are not significantly different among treatments in the 0.05 DMRT test

The treatments: P1 = preventive biopesticide inundation, P2 = biopesticide inundation based on control threshold (CT), P3 = scheduled synthetic pesticide application (weekly), P4 = synthetic pesticide application based on CT, and P5 = without control

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The pod pests found were borers caused by *M. testulalis* (Lepidoptera: Crambidae). The lowest pod borer population occurred in the scheduled synthetic insecticide application treatment (P3), namely 4.1 insect bodies followed by preventive biopesticide inundation treatment (P1), namely 9.9 insect bodies. The application of biopesticides and synthetic pesticides based on CT had not been able to suppress pod borer attack because it was found that the pest population was higher than P3 and P1. The population of pod borer larvae continued to increase if do no control was applied (P5), which reached 20 insect bodies.

The results of this study indicated that scheduled control using synthetic insecticides (P3) and preventive inundation of biopesticides (P1) could be more effective in suppressing the mung bean pod borer population. The efficacy of P1 treatment was thought to be due to the impact of the accumulated application of *Sl*NPV, NSP, and BeBas so that the pod borer population could not develop. Subhasree (2013) reported that the fungus *B. bassiana* and NSP was effective enough to kill pod borer, causing mortality to reach 79%.

Abundance of natural enemies

Insects as natural enemies that were observed, namely five types of predators and three types of parasitoid and other species of parasitoids that had not been identified. Five types of predators were found, namely; *Oxyopes* sp., *Paederus* sp., *Coccinella* sp., *Andrallus* sp., and *Sycanus* sp. (*Table 3*).

Table 3. *Population abundance of natural enemies (predators and parasitoids) per plot of mung beans applied with biopesticides and synthetic pesticides*

Type of natural enemies	Population of natural enemies (insect bodies) per plot at 49 DAP *				
	P1	**P2**	**P3**	**P4**	**P5**
Predator					
1. *Oxyopes* sp.	4.8 b	12.9 a	0.5 c	0.8 c	13.9 a
2. *Paederus* sp	10.2 c	18.5 ab	0.4 d	0.7 d	19.5 a
3. *Coccinella* sp.	4.9 c	13.5 b	0.0 d	1.2 cd	20.4 a
4. *Andrallus* sp.	2.1 a	1.8 ab	0.0 c	0.1 c	1.9 ab
5. *Sycanus* sp.	2.0 a	1.9 a	0.0 c	0.0 c	1.6 ab
Parasitoid					
1. *Trichogramma* sp.	27.8 b	23.5 bc	0.9 d	1.0 d	38.7 a
2. *Telenomus* sp.	21.6 c	31.2 b	1.5 d	0.3 d	41.5 a
3. *Apantheles* sp.	19.5 a	11.5 b	0.5 c	0.9 c	21.9 a
Other species	17.5 ab	21.8 a	2.1 c	2.1 c	23.8 a

*The numbers in the row followed by the same notation are not significantly different between treatments in the 0.05 DMRT test
The treatments: P1 = preventive biopesticide inundation, P2 = biopesticide inundation based on control threshold (CT), P3 = scheduled synthetic pesticide application (weekly), P4 = synthetic pesticide application based on CT, and P5 = without control

There were three types of parasitoid insects identified, namely: *Trichogramma* sp., *Telenomus* sp. and *Apantheles* sp. and there were other species that had not been identified. The populations of these three types of parasitoids were abundant at P5, P2

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and P1 (*Table 3*). The population of *Telenomus* sp. was the highest until it reached 41 insect bodies, namely at P5 then followed by P2, namely 31 insect bodies and P1 as many as 21 insect bodies. The population of *Trichogramma* sp. The highest also occurred in P5, reaching 38 insect bodies, then P1, namely 27 insect bodies and P2 with 23 insect bodies. Meanwhile, P3 and P4 treatments were applied with synthetic pesticides and on a regular basis, it appeared that the parasitoid population was very limited and even almost extinct. This condition was caused by parasitoid insects more susceptible to synthetic pesticides than insect pests. These three types of parasitoids were generally effective in parasitizing eggs and larvae of *S. litura*. Therefore, the population of *S. litura* was lower in treatment P1 and P2 when compared to P5.

Pod damaged

The results of the observation showed that the highest number of empty pods reached 5.5 pods per plant hill that occurred in treatment P5 (without control) (*Fig. 5*). The number of empty pods in P2 and P4 treatments was also quite large, namely 4.5-4.8 pods per plant hill which was not significantly different from the number of pods in P5. The lowest number of empty pods was only 2.5 pods per plants hill which occurred in the scheduled insecticide application treatment (P3) and the number of empty pods was not significantly different from the P1 treatment, namely 3.1 pods per hill.

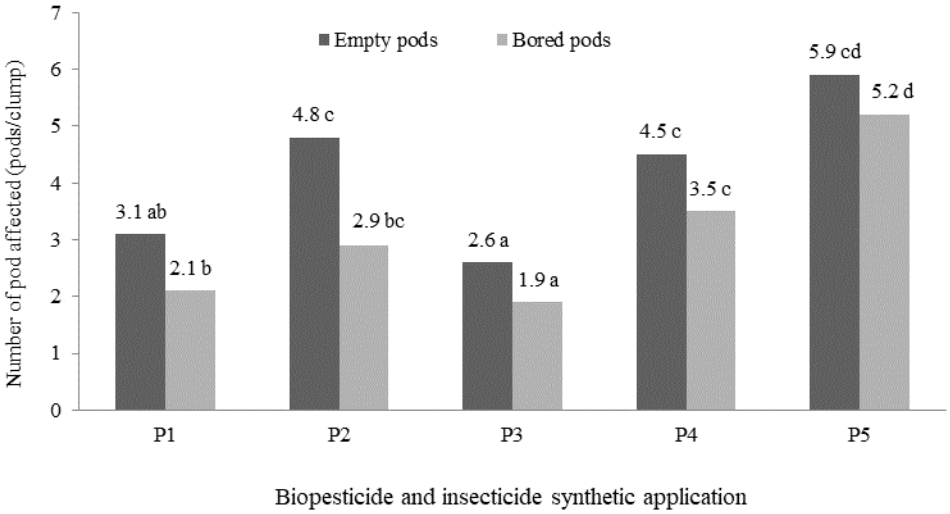

Figure 5. *Number of empty pods and bored pods on mung bean fields applied with biopesticides and synthetic pesticides. The treatments: P1 = preventive biopesticide inundation, P2 = biopesticide inundation based on control threshold (CT), P3 = scheduled synthetic pesticide application (weekly), P4 = synthetic pesticide application based on CT, and P5 = without control*

The results of the observation showed that the number of pods bored by *M. testulalis* was quite low at P1, P2, and P3, which ranged from only 1.8-2.0 pods per plant. The control efficacy in P1 and P2 was due to the use of the biopesticide Virgra and BeBas, which had the ability to kill all insect stages. The highest number of bored pods was found in the uncontrolled treatment (P5), namely up to 5 pods per plant, the number of pods was not significantly different from the application treatment using the insecticide

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cypermethrin based on CT (P4). Thus, the application of cypermethrin based on CT had not been able to suppress pod damage. This condition was due to the larvae of *M. testulalis* developing in the pods so that the synthetic insecticide application was unable to reach the target insects. In addition, the CT value for pod borer caused by *M. testulalis* larvae need to be reviewed so that the control technology set was accurate so that it did not cause losses. Indiati et al. (2021) reported that *M. testulalis* attacks with control application reduced pod damage of mungbean by 48.5% and increased mung bean seed yield by 25% compared to without control.

Seed weight

The research results showed that the average seed weight obtained ranged from 0.71 to 1.03 t/ha (*Fig. 6*). The highest seed weight was obtained from P3, which reached 1.03 t/ha and the weight of the seeds of this treatment was not significantly different from P1, namely 1.02 t/ha. The P2 and P4 treatments, namely the application of biopesticides and synthetic pesticides based on CT, obtained lower seed weight when compared to P1 and P3. Control based on CT, either using biopesticides or synthetic pesticides, had not been able to defend the pods from pests and diseases. This condition occurred, especially the applied biopesticide was unable to kill the pest in a short time, in addition, the application of synthetic pesticides was not able to reach the borer larvae in the pods so that the pests continue to damage the pods.

Seed weight occurred at P5 (without control) was only 0.71 t/ha, by which this condition was caused by many seeds that were empty due to pod sucking bug and being bored by *M. testulalis*. In addition, in the P5 treatment, there were also many whitefly populations whitefly and *M. usitatus* besides *S. litura* armyworm pests.

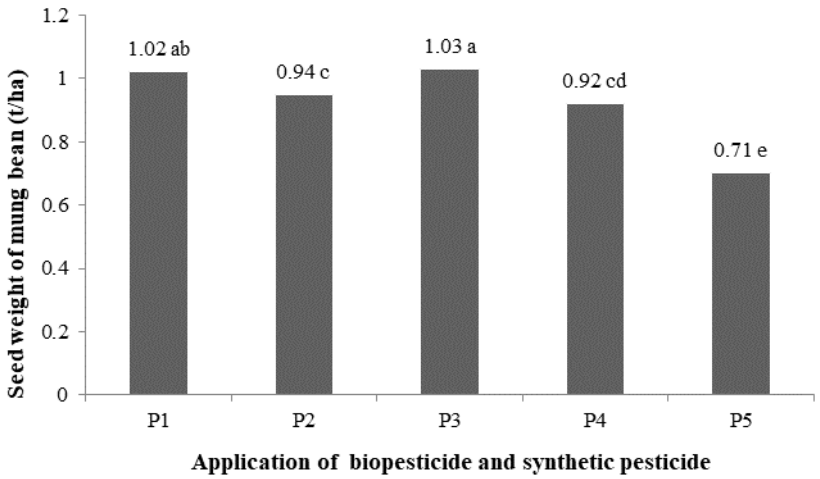

Figure 6. *Average mungbean seed weight (t/ha) applied using biopesticides and synthetic pesticides. The treatments: P1 = preventive biopesticide inundation, P2 = biopesticide inundation based on control threshold (CT), P3 = scheduled synthetic pesticide application (weekly), P4 = synthetic pesticide application based on CT, and P5 = without control*

The intensity of powdery mildew in the two treatments was able to reduce the rate of disease progression because it was lower than the CT value, which was 21% at the time of flowering (Sing et al., 2013). The efficacy of GE occured because the botanical pesticide contained various types of metabolite compounds, such as cineol,

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acetoxychavicol and acetoxyeugenol which function as antimicrobials (Subramanian and Nishan, 2015). Meanwhile, Simarmata (2017) stated that galangal rhizome extract (GE) could suppress the development of rust disease (*Phakopsora euvitis*) up to 70% and anthracnose (*Colletotrichum gloeosporioides*) up to 100%, such as the efficacy of the fungicide Mankozeb.

The low intensity of leaf spot and rust disease was also caused by the role of the Trichol 8, which was an endophytic agent that could suppress the development of leaf disease. This incident was also reported by Shultana et al. (2009) who indicated that the endophytic fungus *T. harzianum* was able to inhibit the development of leaf spot and rust disease. In addition, the effect of the application of NSP which contained azadirahtin, nimbin and salanin compounds functions as a food repellent, affected nerves that produce ecdisone hormone for the moulting process of insects, and reduced insect vertility so that egg production was very limited (Malik et al., 2017).

Empoasca sp. insect was also found with quite high population which was occurred in almost all control treatments. Scheduled preventive inundations of biopesticide (P1) and synthetic insecticide (P3) found the population of *Empoasca* sp. of 7.1 and 12.4 insect bodies, respectively. Meanwhile, the P2 and P4 treatments found the population of *Empoasca* sp. higher than P1 and P3. These insects are quite important leaf-sucking pests because they function as virus vectors in various types of plants (Acosta et al., 2017).

The five types of predators were generalist species that have the ability to prey on various types of major pests. *Oxyopes* sp. and *Paederus* sp. were the predator that occupied the surface of the soil and plant canopy so that the two types of predators had a fairly wide range of prey (pests). Predator *Coccinella* sp., *Andrallus* sp., and *Sycanus* sp. were plant canopy inhabitants that prey on pests that develop on the crown such as *B. tabaci, Aphis* sp., *S. litura, Empoasca* sp., and other pests (Udiarto et al., 2012). Meanwhile, *Oxyopes* sp. has a fairly high predation capacity in preying on almost all types of pests in the order Diptera, Orthoptera, Coleoptera, Hemiptera, Homoptera, and Lepidoptera (Riaz et al*.,* 2014). Khan and Wan (2015) reported that the predator *C. septempunctata* was able to predict the nymphs and adult of *B. tabaci* up to 11 insect bodies/day. Furthermore, Ali et al. (2020) informed that the predator *Paederus* sp., *Coccinella* sp., *Oxyopes* sp. also had the ability to predict insect pests from pod sucker groups up to 9 insect bodies/day.

Abundant predator populations were found in the P5, P2 and P1 treatments, especially the *Paederus* sp. whereas the predator population at P3 and P4 was relatively low even for *Andrallus* sp. and *Sycanus* sp. were not found. Abundance population of the predator *Paederus* sp. due to the predators, the inhabitants of the canopy and the soil surface were very fast moving and immediately fall to the ground if there were vibration or disturbance so that the activity of biopesticide application did not have a negative effect on its survival. The results of the research by Sajap et al. (2001) indicated that the biopesticide from the entomopathogenic virus *Sl*NPV did not affect the survival of the *S. leucomesus* predator, unless the predator had preyed on the host (eggs/larvae) that had been infected with the entomopathogenic virus.

The application of biopesticide BeBas by inundation was able to suppress pod sucking pests as well as the efficacy of the pesticide cypermethrin which was applied regularly. The efficacy of the biopesticide *B. bassiana* is because it can kill all pod sucking bug, both *R. linearis* and *N. viridula*, with a mortality of up to 80% (Prayogo and Tantawizal, 2015).

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Conclusions

Inundation of Trichol 8, SNP, Virgra, BeBas and GE biopesticides which were applied in an integrated preventive manner reduced the attack of major pests in mung beans equivalent to the efficacy of synthetic pesticides which were applied regularly. The application of biopesticides by inundation sustained the survival of predators and parasitoids, while the application of synthetic pesticides destroyed the entire population of natural enemies. The authors recommend for future studies that the control threshold of each pest organism in mungbean needs to be further investigated so that the control time is not too late in order to avoid high yield losses.

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CHARACTERIZATION OF THE REPRODUCTION OF EUROPEAN HAKE (*MERLUCCIUS MERLUCCIUS*, LINNAEUS, 1758) IN ORAN BAY

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Abstract. We have studied the European hake (*Merluccius merluccius*, Linnaeus, 1758) in Oran bay, obtained from fishermen coming to sell their catch at the local markets, in order to characterize the reproduction of this fish and to estimate the impact of local fishing on its population. Males are predominant in catches throughout most of the year, and, on average, males are smaller than females. *Merluccius merluccius* spawns in a partial manner, with asynchronous oocyte maturation, and breeds throughout the year. Determination of length at first sexual maturity (smaller for males) demonstrates earlier puberty in males compared to females. An essential item of information for achieving sustainable fishing is the demonstration that a large part (46%) of caught European hakes has a length below the length at first sexual maturity: they are juveniles. These juveniles are killed before being able to breed, which represents a danger for future fish stocks.
Keywords: *Algeria, reproduction, sexual maturity, sustainable fishing, fish stock*

Introduction

European hake (*Merluccius merluccius*. L) is a species widely present and exploited both in the Atlantic and Mediterranean (Aldebert and Carriès, 1988; Martin, 1991; Oliver and Massutí, 1995). It has been the subject of studies about ecology (Abella et al., 1995; Bouaziz et al., 1998; Murua, 2010; Mellon-Duval et al., 2017; Carrozzi et al., 2019), biology (Pauly, 1980; Aldebert and Recansens, 1995; Murua, 2010; Dominguez, 2007; El Habouz, 2011; Khoufi et al., 2014a; Soykan et al., 2015; Kahraman et al., 2017; Candelma et al., 2018; Uzer et al., 2019; Gül et al., 2019; Carbonara et al., 2019; Girgin et al., 2020), economic interest (Perez-Agundez, 2002) and fishing activity (Gaynilo and Pauly, 1997; Yalçın and Gurbet, 2016; Demirel et al., 2017; Deniz et al., 2020). Its economic value makes it the target of a complex fishing activity composed of several fleets using the most diverse fishing gear (Recasens et al., 1998; Lloret et al., 2001; Lloret and Lleonart, 2002). This represents about 150,000 tonnes of fishes caught by year (FAO, 2007). In Algeria, fishing has been developed in more than 30 ports, with the support of the Algerian government, and the fisheries and fish resources sector have significant potential to contribute to the country's food security, job creation and economic consolidation. The fishing ports of Oran have produced, for example, about 66.5 tonnes of hake in 2010 (Direction des Pêches et des Ressources Halieutiques, personal communication). Hake fishing in Algeria is mainly directed towards the white

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hake (*Merluccius merluccius*), and its minimum market size has been set at 20 cm for Algerian waters according to the Ministry of Fisheries and Fishery Resources of the Republic of Algeria (M.P.R.H, 2016).

Understanding the life cycles of fishes is necessary to achieve sustainable fishing. In order to allow the replenishment of stocks, it is necessary not to catch too many fishes before they have been able to breed. For example, according to Bouaziz et al. (1998), the analysis of hake supplies in Bou-lsmaïl bay has shown that more than 70% of individuals were less than 30 cm in size, with a length at first sexual maturity for females of 30.6 cm. This is alarming because this means that a large quantity of these hakes are young fish caught before they are able to reproduce. To our knowledge, the data on the reproduction of hake in Algeria are limited to those of Bouaziz et al. (Bouaziz, 1992; Bouaziz et al., 1998). Today, our knowledge of the biology of *Merluccius merluccius* (growth and reproduction) is not a sufficient basis for a proper assessment of fish stocks (Morales-Nin et al., 2005), even though studies on the characterization of its reproduction have been carried out in several Atlantic or Mediterranean areas, for example by Bouaziz (1992) or Lahaye (1972). Nevertheless, it is well known that this species is gonochoric and that mature males and females eject gametes in the environmental water where fecundation takes place (Belloc, 1929). Hake has oocytes that do not develop simultaneously (Pérez and Pereiro, 1985; Sarano, 1986; Murua et al., 1998; Murua and Saborido-Rey, 2003; Murua and Motos, 2006), and its spawning activity continues until the death of the fish (Bouhlal, 1973). Note that, as an exception, we have characterized in a previous study a case of hermaphrodism (Belhoucine et al., 2012). Finally, depending on previous studies, this species has been shown to have a spawning period either all year round or discontinuous, and the length at first sexual maturity for females and males greatly varied between studies (as it will be covered in the discussion of the present paper). Hence, in the present study, we have studied the European hake (*Merluccius merluccius*, Linnaeus, 1758), collected from fishermen who came to sell their catch at the local markets of Oran, in order to estimate the impact of local fishing on the European hake population.

Usually, reproduction is characterized by at least two of the following parameters. One of these parameters is the sex ratio which may or may not be close to optimum. Another possibility is to evaluate the physical condition of the animals by classical indexes: hepatosomatic index (useful to estimate whether lipids are accumulated in the liver before being used for gamete production), gonadosomatic index (as it is well known that progression of the weight of gonads during the year allowed assessment of the reproduction period for a given species: gonad weight is at a minimum during biological rest periods, increases during gamete maturation, then decreases for spawning), and the Fulton condition factor (K factor, determined from the relationship between weight and length, which reflects the general state of a fish in function of its physiological activities, is an indicator of fitness). Finally, spawning frequency and fecundity parameters, such as the quantity of mature oocytes in ovaries, age or length at sexual maturity, may be studied.

The present paper presents the estimation of the sex ratio of the European hake caught in Oran bay and the physical condition of these fish. We also focus on results of the characterization of their sexual maturity, by macroscopic and microscopic observations, allowing assessment of length at sexual maturity. For the purposes of environmental management, to achieve sustainable fishing, length at sexual maturity

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should inform on the minimum length for fish catches necessary to avoid a negative impact on fish stocks.

Material and methods

European hake from the Mediterranean Sea were collected monthly for a full year (2017-2018), in Oran bay. Hake fishing is mainly carried out by trawlers, but also by small trades (set nets and bottom longlines) (FAO, 2007), in coastal zones, zones with maximal depth of 500 m. Each month, random sampling was carried out for fish of sizes ranging from 13.6 to 56 cm, when fishermen came to sell their catch at the local markets. A total of 831 specimens were sampled.

Overall measurements

Samples were treated very soon after being caught. All organs were removed by evisceration of the fish. Sex was determined. For each fish, we measured the following parameters:

- Total length (Lt), from the tip of the upper jawbone, adjusted to the lower 1 cm
- Total weight (Wt) and weight of eviscerated fish (We), adjusted to 0.01 g
- Liver weight (Wl, when fresh), adjusted to 0.01 g
- Gonads weight (Wg, when fresh), adjusted to 0.01 g
- Sex determination, observing gonads
- Control of macroscopic stage of sexual maturity and gonad description.

Sex ratio

We have separated the European hake into three categories: immature/undefined sex (immature European hake have gonads similar to thin cords; when mature, gonad volume increases and represents half of the abdominal part), male, or female. Males and females were identified by macroscopic gonad observation in our fish population. Then we have directly calculated the sex ratio as the number of males divided by the number of females. We studied the sex distribution in parallel with the length of each fish. Then the sex ratio was calculated each month of the year from caught fish, in order to follow the dynamic of sex ratio. Significance was statistically assessed with Statistica software (StatSoft Inc 2007), by chi-two, X^2, test of heterogeneity ($p < 0.05$).

Comparison of mean sizes for males or females

To compare these means, we have used Wilcoxon test with R software (R-3.6.1-win, RStudio-1.2.1335).

Hepatosomatic index

In the present paper, for the hepatosomatic and gonadosomatic indexes, we have chosen weight of eviscerated fish as reference to avoid bias due to either the large quantity of fat in the abdominal cavity during some annual periods or gonads/liver weight variations or more or less full digestive tract. Hepatosomatic index was calculated as: weight of liver (g) divided by weight of eviscerated fish (g), multiplied by 100. Then, for each sex, a mean was calculated for each month.

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Gonadosomatic index

This index was calculated as: weight of gonads (g) divided by weight of eviscerated fish (g), multiplied by 100. It was determined separately for males and for females. Then a mean for each month was calculated for each group.

Fulton condition factor (K)

This corresponds to the weight of eviscerated fish (g) divided by the total length (cm) to the power 3, multiplied by 100.

Microscopic observations

Each month, a random sample of females (immature, or mature and at different stages of maturity) was treated for microscopy. Pieces of 50 gonads were fixed in alcoholic Bouin solution just after dissection. Histological sections were done as previously described by Martoja and Martoja-Pierson (1967), with the following protocol:

- Fixation in alcoholic Bouin solution for 4 to 6 days
- Dehydration by alcoholic solutions (ethanol, butanol)
- Inclusion in paraffin wax
- Cutting of 5 micrometer blocks, on a microtome, before displaying on microscopy glass slides (3 per block)
- Staining by green light and hematoxylin
- Observation with a photonic microscope with camera (Leica DM 2000 Wild M 20 and Olympus BH 2, Germany).

Interpretation of these observations was done using the scale of maturity established by Holden and Raitt (1974), maturation starting from stage III for males and females (*Table 1*).

Table 1. *Maturation stages for partial breeders (Holden and Raitt, 1974)*

Stage	State	Description
I	Immature	Ovaries volume is about 1/3 of length of abdominal cavity. Ovaries are pinkish and translucent. Eggs are not visible to the naked eye
II	Virgin during maturation and recovery	Ovaries volume is about 1/2 of length of abdominal cavity. Ovaries are pinkish and translucent. Eggs are not visible to the naked eye
III	Becoming mature	Ovaries volume is about 2/3 of length of abdominal cavity. Ovaries are yellow-pinkish and look rough. Neither transparent nor translucent eggs are visible
IV	Mature	Ovaries volume is about 2/3 of length to total length of abdominal cavity. Ovaries are pinkish orange, with superficial blood vessels visible. Mature and transparent eggs are present
V	After spawning	Ovaries retract to about 1/2 of length of abdominal cavity. Walls are loose. Ovaries may contain remaining eggs which are mature, opaque, disintegrating

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Macroscopic study of sexual maturity

With regard to sexual maturity, determination of stages of maturity was done by macroscopic observations of a large number of male or female gonads. Observed changing characteristics during maturation were color, firmness, quantity of vascularization, volume of gonads in the abdominal cavity, thickness and transparency of the gonad wall. This study was difficult for males, and they were only separated into immature and mature animals. For females, we determined, each month, percentages of stages of maturity. Combined with microscopic observations, there are 5 maturation stages for females.

Length at first sexual maturity

The definition retained for our work was the length at which 50% of the population is mature (Batts, 1972; Conand, 1977; Shung, 1973).

Results

As stated above, we sampled 831 fish (between 12 and 80 fish per month), but 175 were considered as of undefined sex and discarded (21.06%). Among the 656 remaining European hake, 391 were identified as males and 265 as females. Details of the fish catches each month are presented in *Table 2*.

Table 2. *Number of European hake (Merluccius merluccius L., 1758) considered in the present study, and numbers of females or males, caught each month in Oran bay*

Month	Number of considered fish i.e. females + males (total = 656 fish)	Number of females (total = 265)	Number of males (total = 391)
January	67	24	43
February	92	37	55
March	68	39	29
April	68	29	39
May	39	11	28
June	43	19	24
July	37	15	22
August	40	22	18
September	33	10	23
October	31	12	19
November	48	14	34
December	90	33	57

The monthly distribution of caught females or males of European hake shows that the number of females were lower almost all the time (except in March and August), during the year of the study.

The calculated ratio of total number of males divided by total number of females was about 1.47. So, the overall sex ratio compared to the balanced sex ratio of 1 (1 male for 1 female) was significantly different (chi-two test, threshold 95%), with more males than females.

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The overall sex ratio is not precise enough to determine sex distribution in the studied population. It has to be completed by study of sex ratio variations in parallel with animal length.

In order to highlight potential variations of sex ratio as a function of fish length, we grouped our 656 European hake together in 2 cm length classes. Maximum length for females was 56 cm and maximum for males was 48.5 cm, thus females were bigger. Percentages of females or males are displayed by length classes in *Figure 1*.

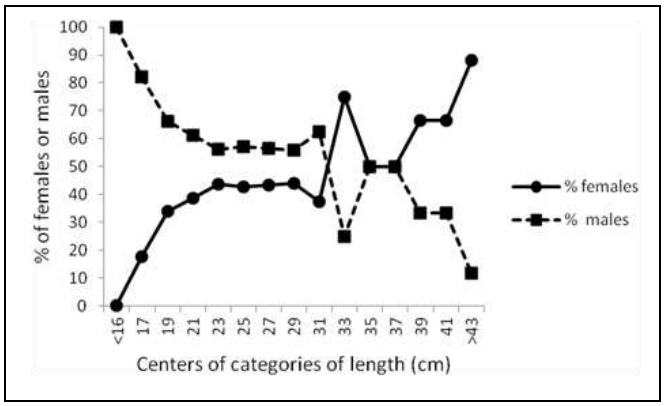

Figure 1. *Percentages of females or males as a function of European hake length, for Oran coasts*

We can see in *Figure 1* that males were more frequent than females for the smallest lengths, from < 16 cm to 30-32 cm. For the largest lengths, from 38-40 cm to maximum size, females were more frequent than males. For length > 49 cm, only females were found in catches.

For our samples, we determined mean size ± SE (standard error) for females of European hake as 28.55 ± 0.55 cm (number of fish = n = 265). Mean size for males was equal to 23.85 ± 0.27 cm (n = 391). Statistical comparison of female and male mean sizes with Wilcoxon test gave a highly significant result (p-value = 3.296 e^{-11}). In other words, females were on average bigger than males along the coasts of Oran.

Mean values of hepatosomatic index (HI) for females or males are presented as a function of month in *Figure 2*. No clear tendency appeared along the year, except that values for females seemed higher than those for males.

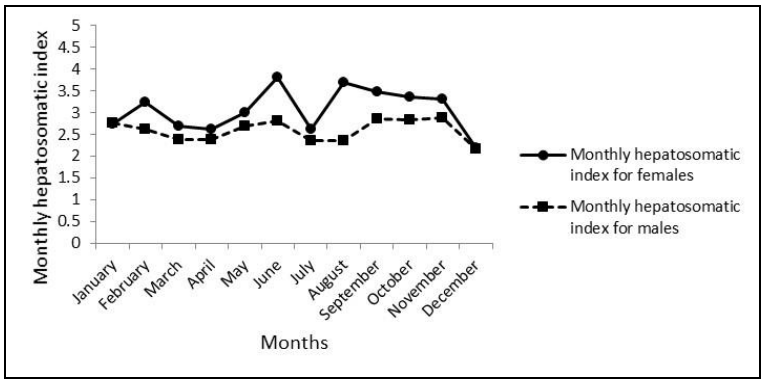

Figure 2. *Monthly progression of hepatosomatic index for female or male European hake, for Oran coasts*

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Mean values of gonadosomatic index (GI) for females or males are presented as a function of month in *Figure 3*. No clear tendency appeared along the year.

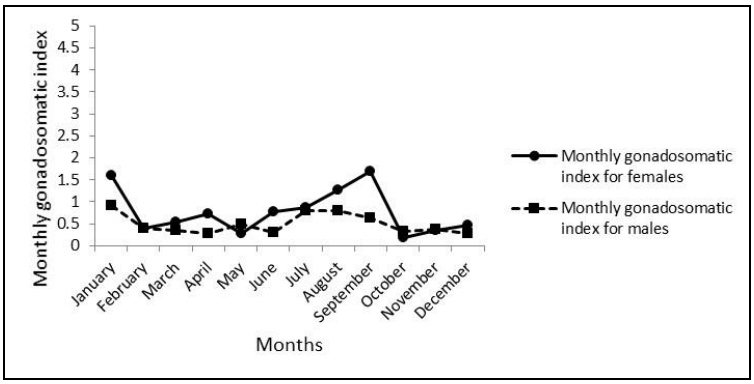

Figure 3. *Monthly progression of gonadosomatic index for female or male European hake, for Oran coasts*

Mean values of Fulton condition factor (K) for females or males are presented as a function of month in *Table 3*. No clear change appeared along the year.

Table 3. *Monthly progression of Fulton condition factor for female or male European hake, for Oran coasts*

Month	Monthly K for females	Monthly K for males
January	0.65	0.66
February	0.69	0.68
March	0.68	0.69
April	0.68	0.67
May	0.69	0.69
June	0.68	0.69
July	0.66	0.67
August	0.67	0.69
September	0.63	0.66
October	0.71	0.68
November	0.74	0.69
December	0.70	0.69

Ovarian dynamics were studied during the sexual cycle of female European hake. The ovary was constituted of conjunctive tissue and follicles. It contained oogonies spread between follicular cells, oocytes in previtellogenesis and oocytes at various stages of vitellogenesis. So, oocyte growth occurred in two phases: the first was previtellogenesis, consisting in organizing the metabolic processes needed for development of germinal cells; the second was vitellogenesis, needed for vitellus accumulation. Each observed ovary contained a set of gametes at different maturation stages. In *Figure 4*, we present photographs of microscopic ovary transverse cross-sections.

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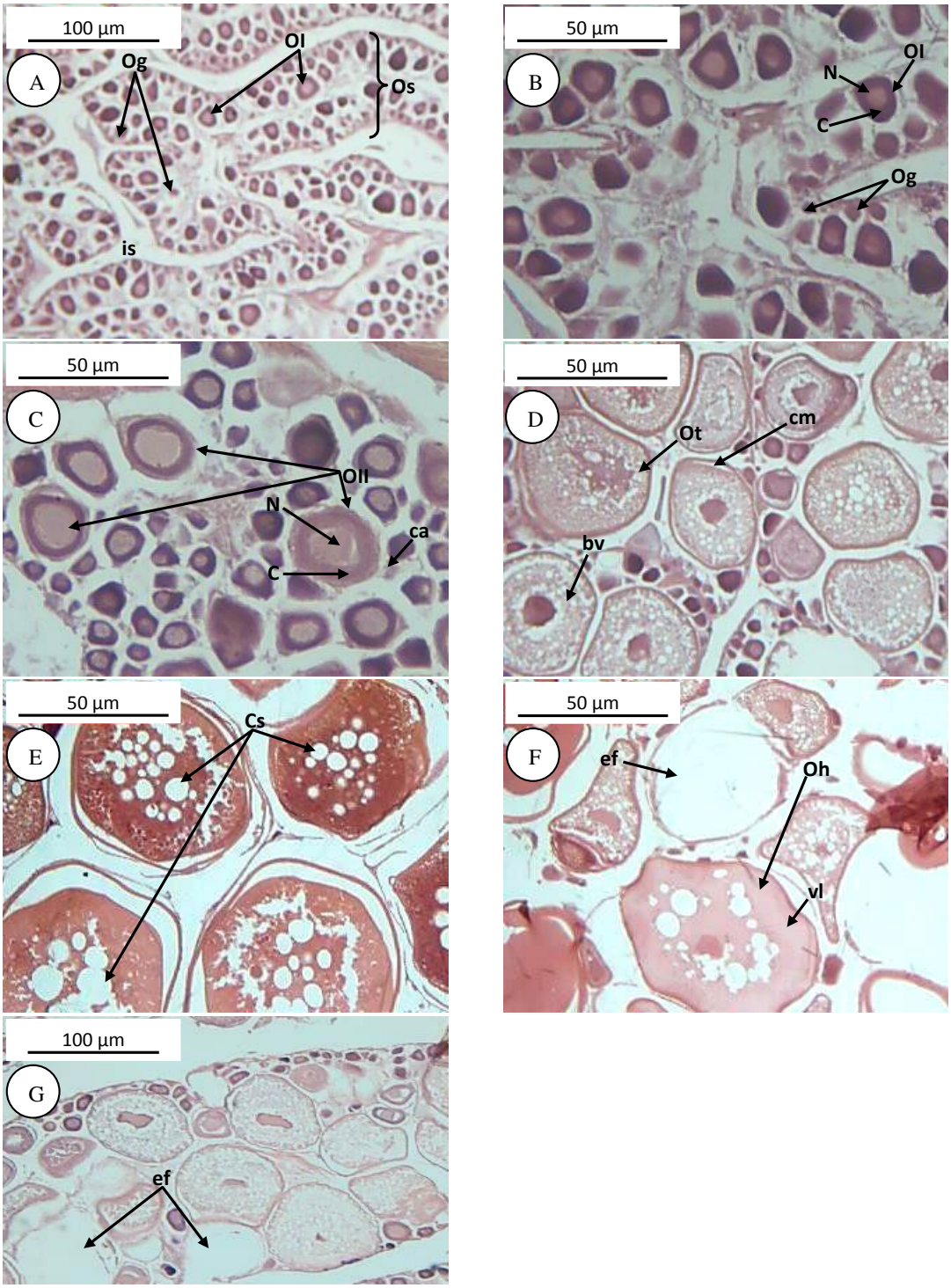

Figure 4. *Histological cross-sections of ovaries of European hake females from the coasts of Oran, for determining the different stages of reproduction. (A) Immature stage I (x0.5); (B) Immature stage I; (C) Beginning of vitellogenesis during stage II; (D) Maturation during stage III; (E) Before spawning period, stage IV; (F) Spawning, stage V; (G) After spawning, stage V. Stage I: ovarian strips (os), interlamellary spaces (is), oogony (Og), nucleus (N), cytoplasm (C), oocyte in previtellogenesis I (OI). Stage II: oocyte II (OII), nucleus (N), cytoplasm (C), cortical alveolus (ca). Stage III: ootide (Ot), cytoplasmic membrane (cm), bladder of vitellus (bv). Stage IV: corpuscles (Cs). Stage V: ootide almost totally hydrated (Oh), vitellus liquefaction (vl), empty follicles (ef)*

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Observations of these microscopic ovary cross-sections, matching the 5 previously described maturation stages, provide a basis for description of these stages as follows:

- Stage I: in *Figure 4A*, we observed piles of spread cells between conjunctive tissues and well-implanted in the epithelium of ovarian strips, separated by interlamellary spaces. Sexual cells were either oogonies (small cells due to mitosis of germ cells) or oogonies developed in oocytes I (in previtellogenesis, of larger size). Magnification of part of this cross-section in *Figure 4B* showed that oogonies have homogeneous, little abundant cytoplasm, oocytes I have large central nucleus and homogeneous and highly stained cytoplasm.
- Stage II: oocytes were starting vitellogenesis, increasing volume and becoming oocytes II (*Fig. 4C*). Cytoplasm became heterogeneous, with traces of lipids in cortical alveolus all around the border.
- Stage III: vitellogenesis continued and oocytes II became higher ootides (*Fig. 4D*). Cytoplasmic membrane was easy to see and cytoplasm was full of small bladders of vitellus. Oogonies, oocytes I and oocytes during vitellogenesis were present.
- Stage IV: ovary was in pre-spawning period (*Fig. 4E*). Bladders of vitellus merged to form corpuscles, starting in the center of the cell. Nucleus was ready for border migration.
- Stage V: *Figure 4F* showed the last maturation stage for gametes, the spawning. Merging of corpuscles full of vitellus went with high hydration, corresponding to vitellus liquefaction just before ovulation, in ootides which became hydrated mature ootides, also called ovums. Overall the gamete grows more than ten times. After ovulation, follicles appeared empty. After spawning, empty follicles degenerated, but we still observed oocytes during the maturation process (*Fig. 4G*).

Results of the macroscopic study completed by microscopic observations, according to the five previously defined maturation stages, are presented in *Figure 5*.

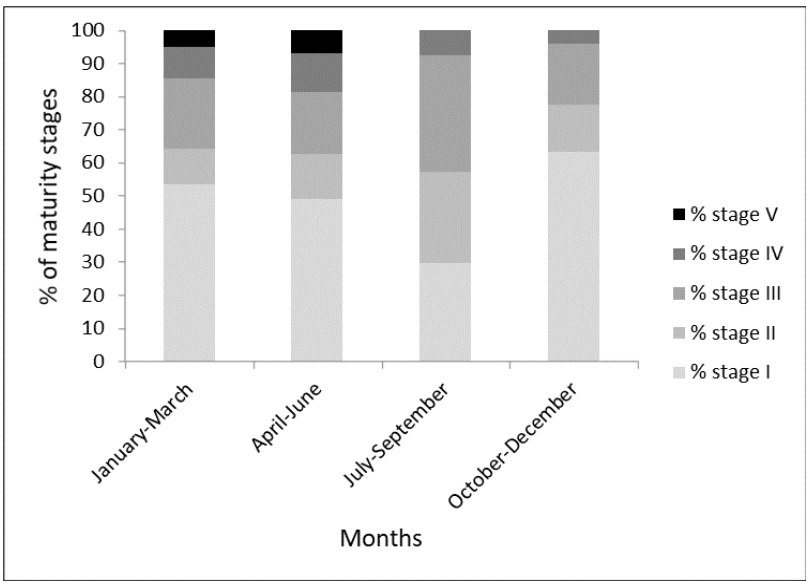

Figure 5. *Progression of sexual maturity stages during the year for female European hake, for Oran coasts*

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Fish of at least four of the five defined maturation stages were present throughout the year, with varying percentages. Note that during July-September and October-December, no females were at stage V. Total (stage III + stage IV) represented females capable of reproduction. We can notice that this total was higher during July-September (42.58%), lower during January-March (31.05%) and April-June (30.5%), and the lowest during October-December (22.44%). So, values are not zero for any time of the year.

Then, the length at first sexual maturity has been estimated by determining percentages of mature fish for each category of animal length, first for females (*Fig. 6*) and secondly for males (*Fig. 7*).

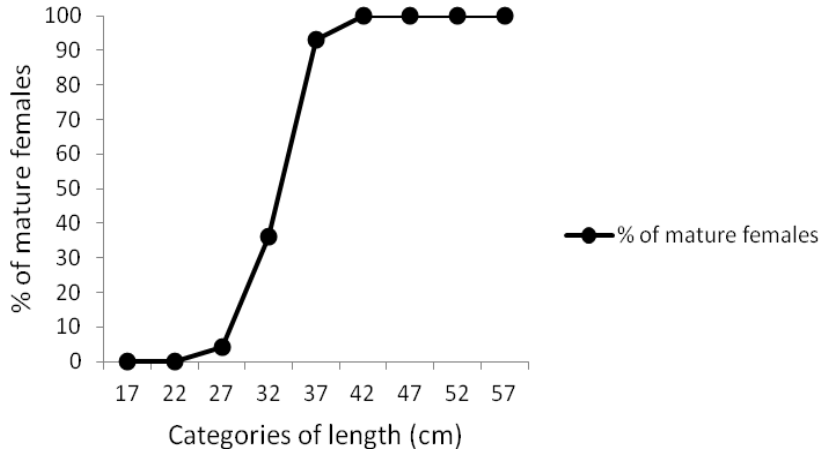

Figure 6. *Determination of length at first sexual maturity for females of European hake, for Oran coasts*

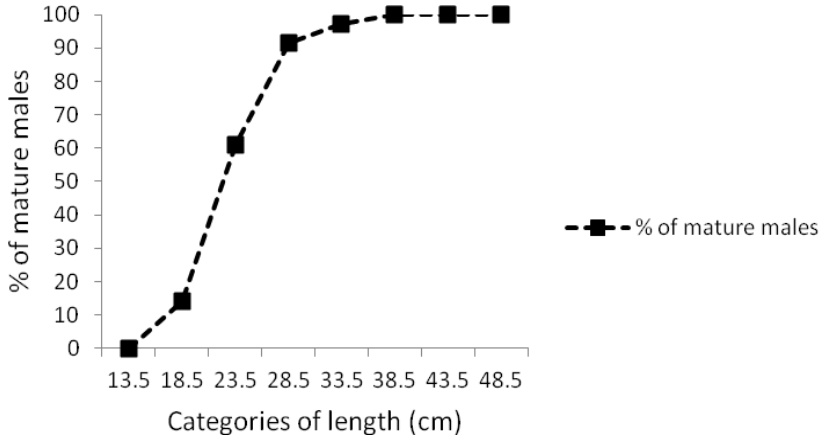

Figure 7. *Determination of length at first sexual maturity for males of European hake, for Oran coasts*

Note that percentages of maturity for females started from 27 cm to reach maximum value at more than 37 cm, and that they increased from 18.5 cm to maximum at more than 33.5 cm for males. Length at first sexual maturity was higher for females, i.e. 33.5 cm, than for males, i.e. 20.5 cm. This 13 cm gap between the two genders revealed the earliness of puberty for males compared to females.

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The most important information provided by determining length at first sexual maturity was that a large part of fish caught along the coasts of Oran were below these length values. Indeed, during this work, we have calculated the number of females, males, or females and males together, having a length below the length at first sexual maturity (*Table 4*).

Table 4. *Numbers of fish of each sex, numbers and percentages of fish smaller than length at first sexual maturity for each sex, for European hake from Oran coasts*

	Females	**Males**	**Total**
Number of fish	265	391	656
Number of fish with length < length at first sexual maturity	191	112	303
Percent of fish with length < length at first sexual maturity (%)	72.08	28.64	46.19

Taken altogether, fish smaller than length at first sexual maturity represented about 46%. This value was dramatically higher dealing only with females (about 72%) and was not negligible dealing only with males (about 29%).

Discussion

In the present study, we have characterized the reproduction of the European hake (*Merluccius merluccius*, Linnaeus, 1758) in Oran bay, from fish collected by fishermen over the course of a year. This was done by assessing the sex ratio, by calculating parameters such as the hepatosomatic and gonadosomatic indexes and Fulton condition factor, by means of macroscopic and microscopic studies of sexual maturity for females, and by measuring length at first sexual maturity.

First of all, sex-ratio is not optimum as it shows that males are predominant throughout most of the year in fishermen's catches. Sex ratio as a function of fish length reveals a majority of males up to 30-32 cm, a majority of females at 38-40 cm or more, and only females are found at lengths > 49 cm. Similar results were obtained by other authors in the Mediterranean Sea, north coast of Tunisia (Khoufi et al., 2012), in the Atlantic, central Morocco (El Habouz et al., 2011), in the Iberian Atlantic (Piñeiro and Sainza, 2003).

Classical indexes have been calculated to estimate fishes' physical state. The hepatosomatic index was calculated because all the energy necessary to obtain gonad maturity and gamete production is thought to come from lipids from hepatic stocks. Authors have previously demonstrated that, for some female fishes, the liver is also responsible for vitellogenine (main protein in oocyte vitellus) synthesis (Nunez-Rogriguez, 1985). Hence, during ovaries development, maturation and when vitellus is accumulated, the HI decreases rapidly (Billard, 1979; Encina and Granado-Lorencio, 1997; Lahaye, 1972). As progression of the weight of gonads during the year allows assessment of the reproduction period for a given species, the gonadosomatic index was calculated. Indeed, it is well known that gonad weight is at a minimum during biological rest periods, increases during gamete maturation and decreases for spawning (Lahaye, 1972). Finally, Fulton condition factor was determined as an indicator of fitness (Bolger and Connolly, 1989). In fact, it is postulated that the heavier an animal is, for a given length, the better is its physical condition. In the present study, as a

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function of the months of the year, no clear change appears for hepatosomatic and gonadosomatic indexes or Fulton condition factor. Hence these indexes and factor do not suggest a specific reproductive period. We assume that some females may lay their eggs not all at the same time. Perhaps their ovaries do not rest after spawning and continue vitellogenesis for a later spawning. For males, storage in the liver may not take place during spermatozoon maturation. Finally, fishes' physical condition seems not to vary in an important way, even during gamete formation or ejection, a result similar to that of Morgan who did not find any relationship between Fulton condition factor and male maturation for codfish (Morgan, 2004).

To investigate more precisely European hake reproduction, microscopic and macroscopic studies of sexual maturity were made. Microscopic study for females showed that each ovary contained a set of gametes at different maturation stages. In fish, two models of spawning have been observed. In the first model, total spawning consists in all oocytes maturing at the same time, see salmon and eel (West, 1990), or groups of oocytes developing in a simultaneous way, see herring, plaice and sole (Horwood, 1990; Le Duff, 1997), and spawned at the same time. In the second model, spawning is realized by series, oocyte maturation being asynchronous, see sardine, Brazilian menhaden, anchovy (Hunter and Goldberg, 1980; Macchi and Acha, 2000; Matsuura, 1998). Our microscopic observations led us to conclude that *Merluccius merluccius* spawns in a partial manner in Oran bay (spawning realized by series), oocyte maturation being asynchronous. This asynchronous oocyte maturation matches observations of other authors for the Mediterranean Sea (Biagi et al., 1995; Nannini et al., 2001; Carbonara et al., 2019) or the Atlantic Ocean (Murua et al., 1998; Murua and Motos, 2006; Murua and Saborido-Rey, 2003; Sarano, 1986). Our macroscopic study for females demonstrated that fish of at least four of the five defined maturation stages were present throughout the year. During July to December no females were at stage V, corresponding to sexual recovery after spawning, and during January to June, only a small percentage at stage V was observed. This might be explained by the fact that, after spawning, almost all female adults return to deep waters and were not caught by fishermen. We highlighted that females capable of reproduction (stages III and IV) were always present. Taken together, the microscopic and macroscopic observations allow us to conclude that females of *Merluccius merluccius* breed throughout the year on the coasts of Oran. Unlike our present results, some studies lead to the conclusion that there is a discontinuous reproductive period, less for the Mediterranean Sea and mainly for the Atlantic Ocean (see references in *Table 5*). But, like us, other authors have concluded that spawning occurs during the whole year (with or without one, two or three more intensive phases), as described mainly for the Mediterranean Sea and less for the Atlantic Ocean (see references in *Table 5*). *Table 5* summarizes data for *Merluccius merluccius* spawning period in various areas.

Finally, length at first sexual maturity provides information on the part of the fish stock able to reproduce and consequently on the replenishment potential of the species. In the present study, it is higher for females (33.5 cm) than for males (20.5 cm). This 13-cm gap between the two genders reveals the earliness of male puberty compared to females. Our observation of length at first sexual maturity lower for males than for females fits well with results from other authors (see *Table 6*), either in Mediterranean Sea or in Atlantic Ocean. Data about length at first sexual maturity for *Merluccius merluccius* in various areas are summarized in *Table 6*.

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In the present work, the most important information provided by determining the length at first sexual maturity is that a large part of fish caught along the coasts of Oran are under the values of length at first sexual maturity. So these individuals are juveniles, responsible for stock sustainability, caught before being able to breed.

Table 5. *Spawning period for Merluccius merluccius in various areas (white: no spawning; grey: spread spawning; black: maximal spawning)*

Sea/Ocean	Area	References	Month											
			J	F	M	A	M	J	J	A	S	O	N	D
Mediterranean Sea	Algeria (Oran)	Present study												
	Algeria (Bou-Ismail)	Bouaziz, 1998												
	Tunisia	Heldt, 1952												
	Tunisia	Bouhlal, 1973												
	Tunisia	Khoufi et al., 2014b												
	Western Mediterranean Sea	Oliver, 1991												
	Western Mediterranean Sea	Morales-Nin and Moranta, 2004												
	France (Gulf of Lion)	Recasens et al., 1998												
	Italy (Adriatic Sea)	Zupanovic, 1968												
	Italy (Adriatic Sea)	Zupanovic and Jardas, 1986												
	Italy (Adriatic Sea)	Arneri and Morales-Nin, 2000												
	Italy (Sardinia)	Carbonara et al., 2019												
	Italy (Tyrrhenian Sea, Adriatic Sea, Ionian Sea)	Carbonara et al., 2019												
	Greece	Tsimenidis and Papaconstantinou, 1985												
	Greece	Mytilineou and Vassilopoulou, 1988												
	Turkey	Soykan et al., 2015												
Atlantic Ocean	Morocco	Maurin, 1954												
	Morocco	El Habouz et al., 2011												
	Spain	Alcázar et al., 1983												
	Spain	Perez and Pereiro, 1985												
	Spain	Piñeiro and Sainza, 2003												
	France	Belloc, 1923												
	France (Bay of Biscay)	Sarano, 1986												
	France (Bay of Biscay)	Alvarez et al., 2004												
	France (Bay of Biscay)	Murua and Motos, 2006												
	Celtic Sea	Fives et al., 2001												

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Table 6. *Length at first sexual maturity for Merluccius merluccius in various areas*

Sea/Ocean	Area	References	Length at first sexual maturity for females (cm)	Length at first sexual maturity for males (cm)
Mediterranean Sea	Algeria (Oran)	Present study	33.5	20.5
	Algeria (Bou-Ismail)	Bouaziz et al., 1998	30.6	21.5
	Tunisia	Khoufi et al., 2014b	29	-
	Eastern Mediterranean Sea	Alheit and Pitcher, 1995	29.5	26.5
	Western Mediterranean Sea	Oliver, 1991	36.3	27.6
	Western Mediterranean Sea	Alheit and Pitcher, 1995	34.5	27
	Spain (Catalonia)	Recasens et al., 2008	35.8	-
	Italy (Tyrrhenian Sea)	Biagi et al., 1995	42.5	27
	Italy (Tyrrhenian Sea)	Recasens et al., 2008	35.1	-
	Italy (Adriatic Sea)	Zupanovic, 1968	26	24
	Italy (Adriatic Sea)	Zupanovic and Jardas, 1986	28	24
	Italy (Sardinia, Tyrrhenian Sea, Adriatic Sea, Ionian Sea)	Carbonara et al., 2019	32	-
	France (Gulf of Lion)	Recasens et al., 1998	38	28.8
	Turkey	Soykan et al., 2015	21.5	25.6
Atlantic Ocean	Morocco	Maurin, 1954	40	40
	Morocco	El Habouz et al., 2011	33.8	28.6
	Spain	Larrañeta, 1970	32.2	24.3
	Spain	Alcázar et al., 1983	54	42
	Spain	Perez and Pereiro, 1985	52.5	38
	Spain	Piñeiro and Sainza, 2003	45.4	32.8
	Spain	Domínguez-Petit et al., 2008	46	-
	North Atlantic	Domínguez-Petit et al., 2008	41	-

Conclusion

To conclude, in Oran bay, our results, from the fishermen's catches studied, show that *Merluccius merluccius* spawns in a partial manner (with asynchronous oocyte maturation) and breeds throughout the year. Determination of length at first sexual maturity (smaller for males) demonstrates the earliness of puberty in males compared to females. During this work, we have also estimated that 46% of caught European hake have a length below the length at first sexual maturity, and thus are juveniles. This information is of crucial importance. It represents a danger for future fish stocks to

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continue to catch such a large quantity of fish killed before being able to breed. If sustainable fishing is to be achieved, appropriate laws will have to be enacted and observed. We suggest that the minimum market size of 20 cm for Algerian waters according to the Ministry of Fisheries and Fishery Resources of the Republic of Algeria (M.P.R.H, 2016) will have to be reconsidered. Furthermore, reducing fishing effort and increasing mesh sizes in the region is recommended for sustainable exploitation of this fishery resource.

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RESPONSES OF FIVE POMEGRANATE (*PUNICA GRANATUM* L.) CULTIVARS TO CONTRASTING WATER AVAILABILITY: LEAF MORPHOPHYSIOLOGICAL AND ANATOMICAL ADAPTATION

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Abstract. This investigation was carried out on five pomegranate cultivars (Wonderful, Manfalouty, Acco, Assuity and 116) to evaluate their performance under two irrigation levels 100% of field capacity (control) and 30% of field capacity (water stress) during two consecutive seasons 2019 and 2020. The results indicated that water stress at 30% of field capacity significantly reduced leaf area, stomatal conductance and transpiration rate in all studied pomegranate cultivars. Manfalouty cultivar recorded the least reduction in leaf area, transpiration rate and stomatal conductance compared to Acco cultivar. Also, Manfalouty cultivar showed the highest increase (32.30%) in the thickness of upper epidermis under 30% of field capacity. On the other hand, lower epidermis showed an increase in all studied cultivars except for 116 cultivar. Moreover, the leaves of both Wonderful and Manfalouty cultivars were characterized by a great thickness of palisade (81.9 and 73.5 µm) and spongy (89.1 and 65.9 µm) tissues under water stress, while the lowest palisade tissue was recorded in Acco cultivar (56.7 µm) and the lowest spongy tissue was recorded in Assuity cultivar (44.4 µm). Furthermore, the highest thickness of xylem tissue was recorded in Manfalouty cultivar (8.70%) and the lowest decrease (6.98%) in phloem tissue. Based on physiological and anatomical characteristics, it could be concluded that Manfalouty cultivar was the most drought tolerant, while Acco cultivar was the most sensitive.
Keywords: *water stress, leaf area, stomatal conductance, transpiration rate, leaf anatomy*

Introduction

Pomegranate (*Punica granatum* L.) belonging to the Punicaceae family, is considered one of the oldest edible fruits (Blumenfeld et al., 2000). It is valued for its nutritional, pharmacological and medicinal benefits and is being used as a herbal treatment for diabetes, cancer, inflammation, blood pressure and many other diseases (Akbarpour et al., 2010; Adhami et al., 2012). Pomegranate production has expanded greatly worldwide due to its hardy nature and xerophytic characteristics that enabled it to cope with adverse and unsuitable climatic and water conditions (Borochov-Neori et al., 2013; Singh et al., 2014), which means it can survive in arid and semi-arid areas even under harsh environmental conditions (Aseri et al., 2008). However, pomegranate tree needs a regular irrigation regime during the dry season to obtain an optimal growth and fruit quality (Holland et al., 2009). Increasing the production and expansion leads to lack of precise available data on pomegranate area and production, although it could be estimated that around 1.5 million tonnes of fruits are produced annually in the world (Holland and Bar-Ya'akov, 2008), and cultivated nearly in more than 302,000 ha, 76% of them found in India, Iran, China, Turkey and USA (Melgarejo et al., 2012). Egypt is one of the most important Mediterranean countries producing and exporting pomegranate fruits with a total cultivated area of about 44809.8 ha and an amount of exports about 122387.2 tonnes (Egyptian ministry of Agriculture and land reclamation, 2019). There are many local

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verities in Egypt like Manfalouty, Assuity, Araby and Nab El-Gamal and other foreign varieties such as Wonderful, 116, Acco, Sweet and Ever sweet.

Facing that agriculture sector consumes about 70% of world fresh water (FAOSTAT, 2016). Limitation of water resources is one of the most important critical factors influencing plant growth specifically in arid and semi-arid regions (Tatari et al., 2020). Worldwide, it is expected that areas of arable land could decrease due to climate change (Harrison et al., 2014; Sherwood and Fu, 2014). Water stress is a serious limiting factor restricting plant performance, growth, and distribution globally (Liu et al., 2011). Exposing plants to such restricting environmental conditions stimulate a series of morphological, biochemical, physiological, and anatomical responses to cope with stressful circumstances and ameliorate plant functions in different ways (Reddy et al., 2004). Mechanisms and strategies to adapt to drought stress vary between cultivars and species. Intrigliolo et al. (2011), Rodriguez et al. (2012) and Parvizi et al. (2016) have shown that in pomegranate, deficit irrigation decreased photosynthesis and transpiration rates due to reduced stomatal conductance. There are few studies assessed anatomical responses of pomegranate leaves to water stress. Besides biochemical and physiological changes, anatomical functions are stimulated in plants leaves enabling them to withstand drought conditions through reducing water loss and maintenance of photosynthetic rates (Hameed et al., 2012).

In this respect, one of the most important strategies to achieve water conserving is to use drought-tolerant species which is more economical, sustainable and eco-friendly. Therefore, in this study we aim to evaluate different responses of some pomegranate cultivars and to understand physiological and anatomical responses in relation to water stress.

Materials and methods

Plant material, growth conditions and treatments

A pot experiment was carried out during two consecutive seasons 2019 and 2020 at the experimental net greenhouse of pomology department Faculty of Agriculture Cairo University, Egypt (30°01'04"N31°12'30"E). Five pomegranate cultivars namely; Wonderful, Manfalouty, Acco, Assuity and 116 were used. All cultivars were propagated by hard wood stem cuttings under net greenhouse conditions, the length and diameter of the cuttings were in the ranges of 25–30 cm and 2–2.5 cm, respectively. Pomegranate cuttings were prepared in 15th February and stored in a moist peat moss for two weeks to promote callusing and root formation. On 1st of March selected cuttings were planted into 10 liter plastic pots filled with washed sandy soil and watered twice a week with tap water (EC: 0.48 ds/m). In the early June, 21 plants of each cultivar for each treatment with an average height of 35-40 cm pruned by lateral shoot removal to uniform plants with single main shoot were subjected to two water stress levels for three months, control (100% of field capacity) and water stress (30% of feild capacity). Field capacity (FC) was calculated using the following equation:

$$FC = \frac{(FW-DW)}{\mathrm{DW}} \mathrm{x}\ 100 \qquad \text{(Eq.1)}$$

where FW was the fresh weight of soil sample and DW was the dry weight of soil sample after oven drying at 85ºC for 3 days (Coombs et al., 1987). *Table 1* shows the results of

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soil and water samples analysis. During the experimental period water levels were maintained by weighing three pots from each treatment every three days and adding proper water volumes. Every second week of the month, experimental plants were fertilized by 0.25 strength of Hoagland solution for macronutrients and full strength for micronutrients (Fozouni et al., 2012).

Table 1. *Estimation of the physical and chemical properties of the experimental soil and irrigation water*

Characteristics	Soil	Water
Particle size distribution %		
Fine sand	44.2	
Coarse sand	41.6	
Silt	11.2	
Clay	3	
Texture	Sandy soil	
EC (dsm^{-1})	1.21	0.48
P^H	7.91	7.53
Soluble cations (meq/l)		
Ca^{+2}	3.8	2.3
Mg^{+2}	1.9	1.4
Na^{+}	60	0.84
K^{+}	0.3	0.18
Soluble anions (meq/l)		
CO_3^{-2}	--	--
HCO_3^{-}	0.37	1.8
Cl^{-}	10	2.5
SO_4^{-2}	1.63	0.24
Soil moisture constants		
Saturation percentage (SP)	24	
Field capacity (FC)	15%	
Wilting point (WP)	6.2	
Available water (AW)	8.8	

At the end of the experimental period (September) the following measurements were recorded.

Leaf area

Leaf area (cm^2): was calculated using the following equation:

$$LA = -0.0477 + 0.0282*L + 0.0842*W + 0.965*L*W \quad \text{(Eq.2)}$$

where, LA is leaf area, W is leaf width and L is leaf length according to Meshram et al. (2012).

Gas exchange measurements

Transpiration rate (T_r) (µg $H_2O/cm^2.s$) and stomatal conductance (g_s) (cm/s) were measured in the 5th and 6th mature leaves from shoot apex using a portable steady-state porometer (LI-1600M, LI-COR, Nebraska, USA) between 10:00 am and 12:00 pm with an air temperature around 30ºC, relative humidity ranged between 35:42% and an active photosynthetic radiation of 1200 mmol m^{-2} s^{-1} according to Surendar et al. (2013).

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Leaf anatomical measurements

A microscopic examination was performed on Leaf samples. Specimens (ca. 1 cm^2) from the middle section of the youngest fully grown leaves were killed and fixed for at least 48 hrs. in F.A.A. (10 ml formalin, 5 ml glacial acetic acid and 85 ml ethyl alcohol 70%). The plant samples were washed in 50% ethyl alcohol, dehydrated in a normal butyl alcohol series and embedded in paraffin wax of melting point 56°C then sectioned to a thickness of 20 microns, double stained with crystal violet-erythrosin and cleared in xylene and mounted in Canada balsam (Nassar and El-Sahhar, 1998). Measurements of the micrographs were detected using light compound microscope (LEICA DM750) and a LEICA ICC50 HD by the Leica Application Suite program.

Statistical analysis

Data were statistically analyzed following the analysis of variance (ANOVA) technique according to split plot design; water stress as the main plot in 2 levels (factor A) and 5 pomegranate cultivars as the sub-plot (factor B). 42 plants of each cultivar were selected and arranged in complete randomized block design. Each treatment was represented by three replicates. Means of the treatments were compared by least significant difference (L.S.D.) at significance level of 0.05 (Duncan, 1955).

Results and discussion

Leaf area

As shown in *Table 2* the results indicate that compared to stress treatment (30% of FC), full irrigated plants (100% of FC) considerably recorded the highest significant values of leaf area during both seasons. Manfalouty cultivar was the least affected by stress conditions among all cultivars and reduced leaf area by 21.20% and 10.55% during first and second seasons respectively, meanwhile Acco cv. was much affected and recorded a reduction by 34.74% and 33.61% in the first and second seasons respectively. Hamdy et al. (2016) found that maximum values of leaf surface area of two studied pomegranate cultivars (Early 116 and Wonderful) were obtained when the plants were irrigated with 100% of field capacity, while the minimum values were recorded at 40% of available water. Moreover, under different irrigation levels 100%, 75% and 50%, it was found that among four pomegranate cultivars Manfalouty cv. recorded the highest values of leaf area during two consecutive seasons (Haleem et al., 2020). Furthermore, it has been reported that reducing irrigation level up to 7 m^3/tree/year decreased leaf area of twenty-year-old pomegranate Manfalouty trees, meanwhile increasing irrigation level up to 15 m^3/tree/year rate recorded the highest leaf area values during two seasons (Khattab et al., 2011). Matthews (1986) found that the drop in leaf area produced by water stress in cantaloupe vines plants was attributable to a decrease in epidermal cell mitotic activity, which resulted in a reduction in the total number of cells. She added that leaves first developed during water stress conditions were the most affected and showed a sever reduction in cell division. While leaves first developed under irrigated conditions then exhibited to stress conditions showed a relative normal cell division and leaf size. Indicating that the majority of cell division took place during and shortly after leaf initiation.

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Table 2. *Effect of water stress on leaf area (cm^2) of five pomegranate cultivars*

Cultivars	1st season		Mean	2nd season		Mean
	Water levels			Water levels		
	100%	30%		100%	30%	
Wonderful	11.130 d	8.100 f	9.615 B	14.213 c	10.450 f	12.332 D
Manfalouty	11.663 cd	9.190 e	10.427 A	14.873 bc	13.303 d	14.088 B
Acco	12.570 b	8.203 f	10.387 A	17.810 a	11.823 e	14.817 A
Assuity	12.173 bc	8.540 ef	10.357 A	15.260 b	12.300 e	13.280 C
116	13.570 a	8.233 f	10.902 A	12.963 d	10.350 f	11.657 E
Mean	12.221 A	8.453 B		15.024 A	11.445 B	

Means with the same letter within each season were significantly equal at L.S.D. 5% level

Leaf gas exchange measurements

In our study, according to the data presented in *Tables 3 and 4*, it can be noticed that water stress at 30% of FC significantly declined transpiration rate (T_r) and stomatal conductance (g_s) in all tested pomegranate cultivars in comparison to full irrigated plants (100% of FC), Manfalouty cv. recorded the lowest reduction in transpiration rate and stomatal conductance by 20.94% and 12.59%, respectively. However, Acco cv. recorded the highest significant reduction in both transpiration rate and stomatal conductance by 69.10% and 68.05%. In line with our findings, Intrigliolo et al. (2011), Rodriguez et al. (2012) and Parvizi (2016) reported a decrease in stomatal conductance under deficit irrigation strategy compared to full irrigation in pomegranate. Also, Pourghayoumi et al. (2017) found that exhibiting five pomegranate cultivars to water stress for 14 days progressively declined net photosynthesis rate (A_n), transpiration rate (T_r) and stomatal conductance (g_s) so as to control water loss via transpiration and maintain leaf turgor. Reduction of leaf gas exchange, stomatal closure, and slowing down photosynthetic activity are the main consequences of drought stress in plants (Hu et al., 2010). This may be due to the reduction in premature leaf senescence, leaf expansion, damaged photosynthetic process, oxidative chloroplast lipids as well as changes in structure of proteins and pigments (Murtaza et al., 2016). On the other hand, it is well known that reducing transpiration rate under stress conditions due to stomatal closure could also decline photosynthetic rate as a consequence of deceasing levels of CO_2 inside stomatal chambers and cells as well as amounts of CO_2 reaching carboxylation sites inside chloroplasts (Xue et al., 2021).

Table 3. *Effect of water stress on transpiration rate (μg $H_2O/cm_2.s$) of five pomegranate cultivars*

Cultivars	Water levels		Mean
	100%	30%	
Wonderful	5.823 a	3.847 b	4.835 A
Manfalouty	5.047 a	3.990 b	4.518 A
Acco	5.447 a	1.683 c	3.565 B
Assuity	5.480 a	3.123 b	4.302 A
116	5.337 a	3.920 b	4.628 A
Mean	5.427 A	3.313 B	

Means with the same letter were significantly equal at L.S.D. 5% level

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Table 4. *Effect of water stress on stomatal conductance (cm/s) of five pomegranate cultivars*

Cultivars	Water levels		Mean
	100%	30%	
Wonderful	0.326 a	0.270 bc	0.298 A
Manfalouty	0.270 bc	0.236 c	0.253 B
Acco	0.313 ab	0.100 e	0.206 C
Assuity	0.320 ab	0.173 d	0.246 B
116	0.306 ab	0.226 cd	0.266 AB
Mean	0.307 A	0.201 B	

Means with the same letter were significantly equal at L.S.D. 5% level

From the microscopic scrutinization of cross sections (*Figure 1*) and tabulated data (*Table 5*), it revealed anatomical differences of leaf pomegranate cultivars under study in response to water stress (30% of FC). There was a decrease in thickness of upper epidermis of the cultivars Wonderful, Acco and 116 by 22.81%, 25.80% and 3.77% respectively, due to low water irrigation. Meanwhile, Manfalouty cv. showed a maximum increase by 32.30% followed by Assuity cv. that increased by 22.63% compared to that at full irrigation (100% of FC). On the opposite, lower epidermis showed an increase at low water level in all studied cultivars except 116 cv. which recorded a decrease by 18.24%. Such increase in thickness of epidermis tissues could impact the loss of water through leaf surface. Similarly, the earlier studies Boughalleb et al. (2012) revealed that thicker epidermis tissues play an important role to minimize nonstomatal water loss through leaf surface under harsh environmental conditions. Palisade and spongy tissues showed an increase in thickness only in Wonderful cv. by 22.23% and 11.23%, respectively, beside spongy tissue of Assuity cv. that increased by 3.25% under water stress conditions. Meanwhile all the cultivars under study showed a reduction in the thickness of palisade and spongy tissues. However, it is worth to mention that Manfalouty cv. had a very low reduction (3.41%) in palisade thickness. Moreover, leaves of both Wonderful and Manfalouty cultivars characterized by great thickness of palisade (81.9 and 73.5 µm) and spongy (89.1 and 65.9 µm) tissues respectively under water stress conditions compared to other cultivars under study. Many studies have mentioned that increase the thickness of palisade tissue could have a role in increasing the number of carboxylation sites as well as a thicker spongy parenchyma may result in improving the diffusion of CO_2 to those sites leading to maintain photosynthesis rate of leaves under low water availability conditions (Ennajeh et al., 2008, 2010; Flexas et al., 2008, 2012). Lamina thickness reduced negatively in all pomegranate cultivars under 30% of FC except Wonderful cv. recorded an increase by 9.89%, this fits with Ennajeh et al. (2010) who observed a total lamina increase under drought conditions in two studied olive cultivars (Chemlali and Meski). Also it is known that succulence of plant leaf thickness is a substantial structural modification to adapt to dry habits (Kaleem and Hameed, 2021). Midvein tissue of Manfalouty cv. was more thickness (733.4 and 714.3 µm) either at full or low water availability conditions respectively, compared to other cultivars under study. However, there was a decrease in midvein thickness of all cultivars except Wonderful cv. which showed an increase by 2.93%, besides noticing that Manfalouty cv. recorded the least decrease by 2.60%. Thicker midvein or least reduction in its thickness may enhance transportation of substances and plant survival under unfavorable conditions (Song et al., 2021).

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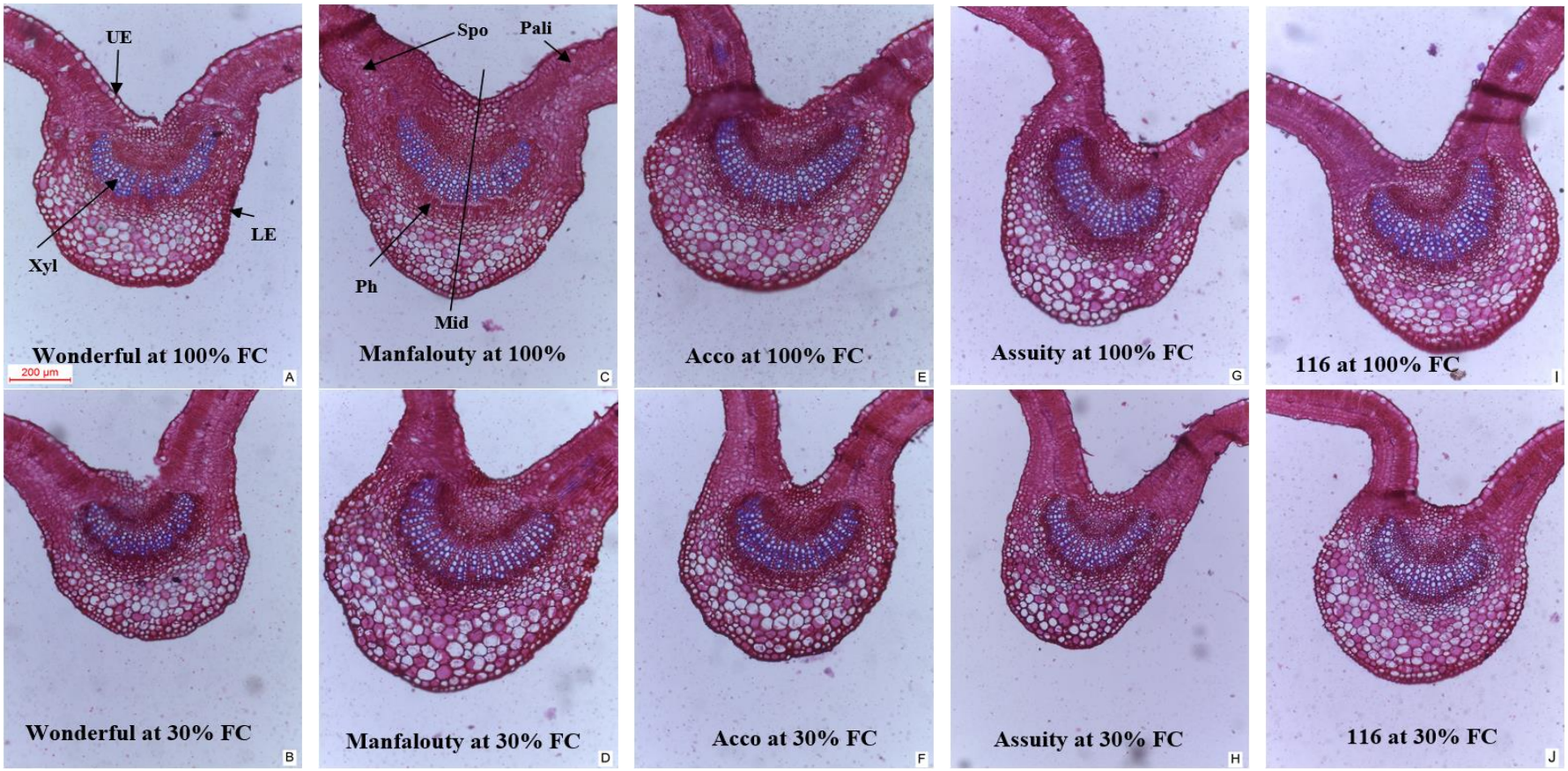

Figure 1. *Comparative leaf anatomy in blade cross-sections (100X) of five pomegranate cultivars Wonderful, Manfalouty, Acco, Assuity and 116.Uper epiderms (UE), Lower epidermis (LE), Midvein thickness (Mid), Palisade thickness (Pali), Spongy thickness (Spo), Xylem thickness (Xyl), Phloem thickness (Ph)*

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Table 5. *Anatomical changes in cross sections of leaves of five pomegranate cultivars under water stress conditions*

Measurements (µm)	Water levels									
	100% of FC					30% of FC				
	Wonderful	Manfalouty	Acco	Assuity	116	Wonderful	Manfalouty	Acco	Assuity	116
Upper epidermis thickness	37.7	22.6	34.1	24.3	34.4	29.1	29.9	25.3	29.8	33.1
Lower epidermis thickness	22.3	29.1	18.6	22.5	30.7	27.5	32.6	28.7	28.0	25.1
Midvein thickness	548.1	733.4	622.1	678.5	658.6	564.2	714.3	587.6	527.7	607.6
Lamina thickness	207.1	216.3	199.0	176.8	210.0	227.6	201.8	165.0	160.0	178.1
Palisade thickness	67.0	76.1	84.9	87.0	65.2	81.9	73.5	56.7	57.8	64.2
Spongy thickness	80.1	88.5	61.4	43.0	79.6	89.1	65.9	54.3	44.4	55.7
Xylem thickness	84.5	117.2	104.5	111.4	135.2	88.2	127.4	97.8	97.3	117.2
Phloem thickness	84.1	73.0	88.4	106.2	62.7	70.7	67.9	61.9	68.3	72.9

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Furthermore, the thickness of xylem tissue of the cultivars Wonderful and Manfalouty with its maximum in Manfalouty cv. (8.70%) could enable maintaining efficient water and nutrients uptake to the leaves, compared to other cultivars that showed a decrease under low water irrigation level. Phloem tissue thickness was decreased under water stress conditions, except 116 cv. that showed an increase by 16.26% and with the lowest decrease in Manfalouty cv. by 6.98%. The thickening of vascular bundles enables for greater flow of water and mineral salts in dry habits, giving the plants proper adaptive characteristics under water restriction conditions (Queiroz-Voltan et al., 2014). Previous results indicating that leaf morpho-anatomical adaptations are spices-specific, Wonderful followed by Manfalouty cultivars were the most adaptable to water stress conditions compared to other studied cultivars.

Conclusion

Water stress reduced leaf area, stomatal conductance and transpiration rate of all studied pomegranate cultivars. Under water stress conditions, Manfalouty cv. recorded a slight decrease in leaf area as a morphological mechanism for drought tolerance. Also, Manfalouty cv. reduced transpiration rate and stomatal conductance as a physiological mechanism for drought tolerance. In addition, Manfalouty cv. showed an increase in anatomical structures of the leaf such as the thickness of upper epidermis, palisade, spongy, xylem tissues as an anatomical mechanism for drought tolerance.

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THE USE OF SCOTS PINE NEEDLES FOR THE ASSESSMENT OF POLLUTION IN THE BALTIC TOWNS IN POLAND

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Abstract. In 2019, the concentration of heavy metals (HMs) in Scots pine needles and soil in the western part of the Polish Baltic coast was analysed. The studies were conducted at 12 test points in 11 seaside locations belonging to two mesoregions: Wolin and Uznam Island, and Trzebiatowskie Coast. The obtained HMs concentration values were compared with anthropogenic and climatic factors identified in the localities. The HM values showed a low degree of pollution both in Scots pine needles as well as the soil. The results demonstrate that needles of *Pinus sylvestris* L. served as an extremely sensitive phytoindicator showing a strong correlation between the content of Cd, Cr, Mn, and Zn, both in Scots pine needles as well as in soil. The statistical analysis confirmed the relationship between HMs concentration in plant and soil material and the number of residents of the seaside localities under analysis. A significant increase in the number of holidaymakers in the summer season did not contribute to exceeding the permissible HMs standards for plants and soil.
Keywords: *phytoindicator, bioaccumulation, biomonitoring, HMs, air and soil pollution*

Introduction

Heavy metals (HMs) constitute atmospheric pollution that is burdensome and hazardous to human health (Idani et al., 2018). The areas most polluted with these elements are urban and industrial areas (Loppi et al., 2019; Mahabadi et al., 2020). It was found that HM pollution contributes to the development of numerous diseases concerning the cardiovascular, respiratory, nervous and reproductive systems (WHO, 2016). Health problems may result directly from HMs when they enter the body with air or an indirect action following a contact with polluted soil and plants. Accumulation of HMs poses a significant risk to health and, consequently, lowers the quality of life in large human settlements. Therefore, in urban areas, the monitoring of toxic HM concentration in all mediums i.e., air, soil and plant is of particular importance (Molnár et al., 2020; Tikhonova et al., 2020). Various plant species, particularly the evergreens e.g. Scots pine needles *Pinus sylvestris* L. are used as bioindicators of air and soil pollution (Mateirć et al. 2016; Turkyilmaz et al., 2018; Schulz et al., 2019; Alaqouri et al., 2020; Aricak et al., 2019, 2020). Plants react to environmental pollution in an observable way due to worsening of their life functions i.e., photosynthesis, respiration, transpiration, accumulation of HMs in tissues, resulting in poor physical condition of the specimens (low growth rate), discolouration or defective organs (Souri et al., 2019).

The western part of the Baltic seaside from Uznam and Wolin Islands to Trzebiatowskie Coast is a unique early post-glacial landscape with protected cliff coast, valuable pine forests (Musiak, 2014). Furthermore, the area also serves cultural purposes and offers an attractive tourism space (Cerić, 2019) despite poor state of transboundary waters in the southern littoral zone of the Baltic Sea (Friedland et al., 2019). The coastal zone is almost completely under nature protection as an ecological corridor for numerous plant and animal species: PLH 320019 - mesoregion Wolin and Uznam, and PLB 320010

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- mesoregion Trzebiatowskie Coast. The area is considered to be only slightly polluted and anthropogenically transformed only to a small extent.

The sustenance of natural biodiversity of the analysed coastal zone is conditioned by low population density in that area, with an exception of Świnoujście - a large border city (at the Polish border with Germany). However, the number of permanent residents in the locations is low, yet during the summer season, the number of people present in that area increases several dozen times and can cause deterioration of the atmospheric, aquatic and soil environment owing to increased heating, food, accommodation and transport needs of the holidaymakers. It was assumed that in localities with higher population, both in terms of permanent residents and seasonal increase, the pollution of plants (Scots pine needles) and soil (in the vicinity of trees from which needles were collected) with HMs would be the highest. High values would be observed in Świnoujście, Międzyzdroje and Dziwnów, the relationships would be particularly noticeable in municipal communes offering the highest number of accommodation establishments i.e., Świnoujście, Dziwnów and Rewal. The aim of the study was the assessment of pollution levels in localities of the Baltic Seaside using Scots pine needles and the analysis of the relationship between the presence of HMs in plant material and HMs level in soil.

Material and Methods

Study area

In 2019 in Poland, the analysis of the chemical composition of HMs in Scots pine needs *Pinus sylvestris* L. and in soil was conducted in 11 seaside localities (1, 2, 4-11) including cities (1, 2, 6), villages (4, 5, 7, 9-12), settlement (8) and a hill (the control point - 3). In terms of natural characteristics, the analysed localities belong to two mesoregions: island - Uznam and Wolin, and land - Trzebiatowskie Coast, both located in the coastal zone of the Baltic Region (*Figure 1*). The selection of the localities was based on their physical characteristics (location).

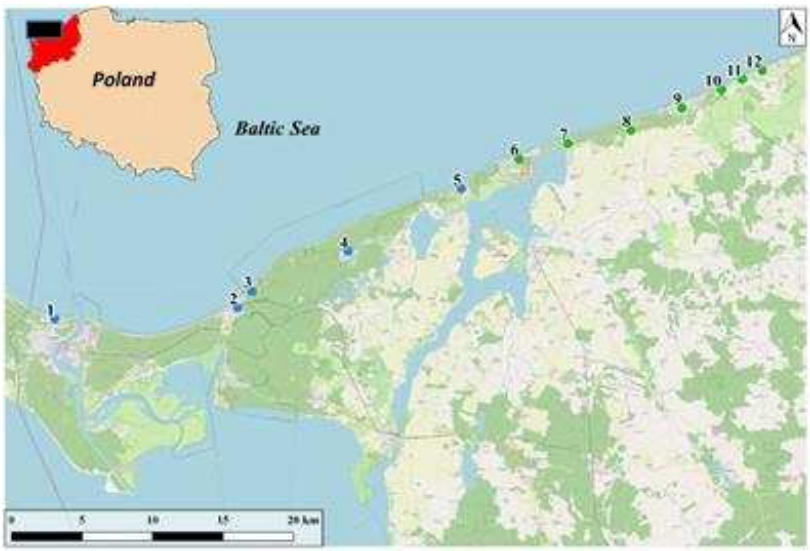

Figure 1. *Location of the studied localities. 1: Świnoujście, 2: Międzyzdroje, 3: Kawcza Mountain, 4: Wisełka, 5: Międzywodzie (in mesoregion Uznam and Wolin), 6: Dziwnów, 7: Dziwnówek, 8: Łukęcin, 9: Pobierowo, 10: Pustkowo, 11: Trzęsacz, 12: Rewal (in mesoregion Trzebiatowskie Coast)*

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Analysis of the physical factors

Among the analysed localities, Świnoujście is the westernmost city, and Rewal village was farthest to the east (*Figure 1, Table 1*). The analysed localities belong to three districts (Gryfice, Kamień Pomorski and Świnoujście) and five municipal communes (Międzyzdroje, Świnoujście, Wolin, Dziwnów and Rewal) - the last two of which were represented by the largest number of localities (4 each) (*Table 1*).

Table 1. *Administrative and physical-geographic factor of studied localities*

District	Commune	No. of loc.	Localities	GPS coordinates
Świnoujście	Świnoujście	1	Świnoujście (c)	53°54′28″N 14°14′51″E
Kamień Pom.	Międzyzdroje	2	Międzyzdroje (c)	53°55′44″N 14°27′05″E
		3	Kawcza Mountain (h)	53°56′23″N 14°27′57″E
	Wolin	4	Wisełka (v)	53°57′57″N 14°34′24″E
	Dziwnów	5	Międzywodzie (v)	54°00′20″N 14°41′49″E
		6	Dziwnów (c)	54°01′03″N 14°44′18″E
		7	Dziwnówek (v)	54°02′04″N 14°48′22″E
		8	Łukęcin (s)	54°02′47″N 14°52′24″E
Gryfice	Rewal	9	Pobierowo (v)	54°03′26″N 14°55′41″E
		10	Pustkowo (v)	54°04′05″N 14°58′08″E
		11	Trzęsacz (v)	54°04′40″N 14°59′34″E
		12	Rewal (v)	54°04′57″N 15°01′07″E

No. of loc.: number of localities (Figure 1), c: city, v: village, s: settlement, h: seaside hill

Biological analysis

One year-old, fully developed needles were collected from lower branches of Scots pines *Pinus sylvestris* L. at the end of August (at the height of the growing season coinciding with the tourist season). The needles were collected from randomly selected tree trees in the vicinity. The age of the specimens was 45 to 50 years, the height from 15 to 18 m and trunk circumference at breast height from 57 to 86 cm. From each tree needles were collected (up to 10 shoots per tree, 100 needles). The samples were collected from trees located in the afforested coastal zone (between the Baltic Sea and the centre of the analysed locality) from the external, sun-lit part of the crown from the side of the buildings (*Figure 2*). While collecting the plant material, a macroscopic analysis of the colour and needle loss on young shoots of Scots pine was conducted.

Figure 2. *Location of plant sampling sites in: a) Międzyzdroje, b) Pobierowo*

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Within the distance of up to 5 m from the trunk of Scots pine from which needles were collected, the soil material from the layer 0-20 cm from six spots was analysed (aggregate samples). The soil tests were also conducted in three replicates.

Chemical analysis

The samples of Scots pine needles were grounded in a laboratory mill after drying. The collected soil material was dried to air-dry condition and passed through a sieve of 1 mm in aperture size. HMs content in soil samples was determined in a mixture of concentrated nitric and perchloric acid, and perhydrol at a ratio of 5:1:1. In the plant samples, HMs content was identified in a mixture of concentrated nitric acid and perhydrol, ratio 4:1. Microwave digestion system mls 1200 mega by Milestone was used for mineralisation. The content of the following HMs: Ni, Co, Mn, Cd, Cu, Cr, Pb and Zn was identified with the use of atomic absorption spectrometer (AAS) ICE 300 by Termo Scientific. The wave length for examined elements were as the following: Cd 228.8 mm, Co 240.7, Cr 357.9, Cu 324.8, Fe 248.3, Mn 279.5, Ni 232.0, Pb 217.0, Zn 213.9.

Soil acidity was determined with the potentiometric method: hydrogen ion concentration pH H_20 in soil suspension in water, and pH KCl in 1M potassium chloride solution (Ostrowska et al., 1991). The permissible standards of HMs pollution of soil and plants were adopted following Kabata-Pendias et al. (1993).

Analysis of climatic elements

The climatic conditions, just as the availability of water, temperature, sunlight exposure and wind force affect the intensity of life functions in plants, e.g. photosynthesis. During the intensive photosynthesis process at high temperature, sunlight exposure and water availability, the presence of HMs in soil, air or water may exacerbate the pollution of plants and, consequently, the accumulation of HMs. Different conditions, i.e. low temperatures, are not conducive to photosynthesis intensification, consequently limiting the presence of HMs within and on plants. The following climatic characteristics were analysed (*Table 2*): temperature [°C], the number of sunny hours in a day - mean values [h], the number of days with rain [day] from January to December together with mean annual values (https://pl.climate...).

Analysis of anthropogenic factors

The number of permanent residents was established for the 11 analysed localities (*Fig. 2*, CSO, 2018). With respect to communes to which particular localities belong, the following numbers are presented in *Table 3* anthropogenic factors as: the number of residents (CSO, 2018), of bed places (Noclegi-e-turysta.pl), of beds provided in July and August of 2019, of tourists in a year (per resort tax) as well as the calculated actual number of tourists (https://szczecin.stat.gov.pl/...).

Statistical analysis

Distribution normality test was conducted with the use of Kolmogorov-Smirnov test. Pearson correlation analysis was conducted to detect relationships between the concentrations of the analysed metals, the location and region from which the biological material was collected, anthropogenic and climatic factors. Analysis of variance (ANOVA) and post-hoc Tukey's test were conducted to investigate the statistically significant differences between the analysed regions and cities.

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Table 2. *Climatic features in the studied localities in the natural units*

	Localities in the Coastal Belt district										
	Island mesoregion - Uznam and Wolin				**Land mesoregion - Trzebiatów Coast**						
Month	**Świnoujście**	**Międzyzdroje**	**Wisełka**	**Międzywodzie**	**Dziwnów**	**Dziwnówek**	**Łukęcin**	**Pobierowo**	**Pustkowo**	**Trzęsacz**	**Rewal**
	***Temperature [av. °C]/ Sunny hours in the day [av. value - h]/ Days with rain [day]**										
I	*2/6/3	2/3/9	3/3/9	2/2/15	2/3/9	2/3/9	2/3/9	2/3/11	1/2/14	2/2/15	2/3/11
II	2/9/3	2/3/7	3/3/7	2/5/11	2/3/7	2/3/7	2/3/7	2/4/8	2/3/10	2/5/11	2/4/8
III	6/10/10	6/6/10	6/6/10	6/6/13	6/6/10	6/6/10	6/6/10	6/6/12	7/6/13	6/6/13	6/6/12
IV	10/10/10	10/9/10	11/9/10	10/8/15	10/9/10	11/9/10	10/9/10	11/10/12	12/8/16	11/8/16	11/10/12
V	15/10/13	15/10/13	15/10/14	15/11/20	15/10/13	15/10/13	15/10/14	16/10/13	16/10/18	16/11/18	16/10/13
VI	18/10/12	18/10/12	19/10/12	18/12/17	18/10/12	18/10/12	18/10/12	19/10/13	19/12/18	19/12/17	19/10/13
VII	21/9/15	21/10/15	22/10/15	21/10/20	21/10/15	21/10/15	21/10/15	22/10/15	22/12/21	22/12/20	22/10/15
VIII	21/5/13	21/10/13	22/10/13	21/10/20	21/10/13	21/10/13	21/10/13	22/10/17	22/10/21	22/10/21	22/10/17
IX	18/4/9	18/9/9	18/9/9	18/8/15	18/9/9	18/9/9	18/9/9	18/9/13	19/8/17	19/8/16	18/8/13
X	12/3/11	12/5/11	13/5/11	12/6/18	12/5/11	12/5/11	12/5/11	12/5/14	13/5/17	13/6/17	12/5/14
XI	8/9/10	8/4/10	9/4/10	8/4/18	8/4/10	8/4/10	8/4/10	8/4/12	8/4/18	8/4/18	8/4/12
XII	4/7/11	4/3/11	5/3/11	4/2/18	4/3/11	4/3/11	4/3/11	4/3/14	4/2/18	4/2/19	4/3/14
av.	11/8/10	11/7/11	12/7/11	11/7/17	11/7/11	12/7/11	11/7/11	12/7/14	12/7/17	12/7/17	12/7/13

I: January, II: February, III: March, IV: April, V: May, VI: June, VII: July, VIII: August, IX: September, X: October, XI: November, XII: December, av. - average

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Table 3. *Anthropogenic factors demonstrated in the communes*

Commune	Anthropogenic factors					
	Nrc	**Nfc**	**Nbc**	**Nbcj-a**	**Nt**	**Ntc**
Świnoujście	41 371	732	10 132	665 047	1 000 000	3 000 000
Międzyzdroje	6 596	81	6 300	290 093	608 696	791 305
Wolin	12 447	83	463	160 231	-	-
Dziwnów	4 024	38	11 074	411 987	323 529	1 764 706
Rewal	3 817	193	13 881	681 680	900 000	1 170 000

Nrc: the number of residents in the commune, Nfc: the number of accommodation facilities in the commune, Nbc: the number of beds places in the commune, Nbcj-a: the number of beds provided in July and August of 2019 in the commune, Nt: the number of tourists in a year in the commune, Ntc: the actual number of tourists in the commune, - no data

Additionally, for the purpose of identifying the differences between the analysed areas, cluster analysis (CA) with Ward's agglomerative clustering method and squared Euclidean distance was used. In order to determine whether needles of Scots pine *Pinus sylvestris* L. can serve as a bioindicator, factor analysis with varimax rotation was conducted. All analyses were conducted using Statistica 13.1 PL. The adopted significance level was $\alpha = 0.05$.

Results

The results of anthropogenic analyses demonstrated that the least populated localities i.e., up to 1000 residents, were predominant among the analysed seaside localities (73%). The average number of permanent residents was approx. 400. Although, in terms of administrative classification, Pobierowo is a village, the number of residents was found to be > 1000. The number of residents in Międzyzdroje and Dziwnów, both localities with city rights, was over 2000. The highest number of permanent residents was found in Świnoujście (40 888), next in: Międzyzdroje (6 504), Dziwnów (2 707), Pobierowo (1 116), Rewal (984), Międzywodzie (681), Wisełka (483), Dziwnówek (391), Łukęcin (146), Pustkowo (125) and Trzęsacz (113).

In the summer season, particularly from July to August, the seasonal influx of holidaymakers and health resort visitors resulted in a substantial increase in the number of people in the analysed municipal communes: 439-times in Dziwnów, 307-times in Rewal, 120-times in Międzyzdroje and 73-times in Świnoujście. Although the number of permanent residents in the analyzed communes was as follows (according to the decreasing number of inhabitants): Świnoujście, Wolin, Międzyzdroje, Dziwnów and Rewal. However, this fact not always corresponded with the influx of tourists. The most visited communes by vacationers were Świnoujście and Dziwnów, with the latter offering the greatest number of most beds, including in hotels, guesthouses and sanatoriums (*Table 3*). The field studies were conducted at the peak of the tourist season i.e., at the end of August. However, the macroscopic observations of needles of Scots pine did not show morphological changes resulting from disruptions of physiological processes due to accumulation of HMs.

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The concentrations of selected HMs determined in samples of Scots pine needles and soil collected from the area in the close proximity to the tree trunks in the analysed localities were variable. The highest average concentrations of all analysed HMs were found with respect to Mn (needles and soil respectively: 31.2 and 27.1 mg/kg) and Zn (40.4 and 6.7 mg/kg). The comparison of localities shows that the highest average HMs values were determined for Świnoujście (needles and soil respectively: 14.3 and 7.0), Międzyzdroje (11.9 and 7.0 mg/kg) and Dziwnów (11.4 and 6.2 mg/kg) (*Table 4a,b*). The layer of ectohumus was insubstantial - up to 8 cm. The obtained results indicated that Cu was significantly different at station 3 (Kawcza Mountain) with all stations, similar to Cr at station 4 (Wisełka). Cd at station 6 (Dziwnów) and Zn at station 7 (Dziwnówek) were significantly different from stations 1-11. For the soil environment, smaller significant differences were found between the studied sites, except for the salinity at site 8 (Łukęcin), which was significantly different from sites 1-12.

The statistical analysis of the results of chemical analyses of Scots pine needles and soil samples showed a normal distribution. The correlation analysis revealed significant statistical correlations between the analysed HMs in needles and heavy metals determined in soils, and the anthropogenic factors, region, locality and only the average precipitation (climatic data - *Table 2*) (*Table 5*). A strong correlation between the region and locality from which the samples were collected was demonstrated for the concentrations of Mn, Cd and Zn in Scots pine needles, as well as Ni, Co and Mn determined in soil samples. Also, the study identified a strong negative correlation between the locality, Pb, pH and soil salinity. With respect to selected anthropogenic indices, a strong correlation was identified between the concentrations of Co, Mn and Zn in Scots pine needles and Mn, Cd, Cr and Zn in soil samples. The average annual number of days with rain demonstrated a very strong negative correlation with Mn and Zn in Scots pine needles and pH of soils, and a very strong correlation with soil salinity (*Table 5*). Rainfall flushes rinse of pollution heavy metals from leaves and thus reduces their amount in plants (Alcock and Morton, 1981). However, according to Hanchi and Rapp (1997), the main part of rainfall remains in the panicle part, and in the study area samples were collected from the lower part of the branches. High values of Mn and Zn found in pine needles, show similar results for Mn only to those shown by Bojarczuk and Oleksy (1994), which indicated a lower amount of Mn and Zn in pine needles growing on contaminated substrates than on control. Although the research showed a low degrees of salinity of the respondents (from 130.7 to 235.2, average 191.3), however according to Šerá (2017) in town places with salt-contaminated soil should not be fitted with: spruce (*Picea*), pine (*Pinus*), linden (*Tilia*) and maples (*Acer*).

The analysis of variance allowed to identify which metals determined in needles of *Pinus sylvestris* L. and soil samples showed a significant difference (*) between the analysed regions - Mn, Cd, Cu, Zn in needles and Ni, Mn, Cu, Cr and soil salinity. Significant differences were also identified with respect to the number of residents in the analysed localities, the number of residents in the communes, the number of accommodation facilities and the number of beds in communities to which the analysed localities belong (*Table 5*).

The analysis of the correlation of metals determined in soils and needles demonstrates a very strong correlation between Cr, Mn, Cd and Zn (respectively 0.987, 0.974, 0.969, 0.911) (*Table 6*).

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Table 4a. *The average values of HMs concentrations in the needles of Scots pine*

No. of loc.	HMs [mg/kg]						
	Ni	**Mn**	**Cd**	**Cu**	**Cr**	**Pb**	**Zn**
1	0.839[7,9-12]	37.350[3-12]	0.052[3,6,12]	4.662[2-6]	2.895[3,7,9,10]	0.587[2-4,7,9-12]	67.718[4,7]
2	0.722[10]	34.739[3,4,7,9-12]	0.042[3,6,12]	2.972[2,3,7,9,11,12]	3.471[3,4,7,9-11]	0.244[1-8]	53.120[4,7,12]
3	0.397[4,5]	50.302[1,2,7,9-12]	0.043[1,2,5,6,8-12]	1.071[1-12]	2.592	0.000[1-6,8-12]	26.241[1,2,6-8,12]
4	0.567[3,7,9-12]	31.122[1,2,7,9-10,12]	0.009[5,6,8,11,12]	1.801[1,3,7,9,11,12]	0.180[1-12]	0.023[1-6,8-12]	24.444[1,2,6-8,12]
5	0.962[3,6-12]	29.032[1,7,9-12]	0.025[3,4,6,12]	2.956[1,3,11,12]	2.147[1-8,11,12]	0.653[2-4,7,9-12]	54.490[7,12]
6	0.993[5,10]	32.315[1,7,9-12]	0.064[1-11]	3.508[1,3,11]	2.990[4,5,8-12]	0.758[2-4,7,9-12]	50.474[4,7]
7	0.656[1,4,5]	31.907[1-8]	0.146[6,12]	3.535[2-4]	3.049[4,5,8-12]	0.000[1-6,8-12]	55.600[1-11]
8	1.242[5,10]	24.270[1,2,9,10,12]	0.034[3,4,6,12]	3.931[3,11]	0.754[1,2,5-8,9,12]	0.496[2-4,7,9-12]	43.316[4,7,12]
9	0.667[1,4,5]	29.099[1-6,8]	0.065[3,6,11]	3.808[2-4]	2.242[1-4,6-11]	0.290[1,3-8]	23.387[7,12]
10	0.460[1,2,4-6,8]	23.919[1-6,8,12]	0.047[3,6,12]	3.948[3]	1.024[1-9-12]	0.286[1,3-8]	21.323[7,12]
11	0.311[1,4,5]	24.254[1-3,4,5]	0.057[3,4,6,12]	3.824[2-6,9]	1.073[1-9-12]	0.298[1,3-8]	30.131[7,12]
12	0.483[1,2,4-6,8]	25.736[1-6]	0.065[1-5,7-11]	4.823[2,3,4,5]	2.121[1-4,5-8,10,11]	0.308[1,3-8]	34.919[2-5,8-11]

Table 4b. *The average values of HMs concentrations and selected physical parameters in the soils around Scots pine trees*

No. of loc.	HMs [mg/kg]							pH	pH	sal.
	Ni	**Mn**	**Cd**	**Cu**	**Cr**	**Pb**	**Zn**	**H_2O**	**KCl**	**µS/cm**
1	0.273[2,4,6]	34.028[4,5,10]	0.447[6,7]	3.488[4,7,8,10,11]	3.212[4,8,11]	3.409[2-5,7-12]	11.515[4,5,7-11]	7.805	7.314[2-5,7,8,10-12]	129.250[4,5,6,7,10-12]
2	0.233[1,4,6,9-12]	32.321	0.397[7]	2.440[8,10]	2.793[4]	3.066[1-5,7-11]	9.616[4,5,7-11]	6.975	6.506[1-5,7,8,10-12]	140.054[4,5,6,7,10-12]
4	0.153[1-9,11]	22.472[1,7,12]	0.255[7]	1.993[1,9]	2.279[1,2,6,9]	2.083[1-4,6,8,9,12]	3.823[1,2,6,12]	5.913	5.062[1,2,6,9]	210.778[1,2,6,8,9,11]
5	0.202[4,6,10-12]	26.072[1,12]	0.270[7]	2.257	2.460	2.970[1,2,6,12]	4.593[1,2,6,12]	6.231	5.569[1,2,6,9]	205.955[1,2,6,8,9,11]
6	0.267[2,4-8]	31.108	0.349[1,7,12]	1.808	2.432[4,8,11]	3.368[4-11]	10.600[4,5,7-11]	7.639	6.900[4-8,10-12]	130.677[4,5,6,7,10-12]
7	0.379[1,4,6,10-12]	21.531[4]	0.419[1-12]	10.281[1,9]	2.252[9]	2.521[1,2,6-9,12]	3.657[1,2,6,12]	5.814	5.377[1,2,6,9]	232.994[1,2,5-9,12]
8	0.239[4,6,10-12]	24.690	0.319[7]	3.418[1,2,9,12]	1.872[1,6,9]	2.259[1-4,6,7,12]	5.724[1,2,6,12]	5.198	4.609[1,2,6-9,12]	272.060[1-12]
9	0.202[4,10,12]	27.411	0.408[7]	3.239[4,7-11]	3.179[4,7-12]	3.621[1-4,6,7,12]	5.747[1,2,6,12]	7.647	7.001[4,5,7-12]	124.835[4,5,6,7,10-12]
10	0.190[2-5,7-9]	23.686[1,12]	0.275[7]	3.498[1,2,9,12]	1.615[9]	2.483[1,2,6,12]	4.570[1,2,6,12]	5.813	5.087[1,2,6,9]	235.161[1,2,5-6-8,9,12]
11	0.191[2-5,7,8]	25.116	0.361[7]	3.125[1,10]	2.202[1,6,9]	2.151[1,2,6,12]	4.485[1,2,6,12]	5.856	5.238[1,2,6,9]	237.091[1,6,8,9,12]
12	0.326[2-5,7-9]	30.108[4,5,10]	0.448[4-7]	4.022[9,10]	2.879[9]	2.558[1,4,5,7-11]	9.287[4,5,7-11]	6.041	5.459[1,2,6,9]	185.653[1,2,5-11]

Co < LOD, n = 3, Ni: nickel, Co: cobalt, Mn: manganese, Cd: cadmium, Cu: copper, Cr: chromium, Pb: lead, Zn: zinc, pH H_2O: soil acidity determined in a solution of hydrogen ions, pH KCl: soil acidity determined in a potassium chloride solution, sal.: salinity, No. of loc.: number of localities (*Figure 1*): 1: Świnoujście, 2: Międzyzdroje, 3: Kawcza Mountain, 4: Wisełka, 5: Międzywodzie, 6: Dziwnów, 7: Dziwnówek, 8: Łukęcin, 9: Pobierowo, 10: Pustkowo, 11: Trzęsacz, 12: Rewal

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Table 5. *Correlation matrix*

Var.	Analysed factors									
	Region	**Lc**	**Nrl***	**Nrc***	**Nfc***	**Nbc***	**Nbcj-a**	**Nt**	**Ntc**	**R**
pn Ni	-0.115	**-0.643**	0.278	0.249	0.053	-0.334	-0.401	**-0.588**	0.480	-0.779
pn Co	**-0.577**	**-0.555**	**0.992**	**0.998**	**0.979**	-0.045	**0.529**	**0.638**	**0.910**	-0.662
pn Mn*	**-0.814**	**-0.992**	**0.723**	**0.683**	**0.498**	**-0.730**	-0.307	-0.007	**0.544**	**-0.980**
pn Cd*	**0.924**	**0.764**	-0.337	-0.290	-0.159	**0.934**	**0.523**	-0.098	0.104	0.547
pn Cu*	0.224	0.443	0.399	0.451	**0.638**	**0.779**	**0.999**	**0.782**	**0.510**	0.293
pn Cr	**-0.648**	**-0.848**	0.144	0.087	-0.134	**-0.951**	**-0.831**	-0.473	-0.067	-0.722
pn Pb	0.281	-0.234	0.273	0.277	0.184	0.233	0.085	-0.386	**0.663**	-0.490
pn Zn*	**-0.762**	**-0.933**	**0.856**	**0.827**	**0.673**	**-0.549**	-0.076	0.145	**0.728**	**-0.975**
s Ni*	**0.643**	**0.861**	-0.166	-0.110	0.114	**0.940**	**0.818**	0.478	0.031	0.746
s Co	**0.739**	**0.741**	-0.069	-0.013	0.165	**0.994**	**0.808**	0.291	0.278	0.530
s Mn*	**-0.878**	**-0.901**	**0.902**	**0.876**	**0.754**	**-0.572**	0.008	0.349	**0.671**	-0.895
s Cd	-0.288	0.186	0.481	**0.512**	**0.677**	0.302	**0.771**	**0.989**	0.261	0.217
s Cu*	-0.028	0.429	0.303	0.345	**0.547**	**0.522**	**0.850**	**0.930**	0.179	0.428
s Cr*	**-0.625**	-0.260	**0.793**	**0.806**	**0.882**	-0.016	**0.619**	**0.946**	**0.515**	-0.234
s Pb	-0.403	**-0.827**	**0.566**	**0.535**	0.341	-0.447	-0.282	-0.316	**0.648**	-0.940
s Zn	-0.357	**-0.656**	**0.829**	**0.823**	**0.719**	-0.090	0.233	0.123	**0.942**	-0.839
s pH H_2O	-0.397	**-0.813**	**0.610**	**0.582**	0.398	-0.398	-0.212	-0.262	**0.701**	-0.939
s pH KCl	**-0.530**	**-0.882**	**0.690**	**0.660**	0.475	-0.480	-0.203	-0.160	**0.709**	**-0.979**
s sal.*	**0.510**	**0.900**	-0.496	-0.455	-0.238	**0.627**	0.456	0.378	**-0.498**	**0.953**

Bold values: statistically significant correlations at $\alpha = 0.05$, * statistically significant differences between regions $\alpha = 0.05$, Var.: variability, Region: island mesoregion (Uznam and Wolin) and land mesoregion (Trzebiatów Coast), Lc: localities (city, village, settlement, hill), Nrl: the number of residents in the localities, Nrc: the number of residents in the commune, Nfc: the number of accommodation facilities in the commune, Nbc: the number of beds places in the commune, Nbcj-a: the number of beds provided in July and August of 2019 in the commune, Nt: the number of tourists in a year in the commune, Ntc: calculated actual number of tourists in the commune, R: average annual rainfall; pn: pine needles, s: soil, Ni: nickel, Co: cobalt, Mn: manganese, Cd: cadmium, Cu: copper, Cr: chromium, Pb: lead, Zn: zinc, pH H_2O: soil acidity determined in a solution of hydrogen ions, pH KCl: soil acidity determined in a potassium chloride solution, sal.: salinity

Table 6. *Correlation between metals in soil and needles of Pinus sylvestris* L.

Metals	**s Ni**	**s Co**	**s Mn**	**s Cd**	**s Cu**	**s Cr**	**s Pb**	**s Zn**	**s pH H_2O**	**s pH KCl**	**s sal.**
pn Ni	0.097	-0.149	0.261	0.135	-0.121	0.292	**0.398**	**0.351**	0.001	**0.526**	**-0.457**
pn Co	-0.116	-0.032	0.197	0.165	-0.096	**0.430**	0.236	-0.020	-0.142	**0.311**	**-0.387**
pn Mn	0.220	-0.203	**0.974**	0.249	0.061	**0.393**	0.276	**0.450**	-0.106	**0.610**	**-0.617**
pn Cd	**0.534**	-0.201	-0.038	**0.969**	**0.708**	0.136	0.142	-0.054	0.037	0.054	-0.020
pn Cu	0.204	0.058	0.279	**0.299**	0.114	0.218	0.131	**0.360**	0.039	0.167	-0.076
pn Cr	**0.383**	**-0.470**	**0.522**	**0.372**	0.234	**0.987**	**0.528**	**0.565**	-0.039	**0.630**	**-0.617**
pn Pb	-0.008	-0.056	**0.311**	-0.079	**-0.395**	-0.013	0.268	**0.425**	-0.023	**0.358**	-0.288
pn Zn	**0.480**	**-0.533**	**0.395**	0.229	0.222	0.210	0.277	**0.911**	0.181	**0.406**	-0.291

Bold values: statistically significant correlations at $\alpha = 0.05$, pn: pine needles, s: soil; Ni: nickel, Co: cobalt, Mn: manganese, Cd: cadmium, Cu: copper, Cr: chromium, Pb: lead, Zn: zinc, sal.: salinity, pH H_2O: soil acidity determined in a solution of hydrogen ions, pH KCl: soil acidity determined in a potassium chloride solution

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Cluster analysis with respect to similarities concerning HMs concentrations in Scots pine needles (*Figure 3a*) allowed the identification of five main groups, in particular: 1) Kawcza Mountain and Wisełka; 2) Rewal, Trzęsacz and Pustkowo; 3) Dziwnówek; 4) Łukęcin, Dziwnów, Międzywodzie and Międzyzdroje; 5) Pobierowo and Świnoujście. Such a classification was statistically significantly most affected by the concentrations of Cr, Mn, Pb and Zn. When detailing the analysis of clusters, it should be noted that: cluster no. 1 was characterized by a low Zn content in the needles (average 25). Cluster no. 2 was characterized by a slight variation in the concentrations of Mn, Cu and Cr. It should be noted, however, that only in the case of Rewal, the values of Cu and Cr were the highest in the selected cluster. In the case of Dziwnówek, its identification as a separate cluster was associated with the highest Zn concentrations in needles, minimum Pb and maximum Cd among those recorded in other points. Cluster no. 4 (Łukęcin, Dziwnów, Międzyzdroje and Międzywodzie) - had the highest concentrations of Pb and high concentrations of Ni. In the case of the Pobierowo / Świnoujście cluster - the highest concentrations of Co in the needles were recorded.

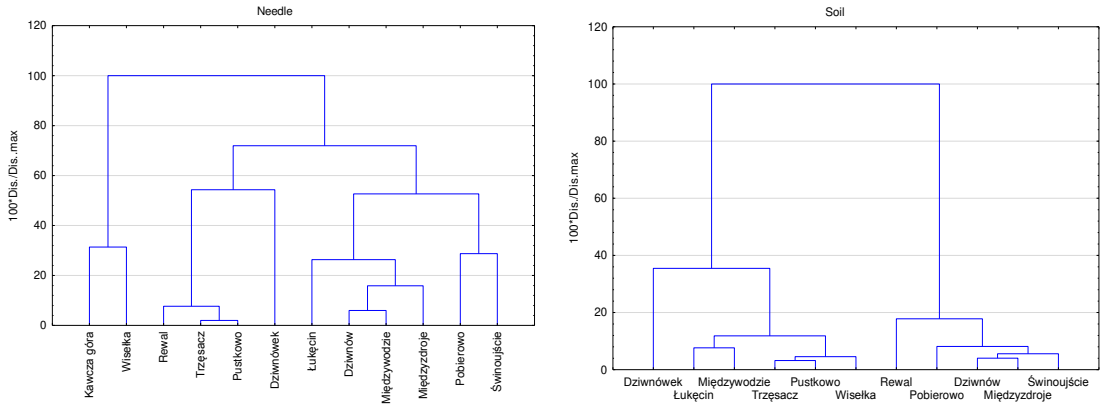

Figure 3. *Similarities in the concentration of HMs in: a) needles Pinus silvestris, b) soil*

With respect to cluster analysis (CA) concerning metal concentrations in soils (*Figure 3b*), the clustering identified the following cities: 1) Dziwnówek; 2) Łukęcin, Międzywodzie, Trzęsacz, Pustkowo and Wisełka; 3) Rewal, Pobierowo, Dziwnów, Międzyzdroje and Świnoujście. In the case of the first cluster, it should be noted that it contains the highest recorded concentrations of Ni and Cu in the soil and a high concentration of Cd with relatively high salinity. Cluster no. 2 contained information on the lowest concentrations of: Mn, Cd, Cr, Zn with simultaneous low pH values and high salinity. Cluster no. 3 grouped localities with the highest concentrations of Mn, Cd, Cr, Pb and Zn in the soil, as well as soils with the lowest salinity and higher pH.

Factor analysis (FA) identified 3 varifactors (*Table 7*). This made it possible to define 99% of variability in the given experimental case. The first factor VF (39.4%) demonstrated strong positive correlations with Mn, Pb and Zn in soils and needles, and with soil pH and the actual number of residents, as well as strong negative correlations with average annual temperature and precipitation values. VF2 (30.5%) showed a strong correlation with Co, Cu in needles, Cd, Cu in soils and the number of commune residents, the number of accommodation facilities and the number of provided accommodation in commune. The third varifactor (29.9%) identified strong correlations between Ni, Cr in soils and Scots pine needles, Cd in needles, the number of beds as well as the region and

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locality. This can be interpreted as: FV1 - a factor demonstrating the relationship between HMs and season in a year and climatic conditions at the actual number of people in a given area; FV2 - a factor identifying seasonal (anthropogenic) pollution with selected HMs; FV3 - a factor which indicates the regional/ urban pollution with the selected HMs i.e., Ni, Cd, Cr and Co.

Table 7. *Results of factor analysis*

Variable	VF1	VF2	VF3
Region	-0.204	-0.507	**0.838**
Localities	-0.649	-0.153	**0.745**
pn Ni	-0.149	-0.385	**0.911**
pn Co	0.599	**0.799**	-0.060
pn Mn	**0.714**	0.222	-0.664
pn Cd	-0.005	-0.202	**0.979**
pn Cu	-0.060	**0.721**	0.690
pn Cr	0.380	-0.313	**-0.870**
pn Pb	**0.882**	-0.239	0.406
pn Zn	**0.786**	0.378	-0.489
s Ni	-0.419	0.310	**0.853**
s Co	-0.060	0.186	**0.981**
s Mn	**0.911**	0.556	-0.563
s Cd	-0.356	**0.925**	0.130
s Cu	-0.448	**0.821**	0.354
s Cr	-0.003	-0.149	**0.989**
s Pb	**0.951**	-0.080	-0.299
s Zn	**0.941**	0.339	0.008
s pH H_2O	**0.967**	-0.025	-0.253
s pH KCl	**0.931**	0.082	-0.357
s sal.	**-0.861**	0.144	0.487
T	**-0.756**	0.197	0.625
R	**-0.839**	-0.161	0.520
Nrl	0.603	**0.777**	-0.181
Nrc	0.589	**0.798**	-0.129
Nfc	0.441	**0.866**	0.025
Nbc	-0.152	0.130	**0.980**
Nbcj-a	-0.021	**0.729**	0.684
Nt	-0.220	**0.970**	0.099
Ntc	**0.799**	0.557	0.226
Var.	11.809	8.982	9.209
Perc. of var	0.394	0.305	0.299
Cumul. var.	0.394	0.693	0.998

pn: pine needles, s: soil; Ni: nickel, Co: cobalt, Mn: manganese, Cd: cadmium, Cu: copper, Cr: chromium, Pb: lead, Zn: zinc, sal.: salinity; R: the average annual rainfall, T: the average annual temperature, Nrl: the number of residents in the localities, Nrc: the number of residents in the commune, Nfc: the number of accommodation facilities in the commune, Nbc: the number of beds places in the commune, Nbcj-a: the number of beds provided in July and August of 2019 in the commune, Nt: the number of tourists in a year in the commune, Ntc: calculated actual number of tourists in the commune, Var.: variability, Perc. of var.: percentage of variability, Cumul. var.: cumulative percentage of variation

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Discussion

The "green" as well as "blue" areas have an important role in improving the quality of life in urban areas (Juranović et al., 2019) and are of particular importance in holiday resort localities with health resort rights. Out of the analysed localities, only Świnoujście can be classified as a health resort. In Poland, only 45 localities have the status of a health and spa resort, whereas to the west of Poland, in Germany, there are 350 health resorts and to the south of Poland - in the Czech Republic and in Slovakia, there are even fewer health resorts than in Poland (30 and 21, respectively). Green areas in parks and urban squares constitute a therapeutic landscape and have a beneficial effect on health and well-being of the holidaymakers and health resorts visitors. Apart from pro-health functions, plant species (or their organs) constituting the urban green, the so-called "green" areas also provide information on the quality and condition of the atmospheric and soil environment. Of particular significance are the bioindicators of elements highly toxic even at low concentrations such as Cd and Pb (Juranović et al., 2019).

The correlation analysis demonstrated statistically significant correlations between the analysed metals in needles and metals identified in soil, and the region – particularly with respect to: Mn (respectively, -0.814 and -0.878 mg/kg), and to needle Zn (-0.762) and Cd (0.924) (*Table 5*). Average values of these HMs were 1 time higher in the island mesoregion, with the exception of Cd present at higher concentrations in Trzebiatowskie Coast mesoregion (*Table 4a,b*). The Mesoregion Wolin and Uznam was the western border of the Polish state. The direction of winds in the coastal belt of the Baltic Sea (Seul et al., 2020) could favour higher values of the analysed HMs in the studied needles and in the soil. Environmental border problems such as pollution with sewage, leakage of hazardous waste or air pollution is common in both countries. Therefore, the environmental border policy pays special attention to cross-border planning with respect to air quality in both countries (Herzog, 2020).

Statistically significant correlations were also found between the analysed HMs in Scots pine needles (Mn -0.992, Zn -0.933, Cr -0.848 and Cd 0.764) and metals determined in soil (Mn -0.901, Pb -0.827 Ni 0.861 and salinity 0.900), and the localities (*Table 5*). This observation is confirmed in the study by Alaqouri et al. (2020) concerning the area of mining and processing magnesite ore in Russia which identified Zn and Pb concentrations in Scots pine needles (respectively, Zn 22.57-53.91 mg/kg, Cd 0.149-0.328 mg/kg) to be statistically significant given the parameters of the age of needles (1 and 2 years old) and the distance of the tree from the emitter (1, 3, 10, 25 km). In Turkey, the study by Aricak et al. (2020) also demonstrated a high dependence of HMs concentration in Scots pine needles, such as: NI, Cr and Zn, on the pollution level due to different distance from the traffic. The values of HMs in Scots pine needles provided by Juranović et al. (2019) in Croatia in Lisičine Arboretum (Cd 0.264 mg/kg, Co<LOD, Cr<LOD, Cu 3.4 mg/kg, Ni 1.27 mg/kg, Pb 0.37 mg/kg, Zn 30.9 mg/kg) can be considered as control values for the area under analysis, indicating substantially lower values, with the exception of Cr and Zn. The location of the study conducted by Parzych et al. (2017) at the University botanic garden in Košice (Slovakia), also predisposes the obtained results to be treated as control values. However, the comparison of these values with the analysed control point - Kawcza Mountain, shows even higher values of metal concentration in Scots pine needles: Ni 19-times, Cd 16-times, Cu 5-times, a Zn 2-times (*Table 4a*). The presence of tourists, therefore the possible greater load on the atmosphere due to meeting the heating and municipal demands of holidaymakers, was not confirmed with the obtained HMs concentration values - neither in needles or in sandy soil samples

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- as the permissible standards were not exceeded (Kabata-Pendias et al., 1993; Niesiobędzka, 2005).

The analysis by Parzych and Jonczak (2014) concerning pollution in Słupsk, located (similarly to the localities investigated in this study) at the seaside a bit farther to the east, showed strong correlations in HMs content between one-year old Scots pine needles and soil for Zn (-0.82) and Pb (-0.71). Mean concentrations of Zn and Pb in needles and soil samples were, respectively: 59.4 and 45.3 mg/kg; 12.2 and 35.8 mg/kg, at pH 6.4-6.8 (KCl-H_2O). The twice as high number of residents in Słupsk in comparison with the most populated localities under analysis - Świnoujście, provides explanation to the 10-times and 4-times increase of the recorded Pb and Zn concentrations in soil at pH 7.3-7.8 (KCl-H_2O), and 21-times higher values of Pb concentrations in Scots pine needles (*Table 4a,b*).

A strong correlation for needles (Mn -0.980, Zn -0.975) and for soil (pH KCl -0.979 and salinity 0.953) with respect to humidity in the analysed area was shown (*Table 5*). Hassan et al. (1970) explained this with an increased Mn and Zn uptake by plants in saline soils (experiment on barley shoots), particularly in urban areas where salt is used to prevent icy conditions or roads. An increase in salinity also results from decomposition of urban and construction waste deposited in soil (Hulisz et al., 2018). Parzych and Jonczak (2013) demonstrated a significant correlation for HMs values between their content in Scots pine needles and rain precipitation for Zn (-0.79) and Cu (-0.34) in dry coniferous forest which, according to Ruszkowska et al. (1996), may affect Zn, Mn, Cd and Ni leeching from sandy soils, particularly of acidic pH (5.1-5.9 pH KCl). The soils of the analysed area can be classified as sandy, with pH values ranging from 4.6 to 7.6. The climatic conditions in own studies conducted with factor analysis (FV1 - *Table 7*) demonstrated the relationship with HMs (mean annual temperature -0.756, mean annual precipitation -0.839), which is explained by Souri et al. (2019) by the uptake of the HMs owing to the functioning of the photosynthetic apparatus adapting to temperature conditions.

The results of own studies allow to assert that Scots pine needles are adequate phytoindicators to be used in the assessment of the quality of atmospheric and soil environment. The analysis of correlations of HMs determined in needles and soil samples demonstrates a very strong correlation between: Mn, Cd, Cr and Zn (respectively, 0.974, 0.969, 0.987 and 0.911) (*Table 6*), which can indicate their bioaccumulation in needles. According to Aricak et al. (2020) Scots pine is a good bioindicator - particularly for monitoring changes in Cr concentrations. Gamrat and Ligocka (2018) in their study on Scots pine needles in Świnoujście showed a positive correlation between chromium and nickel, with R value of 0.858. In the analysed localities, Cr and Ni showed a positive correlation of 0.383, and slightly higher between Cd and Ni (0.534).

The analysis of similarity of the identified groups (*Figure 3a*) showed a group of the lowest (Wisełka and Kawcza Góra) and high (Pobierowo and Świnoujście) HMs concentration in Scots pine needles due to location. Wisełka and Kawcza Góra are located in a large forest complex and, consequently, demonstrate the lowest concentration values of HMs: Ni, Cu, Pb, Ni in needles (respectively, 0.4 and 0.6 mg/kg), Cu (1.1 and 1.8 mg/kg), Pb (0 and 0.023 mg/kg), Zn (26.2-24.4 mg/kg). High concentrations recorded in Pobierowo and Świnoujście (Co 0.003 and Cd 0.052 (*Table 4a,b*) result from the number of residents as well from the ongoing investments in that area. The cluster analysis of HMs concentration in soils (*Figure 3b*) grouped the localities into two main categories: of the lowest number of residents from 681 to 113 (Łukęcin, Międzywodzie,

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Trzęsacz, Pustkowo and Wisełka) and the most populated from 984 to 40 888 (Rewal, Pobierowo, Dziwnów, Międzyzdroje and Świnoujście). Despite a relatively low number of residents (391), Dziwnówek was not included in the group of localities with the lowest number of residents, but formed its own group instead. It was most likely due to close administrative cooperation with Dziwnów. Mustafa et al. (2016) analysed the pollution of pine needles and demonstrated similarities between the industrial localities and background measurements - of a substantially lower number of residents.

In order to sustain the high quality of seaside urban areas which can serve as health resorts, land management principles concerning the area located closest to the seaside, thus under greatest investment pressure (Meller, 2020), must be strictly observed. Especially since holidaymakers are obliged to pay a resort tax when visiting a seaside locality. However, multiannual studies by Šilc et al. (2020) on vegetation on sand dunes on the Adriatic coast, demonstrate a reduction of sand dunes area with an increase of urban and rural areas. Therefore, it is crucial to develop a common concept of sustainable landscape allowing the establishment of permanent, not undergoing transformation, habitats of the coastal flora and fauna, as well as providing the optimal holiday destination for people in seaside areas (Zaucha et al., 2020).

Conclusions

The results demonstrated that needles of Scots pine *Pinus sylvestris* L. proved to be sensitive phytoindicators showing a very strong correlation between the content of selected HMs - Cr, Mn, Cd and Zn, both in needles as well as in soil, which indicates bioaccumulation of these metals in needles. The statistical analysis confirmed the significant dependence of HMs concentrations in plant and soil material on the number of residents of the analysed seaside localities. The demonstrated HMs values showed low level of pollution both in needles as well as soils. A considerable increase in the number of summer holidaymakers did not contribute to exceedance of admissible norms for HMs for plants and soil. However, the highest or one of the highest values for the analyzed elements were found in Świnoujście - the largest and most densely populated town among the surveyed. In the needles, the Cu and Cr pines from sites 3 and 4 (Kawcza Góra, Wisełka) were significantly correlated with all sites, and in the soils it was Pb from the site in Dziwnów. Salinity, as a consequence of retaining the proper state of tracts urban, favors the suggestion that the future studies observe Scots pine's reaction as an indicator of pollution in greater detail bearing in mind its sensitivity.

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UNLOCKING THE POTENTIAL OF ELECTROKINETIC (EK)-ASSISTED PHYTOREMEDIATION ON ACIDIC SLOPE SOIL

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Abstract. The combination of phytoremediation and electrokinetic remediation is an attempt to accelerate the process of soil rehabilitation and overcome the limitations of phytoremediation. This current study was undertaken to assess the influence of electrokinetic-assisted phytoremediation on plant growth and soil quality under acidic conditions. A total of 40 potential slope plants, namely *Melastoma malabathricum* and *Syzygium campanulatum* were grown in 60 pots in the following six treatments; soil (as control – T1); soil + EK (T2); *M. malabathricum* (T3); *M. malabathricum* + EK (T4); *S. campanulatum* (T5); and *S. campanulatum* + EK (T6). After twelve weeks of observation, the EK-assisted treatments (T4 and T6) exhibited increment in soil pH by 4.0% and 5.3%, respectively. The plant growth performance of both species was observed to decline under EK treatments. Despite the lower biomass, the plants treated with EK approach contain higher concentrations of iron (Fe) and aluminum (Al). These plants are also able to improve the soil pH as well as conductivity level in soil, thus, greatly alleviating the soil acidity problem. Further *ex situ* investigation is essential to truly establish the practicality of utilizing both species as phytoremediator with the assistance of electrokinetic remediation.
Keywords: *soil acidity, bioavailability, M. malabathricum, S. campanulatum, combined remediation techniques*

Introduction

Addressing the problem of acidity is one of the topical issues of sloping lands. Generally, slope soil is acidic can be attributed to the naturally prolonged and pervasive weathering of rocks, or developed as the result of anthropogenic activities. Graphitic schist, is one of the common parent materials in Peninsular Malaysia, and the residual soils derived from this rock type is commonly acidic and landslide prone (Kong and Rahman, 2000; Regmi et al., 2016). Moreover, a study by Kong (2017) reported that the cut slope failures frequently occurred in the graphitic schist derived materials, that frequently involving acid sulphate soils and pyrite minerals. The exposure to the warm and wet climates of this country would result in excessive pyrite oxidation and hydrolysis reactions, as part of chemical weathering processes, which will produce sulphuric acid (H_2SO_4) and iron oxides ($Fe(OH)_3$), consequently, contributing to extreme acidic condition of the soil. This may further cause the release of soluble aluminium (Al) due to dissolution of available metals in soil, disintegration of the soil structure, reducing soil stability and eventually, the slope failures (Kong and Rahman, 2000; Sparks, 2003; Nkpadobi et al., 2014). Furthermore, this acidification will also turn the slopes into barren and infertile due to accumulation of metals in soil, hence, ensues lower plant bioavailability and lower plant coverage (Harter, 2007; Bakhshipour et al., 2016; Dislich

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et al., 2017). Subsequently, a reduce plant coverage leads to decrease of biogeochemical cycling restoration, and eventually it expedites soil erosion and degradation (Bowyer, 2006; Dislich et al., 2017). Therefore, a concerted effort to alleviate, remediate and rehabilitate the acidic condition of the slope is indeed essential.

Amongst the rehabilitation strategies, a bioengineering technique using vegetation as protection materials on the slope is regarded as one of the most promising techniques for slope management (Osman and Barakbah, 2011; Bella et al., 2017; Boldrin et al., 2017). In addition, phytoremediation technique is one of the good treatments available in bioengineering to restore the highly acidic slope condition (Patel, 2019). Phytoremediation refers to the inherent ability of certain plant species to accumulate and render harmless the toxic or excessive metals in soils into their vegetative or reproductive parts (Charlesworth et al., 2020; Manoj et al., 2020). Phytoremediation strategies can be classified into four, which are phytostabilization, phytoextraction, phytovolatilization and phytofiltration (Merkel and Hoyer, 2012; DalCorso et al., 2019) and these processes could be varied between the plant species. In general, phytostabilization reduces metal bioavailability in soil, phytoextraction uptakes metals from the soil into the plants and translocate in their aboveground biomass, phytovolatilization releases toxic elements from soil into the atmosphere through plants in harmless volatile form, and phytofiltration absorbs the metals from the hydro-environment through the plant parts (Marques et al., 2009; Jacob et al., 2018; Yan et al., 2020).

Nevertheless, this straightforward approach of phytoremediation is time-consuming with low-efficiency due to the slow plant growth (Ali et al., 2013). Thus, in this study, we proposed combination of electrokinetic-assistance to overcome this limitation. Electrokinetic(EK) remediation approach is an environmental technique that is used to remove contaminants in soil without excavating the soil for the purpose of decontamination (De Battisti and Ferro, 2007; Reddy and Cameselle, 2009). The provision of low-intensity electric potential by the EK process could enhance the bioavailability of excessive metals for desorption and transportation, as well as promoting the plant growth and reducing the period of phytoremediation (Bedmar et al., 2009). The migration of contaminants from the deep soil to plant roots is executed by electromigration and electroosmosis mechanism of the EK (Bedmar et al., 2009). Lower voltage gradient (4 V/cm) applies to the contaminated soil for 8 hours is beneficial to ensure better accumulation of heavymetals/ nutrients near the electrodes area (Kornilovich et al., 2005).

Additionally, selection of suitable remediating species (hyper-accumulators) is also a key, with promising characteristics such as the high tolerance to acidity, fast growing with high biomass, extensive root system, and less nutrient requirement to grow (Manoj et al., 2020), whereby using unsuitable species may result in no changes or adverse effects on the slope soil. More than 450 plant species have been identified as metal hyper-accumulators (Suman et al., 2018), but limited species are suitable for slope treatment plants. Study by Watanabe and Osaki (2001) discovered that *Melastoma malabathricum* was known as the hyper-accumulator plant that usually grows in tropical acid soils. This species can grow well in poor soil conditions; low nutrients, high concentration of aluminium (Al), and low pH level (Osman et al., 2014). Whilst *Syzygium campanulatum* is usually found in the urban and coastal areas and known as a fast-growing plant, which able to grow independently (Roseli et al., 2010). This plant also known for its ability to absorb contaminants from soil such as ammonium nitrate and has higher chances of

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adaptability towards new environment (Ling et al., 2009; Arunbabu et al., 2015). Hence, the above two slope species have been chosen for their potential acidic tolerant plants.

The main objective for this study is to evaluate the effects of electrokinetic-assisted approach to phytoremediation performance and soil acidity improvement, by considering the plant biomass and root growth. Moreover, the concentration of selected metals in the plant tissues, has been determined and the bio-concentration factor (BCF) and the translocation factor (TF) were also assessed.

Materials and methods

Plant material and experimental design

Pot experiment was performed in a glasshouse at Rimba Ilmu, Universiti Malaya Kuala Lumpur, Malaysia (3°7′52.1076″N, 101°39′25.218″E). The glasshouse received a range of Photosynthetically Active Radiation (PAR) between 300-2000 μE mol m^{-2} s^{-1}, relative humidity (RH) and atmospheric temperature of 65-90% and 25-28 ºC, respectively.

A total of 60 pots (27 cm × 20 cm × 17 cm) were filled with silty clay soil with total soil volume of 7803 cm^3. The soil was collected from the cut slope area located at the Bukit Beruntung, Rawang, Selangor. There were six treatments in this study: soil only (control – T1); soil + EK (T2); *M. malabathricum* alone (T3); *M. malabathricum* + EK (T4); *S. campanulatum* alone (T5); and *S. campanulatum* + EK (T6). Each treatment was carried out with ten replicates and monitored for twelve weeks.

Plant materials

The species studied; *M. malabathricum* and *S. campanulatum* were propagated in polybags before being transplanted to the experimental pots. The twenty seedlings of each species with uniform height of 30 cm were selected and transferred into the pots (27 cm × 20 cm × 17 cm) for further investigation. The pots were arranged in a complete randomized design (CRD).

Electrokinetic (EK)-assisted phytoremediation

A pair of copper electrodes were used and were vertically inserted into the soil with 8 cm spacing between each other to allow electroosmotic fluxes induced by the applied electric field in the soil. Plant species was positioned at the middle of the electrodes, where each electrode was 4 cm apart from the plant stem (*Figure 1*). A low intensity direct current (DC) of 4V was applied for 8 hours on/ 16 hours off per day (Cang et al., 2011) for 12 weeks. Each pot was irrigated daily (600 mL) to maintain the soil water content to be at 60% of water holding capacity, and the plants did not receive any fertilization during the experiment.

Parameters measurement

Soil pH and electrical conductivity (EC)

Both the soil pH and electrical conductivity (EC) were measured during initial and end of the experiment. The 10 grams (g) of soil samples were air-dried at room temperature until constant weight and were sieved through a 2-mm mesh sieve. Soil solution was made up of 1:2.5 of soil to ultra-pure water (UPW) and soil pH was determined by using a pH

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meter (PB-10 Sartorius GmbH, Gottingen, Germany). By using the same samples, the volume of 25 mL of UPW was doubled and the samples were then shaken mechanically at 15 rpm for an hour to dissolve the soluble salts. The EC value was recorded using a conductivity benchtop meter with automatic temperature compensation (HI-2315, Hanna Ins., US). Altogether five pots from each treatment were sampled and both parameters were measured in triplicate.

Figure 1. *The vertical insertions of electrodes (yellow arrows) into the soil. Plant species (S. campanulatum) was positioned in between the electrodes*

Plant height, total biomass and root length

Plant height of both species with five replications for each was measured at initial and end of the experiment by using a measuring tape. Plant biomass was recorded at harvest. All individual plant roots were gently washed with tap water to remove soils and then were partitioned into roots and shoots. Samples then were oven-dried at 80 ºC until the constant weight and the dry mass was recorded by using the electronic balance. Five samples of cleaned roots were positioned in a scanner and based on the scanned images, the total root length were determined by using the WinRHIZO software (Pro program, Regent Instruments Inc., Canada) in triplicates.

Soil particle size distribution

Dry soil sample was weighed for 500 g. The weight of the sieves and the pan that would be used during the analysis were also recorded. Each sieves were thoroughly cleaned up before the test. The sieves were assembled in ascending order, in which the larger openings sieve was on top; the No. 4 (4.75 mm) on top and the No. 200 (75 µm) sieve on the bottom of the stack. The soil sample was placed into the top sieve and a cover/ lid was placed over the sieve. The sieves stack was placed on the mechanical shaker and was shaken for 10 minutes. The weight of each sieve and its pan were recorded. The weight of the soil retained on each sieve was calculated to obtained the percentage of particle size distribution of the soil (Choate et al., 2006; Geoengineer, 2021).

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Metal concentrations in soil and plant samples

Iron (Fe) and Al concentrations were determined by using an inductively coupled plasma optical emission spectroscopy (ICP-OES) (Optima 7300 DV, Perkin Elmer, USA). Five samples with the mass of 1 g of each soil and plant part was digested beforehand using microwave (The Titan MPS, Perkin Elmer, USA) following sample preparation system with aqua regia mixture of 65% (v/v) nitric acid (HNO_3) and 37% (v/v) hydrochloric acid (HCl). Duplicate blank and laboratory standards were analysed routinely for quality assurance purposes.

Bio-concentration (BCF) and translocation factors (TF)

The bio-concentration factor (BCF) was calculated as the ratio of metal concentration in the plant at harvest to the concentration of the metal in soil medium (Zhuang et al., 2007) (*Eq. 1*). Whilst the translocation factor (TF) (*Eq. 2*) is defined as the ability of plant to translocate the metals the root to the shoot systems (Padmavathiamma and Li, 2007).

$$BCF = \frac{Metal\ concentration\ in\ the\ plant\ (roots,stems,and\ leaves)}{Metal\ concentration\ in\ the\ soil} \quad \text{(Eq.1)}$$

$$TF = \frac{Metal\ concentration\ in\ shoot\ (stems\ and\ leaves)}{Metal\ concentration\ in\ root} \quad \text{(Eq.2)}$$

Statistical analysis

A one-way analysis of variance (ANOVA) were performed for comparing means between treatments of twelve replications (n=12). Differences between means were compared and a post-hoc analysis of variance was determined using the Tukey's method at 0.05 probability level. Whilst the t-test was analysed for comparing means of initial and end of experiment. These statistical analyses were performed using the SPSS 20.0 statistical package (SPSS Inc, Chicago, IL, USA).

Results and discussion

Physical and chemical characteristics of soil

The study area is underlained by graphitic schist rock type. The surface exposure of graphitic schist to the repetitive wet and dry conditions has accelerated the process of weathering, which contributes to severe soil acidification and the unusual concentrations of metals in the slope soil. Furthermore, the very low soil pH level has resulted in generating acid drainage, as well as the degradation of slope construction materials such as drainage and ditches. Physical and chemical characteristics of the collected soil were presented in *Table 1*.

Fe and Al concentration in soils

In the collected soil (*Table 1*), the anion was dominated by the sulphate (SO_4) whilst for the cations, the iron (Fe) and aluminium (Al) are abundant in the soil. The Fe and Al were further assessed as the target metals due to the fact that vegetation absorbs more positively charged ions (cations) compared to the anions hence, these toxic metals are more closely allied to the topic of phytoremediation.

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Table 1. *Physical and chemical characteristics of the sample soil*

PARAMETER	VALUE / RANGE
pH	3 - 4
Sand (%) (w/w)	16.4
Silt (%) (w/w)	40.4
Clay (%) (w/w)	43.2
Electrical Conductivity (mS cm^{-1})	0.363±0.07
Total SO_4 (mg/kg)	12,587.1±2,555.67
Total iron (Fe) (mg/kg)	23,756.67±3,373.96
Total aluminium (Al) (mg/kg)	20,238.33±882.56

Soil pH and electrical conductivity (EC) changes

After the twelve-week of experiment, T3 recorded significantly highest increment of soil pH value which is by 6.2% whilst the lowest was in T4. Treatments with EK-assisted phytoremediation (T4 and T6) showed the pattern of increment in soil pH (*Figure 2*). These findings demonstrated the good perspectives of the EK assistance in phytoremediation by facilitating the mobility and bioavailability of the contaminants for plants uptake (Cameselle et al., 2013). In addition, increased of pH in acidic soil is also associated with a remarkable decrease in metals concentration in soils (McKee Jr and McKevlin, 1993; George et al., 2012), indicating the ability of the species studied to accumulate the excessive metals in soils. In overall, *S. campanulatum* treated with EK showed a significant increment in soil pH value compared to a slight increment showed by *M. malabathricum* treated with EK.

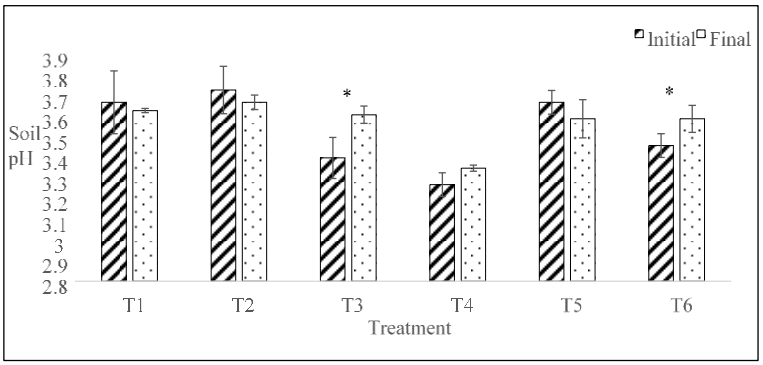

Figure 2. *The soil pH value during initial and end of experiment. Vertical bar represents standard deviation. * denotes significant difference at $p \leq 0.05$, (n=12)*

All treatments presented an increased electrical conductivity (EC) value at the end of experiment with the highest increment was shown in T1 and the lowest in T5 by 177.7% and 34.4%, respectively (*Figure 3*). The higher increment of EC in EK-assisted treatments (T4 and T6) implies that direct current (DC) used in EK had increased the production of metal ions at cathode and anode regions due to electroosmotic flow, thus, increase the conductivity in soil (Chang et al., 2019). In other way, highest conductivity in control (T1) was due to the presence of high metals concentration in soils, contributing to increase the soil acidity (Attah and Regasa, 2013).

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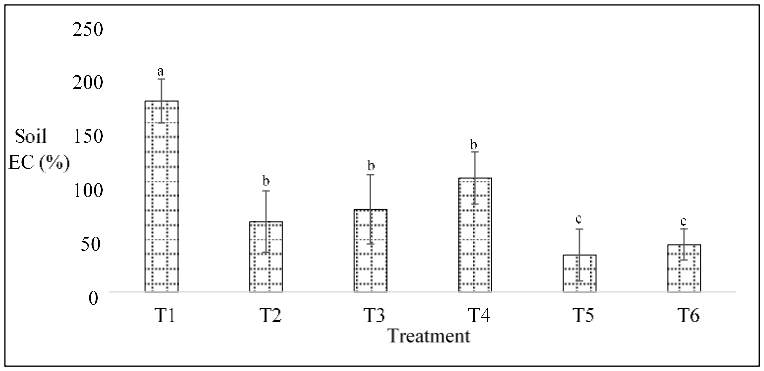
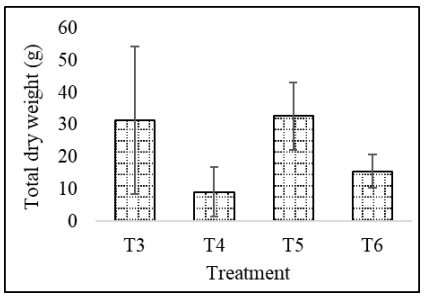


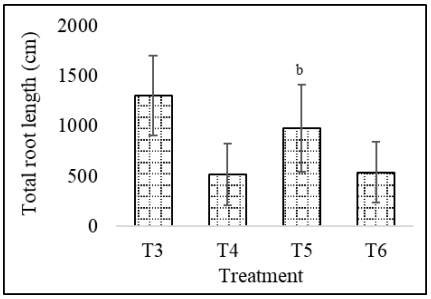
Figure 3. *Percentage of changes (increment) in soil EC among treatments. Different letters showed significant difference at $p \leq 0.05$, (n=12)*

Plant height, total biomass and root length

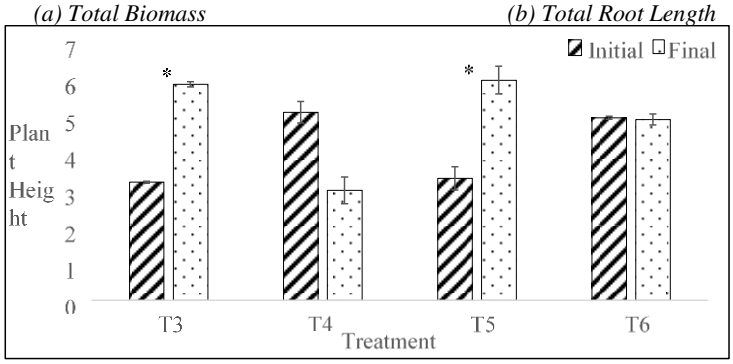
As can be seen in *Figure 4a-c*, the value of total plant biomass, root length, and plant height for both species were significantly higher in non-EK treatments (T5 and T3). The value of dry weight biomass of *M. malabathricum* in T4 was trifold lower than those in T3, whilst the *S. campanulatum* in T6 was 52.4% lower than those in T5. One of the possible reasons for getting poor growth of both plants was due to the greater phytotoxicity of metals on plants (O'connor et al., 2003). Although previous studies allegedly proved that both species are tolerant to highly acidic soils (Watanabe and Osaki, 2001; Osman et al., 2014; Arunbabu et al., 2015), the increased of metal bioavailability in soil by the electric field might provoked higher uptake by plants (Chibuike and Obiora, 2014), thus, interfere the metabolic processes of the plant species.



(a) Total Biomass



(b) Total Root Length



(c) Height

Figure 4. *(a) The Total Biomass, (b) Total Root Length, and (c) Plant Height of the species studied in each treatment. Vertical bar represents standard deviation. Different letters and * denotes significant difference at $p \leq 0.05$, (n=12)*

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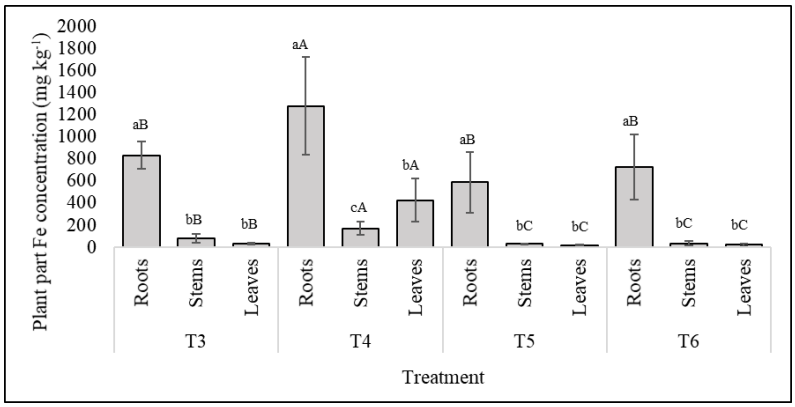
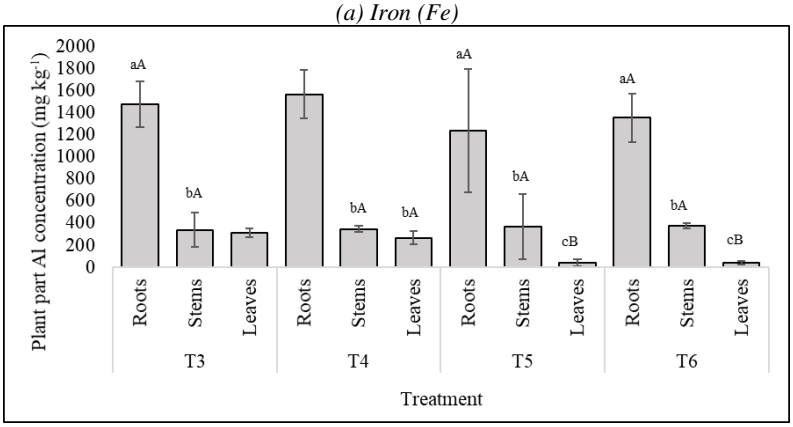
In addition, the applied voltage of 4V in both T4 and T6 exerted a negative effect on total root length of both species studied. Findings by Cang et al. (2011) in Indian mustard species also reported a decline in root biomass in 4V treatment while the bioavailability of heavy metals was enhanced on the similar voltage treatment. We infer that there was a trade-off between the metal bioavailability and the adverse effects of the EK on the plant growth development. Therefore, both plant species are postulated to be EK-sensitive thus, reduced the total root length. Whilst in terms of the plant height, *S. campanulatum* in T6 was seemingly not affected by the EK application, suggesting the independent behaviour of each species against the electric field.

Iron (Fe) and aluminium (Al) in plant

For iron (Fe) concentration in plant parts, the highest concentration value was recorded in roots of *M. malabathricum* in T4 (*Figure 5a*). Similarly, the highest concentration for leaves and stems were also recorded in T4 with 425.23 and 169.53 mg/kg, respectively. For *S. campanulatum*, there was no significant difference was found between the T5 and T6 in all plant parts. Meanwhile, the concentration of aluminium (Al) in all parts (except the plant leaves) of both species in all treatments were similar, either with or without EK treatments (*Figure 5b*). These results imply that *M. malabathricum* and *S. campanulatum* can accumulate Al regardless their poor plant growth performance, showing their capability as Al accumulator plants.



(a) Iron (Fe)



(b) Aluminium (Al)

Figure 5. *Concentration of (a) iron (Fe) and (b) aluminium (Al) in plant parts of each treatment. Vertical bar represents standard deviation. Different lowercase letters indicate significant differences between plant parts for each treatment, and different capital letters indicate a significant difference between treatments for each plant part ($p < 0.05$), (n=12)*

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By comparing the Fe and Al distribution in different plant parts, we can observe that the concentrations of both metals were significantly higher in roots than those in stems and leaves, suggesting that longer cultivation times are necessary for the plants to transport the metals from the roots to the upper parts (Cameselle et al., 2013). Interestingly, despite exhibiting the lowest in total dry weight biomass, *M. malabathricum* (T4) accumulated the greatest amount of metals concentration in its plant organs. Based on this finding, *M. malabathricum* as Al accumulator with high metals accumulation in small volume of biomass, it is recommended that this plant able to be grown in acidic soil condition as it is tolerance to such stressful condition and able to concentrate high level of both essential and toxic elements in their foliage (Mao et al., 2015).

The application of electric field has created the acid front at the anode and base front at the cathode, generating the mass transfer of charged ions towards the electrode regions and allows the ions uptake by the plant roots at the inter-electrode region (FRTR, 2020). Furthermore, the increase of ion mobility in soil would contribute to the charge of the surface becomes less positive, hence, providing less negative sorption plane for the anions in soil (Chad and James, 2019). This latter will transform the metals into a bioavailable form (Roy et al., 2013), thus, improve the acidic condition of the soil.

Bio-concentration (BCF), and translocation factors (TF)

The metal concentrations determined in soil and plant were used to calculate BCF and TF to determine plant phytoremediation potential (Usman et al., 2019). In this study, the calculated bio-concentration factor (BCF) for both Fe and Al demonstrated that *M. malabathricum* in T4 had higher BCF values compared to other treatments (*Table 2*). Both species displayed the BCF values less than 1, in which the species that having BCF more than 1 (≥1) is considered to be ideal hyper-accumulator (Sinha et al., 2007). On a similar note, the translocation factor (TF) values of both metals in all treatments were also less than unity, indicating the plant remediates the metals by phyto-stabilization through concentrating the metals in the root rather than in shoot which suggested ineffective metal transfer (Yoon et al., 2006).

Table 2. *Bio-concentration factor (BCF) and translocation factors (TF) of Fe and Al of each treatment. Different lowercase letters indicate a significant difference between treatments ($p < 0.05$)*

Treatment	Bio-concentration factor (BCF)		Translocation factor (TF)	
	Fe	Al	Fe	Al
T3	0.04 ± 0.013^{b}	0.105 ± 0.048^{a}	0.131 ± 0.061^{c}	0.439 ± 0.18^{c}
T4	0.079 ± 0.032^{a}	0.107 ± 0.062^{a}	0.466 ± 0.2^{d}	0.389 ± 0.114^{c}
T5	0.029 ± 0.009^{b}	0.081 ± 0.026^{b}	0.07 ± 0.033^{a}	0.266 ± 0.097^{b}
T6	0.033 ± 0.016^{b}	0.087 ± 0.037^{b}	0.04 ± 0.019^{a}	0.032 ± 0.01^{a}

Nevertheless, the total amount of >1000 mg/kg of Fe and Al accumulated in both plants (as shown in *Figure 5*) could help to describe the tolerance mechanisms and the ability of both species to accumulate the metals. Additionally, the application of EK field helps in increasing metal bioavailability in the soil, hence, increased the rate of metals uptake by the root, the bioaccumulation as well as the translocation in the plant (Nirola et al., 2015).

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EK assisted phytoremediation and plant adaptability

Phytoremediation rises as a promising environmental friendly technology that can mitigate the acidity problem of the soil. Over the past decade, the electro-kinetic remediation coupled with phytoremediation has been proposed as a feasible alternative to overcome the limitations of phytoremediation such as lower plant bioavailability and long treatment time (Couto et al., 2015; Hassan et al., 2018). In this pot experiment, overall results showed that within the period of twelve weeks, this combined mechanism of EK-phytoremediation reveals remarkable findings in alleviating the acidity and enhance the remediation process of the studied soils.

In terms of soil acidity improvement, the application of EK had a significant positive effect on the pH value increment, may be due to the greater metals uptake in rhizospheric region (Nair et al., 2007). The electric field by EK had generated the electromigration and electrolysis activities in soils, which favour the mobility and bioavailability of the metals. These latter would enhance the phytoextraction by plants, and eventually, improve soil pH and alleviate the soil acidity (Selvi et al., 2019; Vocciante et al., 2021). Besides, the increment of soil EC value was also found greater with EK-assistance (T4 and T6) compared to plant species alone, indicating the combination of the ions in root exudates and electric currents is beneficial to increase the EC, thus, could enhance the transportable metals for removal and nutrients for the plant growth mechanism (Sánchez et al., 2020).

Additionally, we observed that the species studied, both *M. malabathricum* and *S. campanulatum* survived under the concentrated acidic condition (pH 3-4), exhibiting the adaptability of the species towards acidic stress. The amelioration of the soil fertility (increased of pH and EC) resulted in the significantly highest concentration of Fe in *M. malabathricum* in T4, suggesting that this species could be a suitable candidate for the EK-assisted phytoremediation in a real acidic slope condition with a careful operating condition should be selected in order to optimize the phytoextraction and minimize the inhibition of the plant growth (Cameselle et al., 2013). Whilst for the Al, we discovered that the *M. malabathricum* and S. *campanulatum* did not respond to the EK-assistance by having similar Al contents in their plant parts, perhaps due to their characteristic which proven as Al-accumulator plants.

Apart from that, the low BCF and TF values (less than 1) calculated for both species indicates that *M. malabathricum* and *S. campanulatum* are non-hyper-accumulator species encountering the excess amounts of Fe and Al. However, it is evident that the total accumulation of more than 1000 mg/kg of Fe and Al recorded in their plant parts show the effective contribution of EK in metals bioavailability in soils as well as their suitability as phytoremediation candidates.

In regards to the above findings, we discover that the EK-assistance has provide better outcome in improving soil acidic conditions through various mechanism adopted by the selected species to accumulate, translocate, and detoxify metals. The soil pH becomes less acidic by 2.7-4.6%, verifying the importance of electric current to accelerate the metals mobility and enhance the bioavailability in soil, subsequently contributes to the soil fertility improvement. Moreover, the large amounts of metals in bioavailable forms lead to a notable concentration of targeted metals, Fe and Al in the plant parts of *M. malabathricum and S. campanulatum.* Hence, the combination of phytoremediation with electro-kinetic remediation is beneficial not only for remediating the acidity problem of the areas with similar condition but also could help to protect the slope soils against the erosion.

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Conclusion

This study exhibits an assessment of tolerance and suitability of the species studied for phytoremediation potential. It is evident that electro-kinetic application is a promising technique for the enhancement of bioavailability of the metals in soil for the plants uptake, especially in acidic condition. A combination of EK with phytoremediation has positively improved the soil pH and conductivity level in soil, thus, alleviating the soil acidity. Although the plant growth performance has shown adverse effect of EK-assisted treatment, the selected acidic-tolerant slope plants able to accumulate excessive amount of iron (Fe) and aluminium (Al) in their plant parts. However, the results warrant further trials on the field to truly establish the practicality of utilizing both species as phyto-remediator with the assistance of electro-kinetic.

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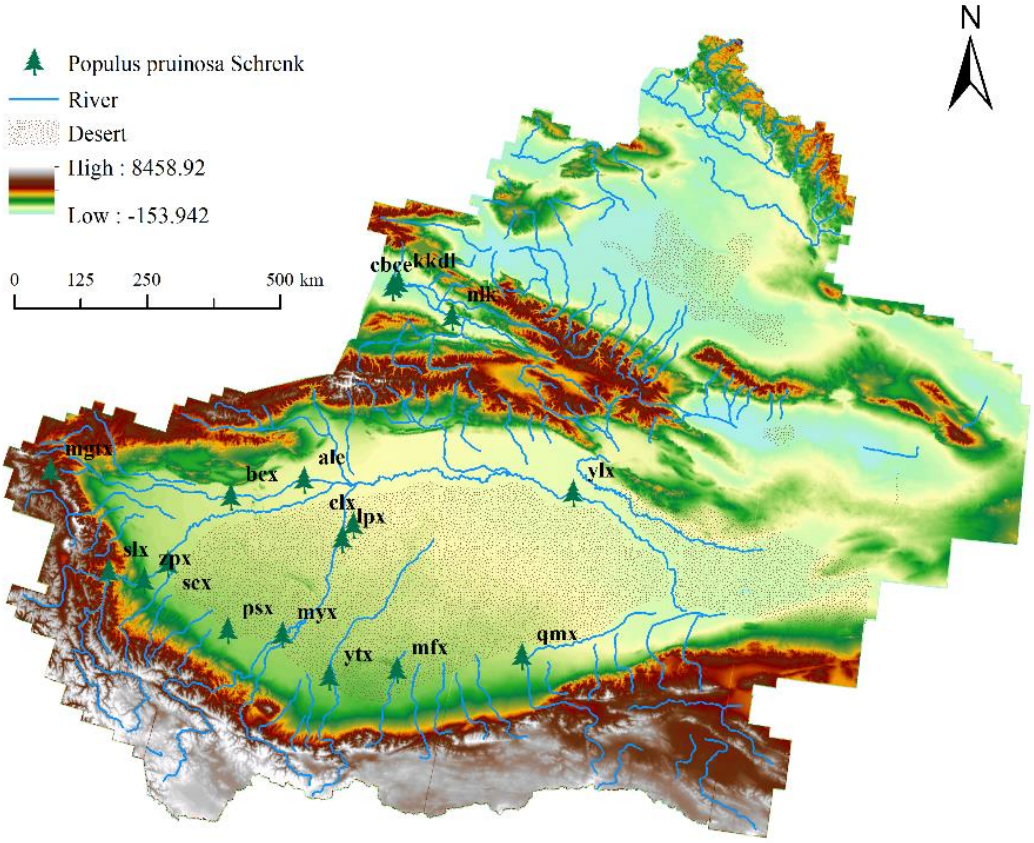

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