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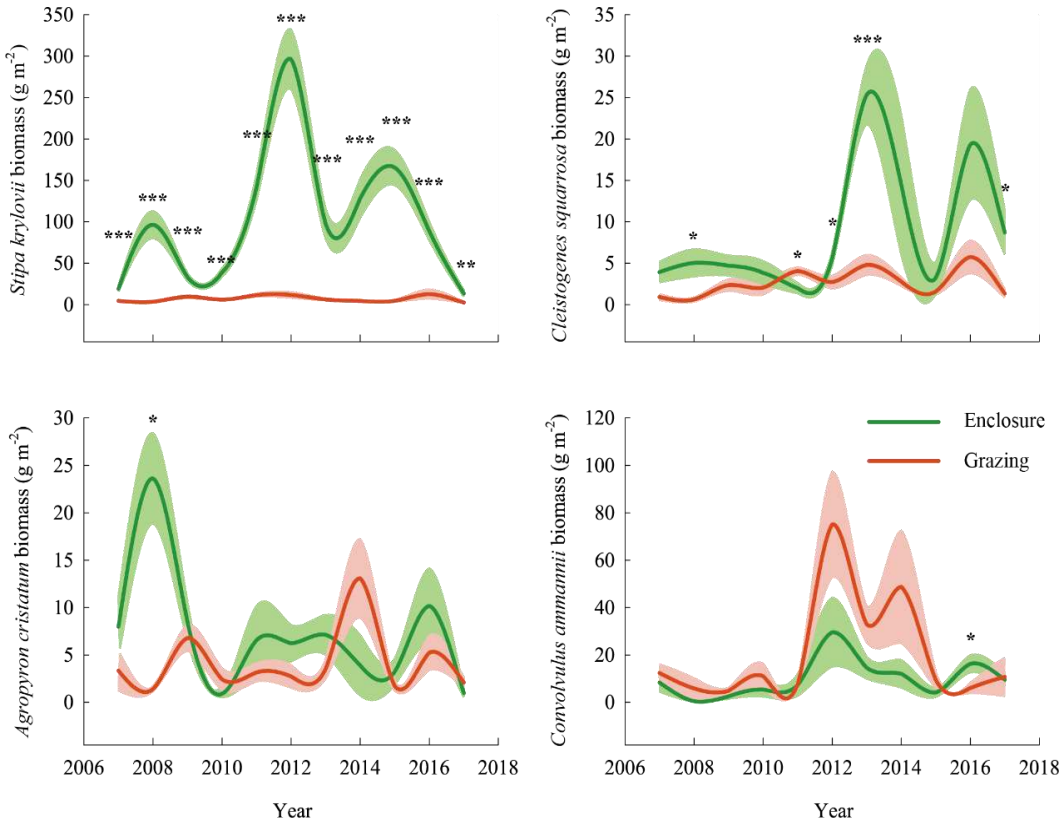

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THE ANTIBIOTIC RESISTANCE PATTERN AND MOLECULAR CHARACTERIZATION OF *bla*CTX AND *bla*TEM GENES OF *E. coli* ISOLATED FROM DIFFERENT HOSTS BASED ON THE RATE OF ANTIBIOTIC CONSUMPTION IN SULAYMANIYAH/IRAQ

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Abstract. Inappropriate use of antibiotics causes the emergence of multidrug resistant bacteria. Therefore, this study investigated the rate of beta- lactamase resistance genes and drug resistance pattern of 87 *E. coli* isolated from feces of different hosts. The rate of resistance to amoxicillin, cefotaxim, ceftriaxone, and meropenem were the highest in *E. coli* of humans compared to other hosts. 18 (20.6%) isolates were positive with *bla*CTX-M and *bla*TEM and 17 (19.5%) of them were found in heavy antibiotic users, but only one *bla*CTX-M gene (1.1%) was discovered in the low antibiotic user group of pigeons. The rate of *bla*CTX-M gene was the highest in *E. coli* isolated from the feces of humans, 13.7%, whereas the highest rate of *bla*TEM was found in *E. coli* of poultry, 3.4%. The highest rate of *bla*CTX-M and *bla*TEM genes were recorded in phylogenetic groups B2 and D at the rate of 61.5% and 60%, respectively. There were sequence similarities between local and foreign beta-lactamase genes, but single nucleotide polymorphism was found in comparison to CTX-M3 isolated in Poland. We conclude that *E. coli* isolated from humans were more resistant to antibiotics compared to other hosts and the rate of CTX-M gene was more common in human hosts.
Keywords: *ESBLs, antibiotic resistance, human, animal and birds*

Introduction

The emergence and spread of multidrug resistant bacteria is on the rise and becoming a real threat to the global public health. Failure of treatment increases morbidity and mortality due to development of resistance to previously effective drug by bacteria (Garcia, 2014). The indiscriminate use of antibiotics is the main reason leading to the increased resistance in bacteria and this has been seen in both humans and animals (Collignon et al., 2013; Chantziaras et al., 2014). Domestic animals, including poultry, have been used as a source of food by humans from ancient times, but it has been considered as one of the potential sources of delivering bacteria carrying beta lactamase resistance genes to humans. This leads to colonization of bacteria in the gut of humans after consuming contaminated meat or gaining bacteria by direct or indirect contact with animals and eventually causing severe infections (Lazarus et al., 2015).

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Escherichia coli (E. coli) is a gram negative facultative anaerobic bacteria and belongs to the family of Enterobacteriaceae (Sorum and Sunde, 2001). These bacteria are mostly found in intestinal tract of humans and animals as part of the normal flora. *E. coli* strains cause different diseases in humans including gastrointestinal infection, urinary tract infection and neonatal meningitis (Todar, 2007). *E. coli* causes food poisoning in humans after consumption of contaminated food by producing lethal toxins that could lead to gastroenteritis. Pathogenic *E. coli* stains are classified into different types based on their virulence properties and serological characteristics (Todar, 2007; Martinez-Medina and Garcia-Gil, 2014). Enteropathogenic *E. coli* (EPEC) is a strain of *E. coli* found in both humans and some other types of animals like dogs, cats, rabbits, and horses. It causes watery diarrhea after producing an adhesion toxin (intimin) which has the ability to bind intestinal cells leading to cell deformities. This variant type of *E. coli* is moderately invasive and stimulates an inflammatory response. Enterotoxigenic *E. coli* (ETEC) strain is found to be implicated in the watery diarrhea in humans especially children in the developing countries and in animals such as sheep, goats, pigs, dogs, cattle, and horses. This strain is not invasive and lodges locally in the lumen of the intestine (Croxen et al., 2013). Enteroinvasive *E. coli* (EIEC) is a variant causing diarrhea only in humans. The patients characteristically have a profuse diarrhea with or without blood and high fever. Enterohemorrhagic *E. coli* (EHEC) strain results in bloody diarrhea with the ability to infect urinary tract and causes acute kidney failure: hemolytic uremic syndrome. It is moderately invasive and produces Shiga toxin that can cause severe inflammatory response. This strain was discovered in both humans and animals (cattle and goats) (Rendón et al., 2007). Enteroaggresive *E. coli* (EAEC) is a noninvasive variant of *E. coli* that can cause watery diarrhea only in humans and has the ability to produce heat-stable enterotoxins (ST) and hemolysin, pore forming toxins. Adherent-Invasive *E. coli* (AIEC) is an invasive strain causing disease in humans and its more common in patients who have Crohn's disease. The strain is able to multiplicate intracellularly after invading intestinal epithelial cells (Martinez-Medina and Garcia-Gil, 2014).

E. coli strains are found to live in the gastrointestinal tract where they develop resistance against antimicrobial agents and able to acquire resistance genes faster than other natural microorganisms because of the selection pressure due to heavy usage of antibiotics (Van Den Bogaard and Stobberingh, 2000; EFSA, 2010). It was recently revealed that propagation of extended spectrum β-lactamases (ESBL) is very rapid with *bla*CTX, one of the common ESBLs, now discovered in different parts of the world. In addition to humans and domestic animals, this resistance gene has been recovered in wild animals and birds in the last few years (Livermore et al., 2007; Coque et al., 2008; Guenther et al., 2010; Pinto et al., 2010; Veldman et al., 2013). Proliferation of this resistance gene in wildlife creates global public health threat at an alarming level. Wild birds attract the attention of the researchers because of their potential roles in transmitting diseases and becoming carriers of bacteria to humans. In addition, wild birds harboring *E. coli* in their intestinal tracts can become a source of dissemination of resistant bacteria (Benskin et al., 2009; Wasiński et al., 2013).

In Iraq, many studies have been conducted to isolate bacteria with resistance genes from human patients, but there are scarce studies on wildlife and free animals including birds. This study focused on a comparative study to investigate and compare the rates of antibiotic-resistant *E. coli* with beta lactamase resistance genes in three classes of antibiotic users. First group was categorized as heavy antibiotic consumers: humans and chicken of

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the poultry houses because of their chronic exposures to antibiotics. Second group was categorized as intermediate antibiotic consumers: free pastured domestic animals and free ranging chickens because of their limited access to antibiotics and the last group was categorized as non-antibiotic users; such as free living birds including sparrows, singing birds, pigeons, and wild animals like turtles, wild rabbits and hedgehogs.

Materials and methods

Sample collection and bacterial isolation

A total of 87 *E. coli* strains were isolated from fresh stool of humans, animals and birds as follows: Humans (healthy) 10 (E1-E10), human patients (diarrhea and UTI) 12 (E11-E22), chicken of poultry houses 11 (E23-E33), free pastured domestic animals 10 (E34-E43), free ranging poultry 14 (E44-E57), wild animals (rabbit, hedgehog, and turtle) 10 (E58-E67), sparrows 8 (E68-E75), pigeons 12 (E76-87) (*Table 1* and *Table 2*). The sample collection was carried out between August and September 2019 in Sulaimani province. One gram of every sample was homogenized with 500 µl of normal saline in a clean container and the mixture was swabbed on selective media, MacCkonkey agar, and incubated at 37 ºC for 20 hours. A typical colony was isolated and streaked again on eosin methylene blue agar (EMB) for further purification and identification of *E. coli*. At the end, the colonies with metallic sheen green characteristic were chosen for the final confirmation by molecular biology technique using polymerase chain reaction (PCR).

Table 1. *Distribution pattern of phylogenetic groups and beta lactamase resistance genes in the E. coli isolates*

Antibiotic (AB) consumers style	Sample No.		A	B1	B2	D	*bla*CTX	*bla*TEM
Heavy AB users	Human (Healthy)	(10)	2	3	5	0	2	1
	Human(Patient)	(12)	0	4	5	3	10	1
	Poultry (house)	(11)	4	5	1	1	0	3
Intermediate AB users	Poultry (Free range)	(14)	4	9	0	1	0	0
	Domestic Animal	(10)	0	9	1	0	0	0
None AB users	Wild Animal	(10)	0	8	2	0	0	0
	Sparrow	(08)	0	8	0	0	0	0
	Pigeon	(12)	0	9	2	1	1	0
	Total	(87)	10	55	16	6	13	5

Antibiotic susceptibility testing

The antimicrobial susceptibility testing of *E. coli* isolates to six classes of beta lactam antibiotics were carried out using Kirby Bauer disc diffusion method following the protocols and guidelines recommended by the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute, 2012). The antibiotics tested in this study were: amoxicillin (AX 25 µg), amoxicillin-clavulanic acid (AMC 30 µg), oxacillin (OX 1 µg), meropenem (MEM 10 µg), ceftriaxone (CRO 30 µg) and cefotaxime (CTX 30 µg) (Bioanalyse, Ankara, Turkey).

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Table 2. *The resistance score of E. coli isolates*

N0.	AX ≤13	AMC ≤13	OX ≤10	MEM≤13	CRO≤13	CTX ≤14	N0.	AX≤13	AMC≤13	OX ≤10	MEM≤13	CRO≤13	CTX ≤14
E1	21	21	13	26	25	30	**E45**	23	15	12	28	26	20
E2	16	13	0	34	25	33	**E46**	14	15	11	30	33	31
E3	20	20	10	31	27	36	**E47**	18	10	2	26	24	28
E4	24	19	0	30	24	29	**E48**	20	16	11	30	27	28
E5	9	20	3	29	9	11	**E49**	18	0	12	30	25	29
E6	25	23	4	31	25	34	**E50**	25	14	15	37	29	33
E7	25	21	2	29	26	34	**E51**	20	10	0	29	22	24
E8	24	21	8	30	25	35	**E52**	21	14	14	29	25	27
E9	15	7	10	15	23	26	**E53**	16	9	7	28	24	26
E10	0	11	9	29	21	9	**E54**	19	19	16	29	23	25
E11	2	0	14	33	25	37	**E55**	15	17	12	31	25	28
E12	15	0	11	28	20	11	**E56**	20	15	17	25	19	25
E13	3	20	7	34	0	23	**E57**	18	15	13	29	24	17
E14	4	19	0	36	0	13	**E58**	19	12	15	26	11	25
E15	0	11	9	28	7	10	**E59**	21	9	10	31	27	29
E16	8	13	1	33	13	17	**E60**	14	7	14	27	23	25
E17	11	11	11	28	8	9	**E61**	20	12	13	29	23	27
E18	0	7	2	34	9	13	**E62**	23	15	6	31	22	30
E19	0	2	0	12	8	5	**E63**	19	12	9	30	24	14
E20	0	19	15	33	7	15	**E64**	20	11	7	31	24	26
E21	6	10	6	28	24	31	**E65**	21	13	0	29	25	26
E22	27	19	13	33	24	31	**E66**	21	16	10	29	24	28
E23	22	19	8	30	21	29	**E67**	21	13	13	30	23	28
E24	13	0	10	35	26	32	**E68**	22	11	7	29	24	26
E25	21	15	0	34	24	31	**E69**	24	14	9	30	25	29
E26	10	13	0	30	27	34	**E70**	21	15	0	30	29	28
E27	23	22	11	32	23	30	**E71**	20	11	13	30	25	29
E28	8	11	9	31	23	32	**E72**	20	12	0	29	31	30
E29	26	22	22	33	29	31	**E73**	18	9	14	28	23	20
E30	28	20	13	31	25	33	**E74**	20	8	11	28	23	27
E31	23	18	13	24	24	28	**E75**	23	14	13	31	26	30
E32	22	14	9	29	26	13	**E76**	14	12	11	30	0	16
E33	25	22	14	27	26	33	**E77**	18	9	2	32	31	31
E34	14	4	12	23	21	26	**E78**	22	8	8	26	27	25
E35	22	19	15	35	24	28	**E79**	13	12	16	30	23	28
E36	15	7	7	34	24	29	**E80**	20	17	9	29	23	29
E37	23	13	0	34	32	30	**E81**	20	11	12	25	23	25
E38	21	17	1	26	22	35	**E82**	19	16	11	26	21	28
E39	23	19	0	30	24	27	**E83**	22	10	18	32	27	29
E40	22	20	13	30	24	31	**E84**	19	15	8	30	27	28
E41	25	14	0	29	29	32	**E85**	26	24	13	34	26	14
E42	7	11	15	37	28	33	**E86**	8	10	12	22	24	30
E43	19	15	2	31	20	15	**E87**	14	13	1	27	9	8
E44	12	16	11	28	20	15							

The resistance score of the isolates were highlighted in red color. The number behind the "≤" is the inhibition zone in mm for the resistance zone. The tested antibiotics were amoxicillin (AX 25 μg), amoxicillin-clavulanic acid (AMC 30 μg), oxacillin (OX 1 μg), meropenem (MEM 10 μg), ceftriaxone (CRO 30 μg) and cefotaxime (CTX 30 μg)

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Molecular biological methods

DNA extraction

Total DNA of *E. coli* was extracted by boiling fresh colonies grown on nutrient media. The boiling was carried out by dissolving one single colony in 150 µl sterilized distilled water in a micro tube and then the mixture was heated at 99.9 °C on a heating block for 15 minutes (Dashti et al., 2009). The mixture was allowed to precipitate at room temperature and the supernatant containing DNA was used for PCR.

E. coli confirmation and phylogenetic grouping

Beta-glucuronidase (*uidA*) gene was used to confirm *E. coli* via PCR amplification of its flanking region. If no *E. coli* isolate harbor (*uidA*) gene, an attempt was made to amplify a second specific *E. coli* marker gene, the universal stress protein (*uspA*). This PCR identification confirmation of *E. coli* was carried out according to Chen and Griffiths, 1998; Heijnen and Medema, 2006; Godambe et al., 2017).

This study used the protocol according to Bonacorsi et al. (2000) to determine a phylogenetic grouping of *E. coli* with some modifications in PCR reactions. The grouping depends on finding and amplifying three genes: the chuA, yjaA and DNA fragment TSPE4.C2. Two of the genes, the chuA and yjaA, were amplified using duplex conventional PCR, but DNA fragment TSPE4.C2 was amplified via singleplex PCR.

Polymerase chain reaction of blaCTX-M and blaTEM-1

Two common extended spectrum beta lactamase resistance genes (ESBLs), *bla*CTX-M (550 bp) and *bla*TEM-1 (1080 bp) (Bradford, 2001) were amplified. Duplex PCR was used to amplify both genes. The primers and protocols used were according to Ahmed et al. (2007, 2009).

PCR condition and Gel electrophoresis

All PCR reactions in this study including multiplex and singleplex were carried out in PCR tubes composed of 20 µl volume of reaction mixture. The volume was prepared by adding 2 µl of DNA template of each isolate into a mixture containing 10 µl of 2X premix *RedTaq* DNA polymerase (SBS Genetech, Beijing, China), each primer with a concentration of 0.25 µM and the final volume attained with distilled water. The following conditions were used to run PCR using applied biosystem thermo cycler (Applied biosystem 2720, California, USA): The run started with 94 °C for 5 min, the run continued with 35 cycles of three different temperatures (94 °C 30 sec, 55 °C 30 sec, 72 °C 30 sec), and finally the PCR reaction was ended with last temperature condition of 72 °C for 7 minutes.

Gel electrophoresis was carried out through running PCR products on DNA agarose gels (2% for phylogenetic grouping genes and 1% for the other genes) at 100 V for 30 minutes. The resolved genes and DNA ladders were visualized under blue light using SmartDoc 2.0 Imaging System (Accuris, NJ, USA) after staining with fluorescent dye, GoodView (SBS Genetech, Beijing, China).

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Sequence analysis

The PCR products of CTX-M genes were gel recovered and purified using gel extraction purification kit (SBS Genetech, Beijing, China). The purified Products were sequenced using Sanger sequencing on ABI 3730XL capillary machine (CHU de Québec-Université Laval, Québec city, Canada). All sequences of this study were submitted to the GenBank National Center for Biotechnology Information (NCBI) via Bankit (Benson et al., 2015) and obtained accession numbers (MN597105-MN597117).

The obtained sequences were aligned to available sequences of CTX-M gene in Genbank using NCBI BLASTn search tool (http://www.ncbi.nlm.nih.gov/). The multi-sequence alignment of the sequences of this current study and those sequences available in the GenBank was carried out using ClustalW multi alignment tool. Phylogenic tree was drawn for all our sequences and retrieved sequences of the GenBank using the neighbor-joining (NJ) method (Phylogeny.fr) (Dereeper et al., 2010).

Results

Isolation and phylogenetic grouping

Eighty-seven (87) *E. coli* isolates were recovered from the different categories of antibiotic users in order to compare the drug resistance pattern to the antibiotics and the rate of the resistance genes. The *E. coli* isolates were recovered using conventional culture method and genetically confirmed using molecular biology techniques. *E. coli* gene marker, beta-glucuronidase (*uidA* 201 bp) was used for the final confirmation. The flanking region of the gene (201 bp) was amplified using *E. coli* specific primers (*Figure 1*). Out of 87 isolates, 83 (95.4%) isolates were positive for *uidA* marker gene. Four isolates remained questionable to be *E. coli*, so the second *E. coli* marker gene, *uspA* was used to identify the isolates. PCR amplification of the universal stress protein (*uspA* 800 bp) was carried out using specific primers for *E. coli uspA* gene. The Four (4.5%) remaining isolates were positive for *uspA* gene therefore all the isolates were confirmed to be *E. coli*.

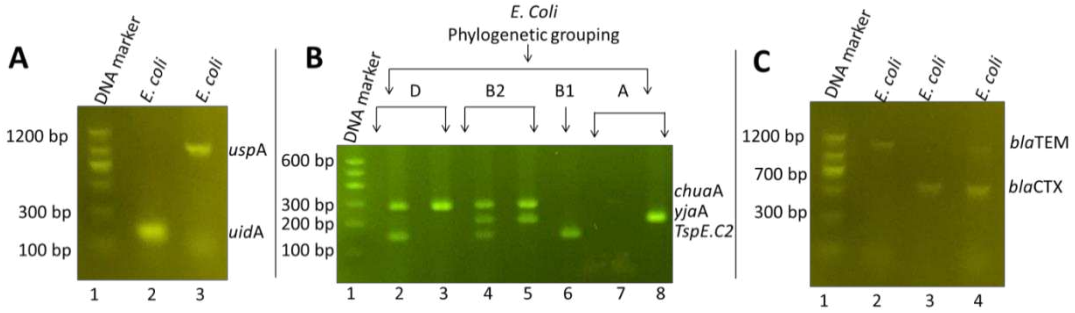

Figure 1. *PCR amplification of different genes of E. coli. (A) PCR amplification of uidA and uspA genes used for the confirmation of E. coli. (B) Phylogenetic grouping* based on the different combinations of chuaA, yjaA, and TspE.C2 (Bonacorsi et al., 2000). (C) PCR amplification of beta lactamase resistance genes, blaTEM, and blaCTX. * The phylogenetic grouping was carried out in two PCR reactions, but two PCR reactions were mixed to clarify and show the combination of different genes of the groups, lane 2 and 3*

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There are four main phylogenetic grouping of *E. coli* strains: A, B1, B2 and D. Commensal strains of *E. coli* belong to group A and B1 (Saralaya et al., 2015). *E. coli* strains in group B2 and to lesser extent group D are virulent extra-intestinal strains (Bonacorsi et al., 2000). All the 87 *E. coli* isolates were subjected to the phylogenetic grouping of *E. coli* based on different combinations of *yja*A, *chu*A, and *TSPE4.C2* genes. Out of the 87 *E. coli* isolates, group B1 was the dominant group with 55 isolates (63.2%) followed by group B2 with 16 isolates (18.3%) and then group A with 10 isolates (11.4%). Group D was the least with just 6 isolates (6.8%) (*Table 1*). Out of 22 isolates recovered from humans, the highest rate 10 (45.4%) were under group B2 while the lowest rate 9% (2 isolates) was in group A. The rate of group B1 in all animals and birds was the highest at 73.8% (48 out of 65), but group D recorded the lowest rate at 4.6% (3 out of 65).

Antibiotic susceptibility testing

E. coli isolates were investigated to determine their antibiotic resistance phenotype against different classes of beta lactam antimicrobials (*Figure 2*). The highest resistance pattern revealed in this study was to oxacillin (1 µg) 54.5%, and the lowest resistance rate was against meropenem (10 µg) 1%. The resistance rate of the other antibiotics was as follows: amoxicillin (25 µg) 19.3%, amoxicillin-clavulanic acid (30 µg) 50.3%, cefotaxime (30 µg) 16.7%., and ceftriaxone (30 µg) 13%. The resistance score of each *E. coli* isolates are shown in *Table 2*.

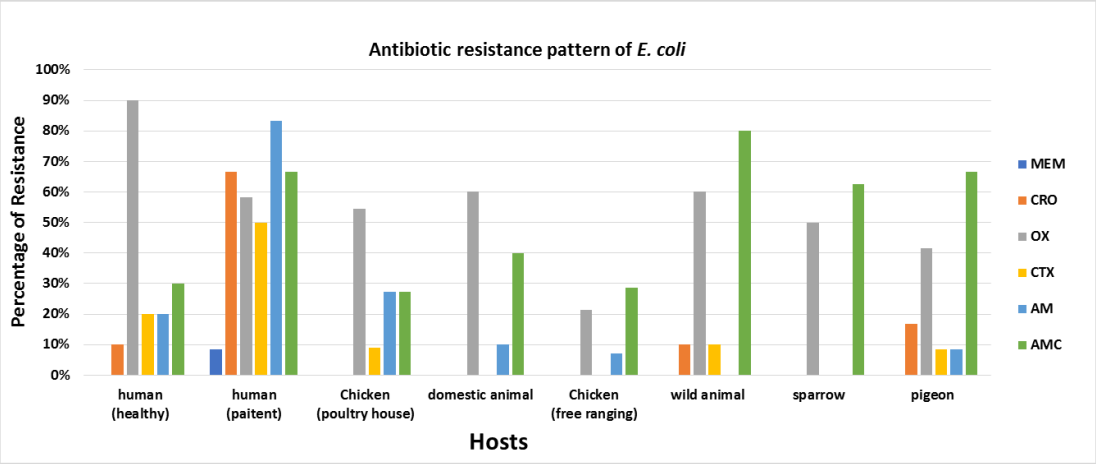

Figure 2. *Antibiotic resistance pattern of E. coli isolates in different hosts*

Antibiotic resistance pattern of E. coli in different hosts

Resistance rate to Oxacillin (1 µg) was high in all different hosts compared to other antibiotics. Resistance rate was the highest in healthy humans (90%) followed by human patients (58%) and chicken of poultry houses (55%), but the lowest rate was in free ranging chickens (21%) (*Figure 2*). *E. coli* isolated from wild animals had the highest rate of resistance to amoxillin-clavulanic acid (30 µg) (80%) among all the hosts followed by human patients (67%) and the lowest rate was in poultry housing chicken (27%). In human patients, the resistance rates of *E. coli* isolates to amoxicillin, ceftriaxone (30 µg), cefotaxim (30 µg), and meropenem (MEM 10 µg) were the highest in human patients

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compared to other hosts at the rates of 83%, 67%, 50%, and 8% for the antibiotics respectively.

The rate of resistance genes, blaCTX-M and blaTEM-1 genes in E. coli isolated from different hosts

CTX-M is a class A beta-lactamase enzyme with a great activity against cefotaxime. It is believed to have evolved from two beta-lactamase enzymes, TEM and SHV after several mutations. More than 172 types of this enzyme have been found (Antonopoulos et al., 2018; Ramadan et al., 2019) and some of these enzymes are active against both ceftazidime and cefotaxime (Boyd et al., 2004; Paterson and Bonomo, 2005). This class of beta-lactamase is mostly found in *E. coli* and *Salmonella enterica*, but they are also present in other members of Enterobacteriaceae (Woodford et al., 2004; Hudson et al., 2014). In this study, a set of primers was used to amplify and sequence *bla*CTX-M gene in order to identify, to family level, the type of CTX-M in the area.

The amplicons of *bla*CTX-M (550 bp) and *bla*TEM-1 (1080 bp) were amplified using gene specific universal primers to determine the rate of the beta lactams resistance genes in the area of the study (*Figure 2*). Out of 87 samples, 18 (20.6%) were positive for the beta lactamase resistance genes, *bla*CTX-M and *bla*TEM-1 (*Table 1*). The rate of *bla*CTX-M was remarkably higher, 13 (14.9%) compared to *bla*TEM 5 (5.7%) isolates (*Table 1*). The highest number of *bla*CTX resistance gene, 10 (11.1%) was discovered in human patients, but the highest rate of *bla*TEM, 3 (3.4%) was found in chickens of poultry houses. In total, 12 (13.7%) *bla*CTX resistance genes were recovered in *E. coli* of heavy antibiotic user hosts (all in human), whereas only one resistance gene (1.1%) was found in none antibiotic consumer hosts and none of the resistance genes were recovered from the intermediate antibiotic users.

Most of the beta lactamase genes, *bla*CTX-M and *bla*TEM were recorded in phylogenetic group B2 and D at the rate of 61.5% and 60%, respectively. The highest rate of *bla*CTX was found in phylogenetic groups B2 and B1 at the rate of 38.4% and 30.7%, followed by group D (23%) and A (7.6%). However, most of the *bla*TEM were located into group D and B1 at equal rate of 40% followed by group B2 (20%) and none of them was recorded in group A (*Figure 3*).

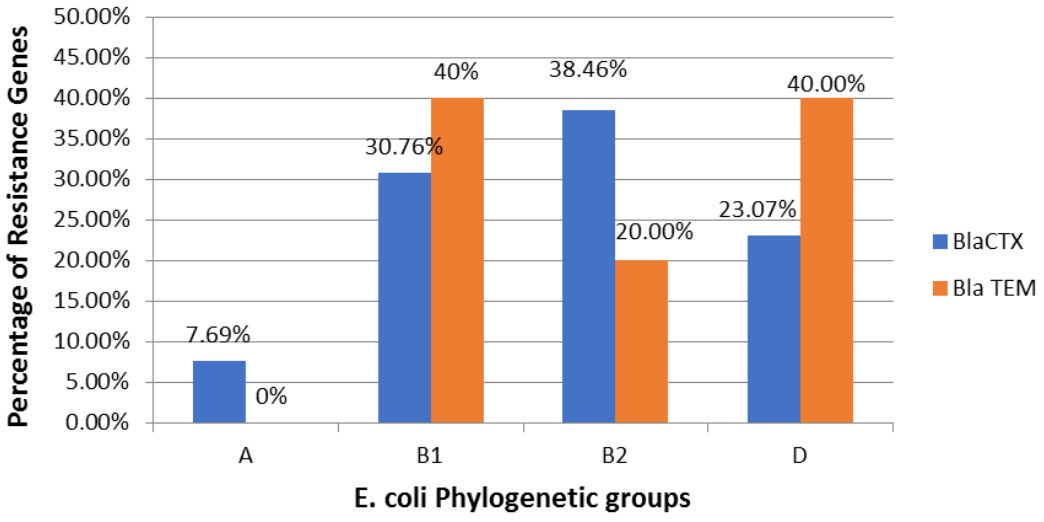

Figure 3. *Distribution of blaCTX and blaTEM resistance genes on E. coli phylogenetic groups*

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Sequence analysis of blaCTX-M

*bla*CTX-M genes isolated in this study were sequenced using sanger sequencing. The results from the sequencing of CTX-M genes recovered from *E. coli* isolates in different hosts were aligned together (*Figure 4*). The results showed that all the sequences were similar to one another with no mutation observed.

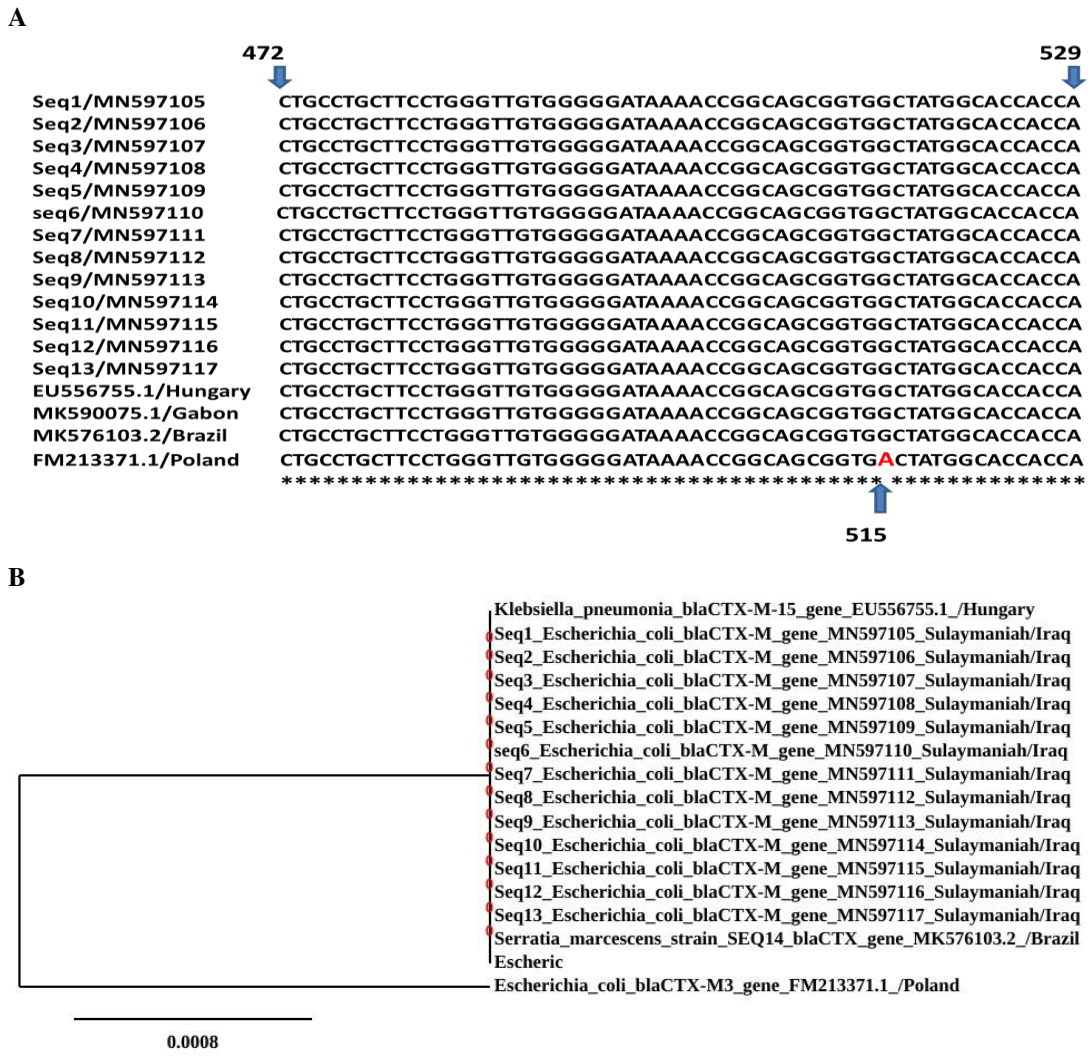

Figure 4. *Multiple sequence alignment and phylogenic tree of blaCTX-M genes recovered from E. coli and some other species. (A) CTX-M gene in E. coli strains isolated from Sulaimaniyah in comparison with published CTX-M genes of E. coli, Klebsiella pneumonia, and Serratia marcescens strains from GenBank. (B) Phylogenetic CTX-M gene in E. coli strains isolated from Sulaimaniyah in comparison with homologous published CTX-M genes in GenBank*

The CTX-M gene sequences of *E. coli* were also compared to available sequences on GenBank using BLAST search. The sequence is completely similar to available sequences deposited in the database from other countries. But interestingly one single nucleotide difference (single nucleotide polymorphism, SNP) was revealed when CTX-M3 gene from our study was compared to that published in Poland, FM213371.1 (*Figure 4*). The single nucleotide polymorphism position was indicated in nucleotide number 515 as indicated in *Figure 4*. The phylogenetic tree showed the degree of

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similarity between local strains with that from Brazilian, Hungarian and Gabon and the SNP observed with the strain from Poland.

Discussion

To our knowledge, this is the first comparative study in Iraq to report the presence of ESBL producing *E. coli* in different antibiotic consumers, heavy antibiotic consumers, intermediate antibiotic consumers, and low antibiotic users. This study was conducted in summer 2019 between September and October. Humans and poultry houses were considered as heavy antibiotic users because they consume large amount of antibiotic for treatment and prophylaxis purposes in the area of the study due to easy access to buy antibiotic over the counter without any drug prescription. Intermediate antibiotic user was used in this study to denote those animals and birds consuming antibiotic in very rare events. Domestic animals and birds are being reared by villagers and are not using antibiotics to rear them except in very necessary cases such as in debilitating diseases. Non-antibiotic user refers to those animals and birds which are not domesticated. They live freely and they are not exposed to antibiotic because they don't have close contact with humans. In this group are wild animals (hedgehog, turtle and rabbit), sparrows and wild pigeons.

The result of this study showed a high percentage of fecal *E. coli* bearing *bla*CTX and *bla*TEM, 20.6% of 87 *E. coli* isolates isolated from feces of humans, animals and birds. Out of 18 beta lactam resistance genes, 13 (14.9%) were *bla*CTX and five (5.7%) were *bla*TEM. The highest rate of *bla*CTX genes 13.7% (12 isolates) were recovered from humans. Most of the resistance genes were found in heavy antimicrobial users, 17 out of 18 resistance genes in 87 isolates (19.5%). The rate of resistance genes was higher in human patients (11.1%) than in healthy humans. There were no beta lactamase resistance genes, *bla*CTX, and *bla*TEM, recorded in intermediate antibiotic users (live stocks and free living poultry) in this study. The high percentage of *E. coli* producing *bla*CTX in human feces is comparable with studies in different countries. This study is totally in agreement with the study of many countries that the rate of *bla*CTX is significantly high (Valentin et al., 2014). The rate of *bla*CTX is 0% in *E. coli* isolated from feces of poultry in the current study and this result is similar to the finding in many countries of Asia and Europe. In The Netherlands *E. coli* harboring *bla*CTX was recorded 0%. In addition, it is comparable with the study in other countries of Europe where the prevalence of *bla*CTX is very low such as in Belgium (2%), and the United Kingdom (12%) (Smet et al., 2008; Randall et al., 2011; Huijbers et al., 2014). Same low record was investigated in Asian countries, Japan and China with low percentage of 0-2%, whereas the finding is different from different studies carried out in Africa. It is concluded in many studies that the rate of *bla*CTX is significantly high and it is one of the most prevalent genes in live stocks and poultry (reviewed in: Alonso et al., 2017). This is however at variance to the finding in Ghana where they recorded high rate of *bla*CTX in poultry, 96% (Falgenhauer et al., 2019). So the geographical distribution of *bla*CTX in this study area is more similar to Asia and Europe and it is the opposite to that of Africa.

For non-antibiotic consumers (wild animals, pigeon, and sparrow) also the lowest prevalence rate of beta lactamase resistance genes was recorded. From the *E. coli* isolated from feces of 30 animals and birds, only one (1.1%) *E coli* isolate producing *bla*CTX gene was recovered in pigeon and there was not any *bla*TEM recorded in this

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category of animals. Comparative study of *E. coli* bearing ESBL in wild birds is not similar in different countries on the same continent. In Europe, the prevalence is very high in some countries while it is very low in others. The rate of *bla*CTX is 0% and 0.7% in Denmark and Poland, respectively, which is more similar to our results. Similar studies showed that prevalence rate was relatively higher in African, Tunisia (10.8%), and in some other countries of Europe (Guenther et al., 2012; Veldman et al., 2013; Alcalá et al., 2016; Ben Yahia et al., 2018). But the rate is much higher in some countries of Europe, in The Netherland (37.8%) and in Spain 74.8% (Stedt et al., 2015).

Sequence analysis of CTX-M revealed that all CTX-M gene sequences recovered in *E. coli* isolates in this study from different hosts were similar depending on multiple sequence alignment and phylogeny tree. Among all the hosts except for humans, only one CTX-gene was discovered in *E. coli* of pigeon (MN597117) and it had 100% sequence similarity with CTX genes of *E. coli* isolated from humans (MN597105-MN597116) (*Figure 4*). The local sequences were then compared to published CTX-M genes of the same strain and other species of bacteria found in the GenBank. The local sequences showed 100% sequence similarities with sequences found in Brazil (MK576103.2), Hungary (EU556755.1), and Gabon (MK590075.1), but there was one nucleotide change in the CTX-M3 found in Poland (FM213371.1) and this difference can be seen in phylogenic tree and multiple sequence alignment in *Figure 4*.

Distribution of *E. coli* phylogenetic groups on different categories of antibiotic consumers carried out in this study. The result showed that the highest rate of heavy antibiotic users was categorized under phylogenetic group B1, 13.7% and B2, 12.6%, while the lowest rate was in group A, 6.8%. Both groups of intermediate and low antibiotic users recorded high rate categorization under group B1 with the rate of 49.4%. Most of the beta lactamase genes, *bla*CTX-M and *bla*TEM were recorded in phylogenetic group B2 and D at the rate of 61.5% and 60%, respectively, and the highest rate of *bla*CTX was found under phylogenetic group of B2, 38.4%. This result is comparable with published conclusion that most pathogenic *E. coli* belong to phylogenetic groups B2 and D and these two groups were prevalent in those human patients who are heavy antibiotic users (Bingen et al., 1998).

Antibiotics have been hitherto effective antibacterial agents used to treat bacterial infection in the field of human and veterinary medicine. Ability of bacteria to produce resistance against antibiotics impedes or delays the treatment which results in economic losses and even morbidity (Grover et al., 2013). In addition, wild birds can become a source of spreading resistant bacteria from one area to another especially to humans (Peirano et al., 2011). Therefore, this comparative study investigated the drug resistance pattern differences between high to low antibiotic users. The multidrug resistance and drug resistance pattern of these tested antibiotics in *E. coli* is significantly important because beta-lactam antibiotics (cephalosporins) especially meropenem (new generation) are known as the frontline antibiotics for the treatment of bacterial infection (Bradford, 2001). Furthermore, bacteria harboring *bla*CTX and *bla*TEM resistance genes become resistant against different classes of beta-lactam antibiotics (Boyd et al., 2004; Paterson and Bonomo, 2005). Therefore, six beta-lactam antibiotics were chosen to investigate the resistance phenotype of *E. coli* in this study, amoxicillin (25 μg), amoxicillin-clavulanic acid (30 μg), oxacillin (1 μg), meropenem (10 μg), ceftriaxone (30 μg) and cefotaxime (30 μg).

The rate of antibiotic resistance was higher in *E. coli* isolated from heavy antibiotic users than in other groups in this study against amoxicillin (25 μg), amoxicillin-

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clavulanic acid (30 μg), oxacillin (1 μg), meropenem (10 μg), ceftriaxone (30 μg) and cefotaxime (30 μg) (*Figure 2*) and followed by wild animals against oxacillin and amoxicillin-clavulanic acid which showed relatively high resistance. In total, the resistance percentage to oxacillin was the highest and the sensitivity against meropenem was remarkably high in all *E. coli* isolates in this study. 29 (33.3%) out of 87 isolates showed multidrug resistance to different antibiotics especially against different antibiotics and the highest rate of multidrug resistance was in heavy antibiotic user group, 15 (17.2%). This multidrug resistance were comparable in different studies in different countries (Forward et al., 2004; Yan et al., 2004; Sunde, 2005; Ahmed et al., 2009; Hansen et al., 2013). *E. coli* isolates bearing *bla*CTX showed resistance to extended spectrum antibiotic, cefotaxim. This finding is consistent with some studies in different countries, Ghana, (Hackman et al., 2014), Ethiopia (Zeynudin et al., 2018) and India (Upadhyay et al., 2015) that most bacteria harboring CTX-M resistance genes are resistant to third generation of cephalosporin.

Conclusion

This study revealed a remarkably high rate of *bla*CTX in *E. coli* isolated from different hosts and it is obviously more predominant in heavy antibiotic user hosts (humans) in Sulaimani region/Iraq. *bla*CTX and *bla*TEM were mostly found in phylogenetic groups B2 and D. Pathogenic *E. coli* isolated from human patients showed higher degree of resistance to antibiotics compared to other hosts. Meropenem (10 μg) antibiotics remains the most active against *E. coli* in all hosts. So *E. coli* living in the intestinal tract of domestic animal, free living poultry, free living flying birds and wild lives are less resistant to antibiotics and less often carry beta lactam resistance genes compared to human. Therefore, those animals and birds do not pose a big threat and risk to human public health in this study area.

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FOLIAR APPLICATION OF DIANELLA ENSATA, AMBROSIA DUMOSA AND MORINGA OLEIFERA IMPROVED BARLEY GROWTH AND YIELD TRAITS UNDER DROUGHT STRESS

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Abstract. Barley (*Hordeum vulgare* L.) is one of the major cereal crops cultivated in Saudi Arabia. A pot experiment was performed to estimate the effect of drought stress on barley crop while natural plants extract were used as a management strategy. The experiment was laid out in a completely randomized design with three repetitions. Pots grown barley were thinned to three plants per pot and allowed to grow undisturbed until the start of reproductive stage. Aqueous extract of flax liliy; *Dianella ensata,* burro-weed; *Ambrosia dumosa* and drumstick tree; *Moringa oleifera* were used along with the control. Drought stress treatments were 40%, 60% and 100% field capacity. The application of *Dianella ensata* and *Moringa oleifera* produced double number of productive tillers and 30-38% recovery for number of spikelets per spike. Severe drought stress reported a substantial decrease (-31%) in number of grains per spike and 1000 grain weight while the application of *Moringa oleifera* has achieved 28% higher number of grains per spike and 24% higher 1000 grain weight. The application of *Moringa oleifera* also reported an increase of 28-35% in leaf area and chlorophyll content. On contrary to other traits, a positive correlation exists between the drought stress and root length.
Keywords: *drought stress, cereal crop, drought mitigation, field capacity, Hordeum vulgare*

Introduction

The global population is continuously increasing and the growth may reach up to 9.7 billion by 2050, so global agriculture is supposed to produce 70% more food in future. In addition, there are other fields for world agriculture to fight, to try to eliminate increasing poverty and hunger, and to restore the diminishing natural agricultural resources as was called for by the food and agriculture organization (FAO, 2009; Bourne, 2009). Crop production is not coping with the increasing demands for food, and this is due to many stressing factors (Alghabari et al., 2015; Fahad et al., 2017, 2018). Scarce rainfall in arid regions is considered the main reason for low crop production, so efforts are needed to manage this problem. Policies should be directed toward planting of drought resistant salt tolerant crops, and crops with short duration together with the application of growth promoting substances (Ihsan et al., 2016a). Naeem et al. (2018) reported a positive role of calcium in drought stress management in maize crop. Daur et al. (2018) studied the positive role of rhizobacteria isolated from the roots of various plants of Saudi Arabia on alfalfa growth and yield.

The annual grain crop barley (*Hordeum vulgare* L.) is one of the cereal crops famous for cultivation in arid and saline regions (Alghabari and Ihsan, 2018). It contains high percentages of carbohydrates, and rich in minerals and vitamins. Barley is considered one of the valuable food resources, and can be used as whole grain as an animal feed for goats, sheeps or processed and making different food products and infant foods. Barley can succeed in arid areas with limited water suuply. Plant growth and yield decreased under drought stress (Fenta et al., 2014), and this reduction in growth and yield is

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attributed to changes in the physiological and phnological parameters of the plant due to the scarcity of water (Ihsan et al., 2016b). There is a continuous reduction in soil water supplies throughout the world, and this is suggested to cause losses in plant production all over the world up to 30% by 2025, compared to the current yields (Zhang, 2011). When planting crops under arid land conditions they face water deficit stress throughout their growth cycle especially at anthesis (Ihsan et al., 2016b; Fahad et al., 2017).

On the other hand climatic conditions are continuously changing, and these changes had negative impact on crop growth and yield due to escalation in drought, salinity and heat stresses intensity. Of these stresses drought can be considered the most devastating abiotic stress (Alghabari et al., 2015; Alghabari and Ihsan, 2018). Drought stress problem increased tremendously in field crops due to climate change, anthropogenic activities, bad crop choice, improper use of land and low irrigation efficiency. Production of new genetically bred drought tolerant crop varieties will help in solving problem of agriculture in arid regions. Plant growth promoting extracts of natural plants would be another suitable option due to their safety and low prices.

In nature there are many drought stress tolerant wild plants with many potentialities that can be used with crop plants (Abbasi et al., 2009), for they represent sources of number of biochemicals which have growth stimulatory characters. There is a limited work on the use of wild plant species as drought stress ameliorators in field crops. Saudi desert is rich in wild flora and efforts should be exerted to discover their growth promoting potential in detail. Drought stress arises from diminish in fresh water resources, reduction in annual rainfall, and inability in storing rain water and generally features of aridity of the region. Drought stress causes a serious damage to field crops particularly at germination and flowering stages. Planting of barley is preferred in arid regions because it is considered a short duration crop and its water requirement is very low, and in spite of that barley crop growth faces severe drought cycles resulting in minimum seed germinaion and final grain yield.

The current study was performed to estimate the impact of drought stress on barley growth, yield and root characteristics. The wild desert plant species water extract was used as a drought stress management strategy. The wild desert plants; *Dianella ensata, Ambrosia dumosa, Moringa oleifera* Lam were compared for drought stress regulation in pot sown barley crop.

Materials and Methods

Layout of the experiment

A model pot experiment was conducted to evluate the response of barley (*Hordeum vulgare*) crop against drought stress and its management through foliar application of natural plants extract (NPE). The experiment was performed at the Hada Al-Sham Agricultural Research Station of King Abdulaziz University Jeddah, Kingdom of Saudi Arabia during 2018. Three NPEs (Flax liliy; *Dianella ensata,* burro-weed; *Ambrosia dumosa,* drumstick tree; *Moringa oleifera*) were used along with the control. These plants were selected based on their adaptibility to arid environment of Saudi Arabia and their properties to withstand drought stress. Drought stress treatments were applied as 40%, 60% and 100% of field capacity (FC) at the onset of reproductiove stage. The experiment was layed out in a completely randomised design with a factorial arrangements. There were three repetitions for each treatment, and a total number of 36

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experimental units were used in this experiment. The used NPE, FC and sequence of treatments are presented in *Table 1*.

Table 1. *Tabulated presentation of treatments; natural plant extract (NPE) and field capacity (FC)*

Sr. No	Treatments	Common name	Botanical name
	Natural Plants Extract (NPE)		
1	NPE1	Control	Water
2	NPE2	Flax lily	*Dianella ensata*
3	NPE3	burro-weed	*Ambrosia dumosa*
4	NPE4	drumstick tree	*Moringa oleifera* Lam
	Drought stress (FC)		
1	FC1	100%	No stress
2	FC2	60%	Moderate stress
3	FC3	40%	Severe stress

Pots preparation

Soil collected from the agriculture fields of Hada Al-Sham research station was mixed with farmyard manure (2:1 ratio) to improve the soil physio-chemical and biological properties of the growing medium. Soil field capacity was determined by using gravimetric method. The irrigation water ratios were 40% FC extreme drought stress, 60% FC moderate drought stress and 100% FC no drought stress were considered as control (non-stressed). The pots were 30 cm tall and 22 cm wide. Each pot was filled with three kg of growth medium. Sowing of barley seeds was carried out on second week of February. At least ten healthy seed (variety; Sanober-96) were sown in each pot.

Treatments application

After 15 days of germination, the number of plants were thinned to three plants per pot and excessive plants were removed. The chemical fertilizer NPK was applied at a rate of 4 g : 2 g : 2 g per pot dissolved with irrigation water. Fertilizers were applied in two split doses at 30 days time interval starting from tillering. Field capacity level of each treatment was adjusted and the exact amount of water was added to each pot under each stress treatment. The soil FC was determined according to the gravimetric method (Alghabari and Ihsan, 2018). The pots were weighted at three days interval and manually irrigated to maintain the required FCs.

Preparation of wild plants extract

Three wild plants (*Dianella ensata, Ambrosia dumosa, Moringa oleifera* Lam) were used to study their response against drought stress in a pot experiment. Two kg air dried biomass of each plant was ground to fine powder and soaked in 5 liters of water for 80 minutes. The mixture was boiled for 3 hours at 100 °C to obtain the concentrate solution, which was then cooled and saved in plastic bottles for further use (Yasmeen et al., 2012).

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Application of wild plants extract

Drought stress was started at the onset of reproductive stage (initiation of spike) and continued untill the maturity stage. The NPEs were exogenously sprayed after diluting the extract 10 times. The NPEs were applied at three times before start of drought stress -10 and after drought stress 20 and 40 days of drought stress application. Plants were harvested at maturity and the following parameters were measured.

Data collection

Data were collected from each pot separately at the time of the harvest of the experiment. Data collection included different growth, yield and root characteristics. Plant height (cm) was measured from the base of the plant to the tip of the spike through a measuring scale. The number of spikes per plant were manually calculated through counting the number of productive tillers. The plant weight was measured from each plant excluding the root biomass and presented in grams. Single spike weight was measured from the spike of the primary tiller. The spike length were measured by following the procedure adoted for the measurement of the plant height. The number of spikelets per spike and number of grains per spike were manually counted from each spike. The 1000 grain weight was recorded by weighting the 100 grains and multiplying it with 10 for each treatment. The flag leaf area was measured through leaf scanner and chlorophyll content through chlorophyll meter. Both characters were measured before the harvest of the plants but after completing the application of treatments. The root dynamics were measured as root length, root fresh and dry biomass accumulation. A measuring scale was used to measure the root length from the base of the stem to the tip of the root. Root fresh weight was taken with the help of digital balance. For the measurement of root dry weight, the fresh root samples were transferred to hot air oven for 72 hours. The temperature was set at 70 °C. The root dry weight was measured after uniform drying of all the root samples.

Statistical analysis

It was a two factor experiment; 1) drought stress and 2) natural plants extract. Drought stress consisted of three levels while plant extracts have four levels. The total number of treatments were twelve. The experiment was run in a completely randomised design with three repetitions. Data were statistically analyzed by using analysis of variance under SAS software, and least significant difference (LSD) test was used to determine and compare treatment means to estimate their significance. Figures are drawn by using MS excel software. Verticle lines on the figures represent the standard error bars.

Results

Growth traits

Table 2 represents the data for the barley growth characteristics. The effect of natural plants extract was highly significant ($p \leq 0.01$) for barley plant height, number of spikes per plant and single plant weight. The effect of drought stress was significant ($p \leq 0.05$) for plant height and plant weight while non-significant ($p \geq 0.05$) for number of spikes per plant. The interaction of natural plants extract and drought stress were highly significnt ($p \leq 0.01$) for plant height and number of spikes per plant while significant ($p \leq 0.05$) for single plant weight.

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Table 2. *Barley plant height (cm) and number of spikes/plant as affeted by the foliar applied natural plant extracts (NPEs) and drought stress (FC)*

Plant extracts	Plant height (cm)			Number of Spikes/plant			Plant weight (g)		
	FC-1	FC-2	FC-3	FC-1	FC-2	FC-3	FC-1	FC-2	FC-3
NPE 1	47.00 f*	44.17 j	40.00 k	4 b	3 c	2 d	8.69 d	6.20 g	4.85 i
NPE 2	50.37 b	48.19 e	45.12 h	5 a	4 b	4 b	10.32 b	7.92 e	5.63 h
NPE 3	49.33 c	47.32 f	44.71 i	5 a	4 b	3 c	9.66 c	7.01 f	5.21 h
NPE 4	52.25 a	48.63 d	46.13 g	5 a	4 b	4 b	11.45 a	8.12 de	6.10 g
NPE	**			**			**		
FC	*			ns			*		
NPE×FC	**			**			*		
LSD	0.35			0.45			0.56		

*; figures differing in letters are statistically significant at p≤0.05, **; significant at p≤0.01, ns; non-significant, LSD; least significant difference. NPE 1; Water, NPE 2; *Dianella ensata*, NPE 3; *Ambrosia dumosa*, NPE 4; *Moringa oleifera* Lam

Drought stress displayed a negative effect on plant growth characteristics and the severity of the negative effect increases with the increase in the level of drought stress. On contrary, the application of natural plants extract has ammeliorated the negtive effects of drought stress and improved plant growth characteristics. The maximum plant height was recorded under normal growth conditions with the application of *Moringa oleifera* leaf extract. Similarly, this treatment reported the maximum plant height at moderate and severe drought stress as compared to control and other natural plants extract treatments. The foliar application of *Moringa oleifera* leaf extract reported 11% increase in plant height under normal condition and 15% at 40% field capacity as compared to control. Number of spike per plant were less affected by the application of natural plants effect and field capacity for 100% FC and 60% FC while the effect was more significant at 40% FC. The foliar application of *Dianella ensata* and *Moringa oleifera* leaf extracts produced a double number of productive tillers as compared to control at 40% FC. The plant weight has sharply decreased with the increase in drought stress levels. The 40% FC exhibited a 44% decrease in plant weight as compared to 100% FC. The application of *Moringa oleifera* leaf extract presented a 26% recovery in plant weight at severe drought stress (40% FC) when compared to control.

Spike characteristics

Spike related characters such as single spike weight, spike length and number of spikelets per spike were presented in *Table 3*. The effect of natural plants extract was highly significant (p≤0.01) for single spike weight and spike length while significant (p≤0.05) for number of spikelets per spike. The effect of drought stress was highly significant (p≤0.01) for single spike weight while significant (p≤0.05) for spike length and number of spikelets per spike. The interaction of natural plants extract and drought stress was highly significant (p≤0.01) for single spike weight and number of spikelets per spike while non-significant (p≥0.05) for the spike length.

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Table 3. *Barley spike characterisitics as affected by drought stress (FC) and natural plant extract (NPE) foliar application*

Plant extracts	Spike weight (g)			Spike length (cm)			No. of Spikelets per spike		
	FC-1	FC-2	FC-3	FC-1	FC-2	FC-3	FC-1	FC-2	FC-3
NPE 1	1.80 b	1.20 b	0.68 c	6.11 d	5.65 c	4.11 c	41.15 c	35.23 e	25.21 h
NPE 2	2.43 a	2.05 a	1.66 b	6.40 b	5.95 b	4.61 b	43.78 a	39.78 cd	32.89 f
NPE 3	2.04 a	1.78 b	1.40 b	6.18 c	5.78 b	4.55 b	42.22 b	38.44 d	30.78 g
NPE 4	2.92 a	2.36 a	1.71 b	6.80 a	6.05 a	4.98 a	44.25 a	40.21 c	34.80 e
NPE	**			**			*		
FC	**			*			*		
NPE×FC	**			ns			**		
LSD	0.98			0.20			1.05		

*; figures differing in letters are statistically significant at p≤0.05, **; significant at p≤0.01, ns; non-significant, LSD; least significant difference. NPE 1; Water, NPE 2; *Dianella ensata*, NPE 3; *Ambrosia dumosa*, NPE 4; *Moringa oleifera* Lam

The application of *Moringa oleifera* leaf extract procured the maximum spike weight and that was statistically non-significant to other three natural plants extract treatments at 100% FC. Spike weight, spike length and number of spikelets per spike were gradually decreased with the application of drought stress irrespective of the application of natural plants extract. At moderate drought stress the application of *Moringa oleifera* leaf extract reported 96% improvement in spike weight as compared to control. The application of *Moringa oleifera* leaf extract displayed 7% and 21% recovery in spike length at moderate and severe drought stress levels as compared to the control. The 40% FC confirmed 39% decrease in number of spikelets per spike as compared to 100% FC. The application of *Dianella ensata* and *Moringa oleifera* plant extracts demonstrated 30% and 38% recovery for number of spikelets per spike as compared to control at severe drought stress respectively.

Grain attributes

Table 4 presented the data of number of grains per spike and 1000 grain weight. Both traits are exeedingly important and directly contribute in final yield. The main effects of natural plant extracts and drought stress were significant (p≤0.05) for number of grains per spike and 1000 grain weight while their interaction was highly significant (p≤0.01). The application of moderate drought stress at 60% FC level presented a slighter decrease (12-14%) while the severe drought stress level at 40% FC reproted a substantial decrease (28-31%) in number of grains per spike and 1000 grain weight, respectively. *Moringa oleifera* reported the maximum number of grains per spike and 1000 grain weight and it was followed by the *Dianella ensata* that was the 2nd best treatment for the mitigation of drought stress on barley grain attributes. Under severe drought stress the application of *Moringa oleifera* leaf extract achieved 28% higher number of grains per spike and 24% higher 1000 grain weight as compared to control.

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Table 4. *1000 grain weight (g) and plant weight (g) as affected by drought stress (FC) and natural plant extract (NPE) foliar application*

Plant extracts	Number of grains per spike			1000 grain weight (g)		
	FC-1	FC-2	FC-3	FC-1	FC-2	FC-3
NPE 1	24.12 e	21.21 h	17.22 j	36.45 c	31.30 f	25.16 h
NPE 2	27.65 b	24.43 e	21.32 h	38.67 a	35.22 d	30.32 g
NPE 3	26.54 c	23.67 f	20.14 i	38.25 b	34.36 e	29.90 g
NPE 4	28.33 a	25.44 d	22.05 g	38.98 a	36.30 c	31.18 f
NPE	*			*		
FC	*			*		
NPE×FC	**			**		
LSD	0.32			0.55		

*; figures differing in letters are statistically significant at p≤0.05, **; significant at p≤0.01, ns; non-significant, LSD; least significant difference. NPE 1; Water, NPE 2; Dianella ensata, NPE 3; Ambrosia dumosa, NPE 4; Moringa oleifera Lam

Flag leaf area and chlorophyll content

Flag leaf attributes are presented in *Figure 1*. Flag leaf area and chlorophyll content play an important role in active photosynthesis and carbon assimilation. Effect of natural plants extract, drought stress and their interaction was significant (p≤0.05) for both flag leaf area and chlorophyll content. The flag leaf area ranged from 25-30 cm^2 for unstressed pots to 17-22 cm^2 for severe drought stressed pots. The values of leaf chlorophyll content ranged from 7-8.9% for unstressed pots to 4.6-6.2% for severe drought stressed pots. The application of *Moringa oleifera* reported an increase of 28-35% in leaf area and chlorophyll content at severe drought stress as compared to control, respectively.

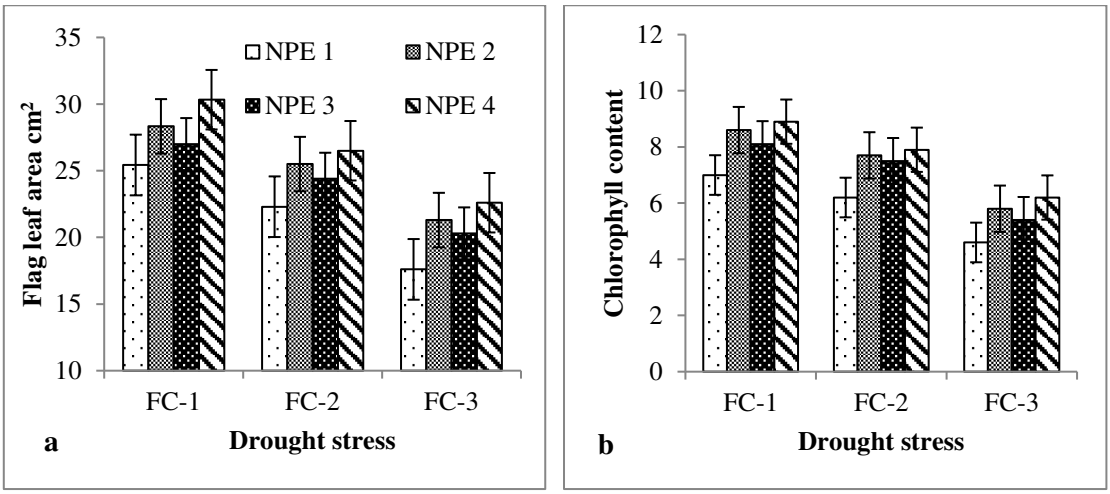

Figure 1. *Effect of drought stress and natural plant extract (NPE) on flag leaf area and chlorophyll content measured two weeks after the application of treatments. NPE 1; Water, NPE 2; Dianella ensata, NPE 3; Ambrosia dumosa, NPE 4; Moringa oleifera Lam. FC is field capacity that represent different levels of drought stress*

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Root dynamics

Root characteristics such as root length, root fresh and dry weight are measured at plant harvest and data is presented in *Figure 2*. The effect of natural plants extract, drought stress and their interaction was significant ($p \leq 0.05$) for root characteristics. Drought stessed displayed a negative effect on root fresh and dry weight while it improved the root length. The effect of natural plant extract was also positive for root length and fresh and dry biomass accumulation. The 40% FC ensured the maximum root length with an increase of 45% as compared to 100% FC. At sever drought, the application of natural plants extract reported an increase of 71-62% in root fresh and dry weight as compared to control. Moringa oleifera and Dianella ensata contributed significantly for the mitigation of drought stress and improvement in root characteristics.

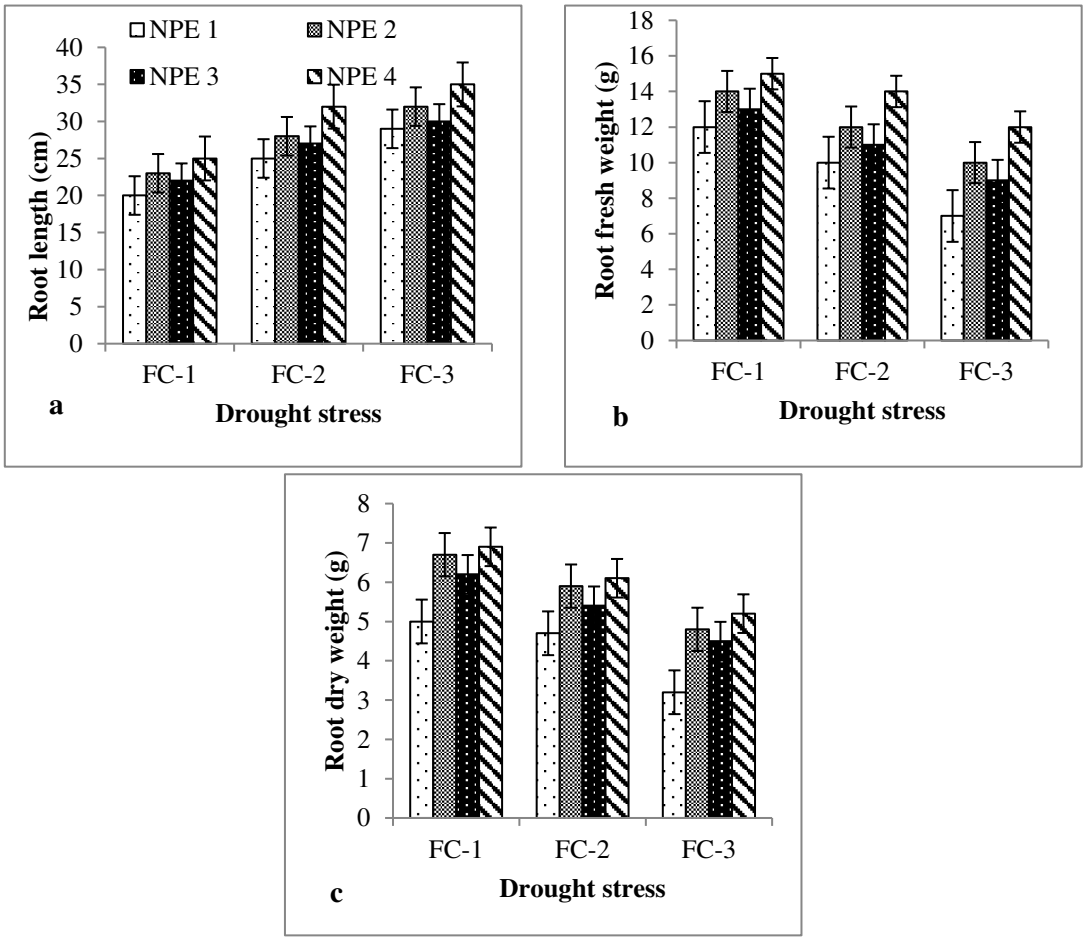

Figure 2. *Effect of drought stress and natural plant extract (NPE) on root length, root fresh and dry weight accumulation. NPE 1; Water, NPE 2; Dianella ensata, NPE 3; Ambrosia dumosa, NPE 4; Moringa oleifera Lam. FC is field capacity that represent different levels of drought stress*

Discussion

Drought is the most intimidating abiotic stress that affects growth and yield of field crops. Drought causes many damaging effects on crop morphological and physiological

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characteristics. It causes alteration in leaf morphology and gives rise to morpho-physiological changes that leads to impaired plant growth and grain development thus reducing plant yield (Alghabari and Ihsan, 2018). Because of drought stress the crop plants face disturbance in their osmotic balance, reduction in stomatal conductance, reduction in photosynthetic activities, lowering rate of membrane electron transport and also rate of evapotranspiration and loss of water from plants increases, and all these processes ultimately lead to reduction of growth and development thus reducing the final crop yield (Srivalli et al., 2003; Allahmoradi et al., 2011; Alghabari et al., 2014, 2015; Hussain et al., 2018). Crop plants under arid land conditions are subjected to water deficit stress especially at reproductive stage.

Barely growth and yield components were significantly affected by the extracts of the three natural plants (*Dianella ensata, Ambrosia dumosa, Moringa oleifera* Lam). These plants may have been containing growth stimulating molecules that may be present in any part of these plants; leaves, roots, stem, flower or fruit. Their presence in the root rhizosphere was demonstrated by many researchers (Bertin et al., 2003; Stock et al., 2019). Ihsan et al. (2015) reported growth inhibitory effects of different plants extract while Alghabari (2018) documented growth stimulatory effects of desert plants extract in mungbean crop. Application of plant based growth regulators is now considered a new agricultural field after its proven positive role in abiotic stress relief, economically viable and environment friendly. The role of the wild and natural plant extracts in reducing effects of drought impact on barley plant growth and yield may be due to their positive role in regulation of moisture supplies to crop plants, and in making nutrients more available to crop plants and enhancing the rate of photosynthesis.

Dianella ensifolia is an evergreen, perennial herb with grass-like leaves growing from a branched, gradually spreading rhizome. It can grow up to 1.5 metres tall, spreading at the roots to form quite large clumps. The plant is sometimes gathered from the wild for use as a medicine, pesticide and dye. *Ambrosia dumosa* is a highly branched shrub 20 to 90 cm in height. It is present in desert areas of the world. It becomes dormant during drought stress, losing all of its leaves to prevent water loss by transpiration. *Moringa oleifera* is a fast-growing, drought-resistant tree native to the Indian subcontinent. It is well adapted to arid lands of Saudi Arabia. A deciduous tree that can reaches a height of 10–12 m.

Many researchers have reported the growth stimulatory effects of different plants in field crops. Cowpea growth, yield and protein content were stimulated by the aqueous extract of *Malva parviflora* and *Artemisia ludia* (Lashin et al., 2013). Application of moringa leaf extract at two weeks interval after emergence has imporved maize plant height, fresh and dry weight, number of grains per cob and 100 grain weight (Biswas et al., 2016). Application of different plants extract controlled wheat leaf rust caused by *Puccinia triticina* and improved grain yield components such as 1000 grain weight (Shabana et al., 2017). The improvement in crops growth and yield by the foliar application of moringa plant aqueous extract suggested that it activated the antioxidants mechanism in plants to enable them to alleviate the oxidative damage caused by drought stress. It may have improved the physiological and molecular attributes in stressed plants that helped them to combat the adverse conditions of environmental stresses. *Moringa oleifera* is rich in amino acids, ascorbate, minerals and zeatin compounds known for their growth promoting potential (Latif and Mohamed, 2016). Chemical analysis of moringa leaf extract showed that GA4 is most likely in crosstalk with auxin is the major growth enhancer (Brockman et al., 2020).

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The ability of plants to resist drought stress and to improve their growth under drought through application of plant growth regulators is because of these extracts act as osmoregulators regulating osmosis in plant species, working as signaling agents, and hence reduces reactive oxygen species production and photorespiration. Overall, improvement in photosynthesis and in nutrient uptake enabled plants to resist and tolerate drought stress. The improvement of barley plants growth and yield under drought stress by application of *Dianella ensata* and *Moringa oleifera* Lam water extracts is due to the stimulatorry role of these extracts through increasing leaf osmotic process, plant water uptake, photo-assimilation and improvement of the plant antioxidant defense system. In future, studies are needed to identify wild plant species that have growth stimulatory effect under drought stress which can be used in field crops to tolerate and resist drought stresses. Reseach is also needed to identify and isolate the compounds that play an active role in drought stress mitigation.

Conclusion

Drought stress significantly affected barley growth and yield. The adversity of the drought stress increased with the decrease in field capacity. Application of wild plants (*Dianella ensata, Ambrosia dumosa, Moringa oleifera* Lam) aqueous extracts improved growth and yield of barley crop. The foliar applied extracts alleviated the negative effects of drought stress through improving crop growth and grain contributing traits that enabled the barley to effeciently absorb water and nutrients. The *Moringa oleifera* was the most promising wild plant extracts. It is recommended to screen and quantify the biomolecultes that have growth promoting effects through chromatographical processes and be produced as commercial commodities for utilization in plant drought tolerance and growth promotion under stressful environment.

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TOPOGRAPHIC INFLUENCES ON THE POPULATION PERSISTENCE OF A TERTIARY RELICT DECIDUOUS TREE *EMMENOPTERYS HENRYI* OLIV. ON MT. TIANMU, EASTERN CHINA

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Abstract. Mt. Tianmu is one of the most important refugia of Tertiary relict plant taxa in Eastern China. We analyzed the habitat characteristics, community structure, demographic structure, and production methods of Tertiary relict deciduous forests at 1,100 m on Mt Tianmu (1,506 m), Zhejiang province, China. *Emmenopterys henryi* mostly occurred in unstable habitats with gap-formation or landslides, and these populations showed a sporadic regeneration pattern. *E. henryi* colonized and established at unstable sites by abundant wind-dispersed seeds. After colonization, *E. henryi* persisted for a long time and dominated in the canopy layer and even reached emergent layer due to its long lifespan and vegetative reproduction capability. It could thus be regarded as an undifferentiated climax pioneering species with an 'r-selected' life history that produced abundant minute seeds and experienced intermittent recruitment and often became pioneer species of secondary succession after moderate disturbance. Here, we discussed conservation strategies for Tertiary relict deciduous trees as climax pioneering species that accounted for the peculiarity of their habitat and population structure in broad-leaved forests.
Keywords: *habitat instability, root sucker, population structure, reproduction methods, regeneration strategy*

Introduction

Tertiary relict floras contain survivors from plant communities that were distributed throughout a large part of the Northern Hemisphere during much of the Tertiary (i.e. 65-15 million years ago (Ma)) (Milne and Abbott, 2002). They are now mainly restricted to warm humid areas (refugia) in southeastern and western North America, East Asia and southwest Eurasia (Milne and Abbott, 2002; Hampe and Arroyo, 2002). A number of deciduous broad-leaved ancient genera, considered to be Tertiary relicts, are found today in subtropical broad-leaved forests of China, such as *Davidia involucrate*, *Tetracentron sinense*, *Cercidiphyllum japonicum* var. *sinense*, *Euptelea pleiospermum*, *Ginkgo biloba, Cyclocrya paliurus*, *Emmenopterys henryi*, *Tapiscia sinensis*, *Fortunearia sinensis* etc. (Del Tredici, 1992; López-pujol et al., 2006; Gong et al., 2008; Wei et al., 2010; Wu et al., 2018). The current populations of these trees are very small and they usually coexist with other evergreen, deciduous and coniferous trees at relatively restricted ranges (Gong et al., 2008; Wu et al., 2018). During the

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Quaternary glaciations, these ancient species experience severely reduced regeneration and survived while most other members of their group were wiped out (Tzedakis et al., 2002; Shen et al., 2002; Tang et al., 2011; Qian et al., 2016). A majority of these species are globally threatened now (López-Pujol et al., 2006). How these relict species survive and persist under current climate conditions or those of rapid climate change and frequent human activities became an important topic for rare and endangered species conservation (Calleja et al., 2009).

Emmenopterys henryi Oliv, belonging to the Rubiaceae family, is an endangered deciduous tree endemic to China (Zhang, 2016). It is characterized by a long lifespan, intermittent flowering with 2-4 year intervals, production of abundant wind-dispersed minute seeds (0.3-0.6 g per 1000 grains) and reduce competitive ability with other deciduous pioneer tree species and evergreen tree species during the juvenile stage (Wang et al., 2002). It is of ancient origin, a relic of the paleotropical flora of the Cretaceous Period, Mesozoic Era, and is considered valuable both for its unique position in the flora of China and in the systematic evolution of the Rubiaceae. *E. henryi* primarily occurs in ravines and mountain valleys at altitudes of 400-1400 m in southwestern China and the Yangtze River Valley. Populations of *E. henryi* have been affected by habitat destruction and over-exploitation in the wild. It has been listed as a threatened species within China due to its lower regeneration. Previous researches mainly focused on chemical components, seed physiology, community structure and genetic structure (Ma and He, 1989; Zhang et al., 2007; Li and Jin, 2008; Guo et al., 2017a,b; Ma et al., 2019). Kang et al. (2007), Guo et al. (2017a) and Ma et al. (2019) also reported that *E. henryi* regenerates through both sexual and asexual modes, but scientific analysis of its regeneration mechanism along the topographic gradients, has not been described. Further studies may be necessary to evaluate potential adaptation of populations to local environmental conditions.

Therefore, this paper focuses on: (1) community structure, distribution pattern and demographic structure of main trees, and (2) the production methods of *E. henryi* population along the topographic gradients, to reveal that how they persisted as related to habitat. The inhabiting habitats, production methods and regeneration strategy of other similar relict trees have been compared to determine whether these relict trees have similar peculiarity for persistence at relatively restricted ranges.

Materials and Methods

Study site

The study was conducted at the Mt. Tianmu Nature Reserve (30°18’30’’ ~ 30°21’37’’N, 119°24’11’’ ~ 119°27’11’’E, Zhejiang Province, P. R. China), which is one of the most famous protected areas in China and throughout the world due to its remarkable number of large, rare and endangered plants. The foothill region is located at 300-350 m a.s.l., which gradually rises to 1,056 m a.s.l. Mt. Tianmu is characterized by a subtropical humid climate (Qian et al., 2002). According to records from weather stations at Chanyuan Temple (350 m a.s.l.) near the base and Xianrending (1,506 m a.s.l.) near the summit of Mt. Tianmu from 1987 to 1996, the average annual temperature is 14.5 ^{o}C and 9.0 ^{o}C, and the average annual precipitation is 1,739 mm and 1,751 mm for Chanyuan Temple and Xianrending, respectively (Da et al., 2009). Because the stratum was affected by tectonic movements and volcanic activity, the study area is made up of steep slopes and irregular terrain, especially many complex

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landscape structures between 900 m and 1,100 m a.s.l. 90% of this area is covered with volcanic rock, and the zonal soils are comprised of red soils (below 600 m a.s.l.), yellow soils (600-1,200 m a.s.l.) and brown yellow soils (above 1,200 m a.s.l.) (Xia, 2004).

Data collection

The population size and distribution region of *E. henryi* on Mt. Tianmu is small by our survey on July to August 2010, which mainly concentrated on the mid-altitude region from 900 m a.s.l. to 1,200 m a.s.l. They were mainly distributed at roadsides, and in valleys, gravel mounds, cliffs, etc. Besides, we also founded that a few of seedlings and saplings of *E. henryi* grow on fallen log of the gap-maker. We established plots by patch sampling in twelve locations representing three micro-topographies (*Table 1*). Plot 1-4 (total 1,600 m^2) was established in an old canopy gaps surrounded by old-growth *Cryptomeria fortunei*. These plots were located on hollow head with an average 20.0° incline. Plots 5-8 (total 1,600 m^2) were established in flood terrace with an average 16.0° incline. Plot 9-12 (total 1,400 m^2) was established in river bed on a seasonally active channel with a mean 23.8° incline. A more detailed description of these three microtopographies was shown in *Table 2* by surveyed.

Table 1. *The geological properties of 12 sampling plots*

Plot	GPS position	Microtopography	Sampling area (m^2)	Altitude (m)	Slope (°)	Aspect
1	30°20′18.4″,119°25′44.8″	Hollow head	400	1062	15	S30E
2	30°20′32.3″,119°26′06.8″	Hollow head	400	1113	10	S65W
3	30°19′52.0″,119°25′45.6″	Hollow head	400	1000	30	NE45
4	30°20′25.5″,119°25′59.5″	Hollow head	400	980	25	S15E
5	30°20′29.8″,119°26′05.6″	Flood terrace	400	1097	10	N10W
6	30°20′29.8″,119°25′58.4″	Flood terrace	400	1080	14	S30W
7	30°21′33.5″,119°25′21.1″	Flood terrace	400	1050	15	NE30
8	30°20′20.8″,119°25′48.2″	Flood terrace	400	1020	25	S79E
9	30°20′27.5″,119°25′59.0″	River bed	400	1064	20	S15E
10	30°21′33.5″,119°25′33.7″	River bed	400	1050	23	NE50
11	30°19′54.3″,119°25′51.4″	River bed	400	856	30	NE45
12	30°20′18.4″,119°25′47.8″	River bed	200	975	22	S74E

Table 2. *The characteristics of three different microtopographies*

Habitat	Hollow head	Flood terrace	River bed
Situation	Upper gentle slope	Lower gentle Slope	Middle gentle slope
Litter cover	High	Low-high	Low
Soil depth	Deep	Medium	Shallow
Soil humidity	Low	High	Low
Disturbance type	Soil erosion	Soil erosion	Soil erosion
Physical stability	Relatively stable	Moderately unstable	Unstable

Because the canopy layer forms at heights above 8 m in these plots, we expressed the strata of the forest as follows: tree layer, 8 m < height (H); subtree layer, 4 m ≤ H ≤ 8 m; shrub layer, 1.5 m ≤ H < 4 m; sapling, 0.5 m ≤ H < 1.5 m; seedlings, H < 0.5 m. In each whole plots, we recorded the species, measured the diameter at breast height (1.5 m above ground; DBH) and the height of trees ≥ 1.5 m. The quantities and heights of seedlings and saplings of *E. henryi* were also identified and measured.

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The plantlets of *E. henryi* were classified into two types, true seedlings from seed-origin and root suckers from adventitious buds on lateral roots, identified by removing the litter and surface soil of lateral roots. Besides, individuals with own root systems in shrub or tree layer were also considered to be seed-orientated. Root suckers remained connected to parent tree for several years and could increase competitively and greater survival under adverse environmental conditions (Ky-Dembele et al., 2007; Beaudet and Messier, 2008).

Data analysis

The dominant species were identified by Ohsawa's dominance analysis method using the relative basal area (RBA) of each species. This analysis is based on the least deviation (*d*) between the share obtained by a given species, as a percentage of the total basal area (x_i), and its calculated share if all species were equally represented (x'):

$$d = 1/N\left\{\sum_{i\in T}(x_i - x')^2 + \sum_{j\in U} x_j{}^2\right\} \quad \text{(Eq.1)}$$

where T is the number of 'top species' in a given dominant-number-model, U is the number of remaining species, and N is the total number of species. For example, in a community dominated by single species, x'=100/T (where T=1), the top dominant's share is 100%. If, however, two species share dominance, the top two dominants share 50%, or if there are three co-dominants, 33.3%, and so on. Species diversity was expressed by Shannon-Wiener Index.

For determining whether these Tertiary relict trees have similar regeneration mechanisms, more or less complete information on the inhabiting habitat, seed mass per 1000 grains, dispersal agent and production methods was available from literatures (Shang et al., 2016). The types of vegetative reproduction were clarified into five types. Seedling sprout means a plantlet of seed origin that was affected by shoot dieback, but re-sprouted from the root collar of the seedling; root sucker means a plantlet arising vertically from superficial lateral root; coppice means a plantlet arising from stumps of cut mature tree in response to logging or non-logging disturbances and which root diameter exceeds 10 cm; water sprout means a plantlet developed from the base of alive mature tree; layer means a plantlet developed from low hanging lateral branch (Ky-Dembele et al., 2007).

Results

Floristic characteristics

Within a total of nearly 4,600 m^2 plots in all habitats, 69 woody species belonging to 54 genera and 37 families were recorded (*Appendix A*). The tree life-forms were deciduous broad-leaved, evergreen broad-leaved, deciduous coniferous and evergreen coniferous, but only 9 species were evergreen. Of the deciduous species, 3 genera (*Pseudolarix, Emmenopterys* and *Cyclocarya*) were endemic to China, 7 genera (*Kalopanax, Acanthopanax*, *Ehretia, Corylopsis, Deutzia, Dendrobenthamia* and *Stachyurus*) were endemic to East Asia. Of the evergreen trees, one genus (*Cunninghamia*) was endemic to China, 3 genera (*Cryptomeria*, *Lithocarpus* and *Orixa*) were endemic to East Asia. Northern temperate deciduous broad-leaved genera such as

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Petrocarya, *Acer* and *Viburnum*, pantropic genera and old-world temperate genera were also appeared. Besides, many relict species such as *E. henryi*, *Cyclocarya paliurus*, *Magnolia cylindrical*, *Pseudolarix amabilis* were co-existed in the community. Hence, the ancient and complexity of community were remarkable that with so many plants of diverse geographic distributions as well as Tertiary relicts.

E. henryi could establish them on different microtopography along habitat instability and become a dominant. In the hollow head with relatively stable, was co-dominated by *P. amabilis*, *C. fortunei* and *E. henryi*; the relative dominance value of *E. henryi* was 15.2-67.6%. In the flood terrace with moderately unstable, *C. fortunei*, *E. henryi* and *P. amabilis* were co-dominant; the relative dominance value of *E. henryi* was 23.7-57.2%. In the river bed, *E. henryi* as the first dominant was co-dominated with *C. fortunei* and the relative dominance value reached to 41.9-97.0%.

Height-class distribution with increasing habitat instability

The height-class frequency distribution of all woody species is shown in *Fig. 1*. In the hollow head, the number of evergreen individuals was more than deciduous and the ratio of evergreen/deciduous was 1.16. Evergreen species such as *Cyclobalanopsis gracilis*, *Lithocarpus harlandii* and *C. fortune* were found in the shrub, subcanopy and canopy layers. Besides, deciduous trees *E. henryi* and *C. paliurus* were also found in all three layers, and the former even reaching the emergent layer (above the canopy, more than 20 m). In the flood terrace, the number of evergreen individuals was decreased, and the ratio of evergreen/deciduous reached to 0.48. They mainly appeared in shrub and subcanopy layer (below 10 m) while *E. henryi* were found in all layers. In the river bed, although *C. fortunei* could reach the emergent layer, evergreen individuals including *L. harlandii* and *C. fortunei* were rare and mainly confined to the shrub layers. The canopy, subcanopy and shrub layers of the forest were occupied by *E. henryi* and other deciduous trees such as *Alangium chinense* and *Bothrocaryum controversum*.

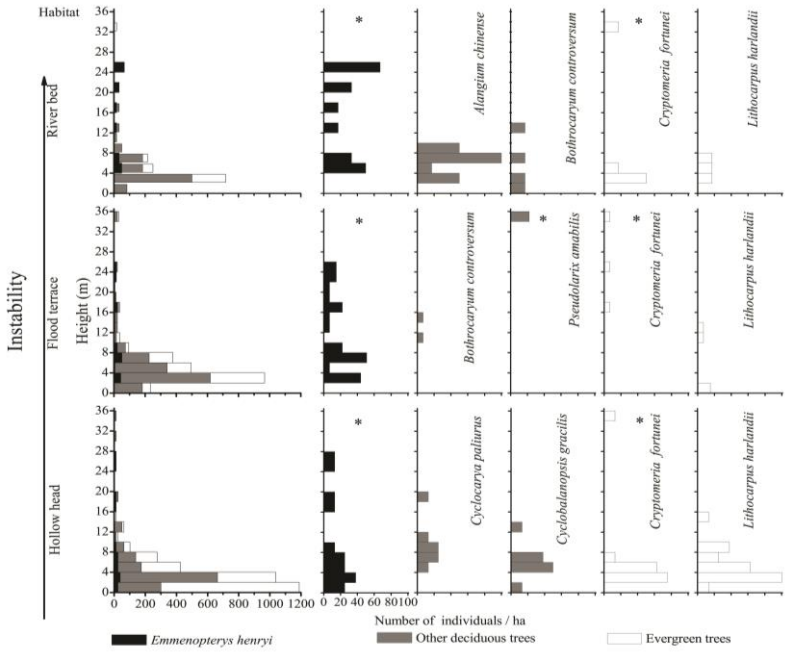

Figure 1. *Height-class frequency distribution of all individuals and main populations with increasing. Dominant species are indicted by an asterisk*

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Population structure with increasing habitat instability

These communities include unimodal, sporadic and L types of size-class frequency distribution (*Fig. 2*). The unimodal type, with a single peak in the intermediate or large size-classes, and fewer if any individuals in small size-classes, suggests a weak regeneration pattern. Evergreen trees *L. harlandii*, *C. gracilis* and *Cunninghamia lanceolata* located on valley and *C. fortunei* appeared in all three habitats were of this type. The sporadic type, with more than one peak in the size-classes, indicates the possibility of good regeneration. *E. henryi* occurred in all three microtopography were of this sporadic type, but the number of populations decreased along habitat instability. Active regeneration is suggested by the L type having the highest frequency in small DBH classes. In hollow head and flood terrace habitats, evergreen trees *L. harlandii*, *C. gracilis* and *C. lanceolata* was of this type, indicating that these evergreen canopy species were suppressed by the dominant deciduous species but their populations could develop.

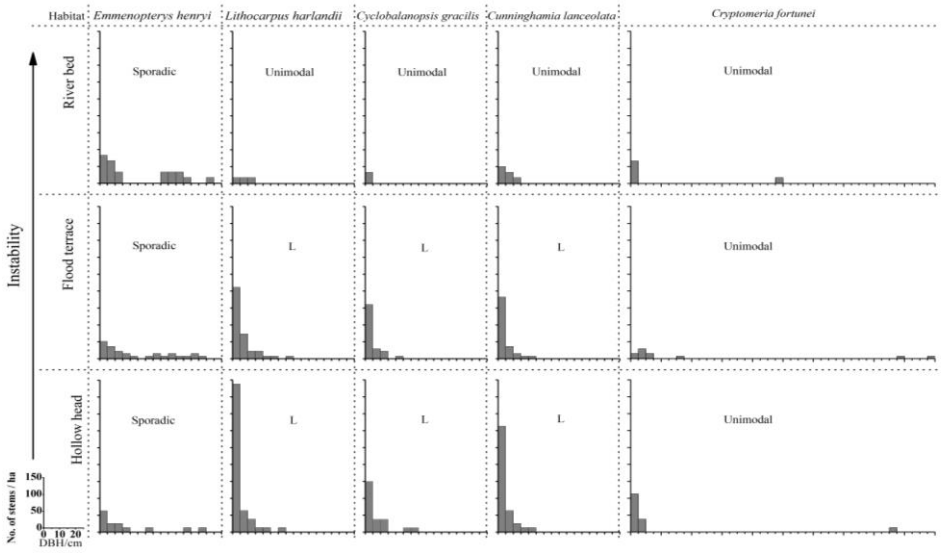

Figure 2. *Size-class frequency distribution for the E. henryi population and main evergreen tree with increasing habitat instability*

Sprouts ratio of E. henryi population at different habitat

Although the reproduction of *E. henryi* was by means of seeds at many habitats, such as gravel mound, canopy gap and fallen-log by our investigation, the resprouter by root sucker were also abundant at hollow head and river bed habitat (*Fig. 3*). In the hollow head, 23 stems (including 14 seedlings and 9 saplings) were found to regenerate successfully from root suckers and 9 individuals were true seedlings or assumable seed-orientated trees. And 2 of these individuals were located on fallen-log. In the river bed, 6 stems originated from root suckers and 6 individuals were true or assumable seed-orientated trees. The suckering stems accounted for 72% of all stems in the hollow head, while 50% in river bed.

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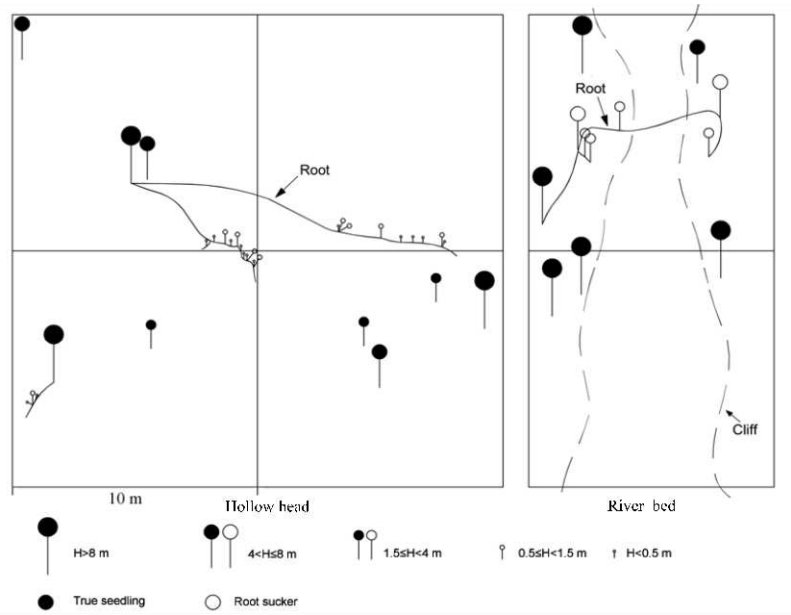

Figure 3. *Vegetative mode of E. henryi population in hollow head and river bed*

Discussion

Reproductive strategy and population persistence of E. henryi in relation to micro-topography

The distribution of *E. henryi* on Mt. Tianmu was primarily at unstable habitats, such as hollow head valleys, flood terrace, fallen logs and gravel-mounds. This was similar to other Tertiary relict species, such as *Euptelea polyandra* in warm-temperature forest of Japan (Sakai and Ohsawa, 1993), *Euptelea pleiospermum*, *Cercidiphyllum japonicum* and *Davidia involucrata* in subtropical evergreen broad-leaved forests of western China (Tang and Ohsawa, 2002; Wei et al., 2010; Wu et al., 2018), *Frangula alnus* subsp. *Baetica* and *Prunus lusitanica* in Iberian Peninsula of Spain (Hampe and Arroyo, 2002; Calleja et al., 2009; Pardo et al., 2018). All these trees seem to require very particular unstable habitats where competition from other trees is limited.

At those unstable habitats, the deciduous pioneer tree *E. henry*i could colonize firstly by mean of abundant minute seeds production (0.3-0.6 g per 1,000 grains) after moderate disturbance (Kang et al., 2007; Shang et al., 2016). Their populations experienced intermittent recruitment as shown by the sporadic type of stem-diameter class frequency distribution, while evergreen trees regenerated more weakly along habitat instability (*Fig. 2*). At hollow head and flood terrace habitat with lower unstable, *E. henryi* can dominate in the canopy layer and even reach the emergent layer due to its long life span (*Fig. 1*; *Appendix A*). When these habitats become more stable, the evergreen trees *L. harlandii*, *C. gracilis* and *C. lanceolata* may become dominants according to the regeneration pattern (*Fig. 2*). At river bed habitat with frequent landslide, *E. henryi* could recruit well due to the possibility of good regeneration while other evergreen trees were not (*Fig. 2*). Here, the *E. henryi* dominated forest in the river bed on Mt Tianmu could be regarded as a topographic climax phenomenon formed in an area of landslide disturbances.

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The results of this investigation showed that asexual reproduction resprouted by long-distance root (root sucker) was the important mechanism of seedling recruitment of *E. henryi* population (*Fig 3*, Guo et al., 2017a). The suckering stems accounted for 72% of all plantlets in hollow head compared to 50% in river bed. This is because although *E. henryi* can colonize at different habitats by the active production of seeds, the seed germination and seedling establishment are influenced by environmental filtering (Zhang et al., 2007; Guo et al., 2017b). The seed germination and seedling recruitment of *E. henryi* at current conditions are hampered by the thick layer of leaf litter in gentle slope habitat except for fallen-log, which agrees with previous findings that seedlings produced by small seeds may be unable to emerge in sites with thicker litter layers (Carlton and Bazzaz, 1998; Castro et al., 1999). After the stage of seedling-establishments, a light-demanding deciduous pioneer tree, *E. henryi* can not compete with modern deciduous pioneer tree species and evergreen tree species due to its reduced competitive ability during the juvenile stage (Zhang et al., 2007; Pulido et al., 2008). On the other hand, resprouts arising from vegetative reproduction grow faster than newly established seedlings due to their well-established root system (Ky-Dembele et al., 2007), providing better resistance to stress in their first years (Deiller et al., 2003), and a stronger competitive advantage (Beaudet and Messier, 2008). It can be considered that root suckers probably contribute to the survival and maintenance of *E. henryi* population by reducing vulnerability to severe disturbance and recruitment failure (Guo et al., 2017a).

Ohsawa (1991) have suggested that most sporadic type species who experienced intermittent recruitment can be regarded as a kind of pioneers in the climax forests and called ‘undifferentiated canopy components’ that between a nomadic pioneer and a climax species. *E. henryi* populations have experienced intermittent recruitment in hollow head and flood terrace habitat with relatively stable (*Fig. 2*). Moreover, *E. henryi* is a deciduous pioneer tree that characterized by a long life span, intermittent flowering with 2-4 year intervals, and production of abundant wind-dispersed minute seeds (0.3-0.6 g per 1,000 grains). Thus we suggested that *E. henryi* can be regarded as an undifferentiated climax pioneering species with an ‘r-selected’ life history.

Persistence mechanism of Tertiary relict deciduous trees as climax pioneering species

A large number of deciduous broad-leaved trees of ancient genera, considered to be Tertiary relicts, occurred in the mid-altitude region of subtropical mountains (*Table 2*; Shang et al., 2016). The Tertiary relict trees seem to require very particular unstable habitats that mainly distributed on valley, forest edges, steep slopes and stream banks (*Table 3*). This is probably due to the importance of differential growth, differential survival and differential dispersal of species as well as its evolutionary factor (Pullio, 2008; Qian et al., 2016).

Since the Quaternary era, the distribution, population size and regeneration capacity of many Tertiary relict plant species has changed greatly (Tzedakis, 2002; Calleja et al., 2009; Zhang et al., 2016). Owing to their reduced competitive ability with modern floras and their ecophysiology (Pulido et al., 2008), these species need to colonize on ‘safe sites’ where competition from other species is limited (Tang and Ohsawa, 2002). The environmental stochasticity in unstable habitats could provide more opportunity for regeneration of seed-orientated species and decrease interspecific competition (García, 2003). Therefore, Tertiary relict trees would recruitment intermittent by abundant minute wind-dispersal seeds (*Table 2*; Zhang et al., 2007; Li et al., 2008).

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Table 3. *Known distribution habitat, vegetation regeneration strategies, and dispersal agent of the main relict deciduous tree in China's subtropical forests*

Relict deciduous trees	Main habitats	Seed mass (g/1,000)	Dispersal agent	Vegetative mode
Annamocarya sinensis	Valley, stream bank	9000-13000	Mammals	Water sprout; Coppice
Bretschneidera sinensis	Humidity valley, stream bank	715-780	Wind	Coppice
Camptotheca acuminata	Stream bank, forest edge	34-45	Wind	Water sprout
Cercidiphyllum japonicum	Steep slope, valley, stream bank	0.75-0.9	Wind	Water sprout
Cyclocarya paliurus	Steep slope, creek valley, forest edge	200	Wind	Water sprout
Davida involucrata	Steep slope, ravine	3400-5500	Mammals	Water sprout; Coppice
Eucommia ulmoides	Valley,dry ravines	58-130	Wind	Root sucker; Coppice
Halesia macgregorii	Steep slope	125-220	Birds	Water sprout
Liquidambar formosana	Valley, stream bank	4.3-6.4	Wind	Water sprout
Liriodendron chinense	Valley	22-35	Wind	Coppice
Nyssa sinensis	Valley, stream,steep slope	125-240	Birds	Water sprout
Pteroceltis tatarinowii	Foothill, forest edge, river bed	21-28	Wind	Water sprout
Tetracentron sinensis	Valley, hillside, rocky ravine	0.1-0.15	Wind	Coppice

Note: Sources: Shang et al., 2016

On the other hand, Tertiary relict trees could sprout new shoots in subtropical area of China, including water sprouts, coppices, and root suckers (*Table 2*). It seems to allow the population to recruitment quickly after disturbance. This supplemental mechanism for population persistence is similar to that of *E. henryi* in our study, *E. polyandra* in Japan and *Rhododendron ponticum* in Mediterranean, which allocate more resources to sprouts than to reproduction by seeds (Sakai et al., 1995; Mejías et al., 2002).

These characteristics are coincident with the general tendency that the Tertiary relict deciduous trees survive and persist well in the unstable scree slopes where competition is not severe, but are unable to thrive in the stable habitats where competition is more rigorous (Tang and Ohsawa, 2002). Consequently, minute easily-dispersed seeds and seedling recruitment supplemented with vegetative reproduction seem to be most important reasons why Tertiary relict deciduous trees have been able to persist at a given site after frequent disturbance (Milne and Abbott, 2002).

Conclusion

Many of Tertiary relict deciduous trees regarded as an undifferentiated climax pioneering species with an 'r-selected' life history has very few individuals remaining. They are of high conservation concern due to their rarity and their phylogenetic uniqueness, and are therefore very important to China and the world. Responsible conservation efforts should aim to maintain present populations of these species and expand their distribution by creating new habitat. Firstly, restoration efforts should focus both on preserving habitats by protecting valley bottoms, stream habitats, and hollows, and also on preserving or restoring the natural disturbance regime. Secondly, we should prohibit salvage logging or clearing of fallen trees so that this wood can

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provide opportunities for the recruitment of seedlings. Thirdly, in order to maintain sufficient genetic variation in a small area, it is important to increase the population of true seedlings (i.e. not resprouts) through protection of seedlings and/or increasing the sowing density. Moreover, plantations could function as starting points for the natural restoration of extirpated populations in unstable habitats. These measures, together with effective legal protections against human disturbance, might help to improve the long-term persistence of Tertiary relict trees in subtropical area of China during future changes in climate.

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APPENDIX

Appendix A. *Floristic composition and RBA (%) of the woody plant in the sample plots*

Parameters	Genus distribution	Plot 1	2	3	4	5	6	7	8	9	10	11	12
Microtopographic type		Hollow head				Flood terrace				River bed			
Plot area (m^2)		400	400	400	400	400	400	400	400	400	400	400	200
Number of plants		186	113	83	123	96	104	244	118	66	280	120	82
Maximum height (m)		35	35	15.5	30	24	36	13	18	32	9	15.5	16
Maximum DBH (cm)		101	69	40.8	129	109	81	18.5	28.5	100	18	59.3	65.8
Average DBH		4.2	5	6.9	7.2	6.2	5	5.9	6.5	5.7	3.3	7.4	6.3
Average Height		5	7	10.3	7.8	8.5	6.9	5.6	6.2	8	3.4	11.8	7.8
Total basal area (m^2/100m^2plot)		4037	5154	2497	6129	5023	3465	2383	1944	4005	1387	2088	4330
Deciduous Coniferous Tree													
Pseudolarix amabilis	China	47.3*	18.1*				37.2*						
Deciduous Broadleaved Tree													
Emmenopterys henryi	China	15.5*	35.3*	67.6*	15.2*	23.7*	33.7*	40.2*	57.2*	41.9*	70.7*	86.9*	97.0*
Bothrocaryum controversum	NTem		0.1	8.2*	<0.1		0.7	5.4		0.4	14.7*	4.1	0.1
Acer henryi	NTem			0.1	<0.1		0.4			0.1	<0.1	<0.1	<0.1
Acer mono	NTem				<0.1					0.2	<0.1	0.3	0.4
Cyclocarya paliurus	China	7.8	1.9	<0.1	3.3		1.1	5.1	13.9*				
Quercus aliena var. acutiserrata	NTem		5.4				0.9						
Toxicodendron vernicifluum	EAs,NAm,dis			5.0	0.3	4.8			1.1				
Kalopanax septemlobus	EAs		2.4										<0.1
Liquidambar acalycina	EAs,NAm,dis	1.4	17.1*										
Padus obtusata	NTem		4.4	4.2									
Ilex macropoda	PanTr			0.1									
Acanthopanax evodiaefolius	EAs				<0.1								
Acer palmatum	NTem		<0.1	3.6									
Acer olivaceum	NTem					<0.1	0.1		0.7				
Acer davidii	NTem					0.2			<0.1				
Ehretia thyrsiflora	EAs					6.2		1.9	5.0				
Juglans cathayensis var. formosana	NTem						2.2						

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Parameters	Genus distribution	Plot											
		1	2	3	4	5	6	7	8	9	10	11	12
Cladrastis wilsonii	EAs,NAm,dis						<0.1		4.2				
Nyssa sinensis	EAs,NAm,dis						2.1						
Fraxinus insularis	NTem								0.1				
Celtis sinensis	PanTr						0.2						
Celtis chekiangensis	PanTr						3.0		4.7				
Alangium chinense	Old World Tr						2.2			1.7	6.5		0.7
Magnolia cylindrica	EAs,NAm,dis						2.0			1.1	<0.1	6.2	<0.1
Diospyros glaucifolia	PanTr									1.0			
Pistacia chinensis	Med to TrAs, Aus.SAm,dis							6.2		0.3			
Deciduous Broadleaved Shrub													
Lindera glauca	TrAs	0.3	0.3		0.9	0.6		0.7					0.1
Symplocos paniculata	PanTr		<0.1		0.1	<0.1	<0.1	<0.1		<0.1	0.1		
Hydrangea chinensis	EAs,NAm,dis		<0.1		0.1	0.2	<0.1	0.1		<0.1	1.9		0.1
Lonicera hemsleyana	NTem		0.1		0.1		0.2	1.1			0.1	0.1	
Lonicera modesta	NTem			<0.1	<0.1	<0.1	<0.1	<0.1					
Euonymus hamiltonianus	NTem		<0.1		0.1			<0.1	0.1				
Dendrobenthamia japonica var. chinensis	EAs	2.1	0.9					2.5	0.7				
Mallotus japonicus var. floccosus	Old World Tr				<0.1	0.1		0.4	2.4				
Lindera fruticosa	TrAs				<0.1	<0.1							
Meliosma flexuosa	TrAs,TrAm,dis		<0.1			0.1		0.1	<0.1				
Meliosma oldhamii	TrAs,TrAm,dis	0.1		9.3*	0.1	0.2		<0.1	7.8				
Clerodendrum trichotomum	PanTr					<0.1		<0.1					
Callicarpa giraldii	PanTr	<0.1		0.5	0.2	0.1	0.2	0.1	0.4				
Styrax obassia	PanTr				0.2								
Corylopsis glandulifera	EAs	<0.1	<0.1										
Viburnum dilatatum	NTem	<0.1	0.1	<0.1	<0.1								
Viburnum erosum	NTem								0.6				
Rhamnus utilis	Cos					<0.1	<0.1	<0.1	0.3				
Rhamnus globosa	Cos					<0.1	<0.1	<0.1	0.6				

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Parameters	Genus distribution	Plot 1	2	3	4	5	6	7	8	9	10	11	12
Photinia beauverdiana	EAs,NAm,dis					<0.1	0.5						
Meliosma veitchiorum	TrAs,TrAm,dis					0.4							
Styrax confusus	PanTr					<0.1							
Aralia chinensis	EAs,NAm,dis					<0.1	0.1			0.1			
Viburnum plicatum var. tomentosum	NTem					0.2	0.1	<0.1		<0.1		0.4	
Sambucus williamsii	NTem,STem,dis					<0.1	<0.1			0.3			<0.1
Phyllanthus glaucus	PanTr					<0.1	<0.1			0.1	2.0		<0.1
Lindera praecox	TrAs					<0.1	0.1	3.0					0.1
Deutzia glauca	EAs					<0.1	<0.1	0.1			<0.1		
Picrasma quassioides	TrAs & Tr Am dis									<0.1			
Stewartia gemmata	EAs,NAm,dis			0.2						1.0	2.7	0.8	
Stachyurus chinensis	EAs									0.1	1.2	0.1	
Evergreen Coniferous Tree													
Cryptomeria fortunei	EAs	16.7*	0.3		61.3*	53.4*	0.3			49.1*			
Cunninghamia lanceolata	China	0.1	0.6		11.6*	3.8	<0.1	17.0*		1.4			
Evergreen Broad-leaved Tree													
Lithocarpus harlandii	EAs	1.0	2.7		5.5	5.4	5.5	15.8*		0.8	<0.1	0.9	0.7
Cyclobalanopsis gracilis	NTem	<0.1					1.1						
Litsea coreana var. sinensis	TrAs,TrAm,dis			1.2	<0.1	<0.1			0.1				
Cyclobalanopsis myrsinifolia	NTem	0.1	8.4*	<0.1	0.6	<0.1	0.1		<0.1				
Evergreen Broad-leaved Shrub													
Daphniphyllum macropodum	TrAs to Tr Af	7.6	1.8		0.2	0.4	5.9			0.4			0.5
Elaeagnus pungens	NTem		<0.1		<0.1		<0.1	0.4					
Orixa japonica	EAs				0.1		<0.1						
Eurya hebeclados	TrAs,TrAm,dis								0.1				
Pittosporum illicioides	Old World Tr												0.2

Note: RBA-relative percent of basal area. Dominant species of each stands are indicated by an asterisk. E-East, N-North, S-South, As-Asia, Tem-Temperate, Cos-Cosmpolitan, Aus-Australasia, Tr-Tropic, Am-America, Med-Mediterranea, dis-disjuncted (Wu, 1991). Dominant species are indicted by an asterisk

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EFFECT OF VARIOUS DOSES OF POTASSIUM ON RESPONSES OF SOYBEAN (*Glycine max* L.) GENOTYPES INFESTED WITH WHITEFLY (*Bemisia tabaci* Genn.)

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Abstract. Soybean may undergo growth disorder due to destructive plant organisms. One of these organisms threatening the productivity of soybean is *Bemisia tabaci* Genn. This research aims to study the response of soybean lines suffering from *B. tabaci* fertilized with four different doses of potassium (K), K0= 0g/kg soil, K1= 0.02789 g/kg soil, K2= 0.055g/ kg soil, K3= 0.083 g/ kg soil. This research used a randomized complete block design with three replications. The genotype difference influenced pod width, pod thickness, number of unfilled pods, number of reproductive nodes, days to flowering, and days to maturity. The variation of dose influenced plant height, pod length, number of unfilled pods, the weight of 50 seeds, and days to maturity. Interaction (genotype x fertilizer) affected many aspects such as plant height, pod length, pod width, pod thickness, number of filled pods, total pods per plant, number of reproductive nodes, seed length, seed weight, and seed thickness, the weight of 50 seeds, days to flowering, and days to maturity. The research result of each genotype showed a different response to every dose.
Keywords: *agronomy, Bemisia tabaci, fertilizer, maturity, honeydew*

Introduction

Many kinds of plant destruction organisms disturb the growth of the soybean plant. Such organisms can be in the form of pests or weeds. *Bemisia tabaci* Genn. is one of the pests of soybean cultivation (Castillo et al., 2011; DEFRA, 2015). Plant destruction organisms attack some parts of the plant, such as leaf, stem, or root. *B. tabaci* is an insect sucking the liquid of leaves and spreads the*Cowpea Mild Mottle Virus* (CpMMV) (Brito et al., 2012; Putnam, 2016). This particular virus can damage the plant structure. Soybean plant infected by CpMMV undergoes leaves spotting, chlorosis, and malformation (Tavasoli et al., 2009; Salaudeen and Aguguom, 2014). The damage to plant structure influences the physiology and metabolism of the plant. The damage to the soybean leaf structure can change the leaf function and the quality, as well as the productivity of soybean Brito et al. (2012).

The efforts to minimize the CpMMV attack carried by *B. tabaci*Genn. have frequently been conducted. One of the efforts is repairing the plant variety. Nowadays, many soybean lines are resistant to CpMMV (Zubaidah et al., 2010). This effort should be continued by fertilizing so that the quality of the soybean line increases. The dose of fertilizer should be adequately applied to streamline the use of fertilizer and maximize productivity. One of the elements needed by the soybean plant in the significant amount

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is potassium (K) since its role is essential for plant growth (Hopkins, 2004; Sczerba et al., 2009).

Potassium (K) is a macromolecule contained in the soil. The high need for the plant to this element can be fulfilled by applying fertilizer containing K until this particular element can be used well by the plant. The use of K influences much to the plant. The role of K is, for example, increasing enzyme activation, reducing the loss of transpiration water through the set of stomata, increasing the production of ATP, helping assimilate translocation, increasing N absorption and protein synthesis, assisting in balancing carbohydrate and protein, increasing the efficiency of photosynthesis process (Marschner, 1995; Pettigrew, 2008), increasing the plant length and plant production (Hamouda et al., 2015), repairing the destructed tissue (Zain and Ismael, 2016), increasing the efficiency of the use of water (McKenzie and Pauly, 2013). K also has a role in strengthening the cell wall, and it is involved in the process of sclerenchyma tissue lignification so that it can enhance the plant resistance to a particular disease (Baiea et al., 2015).

The plant uses K in physiology and metabolism processes. The appropriateness of the use of K dose based on the plant need can streamline the plant's physiology process that will have a positive impact on plant productivity. K determines the resistance, quality, and productivity of the plant by noticing the dose based on the need (Magen, 2008; McKenzie and Pauly, 2013). Plant productivity is in the form of agronomical traits produced. Some agronomical characteristics can be observed such as plant height (Farhad et al., 2010), number of branches, number of total pods per plant, the weight of 50 seeds, number of filled pods, number of unfilled pods, total seeds per plant, the weight of seed per plant, days to flowering, and days to maturity (Magen, 2008; Hamouda et al., 2015), number of reproductive nodes and number of leaves (Baiea et al., 2015). This research was done by using the variation of K dose for soybean genotype attacked by *B. tabaci* so that the response to the plant's agronomica traits can be known.

Methodology

Place and Time of Study

The research was conducted in Kendalpayak Research Station, Indonesian Legume, and Tuber Crops Research Institute, Malang, Indonesia, in November 2016 until January 2017. The preparation was done by filling the soil into a polybag after the soil was dried, sieved, and mixed with manure. The soil used was Entisol soil type. Four seeds of soybean were planted in every polybag and treated based on the fertilizer dose that had been determined. The plant was grown in an open-air condition. The watering and weed cleaning was done once a week until the soybean plants were harvested. The soybean plant is ready to harvest if the pods become yellow or brownish.

Research Design

This research used a randomized complete block design with three replications to analyze the response of the soybean genotype attacked by *B. tabaci.* Soybean genotypes used were UM.4-1, UM.7-2, UM.2-4, UM.7-6, UM.6-2 genotypes, and two check varieties of Gumitir and Wilis. These lines were used as the first factor. The treatments of K dose were K0= 0 g/kg, K1= 0.0278 g/kg, K2= 0.0550 g/kg, and K3= 0.0830 g/kg was used as the second factor. The K was from KCl fertilizer. The basal fertilizing was

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also conducted by using 0.0363 g of nitrogen (N)/kg and 0.0917 g of phosphate (P)/kg in order for the plant can grow optimally. The chemical properties of the soil before the treatments applied are presented in *Table 1*.

Table 1. *Soil chemical properties before treatment were applied*

Soil chemical properties	Value
N (%)	0.08
P_2O_5 (ppm)	188.00
K (Cmol/kg)	0.26
pH	6.70

Data Analysis

The observation was done to the agronomical traits of soybean plant including plant height, number of branches, pod length, pod width, pod thickness, number of filled pods, number of unfilled pods, number total pods, number of reproductive nodes per plant, seed length, seed width, seed thickness, seed weight per plant, the weight of 50 seeds, days to flowering and days to maturity. The digital screw micrometer was used to measure pod length, pod width, and pod thickness, as well as seed length, seed width, and seed thickness after soybean was harvested. The data were analyzed with ANOVA by using SPSS 7.0 program; if the result showed a significant relationship, the LSD test was then conducted.

Results and Discussion

Variations of K fertilizer doses have a different effect on the resistance to the honeydew. The honeydew attack was analyzed, and the results are presented in *Figure 1*. *Figure 1* shows that the K0 dose resulted in the lowest percentage value of the resistance compared to the other doses. This case means that by applying this dose, the soybean became the plant that was most resistant to the honeydew. Honeydew is a secondary metabolite produced by *B. tabaci*. The resistance of the soybean plant by K0 dose was not significantly different from the K2 dose condition. The other doses, such as K1 and K3 doses, resulted in the highest percentage value compared to the other doses; it means that the resistance level was lack (almost close to the slightly resistant category). However, the percentage value resulted was still categorized as resistant. This case shows that the K dose getting higher does not always affect the soybean plant resistance since it is influenced by the other factors as well.

The different response of soybean lines attacked by *B. tabaci* was indicated by the interaction between the soybean lines and variations of K dose affecting significantly to such 12 variables as the plant height, pod length, pod width, pod thickness, number of filled pods, total pods per plant, number of reproductive nodes, seed width, seed thickness, the weight of 50 seeds, days to flowering and days to maturity ($p>0.05$).

Agronomical traits of the plant determine the quality and productivity of the plant. The following sixteen aspects were observed to determine the response of the plant to the fertilizer. The different influence was shown in the use of genotype, fertilizer, and interaction of both of them to the agronomical traits observed. The analysis of variance is presented in *Table 2*.

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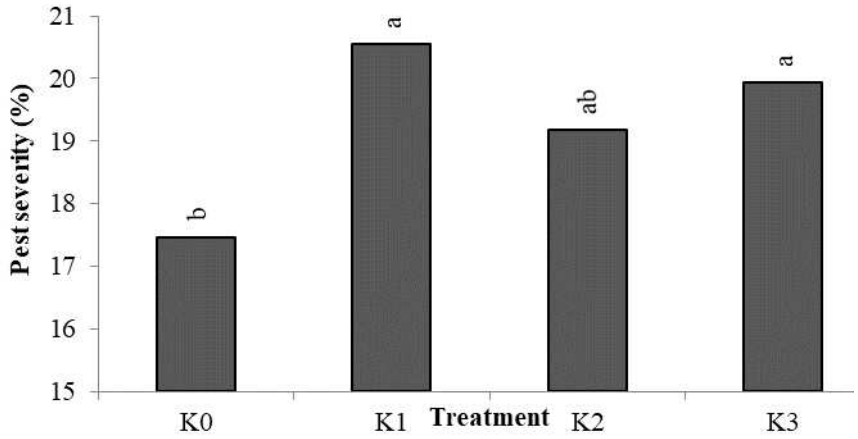

Figure 1. *The effect of variations in potassium (K) dose to the percentage of honeydew's resistance*

Table 2. *Analysis of variance of the agronomy of soybean genotype attacked by B. tabaci*

	MS genotype	MS fertilizer	MS interaction	MS error
Days to flowering	3.83**	0.32	2.74**	0.50
Days to maturity	166.86**	218.42**	149.29**	22.59
Plant height	54.94	309.04**	177.33**	91.25
Number of branches of the main stem	1.34	1.71	0.87	1.07
Pod length	0.08	0.57**	0.94**	0.17
Pod width	1.75**	1.97**	2.10**	0.63
Pod thickness	1.35**	1.13	2.08**	0.61
Number of filled pods	180.15	18.34	239.83**	120.19
Number of unfilled pods	15.48**	16.42**	4.29	5.27
Total pods per plant	254.29	12.45	206.14**	124.43
Number of reproductive nodes	27.47**	1.88	14.67**	7.76
Seed length	809.42	757.21	860.29	945.17
Seed width	0.59	0.21	0.58**	0.33
Seed thickness	0.44	0.07	0.48**	0.29
Weight of 50 seeds	0.36	0.96**	0.88**	0.17
Seed weight per plant	34.45	66.56	43.78	40.94

UM.2-4 with K3 dose showed the longest days to flowering compared to the other doses. However, the K3 dose that with UM.7-2 and 7-6 lines showed the shortest days to flowering compared to the other doses to these lines. UM.4-1 and 6-2 lines flowered quickly after giving K1 dose. A variety of Gumitir flowered quickly by giving K0 dose. Meanwhile, a variety of Wilis flowered quickly after giving K2 dose (*Figure 2*). The speed of days to flowering of a plant is influenced by the plant's metabolism and biochemistry processes. K of a plant increases the plant growth parameter; one of them is flowering initiation (Awon et al., 2012).K can influence the optimum metabolism of the plant so that it can initiate the flowering (Hawkesford et al., 2011). The accuracy of the dose used is the critical factor that should be taken into account. Too much or too little dose can decelerate the flowering process of the plant (Manoj Kumar et al., 2013).

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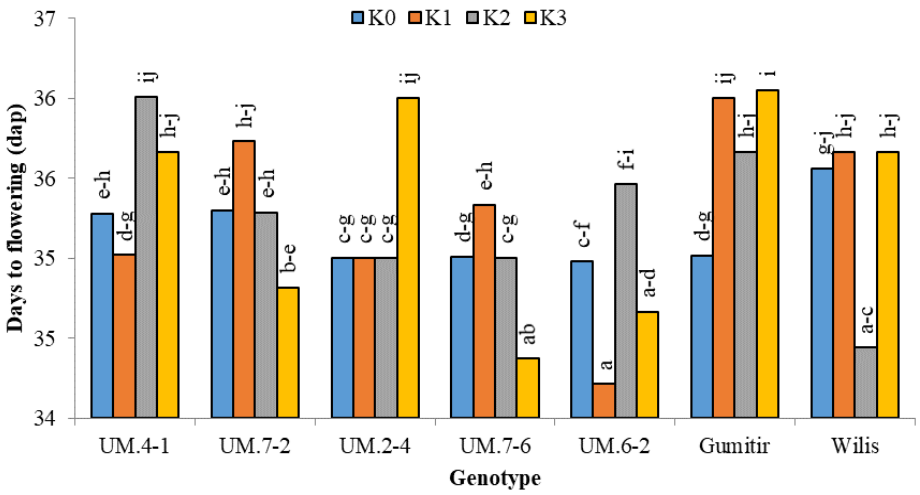

Figure 2. *Interaction of genotype × fertilizer to the days to flowering. Note: dap=day after planting*

The UM.7-2 line with K3 dose produced soybean with the quickest days to maturity compared to the other interactions. Days to maturity in the other interactions (besides UM.7-2 line with K3 dose) showed an insignificant difference (*Figure 3*). The plant growth process determines the speed of days to maturity of the plant. K determines the optimal metabolism performance to initiate the maturity/crop ripening (Hawkeford et al., 2011). If the plant can grow and develop well, absorb and utilize the nutrition optimally; it can do the metabolism process optimally, and the plant can grow and produce its product optimally. Days to maturity is determined by the character shown by the plant (Manoj Kumar et al., 2013). In soybean plants, if the pods become tawny, it means that the plant is ready to harvest.

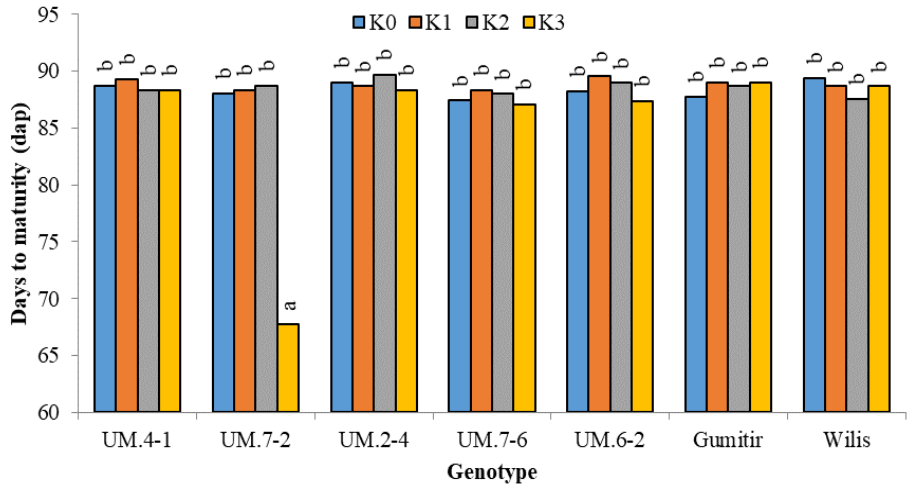

Figure 3. *Interaction of genotype × fertilizer to the days to maturity*

The result of the analysis of plant height showed that the UM.6-2 line with K0 dose produced the highest plant, but when it was K3 dose, it produced the shortest plant. On the contrary, the UM.4-1 line produced the highest plant when applied with K3 dose

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and produced the shortest plant when at K0 dose. Variety of Gumitir showed that the higher the K dose, the plant growth was getting higher as well. The highest plants were in UM.7-2 and 7-6 lines after they were applied K0 dose. Meanwhile, in the UM.2-4 line and the variety of Wilis, the highest plant was produced at the K3 dose (*Figure 4*). K dose and genotype influenced the soybean plant height. The optimum plant height can be reached by giving fertilizer based on the need for soybean genotype. The accuracy in determining the fertilizer dose supports the growth of maximum plant height (Farhat et al., 2010; Yaqoub et al., 2015). A plant needs fertilizer as the nutrition supply; besides, it gets the nutrition from the soil. One of the elements contained in the fertilizer in the treatment is K. K influences the growth and process as well as the metabolism of the plant (Marschner, 1995), including the increase of fission and cell lengthening (Pal et al., 2016). The plant that is growing with an optimum height is one of the direct effects of giving K (Manal et al., 2016). This case was caused by the fulfillment of nutrition needed by the plant by absorbing it from the soil (Xiang et al., 2012). The research results showed that K0 and K3 doses have contributed to influencing the soybean plant height in any genotypes and varieties used. Thereby, we can know that both doses were fitting to the need of some soybean genotypes in initiating the growth of plant height.

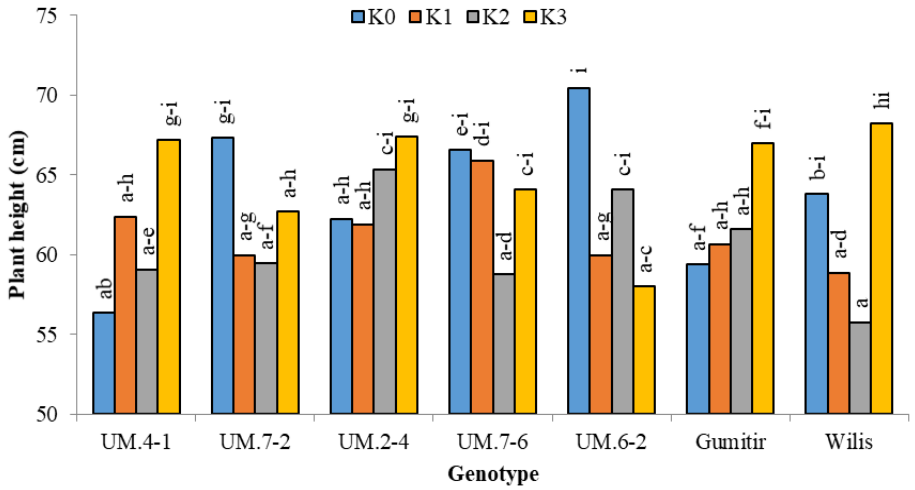

Figure 4. *Interaction of genotype × fertilizer to plant height*

The UM.2-4 line that was applied K1 dose produced soybean with the highest number of reproductive nodes compared to several reproductive nodes resulted from giving the other doses. Giving K1 dose to the UM.7-2 line also resulted in the highest number of reproductive nodes. However, Gumitir with K1 dose resulted in the smallest number of reproductive nodes instead since the highest number of reproductive nodes of this variety was produced in UM.4-1. Another dose like K0 on the UM.6-2 line and the variety of Wilis produced the most significant number of reproductive nodes. Meanwhile, if the K0 dose with UM.7-2, 2-4, and 7-6 lines, they produced the smallest number of reproductive nodes (*Figure 5*). A number of reproductive nodes of a particular plant are closely related to the plant height. Plant height has more areas that can initiate the growth of reproductive nodes rather than the short plant. Thereby, it can be said that higher the plant, the number of reproductive nodes produced will be higher

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as well (Sutrisno and Kuswantoro, 2016).In producing an optimum and high plant, a plant needs sufficient nutrition to facilitate the metabolism process and the growth of the plant. The same thing should be done in forming the reproductive nodes of the plant as well. Giving the right dose of K is related to the nutrition absorption process needed by the plant (Beg and Ahmad, 2012) so that it can support the optimum growth of the reproductive nodes. The fulfillment of K and nutrition required to do the metabolism process can result in the character related to plant productivity.

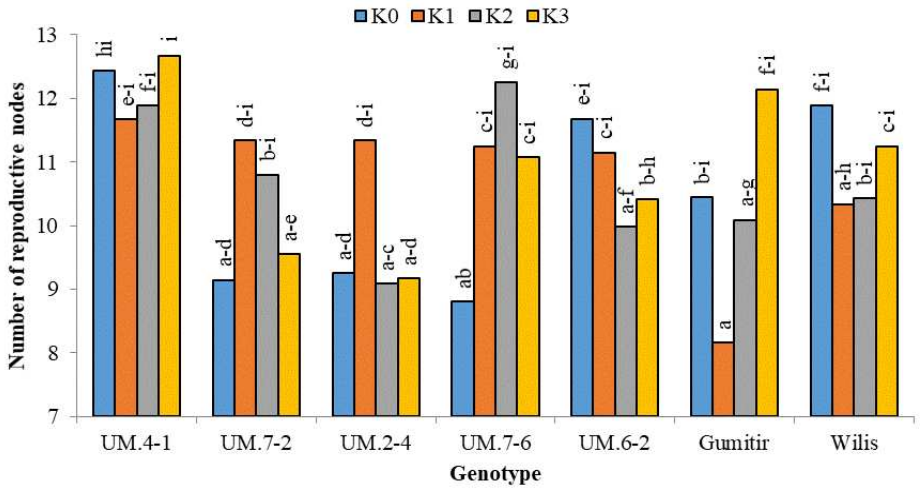

Figure 5. *Interaction of genotype × fertilizer to a number of reproductive nodes*

Figure 6 shows that the UM.2-4 line with K1 dose produced the highest total pods compared to the other treatments. A variety of Gumitir with K1 dose resulted in the smallest number of total pods per plant since the highest number of the total pods had resulted from the K3 dose. In the UM.6-2 line, K0 dose could produce the highest number of total pods per plant, and this condition was similar to the variety of Wilis and UM.4-1line. However, UM.7-2 and 7-6 lines with K0 dose produced the smallest total pods instead. *Figure 4* shows that UM.2-4 lines with K1 dose produced the highest total pods compared to the other doses.

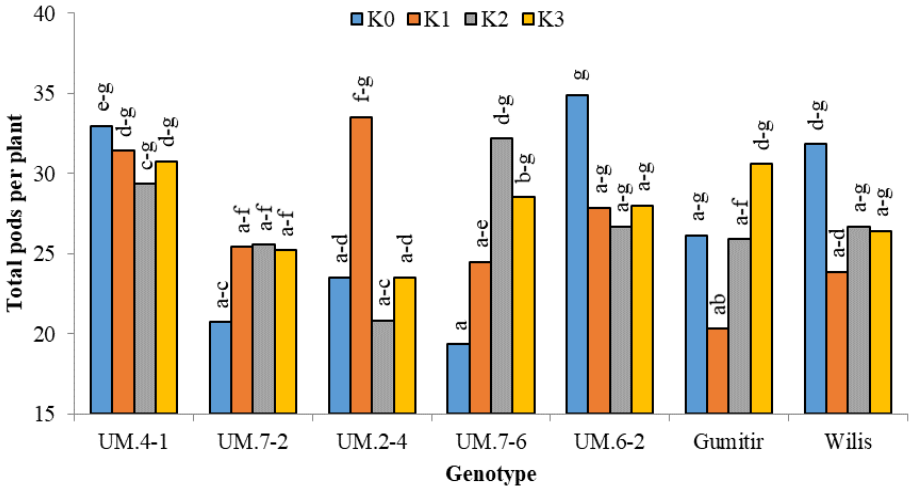

Figure 6. *Interaction of genotype × fertilizer total pods per plant*

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Meanwhile, in the variety of Gumitir, K1 dose produced the lowest total pods per plant since the highest total was resulted after applying K3 dose. In the UM.6-2 line, K0 produced the biggest number of total pods per plant, and it was similar to the variety of Wilis and UM.4-1 lines. UM.7-2 and 7-6 with K0 dose produced the smallest total pods. K3 dose resulted in the biggest total pods only in UM.7-6 line (*Figure 6*). The total pods per plant are correlated to the number of reproductive nodes of a certain plant (Chakma et al., 2015). A large number of reproductive nodes of a certain plant can maximize the growth of pods of soybean plants. The pod is the product of assimilating the storage of the photosynthesis process. Total pods per plant are influenced by the maximum growth and metabolism of the plant. The optimum growth is realized in forming the pods produced by the plant (Xiang et al., 2012).In the growth process, the soybean plant needs nutrition, and it can get the nutrition from the soil or the fertilizer. Total pods will increase in the plant with K with the dose matched with the need for the plant (Chauhan et al., 2013). The appropriateness of K dose is related to the efficiency of its use in the metabolism and biochemistry processed in the plant. The dose that is needed in the various genotypes is based on the need of each soybean genotype.

K0 on to UM.6-2 line and the variety of Wilis, and these lines had the biggest number of filled pods compared to other doses. Meanwhile, UM.7-2 and 7-6 lines had the smallest number of filled pods if they were applied with K0 dose. Both lines resulted in the highest number of filled pods after giving the K2 dose. However, K2 dose caused the lowest number of filled pods in the UM.4-1 line. Meanwhile, UM.2-4 line with K1 had the highest number of filled pods compared to the other dose variations. This case contrasted with the variety of Gumitir with the lowest number of filled pods if applied with a K1 dose (*Figure 7*). A number of filled pods produced showed the plant productivity. Some pods filled seed (filled pod) and unfilled seed (unfilled pod). Soybean plant will be more productive if the number of filled pods is more than the number of unfilled pods. The effectiveness of photosynthesis and assimilate translocation processes in the plant influences number of filled pods (Liesche, 2016). The optimum process is supported by the maximum absorption of nutrition done by the plant (Chakma et al., 2015). K is one of the nutrition needed by the plant. The fulfillment of K within the plant can maximize the assimilate translocation process so that there are many filled pods produced. K as the fertilizer for a plant can increase plant productivity in creating the filled pods (Hussain et al., 2011).

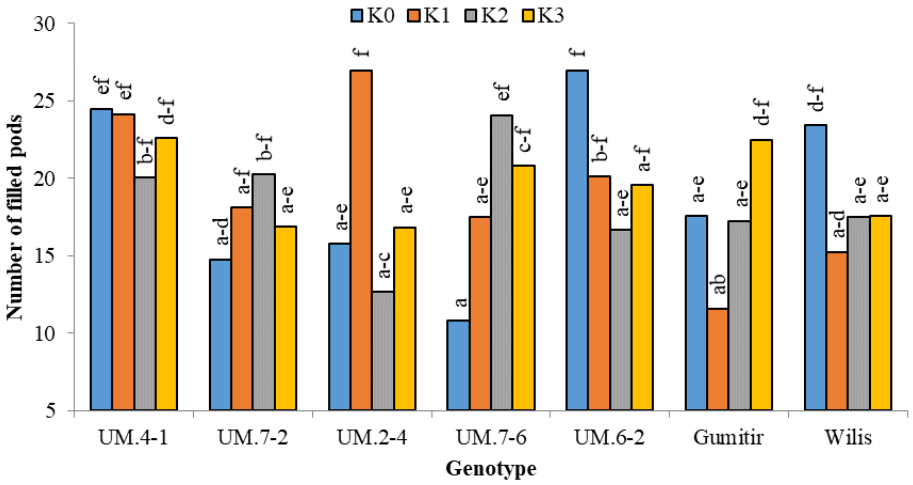

Figure 7. *Interaction of genotype × fertilizer to number of filled pods*

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UM.7-6, 4-1, and 2-4 lines that were applied with K1 dose produced the longest soybean pod. However, K1 dose produced the shortest soybean pod on Gumitir and Wilis. In the UM.7-2 line, K0 dose produced the longest pod compared to the other doses. However, this particular dose produced the shortest pod in UM.7-6 and 6-2 lines (*Figure 8*). The soybean pod produced is one of the plant productivities. Productivity is the result of optimum growth since the need for nutrition can be well fulfilled. The optimum absorption of K supports the growth of the soybean plant so that it can result in the intended productivity (Abbasi et al., 2014). Seed size filled within the pod influences the pod length. This case is closely related to the process of pod filling, forming the filled pod, which is assimilating the translocation process. K helps to optimize the plant assimilate translocation.

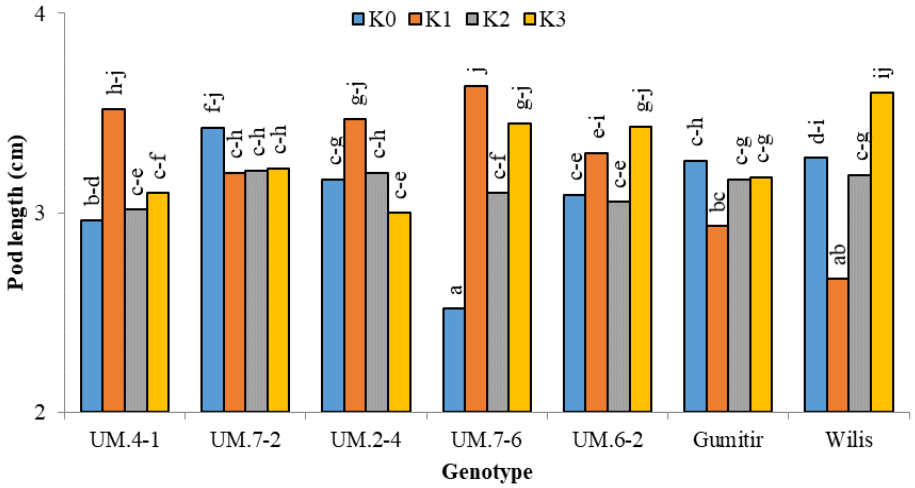

Figure 8. *Interaction of genotype × fertilizer to pod length*

The results showed that in UM.7-6 and 7-2 lines, K0 dose produced the widest soybean pod compared to the other doses. Meanwhile, in the variety of Gumitir, the widest pod was produced after givingK3dose. It was different from the UM.6-2 line, which produced the widest pod after giving K0 and K3 doses. In the other lines, like UM.4-1, 2-4, and variety of Wilis, the difference of pod width resulted did not show a significant difference (*Figure 9*). The soybean plant productivity, as indicated by the pod width, produced as well. The optimum growth is fully supported by the availability of nutrition needed in metabolism and photosynthesis processed. The proper K dose can optimize soybean plant growth (Chen et al., 2007). It was the same with the length and width of soybean pod; they were influenced by the seed size filled within the pod as well. Thereby, the optimum assimilate translocation process truly determines the pod produced with the maximum width. This process can be maximized by fulfilling the need for K in the plant, based on the need of each genotype.

The less the K dose, the pod produced would be thicker in the UM.2-4 line. UM.7-2 line and variety of Wilis with K0 dose also produced the thickest pod. However, in UM.4-1, 6-2, and variety of Gumitir, the thickest pod resulted from K3 dose. The lowest dose resulted in thin pods like those in UM.4-1, 7-6, and 6-2 lines. K1 dose produced the thickest pod in the UM.7-6line. However, this dose produced the thinnest pod in UM.7-2line, the variety of Gumitir and Wilis (*Figure 10*). The thickness of the pod

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produced is correlated to the seed-filled within the pod. The seed quality is obtained from the effectiveness of assimilating translocation from the photosynthesis result by fulfilling the K dose needed by the plant. The photosynthesis process's effectiveness supports the effectiveness of assimilating translocation influencing plant productivity (Yooyen et al., 2015).

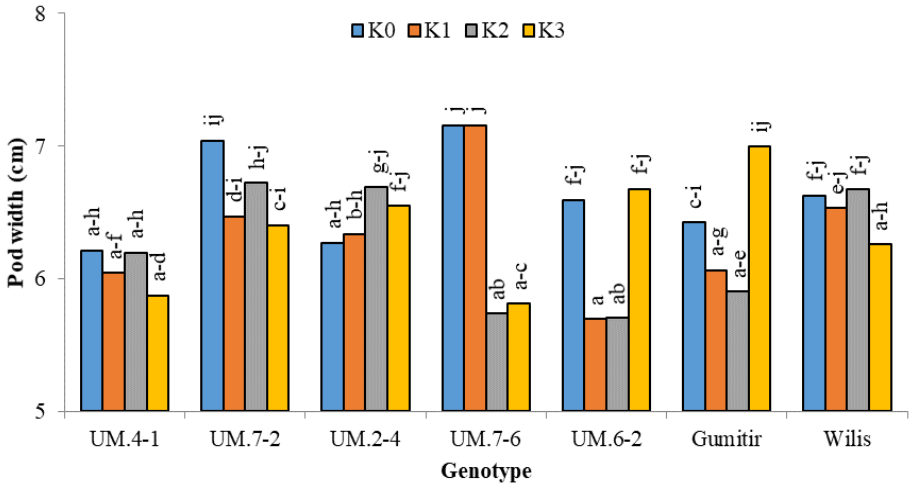

Figure 9. *Interaction of genotype x fertilizer to pod width*

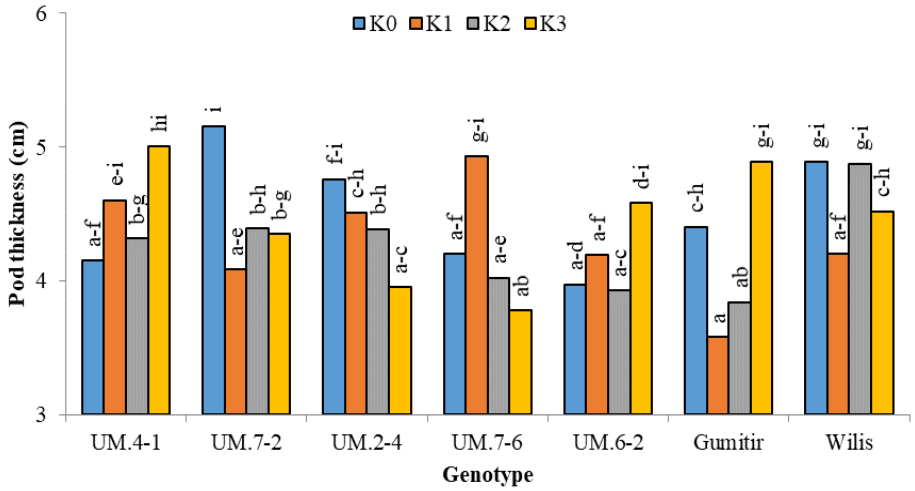

Figure 10. *Interaction of genotype × fertilizer to pod thickness*

The higher the K dose, the seed produced in the UM.2-4 line would be wider as well. K3 dose produced the widest seed in the variety of Gumitir. Meanwhile, in UM.4-1, 7-6, and 6-2 lines, the widest seed was produced after givingK1dose. Besides, the other doses like K0 dose produced the widest seed only in the variety of Wilis (*Figure 11*). The seed width influences plant quality and productivity. The seed is produced by the assimilate translocation as the result of photosynthesis (Ayub et al., 2012). The higher the dose, it will be in line with the productivity resulted (Chakma et al., 2015). Some factors influence the production of seed as the result of plant productivity; one of them is the use of fertilizer. Giving the various doses of K to the plant can initiate the forming

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of seed optimally (Khan et al., 2007; Hosinkhani et al., 2013). The optimum seed is indicated by the thick and wide seed to maximize the plant product and show the effectiveness of the plant productivity.

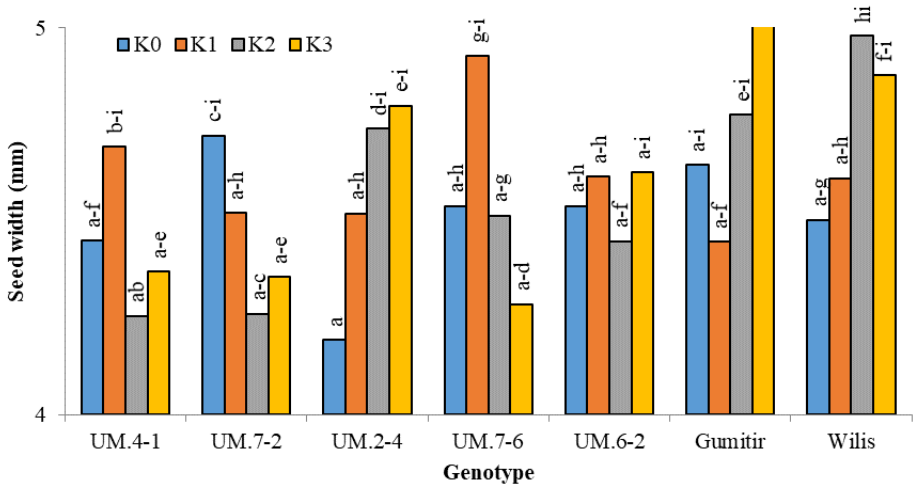

Figure 11. *Interaction of genotype × fertilizer to the seed width*

The UM.2-4 line showed that the higher the K dose, the seed produced would be thicker. UM.6-2line and variety of Gumitir with K3 dose produced the thickest soybean. However, UM.7-2line produced the thickest seed after giving K0 dose. Besides, the other doses, like K1 dose, gave optimum results in the form of thick seed in UM.4-1 and 7-6 lines while K2 dose was optimum in the variety of Wilis (*Figure 12*). The thick seed showed optimum plant productivity. The optimum productivity can be reached by considering the K (Mahadik and Chpde, 2015). The productivity is supported by the increase in photosynthesis and assimilate translocation (photosynthate) to produce the crop. K can increase seed productivity resulted from the soybean plant (Asri and Sonmez, 2010). The dose based on the need for the plant can maximize plant productivity. One of the productivity results is the optimum size of the soybean seed.

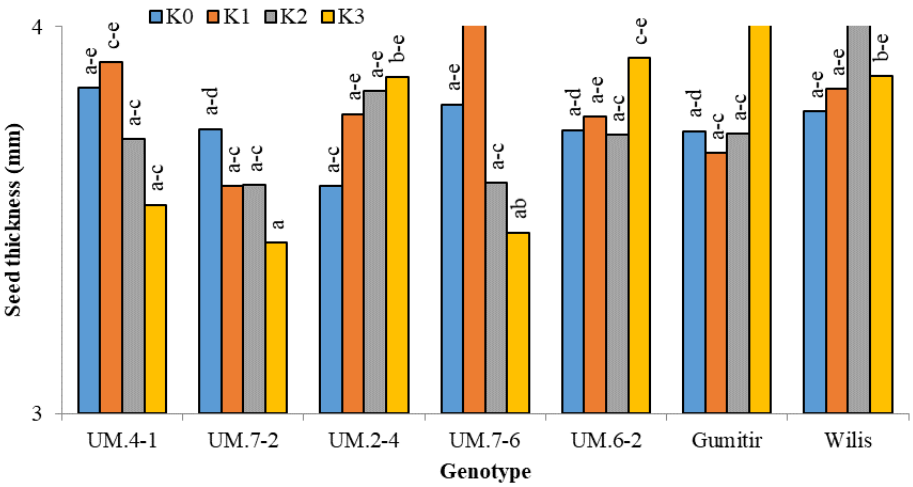

Figure 12. *Interaction of genotype × fertilizer to the seed thickness*

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The results showed that a variety of Wilis, UM.7-2, and 6-2 lines with K0 dose produced the soybean with the heaviest weight of 50 seeds. However, the UM.2-4line that was applied with K0 produced the soybean with the lightest weight of 50 seeds. K2 dose with UM.4-1 line and variety of Gumitir caused the heaviest weight of 50 seeds. However, in a variety of Wilis, K2 dose produced the lightest weight. The UM.2-4 line produced the heaviest weight of 50 seeds when at K1 dose.

On the contrary, when this dose with UM.7-2, 7-6, and 6-2 lines, they produced the lightest seed (*Figure 13*). The maximum photosynthesis and assimilate translocation processes had produced the thick and wide seed with the highest weight of 50 seeds. If those processed were not optimal, the weight resulted would be low. The photosynthesis and assimilate translocation processes were influenced by K supporting the growth and metabolism processes (Weerahewa and David, 2015). Fertilizing by using K can increase the assimilate accumulation so that it increases the plant product as well (Calvante et al., 2015). The plant product was in the form of soybean seed. K is the most effective nutrient in maximizing photosynthesis and photosynthate translocation to increases the weight of the product (Yassen et al., 2010). The increase of plant products can be indicated by the quality of the product resulted. The weight of 50 seeds was influenced by seed thickness and seed width. The thick and wide seed is the result of the intended productivity with high quality. K, in the planting process, influences the quality of the plant product (Dawood et al., 2014; Zhao et al., 2015).

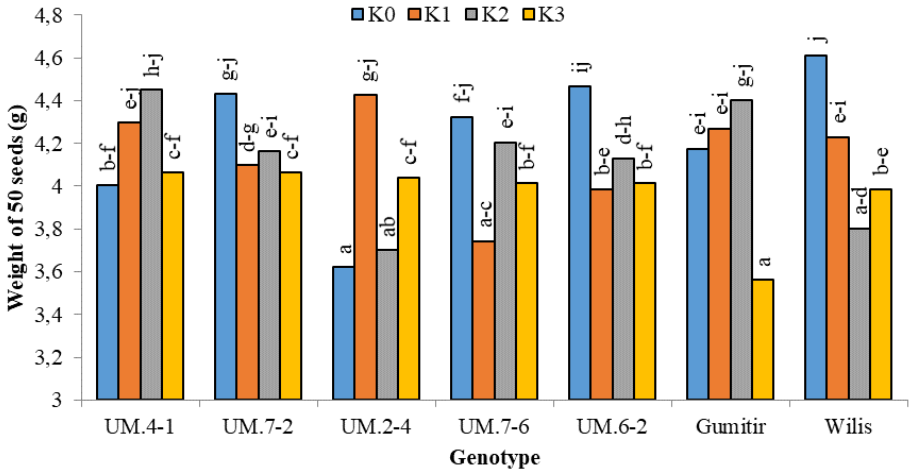

Figure 13. *Interaction of genotype × fertilizer to the weight of 50 seeds*

The responses shown were the effect of giving K to the soybean plant attacked by *B. tabaci*. Fulfilling the need for potassium (K) streamlines its use in influencing the metabolism process (Pettigrew, 2008), and maximizes the plant productivity (Hamouda et al., 2015). The soybean plant productivity was produced from the effectiveness of physiology and biochemistry processes. K is very significant for and needed by the plant. K is available within the soil naturally, and it can be provided by giving fertilizer. The plant absorbs the K within the soil through the transport mechanism of K related to and influences the other mechanisms like water transport (Sczerba et al., 2009) and maintains the cell turgidity that also maintains the leaf form. This case is closely related to the photosynthesis process. The turgid cell can produce the normal shape of the leaf

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so that it can maximize the photosynthesis process (Hopkins, 2004). Photosynthesis produces assimilation that should be translocated from the leaf to the other parts of the plant, such as root, stem, and it will be stored in the food storage. Assimilate translocation can occur optimally if the K dose is appropriate (Calavante et al., 2015; Al-Shaheen et al., 2016). The optimally assimilate translocation can maximize plant growth, influence plant height, the number of reproductive nodes, influencing total pods so that it is stored in the food storage like seed and pod. The quality of seed and pod is influenced very much by the optimally assimilate translocation. In this research, the assimilate translocation was optimal and able to produce long, wide, and thick pods, also thick and wide seed with some filled pods and total pods per plant as well as the weight of 50 seeds.

All research results of each line showed the different responses of every dose. This case was influenced by the genotype, environment (Thilakarathna and Raizada, 2015), the dose in a planting process (Hellal and Abdelhamdi, 2013), and the soil condition (McKenzie and Pauly, 2013). Therefore, a soil test should be done to optimize the use of potassium dose in every variety and reach the intended product (FAO, 2000). This case is because the appropriateness in determining the dose used will influence productivity (Kuswantoro, 2017).

The relationship among the agronomical traits is known by using correlation-regression analysis. The analysis results are presented in *Table 3*.

Table 3. *The Relationship among Agronomical traits*

	DM	PH	NBMS	PL	PW	PT	NCP	NUP	TPP	NRN	SL	SW	ST	W50	SWPP	SM
DF	0.038	0.242	0.089	-0.274	0.109	-0.059	-0.119	0.116	-0.098	0.034	0.018	0.107	0.135	0.157	-0.099	-0.097
DM		-0.037	-0.428	-0.081	-0.140	-0.201	-0.092	-0.071	-0.114	-0.019	0.045	0.192	0.210	-0.288	-0.006	-0.105
PH			0.077	0.138	0.267	0.147	-0.040	-0.275	-0.078	-0.088	0.218	0.235	0.069	-0.022	0.106	-0.145
NBMS				-0.052	0.093	-0.022	-0.030	0.309	0.040	-0.061	0.051	-0.009	-0.045	0.286	0.028	-0.113
PL					-0.037	0.401	0.351	-0.402	0.274	0.281	0.122	0.238	0.114	-0.151	0.521	0.374
PW						0.479	-0.252	-0.289	-0.330	-0.227	0.521*	0.410	0.505*	-0.126	-0.037	-0.196
PT							0.123	-0.264	0.072	0.243	0.501*	0.297	0.400	-0.233	0.253	0.065
NFP								-0.191	0.973**	0.845**	-0.349	-0.097	0.031	0.216	0.766**	0.435
NUP									0.017	0.001	-0.131	-0.121	0.024	0.004	-0.295	-0.008
TPP										0.858**	-0.354	-0.095	0.055	0.219	0.738**	0.427
NRN											-0.180	0.006	0.138	0.051	0.675**	0.553*
SL												0.754**	0.696**	-0.348	0.064	-0.148
SW													0.837**	-0.308	0.327	0.003
ST														-0.390	0.357	0.016
W50															-0.009	-0.057
SWPP																0.406

DF= days to flowering, NUP = number of unfilled pods, DM = days to maturity, TPP = total pods per plant, PH = plant height, NRN = number of reproductive nodes, NBMS = number of branches of the main stem, SL = seed length, PL = pod length, SW = seed width, PW = pod width, ST = seed thickness, PT = pod thickness, W50 = weight of 50 seeds, NCP = number of filled pods, SWPP = seed weight per plant, SM = honeydew

The results showed that there was a significant correlation between some variables. A number of filled pods is related to total pods per plant with a value of 0.973, meaning that if the number of filled pods is getting higher, total pods per plant will be higher as well. The correlation test score between the number of filled pods and number of

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reproductive nodes per plant was 0.845; so there was a significant correlation; if the number of total pods is getting higher, the number of reproductive nodes will increase as well. A large number of reproductive nodes can maximize the pod growth produced in a certain plant (Chakma et al., 2015). This case is influenced by many factors, such as the availability of water and the line of plant type planted (Kuswantoro, 2017).

The number of filled pods is also closely related to seed weight per plant with a score of 0.766. This phenomenon is because the number of filled pods is the form of productivity that resulted from a particular plant. If filled pods produced increase, seed weight per plant would increase as well (Xiang et al., 2012). A large number of filled pods should be continued by the increase in seed and pod size to produce seed weight per plant that significantly increases (Kuswantoro et al., 2014). This phenomenon is because a large amount of filled pods does not always produce seed weight per plant if the seeds produced have a small size.

Total pods per plant were closely related to a number of reproductive nodes with a score of 0.858. It means that the higher total pods per plant produced, the number of reproductive nodes is getting higher as well. The correlation test between total pods per plant and seed weight per plant revealed a score of 0.738. It showed a significant correlation meaning that the higher the total pods per plant, seed weight per plant is getting higher as well. This phenomenon is because the number and weight are in line due to the positive correlation. The number of reproductive nodes is significantly correlated to seed weight per plant with a score of 0.675, meaning that a higher number of reproductive nodes, seed weight per plant, is getting higher .

The three aspects, such as the number of reproductive nodes, number of total pods per plant, and seed weight per plant, showed a significant correlation. This case is because the reproductive nodes are the first spots of soybean pod revealing (Chakma et al., 2015). Reproductive nodes are commonly found in the stem and main branch (Kuswantoro, 2017). This thing determines a large number of pods per plant produced by soybean plants. The number of reproductive nodes is related to several filled pods produced by the soybean plant (Kuswantoro, 2017). Thereby, it will be able to influence seed weight per plant produced as well.

The results of the correlation test between seed length and seed width, seed length and seed thickness and seed width, and seed thickness were 0.754; 0.696; and 0.837, respectively. This case means that there was a significant correlation between seed length and seed width, seed length and seed thickness, and seed width and seed thickness. The longer seed produced, the seed will be wider and thicker as well. Seed length, seed width, and seed thickness influence the quality and productivity resulted (Ayub et al., 2012). The seed is a significant product of the effectiveness of assimilates translocation done by the plant. The seed size is an essential thing in influencing plant productivity. This case is because the seed size determines the seed weight per plant produced directly (Kuswantoro, 2015).

The result of the correlation test also showed a significant correlation between some other variables. Pod width is correlated to seed length and seed thickness with the scores of 0.521 and 0.505, respectively. Pod thickness is also correlated to seed length, which was 0.501. This case means that the wider pod, the seed within it will be longer and thicker as well. The long and thick seed produces the wide seed directly so that it is said that seed is optimally produced (Ayub et al., 2012). Thereby, the wide and thick pods are needed to cover the seeds resulted from the soybean plant.

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Conclusions

Interaction (genotype x fertilizer) affected many aspects such as plant height, pod length, pod width, pod thickness, number of filled pods, total pods per plant, number of reproductive nodes, seed length, seed weight, and seed thickness, the weight of 50 seeds, days to flowering, and days to maturity. Optimal results were obtained if the dosage is given according to the needs of each soybean genotype. The potassium affected plant height and pod length. The increasing level of potassium leads the pest severity to increase.

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CARBOHYDRATE AND LIGNIN CONTENTS IN PERENNIAL RYEGRASS (*LOLIUM PERENNE* L.) TREATED WITH SEA BAMBOO (*ECKLONIA MAXIMA*) EXTRACT AGAINST THE BACKGROUND OF NITROGEN FERTILISATION REGIME

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Abstract. The objective of this study was to determine the effect of *Ecklonia maxima* extract on monosaccharide, structural and non-structural carbohydrate as well as lignin contents in two *Lolium perenne* cultivars against the background of a varied nitrogen fertilisation regime. A field experiment was arranged as a randomised sub-block design (split-split-plot) in Poland. The following factors were examined: biostimulant Kelpak SL applied at 2 $dm^3.ha^{-1}$ and a control (no biostimulant addition); nitrogen application rates of: 0, 50, 100 and 150 kg ha^{-1}; cultivars of *Lolium perenne:* Diament and Gagat. The research reported here demonstrated positive effect of *Ecklonia maxima* extract on *Lolium perenne* chemical composition. Its results showed that structural carbohydrate and lignin contents in grass biomass may be reduced whereas non-structural carbohydrate content may be increased by means of an application of sea alga extract, which results in an improved quality of feed produced from these grasses. The biostimulant applied in combination with each nitrogen rate significantly increased the concentration of monosaccharides and non-structural carbohydrates, and reduced the concentration of structural carbohydrates and lignin in *Lolium perenne* biomass. Increasing nitrogen rates significantly increased cellulose, hemicellulose and lignin contents but they reduced amounts of monosaccharides and non-structural carbohydrates in the test grasses.
Keywords: *Kelpak, biostimulant, fertilisation, grass, monosaccharides, cellulose, hemicellulose, non-structural carbohydrates*

Introduction

As the reliance of agriculture's on chemicals (mineral fertilisers, plant protection agents) may negatively affect both food quality and the natural environment, innovative solutions have been increasingly sought (Niewiadomska et al., 2020). Due to this, in order to reduce such reliance, an alternative solution in the form of natural preparations, which include biostimulants, used as fertilisers is gaining more and more popularity all over the world (Colla and Rouphael, 2015; Sosnowski et al., 2016; Kocira et al., 2018a). An application of natural growth regulators may become one of components of modern agricultural practice. Scientific literature claims that these preparations are environmentally friendly, beneficially affect yield quality and quantity (Godlewska and Ciepiela, 2016; Caruso et al., 2019), and frequently eliminate a large part of biotic and abiotic stress (Rathore et al., 2009; Haider et al., 2012; Sharma et al., 2014). Simultaneously, an application of biostimulants may prove to be economically viable (Rengasamy et al., 2015). Sea algae extracts (division of green, red and brown algae) are biostimulants which have become quite popular in crop plant production (Du Jardin, 2015). *Ecklonia maxima*, a brown alga, is harvested off the coast of South Africa and along the Atlantic coast of Africa. Large amounts of this seaweed are deposited on the seashore, which results in environmental pollution. When collected, the organic matter

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may be utilised to produce the preparation called Kelpak which, in addition to plant hormones (cytokinins (0.03 mg/l) and auxins (11 mg/l), gibberellins, abscisic acid, brassinosteroids), contains polysaccharides (absent in terrestrial plants), amino acids, polyphenols as well as macro- and microelements (Strik et al., 2004; Papenfus et al., 2012; Rengasamy et al., 2015; Rauphael et al., 2017).

Although literature reports claim that sea algae extracts display a positive effect on many species of cultivated plants, the influencing process of those extracts have not be fully understood yet. The effect of biostimulants may, to a great extent, depend on the plant species and cultivar (Sultana et al., 2005; Battacharyya et al., 2015). There is still a paucity of information on an application of biostimulants to meadow plants.

The objective of this study was to determine the effect of *Ecklonia maxima* extract on monosaccharide, structural and non-structural carbohydrate as well as lignin contents in two *Lolium perenne* cultivars against the background of varied nitrogen fertilisation regime.

Materials and Methods

Experimental Design

A field experiment was arranged as a randomised sub-block design (split-split-plot) with three replicates at the Siedlce Experimental Unit of the University of Natural Sciences and Humanities in Poland (52.169° N, 22.280° E) in late April, 2009. The plot area was 10 m^2. The soil of the experimental site represents average soils, Hortic Anthrosol (WRB). Prior to the experiment set-up the characteristics of the soil were as follows: neutral pH (pH in 1n KCl = 7.2), high humus content (3.78%), high available phosphorus and magnesium contents (P_2O_5 - 900 $mg.kg^{-1}$, Mg - 84 $mg.kg^{-1}$) and average total nitrogen and available potassium contents (N - 1.8 $g.kg^{-1}$ DM, K_2O - 190 $mg \cdot kg^{-1}$). Soil chemical analysis was carried out at an accredited laboratory of the Chemical and Agricultural Research Laboratory in Warsaw (Poland). Available phosphorus and potassium in the soil were extracted by means of the Egner-Riehm method (Staugaitis and Rutkauskiene, 2012) and available magnesium - using the Schachtschabel method (Staugaitis and Rutkauskiene, 2012). Phosphorus was determined by the colorimetric method, total nitrogen by the Kjeldahl method and potassium and magnesium by the atomic absorption spectrophotometry AAS. The following factors were examined:

- biostimulant (sea bamboo extract) with the trade name Kelpak SL applied at 2 $dm^3.ha^{-1}$ and a control (no biostimulant addition);
- nitrogen application rate: 0, 50, 100 and 150 kg ha^{-1};
- cultivars of *Lolium perenne*: Diament and Gagat.

The growth stimulant applied in the experiment is an extract from the fastest growing seaweed (kelp) *Ecklonia maxima* harvested off the coast of South Africa. The extract contains, among others, the natural plant hormones auxins (11 mg $L^{-1}$and cytokinins (0.03 $mg.L^{-1}$). The commercial name of the stimulant is Kelpak SL, and it is manufactured by Kelp Products (Pty) Ltd. P.O. Box 325, Simon's Town, the Republic of South Africa.

The sowing amount of *Lolium perenne* L. variety Gagat and Dukat was calculated on the basis of standards developed by Research Centre for Cultivar Testing (COBORU, 2008). Standard cultivation methods were used in the experiment, and additionally the plants were fertilized with various doses of nitrogen and the Kalpak SL biostimulator.

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The grass sowing rate was 38 kg ha^{-1} (TWG - thousand-grain weight – 3.2 g, 1187 kernels per square meter).

In the growing season, when the experiment was set up, neither the nitrogen regime nor the biostimulant were applied. The season was an introductory period when three weed-control cuttings were made. After the second cutting, mineral fertilisation was applied to all the plots at the rates of 30 kg ha N (ammonium nitrate) and 30 kg.ha K_2O (potash salt). Phosphorus was not applied as the soil was rich in available forms of this element. Over the study period (2010-2012), the cutting regime was three harvests per year. Ammonium nitrate was applied three times per year. The total nitrogen amount was split into three equal rates which were applied before each cutting. Phosphorus and potassium needs of the grass were calculated taking into account the expected dry matter yields, the appropriate (from the ruminant nutrition standpoint) mineral contents of hay as well as soil P and K availability.

Phosphorus and potassium fertilisation was applied to all the plots. Phosphorus was applied once as triple superphosphate at a rate of 40 kg ha P_2O_5 in spring. The amount of potassium (160 kg ha K_2O) was split into three equal rates and applied prior to each cutting as 60% potash salt. The biostimulant was sprayed as an aqueous solution; the rate was 2 dm^3 of biostimulant per hectare diluted in water to 400 dm^3. The spraying the rate 2 dm^3 of biostimulant was performed before each cutting: the first application was three weeks before the first cutting, the second one two weeks after the first harvest and the last cutting three weeks after the second harvest.

Weather Conditions

Weather conditions differed during the study period (*Table 1*). Average air temperatures and precipitation sums in all the growing seasons were higher than the long-term means and the precipitation was very unevenly distributed. In 2010 and 2011 rainfall was, respectively, by 115.3 and 80.5 mm higher than the long-term means. It is worth noticing that in July 2011 the precipitation was 4.5 times higher than the long-term mean for July, and it constituted 48% rainfall of the whole growing season. Also in June 2012 precipitation was by 50.5 mm higher compared with the long-term mean for this month. By contrast, high rainfall shortages were recorded in April 2010, September 2011 and 2012.

Table 1. *Meteorological condition in growing season 2010-2012 by meteorological station in Siedlce*

Years	Means air temperatures (^{0}C)						Mean daily air temperature in growing season (^{0}C)
	IV	V	VI	VII	VIII	IX	
2010	8.9	14.0	17.4	21.6	19.8	11.8	15.6
2011	9.8	13.4	18.1	18.2	18.1	14.4	15.3
2012	9.0	14.5	16.4	20.4	18.0	14.2	15.4
Means of many years (2002- 2012)	7.7	10.0	16.1	19.3	18.0	13.0	14.0
Years	**Monthly precipitations (mm)**						**Sum of precipitation in growing season (mm)**
	IV	V	VI	VII	VIII	IX	
2010	10.7	93.2	62.6	77.0	106.3	109.9	459.7
2011	38.1	55.6	44.3	204.2	55.4	26.6	424.2
2012	40.3	59.7	118.7	41.4	64.1	30.8	355.0
Means of many years (2002- 2012)	52.3	50.0	68.2	45.7	66.8	60.7	343.7

Reported by the meteorological station in Siedlce

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Chemical Analysis

During harvest of each cut, green matter from each plot (whole area, that is 10 m^2) was weighed to determine the yield, and 0.5 kg green matter samples of grass were taken to determine the drying-up coefficient and to carry out chemical analyses. The samples were left to dry in a ventilated room. Airy dry matter was weighed (to determine dry matter yield per plot) and was then shredded and ground. The obtained material was subjected to chemical analysis to determine dry matter, monosaccharides, cellulose, hemicellulose, lignin, total protein, crude ash and crude fat. The components were determined in powdered dry plant material placed on Petri dishes by near infrared spectroscopy (NIRS) using a NIRFlex N-500 spectrometer and ready-to-use INGOT® calibration applications. INGOT® is a set of Universal NIR calibrations (adapter to the NIRFlex N-500 data format) for the analysis of raw materials and finished products, e.g. grass. Non-structural carbohydrates were calculated following Virkajärvi et al. (2012):

$$\text{Non-structural carbohydrates} = 1000 - (\text{total protein} + \text{crude ash} + \text{crude fat} + \text{cellulose} + \text{hemicelluloses} + \text{lignin}) \quad \text{(Eq.1)}$$

Statistical Analysis

Means presented in tables 2-6 for each factor (biostimulant: N rate; cultivar; cut) and for interactions between these factors were calculated (for each characteristic studied) using all the data for individual experimental combinations obtained in replicates from each cut in all the study years. The data was processed by means of STATISTICA StatSoft, Inc. (2011) (data analysis software system), version 10 (www.statsoft.com). The program was used to conduct variance analysis (ANOVA/MANOVA). Significance of differences between means for the experimental factors was checked using Tukey's test at the significance level of $\alpha \leq 0.05$.

Results and Discussion

The concentration of monosaccharides, structural and non-structural carbohydrates as well as lignin is an important criterion of forage grass assessment. Monosaccharide content in both *Lolium perenne* cultivars (*Table 2*) depended on all the experimental factors. Regardless of the nitrogen fertilisation regime, cuts and cultivars, the sea alga extract significantly increased monosaccharide content in *Lolium perenne* biomass, which improved the forage quality. Kelpak application increased monosaccharide content by 31.8% in cv. Gagat biomass, and by 40.7% in cv. Diament biomass, which indicates that the effect of the biostimulants was cultivar-related. A positive effect of sea algae extracts on the concentration of the discussed compounds in the biomass of various plant species has been confirmed by Pacholczak et al. (2012), Sridhar and Rengasamy (2011) as well as El-Miniawy et al. (2014). The results of the study reported here indicated a significant interaction of the biostimulant with nitrogen rates. The highest increase in carbohydrate content following *Ecklonia maxima* extract application, amounting to as much as 44.3%, was recorded in the biomass of grasses cultivated in the plots without nitrogen fertiliser. In earlier studies, Godlewska and Ciepiela (2013) demonstrated that the biostimulant Kelpak applied to units without nitrogen fertiliser contributed to an average increase of 42.4% in the sugar content in the biomass of orchard grass and *Braun's festulolium.*

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Table 2. *Content of monosaccharides in Lolium perenne (g.kg^{-1}s.m.) depending on biostimulator, nitrogen dose and cut (mean from years 2010-2012)*

Cut	N dose (kg N • ha^{-1})	Cultivar				Mean		Cultivar		Mean
		Diament		Gagat				Diament	Gagat	
		Dose of Klepak (dm^3 • ha^{-1})				Dose of Klepak		Mean		
		0	2	0	2	0	2			
1	0	117.6 a	174.9 b	124.6 a	165.6 b	121.1 a	170.2 b	146.2 A	145.1 A	145.7 A
	50	108.6 a	154.9 b	117.9 a	148.1 b	113.2 a	151.5 b	131.7AB	133.0 AB	132.4 AB
	100	101.9 a	131.9 b	112.3 a	139.3 b	107.1 a	135.6 b	116.9 B	125.8 AB	121.4 B
	150	94.3 a	125.9 b	107.7 a	130.5 b	98.0 a	128.2 b	110.1 BC	116.1 B	113.1 BC
2	0	98.7 a	147.0 b	114.6 a	163.1 b	106.7 a	155.1 b	122.9 A	138.9 A	130.9 A
	50	95.6 a	132.8 b	103.6 a	138.0 b	99.6 a	135.4 b	114.2 A	120.8 B	117.5 B
	100	54.6 a	74.8 b	65.1 a	77.9 b	59.8 a	76.3 b	64.7 B	71.5 C	68.1 C
	150	76.0 a	106.3 b	87.8 a	114.7 b	81.9 a	110.5 b	91.2 C	101.3 D	96.2 D
3	0	80.4 a	128.2 b	92.0 a	128.0 b	86.2 a	128.1 b	104.3 A	110.0 A	107.1 A
	50	76.5 a	112.1 b	85.1 a	115.1 b	80.8 a	113.6 b	94.3 A	100.1 AB	97.2 B
	100	107.2 a	138.4 b	80.8 a	104.9 b	94.0 a	121.6 b	122.8 B	92.8 B	107.8 A
	150	63.7 a	86.7 b	70.6 a	97.6 b	67.2 a	92.2 b	75.2 C	84.1 BC	79.7 C
Mean	0	98.9 a	150.0 b	110.4 a	152.2 b	104.7 a	151.1 b	124.5 A	131.3 A	127.9 A
	50	93.6 a	133.3 b	102.2 a	133.7 b	97.9 a	133.5 b	113.4 AB	118.0 B	115.7 B
	100	87.9 a	115.0 b	86.1 a	107.4 b	87.0 a	111.2 b	101.5 B	96.7 C	99.1 C
	150	78.0 a	106.3 b	88.7 a	114.3 b	82.4 a	110.3 b	92.2 BC	100.5 C	96.3 C
1	Mean	105.6 a	146.9 b	114.1 a	145.9 b	109.8 a	146.4 b	126.2 A	130.0 A	128.1 A
2		81.2 a	115.2 b	92.8 a	123.4 b	87.0 a	119.3 b	98.2 B	108.1 B	103.2 B
3		81.9 a	116.3 b	82.1 a	111.4 b	82.0 a	113.9 b	99.2 B	96.8 C	98.0 B
Mean		89,6 a	126.1 b	96.3 a	126.9 b	92.9 a	126.5 b	107.9 a	111.6 a	109.8

Different small letters within the same line indicate significant differences. Values in columns for individual factors indicated with different, capital letters differ significantly

Regardless of the remaining factors, increasing nitrogen rates significantly reduced sugar content in the biomass of the test plants, which has been confirmed by results reported by other authors (Godlewska and Ciepiela, 2018). The highest decline in the *Lolium perenne* content of monosaccharides (by 32.8%, on average) compared with the biomass harvested in the unit without nitrogen fertiliser was recorded at the rate of 150 kg N ha^{-1}.

Analysis of results obtained for successive cuts revealed that grasses harvested with the second and third cut contained significantly less monosaccharides compared with their contents determined in the first cut plants (average across years). It can be explained by the fact that plant respiration and utilisation of these compounds is higher at high temperatures.

Non-structural and structural carbohydrate contents as well as lignin content are species-related and exerts a substantial effect on the quality of produced feed. Both an excess and shortage of these compounds in feed fed to ruminants is not recommended. Kelpak application had the same extent of a positive effect on cellulose, hemicellulose and lignin contents in both *Lolium perenne* cultivars (*Tables 3, 4 and 5*), the contents being by, respectively, 6.64, 8.38 and 8.68% lower in the biomass of these grasses. The differences were statistically significant. The obtained results disagree with research by Kocira et al. (2018b) who applied a natural sea alga extract-based biostimulant and obtained a significant increase in the soybean seed content of lignin. The aforementioned discrepancy may result from the fact that the effect of natural biostimulants heavily depends on their concentration, application method and, first and foremost, crop plant species and cultivar (Sultana et al., 2005).

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Table 3. *Content of cellulose in Lolium perenne (g.kg^{-1}s.m.) depending on biostimulator, nitrogen dose and cut (mean from years 2010-2012)*

Cut	N dose (kg N ha^{-1})	Cultivar				Mean		Cultivar		Mean
		Diament		Gagat				Diament	Gagat	
		Dose of Klepak (dm^3.ha^{-1})				Dose of Klepak		Mean		
		0	2	0	2	0	2			
1	0	287 a	270 b	299 a	278 b	293 a	274 b	279 A	289 A	284 A
	50	282 a	265 b	289 a	274 b	285 a	269 b	273AB	281 AB	277AB
	100	275 a	260 b	284 a	266 b	279 a	263 b	267AB	275 B	271 B
	150	268 a	251 b	275 a	254 b	272 a	252 b	260 B	265 BC	262 B
2	0	275 a	259 b	290 a	267 b	283 a	263 b	267 A	279 A	273 A
	50	271 a	254 b	279 a	265 a	275 a	259 b	262AB	272 AB	267AB
	100	265 a	250 b	272 a	255 b	268 a	253 b	257AB	264 B	260 B
	150	259 a	243 b	265 a	241 b	262 a	242 b	251B	253 C	252BC
3	0	272 a	255 b	278 a	261 b	275 a	258 b	263 A	270 A	266 A
	50	265 a	249 b	272 a	255 b	268 a	252 b	257AB	264 AB	260AB
	100	254 a	241 a	264 a	248 b	259 a	245 b	247 B	256 B	252 B
	150	250 a	235 b	258 a	243 b	254 a	239 b	242 BC	250 BC	246BC
Mean	0	278 a	261 b	289 a	269 b	284 a	265 b	270 A	279 A	274 A
	50	272 a	256 b	280 a	265 b	276 a	260 b	264AB	272 AB	268 B
	100	264 a	250 b	273 a	257 b	269 a	253 b	257 B	265 B	261 C
	150	259 a	243 b	266 a	246 b	263 a	244 b	251 BC	256 C	253 D
1	Mean	278 a	261 b	287 a	268 b	282 a	265 b	270 A	277 A	274 A
2		268 a	251 b	276 a	257 b	272 a	254 b	259 B	267 B	263 B
3		260 a	245 b	268 a	252 b	264 a	248 b	252 B	260 B	256 C
Mean		268 a	253 b	277 a	259 b	273 a	256 b	260 a	268 a	264

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Table 4. *Content of hemicellulose in Lolium perenne (g.kg^{-1}s.m.) depending on biostimulator, nitrogen dose and cut (mean from years 2010-2012)*

Cut	N dose (kg N • ha^{-1})	Cultivar				Mean		Cultivar		Mean
		Diament		Gagat				Diament	Gagat	
		Dose of Klepak (dm^3 • ha^{-1})				Dose of Klepak		Mean		
		0	2	0	2	0	2			
1	0	185 a	167 b	189 a	177 b	187 a	172 b	176 A	183 A	180 A
	50	175 a	162 b	184 a	173 b	179 a	167 b	168 A	178 A	173 A
	100	161 a	148 b	172 a	155 b	166 a	152 b	155 B	164 B	159 B
	150	146 a	130 b	147 a	124 b	147 a	127 b	138 C	135 C	137 C
2	0	195 a	179 b	200 a	185 b	198 a	182 b	187 A	192 A	190 A
	50	186 a	175 b	193 a	181 b	190 a	178 b	180 AB	187 A	184AB
	100	181 a	170 b	188 a	179 a	185 a	175 b	175 B	184 AB	180 B
	150	175 a	166 a	186 a	163 b	181 a	165 b	171 B	175 B	173 BC
3	0	191 a	176 b	188 a	177 b	190 a	177 b	184 A	183 A	183 A
	50	180 a	170 a	187 a	175 b	184 a	173 b	175 AB	181 AB	178 A
	100	173 a	160 b	177 a	166 b	175 a	163 b	166 B	172 B	169 B
	150	163 a	136 b	168 a	152 b	165 a	144 b	150 C	160 C	155 C
Mean	0	190 a	174 b	192 a	180 b	192 a	177 b	184 A	186 A	184 A
	50	180 a	169 b	188 a	176 b	185 a	173 b	175 B	182 A	179 A
	100	172 a	159 b	179 a	167 b	176 a	163 b	166 C	173 B	169 B
	150	161 a	144 b	167 a	146 b	165 a	144 b	153 D	157 C	155 C
1	Mean	167 a	152 b	173 a	157 b	170 a	155 b	159 A	165 A	162 A
2		185 a	173 a	192 a	177 b	188 a	175 b	179 B	184 B	181 B
3		177 a	161 b	180 a	168 a	178 a	164 b	169 AB	174 AB	171 C
Mean		176 a	162 b	182 a	167 b	179 a	164 b	169 a	174 a	172

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Table 5. *Content of lignin in Lolium perenne (g.kg^{-1}s.m.) depending on biostimulator, nitrogen dose and cut (mean from years 2010-2012)*

Cut	N dose (kg N.ha^{-1})	Cultivar				Mean		Cultivar		Mean
		Diament		Gagat				Diament	Gagat	
		Dose of Klepak (dm^3.ha^{-1})				Dose of Klepak (dm^3.ha^{-1})		Mean		
		0	2	0	2	0	2			
1	0	40.1 a	37.0 b	41.2 a	37.7 b	40.6 a	37.4 b	38.5 A	39.5 A	39.0 A
	50	38.2 a	36.1 a	40.0 a	36.7 b	39.1 a	36.4 b	37.1 AB	38.4 A	37.8 AB
	100	37.7 a	34.8 b	38.6 a	35.8 b	38.2 a	35.3 b	36.3 AB	37.2 AB	36.8 AB
	150	36.7 a	34.1 b	37.7 a	34.2 b	37.2 a	34.1 b	35.4 B	36.0 B	35.7 B
2	0	41.7 a	38.4 b	44.7 a	40.0 b	43.2 a	39.2 b	40.0 A	42.4 A	41.2 A
	50	40.1 a	38.2 a	42.0 a	38.4 b	41.0 a	38.3 b	39.1 AB	40.2AB	39.7 AB
	100	39.2 a	36.5 b	39.8 a	36.7 b	39.5 a	36.6 b	37.8 AB	38.3 B	38.0 B
	150	38.7 a	35.2 b	39.0 a	35.5 b	38.9 a	35.4 b	37.0 B	37.3 BC	37.1 BC
3	0	38.4 a	35.3 b	39.8 a	36.3 b	39.1 a	35.8 b	36.8 A	38.0 A	37.4 A
	50	36.4 a	34.5 b	38.2 a	34.5 b	37.3 a	34.5 b	35.4 AB	36.4 AB	35.9 AB
	100	35.5 a	33.3 a	36.1 a	33.8 a	35.8 a	33.5 a	34.4 B	35.0 B	34.7 B
	150	35.0 a	31.7 b	36.0 a	32.5 b	35.5 a	32.1 b	33.4 B	34.2 B	33.8 B
Mean	0	40.1 a	36.9 b	41.9 a	38.0 b	41.0 a	37.4 b	38.5 A	40.0 A	39.2 A
	50	38.2 a	36.2 a	40.1 a	36.5 b	39.1 a	36.4 b	37.2 AB	38.3 AB	37.8 AB
	100	37.5 a	34.8 b	38.2 a	35.5 b	37.8 a	35.2 b	36.2 B	36.8 B	36.5 B
	150	36.8 a	33.7 b	37.6 a	34.1 b	37.2 a	33.9 b	35.2 B	35.8 BC	35.5 BC
1	Mean	38.2 a	35.5 b	39.4 a	36.1 b	38.8 a	35.8 b	36.8 A	37.8 A	37.3 A
2		39.9 a	37.0 b	41.4 a	37.7 b	40.7 a	37.4 b	38.5 AB	39.5 A	39.0 A
3		36.3 a	33.7 b	37.5 a	34.3 b	36.9 a	34.0 b	35.0 AC	35.9 B	35.4 B
Mean		38.1 a	35.4 b	39.4 a	36.0 b	38.8 a	35.7 b	36.8 a	37.7 a	37.2

Different small letters within the same line indicate significant differences. Values in columns for individual factors indicated with different, capital letters differ significantly

An interaction of sea alga extract and nitrogen fertilisation was consistently in the direction of reducing the concentration of structural carbohydrates and lignin in both *Lolium perenne* cultivars treated with the biostimulant at each nitrogen fertiliser rate. The strongest interaction was recorded in the plots fertilised with 150 kg N ha^{-1} where Kelpak application contributed to a decline in the *Lolium perenne* content of cellulose, hemicellulose and lignin (respectively, by 7.79, 14.6 and 9.74%). Unfortunately, it is impossible to refer these results to other reports due to lack of literature on this issue.

Regardless of the remaining factors, nitrogen fertilisation contributed to a decline in the *Lolium perenne* content of the discussed compounds. Cellulose and hemicellulose contents in grass biomass declined as nitrogen rates increased, the differences being statistically significant. Similar tendencies were observed for lignin content but significant differences were found only between lignin content in plants harvested from plots without nitrogen fertiliser, and plants fertilised with the nitrogen rate of 150 kg^{-1} ha^{-1}. According to Szkutnik et al. (2012) as well as Ciepiela et al. (2016), high nitrogen rates contribute to a decline in the grass content of raw fibre, which is consistent with the study results reported here.

The concentration of cellulose and hemicellulose in grasses depended significantly on cuts, and differences in the contents of these compounds in the test grass biomass harvested with individual cuts were significant.

Lolium perenne cv. Diament contained less structural carbohydrates and lignin than cv. Gagat but the differences were statistically insignificant.

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Non-structural carbohydrate content in both *Lolium perenne* cultivars as affected by the biostimulant, nitrogen fertilisation and cuts is shown in *Table 6*. Statistical analysis of the study results demonstrated a significant influence of all the experimental factors on non-structural carbohydrate content in the test plant material. The results indicate that there was a uniform positive effect of the biostimulant on the *Lolium perenne* content of the discussed compounds. Grasses treated with Kelpak contained by 9.79% more non-structural carbohydrates compared with their concentration in the biomass of plants which had not been treated with the biostimulant. As far as an interaction between *Ecklonia maxima* extract and nitrogen rates is concerned, there was found a significantly higher non-structural carbohydrate content in both grass species treated with the biostimulant and at each nitrogen rate. The lowest increase in the concentration of the compounds following Kelpak application, amounting to 8.15%, was found in the biomass of plants fertilised with the rate of 50 kg N ha^{-1}, it being the highest (11.9%) in the biomass of plants treated with the biostimulant and fertilised with nitrogen at the rate of 150 kg.ha^{1}.

Table 6. *Content of non-structural carbohydrates in Lolium perenne (g.kg^{-1}s.m.) depending on biostimulator, nitrogen dose and cut (mean from years 2010-2012)*

Cut	N dose (kg N.ha^{-1})	Cultivar				Mean		Cultivar		Mean
		Diament		Gagat				Diament	Gagat	
		Dose of Klepak (dm^3.ha^{-1})				Dose of Klepak (dm^3.ha^{-1})		Mean		
		0	2	0	2	0	2			
1	0	278 a	303 b	262 a	287 b	270 a	295 b	291 A	274 A	283 A
	50	274 a	296 b	253 a	275 b	263 a	286 b	285 A	264 AB	274AB
	100	271 a	289 a	245 a	269 b	258 a	279 b	280 A	257 B	269 B
	150	257 a	281 b	241 a	280 b	249 a	280 b	269 B	260 B	265 B
2	0	240 a	257 a	227 a	240 a	233 a	248 a	248A	233 A	241 A
	50	221 a	241 b	205 a	223 a	213 a	232 a	231 B	214 B	222 B
	100	215 a	232 a	197 a	217 b	206 a	225 a	224 B	207 BC	215 B
	150	191 a	222 b	186 a	204 a	189 a	213 b	207 C	195 C	201 C
3	0	268 a	291 b	237 a	275 b	253 a	283 b	280 A	256 A	268 A
	50	244 a	261 a	229 a	255 b	237 a	258 b	253 B	242 B	247 B
	100	245 a	261 a	221 a	243 b	233 a	252 a	253 B	232 BC	242BC
	150	222 a	243 b	218 a	240 b	220 a	241 b	232 C	229 C	231C
Mean	0	262 a	284 b	242 a	267 b	252 a	276 b	273 A	254 A	264 A
	50	246 a	266 b	229 a	251 b	238 a	258 b	256 B	240 B	248 B
	100	244 a	261 a	221 a	243 b	232 a	252 b	252 B	232 B	242BC
	150	223 a	249 b	215 a	241 b	219 a	245 b	236 C	228 B	232 C
1	Mean	270 a	292 b	250 a	278 b	260 a	285 b	281 A	264 A	273 A
2		217 a	238 b	204 a	221 a	210 a	230 a	227 B	212 B	220 B
3		245 a	264 b	226 a	253 b	236 a	259 b	254 C	240 C	247 C
Mean		244 a	265 b	227 a	251 b	235 a	258 b	254 a	239 b	246

Different small letters within the same line indicate significant differences. Values in columns for individual factors indicated with different, capital letters differ significantly

Regardless of the remaining factors, increasing nitrogen rates reduced the concentration of non-structural carbohydrates in grass biomass, the majority of the differences being statistically significant. The only insignificant differences were recorded between *Lolium perenne* biomass contents of the discussed compounds determined in plots fertilised with the nitrogen rates of 50 and 100 kg ha^{-1}.

Seasonal changes in the concentration of non-structural carbohydrates in the biomass of the harvested plants were observed, the differences being statistically significant. The

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lowest amounts of non-structural carbohydrates were determined in plants harvested with the second cut, them being the highest in the first cut, the differences being significant. Similar relationships were reported by Ciepiela and Godlewska (2015) for orchard grass and Braun's festulolium. As far as *Lolium perenne* cultivars are concerned, cv. Diament were found to contain by 6.28% more non-structural carbohydrates compared with their content determined in cv. Gagat, the difference being significant.

Conclusions

The research reported here demonstrated a positive effect of *Ecklonia maxima* extract on *Lolium perenne* chemical composition. Its results showed that structural carbohydrate and lignin contents in grass biomass were reduced whereas non-structural carbohydrate content increased by means of an application of sea alga extract, which results in an improved quality of feed produced from these grasses. The biostimulant applied in combination with each nitrogen rate significantly increased the concentration of monosaccharides and non-structural carbohydrates, and reduced the concentration of structural carbohydrates and lignin in *Lolium perenne* biomass. Increasing nitrogen rates significantly increased cellulose, hemicellulose and lignin contents but they reduced amounts of monosaccharides and non-structural carbohydrates in the test grasses. The concentration of the examined components in the biomass of *Lolium perenne* plants clearly varied during the growing season. Further studies are needed to develop more comprehensive results and evaluate the impact of different rates of biostimulants.

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VEGETATION DYNAMICS ALONG AN ELEVATIONAL GRADIENT IN TERICH VALLEY, CHITRAL HINDUKUSH RANGE, NORTHERN PAKISTAN

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Abstract. This phytosociological study was carried out during 2016-2019 in Terich valley, Hindukush range, Chitral Pakistan. The objective of this study was to determine the vegetation dynamics along various ecological parameters. The quantitative data of vegetation were collected from five monitoring sites using the systematic-random sampling quadrate method. The size of quadrates was 10 m^2 for the trees, 6 m^2 for shrubs and 1.5 m^2 for herbaceous species. Three communities viz. *Elaeagnus-Prunus-Adiantum*, *Fraxinus-Rosa-Acanthophylum* and *Hippophae-Sophora-Poa* community structures were established at foot-hills. Mid-hills comprised *Ferula-Artemisia-Capparis*, *Prangos-Ribes-Berberis* and *Astragalus*-Astragalus-Eremurus* communities. Community structure was quite different at sub-alpine (*Artemisia-Rhodiola-Rosularia*, *Betula-Scutellaria-Taraxacum*) and alpine zones (*Anaphalis-Cousinia-Kobresia*, *Alajja-Oxyria-Oxytropis*). Soil samples were collected and analyzed to study soil properties. Soils were slightly acidic and had low content of organic matter. Vegetation was found to be under extreme biotic stress. Results also showed that vegetation at higher elevation showed lower species richness.

Keywords: *phytosociology, edaphic parameters, similarity index, species diversity, species richness, maturity index, dry temperate vegetation*

Introduction

Vegetation is an ecological expression of plants growing together in a specific area (Wahab et al., 2010). Vegetation structure depends upon the prevailing environmental conditions (Khan et al., 2011). Plant community is the collection of functional units of species both spatially and temporally. The changes in the community structure are due to seasonal variations and the sampling time (Forzieri et al., 2011; Ali et al., 2018; Hussain et al., 2019). Therefore, the community structure reflects seasonal aspects. The key environmental factors influencing the makeup of the community include over-grazing, deforestation, trampling, construction of buildings, soil erosion, chopping and other natural factors (Ali and Malik, 2010). Vegetation is also influenced by atmospheric humidity, soil moisture, aspect and grazing intensity (Ahmad, 2009; Shaheen and Qureshi, 2011). Vegetation plays a significant role in ecosystem composition and worldwide water balance (Kucharik et al., 2000). Soil is the main factor that determines the different features of vegetation species in particular area (Khan et al., 2010a). Physio-chemical properties of the soil also influence the plants distribution pattern on small scale (Bakkenes et al., 2002). Topography is also an important determinant of vegetation structuring as it alters other environmental factors like temperature, humidity etc. Difference in altitudes, slope and aspect also support different communities (Arora, 2002). The present study was conducted in Terich Valley Chitral of Hindukush range. Five monitoring sites were established viz. Shagrom, Warimon, Zondrangam, Rosh Gol

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and Ghari. On the basis of importance values (IV) 10 different plant communities were established at monitoring site. Different environmental factors such as temperature, soil type, topography and biotic factors influence the composition and the distribution of plant communities in the investigated area. A lot of quantitative phytosociological work has been published from various parts of Pakistan. Ahmed et al. (2006) surveyed different climatic regions of Himalaya and recorded 4 mono-specific forests for analyses of vegetation. Ali et al. (2007) described plants associations and other ecological characteristics of rangelands of Dir. Khan et al. (2010b) observed the physiognomy of *Monotheca buxifolia* forest in Lower Dir with altered elevations and established 6 species associations with *Monotheca buxifolia*. Rashid et al. (2011) recorded the communities structure of Malam Jabba, swat. Ahmed et al. (2011) investigated forests of *Cedrus deodara* in Himalayan and Hindukush mountainous range. Khan et al. (2012) documented the vegetation pattern of forested regions of Chitral. Khan (2012) explored the community structure of *Quercus* species in district Dir. Data were analyzed by Point Centered Quarter method, PC-Ord for cluster classification and Dentrended correspondence analysis for ordination of edaphic and environmental factors. While the major types of *Quercus* sp. vegetation associations were also assessed. Khan et al. (2010c) explored the vegetation-environment relationship of Chitral applying Point Centered Quadrate technique. Mohib Shah (2013) surveyed the phytodiversity and vegetation-environment relationship of Peer Taab graveyard (Swabi). Muhammad et al. (2015) worked on vegetation structure of *Acacia modesta* at various elevational values in Malakand division. Rahman et al. (2016) recorded vegetation composition of *Isodon rugosus* communitie of Khwaza khela, Swat. Khan et al. (2016) worked on pines' forests and their phytosociology in Indus-Kohistan. A total of six groups and four specific plots of *Cedrus deodara* were enlisted. Irshad et al. (2016) recorded the stress on environment about scattering pattern of different species of *Punica granatum* community in district Lower Dir. Ali et al. (2018) documented 15 plant communities, 5 for shrubs, 5 for herbs and 5 for trees of Hindukush Range Swat Pakistan. Ilyas et al. (2018) worked on Kabal Valley, Swat Pakistan to describe the vegetation structures of the sub- tropical range. Hussain et al. (2019) quantified different aspects of vegetation in Koh-e-Safaid Range, Upper Kurram, Pakistan, and summed up seven plant communities of trees, shrubs and herbs, and no studies have addressed the vegetation structure of Terich valley, Chitral a series in the famous Hindukush Mountain range in Pakistan. Being situated on "ecotone zone" this region exhibits special geological features and hence special sort of vegetation distribution. The present study in this region was therefore, aimed to document vegetation structure and characteristics species to inform future conservation strategies.

Materials and methods

Study area

Terich valley is a charming valley of Chitral, Pakistan which provides a wide range of habitat to wild flora. It is located 72º 07′ to 73º 97′ E longitude and 35º 20′ to 36º 55′ N latitude in Chitral. It is bounded on the North by Tajikistan, on the West by Badakhshan, on the South by Nuristan while on the East by District Ghizer of Gilgit-Baltistan (*Fig. 1*). Rugged and uneven terrain characterizes the valley. The climate is cold temperate type. Temperature ranges from -12 ºC in winter to 30 ºC in summer. Phytogeographically, Terich valley lies in the Irano-Turranian floristic region.

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Floristically, the Irano-Turranian region is luxuriant occupied 45.6% of the flora of Pakistan. In Terich valley, Terich Mir (7685 m) the highest peak of the Hindukush range is followed by Saraghrar (7349 m), Shakawar (7116 m), Langar Peak (7100 m) and many other peaks with low altitudes.

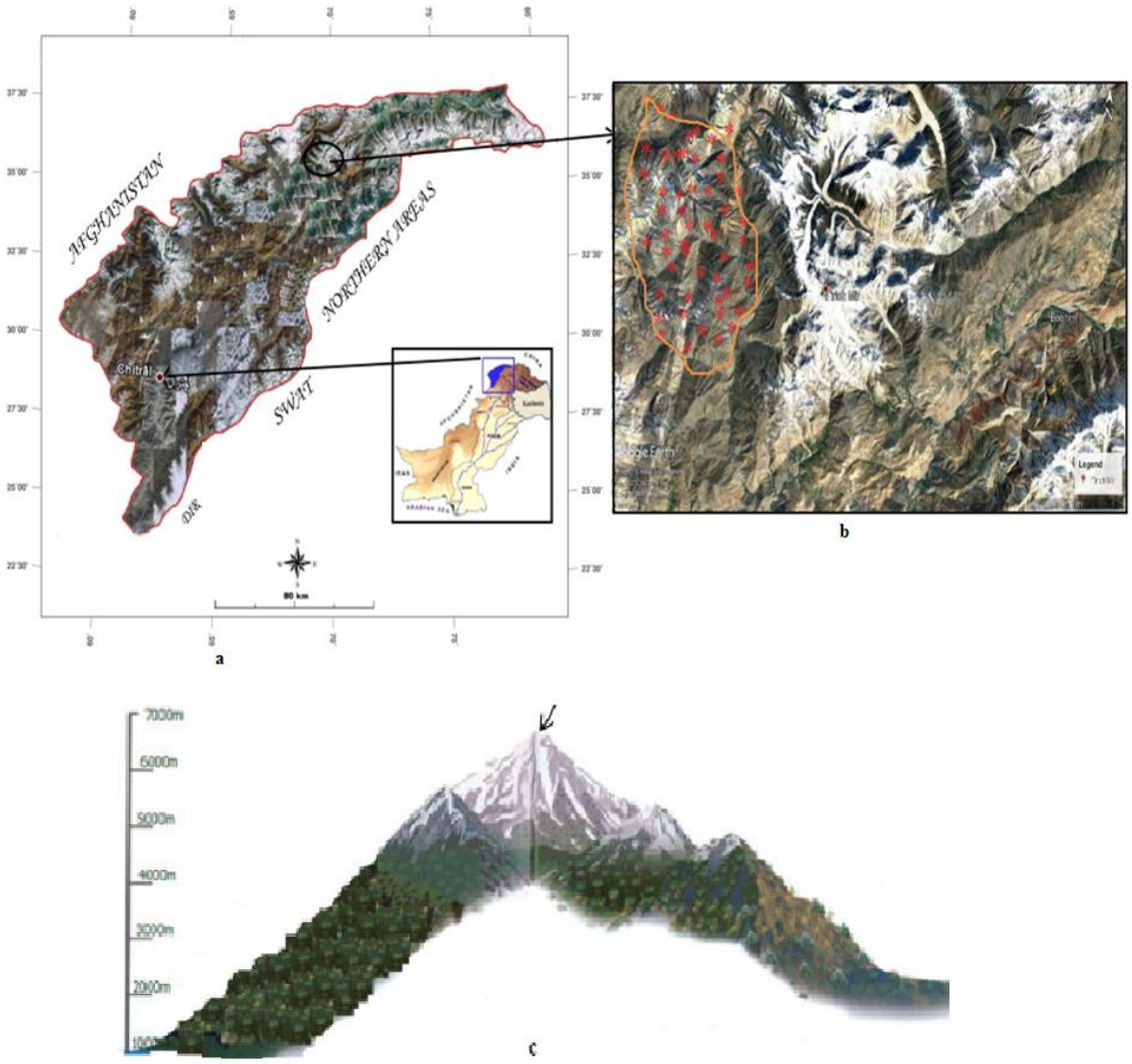

Figure 1. *a) Geo-reference Map of Chitral, b) The study area showing the sampling sites, c) View of Research area along Terich Mir*

Phytosociology

Phytosociological studies were conducted in 5 monitoring sites (*Table 1*). These sites were selected based on altitude, topography, species composition and physiognomic features. Vegetation was evaluated by using 15 (1.5 m^2) quadrats for herbs, 10 (5 m^2) quadrats for shrubs, 5 (10 m^2) quadrats for trees at each monitoring site. Phytosociological attributes viz. Density, cover and frequency of each species was measured and values were changed to relative values to calculate IV (Importance value). The plant communities were established based on highest importance values following (Hussain, 1989; Ahmad and Shaukat, 2012).

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Table 1. *Locations, geographical and ecological characteristics of the sampling sites*

Main Location	Altitude (m)	Slope (°)	Aspect	Soil type	GPS Coordinates	Vegetation
Shagrom	1000-2500	12 17	Foot & Mid-hills	Sandy loam	71.41 35.54	Herbaceous
Warimon	1600-2420	25 29	Foot & Mid-hills		71.46 35.53	Scrubby
Zondrangam	1760-2350	15 32	Foot & Mid-hills		71.05 35.45	Scrubby
Rosh Gol	3440-3650	16 33	North facing & South facing		71.41 35.53	Sub-alpine
Ghari	2950-4200	27 22	North facing & South facing		71.41 35.41	Alpine

Species diversity

Species diversity was obtained by Hussain (1989) method.

$$\text{Diversity Index (D)} = \frac{\sum n(n-1)}{N(N-1)} \quad \text{(Eq.1)}$$

where,
D= Simpson index,
N= Number of individuals of all species,
n= Number of individuals of a species.

The obtained value was subtracted from 1 to obtained Diversity Index.

Species richness

Species richness was calculated after Menhinick (1964) using the following formula.

$$d = \frac{S}{\sqrt{N}} \quad \text{(Eq.2)}$$

where,
d= Species richness,
S= Total number of species in a stand,
N= Total number of individuals in a stands.

Similarity index

Similarity index was calculated by using Sorensen's index (Sorensen, 1948) as modified by Motyka et al. (1950).

$$Is_{mo} = \frac{2W}{A+B} x100 \quad \text{(Eq.3)}$$

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where,
Is= index of similarity of Motyka,
W= Sum of the lowest quantitative value of species common to both stands A and B,
A= Sum of the quantitative value of all species in stands/community A,
B= Sum of the quantitative value of all species in stand/community B.

Maturity index

Community maturity index was obtained by Pichi-Sermolli (1948) method.

$$\text{Degree of maturity index} = \frac{Ft}{N} \quad \text{(Eq.4)}$$

where,
Ft= Frequency values of all species in a stand,
N= Total number of species in a stand.

Importance value index (IVI)

The ecological importance of a species in relation to the community structure was obtained by adding the relative values of density, frequency and cover.

The equation used for the calculation of IVI is given as under:

$$\text{Importance value index (IVI)} = \frac{\text{D3} + \text{C3} + \text{F3}}{3} \quad \text{(Eq.5)}$$

Soil analysis

About 1 kg soil sample (upto a depth of 15 cm) was taken from each five localities of Terich valley. The collected samples were then mixed to get a composite sample, kept in polythene bags and taged appropriately for analyses in the research laboratory. The soil samples analyses were carried out at Pakistan Tobacco Board, Mardan for determination of physio-chemical properties. Soil Texture triangle was used for the identification of texture classes (Bouyoucos, 1936; Brady, 1990). Soil pH was noted by using 1:5 soil water suspensions (Black, 1965). For quantification of organic matter in soil followed Walkley (1947). Lime % ($CaCO_3$) was determined by acid base nutrilization following Rayan et al. (1997). Nitrogen concentration was estimated with Kjeldahl methodology (1983) and Na Concentration was examined by using flame photometery. Potassium and Phosphorus contents were quantified following Olsen and Sommers (1982).

Results

Present study was conducted in Terich valley, Chitral Hindukush range Northern Pakistan during 2016-19. Five selected sites viz. Shagrom, Warimon, Zondrangam, Rosh Gol and Ghari were established. The study area shows a distinctive floristic composition and vegetation pattern. A total of 195 species were documented in five sampling sites. Based on importance value (IV) at Shagrom, Warimon, Zondrangam, Rosh Gol and Ghari from each monitoring sites two communities each were recorded (*Table 2*). The vegetation of the area was mostly scrubby, characterized by herbaceous, shrubby plants and rarely trees.

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Table 2. *Importance value data for plant species*

S.No	Plant Species	Site I	Site II	Site III	Site IV	Site V
A.	**Trees**					
1.	*Betula chitralica* Browicz	0	0	0	27.21	0
2.	*Betula utilis* D.Don	0	0	0	0	17.75
3.	*Cotoneaster affinis* var. *bacillaris* (Lindl.) Schneider	0	12.06	8.19	0	0
4.	*Crataegus songarica* C. Koch.	0	14.42	7.56	0	0
5.	*Crataegus wattiana* Hemsl. & Lace, J .L.	0	0	0	0	11.07
6.	*Elaeagnus angustifolia* var. *angustifolia*	24.31	7.57	10.93	0	0
7.	*Fraxinus xanthoxyloides* (G. Don) DC.	11.66	19.63	0	0	0
8.	*Linaria odora* (M.B.) Fisch.	0	0	8.65	0	0
9.	*Pistacia atlantica subsp. cabulica*	0	0	0	15.83	0
10.	*Prunus griffithii* (Boiss.) C.K.Schneid	10.16	9.53	0	0	0
11.	*Prunus jacquemontii* Hook.f.	19.99	0	0	0	0
12.	*Prunus prostrata* Labill.	7.81	0	0	0	0
13.	*Salix pycnostachya* Andersson.	8.98	0	0	0	0
B.	**Shrubs**					
14.	*Berberis calliobotrys* Aitch.ex Koehne.	0	20.74	0	0	0
15.	*Berberis lyceum* Royle.	18.58	0	0	0	0
16.	*Berberis parkeriana* Schneid.	0	11.91	0	0	0
17.	*Buxus wallichiana* Baill, Monogr.	13.29	0	0	0	0
18.	*Clematis orientalis* L.	8.66	0	0	0	0
19.	*Cotoneaster racemiflorus* (Desf.) Booth ex Bosse	15.3	0	0	0	0
20.	*Cousinia pycnoloba* Boiss.	0	0	0	0	25.74
21.	*Hippophae rhamnoides* Rousi.	0	0	17.36	0	0
22.	*Juniperus communis* L.	1.71	0	0	0	0
23.	*Lonicera myrtillus* Hook. f. & Thoms.	0	0	7.44	0	0
24.	*Rhamnus prostrata* Jacq.ex Parker	0	11.03	9.74	0	0
25.	*Rosa beggeriana* Schrenk.	0	11.81	11.81	0	0
26.	*Rosa ecae* Aitch.	0	0	0	16.10	0
27.	*Rosa webbiana* Wall.ex. Royle.	0	17.93	0	0	0
28.	*Sophora mollis* subsp. *duthiei* (Prain) Ali	0	0	13.86	0	0
29.	*Tamaricaria elegans* (Royle) Qaiser & Ali	0	13.32	10.77	0	0
C.	**Herbs**					
30.	*Acantholimon leptostahyum* Aitch.	0	0	0	14.45	0
31.	*Acantholimon longiflorum* Boiss.	0	0	0	14.73	0
32.	*Acanthophylum laxiflorum* Boiss.	0	17.91	0	0	0
33.	*Adiantum venustum* D. Don	19.66	0	0	0	0
34.	*Alajja rhomboidea* (Benth.) Ikonn.	0	0	0	0	24.63
35.	*Allium caroliniannum* DC.	0	0	0	10.66	0
36.	*Allium chitralicum* Wang & Tang	12.88	0	0	0	0
37.	*Aloitis smithii* Omer.	0	0	0	0	18.95
38.	*Amaranthus viridis* L.	0	12.45	0	0	0
39.	*Anethum gravelons* L.	15.61	0	0	0	0
40.	*Arnebia euchroma* (Royle ex Benth.) I. M. Johnston	0	0	0	13.53	0
41.	*Arnebia griffithii* Boiss., Diagn.	0	0	0	12.12	0
42.	*Arnebia hispidisma* (Lehm.) A. DC	0	0	0	14.77	0
43.	*Artemisia parviflora* Roxb ex. D. Don	0	0	0	22.76	0
44.	*Artemisia persica* Boiss, Diagn.	0	0	0	17.47	0
45.	*Astragalus coluteocarpus* Boiss. ssp. *chitralensis* Wenninger, Mitt. Bot.	0	0	0	0	17.76
46.	*Astragalus minuto-foliolatus* Wendelbo	0	0	0	0	11.79
47.	*Asyneuma strictum* Wendelbo.	0	0	0	10.47	0
48.	*Acantholimon stocksii* Boiss.	14.53	8.87	13.84	0	0

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49.	*Acantholimon longiscapum* Bokhari	0	20.98	0	0	0
50.	*Achillea millefolium* subsp. *chitralensis*	9.47	0	0	0	0
51.	*Adonis aestivalis* L.	10.26	0	0	0	0
52.	*Agrostis nervosa* Nees ex Trin.	9.06	13.09	0	0	0
53.	*Agrostis viridis* Gouan, Hort.	0	11.41	0	0	0
54.	*Alcea nudiflora* (Lindl.) Boiss.	11.63	13.60	0	0	0
55.	*Anemone rupicola* var. *sericea* Hook.f.& Thomson	10.08	13.14	0	0	0
56.	*Anthemis cotula* L.	9.66	11.81	0	0	0
57.	*Artemisia scoparia* Waldst.& Kit.	10.49	0	0	0	0
58.	*Artemisia sieversiana* Ehrh.	17.11	0	0	0	0
59.	*Askellia flexuosa* (Ledb.) W.A. Weber.	11.46	0	0	0	0
60.	*Aster flaccidus* Bunge.	8.047	0	0	0	0
61.	*Astragalus affghanus* Boiss.	0	0	23.43	0	0
62.	*Astragalus amberstianus* Bth.ex. Royle.	0	0	19.69	0	0
63.	*Astragalus imitensis* Ali.	13.69	0	0	0	0
64.	*Astragalus edelbergianus* Sirj & Rech.f.	0	0	21.64	0	0
65.	*Asyneuma strictum* Wendelbo.	0	0	11.74	0	0
66.	*Atriplex schugnanica* Iljin.	7.46	0	0	0	0
67.	*Anaphalis stantonii* Y. Nasir.	0	0	0	0	29.48
68.	*Artemisia brevifolia* Wall ex DC.	0	0	0	0	16.64
69.	*Artemisia elegantissim* Pamp., Nuovo Giorn.	0	0	0	0	18.44
70.	*Asplenium septentrionale* (L.) Hoffm.	0	0	0	0	18.56
71.	*Bromus japonicus* Thunb. ex Murr., Syst.	0	0	0	0	10.9
72.	*Brachypodium distachyon* (L.) P. Beauv.	0	12.77	0	0	0
73.	*Brachypodium sylvaticum* (Huds.) P. Beauv.	0	0	9.16	0	0
74.	*Brassica campestris* L.	16.95	0	0	0	0
75.	*Bromus oxyodon* Schrenk.	0	0	0	12.93	0
76.	*Bunium persicum* (Boiss.) Fedtsch. Rastit	0	6.66	0	0	0
77.	*Brachyactis roylei* (Candolle) Wendelbo.	0	12.21	0	0	0
78.	*Bromus danthoniae* Trin.	11.65	0	0	0	0
79.	*Bromus persicus* Boiss.	0	10.17	0	0	0
80.	*Bromus ramosus* Huds.	0	11.22	9.93	0	0
81.	*Bromus tectorum* L.	10.26	0	0	0	0
82.	*Bupleurum kohistanicum* E. Nasir	0	0	11.20	0	0
83.	*Calendula officinalis* L.	0	6.84	0	0	0
84.	*Calamagrostis decora* Hook.f.	0	0	0	0	13.66
85.	*Crepis multicaulis* Ledeb.var. *congsta*	0	0	0	0	12.91
86.	*Cystopteris fragilis* (L.) Bernh.	0	0	0	0	16.33
87.	*Capsella bursa-pestoris* L.	18.44	0	0	0	0
88.	*Cardaria draba* (L.) Desv	0	0	10.20	0	0
89.	*Carex stenocarpa* Turcz.ex V. Krecz	0	0	13.50	0	0
90.	*Cerastium cerastioides* (L.) Britton.	0	0	0	10.76	0
91.	*Chenopodium botrys* L.	0	9.17	0	0	0
92.	*Chenopodium murale* L.	16.72	0	0	0	0
93.	*Chesneya cuneata* (Benth.) Ali.	0	0	0	0	9.68
94.	*Cirsium griffithii* Boiss.	0	0	0	11.38	11.93
95.	*Cirsium wallichii* var. *glabratum* (Hook. f.) Wendelbo	0	0	13.52	0	0
96.	*Clematis alpina* var. *sibirica* (L.) O. Kuntze, Verh.	0	13.32	0	0	0
97.	*Convulvulus arvensis* L.	9.83	0	0	0	0
98.	*Conyza aegyptiaca* (L.) Dryand. ex Aiton	16.38	0	0	0	0
99.	*Conyza canadensis* (L.) Cronquist.	0	8.30	7.44	0	0

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100.	*Coriandrum stivum* L.	0	9.57	10.26	0	0
101.	*Coronopus didymus* (L.) Sm.	14.26	0	0	0	0
102.	*Crepis aitchisonii* Boiss.	0	0	0	10.24	0
103.	*Crepis multicaulis* Ledeb. var. *congsta*	0	0	0	0	7.46
104.	*Cuscuta villosa* L.	0	0	0	9.79	0
105.	*Cynodon dactylon* (L.) Pers.	18.14	0	0	0	0
106.	*Calamagrostis pseudophragmites* subsp. *speudophragmites* (Hall.f.) Koel.	0	0	13.05	0	0
107.	*Capparis spinosa* L.	15.33	14.4	0	0	0
108.	*Chenopodium foliosum* (Merrich.) Aschers.	8.04	0	0	0	0
109.	*Cichoriun intybus* L.	11.87	0	0	0	0
110.	*Cirsium arvense* (L.) Scop.	8.66	15.23	14.90	0	0
111.	*Codonopsis clematidea* (Schrenk) C.B. Clarke.	8.26	0	0	0	0
112.	*Dicanthium annulatum* Forssk. Stapf.	0	0	9.98	0	0
113.	*Dactylorhiza umbrosa* (Kar. & Kir.) Nevski.	0	0	0	12.93	0
114.	*Draba tibetica* var. *chitralensis* (O. E. Nasir) Jafri.	0	0	0	10.21	0
115.	*Equisetum ramossimum* Desf.	0	7.20	8.59	0	0
116.	*Ephedra gerardiana* Wall.ex Stapf.	0	0	15.87	0	0
117.	*Eremurus stenophyllus* subsp. *stenophyllus* S. I. Ali	0	0	21.04	0	0
118.	*Ferula narthex* Boiss.	19.5	0	0	0	0
119.	*Ferula jaeschkeana* Vatke.	0	0	0	11.29	0
120.	*Ferula hindukushensis* Kitamura.	0	0	0	0	17.71
121.	*Festuca olgae* (Regel) Krivot.	0	0	0	0	12.16
122.	*Fragaria nubicola* (Hook.f.) Lindl.ex Lacaita	0	0	10.37	0	0
123.	*Glycyrrhiza glabra* var. *glandulifera* (Waldst. & Kit.) Boiss.	0	0	9.68	0	0
124.	*Heracleum polyadenum* Rech.f. & Riedl.	0	0	0	0	12.16
125.	*Juniperus excelsa* M. Bieb	0	0	19.69	0	0
126.	*Kobresia laxa* Nees, Contr.	0	0	0	0	22.75
127.	*Koelpinia linearis* Pall. var. *linearis*	0	0	0	0	15.58
128.	*Lactuca serriola* L.	11.89	7.57	0	0	0
129.	*Lagochilus cabulicus* Bth.	0	0	0	0	11.26
130.	*Lindelofia anchusoides* (Lindl.) Lehm.	0	0	8.53	0	0
131.	*Lolium temulentum* L.	0	0	9.6	0	0
132.	*Malcolmia cabulica* var. *topppinii* (O.E. Schulz) Nasir	0	0	8.5	0	0
133.	*Myricatis wallichii* Less.	0	0	0	0	7.26
134.	*Matthiola flavida* Boiss.	0	0	8.59	0	0
135.	*Melica persica* Kunth, Rev. Gram.	0	0	0	10.04	0
136.	*Melilotus officinalis* (L.) Pall., Reise.	0	9.17	0	0	0
137.	*Mentha longifolia* (L.) Huds.	14.88	9.03	0	0	0
138.	*Myosotis avensis* (L.) Hill.	0	0	0	9.39	13.28
139.	*Oxyria digyna* (L.) Hill, Hort.	0	0	0	0	21.35
140.	*Oxytropis chitralensis* Ali.	0	0	0	0	20.96
141.	*Pennisetum flaccidum* Griseb	12.37	6.66	0	0	0
142.	*Poa bulbosa* L.	0	0	0	18.20	0
143.	*Poa pratensis* subsp. *pratensis*	0	0	13.62	0	0
144.	*Polygonatum geminiflorum* Decne.	8.49	7.57	9.34	0	0
145.	*Primula macrophylla* var. *macrophylla* D. Don	0	0	0	11.96	8.25
146.	*Pseudognaphalium luteo-album* (L.), O. M. Hilliard & B. L Burtt	0	0	0	14.52	0
147.	*Pseudomertensia chitralensis* (Riedl) Riedl	0	0	0	0	13.39

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148.	*Psoralea drupaceae* Bunge.	0	0	6.76	0	0
149.	*Prangos pabularia* Lindl.	0	24.82	10.58	0	0
150.	*Piptatherum laterale* (Munro ex Regel) Rozhev	0	0	0	0	13.66
151.	*Poa alpina* L.	0	0	0	0	9.49
152.	*Psychrogeton chitralicus* Grierson.	0	0	0	0	16.13
153.	*Puccinellia minuta* Bor.	0	0	0	0	20.25
154.	*Ranunculus laetus* Wall.ex Hook.f. & Thoms.	0	11.22	12.37	0	0
155.	*Ribes orientale* Desf.	0	21.79	12.34	0	0
156.	*Rheum webbianum* Royle.	0	0	0	13.67	0
157.	*Rhodiola heterodonta* (Hook.f. & Thomson) Boriss.	0	0	0	9.47	0
158.	*Rhodiola wallichiana* (Hook.) S.H. Fu	0	0	0	22.0	0
159.	*Rorippa islandica* (Oeder) Borbas.	0	0	0	9.20	0
160.	*Rosularia adenotricha* subsp. *adenotricha*	0	0	0	9.82	0
161.	*Rosularia alpestris* (Kar & Kir) Boiss.	0	0	0	22.06	8.06
162.	*Rubia tibetica* Hook.f.	0	0	0	16.34	0
163.	*Rumex hastatus* D. Don	10.37	0	0	0	0
164.	*Saussurea leptophylla* Hemsl.	0	0	0	11.03	0
165.	*Scandix pecten-veneris* L.	0	7.75	0	0	0
166.	*Scutellaria multicaulis* Boiss.	0	0	0	20.79	0
167.	*Seriphidium brevifolium* (Wall. ex DC.) Ling & Y. R. Ling	0	0	0	17.79	0
168.	*Seriphidium chitralense* (Podlech)Y. R. Ling	0	0	0	13.17	14.18
169.	*Silene conoidea* L.	0	8.84	0	0	0
170.	*Silene vulgaris* (Moench) Garcke.	0	0	9.22	0	0
171.	*Sisymbrium brassiciforme* C. A. Mey.	0	0	8.53	0	0
172.	*Solanum nigrum* L.	0	6.84	0	0	0
173.	*Solenanthus circinnatus* Ledeb.	0	0	0	9.11	0
174.	*Sonchus asper* (L.) Hill.	0	0	7.93	0	0
175.	*Stellaria decumbens* Edgew.	0	0	5.2	0	0
176.	*Stellaria media* (L.) Vill.	0	10.67	0	0	0
177.	*Stipa chitralensis* Bor.	0	8.99	0	0	0
178.	*Tanacetum chitralense* (Podlech) K.	0	9.67	0	0	0
179.	*Tetrapogon villosus* Desf.	0	16.0	0	0	0
180.	*Thlaspi perfoliatum* L.	0	13.1	0	0	0
181.	*Trisetaria loeflingiana* (L.) Paunero	0	14.3	0	0	0
182.	*Tanacetum griffithii* (C. B. Clarke) Muradyan.	0	0	0	0	19.95
183.	*Taraxacum brevirostre* Hand-Mazz. var. *lanatum.*	0	0	0	13.49	0
184.	*Taraxacum chitralense* Soest.	0	0	0	0	13.36
185.	*Taraxacum officinale* Weber.	0	9.57	6.13	0	0
186.	*Taraxacum tricolor* V. S	0	0	0	15.88	8.82
187.	*Taraxacum wendelboanum* Soest.	0	0	0	0	9.65
188.	*Taraxacum obtusum* (Soest) R. Doll	0	0	0	0	8.74
189.	*Tragopogon gracilis* D. Don.	0	0	0	10.08	0
190.	*Tricholepis toppinii* Dunn.	0	0	0	14.90	16.37
191.	*Tussilago farfara* L.	0	0	0	7.75	0
192.	*Verbascum thapsus* L.	0	0	9.06	0	0
193.	*Verbena officinalis* L.	0	8.30	5.5	0	0
194.	*Xanthium strumarium* L.	0	8.627	7.44	0	0
195.	*Youngia japonica* (L.) DC.	0	12.14	0	0	0

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A. Community structure of foothills vegetation

Shagrom (Site-I)

Elaeganus-Prunus-Adiantum community (EPA)

This plant community was established at Shagrom at an elevation of 1000 m-1550 m having 21 species. *Elaeganus angustifolia* (IV. 24.31), *Prunus jacquemontii* (IV. 19.99), and *Adiantum venustum* (IV. 19.66) were the dominant plant species. Co-dominant species were the *Berberis lyceum*, *Capsella bursa-pestoris*, *Brassica campestris*, *Conyza aegyptiaca*, *Cynodon dactylon* and *Mentha longifolia* (*Table 2*). Soil of this site was basic in nature having pH 8.5 and electrical conductivity 0.4 dsm^{-1}. Similarly soil texture has been reported with 2.6% clay, 38.0% silt and 58.86% sand, respectively. Organic matter was 2.05% followed by calcium carbonate 13%. The nitrogen content 0.1035%, phosphorus 15.28 mg/kg and potassium 114.33 mg/kg was recorded respectively (*Table 3*).

Table 3. *Edaphic variables, mineral content and plant communities at 5 monitoring sites*

Parameters	Foothills			Midhills			Sub-alpine & Alpine			
	S	**W**	**Z**	**S***	**W***	**Z***	**RGN**	**RGS**	**GN**	**GS**
Clay %	2.66	4.56	20.61	5.67	0.81	11.6	6.51	6.3	2.79	2.91
Silt %	38	30.66	38.01	42	18.01	15	24.99	25.9	34.2	30.20
Sand %	58.86	62.86	39.50	51.39	76.41	70.4	72.21	70.2	61.21	61.2
Texture class	**Sandy loam**									
pH	8.5	6.63	8.1	6.67	6.39	8.1	6.6	6.6	7.8	7.5
O-M %	2.05	2.16	2.28	2.1	1.87	1.55	0.81	0.84	0.75	0.78
Lime %	13	16.25	8.4	5.01	5.49	11.4	0.51	0.51	2.7	2.49
Ec $x10^3$ dsm^{-1}	0.4	0.06	0.29	0.12	0.20	0.21	0.48	0.48	0.26	0.36
N %	0.10	0.11	0.11	0.10	0.08	0.08	0.12	0.14	0.12	0.15
P (mg/kg)	15.28	5.87	12.16	20.39	55.67	12.2	14.01	15.9	12	15.99
K (mg/kg)	114.3	96.33	187	116	238	132	183.8	186	84.2	88.2

Key: **S**-Shagrom, **W**-Warimon, **Z**-Zondrangam, **S***-Shagrom, **W***-Warimon, **Z***-Zondrangam, **RGN**-Rosh Gol Northern slope, **RGS-**Rosh Gol Southern slope, **GN-**Ghari Northern slope, **GS-**Ghari Southern slope

Warimon (Site-II)

Fraxinus-Rosa-Acanthophyllum community (FRA)

This community was established at an altitude of 1200 m-1600 m comprised of 30 species. Dominant species of this community includes *Fraxinus xanthxyloides* (IV. 19.63), *Rosa webbiana* (IV. 17.93) and *Acanthophyllum laxiflorum* (IV. 17.92). Co-dominant shrub species include *Crataegus songarica*, *Tamaricaria elegans*, *Rhamnus prostrata*, *Clematis alpina*, and *Cotoneaster affinis* who grew around this community (*Table 2*). Soil pH was 6.6 and electrical conductivity 0.06 dsm^{-1}. The soil consisted of 4.56% clay, 30.66% silt and 62.86% sand, textured with sandy loam. Soil contained 2.16% organic matter and 16.25% calcium carbonate. Nitrogen content was 0.114% which was better than Shagrom community. Phosphorus content was 5.87

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mg/kg and potassium 96.33 mg/kg was also estimated which has a normal range required for the plant growth (*Table 3*).

Zondrangam (Site-III)

Hippophae-Sophora-Poa community (HSP)

This community was established at altitude of 1320 m-1760 m having 32 species. The dominant species of this community was *Hippophae rhamnoides* (IV. 17.36), *Sophora mollis* (IV. 13.86) and *Poa pratensis* (IV. 13.62). The co-dominant species were *Carex stenocarpa*, *Cirsium willichi*, *Cardaria draba*, *Coriandrum sativum*, *Lolium temulentum*, and *Tamaricaria elegans* (*Table 2*). Soil was sandy loam of this site with 20.61% clay, 38.01% silt, and 39.501% sand. The pH was 8.1 and 0.291 dsm^{-1} electrical conductivity was also calculated. Similarly, organic matter 2.28% and calcium carbonate was estimated 8.4%. Nitrogen content was recorded 0.117% followed by phosphorus 12.16 mg/kg and potassium 187 mg/kg, respectively (*Table 3*).

B. Community structure of midhills vegetation

Shagrom (Site-I)

Ferula-Artemisia-Capparis community (FAC)

This community was composed of 26 species located at an altitude of 2250 m-2500 m. *Ferula narthex* (IV. 19.55), *Artemisia sieveriana* (IV. 17.11), and *Capparis spinosa* (IV. 15.33) were the dominant species. The co-dominant species of this community were *Cotoneaster racemiflorus*, *Juniperus communis*, *Cichorium intybus*, *Acantholimon stocksii*, and *Astragalus imitensis* (*Table 2*). The soil pH was 6.6 and electrical conductivity 0.129 dsm^{-1} which represents acidic nature of soil. The soil texture was sandy loam and composed of clay 5.67%, silt 42.0% and sand 51.39%. Organic matter was estimated 2.1% and calcium carbonate 5.01%. The concentration of nitrogen was calculated 0.104%, phosphorus 20.391 mg/kg and 116 mg/kg potassium, respectively (*Table 3*).

Warimon (Site-II)

Prangos-Ribes-Berberis-community (PRB)

Along 2320 m-2420 m altitude *Prangos-Ribes-Berberis*-community was recorded having 22 species. *Prangos pabularia* (IV. 24.82), *Ribes orientale* (IV. 21.79) and *Berberis calliobotrys* (IV. 20.74) were dominant species. The co-dominant species of this community includes *Tetrapogon villosus, Trisetaria loeflingiana, Thlaspi perfoliatum* and *Cirsium arvensis* (*Table 2*). The texture of the soil recorded sandy loamy which contains clay 0.681%, silt 18.01% and sand 76.41%. The pH of the soil was 6.3 and electrical conductivity 0.201 dsm^{-1}. Chemical analysis revealed 1.87% organic matter and 5.49% calcium carbonate. The nitrogen concentration was estimated 0.087% followed by 55.67 mg/kg phosphorus and 238 mg/kg potassium (*Table 3*).

Zondrangam (Site-III)

Astragalus-Astragalus-Eremurus community (AAE)*

This community consisted of 20 species which was reported along 2260 m-2350 m altitude. The dominant species were *Astragalus affghanus* (IV. 23.43), *Astragalus edelbergianus* (IV. 21.64) and *Eremurus stenophyllus* (IV. 21.04). *Juniperus excelsa,*

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Astragalus amberstianus, Acantholimon longiscapum, and *Ephedera gerardiana* were co-dominants (*Table 2*). Chemical analysis of soil revealed 1.551% organic matter, 11.49% calcium carbonate, 0.081% nitrogen, 12.24 mg/kg phosphorus, 132 mg/kg potassium, pH 8.1 and 0.021 dsm^{-1} electrical conductivity. The soil texture represents sandy loam contributed clay 11.67%, silt 15.0% and sand 70.41% (*Table 3*).

C. Community structure of sub-alpine and alpine vegetation

Rosh Gol (Site-IV)

Northern slope

Artemisia-Rhodiola-Rosularia community (ARR)

Artemisia-Rhodiola-Rosularia community was recorded from Rosh Gol along (South facing) of sub-alpine region at an altitude of 3125 m-3440 m. This community was consisted of at had 23 species. The dominant species were *Artemisia parviflora* (IV. 22.76), *Rhodiola wallichiana* (IV. 22.03) and *Rosularia alpestris* (IV. 22.06). *Seriphidium brevifolium, Rosa ecae, Pseudognaphalium luteo-album, Artemisia persica,* and *poa bulbosa* were co-dominants (*Table 2*). Texture of the soil was sandy loam with clay 6.51%, silt 24.99% and sand 72.21%. The soil of this site had 0.81% organic matter and 0.51% calcium carbonate. Amount of nitrogen 0.12%, phosphorus 14.01 mg/kg and potassium 183.8 mg/kg was estimated. The pH was calculated 6.6 and electrical conductivity 0.489 dsm^{-1} (*Table 3*).

Southern slope

Betula-Scutularia-Taraxicum community (BST)

This community consisted of 21 species at Rosh Gol along (North facing) of the sub-alpine region at an altitude of 3320 m-3650 m. The dominant species were *Betula chitralica* (IV. 27.21), *Scutellaria multicaulis* (IV. 20.79) and *Taraxacum tricolor* (IV. 15.88). The co-dominant species were *Tricholepis toppinii, Taraxacum brevirostre, Seriphidium chitralensis,* and *Arnebia hispidisma* (*Table 2*). The pH of soil was 6.6 and electrical conductivity 0.48 dsm^{-1}. The soil was sandy loam with clay 6.3%, silt 25.94% and sand 70.2%. Chemical properties of the soil showed organic matter 0.84% and calcium carbonate 0.51%, respectively. The nitrogen concentration 0.141%, phosphorus 15.99 mg/kg and potassium 186.8 mg/kg was investigated (*Table 3*).

Ghari (Site-V)

Northern slope

Anaphalis-Cousina-Kobresia community (ACK)

This community had 19 species at Ghari site of the alpine zone (South facing) located at an elevation of 2870 m-2950 m. *Anaphalis stantonii* (IV. 29.48), *Cousinia pycnoloba* (IV. 25.74) and *Kobresia laxa* (IV. 22.25) were dominants; while *Puccinellia minuta*, *Psychrogeton chitralicus, Cystopteris fragilis, Ferula hindukushhensis, Koelpinia linearis,* and *Asplenium septentrionale* were co-dominants (*Table 2*). Soil of this site had sandy loam texture with clay 2.79%, silt 34.2% and sand 61.2%. The pH was calculated 7.89 and electrical conductivity 0.2679 dsm^{-1}. Similarly, organic matter 0.75% and calcium carbonates 2.7%. Nitrogen concentration was recorded 0.12%, followed by phosphorus 12 mg/kg and potassium 84.2 mg/kg (*Table 3*).

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Southern slope

Alajja-Oxyria-Oxytropis community (AOO)

Ghari alpine zone (North facing) located at an altitude of 3620 m-4200 m. The community of this site had 23 species. The dominant species of this community included *Allaja rhomboidea* (IV. 24.63), *Oxyria digyna* (IV. 21.35) and *Oxytropis chitralensis* (IV. 20.96). *Tanacetum grifthii, Alotis smithii, Astragalus coluteocarpus,* and *Betula utilis* were co-dominants (*Table 2*). Soil of this site was sandy loam in texture with 2.91% clay, 30.201% silt and 61.2% sand particles. Soil pH was 7.5 and organic matter content was estimated 0.78%. Calcium carbonate was found to be 2.49%. Similarly, concentration of nitrogen 0.15%, phosphorus 15.99 mg/kg and potassium 88.2 mg/kg was also estimated (*Table 3*).

Similarity index

According to Motyka's index of similarity (Motyka et al., 1950), FRA community was found to be 46.1% similar to HSP community. The similarity between PRB community and A*AE community was to be found 28.5%. The analysis showed that similarity between EPA community and that of FRA community was 23.52%. Similarly, index of FAC and PRB communities was 8.33%. EPA community was 7.54% similar to HSP community. Only 5.12% similarity was observed between A*AE community and ACK community. Similarity between ACK, EPA communities and ACK, BST communities was recorded 5% each; followed by similarity of A*AE, EPA communities and ACK, PRB communities 4.87% each. Similarity index was observed for EPA, ARR, EPA, BST, ACK, ARR and ACK, AOO 4.76% each respectively. EPA community showed minimum similarity with PRB community i.e. 4.65%, while the remaining communities had less than 4% similarity index among themselves (*Table 4*). Seasonal variation was the main cause of similarity index between communities. The high similarity index between the communities is due to characteristics such as similarity in composition of nutrients, the proximity of stands to each other that had almost similar habitat conditions in terms of N, P, K, pH and soil from clay loam to sandy loam. The high similarity index among some plant communities during the monsoon and spring seasons might be due to the occurrence of the same shrubs, trees, evergreen perennial herbs and geophytes while most of plant communities dominated by annuals showed least similarity among themselves.

Simpson diversity index

Species diversity was obtained by Hussain (1989) method. Species diversity is affected by various biotic and abiotic factors including elevation, climatic conditions, edaphic characteristics, grazing, browsing, and anthropogenic activities. Species diversity is the main attribute of vegetation dynamics, which reflects the composition and productivity of vegetation. Species diversity calculates the complexity and function within the community. At Site-I Simpson's diversity was found to be 0.1149 followed by Site-II and Site-III with diversity values of 0.159 and 0.1069, respectively. At Site-IV diversity was 0.1004 followed by Site-V with a value of 0.111, Site-VI 0.118, Site-VII 0.127, Site-VIII 0.110 and Site- X had diversity value of 0.174. The lowest value was recorded for Site-IX i.e. 0.106 (*Table 5, Fig. 2*).

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Table 4. *Similarity index for 10 different communities*

Communities	EPA	FRA	HSP	FAC	PRB	AAE	ARR	BST	ACK	AOO
EPA	X	X	X	X	X	X	X	X	X	X
FRA	23.52	X	X	X	X	X	X	X	X	X
HSP	7.54	46.1	X	X	X	X	X	X	X	X
FAC	4.25	3.57	3.44	X	X	X	X	X	X	X
PRB	4.65	3.84	3.70	8.33	X	X	X	X	X	X
AAE	4.87	4.0	3.84	4.34	28.5	X	X	X	X	X
ARR	4.76	3.77	3.63	4.08	4.44	4.65	X	X	X	X
BST	4.76	3.92	3.77	4.25	4.65	4.87	4.54	X	X	X
ACK	5.0	4.08	3.92	4.44	4.87	5.12	4.76	5.0	X	X
AOO	4.54	3.77	3.63	4.08	4.44	4.34	4.37	4.45	4.76	X

Key: **EPA-**Elaeagnus-Prunus-Adiantum community, **FRA-**Fraxinus-Rosa-Acanthophylum community, **HSP-**Hippophae-Sophora-Poa community, **FAC-**Ferula-Artemisia-Capparis community, **PRB-**Prangos-Ribes-Berberis community, **AAE-**Astragalus-Astragalus-Eremurus community, **ARR-**Artemisia-Rhodiola-Rosularia community, **BST-**Betula-Scutellaria-Taraxacum community, **ACK-**Anaphalis-Cousinia-Kobresia community, **AOO-**Alajja- Oxyria-Oxytropis community

Table 5. *Simpson's diversity, among different communities*

Sites	Communities	Simpson's diversity index
Site-I	*Elaeagnus-Prunus-Adiantum*	0.111
Site-II	*Fraxinus-Rosa-Acanthophylum*	0.159
Site-III	*Hippophae-Sophora-Poa*	0.1069
Site-IV	*Ferula-Artemisia-Capparis*	0.1004
Site-V	*Prangos-Ribes-Berberis*	0.111
Site-VI	*Astragalus-Astragalus-Eremurus*	0.118
Site-VII	*Artemisia-Rhodiola-Rosularia*	0.127
Site-VIII	*Betula- Scutellaria-Taraxacum*	0.110
Site-IX	*Anaphalis-Cousinia-Kobresia*	0.106
Site-X	*Alajja-Oxyria-Oxytropis*	0.174

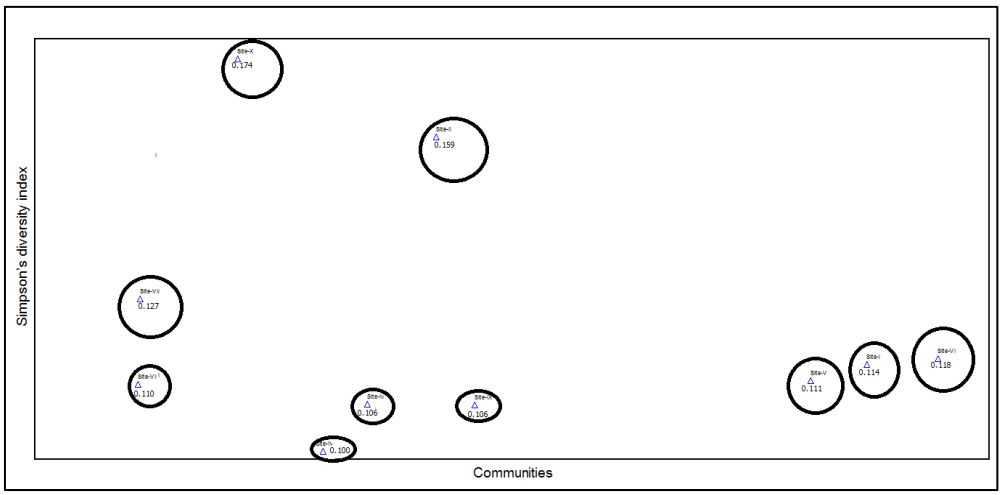

Figure 2. *Simpson's diversity, among communities through PC-Ord*

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Species richness

Species richness was calculated after Menhinick (1964). The species richness of a specific region is the result of various environmental factors such as geography, topography, species pool, area productivity and species competition. In the present study the maximum species richness was recorded 0.132 at Rosh Gol North slope followed by 0.104 at Shagrom Midhills, 0.18 at Ghari south slope and 0.16 at Warimon. The high species richness is directly linked with favorable environmental conditions and soil factors. The low species richness 0.12 was recorded in the foothills of Shagrom. Similarly, midhills of Warimon and the north slope of Ghari contributed 0.11 species richness, followed by foothills 0.10 species richness of Zondrangam and south slope of Rosh Gol respectively (*Table 6, Fig. 3*). Variation in altitude and physicochemical properties of soil may also have profound influence on species richness. The species richness was high during spring but decreased during the monsoon season. This is due to dominance of annuals and geophytes during spring which disappear at the onset of the monsoon season. The low species richness was due to lack of water, high temperature, overgrazing and deforestation. However, in contrast to this, the species richness follows the interpolated richness pattern.

Table 6. *Species richness and maturity index among different communities*

Sites	Communities	SR	MI
Site-I	*Elaeagnus-Prunus-Adiantum*	0.12	31.42
Site-II	*Fraxinus-Rosa-Acanthophylum*	0.16	27.00
Site-III	*Hippophae-Sophora-Poa*	0.10	27.18
Site-IV	*Ferula-Artemisia-Capparis*	0.10	37.69
Site-V	*Prangos-Ribes-Berberis*	0.11	31.36
Site-VI	*Astragalus-Astragalus-Eremurus*	0.12	35.5
Site-VII	*Artemisia- Rhodiola- Rosularia*	0.13	32.60
Site-VIII	*Betula- Scutellaria-Taraxacum*	0.10	37.14
Site-IX	*Anaphalis-Cousinia-Kobresia*	0.11	35.26
Site-X	*Alajja-Oxyria-Oxytropis*	0.18	31.30

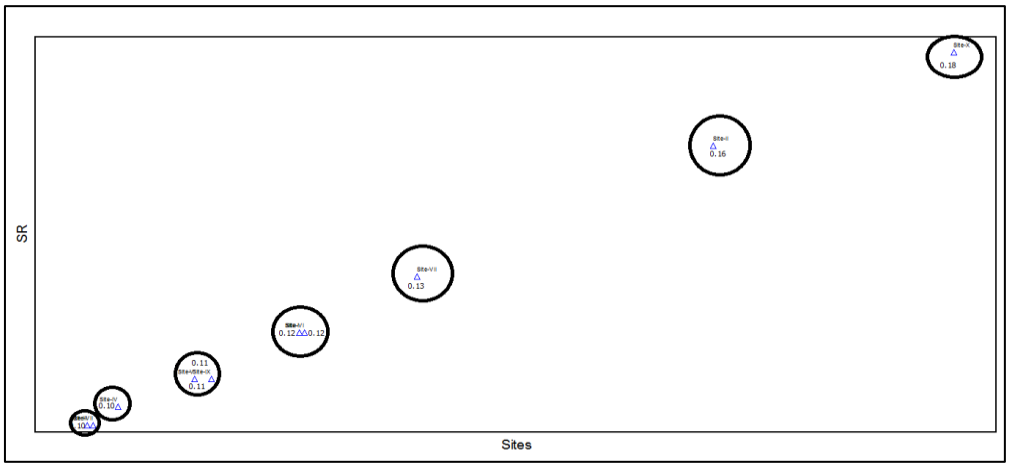

Figure 3. *Species richness among communities through PC-Ord*

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Maturity index

Maturity index was calculated according to Pichi-Sermolli (1948). This is a new index for appraising the vegetation pattern of an area affected by environmental and edaphic conditions. During the present study, index of maturity of communities ranged from 27.0 to 37.69. The highest value of maturity index 37.69 was recorded in the communities of Shagrom. Similarly, maturity index of the communities of south slope Rosh Gol 37.14, followed by the midhills of Zondrangam 35.5. The maturity index of communities in North slope Ghari 35.26 and North slope Rosh Gol was 32.60. Maturity index of the communities of foothills Shagrom was 31.42, while in the midhills communities of Warimon was 31.36. The lowest maturity index 27.18 was reported in south slope communities of Ghari and foothills communities of Warimon 27.0 (*Table 6, Fig. 4*). All the stands were immature which might be due to lesser adaptation to the microclimate of the study sites. This was further enhanced due to high anthropogenic pressures which disturbed the natural balance of the plant communities and prevented them from attaining maturity. Low maturity reflects the heterogeneity within the plant communities as a result of poor adaptability to the ecological conditions of area.

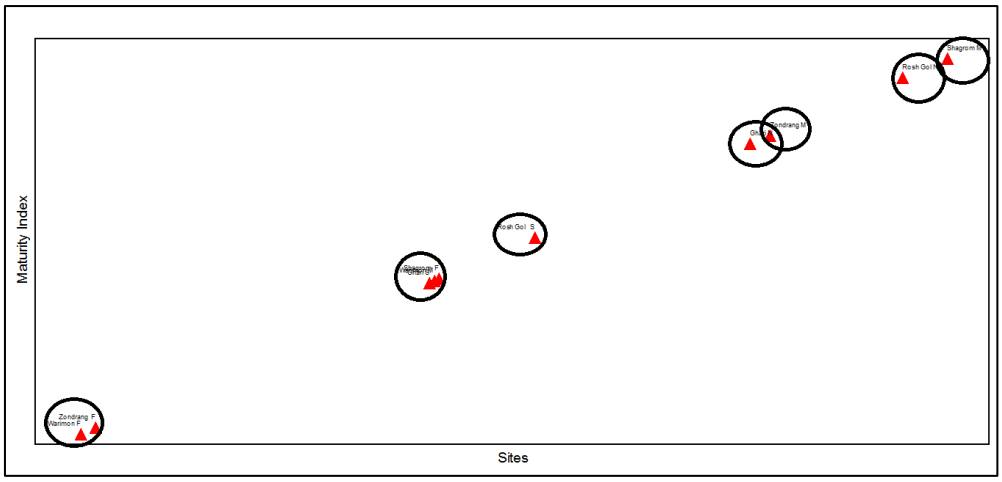

Figure 4. *Maturity index among communities through PC-Ord. Key to abbreviations used in Figs. 2, 3 & 4: Site-I: Shagrom (Foothills), Site-II: Warimon (Foothills), Site-III: Zondrangam (Foothills), Site-IV: Shagrom (Midhills), Site-V: Warimon (Midhills), Site-VI: Zondrangam (Midhills), Site-VII: Rosh Gol Northern slope, Site-VIII: Rosh Gol Southern slope, Site-IX: Ghari Northern slope, Site-X: Ghari Southern slope*

Discussion

During current study the vegetation of Terich valley, was classified into ten plant communities based on importance values (IVs) which is in line with the works of Hussain et al. (2010, 2019), Ali et al. (2018), Srivastava et al. (2016), Gandhi and Sundarapandian (2014), Altay et al. (2012), Zhang and Zhang (2011), Tsiourlis et al. (2009), Liu (2008), Hirst and Jackson (2007) and Oswalt et al. (2006). At Site-I foothills and midhills EPA-FCA communities were established. Distribution of trees was very rare due to lavish wood cutting in this site. Inadequate trees and relatively thick bushes spread close to the lower regions showed the disturbed nature of habitat which is under gigantic biotic pressure. Expanding population and development of

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residential units is the main reason behind the species loss in this locality. Similar communities are reported from their respective study areas by Ali et al. (2018), Hussain et al. (2015), Mehmood et al. (2015), Ilyas et al. (2015) and Rashid et al. (2011). Site-II foothills and midhills had FRA-PRB communities. Species of Trees and shrubs showed a clumped dispersion pattern at these monitoring sites. Effects of soil disintegration were more visible at Site-I and Site-II where dissolved soils had herbaceous individuals like *Conyza aegyptiaca*, *Cynodon dactylon*, *Crataegus songarica*, *Tamaricaria elegans*, *Rhamnus prostrate* and *Verbascum thapsus* which are the indicator species of eroded soils. Similar communities but with different IV are reported by Hussain et al. (2019), Khan et al. (2016), Ummara et al. (2015), Forzieri et al. (2011) and Wahab et al. (2010). Site-III foothills and midhills exhibited HSP-AAE communities. This site had a uniformly spread thick scrubs of *Juniperus, Astragalus, Acantholimon,* and *Ephedera*. Deforestation was likewise not excessively disturbing when contrasted with that of foothills and midhills of Terich valley monitoring sites. At higher altitude the valley was practically undisturbed anyway the environment will confront biotic pressure, particularly the anthropogenic stresses from increasing population in the low laying villages in the next few years. These findings are backed by works of Hussain et al. (2015), Khan et al. (2016), Ali et al. (2015), Ilyas et al. (2015), Sharma et al. (2014), Ali and Malik, (2010) and Wahab et al. (2010). ARR-BST communities were found at north and south slopes of site-IV. This sub-alpine zone supported the richness of *Artemisia parviflora*, *Rhodiola wallichiana*, *Rosularia alpestris, Seriphidium brevifolium, Rosa ecae, Pseudognaphalium luteo-album, Artemisia persica,* and *poa bulbosa.* This is line with findings of Ali et al. (2018), Ilyas et al. (2015), Siddiqui et al. (2011), Ahmed et al. (2011) and Hussain et al. (2004). At north and south slopes of site-V, ACK-AOO communities were recorded from this alpine zone. Plants of this site was found healthy with the special case on Northern slants where deforestation was very clear because of fuel wood extraction by local people for *Betula utilis*. These results are in line with the works of Haq et al. (2015), Naveed (2014), Ilyas et al. (2012), Khan et al. (2010, 2011), and Ahmed et al. (2009).

Conclusion

This research shows vegetation dynamics based on 195 sampling units at 5 monitoring sites. Soil analyses cover 11 parameters with different properties at each site. Variations in edaphic variables, temperature, humidity and slope are due to spatial variations among plant communities at different monitoring sites. Vegetation is endangered in the study region and needs to be conserved until it's too late. The vegetation cover decreases anthropogenic factors, over grazing, natural calamities, and erosion. Extraction of Terich valley trees by local communities, particularly during harsh winters, not only reduces the density of the tree, but also increases the rate of soil erosion. Climate change has not spared vegetation in the Hindukush range and with each passing year, floral diversity is growing. Impacts of anthropogenic pressure for maturity index are evident from the low ratings. Highest similarity index was recorded for community FRA which was found to be 46.1% similar to HSP community. Highest maturity index value was recorded for communities FAC at Site-IV i.e. 37.69 followed by communities BST at Site-VIII i.e. 37.14. Species richness of community AOO was estimated high 0.18 at Site-X. At Site-X Simpson's diversity was found to be maximum for community AOO 0.17 followed by community FRA at Site-II with diversity values

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of 0.15, respectively. Low maturity index values suggest the plant communities' inability to cope with anthropogenic, edaphic, and climatic stresses. Likewise, low values of index of similarity indicate the heterogeneity between plant communities. The structure of vegetation indicates local flora is fighting a war of its survival. Once covered under a thick blanket of vegetation, bare patches are now visible on mountain slopes. Proper management of the grazing should be ensured for understory plant regeneration. To check the erosion due to landsliding and flood, fast-growing plant species must be cultivated to rehabilitate the degraded and fragmented habitat. A comprehensive program must be designed through involving local masses, conservationists, Governmental and Non-Governmental Organizations to take useful measures for threatened plants and their conservation with special focus on endemic species to mitigate their extinction risk.

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RESPONSE OF EUROPEAN BREAD WHEAT TO DIFFERENT VERNALIZATION TREATMENTS UNDER THE ENVIRONMENTAL CONDITIONS OF KURDISTAN-IRAQ

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Abstract. A factorial experiment was conducted in Kurdistan-Iraq to identify responses of European wheat varieties to vernalization treatments at 22ºC, 4ºC and -18ºC for two and four weeks in a Complete Randomized Block Design (CRBD) experiment with three replicates. Sabir-beg had the highest and most desired performance for most of the traits studied including grain yield. The highest spike and grain number were recorded for Hereford and Skalnige, respectively. Incubation temperatures had highly significant effects on most of the traits, including grain yield. Two weeks-vernalization at low temperature is satisfactory for vernalization of these wheat varieties. The interaction between varieties and incubation temperature produced varying effects. Grain number is mainly genotype-related. Sabir-beg×two weeks incubations had spent minimum days to flowering, followed by days to maturity. The varieties were persistent in their interaction with the incubation period. The interaction effect of 4ºC×four weeks incubation advanced flowering, extending flowering-maturation period to give a higher spike number. Harvest index, biomass, and grain yield were highly significant for both interactions of varieties×incubation temperature and varieties×incubation period. The three-factors interaction had a highly significant effect on most traits. The wheat varieties can be developed with more predictable responses to *in vitro* vernalization as an alternative to early sowing.
Keywords: *variety, in vitro, low temperature, VRN gene, grain yield*

Introduction

One of the critical stages in plant development is flowering, that affects crop production. The stage of crop vegetative/reproductive transition is a combination of plant responses to the environment including stresses (Kazan and Lyons, 2014). Optimal products can be obtained by the management of these stages. The time to flowering must coincide with favorable conditions to guarantee the species survival via producing viable seeds. Vernalization, a prolonged period of low temperature, is one of the environmental stimuli that is required to initiate flowering (Michaels, 2009; Dixon et al., 2019). Vernalization, as a plant cue, is required to transit the plant to reproductive stages, which means shifting from the vegetative growth to flowering and seed production (Trevaskis, 2010; Woods et al., 2019). This phenomenon is promoted by a chilling treatment (Chouard, 1960; Muterko and Salina, 2018). The effect of vernalization is to reduce the production period of leaf primordia (Griffiths et al., 1985), and consequently reduce the number of leaves and the tillers initiated on the main shoot (Gott et al., 1955; Levy and Peterson, 1972).

Winter cereals have a quantitative vernalization requirement for flowering (Woods et al., 2019). It has been identified that vernalized plants are more prone to flower earlier than non-vernalized (Robertson et al., 1996). Winter wheat genotypes grown under temperate regions can be vernalized at a seedling stage or even at full grain maturity, during the chilling period of winter (< 4°C) (Clay et al., 2012; Mureşan et al., 2019).

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Bread wheat is one of the most cultivated grain crops occupying a wide range of lands worldwide, its winter varieties are planted in the autumn and they have adequate tolerance to survive freezing temperatures in winter. This adaptation occurs due to the reduction of moisture content and the accumulation of soluble carbohydrates in the crown area of the wheat plant, causing the reduction in growth rate at this stage, providing the plant resistance to frost temperature (Clay et al., 2012). Hence winter wheat varieties usually have higher yield potential than spring varieties planted later in the spring because of their longer growing period and vernalization explosion (Galiba et al., 2009; Li et al., 2013). Response to vernalization is one of the most important factors affecting environmental adaptation of wheat (Wang et al., 1995; Whittal et al., 2018). This action has been found to be under the effect of three genes of VRN1, VRN2, andVRN3, as a major gene for the vernalization in winter wheat. This was mapped using a population of recombinant inbred lines (RILs) generated from two winter wheat cultivars (Yan et al., 2006, 2015). After exposing the plants to non-freezing temperature for certain periods of time the cold-responsive genes are activated (Armonienė et al., 2013). Based on the exposing period of cold temperature to promote flowering on time, winter wheat has three categories; a weak winter type requires less than two weeks, a semi-winter type requires 2-4 weeks exposing of low temperature for flowering: while a strong type requires longer cold exposure (4 weeks and more) for timely flowering (Crofts, 1989). Flowering in wheat occurs after all main-stem leaves have appeared and are fully expanded. It is the consequence of the rate of leaf appearance which is controlled mostly by the temperature of the apical meristem and leaf expansion zones (McMaster and Wilhelm, 2003). *Triticum aestivum* L. (wheat, 2n = 6x = 42) is known to be cultivated across the most variable land area than any other grain crop (Rozbicki et al., 2019).

Vernalization requirement of winter wheat varies according to their genetic construction (Whittal et al., 2018). Varieties obtain sufficient vernalization when sown at an early time in autumn, while in case of late sowing with any reason, varieties will not start flowering on time (Mureşan et al., 2019), making the plant to have reduced flower or have late initiation, making the yield to be reduced quantitatively and qualitatively. The objective of this study was to identify the response of winter wheat varieties to different vernalization treatments in the control environment before planting, aiming at establishing the latest safe sowing date for winter wheat varieties, with a reasonable yield.

Materials and methods

A factorial experiment was conducted in the experimental field of the College of Agricultural Engineering Sciences, University of Sulaimani, Kurdistan of Iraq, located at Lat. 35˚ 34′ 307″; N, Long. 45˚ 21′ 992″; E, 765 m asl. CRBD experiment was conducted for five varieties, four European and one local variety (factor A), see *Table 1*.

Grains were treated with three different temperatures, namely at room temperature (22^{o}C), at laboratory condition, 4^{o}C (in a refrigerator) and at -18^{o}C, in a freezer (factor B), for two different periods of two and four weeks (factor C). The incubation started on 12th November 2018 for four weeks incubation and on 27th November 2018 for two weeks incubation, to be directly sown on 12th December after termination of the designated period, see metrological data in *Table 2*. All treatment combination were sown in three replicates. Each replicate was applied on an area of 0.60 m^2, planting was performed on lines of two-meter length, distanced 30 cm between the lines, and 50 cm between replications.

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Table 1. *Names and the sources of five wheat varieties used in the study*

No.	Name	Pedigree/origin	Source
1	Mariboss	Denmark	DCA - Danish Centre For Food And Agriculture, Aarhus University
2	Skalnige	Denmark	DCA - Danish Centre For Food And Agriculture, Aarhus University
3	Hereford	Denmark	DCA - Danish Centre For Food And Agriculture, Aarhus University
4	Ritraw	Denmark	DCA - Danish Centre For Food And Agriculture, Aarhus University
5	Sabir-beg	Local variety, Iraq	Bakrajo research Station, Sulaimani, Iraq

Table 2. *Meteorological data of Sulaimani- Iraq for the growing season 2018-2019*

Month	Temperature			Rainfall (mm)
	Min. Temp. (C°)	Max. Temp. (C°)	Average Temp. (C°)	
October 2018	9.6	32.0	19.2	48.2
November 2018	6.2	22.0	12.6	99.8
December 2018	-2.6	15.0	5.2	281.8
January 2019	0.8	13.8	5.4	210.6
February 2019	-2.0	17.4	6.8	108.2
March 2019	1.6	21.2	10.8	248.6
April 2019	2.4	26.0	13.2	190.0
May 2019	12.8	33.8	17.6	28.4
Total rainfall (mm)				1215.6

The land was tilled twice, smoothed, and plots were prepared before sowing. The varieties were sown on 12th December 2018 containing 30 treatment combinations in which they were homogenous experimental units. Grains size from all varieties was unified by sieving, to obtain persistent population density in the field. After incubation treatment for designated periods, the grains were planted within the lines for all replicates. Each replicate consisted of 3.5 g with uniform size.

The recommended doses of 80 kg N/ha and 80 kg P_2O_5/ha were applied for the experiment and all other agricultural practices were conducted as required. There was no chemical application (herbicide, herbicide, or pesticide) for the treatments, and the experiment was run under rainfed condition. Some physical and chemical properties of the experimental soil are given in *Table 3*.

Table 3. *Physical and chemical properties of the studied soil*

Soil properties	Soil texture (P.S.D)	Sand ($g.kg^{-1}$)	Silt ($g.kg^{-1}$)	Clay ($g.kg^{-1}$)	E.C. ($dS.m^{-1}$)	pH	O.M. ($g.kg^{-1}$)	$CaCO_3$ ($g.kg^{-1}$)	Total N ($mg.kg^{-1}$)	K^+ (L^{-1})
Values	Clay	41.0	430.50	528.50	0.61	7.32	21.6	107.0	1.07	0.12

Note: E.C.: electronic conductivity; O.M.: organic matter; $CaCO_3$: Calcium Carbonate; K^+: potassium ion

The measurements of agro-morphological traits for growth characteristics, yield, and its components were recorded. Measured traits were:

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- Plant Height: recorded at physiological maturity, height was recorded as the length in centimeters from the soil surface to the tip of spike excluding awns.
- Days to flowering: recorded as the number of days required from sowing till flower initiation.
- Days to maturity: recorded as the number of days required from sowing till physiological maturity.
- Spike number/m^2: calculated as a number of spikes per unit area (m^2).
- Grains/spike: counted as an average of the number of grains per spike for 5 selected spikes randomly per each plot.
- 1000- Kernel weight (g): one thousand grain weight (g) were taken at random from a bulk grain yield of each replicate.
- Biomass yield (ton/ha): recorded as the weight of all biological products of the plant above the soil surface per unit area.
- Harvest index (HI): Counted as a percentage of grain weight to biomass, according to the following equation (Shewry, 1998).

$$H.I. = \frac{\text{Grain yield}}{\text{biomass yield}} \quad \text{(Eq.1)}$$

- Grain yield (ton/ha): calculated as a yield of grains of unit area, converted to ton per hectare.

Data were recorded for the agronomic traits, yield, and its components, and analyzed using XLSTAT 2016 software. The source of variance and comparison between the genotypes were determined. The comparisons of trait means were made using Duncan's multiple range test at 5% level of probability.

Results and Discussion

Studying phenological alteration such as vernalization is essential in winter wheat (*Triticum aestivum* L.), to cope with the adverse effects of global warming on crop production, especially under arid and semi-arid conditions (Shourbalal et al., 2019). It has been indicated that each degree increase in temperature results in the reduction of wheat production by 6% (Asseng et al., 2015). Advancing the process of flowering, through partial phenological alteration in winter wheat, is an important step to reduce the effect of drought (Searle and Coupland, 2004). To induce yield production of winter wheat in mild and dried condition, a period of vernalization is required to initiate reproductive stage following vegetative growth (Heide and Sønsteby, 2015).

The analyzed results indicated a significant variance of the genotypes and their interaction treatments with variable incubation temperature and the exposure period to the incubated temperature (*Table 4*). Vernalization determines flowering and heading times, giving a significant influence on the adaptability of wheat plants to different environmental conditions, that mainly contribute to establishing yield and yield components (Slafer, 2003; Chen et al., 2013). Varieties had a highly significant variance for all the studied traits. The levels of incubation temperature had highly significant effects on days to maturity, spike number/m^2, grain number/spike, 1000 kernel weight, harvest index, and grain yield, while they had a significant effect on days to flowering.

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Table 4. *Analysis of variance for agronomic traits, yield and its components of wheat varieties, incubation temperature and the period of incubation with their interaction effects*

Source	df	Mean square								
		Plant height (cm)	**Days to flowering**	**Days to maturity**	**Spike number/m²**	**Grain number/spike**	**1000 kernel weight (g)**	**Harvest index**	**Biomass yield (ton/ha)**	**Grain yield (ton/ha)**
Varieties (F1)	4	5293.328**	3195.711**	3678.933**	31225.483**	947.763**	441.031**	0.006**	77.354**	6.612**
Incubation temperature (F2)	2	5.204	7.878*	24.1**	4107.011**	128.795**	19.313**	0.018**	0.644	3.133**
Period of Incubation (F3)	1	8.465*	0.178	33.611*	6.4	2.347	15.528	0.00037	1.686	0.015
Replicates	2	15.481**	1.078	1.9	49.244	4.065	1.801	0.001	1.126	0.037
Factor1*Factor2	8	13.498**	7.919**	14.517**	10485.858**	107.45**	16.118**	0.002**	0.965	0.707**
Factor1*Factor3	4	6.787**	2.567	3.556*	1768.761*	20.051*	24.649**	0.011**	3.193**	1.045**
Factor2*Factor3	2	4.238	12.811**	14.144**	291.433	2.028	49.784**	0.016**	4.313**	3.386**
Factor1*Factor2* Factor3	8	6.103**	20.992**	10.172**	3547.253**	27.449**	22.818**	0.005**	4.638**	0.732**
Error	58	105.445	1.561	0.992	570.474	9.122	0.924	0.0003	0.614	0.015
Minimum		70.5	101	145	232	17.6	15.352	0.14	7.066	1.589
Maximum		117.5	143	182	484	55.75	33.992	0.341	16.813	4.968
Mean		84.378	125.644	170.367	350.822	32.989	23.568	0.248	12.52	3.18
Std. deviation		15.55	12.159	13.025	56.71	8.039	5.177	0.048	2.163	0.794

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Period of incubation for different temperatures had no significant effect on the studied traits, except plant height and days to maturity. The interaction of varieties×incubation temperature had a highly significant effect on all traits except for biomass yield. Distribution of the bio-product between sink and source for the wheat varieties was not influenced by the period of incubation. Both interactions of varieties×incubation temperature and varieties× incubation period had similar effects on the studied traits, as harvest index, biomass yield, plant height, and grain yield, and 1000 kernel weight were highly significant for both interactions. A very close pattern was also stated for the interaction between the incubation temperature and the incubation period. The interaction of all three factors showed a highly significant effect on all traits except grain number/spike.

Wheat varieties had a considerable variable effect on the studied characteristics (*Table 5*). Plant height tends to be the highest in Sabir-beg variety (114.861 cm), while other varieties have a close range of height with each other (only 4 cm differences), their values are ranging from 73.8-78,833cm for Maribos and Ritraw, respectively. The local variety (Sabir-beg) spent shorter period to start flowering (101.94 days from sowing), it was earlier in flowering as well than Hereford with a total of 28 days. The same trend was obtained for days to maturity for Sabir-beg and Hereford, respectively. Maximum days required to maturity was referred to Mariboss, spending 179.7 days after sowing. Hereford had the highest number of spike per unit area (413.667 spike/m^2), while the least number of spikes was referred to Mariboss.

Table 5. *Effect of varieties on agronomic traits, yield and its components of wheat varieties*

Varieties	Plant height (cm)	Days to flowering	Days to maturity	Spike number/m^2	Grain number/ spike	1000 kernel weight (g)	Harvest index	Biomass yield (ton/ha)	Grain yield (ton/ha)
Mariboss	73.807 d	133.833 a	179.722 a	311.889 d	28.983 d	22.822 c	0.250 bc	11.701 c	2.905 c
Skalnige	76.370 c	130.778 bc	177.167 b	339.833 c	40.556 a	19.370 d	0.262 ab	10.069 d	2.738 d
Hereford	78.017 b	130.056 c	174.167 d	413.667 a	34.519 c	19.330 d	0.248 c	12.678 b	3.363 b
Ritraw	78.833 b	131.611 b	175.722 c	369.556 b	38.256 b	25.019 b	0.219 d	12.395 b	2.733 d
Sabirbeg	114.861 a	101.944 d	145.056 e	319.167 d	22.633 e	31.298 a	0.264 a	15.756 a	4.162 a

In terms of grain number/spike the highest number of 40.566 grain was recorded for Skalnige variety, followed by Ritraw, however the least number of grain (22.633) was recorded for Sabir-beg variety. A wide range of 1000 kernel weight was also observed for the varieties under study. The maximum of 31.298 g was recorded for Sabir-beg, while the lowest value of 1000 kernel weight was recorded for Hereford and Skalnige, respectively, with nearly 11 g less weight than Sabir-beg. Harvest Index was also significant for the varieties studied. Sabir-beg had the highest harvest index of 0.264, followed by Skalnige, while the lowest harvest index referred to Ritraw. Biomass was also indicated the variation between the varieties under study. Sabir-beg had indicated the highest biomass yield of 15.756 ton/ha, while the minimum of 10.069 ton/ha was recorded for Skalnige. The same pattern of superiority in the grain yield was recorded for Sabir-beg giving a total of 4.162 ton/ha grain yield. The superiority of local wheat varieties to most of the newly introduced genotypes in another study by Ali et al. (2018) compared to some European varieties for the yield and its component highly supports the current result. The genetic variations that drive a wide response of the genotype understudy could serve the development of a new cultivar with high resilience to future climate changes through the introgression of important traits.

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The levels of temperature treatment had variable effects on the studied traits (*Table 6*). Plant height had no significant difference for different temperature levels. Days to flowering was affected with -18ºC incubation, however the incubation at 4ºC had no significant effect on flowering day. Vernalization sensitivity of some of the wheat varieties could be the reason for delaying flowering in certain environmental conditions (Ortiz-Ferrara et al., 1995), making the temperature level of 4ºC to be with less effect to flowering compared to the normal ambient temperature of 22ºC. The same pattern was also observed for days to maturity, spending less days to maturity (166.333 days) for -18ºC incubation. A positive effect of vernalization on days to flowering has been confirmed by other researchers (Sharma et al., 2012). This trend indicates the effect of low-temperature incubation on the vernalization of the varieties under study, giving the desired signal to vegetation period for some days, allowing plants to shift to flowering and maturity period earlier. Hence, in case of impossibility of sowing in the normal sowing times in autumn or at the beginning of winter, the requirement of vernalization could be afforded *in vitro* as a substitute to early sowing. In terms of the spike number per unit area, the low temperature of 4ºC had a significant effect on the spike number (364.233 spike/m^2).

Table 6. *Effect of incubation temperature on agronomic traits, yield and its components of wheat varieties*

Temperature	Plant height	Days to flowering	Days to maturity	Spike number/m^2	Grain number/ spike	1000 kernel weight (g)	Harvest index	Biomass yield (ton/ha)	Grain yield (ton/ha)
22 ˚C	84.266 a	125.300 a	170.833 a	345.533 b	35.297 a	23.701b	0.268 a	12.669 a	3.475 a
4 ˚C	84.839 a	125.400 a	170.933 a	364.233 a	32.383 b	22.707 c	0.256 b	12.376 a	3.232 b
-18 ˚C	84.029 a	122.233 b	166.333 b	342.700 b	31.288 b	24.295 a	0.221 c	12.514 a	2.835 c

A reverse effect was recorded for the vernalization temperatures on the number of grains per spike, as the increased chilling temperature has negatively reduced the grain number/spike. There were also significant differences in the levels of incubation temperature for 1000 kernel weight. Decreasing the temperature to 4ºC had a negative effect on the weight of kernels, while a further reduction in the incubation temperature to -18ºC had increased the weight of 1000 kernels (24.295 g). Harvest index was negatively affected due to the reduced temperature. Total biomass was not affected by different incubation levels. The accumulation of bio-product is more genetically controlled (Kumar et al., 2016; Brinton et al., 2017), while tracking the product to be accumulated to different parts of the plant is a matter of environmental effect in addition to genetic structure. Grain yield was identified to be reduced by decreasing the incubation temperature.

The incubation period for the wheat varieties seems to have a high effect on plant height and days to maturity (*Table 7*). Plant height had a few increases with the extended incubation period, while days to maturity has decreased with the same trends of incubation extension. No significant increase in grain weight was realized by increasing the period of incubation from two to four weeks duration. This result indicates the satisfaction period of two weeks incubation at low temperature for the wheat varieties under study. The varieties under study might be weak winter genotypes and the 2 weeks duration could be satisfactory for vernalization as extending to four weeks duration of a cold explosion being non-necessary for the current genotypes.

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Table 7. *Effect of incubation period on agronomic traits, yield and its components of wheat varieties*

Period of incubation	Plant height (cm)	Days to flowering	Days to maturity	Spike number/m2	Grain number/ spike	1000 kernel weight (g)	Harvest index	Biomass yield (ton/ha)	Grain yield (ton/ha)
Two weeks	84.071 b	169.756 a	13.383 a	351.089 a	33.151 a	23.153 a	0.250 a	12.383 a	3.193 a
Four weeks	84.984 a	170.978 a	11.657 b	350.556 a	32.828 a	23.983 a	0.247 a	12.657 a	3.167 a

The interaction of varieties×incubation temperature for the grain of wheat varieties had different effects on the studied characteristics (*Table 8*). The interaction between Sabir-beg and 4ºC gave the maximum plant height of 115.583 cm, while the minimum plant height of 72.078 cm was referred to as the interaction between Mariboss and 22ºC incubation. Same interaction effect of Sabir-beg×4ºC was recorded for minimum days required to flowering (101.667 days). The interaction of Sabir-beg variety to other incubation temperatures had the same effect on the days to flowering. The maximum number of days required to start flowering (131.5 days) was recorded for the interaction between Mariboss and 22ºC incubation. Like days to flowering the minimum days to maturity was referred to Sabir-beg interaction with different incubation temperatures, requiring 145 days to maturity, however, the maximum days spent to maturity was recorded for Mariboss with the incubation of 22ºC×4ºC. The maximum spike number of 442 spike/m^2 was identified for the interaction of Hereford×22ºC incubation, followed by the interaction of the same variety with -18ºC. The minimum number of spikes/m^2 was recorded for Mariboss interacted with 22ºC incubation. Grain number is one of the yield component traits that was recorded as a maximum of 49.667 grains/spike for the interaction of Skalnige variety with 22ºC incubation.

Table 8. *Interaction effect of varieties × incubation temperature on agronomic traits, yield and its components of wheat varieties*

Varieties× incubation temperature	Plant Height (cm)	Days to flowering	Days to maturity	Spike number/m^2	Grain number/ spike	1000 kernel weight (g)	Harvest index	Biomass yield (ton/ha)	Grain yield (ton/ha)
Mariboss×22ºC	72.078 h	131.500cde	180.833 a	257.000 k	26.400 f	22.235 fg	0.240 efg	11.886 a	2.838 fg
Mariboss×4ºC	73.155 gh	133.833 b	180.667 a	347.667 efg	30.217 e	22.484 f	0.280 abc	11.393 a	3.140 e
Mariboss× -18ºC	76.189 f	136.167 a	177.667 b	331.000 fgh	30.333 e	23.747 e	0.229 g	11.824 a	2.737 g
Skalnige×22ºC	76.833 ef	130.333cef	179.667 a	359.000 def	49.667 a	19.869 h	0.272 bcd	10.133 a	2.758 fg
Skalnige×4ºC	77.989cde	130.167 ef	177.667 b	316.833 hij	37.833 c	17.133 j	0.257 def	10.067 a	2.558 h
Skalnige× -18ºC	74.289 g	131.833 cd	174.167cd	343.667efgh	34.167 cd	21.108 g	0.256 def	10.006 a	2.900 f
Hereford×22ºC	78.083cde	130.333def	174.000cd	442.000 a	33.233 de	18.696 i	0.284 ab	12.300 a	3.818 c
Hereford×4ºC	78.883 c	130.000 ef	173.667 d	380.333 cd	37.033 cd	19.919 h	0.252 defg	12.427 a	3.505 d
Hereford× -18ºC	77.083 def	129.833 f	174.833cd	418.667 ab	33.289 de	19.374 hi	0.207 h	13.307 a	2.767 fg
Ritraw×22ºC	79.167 c	132.000 c	174.667cd	381.667 cd	42.633 b	27.227 c	0.244 efg	12.958 a	3.169 e
Ritraw×4ºC	78.583 cd	131.333cdef	177.500 b	406.667 bc	35.917 cd	21.816 f	0.234 fg	12.362 a	2.895 fg
Ritraw×-18ºC	78.750 cd	131.500cdef	175.000 c	320.333 ghi	36.217 cd	26.015 g	0.180 i	11.866 a	2.134 i
Sabir-beg×22ºC	115.167ab	102.333 g	145.000 e	288.000 j	24.550 fg	30.477 b	0.298 a	16.069 a	4.790 a
Sabir-beg×4ºC	115.583 a	101.667 g	145.167 e	369.667 de	20.917 g	32.185 a	0.260 cde	15.633 a	4.062 b
Sabir-beg× -18ºC	113.833 b	101.833 g	145.000 e	299.833 ij	22.433 g	31.233 ab	0.233 fg	15.565 a	3.634 d

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The minimum grain number/spike referred to the interaction of Sabir-beg with all incubation treatments. From this result, it seems that the grain number trait is mainly genotype-related (Guo et al., 2015), as the incubation treatment has no significant effect on the improvement of this trait. The interaction of Sabir-beg with the 4°C and -18°C incubations showed the maximum weight of 1000 kernel (32.185 g and 31.233 g, respectively). Skalnige interacting with 4°C incubation had the minimum of 1000 kernel weight (17.1333 g). The interaction of Sabir-beg with the 4°C had the maximum record for harvest index, biomass yield and grain yield, while the minimum record refer to Ritraw interacting with -18°C incubation for both traits of harvest index and grain yield. It is clear that Sabir-beg is the most adapted variety to the local environment, requiring less vernalization, which makes the interaction of this variety with low incubation temperatures to give less effect. In terms of biomass yield the minim value (however not significant) referred to the interaction of Skalnige with all incubation temperatures. Less vernalization effect on the European varieties characteristics in this study could refer to the presence of allelic variation (VRN) at vernalization (Whittal et al., 2018). Presence of another allele (VRN4) or recessive VRN allele to VRNI gene could drive the transcription of this gene without severe needs for vernalization period (Goncharov, 2004; Kippes et al., 2015), or they might be semi-winter cultivars that require less period of low incubation temperature for full vernalization, and capable of switching to generative development under local environmental conditions (Goncharov, 2004; Li et al., 2013). Another possibility is that, planting the current varieties by mid of December did not allow the germinated plant to catch enough short day exposure, as a short day phototropism combined with low temperatures is required to permit VRN1 expression for floral transition of the meristem at an earlier period (Hemming et al., 2008; Li and Dubcovsky, 2008; Whittal et al., 2018), as a result no realized differences between the treated vernalization and controlled wheat grains were recorded.

The interaction between varieties and incubation periods had different effects on the studied characteristics (*Table 9*). Plant height had the maximum value of 115.722 cm for the interaction between Sabir-beg and four weeks incubation, followed by the interaction Sabir-beg×two weeks incubations. A minimum plant height was referred to as Mariboss interacted with four weeks incubations. The same interaction of Sabir-beg×2 weeks incubation had spent the minimum days to start flowering (101.778). Regarding plant height, the maximum days required to start flowering was recorded for Mariboss interacting with two and four weeks incubations. The same pattern was also realized for days to maturity. Spike number was identified to be at the maximum rate (423.00 spike/m^2) for Hereford interacting with two weeks incubation, while the least value was recorded for the interaction of Mariboss×2 weeks incubation, giving 296.444 spike/m^2.

A maximum number of 40.978 and 40.133 grain/spike was identified for Skalnige interacting with two and four weeks incubations, while the least number of 22.39 grains/spike referred to Sabir-beg interacting with two weeks interaction. The highest 1000 kernel weight was 31.488 g and 31.109 g for Sabir-beg interacting with two and four weeks incubations, respectively. While the interaction of Hereford with four weeks incubation had the lowest weight of 18.506 g for 1000 grain of wheat. Harvest index was identified to be at a maximum rate of 0.289 for Mariboss interacting with four weeks of incubation. Biomass yield had the maximum value of 15.773 and 15.739 ton/ha for Sabir-beg variety interacting with two and four weeks of incubation, respectively. A minimum value of 9.231 ton/ha was recorded for Skalnige interacting with two weeks incubation. Grain yield also had the same pattern as biomass yield, giving the maximum

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yield of 4.197 and 4.127 ton/ha for the interaction of Sabir-beg with two and four weeks of incubation, respectively. The minimum grain yield was recorded for Mariboss interacting with 2 weeks incubation. The results here indicated the superiority of Sabir-beg interacting with both incubation periods, which surpassed other varieties in the majority of traits. The persistence of the genotypes is a major insight for this experiment, as the incubation of four weeks period had no significant change compared to the two weeks incubation.

Table 9. *Effect of varieties and period of storage interaction on agronomic traits, yield and its components of wheat varieties*

Varieties × Incubation period	Plant height (cm)	Days to flowering	Days to maturity	Spike number/m^2	Grain number/ spike	1000 kernel weight (g)	Harvest index	Biomass yield (ton/ha)	Grain yield (ton/ha)
Mariboss×2 weeks incubation	74.352 f	133.333 a	178.889 b	296.444 f	30.867e	20.521d	0.210 e	11.954cd	2.522 f
Mariboss×4 weeks incubation	73.263 f	134.333 a	180.556 a	327.333 de	27.100 f	25.123bc	0.289 a	11.449de	3.289 c
Skalnige×2 weeks incubation	75.859 e	131.222 a	176.111 c	347.000 cd	40.978 a	19.076 ef	0.281ab	9.231 f	2.838 e
Skalnige×4 weeks incubation	76.881de	130.333 a	178.222 b	332.667 de	40.133ab	19.664 de	0.242 d	10.906 e	2.638 f
Hereford×2 weeks incubation	77.200 d	130.333 a	173.778 e	423.000 a	33.815 d	20.154 d	0.264bc	12.496bc	3.578 b
Hereford×4 weeks incubation	78.833 c	129.778 a	174.556de	404.333 a	35.222cd	18.506 f	0.232 d	12.859 b	3.149 d
Ritraw×2 weeks incubation	78.944 c	131.778 a	174.889 d	372.556 b	37.700bc	24.524 c	0.232 d	12.460bc	2.902 e
Ritraw×4 weeks incubation	78.722 c	131.444 a	176.556 c	366.556 bc	38.811ab	25.514 b	0.207 e	12.330bc	2.563 f
Sabir-beg×2 weeks incubation	114.000b	101.778 a	145.111 f	316.444 ef	22.394 g	31.488 a	0.261c	15.773 a	4.127 a
Sabir-beg×4 weeks incubation	115.722a	102.111a	145.000 f	321.889 e	22.872 g	31.109 a	0.266bc	15.739 a	4.198 a

The effect of different vernalization temperatures and the period of incubation is clarified in *Table 10*. The interaction between incubation temperature and the period of incubation was not significant however the highest plant height of 85.44 cm was recorded for 4^{o}C×4 weeks incubation. The same interaction was significant for days to flowering, recording the least days spent to flowering, while it had the highest and most significant number of days to maturity. It seems from this result that the interaction effect of 4^{o}C incubation with four weeks period had allowed the flowering to be advanced, allowing the plant to stay longer in the flowering stage giving a higher number of spike per unit area with reasonable biomass. Proper management of the flowering period results in higher productivity, however, the highest yield of 3.584 has resulted from the interaction of 4^{o}C×2 weeks incubation. Significant effect of vernalization in wheat has been investigated previously at the temperature below 10^{o}C (Robertson et al., 1996).

Same interaction of 4^{o}C×4 weeks incubation showed the highest potential of 365.333 spikes per square meter, however it is not statistically significant. Grain number/spike was also not significant for the interaction between the incubation temperature and the period of incubation, while the maximum value of 35.43 grain/spike was recorded for the interaction between 22^{o}C×2 weeks incubation. The interaction

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of -18°C×4 weeks incubation showed the highest value of 26.182 g for 1000 kernel weight. The interaction of 4°C×2 weeks incubation gave the highest harvest index of 0.281, while the lowest value referred to the interaction of -18°C×2 weeks incubation. Biomass yield recorded the maximum value of 13.007 followed by 12.931 ton/ha for the interaction of -18°C×4 weeks incubation and 22°C×2 weeks incubation, respectively. Grain yield was identified to reach a maximum of 3.584 ton/ha for the interaction of 4°C×2 weeks incubation, the lowest grain yield of 2.515 ton/ha has been identified for the interaction between -18°C and 2 weeks incubation. This combination treatment between different incubation temperatures and their periods could explore the efficiency of 4°C with two weeks incubation could be satisfactory to induce the vernalization in the wheat varieties under study, as any further extension in the duration of cold treatment would not affect a reduction in the flowering period (Kim et al., 2009).

Table 10. *Effect of temperature and period of storage interaction on vegetative growth, yield and its components of wheat varieties*

Temperature × incubation period	Plant Height (cm)	Days to Flowering	Days to maturity	Spike number/m^2	Grain number/ Spike	1000 kernel weight	Harvest index	Biomass yield	Grain yield
22°C×2 weeks incubation	84.382 a	125.267bc	170.867 b	343.600 a	35.430 a	23.834b	0.267 a	12.931 a	3.481b
22°C×4 weeks incubation	84.149 a	125.333bc	170.800 b	347.467 a	35.163 a	23.568b	0.268 a	12.408ab	3.468b
4°C×2 weeks incubation	84.238 a	126.133ab	169.600 c	363.133 a	32.300 a	23.215b	0.281 a	12.197 b	3.584 a
4°C×4 weeks incubation	85.440 a	124.667 c	172.267 a	365.333 a	32.467 a	22.200c	0.232 b	12.556ab	2.880d
-18°C×2 weeks incubation	83.593 a	125.667 b	168.800 d	346.533 a	31.722 a	22.408c	0.201 c	12.021 b	2.515 e
-18°C×4 weeks incubation	84.464 a	126.800 a	169.867 c	338.867 a	30.853 a	26.182a	0.242 b	13.007 a	3.154 c

There are variable effects on all the traits with the triple interaction effect of varieties×temperature×incubation period (*Table 11*). Sabir-beg interacting with the other two factors showed the best records for the traits plant height, days to flowering, days to maturity, 1000 kernel weight, biomass and grain yield. The reason for surpassing Sabir-beg by the other genotypes either individually or interacting with other factors could refer to the genetic architecture of these genotypes to validate VRN1 alleles to reduce or eliminating the vernalization requirement and able to manage the vernalization stage in a few days at a suitable temperature ranged from 5-20°C (Mureşan et al., 2019). Hereford×4°C×two weeks incubation had a reasonable grain yield after Sabir-beg, recoding 4.546 ton/ha, also the maximum harvest index of 0.327 was recorded for it.

Despite the above results for the interaction of genotypes, incubation temperature, and its period, many other genotypic and environmental factors could be involved in this highly complex biological process. It is identified that mechanisms of vernalization are not just genetically controlled, they are also categorized based on their interactions with the environment (Griffiths et al., 2009). Despite the complexity of genetic and environmental interaction in vernalization operation, other genes are also involved in relation to vernalization, such as VER2 (Yong et al., 2003), which encourage de-vernalization in wheat by silencing the activity of VRN1 gene.

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Table 11. *Effect of the interaction between varieties, temperature and period of storage on vegetative growth, yield and its components of wheat varieties*

Varieties × Temperature × Period of incubation	Plant height	Days to flowering	Days to maturity	Spike number/unit area	Grain number/ Spike	1000 kernel weight	Harvest index	Biomass yield	Grain yield
Mariboss× 22 ˚C × Two weeks	72.578 jk	131.333 cde	181.000 a	252.000 k	26.400 ijk	22.402 ef	0.237 ijkl	12.053 efgh	2.855 jkl
Mariboss × 22 ˚C × Four weeks	71.578 k	131.667 cd	180.667 a	262.000 k	26.400 ijk	22.069 efg	0.243 hijkl	11.720 efghi	2.821 jkl
Mariboss × 4 ˚C × Two weeks	72.589 jk	137.000 b	180.333 a	341.333 fghi	30.533 hi	21.593 fgh	0.243 hijkl	12.369 cdefg	2.999 ijk
Mariboss × 4 ˚C × Four weeks	73.722 ijk	130.667 de	181.000 a	354.000 defgh	29.900 hij	23.374 de	0.317 ab	10.417 ijk	3.282 gh
Mariboss × -18 ˚C × Two weeks	77.888 cde	131.667 cd	175.333 b	296.000 Jk	35.667 efgh	17.568 lm	0.151 o	11.440 fghij	1.712 o
Mariboss × -18˚C× Four weeks	74.489 hij	140.667 a	180.000 a	366.000 defgh	25.000 jkl	29.925 b	0.308 abc	12.209 efgh	3.763 e
Skalnige × 22˚C × Two weeks	77.000 defg	130.333 de	180.000 a	374.000 defg	52.000 a	19.869 hijk	0.272 defghi	10.133 jk	2.758 l
Skalnige × 22˚C × Four weeks	76.667 efgh	130.333 de	179.333 a	344.000 efghi	47.333 ab	19.869 hijk	0.272 defghi	10.133 jk	2.758 l
Skalnige × 4˚C × Two weeks	77.333 cdefg	130.000 de	174.333 bc	340.333 ghi	37.133 defg	16.405 m	0.293 abcdef	9.373 kl	2.739 l
Skalnige × 4˚C × Four weeks	78.644 cde	130.333 de	181.000 a	293.333 jk	38.533 cde	17.860 lm	0.220 lm	10.760 hijk	2.376 m
Skalnige × -18˚C × Two weeks	73.244 ijk	133.333 c	174.000 bc	326.667 hij	33.800 efgh	20.953 fghi	0.278 cdefgh	8.186 l efghi	3.018 ij
Skalnige × -18˚C × Four weeks	75.333 fghi	130.333 de	174.333 bc	360.667 defgh	34.533 efgh	21.263 fgh	0.235 jkl	11.826 bcd	2.781 kl
Hereford × 22˚C × Two weeks	77.833 cdef	130.333 de	174.000 bc	422.000 abc	31.733 ghi	19.196 ijkl	0.279 cdefg	13.800 hijk	3.835 de
Hereford × 22˚C × Four weeks	78.333 cde	130.333 de	174.000 bc	462.000 a	34.733 efgh	18.196 kl	0.289 bcdef	10.800 ghij	3.801 de
Hereford × 4˚C × Two weeks	78.600 cde	130.000 de	173.333 c	398.667 bcd	37.533 cdefg	20.897 fghi	0.327 a	10.911 b	4.546 b
Hereford × 4˚C × Four weeks	79.167 cde	130.000 de	174.000 bc	362.000 defgh	36.533 efg	18.941jkl	0.177 no	13.943 bcdef	2.464 m
Hereford × -18˚C × Two weeks	75.167 ghi	130.667 de	174.000 bc	448.333 a	32.178 fgh	20.368 ghij	0.185 n	12.778 bc	2.353 m
Hereford × -18˚C × Four weeks	79.000 cde	129.000 e	175.667 b	389.000 bcde	34.400 efgh	18.380 kl	0.230 jkl	13.835 bcdef	3.182 hi
Ritraw × 22˚C × Two weeks	79.500 cd	132.000 cd	174.333 bc	380.000 cdefg	42.467 bcd	27.227 c	0.247 ghijkl	12.791 bcde	3.169 hi
Ritraw × 22˚C × Four weeks	78.833 cde	132.000 cd	175.000 bc	383.333 cdefg	42.800 bc	27.227 c	0.241 ijkl	13.124 cdefg	3.169hi
Ritraw × 4˚C × Two weeks	77.500 cdefg	132.000 cd	174.667 bc	384.000 cdefg	33.800 efgh	24.457 d	0.284 bcdef	12.386 defg	3.515 f
Ritraw × 4˚C × Four weeks	79.667 c	130.667 de	180.333 a	429.333 ab	38.033 cdef	19.175 ijkl	0.183 n	12.337 efgh	2.275 m
Ritraw × -18˚C × Two weeks	79.833 c	131.333 cde	175.667 b	353.667 defgh	36.833 efg	21.888 efg	0.166 no	12.204 fghij	2.023 n
Ritraw × -18˚C × Four weeks	77.667cdefg	131.667 cd	174.333 bc	287.000 jk	35.600 efgh	30.141 b	0.195 mn	11.528 a	2.245 m
Sabir-beg × 22˚C × Two weeks	115.000 a	102.333 f	145.000 d	290.000 jk	24.550 jklm	30.477 b	0.301 abcd	15.878 a	4.790 a
Sabir-beg × 22˚C ×Factor-3-C2	115.333 a	102.333 f	145.000 d	286.000jk	24.550 jklm	30.477 b	0.294 abcde	16.261 a	4.790 a
Sabir-beg × 4˚C × Two weeks	115.167 a	101.667 f	145.333 d	351.333 efghi	22.500 klm	32.721 a	0.258 fghijk	15.946 a	4.121 c
Sabir-beg × 4˚C × Four weeks	116.000 a	101.667 f	145.000 d	388.000 bcdef	19.333 m	31.648 ab	0.261efghij	15.320 a	4.004 cd
Sabir-beg × -18˚C × Two weeks	111.833 b	101.333 f	145.000 d	308.000 ij	20.133 lm	31.264 ab	0.224 klm	15.495 a	3.468 fg
Sabir-beg × -18˚C × Four weeks	115.833 a	102.333 f	145.000 d	291.667 jk	24.733 jklm	31.203 ab	0.243 ijkl	15.635 a	3.801 de

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Conclusion

The wheat varieties had a highly significant variance for all the studied traits. Sabir-beg had the highest and most desired performance for most of the traits including grain yield, while Hereford had the highest spike number per unit area, and Skalnige showed the highest grain number/spike. The incubation temperatures had a highly significant effect on most of the traits including grain yield. Days to flowering was reduced with -18^{o}C incubation. The same pattern was also indicated for days to maturity, spending less days to maturity when vernalized at -18^{o}C treatment.

Period of incubation for different temperatures had a significant effect on plant height, days to maturity, and 1000 kernel weight. This result indicates the satisfaction period of two weeks incubation at low temperature for the wheat varieties. Sabir-beg is a local variety that is mostly adapted to the local environment, requiring less vernalization, as the interaction effect of this variety at low incubation temperature had less effect on the studied traits.

The interaction of varieties×incubation temperature had a highly significant effect on all traits except biomass yield. The interaction of 4^{o}C with different wheat varieties had superiority to direct some of the important traits. It also seems that grain number is mainly genotype-related as the incubation treatment has no significant effect on the improvement of this trait. This result assured the necessity of involving the European varieties in field trials to be used directly or in a breeding program to improve the grain number/spike as an important trait related to yield. The high number of 40.566 grain/spike for Skalnige variety compared to 22.633 grain/spike of local variety is a big support for the importance of involving the European variety in the improvement program of wheat in the Kurdistan region of Iraq. The varieties persistency is also an outcome of their interaction with the incubation period, as the incubation of four weeks period had no recognized change on the traits compared to the two weeks incubation. The interaction effect of 4^{o}C×four weeks period had allowed the flowering to be advanced, allowing the plant to stay longer in the flowering and maturation period giving a higher number of spike per unit area, which indicates the effect of low incubation temperature on the varieties under local environmental conditions.

Both interactions of varieties×incubation temperature and varieties×incubation period had a similar effect on the traits studied, as harvest index, biomass yield, and grain yield were highly significant for both interactions, but with less effect on improving the yield. This could refer to the availability of different alleles for VRNI gene, reducing the necessity of vernalization period or reversing its effect. Cold treatment (vernalization) can not alone regulate the flowering initiation, coordinating the responsive interaction of the plant with its environment, it is also necessary to express floral activation. The interaction of all three factors showed a highly significant effect on all the traits. Cultivation of the European varieties under study under local environmental conditions is recommended to boost the increase of grain yield in wheat. *In vitro* vernalization for the grains is a substitute solution for completing the required vernalization period, that might be raised by delaying sowing or unsatisfied cold period during planting and growing season to vernalize such varieties. Further filed trial is suggested in the future, involving more variable wheat genotypes to evaluate the yield resiliency and homeostasis of broad and diverse alleles under local environmental conditions.

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BIOCHEMICAL VARIABILITY OF A DEEP LANDSLIDE-SET LAKE (LAKE TORTUM, ERZURUM/TURKEY)

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Abstract. Lake Tortum is a natural deep and landslide-set lake located in the northeast of Erzurum in the Eastern Anatolia Region of Turkey. The lake is under the influence of anthropogenic activity and pollution from agriculture. This paper, it was aimed to estimate the trophic level of Lake Tortum together with biological and some physicochemical parameters, as well as the external phosphorus load of this lake. The Lake was determined to be mesotrophic according to Secchi depth, total phosphorus concentration and chlorophyll-a tests. External phosphorus load was calculated above the critical phosphorus value determined for lakes. The lake has been shown in the eutrophication phase due to the nutrient inflow results exceeding the lake's loading capacity, the presence of some eutrophic species in the phytoplankton composition, and the periodic increase in blue-green algae. A total of 51 phytoplankton species were identified in the study period, belonging to 12 functional groups. The seasonal succession of dominant functional group is code LM (*Ceratium hirundinella*). The mean value of Q index in Tortum Lake was estimated as 1.88 which pointed out the tolerable ecological quality status.
Keywords: *external phosphorus load, phytoplankton composition, Q index, eutrophication*

Introduction

Global warming and discrepancies in seasonal transitions diminish the freshwater resources each day. In addition to that, available freshwater resources face pollution threats with industrialization and increases in urban population. Determination of the nutrient status of a lake and uncovering the factors affecting it are very important in protecting the present water resources.

Lentic systems are impacted by the rock structure and climate of the catchment, or drainage basin, in which they are located, and each lake has its own dynamics (Wetzel, 2001). Lentic systems have a tendency to transform into ponds and swamps. That process takes a few hundred years in large lakes, but it can happen faster in their smaller counterparts (Tanyolac, 2009). Humanity has an impact on the evolution process of lakes as well. The main pollution sources for lake systems are urban, industrial, and agricultural activities (Metcalf and Eddy, 2002). In addition to those, nuclear power plants and poorly planned hydroelectric power plants are pollution sources. Organic and inorganic pollutants not only disrupt the physicochemical composition of lakes, but they also alter the biological structure (Coban, 2007). Pollutants and climate change have led to increases in eutrophication, fish deaths in some period (expecialy summer months), increasing of invasive species in lakes, carbon dioxide emission from lakes, and methane emission from sediments, as well as an increase in aquatic plants. Besides, excessive water diverted from lakes drops the water level of those lakes (Jessepsen et al., 2009).

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In addition to physicochemical parameters such as N, P, Si, Ca, and Mg content, phytoplankton communities and other aquatic organisms, such as fishes and macrophytes, are used as bioindicators in the determination of the trophic levels of lakes. Various studies have been conducted to determine the trophic levels of lakes by using phytoplankton communities and benthic algae (Thunmark, 1945; Nygaard, 1949; Lepistö and Rosenström, 1998). Those methods were not accepted by other researchers, however, because the phytoplankton communities exist as different species in different regions, and it was not understood whether pollution has any observable effect at all on the species distribution (Padisák et al., 2006). On the other hand, recent studies have illustrated that phytoplankton, macrophytes, benthic organisms, and fishes can be considered as biological parameters in the process of lake trophic level determination.

Reducing the nutrient level in lakes can improve water quality by controlling eutrophication. In addition to the restriction of external nutrients, the nutrients accumulated in the sediments—and especially phosphorus—should be removed in the process of nutrient reduction in lakes, because high nutrient accumulation can alter the biological structure. The most significant step in remediating a lake successfully and permanently is the determination of the corresponding nutrient load of the lake beforehand (Van Damme et al., 2007).

The aims of the study were to determine the trophic level of Lake Tortum, located in the Eastern Anatolian region of Turkey, and the effect of agricultural and other anthropogenic pollutants on the water quality and phytoplankton composition of the lake.

Materials and Methods

Research area

Lake Tortum, a natural landslide-barricaded lake, is situated in northeastern Erzurum. It is between 40° 35′ and 40° 39′ north latitude and at 41° 38′ east longitude, at an altitude of 1012 m and 95 km from the city of Erzurum. It is about 8 km in length and 0.7 km in width, and the average depth is 100 m. The lake has been utilized for touristic and fishing activities. The main water flow to the lake comes from Tortum Stream. The stream, which is approximately 50 km in length, starts in the Mescit, Yıldızdağ, and Ereğli Mountains and collects all the water from a 1900 km^2 catchment in the Tortum District and enters the lake. After its exit, it creates a large waterfall at the shoreline. Later, it merges with the Çoruh River (Kıvrak, 2006; Kıvrak and Gurbuz, 2010).

Research on identification of trophic level on the lake are limited. The first study was carried out in the lake in 1982 for determination of phytoplankton species and some physico-cemical water quality parameters. Another study has been performed on the lake between 2005-2006. In this study, it was investigated if there was changed in plankton species, and finally, in the 2012-2013 period, the structure of the lake's phytoplankton communities and the changes in some water quality parameters were investigated (Altuner, 1982; Kıvrak, 2006; Fakıoglu et al., 2014, 2018). With its 6.45 km^2 lake area, Lake Tortum is a significant lake in Tortum Stream catchment (1653 km^2). The average depth of the lake is 100 m, and its volume is assumed to be 57.6 hm^3. The hydrological and morphometric measurements related to the lake are provided in *Table 1*. Some of the values in the table were provided from DSI (The General Directorate of State Hydraulic Works, Tur, Ministry of Agriculture and Forestry).

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Table 1. *Seasonal variation of some water quality parameters andSecchi dept in Lake Tortum*

Month	Total Hardness (mgL^{-1} $CaCO_3$)		Conductivity ($mS\ cm^{-1}$)		Dissolved oxygen (mgL^{-1})		pH		Water temperature (°C)		Secchi dept (m)	
	Mean	SD*	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Feb	137.56^{F**}	8.02	$.261^{F}$	.002	7.91^{B}	.39	8.35^{D}	.06	4.34^{G}	.16	3.13^{D}	.06
Mar	171.00^{C}	13.16	$.279^{D}$	.003	6.57^{E}	.35	8.40^{D}	.06	6.09^{F}	.59	4.30^{C}	.61
Apr	202.11^{A}	40.77	$.285^{C}$	.007	8.38^{A}	.66	8.52^{B}	.08	7.13^{D}	1.06	2.15^{E}	.74
May	151.72^{E}	15.68	$.281^{C}$	.015	7.20^{C}	.39	8.35	.05	10.33^{C}	2.87	4.07^{C}	2.01
Jun	150.33^{E}	41.06	$.262^{F}$	.024	7.00^{C}	.58	8.53^{B}	.10	10.63^{C}	2.80	7.80^{A}	1.15
Jul	189.22^{B}	16.77	$.305^{B}$	.027	7.10^{C}	1.19	8.58^{A}	.13	14.68^{A}	5.88	3.23^{D}	0.21
Aug	161.44^{D}	20.76	$.319^{A}$	.028	6.70^{D}	1.37	8.46^{C}	.11	15.24^{A}	7.34	3.37^{D}	1.18
Sep	177.50^{B}	29.17	$.320^{A}$	.025	6.15^{D}	.64	8.54^{B}	.10	14.26^{A}	5.73	7.30^{A}	.82
Oct	179.17^{B}	27.17	$.305^{B}$	.017	5.95^{F}	.31	8.52^{B}	.09	11.40^{B}	3.82	4.93^{B}	.21
Nov	157.94^{E}	9.47	$.299^{B}$	.012	5.95^{F}	.57	8.52^{B}	.12	10.11^{C}	2.63	5.23^{B}	.25
Dec	159.89^{E}	13.60	$.277^{D}$	.001	6.30^{E}	.72	8.54^{B}	.18	6.71^{E}	.36	5.20^{B}	1.01
Jan	165.50^{D}	12.70	$.272^{E}$	.001	6.50^{E}	.47	8.48^{C}	.05	5.81^{F}	.04	3.73^{D}	.42

* Standard Deviation, ** The different capital letters in the same column show the differences between months ($p<0.05$)

Fieldwork and sampling

Between February 2017 and January 2018, monthly water and plankton samples were taken in Lake Tortum (Erzurum). A total of 5 stations have been identified through the lake (3 stations) and Tortum Stream (2 stations) (*Fig. 1*). Three stations in the lake and in 5 different depths at each station (surface, 5 m, 10 m, 30 m, and 40 m depth) samples were collected by Ruttner type sampler. Simultaneously, one station at the entrance of Tortum Stream to the lake and one station at its exit were taken water samples for investigation of extarnal phosphorus loading. Phytoplankton samples were taken with the plankton nets for identification of phytoplankton in the lake. To determine the number of phytoplankton, water samples with Lugol solution added, which were taken from both stations and depths of the lake, were brought to the laboratory with polyethylene containers. In order to determine the atmospheric phosphorus loading, presipitations coming to the location of the lake were collected in a sterile container every month and then transferred to the polyester sample bottles of 2 L.

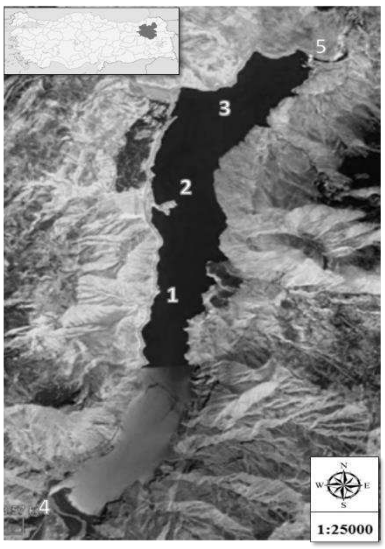

Figure 1. *Location of Lake Tortum and stations*

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Identification and calculation of phytoplankton

Phytoplankton species were identified under a binocular microscope with 100x and 400x magnification using a literature (Hustedt, 1930; Huber-Pestalozzi, 1938, 1942, 1950; Starmach, 1966; Prescott, 1973; Lind and Brook, 1980; Popovski and Pfiester, 1990; Cox, 1996; Komarek and Anagnostidis, 1999; John et al., 2002). The detected species have been checked on www.algaebase.com. Diatoms were investigated after precipitating the water samples with Lugol's solution addition; then nitric and sulfuric acids in equal volumes were utilized for boiling and acid-washing to remove the organics and expose the diatoms' silica skeletons (Round, 1953). For the enumeration of phytoplankton, water samples were first put into 10 mL graduated cylinders, and Lugol's solution was added and left overnight. After that, 3 mL of that solution was conveyed to phytoplankton enumeration rings, and the enumeration was done via an inverted microscope (Utermohl, 1958; Anonymous, 2003).

The Q phytoplankton assemblage index was estimated following Padisák et al. (2006), and ranged from 0 to 5 on a scale according to the WFD (World Framework Drictive) requirements. According to WFD's five grade evaluation system can be evaluated at 0-1: bad; between 1 and 2 as tolerable; 2-3: medium; between 3 and 4 as good, and 4-5: excellent quality (Padisák et al., 2006). For Lake Tortum, the factor numbers described for the Hungarian lake type 1 were used (Padisák et al., 2006). Phytoplankton species constituted more than 5% of total biomass were classified into functional groups according to Reynolds et al. (2002) and Padisák et al. (2009). The Water Framework Directive was adopted by the European Union in 2005. Turkey is a country in the process of integration into the European Union. Therefore, the rules have brought this directive has been implemented in Turkey. In this context, watershed-based study has been conducted in Turkey. In these studies, it was stated that the values developed for Hungarian lake type can be adapted in the use of Q index for in the evaluation of phytoplankton communities in Turkey, as well (Selek and Karaaslan, 2019).

Water quality analyses and chlorophyll-a

Water temperature, dissolved oxygen, pH, conductivity (with YSI Multiparameter), and Secchi depth (with Secchi Disk, 20Ø) were measured in situ. Total hardness analyse in water samples were conducted according to Anonymous (1995). In TP (total phosphorus) analyses, the first part (digestion) was done by using the persulfate decomposition technique. Consequently, free orthophosphate phosphorus (PO_4-P) was analyzed by the ascorbic acid method. Ammonia-nitrogen (NH_3-N) was detected by the Nesslerization method. Nitrite-nitrogen (NO_2-N) was detected by making use of the color formed with diazotization of sulfanilic acid (by nitrite) and the addition of the product to N-1-naphthylethylenediamine dihydrochloride. Its absorbance was then measured via 523 nm wavelength light. Nitrate-nitrogen (NO_3-N) concentration in water samples was determined by the yellow color formed after the nitrate's reaction with brucine sulfate; then the solution's absorbance was measured via 410 nm light (Anonymous, 1995). For chlorophyll *a* detection, 1-L water samples were collected, and the combined water filtration system filtered them through a Whatman GF/C filter. The filtrate was left for 3 to 4 hours and decomposed. After that, it was left in 10 mL of %90 acetone solution, and the centrifuged extract's optical density was monitored in a spectrophotometer in wavelength of 630, 645, and 665 nm light (Strickland and Parsons, 1972).

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External phosphorus loading

The TP loading reaching the lake from the land (TPL, land loading) is equal to the multiplication of the entering waters' phosphorus export coefficients and the catchment area. The phosphorus export coefficient (Ep) is calculated from the TP carried by the streams in a year—flow rate x TP concentration ($kg.yr^{-1}$) divided by the area of the stream in the catchment calculated through planimeter from a standard topographic map (1:50.000) (Kirchner and Dillon, 1975).

$$LL = \sum As\ xEp \quad \text{(Eq.1)}$$

LL: Land loading ($kg.yr^{-1}$),
As: Stream catchment area (km^2),
Ep: Phosphorus export coefficient ($kg.km^{-2}.yr^{-1}$).

The atmospheric phosphorus loding (AL) was calculated according to Dillon and Rigler (1975).

$$AL = P \times TPp \times Ao \quad \text{(Eq.2)}$$

AL: Atmospheric loading ($kg.yr^{-1}$),
P: Annual precipitation ($mm.yr^{-1}$),
TPp: Average TP concentration in rainwater (mgL^{-1}),
Ao: Surface area of the lake (km^2).

$$NL = LL + Al \quad \text{(Eq.3)}$$

NL: Natural loading ($kg.yr^{-1}$),
LL: Land loading ($kg.yr^{-1}$),
AL: Atmospheric loading ($kg.yr^{-1}$).

In Turkey, the phosphorus load coming from domestic wastewater has been determined to be 3–4 g/d/capita, according to the İller Bankası General Specification of Wastewater Treatment Plant Process (Ozden, 2002). As a result, artificial or domestic load was calculated as (DL).

$$DL = N \times \frac{\frac{3.5g}{day}}{capita} \times 365\ days \quad \text{(Eq.4)}$$

DL: Domestic load ($kg.yr^{-1}$),
N: Basin population (individual).

The phosphorus load reaching the lake ($kg.yr^{-1}$) is equal to the sum of natural and domestic loads.

$$TPL = NL + DL \quad \text{(Eq.5)}$$

TPL: Total phosphorus load ($kg.yr^{-1}$),
NL: Natural load ($kg.yr^{-1}$),
DL: Domestic load ($kg.yr^{-1}$).

On the other hand, the total phosphorus loading factor is equal to the TP load divided by the lake's surface area.

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$$Lp = TPL \div Ao \quad \text{(Eq.6)}$$

Lp: Total phosphorus loading factor ($kg.km^{-2}.yr^{-1}$),
TPL: Total phosphorus load ($kg.yr^{-1}$),
Ao: Surface area of the lake (km^2).

Vollenweider (1976), emphasized that water residence time is also important in the critical load formula. It was found that the critical phosphorus concentration should be between 10 mg/m^3 and 20 mg/m^3. According to that, the following formula is used to calculate critical phosphorus loading:

$$Lcritical = 10q\left(1 + \sqrt{tw}\right) \quad \text{(Eq.7)}$$

Lcritical: Critical phosphorus loading ($mg.m^{-2}.yr^{-1}$),
q: Flushing rate ($m.yr^{-1}$),
tw: Hydraulic retention time (year).

The permit water loading ($g.m^{-2}.yr^{-1}$) and the critical loading value ($g.m^{-2}.yr^{-1}$) was determined by the equations, used to be flushing rate (q, $m.yr^{-1}$), given below (Chapra and Tarapchak, 1976):

$$Lpermit = 0.011(q + 12.4) \quad \text{(Eq.8)}$$

$$Lcritical = 0.025(q + 12.4) \quad \text{(Eq.9)}$$

Critical phosphorus loading could also be calculated by using field phosphorus loading ($g.m^{-2}.yr^{-1}$), chlorophyll-a (mgL^{-1}) and flushing rate (q, $m.yr^{-1}$) (Chapra and Tarapchak, 1976):

$$Lcritical = 0.0055\ (chl\ a)^{0.69}\ (q + 12.4) \quad \text{(Eq.10)}$$

Statistics

The monthly, stationary, and depth-related changes in the data taken from the three selected stations' surface, 5 m, 10 m, 20 m, 30 m, and 40 m depths were examined with IBM SPSS 20 software. To understand the collective effect of the stations and seasons, water temperature, dissolved oxygen, pH, conductivity, Secchi disk, total hardness, NH_3-N, NO_2-N, NO_3-N, TP and PO_4-P were analyzed by the use of three-factorial Analysis of Variance (ANOVA). The Duncan test was employed to determine the intergroup differences. Canonical Correspondence Analysis was done with XLSTAT software.

Results

Physico-chemical parameters of Lake Tortum

The average depth of the Lake Tortum (100 m) was provided from DSI (The General Directorate of State Hydraulic Works, Tur, Ministry of Agriculture and Forestry). The depth of Secchi was determined between 3 m and 5 m throughout the year. The lowest value (2.15±0.74 m) of Secchi depth was measured in April and the highest value (7.80±1.51 m) in June during the clean water phase. The mean Secchi depth was 4.42 m.

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Stationary and depth-related changes in water temperature, dissolved oxygen, pH, conductivity and total hardness values were statistically significant ($p < 0.05$). Water temperature, dissolved oxygen, pH, conductivity, and total hardness mean values in the lake were 10.64 ºC, 6.81 mgL^{-1}, 8.47, 0.289 $mS\ cm^{-1}$, and 166 mgL^{-1} $CaCO_3$, respectively (*Table 1*).

The catchment area of Lake Tortum, shows microclimate characteristics (Duman, 2009), so the lake not ice-covered in winter, and the mean water temperature in the winter months was measured as 6 °C. The thermal stratification in spring and autumn periods was weak, but a considerable stratification was observed in summer. Thus, Lake Tortum belongs to the warm monomictic lake class in Wetzel's (2001) classification of the lakes according to their stratification.

Detected lowest dissolved oxygen value was (5.6 mgL^{-1}) in September and the highest value (8.65 mgL^{-1}) in April. The dissolved oxygen value in the lake water supply and the drainage water of the lake was also higher than the lake and the average minimum and maximum values were found to be 5.06 mgL^{-1} and 11.03 mgL^{-1}, respectively (*Table 1*). The lowest water temperature value was detected at the 1st station (4.1 ºC) in February, at a depth of 5 and 10 m, and the highest water temperature value at the 1st station (25.4 ºC) on the surface in August. Depending on the depth of the Tortum Lake water temperature, a significant stratification was detected in the summer and autumn seasons. In spring, the water temperature value on the surface was measured at 10.26 ºC, and as the depth increased, a decrease of 1 ºC was observed in the water temperature value. During the winter period, the water temperature value ranged between 5.37 ºC and 5.8 ºC, respectively, at depths of 0-40 m. At the stations that feed the lake and discharge the lake water, the water temperature value was determined as the lowest 3.4 ºC and 4.8 ºC, the highest 24.1 ºC and 25.1 ºC, respectively.

The mean pH value was measured as 8.48 ± 0.12. According to Turkish Environmental Legislation Inland Water Resources Classification, the lake was identificated as first class waters quality (Anonymous, 2012). pH was measured in June at its lowest value in the 1st station (8.0) and the highest value in February at the depth of 10 m (8.9) in June. At the 2nd station, the lowest value (8.1) was found at the surface in July at the surface depth in July (8.79). The lowest value at the 3rd station (8.32) was determined at the depth of May 20 m, the highest value (8.7) at 0 m and at a depth of 5 m in July (*Table 1*).

The difference between TP, PO_4-P, NH_3-N, NO_2-N and NO_3-N concentration values between stations, months and depths was found to be statistically significant ($p < 0.05$). TP, PO_4-P, NH_3-N, NO_2-N, and NO_3-N (mean± SD) were 0.31 ± 0.03 mgL^{-1}, 41.05 ± 2.65 μgL^{-1}, 0.14 ± 0.02 mgL^{-1}, 0.55 ± 0.08 mgL^{-1}, and 1.10 ± 0.02 mgL^{-1}, respectively.

TP values were 0.33 ± 0.03 mgL^{-1}, 0.34 ± 0.04 mgL^{-1}, and 0.27 ± 0.03 mgL^{-1} in the first, second, and third stations, respectively. PO_4-P showed its lowest value (0.0 ± 0.0μgL^{-1}) at all stations except for the months of July, August, and January. The highest values in the first, second, and third stations were 77.84 ± 0.09 μgL^{-1}, 39.20 ± 0.01 μgL^{-1}, and 47.16 ± 0.02 μgL^{-1}, respectively (*Table 2, Table 3*).

For differences of NH_3-N at the stations, the lowest value at the first station was found in January in all depths; on the other hand, the highest value was 0.25 ± 0.01 mgL^{-1} at 10 m, 30 m, and 40 m depths in March. At the second station, the lowest value was 0.06 ± 0.01 mgL^{-1} on the surface in June, and the highest value was 0.43 ± 0.02 mgL^{-1} at 40 m depth in January. At the third station, the lowest value was 0.0 ± 0.0 mgL^{-1} at the surface in August, and the highest value 0.45 ± 0.02 mgL^{-1} was at 5 m depth in January (*Table 4*).

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Table 2. *Change of Total Phosphorus (TP) values depending on months, stations and depth on Tortum Lake (Mean ± SD, mgL^{-1}) (n = 4)*

St	Depth	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan
1	0	0.00±0.0^{Gdg**}	0.04±0.02^{Fcd}	0.08±0.02^{Faa}	0.0001±0.0^{Gaa}	**1.16±0.0^{Adc}**	0.96±0.02^{Aaa}	0.24±0.01^{Dac}	0.28±0.01^{Dbf}	0.20±0.01^{Eaa}	0.80±0.05^{Baa}	0.28±0.02^{Dde}	0.48±0.01^{Cab}
	5	0.02±0.01^{Fff}	0.06±0.02^{Fcd}	0.24±0.02^{Dcb}	0.0001±0.0^{Gaa}	0.81±0.03^{Abb}	0.70±0.02^{Bcc}	0.13±0.01^{Ebd}	0.44±0.01^{Cae}	0.26±0.01^{Daa}	0.44±0.01^{Cbb}	0.47±0.02^{Ccd}	0.24±0.01^{Dbd}
	10	0.00±0.0^{Gdg}	0.20±0.02^{Dbc}	0.08±0.01^{Edc}	0.0001±0.0^{Faa}	0.92±0.04^{Aaa}	0.80±0.01^{Bbb}	0.07±0.01^{Ece}	0.46±0.01^{Eae}	0.0±0.0^{Gbb}	0.40±0.01^{Ccd}	0.93±0.01^{Add}	0.24±0.01^{Dcf}
	20	0.04±0.01^{Fbe}	0.20±0.02^{Dbc}	0.36±0.02^{Cbb}	0.0001±0.0^{Gaa}	0.48±0.03^{Bed}	0.90±0.02^{Aaa}	0.04±0.01^{Fce}	0.49±0.01^{Dad}	0.16±0.01^{Ecd}	0.27±0.01^{Ede}	0.24±0.02^{Bbx}	0.17±0.01^{Dbd}
	30	0.03±0.01^{Fbe}	0.40±0.02^{Cab}	0.47±0.02^{Caa}	0.0001±0.0^{Gaa}	0.59±0.03^{Bcd}	1.03±0.05^{Aaa}	0.04±0.01^{Fce}	0.52±0.01^{Bad}	0.0±0.0^{Gcd}	0.16±0.01^{Ede}	0.56±0.01^{Bbc}	0.25±0.02^{Dbd}
	40	0.00±0.0^{Gdg}	0.40±0.02^{Cab}	0.32±0.02^{Gbb}	0.0001±0.0^{Gaa}	0.40±0.03^{Bde}	0.85±0.05^{Abb}	0.29±0.01^{Cbb}	0.43±0.01^{Bae}	0.27±0.01^{Caa}	0.27±0.01^{Ccd}	0.4±0.01^{Def}	0.28±0.02^{Cbd}
2	0	0.00±0.0^{Gdg}	0.52±0.02^{Caa}	0.0001±0.0^{Gad}	0.0001±0.0^{Gaa}	1.56±0.05^{Aaa}	0.72±0.05^{Bbc}	0.90±0.01^{Aaa}	0.60±0.01^{Abc}	0.11±0.01^{Ebb}	0.07±0.01^{Fdf}	0.44±0.02^{Dbd}	0.12±0.01^{Ebe}
	5	0.00±0.0^{Gdc}	0.41±0.02^{Cbb}	0.0001±0.0^{Gad}	0.0001±0.0^{Gaa}	0.91±0.03^{Aaa}	0.92±0.04^{Aaa}	0.16±0.01^{Ebd}	0.50±0.01^{Bcd}	0.19±0.01^{Eab}	0.2±0.01^{Fce}	0.5±0.01^{Fdf}	0.31±0.02^{Dac}
	10	0.03±0.0^{Fbe}	0.16±0.02^{Ecc}	0.0001±0.0^{Gad}	0.0001±0.0^{Gaa}	0.52±0.03^{Ccd}	0.95±0.04^{Aaa}	0.18±0.01^{Ebd}	0.68±0.04^{Bac}	0.10±0.01^{Cbb}	0.52±0.01^{Cab}	0.32±0.01^{Dce}	0.36±0.03^{Dac}
	20	0.00±0.00^{Fdg}	0.12±0.02^{Ecc}	0.0001±0.0^{Fad}	0.0001±0.0^{Faa}	0.40±0.03^{Dde}	1.13±0.06^{Baa}	0.00±0.0^{Fdf}	0.72±0.04^{Cab}	0.15±0.01^{Eab}	0.12±0.01^{Ebe}	**2.08±0.05^{Aaa}**	0.15±0.02^{Ebe}
	30	0.03±0.01^{Fbe}	0.19±0.02^{Ecc}	0.0001±0.0^{Gad}	0.0001±0.0^{Gaa}	0.55±0.03^{Bcd}	0.76±0.03^{Abc}	0.02±0.01^{Fce}	0.70±0.04^{Aab}	0.0±0.0^{Gdd}	0.00±0.0^{Gdg}	0.35±0.02^{Cce}	0.29±0.02^{Dad}
	40	0.00±0.0^{Gdg}	0.21±0.02^{Ccc}	0.0001±0.0^{Fad}	0.0001±0.0^{Faa}	0.63±0.03^{Bbc}	0.76±0.03^{Abc}	0.04±0.01^{Ece}	0.79±0.04^{Aab}	0.06±0.01^{Ecc}	0.12±0.01^{Dbe}	0.56±0.01^{Bbc}	0.19±0.01^{Cbe}
3	0	0.04±0.01^{Fcd}	0.23±0.01^{Eac}	0.0001±0.0^{Gad}	0.0001±0.0^{Gaa}	0.6±0.03^{Fdf}	0.75±0.03^{Abc}	0.23±0.01^{Dbc}	0.57±0.02^{Bad}	0.15±0.01^{Ebb}	0.00±0.0^{Gcg}	0.36±0.01^{Cbe}	0.13±0.01^{Ebe}
	5	0.05±0.01^{Fbc}	0.25±0.01^{Dac}	0.0001±0.0^{Gad}	0.0001±0.0^{Gaa}	0.69±0.03^{Bbc}	0.94±0.04^{Aaa}	0.37±0.01^{Cab}	0.60±0.01^{Bac}	0.12±0.01^{Ebb}	0.12±0.01^{Eae}	0.36±0.01^{Cbe}	0.11±0.01^{Ebe}
	10	0.06±0.0^{Ebb}	0.06±0.01^{Ecd}	0.0001±0.0^{Fad}	0.0001±0.0^{Faa}	0.78±0.03^{Bab}	0.93±0.04^{Aaa}	0.31±0.01^{Cab}	0.40±0.01^{Cbe}	0.0±0.01^{Fdd}	0.06±0.01^{Ebe}	0.36±0.02^{Cbe}	0.16±0.01^{Dbe}
	20	0.08±0.00^{Aaa}	0.15±0.02^{Ebc}	0.0001±0.0^{Gad}	0.0001±0.0^{Gaa}	0.47±0.02^{Cce}	**1.01±0.05^{Aaa}**	0.24±0.01^{Dbc}	0.59±0.01^{Bad}	0.04±0.01^{Fcc}	0.12±0.01^{Eae}	0.34±0.03^{Dbe}	0.04±0.0^{Fcf}
	30	0.08±0.0^{Faa}	0.06±0.01^{Fcd}	0.0001±0.0^{Gad}	0.0001±0.0^{Gaa}	0.10±0.01^{Edf}	0.96±0.04^{Aaa}	0.05±0.01^{Fce}	0.47±0.01^{Cae}	0.24±0.01^{Daa}	0.04±0.01^{Fbf}	0.54±0.02^{Bac}	0.25±0.02^{Dbd}
	40	0.02±0.01^{Dde}	0.16±0.05^{Cbc}	0.00±0.00^{Ebe}	0.0001±0.0^{Eaa}	0.49±0.03^{Bce}	0.90±0.04^{Aaa}	0.23±0.01^{Cbc}	0.50±0.01^{Bad}	0.04±0.01^{Dcc}	0.12±0.01^{Cae}	0.56±0.02^{Bac}	0.47±0.03^{Cab}

** A, B, C, D ..: Capital letters show the difference between the months for each station and the difference between the months carrying different capital letters on the same line is statistically significant (p <0.05),

a, b, c, d ..: Lower case letters show the difference between the depths for each station and the difference between the depths carrying different lower case letters in the same line is statistically significant (p <0.05),

a, b, c, d ..: Italic letters show the difference between stations for each station and the difference between the stations carrying different italic letters in the same line is statistically significant (p <0.05)

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Table 3. *Change of Orthophosphate Phosphorus (PO_4-P) values depending on months, stations and depth on Tortum Lake (Mean ± SD, mgL^{-1}) (n = 4)*

St	Depth	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan
1	0	0.00±0.0^{Dba}	6.82±0.02^{Cac}	17.61±0.01^{Bdc}	13.64±0.08^{Bbb}	18.18±0.01^{Bbc}	25.00±0.01^{Abb}	0.00±0.0^{Bde}	1.14±0.01^{Cbb}	0.00±0.0^{Dcc}	6.25±0.02^{Cbb}	13.07±0.09^{Bab}	9.09±0.01^{Ccb}
	5	1.14±0.01^{Cab}	0.00±0.0^{Ddd}	9.09±0.02^{Ced}	27.27±0.09^{Aaa}	17.61±0.01^{Bbc}	30.11±0.02^{Aab}	10.80±0.05^{Bbc}	0.00±0.0^{Dcc}	10.80±0.05^{Baa}	4.55±0.02^{Cbb}	13.07±0.08^{Bab}	4.55±0.01^{Ccb}
	10	0.00±0.0^{Dab}	0.00±0.0^{Ddd}	0.57±0.01^{Dfe}	0.00±0.0^{Dcd}	25.00±0.01^{Aab}	34.09±0.02^{Aaa}	6.82±0.05^{Ccd}	0.00±0.0^{Dcc}	0.00±0.0^{Dcc}	9.09±0.02^{Cbb}	14.20±0.07^{Bab}	10.80±0.01^{Bbb}
	20	0.00±0.0^{Dba}	4.55±0.05^{Cbc}	50.57±0.05^{Abb}	0.00±0.0^{Dcd}	4.55±0.01^{Ccd}	22.73±0.02^{Bbb}	19.89±0.04^{Bac}	3.98±0.01^{Cab}	1.70±0.01^{Cbb}	21.59±0.09^{Baa}	2.84±0.01^{Ccb}	6.82±0.01^{Ccb}
	30	0.00±0.0^{Dba}	2.27±0.05^{Ccc}	**77.84±0.09^{Aaa}**	0.00±0.0^{Dcd}	3.98±0.02^{Ccd}	31.82±0.02^{Aab}	14.20±0.04^{Bbc}	0.00±0.0^{Dcc}	1.14±0.01^{Cbb}	0.00±0.0^{Dcc}	13.64±0.01^{Bba}	10.80±0.01^{Bba}
	40	0.00±0.0^{Dba}	1.70±0.03^{Ccc}	29.55±0.08^{Acb}	0.00±0.0^{Dcd}	10.23±0.01^{Bbc}	36.36±0.02^{Aaa}	32.39±0.02^{Aaa}	0.00±0.0^{Dcc}	6.25±0.01^{Cbb}	13.64±0.09^{Bba}	6.82±0.01^{Ccb}	20.45±0.01^{Baa}
2	0	0.00±0.0^{Dba}	29.55±0.05^{Aaa}	15.91±0.08^{Bac}	25.57±0.05^{Aaa}	14.77±0.01^{Bac}	28.98±0.01^{Abb}	25.00±0.02^{Aab}	0.00±0.0^{Dcc}	9.09±0.02^{Cbb}	6.25±0.01^{Cab}	2.27±0.01^{Cac}	4.55±0.01^{Cbb}
	5	0.57±0.01^{Daa}	23.30±0.05^{Aab}	14.77±0.05^{Bab}	18.18±0.01^{Bbb}	13.64±0.01^{Bac}	34.09±0.01^{Aaa}	15.91±0.01^{Bbc}	0.00±0. 0^{Dcc}	5.68±0.05^{Cbb}	2.27±0.01^{Cab}	0.00±0.0^{Dbd}	15.91±0.01^{Baa}
	10	0.00±0.0^{Dba}	9.09±0.01^{Ccc}	9.09±0.08^{Cbd}	19.89±0.01^{Bbb}	15.34±0.01^{Bac}	21.59±0.01^{Abb}	26.14±0.01^{Aab}	0.00±0.0^{Dcc}	0.00±0.0^{Dcc}	6.25±0.01^{Cab}	0.00±0.0^{Dbd}	4.55±0.01^{Cbb}
	20	0.00±0.0^{Dba}	6.82±0.01^{Bbb}	10.23±0.08^{Bac}	3.41±0.01^{Ccc}	3.41±0.01^{Cbd}	25.57±0.01^{Abb}	15.91±0.01^{Bbc}	0.00 ±0.0^{Dcc}	2.27±0.05^{Cbb}	4.55±0.01^{Cab}	0.00±0.0^{Dbd}	4.55±0.01^{Cbb}
	30	0.00±0.0^{Dba}	10.80±0.03^{Cbb}	0.57±0.01^{Dcf}	4.55±0.02^{Ccc}	0.00±0.0^{Dbe}	31.82±0.01^{Aaa}	35.23±0.01^{Aaa}	0.00±0.0^{Dcc}	0.00±0.0^{Bcc}	5.68±0.01^{Cab}	0.00±0.0^{Dbd}	13.07±0.01^{Baa}
	40	0.00±0.0^{Dba}	11.93±0.05^{Bab}	0.00±0.0^{Daf}	5.68±0.05^{Ccc}	0.57±0.01^{Dbe}	**39.20±0.01^{Aaa}**	22.73±0.01^{Bbb}	0.00±0.0^{Dcc}	13.64±0.05^{Baa}	6.82±0.01^{Cab}	0.00±0.0^{Dbd}	20.45±0.01^{Baa}
3	0	0.00±0.0^{Dba}	13.07±0.02^{Bab}	0.00±0.0^{Daf}	9.09±0.04^{Cbc}	10.80±0.01^{Bac}	22.73±0.02^{Abb}	23.86±0.0^{Abb}	0.00±0.0^{Dcc}	0.00±0.0^{Dac}	1.14±0.01^{Cbb}	0.00±0.0^{Dad}	17.61±0.01^{Baa}
	5	0.00±0.0^{Dba}	14.20±0.02^{Bab}	0.00±0.0^{Daf}	17.61±0.01^{Bab}	14.20±0.01^{Bac}	26.70±0.02^{Abb}	11.93±0.01^{Bcc}	0.00±0.0^{Dcc}	0.00±0.0^{Dac}	6.82±0.01^{Cab}	0.00±0.0^{Dad}	8.52±0.01^{Ccb}
	10	0.00±0.0^{Dba}	3.41±0.03^{Cbc}	0.00±0.0^{Daf}	9.09±0.01^{Cbb}	14.77±0.01^{Bac}	29.55±0.02^{Abb}	27.27±0.01^{Abb}	0.00±0.0^{Dcc}	0.00±0.0^{Dac}	2.27±0.01^{Cbb}	0.00±0.0^{Dad}	4.55±0.01^{Ccb}
	20	0.00±0.0^{Dba}	8.52±0.04^{Cbc}	0.00±0.0^{Daf}	0.00±0.0^{Dcd}	0.00±0.0^{Dbe}	**47.16±0.02^{Aaa}**	15.34±0.01^{Bcc}	0.00±0.0^{Dcc}	0.00±0.0^{Dac}	6.25±0.01^{Cab}	0.00±0.0^{Dad}	13.64±0.01^{Bba}
	30	1.14±0.03^{Cab}	3.41±0.02^{Ccc}	0.00±0.0^{Daf}	0.00±0.0^{Dcd}	0.00±0.0 ^{Dbe}	37.50±0.02^{Aaa}	34.09±0.01^{Abb}	0.00±0.0^{Dcc}	0.00±0.0^{Dac}	1.14±0.01^{Cbb}	0.00±0.0^{Dad}	22.16±0.01^{Baa}
	40	0.00±0.0^{Dba}	9.09±0.01^{Cbc}	0.00±0.0^{Daf}	0.00±0.0^{Dcd}	0.00±0.0 ^{Dbe}	29.55±0.02^{Bbb}	44.32±0.01^{Aaa}	2.27±0.01^{Cbb}	0.00±0.0^{Dac}	6.82±0.01^{Cab}	0.00±0.0^{Dad}	25.00±0.01^{Baa}

** A, B, C, D ..: Capital letters show the difference between the months for each station and the difference between the months carrying different capital letters on the same line is statistically significant (p <0.05),

a, b, c, d ..: Lower case letters show the difference between the depths for each station and the difference between the depths carrying different lower case letters in the same line is statistically significant (p <0.05),

a, b, c, d ..: Italic letters show the difference between stations for each station and the difference between the stations carrying different italic letters in the same line is statistically significant (p <0.05)

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Table 4. *Change of Ammonia-Nitrogen (NH_3-N) values depending on months, stations and depth on Tortum Lake (Mean ± SD, mgL^{-1}) (n = 4)*

St	Depth	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan
1	0	0.07±0.011^{Cbb*}	0.24±0.01^{Aaa}	0.17±0.01^{Baa}	0.20±0.01^{Aaa}	0.04±0.01^{Caa}	0.10±0.01^{Baa}	0.11±0.01^{Baa}	0.10±0.01^{Baa}	0.10±0.01^{Bcb}	0.13±0.01^{Bba}	0.13±0.01^{Baa}	0.00±0.0^{Dad}
	5	0.08±0.011^{Cbb}	0.24±0.01^{Aaa}	0.17±0.01^{Baa}	0.17±0.01^{Aba}	0.05±0.01^{Caa}	0.08±0.01^{Cba}	0.12±0.01^{Baa}	0.12±0.01^{Baa}	0.08±0.01^{Cdc}	0.12±0.01^{Bba}	0.13±0.01 ^{Baa}	0.00±0.0 ^{Dad}
	10	0.08±0.011^{Cbb}	**0.25±0.01^{Aaa}**	0.18±0.01^{Baa}	0.14±0.01^{Bba}	0.05±0.01^{Caa}	0.11±0.01^{Baa}	0.10±0.01^{Baa}	0.11±0.01^{Baa}	0.21±0.01^{Aaa}	0.11±0.01^{Bba}	0.15±0.01 ^{Baa}	0.00±0.0 ^{Dad}
	20	0.08±0.01^{Cbb}	0.24±0.01^{Aaa}	0.17±0.01^{Baa}	0.16±0.01^{Bba}	0.09±0.01^{Caa}	0.12±0.01^{Baa}	0.13±0.01^{Baa}	0.13±0.01^{Baa}	0.16±0.01^{Bbb}	0.12±0.01^{Bba}	0.12±0.01 ^{Baa}	0.00±0.0 ^{Dad}
	30	0.06±0.01^{Cbb}	**0.25±0.01^{Aaa}**	0.16±0.01^{Baa}	0.15±0.01^{Bba}	0.08±0.01^{Caa}	0.12±0.01^{Baa}	0.13±0.01^{Baa}	0.14±0.01^{Baa}	0.10±0.01^{Bcb}	0.16±0.01^{Bba}	0.16±0.01 ^{Baa}	0.00±0.0 ^{Dad}
	40	0.10±0.01^{Baa}	**0.25±0.01^{Aaa}**	0.18±0.01^{Baa}	0.19±0.01^{Bba}	0.08±0.01^{Caa}	0.10±0.01^{Baa}	0.13±0.01^{Baa}	0.10±0.01^{Baa}	0.14±0.01^{Bcb}	0.13±0.01^{Bba}	0.12±0.01 ^{Baa}	0.00±0.0 ^{Dad}
2	0	0.08±0.01^{Cbb}	0.23±0.01^{Aaa}	0.18±0.01^{Baa}	0.17±0.01^{Baa}	0.06±0.01^{Caa}	0.15±0.01^{Baa}	0.09±0.01^{Cba}	0.09±0.01^{Cba}	0.13±0.01^{Bab}	0.14±0.01^{Baa}	0.15±0.01 ^{Baa}	0.09±0.01^{Ccc}
	5	0.08±0.01^{Cbb}	0.24±0.01^{Aaa}	0.18±0.01^{Baa}	0.19±0.01^{Baa}	0.07±0.01^{Caa}	0.11±0.01^{Baa}	0.12±0.01^{Baa}	0.15±0.01^{Baa}	0.11±0.01^{Bab}	0.14±0.01^{Baa}	0.18±0.01 ^{Baa}	0.13±0.02^{Bbb}
	10	0.08±0.01^{Cbb}	0.25±0.01^{Aaa}	0.14±0.01^{Baa}	0.19±0.01^{Baa}	0.07±0.01^{Caa}	0.13±0.01^{Baa}	0.12±0.01^{Baa}	0.11±0.01^{Baa}	0.15±0.01^{Bab}	0.12±0.01^{Baa}	0.16±0.01 ^{Baa}	0.34±0.02^{Aaa}
	20	0.09±0.01^{Cbb}	0.21±0.01^{Aaa}	0.18±0.01^{Baa}	0.16±0.01^{Baa}	0.08±0.01^{Caa}	0.11±0.01^{Baa}	0.12±0.01^{Baa}	0.11±0.01^{Baa}	0.13±0.01^{Bab}	0.13±0.01^{Baa}	0.16±0.01 ^{Baa}	0.09±0.01^{Ccc}
	30	0.07±0.01^{Cbb}	0.23±0.01^{Aaa}	0.18±0.01^{Baa}	0.18±0.01^{Baa}	0.07±0.01^{Caa}	0.13±0.01^{Baa}	0.12±0.01^{Caa}	0.15±0.01^{Baa}	0.11±0.01^{Bab}	0.13±0.01^{Baa}	0.18±0.01 ^{Baa}	0.10±0.01^{Bbb}
	40	0.07±0.01^{Dab}	0.24±0.01^{Baa}	0.17±0.01^{Caa}	0.14±0.01^{Caa}	0.08±0.01^{Daa}	0.11±0.01^{Caa}	0.12±0.01^{Caa}	0.12±0.01^{Caa}	0.10±0.01^{Cab}	0.14±0.01^{Baa}	0.11±0.01^{Caa}	**0.43±0.02^{Aaa}**
3	0	0.06±0.01^{Cbb}	0.18±0.01^{Bbb}	0.10±0.01^{Baa}	0.14±0.01^{Baa}	0.07±0.01^{Caa}	0.13±0.01^{Baa}	0.00±0.0^{Dbb}	0.17±0.01^{Baa}	0.10±0.01^{Bab}	0.14±0.01^{Caa}	0.13±0.01^{Baa}	**0.43±0.01^{Aaa}**
	5	0.09±0.01^{Cbb}	0.19±0.01^{Bbb}	0.15±0.01^{Baa}	0.18±0.01^{Baa}	0.06±0.01^{Caa}	0.11±0.01^{Baa}	0.11±0.01^{Baa}	0.17±0.01^{Baa}	0.12±0.01^{Dbc}	0.15±0.01^{Baa}	0.14±0.01^{Baa}	**0.45±0.02^{Aaa}**
	10	0.10±0.01^{Cbb}	0.22±0.01^{Baa}	0.12±0.01^{Daa}	0.21±0.01^{Baa}	0.05±0.01^{Daa}	0.11±0.01^{Caa}	0.11±0.01^{Caa}	0.16±0.01^{Caa}	0.09±0.01^{Dbc}	0.13±0.01^{Baa}	0.14±0.01^{Caa}	0.40±0.02^{Aaa}
	20	0.11±0.01^{Cbb}	0.20±0.01^{Baa}	0.16±0.01^{Baa}	0.17±0.01^{Baa}	0.08±0.01^{Caa}	0.09±0.01^{Cba}	0.12±0.01^{Baa}	0.17±0.01^{Baa}	0.14±0.01^{Bab}	0.13±0.01^{Caa}	0.15±0.01^{Baa}	0.42±0.02^{Aaa}
	30	0.09±0.01^{Cbb}	0.27±0.01^{Baa}	0.17±0.01^{Baa}	0.20±0.01^{Baa}	0.07±0.01^{Caa}	0.11±0.01^{Baa}	0.12±0.01^{Baa}	0.17±0.01^{Baa}	0.12±0.01^{Bab}	0.12±0.01^{Baa}	0.14±0.01^{Baa}	0.39±0.02^{Aaa}
	40	0.08±0.01^{Dbb}	0.24±0.01^{Baa}	0.15±0.01^{Caa}	0.19±0.01^{Cba}	0.07±0.01^{Daa}	0.07±0.01^{Dba}	0.12±0.01^{Caa}	0.16±0.01^{Caa}	0.12±0.01^{Cab}	0.13±0.01^{Caa}	0.16±0.01^{Caa}	0.42±0.02A^{aa}

** A, B, C, D ..: Capital letters show the difference between the months for each station and the difference between the months carrying different capital letters on the same line is statistically significant (p <0.05),

a, b, c, d ..: Lower case letters show the difference between the depths for each station and the difference between the depths carrying different lower case letters in the same line is statistically significant (p <0.05),

a, b, c, d ..: Italic letters show the difference between stations for each station and the difference between the stations carrying different italic letters in the same line is statistically significant (p <0.05)

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When the NO_3-N values were examined according to different months and stations, the lowest mean value at the first station was 0.02 ± 0.01 mgL^{-1} in January, while the highest value was 6.06 ± 0.05 mgL^{-1} in September. The lowest mean value at the second station was 0.01 ± 0.01 mgL^{-1} on January, while the highest value was 5.59 ± 0.02 mgL^{-1} in September. The lowest mean value at the third station was 0.0 ± 0.0 mgL^{-1} in August and January, while the highest value was 9.45 ± 0.05 mgL^{-1} in September. The mean values of NO_3-N at the first, second, and third stations were 1.05 ± 0.02 mgL^{-1}, 0.99 ± 0.05 mgL^{-1}, and 1.27 ± 0.03 mgL^{-1}, respectively (*Table 5*).

NO_2-N values were lowest in February, March, and January for all stations and were highest in December. In March, July, and January, the second and third stations NO_2-N values for all depths were 0.0 ± 0.0 mgL^{-1}. During the same period at the first station, a concentration of 0.38 ± 0.01 mgL^{-1} was measured, at the surface only. The largest NO_2-N values at the first, second, and third stations were detected as 4.38 ± 0.01 mgL^{-1}, 4.69 ± 0.01 mgL^{-1}, and 5.63 ± 0.01 mgL^{-1}, respectively (*Table 6*).

External phosphorus loading in Lake Tortum

The land phosphorus load was calculated by the multiplication of phosphorus export coefficient (kg yr^{-1}) and catchment area (km^2). Tortum Stream continuously supplies water to Lake Tortum from its catchment area of 16.534 km^2. The phosphorus export coefficient (Ep) was calculated to be 0.07934 kg km^{-2} according to Kirchner and Dillon (1975) methods. The morphometric and hydrological parameters of Lake Tortum have been supplyed from DSI (*Table 7*).

The land phosphorus load was found to be 1312 kg yr^{-1} (*Equation 1*), while the atmospheric loading (AL) is 31 kg yr^{-1} (*Equation 2*). The natural phosphorus load is 1343 kg yr^{-1} (*Equation 3*), whereas the domestic phosphorus load is 9707 kg yr^{-1} (*Equation 4*). The external TP loading to the Lake Tortum is 11050 kg yr^{-1}. The TP load coeficient was computed to be 1.71 $g.m^{-2}.yr^{-1}$ (*Equation 6*). According to the formulas developed by Chapra and Tarapchak (1976) and Vollenweider (1976) from the calculated phosphorus loadings of Lake Tortum measured in 2017.

$$\begin{aligned} L_{critical} &= 10 \text{ x } 74.42\ (1+0.34641) \\ &= 1002 \text{ mg.m}^{-2}.\text{yr}^{-1} \\ &\sim 1.002 \text{ g.m}^{-2}.\text{yr}^{-1} \end{aligned} \quad \text{(Eq.7)}$$

$$\begin{aligned} L_{permit} &= 0.011\ (74.42 + 12.4) \\ &= 0.96 \text{ g.m}^{-2}.\text{yr}^{-1} \end{aligned} \quad \text{(Eq.8)}$$

$$\begin{aligned} L_{critical} &= 0.025\ (74.42 + 12.4) \\ &= 2.17 \text{ g.m}^{-2}.\text{yr}^{-1} \end{aligned} \quad \text{(Eq.9)}$$

$$\begin{aligned} L_{critical} &= 0.0055\ (0.04)^{0.69}\ (74.42 + 12.4) \\ &= 0.05 \text{ g.m}^{-2}.\text{yr}^{-1} \end{aligned} \quad \text{(Eq.10)}$$

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Table 5. *Change of Nitrate-Nitrogen (NO_3-N) values depending on months, stations and depth on Tortum Lake (Mean ± SD, mgL^{-1}) (n = 4)*

St	Depth	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan
1	0	0.09±0.01^{Cbb*}	0.05±0.01^{Cbc}	0.02±0.01^{Cad}	0.14±0.01^{Bcd}	0.27±0.01^{Bbc}	0.26±0.01^{Bbd}	0.12±0.01^{Bbb}	1.04±0.01^{Acb}	6.22±0.01^{Aaa}	2.49±0.01^{Abb}	0.09±0.01^{Cbb}	0.03±0.01^{Aa}
	5	0.10±0.02B^{aa}	0.00±0.01^{Dcd}	0.02±0.01^{Cad}	0.32±0.01^{Bbc}	0.15±0.01^{Bcd}	0.16±0.01$_{Bcd}$	0.00±0.0^{Dcd}	1.59±0.05^{Acb}	2.77±0.01^{Abb}	3.53±0.01^{Aab}	0.08±0.01^{Cbb}	0.00±0.01^{Dbb}
	10	0.10±0.02^{Baa}	0.06±0.01^{Cbc}	0.03±0.01^{Cad}	0.47±0.01^{Bbb}	0.20±0.01^{Bcc}	0.17±0.01^{Bcd}	0.00±0.0 ^{Dcd}	1.87±0.05^{Acb}	2.70±0.01^{Abb}	3.32±0.01^{Aab}	0.08±0.01^{Cbb}	0.03±0.01^{Caa}
	20	0.07±0.02^{Cbb}	0.19±0.01^{Bab}	0.06±0.01^{Cad}	0.56±0.01^{Bab}	0.19±0.01^{Bcd}	0.07±0.01^{Cde}	0.00±0.0 ^{Dcd}	**15.69±0.05^{Aaa}**	4.84±0.01^{Bab}	4.42±0.01^{Baa}	0.09±0.01^{Cbb}	0.02±0.01^{Caa}
	30	0.10±0.02^{Baa}	0.15±0.01^{Bab}	0.06±0.01^{Cad}	0.48±0.01^{Bbb}	0.31±0.01^{Bbb}	0.13±0.01^{Bab}	0.00±0.0 ^{Dcd}	3.25±0.01^{Bbb}	0.00±0.0^{Dcc}	0.62±0.01^{Bcc}	0.31±0.02^{Baa}	0.02±0.01^{Caa}
	40	0.09±0.01^{Cbb}	0.07±0.01^{cbc}	0.04±0.01^{Cad}	0.44±0.01^{Bbb}	0.42±0.01^{Baa}	0.68±0.01^{Bab}	0.26±0.01^{Baa}	12.93±0.05^{Aaa}	0.00±0.0^{Dcc}	0.28±0.01^{Bcc}	0.04±0.01^{Cbb}	0.02±0.01^{Caa}
2	0	0.21±0.03^{Baa}	0.22±0.02^{Baa}	0.16±0.01^{Bcc}	0.37±0.01^{Bcc}	0.29±0.01^{Bbc}	0.38±0.01^{Bcc}	0.15±0.01^{Bab}	1.87±0.05^{Acb}	4.36±0.01^{Aab}	3.39±0.01^{Aab}	0.20±0.02^{Baa}	0.01±0.01^{Caa}
	5	0.24±0.03^{Baa}	0.14±0.01^{Bbb}	0.21±0.03^{Bbb}	0.53±0.01^{Bab}	0.31±0.01^{Bab}	0.42±0.01^{Bcc}	0.00±0.0^{Dbd}	4.08±0.01^{Abb}	4.42±0.01^{Aab}	1.38±0.01^{Abb}	0.07±0.01^{Cbb}	0.02±0.01^{Caa}
	10	0.20±0.03^{Bba}	0.19±0.01^{Baa}	0.23±0.02^{Baa}	0.48±0.01^{Bbb}	0.23±0.01^{Bbc}	0.23±0.01^{Bdd}	0.00±0.0^{Dbd}	3.53±0.01^{Abb}	4.91±0.01^{Aab}	3.94±0.01^{Aab}	0.05±0.02^{Baa}	0.00±0.0^{Dbb}
	20	0.23±0.03^{Baa}	0.22±0.02^{Bbb}	0.23±0.02^{Baa}	0.46±0.01^{Bbb}	0.26±0.01^{Bbc}	0.83±0.01^{Baa}	0.00±0.0^{Dbd}	4.22±0.01^{Abb}	1.59±0.01^{Aab}	0.83±0.01^{Bcc}	0.33±0.01^{Cbb}	0.00±0.0^{Cbb}
	30	0.19±0.01^{Baa}	0.15±0.02^{Bbb}	0.17±0.02^{Bbb}	0.43±0.01^{Bbb}	0.29±0.01^{Bbc}	0.27±0.01^{Bdc}	0.00±0.0^{Dbd}	3.87±0.01^{Abb}	0.28±0.01^{Bcb}	0.00±0.0^{Ddd}	0.06±0.01^{Cbb}	0.00±0.0^{Dbb}
	40	0.24±0.03^{Baa}	0.11±0.01^{Bab}	0.31±0.03^{Baa}	0.68±0.01^{Bbb}	0.35±0.01^{Bab}	0.79±0.01^{Bba}	0.00±0.0^{Dbd}	**15.97±0.05^{Abb}**	0.00±0.0^{Ccc}	0.41±0.01^{Bcc}	0.28±0.01^{Baa}	0.00±0.0^{Cbb}
3	0	0.15±0.03^{Bba}	0.19±0.02^{Dcd}	0.20±0.03^{Bbb}	0.60±0.01^{Bbb}	0.16±0.01^{Bbd}	0.39±0.01^{Bac}	0.00±0.0^{Cbd}	3.25±0.01^{Acb}	2.97±0.01^{Abb}	1.45±0.01^{Abb}	0.40±0.03^{Baa}	0.00±0.0^{Cab}
	5	0.16±0.03^{Baa}	0.00±0.0^{Cbc}	0.18±0.03^{Bbb}	0.54±0.01^{Bbb}	0.33±0.01^{Bab}	0.38±0.01^{Bac}	0.01±0.01^{Cac}	8.85±0.02^{Abb}	6.64±0.01^{Aaa}	2.90±0.01^{Aab}	0.05±0.01^{Cbb}	0.00±0.0^{Dab}
	10	0.16±0.02^{Baa}	0.03±0.01^{Dbc}	0.20±0.03^{Bbb}	0.37±0.01^{Bcc}	0.31±0.01^{Bab}	0.18±0.01^{Bbd}	0.00±0.0^{Dbd}	5.05±0.03^{Abb}	3.87±0.01^{Abb}	2.70±0.01^{Aab}	0.05±0.01^{Cbb}	0.00±0.0^{Dab}
	20	0.11±0.01^{Cba}	0.05±0.01^{Ebc}	0.27±0.02^{Cab}	0.42±0.01^{Bcb}	0.30±0.01^{Cab}	0.26±0.01^{Cbd}	0.00±0.0^{Ebd}	4.98±0.03^{Abb}	0.83±0.01^{Bcb}	0.28±0.01^{Ccc}	0.06±0.01^{Dbb}	0.00±0.0^{Eab}
	30	0.11±0.01^{Dba}	0.08±0.01^{Gcd}	0.29±0.01^{Dab}	0.96±0.01^{Caa}	0.33±0.01^{Dab}	0.41±0.01^{Dac}	0.00±0.0^{Ebd}	16.18±0.02^{Aaa}	0.00±0.0^{Fdc}	1.59±0.01^{bbb}	0.03±0.01^{Dbb}	0.00±0.0^{Fab}
	40	0.13±0.03^{Eba}	0.00±0.0^{Gcd}	0.29±0.01^{Dab}	0.68±0.01^{Cbb}	0.37±0.01^{Dab}	0.32±0.01^{Dac}	0.00±0.0^{Gbd}	**18.39±0.05^{Aaa}**	0.90±0.01^{Bcb}	0.00±0.0^{gdb}	0.07±0.01^{Fbb}	0.00±0.0^{Gab}

** A, B, C, D ..: Capital letters show the difference between the months for each station and the difference between the months carrying different capital letters on the same line is statistically significant (p <0.05),

a, b, c, d ..: Lower case letters show the difference between the depths for each station and the difference between the depths carrying different lower case letters in the same line is statistically significant (p <0.05),

a, b, c, d ..: Italic letters show the difference between stations for each station and the difference between the stations carrying different italic letters in the same line is statistically significant (p <0.05)

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Table 6. *Change of Nitrite-Nitrogen (NO_2-N) values depending on months, stations and depth on Tortum Lake (Mean ± SD, mgL^{-1}) (n = 4)*

St	Depth	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan
1	0	0.00±0.0^{Dcc*}	0.38±0.01^{Baa}	0.55±0.01^{Bbc}	1.27±0.01^{Aab}	0.03±0.01^{Cce}	0.66±0.01^{Baa}	0.50±0.01^{Baa}	0.23±0.01^{Bdd}	1.41±0.01^{Aaa}	0.56±0.01^{Bcb}	1.23±0.01^{Cab}	0.47±0.01^{Baa}
	5	0.00±0.0^{Bcc}	0.00±0.0^{Dbb}	0.75±0.01^{Bbc}	2.00±0.01^{Aaa}	0.03±0.01^{Cce}	0.13±0.01^{Bbb}	0.38±0.01^{Bab}	0.36±0.01^{Bdb}	0.63±0.01^{Bbb}	0.80±0.01^{Bbb}	1.19±0.01^{Cab}	0.00±0.0^{Dbc}
	10	0.00±0.0^{Ccc}	0.00±0.0^{Cbb}	0.75±0.01^{Bbc}	0.41±0.01^{Bbc}	1.13±0.01^{Aaa}	0.14±0.01^{Cbb}	0.42±0.01^{Baa}	0.42±0.01^{Bdb}	0.61±0.01^{Bbb}	0.75±0.01^{Bbb}	1.13±0.01^{Cab}	0.42±0.01^{Baa}
	20	0.00±0.0^{Dcc}	0.00±0.0^{Dbb}	1.95±0.01^{Aab}	0.14±0.01^{Bbd}	0.22±0.01^{Bbd}	0.06±0.01^{Ccc}	0.28±0.01^{Bbb}	3.55±0.01^{Aaa}	1.09±0.01^{Aaa}	1.00±0.01^{Aaa}	1.25±0.01^{Cab}	0.23±0.01^{Bab}
	30	0.30±0.01^{Caa}	0.00±0.0^{Cbb}	2.06±0.01^{Aaa}	0.00±0.0^{Dcf}	0.09±0.01^{Cce}	0.00±0.0^{Ddd}	0.06±0.01^{Ccc}	0.73±0.01^{Bcc}	0.00±0.0^{Dcd}	0.14±0.01^{Bcc}	**4.38±0.01^{Aaa}**	0.23±0.01^{Bab}
	40	0.09±0.01^{Cbb}	0.00±0.0^{Dbb}	1.50±0.01^{Aab}	0.00±0.0^{Dcf}	0.17±0.01^{Bbd}	0.09±0.01^{Ccc}	0.13±0.01^{Bbb}	2.92±0.01^{Abb}	0.00±0.0^{Dcd}	0.06±0.01^{Cdd}	0.63±0.01^{Bce}	0.22±0.01^{Bab}
2	0	0.09±0.01^{Cbb}	0.00±0.0^{Dab}	1.08±0.01^{Aab}	0.98±0.01^{Aac}	0.63±0.01^{Bab}	0.09±0.01^{Cac}	0.47±0.01^{Baa}	0.42±0.01^{Bcd}	0.98±0.01^{Abd}	0.77±0.01^{Bab}	2.81±0.01^{Abb}	0.20±0.01^{Bab}
	5	0.16±0.01^{Baa}	0.00±0.0^{Cab}	1.25±0.01^{Aab}	0.81±0.01^{Bac}	0.53±0.01^{Bab}	0.00±0.0^{Dbd}	0.41±0.01^{Baa}	0.92±0.01^{Bbc}	1.00±0.01^{Aaa}	0.31±0.01^{Bbc}	0.92±0.01^{Bbd}	0.25±0.01^{Bab}
	10	0.13±0.01^{Baa}	0.00±0.0^{Cab}	1.16±0.01^{Aab}	0.38±0.01^{Bbd}	0.61±0.01^{Bab}	0.00±0.0^{Cbd}	0.28±0.01^{Bbb}	0.80±0.01^{Bbc}	1.11±0.01^{Aaa}	0.89±0.01^{Bab}	0.73±0.01^{Bcd}	0.00±0.0^{Cbc}
	20	0.06±0.01^{Cbb}	0.00±0.0^{Cab}	0.75±0.01^{Bbc}	0.05±0.01^{Cce}	0.05±0.01^{Ceb}	0.00±0.0^{Dbd}	0.06±0.01^{Ccc}	0.95±0.01^{Bbc}	0.36±0.01^{Bcb}	0.19±0.01^{Bbc}	**4.69±0.01^{Aaa}**	0.00±0.0^{Dbc}
	30	0.06±0.01^{Bbb}	0.00±0.0^{Cab}	0.94±0.01^{Abc}	0.19±0.01^{Bbd}	0.00±0.0^{Cbe}	0.00±0.0^{Cbd}	0.06±0.01^{Bcc}	0.88±0.01^{Abc}	0.06±0.01^{Bdc}	0.00±0.0^{Cde}	0.78±0.01^{Acd}	0.00±0.0^{Cbc}
	40	0.09±0.01^{Cbb}	0.00±0.0^{Dab}	0.67±0.01^{Bbc}	0.00±0.0^{Ddf}	0.00±0.0^{Ccf}	0.00±0.0^{Dbd}	0.00±0.0d^{Ddd}	3.61±0.01^{Aaa}	0.00±0.0^{Ded}	0.09±0.01^{Ccd}	3.91±0.01^{Aba}	0.00±0.0^{Dbc}
3	0	0.06±0.01^{Bab}	0.00±0.0^{Dab}	0.75±0.01^{Bac}	0.44±0.01^{Bbc}	0.22±0.01^{Dcf}	0.00±0.0^{Dad}	0.14±0.01^{Bbb}	0.73±0.01^{Bbc}	0.67±0.01^{Bbb}	0.33±0.01^{Bbc}	**5.63±0.01^{Aaa}**	0.00±0.0^{Dac}
	5	0.03±0.01^{Cab}	0.00±0.0^{Dab}	0.75±0.01^{Bac}	1.06±0.01^{Aab}	0.42±0.01^{Bbd}	0.00±0.0^{Cad}	0.47±0.01^{Baa}	2.00±0.01^{Aab}	1.50±0.01^{Aaa}	0.66±0.01^{Bab}	0.73±0.01^{Bbd}	0.00±0.0^{Dac}
	10	0.05±0.01^{Cab}	0.00±0.0^{Dab}	0.75±0.01^{Bac}	0.44±0.01^{Bbc}	0.44±0.01^{Bac}	0.00±0.0^{Dad}	0.34±0.01^{Bba}	1.14±0.01^{Aac}	0.88±0.01^{Bbb}	0.61±0.01^{Bab}	0.72±0.01^{Bbd}	0.00±0.0^{Dac}
	20	0.00±0.0^{Bbc}	0.00±0.0^{Dab}	0.56±0.01^{Bbc}	0.19±0.01^{Bcd}	0.17±0.01^{Bac}	0.00±0.0^{Dad}	0.06±0.01^{Ccc}	1.13±0.01^{Aac}	0.19±0.01^{Bbb}	0.06±0.01^{Ccd}	0.88±0.01^{Bbd}	0.00±0.0^{Dac}
	30	0.00±0.0^{Cbc}	0.00±0.0^{Cab}	0.50±0.01^{Bbc}	0.19±0.01^{Bcd}	0.00±0.0^{Bbd}	0.00±0.0^{Cad}	0.00±0.0^{Cdc}	3.66±0.01^{Aaa}	0.00±0.0^{Ccd}	0.36±0.01^{Bbc}	0.48±0.01^{Bce}	0.00±0.0^{Cac}
	40	0.00±0.0^{Cbc}	0.00±0.0^{Cab}	0.56±0.01^{Bbc}	0.00±0.0^{Cdf}	0.00±0.0^{Ccf}	0.00±0.0^{Cad}	0.00±0.0^{Cdc}	4.16±0.01^{Aaa}	0.20±0.01^{Bbd}	0.00±0.0^{Cde}	0.95±0.01^{Bbd}	0.00±0.0^{Cac}

** A, B, C, D ..: Capital letters show the difference between the months for each station and the difference between the months carrying different capital letters on the same line is statistically significant (p <0.05),

a, b, c, d ..: Lower case letters show the difference between the depths for each station and the difference between the depths carrying different lower case letters in the same line is statistically significant (p <0.05),

a, b, c, d ..: Italic letters show the difference between stations for each station and the difference between the stations carrying different italic letters in the same line is statistically significant (p <0.05).

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Table 7. *Some morphometric and hydrological parameters of Lake Tortum*

Parameters	Symbol	Formula	Value	References
Drainage area (km^2)	Ad	-	1653	Supplied from DSI
Surface area (km^2)	Ao	-	6.45	Supplied from DSI
Drainage area/Surface area	-	Ad/Ao	256.28	Calculated from formula
Lake volume (10^6 m^3)	V	-	57.6	Supplied from DSI
Mean depth (m)	z	-	100	Supplied from DSI
Total outflow (10^6 m^3)	Q		480	Supplied from DSI
Flushing rate (yr^{-1})	p	p = Q/V	8.33	Calculated from formula
Hydraulic retention time (year)	tw	tw = 1/p	0.12	Calculated from formula
Water load (m yr^{-1})	q	q = Q/Ao	74.42	Calculated from formula
Critical phosphorus loading (g $m^{-2}yr^{-1}$)	Lp	Lp =TPL/Ao	1.71	Calculated from formula

Chlorophyll-a

The changes in the value of chlorophyll-a in different months, stations, and depths were found to be statistically significant ($p < 0.05$). The mean chlorophyll-a value of the lake was 0.04 mgL^{-1}, and the lowest value was measured as 0.01 mgL^{-1} in the months of February 2017 and January 2018, whereas the highest value, 0.15 mgL^{-1}, was detected in April. When the changes according to the stations and depths are considered, the lowest value at the first station (0.0 ± 0.0 mgL^{-1}) was found at all depths in February and at all depths except the surface in January. The highest value, on the other hand, was 0.31 ± 0.01 mgL^{-1} in April at 10 m depth. At the second station, the lowest value (0.0 ± 0.0 mgL^{-1}) was found at all depths in January and February. The highest value, on the other hand, was 0.27 ± 0.02 mgL^{-1} in April at 5 m depth. At the third station, the lowest value (0.0 ± 0.0 mgL^{-1}) was observed in November and December, whereas the highest value (0.26 ± 0.01 mgL^{-1}) was in April and at the surface.

Strong correlations were found between chlorophyll-a and NH_3-N and PO_4-P. On the contrary, the correlation was weak with other parameters. Additionally, there was a negative correlation with TP and NO_3-N (*Table 8*).

Phytoplankton composition and biodiversity in Lake Tortum

In this study, 51 phytoplankton species in total were detected in Lake Tortum; they were Bacillariophyta (38), Charophyta (2), Chlorophyta (7), Cyanobacteria (3) and Pyrrophyta (1). Throughout the investigation period, the most detected species was *Ceratium hirundinella.* In addition to these, there was a specific increase in species *Microcystis aeruginosa* and *Oscillatoria limosa* (*Table 9*).

Pyrrophyta were detected throughout the study period at the first station. On the other hand, Cyanobacteria species showed an increase only in the autumn. At the second station, all species increased in number in April. A sharp decrease in the number of all species was observed in the summer months. Later, especially in October, the number of individuals from Bacillariophyta, Cyanobacteria, and Pyrrophyta increased. Charophyta had an increase in their number in January. At the third station, Bacillariophyta were observed throughout the study period. Bacillariophyta, Cyanobacteria, and Pyrrophyta were detected increase in the number of species in April, September and October. Additionally, Cyanobacteria were more frequently detected in February and March than were the other division (*Fig. 2*).

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Table 8. *Correlation is TP, PO_4-P, NH_3-N, NO_3-N, NO_2-N and Chl-a*

		Correlations					
		TP	**PO_4-P**	**NH_3-N**	**NO_2-N**	**NO_3-N**	**Chl-a**
TP	Pearson Correlation	1	.292**	-.169**	.202**	.107**	-.060
	Sig. (2-tailed)		.000	.000	.000	.002	.076
	N	864	864	864	864	864	864
PO_4-P	Pearson Correlation	.292**	1	.023	-.094**	-.211**	.123**
	Sig. (2-tailed)	.000		.508	.006	.000	.000
	N	864	864	864	864	864	864
NH_3-N	Pearson Correlation	-.169**	.023	1	-.012	-.030	.151**
	Sig. (2-tailed)	.000	.508		.735	.384	.000
	N	864	864	864	864	864	864
NO_2-N	Pearson Correlation	.202**	-.094**	-.012	1	.570**	.060
	Sig. (2-tailed)	.000	.006	.735		.000	.078
	N	864	864	864	864	864	864
NO_3-N	Pearson Correlation	.107**	-.211**	-.030	.570**	1	-.042
	Sig. (2-tailed)	.002	.000	.384	.000		.218
	N	864	864	864	864	864	864
Chl-a	Pearson Correlation	-.060	.123**	.151**	.060	-.042	1
	Sig. (2-tailed)	.076	.000	.000	.078	.218	
	N	864	864	864	864	864	864

** Correlation is significant at the 0.01 level (2-tailed)

Table 9. *Phytoplankton taxa identified in Lake Tortum, 2018*

Bacillariophyta	*N. recta* Hantzsch ex Rabenhorst
Aulacoseira granulata (Ehrenberg) Simonsen	*N. sublinearis* Hustedt
A. islandica (Ehrenberg) Simonsen	*Pantocsekiella ocellata*(Pantocsek) K.T.Kiss & Ács in Ács & al.
Campylodiscus bicostatus W.Smith ex Roper	*Rhopalodia gibberula* (Ehrenberg) Otto Müller
C.noricus Ehrenberg ex Kützing	*Stephanodiscus neoastraea* Hakansson & Hickel
Cocconeis placentula Ehrenberg	*Surirella brebissonii* Krammer & Lange-Bertalot
Cyclotella meneghiniana Kützing	*S. elegans* Ehrenberg
Cymbella affinis Kützing	*Tryblionella scalaris* (Ehrenberg) Siver & P.B.Hamilton
Diatoma ehrenbergii Kützing	*Ulnaria ulna* (Nitzsch) Compère
D. vulgaris Broy	**Chlorophyta**
Eunotia minor (Kützing) Grunow in Van Heurck	*Asterionella formosa* Hassall
Fistulifera saprophila (Lange-Bartalot &Bonik) Lange-Bartalot	*Botryococcus braunii* Kützing
Fragilaria capucina Desmazières	*Chlamydocapsa planctonica* (West & G.S. West) Fott
Gomphonella olivacea (Hornemann)Rabenhorst	*Chlamydomonas microsphaerella* Pascher & Jahoda
Gyrosigma acuminatum (Kützing) Rabenhorst	*Chlorotetraedron incus* (Teiling) Komárek & Kovácik
Melosira varians C.Agardh	*Monoraphidium contortum* (Thuret) Komárková-Legnerová in Fott
Navicula angusta Grunow	*Scenedesmus ellipticus* Corda
N. cincta (Ehrenberg) Ralfs in Pritchard	**Charophyta**
N. cryptocephala Kützing	*Staurastrum cingulum* (West & G.S. West) G.M. Smith
N. lanceolata Ehrenberg	*S. diacanthum* A.Lemaire
N. menisculus Schumann	**Cyanobakteri**
N. minima Grunow in van Heurck	*Anabaenopsis circularis* (G.S.West) Woloszynska & V.V.Miller in V.V.Miller
Nitzschia bacilliformis Hustedt	*Microcystis aeruginosa* (Kützing) Kützing
N. communis Rabenhorst	*Oscillatoria limosa* C.Agardh ex Gomont
N. commutata Grunow	**Pyrrophyta**
N. dissipata (Kützing) Rabenhorst	*Ceratium hirundinella* (O.F.Müller) Dujardin
N. hantzschiana Rabenhorst	
N. palea (Kützing) W.Smith	
N. radicula Hustedt	

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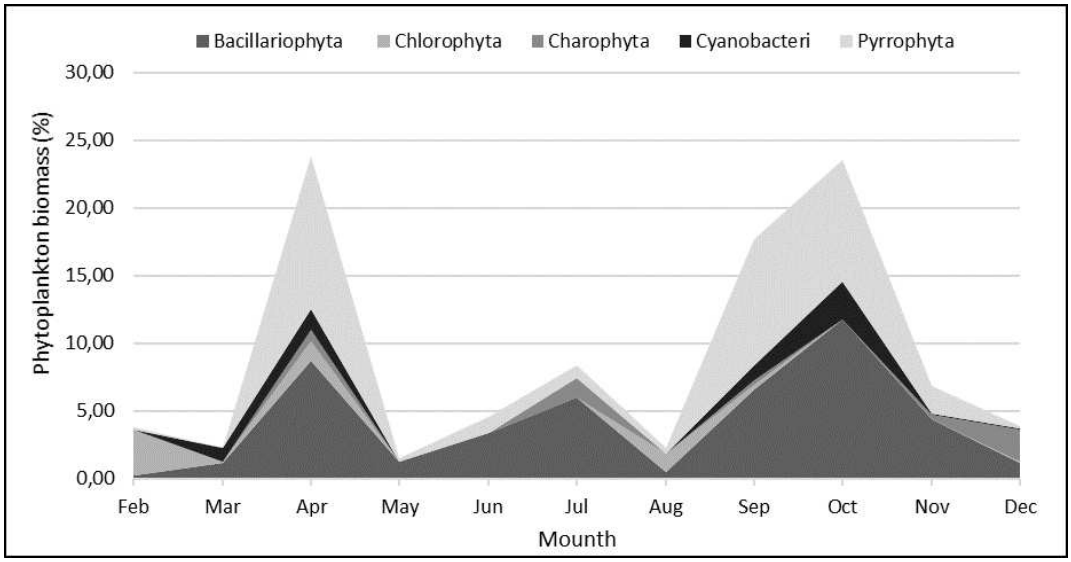

Figure 2. *Phytoplankton composition of Lake Tortum*

The dominant functional groups were both LM (*Ceratium hirundinella*) and MP (pennate diatoms) in the Lake Tortum in 2017-2018. *Ceratium hirundinella,* which is the only species found continuously during the research period, showed an increase in the autumn periods. *Cyclotella ocellata* (D) showed numerical increases in October and November. The Pennad diatoms, *Fragilaria capucina* (MP), were observed intensively in September. The other pennad diatoms *N. saprophila* and *N. palea* (MP) were idendificated in spring and early summer. In addition, *Microcystis aeruginosa* (LM) was found to be increasing in April, May and in October and *Oscillatoria limosa* (MP) was observed intensively in April (*Fig. 3*). the Q index indicated tolerable (mean 1.88) ecological conditions. The Q quality index based on stations (1, 2 and 3) were 1.79 (tolerable), 2.21 (medium) and 1.63 (tolerable), respectively (*Fig. 4*).

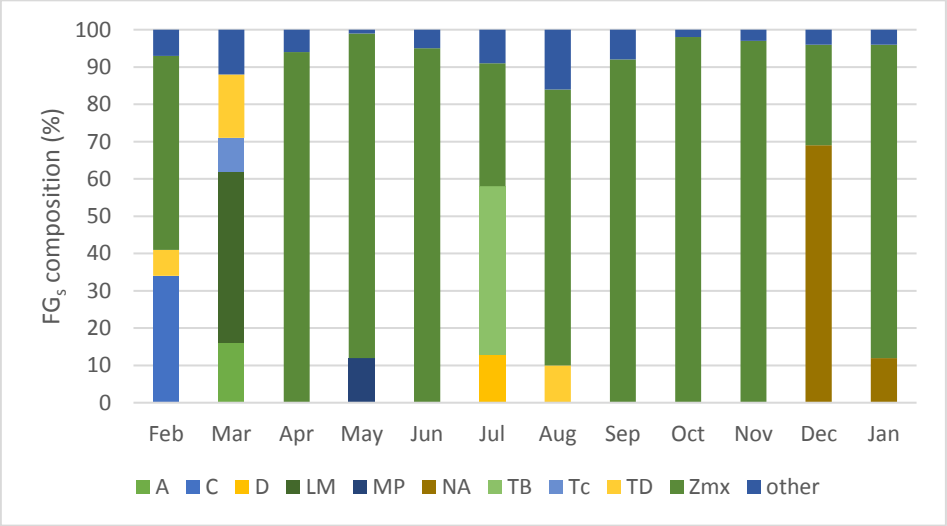

Figure 3. *Seasonal succession of phytoplankton functional groups (FGs) in in Lake Tortum. "Others" mean phytoplankton species that constituted less than 5% of the total biomass*

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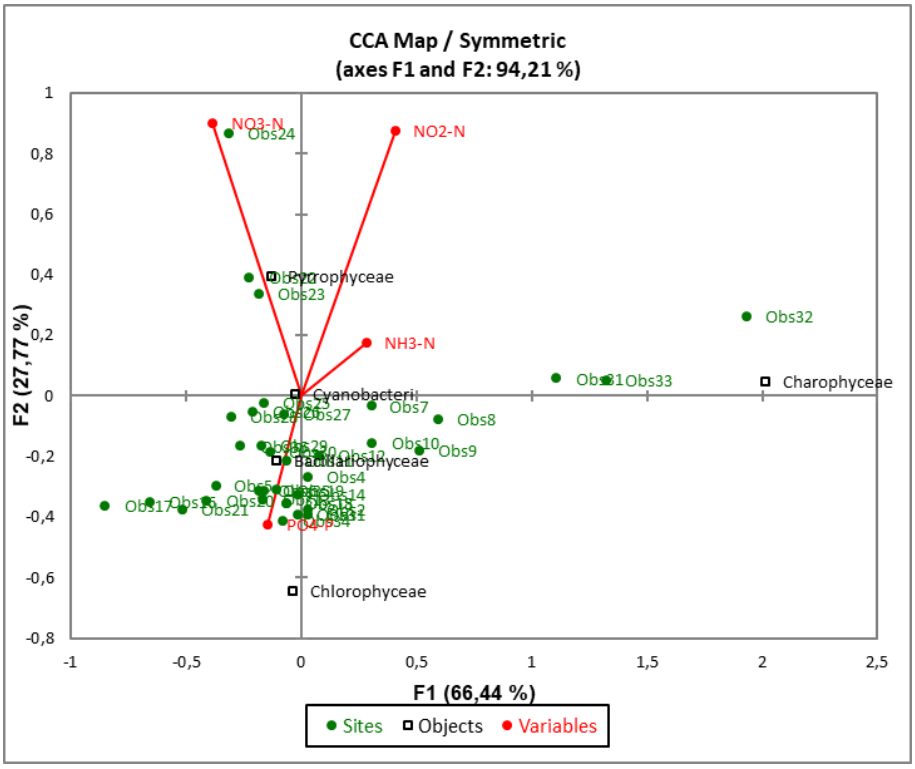

Figure 4. *Species-conditional triplot based on a canonical correspondence analysis of the example phytoplankton data displaying 27.77% of the inertia (= weighted variance) in the abundances and 94.21% of variance in the weighted averages and class totals of species with respect to the environmental variables*

Discussion

Lake Tortum is a landslide lake; moreover, it is a deep and open lake (Tanyolac, 2009). According to measurements of DSI, the mean depth of lake adopted as 100 m. Previous measurements of mean depth were 110 m in 1982 (Altuner, 1982), 100 m in 2003 (Kıvrak, 2006), and 80 m in 2013 (Fakıoglu et al., 2014). This lake is the major source for the Tortum Waterfall; for that reason, the regulator in the discharge region of the lake is opened during periods of drought to feed the waterfall. Thus, there may be temporary reductions in the water level. Nevertheless, the studies done in Lake Tortum indicate a 10 m reduction in depth since 1982.

In this study period, a weak thermal stratification in the lake has been observed. Even though mean water temperature value was measured 6.81 ± 1.00 ºC in this study, it was measured in 2003 and 2013 as 12.85 ± 5.75 ºC and 10.5 ºC, respectively (Kıvrak, 2006; Fakıoglu et al., 2014). Warm winters are especially critical to temperate in monomictic lakes, because the temperature affects the duration of the winter and its strength and, consequently, the thermal budget of the lake and the vertical distribution of nutrients and dissolved oxygen (Stratile et al., 2003).

The mean dissolved oxygen value in Tortum Lake was found as 6.81 ± 1.00 mgL^{-1} during the study. When there was mixing in the lake, an increase in dissolved oxygen concentration at the 40 m depth was observed. According to Turkish Environmental Legislation for Inland Water Resources Classification, the lake was identificated as II. class waters quality (Anonymous, 2012). The average dissolved oxygen value was found

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12.85 ± 5.75 mgL^{-1} in 2006 and 10.5 mgL^{-1} in 2013 (Kıvrak, 2006; Fakıoglu et al., 2013). Dissolved oxygen value has droped significantly since 2006. Increased organic load in the past years and subsequent organic degradation may have caused a decrease in dissolved oxygen value. However, fishing in the lake has been banned in the past 5 years, which has increased the fish population. In this case, it is thought that it will affect the decrease in oxygen value.

The lakes being in the easily melted area are hard-water lakes and forthis type of lakes the pH is close to 8.5 (Tanyolaç, 2009). Tortum Lake is located in the basin where landslide events are seen too much and the average pH value of lake was measured as 8.48. And, according to the total hardness classification of Lawson (1995) for lakes, water of Lake Tortum is in the hard water classification. Plant biomass is particularly high in moderately hard water bodies (Tanyolac, 2009). Therefore, biodiversity in the lake is very low, only two types of chlorophytes were observed in the littoral region, and only 51 phytoplankton species were identified in the lake.

The mean electrical conductivity was 0.289 ± 0.03 mS cm^{-1}. In another study in Lake Tortum in 2006, the electrical conductivity was found to be 0.308 mS cm^{-1} (Kıvrak, 2006). Natural lakes with discharge points typically have electrical conductivity values between 0.1 and 1 mS cm^{-1}. (Ozturk, 2014). It is possible to establish a connection between total dissolved solids concentration and electrical conductivity. The more ion and total dissolved solids concentrations in water, the higher electrical conductivity it has (Metcalf and Eddy, 2002). Tortum Stream carries almost 2.5 million m^3 of suspended sediment carries to the lake every year (Kopar and Sevindi, 2013).

In deep lakes, changes in depth and vertical stratification alter the water quality (Salmoso et al., 2002). In this study, according to the trophic status range of natural lakes reported by Wetzel (2001) and Anonymous (1982), Tortum Lake, Secchi depth (4.15 m), chlorophyll-a (0.04 ± 0.0005 mgL^{-1}) and total phosphorus concentration (0.31 ± 0.03 mgL^{-1}), was calculated as mesotrophic. In 1982, the lake was reported to be in an oligotrophic state (Altuner, 1982), and it was pointed to be oligo-mesotrophic in 2003, as well (Kıvrak, 2006). Unfortunately, the studies on this lake are very limited. In addition, not all physico-chemical parameters of the lake have been studied in these previous studies. However, these studies cover only certain periods, not every period. Although it makes it difficult for us to understand the change in the lake very clearly, collecting the data of the lake is important for the future of the lake.

In terms of TP values, the lake's trophic level changed from the oligotrophic to the mesotrophic level. In deep lakes, such trophic level changes are under the control of both climatologic and anthropologic variables (Salmoso et al., 2002). The highest TP value was seen on the surface at the first station. At the second and third stations, especially during the time when the mixing is strong (such as in summer months), the 40 m depth showed high TP values. Phosphorus has a tendency to precipitate on sediments in the particle state (Pulatsu and Topcu, 2012). Besides, the TP also depends on the geologic structure of the region and on organic matter entry into the water (Wetzel, 2001; Tanyolac, 2009). The mean orthophosphate concentration was found to be 0.1 ± 0.01 mgL^{-1} in this study. That value was 0.05 mg/L in a study done in 2003 in Lake Tortum (Kıvrak, 2006). According to Wetzel (2001), increased zooplankton numbers come from increased orthophosphate concentration, whereas Ozkundakçı (2014) claimed that they were affected by agricultural pollution. Zooplankton species and number were observed quite abundant over the study period. While a fraction of the phosphorus is expected to precipitate on sediments in deep and monomictic Lake Tortum, another part

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again returns to the water column according to the sedimentation, disintegration, and transformation ratios. Moreover, the concentrations of Fe, Al, Cd, and Pb elements were found to be low in heavy metal analyses conducted in Lake Tortum water, yet the potential ecological risk coming from those elements together has been determined to be moderate (Kaya et al., 2017). The increase in total phosphorus value in the lake, led to an increase in the number and variety of the primary producers of the lake. At the first station, where the lake is fed, the chlorophyll *a* value in summer months at the 40 m depth was found to be 0.03 ± 0.01 mgL^{-1}, while at other stations in the spring months and in the epilimnion stratum (the upper thermal layer of the lake), it was 0.13 ± 0.02 mgL^{-1}. Similar situations were also observed in other deep lakes, Lakes Gordo and Iseo; in both of them, when the temperature rose in the summer months, both the algae biomass and the level of nutrient salts increased (Salmaso, 2002).

In this study, the NH_3-N mean value was found to be 0.14 ± 0.07 mgL^{-1}. Fakıoglu et al. (2014) and Kıvrak (2006) were found that value to be 0.15 mgL^{-1} and 0.19 ± 0.01 mgL^{-1} in 2013 and 2006, respectively. The mean NO_2-N concentration and NO_3-N concentration were calculated 0.55 ± 0.08 mgL^{-1}, 1.10 ± 0.02 mgL^{-1}, respectively. An increase was observed for those values in comparison to NO_2-N (0.07 mgL^{-1}, 0.002 ± 0.0 mgL^{-1}) and NO_3-N (0.48 mgL^{-1}, 0.08 ± 0.01 mgL^{-1}) values found in 2013 and 2003. In this case, we can say that the amount of ammonia-nitrogen coming to the lake is decreased or the ammonia-nitrogen might be converted to nitrite-nitrogen by the nitrobacterias. Approximately 92% of the villages around the lake were built on the mountain slopes. The lands that have flatness are used as agricultural lands which contain rich alluvium material (Kopar and Sevindi, 2013). In addition, the increase in nitrite concentration is important as it is an indicator of pollution caused by organic or industrial waste (Pulatsu et al., 2014).

Reduction of the external phosphorus load to the lake is a very crucial step in eutrophication control. There are several studies illustrating the success of that process (Cole, 1983; Oenema, 1991). In Lake Tortum, phosphorus loading was calculated to be 11050 kg yr^{-1} (*Equation 5*). That value is much higher than the critical phosphorus loading value calculated by the formula of Vollenweider (1976). Two main factors on the external phosphorus loading are agricultural activities and the absence of sewer systems (Bronmark and Hansson, 2017). There have been intense reclamation and road expansion activities in recent years on Tortum Stream. Besides, sewer systems are absent in many regions in the vicinity of Tortum Stream and its tributaries. This study found the main component of the TP to be the artificial artifical phosphorus loading. The domestic load was calculated 9707 kg yr^{-1} (*Equation 4*). In the studies carried out in Mogan Lake, domestic phosphorus load was found 2998 kg yr^{-1} in 2002 (Pulatsü and Aydın, 1997). In another study conducted on the same lake, domestic phosphorus load was determinated 8084 kg yr^{-1} in 2004 (Fakıoglu and Pulatsu, 2004). Domestic phosphorus load value of the study was found to be higher than those values in Lake Mogan, which is a eutrophic lake status. While Lake Mogan is not only a shallow lake, but also under effect of agriculture and anthropogenic pollution, Tortum Lake is both deep and many of these effects are not seen on the shore of the lake. Nevertheless, the external phosphorus load calculated for Tortum Lake was found to be high. This is thought to be a threat to the lake. On the other hand, for the potential ecological risk evaluation of the lake, it was found to be under moderate risk threat; however, while the contamination load risk should be 0, it was found between 0 and 1 (Kaya et al., 2017).

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The phosphorus export coefficient was calculated to be 1.71 g m^{-2} yr^{-1} (*Equation 6*) for Lake Tortum in this study. Even though that value is less than the critical load of 2.17 g m^{-2} yr^{-1} (*Equation 9*), it is still higher than the permissible phosphorus export coefficient (0.96 g m^{-2} yr^{-1}, *Equation 8*). Kirchner and Dillon (1975) considered a field with a phosphorus export coefficient between 1.41 and 14.88 g m^{-2} yr^{-1} to be a forest field and pasture. Lake Tortum is in that category. However, the lake was determinated as oligotrophic status according to the Dillon and Rigler (1975) classification. The phosphorus export coefficient was found to be lower than the critical value of Wetzel (2001) for agricultural and forestry fields.

The reaction of deep lakes to the reduction of external nutrient load has been reported to be faster than that of shallow lakes (Beklioğlu, 1999). Harper (1992) stated that permissible phosphorus loading is 0.4 g m^{-2} yr^{-1} and that hazardous phosphorus loading is 0.8 g m^{-2} yr^{-1} for 100 m deep lakes. Thus, Lake Tortum's phosphorus load is higher than the hazardous level. Even though the nutrient status of the lake was determined to be mesotrophic in terms of TP concentration, unless the necessary precautions are taken the lake might rapidly transform into a eutrophic state. In another deep lake, Lake Bolsena, it was stated that remediation of the lake will take a very long time due to the increase in the external phosphorus loading and the fact that the increase also raised algae production and mineralization in the hypolimnion, the lower thermal layer of the lake (Mosello et al., 2018).

Phytoplankton communities react to the changes in the lake environment rapidly, while they are also in a rivalry for seasonal succession, which is why alterations in the environmental conditions lead to huge compositional diversity (Scheffer et al., 2003). In this study, 51 phytoplankton were detected. Altuner (1982) investigated the phtoplankton in the lake in 1981, and 35 species in total were detected from Bacillariophyta, Chlorophyta, Cyanophyta, Dinophyta, and Crysophyta. Another researcher examined the phytoplankton community in the lake in 2006 and found only 12 species, mainly from Bacillariophyta (Kıvrak, 2006). Lake Salda and Lake Burdur are a deep and highly alkaline lakes of Turkey. These lakes were found at the oligotrophic level due to low nutrient and chlorophyll-a measurements. In both lakes, the taxa of phytoplankton were low (15 taxa in Lake Salda, 21 taxa in Lake Burdur) (Kazanci et al., 2004; Girgin et al., 2004).

The dominant phytoplankton species change according to the nutrient level of the lake. In Lake Tortum, species *Microcystis aeruginosa* shows an increase in number from time to time. Desmidiaceae and centric diatoms indicate that the lake is oligotrophic, and pennate diatoms and cyanobacteria, on the other hand, are an indicator of a eutrophic lake (Harper, 1992). Species from both groups have been observed in Lake Tortum. The phytoplankton and benthic algae compositions were detected in 1979–1981 as oligotrophic. The researchers observed a high number of *Cyclotella kützingenia,* a centric diatom, and, albeit in low numbers, *Ceratium hirundinella* and *Microcystis* spp. (Altuner, 1982). On the other hand, in a study done in 2002–2003, the dominant species were determined to be *Chlamydomonas microsphaerella*, *Cyclotella krammeri*, *C. glomerate*, and *Ceratium hirundinella*, while the rare species were *Stephanodiscus rotula*, *Fragilaria ulna*, *Cocconeis placentula*, *Cymbella affinis*, *Navicula salinarum*, *Carteria* spp., *Staurastrum vestitum*, *Trachelomonas volvocina*, and *Peridinium cinctum* (Kıvrak, 2006).

The most detected species in our study was *Ceratium hirundinella*. The second and third were *Cyclotella meneghiniana* and *Fragilaria capucina*. According to Reynolds et

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al. (2002) Cyclotella spp. generally found in oligotrophic lakes, but Padisák et al. (2009) reported that this species has mesotrophic or eutrophic lakes. Although *Ceratium hirundinella* was reported as a species found in nutrient-rich oligotrophic and mesotrophic lakes in summer months (Reynolds et al., 2002), the highest number they reached in this study was seen in October. Some researchs were turn *C. hirundinella* considered as oligotrophic and mesotrophic lakes; while others were evaluated as eutrophic to hypertrophic, small to medium-sized lakes (Padisák et al., 2009; Aboim et al., 2020). However, Dinoflagellata taxa often have been found in eutrophic and mesotrophic lakes of Turkey (Gönülol and Obalı, 1986; Taş et al., 2002; Yerli et al., 2012). Moreover, the indicators of nutrient-rich turbid lakes, *Ulnaria ulna* and *Nitzschia* spp. (Padisák et al., 2006), were also detected in this study. Besides, *Anabaena elenkinii* (code: H1), *Microcystis aeruginosa* (code: LM), and *Oscillatoria limosa* (code: MP), belonging to Cyanobacteria, and *Chlamydomonas microsphaerella* and *Asterionella formosa* (code: C), belonging to Chlorophyta, were also observed. They are indicators of eutrophic water bodies (Kıvrak, 2006). Reynolds et al. (2002) indicate that *Asterionella formosa*, *Aulacoseira granulata* (code: P), and cyanobacteria are typical indicators of eutrophic lakes. In Lake Tortum, *Asterionella formosa, Aulacoseira granulata*, and *Microcystis aeruginosa* species were detected. In 2014, a yellow area was spotted in the lake, and, when it was investigated, a rapid increase in the numbers of *Asterionella formosa* was found.

When functional groups were examined on a month-by-month scale, dominant functional groups were found to be LM (*C. hirundinella,* Microcystis) in April, October and November, MP (Diatome, Navicula, Cymbella, Oscillatoria) in March, C (*Asterionella formo*sa) in Februvary, B (*Cyclotella meneghiniana*) in March and TB (Fragilaria, Nitzschia) in July. In Lake Mogan, the dominant phytoplankton functional groups of the study period were X2, H1, Y, LM, F, Lo, M, W1, C, P, X1, and S1 and the Q index was indicated a moderate ecological status for Lake Mogan (Demir et al., 2014). Lake Tortum was determined to be mesotrophic according to chemical parameter and chlorophyll-a. Hovewer, Q index was indicated tolerable ecological quality status. The reason for this is that *C. hirundinella*, which is located in Lake Tortum, is evaluated as a functional group in not only oligotrophic but also eutrophic lakes.

Q index has changed in bad ecology except in February and August (clean water phase) in 1th station due to seasonal change in stations. Similarly, it was observed in 3rd station, but this station was in excellent ecological condition in July. It is thought to be the reason that the high phosphorus loading to the lake affects the biodiversity in the 1st station, while filling the lake in 3rd station and the use of these areas as recreational areas. The 2nd station is in the moderate ecological state. Since Tortum Lake is in the deep lake class, it is thought that the ecological status of this station is better than the others. Water quality variables and phytoplankton were examined within the Jequitinhonha River lower course. Results indicated that chemical oxygen demand, dissolved aluminum, and turbidity were the main factors which influenced phytoplankton community structure and composition (Aboim et al., 2020).

In this study, the highest number of total phytoplankters by count was found in April whereas the lowest count was in January. In Lake Tortum, the TP load was found to be higher than the critical phosphorus load. In addition to that, NO_2-N and NO_3-N were also high in concentration. As a result, both the species biodiversity and their numbers increased. In the development of Charophyta was effected NO_3-N value and in Pyrrophyta were found to play a role NO_3-N and NH_3-N values. TP and PO_4-P were found to be a

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limiting factor in species in Bacillariophyta and Chlorophyta. Cyanobacteria group algae were calculated as limiting both nitrogen and phosphorus fractions (*Fig. 5*). However, Pedators are known to be the main factor in the phytoplankton species diversity and numerical increase as well as nutrient salts. After the rapid increase in the number of phytoplankton in April, there was a sharp decline in May, which can be attributed to the grazing pressure coming from zooplankton at that time, which was also seen in October. It has been reported that, after the increase in phytoplankton and subsequent decrease in nutrients and the filtration of the zooplankton, a clear-water state can arise (Lampert and Sommer, 1997). On the other hand, after the increase in the TP load and, consequently, the increase in phytoplankton and suspended algae (and production) in the lake, there may be a shift in phytoplankton species to the toxic ones or the ones that have not been grazed efficiently by hunter species. That may result in an algal bloom. In addition to that, the increase in phosphorus loading causes an increase in production, biomass, and microalgae composition (Dogan-Saglamtimur and Saglamtimur, 2018).

Conclusion

It is important to protect and to continuously monitor inadequate freshwater resources and to control the changes that can occur in the lakes as well. Deep and meromictic lakes are especially problematic in regard to restoring their trophic states. It is reported that, in temperate climates where meromictic lakes are prevalent, global warming has a stronger and more steady impact on stratification and, consequently, on lake restoration (Lepori et al., 2018). The works done on Lake Tortum, such as island creation by adding earth and degradation of the shoreline, along with the destructions on Tortum Stream, have significantly damaged the lake ecosystem. Lake Tortum's contamination increases every day. For that reason, species composition in the lake has transformed with time, and more and more eutrophic species have started to appear and become dominant species. In conclusion, the detection of the mechanisms affecting the ecology, hydrology, and morphology of deep lakes is critical for protecting these aquatic systems.

In the future studies are recommend that comprehensive determination of carbon, nitrogen and phosphorous substance loads entering the lake from external point and spread sources, and the use of lake models (mathematical model), including bioprocesses (carbonaceous removal, nitrification, denitrification, photosynthesis) and other physical (sedimentation, resuspension) and chemical processes (adsorption, chemical precipitation) that have an impact on the consumption and production of the lake.

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STAND STRUCTURE AND SPATIAL DISTRIBUTION OF TREES AT DIFFERENT DEVELOPMENTAL STAGES AND STAND LAYERS IN MIXED STANDS IN ARTVIN REGION, TURKEY

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Abstract. The objective of this study is to determine the stand structure and spatial distribution patterns in rare mixed stands of Oriental spruce (*Picea orientalis* L.), Scots pine (*Pinus sylvestris* L.) and Caucasian fir (*Abies nordmanniana* (Stev.) Spach. subsp. *nordmanniana*). The study was conducted in Ovacik Forests of Artvin, Turkey in 2016. To evaluate stand structure, the distribution of tree species was determined according to developmental stage (seedling- thicket stage; sapling- pole stage; small wood stage; medium - large wood stage) and vertical structure (overstory, middlestory and understory). Spatial distribution of trees was analyzed using Ripley's K function in ArcGIS. Results showed that all three species contributed to the mixed stand. Caucasian fir dominated in in all plots and plots had a layered structure. Spatial analyses revealed that all trees showed clumped distributions ($p<0.01$) at smaller scales. Scots pine and Caucasian fir mostly showed random distribution whereas Oriental spruce showed a clumped distribution at 0–7 m scales. Each of the tree species at the medium wood developmental stage showed random distributions at all scales. Clumped distribution was observed in the middle and understory while trees in the overstory layer were randomly distributed.
Keywords: *spatial pattern, layered structure, Scots pine, Oriental spruce, Caucasian fir*

Introduction

Spatial distribution can be described as the data set of points positioned in an almost planar area (Gabriel et al., 2013). It is possible to use spatial distribution to express how forest trees are distributed since they are obtained by the analysis of point data (Montes et al., 2008). In the context of forestry, spatial distribution can be described as the point distribution pattern of trees in a stand or forest area (Dale, 2000).

In spatial distributions that are grouped into two classes, as intra- and inter-species, points belonging to the same species represent intra-species (univariate) distributions and points belonging to different species represent inter-species (multivariate) distributions. Intra-species distributions are classified as random, clumped or regular while interspecies spatial distributions are classified as segregated or aggregated (Ripley, 2005).

Spatial distribution of trees is important in investigating forest regeneration methods, as it reflects the spatial distribution of seedlings (Paluch, 2006). In other words, the spatial distribution of trees reflects the element that triggers regeneration and the subsequent arrival of seedlings at the site. Therefore, it can be said that understanding the spatial distribution of forest trees is important in maintaining the continuity of silvicultural systems.

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Spatial distribution of trees is important in defining stand structure (Gadow et al., 2012). The spatial patterns of trees in mixed stands determines their mixing patterns. Since the crowns are adjacent in single-layered mixtures, this mixing pattern is also called a horizontal mixture. In multi-layered mixtures, crowns of tree species in the mixture are overstory, understory or middle story, relative to each other and therefore this mixing pattern is also termed a vertical mixture (Genc et al., 2012). Random, clumped and regular distributions can be referred to as tree-wise, group-wise and row-wise mixtures, respectively, to characterize horizontal mixing patterns in mixed stands (Del Rio et al., 2015).

Tree species are located randomly in mixed stands in tree-wise mixtures while, in group-wise mixtures, tree species are scattered in clumps or groups within the stand. In row-wise mixtures, distribution is regular because there is a particular distance between trees and a regular row-wise mixture is usually observed in artificial regeneration sites (Genc et al., 2012).

Due to the mountainous and rough terrain in forests of the Artvin region, stand structures and mixing types change over even short distances. Such changes are observed especially with respect to tree species and mixing rates. Ovacik forests located in the Artvin region show good examples of such forests, which are also forests with the least human intervention. For these reasons, Ovacik forests have been the subject of this present study.

In this study, we tested changes in stand structure and the hypothesis that spatial patterns of trees will differ, depending on development stage and stand layer, in mixed stands which have different mixing ratios of Scots pine, Oriental spruce and Caucasian fir. Accordingly, mixing patterns were characterized in relation to the distribution patterns of the three tree species.

Materials and methods

Study area

The study was conducted in the Ovacik Forest Management Unit of the Ardanuc Forest Enterprise of Artvin Forest Regional Directorate, Turkey (*Fig. 1*) in 2016. The site is a natural mixed forest of Scots pine, Oriental spruce and Caucasian fir (*Fig. 2*). The characteristics of the study site are given in *Table 1*.

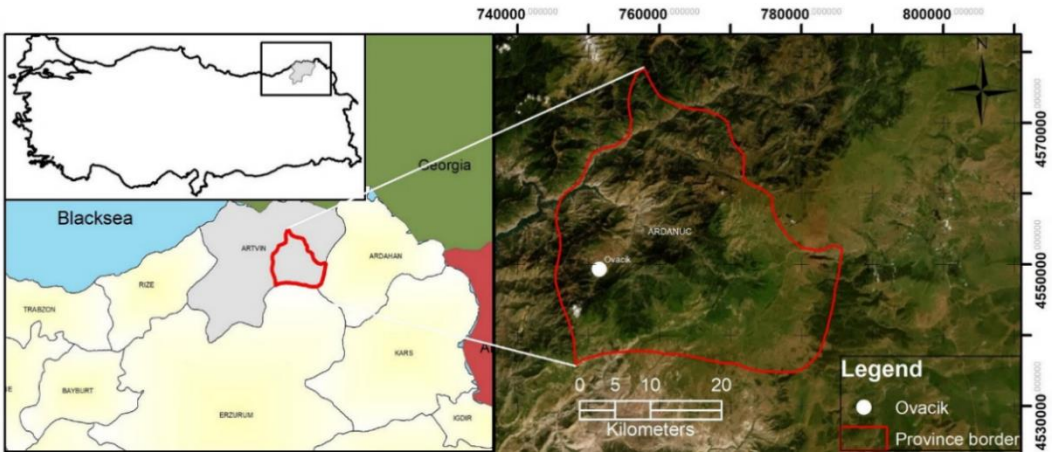

Figure 1. *Locations of study area*

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Figure 2. *Photo about samplings sites*

Table 1. *Characteristics of the study site.*

Characteristics	**Plot 1**			**Plot 2**			**Plot 3**		
Plot Size (ha)	0.25			0.25			0.25		
Exposure	West			West			West		
Altitude (m)	1763			1742			1707		
Lon (E)	41°59'20.46"			41°59'18.27"			41°59'16.07"		
Lat (N)	41° 1'25.10"			41° 1'24.93"			41° 1'24.70"		
Species in Mixture	Scots pine	Oriental spruce	Caucasian fir	Scots pine	Oriental spruce	Caucasian fir	Scots pine	Oriental spruce	Caucasian fir
Stand Basal Area (BA) (m^2 ha^{-1})	16.07	7.77	19.42	19.93	10.83	16.03	15.19	13.5	15.66
Percentage of species in BA (%)	37.15	17.96	44.89	42.59	23.15	34.26	34.24	30.45	35.31
Number of trees (ha^{-1})	132	248	384	184	356	488	148	348	356
Mean Diameter at Breast Height (DBH) (cm)	36.88	17.26	23.26	34.37	16.16	17.28	34.59	18.86	20.71
Maximum DBH (cm)	67	47	49	62	60	49	54	61	50
Mean height of the 100 largest trees ha^{-1} (m)	18.4			24.8			23.4		

Data collection

Three 0.25-ha plots were randomly selected within the stand. The plots used in this study were square to accurately determine the spatial distribution of the trees. Plots were typically located on sites with 40–60% slopes. Since the plots would be regarded as 50 m × 50 m, the length of the plots perpendicular to the contour lines in sloped sites was determined by taking the cosine of the angle between stems (Q). Accordingly, the length of the plot sides was calculated using the following equation (*Eq. 1*).

$$Plot\ side\ length\ =\ 50\ m/Cos\ (Q) \qquad \text{(Eq.1)}$$

All trees, including cut, fallen and standing dead trees with DBH greater than 4 cm, were numbered. Measurements of DBH, tree height and distance-angle between stems were carried out in every plot to determine the coordinates of individual trees. The tree-to-tree distance-angle measurement method was used because of the convenience of

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central tree selection and the practicality of the procedure (Ripley, 2005). All live, standing dead, fallen stems and stumps were considered for distance-angle measurements in each plot. Trees in each plot were classified into classes with a 3-cm range based on their DBH, and the height of three trees per species in each diameter class was measured by using the Blume-Leiss (Baumhöhenmesser-von-Bloeck) hypsometer (manufactured in Oranienburg, Brandenburg Germany), accurate to within 1 cm.

Data analysis

The mean DBH values, which are used to differentiate stand development stage diameter thresholds in forest management (Genc et al., 2012), were used to determine the number of Scots pine, Oriental spruce and Caucasian fir trees in the plots at each development stage (*Table 2*), namely seedling stage (a), sapling stage (b), small wood stage (c) and medium to large wood stage (d). The number of trees and basal area calculated for each plot were grouped according to development stage DBH intervals for each species, and their numbers and proportions were determined.

Table 2. *DBH ranges of development stages*

Development Stage*	Symbols	DBH Ranges
Seedling - thicket stage	a	$d_{1.3} < 8$ cm
Sapling - pole stage	b	$8.0 < d_{1.3} < 19.9$ cm
Small wood (tree) stage	c	$20.0 < d_{1.3} < 35.9$ cm
Medium - large wood stage	d	$36.0 \leq d_{1.3}$

* In the later parts of the article, symbols of the development stages have been used

The DBH and height values measured for each species were regressed against one another using the SPSS program (IBM, Armonk, NY, USA). The S regression model was considered to be the most appropriate model to estimate the height of trees which were not directly measured. The S model consists of the following equations (*Eqs. 2-3*).

$$y = e^{b_0 + b_1/x} \quad \text{(Eq.2)}$$

$$lny = b_0 + b_1/x \quad \text{(Eq.3)}$$

where y is the estimated height, e is the natural log (2.71), x is the measured DBH (i.e. the independent variable) and b_0 and b_1 are the coefficients of the regression equation.

There is a systematic error in the *Eq. 2* because the coefficients were calculated using logarithm-transformed data. The values obtained using these regression equations need to be multiplied by a correction factor to remove this systematic error. The correction factor used in this study (f) is calculated using the Baskerville (1972) equation (*Eq. 4*), below which "e" and "Syx" represent the natural log and the standard error of the equation, respectively.

$$f = e^{\frac{S_{y.x}^2}{2}} \quad \text{(Eq.4)}$$

The vertical arrangement of a stand is characterized by determining the layering of the canopy using physical data of tree height or vertical extent (Morsdorf el al., 2010). The

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classification, based on the vertical status of a tree, was taken into account to calculate the proportions of the number of trees within each stand layer for determining the vertical mixture in the plots. According to this, trees with heights greater than two-thirds of the maximum height were classified as overstory (O) whereas trees whose heights fell between one-third and two-thirds of the maximum height were categorized as middlestory (M), with trees whose heights were less than one-third of the maximum height being grouped into the understory (U) class. Stand dominant height, determined as the mean height of the highest 100 trees per hectare, was calculated for each plot.

Analysis of spatial distributions

The coordinates of the trees were grouped according to species, developmental stage and stand layer difference in order to generate the spatial distribution maps. Point distribution data files and species distribution maps were generated in ArcGIS 10.2.1 software released by Environmental Systems Research Institute (ESRI), using these grouped coordinates.

Spatial distribution analyses were conducted using the multi-distance spatial cluster analysis method in ArcGIS software. ArcGIS software uses Ripley's K function (Ripley, 2005) in multi-distance spatial cluster analysis (Mitchell, 2005). The null hypothesis is that all trees at the site display random distribution. Confidence intervals were used to test the reliability of the observed and expected values and to determine significant deviations from the complete spatial randomness pattern. Confidence intervals was constructed at the 99% confidence level (99 simulations).

Spatial analysis was focused on developmental stages (a, b, c, and d) and stand layers (O, M, and U) for all trees (A) and individual tree species (Scots pine, Oriental spruce and Caucasian fir). In the tables and figures, spatial analysis results were labeled for each tree species according to their developmental stages and stand layers. For example, results of spatial analysis for all trees (A) at the seedling stage (a) were labeled as A(a) and the understory (U) for all trees were labeled as A(U).

Results and discussion

Stand structure

The number of trees in plots 1, 2 and 3 were 764, 1028 and 852 trees ha^{-1}, respectively, and the basal area in plots 1, 2 and 3 were 43.26, 46.9 and 44.35 m^2 ha^{-1}, respectively. All three species contributed to the mixture in all of the plots in terms of tree number and basal area. Even though Caucasian fir represented the main stand species in terms of number in each of the plots, Scots pine dominated only in plot 2 in terms of basal area (*Table 3*).

The greatest basal area occurred in stages c (small wood stage) and d (medium to large wood stage) in all of the plots. An examination of species presence by number and basal area in the plots showed that Oriental spruce and Caucasian fir were present at each development stage, while Scots pine was mainly concentrated at stages c and d, with few individuals at stage b and almost none in stage a. Similar to these results Gokturk and Demirci (2017) stated that in the majority of the mixed stands of the scots pine, oriental spruce and Caucasian fir there was little or no scots pine youths and thickets. This result showed that the number of seedlings was affected by the stand structure as reported by Kara and Topacoğlu (2018) and Paluch et al. (2019). Pretzsch et al. (2015) reported that

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the absence of Scots pine seedlings from stands where it contributes to the mixture is related to the amount of light. Scots pine seedlings needs minimum 35% light to achieve 50% of the growth that would be achieved in full light, while spruce and fir needs 20% and 15% light, respectively (Hale, 2004). The crown cover and branching, and the needle density of Oriental spruce and Caucasian fir were greater than for Scots pine. Therefore, the top canopy of Oriental spruce and Caucasian fir was opaque compared to the Scots pine canopy, resulting in light intensities less than required for growth of Scots pine seedlings in the lower canopy.

Table 3. *Mixing rates of trees by species and development stages (DS) in the plots*

Species	DS	Number of Trees (trees ha^{-1})			Number of Trees (%)			Basal Area (m^2 ha^{-1})			Basal Area (%)		
		1	2	3	1	2	3	1	2	3	1	2	3
Oriental spruce	a	28	64	52	3.66	6.23	6.1	0.08	0.2	0.19	0.19	0.43	0.42
	b	136	192	152	17.8	18.68	17.84	1.62	2.25	1.97	3.74	4.81	4.44
	c	72	80	108	9.42	7.78	12.68	4.31	4.41	5.57	9.97	9.42	12.55
	d	12	20	36	1.57	1.95	4.23	1.76	3.97	5.78	4.07	8.48	13.03
Subtotal		248	356	348	32.46	34.63	40.85	7.77	10.83	13.5	17.96	23.15	30.45
Scots Pine	a	0	0	4	0	0	0.47	0	0	0.01	0	0	0.02
	b	12	28	8	1.57	2.72	0.94	0.13	0.44	0.16	0.31	0.95	0.37
	c	48	68	68	6.28	6.61	7.98	3.05	4.88	4.83	7.04	10.43	10.89
	d	72	88	68	9.42	8.56	7.98	12.89	14.6	10.19	29.79	31.21	22.97
Subtotal		132	184	148	17.28	17.9	17.37	16.07	19.93	15.19	37.15	42.59	34.24
Caucasian fir	a	20	84	32	2.62	8.17	3.76	0.04	0.29	0.11	0.1	0.61	0.25
	b	152	252	156	19.9	24.51	18.31	2.19	3.23	2.04	5.06	6.91	4.61
	c	144	104	128	18.85	10.12	15.02	8.48	6.14	7.77	19.6	13.13	17.52
	d	68	48	40	8.9	4.67	4.69	8.71	6.37	5.73	20.13	13.61	12.93
Subtotal		384	488	356	50.26	47.47	41.78	19.42	16.03	15.66	44.89	34.26	35.31
A*	a	48	148	88	6.28	14.40	10.33	0.12	0.49	0.31	0.28	1.06	0.70
	b	300	472	316	39.27	45.91	37.09	3.94	5.92	4.17	9.11	12.66	9.40
	c	264	252	304	34.55	24.51	35.68	15.84	15.43	18.17	36.62	32.98	40.97
	d	152	156	144	19.90	15.18	16.90	23.36	24.94	21.7	54.00	53.30	48.93
Total		764	1028	852	100	100	100	43.26	46.79	44.35	100	100	100

*A emphasizes all tree species in the plots

It is clear that Scots pine seedlings cannot find an opportunity to become established because of insufficient light following the emergence of Oriental spruce and, in particular, Caucasian fir seedlings at the site. It is evident that the growth conditions are not favorable for Scots pine under Caucasian fir and Oriental spruce cover.

Caucasian fir dominated in terms of number of trees in plot 2, where mature Scots pines dominated in terms of basal area. This situation may lead to misleading assumptions when the continuity of the mixture is considered. For example, Scots pine, which dominates in terms of basal area, can be thought to have continuity in the mixture and that regeneration can occur without considering the risk of the species disappearing at the site. As a result of the reality of the situation, the continuity of the mixture may be disrupted.

The S regression model, which characterizes the relationship between the height and DBH of Caucasian fir, Oriental spruce and Scots pine trees, was significant at p<0.001 in

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all plots across each species. The heights of the trees were predicted by using the coefficients estimated from the regression analyses (*Table 4*) in the S regression model.

Table 4. *Stand top heights and coefficients of the S model*

Sample Plots	Correction Factor (f)	Species	Coefficients		R^2	Standard Error	F-Ratio	Significance Level
			b0	b1				
1	1.0302	Scots Pine	3.062	-9.836	0.57	0.24	11.91	0.013
	1.0119	Oriental spruce	3.485	-21.941	0.888	0.15	96.408	0.000
	1.0240	Caucasian fir	3.145	-11.011	0.715	0.22	41.078	0.000
2	1.0060	Scots Pine	3.432	-12.208	0.918	0.11	113.227	0.000
	1.0112	Oriental spruce	3.293	-6.905	0.458	0.15	9.444	0.013
	1.0158	Caucasian fir	3.618	-16.508	0.886	0.18	101.687	0.000
3	1.0147	Scots Pine	3.395	-12.512	0.889	0.17	105.375	0.000
	1.0065	Oriental spruce	3.396	-10.075	0.452	0.11	9.257	0.014
	1.0383	Caucasian fir	3.303	-9.102	0.454	0.27	14.298	0.002

When the distribution of tree number proportions in stands was evaluated, results showed that the proportion of tree numbers in each stand layer in all plots was greater than 10%. Caucasian fir dominated the overstory in plots 1 (32.46%) and 3 (22.54%) in terms of the number of trees whereas Scots pine and Oriental spruce dominated in plot 2 (15.12% for each species). In addition, Scots pine, Oriental spruce and Caucasian fir were largely located in the overstory in terms of tree numbers and basal area mixing ratios (*Table 5*).

Table 5. *Mixing ratios of species in the plots*

Sample Plot	Stand Layer	Number of Trees (%)				Basal Area (%)			
		Scots pine	Oriental spruce	Caucasian fir	*Total*	Scots pine	Oriental spruce	Caucasian fir	*Total*
1	Overstory	15.71	9.95	32.46	*58.12*	36.84	13.39	41.77	*92.00*
	Middlestory	1.57	5.76	13.09	*20.42*	0.31	2.44	2.88	*5.64*
	Understory	0.00	16.75	4.71	*21.47*	0.00	2.13	0.24	*2.36*
	Total	17.28	32.46	50.26	*100.00*	37.15	17.96	44.89	*100.00*
2	Overstory	15.12	15.12	14.73	*44.96*	41.60	20.25	26.71	*88.56*
	Middlestory	2.33	18.22	15.50	*36.05*	0.90	2.83	5.67	*9.39*
	Understory	0.39	1.16	17.44	*18.99*	0.05	0.05	1.95	*2.06*
	Total	17.83	34.50	47.67	*100.00*	42.55	23.13	34.33	*100.00*
3	Overstory	15.96	21.60	22.54	*60.09*	33.86	27.76	31.91	*93.52*
	Middlestory	0.94	13.15	15.49	*29.58*	0.37	2.27	3.15	*5.79*
	Understory	0.47	6.10	3.76	*10.33*	0.02	0.42	0.25	*0.69*
	Total	17.37	40.85	41.78	*100.00*	34.24	30.45	35.31	*100.00*

Each of the three species consisted of less than 10% in terms of basal area of the middle and understory layers across all plots. The dominant species in the overstory in terms of basal area were Caucasian fir (41.77%) in plot 1 and Scots pine (41.60 and 33.86%) in plots 2 and 3, respectively. Even though the proportion of Scots pine in the upper layer was very high, there was almost no Scots pine in the middle and understory layers. Oriental spruce and Caucasian fir dominated the middle layer and the understory in terms of basal area as well as in terms of the number of trees.

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Spatial patterns of tree species at different developmental stages

When the three tree species in plot 1 were analyzed together, the pooled trees (labeled A) showed clumped distribution at scales 2–8 m (*Table 6, Figs. 2, 3*). Similarly, A(c) (i.e. trees of all species at small wood stage) showed a clumped distribution at scales 5–11 m, while A(a), A(b) and A(d) were randomly distributed at all scales. Oriental spruce was distributed at random over all scales while the distribution of Scots pine and Caucasian fir were clumped at scales 2–3 m and 4–11 m, respectively. When the tree species were analyzed at different developmental stages, Caucasian fir (c) showed clumped distribution at scales 4–12 m, while all three tree species at the other developmental stages showed random distribution (*Table 6, Figs. 3, 4*).

Table 6. *Spatial cluster analyses result for plot 1*

Species	Scale (m)													
	0	1	2	3	4	5	6	7	8	9	10	11	12	13<
A	r	r	r	c	c	c	c	c	c	r	r	r	r	r
a	r	r	r	r	r	r	r	r	r	r	r	r	r	r
b	r	r	r	r	r	r	r	r	r	r	r	r	r	r
c	r	r	r	r	r	c	c	c	c	c	c	c	r	r
d	r	r	r	r	r	r	r	r	r	r	r	r	r	r
Scots pine	r	r	c	c	r	r	r	r	r	r	r	r	r	r
a														
b														
c	r	r	r	r	r	r	r	r	r	r	r	r	r	r
d	r	r	r	r	r	r	r	r	r	r	r	r	r	r
Oriental spruce	r	r	r	r	r	r	r	r	r	r	r	r	r	r
a	r	r	r	r	r	r	r	r	r	r	r	r	r	r
b	r	r	r	r	r	r	r	r	r	r	r	r	r	r
c	r	r	r	r	r	r	r	r	r	r	r	r	r	r
d	r	r	r	r	r	r	r	r	r	r	r	r	r	r
Caucasian fir	r	r	r	r	c	c	c	c	c	c	c	c	r	r
a														
b	r	r	r	r	r	r	r	r	r	r	r	r	r	r
c	r	r	r	r	c	c	c	c	c	c	c	c	c	r
d	r	r	r	r	r	r	r	r	r	r	r	r	r	r

"A" symbolizes all three species, "r" random distributions, "c" clumped distributions. Blank rows indicate that spatial analysis are not performed due to insufficient number of stems. a, b, c, and d means seedling - thicket stage, sapling-pole stage, small wood (tree) stage and medium- large wood stage

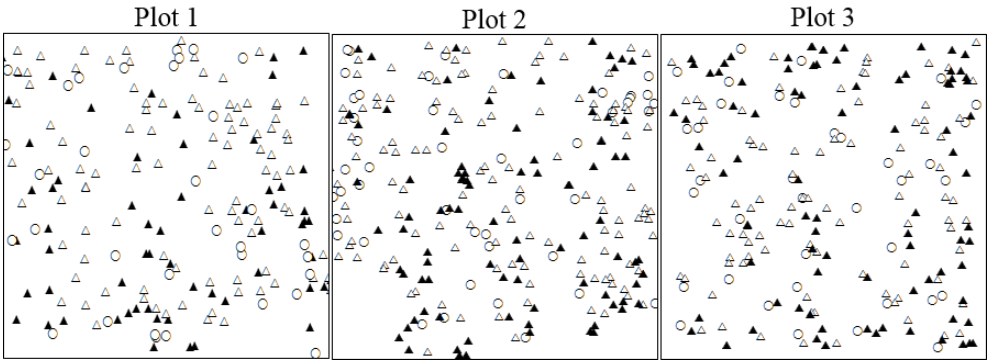

Figure 3. *Spatial distributions of tree species at each study plot (circle: Scots pine, closed triangle: Oriental spruce, triangle: Caucasian fir)*

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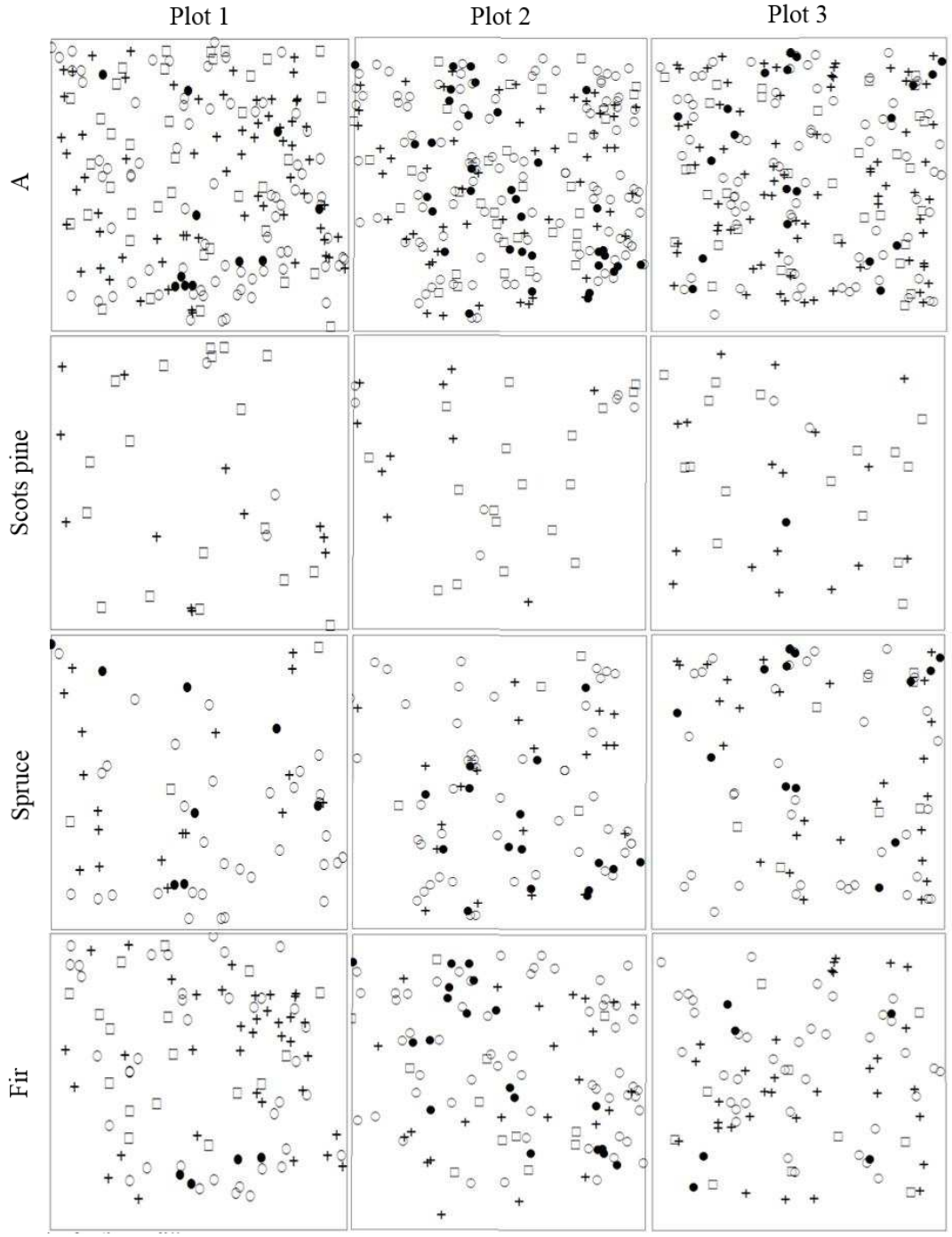

Figure 4. *Distribution maps of tree species at four development stages (closed circle: seedling-thicket stage, circle: sapling-pole stage, plus: small wood stage, square: medium and large wood stage, A symbolizes all scots pine, Oriental spruce and Caucasian fir trees)*

The three tree species together (A) in plot 2 displayed clumped distribution at ≤ 7 m scales. Similarly, Scots pine and Oriental spruce showed clumped distributions at scales 2–6 m and 2–7 m, respectively, while Caucasian fir showed random distribution at all scales. When the tree species in plot 2 at different developmental stages were analyzed, A (a) and Caucasian fir (a) showed clumped distribution at all scales, while Scots pine

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(b), Oriental spruce (b) and Caucasian fir (b) showed clumped distribution at the lower scales. Trees at the other developmental stages showed random distributions at all scales (*Table 7, Figs. 3, 4*).

Table 7. *Spatial cluster analyses result for plot 2*

Species	Scale (m)													
	0	1	2	3	4	5	6	7	8	9	10	11	12	13<
A	c	c	c	c	c	c	c	c	r	r	r	r	r	r
a	c	c	c	c	c	c	c	c	c	c	c	c	c	c
b	c	c	c	c	c	c	c	c	c	c	c	c	r	r
c	r	r	r	r	r	r	r	r	r	r	r	r	r	r
d	r	r	r	r	r	r	r	r	r	r	c	c	r	r
Scots pine	r	r	r	c	c	c	c	r	r	r	r	r	r	r
a														
b	r	r	r	c	c	c	c	r	r	r	r	r	r	r
c	r	r	r	r	r	r	r	r	r	r	r	r	r	r
d	r	r	r	r	r	r	r	r	r	r	r	r	r	r
Oriental spruce	c	c	c	c	c	c	c	c	r	r	r	r	r	r
a	r	r	r	r	r	r	r	r	r	r	r	r	r	r
b	c	c	c	c	c	r	r	r	r	r	r	r	r	r
c	r	r	r	r	r	r	r	r	r	r	r	r	r	r
d														
Caucasian fir	r	r	r	r	r	r	r	r	r	r	r	r	r	r
a	c	c	c	c	c	c	c	c	c	c	c	c	c	c
b	r	r	r	c	c	c	c	c	c	r	r	r	r	r
c	r	r	r	r	r	r	r	r	r	r	r	r	r	r
d	r	r	r	r	r	r	r	r	r	r	r	r	r	r

"A" symbolizes all three species, "r" random distributions, "c" clumped distributions. Blank rows indicate that spatial analysis are not performed due to insufficient number of stems. a, b, c, and d means seedling - thicket stage, sapling-pole stage, small wood (tree) stage and medium- large wood stage

The three tree species (A) in plot 3 displayed a clumped distribution at the 2–5 m scales as in the other sample plots, when the tree species were analyzed together. Scots pine and Caucasian fir showed random distribution at all scales, whereas Oriental spruce showed a clumped distribution at the 2–7 m scales. A (b) showed a clumped distribution at the 2-4 m scales, whereas trees (A) at other developmental stages showed random distributions at all scales. Clumped distributions were observed in Oriental spruce (a) and Caucasian fir (c) at scales of 2–5 m and 9–12 m, respectively, while random distributions were observed at other developmental stages for each of the three tree species (*Table 8, Figs. 3, 4*).

The spatial distribution of trees within mixed stands may differ according to the silvicultural characteristics of the species present in the mixture and their mixing rates. Therefore, the species found at the site, their developmental stages and their frequencies are the determining factors in assessing spatial distribution (Gokturk, 2013). The spatial patterns displayed by the species were similar even though the number and basal area values of the trees differed.

Kazempour Larsary et al. (2018) stated that identifying spatial patterns of trees according to development stages is a first step in understanding regeneration process. Seedlings mostly emerge in clusters (Mason et al., 2007; Pardos et al., 2007). Stem exclusion takes place in these clusters over time as a result of competition for nutrients

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and light. The subsequent decrease in the number of stems results in these clusters being replaced by a random distribution (Kazempour Larsary et al., 2018; Muhamed, 2019). Therefore, tree seedlings display a clumped distribution initially, which develops over time into a random distribution (Gu et al., 2019). In the present study, it has been determined that each tree species at the growth stage d showed a random distribution at all scales.

Table 8. *Spatial cluster analyses result for plot 3*

Species	Scale (m)													
	0	1	2	3	4	5	6	7	8	9	10	11	12	13<
A	r	r	c	c	c	c	r	r	r	r	r	r	r	r
a	r	r	r	r	r	r	r	r	r	r	r	r	r	r
b	r	r	c	c	c	r	r	r	r	r	r	r	r	r
c	r	r	r	r	r	r	r	r	r	r	r	r	r	r
d	r	r	r	r	r	r	r	r	r	r	r	r	r	r
Scots pine	r	r	r	r	r	r	r	r	r	r	r	r	r	r
a														
b														
c	r	r	r	r	r	r	r	r	r	r	r	r	r	r
d	r	r	r	r	r	r	r	r	r	r	r	r	r	r
Oriental spruce	r	r	c	c	c	c	c	c	r	r	r	r	r	r
a	r	r	r	c	c	c	r	r	r	r	r	r	r	r
b	r	r	r	r	r	r	r	r	r	r	r	r	r	r
c	r	r	r	r	r	r	r	r	r	r	r	r	r	r
d	r	r	r	r	r	r	r	r	r	r	r	r	r	r
Caucasian fir	r	r	r	r	r	r	r	r	r	r	r	r	r	r
a	r	r	r	r	r	r	r	r	r	r	r	r	r	r
b	r	r	r	r	r	r	r	r	r	r	r	r	r	r
c	r	r	c	c	r	r	r	r	r	c	c	c	c	r
d	r	r	r	r	r	r	r	r	r	r	r	r	r	r

"A" symbolizes all three species, "r" random distributions, "c" clumped distributions. Blank rows indicate that spatial analysis are not performed due to insufficient number of stems. a, b, c, and d means seedling - thicket stage, sapling-pole stage, small wood (tree) stage and medium- large wood stage.

The random distribution observed in the plots of Scots pine, which is a typical shade-intolerant tree, can be attributed to the fact that most of the Scots pine trees at the sites are at the medium-wood stage, d. The findings of Pardos et al. (2007) and Gu et al. (2019) support the observed distribution of Scots pine at our site. Pardos et al. (2007) reported that Scots pine seedlings emerged at a site in clusters. In the current study, unfortunately, there were no pine seedlings in any of the sample plots. For this reason, spatial analysis could not be performed for developmental stage a in Scots pine.

Several studies have showed that Caucasian firs can occupy a site because their seedlings can grow under the canopy (Grassi et al., 2004; Nagel et al., 2006; Hofmeister et al., 2008). Based on this, the random distribution observed in Caucasian fir at our sites can be attributed to its ability to colonize a site.

Aldrich et al. (2003) reported that the tendency of tree species to clumped distribution increases as their shade tolerance increases. The clustering tendency observed in Oriental spruce in our study can be attributed to its shade tolerance. Even though the shade-tolerant

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trees of Oriental spruce and Caucasian fir are treated as similar species in silvicultural applications in Turkey, Caucasian firs may display a distribution pattern different to that of Oriental spruces, because their seedlings exhibit a denser presence under the canopy compared to being restricted to gaps within the stand (Grassi et al., 2004). Despite the fact that Gokturk (2013) assigned the random distribution observed in Caucasian fir to its low stem number at a site, the dominance of Caucasian firs in terms of number of trees in all plots in the present study site supports the observation that Caucasian firs tend to display a random distribution.

Spatial patterns of tree species at different stand layers

Spatial analysis results showed that stem distribution patterns of trees at different stand layers differed among the species. When the tree species at different stand layers were analyzed together, A (O) showed random distribution at all scales, whereas a clumped distribution was observed at A (M) and A (U) in plot 1 (*Table 9*).

Table 9. *Spatial cluster analyses of tree species at stand layers for plot 1*

Stand Layer		Scale (m)													
		0	1	2	3	4	5	6	7	8	9	10	11	12	13<
A	O	r	r	r	r	r	r	r	r	r	r	r	r	r	r
	M	r	r	r	r	r	c	c	c	c	c	r	r	r	r
	U	r	r	c	c	c	c	c	r	r	r	r	r	r	r
Scots pine	O	r	r	r	r	r	r	r	r	r	r	r	r	r	r
	M														
	U														
Oriental spruce	O	r	r	r	r	r	r	r	r	r	r	r	r	r	r
	M	r	r	r	r	r	r	r	r	r	r	r	r	r	r
	U	r	r	r	r	r	r	r	r	r	r	r	r	r	r
Caucasian fir	O	r	r	r	r	r	r	r	c	c	c	c	c	c	r (c)
	M	r	r	r	r	r	r	r	r	r	r	r	r	r	r
	U														

"A" symbolizes all three species, "r" random distributions, "c" clumped distributions, while "r(c)" means more random points than clumped. Blank rows indicate that spatial analysis are not performed due to insufficient number of stems. O, M and U means overstory, middlestory and understory

Similar findings were reported by Sotiris and Alan (2005), who showed that the larger trees in a mixed stand showed a random distribution whereas the smaller trees showed a clumped distribution. Similarly, Hao et al. (2007) observed that the trees in the overstory and middle story layers showed a random distribution, while the trees in the understory layer exhibited a clumped distribution. Unlike the situation in plot 1, A (O) showed a clumped distribution at scales 7–13 m and 2–4 m in plots 2 and 3, respectively (*Tables 10, 11*).

In each of the plots, because of the absence of sufficient numbers of Scots pine in the middle story and understory layers (*Figure 5*), spatial analysis could not be carried out. Scots pine were substantially present in the overstory and showed random distribution at all scales (*Tables 9, 10 and 11*). Oriental spruce was randomly distributed in all stand layers and at all scales in plots 1 and 2, whereas a significantly clumped distribution was observed only for Oriental spruce (U) at the 3–7 m scales in plot 3. In the overstory layer,

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a clumped distribution was observed only in Caucasian fir. Caucasian fir (O) showed a clumped distribution at 7–12 m scales in plot 1 and at 2–11 m scales in plot 3, whereas it showed random distribution at all scales in plot 2.

Table 10. *Spatial cluster analyses of tree species at stand layers for plot 2*

Stand Layer		Scale (m)													
		0	1	2	3	4	5	6	7	8	9	10	11	12	13<
A	O	r	r	r	r	r	r	r	c	c	c	c	c	c	c(r)
	M	r	c	c	r	r	c	c	c	c	c	c	c	r	r
	U	r	c	c	c	c	c	c	c	c	c	c	c	c	r(c)
Scots pine	O	r	r	r	r	r	r	r	r	r	r	r	r	r	r
	M														
	U														
Oriental spruce	O	r	r	r	r	r	r	r	r	r	r	r	r	r	r
	M	r	r	r	r	r	r	r	r	r	r	r	r	r	r
	U														
Caucasian fir	O	r	r	r	r	r	r	r	r	r	r	r	r	r	r
	M	r	r	r	r	r	r	r	r	r	r	r	r	r	r
	U	r	r	r	r	c	c	c	c	c	c	c	c	c	r(c)

"A" symbolizes all three species, "r" random distributions, "c" clumped distributions, while "r(c)" means more random points than clumped and "c(r)" means more clumped points than random points. Blank rows indicate that spatial analysis are not performed due to insufficient number of stems. O, M and U means overstory, middlestory and understory

Table 11. *Spatial cluster analyses of tree species at stand layers for plot 3*

Stand Layer		Scale (m)													
		0	1	2	3	4	5	6	7	8	9	10	11	12	13<
A	O	r	r	c	c	c	r	r	r	r	r	r	r	r	r
	M	r	r	r	r	r	r	r	r	r	r	r	r	r	r
	U	r	r	r	r	r	r	r	r	r	r	r	r	r	r
Scots pine	O	r	r	r	r	r	r	r	r	r	r	r	r	r	r
	M														
	U														
Oriental spruce	O	r	r	r	r	r	r	r	r	r	r	r	r	r	r
	M	r	r	r	r	r	r	r	r	r	r	r	r	r	r
	U	r	r	r	c	c	c	c	r	r	r	r	r	r	r
Caucasian fir	O	r	r	c	c	c	c	c	c	c	c	c	c	r	r
	M	r	r	r	r	r	r	r	r	r	r	c	c	c	c (r)
	U														

"A" symbolizes all three species, "r" random distributions, "c" clumped distributions, while "c(r)" means more clumped points than random points. Blank rows indicate that spatial analysis are not performed due to insufficient number of stems. O, M and U means overstory, middlestory and understory

The results of spatial analysis of the tree species at different stand layers showed that clumped distribution was observed in the middle and understory layers, whereas trees at the overstory layer were randomly distributed. Gokturk (2013) also reported that a clumped distribution of the trees was observed in the middlestory and understory,

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whereas, toward the overstory, the random nature of the tree distribution increased. The findings of Hanewinkel (2004) and Hao et al. (2007) support these findings. Hanewinkel (2004) found that the trees in the overstory were distributed randomly whereas the trees in the middlestory or understory layers were distributed in a clumped manner. In the present study, a clumped distribution was more commonly seen when all tree species were analyzed together rather than when the species were analyzed individually.

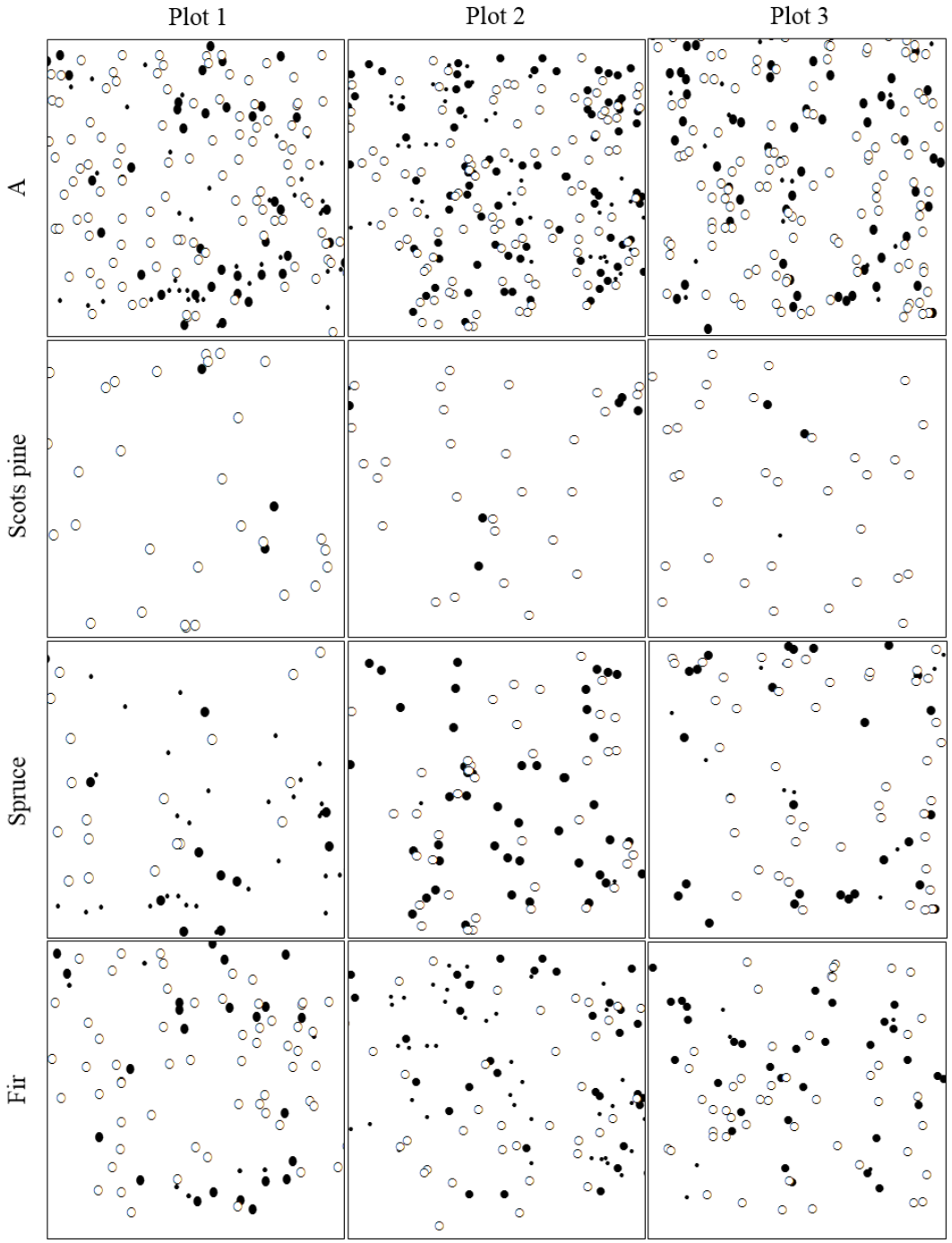

Figure 5. *Distribution maps of tree species at stand layers (circle: overstory, closed circle: middlestory, small closed circles: Understory, A symbolizes all scots pine, Oriental spruce and Caucasian fir trees)*

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Martens et al. (2000) stated that, with the increase in the height of the trees, the amount of light reaching the ground under the stand decreased. Based on this statement, it can be said that light plays an important role in the reduction of clumped distributions in the sample areas. Although clumped distributions would be expected to decrease in the lower layers due to a lack of light, clustering continues because the species colonizing these layers are shade-tolerant Oriental spruce and Caucasian fir. The number of individuals at the seedling-thicket and sapling-pole stages in the lower layers is an important factor determining the clumped distribution.

Conclusions

In the present study, the spatial distribution patterns of trees, based on development stages and on stand layers, were determined in Scots pine, Oriental spruce and Caucasian fir in a mixed stand. Because the quantity of trees affects the spatial distributions, the number of trees and the stand structures were identified prior to the spatial analyses. Caucasian fir dominated in terms of tree numbers in all plots. Findings showed that Oriental spruce and Caucasian fir were present at every developmental stage whereas Scots pines were mainly concentrated at the c and d development stages and in the upper layer. There was almost no Scots pine in the middle and understory layers. In the middle story and the understory, Oriental spruce and Caucasian fir dominated in terms of both basal area and number of trees. These results shows that the scots pine gradually disappears from mixtures. In order to maintain the continuity of the mixtures, scots pines must be preserved in silvicultural cuttings in these mix stands.

The stands in all plots had layered canopies, resulting in both horizontal and vertical mixing. Spatial distribution analyses revealed that Caucasian firs displayed only random distribution, and Scots pines exhibited mostly random distribution whereas Oriental spruces tended toward a clumped distribution. Random distributions were observed at all scales in the medium wood development stages of all three species, whereas clumped distributions were generally seen at other development stages. Trees in the middlestory and understory layers showed clumped distribution while trees in the overstory layer were randomly distributed. It could be determined, on the basis of these distributions, that Scots pine and Caucasian fir had a tree-wise mixture whereas Oriental spruce displayed a group-mixing pattern. Studies about species interactions with the increasing sampling size and number in spruce-fir-scots pine mixed stands should be done for to be able to discuss the underlying factors behind the reported results.

The spatial distributions of trees in the mixed stands of Scots pine, Oriental spruce and Caucasian fir can be retained or they can be altered, based on the needs of the individual species, by silvicultural interventions. Changed spatial distributions also imply changes in mixing patterns. Therefore, it is important to identify the target mixing pattern and then to adjust intervention intensity accordingly, by means of silvicultural practices. Before the silvicultural cuttings, it is necessary to conduct studies for determination of spatial and mixing patterns based on spatial analysis and the results of these studies should be taken into consideration for to ensure sustainability of these stands.

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COMPARISON OF PB^{2+} ADSORPTION AND DESORPTION BY SEVERAL CHEMICALLY MODIFIED BIOCHARS DERIVED FROM STEAM EXPLODED OIL-RAPE STRAW

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Abstract. To deal with lead (Pb^{2+}) contamination, four engineered biochar materials: NaOH modification of biochar (BC_{Na}), $KMnO_4$ impregnation of biochar (BC_{Mn}), hydroxyapatite modified biochar (BC_{HA}), and chitosan modification of biochar (BC_C), were compared for their adsorption and desorption capabilities. Steam exploded oil-rape straw was selected as the biomass material. Scanning Electron Microscopy-Energy Dispersive Spectroscopy (SEM-EDS) examination of biochars revealed that the modified adsorbents surface was covered by the respective modifying mineral. Adsorption and desorption studies were executed to examine the properties of adsorbents and the focal adsorption/desorption mechanism. The findings revealed that Mn oxides and hydroxyapetite nano-particles were conceded well within the biochar structure and formed inner sphere complexes perhaps with oxygen bearing functional groups. The extra adsorption sites formed by the impregnated respective minerals play the crucial roles in Pb^{2+} adsorption in an aqueous solution. Pb^{2+} adsorption isotherm experiments by BC_{Mn} was well determined by the Langmuir model with the highest adsorption capacity of BC_{Mn} being 70.92 mg g^{-1} followed by the BC_{HA} (49.26 mg g^{-1}). While, the adsorption kinetics of Pb^{2+} by all adsorbents were well determined by pseudo-second-order kinetics with the highest adsorption capacity of BC_{Mn} being 22.61 g (mg h)$^{-1}$. $KMnO_4$ and hydroxyapatite modified biochars exhibited very promising physicochemical and adsorptive properties for adsorbing divalent metal Pb^{2+} and might thus have high potential as a soil amendment and an alternative adsorbent for environmental remediation.
Keywords: *biochar, modification, heavy metals, kinetics, isotherms*

Introduction

Lead (Pb^{2+}) is considered as one of the most deleterious environmental pollutants because of its persistence and non-degradability nature in the terrestrial ecosystem (Wang et al., 2015a). Pb^{2+} contamination of soil and water is reputed all over the world as one of the key substantial problem in our ecosystem (Arshadi, 2015). Heavy metals including Pb^{2+} in aquatic system has also been revealed in Russia (Snakin and Prisyazhnaya, 2000), the USA (Triantafyllidou et al., 2014), India (CPCB, 2008), France (Ayrault et al., 2012), China (Li et al., 2012), Argentina (Kohn et al., 2001), Korea (Lee et al., 2005) and numerous other countries. A huge amount of Pb-contained waste-water is inescapably discharged from mining-related industries annually in China, as it is one of the key producer and consumer of Pb^{2+} in the world (Zhang et al., 2012). Pb^{2+} also can be espoused and concerted in organisms (Mohan et al., 2014a) and proposes deleterious effects on living organisms (Lu et al., 2012). Furthermore, Pb toxicity has serious implication on human health including damage of

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the reproductive and central nervous system, hypertensive toxicity which ultimately leads towards fatality (Zhang et al., 2019).

Numerous conservative and modern practices have been passed on for Pb^{2+} removal, for instance; ion exchange, electrocoagulation, membrane filtration, precipitation and adsorption (Malamis et al., 2010). Adsorption has been identified to be an influential, meek and efficient methodology for Pb^{2+} removal (Inyang et al., 2012). There is still a crucial requirement for designing an environment friendly and low-cost adsorbent for reducing Pb^{2+} pollution in terrestrial environment. Biochar as one of the biosorbents has recently gained increasing attention in removal and bioavailability decrement of heavy metals in soil and water medium as well (Wang et al., 2015b; Rechberger et al., 2017). Bundles of novel measures have been established for improving biochar adsorption capacity for heavy metals through improving surface characteristics (Mejias Carpio et al., 2014), effective functional groups (Becidan et al., 2017), hydrophobic/hydrophilic characters (Chen et al., 2014) and surface charges etc. (Samsuri et al., 2013). In addition, the use of diverse engineering approaches in biochar production, such as treatment of biomass or modification of biochar surface has ensued in numerous great proficiencies and efficient novel modified biochars with adsorption capacities equivalent to or even better than that of a few commercially available activated carbons.

The pristine biochar can be significantly modified with impregnation approaches; metal salts/oxides of minerals can be mixed with biochars (BCs) to expedite physical/chemical bonding of metal ions in the porous structure of biochars. To further improve the metal adsorption/sorption proficiency of biochars, these biosorbents have been pretreated or modified prior to pyrolysis process. The distinct improvement of divergent biochar materials has been revealed in previous studies (Rajapaksha et al., 2016; Sizmur et al., 2017), where improvement of metal adsorption has been analytically proved. To further improve the adsorption of biomass-derived biochars, biomass pretreatment is led to increase porosity because biomass fractionation process is beneficial for the succeeding activation process (Harun and Danquah, 2011; Rizwan et al., 2020).

In the current years, several technical innovations and applications of steam explosion were reported (Jia et al., 2013; Chen and Peng, 2014; Liu et al., 2014; Huang et al., 2015; Chen et al., 2019a). Steam explosion consequences in the hemicelluloses being hydrolyzed, the cellulose and lignin is marginally depolymerised, which aid in binding particles collectively during the densification process. Our previous group study (Chen et al., 2019a) revealed that steam explosion could remarkably change the physicochemical properties of typical agricultural feedstocks such as oil-rape, wheat, rice, maize and cotton straws and their derived biochars. In the current study, the most effective feedstock of oil-rape straw was selected for further mineral impregnation and biochar modification as well. It was hypothesized that chemical modification following steam explosion would fabricate the novel biochar with satisfactory adsorption and desorption properties. Apparently, this is the first study on modification of biochar/biomass following steam explosion of oil-rape straw. The particular objectives of the present study were thus (1) to quantify the adsorption and desorption characteristics of the engineered biochars for Pb^{2+}, (2) and then to recommend a measure for preparing the novel engineered biochar.

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Materials and methods

Oil-rape straw pretreatment and biochar production

Oil-rape straw residues were collected from Shang Zhuang experimental station, China Agricultural University, Beijing (40.14°N, 116.18°E). The details of the steam explosion (SE) pretreatment and biochar synthesis were described previously in Chen et al. (2019a). Briefly, oil-rape straw was steam-exploded for 2 min at 210°C and 2.5 MPa using a QB-200 platform, in Hebei Heavy-Duty Mechanical Factory. The steam exploded straw was distributed into two bulks. One bulk of steam exploded oil-rape straw was kept in a stainless steel reactor and heated in a muffle furnace at 500℃ for 2 h under N_2 flow (10 psi). The solid residues in the reactor were obtained and denoted as BC.

Synthesis of engineered biochars

The above described prepared biochar (BC) was further modified with chitosan and NaOH. The detailed protocol for the preparation of engineered biochars was described in our previous study (Chen et al., 2019b). Briefly, three g of chitosan was initially dissolved in 180 mL acetic acid (2%) solution, and further mixed with 3 g BC while stirring for 30 min at a rotatary shaker. The obtained suspension was then drop-wise added into a 900 mL NaOH (1.2%) solution and further retained for 12 hours. The obtained biochar material (named as BC_C) was further washed with deionized water to eliminate the surplus sodium hydroxide till the pH value became neutral and afterwards the obtained product was oven dried at 70℃ for 24 h (Zhou et al., 2013).

The NaOH-modified biochar (BC_{Na}) was synthesized according to Li et al. (2017). Briefly, 10 of the BC were mixed with 100 mL of 2 M NaOH solution with vigorous stirring for 12 h at 100℃. The biochar was oven-dried as described above followed by washing 3 times with 0.01 M $NaHCO_3$ solution to remove impurities and further 3 times washing with deionized water until the pH value reached pH 7.

Second bulk of steam exploded oil-rape straw was used for further modification. A nano-hydroxyapatite suspension solution was prepared by adding two grams of nano-hydroxyapatite mineral powder to 500 mL deionized water followed by the ultra-sonication of the mixture for 30 minutes. Ten grams of steam exploded oil-rape straw were agitated with the nano-hydroxyapatite suspension for 1 h, then oven dried at 60℃ for 24 hours. The hydroxyapatite-pretreated straw was kept in a quartz tube and then slowly pyrolized as described above (Yao et al., 2014; Yang et al., 2016). The solid residue was denoted as BC_{HA}.

One hundred grams of the SE oil-rape straw were vigorously agitated with 1000 ml 2% $KMnO_4$ solution at 80℃ for 3 h and then ultra-sonicated for 20 min, then oven-dried at 105℃ for overnight. The straw was slowly pyrolized as described above. The collected biochar (denoted as BC_{Mn}) was rinsed 3 times with 0.01 M $NaHCO_3$ solution and further 3 times with deionized water (Li et al., 2017). The details and study circumstances of studied biochars are given in *Table 1*.

Adsorption kinetics and isotherm studies

Adsorption isotherms were determined for Pb^{2+} by using the identical protocol as described in a previous study (Wang et al., 2015a), with minor modifications. A range of Pb^{2+} (25 mL, 1–250 mg L^{-1}) sorbate concentrations of solution and 24 hours contact time period was set in the adsorption isotherms study. Nitrate salts were used for

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preparing all solutions. Concisely, 0.1 g of biochar in a 25 mL Pb^{2+}, three drops of phenol were added in each sample to prevent microbial growth. Hence, the sorbent concentrations were 2.5 g L^{-1} for all treatments. In addition, to prevent metal precipitation, the initial pH values of the Pb^{2+} solutions were adjusted to 5 in all cases, by using 0.01 M HCl and 0.01 M NaOH solutions. In a former study (Li et al., 2017) it was revealed that the highest adsorption capacities were obtained at pH 5.0, hence this pH was used in the current study. The isotherms and kinetics tests were executed in triplicates and mean values were used for further data analysis.

Table 1. *Tested biochars and study circumstances are given as:*

Tested Biochar	Abbreviation	Study circumstances
Pristine Oil-rape straw biochar	BC	Oil-rape straw was pyrolyzed at 500℃ for 2 h under N_2 flow (10 psi).
Steam Exploded-Pretreated	BC_{SE}	Oil-rape straw was steam-exploded for 2 min at 210°C and 2.5 MPa.
$KMnO_4$-Impregnated	BC_{Mn}	Hundred grams of steam exploded rape straw was mixed with 1000 ml 2% $KMnO_4$ solution prior to biochar production.
NaOH-Modified	BC_{Na}	Ten grams of biochar was mixed with 100 ml of 2M NaOH solution, stirred vigorously at 100℃ for 12 h.
Hydroxyapatite-Modified	BC_{HA}	Two grams of hydroxyapatite powder was dissolved in 500 ml deionized (DI) in ultrasonic condition. Ten grams of steam exploded oil-rape straw were agitated with the nano-hydroxyapatite suspension for 1 h (prior to biochar production).
Chitosan-Modified	BC_C	Three grams of chitosan was first dissolved in 180 ml of acetic acid (2%), and 3 g of the as-is biochar was added to this solution.

Investigation of Pb^{2+} (50 mg L^{-1}) adsorption kinetics by biochars was carried out following the methods described above. Batch adsorption tests were conducted in triplicates using 50 ml centrifuge tubes on a rotatory shaker at 180 rpm. At each sampling time (0-24 h), the suspensions were collected and promptly filtered by using 0.22 ml pore size nylon membrane filters (GE cellulose nylon membrane). The Pb^{2+} ions concentrations in the resulting supernatant were determined by using inductively coupled ICP-OES (Optima 2300, Perkin-Elmer SCIEX, USA).

Desorption study

Desorption experiments were executed to examine, if biochar adsorption of Pb^{2+} was reversible and the Pb^{2+} immobilization aptitude of biochar was assessed. For desorption experiment, all Pb^{2+} loaded biochar samples were shaken for 24 h with 0.01 M HNO_3 (background electrolyte at pH = 5.00); (Trakal et al., 2014b), 0.01 M $CaCl_2$ (solution simulating ‘‘bioavailable form’’ of metal; Houba et al., 1996); and finally 0.01 $NaNO_3$ (solution exhibiting ‘‘geochemically active form’’ of metals; Tipping et al., 2003) to assess potential metal desorption. The aqueous solution phase was then separated from the sorbent using a centrifuge and promptly filtered by using 0.22 ml pore size nylon membrane filters. The residual concentrations of Pb^{2+} ions in the resulting supernatant were then determined using ICP-OES.

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Calculations

The adsorption amount (q_t) was determined according to the equation given below:

$$q_t = \frac{(C_0 - C_t)\,V}{m} \quad \text{(Eq.1)}$$

Here, q_t denotes the maximum adsorption quantity of metal ions at a specific time t (mg/g), m represents the mass of biochar used (g), V denotes the volume of solution used (dm^3), and C_0 and C_e represents the initial and equilibrium concentration of the Pb^{2+} ions (mol/dm^3), respectively. The Langmuir model is expressed as (Langmuir, 1916; Aksu and Isoglu, 2005):

$$q_e = \frac{q_0 K_L C_e}{1 + K_L C_e} \quad \text{(Eq.2)}$$

Here, q_e denotes the maximum adsorption capacity at equilibrium stage (mg/g), and C_e represents the concentration of Pb^{2+} ions at equilibrium (mg/dm^3). While, q_0 (mg/g) and K_L are constant of the Langmuir equation (dm^3/mg) and both these constants can be calculated from its linear form. The Freundlich model can be expressed as below (Freundlich, 1906; Zhang et al., 2011):

$$q_e = K_F C_e^{1/n} \quad \text{(Eq.3)}$$

Pseudo second order model can be given as the following equation (Blanchard et al., 1984; Ho and McKay, 1998).

$$\frac{t}{q_t} = \frac{1}{K_2 q_e^2} - \frac{t}{q_e} \quad \text{(Eq.4)}$$

Pseudo first order kinetic model equation can be expressed as:

$$log(q_e - q_t) = log(q_e) - \frac{k_1 t}{2.303} \quad \text{(Eq.5)}$$

Here, q_e and q_t represent quantities of Pb^{2+} ions adsorbed at equilibrium stage and at specific time t, respectively. k_1 and k_2 are the pseudo-first and second-order rate constants for the adsorption process.

Data analysis

All the tests were carried out in triplicates and the mean data was reported to plot adsorption kinetics and isotherms. The obtained data was examined statistically by using Statistics 8.1 software. The variability in the data was determined as the standard deviation and threshold of significance was $p < 0.05$.

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Results

Morphological characteristics of the studied biochar

To investigate the surface morphology characteristics of the studied biochars, SEM-EDS images of the pristine and numerous engineered biochars used in this study were obtained (*Figs. 1, 2 and 3*). The results displayed that porous structure exists on the biochar that sustains the disordered pattern of the pristine oil-rape straw cell morphology. As it is evident from the SEM images there is deposition of Mn, Na and Ca/ P on BC_{Mn}, BC_{Na} and BC_{HA} respectively. On the other hand, there is no obvious deposition on pristine and SE biochars (*Fig. 1a,b*). Furthermore, pristine biochar structure was disturbed, fragmented, and even shattered after SE pretreatment, which had a substantial impact on the structure of the corresponding biochars.

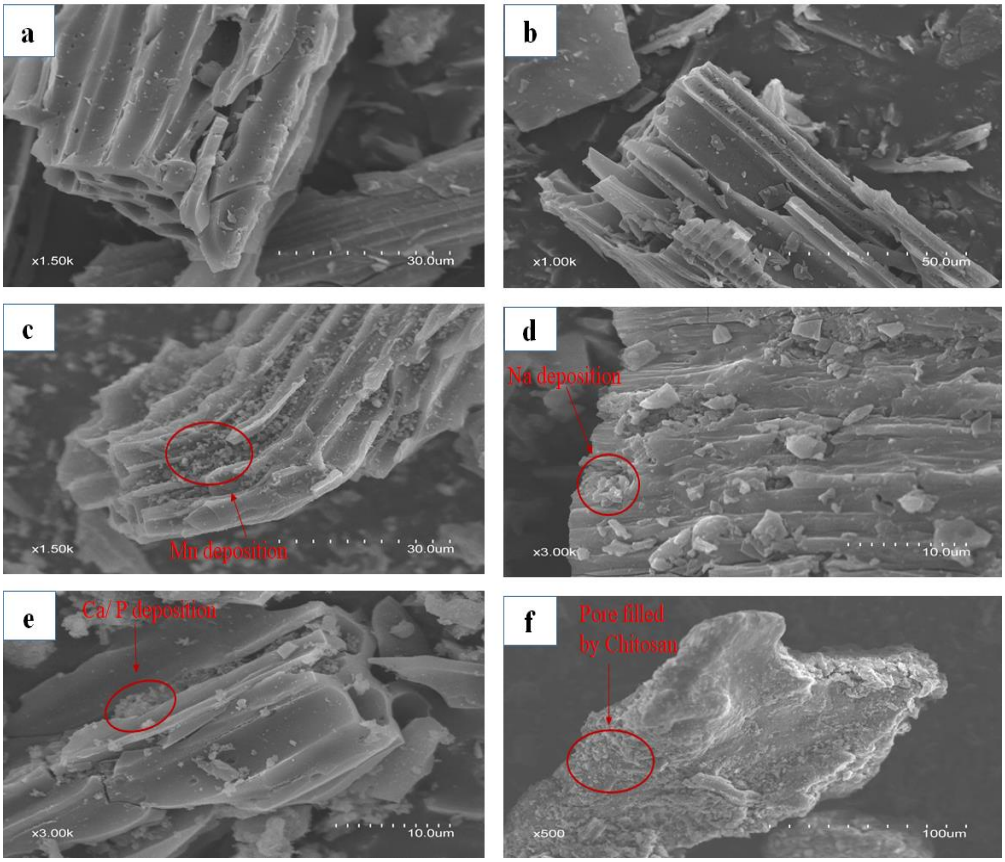

Figure 1. *SEM analysis of the biochars derived from pristine (BC) (a), SE pretreated (BC_{SE}) (b), $KMnO_4$-impregnated (BC_{Mn}) (c), NaOH- (BC_{Na}) (d), hydroxyapatite (BC_{HA}) (e), and chitosan (BC_C) (f)*

Additionally, the findings of surface elemental examination by using the EDS spectra obviously confirmed the higher concentration of respective various mineral contents in terms of weight and atomic percentages (*Figs. 2 and 3*). After modification, the higher concentration of these minerals depicts that these minerals were successfully impregnated on the respective biochar surface. Moreover, EDS elemental composition analysis of biochars further affirmed that biochars not just have greater amounts of oxygen and carbon, yet they additionally contain substantial amounts of slag

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components such as P, Cl, P and K amongst others (*Figs. 2 and 3*). The accumulation of Mn oxides on the BC_{Mn} surface, which was further confirmed by SEM-EDS analysis and these could provide more adsorption sites for Pb^{2+}. SEM-EDS analysis of studied BCs also confirmed the significant concentration of potassium and chlorine in the respective biochar. The SEM images of BC_{HA} is identical to that of BC, with the exemption that the amounts of P and Ca elements enhanced on the surface of biochar.

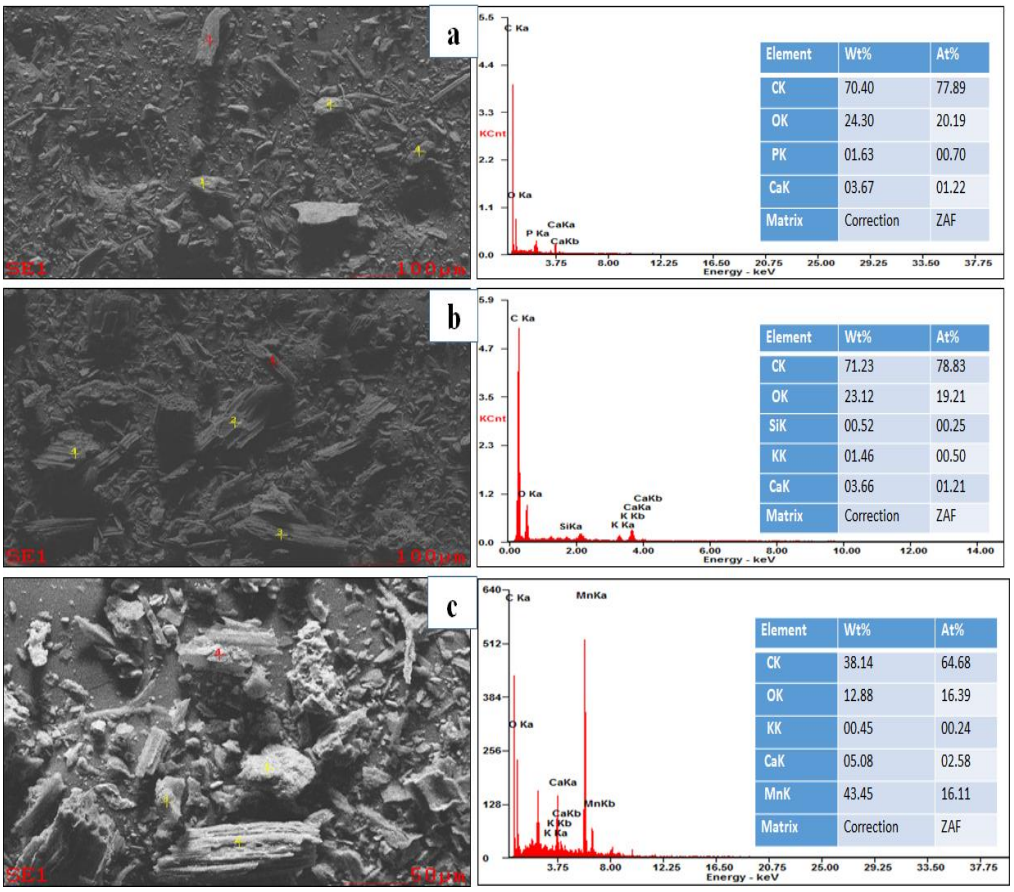

Figure 2. *EDS analysis of the biochars derived from pristine (BC) (a), SE pretreated (BC_{SE}) (b), and $KMnO_4$-impregnated (BC_{Mn}) (c), and their corresponding EDS spectra with atomic elemental ratio*

Adsorption isotherms

In order to check the distribution of the adsorbate molecules between the liquid and the solid phases in equilibrium state, the adsorption equilibrium isotherm is necessary (Almeida et al., 2009). We performed Pb^{2+} batch adsorption experiment and the obtained experimental data was fitted in Langmuir and Freundlich models. In the present study linear forms of isotherm models were being employed instead of non linear forms as 95 % of liquid-phase adsorption studies used non linear forms (*Fig. 4, Table 2*). The data obtained from isotherm models was well fitted by using Langmuir models and 0.99 R^2 value was obtained for approximately all samples (*Table 2*). The Pb^{2+} adsorption hiked dramatically when the equilibrium solution concentration of Pb^{2+} was less than 30 mg L^{-1}.

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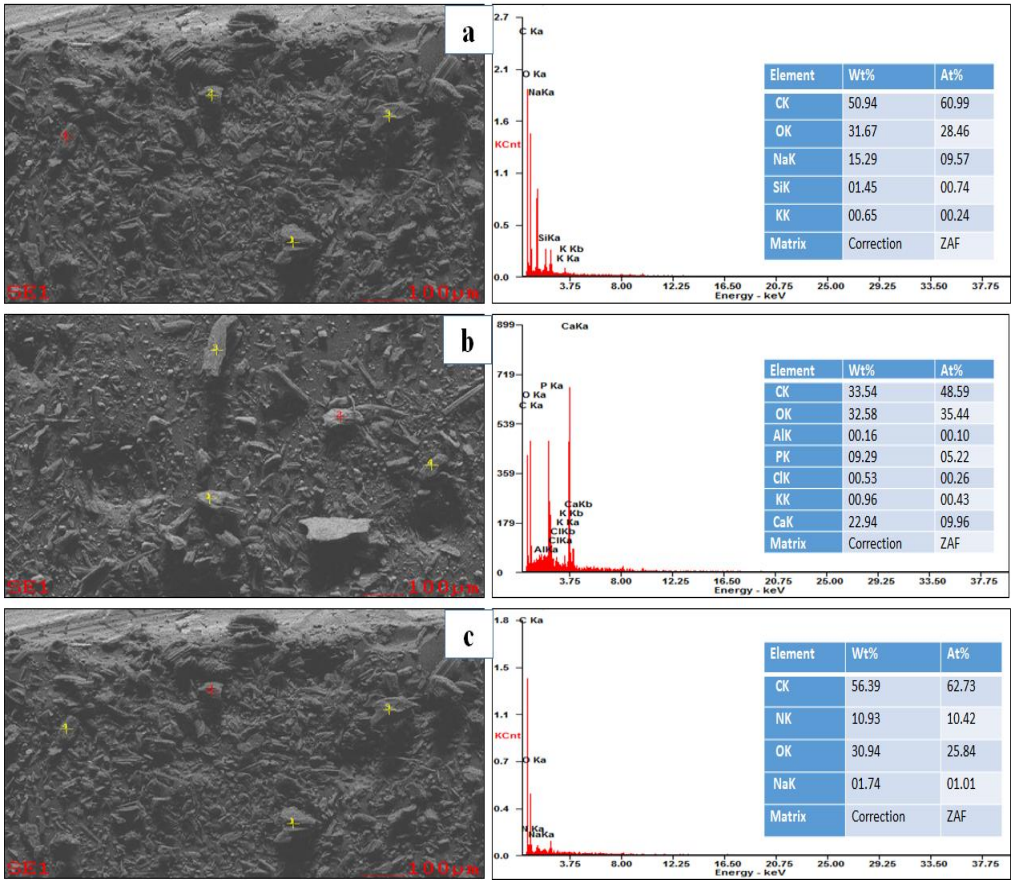

Figure 3. *EDS analysis of the biochars derived from NaOH- (BC_{Na}) (a), hydroxyapatite (BC_{HA}) (b), and chitosan (BC_C) (c) and their corresponding EDS spectra with atomic elemental ratio*

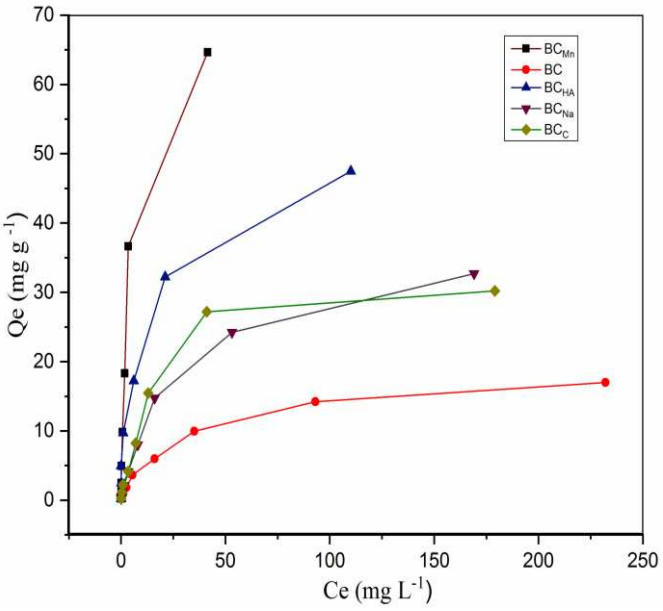

Figure 4. *Adsorption isotherms for Pb^{2+} on pristine (BC), $KMnO_4$- (BC_{Mn}), hydroxyapatite- (BC_{HA}), NaOH- (BC_{Na}) and chitosan-modified (BC_C) biochars (Qe is the adsorbed Pb^{2+} per unit mass of biochar, Ce is the equilibrium solution concentration)*

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Table 2. *The isothermal parameters calculated from both Langmuir and Freundlich equations for Pb^{2+} adsorption by pristine (BC), $KMnO_4$- (BC_{Mn}), hydroxyapatite- (BC_{HA}), NaOH- (BC_{Na}) and chitosan-modified- (BC_C) biochars*

Biochars	Langmuir			Freundlich		
	Q_{max}	K_L	R^2	1/n	K_F	R^2
BC	18.32	0.05	0.99	0.56	1.11	0.98
BC_{Mn}	70.92	0.24	0.98	0.71	9.92	0.89
BC_{Na}	37.04	0.04	0.99	0.67	1.6	0.98
BC_{HA}	49.26	0.18	0.99	0.49	6.37	0.75
BC_C	32.89	0.07	0.99	0.65	1.94	0.96

Q_{max} is the maximum adsorption capacity (mg g^{-1}). K_L is the Langmuir constant related to the sorption energy (L mg g^{-1}). 1/n is the Freundlich constant associated to surface heterogeneity. K_F is the Freundlich constant associated to sorption capacity ($mg^{(1-n)} L^n g^{-1}$)

In contrast the Pb^{2+} adsorption inclined to plateau when equilibrium concentration of Pb^{2+} was more than 30 mg L^{-1}. Adsorption capacities (q_e, mg g^{-1}) of the engineered biochars were raised significantly, as compared with the pristine biochar at this concentration. The results are consistent with a previous study (Foo and Hameed, 2010). The adsorption capacity of BC_{Mn}, BC_{HA}, BC_{Na} and BC_C is 287.12%, 168.89%, 102.18%, and 79.53%, respectively, much higher than BC. The maximum adsorption capacities of the biochars were observed in the following order: $BC_{Mn} > BC_{HA} > BC_{Na} > BC_C > BC$.

Table 2 shows the correlation coefficient, Langmuir and Freundlich models parameters. It is obvious that marginally better fits were attained by the use of the Langmuir model as compared to those attained from the Freundlich model, proposing that Pb^{2+} adsorption on these biochar was more constant with the Langmuir model rather than with the Freundlich model. Consequently, it was presumed that the adsorption happened mainly in monolayers, or occurred through fixed number of equal and energetically corresponding sites on the surface of biochar. In particular, Mn-modified biochar (BC_{Mn}) revealed the highest potential for Pb^{2+} adsorption, in which Q_{max} and K_L values were 3.87 times and 4.8 times (respectively) as high as those of virgin biochar.

Adsorption kinetics

Kinetic models were used to access the effect of contact time on the quantity of adsorbed Pb^{2+} on biochar and all the parameters of kinetic equations are given in *Table 3.* In the initial hours, the adsorption rate was very high. The rate of adsorption subsequently declined with the loom in equilibrium concentrations. Twenty-four hours were considered enough to confirm that the adsorption has reached equilibrium stage. Pseudo first order and pseudo second order models were used to evaluate the adsorption mechanism, (*Table 3*). The obtained regression coefficients (R^2) after using pseudo first order were 0.79-0.96 and the fitted model perceived a poor fit in the experimental data (*Table 3*). The pseudo second order equation fitted the kinetics data well and the regression coefficient of most samples was more significant (*Table 3*). The calculated q_e accorded the experimental data well, specifying the Pb^{2+} adsorption on engineered biochars follows the pseudo second order model which presumes chemisorption mechanism.

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Table 3. *Fitting parameters for the kinetic equations that describe Pb^{+2} adsorption on pristine (BC), $KMnO_4$- (BC_{Mn}), hydroxyapatite- (BC_{HA}), NaOH- (BC_{Na}) and chitosan-modified (BC_C) biochars*

Biochars	Pseudo-first-order model			Pseudo-second-order model		
	q_e	K_1	R^2	qe	K_2	R^2
BC	8.32	0.037	0.91	11.78	0.03	0.95
BC_{Mn}	18.93	1.24	0.79	22.61	0.78	0.96
BC_{Na}	11.04	0.42	0.89	13.67	0.36	0.91
BC_{HA}	14.26	0.18	0.81	16.49	0.37	0.96
BC_C	10.89	0.08	0.90	12.65	0.05	0.92

Where, q_e is calculated data, (mg g^{-1}). K_1 is the rate constant for pseudo-first-order adsorption (L h^{-1}). While, K_2 is the rate constant for pseudo-second-order (g (mg h)$^{-1}$)

The Pseudo second-order equation determined the adsorption of Pb^{2+} on the surfaces of BC, BC_{Mn}, BC_{HA}, BC_{Na} and BC_C as well (*Table 3*). Adsorption rate constant (K) for various chars was in the following orders: BC_{Mn} > BC_{HA}> BC_{Na} > BC_C > BC. The rate constant is higher for BC_{Mn} (0.78 g (mg h^{-1}) than for BC (0.03 g (mg h^{-1}), suggesting the rapid adsorption of Pb^{2+} to BC_{Mn} (*Table 3*).

Desorption capacity

A post desorption test was piloted to examine the stability of the adsorbed metals onto/into these adsorbents (closely related with variable adsorption mechanism of each biochar). In the beginning, all the loaded Pb^{2+} was desorbed from every examined biochar and most of the adsorbed Pb^{2+} was detached from BC_{Mn}, BC_{HA}, BC_{Na}, BC_C and BC using the 0.1 M HNO_3. The desorption aptitude of all the biochars was much higher with HNO_3 extractant (*Fig. 5*). As 98.31%, 89.42%, 91.56%, 93.98% and 95.71% of metal desorption occurred in BC, BC_{Mn}, BC_{HA}, BC_{Na}, and BC_C, respectively. While, 9-11% of pre-loaded Pb^{2+} in BC_{Mn}/ BC_{HA} could be fixed in the studied biochars after desorption is anticipated to expose the ‘‘geochemically active form’’ of metals (Tipping et al., 2003).

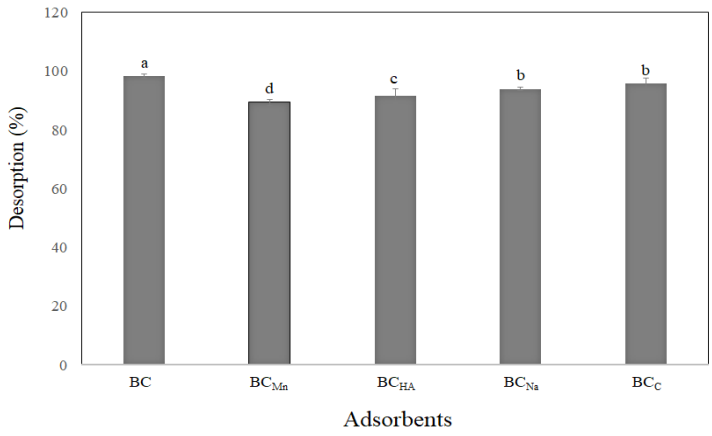

Figure 5. *Pb^{2+} desorption capacity by pristine (BC), $KMnO_4$- (BC_{Mn}), hydroxyapatite- (BC_{HA}), NaOH- (BC_{Na}) and chitosan-modified (BC_C) biochars by using 0.1 N HNO_3 solution. Experimental conditions: pH 5.0, initial concentration 50 mg/L, agitation rate 180 rpm, temperature 25°C, contact time 24 h*

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Next, metal desorption was carried out using 0.01 M $CaC1_2$ for the assessment of bioavailable metal forms (Houba et al., 1996), and desorption is variable for all the studied biochars (*Fig. 6*). Pb^{2+} was desorbed at lower rate (2.67%-4.34%) in all types of biochars. Obviously, BC has much desorption capacity (4.34%) and BC_{Mn} has the lowest one (2.67%). Finally, background electrolyte of 0.1 M $NaNO_3$ having pH 5.00 was used for desorption of Pb^{2+} from biochars (*Fig. 6*). Results exhibited that Pb-desorption was trivial in all cases (<1.83%), which was caused by the contrasting behaviour of Pb^{2+} (higher stability and affinity to organic matter in Pb^{2+} at a specific pH) and by variable metal adsorption mechanisms.

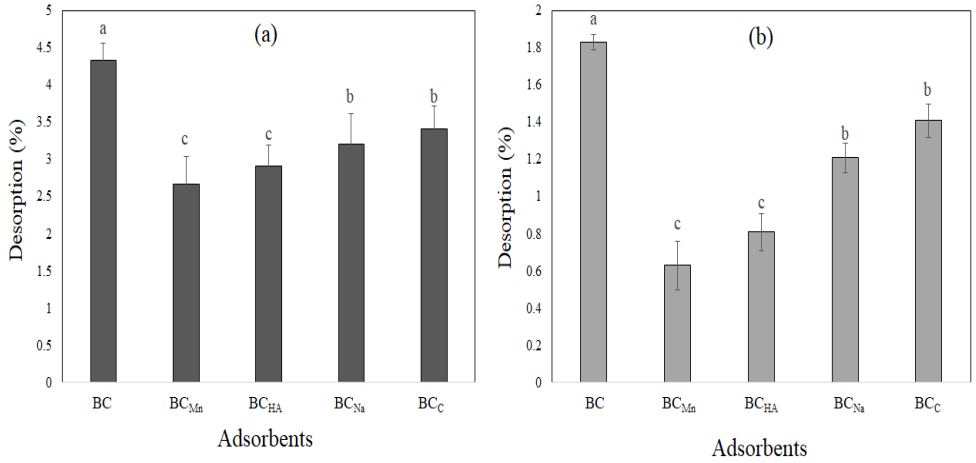

Figure 6. *Pb^{2+} desorption capacity by pristine (BC), $KMnO_4$- (BC_{Mn}), hydroxyapatite- (BC_{HA}), NaOH- (BC_{Na}) and chitosan-modified (BC_C) biochars by using 0.1 N $CaCl_2$ (a) and 0.1 M $NaNO_3$ (b). Experimental conditions: pH 5.0, initial concentration 50 mg/L, agitation rate 180 rpm, temperature 25°C, contact time 24 h*

Discussion

Our results revealed that the surface of biochars derived from SE-treated feedstock became coarser, compared to the smooth surface, clear anatomy, and distinctive pore structure in the biochars derived from the pristine feedstock. Our results are consistent with a previous study (Chen et al., 2019a). The accumulation of small particles on the BC_{Mn} surface is very obvious in SEM-EDS images, and is probably due to potassium permanganate. Due to Mn oxides formation, the porous structure was obstructed by these Mn oxide particles that are formed during pyrolysis (Petit et al., 2010). Various compounds of Mn oxides with various phases like β-MnO_2, δ-MnO_2, and MnO_2- coated sand have been reported to have strong affinity for various divalent metal cations (Tripathy and Kanungo, 2005). This might enhance the surface area of respective biochar and expose additional adsorption sites for Pb^{2+} (Chia et al., 2015).

All the four modified biochars had significantly greater maximum adsorption capacities (Q_{max}) and K_L values than the pristine biochar, which could direct that both feedstock pretreatment and biochar modification significantly increase the adsorption capacity of Pb^{2+}. The Langmuir model, presumes that the adsorptions of metal happened on a homogenous surface by monolayer adsorption deprived of any interaction between the adsorbed ions and has been used efficiently in many monolayer adsorption processes. Whereas, the Freundlich model presumes that the adsorption of metal ions

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took place on heterogeneous surfaces and sorption was multilayer. As evident from *Table 2* the adsorption isotherms are well described by the Langmuir model, suggesting that adsorption could be mono-molecular. As cited earlier, the adsorption/sorption efficiency differed not merely amongst tested metals, but usually differed among all the studied biochars. This might be because of the diverse adsorption mechanisms of respective metals on the studied biochars. As it is reported ion exchange, complexation and physical adsorption are liable for adsorption and sorption of metal on the surface of biochar (Sohi et al., 2010; Lu et al., 2012; Ahmad et al., 2014). However, these metal ions adsorption mechanisms are different for different kinds of biochars.

The adsorption proficiency of Pb^{2+} by these five adsorbents enhanced with time and then plateaued when equilibrium was attained. In the early stages adsorption capacity of Pb^{2+} by the pristine and modified biochars was much faster, probably because adsorption mainly took place on the external surfaces of biochars (Li et al., 2017). Pb^{2+} diffused into the carbon pores with the passage of time and further reacted with interior active sites of the carbon skeleton, where the adsorption process is comparatively slow (Babel and Kurniawan, 2003). BC_{Mn} and BC_{HA} adsorbed Pb^{2+} faster than BC and reached the equilibrium stage within only four hours. On the other hand, BC, BC_{Na} or BC_C needed eight hours to attain adsorption equilibrium. The aptitude of BC_{Mn} to grasp fast and proficient adsorption equilibrium which is extensively employed for the treatment of heavy metals contaminated water particularly in emergency conditions. Results of our investigation are in accordance with those of Ofomaja et al. (2010) who indicated that the sorption kinetics of heavy metals is considerably dependent on the physiochemical properties of biochar.

Solute-uptake rate is determined by adsorption kinetics which in turn governs the time of adsorption process (Ofomaja et al., 2010; Betts et al., 2013). Validation of the sorption kinetics model and the potential rate controlling steps was checked by pseudo first (Lagergren, 1898) and pseudo second order models (Ho and McKay, 2000). These parameters are beneficial for the selection of the optimal operating conditions. Furthermore, the adsorption capacities attained from pseudo-second-order model fitting are reconcilable with the experimental data, along with a chemisorption rate-controlling mechanism, where the limiting step is a physicochemical sorption or adsorption process including valence forces through the sharing or exchange of electrons between the sorbent and the sorbate (Vijayaraghavan and Yun, 2008). Findings of the current study are also in line with the observation of Li et al. (2017). Thus, this study speculated that these modified biochars may have higher potential for adsorption of pollutants as compared to pristine biochar. In addition, oil-rape straw might be suitable to SE pretreatment for preparing novel biochar for waste-water treatment and other environmental applications.

Following the results of an earlier study (Anastopoulos et al., 2015), 0.1 N HNO_3 was perceived as the best desorbing solution among numerous solutions. This proposes a dominant role for exchange sites in determining the adsorption properties of the various adsorbents. It is presumed that higher concentration of protons (H^+) of 0.1 N HNO_3 solution triggered intense competition of the H^+ between the metal ions for the adsorbent's exchange sites throughout the ion exchange process, and to the resultant desorption of Pb^{2+} ions adsorbed on exchange sites of the adsorbents surface (Anastopoulos et al., 2015). Obtained results of desorption study are most probably because of the predominant weak Pb π-bindings with poly-organic chains, e.g. physical adsorption and, in contrast, due to the stronger fixing of metals into the engineered

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biochar structures caused mostly by the cation release (particularly for BC_{Mn}). These results support the findings about the very strong fixation of Pb^{2+} to the structure of BC_{Mn}, BC_{HA}, BC_{Na} and BC_C (ion exchange adsorption mechanism). This partial desorption of adsorbed Pb^{2+} in the biochar after all extractions might be described by surface complexation reactions (Namgay et al., 2010). The current study, recommends that this often perceived partial extraction using $NaNO_3$ and $CaCl_2$ might be the result of strong bonds to biochar surfaces that would make adsorbed Pb^{2+} unavailable. So our study recommends that Pb^{2+} adsorption is not entirely reversible using $CaCl_2$ and $NaNO_3$ regardless of modifying agents, in contrast, it can be accomplished with HNO_3 as an extractant. Our findings are in accordance with previous studies (Trakal et al., 2014; Anastopoulos et al., 2015). Thus the current study, may reveal that the adsorbed Pb^{2+} was much more stable on the modified biochar with a lower desorption rate, as compared to the pristine biochar.

Conclusion

To summarize, $KMnO_4$ and nano-hydroxyapatite modification significantly improved the adsorption capacities of biochar for Pb^{2+}. The highest adsorption capacity for Pb^{2+} was exhibited by Mn and nano-hydroxyapatite modified biochars (70.92 and 49.26 mg g^{-1}, respectively), as compared to pristine biochar. Additional adsorption sites on engineered biochars appeared to play more significant role in Pb^{2+} adsorption instead of specific surface area of biochars. It can be concluded that cation-π bonding and cation exchange are the key mechanisms responsible for the highest adsorption capacities of studied biochars. Surface precipitation, surface electrostatic attraction and surface complexation are responsible for higher adsorption capacity in BC_{Na}. Conversely, BC_C exhibiting lower adsorption capacity might be caused by plenty of protons that obstructed the approach of Pb^{2+} ions, prompting to the decreased adsorption of Pb^{2+}. Thus, the manganese oxide impregnated biochar might provide an efficient way to elevate Pb^{2+} removal from aqueous medium. Although, various metal removal mechanisms are involved in the current study, but ion exchange mechanism is a crucial one among others. Furthermore, ion exchange mechanism revealed solid binding of adsorbed metal as affirmed by the post desorption of fully metal loaded biochars. To conclude, these biochars showed much promising adsorptive properties for divalent metals and might thus have a high potential as a soil amendment and an alternative adsorbent for environmental remediation. Further competitive adsorption and desorption studies are necessary in order to accurately estimate the heavy metal adsorption/desorption capabilities of biochar in natural environments.

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METABOLOMICS ANALYSIS OF *ANABASIS APHYLLA* SEEDLINGS UNDER COLD STRESS

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Abstract. Cold severely affects plant growth and metabolism. The analysis of the characteristics of metabolite variations in seedlings under cold stress can help understand the physiological mechanism of *Anabasis aphylla* when responding to stress. In this study, gas chromatography-mass spectrometry technology was used to systematically analyze the changing characteristics of the metabolite profiles of *A. aphylla* under cold stress. A total of 116 types of differential metabolites were stably detected, of which 41 were up-regulated, while 75 were down-regulated. The metabolites of carbohydrates, amino acids, and organic acids formed the majority. The results showed that cold stress had a significant impact on the differential metabolites of *A. aphylla* seedlings. Via enrichment of metabolites such as γ- aminobutyric acid, α- ketoglutaric acid, and 1-kestose, *A. aphylla* could reduce the extent of damage caused by cold stress. The results of this study provide a basis for further analysis of the molecular and physiological mechanisms of the cold stress response of *A. aphylla*.
Keywords: *desert plant, differential metabolite, low temperature, carbohydrate, amino acids, organic acids*

Introduction

Cold is one of the most important abiotic stress factors to affect plant growth, plant development, geographical distribution, and crop yield. For cold-sensitive plants, cold stress from 1 to 10 °C can cause disorders in physiological processes such as water condition, mineral nutrition, photosynthesis, respiration, and metabolism, resulting in severe damage and even death of the plants (Wu et al., 2015). However, after a long evolutionary process, many plants that grow in a relatively low-temperature environment developed a well-established defense mechanism adapting to adverse environmental conditions. Studies have shown that the adaptation of plants to cold is related to the regulation of several metabolic pathways, particularly in the regulation of carbon metabolism, photosynthesis, and membrane lipids (Li, 2015). In Junggar basin of China, some desert plants have low temperature germination feature during snow-melting during the snow-melting period of early spring, these desert plants include *H. ammodendron*, *Haloxylon Persicum*, *Anabasis elatior*, *Lepidium apetalum* and *Anabasis aphylla* (Huang et al., 2003; Han et al., 2011; Peng et al., 2018). In the field, we found that germination speed of *Anabasis aphylla* was higher than other species, while its seedling regeneration probability is very low. Therefore, study on regeneration disorder mechanism of *A. aphylla* seedling is necessary for desert vegetation protection.

Anabasis aphylla is a semi shrub belonging to the Chenopodiaceae family. It is an important type of plant community for windbreak, sand fixation, and for the protection of the local ecological environment as well as surrounding land resources (Wang et al., 2017). The snow-melting period at Junggar basin happens within a 10-day period in March. During this time, the average daytime temperature is above 0 °C and the

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maximum temperature is maintained at 7-14 °C, while the night-time minimum temperature drops below 0 °C (Zhou et al., 2009). Consequently, the ground surface is in a repeated freeze-thaw state during the snow melting period (Gornish et al., 2015). During field observations, we discovered that a large quantity of *A. aphylla* seedlings had already germinated under the snow at the snow-melting period, which indicates that *A. aphylla* has already adapted to the cold. In recent years, comparative transcriptomics, proteomics, and metabolomics studies combined with mutation analysis of cold resistance have significantly increased our understanding of the complex network of signaling pathways of the cold training process and the necessary molecular changes (Janda et al., 2014). Via proteomics studies, we found that the adaption of *A. aphylla* to cold was primarily achieved through changes in the energy metabolism. The proteins associated with the energy metabolism could be identified, such as the fructose-bisphosphate proteins, malate dehydrogenase proteins, citrate synthase proteins, oxygen-evolving enhancer protein, and photosynthetic proteins. However, the proteomic study mainly focused on the classification and identification of the associated proteins as well as on the analysis of the interaction between proteins and the function of the proteins, it cannot intuitively describe the physiological state of the plants under stress, while a plant metabolomics study is conducive to the overall study of the response of a biological system to changes in genes or the environment. The overall changes of the metabolites can directly reflect the physiological and pathological states of the organism and help to uncover complex plant stress response mechanisms (Teng et al., 2011). At present, metabolomics studies of plants under stress have been widely applied in species such as *Arabidopsis thaliana*, rice, potatoes, and *Festuca arundinacea* (Tarryn et al., 2016; Lin et al., 2017; Aliferis and Jabaji, 2012; Li et al., 2016). Studies have shown that under environmental conditions such as drought, salinity, or extreme temperature, the plant amino acid content increased significantly (Lanzinger et al., 2015; Annick et al., 2016), while the contents of several carbohydrates such as sucrose, lactose, and trehalose were also significantly increased induced by stress (Min et al., 2014; Wingler and Roitsch, 2008). Further studies have revealed that under adverse conditions, the contents of organic acids involved in the tricarboxylic acid (TCA) cycle showed increasing characteristics of metabolite changes at an early stage, while decreasing characteristics at a later stage (Widodo et al., 2009). However, a metabolomics study of *A. aphylla* seedlings under cold stress has not been reported yet. In this study, metabolomics technology was utilized for an analysis of the differential metabolites of *A. aphylla* seedlings under cold stress, thus providing a theoretical basis to explore the complex metabolic processes as well as their products and the secondary metabolic network structure.

Materials and methods

Plant materials and stress treatments

In the northwestern of China, it is mainly distributed around the piedmont alluvial fan, the Gobi Desert, and sand dune gravels areas (*Fig. 1*). *A. aphylla* seeds were obtained from the southern edge of the Gurbantünggüt Desert (E84°49′-85°23′, N45°21′-45°40′) in Xinjiang, China. All collected *A. aphylla* seeds were sterilized by soaking them in 10% H_2O_2 for 30 min, after which they were rinsed with distilled water. For the control treatment, the sterilized seeds were germinated on filter paper discs soaked with distilled water in Petri dishes at room temperature and a 16-h light/8-h dark

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photoperiod until seedlings were approximately 3 cm in length. For the cold treatment, 3-cm-long seedlings were kept in a -3 °C growth chamber for 12 h each day for one week. For each treatment, 3-g seedlings were selected as samples and quickly frozen in liquid nitrogen individually and stored at -80 °C for metabonomics analyses. Both treatments had eight biological replicates.

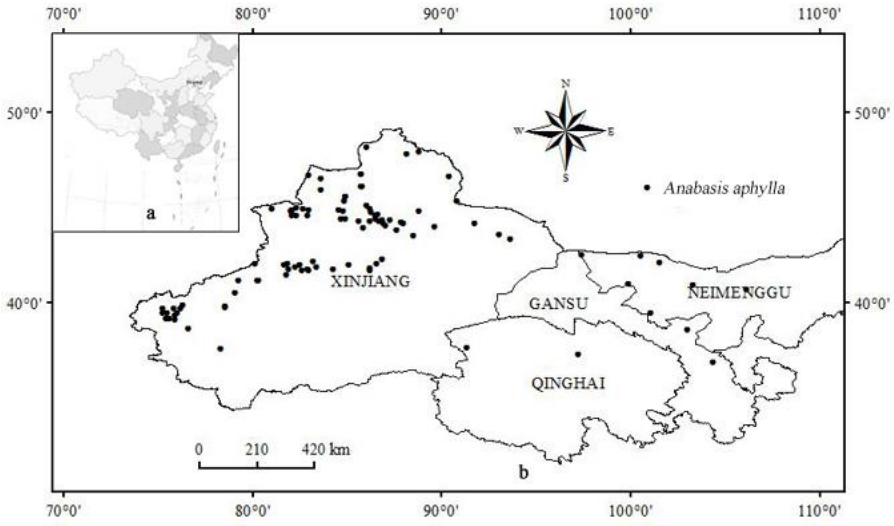

Figure 1. *Distribution of A. aphylla in northwestern China. (a) Map of China; (b) distribution of A. aphylla in northwestern China*

Sample pretreatment

Precision weighed 60 mg *A. aphylla* samples into 1.5 mL centrifugal tube, after adding 360 μL cold methanol and 40 μL internal standard (L-2-chlorine-phenylalanine, 0.3 mg mL^{-1}, methanol configuration) and homogenate (ground in Tissuelyser-48 (60 Hz, 2 min)). Then extracted by homogenate ultrasound at 30 min and sequentially added 200 μL chloroform and 400 μL water, again 30 min extracted by ultrasound and centrifuged for 10 min (14000 rpm, 4 ℃) at cold. Thereafter, collected 500 μL supernatant placed into glass derivative bottle, then dried with rapid centrifugal concentrator and added 80 μL methoxy pyridine hydrochloride solution (15 mg. mL^{-1}), after 2 min vorticity shock later, carried on oximation reaction 90 min in 37 ℃ shock incubator, then removed and 80 μL of N,O-bis trifluoroacetamide (BSTFA) containing 1% trimethylsilyl chloride (TMCS) derivative reagent and 20 μL n-hexane were added, after 2 min of vortex oscillation, reaction 60 min at 70 ℃. Later the samples were taken and placed at room temperature for 30 min for gas chromatography-mass spectrometry (GC/MS) metabolomics analysis (Lin et al., 2017).

GC/MS analysis

Each 1 μL aliquot of the derivatized solution was injected in split less mode into the Agilent 7890A-5975C GC-MS system (Agilent, USA). Separation was carried out on a non-polar DB-5 capillary column (30 m × 250 μm I.D., J&W Scientific, Folsom, CA), with high purity helium as the carrier gas at a constant flow rate of 1.0 mL min^{-1}. The GC temperature programming began at 60 °C, followed by 8 °C min^{-1} oven temperature

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ramps to 125 °C, 4 °C min^{-1} to 210 °C, 5 °C min^{-1} to 270 °C, and 10 °C min^{-1} to 305 °C, and a final 3 min maintenance at 305 °C. The electron impact (EI) ion source was held at 260 °C with a filament bias of -70 V. Full scan mode (m/z 50−600) was used, with an acquisition rate of 20 spectrum/second in the MS setting.

Quality control (QC) sample

QC sample was prepared by mixing aliquots of the all samples to be a pooled sample, and then analyzed using the same method with the analytic samples. The QCs were injected at regular intervals (every eight samples) throughout the analytical run to provide a set of data from which repeatability can be assessed.

Data analysis

The acquired MS data from GC-MS were analyzed by ChromaTOF software (v 4.34, LECO, St Joseph, MI). Briefly, after alignment with Statistic Compare component, the CSV file was obtained with three-dimension data sets including sample information, retention time-m/z and peak intensities, and the internal standard was used for data quality control (reproducibility). After internal standards and any known pseudo positive peaks, such as peaks caused by noise, column bleed and BSTFA derivatization procedure, were removed from the data set, and the peaks from the same metabolite were combined, the detectable metabolites of samples in GC-MS were 293 in total. The data set was normalized using the sum intensity of the peaks in each sample.

The data sets resulting from GC-MS were separately imported into SIMCA-P + 14.0 software package (Umetrics, Umeå, Sweden). Principle component analysis (PCA) and (orthogonal) partial least-squares-discriminant analysis (PLS-DA) were carried out to visualize the metabolic alterations among experimental groups, after mean centering and unit variance scaling. Variable importance in the projection (VIP) ranks the overall contribution of each variable to the (PLS-DA) model, and those variables with VIP > 1.0 are considered relevant for group discrimination.

In this study, the default 7-round cross-validation was applied with 1/seventh of the samples being excluded from the mathematical model in each round, in order to guard against overfitting.

Metabolite identification

All of the differentially expressed compounds in treated group were selected by comparing the compounds in the treated group with the control using the multivariate statistical method and Wilcoxon-Mann-Whitney test. Metabolites with both multivariate and univariate statistical significance (VIP > 1.0 and $p < 0.05$) were annotated with the aid of available reference standards in our lab and the NIST 11 standard mass spectral databases and the Feinh databases linked to ChromaTOF software. The similarity of more than 70% can be considered as reference standards.

Results

Chromatograms of cold stress and control treatment

Figure 2 shows the result of overlapping GC-MS total ion current (TIC) of the quality control samples (QC). This was a preliminary study of the reproducibility of the method.

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Figure 2 shows that the reproducibility of the retention time and the corresponding intensity of *A. aphylla* QC sample mass peaks were very good, indicating the whole analysis method to be stable and reliable, making it useful for subsequent analysis. The typical TIC diagram of each set of sample is also presented (*Figs. 3* and *4*).

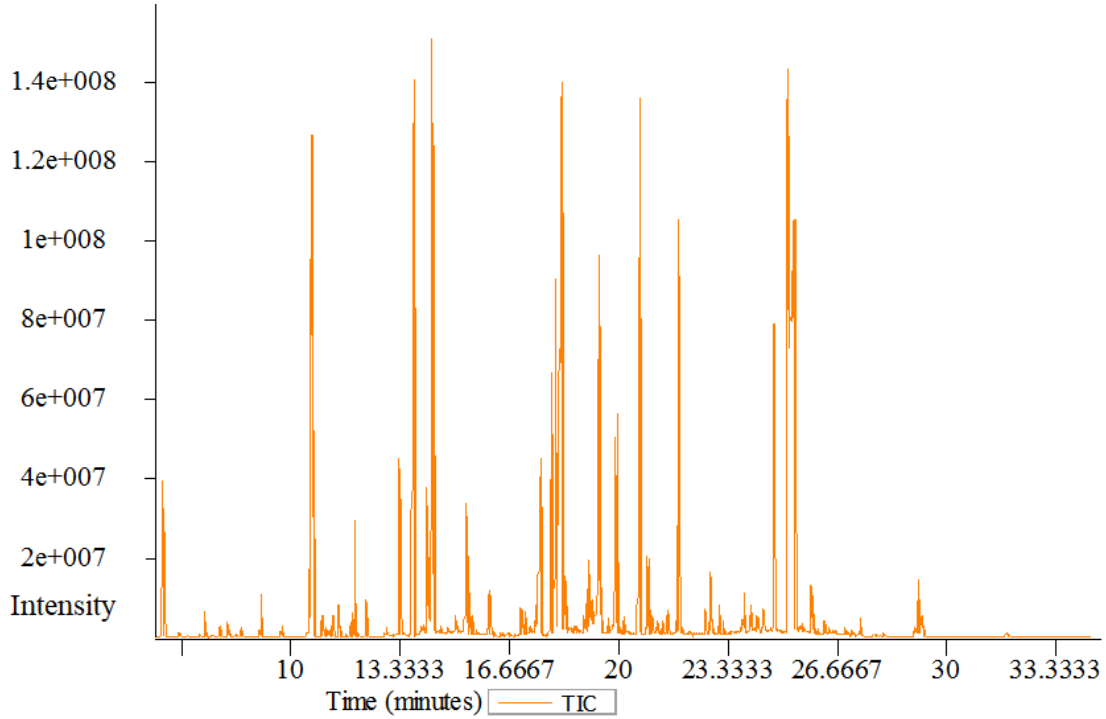

Figure 2. *Overlap total ion chromatography (TIC) of the quality control samples (QC)*

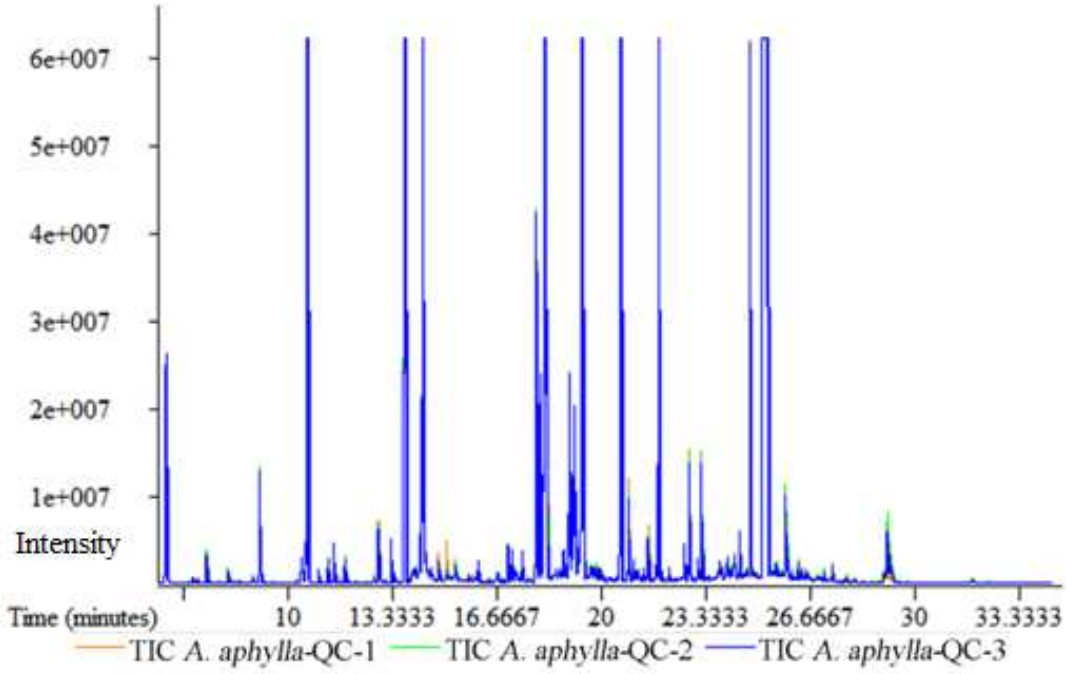

Figure 3. *Total ion chromatography (TIC) of A. aphylla from cold stress samples*

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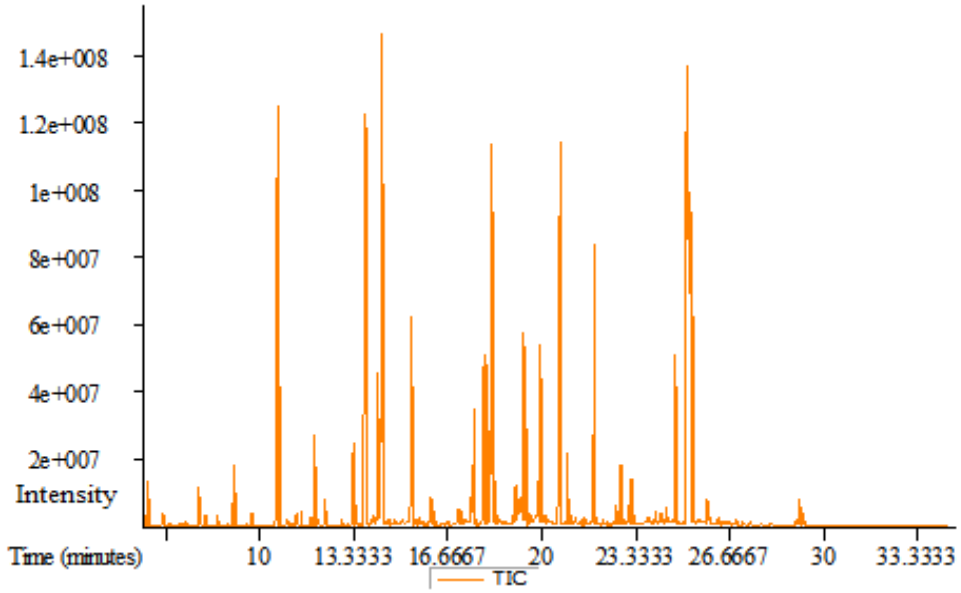

Figure 4. *Total ion chromatography (TIC) of A. aphylla from control treatment samples*

PCA, PLS-DA, and OPLS-DA analysis of A. aphylla seedlings under cold stress

PCA, PLS-DA, and OPLS-DA analyses were conducted on the *A. aphylla* samples in both the cold treated group and the normal temperature control group, and the scores of the two groups are shown in *Figures 5, 6,* and *7*. The PCA score diagram shows that the results of the eight duplicates from the cold treated group and the control group were well divided into two groups and were clustered together respectively, indicating that the error of the pretreatment method and the instrument analysis system were relatively small and that the reliability of the data was high, indicating that the method can be used for subsequent analysis. Using PLS-DA and OPLS-DA for further analysis of the GC-MS data from the control group and the treatment group, the results showed that the cold treated *A. aphylla* seedlings were clearly separated from the control group. Both the fitting degree and the predictive value of each model indicated good reliability of the models and the metabolites of the *A. aphylla* seedlings under cold stress showed significant changes compared to the control group.

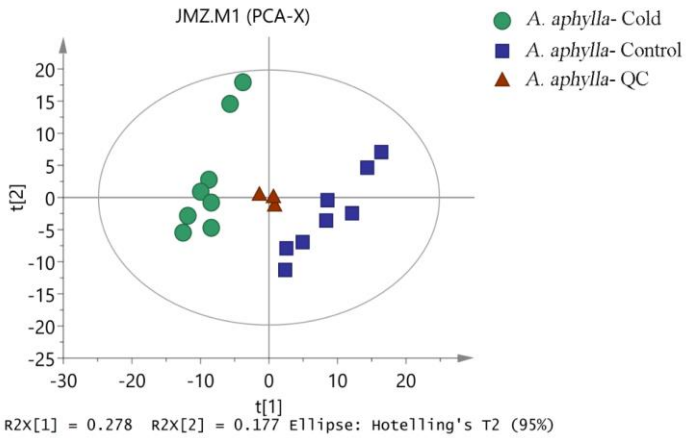

Figure 5. *PCA scatter plot of cold stress and control treatment A. aphylla samples*

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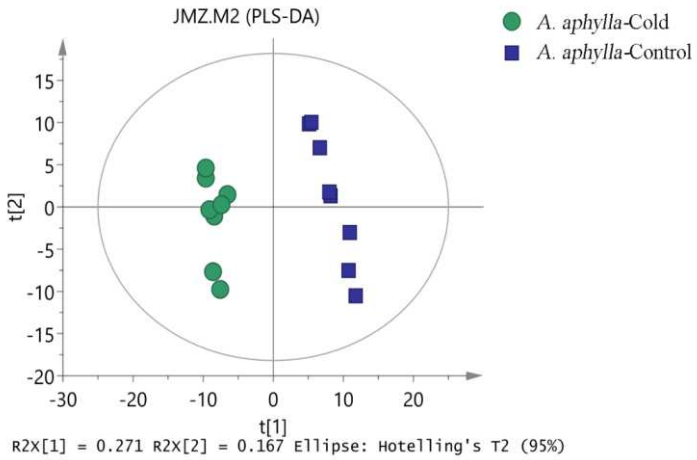

Figure 6. *PLS-DA scatter plot of cold stress and control treatment A. aphylla samples*

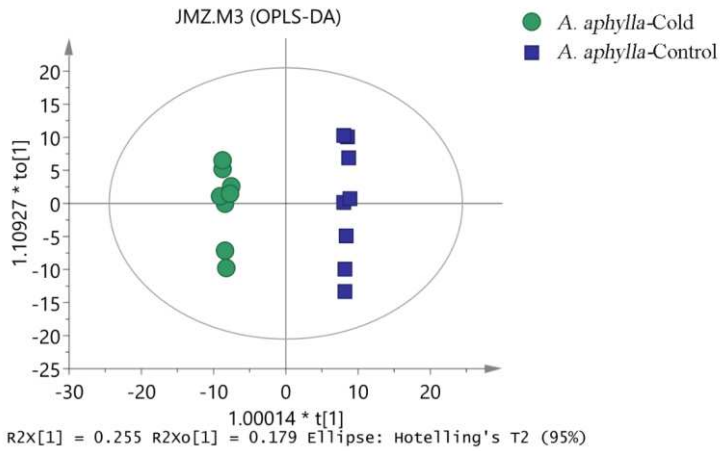

Figure 7. *OPLS-DA scatter plot of cold stress and control treatment A. aphylla samples*

Differential metabolite analysis under cold stress

293 metabolites were detected from the samples of the treated group and the control group, of which 116 metabolites with VIP > 1, $p < 0.05$ were screened out as differential metabolites. Among these, 41 were up-regulated, while 75 were down-regulated. The mass-to-charge ratio and the abundance of the characteristic fragmentation pattern of each chemical compound were compared to the standard fragmentation pattern in the NIST database and the Feihn metabolomics database to obtain the name of metabolites. The detected metabolites mainly included organic acids, amino acids, carbohydrates, alkaloids, phytohormones, organic bases, quinones, flavonoids, and other substances. Among these, the most abundant identified materials were amino acids, organic acids, and carbohydrates, including 21, 15, and 8 different types of substances in each category, respectively (*Table 1*); therefore, the materials in the three categories were the main focus for subsequent discussion and analysis. The metabolites in the focused discussion are listed in *Figure 8*. Some of these metabolites are associated with the ornithine cycle and the TCA cycle, and these identified metabolites are labeled in green in the diagram (*Figs. 9* and *10*).

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Table 1. *Identified sugars, amino acids and organic acids species in A. aphylla seedlings under cold stress*

Metabolites	R.T. (min)	Quant mass	VIP value	FC (cold/control)	*p* value (*t* test)	Up/down
Saccharide						
Ribose	16.05798	307	1.01775	2.05672	0.02891	↑
1-Kestose	17.92873	438	1.35691	3.31756	0.00054	↑
D-Talose	19.40036	160	1.44748	7.50492	0.0001	↑
Tagatose	18.96745	307	1.45601	8.46888	0.00007	↑
Sedoheptulose	24.60103	333	1.10726	0.65934	0.02607	↓
Trehalose	26.08805	332	1.19952	0.80285	0.00735	↓
Cellobiose	22.49745	308	1.32735	0.14212	0.00067	↓
Melezitose	24.79442	199	1.48625	0.4118	0.00005	↓
Amino acids						
Isoleucine	10.94246	158	1.49586	1.69923	0.00006	↑
γ-aminobutyric acid	14.30521	304	1.40075	1.23911	0.00073	↑
Beta-Glutamic acid	14.03546	217	1.36979	1.50227	0.00081	↑
Phenylalanine	15.50448	91	1.30639	1.38467	0.00572	↑
Cycloleucine	15.18698	156	1.18226	2.17144	0.00577	↑
Proline	11.03825	142	1.12251	1.22459	0.03674	↑
Asparagine	13.3133	128	1.45597	0.5259	0.00018	↓
Sarcosine	8.40767	116	1.4032	0.22582	0.00083	↓
Citrulline	18.34069	256	1.31285	0.76186	0.00111	↓
Alanine	7.82152	116	1.26208	0.40557	0.00501	↓
Histidine	19.9032	317	1.18169	0.81064	0.00984	↓
Ornithine	15.34038	316	1.14275	0.75554	0.00987	↓
L-homoserine	13.17861	218	1.17977	0.75721	0.02349	↓
Aspartic acid	14.14461	232	1.06868	0.73052	0.02481	↓
O-acetylserine	12.40576	174	1.06425	0.81941	0.03432	↓
Threonine	12.27561	293	1.08858	0.88137	0.03566	↓
Methionine	13.51492	256	1.54807	0.65116	0.00001	↓
Trans-4-hydroxy-L-proline	11.21533	140	1.55369	0.25781	0	↓
L-Cysteic acid	14.40056	241	1.39356	0.65681	0.00074	↓
L-kynurenine	23.18388	322	1.22941	0.49746	0.00801	↓
Xanthurenic acid	22.55864	304	1.07547	0.47683	0.01136	↓
Organic acid						
Malonic acid	12.46042	307	1.44983	2.35443	0.00009	↑
Saccharic acid	21.47683	333	1.44611	2.00206	0.00018	↑
Tartaric acid	19.97148	275	1.34254	1.63333	0.00047	↑
Palmitic acid	21.43681	313	1.30646	1.20218	0.00616	↑
Alpha-ketoglutaric acid	14.79833	304	1.24162	1.74297	0.00657	↑
4-hydroxybutyrate	10.04681	233	1.23565	1.46819	0.00927	↑
3-Hydroxypropionic acid	8.52303	177	1.19535	1.32059	0.01215	↑
3,4-dihydroxybenzoic acid	7.5046	313	1.03061	2.13249	0.02131	↑
Benzoic acid	10.32072	179	1.02761	2.22846	0.02507	↑
Mucic acid	17.14024	333	1.56701	1.53627	0	↑
Gluconic acid	20.87028	333	1.37666	0.47108	0.00025	↓
Citramalic acid	13.46879	247	1.38831	0.7327	0.00092	↓
Glutaric acid	12.58547	261	1.31443	0.52929	0.00155	↓
Lactic acid	7.1533	190	1.16019	0.49001	0.01089	↓
Gallic acid	9.51771	370	1.19889	0.75783	0.01563	↓

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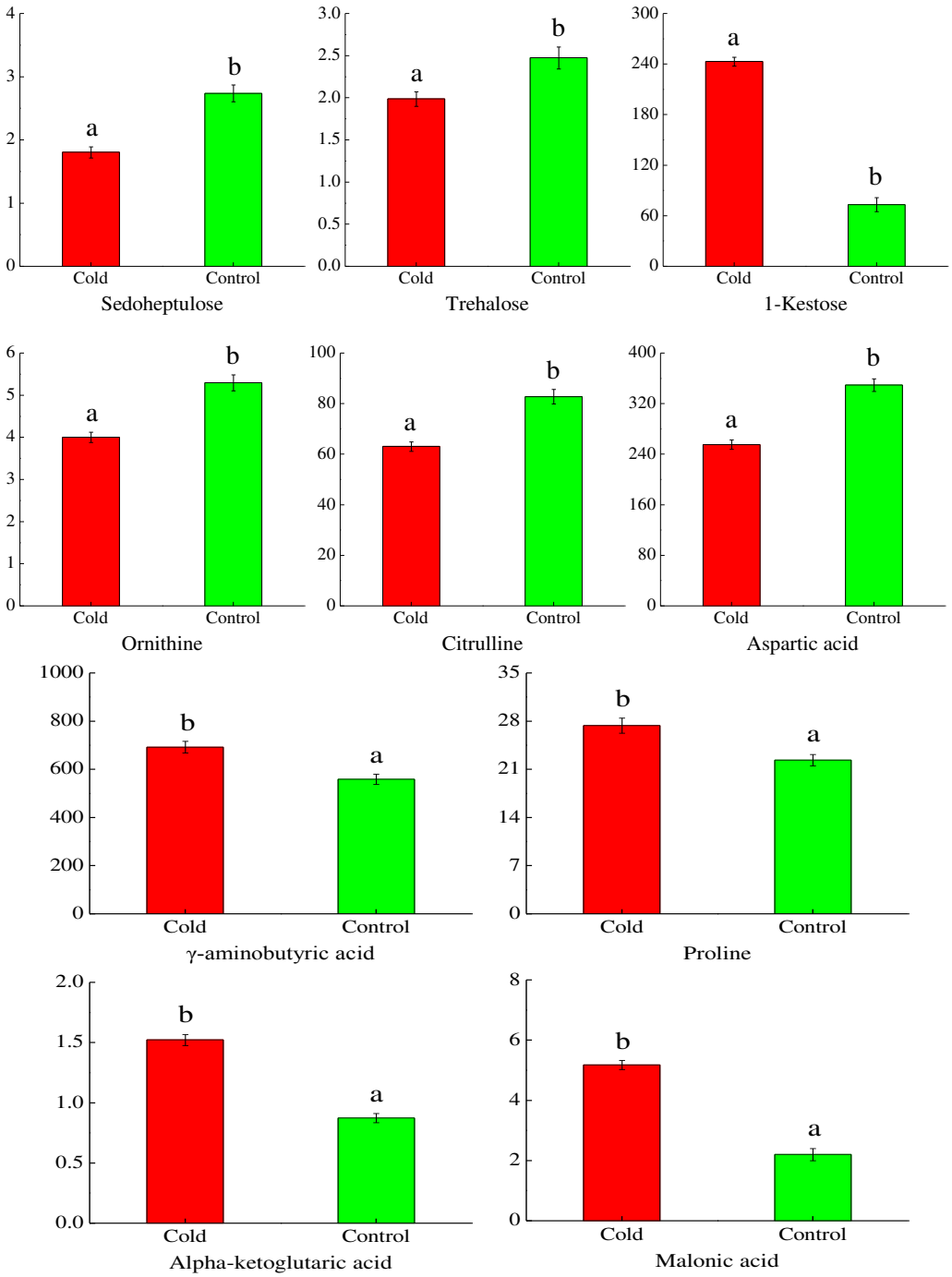

Figure 8. *Sedoheptulose, trehalose, 1-Kestose, ornithine, citrulline, aspartic acid, 4-aminobutyric acid, proline, alpha-ketoglutaric acid and malonic acid content in A. aphylla under cold stress and control treatment*

Discussion

During growth and development, plants are often subjected to stresses such as environmental stress (temperature and moisture), biological stress (diseases, pests, and weeds), and nutritional deficiency stress (nitrogen, phosphorus, and potassium) (Hans et al., 2006). Plants can defend themselves against biotic and abiotic stress factors by

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regulating their metabolic network to induce the production of a series of specific metabolites as response (Teng et al., 2011). Metabolomics can qualitatively and quantitatively analyze all metabolites within a specified biological sample under given conditions (Oliver et al., 2000). Therefore, using metabolomics to analyze changes of metabolites to study the metabolic regulation of plants under stress has gained increasing attention (Vicent et al., 2013).

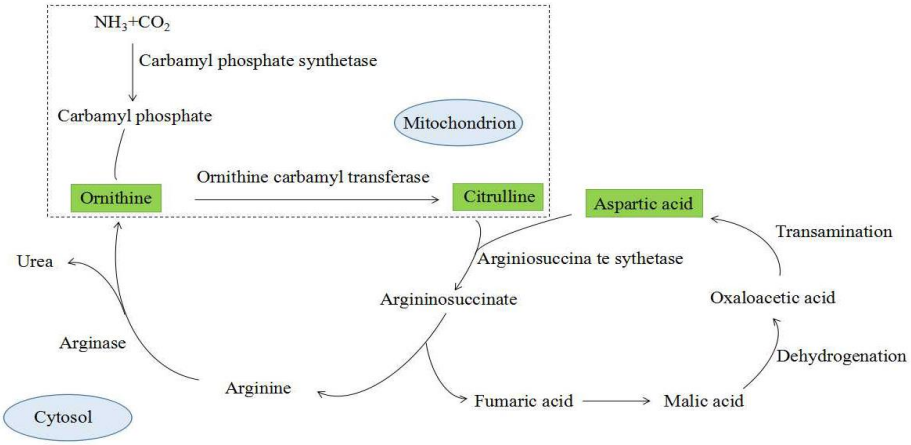

Figure 9. *Identified three amino acid metabolites are involved in the ornithine cycle*

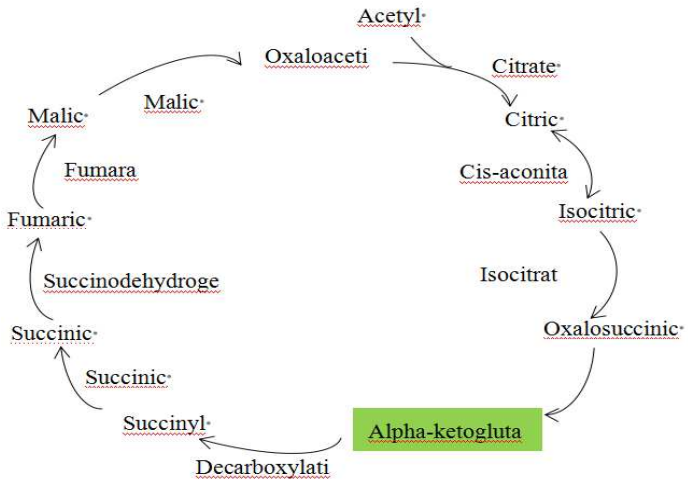

Figure 10. *Identified organic acid metabolites are involved in the TCA cycle*

Effects of cold on carbohydrate metabolites of A. aphylla seedlings

Carbohydrates play an important role in the life process, and it is the main source of energy required for all life to maintain alive. Our study on differential metabolites of *A. aphylla* seedlings under cold stress showed that carbohydrates played very important roles in the response of *A. aphylla* seedlings to cold stress. Among these, tagatose, D-talose, ribose, and 1- kestose accumulated in the seedlings under cold stress, while melezitose, cellobiose, trehalose, and sedoheptulose showed a trend of down-regulation. D- talose is an aldohexose, tagatose is a sweetening agent, often used in food and drug

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industry, and cellobiose is the hydrolysis product of cellulose, which also forms the basic structural unit of cellulose. Many studies have focused on cellobiohydrolase; however, the significance of cellobiose in plants has been rarely reported (Fan et al., 2018). Melezitose has been identified from the nectar secretion of plants such as the North American pinon pine, the Douglas fir, leguminous plants, and the poplar (Aderkas et al., 2012; Tian et al., 2018). Some researchers have also indicated that melezitose was once isolated from the leave secretion of the Yunnan pine. It is mostly used in the food industry (Mu and Lu, 1984). Therefore, only sedoheptulose, trehalose, and 1-kestose, which play important roles in plants, were discussed here. Sedoheptulose belongs to the heptoses and is an important primary and secondary metabolite in the pentose phosphate cycle, photosynthesis, other carbohydrates synthesis, and the biosynthesis of shikimic acid and aromatic ammonia (Xie, 2009). Studies reported that as the primary product of plant photosynthesis, sedoheptulose may be converted from 1,7-diphosphate sedoheptulose produced from carbon source through the Calvin cycle and the aldolase reaction at the end. This is not only the main storage form of carbon in plants, it also plays a key role in the carbon balance of plant. Sedoheptulose in plant mainly exists in the forms of 7-phosphate sedoheptulose and 1,7-diphosphate sedoheptulose and is involved in plant photosynthesis (Keith, 2017). Lee et al. have described sedoheptulose in the leaves of *Cornus oficinalis* of Comaceae, and it was also found in *Sedum spectabile* of Crassulaceae (Lee et al., 1989; Tolbert and Zill, 1954). Trehalose is a non-reducing disaccharide, widely distributed in organisms. Genome sequencing results showed that trehalose biosynthesis genes are widespread in plants (Lunn et al., 2014). In most plants however, the content of trehalose is very low, indicating that trehalose may not directly participate in the plant metabolism, but may be involved in signal regulation, metabolic regulation, and gene expression regulation processes (Paul et al., 2008). The synthesis, decomposition, and regulation of trehalose are important components of the plant sugar signal transduction pathway (Chen et al., 2014a). Studies have found that trehalose has a significant effect on the protection of *Volvariella volvacea* under cold. Furthermore, a large number of studies have shown that in stress conditions such as freezing and refrigeration, trehalose can prevent protein denaturation and protect cells from damage (Wang et al., 2008). However, Veluthambi et al. suggested that too much trehalose can severely inhibit the growth of plants. This would be because the excessive amount of trehalose impairs sugar utilization and reduces phloem transport processes (Veluthambi et al., 1982). In this study, in the seedlings of *A. aphylla*, trehalose was found to be down-regulated under cold stress, which is similar to the results of previous studies (Maria et al., 2013). 1-kestose is a linear inulin-type fructan and fructan is the most widely distributed biopolymer in nature. The fructan metabolism is an important means for regulating environmental stress in plants and the accumulation of fructan in plants can enhance their stress resistance (Wu et al., 2011). Studies have shown that fructan is mainly distributed in plant cell vacuoles. Under cold or cold conditions, fructan in the vacuoles is de-polymerized and releases large amounts of free fructose, which can reduce the water freezing point in plant tissues, thus acting as a vacuole osmotic buffer and cold protective agent in plants (Wang, 2000). Scholars have reported that the winter wheat varieties with strong cold resistance have higher fructan contents; after the introduction of the levansucrase gene that synthesizes fructan into sugar beet and tomato, the obtained transgenic plants not only exhibited cold resistance, but also expressed functions of drought resistance, salt tolerance, and improved fruit quality (Tognetti et

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al., 1989; Sevenier et al., 1998; Wang et al., 2004). The contents of the metabolites sedoheptulose and trehalose detected in this study under cold conditions were 0.66 times and 0.80 times that of the contents under normal temperature, respectively. However, under cold, the content of 1-kestose was 3.32 times that of the content under normal temperature, indicating that cold stress inhibits the photosynthesis and carbohydrate metabolism in *A. aphylla* seedlings but does not affect the normal growth of plants. *A. aphylla* may adapt to a cold environment by accumulating 1-kestose or other carbohydrate metabolites.

Effects of cold on amino acid metabolites of A. aphylla seedlings

During the metabolic regulation process of responding to abiotic stress, the synthesis and decomposition of metabolites in plants are in a delicate dynamic state of equilibrium in order to swiftly respond to the changes of the external environment and to maintain the normal metabolism of the plant (Zhou et al., 2017). In addition to carbohydrate substances, the contents of amino acids in *A. aphylla* under cold stress also differed. As the basic components of the biological functional macromolecule proteins and the important nitrogen metabolites in plants, amino acids play important roles in the nitrogen metabolism and in plant stress resistance (Song et al., 2012). By comparing *A. aphylla* seedlings under cold treatment to the control under normal temperature, a large number of amino acids and amino acid metabolic intermediates revealed to be produced under cold stress. Among these, only few amino acids were up-regulated such as isoleucine, γ-aminobutyric acid, proline, cycloleucine, phenylalanine, and β- glutamic acid, while asparagines, L-homoserine, O-acetyl serine, methionine, 4-hydroxybutyrate, cysteic acid, threonine, aspartic acid, ornithine, histidine, citrulline, alanine, sarcosine, and intermediate metabolites of tryptophan, kynurenine, and xanthurenic acid were down-regulated.

γ-aminobutyric acid (GABA) is a non-protein amino acid found in many organisms and it is an important component of the free amino acid pool, which is widespread in every part of the plant (Yang et al., 2014). Under normal growth conditions, the GABA content is relatively low in plant tissue; however, under conditions of environment stress, physiological stress, or insect pest and other adverse conditions, the concentration of GABA will sharply increase. This reduces the damage of adverse conditions to plants, while enhancing plant resistance under stress through the stabilization of the cell membrane structure, reduction of activity oxygen damage, and regulation of biological macromolecule synthesis (Fait et al., 2008). Two major ways exist to synthesize and transform GABA in plants: glutamic acid decarboxylation and polyamine degradation. Glutamic acid metabolism plays an important role in plant growth, while GABA, as a glutamic acid enzymatic product, also plays an integral role in the life cycle of plants (Francisco et al., 2014). A study by Shelp et al. (2006) reported that plant endogenous GABA could stabilize and protect the thylakoid membrane against freeze damage *in vitro*, while exogenous GABA treatment could reduce the occurrence of cold damage and reduce the damage to plants. Proline is one of the components of plant proteins and it is widely distributed in plants in a free state. When the ambient temperature is lowered, the accumulation of proline plays an important role for protecting the stability of the cytoplasmic membrane of plants and for scavenging free radical damage (Matysik et al., 2002). Therefore, many researchers regard the content of proline in plants as one of the indexes to determine the cold hardiness of plants. It is generally assumed that the accumulation of proline under stress

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may be a great help for plants to recover after stress relief since proline can be used as both carbon source and nitrogen source, and it can also provide a large amount of energy for cell recovery (Sun et al., 2017). Many studies have shown that under cold, proline contents in plants such as *Festuca arundinacea*, tea tree, *Cinnamomum camphora*, and *Hibiscus syriacusplants* showed a significant increase compared to control groups, indicating that plants can adjust their proline content in response to adverse environmental conditions of cold (Li et al., 2016; Yang et al., 2004; Wen, 2017; Li and Zou, 2016).

The ornithine cycle in plants plays an important role in their adaptation to cold stress. Except for a few free amino acids that are increased during the accumulation, the majority of the amino acids were down-regulated under cold stress. Aspartic acid is the precursor of the biosynthesis of amino acids such as threonine, isoleucine, and methionine, as well as purine and pyrimidine. Ornithine, citrulline, and aspartic acid participate in the *in vivo* biosynthesis process of ornithine in plants. During this process, arginine is hydrolyzed into ornithine and urea via arginase; the carbamoyl group on the carbamyl phosphate is transferred onto ornithine, thus forming citrulline by ornithine transcarbamylase. Citrulline is then transported from the mitochondria to the cytoplasm and is condensed with aspartic acid to form argininosuccinic acid. Argininosuccinic acid is decomposed into arginine via argininosuccinase, releasing fumaric acid. The fumaric acid that is generated from the ornithine cycle not only connects the ornithine cycle and the citric acid cycle, it can also be decomposed by urease to produce ammonia, which can be used in the synthesis of other nitrogen-containing compounds such as nucleic acids, hormones, chloroplast, heme, amine, and alkaloids (He et al., 2014). The results of this study showed that cold stress significantly affected the amino acid metabolism, and weakened the ornithine cycle process, while differential metabolites such as isoleucine, β-glutamic acid, phenylalanine, cycloleucine, γ-aminobutyric acid, and proline were significantly up-regulated, indicating that *A. aphylla* seedlings had adapted to the cold environment via accumulation of these amino acids. The reason for the decreases in the contents of the majority of amino acids might be due to an inhibition of the metabolism under cold, resulting in amino acid decomposition.

Effects of cold on organic acid metabolites of A. aphylla seedlings

Cold stress induced the accumulation of a large quantity of organic acids in *A. aphylla* seedlings. The content of organic acids directly reflects plant growth and metabolic activity (Zhao et al., 2013). Malonic acid, also known as maleic acid, is a succinic acid analogue that can be used as a competitive inhibitor of succinate dehydrogenase, thus inhibiting the corresponding respiratory process (Ye et al., 2012). In our study, the content of malonic acid in *A. aphylla* seedlings under cold stress was increased by 2.35 times compared to the control group. This indicates that the respiration of *A. aphylla* seedlings was decreased and the metabolic activity was reduced. Some studies have also reported that plant respiration was inhibited under the treatment of high concentration of malonic acid. At the same time, the consumption of soluble solids was decreased, enabling plants to slow their growth under stress to survive the hazards caused by an adverse environment (Guo et al., 2016).

Abiotic stress induces the production of large quantities of reactive oxygen species in plants, which may activate proteases and induce excessive production of NH_4^+ in cells. To avoid surplus of NH_4^+, plants usually convert excessive NH_4^+ into glutamic

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acid/glutamine via transamination or convert it into α-ketoglutaric acid via glutamate dehydrogenase, entering the TCA cycle (Dai et al., 2010). Our results indicate that compared to the control group, the content of α-ketoglutaric acid was significantly increased under cold stress in the TCA cycle metabolism, indicating that the cold does not inhibit the TCA cycle, and that the energy metabolism was not compromised. Since the TCA cycle is the hub of communication among the metabolisms of carbohydrates, lipids, and amino acids, cold stress cannot only affect carbohydrate, lipid, and protein metabolisms, but can also directly affect the synthesis of several amino acids. Among the identified 15 organic acids, the relationships between many of these organic acids and plant stress resistance remain unknown. Tartaric acid is a dicarboxylic acid, and it exists in plants such as grapes, bananas, and tamarind, and is commonly used as antioxidant synergist, retarder, and chelating agent. Some studies suggest that tartaric acid is a good antidote, which is important for the germination and growth of plant seeds under heavy metal stress (Chen et al., 2014b). Some of the organic acids such as 4-hydroxybutyrate, mucic acid, glutaric acid, gallic acid, and 3-hydroxypropionic acid are also commonly used for chemical production, medicine and health care, food processing, and in research of separation and extraction technologies. The applications and significances of these organic acids in plants still require further exploration.

Conclusion

Gas chromatography-mass spectrometry technology was used in this study to analyze the metabonomic changes in *A. aphylla* seedlings under cold stress. A total of 116 differential metabolites were identified, including organic acids, amino acids, carbohydrates, alkaloids, auxins, organic bases, quinone compounds, flavonoid compounds, and other substances. In this study, most of the differential metabolites found in *A. aphylla* seedlings under cold stress showed a down-regulated trend. Through the analysis of carbohydrates such as sedoheptulose and trehalose, various amino acids, and the organic acid malonic acid, it was found that photosynthesis, respiration, carbohydrate metabolism and ornithine cycle of *A. aphylla* seedlings were all inhibited under cold stress; however, at the same time, *A. aphylla* seedlings were able to lower the extent of damage caused by adversity via accumulation of metabolites such as γ-aminobutyric acid, α-ketoglutaric acid, and 1-kestose. The results of this study provide a foundation for further analysis of the molecular and physiological mechanisms of the response of *A. aphylla* to cold stress.

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MORPHO-PHYSIOLOGICAL RESPONSE OF HYBRID MAIZE (*Zea mays* L.) TO SALT STRESS IN COMBINATION WITH EXOGENOUS CALCIUM APPLICATIONS

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Abstract. Maize, the third most important cereal crop around the globe, is hampered by devastating abiotic factors including salt stress. Calcium is known to increase plant tolerance to biotic and abiotic stresses by influencing physiological processes. The current study was carried out to investigate the genetic variation of maize hybrids against salt stress in combination with exogenous application of calcium (Ca^{2+}) in a hydroponic culture. Three levels of salinity, each with NaCl (0 mM, 75 mM, and 150 mM) and Ca^{2+} (0 mM, 2.5 mM, 5 mM) were applied to maize hybrids in three replications and data were recorded for key morpho-physiological traits. The results showed that all concentrations of NaCl significantly affect the maize hybrids with Sarhad White Azad Jammu Kashmir (SWAJ 6,7) and Syngenta 8441 being the least affected by both 75 mM and 150 mM NaCl concentrations. The exogenous application of 2.5 mM Ca^{2+} with 75 mM salt concentration showed significant effects on all morpho-physiological traits of maize hybrids. Better growth performance, maintenance of nutrient contents, lower accumulation of toxic sodium ions and lower Na^+/ K^+ in SWAJ6,7 and Syngenta 8441 indicate that these maize hybrids are more tolerant to salinity stress than the others under study.
Keywords: *hydroponic, ion contents, Zea mays* L.), *NaCl, proline*

Introduction

Soil salinity is one of the key environmental constraints that limit the production of crop throughout the world (Jiang et al., 2018). In arid and semiarid regions, the soil salinity more severely affects crop production due to global climate changes and as consequences of irrigation practices (Farooq et al., 2015). In Pakistan around 6.8 million hectares of land is affected by salinity. Therefore, major agricultural area remains uncultivated (Chaudhary et al., 2017). Saline soils have high pH and electrical conductivity (EC > 4.0 dS/m) and are usually rich in sodium, chloride and sulfate ions, with high absorption rate of sodium (Flowers and Flowers, 2005). In the rhizosphere, osmotic pressure due to high saline condition reduces mineral and water uptake by the plant, which ultimately affect primary and secondary metabolism of the plant (Hendawy and Khalid, 2005). The cumulative response of meristematic and expanding cells to salt stress is the inhibition of shoot growth which is observed primarily under salinity stress (Shoresh et al., 2011). Salt stress causes various morphological and metabolic modifications in plants by creating osmotic stress and ionic imbalance thus disrupts the vital cellular functions (Taylor et al., 2004; Yildirim et al., 2006). Osmotic stress leads to the reduced water absorption by roots and increase water loss from leaves. This

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mechanism is called hyperosmotic stress and it leads to various physiological modifications in plants such as nutrient imbalance, decreased photosynthetic activity, decreased stomatal apertures, membranes interruptions, modifications in antioxidant enzymes and failure to detoxify reactive oxygen species (ROS) (Soliman and El-Shaieny, 2014). The formation of ROS due to salt stress interrupts vital cellular functions by causing damage to various cell components such as DNA, protein and lipids (Gupta and Huang, 2014). Plant tissues exposed to saline soil accumulate more Na^+ and Cl^- which is one of the most detrimental effect as it causes severe ionic imbalance in plants by decreasing uptake of important mineral ions which are essential for the growth and development of plant (James et al., 2011). Reduced water permeability and aquaporin abundance of membrane under salinity stress is restored by calcium treatment (Martínez-Ballesta et al., 2008).

Calcium, an essential plant nutrient is responsible for the maintenance of structure and functions of plant membranes. It also increases the stability of cell wall structures, selectivity and transport of ions and improves the metabolic activities in plants. Calcium as second messenger plays a crucial role in signal transduction in response to developmental and environmental signals (Guo et al., 2019). Almost half of the cellular Ca^{2+} is bound mainly to carboxyl groups of pectins in the cell wall (Cramer, 2002). Previous studies indicated that salt induced inhibition of growth is improved by high content of Ca^{2+} bound to pectin on cell wall (Madea et al., 2005). Increased Na^+ ions due salinity stress reduces calcium availability and its transport to growing regions of the plant (Shoresh et al., 2011). The reduction of Ca^{2+} concentration in salinized plants severely effects the physiological function. The addition of supplemental Ca^{2+} provide protection by stabilizing on the cell-wall components (Tuna et al., 2007).

Maize (*Zea mays* L.), an important cereal crop is grown under wide range of soil and climatic conditions. It is an important C4 plant belongs to the grass family Poaceae (Farooq et al., 2015). About 21% of the total maize grain produced is consumed as food throughout the world (Chaudhary et al., 2017). The larger producer of maize is the United States with annual yield of about 4096190 thousand million tons. In Pakistan average yield of maize is 4268 kg/ha (Economic Survey of Pakistan, 2012-13). Various biotic and abiotic stresses extremely affect the yield potential of maize crop. Among abiotic stresses, salinity reduces the maize yields by causing numerous biochemical and physiological changes in plants (Zafar-ul-Hye et al., 2014). Maize is found to be moderately sensitive to salt stress. However, extensive genetic variation exists intra-specifically in maize for salt resistance (Mansour et al., 2005). The current study was aimed to explore the genetic variation of five different maize hybrids against salt stress in combination with exogenous application of Ca^{2+}.

Materials and Methods

In order to investigate the influence of exogenously applied $CaSO_4$ as calcium source on five maize hybrids under salt stress, an experiment was conducted in 2018 under hydroponic conditions in the greenhouse of COMSATS University Islamabad, Abbottabad Campus, Pakistan. Maize hybrids used in current study were:
COMSATS Random Mating 8 (CTRM 8), Pirsabak Experimental Variety 2 (PSEV 2), Sarhad White Azad Jammu Kashmir 6, 7 (SWAJ 6, 7) and Syngenta 8441.

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Plant growth and experimental treatments

The seeds of five maize hybrids (CTRM 8, PSEV 2, SWAJ 6-7, Syngenta 8441 and XPW3) were sterilized with 1% HOCl for 10 minutes and rinsed with distilled water, the process being repeated 3 times. The seeds were germinated on moist thin sheets of foam at 25°C in an incubator. After germination, uniform sized seedlings were transferred in constantly aerated pots containing 1/3rd strength Hoagland's solution. Plants were adapted to full strength nutrient solution in 25% increments every second day in similar way as described by Richter et al. (2015). The experiment was carried out in a completely randomized design (CRD). Plants were subjected to different concentrations of NaCl along with application of Ca^{2+} as foliar at four leaf stage. Seven different treatment combinations with three replicates per treatment were used. Treatment combinations were as follow:

T1: Control (Hoagland solution alone); T2: Salt Stress (75 mM NaCl); T3: Salt stress and foliar (75 mM NaCl + 2.5 mM Ca^{2+}); T4: Salt stress and foliar (75 mM NaCl + 5 mM Ca^{2+}); T5: Salt Stress (150 mM NaCl); T6: Salt stress and foliar (150 mM NaCl + 2.5 mM Ca^{2+}); T7: Salt stress and foliar (150 mM NaCl + 5 mM Ca^{2+}).

Growth parameters

Growth parameters were scored at seventh day of treatments application by measuring leaf number, leaf area, shoot length and maximum root length. Fresh weights of plants were taken immediately after harvesting. For dry weight, plant organs were placed in an incubator at 75℃ for 72 hrs (Sun et al., 2018). Leaf area was calculated according to *Equation 1*:

$$A = LL * W * K \quad \text{(Eq.1)}$$

where,
LL represents leaf length,
W is the maximum width and
K is a shape factor with value 0.75.

Chlorophyll content

Chlorophyll extraction was done by grinding 0.1 g of fresh leaves in 15 ml of acetone (80%). This mixture was then subjected to centrifugation at 5000 rpm for 10 minutes at 4°C. Supernatant from each sample was collected until the residue was turned into colorless mixture. The chlorophyll content was measured using spectrophotometer at wavelength of 645 nm and 663 nm (Nayyar et al., 2005).

Relative water content

Relative Water content (RWC) was determined according to *Equation 2* as described by Loutfy et al. (2012).

$$RWC(\%) = [(FW - DW)/(TW - DW)] * 100 \quad \text{(Eq.2)}$$

where,
FW represents fresh weight,
TW is Turgid weight and
DW is Dry weight.

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Electrolyte leakage

For determination of electrolyte leakage (EL), 10 leaf discs of about 10 mm diameter from expanded leaves of all maize hybrids were taken and rinsed with distilled water to get rid of electrolytes adhered to the surface. These leaf discs were incubated at 10°C after placing them in glass tubes filled with 10 ml of distilled water for 24 hr. The initial electrical conductivity (EC1) of the solution was measured using conductometer. To release all electrolytes, these tubes were heated at 95°C in water bath for 20 min followed by cooling at room temperature. After that final electrical conductivity (EC2) was determined.

EL was calculated using *Equation 3* (Yildirim et al., 2009).

$$EL = \left(\frac{EC1}{EC2}\right) * 100 \quad \text{(Eq.3)}$$

Plant mineral ion contents

For determination of mineral ions, 100 mg of dried plant samples (roots and leaves) were heated for about 5 hrs in furnace at 520°C for ash formation. The ash of each plant sample was then solubilized in nitric-perchloric acid mixture (5:1) and volume was raised to 15 ml using distilled water. Afterwards filtered suspensions were used to determine the concentrations of Na^+, K^+, Ca^{+2} and Mg^{+2} using atomic absorption spectrometry. For standard curves, different concentrations of Ca^{+2}, K^+, Na^+ and Mg^{+2} were prepared by diluting stock solution of $CaSO_4$, KCl, NaCl and $MgSO_4$. The standard curve was used to determine the content of each element in the plant organs (roots and leaf) and expressed in mg g^{-1} dw (Qadir, 2016).

Proline contents

Leaf proline content was extracted and analyzed by the method of Bates et al. (1973). 0.1 g of fresh leaf of all maize hybrids were ground using liquid nitrogen in a mortar. 1 ml of sulfosalicylic acid (3% w/v) was added to the resulted homogenate powder of leaves and then filtered. The filtrate was reacted with 1 ml of glacial acetic acid (GAA) and 1 ml of ninhydrin reagent (1.25 mg Ninhydrin in 30 ml of GAA and 20 ml 6 M H_3PO_4) and placed in an incubater for 1 hr at 95°C. To terminate the reaction, reaction mixture containing tubes were kept in ice bath for 5 min followed by vigorous mixing with 2 ml toluene. After warming at 25°C, the chromophore was measured at 520 nm. L-proline was used as a standard.

Statistical analysis

For statistical analysis, results of all experiments were expressed as mean ±standard deviation (SD) of three replicates. Data were subjected to one-way analysis of variance (ANOVA) and LSD multiple comparison test ($P < 0.05$) using the SPSS statistical package. Significant differences are indicated by alphabets in figures and tables.

Results

Growth parameters of maize under salt stress and Ca^{2+} treatments

The growth parameters of maize seedlings were evaluated by measuring leaf area, root length and shoot length of plants under salt stress. Effect of salt stress and calcium on

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growth parameters of five maize hybrids (CTRM8, PSEV2, SWAJ 6, 7, Syngenta 8441 and XPW3) are listed in *Table 1*. Under salt stress all five Maize hybrids react differently. Salt addition showed significant effect on growth parameters like leaf area, root length and shoot length of plants. The maximum decrease in these parameters was observed in CTRM 8 and XPW 3 whereas maximum shoot length, maximum root length and leaf area was exhibited by SWAJ 6, 7 and Syngenta 8441 which showed improved growth traits. However, effects of salinity were significantly alleviated in all five maize hybrids by foliar application of 2.5 mM Ca^{2+}. Although application of 5 mM Ca^{2+} also improved plant growth but the effect was not significant as compare to 2.5 mM Ca^{2+} (*Table 1*).

Table 1. *Shoot length (cm), Root length (cm), and Leaf area (m^2) concentration in leaves of all five Maize hybrids in response to salinity and Ca^{2+} concentrations*

Maize hybrid	Treatment	Shoot length	Root length	Leaf area
CTRM8	T1	31.00 CD	21.33 G	21.85 IJ
	T2	22.68 I	17.00 IH	13.79 L
	T3	32.50 C	19.83 H	27.55 D
	T4	30.33 D	15.67 I	25.72 F
	T5	15.68 KL	13.33 J	13.34 L
	T6	30.17 D	17.17 IH	24.04 FG
	T7	24.00 G	11.83	17.23 K
PSEV2	T1	33.50 BC	18.17 H	24.38 FG
	T2	22.67H	16.17 I	18.55 IK
	T3	33.33 BC	21.50 G	26.58 E
	T4	30.00 E	18.33 H	25.09 F
	T5	18.50 K	12.67 J	18.77 IK
	T6	32.00 D	16.17 I	25.96 EF
	T7	27.00 EF	8.17 M	18.19 IK
SWAJ 6, 7	T1	34.67 C	33.50 A	26.46 E
	T2	28.17 F	16.83 I	23.04 G
	T3	33.17 BC	32.50 B	31.64 B
	T4	31.00 CD	25.67 E	26.83 E
	T5	20.50 J	17.67 H	18.86 IK
	T6	33.17 BC	27.00 D	25.24 F
	T7	31.67 D	16.17 I	25.08 F
Syngenta 8441	T1	38.17 A	33.83 A	28.88 CD
	T2	27.00EF	25.57 E	26.06 E
	T3	37.00 AB	32.67 B	33.26 A
	T4	33.50 BC	23.33 F	30.14 BC
	T5	23.83 GH	26.67 DE	23.63 G
	T6	33.67 CD	21.00 G	29.67 C
	T7	36.17 B	28.83 C	27.75 D
XPW3	T1	27.00 EF	26.50 DE	21.85 H
	T2	24.83 H	14.50	17.08 K
	T3	29.33 DE	18.67 H	22.74 GH
	T4	25.33 FG	14.17 K	22.35 GH
	T5	15.83 KL	13.00 K	14.93 KL
	T6	26.67 G	14.33 K	20.13 IJ
	T7	24.17 H	12.67 L	19.55 I

Mean values followed by different letters within each column differ significantly at $P \leq 0.05$. T1: Control (non- saline); T2: 75 mM NaCl; T3: 75 mM NaCl + 2.5 mM Ca^{2+}; T4: 75 mM NaCl + 5 mM Ca^{2+}; T5: 150 mM NaCl; T6: 150 mM NaCl + 2.5 mM Ca^{2+}; T7: 150 mM NaCl + 2.5 mM Ca^{2+}

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Leaf chlorophyll content in maize under salt stress and Ca^{2+} treatments

Salt stress showed significant effect on total leaf chlorophyll contents of maize hybrids. Increase in salt stress resulted decrease in chlorophyll contents of maize leaves. It is found from the results that the high levels of salinity (150 mM NaCl) induced reduction in the total chlorophyll contents of leaves significantly in comparison to leaves of control plants. However, Ca^{2+} application on plants under salt stress increased the leaf chlorophyll contents (*Table 2*). Importantly, 2.5 mM Ca^{2+} treatments showed significantly higher total chlorophyll contents compared to that of 5 mM Ca^{2+} treatments.

Table 2. *Total leaf chlorophyll contents (mg g^{-1} FW), RWC (%), and Electrolyte leakage (%) concentration in leaves of all five Maize hybrids in response to salinity and Ca^{2+} concentrations*

Maize hybrid	Treatment	Total chlorophyll (mg g^{-1} FW)	Relative water (%)	Electrolyte leakage (%)
CTRM8	T1	15.19 C	77.09 I	22.61 R
	T2	9.42 F	72.32 J	61.67 D
	T3	13.58 D	86.60 D	33.93 N
	T4	11.80 E	80.56 H	29.61 P
	T5	6.79 G	60.14	68.63 A
	T6	10.88 EF	78.73 I	65.26 B
	T7	10.35 EF	59.60 N	63.15 C
PSEV2	T1	17.73 B	84.34 E	25.33 Q
	T2	12.01 DE	70.14 K	58.54 E
	T3	15.79 C	96.34 A	36.33 M
	T4	13.55 D	79.25 H	32.33 N
	T5	8.51 F	61.26 M	63.33 C
	T6	13.43 D	77.16 I	61.33 D
	T7	14.06 CD	65.67 L	59.33 E
SWAJ 6, 7	T1	19.38 A	90.85 C	20.00 S
	T2	13.16 D	73.05 J	46.39 K
	T3	16.26 BC	93.31 B	31.00 O
	T4	14.45 CD	85.85 E	27.53 Q
	T5	10.56 EF	61.81 M	56.27 F
	T6	15.10 C	81.84 G	49.38 I
	T7	13.59 D	72.01 J	46.05 K
Syngenta 8441	T1	19.60 A	93.68 B	19.32 S
	T2	13.68 D	80.27 H	45.21 L
	T3	16.63 BC	96.76 A	30.32 P
	T4	14.99 CD	86.05 D	26.42 Q
	T5	11.75 E	68.34 L	55.15 G
	T6	14.40 CD	81.66 G	48.00 J
	T7	15.40 C	67.06 L	45.00 L
XPW3	T1	17.46 B	85.25 D	23.08 R
	T2	10.51 EF	65.67 L	59.68 E
	T3	14.97 C	89.66 C	33.74 N
	T4	14.42 CD	85.29 D	30.26 P
	T5	6.53 G	49.77 P	69.83 A
	T6	9.93 F	83.82 F	51.43 H
	T7	8.38 F	54.29 O	59.80 E

Mean values followed by different letters within each column differ significantly at $P \leq 0.05$. T1: Control (non-saline); T2: 75 mM NaCl; T3: 75 mM NaCl + 2.5 mM Ca^{2+}; T4: 75 mM NaCl + 5mM Ca^{2+}; T5: 150 mM NaCl; T6: 150 mM NaCl + 2.5 mM Ca^{2+}; T7: 150 mM NaCl + 2.5 mM Ca^{2+}

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RWC of maize under salt stress and Ca^{2+} treatments

Salt stress significantly reduced RWC in all maize hybrids with more prominent effect in CTRM8 and XPW3 in comparison with the control conditions. Salt-induced water imbalance resulted in decrease RWC. However, foliar application of 2.5 mM and 5 mM Ca^{2+} on salt-stressed maize plants increased RWC significantly in comparison to plants under salt stress only (*Table 2*).

Electrolyte leakage under salt stress and Ca^{2+} treatments

The increasing salt concentrations enhanced EL value of all maize hybrids (*Table 2*). The highest EL was observed in CTRM 8 and XPW3 in comparison to SWAJ 6, 7 and Syngenta 8441 under all stress conditions. However, supplemented Ca^{2+} reduced MP of salt stressed plants compared to plants without Ca^{2+} under salt stress. The application of Ca^{2+} significantly improved MP by decreasing the electrolyte leakage under salt stress.

Na, K^+, Ca and Mg contents of leaves and roots under salt stress and Ca^{2+} treatments

Salinity has very clear effects on ionic composition of maize. Maize genotype SWAJ 6, 7 and Syngenta 8441 showed better chemical attributes at all levels of salt stress. In all five maize hybrids, Na^+ accumulation in leaves and roots was proportional to the NaCl concentration in the medium applied exogenously. Significant increase of Na^+ levels was found in roots and leaves of all maize hybrids under treatments. This increase was more obvious in the leaves of XPW 3 than that in SWAJ 6, 7 and Syngenta 8441 hybrids (*Fig. 1a*). The highest concentration of Na^+ were observed in the roots of SWAJ 6, 7 and Syngenta 8441 at 150 mM NaCl treatment in combination with 2.5 and 5 mM foliar Ca^{2+} applications (*Fig. 1b*). Sodium accumulation in roots and leaves of maize hybrids under combined treatment of NaCl + Ca^{2+} was found less than sodium in plants under treatment of NaCl alone. Ca^{2+} application thus alleviates the hyperaccumulation of Na^+ ions in plants under salt stress (*Fig. 1a,b*).

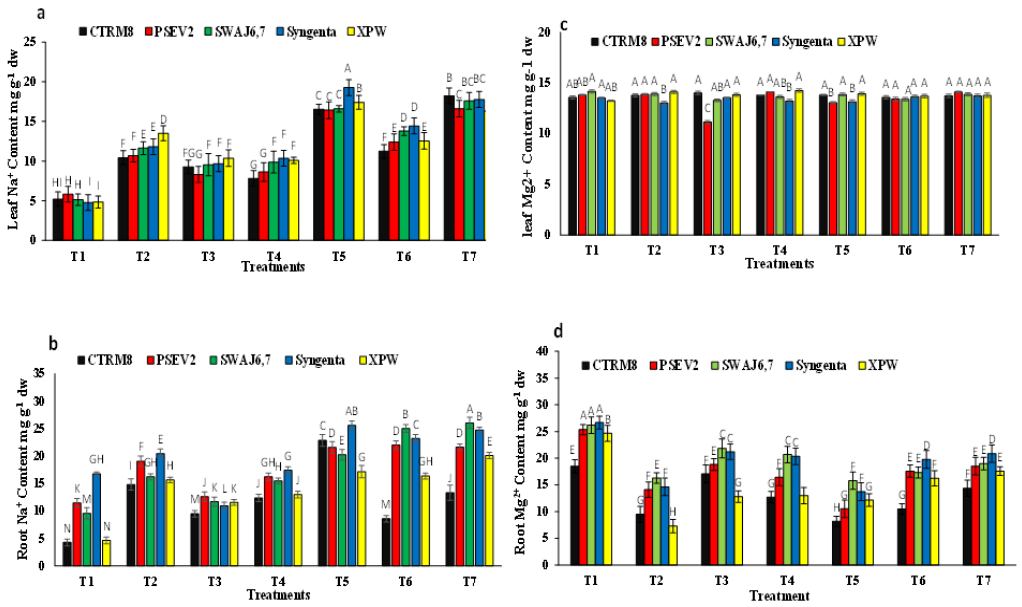

Figure 1. *The Na concentration of leaf (a) and root (b) Mg concentration of leaf (c) and root (d) of five maize hybrids (CTRM8, PSEV2, SWAJ 6, 7, Syngenta 8441, XPE3) in response to salinity and Ca^{2+} supplement. Values are means ± SE of three replicates*

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Mg^{2+} content of leaf showed no significant effect under salt stress and foliar application of Ca^{2+} under salinity did not change the Mg^{2+} contents in leaf (*Fig. 1c*). However, salinity showed negative effect on root Mg^{2+} contents. Foliar Ca^{2+} application enhanced concentration of Mg in roots of all maize hybrids under salt stress (*Fig. 1d*).

Salt stress had induced noticeable variations in Ca^{2+} and K^+ contents in leaves and roots of all maize hybrids. In comparison to that of control plants, all five maize hybrids showed decline in K^+ and Ca^{2+} contents of roots and leaves under salt stress. This decrease in K^+ and Ca^{2+} was more pronounced in CTRM8 and XPW3 as compared to SWAJ 6, 7 and Syngenta 8441 maize hybrids. Supplemental Ca^{2+} enhanced the K^+ and Ca^{2+} concentrations of leaves and roots of maize plants under salt stress. At all salinity levels, maintenance of high level of K^+ was better in SWAJ 6, 7 and Syngenta 8441 in comparison to CTRM8 and XPW3 (*Fig. 2*).

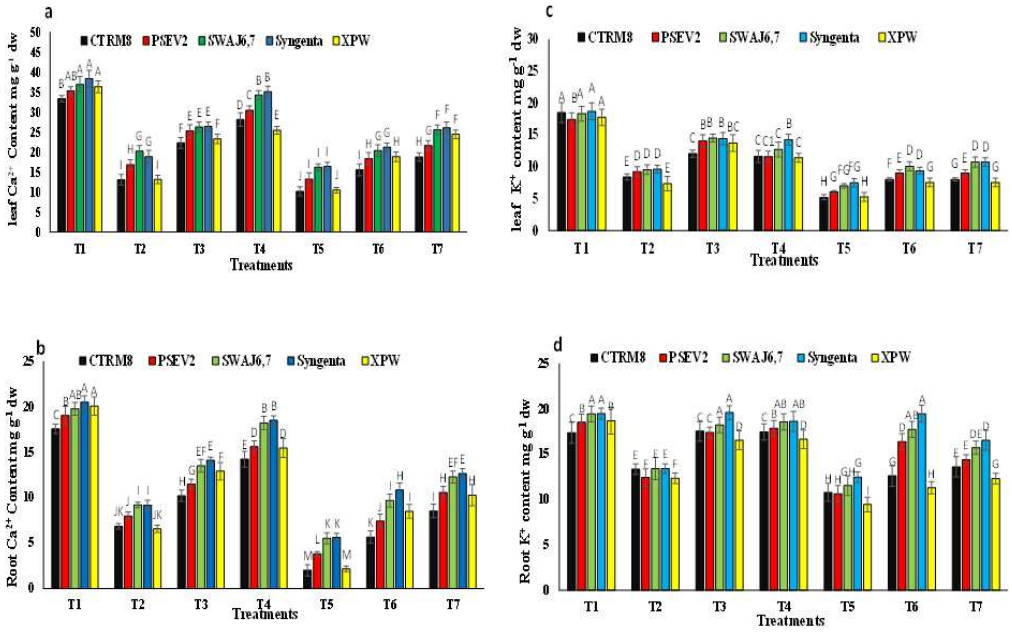

Figure 2. *The Ca concentration of leaf (a) and root (b) K concentration of leaf (c) and root (d) of five maize hybrids (CTRM8, PSEV2, SWAJ 6, 7, Syngenta 8441, XPE3) in response to salinity and Ca^{2+} supplement. Values are means ± SE of three replicates*

For all five maize hybrids, Na^+/K^+ ratio increased dramatically under salt stress with more notable effect in leaves of CTRM8 and XPW3 (*Fig. 3*). Supplemental Ca^{2+} resulted significant decline in Na^+/K^+ ratio of maize leaves under salt stress.

Proline contents of maize leaves under salt stress and Ca^{2+} treatments

Analysis of proline contents of maize leaves indicated that application of salt resulted in increased proline contents in all maize hybrids with more pronounced effect in CTRM8 and XPW3 as compare to SWAJ 6, 7 and Syngenta 8441. Under salt stress, proline contents in maize enhanced significantly (*Fig. 4*). However, proline concentration was markedly decreased in plants with exogenous application of 2.5 mM and 5mM concentrations of Ca^{2+} under salt stress. The less proline contents were found in plants treated with 2.5 mM Ca^{2+}.

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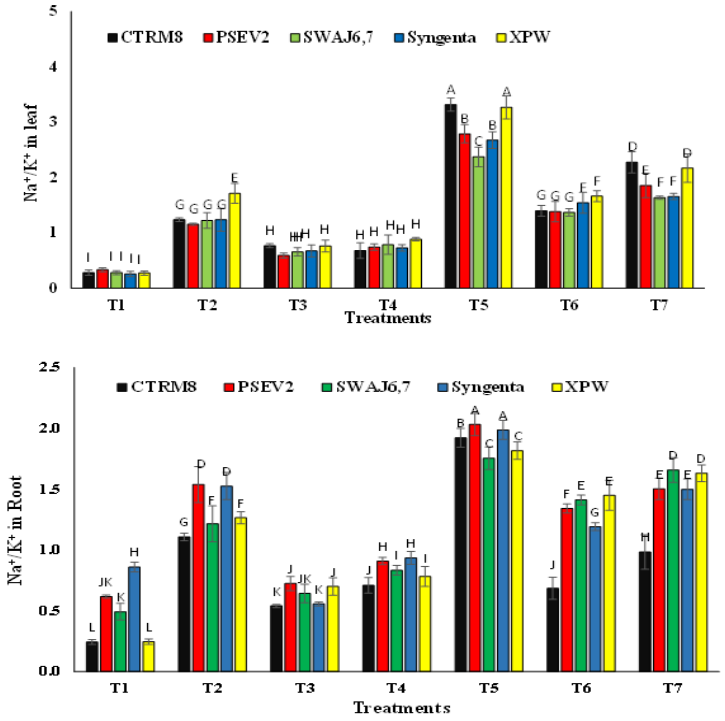

Figure 3. *The Na/K Ratio in roots (a) and leaf (b) of leaf of five maize hybrids (CTRM8, PSEV2, SWAJ 6, 7, Syngenta 8441, XPE3) in response to salinity and Ca^{2+} supplement. Values are means ± SE of three replicates*

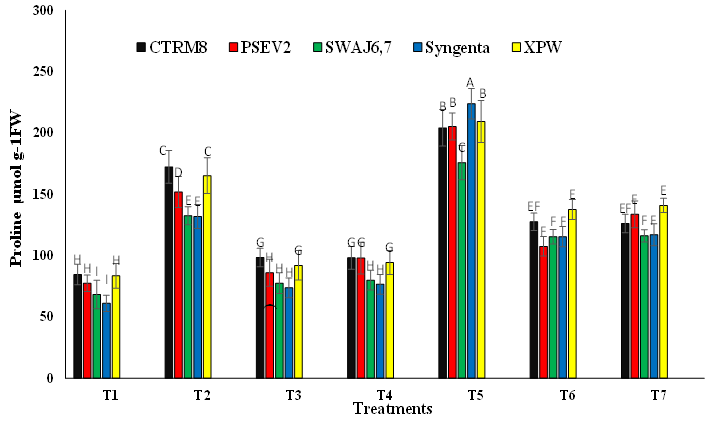

Figure 4. *Proline content of of five maize hybrids (CTRM8, PSEV2, SWAJ 6, 7, Syngenta 8441, XPE3) in response to salinity and Ca^{2+} supplement. Values are means ± SE of three replicates*

Discussion

The current study was carried out to investigate the genetic variation of five maize hybrids against salt stress in combination with exogenous application of calcium (Ca^{2+}). The decrease in plant growth parameters such as shoot length, root length, leaf area, plant weight (fresh and dry) due to salt stress, confirming many prior findings (Mulholland, 2002; Yurtseven et al., 2003; Agong et al., 2004; Ahmad, 2012). Excessive Na^+ accumulation in presence of NaCl resulted in nutritional and metabolic imbalances due

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to ion toxicity which led to reduction in growth parameters of maize hybrids. It was reported that higher accumulation of Na^+ ions is the main cause of decreased plant growth and metabolism (Zhu, 2002). The genotypes with less concentration of Na^+ produced more biomass as confirmed by Munns and James (2003).

Ca^{2+} ion shows ameliorating effect on growth parameters of plants under salt stress may be by competing with Na^+ ion for binding with membranes. Ca^{2+} application minimized the harmful effects of NaCl and enhanced plant growth of all five maize hybrids. Previous studies also reported that exogenous Ca^{2+} application increased plant growth and tolerance to salt stress in *Cassia angustifolia* (Senna) (Arshi et al., 2005), *Eleusine coracana* (finger millet), *Thinopyrum ponticum* (wheatgrass) and *Lolium perenne* (ryegrass) (Sima et al., 2009), *Cakile maritime* (Sea Rocket) (Amor et al., 2010), *Zea mays* (Maize) (Shoresh et al., 2011), *Cunninghamia Lanceolata* (Chinese fir) (Liu et al., 2014), *Lycopersicon esculentum* (Tomato) (Parvin et al., 2015) and *Calligonum mongolicum* (Xu et al., 2017). Ameliorative effect of calcium by enhancing the vegetative growth and total dry weight of plants under salt stress has also been confirmed by Manivannan et al. (2007). Improved growth performance of SWAJ 6, 7 and Syngenta 8441 under salt stress indicate that these maize hybrids are more tolerant to salinity stress than CTRM8, PSEV2 and XPW3.

Salt stress showed significant effect on total leaf chlorophyll contents of maize hybrids. Increase in salt stress resulted decrease in chlorophyll contents of maize leaves. It is found from the results that the high levels of salinity (150 mM NaCl) induced reduction in the total chlorophyll contents of leaves significantly in comparison to leaves of control plants. Netondo et al. (2004), Amini and Ehsanpour (2006) and Naher (2014) also reported similar findings. Moreover, chlorophyll contents were less affected in SWAJ 6, 7 and Syngenta 8441 than CTRM8 and XPW3 indicated that they were able to maintain photosynthetic capacity under salt stress. However, Ca^{2+} application on plants under salt stress increased the leaf chlorophyll contents (*Table 2*). A range of plant species responses from positive to negative effects to supplemental Ca^{2+} have been described in previous studies (Cramer, 2002; Cabot et al., 2009). Nevertheless, total leaf chlorophyll significantly increased by supplemental Ca^{2+} under salt stress (*Table 2*). Similar responses were found in other plants such as *Cucumis sativus* L. (Cucumber), *Fragaria ananassa* (strawberry) and *Vigna radiate* (Mung bean) (Kaya and Higgs, 2002; Kaya et al., 2002; Manivannan et al., 2007). Importantly, 2.5 mM Ca^{2+} treatments showed significantly higher total chlorophyll contents compared to that of 5 mM Ca^{2+} treatments.

Reduction in RWC in salt stressed plant is a common phenomenon. In present study all five maize hybrids showed decreased RWC under salt stress with less significant effect under mild stress of 75 mM NaCl in Syngenta 8441 and SWAJ 6, 7. Similar water shortage induced by salt was observed in *Oryza sativa* and *Zea mays* affected with salt (Cicek and Cakirlar, 2002; Tuna et al., 2008). Plants supplemented with Ca^{2+} showed improved RWC may be due to water retention under salt stress. Similar results were reported by Tahjib-Ul-Arif et al., (2018).

Membrane permeability (MP) can be determined by measuring electrolyte leakage (EL). Plasma membranes are primarily affected by ion-specific salt injury (Mansour et al., 2005). Hence, for the identification of salt tolerant plants, determination of electrolyte leakage from plasma membranes is an important criterion (Ashraf and Ali, 2008). In present study, all maize hybrids showed increase in EL under salt stress which is an indication of membrane dysfunction. The increased permeability of cellular membranes for electrolytes and ions is the clear indication of membrane dysfunction caused by salt

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stress and it can be measured by the electrolytes and efflux (Qadir, 2016). The highest EL was observed in CTRM 8 and XPW3 in comparison to SWAJ 6, 7 and Syngenta 8441. Demidchik et al. (2014) stated that efflux of K^+ is mainly related to electrolyte leakage. However, supplemented Ca^{2+} reduced MP of salt stressed plants compared to plants without Ca^{2+} under salt stress. The application of Ca^{2+} significantly improved MP by decreasing the electrolyte leakage under salt stress.

Salinity has very clear effects on ionic composition of maize. Significant increase of Na^+ levels was found in roots and leaves of all maize hybrids under treatments. This increase was more obvious in the leaves of XPW 3 than that in SWAJ 6, 7 and Syngenta 8441 hybrids (*Fig. 1a*). The highest concentration of Na^+ were observed in the roots of SWAJ 6, 7 and Syngenta 8441 at 150 mM NaCl treatment (*Fig. 1b*). Similar response was also reported in other plants such as Malus species, *Myrtus Communis* L. (Myrtle), Citrus and *Pistacia vera* (Pistachio) (Liu et al., 2012; Acosta-Motos et al., 2015; Martínez-Alcántara et al., 2015; Rahneshan et al., 2018).

Salt stress had induced noticeable variations in Ca^{2+} and K^+ contents in leaves and roots of all maize hybrids. In comparison to that of control plants, all five maize hybrids showed decline in K^+ and Ca^{2+} contents of roots and leaves under salt stress. This decrease in K^+ and Ca^{2+} was more pronounced in CTRM8 and XPW3 as compared to SWAJ 6, 7 and Syngenta 8441 maize hybrids. Supplemental Ca^{2+} enhanced the K^+ and Ca^{2+} concentrations of leaves and roots of maize plants under salt stress, similar to findings in other plant species (Tuna et al., 2007; Nedjimi and Daoud, 2009; Cabot et al., 2009; Kwon et al., 2009). At all salinity levels, maintenance of high level of K^+ was better in SWAJ 6, 7 and Syngenta 8441 in comparison to CTRM8 and XPW3 (*Figure 2*).

Increase in salinity levels lead to increased uptake of Na^+ which dramatically increased Na^+/K^+ ratio in roots and leaves of all maize hybrids. For all five maize hybrids, Na^+/K^+ ratio increased dramatically under salt stress with more notable effect in leaves of CTRM8 and XPW3 (*Fig. 3*). Supplemental Ca^{2+} resulted significant decline in Na^+/K^+ ratio of maize leaves under salt stress. It is documented that in salt-stressed plants, Ca^{2+} sustains K^+/ Na^+ selectivity and K^+ transport at the plasma membrane. Exogenous Ca^{2+} can reduce K^+ loss and enhance its uptake in plants under salt stress by affecting different channels as demonstrated by several previous studies (Shabala and Newman, 2000; Maathuis and Sanders, 2001; Shabala et al., 2003, 2005, 2006). This indicates that Ca^{2+} amelioration of salt stress is a critical process in K^+ transport regulation that may induce growth in plants. Better chemical attributes of SWAJ 6, 7 and Syngenta 8441 at all levels of salt stress could be the reason of their tolerance to salt stress.

Proline is considered as a source of nitrogen and carbon for rapid recovery of plants under salt stress. It acts as free radical scavenger and not only adjust the osmotic pressure of plant but also found to involve in stabilization of membranes and some macromolecules in plant cells. Less accumulation of proline contents in salt stressed maize seedlings treated with calcium shows protective role of calcium in plants by reducing osmotic pressure caused by salt stress (Hoque et al., 2007). Therefore, plants accumulated less proline contents under salt stress with application of calcium. Accumulation of proline is the primary defense response in many plants under salt stress to adjust the osmotic potential as reported in various salt sensitive/ tolerant cultivars such as *Zea mays* (Maize), *Vigna radiate* (Mung bean), *Sesamum indicum* (sesame), *Setaria italica* L. (foxtail millet) and *Pistacia vera* (Pistachio) (Mansour et al., 2005; Misra and Gupta, 2005; Koca et al., 2007; Veeranagamallaiah et al., 2007; Chelli-Chaabouni et al., 2010). Some studies showed that under salt stress, tolerant

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cultivars accumulate more proline than the salt sensitive cultivars (Misra and Gupta, 2005; Rahneshan et al., 2018). However, proline accumulation is also controversial with reference to tolerance and osmotic adjustment in plants exposed to undesirable environmental conditions (Ghars et al., 2008) as indicated by some studies where more proline accumulated in salt sensitive cultivars than the salt tolerant cultivar (Claussen, 2005). In current study proline accumulation was significantly higher in maize hybrids evaluated as salt sensitive (CTRM8 and XPW3) than in salt tolerant (SWAJ 6, 7 and Syngenta 8441). These results suggest that loss of cell homeostasis is responsible for excessive proline accumulation in salt sensitive hybrids. Similar results have been reported in previous studies where salt sensitive genotype of *Sorghum bicolor* L. (Sorghum kaya) (Wheat Arta) (Akbari et al., 2016) accumualted more proline under salt stress. These findings indicate that more proline accumulation is due to loss of cell homoeostasis. Moreover, these results suggest that salt tolerance is not fully reflected by proline accumulation itself in plants; however, proline accumulation could be an indicator of stress.

Conclusion

The overall results indicate that the growth parameters of all five maize hybrids were affected significantly by different salt stresses. However, better growth performance, maintenance of nutrient contents, less accumulation of toxic sodium ions and lower Na^+/K^+ in SWAJ 6, 7 and Syngenta 8441 indicate that these maize hybrids are more tolerant to salinity stress than all others. It is also concluded that the foliar application of Ca^{2+} against salt stresses improved the growth parametrs of hybrids. Therefore, it can be concluded that the salt stress can be mitigated by the exogenous application of Ca^{2+} in crop plants. Further, the experiment might be repeated in different locations with different maize hybrids for making concrete recommendations.

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EFFECT OF POMEGRANATE EXTRACT GALLIC ACID ON THE PROLIFERATION OF PROSTATE CANCER CELLS BY PROMOTING THE EXPRESSION OF IGFBP7

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Abstract. Objective: Pomegranate extract gallic acid inhibits the proliferation of prostate cancer PC-3 cells by promoting IGFBP7 expression. Methods: PC-3 cells were divided into six groups: control group (NC), blank control group (BL), IGFBP7 overexpression group (IGFBP7), Pomegranate extract gallic acid group (5.0 μmol/L, 10.0 μmol/L, 20.0 μmol/L). Cell proliferation was detected by MTT assay; apoptosis of each group was analyzed by flow cytometry; the expression levels of IGFBP7, AKT and mTOR were assessed by Western blot. Results: The proliferative plural of PC-3 cells in IGFBP7 group and pomegranate gallic acid group were significantly lower than those in NC and BL groups ($P < 0.05$). The pomegranate gallic acid group showed dose- dependence. The results of Western blot analysis showed that IGFBP7 group and The levels of IGFBP7 in the pomegranate extract gallic acid group were significantly higher than those in the NC and BL groups ($P < 0.05$), while the levels of AKT and mTOR plural were significantly lower than those in the NC and BL groups ($P < 0.05$). Pomegranate extract gallic acid showed dose-effect further elaborated within each group. Conclusion: Pomegranate extract gallic acid can inhibit the expression level of AKT/mTOR protein by enhancing the expression level of IGFBP7, thereby inhibiting the AKT/mTOR signaling pathway, the result is an inhibitory effect on the proliferation of prostate cancer PC-3 cells.
Keywords: *pomegranate extract gallic acid, IGFBP7 protein, AKT/mTOR protein, PC-3 cell, prostate cancer*

Introduction

Pomegranates (*Punica granatum* L.) are widely distributed and are cultivated in large quantities in China, India and Asia, Africa, Europe along the Mediterranean Sea, and California, USA. Among them, the key production areas of Chinese pomegranates are mainly fruit production, including Lintong, Ganxian, and Sanyuan in Shanxi Province, Zaozhuang in Shandong Province, Suzhou, Nanjing, Xuzhou, Pi xian in Jiangsu Province, Mengzi, Qiaojia, Jianshui, Chenggong in Yunnan Province, Yecheng Pomegranate in Xinjiang, Huili in Sichuan, Huaiyuan in Anhui Province, Xiaoxian, Suixi, Chaoxian (now Chaohu area of Hefei City), etc., as a common fruit, has many biological uses in areas like edible, medicine and health care, especially against cancer, cardiovascular and cerebrovascular diseases, liver disease and inflammatory infection certain treatment and prevention effects. The effective components of pomegranate are mainly the polyphenols in its fruit, and the pomegranate extract gallic acid is one of the main phenolic substances. It had many biological activities such as antibacterial, anti-inflammatory, anti-oxidation, liver function protection and anti-tumor. Function (Feng,

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2014; Lee, 2017; Pang, 2017; Rasool, 2010). Recent studies have shown that polyphenols have a positive significance in inhibiting the proliferation and migration of tumor cells (Panth, 2017; Momtaz, 2017). At present, prostate cancer is the primary factor that endangers male health in the world. The incidence of males is the second in the world, and the incidence of developed countries is the first. It ranks among the highest rates of male genitourinary malignant tumors (Wang, 2019). In this study, the mechanism of action of pomegranate extract gallic acid on prostate cancer tumor cells was explored. Different concentrations of pomegranate extract gallic acid were used to treat prostate cancer PC-3 cells. This provides a theoretical basis for the development and application of pomegranate extract gallic acid.

Materials and methods

Cell line

The prostate cancer PC-3 cell line was purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences.

Experimental drugs and related reagents

Pomegranate extract gallic acid (GA) was purchased from Nanjing Surang Pharmaceutical Technology Development Co., Ltd., with a mass fraction of 99%(China); fetal bovine serum, DMEM high glucose medium, and trypsin were purchased from Sigma (USA); rabbit anti-human IGFBP7 was purchased from Abcam (UK); Annexin V/PE Apoptosis Detection Kit, MTT Cell Proliferation and Cytotoxicity Assay Kit from Shanghai Biyuntian Biotechnology Co., Ltd (China).

Experimental methods

PC-3 cells were divided into six groups: control group (NC), blank control group (BL), IGFBP7 overexpression group (IGFBP7), 5.0 μmol/L pomegranate extract gallic acid group (5.0 μmol/L GA), 10.0 μmol/L pomegranate extract gallic acid group (10.0 μmol/L GA), and 20.0 μmol/L pomegranate extract gallic acid group (20.0 μmol/L GA). The NC group was cultured in DMEM high-glucose medium; the BL group was transfected with blank plasmid in PC-3 cells and cultured in DMEM high glucose medium (Johnson, 2018; Zephania, 2019); IGFBP7 overexpression group was transfected with IGFBP7 plasmid on PC-3 cells. The cells were cultured in DMEM high glucose medium. The pomegranate extract gallic acid group was not transfected with PC-3 cells. The concentration of pomegranate extract gallic acid in the medium was 5.0 μmol/L, 10.0 μmol/L and 20.0 μmol/L, respectively. The culture medium of each group was incubated for 24 h in an incubator with 5.0% CO_2 and a temperature of 37 °C.

IGFBP7 transfected cells (Hu, 2017)

PC-3 cells were inoculated into 24-well plates, and the cells were observed to be in good condition after 48 h, that is, adherent growth, when the cell fusion rate reached 60% to 80%, prepared for transfection. Lipofectamine TM 2000 transfection kit (Invitrogen, USA), IGFBP7 and internal reference primers were designed and synthesized by Shanghai Shenggong Biotech Co., Ltd. to transfect PC-3 cells and stably express IGFBP7 gene.

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MTT assay to detect cell proliferation (Maleki, 2020)

Six groups of cells were taken, trypsinized, and inoculated into a 96-well plate to adjust the cell density to 1.5×10^4/well, and continue to incubate. The cultures were stopped at 12, 24 and 48 h, and 20 μL of MTT was added. The culture was continued for 4 h. 150 μL of dimethyl sulfoxide was added and shaken for 10 min. The OD value of each well was measured by a microplate reader at 570 nm.

Apoptosis detection apoptosis rate (Guo, 2016)

PC-3 cells in logarithmic growth phase were inoculated into 96-well plates for 24 h. The NC group, the BL group and the IGFBP7 transfection group were cultured in DMEM medium, and the treatment group was added with 5.0 μmol/L, 10.0 μmol/L and 20.0 μmol/L pomegranate gallic acid for 24 h, and the Annexin V/PE apoptosis detection kit was used. The cells were prepared in strict accordance with the instructions and the apoptosis rate of the above six groups of prostate cancer PC-3 cells was analyzed by BD FACSCanto II flow cytometry.

Western blot detection of total protein extracted from each group of cells (Wei, 2016)

The protein concentration was determined by the BCA method. After treatment with different concentrations of pomegranate gallic acid for 24 h, PC-3 cells were collected and washed twice with cold PBS. The resulting cells were then lysed on ice for 10 min. After centrifugation at 12000 × g for 10 min at 4 °C, the supernatant was transferred to a fresh tube and stored at -70 °C. The protein concentration was determined by using a BCA test kit, and 50 μg of each well was loaded, and the sample was separated by SDS-PAGE at a concentration of 12%, transferred, blocked, and added with a primary antibody at 4 °C overnight. Wash the membrane, add the secondary antibody, incubate for 1 h at room temperature, and wash the membrane with TBST. The band gradation value was determined by adding the illuminant using ImageJ software.

Statistical processing and analysis (Guo, 2016)

The results were processed and statistically analyzed using the SPSS19.0 statistical software package. The mean ± standard deviation ($\overline{X} \pm s$) was used. The pairwise comparison was performed by LSD-t test; the count data was expressed by the rate value. The comparison was performed using the χ 2 test. $P < 0.05$ indicates that the difference was statistically significant.

Results

PC-3 cell inhibition results

The results of six groups of cells after different treatments inhibited PC-3 cells. The IGFBP7 overexpression group and the three GA groups (5.0 μmol/L group, 10.0 μmol/L group and 20.0 μmol/L group) were significantly higher than the BL and NC group; and compared with the BL and NC groups, there are statistical differences between the three GA groups ($P < 0.05$), IGFBP7 overexpression group and the three GA groups showed obvious time-effect relationship and dose-effect relationship (see *Table 1*).

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Table 1. *Inhibition of PC-3 cells in six groups after different treatments ($\bar{x} \pm s$)*

Groups	Number of samples	12 h	24 h	48 h
		OD value	OD value	OD value
NC group	5	0.45 ± 0.02	0.56 ± 0.03	0.87 ± 0.03
BL group	5	0.46 ± 0.02	0.57 ± 0.03	0.85 ± 0.04
IGFBP7 transfection group	5	0.21 ± 0.03a,b	0.25 ± 0.08a,b	0.33 ± 0.08a,b
5.0 μmol/L group	5	0.54 ± 0.03a,b	0.59 ± 0.08a,b	0.69 ± 0.08a,b
10.0 μmol/L group	5	0.39 ± 0.02a,b,aa	0.45 ± 0.08a,b,aa	0.48 ± 0.08a,b,aa
20.0 μmol/L group	5	0.26 ± 0.04a,b,aaa	0.30 ± 0.05a,b,aaa	0.46 ± 0.05a,b,aaa

a: $P < 0.05$, the difference from the NC group was statistically significant
b: $P < 0.05$, the difference from the BL groups was statistically significant
aa: $P < 0.05$, the difference was statistically significant compared with the 5.0 μmol/L GA group
aaa: $P < 0.05$, the difference was statistically significant compared with the 10.0 μmol/L GA group

Apoptosis detection

Compared with the NC group (4.84 ± 0.45) and the BL group (5.16 ± 0.38), the apoptosis rates of PC-3 cells in the IGFBP7 overexpression group and the three GA groups (40.52 ± 1.76, 15.98 ± 1.89, 30.36 ± 1.7 and 39.04 ± 1.43) increased significantly. Compared with NC group and BL group, the difference between IGFBP7 overexpression group and the three GA treatment group was statistically significant ($P < 0.05$), and there was a significant dose-effect relationship between with the three GA groups. The result is shown in *Figure 1*.

Comparison of six groups of IGFBP7 protein levels

Western blot method was used to detect the effect of different treatments on the expression of IGFBP7 protein in PC-3 cells. Compared with NC and BL groups, the results showed that the expression levels of IGFBP7 protein by IGFBP7 overexpression group and the three GA groups (5.0 μmol/L group, 10.0 μmol/L group and 20.0 μmol/L group) was significantly increased; and compared with BL and NC groups, the three GA groups are statistical differences between groups ($P < 0.05$). The result is shown in *Figure 2*.

Comparison of six groups of AKT protein levels

Western blot method was used to detect the effect of different treatments on the expression of AKT protein in PC-3 cells. Compared with NC and BL groups, the results showed that the expression levels of AKT protein by IGFBP7 overexpression group and the three GA groups (5.0 μmol/L group, 10.0 μmol/L group and 20.0 μmol/L group) were significantly reduced. And compared with the BL and NC groups, the three GA groups are statistical differences between groups ($P < 0.05$). The result is shown in *Figure 3*.

Comparison of six groups of mTOR protein levels

Western blot method was used to detect the effect of different treatments on the expression of mTOR protein in PC-3 cells. Compared with NC and BL groups, the results showed that the expression levels of mTOR protein by IGFBP7 overexpression group and the three GA groups (5.0 μmol/L group, 10.0 μmol/L group and 20.0 μmol/L group) were significantly reduced. And compared with the BL and NC groups, the three GA groups are statistical differences between groups ($P < 0.05$). The result is shown in *Figure 4*.

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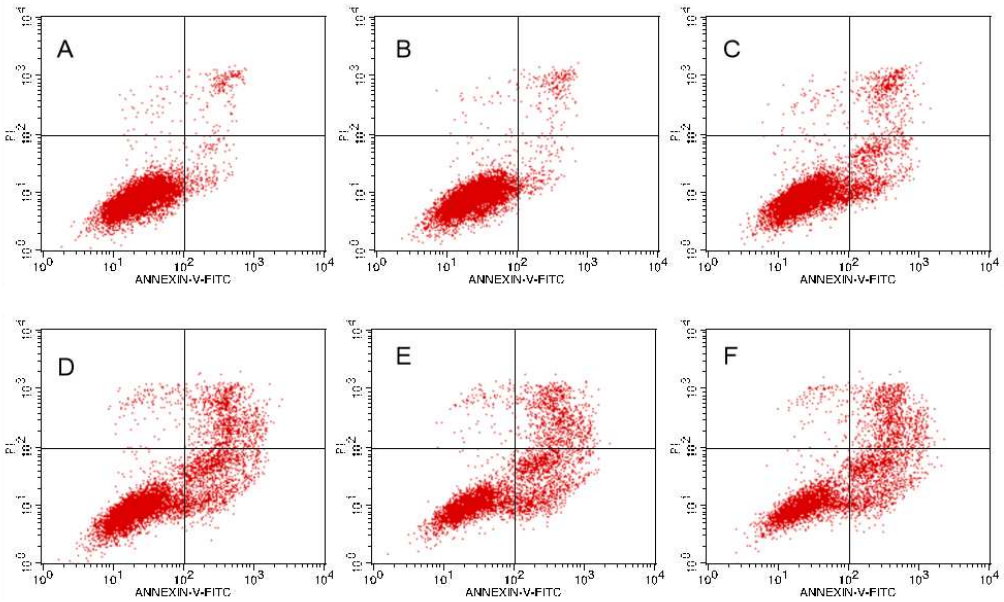
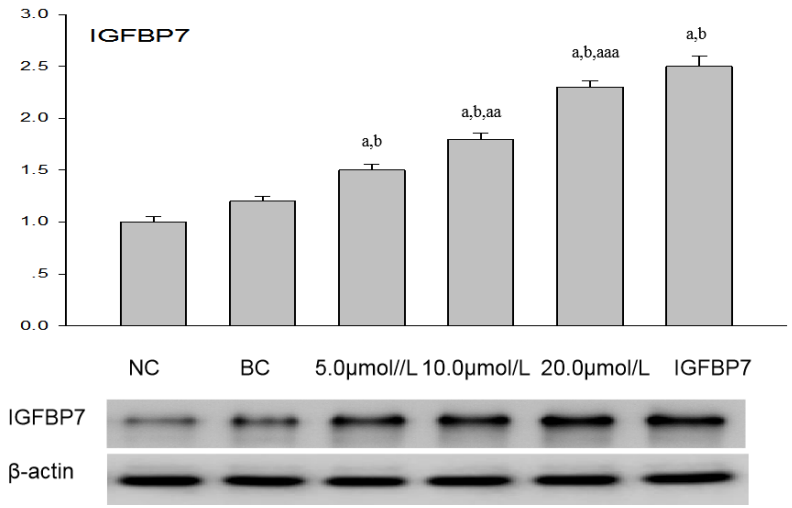


G

groups	NC group	BL group	IGFBP7 group	5.0μmol/L group	10.0μmol/L group	20.0μmol/L group
UL(%)	0.61±0.03	0.48±0.03	1.86±0.12	0.92±0.03	1.6±0.05	1.99±0.05
UR+LR(%)	4.84±0.45	5.16±0.38	40.52±1.76^{a,b}	15.98±1.89^{a}	30.36±1.76^{ab,aa}	39.04±1.43^{ab,aa}
LL(%)	94.55±1.89	94.36±2.14	57.61±2.01	83.1±2.33	66.03±2.98	59.07±2.17

a: P<0.05, the difference from the NC group was statistically significant
b: P<0.05, the difference from the BL groups was statistically significant
aa: P<0.05, the difference was statistically significant compared with the 5.0μmol/L GA group
aaa: P<0.05, the difference was statistically significant compared with the 10.0 μmol/L GA group

Figure 1. *Comparison of apoptosis in six groups. (A is NC groups; B is BL groups; C is IGFBP7 transfection group; D is 5.0 μmol/L GA group; E is 10.0 μmol/L GA group; F is 20.0 μmol/L GA group)*



a: P < 0.05, the difference from the NC group was statistically significant
b: P<0.05, the difference from the BL groups was statistically significant
aa: P<0.05, the difference was statistically significant compared with the 5.0μmol/L GA group
aaa: P<0.05, the difference was statistically significant compared with the 10.0 μmol/L GA group

Figure 2. *Comparison of six levels of IGFBP7 protein levels*

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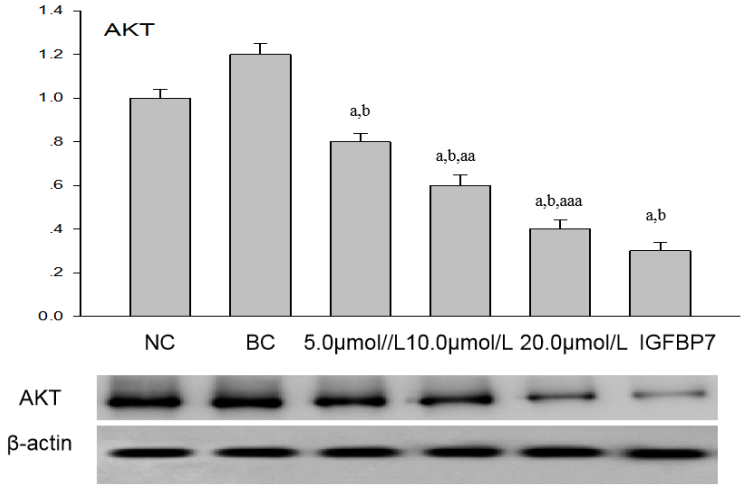
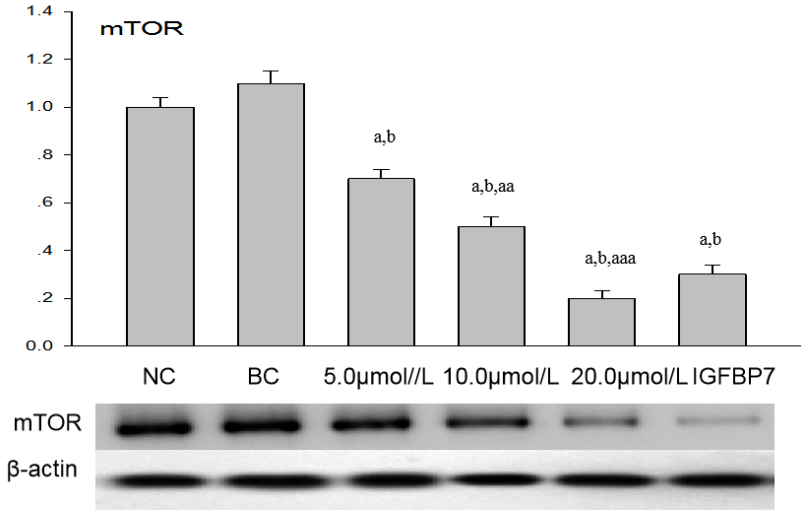


a: P < 0.05, the difference from the NC group was statistically significant
b: P<0.05, the difference from the BL groups was statistically significant
aa: P<0.05, the difference was statistically significant compared with the 5.0μmol/L GA group
aaa: P<0.05, the difference was statistically significant compared with the 10.0 μmol/L GA group

Figure 3. *Comparison of six levels of AKT protein levels*



a: P < 0.05, the difference from the NC group was statistically significant
b: P<0.05, the difference from the BL groups was statistically significant
aa: P<0.05, the difference was statistically significant compared with the 5.0μmol/L GA group
aaa: P<0.05, the difference was statistically significant compared with the 10.0 μmol/L GA group

Figure 4. *Comparison of six levels of mTOR protein levels*

Discussion

IGFBP7, as a member of the IGFBP superfamily, plays an extremely important role in metabolic processes such as cell differentiation, proliferation and growth. A large number of studies have confirmed that the expression of IGFBP7 is related to the occurrence of various cancers (Wei, 2016; Chen, 2017; Kim, 2018; Akiel, 2017; Benassi, 2015), which is Many tumor suppressor factors. The data shows that IGF can bind to PI3K to produce protein kinase phosphorylation, activate the Akt/mTOR signaling pathway, and thereby promote cell proliferation, while IGFBP7 is an IGF binding protein 7 factor, which inactivates PI3K protein, inhibits Akt/mTOR signaling

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pathway, and produces cell inhibition role (Zhao, 2017; Xu, 2013). The results of this study suggest that the level of expression of IGFBP7 protein in prostate cancer PC-3 cells after treatment with different concentrations of pomegranate extract gallic acid group (5.0 μmol/L group, 10.0 μmol/L group and 20.0 μmol/L group) and IGFBP7 overexpression group was significantly higher than the untreated group, but the corresponding AKT protein and mTOR protein expression levels were significantly lower than the untreated group. This result confirmed that the mechanism of pomegranate extract gallic acid inhibiting the proliferation of prostate cancer PC-3 cells may firstly enhance the expression of IGFBP7 protein, and then weaken the expression levels of AKT protein and mTOR protein, causing negative feedback of AKT/mTOR signaling pathway. It is related to regulation and eventually produces the inhibitory effect of tumor cells. However, whether the regulation of AKT/mTOR signaling pathway by gallic acid from pomegranate extract also blocks PI3K protein expression still needs further verification.

In the process of cell proliferation, differentiation, apoptosis and invasion, the AKT/mTOR signaling pathway plays a key role, and is an important signaling pathway in the process of tumor development (Wang, 2019; Zhao, 2019; Gasparri, 2018; Li, 2019). Data show that in the signal pathway of P13K/Akt/mTOR, the upstream pathway of mTOR, P13K is phosphorylated by AKT protein to activate mTOR gene expression and promote tumor cell apoptosis (Gao, 2016; Johnson, 2018). The results suggest that the apoptosis rate of PC-3 cells treated with IGFBP7 protein expression group and pomegranate extract gallic acid group (40.52 ± 1.76, 15.98 ± 1.89, 30.36 ± 1.76, 39.04 ± 1.43) was significantly higher than the untreated NC and BL groups (4.48 ± 0.45, 5.16 ± 0.38), this result indicates that PC-3 cell apoptosis is related to AKT/mTOR signaling pathway. In addition, the inhibitory effect on prostate cancer PC-3 cells has a significant dose-effect and time-effect relationship with the concentration and action time of pomegranate extract gallic acid. There were also some defects in this paper. Considering that pomegranate gallic acid also has certain toxicity to cells, the concentration is relatively low, and whether 20 μmol/L was its maximum effective concentration remains to be further discussed.

Conclusion

The study found that naturally-derived polyphenolic compounds play an important role in inhibiting the proliferation and migration of tumor cells, but there are certain differences in the reports of their mechanism of action (Wang, 2017; Estrela, 2017; Amani, 2017). This experiment used different concentrations of pomegranate extract gallic acid to treat prostate cancer PC-3 cells. It was found that pomegranate extract gallic acid can inhibit the expression level of AKT/mTOR protein by stimulating the expression of IGFBP7 and inhibit the AKT/mTOR signaling pathway Therefore, it has the effect of inhibiting the proliferation ability of PC-3 cells, and there is a significant dose-effect and time-effect relationship between this action trend and its concentration and action time. Studies have shown that compared with the NC and BL groups, the proliferation of PC-3 cells in the IGFBP7 transfection group and the three groups of pomegranate extract gallic acid was significantly reduced. The proliferative capacity of cells, as for the molecular mechanism of this result needs to be further explored.

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CAN WE EXPLOIT SUPERNUMERARY SPIKELET AND SPIKE BRANCHING TRAITS TO BOOST BREAD WHEAT (*Triticum aestivum* L.) YIELD?

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Abstract. Maintaining population growth and competitiveness of arable lands is forcing plant scientist to develop novel ways to enhance grain yield per plant. Several studies on wheat have demonstrated the manipulation of the number (supernumerary spikelet) and arrangement (spike branching) of spikelets. Several genes (*FZP, ndsu, mrs1*, *qTS2A-1, bh, Ppd-1, bh^t*, *bh^m, sb1, sb2, TFL*) controlling supernumerary spikelet and spike branching trait have been reported. Different supernumerary spikelet and branched head wheat germplasm sources (Fen 33, SG203, R107, 166 Schakheli, AUS15910, Kalyan Sona, SWP-BSW1, BS-33, Yupi branching, WCB617 etc.) are also available in the world. However, little is known about the genetic underpinnings, mechanism, plant signaling and physiological aspects of these traits in wheat. Further, these traits are negatively correlated with grain weight and number of tillers per plant and are highly influenced by environmental factors, even tetraploid and hexaploid wheats with reported tendencies of ear branching show different expressions in different environments. In this review, which is a first review report to our knowledge, we have reported the possibilities to exploit these traits to double the number of grains per spike through the use of available supernumerary and branched head germplasm resources and how plant scientists can overcome the negative correlations to develop a sustainable phenotype.
Keywords: *branched head, environmental factors, food security, germplasm sources, grain yield, spike architecture*

Introduction

World food security is challenged by climate changes and loss of arable land due to urbanization and ever increasing population (Godfray et al., 2010). Therefore, an increase in cereal grain production is required to feed the growing population which is anticipated to rise up to 9 billion by 2050. Bread wheat (*Trticum aestivum* L.) is one of the most needed food crop in the world. Its yield in terms of grain production/plant mainly depends on the structural design of the inflorescence. Wheat scientists consider that managing spike architecture is one possible way to increase per plant yield. Dobrovolskaya et al. (2015) reported that genetic determinism of inflorescence development may permit new spike architecture to be designed, with the aim of improving grain production. New spike morphology may lead towards increase in the number of spikelets in the spike i.e. making selections for wheat forms with

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supernumerary spikelet and branched spike. These traits are natural variation in wheat inflorescence and holds great potential for boosting bread wheat yield by doubling the number of grains per spike (Li and Zhao, 2000). However, spike branching is negatively correlated with kernel weight and number of tillers per plant. Wheat breeders observed that low kernel weight/size is very problematic and hard to overcome in the branched or supernumeric spikes (Yen et al., 1993). Furthermore, stability of the lines to express spike branching trait is influenced by environmental dynamics such as vernalization, temperature, time of sowing, nutrients availability and photoperiod (Swaminathan et al., 1966; Pennell and Halloran, 1984a). Fortunately, some wheat germplasm is available that show stable expression of branching trait which can be exploited to create desired variation in the architecture of wheat inflorescence and to boost the grain yield. Studies have shown that it is practical to utilize genes underlying spike branching character to develop stable cultivars with more kernel weight and tillering capacity (Poursarebani et al., 2015). The objectives of this review are to: (1) highlight the importance of supernumerary spikelet and spike branching trait and their potential utilization in improving wheat crop productivity, (2) describe the detailed genetic basis including underlying genes, evolution and mechanism of supernumerary spikelets and spike branching trait development in wheat, (3) understand the genetic basis, morpho-physiological, biochemical and molecular aspects, (4) provide knowledge regarding expression of these characters, some stable materials and isogenic lines developed for pyramiding different underlying genes, (5) in the end, environmental factors affecting the expression of supernumerary spikelet and spike branching traits and prospects are highlighted.

Genetics of Supernumerary Spikelet and Spike Branching

The branching appearance in wheat inflorescence is due to a spontaneous mutation that has been recognized since prehistoric times (Tschermak, 1914; Sharman, 1944). A mutant with adventitious branching in the ears was found in nature in *T. turgidum* var. mirabile Korn (2n = 28). In 1957, a mutant with ear branching was isolated from M_2 progeny of *T. aestivum* var. N. P. 797 (2n = 42) treated with 10 μC. per seed of S^{35}. Swaminathan (1966) observed that this mutant had about 15% pollen sterility and 30% seed sterility and gave rise to both normal and branched ears when seeds were sown from open pollinated heads. The proportion of branched ears was found 9 to 54% in different years. Later on, spike branching and supernumerary spikelets has been witnessed in diploid (*T. monococcum*, bh^m locus; chromosome $2A^mS$; Amagai et al., 2014), tetraploid (*T. turgidum*, bh^t locus; chromosome 2AS; Klindworth et al., 1997; Poursarebani et al., 2015) and hexaploid wheat (*T. aestivum*, *bh* genes; chromosome 2AS, 2BS, 2D, 4A, 4B, 5A; Peng et al., 1998; Dobrovolskaya et al., 2015). The loci maintaining the branchless inflorescence are located in syntenic chromosome positions in all the respective genomes. This shows that branch repression in wheat is controlled by major orthologous genes and defects in these genes are responsible for the formation of lateral branching (Poursarebani et al., 2015). These branched heads are distinct in phenotype from the supernumerary spikelets having additional spikelets per node of rachis (Pennell and Halloran, 1983). Fundamental genetic factors for supernumerary spikelet phenotype found to be assorted and exemplified for paired spikelet phenotype (Boden et al., 2015) or multi–rowed spike (*mrs*) locus (Dobrovolskaya et al., 2009). Echeverry-Solarte et al. (2014) reported seven QTLs controlling supernumerary spikelet development located on 2D, 5B, 6A, 6B and 7B chromosome. All the reported genetic factors for supernumerary spikelet and spike branching trait in different wheat germplasm are summarized in *Table 1*. Despite of

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enduring efforts of scientific community, the phenomenon of laterally formed branches has always remained elusive in wheat.

Table 1. *List of the genes responsible for supernumerary spikelet and spike branching in wheat*

Gene/locus name	Symbol	Chromosome Location	Controlled trait	Wheat species	Reference
branched head	bh^m	$2A^mS$	Branched head	*T. monococcum*	Amagai et al. (2014)
branched head	bh^t	2AS	Branched head	*T. turgidum*	Klindworth et al. (1997), Poursarebani et al. (2015)
Frizzy Panicle	*FZP*	2AS,2BS,2DS	Supernumerary spikelet	*T. aestivum*	Dobrovolskaya et al. (2015)
QSS-NDSU	*ndsu*	2D,5B,6A,6B,7B	Supernumerary spikelet	*T. aestivum*	Echeverry-Solarte et al. (2014)
monstrosum spike	*mrs1*	2DS	Multi-row spikelet	*T. aestivum*	Dobrovolskaya et al. (2009)
triple spikelet	*qTS2A-1*	2A	Supernumerary spikelet	*T. aestivum*	Li et al. (2011)
Bh genes	*bh*	2D,4A,4B,5A	Branched head	*T. aestivum*	Peng et al. (1998)
Photoperiod D-1	*Ppd-1*	2D	Paired spikelet	*T. aestivum*	Boden et al. (2015)
spike branching	*sb1, sb2*	2A,2D	Spike branching	*T. aestivum*	Zhang et al. (2012)
LEAFY-like gene	*TFL*	-	Spike branching	*T. aestivum*	Wang et al. (2017)

Evolution & Domestication

Modern form of cultivated wheat is one of the oldest cereal crops opted by the early farmers for domestication and improvement (Gross and Olsen, 2010). This domestication represents the course of selection and adaptation of wild relatives based on their versatile genetic background to meet the human needs like taste, yield, cultivation and harvest methods (Gepts, 2004; Lenser and Theiben, 2013). Archeological evidences show that the domestication of wheat started 10000 to 12000 years ago (Doebley et al., 2006) in the Fertile Crescent (Glemin and Bataillon, 2009). The set of traits altered during this process is commonly known as domestication syndrome included alteration in architecture of plant, depletion of toxin compounds, compact growth habit, early maturity and other yield related traits (Konopatskaia et al., 2016). Besides grain yield, shape, and color spike morphological characteristics like spike shape, presence or absence of awns, spike branching and supernumerary spikelets per spike were the key factors which remained the subject of interest for early farmers and breeders.

No literature is reported about the evolution and domestication of branched spike or supernumerary spikelets per spike. The possible reason is the low yield as compared to branchless wheat and that's why early farmers and breeder were reluctant to select branched wheat. However, many scientists reported the presence of branched wheat

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spike as abnormality in spike morphology which appeared rarely in *Triticum* species (Dobrovolskaya et al., 2017). Pliny the Elder has mentioned the presence of branched spike in *T. trugidum* 2000 years ago (23-79 AD) under the name of Ramosum and Centigranum (de Candolle, 1883). Some other names like "Miracle wheat", "Mummy", "Egyptian", "Jerusalem wheat", and "Seven-headed" has also been reported in the literature to describe the branching pattern of this tetraploid wheat (Dahlgren, 1922; Dorofeev and Korovina, 1979). This branching pattern in tetraploid wheat is due to the natural mutation which has been known since ancient times (Poursarebani et al., 2015). The character of branched spike is usually exhibited by the tetraploid wheats, that's why all the members of tetraploid wheat having branched spike are classified in a separate group named as *T. turgidum convar. compositum* (L.f) A. Filat. It is noteworthy that the members of this group possess unique spike morphology which is characterized by the development of additional spikelets on rachis nodes or spikelets appear on the extended rachilla in the form of branching especially in the lower portion of the spike (Dobrovolskaya et al., 2009). This type of branching is known as turgidum type of branching (Dobrovolskaya et al., 2017). The other species of genus *Triticum* in which branched spikes have been observed are *T. dicoccum, T. polonicum*, *T. spelta* and *T. vulgare* (Masubuchi, 1974; Wang et al., 2016).

Wheat Germplasm Sources with Supernumerary Spikelet and Spike Branching Trait

Researchers emphasized that current performance of wheat is generally supposed to be sink regulated (Miralles and Slafer, 2007; Lawlor and Paul, 2014). Therefore, spike branching is important for increasing sink capacity and improving the yield potential. Many researchers in the world have developed and maintained germplasm sources/varieties with supernumerary spikelet and spike branching traits. These lines differ in giving stable expression in all kind of favorable and unfavorable environments. However, some stable lines are also present in the world. The detail of such germplasm is presented in *Table 2* and pictorial view of supernumerary spikelet and branched head wheat along with normal spike wheat is given in *Figure 1*.

Role of Phytohormones and Growth Regulators in the Development of Supernumerary Spikelets

In hetero branching spike the supernumerary spikelet attribute was influenced by light intensity, varied temperature, nutrients balance (Koric, 1975; Peanell and Halloran, 1983) and plant hormones (McSteen and Leyser, 2005). Plant growth and development regulated by natural and synthetic plant growth regulators are being implemented as potential investigation tool to elucidate plant responses on physiological basis or to explore the mechanisms of biochemical control. Among many plant hormones, auxin plays a cardinal role to promote polar growth of floral meristems and all other organ primordia (Cheng and Zhao, 2007; Benjamins and Scheres, 2008; Delker et al., 2008). Localized auxin biosynthesis is resulted from activation of YUC (YUCCA flavinmonooxygenase) genes which indicates that auxin biosynthesis is also necessary for initiation of floral meristem (Zhao, 2008). It is well documented that the role of auxin transport and biosynthesis in axillary meristem initiation is found in monocots (Poursarebani et al., 2015).

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Table 2. *Wheats with stable branched head/supernumerary spikelet trait*

Line name	History/Country of origin	Reference
Gandilyan-2	From *T. durum*/Armenian Agriculture Academy, Armenia	Amagai et al. (2017)
W-589 Osiris	From *T. turgidum*/John Innes Centre, Norwich, UK.	Amagai et al. (2017)
TRI 5200, TRI 900 and TRI 1777	From *T. dicoccon*/Leibniz Institute of plant genetics and crop plant research, Gatersleben, Germany	Amagai et al. (2017)
k-39297	From *T. polonicum*/Institute of Cytology and Genetics, Novosibisrk, Russia.	Amagai et al. (2017)
PI 623936 PI 349056	From *T. durum & turgidum*/National Small Grain Collection, Aberdeen Idaho, USA	Amagai et al. (2017)
R107	From *T. durum*/Agrotest Fyto Ltd. Czech Republic	Amagai et al. (2017)
WCB617	Source for the supernumerary spikelet phenotype maintained by North Dakota State University	Echeverry-Solarte et al. (2014)
MC1611	Induced mutant of bread wheat with supernumerary spikelet trait/Altai Research Institute of Agriculture, Russia)	Dobrovolskaya et al. (2014)
Fen33	Interspecific hybrid of *T. turgidum* × *T. aestivum*/Northwestern wheat zone, China	Zhang et al. (2012)
NILs (DD, DR, SR1, SR2)	BC populations of Fen33 (*sb* gene donor, non-recurrent parent) and Taishan (recurrent parent)/Shandong Agricultural University, China	Zhang et al. (2012)
SG203	Elite line selected from a cross between Fen33 and Weimai (common cultivar of wheat zone in Huanghuai, China)	Zhao et al. (2012)
166-Schakheli	Branched spike wheat selected from a cross between 171ACS (a complex wheat line) and Bereketli-95 (durum wheat variety *T. durum* Desf.)/ Genetic Resources Institute, Baku, Azerbaijan	Aliyeva and Aminov (2011)
Yupi branching	Bread wheat selection which stably expresses supernumerary spikelet character/Sichuan Agricultural University China	Peng et al. (1998)
BS33	1st branched spike stable cultivar in the world derived from *T. turgidum* × *T. aestivum* cross/ maintained by Crop Genetics Institute, Taiyuan	Yuan et al. (1994)
SWP-BSW1	Landrace accession of *T. turgidum* with spike branching trait/Crop development center, University of Saskatchewan, Canada	Hucl and Fowler (1992)
AUS15916	Supernumerary spikelet wheat from *T. aestivum*/CIMMYT, Mexico	Pennell and Halloran (1984b)
AUS15910 AUS4531	Supernumerary spikelet wheat from *T. aestivum*/Brumley Plant Sciences Center, Victoria, Australia	Pennell and Halloran (1983)
AUS17335 AUS17336	Supernumerary spikelet wheat from *T. aestivum*/Zagreb, Yugoslavia	Koric (1973)
Kalyan Sona	Indian wheat derived from Mexican stock with ear branching came from *T. turgidum*	Rawson and Ruwali (1972a)

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Figure 1. *Pictorial view of normal spike along with supernumerary spikelet and branched spike wheat*

Plant architecture and reproduction is dependent on axillary meristems that developed in axils of plant leaves. In reproductive growth, flowering branches or flowers produced from axillary meristems, thus during development of grass inflorescence, axillary meristems give rise to branches and spikelets before they give rise to flowers (McSteen et al., 2000; Bommert et al., 2005). Plant hormones and transcription factors controlled the uniqueness and determinacy of various kind of meristem (Bortiri and Hake, 2007; Thompson and Hake, 2009; McSteen, 2009). Hormones play a significantly important role to regulate the branching of shoot and inflorescence (McSteen and Leyser, 2005; Beveridge, 2006; Barazesh and McSteen, 2008; Ongaro and Leyser, 2008). Axillary meristem initiation required auxin for both vegetative and inflorescence development while cytokinin controlled the volume of meristem and consequently affects branching indirectly (Shani et al., 2006; Kyozuka, 2007).

Findings of Tetsuka (2001) provided important information regarding the function of chemical metabolism associated to the genetic aspects and photoperiodism in stimulating the supernumerary spikelet differentiation, by showing overproduction of endogenous concentration of gibberellic acid, nucleic acids and phosphatide in wheat mutant Norin52. Likewise, exogenous application of gibberellic acid and nucleic acids had positively affected the supernumerary spikelets differentiation indicating their role on spikelet developmental phase by influencing chemical metabolism while phosphoric substances and their derivative nucleic-like compound might show their efficacy on differentiation of supernumerary spikelets and rachis branch development. In autumn sowing, exogenously applied uracil and thymine had significantly promoted supernumerary spikelets formation, hence uracil induced the differentiation of the side-type supernumerary spikelets and the thymine stimulated the double-type supernumerary spikelets. Pearce et al. (2013) reported that wheat genotypes expressing *VRN1* has positively responded to exogenous application of gibberellins in terms of accelerated spike development. The *VRN1* and gibberellic acid's simultaneous existence leads toward up-regulation of floral meristems identity genes '*LEAFY and SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1-1'*.

It is well documented that character of branched rachis was effectively modulated by exogenous auxin which induced interratic branched rachis. Foliage application of Indole-3-Acetic Acid (IAA) and Indole-3-Butyric Acid (IBA) (0.1 mM) after two ridge stage of shoot apex, induced maximum increase in quantity of branched rachis by 82.6 and 78.3% respectively while 2,4-D and Naphthalene acetic acid (NAA) applied at concentration of 1

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mM produced 57.6 and 46.7% more branched rachis in comparison with untreated plants. Likewise, whole spike subjected to 1 mM NAA and 2,4-D illustrates improvement in the number of spikelet 57.2% and 53.4%, correspondingly over non-sprayed plants. Moreover, application of auxins stimulated the expression of *TFL* gene during the occurrence of branched rachises that designates the central role to auxins in directing the branched spike development and *TFL* might be correlated with this development of branched rachises in wheat. The newly developed young spike had high level of Indole acetic acid in genotypes having branched spike as compared to normal spike wheat lines. It might be attributed to higher relative expression of *TaIAR3*, an auxin synthesis related gene, in wheat line having branched spike as compared to normal spike lines (Wang et al., 2017). Therefore, it can be speculated that higher IAA contents might be involved in the development and growth of branched spike in wheat. Floral identity in newly formed floral primordial was stimulated by floral meristem identity genes *LFY* and APETALA1 (AP1) in terms of establishment and maintenance of floral identity (Simon et al., 1996). Thus, polar transport of auxin and *LFY* coordinately alter flower development by regulating genes needed for growth of the floral organs and might be contributed to branched spike (Yamaguchi et al., 2014). It is also reported that higher level of endogenous IAA positively influenced the expression of *FLO/LFY* in juvenile vegetative shoot apices and leaf primordia (Wang et al., 2017). It might play a key role in development of inflorescence architecture and regulation of floral meristems initiation in several plant species (Kyozuka et al., 1998; Bomblies et al., 2003).

Molecular Characterization

Efforts were started in the last century to get the better understanding of genetics of supernumerary or branched spike in tetraploid wheat. The study revealed that trait is regulated by one recessive gene designated as *bh*, *branched head* (Percival, 1921; Sharman, 1944). Later on same type of gene action was observed in diploid (*T. monococcum*) and hexaploid wheat. The gene(s) controlling this trait is resided on the homeologous group 2 which is also affected by other environmental factors (Penell and Halloran, 1983; Klindworth et al., 1990, 1997). Further study in hexaploid wheat elucidated that branching spike is controlled by three different genes names as *Normalizator* (*Nr*), *Ramifera* (*Rm*) and *Tetrastichon* (*Ts*). Among these genes, *Rm* and *Ts* complement each other to produce the branched spike phenotype while the *Nr* acts as dominant repressor of branched spike (Koric, 1973). Further studies also supported that spike branching in hexaploid wheat is regulated by three different genes (Penelle and Halloran, 1983; Dencic, 1988). Recently, Dobrovolskaya et al. (2015) showed that the formation of supernumerary spikelets or spike branching in bread wheat is under the control of *WHEAT FRIZZY PANNICLE* (*WFZP*) resided on the short arm of homeologous group 2. The molecular characterization of orthologue genes of *WFZP* in other members of Poaceae family (maize, rice, barley, and sorghum) suggest that this gene belongs to *ERF* (ethylene-responsive element-binding factor) family of *AP2* transcription factors (Chuck et al., 2002) which are meant to function as repressor to the formation of lateral branching of meristem (Komatsu et al., 2003; Zhang et al., 2017). Detailed structural and functional analysis of wheat *WFZP* genes revealed the presence of mutations in two homeologous genes i.e. *TaWFZR-2A* and *TaWFZR-2D* which are responsible for the formation of branched spike in hexaploid wheat (Dobrovolskaya et al., 2015). Another study in tetraploid wheat (*T. trugidum*) found a mutant allele of *WFZP-A* designated as *TtBH-A1* having an amino acid substitution in the DNA binding domain to produce branched spike (Poursarebani et al., 2015). In tetraploid wheat, two types of spike branching phenotypes are found which are

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named as "true spike ramification" and "false-true spike ramification" which have some resemblances in their phenotypes. *TtBh-A1* is mainly responsible for the true spike ramification phenotype while false-true spike ramification is under the regulation of another recessive allele designated as *SHAM RAMIFICATION 2 (SHR2)* and both are located at chromosome 2AL (Amagi et al., 2014). However, the information regarding structural and functional pathway of *SHR2* is still lacking (Dobrovolskaya et al., 2017). Recently, Dixon et al. (2018) elaborated the genetic factors controlling the formation of paired spikelets, a form of branched spike in hexaploid wheat. The study shows that *TEOSINTE BRANCHED1* (*TB1*) an ortholog of maize domestication gene locus interacts with *FLOWERING LOCUS T1* (*FT1*) to regulate the inflorescence architecture in bread wheat.

Although several studies have elaborated the inheritance and genetics of branched spike or supernumerary spike in wheat but a very few studies elaborated the molecular markers linked to these traits (Echeverry-Solarte et al., 2014). Dobrovolskaya et al. (2009) reported the mapping of a major locus controlling multi-row spike (*mrs*) in wheat on chromosome 2D which is linked to microsatellite locus *Xwmc453*. Similarly, a QTL flanking two SSR markers (*Xgwm275* and *Xgwm122*) was mapped on chromosome 2A in Tibetan wheat (Li et al., 2011). In *T. durum* Desf. var. ramosoobscurum Jakubz. "Vetvistokoloskaya" R-107 three F_2 mapping populations were used to map the branched head spike. The results depicted that the trait of branched head was located on chromosome 2A flanked proximally by the molecular marker *Xgwm425* (Haque et al., 2012). In *T. monococcum* the locus of branched head *(bh^m)* was mapped on chromosome $2A^m$ along with co-segregating marker *Xgwn122* present at the distal end of the gene (Amagi et al., 2014). The detailed mechanism of supernumerary and spike branching trait development in wheat based on above discussion is presented in *Figure 2*.

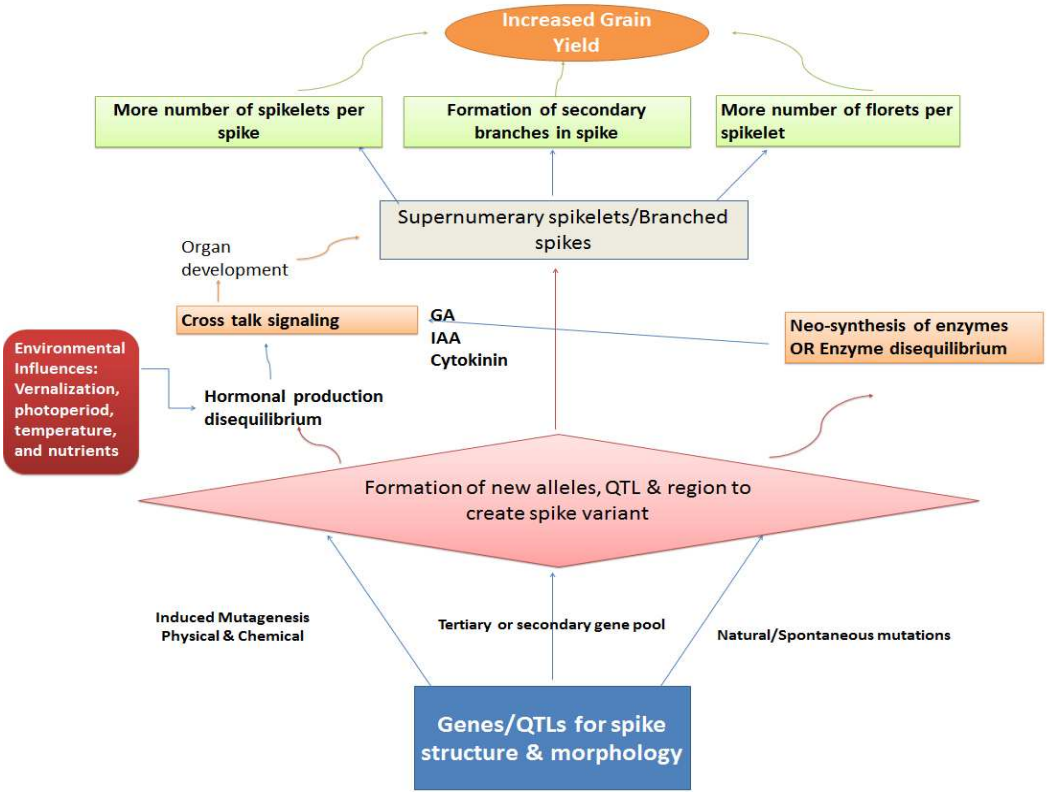

Figure 2. *Mechanism of Supernumerary Spikelet and Branched Spike Development*

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Grain Yield and Quality of Normal vs. Supernumerary Spikelet and Branched Spike

The impact of supernumerary spikelet on other agronomically important yield contributing traits is not clearly mentioned in literature; however, some studies have found that branched head genotypes are negatively correlated with yield contributing traits including reduced tillering, low grain weight, some sterile florets, delayed heading and maturity (Hucl and Fowler, 1992) and ultimately less grain yield (Zhang et al., 2012). However, some branched genotypes defy these generalizations (Martinek and Bednar, 1998). For example, some lines in Tibetan Triple-Spikelet wheat have good grain production with more than 120 seeds per spike and appropriate thousand grain weight (Yang et al., 2005). Miracle wheat, a highly stable phenotype with branched spikes has a higher yield potential as compared to unbranched tetraploid wheats (Poursarebani et al., 2015). Further, it has also been observed that grain yield of such genotypes can be enhanced by improving the fertility of sterile florets (Pennell and Halloran, 1984a,b).

Quality of supernumerary grains is extremely important for the end users. Traits like; starch (amylose and amylopectin), proteins and gluten protein contents are important to focus. Limited information is available about the quality of these grains. Few of the recent studies of genome wide association (GWA) revealed that gluten protein contents were positively correlated with supernumerary spikes (Echeverry-Solarte et al., 2015). Singh et al. (2018) pointed out that next generation sequencing coupled with haplotype-based approaches can be helpful to detect the real changes in multiple environments. These results are encouraging for the plant scientists; however, further studies are required to explain the quality of grains and their products.

Influence of Environmental Conditions

The degree of supernumerary spikelet and ear branching appearance in wheat is strongly associated to environmental effects. Tendency of both tetraploid and hexaploid wheats towards branched headings differs under different vernalization, photoperiod, temperature, moisture and nutrient regimes (Sharman, 1967; Pennell and Halloran, 1984a,b).

Vernalization

Ups and downs in the required vernalization of wheat affect the potential seed number per head. Similarly, the branched spike and supernumerary spikelet expression is influenced by vernalization. Pennell and Halloran (1984a) studied two tetraploid and six hexaploid wheats with branched spike tendency and found that the expression differed in lines with strong vernalization response. They also indicated high stability in the expression of supernumerary spikelet in certain genotypes especially under different environments and their potential to incorporate this character in breeding material or commercial wheat varieties to increase grain number per head.

Photoperiod

Day length is also crucial to express the potential of supernumerary spikelets into grain yield. Pennell and Halloran (1984b) reported that wheats with tendency of branched heads develop more supernumerary spikelets in short photoperiod

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environment (9-14 h) with weak vernalization. The expression of this character was high in long photoperiod environment (24 h) lines having strong vernalization response. Sharman (1967) also reported significant expression under short photoperiods whereas Rawson and Ruwali (1972b) found that the initiation of supernumerary spikelet takes longer photoperiod than in normal wheat. Fedorov (1955) also indicated length of the day as main conducive factor.

Temperature/Time of Sowing

The variations in spike morphology especially spike branching is strongly associated with time of sowing and mean minimum and maximum temperature at the time of heading. Sharman (1967) reported that the branching in wheat is caused by a recessive gene and appear only in normal or low temperature conditions and inhibited at longer day length or high temperature. Pennell and Halloran (1984b) also endorsed these findings. Meena et al. (2008) reported the occurrence of hetero-branching spike in bread wheat at different temperature and sowing times. The mean minimum and maximum temperatures at heading stage during early sown crop were 6.90^{o}C and 22.79^{o}C while the corresponding figures under late sown conditions were 8.39^{o}C and 24.09^{o}C. They characterized the most striking morphotypes having two fully developed spikes on a single culm. Such morphotypes were expressed only in early sown F_2 generation. There was no such type of segregation observed in F_2 material sown late in December. It may be due to low heritability of the character and threshold controlled by one or more recessive genes at various temperatures.

Nutrients

Appearance of branched spike is not limited to environmental conditions and is hereditary (Masubuchi, 1974). This trait is strongly influenced by nutritional conditions. Vigolov's (1954) reported that branched spikes can be obtained from fertile soils. Ryzei (1950) also produced branched wheat after removing the majority of the spikelets and gave abundant nutrition to the remaining spikelets. Ryzei (1950), Muhin (1952) and Vigolov (1954) observed nutritional conditions as main influencing factors on the plant to produce branched heads. Reduced grain weight or size in branched spike might be the result of other factors like less nutrition, or compact grain in a spike (Koric, 1975).

Future Prospects

Genome Wide Association Mapping

Spike length, grain number and size have extensively been studied in the recent to identify the genomic regions associated with these traits (Singh et al. 2018; Rehman-Arif et al. 2020; Shokat et al. 2020). Studies investigated the underlying QTLs/genes in branched genotypes and studied the association of agronomically important traits (AIT) in these genotypes on the basis of phenotypic analysis. Some molecular marker studies also identified genetic regions controlling the spike branching trait (Li et al., 2011; Echeverry-Solarte et al., 2014), but to date only one study investigated the association of these genetic regions with AIT using genome wide association mapping (GWAS). Echeverry-Solarte et al. (2015) evaluated several recombinant inbred lines from a cross between WCB414×WCB617, a normal and branched spike phenotype, respectively.

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They identified QTLs associated with nine spike related traits and 10 AIT, and found chromosome 2DS rich for these characters. Therefore, GWAS can be used to detect QTLs for spike branching, supernumerary spikelet and AIT of bread wheat. It can also identify common genomic regions with QTL for spike related and AIT and also to explore novel alleles with possible use in breeding wheat improvement programs.

Next Generation Sequencing

In comparison to 1950s, a massive increase in our information about the biological basis of plant productivity has opened new prospects for novel breeding strategies. The large and complex genome of bread wheat represents a big challenge, but at the same time offers a large reservoir of genes that can be targeted to breed for desired traits (Nadolska-Orczyk et al., 2017). Detailed description and an analysis of the reference sequence of the hexaploid wheat genome by International Wheat Genome Sequencing Consortium (IWGSC), paved the way for the development of wheat varieties resilient to climate changes, endowed with higher yields, improved nutritional quality and sustainability (Appels et al., 2018). Now, genes involved in supernumeric wheat can be easily exploited following reference sequence. Next generation sequencing (NGS) can be used to sequence these genes and to understand the mechanism of supernumerary spikelet development at genetic level. This technique can also pave the way for expression analysis of these genes in the source material or otherwise their transformation in the elite bread wheat germplasm.

Gene Pyramiding

Pyramiding of elite alleles for dynamic development of spike branching in bread wheat can be rewarding. Even researchers have combined different spike branching genes to develop near isogenic lines in wheat (Zhang et al., 2012). However, very little research has been focused on the effects of pyramiding spike branching genes on AIT in bread wheat. This is due to lack of suitable materials and unfavorable environmental influences (Zhao et al., 2012). These results are encouraging for the plant scientists; however, further studies are required to explain the effects of combining spike branching genes on agronomically important traits in bread wheat.

Speed Breeding

Recent advances in generation advancement in plant breeding research i.e. “Speed Breeding” by Watson et al. (2018) can be potentially utilized to understand the optimum environment required for the expression of supernumerary or spike branching trait in wheat. Speed breeding protocols can be used for modeling the ideal photoperiod, temperature and nutrient conditions that favor the expression of these traits. Hence, genetic control of spike branching in relation with environmental influences can be exploited and rationalized.

Conclusion

Supernumerary spikelet and branched spikes holds enormous potential for boosting number of grains/spike and ultimately wheat yield. These are genetically controlled and can be exploited in future breeding programs. Although some negative correlations among other yield components and environments put hindrance to use these traits for

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the development of commercial high yielding wheat cultivars. However, possibilities are there to break these strong linkages through plant improvement approaches and by modeling the ideal photoperiod, temperature and nutrient conditions that favor the expression of these traits. Recent approaches of next generation sequencing can be helpful to investigate these traits more precisely. Likewise, certain physiological aspects, along with plant signaling are yet to be covered. Additionally, workload management from source to sink is also not been studied yet. Despite of all missing information, still these traits can be exploited and their maintained germplasm sources can be utilized to improve yield potential in this globally important crop.

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COMPARATIVE STUDY FOR SIX DURUM WHEAT CULTIVARS (*TRITICUM DURUM* L.) CONDUCTED DURING FIVE GROWING SEASONS FOR GRAIN YIELD AND ITS COMPONENTS

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Abstract. Six cultivars of durum wheat (*Triticum durum* L.), namely Iraq-7, Acsad-65, Cham-1, Ovanto, Crezo and Simeto, were evaluated the five different environments (seasons) in Sulaimani region-Iraq from (2014-2015 until 2018-2019) using Completely Randomized Block Design (CRBD) with three replications in order to study correlations, path analysis, stability and genotypic resultant for all studied traits which were grain yield and its components. The results of combined analysis across the five seasons as summarized below: the mean squares due to cultivars and the interaction between cultivars × seasons were highly significant for all characteristics. The Simeto cultivar showed the best value for grain yield and most its important components. The correlation between grain yield and spike weight m^{-2} and harvest index was positive and highly significant, while it was positive and significant with grain weight $spike^{-1}$ as the average of all seasons. Maximum positive direct effect for grain yield was recorded to be spike weight m^{-2}, while maximum positive indirect effect was recorded by spike weight m^{-2} via harvest index as the average of all seasons. The highest stability and genotypic resultant value among all traits recorded by biological yield via Crezo cultivar reached 0.829 and 0.951 respectively.
Keywords: *grain yield, correlation, path analysis, stability, genotypic resultant*

Introduction

Wheat (*Triticum durum* L.), is grown in all regions of Iraq, mostly under rain fed conditions including Kurdistan regions. Therefore, annual production is affected to large extent by the annual and seasonal distribution of precipitation, environmental states and crop managements like sowing time, soil fertility, etc. Like to the other crops, increasing the potential of yield is an important target of durum wheat improvement programs production. However, durum wheat yields in most production regions seem to be no more than the potential yields of the cultivars and far below the theoretical maximum yields (Rharrabti et al., 2003). For the production of high-quality durum wheat, dry environments are necessary, with warm days and cold nights during the growing season so that large grains are obtained with yellow color, vitreous kernels (more than 95%), hard texture and high test weight (about 82 kg hL^{-1}), alongside high protein content (greater than 10%) and strong gluten (greater than 30% wet gluten), which gives elasticity to dough for industrial use (Acevedo and Silva, 2007). As Borlaug and Dowswell (1997) observed, "The only way for agriculture to keep pace with population and alleviate world hunger is to increase the intensity of production in those ecosystems that lend themselves to sustainable intensification, while decreasing intensity of production in the more fragile ecosystems." By 2020, "The world's farmers will have to produce 40% more grain... most of which will have to come from yield increases" (Pinstrup-Andersen et al., 1999). Production increases can originate from

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various sources: (1) genetic gains in GYP; (2) genetic gains in tolerance to a biotic and biotic stresses; (3) gains through improved and novel CMR production techniques and technological gains related to optimizing permanent and variable factors of the environment; and (4) synergistic effects among these factors. Of these, GYP and GYP improvement occupy center stage in research (Pfeiffer et al., 2000). The improved genotypes are evaluated in multi-environment trials to test their performance across different environmental conditions. In most trials, crop yield fluctuates due to suitability of genotypes to different conditions which is known as genotype × environment interaction (G×E) (Kang, 1998). Yield and other quantitative traits of crop plants, are among the most important in studying genotypes grown in multi-environments. In this kind of studies, it is important to differentiate the best genotype in term of performance and stability across environments (El-Sahookie and Al-Rawi, 2011). The various phenotypic homeostasis measures were used to evaluate the echo of genotypes when grown in different environments. Summarized genotype-by-environment interaction through homeostasis and genotypic resultant (El-Sahookie and Al-Rawi, 2011). Statistically concept according to Estimates of (H%) and (GR) according to (El-Sahookie, 1990), who mentioned if the value of homeostasis is less than 85%, it means that the cultivar was unstable across environments, and if the value of genetic resultant was high and close to unity, it means that the cultivar has a good performance under varying environments. The development of high-yielding cultivars requires a thorough knowledge of the existing genetic variation for yield and its components (Shukla et al., 2006).

The objective of the study is to estimate the inheritance between cultivars and environment and finding stability for selecting a cultivar suits the environmental conditions prevailing in the region.

Materials and methods

Six durum wheat cultivars were used as plant material in this study (Iraq-7, Acsad-65, Cham-1, Ovanto, Crezo and Simeto). The traits were conducted during the winter season of 2014-2015 until 2018-2019 at University of Sulaimani, Sulaimani-Iraq. (Latitude: 35° 33' N; Longitude 45° 27' Est. altitude of approximately 830 m). At each experimental season all cultivars were sown according to Completely Randomized Block Design with three replications, according to the following linear modeling (Al-Mohammad and Al- Yonis, 2000). Wheat was sown in autumn at sowing density of 450 plants m^{-2}. Each experimental plot was consisted of 6 rows, 5 m in length with 0.25 cm apart rows. The experiments were sown at (November 15 2014, December 10 2015, November 12 2016, November 16 2017 and December 5 2018) respectively. The recommended doses of fertilizer was used which was 40×40 kg nitrogen and P_2O_5/ha respectively. All phosphorus fertilizer and half of nitrogen fertilizer were applied at the Zadoks growth stage 25. Recorded data were subjected to the analysis of variance ANOVA using MSTATC software. Relative magnitude of year, genotypes and their interactions attributed to total sum of squares were calculated as percentage (Akcura et al., 2006). Stability analysis and genotypic resultant were performed for each trait studied. The correlation and path analysis were conducted. The experiments were harvested at (June 6 2015, June 9 2016, June 5 2017, June 8 2018 and June 10 2019) respectively.

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Studied traits: Spikes number m^2, Spike weight m^2 (g), Average spike weight (g), Spike length (cm), Grains number $spike^{-1}$, 1000-grain weight (g), Grain yield t h^{-1}, Biological yield t h^{-1} and Harvest index.

From each plot at each block ten individual plants were chosen randomly to study these traits from the guarded rows.

Climate conditions of Sulaimani region

The climate of Sulaimani governorate is semi-arid environment: wet and cold in winter dry and hot in summer; During July and August, the average temperature is between 39-43 °C, and overwhelmingly amount to nearly 50 °C. Autumn means high temperatures are 20-30 °C in October, cooling slightly in November. Precipitation is limited to winter and spring months, and the overall average annual rainfall of 550-700 mm was at Sulaimani city. An overview of experimental conditions is given in *Table 1*.

Table 1. *Metrological data for experimental location during five winter seasons*

Months	2014-2015	2015-2016	2016-2017	2017-2018	2018-2019
September	-	15.2	-	-	-
October	43.2	114.2	-	10.0	48.2
November	151.6	197.2	6.0	114.6	99.8
December	128.6	75.8	149.4	22.2	281.8
January	100.0	110.6	39.8	72.4	210.6
February	65.0	76.2	105.0	323.0	108.2
March	98.4	171.8	121.0	44.6	248.6
April	25.8	57.6	70.0	98.6	190.0
May	19.8	12.2	20.0	70.4	28.4
Total	632.4 mm	830.8 mm	511.2 mm	755.8 mm	1215.6 mm

Results

Data represented in *Table 2* illustrate the mean squares for variance sources of all characters. It was observed that the differences among cultivars were highly significant for all characters at the first and the second season 2014-2015 and 2015-2016.

Concerning to the third season 2016-2017 the differences among cultivars were highly significant for all characters except weight of $spike^{-1}$ and biological yield which were significant only.

Data recorded for the fourth season 2017-2018 represent in the same table confirmed that the differences among cultivars were highly significant for spikes number m^{-2}, spikes weight m^{-2}, spike length and 1000-grain weight, while it was significant for average spike weight, grains number $spike^{-1}$, grains weight $spike^{-1}$, grain yield and biological yield, whilst the differences among cultivars were not significant for harvest index.

Regarding to the last season 2018-2019 as represent in the same table it was observed that the differences between cultivars were significant for all characters except spike length and grain yield which recorded highly significant differences among cultivars and the differences were not significant for harvest index.

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Data due to combine analysis for the average of all seasons represent in *Table 1,* confirming that the effect of seasons were highly significant on all characters except harvest index which responded significantly to seasonal effect. The mean squares due to cultivars and the interaction between cultivars were highly significant for all characters as combine analysis. From the same table it was noticed that the interaction between cultivars and seasons were highly significant for all characters, the analysis of genotype by environment interactions is of the primary importance for most crops (Ceccarelli, 1996; Annicchaiarico, 2002; Voltas et al., 2002 and Rodriguez et al., 2008).

Table 2. *Mean squares of variance analysis in durum wheat genotype for studied characters*

S.0.V	df	Spikes no. m^2	Spike wt. m^2 (g)	Average spike wt. (g)	Spike length (cm)	Grains no. $spike^{-1}$	Grains wt. $spike^{-1}$ (g)	1000-grain wt. (g)	Grain yield t h^{-1}	Biological yield t h^{-1}	Harvest index
2014-2015											
Block	2	3286.500	1847.056	0.006	0.245	19.056	0.006	5.541	0.220	0.595	0.003
Treat	5	9856.667**	62965.289**	0.169**	3.235**	120.889**	0.256**	66.572**	1.215**	2.281**	0.012**
Error	10	33.367	90.789	0.004	0.092	1.389	0.019	0.089	0.076	0.029	0.001
2015-2016											
Block	2	2888.667	865.722	0.019	0.062	14.000	0.021	4.641	0.231	0.466	0.0004
Treat	5	1709.833**	28236.222**	0.188**	0.235**	42.900**	0.233**	117.151**	1.985**	0.905**	0.008**
Error	10	438.600	66.389	0.044	0.050	7.400	0.004	0.202	0.047	0.079	0.0003
2016-2017											
Block	2	861.166	17914.905	0.187	0.242	33.371	0.147	0.270	0.086	0.825	0.001
Treat	5	5379.733**	79499.022**	0.917**	2.081**	117.397**	0.557*	147.865**	2.264**	2.116*	0.010**
Error	10	867.900	2084.027	0.091	0.083	19.393	0.110	0.577	0.346	0.501	0.001
2017-2018											
Block	2	1058.793	124.965	0.106	1.186	35.337	0.144	12.170	0.089	1.433	0.220
Treat	5	12953.320**	7438.100**	0.371*	1.211**	31.965*	0.331*	91.731**	0.439*	2.285*	0.002$^{n.s}$
Error	10	2052.675	2260.291	0.074	0.112	8.109	0.072	8.780	0.093	0.595	0.001
2018-2019											
Block	2	3731.348	1442.727	0.068	0.564	20.604	0.340	2.053	0.083	0.355	0.001
Treat	5	12700.851*	12465.632*	0.688*	1.939**	77.627*	0.343*	27.534*	0.984**	21.552*	0.011$^{n.s}$
Error	10	2559.818	2872.216	0.183	0.151	15.513	0.076	5.630	0.168	4.839	0.004
Averages											
Seasons	4	14720036.246**	676185.763**	7.339**	29.532**	294.317**	4.483**	1578.644**	40.795**	239.182**	0.162*
B/S E(a)	10	2277.671	4783.493	0.078	0.460	27.640	0.109	4.935	0.160	0.634	0.046
Cultivars	5	22654.280**	52540.082**	1.248**	2.495**	169.231**	0.979**	259.186**	3.385**	5.434**	0.021**
C×S	20	5714.620**	48397.843**	0.272**	1.552**	55.387**	0.148**	47.917**	0.875**	5.926**	0.006**
E/S E(b)	50	1081.675	1040.842	0.079	0.098	12.528	0.051	3.056	0.152	1.174	0.002

Data in *Table 3* illustrate the means of grain yield and its related components for all seasons and their averages. Concerning to the first season 2014-2015, the Smeto cultivar produced the highest value for spike wt. m^2, average spike wt., spike length, grains wt. $spike^{-1}$, 1000-grain wt, grain yield and harvest index reached 736.000 g, 1.920 g, 10.833 cm, 1.613 g, 30.517 g, 4.110 t h^{-1} and 0.439 respectively, while Crezo cultivar exhibited the highest value for spikes number m^{-2} and grains number $spike^{-1}$ reached 424.000 spikes and 52.000 grains. The highest value for biological yield was 9.867 t h^{-1} produced by Cham-1 cultivar. The lowest value for almost all characters exhibited by Ovanto cultivar with the exception of harvest index which was showed by Cham-1 cultivar.

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Table 3. *Means of grain yield components for five successive seasons and their average of durum wheat cultivars*

Cultivars	Spikes no. m^2	Spike wt. m^2 (g)	Average spike wt. (g)	Spike length (cm)	Grains no. $spike^{-1}$	Grains wt. $spike^{-1}$ (g)	1000-grain wt. (g)	Grain yield t h^{-1}	Biological yield t h^{-1}	Harvest index
2014-2015										
Iraq-7	419.667	509.667	1.307	8.487	38.000	0.893	24.120	3.033	8.407	0.361
Acsad-65	332.333	501.000	1.550	9.133	46.333	1.303	23.370	2.987	8.010	0.373
Cham-1	349.333	446.000	1.427	9.367	38.000	1.173	20.433	2.553	9.867	0.259
Ovanto	278.667	346.333	1.300	8.033	37.667	0.867	17.603	2.327	7.493	0.312
Crezo	424.000	673.667	1.657	10.167	52.000	1.400	27.800	3.410	8.477	0.403
Simeto	400.000	736.000	1.920	10.833	48.667	1.613	30.517	4.110	9.357	0.439
LSD	10.509	17.335	0.116	0.552	2.144	0.254	0.543	0.501	0.308	0.060
2015-2016										
Iraq-7	312.000	621.333	2.260	6.767	46.667	1.686	35.303	4.990	14.910	0.335
Acsad-65	300.000	495.333	1.983	6.733	38.667	1.149	36.953	4.133	16.077	0.257
Cham-1	274.667	430.000	2.007	7.000	42.000	1.198	32.370	3.290	15.097	0.218
Ovanto	293.000	471.667	2.083	7.400	41.000	1.387	43.863	3.800	15.853	0.240
Crezo	250.000	334.667	1.723	7.133	46.667	1.175	32.110	2.970	14.890	0.199
Simeto	261.333	536.333	2.453	7.333	38.000	1.790	47.280	4.840	15.923	0.304
LSD	21.610	14.823	0.382	0.408	4.949	0.121	0.817	0.395	0.513	0.030
2016-2017										
Iraq-7	463.333	876.110	2.183	7.583	42.267	1.545	32.867	5.106	9.880	0.524
Acsad-65	414.000	771.500	1.814	5.690	35.567	1.249	36.040	3.728	9.127	0.411
Cham-1	458.000	564.617	1.466	5.133	33.167	1.317	30.547	3.738	9.073	0.409
Ovanto	430.667	864.717	1.988	5.917	35.233	1.412	35.647	4.562	10.300	0.439
Crezo	366.000	1060.690	2.975	6.150	50.100	2.096	45.727	6.004	11.200	0.547
Simeto	330.667	878.073	2.645	5.700	40.867	2.040	47.773	4.866	10.607	0.453
LSD	83.209	83.052	0.550	0.526	10.002	0.522	1.382	1.118	1.041	0.125
2017-2018										
Iraq-7	437.222	694.939	1.500	7.773	42.867	1.250	28.599	4.437	12.206	0.364
Acsad-65	415.556	624.789	1.405	5.850	41.867	1.305	31.110	4.079	11.642	0.350
Cham-1	377.222	636.872	2.072	6.530	46.800	1.498	32.016	3.981	10.819	0.368
Ovanto	295.000	584.972	1.871	6.417	42.133	1.662	38.896	3.281	10.427	0.315
Crezo	335.000	617.683	2.250	6.397	49.667	2.064	41.330	4.136	11.212	0.369
Simeto	273.334	547.400	2.155	6.587	47.400	1.938	40.824	3.981	9.761	0.408
LSD	75.253	17.335	0.495	0.608	5.181	0.489	5.391	0.555	1.403	0.051
2018-2019										
Iraq-7	474.815	953.792	2.919	8.177	47.700	2.364	49.228	7.558	15.254	0.496
Acsad-65	418.519	868.222	2.743	6.153	45.300	2.328	51.524	6.529	16.323	0.411
Cham-1	445.926	879.507	2.827	5.890	45.467	2.025	48.207	6.326	16.273	0.391
Ovanto	298.518	868.637	3.353	6.857	56.633	2.619	45.938	6.779	16.305	0.420
Crezo	368.889	993.593	3.605	6.433	51.033	2.735	53.752	7.465	22.478	0.335
Simeto	354.074	1007.759	3.933	6.670	56.100	2.978	53.114	7.633	15.804	0.486
LSD	68.819	97.500	0.778	0.707	7.166	0.500	4.317	0.746	4.002	0.108
Average										
Iraq-7	421.407	731.156	2.034	7.754	43.500	1.548	34.023	5.025	12.131	0.413
Acsad-65	376.082	652.168	1.899	7.712	41.547	1.467	35.799	4.291	12.236	0.351
Cham-1	381.030	591.399	1.960	6.784	41.087	1.442	32.715	3.978	12.226	0.325
Ovanto	319.170	627.265	2.119	7.925	42.533	1.589	36.389	4.150	12.076	0.344
Crezo	348.778	736.060	2.442	7.256	49.893	1.894	40.144	4.203	13.651	0.308
Simeto	323.882	738.913	2.621	7.425	46.207	2.072	43.902	5.086	12.290	0.414
LSD	24.127	23.667	0.206	0.229	2.597	0.166	1.282	0.286	0.795	0.033

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Concerning to the second season 2015-2016, the Iraq-7 cultivar produced the maximum values for spikes number m^{-2}, spikes weight m^{-2}, grains number $spike^{-1}$, grain yield and harvest index reached 312.000 spikes, 621.333 g, 46.667 grains, 4.990 t h^{-1} and 0.335 respectively, while the highest value for biological yield was 16.077 t h^{-1} produced by Acsad-65. The Simeto cultivar gave the highest value for average spike weight, spike length, grains weight $spike^{-1}$ and 1000-grain weight reached 2.453 g, 7.333 cm, 1.790 g and 47.280 g, respectively. The lowest values for most characters produced by Crezo cultivar.

Regarding to the third season 2016-2017, the means of the characters represent in the same table confirmed that the highest value for most characters such as spike weight m^{-2}, average spike weight, grains number $spike^{-1}$, grain weight $spike^{-1}$, grain yield, biological yield and harvest index produced by Crezo cultivar reached 1060.690, 2.975, 50.100, 2.096, 6.004, 11.200 and 0.547 respectively, while the highest value for spikes number m^{-2} and spike length recorded by Iraq-7 reached 463.333 spikes and 7.583 cm respectively, but Smeto produced the highest weight of 1000-grain reached 47.773 g. Cham-1 cultivar recorded the lowest value for most characters.

Data represent in the same table respect to the fourth season 2017-2018, ratified that Iraq-7 recorded the highest value for spike number m^{-2}, spike weight m^{-2}, spike length, grain yield and biological yield reached 437.222 spikes, 694.939 g, 7.773 cm, 4.437 t h^{-1} and 12.206 t h^{-1} respectively. While Crezo cultivar recorded the highest value for average spike weight, grains number $spike^{-1}$, grains weight $spike^{-1}$ and 1000-grain weight reached 2.250 g, 49.667 grains, 2.064 g and 41.330 g respectively, whilst the highest value for harvest index was 0.408 recorded by Simeto cultivar. The lowest value for most characters produced by Acsad-65 and Simeto cultivars.

Concerning to the fifth season 2018-2019, it was illustrate that Iraq-7 showed maximum value for spike number m^{-2}, spike length and harvest index reached 474.815 spike, 8.177 cm and 0.496 respectively, while the highest grains number $spike^{-1}$ was 56.633 recorded by Ovanto cultivar and for 1000-grain weight and biological yield the highest values were 53.752 g and 22.478 t h^{-1} recorded by Crezo cultivar respectively. The Smeto cultivar recorded the highest value for spike weight m^{-2}, average spike weight, grains weight $spike^{-1}$ and grain yield reached 1007.759 g, 3.933 g, 2.978 g and 7.633 t h^{-1} respectively. Acsad-65 and Cham-1 cultivars recorded the lowest value for most characters.

Data recorded for the average of all seasons represent in the same table, confirming that the Simeto cultivar recorded the highest value for most characters including spike weight m^{-2}, average spike weight, grains weight $spike^{-1}$, 1000-grain weight, grain yield and harvest index reached 738.913 g, 2.621 g, 2.072 g, 43.902 g, 5.086 t h^{-1} and 0.414 respectively, while Iraq-7 showed the highest value for spikes number m^{-2} reached 421.407 spikes, while for spike length the highest value was 7.925 cm recorded by Ovanto cultivar and for both grains number $spike^{-1}$ and biological yield the highest values were 49.893 grains and 13.651 t h^{-1} respectively. The lowest value for most characters recorded by Cham-1 cultivar as the average of all locations.

Data in *Table 4* explain the effect of seasons on grain yield and its components of durum wheat cultivars, signifying that the highest values for most characters recorded at the fifth season 2018-2019 including spike weight m^{-2}, average spike weight, grains number $spike^{-1}$, grains weight $spike^{-1}$, 1000-grain weight, grain yield and biological yield reached 928.585 spikes, 3.230 g, 50.372 spikes, 2.508 g, 50.294 g, 7.048 t h^{-1} and 17.073 respectively. While the lowest value for most characters exhibited by the first season 2014-2015.

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Table 4. *Effect of seasons on grain yield and its components of durum wheat cultivars*

Seasons	Spikes no. m^2	Spike wt. m^2	Average spike wt. (g)	Spike length (cm)	Grains no. $spike^{-1}$	Grains wt. $spike^{-1}$ (g)	1000-grain wt. (g)	Grain yield t h^{-1}	Biological yield t h^{-1}	Harvest index
2014-2015	367.333	535.445	1.527	9.337	43.445	1.208	23.974	3.070	8.602	0.356
2015-2016	281.833	481.556	2.085	7.061	42.167	1.398	37.980	4.004	15.458	0.259
2016-2017	410.445	835.617	2.179	6.029	39.534	1.610	38.100	4.667	10.031	0.464
2017-2018	355.556	617.776	1.876	6.592	45.122	1.620	35.463	3.983	11.011	0.362
2018-2019	393.457	928.585	3.230	6.647	50.372	2.508	50.294	7.048	17.073	0.423
$LSD_{0.05}$	35.444	51.365	0.207	0.504	3.904	0.245	1.650	0.297	0.591	0.159

Data represented in *Table 5* illustrate stability and genotypic resultant across all seasons for all studied characters. Highly significant interaction due to cultivars and seasons for all studied characters were recorded from this table variant values of stability and genetic resultant for all characters were represented. Iraq-7 cultivar recorded the highest stability for spike number m^{-2} reached 0.643, while the highest genotypic resultant was o.637 recorded by Cham-1 for the same trait. The Simeto cultivar exhibited the highest value for stability and genotypic resultant due to traits spike weight m^{-2}, average spike weight, grain weight $spike^{-1}$, 1000-grain weight, grain yield and harvest index reached 0.690 and 0.750, 0.574 and 0.690, 0.523 and 0.649, 0.633 and 0.748, 0.638 and 0.729, 0.634 and 0.714 respectively. Ovanto cultivar showed the highest value for stability and genotypic resultant due to spike length reached o.801 and 0.824 respectively. The highest value for stability and genotypic resultant for both grains number $spike^{-1}$ and biological yield recorded by Crezo cultivar reached 0.739, 0.838 and 0.829, 0.951 respectively.

Table 5. *Stability (H) and genotypic resultant (GR) of treats a cross 5 years*

Variety	H and GR	Spikes no. m^2	Spike wt. m^2	Average spike wt. (g)	Spike length (cm)	Grains no. $spike^{-1}$	Grains wt. $spike^{-1}$ (g)	1000-grain wt. (g)	Grain yield t h^{-1}	Biological yield t h^{-1}	Harvest index
Iraq-7	H	0.643	0.687	0.451	0.796	0.701	0.361	0.527	0.634	0.808	0.632
	GR	0.416	0.739	0.421	0.826	0.691	0.335	0.482	0.715	0.823	0.708
Acsad-65	H	0.600	0.649	0.412	0.795	0.687	0.326	0.550	0.571	0.809	0.571
	GR	0.624	0.622	0.359	0.820	0.647	0.286	0.530	0.550	0.832	0.549
Cham-1	H	0.605	0.612	0.430	0.767	0.683	0.314	0.508	0.537	0.809	0.523
	GR	0.637	0.533	0.387	0.696	0.636	0.271	0.447	0.480	0.831	0.452
Ovanto	H	0.528	0.635	0.473	0.801	0.694	0.378	0.558	0.557	0.738	0.555
	GR	0.466	0.586	0.460	0.849	0.669	0.360	0.546	0.518	0.552	0.515
Crezo	H	0.568	0.689	0.543	0.782	0.739	0.478	0.599	0.562	0.829	0.587
	GR	0.548	0.746	0.608	0.759	0.836	0.542	0.647	0.530	0.951	0.586
Simeto	H	0.535	0.690	0.574	0.787	0.718	0.523	0.633	0.638	0.810	0.634
	GR	0.479	0.750	0.690	0.782	0.752	0.649	0.748	0.729	0.836	0.714

Simple correlation coefficients among characters were representing in *Table 6* for all seasons. Regarding to the first season 2014-2015 (in *Table 6a*) which illustrate the correlation among characters and the path analysis to determine the direct and indirect effect of yield components on grain yield. Grain yield recorded highly significant and positive correlation with all its components except biological yield. Highly significant and positive correlations were recorded between spikes number m^{-2} with all characters except

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average spike weight, grain weight spike^{-1} and biological yield which were not significant. Spike weight m^{-2} recorded highly significant and positive correlation with all characters except biological yield, which was not significant. Average spike weight produced highly significant and positive correlation with all characters except spike number m^{-2} and biological yield which were not significant. Highly significant and positive correlations were recorded between spike length and the other characters. Grains number spike^{-1} recorded highly significant and positive correlation with all characters except spike number m^{-2} which was only significant and biological yield which was not significant. Grain weight spike^{-1} showed highly significant and positive correlation with all characters except biological yield which was only significant and spikes number m^{-2} which was not significant. 1000-grain weight recorded highly significant and positive correlation with all characters except biological yield which was not significant.

Table 6a. *Simple correlation coefficient among each pairs of traits at 2014-2015*

Characters	Grain yield t h^{-1}	Spikes no. m^2	Spike wt. m^2	Average spike wt. (g)	Spike length (cm)	Grains no. spike^{-1}	Grains wt. spike^{-1} (g)	1000-grain wt. (g)	Biological yield t h^{-1}	Harvest index
Grain yield t h^{-1}	1									
Spikes no. m^2	0.709	1								
Spike wt. m^2	0.968	0.786	1							
Average spike wt. (g)	0.900	0.438	0.893	1						
Spike length (cm)	0.859	0.583	0.914	0.950	1					
Grains no. spike^{-1}	0.763	0.484	0.837	0.834	0.799	1				
Grains wt. spike^{-1} (g)	0.820	0.420	0.852	0.971	0.961	0.853	1			
1000-grain wt. (g)	0.982	0.806	0.990	0.862	0.865	0.806	0.811	1		
Biological yield t h^{-1}	0.349	0.415	0.405	0.420	0.617	0.063	0.487	0.359	1	
Harvest index	0.896	0.579	0.845	0.755	0.628	0.802	0.649	0.881	-0.100	1

Path

Characters	Spikes no. m^2	Spike wt. m^2	Average spike wt. (g)	Spike length (cm)	Grains no. spike^{-1}	Grains wt. spike^{-1} (g)	1000-grain wt. (g)	Biological yield t h^{-1}	Harvest index
Spikes no. m^2	0.000	0.000	0.000	0.167	-0.109	0.000	0.000	0.116	0.535
Spike wt. m^2	0.000	0.000	0.000	0.262	-0.189	0.000	0.000	0.113	0.781
Average spike wt. (g)	0.000	0.000	0.000	0.273	-0.188	0.000	0.000	0.117	0.698
Spike length (cm)	0.000	0.000	0.000	0.287	-0.180	0.000	0.000	0.172	0.580
Grains no. spike^{-1}	0.000	0.000	0.000	0.229	-0.225	0.000	0.000	0.018	0.741
Grains wt. spike^{-1} (g)	0.000	0.000	0.000	0.276	-0.192	0.000	0.000	0.136	0.600
1000-grain wt. (g)	0.000	0.000	0.000	0.248	-0.182	0.000	0.000	0.100	0.815
Biological yield t h^{-1}	0.000	0.000	0.000	0.177	-0.014	0.000	0.000	0.279	-0.093
Harvest index	0.000	0.000	0.000	0.180	-0.181	0.000	0.000	-0.028	0.924

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From the same table the path coefficient analysis for the direct and indirect effect of yield components on grain yield represent in the same table due to the first season. Maximum positive direct effect in grain yield was 0.924 recorded by harvest index and followed by spike length with 0.287. Maximum indirect effect in grain yield 0.815 recorded by harvest index via 1000-grain weight and followed by 0.781 for also harvest index via spike weight m^{-2}.

At the second season (*Table 6b*) highly significant and positive correlation were recorded between grain yield and spike weight m^{-2}, average spike weight, grain weight $spike^{-1}$ and harvest index, while the correlation was significant and positive between grain yield with spike number m^{-2} and 1000-grain weight. Spike number m^{-2} recorded highly significant and positive correlation with spike weight m^{-2}, while significant and positive correlation was recorded between spike number m^{-2} with spike length and harvest index.

Table 6b. *Simple correlation coefficient among each pairs of traits at 2015-2016*

Characters	Grain yield t h^{-1}	Spikes no. m^2	Spike wt. m^2	Average spike wt. (g)	Spike length (cm)	Grains no. $spike^{-1}$	Grains wt. $spike^{-1}$ (g)	1000-grain wt. (g)	Biological yield t h^{-1}	Harvest index
Grain yield t h^{-1}	1									
Spikes no. m^2	0.522	1								
Spike wt. m^2	0.953	0.715	1							
Average spike wt. (g)	0.881	0.266	0.827	1						
Spike length (cm)	-0.180	-0.553	-0.306	0.172	1					
Grains no. $spike^{-1}$	-0.247	0.006	-0.157	-0.423	-0.259	1				
Grains wt. $spike^{-1}$ (g)	0.843	0.136	0.751	0.907	0.246	-0.080	1			
1000-grain wt. (g)	0.546	0.004	0.401	0.717	0.637	-0.673	0.631	1		
Biological yield t h^{-1}	0.310	0.156	0.208	0.356	0.260	-0.917	0.085	0.748	1	
Harvest index	0.984	0.537	0.961	0.847	-0.244	-0.081	0.855	0.424	0.136	1

Path

Characters	Spikes no. m^2	Spike wt. m^2	Average spike wt. (g)	Spike length (cm)	Grains no. $spike^{-1}$	Grains wt. $spike^{-1}$ (g)	1000-grain wt. (g)	Biological yield t h^{-1}	Harvest index
Spikes no. m^2	0.083	0.000	0.000	0.245	0.001	0.124	0.000	0.069	0.000
Spike wt. m^2	0.059	0.000	0.000	0.135	-0.018	0.685	0.000	0.092	0.000
Average spike wt. (g)	0.022	0.000	0.000	-0.076	-0.050	0.827	0.000	0.158	0.000
Spike length (cm)	-0.046	0.000	0.000	-0.443	-0.030	0.225	0.000	0.115	0.000
Grains no. $spike^{-1}$	0.000	0.000	0.000	0.115	0.117	-0.073	0.000	-0.406	0.000
Grains wt. $spike^{-1}$ (g)	0.011	0.000	0.000	-0.109	-0.009	0.912	0.000	0.037	0.000
1000-grain wt. (g)	0.000	0.000	0.000	-0.282	-0.079	0.576	0.000	0.331	0.000
Biological yield t h^{-1}	0.013	0.000	0.000	-0.115	-0.108	0.077	0.000	0.443	0.000
Harvest index	0.045	0.000	0.000	0.108	-0.010	0.780	0.000	0.060	0.000

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Spike weight m^{-2} showed highly significant and positive correlation with average grain weight $spike^{-1}$ and harvest index. Average spike weight recorded highly significant and positive correlation with grain weight $spike^{-1}$, 1000-grain weight and harvest index. Spike length showed highly significant and positive correlation with 1000-grain weight. Grain number $spike^{-1}$ produced highly significant and positive correlation with 1000-grain weight and biological yield. Highly significant and positive correlation was recorded between grain weight $spike^{-1}$ with 1000-grain weight and harvest index. 1000-grain weight showed highly significant and positive correlation with biological yield.

From the same table and the second season the path analysis confirmed that the maximum direct effect in grain yield recorded by grain weight $spike^{-1}$ with 0.912 and followed by biological yield 0.443, while maximum positive indirect effect was 0.827 recorded by grain weight $spike^{-1}$ via average spike weight and followed by 0.780 for also grain weight $spike^{-1}$ via harvest index.

Concerning to the third season 2016-2017 (*Table 6c*) the grain yield recorded highly significant and positive correlation with all characters except spike number m^{-2} and spike length which were significant only. Spike number m^{-2} recorded highly significant and negative correlation with average spike weight, grain weight $spike^{-1}$, 1000-grain weight and biological yield, but it correlated significantly and negatively with spike weight m^{-2} and grain number $spike^{-1}$. Spike weight m^{-2} produced highly significant and positive correlation with average spike weight, grain number and weight $spike^{-1}$, 1000-grain weight, biological yield and harvest index, while it correlated significantly and positively with spike length. Average spike weight showed highly significant and positive correlation with grain number and weight $spike^{-1}$, 1000-grain weight, biological weight and harvest index. Spike length correlated significantly and positively with grain number $spike^{-1}$ and harvest index. There are highly significant and positive correlations between grain number $spike^{-1}$ with each of grain weight $spike^{-1}$, 1000-grain weight, biological yield and harvest index. Grain weight $spike^{-1}$ recorded highly significant and positive correlation with 1000-grain weight, biological yield and harvest index. 1000-grain weight showed highly significant and positive correlation with biological yield. Biological yield gave highly significant and positive correlation with harvest index.

From the same table, it was noticed that harvest index produced maximum direct effect value in grain yield reached 0.638 and followed by 0.491 for biological yield. The highest indirect effect value on grain yield recorded by harvest index via grain number $spike^{-1}$ reached 0.592 and followed by 0.513 for harvest index also via spike weight m^{-2}.

Concerning to the fourth season 2017-2018 (*Table 6d*), the correlation between each pairs of characters represent in the same table. Grain yield recorded highly significant and positive association with spike number and weight m^{-2} and biological yield, while it correlated significantly and positively with spike length and harvest index, but it correlated significantly and negatively with 1000-grain weight. Spike number m^{-2} produced highly significant and negative association with average spike weight, grain weight $spike^{-1}$ and 1000-grain weight, and correlated high significantly and positively with spike weight m^{-2} and biological yield. Spike weight m^{-2} recorded highly significant and positive correlation with spike length and biological yield, but it correlated high significantly and negatively with grain weight $spike^{-1}$ and 1000-grain weight, and also recorded significant correlation and negative correlation significant with average spike weight. Average spike weight produced highly significant and positive correlation with grain number and weight $spike^{-1}$ and 1000-grain weight, while it correlated high significantly and negatively with biological yield, whilst the correlation between

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average spike weight and harvest index was significant and positive. Highly significant and positive correlation was recorded between grain number spike^{-1} and each of grain weight spike^{-1}, 1000-grain weight and harvest index. Grain weight spike^{-1} showed highly significant and positive correlation with 1000-grain weight, while it correlated high significantly and negatively with biological yield and also correlated significantly and positively with harvest index. 1000-grain weight recorded highly significant and negative correlation with biological yield.

From the same table the path analysis indicated that the harvest index recorded the highest direct effect in grain yield reached 1.024 and followed by 0.973 for spike weight m^{-2}. The highest indirect effect value on grain yield was 0.896 recorded by spike weight m^{-2} via biological yield and followed by 0.703 for harvest index via grain number spike^{-1}.

Table 6c. *Simple correlation coefficient among each pairs of traits at 2016-2017*

Characters	Grain yield t h^{-1}	Spikes no. m^2	Spike wt. m^2	Average spike wt. (g)	Spike length (cm)	Grains no. spike^{-1}	Grains wt. spike^{-1} (g)	1000-grain wt. (g)	Biological yield t h^{-1}	Harvest index
Grain yield t h^{-1}	1									
Spikes no. m^2	-0.445	1								
Spike wt. m^2	0.899	-0.556	1							
Average spike wt. (g)	0.909	-0.766	0.911	1						
Spike length (cm)	0.536	0.260	0.498	0.318	1					
Grains no. spike^{-1}	0.945	-0.508	0.864	0.917	0.484	1				
Grains wt. spike^{-1} (g)	0.835	-0.810	0.739	0.946	0.145	0.839	1			
1000-grain wt. (g)	0.624	-0.977	0.704	0.884	-0.091	0.665	0.899	1		
Biological yield t h^{-1}	0.910	-0.676	0.888	0.930	0.244	0.808	0.890	0.804	1	
Harvest index	0.940	-0.192	0.804	0.771	0.727	0.927	0.667	0.389	0.715	1

Path

Characters	Spikes no. m^2	Spike wt. m^2	Average spike wt. (g)	Spike length (cm)	Grains no. spike^{-1}	Grains wt. spike^{-1} (g)	1000-grain wt. (g)	Biological yield t h^{-1}	Harvest index
Spikes no. m^2	0.000	0.024	0.000	-0.007	0.000	0.000	-0.008	-0.332	-0.123
Spike wt. m^2	0.000	-0.043	0.000	-0.013	0.000	0.000	0.006	0.436	0.513
Average spike wt. (g)	0.000	-0.039	0.000	-0.008	0.000	0.000	0.007	0.457	0.492
Spike length (cm)	0.000	-0.021	0.000	-0.025	0.000	0.000	-0.001	0.120	0.464
Grains no. spike^{-1}	0.000	-0.037	0.000	-0.012	0.000	0.000	0.005	0.397	0.592
Grains wt. spike^{-1} (g)	0.000	-0.032	0.000	-0.004	0.000	0.000	0.007	0.437	0.426
1000-grain wt. (g)	0.000	-0.030	0.000	0.002	0.000	0.000	0.008	0.395	0.249
Biological yield t h^{-1}	0.000	-0.038	0.000	-0.006	0.000	0.000	0.007	0.491	0.457
Harvest index	0.000	-0.034	0.000	-0.019	0.000	0.000	0.003	0.351	0.638

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Table 6d. *Simple correlation coefficient among each pairs of traits at 2017-2018*

Characters	Grain yield t h^{-1}	Spikes no. m^2	Spike wt. m^2	Average spike wt. (g)	Spike length (cm)	Grains no. $spike^{-1}$	Grains wt. $spike^{-1}$ (g)	1000-grain wt. (g)	Biological yield t h^{-1}	Harvest index
Grain yield t h^{-1}	1									
Spikes no. m^2	0.655	1								
Spike wt. m^2	0.618	0.911	1							
Average spike wt. (g)	-0.232	-0.735	-0.549	1						
Spike length (cm)	0.459	0.314	0.574	-0.161	1					
Grains no. $spike^{-1}$	0.232	-0.413	-0.280	0.881	-0.070	1				
Grains wt. $spike^{-1}$ (g)	-0.252	-0.821	-0.688	0.887	-0.276	0.788	1			
1000-grain wt. (g)	-0.478	-0.917	-0.811	0.784	-0.366	0.581	0.957	1		
Biological yield t h^{-1}	0.627	0.925	0.921	-0.687	0.370	-0.369	-0.656	-0.738	1	
Harvest index	0.511	-0.304	-0.306	0.473	0.174	0.691	0.557	0.400	-0.292	1

Path

Characters	Spikes no. m^2	Spike wt. m^2	Average spike wt. (g)	Spike length (cm)	Grains no. $spike^{-1}$	Grains wt. $spike^{-1}$ (g)	1000-grain wt. (g)	Biological yield t h^{-1}	Harvest index
Spikes no. m^2	0.000	0.887	0.000	-0.106	0.061	0.000	0.125	0.000	-0.311
Spike wt. m^2	0.000	0.973	0.000	-0.194	0.041	0.000	0.111	0.000	-0.313
Average spike wt. (g)	0.000	-0.534	0.000	0.055	-0.130	0.000	-0.107	0.000	0.484
Spike length (cm)	0.000	0.559	0.000	-0.338	0.010	0.000	0.050	0.000	0.178
Grains no. $spike^{-1}$	0.000	-0.272	0.000	0.024	-0.147	0.000	-0.080	0.000	0.707
Grains wt. $spike^{-1}$ (g)	0.000	-0.670	0.000	0.093	-0.116	0.000	-0.131	0.000	0.571
1000-grain wt. (g)	0.000	-0.789	0.000	0.124	-0.085	0.000	-0.137	0.000	0.410
Biological yield t h^{-1}	0.000	0.896	0.000	-0.125	0.054	0.000	0.101	0.000	-0.299
Harvest index	0.000	-0.297	0.000	-0.059	-0.102	0.000	-0.055	0.000	1.024

From the (*Table 6e*), the correlation between each pairs of characters was illustrated during the fifth season 2018-2019. Highly significant and positive correlation was recorded between grain yield and each of spike weight m^{-2}, average spike weight, spike length and grain weight $spike^{-1}$, but highly significant and negative correlation was observed between grain yield and spike number m^{-2}, whilst the correlation between grain yield and each of grain number $spike^{-1}$ and 1000-grain weight was significant and positive. Highly significant and negative correlation was recorded between spike number m^{-2} with each of average spike weight and grain number and weight $spike^{-1}$. Spike weight m^{-2} recorded highly significant and positive correlation with each of average spike weight, grain weight spike and 1000-grain weight. Average spike weight recorded highly significant and positive correlation with each of grain number and

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weight spike^{-1}, while with 1000-grain weight it correlated significantly and positively. Concerning to spike length highly significant and positive correlation was observed with harvest index. Grain number spike^{-1} correlated high significantly and positively with grain weight spike^{-1}. Grain weight spike^{-1} showed significant and positive correlation with 1000-grain weight. 1000-grain weight recorded significant and positive correlation with biological yield, but biological yield recorded highly significant and negative correlation with harvest index.

The path analysis for the last season represent in the same table, confirmed that the maximum direct effect in grain yield was 0.658 recorded by spike length and followed by 0.630 for average spike weight, while maximum positive indirect effect in grain yield was 0.588 recorded by average spike weight via grain weight spike^{-1} and followed by 0.534 for also average spike weight via grain number spike^{-1}.

Table 6e. *Simple correlation coefficient among each pairs of traits at 2018-2019*

Characters	Grain yield t h^{-1}	Spikes no. m^2	Spike wt. m^2	Average spike wt. (g)	Spike length (cm)	Grains no. spike^{-1}	Grains wt. spike^{-1} (g)	1000-grain wt. (g)	Biological yield t h^{-1}	Harvest index
Grain yield t h^{-1}	1									
Spikes no. m^2	-0.092	1								
Spike wt. m^2	0.917	-0.051	1							
Average spike wt. (g)	0.671	-0.688	0.739	1						
Spike length (cm)	0.630	0.217	0.312	0.016	1					
Grains no. spike^{-1}	0.464	-0.869	0.366	0.848	0.168	1				
Grains wt. spike^{-1} (g)	0.732	-0.693	0.700	0.935	0.162	0.838	1			
1000-grain wt. (g)	0.529	0.053	0.728	0.457	-0.185	-0.026	0.501	1		
Biological yield t h^{-1}	0.229	-0.251	0.388	0.357	-0.293	0.060	0.293	0.521	1	
Harvest index	0.391	0.200	0.184	0.048	0.665	0.200	0.161	-0.152	-0.805	1

Path

Characters	Spikes no. m^2	Spike wt. m^2	Average spike wt. (g)	Spike length (cm)	Grains no. spike^{-1}	Grains wt. spike^{-1} (g)	1000-grain wt. (g)	Biological yield t h^{-1}	Harvest index
Spikes no. m^2	0.205	0.000	-0.433	0.143	0.000	0.000	0.016	-0.022	0.000
Spike wt. m^2	-0.010	0.000	0.465	0.205	0.000	0.000	0.222	0.034	0.000
Average spike wt. (g)	-0.141	0.000	0.630	0.011	0.000	0.000	0.140	0.032	0.000
Spike length (cm)	0.044	0.000	0.010	0.658	0.000	0.000	-0.056	-0.026	0.000
Grains no. spike^{-1}	-0.178	0.000	0.534	0.110	0.000	0.000	-0.008	0.005	0.000
Grains wt. spike^{-1} (g)	-0.142	0.000	0.588	0.106	0.000	0.000	0.153	0.026	0.000
1000-grain wt. (g)	0.011	0.000	0.288	-0.121	0.000	0.000	0.305	0.046	0.000
Biological yield t h^{-1}	-0.051	0.000	0.225	-0.192	0.000	0.000	0.159	0.089	0.000
Harvest index	0.041	0.000	0.030	0.438	0.000	0.000	-0.047	-0.071	0.000

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The simple correlation coefficient among each pairs of traits for the average of all seasons represent in (*Table 6f*), confirmed that the grain yield recorded highly significant and positive correlation with spike weight m^{-2} and harvest index, while with grain weight $spike^{-1}$ recorded significant and positive correlation.

Table 6f. *Simple correlation coefficient among each pair of the average of all seasons*

Characters	Grain yield t h^{-1}	Spikes no. m^2	Spike wt. m^2	Average spike wt. (g)	Spike length (cm)	Grains no. $spike^{-1}$	Grains wt. $spike^{-1}$ (g)	1000-grain wt. (g)	Biological yield t h^{-1}	Harvest index
Grain yield t h^{-1}	1									
Spikes no. m^2	0.160	1								
Spike wt. m^2	0.753	0.019	1							
Average spike wt. (g)	0.435	-0.616	0.688	1						
Spike length (cm)	0.340	-0.095	0.224	-0.104	1					
Grains no. $spike^{-1}$	0.233	-0.335	0.778	0.828	-0.110	1				
Grains wt. $spike^{-1}$ (g)	0.484	-0.589	0.733	0.994	-0.057	0.827	1			
1000-grain wt. (g)	0.434	-0.685	0.656	0.938	0.062	0.737	0.963	1		
Biological yield t h^{-1}	0.170	0.375	0.498	0.270	-0.558	0.516	0.308	0.230	1	
Harvest index	0.964	0.116	0.898	0.544	0.362	0.454	0.596	0.538	0.282	1

Path

Characters	Spikes no. m^2	Spike wt. m^2	Average spike wt. (g)	Spike length (cm)	Grains no. $spike^{-1}$	Grains wt. $spike^{-1}$ (g)	1000-grain wt. (g)	Biological yield t h^{-1}	Harvest index
Spikes no. m^2	-0.228	0.040	0.000	0.051	0.451	0.000	0.000	-0.154	0.000
Spike wt. m^2	-0.004	2.129	0.000	-0.120	-1.047	0.000	0.000	-0.204	0.000
Average spike wt. (g)	0.141	1.465	0.000	0.056	-1.116	0.000	0.000	-0.111	0.000
Spike length (cm)	0.022	0.477	0.000	-0.537	0.148	0.000	0.000	0.229	0.000
Grains no. $spike^{-1}$	0.076	1.655	0.000	0.059	-1.347	0.000	0.000	-0.212	0.000
Grains wt. $spike^{-1}$ (g)	0.135	1.559	0.000	0.031	-1.114	0.000	0.000	-0.126	0.000
1000-grain wt. (g)	0.156	1.397	0.000	-0.033	-0.993	0.000	0.000	-0.094	0.000
Biological yield t h^{-1}	-0.086	1.061	0.000	0.300	-0.695	0.000	0.000	-0.410	0.000
Harvest index	-0.026	1.912	0.000	-0.194	-0.612	0.000	0.000	-0.116	0.000

Spike number m^{-2} showed highly significant and negative correlation with each of average spike weight, grain weight $spike^{-1}$ and 1000-grain weight. Spike weight m^{-2} produced highly significant and positive correlation with average spike weight, grain number and weight $spike^{-1}$, 1000-grain weight and harvest index, whilst with biological yield and positive correlation was recorded. There are highly significant and positive correlation between average spike weight and each of grain number and weight $spike^{-1}$

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and 1000-grain weight, while significant and positive correlation was recorded between average spike weight and harvest index. Spike length recorded significant and negative correlation with biological yield. Highly significant and positive correlation was recorded between grain number spike^{-1} with each of grain weight spike^{-1} and 1000-grain weight, but it correlated significantly and positively with each of biological yield and harvest index. Grain weight spike^{-1} showed highly significant and positive correlation with each of 1000-grain weight and harvest index. Finally, 1000-grain weight recorded significant and positive correlation with harvest index.

The path analysis for the studied characters as the average of all seasons was illustrated in the same table, confirming that the spike weight m^{-2} recorded maximum positive direct effect in grain yield reached 2.129, while maximum negative direct effect was -1.347 recorded by grain number spike^{-1}. Maximum positive indirect effect in grain yield was 1.912 recorded by spike weight m^{-2} via harvest index and followed by 1.655 for also spike weight m^{-2} via grain number spike^{-1}. Maximum negative indirect effect in grain yield was -1.116 produced by grain number spike^{-1} via average spike weight and followed by -1.114 for also grain number spike^{-1} via grain weight spike^{-1}.

Discussion

The results of variance analysis for spikes number m^{-2}, spike length, grains number spike^{-1}, spike weight, 1000-grain weight and grain yield are given. Effects of locations and years on investigated traits were statistically significant ($P < 0.01$), except for year effects on spike weight. Differences among the genotypes were significant for all investigated traits. Genotypes × environment interactions were found to be significant for all investigated traits except for spikes number m^{-2}. The results of the combined analysis of variance showed a strong influence of the locations on spikes number m^{-2}, grains number spike^{-1}, spike weight, 1000-grain weight and grain yield. Genotypic effects were mainly observed for spike length. Gradual changes in yield and yield components were determined by the genotype and also by the environment (Moragues et al., 2006).

Two years averaged values of yield components and grain yield. The reason of upper grain yield at Sivas-Ulas could be also short period of dry matter production and nutrition conditions (Rharrabti et al., 2003). Negative effects on spikes m^{-2} were minor and 1000-grain weight could be maintained. The simultaneous increase in both spikes m^{-2} and grains spike^{-1} produced the highest increase in grains m^{-2}, grain yield plant^{-1}, and biomass (Pfeiffer et al., 2000). For instance, in durum wheat growing in Mediterranean environments, grain weight was superior in modern cultivars in Turkey (Koç et al., 2003), but remained unchanged in Italian and Spanish cultivars from the 20th century (Royo et al., 2007). In bread wheat, grain weight has been reduced (Trethowan et al., 2007; Matus et al., 2012; Guarda et al., 2012) or has not changed (Zhou et al., 2007) with genetic improvement. The increase in grain yield was a consequence of a greater grains number m^{-2} and higher grain weight in the more modern cultivars. The test weight was lower in the 1960s and increased curvilinear with year of cultivar release. The yield progress of a set of advanced lines evaluated between 2006 and 2015 was very high, due to genetic progress, but this was also due to management improvements, particularly adjustment of fertilization practices conducted during the first three years. Unlike other Mediterranean agro environments, a longer growing cycle together with taller plants seems to be related to the increase in the grain yield of durum

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wheat during recent decades (Alejandro et al., 2019). Hence, future crop improvement has to emphasize grain yield potential (grain yield plant^{-1}), yield stability, and user preferences in concerted, interdisciplinary approaches. Issues of environmental sustainability must be an integral part of the research agenda. To achieve these goals, crop breeding at CIMMYT aims to protect high genetic yield potential as a prerequisite of broad adaptation through incorporating resistance to a biotic and biotic stresses.

This strategy capitalizes on newer empirical methods. The analysis revealed that improvements in grain yield plant^{-1} (Pfeiffer et al., 2000). Grain yield growth rates must match future demands for food. To achieve projected production levels, breeding for realized grain yield should emphasize enhancement of yield per se and grain yield stabilization through integrated, interdisciplinary approaches that take into account environmental sustainability. This challenge requires concerted, complementary efforts to gather a critical mass of scientists and achieve essential operational sizes; sound hypotheses and strategies, translated into breeding objectives; free exchange of Germplasm and information; and dynamic cooperation among the global community of scientists. Each one of these requirements must be met if we are to accomplish our common mission: the alleviation of poverty in developing countries (Pfeiffer et al., 2000). Higher grain yields are associated with higher grain weight (v4), which resulted from early flowering (v1), and so more emphasis should be given to these traits for the improvement of yield potential in durum wheat under highland rain fed conditions. Positive correlation of stability variance (σ^2) with v4 component indicated that the grain weight is the main contributor towards GE interaction for grain yield in rain fed durum wheat (Mohammadi et al., 2016).

In modern durum wheat resulted from higher biomass, primarily through an increased grains number m^{-2} via an augmented spikes number m^{-2} and/or grains spike^{-1}. Spike weight and grain biomass production rate per day increased, while 1000-grain weight decreased (Pfeiffer et al., 2000). Earlier efforts to increase biomass focused on manipulating spikes m^{-2} and later by augmenting the grains number spike^{-1}, both of which are suitable traits in phenotypic selection. The avenue of selecting for grains m^{-2} via a higher grains number spike^{-1} proved superior in raising grain yield plant^{-1}. The balance in yield components may have approached a near optimal constellation, as results from crop comparison suggest. With limited scope for increasing the partitioning of assimilates to the grain, future progress has to be based on increased biomass (Pfeiffer et al., 2000). The maximum values of harvest index (0.53) found in the current work were higher than those reported by Royo et al. (2007). The correlation matrix among the agronomic traits of the 10 cultivars evaluated during three growing seasons indicated that grain yield showed a positive and significant correlation with grain m^{-2} ($p < 0.05$) and 1000-grain weight ($p < 0.001$). Plant height showed a negative and highly significant ($p < 0.001$) correlation with 1000-grain weight and harvest index. Spike m^{-2} had a positive correlation with grain m^{-2} but a negative correlation with grain spike^{-1}. There was a positive relationship between grain yield and spikes number m^{-2} together, whereas spike lengths were negatively correlated to grain yield.

The results of this study also imply that Line-5 and cultivar Gidara among genotypes were the most stable cultivars and can be used as breeding materials. The spikes number m^{-2} and spike length could be adequate to introduce the differences among genotypes (Sakin et al., 2011). The stability parameters showed a wide range of variation between cultivars for grain yield. By simultaneous selection for yield and stability the cultivars Crezo and Iraq-7 had the best values according to most parameters of stability; hence, it

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has a wide adaptability over a range of environments of rainfall conditions in Sulaimani, Kurdistan-Iraq (Aziz et al., 2015). The old cultivar Rash gull was characterized by a minimal responsiveness to improved environmental conditions, showing an almost stable grain yield in agreement with the concept of stability. Crezo and Iraq-7 cultivars had the best values according to the most parameters of stability (bi, S_2di, Pi, DFM, EV and mean); hence, it has a wide adaptability over a range of environments and may be considered as a future wheat cultivar for wide range cultivation under varying of rainfall conditions in Iraq (Aziz et al., 2015).

Improved yield stability, as evidenced by the correlation of grain weight and plant height with stability variance of yield. This indicates that the key strategies for yield stability improvement are most likely to be the grain weight and plant height under rain fed conditions. High yielding breeding lines at warm and moderate cold locations had good tolerance ability throughout the whole stress season especially to terminal drought and heat stresses. The cold stress was more dominant than drought stress at cold locations, as none of the breeding lines did not surpass the bread wheat old variety (beard wheat) cultivar with good tolerance to cold stress and widely adapted to highland rain fed regions indicating no genetic gain for cold tolerance in breeding lines compared to this popular cultivar. Mean yield of five top yielding breeding lines at warm location was 2469 kg ha^{-1} and at moderate location was 1930 kg ha^{-1} and top old variety (G25) at warm and moderate cold locations produced 1884 and 1624 kg ha^{-1}, respectively. These results indicated yield improvements equal to 40 and 18% for first five top yielding breeding. The results also clearly indicated that higher grain yields are associated with higher grain weight (v4), so more emphasis should be given to these traits for the improvement of yield in durum wheat under rain fed conditions. Selection for high value grain weight resulting from yield stability in breeding lines which is a major step towards facilitating the increasing a biotic stress expected from the predicted climate change. In conclusion, path analysis provided a useful picture for understanding GE interaction and grain yield components compensation in rain fed durum wheat, and hence these traits may be taken as indices of selection purposes. The responses of the individual genotypes did not reveal a common structure that would explain genotypic differences in tolerance to environmental stresses. However, the determination of genotypic strategies that maximize tolerance to environmental stresses deserves further research (Mohammadi et al., 2016).

The results of combined analysis of variance showed a strong influence of the locations on plant height, spikes number m^{-2}, grains number $spike^{-1}$, spike weight, 1000-grain weight and grain yield. Genotypic effects were mainly observed for spike length (Sakin et al., 2011). Strong influence of environmental conditions on spikes number m^{-2}, grains number $spike^{-1}$, spike weight, 1000-grain weight and grain yield. Genotypic effects were mainly observed for spike length. Diyarbakir location which had higher average rains and temperatures in the experimental years resulted better ecological conditions for durum wheat cultivation when compared with that of Tokat and Sivas locations. The highest grain yield was obtained from Line 299, whereas the lowest grain yield was obtained from Line-Gdem-2-1. Line-4 and cultivar Gidara can be considered as judged by their *bi* values and adaptation classifications, whereas genotype line 5 can only be considered stable by the *S2 d* value. Line 5 and cultivar Gidara were both stable in yield ability and also appeared in the stable group based on the cluster analysis. In the first principal component spikes number m^{-2} and spike length were the most important traits contributing to variation that obtained about 44.3%. There was a positive

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relationship between grain yield and spikes number m^{-2} together, whereas spike length was negatively correlated to grain yield. The results of this study also imply that Line 5 and cultivar Gidara among genotypes were the most stable cultivars and can be used as breeding materials. The spikes number m^{-2} and spike length could be adequate to introduce the differences among genotypes. (Sakin et al., 2011).

Environmental variations seemed to be of importance in determining performance, and therefore, evaluation based on several years and locations is a necessary strategy to be pursued in the breeding program (Yue et al., 1997). Year to- year climatic variation has a great impact on the degree of stress experienced by crops, hence the use of testing environments to represent stressed target environments. Since each environment consists of a combination of various factors, in other words, cold and drought stresses that influence adaptation and stability performance, it is difficult to specify all the differences between environments in relation to these factors (Chapman et al., 1997). High yield of durum wheat under fluctuation environments requires not only high yield in a unique environment, but also the stability of relatively high yield across varied environments (Mohammadi et al., 2016). The main purpose of multi-environment experiments in durum wheat is to identify superior varieties based on multiple traits and mega environments. Given the unpredictable environmental factors in the GE interaction studies, different models (GE, GE interaction, and AMMI), were developed to elucidate the effect of genotype, environment, or interaction; they are still used in breeding studies (Kendal, 2019; Kendal and Sayar, 2016).

In addition, the GT biplot technique has been used for a long time by many researchers to understand the effect of genotype and environment on the relationships between agronomic, physiological and quality characters, and yield (Yan and Tinker, 2006; Kendal and Dogan, 2015; Akcura et al., 2016; Oral et al., 2018). The GT biplot is used to compare varieties based on multiple traits and to define them based on these traits. This technique does not suffice to determine the effect of combining all the traits on yield under multiple environmental conditions, while the relationship between each trait and yield can be determined. Therefore, the GYT biplot technique has been developed to determine the effect of combining all traits with yield under multiple environmental conditions. However, publications based on multiple traits combined with grain yield (GYT) in different environments to evaluate the varieties are limited (Yan and Frégeau-Reid, 2018). Genotype×environmental interaction (GEI) is an important consideration in plant breeding programs because it reduces the progress from selection in any one environment (Hill, 1975). Significant GEI results from the changes in the magnitude of differences between genotypes in different environments or changes in the relative ranking of the genotypes. Consistent performances across different sites and/or years are referred to as stability. Partitioning GEI into stability statistics assignable to each genotype evaluated across a range of environments is useful in selecting stable genotypes. Different stability estimates are proposed to measure the stability of genotypes tested under a wide range of environments (Fernandez et al., 1989; Hill, 1975; Pritts and Luby, 1990).

Conclusion

It was observed clearly that the performance of each cultivar was differed from location to other depending on the climatic condition, referring to positive response of this cultivar to favorable environmental factors of that location. The presence of the

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genotype × environment interaction was indicated by changes in relative rankings over environments. The stability pattern revealed by the analysis indicated that the tested wheat genotypes are narrowly adapted, and no genotype was found to have high grain yield plant^{-1} performances in all environments. The development of high-yielding cultivars requires a thorough knowledge of the existing genetic variation for yield and its components for our cultivars under the study. The best stability and genotypic resultant recorded by Crezo cultivar due to the biological yield character at different environments, indicating this genotype had high performance at different environments and should be not disregarded in future studies. The Simeto cultivars, exhibited the best results for yield and most its components across all environments, could be used in future breeding programs to increase yield ability under normal and drought stress conditions, respectively. The stability parameters showed a wide range of variation between cultivars for grain yield. By simultaneous selection for yield and stability the cultivars Crezo and Iraq-7 had the best values according to most parameters of stability; hence, it has a wide adaptability over a range of environments of rainfall conditions in Sulaimani, Kurdistan-Iraq. In the view of present results it was concluded that environment plays an important role in correlation among characteristics.

Recommendation

Carrying out more investigations for cultivars with survival potentials in the prevailing climate conditions in the region, and under different environmental conditions to ensure their yield stability and to estimate their performances under different cultural practices. For more understanding of the environment, it is recommended to increase some locations under different planting dates to insure the stability and genotypic resultant of more cultivars.

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GENETIC ANALYSIS FOR SEVEN PEA CULTIVARS (*PISUM SATIVUM* L.) USING LINE × TESTER METHOD FOR SEED YIELD AND ITS COMPONENTS IN F_5 GENERATION UNDER SULAIMANI CONDITIONS

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Abstract. Seven pea cultivars (*Pisum sativum* L.) were crossed in a line × tester mating design. The 12 F_5 generation and their parents were evaluated using a completely randomized block design with three replications in Kurdistan Reign, Iraq at Qlyasan Agricultural Research Station, University of Sulaimani, during the winter season of 2015-2016. Heritability in a broad sense was high for all treats except number and seed weight pod^{-1} which were low, while in narrow sense it was low for all treats except average pod weight and 100-seed weight which were high. Highly significant and positive correlation was found between seed weight plant^{-1} with pod number plant^{-1}, while highly significant and negative correlation was recorded between seed weight plant^{-1} with pod length and seed weight pod^{-1}. Seed number pod^{-1} recorded maximum direct effect in seed weight plant^{-1}, while maximum positive indirect effect in seed weight plant^{-1} recorded by pod length via pod number plant^{-1}. The present study was conducted to find out the relative importance of various yield traits for seed yield and to evaluate promising genotypes by mean of correlation and to find genetically diverse genotypes which can be used further in a various breeding program in developing wide yielding varieties.
Keywords: *gene action, heterosis, heritability, combining ability, correlation and path analysis*

Introduction

Pea (*Pisum sativum* L.) is a self-pollinated, diploid crop with 14 chromosomes (2n = 14). Pea originates from Near East and Mediterranean regions. It is one of the world's oldest crops cultivated as early as 9,000 years ago for human consumption and animals feed (Askander et al., 2018). Peas is one of the four of the most important cultivated legume and largest world's legume crop in the production after soybean, peanuts and dry beans (Yoshida et al., 2007 and Smykal et al., 2012).

Pea (*Pisum sativum* L.) is an important legume grown as a garden and field crop throughout the temperate regions of the world. Pea is valued primarily for the nutritional quality of its seeds. Pea protein is low in sulfur containing amino acids i.e. cysteine and methionine, but rich in lysine and other essential amino acids (Ceyhan and Avci, 2005). It is the source of protein having essential amino acids that have high nutritional values for resource poor households (Nawab et al., 2008). Moreover, some important minerals such as calcium, phosphorus and iron are also present in abundant quantities in pea which are lacking in cereals (Haque et al., 2014). Pea contains 20-25% starch, 4-10% sugar, 0.6-1.5% fat and 2-4% minerals (Makasheva, 1983). The present nutritional situation is a matter of great concern because most of the people are suffering from malnutrition (Mahbub et al., 2016).

The line × tester analysis method is used to breed both self and cross-pollinated plants and to estimates favorable parents and crosses, and their general and specific combining abilities (Kempthorne, 1957). Combining ability analysis is an important

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tool for the selection of desirable parents together with the information regarding nature and magnitude of gene effects controlling quantitative traits (Basbag et al., 2007). General combining ability and specific combining ability which identify the hybrids with high yield are the most important criteria in breeding programs (Ceyhan, 2003).

The knowledge of combining ability and nature of gene action is necessary for the selection of best parents for hybridization in order to improve the existing cultivars. It is also necessary to know the performance of a cross combination in comparison to the parents involved in the hybrids (Tampha et al., 2018).

The objective of this study was to measure the phenotypic variability of these traits, to obtain the general and specific combining ability, and to estimate heritability, heterosis, correlation and path analysis assess their potential use in breeding for winter season that suits the environmental conditions prevailing in the region.

Materials and methods

The present investigation was conducted at Qlyasan locations, in Kurdistan Region-Iraq, Agricultural Research Station, College of Agricultural Engineering Sciences-University of Sulaimani located (Lat 35° 34′ 307″; N, Long 45° 21′ 992″; E, 765 masl) 2 km North West of Sulaimani City, during (2015-2016). The experimental material comprises seven pea cultivars (*Pisum sativum* L.). Three cultivars (1/Avolla, 2/America, 3/Jeza) implemented as females, hereafter designated as lines, and four cultivars (4/Joneor, 5/Packland, 6/Arvena, 7/Samara) were used as males, fixed as testers (*Table 1*). All parental cultivars were crossed to produce 12 F_1 crosses according to the line × tester mating design assessed by Kempthorne (1957). F_1 seeds were sown in the field, along with their parents, in Complete Randomize Block Design with three replication. Each plot comprised one row of 2 m length with space of 40 cm between rows and seeds were placed 20 cm apart. Five competitive plants (excluding border plants) were chosen and data were recorded for: Pods number plant^{-1}, pods weight plant^{-1}, pod length, average pod weight, seeds number pod^{-1}, seeds weight pod^{-1}, 100-seed weight, biological weight plant^{-1} and seeds weight plant^{-1}.

Table 1. *Studied breeding materials*

Line parent	Tester parent			
	4- Joneor	**5- Packland**	**6- Arvena**	**7- Samara**
1-Avolla	1 × 4 Avolla × Joneor	1 × 5 Avolla × Packland	1 × 6 Avolla × Arvena	1 × 7 Avolla × Samara
2-America	2 × 4 America × Joneor	2 × 5 America × Packland	2 × 6 America × Arvena	2 × 7 America × Samara
3-Jeza	3 × 4 Jeza × Joneor	3 × 5 Jeza × Packland	3 × 6 Jeza × Arvena	3 × 7 Jeza × Samara

Genetic parameters: general combining ability (gca) and effects, specific combining ability (sca) and effects, heterosis percentage as the F_1s deviation from average parental values, heritability in broad sense, heritability in narrow sense, average degree of dominance (ā), correlation and path coefficient analysis. The collected data were submitted to analysis of variance as proposed by Al-Mohammad and Al-Yonis (2000) to estimate significant differences among cultivars. Combining ability effects are very

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important genetic parameters in determining the next phase of breeding programs. They were computed according to the line × tester method (Singh and Chaudhary, 1985) (*Table 2*).

Table 2. *Analysis of variance according to line × tester design (Singh and Chaudhary, 1985)*

S.O.V	d.f	SS	MS
Replicates	*(r-1)*	$SS_R = \frac{\sum Y_{.k}^2}{g} - C.F\ (Over\ all)$	*MSR*
Genotypes	*(g-1)*	$SS_G = \frac{\sum\sum C_{ij}^2 + \sum\sum P_{ii.}^2}{r} - C.F\ (Over\ all)$	*MSG*
Parents	*(P-1)*	$SS_P = \frac{\sum\sum P_{ii.}^2}{r} - C.F\ (Parents)$	*MSP*
Crosses	*(Lt-1)*	$SS_C = \frac{\sum\sum C_{ij}^2}{r} - C.F\ (Crosses)$	*MSC*
Parents vs. Crosses	*1*	$SS_{P\ vs.\ C} = SS_G - SS_p - SS_C = C.F\ (Parents) + C.F\ (Crosses) - C.F\ (Over\ all)$	*MSP vs. C*
Lines	*(L-1)*	$SS_L = \frac{\sum C_{i.}^2}{rt} - C.F\ (Crosses)$	*MSL*
Testers	*(T-1)*	$SS_T = \frac{\sum C_{.j}^2}{rl} - C.F\ (Crosses)$	*MST*
Lines × Testers	*(L-1)(T-1)*	$SS_{L\ x\ T} = SS_C - SS_L - SS_T$	*MSL × T*
Error	*(r-1)(g-1)*	*SSe = SST - SSR - SSG*	*MSe*
Total	*(rg-1)*	$SS_{Total} = \sum Y_{ijk}^2 - C.F\ (Over\ all)$	

R = replication; L = line; T = tester; G = genotypes

Results

Data in (*Table 3*) illustrate the significance of ANOVA table components due to studied characters. The mean squares due to genotypes and parents were highly significant for all characters, while the mean squares due to parent's × crosses were highly significant for only pod weight plant^{-1}, pod length, biological yield plant^{-1} and seed weight plant^{-1}. The mean squares due to crosses were highly significant for all characters except seed number pod^{-1} which was significant only. The mean squares due to lines, there were highly significant mean squares for all characters except seed number and weight pod^{-1} which were only significant. The mean squares estimated for testers were highly significant for all characters except pod length which was significant only, but the characters seed number pod^{-1} and 100-seed weight were not significant. The mean squares due to line × tester were highly significant for all characters except average pod weight^{-1}, seed number pod^{-1}, seed weight pod^{-1} and 100-seed weight which were not significant.

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Table 3. *ANOVA table for the studied characters*

S.O.V	d.f	M.S								
		Pods no. plant^{-1}	**Pods weight plant^{-1} (g)**	**Pod length (cm)**	**Average pod weight (g)**	**Seeds no. pod^{-1}**	**Seeds weight pod^{-1} (g)**	**100-seed weight (g)**	**Biological weight plant^{-1} (g)**	**Seeds weight plant $^{-1}$(g)**
Blocks	2	13.806	6.339	0.684	0.001	0.989	0.001	1.739	5.183	0.104
Genotype	18	** 185.155	** 97.461	** 5.010	** 0.077	** 0.930	** 0.027	** 53.854	** 171.552	** 11.968
Parents	6	** 244.543	** 108.926	** 6.635	** 0.116	** 1.908	** 0.046	** 74.543	** 283.103	** 23.206
Parents × Crosses	1	n.s 2.087	** 200.246	** 8.949	n.s 0.070	n.s 0.165	n.s 0.015	n.s 0.016	** 414.141	** 2.981
Crosses	11	** 169.404	** 81.863	** 3.765	** 0.056	* 0.467	** 0.018	** 47.463	** 88.654	** 6.655
Lines	2	** 315.131	** 153.621	** 6.583	** 0.109	* 1.285	* 0.028	** 249.440	** 30.184	** 7.977
Testers	3	** 160.380	** 46.237	* 4.324	** 0.121	n.s 0.264	** 0.026	n.s 2.876	** 131.000	** 6.503
Lines × Testers	6	** 125.340	** 75.756	** 2.546	n.s 0.007	n.s 0.296	n.s 0.010	n.s 2.431	** 86.970	** 6.291
Error	36	0.582	0.623	0.592	0.004	0.223	0.005	1.088	0.359	0.159

Data in (*Table 4*) explain the average of studied characters for parents and their F_5 crosses. Maximum value for pod number plant^{-1} produced by the cross 1 × 6 with 35.256, while for pod weight plant^{-1} it was produced by the cross 2 x 4 with 35.125 g, whist for average pod weight produced by the cross 2 x 5 with 1.493 g. Maximum value for pod length, seed weight pod^{-1} and biological weight plant^{-1} produced by the cross 2 × 6 with 8.000 cm, 1.281 g and 40.375 g respectively. The cross 2 × 7 showed maximum value for 100-seed weight with 23.392 g. The cross 3 × 6 accepted the highest value for pod length and seed number pod^{-1} with 8.000 cm and 6.033. Maximum value for see weight plant^{-1} produced by the cross 1 × 4 with 17.338 g. The same table illustrates the average of characters due to the parents, parent 2 produced the highest value for pod length and 100-seed weight with 8.000 cm and 28.253 g. Parent 3 exhibited the highest value for seed number pod^{-1} and seed weight pod^{-1} with 6.833 and 1.277 g. The highest value due to pod number plant^{-1}, biological weight plant^{-1} and seed weight plant^{-1} produced by parent 6 with 36.929, 44.195 g and 20.268 g. Parent 7 gave the highest value for pod weight plant^{-1} and average pod weight with 30.389 g and 1.577 g. The lowest value due to almost most characters produced by parent 5 and 6.

Table 5 explains the heterosis values estimated as the percentage of F_5s deviated from mid parental values. Maximum positive heterosis values for pod number plant^{-1}, average pod weight and seed number pod^{-1} were 20.115, 7.207 and 2.719% respectively produced by the cross 2 × 5, while the cross 2 × 6 produced maximum positive value for seed weight pod^{-1} and 100-seed weight with 5.031 and 3.932%. The cross 3 × 5 gave the highest positive heterosis value for pod weight plant^{-1} and biological weight plant^{-1} with 22.989 and 23.703%. The highest heterosis positive value for pod length was 11.364% produced by the cross 3 × 6, while for seed weight plant^{-1} it was 4.401% produced by the cross 1 × 4. The cross 3 × 7 showed maximum negative heterosis values for most characters. The positive value due to heterosis ratify the over dominance gene effect for the parent with high value, while the negative heterosis value signify the partial dominance gene effect for the parent with low value.

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Table 4. *Averages of studied characters for parents and their F_5 crosses*

Crosses and parents	Pods no. plant^{-1}	Pods weight plant^{-1} (g)	Pod length (cm)	Average pod weight (g)	Seeds no. pod^{-1}	Seeds weight pod^{-1} (g)	100-seed weight (g)	Biological weight plant^{-1} (g)	Seeds weight plant $^{-1}$(g)
1 x 4	34.822	26.862	5.000	1.117	5.333	1.037	14.372	35.070	17.338
1 x 5	24.087	28.472	6.333	1.350	4.967	1.213	14.320	35.134	13.150
1 x 6	35.256	24.663	5.000	1.231	5.133	1.036	14.175	40.219	15.249
1 x 7	27.858	26.385	5.333	1.079	5.167	1.049	16.122	35.249	14.517
2 x 4	33.441	35.125	5.667	1.394	4.667	1.134	22.306	20.467	14.317
2 x 5	23.185	23.147	5.333	1.493	5.267	1.178	23.075	33.177	14.085
2 x 6	17.999	27.508	8.000	1.375	5.433	1.281	22.467	40.375	13.350
2 x 7	24.432	30.379	7.000	1.276	5.167	1.126	23.392	40.261	14.458
3 x 4	24.531	23.320	5.333	1.213	5.600	1.086	14.044	34.088	14.271
3 x 5	29.269	29.780	7.000	1.448	6.000	1.176	16.793	40.249	15.202
3 x 6	16.073	19.734	8.000	1.380	6.033	1.177	15.085	35.158	11.132
3 x 7	11.256	15.139	7.000	1.128	5.200	1.078	14.439	35.318	13.195
1	26.592	25.299	5.000	1.048	5.000	1.131	14.118	25.145	15.145
2	15.400	23.470	8.000	1.156	5.000	1.206	26.253	35.136	11.166
3	26.188	20.286	7.000	1.403	6.833	1.277	16.119	27.264	13.239
4	30.035	20.336	5.000	1.081	5.200	0.975	13.171	36.395	14.334
5	10.296	10.742	4.333	1.162	4.500	1.111	21.119	14.057	14.409
6	36.929	23.412	4.000	1.095	5.500	0.927	12.575	44.195	20.268
7	28.073	30.389	4.667	1.577	4.500	1.051	19.242	26.472	14.079
LSD ($p \leq 0.05$)	1.263	1.307	1.274	0.105	0.782	0.117	1.727	0.993	0.660

Table 5. *% Heterosis values for F_5 crosses of the studied characters*

Crosses	Pods no. plant^{-1}	Pods weight plant^{-1} (g)	Pod length (cm)	Average pod weight (g)	Seeds no. pod^{-1}	Seeds weight pod^{-1} (g)	100-seed weight (g)	Biological weight plant^{-1} (g)	Seeds weight plant^{-1} (g)
1 x 4	5.747	4.4310	0.000	1.221	1.144	-0.388	1.332	3.494	4.401
1 x 5	7.649	14.500	8.929	5.543	1.140	2.052	-4.681	19.812	-2.753
1 x 6	2.751	0.315	2.778	3.717	-0.556	0.158	1.553	4.001	-3.470
1 x 7	0.481	-1.310	2.586	-4.457	2.193	-0.974	-0.836	9.145	-0.162
2 x 4	11.801	15.092	-3.205	6.149	-2.124	0.998	3.290	-10.694	3.073
2 x 5	20.115	8.827	-3.378	7.207	2.719	0.428	-0.645	8.721	2.536
2 x 6	-7.802	4.338	8.333	5.533	0.873	5.031	3.932	0.447	-3.765
2 x 7	3.100	3.203	2.632	-1.655	2.193	-0.052	0.708	7.675	3.635
3 x 4	-3.184	3.703	-2.778	-0.584	-1.731	-0.888	-1.026	1.774	0.879
3 x 5	15.112	22.989	5.882	3.223	1.471	-0.363	-2.452	23.703	2.493
3 x 6	-12.267	-2.420	11.364	2.615	-0.541	1.713	1.286	-0.400	-8.389
3 x 7	-14.628	-10.062	5.000	-6.074	-2.059	-1.837	-4.584	7.8623	-0.850
S.E	3.074	2.580	1.423	1.236	0.500	0.524	0.795	2.619	1.101

*S.E: standard error of the estimation

Table 6 illustrates the estimation of gca effect for parents due to studied characters. Maximum positive gca effect for pod number plant^{-1} was 5.747 produced by tester parent 4, while for pod weight plant^{-1} and 100-seed weight were 3.164 and 5.261 respectively produced by line parent 2. Maximum positive gca effect value for pod length and biological weight plant^{-1} were 0.750 and 3.187 respectively produced by tester parent 6. The tester parent 5 showed maximum positive gca effect value for average pod weight with 0.140. The line parent 3 exhibited the highest positive gca

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effect for seed number pod^{-1} with 0.387. The tester parent 5 showed maximum gca effect value for seed weight pod^{-1} with 0.058, while for seed weight plant^{-1} was 1.120 produced by tester parent 4. Maximum negative gca effect value for pod number plant^{-1} was -4.902 and for pod weight plant^{-1} was -3.883 produced by tester parent 3, while for 100-seed weight and biological weight plant^{-1} was -2.802 and -5.522 produced by line parent 1 and tester parent 4 respectively. The positive value of gca indicated to the tendency of these parents to increase the value of this character, while the negative gca value indicate to the ability of these parents to reduce the value of this character.

Table 6. *Estimation of general combining ability (gca) effect for the parents of the studied characters*

Parents	Pods no. plant^{-1}	Pods weight plant^{-1} (g)	Pod length (cm)	Average pod weight (g)	Seeds no. pod^{-1}	Seeds weight pod^{-1} (g)	100-seed weight (g)	Biological weight plant^{-1} (g)	Seeds weight plant^{-1}(g)
1	5.322	0.719	-0.833	-0.096	-0.181	-0.047	-2.802	1.021	0.875
2	-0.420	3.164	0.250	0.094	-0.197	0.049	5.261	-1.827	-0.136
3	-4.902	-3.883	0.583	0.002	0.378	-0.002	-2.459	0.806	-0.739
S.E lines	0.220	0.228	0.222	0.018	0.136	0.020	0.301	0.173	0.115
4	5.747	2.559	-0.917	-0.049	-0.131	-0.046	-0.642	-5.522	1.120
5	0.330	1.257	-0.028	0.140	0.081	0.058	0.513	0.790	-0.043
6	-2.075	-1.908	0.750	0.038	0.203	0.034	-0.307	3.187	-0.945
7	-4.002	-1.908	0.194	-0.129	-0.153	-0.047	0.435	1.546	-0.132
S.E testers	0.254	0.263	0.256	0.021	0.157	0.023	0.348	0.200	0.133

The estimation of the sca effect of crosses represent in (*Table 7*). The highest positive effect for seed number pod^{-1} produced by the cross 1 × 4 with 0.314, while the cross 1 × 5 showed maximum positive sca effect for pod length and seed weight pod^{-1} with 0.944 and 0.071 respectively, but for average pod weight it was 0.058 produced by the cross 2 × 4 and for biological weight plant^{-1} it was 5.145 produced by the cross 2 × 7. The cross 3 × 5 produced maximum sca effect value for pod number plant^{-1}, pod weight plant^{-1}, 100-seed weight and seed weight plant^{-1} with 8.657, 6.530, 1.189 and 1.795 respectively. Maximum negative sca effect value for pod number plant^{-1} and seed weight plant^{-1} was -6.748 and -1.871 produced by the cross 1 × 5, while for pod length and seed weight pod^{-1} it was -1.167 and -0.082 produced by the cross 1 × 6. The cross 2 × 4 showed maximum negative sca effect for biological weight plant^{-1} with -7.581 and for pod weight plant^{-1} it was -7.150 produced by the cross 2 × 5, while for average pod weight it was -0.048 produced by the cross 2 × 6, but for both seed number plant^{-1} and 100-seed weight it was -0.356 and -1.086 respectively produced by the cross 3 × 7.

The estimation of some genetic parameters for the studied characters represent in (*Table 8*). The ratio of $\sigma^2_{gca}/\sigma^2_{sca}$ for all characters was less than unity except average pod weight and 100-seed weight which were more than unity. The average degree of dominance was more than unity for all characters except average pod weight and 100-seed weight, this indicate to the over dominance gene effect in controlling the inheritance of most characters. Heritability in broad sense was found to be high for most characters except the characters seed number pod^{-1} and seed weight pod^{-1} in which they found to be low. The estimation of heritability in narrow sense was low for all characters except the characters average pod weight and 100-seed weights which were recorded high estimation.

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Table 7. *Estimation of specific combining ability (sca) for the F_1 crosses of the studied characters*

Crosses	Pods no. plant^{-1}	Pods weight plant^{-1} (g)	Pod length (cm)	Average pod weight (g)	Seeds no. pod^{-1}	Seeds weight pod^{-1} (g)	100-seed weight (g)	Biological weight plant^{-1} (g)	Seeds weight plant^{-1} (g)
1 x 4	-1.431	-2.293	0.500	-0.028	0.314	-0.001	0.266	4.174	1.155
1 x 5	-6.748	0.620	0.944	0.016	-0.264	0.071	-0.941	-2.073	-1.871
1 x 6	6.825	-0.025	-1.167	-0.001	-0.219	-0.082	-0.265	0.614	1.130
1 x 7	1.355	1.698	-0.278	0.014	0.169	0.012	0.940	-2.715	-0.414
2 x 4	2.929	3.526	0.083	0.058	-0.336	-0.000	0.138	-7.581	-0.855
2 x 5	-1.909	-7.150	-1.139	-0.031	0.053	-0.060	-0.249	-1.183	0.075
2 x 6	-4.690	0.376	0.750	-0.048	0.097	0.068	-0.036	3.619	0.243
2 x 7	3.670	3.248	0.306	0.021	0.186	-0.007	0.147	5.145	0.538
3 x 4	-1.498	-1.233	-0.583	-0.030	0.022	0.002	-0.404	3.407	-0.299
3 x 5	8.657	6.530	0.194	0.016	0.211	-0.011	1.189	3.256	1.795
3 x 6	-2.134	-0.352	0.417	0.049	0.122	0.014	0.301	-4.232	-1.373
3 x 7	-5.025	-4.945	-0.028	-0.035	-0.356	-0.005	-1.086	-2.431	-0.123
S.E	0.623	0.644	0.628	0.0517	0.386	0.057	0.852	0.490	0.325

Table 8. *Estimation of some genetic parameters for the studied characters*

Parameters	Pods no. plant^{-1}	Pods weight plant^{-1} (g)	Pod length (cm)	Average pod weight (g)	Seeds no. pod^{-1}	Seeds weight pod^{-1} (g)	100-seed weight (g)	Biological weight plant^{-1} (g)	Seeds weight plant $^{-1}$(g)
σ^2 e	0.582	0.623	0.592	0.004	0.223	0.005	1.088	0.359	0.159
σ^2_{gca}	2.851	0.395	0.079	0.003	0.011	0.000	2.914	0.109	0.024
$\sigma^2_{sca} = \sigma^2_D$	41.586	25.044	0.652	0.001	0.024	0.002	0.448	28.870	2.044
$\sigma^2_{gca}/\sigma^2_{sca}$	0.069	0.016	0.121	3.756	0.458	0.281	6.509	0.004	0.012
σ^2_A	5.702	0.790	0.158	0.006	0.022	0.001	5.828	0.218	0.047
ā	3.819	7.961	2.874	0.516	1.478	1.886	0.392	16.280	9.309
$h^2_{b.s}$	0.988	0.976	0.578	0.645	0.172	0.356	0.852	0.988	0.929
$h^2_{n.s}$	0.119	0.030	0.113	0.569	0.082	0.128	0.791	0.007	0.021

σ^2e: mean squares of experimental error or (environmental variance); σ^2_{gca}: the variance of general combining ability; σ^2_{sca}: the variance of specific combining ability; $^*\sigma^2$A: additive variance *ā: average degree of dominance; $h^2_{b.s}$: heritability in broad sense; $h^2_{n.s}$: heritability in narrow sense

Data in (*Table 9*) explain the simple correlation coefficient among all characters. The character pod number plant-1 correlated high significantly and positively with pod weight plant-1 and seed weight plant-1 r = 0.597 and 0.690 respectively, while there were significant and negative correlation between pod number plant-1 with pod length and seed weight pod-1 r = -0.512 and -0.485 respectively. The character pod length exhibited highly significant and positive correlation with seed weight pod-1 r = 0.743, but it correlated high significantly and positively with seed weight plant-1 r = -0.702. The character average pod weight recorded significant and positive correlation with seed weight pod^{-1} r = 0.519. Seed weight pod^{-1} produced significant and positive correlation with 100-seed weight r = 0.493, but it recorded highly significant and negative correlation with seed weight plant^{-1} r = -0.666.

Data in (*Table 10*) explain the path coefficient analysis indicated to the direct and indirect effect of the character seed weight plant^{-1}. The character seed number pod^{-1} recorded maximum positive direct effect in seed weight plant^{-1} with 0.428 and followed by biological weight plant^{-1} with 0.322. Maximum negative direct effect in seed weight plant^{-1} recorded by pod length with -1.002 and followed by average pod weight with -

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0.240. Maximum positive indirect effect in seed weight plant^{-1} recorded by pod length via pod number plant^{-1} with 0.513 and followed by pod weight plant^{-1} via pod number plant^{-1} with 0.184. Maximum negative indirect effect was -0.744 recorded by pod length via seed weight pod^{-1} and followed by -0.439 recorded by pod length via seed number pod^{-1}.

Table 9. *Correlation coefficient among the studied characters*

Characters	Pods no. plant^{-1}	Pods weight plant^{-1} (g)	Pod length (cm)	Average pod weight (g)	Seeds no. pod^{-1}	Seeds weight pod^{-1} (g)	100-seed weight (g)	Biological weight plant^{-1} (g)	Seeds weight plant $^{-1}$ (g)
Pods no. plant^{-1}	1.000								
Pods weight plant^{-1} (g)	0.597 **	1.000							
Pod length (cm)	-0.512 *	0.062 n.s	1.000						
Average pod weight (g)	-0.021 n.s	0.390 n.s	0.294 n.s	1.000					
Seeds no. pod^{-1}	0.070 n.s	-0.135 n.s	0.438 n.s	0.160 n.s	1.000				
Seeds weight pod^{-1} (g)	-0.485 *	0.089 n.s	0.743 **	0.519 *	0.341 n.s	1.000			
100-seed weight (g)	-0.385 n.s	0.203 n.s	0.364 n.s	0.381 n.s	-0.311 n.s	0.493 *	1.000		
Biological weight plant^{-1} (g)	0.267 n.s	0.215 n.s	0.286 n.s	-0.101 n.s	0.367 n.s	-0.162 n.s	-0.240 n.s	1.000	
Seeds weight plant $^{-1}$ (g)	0.690 **	0.151 n.s	-0.702 **	-0.330 n.s	-0.054 n.s	-0.666 **	-0.408 n.s	0.224 n.s	1.000

Table 10. *Path coefficient analysis among the studied characters*

Characters	Pods no. plant^{-1}	Pods weight plant^{-1} (g)	Pod length (cm)	Average pod weight (g)	Seeds no. pod^{-1}	Seeds weight pod^{-1} (g)	100-seed weight (g)	Biological weight plant^{-1} (g)
Pods no. plant^{-1}	-0.078	0.184	0.513	0.005	0.030	0.024	-0.074	0.086
Pods weight plant^{-1} (g)	-0.047	0.308	-0.062	-0.094	-0.058	-0.004	0.039	0.069
Pod length (cm)	0.040	0.019	-1.002	-0.071	0.187	-0.037	0.070	0.092
Average pod weight (g)	0.002	0.120	-0.294	-0.240	0.068	-0.026	0.073	-0.032
Seeds no. pod^{-1}	-0.005	-0.042	-0.439	-0.038	0.428	-0.017	-0.059	0.118
Seeds weight pod^{-1} (g)	0.038	0.027	-0.744	-0.125	0.146	-0.050	0.094	-0.052
100-seed weight (g)	0.030	0.063	-0.365	-0.092	-0.133	-0.025	0.191	-0.077
Biological weight plant^{-1} (g)	-0.021	0.066	-0.287	0.024	0.157	0.008	-0.046	0.322

Discussion

Analysis of variance for the mean sum of square due to parents and crosses showed significant differences for all the characters studied indicating the presence of variability among parents and crosses. The mean sum of square due to progenies was significant for all the characters studied. There were no significant different among the lines. There were significant differences among the testers for seed yield plant^{-1}, 100-seed weight and harvest index. The line × tester interaction gave significant differences for all the characters studied indicating the diversity among crosses and the prevalence of non-

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additive variance. Parents versus crosses also showed significant differences for all the characters. The present findings were quite similar with that of Ceyhan et al. (2008), Kumar et al. (2009) and Kumar et al. (2016).

Al-Hamdany (2014) reported that the General Combining Ability was significant for seed yield, 100-seed weight and pods weight but non-significant for seeds pod^{-1}, while sca for most characters was significant in pea. Tawfiq and Abdulla (2014) carried out genetic analysis between seven pea in a half diallel crosses and showed that the variance due to specific combining ability was larger than that of general combining ability for some studied characters, while the gca/sca variance ratio to be more than one in most studied traits, indicate the importance of additive gene effect in the inheritance of all characters.

Singh and Mishra (2002) derived information on combining ability in 10 × 10 diallel set. The mean sum of squares due to gca and sca variances were highly significant for all the characters except seeds pod^{-1}. Pandey et al. (2006) reported that combining ability analysis showed significant difference for gca and sca variance for all the characters. Parent Lincoln appeared to be one of the best combiners for all the traits. The mean squares for general combining ability were observed higher than those of specific combining ability in all the characters (Singh et al., 2007).

Significant means squares due to line, crosses, half diallel, parents Vs crosses observed for yield its contributing traits. The gca/sca variance ratio were less than unity for seeds pod^{-1}, yield plant^{-1}, 100-seed weight, indicating predominance of additive gene effects for these character, therefore, it is suggested that selection in F_1 generation may be either following progeny or simple recurrent selection (Suman et al., 2017).

All the morphological traits showed highly significant variations among the genotypes and the variations could be used in plant improvement program. Pod length, 100-seed weight, pods plant^{-1}, seeds plant^{-1} and seed yield plant^{-1} were controlled by additive gene action and selection for the improvement of these traits could be effective. Furthermore, among studied traits, four traits *viz,* 100-seed weight, seeds pod^{-1} and seeds plant^{-1} showed highly positive correlation and positive direct effect on seed yield. Therefore, emphasis should be given on these traits during selection in breeding program in order to increase seed yield (Khan et al., 2017).

F_1 crosses had more seed yield, more plant height, more pods plant^{-1}, more seeds pod^{-1}, a greater pod yield and more 100-seeds weight than the average of their parents. Heterosis was found to be significant for seed yield and its components. The values reported in this study are in agreement with the values of heterosis obtained by Lejeune-Henaut et al. (1992), Mishra et al. (1993), Sarawat et al. (1994) and Ceyhan (2003) which is attributable to non-additive gene effects rather than over dominant ones.

It was found that the magnitude of sca variance (σ^2_s) was higher than gca variance (σ^2_g) for all the characters under studied. Hence, the ratio of σ^2_g/σ^2_s was less than unity for all the characters indicating the predominant role of non-additive gene action for all the characters understudied. Similar results were also reported by Ceyhan (2006), Bora et al. (2009), Esposito et al. (2013) and Suman et al. (2017). The combining ability analysis has been the most important and efficient tool in choosing the desirable parents for hybridization programs. This technique makes it possible to classify the parental lines in terms of superiority in cross combinations and the gene action involved in the inheritance of different characters. Therefore, analysis of combining ability has been the most important and efficient tool in selecting the desirable parents for a hybridization program (Sharma et al., 2013). Singh et al. (2010) observed higher values of variance

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due to gca for pod length, and green pod yield plant^{-1} showed presence of additive gene action while it was non additive for pods number plant^{-1} based on both the generations.

The sca variance component was predominant indicating the importance of non-additive gene effects for all the characters except for peas pod^{-1} and pod yield which were influenced by additive gene action, suggesting their improvement through pure line selection (Kalia and Sood, 2009). Genes are the functional units that govern the development of various characters of an individual. Gene action refers to the behavior or mode of expression of genes in a genetic population. Genes control synthesis of proteins which in turn control expression of various traits of organisms. Knowledge of gene action in plant breeding helps in the selection of parents for use in the hybridization programs and also in the choice of appropriate breeding procedure for the genetics improvement of various quantitative characters (Sharma et al., 2013).

The knowledge of gene action is very useful to a plant breeder in the selection of parents for hybridization, the estimation of some other genetic parameters and choice of breeding procedures for the genetic improvement of various quantitative characters. In an autogamous crop exploitation of non-additive genetic variance as such would be impractical. Since, the research investigation exhibited that earliness and yield attributing traits were predominantly controlled by additive gene effects, simple selection procedure like single seed descent would be effective for isolating short duration progenies in advanced generations. Simple progeny selection may be followed for selecting transgressive segregate in later generations for developing genotypes having long pods. The cross combinations involving poor × poor, good × good and poor × good general combining parents with highest significant sca effects may be obtained for different horticultural traits. Crosses having both the parents as poor general combiners may involve dominance × dominance or epistatic interaction. Such crosses may not give good transgressive segregate in later generation. The crosses involving good × good general combiners and showing high sca effects could be utilized for the purpose of developing high yielding genotypes and obtaining transgressive segregate in F_2 generation (Sharma et al., 2013).

Dhillon et al. (2006) reported additive and non-additive gene effects governed the inheritance of all the studied characters. The additive gene effects were more pronounced for pods number plant^{-1} and pod length, whereas the non-additive gene effects were more pronounced for seeds number pod^{-1}, pod yield plant^{-1}. Sharma and Sharma (2012) observed the prevalence of over dominance for most of the traits. Low estimates of narrow sense heritability indicated the presence of non-additive gene action for most traits. These characters also exhibited medium to high level of heritability and the selections in segregating generation could be effective for evolving early maturing types.

Sharma and Bora (2013) reported higher values of heritability in broad sense and genetic gain indicating that the additive gene actions are important in determining the characters *viz*. 100 green pod weights and pod yield revealed. Therefore, selection program based on these characters would be more effective in improving yield parameters of garden pea. Combining ability analysis for six physiological characters in pea revealed in some traits by additive gene action, while both additive and non-additive gene actions were important for controlling some studied traits as found by Sirohi and Singh (2013).

Seed yield plant^{-1} exhibited highly significant and positive correlation with 100-seed weight (0.3775), pods number plant^{-1} (0.3524), harvest index (0.3270), seeds number pod^{-1} (0.3262) and biological yield (g) (0.2828). The occurrence of negative as well as positive indirect effects on yield by one or another character presents a complex

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situation where a compromise balance is required to attain proper balance of different yield components, for determining the ideotype of seed yield in field pea. The highest positive direct effect on seed yield plant^{-1} was exerted by harvest index (0.7902) followed by biological yield (g) (0.7820), 100-seed weight (0.3406), seeds number pod^{-1} (0.2788) and pods number plant^{-1} (0.2622) (Srivastava et al., 2018).

The genotypic correlation coefficient between different characters was generally similar in sign and nature to the corresponding phenotypic correlation coefficients in the experiment as found by Tyagi et al. (2012) and Pooja et al. (2015) also. However, in general, genotypic correlation coefficients were higher in magnitude from the corresponding phenotypic correlation coefficient values. Similar, results have been reported by Mahant et al. (2001), Arya et al. (2004) and Kumar et al. (2003). High positive direct contribution to seed yield plant^{-1} was exhibited by harvest index and biological yield plant^{-1}. However, 100-seed weight exhibited positive considerable direct effect on seed yield plant^{-1}. Patel et al. (2006) also reported positive correlation between seed yield plant^{-1} with pods number plant^{-1} and pod length at genotypic and phenotypic level. Ghobary (2010) also obtained results that revealed; important yield components effect directly like biological yield, harvest index and 100-seed weight. The simple correlation Coefficient among characters and path analysis between seeds weight plant^{-1} and other characters. It was observed that the character seeds weight plant^{-1} correlated positively and highly significantly with the character pods number plant^{-1}, pods weight plant^{-1}, biological weight plant^{-1} and harvest index recording (0.857, 0.839, 0.694 and 0.505) respectively. The character biological weight plant^{-1} and harvest index exhibited maximum positive direct effect in seeds weight plant^{-1} recording (0.630 and 0.456) respectively, the character pods number plant^{-1} showed the highest positive indirect effect in seeds weight plant^{-1} via harvest index recording (0.191) (Tofiq et al., 2015).

All the characters showed moderate to low phenotypic and genotypic coefficient of variation. Genotypic coefficient of variation was the highest for 100-seed weight (37.24) followed by seeds plant^{-1} (15.27). 100-seed weight had the highest heritability (95.97). Pod length, 100-seed weight, pods plant^{-1} and seeds plant^{-1} showed significant positive genotypic and phenotypic correlation with seed yield. In path analysis, 100-seed weight, seeds number pod^{-1} and seeds plant^{-1} showed positive direct effect on yield. Considering genetic variability, correlation and path analysis, emphasis should be given on pod length, seeds pod^{-1}, seeds plant^{-1} and 100-seed weight during breeding program to improve seed yield of pea (Khan et al., 2017).

Conclusion

The preponderance of non-additive type of gene action clearly indicated that selection of superior plants should be postponed to later generation. It could be clear that non-additive genetic variance is considered to be the major source of the total genetic variance responsible for the inheritance of most of the studied characters except average pod weight and 100-seed weight. The crosses involving (good × good) general combiners and showing high sca effects could be utilized for the purpose of developing high yielding genotypes and obtaining transgressive segregation in F generation. The high values of average degree of dominance for almost all of the studied characters which were more than unity confirm the superiority of non-additive gene effect in the inheritance of the studied characters which make the hybridization to be the method to improve these characters.

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Recommendation

Continuous breeding programs are required to reveals pea cultivars for high yielding crosses like Avolla × Joneor. Further works is recommended to fix the desirable genes respect both forage and seed yield. More investigation were required to evaluate more pea cultivars at autumn and spring seasons which survival potential to the climatically conditions prevailing in Sulaimani region.

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COMPATIBLE STEM VOLUME AND TAPER EQUATIONS FOR FIVE MAJOR TREE SPECIES IN NORTHEAST CHINA

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Abstract. Compatible segmented taper and volume equations were developed for Dahurian larch, Korean spruce, Manchurian fir, planted Dahurian larch and Mongolian pine in northeast China. The model form developed by Max and Burkhart (1976) was fitted to the data of 720 sample trees. The data was randomly split into two sets for each species: 80% of total data was used for model fitting, and 20% of total data was reserved for model validation. The proposed equation provided good results for the whole tree and different stem sections. Mean prediction errors for diameter and volume were less than 1.9 cm and 0.005 m^3, respectively. The recommended model provided adequate diameter and merchantable volume estimates against ten relative height classes, examined for each species. Additionally, the model's estimates were superior to the existing volume tables for these commercial species, particularly for Korean spruce, Manchurian fir, and planted Dahurian larch.
Keywords: *segmented models, taper and volume, Dahurian larch, Korean spruce, Manchurian fir, Mongolian pine*

Introduction

China has assigned a strategic position to forestry development and ecological improvement, being pivotal factors in the sustainable development of the society and economy. A significant increase of 8.94% has been achieved in the total forest cover during the past forty years. However, sustainable forestry development remains a rigorous challenge to bridge supply and demand conflicts and meet other national and international commitments (Jia and Qu, 2011; Zeng et al., 2015). At the same time, plenty of room for improvement is available in terms of multiple products and services of the forests. Therefore, sustainable forest management is declared as a core objective of the forestry research for which accurate predictions of the standing volume are a prerequisite.

Distributed over an area of 32.71 million hectares with 28.18 billion m^3 of standing volume, northeast China is the largest forested area in the country (Zeng et al., 2015). It is recognized as a national base of wood products and a region of ecological significance. This region covers 30% of the total area, and almost half of the national ecosystem carbon is stored in these forests (Zhang et al., 2018).

Natural and planted Dahurian larch (*Larix gmelinii*), Korean spruce (*Picea koraiensis*), Manchurian fir (*Abies nephrolepis*), and planted Mongolian pine (*Pinus sylvestris*) are dominant commercial tree species in NE China. The forests of *Larix gmelinii*, *Picea koraiensis*, and *Abies nephrolepis*, and plantations of *Larix gmelinii* and *Pinus sylvestris* occupy 10.6 million ha, 4.3 million ha, 3.1 million ha, 2.8 million ha, and 0.2 million ha area, respectively. The corresponding standing volume of these species is 955.2 million m^3, 1001.6 million m^3,1135.6 million m^3, 163.9 million m^3, and 13.5 million m^3 (Chinese Ministry of Forestry, 2009). *Larix gmelinii* is the dominant tree

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species of Larix forests and covers 55% of the total area, 75% of total volume (Zhou et al., 2002). *Picea koraiensis* is the leading species of Picea forests with *Abies nephrolepis* as a major associate in NE China.

China has the largest forest plantations in the world; over an area of 79 million ha, that amounts to 25% of the world's total (FAO, 2015; Payn et al., 2015). Dahurian larch is generally planted after fire incidents and logging due to its fast growth and cold tolerance. The growth rate of these plantations is an important indicator to assess the forest recovery process and carbon sequestration potential (Jia and Zhou, 2018). Mongolian pine (*Pinus sylvestris* L. var. *mongolica* Litv.), a geographical variety of Scots pine (*Pinus sylvestris* L.) is highly tolerant to cold, drought, and soil infertility. It has been widely planted in the Three-North (north, northeast, northwest) project of China for timber production, windbreaks, and soil and water conservation (Zhang et al., 2019). Measurement tools are required for the sustainable management of these species that should allow for the industrial and ecological advances in Chinese forestry.

As a measurement tool, taper models are an essential component in prevailing forest management and planning systems (Heidarsson and Pukkala, 2011). Generally, taper models use the diameter at breast height, total height, and height above ground as independent variables, since these measurements can be easily recorded during routine forest inventories (Brooks et al., 2008). Such models can estimate (i) diameter at any height; (ii) total volume; (iii) merchantable volume and merchantable height; (iv) and volume of a log from any section of the stem (Kozak, 2004). Additionally, taper models are utilized in timber quality studies, decisions support systems, assessing the impact of silvicultural treatment on stem taper, and in the modelling of carbon allocation in different stem sections (Deleuze and Houllier, 2002; Ikonen et al., 2003; Younger et al., 2008).

Taper models have been a topic of interest in forest mensuration and management studies for over a century (Fang and Bailey, 1999). Newnham (1988) has described two reasons that bring taper models into topicality. Firstly, no single theory can satisfactorily explain all types of stem forms. Secondly, taper models are flexible tools for total and merchantable volume estimates that can comply with the changes in market trends and product classification. Practically, this flexibility of taper models keeps them under perpetual studies.

Numerous taper models of different forms have been developed over the years. A detailed discussion is available in the literature regarding the types, evolution, and comparison of these models (Rojo et al., 2005; Diéguez-Aranda et al., 2006; Sakici et al., 2008; Crecente-Campo et al., 2009; Özcelik and Crecente-Campo, 2016; Burkhart et al., 2019). The approaches of variable form taper equations and segmented taper equations have substantiated their superiority in different studies, e.g. (Barrio Anta et al., 2007; Li and Weiskittel, 2010; Schröder et al., 2014; Lumbres et al., 2016; Özcelik and Dirican, 2017; Sakici and Ozdemir, 2018). Generally, variable form taper functions provide the lowest range of local bias and maximum precision. However, they are unable to calculate total stem volume or log volume and merchantable height. Numerical integration and iteration procedure is required to calculate these variables. Alternatively, segmented taper functions can be integrated to calculate volume, and merchantable heights can be obtained directly from the equation (Kozak and Smith, 1993).

The integration of a taper equation can provide volume estimates to any merchantability limit, as well as to the total tree height. Taper and volume equations are compatible when total volume calculated by the integration of a taper equation is identical to the results of the volume equation (Demaerschalk, 1972). The primary benefit of a

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compatible taper volume system is to achieve consistent results from both taper and volume equations (Burkhart and Tomé, 2012; Özcelik and Göceri, 2015).

There are some references to taper and volume studies of *Pinus sylvestris* (natural forests) in Europe, the USA, and Turkey (e.g., Laasasenaho, 1982; Westfall and Scott, 2010; Özcelik and Brooks, 2012). In China, *Larix gmelinii*, *Picea koraiensis*, *Abies nephrolepis*, and *Pinus sylvestris* L. var. *mongolica* have been studied, but the investigations were focussed on biomass and climate change (Zhou et al., 2002; Wang et al., 2012, 2018; Dong et al., 2014; Ma et al., 2014). A segmented taper equation was also presented for planted *Larix gmelinii* in NE China (Jiang and Liu, 2011). However, compatible taper and volume equations are yet to be developed for these dominant tree species in NE China.

At present, volume estimation is based on the volume tables, which were developed more than three decades ago (Heilongjiang Forest Bureau, 1981). The species under analysis provide timber for building logs, railway sleepers, construction, shipbuilding, and plywood and veneer. Other uses include soundboards in the musical instrument, boxes, and raw materials for the pulp industry. The versatility in their uses requires accurate estimates of the diameters and volumes to different merchantability limits. The conventional volume tables are no longer adequate for volume estimation in fluctuating market trends and product specifications.

The objective of this study was to develop a volume equation that can correctly estimate the tree volume to any merchantable size. A widely recognized and flexible taper equation presented by Max and Burkhart (1976) was evaluated for five commercial tree species of NE China. The total volume estimates, obtained from the proposed equation, were also compared with the prevailing volume tables.

Material and methods

Study area

This study was conducted at Lilin forest farm (129°15′-129°30′ E, 48°74′-49°9′ N) covering an area of covering an area of 8128 hm^2 and Jinsha forest farm (130°06′-131°58′E, 45°16′-46°37′ N), with an area of 6651 hm^2 located in Wuying forest bureau and Qitaihe City, respectively in Heilongjiang province, NE China. The sample trees of Dahurian larch, Korean spruce, and Manchurian fir were selected from the natural stands in the Lilin forest farm. It falls in the cold temperate forest with a continental monsoon climate. The mean annual precipitation fluctuates from 550 to 600 mm, and the mean annual temperature ranges from 0°C to 2°C. The predominant forest types are *Larix gmelinii*, *Picea koraiensis*, *Abies nephrolepis*, and deciduous broadleaf mixed forest (Tan et al., 2007).

The sample trees of planted Dahurian larch and Mongolian pine were selected from the Jinsha forest farm (*Figure 1*). This area is located in the middle temperate humid climate zone, with four distinct seasons and uneven distribution of precipitation in each season. The mean annual precipitation is 549 mm, and the mean annual temperature is 4.0℃. The typical soil type of the study area is mainly dark brown soil (Burger and Shidong, 1988).

A sample of 770 trees was collected from natural stands and plantations, covering the existing range of stand densities, conditions, and sites. Diameter at breast height (D) was measured to the nearest 0.1 cm. Later, the trees were felled to measure the total height (H) to the nearest 0.1 m. The diameter outside bark (d) was measured at the heights (h) of 0.3,

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0.6, 1, 1.3, 2, 3 m, and then at an interval of 1 m for the rest of the stem. The data were randomly split into two sets for each species: 80% of total data (617 sample trees) for model fitting and 20% of total data (153 sample trees) for model validation. Descriptive statistics for both data sets are shown in *Tables 1 and 2*. The overlapping bolts method was used to calculate the actual volume of each bolt and tree (Bailey, 1995).

Figure 1. *Image of Lilin forest farm and Jinsha forest farm Data collection*

Table 1. *Fitting data summary statistics for five commercial tree species in NE China*

Natural species	Variable	Mean	SD	Minimum	Maximum
Dahurian larch (n = 110 trees)	*D* (cm)	24.37	5.64	8.6	40.2
	H (m)	18.67	2.57	8.9	24.6
	d (cm)	17.61	8.89	1.0	50.4
	h (m)	7.90	6.03	0.3	24.0
Korean spruce (n = 124 trees)	*D* (cm)	29.38	6.89	9.1	49.5
	H (m)	21.59	3.90	6.8	30.7
	d (cm)	22.37	9.65	1.2	58.6
	h (m)	9.32	7.10	0.3	30.0
Manchurian fir (n = 145 trees)	*D* (cm)	25.49	5.92	5.5	40.2
	H (m)	19.34	3.25	6.9	25.9
	d (cm)	19.09	8.81	1.1	47.0
	h (m)	8.36	6.40	0.3	25.0
Planted species					
Dahurian larch (n = 114 trees)	*D* (cm)	23.78	2.78	16.90	32.4
	H (m)	20.44	1.47	14.9	23.9
	d (cm)	16.70	7.25	1.00	43.0
	h (m)	9.14	6.31	0.30	23.0
Mongolian pine (n = 124 trees)	*D* (cm)	28.50	3.34	18.6	36.6
	H (m)	17.99	1.06	1480	22.5
	d (cm)	19.67	8.82	0.80	40.2
	h (m)	7.95	5.55	0.30	22.0

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Table 2. *Validation data summary statistics for five commercial tree species in NE China*

Natural species	Variable	Mean	SD	Minimum	Maximum
Dahurian larch (n = 27 trees)	*D* (cm)	25.12	4.29	19.40	36.6
	H (m)	19.37	1.76	14.50	22.6
	d (cm)	18.23	8.50	0.60	44.2
	h (m)	8.22	6.15	0.30	22.0
Korean spruce (n = 31 trees)	*D* (cm)	28.85	7.66	20.40	47.1
	H (m)	21.40	2.76	17.70	28.2
	d (cm)	21.99	9.92	1.00	45.10
	h (m)	9.21	6.87	0.30	28.00
Manchurian fir (n = 36 trees)	*D* (cm)	22.18	3.89	10.60	30.50
	H (m)	18.46	2.37	10.90	22.20
	d (cm)	16.93	7.44	1.10	41.90
	h (m)	7.88	6.01	0.30	22.00
Planted species					
Dahurian larch (n = 28 trees)	*D* (cm)	23.85	2.96	17.10	29.9
	H (m)	21.01	1.10	18.70	23.0
	d (cm)	16.88	7.05	1.00	36.5
	h (m)	9.39	6.45	0.30	22.0
Mongolian pine (n = 31 trees)	*D* (cm)	28.61	3.66	22.00	35.0
	H (m)	18.43	1.14	15.80	20.8
	d (cm)	19.81	8.79	1.00	41.4
	h (m)	8.10	5.66	0.30	20.0

Data analysis

The segmented model of Max and Burkhart (1976) was selected for this analysis based on its good results for many tree species. For example: Appalachian hardwood species in Virginia (Martin, 1981); *Pinus taeda* in East Texas (Coble and Hilpp, 2006); important conifer species in Turkey (Brooks et al., 2008); *Larix kaempferi* in South Korea (Doyog et al., 2017); and *Betula alnoides* in South China (Tang et al., 2017). This model contains three sub-functions, combined in a single equation to represent the top, middle, and bottom stem sections as a cone, paraboloid, and neiloid frustums. The equation is of the form:

$$\frac{d^2}{D^2} = b_1(Z-1) + b_2(Z^2-1) + b_3(a_1 - Z)^2 I_1 + b_4(a_2 - Z)^2 I_2 \qquad \text{(Eq.1)}$$

where:

$$I_i = \begin{cases} 1 & Z \le a_i \\ 0 & Z > a_i \end{cases} \quad i = 1,2$$

$Z = h/H$, h = height above the ground to the measurement point (m), H = total tree height (m), D = diameter over bark at breast height (cm), d = diameter over bark (cm) to the measurement point at height h, a_i = joining points to be estimated from the data, and b_i are regression coefficients (i = 1,2,3,4).

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The volume equation derived through the integration of Max and Burkhart (1976) taper equation is:

$$V = KD^2H\begin{Bmatrix} b_2/3(Z_u{}^3 - Z_l{}^3) + b_1/2(Z_u{}^2 - Z_l{}^2) - (b_1 + b_2)(Z_u - Z_l) \\ -b_3/3[(a_1 - Z_u)^3 J_1 - (a_1 - Z_l)^3 K_1] \\ -b_4/3[(a_2 - Z_u)^3 J_2 - (a_2 - Z_l)^3 K_2] \end{Bmatrix} \quad \text{(Eq.2)}$$

where:

$$J_i = \begin{cases} 1 & Z_u \le a_i \\ 0 & Z_u > a_i \end{cases} \qquad K_i = \begin{cases} 1 & Z_l \le a_i \\ 0 & Z_l > a_i \end{cases}$$

$K = 0.0000785$, $Z_l = h_l/H$, $Z_u = h_u/H$, h_l = lower height of interest, and h_u = upper height of interest. All other variables, as previously defined.

Simultaneous fitting of taper and volume equations was carried out to minimize the diameter and volume prediction errors at the same time. The parameters used in taper and volume equations were the same. Both equations were fitted independently for all species using SAS PROC MODEL (SAS Institute Inc., 2008). Correlated error structure of the data was not considered in the SAS MODEL procedure. Prediction accuracy is slightly influenced even if the error structure is accounted for during the fitting procedure (Kozak, 1997).

Data evaluation

Three goodness-of-fit statistics, mean absolute bias (MAB), the standard error of the estimate (SEE), and fit index (FI) were tested. The notations for these statistics is as under:

$$MAB = \frac{\sum_{i=1}^{n} |y_i - \hat{y}_i|}{n} \quad \text{(Eq.3)}$$

$$SEE = \sqrt{\frac{\sum_{i=1}^{n} (y_i - \hat{y}_i)^2}{n-k}} \quad \text{(Eq.4)}$$

$$FI = 1 - \left[\frac{\sum_{i=1}^{n} (y_i - \hat{y}_i)^2}{\sum_{i=1}^{n} (y_i - \bar{y}_i)^2} \right] \quad \text{(Eq.5)}$$

where: y_i , $\hat{y}_i$ stand for the measured and predicted values of the i^{th} observation, $\bar{y}_i$ is the mean of $\hat{y}_i$, n symbolize the total number of observations, and k is the number of model parameters.

Comparison of taper equations among species

The method of the non-linear extra sum of squares was used to observe whether different taper functions would be needed for different species (Neter et al., 1990). In this method, fitting of full and reduced models (*Eq. 6*) is required. It has been implemented to assess the need for separate models for different species (Rivas et al., 2004; Brooks et al.,

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2008; Özcelik et al., 2016). The full model constitutes a separate set of parameters for each species, while the reduced model involves the same set of parameters for all species. The full model is attained by expanding all global parameters with a dummy variable and an associated parameter to distinguish the species. The significance of the comparison between full and reduced model is based on *F*-test of the formula:

$$F = \frac{(SSE_R - SSE_F)/(df_R - df_F)}{SSE_F/df_F} \quad \text{(Eq.6)}$$

where: SSE_R, SSE_F, df_R, and df_F are the error sum of squares and degrees of freedom for reduced and full models, respectively. The non-linear extra sum of squares follows an *F*-distribution.

Generally, *F*-test (*Eq. 6*) is believed to be significant, if the *P*-value is below 0.05. In this case, taper equations are not the same across species, and more tests are required to evaluate the differences. Since any possible combination of the species can produce these differences, the *F*-test of full and reduced models (*Eq. 6*) was carried out for all possible pairs of species.

Results and Discussion

Taper equations

Simultaneous fitting was performed to obtain the parameter estimates of taper and volume equations for each species. All parameters were significant at $P<0.0001$. *Table 3* highlights the values of MAB, SEE, and FI for the diameters of each species. Above 95% of the total variation was explained for the prediction of diameter. The estimated SEEs were less than 1.9 cm for all species. The plots of residuals against the predicted diameters are shown in *Fig. 1*. Most of the residuals were clustered near the centre of the data points. There was no apparent increase in the error variance with the increase of tree size.

Table 3. *Fit statistics for compatible taper and volume equation system for five species*

Species	Taper (cm)			Volume (m^3)		
	MAB	SEE	FI	MAB	SEE	FI
Dahurian larch	1.1317	1.4083	0.9703	0.0003	0.0025	0.9602
Korean spruce	0.1755	1.6977	0.9685	0.0004	0.0042	0.9715
Manchurian fir	0.1646	1.8650	0.9552	0.0004	0.0043	0.9500
Planted Dahurian larch	0.0430	1.1502	0.9749	0.0001	0.0023	0.9681
Planted Mongolian pine	0.0047	1.1392	0.9833	0.0001	0.0027	0.9767

Note: MAB, Mean absolute bias; SEE, Standard error of the estimate; FI, Fit index

The performance of the Max and Burkhart equation was also evaluated for different stem sections by using ten relative height (*h/H*) classes for all species. The statistics of MAB and SEE were calculated for diameter and volume prediction (*Tables 4, 5*). The SEE values were relatively smaller for Dahurian larch, planted Dahurian larch, and Mongolian pine for larger (>10%) relative height classes (*Table 4*). However, the values of MAB and SEE were higher for Dahurian larch, Korean spruce, and Manchurian fir near the ground.

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Table 4. *Diameter (cm) mean absolute bias (MAB) and standard errors of the estimate (SEE) by relative height (RH) classes for five tree species*

RH	Dahurian larch			Korean spruce			Manchurian fir			Planted Dahurian larch			Planted Mongolian fir		
	n	MAB	SEE	n	MAB	SEE	n	MAB	SEE	n	MAB	SEE	n	MAB	SEE
0.0-0.1	642	0.5442	2.0017	689	0.3284	1.9774	440	0.2941	1.5689	528	0.1550	1.1253	498	-0.0693	0.9040
0.1-0.2	227	-0.1586	0.7675	266	0.1308	1.4880	160	0.1065	0.9463	227	0.0247	0.7524	246	0.1686	0.7904
0.2-0.3	228	-0.2516	0.9290	257	0.0030	1.5774	155	0.1049	1.1418	228	-0.1729	0.9097	238	-0.1638	0.9252
0.3-0.4	223	-0.1140	1.0099	258	0.1612	1.5811	151	0.1432	1.3677	223	-0.1195	1.0101	208	-0.2388	0.9750
0.4-0.5	233	0.0659	1.1055	260	0.2664	1.5534	162	0.2375	1.6060	233	-0.0005	1.1031	230	-0.1508	1.0279
0.5-0.6	234	0.1914	1.2392	258	0.3375	1.6261	152	0.1960	1.8235	234	0.0931	1.2281	225	0.2017	1.1664
0.6-0.7	235	0.2485	1.2696	261	0.3640	1.7660	159	0.1229	2.1620	235	0.1608	1.2552	227	0.4194	1.3843
0.7-0.8	226	0.1364	1.4071	252	0.2472	1.9981	154	0.0052	2.4983	226	0.1261	1.4024	206	0.1331	1.6222
0.8-0.9	230	-0.1811	1.3132	263	0.0540	2.2425	155	-0.0799	2.6593	230	-0.0897	1.3020	221	-0.2381	1.5301
0.9-1.0	226	0.0794	1.3449	246	0.1880	2.8647	165	0.2691	2.5956	226	0.1063	1.3442	217	0.0584	1.1705
All	2704	0.1317	1.4083	3010	0.2251	1.9064	1853	0.1646	1.8650	2590	0.0430	1.1502	2516	0.0047	1.1392

Note: The last row represents the overall mean of MAB and SEE

Table 5. *Volume (m^3) mean absolute bias (MAB) and standard errors of the estimate (SEE) by relative height (RH) classes for five tree species*

RH	Dahurian larch			Korean spruce			Manchurian fir			Planted Dahurian larch			Planted Mongolian fir		
	n	MAB	SEE	n	MAB	SEE	n	MAB	SEE	n	MAB	SEE	n	MAB	SEE
0.0-0.1	642	0.0009	0.0025	689	0.0008	0.0037	440	0.0005	0.0022	528	0.0004	0.0016	498	-0.0001	0.0013
0.1-0.2	227	-0.0006	0.0026	266	0.0000	0.0072	160	0.0000	0.0040	227	-0.0001	0.0025	246	0.0007	0.0031
0.2-0.3	228	-0.0007	0.0030	257	-0.0002	0.0071	155	0.0003	0.0045	228	-0.0005	0.0029	238	-0.0007	0.0035
0.3-0.4	223	-0.0001	0.0029	258	0.0007	0.0066	151	0.0006	0.0052	223	-0.0002	0.0029	208	-0.0007	0.0034
0.4-0.5	233	0.0004	0.0031	260	0.0009	0.0060	162	0.0007	0.0055	233	0.0002	0.0030	230	-0.0002	0.0033
0.5-0.6	234	0.0006	0.0030	258	0.0009	0.0057	152	0.0006	0.0056	234	0.0004	0.0030	225	0.0008	0.0035
0.6-0.7	235	0.0006	0.0026	261	0.0010	0.0054	159	0.0004	0.0058	235	0.0005	0.0026	227	0.0011	0.0036
0.7-0.8	226	0.0002	0.0022	252	0.0004	0.0049	154	0.0003	0.0053	226	0.0003	0.0022	206	0.0002	0.0028
0.8-0.9	230	0.0000	0.0014	263	0.0001	0.0042	155	0.0003	0.0039	230	0.0001	0.0014	221	0.0000	0.0016
0.9-1.0	226	0.0001	0.0007	246	0.0002	0.0024	165	0.0002	0.0019	226	0.0001	0.0007	217	0.0001	0.0005
All	2704	0.0003	0.0025	3010	0.0005	0.0054	1853	0.0004	0.0043	2590	0.0001	0.0023	2516	0.0001	0.0027

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As a whole, the standard errors of the estimates were higher for 0-10% and above 60% relative heights in all species. These specific relative heights are connected with the butt swell and the point corresponding to the base of the live crown for the sampled trees (Jiang et al., 2005). Previously, this trend was also noticed for other species (Özcelik and Brooks, 2012; Özcelik and Crecente-Campo, 2016).

Volume equations

The values of MAB, SEE, and FI for total stem volume are given in *Table 3*. Above 96% of the total variance of the volume was explained in all species except Manchurian fir, where 95% of the total variance was indicated. The value of SEE was less than 0.005 m^3 for all species. The proposed equation was also examined for volume estimation of different stem sections (*Table 5*). The range of SEE was 0.0005 m^3 to 0.0072 m^3 depending upon the species and relative height classes. As compared to Korean spruce and Manchurian fir, the average error was lower and almost the same for Dahurian larch and planted species. Most of the residuals were accumulated around zero, showing that the predictions were not biased (*Fig. 1*).

The average errors (SEE) in diameter and volume predictions correspond to the previous evaluations of the Max and Burkhart equation. Brooks et al. (2008) and Özcelik and Brooks (2012) reported the maximum SEE of 2.83 cm and 2.53 cm, respectively, in diameter prediction by relative heights for major commercial trees in Turkey. In our case, the SEE was 2.86 cm for this variable. However, the standard errors were lower for the estimates of total stem diameter, total volume, and sectional volume in this study. Doyog et al. (2017) recorded the SEE in diameter predictions of Japanese larch as 1.74 cm for total stem and 2.38 cm for different sections, lower than this study. Conversely, the values for volume estimates were higher compared with our results. Interestingly, the analysis by Jiang et al. (2005) showed a similar tendency for yellow poplar in West Virginia.

A comparison was also carried out between total volume estimates by the proposed model and existing volume tables for the species analysed (*Table 6*). Total volume predictions based on the validation data were better than the estimates of volume tables in terms of the MAB and SEE for all species. Particularly for Korean spruce, Manchurian fir, and planted Dahurian larch, the SEE was 30%, 33%, and 24% less than the volume tables. The plots of total volume residuals for the proposed equation and the prevailing volume tables exhibited higher prediction errors in volume tables for all species (*Fig. 2*). In general, the predictions were larger for bigger trees. This model not only provides a better prediction of total volume but can also predict volumes to any specific height. The available volume tables are devoid of this useful characteristic. Finally, the suggested taper and volume equations were refitted to the combined data (fitting and validation datasets). Species wise parameter estimates are depicted in *Table 7*.

Fitting results with the combined data for full and reduced models based on the proposed *equation (6)* are shown in *Table 8*. The *F*-statistic computed by *equation (6)* was 241.51, which indicates a probability of Type I error of less than 0.0001. Thus, taper equations were not the same for different species. *F*-tests were exercised for each possible pair of the species to detect the differences. All of the ten possible paired comparisons produced significant *F*-values, indicating the nonconformity of one equation for different species. Therefore, separate parameter estimates by species and stand origins (planted vs. natural) were developed.

The Max and Burkhart equation has delivered reliable results in many studies (Martin, 1981; Coble and Hilpp, 2006; Brooks et al., 2008; Özcelik and Brooks, 2012; Doyog et

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al., 2017; Tang et al., 2017). Among five equations, this model provided the most consistent results in predicting the diameter, height, and volume of Appalachian tree species in Virginia (Martin, 1981). Coble and Hilpp (2006) recommended it for diameter and volume estimation of loblolly pine in East Texas. Out of 28 taper equations, the Max and Burkhart equation was the best-segmented model for *Betula alnoides* in South China (Tang et al., 2017). It was recommended for major commercial tree species of Turkey and Japanese larch in South Korea (Brooks et al., 2008; Doyog et al., 2017).

Table 6. *Comparison of total volume MAB and SEE for the proposed Max and Burkhart model and existing total stem volume table estimates for five commercial species*

Species and Models	MAB (m^3)	SEE (m^3)
Natural species		
Dahurian larch		
Max and Burkhart model	-0.0134	0.0515
Existing volume table	0.0427	0.0577
Korean spruce		
Max and Burkhart model	0.0249	0.0583
Existing volume table	0.1506	0.1926
Manchurian fir		
Max and Burkhart model	0.0177	0.0313
Existing volume table	0.0821	0.0955
Planted species		
Dahurian larch		
Max and Burkhart model	-0.0109	0.0354
Existing volume table	0.1442	0.1493
Mongolian pine		
Max and Burkhart model	-0.0393	0.0689
Existing volume table	0.0765	0.1023

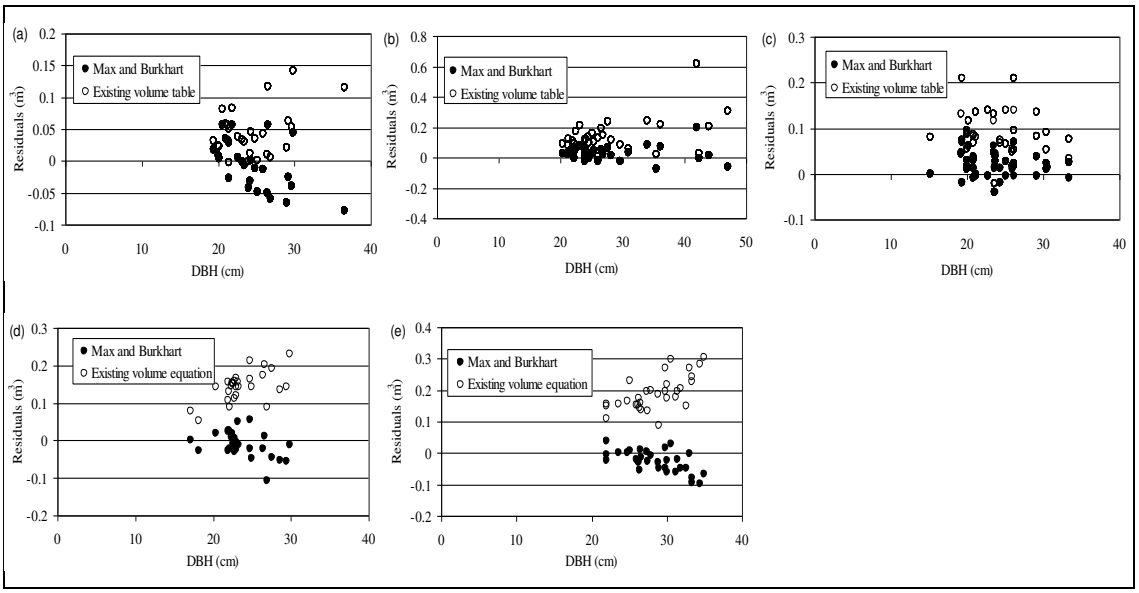

Figure 2. *Total volume residuals (validation data) for the proposed Max and Burkhart volume model and the existing total volume tables for (a) Dahurian larch, (b) Korean spruce, (c) Manchurian fir, (d) Planted Dahurian larch, and (e) Planted Mongolian pine, respectively. Note: Planted Dahurian larch:* $V=0.00013884773(dbh)^{2.4356927S}$*; Planted Mongolian pine:* $V=0.0001611358(dbh)^{2.2206393}$

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Table 7. *Parameter estimates for Max and Burkhart model for five species based on all sample data*

Parameter	Dahurian larch	Korean spruce	Manchurian fir	Planted Dahurian larch	Planted Mongolian pine
b_1	-3.7441	-2.3407	-3.1631	-4.8482	-5.2379
b_2	1.7342	0.6818	1.1547	2.2595	2.5409
b_3	-1.7395	-0.9794	-1.4586	-2.2460	-2.5964
b_4	91.390	147.859	117.821	618.454	73.603
a_1	0.7752	0.8460	0.8303	0.8339	0.8118
a_2	0.0936	0.0714	0.0715	0.0441	0.0923

Table 8. *F-test for Full and Reduced models using Max and Burkhart model for the species analysed*

Model	n	Full Model		Reduced Model		$df_R - df_F$	*F*-value	*P*-value
		SSE_F	df_F	SSE_R	df_R			
Combined	13668	35307.21	13643	47182.50	13662	19	241.51	<0.0000
DL-KS	5395	17792.10	5383	22788.42	5389	6	251.93	<0.0000
DL-MF	5552	17821.21	5540	23289.14	5546	6	283.29	<0.0000
DL-PDL	4975	10359.23	4963	10833.99	4969	6	37.90	<0.0000
DL-PMP	4901	10186.86	4889	10869.98	4895	6	54.64	<0.0000
KS-MF	6177	21703.96	6165	22053.46	6171	6	16.54	<0.0000
KS-PDL	5600	14265.63	5588	16570.82	5594	6	150.49	<0.0000
KS-PMP	5526	16105.30	5514	19665.18	5520	6	203.13	<0.0000
MF-PDL	5757	14271.08	5745	16973.27	5751	6	181.29	<0.0000
MF-PMP	5683	16134.41	5671	19841.96	5677	6	217.19	<0.0000
PDL-PMP	5106	6660.40	5094	7169.40	5100	6	64.88	<0.0000

Note: DL, Dahurian larch; KS, Korean spruce; MF, Manchurian fir; PDL, Planted Dahurian larch; and PMP, Planted Mongolian pine. F-values were calculated by *equation (6)*. SSE_F, df_F, SSE_R, and df_R are the sum of squared errors and the degrees of freedom

Conclusions

This study presents an initial attempt to develop compatible taper and volume equations for Dahurian larch, Korean spruce, Manchurian Fir, and planted Dahurian larch and Mongolian pine in NE China. Fitting of the taper and volume equations was performed simultaneously to confirm the numeric consistency. The model of Max and Burkhart behaved consistently in terms of fit statistics, sectional performance, and graphical interpretation for diameter and volume estimates. The prediction of total stem volume by the proposed equation was superior to volume tables in vogue for the species under study. As an additional attribute, this model can also be applied in the field for the estimation of stem volume to any specific height.

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GRAZING REDUCES BIOMASS FLUCTUATIONS OF RANGELAND PLANTS: AN 11-YEAR COMPARISON OF GRAZING VS. ENCLOSURE

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Abstract. Enclosure age induces technical difficulties related to establishing recovery measures for degraded rangeland biomass. The long-term and continuous enclosure of a degraded rangeland may provide strong theoretical support for this practice. In this study, enclosure measures were established to monitor the plant communities in the long-term grazing rangelands of Xilamuren in the Inner Mongolian Plateau, China, for 11 years. This study found that the number of species decreased in the enclosure rangeland every year, especially the number of perennial forbs. Grazing reduced the interannual fluctuation in the plant community biomass, and enclosure increased the biomass of perennial bunchgrasses and perennial rhizomatous grasses. In the enclosure treatment, community biomass began to decrease in 2014 (an extreme drought year), and there was no significant difference in biomass between the enclosure and grazing treatments in the 11th year. Our results indicate that grazing maintains grassland species and reduces the interannual fluctuations of biomass, and enclosure increases the risks of plant communities coping with extreme drought climates.
Keywords: *species, plant functional groups, community, precipitation, Inner Mongolian Plateau*

Introduction

Grassland ecosystems are one of the largest ecosystems in the world (White et al., 2000). Pasture-based grazing not only provides human beings with products of direct economic value, such as meat, milk, skin, and wool, but also has the extremely important service functions of maintaining the relative constancy of atmospheric components, improving climate, maintaining the biological gene bank, and fixing CO_2, soil and water conservation (Steffens et al., 2008; Reszkowska et al., 2011), among others (Sala and Paruelo, 1997; White et al., 2000). China has the third largest area of grasslands and rangelands in the world (3.9×10^8 ha, occupying 41% of the total land area of China). However, over the past half century, the sharp increase in the number of livestock and human activities have been the most important factors in reducing grassland vegetation coverage, biomass and biodiversity (White et al., 2000; Schönbach et al., 2011). Therefore, it is necessary to study the restoration and rational utilization of degraded rangelands (Nan, 2005).

Existing theory suggests that excessive livestock carrying capacity is an important cause of rangeland degradation. Therefore, the comprehensive interference of feeding, trampling and defecation of livestock can be reduced or eliminated by reducing the stocking rate of grazing livestock or encircling animal husbandry. The original degraded rangeland plant community can recuperate and promote seedling

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germination and growth to improve rangeland productivity. In theory, it should be an ideal measure for rangeland restoration. However, Ruiz-Jaen (2005) stated that most grassland recovery programs rarely last for more than 5 yr. Conversely, long-term grassland observations may be established by replacing time with space (historical background). Although this method can adequately solve the problem regarding time, there are certain hidden dangers; that is, the rangelands can vary in microenvironment, terrain, heterogeneity, climate and other factors. Thus, long-term continuous research in the same study area (grazed vs. enclosed) is needed to statistically evaluate potential differences.

Rangeland plants are also vulnerable to climatic factors, especially extreme climatic variation. Previous research has shown that the climatic characteristics from January to July were primary factors driving plant community changes (Bai et al., 2004). Westoby (1989) noted that the transition of community succession required particular rainfall events (such as rare heavy rain) to drive a change in community composition. This finding is of great interest to us, and we wondered whether extreme drought will also change the characteristics of a community during a long-term enclosure experiment. As the direct manager of rangeland plant community composition and diversity, the quantitative change of herbivores will lead to a series of cascading effects (Bai et al., 2004) of multispecies, plant functional groups and intercommunity feedback regulation in rangeland ecosystems. This has piqued our interest, and we propose that rangeland plant communities facing extreme drought will also have a significant impact, and grazing and enclosure plant communities will show different responses.

In our study, the research determine site was a severely degraded grazed rangeland. Annual dynamic changes in plant communities in long-term (11-yr) enclosed areas of the rangeland were studied to 1) frequency dynamic changes in species and plant functional groups under grazing and no grazing; (2) dynamic changes in plant functional group biomass and community biomass due to grazing and no grazing; and (3) potential factors that drive changes in community biomass.

Materials and methods

Study area

The research site was located in the Xilamuren rangeland (111°12′E, 41°21′N) (desert steppe.) in Darhan Muminggan United Banner, Baotou, Inner Mongolia, China (*Fig. 1*). This region has a gentle topography with a mean elevation of 1468 m. The soil type was chestnut soil with a soil organic matter layer of 5-10 cm. From 2007 to 2017, the mean annual precipitation was 273 mm (*Table 1*). Between 60 and 80% of precipitation occurred during the plant growth season (May to October). The annual evaporation was 2526 mm. The mean temperature by month remained similar from 2007 to 2017. The mean temperature was 4.2 ℃ (maximum 38.0 ℃; minimum -39.4 ℃) (*Table 1*). The coverage of plant communities ranged from 39% to 68%. The dominant species was Stipa krylovii (Roshev.), and the codominant species were Leymus chinensis (Trin.) and Agropyron cristatum (Linn.). Historically, rangelands were mainly used by nomads. Since the implementation of the grassland contract system in 1988, local herdsmen have changed from traditional nomadic life to long-term settled grazing. No utilization was performed between 1988 and 2006, and the enclosure experiment was initiated in 2007.

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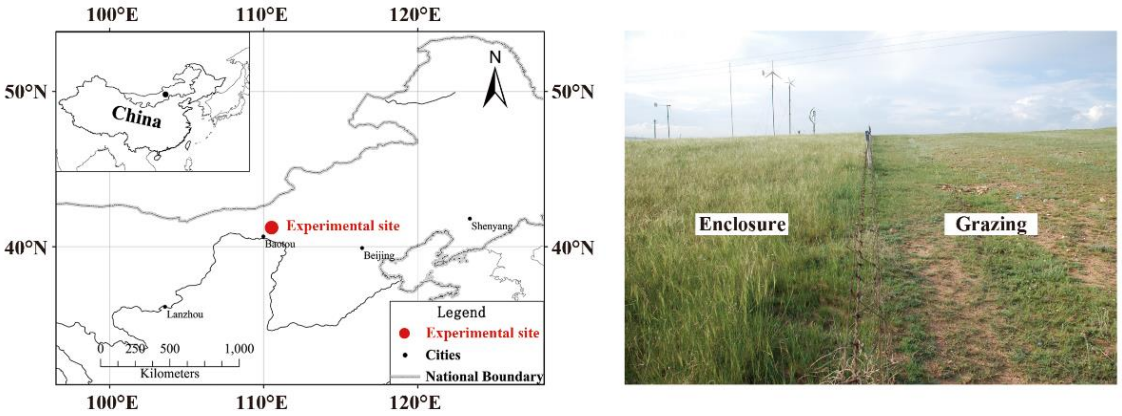

Figure 1. *The location of experimental site and experiment treatment*

Table 1. *Annual precipitation distribution map of the study site*

Year	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017
Annual precipitation (mm)	231.40	372.70	200.20	274.90	258.60	442.30	278.10	184.95	265.20	264.80	228.00
Average temperature of plant growing season (℃)	19.19	20.24	19.59	18.44	19.05	19.64	18.61	19.47	19.86	19.55	19.88

Study site setting

For the enclosure treatment, three 40 ha enclosures were established in the rangeland in early May 2007. No livestock entered these enclosures during the 11-yr period. Sites were selected for similarity of natural habitat factors, including slope and slope aspect. For the grazed treatment, three sites were closed to the enclosure treatments, and each site area was 40 ha. For the grazed treatment, three 40-ha sites were selected close to the enclosure treatments. Grazing was by sheep only, and the stocking rate of each site was 0.30 sheep ha^{-1} $month^{-1}$, resulting in a 50 to 55% grass utilization rate (Wang et al., 2014). Sites were similar in topography and landforms to the enclosure sites. Grazing took place from May to October every year. In the evening, the flock was allowed in the site, with no supplementary feeding.

Sampling method

Changes in vegetation were determined in August, which is the peak biomass in desert rangelands. To determine the biomass of each plant species present, ten 1 m^2 quadrats were regularly placed along the transect in a Z-shaped orientation. The transect lines were 200 m long and laid 5 m away from the boundary enclosure to avoid edge effects. Along each transect, 1 m^2 quadrats were laid at intervals of 20 m. For each quadrat, live and dead aboveground biomass was clipped at the ground level, and dead parts were removed (Bai et al., 2012). Plants were clipped by species in each quadrat and numbered. The fresh materials were brought back to the laboratory and put in an oven at 105 ℃ for 10 min. Then, samples were dried to constant weight in a drying cabinet at 65 ℃, and finally, the dry sample was weighed. A total of 60 quadrats were investigated each year. The data for this study were collected from 2007 to 2017, with 660 quadrats in total.

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Plant functional groups

According to the existing literature and the plant types in the experimental fields, plants were divided into five functional groups (Bai et al., 2004): perennial bunchgrasses, perennial rhizomatous grasses, perennial forbs, shrubs and subshrubs, and annual or biennial herbs (*Table 2*).

Table 2. *Existing plants of the study site*

Species code	Species name	Plant functional groups	Species code	Species name	Plant functional groups
1	*Stipa krylovii*	PB	31	*Iris tenuifolin*	PF
2	*Cleistogenes squarrosa*	PB	32	*Chamaerhodos trifida*	PF
3	*Agropyron cristatum*	PB	33	*Thalictrum petaloideum*	PF
4	*Koeleria cristata*	PB	34	*Allium bidentatum*	PF
5	*Cleistogenes songorica*	PB	35	*Polygala tenuifolia*	PF
6	*Stipa breviflora*	PB	36	*Iris lactea*	PF
7	*Leymus chinensis*	PR	37	*Haplophyllum dauricum*	PF
8	*Kochiaprostrata*	SS	38	*Oxytropis leptophylla*	PF
9	*Ptilotricum canescens*	SS	39	*Carex duriuscula*	PF
10	*Thymus mongolicus*	SS	40	*Cirsium setosum*	PF
11	*Caragana stenophylla*	SS	41	*Astragalus galactites*	PF
12	*Artemisia frigida*	PF	42	*Taraxacum mongolicum*	PF
13	*Heteropappus altaicus*	PF	43	*Allium tenuissimum*	PF
14	*Allium mongolicum*	PF	44	*Leymus secalinus*	PF
15	*Hedysarum brachypterum*	PF	45	*Scorzonera pseudodivaricata*	PF
16	*Convolvulus ammannii*	PF	46	*Phlomis dentosa*	PF
17	*Stellera chamaejasme*	PF	47	*Lappula myosotis*	AB
18	*Artemisia argyi*	PF	48	*Plantago depressa*	AB
19	*Bupleurum scorzonerifolium*	PF	49	*Chenopodium aristatum*	AB
20	*Arenaria juncea*	PF	50	*Eragrostis pilosa*	AB
21	*Potentilla verticillaris*	PF	51	*Artemisia anethifolia*	AB
22	*Cymbaria dahurica*	PF	52	*Corispermum declinatum*	AB
23	*Potentilla bifurca*	PF	53	*Neopallasia pectinata*	AB
24	*Gentiana dahurica*	PF	54	*Chenopodium iljinii*	AB
25	*Androsace incana*	PF	55	*Orostachys fimbriatus*	AB
26	*Potentilla acaulis*	PF	56	*Euphorbia humifusa*	AB
27	*Potentilla tanacetifolia*	PF	57	*Lepidium apetalum*	AB
28	*Melissilus ruthenicus*	PF	58	*Salsola collina*	AB
29	*Dracocephalum heterophyllum*	PF	59	*Chenopodium glaucum*	AB
30	*Sibbaldia adpressa*	PF			

PB, perennial bunchgrasses; PR, perennial rhizome grass; SS, shrub and semishrubs; PF, perennial forbs; and AB, annuals and biennials

Statistical analysis

Independent sample t-tests were used to examine and compare the plant community biomass, biomass of each functional group and biological differences in dominant species in the enclosure and grazing rangelands in the same year. Analysis was completed in SAS 9.0 (SAS Institute Inc., Cary NC, USA).

A regression equation was constructed between community biomass and grazing treatment, annual precipitation, perennial bunchgrasses, perennial rhizomatous grasses, perennial forbs, shrubs and subshrubs, and annual or biennial herbs by using partial

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least squares. According to the obtained regression equation, variables important in prediction (*VIP*) can be identified.

$$VIP_j = \sqrt{\frac{q\sum_{h=1}^{m} r^2(Y,t_h)w_{hj}^2}{\sum_{h=1}^{m} r^2(Y,t_h)}} \quad \text{(Eq.1)}$$

In *Equation1, q* is the number of independent variables, $r(Y,t_h)$ is the covariance of two observational variables, and w_{hj} is component *j* of axis w_h.

The larger the *VIP* of the independent variable is, the stronger the effect of the independent variable on the community biomass. If the *VIP* of the independent variable is > 1, the independent variable is considered an important index that affects the community biomass, and if the *VIP* of the independent variable is < 0.5, the independent variable is considered an unimportant index that does not affect the community biomass (Li et al., 2015). The above statistical analysis was completed in SAS 9.0 (SAS Institute Inc., Cary NC, USA).

To further analyze factors driving biomass changes in rangeland plant communities, we selected variables with *VIP* > 1 and defined these variables as observable variables, and we defined plant functional groups as potential variables. A structural equation model was constructed for statistical analysis with the maximum likelihood as the estimation method (Bansal et al., 2014). The above statistical analysis was completed in Amos 20 (IBM, SPSS, Armonk, NY, USA).

Results

Frequencies of species and functional groups

Fifty-nine plant species were found in the 11 years of field observation, among which the frequencies of *Stipa krylovii*, *Cleistogenes squarrosa*, *Agropyron cristatum* and *Convolvulus ammannii* were always higher than 0.1 (*Fig. 2*). First, compared with grazed rangeland, frequencies of *Artemisia frigida*, *Heteropappus altaicus* and *Allium mongolicum Regel* in the enclosure treatment decreased year by year, which is contrary to patterns observed for *Koeleria cristata var. poaeformis* and *Salsola collina*. Second, the total numbers of plant species decreased every year under the grazing treatment (*Fig. 2*), while the numbers of plant species remained stable at approximately 10 species under the grazing treatment. From the perspective of plant functional groups, the frequencies of perennial bunchgrasses were always higher than 0.8 in the 11 years. Compared with the enclosure treatment, interannual changes in the frequency of perennial rhizomatous grasses were relatively higher under the grazing treatment than under the enclosure treatment. This is opposite to patterns observed for annual and biennial herbs.

Biomass of common species, plant functional groups and communities

Over the 11 years, *Stipa krylovii*, *Cleistogenes squarrosa*, *Agropyron cristatum* and *Convolvulus ammannii* were all found in both grazed and enclosed treatments. The biomass of *Stipa krylovii* was higher under the enclosure treatment than under the grazed treatment ($P < 0.01$), which reached a maximum value in 2012 (*Fig. 3*). The

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range of fluctuation in interannual biomass of the common species under the grazed treatment was lower than when enclosure was allowed. Additionally, *Stipa krylovii*, *Cleistogenes squarrosa*, and *Agropyron cristatum* are perennial bunchgrasses. The results of the plant functional groups showed that the biomass of perennial bunchgrasses and perennial rhizomatous grasses significantly increased in the enclosure treatment. However, the biomass of perennial forbs decreased (*Fig. 4*). Community biomass results showed that ranges of interannual fluctuations of community biomass were similar under the grazing and enclosure treatments. The community biomass maximum value in 2012 was over 600% higher than that in 2017 (minimum value), where enclosure occurred, but the community biomass under grazing was relatively low. However, the range of interannual fluctuation in community biomass under grazed treatment was relatively lower than when enclosure occurred. In the first 10 yrs, community biomass under the enclosure treatment was significantly higher than that under the grazed treatment ($P < 0.01$), but there was no significant difference between the two treatments in the 11th year; however, community biomass in the enclosure treatment began to decrease in 2014.

Biomass of plant functional groups and communities between 2007 and 2017

The results of the independent sample t test showed that over the 11 years, the community biomass under enclosure decreased 41.17% ($P < 0.05$). The difference was mainly reflected in the perennial bunchgrasses and perennial rhizome grasses. Compared with the perennial bunchgrasses and perennial rhizome grasses in 2007, the perennial bunchgrasses and perennial rhizome grasses decreased 26.61% and 49.56% ($P < 0.05$) in 2017. However, under the grazing treatment, there was no significant difference between the biomass of communities and functional groups in 2007 and 2017 (*Fig. 5*).

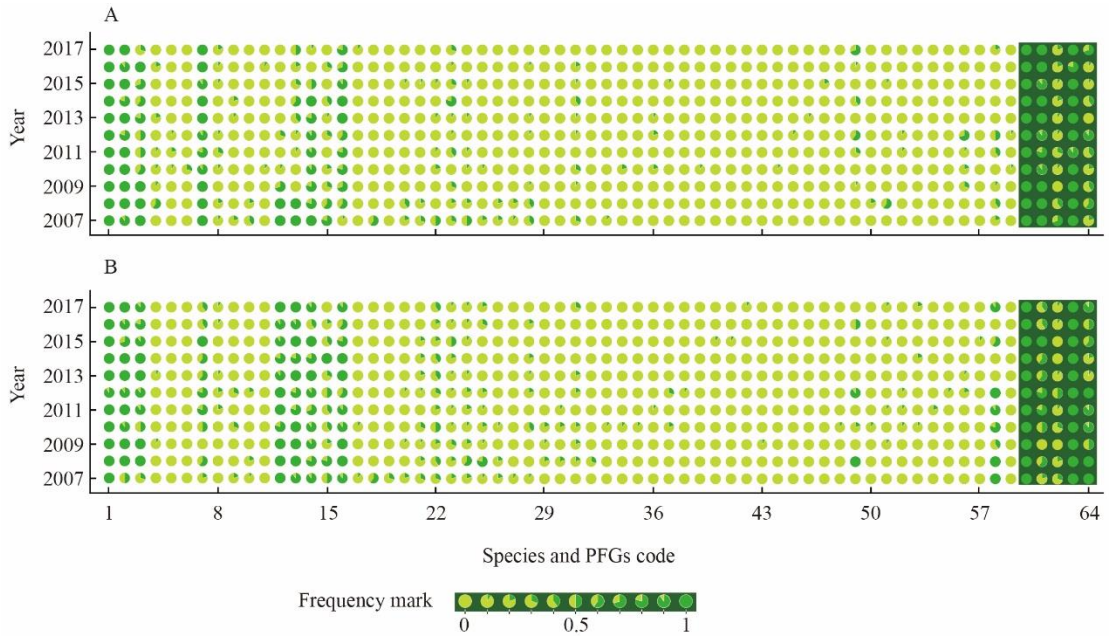

Figure 2. *Interannual variability in the frequencies of species and functional groups. A and B indicate enclosure and grazing, respectively. Codes 1-59 indicate the plant species; see Table 2 for details. Codes 60-64 (green shadow) indicate perennial bunchgrasses, perennial rhizome grasses, shrubs and semishrubs, perennial forbs, and annuals and biennials*

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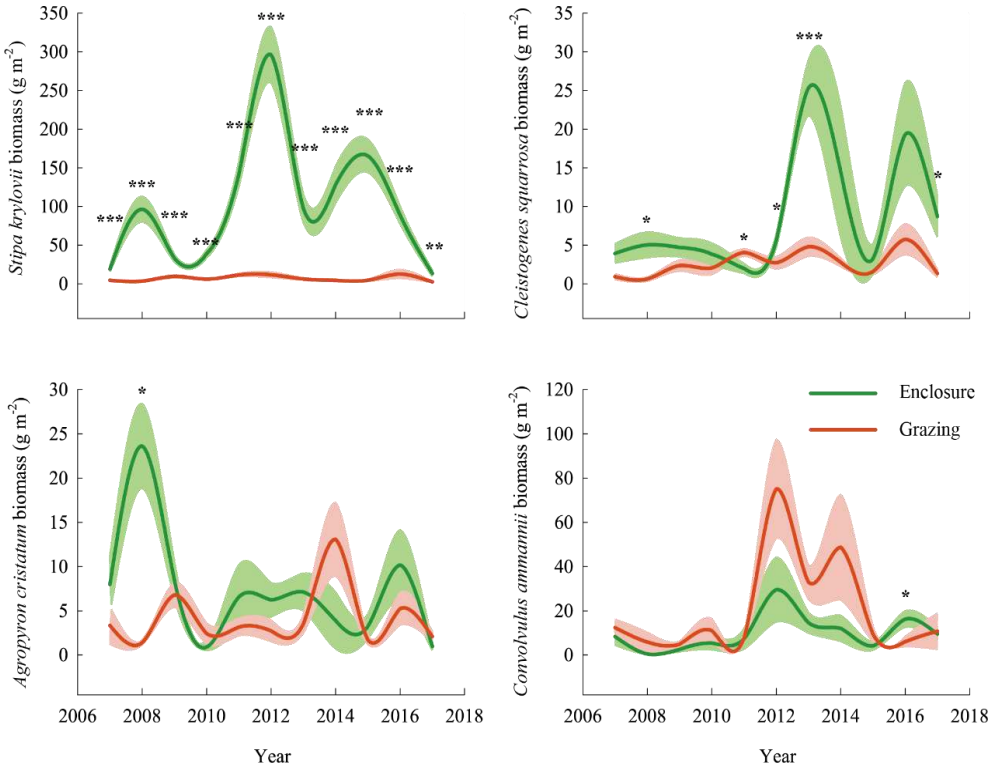

Figure 3. *Annual variation in the biomass of dominant plant species under enclosure and grazing treatments. The shadowed part represents the standard error. *, P < 0.05; **, P < 0.01; ***, P < 0.0001*

Driving factors for biomass

Regression equations were constructed between community biomass and five plant functional groups, annual precipitation and grazing treatment by using partial least squares, which were used to calculate the VIP value for each factor that drives the changes in community biomass. The results showed that perennial bunchgrasses, perennial rhizomatous grasses, precipitation and grazing were important indexes (VIP > 1) that affected changes in community biomass (*Fig. 6A*). The selected indexes were used to construct the structural equation model in this study as indicated by nonsignificant P values (*Fig. 6*). The results of this model showed that grazing had a negative effect on the biomass of the plant functional groups and indirectly affected the community biomass ($R^2 = 0.90$) (*Fig. 6B*). However, precipitation had a positive effect on the biomass of the plant functional groups and indirectly affected the community biomass.

Discussion

Compared with the grazed rangeland, the numbers of species in the enclosure plant communities decreased every year, which is in contrast to the results of other studies (Loeser et al., 2007; Liu et al., 2016, 2017). These differences may be caused by the following factors: a) Dominant species: the competitive advantages of the dominant species significantly increased when grazing was excluded, which increased the biomass of perennial bunchgrasses and perennial rhizomatous grasses, especially bunchgrasses (e.g., *S. krylovii*, *C. squarrosa* and *L. chinensis*). Moreover, when this

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rangeland is supplied limited resources, increases in dominant species inevitably lead to decreases in survival chances of other species (e.g., *A. frigida*, *H. altaicus* and *A. mongolicum Regel*). In addition, in the grazing treatment, the biomass of perennial bunch grasses was always at a low level, which may be related to the selective feeding of herbivores and the palatability of the plants themselves. Koerner et al. (2018) suggests that when herbivores reduce the abundance (biomass, coverage) of dominant species (for example, because dominant plants are delicious), additional resources can be used to support new species, thereby increasing biodiversity. b) Habitat homogenization: Livestock disturbance causes rangeland to produce a variety of habitat patches to ensure that plant species in different successional stages will coexist; however, large livestock disturbances were removed from the enclosed rangeland, which resulted in habitat homogenization, resulting in a decrease in the number of species. c) Species migration: Under grazed treatment, plant seeds can spread effectively with the help of anemophily (wind), insects and large herbivores, increasing the chances of survival of the species, which in turn increases the number of species. Under enclosure treatment, the spread of plant seeds can occur only through anemophily or insects, which greatly limits the spread of species. In this study, the biomass of *S. krylovii* under the grazing treatment was 3496% higher than that under the enclosure treatment. The spatial distribution data are not presented in this study, but in the 1 m^2 quadrat, the biomass of *S. krylovii* reached 296.19 ± 36.00 g in 2012, and limited spatial dispersion (small-scale diffusion) could have caused strong spatial aggregation of the population. The aggregation of plants caused by limited spatial transmission can make the population appear to have a patchy distribution (Webb and Peart, 2000). However, the occurrence of small-scale plant aggregations in relatively harsh habitats may lead to competition and self-thinning of plants (Javier, 2012) and may even lead to the death of young plants, leading to a decrease in the number of species in the enclosure communities every year.

Enclosure increased community biomass, which was mainly because disturbance by foraging, trampling and defecation of livestock was eliminated by enclosing the rangeland. Thus, dominant species in the community that were previously strongly impacted by livestock could quickly exert their competitive advantages to change the species composition of the community. Related results have shown that fencing enclosures increased the biomass of grassland plant communities (Han et al., 2015; Kohyani et al., 2011; Lu et al., 2015). However, in our study, we found that the range of fluctuation in the interannual biomass of plant communities under grazing was relatively lower than that of plant communities in the enclosure treatment because livestock disturbance may stimulate supercompensation effects in plants that drive them to conduct an effective carbon assimilation process. In addition, during long-term livestock disturbance, plant communities may be in the “intermediate disturbance” stage, which reduces competition effects of specific plants in the plant communities, enables nondominant species to utilize growth space (Altesor et al., 2005), and increases community biomass, which is consistent with the results of this study. From the perspective of plant functional groups, the structural equation model also shows that grazing livestock can indirectly regulate the biomass of plant communities by affecting plant functional groups, such as perennial bunchgrasses and perennial rhizomatous grasses, thus releasing competition among different plant functional groups. It makes it in a relatively stable state for a long time, which verifies the hypothesis of a compensation effect between functional groups in the community (Bai et al., 2004).

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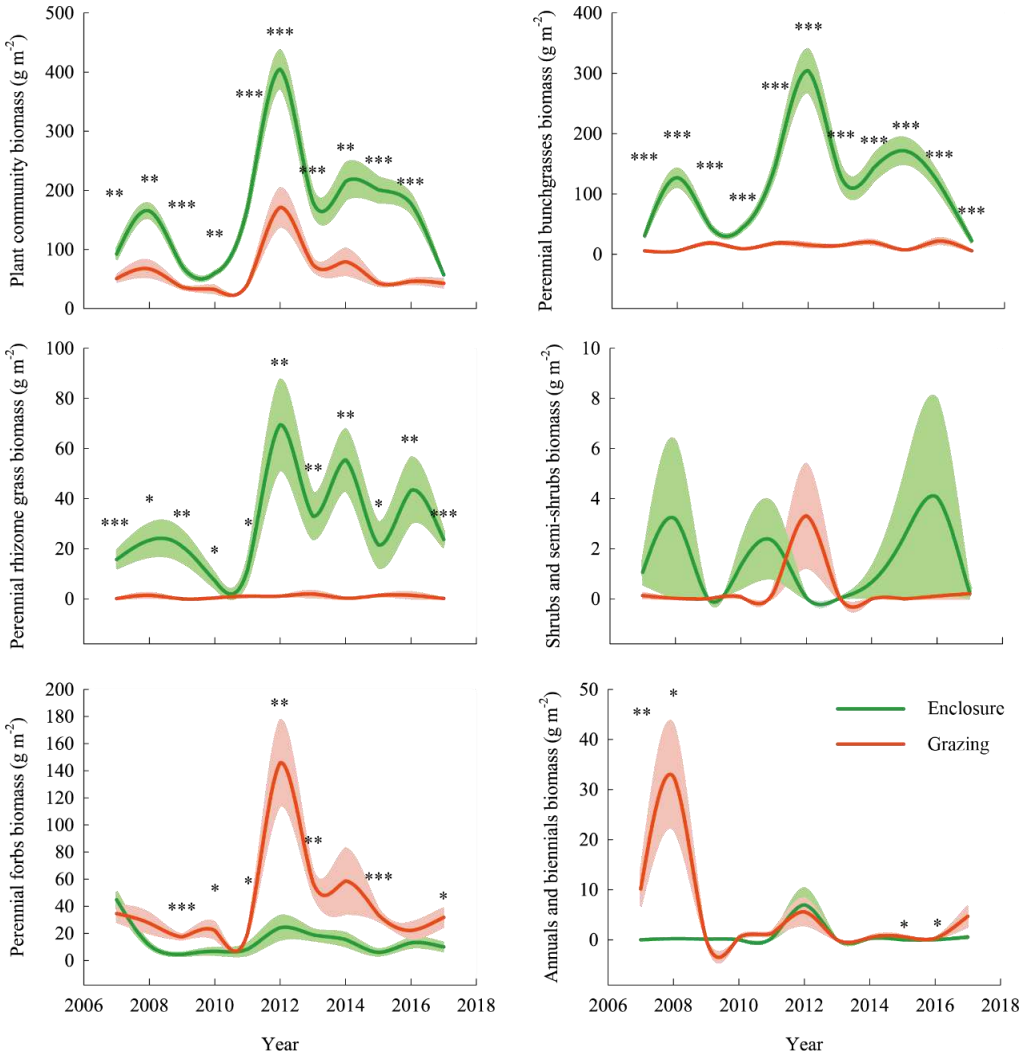

Figure 4. *Annual variations in plant functional groups and community biomass under enclosure and grazing treatments. The shadowed part represents the standard error. *, P < 0.05; **, P < 0.01; ***, P < 0.0001*

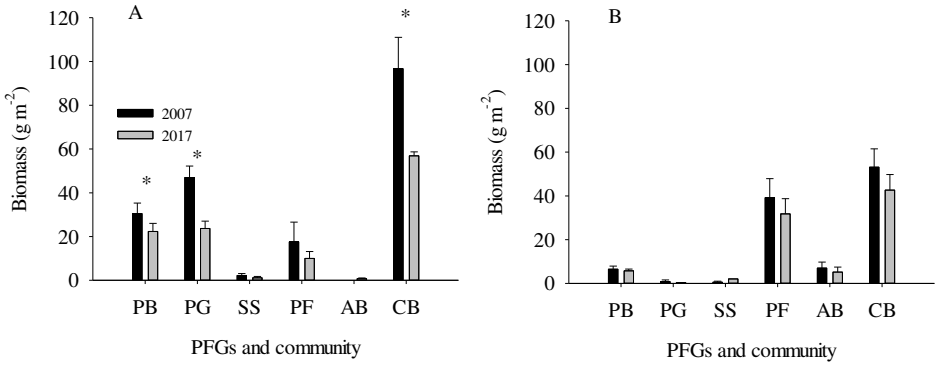

Figure 5. *Biomass of community and functional groups under enclosure and grazing in 2007 and 2017. A and B indicate enclosure and grazing, respectively. PB, PG, SS, PF, AB, CB indicate perennial bunchgrasses, perennial rhizome grasses, shrubs and semishrubs, perennial forbs, and annuals and biennials and community. * indicates a significant difference between 2007 and 2017. No mark indicates no difference between 2007 and 2017*

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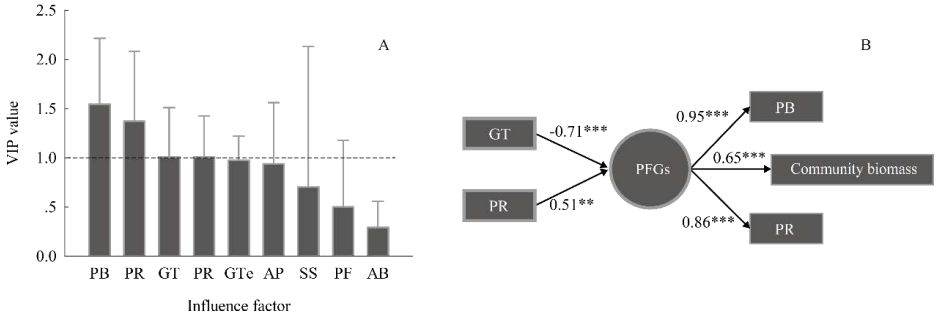

Figure 6. *Screening of the factors driving the changes in biomass and the final structural equation model of biomass. The results of the structural equation model fitting: $\chi^2 = 2.833$, df = 5, P = 0.051. PB, perennial bunchgrasses; PR, perennial rhizomatous grasses; PF, perennial forbs; SS, shrubs and subshrubs; AB, annual or biennial herbs; GT, grazing treatment; AP, annual precipitation; PFGs, plant functional groups; PR, precipitation; GTe, average temperature of the plant growing season*

By screening the important indexes that affect community biomass, we found that grazing affected community biomass. However, we also found that biomass fluctuations always increased and decreased with changes in annual precipitation, and excluding grazing increased the effect of annual precipitation on rangeland plants (Wang et al., 2014). Further analysis showed that the community biomass where grazing was excluded was higher than that under the grazed treatment from 2007 to 2016, and there was no difference between the two treatments in 2017. However, the community biomass in enclosure areas began to decrease in 2014. These data are similar to the research results of Bai et al. (2004). The biomass of rangeland plants decreases during extreme drought years and cannot be effectively increased in the following several years except after extreme precipitation (Westoby, 1989). This impact may be because in the Inner Mongolian desert grassland where there are four distinct seasons, the aboveground branches of perennial bunchgrasses usually survive only one growing season, while underground organs can survive for many years (Li et al., 2012). These underground vegetative organs (bud banks) play a decisive role in the reproduction and survival of plant populations (Hartnett et al., 2006). However, extreme droughts can cause devastating damage to underground organs. In addition, under such drought conditions, the quantity of microorganisms decreases, their activity weakens, and the mineralization rate and fluxes of carbon and nitrogen in the soil decrease significantly (Bloor and Bardgett, 2012). This in turn affects the growth, development, and reproduction of rangeland plants so that they cannot be restored for a long time. This result provides a warning that long-term exclusion of livestock seems to increase vulnerability when extreme drought occurs, and if these rangelands face successive years of drought or long-term drought, plant communities that have remained enclosed for long periods of time will be more vulnerable than those in grazed rangelands, which may result in more severe degeneration. That is, excluding grazing may increase the risks of plant communities coping with global changes.

Conclusion

Based on the analysis of the plant community biomass in grazed and enclosed rangelands for 11 consecutive years in Inner Mongolia, it is concluded that grazing

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reduces plant community biomass and the range of interannual fluctuation in biomass, while enclosure increases the biomass of perennial bunchgrasses and perennial rhizomatous grasses, and the difference was mainly reflected in the perennial bunchgrasses and perennial rhizome grasses. Therefore, it is of great significance for the ecology and management of desert grassland to set a reasonable enclosure. In addition, precipitation had a positive effect on the biomass of the plant functional groups and indirectly affected the community biomass. Our results also indicated that in the face of extreme climate, enclosure increases the impact of extreme precipitation on plant communities and increases risks associated with extreme drought conditions. This indicated that in the face of future climate change, especially precipitation and seasonal changes, long-term enclosure is not an appropriate measure for rangeland restoration.

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

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HIGH-FREQUENCY INDUCTION OF MULTIPLE SHOOTS AND PLANT REGENERATION FROM COTYLEDONARY NODE EXPLANTS OF TONGKAT ALI (*EURYCOMA LONGIFOLIA* JACK)

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Abstract. *Eurycoma longifolia* Jack is traditionally used as an aphrodisiac and health supplement for various diseases. Due to its potential commercial value as a plantation crop as well as to conserve its germplasm, it is necessary to establish a suitable protocol of propagation as a better alternative for mass production. Hence, this study describes an efficient and reproducible *in vitro* regeneration system of *E. longifolia.* Cotyledonary node explants were excised from 2-week-old *in vitro* seedlings and cultured on Murashige and Skoog (MS) medium supplemented with different concentrations of 6-benzyl aminopurine (BAP), kinetin (KIN) and thidiazuron (TDZ). In addition, various concentrations of indole-3-butyric acid (IBA) and α- naphthaleneacetic acid (NAA) were tested for *in vitro* rooting of shoots. From the results, it was observed that 1.0 mgL^{-1} of BAP induced the highest percentage of shoot formation (76.7%) from cotyledonary node explants. The best rooting response was observed on half-strength MS medium containing 0.5 mgL^{-1} IBA with an average of 3.2 roots per shoot. Regenerated plantlets were successfully acclimatized to *ex vitro* conditions with an 85% survival rate. Overall, this *in vitro* regeneration protocol provides a rapid technique that can be utilized for commercial propagation and genetic transformation of this medicinal plant.
Keywords: *seed germination, shoot multiplication, in vitro rooting, acclimatization, cytokinin and auxins*

Introduction

Medicinal plants have been an essential part of the ethnobotanical aspect of the people around the world since ancient times. Today, the majority of people are still relying on traditional remedies to meet their primary health care needs (Parveen et al., 2010; Uprety et al., 2012; Alsarhan et al., 2014; Jamshidi-Kia et al., 2018). *Eurycoma longifolia* Jack or Tongkat Ali as locally known in Malaysia is an important medicinal plant belonging to the family Simaroubaceae. Due to its diverse medicinal values, every part of the plant is used as medicine, especially the roots (Rahmawati and Esyanti, 2014; Yahya et al., 2015). The traditional use of the Tongkat Ali root extracts as anti-inflammatory and analgesic remedies is well established. Studies have revealed that the root extracts of Tongkat Ali have antimalarial, cytotoxic, aphrodisiac, antioxidant, anti-tumor, anti-inflammatory, anti-pyretic, and anti-amoebic properties, and also it has been applied in the treatment of diverse conditions such as fatigue, impotence, loss of sexual desire, high blood pressure and fever (Bhat and Karim, 2010; Rehman et al., 2016). Conventional propagation of Tongkat Ali via seed germination is considered as the most common method for the plant propagation, but as a woody plant, it is difficult for several reasons

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such as low rate of seed germination and poor flowering (Keng et al., 2002; Rahmawati and Esyanti, 2014; Thu et al., 2016).

Being a recalcitrant plant, the seeds took a long time to germinate and have the lowest percentage of germination due to immaturation of the zygotic embryo (Ayoba et al., 2013; Zeng et al., 2014). Furthermore, Tongkat Ali roots were harvested after 4-7 years of cultivation, so the production of the roots is time-consuming, and fluctuated depending on the seasons (Chua et al., 2011). Therefore, the tissue culture technique was an urgent need for rapid propagation on a commercial scale to meet the pharmaceutical industry demand (Lulu et al., 2015). Previously, efforts have been conducted on Tongkat Ali regeneration using different explants such as shoot tips, roots, stems, leaves, and cotyledons (Hussein et al., 2005, 2012; Mahmood et al., 2010; Rodziah and Madihah, 2015). However, regeneration protocols on Tongkat Ali are not well developed; mainly due to the recalcitrant nature of plants. Direct shoot regeneration through the organogenesis provides a better solution for propagation within a short period of time and less soma clonal variability (Juturu et al., 2015).

Development of suitable *in vitro* regeneration protocols is one of the major prerequisites for improvement of genetic characters of plants using biotechnological methods (Venkatachalam and Kavipriya, 2012; Singh et al., 2015). Although *in vitro* propagation was an alternative method for mass production and conservation, studies had shown that shoot induction of recalcitrant species still rare due to its woody nature (Hussein et al., 2012; Isah, 2016). As an important plant in folk medicinal practices, an efficient propagation protocol is urgently needed to fulfil the market demand for Tongkat Ali. *In vitro* micropropagation provides an alternative solution for obstacles faced by the conventional method of propagation. It can also be applied as a strategy for conservation and utilization of genetic resources (Groach and Singh, 2015; Singh, 2018). Thus, the current study reported a rapid micropropagation system of *E. longifolia* through cotyledonary node explants by using different concentrations of various plant growth regulators.

Material and methods

Plant material and seed germination

The plant material used in this study was provided by Institute of Bioscience, University Putra Malaysia. All experimental procedures were carried out at Plant Biochemistry and Biotechnology Laboratory of Biochemistry Department at University Putra Malaysia. Matured ripe dark-red fruits of Tongkat Ali were washed with detergent and rinsed under running tap water for 30 minutes. Then the epicarp and mesocarp of the fruits were manually removed (*Fig. 1B*). Surface-sterilization of seeds was carried out in laminar-flow hood according to procedure described by Mahmood et al. (2010) as follows: seeds soaked for 5 minutes in 70% (v/v) ethanol; and then submersed for 20 minutes in 20% (v/v) Clorox® plus two drops of Tween-20; after that the seeds were rinsed with sterile distilled water five times. *In vitro* seed germination was carried out to ensure aseptic growth conditions of seedlings that would be used as a source of explants (cotyledonary nodes). After sterilization, the seed coat was removed to accelerate the rate of germination and help to avoid any phenolic compounds that naturally released in culture media from seed coats (*Fig. 1C,D*). Embryos were inoculated into 15 × 2.5 cm vials containing 20 mL of full-strength Murashige and Skoog (1962) (MS) medium without plant growth regulators (PGRs) (*Fig. 1E,F*).

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Figure 1. *Preparation of seeds for in vitro germination, (A) Eurycoma longifolia fruits, (B) removing the epicarp and mesocarp (C, D) removing of the seed coat (E) isolated embryos ready for inoculation (F) inoculation of the embryo on germination media (Bars =1 cm)*

Culture medium and conditions

All experiments were conducted using MS medium with the addition of 3% sucrose, and various concentrations of PGRs. A medium without any PGRs was included as a control. Culture medium solidified with 0.25% Gelrite, and the pH of culture medium was set to 5.8 using HCl or 1N NaOH prior autoclaving for 20 minutes at 121ºC and 1.06 kg cm^{-2} pressure. All cultures were initially maintained in the growth room in the dark at 25 ± 2°C for 1 week, then transferred to 8 h dark and 16 h light photoperiod supplied by cool white fluorescent bulbs (35 µmol m^{-2} s^{-1} photon flux density). Relative humidity was maintained at 60%.

Shoot induction from cotyledonary node explants

Cotyledons nodes were excised from 14-day-old *in vitro* raised seedlings (*Fig. 2C*) under sterile conditions and used as explants. The cotyledonary node explants with intact embryonic axis were cultured on MS medium supplemented with different concentrations (0.2, 0.5, 1.0, 2.0 and 3.0 mgL^{-1}) of 6-benzylaminopurine (BAP) and kinetin (KIN) as well as thidiazuron (TDZ) at concentrations (0.1, 0.2, 0.5, 1.0 and 2.0 mgL^{-1}). Data on shoot regeneration percentage, number of shoots per explant and shoot length were frequently recorded every week of culture. For shoots proliferation, the shoots were regularly sub-cultured at six-week intervals onto the fresh MS medium containing the same cytokinin composition. After harvesting the multiple shoots, the original cotyledonary nodes were recultured on a fresh medium for further shoot multiplication.

In vitro rooting and acclimatization

In order to obtain a complete plantlet, regenerated shoots (2.5 - 4.5 cm in length) were aseptically excised and transferred to rooting medium comprising of half-strength MS medium without auxins or with auxins such as indole-3-butyric acid (IBA) or naphthaleneacetic acid (NAA) at different concentrations (0.2, 0.5, 1.0 and 2.0 mgL^{-1}). The observations on root induction percentage, root number and root length were recorded weekly. Acclimatization was initially carried out in the laboratory as follows: plantlets that have vigorous shoots with a well-developed root system were gently

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removed from the culture medium and washed carefully with autoclaved distilled water to remove the traces of nutrient medium from the roots. Then the plantlets potted in 7.5 cm plastic pots containing an autoclaved jiffy-7 medium. Plantlets were watered and immediately covered with transparent polyethylene bags to maintain high humidity around the plantlets. The plantlets were maintained in the growth room at 25 ± 2ºC in 16 h light and 8 h dark photoperiod (35 µmol m^{-2} s^{-1} photon flux density supplied by cool white fluorescent bulbs). After two weeks the humidity around the plantlets was gradually reduced by puncturing the polyethylene bags with small holes. Finally, the polyethylene bags were removed and plantlets were shifted to big plastic 18cm-pots containing garden soil. These plantlets were then kept under natural day light conditions (12/12-h of light/ dark photoperiod) at the greenhouse for normal growth.

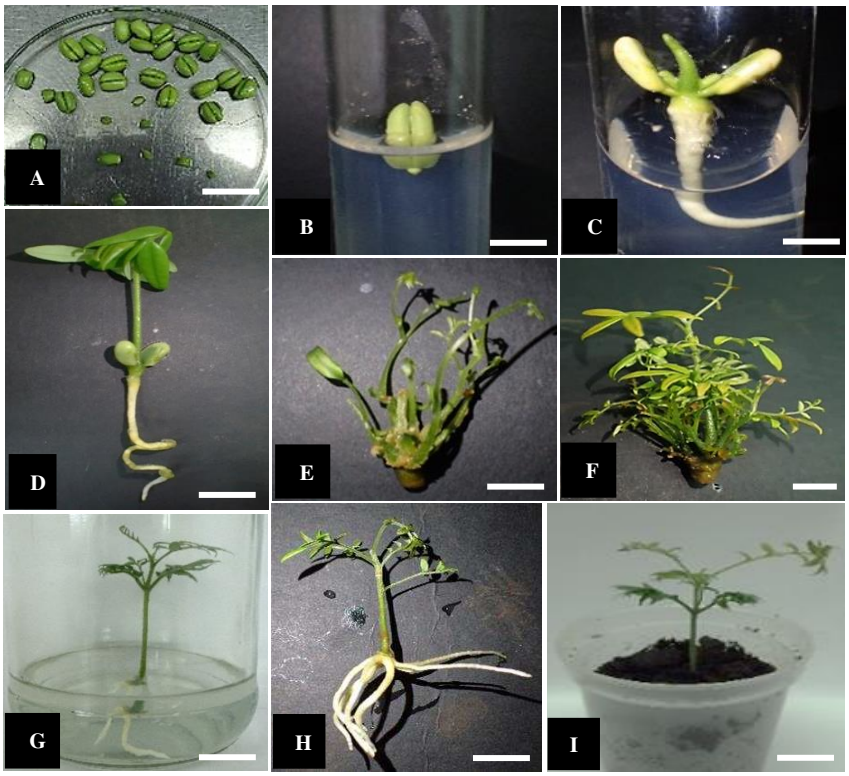

Figure 2. *Multiple shoots formation from a cotyledonary node explants of Eurycoma longifolia. (A) removing the epicarp and mesocarp of the fruits and seed coat from the seed. (B) seed cultured on MS medium with different concentration of cytokinin. (C) seed germination. (D) formation of multiple shoots. (E) shoot proliferation from a cotyledonary node explant on MS BAP 1.0 mgL^{-1}. (F) In vitro rooting of shoots on MS medium supplemented with 0.5 mgL^{-1}IBA. (G) formation of the root system after 6 weeks. (H) plantlet transferred to jiffy-7 potting medium. (I) acclimatized plantlets (Bars =1 cm)*

Statistical analysis

All experiments were designed as a complete randomized design (CRD) with 10 replicates and repeated three times. The percentage of seed germination was calculated with the following *Equation 1* (Keng et al., 2002).

$$\frac{n}{N} x\ 100\% \qquad \text{(Eq.1)}$$

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In *Equation 1,* n is the number of seeds germinated and N is the total number of seeds used in each germination treatment. The observations on the treatments were recorded weekly. Finally, the data obtained were subjected to ANOVA (one-way analysis of variance) to examine whether they were statistically significantly different from each other or not using Minitab software. Means were subjected to Tukey's test ($p \leq 0.05$) and the results were expressed as means ± standard errors (SE).

Results

Effects of plant growth regulators on shoot bud induction

Types of plant growth regulators (PGRs) and their concentrations have been reported to influence organogenesis in plants, as evidenced in this study with the multiplication of shoots from cotyledonary node explants. As shown in *Table 1*, frequency, number and length of multiple shoots formed varied significantly with PGRs supplementations. Variance analysis revealed that shoot proliferation rates differed significantly ($P \leq 0.05$) based on concentrations and type of cytokinin used. The three cytokinins, namely BAP, KIN and TDZ at different concentrations were tested for their ability to induce multiple shoots. Multiple shoots were initially formed from cotyledonary node explants within 15 to 20 days in the presence of cytokinins (*Fig. 2D*). These multiple shoots were induced by supplementing the media with 0.2 - 3.0 mgL^{-1} BAP, KIN or 0.1 - 2.0 mgL^{-1} TDZ. Cotyledonary node explants cultured on MS medium without the PGRs (as a negative control) did not give response to shoot buds formation. Among different cytokinins tested, BAP at a concentration of 1.0 mgL^{-1} showed the highest frequency of multiple shoots induction (76.7%) with the maximum shoot number (4.87±0.70) shoot per explant compared to other concentrations of KIN or TDZ (*Table 1, Fig. 2E*). For shoot proliferation, cotyledonary nodes were regularly subcultured onto fresh medium of the same composition of PGRs.

Table 1. *Effect of different cytokinins concentrations on shoot induction from cotyledonary node explants of Eurycoma longifolia*

Cytokinin concentration (mgL^{-1})			Shoot induction (%)	No. of shoot/ explant (mean ± SE)	Shoot length (cm) (mean ± SE)
BAP	KIN	TDZ			
0	0	0	0.0 d	0.0 d	0.0 c
0.2	-	-	26.67±0.08 bcd	0.93±0.35 cd	0.34 ± 0.13 bc
0.5	-	-	63.33±0.08 ab	1.57±0.39 bcd	0.95 ± 0.24 bc
1.0	-	-	**76.67±0.07 a**	**4.87±0.70 a**	**2.62 ± 0.29 a**
2.0	-	-	60.00±0.09 abc	2.87±0.45 b	1.04 ± 0.18 b
3.0	-	-	36.67±0.08 bcd	1.13±0.39 bcd	0.57 ± 0.20 bc
-	0.2	-	13.33±0.06 d	0.67±0.30 cd	0.32 ± 0.14 bc
-	0.5	-	26.67±0.08 bcd	1.03±0.28 bcd	0.71 ± 0.19 bc
-	1.0	-	36.67±0.08 bcd	1.08±0.38 bcd	2.13 ± 0.31 a
-	2.0	-	26.67±0.08 bcd	1.33±0.31 bcd	0.80 ± 0.19 bc
-	3.0	-	16.67±0.06 d	0.53±0.22 cd	0.38 ± 0.15 bc
-	-	0.1	13.33±0.06 d	1.00±0.33 bc	0.82 ± 0.26 bc
-	-	0.2	23.30±0.07 cd	2.30±0.53 bcd	0.82 ± 0.17 bc
-	-	0.5	30.00±0.08 bcd	1.47±0.38 bcd	0.64 ± 0.16 bc
-	-	1.0	20.00±0.07 d	0.90±0.41 cd	0.43 ± 0.18 bc
-	-	2.0	0.0 d	0.0 d	0.0 c

*Means within a column that do not share a letter are significantly different at $p \leq 0.05$ using Tukey's test

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Based on the current study, BAP was more efficient cytokinin in *E. longifolia's* multiple shoot induction and proliferation, where the number of shoots per explant was significantly higher at 1.0 mgL^{-1} BAP treatment as compared to KIN and TDZ. In general, increased concentrations of plant growth regulators over the optimum concentration resulted in reducing the frequency of shoot induction and the number of shoots per explant (Rahimi et al., 2013; Khan et al., 2015; Kazeroonian et al., 2018). Our study also shows that the TDZ was less effective than BAP or KIN and the shoots formed from the TDZ-containing MS, were stunted and failed to elongate even when the shoots were subcultured on PGR-free MS medium. Moreover, a small amount of callus was observed on some explant cultured on the TDZ-containing medium. Low concentrations of TDZ also inhibited shoot elongation as compared to the BAP treatments; hence shoot induction and elongation with BAP treatment was preferred.

Rooting of the shoot and plantlets acclimatization

Different concentrations of IBA and NAA were added to half-strength MS medium to induce rooting in *E. longifolia*. In this study, regenerated shoots with 2.5 - 4.5 cm in length were excised from the cotyledonary nodes and transferred to the half-strength MS medium supplemented with different concentrations of IBA and NAA or without any auxins (*Table 2*). Initiation of root was observed even from the shoot cultured on half-strength MS medium without auxins with an average number of 0.26 ± 0.15 roots per shoot after three weeks. Although the root formation was observed in MS medium without auxins, the presence of auxins was required to induce a higher number of roots. *Table 2* shows that, out of the four concentrations of IBA tested, 0.5 mgL^{-1} of IBA has proven to be the best concentration for rooting, which represents the highest value of rooting (3.20 ± 0.50). Moreover, the root length was the longest (3.66 ± 0.52 cm) and developed relatively normal in the same concentration of IBA (*Fig.2F,G*).

Table 2. *Effect of different concentrations of IBA and NAA in ½ MS medium on root induction from in vitro shoot of Eurycoma longifolia*

Auxin concentration (mgL^{-1})		Rooting respons (%)	No. of root/ shoot (mean ± SE)	Root length (cm) (mean ± SE)
IBA	NAA			
0	0	20.00±0.10 b	0.26±0.15 e	0.86±0.46 cd
0.2	-	33.33 ±0.12 ab	0.53±0.21 cd	1.04±0.40 c
0.5	-	**80.00±0.10 a**	**3.20±0.50 a**	**3.66±0.52 a**
1.0	-	40.00±013 ab	0.80±0.29 bc	1.28±0.0.43 c
2.0	-	26.70±0.11 b	0.41±0.19 d	0.90±0.40 cd
-	0.2	15.13±0.09 b	0.40±0.18 d	0.62±0.15 d
-	0.5	23.20±0.10 b	1.02±0.43 b	0.90±0.30 cd
-	1.0	30.33±0.11 b	1.43±0.34 b	2.20±0.30 b
-	2.0	19.10± 0.10 b	0.70±0.28 bc	1.26±0.40 c

*Means within a column that do not share a letter are significantly different at $p \leq 0.05$ using Tukey's test

In the current study, it was observed that no callus was induced around the shoot's bases, whereas the roots were initiated directly from the shoot (*Fig. 2H*). Increasing the level of IBA had caused a steady decline in the root formation compared to the medium without auxin (control), which emphasized the fact that a high concentration of auxin in tissue culture conditions could affect root development. It was observed that the platelets

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which had developed root systems before being transferred to soil, seemed to be well-established in the acclimatization stage. In this study, the acclimatization of *in vitro* plantlets with *ex vitro* condition was successfully carried out. Initially the plantlets were transferred to the plastic pots (7.5 cm diameter) containing an autoclaved jiffy-7 medium and covered with transparent polyethylene bags to maintain high humidity and kept in the growth room for the first 2 weeks. The data has shown that 85% of plantlets survived and developed into grown plants when transferred into the soil. No variation in leaf morphology was recorded when they were compared to *in vivo* plants (*Fig. 2I*).

Discussion

Direct shoot regeneration protocol provides a potential rapid technique that can be utilized in genetic transformation. In this study, multiple shoots formation was induced from cotyledonary node explants. It has been reported that cotyledonary nodes were the optimal *in vitro* explants for shoot production in other plants such as *Cassia sophera* (Parveen and Shahzad, 2010), *Sassurealappa Clarke* (Groach and Singh, 2015) and *Cucumis sativus* L. (Venkatachalam et al., 2018). In this study cytokinins such as; BAP, KIN and TDZ were tested for their *in vitro* effects and showed different responses of shoot formation. BAP at low concentration of 0.2 mgL^{-1} BAP showed only 20% shoot induction with an average of 0.83 shoots number. However, BAP at 1.0 mgL^{-1} produced the highest shoot induction (76.7%), with 4.87 number of shoots. Nayak et al. (2013) reported that in *Withania somnifera* the best result was recorded with 1.0 mgL^{-1} BAP and 100% of the cotyledonary nodes exhibited shoot initiation within 12 days. In addition, Kumar and Chandre (2009) reported that among the three cytokinins (BAP, KIN and TDZ) used, BAP was the best cytokinin for inducing the maximum number of shoots from the apical meristem of *Stylosanthes seabrana*. Their result also showed that 1.0 mgL^{1} KIN induced 63.3% multiple shoots with a lower number of shoots (2.40 shoot per explant) on average.

Shoot induction decreased (36.7%) with an average of shoot number 1.13±0.39 at a higher concentration of BAP concentration (*Table 1*). Venkatachalam and Kavipriya (2012) reported a similar result of the frequency of shoot regeneration from the cotyledonary node of *Arachis hypogaea* L. using two cytokinins BAP and KIN with different concentrations. KIN induced the lower percentage of the shoot. Similar observations on the effectiveness of BAP at lower concentration detected in several plant species such as *Ricinus communis* (Alam et al., 2010); *Lens culinaris* (Bermejoa et al., 2012); *Psoralea corylifolia* (Pandey et al., 2013) and *Vitex negundo* (Groach et al., 2014).

Formation of stunted shoots was a common problem with MS medium containing TDZ, as have been reported for several plant species, such as *Cassia siamea* (Parveen et al., 2010); *Withania somnifera* (Nayak et al., 2013); *Elaeocarpus blascoi* (Siva et al., 2015) and *Platanus acerifolia* (Bao et al., 2017). However, prolonged exposure of regenerated shoots to medium supplemented with high concentrations of TDZ resulted in distortion in the shoots. Similar observations have also been reported in other studies (Parveen and Shahzad, 2010; Ahmed and Anis, 2012; Dewir et al., 2018). The proliferation of multiple shoots could be stimulated by adding cytokinins to the regeneration medium, but it can inhibit their further growth and elongation. Therefore, elongation of the shoot could be consistently inhibited by TDZ due to its high cytokinin activity (Kumar and Reddy, 2012; Siddique et al., 2015). Similarly, in several studies on other plant species such as *Cassia sophera* (Parveen and Shahzad, 2010), *Curculigo*

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latifolia (Babaei et al., 2014) and *Jatropha curcas* (Aishwariya et al., 2015) it has been reported that the higher concentrations of TDZ resulted in stunted shoot and callus production.

It has been reported that with an increase in concentrations of cytokinins, the responding frequency of explants and the number of shoot regeneration was increased up to certain limits and then reduced markedly (Naing et al., 2015; Seo et al., 2017; Kumari and Harsh, 2018). Cotyledonary nodes as an explant have been also reported to be an efficient starting material to induce multiple shoots in many of other plant species such as *Sterculia urens Roxb* (Devi et al., 2011), *Citrullus colycynthis* (Meena et al., 2014) and *Mucuna bracteata* DC. (Aziz et al., 2018). These nodes had a better regeneration response compared to the other parts of the plant due to its regenerative axillary meristem cells (Hsieh et al., 2017).

Rooting of *in vitro* regenerated shoots is an important step for successful *in vitro* regeneration of whole plantlets (Toppo et al., 2012; Shekhawat et al., 2015). It produces the whole plant that can survive in *ex vitro* conditions. Roots could be only induced from the shoots which are not succulent or fragile. Development of roots should be directly from the base of the shoot and no callus should be formed to provide a good vascular connection between shoot and root (Anwar et al., 2010). In general, auxins are used for root induction, but they might prevent the continued growth of roots if they remain on the same rooting medium (Harahap et al., 2014). IBA is an auxin that is widely used for root induction in *in vitro* cultures (Baque et al., 2010; Patel et al., 2014). A study conducted by Hussein et al. (2005) on shoot regeneration from shoot tip explants of *Eurycoma longifolia* found out that, out of different concentrations of IBA tested only 0.4 and 0.5 mgL^{-1} were able to induce roots from the *in vitro* plantlets. This is in agreement with our study, which shows that 0.5 mgL^{-1} of IBA is the best concentration for root induction of *E. longifolia*. IBA at 0.5 mgL^{-1} produced the highest percentage of roots (80%) with an average 3.20 root per shoot (*Table 2*). Kumar and Nand (2015) reported a similar observation with the same concentration of auxin (IBA 0.5 mgL^{-1}) in half-strength MS medium for root induction from *Asteracantha longifolia*. However, NAA is more potent than IBA and IAA for the induction of adventitious roots from leaf explants of *E. longifolia* (Hussein et al., 2012).

The superiority of IBA over the other auxins in the induction of root has been previously reported in other studies (Parveen and Shahzad, 2010; Hussein et al., 2012; Agarwal et al., 2015; Bohra et al., 2016). A previous study by Hussein et al. (2005) on *E. longifolia* reported that MS medium supplemented with NAA or IAA, tended to form callus at shoot's bases. Whereas, only IBA showed a sign of direct root formation. These observations could be due to the fact that IBA is more resistant to chemical degradation than other auxins during the autoclaving of culture media, or it might be due to the location of auxin receptors of the plant tissues, where different types of auxin react differently based on the position of its receptor (Nissen and Sutter, 1990; Zhao et al., 2014; Andujar et al., 2019).

Micropropagated plantlets, which are developed in a controlled microenvironment might desiccate and die if they were directly placed at a low level of humidity or higher light level that is stressful as compared to *in vitro* conditions. Moreover, during the acclimatization process the plantlets have poor photosynthetic capability and the leaves act as a source of carbohydrates for the newly developing leaves. This poor photosynthetic capability could cause the deaths of some of the micropropagated plantlets (Chaari-Rkhis et al., 2015). Syafiqah et al. (2017) suggested that the usage of 100% jiffy

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was the suitable potting medium for the acclimatization of *Labisia pumila* plantlets. Yahya et al. (2015) reported that jiffy-7 was the best potting media for Tongkat Ali acclimatization. Their results showed that 100% of plantlets survival was obtained with jiffy-7 medium.

Conclusion

In conclusion, the present study described a rapid and simple protocol for mass propagation. The result showed that the high frequency of multiple shoot induction was possible from the cotyledonary node explants of *E. longifolia* plant. The complete regeneration system described here can be achieved within 12 weeks; a much shorter period as compared to the germination in forest sandy soils in nature which normally takes eight months to grow up to the same height of the *in vitro* propagated plants. This protocol would be beneficial for large-scale propagation that could meet the increasing demand of the pharmaceutical industry. However, it can be used to perform further experiments to obtain transgenic plants or other biotechnological approaches.

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THE EFFECTS OF RETURNING FARMLANDS TO FORESTS OR PASTURES ON SOIL ANIMAL DIVERSITY AND ITS REGIONAL DIFFERENTIATION CHARACTERISTICS IN CHINA: A META-ANALYSIS

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Abstract. A meta-analysis was conducted to investigate the effect of returning farmlands to forests or pastures on soil animal diversity and its regional differentiation characteristics in China. Overall, based on the Shannon-Wiener index and Simpson index values, we found that returning farmland to forest (pasture) had an important effect on soil animal diversity. Forests and pastures might have a higher species richness and lower species evenness of soil animals than farmland. The effect of returning farmland to forest on soil animal diversity was greater than the effect of a return to pasture, although the effects varied between different land use/cover changes. The Shannon-Wiener index and Simpson index values showed different regional characteristics of soil animals when returning farmland to forest (pasture) in North China, South China, Northwest China and Qinghai-Tibet China. Returning farmland to forest (pasture) should be more conducive to increasing soil animal species richness in high-temperature regions and increasing soil animal species evenness in low-temperature regions. Furthermore, in low-temperature regions, returning farmland to forest affected the soil animal diversity more than returning farmland to pasture. Returning farmland to forest was more conducive in improving soil animal diversity synthetically in dry regions, and the changes in soil animal diversity due to the effect of returning farmland to pasture were not evident between dry and rainy regions in China.
Keywords: *soil fauna, Shannon-Wiener index, Simpson index, land use, land cover, species richness, species evenness, meta-analysis*

Introduction

Currently, with global attention focused on biodiversity and its protection, soil animal diversity and its ecological service functions have attracted widespread international and local attention from scholars (Bardgett and Cook, 1998; Shao et al., 2015). Soil animals are an integral component of soil ecosystems and play the role of consumers and decomposers in ecosystems (Albers et al., 2006). These animals have important effects on soil formation, soil structure, fertility conservation and energy flow (Bardgett and Wardle, 2010). Environmental changes and external disturbances could affect the composition and distribution of the soil animal community (Ponge et al., 2013; Baretta et al., 2014).

Land use/cover changes affect the input of litter, leading to changes in soil physicochemical properties, and change the characteristics of the soil animal

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community (Jangid et al., 2011; Liu et al., 2011). Many related studies have shown that vegetation type, soil organic matter, and soil physicochemical properties have important effects on soil animal community structure. For example, changes in land use patterns from forest to farmland radically change vegetation types, resulting in changes in a number of environmental factors, such as soil organic matter, soil temperature and water, and thus changing the structure and composition of the soil animal community (Tan et al., 2008; Yin et al., 2010). Some researchers suggested the richness and diversity of soil animals were influenced by a wide range of agricultural and other land use practices (Baker, 1998), and land-use intensification strongly influenced biodiversity by altering habitat heterogeneity, the distribution of habitat types and their expanse (Eggleton et al., 2005). The richness of different soil animal groups showed contrasting relationships with location and land-use type (Birkhofer et al., 2012).

Returning farmland to forest (pasture) is a China's governmental environmental program that converts arable land to forestry plantation or permanent pasture in order to protect and improve the ecological environment with the principle of sustainable development. Returning farmland to forest (pasture) is a major ecological restoration policy for controlling soil erosion and desertification in China. The process prevents soil erosion by ending land cultivation operations, planting trees and grass, restoring vegetation and controlling soil erosion (Li et al., 2008). The implementation of this policy can cause large-scale and transformative changes in surface cover, but due to the difference of surface vegetation between forest and pasture, the distribution of litter and underground organic matter shows obvious differences, which has a significant different effect on the regional ecological environment, and subsequently affects differently the habitat conditions and diversity of soil animals (Yang, 2004; Liu and Men, 2009). Land use changes across China demonstrated significantly temporal and spatial differences during the last two decades (1990–2010). The area of farmland decreased in the South China and increased in North China. Forest decreased first, and then increased. Pasture continued decreasing (Sun et al., 2018). Thus, increasing interests focused on the effect of land use changes on soil animal diversity in China. Chinese scholars have conducted a series of studies on the changes in soil animal diversity under different land use/cover changes, such as changes between farmland, forest and pasture (Liu et al., 2011; Zhang et al., 2014; Wei et al., 2014). However, due to the complexity of the underground soil ecosystem, previous studies have failed to draw comprehensive conclusions regarding the effect of land use/cover changes on soil animal diversity or to clarify soil animal diversity after the implementation of the policy of returning farmland to forest (pasture) in China. Therefore, correctly and scientifically evaluating the effect of land use/cover change on soil animal diversity has great significance for the scientific management of land, the protection of soil animal diversity and the maintenance of soil ecological function. In addition, China's four geographic regions are North China, South China, Northwest China and Qinghai-Tibet China. The regional characteristics are significant, which include topography, climate and vegetation types, etc. The above factors have important effects on soil animal diversity. So here, regional differentiation characteristics of the effect on soil animal diversity under the policy of returning farmland to forest (pasture) in China is investigated. A meta-analysis is a comprehensive statistical analysis of different studies based on the same research topic (Sun et al., 2015). Meta-analyses have been applied in ecological studies in China (Tian et al., 2014; Zhang et al., 2015; Zhao et al., 2017).

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However, there have been no reports of meta-analyses on the effect of returning farmland to forest (pasture) on soil animal diversity. Based on a meta-analysis, the effect and regional differentiation characteristics of land use/cover change on soil animal diversity caused by the policy of returning farmland to forest (pasture) in China were comprehensively analysed.

Materials and methods

Data collection and selection criteria

We collected data on the effect of returning farmland to forest (pasture) on soil animal diversity in Chinese farmland from peer-reviewed articles published in both Chinese and English language journals from 1999 to 2018. The keywords used for the search included the following: returning farmland to forest, returning farmland to pasture, land use, land cover, vegetation types, soil animal, soil fauna, diversity, farmland, forest, and pasture. The articles in Chinese were collected from the China National Knowledge Infrastructure (CNKI) database, and those in English were from ISI-Web of Science and Google Scholar. Articles were only included if they met the following criteria: 1) the study area needed a detailed geographical location; 2) there was at least one land use change that included returning farmland to forest, returning farmland to pasture or converting forest to pasture; 3) there was at least one detailed chart or description of the soil animal diversity index (Shannon-Wiener or Simpson) before and after the land use change; 4) the soil animal sampling methods, determination methods, and diversity analysis and calculation methods were identical; 5) the means and sample sizes had to be reported; and 6) returning farmland to forest (pasture) could be represented by two different land use types as long as they were located in adjacent sites. The process was conducted following the flowchart diagram presented in *Fig. 1* (Moher et al., 2009), and a total of 60 peer-reviewed articles were selected for our analysis (*Appendix*). Then in those 60 articles, the data of soil animal group number and density was collected for meta-analysis to investigate the change before and after land use change.

Data grouping

To investigate the regional differentiation characteristics of the effect of returning farmland to forest (pasture) on soil animal diversity, the selected data were grouped. For China, according to the comprehensive characteristics of geographical location, physical geography and human geography, the data were divided into four groups: North China, South China, Northwest China and Qinghai-Tibet China, which the boundary between North China and South China is Qinling and Huaihe, the boundary between North China and Northwest China is the Greater Khingan Mountains and the Great Wall, and the boundary between Northwest China and Qinghai-Tibet China is Kunlun Mountains and Qilian Mountains (Chi et al., 2015). In order to further investigate the regional differentiation characteristics, we divided the regions according to annual average temperature (< 12ºC and > 12ºC) and annual rainfall (< 200 mm, 200-400 mm, 400-800 mm and > 800 mm) because of the annual average temperature of study regions and the division base of semi-arid, semi-humid and humid region in China.

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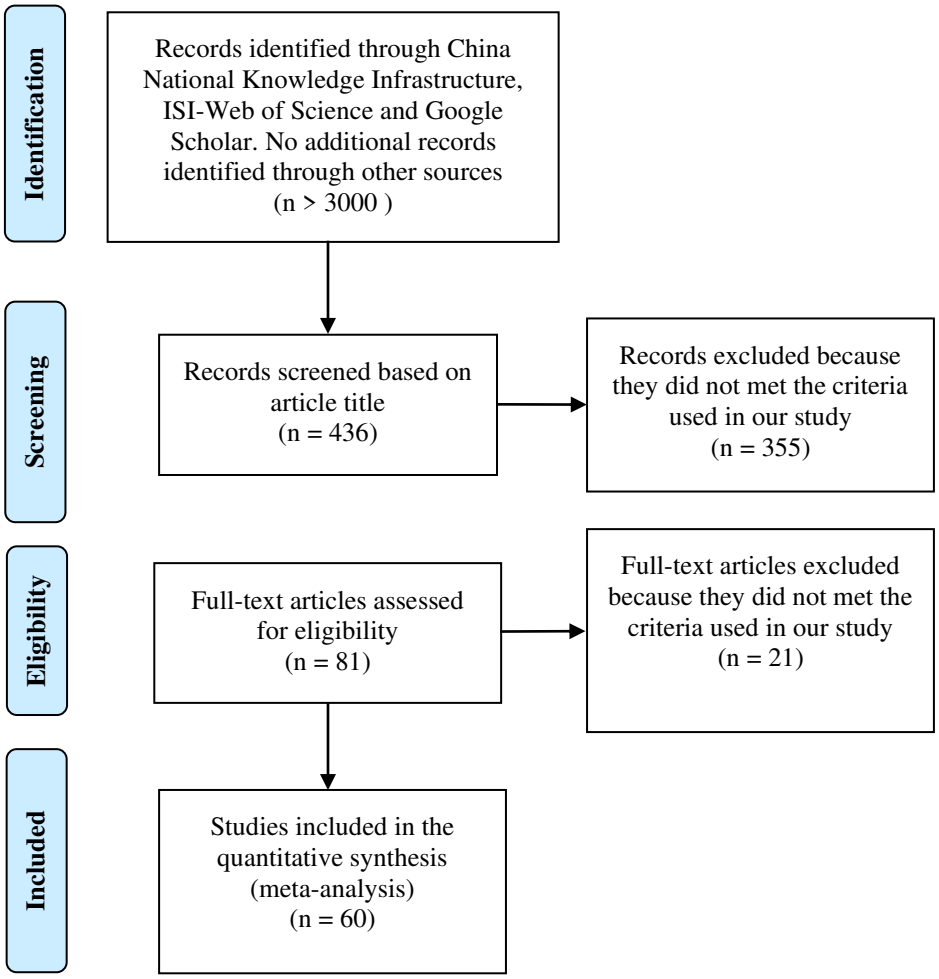

Figure 1. *Flowchart diagram of the process used to obtain the literature data to build the database in our study*

Data analysis

The Shannon index (Shannon, 1948) and Simpson index (Simpson, 1949) were selected as our soil animal diversity metrics because they are often used for analyzing soil animal alpha diversity in soil ecology (Bardgett and Cook, 1998; Wang et al., 2018). Although the Shannon index and Simpson index consider both species richness and species evenness, the Simpson index is more sensitive to community species evenness (Magurran, 1988; Xu et al., 2011). A large Simpson index value indicates that soil animals are unevenly distributed and the ecological functions of dominant species are prominent. The simultaneous increase in the Shannon index and decrease in the Simpson index indicate an increase in soil animal diversity. However, it is possible that the Shannon index and Simpson index increase simultaneously under the same treatment, which generally indicates potential species evenness loss. The Shannon index and Simpson index are calculated using the following *equations*:

$$\text{Shannon index} = -\sum_{i=1}^{S} p_i \ln p_i \qquad \text{(Eq.1)}$$

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$$\text{Simpson index} = \sum_{i=1}^{S} {p_i}^2 \quad \text{(Eq.2)}$$

where p_i is the proportion of individuals of category "i " to the total number of individuals in the community and S is the number of groups.

The variance in each index and the sample sizes of the corresponding research were included in our study. Web Plot Digitize software 3.12 (Rohatgi, 2012) was used to extract the data from the articles that provided only graphics. The variances were obtained by contacting the authors of the articles when the variance data were missing. If the variance data could not be obtained in some articles, the proportion of the variance to the average value of the existing data was used as the standard for calculating the variance based on previous studies (Skinner et al., 2014).

In ecological studies, the meta-analysis effect size was generally shown by the natural logarithm of the response ratio (R), and the objective regulations reflected in selected research data were obtained by using weighted integral method (Hedges, 1999). In this study, the ratio of the average index (X_t, X_c) of soil animal diversity before and after returning farmland to forest (pasture) was taken as the response ratio, and the natural logarithm (ln$R$) was taken as the effect size.

$$\ln R = \ln \frac{X_t}{X_c} = \ln X_t - \ln X_c \quad \text{(Eq.3)}$$

where R is the response ratio, ln$R$ is the effect size, X_t is the mean value of each index before returning farmland to forest (pasture) and X_c is the mean value of each index after returning farmland to forest (pasture). The effect size of each data pair was obtained by meta-analysis, and the weighted mean effect size, lnR_{++}, and its 95% confidence intervals were obtained by calculating the weight based on the standard deviation.

The effect of returning farmland to forest (pasture) on soil animal diversity was significant ($P < 0.05$) if all confidence intervals were greater than 0. Conversely, there was a significant negative effect ($P < 0.05$) if the confidence intervals were all less than 0. If 0 was within the confidence interval, returning farmland to forest (pasture) had a nonsignificant effect on soil animal diversity. We also used the percentage transformed from the mean effect size to explain the response of soil animal diversity on returning farmland to forest (pasture):

$$\left(e^{\ln R_{++}} - 1\right) \times 100\% = \left(R_{++} - 1\right) \times 100\% \quad \text{(Eq.4)}$$

Publication bias was quantified by rank-correlation methods, which included Kendall's tau and Spearman's rank-order method; a $P < 0.05$ indicates the existence of publication bias (Begg and Mazumdar, 1994). Rosenthal's fail-safe method (Rosenthal, 1979) was used to evaluate the publication bias results (*Table 1*). When the fail-safe number is considerably larger than $5k + 10$ (where k is the original number of studies), the result is not affected by publication bias and the meta-analysis result is reliable (Hoeve et al., 2012). In this study, Metawin 2.1 software (Rosenberg et al., 2000) was used for the meta-analysis, and Origin 8.5 software was used for generating the figures.

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Table 1. *Results of publication bias were tested and evaluated. FLF represents returning farmland to forest; FLP represents returning farmland to pasture; and FP represents the conversion from forest to pasture*

Categories		Kendall's Tau method	Spearman's rank-order method	The Rosenthal's fail-safe method
		P-value	P-value	Fail-safe number
Shannon index	FLF	0.7266	0.6821	-
	FLP	0.5912	0.5492	-
	FP	0.4925	0.5999	-
Simpson index	FLF	0.3484	0.4561	-
	FLP	0.6833	0.6332	-
	FP	**0.0058**	**0.0096**	1449 >> 5*k* + 10

Results

Effect of returning farmland to forest (pasture) on soil animal diversity

Across all studies, the Shannon-Wiener index and Simpson index showed different responses by soil animals to returning farmland to forest (pasture) (*Fig. 2*). The Shannon-Wiener index results showing the effect of returning farmland to forest (pasture) on soil animals are shown in *Fig. 2a*. On average, the Shannon-Wiener index value for forest was 18.35% higher than that of farmland, which was significant; however, compared with farmland, the mean effect size of the Shannon-Wiener index value for pasture was positive, but the effect was nonsignificant. In addition, the Shannon-Wiener index value significantly decreased by 9.71% from forest to pasture. The effect of returning farmland to forest (pasture) on soil animals by the Simpson index is shown in *Fig. 2b*. According to *Fig. 2b*, the Simpson index changed significantly with the conversion of land use types, which was caused by returning farmland to forest (pasture). On average, the Simpson index significantly increased by 2.14% and 3.38% in changing farmland to forest and pasture, respectively. In addition, the Simpson index value for forest was 4.32% lower than that of pasture. According to *Fig. 3*, the group number and density of soil animals significantly increased by 9.31% and 46.69%, 3.86% and 62.24% when returning farmland to forest and pasture, respectively.

Regional differentiation characteristics of the effect of returning farmland to forest (pasture) on soil animal diversity

The Shannon-Wiener index and Simpson index values showed different regional characteristics of returning farmland to forest (pasture) on soil animals in China (*Fig. 4*). According to *Fig. 4a*, in North and South China, the Shannon-Wiener index value for forest was 34.21% and 17.75% higher than that of farmland, respectively, which was significant. However, compared with farmland, the mean effect size of the Shannon-Wiener index value for forest was negative, and the effect was nonsignificant in Northwest China. The Simpson index value significantly increased by 5.68% and 3.37% when returning farmland to forest in North and South China, respectively, but significantly decreased by 10.64% in Northwest China. According to *Fig. 4b*, in North and Qinghai-Tibet China, the Shannon-Wiener index value for pasture was 50.92% and 25.59% higher than that for farmland, respectively. In Northwest China, the Shannon-Wiener index value for pasture significantly decreased by 21.05%, and the effect was nonsignificant despite the negative mean effect size in South China. When

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returning farmland to pasture, the Simpson index value significantly decreased by 9.36% and 9.62% in North and South China, respectively. In Qinghai-Tibet China, the Simpson index value for pasture significantly increased by 63.57%, and the mean effect size was positive. However, the effect was nonsignificant in Northwest China. According to *Fig. 4c*, in the conversion from forest to pasture, the mean effect size of the Shannon-Wiener index was negative, and the effects were nonsignificant in North and South China. The Shannon-Wiener index value for pasture was 14.52% lower than that for forest in Northwest China. The Simpson index value for pasture significantly decreased by 1.93% in South China and significantly increased by 23.06% in Northwest China. In addition, the effect was nonsignificant in North China.

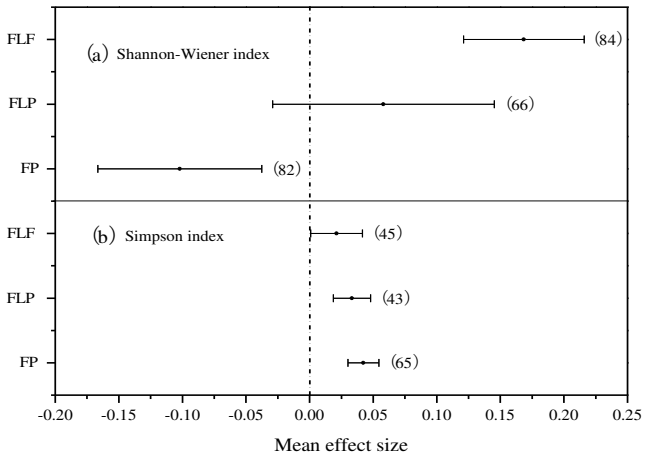

Figure 2. *Effect of returning farmland to forest (pasture) on the Shannon index (a) and Simpson index (b) values. The error bars represent 95% confidence intervals (CIs). The solid circle represents the mean effect size. The numbers represent the number of observations, and the dashed line is a mean effect size of 0. FLF represents returning farmland to forest; FLP represents returning farmland to pasture; and FP represents the conversion from forest to pasture*

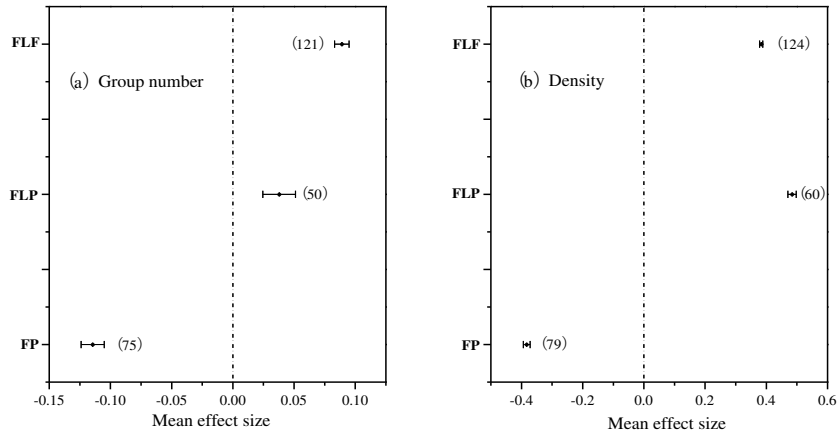

Figure 3. *Effect of returning farmland to forest (pasture) on the group number (a) and density (b). The error bars represent 95% confidence intervals (CIs). The solid diamonds represent the mean effect size. The numbers represent the number of observations, and the dashed line is a mean effect size of 0. FLF represents returning farmland to forest; FLP represents returning farmland to pasture; and FP represents the conversion from forest to pasture*

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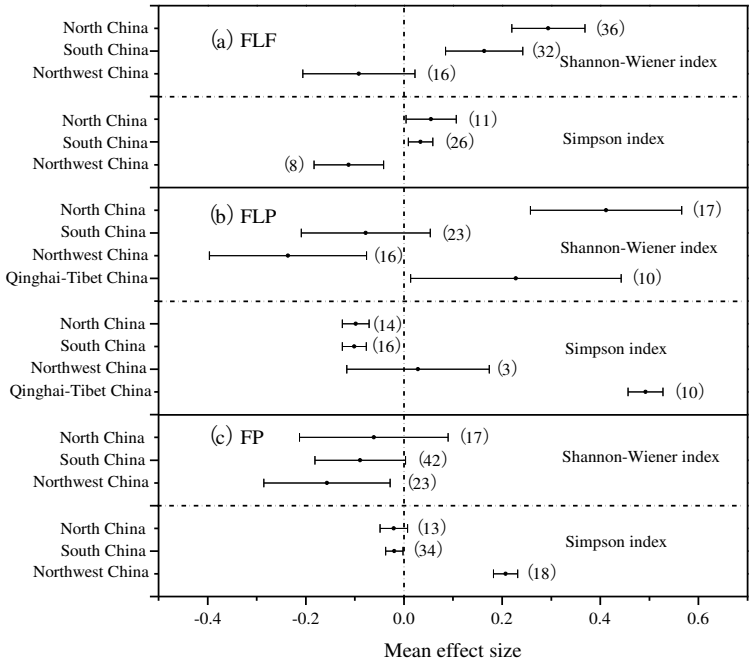

Figure 4. *Regional characteristics of the effect on the Shannon index and Simpson index values of FLF (a), FLP (b) and FP (c). The error bars represent 95% confidence intervals (CIs). The solid circle represents the mean effect size. The numbers represent the number of observations, and the dashed line is a mean effect size of 0. FLF represents returning farmland to forest; FLP represents returning farmland to pasture; and FP represents the conversion of forest to pasture*

According to *Fig. 5*, in North China, returning farmland to forest had significant positive effects on the group number and density of soil animals, 29.62% and 124.13%, respectively. Moreover, returning farmland to pasture had significant positive effects on group number and density, with percentages of 65.68% and 164.86%, respectively. In South China, returning farmland to forest had significant positive effects on the group number and density of soil animals, with percentages of 23.59% and 56.85%, respectively. Moreover, the group number of pastures was 10.97% higher than that of farmland, which was significant. However, the effect of density was nonsignificant compared with the effect of farmland. In Northwest China, returning farmland to forest decreased the group number and density significantly by 13.84% and 11.44%, respectively, and returning farmland to pasture changed the percentages of the group number and density were -11.88% and 16.78%, respectively. In Qinghai-Tibet China, returning farmland to pasture changed the percentages of the group number and density were 7.88% and 84.81%, respectively.

Regional differentiation characteristics caused by temperature and rainfall

When the average temperature was < 12ºC and > 12ºC, returning farmland to forest significantly increased the Shannon index value by 18.60% and 17.72%, respectively (*Fig. 6a*). Moreover, the Simpson index value significantly decreased by 10.13% when the average temperature was < 12ºC, and then significantly increased by 41.30% (*Fig. 6d*). In addition, the group number and density changed by -3.79% and 11.28% when the average temperature was < 12ºC, and increased by 26.23% and 56.87% when the average temperature was > 12ºC (*Fig. 7a* and *7d*). Returning farmland to pasture

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significantly increased the Shannon index value by 12.73% and significantly decreased the Simpson index by 7.50% when the average temperature was < 12ºC. Moreover, the return significantly increased the Simpson index by 31.48% and had a nonsignificant effect on the Shannon index when the average temperature was > 12ºC (*Fig. 6b* and *6e*). In addition, the group number and density changed by -9.49% and 43.21% when the average temperature was < 12ºC, and they increased by 19.50% and 91.08% when the average temperature was > 12ºC (*Fig. 7b* and *7e*).

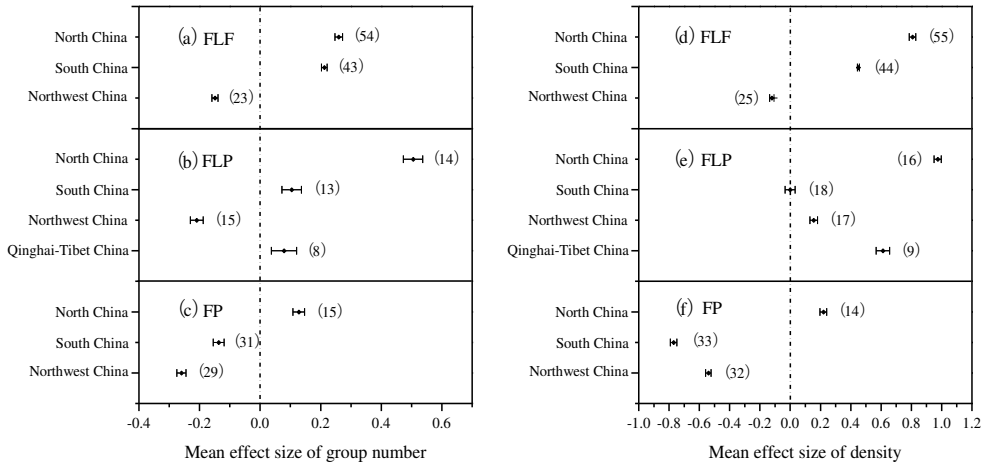

Figure 5. *Regional characteristics of the effect on the group number of FLF (a), FLP (b) and FP (c) and density of FLF (d), FLP (e) and FP (f). The error bars represent 95% confidence intervals (CIs). The solid diamonds represent the mean effect size. The numbers represent the number of observations, and the dashed line is a mean effect size of 0. FLF represents returning farmland to forest; FLP represents returning farmland to pasture; and FP represents the conversion from forest to pasture*

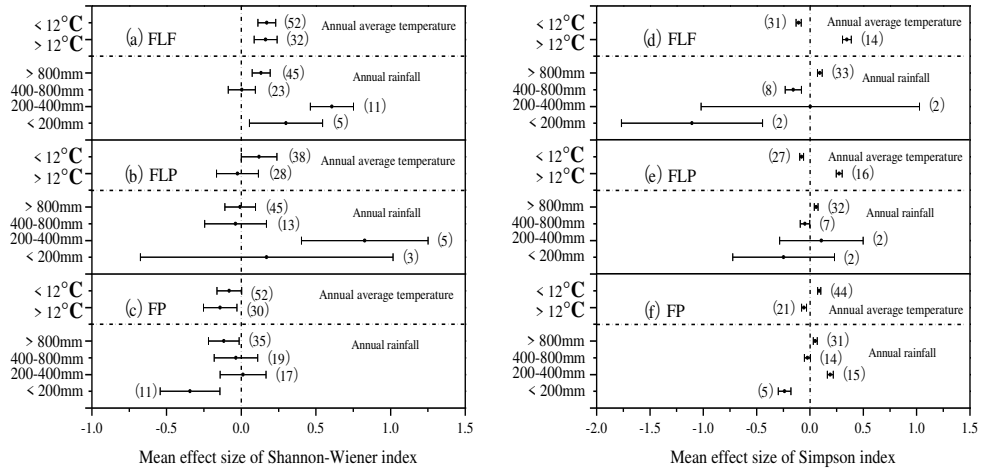

Figure 6. *Effect of returning farmland to forest (pasture) on the Shannon index values of FLF (a), FLP (b) and FP (c) and the Simpson index values of FLF (d), FLP (e) and FP (f) with changes in temperature and rainfall. The error bars represent 95% confidence intervals (CIs). The solid circle represents the mean effect size. The numbers represent the number of observations, and the dashed line is a mean effect size of 0. FLF represents returning farmland to forest; FLP represents returning farmland to pasture; and FP represents the conversion from forest to pasture*

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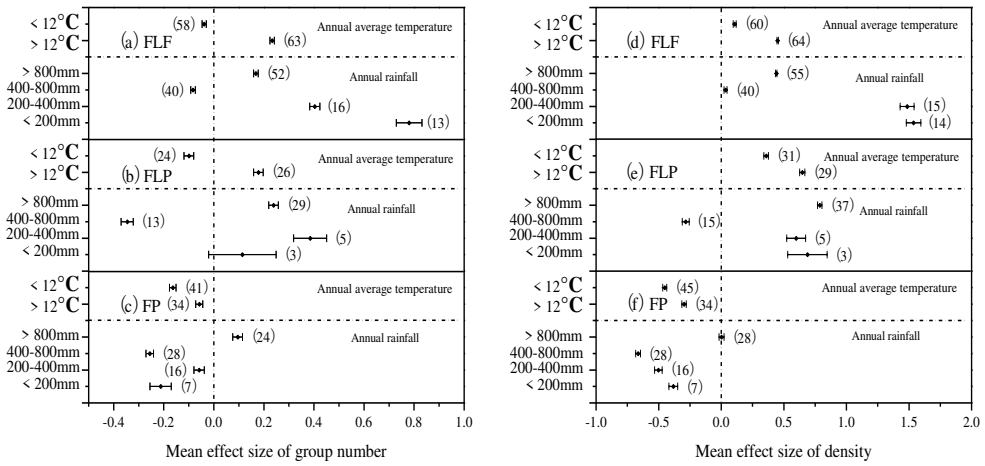

Figure 7. *Regional characteristics of the effect on the group number of FLF (a), FLP (b) and FP (c) and density of FLF (d), FLP (e) and FP (f) with the temperature and rainfall changes. The error bars represent 95% confidence intervals (CIs). The solid diamonds represent the mean effect size. The numbers represent the number of observations, and the dashed line is a mean effect size of 0. FLF represents returning farmland to forest; FLP represents returning farmland to pasture; and FP represents the conversion from forest to pasture*

With an increase in annual rainfall, returning farmland to forest significantly increased the Shannon index by 34.94%, 83.5%, and 14.24%, except for the nonsignificant effect at 400-800 mm (*Fig. 6a*). The Simpson index significantly decreased by 66.91% and 14.45%, at amounts < 200 mm and 400-800 mm and then increased significantly by 9.67% at amounts > 800 mm, but there was a nonsignificant effect at amounts from 200-400 mm (*Fig. 6d*). In addition, the group number changed by 118.17%, 49.68%, -7.98% and 18.3%, and the density changed by 364.80%, 341.57%, 3.51% and 55.22% when the amount was < 200 mm to > 800 mm (*Fig. 7a* and *7d*). Returning farmland to pasture significantly increased the Shannon index by 128.71% at amounts from 200-400 mm but had a nonsignificant effect in other regions (*Fig. 6b*). The Simpson index decreased significantly by 4.59% at amounts between 400-800 mm and then increased significantly at amounts > 800 mm, but there was a nonsignificant effect in other regions (*Fig. 6e*). In addition, the group number changed by 46.89%, -29.25% and 26.92% at amounts from 200-400 mm to > 800 mm, and the density changed by 98.97%, 81.92%, -24.72% and 119.71% at amounts from < 200 mm to > 800 mm. Returning farmland to pasture had a nonsignificant effect on the group number at amounts < 200 mm (*Fig. 7b* and *7e*).

Discussions

Returning farmland to forest

Under China's governmental environmental program of returning farmland to forest, a large amount of farmland has been converted to forest (Wang et al., 2011). The conversion of land use types has changed the cycling of soil organic matter (Detwiler, 1986; Zhao et al., 2014), the soil physicochemical properties (Adejuwon and Ekanade, 1988; Toohey et al., 2018) and the soil animal habitat (Eggleton et al., 2005). As a result, there have been important effects on soil animal diversity. The results of the

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present study confirm that returning farmland to forest has an important effect on soil animal diversity, as indicated by the Shannon-Wiener index and Simpson index values, and indicate that returning farmland to forest increases soil animal richness. However, returning farmland to forest might have decreased the soil animal evenness and increased the proportion of dominant species. This result was consistent with previous studies regarding the effects of changes from farmland to forest on soil animal diversity. Bartz et al. (2014) found that forest provided better soil conditions for the development of a higher diversity of soil animal compared to farmland. Okwakol (2010) also reported that forest had a higher soil animal diversity compared with farmland.

Returning farmland to pasture

Returning farmland to pasture had an important effect on soil animal diversity, but its effect on the Shannon index and Simpson index values were inconsistent (*Fig. 2*). The Simpson index value significantly increased by 3.38%, which indicated that soil animal evenness decreased and the proportion of dominant species increased. The above results show that the nonsignificant effect on the Shannon index might have been caused by the comprehensive results of an increase in species richness and a decrease in species evenness, which was suggested by the results of our study (*Fig. 2*). Similar results were reported by Cluzeau et al. (2012), who demonstrated most of the soil animal groups exhibited lower values of abundance and community richness in farmland than in pasture. In addition, regarding the conversion from forest to pasture, the Shannon index value decreased and the Simpson index value increased, indicating a decrease in soil animal diversity (-9.71%, 4.32%). This was consistent with the results obtained by Callaham et al. (2006). The group number and density of soil animals significantly decreased, which might have been caused by the lower soil animal richness and soil animal evenness of pastures compared with forests because the distribution patterns of the soil animal community changed with the change in surface vegetation cover (Vohland and Schroth, 1999; Yin et al., 2017).

Regional differentiation characteristics

There were significant differences in topography, climate, soil properties and vegetation types in North China, South China, Northwest China and Qinghai-Tibet China, which had important effects on soil animal diversity. So the effect on soil animal diversity showed different regional characteristics when returning farmland to forest (pasture). In North China, returning farmland to forest increases soil animal species richness, and soil animal species evenness might decrease. While returning farmland to pasture increased both the species richness and species evenness of soil animals, returning farmland to pasture significantly increased the soil animal diversity. The effect on soil animal diversity was nonsignificant because the changes in the Shannon index and Simpson index values were nonsignificant in the change from forest to pasture even though the species richness increased. In South China, returning farmland to forest has an important effect on soil animal diversity, which might have been caused by the increase in species richness and the decrease in species evenness of soil animals. Returning farmland to pasture increased soil animal diversity, which might be caused by the highly significant increases in species richness and species evenness even though the effect indicated by the Shannon index was nonsignificant. In addition, the changes in soil animal diversity might have been caused by both a decrease in species richness and an increase in species evenness from the conversion of forest to pasture. In

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Northwest China, the increase in soil animal diversity might have been caused mainly by the increase in species evenness when returning farmland to forest. Returning farmland to pasture can decrease soil animal diversity largely due to a decrease in species richness. With the conversion from forest to pasture, the change percentages of the group number and density were -12.77% and -41.75%, respectively (*Fig. 5*). The Shannon index value decreased significantly, and the Simpson index increased significantly, which might have been caused by the significant decrease in species richness and species evenness. In Qinghai-Tibet China, returning farmland to pasture has an effect on soil animal diversity, which was possibly caused by the increase in species richness and decrease in species evenness. The above regional differentiation characteristics were supported by some studies of land use change affecting soil animal diversity in other countries and regions, which indicated that the effect of land use change on soil animal diversity showed different regional differences with the change of geographical environment (Rossi and Blanchart, 2005; Karanja et al., 2009; Begum et al., 2013; Steinwandter et al., 2017).

Effect of temperature and rainfall

In China, the effect on soil animal diversity showed different regional characteristics when returning farmland to forest (pasture) because of differing regional natural conditions. The regional climate conditions mainly include temperature and rainfall, which have important effects on the cycling of soil organic matter and soil physicochemical properties (Kirschbaum, 1995; Mani et al., 2018). Therefore, to further discuss the regional differentiation characteristics, we divided the regions according to the annual average temperature and annual rainfall in China.

Returning farmland to forest (pasture) should be more conducive to increasing soil animal species richness in high-temperature regions and increasing soil animal species evenness in low-temperature regions. The improvement of plant community coverage and vegetation height after converting farmland greatly improved the survival conditions of soil animals and attract more soil animals to inhabit the land and survive (Mathieu et al., 2004). Additionally, the conversion was very beneficial for the concentration of dominant species. This benefit was proved by our study results, i.e., the proportion of dominant species increased in high-temperature regions. Returning farmland to pasture had a nonsignificant effect on the Shannon index when the average temperature was > 12ºC, which might be caused by the comprehensive effect of both the increase in species richness and the decrease in species evenness. Furthermore, in low-temperature regions, returning farmland to forest affected soil animal diversity more than a return to pasture because the mean effect size of the Shannon index decreased and the Simpson index increased from forest to pasture when the annual average temperature was < 12ºC.

With an increase in annual rainfall, the effect of returning farmland to forest on species richness and evenness gradually decreased and was conducive to the concentration of dominant species. Our study found that arid regions were more conducive to improving soil animal diversity when returning farmland to forest because soil animals are more sensitive to vegetation changes under drought conditions (Schlaghamerský et al., 2014). Alternatively, the result might be because of the lower amount of data in the meta-analysis. While the regional differentiation characteristics of the effect of returning farmland to pasture on soil animal diversity were not evident with the increase in annual rainfall, returning farmland to forest can increase soil animal

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diversity better the conversion to than pasture at amounts < 200 mm. The Simpson index value increased significantly at amounts of 200-400 mm, which could be interpreted by the increase in dominant species. At amounts > 800 mm, the Shannon index value decreased, which can be interpreted by the complicated comprehensive effect of both the increase in species richness and the decrease in species evenness. In particular, at amounts of 400-800 mm, returning farmland to forest (pasture) decreased the species richness but improved the species evenness. The specific reasons require further study.

Conclusions

Based on the results of our meta-analysis, the effects of returning farmland to forest (pasture) on soil animal diversity and its regional differentiation characteristics are complicated. The conclusions are as follows: (1) Overall, returning farmland to forest (pasture) had an important effect on soil animal diversity, which included the increase in species richness and the decrease in species evenness, and forests had a higher soil animal diversity than pastures; (2) In China, the effect of soil animal diversity showed different regional characteristics that largely depended on the specific geographic region and climate conditions, mainly temperature and rainfall, when returning farmland to forest (pasture).

In our study, a meta-analysis was used to investigate the effect of returning farmland to forest (pasture) on soil animal diversity, but in an actual underground ecosystem, many factors have different and comprehensive effects on soil animal diversity. Therefore, the analysis of regional differentiation characteristics and mechanisms requires further study. In addition, the reliability of some results may be reduced because effective data could not be obtained or the amount of data was relatively small, and therefore, future studies of these topics require further improvement.

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APPENDIX

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EVALUATION OF MASS TRANSFER EVAPOTRANSPIRATION MODELS UNDER SEMIARID CONDITIONS USING MCDM APPROACH

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Abstract. The selection of suitable reference evapotranspiration (ETo) models in case of climatic data scarcity is a challenging task as it plays a pivotal role in agriculture and water resource management. Therefore, the research work deals with selecting the appropriate mass transfer reference evapotranspiration model using multi criteria decision technique (MCDM) in a semi-arid region of the southern part of Kingdom of Saudi Arabia i.e., Abha. The ten mass transfer methods with ten criteria (statistical indices) using available weather parameters from 1980 to 2018 have been illustrated in this study. Models were calibrated (1980-2006) and validated for the period (2007-2018). The objective weight was computed by criteria importance through inter criteria correlation (CRITIC) method and performance score by weighted sum model (WSM), weighted product model (WPM), weighted aggregates sum product assessment (WASPAS) and evaluation based on distance from average solution (EDAS) methods which in turn rank the evapotranspiration method. The rankings obtained from MCDM techniques were validated with ranking by GPI method using spearman ranking coefficient. The result from MCDM shows that Saif model is the best model and that also GPI yielded same result. The methodology applied in this study can be adopted in any other region which in turns proved to be beneficial for crop cultivators, crop advisors, researchers, and water resource management.
Keywords: *water management, CRITIC, WSM, WPM, WASPAS, EDAS*

Introduction

The reliable estimation of reference evapotranspiration (ETo) is a crucial part of regional water resources planning, net irrigation requirement, agriculture water requirements and to model the climate change effect (Pandey et al., 2016). The ETo can be measured directly by lysimeter, but the procedure is time consuming and expensive (Mehdizadeh, 2018). The ETo could be indirectly calculated using a site-specific energy balance or empirical models that typically includes meteorological data, altitude, and latitude. Of the indirect methods, FAO56-PM is the most effective method for the precise estimation of ETo (Allen et al., 1998; Berti et al., 2014). However, it demands high and reliable data quality which is difficult to achieve (Valiantzas, 2013). The precise quantification of the ETo forecasts is dependent on meteorological input data (Allen, 2008). The lack of climate data leads to the need for a simple empirical equation requiring less climate parameters, such as mass transfer, radiation and temperature methods (Sentelhas et al., 2010). Researchers in different regions such as India, Bosnia, Africa, China and Saudi Arabia have developed and applied many empirical equations (Pandey and Pandey, 2018; Cadro et al., 2017; Djaman et al., 2017; Lang et al., 2017;

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Alblewi et al., 2015) as an alternative to lysimeter or standard FAO56-PM. However, mass transfer-based models involving lesser climatic parameters are among the most commonly used (Valipour, 2017). Such empirical equation should be assessed and validated against lysimetric or standard FAO56-PM technique due to regional constraints (Bogawski and Bednorz, 2014). In previous research, mass transfer methods typically only include evaluation of the studied model. Few research conducted evaluation calibration as well as validation of mass transfer equation (Djaman et al., 2016). In addition, rankings using performance assessment of different models are rarely studied against Standard FAO56-PM (Almorox et al., 2015). Alternatively, previous studies adopt few questionable statistical indices for performance evaluation of reference evapotranspiration, such as RMSE or MBE (Muhammad et al., 2019). Therefore, multiple statistical indices must be used to assess the performance of the mass transfer equation in order to obtain a realistic result. However, it is a challenging task for decision-makers (DMs) to find optimum decision. Therefore, powerful tool is desired for the final selection. Recently, researchers applied multi-criteria decision making (MCDM) techniques in the field of water resource management (Minatour et al., 2015; Makropoulos et al., 2008). To date, different MCDM methods have been developed for ranking purpose (Mardani et al., 2016) such as Water reservoirs (Srdjevic et al., 2004), urban water management (Zarghami et al., 2008), groundwater management (Pietersen, 2006), water conservation (Janssen et al., 2005), and irrigation planning (Gupta et al., 2000). Senent-Aparicio et al. (2017) assesses the effects of climate change in the Segura river basin (SE Spain) using SWAT and Fuzzy TOPSIS. Many studies have been carried out in the Kingdom of Saudi Arabia to estimate various ETo models against the FAO-56 Penman Monteith models (Abo-Ghobar and Mohammad, 1995; Al-Omran et al., 2004; ElNesr et al., 2010; Islam et al., 2019 a, b; Islam et al., 2020). In the study region, the ranking of mass transfer-based equation was rarely studied using MCDM. The goal of this study is to estimate (evaluate) ETo using ten mass transfer equations for the period between 1980-2018 and to further improve its efficiency, it is calibrated for the period between 1980-2006 and validated against standard FAO56-PM for the period between 2007-2018 and finally ranked by MCDM technique. The suggested methodology in the present study could be used in future for selecting best reference evapotranspiration model as a substitute to standard FAO56-PM in any region around the world. Also, the calibration improves the preciseness of ETo estimation. Moreover, the best selected model for estimating ETo could be used by agriculturist, hydrologist, policy and decision makers for the strategic planning of water resource management in the future.

Materials and methods

The present research was conducted to evaluate the performance of mass transfer-based ETo under limited climatic condition against standard FAO56-PM during evaluation (1980-2018), calibration (1980-2006) and validation (2007-2018) under semi-arid scenario Abha, KSA. The weather data is taken from Abha meteorological department at GPS location 18°14’N, 42°39’E. Such partitioning of calibration and validation period is attributed to the need for more data to train the algorithms, as suggested by Valipour (2015). The performance of these equations has been based on ten statistical criteria. The model ranking was based on MCDM techniques, where weightage is obtained by CRITIC method, while ranking by WSM, WPM, WASPAS

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and EDAS method. The ranking results from MCDM were compared with the outcome of GPI. The spearman ranking correlation was used to check the accuracy of the MCDM ranking. The fundamental objective of this study work is to choose the best model in the study region to replace the FAO56-PM model since this model required several climatic parameters that were sometimes difficult to achieve in the mountainous Abha region due to signal connectivity problems. Moreover, to select FAO56-PM as a standard against mass transfer models. The first important step is to validate its accuracy with respect to experimentally measured data.

The stepwise methodology adopted in the study is described by the flowchart in *Figure 1*.

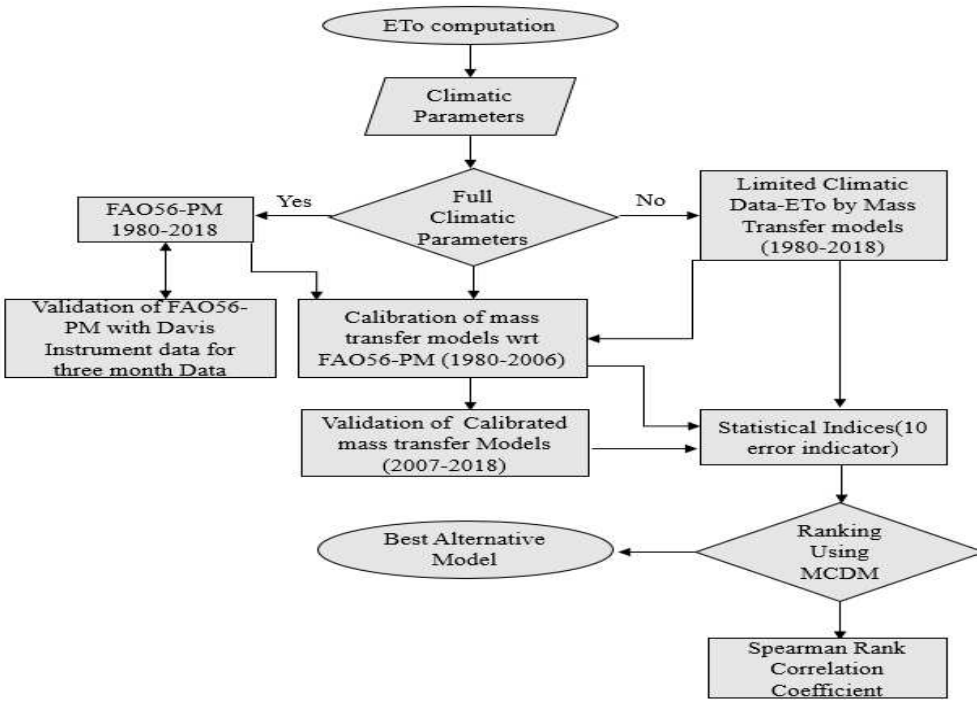

Figure 1. *Methodology (stepwise computation)*

Experimental setup

For the validation of the FAO56-PM model The measurement of the Davis Vantage Pro2 weather station at GPS location 18°15'06"N, 42°33'27"E was taken for a period of three months, i.e. from February to April, 2019. The schematic diagram for the weather station is given in *Figure 2*. The integrated instrument includes all sensors – anemometer, rain collector, temperature, humidity and solar irradiance – for measuring all required climatic parameters as well as measured evapotranspiration. The specific aim of reading from the Davis instrument is to verify the result from estimates of FAO56-PM using climatic data obtained from measurement of sensors against measured ETo from the weather station so that it can be used as a reference for other mass transfer model, as the FAO56-PM model is used in this analysis for evaluation, calibration and validation purposes. The outcome of plots of estimated FAO56-PM against measured ETo from Davis instrument reading (*Fig. 3*) indicates that there is a strong association between two readings with a very small error as indicated from the figure.

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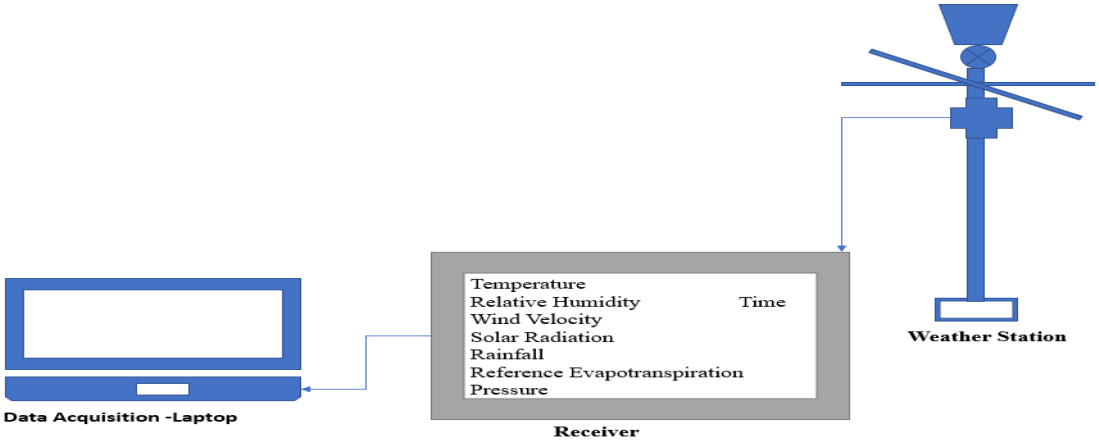

Figure 2. *Schematic diagram for experimental setup of Davis weather station*

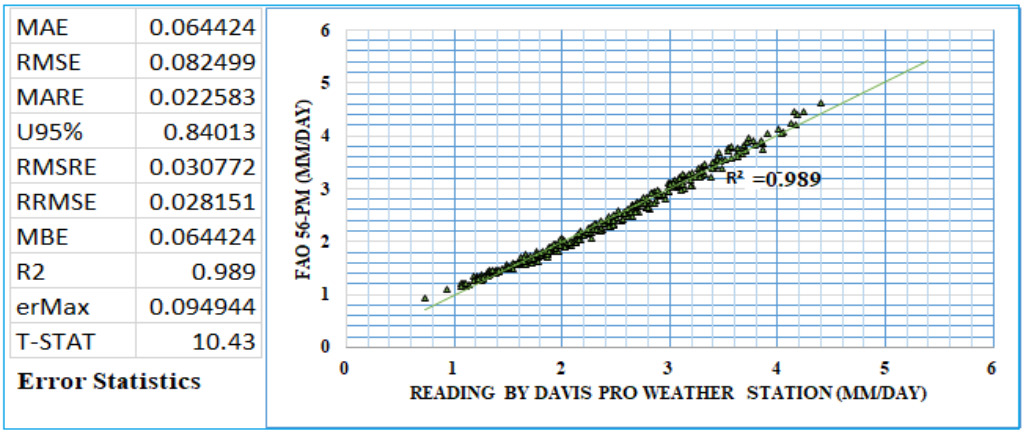

Figure 3. *Validation of FAO56-PM with Davis weather station (Abha)*

FAO56-PM and mass transfer model description

The estimation of reference evapotranspiration was done using well-recognized model i.e., FAO56-PM as given by *Equation 1* as well as ten mass transfer equation as given by *Equations 2-11* under data limitation in the study region between 1980-2018. Models selected for the study region are largely accepted under similar climatic conditions. Models are mentioned below.

Standard model (FAO 56-PM) (Allen et al., 1998)

$$ETo = \frac{0.408\times\Delta\times(Rn-G)+\gamma\times\left(\frac{900}{T+273}\right)\times u_2\times(e_s-e_a)}{\Delta+\gamma\times(1+0.34u_2)} \quad \text{(Eq.1)}$$

Empirical models (mass transfer)

Dalton (1802)

$$ETo = (0.3648 + 0.07223u_2) \times (e_s - e_a) \quad \text{(Eq.2)}$$

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Trabert (1896)

$$ETo = 0.408 \times 0.3075 \times \sqrt{u_2} \times (e_s - e_a) \quad \text{(Eq.3)}$$

Meyer (1926)

$$ETo = (0.375 + 0.05026u_2) \times (e_s - e_a) \quad \text{(Eq.4)}$$

Rohwer (1931)

$$ETo = 0.44(1 + 0.27u_2) \times (e_s - e_a) \quad \text{(Eq.5)}$$

Penman (1948)

$$ETo = 0.35 \times (1 + 0.24u_2) \times (e_s - e_a) \quad \text{(Eq.6)}$$

Albrecht (1950)

$$ETo = (0.1005 + 0.297u_2) \times (e_s - e_a) \quad \text{(Eq.7)}$$

Brockamp (1963)

$$ETo = (0.543u_2^{0.456}) \times (e_s - e_a) \quad \text{(Eq.8)}$$

WMO (1966)

$$ETo = (0.1298 + 0.0934u_2) \times (e_s - e_a) \quad \text{(Eq.9)}$$

Mahringer (1970)

$$ETo = 0.15072 \times \sqrt{3.6u_2} \times (e_s - e_a) \quad \text{(Eq.10)}$$

Saif (2019b)

$$ETo = (0.37 + 0.72u_2) \times (e_s - e_a) \quad \text{(Eq.11)}$$

where ETo is in mm day^{-1}. Rn and G represent net radiation and heat flux density of soil (MJm^{-2} day^{-1}) respectively. u_2 represent the velocity of wind at 2 m height (m s^{-1}). T represents mean temperature at height of 2 m (°C). (e_s–e_a) (kPa) represent vapour pressure deficit. Δ and γ denoted vapor pressure curve (slope) and psychrometric constant (kPa °C^{-1}) respectively. RH_{mean} = Mean Relative Humidity (%).

Calibration and validation of ET_O equations

To calibrate Mass Transfer Models. The graph between mass transfer equation and standard FAO56-PM equation was plotted and regression analysis was performed (Allen et al., 1998). The calibration technique adopted was defined by *Equation 12:*

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$$ET_{FA056-PM} = a.ET_{EMP} + b \quad \text{(Eq.12)}$$

where $ET_{FAO56\ PM}$ denotes estimated result by FAO56-PM model while ET_{EMP} denotes the various empirical equation using in the present study (10-mass transfer equations). The constant a (slope) and b (intercept) called as calibrated empirical coefficients. The calibrated equations must have slope (a) close to unity while intersept (b) should be near to zero for best result. In order to estimate calibrated coefficient a (slope) multiply the slope of a regression line by inversing the slope in order to make the slope of equation closer to unity. Also to get b (intercept) closer to zero opposite sign value of intercept was added for new regression equation (Xu et al., 2013).

Evaluation criteria and global performance index (GPI)

For the GPI computation (*Eq. 13*), various statistical indices (error indictor) as described by *Equations 14-23* is required prior to the estimation of GPI. (Ali et al., 2019). The ideal value of all indices equals zero except for R^2 it is taken as 1. Despotovic et al. (2015) used the concept of GPI by normalizing the errors between the scale of 0 to 1 and further subtracting it from the equivalent medians then adding up the differences so obtained using the weight factors. The expression for GPI for the i^{th} model is as follows:

$$GPI_i = \sum_{j=1}^{10} \alpha_j \left(\tilde{y}_j - \tilde{y}_{ij}\right) \quad \text{(Eq.13)}$$

where α_j depends on statistical values (+1 value for recommended value 0 and -1 for recommended value 1 (e.g. R^2). $\tilde{y}_j$ and $\tilde{y}_{ij}$ are the median and scaled values, respectively.

Willmott and Matsuura (2005) applied MAE in their study as given by *Equation 14*.

Mean absolute error (MAE)

$$MAE = \frac{1}{n}\sum_{i=1}^{n}\left|ET_{O,Mi} - ET_{o,FAO56-PM}\right| \quad \text{(Eq.14)}$$

Root mean square error

$$RMSE = \left[\frac{1}{n}\sum_{i=1}^{n}\left(ET_{O,Mi} - ET_{o,FAO56-PM}\right)^2\right]^{\frac{1}{2}} \quad \text{(Eq.15)}$$

Mean absolute relative error (MARE)

$$MARE = \frac{1}{n}\sum_{i=1}^{n}\left|\frac{ET_{O,Mi} - ET_{o,FAO56-PM}}{ET_{O,Mi}}\right| \quad \text{(Eq.16)}$$

In the modelling of solar radiations, Behar et al. (2015) and Gueymard (2014) used U_{95} as given by *Equation 17:*

Uncertainty at 95%

$$U_{95} = 1.96(SD^2 + RMSE^2)^{\frac{1}{2}} \quad \text{(Eq.17)}$$

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Root mean squared relative error

$$RMSRE = \sqrt{\frac{1}{n}\sum_{i=1}^{n}\left(\frac{ET_{O,Mi}-ET_{o,FAO56-PM}}{ET_{O,Mi}}\right)^2} \quad \text{(Eq.18)}$$

Also in the modelling of global solar radiation, Li et al. (2013) applied RRMSE as given by *Equation 19:*

Relative root mean square error

$$RRMSE = 100 \times \frac{\sqrt{\frac{1}{n}\sum_{i=1}^{n}\left(ET_{O,Mi} - ET_{o,FAO56-PM}\right)^2}}{\sum_{i=1}^{n} ET_{O,Mi}} \quad \text{(Eq.19)}$$

Mean bias error

$$MBE = \frac{1}{n}\sum_{i=1}^{n}\left(ET_{O,Mi} - ET_{o,FAO56-PM}\right) \quad \text{(Eq.20)}$$

Correlation coefficient

$$R^2 = 1 - \frac{\sum_{i=1}^{n}\left(ET_{O,Mi} - ET_{o,FAO56-PM}\right)^2}{\sum_{i=1}^{n}\left(ET_{O,Mi} - ET_{O,Mi_{av}}\right)^2} \quad \text{(Eq.21)}$$

Maximum absolute relative error

$$erMAX = max\left(\left|\frac{ET_{O,Mi} - ET_{o,FAO56-PM}}{ET_{O,Mi}}\right|\right) \quad \text{(Eq.22)}$$

Moreover, Stone (1993) and Mulaudzi et al. (2015) applied t-statistics in the evaluation of solar radiation as shown by *Equation 23:*

$$t = \left[\frac{(n-1)MBE^2}{RMSE^2 - MBE^2}\right]^{\frac{1}{2}} \quad \text{(Eq.23)}$$

Multi criteria decision technique

For the implementation of MCDM techniques the weightages were computed using CRITIC method and for measuring the performance score of various empirical equations (alternatives), the models i.e., WSM, WPM, WASPAS and EDAS were used using the same statistical indices as implemented in GPI (criteria). Of all the criteria, the R^2 criteria is referred to as beneficial criteria and the other nine are non-beneficial criteria. The performance values (the higher the value the better the model will be) of the different ETo models will determine the promising model in the Abha region, which is one of the novelty in this research work. The adopted technique is as defined by *Figure 4*.

Objective weight

The objective weight is computed by criteria importance through inter criteria correlation (CRITIC) method. The method is first proposed by Diakoulaki et al. (1995)

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and it is objective weighting methods. In order to find the contrast between criteria correlation analysis are done (Adalı and Işık, 2017). There is m feasible alternatives, A_i (i = 1, 2, …, m) and n evaluation criteria C_j (j = 1, 2, …, n) in the problem. The stepwise methodology is described below:

Step 1 To establish decision matrix X showing alternatives performance as compared to various criteria.

Step 2 The normalization of decision matrix is done using *Equation 24:*

$$r_{ij} = \frac{x_{ij-x_j^{min}}}{x_j^{max} - x_j^{min}} \quad \text{(Eq.24)}$$

where r_{ij} represent performance value of normalized decision matrix of i^{th} alternative on j^{th} criterion.

Step 3 The weight of the j^{th} criterion (w_j) is obtained as by *Equation 25,* where C_j is given by *Equation 26*:

$$w_j = \frac{c_j}{\sum_{i=1}^{m} c_j} \quad \text{(Eq.25)}$$

where

$$C_j = \sigma_j \sum_{i=1}^{m} (1 - r_{ij}) \quad \text{(Eq.26)}$$

C_j is the quantity of information contained in j^{th} criterion, σ_j is standard deviation of the j^{th} criterion and r_{jj}' is the correlation coefcient between j^{th} and j'^{th} criteria.

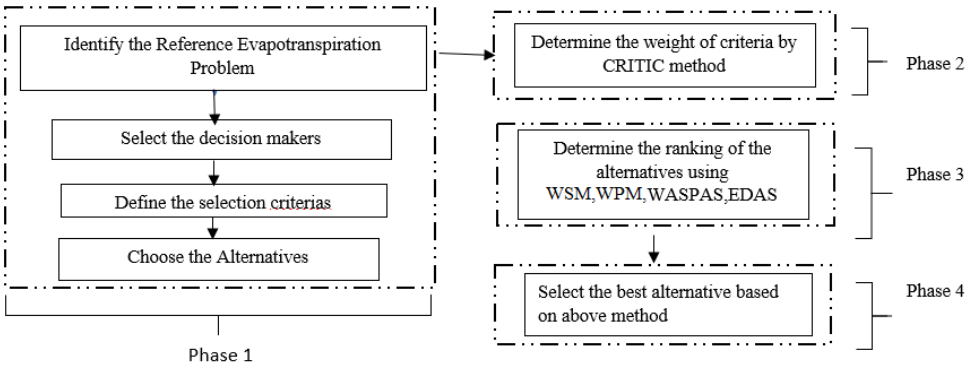

Figure 4. *Application of MCDM in selecting best ETo model*

Performance score

Weighted sum model (WSM) (Mann and Evangelos, 1989): The importance of i^{th} alternative by WSM technique is computed using *Equation 27:*

$$Q_i^{(1)} = \sum_{j=1}^{n} \bar{x}_{ij} w_j \quad \text{(Eq.27)}$$

Weighted Product Model (WPM) (Mann and Evangelos, 1989): The importance of i^{th} alternative by WPM technique is computed using *Equation 28:*

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$$Q_i^{(2)} = \prod_{j=1}^{n} (\bar{x}_{ij})^{w_j} \quad \text{(Eq.28)}$$

Weighted Aggregates Sum Product Assessment (WASPAS) method: A joint generalized criterion of weighted aggregation of additive and multiplicative method is then proposed as given by *Equation 29* (Šaparauskas et al., 2011).

$$Q_i = 0.5Q_i^{(1)} + 0.5Q_i^{(2)} = 0.5 \sum_{j=1}^{n} \bar{x}_{ij}\, w_j + 0.5 \prod_{j=1}^{n} (\bar{x}_{ij})^{w_j} \quad \text{(Eq.29)}$$

Evaluation based on Distance from Average Solution (EDAS) method:

The methodology is adopted as studied by Keshavarz et al. (2015). The stepwise computation by EDAS method is described below:

Step 1. Criteria and alternatives are decided based on need of problem.

Step 2. Decision matrix of X based on selected criteria and alternatives are established as given by *Equation 30:*

$$X = |X_{ij}|_{n\times m} = \begin{bmatrix} x_{11} & x_{12} \dots & x_{1m} \\ \vdots & \vdots & \vdots \\ x_{n1} & x_{n2} \dots & x_{nm} \end{bmatrix} \quad \text{(Eq.30)}$$

where x_{ij} represents the value of i^{th} alternative with respect to j^{th} criterion based on performance.

Step 3. AV based on all criteria are determined using *Equations 31-32:*

$$AV = [AV_j]_{1\times m}, \qquad j = 1, \dots\dots, m. \quad \text{(Eq.31)}$$

$$AV_j = \frac{\sum_{i=1}^{n} X_{ij}}{n}, j = 1, \dots\dots, m. \quad \text{(Eq.32)}$$

Step 4. The PDA and NDA matrices are calculated based on the type of Criteria as given by *Equations 33-38:*

$$PDA = [PDA_{ij}]_{n\times m} \quad \text{(Eq.33)}$$

$$NDA = [NDA_{ij}]_{n\times m} \quad \text{(Eq.34)}$$

If criterion j is benefit criteria,

$$PDA_{ij} = \frac{max\left(0, (x_{ij} - AV_j)\right)}{AV_j} \quad \text{(Eq.35)}$$

$$NDA_{ij} = \frac{max\left(0, (AV_j - x_{ij})\right)}{AV_j} \quad \text{(Eq.36)}$$

If criterion j is non beneficial criterion,

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$$PDA_{ij} = \frac{max\left(0, \left(AV_j - x_{ij}\right)\right)}{AV_j} \quad \text{(Eq.37)}$$

$$NDA_{ij} = \frac{max\left(0, \left(x_{ij} - AV_j\right)\right)}{AV_j} \quad \text{(Eq.38)}$$

Here, PDA_{ij} and NDA_{ij} indicate the positive and negative distances of i^{th} alternative from AV in terms of jth criterion, respectively.

Step 5. Weighted sum of PDA and NDA for all alternatives are determined by using *Equations 39-40:*

$$SP_i = \sum_{j=1}^{m} w_j \, PDA_{ij} \quad \text{(Eq.39)}$$

$$SN_i = \sum_{j=1}^{m} w_j \, NDA_{ij} \quad \text{(Eq.40)}$$

Here W_j indicates the weight of j^{th} criterion.

Step 6. For all alternatives, SP and SN values are normalised by using *Equations 41-42*):

$$NSP_i = \frac{SP_i}{\max\left(SP_i\right)} \quad \text{(Eq.41)}$$

$$NSN_i = 1 - \frac{SN_i}{\max\left(SN_i\right)} \quad \text{(Eq.42)}$$

Step 7. Appraisal score (AS) for all alternatives are calculated as:

$$AS_i = \frac{1}{2}\left(NSP_i + NSN_i\right) \quad \text{(Eq.43)}$$

where $0 \leq AS_i \leq 1$.

Step 8. According to the obtained AS, alternatives are ranked in descending order. The alternative with the highest AS is the best one among the other alternatives.

Results and discussion

This study presents a comparison (evaluation 1980-2018, calibration 1980-2006 and validation 2007-2018) of the selected ten mass transfer reference equations (alternatives) to the standard FAO56-PM. The models of mass transfer based were chosen in the study because of their rigor and comprehensiveness as defined by various researchers in the field of water resource management (e.g. Ali and Shui, 2009; Tabari et al., 2013). Average Monthly ETo per day (mm/day) during evaluation, calibration and validation period is given in *Tables 1-3* while the seasonal variation as shown by *Table 4*. The performance of different mass transfer equations was evaluated through

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ten statistical indices (criteria) as described in the methodology. The criterion weight of the different indices was determined using the CRITIC method, whereas four MCDM techniques i.e., WSM WPM, WASPAS and EDAS were used to rank the mass transfer method. In addition, the GPI ranking has been computed to validate the result using MCDM techniques. The rank correlation coefficient for Spearman was determined between MCDM and GPI technique to check the accuracy of MCDM technique. When comparing the reference evapotranspiration by selected mass transfer model with standard FAO56-PM in terms of correlation coefficient and error indices (*Fig. 5*) during the evaluation process, it was observed that all selected equations bears a strong correlation (R^2 ranges 0.89 to 0.96) with FAO56-PM with the highest correlation observed in the Saif model and lower by Trabert model. In addition, when evaluating the output of the same equations in terms of the error indicator, Saif model achieved the highest precision. The results assessed from 1980-2018 showed that some of the method of mass transfer performed better without calibration like Albrecht and Saif model, which is in agreement with Islam et al. (2019b). Similar findings were previously obtained after evaluating six ETo equations for the Senegal River Delta (Djaman et al., 2016). The results are in accordance with the analysis done by Djaman et al. (2015) under Sahelian conditions in the Senegal river valley. These models use the temperature and wind speed observation to estimate the ETo values (Shiri, 2018). Though in certain regions such models may provide accurate results (Xu and Singh, 2002; Tabari et al., 2013). The accuracy of the results of these models as described in Kiafar et al. (2017) may be reduced by low aerodynamic effects. Moreover, wind speed and air temperature were determined at different altitudes resulting in a significant number of related or equivalent equations. Therefore, it will be difficult to apply data from one location and/or height to another and apply a model developed in a specified region at another location with certainty (Shiri, 2018). Like the other ETo models, local calibration is a big drawback of such models. To overcome such problems, the comparison was made again with regard to correlation coefficients and error indicators while calibrating the whole equation against standard FAO56-PM (*Fig. 6*), All chosen equations were shown to have a high correlation (R^2 ranges 0.91 to 0.98) with the FAO56-PM with the highest correlation detected in the Saif model and lower in the Meyer and Trabert model. The Saif model also achieved the highest accuracy while observing the performance of the same equations in terms of the error indicator. The models are calibrated similar to the studies of Irmak et al. (2003) and Xu and Singh (2001). It has been found from the inspection of the output during calibration that the model calibration significantly enhanced the efficiency of all equations. Also same result noticed while validating the calibrated equations (*Fig. 7*) with (R^2 ranges from 0.872 to 0.921). The high correlation observed by Saif model and lower by Trabert model. Which is similar to result obtained by Bogawski and Bednorz, 2014 in study in Poland. Conversely Meyer equation perform better in north-western Ontario, Canada (Singh and Xu, 2002) Also while observing the performance of same equations in terms of error indicator the highest accuracy was achieved by Saif model. The result obtained is in agreement with Islam et al. (2019b). Additionally, the findings of the research are in agreement with Kisi and Zounemat Kermani (2014). From the study it has been confirmed that in some region overestimated the ETo by Mass transfer (Valipour, 2015; Winter et al., 1995) while other underestimated (Tabari et al., 2013; Djaman et al., 2015). Azhar and Perera (2011) and Zhai et al. (2010) calibrated ETo models and concluded that calibration can be used to modify ETo with multi-station data to improve its accuracy. Bormann (2011)

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examined various models of mass transfer to examine climate change in Germany and noticed a substantial difference in the performance of all models. Therefore, MCDM technique was applied to rank different model based on performance.

Table 1. *Average monthly ETo per day (mm/day) during evaluation period 1980-2018*

Month	FAO56-PM	Dalton	Trabert	Meyer	Rohwer	Penman	Albrecht	Brockamp	WMO	Mahringer	Saif
Jan	1.84	0.4	0.15	0.36	0.54	0.54	0.67	0.61	0.28	0.34	1.72
Feb	1.91	0.38	0.14	0.34	0.52	0.52	0.66	0.59	0.27	0.32	1.69
Mar	1.95	0.39	0.15	0.35	0.54	0.54	0.7	0.61	0.29	0.34	1.79
Apr	2.59	0.59	0.22	0.54	0.8	0.78	0.94	0.9	0.4	0.49	2.42
May	2.84	0.76	0.27	0.71	1.03	0.99	1.13	1.14	0.5	0.62	2.92
Jun	3.85	1.09	0.39	1	1.47	1.43	1.66	1.64	0.72	0.9	4.29
Jul	3.18	0.86	0.31	0.79	1.16	1.13	1.34	1.3	0.57	0.71	3.44
Aug	3.12	0.84	0.3	0.77	1.13	1.1	1.31	1.26	0.56	0.69	3.37
Sep	3.52	0.96	0.34	0.88	1.29	1.24	1.43	1.43	0.63	0.78	3.69
Oct	2.89	0.73	0.26	0.68	0.97	0.93	1.04	1.08	0.46	0.59	2.69
Nov	1.62	0.38	0.13	0.35	0.51	0.49	0.55	0.56	0.24	0.31	1.42
Dec	1.5	0.35	0.12	0.33	0.47	0.45	0.49	0.52	0.22	0.28	1.28

Table 2. *Average monthly ETo per day (mm/day) during calibration period 1980-2006*

Month	FAO56-PM	Dalton	Trabert	Meyer	Rohwer	Penman	Albrecht	Brockamp	WMO	Mahringer	Saif
Jan	1.36	1.29	1.34	1.28	1.31	1.35	1.47	1.35	1.39	1.36	1.45
Feb	1.99	1.71	1.77	1.69	1.74	1.78	1.94	1.77	1.84	1.79	1.92
Mar	1.90	1.70	1.76	1.68	1.73	1.77	1.93	1.76	1.83	1.78	1.91
Apr	2.80	2.48	2.53	2.47	2.50	2.54	2.66	2.53	2.58	2.55	2.64
May	2.58	2.53	2.53	2.55	2.53	2.53	2.53	2.54	2.53	2.54	2.53
Jun	4.18	4.14	4.22	4.13	4.18	4.23	4.42	4.21	4.30	4.23	4.39
Jul	3.55	3.74	3.72	3.77	3.73	3.73	3.70	3.74	3.72	3.73	3.71
Aug	3.18	3.31	3.29	3.33	3.30	3.30	3.28	3.31	3.29	3.31	3.29
Sep	3.85	3.93	3.91	3.96	3.92	3.91	3.89	3.92	3.91	3.92	3.89
Oct	3.87	3.51	3.58	3.50	3.54	3.59	3.75	3.58	3.65	3.60	3.73
Nov	1.77	1.75	1.75	1.76	1.75	1.75	1.77	1.77	1.76	1.77	1.76
Dec	2.06	2.10	2.04	2.14	2.09	2.06	1.95	2.07	2.02	2.06	1.97

Table 3. *Average monthly ETo per day (mm/day) during validation period 2007-2018*

Month	FAO56-PM	Dalton	Trabert	Meyer	Rohwer	Penman	Albrecht	Brockamp	WMO	Mahringer	Saif
Jan	1.52	0.36	0.18	0.33	0.48	0.47	0.56	0.53	0.26	0.31	1.40
Feb	1.99	0.43	0.21	0.39	0.58	0.58	0.73	0.65	0.32	0.37	1.81
Mar	2.31	0.53	0.24	0.47	0.71	0.71	0.89	0.80	0.39	0.46	2.23
Apr	2.42	0.59	0.26	0.54	0.79	0.77	0.89	0.88	0.40	0.50	2.24
May	2.61	0.77	0.31	0.72	1.02	0.97	1.05	1.11	0.49	0.62	2.68
Jun	3.48	1.06	0.42	0.98	1.40	1.34	1.48	1.54	0.68	0.86	3.79
Jul	3.23	0.98	0.40	0.91	1.31	1.26	1.41	1.45	0.64	0.81	3.61
Aug	2.78	0.83	0.34	0.77	1.09	1.05	1.15	1.20	0.54	0.67	2.94
Sep	3.42	0.98	0.39	0.91	1.31	1.26	1.41	1.45	0.64	0.80	3.61
Oct	2.92	0.75	0.31	0.69	0.98	0.94	1.01	1.08	0.48	0.60	2.58
Nov	1.56	0.43	0.19	0.40	0.55	0.52	0.55	0.60	0.27	0.34	1.39
Dec	1.41	0.36	0.17	0.33	0.47	0.45	0.50	0.51	0.24	0.30	1.24

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Table 4. *Seasonal average variation of ETo per day (mm/day) during period 1980-2018*

Month	FAO56-PM	Dalton	Trabert	Meyer	Rohwer	Penman	Albrecht	Brockamp	WMO	Mahringer	Saif
Winter	1.90	0.39	0.15	0.35	0.54	0.53	0.68	0.60	0.28	0.33	1.73
Spring	3.09	0.81	0.29	0.75	1.10	1.07	1.25	1.23	0.54	0.67	3.21
Summer	3.27	0.88	0.32	0.81	1.19	1.16	1.36	1.33	0.59	0.73	3.50
Autumn	2.00	0.49	0.17	0.45	0.65	0.62	0.69	0.72	0.31	0.39	1.80

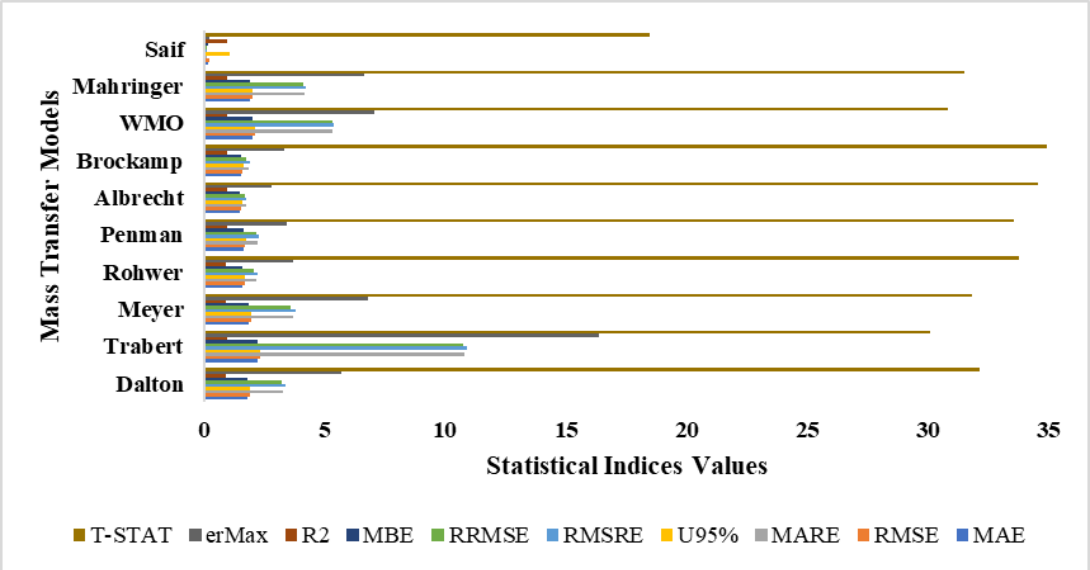

Figure 5. *Error indices of all model during evaluation*

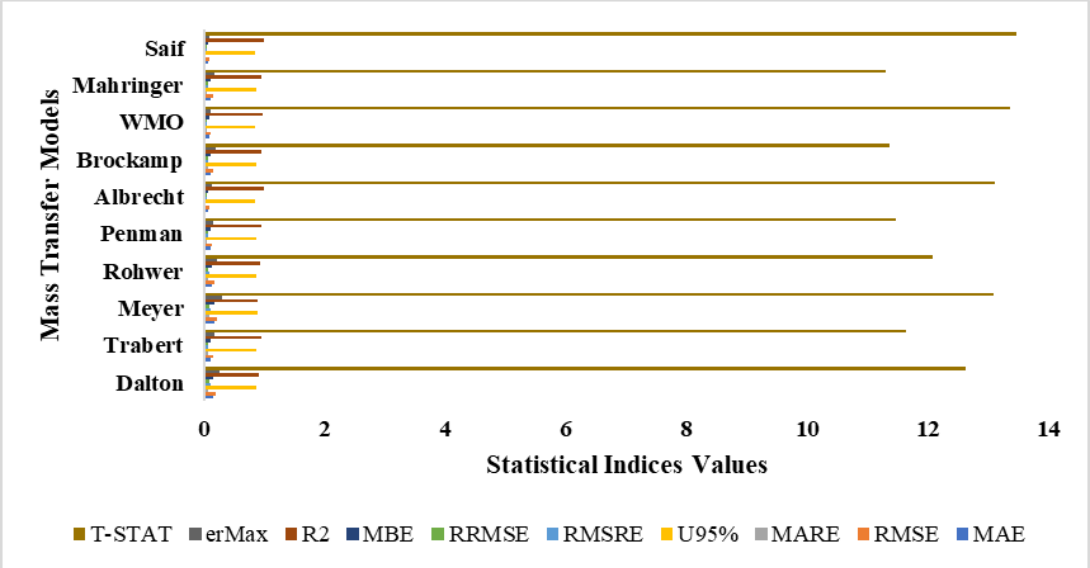

Figure 6. *Error indices of all model during calibration*

The models are ranked on the basis of multi-criteria decision-making techniques after evaluating, calibrating and validating the mass transfer equation against FAO56-PM model. The weightage was estimated by the CRITIC model as shown by *Figure 8*.

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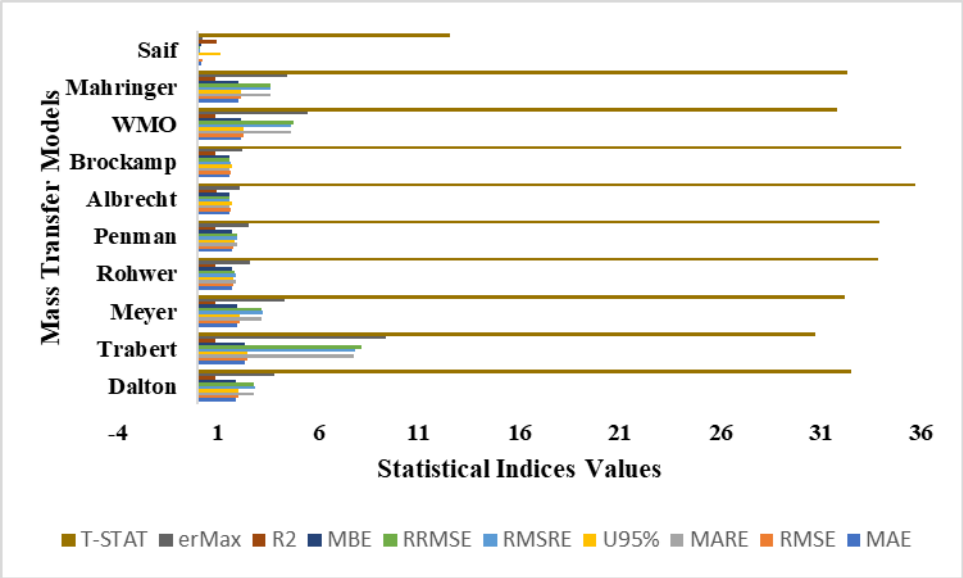

Figure 7. *Error indices of all model during validation*

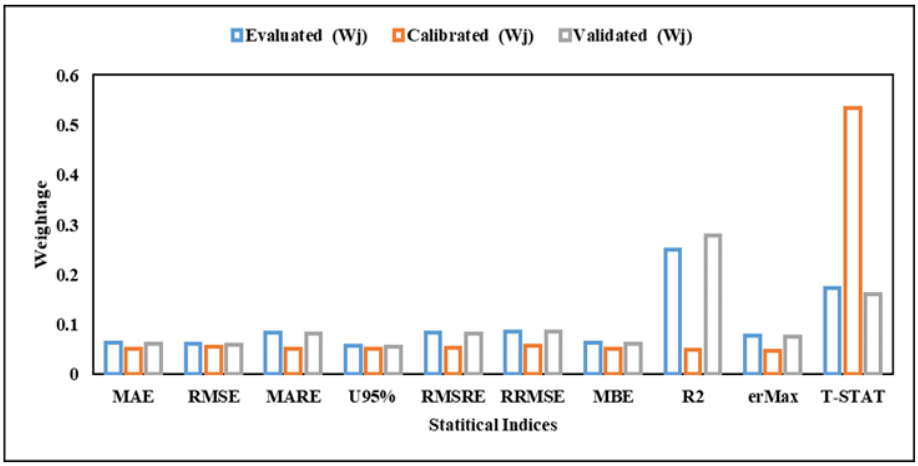

Figure 8. *Weightage by CRITIC method*

The performance score obtained by WSM, WPM, WASPAS, EDAS Models during evaluation calibration and validation. Furthermore, the performance score for validation as described in *Figures 9-11* is computed by GPI. The findings clearly show that the Saif model is the one that better fits the study region. The ranking of the MCDM technique shows that Saif model is the best model in the study area. In addition, GPA validates the same result as indicated (*Fig. 12*). Trabert model showed equally worst performance during evaluation, calibration and validation respectively. Also, spearman's ranking correlation is estimated between MCDM and GPI as shown in *Table 5*. This study finds strong concordance among the results of three different MCDM methods. Although the low-ranked alternatives were not always positioned alike, the best and second-best choices were the same for all three results. Similarly, the least preferred alternative was also the same. The important fact to note here is, despite the variety of data synthesis procedures and level of intricacy involved with these methods, the suggestions were

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similar for the preferred alternatives. Since most of the water supply related problems seek to find the best suitable alternatives (Hajkowicz and Higgins, 2008; Sikder et al., 2015; Tirth et al., 2020), it could be inferred from the findings of the study that both simple and comprehensive MCDM methods yield the same output.

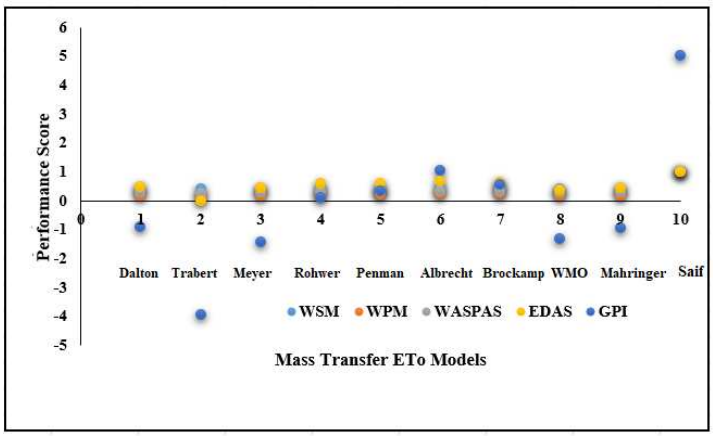

Figure 9. *Performance score by different method during evaluation*

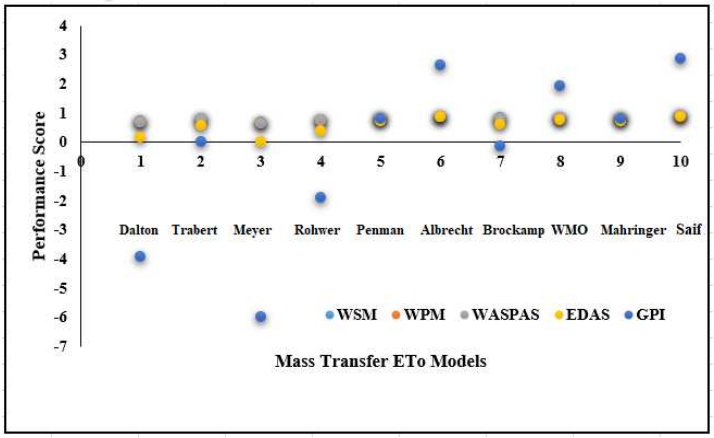

Figure 10. *Performance score by different method during calibration*

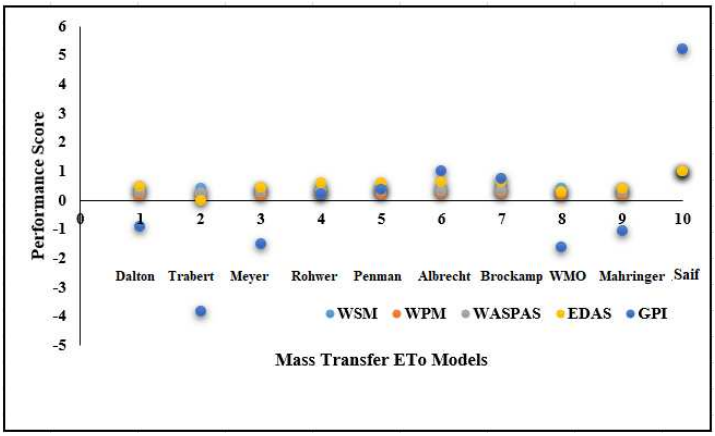

Figure 11. *Performance score by different method during validation*

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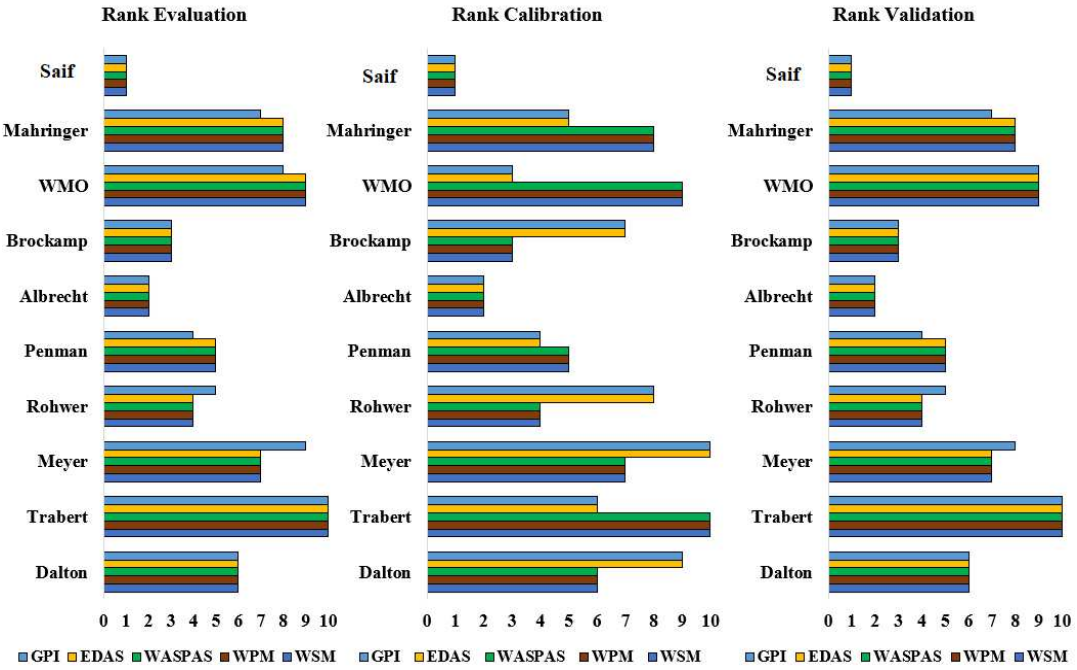

Figure 12. *Ranking using MCDM and GPI model*

Table 5. *Spearman's rank correlation coefficient values*

	WSM	WPM	WASPAS	EDAS	GPI
Evaluation					
WSM		0.92	0.89	0.92	0.88
WPM			0.99	1.00	0.96
WASPAS				0.99	0.99
EDAS					0.96
Calibration					
WSM		0.96	1.00	0.96	0.94
WPM			0.96	1.00	0.98
WASPAS				0.96	0.94
EDAS					0.98
Validation					
WSM		0.89	0.94	0.89	0.95
WPM			0.99	1.00	0.98
WASPAS				0.99	0.99
EDAS					0.98

Conclusions

This study was conducted to evaluate (period: 1980-2018), calibrate (period: 1980-2006) and further validate (period: 2007-2018) ten reference evapotranspiration models against standard FAO56 PM model in southern region (Abha) of Saudi Arabia. The performance scores (ranking) of aforementioned alternative models were computed based on ten statistical indices. The overall effect of these ten statistical indices were

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computed by multicriteria decision technique such as CRITIC (weightage of statistical indices), WSM, WPM, WASPAS and EDAS (ranking of alternative models). The ranking by MCDM technique were compared by GPI ranking using spearman ranking coefficient. The following inference can be made after achieving aforementioned objectives:

1. This work provides an integrated decision support tool for evaluating water resource management strategies

2. The calibrated equation performs better and hence provide consistent result.

3. The ranking by MCDM techniques (WSM, WPM, WASPAS and EDAS) shows that Saif model gives best performance while estimating reference evapotranspiration also GPI confirms the same.

4. MCDM models proved to be a versatile technique for selecting the most promising model in the study region.

5. The results are likely to help minimize the error in estimating reference evapotranspiration, and in addition the approach implemented in this study can be used in regions around the world with similar topography and climatic conditions.

6. Further research are required to assess the impact of using a reduced data set for daily ETo estimation hourly. In addition, in future the seasonal shifts in ETo must also be examined. Additionally, similar problem can be ranked by other available MCDM methods in the future.

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

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FIELD EVALUATION OF RHIZOBACTERIAL INOCULANTS IN COMBINATION WITH HUMIC SUBSTANCES TO IMPROVE SEED AND OIL YIELDS OF SAFFLOWER (*CARTHAMUS TINCTORIUS* L.) UNDER IRRIGATED AND RAINFED CONDITIONS

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Abstract. The present study was evaluated the application of humic acid (HA), which possess multifaceted biological action, and plant growth promoting rhizobacteria (PGPR) inoculation to improve the morphologic performance, yield and seed quality of safflower under different water regimes in the Eastern Anatolia region of Turkey. In a 2-year field trial, the two humic acid level (200 and 400 kg ha^{-1}) and the two rhizobacterial strains (*Bacillus megatorium* M3 and *Bacillus subtilis* OSU142) in together with control treatments were evaluated under irrigated and rainfed conditions using randomized complete block design with a split-split plot arrangement. Bacterial strains and humic acid levels highly increased plant growth, seed and oil yields, and seed nutrient contents under irrigated and rainfed conditions. However, the combined application of the OSU142 strain with 400 kg HA ha^{-1} resulted in the greatest enhancement of safflower growth and yield under irrigated conditions, and seed and oil yields increased by 101.8 and 127.6% with treatment of 400 HA + OSU14 respectively. Moreover, the integrated use of humic acids and PGPR inoculation relatively showed additive effect in the growth and yields of safflower under rainfed conditions, and increased by 186.5 with treatment of 400 HA + OSU142 and 257.1% with treatment of 400 HA + M respectively.
Keywords: *safflower, PGPR, humic acids, energy crop, agricultural sustainability, crop performance*

Introduction

Safflower (*Carthamus tinctorius* L.) is a dryland oilseed crop with a very long cultivation history. It is widely grown for food, medicinal, cosmetic and biofuel purposes over a wide range of geographical areas from Far East to American continent (Ekin, 2005). Nowadays, scientific interest in this species has gained importance as a result of mainly their utilization increase in human nutrition and non-food production due to their high-quality vegetable oil. In safflower oil, the major fatty acids are linoleic and oleic acids and their relative proportions determine the safflower oil's functional properties and nutritional value (Cao et al., 2013; Rapson et al., 2015). On the basis of the nutritional value of safflower oil, oil quality, in fact, shows similarity to that of olive oil and is valued for human health reasons since the high unsaturated fatty acids (linoleic and oleic acids) contents leads to significant reduction in blood cholesterol levels (Dajue and Mündel, 1996; La Bella et al., 2019). In this regard, safflower would seem to be more favorable when compared to those of the oils of major oilseed crops such as sunflower, rapeseed, maize and soy commonly used for food and non-food sector in the semi-arid and marginal croplands of the world and central-eastern Turkey (Ekin, 2005; Kizil et al., 2008; Ozturk et al., 2008). In general, oilseed crops for application in the non-food sector, especially regarding biofuel research and development projects, have attract intensive attention in recent years in Turkey. In this regard, safflower appears to be more suitable as a dryland oilseed crop that can adapt

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highly to chancing climate and soil conditions for the agriculture and biofuels industry among other widely cultivated crops (Nosheen et al., 2018). However, it is characterized by spiny nature and low seed yield which have devitalized farmers from taking on its cultivation in the many countries including Turkey in dryland farming (Ekin, 2005; La Bella et al., 2019).

To maintain high yielding potential in safflower cultivation is firstly required water stress and soil fertility management in semi-arid and highland regions typically characterized by variable and unpredictable inadequate rainfall, poor moisture storing capacity of soils, large diurnal ranges in temperature and frequent strong winds (Ozturk et al., 2008). Due to readily deterioration of soil, water and other environmental resources in those regions compared to agriculture areas in different regions of the world, future food and energy demand and its created pressures are likely to further exacerbate the damaged soil health and effects of drought (Somerville and Briscoe, 2001). Therefore, it is imperative to protect soil health and productivity and to enhance the water stress tolerance of crops under the changing environmental conditions. In particular, drought tolerance will likely become increasingly significant for producing steady yields in all oilseed crops under global climate change (Comas et al., 2013). Although the safflower is well adapted to dryland cropping system, its agronomic performance and seed quality is particularly dependent upon climatic factors such as temperature and rainfall in flowering and seed maturation stages, and is largely affected by water stress conditions in these periods (Weiss, 1983; Kumar et al., 2016; La Bella et al., 2019). There are yet no economically current technological vehicles to facilitate plant production under drought. But the introduction of novel techniques to increase water stress tolerance is crucial for increasing safflower oil-based food and biofuel production. In this context, the role of beneficial rhizosphere microorganisms is gaining premium importance in the development of climate change resilient agriculture and stress management.

The rhizosphere of plants, the interface between plant roots and soil, is widely colonized by soil microorganisms (e.g. plant growth promoting rhizobacteria, PGPR). After inoculated on seed, PGPR can successfully colonize plant roots and improve plant growth directly by either releasing of plant growth stimulating compounds (e.g. phytohormones such as cytokines or auxins) or improving in nutrient uptakes (e.g. N_2 fixing, P-solubilizing or siderophore release increasing nitrogen, phosphorus and iron availability, respectively) or indirectly by decreasing the harmful effects of pathogens via synthesis of antibiotics. Thus, the plant-bacteria symbioses could stimulate plant growth and yield by reducing dependency on traditional fertilizers, enhance the plant tolerance to both biotic and abiotic stresses, and serve to keep soil productivity and environmental health. However, the density and structure of the rhizosphere bacteria in the soil are dictated by soil pH, organic matter content, water and nutrient availability throughout the root surface (Bossio et al., 1998; Drenovsky et al., 2004; Garcia-Pausas and Paterson, 2011; Backer et al., 2018). On the other hand, biogeographical patterns including topographic and climatic variations have also the powerful impacts on density of bacterial community (Kristin and Miranda, 2013). In the world, several PGPR species (*Azotobacter, Azosprillum, Bacillus, Serratia, Enterobacter, Pseudomonas, Klebsiella* and *Variovorax*) as rhizosphere-colonizing microorganisms were the subject of intense study in the field or laboratory in various areas of the many countries in recent years. In this context, it was generated valuable information on the various field crops, such as sugar beet and barley (Cakmakci et al., 200 2006), sugarcane (Silva et al.,

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2017), sunflower (Shadid et al., 2012), wheat (Rosas et al., 2009; Hungria et al., 2010; Rana et al., 2012; Chandra et al., 2019), rice (Lucas et al., 2009), bean (Hoyos-Carvajal et al., 2009), canola (El-Howeity and Asfour, 2012), maize (Thonar et al., 2017) and soy (Cassán et al., 2009). However, each plant's responses to rhizobacteria cannot be expressed by admitted valid comprehension and this actually presents both an opportunity and a challenge. The capability of soil bacteria to reveal favorable effects on plant growth can be impaired under field conditions since other exterior factors come into play (Nelson, 2004; Backer et al., 2018; Chandra et al., 2019). Therefore, it has recently been demonstrated that the integrated use of humic substances and PGPR in agricultural practices could be envisaged as environmental-friendly technology to promote crop yield and quality. In addition, this sustainable agricultural approach can be a good means to enhance vegetable oil and biofuel production in water-deficit regions.

Humic substances, consisting of soil organic matter decomposition, are of crucial important for plant physiology and environmental protection by improving structure and fertility of soils and their resistance to erosion. However, they are a valuable complementary of soil microbiota, since they have natural hormone-like structure and exhibit biological activities. In addition, humic substances are show capacity to regulate the uptake and transport of nutrients to plants, improve crop yield and plant resistance to stress and effect root hair formation and lateral root development. Due to humic substances have a complex structure, which explains their versatile biological activity, the studies have majored on their interactions and structure with plants and associated rhizosphere microorganisms such as PGPR in controlled laboratory experiments. However, critical assessments of such interactions under real field conditions are necessary to quantify the current benefits on productivity, in view of improving practical modern technologies for advanced and sustainable agricultural systems purposed in enhancing crop yield and quality. Some authors (Canellas et al., 2013; Esringu et al., 2016; Schoebitz et al., 2016; Silva et al., 2017) suggested that co-application of humic substances and PGPR inoculation in several crop species grown under field conditions can increase significantly macro- and micro nutrient uptakes and root growth, which in turn led to the increase crop yield. Moreover, these authors reported that stimulation of biological activity by humic acid can further enhance nutrient cycling through the action of microorganisms. Another reason given for increased crop yield, was also attributed to be highly adaptation of plant to several stress conditions by integrated use of humic substance and PGPR (Nardi et al., 2009; Dobbss et al., 2010; Busato et al., 2012; Puglisi et al., 2013; Canellas and Olivares, 2014; Olivares et al., 2015). Previous studies also reported that inoculation of specific plant growth promoting bacteria species like N_2-fixing *Bacillus subtilis* and phosphorus solubilizing *Bacillus megatorium* instead of chemical fertilizers can serve as an environmental friendly alternative application and improve plant nutrition by increasing N and P uptake by plants (Cakmakci et al., 2001; Esitken et al., 2003). But, up till now no data are present in respect of the use of this *Bacillus* strains in safflower plants. However, only limited data are available with respect to the use of *Pseudomonas, Azosprillum* and *Azotobacter* spp., which were used to increase nitrogen uptake (Mirzakhani et al., 2009; Soleymanifard and Sidat, 2011; Sharifi et al., 2017; Nosheen et al., 2018), root morphology (Nosheen et al., 2011), and protein quantity and quality of seeds (Nosheen et al., 2016) in safflower.

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Safflower is an important oilseed plant that grows optimally in dry conditions, but is negatively affected by drought in the phenological periods (i.e. germination, stem elongation and branching, flowering), which are the most critical periods in terms of plant moisture requirement (Dajue and Mündel, 1996; Ekin, 2005). Thus, the combined use of humic acids and PGPR inoculation was explored as a possibility for improving agronomic performance of the safflower under irrigated and rainfed conditions in arid and semi-arid regions.

Materials and methods

Site characterization

The safflower (Carthamus tinctorius L.) variety Remzibey-05 was investigated for its response to plant growth promoting rhizobacteria inoculation and humic acid application under irrigated and rainfed field conditions. The field experiment was carried out at the research farm located in Ahlat district (38° 46' N and 42°30' E with an altitude of 1722 m) in the Eastern Anatolia region of Turkey during the cropping seasons 2010 and 2011. The climate of experimental area is continental with 562.6 mm total rainfall in long-term average (1958-2017), concentrated in winter. Annual mean air temperature and relative humidity values are 9.3 °C and 63.8%, respectively. Maximum temperature is 22.8 °C in August and minimum temperature is -2.5 °C in January. The weather data were collected from weather station close to the fields (*Table 1*). In 2010 and 2011 years, mean temperature values were 16.5 °C and 16.0 °C and amounts of rainfall were 291.0 mm and 269.4 mm, respectively. The soil of experiment site was a silt-clay-loam, including 1.60% organic matter and pH 7. total N 0.15 g/kg, 6.8% CaCO 1.16 dS/m electrical conductivity (EC), 0.15 g kg^{-1} total N, 7.95 mg kg^{-1} available P, 3.30 mg kg^{-1} available Mn, 196 mg kg^{-1} available K, 5.85 mg kg^{-1} available Fe, 1.44 mg kg^{-1} available Zn and 0.59 mg kg^{-1} available Cu.

Plant material and experimental design

Safflower seeds were obtained from the Transitional Zone Agricultural Research Institute, Republic of Turkey Ministry of Agriculture and Forestry. The Remzibey-05 is an early-maturing safflower variety with Turkey of origin, drought-tolerant, spiny, open-pollinated, yellow flower color, white seed color, 32-35% oil content, high-oleic oil type and approximately 1.00 t ha^{-1} dryland seed yield. The field experiments lay out was split-split plot design in randomized complete block with three replicates, with water regimes (irrigated and rainfed) as the main plot, humic acid (Control: without the addition of humic acid, 200 kg ha^{-1} and 400 kg ha^{-1}) as the sub plots, and PGPR strains (Uninoculated control, *Bacillus megatorium* M3 and *Bacillus subtilis* OSU-142) as the sub-subplots. The humic acid doses were prepared by Agro-Lig™ (commercial products - 75% total organic matter, 65% total humic acid derived from leonardite, 22% max moisture, pH 3.5-5.5) in granule form.

Bacterial strains, culture media and seed bacterization

Gram-positive, non-pathogenic *Bacillus subtilis* OSU-142 and *Bacillus megatorium* M3 strains were kindly obtained from Atatürk University-Turkey. In the previous studies, strains used in this study were determined that they showed capacity to grow in N-free conditions, for hormones (IAA, GA_3),1-aminocyclopropane-1carboxylate (ACC)

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deaminase and siderophore production, and to solubilize phosphate and to N_2-fixing (Cakmakci et al., 2001; Orhan et al., 2006). For the inoculation of safflower seeds, bacterial cultures were grown on nutrient agar for routine use. Subsequently, single colony was transferred to 500 mL flasks containing nutrient broth. The cultures were incubated on a rotating shaker (150 rpm) overnight at 28 °C aerobically, and then bacterial suspensions were diluted to final concentration at cell density of 1 x 10^9 colony forming unit ml^{-1} by sterilized and distilled water (sdH_2O). Safflower seed surface was disinfected by dipping first in 3% (v/v) sodium hypochlorite for 3 min and then 95% ethanol for 1 min. After surface disinfection, the seeds were washed 3–4 times with sdH_2O. Approximately, 5 g sugar (50 mg mL^{-1}) was added to each Erlenmeyer flasks, and the surface-sterilized seeds were soaked separately in this suspension. The seeds were treated with the bacteria in the flasks by shaking at 80 rpm for 2 h. For the uninoculated control, seeds were treated with sterile nutrient broth supplemented with sugar. After shaking, the seeds were taken out and air-dried on sterile Whatman filter paper sheets overnight.

Table 1. *Mean, maximum and minimum temperature (°C), monthly rainfall (mm) and relative humidity (%) trends in the experimental site (Ahlat, Turkey) during 201 2011 and long-term average (LTA: 1958-2017)*

Year	Month	T_{mean} (°C)	T_{max} (°C)	T_{min} (°C)	Rainfall (mm)	R. Humidity (%)
2010	April	7.3	14.5	3.4	154.4	71.0
	May	11.4	16.9	7.3	106.2	65.8
	June	18.3	24.7	11.9	28.0	50.4
	July	22.8	29.3	16.1	1.8	37.3
	August	22.5	29.2	15.7	0.6	35.6
Season (M/T)*		**16.5**	**22.9**	**10.8**	**291.0**	**52.0**
Yearly (M/T)		**10.9**	**16.6**	**5.2**	**399.0**	**59.6**
2011	April	6.9	11.0	3.3	159.0	71.0
	May	11.2	16.6	6.7	90.0	69.1
	June	17.6	23.6	11.5	15.6	52.1
	July	22.3	28.9	15.8	3.2	41.3
	August	22.0	28.4	15.4	1.6	40.4
Season (M/T)		**16.0**	**21.7**	**10.5**	**269.4**	**54.8**
Yearly (M/T)		**8.6**	**13.7**	**4.0**	**566.6**	**56.4**
LTA	April	6.9	11.4	2.8	87.1	69.4
	May	13.1	17.0	7.1	70.2	65.0
	June	18.9	23.3	11.2	28.7	55.6
	July	21.5	28.0	15.3	8.3	49.4
	August	22.8	28.3	15.4	5.7	47.7
Season (M/T)		**16.1**	**21.6**	**10.4**	**136.2**	**57.4**
Yearly (M/T)		**9.3**	**14.2**	**4.5**	**562.6**	**63.8**

* M: Mean, T: Total

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Main cultivation practices and measurement of morphologic and yield data

The field experiment was carried out under rainfed and irrigated conditions. After soil was ploughed and harrowed, safflower seeds were sown at 40 cm row spacing on early April in both years. Overall, plant density was 40 viable seeds m^{-2} and sub-subplots area was 16 m^2. At the start of the field experiment, a basal dose of N (urea) and P (Triple Super Phosphate), at rates of 300 and 150 kg ha^{-} which might support root morphology and plant growth improvements in safflower (Nosheen et al., 2011), were applied to the plants. Humic acids were applied in the soil according to layout at sowing time and then mixed well in the soil. In the study, irrigation was applied based on the phenological periods (stem elongation and flowering), which are the most critical periods in terms of plant moisture requirement in safflower. This application called as supplemental irrigation, as a notion, refers to providing significant increases in yield by applying irrigation water in support of rainfall in plants grown under rainfall conditions as the most accurate approach in irrigation water management in the arid and semi-arid regions. In the study, the soil moisture has been allowed to fluctuate only at field capacity range, so soil water potential was controlled in the range between -10 and -20 kPa as indicated by Tensiometer at a depth of 15 cm below the soil surface in the irrigated treatment. Water was applied uniformly across all irrigated plots. Each of irrigations applied in the stem elongation (June) and flowering (July) growth periods represented a total of approximately 48 mm and 80 mm in 201 and 56 and 85 mm rainfall equivalent, respectively. Therefore, the total quantity of water received in irrigation conditions was 419.0 mm and 410.4 mm in the five-month cycle in 2010 and 2011 years, respectively. Weeds were controlled by hand. Safflower plants were harvested at the physiological maturity in mid-August in both years. Seed moisture content was also below 8.00%.

Morphologic and yield characterization were performed following the guide of International Plant Genetic Resources Institute for safflower. Each treatment was evaluated for 5 pre-harvest and 17 post-harvest traits. The pre-harvest traits were stem diameter (mm), plant height (cm), number (no.) of primary branches per plant, capitilum diameter (cm) and number (no.) of capitula per plant. Data was recorded for twenty healthy plants in each of 54 experimental plots. The post-harvest traits were number (no.) of seeds per capitulum, fertile and sterile seed percentage (%),1000-seed weight (g), seed oil and protein contents (%), seed, oil and protein yields (t ha^{-1}) and mineral composition of safflower seeds (mg/kg). Plant height was measured as main shoot length from soil surface to the uppermost capitulum of the plant. Diameters of stem and capitilum were measured by digital caliper. The total number of capitula and branches was recorded as number of capitilum and primary branches per plant. Thousand seed weight was measured as seed weight of 1000 achenes from each of plants in grams. Seed yield was recorded at the harvest area of 7.2 m^2 after removing the two rows border plants all around. Seed oil, protein and nutrient contents were analyzed in three replicates. Oil content was measured by Soxhalet extraction method using hexane as solvent. Protein content in seed samples was calculated from nitrogen using the conversional factor of 6.25 and determined by the Kjeldahl method for nitrogen content measurement in safflower. Magnesium (Mg), potassium (K), calcium (Ca), phosphorus (P), zinc (Zn), manganese (Mn), copper (Cu) and iron (Fe) contents in seed samples were determined by wet-digesting the ground seeds using HNO_3:$HClO_4$ (6:2 v/v) by Advanced Microwave Digestion System. Subsequently, mineral nutrients composition was evaluated by Inductively Coupled Plasma Optical Emission Spectrometry (iCAP 6000 SERIES, ICP Spectrometer) as per manufacturer's instructions.

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Statistical analysis

In order to reveal if data are suitable for normal distribution and homogeneity, the Kolmogorov-Smirnov normality and Bartlett homogeneity tests were used. Afterwards, analysis of variance (ANOVA) was used to compare the effects of water regimes, humic acids and PGPR treatments interactions for 2 years using PROC GLM of SAS 9. Mean comparisons were conducted using Duncan multiple comparison test (DMRT). All the experiments were performed in three replicates and data were presented as descriptive statistics (means; n = 3). Since there were no significant year x treatment interactions for the tested most traits ($p < 0.05$), 2-year data were combined and used for the mean comparisons.

Results

Analysis of pre-harvest morphologic traits

The analysis of combined ANOVA showed significant differences by the tested factors (humic acids and PGPR inoculation) under both irrigated and rainfed conditions, as pointed by variations in multiple biometric characteristics of safflower growth (*Table 2*). Results from analysis of pre-harvest morphologic traits indicated that plant height significantly differed between soil moisture conditions. On average, the highest plant height was recorded in irrigated conditions. Plant height significantly ($P < 0.01$) increased with increasing doses of humic acid. Likewise, inoculations of OSU142 and M3 significantly ($P < 0.01$) increased plant height by 26.20 and 15.57%, over that of the uninoculated control, respectively. Significantly ($P < 0.01$) interactions were observed for W x HA, W x P, HA x P and W x HA x P (*Table 2*).

The mean values for the W x P and W x HA interactions (*Table 3*) revealed that, for both irrigated and rainfed conditions, the OSU142 strain and the 400 kg HA ha^{-1} treatment had the highest values for plant height, over their respective control treatments. Similarly, the interaction of HA x P (*Table 4*) indicated that humic acid and rhizobacterial inoculation increased the plant height, with 400 kg HA ha^{-1} and the OSU142 strain being the most effective. Moreover, there was significant ($P < 0.01$) W x HA x P interaction (*Fig. 1*) for plant height, and the highest plant height was recorded for plants treated with 400 HA + OSU142 in irrigated condition maintaining an increase of 47.8%, compared to their respective non-HA uninoculated control.

Significant differences ($P < 0.01$) in the stem diameter and number of primary branches per plant of safflower were observed for water regimes, HA, and PGPR inoculation (*Table 2*). Irrigation and HA applications considerably increased the both stem diameter and number of primary branches per plant of safflower, with the highest values noted for the 400 HA ha^{-1} treatment. PGPR inoculation also significantly increased stem diameter and number of primary branches, with higher values noted for OSU142 (*Bacillus subtilis*) than for M3 (*Bacillus megatorium*) and the uninoculated control. In addition, there were significant ($P < 0.01$) W x P and HA x P interactions for the stem diameter of safflower (*Table 2*). Mean values for the W x P interaction (*Table 3*) suggested that the highest stem diameter (7.97 mm) was for the OSU142 strain under irrigated conditions maintaining an increase of 22.7%, over their respective control treatment. The interaction of HA x P (*Table 4*) revealed that, for the 200 or 400 HA + OSU142 and the 400 HA + M3 treatments had the highest values for stem diameter (7.5 7.39 and 7.50 mm, respectively).

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Table 2. *Pre-harvest morphological traits of safflower affected by humic acid application and bacterial inoculation under different water regimes (means of the combined years)*[1]

	Plant height (cm)	Stem diameter (mm)	No. of branches/plant	No. of capitula/plant	Capitilum diameter (cm)
WATER REGIMES (W)					
Irrigated	73.8 a	7.39 a	9.5 a	11.6 a	2.51 a
Rainfed	59.5 b	5.64 b	5.9 b	8.5 b	2.28 b
HUMIC ACID (HA)					
0	59.7 c	5.97 c	6.9 c	7.8 c	2.24 c
200	68.2 b	6.58 b	7.8 b	10.6 b	2.42 b
400	72.0 a	7.00 a	8.5 a	11.8 a	2.53 a
PGPR STRAINS (P)					
Uninoculated	58.0 c	5.78 c	6.8 c	8.6 c	2.29 c
OSU142	73.2 a	7.15 a	8.5 a	11.5 a	2.51 a
M3	68.7 b	6.60 b	7.7 b	10.1 b	2.40 b
SIGNIFICANCE					
Source	**df**	**Mean square**			
Year (Y)	1	288.12*	1.13^{ns}	21.87*	32.34**	0.0800^{ns}
W	1	5532.67^{**}	82.51^{**}	347.76^{**}	251.16^{**}	1.52^{**}
Y x W	1	28.83 *	0.08^{ns}	0.71^{ns}	0.46^{ns}	0.0003^{ns}
HA	2	1417.95^{**}	9.60^{**}	22.83^{**}	156.20^{**}	0.74^{**}
Y x HA	2	16.83*	0.12^{ns}	0.22^{ns}	0.87^{ns}	0.0001^{ns}
P	2	2199.35^{**}	17.24^{**}	23.93^{**}	75.69^{**}	0.41^{**}
Y x P	2	6.39^{ns}	0.02^{ns}	0.19^{ns}	0.84^{ns}	0.0016^{ns}
W x HA	2	74.41^{**}	0.01^{ns}	0.11^{ns}	4.50^{**}	0.07^{**}
Y x W x HA	2	19.68*	0.11^{ns}	0.28^{ns}	0.46^{ns}	0.0005^{ns}
W x P	2	299.87^{**}	1.34^{**}	0.06^{ns}	4.69^{**}	0.02^{**}
Y x W x P	2	0.27^{ns}	0.07^{ns}	0.17^{ns}	0.21^{ns}	0.0010^{ns}
HA x P	4	62.72^{**}	1.49^{**}	0.25^{ns}	1.37^{**}	0.009^{*}
Y x HA x P	4	6.77^{ns}	0.16^{ns}	0.04^{ns}	0.14^{ns}	0.0013^{ns}
W x HA x P	4	45.01^{**}	0.26^{ns}	0.47^{ns}	3.17^{**}	0.004^{ns}
CV (%)		5.09	9.24	6.23	4.24	5.33

[1]The means were obtained from combined analysis of variance. ns, *, and ** represent nonsignificant and significant at $P \leq 0.05$ and $P \leq 0.01$ according to DMRT, respectively. CV: coefficient of variations

Number of capitula per plant and capitilum diameter for safflower were severely decreased by rainfed condition, whereas it increased with the application of HA and PGPR inoculation (*Table 2*). Furthermore, significant ($P < 0.01$) interactions were observed for W x HA, W x P and HA x P for these parameters. The W x HA and W x P interactions (*Table 3*) revealed that the highest number of capitula per plant and capitilum diameter for safflower was for the 400 kg HA ha^{-1} treatment and the OSU142 inoculation under the irrigated conditions, whereas the lowest values were observed for the without HA control and the uninoculated control under rainfed conditions. Likewise, the HA x P interaction showed that OSU142 significantly enhanced number of capitula per plant and capitilum diameter in the 400 kg HA ha^{-1} treatment by 104.6% and 22.2% over that of without HA and the uninoculated control, respectively. Mean values for the

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W x HA x P interaction (*Fig. 2*) suggested that the highest number of capitula per plant was recorded for plants treated with 400 HA + OSU142 under irrigated conditions maintaining an increase of 63.6%, whereas the plants submitted to rainfed condition showed considerably increase rate of 181.4% in number of capitula per plant in the 400 HA + OSU142 treatment, over their respective control treatments.

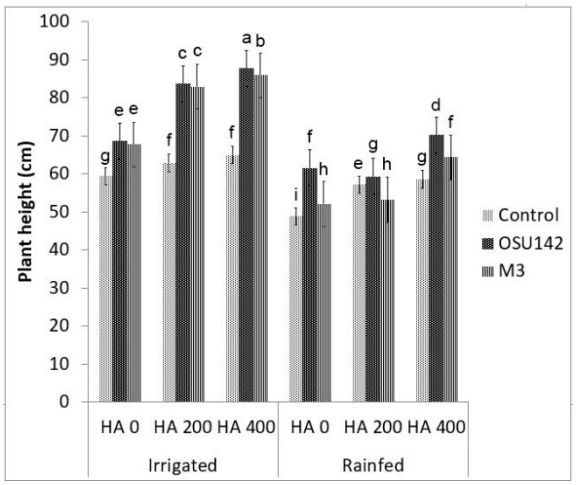

Figure 1. *The interactive effect of water regimes, humic acids and PGPR on the plant height of safflower. Different letters along with water regimes, humic acids and PGPR treatments indicate significant differences in access treatment means from three replications tested at P ≤ 0.05 according to DMRT*

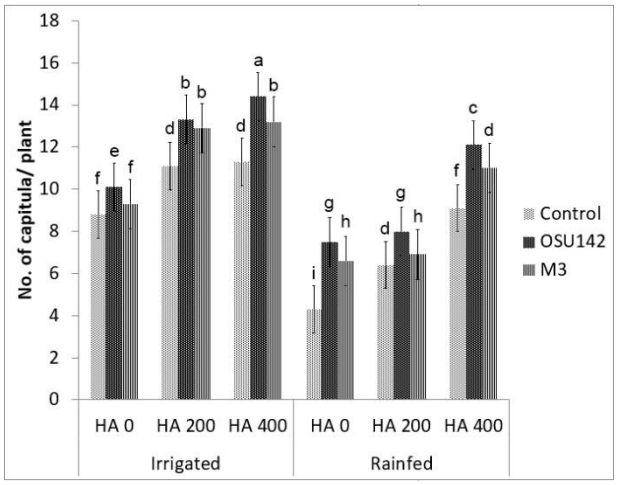

Figure 2. *The interactive effect of water regimes, humic acids and PGPR on the number of capitula per plant of safflower. Different letters along with water regimes, humic acids and PGPR treatments indicate significant differences in access treatment means from three replications tested at P ≤ 0.05 according to DMRT*

Analysis of post-harvest seed traits

The post-harvest seed traits of safflower (number of seeds per capitulum, thousand seed weight, and fertile and sterile seed percentage) were significantly ($P < 0.01$) affected by all the treatments (*Table 5*). Irrigation application considerably increased the

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number of seeds per capitulum, thousand seed weight, and fertile seed percentage, whereas the highest sterile seed percentage was noted for the rainfed condition. HA and rhizobacterial inoculation increased all the seed traits of safflower, except for sterile seed percentage. *Table 5* shows the significant ($P < 0.01$) interactions of W x HA, W x P, HA x P and W x HA x P for number of seeds per capitulum.

Table 3. *Interaction between humic acid (HA) and water regimes (irrigated and rainfed); PGPR strain and water regimes on pre-harvest traits of safflower*

Factors	Plant height (cm)		Stem diameter (mm)		No. of capitula/ plant		Capitulum diameter (cm)		No. of seeds/ capitulum		1000 seed weight (g)		Seed yield (t ha^{-1})		Oil yield (t ha^{-1})	
	I	R	I	R	I	R	I	R	I	R	I	R	I	R	I	R
HA (kg ha^{-1})																
0	65.3 ^{c}	54.2 ^{e}	6.85	5.09	9.4 ^{d}	6.1 ^{f}	2.41 ^{c}	2.08 ^{e}	28.1 ^{c}	23.9 ^{d}	38.2 ^{b}	36.3 ^{c}	1.89 ^{c}	1.02 ^{e}	0.61 ^{c}	0.29 ^{e}
200	76.5 ^{b}	59.8 ^{d}	7.47	5.68	12.4 ^{b}	7.4 ^{e}	2.54 ^{b}	2.31 ^{d}	34.4 ^{a}	24.4 ^{d}	41.5 ^{a}	38.8 ^{b}	2.83 ^{b}	1.37 ^{d}	0.99 ^{b}	0.41 ^{d}
400	79.6 ^{a}	64.4 ^{c}	7.85	6.14	13.0 ^{a}	10.7 ^{c}	2.60 ^{a}	2.44 ^{c}	32.5 ^{b}	28.2 ^{c}	42.4 ^{a}	39.7 ^{b}	3.04 ^{a}	1.87 ^{c}	1.10 ^{a}	0.60 ^{c}
PGPR STRAIN																
Uninoculated	62.4 ^{d}	53.6 ^{f}	6.51 ^{c}	5.06 ^{e}	10.4 ^{c}	6.8 ^{e}	2.43 ^{c}	2.15 ^{e}	29.6 ^{b}	24.0 ^{e}	39.2	36.8	2.09 ^{c}	1.15 ^{f}	0.69 ^{b}	0.33 ^{e}
OSU142	80.1 ^{a}	66.4 ^{c}	7.97 ^{a}	6.35 ^{c}	12.6 ^{a}	10.4 ^{c}	2.59 ^{a}	2.42 ^{c}	33.2 ^{a}	28.5 ^{c}	41.7	39.2	2.92 ^{a}	1.82 ^{d}	1.01 ^{a}	0.57 ^{c}
M3	78.9 ^{b}	58.4 ^{e}	7.70 ^{b}	5.52 ^{d}	11.8 ^{b}	8.5 ^{d}	2.52 ^{b}	2.27 ^{d}	32.1 ^{a}	26.2 ^{d}	41.3	38.8	2.75 ^{b}	1.41 ^{e}	0.98 ^{a}	0.46 ^{d}

Means having different letters are significantly different at $P \leq 0.05$ according to DMRT

Table 4. *Interaction between humic acid (HA) and PGPR strain on growth and yield of safflower*

HA (kg ha^{-1})	PGPR strain	Plant height (cm)	Stem diameter (mm)	No. of capitula per plant	No. of seeds per capitulum	Capitilum diameter (cm)	Seed yield (t ha^{-1})	Oil yield (t ha^{-1})	Protein yield (t ha^{-1})
0	Uninoculated	54.1 h	5.37 d	6.5 f	24.8 b	2.16 f	1.20 f	0.35 f	0.15 f
	OSU142	65.2 d	6.55 b	8.8 d	27.0 b	2.32 d	1.68 d	0.52 e	0.23 d
	M3	60.0 f	6.00 c	8.0 e	26.2 b	2.25 e	1.48 e	0.49 e	0.19 e
200	Uninoculated	58.1 g	5.88 c	9.0 d	26.8 b	2.30 d	1.69 d	0.55 e	0.25 d
	OSU142	75.4 b	7.54 a	12.3 b	33.2 a	2.56 b	2.47 a	0.84 b	0.35 b
	M3	71.2 c	6.33 bc	10.4 c	31.7 a	2.41 c	2.03 c	0.71 c	0.27 c
400	Uninoculated	61.8 e	6.11 c	10.2 c	27.2 b	2.41 c	1.97 c	0.64 d	0.29 c
	OSU142	79.0 a	7.39 a	13.3 a	30.5 a	2.64 a	2.64 a	0.92 a	0.39 a
	M3	75.2 b	7.50 a	12.1 b	31.3 a	2.53 b	2.44 b	0.87 b	0.32 b

Means having different letters are significantly different at $P \leq 0.05$ according to DMRT

The W x HA interaction (*Table 3*) revealed the highest number of seeds per capitulum for the 200 kg HA ha^{-1} treatment under irrigated condition, from that of the non-HA treatment. Conversely, it was also noted increased with increasing humic acid under rainfed condition. The W x P interactions means (*Table 3*) indicated the highest number of seeds per capitulum of safflower was noted for all the bacterial inoculations under irrigated conditions, whereas the plants inoculated with the OSU142 strain increased the number of seeds per capitulum under both irrigated and rainfed conditions by 12.2 and 18.8%, respectively, over their respective control treatments. Similarly, HA x P interactions means (*Table 4*) revealed that all the bacterial inoculations significantly increased the number of seeds per capitulum in all levels of HA treatment, from that of the control. In addition, mean values for the W x HA x P interaction (*Fig. 3*) revealed

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that the applications of 200 HA + M3 and 200 HA + OSU142 under irrigated conditions resulted in the highest number of seeds per capitulum by increases of 41.4% and 36.5%, whereas the plants submitted to rainfed condition showed considerably increase rates of 28.7% and 27.0% in number of capitula per plant in the 400 HA + M3 and 400 HA + OSU142 treatments, over their respective control treatments.

Table 5. *Post-harvest seed traits of safflower affected by humic acid application and bacterial inoculation under different water regimes*

	No. of seeds/capitulum	1000 seed weight (g)	Fertile seed percentage (%)	Sterile seed percentage (%)
WATER REGIMES (W)				
Irrigated	31.7 a	40.7 a	95.90 a	4.12 b
Rainfed	26.3 b	38.3 b	94.36 b	5.67 a
HUMIC ACID (HA)				
0	26.0 b	37.4 b	94.54 c	5.50 a
200	30.6 a	40.6 a	95.14 b	4.85 b
400	30.3 a	40.6 a	95.71 a	4.34 b
PGPR STRAIN (P)				
Uninoculated	26.9 b	38.0 b	94.00 b	6.05 a
OSU142	30.2 a	40.4 a	95.70 a	4.36 b
M3	29.8 a	40.0 a	95.69 a	4.30 b
SIGNIFICANCE				
Source	**df**	**Mean square**		
Year (Y)	1	0.68^{ns}	1.14^{ns}	1.18^{ns}	2.15^{ns}
W	1	778.70^{**}	165.51^{**}	64.79^{**}	64.68^{**}
Y x W	1	38.16^{*}	0.63^{ns}	0.99^{ns}	0.98^{ns}
HA	2	240.04^{**}	134.56^{**}	12.13^{**}	12.23^{**}
Y x HA	2	6.31^{ns}	0.69^{ns}	1.14^{ns}	1.46^{ns}
P	2	113.34^{**}	58.55^{**}	34.49^{**}	35.71^{**}
Y x P	2	11.35^{ns}	0.45^{ns}	4.22^{*}	3.48^{ns}
W x HA	2	35.76^{**}	9.67^{*}	0.35^{ns}	0.19^{ns}
Y x W x HA	2	9.04^{ns}	1.90^{ns}	0.43^{ns}	1.71^{ns}
W x P	2	20.82^{**}	0.06^{ns}	0.38^{ns}	0.13^{ns}
Y x W x P	2	1.23^{ns}	3.77^{ns}	1.75^{ns}	0.91^{ns}
HA x P	4	25.67^{**}	2.25^{ns}	0.91^{ns}	0.44^{ns}
Y x HA x P	4	4.36^{ns}	1.34^{ns}	0.99^{ns}	0.67^{ns}
W x HA x P	4	17.46^{**}	1.21^{ns}	1.55^{ns}	1.73^{ns}
CV (%)		6.76	5.06	8.19	13.8

The means were obtained from combined analysis of variance. ns, *, and ** represent nonsignificant and significant at $P \leq 0.05$ and $P \leq 0.01$ according to DMRT, respectively. CV: coefficient of variations

Significant differences ($P < 0.01$) in the 1000 seed weight, fertile and sterile seed percentage of safflower were observed for water regimes, HA, and bacterial inoculation (*Table 5*). A higher 1000 seed weight and fertile seed rate were noted for the irrigation treatment than for the rainfed condition. However, irrigation treatment induced a marked decline in sterile seed rate, with the lowest value noted for the rainfed

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conditions. On the other hand, all HA and PGPR treatments enhanced the 1000 seed weight by 8.6 and 6.3%, respectively, over that of control (no HA or uninoculated). Similarly, fertile seed percentage significantly increased with increasing doses of HA and all the PGPR inoculations, whereas it considerably reduced the sterile seed percentage of safflower. The interactions between HA and PGPR were not significant for these parameters, but W x HA interaction was significant ($P < 0.05$) for 1000 seed weight only (*Table 5*). The highest values for 1000 seed weight were noted for all the HA doses under irrigated condition, maintaining an increase from 8.7 to 11.0%, compared to the control (*Table 3*).

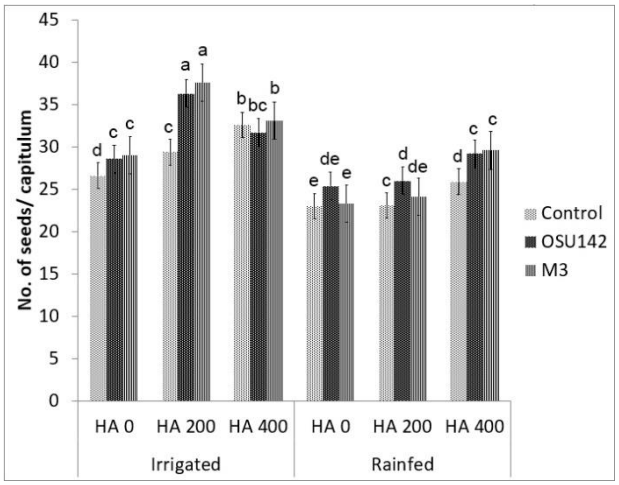

Figure 3. *The interactive effect of water regimes, humic acids and PGPR on the number of seeds per capitulum of safflower. Different letters along with water regimes, humic acids and PGPR treatments indicate significant differences in access treatment means from three replications tested at $P \leq 0.05$ according to DMRT*

Analysis of seed quality and yield traits

Seed oil and protein contents were significantly affected by all the treatments (*Table 6*). Irrigation significantly ($P < 0.01$ and $P < 0.05$) increased the seed oil and protein contents, respectively. Seed oil content significantly ($P < 0.01$) increased with increasing HA doses, while the highest seed protein contents noted for both HA doses compared to control plants. Rhizobacteria inoculation significantly ($P < 0.01$) increased seed oil and protein contents, with the highest oil content noted for M3 and with a higher protein content noted for OSU142 than for M3 and the uninoculated control.

Seed yield of safflower was significantly ($P < 0.01$) affected by water regimes, HA, and rhizobacterial strains (*Table 6*). For different water regimes, irrigation treatment induced a marked increase in the seed yield, with the lowest value noted for the rainfed conditions. Likewise, increasing doses of HA and PGPR inoculation (OSU142 > M3) increased seed yield (*Table 6*). Moreover, W x HA, W x P, HA x P and W x HA x P ($P < 0.01$) interactions were significant. The W x HA interaction (*Table 3*) revealed that the highest seed yield (3.04 t ha^{-1}) was recorded for 400 kg HA ha^{-1} under irrigated condition, and the lowest yield (1.02 t ha^{-1}) was recorded for the non-HA control under rainfed condition. The 400 kg HA ha^{-1} treatment significantly enhanced the seed yield by 60.8% and 83.3% under irrigated and rainfed condition, respectively, over that of

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their respective non-HA control. The W x P interaction (*Table 3*) revealed that OSU142 was a potential strain for improving seed yield under both irrigated and rainfed conditions, with highest yield (2.92 t ha^{-1}) noted for the irrigation treatment. Similarly, the HA x P interaction (*Table 4*) showed that OSU142 in all the HA rates considerably increased seed yield by 105.8 and 119.8%, respectively, over that of the non-HA and uninoculated control. Moreover, the W x HA x P interaction (*Fig. 4*) revealed that the highest seed yield (3.37 t ha^{-1}) was recorded for 400 HA + OSU142 treatment under irrigated conditions, with increase of 101.8% and followed by 400 HA + M3 (89.8%) treatment, compared to their respective control. However, the treatments of 400 HA + OSU14 200 HA + OSU142 and 400 HA + M3 were resulted for the higher seed yields (2.1 1.95 and 1.91 t ha^{1}) under rainfed conditions, with increases of 186. 163.5 and 158.1%, respectively, compared to their respective control.

Table 6. *Seed yield and quality traits of safflower affected by humic acid application and bacterial inoculation under different water regimes*

	Seed content (%)		Yield (t ha^{-1})		
	Oil	**Protein**	**Seed**	**Oil**	**Protein**
WATER REGIMES (W)					
Irrigated	34.55 a	13.96 a	2.59 a	0.89 a	0.36 a
Rainfed	30.47 b	13.47 b	1.44 b	0.44 b	0.20 b
HUMIC ACID (HA)					
0	30.64 c	12.75 b	1.45 c	0.45 c	0.19 c
200	32.93 b	14.10 a	2.10 b	0.70 b	0.31 b
400	33.97 a	14.30 a	2.46 a	0.85 a	0.35 a
PGPR STRAIN (P)					
Uninoculated	30.73 c	13.75 b	1.62 c	0.51 c	0.23 c
OSU142	32.93 b	14.27 a	2.37 a	0.79 a	0.34 a
M3	33.89 a	13.14 b	2.08 b	0.72 b	0.28 b
SIGNIFICANCE					
Source	**df**	**Mean square**			
Year (Y)	1	21.78^{*}	10.70^{ns}	77.48^{*}	15.33^{*}	3.51^{ns}
W	1	450.18^{**}	6.35^{*}	2665.11^{**}	436.21^{**}	54.57^{**}
Y x W	1	0.83^{ns}	0.01^{ns}	0.57^{ns}	0.82^{ns}	0.14^{ns}
HA	2	104.62^{**}	25.45^{**}	756.79^{**}	120.01^{**}	20.56^{**}
Y x HA	2	0.08^{ns}	0.44^{ns}	0.59^{ns}	0.09^{ns}	0.006^{ns}
P	2	94.44^{**}	11.75^{**}	372.89^{**}	58.81^{**}	7.78^{**}
Y x P	2	0.24^{ns}	1.35^{ns}	0.15^{ns}	0.08^{ns}	0.10^{ns}
W x HA	2	1.50^{ns}	0.94^{ns}	15.69^{**}	5.04^{**}	0.35^{ns}
Y x W x HA	2	0.10^{ns}	0.72^{ns}	0.21^{ns}	0.02^{ns}	0.03^{ns}
W x P	2	3.49^{ns}	8.33^{*}	14.53^{**}	2.50^{**}	0.36^{ns}
Y x W x P	2	2.21^{ns}	1.35^{ns}	0.44^{ns}	0.26^{ns}	0.07^{ns}
HA x P	4	1.14^{ns}	6.18^{*}	10.21^{**}	1.83^{**}	0.06^{ns}
Y x HA x P	4	0.72^{ns}	0.08^{ns}	0.75^{ns}	0.18^{ns}	0.02^{ns}
W x HA x P	4	0.17^{ns}	0.39^{ns}	5.84^{**}	1.42^{**}	0.17^{ns}
CV (%)		5.95	8.86	5.53	7.54	9.15

The means were obtained from combined analysis of variance. ns, *, and ** represent nonsignificant and significant at $P \leq 0.05$ and $P \leq 0.01$ according to DMRT, respectively. CV: coefficient of variations

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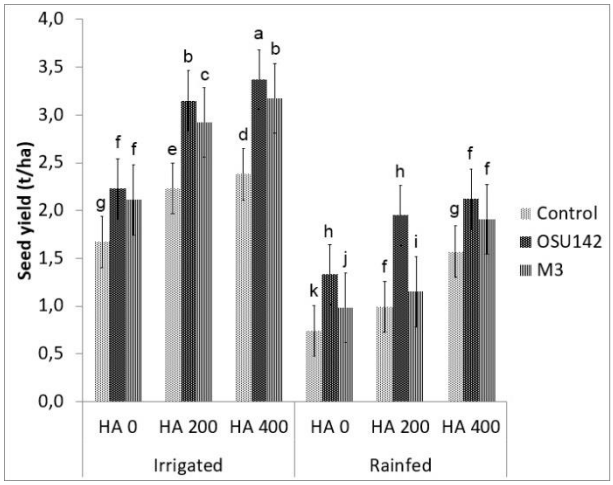

Figure 4. *The interactive effect of water regimes, humic acids and PGPR on the seed yield (t ha^{-1}) of safflower. Different letters along with water regimes, humic acids and PGPR treatments indicate significant differences in access treatment means from three replications tested at P ≤ 0.05 according to DMRT*

Results for the oil and protein yields showed trends similar to that seen for seed yield. Significant differences ($P < 0.01$) in the oil and protein yields of safflower were observed for water regimes, HA, and bacterial inoculation (*Table 6*). For different water regimes, the highest oil and protein yields were recorded for irrigated condition with 0.89 and 0.36 t ha^{-} respectively. The increasing doses of HA (400 HA > 200 HA) and PGPR inoculation (OSU142 > M3) significantly ($P < 0.01$) improved oil and protein yields (*Table 6*). Furthermore, significant ($P < 0.01$) interactions were observed in W x HA, W x P, HA x P and W x HA x P for oil yield. The W x HA interaction (*Table 3*) showed that the highest oil yield (1.10 t ha^{-1}) was recorded for 400 kg HA ha^{-1} under irrigated condition, and the lowest yield (0.29 t ha^{-1}) was recorded for the non-HA control under rainfed condition. Likewise, the W x P interaction (*Table 3*) revealed that OSU142 and M3 strains enhanced oil yield under both irrigated and rainfed conditions, with highest yield (1.01 and 0.98 t ha^{-} respectively) noted for the irrigation treatment. Furthermore, the HA x P interaction (*Table 4*) showed that the highest oil yield was observed for 400 HA + OSU142 treatment. Moreover, the W x HA x P interaction (*Fig. 5*) revealed that the highest oil yields were recorded for 400 HA + OSU14 400 HA + M3 and 200 HA + OSU142 treatments under irrigated conditions, with increases of 127. 119.1 and 108.4%, compared to their respective control, whereas the treatments of 400 HA + M3 and 400 HA + OSU142 were resulted in the higher seed yields under rainfed conditions, with increases of 257.1 and 226.5%, respectively, compared to their respective control.

Elemental analysis of safflower seeds

Macro- and micronutrients contents (P, K, Ca, Mg, Fe, Zn, Cu and Mn) of safflower seeds were significantly ($P < 0.01$) affected by water regimes, HA, and PGPR strains (*Table 7*). A higher P, K, Ca, Fe, Cu and Mn contents were noted for the irrigation treatment than for the rainfed condition, whereas rainfed condition induced a marked increase in Mg and Zn contents, with the lowest value noted for the irrigation treatment.

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However, the seeds in both HA and PGPR treated plants generally had higher macro- and micronutrient contents compared to the control (no HA or uninoculated) plants.

Table 7. *Mineral composition (mg kg^{-1}) of safflower seeds affected by humic acid application and bacterial inoculation under different water regimes*

	P	K	Ca	Mg	Fe	Zn	Cu	Mn
WATER REGIMES (W)								
Irrigated	4889.94 a	4536.54 a	2500.12 a	1339.86 b	58.01 a	38.48 b	16.49a	13.16a
Rainfed	4449.45 b	4088.63 b	2287.45 b	1610.77 a	52.14 b	43.19 a	14.71b	12.15b
HUMIC ACID (HA)								
0	4071.87 c	3775.07 c	2151.89 c	1327.10 c	50.01 c	39.71 b	16.23a	11.68c
200	4842.96 b	4464.84 b	2431.53 b	1534.07 b	56.81 b	42.51 a	15.51b	12.87b
400	5094.25 a	4697.72 a	2597.78 a	1564.77 a	58.47 a	40.28 b	15.06c	13.41a
PGPR STRAIN (P)								
Uninoculated	4126.31 c	3732.09 c	1962.34 c	1201.17 c	47.87 c	36.94 c	16.58a	11.51c
OSU142	4653.25 b	4688.32 a	2775.90 a	1696.44 a	64.16 a	41.37 b	15.44b	13.74a
M3	5319.52 a	4517.35 b	2442.96 b	1528.34 b	53.28 b	44.21 a	14.78c	12.73b
SIGNIFICANCE								
Source	**df**	**Mean square**						
Year (Y)	1	440755.39*	209896.62*	1572725.90*	152026.53*	82.88*	142.18*	25.49*	4.58*
W	1	5238800**	5416687**	1219954**	1981519**	948.44**	597.18**	85.45**	27.55**
Y x W	1	18198.74^{ns}	46276.09*	11092.35^{ns}	4048.25^{ns}	0.58^{ns}	1.00^{ns}	1.72^{ns}	0.03^{ns}
HA	2	10217855**	8289593**	1827926**	601632**	723.70**	78.69**	12.68**	28.29**
Y x HA	2	21474.17*	2571.70^{ns}	16159.02*	6990.01^{ns}	2.36^{ns}	8.18*	0.27^{ns}	0.26^{ns}
P	2	13119598**	9361325**	6022263**	2283541**	2478.5**	484.90**	29.96**	45.08**
Y x P	2	1539.32^{ns}	9289.72^{ns}	64407.37^{ns}	13745.38*	0.53^{ns}	0.20^{ns}	1.88^{ns}	0.009^{ns}
W x HA	2	1497^{ns}	60^{ns}	3502^{ns}	236790**	44.24**	2.88^{ns}	3.74**	0.05^{ns}
Y x W x HA	2	177.42^{ns}	1748.94^{ns}	15377.23*	2486.49^{ns}	2.05^{ns}	0.93^{ns}	0.16^{ns}	0.04^{ns}
W x P	2	14764^{ns}	4269^{ns}	168032**	34126**	17.84**	26.84**	0.44^{ns}	0.34^{ns}
Y x W x P	2	2734.64^{ns}	1991.92^{ns}	1504.53^{ns}	2727.04^{ns}	1.50^{ns}	0.38^{ns}	0.74^{ns}	0.17^{ns}
HA x P	4	15701*	29615**	93578**	22250**	126.21**	34.14**	1.32^{ns}	1.49**
Y x HA x P	4	13354.16*	6223.89^{ns}	36666.32*	2255.49^{ns}	0.90^{ns}	1.29^{ns}	0.41^{ns}	0.11^{ns}
W x HA x P	4	33564**	1482^{ns}	3891^{ns}	18616**	30.61**	7.07**	1.33^{ns}	1.54**
CV (%)		4.53	4.49	5.64	7.88	5.15	5.97	5.09	7.66

The means were obtained from combined analysis of variance. ns, *, and ** represent nonsignificant and significant at $P \leq 0.05$ and $P \leq 0.01$ according to DMRT, respectively. CV: coefficient of variations

For macronutrient contents (P, K, Ca and Mg), the individual effects of all treatments were significant ($P < 0.0$ *Table 7*). All macronutrient contents increased with increasing doses of HA (400 HA > 200 HA), whereas the M3 inoculation noted for the highest P content, and the OSU142 inoculation noted for the highest K, Ca and Mg contents. For these parameters, significant interactions were observed in W x HA ($P < 0.01$) for Mg content, W x P ($P < 0.01$) for Ca and Mg contents, HA x P ($P < 0.01$ and $P < 0.05$) for all the macronutrients, and W x HA x P ($P < 0.01$) for P and Mg contents (*Table 7*). The W x HA interaction (*Table 8*) showed that the highest Mg content (1750.60 mg kg^{-1}) was recorded for 200 kg HA ha^{-1} under rainfed condition, and the lowest value (1272.78 mg kg^{-1}) was recorded for the non-HA control under irrigated condition.

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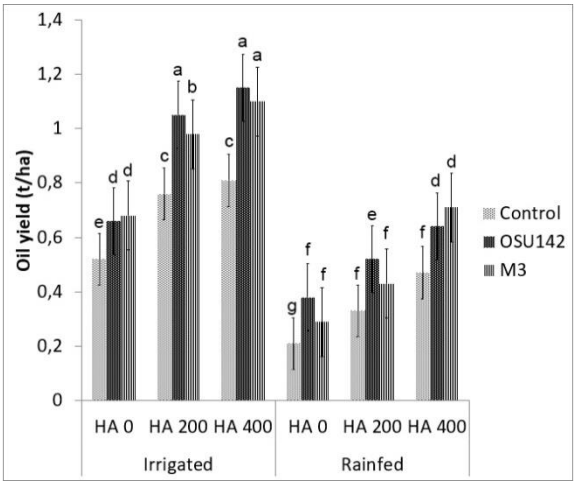

Figure 5. *The interactive effect of water regimes, humic acids and PGPR on the oil yield (t ha^{-1}) of safflower. Different letters along with water regimes, humic acids and PGPR treatments indicate significant differences in access treatment means from three replications tested at $P \leq 0.05$ according to DMRT*

Table 8. *Interaction between humic acid (HA) water regimes (Irrigated and Rainfed); PGPR strain and water regimes on nutrient content (mg kg^{-1}) of safflower seeds*

Factors	Calcium		Magnesium		Iron		Zinc		Copper	
	I	R	I	R	I	R	I	R	I	R
HA (kg ha^{-1})										
0	2074.44	2817.22	1272.78 ^{f}	1381.41^{d}	54.25 ^{d}	45.78 ^{e}	37.14	42.29	17.06 ^{a}	15.40 ^{c}
200	2520.21	3296.56	1317.56^{e}	1750.60^{a}	59.27 ^{b}	54.36 ^{d}	40.07	44.95	16.11 ^{b}	14.92 ^{c}
400	2738.88	3481.12	1429.25^{c}	1700.29^{b}	60.68 ^{a}	56.28 ^{c}	38.25	42.32	16.30 ^{b}	13.82 ^{d}
PGPR STRAIN										
Uninoculated	2133.98 ^{e}	1790.70 ^{f}	1100.67 ^{f}	1301.66^{e}	51.65 ^{d}	44.10 ^{f}	33.71 ^{e}	40.16 ^{d}	17.54	15.63
OSU142	2811.25 ^{a}	2740.54 ^{b}	1549.11^{c}	1843.75^{a}	66.74 ^{a}	61.59 ^{b}	39.87 ^{d}	42.87 ^{b}	16.20	14.68
M3	2554.81 ^{c}	2331.11 ^{d}	1369.80^{d}	1686.88^{b}	55.82 ^{c}	50.74 ^{e}	41.89 ^{c}	46.55 ^{a}	15.74	13.83

Means having different letters are significantly different at $P \leq 0.05$ according to DMRT

Likewise, the W x P interaction (*Table 8*) revealed that OSU142 strains enhanced Ca content of seeds under both irrigated and rainfed conditions, with highest value (2811.25 mg kg^{-1}) noted for the irrigation treatment, whereas the highest Mg content of seeds (1843.75 mg kg^{-1}) noted for OSU142 inoculation under rainfed conditions. Furthermore, the HA x P interaction (*Table 9*) showed that the highest K, Ca and Mg contents were observed for 400 HA + OSU142 treatment, whereas the highest P content noted for 400 HA + M3 treatment.

Moreover, the W x HA x P interactions revealed that the highest phosphorus content was recorded for 400 HA + M3 under irrigated conditions, with increase of 56.3%, whereas the highest Mg content was recorded for 400 HA + OSU142 under rainfed conditions, with increase of 96.6%, compared to their respective control (*Fig. 6a* and *b*).

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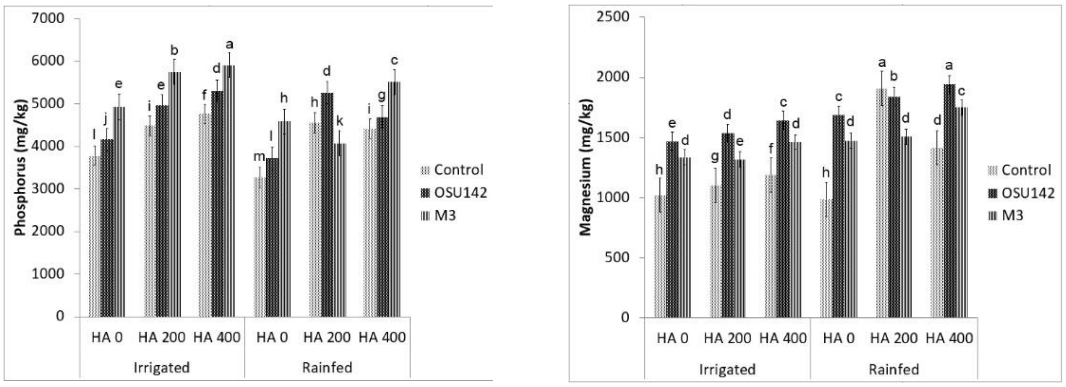
Table 9. *Interaction between humic acid (HA) and PGPR strain on nutrient content (mg kg^{-1}) of safflower seeds*

HA (kg ha^{-1})	PGPR strain	P	K	Mg	Ca	Fe	Zn	Mn
0	Uninoculated	3523.43 h	3207.32 h	1001.32 f	1627.45 g	38.48 h	33.97 f	10.09 d
	OSU142	3938.49 g	4108.40 e	1575.24 c	2629.59 c	61.26 c	41.12 c	13.02 b
	M3	4753.68 d	4009.47 f	1402.71 d	2198.62 e	50.31 g	44.08 b	11.93 c
200	Uninoculated	4274.58 f	3912.74 g	1303.02 e	2082.94 f	51.46 f	39.03 d	12.07 c
	OSU142	4755.06 d	4866.69 c	1720.93 b	2735.98 b	65.10 b	42.00 c	13.70 b
	M3	5499.22 b	4615.10 d	1578.16 c	2475.66 d	53.90 e	46.51 a	12.88 b
400	Uninoculated	4580.91 e	4076.21 e	1299.14 e	2176.64 e	53.68 e	37.82 e	12.35 c
	OSU142	4996.18 c	5089.85 a	1791.13 a	2962.11 a	66.14 a	40.97 c	14.50 a
	M3	5705.65 a	4927.46 b	1604.04 c	2654.58 c	55.61 d	47.07 c	13.37 b

Means having different letters are significantly different at P ≤ 0.05 according to DMRT



(a) (b)

Figure 6. *Phosphorus content (mg kg^{-1}) (a) and magnesium content (mg kg^{-1}) (b) in seed at humic acid treatments and rhizobacterial inoculation and their interaction under the irrigated and rainfed conditions. Different letters along with water regimes, humic acids and PGPR treatments indicate significant differences in access treatment means from three replications tested at P ≤ 0.05 according to DMRT*

For micronutrient contents (iron, zinc, copper and manganese), the individual effects of all treatments were significant (P < 0.0 *Table 7*). Iron and manganese contents increased with increasing doses of HA (400 HA > 200 HA), whereas the highest zinc and copper contents noted for 200 kg HA ha^{-1} and non-HA control, respectively. The OSU142 and M3 inoculations significantly (P < 0.01) increased contents of iron, zinc and manganese, whereas it marked decline in copper content with the highest value noted for the uninoculated control. For these parameters, significant (P < 0.01) interactions were observed in W x HA for iron and copper contents, W x P for iron and zinc contents, HA x P for iron, zinc and manganese contents, W x HA x P for iron, zinc and manganese contents (*Table 7*). The W x HA interaction (*Table 8*) showed that the highest iron (60.68 mg kg^{-1}) and the lowest copper (13.82 mg kg^{-1}) contents were recorded for 400 kg HA ha^{-1} under irrigated condition, whereas the highest copper content (17.06 mg kg^{-1}) was recorded for the non-HA control under irrigated condition.

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Likewise, the W x P interaction (*Table 8*) revealed that OSU142 strains improved iron content of seeds under both irrigated and rainfed conditions, with highest value (66.74 mg kg^{-1}) noted for the irrigation treatment, whereas the highest zinc content of seeds (46.55 mg kg^{-1}) noted for M3 inoculation under rainfed conditions. Furthermore, the HA x P interaction (*Table 9*) indicated that the highest iron and manganese contents were observed for 400 HA + OSU142 treatment, whereas the highest zinc content noted for 200 HA + M3 treatment. Similarly, the W x HA x P interactions (*Fig. 7a, b* and *c*) revealed that the highest zinc content was recorded for 200 HA + OSU142 under rainfed condition, whereas the highest iron and manganese contents were recorded for 400 HA + OSU142 under irrigated conditions, with increases of 51.3 and 41.9%, respectively, compared to their respective controls.

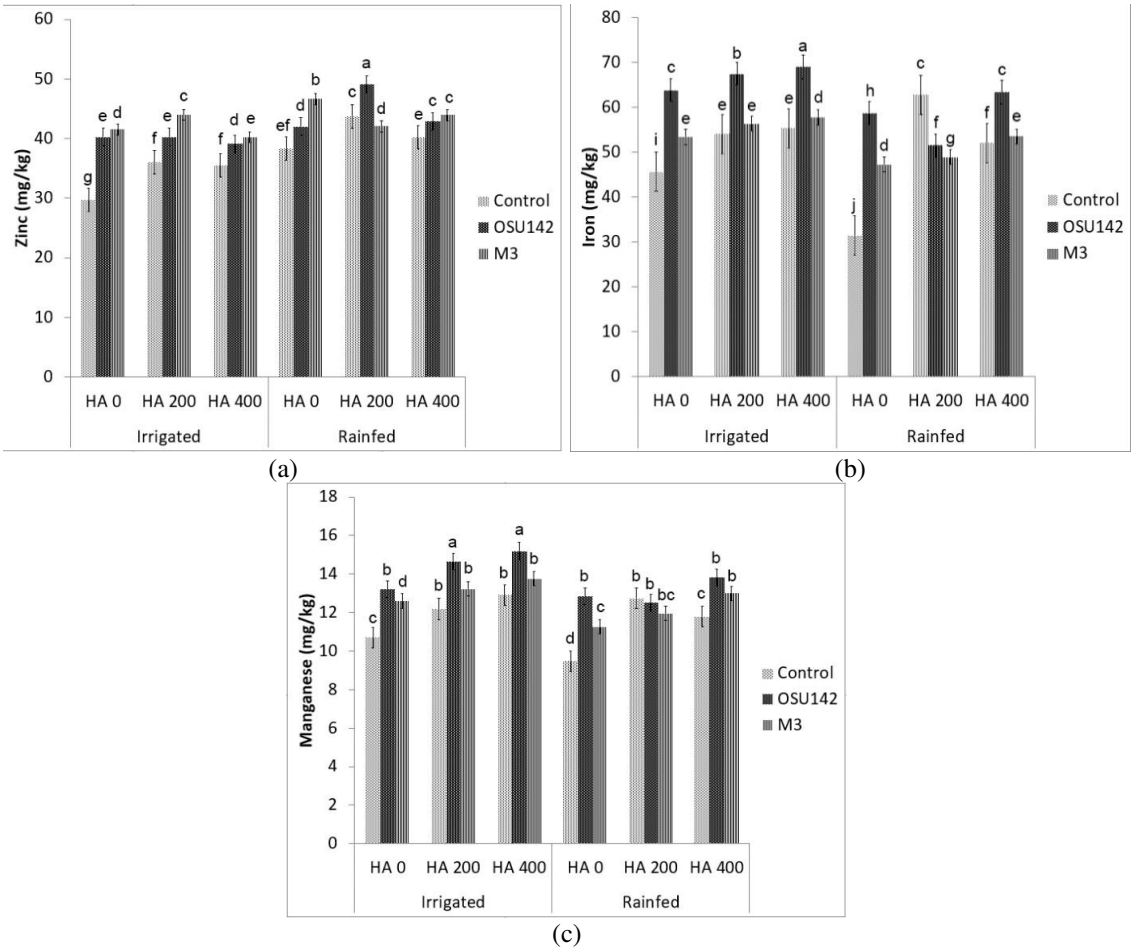

Figure 7. *Zinc content (mg kg^{-1}) (a), iron content (mg kg^{-1}) (b), manganese content (mg kg^{-1}) (c) in seed at humic acid treatments and rhizobacterial inoculation and their interaction under the irrigated and rainfed conditions. Different letters along with water regimes, humic acids and PGPR treatments indicate significant differences in access treatment means from three replications tested at $P \leq 0.05$ according to DMRT*

Discussion

In this two-year field study, humic acid (200 and 400 kg ha^{-1}) and two rhizobacterial strains (*Bacillus megatorium* M3 and *Bacillus subtilis* OSU-142) were evaluated for

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their effect on the growth, yield and quality of safflower under irrigated and rainfed conditions. The results indicated that humic acid and rhizobacterial inoculation under irrigated conditions significantly increased the growth and yield of safflower. However, the combined application of rhizobacterial strains and humic acid alleviated the negative effects of water deficit on growth and yield of safflower under rainfed conditions, thereby presented an improved strategy for the remediation of water stress in the safflower production in arid and semi-arid regions.

Nowadays, worldwide studies are intensively maintained to isolate and characterize the PGPR strains for improving crop production in sustainable agriculture and several stress conditions. Many PGPR species, including *Azotobacter, Azosprillum, Bacillus, Serratia, Enterobacter, Pseudomonas, Klebsiella* and *Variovorax* etc., improve plant growth by releasing of plant growth stimulating compounds (e.g. phytohormones such as IAA, cytokines or auxins) or improving in nutrient uptakes (e.g. N_2 fixing, P-solubilizing or siderophore production) or enhancing in plant tolerance to both biotic and abiotic stresses (Glick, 1995; Nelson, 2004; Kristin and Miranda, 2013; Backer et al., 2018). In the present study, OSU-142 (*Bacillus subtilis)* and M3 (*Bacillus megatorium*) strains, having properties of which were mentioned above, were tested for agronomic performance of safflower in the field conditions. Between the tested two bacteria, the N_2-fixing OSU-142 strain was determined the most effective in growth promotion (esp. plant height, stem and capitilum diameters, and number of capitula), and yields of seed, oil and protein in safflower. It was observed no difference between bacteria in terms of seed characteristics (e.g. number of seeds, 1000 seed weight, and fertile and sterile seed ratios) of safflower, whereas P-solubilizing M3 strain was showed the greatest performance in oil content improvement of seeds. Both of these strains have IAA and ACC deaminase production (Cakmakci et al., 200 2006), and these properties might be the cause of the noted plant growth promotion (Arshad et al., 2008; Glick, 2014; Vejan et al., 2016; Chandra et al., 2019). This potential relationship is supported by Belimov et al. (2009) and Chandra et al. (2019) who observed that inoculations of pea and wheat with *Variovorax* sp., possessing IAA, ACC deaminase, and N_2-fixing and P-solubilizing activities, significantly increased the shoot biomass of pea and yield characteristics of wheat. Similar effects of PGPR inoculation on plant growth promotion have been observed in the several studies in sugar beet and barley (Cakmakci et al., 200 2006), sugarcane (Silva et al., 2017), sunflower (Shadid et al., 2012), wheat (Rosas et al., 2009; Hungria et al., 2010; Rana et al., 2012; Chandra et al., 2019), rice (Lucas et al., 2009), bean (Hoyos-Carvajal et al., 2009), canola (El-Howeity and Asfour, 2012), maize (Thonar et al., 2017) and soy (Cassán et al., 2009). Moreover, the superior performance of OSU14 compared to that of M could be due to its N_2-fixing ability converting atmospheric nitrogen (N_2) into ammonia (NH_3) or related nitrogenous compounds in soil, which helps the plant in nitrogen acquisition (Cakmakci et al., 200 2006). Additionally, biological nitrogen fixation is considered to be an essential to plants because fixed inorganic nitrogen compounds are required for the biosynthesis of all nitrogen-containing organic compounds, such as amino acids and proteins, nucleoside triphosphates and nucleic acids. Hence, the greater nitrogen uptake by plants directly affects plant growth. In the study, the effect of M3 strain showing the higher performance in increasing oil contents of safflower seed could be due to its phosphate solubilizing ability, which one of the most important traits associated with plant phosphate nutrition. The previous studies also indicated that enhanced phosphate acquisition by phosphate solubilizing microorganisms positively affect seed oil

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concentration of oilseed crops (Mirzakhani et al., 2009; Shahid et al., 2012; Sharifi et al., 2017; Nosheen et al., 2018; Chandra et al., 2019).

In the current study, the humic acid and bacterial inoculation considerably increased the morphologic, seed and yield characteristics of safflower in the field conditions. The results revealed that morphologic characteristics, such as plant height, stem diameter, numbers of branches and capitula, and capitulum diameter, and yields of seed, oil and protein were significantly affected by both humic acid and bacterial inoculation, thus their highest values obtained from 400 kg HA ha^{-1} and OSU142 strain, respectively. Similarly, the seed characteristics and seed protein concentration of safflower treated by humic acid and bacterial inoculation significantly increased compared to control, but showed no difference between HA doses or PGPR strains. Overall, the enhanced safflower growth due to humic acids and PGPR might be attributed to their beneficial effects on the plant growth, which affects because of promotion of root morphology, nutrient uptake, and soil biological activity etc. (Schmidt et al., 2007; Puglisi et al., 2013; Canellas and Olivares, 2014). The plants treated with 400 kg HA ha^{-1} exhibited considerably increased morphologic characteristics of safflower, which probably directly affected the root growth, especially lateral root emergence (Nardi et al., 2009), and indirectly improved photosynthetic rate, leading to increased growth and yield of safflower (Puglisi et al., 2013; Canellas and Olivares, 2014). This proposed explanation is supported by those of Canellas et al. (2013), who noted significantly enhanced the rate of net photosynthesis in maize in response to the increase of the humate concentration under greenhouse condition. Similarly, different endophytic rhizobacteria species have been also reported that increased the photosynthetic rate, stomatal conductance, transpiration velocity, water utilization efficiency in rice, which might support plant growth improvement (Chi et al., 2005). More recently, Schoebitz et al. (2016), Esringu et al. (2016) and Olivares et al. (2015) have reported significant growth and yield improvements in blueberry, Hungarian vetch and tomato, respectively, due to humates and PGPR inoculation under field conditions.

Although the studies recently reviewed basic mechanisms and synergistic effects produced by integrated use of humic substances and PGPR on nutrient uptake, growth and yield of various crops under different stress conditions (Baldatto et al., 2010; Glick, 2014; Esringu et al., 2016; Schoebitz et al., 2016; Silva et al., 2017; Olivares et al., 2017), their effects has not been previously reported in safflower under different water regimes. In the study, the enhanced growth and yield of safflower might be due to similar synergistic effects of humic acids and PGPR under irrigated and rainfed conditions. Similar to the present results, humic substances widely reported to having potential as enhancer of PGPR efficiency (Esringu et al., 2016; Schoebitz et al., 2016; Silva et al., 2017). Cakmakci et al. (2006) observed a positive correlation among soil organic matter content, PGPR strains and growing stage, and reported that the effect of PGPR was greater in the high soil organic matter content, and at early plant growth stages than at the later. In the previous studies, it has also been notified that the combined use of PGPR and humic substances increased productions of maize grains by 65%, tomato fruits by 87.1% and potato tubers by 140% under field conditions (Canellas et al., 2013; Olivares et al., 2015; Ekin, 2019).

Safflower is an extensively branching oilseed plant, and dry matter accumulation in connection with seed yield depends not only on plant height but also on numbers of branch and capitula, and some morphological characteristics sensitive to water deficit stress (Dajue and Mündel, 1996). In the current study, the plant height, number of

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capitula per plant and number of seeds per capitulum were the highest for safflower plants treated with integrated use of humic acids and PGPR under irrigated conditions. However, their effects noted for relatively higher for safflower yield characteristics than for irrigated plants under rainfed conditions. Under rainfed conditions, the combined use of humic acid and PGPR helped the safflower plants acclimatize to water-deficit (rainfed) conditions by improving a variety of morphologic and yield characteristics, including plant height, stem diameter, number of capitula, capitilum diameter, number of seeds, 1000 seed weight and fertile seed percentage, which cause for enhancing seed and oil yields. The improved safflower growth and yield by bacterial inoculation and humic acids under rainfed conditions might be correlated with the enhancement in plant tolerance to drought by increasing their water content, which can be attributed to the enhancement of root growth by humic substances and PGPR inoculation. In addition, it has also been reported that PGPR could enhance the plant growth under stress conditions by biosynthesis of phytohormones (IAA, GA, and cytokines), producing the enzyme ACC deaminase, fixing asymbiotic nitrogen, and solubilizing of phosphates and other nutrient elements (Glick, 2014). This proposed explanation is consistent with the results of Sahin et al. (2015), who stated N_2-fixing, P-solubilization, and producing of IAA, GA and SA of PGPR has been basic factors improving nutrient uptake and growth of lettuce. They also reported that PGPR inoculation significantly increased growth and yield of lettuce plants under lower and well-watered conditions, and alleviated the harmful effects of lower irrigation conditions. Similar results were noted by Rubin et al. (2017) and Mutumba et al. (2018), who reported that plants were highly responsive to bacterial inoculation under well-watered conditions, but the effect was relatively higher under drought conditions. Other previous studies have also reported improving effects of PGPR inoculation on the yield and growth of different crops grown under drought stress (Vivas et al., 2003; Arkhipova et al., 2007; Marulanda et al., 2009; Sandhya et al., 2010).

In the current study, the interactive effect of humic acids and PGPR inoculation on nutrient contents of safflower seeds was studied in the natural field conditions. In general, the nutrient uptake of safflower plants except for Mg and Zn contents was lower in rainfed conditions, compared to the irrigated conditions. However, humic acids and PGPR inoculation considerable improved macro and micro nutrient contents of seeds except copper under both irrigated and rainfed conditions. In addition, K, Ca, Mg, Fe and Mn concentrations of the seeds noted for the highest for plants treated with OSU142 strain, whereas P and Zn concentrations in the M3 strain. In the study, the specialty of M3 strain highly responded to enhancing especially phosphorus and zinc uptake could be attributed to its P-solubilizing ability and affecting root growth and lateral root formation by producing plant hormones such as IAA in the rhizosphere (Cakmakci et al., 2001; Chandra et al., 2019). Likewise, N_2- fixing ability and phytohormone production of OSU142 strain also might have been one of the main factors improving growth and mineral nutrient uptake of safflower. Moreover, the combined use of humic acids and PGPR also alleviated the harmful effects of water deficit conditions on the mineral nutrient uptake. Under rainfed conditions, the reduction in nutrient uptake may be due to a notable decrease in root cation exchange capacity of the plants, which can accumulate some ions, organic and amino acids to avoid harmful effects of abiotic stress conditions in dry environments (Sahin, 2015). Similarly, the reduction in copper content may be attributed to strongly retain in the soil after the application of humic acids (Rong et al., 2020). Nowadays, the applications of

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humic acid and PGPR have been widely used to improve soil functions, to mobilize or immobilize cations and metal ions in soil, leading to increased nutrient uptake from soils (Rubin et al., 2017; Rong et al., 2020). In the study, the improvement in the nutrient concentrations of safflower seeds may be due to more effective mobilizing mineral nutrients from the soil because of enhanced excretion of organic acids through rhizosphere bacteria (Glick, 1995; Biswas et al., 2000; Chandra et al., 2019). Another possible reason for the improved nutrients from the PGPR is the roots enhancement by producing phytohormones, resulting in a larger root surface, and therefore, has positive effects on plant acquisition of nutrients and water (Vardharajula et al., 2011; Wang et al., 2012). In addition, an increase in zinc uptake because of root-induced changes (i.e. enhanced root growth, surface area and activity) in the rhizosphere after the application of PGPR has also been reported in lettuce (Sahin et al., 2015). More recently, many studies have reported that the integrated use of humic acids and bacterial inoculation would be provide the higher nutrient uptake and plant performance in the various stress conditions, and ultimately result in vigorous, well-established and healthy plants (Esringu et al., 2016; Silva et al., 2017; Olivares et al., 2017). Similarly, Baldatto et al. (2010) and Schoebitz et al. (2016) also reported that inoculation of PGPR in combination with humic substances increased root and shoot biomass of pineapple and blueberry plants, and nutrient contents increased by 80% K, 131% P and 132% N in pineapple, and 55% N and 56% K in blueberry, compared to uninoculated control.

Conclusions

In conclusion, the current study comprehensively evaluated the effect of humic acids (200 and 400 kg ha^{-1}) and two promising *Bacillus* spp. strain on the growth, yield and quality of safflower under rainfed and irrigated field conditions. The study revealed that 400 kg humic acid ha^{-1} with the *Bacillus subtilis* OSU142 strain was the most effective combination for growth, seed and oil yields, and seed quality of safflower under irrigated conditions. Moreover, the integrated use of humic acids and PGPR alleviated the unfavorable effects of water deficit under rainfed conditions, and presented an improving strategy for the remediation of water stress in the safflower production in arid and semi-arid regions. The present results suggest that the combined use of humic acids and PGPR can be used as a biotechnological intervention for the alleviation of water deficit stress in safflower production in the arid and semiarid regions.

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EFFECT OF DIFFERENT CHEMICAL RIPENERS ON SUGARCANE (*SACCHARUM OFFICINARUM* L.) QUALITY, SUGAR YIELD AND RATOONING ABILITIES

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Abstract. Field experiments were conducted to evaluate the impact of various chemical ripeners (Ethephon, Glyphosate, Sulfometuron-methyl) on sugarcane cultivars (HSF-242, NSG-311, HSF-240, NSG-555). Each chemical ripener (200 ppm) was applied as a foliar spray 15, 30 and 45 days before cane harvesting for planted crops and ratoons were collected. Chemical ripeners significantly enhanced the quality and sugar yielding traits including harvest index, juice extraction and purity, sugar recovery % cane and total sugar yield for planted crop especially when treatment was applied 45 days before cane harvesting. Changes in antioxidant activities indicated the influence of various chemical ripeners on the planted crop. Chemical ripeners were imperative to create short-term changes for early cane maturity and sugar yielding abilities. Performance of ratooning crop was normal concerning growth, yield and antioxidant activities according to its existing genetic makeup showing non-significant effect of chemical ripeners. It was concluded that chemical ripeners can be suitable to boost up sugar yielding characteristics by inducing early cane maturity for a short duration before cane harvesting and it will have no effect on lateral life cycle span and its ratooning abilities.
Keywords: *glyphosate, ethephon, sulfometuron-methyl, sugar production*

Introduction

Sugarcane (*Saccharum officinarum* L.) is considered as an industrial crop for the production of sugar (Neliana et al., 2019). Sugarcane is one of the world's major food-producing crops, providing about 75% of sugar in the world for human consumption (De Souza, 2008). Sugarcane is rich in sucrose which is accumulated in stalk internodes and is used to manufacture many industrial goods such as furfural, alcohol, dextrans etc. and some other natural pharmaceutical products (Ma et al., 2005).

Chemical ripeners (Ethephon, ethyl-trinexapac, glyphosate and sulfometurom methyl) are classified as growth retardants and growth inhibitors as described by Leite et al. (2011). Chemical ripening of sugarcane is an important component to profitable sugar production throughout the world. Harvesting of sugarcane often begins before the sugarcane reaches the desirable maturity level (Dalley and Richard-Junior, 2010). The main advantage of chemical ripeners is that they can suppress stalk and leaf growth much more rapidly and consistently than natural processes such as reduced temperatures or limiting soil moisture (Van Heerden et al., 2015). Ethephon, an ethylene releasing compound, was the first growth regulator in the early 1960s used for crop management and post-harvest quality in a wide range of agricultural, horticultural and forestry. The application of Ethephon in sugarcane has accelerated ripening, increased the overall sugar yield, and inhibited flowering (De Almeida and Caputo, 2012).

Glyphosate, an amino acid synthesis inhibitor, applied at sub-lethal doses has been widely used to increase sucrose levels in sugarcane (Solomon and Li, 2004). Glyphosate

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(N-phosphonomethyl glycine, $C_3H_8NO_5P$) is the analogue of glycine. It is a highly used herbicide throughout the world because it is an efficient killer of weeds, less toxic and available at low cost (Goscinny and Hanot, 2012). Leite et al. (2009) noted that ripener application for early harvest sugarcane led to an increase in technological quality, although sugar yield had been significantly affected, which positively contributed to the profit per unit area. El-Hamd et al. (2013) found that glyphosate application increased total soluble solids in cane juice but other quality parameters viz. sucrose content also increased proportionately. The introduction of sulfometuron-methyl, which overcomes some of the disadvantages of other ripeners, is therefore timely. Sulfometuron-methyl is a grass herbicide that showed promise as a chemical at low rates of application (Almendares et al., 2013). Many studies reported that sulfometuron-methyl regarding its potential ripening effect in sugarcane varieties, causes no damage to sugarcane production (t ha^{-1}) or the agronomic characteristics of the culture (Silva et al., 2007; Leite et al., 2010).

There is a lack of information about the use of chemical ripeners in Pakistan for sugarcane. There are different studies in few countries about the effective use of chemical ripeners for early cane ripening with higher sugar yield but there is no information and use of chemical ripeners in Pakistan. Secondly, residual effects of chemical ripeners has not been evaluated on sugarcane ratooning abilities. This study was conducted first time in Pakistan to find the efficacy of chemical ripeners on sugar yielding attribute on planted crop as well as its impact on the ratooning abilities.

Materials and methods

Experiments were carried out at Shakarganj Sugar Research Institute (SSRI) Jhang and University of Gujrat, Pakistan during 2018-19. Sowing of four sugarcane cultivars i.e. HSF-242, NSG-311, HSF-240 and NSG-555 was done in two sowing seasons i.e. spring and autumn during 2018 and 2019. Experimental design was RCBD (Plot size 30 × 30 feet beds) with four replicates. A seed rate of 75000 double-bedded setts per hectares was used with 2.5 feet row spacing. Seed was treated with hot water at 52 °C for 30 min and fungicide for better germination and to control sugarcane diseases. Soil insecticide was also applied in the soil to control termites. Double-cut setts were placed end to end in furrows at a depth of about 10 cm and covered with 5 cm soil. Immediate irrigation was applied after planting. Each chemical ripener was sprayed at cane formation and elongation phase. These concentrations have not been studied for sugarcane crop in previous research and with the treatment intervals of 15, 30 and 45 before cane harvesting. Researcher used less or more than 200 ppm concentrations of different chemical ripeners in sugarcane and used single dose application that is why this interval of treatments was applied.

The following treatments were applied on planted crop:

T0 = Control
T1 = 200 ppm Ethephon spray (15 days before harvesting)
T2 = 200 ppm Ethephon spray (30 days before harvesting)
T3 = 200 ppmEthephon spray (45 days before harvesting)
T4 = 200 ppm Glyphosate spray (15 days before harvesting)
T5 = 200 ppm Glyphosate spray (30 days before harvesting)
T6 = 200 ppm Glyphosate spray (45 days before harvesting)
T7 = 200 ppm Sulfometuron-methyl spray (15 days before harvesting)

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T8 = 200 ppm Sulfometuron-methyl spray (30 days before harvesting)
T9 = 200 ppm Sulfometuron-methyl spray (45 days before harvesting)

The following quality, sugar yielding and antioxidant activities were determined for planted crop in January-February, 2019:

1. Harvest Index (%)
2. Juice Extraction (%)
3. Juice purity (%)
4. Sugar recovery % cane
5. Sugar yield (t ha^{-1})
6. Antioxidant activities (CAT, POD and SOD)

After the cane harvesting, ratoon was kept from the planted crop at which chemical ripeners were sprayed and the following parameters were studied in January-February, 2020:

1. Ratoon Sprouting (%)
2. Number of tillers per plant
3. Mill-able canes (t ha^{-1})
4. Juice Extraction (%)
5. Juice purity (%)
6. Sugar recovery % cane
7. Sugar yield (t ha^{-1})
8. Total cane yield (t ha^{-1})
9. Antioxidant activities (CAT, POD and SOD)

Harvest index (HI) was calculated for planted crop using this formula:

$$HI\ (\%) = \text{Stripped cane yield/Unstripped cane yield} \times 100$$

Juice extraction % was calculated both for planted and ratooning crop by this formula:

$$\text{Juice extraction } (\%) = \text{Juice weight (g) / Filter cake weight (g)} \times 100$$

Sugar recovery % of cane for planted and ratooning crop was calculated by using the formula as follows:

$$\text{Sugar recovery percentage} = [S - 0.4\ (B - S)] \times 0.73$$

where: B = Brix percentage, S = sucrose percentage, 0.4 and 0.73 constant factors.

Total sugar yield (t ha^{-1}) was calculated for each treatment by the following method:

$$\text{Total sugar (t ha}^{-1}\text{)} = \text{Sugar recovery} \times \text{Stripped-cane yield}/100$$

Estimation of CAT, POD and SOD activities were determined by the procedure of Chance and Maehly (1955).

Data were analyzed statistically using analysis of variance (ANOVA) technique using Ministate-C software and significant mean separation was done at $P \leq 0.05$ using Tukey's test.

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Results

The following results were obtained by the applications of chemical ripeners in planted and ratooning crop.

Harvest index (HI) % for planted crop

Effect of chemical ripeners was highly significant in sugarcane for harvest index (*Table 1*). There were significant variations among sugarcane cultivars for HI and its interaction between ripeners and cultivars. Higher HI value was noted in cultivar HSF-240 with the treatment of Ethephon and Sulfometuron-methyl that was applied 45 days before cane harvesting. Cultivar NSG-555 showed the lowest HI values for all the treatments of ripeners (*Fig. 1A*). Overall, all the treatments that were applied 45 days before harvesting had the highest values for HI as compared to other treatments (*Table 2*). HI increased because chemical ripeners helped to produce maximum number of stripped cane.

Juice purity (%) in planted crop

It was noted from the results that the effect of chemical ripeners was highly significant for juice purity % of sugarcane. Variations among cultivars were significant while the interaction between ripeners x cultivar was highly significant (*Table 1*). Higher juice purity % was calculated in NSG-555 and the lowest juice purity was present in HSF-242 (*Table 2*). Applications of 200 ppm of Glyphosate and Ethephon that were applied 45 days before harvesting showed better results for juice purity (%). All the chemical ripeners applied 45 days before harvesting increased juice purity % (*Fig. 1C*).

Sugar recovery % of cane in planted crop

Sugar recovery % of cane significantly increased with the applications of chemical ripeners. There were highly significant results for sugar recovery % of cane among cultivars as well as in interactions of ripeners x cultivar (*Table 1*). The changes in sugar recovery % of cane was due to the changes created by chemical ripeners in juice extraction and purity %. Higher sugar recovery % of cane was obtained in NSG-555 by the applications of 200 ppm Glyphosate that were applied 45 days before cane harvesting. HSF-242 produced the lowest sugar recovery % of cane (*Fig. 2A*). All the chemical ripeners increased the sugar recovery % of cane but the treatments applied before 45 days were the best sugar recovery % of cane (*Table 2*).

Juice extraction (%) from planted crop

Data presented for juice extraction showed that chemical ripeners had highly significant juice extraction % (*Table 1*). Interaction of ripeners x cultivar were also highly significant. Cultivars had also highly significant response to chemical ripeners. Juice extraction % was increased by the treatments of chemical ripeners (*Fig. 1B*). Higher juice was extracted from HSF-240 with the applications of 200 ppm Ethephon that was applied 45 days before harvesting. Overall, NSG-555 cultivar had the highest juice extraction while the lowest juice quantity was extracted from HSF-242. From the results it was apparent that chemical ripeners produced early maturity and increased the production of juice in sugarcane (*Table 2*). All the chemicals applied 45 days before

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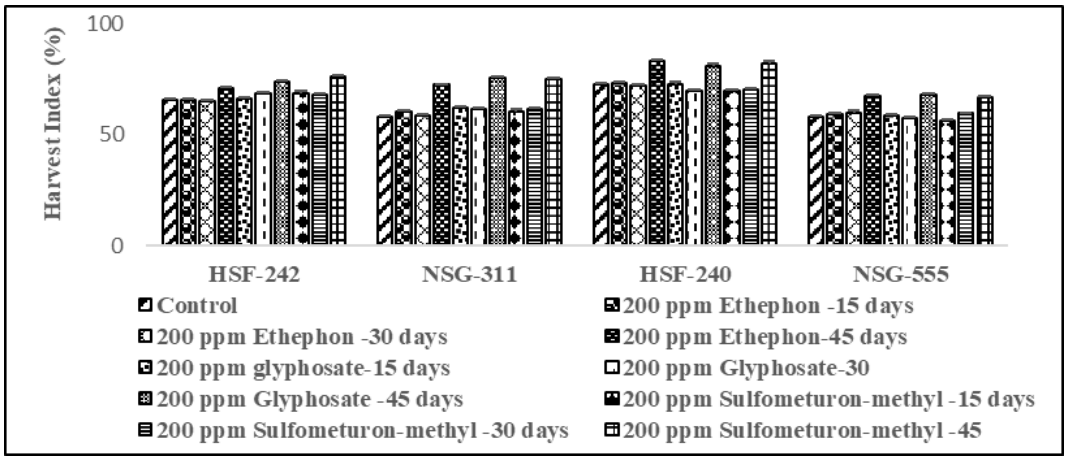
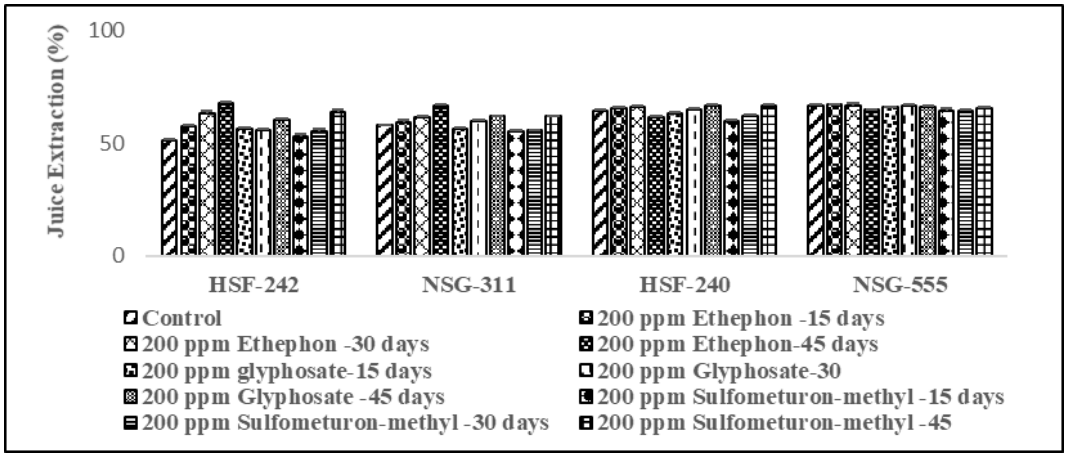
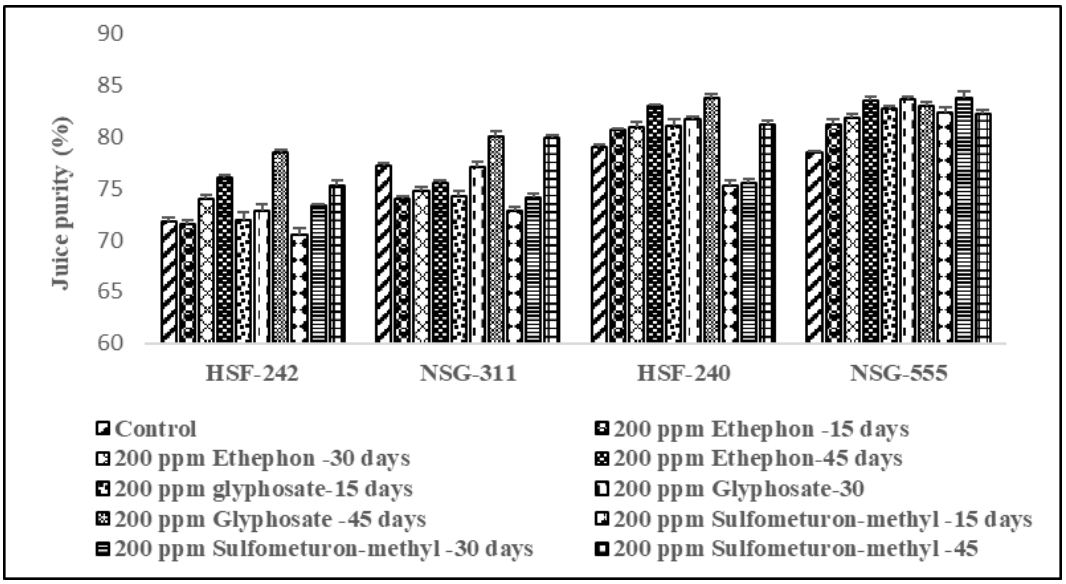
harvesting were the best in juice extraction as compared to the treatments sprayed 15 and 30 days before cane harvesting.



A: Harvest index



B: Juice extraction



C: Juice purity

Figure 1. *Effect of different chemical ripeners on quality attributes of sugarcane cultivars in planted crop*

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Table 1. *Mean squares (MS) from the analysis of variance (ANOVA) for quality and antioxidant activities of planted sugarcane cultivars under the applications of chemical ripeners*

Source	df	Harvest index (%)	Juice extraction (%)	Juice purity (%)	Sugar recovery % cane	Total sugar yield (t ha^{-1})	Peroxidase (POD) activities	Catalases (CAT) activities	Superoxide dismutase (SOD) activities
Main effects Ripeners (Rip)	9	62.579***	97.439***	52.524***	0.522***	479.487***	0.005**	0.076ns	0.029*
Treatment time (T)	2	32.765*	101.304**	78.364***	3.789***	186.398***	0.765*	0.345**	0.0523*
Cultivars (Cv)	3	1379.752***	485.440***	626.596**	6.256***	8236.089***	0.076**	0.150**	1.150**
Interactions Rip x T	18	2050.401*	9870.96**	4115.991***	1.977858**	89375.42***	0.0038*	0.0262ns	0.0015*
Rip x Cv	27	86343.5**	47300.79***	32911.33***	3.265***	3949098***	0.00038**	0.0114*	0.0333*
T x Cv	6	45207.57*	49177.01**	49102.57***	23.703**	1535191***	0.0581*	0.0517*	0.0601*
Rip x T x Cv	54	2829045*	4791759**	2579063**	12.373***	7386722**	0.00029*	0.0039*	0.0017*
Error	40	4237.943	2346.421	452.614	74.043	103.011	1.973	0.967	0.456
Total	159								

ns = non-significant and *, **, *** = significant at P < 0.05, 0.01, 0.001 probability levels

Sugar yield (t ha^{-1}) of planted crop

Sugar yield was highly significantly affected by the applications of chemical ripeners. Sugar yield increased in all the cultivars by the treatments of chemical ripeners. Effect of chemical ripeners for cultivars and their interactions (ripeners x cultivar) also yielded highly significant results (*Table 1*). Higher sugar yield was obtained from NSG-555 and the lowest sugar yield was calculated in HSF-242. Higher sugar yield was noted by the applications of 200 ppm Sulfometuron-methyl and Glyphosate that was applied 45 days before cane harvesting. All the chemical ripeners applied 45 days before cane harvesting were the best for the production of sugar as compared to other treatments (*Fig. 2B*). As the chemical ripeners affected the juice extraction, juice purity and sugar recovery % of cane that resulted higher production of sugar (*Table 2*).

Antioxidant activities in planted crop

Antioxidant activities i.e. peroxidase (POD), catalases (CAT) and superoxide dismutase (SOD) were determined by evaluating the effects of Ethephon, Glyphosate and Sulfometuron-methyl. Effect of chemical ripeners was significant on POD activities in sugarcane (*Table 1*). Higher POD activities were noted in cultivar HSF-242 and the lowest was noted in HSF-240 (*Fig. 3A*). Maximum changes in POD was noted by the applications of Ethephon that were applied 30 and 45 days before cane harvesting. Cultivar HSF-240 had higher POD activities for Sulfometuron-methyl applications. There was a non-significant effect of chemical ripeners for CAT activities, however there were significant variations among cultivars (*Table 1*). Higher values of CAT were noted in HSF-240 and the lowest in cultivar HSF-242 (*Fig. 3B*). SOD activities were significantly changed by Chemical ripeners (*Table 2*). Maximum variations were noted in HSF-240 and the lowest values were noted in HSF-242 (*Fig. 3C*). Maximum value of

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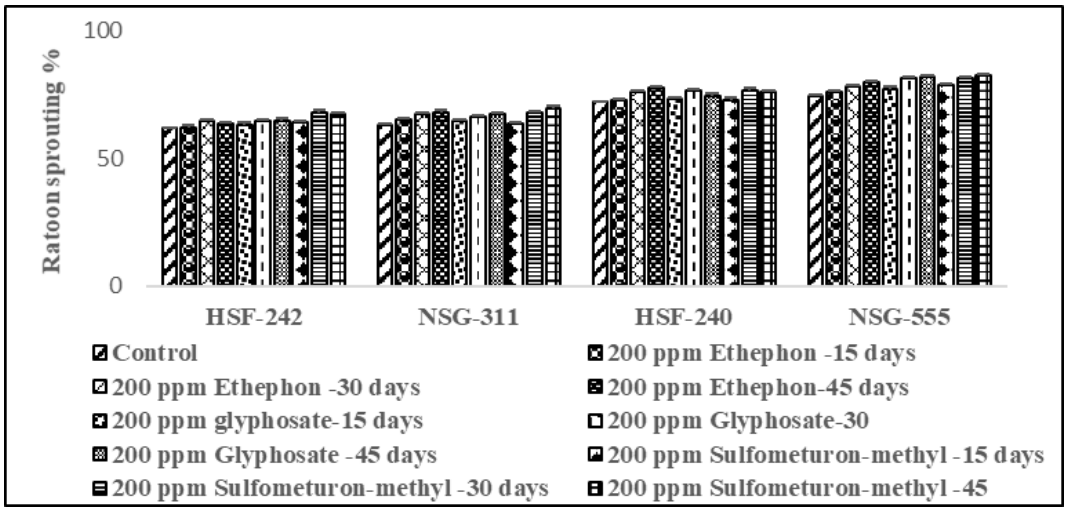
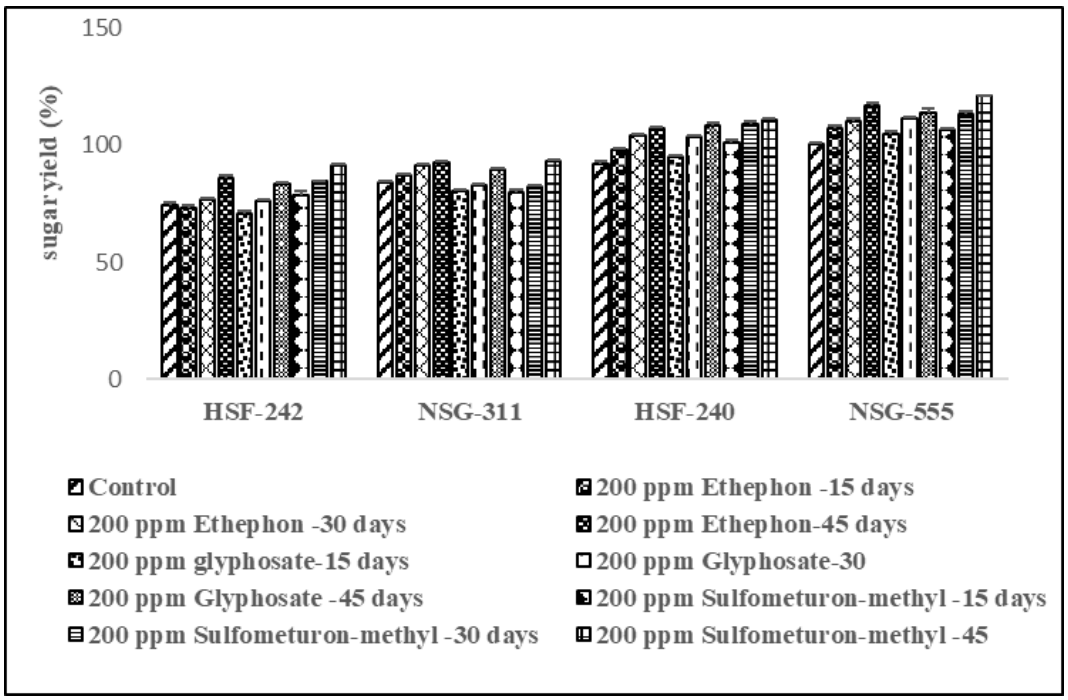
SOD was noted by the treatment of Glyphosate that was applied 45 days before cane harvesting.

Ratoon sprouting (%)

Data for ratoon sprouting of sugarcane crop is given in *Table 3*. Effect of chemical ripeners was non-significant on ratoon sprouting, however there was significant variations among cultivars. High sprouting % was noted in NSG-555 and the lowest in HSF-242 (*Fig. 4A*). Ethephon treatment applied 45 days before cane harvesting were the best as compared to other treatments.



A: Sugar recovery % of cane



B: Sugar yield

Figure 2. *Effect of different chemical ripeners on sugar yielding attributes of sugarcane cultivars in planted crop*

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Table 2. *Mean comparison of significant results using Tukey's test for different variable of sugarcane cultivars in planted crop*

Cultivar	Treatments	Harvest index %	Juice extraction %	Juice purity %	Pol %	Sugar recovery %	Sugar yield (t ha^{-1})	Peroxidase (POD)	Catalases (CAT)	Superoxide dismutase (SOD)
HSF-242	Control	65.62±1.2 BC	51.22±0.99C	71.82±2.1 BC	14.21±1.1 D	9.12±0.91 E	74.32±3.4 D	0.892±0.03C	1.027±0.04A	0.665±0.02C
	Ethephon (15 days)	65.61±2.2BC	57.35±1.2 B	71.57±2.4 BC	14.66±1.7D	9.97±0.76 CD	72.91±2.8 D	0.895±0.01C	1.037±0.03A	0.667±0.02C
	Ethephon (30 days)	65.41±1.9 BC	63.11±2.3 A	74.05±1.9 B	15.19±0.98 C	10.19±0.97 C	76.81±4.2 C	0.912±0.04B	1.025±0.04A	0.721±0.01B
	Ethephon (45 days)	71.15±1.4 A	67.85±2.2 A	76.06±2.4 A	16.51±1.2 B	11.23±1.01 A	86.05±4.4 AB	0.991±0.04B	1.042±0.06A	0.742±0.04A
	Glyphosate (15 days)	66.17±2.1 B	56.32±1.7 B	71.97±1.7 BC	16.54±1.6 A	10.21±0.92 BC	70.87±2.5 D	1.010±0.02 A	1.051±0.03A	0.665±0.04C
	Glyphosate (30 days)	68.57±3.1 B	56.01±1.8 B	72.82±2.1 B	16.68±1.7B	10.39±0.73 BC	75.98±2.1 C	1.007±0.03A	1.061±0.04A	0.712±0.03B
	Glyphosate (45 days)	73.81±2.7 A	60.47±2.0 B	78.55±2.7 A	16.93±0.72 A	10.51±0.72 B	83.63±3.4 B	1.012±0.04 A	1.034±0.01A	0.752±0.02A
	Sulfometuron-methyl (15 days)	68.65±3.1 B	53.05±1.1 C	70.55±1.8 C	16.29±1.7 BC	10.06±0.88 C	78.83±2.2 C	1.060±0.02 A	1.067±0.04A	0.677±0.05C
	Sulfometuron-methyl (30 days)	67.87±1.4 B	55.35±1.5 C	73.31±1.4 B	16.44±1.5 B	10.25±0.54 BC	84.41±2.8 B	1.037±0.03 A	1.064±0.03A	0.714±0.06B
	Sulfometuron-methyl (45 days)	76.22±2.2 A	63.82±2.3 A	75.34±2.1 A	17.02±1.9 A	10.99±0.62 AB	91.41±3.8 A	1.061±0.04 A	1.072±0.05A	0.732±0.02A
NSG-311	Control	58.27±1.0 C	57.92±1.3 B	77.25±3.2 A	14.06±1.5D	9.06±0.43 D	83.76±3.8 B	0.996±0.03B	1.102±0.04B	0.815±0.03BC
	Ethephon (15 days)	60.17±1.3 C	59.35±1.2 B	73.97±2.9 BC	15.16±0.99C	10.16±0.76 BC	86.82±3.9 AB	0.967±0.04B	1.092±0.03B	0.835±0.04B
	Ethephon (30 days)	58.75±1.6 C	61.51±2.1 B	74.77±3.5 B	16.30±1.1B	10.30±0.73 B	91.15±4.3 A	1.011±0.05A	1.107±0.06A	0.869±0.03A
	Ethephon (45 days)	72.55±2.3 A	66.93±1.3 A	75.55±2.6 B	16.40±0.94B	11.40±0.92 AB	92.29±3.9 A	1.015±0.05A	1.101±0.02A	0.887±0.03A
	Glyphosate (15 days)	62.42±1.1 B	56.32±0.92 C	74.21±1.8 B	15.24±1.2C	10.24±0.84 B	80.28±3.4 BC	0.895±0.03C	1.102±0.04B	0.841±0.05B
	Glyphosate (30 days)	61.45±1.4 B	59.97±B	77.12±2.3 A	15.36±1.3C	10.36±0.34 B	82.56±2.8 B	1.017±0.04A	1.107±0.03A	0.872±0.04A
	Glyphosate (45 days)	75.61±2.4 A	61.82±1.1 B	80.02±3.3 A	16.40±1.5B	10.40±0.56 B	89.69±3.3 A	1.027±0.03A	1.112±0.08A	0.875±0.04A
	Sulfometuron-methyl (15 days)	60.65±1.7 B	55.25±0.82 C	72.82±2.2 C	15.16±0.99C	10.16±0.74 BC	80.05±4.1 BC	0.937±0.05BC	1.115±0.03A	0.807±0.02C
	Sulfometuron-methyl (30 days)	61.41±2.6 B	55.61±0.91 C	74.13±1.8 B	16.32±1.3B	10.32±0.65 B	82.17±3.7 B	1.011±0.05A	1.117±0.06A	0.825±0.03B
	Sulfometuron-methyl (45 days)	75.33±1.1 A	61.97±1.1 B	79.92±2.4 A	17.10±1.8A	11.10±0.93 A	92.97±4.1 A	1.027±0.04A	1.121±0.05A	0.881±0.01A
HSF-240	Control	72.82±1.9 B	64.41±1.4 A	79.02±1.9 AB	14.31±0.96C	9.44±0.48 E	91.96±2.8 C	0.865±0.03D	1.112±0.05AB	0.907±0.05CD
	Ethephon (15 days)	73.37±2.2 B	65.35±2.1 A	80.71±2.8 A	16.06±1.6AB	10.98±1.12 B	97.73±5.2 B	0.876±0.04D	1.131±0.07A	0.932±0.04B
	Ethephon (30 days)	72.02±1.6 B	66.05±2.2 A	80.95±3.2 A	15.64±1.1B	10.70±0.74 B	103.88±4.8 A	0.875±0.02D	1.137±0.08A	0.907±0.06B
	Ethephon (45 days)	83.45±3.2 A	61.57±3.1 B	82.95±2.7 A	16.99±1.4A	11.67±0.86 A	107.36±5.4 A	0.935±0.02B	1.132±0.03A	1.011±0.03A
	Glyphosate (15 days)	72.65±2.8 B	63.22±2.4 B	81.05±1.7 A	14.52±0.89C	10.34±0.44 D	94.84±3.9 B	0.941±0.01B	1.151±0.05A	0.917±0.04B
	Glyphosate (30 days)	70.02±1.7 C	65.17±1.8 A	81.75±2.8 A	15.69±0.99B	10.48±0.56 C	103.45±4.2 A	0.950±0.03B	1.135±0.05A	1.015±0.04A
	Glyphosate (45 days)	81.02±2.2 A	66.72±1.1 A	83.82±3.2 A	14.81±1.3C	10.61±0.73 C	108.50±5.6 A	0.901±0.04C	1.153±0.06A	1.115±0.05A
	Sulfometuron-methyl (15 days)	69.85±1.8 C	59.61±0.91 BC	75.32±3.1 B	15.21±1.1BC	10.35±0.81 D	101.23±3.8 A	1.001±0.04A	1.135±0.05A	0.857±0.04C

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	Sulfometuron-methyl (30 days)	70.52±2.2 C	62.02±1.1 B	75.52±2.2 B	16.22±1.3A	11.10±0.93 AB	108.77±5.5 A	0.977±0.03B	1.112±0.03B	1.101±0.03A
	Sulfometuron-methyl (45 days)	82.07±3.1 A	66.37±1.0 A	81.15±3.4 A	16.56±1.6A	11.23±0.99 A	110.67±5.0 A	1.031±0.04A	1.123±0.04B	1.061±0.06A
NSG-555	Control	57.82±2.4 B	66.82±2.1 A	77.51±1.8 B	15.22±1.2C	10.01±0.43 D	100.26±4.7 C	0.796±0.02D	1.123±0.03B	0.997±0.04D
	Ethephon (15 days)	59.21±1.5 B	67.10±3.2 A	81.25±2.5 A	16.31±1.4B	11.45±0.82 B	106.97±5.4 BC	0.806±0.03D	1.141±0.06A	1.072±0.03C
	Ethephon (30 days)	59.87±1.1 B	66.87±3.3 A	81.91±3.4 A	16.27±1.6B	11.13±0.77 BC	110.18±4.8 B	0.812±0.02D	1.132±0.07AB	1.117±0.07B
	Ethephon (45 days)	67.32±1.5 A	64.65±1.4 A	83.52±2.6 A	17.21±1.9A	12.12±1.01 A	116.85±5.6 A	0.822±0.03BC	1.161±0.02A	1.145±0.07A
	Glyphosate (15 days)	58.85±2.2 B	65.77±2.3 A	82.72±2.9 A	17.43±1.7A	11.66±0.94 B	104.74±4.5 BC	0.841±0.04C	1.132±0.06AB	1.115±0.06B
	Glyphosate (30 days)	57.42±2.1 B	66.71±1.6 A	83.67±1.9 A	16.54±1.9B	10.76±0.82 C	111.02±4.3 B	0.853±0.03C	1.157±0.04A	1.147±0.05A
	Glyphosate (45 days)	67.87±2.3 A	65.97±2.4 A	83.05±3.1 A	17.63±2.1A	11.44±0.92 B	113.95±5.9 B	0.912±0.04A	1.152±0.05A	1.135±0.06A
	Sulfometuron-methyl (15 days)	56.57±1.7 BC	64.12±3.4 A	82.42±3.7 A	15.22±1.4C	10.69±0.74 C	106.68±4.3 BC	0.847±0.02C	1.132±0.02AB	1.045±0.07C
	Sulfometuron-methyl (30 days)	59.65±1.2 B	64.17±2.8 A	83.82±2.4 A	16.18±1.5B	11.21±0.85 BC	113.08±3.9 A	0.881±0.04B	1.123±0.03B	1.105±0.05B
	Sulfometuron-methyl (45 days)	66.77±2.2 A	65.52±1.8 A	82.27±2.2 A	17.52±1.4A	11.41±0.90 B	120.73±4.9 A	0.923±0.05A	1.133±0.04AB	1.137±0.08A

±(SE) = Standard error
In a column, means with different capital letters are statistically significant as determined by Tukey's test at $P \leq 0.05$

Table 3. *Mean squares (MS) from the Analysis of Variance (ANOVA) for ratooning growth and yield attributes of sugarcane cultivars under the applications of chemical ripeners*

Source	df	Ratoon sprouting (%)	No. of tillers/plant	Mill-able canes	Ratoon cane yield
Main effects Ripeners (Rip)	9	1536.016ns	2.091 ns	1379.649ns	27199.380ns
Treatment time (T)	2	456.671ns	4.786ns	341.132ns	1421.872ns
Cultivars (Cv)	3	193.219 *	1.655 *	384.404 **	4622.580**
Interactions Rip x T	18	67388.09ns	256.6953ns	291993.15ns	2148017ns
Rip x Cv	27	296787.5*	3.460605ns	530342.6*	1260006ns
T x Cv	6	8476.904ns	203.1711ns	81356.42*	3650593ns
Rip x T x Cv	54	13020660*	424.8308ns	1120400*	997643ns
Error	40	8657.149	860.910	1087.413	5081.462
Total	159				

ns = non-significant and *, ** = significant at $P < 0.05$, 0.01 probability levels

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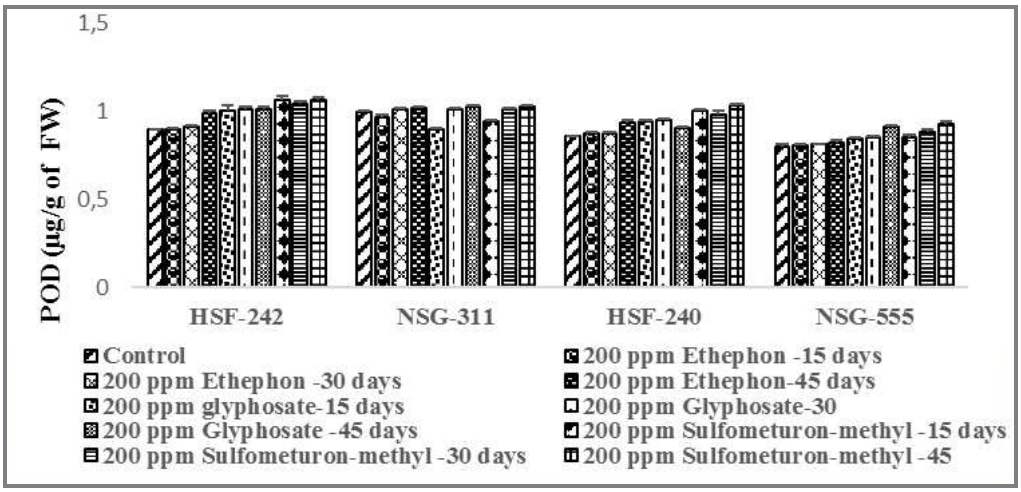
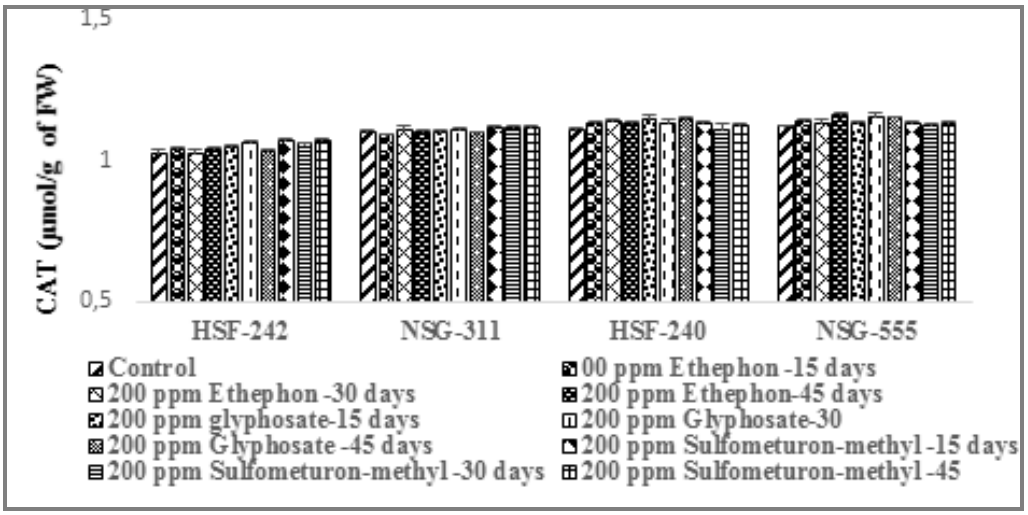
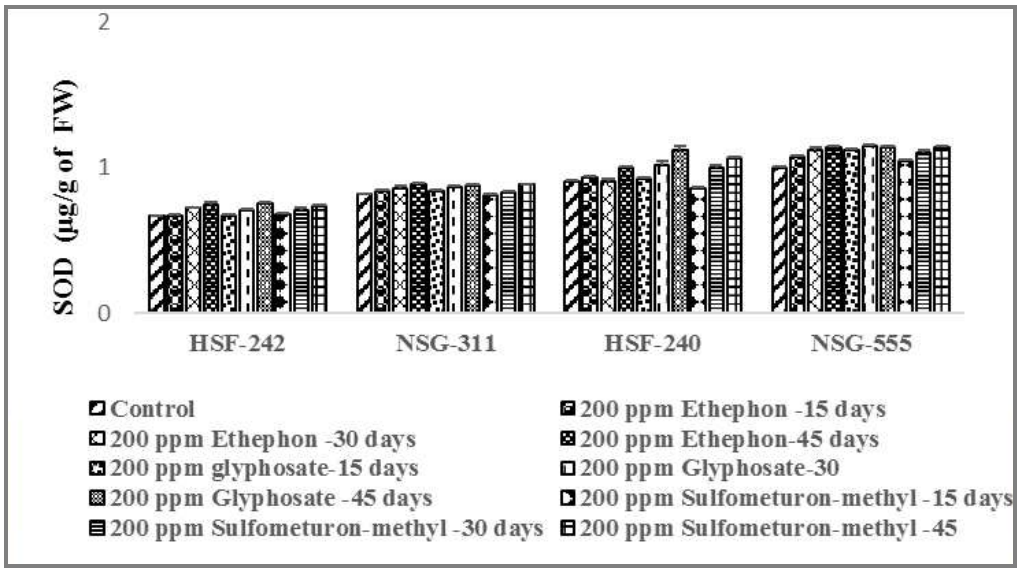


A: Peroxidase (POD)



B: Catalases (CAT)



C: Superoxide dismutase (SOD)

Figure 3. *Effect of different chemical ripeners on antioxidant activities of sugarcane cultivars in planted crop*

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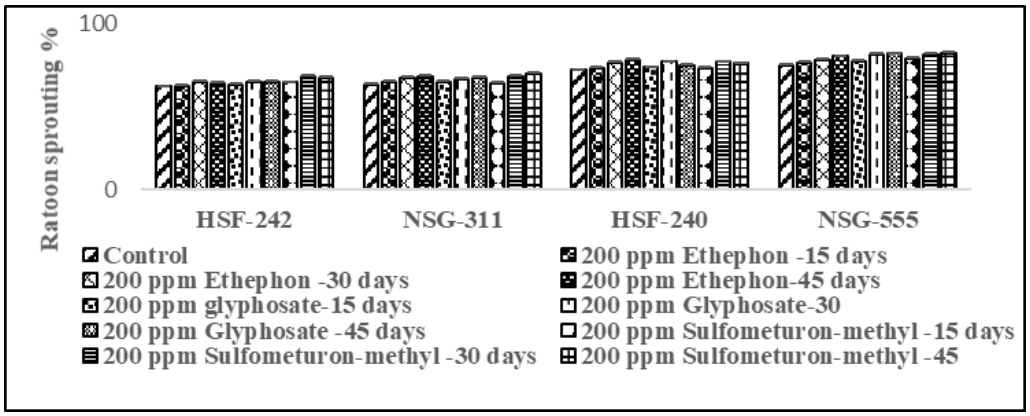
No. of tillers/plant of ratooning

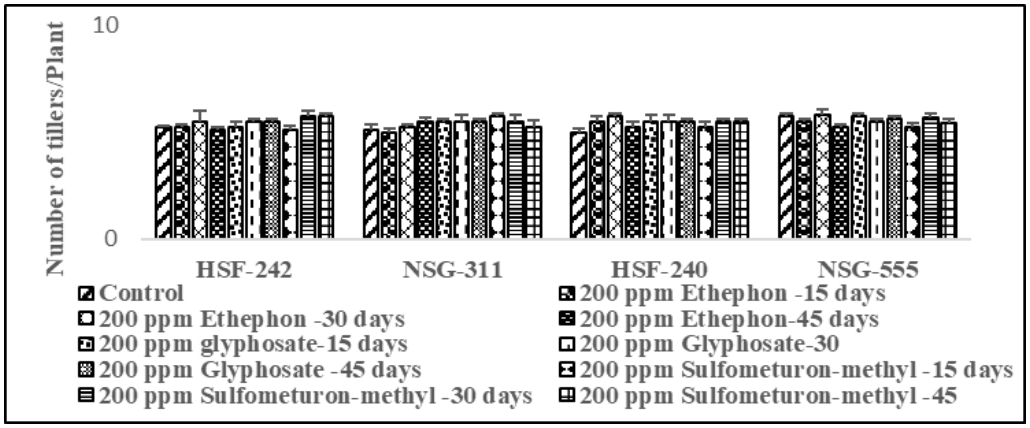
Effect of chemical ripeners was non-significant on number of tillers/plant in ratooning crop, however there was significant variations among sugarcane cultivars (*Table 3*). Higher number of tillers was noted in NSG-555 cultivar and the lowest number of tillers was counted in HSF-242 (*Fig. 4B*).

Number of millable canes of ratooning

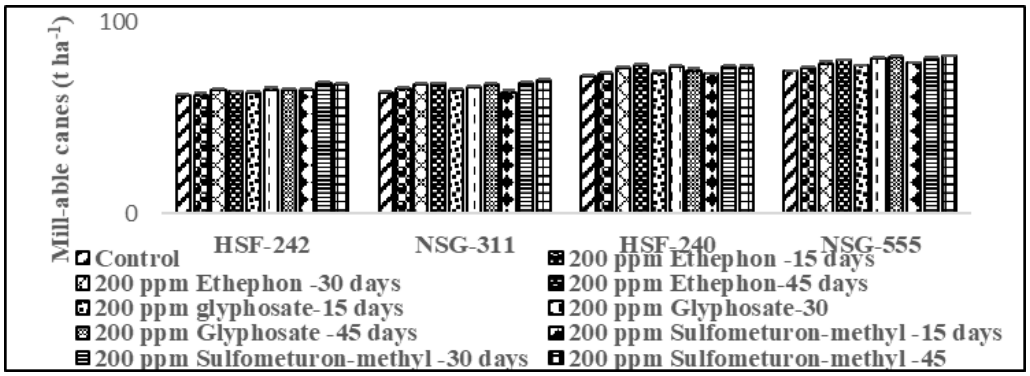
There was a non-significant effect of chemical ripeners on millable canes counted in ratooning crop (*Table 3*). Higher number of millable canes were counted in cultivar HSF-240 and the lowest number of millable cane was present in NSG-555 (*Fig. 4C*). Sulfometuron-methyl showed better results for millable canes in HSF-242.



A: Ratoon sprouting



B: Number of tiller/plant



C: Millable canes

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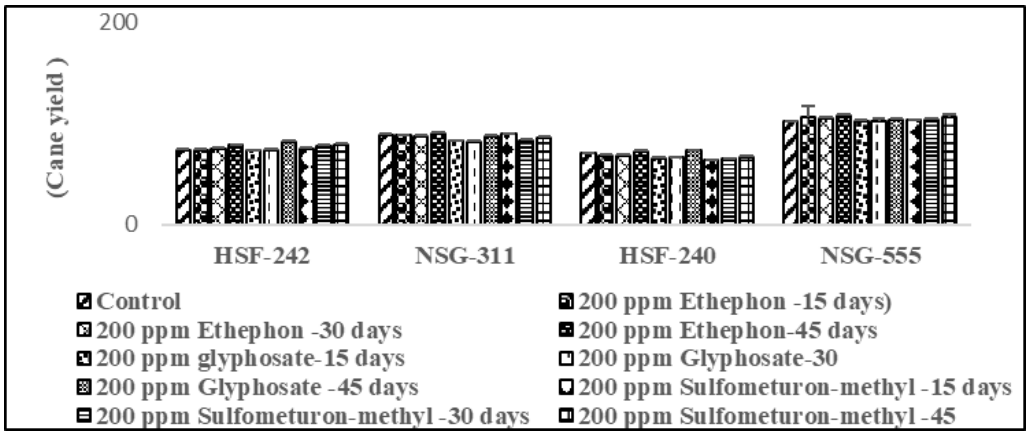

D: Cane yield

Figure 4. *Effect of different chemical ripeners on ratooning growth and yield of sugarcane cultivars*

Cane yield (t ha^{-1}) of ratooning

Data for ratoon cane yield is given in *Table 3*. Effect of chemical ripeners was non-significant on cane yield, however there was significant variations among cultivars. Higher cane yield was calculated in NSG-555 and the lowest in HSF-240 (*Fig. 4D*). Chemical ripeners had non-significant effects on ratoon sprouting, number of tillers and millable canes due to that cane yield was also not affected.

Juice extraction (%) from ratooning

Table 4 showed that there were non-significant results for juice extraction % for ratooning crop. It was apparent that chemical ripeners did not affect the growth and quality attributes. It only affected the quality attributes during the maturity of planted crop by the applications of chemical ripeners. HSF-240 cultivar was the best in juice extraction during ratooning harvest (*Fig. 5A*).

Table 4. *Mean squares (MS) from the analysis of variance (ANOVA) for quality and antioxidant activities of ratooning sugarcane cultivars under the applications of chemical ripeners*

Source	df	Juice extraction	Juice purity	Sugar recovery % cane	Total sugar yield	Peroxidase (POD) activities	Catalases (CAT) activities	Superoxide dismutase (SOD) activities
Main effects Ripeners (Rip)	9	71.248ns	37.654*	68.934ns	49.694ns	0.065ns	0.0762ns	0.023ns
Treatment time (T)	2	43.872ns	122.762ns	211.643ns	78.973ns	0.0342ns	0.0341	0.0642ns
Cultivars (Cv)	3	376.423**	576.976**	387.459*	623.434*	1.561*	1.254*	1.150**
Interactions Rip x T	18	3125.792ns	4622.48 ns	14589.4 ns	3924.484 ns	0.0022 ns	0.0025 ns	0.0014 ns
Rip x Cv	27	26819.39*	21725.45**	26709.1*	30980.93*	0.1014ns	0.0955ns	0.0264ns
T x Cv	6	16514.43ns	70830.73*	82002.99ns	49234.45*	0.0533ns	0.042ns	0.0738ns
Rip x T x Cv	54	117662*	2667060*	5652794ns	2446657ns	0.0034ns	0.0032ns	0.0016ns
Error	40	234.324	265.764	558.695	333.421	1.057	0.778	0.634
Total	159							

ns = non-significant and *, ** = significant at P < 0.05, 0.01 probability levels

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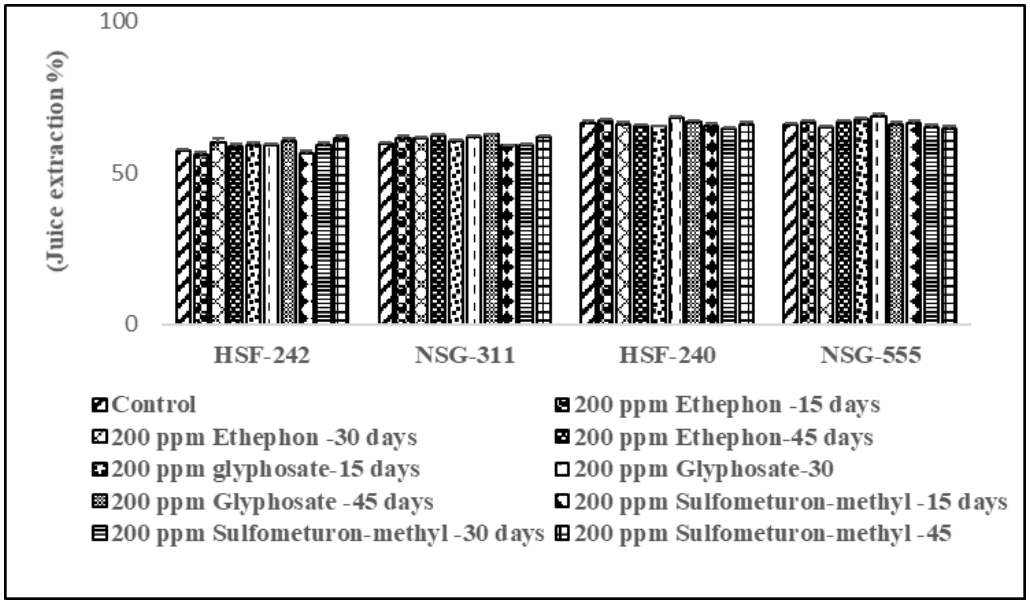
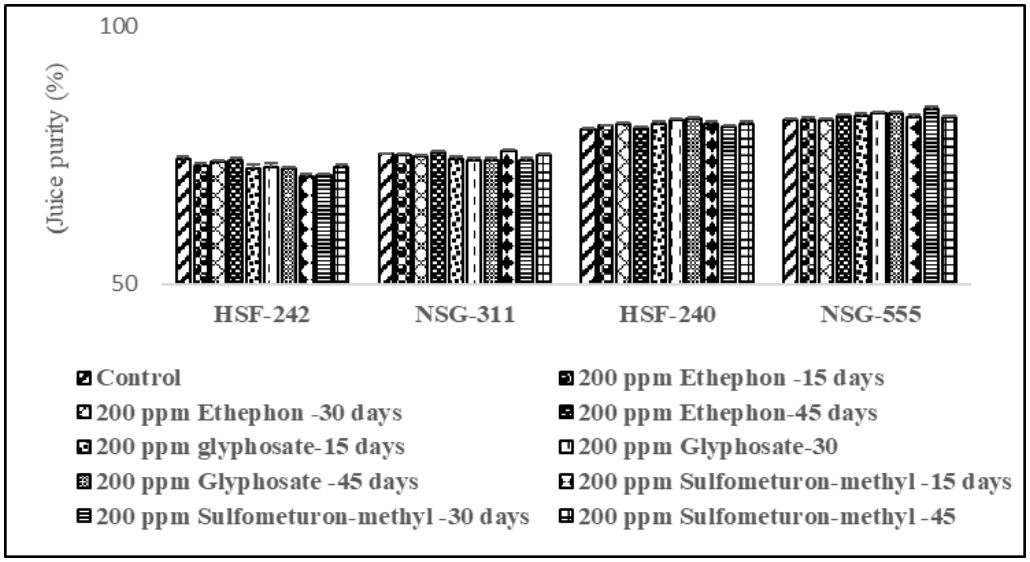


A: Juice extraction



B: Juice purity

Figure 5. *Effect of different chemical ripeners on juice extraction and purity of sugarcane cultivars in ratooning crop*

Juice purity (%) in ratooning

Effect of chemical ripeners was significant on juice purity % for sugarcane (*Table 4*). HSF-242 showed the maximum variations for juice purity in response to chemical ripeners especially Ethephon applied 45 days before harvesting. There were also significant variations among cultivars in response to chemical ripeners. The highest juice purity % was noted in NSG-555 and the lowest in HSF-242 (*Fig. 5B*).

Sugar recovery % of cane in ratooning

There was a non-significant effect of chemical ripeners on sugar recovery % of cane (*Table 4*). Higher sugar recovery % of cane were calculated in cultivar NSG-555 and

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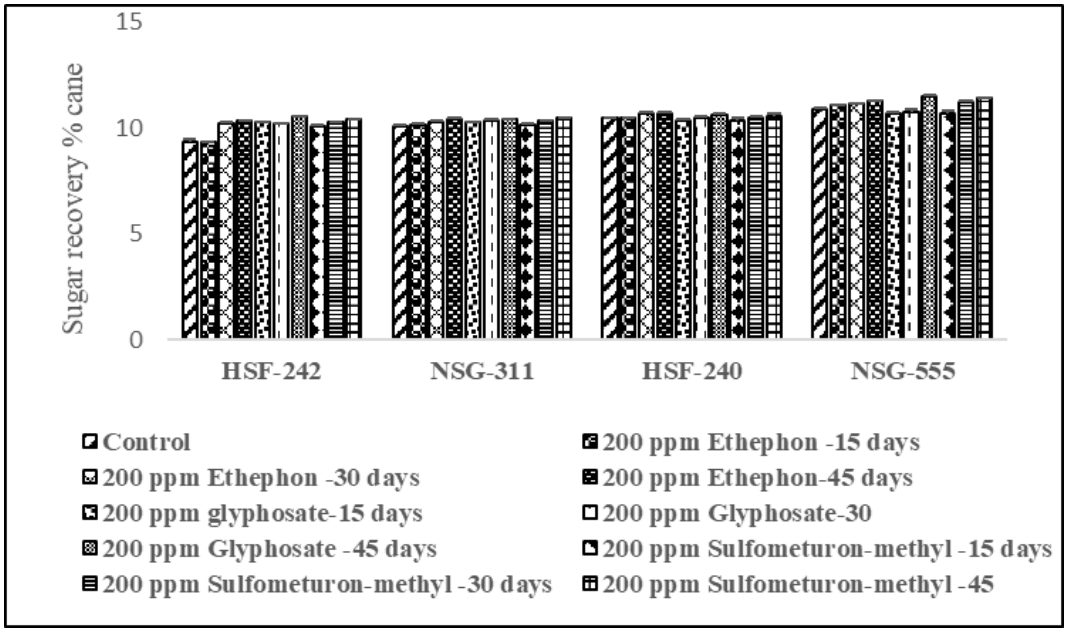
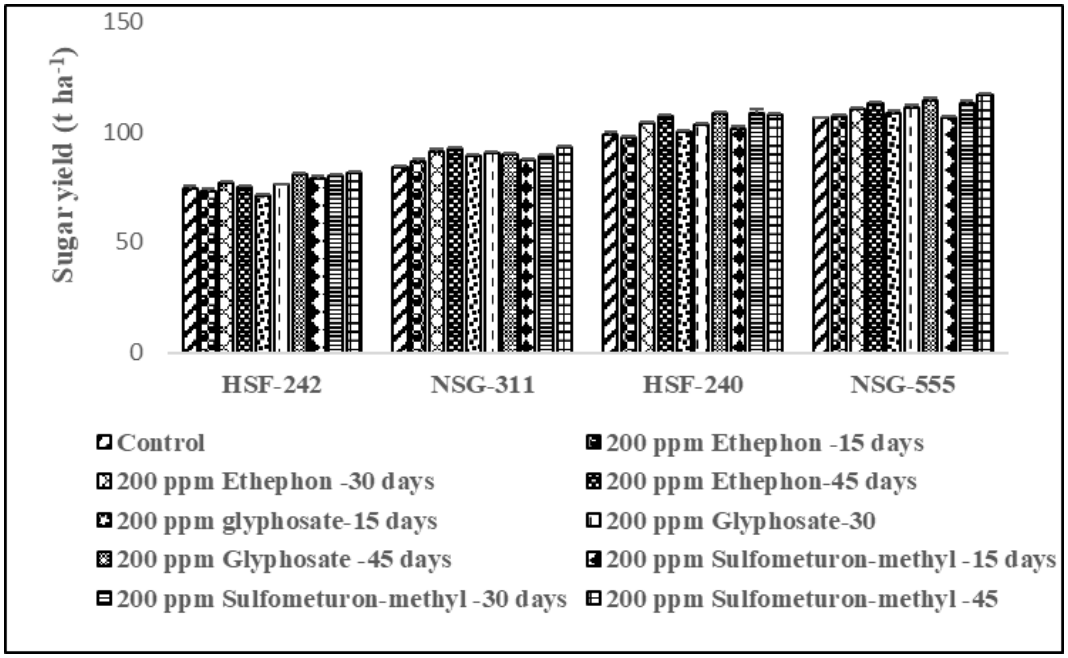
the lowest in HSF-242 (*Fig. 6A*). Results for sugar recovery % of cane was non-significant as other quality related parameters were also non-significant that also resulted no change in sugar recovery % of cane.



A: Sugar recovery % of cane



B: Sugar yield

Figure 6. *Effect of different chemical ripeners on sugar yielding attributes of sugarcane cultivars in ratooning crop*

Total sugar yield (t ha^{-1}) of ratooning

Results for total sugar yield for ratooning crop was similar as in the case of sugar recovery % of cane. There were non-significant results for total sugar yield (*Table 4*).

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Higher total sugar yield was calculated in cultivar NSG-555 and the lowest in HSF-242 (*Fig. 6B*). All the quality parameters had non-significant results for chemical ripeners that also resulted non-significant effects for total sugar yield.

Antioxidant activities in ratooning

There were non-significant effects for antioxidant activities i.e. peroxidase (POD), catalases (CAT) and superoxide dismutase (SOD). As on ratooning crop there was not any influence of chemical ripeners for growth, yield and quality that also affected antioxidant activities (*Table 4*). These results showed there was no change in ratooning crop towards any stimulant, its growth was normal depending upon the genetic makeup of sugarcane cultivars. Data related to antioxidant activities of POD, CAT and SOD is presented in *Figure 7*.

Discussion

Results have shown that chemical ripeners increased the harvest index (HI) of sugarcane. It might be due to the conversion of cane formation and elongation phase into ripening phase by Ethephon and Sulfometuron-methyl. Kapur et al. (2013) found the variations for HI in sugarcane between 66-81% among different cultivars with the applications of chemical ripeners. HI is a useful parameter to assess the suitability of different sugarcane cultivars for various products in the industry that can vary under the influence of different chemicals and stresses (Naidu and Venkataramana, 1989). It was noted that all the quality and sugar yielding attributes including juice extraction and purity, sugar recovery and yield was increased by the applications of different chemical ripeners. Chemical ripeners or herbicides can affect the production of ethylene that can induce early maturity. Lee and Dumas (1982) found the changes in ethylene production in tobacco with the applications of Glyphosate. Glyphosate beneficially increased the sucrose contents in sugarcane (McDonald and Jackson, 2001). Changes in ethylene level directly or indirectly regulate the lifespan of plants. Ethylene is tightly correlated with the biosynthesis of volatile organic compounds to create early ripening (Iqbal et al., 2017).

Karmollachaab et al. (2016) observed a beneficial increase in juice pole and brix value in sugarcane by applying Glyphosate and Ethephon for 40 and 65 days respectively. Most of the studies have shown that chemical ripeners inhibited the growth and enhanced the early cane ripening process in various crops including sugarcane. Ethephon inhibited the growth but it also enhanced the tillering and emergence of ratoon with rapid maturity. Different cultivars of sugarcane respond in different ways to Ethephon when it is used as a ripener (Castro et al., 2001; Silva et al., 2007). By applying sulfometuron-methyl, a reduction in pith process (50 to 60%) was noted with increased sugar yield (Castro et al., 1996). Studies have claimed that sulfometuron-methyl, as a ripener showed consistent improvement in sugarcane brix, pol and reduced pith process (Caputo et al., 2008). Li et al. (2004) compound ripener in sugarcane had an effect on stick's digestion, development and sugar accumulation. It was noted that sulfometuron-methyl did not affect the sugarcane crop yield (t ha^{-1}) and the agronomic features of the crop but was useful for early ripening (Silva et al., 2007; Leite et al., 2010). Different factors can affect the role of chemical ripeners as Solomon and Li (2004) found that cultivar of sugarcane, functional stage of crop at the time of application of ripeners, application rate of chemical, type or combination of ripener and the conditions of growth prior to or after application affected the response of sugarcane to ethephon and glyphosate.

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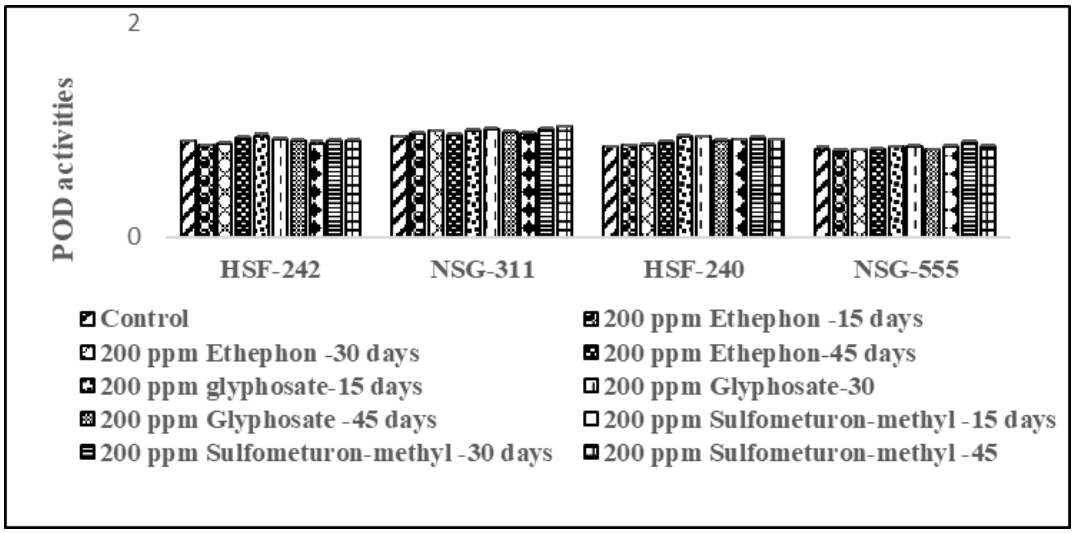
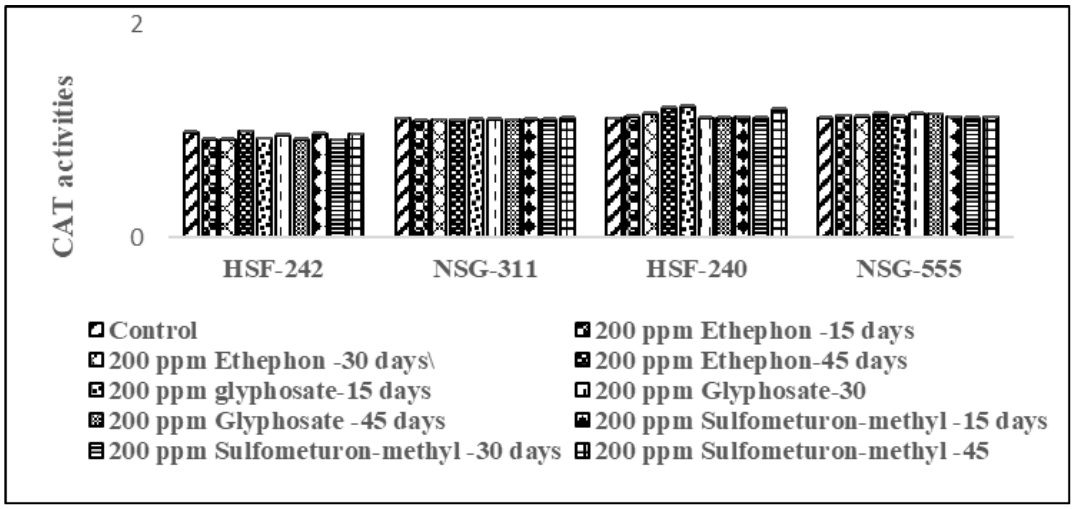
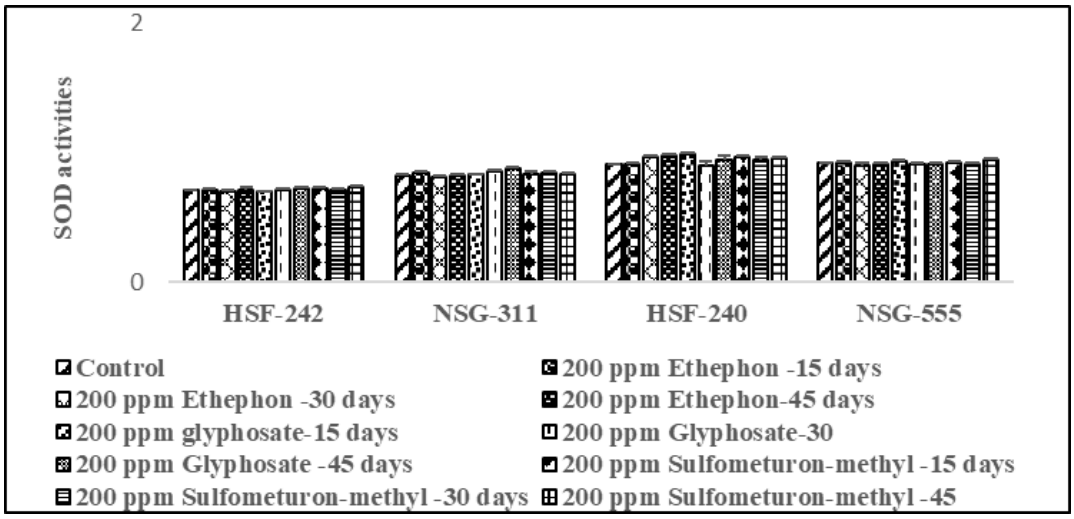


A: Peroxidase (POD)



B: Catalases (CAT)



C: Superoxide dismutase (SOD)

Figure 7. *Effect of different chemical ripeners on antioxidant activities of sugarcane cultivars in ratooning crop*

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During this study the change in antioxidant activities were also noted. Activities of enzymes (CAT, POD and SOD) are a significant index to foretell the plant responses to the changing environments and chemicals (Sen and Mukherji, 2009). In plants, antioxidants activities may act like a defense line for many troublesome conditions and induce early ripening (Lohrmann et al., 2004). Moreira et al. (2020) noted that the Glyphosate (0.15 L ha^{-1}) and ethephon (0.33 L ha^{-1}) provided the highest CAT and POD activities without affecting SOD activities of sugarcane. Gill and Tuteja (2010) found that glyphosate increased the CAT activities in sugarcane varieties SP80-1842 and SP80-3280.

In the present studies, it was noted that chemical ripeners had only positive increasing effects for quality and sugar yield for planted crop. It showed non-significant effect on the ratooning abilities, its yield and quality attribute. Ethephon anticipated harvesting stage by minimum 21 days and its residual effect lasted for 60 to 90 days after it had been applied (Caputo et al., 2008). It is well known that chemical ripeners are useful for early cane ripening as well as to enhance the sugar contents and yield in sugarcane but there are some reports showing the negative effect on ratooning abilities. Didier et al. (2017) noted that the applications of glyphosate reduced the sprouting and growth of ratooning crop of sugarcane. Chemical ripener interacted with the production of ethylene for early cane maturity at planted crop but its residual effect was not sustained in ratooning. It was noted that glyphosate negatively affected ratoon and number of stalk. The number and height of cane stalks per unit surface area was reduced (Dalley and Richard-Junior, 2010). Caputo et al. (2008) reported that ethephon showed non-significant effects on sprouting of sugarcane ratoon. Different research studies have reported that non-significant response to sulfometuron-methyl for ratoon of sugarcane. Silva et al. (2007) noted an enhancing effect on the tillering abilities of sugarcane under applications of various chemical ripeners.

Conclusion

It was concluded that chemical ripeners can be suitable to boost up the sugar yielding characteristics by creating early cane maturity for short term duration before cane harvesting of the crop and it will have no effect on lateral life cycle span and ratooning abilities of the crop.

Recommendations

It is recommended that chemical ripeners should be utilized to induce early cane maturity in order to achieve higher sugar production from sugarcane.

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METHANE POTENTIAL AND RESPIRATION INTENSITY OF WASTES AND AGRICULTURAL BYPRODUCTS

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Abstract. Biogas production can support waste management efficiently – such an integrated system may reflect the approaches of a circular economic strategy. Our purpose was to assist in this task through the quantification of the methane potential of several substrates in Hungary, and by an investigation of the correlation between methane potential values and other biological-chemical parameters. The experimental substrates (n=10) were wastes and agricultural byproducts. Mesophilic anaerobic fermentation tests were carried out with the instrument AMPTS II in three parallel runs. Substrate from municipal solid waste was excluded, thus the number of parallel tests was nine. In addition, the following properties were assessed (n=3): organic matter, total solid content, volatile solid content (calculated), pH, C/N ratio, and respiration intensity. The methane potential values of the investigated substrates confirmed that Hungary has considerable potential for biogas production. Significant correlation was detected between methane potential (an anaerobic process) and respiration intensity (an aerobic process): the correlation was linear at R^2=0.9899. This phenomenon is very important and the finding is innovative although further research is required.
Keywords: *aerobic degradation, anaerobic degradation, biogas, manure, municipal solid waste*

Introduction

Environmental pollution, the uncertainty surrounding energy supplies, human and animal hygiene, and promoting the security of the food industry require complex and sustainable solutions that include treating and utilizing wastes and byproducts. Treating such materials using anaerobic fermentation has two main advantages: biogas is suitable for producing electricity and thermal power, and the composted digestate as a stable product has auspicious agronomic properties. Accordingly, the proportion of renewable energy that is produced and used can be increased, while the composted digestate is suitable for substituting synthetic fertilizers in agricultural practice, and the effective treatment of wastes and their byproducts is thereby supported.

Research on anaerobic fermentation technology is going through a revolution. The selection of suitable input materials, the preparation methods for substrates, the adjustment of circumstances during the sensitive process – all of these aspects require further scientific research before biogas plants can be operated effectively (Angelidaki

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and Ahring, 1992, 1993, 1994; Cheng and Zhong, 2014; Tabatabaei et al., 2018). In this paper the results of methane potential tests are presented. One of our purposes was to characterize the potential substrates in Hungary for biogas production and contribute to the establishment of a scientific database related to the Hungarian conditions. It is important to know the properties of the substrates found in Hungary, because the country possess's significant unutilized potential in biogas production. An additional purpose was to identify the correlation between methane potential (MP) and respiration intensity (AT_4) value of a substrate. The goal of it was to find a faster method for determination of suitability of potential substrate materials for biogas production. Carbon-to-Nitrogen ratio (C/N) is not proper in all of the cases (for example in the case of heterogeneous materials). Traditional MP tests need one month, however respiration intensity tests can be performed in 4-5 days.

Present results of methane potential tests

In the last few years, a number of studies about biomethane potential (BMP) tests have been carried out to demonstrate biodigestability, methane yield, reaction-rate kinetics, the extent of anaerobic activity, the influence of pretreatment, and the effects of mixing different viscosities (André et al., 2016; Wang et al., 2017a; Passos et al., 2017; Raj Paudel et al., 2017; Siddique et al., 2018). Many types of substrates, such as solid household waste, pulp and paper mill sludge, wastewater sludge, commercial foodwaste, livestock materials (manures) and agricultural byproducts, and plant residues have been investigated (Wang et al., 2012; Wei et al., 2015; Hidalgo and Martín-Marroquín, 2015; Pecorini et al., 2016; Ebner et al., 2016; Neshat et al., 2017). Pecorini and his colleagues (2016) also reported that BMP tests can contribute to characterizing and evaluating the optimal performance of a full-scale anaerobic digestion (AD) process.

Li et al. (2015) reported the laboratory scale methane potential (MP) tests of pig-, dairy-, cattle-, and rabbit manure. Based on the volatile solid (VS) (referred to dry matter; d.m.) and pH values of the substrates, these samples may be suitable for biogas production, although their C/N ratio and cellulose and lignin contents were not found to be adequate. Their analysis confirms that manures from different types of livestock may have very different properties, leading to variable performance in biogas production. During BMP tests different organic loading rates (OLR) were also investigated: 8, 16, 32, and 64 g VS/l. Results show that the maximum point of the methane potential curve was reported increasingly later in time, and cumulative methane potential decreased due to the increasing OLR. A further important result is the observation of a decrease in the degradation of organic material while OLR increases. De la Rubia et al. (2009) explained this phenomenon by claiming that high substrate concentration can lead to the inhibitory effects of ammonium-nitrogen. Another possible reason is the decrease in inoculum-to-substrate ratio. Dairy manure produced the lowest amount of methane because of its fibre content. Research by Wu et al. (2017) confirmed the results of Li and his colleagues: swine manure contains cellulose and hemicellulose in high proportions, which compounds have a low hydrolysis rate, therefore their biodegradability is also low. Thermal pretreatment of carbohydrates increases methane production: without pretreatment the MP of swine manure is 201.9 ml CH_4/gVS; however, three-day thermal treatment may increase MP by 39.5%.

Hardly biodegradable carbohydrates are present in significant proportions also in the organic fraction of municipal solid waste (OFMSW). OFMSW investigated by Ventorino et al. (2018) contained 30% cellulose, 21% hemicellulose, 15% starch, 12%

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pectin and 20% lignin. Ventorino performed medium-scale BMP tests (digesters were 100 l in capacity). According to their results, mechanical pretreatment efficiently increases biogas production. Krause et al. (2018) sorted an OFMSW fraction into its components and analyzed them separately, creating opposing results: in the case of certain carbohydrates, decreasing the size of pieces did not have a significant effect on MP. An important difference between the two types of examination is the method: Ventorino (2018) used dry fermentation, while Krause (2018) used wet fermentation. Dry fermentation is recommended if the dry matter content of the substrate is over 15% (Li et al., 2011; Ge et al., 2016; Chiumenti et al., 2018). However, this technology is not in practice routinely used. Riya et al. (2018) performed dry fermentation tests using 20 l volume and a semi-batch run. He found that the operation of the reactor can be stable if total solid (TS) content is between 18-27%.

However, it should be mentioned that the determination of methane potential is lacking with regard to the standardization of protocols (André et al., 2018). The values obtained by different laboratory procedures represent the methane potential stated in the literature that is used to dimension the units, thus sometimes resulting in under- or over-sizing. An interlaboratory assay in France involving eleven laboratories showed that interlaboratory reproducibility had a coefficient of variation (CV) of 20% and an interlaboratory repeatability with a CV of 10% for a given substrate, although different protocols were used in the laboratories (Cresson et al., 2015). For any given protocol and substrates, interlaboratory reproducibility had a CV of 17%, and a CV of 5% for intralaboratory reproducibility. In addition, anaerobic fermentation is an extremely complex process which is influenced significantly by biological-chemical factors, living and non-living agents, and the interactions between these (Ebner et al., 2016; Tsapekos et al., 2017). The more influential physical-chemical parameters are temperature, pH, C/N ratio, OLR, alkalinity, and the concentration of volatile fatty acids (VFAs) (Tufaner and Avsar, 2016; Koch et al., 2017; Ventorino et al., 2018). Nevertheless, a new European interlaboratory assay is taking place and should soon provide new conclusions in this aspect (Holliger et al., 2016).

Alternative parameters for characterization instead of BMP

Although BMP tests can be carried out very simply by using AMPTSII (Automatic Methane Potential Test System), this process takes at least 3-4 weeks, thus it would be practical and useful to be able to characterize potential substrates with analytical methods which take less time. Moreover, if such an analysis can be standardized, the reproducibility and reliability of results could be improved.

According to Wang et al. (2017b), the determination of the available carbon to nitrogen ratio (AC/N) may be an alternative way to appraise the suitability of potential substrates for biogas production. This procedure is particularly efficient in the case of substrates which contain slowly biodegradable compounds. However, all types of manure and MSW contain such components to some degree.

NIR spectroscopy (Near Infrared Reflectance Spectroscopy) can also be suitable for determining MP quickly and precisely (Triolo et al., 2014). NIRS is a non-destructive, real-time measurement which has also been used in biofuel technology over the last decade. In addition, there are a few studies regarding the application of NIRS in the estimation of methane potential – these are based on the following substrates: MSW; MSW mixed with agroindustrial wastes; and meadow grass (Lesteur et al., 2011; Raju et al., 2011; Doublet et al., 2013), respectively. The goal of Triolo and his colleagues was

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to set up a robust model for predicting BMP based on NIR spectra. Their study is satisfactory and the related model can be used for the estimation of BMP. However, it should be mentioned that NIRS requires specific user knowledge, and a reference spectra database must be set up for calibration.

Standardized respiration intensity can be a further alternative for appraising MP. One suitable piece of equipment for performing such measurement is the so-called OxiTop®. Its scope is limited to determining the biodegradable organic content of mechanically-biologically treated wastes (there are a lot of cases when traditional analytical methods – total organic carbon, dissolved organic carbon, loss on ignition – are not suitable for determining this).

Tests using OxiTop®

Standardized German and Austrian methods for determining respiration activity (AT_4) are based on equipment made by Sapromat®. This technology is associated with several advantages (fluctuations in atmospheric pressure do not affect the measurements; oxygen is not a limiting factor; a great amount of data can be fixed; its operation is flexible), although the use of OxiTop® has become increasingly frequent over the last few years. The reason for this is the fact that OxiTop® is rather user-friendly and cheaper. It is, however, necessary to execute comparison tests in order to confirm the hypothesis that the two methods are equivalent. Binner et al. (2012) studied wastes (n=169) using both methods to the determine statistical relationship between them. Results showed that the correlation was good: R^2=0.987. The coefficient of determination was also appropriate in the case of low reaction wastes (AT_4 < 20 mg O_2/g d.m.): R^2 = 0.983. This research confirms that Sapromat® can be substituted by OxiTop®.

This is important for us, because our purpose was to find an appropriate method to prise the suitability of substrates for biogas production. C/N ratio is not proper in all of the cases (for example in the case of heterogeneous materials). Traditional MP tests need one month, however respiration intensity tests can be carried out in 5 days. Further purpose was to characterize substrate materials in Hungary.

Materials and methods

Experimental materials

Substrates

The samples were different manures and wastes; their abbreviations are listed in *Table 1*.

Table 1. *Abbreviations of the substrates*

Name of the samples	Abbreviation
Cattle manure with litter 1	CMwL 1
Cattle manure with litter 2	CMwL 2
Liquid cattle manure with straw	LCMwS
Swine manure	SM
Spent mushroom compost	SMC
Cattle manure fresh-compost	CMC
Liquid cattle manure with shredded straw	LCMwSS
Liquid swine manure with straw	LSMwS
Separately collected green waste	GW

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CMwL1 and CMwL2 are littered manures, while LCMwS and LCMwSS are liquid cattle manures. The difference between them is the method of pretreatment. We mixed these liquid manure samples with straw, but in the case of LCMwSS the straw was shredded into pieces of 2 cm size. SMC refers to spent mushroom. CMC is fresh cattle manure compost. SM is swine manure, while LSMwS is liquid swine manure with straw. Municipal solid waste (MSW) was treated using mechanical-biological methods. We investigated the fraction which is smaller than 25 mm. Green waste (GW) was supplied by a regional waste collection company. In the case of liquid manures, tests were executed after mixing them with straw in proportions of 1:1 by weight. The biodegradability of straw was not increased in advance (for example, using aerobic methods) to enhance hydrolysis. Straw was only shredded in the case of LCMwSS.

Inoculum

We obtained the inoculum from the mesophilic reactor of an operating plant in Hungary. The substrates of this plant are agricultural materials (animal manure and plant residue) and industrial food byproducts.

Experimental methods

BMP tests with AMPTSII

The anaerobic digestion study was carried out in AMPTS II, produced by Bioprocess Control AB. Comparison tests were performed using AMPTS II for determining BMP. Results confirmed the reliability of the method (Kleinheinz and Hernandez, 2016).

Before the tests, BMP reactors were purged with high purity nitrogen for three minutes to remove oxygen from the reactor head-space. The exact weight of substrates and inoculum was calculated by software based on the volatile solid content (VS) and the inoculum-to-substrate ratio (I/S). The latter was always 2:1. The total volume of substrate and inoculum in the reactors was always 400 ml, therefore the volume of the gas that was produced was 200 ml.

Blanks and positive blanks (references) were measured in every sequence in parallels of 3-3, thus we could measure only three different substrates (also arrayed in 3-3 parallels) in one sequence. In the case of blank measuring, only inoculum was entered into the reactors after weighing. In the case of positive blanks, we used microcrystalline cellulose, a product made by Molar Chemicals Kft. The positive blank was used as a reference with regard to identifying the biologically activity of the inoculum.

The length of time for one sequence was 20-30 days. Tests were stopped if the volume of methane produced in one day was less than 20 ml.

Further measurements

Before starting the tests it was necessary to determine the total solid (TS) and the organic matter (OM) content of the substrates and the inoculum; furthermore, to calculate VS content. The calculation of VS was accomplished according to *Eq.1*.

$$VS(\%) = (m_{dried} - m_{burned}) / m_{wet} \quad \text{(Eq.1)}$$

These data were necessary for determining the exact weight of the substrates and the inoculum to be put into the reactors. Total solid measurement was done based on the standard MSZ EN 13040:2008. Measurements of organic matter were based on the

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method of loss on ignition (LOI) (MSZ EN 15935:2013). Further analytical measurements were carried out: C/N ratio, respiration intensity, and pH. Measurements of carbon and nitrogen content were performed using Elementar Vario Macro, the standard for which was MSZ EN ISO 16948:2015. Respiration intensity values were determined by using OXITOP®. Originally, the procedure was standardized to measure the biological stability of wastes, although it has been confirmed that OXITOP® can be efficiently and reliably used for another purposes (Binner et al., 2012; Malinska, 2016). The temperature at which measurement was taken was 20°C, and the duration of the tests was seven days (accordingly, the abbreviation for this measurement in the following parts of the paper is AT_7). pH was measured according to the standard MSZ EN 13037:2012. Every analytical measurement was carried out in three replications.

Statistical methods

The statistical analyses were performed using PAST3 statistical program version 1.0.0.0. Three independent replicates were used for each measurement. One exception is the BMP measurements of municipal solid waste (n=9). We did polynomial regression calculation on BMP curves. We accomplished normality tests and Levene's tests on methane potential, respiration intensity, carbon-to-nitrogen ratio and organic matter data. Valid normality and homogeneity of variance are minimum assumptions to doing ANOVA analysis on data. ANOVA analysis could not be used because of the violation of the assumptions, therefore non-parametric Kruskal-Wallis tests were accomplished on data.

Relationship between the four mentioned parameters (MP, AT_7, C/N, OM) was checked by cluster analysis.

Results

TS, VS, OM, C/N ratio, pH and respiration intensity results for the substrates can be seen in *Table 2*. The values represented are the averages of three replicate measurements in every case. VS is a calculated parameter (*Eq.1*). Relative deviation values (SD%) are represented in parenthesis for every substrate and every measurement. Relative deviation value of LSMwS in the case of TS measurements is outstanding. Probably the reason of that is the mistake of sample weighing (differences in the straw content). Relative deviation value of MSW in case of the pH measurements is also higher by an order of magnitude than the other pH values. The reason is the characteristic of MSW sample – MSW is very heterogenous so the number of replicates was not enough in this case.

From the perspective of anaerobic digestion, the pH values for substrates of agricultural origin were neutral or weakly alkaline, therefore adjustment of pH was not necessary. In the case of MSW, pH was low (5.44; SD%=10.4%), but we did not add sodium-hydroxide because Na^+ ions in high concentration may inhibit the process of anaerobic fermentation. The C/N ratio was outside the acceptable range (20-30) in the case of four substrates: SMC and CMC had a slightly lower value (13.9 and 11.6, respectively), while LCMwS and LCMwSS substrates had a higher C/N ratio than optimal (34.1 and 73.2, respectively). BMP and respiration intensity (AT_7) values can be seen in *Table 3*. The number of parallel measurements was also three. In the case of BMP measurements of MSW the number of replicates was 9 because of its heterogenity.

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Table 2. *Characterisation of the substrates*

	TS [%] (SD%)	**OM [%] (SD%)**	**VS [%]**	**C/N ratio (SD%)**	**pH (SD%)**
Inoculum (n=3)	5.9 (5.2%)	80.5 (1.3%)	4.7	-	7.90 (0.8%)
CMwL 1 (n=3)	18.2 (4.5)	87.5 (0.9%)	15.9	20.7 (0.9% / 1.8%)	8.46 (0.5%)
CMwL 2 (n=3)	14.7 (3.5%)	84.2 (0.9%)	12.4	23.9 (0.3% / 6%)	8.37 (1.4%)
LCMwS (n=3)	7.2 (12.3%)	89.6 (1.3%)	6.5	34.1 (1.8% / 10.5%)	7.98 (1.1%)
SM (n=3)	24.8 (3.9%)	88.1 (2.8%)	21.9	22.3 (0.3% / 6.9%)	8.16 (0.7%)
SMC (n=3)	34.6 (2.0%)	53.5 (4.0%)	18.5	13.9 (5.4% / 1.9%)	7.04 (0.5%)
CMC (n=3)	38.1 (19.8%)	59.9 (29.4%)	22.0	11.6 (3.2% / 2.3%)	7.17 (0.5%)
LCMwSS (n=3)	5.6 (9.9%)	87.5 (4.8%)	4.9	73.2 (2.1% / 10.5%)	8.00 (0.7%)
LSMwS (n=3)	18.5 (53.3%)	82.0 (0.6%)	15.2	21.3 (0.8% / 10.6%)	6.72 (1.7%)
GW (n=3)	45.4 (6.1%)	56.9 (0.9%)	25.8	18.1 (2.2% / 1.4%)	7.22 (0.9%)
MSW (n=3)	47.54 (7.0%)	59.57 (3.3%)	28.3	23.0 (3.7% / 2.0%)	5.44 (10.4%)

Table 3. *Average BMP and respiration intensity values of the substrates. Relative deviation values (SD%) are represented in parenthesis*

Abbreviation	**Average BMP [Nml/gVS] (SD%)**	**Duration of AD [days]**	**AT_7 [mg O_2/g d.m.] (SD%)**
CMwL 1 (n=3)	173.0 (3.1%)	29	58 (6.6%)
CMwL 2 (n=3)	149.4 (15.4%)	29	41 (9.9%)
LCMwS (n=3)	370.1 (6.4%)	29	159 (2.8%)
SM (n=3)	136.2 (2.9%)	29	46 (5.4%)
SMC (n=3)	48.4 (5.4%)	19	9 (8.7%)
CMC (n=3)	135.9 (30.2%)	19	40 (22.1%)
LCMwSS (n=3)	250.06 (3.3%)	19	98 (4.8%)
LSMwS (n=3)	156.3 (14.4%)	20	50 (9.2%)
GW (n=3)	72.81 (17.0%)	20	7 (12.6%)
MSW	325.71 (16.7%. n=9)	19	137 (3.7%. n=3)

AMPTS II equipment saves data in continuously cumulated form, although separately, on a daily basis. Accordingly, we can characterize every single day according to the amount of methane that was produced and the differences in the three parallel measurements. The standard deviation of the blank measurements were between 1.3 – 14.87% in every measuring sequence. Positive blank measurements also confirmed the microbiological activity of the inoculum in every measuring sequence (0.2 – 10.6%).

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Based on the results concerning respiration intensity, SMC and GW substrates contained biologically degradable organic matter in low amounts (9 and 7 mg O_2/g d.m., respectively), and their average BMP values are in line with this finding. LCMwS is outstanding from the point of view of respiration intensity (159 mg O_2/g d.m.); however, MSW and LCMwSS also showed high activity (137 and 98 mg O_2/g d.m., respectively). CMC and CMwL2 substrates (which contained nutrients hardly available to microorganisms in anaerobic digestion processes based on the shape of BMP curves (*Fig. 1*), standardized curves (*Fig. 2*), and gradient values (*Table 4*)) showed medium respiration intensity (40 mg O_2/g d.m. (22.1%) and 41 mg O_2/g d.m. (9.9%), respectively). Further results concerning the respiration intensity of all of the substrates and their standard deviations can be seen in *Table 3*. Relative deviations of CMC in the case of BMP and AT_7 measurements (30.2% and 22.1% respectively) are outstanding. CMC sample was a fresh compost, so the reason probably is the heterogenity.

The average methane potential curves of the substrates can be seen in *Fig. 1*.

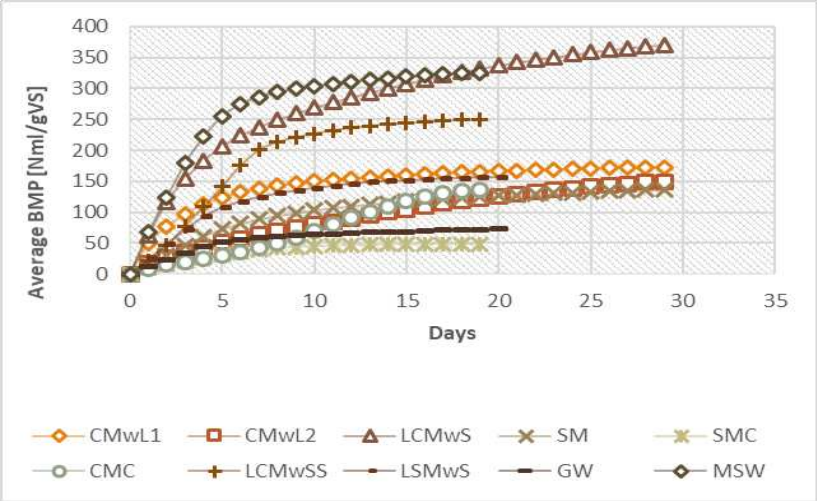

Figure 1. *BMP curves of the substrates*

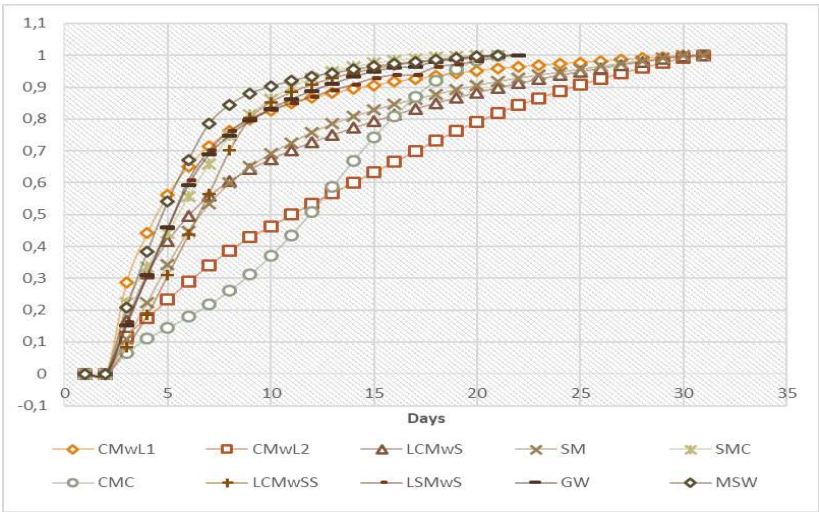

Figure 2. *Standardized methane potential curves*

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Table 4. *Gradiation values of the initial phase (first 5 days) of MP curves*

	y	R^2
CMwL1	23.661	0.9299
CMwL2	9.7244	0.9754
LCMwS	41.018	0.9650
SM	15.386	0.9997
SMC	6.0845	0.9778
CMC	5.6638	0.0983
LCMwSS	31.116	0.9983
LSMwS	21.942	0.9940
GW	10.984	0.9997
MSW	51.306	0.9865

The shape of the BMP curve for substrate CMwL1 refers to the right conditions for anaerobic digestion. Based on the shape of the BMP curve of substrate CMwL2 (it has a moderate gradient), it is clear that the organic matter content is hardly available to microorganisms. Considering the results of the previous analytical measurements, there is significant difference between the two substrates in terms of AT_7. These seven-day and aerobic tests also showed the low availability of nutrients for microorganisms in the substrate-inoculum mixes (*Table 3*). In the case of LCMwSS, the high C/N value (73.2) indicated in forward inhibitor processes during AD, which assumption is confirmed by the shape of the BMP curve. The results for the CMC substrate are also expressed in average values, which is misleading: BMP (*Table 3*), respiration intensity (*Table 3*) and OM content (*Table 2*) results for CMC are associated with a notable standard deviation. In the case of MP tests, two measurements of the three replications showed the following: gas production started on the fifth day, and at the end of the test the final and cumulated methane yields showed more than 30% standard deviation relative to each other. Results of previous measurements of SMC substrate (low OM content (53.5%), C/N ratio (13.9) and respiration intensity (40 mg O_2 /g d.w.)) also indicated inhibition of microbial gas production. MSW is a strongly heterogeneous substrate, although the actual results also confirm its significant biodegradable material content. The average BMP of the nine MSW measurements (325.71 Nml/gVS, SD=16.7%, n=9) is in line with finding in the literature (Jokela et al., 2005; Liu et al., 2008; Pecorini et al., 2016; Krause et al., 2018; Ventorino et al., 2018).

Standardized curves were made from the standardized BMP values (calculation was based on the average values), and shown in *Figure 2*. In this way, the quality of degradation processes in the single reactors can be compared easily.

It can also be seen that CMC and CMwL2 substrates contained only weakly available nutrients (*Fig. 2*). The initial gradient of the curves (*Table 4*) indicates the previous phenomenon in numbers. Based on the standardization, there was no significant difference in the quality of microbial degradation processes between the nine parallel measurements of MSW (*Fig. 3*). Final and cumulated methane production varied between 228.86 – 385.57 Nml/gVS (SD%=16.7%) because of the quantity of added OM; however, biochemical processes continued very similarly, despite the heterogeneity of the MSW substrate.

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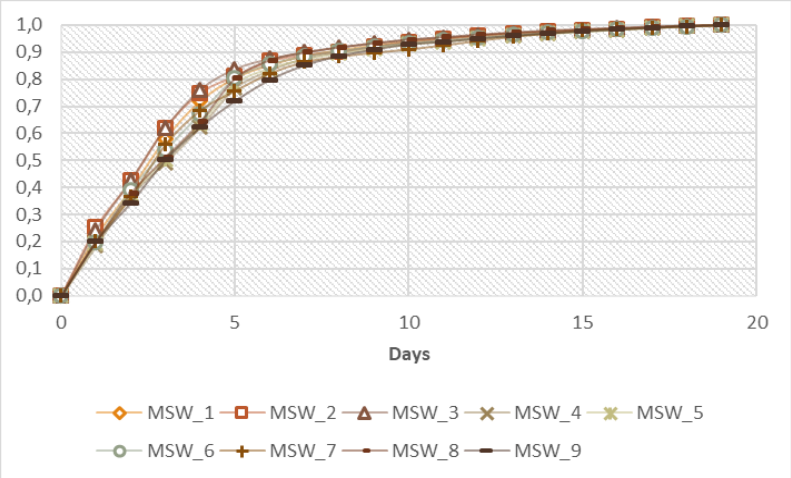

Figure 3. *Standardized curves of the nine parallel measurements of MSW*

The gradients of the initial five days of methane potential curves, and also the accuracy values for linear trend-lines can be seen in *Table 4*. The initial intensity of methane production shows the availability of organic matter in the substrate. Based on this conception, the OM content of CMC and SMC substrates was hardly available, although in case of CMC, after the first step of AD (hydrolysis), aerobic and anaerobic degradation also occurred (AT_7 40 mg O_2/g d.m., BMP 135.9 Nml/gVS). Degradation tests for SMC showed (AT_7 9 mg O_2/g d.m., BMP 48.39 Nml/gVS) that its biologically degradable organic matter content was minimal. Total organic matter content was measured with the 'loss on ignition' method; the result was also low: 53.5%.

Very important experience during our experiments is the correlation between BMP and AT_7 measurement. Respiration intensity measurement is based on aerobic biochemical processes, so the composition of the microbial consortium is different than under anaerobic conditions. However, data from these measurements (*Table 3*) showed strong correlation with BMP results from anaerobic digestion tests (*Table 3*). The correlation between the two kinds of data can be seen in *Fig. 4*. The quality markers of the linear correlation between the two parameters are: r=0.9955, R^2=0.9911, t=29.81, p=1.7372E-09, permutation p=0.0001. The results of these tests confirm our hypothesis: previous respiration intensity tests of potential substrate materials indicate their suitability for biogas production. This is very useful in practice because BMP tests are very time consuming. Performing respiration intensity tests is more time-efficient.

Statistical analyses

The first step of the statistical analysis involved doing polynomial regression fittings (second, third and fourth order) on BMP curves. According to the qualitative parameters different orders were fitted for the certain curves. Second-order regression was applied in the following three cases (*Table 5*): CMwL2, SMC, and CMC. Third-order regression was applied in the case of SM, LSMwS, and GW, and fourth-order in the case of CMwL1, LCMwS, LCMwSS, and MSW.

Normality tests were carried out on BMP, respiration intensity, C/N ratio, and OM content results (*Tables 6 and 7*). Data of AT_7 and C/N ratio measurements were well-modeled by normal distribution in case of all substrates.

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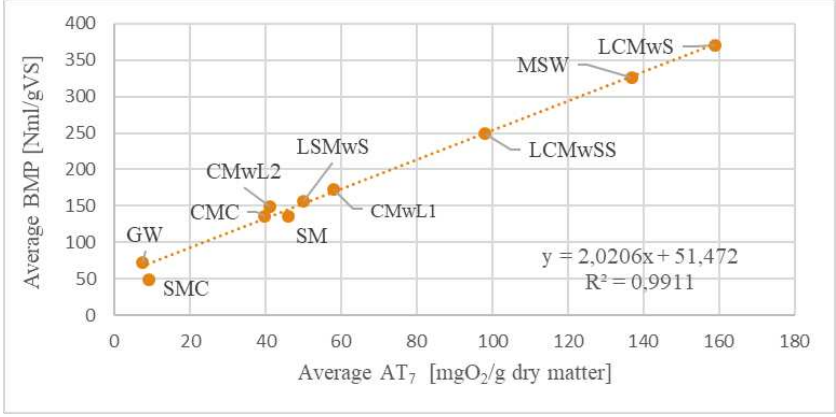

Figure 4. *Relationship between respiration intensity and BMP results*

Table 5. *Data of polynomial regression tests accomplished on the average BMP curves*

Samples	Linear regression of the initial phase		POLYNOMIAL REGRESSION OF BMP CURVES								
			Order 2			Order 3			Order 4		
	Y	R2	Akaike IC	p	R2	Akaike IC	p	R2	Akaike IC	p	R2
CMwL1	23.6610	0.9299	5762.60	4.48E-13	0.8783	1707.00	6.81E+19	0.9641	445.64	4.78E-25	0.9908
CMwL2	9.7244	0.9754	161.16	5.31E-35	0.9971	105.96	1.05E-35	0.9982	44.283	1.58E-39	0.9994
LCMwS	41.0180	0.9650	5809.50	4.80E-20	0.9674	3540.30	2.66E-24	0.9862	427.47	3.66E-31	0.9977
SM	15.3860	0.9997	1496.00	4.09E-20	0.9634	174.88	3.89E-31	0.9959	41.603	1.82E-38	0.9992
SMC	6.0845	0.9778	65.36	2.80E-16	0.9852	13.58	4.69E-23	0.9986	15.576	3.18E-21	0.9986
CMC	5.6638	0.0983	434.96	1.56E-17	0.9895	135.22	3.03E-20	0.9969	32.515	3.16E-24	0.9995
LCMwSS	31.1160	0.9983	2000.80	2.93E-16	0.9851	1422.80	5.12E-16	0.9895	649.38	3.28E-17	0.9952
LSMwS	21.9420	0.9994	1345.70	3.01E-14	0.9686	126.74	6.60E-22	0.9972	74.626	2.52E-22	0.9985
GW	10.9840	0.9997	402.80	7.54E-13	0.9550	49.10	5.20E-20	0.9953	36.344	5.68E-20	0.9970
MSW	51.3060	0.9865	3782.90	1.05E-11	0.9489	703.03	2.02E-16	0.9906	237.08	1.21E-18	0.9969

Table 6. *Results of normality tests accomplished on BMP and respiration intensity data*

		CMwL 1	CMwL 2	LCMwS	SM	SMC	CMC	LCMwSS	LSMwS	GW	MSW
BMP	N	3	3	3	3	3	3	3	3	3	9
	Shapiro-Wilk W	0.8139	0.9855	0.7789	0.7522	0.9087	0.7565	0.9930	0.9166	0.9387	0.9132
	p(normal)	0.1482	0.7694	0.0650	0.0048	0.4139	0.0144	0.8400	0.4405	0.5222	0.4287
	Anderson-Darling A	0.4073	0.2057	0.4510	0.4850	0.2936	0.4794	0.1973	0.2844	0.2589	0.2884
	p(normal)	0.1122	0.5593	0.0773	0.0579	0.2910	0.0607	0.5943	0.3138	0.3860	0.3037
	p(Monte Carlo)	0.1470	0.7734	0.0656	0.0039	0.4077	0.0153	0.8434	0.4333	0.5298	0.4227
	Jarque-Bera JB	0.5179	0.3126	0.5287	0.5312	0.4397	0.5311	0.2967	0.4295	0.3975	0.4340
	p(normal)	0.7718	0.8553	0.7677	0.7667	0.8027	0.7668	0.8621	0.8068	0.8197	0.8049
	p(Monte Carlo)	0.1499	0.7693	0.0651	0.0050	0.4049	0.0144	0.8479	0.4428	0.5307	0.4282
		CMwL 1	**CMwL 2**	**LCMwS**	**SM**	**SMC**	**CMC**	**LCMwSS**	**LSMwS**	**GW**	**MSW**
AT7	N	3	3	3	3	3	3	3	3	3	3
	Shapiro-Wilk W	0.9209	0.9893	0.9996	0.9548	0.8952	0.9892	0.9124	0.9979	0.8845	0.9669
	p(normal)	0.4555	0.8024	0.9611	0.5906	0.3704	0.8011	0.4261	0.9125	0.3378	0.6503
	Anderson-Darling A	0.2794	0.2014	0.1900	0.2405	0.3094	0.2016	0.2893	0.1918	0.3220	0.2267
	p(normal)	0.3269	0.5766	0.6285	0.4469	0.2552	0.5760	0.3014	0.6196	0.2296	0.4980
	p(Monte Carlo)	0.4563	0.8023	0.9594	0.5944	0.3678	0.8057	0.4258	0.9133	0.3411	0.6632
	Jarque-Bera JB	0.4236	0.3046	0.2822	0.3711	0.4557	0.3049	0.4350	0.2859	0.4672	0.3494
	p(normal)	0.8091	0.8587	0.8684	0.8306	0.7962	0.8586	0.8045	0.8668	0.7917	0.8397
	p(Monte Carlo)	0.4542	0.7999	0.9601	0.5871	0.3667	0.8004	0.4230	0.9097	0.3398	0.6526

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Table 7. *Results of normality tests accomplished on C/N ratio and OM data*

		CMwL 1	CMwL 2	LCMwS	SM	SMC	CMC	LCMwSS	LSMwS	GW	MSW
C/N	N	3	3	3	3	3	3	3	3	3	3
	Shapiro-Wilk W	1.0000	0.8789	0.8399	0.9181	0.8995	0.9643	0.8538	0.9927	0.9231	1.0000
	p(normal)	1.0000	0.3212	0.2139	0.4458	0.3839	0.6369	0.2506	0.8362	0.4633	1.0000
	Anderson-Darling A	0.1895	0.3287	0.3755	0.2826	0.3044	0.2296	0.3588	0.1977	0.2769	0.1895
	p(normal)	0.6307	0.2171	0.1451	0.3184	0.2661	0.4867	0.1678	0.5927	0.3337	0.6307
	p(Monte Carlo)	1.0000	0.3223	0.2032	0.4492	0.3832	0.6359	0.2447	0.8305	0.4627	1.0000
	Jarque-Bera JB	0.2813	0.4728	0.5041	0.4274	0.4508	0.3541	0.4945	0.2974	0.4206	0.2813
	p(normal)	0.8688	0.7895	0.7772	0.8076	0.7982	0.8377	0.7810	0.8618	0.8103	0.8688
	p(Monte Carlo)	1.0000	0.3150	0.2141	0.4498	0.3811	0.6415	0.2497	0.8321	0.4554	1.0000
		CMwL 1	**CMwL 2**	**LCMwS**	**SM**	**SMC**	**CMC**	**LCMwSS**	**LSMwS**	**GW**	**MSW**
OM	N	3	3	3	3	3	3	3	3	3	3
	Shapiro-Wilk W	0.9985	0.7500	0.7500	0.8480	0.8300	0.8791	0.8547	0.9134	0.8196	0.9206
	p(normal)	0.9265	0.0000	0.0000	0.2351	0.1884	0.3217	0.2530	0.4295	0.1623	0.4545
	Anderson-Darling A	0.1911	0.4878	0.4878	0.3657	0.3876	0.3285	0.3577	0.2881	0.4003	0.2798
	p(normal)	0.6228	0.0565	0.0565	0.1580	0.1305	0.2174	0.1694	0.3044	0.1191	0.3260
	p(Monte Carlo)	0.9283	0.0001	0.0001	0.2404	0.1855	0.3246	0.2485	0.4255	0.1625	0.4549
	Jarque-Bera JB	0.2846	0.5312	0.5312	0.4987	0.5100	0.4727	0.4938	0.4337	0.5154	0.4241
	p(normal)	0.8674	0.7667	0.7667	0.7793	0.7749	0.7895	0.7812	0.8050	0.7728	0.8089
	p(Monte Carlo)	0.9291	0.0001	0.0001	0.2361	0.1867	0.3210	0.2509	0.4379	0.1620	0.4563

In the case of BMP results, data of three substrates were not well-modeled by normal distribution: SM, LCMwS and CMC. The non-compliance of the results of BMP for SM and CMC substrates may originate from the low number of parallel measurements. The CMC substrate was heterogeneous, as represented in the values for standard deviation.

In terms of OM content, another two substrates were not well-modeled by normal distribution: CMwL2, and LCMwS. This non-compliance may also be due to their heterogeneity and the low number of parallel measurements.

However homogeneity of variance is also an assumption of the ANOVA analysis, therefore we applied Levene's test to test the null-hypothesis that the variance is equal across groups. Both of normality and homogeneity of variance need to be valid on data for applying ANOVA analsyis. Calculations of Levene's tests were done from means and also from medians. (p) values calculated from means are the following: BMP 0.001029, AT_7 0.02819, C/N 0.0001289 and OM 3.24E-04. (p) values calculated from medians are the following: BMP 0.3207, AT_7 0.194, C/N 0.5165 and OM 0.3059. These results of Levene's tests mean a violation for the whole amount of data, therefore one-way ANOVA could not be applied for analyzing.

Calculated parameters of Kruskal-Wallis tests on measurement data are the following. For BMP: H(chi2)=25.9, Hc(tie corrected)=25.9, p=0.002119. For AT_7: H(chi2)=27.51, Hc(tie corrected)=27.51, p=0.00115. For C/N: H(chi2)=26.79, Hc(tie corrected)=26.79, p=0.001516. For OM: H(chi2)=25.84, Hc(tie corrected)=25.87, p=0.002145. These result mean that there was a significant difference between sample medians in all of the measurements (BMP, AT_7, C/N, OM).

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Based on cluster analysis, there is direct correspondence between BMP and respiration intensity: the correlation is 0.9986 (*Fig. 5*).

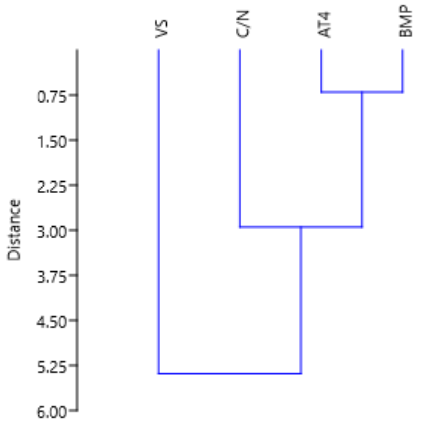

Figure 5. *Cluster analysis between the investigated parameters*

Discussion

Our results are in accordance with the results published in 2015 by Li et al. In these investigations, some substrates had an overly high or low C/N ratio. In such cases, it is expedient to mix the substrate with material which has other chemical characteristics in order to meet the criteria for biochemical processes and effective reactor operating (for example straw). The liquid manure substrates represented in this study were mixed with straw or shredded straw (LCMwS, LCMwSS, LSMwS). The BMP value of liquid manure mixed with previously shredded straw was lower than the same liquid manure mixed with straw (without shredding) (*Table 3*). However this result are in contrast with general findings in the literature (Neshat et al., 2017; Raj Paudel et al., 2017) concerning the effects of mechanical pretreatment. Probably the degree of shredding was small in the case of our experiment.

Fiber-characterised compounds (cellulose, lignin, hemi-cellulose) are hardly biodegradable (especially in terms of anaerobic degradation). This is the reason for the low BMP values of substrates which contain fibre in significant amounts (SM). However, prior aerobic treatment may increase methane production, but this was not investigated in the present study. Among the substrates investigated in this study, only SM contains fibre in significant amounts. According to Wu et al. (2017), cellulose and hemi-cellulose are significant components of swine manure. We could not measure fibre content, although the values for BMP and AT_7 for SM were notably lower than the results for cattle manures.

Based on the research of Krause et al. (2018) and Ventorino et al. (2018), weakly biodegradable carbohydrates are also present in the organic fraction of MSW in notable amounts. However, it is very important to take into consideration that the composition of MSW can be very different in certain regions. Besides this, the organic fraction in MSW is high in most cases – indicating that we can create value and products during the aerobic and / or anaerobic treatment of MSW. Because of its complex composition and heterogeneity, at least mechanical pretreatment is necessary before loading the reactors. Regarding weakly biodegradable compounds (carbohydrates of big molecular

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weight), we suggest mechanical-biological treatment prior to anaerobic digestion. In terms of the methane potential of the MSW substrate investigated in this study (325.71 Nml/gVS, SD=16.7%), this type of waste is definitely suitable for biogas production. This experience is totally in line with literature (Liu et al., 2008; Pecorini et al., 2016; Ventorino et al., 2018). On the other hand, municipal solid waste may contain organic and inorganic pollutants, therefore digestate obviously cannot be used in agriculture, nor after composting. Digestate from municipal solid waste must be incinerated. However, nowadays this process can be clean and environmentally safe, moreover, heat power and electricity can be produced from it. The MP values of the investigated substrates are in accordance with literature (Wang et al., 2012, 2017a; Hidalgo and Martín-Marroquín, 2015; Wei et al., 2015; André et al., 2016; Pecorini et al., 2016; Passos et al., 2017; Raj Paudel et al., 2017; Neshat et al., 2017; Siddique et al., 2018).

Correlation – with a good correlation coefficient – between data for respiration intensity and methane potential was not evident and was not written in the literature earlier. A basic difference between the two processes is the presence of oxygen, and therefore the difference in the microbial consortium of the biochemical process, thus it appears that degradation of the biodegradable fraction of the substrates occurred in two different biochemical ways. Despite this, we found linear correlation between the results of the two different examinations. This is an important fact, since respiration intensity tests with OxiTop® take a shorter time (4-7 days) than methane potential tests (at least one month).

Conclusion

According to the results of our laboratory-scale methane potential tests, the investigated substrates may be suitable for biogas production in full-scale conditions (in some cases, co-digestion may be needed). Hungary does have a significant potential in biogas production which is unutilized presently. The results of this study are especially important with regard to the establishment of a Hungarian scientific database which contains information about the biodegradability and other biological-chemical-physical properties of potential substrate materials. Henceforward we improve and enrich the database with further measurement data of substrates. It is also important to investigate the affects of pretreatments and certain adjustments (pH, OLR, etc.).

Considering the results of our investigation into correlation, there is a linear relationship between data concerning the aerobic and anaerobic parameters of wastes and agricultural byproducts. This finding can contribute to further developmental endeavors. The aerobic respiration intensity measurements need much less time than biomethane potential tests. Prising the suitability of potential substrates for biogas production can be done more effectively using the linear relationship represented in this study. However further research on this correspondence with bigger number of investigated samples is important (and with more replicates), and also pilot scale experiments are necessary.

Nowadays law on environment and on waste management is getting more and more strict in the European Union, so the results displayed may play an important role to support Hungarian developments on these fields.

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Author Contributions. Zsuzsanna Uveges (Assistant Researcher at NARIC IAE and Ph.D student at SZIU) planned the experiments and took part in executing BMP tests. She did the evaluation of measurement data and drew conclusions. Ádám Ragoncza, Dr. (CEO, Nawaro Inc.) was responsible for sample procurement and took part in executing BMP tests. He also gave consultations about exterior technical experiences. Zsolt Varga (Research Engineer, Profikomp Environmental Technologies Inc.) conducted all of the respiration intensity tests. László Aleksza, Dr. (CEO, Profikomp Environmental Technologies Inc.) ensured professional supervision and coordinating of work.

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EVALUATION OF COLOUR CHANGES, SURVIVAL RATE AND LIFE SPAN OF THE CONFUSED SAP BEETLE (*Carpophilus mutilatus*) (COLEOPTERA: NITIDULIDAE) IN DIFFERENT CONCENTRATIONS OF CARBON DIOXIDE (CO_2)

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Abstract. This study conducted in a rearing room (RR) (300-410 ppm) and in an open roof ventilation greenhouse system (ORVS) (800-950 ppm). No changes observed on *Carpophilus mutilatus* colouration after treatment in the ORVS. The survival rate increased from 61.59% in the F1 to 73.05% in the F2 generation reared in the RR. However, a sharp decline was observed from 27.05% in F1 to 1.5% in F2 in the ORVS. There was significant difference in number of individuals between RR and ORVS in F1 and F2 (F 12.76 p= 0.001< 0.05). The life span of F1 and F2 in the RR took about 46 days to complete; 7-21 days from adult to larvae stage, 5-15 days from the larval to pupal stage and 3-10 days from adult to pupal stage. Whereas in ORVS, F1 and F2 took about 30 and 22 days, respectively to complete their life cycles; that is 7-14, 7-14 days (adult to larval stage), 5-10, 0-5 days (larval to pupal stage) and 3-6, 0-3 days (pupal to adult stage), respectively. These data can be used to describe the changes in *C. mutilatus* due to global warming effects, as CO_2 could be one of the main factors affecting the growth and development.
Keywords: *morphology, biology, climate change, insects, global warming*

Introduction

The life cycle and life span of insects depend on a variety of factors including biotic and abiotic factors. Biological information regarding the life cycle, life span and mortality rates of important groups of insects, especially pests and natural enemies, are crucially important for the biological control program (Gurr et al., 2000; Khaliq et al., 2014). These parameters are usually studied under laboratory conditions where the insects are reared in cages closely representing their native habitat, with the advantage that the studied parameters and test animals are well controlled and the trials can be carried out more easily than in the field (Leppla, 2009). Insect rearing in the laboratory is important as the basis for developing a cost-effective large-scale mass-rearing in Integrated Pest Management (IPM) (Echegaray and Cloyd, 2013). Nitidulid beetles in the genus *Carpophilus* are important pests of dried fruit worldwide but have recently also become serious pests of ripening stone fruit in southern Australia (James and Vogele, 2000), as well as palms, including dates and oil palm fruits (Blumberg, 2008; Nor Atikah et al., 2019). Adult beetles damage fruit either by chewing through the skin, usually at the stem end, or entering from the sites of mechanical damage.

Taxonomists also implemented a rearing method as the first step for species identification, especially at the early stage. The insect larvae and pupae need to be reared into adult stage in order to identify the species correctly (Gibbs et al., 2015). However, insect rearing methods often require optimum conditions that correlate with their natural

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habitats so that the insects are healthy, free from disease and can reproduce for future generations. Therefore, factors such as environmental conditions, food sources and rearing chambers should be appropriate, simple and cost-effective (Cohen, 2018). The rearing of nitidulid beetles in the laboratory had been studied to determine their diets (Dowd, 1987; Peng and Williams, 1990; Dowd and Weber, 1991) and under different temperature conditions (De Guzman and Frake, 2007; Cuthbertson et al., 2008) to measure their survival, development and reproduction rates (Tsukada et al., 2005; Okada and Miyatake, 2007; Meikle and Patt, 2011).

Greenhouse gases such as carbon dioxide, ethane and nitrous oxide are often associated with global warming (Cox et al., 2000; Root et al., 2003; Ainsworth and Long, 2005; Meinshausen et al., 2009). CO_2 accounts for about 82% of greenhouse gas emissions compared to other gases (EPA's Greenhouse Gas Inventory 2017). The CO_2 gas that is trapped in the atmosphere captures heat and prevents it from being released, which can lead to global warming. This phenomenon affects biological changes in many organisms such as animals and plants (Hunter, 2001; Peñuelas et al., 2002; Mondor and Tremblay, 2010; DeLucia et al., 2012; Khaliq et al., 2014). Insects are expected to be more vulnerable and sensitive to environmental changes as they are ectothermic organisms and have a short lifespan (Bale et al., 2002). For example, long-term studies on Lepidoptera found that the life cycles of this insect group are shorter as a result of global temperature changes (Roy and Sparks, 2000; Peñuelas et al., 2002; Wallis de Vries and van Swaay, 2006). The distribution of the lepidopteran species from the family Geometridae was elevated by 67 meters of altitude over 42 years due to increasing annual temperatures (Chen et al., 2009).

Previous studies, mostly conducted between 1980-90, included reports on the interactions between herbivorous insects and plants exposed to high concentrations of CO_2 gas (Agrell et al., 2000; Chen et al., 2005; Dáder et al., 2016). The oviposition behaviour of *Cactoblastis cactorum* (Stange, 1997, 1999), the feeding behaviour of the larvae of *Diabrotica virgifera* and *Helicoverpa armigera* (Rasch and Rembold, 1994; Bernklau and Bjostad, 1998) and the host-searching behaviour of mosquitoes (Gillies, 1980; Eiras and Jepson, 1991), all were reportedly influenced by the concentration of CO_2 gas as one of the most important environmental parameters. Most of these studies have been conducted in specialized rooms, especially in greenhouses or closed rooms with specific controlled parameters (Kimball et al., 2002).

In most studies, the CO_2 concentration was usually raised to twice the ambient level, or monitored between 700-720 ppm (Hughes and Bazzaz, 2001; Veteli et al., 2002; Johns et al., 2003). Therefore, our objectives were to investigate the effect of increasing CO_2 levels on the colour changes, mortality rates, life cycle and life span of the oil palm pest, the nitidulid beetle *Carpophilus mutilatus* reared under two different conditions, i.e. within the Rearing Room (RR) and in the Open Roof Ventilation Greenhouse System (ORVS). The results are expected to be very useful in predicting the future changes in the population of nitidulid beetles, particularly the pest species of our oil palm crop (Blumberg, 2008; Nor Atikah et al., 2019).

Materials and Methods

Trap design

The nitidulid beetle trap in the field was designed using a 1.0 L transparent plastic container with a cover. A 10.2 cm x 5.1 cm window was cut 5 cm from the top edge of the trap and then covered with a muslin cloth for air ventilation. The trap was filled with

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dried soil up to 10 cm from its base. A rope was fastened to both sides of the trap to hang it onto a palm tree. Ripe bananas were used as food bait (*Figure 1*).

Figure 1. *Trap of C. mutilatus*

Cultural sampling of C. mutilatus

The sampling of *C. mutilatus* was conducted in September 2015 at an oil palm plantation in Felda Lui Muda, Negeri Sembilan, in the west coast of Peninsular Malaysia (GPS: latitude 3.013396 longitude 102.379504). The traps were used to obtain live samples of *C. mutilatus* for rearing process in the Rearing Room (RR). In the sampling area, only mature palm trees (aged 18 years and above) were selected for trapping. Three traps were hanged randomly 1 m above ground at 50 m apart from each other. The traps were inspected every three days and each trap was placed in a container containing ripe fruits and water. The trapped nitidulid beetles were taken to the Centre for Insect Systematics (CIS) laboratory, Universiti Kebangsaan Malaysia (UKM) for species identification. Only beetles from *C. mutilatus* species were selected for the rearing process in the rearing room.

Rearing process of C. mutilates in the Rearing Room (RR)

The rearing of *C. mutilatus* was conducted in the Rearing Room at the Biology Building, Faculty of Science and Technology, UKM following the method by Nur Hasyimah et al. (2018). The room temperature and humidity were controlled at 28-32 °C and 77-85%. A 19 x 14 x 12 cm transparent plastic container was used to rear the *C. mutilatus* specimen samples. The container was covered using a muslin cloth to prevent the beetles from escaping and a 3 cm depth of soil medium was placed inside each container to simulate the original natural habitat of *C. mutilatus* and to maintain moisture. In this study, the selected culture room was a closed system that was used exclusively for insect rearing and was also free from chemical contamination and other animals or insects. The room also had a good ventilation system to maintain the

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temperatures at 28-32 °C and 77-85% humidity. Controlling temperature and humidity at appropriate levels is important to prevent fungus and other diseases that could affect the rearing process of the beetles (Singh, 1982).

A total of 200 test beetles were used for the experiment with 20 randomly selected individuals of *C. mutilatus* species placed in 10 different containers (replicates). In this study, only 20 adult beetles were placed in each culture container to prevent overpopulation, which could lead to a reduction in survival rates due to injury and lack of phosphorylation. The 3 cm depth of soil medium in the culture container was important for the pupal stage of the nitidulid beetles, which would also involve the process of fertilizing the soil during the developmental stages of their life cycle (Myers, 2001).

Once the larvae reach their optimum growth, they will excavate up to 2.5-7.5 cm depth in the soil to provide space for the pupal stage (Capinera, 2001). Water was sprayed every three days to maintain moisture in the container and ripe banana fruit was provided as a food source. The diet or food source provided is an important factor for optimal growth. In this study, mature palm fruit was given to the adult beetles as a food source. Palm fruit also provided a suitable medium for egg-laying and larval growth of the nitidulid beetles. The female would deposit its eggs in the mesocarp and in the early stage of development the larvae would eat and crawl within the fruit before exiting and searching for soil to continue with further pupal development (Glazer et al., 2007). Adult beetles would enter the palm fruit on the calcareous side and obtain food through the fibers (Blumberg, 2008).

The temperature and humidity parameters were monitored every three days using a Digital Hygrometer, i.e. a MEXTECH TM-2 model (Global Instruments, new Delhi, India) while CO_2 concentration was monitored using a CO_2 Meter, i.e a 8802-EN-00 version (BENETECH, China). The CO_2 concentration in the RR was between 300-410 ppm.

Rearing process of C. mutilatus in the Open Roof Ventilation Greenhouse (ORVS)

The same rearing process as above was used for *C. mutilatus* trial specimens placed in ORVS following the method by Nur Hasyimah et al. (2018). The temperature and humidity of the ORVS were controlled using a computerized system and ranged between 25-45 °C and 37-87%, respectively. Abiotic parameters such as temperature, air humidity and CO_2 concentration were monitored every three days through readings on the system's screen display. In ORVS, the CO_2 treatment was given daily from 9-11 am. Pure CO_2 spraying was continued for two hours at a concentration of 800–950 ppm. After two hours, the CO_2 levels inside the ORVS was almost equal to the CO_2 levels outside. The gas was supplied through a cylinder connected to the air-conditioning system for open-air chambers and vents. The CO_2 concentration was regulated by dilution with air produced by an air blower. CO_2 analysers were used to monitor CO_2 concentrations and the automatically controlled ORVS systems monitored by the Climate Change Institute (IPI), UKM.

Monitoring the life cycle and life span of C. mutilatus

The life cycle of *C. mutilatus* was monitored daily and the emergence of the first instar larvae was recorded. The larvae were promptly isolated into different containers containing the soil medium as described earlier. Larvae that had turned into pupae were removed from the soil and placed in different containers until they became adult beetles. Observations were conducted throughout the life cycle of the beetle up to the adult stage. The number of individuals at each stage (survival rates), lifespan and body colour changes

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were observed and judiciously recorded. In each stage, five individuals were selected and the colour changes were observed every two days under a microscope.

Data analysis

Two-ANOVA was used to determine any significant differences and variations between the test individuals observed between RR and in ORVS and in the F1 and F2 generations. The analysis was implemented by using Minitab17.

Results

Effect of the different CO_2 concentrations on the external morphology (body colour) of C. mutilatus in RR and ORVS

Life cycle was observed starting from the instar larval stage 1. No obvious change in body colour was observed in *C. mutilatus* reared in RR and in ORVS, even at elevated CO_2 levels. The short life cycle and morphology of *C. mutilatus* from the larval stage are as follows;

The larvae were milky-white colour on the first day of hatching and would turn into yellow when they reach the active stage and started searching for food (*Figures 2a-b*), while the pupae were white in the early stage, and would turn into brown upon reaching the final stage, and before emerging as adults (*Figures 2c-d*). Adult beetles of this species were dark brown or deep brown in colour (*Figures 2e-f*).

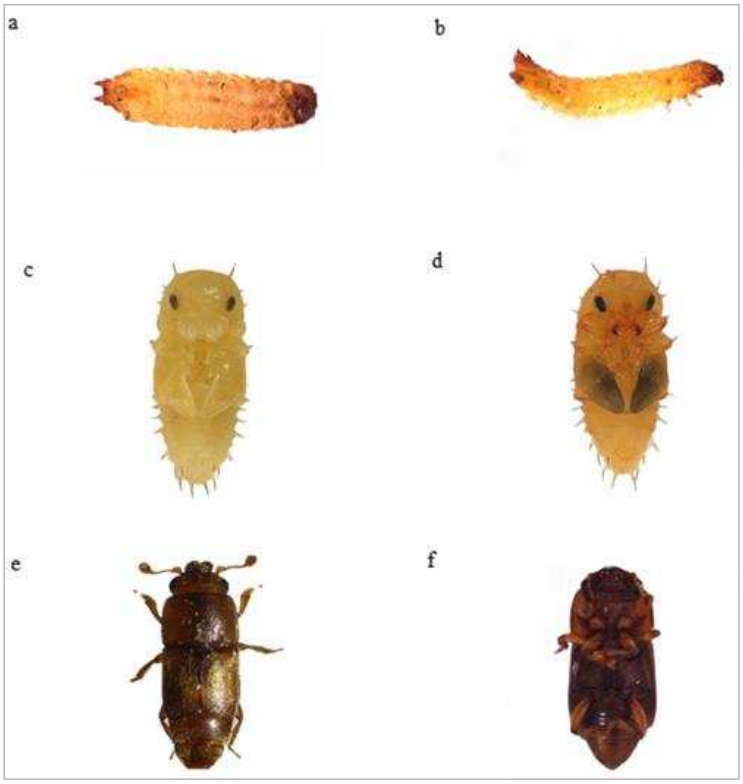

Figure 2. *External morphology of C. mutilatus a) Larval stage (ventral) b) Larval stage (c) Early stage d) Pupa stage e) Adult stage (dorsal) f) Adult stage (ventral), taken from the ORVS system*

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Effect of different CO_2 concentrations on the number of individuals, life cycle and life span of C. mutilatus

The number of live *C. mutilatus* individuals at each developmental stage was recorded in the RR and ORVS systems. The CO_2 levels were between 300-410 ppm in the RR, and 800-950 ppm in the ORVS. In the RR, the average live individuals obtained for F1 was 2283 at the larval stage, 1727 at the pupal stage, and 1406 individuals that had successfully emerged into adults. For the F2 generation, from 1406 F1 adults, 5162 larvae, 4077 pupae and 3771 adults had successfully emerged (*Table 1*). In the ORVS, the average live individuals obtained for F1 generation was 244 larvae, 99 pupae and 66 adults. As for F2, 66 adults from F1 had produced 34 larvae and 13 pupae, while only one pupa had successfully emerged into the adult stage. In the RR, the species survival rate in the F1 generation was 61.59% and had increased to 73.05% in the F2 generation. The percentage of species survival rate in ORVS showed a sharp decline from 27.05% in F1 to 1.5% in the F2 generation. There was a significant difference in the number of individuals between RR and ORVS in the F1 and F2 generations ($F\ 12.76$, $p=0.001$, $p < 0.05$) (*Figure 3*).

Table 1. *Number of C. mutilatus individuals of F1 and F2 in the RR and ORVS systems*

Generation		1st Generation (F1)			2nd Generation (F2)		
Room	**CO_2 levels (ppm)**	**Larvae**	**Pupae**	**Adult**	**Larvae**	**Pupae**	**Adult**
RR	300-410 ppm	2283	1727	1406	5162	4077	3771
ORVS	800- 950 ppm	244	99	66	34	13	1

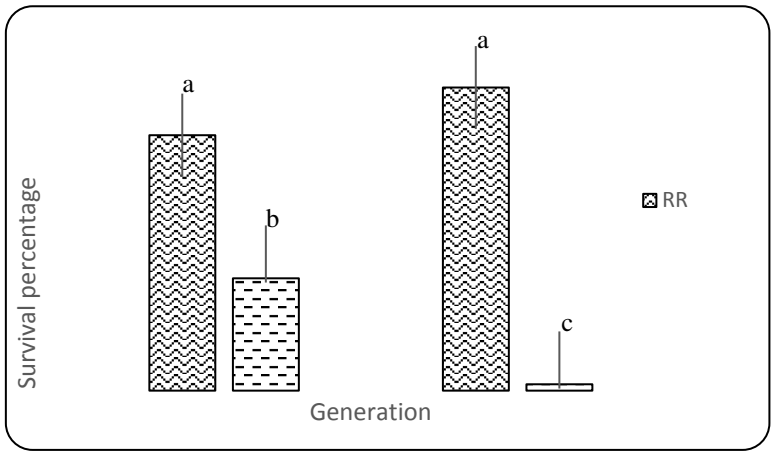

Figure 3. *Percentage of C. mutilatus F1 and F2 survival rates in RR and ORVS systems*

The life span of *C. mutilatus* F1 and F2 in RR took about 46 days to complete. In the RR, *C. mutilatus* took 7-21 days from adult to larval stage, 5-15 days from larval to pupal stage, and 3-10 days from pupal to adult stage. However, in ORVS, the F1 took about 30 days to complete its life cycle, i.e. 7-14 days from adult to larva, 5-10 days from larval to pupal stage, and 3-6 days from pupal to adult stage. However, in the F2 generation, *C. mutilatus* in ORVS had a shorter life cycle of 22 days, i.e. 7-14 days from adult to larval stage, 0-5 days from larval to pupal stage, and 0-3 days from pupal to adult stage (*Figure 4*).

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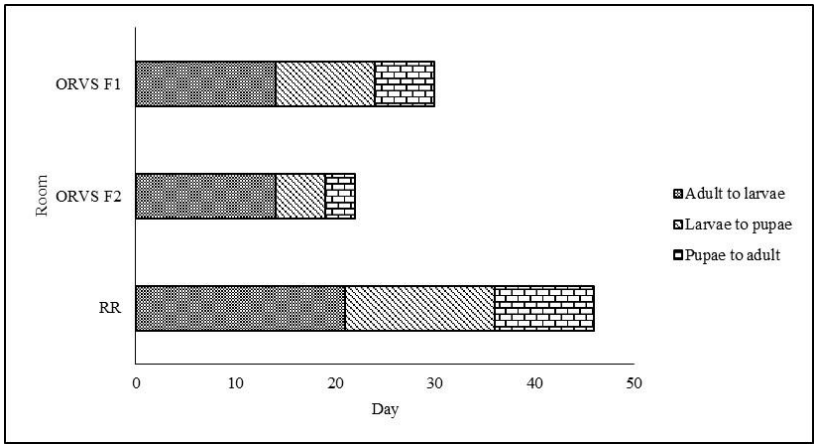

Figure 4. *The lifespan of C. mutilatus in RR and ORVS according the developmental stages*

According to *Table 2*, the number of individuals at each life stage in RR and ORVS also significantly differed. In RR, the adult to larva stage showed the highest number of individuals (1549 of F1 and 3147 of F2) compared to ORVS, recorded on day 8-14. In ORVS, the highest number of individuals recorded was 187 of F1 and 30 of F2, on days 0-7. The larval stage in RR was also recorded on days 6-10, with 994 and 2715 individuals of F1 and F2, respectively, while in ORVS the highest number of individuals was recorded on day 0-5, with 86 of F1 and 13 of F2, respectively. However, the pupal to adult stages in both RR and ORVS systems recorded the highest number of individuals on day 0–3, even though only one individual was recorded in F2 of ORVS.

Table 2. *Number of individuals according to the developmental stages of F1 and F2 of C. mutilatus in RR and ORVS systems*

		Growth development (Days)								
Room	**Generation**	**Adults to larvae**			**Larvae to pupae**			**Pupae to adults**		
	Day	**0-7**	**8-14**	**15-21**	**0-5**	**6-10**	**11-15**	**0-3**	**4-6**	**7-10**
RR	F1	592	1549	142	556	994	117	992	305	109
	F2	1536	3147	479	1217	2715	145	2774	822	175
ORVS	F1	187	57	0	86	13	0	61	5	0
	F2	30	4	0	13	0	0	1	0	0

Discussion

According to Hunter (2001), the Free-Air Carbon Dioxide Enrichment (FACE) and Open-Top Chamber (OTC), i.e. resembling ORVS, are two effective systems for studying long term interactions of plants and insects. In both systems, the CO_2 has been raised abnormally for the purpose of the ecological study, which is believed to have significant influence on the distribution, abundance and performance of insects that feed on plants (herbivorous insects). Furthermore, the elevated levels of CO_2 in the atmosphere could exert various effects on many insects either directly or indirectly, leading to marked changes in their life cycle, physiological and behavioural aspects (Lake and Wade, 2009; Yuan et al., 2009).

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From this study, no colour change was detected after exposure of the test beetle individuals to high concentrations of CO_2 in the ORVS system. According to Zeuss et al. (2014), colour changes in butterflies would involve a change from lighter to darker coloured in cooler climate, and vice versa at warmer climates when exposed to higher CO_2 levels. In our study, despite the different CO_2 levels in the culture chambers, no changes were observed based on the morphology (body colour) of each stage of the life cycle. According to Roulin (2014), the increment of CO_2 led to a rise in temperature together with the UV radiation. However, the *C. mutilatus* has been categorised as a dark beetle species which is not affected by changes in CO_2 levels due to its resistance by the production of melanin. This pigment has become a useful indicator to study the adaptation of insects to climate change. The life cycle of the *C. mutilatus* species occurs mainly inside the ripe oil palm fruit to complete the larval stages before hibernating in the soil during the pupal stage (Nor Atikah et al., 2019).

Furthermore, previous study results indicated that increase in CO_2 could also reduce insect abundance by 22%, increase the nutritional rate and life span of certain insects by 17% and 4%, as well as lowering the growth rate and weight of pupae by 9% and 5% (Stiling and Cornelissen, 2007). In our study, significant differences in the number of individuals or abundance in the RR and ORVS systems indicate that high CO_2 concentrations could have induced changes in the hatching rate of the nitidulid beetle, *C. mutilatus*. This is proven by the differences in the survival rates of this species in both culture systems.

The survival rate of *C. mutilatus* in ORVS was lower than in RR, where the rate had also decreased in the F1 and F2 generations. However, the results of this study differ from studies on *C. dimidiatus* by Odeyemi et al. (2004) and *C. hemipterus* by Gbaye and Odeyemi (2005). Dáder et al. (2016) reported that the aphid, *Myzus persicae* showed lower survival rates from eggs to adulthood and shorter larval stages in ambient conditions, which was contradictory to our own findings. The egg stage of *C. dimidiatus* showed the highest level of tolerance to high levels of CO_2 compared to the other stages in its life cycle. The mortality rate of the eggs was 13.3% lower than that of the larvae and pupae at 50.0% and 33.3%, respectively, while 100% of the adult beetles died after exposure to high CO_2 levels for six hours (Odeyemi et al., 2004).

Carpophilus hemipterus also recorded the lowest egg mortality rate of 26.7% compared to 86.7%, 100.0% and 90.0% in larval, pupal and adult stages, respectively. However, in our study the egg stage was not successfully obtained from the emerged adults. Most insect eggs require less oxygen to survive compared to the larval, pupal and adult stages. The egg tolerance rate is due to an impermeable layer of egg coral structure that is not present at other stages (Chapman, 1971). In this study, the eggs stages were unsuccessfully collected and thus, not evaluated. However, the percentage of emergence in the pupal and adult stages were higher in larval, continued with pupal and then in adult stages in both conditions and in both generations.

Insects adapt differently when their habitat is changed. At higher CO_2 concentrations in ORVS, the life span of *C. mutilatus* was shorter than in the RR. The *C. mutilatus* F1 and F2 generations also survived, the second generation having a shorter life span than the first generation. The effect of increasing CO_2 concentration either in ambient conditions or in the system is usually associated with the interactions of plants and insects. This is because by increasing CO_2 the nitrogen cycle is affected and thus, resulting in a decrease in the C:N ratio in the plants (Ainsworth and Long, 2005; Oehme et al., 2013; Ryan et al., 2014). As a result of the changes, macronutrients such as calcium, magnesium

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and phosphorus would decrease due to the lack of water supply from the soil (Taub and Wang, 2008). Therefore, the lack of nutrients and plant quality due to the changes in CO_2 concentration would indirectly affect insects through their nutritional responses (Hughes and Bazzaz, 2001; Himanen et al., 2008; Stiling et al., 2013).

Our results showed that the life span of *C. mutilates* was shorter in ORVS compared to the RR, indicating that the life span of the beetles treated with elevated CO_2 above the ambient levels had been shortened, and this effect was not limited to F1 but was also observed in the F2 generation. However, this result was contradictory to those of previous studies, which reported that insects tended to reduce their growth rates and extend their life span in order to adapt to high CO_2 levels (Goverde and Erhardt, 2003). Insects from *Helicoverpa armigera* and *Orygia leucostigma* species also reportedly extended their lifespan when exposed to elevated CO_2 levels (Agrell et al., 2000; Chen et al., 2005).

The rise of CO_2 levels as a greenhouse gas (GHG) would affect the atmospheric temperature, and both abiotic factors could significantly affect the growth and development of many insect species (Agrell et al., 2000; Goverde and Erhardt, 2003; Mondor and Tremblay, 2010). Although some species are not affected by CO_2 changes, but as exothermic organisms, most insects are more sensitive and respond quickly to changes in temperature that affect their life cycles and growth rates (Bale et al., 2002). Insects also respond to rising temperatures by increasing their rates of growth, reproduction and mortality (DeLucia et al., 2012).

The development of nitidulid beetle species, namely *C. humeralis*, *C. hemipterus* and *C. mutilatus* at different temperatures had been studied by James and Vogele (2000), who reported that the lifespan of these three species were shorter at higher temperatures, i.e. 14-18 days at 32.5 °C compared to 47-65 days at 20 °C. These findings, however, contradictory to our results that in RR under ambient conditions (28-32 °C), the total lifespan of *C. mutilatus* was 46 days, whereas for ORVS it was 30 days. The mortality rate for each species was the lowest at temperature between 25-30 °C. The life span of *C. hemipterus* also exhibited similar effects when exposed to different temperature ranges where the growth rates for eggs, larvae and pupae were longer at low temperatures (18 °C) than at high temperatures, (30 °C). However, the mortality rate has increased with increasing temperature (Tsukada et al., 2008). At low temperatures of -10 °C to -8 °C, the mortality rates of *C. mutilatus* and *C. hemipterus* were higher with 100% mortality (Donahaye et al., 1991).

Conclusion

The nitidulid beetle *C. mutilates,* was selected as a model species to evaluate the colour changes, survival rate and life span of *C. mutilatus* (Coleoptera: Nitidulidae) in different concentrations of carbon dioxide (CO_2). This species is interesting because its life cycle from the egg to larval stages is spent inside the oil palm fruit. From this study, no significant changes were observed in the coloration of the larval to adult stages of *C. mutilatus* after exposure to treatment with high ambient CO_2 levels. In the RR, the species survival rate in the F1 generation was 61.59% and had increased up to 73.05% in the F2 generation. However, the survival rate in ORVS showed a sharp decline from 27.05% in F1 to 1.5% in F2 generation. Two-ways ANOVA shows there was significant difference in number of individuals between RR and ORVS in F1 and F2 (F 12.76, $p=0.001$, $p < 0.05$). The life span of F1 and F2 of *C. mutilatus* in RR took about 46 days to complete; i.e. 7-21 days from adult to larval stage, 5-15 days from larval to pupal stage, and 3-10

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days from adult to pupal stage. However, in ORVS, the F1 and F2 generations of *C. mutilatus* took about 30 and 22 days, respectively, to complete their life cycles; i.e. 7-14, 7-14 days from adult to larval stage, 5-10, 0-5 days from larval to pupal stage, and 3-6, 0-3 days from pupal to adult stage, respectively. This data can be used to describe the changes in *C. mutilatus* caused by global warming effects, as CO_2 could be one of the main factors affecting the species development. Further ecological study in the field (oil palm plantation) is suggested to relate the growth and development of the nitidulid beetles with other abiotic factors such as temperature, humidity, light intensity and also the most important parameter, i.e. CO_2 concentration in order to validate the effects on and biological responses of *C. mutilatus* to climate changes in the natural environment.

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EFFECTS OF SEWAGE SLUDGE APPLICATIONS TO AGRICULTURAL SOIL ON THE BIOCHEMICAL PARAMETERS OF FABA BEAN (*FABA SATIVA* BERNH.), WHEAT (*TRITICUM AESTIVUM* L.), SPINACH (*SPINACIA OLERACEA* L.) AND CUCUMBER (*CUCUMIS SATIVUS* L.) CROPS

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Abstract. This study aimed to assess the sewage sludge (SS) amendments to agricultural soil on the biochemical composition of four plants (faba bean, wheat, spinach and cucumber). The applied SS rates were 0, 10, 20, 30, 40 and 50 g/kg soil. Plant leaves 45 days after SS treatments were used in biochemical parameter analyses. Starch decreased in spinach from 179.34 to 41.38 mg/g DW with SS treatments and increased in faba bean, wheat and cucumber at 10, 20, 30 and 40 g/kg rates. Water soluble carbohydrates and total non-structural carbohydrates declined in the four plants with SS treatments. Total lipids increased in faba bean and spinach up to 41.94 and 7.89 mg/g FW, respectively, at the SS amendment of 50 g/kg, while in wheat and cucumber the increase was detected only at 40 and 50 g/kg rates. In spinach, the accumulation of proline started at SS treatment of 10 g/kg, while, for wheat and cucumber the increase of proline was started at 20 g/kg SS. The highest proline accumulation was 0.96 mg/g FW in spinach at 50 g/kg SS rate. SS did not affect phenol content in faba bean and spinach with any treatment, whereas, the phenol declined in wheat and cucumber.
Keywords: *carbohydrates, economic plants, environmental pollution, phenol, proline*

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Introduction

Application of sewage sludge (SS) in the agricultural sector as soil fertilizers is in several countries (Singh and Agrawal, 2008 and 2010). The municipal SS is a source of macro- and micro-nutrients for crops cultivated in SS amended agricultural soil (Ahmed et al., 2010; Abdul Khaliq et al., 2017). Soil amendment with SS at different rates has improved the growth and yield of several crops including faba bean (*Faba sativa* Bernh.), wheat (*Triticum aestivum* L.), spinach (*Spinacia oleracea* L.) and cucumber (*Cucumis sativus* L.) (Eid et al., 2017a, b, 2018, 2019). Bioaccumulation and translocation of certain heavy metals in tissues of crops cultivated in soil amended with sewage sludge were analyzed in several plants (Kabata-Pendias, 2011; Bourioug et al., 2014; Eid et al., 2020a, b).

Chemical constituents of plants grown in soils amended with SS play a vital role in plant physiology as biochemical responses to abiotic stresses (Sharma et al., 2018). Carbohydrates are major components in plant tissues since they are related to the primary metabolism. Carbohydrates content has been monitored in several crops when cultivated in SS amended soils (Han et al., 2004). Elevation of nitrogen, phosphorus and potassium content and other macro-nutrients in plants grown in agricultural soil supplemented with SS has been reported in several studies (Hue, 1988; Bourioug et al., 2014; Kępka et al., 2016). Proline content has been investigated wildly as protectant to environmental toxicity stress in many crops (Boudjabi et al., 2015). Phenolic compounds in plants such as *Beta vulgaris* and *Albizia lebbek* were reported in response irrigation with municipal waste water and heavy metal toxicity (Tripathi and Tripathi, 1999; Singh and Agrawal, 2010). Biochemical composition analyses of the four economic plants under investigation is essential since leaves of spinach, seeds of faba bean, grains of wheat and fruits of cucumber are used for human consumption and the hay produced from wheat and faba bean is used for animal feeding.

The objective of the current investigation is to determine the effect of SS application to agricultural soils on the content of starch, water soluble carbohydrates (WSC), total non-structural carbohydrates (TNC), total lipids, proline and phenol in leaves of faba bean, wheat, spinach and cucumber plants.

Materials and methods

Soil-sewage sludge treatments

Four plant species, namely, faba bean (*Faba sativa*), wheat (*Triticum aestivum*), spinach (*Spinacia oleracea*) and cucumber (*Cucumis sativus*) were used to assess the effects of soil amendments with SS on biochemical composition of leaves of the tested crops. The rates of soil-sewage sludge amendments were zero (control), 10, 20, 30, 40, and 50 g SS/kg soil. The detailed analysis the physic-chemical properties of the applied SS used in the current study was previously performed; salinity 2.07; pH 6.38; organic matter 65.1%; the heavy metals concentrations were Cd (1.17 mg/kg), Co (27.9 mg/kg), Cr (179.1 mg/kg), Cu (162.6 mg/kg), Fe (25.4 mg/g), Mn (595.7 mg/kg), Ni (138.7 mg/kg), Pb (671.2 mg/kg) and Zn (667.6 mg/kg) (Eid et al., 2017a, b, 2018, 2019).

Plant materials and growth conditions

The broad bean, wheat, spinach (Superdane 7 Star, F1 Hybrid, Denmark) and cucumber seeds were obtained from local markets in Abha City. The seeds were planted

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in plastic pots filled with 4 kg of each respective SS-soil mixture. Three biological replicates were used. The broad bean and wheat were grown for 80 days, while, spinach and cucumber were grown for 50 days. *Figure 1* shows the experimental setup of the four plants grown in pots represent the rates of sewage sludge-soil amendment rates. The greenhouse conditions were natural day/night regime and the plants were watered when needed (Eid et al., 2017a, b, 2018, 2019).

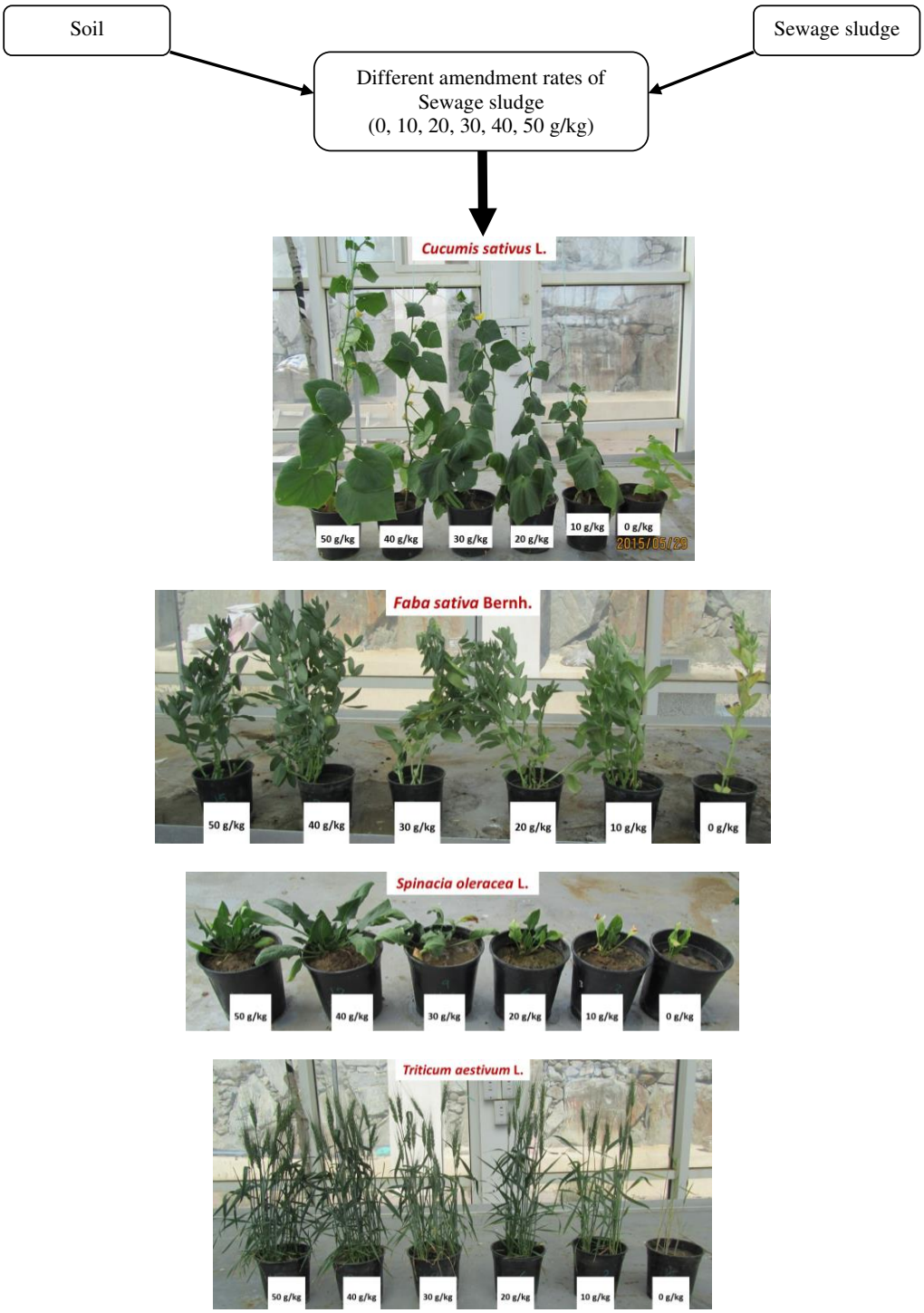

Figure 1. *A demonstration of the experiment steps with the four plants in pots represents the treatments of sewage sludge-soil amendment rates*

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Biochemical analyses

Fully expanded fresh leaves from all the replicates for four species were sampled manually at 45 days, first washed under the running water and some kept in freezer at −20 °C for further estimation of total lipids, proline and phenol. Some leaves were dried in the oven at a temperature of 60 °C. Dried leaf materials were ground using a metal-free plastic mill into particles less than 0.4 mm for carbohydrate analysis. Total lipids concentration was measured following the method of Byreddy et al. (2016). Proline concentration was measured by the method of Bates et al. (1973). Total phenol concentration was measured following the method of Bray and Thorpe (1954). Water-soluble carbohydrates (WSCs) were extracted from 0.25 g of ground material with hot distilled water, while total non-structural carbohydrates (TNCs) were extracted from another 0.25 g of ground material using diluted H_2SO_4 following the method of Smith et al. (1964). WSC and TNC concentrations (mg/g DW) in the extracted solutions were measured by spectrophotometry using the phenol-H_2SO_4 colorimetric method as described in Granéli et al. (1992), where the standard was glucose. The starch concentration was calculated according to *Equation 1:*

$$\text{Starch (mg/g DW)} = 0.9 \times (\text{TNC conc. (mg/g DW)} - \text{WSC conc. (mg/g DW)}) \quad \text{(Eq.1)}$$

where TNC is the total non-structural carbohydrates and WSC is the water-soluble carbohydrates

Data analyses

Because absorption, accumulation and tolerance to heavy metals vary between different crops and at different levels of SS amendments (see Eid et al., 2017b) and hence heavy metal effects on the concentrations of total lipids, proline, phenol, WSC, TNC, and starch concentrations may vary between these different crops. Therefore, significant differences in the total lipids, proline, phenol, WSC, TNC, and starch concentrations between the spinach, cucumber, faba bean and wheat plants grown in soils amended with different rates of SS were evaluated using a two-way analysis of variance (ANOVA). The data were examined for their homogeneity of variance and normality of distribution, and when necessary, the data were log-transformed before a two-way ANOVA was performed. Significant difference between means among the five SS treatments were identified using the Tukey's HSD test at $p < 0.05$. Statistica 7.1 was used to process all of the statistical analyses (Statsoft, 2007).

Results and discussion

Application of sewage sludge to the agricultural soil has been reported to increase the growth and biomass of several crops. The growth of the four plants used in the current study was promoted when grown in soil amended with SS (Eid et al., 2017a, b, 2018, 2019). The biochemical parameters of leaves from the treated plants were determined. *Figure 2* illustrates the effect of SS treatments on starch content (mg/g DW) in faba bean, wheat, spinach and cucumber leaves. Spinach showed the highest content of starch as compared to faba bean, wheat and cucumber, however, the treatment with SS decreased the starch concentration significantly in spinach leaves with all SS amendment treatments from179.34 to 41.38 mg/g DW. For faba bean there

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was an increase in starch concentration at all SS treatments with obvious significant increase at amendments rates of 10 and 20 g/kg with concentrations of 73.07 and 89.56 mg/g DW, respectively. Starch concentration in leaves of wheat and cucumber increased at treatment rates of 10, 20, 30 and 40 g/kg with the highest concentrations at SS treatment of 20 g/kg with 100.42 mg/g DW of wheat and 95.86 mg/g DW of cucumber then starch concentrations were declined significantly at the highest SS treatment rate of 50 g/kg. Stimulation of carbohydrates as response to SS application has been reported in bean (Zeid and Abou El Ghate, 2007). The enhancement of starch synthesis in plants treated with sewage sludge could be a result of increasing photosynthetic pigments, i.e. chlorophyll, with the organic matter, macro- and micro-nutrients in SS which result in increasing of the starch biosynthesis (El-Maghraby and Gomaa, 1992).

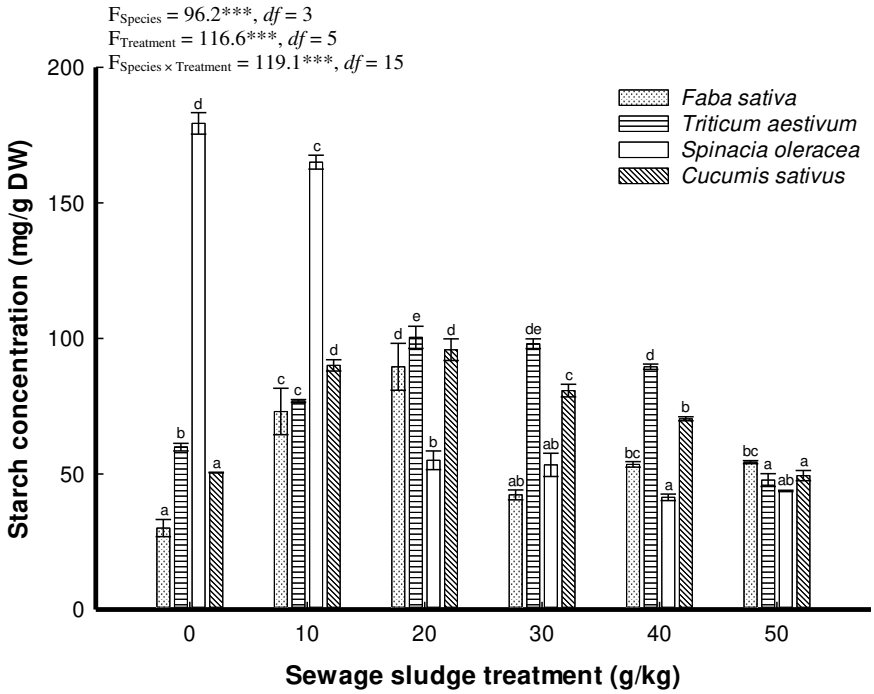

Figure 2. *Starch concentration (mean ± standard error) in leaves of the four plant species Faba sativa, Triticum aestivum, Spinacia oleracea and Cucumis sativus grown in soil amended with different sewage sludge treatment rates. Means (for each plant species) with different letters are significantly different at $p < 0.05$ according to Tukey's HSD test. ***: $p < 0.001$, df: degrees of freedom*

The concentrations of water soluble carbohydrates (*Fig. 3*) and total non-structural carbohydrates (*Fig. 4*) were inhibited in the four plant leaves due to soil amendment with SS at all treatment rates. It has been reported that total organic carbon was increased in response to SS treatments (Abdul Khaliq et al., 2017). Soluble sugar contents in sunflower plant grown in SS amended soil were increased significantly and suggested to play role in defense mechanisms that triggered by heavy metals in the SS (Belhaj et al., 2016). In maize plants, sugars content, proline and antioxidant enzymes activity were increased when the plant irrigated with sewage water (Abdel Latef and Sallam, 2015).

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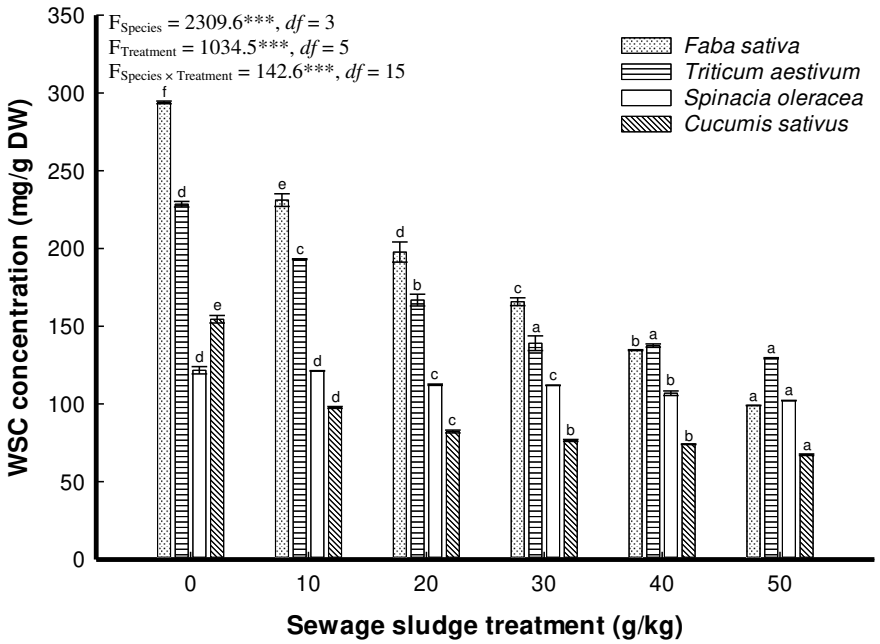

Figure 3. *Water soluble carbohydrates (WSC) concentration (mean ± standard error) in leaves of the four plant species Faba sativa, Triticum aestivum, Spinacia oleracea and Cucumis sativus grown in soil amended with different sewage sludge treatment rates. Means (for each plant species) with different letters are significantly different at $p < 0.05$ according to Tukey's HSD test. ***: $p < 0.001$, df: degrees of freedom*

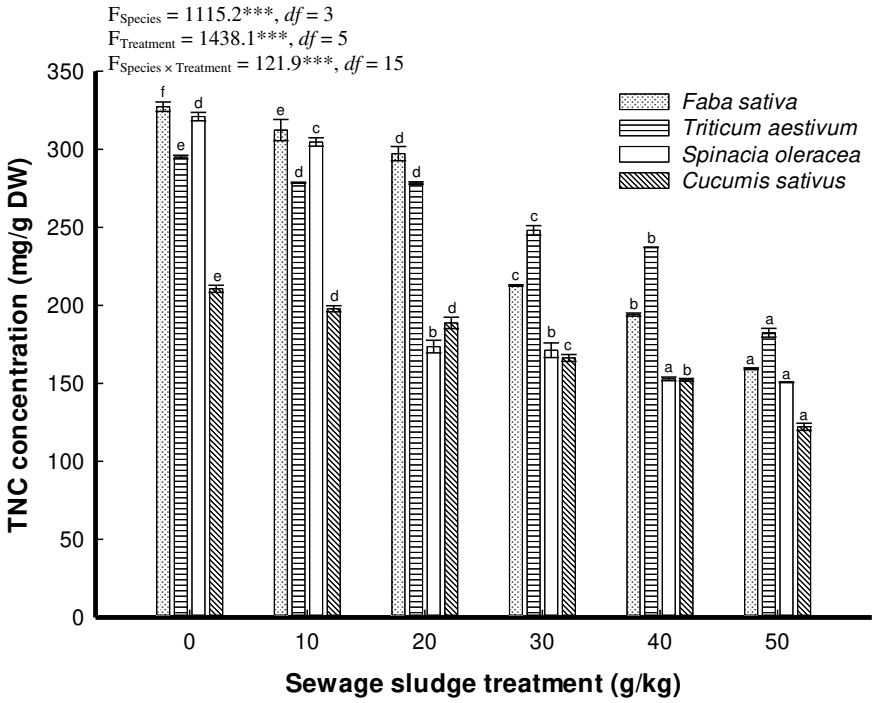

Figure 4. *Total non-structural carbohydrates (TNC) concentration (mean ± standard error) in leaves of the four plant species Faba sativa, Triticum aestivum, Spinacia oleracea and Cucumis sativus grown in soil amended with different sewage sludge treatment rates. Means (for each plant species) with different letters are significantly different at $p < 0.05$ according to Tukey's HSD test. ***: $p < 0.001$, df: degrees of freedom*

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Total lipids concentrations in leaves of the four analysed plants are demonstrated in *Figure 5*. In both faba bean and spinach, the increase of total lipids was gradual and the significant levels were detected on SS treatment rates of 20 g/kg and above. For the wheat and cucumber, the significant increase of total lipids content in leaves was observed only at high SS treatment rates of 40 and 50 g/kg. Increasing of lipid peroxidation and proline content has been detected in tomato seedling grown in soil amended with SS (Elloumi et al., 2016). Several studies reported detoxification processes after exposure to SS which indicated using lipid peroxide assays in plant tissues and associate that with uptake of heavy metals from SS (Wyrwicka and Urbaniak, 2016).

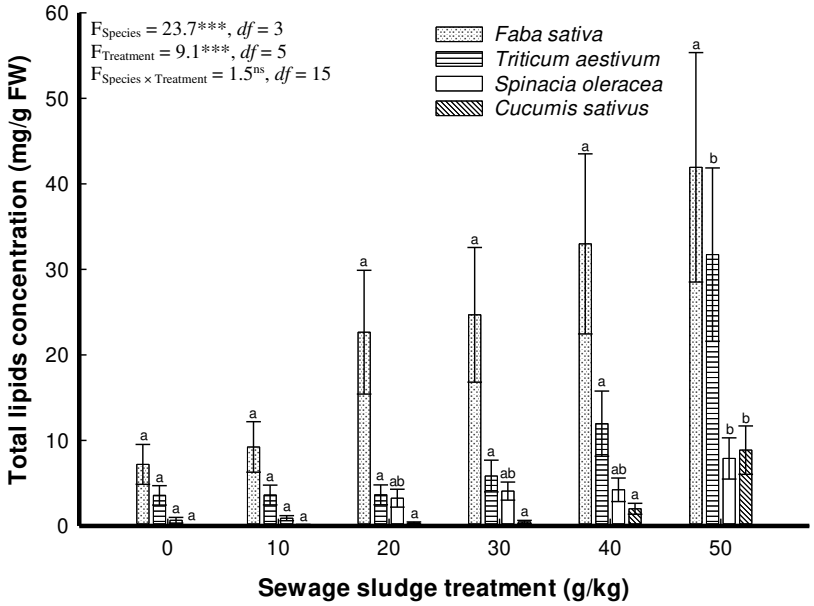

Figure 5. *Total lipids concentration (mean ± standard error) in leaves of the four plant species Faba sativa, Triticum aestivum, Spinacia oleracea and Cucumis sativus grown in soil amended with different sewage sludge treatment rates. Means (for each plant species) with different letters are significantly different at $p < 0.05$ according to Tukey's HSD test. ***: $p < 0.001$, ns: not significant (i.e., $p > 0.05$), df: degrees of freedom*

The amino acid proline is known to be accumulated in plant tissues as response of abiotic stresses on several crops (John et al., 2009). Proline concentration in leaves of the four plants was assessed (*Fig. 6)*. The proline concentration was in the same level in control plants. In faba bean, the increase of proline was slight at all SS treatment rates. The application of SS amendment rates stimulated the biosynthesis and accumulation of proline in leaves of wheat, spinach and cucumber plants. In spinach, the significant increase level of proline started at SS treatment of 10 g/kg with concentration of 0.39 mg/g FW to 0.96 mg/g FW at SS treatment 50 g/kg, while, in case of wheat and cucumber the significant increase of proline was detected at 20, 30, 40 and 50 g/kg SS treatment rates. It has been reported that proline may play a role in protection of enzymes, cellular organelles and adjust the osmotic pressure of plant cells under environmental stress conditions (Dhir et al., 2004; Tantrey and Agnihotri, 2010). The stress-protective role of proline was documented in wheat plants treated with cadmium (Asgharipour et al., 2011). Proline was accumulated in wheat plants cultivated in desert reclaimed soil amended with SS (Mazen et al., 2010).

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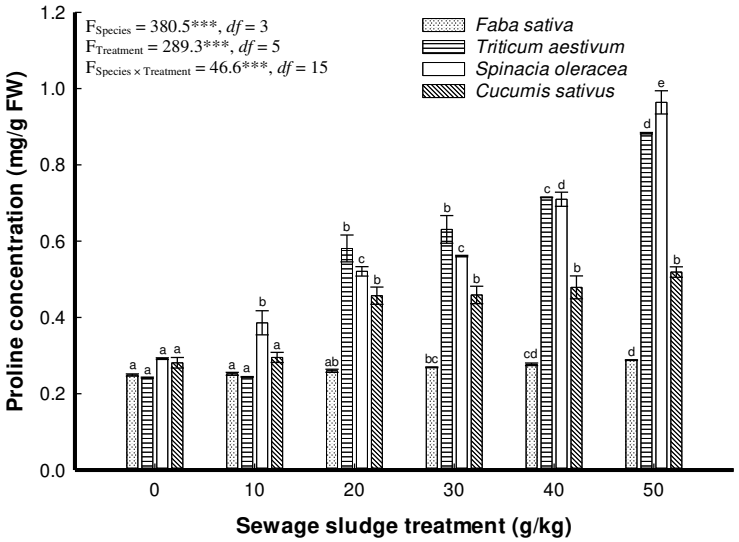

Figure 6. *Proline concentration (mean ± standard error) in leaves of the four plant species Faba sativa, Triticum aestivum, Spinacia oleracea and Cucumis sativus grown in soil amended with different sewage sludge treatment rates. Means (for each plant species) with different letters are significantly different at p < 0.05 according to Tukey's HSD test. ***: p < 0.001, df: degrees of freedom*

Figure 7 presents the relationship between SS treatment rates and phenol concentrations in leaves of faba bean, wheat, spinach and cucumber. SS amendment did not affect the phenol content in faba bean and spinach at all treatment rates. On the other hand, phenol concentration in leaves of wheat and cucumber was declined with SS treatment. The decline was at rates of 40 and 50 g/kg in wheat and was at all SS treatment rates in case of cucumber plant.

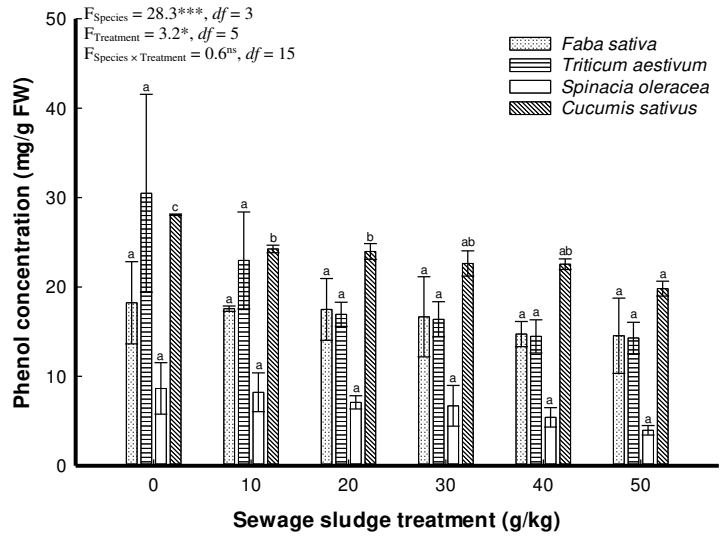

Figure 7. *Phenol concentration (mean ± standard error) in leaves of the four plant species Faba sativa, Triticum aestivum, Spinacia oleracea and Cucumis sativus grown in soil amended with different sewage sludge treatment rates. Means (for each plant species) with different letters are significantly different at p < 0.05 according to Tukey's HSD test. ***: p < 0.001, *: p < 0.05, ns: not significant (i.e., p > 0.05), df: degrees of freedom*

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Phenolic compounds have been known in plant defense mechanisms against abiotic stresses such as heavy metal toxicity and to biotic stresses such as phytopathogenic fungi and bacteria in several plants (Singh and Agrawal, 2010; Daayf et al., 2012; El-Bebany et al., 2013). Thus, diminishing phenol content in plants grown in soil amended with SS may inhibit their resistance to pathogens.

Conclusions

The current investigation assessed the biochemical parameters in four crops (faba bean, wheat, spinach and cucumber) cultivated in agriculture soil amended with SS. The results revealed that the response at the biochemical level is differing according to the plant species and SS application rate. In spinach, starch was decreased significantly with SS treatments, whereas, increased in faba bean, wheat and cucumber at 10, 20, 30 and 40 g/kg SS amendment rates. The concentrations of water soluble carbohydrates and total non-structural carbohydrates were declined in all the tested plants as response to SS amendments. Total lipids were increased in all plants depend on the SS application rates. The increase of total lipids started at 20 g/kg SS treatment in faba bean and spinach, whereas, at 40 and 50 g/kg SS amendments in wheat and cucumber. The amino acid proline accumulated significantly in spinach, wheat and cucumber at all SS treatment rates, while the changes were not significant in faba bean. There were no significant differences in phenol content in faba bean and spinach. The phenol content was decreased in wheat and cucumber leaves with SS treatment. Application of SS to agricultural soils alters the chemical composition of crops. The application of SS at low amendment rates from 10 to 40 g/kg could be useful, whereas, the highest amendment rate, i.e. 50 g/kg is not recommended because of the negative effects on the biochemical composition of the plant, in addition to the possible accumulation of heavy metals in plant materials that may be used for human food or animal feed. The SS amendments could be oriented according to the crop consuming purposes. Future experiments on successive applications of SS in agricultural sites are needed to explore the long-term effects of SS on crops.

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PLANT COMMUNITY COMPOSITION ALTERED BY LONG-TERM NITROGEN ADDITION HAS MINOR CONTRIBUTION TO PLANT NUTRIENT STATUS AT THE COMMUNITY LEVEL

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Abstract. Short-term nitrogen (N) deposition has exhibited great impacts on plant internal nutrient cycling in forest, grassland and desert ecosystems. However, the responses of plant nutrient status to chronic N deposition are not well understood, especially in alpine grasslands limited by soil N availability. This study used an N addition experiment that had been implemented since 2009 in the Tianshan mountains, northwest China, to investigate the effects of N addition at five levels (0, 1, 3, 9 and 15 g N m^{-2} $year^{-1}$) on the leaf nutrient concentrations and stoichiometric ratios of dominant perennial grasses (*Leymus tianschanicus*, *Festuca ovina*, *Agropyron cristatum and Koeleria cristata*) and forbs *(Potentilla anserina* and *Potentilla bifurca)* at the species, functional group and community levels. The results showed that increasing N addition significantly enhanced soil available N concentrations and soil available N:P ratios but had no detectable impacts on soil available phosphorus (P) concentrations. Nitrogen addition significantly reduced forb relative biomass, ranging from 13% in control plots versus 2% in the highest N addition plots. Furthermore, N addition increased the foliar N concentrations and N:P ratios for all the species, decreased foliar P concentrations for grasses, and had no significant effects on P concentrations in forbs. Plant carbon (C) concentrations remained relatively stable, resulting in reduced C:N as N addition increased. In contrast, N addition consistently increased leaf N concentrations, C:P and N:P and decreased leaf P concentrations and C:N at the community level. Regression analysis showed that soil available N and N:P ratios, rather than soil available P, were key parameters controlling plant N and P concentrations and stoichiometric ratios. Foliar N and P concentrations showed divergent responses to long-term N addition at the species and community levels, which implied that plant N and P cycling decoupled under N deposition. Grasses were more sensitive to N addition than forbs, resulting in significantly altered plant community composition.

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These findings are important for understanding plant nutrient allocation strategies and how plants adapt to environmental changes, such as N deposition in alpine ecosystems.
Keywords: *aboveground biomass, alpine grassland, community composition, ecological stoichiometry, nutrient cycling, nitrogen addition*

Introduction

Ecological stoichiometry has been used to study the balance of multiple elements in many ecological processes (Elser et al., 2007). Plant carbon (C): nitrogen (N): phosphorus (P) stoichiometric ratios are closely related to ecosystem processes, such as nutrient cycling (Sterner et al., 2002; Lü et al., 2013; Li et al., 2016), litter decay (Manzonl et al., 2010), plant community structure and stability (Yu et al., 2010), and microbial composition (Güsewell et al., 2009). Previous studies have reported that plants tend to show stoichiometric variability in response to environmental disturbance, for instance, N deposition and P deposition (Sistla et al., 2015), but this flexibility varies across different species (Sardans et al., 2015). Therefore, determining the sensitivity of plant ecological stoichiometry is critical to clarify ecosystem patterns, processes and functions to N deposition.

Unprecedented increases in atmospheric nitrogen (N) deposition have occurred in drylands of China from 1980 to 2010 and are predicted to double by 2050 (Gu et al., 2015). Nitrogen is one of the primary limiting nutrients in grasslands; N addition not only facilitates plant growth but also enhances primary productivity (Bai et al., 2010). Moreover, increasing N availability has significant effects on plant internal nutrient cycling (Burton et al., 2012). The increase in leaf N concentration due to N addition has been extensively examined in grasslands (Lü et al., 2013; Li et al., 2016), wetlands (Mao et al., 2012), deserts (Zheng et al., 2018; Li et al., 2019) and forest ecosystems (Huang et al., 2019). However, large uncertainties exist regarding the impacts of N addition on leaf P concentrations. The effects of N addition on leaf P concentrations have been shown to be positive (Menge and Field, 2007), neutral (Kozovits et al., 2007; Lü et al., 2012), or negative (Li et al., 2016). Furthermore, nutrient traits in plants and their response to nutrient addition vary greatly among different species (Han et al., 2014; Hou et al., 2017; Wang et al., 2018; Lü et al., 2018). Studies in alpine grassland ecosystems are relatively scarce, and limited data are available regarding how enhanced N inputs affect leaf nutrient status. For example, N deposition enhanced the leaf P concentrations of *Seriphidium rhodanthum* but did not have a significant effect on that of *Stipa capillata* in alpine grasslands (Li et al., 2016). In contrast, Peng et al. (2017) found that N addition consistently decreased leaf P concentrations in alpine grasslands across a two-year period. Therefore, the existence of the potential divergent responses of plant nutrient status at the species level to N addition can constrain our understanding regarding the effects of N addition on plant internal nutrient cycling. More empirical evidence is needed to determine the effects of chronic N deposition on the nutrient status of plants in alpine grasslands.

To date, N addition affecting the plant nutrient status at the species level has been extensively investigated (Li et al., 2016, 2020; Hou et al., 2017), but the responses at the functional group and community levels remain limited. It is well documented that N deposition has significant effects on plant community composition and that plant nutrient status at the species level does not accurately represent the community level nutrient status (Han et al., 2014; Wang et al., 2018). N addition has significantly increased N concentrations and the N:P ratio at the community level (Hou et al., 2017; Wang et al., 2018), yet Lü et al. (2018) reported that N addition had no impact on the leaf N:P ratio at

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the community level. Moreover, positive, negative and neutral responses of P concentrations to N addition have been shown (Hou et al., 2017; Wang et al., 2018). Considering these inconsistent results, more experimental evidence is needed to obtain a general conclusion of plant ecological stoichiometry at the community level in response to enhanced N deposition. Furthermore, the duration of N addition is relatively short, and plant ecological stoichiometry to long-term N addition is still unknown in alpine grassland ecosystems.

Plant N and P statuses are tightly coupled with soil nutrient availability (Ågren et al., 2012). A recent study reported that N addition led to the decoupling of plant internal N and P cycling in a meadow steppe (Feng et al., 2019). This occurred because N addition significantly enhanced soil N availability and then led to soil acidification affecting plant-soil stoichiometry. Previous studies showed that N addition consistently enhanced leaf N concentrations and decreased the C:N ratio, thus influencing litter decay (Manzonl et al., 2010). Leaf N:P ratios have been used to determine plant nutrient limitation (Güsewell et al., 2004; Zhan et al., 2017). Nitrogen addition significantly increased leaf N:P ratios and finally resulted in plant growth limited by soil P availability (Zheng et al., 2018; Huang et al., 2019). Compared with stoichiometric variability at the species level, that at the community level is more difficult to reach a general conclusion for the following reasons. On the one hand, the plant nutrient status response to N addition is greatly dependent on species identity (Lü et al., 2012). On the other hand, N addition changes plant community composition, and the relative aboveground biomass significantly impacts plant nutrient status at the community level (Wang et al., 2018). There was a new finding showing that N addition significantly enhanced leaf N concentrations for grasses and forbs at the functional group level and had non-significant effects on P concentrations. However, the responses of leaf N:P ratios to N addition presented positive effects for grasses and neutral effects for forbs (Zhang et al., 2018). Increased N concentrations and negligible effects on P concentrations showed divergent responses of foliar N and P to N addition. Furthermore, studies regarding how plant nutrient status adapts to environmental disturbance are relatively limited. Simultaneously, how stoichiometric flexibility responds to N addition is highly variable from species to community. Therefore, more experimental evidence of plant nutrient status responses to long-term N addition is necessary, especially in alpine grassland ecosystems.

The Tianshan Mountains have been regarded as one of the areas critical for global biodiversity protection in temperate arid regions. The current total N deposition has been estimated at 8 kg N ha^{-2} yr^{-1} (Li et al., 2015). Previous studies have shown that N addition has great consequences on plant and soil C and N cycling. For instance, N addition tends to increase soil N_2O emissions and decrease methane uptake (Li et al., 2012; Yue et al., 2016); such results have mainly emerged from enhanced soil N availability by N addition. In addition, N addition generally enhanced plant growth and significantly increased plant N uptake (Li et al., 2015) because plant growth could be limited by soil available N. Studies regarding the effect of altered plant community composition and increased aboveground biomass due to N addition on plant ecological stoichiometry at the functional group and community levels in alpine grassland ecosystems are limited.

Therefore, a field experiment was designed to examine the effect of various N addition levels on leaf C, N and P concentrations and their stoichiometric ratios at the species, functional group, and community levels by taking advantage of an 11-year experiment with various N addition levels (0, 1, 3, 9 and 15 g N m^{-2} $year^{-1}$) in an alpine grassland in northwest China. We tested the following hypotheses: (1) long-term N addition increased

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leaf N concentrations from species to community because of significantly enhancing soil N availability by N addition but (2) decreased P concentrations primarily because of the non-significant effects of N addition on soil P availability, and (3) N addition generally mitigates N limitation but exacerbates P limitation based on the enhanced leaf N:P ratio.

Materials and Methods

Study site and experimental set up

This field experiment was conducted at the Bayinbuluk Grassland Ecosystem Research Station (42°18′-43°34′ N, 82°27′-86°17′ E), located on the south slope of the middle section of Tianshan Mountain in northwestern China. The long-term average (1981–2012) is 282.3 mm, with 60% of precipitation occurring in the growing season from May to September, and the mean annual temperature is −4.8 °C (Li et al., 2015). Furthermore, this natural grassland has a mean altitude of 2,500 m and a total area of approximately 23,000 km^2, belonging to the typical alpine and dry grassland. The soil is a Camisole according to the Food and Agriculture Organization soil classification system, with a high organic matter and N content and a low P content. Due to disturbance from human activities, the current atmospheric nitrogen deposition is approximately 8 kg N ha^{-1} yr^{-1}. The dominant grass species in the ecosystem are *Leymus tianschanicus, Festuca ovina, Agropyron cristatum and Koeleria cristata*, contributing approximately 87% to the aboveground biomass (*Fig. 1*).

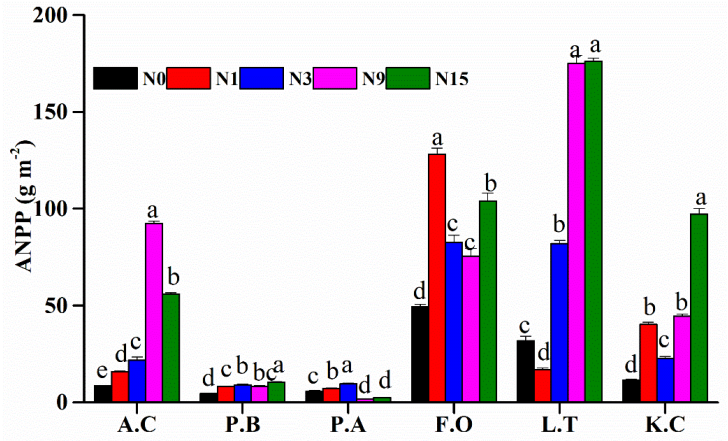

Figure 1. *Responses of plant aboveground biomass to N addition. Error bars + SE. Bars with different letters show significant differences (P <0.05) between the treatments. Leymus tianschanicus (L. T), Festuca ovina (F. O), Agropyron cristatum (A. C), Koeleria cristata (K. C), Potentilla anserina (P. A), and Potentilla bifurca (P.B)*

N addition experiments have been carried out since 2009. There were five treatments: control (N0), 10 (N1), 30 (N3), 90 (N9), and 150 (N15) kg N ha^{-1} yr^{-1}. Each treatment was established with four replicates, resulting in a total of 20 plots. Each 8 × 4 m plot was arranged in a randomized block design, and each plot was separated by a 1 m buffer. Nitrogen was added to the plots as purified NH_4NO_3 in two split doses applied in early May and June each year. A weighed amount of NH_4NO_3 was dissolved in 8 L water and

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applied to each block using a sprayer to distribute the fertilizer evenly. The same amount of water was added to the control plots without N fertilizer.

Plant and soil sampling and chemical measurements

In mid-August 2019, aboveground biomass was sampled by clipping all plants in each plot using a 0.5 × 0.5 m quadrat at peak biomass time. The plant samples were harvested, sorted into species and then oven-dried at 65 °C for 48 h. The dry mass of all living plants in each quadrat was used to estimate the aboveground species biomass based on the four replicates for each treatment. Aboveground net primary production (ANPP) was estimated as the peak aboveground biomass during the growing season (Bai et al., 2010).

Leaf samples were collected from the dominant grass species *Leymus tianschanicus, Festuca ovina*, *Agropyron cristatum, and Koeleria cristata*, and perennial forbs of *Potentilla multifida* and *Potentilla bifurca* were also collected for chemical analysis. All plant species were divided into two plant functional groups: grasses and forbs. These species represented almost 95% of the total aboveground biomass. Similar leaves were randomly selected in the subplot (2 m × 2 m) from the center of each plot. The leaves were oven-dried at 65 °C to reach a constant weight, ground, and then mixed evenly for elemental analysis. Plant C and N contents were determined with an elemental analyzer (Vario micro, Hanau, Germany). The total P concentrations were measured by wet digestion with H_2SO_4+$HClO_4$. C, N and P concentrations were expressed as the nutrient content per unit mass (mg g^{-1}).

Soil samples (0–10 cm depth) were collected from each plot in August 2019. In the center of each plot at least 2 m away from the edge of the plot, five soil cores were collected using a 3 cm diameter soil auger and were mixed into a single composite sample. All soil samples were sieved through a 2 mm mesh to remove roots and litter. Soil available N was extracted with 50 ml of 2 M KCl solution. Then, the filtered soil extract was used to determine alkaline nitrogen concentrations with a Kjeldahl distillation system (VAP-45, Gerhaedf, German). Available soil P was measured by extracting soil with 0.5 M $NaHCO_3$ (pH = 8.5), followed by analysis with the molybdenum blue-ascorbic acid method (Olsen et al., 1954).

Calculation of data and statistical analysis

Plant nutrient concentrations at the functional group and community levels were estimated as species relative biomass as a weighting factor, and the C, N, and P concentrations and nutrient stoichiometric ratio were calculated for each species in every plot (Lü et al., 2018).

All statistical analyses were performed in SPSS version 23.0 (SPSS Inc. Chicago, IL, USA). Data were tested for normality using the Kolmogorov-Smirnov test and Levene's test for equality of error variance. One-way analysis of variance (ANOVA) was applied to test whether the N treatment influenced the plant nutrient concentrations and stoichiometric ratios of each species, with N treatment as a fixed factor. Differences among treatments were analyzed by Tukey's multiple comparison post hoc test. Linear regression analyses were conducted to determine the relationships between soil available nutrient concentrations, plant nutrient concentrations and stoichiometric ratios. All figures were constructed in Origin 9.0.

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Results

Responses of soil available nutrients and ANPP

Nitrogen addition significantly enhanced the soil available N concentration, ranging from 189.62±10.1 mg kg^{-1} in the control plots (N0) to 267.40±4.0 mg kg^{-1} in the highest N addition plots (N15). However, N addition showed no significant effect on soil P availability, with a range from 7.52±0.56 to 8.62±0.9 mg kg^{-1} (*Fig. 2*). The soil available N:P ratio significantly increased across the N addition gradient (*Fig. 2*). N addition significantly enhanced ANPP for grass and decreased biomass for forb species, but the response of magnitude varied highly among species (*Fig. 1*).

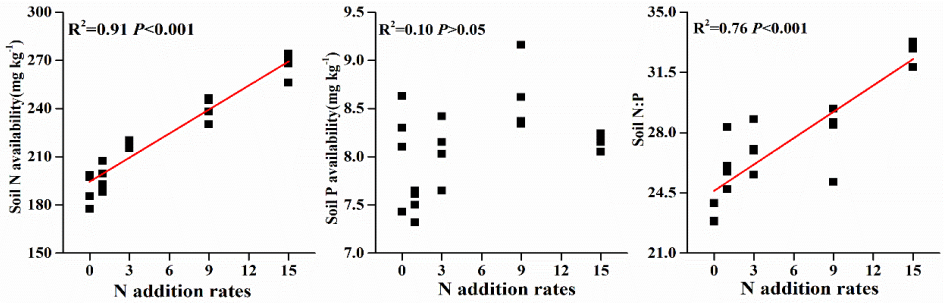

Figure 2. *Relationships between nitrogen (N) addition rates and soil available N and phosphorus (P) concentrations and available soil N:P ratios in the alpine grassland*

Responses of nutrient status at the species, plant functional group and community levels

The nitrogen addition rate and species identity had significant effects on leaf N and P concentrations and the N:P ratio (*Fig. 3*, *Table 1*). N addition significantly increased the leaf N concentrations of the six species (*Fig. 3*). In contrast, the P concentrations in the leaves of the four plant species significantly decreased and had no effects on P.A and P.B. Plant C concentrations remained relatively stable across N addition treatments (*Fig. 3*, *Table 1*). N addition significantly enhanced the N:P ratio but reduced the C:N ratio for all grass species (*Fig. 3*). The ratios of C to P were insignificantly affected by P.A and P.B but significantly increased for other species (*Table 1*).

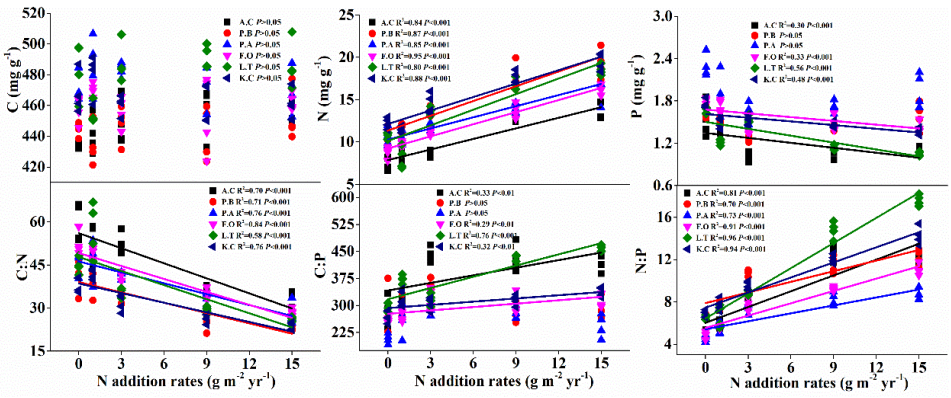

Figure 3. *Relationships between plant nutrient concentrations or stoichiometric ratio and N addition rates at the species level. Leymus tianschanicus (L.T), Festuca ovina (F.O), Agropyron cristatum (A.C), Koeleria cristata (K.C), Potentilla anserina (P.A), Potentilla bifurca (P.B)*

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Table 1. *Results of a one-way analysis of variance (ANOVA) of the dependence of leaf nutrient concentrations and stoichiometric ratios on N addition. The F-ratios are presented together with their level of significance. Leaf C, N and P concentrations are represented. The leaf C:N, C:P and N:P ratios were also represented as mass ratios from species to community*

Plant types	C	N	P	C:N	C:P	N:P
A.C	0.91	53.53**	13.41**	20.32**	19.59**	80.52***
K.C	1.00	42.72**	5.85*	18.38**	2.66	82.21***
P.A	1.00	24.16**	1.43	15.77**	0.85	46.17***
P.B	0.95	107.56***	0.57	48.59**	0.31	172.29***
F.O	1.29	124.96***	184.95***	31.09**	161.9***	243.16***
L.T	2.11	66.94**	24.08**	54.15**	10.16*	128.61***
Forbs	0.98	48.37**	1.82	53.57**	1.59	84.75***
Grasses	1.16	222.65***	55.53***	65.54***	22.25*	297.88***
Community	3.20	246.2***	66.4***	72.0***	26.0**	342.9***

Grasses species: *Leymus tianschanicus* (L.T), *Festuca ovina* (F.O), *Agropyron cristatum* (A.C) and *Koeleria cristata* (K.C), Forbs species: *Potentilla anserina* (P.A) and *Potentilla bifurca* (P.B), Community= Grasses species+ forbs species. ns indicates no significant difference. **P* < 0.05. ***P* < 0.01. ****P* < 0.001

Nitrogen addition had no significant impacts on C concentrations and increased N concentrations but decreased P concentrations at both the plant functional group and community levels (*Fig. 4*, *Table 1*). Furthermore, N addition significantly enhanced the ratios of C to P and N to P but significantly reduced the ratios of C to N at the community level.

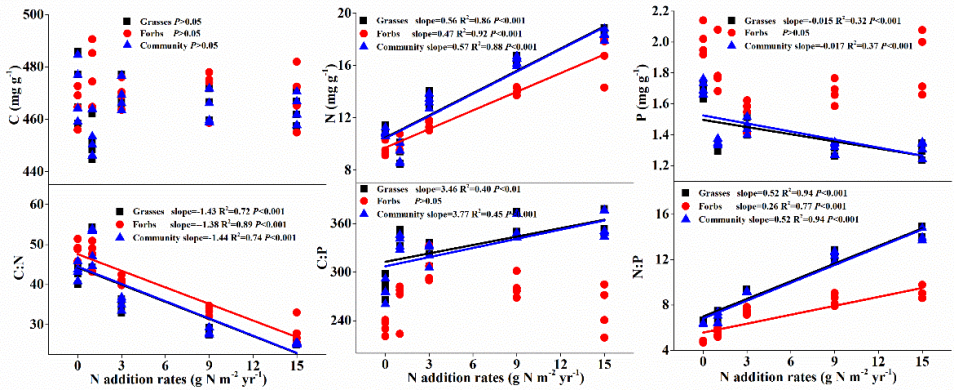

Figure 4. *Relationships between plant nutrient concentrations or stoichiometric ratio and N addition rates at the functional group and community levels. Leymus tianschanicus (L.T), Festuca ovina (F.O), Agropyron cristatum (A.C), Koeleria cristata (K.C), Potentilla anserina (P.A), Potentilla bifurca (P.B)*

Relationship between leaf nutrient status and soil available nutrients

For all species, the results showed that altered soil nutrient availability had non-significant effects on C concentrations. Leaf N concentrations and ratios of N to P were positively correlated with soil available N, while P concentrations and ratios of C to N were negatively correlated with soil available N concentrations (*Fig. 5*). Soil available N:P showed similar effects on leaf nutrient status with soil N availability. There was a

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non-significant linear relationship between P concentrations in leaves and soil P availability, but a positive relationship was observed between N concentrations and soil P availability among 4 out of 6 species. However, soil available N:P significantly affected PTP and the plant nutrient stoichiometric ratio (*Fig. 5*).

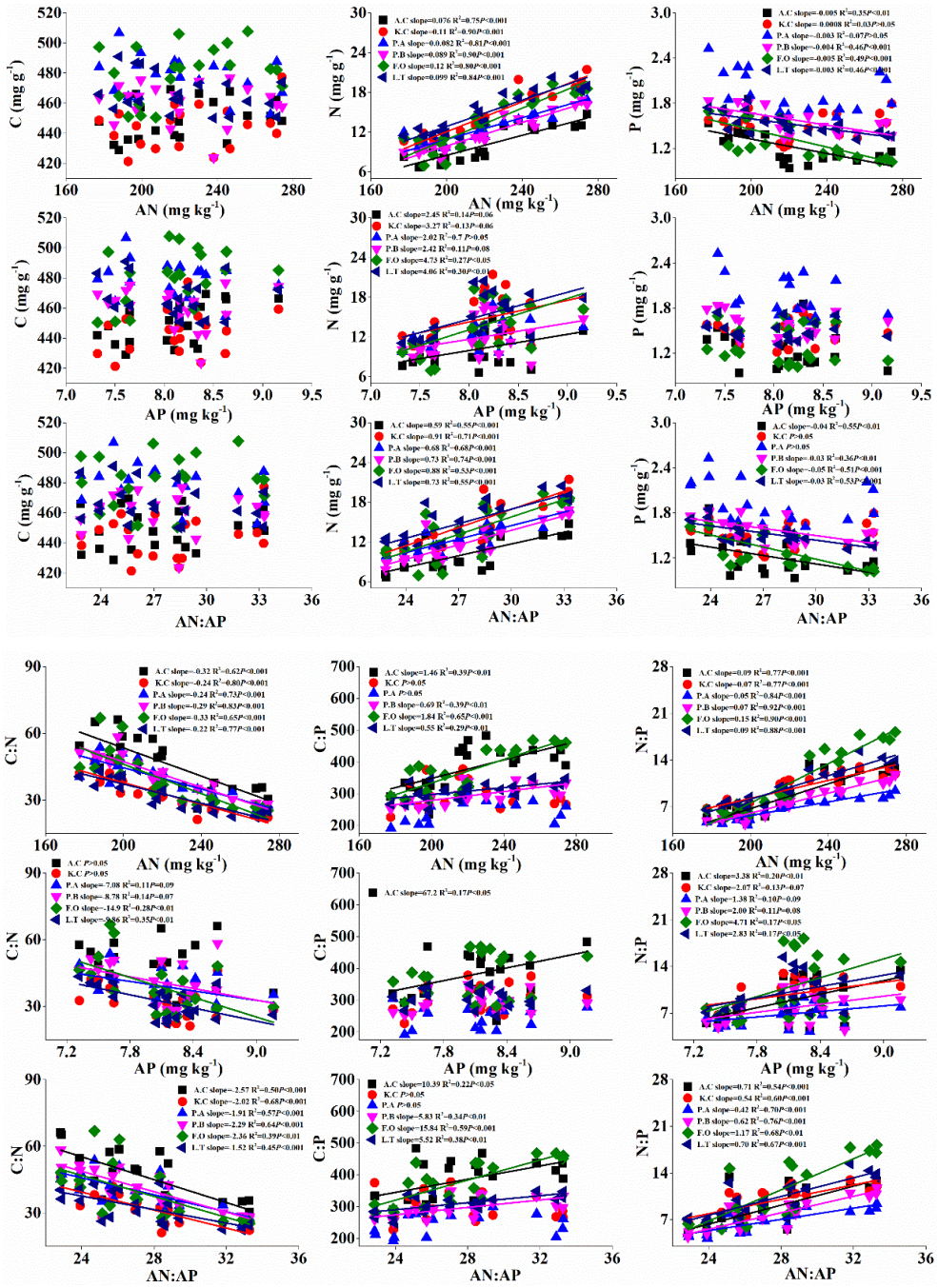

Figure 5. *Relationships between plant nutrient concentrations or stoichiometric ratio and soil available nutrients (AN, AP and AN:AP) at the species level. Leymus tianschanicus (L. T), Festuca ovina (F. O), Agropyron cristatum (A. C), Koeleria cristata (K. C), Potentilla anserina (P. A), Potentilla bifurca (P.B). Soil available nitrogen (AN), soil available phosphorus (AP) and soil AN:AP ratios (AN:AP)*

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At the community level, soil N availability had no impacts on C concentrations but increased N concentrations, C:P and N:P ratios; at the same time, it decreased the P concentrations and C:N ratio. Soil available P did not affect the C concentrations, P concentrations or C:P ratio (*Fig. 6*). The leaf N concentration and N:P ratio were positively correlated with the soil available P, and the C:N ratio was negatively correlated. The soil available N:P ratio revealed constant effects on the leaf nutrient status with soil N availability.

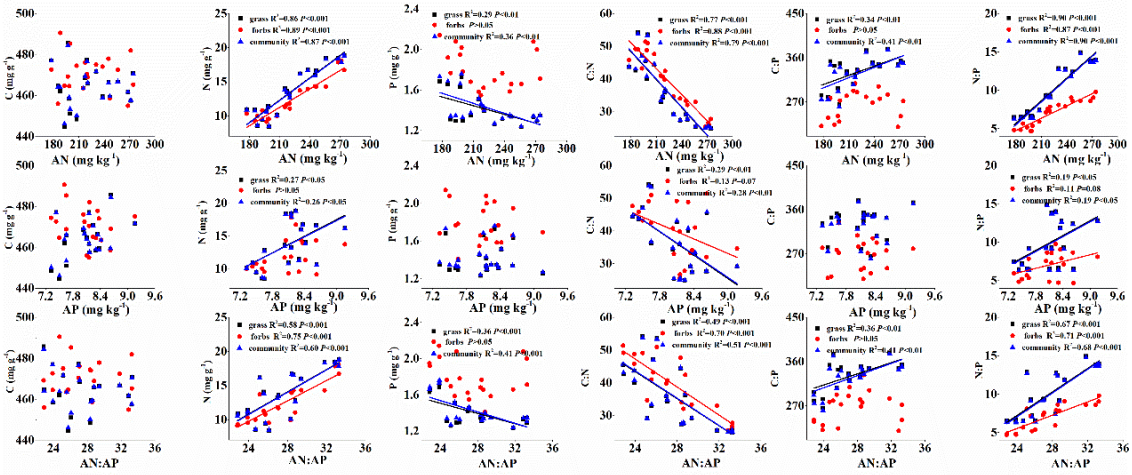

Figure 6. *Relationships between plant nutrient concentrations or stoichiometric ratios and soil available nutrients (AN, AP and AN:AP) at the functional group and community levels. Grass: Leymus tianschanicus, Festuca ovina, Agropyron cristatum and Koeleria cristata. Forbs: Potentilla anserina and Potentilla bifurca. Community= grass + forbs. Soil available nitrogen (AN), soil available phosphorus (AP) and soil AN:AP ratios (AN:AP)*

Discussion

Responses of species biomass to N addition

Nitrogen addition significantly increased soil N availability and had non-significant effects on soil available P, resulting in a higher soil AN:AP ratio. This is consistent with a study from alpine steppe (Peng et al., 2017). Altered soil available N significantly promoted grass species growth and enhanced plant biomass from 101.4±19.1 $g \cdot m^{-2}$ to 307.7±54.4 $g \cdot m^{-2}$ for the average of all N treatments. However, N addition had different effects on forb species biomass depending on species identity. Previous studies have reported that N addition significantly increased grass species biomass but had positive, negative and negligible impacts on forb species biomass (Han et al., 2014; Hou et al., 2017; Wang et al., 2018). These findings indicated that N is the limiting factor of plant growth in this grassland. The biomass responses of forb species against N addition indicated less sensitivity compared to grass species in our study. One possible reason could be that N addition might have increased the height of grass species, which would have restricted the availability of light in the lower layer of the forb species, resulting in limited plant growth (Bai et al., 2010; Zhou et al., 2018). In our study, N addition enhanced the biomass of dominant grass species and reduced the biomass of non-dominant forb species, which significantly affected the plant community composition and increased the risk of loss of forb species. Nitrogen deposition showed a positive impact

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by promoting plant growth and significantly improving plant aboveground biomass for forage supply, but its negative impact appeared in the form of alteration of plant community structure that resulted in loss of biodiversity (Bai et al., 2010; Wang et al., 2018).

Effects of N addition on the nutrient status of leaves and stoichiometric ratios at the species level

In the control plots, plant nutrient status revealed wide variation among species. In all species, the average concentrations of leaf C (463.11 mg g^{-1}), N (10.12 mg g^{-1}) and P (1.77 mg g^{-1}) were partly consistent with the results of previous studies on grasslands in China (He et al., 2006, 2008). In our study, plant N content was significantly lower compared to grassland in northern China, but our findings are supported by the results from alpine grasslands (Peng et al., 2017). Additionally, our results are also comparable with those of a previous study from alpine grasslands where multiple sites across the Tibetan Plateau have been tested (Zhou et al., 2020). It can be inferred that reduced nutrient accumulation in leaves could be the result of declined rates of plant absorption and utilization under drier and colder environments (Aerts et al., 1996). Moreover, the relatively low concentration of soil available N under conditions of less rainfall and low temperature would be the reason for the low plant N concentration in this alpine grassland (Tian et al., 2010). This can result not only from the rate of plant absorption and utilization slowing down in drier and colder environments and reducing leaf nutrient accumulation (Aerts et al., 1996) but also from the fact that the soil available N concentration is relatively low because of limited precipitation and low temperature (Tian et al., 2010), finally resulting in lower plant N concentrations in this alpine grassland.

N addition did not show any significant impact on plant C concentrations, but it increased plant biomass and led to more C sequestration in plants. Our results showed that N addition significantly increased leaf N concentrations. Increases in plant N concentrations following N addition have been well documented in many previous studies (Xia and Wan, 2008; Peng et al., 2017; Hou et al., 2017; Wang et al., 2018). It seems that soil available N significantly increased after N addition, which provided an adequate soil nutrient supply for plant growth. This also implies that plant growth is affected by N availability but that plants can exhibit luxury uptake when N is not a limiting factor (Sistla et al., 2015), hence carrying higher N concentrations. On the other hand, N addition significantly reduced the P concentration for grass species but did not show any effect on the P concentrations of forb species. In contrast, many previous studies indicated an increase in plant P concentration in response to N addition (Lü et al., 2013; Hou et al., 2017; Wang et al., 2018). Simultaneously, many studies also found that N addition significantly increased soil P availability in grassland ecosystems (Lü et al., 2013; Han et al., 2014; Li et al., 2016). This difference can be explained as follows. First, N addition significantly increased plant aboveground biomass for grass species in our study, which would have partly diluted the P content in plants (Hou et al., 2017). Second, N addition had an ignorable impact on the soil available P content and soil alkaline phosphatase activity, which would have direct effects on reducing the plant P concentrations. Hence, N addition had positive, negative and neutral effects on plant P concentrations (You et al., 2018), which partly supports our findings that N addition decreased or had no effect on plant P concentrations depending upon species identity. For instance, N fertilization decreased P concentrations in leaves of dominant grasses by 40% in an annual grassland

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(Menge and Field, 2007), while it had neutral effects on plant P concentrations for forb species in semiarid grasslands (Hou et al., 2017).

Our results clearly demonstrated that the N:P ratio in leaves was enhanced after N addition. These findings are in accordance with previous studies (Lü et al., 2013; Zhan et al., 2018), where they found that increased N deposition can induce a shift from N to P limitation or N-P colimitation. Furthermore, plant N and P concentrations showed opposite responses to each other, indicating nutrient decoupling in plants under N deposition. These outcomes are not consistent with previous reports that observed that plant N and P are tightly coupled in grassland ecosystems (Agren et al., 2012; Lü et al., 2013). It is possible that altered soil nutrient availability by N addition could have reduced the leaf C:N ratio, which would have promoted litter decomposition (Manzonl et al., 2010), because enhanced litter quality significantly promoted litter decomposition (Manzonl et al., 2010). Moreover, an increase in N inputs through fossil fuel combustion and cropland fertilization can greatly enhance the substrate nutrient availability for plants, which may also result in nutrient decoupling in plants, thus negatively influencing the plant community structures (Liu et al., 2015). For grass species, the responses of plant N and P concentrations and stoichiometric ratios following N addition could be controlled by soil N availability and soil available N:P, and the data suggest that the N:P ratio in plants and soils was strongly linked. Our results supported the idea that increasing N deposition not only induces changes in N concentrations directly but also leads to an indirect alteration in the soil available N:P ratio (Penuelas et al., 2012).

Effects of N addition on the nutrient status of leaves and stoichiometric ratios at the functional group and community levels

Our results indicated that leaf C remained relatively stable along the N addition levels at the functional group and community levels. However, N and P in leaves varied greatly among different plant functional groups. Nitrogen addition increased the N concentration and decreased the P concentration in leaves for the grass functional group; however, it showed a non-significant impact on N and P for the forb functional group. Nonetheless, N addition consistently increased the N concentration and decreased the P concentration at the community level. The responses of P concentration towards N addition were negative and neutral at functional group levels; thus, N addition did not enhance plant P uptake. These results are in line with the findings of (Hou et al., 2017; Wang et al., 2018), who described that N addition significantly increased the P concentration in grass species but had no impact on the P concentration of forb functional groups or community levels. This might have occurred because N addition did not affect soil phosphatase activity, leading to non-significant changes in soil P availability under long-term N addition. Furthermore, dominant grass species drove community-level N and P concentrations, which is also consistent with previous reports from typical steppe (Lü et al., 2018) and meadow steppe (Wang et al., 2018). The nutrient and stoichiometric responses of grass species to N addition are more sensitive than those of forb species, and the biomass of grass species was significantly enhanced, which could be a mechanism by which plants adapt to environmental changes, such as N deposition (Yu et al., 2010).

Nitrogen addition consistently decreased the C:N ratio and increased the N:P ratio in leaves at the functional group and community levels. Lower C:N ratios generally show fast decomposition (Hou et al., 2017). In our study, N addition significantly enhanced leaf quality and contributed to decomposition in this alpine grassland. N:P ratios have been used to determine relative nutrient limitation (Zhan et al., 2017) and regulate litter

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decomposition processes (Güsewell and Gessner, 2009). The enhancement of N:P ratios following N addition led to the decoupling of plant N and P, which agreed with many studies from diverse ecosystems, such as grasslands (Wang et al., 2018), wetlands (Mao et al., 2012) and desert ecosystems (Huang et al., 2018), indicating that N addition may alleviate N limitation and intensify P limitation. However, Han et al. (2014) found that N addition significantly increased P concentrations and C:P ratios at the species level, and this phenomenon was not observed at the community level. Furthermore, N addition significantly increased plant aboveground biomass, but only in one of the six species with the largest biomass was at the highest N addition, which implied that the growth of most species was not limited by N availability. If plant growth is limited by N, N addition will further promote plant growth. Considering the current high N deposition in China, plant growth may be limited by soil P availability in grassland ecosystems because the soil available P content was relatively lower than that in other regions (Han et al., 2005; Zhang et al., 2005). In brief, our results suggested that the N and P of plants from the species to community levels revealed divergent responses to long-term N addition in this alpine grassland, but the specific mechanism still needs to be explored further.

Soil available N and available N:P ratio as determining factors for plant nutrient concentrations and stoichiometric ratios

It has been well established that soil available N is a strong predictor of plant nutrient concentrations and stoichiometric ratios (Lü et al., 2013; Zheng et al., 2018). In our study, we confirmed that N addition directly affected plant N and P concentrations and stoichiometric ratios at the species and community levels by enhancing soil available N concentrations rather than through soil available P in this alpine grassland, which is in agreement with the results reported from alpine steppe ecosystems (Peng et al., 2017). Compared with soil available N concentrations, the effects of soil available P on plant nutrient concentrations and stoichiometric ratios could be considered minimal because long-term N addition did not cause significant changes in soil P availability among treatments.

Our results suggested that altered soil AN:AP ratio significantly affected plant nutrient status. For instance, plant N concentrations showed a significant positive relationship with the soil AN:AP ratio, but plant P concentrations had a significantly negative relationship with the soil AN:AP ratio at the species and community levels. This indicated that the responses of plant nutrient concentrations and stoichiometric ratios to N addition could be controlled by the soil available N:P ratio, and this N:P ratio in plants and soil is strongly coupled (Lü et al., 2013; Li et al., 2020). In desert grassland ecosystems, Zheng et al. (2018) found a positive linear correlation between the plant available N:P ratio and the leaf N:P ratio. In addition, Zhan et al. (2017) discovered that the leaf N:P ratio is positively correlated with the soil available N:P ratio across N addition treatments. These findings highlight the importance of the soil available N:P ratio in controlling plant nutrient status in alpine grassland ecosystems. This means that enhancing N deposition would change the plant community composition and plant internal nutrient cycling through direct alteration in soil N availability and by indirect changes in soil nutrient stoichiometry.

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Conclusion

The divergent responses of plant N and P concentrations in leaves to long-term N addition at the species and community levels implied that plant N and P cycling decoupled under N deposition. Grasses were more sensitive to N addition than forbs, resulting in significantly altered plant community composition. Moreover, soil available N and the soil available N:P ratio, rather than soil available P, were the key environmental parameters that controlled the N and P concentrations and stoichiometric ratios in plants. These findings are important for understanding plant nutrient allocation strategies and how plants adapt to environmental changes in alpine ecosystems. Altered plant community composition of forb species by N addition has minor influences on leaf nutrient status. Briefly, these findings have important implications for understanding the influence of N deposition on grassland nutrient cycling. In the future, biogeochemical models should consider the divergent responses of plant nutrient cycling to nutrient changes.

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BIOMASS CHARACTERISTICS AND GROWTH ANALYSIS OF VARIOUS COMPONENTS OF *ACHNATHERUM INEBRIANS* (DRUNKEN HORSE GRASS) POPULATION IN DIFFERENT HABITATS

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Abstract. *Achnatherum inebrians* (drunken horse grass) is one of the main poisonous grasses in Chinese grasslands, and it is mainly distributed in the degenerated grassland in the northern pastoral area. Individual plants grown in desert, steppe and meadow ecosystems were collected from July to August in Xinjiang, China. The results showed that there was no significant difference in the inflorescences, stems and reproductive biomass in the desert, steppe and meadow ecosystems, while the leaf, above- and below-ground vegetative and total biomass in the desert was significantly lower than that in the steppe and meadow habitats. The allocation of the biomass into the leaf was the most and the inflorescences was the least in three habitats. The biomass of inflorescences/stems increased in all three habitats, but there was a certain fluctuation in the biomass of other components. The biomass of *A. inebrians* in steppe and meadow habitats distributed more resources into the above-ground part concerning the accumulation of total biomass, while the opposite trend was observed in the desert. In the process of growth, the resources in desert and grasslands decreased reproductive allocation and more resources were allocated into vegetative biomass. It can be seen that there are differences in growth characteristics, and biomass allocation of *Achnatherum inebrians* grown in different habitats, which shows the flexible growth and resource allocation strategies of *Achnatherum inebrians* in different habitats.
Keywords: *biomass; invasive plants, reproductive biomass allocation, vegetative biomass allocation, heterogeneous habitats*

Introduction

Growth and reproduction are two of the most fundamental life-history functions in plants (She et al., 2017). The pattern of resource allocation to sexual vs asexual growth is a core component of studies of plant life history (Bonser and Aarssen, 2009; Weiner et al., 2009). Plants can modify their biomass allocation patterns to adapt to different environments (Guo et al., 2012). Biomass is one of the basic biological and functional characteristics of plants and is the basic embodiment of material and energy accumulation (Hao et al., 2013). The pattern of the distribution of resources in plants to various organs is the main focus of studies of changes in plant growth patterns, while environmental changes will affect the distribution of biomass among various organs to varying degrees (Fan et al., 2017). Modular allocation in an organism refers to the proportion of assimilated resources used in roots, stems, leaves, inflorescences, fruits and other organs during plant growth and reproduction (Xiao et al., 2014). It reflects the ecological strategies of plants in different habitats (Weiner, 2004) and is the result of long-term adaptation to the environment (Poorter et al., 2012; Wang et al., 2017). As a functional index, the value representing plant modular allocation not only reflects the material circulation and energy flow in the ecosystem but also has very important

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significance in the composition of ecosystem structure (Bloom et al., 1985; Hovenden et al., 2014; Luo et al., 2017). Therefore, the measurement of the biomass of plant components is of great significance in ecosystem research (Wang et al., 2015).

Invasive plants represent a serious threat to the structure and function of invaded ecosystems (Si et al., 2013). Approximately 30-40% of the world's endangered plants are disturbed and affected by exotic species (Walker et al., 1997), and invasive plants change the direction of community succession by altering environmental conditions. Invasive plants undergo a series of interactions with the environment during the invasion process; these interactions play an important role in further expanding the scope of plant invasions (Alpert et al., 2000; Liu et al., 2010). To study the influence of the invaded environment on the spread and distribution of invasive plants is helpful to reveal the rules of successful invasion in a particular location (Zhu et al., 2018). The distribution of an invasive alien plant is not only related to the biological characteristics and origin of the species itself but is also closely related to the environment in the invaded area (Wu et al., 2006). Invasive plants can use different reproductive strategies to colonize new areas. Ramets, which are produced via asexual reproduction, have higher growth and survival rates than seedlings, which are produced as a result of sexual reproduction, but their dispersal is generally restricted to short distances (Holsinger, 2000; Barrett et al., 2008). Consequently, exotic species using both strategies (sexual reproduction and asexual reproduction) may show greater invasion success than others (Winkler and Fischer, 2002). Identifying the relative contributions of each reproductive mode to the propagation of an invasive species is crucial for devising efficient control strategies. Control methods based on the misunderstanding of dispersal mechanisms risk failure because such efforts do not target plant parts from which new populations arise (Albert et al., 2015). Invasive plants are divided into narrow expansion and broad expansion. Broad expansion includes the expansion of native species and the expansion of alien species. *Achnatherum. Inebrians* (drunken horse grass) belongs to grassland species, if it spreads in the grassland, it belongs to local expansion, and to deserts and meadows belongs to local invasion.

A. Inebrians is a perennial herb belonging to *Achnatherum* Beauv. in Gramineae. It is one of the main toxic weeds in the natural grassland in north-western China. It is mainly distributed in Gansu, Inner Mongolia, Qinghai, Tibet, Xinjiang and other areas in China and is toxic to sheep, cattle, horses and other domestic animals (Ren, 1954). As early as 1876, American scholars named the *A. inebrians* collected in Inner Mongolia *Stipa inebriants* (Hance, 1876). Keng (1959) changed the classification status from *Stipa* to *Achnatherum* and named the plant *A. inebrians*; this name is still in use today. Due to its strong stress resistance and toxicity, it is not eaten by local livestock and gradually spreads. The direct economic loss caused by the decrease in grass production alone exceeds US$15 million per year, and this species has become the main limiting factor for the healthy development of animal husbandry in the grassland (Li et al., 2018). In recent decades, with the aggravation of grassland degradation, the harm caused by *A. inebrians* to livestock has gradually gained people's attention (Ren, 1954). Scholars have conducted a great deal of research on its botanical characteristics, eco-biological characteristics (Wang et al., 1991; Ji, 2009), endophytic fungi (Li et al., 1996; Chen et al., 2016), control and utilization (Yang et al., 2015; Jin et al., 2017). *A. inebrians* is not distributed only in steppes and meadows, like *Aconitum leucostomum*, or only in deserts, like *Anabasis aphylla*. *A. inebrians* species is distributed in deserts, steppes and meadows. Therefore, this study aims to investigate the quantity and biomass

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characteristics of components, vegetative growth and reproductive growth of *A. Inebrians*, as well as the changes of biomass allocation between the above-ground and the below-ground, and discusses the propagation characteristics and adaptation strategies of the plants in different habitats.

Materials and methods

Study site

The studied areas are located in Heijiagou and Xiejiagou of in Urumqi City, Xinjiang, China (*Fig. 1*). Among them: Heijiagou is located in the low hills of the shallow mountain belt in the middle of the northern slope of Tianshan Mountain. The annual average precipitation is 221.3 mm, the annual average evaporation is 1765.4 mm, the annual average temperature is 4.3 °C, the annual frost-free period is about 129 days, and the soil is mountain brown desert soil. Xiejiagou is located in the middle and low mountain zone of the northern slope of Tianshan Mountain, with an annual average precipitation of 388.7-535.9 mm, an annual average evaporation of 1141.7-1564.9 mm, an annual average temperature of 2.1-3.3 °C and a frost-free period of 100-113 days. The soil is mountain chestnut soil. In terms of utilization, it is the spring and autumn grazing grassland for local livestock and sheep.

Heijiagou and Xiejiagou in Urumqi county correspond to three grassland types of desert, steppe and meadow respectively, forming three different environmental sample areas. These areas are typical areas invaded by *A. inebrians*, where this species has become the dominant species in the community (*Table 1*).

Research methods

Samples were collected during the fruiting stage (July to August 2018) of *A. inebrians* in desert, steppe and meadow areas. Each habitat included 3 parallel transects with a spacing of more than 10 m. Each transect had 5 plants with relatively consistent plant height (*Table 2*) that were more than 5 m apart, i.e., 15 plants were taken from each habitat, for a total of 45 plants.

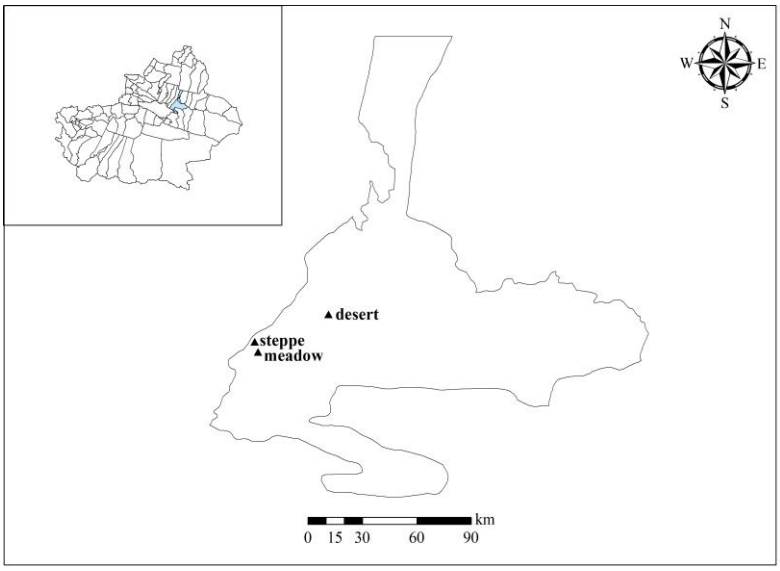

Figure 1. *Location of the study sites*

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Table 1. *Sample information of three habitats (mean ± SD)*

Grassland type	Community information						Geographic information		
	Species composition		Height (cm/plant)	Coverage (%)	Density (plant/m^2)	Plant biomass (g/plant)	Altitude (m)	Geographic coordinates	
	Before degradation	After degradation (important value)							
Desert	*Nanophyton erinaceum* + *Anabasis brevifolia*	*Achnatherum inebrians* (0.423) + *Peganum harmala* (0.247) + *Carex turkestanica* (0.119)	111.30± 35.06	34.93± 7.19	118.22± 65.27	154.54± 41.02	1353	N 43°39′	E 87°23′
Steppe	*Caragana acanthophylla* + *Seriphidium borotalense* + *Carex turkestanica*	*Achnatherum inebrians* (0.537) + *Carex turkestanica* (0.280)	111.75± 20.11	66.58± 8.23	165.91± 34.01	220.98± 120.09	1660	N 43°31′	E 87°01′
Meadow	*Iris ruthenica* + *Poa angustifolia*	*Achnatherum inebrians* (0.470) + *Carex turkestanica* (0.137) + *Potentilla bifurca* (0.063)	165.59±5 2.63	59.62± 16.85	277.67± 165.86	226.46± 70.28	2180	N 43°28′	E 87°02′

Height: the scale measures the natural height of each species. Coverage: acupuncture method to determine species coverage. Density: the density of species is determined by the counting method, expressed as the number of species in a unit area. Biomass: fresh weight above and below ground for each plant in the sample. Important value = (relative height + relative density + relative coverage + relative biomass) / 4

Table 2. *Basic plant height parameters of A. inebrians*

Treatments	Max (cm)	Min (cm)	Mean (cm)	5%	1%	Standard deviation	Coefficient of variation (%)
Desert	109.00	95.00	101.33	a	A	4.87	4.80
Steppe	112.00	95.00	105.00	a	A	5.57	5.30
Meadow	112.00	95.00	105.40	a	A	4.78	4.53
Mean (N = 15)	112.00	95.00	103.91			5.30	5.10

Different small letters and large letters mean significant difference at 0.05 and 0.01 level

After measuring the plant height and inflorescence length in the field, the below-ground system was excavated and the above-ground parts were removed. When sampling, care was taken to maintain the integrity of each branch. After the roots were washed clean, the whole plant was taken back to the laboratory for air drying. Then, the dry weight of the roots, stems, leaves and inflorescences were measured individually with a 1/10,000 electronic balance.

Data analyses

One-way analysis of variance (ANOVA) and Pos hoc-LSD was used to analyze data utilizing SPSS 22.0 statistical analysis software. Relationships between vegetative and reproductive, above-ground and below-ground biomass allocation and total biomass were evaluated using regression analysis. All the quantitative relations were carried out regression analysis of the linear function $y = a + bx$, the power function $y = ax^b$, and the exponential function $y = ae^{bx}$, The Duncan test at the 5% confidence level was used for comparisons. The most correlated one was used as its description model (Liu, 2004). The figure was drawn with Origin 8.0. Inflorescence, leaf, stem, root, above-ground,

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reproductive, vegetative biomass allocation were calculated as follows (Liu et al., 2012; Tian et al., 2018):
Inflorescence biomass allocation (%) = (Inflorescence biomass/total biomass) ×100%
Leaf biomass allocation (%) = (leaf biomass/total biomass) ×100%
Stem biomass allocation (%) = (stem biomass/total biomass) ×100%
Root biomass allocation (%) = (root biomass/total biomass) ×100%
Above-ground biomass allocation (%) = (above-ground biomass/total biomass) ×100%
Reproductive biomass allocation (%) = (reproductive biomass/total biomass) ×100%
Vegetative biomass allocation (%) = (vegetative biomass/total biomass) ×100%

Results

Comparison of the biomass of different plant parts among three habitats

There was no significant difference in the inflorescences and stems biomass of *A. inebrians* in the three habitats ($P > 0.05$), but the leaves, roots and total biomass in the desert was significantly lower than that in the other two habitats ($P < 0.05$); there was no significant difference in the reproductive biomass in the three habitats ($P > 0.05$), but the vegetative biomass in the desert was significantly lower than that in the other two habitats ($P < 0.05$); the above and below-ground biomass showed significant difference in the desert, it was smaller than the other two habitats ($P < 0.05$) (*Fig. 2*).

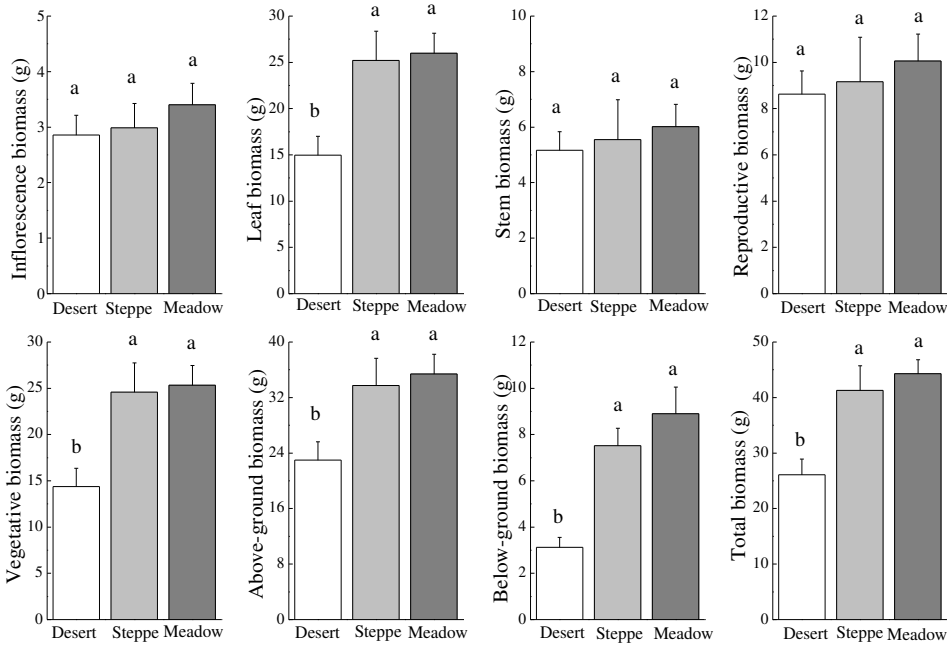

Figure 2. *Biomass of different parts of A. inebrians in three different habitats (g/plant). The error bars indicate standard error. Means not sharing a letter in common differ significantly at 5% probability level. N = 15*

The inflorescences, leaves, stems and roots biomass of *A. inebrians* in the desert represented 13.56%, 53.35%, 20.99%, and 12.11% of the total biomass of the plant, respectively, and biomass partitioning to the leaves and roots increased by approximately 5.67% and 8% of the total biomass in steppe and 8.55% and 5.47% of

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the total biomass in meadow, respectively, while the biomass allocation to stems and inflorescences (approximately -7.45% and -6.24% in steppe, -7.18% and -6.84% in meadow, respectively) decreased accordingly, The average inflorescences, leaves, stems and roots biomass allocation in the three habitats were 9.23%, 57.68%, 15.73% and 17.36%, respectively (*Fig. 3*).

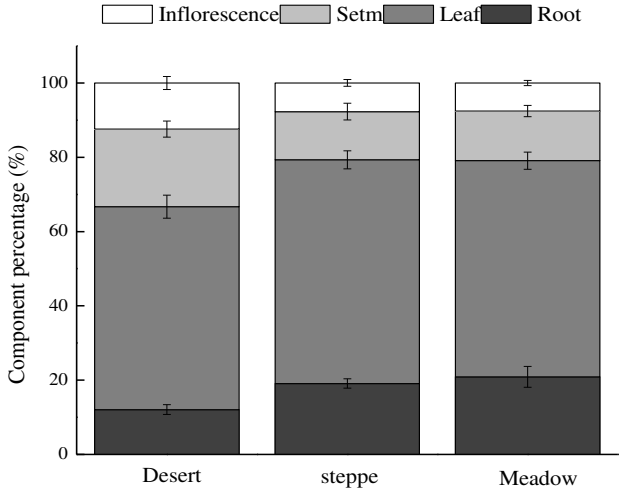

Figure 3. *Biomass percentages of different organs in relation to the total biomass of A. inebrians in three different habitats. The error bars indicate standard error. Means not sharing a letter in common differ significantly at 5% probability level. N = 15*

Changes of biomass of components in three habitats

Regression analysis of the root-leaf biomass, root-stem biomass, leaf-stem biomass, stem-inflorescence biomass and root-above-ground biomass correlations showed that there were similar positive linear correlations in desert and steppe, which reflected the structural and functional consistency and indivisibility of the individual plant organs. The coefficient of determination for the regressions including stem-inflorescence biomass showed a better fit across all three habitats than those involving the other organs, with R^2 values ranging from. The R^2 values of the root-leaf biomass, root-stem biomass, leaf-stem biomass, stem-inflorescence biomass, above-, root ground biomass and reproductive-vegetative biomass relationships fell within the ranges of 0.187-0.614, 0.001-0.231, 0.017-0.268, 0.347-0.636, 0.481-0.691 and 0.108-0.321, respectively, across all habitats, There are some differences in the biomass relationship of each component in the meadow habitat (*Fig. 4; Table 3*).

Relationship between the above- and below-ground biomass allocation and the total biomass of A. inebrians in three different habitats

The above-ground biomass allocation increased with the increase of the total biomass in steppe and meadow, while the above-ground biomass distribution decreased, indicating that more resources were allocated to the above-ground part with the accumulation of the total biomass of *A. inebrians*, while the change rule in desert habitat was just the opposite (*Fig. 5*). The determination coefficient R^2 of above-ground biomass allocation in each habitat was between 0.018 and 0.235, respectively. The coefficient R^2 of below-ground biomass allocation was between 0.011 and 0.235.

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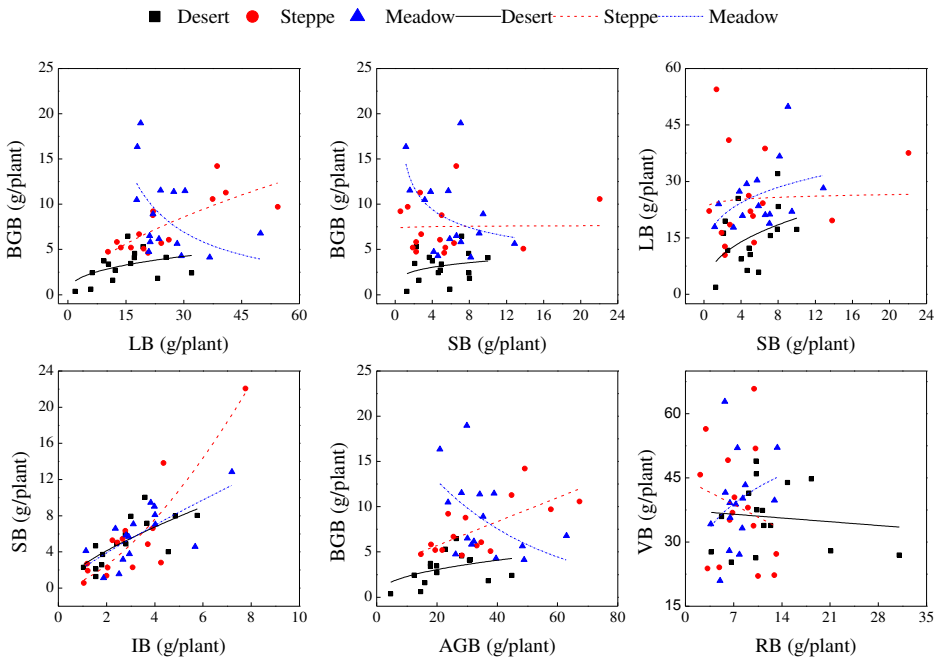

Figure 4. *The relationships between of the different organs biomass on the A. inebrians. The error bars indicate standard error. Means not sharing a letter in common differ significantly at 5% probability level. N = 15. BGB-below-ground biomass, SB-stem biomass, LB-leaf biomass, IB-inflorescence biomass, AGB-above-ground biomass, RB-reproductive biomass, VB-vegetative biomass*

Table 3. *The relationships between paired biomass parts of A. inebrians in three different habitats. The regression equations are from regression analysis. Probability (P) values denote significance levels from regression equation*

	Habitat	Function	df	R^2	F value	*P* value
Below-ground biomass vs leaf biomass	Desert	Power	15	0.533	14.803	0.002
	Steppe	Power	15	0.614	18.809	0.001
	Meadow	Power	15	0.187	3.167	0.099
Below-ground biomass vs stem biomass	Desert	Power	15	0.142	2.144	0.167
	Steppe	Linear	15	0.001	0.013	0.910
	Meadow	Power	15	0.231	3.900	0.070
Leaf biomass vs stem biomass	Desert	Power	15	0.268	14.941	0.002
	Steppe	Power	15	0.017	0.213	0.652
	Meadow	Power	15	0.188	2.679	0.126
Stem biomass vs inflorescence biomass	Desert	Power	15	0.589	5.711	0.033
	Steppe	Power	15	0.636	22.679	0.000
	Meadow	Power	15	0.347	1.483	0.005
Above-ground biomass vs below-ground biomass	Desert	Power	15	0.679	11.129	0.005
	Steppe	Linear	15	0.691	11.892	0.004
	Meadow	Exponential	15	0.481	3.905	0.070
Reproductive biomass vs vegetative biomass	Desert	Linear	15	0.108	0.155	0.700
	Steppe	Linear	15	0.215	0.629	0.442
	Meadow	Power	15	0.321	1.493	0.243

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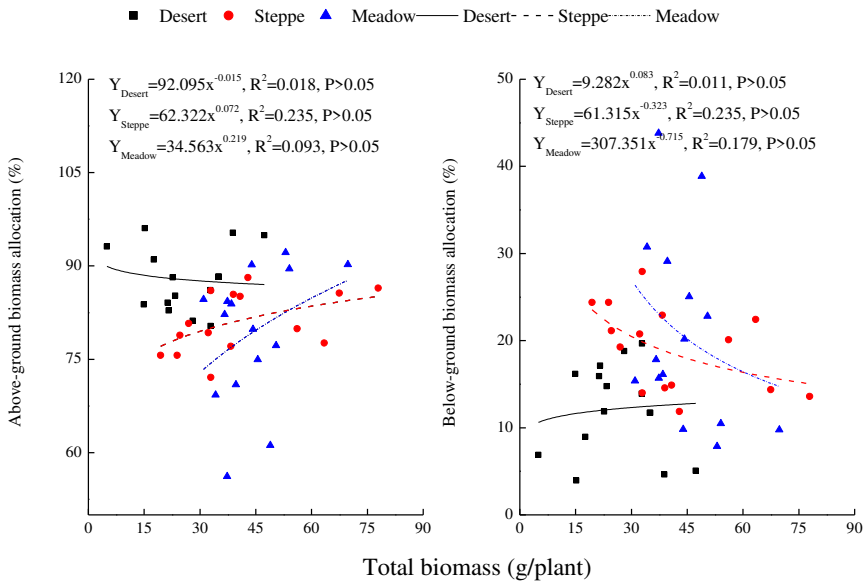

Figure 5. *Relationship between above-ground biomass, below-ground biomass and total biomass in A. inebrians. The error bars indicate standard error. Means not sharing a letter in common differ significantly at 5% probability level. N = 15*

Relationship between the reproductive and vegetative biomass allocation and the total biomass of A. inebrians in three different habitats

Reproductive allocation decreased with the increase of total biomass in desert and meadow habitats, but increased in steppe habitats. With the increase of total biomass, the nutrient distribution of three habitats showed an increasing trend. The coefficient R^2 of reproductive biomass allocation was 0.089-0.326 for each habitat, and vegetative biomass allocation 0.262-0.835 for each habitat. It can be seen that in all three habitats, the reproductive allocation of *A. inebrians* decreased and more resources were allocated for vegetative growth (*Fig. 6*).

Discussion

Biomass is an important indicator that reflects the interaction between plants and the environment. It is the embodiment of plant adaptability to the environment and its growth and development trends. Individual and modular organism biomass, are also the embodiment of the ecosystem's ability to obtain energy (Yu et al., 2001). The comparison of the biomass of each component in different habitats showed that there was no significant difference between the biomass of inflorescence and stem in the three habitats ($P > 0.05$), but the total, biomass of leaf and root in the desert habitat were significantly lower than those in other two habitats ($P < 0.05$). The results showed that the difference in total biomass mainly came from leaves and roots, which were the above-ground photosynthetic organs and the below-ground nutrient absorbing organs. The difference of environment mainly affected the biomass of the two components. The biomass of vegetative was significantly lower than that of the other two habitats ($P < 0.05$), but the biomass of reproductive was not significantly different ($P > 0.05$). It can be seen that under the fragile environment, the growth of *A. inebrians* decreased. The above-ground biomass was significantly lower than those in the other two habitats

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($P < 0.05$), and the overall trend of above-ground components was that the above-ground components were smaller than those in the other two habitats ($P < 0.05$). The cumulative benefits of each component, especially the significant reduction of leaf biomass, led to the significant above-ground components biomass. It was lower than other habitats ($P < 0.05$); therefore, the total biomass showed the biomass of sacrificing leaves, vegetative branches and even roots, maintaining the growth of reproductive branches and inflorescences, and maintaining sexual reproduction.

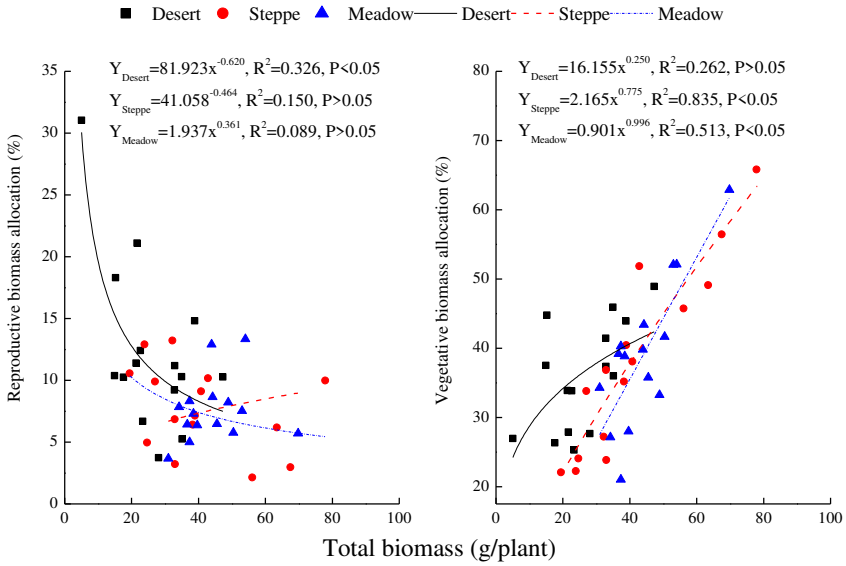

Figure 6. *Relationship between reproductive allocation, vegetative allocation and total biomass in A. inebrians. The error bars indicate standard error. Means not sharing a letter in common differ significantly at 5% probability level. N = 15*

The proportion of the biomass of various components to the total biomass represents the distribution ratio of assimilates to different organs and the coordinated relationships of various components during the growth process (Wang et al., 1995). In terms of biomass allocation, the biomass allocation into inflorescences and stems in the desert habitat was significantly higher than that in the steppe and meadow habitats, and the biomass allocation into the roots showed the opposite trends ($P < 0.05$). The proportion of leaf is the largest in biomass allocation of all habitats, Similar results have been obtained from previous studies (Yang et al., 2003). The mean value of the biomass allocation of each component in the three habitats indicated that leaf biomass allocation > root biomass allocation > stem biomass allocation > inflorescence biomass allocation; all of these differences were significant ($P < 0.05$) (*Fig. 3*). In terms of function, the leaves of plants are the production organs of nutrients, and the stems have multiple functions, such as nutrient transport and storage as well as supporting the leaves in expanding the growth space (Arenas et al., 2002). The distribution ratio to leaf biomass is the largest for all three habitats, and the distribution ratio to inflorescences biomass is the smallest. The pattern of biomass distribution in the three habitats is as follows: first to the leaves, then to the roots, and finally to the stem and inflorescences. This reflects that *A. inebrians* shows high ecological plasticity and strong adjustability. In response to different environments, *A. inebrians* uses different growth strategies to ensure its growth, development and reproduction.

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The coefficient of determination for the regressions including stem- inflorescence biomass showed a better fit across all three habitats than those involving the other organs, with R^2 values ranging from 0.347 to 0.636. The differences in the species index and organ size may be due to the distribution of photosynthetic products, water and nutrients between the above-ground parts and roots (*Fig. 4; Table 3*).

It is generally believed that high root biomass allocation ratio is a common feature of plants responding to below-ground resource deficit in the process of desert succession (Gill et al., 2002). The above-ground biomass allocation increased with the increase of total biomass, but the below-ground biomass allocation showed the opposite trend. This reflects the growth law of the *A. inebrians*, with the accumulation of biomass, the *A. inebrians* in steppe and meadow habitat preferentially distributes more biomass into above-ground components by reducing the input of below-ground roots, which is an important way to ensure the sexual reproduction and population diffusion of plants (Li et al., 2009); In the desert environment, the above-ground biomass distribution decreases with the increase of total biomass, while the below-ground biomass distribution decreases with the total growth. The growth of root system is enhanced by the increase of biomass, so as to ensure the water and inorganic nutrition needed for growth and reproduction in arid environment. Similar results have been obtained from previous studies (Zhao et al., 2017; Zhou., 2015) (*Fig. 5*).

The biomass allocation of vegetative, reproductive allocation and total biomass allocation reflect the resource allocation strategies between sexual and asexual reproduction (Hong et al., 2007; Zhang et al., 2004). The results showed that the reproductive allocation of *A. inebrians* in desert and steppe decreased, while that of meadow increased. The biomass distribution of vegetative branches and the total biomass of three habitats increased. On the one hand, the spatial characteristics of desert, grassland and meadow were related to the environmental conditions. Compared with desert and steppe habitat, meadow habitat can provide enough space for the spread of the *A. inebrians*, and the light is sufficient. The most direct and effective growth strategy for the *A. inebrians* to rapidly occupy such a favorable space is asexual reproduction. On the other hand, after entering the invasion area and settling down, the population regeneration of *A. inebrians* is mainly carried out by vegetative reproduction; for the allocation of reproductive branches, *A. inebrians* is also kept at a medium average level, which shows that *A. inebrians* has strong sexual reproduction ability, which is similar to the previous research results (Hong et al., 2010). Therefore, this study further speculated that the local spread and diffusion of the *A. inebrians* in the invasion area, the population renewal mainly through nutrition reproduction, long-distance transmission, mainly through seeds, which need further study to investigate (*Fig. 6*).

Conclusion

There was no significant difference in the biomass of inflorescences, stems and reproductive branch of *A. inebrians* among the three different habitats of desert, steppe and meadow ($P > 0.05$), but the biomass of other components in the desert was significantly lower than those in the other two habitats ($P < 0.05$). The allocation of leaves biomass was the most and inflorescence was the least in three habitats, and the biomass of reproductive and vegetative, above-ground and below-ground biomass increased with the increase of total biomass. In the steppe and meadow habitats, more

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energy is preferentially distributed to the above-ground components by reducing the input of below-ground biomass, but in the desert habitats, the opposite trend was observed.

In this paper, we only analyse the effect of different habitats on biomass and biomass allocation of *A. inebrians*. The allocation of biomass in the population of *A. inebrians* was neglected the influence of disturbance degree and genetic characteristics on them, and whether the difference of the relationship among the components of *A. inebrians* is related to their location Is it related to the level of phylogeny at present, certain regularity can be studied from these angles in the future Growth strategy of *A. inebrians* population.

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DEVELOPING A pH MODEL USING ARTIFICIAL NEURAL NETWORK AND VISUAL MODFLOW TO EVALUATE GROUNDWATER QUALITY

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Abstract. Groundwater pollution cause excessive levels of water quality parameters such as pH, Total Dissolved Solids (TDS), hardness etc, therefore, it is a severe problem for people. The present study describes the prediction of pH in groundwater using Artificial Neural Network (ANN) model and visual MODFLOW. It is observed that the pH level of study area is lower than the standard limit so that the water turns to acidic. The water is not suitable for drinking purposes thus a prediction model is needed to rectify this issue. A smart prediction modelfor pH was developed using ANN. Selected input variables were Hardness, Calcium (Ca), Magnesium (Mg), Sodium (Na), Potassium (K), Nitrite (NO_3), Chloride (Cl) and Sulphate (SO_4)and the best performance value was 0.025637. Also to simulate the groundwater of the study area, visual MODFLOW model was created.Using this model, pH is simulated for 365 days. From the calibration plot, it is known that the correlation coefficient of the observed and the simulated value is 0.92. From the simulation it is concluded that the pH level was almost the same for 365 days.
Keywords: *Mondaikadu, water quality, solute transport, simulation, prediction*

Introduction

Groundwater has been essential to sustain India's economy and to fulfill its domestic needs, and it also has agricultural and industrial use. Around 33% of the total population relies upon the utilization of groundwater for drinking (Nickson et al., 2005).Groundwater accounts for about 98% of all of the planet's available fresh water, and it is about 60 times more concentrated than the fresh water present in lakes and streams. India is the world's biggest groundwater user, with a yearly use of roughly 230 cubic kilometers for each annum (World Bank, 2012). The nation as a whole hasanet annual groundwater supply of 398 billion cubic meters (Water and Related Statistics, 2015). More than 60% of farming and 85% of drinking water supplies depending on it, groundwater is a basic asset for Indian rural zones.

Nowadays, groundwater pollution is considered as a world level issue. Many organizations are taking serious actions against this issue and creating regular monitoring programmes. A research studies on various features of groundwater, such as, storage potential, hydrogeology, water quality, vulnerability, and sustainability and

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so on was conducted by many researchers (Chapagai et al., 2010; Pandeyet al., 2011, 2012). Particularly the quality of water is considered a key contributor to various diseases. In general, groundwater is less vulnerable to pollution as compared to surface water. Contamination of groundwater is due to the presence of certain pollutants in groundwater which exceed limits prescribed for drinking water. Arsenic, nitrate and carbon, which are of a geogenic origin, are the widely found pollutants. Many pollutants include bacteria, phosphates, and heavy metals that arise from human activities like domestic waste, agricultural practices, and industrial effluents. Groundwater quality may change from place to place as well as season to season. Weak environmental management schemes in factories contribute to water discharges of hazardous and agricultural wastes. This resulted in the contamination of those surface and groundwaters from which water is drawn for irrigation and household use (Pai and Mulye, 2016). Landuse pattern is one of the reasons for groundwater contaminations.

Many intelligent tools, such as linear and non-linear tools are successfully used to predict the water quality. Nowadays artificial neural networkis used to find a solution for many cases of water quality problems.Wagh et al.(2016) used the ANN model to predict sodium adsorption ratio (SAR) values, residual sodium carbonate, magnesium adsorption ratio, Kellys ratio and sodium percentage in Nanded tehsil ground water.It is found from the results that there is a strong agreement between the actual data and the ANN outputs for indices of irrigation suitability for training and testing datasets.Mohammadi et al. (2016) utilized ANN model to simulate concentrations of fluoride in groundwater resources in Khaf and surrounding villages based on the water's physical and chemical properties.The best results were observed by the MLP1 model with eight parameter inputs such as root mean square error (RMSE) and correlation coefficient of real and expected outputs.The simulation results from the MLP1 test stage as well as the high correlation between experimental and projected data suggested that this model can be used to predict fluoride concentrations in groundwater supplies with its high confidence coefficient.Kheradpisheh et al. (2015) developed the artificial neural network for Cl^-, EC, SO_4^{2-} and NO_3^- in order to assess the influence of the key input parameters, the test results were estimated and compared with the measured values.Palani et al. (2008) predicted the quantitative characteristics of the water bodiesusing ANN. The ANN model was developed at any location in the domain of interest for fast assessment and selected water quality variableswere forecasted. The input parameters measured at other locations serve as respective variables. Input variables include salinity, temperature, dissolved oxygen, and chlorophyll-alpha.The findings demonstrate the great potential of the ANN for simulating variables in water quality.

During the summer, winter and monsoon seasons, the level of pH in the study area is much less (5.4 to 6.2) when compared to the World Health Organization (pH 6.5-8.5) (WHO, 2008)and Indian Standards(pH 6.5-8.5) (BIS, 2009),therefore the water becomes acidic in nature and the quality of the water is degraded. The reason for low pH is due to the coconut retting zone of the study area (Jessy Mol and Baskaran, 2017). Since the level of pH is decreasing, modelling of pH is needed to find out the pH level in the future.The novel aspect of this study is the Visual MODFLOW combined with MT3DMS,it is used for the groundwater quality model, where the coconut retting zone of the study area is taken as the recharge zone for boundary conditions in MODFLOW. Therefore, the influence of the retting zone on the pH level can be predictedvery easily.

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Materials and Methods

Study area

The area taken for study is in Mondaikadu Panchayat of Kanyakumari District, Tamil Nadu, India. It lies between $8^0$9'47" latitude and $77^0$16'48.09" longitude. The town averages 25 meters above Mean Sea Level (MSL). It is a coastal area of Kanyakumari District. The soil is usually red loam but in some areas there is also sandy loam soil. The average annual rainfall is 1456.8mmwith 79.7 rainy days. The precipitation is dispersed from April to December, and goes under three seasons, to be specific hot weather seasonal rainfall, South West monsoon and North East monsoon (ENVIS centre Tamil Nadu). The study area map is shown in *Fig. 1*.

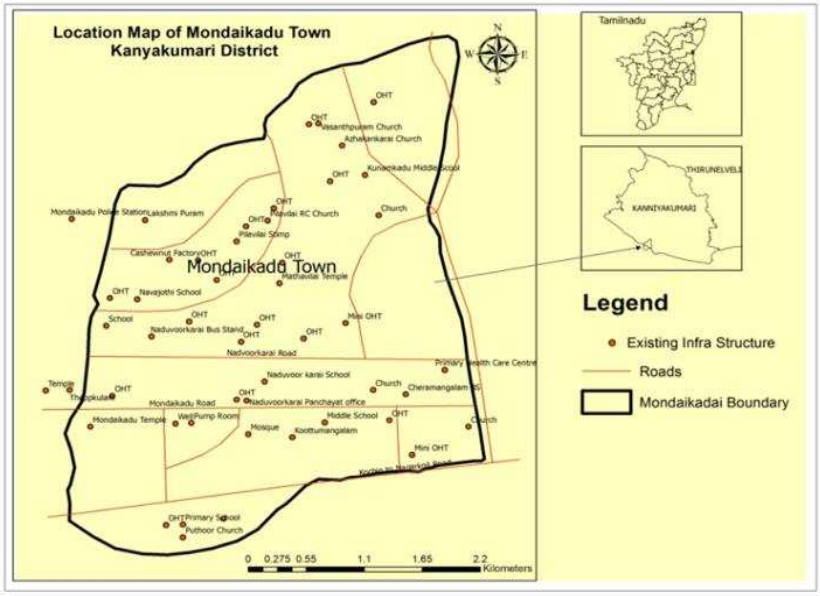

Figure 1. *Study area map*

Sample collection and analysis

Collection of samples is the key aspect of any type of scientific study. Twenty sampling points were chosen from the study area and samples for the three seasons were collected, namely winter (October to February), summer (March to May) and monsoon (June to September). For three years (2017-2019) the samples were collected from the study area, so that the total number of samples collected was 180. The approximate depth of the sampling was 50m. After 10 minutes of pump start, the samples were collected directly from the bore wells. The unstable parameters turbidity, pH and temperature were tested using digital meters at the sampling points. Other parameters were tested in a laboratory as per APHA (1998). Temperature, pH and turbidity were determined at the sampling site with a thermometer, systronics pH meter and Nephlometric turbidity meter respectively. Calcium and magnesium were determined titrimetrically using standard EDTA solution and total chloride by volumetric titration method. Sodium and Potassium were estimated using flamephotometry, Nitrite was determined using ion selective electrode.

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Artificial neural network (ANN)

ANN is a tool for mathematical modelling. It works based on neural processing in the human brain. In the human brain, each neuron is connected as a network. In the same manner, in the neural network, the layout has a set of neurons and they are linked and organized. The information passes through a neural network, from the input to the output. The Network consists of interconnected node layers. These nodes are called neurons which function as elementary units of processing. From the various inputs each neuron receives the information and produces an output by the value. Its activation function happens when the weighted whole of its information is the statement (Kilicaslan et al., 2014).Every layer is related by its respective weights to the next layer (Nasr et al., 2012).*Eq.1* is used to calculate the weighted sum of the layer.

$$y_j = \sum_{i=1}^{n} W_{ij} x_i + \theta \quad \text{(Eq.1)}$$

where yj is the weighted sum of the jth neuron for the input data obtained from the n-neuron proceeding layer, Wij is the weight between the ith neuron and the jth neuron in the proceeding layer, xi is the contribution of the ith neuron in the proceeding layer and θ may be biased by the jth neuron. The input layer of the network is used to feed the data to the network; the hidden layer of the network is used to act as a collection of features detector, and the output layer is used to get out the result (Seyamand Mogheir, 2011).

In the forward direction, the input signal passes through the network from one to another layer. This process is named as Multi Layer Perception (MLP). Back Propagation Algorithm is used in MLP to solve the problems. This algorithm consists of Feed Forward and Feed Backward Network (Subbarayan, 2013).Feed Forward Back Propagation Network is mostly used in many water resources problems.The optimal weight is calculated by minimizing the Mean Square Error (MSE) of the training stage (Nasr et al, 2013). Mean Square Error is calculated by *Eq.2*.

$$MSE = \frac{1}{n} \sum_{1}^{n} (T_i - Out_i)^2 \quad \text{(Eq.2)}$$

where T_i and the out_i are the desired neural network target and output for the ith neuron respectively, n is the number of samples in the network. Neural networks have three stages; these are training stage, validation stage and testing stage. In the training stage, the network's weight and bias are computed. Learning functions are used at this stage to update weight and bias of the layer. Here, the trained network is validated. After that, the data set is used to examine the generalization of the network. This stage is the testing stage.

The network is classified as two types namely, supervised and unsupervised classification. Supervised classification means that the input and output data are given and the error is calculated for the predicted output and the output given to the network. Mostly used training function is trainlm function, Levenberg – Marquardt back propagation (trainlm) function locates the minimum of a multivariate equation it can be represented as the sum of the non-linear real-evaluated functions squares. It's an iterative technique that operates in such a way that every iteration of the algorithm is always reduced by output level. This component makes trainlm the quickest training algorithm for reasonably sized networks (Sharma and Venugopalan, 2014). This

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training function is often capable of getting lower mean square errors than the other training function (Kumar, 2010).

Visual MODFLOW model

Visual Modflow is used to simulate the Groundwater flow because of its most comprehensive and user-friendly modeling environment. MT3DMS is one of Visual Modflow's problem solving tool which is used to evaluate the simulation of contaminant transport. This model is completely incorporated, combining the strong analytical tools with a clear menu structure.In model creation or analysis of results, the model info parameters and results can be envisioned in 2D and 3D at any minute. The mathematical equation for groundwater model is given by *Eq.3*.

$$\frac{\partial}{\partial x}\left\{Kx\frac{\partial h}{\partial x}\right\}+\frac{\partial}{\partial y}\left\{Ky\frac{\partial h}{\partial y}\right\}+\frac{\partial}{\partial x}\left\{Kz\frac{\partial h}{\partial z}\right\}=Ss\frac{\partial h}{\partial t} \quad \text{(Eq.3)}$$

where, Kx, Ky are the Hydraulic conductivity of the x and y directions respectively, Kz is the Hydraulic conductivity of the z direction, x ,y, z are the Cartesian coordination, h is the Hydraulic head, Ss is the Specific storage of the porous material and t is the Time.

Transport equation

The equation represents motion of the solute mass transport through a volume of control. It indicates that the amount of masses absorbing or producing solute with the volume of control must be equal to a shift of solvent concentration with the volume of control. *Eq.4* gives the solute transport equation that is implanted in MT3D used for modeling.

$$\frac{\partial C}{\partial t}=\left[\frac{\partial}{\partial x_i}\left(D_{ij}\frac{\partial C}{\partial x_j}\right)\right]-\left[\frac{\partial}{\partial x_i}(V_i C)\right] \quad \text{(Eq.4)}$$

where,

V_i = Seepage velocities in x, y, z directions, m/s (LT^{-1}),
D_{ij} = Dispersion coefficients, m^2 /sec (L^2 T^1),
C = Solute concentration, mg/m^3 (ML^3),
t = Time.

Input data

The detailed hydrogeological description of the study area was collected to allocate the required modeling inputs. The data required to input the MODFLOW for groundwater modelling areelevation, quality data, observation well details, subsurface thickness, hydraulic head, contaminant concentration, conductivity, storage properties, dispersion coefficient, evapo-transpiration, recharge and Boundary conditions.

Simulation

A stress cycle is characterized as a time period during which all system stresses are constant. Design Visual Modflow was utilizing the data from the first time period of each given boundary condition for a steady state simulation. For transient simulations,

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all of the data described in different time period for each boundary condition and well was automatically merged into the stress period format provided by Modflow.

Results and Discussion

ANN model for pH

A back-propagation Feed Forward Neural Network (FFNN) algorithm is used to compare the relationship between input and output. This model is performed using MatlabTool box for ANN (MATLABand StatisticsToolbox, 2012). Based on prior research by Sathyamurthy (2013), the ANN inputs for pH are identified. The selected output is pH and the input variables are Hardness, Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, NO_3^{-},Cl^{-} and SO_4^{2-}. Several trials were conducted for each group to attain the suitable network structure until the appropriate training rate, the number of hidden layers and the number of neurons per hidden layer was reached. The best structure is the one that produces the minimum MSE in both the training and testing data (Nasr and Zahran,2014). The algorithm for back propagation minimizes the Mean Square Error in the output layer between the observed and the predicted output. The structure resulting in a minimal number of errors is the one selected as shown in figure. The network properties are as follows:

- Data Used: 160 for training 20 for testing.
- Network input: Hardness, Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, NO_3^{-}, Cl^{-} and SO_4^{2-}.
- Network output: pH.
- Network type: feed-forward back –propagation.
- Training function: Levenberg–Marquardt algorithm (TRAINLM).
- Adaptation learning function: LEARNGDM.
- Performance function: MSE.
- Epochs: 1000.
- Number of neurons: 25, *Fig. 2* shows the neural network diagram of the model.

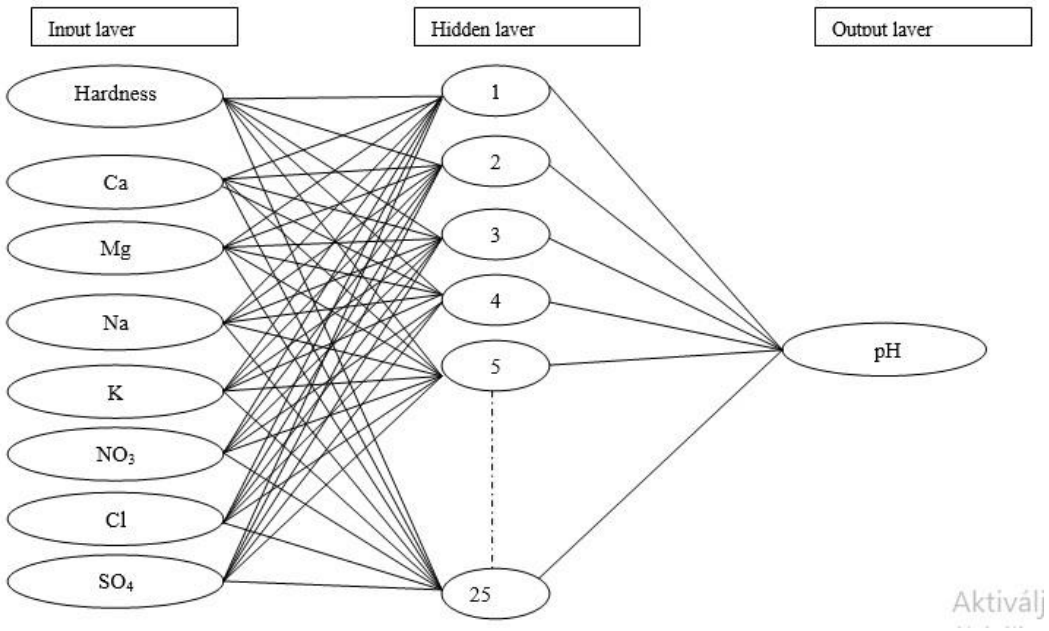

Figure 2. *Neural Network Diagram of pH*

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To terminate the network training, the gradient's magnitude and the iterations of validation checks is used. The number of validity checks is 9,which is the right value to quit training. The output plot indicates the function's value as opposed to the iteration number in terms of preparation, evaluation, and test behaviors. Based on the MSE the best validation performance is 0.025637 at epoch 3 as shown in *Fig. 3*. Since the validation and test graphs are close, the training did not pose any major problems or over fit. During preparation, each layer neuron changes its weight vector toward the input vector group nearest to it.

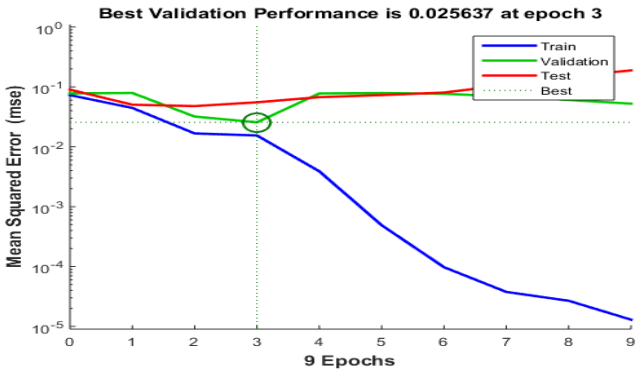

Figure 3. *Training performance graph*

As shown in *Fig. 4*, for training, validation, and testing, a linear regression analysis is performed to find out the correlation between the network outputs and goals. The dotted line represents the optimal outcome in each map, i.e. outputs = goals, while the dark line indicates the best linear regression that suits. The effects of regression (R-value), respectively, are 0.96, 0.99 and 0.98 for preparation, validation and research. Those results corresponded to a cumulative 0.96.

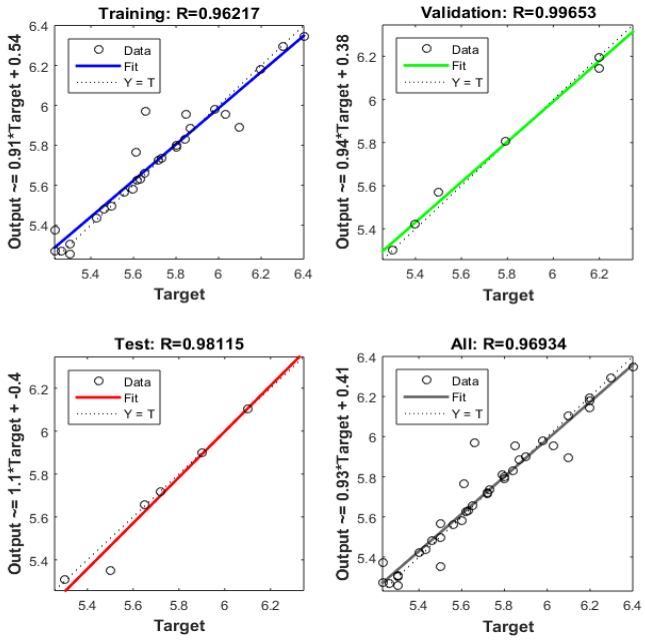

Figure 4. *Regression plot for training, validation and testing*

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After training, validation and testing of the network, the created network can be used to predict the pH via the new input data. From *Fig. 5* it was found that the experimental pH analysis is similar to the expected data determined from the network and this confirms this model's validity. *Fig. 6* shows the linear relationship between the observed and predicted pH and it found R^2 0.927. From this, it is inferred that the model established is apt to predict groundwater pH.

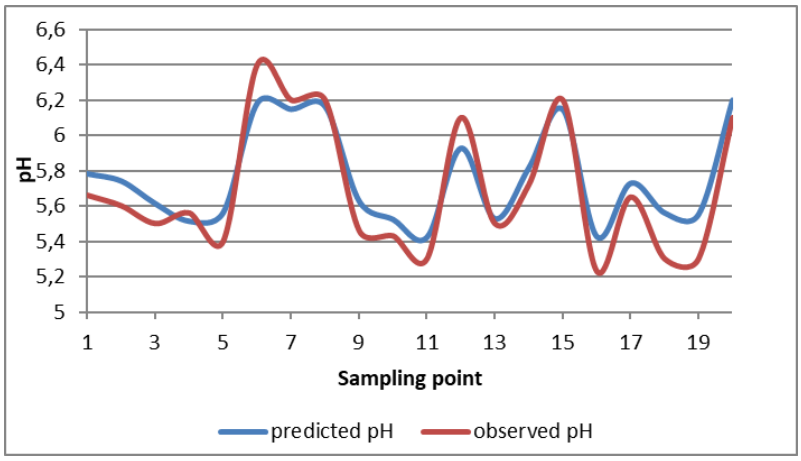

Figure 5. *Comparison of observed and predicted pH*

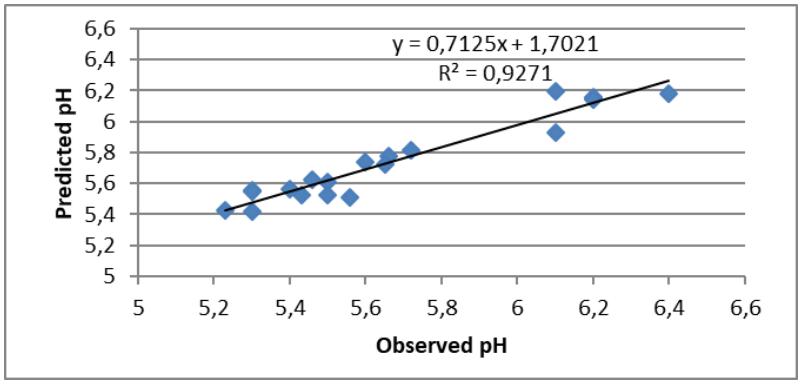

Figure 6. *Linear relation between observed and predicted EC*

MODFLOW model

Modeling of groundwater flow and transport is performed using Visual MODFLOW and MT3DMS tools (Harbaugh, 2005). The three Visual MODFLOW sections are Input, Run, and Output. The groundwater parameters and aquifer properties are given in the input section as input to the model. Based on the specifications, the correct engine and solver parameters are selected in the run portion, and the model is simulated.

Input of transport modeling

Hydro-geological characterization of the study area is performed to allocate the required modeling data. *Fig. 7* shows output obtained from the model, such as location of pumping wells and concentration wells and boundary conditions. The assigned

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boundary conditions of the study area are concentration and recharge boundary conditions. Since the pH level of the study area is less than the drinking water standards (WHO) the level of pH is given as the input to the concentration wells.

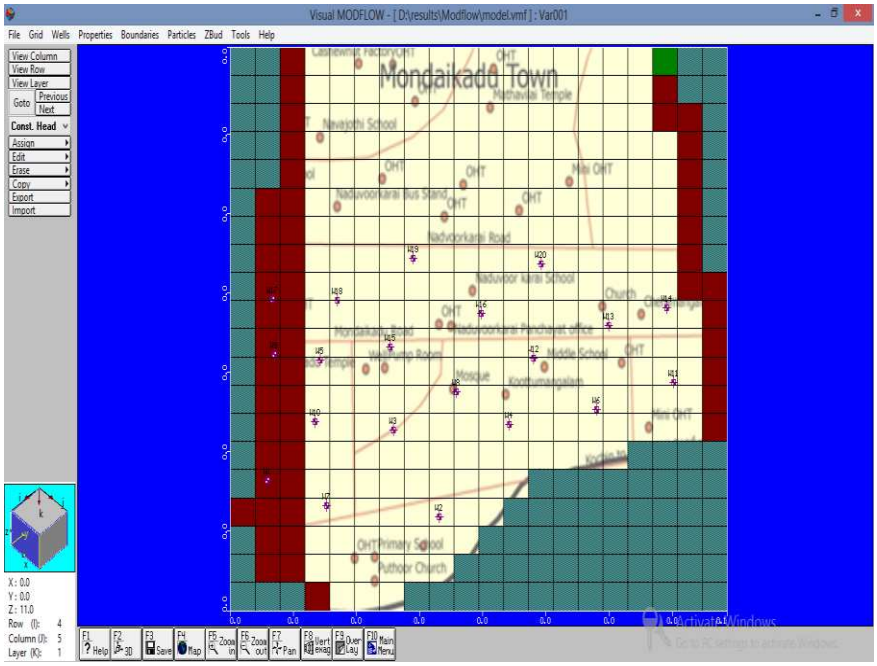

Figure 7. *Boundary conditions of study area*

Model simulation

The results of the transport contamination were simulated using the following assumptions.

Run Type	-	Transient flow
Time Steps	-	10 stress periods
Simulation Period	-	365 days
Solver	-	WHS solver
Layer	-	Single layer of unconfined aquifer

Model output

In *Fig. 8* the flow direction is initially from the south side towards the other three sides of the study area, because the south side is given as the recharge zone of the study area. The vectors of velocities represent the speed and direction of the particle of water as it travels through the field of flow.

Calibration of model

The pH is simulated and calibrated for 365 days; the calibration plot between the observed and the predicted pH is shown in *Fig. 9*. It shows the observed pH is 5.68 and the calculated pH is 5.35 same as the observed value of pH for another sample is 5.15 and observed value is 5.20. The correlation coefficient between the observed and the calculated value is 0.92; this value is nearer to 1, and therefore the model is calibrated in a correct manner.

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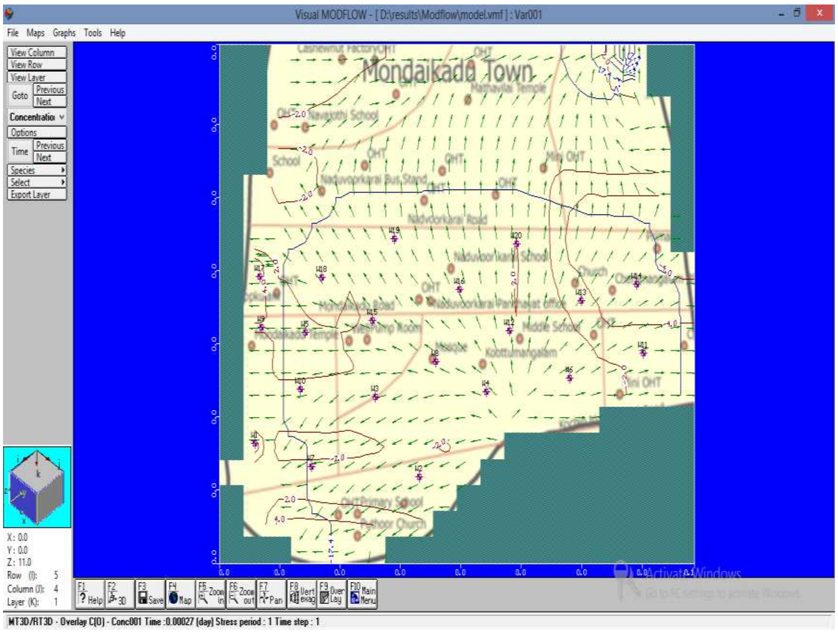

__Figure 8.__ Flow directions and contour lines

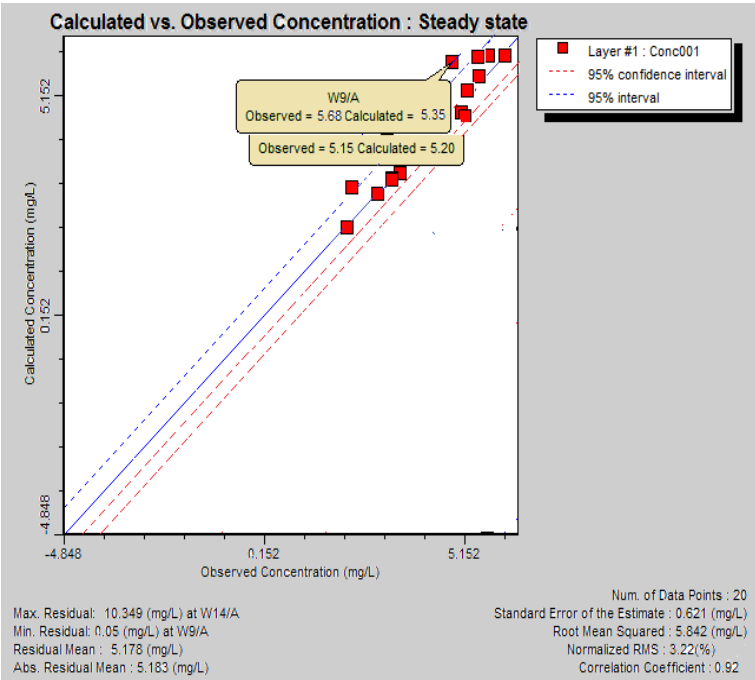

__Figure 9.__Model Calibration Chart

Conclusions

The pH was predicted by ANN model, using Hardness, Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, NO_3^{-}, Cl^{-} and SO_4^{2-} as inputs. The best validation performance was 0.025637. Since the ANN model is based on the output data, the approach can be used for the other basins of study area, depending on the availability of data. To simulate the pH, Visual MODFLOW

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model was used. The flow concentration was simulated for 365 days, and the correlation coefficient of the observed and the simulated value was 0.92. The decision makers and the groundwater managers associated with Mondaikadu will benefit if they use the findings of this paper as a decision support tool. It is recommended that the research can be done in the future by implementing various artificial recharge structures in the study area to enhance the groundwater quality.

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EFFECTS OF STRAW DECOMPOSITION ON AGGREGATE COMPOSITION AND AGGREGATE-ASSOCIATED ORGANIC CARBON IN DIFFERENT SOIL MINERAL TYPES

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Abstract. Straw is an important organic resource that could be recycled, however, few studies investigated the effects of straw decomposition on aggregate stability and organic carbon (OC) of different mineral soils. In this study, we conducted a constant temperature incubation experiment for 120 days in three different mineral soils consisting of two treatments: control soil and straw-amended soil. The results showed that straw addition promoted a significant increase of >2 mm water-stable macroaggregates as well as a significant decrease of microaggregates (<0.25 mm) compared with control soils (P<0.05). From day 15 to day 120, the three straw-amended soils significantly increased in mean weight diameter (MWD) and geometric mean diameter (GMD). The OC concentration of different particle-size aggregates in straw-amended soils increased significantly during the early 15 days of incubation, then gradually decreased during the later stages. After 120 days of incubation, the contribution of OC of macroaggregates to soil organic carbon in fluvo-aquic soil reached the highest (66.14%). Among them, the fluvo-aquic soil with 2:1 clay mineral showed better improvement of aggregate stability and OC concentration than red soil during straw decomposition. These results confirm the differences and benefits of straw application for soil structure and quality of different clay mineral types.
Keywords: *straw-amended soil, incubation, water-stable aggregates, aggregate stability, soil organic carbon*

Introduction

Soil aggregate is a special organic-inorganic-biological complex with multi-level structure or fractal characteristics. Its formation and stability process are extremely complex. As the basic unit of soil structure, it features complicated formation and stability process which is not only affected by the material composition of the soil itself, but also by human activities and other factors (Verchot et al., 2011; Zhang et al., 2014; Rabot et al., 2018). Quality soil aggregate plays an important role in regulating soil properties, as well as maintaining soil fertility and ecological environment function. It is not only conducive to the efficient use of water and fertilizer and crop growth, but also to improve soil erosion resistance and carbon sequestration. As its stability is closely related to many soil properties and the ecological environment, it

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plays a vital role in maintaining the sound and sustainable development of soil and the protection of ecological environment (Blanco-Canqui and Lal, 2004; Abiven et al., 2007; Bandyopadhyay and Lal, 2014; Wang et al., 2017). On the contrary, the decrease of soil structure stability will not only reduce the water and fertilizer utilization efficiency of the soil, but it also affects crop yield by increasing the risk of soil degradation and soil erosion, and destroying the sound and sustainable development of soil (Lal, 2008; Li et al., 2017; Vaezi et al., 2017). Therefore, the research on the structural stability and function of soil aggregates has always been one of the main focuses in the field of soil science.

Soil organic carbon (SOC) is the main factor of carbon balance in terrestrial ecosystems, and its small fluctuation may have a significant impact on greenhouse gas emissions and even carbon balance in terrestrial ecosystems (Vanhala et al., 2008; Lu et al., 2013). According to the research, there is a close relationship between SOC and aggregates, and the interaction between the formation of aggregates and the carbon sequestration is of great significance to promote soil carbon sequestration. As the main source of SOC, exogenous new carbon contains a variety of nutrient elements, whose direct addition can increase the number and activity of soil microorganisms, and promote the accumulation of SOC (Fonte et al., 2009; Blaud et al., 2012; Haydu-Houdeshell et al., 2018). The stability of soil aggregates mostly refers to the water stability of soil aggregates. The content and stability of water-stable aggregates play an important role in evaluating the stability of soil structure and soil anti-erosion ability. SOC is one of the most important factors that affect the structure of soil aggregates. As an important cementing substance in the formation of soil aggregates, it plays an important role in the formation of aggregates with different number and particle size distribution, promoting the aggregation of aggregates and the stability of water-stable aggregates (Chivenge et al., 2011; Ji et al., 2014; Zhu et al., 2017). At the same time, OC can be found mainly in aggregates, and soil aggregation can provide physical protection for SOC. Therefore, relevant scholars regard the stability of soil aggregates or the carbon sequestration capacity of soil as an important index to evaluate soil physical properties and SOC pool. They think that the improvement of the stability of soil aggregates, the quantity and quality of aggregates, as well as the content of SOC pool plays an important role in promoting the sound and sustainable development of agriculture and carbon cycle (Peth et al., 2008; Bimüller et al., 2016).

Straw is an important organic resource that can be recycled, at present, great importance has been attached to the sustainable development of agriculture by adopting the conservation tillage method with straw amendments or mulching as auxiliary measures. The application of straw can increase the concentration of SOC and promote the recycling of straw nutrients in the soil. The organic cement produced after straw decomposition can also promote the formation of soil aggregates, strengthen the cohesion between soil particles, and improve the structure of soil aggregates (Blanco-Canqui and Lal, 2004; Abiven et al., 2009; Blanco-Moure et al., 2012). For example, Gu et al. (2014) studied the effect of corn straw treatment on aggregate composition and OC concentration of aggregates in brown soil, which showed that the addition of corn straw significantly increased the content of >2000 μm water-stable aggregates, reduced the content of 250-2000 μm and <250 μm aggregates, and increased the stability of aggregates and OC concentration of aggregates. Tang et al. (2011) studied the effect of plant residue decomposition on the quantity and stability of red soil aggregates, which showed that straw treatment significantly promoted the formation of >2000 μm and

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250-2000 μm water-stable aggregates, and improved the stability of aggregates. According to the above studies, straw addition has a great influence on the distribution, stability and OC concentration of soil aggregates. Soil aggregation processes were very complicated, different types of soil contain different clay minerals, which will affect the agglomeration of aggregates and the retention of OC, it is necessary to explore the agglomeration of aggregates and the retention of OC with the application of straw after the application of straw in different types of soil. However, there are few reports about the effect of straw decomposition on aggregates stability and aggregate-associated OC of different types of soil. It is difficult to distinguish the difference about straw decomposition on the distribution and stability of aggregates and aggregate associated OC of different types of soil, which requires further study. To this end, this paper studies the effect of straw application on the distribution and stability of water-stable aggregates in three different types of soil, and explores the distribution law of OC in different particle-size aggregates and the contribution rate of aggregate-associated OC during the process of rice straw decomposition with disturbance-free indoor incubation experiment. This study aims to provide theoretical support for the improvement of aggregate structure and OC in different types of soil.

Materials and Methods

Site description and experimental samples

The three typical soil samples were red soil (R Soil), fluvo-aquic soil (FA Soil) and lime concretion black soil (LCB Soil) in China, which were collected in their respective test stations in September 2014 with a sampling depth of 0-30 cm (*Fig. 1*). Five sampling points were evenly mixed and then quartered for sampling. Specifically, the sampling area of R soil is located in Yingtan R Soil Ecological Experimental Station of Chinese Academy of Sciences, Jiangxi Province, China (116°55′E, 28°15′N). The soil type is R soil developed from Quaternary red clay and the parent rock mineral types are mainly iron-aluminum oxides and 1:1 clay mineral, which is classified as Ultisol in the USDA soil taxonomy; the sampling area of FA soil is located in Fengqiu Ecological Experimental Station, Henan Province, China (114°24′E, 35°00′N). The soil type is FA soil developed from Yellow River sedimentation and the mineral type is mainly 2:1 clay mineral with high calcium carbonate content, which is classified as Inceptisol (USDA); the sampling area of the LCB soil (Vertisol) is located in Yangliu Experimental Site, Suixi, Anhui Province, China (116°46'E, 33°37'N), and the mineral type is mainly 2:1 clay mineral with montmorillonite as the main component (Lv and Li, 2006). After the coarse plant residues, large gravel and other impurities were removed from the collected soil, the soil was brought back to the laboratory for natural air drying in a cool and ventilated place, and then it was gently forced apart along the natural fragile zone so that it could pass through the 2 mm sieve. Special attention was paid to minimize the disturbance to the soil during the collection and transportation process to avoid the damage to the aggregates.

The basic properties of the three typical types of soil were shown in *Table 1*. The straw samples used in this experiment were rice straw cultivated in greenhouse. After 115 days of sowing, the above ground part of rice plants was obtained. After being dried at 60 ˚C, the collected rice plant residues were crushed and put into bags after 0.25 mm sieving. The C and N concentration of rice straw was 396.5 g kg^{-1} and 16.5 g kg^{-1}, C/N ratio was 24.03.

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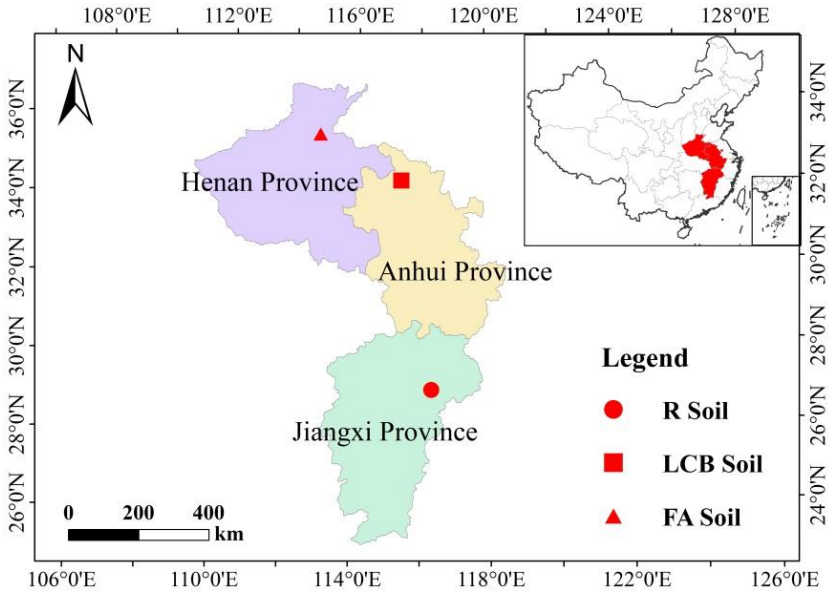

Figure 1. *Location of the sampling site*

Table 1. *Properties of studied soil*

Soil Type	Organic Carbon (g kg^{-1})	Total N (g kg^{-1})	pH	Sand (%)	Silt (%)	Clay (%)	Texture (USAD)
R Soil	9.86±0.23	1.12±0.01	4.62±0.01	36	20	44	clay
FA Soil	5.38±0.86	0.57±0.05	8.68±0.07	23	32	45	clay
LCB Soil	10.45±0.33	0.90±0.05	6.34±0.02	30	33	37	clay loam

R Soil, red soil; LCB Soil, lime concretion black soil; FA Soil, fluvo-aquic soil

Experimental design

Self-designed experiment was conducted under three types of soil during different incubation periods. The experiment consisted of two treatments: no straw (control soil) and 1% straw (straw-amended soil). Each treatment was set with three repetitions. Specifically, three typical soil samples were sieved with a 2 mm sieve and 300 g were weighed into a 2 L plastic culture flask, 3 g of rice straw were added into the flasks, rice straw was evenly distributed in the test soil, then water was added to 70% of the maximum soil water capacity, and samples were incubated in a constant temperature incubator at 28°C. Meanwhile, the control treatment was also prepared. During the whole incubation period, the daily ventilation was ensured and the weight was measured every week to keep the constant soil moisture content. Then three repeated treatments of soil were carried out after 15, 60 and 120 days of incubation to determine the aggregate-associated OC, water-stable aggregates and other indicators.

Measurement indexes and methods

The aggregate-associated OC was measured by potassium dichromate oxidation-outer heating method; soil pH value was measured by potential method (soil water ratio 2.5:1); soil clay and silt content were measured by pipette method (Tiessen and Moir, 1993; Bao, 2000). Total carbon content of straw was measured by C/N element analyzer;

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distribution and stability of soil aggregates were measured by wet sieving method, and water-stable aggregates with particle sizes of >2, 0.25-2, 0.053-0.25 and <0.053 mm were obtained (Nimmo et al., 2002; Six et al., 2004). The detailed calculation equations of stability index $R_{0.25}$ (>0.25 mm water-stable macroaggregates), geometric mean diameter (GMD), soil unstable aggregate exponent (E_{LT}), mean weight diameter (MWD), fractal dimension (D) and contribution rate of soil water-stable aggregate OC were extracted from relevant references and calculated as the following equations (Liu et al., 2012; An et al., 2016; Zhu et al., 2017; Xue et al., 2019).

$$\mathrm{MWD} = \frac{\sum_{i=1}^{n}(\overline{x_i}w_i)}{\sum_{i=1}^{n} w_i} \quad \text{(Eq.1)}$$

$$\mathrm{GMD} = \exp\left(\frac{\sum_{i=1}^{n} w_i \ln \overline{x_i}}{\sum_{i=1}^{n} w_i}\right) \quad \text{(Eq.2)}$$

$$E_{LT} = \frac{M_T - R_{0.25}}{M_T} \times 100\% \quad \text{(Eq.3)}$$

$$\frac{M(r < \overline{x_i})}{M_{\mathrm{T}}} = (\frac{\overline{x_i}}{x_{\max}})^{3-D} \quad \text{(Eq.4)}$$

where n denotes the number of aggregate size fractions, $\overline{x_i}$ is the mean diameter of aggregates retained in the ith sieve, W_i is the aggregate weight retained in the ith sieve, $M(r \leq x_i)$ is the weight of aggregates with a fraction diameter less than or equal to x_i, and M_{T} is the gross weight of aggregates.

Statistical analyses

The data were organized via Microsoft Excel 2013, the mapping was made by SigmaPlot10.0, the one-way analysis of variance of experimental data was conducted through SPSS22.0 and the least significant range (LSD) method was used for multiple comparison, with P<0.05 indicating significance level.

Results

Effects of rice straw addition on the distribution of soil water-stable aggregates in different mineral types

The addition of rice straw in the three types of soil showed a significant effect on the dynamic changes of water-stable aggregates during the whole incubation period in the three different mineral soils (*Fig. 2*), the water-stable macroaggregates (>0.25 mm) significantly increased, and the water-stable microaggregates (<0.25 mm) significantly reduced (P<0.05). The three types of control soil mainly contained water-stable microaggregates in the incubation process, and the proportion of >2 mm aggregates was very little, which was significantly different (P<0.05) from the other three particle-size aggregates; from day 15 to day 60, the number of macroaggregates showed no significant difference of the control treatment, after 120 days of incubation, for the

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control soil, the proportion of water-stable macroaggregates increased slightly, but the water-stable microaggregates were still dominant.

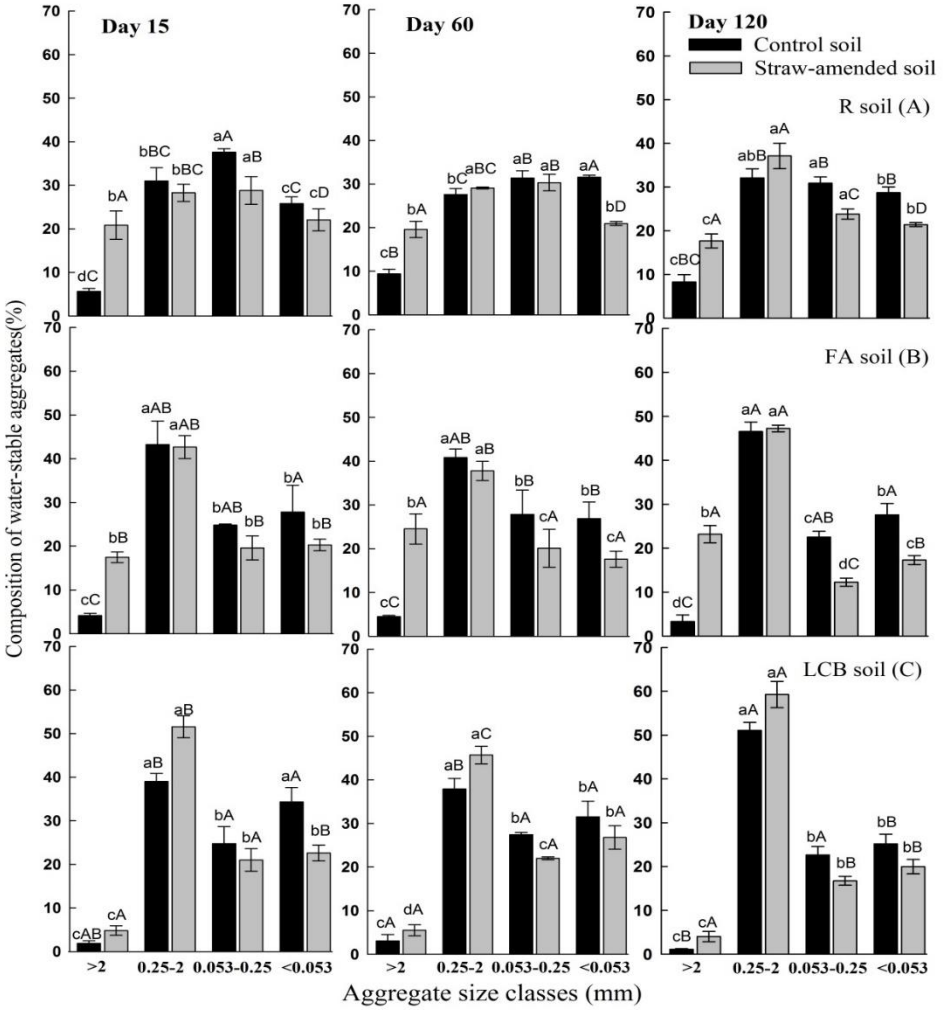

Figure 2. *Water-stable aggregate size distribution under different incubation periods (%). Different lowercase letters indicate significant differences among different particle-size aggregates under same treatments for the same incubation period (P<0.05). Different uppercase letters indicate significant differences among the same particle-size aggregates in the same soil type under different treatments for different incubation periods (P<0.05). $R_{0.25}$, >0.25 mm aggregates (water-stable macroaggregates). R Soil, red soil; LCB Soil, lime concretion black soil; FA Soil, fluvo-aquic soil*

Compared with the control soil, the three types of straw-amended soil showed a significant increase in >2 mm water-stable macroaggregates and remarkable decrease in 0.053-0.25 mm and <0.053 mm water-stable microaggregates during the early 15 days of incubation (*Fig. 2*). The proportion of macroaggregates ($R_{0.25}$) was increased by 270.3, 108.3 and 113.5% for the R soil (*Fig. 2A*), 598.2, 449.6 and 321.3% for the FA soil (*Fig. 2B*), and 157.2, 79.5 and 265.5% for the LCB soil after 15, 60, and 120 days of incubation (*Fig. 2C*), respectively, from day 15 to day 120 the amounts of newly formed >2 mm macroaggregates were significantly larger in the FA soil than in the R soil and in the LCB soil. With the increase of incubation time, the water-stable

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macroaggregates ($R_{0.25}$) of R soil and LCB soil increased more rapidly during the early incubation stage (within 15 days) than during the later stage (from day 15 to day 60). Among them, the water-stable macroaggregates of FA soil showed a continuous increase, and that of R soil and LCB soil showed a trend of increase first, then a slight decrease and then an increase during the whole incubation period. After 120 days of incubation, >2 mm water-stable aggregates in straw-amended R soil, LCB soil and FA soil were 113.5%, 298.0% and 595.8% higher than those in the control soil with the largest increase in FA soil. The 0.25-2 mm water-stable aggregates in straw-amended R soil, LCB soil and FA soil were 15.6%, 16.0% and 1.5% higher than that in the control soil with the largest increase in LCB soil. After 120 days of incubation, the order of water-stable macroaggregate in three types of soil was: FA soil > LCB soil > R soil, with a proportion of 70.4%, 63.3% and 54.8%, respectively, and the water-stable macroaggregates accounted for the majority in all straw-amended soil. The water-stable macroaggregate in R soil, LCB soil and FA soil increased by 35.6%, 22.0% and 41.1%, respectively after 120 days of incubation compared with the control soil, with the most obvious increase proportion in the FA soil.

Effects of rice straw addition on MWD, GMD, D and E_{LT} of three different mineral soils

Water-stable aggregate stability is an important indicator of soil structure, the addition of rice straw had a different positive effect on the stability of three types of soil aggregates during the incubation (*Table 2*). During the 120-days incubation period, MWD and GMD values of three types of straw-amended soils were significantly higher than those of the control soil, and E_{LT} and D values were significantly lower than those of the control soil (P<0.05). However, from day 0 to day 120, the differences of MWD, GMD, E_{LT} and D values of all control soils in different incubation periods were not very obvious. With the extension of incubation period, the MWD, GMD, E_{LT} and D values of the straw-amended soil at day 120 showed better changing trend than at day 15 and day 60, and the soil structural stability enjoyed continuous improvement. After 120 days of incubation, compared with the corresponding figures in the control treatment, the straw-amended FA soil's MWD and GMD increased by 59.4% and 90.3%, respectively, and its D and E_{LT} decreased by 8.1% and 40.7% respectively; the straw-amended R soil's MWD and GMD increased by 40.0% and 53.8% respectively, and its D and E_{LT} decreased by 3.8% and 24.2%; and the straw-amended LCB soil's MWD and GMD increased by 21.9% and 36.4%, respectively, and its D and E_{LT} decreased by 1.7% and 23.2%. Apparently, the FA soil showed the most prominent change of aggregate stability compared with R soil and LCB soil.

Effect of straw addition on the concentration of soil aggregate-associated organic carbon in different mineral types

Compared with the control treatment, the OC concentration of different particle-size aggregates in different types of straw-amended soil increased significantly (P<0.05), and the distribution of OC in different particle-size aggregates was significantly different (*Fig. 3*). During the whole incubation period, the OC concentration of the straw-amended soil aggregate was the highest after 15 days of incubation. During the later incubation stage (from day 15 to day 120), the OC concentration of the straw-amended soil aggregates decreased gradually. In general, the increase of OC concentration in microaggregates of three types of straw-amended soil was larger than that in macroaggregates, and the concentration of OC in water-stable microaggregates

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was higher than that in macroaggregates. In addition, with the extension of incubation period, these were few changes in the aggregate-associated OC concentration of the control treatment. The analysis results from aggregate-associated OC indicated that straw application had main effect on the improvement of aggregate-associated OC in different mineral soils, while incubation time and soil type had little effect on aggregate-associated OC in general (*Fig. 3*).

Table 2. *Effects of rice straw on aggregate stability index*

Soil type	Time (days)	Treatments	Wet sieving			
			MWD (mm)	GMD (mm)	*D*	E_{LT}/%
R Soil	15	control soil	0.53±0.02b	0.25±0.01c	2.94±0.01a	63.4±3.73a
		straw-amended soil	0.79±0.06a	0.36±0.04b	2.76±0.04b	50.9±5.23b
	60	control soil	0.56±0.02b	0.24±0.01c	2.90±0.01a	63.0±2.45a
		straw-amended soil	0.78±0.04a	0.36±0.02b	2.77±0.02b	51.3±2.06b
	120	control soil	0.59±0.01b	0.26±0.01c	2.91±0.02a	59.6±3.72a
		straw-amended soil	0.82±0.01a	0.40±0.01a	2.80±0.02b	45.2±4.49b
LCB Soil	15	control soil	0.53±0.01d	0.24±0.01d	2.97±0.01a	59.1±0.61a
		straw-amended soil	0.72±0.01b	0.38±0.01b	2.91±0.02bc	43.6±1.77bc
	60	control soil	0.55±0.05d	0.25±0.03d	2.95±0.02ab	59.0±3.24a
		straw-amended soil	0.67±0.04bc	0.33±0.03c	2.90±0.02c	48.8±2.99b
	120	control soil	0.64±0.02c	0.33±0.02c	2.98±0.01a	47.8±1.84b
		straw-amended soil	0.78±0.03a	0.45±0.03a	2.93±0.02bc	36.7±2.57c
FA Soil	15	control soil	0.62±0.06c	0.30±0.05c	2.93±0.01a	52.6±5.91a
		straw-amended soil	0.87±0.05b	0.45±0.04b	2.67±0.02b	39.9±3.70b
	60	control soil	0.60±0.02c	0.29±0.01c	2.92±0.01a	54.7±2.26a
		straw-amended soil	0.96±0.07ab	0.51±0.06b	2.51±0.01c	37.7±4.86b
	120	control soil	0.63±0.04c	0.32±0.03c	2.95±0.03a	50.2±2.07a
		straw-amended soil	1.03±0.03a	0.59±0.02a	2.55±0.04c	29.7±1.87c

Different lowercase letters indicate significant differences among the same soil type under different treatments for different incubation periods (P<0.05). R Soil, red soil; LCB Soil, lime concretion black soil; FA Soil, fluvo-aquic soil

For the transport and distribution of OC in aggregates, compared with the control soil, when the straw-amended soil was incubated for 15 days, the concentration of OC in the >2, 0.25-2, 0.053-0.25 and <0.053 mm aggregates of FA soil increased by 42.7%, 11.2%, 123.6% and 60.3%, respectively (*Fig. 3A*); the concentration of organic carbon in the >2, 0.25-2, 0.053-0.25 and <0.053 mm aggregates of R soil increased by 41.5%, 8.1%, 21.5%, and 11.5%, respectively (*Fig. 3B*); the concentration of OC in the >2, 0.25-2, 0.053-0.25 and <0.053 mm aggregates of LCB soil increased by 21.4%, 25.4%, 34.7% and 50.0%, respectively, the aggregate-associated OC in FA soil increased the most (*Fig. 3C*). From day 15 to day 120, the aggregate-associated OC of all types of straw-amended soil decreased gradually, the OC decomposition rate in macroaggregates was faster than that of microaggregates. In the incubation period of day15 to day 120, it was found that for the three types of soil, the increase of OC in water-stable microaggregates was larger than that in macroaggregates, and the differences of OC concentration between macroaggregates and microaggregate showed a decreasing trend.

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After 120 days of the incubation, except the <0.053 mm aggregate in LCB soil, the OC of other aggregates fraction in three types of soil was still significantly (P<0.05) higher than that in the control soil. The results showed that rice straw addition could improve the OC concentration of all aggregates in the three types of soil, and the increase of OC in microaggregates was significantly greater than that in the macroaggregates, but the increase degree of OC in aggregates of different types of soil was different during the 120 days of incubation period.

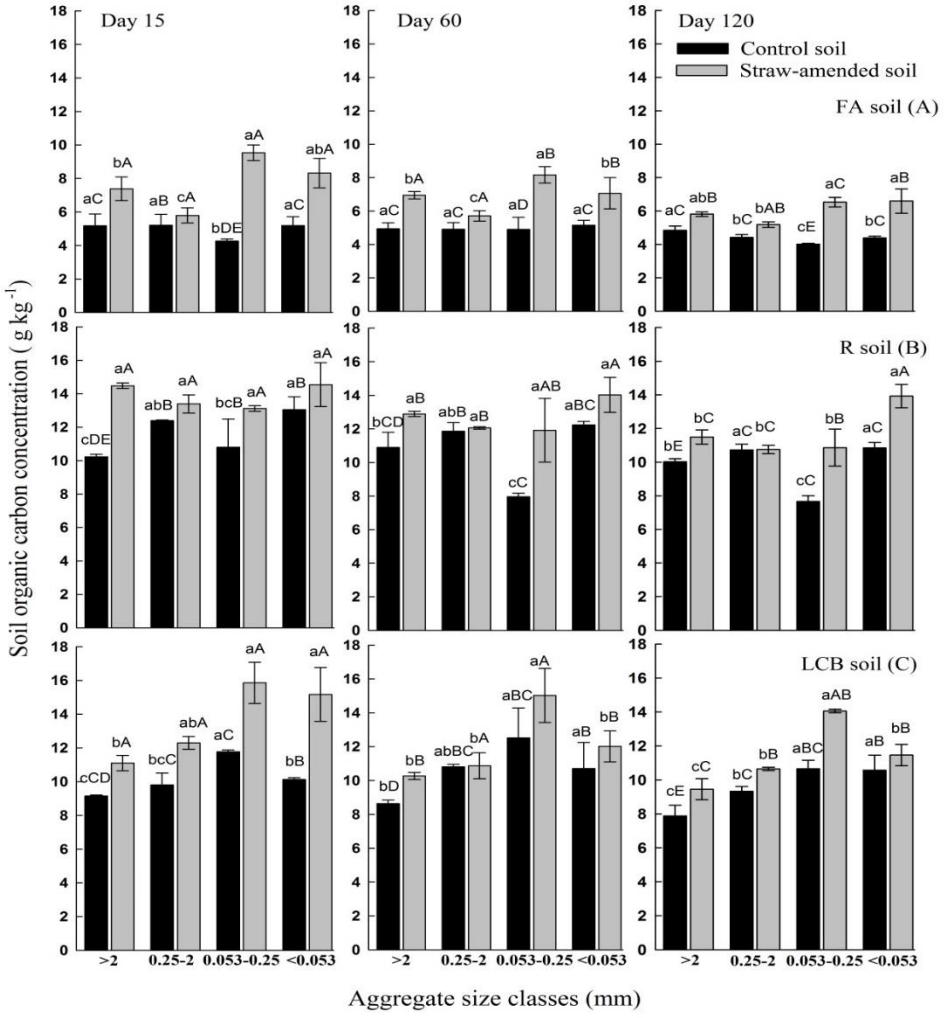

Figure 3. *Concentration of aggregate-associated organic carbon in different types of soil during different incubation periods. Different lowercase letters indicate significant differences among different particle-size aggregates under the same treatment for the same incubation period (P<0.05), and different uppercase letters indicate significant differences among the same particle-size aggregates in the same soil type under different treatments for different incubation period (P<0.05). R Soil, red soil; LCB Soil, lime concretion black soil; FA Soil, fluvo-aquic soil*

Contribution rate of organic carbon in different particle-size aggregates of three types of soil to the soil organic carbon

Straw addition also had a great impact on the contribution rate of OC in different particle-size aggregates to the SOC (*Table 3*). For the control soil, there was slight

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change in the contribution rate of OC in different particle-size aggregates to the SOC, which was mainly reflected in the contribution rate of water-stable microaggregate OC to SOC. Compared with the control soil, after 15 days of incubation, the three straw-amended soil types showed a significant increase in the contribution rate of >2 mm water-stable macroaggregates and significant decrease (P<0.05) in the contribution rate of 0.053-0.25 mm and <0.053 mm water-stable microaggregates. After 120 days of incubation, the contribution rate of OC in >2 mm aggregates of R soil, LCB soil and FA soil increased by 105.6%, 290.8% and 541.0%, respectively; the contribution rate of OC in >0.25 mm aggregates of R soil, LCB soil and FA soil increased by 18.6%, 20.7%, and 29.4%, respectively; and the contribution rate of water-stable macroaggregates in the three types of soil reached 51.98%, 59.07%, 66.14%, respectively; the highest contribution rate of water-stable macroaggregates to the SOC was found in the FA soil and the lowest of that was found in the R soil.

Table 3. *Contributing rates of water-stable aggregates carbon of different particle-size to soil organic carbon (%)*

Soil type	Time (days)	Treatments	Aggregate size			
			>2 mm	0.25-2 mm	0.053-0.25 mm	<0.053 mm
R Soil	15	control soil	4.85±0.35bD	32.54±4.35aAB	34.23±4.83aA	28.38±0.88aC
		straw-amended soil	21.84±2.48bA	27.47±1.65aC	27.48±3.48aBC	23.2±2.36abD
	60	control soil	9.61±1.31dC	30.71±1.97bAB	23.45±1.94cAB	36.23±0.53aA
		straw-amended soil	20.07±1.02cAB	27.94±0.76aBC	28.56±2.01aB	23.43±2.53bD
	120	control soil	8.52±1.77cC	35.32±3.17aA	24.28±1.96bBC	31.89±0.61aB
		straw-amended soil	17.52±0.89dB	34.46±2.16aA	22.27±0.84cC	25.75±0.95bCD
LCB Soil	15	control soil	1.63±0.21cAB	36.82±2.63aB	28.07±2.63bAB	33.46±2.93abA
		straw-amended soil	3.96±0.53cA	47.34±4.78aA	24.96±3.84bA	23.74±1.68bB
	60	control soil	2.38±0.24cA	36.76±2.69aB	30.79±2.59bA	30.06±3.27bA
		straw-amended soil	4.68±0.49cA	41.27±3.05aB	27.47±3.75bA	26.58±2.27bA
	120	control soil	0.87±0.06cB	48.05±2.56aA	24.27±1.47bB	26.82±1.94bB
		straw-amended soil	3.40±0.30cA	55.67±3.36aA	20.77±1.54bA	20.16±1.66bB
FA Soil	15	control soil	4.27±0.04cB	45.37±1.53aA	21.40±1.18bB	28.96±2.14bA
		straw-amended soil	17.70±2.46bA	33.80±2.73aB	25.49±2.90abA	23.02±1.39abB
	60	control soil	4.40±0.26cB	40.19±4.93aA	27.74±3.90bA	27.68±2.51bA
		straw-amended soil	25.42±4.96bA	32.05±2.26aB	24.11±3.18bA	18.42±4.61bB
	120	control soil	3.66±0.42dB	47.47±2.41aA	20.90±1.22cA	27.97±2.89bA
		straw-amended soil	23.46±1.98bA	42.68±1.91aB	13.92±0.80cB	19.94±3.03bB

Different lowercase letters indicate significant differences among different particle-size aggregates under the same treatments for the same incubation period (P<0.05), and different uppercase letters indicate significant differences among the same particle-size aggregates in the same soil type under different treatments for different incubation periods (P<0.05)

Correlation between water-stable macroaggregates($R_{0.25}$) and mean weight diameter (MWD) in different mineral soils

There was a significant positive correlation between the >0.25 mm water-stable macroaggregates ($R_{0.25}$) of the three types of soil and MWD (*Fig. 4*; R Soil, R^2=0.9446, P<0.0001; FA Soil, R^2=0.9610, P<0.0001; LCB Soil, R^2=0.9758, P<0.0001), indicating

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that the more the $R_{0.25}$, the larger the MWD value of soil aggregates, and the greater water stability of soil aggregates and the more stable of the soil structure. In general, as the addition of rice straw to three different types of soil could increase the proportion of macroaggregate and its water stability, it was an effective way to improve the physical and chemical properties of soil and enhanced the erosion resistance, but the improvement degree of aggregate stability of different mineral soils was different.

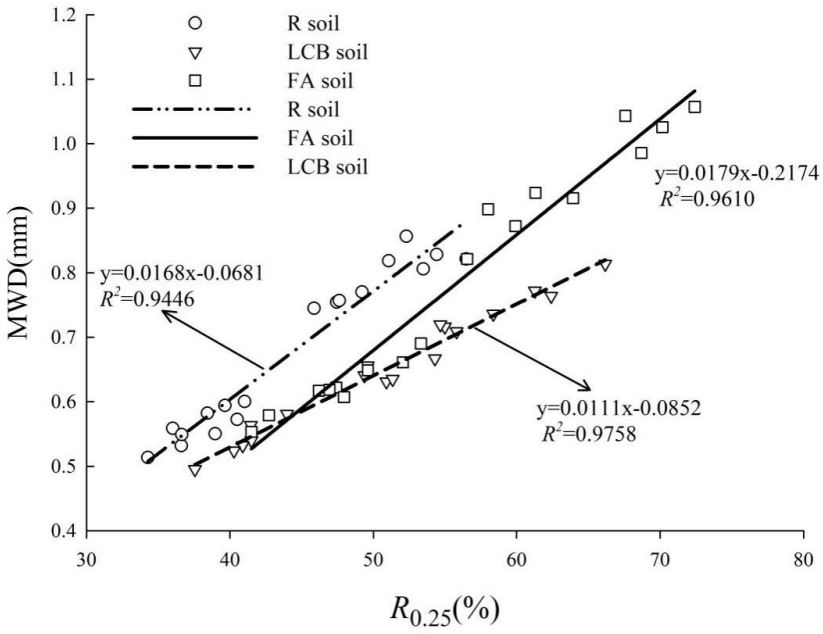

Figure 4. *Correlation between the proportion of >0.25 mm water-stable aggregates ($R_{0.25}$) and mean weight diameter (MWD). R Soil, red soil; LCB Soil, lime concretion black soil; FA Soil, fluvo-aquic soil*

Discussions

Effect of rice straw addition on distribution and stability of water-stable aggregates in different types of soil

The results of this study showed that rice straw addition had a significant impact on the distribution and stability of three different types of soil aggregates. For the three types of straw-amended soil, the proportion of >2 mm and 0.25-2 mm water-stable macroaggregates increased significantly, and the proportion of 0.053-0.25 mm and <0.053 mm water-stable microaggregates decreased significantly during the incubation period of day 15 day 60 and day 120 compared to the control treatment (*Fig. 2*) (P<0.05). As a new exogenous carbon source, rice straw could increase the input of soil organic materials. The research showed that the increase of exogenous organic materials improved the activity of microorganisms and enzymes, and promoted the growth of microbial hyphae. With the growth of fungal mycelium and the decomposition of other microorganisms, the organic materials would be transformed to humus which was an important cement for the formation of aggregates. The humus can promote the combination of soil particles and minerals, with a stimulating effect on the formation of aggregates to promote microaggregates to be formed into macroaggregates (Verchot et al., 2011; Cui et al., 2011). The results of the experiment were similar to those of Sodhi

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et al. (2009), who found that rice straw compost could significantly increase the proportion of >0.25 mm water- stability macroaggregates in sandy loam. However, straw addition had different distribution and migration of three types of soil aggregates at different particle sizes, the amounts of newly formed >2 mm macroaggregates were significantly larger at the early incubation stage (within 15 days) than later incubation stage (from day 60 to day 120). After 120 days of incubation, the >2 mm water-stable aggregates in straw-amended R soil, LCB soil and FA soil were 113.5%, 298.0% and 595.8% higher than those in the control soil with the largest increase in FA soil and their concentrations were 17.68%, 4.02% and 23.18%, respectively. The order of the proportion of water-stable macroaggregate in the three types of soil was FA soil > LCB soil > R soil, and the increasing proportion of water-stable macroaggregate in FA soil was the most obvious from day 15 to day 120 (*Fig. 2*). On the one hand, the reason may be that the added rice straw was more finely crushed, and the finer the straw was, the easier it was to be used by microorganisms, and the closer it would be combined with the soil. After the straw was decomposed, it could provide rich carbon source for the microorganisms in the soil, stimulate the microbial activity, and lead to the increase of water-stable macroaggregates. On the other hand, the FA soil and LCB soil were 2:1-type clay minerals mainly composed of montmorillonite with high constant surface charge and large specific surface, but R soil was a highly weathered soil mainly composed of iron aluminum oxide and 1:1 clay mineral with large crystal particles and relatively small specific surface. ANOVA results of control soils from *Figure 2* showed that incubation time and soil types were not the major factors on the formation of aggregates, while according to ANOVA results of straw-amended soils, straw application was considered as the main effect on the formation of aggregates compared with control soils. According to the multilevel aggregate formation theory, 2:1 clay minerals (FA soil and LCB soil) had stronger adsorption capacity for organic matter than 1:1 clay minerals (R soil), and the organic binding agents play dominant roles in the formation and stabilization of soil aggregates with 2:1 clay minerals. Compared with R soil, FA soil and LCB soil would be more conducive to the formation of water-stable macroaggregates (Tisdall and Oades, 1982; Lugato et al., 2010).

MWD, GMD, D and E_{LT} of soil aggregates can better reflect the size distribution transformation, aggregation and erosion resistance of soil aggregates. The larger MWD and GMD values as well as the smaller D and E_{LT} values that indicate the higher average aggregation, the better structure and stability of the soil, and the stronger erosion resistance (Stoops, 2003; Wang et al., 2013; Tang et al., 2016). Compared with the control soil, the MWD and GMD values of water-stable aggregates in three types of straw-amended soil increased significantly and the D and E_{LT} values in them decreased significantly. The differences of MWD, GMD, E_{LT} and D values of three types of soil in different incubation periods (from day 0 to day 120) were not always significantly in the control soil, but the MWD, GMD, E_{LT} and D values of straw-amended soil showed continuous improvement during the 120 days of incubation period, and the change of aggregate stability of FA soil was the most obvious at 120-day compared with LCB soil and R soil (*Table 2*). There was a significant positive correlation between $R_{0.25}$ and MWD in three mineral soils, which indicated that as $R_{0.25}$ increased due to straw application, the MWD values also increased. Straw is an important resource of SOC, which promotes the aggregation and water stability of aggregates. The difference of determination coefficient in correlation analysis showed that the improvement effect on LCB soil and FA soil, which were mainly composed of 2:1 clay mineral, was better (*Fig.*

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4). These results showed that with the addition of rice straw and the extension of incubation time, which improved the activity of microorganisms and enzymes, soil microorganisms can transform plant residues into organic binding agents, promoted the agglomeration of soil aggregates, and the degree of aggregation and stability of the three types of soil were significantly enhanced, and the soil structure was improved. Among them, the change of the stability of the FA soil was significant, which was consistent with the change of the macroaggregates, and the FA soil also enjoyed the best improvement of aggregate structural stability because FA soil was dominated by 2:1 clay mineral with high calcium carbonate contents, the content of silt and clay particles was greater than 77%, and the organic binding agents play dominant roles in the stabilization of soil aggregates. This was similar to the research results of Lu et al. (2014) and Sodhi et al. (2009). Sodhi et al. (2009), which concluded that straw composting treatment could significantly improve MWD and soil structural stability of sandy loam; Lu et al. (2014) found that rice husk biochar treatment could not only significantly improve the proportion of 2-5 mm and 0.25-0.5 mm macroaggregates of modified clay, but also reduce the proportion of <0.25 mm microaggregates, significantly improving MWD and GMD.

Effect of rice straw addition on distribution and contribution rate of organic carbon in different particle-size aggregates

The content of water-stable aggregates is an important indication to evaluate the stability of soil structure and erosion resistance, and relevant experimental studies had also revealed that the distribution of OC in different particle-size aggregates was different and the stability of OC in different particle-size aggregates was also different. The possible reason lies in that the distribution of exogenous new carbon in aggregates is different due to factors such as incubation conditions, soil types and types of exogenous materials (Tripathi et al., 2014; Zhang et al., 2016). The results of this study showed that the OC concentration of three types of straw-amended soil in four different particle-size aggregates was significantly (P<0.05) higher than that of the control soil during the 120 days of incubation periods, and there was a certain difference in the OC concentration of different particle-size aggregates (*Fig. 3*). For the transport and distribution of aggregate-associated OC, compared with the control soil, when the straw-amended soil was incubated for 120 days, the concentration of OC in the >2, 0.25-2, 0.053-0.25 and <0.053 mm aggregates of FA soil increased by 20.0%, 17.4%, 62.6% and 50.4%, respectively; the concentration of OC in the >2, 0.25-2, 0.053-0.25 and <0.053 mm aggregates of R soil increased by 14.7%, 0.2%, 41.9%, and 28.4%, respectively; the concentration of OC in the >2, 0.25-2, 0.053-0.25 and <0.053 mm aggregates of LCB soil increased by 19.9%, 14.1%, 32.0% and 8.4%, respectively (*Fig. 3*). The results showed that the lower the initial organic carbon concentration of soil aggregates was, the larger the increase range of the aggregate-associated OC was, the OC concentration increased rapidly during the early incubation stage (within 15 days) in all three amended soils, especially, the FA soil increased the most, and the OC concentration of the straw-amended soil aggregates decreased gradually from day 15 to day 120.

The increase range of the OC concentration of water-stable microaggregate was significantly larger than that of the macroaggregate, and the increase range of aggregate-associated OC in FA soil was the largest from day 15 to day 120, and the OC concentration in the water-stable microaggregates of the three types of soil was

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generally higher than that of in the macroaggregates. This was similar to the results of Lee's study. Lee et al. (2009) studied the effect of different fertilization treatments on SOC and found that straw composting significantly increased the OC concentration of aggregates, and the OC concentration of water-stable microaggregates was higher than that of water-stable macroaggregates. The reason may be that the mineral types and clay content of the three types of soil were different, 2:1 clay minerals had stronger adsorption capacity for organic matter than 1:1 clay minerals. Due to the influence of biological, chemical and environmental factors, with the extension of incubation period, the aggregate-associated OC was gradually decomposing, OC available for microorganisms will decrease, and the straw crushing was relatively fine, which would have different effects on the increase and sequestration of OC of different particle-size aggregates. Moreover, the carbon sequestration by the microaggregates was not easy to decompose as it was protected physically and had biochemical resistance. Therefore, the OC of the microaggregates was more durable and stable in the soil. The FA soil and LCB soil contained higher silt and clay particles content with high constant surface charge and large specific surface, which would contribute to the increase and sequestration of the OC in the water-stable microaggregate. Therefore, the straw addition was of great significance to improve the soil structure and stability, increase OC concentration of soil aggregates, and improve carbon sequestration capacity (Six et al., 2000; Dimoyiannis, 2012; Song et al., 2019).

Compared with the control soil, after 15 days of incubation, all straw-amended soil types showed a significant increase in the contribution rate of water-stable macroaggregates and remarkable decrease (P<0.05) in the contribution rate of water-stable microaggregates. After 120 days of incubation, the contribution rate of OC in water-stable macroaggregates of R soil, LCB soil and FA soil was 51.98%, 59.07%, 66.14%, respectively. The FA soil enjoyed the largest contribution rate of OC in water-stable macroaggregates to SOC (*Table 3*). The reason for the decrease of the contribution rate of OC in water-stable microaggregates may be that the addition of rice straw promoted the activity of microorganisms and enzymes, increased the organic cementing substance, and helped the water-stable microaggregates to continuously bond and aggregate into water-stable macroaggregates, and the distribution proportion of >2 mm and 0.25-2 mm water-stable macroaggregates increased significantly. The increase extent of water-stable macroaggregates was larger than that of OC concentration in water-stable microaggregates, and the distribution proportion of water-stable microaggregates in the whole aggregates was relatively low.

Conclusions

Rice straw application resulted in significant effect on water-stable aggregates and aggregate-associated OC of the three typical types of soil during the whole incubation stage, which promoted the aggregation of water-stable microaggregates to macroaggregates. After 120 days of incubation, the proportion of water-stable macroaggregates in R soil, LCB soil and FA soil reached 54.8%, 63.3% and 70.4%, respectively, becoming the dominant aggregates, significantly improving the MWD and GMD of soil water-stable aggregates, and reducing the D and E_{LT} values (P<0.05). Furthermore, MWD and $R_{0.25}$ showed a significant positive correlation (P<0.001). These results suggest that the addition of straw enhanced the degree of aggregation of water-stable aggregates and improved soil aggregate stability, the increase degree of

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aggregates content and stability was different, probably due to stronger adsorption capacity and high constant surface charge in 2:1 clay minerals than 1:1 clay minerals.

The straw addition also increased OC concentration in different particle-size aggregates of three types of amended soil, and the OC concentration increased rapidly during the early 15 days of incubation in the three amended soils, the contribution rate of organic carbon in >2 mm water-stable macroaggregates also significantly improved. Meanwhile, the increase of OC in water-stable microaggregates was larger than that in macroaggregates in all straw-amended soil. In conclusion, the addition of rice straw to three different types of soil was an effective way to increase the aggregates stability and aggregate-associated OC concentration, and the increase degree of aggregates stability and OC concentration of different types of soil was different, which could play a more important role in FA soil than in R soil and LCB soil.

This study analyzed the effects of straw addition on the distribution and stability of water-stable aggregates and aggregate associated OC in three different soil clay mineral types. Microbial processes related to decomposition of plant debris or root growth can greatly promote the formation of soil aggregates and nutrient decomposition. Therefore, the influence of straw addition on soil microbial biomass and microbial activity at different incubation stage should be further studied, and main factors affecting soil aggregates formation are also needed to be noticed. Additionally, the effect of straw addition on soil interparticle forces (electrostatic repulsive force, van der Waals attractive force and surface-hydration repulsive force) in different mineral types should be considered because they can reveal the relationship between the interparticle forces and soil structural stability. These further studies help to provide theoretical support for soil quality improvement in different mineral types.

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IN VITRO LEISHMANICIDAL, ANTIBACTERIAL, ANTIFUNGAL, ANTICANCER (MCF-7, 3T3 AND HELA CELL LINES) ACTIVITIES OF EXTRACT AND FRACTIONS OF *PEROTIS HORDEIFORMIS* AND GC-MS ANALYSIS OF *PEROTIS HORDEIFORMIS* WHOLE PLANT BUTANOL FRACTION (PHWBF)

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Abstract. In this research study, leishmanicidal, antibacterial, antifungal, anticancer (MCF-7, 3T3 and HeLa cell lines) activities and GC-MS studies of *Perotis hordeiformis* extract and fractions were examined. Leishmanicidal bioassay, 96 Well Plate Method, Agar tube dilution method and MTT assay were the methods used for leishmanicidal, antibacterial, antifungal and anticancer activities. *Perotis hordeiformis* whole plant butanol fraction (PHWBF) exhibited leishmanicidal activity with IC_{50} 53.31 ± 0.59. *Perotis hordeiformis* whole plant hexane fraction (PHWHF) showed activity against bacteria such as *Staphylococcus aureus* having an inhibition percentage of 58.5%. *Perotis hordeiformis* whole plant methanol extract (PHWME) showed activity against fungi such as *Microsporum canis and Fusarium lini* having an inhibition percentage of 55% and 50%, respectively. *Perotis hordeiformis* whole plant hexane fraction (PHWHF) showed activity against *Aspergillus niger* having an inhibition percentage of 40% while *Perotis hordeiformis* whole plant aqueous fraction (PHWAF) showed activity against *Microsporum canis and Fusarium lini* having an inhibition percentage of 100% and 40%, respectively. *Perotis hordeiformis* whole plant butanol fraction (PHWBF) showed activity against cancer cell lines such as HeLa cell line and MCF-7 cell line with an inhibition percentage of 55% and 48%, respectively. Other extract and fractions were less active against cancer cell lines. GC-MS analysis showed 8 compounds in *Perotis hordeiformis* whole plant butanol fraction (PHWBF) which exhibited leishmanicidal and anticancer activities.

Keywords: *leishmaniasis, bacterial strains, fungal strains, cell lines, PHWME, PHWHF, PHWAF, PHWBF*

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Introduction

Traditional plants are the main sources of phytochemicals, used for the preservation of human health and alleviating infectious diseases of mankind since prehistoric times. At present, the entire world has interest in green medicines and demands medicines originating from traditional plants rather than from a synthetic source. This is due to the fact that traditional drugs are safer than synthetic medicines which have toxicity and side effects. This stimulates the researchers to develop new medicines against microbes (Cordell et al., 2000; Nair et al., 2007). The drugs which are synthesized, are expensive, have side effects and the diseases are not properly treated. Hence, new antimicrobial agents are needed to be developed from traditional plant sources (Sieradzki et al., 1999; Dabur et al., 2007). According to the WHO, 80% of the population of the world use traditional medicines to cure infectious diseases (WHO, 1993). The compounds which are extracted from plant sources are more than 50% of current drugs (Baker et al., 1995). The medicinal plant, *Perotis hordeiformis* is a short lived perennial or annual and belongs to the Poaceae family. Sandy places are the main locations for the presence of this ethnomedicinal plant. This traditional plant is mainly distributed in Nepal, Thailand, Indonesia, India, Pakistan, Sri Lanka and Myanmer. This plant has significant antileishmanial, cytotoxic and antioxidant activities. *Perotis hordeiformis* has close resemblance with *Perotis indica* (Baloch et al., 2013). In this study, *Perotis hordeiformis* whole plant extract and fractions are used against leishmania major, six bacteria, five fungi and three cancer cell lines.

Materials and Methods

Plant material

Perotis hordeiformis whole plant was the plant material in this analysis.

Extraction

The medicinal plant *Perotis hordeiformis* was collected from Soorab, Balochistan, Pakistan and was authenticated by Prof. Dr. Rasool Bakhsh Tareen, Department of Botany, UoB, Quetta, Pakistan. *Perotis hordeiformis* was kept for one month under the shade and then powdered in a grinder. 1.5 kg powdered *Perotis hordeiformis* was macerated in 14 L of methanol for the period of seven days and then the mixture was filtered, and vaporized in a rotary evaporator. The crude extract of *Perotis hordeiformis* whole plant methanol extract (PHWME) was 24.52 g.

Fractionation of crude extract

The methanolic crude extract was fractionated with n-hexane and aqueous solvents in a separatory funnel and vaporized in a rotary evaporator to form *Perotis hordeiformis* whole plant hexane fraction (PHWHF) 8.1 g and *Perotis hordeiformis* whole plant aqueous fraction (PHWAF) 17.3 g. *Perotis hordeiformis* whole plant aqueous fraction (PHWAF) was fractionated with butanol to form *Perotis hordeiformis* whole plant butanol fraction (PHWBF) 4 g (Bakht et al., 2013; Achakzai et al., 2016, 2019).

Leishmanicidal bioassay

At 3000 rpm for 10 min, the leishmanial parasite such as promastigotes was sedimented. This parasite was counted by Neubaur chamber, diluted to a concentration

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of $1x10^6$ with fresh medium. In 96 well plate, 180 uL of parasite culture was added. The sample with the concentration of 20 uL was added and then serially diluted till final concentration of sample 1 ug/mL. $1x10^6$ cells/mL of parasite density was kept for negative control while for positive control, it varied. The 96 well plate including parasites, sample, positive and negative control was kept in an incubator between 21 to 22 ºC for the duration of 72 h. With the help of Neubaur chamber, parasites with IC_{50} values were counted (Atta-ur Rahman et al., 2001).

Antibacterial assay

96 well plate method

For the growth of bacterial strains, Muller Hinton medium was used. McFarland turbidity index with 0.5 was used for the adjustment of inoculums. In DMSO, extracts/fractions were added and from this stock solution were formed. In wells, media and samples were added while control wells were without extracts and fractions. Up to 200 uL, the wells were filled. $5x10^6$ cells were added in both control and test wells, which were then sealed with parafilm and kept in an incubator for 18-20 h. Alamar Blue Dye was added to all wells, which were then shaken for 2-3 h at 80 RPM. Blue to pink color change of dye indicated the growth of bacteria. Absorbance was recorded at 570 nm with ELISA reader (Pettit et al., 2005).

Antifungal assay

Agar tube dilution method

In this assay, in 1 mL of DMSO, 24 mg of sample was dissolved. SDA with the concentration of 32.5 g was dissolved in 0.5 L of Distilled water. With the help of steam, this growth medium was completely dissolved. This medium with 4 mL was poured in tubes with screw cap, autoclaved for 15 min at 121 ºC, cooled till 50 ºC. 66.6 uL of sample was loaded into non-solidified SDA. At room temperature, in a slanting position, tubes were solidified. Fungus was inoculated with 4 mm diameter into tubes. In other media, reference antifungal drug and DMSO were used as positive and negative control. Tubes were kept in an incubator for one week at 27-29 ºC, and examined twice in a week (Choudhary et al., 1995).

Calculating Inhibition % of fungal growth

$$Inhibition\% = 100 - \frac{linear\ growth\ in\ test\ (mm)}{linear\ growth\ in\ control\ (mm)} \times 100 \qquad \text{(Eq.1)}$$

MTT assay

In this study, cancer cell lines were cultured in Dulbecco's Eagle modified medium with 10% FBS, 2% antibiotics were used and then kept in 5% CO_2 in incubator at 37 °C. After the development of confluency, cell lines were harvested. In a 96 well flat, 5×10^4 cells/well were added and then after one day, sample with the concentration of 50 ug/mL was added, and kept for 48 hours in an incubator. The sample was removed after incubation. MTT with the concentration of 0.5 mg/mL was added, kept at 37 °C for hours in an incubator. Formazan crystals were formed when MTT was reduced. With the help of 100 ul DMSO, Formazan crystals were dissolved. Micro-plate reader was used for recording absorbance at 570 nm (Spectra Max plus, Molecular Devices,

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CA, USA). In this assay, doxorubicin was used as a standard drug. The decrease in viable cells or percent inhibition was calculated with the help of the following formula:

$$\%\ Inhibition = 100 - \frac{mean\ of\ O.D.\ of\ test\ compound - mean\ of\ O.D.\ of\ negative\ control}{mean\ of\ O.D.\ of\ positive\ control - mean\ of\ O.D.\ of\ negative\ control} \times 100 \quad \text{(Eq.2)}$$

For the calculation of IC_{50} 20 mM stock solution of extracts/fractions were diluted into working solution with 50 uM and then in order to get less than 50 percent inhibition, working solution was further diluted in serial dilutions. With the help of EZ-fit5 software, IC50 was calculated (Scudiere et al., 1988).

Gas chromatography mass spectrometry (GC-MS) analysis triple quadrupole acquisition method MS parameters

For identification and quantification of *Perotis hordeiformis* compounds: 2 ul of *Perotis hordeiformis* extract or fraction was directly injected into the gas chromatograph mod.6890N Network GC System (Agilent Technologies Palo Alto, CA) together in the presence of mass spectrometer mod. 5973 Network Mass Selective Detector (Agilent Technologies Palo Alto, CA) and furnished in the presence of a column HP-5MS (30 m length, 0.25 mm interior diameter, 0.25 um film width Agilent Technologies, Palo Alto, CA). Helium gas was off. Injection was made into a split-splitless injector (split ratio 30:1) at 250 ºC. The oven program was the following: 70 ºC for 3 min then 6 ºC /min to 180 for 5 min, then 6 ºC /min to 280 ºC for 10 min, then 8 ºC /min to 290 ºC for 20 min. The MSD transfer line was set at a temperature of 250 ºC; MSD temperature quadrupole was of 150 ºC and ionization temperature was 230 ºC, Mass spectra were seventy electrovolts and scan achievement was accomplished in the series between thirty-five and 300 m/z. The identification of the components of the *Perotis hordeiformis* extract or fraction was assigned by matching their mass spectra with those available in the libraries NIST 02 and WILEY (El-Wakil et al., 2015).

Results and Discussion

Perotis hordeiformis whole plant butanol fraction (PHWBF) exhibited leishmanicidal activity with IC_{50} 53.31 ± 0.59. None of other *Perotis hordeiformis* extract and fractions exhibited leishmanicidal activity. Leishmanicidal activities of extract and fractions of *Perotis hordeiformis* are shown in *Table 1*.

Table 1. *Leishmanicidal analysis of extract/fractions of whole plant of Perotis hordeiformis*

Extract/Fractions	**IC_{50} (ug/mL) ± S.D.**
PHWME	>100
PHWHF	>100
PHWAF	>100
PHWBF	53.31 ± 0.59 moderate activity
Standard (Pentamidine)	4.08 ± 0.8
Standard (Amphotericin B)	0.30 ± 0.4

Perotis hordeiformis whole plant hexane fraction (PHWHF) showed activity against bacteria such as *Staphylococcus aureus* having an inhibition percentage of 58.5%.

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Perotis hordeiformis whole plant methanol extract (PHWME), *Perotis hordeiformis* whole plant aqueous fraction (PHWAF) and *Perotis hordeiformis* whole plant butanol fraction (PHWBF) showed no antibacterial activity. The antibacterial activities of *Perotis hordeiformis* whole plant extract and fractions are shown in *Table 2*.

Table 2. *Antibacterial activities of Perotis hordeiformis whole plant extract and fractions*

	Escherichia coli (%) **Inhibition**	Bacillus subtilis (%) **Inhibition**	Shigella flexenari (%) **Inhibition**	Staphylococcus aureus (%) **Inhibition**	Pseudomonas aeruginosa (%) **Inhibition**	Salmonella typhi (%) **Inhibition**
PHWME	-	19%	-	7.2%	-	-
PHWHF	-	11.5%	-	58.5%	-	-
PHWAF	-	-	-	-	-	-
PHWBF	-	-	-	-	-	-
Standard (ofloxacin)	87.6%	95.6%	-	93.7	95.5%	96.2%

Perotis hordeiformis whole plant methanol extract (PHWME) showed activity against fungi such as *Microsporum canis and Fusarium lini* having an inhibition percentage of 55% and 50%. *Perotis hordeiformis* whole plant hexane fraction (PHWHF) showed activity against *Aspergillus niger* having an inhibition percentage of 40% while *Perotis hordeiformis* whole plant aqueous fraction (PHWAF) showed activity against *Microsporum canis and Fusarium lini* having an inhibition percentage of 100% and 40%. *Perotis hordeiformis* whole plant butanol fraction (PHWBF) showed no antifungal activity. The antifungal activities of *Perotis hordeiformis* whole plant extract and fractions are shown in *Table 3*.

Table 3. *Antifungal activities of Perotis hordeiformis whole plant extract and fractions*

	Candida albicans (%) **Inhibition/MIC**	Trichphyton rubrum (%) **Inhibition/MIC**	Aspergillus niger (%) **Inhibition/MIC**	Microsporum canis (%) **Inhibition/MIC**	Fusarium lini (%) **Inhibition/MIC**
PHWME	0%	0%	0%	55%	50%
PHWHF	0%	0%	40%	0%	20%
PHWAF	12.5%	0%	30%	100%	40%
PHWBF	0%	0%	0%	0%	0%
Standard (Miconazole) Mic (ug/mol)	113.5	97.8	20.70	98.1	73.50

Perotis hordeiformis whole plant butanol fraction (PHWBF) showed anticancer activity against HeLa cell line and MCF-7 cell line with percent inhibition 55% and 48%. Other extract and fractions are less active against cancer cell lines. The anticancer activities of extract and fractions of whole plant of *Perotis hordeiformis* are shown in *Table 4*.

Molecular formula, molecular mass, structure, m/z and RT of compounds 1-8 of *Perotis hordeiformis* whole plant butanol fraction (PHWBF) are shown in *Tables 5 and 6* while mass spectra interpretation of compounds 1-8 of *Perotis hordeiformis* whole plant butanol fraction (PHWBF) are shown in *Tables 7 and 8*.

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Table 4. *Anticancer activities of extract and fractions of whole plant of Perotis hordeiformis*

	MCF-7 **(%) Inhibition**	3T3 **(%) Inhibition**	HeLa **(%) Inhibition**
PHWME	12%	25%	36%
PHWHF	8%	32%	32%
PHWAF	16%	25%	6%
PHWBF	48%	25%	55%
Standard Doxorubicin	87.6%	71%	70%

Table 5. *Molecular formula, molecular mass, structure, m/z and RT of compounds 1-4 of Perotis hordeiformis whole plant butanol fraction (PHWBF)*

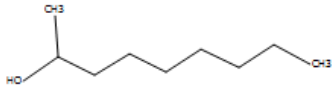
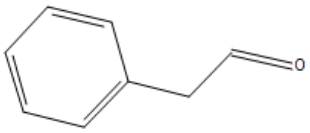
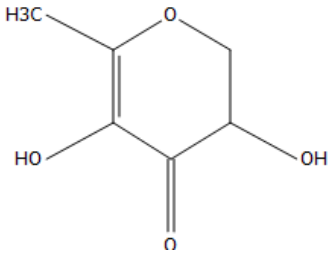
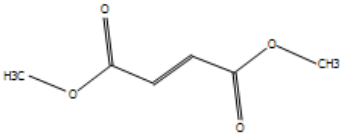
compd	**Molecular Formula**	**Molecular Mass**	**Structure**	**m/z**	**RT**
1	C9H20O	144		45.1	6.681
2	C8H8O	120		91	7.503
3	C6H8O4	144		43.1	9.983
4	C6H8O4	144		53.1	12.39

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Table 6. *Molecular formula, molecular mass, structure, m/z and RT of compounds 5-8 of Perotis hordeiformis whole plant butanol fraction (PHWBF)*

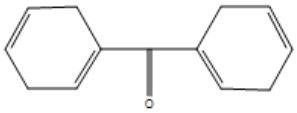
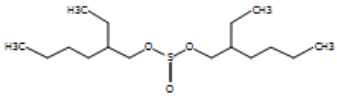
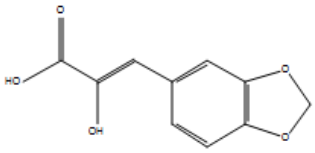
compd	Molecular Formula	Molecular Mass	Structure	m/z	RT
5	C11H24O	172		43.1	17.44
6	C13H10O	182		104.9	20.61
7	C16H34O3S	306		57.1	35.25
8	C10H8O5	208		135	68.13

Table 7. *Mass spectra of compounds 1-4 of Perotis hordeiformis whole plant butanol fraction (PHWBF)*

compd	m/z (% Relative abundance)
1	144(M^+), 126(2365.9), 69(2401), 57.1(3606.8), 56.1(2876.3), 55.1(1849.7), 54(2775.9), 53.1(3732.9), 45.1(8743.3), 44.1(4950.7), 41.1(2064.4)
2	119.9(M^+],13185), 91.9(21295), 91(83166), 88.9(4417.4), 64.9(18968), 62.9(7995.1), 62(3067.6), 51.1(6416.9), 50.1(3636.7), 39.1(2357.5)
3	144(M^+],58661.2), 101(56580), 73(39808.6), 72(47429.7), 58(7389.8), 55.1(43751.8), 45.1(39179.1), 44.1(108117.2), 43(145948), 42.1(7752.4)
4	144(M^+), 113.9(2272.7), 112.9(3354), 98(2209.6), 85(2140.8), 71(2930), 68(1637.4), 56.1(1855.7), 53.1(4503.1), 52(1566.3), 51.1(3247.2)

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Table 8. *Mass spectra of compounds 5-8 of Perotis hordeiformis whole plant butanol fraction (PHWBF)*

compd	m/z (% Relative abundance
5	172(M^+), 97(1101.3), 84(1288.9), 83(2228.8), 70(2031.5), 69(4248.8), 57.1(4541.2), 56.1(5445.9), 55.1(5725.6), 43.1(8861.9), 41.1(2510.4)
6	181.9(M^+],4016.4), 105.9(1127.1), 104.9(11055), 91(1654.5), 87(6040.8), 76.9(7954.4), 75.9(1546), 53.1(1837), 51.1(4023.6), 50.1(1050.9)
7	306(M^+), 135(6436.7), 112.9(13869), 111.9(6419.7), 88.9(16427), 71(27183), 70(11703), 57.1(33305), 55.1(9046.4), 43.1(13743), 41.1(6340.2)
8	208.9(M+1],672.6), 207.9(M^+],771.6), 196.9(816.7), 149(546.4), 135(1655.4), 104.9(543.5), 95.9(770.1), 76.9(541.6), 75(774.9), 44.1(829.8)

Conclusion

In this research study, *Perotis hordeiformis* whole plant butanol fraction (PHWBF) exhibited leishmanicidal activity with IC_{50} 53.31 ± 0.59. None of other extract and fractions of *Perotis hordeiformis* exhibited leishmanicidal activity. *Perotis hordeiformis* whole plant hexane fraction (PHWHF) showed activity against bacteria such as *Staphylococcus aureus* having an inhibition percentage of 58.5%. *Perotis hordeiformis* whole plant methanol extract (PHWME), *Perotis hordeiformis* whole plant aqueous fraction (PHWAF) and *Perotis hordeiformis* whole plant butanol fraction (PHWBF) showed no antibacterial activity. *Perotis hordeiformis* whole plant methanol extract (PHWME) showed activity against fungi such as *Microsporum canis and Fusarium lini* having an inhibition percentage of 55% and 50%, respectively. *Perotis hordeiformis* whole plant hexane fraction (PHWHF) showed antifungal activity against *Aspergillus niger* having an inhibition percentage of 40% while *Perotis hordeiformis* whole plant aqueous fraction (PHWAF) showed antifungal activity against *Microsporum canis and Fusarium lini* having an inhibition percentage of 100% and 40%, respectively. *Perotis hordeiformis* whole plant butanol fraction (PHWBF) exhibited no antifungal activity. *Perotis hordeiformis* whole plant butanol fraction (PHWBF) showed anticancer activity against HeLa cell line and MCF-7 cell line with an inhibition percentage 55% and 48%, respectively. Other extract and fractions are less active against cancer cell lines. GC-MS analysis showed 8 compounds in *Perotis hordeiformis* whole plant butanol fraction (PHWBF) which exhibited leishmanicidal and anticancer activities. In the near future, in the Institute of Biochemistry, University of Balochistan, Quetta, Pakistan, the compounds present in the *Perotis hordeiformis* whole plant butanol fraction (PHWBF) will be isolated and tested against cancer cell lines and leishmaniasis and will lead to drug development with least toxicity and side effects.

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SNOW COVER DISTRIBUTION'S CORRELATION WITH CLIMATIC FACTORS IN NORTHEAST CHINA'S MOLLISOL REGION

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Abstract. Snow cover greatly influences climate in Northeast China, and it is one of the most relevant parameters to analyze climate change. The Mollisol area of Northeast China is one of the three seasonal snow areas in the country. This study investigates the spatial distribution characteristics and variation trends of monthly snow depth over a 30-year period. Linear regression analysis was applied for each factor. The results indicated a non-significant decreasing trend of snow depth in the mountainous area. Snow depth in the plains increased by 0–0.4 cm/year. The spatial distribution of first, last, and cover days of snow, and the maximum depth of snow cover were consistent in their correlation with snow cover depth. The snow cover gradually decreased with increasing temperature, latitude, and change in topography (altitude) from northeast to southwest. In terms of snow cover days, the highest correlation was obtained for temperature during the snow accumulation and melting periods. In contrast, there was no correlation with precipitation. The volume of snowmelt water had a significant change every 10 years.
Keywords: *snow depth, snow cover days, climate change, snowmelt water volume, spatiotemporal distribution*

Introduction

Accumulated snow is an important source of freshwater in the Mollisol area of northeast China, where its average depth is 2–20 cm. Snowmelt provides water for industrial, agricultural, and domestic supplies in spring. Owing to approximately 100 years of intensive and uncontrolled development, utilization of water resources and soil erosion in this area are intensive. The combined effects of snowmelt runoff, freezing–thawing cycles, and incomplete thawed layer cause slope erosion and rill formation. With higher number of confluences, rills develop into ephemeral and young gullies (Xu et al., 2019). Therefore, it is important to analyze the spatiotemporal variation of snow depth in the Mollisol area of northeast China.

In recent decades, remote sensing has been effectively utilized to monitor snow dynamics at regional and global scales (Li et al., 2018). In April 1960, the Television Infrared Observation Satellite TIROS-1 was used to monitor snow cover in Canada for the first time. The optical sensor was mainly used for monitoring snow areas and albedos, but that was restricted by weather conditions. Quantitative remote sensing was then applied to solve the issue of cloud cover and achieve better accuracy of remotely sensed data (Basang et al., 2017; Chang et al., 2017). In recent years, passive microwave remote sensing with features of all-weather, multi-polarization, and limited penetration of the underlying surface of the earth has been widely used for snow monitoring. When compared with optical sensor satellites, microwave remote sensing satellites are more accurate and timelier in the acquisition of snow depth, snow–water

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equivalent, and snow cover. A common passive microwave sensor satellite includes a Scanning Multichannel Microwave Radiometer (SMMR), the Defense Meteorological Satellite Program (DMSP), and a series of special microwave imaging detectors such as the Special Sensor Microwave Imager (SSM/I) and Advanced Microwave Scanning Radiometer-EOS (AMSR-E) (Josberger et al., 2017; Tang et al., 2019).

Snow covers present high albedo and thermal insulation, and they are important for the global climate system and hydrological cycles (Brown et al., 2007; Li et al., 2019; Zhang, 2005). Snow affects the relationship between ground systems and climate, thereby influencing climate change (Scipión et al., 2013; Fassnacht et al., 2018; Tan et al., 2019). The accumulation of snow in arid and semiarid areas provides water for the ecosystem and human society. Snow depth is an easy parameter to measure and an important characteristic of snow cover (Liu et al., 2018), and it can be used to derive snow–water equivalent. In this calculation, the spatial variation of snow density is less significant than snow depth (Fassnacht et al., 2013; López-Moreno et al., 2013). Area and duration of snow cover and snow–water equivalent have decreased because of global warming (Kunkel et al., 2016). Foster et al. (1997) observed that the snow area in South America has decreased cyclically in the past 25 years using remote sensing technology. The period of snowmelt greatly influences the amount of water resources, climate change in surroundings, and downstream areas. Snow accumulation and ablation are diverse in different regions, and the estimation of snow–water equivalent can improve the accuracy of snow forecast (Kuraś et al., 2008; Dai et al., 2012). Therefore, the spatiotemporal distribution and variation of snow cover should be studied on a regional scale.

Precipitation and temperature are the main factors affecting the period of snow accumulation and ablation (Martinec and Rango, 1981; Waqas and Athar, 2019). Tachibana et al. (2007) made a preliminary estimation for Tokyo and nearby areas in Japan and discovered that snow cover did not significantly change with time. By analyzing the characteristics related to snow cover duration, Lethers and Luff (1997) observed that snow cover duration was highly correlated with precipitation and temperature. Schöner et al. (2019) reported that snow depth is mainly influenced by temperature at low altitudes and precipitation at high altitudes. They used snow–water equivalent data from 2002–2009 to interpret snow depth in northeast China by correlating snow cover with temperature and precipitation. The results showed that snow depth gradually decreased over time and temperature was the main influencing factor for snow cover change (Zhang, 2010). A study on the first and last days of snow cover over the past 42 years in the Greater Khingan Range indicated that the first day of snow cover was delayed, the last day was advanced, and the overall duration of snow cover was shorter (Chang, 2018). Snow cover is an important part of a climate system, and it serves as an indicator of climate change and overall climate. Accordingly, climate change also has an impact on the generation and maintenance of snow cover, and the changes in snow cover result from the combination of temperature, radiation, precipitation, altitude, and other climatic factors (Li et al., 2019; Orsolini et al., 2019). In this study, seasonal snow cover is classified into snow accumulation and snowmelt periods according to the variation and trend in the monthly snow depth over a 30-year period (Aizen et al., 1995). The main factors affecting snow cover duration are analyzed. The results lay a foundation to study the utilization of water resources based on snowmelt in the Mollisol areas of China under the prevalent climate change conditions, and provide data support for agricultural production and ecological environment monitoring.

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Materials and methods

Study area

Mollisol areas are mainly distributed in the northeast plain of China. Shown in *Figure 1,* the study area is located between longitudes 119°01′ and 135°06′ and latitudes 30°48′ and 53°33′, and it covers 108.53×10^4 km^2 (Wang et al., 2020). Due to the large-scale cultivation in the Mollisol areas of northeast China, serious soil and water loss has occurred. Soil erosion in the study region has been detected in 27.5×10^4 km^2, which represents approximately 27% of the total area. The study area has an average altitude of 446.811 m, mean annual air temperature of -5–11 °C, and annual precipitation of approximately 300–1000 mm varying from the west to east. Considering the regionalization based on water and soil conservation, there are six secondary regions within the Mollisol area according to dominant landforms: the Greater and Lesser Khingan Range, mountainous and hilly region in the southeast of the Greater Khingan Range, mountainous and hilly part of the northeast, Hulun Buir hilly plain, Songliao plain sandy soil, and Changbai and Wanda mountain hilly (MWR, 2012).

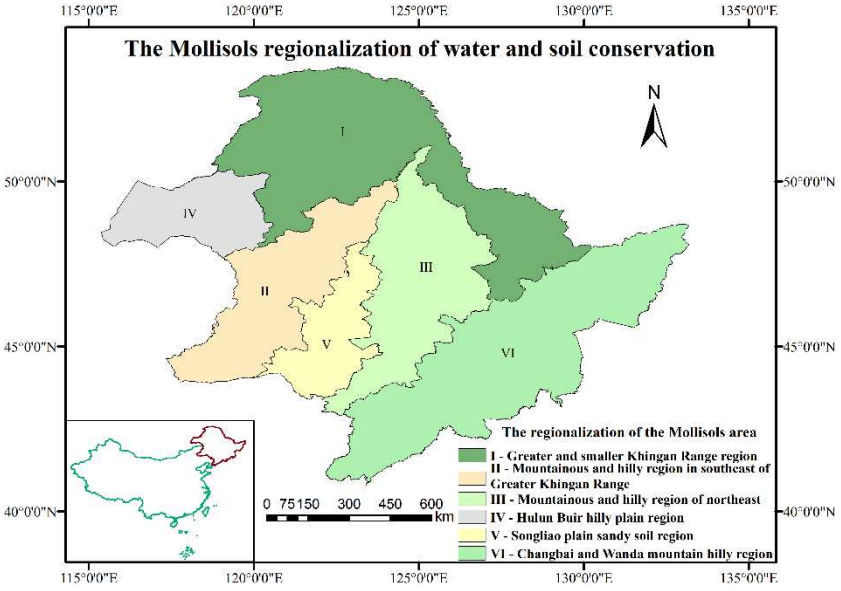

Figure 1. *Study area*

Data

A long-term series (1978–2016) of daily snow depth in China (http://westdc.westgis.ac.cn/) (Nie et al., 2019) was used for analyzing the snow cover (Dai and Che, 2014). Meteorological data was obtained from the national meteorological science center of China (http://data.cma.cn/). As shown in *Table 1,* the daily meteorological data was downloaded and collated from 71 meteorological stations, and it included precipitation, temperature, wind speed, and solar radiation from 1987 to 2016. Quality control was performed to evaluate the homogeneity of the series. This control involved detection of any breaks caused by changes in the location or in the instruments of the stations. In addition, any questionable values were removed, such as daily precipitation amounts lower than zero, until the series showed continuity for several years and gaps lower than <10% (Aguilar et al., 2005).

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Table 1. *Information of the meteorological stations used in this study*

Code	Station	Lon./deg	Lat./deg	Altitude/m	Code	Station	Lon./deg	Lat./deg	Altitude/m
50136	Mohe	122.52	52.97	438.5	54157	Siping	124.38	43.12	179.5
50246	Tahe	124.72	52.35	361.9	54161	Changchun	125.22	43.90	236.8
50349	Xinlin	124.40	51.67	501.5	54181	Jiaohe	127.33	43.70	295.0
50353	Huma	126.63	51.73	173.9	54186	Dunhua	128.20	43.37	524.9
50442	Jiagedaqi	124.12	50.40	371.7	54195	Wangqing	129.78	43.30	244.8
50468	Aihui	127.47	50.25	166.4	54273	Huadian	126.75	42.98	263.3
50557	Nenjiang	125.23	49.17	242.2	54276	Jingyu	126.80	42.40	570.0
50564	Sunwu	127.35	49.43	234.5	54284	Donggang	127.50	42.15	774.2
50656	Beian	126.50	48.25	278.4	54285	Erdao	128.12	42.40	731.7
50658	Keshan	125.88	48.05	236.0	54292	Yanji	129.50	42.87	257.3
50742	Fuyu	124.48	47.80	162.7	54363	Tonghua	125.90	41.68	402.9
50745	Qiqihaer	123.92	47.38	147.1	54374	Linjiang	126.88	41.80	379.7
50756	Hailun	126.87	47.45	247.4	54377	Jian	126.22	41.15	225.1
50774	Yichun	128.83	47.70	264.8	54386	Changbai	128.18	41.42	775.0
50788	Fuyin	131.98	47.23	66.4	54254	Kaiyuan	124.05	42.53	98.2
50844	Tailai	123.45	46.40	138.8	54259	Qingyuan	124.87	42.07	224.0
50853	Beilin	126.97	46.62	179.6	54346	Benxi	123.78	41.30	185.4
50854	Anda	125.32	46.38	149.3	54351	Fushun	124.07	41.92	118.5
50862	Tieli	127.98	46.98	206.0	54486	Xiuyan	123.28	40.33	97.7
50873	Boli	130.30	46.78	82.0	54493	Kuandian	124.78	40.72	260.1
50877	Yilan	129.58	46.30	100.1	54497	Dandong	124.33	40.03	13.8
50888	Baoqing	132.17	46.38	79.6	50425	Flow Down	120.18	50.25	581.4
50950	Zhaozhou	125.25	45.70	148.7	50434	Tulihe	121.68	50.48	732.6
50953	Haerbin	126.57	45.93	118.3	50514	Manchuria	117.32	49.58	661.8
50963	Tonghe	128.73	45.97	108.6	50527	Hailar	119.70	49.25	649.6
50968	Shangzhi	127.97	45.22	189.7	50548	Xiaoergou	123.72	49.20	286.1
50978	Jixi	130.92	45.30	272.5	50603	Xin Barag right Banner	116.82	48.68	542.4
50983	Hulin	132.97	45.77	100.2	50618	Xin Barag left Banner	118.27	48.22	642.0
54094	Mudanjiang	129.67	44.50	305.7	50639	Zhalantun	122.73	48.00	306.5
54096	Suifenhe	131.17	44.38	567.8	50727	Arshaan	119.93	47.17	997.2
50936	baicheng	122.83	45.63	155.3	50834	Suolun	121.22	46.60	499.7
50948	Qianan	124.02	45.00	146.3	54026	Jarud Banner	120.90	44.57	265.0
50949	Qianguo	124.87	45.08	136.2	54027	Bairin Left Banner	119.40	43.98	486.2
54041	Tongyu	123.07	44.80	150.0	54115	Linxi	118.03	43.63	825.0
54049	Changling	123.97	44.25	188.9	54134	Kailu	121.28	43.60	241.0
54063	Fuyu	126.00	44.97	196.8	54135	Tongliao	122.27	43.60	178.7
54142	Shuangliao	123.53	43.50	114.9					

Average monthly snow depth (AMSD)

The average monthly snow depth (AMSD) was calculated as the ratio of monthly accumulated snow depth to the number of snow cover days. AMSD values from October of each year to May of the subsequent year were calculated (Baronetti et al., 2019). The average monthly variation rate (AMVR) was analyzed. The linear change

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tendency of each climate factor was analyzed using the linear regression analysis method with one variable. A confidence level of $p < 0.05$ was used to evaluate the significance of the trends (Shafiq et al., 2018; Yang et al., 2019). The linear regression equation is as follows:

$$b = \frac{n\sum_{i=1}^{n} x_i y_i - \sum_{i=1}^{n} x_i \sum_{i=1}^{n} y_i}{n\sum_{i=1}^{n} x_i^2 - (\sum_{i=1}^{n} x_i)^2} \quad \text{(Eq.1)}$$

where $y_i = b \times x_i + a$, $a = \bar{y} - b\bar{x}$, y_i is the AMSD data of the snow cover period of 1978–2016, x_i is the time series, a is the regression constant term, and b is the regression coefficient. In this study, b is used to quantitatively analyze the linear change tendency of climatic factors.

Duration and first and last days of snow cover

The first day of snow cover (SFD) is the 5th day of the first spell of a 5-day consecutive snow fall in autumn or winter, and the end day snow cover (SED) is the last day of the last 5-day consecutive snow fall in the spring of the subsequent year. The snow cover duration (SCD) is defined as the sum of days with a snow depth of more than 1 cm (Gusain et al., 2016; Mo et al., 2017; Orozco et al., 2019).

Snow cover duration and climate change

The correlation between snow cover duration and temperature, precipitation, radiation, altitude, and latitude were analyzed for 1987–2016 according to the methods provided by Liu and Chen (2000) and Buisan et al. (2015).

Periodic changes in snowmelt water volume

Data on snowmelt water volume was obtained from the maximum depth in the snow accumulation period during 1987–2016. The average snow density was 0.298 g/cm^3 (Ma, 2017). The Morlet wavelet method was used to analyze the multitemporal and spatial scale variation of snowmelt water volume. The continuous wavelet transform was as follows:

$$V_{volum} = \sum_{1}^{s} 0.298 \times area \times SD_{\max} \quad \text{(Eq.2)}$$

where V_{volum} is the volume of snowmelt water, *area* is a pixel area, and SD_{max} is the maximum snow depth.

$$W_f(a,b) = \frac{1}{\sqrt{a}} \Delta t \sum_{k=1}^{N} f(k\Delta t)\bar{\psi}(\frac{t-b}{a}) \quad \text{(Eq.3)}$$

where $W_f(a,b)$ can reflect the characteristics of time domain parameter b and frequency domain parameter a, Δt represents the sampling interval, and $f(k\Delta t)$ represents the output of the filter through the impulse response (Slavie et al., 2003).

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Results and discussion

Temporal and spatial variation of snow depth

Based on the seasonal changes in northeast China, the hydrological year (HY) was defined as the period from December 1 to September 30 of the next year. The snow cover period in the Mollisol region begins in early October and ends in early May. The spatiotemporal distribution of AMSD is shown in *Figure 2*. Snow accumulation was lower in October and November. Snowfall first appeared in Mohe and Tulihe of the Greater Khingan Range, where the AMSD was 0–5 cm, and accumulation was rapid due to the influence of temperature. In February, the maximum value of AMSD was observed with an increasing trend from southwest to northeast. Owing to the influence of topography, AMSD in the Greater Khingan Range was 15–30 cm, and in the Changbai and Wanda mountain region it was 10–15 cm. However, the values were lower in the plain and low-altitude regions. From mid-March, snow depth began to decrease and the snow completely melted in early May. The AMSD value of the Mollisol region became below the effective snow depth within May and October.

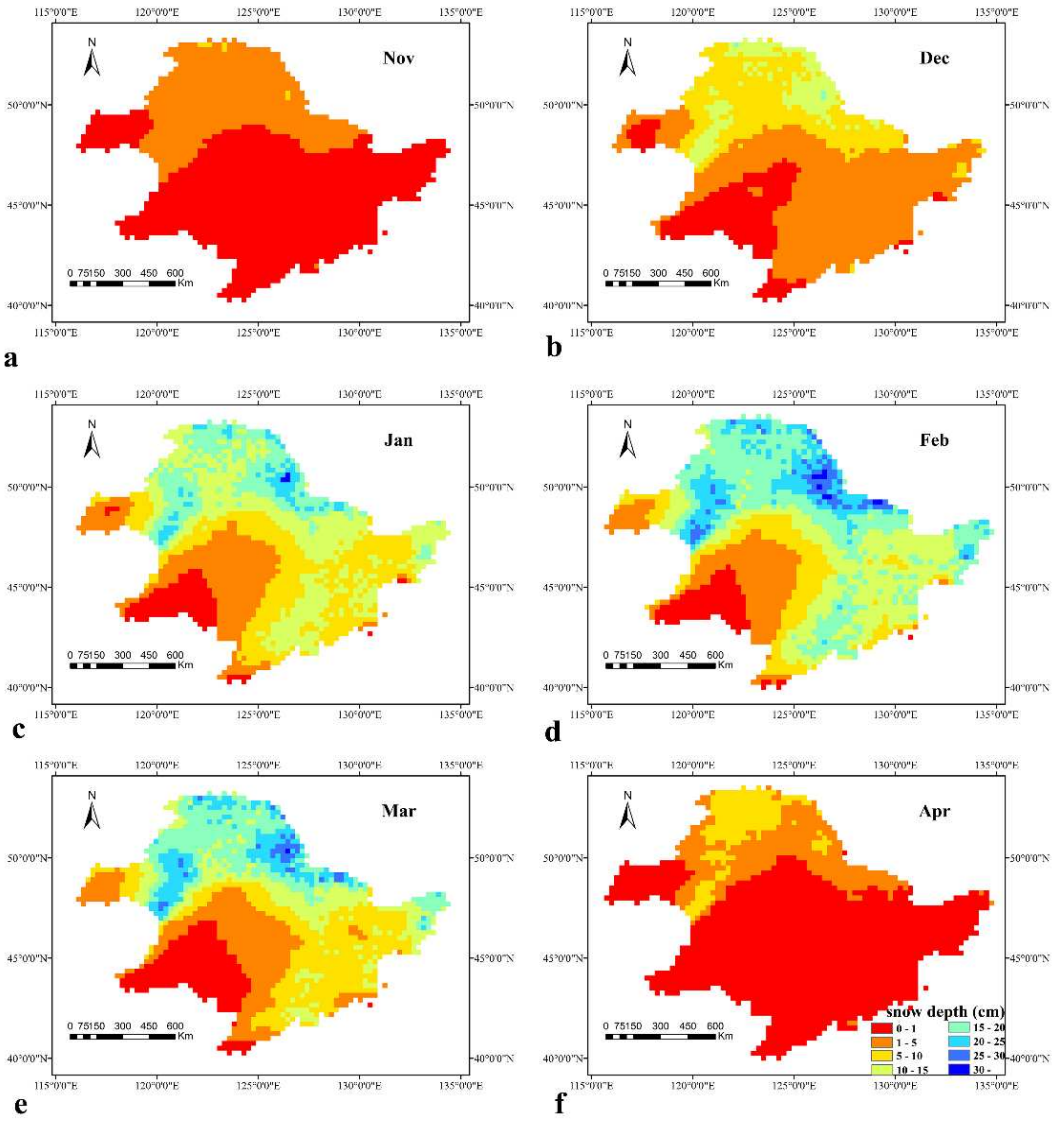

Figure 2. *Average monthly snow depth (AMSD) in the study area*

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The AMVR values for the 30-year period considered in the study are shown in *Figure 3*, which was calculated by *Equation 1*. The AMVR in the Mollisol region increased by 0–0.1 cm/year in November with no significant variation throughout the area. Large areas with significant increase of above 0.2 cm/year occurred in the mountainous and hilly regions of northeast China in December. In January, the AMVR shows an insignificant increasing trend in snow depth for most areas, particularly in the mountainous and hilly region of northeast at 0–0.1 cm/year. In February and March, an insignificant decrease is evidenced in large areas of the Greater and Lesser Khingan Range, while a significant increase occurs in the mountainous and hilly region of northeast and the Sanjiang plain, with variations higher than 0.2 cm/year. In April, increasing and decreasing trends each occupied approximately half of the study area, in which the southern part of the Songliao plain's sandy soil region presented a significant decreasing trend.

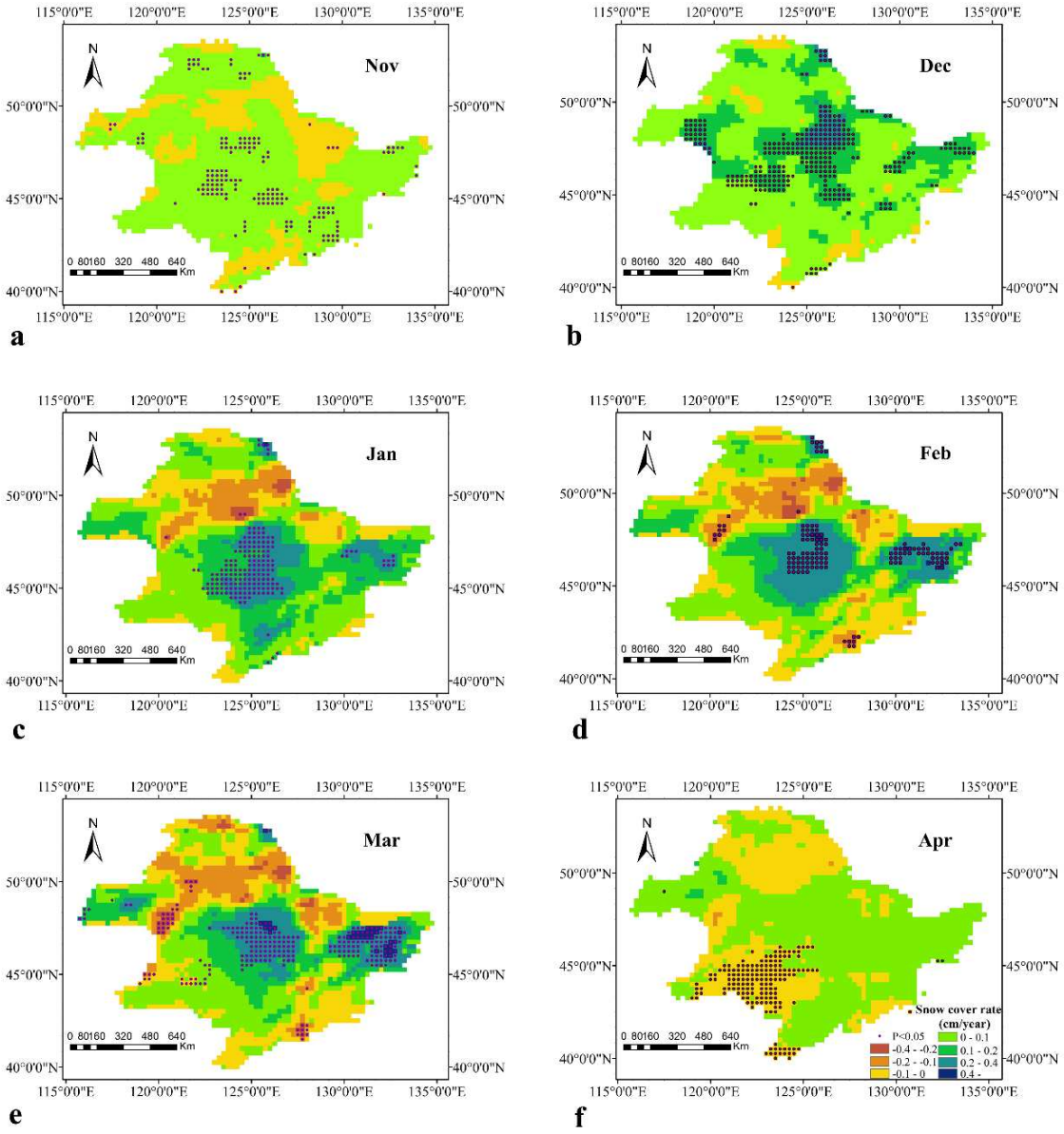

Figure 3. *Average monthly variation rate (AMVR) of snow depth in the study area*

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Temporal and spatial variation of snow cover days

The variations of monthly average and maximum snow depths in the Mollisol region shown in *Figure 4* were consistent. The snow depth was shallow from October to November with a low rate of increase. From October to February, the snow depth increased at a fast rate and reached its maximum in February. Therefore, this period was defined as the snow accumulation period. From March to May, the depth of snow decreased continuously with a short duration of snowmelt. This period was defined as the snow ablation period.

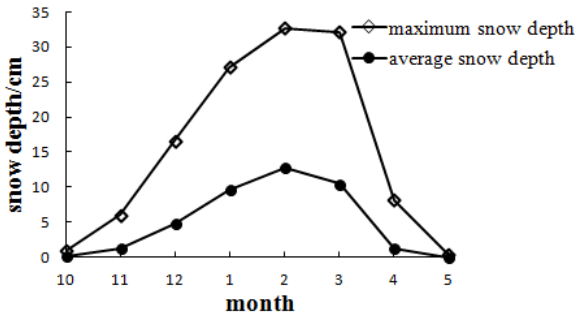

Figure 4. *30-year variation in monthly snow depth*

As shown in *Figure 5a*, during the 30-year period, the first day of snow appeared in the Greater Khingan Range of the Mollisol region from the 12th to 30th day, followed by the Lesser Khingan Range region from the 30th to 60th day. The first day of snow in the Changbai and Wanda mountain hilly region and mountainous and hilly region of northeast occurred between the 60th and 90th day. In the Songliao plain sandy soil region and mountainous and hilly region in the southeast of the Greater Khingan Range, the first day of snow occurred between the 60th and 90th day. It was delayed from north to south, and there was no snow in the south of the hilly region. The first day of snow in the Hulun Buir hilly plain region occurred between the 90th and 150th day. *Figure 5b* shows that the last day of snow was delayed the most in the Greater and Lesser Khingan Range, where it occurred between the 200th and 220th day. For the Changbai and Wanda mountain hilly region, mountainous and hilly region of northeast, and Hulun Buir hilly plain region, it occurred between the 180th and 200th day. The last day of snow for the Songliao plain sandy soil region and hilly region in the southeast of Greater Khingan Range occurred progressively from north to south.

As shown in *Figure 5c*, the snow cover plural for the northern part of the Greater Khingan Range, lesser Khingan Range and Changbai and Wanda mountain hilly region, and Hulun Buir hilly plain region were 180–200, 150–180, and 120–150 days, respectively. The snow cover duration in the mountainous and hilly region southeast of the Greater Khingan Range, Songliao plain sandy soil region, and mountainous and hilly region of northeast advanced from north to south (Whetton et al., 1996). The annual average of maximum snow depth in the Greater and Lesser Khingan Range, north region, Changbai and Wanda mountain hilly region, and mountainous and hilly region of northeast were 20–30, > 30, 10–20, and 5–10 cm, respectively. In addition, the annual average of maximum snow depth in the Songliao plain sandy soil region and mountainous and hilly region southeast of the Greater Khingan Range decreased from north to south.

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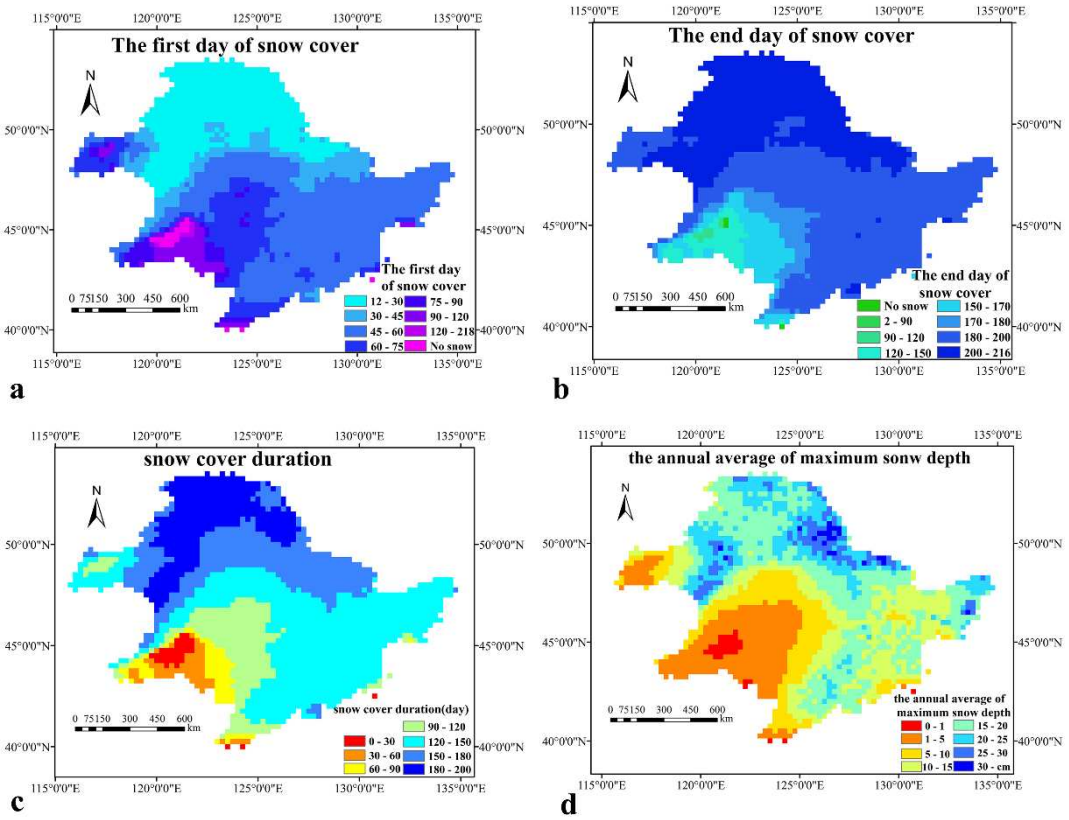

Figure 5. *Snow cover in the study area during the study period: (a) first day of snow cover, (b) last day of snow cover, (c) duration of snow cover, and (d) annual average snow depth*

Snow cover days and climate change

Linear regression analyses were performed to correlate average temperature, total precipitation, and latitude and altitude of meteorological stations with snow cover days and accumulation period, and the results are presented in *Figure 6*. Temperature presented a negative correlation with snow cover days during the snow accumulation period (*Fig. 6a*). No significant correlation was observed between total precipitation and snow cover days (*Fig. 6b*). The latitude (*Fig. 6c*) and altitude (*Fig. 6d*) of the meteorological stations were positively correlated with snow cover days during the accumulation period. Temperature was considered the main influencing factor for snow cover days during the accumulation period because its correlation value was the highest, at $R^2 = 0.55$. The correlation between environmental factors and number of snow cover days during the snow accumulation period were, in order of significance: average temperature > latitude of meteorological station > altitude of meteorological station > total precipitation.

Linear regression analyses were also performed to correlate average radiation, average temperature, and latitude and altitude of meteorological stations with snow cover days for the snow melt (ablation) period, and the results are presented in *Figure 7*. In the Mollisol region of northeast China, there was no correlation between total precipitation and snow cover days during the snow ablation period. However, there was a negative correlation between average temperature (*Fig. 7a*) and average radiation (*Fig. 7b*) with snow cover days. Altitude (*Fig. 7c*) and latitude (*Fig. 7d*) of

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meteorological stations were positively correlated with snow cover days. The correlation with temperature during the snow ablation period was the highest, at $R^2 = 0.66$, which indicates that temperature presents the most influence on the number of snow cover days during the snow ablation period. The correlation with environmental factors were, in order of significance: average temperature > latitude of meteorological station > average solar radiation > altitude of meteorological station.

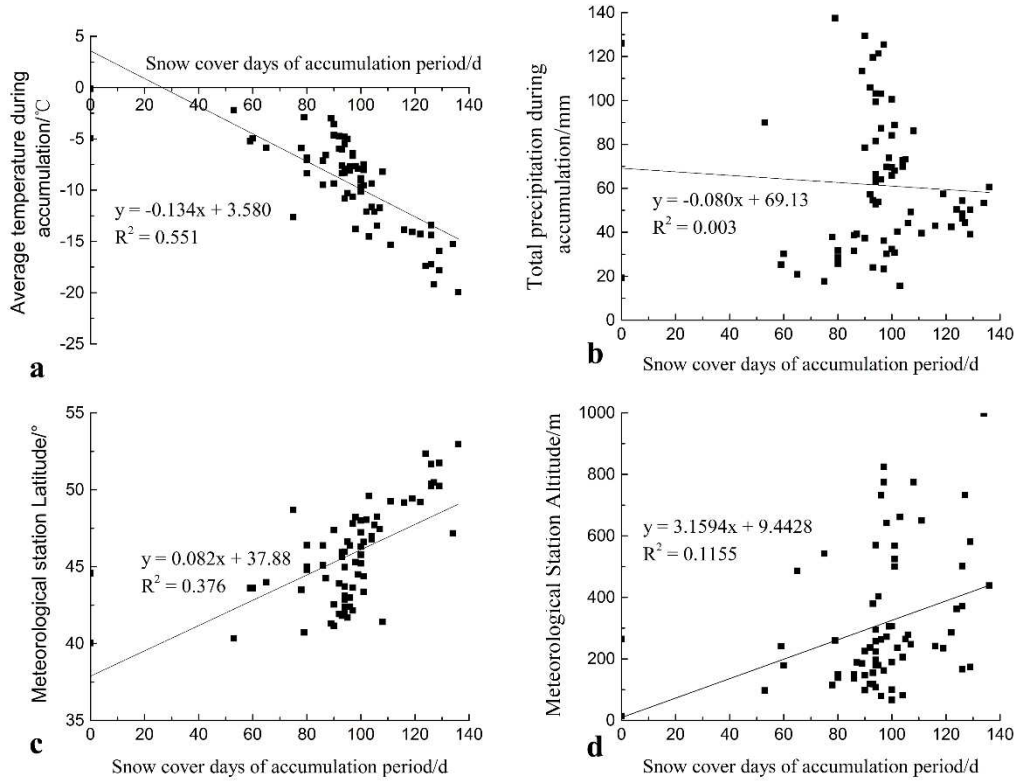

Figure 6. *Correlation between snow cover days and climatic factors in the snow accumulation period: (a) average temperature, (b) total precipitation, (c) latitude of meteorological station, and (d) altitude of meteorological station*

Time scale of snowmelt water volume

Figure 8a shows the volume diagram of the snowmelt water in the Mollisol region of northeast China, which is calculated by *Equation 2*. The annual volume of snowmelt water did not experience a continual increase or decrease, and the volume of snowmelt water varied by 2.764×10^9 m^3 every ten years. In the northeast China, the long-term variability of snow cover is marked by a stochastic oscillation superimposed on a small increasing trend over the past 30 years. No abrupt change was found in snow cover. In addition, the minimum and maximum snowmelt water volume occurred at 35.089×10^9 and 97.646×10^9 m^3 in 2008 and 2013, respectively. *Figure 8b* shows the wavelet analysis diagram of the snowmelt water volume in the studied region, which is calculated by *Equation 3,* and it indicates that the variation period was the most obvious in 3–5 years. Before 2005, the annual variation period of snowmelt water volume in 10 years was obvious. After 2005, snowmelt water volume showed a variation period of 5–7 years, and this period gradually decreased.

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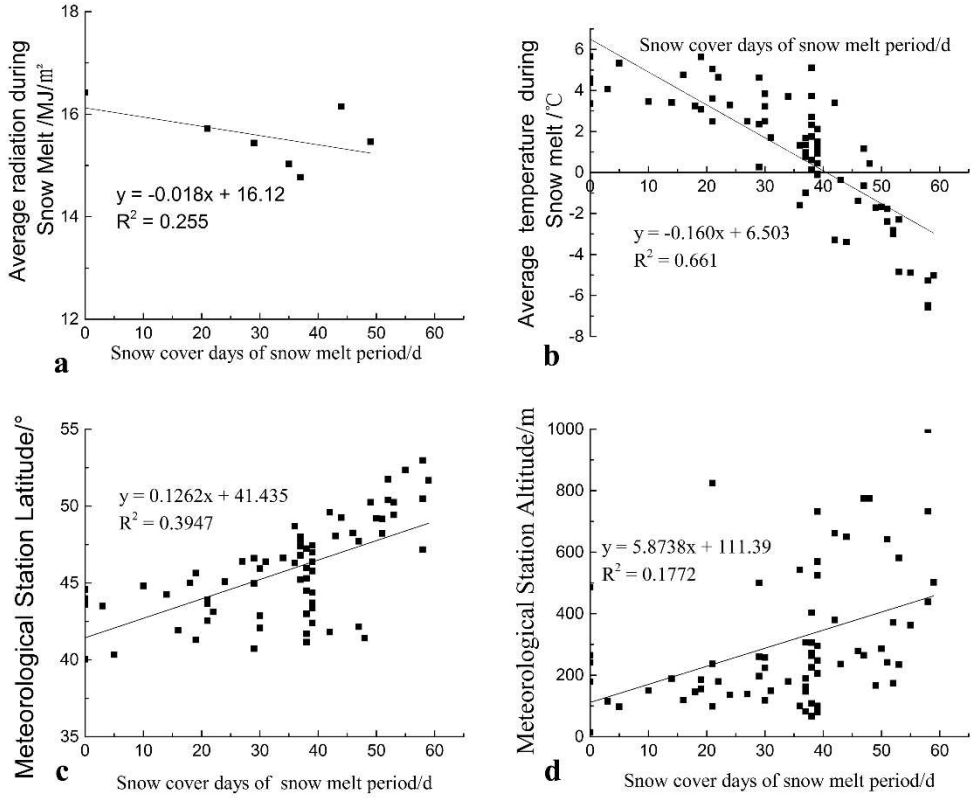

Figure 7. *Correlation between snow cover days and climatic factors in the snow melt period: (a) average radiation, (b) average temperature, (c) latitude of meteorological station, and (d) altitude of meteorological station*

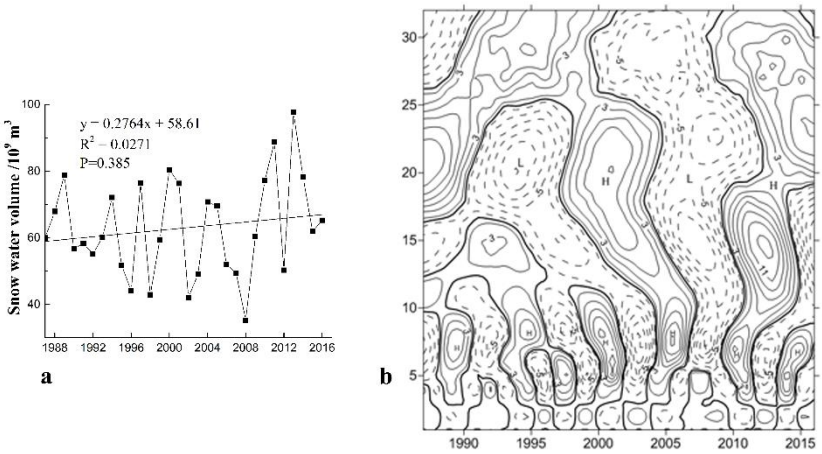

Figure 8. *Morlet wavelet analysis of snowmelt water volume*

Trujillo and Molotch (2014) report that the snow–water equivalent curve can represent hydrological indexes in a HY (Egli and Jonas, 2009; De Gregorio et al., 2019; Yang et al., 2019). Accordingly, the snow accumulation and ablation periods varied in different regions. In this study, the spatiotemporal distribution showed that snow cover began to accumulate in mid-November, reached the maximum depth by the end of

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February of the subsequent year, and completely melted in late March. The distribution pattern was consistent in terms of average maximum snow depth and number of snow cover days, and had the strong spatial heterogeneity in the whole area.

The results of this study contradict the trend of decreasing maximum snow cover in the Northern Hemisphere (Mote, 2003). The volume of snowmelt water in the Mollisol region of northeast China had no significant increase during the 30-year period. In terms of average snow depth under the global warming scenario, the Greater and Lesser Khingan Range and the Changbai and Wanda mountain hilly region showed a decrease, and the plain region showed an increase. The decrease in average snow depth at high attitudes is similar to those of previous studies conducted in the Alps and the Tianshan mountains (Li et al., 2019; Schöner et al., 2019). Meteorological data are mainly used to study the changes of snow depth, and the variation rate of snow depth is consistent with station observations (Tan et al., 2019). The results showed that the spatial variation of snow depth had no significant change.

Studies have shown that there is a strong correlation between spring temperatures and streamflow (Barnett et al., 2005; Niedzielski et al., 2019; Yang et al., 2020). Temperature and precipitation influence the duration of snow accumulation and ablation periods in high altitude mountains (Hantel et al., 2000). These studies reveal that average temperature and total precipitation can affect the entire snow season (Qin et al., 2006). However, in the present study, the results show that temperature was the main factor influencing snow accumulation and ablation periods. The difference may be due to the inconsistent length of time series, and the discrepancy in the selected sites and analytical methods. The findings also revealed that there was no correlation between precipitation and snow cover duration.

Conclusion

In the present study, the spatiotemporal distribution characteristics of snow cover during a 30-year period in the Mollisol region of northeast China were analyzed. Their correlation with climatic factors were also investigated. Data on the maximum snow cover depth was used to analyze the variation period of snowmelt water volume in the region. The main conclusions are as follows.

(1) The spatial distribution of snow depth was consistent with the secondary regionalization of the Mollisol region, and the maximum average monthly snow occurred in the Greater Khingan Range. Snow depth was largely affected by topographic factors, and it was relatively shallow in the plain region. The snow depth gradually increased from southwest to northeast, and the AMSD showed an increasing trend in most parts of the study region. However, the south region of the Greater and Lesser Khingan Range and the Changbai and Wanda mountain hilly region showed a decreasing trend of 0–0.4 cm/year. In contrast, the plain region showed an increasing trend of 0–0.4 cm/year.

(2) According to the variation pattern of monthly snow depth in the Mollisol region, the AMSD reached its maximum value in February. Therefore, the snow cover duration was divided into snow accumulation and ablation periods. Regarding correlation with snow cover days, temperature presented the highest positive value. The latitude and altitude of the meteorological stations also presented positive correlations with the number of snow cover days. In contrast, precipitation presented a non-significant correlation, and solar radiation presented a negative correlation.

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(3) The first snow cover day appeared in the northern area of the Greater Khingan Range, and the duration of the snow cover was long. The onset of the first snow cover day occurred last in the mountainous and hilly region in the southeast of the Greater Khingan Range, where the duration of snow cover was short. The duration of snow cover and distribution of snow depth consistently increased from southwest to northeast within the study area.

(4) The amount of snowmelt water in the Mollisol region showed an overall increasing trend, and the 3–5-year variation period was the most significant, while the 5–10-year variation period was not significant, and the cycle gradually decreased. Additionally, the accurate start time of the snow ablation remains to be further studied in the follw-up study.

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

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EFFECTS OF FERTILIZATION ON SOIL WATER USE EFFICIENCY AND CROP YIELD ON THE LOESS PLATEAU, CHINA

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Abstract. The selection of appropriate fertilizers influences agricultural production, especially in water-limited conditions such as those on the Loess Plateau (China). In this study, we aimed to determine the optimum method for the fertilization of dryland crops. In a two-year split-plot experiment, we tested eight fertilization treatments with different combinations of organic and inorganic fertilizers and a no-fertilizer control. Crop yield, soil water storage, and soil water use efficiency (WUE) were assessed pre-sowing and post-harvest in 2017 and 2018. The results showed that soil water storage was decreased by fertilization, while the WUE and crop yield were both increased: compared to the control, WUE was 6–39% higher in 2017 and 8–39% higher in 2018, while crop yield was 3–21% higher in 2017 and 1–19% higher in 2018. The maximum effect on WUE and crop yield was achieved when a combination of organic fertilizer and either nitrogen or phosphate fertilizer was applied. Linear regression analysis revealed that soil water consumption (ET) was significantly positively correlated with crop yield. Our results provide a scientific basis for rational crop fertilization on the Loess Plateau and suggest that a combination of organic and inorganic fertilizers is the most appropriate.
Keywords: *inorganic fertilization, organic fertilization, agricultural production, soil water storage, rain-fed agricultural*

Introduction

In the world's typical traditional dry farming regions, scarce precipitation and insufficient water for irrigation have become the main factors limiting the improvement of agricultural productivity (Hamdy, 2003). Different soil water and fertilizer conditions are known to have important effects on crop growth, dry matter distribution, and crop yield (Yan, 2015). For instance, insufficient soil water affects the synthesis and transport of nutrients, reducing crop yield and quality, while inadequate fertility can affect soil water absorption and utilization (Zhang et al., 2007; Wu et al., 2019). The availability of soil water has complex interactions with soil microbial activities, soil physics and chemistry, and plant physiological and biochemical processes. In addition, soil water and fertilizers are important material resources in agricultural production. Therefore, the study of the relationship between fertilization and soil water is a key research area in crop yield and agricultural economic development (Huang, 2010).

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Improving crop yield and soil water use efficiency (WUE) through rational fertilization strategies under water-limited conditions is a hot research topic (Guo et al., 2019). Many studies have shown that the effects of the addition of soil water and the application of fertilizer on crop yield are strongly dependent on the pre-existing soil water and fertility (Fan et al., 2005).

Some studies in the Loess Plateau have found that the effect of fertilization on crop yield is more significant when the soil's natural water level is low. With the improvement in soil fertility, soil water also increased, suggesting that soil water and fertilizer have a coupling effect on crop yield (Krbel et al., 2012; Tang et al., 2016). In another study, fertilizer application boosted soil WUE under all soil water conditions, but especially when soil water levels were already sufficient (Xue, 2009; De, 2013). Compared with the non-fertilization treatment, crop yield increased significantly when fertilized, but the yield difference was not obvious under drought conditions (Tahir, 2012). Soil water and fertilization have also been shown to have extremely significant effects on crop soil water consumption and soil WUE (Tang et al., 2016). Fertilization can significantly inhibit plant transpiration and soil water loss, increase the drought resistance and soil water consumption of crops, and increase soil WUE (Laxminarayana et al., 2011; Belay, 2002) Fertilization can also reduce the consumption of ineffective water in the soil, increase the water utilization rate, and boost the crop's ability to use the deep-layer soil water (Chen et al., 2016; Ren et al., 2019).

The Loess Plateau is a typical traditional dry farming region; it is located in a semi-arid/semi-humid climate zone and has undergone serious soil erosion. Due to the low precipitation level, its uneven seasonal distribution, and the low soil organic matter content, nutrients and moisture have become the key factors restricting agricultural production in the area. Precipitation is a natural event which determines the fluctuation in dryland crop yield. Careful fertilization that correctly influences the soil nutrient cycle can ensure high nutrient uptake of the crops and enhance soil fertility. Conversely, excessive fertilization has a limited effect on increasing yield and undermines the soil–crop system nutrient balance. Despite the importance of this issue in the Loess Plateau region, there are few reports that have directly looked at the relationship between fertilization and WUE in this area.

Our objective was to fill this gap in knowledge and to achieve a science-based recommendation for the fertilizer that is the most appropriate to the drylands on the Loess Plateau. In this study, we report the changes in crop yield and WUE under different fertilization scenarios in two consecutive years in which different crops were planted on the same study plots. We hypothesized that (1) fertilization would significantly increase soil water storage, WUE, and crop yield; and (2) the impact of an organic fertilizer would be higher than that of inorganic fertilizer.

Materials and methods

Basic geographic information and experimental soil conditions

The field experiment was conducted in 2017 and 2018 at a study site located in the middle of the Loess Plateau (36°51′N, 109°18′E). The site (*Fig. 1*) is located 1068 m above sea level; it has a mean annual temperature of 8.8 °C and an annual precipitation level of 500.0 mm in the last 10 years. The recorded annual precipitation was 557.6 mm in 2017 and 536.3 mm in 2018. The precipitation during the growth period (between March and September) was 443.1 mm in 2017, accounting for 79.47% of the total

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annual precipitation; the respective values were 503.1 mm and 93.8% in 2018. The climate details are shown in *Figure 2*. The soil type at the study site was loess soil; basic soil physical and chemical properties were measured at the depth of 0–40 cm before sowing in two years of experimentation (*Table 1*).

Figure 1. *The test field*

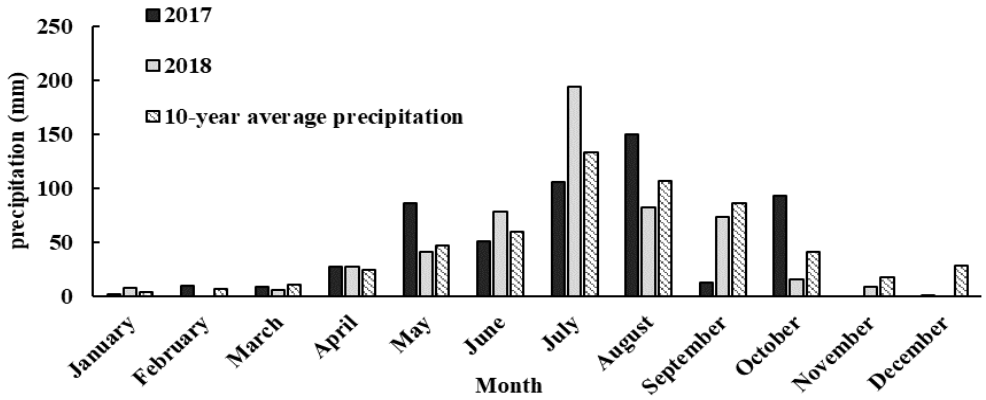

Figure 2. *Average monthly precipitation in Ansai County between 2017 and 2018*

Table 1. *Basic physical and chemical properties of soil (0-40 cm soil layers)*

Year	Bulk density	SOM	TN	TP	pH
2017	1.327	4.053	0.217	0.422	8.31
2018	1.334	4.123	0.196	0.478	8.32

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Fertilizer treatments

Organic and inorganic fertilizers were used. The nitrogen fertilizers (N) were urea and diammonium phosphate, the phosphate fertilizer (P) was diammonium phosphate, the potassium fertilizer (K) was potassium chloride, and the organic fertilizer (M) was sheep manure. Organic fertilizer treatments were the following: M, MN, MP, and MNP. Inorganic fertilizer treatments were NP, NK, PK, and NPK. In addition, a no-fertilizer treatment was used as the control CK. The fertilizers were spread evenly on the soil surface before sowing. Nine fertilization practices are shown in *Table 2*.

Table 2. *Experimental fertilization*

Treatment	Illustration
M	Sheep manure (0.75 kg/m^2)
MN	Sheep manure (0.75 kg/m^2) + Urea 0.021 kg/m^2
MP	Sheep manure (0.75 kg/m^2) + Diammonium phosphate (0.017 kg/m^2)
MNP	Sheep manure (0.75 kg/m^2) + Urea (0.021 kg/m^2) + Diammonium phosphate (0.017 kg/m^2)
NP	Urea 0.021 kg/m^2 + Diammonium phosphate (0.017 kg/m^2)
NK	Urea (0.021 kg/m^2) + Potassium sulfate (0.012 kg/m^2)
PK	Diammonium phosphate (0.017 kg/m^2) + Potassium sulfate (0.012 kg/m^2)
NPK	Diammonium phosphate (0.017 kg/m^2) + Urea (0.021 kg/m^2) + Potassium sulfate (0.012 kg/m^2)
CK	No fertilizer

Experiment design

Local heat and moisture conditions allow crops to only grow once a year. The planting mode used millet-soybean rotations, so millet was sowed on May 2 in 2017, and soybeans on May 4 in 2018. The millet cultivar was Hongyang 7. The soybean variety planted was Zhonghuang 35. Millet sowing in 2017 was quantitative sowing, planting density was 15 plants/m^2. The sowing method was drill, spraying 10% Monosulfuron 0.023 kg/m^2 on May 24 for chemical weeding in the experimental plot. In 2018, soybean planting density was 22 plants/m^2. At the end of May, when two compound leaves of soybean were grown after seedling emergence, 5% imazethapyr 0.0001 kg/m^2 was applied to soybean to remove weeds. Each of the 9 treatments was allocated 4 plots, yielding a total of 36 plots. Each plot was 3.5 m long and 8.57 m wide (plot area of 30 m^2). The plots were arranged in random blocks (*Fig. 3*). The soil fertility and environmental conditions in all plots were uniform. Organic, potash, and phosphate fertilizers were each applied once, while urea fertilizer was applied at a rate of 136 g per plot, and the remaining 500 g nitrogen fertilizer was applied during the flowering period. The millet was fertilized with urea on April 27th, 2017, and supplementary nitrogen fertilizer was added on July 3rd, 2017. Urea fertilizer was added to soybeans on April 28th, 2018, and supplementary nitrogen fertilizer was added on July 26th, 2018. The 9 fertilization treatments are shown in *Figure 3*.

Soil sampling and crop harvesting

Soil sample collection method: Taking each plot as a unit, the soil sampling point was more than 1 m of the boundary of the sample plot. Sampling points were randomly selected forming an S shape. Surface debris was then removed, and soil samples were collected. All soil samples from the same sampling point were combined and passed

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through a 2 mm sieve. Samples were stored and labelled after the relevant sampling information had been recorded. In all plots, the soil water level was measured every 10 cm up to the depth of 100 cm with a neutron moisture meter (Neutron moisture meter, Model CS830, Rurui Technology Company, Guangzhou, China). The weight water consumption was multiplied by the soil bulk density to obtain the volumetric water consumption (Wang et al., 2016). The soil water consumption was determined using the oven-drying method. The soil samples were brought back to the laboratory; plant roots and other impurities were removed, and the percentage of soil moisture in weight was measured (Lu et al., 2019). The soil bulk density was measured using a soil bulk sampler (Soil bulk sampler, Model JC-8028, Juchuang, Qingdao, China) for every soil sample. The soil pH was measured using a pH meter (pH meter, Model PHS-25, LEICI, Nanjing, China) for every soil sample.

9 MP	8 MNP	7 CK	6 NP	5 NK	4 PK	3 NPK	2 M	1 MN
18 MNP	17 M	16 NK	15 NPK	14 CK	13 NK	12 PK	11 MN	10 MP
27 M	26 MN	25 NPK	24 PK	23 NP	22 NK	21 CK	20 MP	19 MNP
36 MN	35 MP	34 PK	33 NK	32 NPK	31 CK	30 NP	29 MNP	28 M

Figure 3. *Test plot layout. M, organic fertilizer; MN, organic fertilizer combined with nitrogen fertilizer; MP, organic fertilizer combined with phosphate fertilizer; MNP, organic fertilizer combined with nitrogen and phosphate fertilizer; NP, nitrogen fertilizer combined with phosphate fertilizer; NK, nitrogen fertilizer combined with potassium fertilizer; PK, phosphorus combined with potassium fertilizer; NPK, nitrogen combined with phosphate and potassium fertilizer; CK, no-fertilizer control*

At the end of the growing season, 3 quadrats (1 m × 1 m) were harvested in each plot by cutting at the soil surface level; the harvested plants were dried in an oven (75 °C, 24 h), weighed, and their value was converted to plot yield. Final crop yield was obtained by harvesting 4 replicates of each treatment. The 2017 millet was harvested on October 12th, and the 2018 soybean crop was harvested on October 4th. In 2017, soil moisture content before sowing was measured on April 26th, and post-harvest moisture was measured on October 13th. In 2018, soil moisture content before sowing was measured on April 20th, and post-harvest moisture was measured on October 5th.

Index calculations

The requisite indices were calculated according to the following formulae.

$$Sw = H_i \times D_i \times B \times 10 \quad \text{(Eq.1)}$$

where: Sw (mm) is the sum of soil water stored in different soil layers (Lu et al., 2019); D_i (g cm^{-3}) is the soil bulk density in different soil layers; H_i (cm) is the soil depth; and B (%) is the percentage of soil moisture in weight.

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$$ET = W_1 - W_2 + P \quad \text{(Eq.2)}$$

where: ET (mm) is the water consumption during the crop-growing season (Xu et al., 2020a); W_1 (mm) is the water storage at a soil depth of 0–100 cm at sowing; W_2 (mm) is the water storage at a soil depth of 0–100 cm during harvest; P (mm) is the precipitation measured during the crop growth period.

$$\text{WUE} = {}^{Y}/_{ET} \quad \text{(Eq.3)}$$

where: WUE is water use efficiency (kg m^2 mm^{-1}) (Adimassu et al., 2017); Y (kg m^2) is the yield per unit plot.

Statistical analysis

One-way ANOVA analysis was used to examine the effects of fertilization treatment on soil water storage, soil WUE, and crop yield ($P < 0.05$). Before the analysis, we performed a normality and homogeneity test of the data. Linear regression analysis was used to show the relationship between crop yield and soil WUE. All statistical analyses were performed using the software package IBM SPSS Statistics (version 26.0). The differences between the treatments were calculated using the least significance difference test at a 0.05 probability level. Figures were prepared using Origin 9.0.

Results

Soil profile water content

In 2017, before sowing, the soil moisture water content in the 0–10 cm soil layer of the M, MN, MP, and NK plots was higher than that in the CK plots, while in the 10–100 cm soil layers the soil moisture water content of all the fertilization treatments was lower than that of the CK (*Fig. 4a*). The soil moisture water content of all fertilization treatments in the 0–10 cm soil layer was higher than that of CK after the crop harvest in 2017 and before sowing in 2018, and the soil moisture water content of all fertilization treatments in the 10–100 cm soil layers was lower than that of CK (*Fig. 4b, c*). Furthermore, after the crop harvest in 2018 the soil moisture water content of the M, MN, MP, and NPK treatments in the 0–10 cm soil layer was higher than that of the CK treatment, and in the 10–100 cm soil layers the soil moisture water content of all the fertilization treatments was lower than that of the CK treatment (*Fig. 4d*).

Soil water storage

In 2017, the soil water storage recorded in the CK plots exceeded that found in fertilized plots; it was 1.22–21.11 mm higher than that of all of the fertilization treatments, both before sowing and after harvest. Meanwhile, the soil water storage of the MN, MP, and MNP plots was higher than that of the other fertilization treatments, both before sowing and after harvest (*Fig. 5a*). In 2018, the soil water storage of the CK plots was greater than that of the fertilization treatments after harvest; meanwhile, the soil water storage in the MN, MP, MNP, and NPK plots was higher than in the other fertilization treatments, both before sowing and after harvest (*Fig. 5b*).

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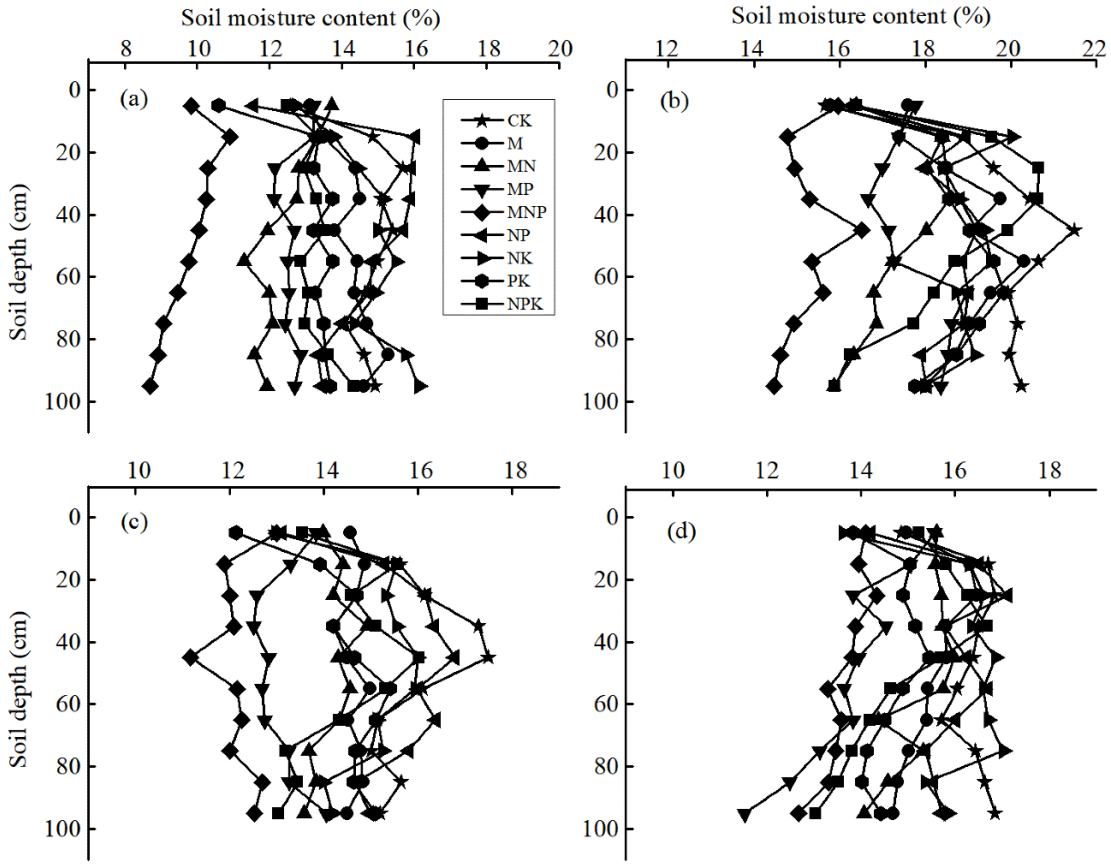

Figure 4. *Soil moisture water content (mean) in 2017 before sowing (a), 2017 after harvest (b), 2018 before sowing (c), and 2018 after harvest (d) in different fertilization treatments. Notes: M, organic fertilizer; MN, organic fertilizer combined with nitrogen fertilizer; MP, organic fertilizer combined with phosphate fertilizer; MNP, organic fertilizer combined with nitrogen and phosphate fertilizer; NP, nitrogen fertilizer combined with phosphate fertilizer; NK, nitrogen fertilizer combined with potassium fertilizer; PK, phosphorus combined with potassium fertilizer; NPK, nitrogen combined with phosphate and potassium fertilizer; CK, no-fertilizer control*

Crop yield and soil water consumption (ET)

Overall, higher crop yield was recorded after fertilization. In 2017, the crop yields of the M, MN, MP, MNP, NP, NK, and NPK plots were 15%, 11%, 8%, 21%,8%, 5%, and 11% greater, respectively, than that after CK treatment. In 2018, the crop yield of the M, MN, MP, MNP, NP, NK, and NPK plots was higher than that of the CK plots by 11%, 14%, 15%, 19%, 7%, 3%, and 11%, respectively (*Fig. 6*).

In 2017, the water consumption (ET) recorded in the CK plots lower than the other fertilized plots; approximately 12.83–47.73 mm lower than that of all fertilization treatments. Meanwhile, the soil water consumption of the MN, MP, and MNP plots was higher than that of other fertilization treatments, both before sowing and after harvest (*Fig. 7*). In 2018, the soil water consumption of the CK plots was lower than that of the fertilization treatments; meanwhile, the soil water storage in the MN, MP, MNP, and NPK plots was higher in other fertilization treatments (*Fig. 7*).

The linear regression showed that the ET was significantly positively correlated with crop yield ($R^2 = 0.846$, $P < 0.001$) (*Fig. 8*).

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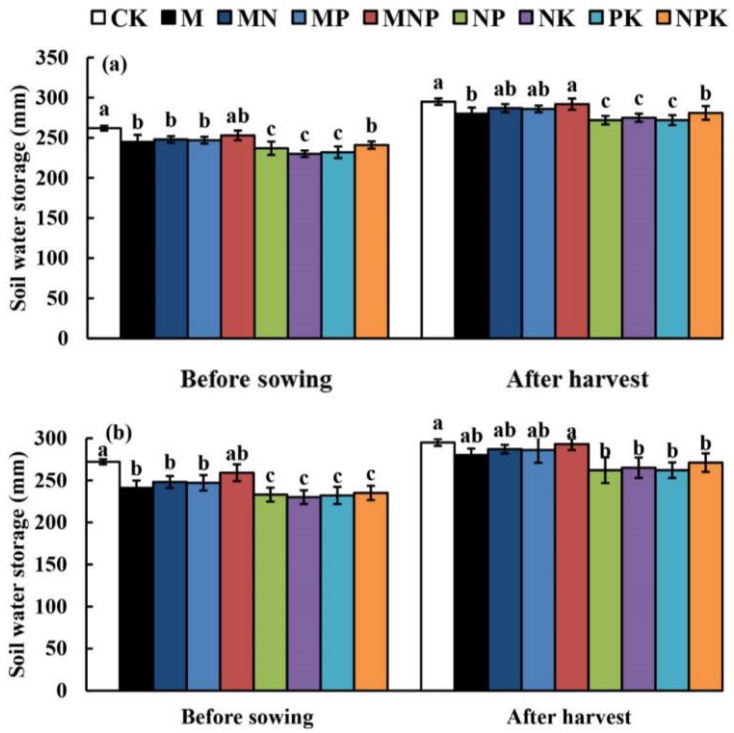

Figure 5. *The soil water storage (mean ± standard error) recorded in 2017 (a) and 2018 (b) for the different fertilization treatments. Values that are in the same column and the same year followed by different letters indicate significant differences at the 5% level (Duncan $P < 0.05$). M stands for organic fertilizer; MN, organic fertilizer combined with nitrogen fertilizer; MP, organic fertilizer combined with phosphate fertilizer; MNP, organic fertilizer combined with nitrogen and phosphate fertilizer; NP, nitrogen fertilizer combined with phosphate fertilizer; NK, nitrogen fertilizer combined with potassium fertilizer; PK, phosphorus combined with potassium fertilizer; NPK, nitrogen combined with phosphate and potassium fertilizer; CK, no-fertilizer control*

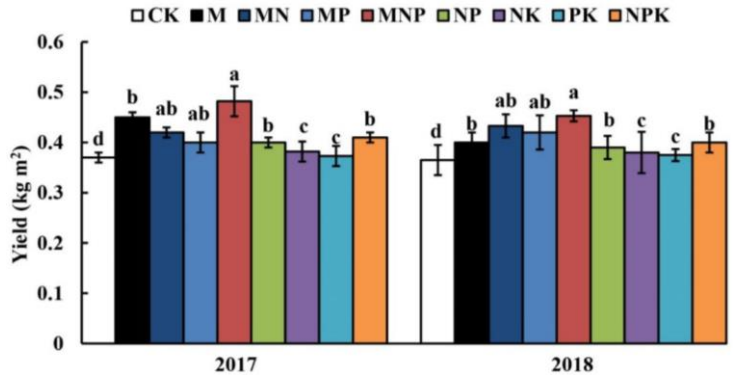

Figure 6. *The crop yield (mean ± standard error) at different fertilization treatments in 2017 and 2018. Values in the same column and the same year followed by different letters indicate significant differences (Duncan $P < 0.05$). M stands for organic fertilizer; MN, organic fertilizer combined with nitrogen fertilizer; MP, organic fertilizer combined with phosphate fertilizer; MNP, organic fertilizer combined with nitrogen and phosphate fertilizer; NP, nitrogen fertilizer combined with phosphate fertilizer; NK, nitrogen fertilizer combined with potassium fertilizer; PK, phosphorus combined with potassium fertilizer; NPK, nitrogen combined with phosphate and potassium fertilizer; CK, no-fertilizer control*

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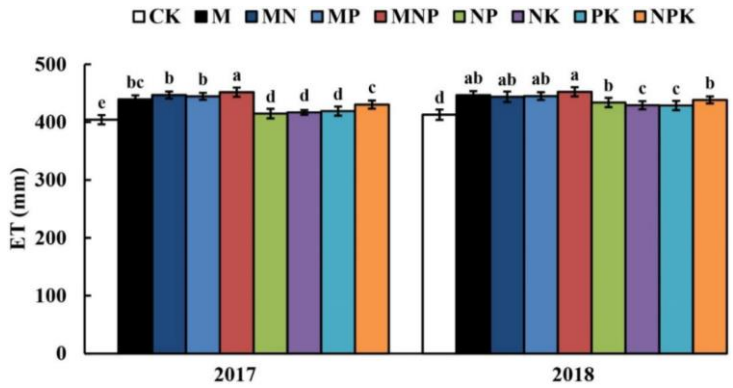

Figure 7. *Water consumption (ET) (mean ± standard error) at different fertilization treatments in 2017 and 2018. Values in the same column and year followed by different letters indicate significant differences (Duncan $P < 0.05$). M, organic fertilizer; MN, organic fertilizer combined with nitrogen fertilizer; MP, organic fertilizer combined with phosphate fertilizer; MNP, organic fertilizer combined with nitrogen and phosphate fertilizer; NP, nitrogen fertilizer combined with phosphate fertilizer; NK, nitrogen fertilizer combined with potassium fertilizer; PK, phosphorus combined with potassium fertilizer; NPK, nitrogen combined with phosphate and potassium fertilizer; CK, no-fertilizer control*

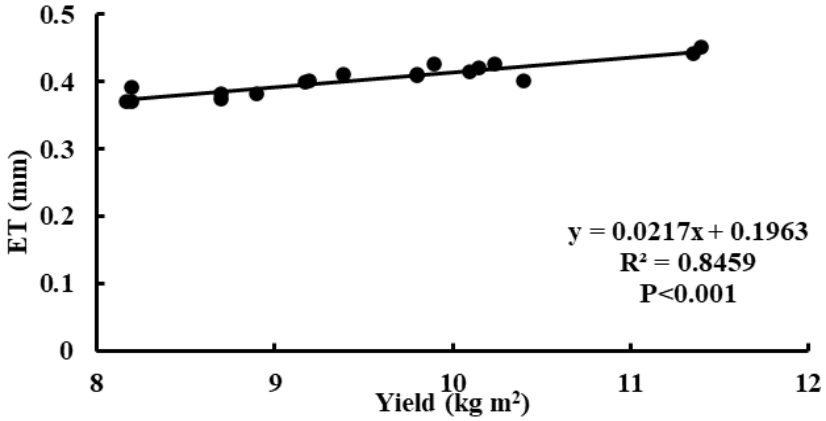

Figure 8. *The correlation between crop yield and soil water consumption (ET)*

Soil water use efficiency (WUE)

Overall, higher WUE was recorded after fertilization. In 2017, in comparison to the CK plot, the WUE of the M, MN, MP, MNP, NP, PK, and NPK plots was greater by 21%, 23%, 26%, 39%, 12%, 6% and 20%, respectively (*Fig. 9*). In 2018, the WUE of the M, MN, MP, MNP, NP, PK, and NPK plots was greater than that of the CK plots by 15%, 24%, 25%, 39%,12%, 8% and 20%, respectively (*Fig. 9*).

Discussion

Improving WUE is a major goal of agricultural development in arid and semi-arid areas. Appropriate fertilizer application can promote crop growth and development, encourage the growth of deep roots, and enhance crop use of deep soil water (Ameen et al., 2019; Tamaki, 1997). We tested whether the application of organic and inorganic

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fertilizers will improve soil WUE and found that fertilization was closely linked to soil water storage and WUE (Samuelson, 2018; Zhang et al., 2019). In 2017 and 2018, soil WUE increased by 6–39%, and 8–39%, respectively, compared with the no-fertilization control, supporting Hypothesis 1. However, the soil water content and water storage showed an overall reduction compared with the no-fertilization treatment; this result is in line with those of the previous studies but contradicts Hypothesis 1.

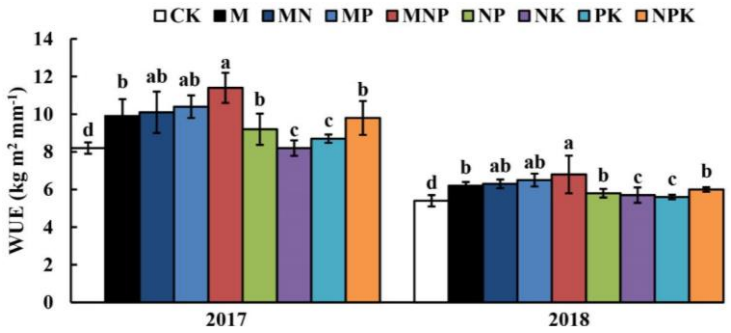

Figure 9. *Soil water use efficiency (WUE) (mean ± standard error) of the different fertilization treatments in 2017 and 2018. Values in the same column and year followed by different letters indicate significant differences (Duncan $P < 0.05$). M, organic fertilizer; MN, organic fertilizer combined with nitrogen fertilizer; MP, organic fertilizer combined with phosphate fertilizer; MNP, organic fertilizer combined with nitrogen and phosphate fertilizer; NP, nitrogen fertilizer combined with phosphate fertilizer; NK, nitrogen fertilizer combined with potassium fertilizer; PK, phosphorus combined with potassium fertilizer; NPK, nitrogen combined with phosphate and potassium fertilizer; CK, no-fertilizer control*

Multiple mechanisms and factors are at play in the effect of fertilization on crop water use. Previous studies have found that soil WUE follows the same changing characteristics as the leaf area index (De et al., 2013; Ibrahim et al., 2015; Krbel, 2012). Firstly, appropriate fertilization treatment can significantly increase the total amount of crop roots, enhance root vitality, and expand the space available to roots to absorb soil moisture and nutrients; it can also increase leaf area, boost the photosynthetic rate, reduce evaporation, and achieve maximum efficiency in the utilization of limited moisture in semi-arid areas (Wang, 2013; Liu et al., 2010). It is because fertilization promotes the vigorous growth of crops that the water consumption of ground evaporation is reduced (Sheoran et al., 2017). Therefore, after fertilization, the rapid growth of the crops increases soil water consumption and decreases soil water storage and content, while soil WUE increases. Secondly, the growth of the vegetation and the expansion of plant stems and leaves after fertilization results in a stronger plant cover on the ground surface, slowing down the water consumption in the form of surface evaporation, which also increases the plant water use. In addition, the abundant rainfall in the late filling stage (*Fig. 2*) is likely to have enabled the plants to fully exploit the available soil nutrients. This explains why the water consumption of the fertilized plots was significantly greater than that of the unfertilized control.

The ultimate goal of increasing crop WUE is an enhanced yield, which can be achieved when the right balance is struck between the source and sink conditions (Zhang et al., 2019; Amoah et al., 2012). Fertilization is an important agronomic measure for increasing source characteristics such as dry matter accumulation and sink characteristics such as crop yield (Chaab, 2011; Meng et al., 2012). Our results show

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that higher crop yield can be achieved by fertilization. In this study, the yield of plants treated with additional organic and inorganic fertilizers was significantly higher than that of the no-fertilization control. This is in line with the results of previous studies showing an overall increase in dry matter accumulation after fertilization.

Fertilization can enhance crop yield via a variety of pathways. It is known that low soil nutrient availability limit growth (Kurwakumire et al., 2014). Fertilization increases the amount of available nutrients in the soil, boosting the absorption and utilization of soil nutrients by plants. In addition, chlorophyll is essential for photosynthesis, and its content in leaves reflects the level of photosynthesis (Roland et al., 2012). Nutrients are required for chlorophyll production (Wang and Jin, 2007). Appropriate application of fertilizers can increase chlorophyll content in plant tissues, as demonstrated previously (Xu et al., 2020b); this promotes the growth of above-ground parts, thereby increasing biomass accumulation. Some studies have shown that fertilization can achieve higher yields through simultaneously improving cooperatively improving the indicators of crop dry matter accumulation, dry matter transport capacity of vegetative organs, and photosynthetic compound accumulation (Eisvand, 2018).

It is likely that both nutrient availability and water consumption are involved in the enhancement of crop yield observed after the application of fertilizers. This is consistent with our finding of a significant positive correlation between crop yield and soil ET, suggesting that the two occur synergistically after fertilization. Studies have shown that nutrients can increase water uptake, transport, and overall content in plants, thereby increasing yields (Tamaki, 1997). A 2006 study reported that the addition of organic and inorganic fertilizers increased the soil nutrient supply and the soil WUE during the crop growth period, benefitting plant growth and development, enhancing photosynthesis, and thus increasing plant biomass accumulation (Li et al., 2018). A positive feedback loop mechanism may be involved: it has been shown that increases in biomass promote transpiration, which indirectly promotes root growth and photosynthesis, thereby further increasing crop yields. Drought and nutrient deficiencies are typical characteristics of the Loess plateau and can have significant negative effects on crop yields (Amoah et al., 2012). Our study provided a preliminary look at the effect of different types of fertilization on crop production and WUE. There is scope for future studies to explore the complex mechanisms that drive the yield changes observed under different fertilization modes.

In recent years, owing to many countries' emphasis on the protection of the ecological environment and the quality and safety of agricultural products, more and more scientists have focused their research on the rational use of organic fertilizers (Sadeghzadeh and Rengel, 2011). We found that the use of organic fertilizers improved soil water efficiency and crop growth more than the use of inorganic fertilizers, which supported Hypothesis 2. One potential reason for this result is that organic fertilizers can improve the functional diversity of microbial communities in crop rhizosphere soils (Cheng et al., 2015; Wang, 2009) and are therefore superior to inorganic fertilizers in terms of their effect on plant nutrient absorption and transformation. For instance, Wang et al. (2012) found that the combined application of NPK fertilizers and organic fertilizer can make crops have improved yields and results in higher yields than NPK fertilizers alone. In addition, some studies have found that the nutrient release of organic fertilizer is slow and thus more helpful for subsequent improvement of soil fertility (Ren et al., 2019). Our results showed that the treatments that increased production and improved water use the most were the

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combination of organic fertilizer with N and P fertilizer in 2017 and 2018. Further experiments are needed to quantify the economic benefits of organic fertilizer and elucidate its relative effects on different crop species. Additional factors such as ecological and environmental protection, sustainable soil use, and economic output benefits should be comprehensively considered when making definitive recommendations on the application of organic and inorganic fertilizers.

Conclusion

Our study sheds light onto the effect of organic and inorganic fertilizers on soil water use and agricultural production in rain-fed agricultural areas on the Loess Plateau (China). We examined the variations in soil WUE and crop yield under nine fertilization treatments. Our results revealed that fertilization increases soil WUE and crop yield but decreases soil water storage. The treatments combining organic fertilizer with N and P fertilizer had the strongest positive effect on soil WUE and crop yield. Based on our results, we therefore recommend the use of organic fertilizers in arid and semi-arid areas as a method for improving soil WUE and crop yield. Further studies should conduct similar analyses over a longer study period and with different crop species and attempt to elucidate the mechanisms at play in the effect of fertilization on crop yield.

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SALT TOXICITY IN A NATURAL HABITAT INDUCES STRUCTURAL AND FUNCTIONAL MODIFICATIONS AND MODULATE METABOLISM IN BERMUDA GRASS (*CYNODON DACTYLON* [L.] PERS.) ECOTYPES

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Abstract. We investigated salt toxicity effects and related physiological as well as anatomical changes for adaptation in *Cynodon dactylon*. Eleven ecotypes from different areas of Pakistan were examined for their survival capacity in a controlled environment. The experiment was designed in a two factor Completely Randomized Design (ecotypes and 4 salt levels 0, 100, 200, 300 m*M* NaCl). Differently adaptive populations displayed specific structural and functional adaptations with respect to photosynthesis and photosynthetic pigments to withstand salinity. The ecotypes from saline and waterlogged areas exhibited higher Photosynthetic rate to same level of increase in salinity level. Transpiration rate of all ecotypes showed variations with different salinity levels and stomatal conductance increased in all ecotypes with increasing salinity. Similarly, all ecotypes responded significantly as far as chlorophyll contents were concerned. Most ecotypes consistently showed high leaf blade thickness and sclerenchyma thickness with elevated salinity except for plants collected from saline, waterlogged and salt affected wasteland. Different salt levels decreased the metaxylem cell area while phloem area increased in the ecotypes correspondingly. *Cynodon* ecotypes possessed low abaxial stomatal density at 300 m*M* salinity. Overall, physiological and anatomical adaptations to saline environments appeared very specific in grasses and supported life cycle under salt stress.

Keywords: *anatomical changes, Bermuda grass, photosynthetic pigments, salinity, sclerification*

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Introduction

Plants, as sessile organisms, are often subjected to various environmental stresses i.e. biotic and abiotic causing massive structural and functional changes (Khalid et al., 2020; Mickelbart et al., 2015; Pandey et al., 2017). Growth and all other plant activities depend on photosynthesis, photosynthetic pigments and photosynthetic machinery (Ashraf and Harris, 2013; Cardona et al., 2018). But these cellular systems are damaged by abiotic and biotic stresses (Gururani et al., 2015; Mohanty et al., 2006) especially the degradation of chlorophyll pigmentation due to alteration in gene expression (Dutta et al., 2009; Noman and Aqeel, 2017; Sharma et al., 2019) that limits photosynthesis but also reduce plant growth and yield (Ali and Ashraf, 2011; Arshad et al., 2016; Khalid et al., 2020, 2018; Thuynsma et al., 2016). The salinization of irrigated lands in arid/semi-arid areas is one of the major causes of low crop production in many parts of the world (Araus et al., 2010; Hussain et al., 2019).

Cynodon dactylon (L.) Pers. (Bermuda grass, locally known as Khabbal grass) is distributed abundantly in tropical and warm temperate areas (Shi et al., 2012) throughout the world between 45°N and 45°S (Ling et al., 2015). It tolerates high temperatures, drought, and salinity (Chen et al., 2015; Hu et al., 2015). It has C_4 photosynthesis pathway (Edwards et al., 2004) which is a complex adaptation evolved from C_3 photosynthesis (Carmo-Silva et al., 2008). The C_4 adaptation is extremely successful in the monocotyledonous families i.e., Poaceae and Cyperaceae (Besnard et al., 2009; Sage, 2004). Salinity tolerance, especially in grasses, depends on plant morpho-physiological features (Bahrani et al., 2010; Volkov and Beilby, 2017). In different *C. dactylon* ecotypes salinity ranges from 6 to 10 dS m^{-1}, and the different species are classified as semi-tolerant to tolerant to drought stress (Uddin et al., 2012).

Physiological adaptations are important criteria for salt tolerance selection (Ashraf, 2004; Gupta and Huang, 2014; Hernández, 2019), but anatomical modifications under high salinities are also crucial (Naz et al., 2014; Noman et al., 2017, 2012; Ye et al., 2015). Photosynthesis is affected by leaf anatomy and CO_2 exchange. Structural features of the leaf contributing to the maintenance of the high CO_2 concentration in the chloroplast stroma may have been selected during evolution (Terashima et al., 2011). Stomata and stomatal activities are much important for the photosynthetic activities and reduction in net photosynthetic rate, stomatal conductance, intercellular CO_2 concentration, quantum efficiency of PSII, and non-photochemical quenching in *C. dactylon* is directly linked to non-stomatal activities (Bhuiyan et al., 2015).

A typical respond to salt stress is the development of thick leaves due to the thickening of the cortex and mesophyll cells (Boughalleb et al., 2009; Geldner, 2013). The structural alterations in vascular bundles, the nature of lignified tissues are all thought to be very helpful in allowing the plant to fight against various environmental stresses (Noman et al., 2014; Qaderi et al., 2019; Zwieniecki et al., 2003). The emptying of the vacuoles of large bulliform cells induces leaf curling and the rolling of leaves that can be distinctively considered as an important adaptation against salinity-induced physiological drought stress for the water conservation and lowering the rate of transpiration (Alvarez et al., 2008; Balsamo et al., 2006; Hameed et al., 2013). Besides, the increased density and size of trichomes may also provide the additional benefit of enabling a plant to withstand environmental stresses like drought and salinity. Increasing the density of micro-hairs under conditions of increased salt may be one of the most effective mechanisms for salinity tolerance in Bermuda grass (Hu et al., 2015).

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It was hypothesized that differently adaptive populations might have some specific structural and functional adaptations with respect to photosynthesis and photosynthetic pigment to withstand salt stress condition. The investigation was focused on the leaf anatomical changes as dermal tissues (epidermis, stomatal density and area, trichome length, and leaf hairiness), vascular tissues (large metaxylem vessels), and mechanical tissues (sclerenchyma) that influence photosynthesis and changes in chlorophyll pigments and gas exchange characteristics under salt stress conditions.

Materials and methods

Selected ecotypes of Cynodon dactylon

Various ecotypes of *C. dactylon* were selected from the Punjab, Pakistan from different ecological regions as reported by Tufail et al. (2017). The ecotypes (DF-SD) and (S-HS) were selected from saline arid regions. Ecotypes (KL-HS), (UL-HS), and (KKL-S) were collected from saline lakes in the Salt Range. Ecotypes (S-SW) and (T-SW) were from saline waterlogged areas. Two ecotypes were from salt-affected wasteland, (PA-HS) and (PA-RF). Two ecotypes were from non-saline well irrigated habitats, (MG-RB) and (BG-NS) (*Table 1; Fig. 1*).

Table 1. *Various ecotypes of Cynodon dactylon L. from the Punjab, Pakistan*

Sr. No.	Sampling sites	Ecotypes	Latitude (N)	Longitude (E)
1	Derawar Fort-Saline Desert	DF-SD	28° 75′	71° 33′
2	Muzaffargarh-River Bank	MG-RB	30° 08′	71° 19′
3	Khabbeki Lake-Hyper Saline	KL-HS	32° 29′	72° 34′
4	Ucchali Lake-Hyper Saline	UL-HS	32° 32′	72° 11′
5	Kalar Kahar Lake-Saline	KKL-S	32° 47′	72° 42′
6	Treemu-Saline Wetland	T-SW	31° 31′	72° 33′
7	Sahianwala-Saline Wetland	S-SW	31° 38′	73° 14′
8	Sahianwala-Hyper Saline	S-HS	32° 13′	73° 29′
9	Pakka Anna Hyper Saline	PA-HS	31° 14′	72° 48′
10	Pakka Anna Reclaimed Field	PA-RF	31° 98′	72° 99′
11	Botanic Garden-Non Saline	BG-NS	31° 36′	73° 35′

Cynodon dactylon culture and salt treatment

Naturally adapted populations of *C. dactylon* from several regions of the Punjab, Pakistan were established in the Botanic Garden, University of Agriculture, Faisalabad, in non-saline soil. The plants were kept under full sunlight and irrigated daily up to their establishment in Faisalabad environment. Randomly selected ramets from each ecotype of equal size (with two mature up tillers) were detached, fixed and grown in aerated hydroponics using half-strength Hoagland's nutrient solution (Hoagland and Arnon, 1950) for eight weeks. Air pumps were used for aeration of hydroponic culture system for about 12 h daily. Ten ramets from each population were selected for each replication and fixed in the pores of thermophores (mineral fiber) sheets for each experimental unit, which was placed on hydroponic culture solution.

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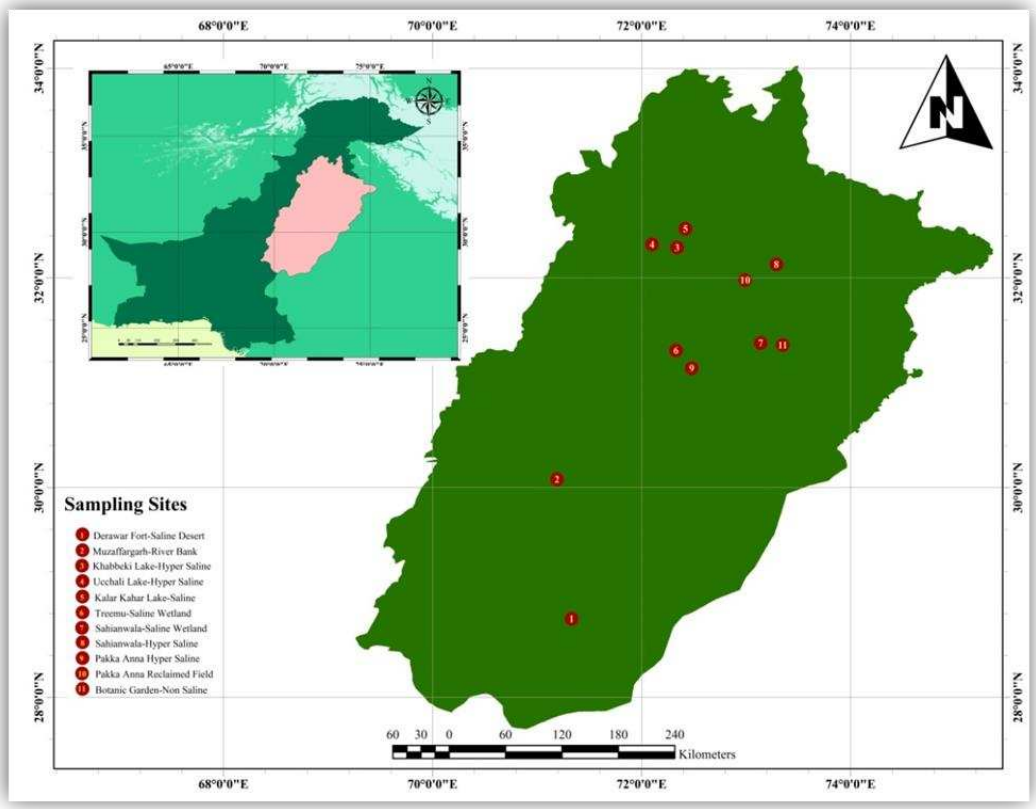

Figure 1. *Map of the Punjab, Pakistan showing ecotypes of Cynodon dactylon from different sampling sites*

Four salinity levels were maintained during the experiment viz. control (no salinity treatment), 100, 200 and 300 mM of NaCl salinity in solution culture up to flowering stage. After 8-weeks, plants were washed thoroughly and used for studying the various anatomical characteristics.

The ecotypes were subjected to salt stress after their establishment in the Faisalabad environment. The experiment for salt stress was arranged in two factor-factorial (ecotypes and salt levels) completely randomized design with ten replications and 0 (control), 100, 200, and 300 mM NaCl levels in aqueous culture media containing half-strength Hoagland's medium.

Leaf anatomical studies

Transvers sections of the newly grown leaves from three plants as replicates were made by the free hand sectioning by serial dehydrations in ethanol using double standard staining (Safranin and fast green) technique. Plant material was preserved in the Formalin Acetic Alcohol (FAA) fixative for 48 h and subsequently transferred to acetic alcohol (v/v acetic acid 25%, and ethanol 75%) solution for long term storage.

Photographs were taken by a camera-equipped light microscope (Meiji Techno: MT4300H USA). Parameters were recorded as: Leaf blade thickness (µm), Epidermal cell area (µm^2), Sclerenchymatous thickness (µm), Bulliform cell area (µm^2), Vascular Bundle area (µm^2), Metaxylem area (µm^2), Phloem area (µm^2), Trichome length (µm) and density, Stomatal area (µm^2) and density.

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Photosynthetic parameters

Measurements of net CO_2 Assimilation Rate (*A*), Transpiration (*E*) and Stomatal Conductance, Sub Stomatal CO_2 concentration and Water Use Efficiency (calculated as WUE = A/E) were recorded by using LCA-4 ADC portable infrared gas analyzer (Analytical Development Company, Hoddesdon, England). The gas exchange measurements were performed *in situ* from 10:30 a.m. to 12:30 p.m. with specific specifications/adjustments as reported by Schiavon et al. (2016) and Tamayo et al. (2001).

Photosynthetic pigments

The chlorophyll a, b and carotenoids were determined according to the method of Arnon (1949).

Statistical analysis

The data were subjected using analysis of variance in completely randomized design. Redundancy analysis (RDA) was performed using Conoco 4.5 computer software.

Results

Photosynthetic attributes

Gas exchange attributes

The net CO_2 assimilation rate (*A*) decreased in the ecotypes of *C. dactylon* KL-HS, UL-HS, KKL-S, S-HS, PA-RF and BG-NS (*Table 1*) with increase in the concentration of salinity. The ecotypes from T-SW and S-SW responded the best to the maximum salinity level with higher net CO_2 assimilation rates (*Fig. 2*).

The transpiration rate (*E*) of ecotypes of *C. dactylon* from S-HS, PA-RF, and BG-NS decreased as the salt stress level increased (*Fig. 2*). Ecotypes from DF-SD, KKL-S, T-SW, S-SW showed slightly decreased transpiration rate at salinity levels (100 and 200 mM NaCl) but increased at 300 mM NaCl. The transpiration rate for the PA-HS ecotype peaked at 100 mM NaCl but decreased at higher levels of salt stress (*Fig. 2*).

The stomatal conductance of ecotypes from the DF-SD, M-RB, KL-HS, KKL-S, and BG-NS increased as the salinity level increased (*Fig. 2*). There was no significant variation in sub stomatal CO_2 concentration, for many of the experimental ecotypes, except from DF-SD, KL-HS, UL-HS and PA-RF exhibited reduced sub stomatal CO_2 concentrations at the highest experimental salinity level (300 mM NaCl) (*Fig. 2*).

Ecotypes from the DF-SD, M-RB, KL-HS, UL-HS, and BG-NS exhibited a quadratic response of water use efficiency to salinity. S-SW, S-HS, PA-HS exhibited quadratic responses and exhibited the highest levels of water use efficiency at 300 mM NaCl (*Fig. 2*).

Photosynthetic pigments

DF-SD, S-HS, and BG-NS exhibited slightly increased chlorophyll a levels at the 100 and 200 mM NaCl levels, and exhibited maximum reduction in the pigment at 300 mM NaCl (*Fig. 2*).

The chlorophyll b response was, for many of the experimental ecotypes, higher at the 100 or 200 mM NaCl levels. M-RB exhibited increasing chlorophyll b levels as salinity increased. KL-HS, UL-HS, and S-SW exhibited peak levels of chlorophyll b at 100 mM

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NaCl (*Fig. 2*). DF-SD, PA-RF. T-SW, S-HS, PA-HS, and BG-NS exhibited maxima at the 200 mM NaCl level (*Fig. 2*).

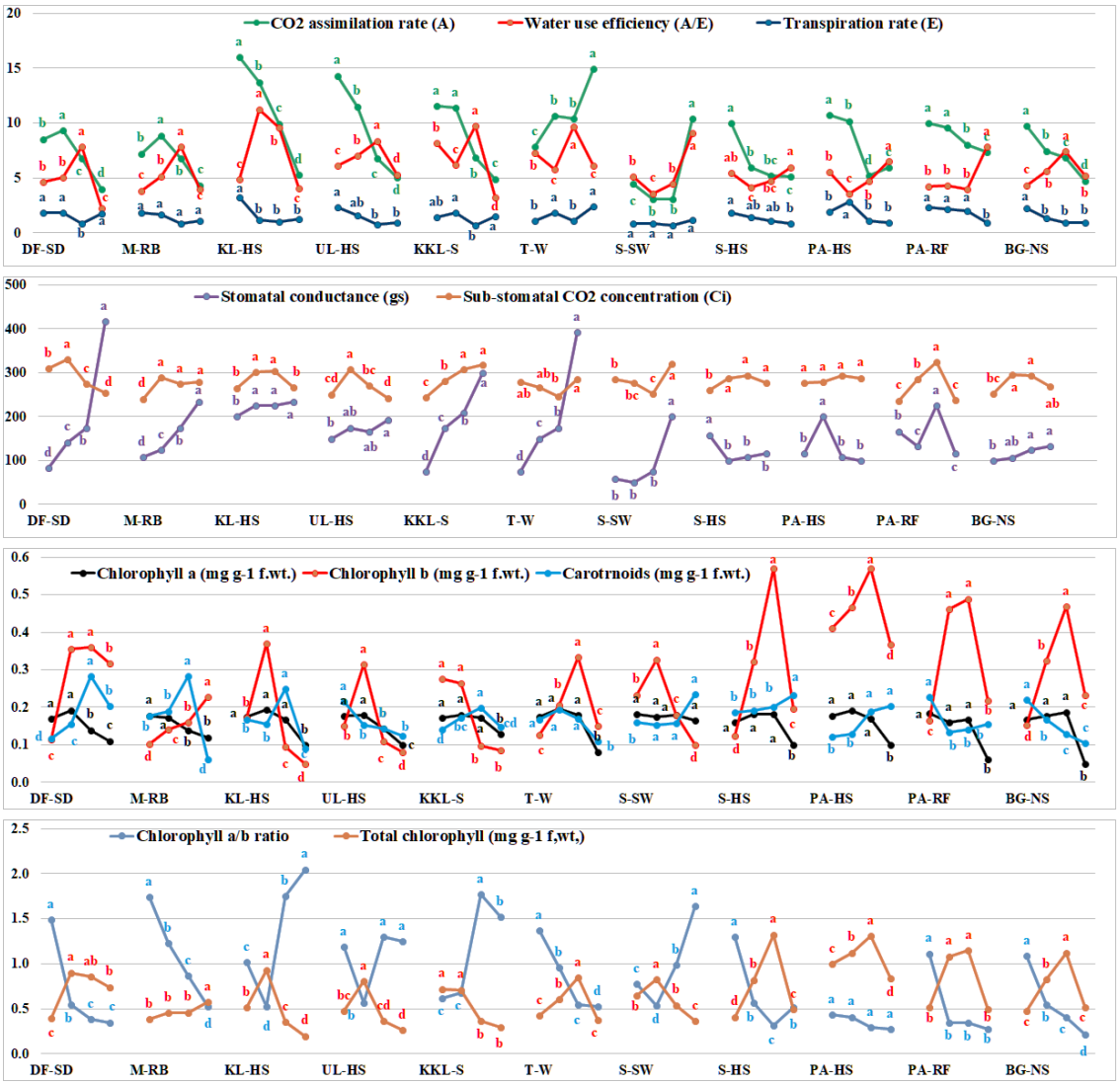

Figure 2. *Series plots of gas exchange characteristics and photosynthetic pigments under four salt levels (0, 100, 200, 300 mM) NaCl. DF-SD (Derawar Fort-Saline Desert), MG-RB (Muzaffargarh-River Bank), KL-HS (Khabbeki Lake-Hyper Saline), UL-HS (Ucchali Lake-Hyper Saline), KKL-S (Kalar Kahar Lake-Saline), T-SW (Treemu-Saline Wetland), S-SW (Sahianwala-Saline Wetland), S-HS (Sahianwala-Hyper Saline), PA-HS (Pakka Anna Hyper Saline), PA-RF (Pakka Anna Reclaimed Field) and BG-NS (Botanic Garden-Non Saline)*

The amount of leaf carotenoid pigments indicated different patterns of response in the experimental ecotypes at the experimental levels of salinity. While ecotypes from S-SW, S-HS and PA-HS exhibited increasing leaf carotenoid compounds due to salinity. DF-SD, M-RB, and KL-HS responded with higher amounts of carotenoids at 200 mM NaCl (*Fig. 2*).

Chlorophyll a and b ratio also decreased with increase in salinity level in ecotypes of *C. dactylon* from DF-SD, M-RB, T-SW, PA-HS, PA-RF and BG-NS (*Fig. 2*). Furthermore KL-HS, UL-HS and S-SW exhibited higher amounts of total chlorophyll at

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100 mM NaCl. Ecotypes from T-SW, S-HS, PA-HS, PA-RF and BG-NS exhibited higher amounts of total chlorophyll at 200 mM NaCl (*Fig. 2*).

Leaf anatomy

Many of the ecotypes of *C. dactylon* exhibited consistently increased leaf blade thickness with increased salt stress, except ecotypes from S-SW, S-HS, and PA-HS, which exhibited gradual increases of leaf blade thickness up to 200 mM NaCl stress but reduced leaf thickness at the maximum experimental salinity level. In the ecotypes from BG-NS, the maximum leaf blade thickness was observed at 100 mM NaCl, and increasing levels of salinity reduced leaf blade thickness (*Fig. 3*).

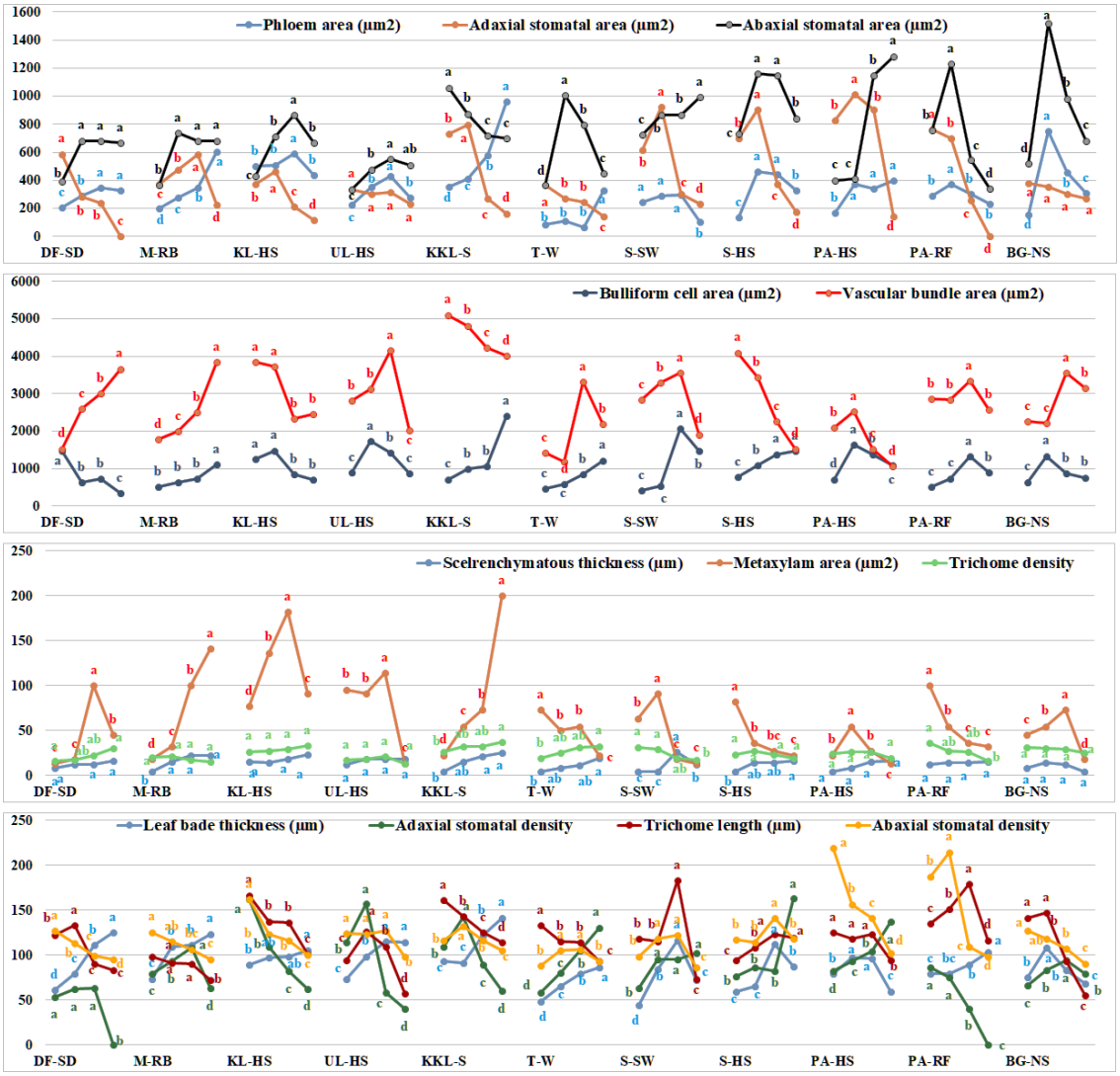

Figure 3. *Series plots of leaf anatomical attributes under four salt levels (0, 100, 200, 300 mM) NaCl. DF-SD (Derawar Fort-Saline Desert), MG-RB (Muzaffargarh-River Bank), KL-HS (Khabbeki Lake-Hyper Saline), UL-HS (Ucchali Lake-Hyper Saline), KKL-S (Kalar Kahar Lake-Saline), T-SW (Treemu-Saline Wetland), S-SW (Sahianwala-Saline Wetland), S-HS (Sahianwala-Hyper Saline), PA-HS (Pakka Anna Hyper Saline), PA-RF (Pakka Anna Reclaimed Field) and BG-NS (Botanic Garden-Non Saline)*

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Sclerenchyma thickness was consistently high where plants were subjected to 300 mM NaCl, except in the ecotypes from BG-NS, where the maximum sclerenchyma thickness was observed at 100 mM NaCl. With the increase of salinity a reduction of sclerenchyma thickness was observed, but S-SW exhibited a gradual increase of sclerenchyma thickness up to 200 mM NaCl stress, but reduced sclerenchyma thickness at 300 mM NaCl (*Fig. 3*).

The ecotypes of *C. dactylon* from M-RB, KKL-S, T-SW, and S-HS exhibited that bulliform cell area increased as salinity increased while bulliform cell area of DF-SD were described by a third order polynomial or sinusoid (*Fig. 3*). Ecotypes from KL-HS, UL-HS, PA-HS and BG-NS showed increased bulliform cell area at 100 mM NaCl stress, but above the 100 mM NaCl level, bulliform cell area decreased with increase in salinity. Furthermore, ecotypes from S-SW and PA-RF exhibited a quadratic response of bulliform cell area that peaked at 200 mM NaCl and decreased at the maximum experimental salinity level (*Fig. 3*).

Ecotypes from DF-SD and M-RB exhibited Vascular Bundle (VB) area that increased as salinity increased, while a negative linear component described the KL-HS, KKL-S and S-HS VB area in response to salinity. Ecotypes from UL-HS, T-SW, S-SW, PA-RF, and BG-NS exhibited marked induction of VB area at 200 mM NaCl stress, but reduced areas at 300 mM NaCl. Ecotypes from PA-HS exhibiteda quadratic response for VB area to salt stress that peaked at 100 mM NaCl (*Figs. 3, 4*).

Metaxylem cell area decreased in response to salinity, in the ecotypes from T-SW, S-HS and PA-RF, while M-RB and KKL-S exhibited increasing metaxylem area with the increasing salinity. The metaxylem area of ecotypes DF-SD, KL-HS, UL-HS, and BG-NS peaked at 200 mM NaCl stress. The metaxylem area of ecotypes from S-SW and PA-HS peaked at 100 mM NaCl (*Figs. 3, 4*).

Ecotypes from M-RB, KKL-S, T-SW, and PA-HS exhibited higher phloem areas at the highest experimental salinity level (300 mM NaCl). The phloem area of ecotypes from PA-RF and BG-NS peaked at 100 mM NaCl (*Figs. 3, 4).*

Most of the ecotypes of *C. dactylon* showed reduced stomatal density at the 300 mM NaCl level, (*Figs. 3, 5*). Stomatal cell area decreased in response to salinity in ecotypes from DF-SD, M-RB, KL-HS, and PA-HS. S-HS exhibited peak induction of stomatal density at 200 mM NaCl, and T-SW and PA-RF exhibited peak stomatal density at 100 mM NaCl stress (*Figs. 3, 5*).

Trichome length decreased in response to salinity in the ecotypes from M-RB, KL-HS, KKL-S, and T-SW (*Figs. 3, 6*). Trichome density increased in response to salinity in the ecotypes from DF-SD, KL-HS, KKL-S, and T-SW but decreased due to salinity in the ecotypes from M-RB, S-SW, PA-RF and BG-NS (*Figs. 3, 6*).

Redundancy (RDA) ordination

RDA ordination biplot for leaf anatomy

The RDA ordination biplot (*Fig. 7*) showed DF-SD showed the association with BCA at control and with Trichome Length (TRL), Abaxial Stomatal Density (ASD), Vascular Bundle Area (VBA), Trichome Density (TRD), Adaxial Stomatal Area (ADA), Sclerenchymatous Thickness (SCT), Leaf Blade Thickness (LBT), Adaxial Stomatal Density (ADD) and Metaxylem Area (MXA) at minimum salinity level 100 mM NaCl while from M-RB showed the same association up to maximum salinity level 300 mM NaCl and with Phloem area (PHA) at moderate salinity level

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200 mM. Ecotypes from KL-HS showed association with PHA up to maximum salinity level 300 mM, however from UL-HS showed association with TRL, LBT, SCT and TRD at moderate salinity level 200 mM and with VBA, MXA at maximum salinity level 300 mM. Ecotype from KKL-S showed association with VBA, MXA at 0, 200 mM, with TRL, ASD, VBA, TRD, ADA, SCT, LBT, ADD and MXA at minimum salinity level 100 mM. Ecotype from T-SW showed association with ADA at control (*Fig. 7*).

Figure 4. *Leaf blade transverse sections of Cynodon dactylon ecotypes collected from the Punjab, Pakistan*

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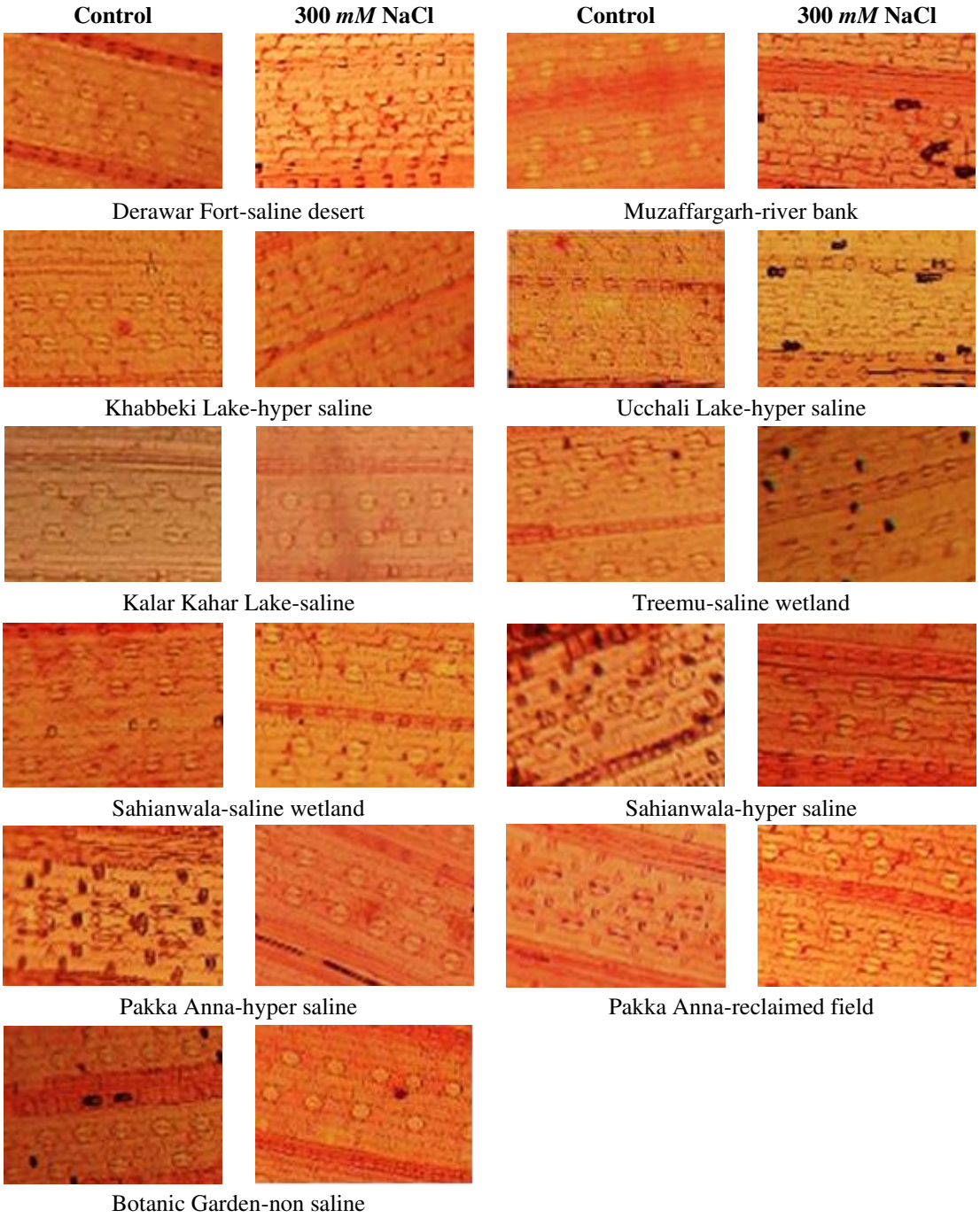

Figure 5. *Surface view of leaf epidermis of Cynodon dactylon ecotypes collected from the Punjab, Pakistan*

Ecotypes from S-SW showed association with ASD at control 0, 100 mM NaCl, with TRL at minimum salinity level 100 mM, with BCA at maximum salinity level 300 mM, however from S-HS showed association with VBA at control 0 mM NaCl BCA while up to maximum salinity level 300 mM showed association with BCA. Ecotypes from PA-HS showed association with ADA and ASA up to maximum salinity level 300 mM while PA-RF showed association with PAR at 0 mM salinity and with VBA, MXA and ADA at maximum salinity level 300 mM salinity. Ecotypes from BG-NS showed

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association with PHA, ADD, LBT and SCT at control 0 mM NaCl and with PHA up to maximum 300 mM salinity level (*Fig. 7*).

Figure 6. *Leaf margins of Cynodon dactylon ecotypes collected from the Punjab, Pakistan*

RDA ordination biplot for photosynthetic characteristics

RDA ordination biplot (*Fig. 8*) showed the effect of photosynthetic characteristics on the ecotypes of *C. dactylon*. The ecotypes from DF-SD and S-SW showed the

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association with Chlorophyll-a (Chl-a), Chlorophyll-b (Chl-b), Caroteniod (CAR), Total Chlorophyll (Chl-T) and Transpiration rate (*E*) at control while the ecotypes from DF-SD and KL-HS with *gs* at moderate salinity level 200 mM. Ecotypes from M-RB and UL-HS showed no noticeable association along salinity gradient. Ecotypes from KKL-S showed association with *A* at 0 mM NaCl, with *gs* at minimum salinity level 100 mM however from T-SW showed association with *A/E* and *Ci* at control. Ecotypes from S-SW showed association with *A/E* at minimum 100 mM salinity level and with *A* and *Ci* at moderate salinity level 200 mM. However from S-HS showed association with *gs* at control 0 mM NaCl, with *A* and *Ci* at minimum salinity level 100 mM NaCl, with Chl-T, *E*, Chl-a and Chl-b at moderate salinity level 200 mM NaCl while at maximum salinity level 300 mM showed association with *A/E* (*Fig. 8*).

Ecotypes from PA-HS showed association with *A* at control 0 mM NaCl, with Chl-T, *E*, Chl-a and Chl-b at moderate salinity level 200 mM NaCl and with Chl-T, Chl-a Chl-b, CAR, *A, E* and chlorophyll a/b at maximum salinity level 300 mM NaCl, while PA-RF showed association with *A/E* at maximum salinity level 300 mM NaCl, however from BG-NS showed association with *Ci* at control 0 mM NaCl, with *A, Ci* at minimum salinity level 100 mM NaCl (*Fig. 8*).

Discussion

Across the world, increasing soil salinity is a serious threat for the plants (Habib et al., 2016) with respect to modifications in their physiological, biochemical and anatomical attributes. Ecotypes are the species that have long term genetic variations among their populations (Sridevi et al., 2012) and showed structural, functional and geographical variations that leads to genetic variance (Johnson, 2010; Phillips et al., 2015). Morphological, anatomical, physiological and biochemical adaptive markers are of prime importance to study the adaptive mechanism in differentially adapted ecotypes against abiotic and biotic stress (Hameed et al., 2011; Naz et al., 2009). Therefore, ecotypes of *Cynodon dactylon* were collected from various highly saline, moderately saline, marshy, waterlogged and non-saline areas to evaluate adaptation strength and extent of involvement of these adaptations in plant survival.

C. dactylon is also considered as halophytic grass due to strong resistance against salinity (Marcum et al., 2005; Pessarakli, 2015). In view of stresses *"escape"* or *"tolerance"* is the most prevailing phenomenon in adaptation (Sunkar, 2010; Witcombe et al., 2008). Salt tolerant grasses can minimize the detrimental effects of high salinity by showing a series of structural and functional modifications in anatomical and physiological characteristics of plants (Zhou et al., 2015) similar to our findings e.g. as lignification around the vascular region (Alam et al., 2015), increased thickness (succulence) of epidermis, midribs and cortical parenchyma (Vijayan et al., 2011), increased sclerenchyma in the leaves (Noor et al., 2015), reduced leaf area (Monteverdi et al., 2008), greater density of salt secreting glands and hairs on the leaf surface (Farooq et al., 2015), enlarged bulliform cells that help in leaf rolling to avoid water loss (Alvarez et al., 2008), increased stomatal density with decreased stomatal size (Hameed et al., 2013).

Our findings of adaptaive responses get advocacy from the subrized exodermis and endodermis, thick sclerenchymatous tissues around vascular bundle, large mesophyll cells and large cortical aerenchyma cells that are reportedly developed against stress (Noman et al., 2014; Noman et al., 2012; Yang et al., 2011). Tolerance level of *C. dactylon* to environmental stresses varies from highly sensitive to tolerant grass. Increased succulence

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for water conservation and toxic ion accumulation, excretory structures like micro-hairs for toxic ion exclusion (Colmer and Flowers, 2008), sclerification for desiccation tolerance and minimizing water loss, and wider metaxylem vessels for efficient water and nutrient conduction (Ali et al., 2009; Naz et al., 2014; Noman et al., 2017).

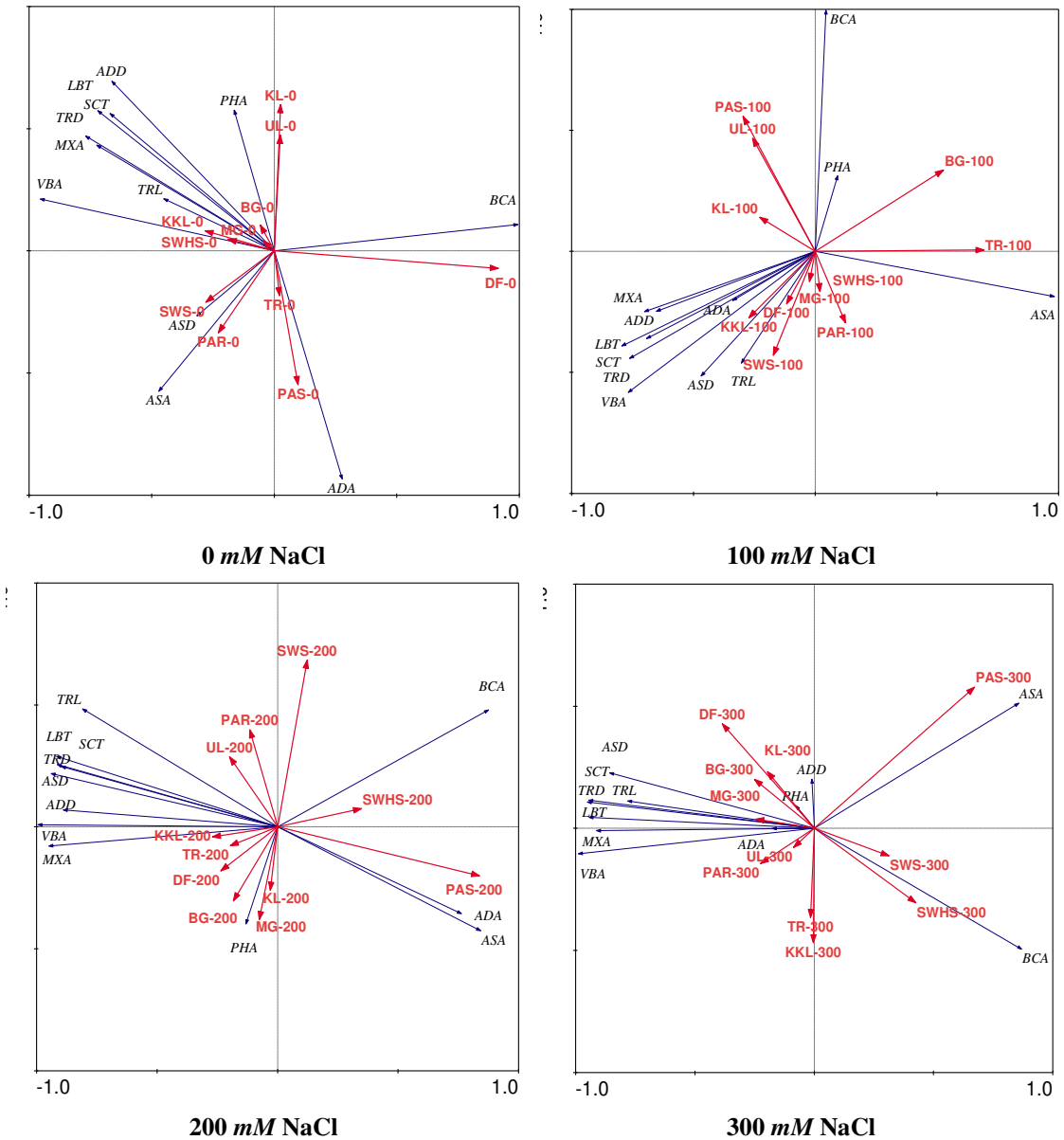

Figure 7. *RDA ordination biplot showing the effect of leaf anatomical characteristics on the ecotypes of Cynodon dactylon from various region of Punjab. DF (Derawar Fort), MG (Muzaffargarh), KL (Khabbeki Lake), UL (Ucchali Lake), KKL (Kalar Kahar Lake), T-SW (Treemu-Saline Wetland), SWS (Sahianwala-Saline Wetland), SWHS (Sahianwala-Hyper Saline), PAS (Pakka Anna Saline), PAR (Pakka Anna Reclaimed) and BG (Botanic Garden). Leaf anatomical characteristics are abbreviated as LBT: Leaf blade thickness, SCT: Sclerenchymatous thickness, BCA: Bulliform cell area, VBA: Vascular bundle area, MXA: Metaxylem area, PHA: Phloem area, TRL: Trichome length, TRD: Trichome density, ASA: Abaxial stomatal area, ASD: Abaxial stomatal density, ADA: Adaxial stomatal area and ADD: Adaxial stomatal density*

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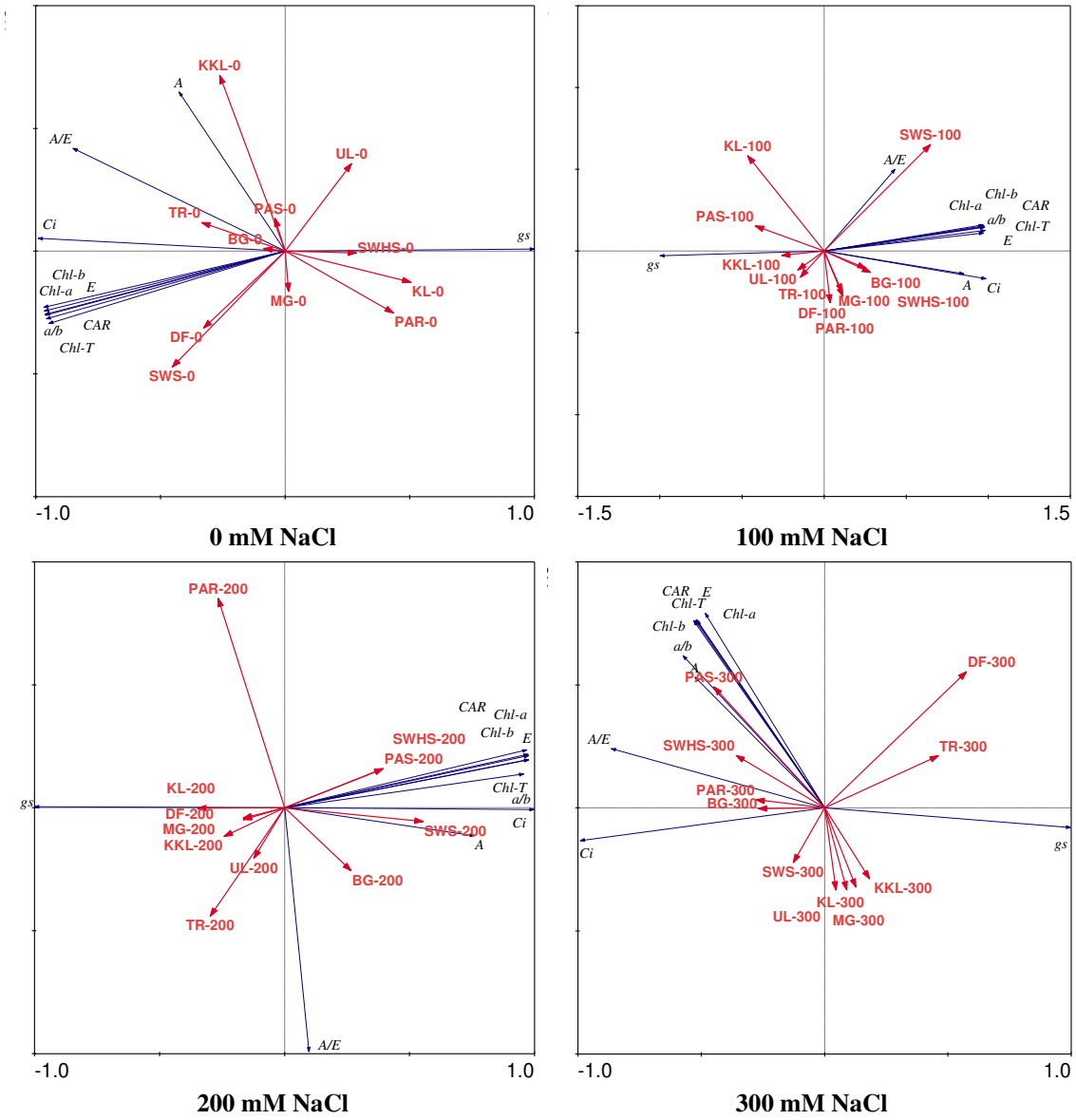

Figure 8. *RDA ordination biplot showing the effect of photosynthetic characteristics on the ecotypes of Cynodon dactylon from various region of Punjab. DF (Derawar Fort), MG (Muzaffargarh), KL (Khabbeki Lake), UL (Ucchali Lake), KKL (Kalar Kahar Lake), T-SW (Treemu-Saline Wetland), SWS (Sahianwala-Saline Wetland), SWHS (Sahianwala-Hyper Saline), PAS (Pakka Anna Saline), PAR (Pakka Anna Reclaimed) and BG (Botanic Garden). Photosynthetic characteristics are abbreviated as: A: Net-CO_2 assimilation rate, E: Transpiration rate, gs: Stomatal conductance, Ci: Sub-stomatal CO_2 concentration, A/E; Water use efficiency, Chl-a, Chlorophyll a, Chl-b, Chlorophyll b, a/b: Chlorophyll ratio a/b, Chl-T: Total Chlorophyll and CAR: Carotenoids*

Leaf blade thickness is a critical adaptation against harsh environment (Hameed et al., 2011). We noticed invariably increased in this characteristic among all ecotypes of *C. dactylon* under salt stress, mainly due to increased sclerification and bulliform cell area. Therefore, our finding is in accordance with the facts that sclerification is the immediate response of plants when exposed to moisture limiting environments. This process provides mechanical strength to soft and delicate tissue avoiding from collapse

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(Al-maskri et al., 2013; Leroux et al., 2015) and at the same time preventing undue water loss through leaf surface (Toon et al., 2015) and hence important for water conservation.

Additionally, we recorded increasing Metaxylem area with increased along with increased salinity levels, at least in more tolerant ecotypes of *C. dactylon.* This adaptive feature has earlier been reported for different plants facing salt stress, e.g., *Alternanthera bettzickiana* (Younis et al., 2013)*, Juncus* species (Al Hassan et al., 2015) and Cattail (Correa et al., 2015).

Increased density of micro-hairs with increasing salt level may be one of the most effective mechanism for salinity tolerance in this salt excretory halophyte (Hu et al., 2015). A substantial increase in trichome density and length was recorded in relatively more tolerant ecotypes of *C. dactylon,* which is regarded as an important ecological adaptation. The increased density and size of trichomes in this case provide additional benefit to a plant to cope with environmental stresses like drought and salinity. Stomatal area on abaxial leaf invariably increased under salinity in all cases except in ecotype from KKL-S but density decreased on both surfaces. In *C. dactylon,* abaxial leaf surface is directly exposed to sun and in that condition stomatal regulation is very important in controlling transpiration rate.

Photosynthetic parameters were severely affected in all grasses (Alam et al., 2015; Ali et al., 2015) and particularly in ecotypes collected from low and moderate salinities. Photosynthetic parameters, in particular net CO_2 assimilation rate, transpiration rate, water use efficiency decreased in most of ecotypes up to maximum salinity level 300 Mm except T-W. Stomatal conductance was observed higher in most of ecotypes with the increase of salinity except S-SW and some reports (Manuchehri and Salehi, 2015; Noman et al., 2018; Yu et al., 2013) have already mentioned these attributes supportive in halophytic or highly salt tolerant grasses.

Reduction in chlorophyll contents as a result of high salinity is well documented by many researchers (Amareh et al., 2015). However, stability in these parameters (chl a, chl b and carotenoids) in more tolerant ecotypes might have contributed to increased salt tolerance. Stimulated concentration of photosynthetic pigments chl b was observed in most of the ecotypes except UL-HS, KKL-S and S-SW and chlorophyll a and carotenoids showed reduction in most of ecotypes and showed increase Chl a pigment in sensitive ecotypes T-W and BG-NS and carotenoids in MG-RB at 200 mM NaCl indicates the better adaptation of these populations as also reported by (Takahashi and Badger, 2011)

Physiological mechanisms such as repression in transpiration loss, improved water-use efficiency, maintenance of turgor potential, deep root system, upregulation of antioxidants, stomatal regulation, and photosynthetic rate at low water potential, and synthesis of osmolytes/osmoregulation help plants to sustain growth and biomass production under stress condition as reported by Farooq et al. (2012), Khalid et al. (2019), Shafiq et al. (2014) and Zafar et al. (2016).

Conclusion

Cynodon dactylon is a widespread grass that has an excellent potential to inhabit a variety of habitats. Differently adapted ecotypes of this grass are independently evolved during the long evolutionary history and it is confirmed by their specific adaptive mechanism for salinity tolerance under similar controlled environments. The changes in

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structural (Sclerenchyma bulliform cells, trichomes, broad metaxylem) and physiological (repression of transpiration, stability in chlorophyll contents) attributes confirms it's potential to thrive well in different conditions and laid the basis for future investigations. *Cynodon dactylon* being considered as best halophytes and have mechanistic ability to tolerance salinity, it is recommended for farmers to use it as an alternative fodder crop for salt effected areas/land.

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EFFECT OF CHITOSAN PRETREATMENT ON SEEDLING GROWTH AND ANTIOXIDANT ENZYME ACTIVITY OF SAFFLOWER (*Carthamus tinctorius* L.) CULTIVARS UNDER SALINE CONDITIONS

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Abstract. Today, salinity has become a major problem in agricultural areas all over the world. However, plants develop a defense mechanism against stress by increasing various antioxidant enzyme activities in order to tolerate salt and other stress factors. In addition to this mechanism, the effects of stress are attempted to be reduced by various applications that increase enzyme activities. One of these applications is chitosan application. In this study, 4 different doses of chitosan pretreatment (0 (control) (Ch1), 0.2% (Ch2), 0.4% (Ch3), 0.6% (Ch4)) were applied to safflower cultivars (Balcı, Linas, Remzibey) for 4 hours under laboratory conditions. For each chitosan application, 50 seeds were germinated under saline conditions (0 (control) (S1), 50 mM (S2), 100 mM (S3), 150 mM (S4)) in petri dish. As a result of the study, it has been determined that chitosan applications provide increases in seedling length, root length, seedling wet weight, root wet weight, germination percentage, total chlorophyll, carotenoid, β-carotene and lycopene parameters. In the study, it was determined that the most effective chitosan application was Ch3 in terms of the properties examined in stress conditions. According to the research results, it was concluded that chitosan can be considered as a natural material that can positively affect in the defense mechanism of plants under stress conditions.
Keywords: *abiotic stress, environmental stress, damage, seed germination, seedling development*

Introduction

High soil salinity is an serious factor that limits agricultural production in many regions of the world (Yamaguchi and Blumwald, 2005). Salt soil conditions are also one of the most important environmental stress factors that damage plants significantly (Bulgari et al., 2019; Jafari and Garmdareh, 2019). In addition, stress factors that plants are exposed to divided into two types abiotic and biotic (Bulgari et al., 2019). Salinity, one of the abiotic stress factors, affects plants in two ways. Firstly it causes a decrease in soil water content through osmotic stress. In this way, the water intake of the plant is restricted. The second is that it causes excessive ion intake. In particular, it increases the uptake of Na^+ and Cl^- ions (Abogadallah, 2010). Various environmental stresses cause oxidative damage in plants, causing damage and even death of the plant's cells (Sharma et al., 2012). A variety of reactive oxygen species (ROS) are usually produced in plants under stress conditions. Plants develop molecular defense systems to avoid the effects of damage caused by ROS and limit ROS formation (Rejeb et al., 2014). To reduce oxidative damage in ROS cells, plants develop a defense system containing antioxidant enzymes as well as reduced glutathione, tocopherol, carotenoids and flavonoids such as non-enzymatic ascorbate (Nú˜nez et al., 2003; Azevedo-Neto et al., 2006).

Although germination of seed is one of the critical stages for seedling development and successful crop production, it is a complex process that is very sensitive to the negative effects of environmental conditions (Almansouri et al., 2001; Fan et al., 2013; Kataria et al., 2017). The negative effects of salt application on seed germination and

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seedling development occur as physiological and biochemical changes such as osmotic stress, ion toxicity and oxidative damage (Yu et al., 2013; Alsaeedi et al., 2017; Fang et al., 2017). In plants, salt tolerance can be increased with some environmental applications as well as genetic mechanisms (Razzaq et al., 2020). Therefore, pretreatment applications to seed stimulate the metabolic processes of germination and increase the performance of the seed against various environmental conditions (Jisha et al., 2013; Kataria et al., 2017).

Recently, with the use of biostimulants, the resistance of plants to abiotic stress conditions has been increased and thus agricultural production and quality increase has been provided (Boehme et al., 2008; Mahdavi et al., 2011; Safikhan et al., 2018).

Biostimulators are defined as substances that stimulate the development of the plant obtained from various organic and inorganic substances and also play an important role in reducing the effects of abiotic stress (Boehme et al., 2008; Mahdavi et al., 2011; Du Jardin, 2015). Biostimulant application is one of the approaches to reduce abiotic stress and increase the yield and quality of the product in most plants (Safikhan et al., 2018). Today, one of the biostimulant applications is chitosan application. It is a natural, non-toxic biopolymer obtained by deacetylation of chitosan chitin (Katiyar et al., 2015; Younes and Rinaudo, 2015). In addition, the Crustacea family of the chitin is an important ingredient in crustaceans (crab, shrimp, crayfish, etc.) (No et al., 2002; Gürsoy et al., 2018). It is also stated by the researchers that the chitin is a natural aminopolysaccharide that is abundant in nature (Ravi Kumar, 2000). In addition to being biologically renewable, chitosan is biodegradable, biocompatible, antigenic and non-toxic, and biofunctional structure, and this polymer and the materials obtained using this polymer have been used in biomedical applications such as wound dressing material and drug delivery systems (Kim et al., 2007; Hosseinnejad and Jafari, 2016; Muxika et al., 2017).

In plants, it has been reported that antimicrobial activity, stimulating plant growth and development, inducing chitinase activity and increasing seed yield in the seed coating (Tay, 1993; Tham, 2001; Vasyukova, 2001; Devlieghere et al., 2004). Plant growth, seed germination, chlorophyll content and ion uptake can be increased with chitosan application (Ahmed et al., 2020).

Safflower (*Carthamus tinctorius* L.) is an annual medicinal and aromatic oilseed crop (Kumar and Kumari, 2005; Moghadam and Mohammadi, 2014; Golkar and Taghizadeh, 2018). In addition, it is an oil plant with flowers in yellow, red, orange, colors, with and without thorns, resistant to drought, with an average oil rate of 30-50% (Gürsoy, 2019).

In this study, it was aimed to determine the effect of chitosan pretreatment on the development of safflower cultivars, photosynthetic activity and antioxidant enzyme activities in saline conditions.

Materials and methods

Research material and growth conditions

Safflower cultivars (Balci, Linas, Remzibey) were obtained from the Central Field Crops Research Institute, Ankara, Turkey. The research was carried out at the Aksaray University Scientific and Technological Research Laboratory (ASÜBTAM). Before commencing the experiment, seeds of cultivars were kept in 5% sodium hypochlorite solution for 5 minutes for surface sterilization. Then washed with pure water and subjected to 4 hours priming with different concentrations of chitosan (Ch) solutions. Chitosan (Sigma-Aldrich, medium molecular weight, at 85% -acetylated, viscosity 270 cP, CAS: 9012-76-4) after dissolving in 0.1% acetic acid of commercially available

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chitosan [0 (control) (Ch1), 0.2% (Ch2), 0.4% (Ch3), 0.6% (Ch4)]. For each Ch dose, 50 seeds were placed in sterile petri dishes on Whatman No:1 blotting papers and 10 mL of different doses of salt (0 (control) (S1), 50 mM (S2), 100mM (S3), 150mM (S4)) concentrations were added from solutions containing NaCl (Merck). Only water was added to the control petri dish. In order to prevent evaporation the petri dishes are wrapped with parafilm. The petri dishes were left to germinate at 24 ± 1^0C. The research randomized plots experimental desing were made with 3 replication according to the trial pattern. Measurements and observations were made on the 14th day of the study.

Germination percentage (%)

Germination percentage was calculated using the formula below.

$$\text{Germination\%} = (\text{number of germinated seeds} / \text{total number of seeds}) \times 100 \quad \text{(Eq.1)}$$

(Siddiqi et al., 2007)

Chlorophyll (mg/g)

The young leaf samples (0.25 g) from each safflower cultivar were filtered after homogenizing in 80% acetone (Merck) and the extracts were filtered with 25 ml with acetone. These samples were read at 663 nm and 645 nm wavelength (Beckman coulter DU 730 Life Sciences UV / VIS Spectrophotometer) followed by calculation of chlorophyll using the formula given below (Lichtenthaler, 1983; Amira and Qados, 2011; Kabay and Şensoy, 2016). Before each reading, the device was reset using blind reading.

$$\text{Chlorophyll a (mg/g)} = (12.7*663\text{ nm})-(2.69*645\text{ nm})*V/W*10000 \quad \text{(Eq.2)}$$

$$\text{Chlorophyll a (mg/g)} = (22.91*645\text{ nm})-(4.68*663\text{ nm})*V/W*10000 \quad \text{(Eq.3)}$$

$$\text{Total Chlorophyll} = \text{Chlorophyll a} + \text{Chlorophyll b} \quad \text{(Eq.4)}$$

Carotenoid (mg/g)

The carotenoid amount was determined according to the Jaspars formula by reading the extract used in determining the chlorophyll amount at 450 nm wavelength (Beckman Coulter DU 730 Life Sciences UV / VIS Spectrophotometer) (Turfan, 2017).

$$\text{Carotenoid} = (4.07\times A450-(0.0435\times\text{Chlorophyll a}+0.367\times\text{Chlorophyll b}) \quad \text{(Eq.5)}$$

β-Carotene (mg/g)

100 mg sample was homogenized for 1 minute in a mixture of 10 ml acetone-hexane (92:3) and filtered. The absorbance of the filtrate at 453, 505 and 663 nm was recorded. It is preferred in the calculation of carotene amount. (Turfan, 2017).

$$\text{mg β-Carotene/100 mg} = 0.0458 \text{ x } A663+0.372 \text{ x } A505-0.0806 \text{ x } A453 \quad \text{(Eq.6)}$$

Lycopene (mg/g)

100 mg sample was homogenized for 1 minute in a mixture of 10 ml acetone-hexane (92:3) and filtered. The absorbance at 453, 505 and 663 nm was recorded in the filtered

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extract. The following formula was used to determine the amount of lycopene (Turfan, 2017).

$$\text{mg Lycopene/100 mg}=0.216xA663–0.304xA505+0.452xA453 \quad \text{(Eq.7)}$$

Statistical analysis

The experimental data obtained at the end of the research, was subjected to analysis of variance using MSTAT-C computer software. Duncan test was applied to determine the significance levels of the differences between means of applications.

Results

A statistically significant difference was determined at the level of $P < 0.01$ in Cultivars × Salt doses× Chitosan doses triple interaction (*Table 1*) in all the features studied.

Table 1. *Variance analysis results on the effect of chitosan pretreatment on seedling growth and antioxidant enzymes of safflower cultivars under saline conditions*

Variation sources	D f	Seedling Length		Root Length		Seedling Wet Weight		Root Wet Weight	
		Mean square	F	Mean square	F	Mean square	F	Mean square	F
Cultivar	2	0.185	1.85	0.02	1.64	0.002	5.78**	0.0005	3.66*
Salt	3	13.21	132.57**	0.547	44.69**	0.023	59.05**	0.003	28.00**
Cultivar×Salt	6	1.123	11.26**	0.038	3.104**	0.001	3.26**	0.002	13.42**
Chitosan	3	11.04	110.76**	1.702	139.15**	0.025	65.52**	0.015	122.46**
Cultivar×Chitosan	6	0.061	0.613	0.075	6.12**	0.001	1.38	0.001	6.98**
Salt×Chitosan	9	1.38	13.88**	0.239	19.52**	0.002	6.03**	0.001	9.70**
Cultivar×Salt×Chitosan	18	0.3	3.01**	0.068	5.546**	0.002	4.39**	0.001	8.25**
Error	96	0.1		0.012		0.0003		0.0001	
Total	143								
CV%		3.88		3.4		5.59		7.24	

Variation sources	D f	Germination percentage		Total chlorophyll		Carotenoid		β -Carotene		Lycopene	
		Mean square	F	Mean square	F	Mean square	F	Mean square	F	Mean square	F
Cultivar	2	1.646	4.74*	0.024	0.3922	0.25	6.18**	0.044	32.55**	0.008	9.18**
Salt	3	18.94	54.62**	7.145	115.54**	0.239	5.92**	0.134	98.147**	0.02	24.74**
Cultivar× Salt	6	1.741	5.02**	0.533	8.62**	0.251	6.22**	0.012	8.57**	0.003	3.58**
Chitosan	3	15.45	44.57**	8.061	130.35**	5.164	127.95**	0.313	229.61**	0.118	142.33**
Cultivar× Chitosan	6	0.137	0.395	0.124	2.008	0.343	8.48**	0.017	12.62**	0.002	2.378*
Salt×Chitosan	9	2.144	6.184**	0.634	10.24**	0.792	19.62**	0.065	47.69**	0.031	37.68**
Cultivar×Salt×Chitosan	18	0.882	2.543**	0.147	2.38**	0.325	8.051**	0.019	13.66**	0.003	4.03**
Error	96	0.347		0.062		0.04		0.001		0.001	
Total	143	----------		----------		----------		----------		----------	
CV%		0.60		10.02		6.16		5.96		4.74	

** $P<0.01$, *$P<0.05$

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When the seedling length feature is examined (*Table 2*), the longest seedling length was obtained from Balcı variety in S1 Ch3 application as 10.00 cm. The minimum seedling length was obtained in S4 Ch4 application and Balcı variety. Generally, the longest seedling length in all cultivars has been obtained in Ch3 application, and it is determined that chitosan is effective in eliminating the harmful effects of salt while increasing the salt doses. Chitosan application seems to inhibit the effects of salt stress, causing the seedling to grow higher. However, chitosan application had different effects on varieties. For example, in S1 and S4 applications in Balcı cultivar, it is seen that the seedling height is longest in Ch3. The same is true for the Linas variety. In Remzibey cultivar, Ch3 was found to be the longest seedling length only in S1 application, and in all other salt doses, the longest seedling length was detected in Ch1 application. In the Remzibey cultivar, a positive effect of chitosan applications against salt doses in terms of seedling length could not be determined. Cultivars × S doses × Chitosan doses interaction caused significant difference in seedling length feature at $p<0.01$ level. In this respect, when the study results were examined, it was determined that the harmful effects of salt doses were inhibited by interaction with chitosan. Similarly, salt doses interacted with varieties and showed different reactions in each variety.

Table 2. *Average values the effect of chitosan doses applied to safflower cultivars on saline length (cm) under saline conditions*

Cultivars	Seedling Length (cm)							
	S1				S2			
	Ch1	Ch2	Ch3	Ch4	Ch1	Ch2	Ch3	Ch4
Balcı	9.53 ab	7.52 n-r	10.00 a	7.82 ı-q	9.35 a-c	8.47 d-l	8.63 c-ı	7.94 h-p
Linas	9.56 ab	8.54 d-j	9.89 a	8.22 d-n	8.82 b-f	7.77 j-q	8.21 d-n	7.90 h-q
Remzibey	8.90 b-e	8.68 c-h	9.74 a	7.96 g-p	8.75 c-g	7.62 m-r	7.63 m-r	7.52 n-r
	S3				S4			
	Ch1	Ch2	Ch3	Ch4	Ch1	Ch2	Ch3	Ch4
Balcı	8.23 d-n	7.74 j-q	7.26 p-s	7.74 j-q	7.74 j-q	6.74 st	8.10 e-o	6.40 t
Linas	8.92 b-d	7.68 k-r	8.30 d-n	8.07 f-p	7.84 ı-q	7.12 q-t	7.86 ı-q	6.45 t
Remzibey	8.86 b-f	7.66 l-r	8.50 d-k	8.36 d-m	8.21 d-n	7.29 o-s	7.93 h-q	6.92 r-t
LSD%1	0.6786							

According to the Duncan test dissimilar letters in the column show different groups

In terms of root length (*Table 3*), the longest root length (3.80 cm) was detected in Remzibey variety and S1 Ch1 application. The shortest root length was determined as 2.59 cm in Remzibey cultivar and S4 Ch4 application. It is seen that root length increases with increasing Ch doses in all cultivars. However, this increase didn't occur only in the S1 dose, and it is the dose that does not apply salt since the S1 dose is a control application. In other words, with the application of salt, the effectiveness of chitosan has been revealed and it has been effective in lengthening the root length by inhibiting the negative effects of salt. Although this effect was determined in all varieties, the most effective chitosan application was determined as Ch3. Cultivars × S doses × Chitosan doses were determined statistically significant differences in root length characteristics in terms of $p<0.01$. With this interaction in all cultivars, root length feature was affected and increased due to salt doses and chitosan doses.

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Table 3. *Average values of the effect of chitosan doses applied to safflower varieties under saline conditions on root length (cm)*

Cultivars	Root Length (cm)							
	S1				S2			
	Ch1	Ch2	Ch3	Ch4	Ch1	Ch2	Ch3	Ch4
Balcı	3.30 e-k	3.39 d-j	3.64 a-d	3.22 g-n	3.11 j-p	3.20 g-n	3.46 b-g	3.16 h-p
Linas	3.29 e-l	3.30 e-k	3.40 c-ı	3.23 f-n	3.00 l-q	3.20 g-n	3.60 a-d	3.17 h-o
Remzibey	3.80 a	3.42 c-h	3.50 b-f	3.14 h-p	3.11 j-p	3.13 ı-p	3.64 a-d	3.06 k-q
	S3				S4			
	Ch1	Ch2	Ch3	Ch4	Ch1	Ch2	Ch3	Ch4
Balcı	2.98 n-q	3.04 k-q	3.72 ab	2.97 n-q	2.99 m-q	3.07 k-q	3.45 b-g	2.63 r
Linas	2.97 n-q	3.15 h-p	3.66 abc	2.99 m-q	2.88 pq	3.52 b-e	3.22 g-n	2.80 qr
Remzibey	3.04 k-q	3.15 h-p	3.27 e-m	3.06 k-q	2.90 o-q	3.65 a-d	3.48 b-g	2.59 r
LSD%1	0.2351							

*According to the Duncan test dissimilar letters in the column show different groups

Seedling wet weight (*Table 4*) was determined as the most seedling wet weight of 0.45 g in Linas cultivar in S1 Ch3 application. The minimum seedling wet weight was determined as 0.28 g in S4 Ch1 application, that is, when S doses are highest but Ch dose is control, ie 0%. In all other applications, seedling wet weight increased with Ch application. Cultivars × S doses × Chitosan doses were determined statistically significant differences in seedling wet weight in terms of interaction with $p<0.01$. All cultivars were significantly affected by S and Ch doses. While the application of Ch3 in S2, S3 and S4 in the Balcı cultivar causes the formation of seedling wet weight the most, Ch3 application in all salt doses in Linas and in S2 and S3 in Remzibey were effective in obtaining the seedling wet weight the most. In S1 and S4, the dose of Ch3 was more effective.

Table 4. *Average values the effect of chitosan doses applied to safflower varieties under saline conditions on seedling wet weight (g)*

Cultivars	Seedling Wet Weight (g)							
	S1				S2			
	Ch1	Ch2	Ch3	Ch4	Ch1	Ch2	Ch3	Ch4
Balcı	0.41 a-c	0.34 g-o	0.38 c-g	0.34 g-o	0.33 h-p	0.40 b-e	0.44 ab	0.31 k-p
Linas	0.35 g-n	0.40 b-f	0.45 a	0.35 g-n	0.34 g-n	0.37 c-j	0.41 b-d	0.33 h-p
Remzibey	0.35 g-n	0.38 c-h	0.44 ab	0.35 f-m	0.31 k-p	0.37 c-j	0.36 d-k	0.33 g-o
	S3				S4			
	Ch1	Ch2	Ch3	Ch4	Ch1	Ch2	Ch3	Ch4
Balcı	0.31l-p	0.35 f-m	0.38 c-g	0.35 g-n	0.30 m-p	0.36 e-l	0.35 g-n	0.32 j-p
Linas	0.32 j-p	0.35 g-n	0.37 c-ı	0.35 g-n	0.30 n-p	0.33 h-p	0.34 g-n	0.31 k-p
Remzibey	0.32 ı-p	0.36 e-l	0.35 g-n	0.35 g-n	0.28 p	0.32 j-p	0.33 h-p	0.29 op
LSD%1	0.04183							

* According to the Duncan test dissimilar letters in the column show different groups

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In the root wet weight feature (*Table 5*), the maximum root wet weight was determined in Remzibey cultivar in Ch3 application in S1. Although S1 was a control dose, Ch affected root caused an increase in wet weight. However, in other S doses, the root wet weight increased with the increase of Ch. In terms of root wet weight, the most effective Ch doses were Ch2 and Ch3. Cultivars × S doses × Chitosan doses interaction showed statistically significant difference at $p<0.01$ in terms of root wet weight.

Table 5. *Average values of the effect of chitosan doses applied to safflower varieties under saline conditions on root wet weight (g)*

Cultivars	Root Wet Weight (g)							
	S1				S2			
	Ch1	Ch2	Ch3	Ch4	Ch1	Ch2	Ch3	Ch4
Balcı	0.14 g-m	0.12 l-n	0.15 f-l	0.16 d-ı	0.17 d-f	0.15 e-k	0.21b	0.14 g-m
Linas	0.16 d-h	0.14 f-l	0.18 cd	0.15 e-k	0.17 d-f	0.18 cd	0.18 c-e	0.14 g-m
Remzibey	0.15 e-k	0.15 d-j	0.24 a	0.16 d-h	0.13 j-n	0.17 d-f	0.16 d-h	0.14 g-m
	S3				S4			
	Ch1	Ch2	Ch3	Ch4	Ch1	Ch2	Ch3	Ch4
Balcı	0.14 h-n	0.15 d-j	0.17 d-f	0.15 f-l	0.12 l-n	0.13 ı-n	0.18 cd	0.13 ı-n
Linas	0.13 ı-n	0.16 d-h	0.20 bc	0.15 e-k	0.11 n	0.15 e-k	0.17 d-g	0.12 l-n
Remzibey	0.11 mn	0.16 d-h	0.21 ab	0.12 k-n	0.15 f-l	0.15 d-j	0.15 d-j	0.11n
LSD%1	0.02351							

* According to the Duncan test dissimilar letters in the column show different groups

In germination percentage (*Table 6*), the highest germination rate was determined as 99.97% in Balcı cultivar in S2 Ch1 application. The least germination was detected in Linas cultivar and Ch4 application with 95.73%. Cultivars × S doses × Chitosan doses interaction showed statistically significant difference in $p<0.01$ level in terms of germination percentage. In all cultivars, the germination rate decreased with increasing salt doses. However, an increase in germination rate was determined in all cultivars and all S doses with Ch applications. For example, in the Remzibey cultivar, it is seen that the germination percentage is highest with the application of Ch3 at the dose of S4. Similarly, the most germination was determined in S3 and S4 in Ch3 in Balcı cultivar.

In terms of total chlorophyll (*Table 7*) Cultivars × S doses × Chitosan doses interaction showed statistically significant difference at $p<0.01$ level. Differences between varieties were determined with chitosan applications in applied salt doses. Although chlorophyll amount is expected to decrease with increasing salt doses, it has been determined that it increases with chitosan applications. Chitosan had a positive effect by eliminating the negative effect of salt by playing an encouraging role. The highest chlorophyll content was obtained in S1 and Ch1 and the lowest chlorophyll was determined in S4 Ch4 as 1.04 mg g^{-1}. However, the effects of S and Ch doses on varieties were in different ways. While S3, S3 and S4 increase Ch3 chlorophyll in the Balcı cultivar, it is determined that the application of Ch2 in S1 and S2 and Ch4 in S3 and S4 are more effective in linas cultivar. In Remzibey variety cultivar, Ch3 was determined as the most effective application dose in S2, S3 and S4.

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Table 6. *Average values of the germination percentage (%) of chitosan doses applied to safflower varieties under saline conditions*

Cultivars	Germination percentage (%)							
	S1				S2			
	Ch1	Ch2	Ch3	Ch4	Ch1	Ch2	Ch3	Ch4
Balcı	99.60 a-c	98.73 a-ı	99.60 a-c	98.40 b-k	99.97 a	98.80 a-ı	98.40 b-k	98.57 a-j
Linas	99.57 a-d	99.10 a-f	99.80 ab	97.57 f-q	98.43 b-k	97.97 e-p	98.13c-m	98.07 d-o
Remzibey	99.43 a-e	98.80 a-ı	98.33 b-l	98.10 c-n	97.90 e-p	96.90 k-s	98.27 c-l	97.73 f-p
	S3				S4			
	Ch1	Ch2	Ch3	Ch4	Ch1	Ch2	Ch3	Ch4
Balcı	97.43 h-q	97.43 h-q	98.97 a-h	96.60 n-s	97.53 g-q	96.57 o-s	98.40 b-k	96.50 p-s
Linas	98.07 d-o	96.83 l-s	98.40 b-k	97.03 j-s	98.00 e-p	96.73m-s	97.73 f-p	95.73 s
Remzibey	97.33 ı-r	96.20 q-s	99.03 a-g	96.93 k-s	98.07 d-o	98.07 d-o	98.87 a-ı	95.87 rs
LSD%1	1.264							

* According to the Duncan test dissimilar letters in the column show different groups

Table 7. *Average values the effect of chitosan doses applied to safflower varieties under saline conditions on total chlorophyll (mg g^{-1} FW)*

Cultivars	Total chlorophyll (mg g^{-1})							
	S1				S2			
	Ch1	Ch2	Ch3	Ch4	Ch1	Ch2	Ch3	Ch4
Balcı	3.92 a	2.81 c-k	1.95 n-q	2.13 l-q	3.31 bc	2.41 g-p	3.23 b-d	2.37 ı-p
Linas	3.32 bc	3.04 b-g	2.93 c-j	3.02 c-h	3.00 c-ı	2.33 j-q	2.95 c-j	2.04 m-q
Remzibey	3.64 ab	3.18 b-e	3.08 b-f	3.00 c-ı	2.80 c-k	2.05 m-q	2.60 d-m	2.09 l-q
	S3				S4			
	Ch1	Ch2	Ch3	Ch4	Ch1	Ch2	Ch3	Ch4
Balcı	3.31 bc	1.95 n-q	2.70 c-l	1.99 m-q	2.57 e-n	1.92 o-q	2.23 k-q	1.30 rs
Linas	3.26 bc	1.92 o-q	2.51 f-o	1.84 p-r	2.26 k-q	1.93 n-q	2.15 l-q	1.14 s
Remzibey	3.08 b-f	2.04 m-q	2.39 h-p	1.72 qr	2.55 e-o	1.84 p-r	2.26 k-q	1.04 s
LSD%1	0.5343							

* According to the Duncan test dissimilar letters in the column show different groups

When the amount of carotenoid is examined (*Table 8*), the most carotenoid amount is determined as S4 Ch3 as 4.09 mg g^{-1}. The minimum amount of carotenoids was determined as 1.59 mg g^{-1} in S1 Ch1. It was determined that as the S doses increased, the amount of carotenoids increased in all cultivars. However, it is seen that this increase is higher in Ch3. Cultivars × S doses × Chitosan doses interaction showed statistically significant difference at $p<0.01$ in terms of carotenoid amount. In all varieties, especially in S2, S3 and S4, the carotenoid decreased slightly in Ch2, but increased in Ch3.

When examined in terms of β-carotene (*Table 9*), the most β-carotene was found as S4 and Ch3 application in Balcı cultivar as 0.86 mg g^{-1}. It was determined as at least β-carotene 0.24 mg g^{-1} FW in S1 Ch1 application and Balcı variety. However, the

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interaction of Cultivars × S doses × Chitosan doses showed a statistically significant difference in $p<0.01$ level in terms of β-carotene. In terms of β-carotene, the effects of S and Ch doses on varieties were determined, and the highest β-carotene amount in Ch3 was determined in all S doses. Especially in S3, which has the highest salt dosage, the highest β-carotene was determined in all cultivars.

Table 8. *Average values the effect of chitosan doses applied to safflower varieties on saline conditions on the amount of carotenoid (mg g^{-1} FW)*

Cultivars	Carotenoid (mg g^{-1} FW)							
	S1				S2			
	Ch1	Ch2	Ch3	Ch4	Ch1	Ch2	Ch3	Ch4
Balcı	1.59 r	2.67 n-q	3.77 a-e	3.66 a-h	2.96 k-p	2.66 o-q	3.68 a-f	3.66 a-g
Linas	2.21 q	2.84 m-p	3.65 a-h	3.59 a-ı	3.59 a-ı	2.85 m-p	3.58 a-j	3.27 e-m
Remzibey	3.68 a-f	2.72 n-p	3.77 a-e	3.60 a-ı	3.60 a-ı	3.09 ı-o	3.41 c-l	3.55 b-j
	S3				S4			
	Ch1	Ch2	Ch3	Ch4	Ch1	Ch2	Ch3	Ch4
Balcı	2.96 k-p	3.62 a-h	3.45 c-k	3.33 d-m	2.91 l-p	3.31 d-m	3.81 a-d	3.24 f-m
Linas	3.14 h-o	3.07 j-o	3.81 a-d	3.14 h-o	2.56 pq	3.15 g-o	3.99 ab	3.26 e-m
Remzibey	2.94 k-p	3.27 e-m	3.88 a-c	3.17 f-n	2.65 o-q	3.01 k-p	4.09 a	3.03 k-p
LSD%1	0.4292							

* According to the Duncan test dissimilar letters in the column show different groups

Table 9. *Average values the effect of chitosan doses applied to safflower varieties on saline conditions on the amount of β-carotene (mg g^{-1} FW)*

Cultivars	β-Caroten (mg g^{-1} FW)							
	S1				S2			
	Ch1	Ch2	Ch3	Ch4	Ch1	Ch2	Ch3	Ch4
Balcı	0.24 p	0.50 ı-l	0.69 bc	0.69 bc	0.39 no	0.37 o	0.65 b-f	0.65 b-e
Linas	0.69 b	0.42 m-o	0.69 b	0.64 b-f	0.51 ı-l	0.48 k-m	0.84 a	0.68 b-e
Remzibey	0.52 ı-l	0.36 o	0.66 b-e	0.60 e-h	0.55 h-k	0.48 j-m	0.71b	0.63 b-g
	S3				S4			
	Ch1	Ch2	Ch3	Ch4	Ch1	Ch2	Ch3	Ch4
Balcı	0.68 b-d	0.67 b-e	0.66 b-e	0.65 b-e	0.61 c-h	0.68 b-e	0.86 a	0.54 h-k
Linas	0.69 bc	0.69 bc	0.82 a	0.65 b-f	0.57 f-ı	0.71 b	0.82 a	0.56 g-j
Remzibey	0.65 b-f	0.65 b-f	0.85 a	0.60 d-h	0.55 h-k	0.70 b	0.80 a	0.45 l-n
LSD%1	0.0678							

* According to the Duncan test dissimilar letters in the column show different groups

When the study results are examined in terms of lycopene (*Table 10*), the most lycopene was determined as 0.81 mg g^{-1} in Linas and Remzibey varieties in S4 and Ch3. The least lycopene was determined as 0.44 mg g^{-1} in S2 Ch1. Especially in S3 and S4 doses in which salt doses increased, the highest lycopene in Ch3 was determined. As in carotene, S4, which has a high salt dose in lycopene, has the highest lycopene content in all varieties in Ch3 application.

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Table 10. *Average values the effect of chitosan doses applied on safflower varieties on saline conditions on the amount of lycopene (mg g^{-1} FW)*

Cultivars	Lycopene (mg g^{-1} FW)							
	S1				S2			
	Ch1	Ch2	Ch3	Ch4	Ch1	Ch2	Ch3	Ch4
Balcı	0.55 f-k	0.60 d-g	0.60 c-g	0.69 b	0.44 l	0.60 c-g	0.63 b-f	0.62 b-g
Linas	0.51 ı-l	0.57 d-ı	0.62 b-g	0.64 b-e	0.49 kl	0.64 b-e	0.60 c-g	0.63 b-f
Remzibey	0.52 h-k	0.64 n-d	0.60 d-g	0.64 b-d	0.54 g-k	0.61 b-g	0.59 d-ı	0.64 b-e
	S3				S4			
	Ch1	Ch2	Ch3	Ch4	Ch1	Ch2	Ch3	Ch4
Balcı	0.49 j-l	0.60 d-h	0.60 d-h	0.59 d-h	0.56 e-k	0.60 c-g	0.78 a	0.56 d-k
Linas	0.55 f-k	0.60 c-g	0.68 bc	0.57 d-ı	0.62 b-g	0.57 d-j	0.81a	0.56 d-k
Remzibey	0.54 g-k	0.60 c-g	0.78 a	0.59 d-h	0.63 b-f	0.58 d-ı	0.81a	0.59 d-h
LSD%1	0.06786							

* According to the Duncan test dissimilar letters in the column show different groups

Discussion

In this study, in which different doses of salt were applied with different doses of chitosan pretreatment to the safflower varieties, although the results were evaluated, there was a decrease in some morphological features due to the increase of salt doses, and with the application of chitosan, the characteristics of seedling length, root length, root wet weight, seedling wet weight and germination rate an increase was observed. However, there are increases in biochemical properties with the effect of chitosan application in stress conditions. The most effective chitosan dose was determined as Ch3 in all the properties studied. Ma et al. (2012) reported that application of 0.0625% oligochitosan to the nutrient solution in which wheat seeds were grown reduces the negative effect of salt stress. Jabeen and Ahmad (2013), applied chitosan to sunflower and safflower varieties under salty conditions. As a result of the study, they reported that low concentration of chitosan application caused increase in germination parameters of both cultivars. In the study conducted by Sheikha and Al-Malki (2011), they reported that chitosan applications had an effect on the plant's growth parameters, such as seedling and root height, wet and dry weight. Al-Tawaha et al. (2018), 81.94 cm in plant height control application, 84.06 cm in 30 mg/L chitosan application, 84.38 cm in 60 mg/L chitosan application, 84.81 in 60 mg/L chitosan application, they reported that they had determined in cm. Salt stress affects chlorophyll metabolism and causes a significant decrease in chlorophyll production (Qin et al., 2019). Chlorophyll content is widely used in plants as an indicator of abiotic stress tolerance. In addition, chlorophyll decreases in plants exposed to stresses such as salinity, as a result of which growth is delayed (Safikhan et al., 2018). In recent studies, it has been reported by researchers that chitosan increases chlorophyll content in soybean and peanuts (Dzung, 2005). Yahyaabadi et al. (2016), applied chitosan to fenugreek plants under salt stress. As a result of the study, they reported that the application was effective in promoting plant growth by reducing salt stress on the water content of the leaf and photosynthetic pigment parameters. Turfan (2017), applied various abiotic stresses (salt, heavy metal, drought and lime) to the spinach plant. As a result of the study, it was reported that chlorophyll a, b, total chlorophyll and carotenoid,

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β-carotene and lycopene content increased in drought and lime stress applications. Stahl and Sies (2003), reported that carotenoids are pigments that play a very important role in the protection of plants against photooxidative processes in plants and are antioxidants that play an extremely active role in eliminating the harmful effects of free oxygen radicals. In addition to having antioxidant capacity, carotenoids also play a very important role in reacting to various stress conditions (Boba et al., 2011). In plants under stress, the amount of photosynthetic pigment consisting of chlorophyll a, chlorophyll b, total chlorophyll and carotenoid varies depending on factors such as species, type of stress, duration of stress, the period of the plant in the life cycle, and the intensity of stress (Turfan, 2017). Linic et al. (2019), it is suggested that carotenoids, which are specific metabolites, can play a positive role as natural substances in stress management in tolerant species. Rahman et al. (2018), reported that the application of chitosan to the strawberry plant in the form of a spray causes an increase in the carotenoid content of the plant. ß-carotene is an organic red-orange colored pigment that is abundant in plants (Pop et al., 2019). Falcinelli et al. (2017), reported that appropriate doses of salt cause increased phenolic compounds in rapeseed. He et al. (2020), reported that NaCl and $CaCl_2$ application can be used as a strategy that will increase the antioxidant activity through the accumulation of carotenoids as a result of their study in corn plant. In this study, it was determined that pre-application of chitosan to the seeds of safflower varieties against salt stress promotes the strengthening of the defense system by causing the enzyme activities of the varieties to increase.

Conclusion

In this study, besides the negative effects of salt doses, the positive effects of chitosan application, safflower varieties were affected at different levels. The most advantageous results were obtained from the Remzibey variety in terms of morphological (seedling length, root length, seedling wet weight, root wet weight, germination percentage) and biochemical (chlorophyll, carotenoid, β-carotene and lycopene) parameters. In addition, it has been determined that chitosan plays a role in promoting the increase of enzyme activities, which is the defense mechanism of plants under stress conditions. In this study, the third dose of chitosan (Ch3) caused the most advantageous results. As a result of the study, it was concluded that chitosan can be evaluated as a natural material that can be effective in the defense mechanism of plants under stress conditions. It has been concluded that with the pretreatment of chitosan in regions with salty soil conditions in the world, the germination and seedling development of plants can be increased accordingly it was concluded that yield and quality can be increased. However, applications should be made in other plants and under various stress conditions and their results should be evaluated.

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SPATIOTEMPORAL CHARACTERISTICS OF GROUNDWATER DEPTH IN XINGTAI CITY ON THE NORTH CHINA PLAIN: CHANGING PATTERNS, CAUSES AND PREDICTION

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Abstract. The analysis of spatiotemporal characteristics for groundwater depth can provide a theoretical basis for comprehensive management of groundwater and sustainable development of ecology and environment. The paper investigated the changing patterns of groundwater depth, and further explored the linear and nonlinear relationships between groundwater depth and four potential influencing factors using grey relational analysis and wavelet coherence during 2000-2017. The multiple linear regression and Random Forest (RF) models were proposed to predict the groundwater depth. Taking Xingtai city on the North China Plain as an example, the results indicate that: (1) The groundwater depth in a shallow aquifer has deepened significantly after 2006, and decreased from northwest to southeast. A clear upward trend was detected in the southeast region of the study area. (2) The change of groundwater depth is related to the consumption during agricultural irrigation period and the supply of heavy rainfall during flood season, and the deepest groundwater depth was detected in June. (3) The cross-correlation analysis demonstrated that main influencing factors of groundwater depth are precipitation and temperature. (4) The comparison between actual value and prediction value indicated that the RF model has better accuracy in predicting the groundwater depth than the multiple linear regression model.
Keywords: *groundwater comprehensive treatment, spatial and temporal variability, non-parametric Mann-Kendall test, grey relational analysis, wavelet coherence, random forest model*

Introduction

Groundwater is one of important sources of water for humans, especially in the arid and semi-arid areas (e.g., Algeria, Australia, China, Egypt, and Western United States) where water resources are insufficient, or the surface water is heavily polluted, due to serious ecological and environmental issues. Water for human life, agricultural irrigation and industrial activities mainly depends on the exploitation of groundwater resources (Alley et al., 2002; Oki et al., 2006; Wada et al., 2012). Globally, the groundwater accounts for 29.9% of all global freshwater resources (Shiklomanov, 2000; Li and Qian, 2018) and the shortages of water resource have become one of the most important challenges to humankind (Robertson and Sharp, 2013; Wang et al., 2016). Water resources problems are common throughout China (Jiang, 2009), especially, the North China Plain (NCP), which has serious groundwater problems including the overexploitation of groundwater or the groundwater pollution. The NCP is the biggest plain of China, and its aquifer system extends about 122,000 km^2, supporting over 11% of China's population and 14% of arable land (Pang et al., 2020). The groundwater has provided more than 70% of the NCP's total water supply, which can support

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agricultural irrigation, daily life, and economic development (Liu et al., 2011). However, the overexploitation of groundwater has accelerated since 1970s, and NCP has gradually become one of the most groundwater over-pumped zones around the world, with a long belt along the Beijing-Shijiazhuang-Xingtai area (Shah et al., 2003; Zhang and Li, 2013). The groundwater depth has declined more than 10-60 m in 40% of the entire NCP (Zheng et al., 2010). To avoid the problem of groundwater overexploitation, the government has already taken action to reduce groundwater consumption and adopt measures to adjust agricultural planting structure. For more scientific and effective management, it is important to analyze the changing patterns and causes of groundwater depth. The spatiotemporal evolution of groundwater systems is affected by many factors, including both natural and man-made factors, such as meteorological and hydrological conditions, geological environment, economic development, industrialization, and urbanization. Various measures have been taken to change the balance between groundwater consumption and supply. The analysis of the changes and causes of groundwater depth under the multiple influencing factors are thus of great significance for sustainable development of groundwater resources.

Many previous studies have focused on the changes of groundwater depth, to explore the ecological environment of groundwater resources, and to find a sustainable development path (Ebraheem et al., 2004). Generally, the studies on the spatial-temporal characteristics of groundwater depth have mainly combined with mathematical models. E.g., Manap et al. (2014) drew a map of groundwater potential in the Langat Basin, Malaysia based on probabilistic-based frequency ratio model (FR), and analyzed the spatial relationships between groundwater depth and various hydrological elements. The results indicated that FR model is a high precision way to validate the groundwater potential map (Manap et al., 2014). Naghibi et al. (2016) used the boosted regression tree (BRT), classification and regression tree (CART), and Random Forest (RF) models in conjunction with GIS to draw a groundwater potential mapping, and evaluated the simulation accuracy of the three models. The results showed that the BRT model produced the best prediction value in predicting locations of springs (Naghibi et al., 2016). With the development of urbanization, the impact of climate change and human life on groundwater continues to aggravate. Research scholars focus on the analysis of impact on groundwater changes from both natural factors and human activities. Green et al. (2011) proposed exploring the relationship between groundwater systems and climate change from two aspects: groundwater quality and groundwater volume (Green et al., 2011). Vidal et al. (2000) analyzed the spatiotemporal characteristics of groundwater chemical composition from the perspective of groundwater quality (Vidal et al., 2000). In addition, the groundwater system environment is complicated, and analyzing the potential influencing factors of groundwater is an important research direction. The RF model has been proved to be useful in discovering potential influence factors and predicting target value, and it can improve the prediction accuracy without a significant increased calculation. Iverson et al. (2008) used RF model to draw groundwater potential maps (GPMs), proving that the model is an effective method for drawing GPMs (Iverson et al., 2008).

The objectives of this study include: (1) To analyze the characteristics of groundwater depth from aspects of inter-annual, intra-annual, depth variation and spatial distribution. The non-parametric Mann-Kendall test is used to analyze the temporal and spatial trends and the catastrophe point of groundwater depth. (2) To further reveal the physical mechanism behind the changes of groundwater depth and its

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influencing factors, this paper explored the spatiotemporal characteristics of groundwater depth and relationships between several main influencing factors. The grey relational analysis and wavelet coherence are used to analyze the main influencing factors and their potential relationships. The multiple linear regression and RF models are used to predict the groundwater depth using the influencing factors as predictors, and the accuracy of two models are further compared. (3) To investigate the characteristics and causes of groundwater depth change, taking Xingtai city on the North China Plain as an example, which should be of great significance to coordinate the development of groundwater resources and maintain the stability of the water ecology.

Material and methods

Study area

The NCP is located in the middle part of the North China, and it refers to the region bordered to the north of the Yanshan Mountains, and west of the Taihang Mountains, to the south by the Yellow River, and to the northeast by the Bohai Gulf (Zhang et al., 2003). The NCP includes all the plains of the northern parts of Shandong and Henan Provinces, Beijing Municipality, Tianjin Municipality and Hebei province. Xingtai city is located at the central and southern part of Hebei province, and the surface water resources are scarce and groundwater is serious overexploited (Li et al., 2005; Sun et al., 2010). *Figure 1* shows the locations of the study area and spatial distribution of the groundwater monitoring stations.

Figure 1. *Study area and locations of groundwater monitoring stations in the shallow aquifer of Xingtai city, China*

Xingtai city is located between the longitude of 113°52′ - 115°49′ E and the latitude of 36°50′ - 37°47′ N. It has a total of 18 counties and cities with a total area of 12,456 km^2, of which the plain area is 8911 km^2 and the mountain area is 3545 km^2. Xingtai city has a temperate continental monsoon climate. The average temperature is about 13.1℃, and the precipitation is about 470 mm per year which rainfall is concentrated in summer. The types of groundwater mainly include confined water and

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phreatic water. The confined water is generally below 100 m and is hard to mine. The groundwater in the shallow aquifer is the main source of agricultural and domestic water. This study mainly focuses on the groundwater in the shallow aquifer. Xingtai city is located in the transition zone from the mountain area to the plain area. The shallow groundwater monitoring stations are distributed in the eastern plain, including the water resources area of Luxi Plain, Heilonggang District, and Luxi District.

Data collection

The groundwater depth data were obtained from the Hydrological and Water Resources Survey Bureau of Xingtai city, Hebei province. The groundwater depth data from 41 monitoring stations in the plain area of Xingtai city during 2000-2017 were obtained to analyze the groundwater depth characteristics of the shallow aquifer. The Shapefile of the study area was downloaded from the Global Aviation Data Management (GADM) at https://gadm.org/download_country_v3.html to analyze the spatial distribution of groundwater depth using ArcGIS. The nature factors that might affect the groundwater depth such as precipitation, temperature, and evapotranspiration (ET) data from nine counties were used in the study. These data are obtained from Xingtai city meteorological bureau. The Gross Domestic Product (GDP) of Xingtai city was selected as the artificial factor affecting the depth of groundwater, which collected from the statistical yearbook of Xingtai city for the period of 2000-2017.

Research methodology

Non-parametric Mann-Kendall test

The trends of groundwater depth for 41 monitoring stations were analyzed using the non-parametric Mann-Kendall test method from 2000 to 2017. The Kendall's *S* is calculated using *Equation 1*.

$$S = \sum_{k=1}^{n-1} \sum_{j=k+1}^{n} \mathrm{sgn}(x_j - x_k) \qquad \mathrm{sgn}(x_j - x_k) = \begin{cases} 1, & if\,(x_j - x_k) > 0 \\ 0, & if\,(x_j - x_k) = 0 \\ -1, & if\,(x_j - x_k) < 0 \end{cases} \quad \text{(Eq.1)}$$

where *n* is the length of *X*, in this experiment, *n=18*.

The standardized *Z* statistic is provided in *Equation 2* (Shadmani et al., 2012; Jiang et al., 2015; Jiang et al.,2020).

$$Z = \begin{cases} \dfrac{S-1}{\sqrt{Var(S)}}, & if \quad S > 0 \\ 0, & if \quad S = 0 \\ \dfrac{S+1}{\sqrt{Var(S)}}, & if \quad S < 0 \end{cases} \quad \text{(Eq.2)}$$

The formula is normal distribution. *Var(S)* is the variance of statistic *S*. The positive values of *Z* indicate increasing trends of groundwater depth, negative values for decreasing trends, and *Z=0* is stationary. The null-hypothesis at α significance level

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will be rejected if $|Z| > Z_{1-\alpha/2}$, indicating statistically significant trends. Two significance levels of 10% and 5% are used in this paper to provide trends of groundwater depth, which corresponding the $|Z|$ is greater than 1.64 and 1.96.

Wavelet coherence

The wavelet analysis is used to reveal the nonlinear relationship between groundwater depth and its influencing factors. The wavelet coherence (WTC) is to detect regions with significant coherence (Jiang et al., 2018; Jiang et al., 2019b; Yan et al., 2020). The wavelet defines two time series X_n and Y_n as $W^{XY} = W^X W^{Y*}$, where $*$ is complex conjugate. The power spectrum P_k^X and P_k^Y of X_n and Y_n are defined as *Equation 3*.

$$D(\frac{\left|W_n^X(s)W_n^{Y*}(s)\right|}{\sigma X \sigma Y} < p) = \frac{Z_v(p)}{v}\sqrt{P_k^X P_k^Y} \quad \text{(Eq.3)}$$

where $Z_v(P)$ is the confidence degree of *P*, which is the square root of the wavelet spectrum.

The phase angle of the wavelet coherence is defined as *Equation 4*.

$$a_m = \arg(X, Y) = \arg\left[\sum_{i=1}^{n}\cos(a_i), \sum_{i=1}^{n}\sin(a_i)\right] \quad \text{(Eq.4)}$$

The standard deviation of the wavelet phase angle is defined as *Equation 5*.

$$s = \sqrt{-2\ln(R/n)} = \sqrt{-2\ln(\sqrt{X^2+Y^2}/n)} \quad \text{(Eq.5)}$$

The WTC $R_n^2(S)$ is the square of the of X_n and Y_n, and $R_n^2(S) \in [0,1]$, normalized by the smoothed wavelet power, where $[\cdot]$ smooth the wavelet spectrum in different time and scale, calculated using *Equation 6* (Jiang et al., 2014).

$$R_n^2(s) = \frac{\left|S\left[s^{-1}W_n^{XY}(s)\right]\right|^2}{S\left[s^{-1}\left|W_n^X(s)\right|^2\right] \bullet S\left[s^{-1}\left|W_n^Y(s)\right|^2\right]} \quad \text{(Eq.6)}$$

where $W_n^X(s)$ and $W_n^Y(s)$ are the wavelet transform of X_n and Y_n. The statistical significance level of the WTC is estimated using Monte Carlo methods. The smoothing operator S of $W_n^{XY}(s)$ in numerator and wavelet power spectrum (WPS) in denominator is given in *Equation 7* (Jevrejeva et al., 2003; Jiang et al., 2019a).

$$S(W) = S_{scale}\{S_{time}[W_n(s)]\} \quad \text{(Eq.7)}$$

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where S_{scale} and S_{time} are denoted smoothing along the wavelet scale axis and in time. S_{scale} and S_{time} are defined as *Equation 8* and *Equation 9*.

$$S_{scale}(W)\Big|_s = \left(W_n(s) * c_1 \frac{-t^2}{2s^2}\right)\Big|_s \quad \text{(Eq.8)}$$

$$S_{time}(W)\Big|_n = \left(W_n(s) * c_2 \Pi(0.6s)\right)\Big|_n \quad \text{(Eq.9)}$$

where c_1 and c_2 are the normalized constants, Π is the rectangle function, and 0.6 is the scale de-correlation length for the Morlet wavelet.

Grey relational analysis

The grey relational analysis is used to analyze the degree of correlation between the influencing factors and groundwater depth, and to determine the main factors. The main evaluation model is determined in *Equation 10*.

$$R = Y \times W \quad \text{(Eq.10)}$$

where R is the comprehensive evaluation result vector of indicators, Y is the evaluation matrix of indicators, and W is the weight vector of indicators. The influencing factors selected in this study are rainfall, temperature, evapotranspiration, and GDP. The target value vector is determined in *Equation 11*.

$$F = \left[x_1^i, x_2^i, x_3^i, x_4^i\right] \quad \text{(Eq.11)}$$

where x_k^i is the original value of the index k in year i, and $(i = 1,2,\cdots 18), (k = 1,2,\cdots,4)$.

The physical meanings of the indicators are different, so the dimensions of the data are not the same. It is not easy to directly compare them, and it's necessary to perform the dimensionless processing of the data. The *Equation 12* is used for indicator normalization.

$$C_k^i = \frac{x_k^i - x_{k1}}{x_{k2} - x_k^i} \quad \text{(Eq.12)}$$

where x_{k1} is the minimum value of the k index, and x_{k2} is the maximum value of the k index.

The comprehensive evaluation is calculated by the correlation coefficient between each comparison column and the corresponding element in the reference column, which is determined as *Equation 13*.

$$\xi_i(k) = \frac{\min\limits_i \min\limits_k \left|C_k^* - C_k^i\right| + \rho \max\limits_i \max\limits_k \left|C_k^* - C_k^i\right|}{\left|C_k^* - C_k^i\right| + \rho \max\limits_i \max\limits_k \left|C_k^* - C_k^i\right|} \quad \text{(Eq.13)}$$

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where ρ is the resolution coefficient, and $0<\rho<1$. Normally, $\rho=0.5$.

The correlation degree refers to the average value of the correlation coefficient between each influencing element and the corresponding element in the reference column, which is determined as *Equation 14*.

$$r_i = \frac{1}{m}\sum_{k=1}^{m}\xi_i\ (k) \qquad \text{(Eq.14)}$$

According to r_i, factors related to the reference sequence are obtained. A larger r_i indicates that the factor is more closely related to the dependent variable.

Random forest (RF) model

The statistical model and artificial intelligence model are two common types of predictive models (Breiman et al., 2010). The statistical models take a long time to analyze the data of long-term series, and it is difficult to accurately predict the trend of the dependent variable. The artificial intelligence model has strong fitting ability and high intensive reading, so the random forest model is used to predict the groundwater depth in this study. Breiman et al. (2001) proposed the random forest model based on the regression tree model, which is widely used to explain the effect of multivariate on the dependent variable and to predict the dependent variable. The principle of RF model is to randomize the use of variables (columns) and data (rows), so that the classification trees can be generated, which can significantly improve the prediction accuracy (Van et al., 2014).

There are two key indicators in the RF model including the average reduction of standardity and Gini coefficient (Hong et al., 2017), indicating the number of trees (ntree) and the number of variables (mtry). The ntree changes according to dataset size, for datasets that are small, 50 trees may suffice. However, large datasets might require 100 or more trees. The ntree is often set to 100 in experiments (Bahareh et al., 2019). In this experiment, we randomly selected 70% of the dataset to calibrate the RF model. The remainder (30%) was used for accuracy testing. Variable importance is obtained from *Equation 15*.

$$VI_{mp}\left(Y_j\right) = \frac{1}{N}\sum_t errOOB_t^j - errOOB_t \qquad \text{(Eq.15)}$$

In *Equation 15*, N is the total number of trees. The function $VI_{mp}(Y_j)$ indicates the importance of the variable. The term $errOOB_t$ is the prediction error when all factors are considered, whereas $errOOB_t^j$ is the error after removing the j variable.

The RF model determines the importance of each variable based on the impact of the overall error on the model. By fitting the correlation between variables, it can be applied to different types of variables and missing values (Pourghasemi et al., 2013). The RF model can avoid over fitting and bias due to variable diversity (Rahmati et al., 2016). The Python is used for programming. The groundwater depth is used as independent variable, and the evolution factors such as precipitation, temperature, evapotranspiration, and GDP that affect the depth of groundwater are used as independent variables, to predict the groundwater depth for the period of 2000-2017.

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Results and discussion

Spatial and temporal variability of groundwater depth

Temporal distribution of interannual variation of groundwater depth

Figure 2 shows the relationship between groundwater depth and precipitation in Xingtai city from 2000 to 2017, based on the average groundwater depth and precipitation data monitored at the observation wells in the study area. It can be seen that the fluctuation of groundwater depth is generally consistent with the change of annual precipitation, indicating that precipitation is one of the influencing factors of groundwater depth. From the trend line, it can be seen that the groundwater depth in Xingtai city has deepened, and the Person's correlation coefficient is 0.95 showed that the changes were statistically significant. The groundwater depth has dropped 5.56 m from 16.75 m in 2000 to 22.31 m in 2017. The decrease rate of groundwater depth is 0.7 m/a, and the descending rate is relatively large. The groundwater depth has been in a continuous downward trend from 2003 to 2012. The depth has slightly rebounded from 2015 to 2017, mainly because Hebei province unveiled effective engineering and non-engineering measures for groundwater overexploitation control in 2014.

The uneven distribution of water resources in Xingtai city, and the conditions of the groundwater depth also varied in counties. According to the counties of each monitoring station, the annual average groundwater depth in each county is calculated from 2000 to 2017, as shown in *Figure 3*. The groundwater depth in Heilonggang Plain including Julu county, Guangzong county, Nangong county and Wei county, is relatively shallow and the variations are within 5 m from 2000 to 2017. However, the depth is deep and changes more than 10m in the Luxi Plain including Nanhe, Longyao and Ren counties. The Longyao county has the maximum change of groundwater depth, and it has declined 1.97 m/yr.

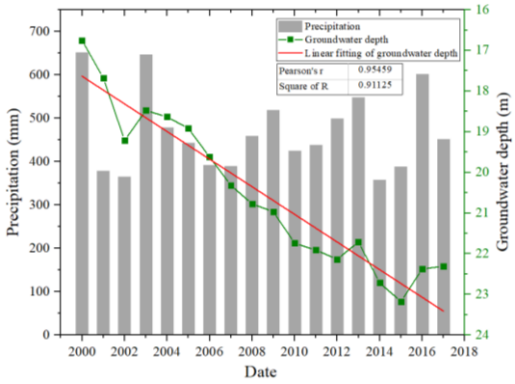

Figure 2. *Average groundwater depth and precipitation*

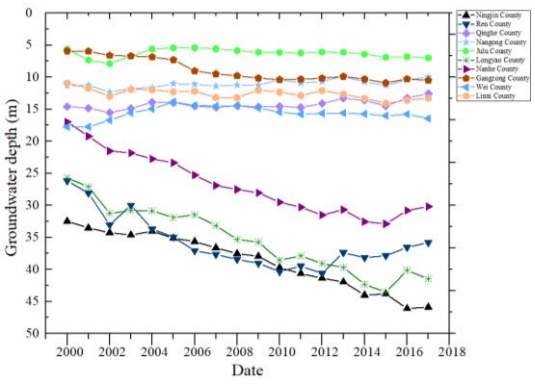

Figure 3. *Groundwater depth of counties*

Spatial distribution of interannual variation of groundwater depth

Figure 4 shows the spatial distribution of interannual changes in the groundwater depth in Xingtai city at 2000, 2005, 2010 and 2017 using Kriging interpolation method. It can be seen that the groundwater depth in the northwest of the study area is deeper than that in the southeast region. The annual average groundwater depth has increased from 18.25 m to 23.97 m, indicating an overall deepening trend. In 2000, the

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groundwater depth in northwestern areas of Ningjin county and Longyao county were deeper than in other counties, and the groundwater overexploitation was serious. However, it rebounded significantly in 2005. The overall groundwater depth of Julu County and Guangzong county was relatively shallow in 2000. As the overexploitation has increased, the groundwater depth has gradually deepened. The groundwater depth in the northwestern part of Nangong city was shallow in 2000, but the increase of overexploitation had decreased by 2015. It has rebounded under the implementation of overdraft control measures during 2015-2017. In the southeast of Wei County and the northwest of Linxi County, the groundwater depth dropped sharply in 2010.

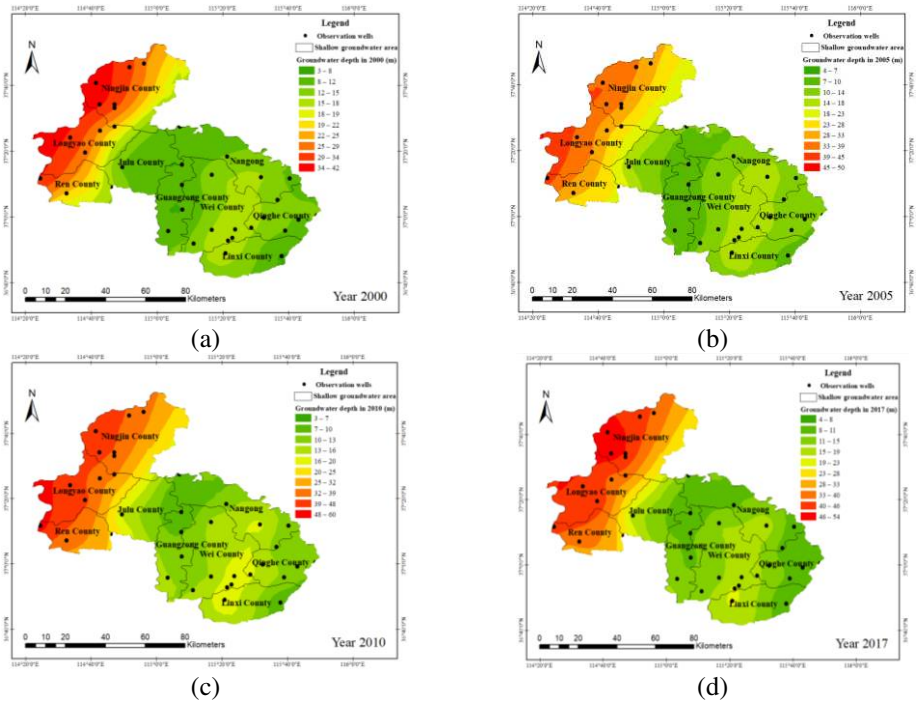

Figure 4. *Spatial distribution of interannual changes in groundwater depth (a) Groundwater depth in 2000; (b) Groundwater depth in 2005; (c) Groundwater depth in 2010; (d) Groundwater depth in 2017*

The results show that the groundwater depth in Xingtai city has deepened for the period of 2000-2017, and the rate of change has increased from 2000 to 2017, which is mainly consistent with previous results. For example, Li et al. (2014) found that the groundwater levels showed an overall decreasing trend over the past 50 years, and an increase in the groundwater depth at a rate of approximately 0.36 m/a.

Intraannual variability of groundwater depth

Figure 5 shows the trends of monthly groundwater depth in two typical years including 2016 and 2017, to analyze the intral-annual variations of groundwater depth in Xingtai city. The trends of groundwater depth in different years are similar, and June groundwater depth is the deepest. The annual spring irrigation period is from March to June, and agricultural irrigation is mainly from the exploitation of shallow groundwater, which made the groundwater depth deeper. With the increase of rainfall from July to September, the high permeability of the aquifer makes the groundwater recharged and

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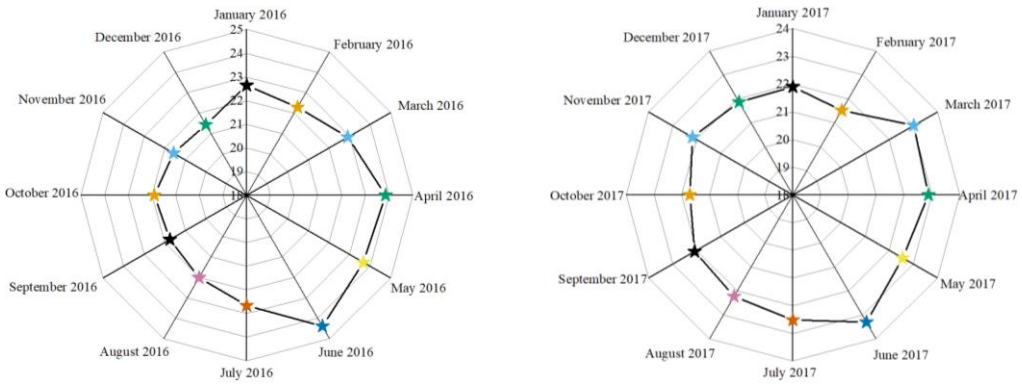
the groundwater depth rebounded. Crops like winter wheat are at the seedling stage from October to December, during which time the water demand is reduced, domestic water is stable, irrigation and replenishment are in a relatively balanced period, and thus the groundwater level is basically stable during this period. The trend throughout the year shows that the change of groundwater depth is related to the consumption during agricultural irrigation period and the replenishment of rainfall during flood season.



(a) (b)

Figure 5. *Trend of groundwater depth in typical years (a) Trend of monthly groundwater depth in 2016; (b) Trend of monthly groundwater depth in 2017*

The results showed that during the crops growing season from March to June, the groundwater depth is deep, which is mainly consistent with previous results. For example, Wang et al. (2009) found that the lowest water table was in the dry season with strong evaporation and less precipitation. Zheng et al. (2019) demonstrated that cultivation intensified water evaporation in the top soil layer (upper 10-20 cm), and the groundwater depth is reduced. The results showed that from July to September, the groundwater depth is recovered due to the increase of the rainfall, which is also mainly consistent with previous results. Wang et al. (2009) detected that the water table increases rapidly due to recharge by precipitation.

Spatial distribution of trends and abrupt change of groundwater depth

Figure 6 shows the spatial distributions of the trends of groundwater depth groundwater depth trends for 41 monitoring stations in the shallow aquifer of Xingtai city from 2000 to 2017 using the non-parametric Mann-Kendall test. The positive magnitude indicates the increasing trend of groundwater depth, and vice versa for decreasing trend. The results showed that eleven stations had downward trends in groundwater depth (the blue downward triangle), indicating that the groundwater depth has become shallower. All of them are distributed in the southeast of Xingtai city. Four stations that had significant decreasing trend at 10% significant level were located in the northern Guangzong county, central Nangong city, southern Wei county and northern Qinghe county, indicating clear rising trends of groundwater depth. However, there are six stations located in the middle of the shallow area have increasing trends. The stations located in the west and south of the shallow area shown significant upward trends (the purple upward triangle) at 5% significant level, indicating that the

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groundwater depth will continue to decline, and the groundwater in these regions are overexploited which should be emphasized for governing.

Figure 7 shows the result of abrupt change of the groundwater depth using Mann-Kendall test. It can be seen that the groundwater depth in Xingtai city has increased since the 21st century (*UF*>0, *UB*>0), and it has significantly increased at significant level of 5% after 2005, indicating that the amount of groundwater exploitation has significantly increased since 2005. The results are mainly consistent with previous results. For example, Liu et al. (2017) found that the groundwater level continues to decline in the 21st century. At the same time, a sudden change in groundwater depth occurred in 2006, and the problem of groundwater overexploitation are beginning to be prominent.

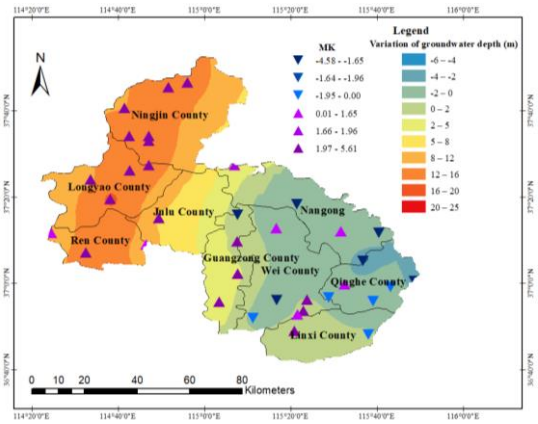

Figure 6. *Non-parametric Mann-Kendall test of groundwater depth in shallow aquifer*

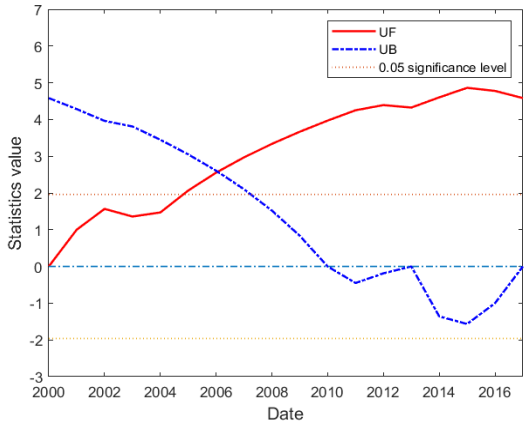

Figure 7. *Mann-Kendall abrupt change test of groundwater depth*

The influencing factors of groundwater depth and their relationships

Correlation and wavelet coherence analysis

The recharge of groundwater system in Xingtai city of Hebei province mainly originated from precipitation, river and irrigation infiltration. The excretion is mainly from groundwater exploitation and evapotranspiration of shallow aquifer groundwater. In addition, some potential factors also affect the change of groundwater depth. The annual average precipitation, evapotranspiration of shallow aquifer groundwater, annual average temperature, and GDP of Xingtai city are selected as influencing factors in this following analysis.

The importance of the influencing factors is obtained by using the gray relational analysis. From the correlation between the influencing factors and the groundwater depth, it can be obtained that the main influencing factor of the groundwater depth is the precipitation, followed by the temperature, and the correlation degrees are 0.9965 and 0.7193, respectively. It is indicated that increasing the amount of groundwater recharge is the first task to improve the groundwater overexploitation problems. The correlation degree between GDP of Xingtai city and the groundwater depth is 0.5652. The correlation coefficient between the evapotranspiration of shallow groundwater and the groundwater depth is 0.5192.

The wavelet coherence is further used to analyze the nonlinear relationships between groundwater depth and each influencing factor including rainfall, temperature (Tem),

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evapotranspiration, GDP, as shown in *Figure 8*. The results are compared with the importance scores obtained from the grey relational analysis. It can be seen that the groundwater depth is negatively correlated with the rainfall, and negatively correlated with the temperature. The groundwater depth is negatively correlated with GDP in Xingtai city for the period of 2000-2017.

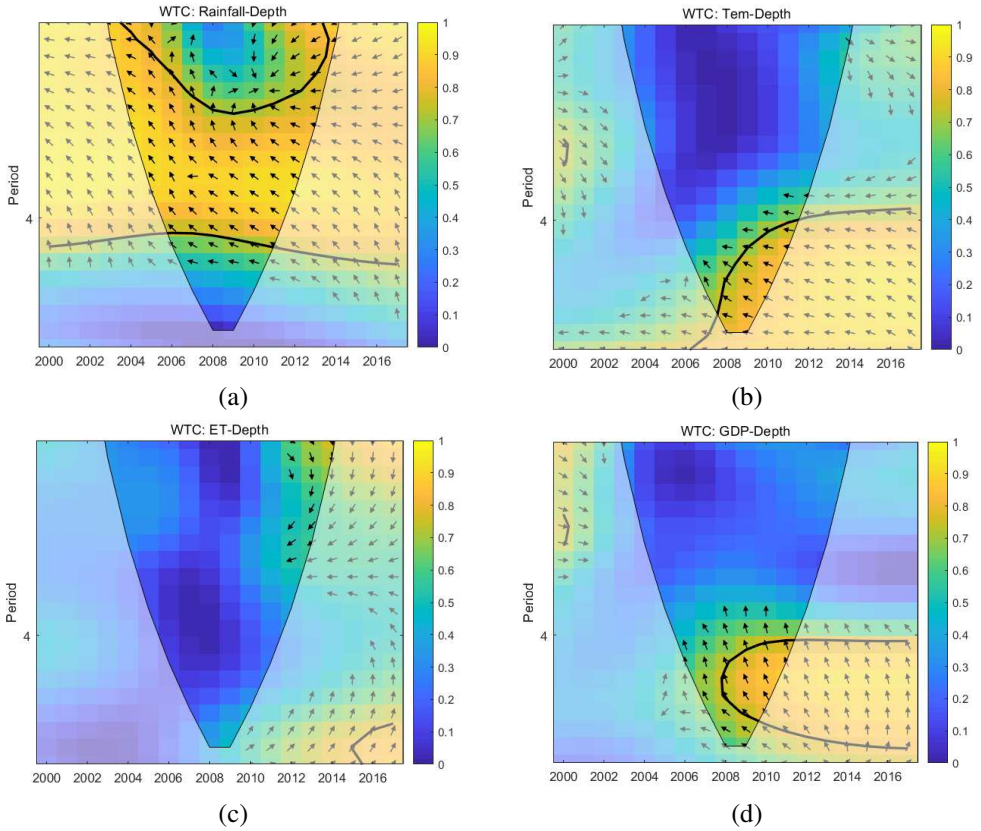

Figure 8. *The WTCs between groundwater depth and (a) Rainfall, (b) Temperature (Tem), (c) evapotranspiration, (d) GDP in Xingtai city for the period of 2000-2017*

The results revealed in *Figure 8* using wavelet coherence are generally consistent with the results using gray correlation analysis, indicating that the wavelet coherence method should be more feasible to investigate the nonlinear relationships between groundwater depth and influencing factors. The results indicated that precipitation is the mainly influencing factor of the groundwater depth, which is mainly consistent with previous results. For example, Liu et al. (2016) showed that large rainstorms can play an important role in regional shallow groundwater resources to recharge the groundwater. Meng et al. (2015) found that the precipitation infiltration recharge is closely related to the water table decrease. The results of the relationship between temperature and groundwater depth is mainly consistent with the previous results of Zhang et al. (2014), who detected that the average temperature of NCP had a close relationship with groundwater depth. The results showed that there is no significant correlation between evapotranspiration and groundwater depth, which is mainly concurred with Bai et al. (2017), who demonstrated that the total agriculture ET stayed relatively stable, in spite of the great adjustment of cropping pattern to reduce the exploitation of groundwater.

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Tian et al. (2016) found that the factors such as the evaporation are less relevant to the groundwater depth variations, which concurred with our results.

Prediction of groundwater depth

The precipitation, temperature, evapotranspiration and GDP of Xingtai city are used as independent variables in multiple linear regression model, and the groundwater depth is dependent variable. The multiple linear regression is given in *Equation 16*, which can be obtained using Statistic Package for Social Science (SPSS) software.

$$y = -0.005x_1 - 1.703x_2 - 0.001x_3 + 0.003x_4 + 45.911 \quad \text{(Eq.16)}$$

where x_1 is the average annual precipitation, x_2 is the average annual temperature, x_3 is the evapotranspiration of shallow aquifer groundwater and x_4 is the GDP of Xingtai city for the period of 2000-2017.

The relative error is used to evaluate the simulation accuracy of the regression equation. *Figure 9a* shows the changes of the measured and predicted values of groundwater depth using multiple linear regression. It can be seen that the absolute value of the relative error of the groundwater depth during eighteen years is within 7%, and the relative error in 2000, 2001 and 2008 exceeded 5%. The correlation coefficient between prediction and actual values is 0.94. It shows that the predicted value obtained by the multiple linear regression model is consistent with the actual value, which should be an effective method for predicting the depth of groundwater.

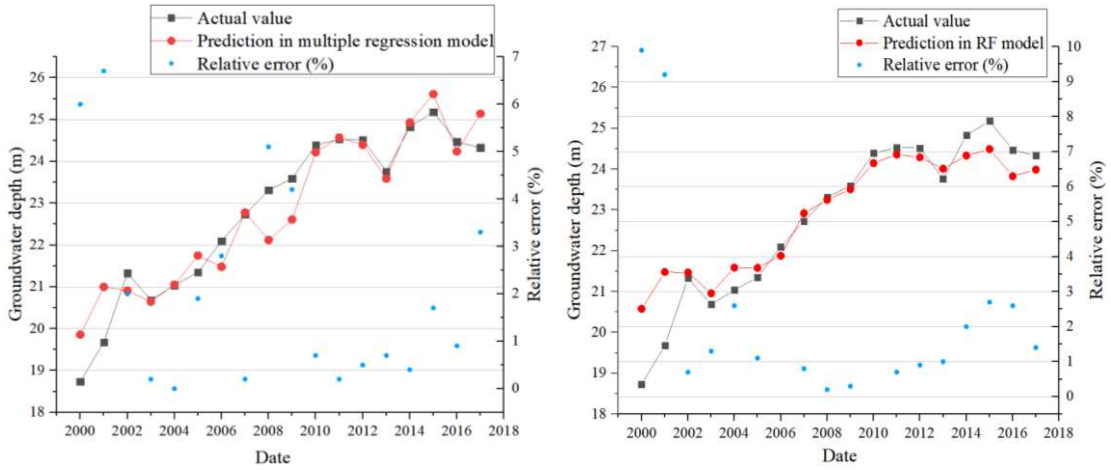

Figure 9. *The prediction and the actual value of groundwater depth by using (a) Multiple linear regression, and (b) RF model*

Similarly, the above four factors are used as independent variables to establish a RF model to predict the groundwater depth, as shown in *Figure 9b*, which showed that the prediction can better fit the actual value, and the correlation coefficient between the actual value and the prediction value is 0.97. Except for the relative error is 9% and the standard deviation is 1.2 in 2000 and 2001, the absolute relative errors of the prediction and actual values are less than 3%, and the standard deviation is less than 0.5 in other years. The prediction error and the standard residual value are small, indicating that the

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RF model is more accurate in analyzing the changes of dependent variables under the influence of multiple factors and predicting the dependent variable.

After comparing the prediction values obtained by two models, it can be concluded that the multiple linear regression is an effective method to predict the groundwater depth using precipitation, temperature, evapotranspiration and GDP as predictors (*Figure 9a*). However, the random forest model has higher accuracy than that of multiple linear regression (*Figure 9b*), which is mainly concurred with Chen et al. (2019), who found that the prediction value of the RF model are ideal in the research of long time series.

Conclusions

The paper investigated the spatial and temporal characteristics of groundwater depth for the period of 2000-2017 in a typical city in NCP. The linear and nonlinear relationships between groundwater depth and influencing factors including precipitation, temperature, evapotranspiration and GDP were examined using gray correlation analysis and wavelet coherence. The multiple linear regression and random forest models were used to obtain the predictions of groundwater depth using the influencing factors as potential predictors. The main conclusions are summarized as follows:

(1) The groundwater depth in shallow aquifer of Xingtai city has generally deepened since the 21st century, and it has significantly increased after 2005. There has been a sudden change of groundwater depth in 2006. The problem of groundwater overexploitation has become prominent. The groundwater depth varied in different regions and different time. In the northwest, the groundwater depth was deep and still had a tendency to deepen. Therefore, it should be the key region for groundwater treatment. After unveiling the groundwater overdraft control measures in 2014 in Hebei province, the groundwater depth in shallow aquifer recovered from 2015 to 2017. In the eastern region, the groundwater depth has rebounded significantly.

(2) The change of groundwater depth is related to the consumption during agricultural irrigation period and the replenishment of rainfall during flood season. The deepest groundwater depth was detected in June. With the increase of precipitation, the groundwater depth rebounded from July to September. The irrigation water and groundwater recharge were in a relatively balanced at the seedling stage from October to December, during which time the groundwater depth was basically stable.

(3) The main factors that affected the depth of groundwater in shallow aquifer in Xingtai city were precipitation, temperature, GDP and evapotranspiration. The grey relational and wavelet coherence analysis showed that the temperature and precipitation had the most significant impacts on groundwater depth. There was a negative correlation between temperature and groundwater depth. The precipitation was negatively correlated with groundwater depth. There was a negative correlation between GDP and groundwater depth. However, there was no significant correlation between evapotranspiration and groundwater depth.

(4) The groundwater depth was predicted using two models including the multiple regression and RF model. The correlation coefficient between the predictive value and the actual value using the multiple linear regression model was 0.94, and 0.97 for RF model, indicating that the RF model should be more suitable to predict the groundwater depth than multiple regression, mainly because the nonlinear relationships between

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groundwater depth and its influencing factors, which were also revealed by the correlation analysis.

(5) In the process of excavating the influencing factors of groundwater depth, this paper mainly considered four factors including precipitation, temperature, GDP, and evapotranspiration. However, the change of groundwater is complicated, and it will be affected by other potential factors. Future studies are recommended to consider other influencing factors that might affect the groundwater depth. In addition, time series should be extended in future studies, in order to define cycles and analyze the relevance more accurately.

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RESTORATION OF Cu^{2+}-CONTAMINATED PURPLE SOIL BY APPLYING YEASRACT AND INTERPLANT

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Abstract. Beer yeast powder was added to purple soil (PS) at a 1‰ mass ratio to prepare mixed soil samples (PS_B) to investigate the enhancement effect of fungus in the restoration of Cu^{2+}-polluted PS by sudangrass (*Sorghum sudanense* (Piper) Stapf.) and the intercropping of sudangrass and calliopsis (*Cosmos bipinnata* Cav.). Then, indoor potted experiments were performed to explore the physiological indices and Cu^{2+} accumulation in sudangrass and calliopsis in different Cu^{2+}-polluted PS and PS_B. Three key results were observed. (1) PS_B promoted the germination rate of sudangrass but inhibited that of calliopsis; the germination rates of sudangrass planted alone were higher than those of the intercropped ones under the same Cu^{2+} pollution concentration. Sudangrass grew well on PS_B, and its height was higher than that of calliopsis under intercropping conditions. (2) The aboveground and underground biomasses of sudangrass planted in PS_B by monoculture were higher than those by intercropping. The correlation of plant physiological indices in PS_B was higher than that in PS. (3) Cu^{2+} content in roots was higher than that in cormus. Soil polluted with 75 mg/kg Cu^{2+} was beneficial to the enrichment of plant, and the translocation coefficient was the largest in PS_B.
Keywords: *beer yeast powder, plant intercropping, phytoremediation, physiological characteristics, contamination accumulation*

Introduction

The discharge of livestock raising, industrial, and agricultural wastewater has caused serious heavy metal pollution of soil (Li et al., 2017; Barsova et al., 2019). The pollution control work became a difficult and important topic in the current research (Deng et al., 2020). Soil microbes can not only promote plant growth but also enhance the absorption of heavy metal ions by pollution-repairing plants (Liu et al., 2013). Therefore, adding microbes to the soil to improve the plants' remediation ability bears significance for the research of combined plant–microorganism remediation of soil pollution.

Phytoremediation has been the focus of studies and applied by many researchers due to its low cost and easy operation (Zheng and Yuan, 2017; Acosta et al., 2018). Seed germination rate and plant growth are promoted under low-concentration copper pollution (Li et al., 2018), but when Cu^{2+} levels in the soil exceeds a certain load, the growth of

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plants will be seriously affected (Jin et al., 2012; Fu et al., 2016). *Elsholtzia splendens* Nakai and *Commelina communis* L. show high tolerance to Cu^{2+}-contaminated soil, whereas *Commelina communis* L. can normally grow in soil with a Cu^{2+} concentration of 500 mg/kg (Liu et al., 2006, 2014). In the phytoremediation of Cu^{2+}-contaminated soils, biomass directly determines the efficiency of restoration (Peng and Yang, 2005), whereas root exudates promote the root absorption of Cu^{2+} (Xu et al., 2017). Sudangrass (*Sorghum sudanense* (Piper) Stapf.) grows vigorously, has well-developed roots, and shows strong adaptability and resilience (Jia et al., 2014); thus, it is widely used in soil remediation and pollution control. The Cu^{2+} accumulation of sudangrass root reaches 673 mg kg^{-1} (Wang, 2014). Cropping patterns have observable effect on plant biomass and its adsorbability to heavy metals (An et al., 2011; Singh et al., 2012; Li et al., 2013; Wei et al., 2015), whereas intercropping can change the types and quantity of rhizospheric microorganisms (Nai et al., 2013), soil enzyme activity in the rhizosphere (Zhang et al., 2015), plant growth, and nutrient conditions and ultimately influence the absorption effect of heavy metals by plants (Su et al., 2010). Therefore, adjusting the planting pattern is one of the important ways to improve the effects of phytoremediation.

Microbial remediation technology has become an important topic in the treatment of heavy metal pollution (Xue et al., 2012; Jacob et al., 2018). Microorganisms present a good adsorption effect on heavy metal ions with the advantages of large specific surface area, rapid reproduction, and strong metabolic capacity (Cao et al., 2016), and their repair mechanisms mainly include biosorption and biotransformation (Xue et al., 2012). Cao and Cheng (2004) reported that at low Cu^{2+} concentration ($\leq$ 5 mg/L) in the soil environment, microorganisms exhibited a good repair performance for Cu^{2+} pollution, and the removal (consolidation) rate reached 25%–60%. Stanila et al. (2016) noted that beer yeast has a high adsorption capacity and adsorption rate for copper ions, and the adsorption equilibrium can be reached in 10 min. Studies demonstrated that the plant-microbial repair system is beneficial to the remediation of heavy-metal contaminants in soil (Li et al., 2015).

The phytoremediation, microbial remediation, and combined repair of Cu^{2+} in soil all achieve good outcomes. The advantage of intercropping can be further exploited if the combined microorganism–plant repair is optimized. The repair effect of Cu^{2+} can be improved to a great extent due to the no-increase repair cost. Minimal exploration was conducted in this area. In this paper, beer yeast was used to strengthen the intercropping of sudangrass and calliopsis (*Cosmos bipinnata* Cav.) for repairing different degrees of Cu^{2+} pollution of purple soil (PS). By measuring the changes in physiological characterization of sudangrass and calliopsis under different Cu^{2+} conditions and analyzing the combined repair effect of applying yeasract and interplant on Cu^{2+} pollution, this study can provide a reference for microorganism–plant intercropping to repair heavy metal-contaminated soil.

Materials and methods

Experimental materials

Potted soil (PS; a kind of soil formed on weathered materials of purple rock, and it maintains the physical and chemical properties of the parent material) was collected from the test field of China West Normal University. After multi-point sampling, the soil samples were mixed evenly, dried, and crushed, and the physicochemical properties of the soil samples were measured. *Table 1* shows the results.

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Table 1. *Basic properties of the test soil*

Soil layer (cm)	pH value	CEC (mmol/kg)	TOC content (g/kg)	Cu^{2+} content (mg/kg)
0~30	8.08	288.46	16.66	18.60

The fungus powder was tested with beer yeast, which is a yellow-brown powder and granule with more than 60 million active bacteria per gram and less than 10% water content. The beer yeast was purchased from Guangzhou Pengxiang Agriculture Co., Ltd.

The test plants were sudangrass and calliopsis. The seeds with impurities, worm corrosion, and low maturity were discarded. A total of 960 full seeds of sudangrass and 120 full seeds of calliopsis were selected and soaked in 1% potassium permanganate solution for 15 min, rinsed with tap water, and three times by deionized water, and finally dried with a filter paper.

Copper pollution concentration was expressed as Cu^{2+}; the reagent was analytically pure $CuSO_4 \cdot 5H_2O$ and purchased from Xilong Chemical Industry Co., Ltd.

Experimental design

Beer yeast powder (B) was mixed with PS at a mass ratio of 1‰ to form the amended soil sample (PS_B). Three groups of indoor pot experiment of PS-S (single sudangrass planted on PS), PS_B-S (single sudangrass planted on PS_B), and PS_B-M (sudangrass and calliopsis intercropping planted on PS_B) were implemented. Among PS_B-M, PS_B-MS (sudangrass in sudangrass–calliopsis intercropping) and PS_B-MC (the calliopsis in sudangrass–calliopsis intercropping) were measured separately.

The Cu^{2+} content of soil in the pot experiments was set up for four treatments: 0, 75, 100 and 125 mg/kg soil. Each experiment was repeated thrice.

Plastic pots with a diameter of 43 cm, a width of 15.5 cm, and a height of 11 cm were loaded with 2.0 kg soil samples. Then, 500 mL Cu^{2+} solutions of different concentrations (0, 300, 400, and 500 mg/L) were added to the 36 pots in order. After the Cu^{2+} solution was permeated uniformly, 30 plant seeds (the number of sudangrass and calliopsis were 20 and 10 in the intercropping experiment, respectively) were sown uniformly at a depth of 2 cm below the soil surface of each pot. Then, the pots were watered at 100 mL volume per week after germination (four times in total). During the growth period, the germination rate and plant height of sudangrass and calliopsis were measured, and the biomasses of aboveground and underground parts were measured after harvest.

Experimental method

Determination of germination rate and plant height

The germination rates of sudangrass and calliopsis seeds and the number of seed germination was recorded every day. When the number of germinating seeds remained unchanged for two consecutive days, the germination was considered complete, and the germination rate was computed. The formula for germination rate is:

$$\text{Germination rate} = (\text{germination number of seeds} / \text{total number of seeds}) \times 100\% \quad \text{(Eq.1)}$$

The average plant height of sudangrass and calliopsis was measured every 3 days after germination (10 times in total). The plant height of all plants (sudangrass and calliopsis)

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was measured separately. Then, the highest and lowest 10% scores were removed, and the average value of the remaining plants was calculated to obtain the average plant height.

Determination of biological indicator

All the plants were harvested after 30 days of growth. Then, the plant samples were divided into aerial (stems and leaves) and underground parts (roots). The soil and dirt that attached to the plant samples were thoroughly rinsed off with tap water, followed by rinsing with distilled water for three times. Then, the water was filtered out, and the fresh weight was determined. After weighing, the samples were individually loaded into a kraft bag and cured in an oven at 105 °C for 30 min and then dried to constant weight at 85 °C. The dry weight of the aerial parts and root parts was determined.

Determination of Cu^{2+} in plants

The leaves and root samples of the dried plant were grinded and passed through a 100-mesh nylon sieve. Cu^{2+} contents in plant samples were determined via Hitachi Z-5000 (Japan) flame atomic absorption spectrophotometry, and background absorption was corrected through Zeeman effect.

Data processing

SPSS 16.0 statistical analysis software was used to process the experimental data for variance and correlation analysis. SigmaPlot 10.0 software was adopted to improve data plotting. The data were expressed as the means with standard deviation, and different letters indicate significant differences among various amendments. Analysis of variance was performed to determine the effects of amendments, followed by Tukey's honestly significant difference test. Differences of $p < 0.05$ were considered significant.

Results and discussion

Effect of Cu^{2+} on the germination rate

Figure 1 shows the germination rate of plant seeds under different Cu^{2+}-contaminated soils. With the increase in Cu^{2+} concentration, the seed germination rates decreased in PS-S and PS_B-MC, whereas it increased first and then decreased in PS_B-S and PS_B-MS. When the concentration of Cu^{2+} was 0 mg/kg, the germination rate of PS-S was the highest (90%), that is, it was 1.13 times higher than that of other treatments. When the concentration of Cu^{2+} increased to 75 mg/kg, the seed germination rate of PS_B-S and PS_B-MS increased by 10.00% and 5.00% compared with 0 mg/kg, respectively. Meanwhile, the PS-S and PS_B-MC decreased by 20.00% and 10.00% compared with 0 mg/kg, respectively. When the concentration of Cu^{2+} was 100 mg/kg, the PS_B soil showed a strong promoting effect on the seed germination of sudangrass (PS_B-S and PS_B-MS), and the germination rate increased by 16.67% and 15.00% compared with that of 0 mg/kg. However, the germination rate decreased under the 125 mg/kg Cu^{2+} treatment.

Under the same Cu^{2+} concentration, the plant germination rate was expressed as PS_B-S > PS_B-MS > PS-S > PS_B-MC. When Cu^{2+} concentration was 0–125 mg/kg, the germination of sudangrass was promoted, but calliopsis was inhibited in PS_B. In general, PS_B soil samples had a promoting effect on the seed germination of sudangrass and inhibiting effect on calliopsis.

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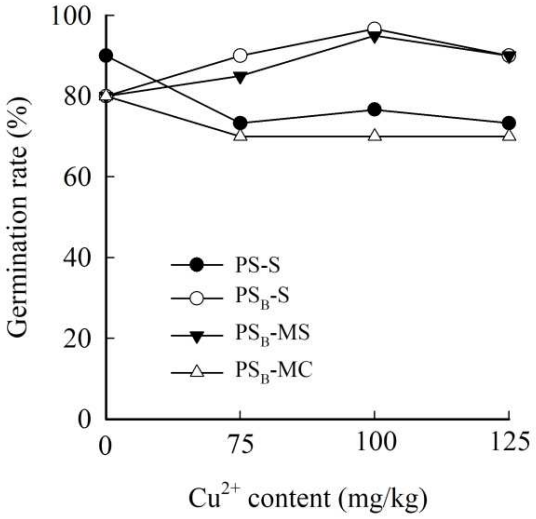

Figure 1. *Effect of Cu^{2+} content on the germination rate of the studied plants*

The germination of plant seeds is affected by pollution stress. Low concentration of pollution stress can improve the germination rate of plant seeds. With the increase in pollution concentration, the inhibiting effect of seed germination will gradually increase until germination cannot be achieved (Salian et al., 2018; Li et al., 2018). The addition of beer yeast can increase the microbial activity in soil, and at the same time, microorganisms can metabolize certain pollutants (Riaz-ul-Haq and Shakoori, 2000; Liu et al., 2013), thus reducing the inhibitory effect of pollutants on seed germination rate. Therefore, for PS-S treatment, 75 mg/kg Cu^{2+} treatment inhibited the germination rate of sudangrass, whereas for PS_B-S and PS_B-MS, the germination rate increased continuously with the Cu^{2+} content from 0 mg/kg to 100 mg/kg and reached the maximum under 100 mg/kg treatment, indicating that the addition of beer yeast played a role in promoting seed germination. Cu^{2+} inhibited the seed germination rate of calliopsis planted by intercropping, indicating the existence of inter-species survival competition (Sofo et al., 2013), which is consistent with the results of previous studies.

Effect of Cu^{2+} on plant height

Figure 2 shows that after germination (3 days later), the plant height gradually increased with prolonged growth period. The plant height of PS-S increased rapidly within 6 days after germination, with an average daily growth of 1.64 cm and a maximum of 1.98 cm. However, the growth rate was slow at 9–30 days and remained stable after the 15th day. The growth rate of PS_B-S increased the fastest with the increase in growth days and reached a maximum of 26.2 cm. For PS_B-M, the height of PS_B-MS was significantly higher than that of PS_B-MC. The plant height treatments were higher in 0 and 75 mg/kg Cu^{2+} than that in 100 and 125 mg/kg Cu^{2+}. At different concentrations of Cu^{2+}, sudangrass grew well on PS_B soil samples, and the height of calliopsis was lower than that of sudangrass under intercropping conditions, which indicates that bear yeast can promote the growth of sudangrass. The plant height and germination rate of the two plants had a similar relationship. After the addition of beer yeast to soil samples, the plant height of sudangrass significantly increased, but that of intercropping plants was inhibited.

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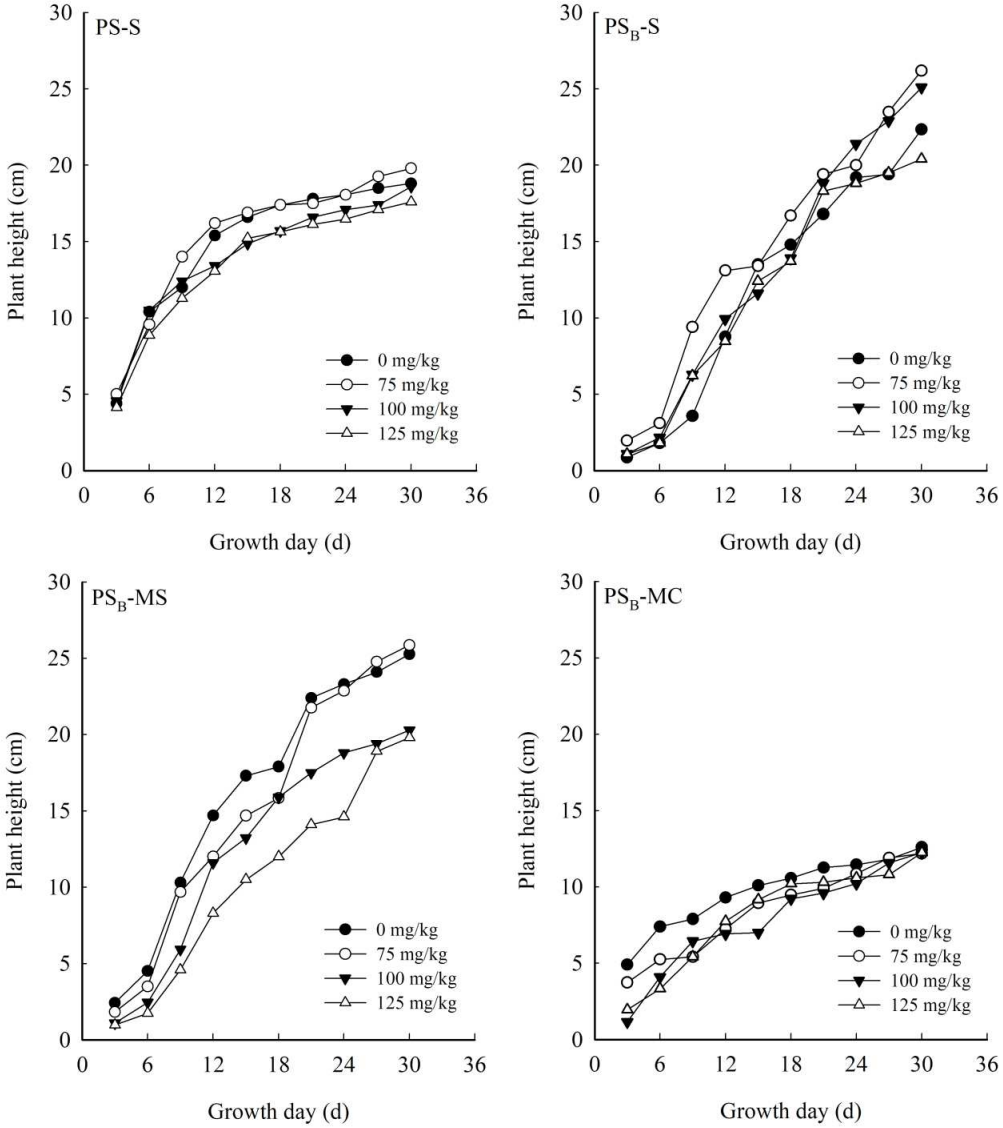

Figure 2. *Average plant height of sudangrass and calliopsis in 30 days*

Fresh and dry weights of aerial parts

The fresh and dry weights of PS-S, PS_B-MS and PS_B-MC increased first and then decreased with the increase in Cu^{2+} content of potted soil. The values reached the maximum at 75 mg/kg Cu^{2+} and 1.34–1.36 (PS-S), 1.43–1.58 (PS_B-MS), and 2.11–2.40 (PS_B-MC) times higher than that of 0 mg/kg Cu^{2+} (*Fig. 3*). The fresh and dry weights of PS_B-S showed a fluctuating trend with the increase in Cu^{2+} content. When the content of Cu^{2+} was 100 mg/kg, the fresh and dry weights of PS_B-S were the highest at 4.97 and 0.64 g, respectively, and significantly differed from those of other treatments ($p < 0.05$).

For the single-planting experiments, PS_B promoted the aboveground biomass growth of sudangrass, and the maximum biomass was reached at the Cu^{2+} content of 100 mg/kg. For the intercropping plants, the maximal promotion of the aboveground biomass was recorded at the Cu^{2+} content of 75 mg/kg. In summary, the aboveground biomass of sudangrass planted in PS_B by monoculture was higher than that planted by intercropping.

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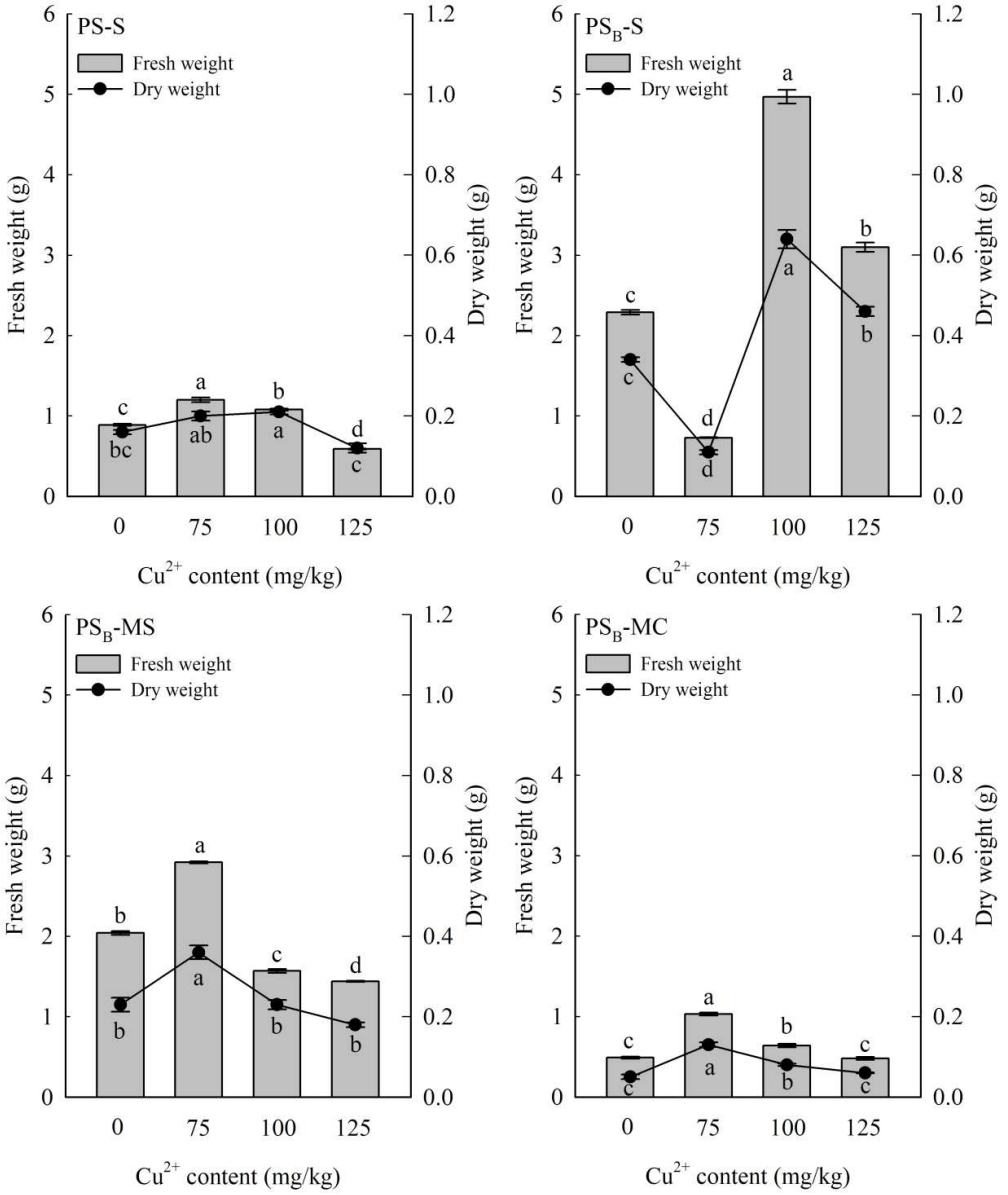

Figure 3. *Fresh and dry weights of aboveground parts of sudangrass and calliopsis. Note: Different lowercase letters indicate significant difference under various treatments at 0.05 levels (the same meaning of different lowercase letters in the following figures)*

Plant biomass is closely related to the overall growth status. Except for PS_B-S, the biomass of plants under 75 mg/kg treatment was high on the whole, indicating that low-concentration pollution stress is conducive to the accumulation of plant biomass. The biomass of PS_B-S was the lowest at 75 mg/kg Cu^{2+} and increased significantly at 100 mg/kg Cu^{2+}. This result is related to the tolerance of microorganisms to pollutants, and it is consistent with the research results by Boiko et al. (2020).

Fresh and dry weights of underground parts

With the increase in Cu^{2+} content, the root fresh and dry weights of PS-S and PS_B-S increased, reached the peak value at 100 mg/kg Cu^{2+}, and then decreased at 125 mg/kg

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Cu^{2+} (*Fig. 4*). The fresh and dry weights of roots of PS_B-MS and PS_B-MC were the highest under the 75 mg/kg Cu^{2+} treatment. A corresponding relationship was observed between the plant's root fresh and dry weights at different Cu^{2+} concentration treatments. The fresh and dry weights of intercropped sudangrass were significantly higher than those of calliopsis, indicating that sudangrass dominated the competition, and its root system had strong adaptability to Cu^{2+}-polluted PS. However, the plant roots grew poorly under Cu^{2+} concentration of 100–125 mg/kg, indicating that high concentrations of Cu^{2+} inhibited plant root growth. At 75 mg/kg Cu^{2+}, the intercropped plants showed better growth and stronger adaptability, but their underground biomass was still inhibited compared with the monoculture of sudangrass.

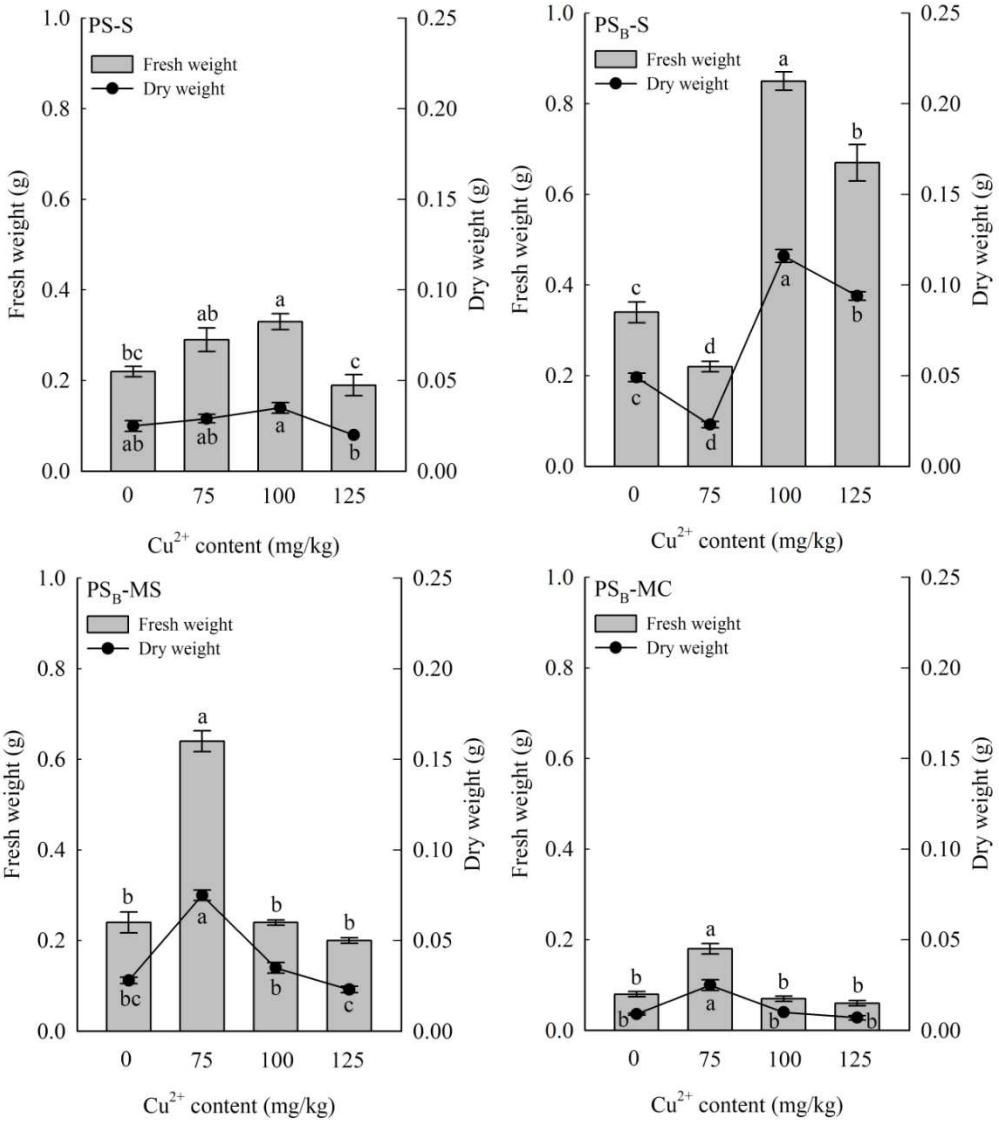

Figure 4. *Fresh and dry weights of roots of sudangrass and calliopsis*

Compared with the aboveground plant parts, plant roots are directly exposed to pollutants and have a higher tolerance to pollution. The root biomass was the largest under the treatment of 100 mg/kg Cu^{2+} for sudangrass monocultures. For the intercropped plants,

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the two kinds of plant roots were influenced not only by pollution tolerance but also their competition relationship. The root biomass of intercropped plants was the largest under 100 mg/kg Cu^{2+} treatment.

Correlation analysis of physiological indices

Table 2 shows the positive correlation between various physiological indicators of sudangrass and calliopsis. No significant correlation was observed between plant height and other indicators, whereas a significant correlation was noted between dry and wet biomass. As for PS-S, the aboveground dry weight was significantly related to the root fresh and dry weights. For PS_B-S, PS_B-MS and PS_B-MC, a significant correlation existed between any two of the aboveground fresh weight, aboveground dry weight, root fresh weight, and root dry weight. An extremely significant correlation was recorded between the aboveground and root dry weights of PS_B-MS and between the aboveground fresh weight and root dry weight of PS_B-MC. The overall correlation of plant physiological indices in PS_B-S was higher than that in PS. For the same plant, the change in root and aboveground biomass was closely related to plant pollution tolerance. Therefore, compared with PS, PS_B showed a higher correlation between the aboveground and underground plant parts, which also indicates that microorganisms promoted the tolerance of plants to pollution.

Table 2. *Correlation analysis of physiological indexes of sudangrass and calliopsis*

Correlation coefficients		Plant height (cm)	Above ground fresh weight (g)	Above ground dry weight (g)	Root fresh weight (g)	Root dry weight (g)
PS-S	Plant height (cm)	1	0.5547	0.2513	0.0904	0.0593
	Above ground fresh weight (g)		1	0.9010*	0.7372	0.6442
	Above ground dry weight (g)			1	0.9525*	0.8885*
	Root fresh weight (g)				1	0.9560*
	Root dry weight (g)					1
PS_B-S	Plant height (cm)	1	0.1585	0.2455	0.1209	0.1926
	Above ground fresh weight (g)		1	0.9861**	0.9156*	0.9316*
	Above ground dry weight (g)			1	0.9198*	0.9531*
	Root fresh weight (g)				1	0.9897**
	Root dry weight (g)					1
PS_B-MS	Plant height (cm)	1	0.5340	0.3605	0.2220	0.2122
	Above ground fresh weight (g)		1	0.9032*	0.8807*	0.8271*
	Above ground dry weight (g)			1	0.9509*	0.9725**
	Root fresh weight (g)				1	0.9778**
	Root dry weight (g)					1
PS_B-MC	Plant height (cm)	1	0.0497	0.1243	0.0010	0.0033
	Above ground fresh weight (g)		1	0.9795**	0.9231*	0.9706**
	Above ground dry weight (g)			1	0.8508*	0.9109*
	Root fresh weight (g)				1	0.9865**
	Root dry weight (g)					1

Note: ** or * indicates that the correlation coefficient is significant at p=0.01 or p=0.05 level (r=0.959 or r=0.878 when the degree of freedom f=3)

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Changes in Cu^{2+} Content of Plant Samples

Table 3 shows the content of Cu^{2+} in plant cormus (leaves and stems) and roots under different soil treatments. Cu^{2+} content in the cormus and roots of plants increased with the increase in soil Cu^{2+} concentration, with the maximum values reaching 8.82 (cormus in PS_B-MC) and 30.15 mg/kg (roots in PS_B-MC). Cu^{2+} content in the root was higher than that in the cormus, which indicates that the plant root system was more likely to enrich Cu^{2+}; this result was consistent with the findings of Wang et al. (2014). The enrichment coefficient of cormus and roots was the largest under the soil treatment of 75 mg/kg Cu^{2+}, indicating that the 60-day-grown plants were more capable of enriching low-concentration pollution. The enrichment coefficient showed the trend of PS_B-MC > PS_B-MS > PS_B-S > PS-S of cormus, whereas the trend was PS_B-MC > PS_B-S > PS_B-MS > PS-S for the roots. The largest translocation coefficient was observed on PS_B-MC, indicating that calliopsis was more capable of Cu^{2+} transport than sudangrass.

Table 3. *Remediation effect of Cu^{2+} on potted plants*

Treatmengts (mg/kg)		Overground part (cormus)		Underground part (roots)		Translocation coefficient (%)
		Cu^{2+} content (mg/kg)	Enrichment coefficient (%)	Cu^{2+} content (mg/kg)	enrichment coefficient (%)	
PS-S	75	2.60	3.47	14.82	19.76	17.54
	100	2.71	2.71	18.90	18.90	14.34
	125	2.76	2.21	19.69	15.75	14.02
PS_B-S	75	4.85	6.47	20.60	27.47	23.54
	100	5.05	5.05	21.81	21.81	18.57
	125	5.88	4.70	23.48	18.78	16.52
PS_B-MS	75	6.30	8.40	20.76	27.68	30.35
	100	6.56	6.56	24.74	24.74	24.09
	125	7.35	5.88	30.15	24.12	17.74
PS_B-MC	75	7.31	9.75	18.86	25.14	39.13
	100	8.07	8.07	23.68	23.68	34.12
	125	8.82	7.06	27.33	21.86	28.61

The above results were mainly due to the direct contact of root system to Cu^{2+}. Thus, the Cu^{2+} content of the root system was higher than that of the cormus. The enrichment and transport coefficients of intercropped plants were higher than that of monocultures and mainly caused by the competition between sudangrass and calliopsis. The competition involved the nutrients and pollution elements.

Conclusions

In this paper, the enhancement effect of fungus in the restoration of Cu^{2+}-polluted PS by sudangrass and the intercropping of sudangrass and calliopsis was studied by indoor potted experiments. The main conclusions are summarized as follows: PS_B promoted the germination rate of sudangrass, and the germination rates of sudangrass planted alone were higher than that of those planted by intercropping. Sudangrass grew well on PS_B, and the height of calliopsis was lower than that of sudangrass under intercropping conditions. The biomass of sudangrass planted in PS_B by monoculture was higher than

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that by intercropping. Cu^{2+} content in roots was higher than that in the cormus. The enrichment coefficient of cormus and roots was the largest under the soil treatment of 75 mg/kg Cu^{2+}.

The results of this study provide a reference for microorganism–plant intercropping to repair heavy metal-contaminated soil. Based on the conclusions obtained in this study, future research can be further discussed in the following aspects: (1) determination of optimum microbial dosage and its combination with plants; (2) collocation and selection of intercropped plants; (3) effects of microorganisms on plant rhizosphere and their combined effects on contaminants.

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INTER- AND INTRASPECIFIC LEAF TRAIT VARIATION INDUCED BY THE LOCAL ENVIRONMENT IN A MONTANE BROAD-LEAVED FOREST IN WESTERN CHINA

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Abstract. Understanding how forest communities respond to environmental factors via intra- and interspecific variation in leaf functional traits is a fundamental challenge in forest ecology. Especially, little is known about the degree to which trees respond to environmental factors at the forest community level. To fill this gap, an experiment across 34 plots was conducted in a subtropical montane broad-leaved forest in western China. Based on 327 trees of 27 species, intra- and interspecific variations in leaf morphological and chemical traits were measured, and their relationship with environmental factors was determined. Canonical correlation analysis (CCA) was used to assess the relationships between the local environment and trait variation. Our results show that leaf morphological traits are related to chemical traits. The contribution of interspecific variability was dominant between species and communities, but intraspecific variability explained a large amount of variation (35.9%–56.2%) in chemical traits, despite the fact that high levels of species turnover were observed at the forest community level. Leaf traits showed responses to local environmental variables, with tree size being most strongly correlated. Our findings emphasize that leaf functional traits are correlated with environmental gradients. Therefore, to study the ecological process in subtropical forests using traits-based approaches, researchers need to account for their considerable intraspecific variability.
Keywords: *leaf morphological traits, leaf chemical traits, interspecific and intraspecific variability, tree size, soil properties, community ecology*

Introduction

Leaf functional traits including morphological traits, chemical traits, physiological traits, and symptoms, balance leaf construction costs against growth potential, reproduction and survival (Violle et al., 2007; Diaz et al., 2016). Foliar morphological traits reflect structural and physical characteristics, mainly including leaf mass, size, morphology and water status (Bussotti and Pollastrini, 2015). For instance, specific leaf area (SLA) is positively related to relative growth rates, leaf turnover rates, foliar nutrient concentrations and photosynthetic capacities in plant community assembly (Wright et al., 2004). Leaf dry matter content (LDMC) in particular has been regarded as an important component of the evolutionary history of species (Shipley et al., 2007; Messier et al., 2010). Foliar chemical traits, characterizing the mineral nutrition status, have been used as important parameters to recognize critical ecological processes of community assembly and species coexistence, and ecosystem structure and function (Aerts and Chapin, 2000; Wright et al., 2004). For example, leaf nitrogen content (LNC) is an extremely relevant ecological index, which is connected to photosynthesis, nutrient cycling, belowground diversity and water quality (Niinemets, 2010). By working with functional traits and their variation within and among communities,

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researchers shed generalisable light on community assembly and ecosystem processes, one example is that three quarters of trait variation is captured in a two-dimensional global spectrum of plant form and function by analyzing variation in six major traits with the largest sample of global vascular plant species (Diaz et al., 2016). Traditionally, most previous studies operating on the mean values of species traits, focused on interspecific differences more between co-occurring species than within species (Wright et al., 2004; Mcgill et al., 2006; Cornwell et al., 2008). However, to date there is now growing evidence that intraspecific variability, can have significant effects on many ecological and evolutionary processes (Violle et al., 2012), moreover, sometimes the extent of intraspecific trait variation is similar to or greater than interspecific variation within and among plant communities. For example, many studies have demonstrated the importance of intraspecific variability for the maintenance of species coexistence, the dynamics of communities and the ecosystem properties in tropical forest, subtropical forest and grass ecosystems (Albert et al., 2010a; Messier et al., 2010; Bolnick et al., 2011). As such we need to consider that moving beyond the species mean approach by focusing on individual traits may improve our predictive ability of community ecology (Violle et al., 2012).

Trait-based community assembly rules have shed light on that environmental variation (or environmental filter) plays in shaping plant community functional trait composition (Albert et al., 2010a; Auger and Shipley, 2013). As the matter of fact, many studies have examined correlations in leaf traits with environmental variables like regional climate, local soil conditions and biotic interactions (Santiago and Wright, 2007; Atkin et al., 2008; Ordoñez et al., 2009), which can influences plant functional diversity and ecosystem function through primary production, carbon sequestration, trophic transfer and litter decomposition (Cornwell et al., 2008; Sedjo and Sohngen, 2012). To our knowledge, along environmental gradients the variation of many popular indices (e.g. aggregated trait averages) reflecting the functional characteristics of locally dominant species in ecological communities can be as a consequence of both species turnover and intraspecific trait variability (Lepš et al., 2011). In other words, not only can trait values among species vary in response to the environment (i.e., niche breadth) via phenotypic plasticity (Ashton et al., 2010), but intraspecific variability can also display different idiosyncratic responses (Albert et al., 2011). Especially, recent work also shows that an increasing interest in accounting for intraspecific functional trait variability on a regional to global scale, the response of functional, ecophysiological or demographic traits to environmental gradients (Mcgill et al., 2006; Hausch et al., 2018; Li et al., 2018). For instance, most studies dealing with intraspecific functional variability have focused on indirect gradients (e.g., altitude, latitude or longitude), which are unknown combinations of multiple direct environmental gradients that impact plant physiology (e.g., temperature, nutrient availability) (Cordell et al., 1998; Albert et al., 2015). Moreover, Violle et al. (2012) reported that the relative contribution of intraspecific trait variation to shifts in community-average trait values along environmental gradients reflects the importance of within-species trait responses to environmental stress. However, Lajoie and Vellend (2015) suggested that the relative contribution of intraspecific variation and species turnover to total trait variation along environmental gradients is poorly understood. For example, on account of the scale of environmental heterogeneity relative to the size of individual plants, the potential for individuals to express genetic and plastic trait differences across different environments, thus the relationship

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between spatial grain (plot or sampling unit size) and the relative extent of intraspecific trait variation within communities is more difficult to predict (Siefert et al., 2015). Therefore, how intraspecific variation is influenced by environmental gradients, this issue might be dependent on the functional traits measured, the spatial scale of observation, and the study habitat type (Luo et al., 2016).

Montane broad-leaved forests in the southwest Sichuan are known for their high biodiversity. Because of the complicated geomorphological features and climate conditions, they shape abundant tree species and large habitat heterogeneities. Moreover, these forest communities appear tremendously diverse ecological characteristics of their spatial structure, functions and dynamics, with wide ranges in growth rates and shade tolerance (Zhao et al., 2009). But to date little is known about the ecological processes of structural and functional features of these broad-leaved forests and their effects of large range of environmental factors in this region (Zhao et al., 2009). Therefore, this study centered on leaf functional traits and its responses to environmental gradients (especially local soil properties and stand structures) in forest communities. As we now know foliar morphology correspond to the fundamental tradeoff in leaf construction costs vs. light-intercepting surface area and foliar chemical compounds influence the nutrient cycling and photosynthetic machinery of forest ecosystems (Wright et al., 2004). Hence, in this study from two aspects of foliar morphology and foliar chemistry we discuss the following questions: (1) How variable are the leaf morphological and chemical traits across individuals and communities? (2) Whether the contribution of intraspecific variation is lower than that of interspecific variation, and whether these relative contributions would differ among traits? (3) Whether differences and relationships between leaf functional traits and environmental gradients had existed among communities, and how do leaf traits vary in response to environmental gradients (i.e., soil properties and stand structures)? To address these questions, we measured leaf functional traits, soil properties and stand structures of 34 plots collected from the montane broad-leaved forest in western China.

Materials and methods

Study site

This study was conducted in Shangli town of Ya'an City (30°11'N, 103°5'E, 900-1800 m a.s.l., *Fig. A1*), within the montane broad-leaved forest region. The area is a geomorphologic complex located in southwest Sichuan, western China. The study area is characterized by a subtropical humid monsoon climate. The mean annual temperature is 16.1 ℃ and the mean annual precipitation is 1772.2 mm (Zhao et al., 2009; Zhou et al., 2018). Annual average sunshine is 1019.9 h, with an average of 289 frost free days. The soils are derived from sandy mudstone and mudstone substrates and contain >5% organic matter and >2% nitrogen (N) content. Local conditions for plant growth are strongly P-limiting, with soil total phosphorus (P) and available P of 0.5 g/kg and 12.5 mg/kg, respectively. The dominant tree species in this region include *Machilus pingii*, *Machilus ichangensis*, *Phoebe zhennan*, *Castanopsis fargesii*, *Quercus serrata* and *Photinia beauverdiana*. Dominant shrub species include *Camellia oleifera*, *Dichroa febrifuga, Eurya groffii*, *Eurya glaberrima* and *Ficus heteromorpha*. Herbaceous species were represented by *Setaria plicata*, *Iris japonica*, *Hosta plantaginea*, *Pilea notate*, *Pteridium aquilinumvar* and *Latiusculum* (*Table A2*).

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Field survey and leaf traits measurements

Data of forest communities were collected in forest plots from May to August 2017. According to the distributed characters of this study forest, 34 plots (20*20 m) were randomly sampled (*Fig. A1; Table A3*), with a minimum distance of 100 m from the outer forest margins, and a minimum distance of 250 m relative to one another. At each plot, each tree with a diameter at breast height (DBH) > 3 cm was recorded, overall, 2067 trees of 76 species were identified to the species level in total sampling areas. The procedure of leaf trait collection and measurements is described and explained below. One species was randomly selected in a plot with three mature and unshaded individuals based on DBH > 15 cm, of the 76 species in the survey data, sufficient trait data were collected for 327 trees of 27 species. In the field, leaves from these three individuals per species were assessed for chlorophyll content with a SPAD-502 meter (Konica Minolta, Tokyo, Japan), averaging five measurements taken on different parts of the leaf lamina. Next, we collected 10 in-tact leaves per individual tree for other trait measurements. In the laboratory, the fresh mass of each leaf was measured immediately with an SE202F electronic balance (Ohaus Corp., Parsippany, NJ, US). Leaves were scanned with a scanner (CanonScan LiDE 210, Canon Inc., Tokyo, Japan) and leaf area was calculated by using Image J (Pérez-Harguindeguy et al., 2013). An electronic digital caliper was used to measure leaf thickness (mm) at the center of the lamina by avoiding the major leaf veins. Leaves were dried to a constant weight at 70 ℃ for at least 3 days and then weighed. Specific leaf area (SLA), leaf dry matter content (LDMC), and leaf density (LD) were also calculated. Leaf N content (LCN) was determined with the Kjeldahl method and leaf P content (LCP) with spectrophotometry (Bao, 2000). Foliar N concentrations per unit leaf area (N_{area}) and foliar P concentrations per unit leaf area (P_{area}) were also obtained. All the leaf traits are described in *Tables A1* and *A2*.

Characterization of the stand structures and soil properties

The local environmental conditions of these 34 plots were focused on diameter at breast height (1.3 m, DBH), tree height, forest crown and stem density, and so on. In this study, we employed terms of forest stand structure to describe plot structures characteristics, which convey much information about the size distribution of trees, forest stand structure is the capital importance for understanding forest ecosystem structure and function. In each 20*20 m plot, censuses of all living trees with DBH > 3.0 cm were performed and the following parameters were recorded. DBH was measured using a caliper, height was measured with a vertex hypsometer; crown projection was inventoried in four cardinal directions. The basal area (BA), Shannon–Weaver's index of diversity (SHI) (Shannon and Weaver, 1949) and Pielou's evenness index (EVE) (Pielou, 1975) were computed for all sampled plots.

One soil sample per plot was taken using an auger, soil water content (here refers to actual water content, was calculated as (wet soil weight - dry soil weight)/dry soil weight) was quantified using the gravimetric method (Bao, 2000). An additional five profiles of the top 20 cm of depth were also collected in each plot and further mixed to make a combined soil sample per plot using this for chemical analysis. For each plot, soil samples were pooled, homogenized, air-dried and sieved (2 mm) for further analyses in the laboratory. Soil organic matter was determined by the Walkley and Black method and total N was determined by Kjeldahl digestion. Available P was estimated by the Olsen method; total K was extracted with 1 M ammonium acetate and

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determined by atomic absorption spectrophotometry (Bao, 2000). The characters of stand structures and soil properties are described in *Tables A1* and *A3*.

Statistical analyses

Linear mixed models were used to analyze single-traits among individual species. Models were calibrated for each functional trait using individual trait measurements and included either no fixed effects (written as fixed ~1, m0) or a species fixed effect (fixed ~Species, m1) (Bolker et al., 2009; Albert et al., 2010b), to reflect interspecific, intraspecific variance, then we calculated a measure of explained variation based on the variances for the different models following (Xu, 2003): $R^2 = 1 - (\sigma m1)^2 / (\sigma m0)^2$, where σm1 and σm0 are the estimated error standard deviations under models m1 and m0 respectively. Variances were estimated by maximizing the restricted log-likelihood (REML).

According to Dodélec and Chessel (1991) and Albert's method (Albert et al., 2010a), we conducted the principal component analysis (PCA) to disentangle multidimensional structure within the leaf trait space at individual and community level. The relationships between leaf morphological traits and chemical traits were also explored by standardized major axis (SMA) regressions, a statistical tool highly recommended for allometric studies (Warton et al., 2006), using the PCA axis of morphological or chemical traits. There is an interest in knowing the slopes, which are fitted by minimizing the sums of squares of errors in X and Y dimensions, indicating the magnitude of the scaling between the variables. SMA regressions were performed using SMATR software (Falster et al., 2006).

To examine functional trait variations at the forest community level, all leaf traits were weighted by the relative abundance of each species to calculate the community weighted means (CWM) according to Lepš et al.'s (2011) method. We calculated three types of CWM parameters: (1) specific average trait values were calculated for each plot using the trait average of each species measured overall individuals of that species in that specific plot, which reflects the effect of both species' inter- and intraspecific trait variation; (2) fixed trait values were calculated for each plot using the single trait average of each species measured overall individuals of that species in the study, which changes in value among plots that are only due to interspecific trait variation; (3) intraspecific variability trait values were calculated from the differences between specific and fixed average traits and permit an estimation of the pure effects of the intraspecific variability. Specifically, to quantify how much intra- and interspecific variability, this can be employed three community parameters (fixed and specific averages and their difference) to run three parallel ANOVAs for each functional trait. Then, from outcomes of the preceding analyses, we partitioned inter- and intraspecific trait variability effects on plot-level traits values among plots, and used the method that the sum of squares of species trait variance for all plots ($SS_{specific}$) was decomposed into three different components which include fixed (SS_{fixed}) effects, intraspecific ($SS_{intraspecific}$) effects and covariation (SS_{cov}) effects, the equations as following: $SS_{cov} = SS_{specific} - SS_{fixed} - SS_{intraspecific}$ (Lepš et al., 2011). Where, $SS_{specific}$ was the 'total' variation of community trait averages originating from the specific averages. SS_{fixed} and $SS_{intraspecific}$ came from fixed averages, intraspecific trait variability above-mentioned ANOVAs respectively. SS_{cov} was Covariations of the species turnover (SS_{fixed}), intraspecific variability ($SS_{intraspecific}$) (more details in Lepš et al., 2011; Carlucci et al., 2015; Luo et al., 2016).

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Finally, we used canonical correlation analysis (CCA) to quantify correlations between two sets of multidimensional variables (Tabachnick and Fidell, 2012). As leaf traits and environmental factors are multivariate in nature, an analytic approach that allows for multiple independent variables is thus preferred (Bajorski, 2012). The use of the canonical correlation for this study enabled a more in-depth analysis of the relationships between stand structure, soil factors, and leaf traits than would have been possible with univariate statistical procedures such as multiple regressions. Therefore, we can assume that two sets of random variables, X (leaf traits) with p variables and Y (environmental factors) with q variables, have means of zero. Let n be the number of observations, and let m be n-1. Then, we use two aggregate variables U and V to express X and Y in new linear combinations as U = aX, V = bY. Using PCA's idea, we will try to find the coefficient sets a and b, which lead to the maximum covariance of cov(U,V). The main steps includes (1) getting the covariance matrix cov(U,V), (2) employing Lagrange Multiply Method to maximize cov(U,V), and (3) getting U, V, a, b and their corresponding eigenvalues. From CCA, we can obtain the key values such as canonical correlation coefficients, explanation proportion, and significance testing value (Tabachnick and Fidell, 2012). Linear mixed analyses was conducted using the packages 'ape' (Paradis et al., 2004), the principal component analysis was performed using the packages 'FactoMinerR' (Lê et al., 2008) and the canonical correlation analysis was performed using the packages 'vegan' (Dixon, 2003) in the R (R 3.5.3 version).

Results

Leaf traits variations of the individuals of all species

Principal component analysis on individual data produced variation and structure of the leaf morphological and chemical trait space. Morphologic traits were positively correlated among themselves (*Table A4*). The data set was structured by a strong first axis (58.6% of the variance) that was primarily correlated with LDMC and SLA (*Fig. 1a*). The second axis explained 28.6% of the variance and to some extent was correlated weakly with the leaf area (LA) and LD (*Fig. 1a*). Multi-trait variation of chemical traits representing the first PCA component (43.2% of the variance) was mainly driven by leaf chlorophyll (LChl), and the second axis (36.5% of the variance) was driven by leaf N content (LCN), and they were both positively correlated among themselves (*Fig. 1c*).

The SMA regressions between morphological traits and chemical traits were largely significant relationships ($R^2 = 0.51$, $F = 284.3$, $P < 0.0001$, *Fig. 2a*). In addition, the morphological traits were significantly correlated with chemical traits such as LChl, LCN, leaf N content per area (N_{area}), and leaf P content per area (P_{area}) (*Fig. 3*). They were negatively related to leaf morphology, except for LCN. Similarly, the chemical traits were significantly correlated with the morphology related traits such as LA, SLA, LDMC, and LD (*Fig. 3*).

Leaf morphological and chemical traits led to similar results, with a partition around 80% vs. 20% for interspecific vs. intraspecific variability (*Fig. 4*). There were small differences between traits, with LD (up to 24.7%, *Fig. 4*) and P_{area} (up to 24.9%, *Fig. 4b*) showing relatively more intraspecific variability. Total variances are about 60% for leaf morphological and chemical traits, except for leaf thickness (LT) (up to 97.7%, *Fig. 1a*), LA (up to 79.7%, *Fig. 1a*) and LChl (up to 73.7%, *Fig. 4*).

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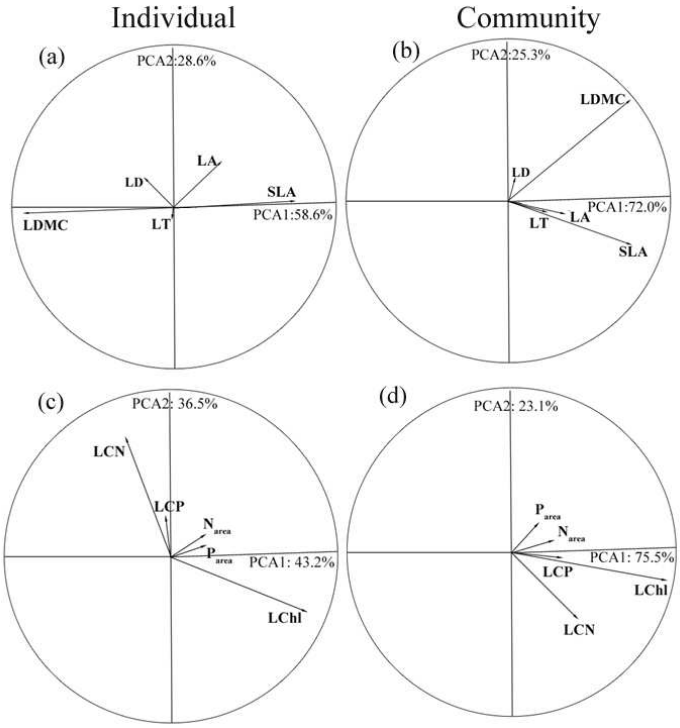

Figure 1. *Multidimensional structure within the trait space showing (a) leaf morphology trait at individual level, (b) leaf morphology trait at community level (c) leaf chemistry trait at individual level, (d) leaf chemistry trait at community level. Variables (leaf traits) used for the PCA are displayed with their vector. See Table A1 for abbreviations of traits*

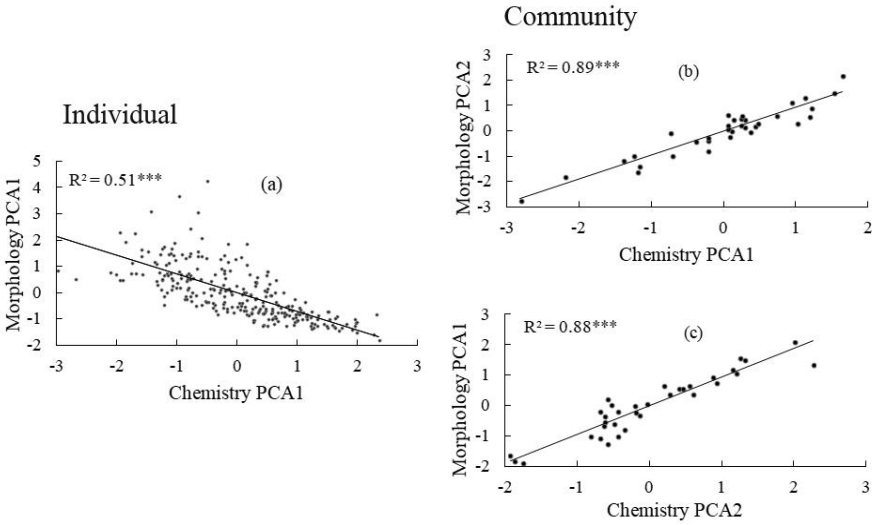

Figure 2. *Standardized major axis (SMA) regressions between leaf morphological traits and chemical traits at the between-species and communities. (a) Species analyses, only morphology PCA1 and chemistry PCA1 showed significant relationships which R^2 was 0.51, $P < 0.0001$. (b) Community analyses morphology PCA2 and chemistry PCA1 showed significant relationships which R^2 was 0.89, $P < 0.0001$. (c) Community analyses morphology PCA1 and chemistry PCA2 showed significant relationships which R^2 was 0.88, $P < 0.0001$. Squared correlation coefficient (R^2) is given and SMA regression line is plotted when significant. **$P < 0.01$, ***$P < 0.001$*

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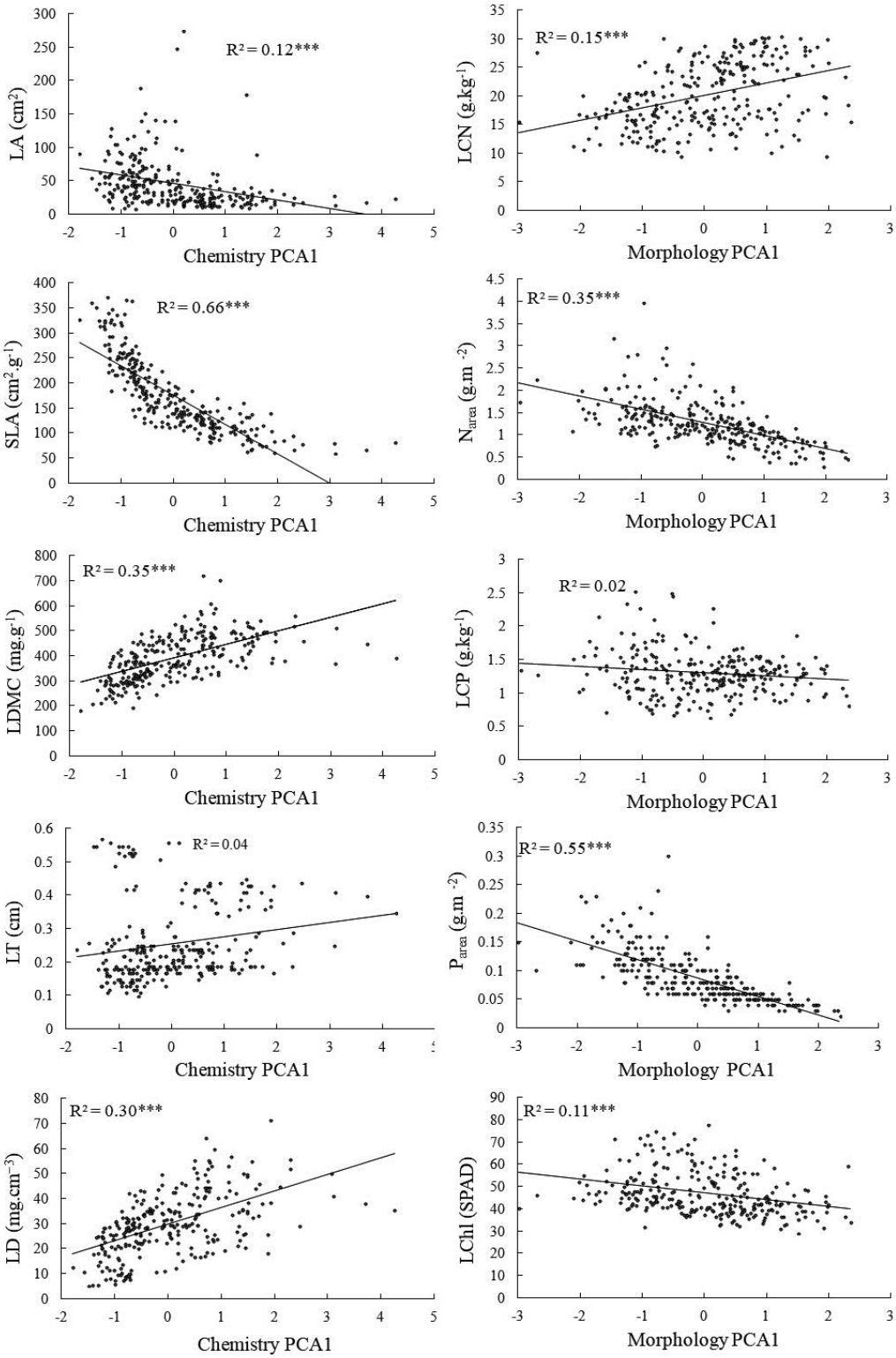

Figure 3. *Relationships at the between-species are morphology related traits (LT, LA, SLA, LDMC and LD) and chemical traits (chemistry PCA1) (left column), and chemistry related traits (LChl, LCN, N_{area}, LCP, P_{area}) and morphological traits (morphological PCA1) (right column) using standardized major axis (SMA) regressions. Squared correlation coefficient (R^2) is given and SMA regression line is plotted when significant. **P < 0.01, ***P < 0.001. See Table A1 for abbreviations of traits*

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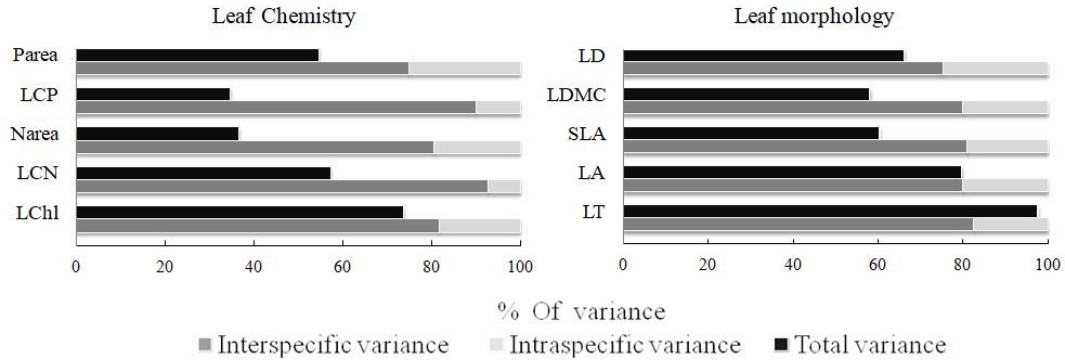

Figure 4. *Variance decomposition in interspecific and intraspecific contributions for single-trait of between-species. Leaf morphological traits analyses (left) and leaf chemical traits analyses (right) resulting from mixed models, the black bars are the total variance resulting from general line mixed models. See Table A1 for abbreviations of traits*

Leaf traits variations of the communities

At the community, PCAs of variation and structure of the leaf morphological and chemical trait were similar to PCA results at the species level. For the morphologic traits, PCAs results showed that the data set was structured by a strong first axis (72.0% of the variance) that was mainly explained by LDMC and SLA. A second axis (25.3% of the variance) was explained largely by LA and LD (*Fig. 1b*). Multi-trait variation of chemical traits represented the first PCA component (75.5% of the variance) and was mainly driven by LChl and LCN (*Fig. 1d*).

The SMA regressions indicated that morphology PCA2 was significantly correlated with chemistry PCA1 (*Fig. 2b*, R^2 was 0.89, $P < 0.0001$), and morphology PCA1 was significantly correlated with chemistry PCA2 (*Fig. 2d*, R^2 was 0.88, $P < 0.0001$). Chemistry PCA1 was significantly correlated with LDMC and LD, and chemistry PCA2 was significantly correlated with LT, LA, and SLA (*Fig. 5*, left column). Similarly, morphology PCA1 was significantly correlated with LChl and LCN, and morphology PCA2 was significantly correlated with LCN, N_{area}, LCP, and P_{area} (*Fig. 5*, right column).

Interspecific variability contributed to a greater proportion of explained variation to the functional shift than did intraspecific variability for leaf morphological and leaf chemical traits. For LT and LChl, the CWM trait variation was almost completely generated by interspecific variation, which accounted for 98.3% and 93.8% of the total variation, respectively. However, the contribution of intraspecific variation was greater than interspecific variation for LCN (56.2% vs. 21.0%), and there was a positive covariation between interspecific and intraspecific variation among the plots (*Table 1*). This, however, was not the case for LT (-2.3%) and LChl (-7.2%).

Intra- and inter-specific variability of CWM along different environmental axes

A canonical correlation analysis was conducted to evaluate the multivariate shared relationship between the leaf traits and the environmental groups, which yielded the first canonical variables with squared canonical correlations (Canonical R^2) and Wilks' Lambda values (*Table 2*). To total variability and interspecific variability, the full model across the first canonical variables was statistically significant except for leaf chemistry and soil properties. Meanwhile, for intraspecific variability, leaf morphology was

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significantly correlated with soil properties (F = 5.29, $p < 0.0001$), and leaf chemistry were significantly correlated with stand structures (F = 1.63, $p = 0.02$).

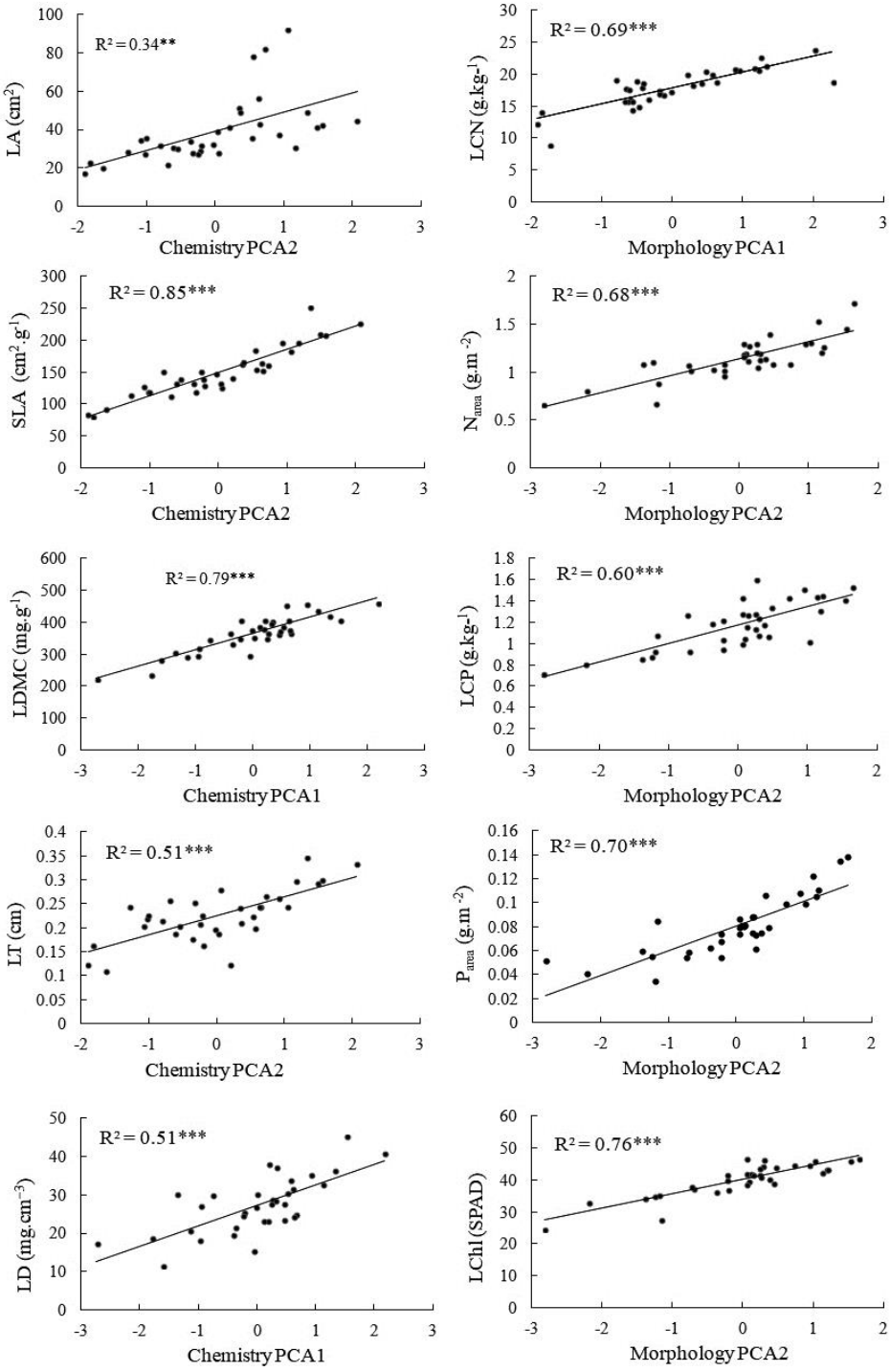

Figure 5. *Relationships in community are morphology related traits (LT, LA, SLA, LDMC and LD) and chemical traits (chemistry PCA1 or PCA2) (left column), and chemistry related traits (LChl, LCN, N_{area}, LCP, P_{area}) and morphological traits (morphological PCA1 or PCA2) (right column) using standardized major axis (SMA) regressions. Squared correlation coefficient (R^2) is given and SMA regression line is plotted when significant. **P < 0.01, ***P < 0.001. See Table A1 for abbreviations of traits*

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Table 1. *The proportion of interspecific variation, intraspecific variation and covariation effects contributing to the variance in community weighted mean trait values (unit: %). See Table A1 for abbreviations of traits*

Group	Traits	Interspecific variation effect	Intraspecific variation effect	Covariation effect
Leaf morphology	LT	100.26	4.02	-4.28
	LA	59.67	15.33	25
	SLA	63.22	22.29	14.49
	LDMC	82.23	16.16	1.61
	LD	67.41	21.41	11.18
Leaf chemistry	LChl	93.84	13.32	-7.16
	LCN	21.03	56.19	22.78
	N_{area}	49.74	42.78	7.48
	LCP	40.29	36.16	23.55
	P_{area}	46.55	35.91	17.54

Table 2. *Canonical correlation between leaf trait functions (morphology and chemistry) and environmental variables (stand structures and soil properties) for their first canonical variables*

	Leaf traits	Environmental variables	Proportion	Canonical R^2	Wilks' Lambda
Total variability	Morphology	Stand structures	0.64	0.83 ***	0.023
		Soil properties	0.71	0.99***	0.0001
	Chemistry	Stand structures	0.34	0.69 ***	0.021
		Soil properties	0.58	0.41	0.380
Interspecific variability	Morphology	Stand structures	0.60	0.77 **	0.043
		Soil properties	0.83	0.76 ***	0.137
	Chemistry	Stand structures	0.56	0.72 **	0.062
		Soil properties	0.68	0.57	0.259
Intraspecific variability	Morphology	Stand structures	0.58	0.72	0.066
		Soil properties	0.99	0.99 ***	0.0002
	Chemistry	Stand structures	0.54	0.69 **	0.074
		Soil properties	0.45	0.22	0.560

**P < 0.01, ***P < 0.001. Canonical R^2 is squared canonical correlations, Wilks' lambda is Wilks' lambda (likelihood ratio) statistic

Stand structures and soil properties significantly influenced leaf morphology and leaf chemistry (*Tables 3, 4, 5*). For instance, the total variation in LA, LDMC, and LCN were all significantly correlated with soil properties, which were mostly explained by soil water and soil P content (*Table A5*). Meanwhile, SLA, LD and all variables of leaf chemistry were significantly correlated with stand structures, which were mainly supported by tree sizes (e.g. H and DBH) (*Tables 3* and *A5*). Furthermore, soil properties explained 29.3% of the total variance in leaf morphology and stand structures explained 19.6% and 30.2% of the total variance in leaf morphology and chemistry (*Fig. 6*). In the same situation, the interspecific variation of LA and LCN were all

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significantly correlated with soil properties, which were mainly supported by soil water and soil P content (*Table A6*). Specific leaf area, LChl, LCN, and LCP were significantly correlated with stand structures that mainly contributed by BA and DBH (*Tables 4* and *A6*). Soil properties explained 18.4% of the interspecific variance in leaf morphology, stand structure explained 26.89% and 31.7% of the interspecific variance in leaf morphology and chemistry (*Fig. 6*). For intraspecific variance, LA, LDMC, and LCN were all significantly correlated with soil properties, and SLA, LD, LCN, LCP, and Parea were significantly correlated with stand structures defined by stand tree height (*Tables 5* and *A7*). 40.7% of intraspecific variation of leaf morphology was explained by soil properties, and 20.9% of leaf chemistry was explained by stand structures (*Fig. 6*).

Table 3. *Univariate multiple regressions of community-weighted mean traits values in total variability by soil properties and stand structures in CCA. R^2 is squared partial correlations, F is approximation or upper bound, and Pr > F is the probability level. See Table A1 for abbreviations of traits*

Variables		Soil properties			Stand structures		
		R^2	F	Pr > F	R^2	F	Pr > F
Leaf morphology	LT	0.41	1.4	0.30	0.34	1.35	0.26
	LA	0.90	17.35	0.00	0.37	1.56	0.18
	SLA	0.44	1.57	0.25	0.52	2.86	0.02
	LDMC	0.98	109.52	<.0001	0.40	1.80	0.12
	LD	0.30	0.85	0.55	0.51	2.77	0.02
Leaf chemistry	LChl	0.11	0.71	0.62	0.64	4.84	0.00
	LCN	0.36	3.08	0.02	0.66	5.12	0.00
	N_{area}	0.03	0.19	0.97	0.46	2.29	0.05
	LCP	0.09	0.57	0.72	0.58	3.70	0.00
	P_{area}	0.07	0.42	0.83	0.47	2.37	0.04

Table 4. *Univariate multiple regressions of community-weighted mean traits values in interspecific variability by soil properties and stand structures in CCA. R2 is squared partial correlations, F is approximation or upper bound, and Pr > F is the probability level. See Table A1 for abbreviations of traits*

Variables		Soil properties			Stand structures		
		R^2	F	Pr > F	R^2	F	Pr > F
Leaf morphology	LT	0.11	0.71	0.62	0.37	1.57	0.18
	LA	0.62	8.96	<.0001	0.41	1.84	0.11
	SLA	0.31	2.49	0.05	0.54	3.17	0.01
	LDMC	0.11	0.72	0.62	0.45	2.22	0.06
	LD	0.12	0.75	0.59	0.42	1.95	0.09
Leaf chemistry	LChl	0.18	1.25	0.31	0.66	5.15	0.00
	LCN	0.54	6.51	0.00	0.47	2.39	0.04
	N_{area}	0.08	0.50	0.77	0.43	2.00	0.08
	LCP	0.12	0.78	0.57	0.47	2.34	0.05
	P_{area}	0.13	0.80	0.56	0.39	1.73	0.14

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Table 5. *Univariate multiple regressions of community-weighted mean traits values in intraspecific variability by soil properties and stand structures in CCA. R2 is squared partial correlations, F is approximation or upper bound, and Pr > F is the probability level. See Table A1 for abbreviations of traits*

Variables		Soil properties			Stand structures		
		R^2	F	Pr > F	R^2	F	Pr > F
Leaf morphology	LT	0.31	0.90	0.52	0.31	1.18	0.35
	LA	0.67	4.05	0.03	0.24	0.86	0.58
	SLA	0.61	3.14	0.06	0.37	1.56	0.19
	LDMC	0.99	144.19	<.0001	0.30	1.12	0.38
	LD	0.31	0.89	0.53	0.55	3.30	0.01
Leaf chemistry	LChl	0.09	0.55	0.74	0.22	0.75	0.66
	LCN	0.16	1.06	0.41	0.53	3.00	0.02
	N_{area}	0.16	1.10	0.38	0.34	1.35	0.26
	LCP	0.07	0.42	0.83	0.58	3.69	0.01
	P_{area}	0.08	0.46	0.81	0.49	2.60	0.03

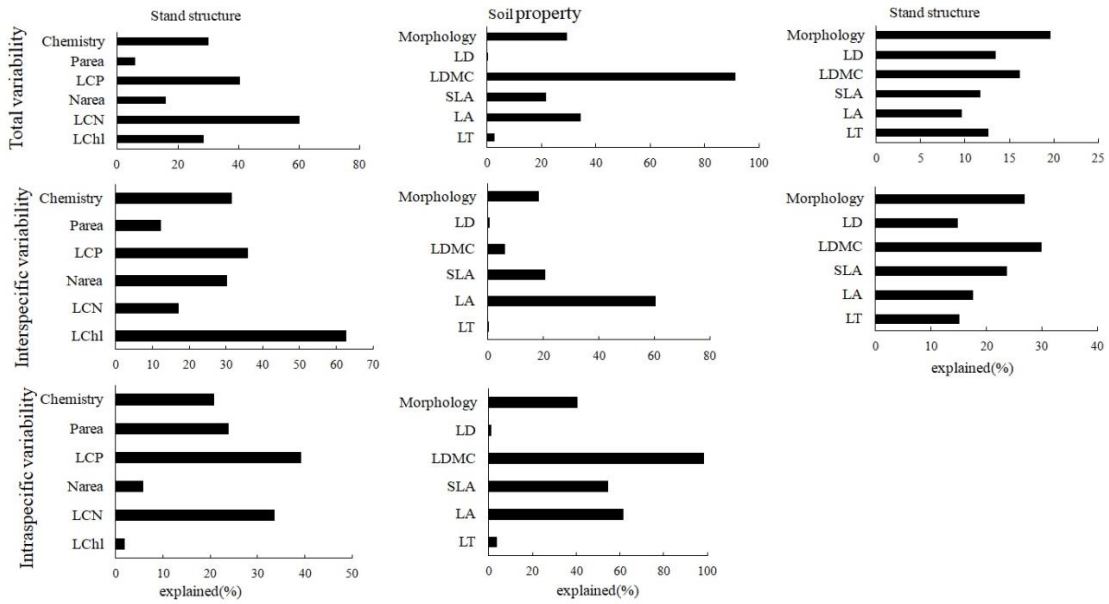

Figure 6. *The relative contribution of total, interspecific and intraspecific variability of CWM trait values along different environmental axes. (According to canonical correlation analysis, the results showing the significance level is listing, seeing Table 2). See Table A1 for abbreviations of traits*

Discussion

Intra- and interspecific variability of leaf traits

Our findings indicate that leaf morphology traits of the between-species for two independent axes of trait covariation together accounted for approximately 87% of the variance. Likewise, at the communities, roughly 97% of the variance was explained in multidimensional trait space (*Fig. 1*). The first axis alone accounted for 58.6% of

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variance at between-species and 75% of variance at the community, which depicted light capture dimension of the plant strategy spectrum and light availability (LDMC and SLA) (Wright et al., 2004; Lusk et al., 2008), and the second axis accounted for 28.6% of variance at between-species and 25.3% of variance at the community, which described investment physical attributes (LA and LD) (Chauvin et al., 2018).

For chemical trait spaces of the between-species and communities, the first axis reflected the chemical attributes, which is integral to the proteins of the photosynthetic machinery. The second axis represents an investment in foliar nutrition, which is essential to many of the chemical compounds involved in leaf structure and metabolism (Wright et al., 2004). Other results from The SMA regression analysis demonstrated that morphological traits were reciprocally correlated with chemical traits (*Figs. 2, 3, 5*). In fact, these traits are often found to be strongly correlated with each other across species and communities. For example, evergreen leaves are often sclerophyllous and associated with a lower SLA, lower leaf N content, and lower mass-based photosynthetic capacity (Curtis and Ackerly, 2010; Kröber et al., 2015). This trend is in line with the leaf economics spectrum (LES) that characterizes ecological strategies with quick to slow payback of investments of nutrients and dry mass (Wright et al., 2004; Osnas et al., 2013). At the leaf level, SLA, leaf dry matter content, and leaf N concentration has been employed to predict accurately the maximum photosynthetic rates of a wide range of species (Reich et al., 1997). At the plant level, all three traits have been found to be involved in a fundamental tradeoff between a rapid production of biomass and efficient conservation of nutrients (Poorter and Jong, 1999). And SLA (or related leaf traits) and LCN significantly impacted on primary productivity and nutrient cycling at the ecosystem level (Aerts and Chapin, 2000).

The importance of intraspecific variance has been neglected in community ecology for a long time (Bolnick et al., 2011). Recently, however, there has been greater attention paid to intraspecific variability, which has underlined the developments to integrate variation in both the intraspecific as well as interspecific levels in trait-based community ecology (Violle et al., 2012). Despite this increase in attention, understanding how intraspecific variation influences such functional shifts is not well understood in montane broad-leaved forests. Our results show that intraspecific variability of both leaf morphological and chemical traits account for roughly 20% of the variation, and interspecific variability explained 80% in functional turnover (*Fig. 4*). These findings illustrate that most of the variance in raw trait values was explained by differences between species. Intraspecific variance accounted for a smaller part of the total variance, resulting in either from genetic variation or phenotypic plasticity (Albert et al., 2011), in line with the growing consensus that intraspecific trait variation is not negligible (Albert et al., 2010b). This result could reflect the dissimilarity in high species turnover in montane broad-leaved forests (Luo et al., 2016).

Based on community-weighted mean traits values, the interspecific variation of leaf morphologic traits was the main contributor to functional trait change (*Table 2*). For chemical traits, intraspecific variability of LCN, N_{area}, LCP, P_{area} accounted for 56.2%, 42.8%, 36.2% and 35.9% variation, respectively. This was in accordance with previous results that intraspecific effects are often comparable to, and sometimes stronger than, species effects. These effects tend to be larger for direct ecological responses, whereas intraspecific effects and species effects tend to be similar for indirect responses; intraspecific effects are especially strong when indirect interactions alter community composition (Des Roches et al., 2018). Chemical functional traits are associated with

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photosynthetic rate and nutrient cycling (Wright et al., 2004; Pérez-Harguindeguy et al., 2013), high variability in leaf chemical trait responses to stress or environmental gradients has been reported in previous studies (Auger and Shipley, 2013; Siefert et al., 2015). Finally, chemical traits are typically greater than leaf morphologic traits in broad-leaved forests, indicating that the relative contribution of intraspecific variation largely influences local functional composition, the maintenance of species coexistence, and the dynamics of communities (Lichstein et al., 2007; Bolnick et al., 2011; Courbaud et al., 2012; Violle et al., 2012; Luo et al., 2016).

Influence of environmental factors on intra- and interspecific trait variability

We found that soil properties significantly showed relationships with leaf morphology in total variance, interspecific variance, and intraspecific variance (*Table 2*), Soil properties accounted for 29.3%, 18.4% and 40.7% of the variation in these variables, respectively (*Fig. 6*). This result is interesting given that soil nutrient conditions (i.e., total soil N and total organic C) are the most important explanatory factors for SLA variation at both intra- and interspecific levels (He et al., 2018). In particular, LDMC of intraspecific variance was more significant than SLA with soil properties, and soil properties explained roughly 98% of variation intraspecific variance (*Fig. 6*). In other words, LDMC was a better predictor than SLA for describing the relationships between leaf morphologic traits and soil properties. Though LDMC and SLA were both correlate with nutrient availability, LDMC varied independently from leaf thickness and was also strongly correlated with resource availability and with relative growth rate (Garnier et al., 2004; Roche et al., 2004; Fortunel et al., 2009).

Leaf dry matter content has been recommended as a more reliable correlate of soil fertility in biomes not subject to severe water limitation (Vendramini et al., 2002). Another reason is that in montane broad-leaved forests, the shaded leaves have high SLA that results in the optimization of light capture rather than being associated with high soil fertility (Hodgson et al., 2011). Likewise, abundance-weighted LDMC, as opposed to SLA, was the superior predictor of aboveground net primary production (Smart et al., 2017). In addition, leaf area (LA) of inter- and intraspecific variance was related to soil attributes (*Tables 4, 5*), this indicated increasing in LA had greater access to light, leading to take advantage of increased soil resource availability, especially for tall species (Siefert and Ritchie, 2016), in line with the dominant plasticity mechanism, which predicts that competitive species have strong phenotypic plasticity to maximize resource capture and competitive ability (Ashton et al., 2010).

With regard to the relationships between leaf chemical traits and soil properties, our finding was that two categories showed inconspicuous relevance for the first canonical variable (*Table 2*). This result hints at the idea that soil properties had no systematic effect on foliar nutrient status, which might be because plants are able to constrain the flexibility of nutrient concentrations (Sistla et al., 2015). Namely, a change in soil nutrient availability did not necessarily imply a change in foliar nutrient status, especially when deficiencies were moderate (Luiro et al., 2009). Another explanation is that gradients of nutrient availability in soils were generally narrow, making investigations of the role of soil fertility in nutrient remobilization difficult (Achat et al., 2018). Additionally, their relationships were directly or indirectly affected by soil properties, soil types, parent materials, and climate gradients, even though their gradients were not very wide in our study sites (Augusto et al., 2017; Achat et al., 2018).

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From a stand structural perspective, we suggest that leaf traits in interspecific and intraspecific variance were significantly correlated with stand characteristics. Previous studies on height and crown-related changed in leaf morphological, chemical and photosynthetic traits between and within species (Chmura and Tjoelker, 2008; Burgess and Dawson, 2010; Kenzo et al., 2016). For instance, mass- and area-based leaf N decreased, and specific leaf area (SLA) increased with increasing canopy depth, and SLA and leaf N -trait gradients showed variations between loblolly pine and slash pine (Kenzo et al., 2016). The intraspecific variance of leaf traits (e.g., LDMC, SLA) may also be related to aboveground biomass, which was potentially driven by the functional identity of tree height at different forest strata as well as at whole-community (Ali and Yan, 2018). In general, plant life history strategies suggest that taller species respond more strongly to leaf photosynthetic capacity (e.g. SLA, N and P content) because they potentially experience increasing light levels as they grow to the canopy. In contrast, small-statured species may remain in the shaded understory for their whole life cycle, and those results are supported by studies of the effect of light, nutrients and other environmental factors on plant leaf traits and their relationships (Santiago and Wright, 2007; Mao et al., 2017).

Our study showed that leaf N content per area (N_{area}) and leaf P content per area (P_{area}) (high values for acquisitive strategies) are strongly related to stand height and DBH in their intra- and inter-specific variance (*Tables A5, A6, A7*). Leaf density (LD) and dry matter content (LDMC) (high values for conservative strategies) are strongly in their interspecific variance responses to different stand height and DBH (*Tables A5, A6, A7*). Those findings that leaf traits consistently represent inter- and intraspecific variations in the studied forest have important implications in the individual plant strategies, community assembly and ecosystem function (Siefert et al., 2015). In addition, LDMC showed high contributions of interspecific variance to the changes along the stand structural gradient (*Fig. 6*). Those results hint at the fact that leaf traits and bivariate leaf trait relationships are modified by plant size, although their positive correlations generally remain invariant. However, a plant size effect on leaf traits relationships has scarcely been examined, although it may be critical to understanding plant life history strategies, especially for subtropical forests (Liu et al., 2010).

Conclusion

Our results revealed that the contribution of interspecific variability was dominant for leaf morphological and chemical traits at between-species and communities, despite the high level of intraspecific variability for leaf chemical traits (e.g. LCN, N_{area}, LCP, P_{area}) at communities in montane broad-leaved forest, which suggest that intraspecific functional variability should be a concern for ecologists. We also observed that intently relationships between leaf morphological and chemical traits presented by leaf assemblages at between-species and communities. Traits that are coordinated within plant strategies (leaf economics spectrum) showed that total, intra- and inter-specific variability responses to environmental gradients (stand structures and soil properties) when considered for the communities. Our findings highlight the structure of inter- and intraspecific variability in actual communities acts as a signature of community assembly processes, and alters with environmental gradients. In the future, we need more experiments to better understand the influence of sampling effort and design on the quantification of the absolute and relative amount of intraspecific trait variability

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within and among communities, and disentangle the extent and consequences of plastic and genetic trait variation at the community and ecosystem levels. Most importantly, the researchers should perform a more systematic evaluation of the effects of inter- and intraspecific variability on functional diversity indices and their relationships to environmental variables or other community properties on local scales.

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Conflict of interests. The authors declare that there is no conflict of interests.

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APPENDIX

Table A1. *List of the leaf functional traits and environmental factors (stand structures and soil properties) considered in this study*

Group	Traits	Abbreviation	Unit	Min	Max	Mean	STD	Kurtosis	Skewness
Leaf morphology	Leaf thickness	LT	cm	0.1	0.57	0.25	0.11	1.25	0.58
	Leaf area	LA	cm^2	8.32	274.91	45.56	36.85	2.51	9.47
	Leaf dry matter content	LDMC	$mg.g^{-1}$	180.63	719.33	392.09	90.57	0.3	0.1
	Leaf density	LD	$mg.cm^{-3}$	5.17	71.29	29.7	12.2	0.3	0.07
	Specific leaf area	SLA	$cm^2.g^{-1}$	59.33	370.85	174.87	72.29	0.72	-0.18
Leaf chemistry	Leaf chlorophyll	LChl	SPAD	29.2	77.9	47.17	9.66	0.93	0.41
	Leaf nitrogen content	LCN	$g.kg^{-1}$	9.38	30.47	20.1	5.63	0.12	-1.12
	Leaf nitrogen content per area	N_{area}	$g.m^{-2}$	0.3	3.98	1.29	0.5	1.34	3.9
	Leaf phosphorus content	LCP	$g.kg^{-1}$	0.63	2.52	1.3	0.33	0.86	1.75
	Leaf phosphorus content per area	P_{area}	$g.m^{-2}$	0.02	0.3	0.09	0.04	1.36	2.62
Stand structures	Tree height	H	m	5.85	18.64	10.21	2.25	1.26	5.24
	Maximum height	H_{max}	m	11.34	812.5	42.78	136.05	5.82	33.95
	Basal area	BA	cm^2	363	2236.18	1099.37	416.74	0.77	1.62
	Canopy size	CA	m^2	39.6	140.4	67.79	18.05	1.8	6.97
	Diameter at breast heigh	DHB	cm	7.75	17.84	12.97	2.58	-0.05	-0.53
	Stand density	DEN	$N.ha^{-1}$	650	2350	1315.44	418.65	0.4	-0.21
	Shannon index	SHI		1.24	2.47	2.02	0.25	-0.63	1.52
	Evenness index	EVE		0.3	0.67	0.5	0.08	-0.17	0.27
Soil properties	Soil water content	SCW	%	3.94	8.34	6.09	1.2	0.1	-0.66
	Soil nitrogen content	SCN	%	0.62	2.27	1.17	0.45	0.85	-0.15
	Organ matters	OM	%	0.44	6.21	2.89	1.51	0.67	-0.28
	Soil potassium content	SCK	$mg.kg^{-1}$	26.57	88.13	51.79	15.45	0.68	0.36
	Soil phosphorus content	SCP	$g.kg^{-1}$	0.05	0.14	0.08	0.02	1.03	0.36

Table A2. *List of the sampled species and their mean values of leaf functional traits. See Table A1 for abbreviations of traits*

Species	Family	Genus	LT	LA	SLA	LD	LDMC	LCN	LCP	LChl	N_{area}	P_{area}
Liquidambar formosana	Hamamelidaceae	Liquidambar	0.14	51.54	233.64	29.23	312.99	27.32	1.40	36.90	1.19	0.06
Acer davidii	Aceraceae	Acer	0.14	98.55	229.46	15.23	307.65	21.56	1.32	43.04	0.97	0.06
Alangium chinense	Alangiaceae	Alangium	0.11	95.04	264.33	18.93	287.79	18.46	1.40	42.00	0.72	0.05
Betula luminifera	Betulaceae	Betula	0.18	36.68	252.47	33.12	318.82	18.05	1.47	44.30	0.72	0.06
Bothrocaryum controversum	Cornaceae	Bothrocaryum	0.18	52.57	197.96	21.84	313.74	19.45	1.21	50.68	1.00	0.06
Camptotheca acuminate	Nyssaceae	Caimptotheca	0.27	77.51	264.67	8.99	279.17	21.79	1.24	46.99	0.90	0.05

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Castanopsis fargesii	Fagaceae	Castanopsis	0.19	19.87	113.15	87.42	484.14	12.83	1.66	46.58	1.16	0.15
Castanopsis platyacantha	Fagaceae	Castanopsis	0.18	22.59	122.52	80.08	495.95	16.86	1.59	46.81	1.40	0.13
Cinnamomum septentrionale	Lauraceae	Cinnamomum	0.30	45.65	144.42	20.11	408.47	18.04	1.20	48.99	1.47	0.11
Cyclobalanopsis glauca	Fagaceae	Cyclobalanopsis	0.24	93.64	254.03	10.37	342.17	12.86	1.09	35.00	0.56	0.04
Diospyros kaki	Ebenaceae	Diospyros	0.25	58.50	285.65	11.16	244.91	16.33	1.13	39.88	0.67	0.05
Elaeocarpus japonicus	Elaeocarpaceae	Elaeocarpus	0.35	30.90	128.63	24.54	394.40	21.80	1.51	54.63	1.76	0.12
Eurya loquaiana	Theaceae	Eurya	0.40	15.81	113.68	42.17	395.44	20.55	1.31	63.50	1.97	0.12
Ilex chinensis	Aquifoliaceae	Ilex	0.43	14.86	133.41	41.39	398.69	21.91	1.25	53.24	1.92	0.11
Kalopanax septemlobus	Araliaceae	Kalopanax	0.26	216.11	168.16	5.43	448.93	23.49	1.35	41.40	1.41	0.08
Liquidambar formosana	Hamamelidaceae	Liquidambar	0.14	44.56	300.43	27.02	243.76	27.76	1.34	35.10	0.92	0.04
Machilus ichangensis	Lauraceae	Machilus	0.24	53.13	147.53	18.94	362.26	14.90	0.87	47.09	1.04	0.06
Mallotus japonicus	Euphorbiaceae	Mallotus	0.20	123.28	211.56	9.97	363.40	20.57	1.42	49.61	0.99	0.07
Padus wilsonii	Rosaceae	Padus	0.19	45.03	191.08	29.91	377.11	23.53	1.22	37.93	1.29	0.07
Phoebe zhennan	Lauraceae	Phoebe	0.21	24.33	136.88	67.03	519.30	18.48	1.59	41.85	1.38	0.12
Photinia beauverdiana	Rosaceae	Photinia	0.54	36.11	234.93	12.66	368.86	27.02	1.18	41.51	1.21	0.05
Platycarya strobilacea	Juglandaceae	Platycarya	0.17	22.28	216.93	87.16	485.59	21.97	1.22	42.13	1.23	0.06
Quercus serrata	Theaceae	Eurya	0.37	12.53	131.77	48.66	338.43	22.80	1.14	54.80	1.73	0.09
Rhododendron stamineum	Ericaceae	Rhododendron	0.36	29.77	92.48	24.35	391.44	21.86	1.87	73.10	2.32	0.21
Rhus chinensis	Anacardiaceae	Rhus	0.17	56.40	218.40	22.24	319.92	25.20	1.36	37.38	1.30	0.08
Schima superba	Theaceae	Schima	0.43	40.08	220.87	9.55	244.05	19.84	1.53	35.60	0.92	0.07
Symplocos setchuensis	Symplocaceae	Symplocos	0.26	29.75	101.65	30.77	357.04	18.04	1.42	63.80	1.85	0.15

Table A3. *List of main local environmental conditions of the sampled plots. See Table A1 for abbreviations of environmental Variables and X, Y are the GPS positions of the sampling sites*

Plot number	X	Y	H	DHB	DEN	OM	SCW
1	606730	3342355	5.89	7.75	2000	1.30	4.42
2	607442	3342433	9.23	12.14	1225	1.88	5.22
3	607117	3342600	18.64	15.14	2050	0.96	5.68
4	606070	3341997	10.85	15.35	1125	4.82	8.34
5	606070	3341997	10.48	16.25	1225	5.50	6.33
6	605797	3343515	9.76	14.83	2350	6.21	6.78
7	605779	3343579	8.58	13.72	1600	5.18	7.62
8	605819	3343656	10.85	14.30	1450	6.05	5.39

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9	605310	3343434	9.89	13.92	1600	3.89	6.72
10	605293	3343421	7.62	8.65	950	4.65	4.32
11	605315	3343441	5.85	9.57	1500	4.76	5.34
12	604510	3344733	11.45	13.75	1700	1.51	7.77
13	604465	3344811	9.83	12.70	700	2.69	6.22
14	605603	3342480	8.79	11.00	1075	1.25	3.94
15	606525	3342562	13.23	13.82	1250	2.01	4.74
16	605542	3342588	11.54	10.64	1475	4.24	3.94
17	605418	3342052	9.90	9.81	1325	2.84	6.78
18	605363	3342035	11.85	17.31	675	1.98	5.01
19	605316	3341863	9.21	13.53	650	2.82	5.72
20	605235	3341875	8.08	11.22	975	1.97	5.27
21	605235	3341875	10.61	15.33	1225	2.69	6.05
22	605297	3341338	12.26	17.84	725	2.72	6.79
23	605254	3340746	12.11	15.74	1025	2.34	5.08
24	605286	3340796	12.42	17.53	800	2.96	5.75
25	605396	3340840	10.73	12.86	1050	2.91	8.07
26	605456	3340905	11.53	8.91	1725	3.70	6.99
27	605540	3340871	9.70	10.72	950	2.16	5.45
28	602463	3342556	10.10	12.72	1275	2.45	7.96
29	602454	3342382	9.78	11.10	1075	0.67	7.96
30	602542	3342373	10.55	12.38	1675	0.44	6.39
31	602615	3342629	7.76	13.25	1725	2.13	5.82
32	602582	3342925	9.46	13.84	1825	2.13	6.03
33	602589	3342973	8.85	12.91	1475	2.23	6.53
34	602586	3343161	9.67	10.41	1275	2.34	6.79

Table A4. *Pearson correlation coefficients for species data from the PCA analysis (Fig. 1). The significance level is as follows: * $P < 0.05$, ** $P < 0.01$. See Table A1 for abbreviations of traits*

		Leaf morphology				**Leaf chemistry**				
		LA	**SLA**	**LDMC**	**LD**	**LChl**	**LCN**	**N_{area}**	**LCP**	**P_{area}**
Leaf morphology	LT	0.3	0.75**	0.12	-0.35*	0.42*	0.74**	0.07	0.21	-0.19
	LA		0.48**	0	-0.3	0.06	0.58**	-0.06	0.21	-0.22
	SLA			-0.12	-0.54**	0.19	0.78**	-0.32	0.18	-0.44**
	LDMC				0.76**	0.77**	0.25	0.79**	0.76**	0.83**
	LD					0.56**	-0.18	0.78**	0.51**	0.92**
Leaf chemistry	LChl						0.52**	0.72**	0.63**	0.58**
	LCN							0.23	0.51**	-0.05
	N_{area}								0.57**	0.83**
	LCP									0.72**

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Table A5. *Univariate multiple regression statistics for predicting environmental factors from the leaf traits in total variability in CCA. R^2 is squared partial correlations, F is approximation or upper bound, and Pr > F is the probability level. See Table A1 for abbreviations of traits. The significant factors are specified by bold values (i.e. p-value < 0.05)*

Environmental factors		Chemical traits			Morphological traits		
		R^2	F	Pr > F	R^2	F	Pr > F
Soil properties	SCN	0.10	0.60	0.704	**0.95**	**27.78**	**<.0001**
	OM	0.13	0.84	0.531	**0.96**	**40.33**	**<.0001**
	SCK	0.06	0.35	0.877	**0.72**	**3.90**	**0.034**
	SCW	**0.17**	**1.18**	**0.344**	**0.79**	**5.75**	**0.010**
	SCP	**0.33**	**2.71**	**0.041**	**0.93**	**20.50**	**<.0001**
Stand structures	H	**0.58**	**7.79**	**0.000**	**0.56**	**5.82**	**0.001**
	H_{max}	**0.46**	**4.73**	**0.003**	**0.47**	**3.91**	**0.006**
	BA	**0.44**	**4.42**	**0.004**	0.28	1.73	0.152
	CA	0.20	1.42	0.249	0.21	1.16	0.354
	DHB	**0.40**	**3.79**	**0.010**	**0.45**	**3.69**	**0.008**
	DEN	0.25	1.84	0.138	0.20	1.10	0.386
	SHI	**0.45**	**4.58**	**0.004**	0.14	0.73	0.631
	EVE	0.29	2.24	0.079	0.13	0.66	0.684

Table A6. *Univariate multiple regression statistics for Predicting environmental factors from the leaf traits in interspecific variability in CCA. R^2 is squared partial correlations, F is approximation or upper bound, and Pr > F is the probability level. See Table A1 for abbreviations of traits. The significant factors are specified by bold values (i.e. p-value < 0.05)*

Environmental factors		Chemical traits			Morphological traits		
		R^2	F	Pr > F	R^2	F	Pr > F
Soil properties	SCN	0.09	0.55	0.739	0.12	0.63	0.707
	OM	0.06	0.36	0.870	0.07	0.33	0.917
	SCK	0.07	0.45	0.810	0.23	1.38	0.259
	SCW	**0.33**	**2.79**	**0.036**	**0.42**	**3.25**	**0.016**
	SCP	**0.40**	**3.68**	**0.011**	**0.35**	**2.47**	**0.049**
Stand structures	H	0.31	2.57	0.050	0.32	2.09	0.087
	H_{max}	0.13	0.83	0.539	0.09	0.45	0.838
	BA	**0.48**	**5.08**	**0.002**	**0.45**	**3.62**	**0.009**
	CA	0.26	1.98	0.113	0.30	1.93	0.112
	DHB	0.38	3.46	0.015	**0.46**	**3.79**	**0.007**
	DEN	0.25	1.90	0.127	0.23	1.38	0.260
	SHI	0.13	0.84	0.535	0.20	1.12	0.375
	EVE	0.14	0.93	0.474	0.17	0.89	0.515

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Table A7. *Univariate multiple regression statistics for predicting environmental factors from the leaf traits in intraspecific variability in CCA. R2 is squared partial correlations, F is approximation or upper bound, and Pr > F is the probability level. See Table A1 for abbreviations of traits. The significant factors are specified by bold values (i.e. p-value < 0.05)*

Environmental factors		Chemical traits			Morphological traits		
		R^2	F	Pr > F	R^2	F	Pr > F
Soil properties	SCN	0.06	0.35	0.876	0.33	0.75	0.624
	OM	0.15	0.99	0.439	**0.70**	**3.45**	**0.047**
	SCK	0.03	0.18	0.969	0.38	0.93	0.517
	SCW	0.15	1.00	0.436	0.43	1.15	0.407
	SCP	0.15	0.95	0.462	0.27	0.55	0.759
Stand structures	H	0.45	4.62	0.003	**0.41**	**3.08**	**0.020**
	H_{max}	0.21	1.48	0.227	**0.53**	**5.12**	**0.001**
	BA	0.16	1.05	0.407	0.29	1.82	0.133
	CA	0.14	0.90	0.496	0.22	1.29	0.297
	DHB	0.22	1.57	0.202	0.08	0.38	0.885
	DEN	0.11	0.71	0.624	0.15	0.80	0.580
	SHI	0.41	3.81	0.009	0.12	0.60	0.727
	EVE	0.29	2.25	0.077	0.11	0.57	0.752

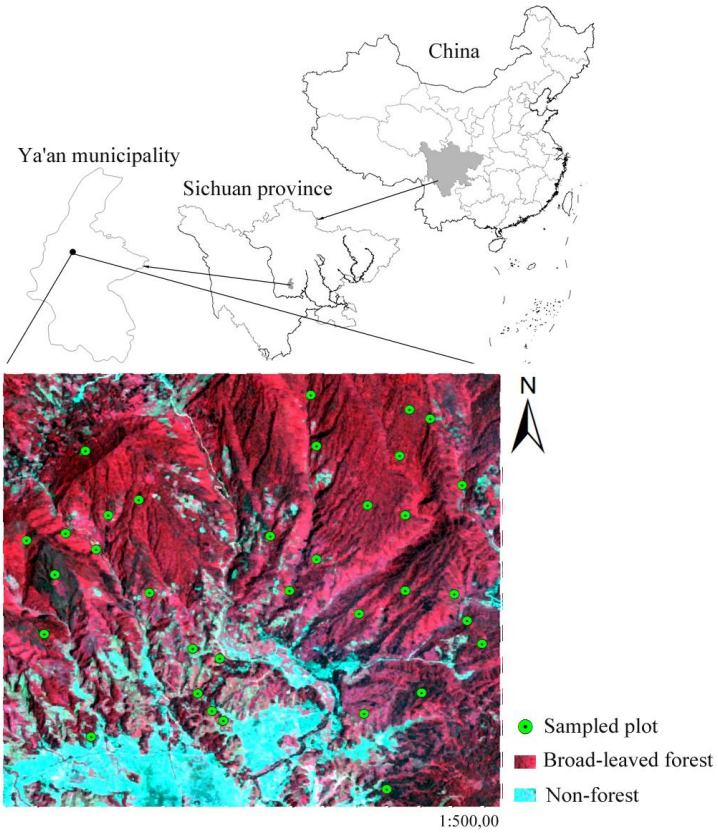

Figure A1. *Map of the study site in Shangli town of Ya'an city, southwest Sichuan, western China. 34 plots are shown used green dot. Data obtained from the National Fundamental Geographic Information System (NFGIS, http://ngcc.sbsm.gov.cn/), then edited using ArcGIS 10.2 (ESRI, Redlands, CA, USA)*

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HOST AND OVIPOSITIONAL PREFERENCE OF RICE WEEVIL (*SITOPHILUS ORYZAE)* DEPENDING ON FEEDING EXPERIENCE

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Abstract. Rice weevil, *Sitophilus oryzae* is one of the most devastating primary pests of stored grains. Adults feed mainly on endosperm, while larvae feed on germ, resulting in reduced germination and nutritional value of kernels. The influence of a commodity condition on the food preferences of *S. oryzae* has been well documented, but the influence of previous feeding experience ("natal habitat preference induction" – NHPI theory) has not yet been confirmed. This research aims to test the NHPI theory, to study the behavioural responses of *S. oryzae* virgin males and females to different grains (maize, wheat and barley) depending on rearing substrate and feeding history and to test host, feeding and oviposition preference. In a "Choice test", the host preference was determined based on the adult distribution on specific grains, feeding preferences based on grain damage (%) and grain loss (%), and ovipositional preference based on the progeny production. The results indicate that host, feeding and ovipositional preference of *S. oryzae* was not dependent on the previous feeding experience. Maize was the most preferential grain, regardless on the rearing substrate, for both male and female weevils. Grain damage, weight loss and progeny production were higher on maize, regardless on the rearing history.
Keywords: *maize, grains, storage pest, food attractiveness, progeny*

Introduction

In general, annual grain losses in storages due to the presence of insect pests approach 15% (Joshi et al., 1991), while the maximum grain loss attributed to a single weevil species reached 19% in wheat and nearly 57% in rice (Banerjee and Nazimuddin, 1985).

Rice weevil, *Sitophilus oryzae* (Linnaeus, 1763), (Coleoptera: Curculionidae) is one of the most economically damaging primary pests of stored grains, primarily barley, maize, rice, and wheat (Atwal and Dhaliwal, 2002). This pest causes the highest damage to grains that are stored at 25 – 30 °C and at low relative humidity (Batta, 2004). Adult weevils feed mainly on endosperm, reducing the carbohydrate content while larvae feed preferentially on germ, resulting in reduced germination and nutritional value due to removal of a large percentage of the proteins and vitamins. Nonetheless, the kernel

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damage caused by *S. oryzae* enables other species, secondary pests (external feeders) to additionally damage the grain. Similarly, Zakladnoi and Ratanova (1987) reported that during the development, larvae consume about 50% of the total grain weight. Additionally, seed germination is reduced and infested grain is more susceptible to infestation by other associated pests and pathogens. Although polyphagous pests consume and develop on a wide range of hosts, their behaviour is influenced by several physical and chemical factors. The influence of a commodity condition on the food preferences of *S. oryzae* has been well documented, but available data on the influence of previous feeding experience (at a larval stage) on food preference are scarce (Trematerra et al., 2013). Some authors report that a previous experience on host plants can modify insects feeding behavior. Since the induction of insect's preference to host plants varies depending on both, plant and insect species, it is necessary to investigate each insect-host plant interaction to determine if this phenomenon occurs or not (Boica Junior et al., 2016).

The most comprehensive review on the storage insect-host plant interactions was published by Trematerra et al. (2013) who summarized theories on insect host preference. As mentioned by Trematerra et al. (2013), theories like Hopkins host selection principle (Dethier, 1954), neo-Hopkins principle (Jaenike, 1983) and "chemical legacy hypothesis" (Corbet, 1985) tend to explain how can host preferences be induced without being genetically fixed. The Hopkins host selection principle (Hopkins, 1917) assumes that a memory of the feeding substrate is formed during the larval stage, stored in the central nervous system and transferred across metamorphosis to the adult stage. This phenomenon is called "preimaginal conditioning" (Thorpe and Jones, 1937; van Hemden et al., 1996; Barron, 2001; Blackiston et al., 2008). The "neo-Hopkins principle" (Jaenike, 1983) postulates that the host preference is determined at the adult stage shortly after the emergence from pupa ("early adult experience") (Van Emden et al., 1996). To date, the most comprehensive theory on the induction of host plant preferences is the "chemical legacy hypothesis" (Corbet, 1985). Based on this theory, small amounts of molecules of environmental chemicals inside the insect body or on the body surface at larval or pupal stage are assumed to influence the adult's food preferences. In this case, induction might happen in any developmental stage by contact with traces of chemicals transferred from an earlier stage. In this theory, no "learning" and no "memory" transfer among stages is required.

Mentioned theories, that were based on the results from several studies, support the idea that the experience with plant chemical residuals at the larval and/or adult stage can influence adult preference. However, in most studies, it has been difficult to identify the specific factors by which larval experience has changed adult behaviour because several learning mechanisms are involved. As Dethier noted in 1982, the mechanisms by which natal experiences affect later preferences vary. Therefore, he endorsed the more general term to describe situations in which experience with particular stimuli (plant chemical) increases preferences for the same stimuli "preference induction" or "natal habitat preference induction" - NHPI (Davis and Stamps, 2004). Namely, NHPI is an umbrella concept that encompasses a few more specialized terms, such as the Hopkins host selection principle and the "chemical legacy".

According to the NHPI hypothesis, phytophagous insect females prefer to lay their eggs on the host species on which they developed as larvae (Schoonhoven et al., 1998). As mentioned by Davies (2004), at this point, NHPI in the strict sense has been observed in relatively few species, so additional studies of this phenomenon are

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necessary. Even though there are numerous studies on the factors that affect the behaviour of *S. oryzae* in relation to previous experience at the adult stage, there is still inadequate information about the effect of natal exposure to specific nutrient medium. Food selection under natural conditions is influenced by various chemical and physical factors that affect insects' behaviour, ecological interactions and developmental or physiological status. Effects of feeding experience on food preference have been shown in various phytophagous insects, including stored-grain pests, as reported by Davis and Stamps (2004).

The relationship between preference of ovipositing females to certain plant species, growth, survival, and reproduction of offspring on those plants has been a central problem in the theory of insect-plant interactions. For *Sitophilus zeamais* (Motschulsky, 1855), it has been confirmed that females tend to lay eggs on maize kernels that are subjected to multiple visits, which emphasizes the significance of "already visited" kernels (Danho and Haubruge, 2003). However, for stored-product weevils, it is not clear what is the benefit from the oviposition on already-visited and infested kernels. Generally, it is considered that stored-product weevils differ significantly in food-mediated oviposition selection. As reported by Danho and Haubruge (2003), seed beetles like *S. zeamais,* can lay multiple eggs in a seed, increasing the competition among the larvae, while females of other species like bruchids, usually avoid competition during oviposition. As reported by Russell (1968), "kernel size" is a factor that also influences ovipositional preference of grain weevils and should not be overlooked.

As mentioned earlier, the influence of a specific commodity condition on *S. oryzae* development and preference depending on food quality has been well documented, but there are only a few available information for the nutritional preferences in relation to previous experience of food at larval stage. According to the NHPI hypothesis, phytophagous insect females prefer to lay eggs on the plant species on which they developed as larvae. This research aims to study the behavioral responses of virgin *S. oryzae* male and female adults to different grains on which they were not reared, and to test the NHPI hypothesis. Additionally, this work aimed to gain the information on host, feeding and oviposition preference of *S. oryzae*.

Material and methods

This work aimed to assess the feeding preference of *S. oryzae* weevils to different grains, in relation to previous feeding experience, as well as to test the NHPI hypothesis on this species. The influence of one factor- feeding experience at larval stage (i.e. rearing substrate) on host, feeding and oviposition preference of adults was assessed based on following indices: male and female distribution on different offered grains in a "Choice test", grain damage (%), remained grain weight (g), weight loss (g) and progeny production (male and female weevils).

Insect culture

S. oryzae weevils were reared for 10 generations on wheat kernels, variety Pobeda (wheat-reared weevils) and dent type maize kernels, hybrid NS640, (maize-reared weevils), in controlled conditions, at 27 ± 1 °C, 70 ± 5% RH and in continuous darkness (Trematerra et al., 2013).

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Newly emerged, less than 12-h-old adults of *S. oryzae,* were collected from rearing jars containing wheat and maize. The weevils were sexed for the assay, individually according to the shape of the rostrum, which is distinctly longer, narrower and smoother in the females than in males (Halstead 1963) and pronotum characters (Nardon and Nardon, 2002). Weevils were starved 24 h before setting up the experiment.

Host and feeding preference

In a "Choice test", host and feeding preference parameters were determined.

The host preference was assessed based on the mean number of weevils in each Petry dish (males, females and males + females), attracted to different grains, after each exposure period. To determine the response of *S. oryzae* males and females to different grains, 20 sexed weevils were placed from the rearing substrate in the center of test arena, a plastic container with a lid (60×40×25 cm). Four treatments containing 100 g of wheat, maize and barley were offered in separate Petri dishes (Ø 9 cm). Three Petri dishes were placed in the test arena and the assay was carried out for 50 days, but the readings were made after 12, 36 and 60 h, and 7, 25 and 50 days.

Feeding preference parameters included: grain damage, remained grain weight and weight loss (expressed in %). Damaged grains were separated manually from undamaged grains using a magnifying glass and were weighed, separately. Percent of grain damage was calculated using the following formula:

$$\text{Grain damage } (\%) = \frac{\text{number of damaged grains}}{\text{total number of grains}} \text{ x } 100 \qquad \text{(Eq.1)}$$

Upon the conclusion of the experiment (50 days) and after the new generation emergence, weight loss was calculated based on the weight at the beginning of the experiment and weight of grains after the exposure of 50 days and the removal of insects. Experiment was replicated six times.

Ovipositional preference and progeny production

Ovipositional preference was assessed based on the progeny production. From each substrate, and replicate, after the termination of the feeding preference assay (25 days), the adults were removed and Petri dishes with grains were closed and maintained at the same temperature for an additional period, up to 50 days from the beginning of the experiment. After 50 days, dishes were opened, and live adult progeny was counted.

All experiments were performed at 27 ± 1 °C, 70 ± 5% RH, in continuous darkness, as the rearing of parenteral population. Experiments were set in six replications.

Statistical analysis

The differences between distribution percent of weevils on different grains, the amount of consumed food and difference in progeny production were tested using Bonferroni test and Students T test in statistical Software SPSS 17, for the confidence interval of 95%. To determine the strength of the differences between groups the effect size was calculated using Eta squared test (Cohen, 1992).

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Results

Host and feeding preference

The number of weevils in different commodities significantly differed among recording periods. When provided with a choice of grains, wheat-reared males and females showed higher preference to maize and barley, compared to the wheat. In the first reading (after 12 h), the distribution of wheat-reared S. oryzae females in Petri dishes was 73% on maize, 22% on barley and 5% on wheat. This trend remained throughout the experiment. In the same period, males from wheat-reared population were distributed as follows: 67% of individuals on maize, 32% on barley and 1% on wheat (*Fig. 1*). The difference in distribution was statistically highly significant in both cases ($F = 466.7^{**}$, 740.3^{**}, $p < 0.01$, respectively). After 25 days, the total amount (100 g) of maize kernels was consumed in all replicates and both male and female weevils migrated mainly to barley (average 81% of individuals) and in smaller precent to wheat (19% of individuals). The difference was highly significant ($t = 387.8^{**}$, $p < 0.01$).

Maize-reared weevils were predominantly found on maize, followed by barley, while wheat kernels did not attract adults. Weevils already chose maize kernels in the first assessment, after 12 h (*Fig. 1*), and the number of both males and females on maize remained high during the entire observation period. However, slightly higher preference of males than females for maize kernels was recorded. After 12 h, the distribution of females was 93% on maize and 7% on barley, while males were distributed 89% on maize, 10% barley and 1% on wheat. The difference was highly significant ($t = 582.1^{**}$; $F = 962.5^{**}$, $p < 0.01$, respectively). This trend was more evident when pairs (the presence of male and female weevils simultaneously) were considered. Such distribution of individuals remained the same until the observation on the day 25. After 25 days, the entire amount of maize kernels was consumed and the weevils migrated mainly on barley (95% of weevils), and only 5% on wheat (in total, regardless on the sex). The difference was highly significant ($t = 744.1^{**}$, $p < 0.01$).

In the "Choice" test, after 50 days, in Petry dishes containing maize-reared weevils, the grain damage in maize (98.8%) was statistically higher compared to both barley (34.3%) and wheat (6.7%). The damages significantly differed from wheat-reared population ($F = 1481.0^{**}$, $p < 0.01$, Eta Squared 1.00) and the amount of consumed grains after 50 days is presented in *Table 1,* as well as the results on the remained grain weight and weight loss (%). The highest grain loss in wheat-reared case was evident in maize (84.3%), followed by barley (43.6%), while the lowest loss occurred in wheat (23.5%). The difference is statistically highly significant ($F = 167.4^{**}$, $p < 0.01$, Eta Squared 1.00).

Ovipositional preference and progeny production

Ovipositional preference was determined based on the progeny production. Significantly higher emergence of males and females from maize and barley grains, compared to the wheat, was recorded from maize-reared population (*Table 1*). On average, 18.62 males and 19.33 females emerged from maize, 12.71 males and 11.85 females emerged from barley, whereas 1.5 males and 2.0 females emerged from wheat. The differences between the number of emerged weevils from different substrates were significantly higher in maize-reared population, both for males ($F = 141.0^{**}$, Eta squared 1.00, $p < 0.01$) and females ($F = 530.0^{**}$, Eta squared 0.2, $p < 0.01$) as well as in a total number of emerged weevils ($F = 1377.6^{**}$, Eta squared 1.00, $p < 0.01$). However, the difference in sex emergence was not significant on certain grains.

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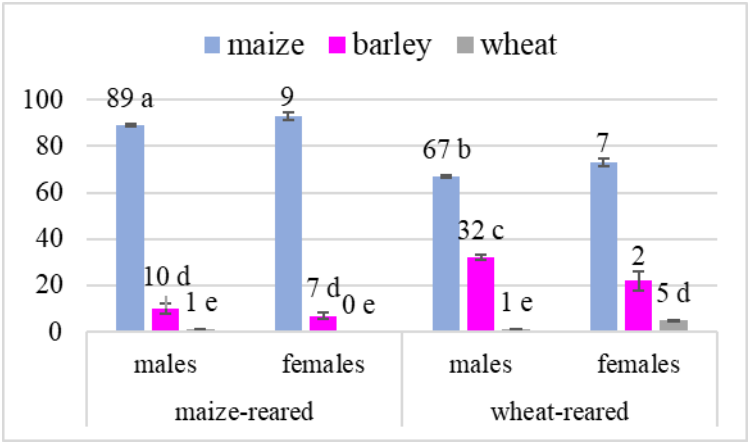

Figure 1. *Distribution of Sitophilus oryzae males and females after 12 h on different cereal grains*

From wheat-reared population, 9.8 males and 10.7 females emerged on maize, 7.6 males and 8.2 females on barley, while no progeny production was recorded on wheat (*Table 1*). The differences between sexes were not statistically significant, nor were the differences between maize and barley. However, the total differences, including wheat emerged progeny, were statistically highly significant for all, males (F = 1544.7**, Eta squared 0.94, $p < 0.01$), females (F = 5644.0**, Eta squared 0.63, $p < 0.01$) and total progeny (F = 3214.8**, Eta squared 1.00, $p < 0.01$).

Table 1. *Host and feeding preferences of maize- and wheat-reared S. oryzae adults on different grains after 50 days*

	Treatment	Grain damage (%)	RGW (g)	CGW (g)	Weight loss (%)	F1 progeny males[#]	F1 progeny females[#]	F1 total
Maize-reared	Maize	98.8 ± 0.1 a	1.3 ± 0.7 c	98.7 ± 8.2 a	98.7 ± 5.8 a	18.6 ± 3.1 a	19.3 ± 3.1 a	37.9 ± 1.1 a
	Wheat	6.7 ± 0.2 c	90.4 ± 4.1 a	9.6 ± 2.1 c	9.6 ± 1.1 c	12.7 ± 2.5 a	11.8 ± 2.6 b	24.5 ± 0.9 b
	Barley	34.3 ± 1.5 b	66.4 ± 3.6 b	33.6 ± 4.6 b	33.6 ± 6.2 b	1.5 ± 3.6 b	2.0 ± 0.2 c	5.2 ± 0.7 c
	F value	1481.0**	4599.0**	2141.00**	2141.0**	141.0**	530.0**	1377.6**
	Eta squared	1.000	1.000	1.000	1.000	0.994	0.183	0.998
Wheat-reared	Maize	84.3 ± 0.7 a	14.7 ± 2.7 c	85.3 ± 9.1 a	85.3 ± 2.5 a	9.8 ± 0.8 a	10.7 ± 2.3 a	20.5 ± 3.2 a
	Wheat	23.5 ± 1.3 c	64.4 ± 3.4 a	35.4 ± 3.3 c	35.4 ± 5.5 c	7.6 ± 1.1 a	8.2 ± 1.1 a	15.8 ± 2.4 a
	Barley	43.6 ± 2.1 b	24.6 ± 1.1 b	75.4 ± 7.4 b	75.4 ± 8.1 b	0.0 ± 0.0 a	0.0 ± 0.0 b	0.0 ± 0.0 b
	F value	167.4**	276.0**	654.2**	654.2**	1544.7**	5644.0**	3214.8**
	Eta squared	1.000	1.000	1.000	1.000	0.940	0.630	0.990

Value ± SD; F value – Bonferroni test; Eta squared – testing effect size; Values with the same letter are on the same level of significance for the confidence interval 95%; ** $P < 0.01$; * $P < 0.05$; NS - $P > 0.05$; RGW –remained grain weight; CGV – consumed grain weight; [#] progeny is presented as the number of offspring specimens

Discussion

Cereal grains differ significantly in attractiveness to *S. oryzae*, but this effect depends on several factors (Baker, 1988). This study tended to determine the main factors influencing host, feeding and oviposition preference of *S. oryzae*, in relation to Natal habitat preference induction hypothesis (NHPI) and/or food attractiveness.

In phytophagous insects, the NHPI predicts that females prefer to lay their eggs on the same host species on which they developed as larvae. NHPI hypothesis is based on

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the fact that experience with a natal habitat, namely food they developed on, shapes the habitat preferences of adults. However, since scientists used different terms to describe this phenomenon (Immelmann, 1975; Jaenike, 1983; Barron, 2001) it is unclear how frequently NHPI occurs and what are the implications of its occurrence. Results from several studies suggest that experience with plant chemical compounds (residuals) at the larval and/or pupal stage can influence adult preference to specific food. However, in most studies, the specific factors by which larval or pupal experience has changed adult behaviour were not identified because several learning mechanisms are involved.

Results on the host preference determined in this study indicate that wheat-reared *S. oryzae* adults preferred maize (43% female and 39% male individuals), to barley (35% female and 32% male individuals) and wheat (22% female and 29% male individuals). Maize-reared individuals, both males and females, had higher preference for maize (89% males and 93% females), less for barley (10% males and 7% females), while wheat was of the least favourable choice (1% of males). These findings are in accordance with Trematerra et al. (2013) who reported that inequality of maize, rice, barley and wheat in experiments indicate the fact that the preference is also influenced by genetic predispositions and that there are several factors that determine the behavioral response of *S. oryzae* to specific semiochemicals from food that are not related with natal exposure. On the other hand, this inequality could also be caused by the fact that maize, rice, barley and wheat kernels release different concentrations of odour. Also, as suggested by Trematerra et al. (2013), males visited more different food sources than females, which can be attributed to males pronounced mobility compared to females when food is available. Also, repeated "visits" by males could have occurred which is probably related to an increased probability in finding a suitable mating partner (Campbell, 2005; Guedes et al., 2010).

Price et al. (2011) reported that polyphagous herbivores use multiple host-plants for feeding and/or oviposition which is considered an evolutionary adaptation that enabled these species to adapt to variable environments. Unfortunately, the mechanisms of host-plant choice are not always easily defined and many factors are of importance, larval physiology, natural enemies, reproductive behaviour etc. (Bernays, 2001; Forister and Wilson, 2013; Bernays and Grahm, 2013). Female should choose the most suitable host plant for oviposition and for her offspring to develop. However, as reported by Nanthagopal and Uthamasamy (1989), female may make host choice decisions based on factors influencing her survival, such as nutritional quality of the plant.

For phytophagous insects such as *S. oryzae*, oviposition and food choice decisions are essentially the same (Singer et al., 1992). There is some evidence that oviposition preference and performance of offsprings can be correlated with heritable variability for oviposition preference as reported by Singer et al. (1988) for phytophagous insects in general, and by Fox (1993) for bruchids.

Based on the results of our study, previous feeding experience on feeding behaviour of *S. oryzae* virgin males and males was only confirmed in the case of maize-reared populations. Trematera et al. (2013) concluded that larval experience does not affect host preference in *S. oryzae* adults and also, it is not determinative in food selection. However, it should be mentioned that presented results correspond the specific grain used in the experiment (i.e. varieties) and no generalization should be made, since the data obtained may not be transferable to other commodities. The higher preference of maize and barley compared to wheat might be also due to a release of different concentrations of volatile compounds from maize, rice, barley and wheat kernels. Plant-

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borne volatiles play a role in food and host location, routing insect orientation and searching behaviour (Dicke and Baldwin, 2010). Germinara et al. (2008) indicate that for example, granary weevil adults can respond with different behavior to a wide range of cereal volatiles and that response may change depending on the concentration. Host finding behavior of weevils will depend on the balance of positive and negative volatile stimuli from grain as the relative concentrations of volatiles may change during storage. According to Visser (1986), phytophagous insects use volatiles from plant materials to locate suitable substrates and, as stated by Kanaujia and Levinson (1981) the presence of phagostimulatory compounds are considered crucial in the infestation process in storage pests. Additionally, Levinson and Kanaujia (1982) report that *S. granarius* male and female respond to various extracts from stored winter wheat. We can speculate that, since NHPI theory was not proven in our study, the food attractiveness, as a result of nutritive value and the presence of certain volatiles, was the factor influencing host, food and oviposition preference of *S. oryzae* to maize, primarily.

In our study, the feeding preference of *S. oryzae* towards wheat, maize, and barley was tested under "choice" conditions. Grain damage, grain weight loss and progeny production differed significantly among the various selected host grains. Grain weight loss was found to be the greatest in maize (98.8%) and the lowest in wheat (6.7%), for the population reared on maize. Subedi et al. (2009) also confirmed that, wheat was the least attractive host for *S. oryzae* compared to other cereals (rice, barley and maize). These authors reported that the greatest grain damage was observed in polished rice (18.75%) and less in wheat (16.25%) in a free-choice test. However, these findings are opposite to the results presented by Ansari (2003) where damages in wheat were the highest (67.78%), while in maize were significantly lower (40.97%). Subedi et al. (2009) reported that wheat was the most preferred host under no-choice conditions, however, when insects were offered a choice of polished rice and wheat, polished rice was the most preferred choice in "choice test". *S. oryzae* thus preferred polished rice under free-choice and wheat under no-choice.

Ovipostion and progeny production were higher on maize, regardless on the rearing history. In this study, the progeny production in maize-reared weevils was the highest on maize (37.9 in total), followed by barley (24.5 individuals; males + females) while the lowest on wheat (3.5 individuals). On wheat-reared population, the situation was similar, so in total (males + females) 20.5 weevils emerged from maize, 15.8 from wheat and none from barley. Our results also suggest that larger kernels, like maize are more desirable hosts for *S. oryzae*, comparing to small-kernel hosts, such as barley and wheat. Stejskal and Kucerova (1996) and Akhter et al. (2017) have demonstrated that *S. oryzae* prefers large kernels for oviposition than smaller, because they can contain more than one egg, comparing to smaller ones. Akhter et al. (2017) showed that *S. oryzae* adults preferred to lay larger number of eggs on pulse, which are considerably larger in size than other tested grains, rice and wheat. Russell (1968) also observed that weevil preferred to lay eggs in grains of larger size. Some other factors besides grains size also affect host and ovipositional preference. For example, seed with smooth surface are more preferred than rough and spiny ones (Salunkhe and Jadhav, 1982). Also, hardness of grain is very important factor which affects the oviposition rates (Teotia and Singh, 1968). Females accepted large kernels more quickly than small kernels and this contributed to increased oviposition in large kernels. The increase in the number of eggs per kernel appears to result from an increase in number of visits resulting in oviposition rather than an increase in the number of eggs laid during a visit (Campbell, 2002.)

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Conclusion

This study tended to determine the main factors influencing host, feeding and oviposition preference of *S. oryzae*, in relation to NHPI and/or food attractiveness.

The results of this study reveal that host, feeding and ovipositional preference of rice weevil, *Sitophilus oryzae* was not depending on the previous feeding experience. Host preference, based on the distribution of females and males on different grains, grain damage, grain weight loss and progeny production differed significantly among selected host grains. Maize was the most preferential grain, in all aspects, regardless on the rearing substrate, for both male and female weevils. Grain damage, weight loss and progeny production were higher on maize, regardless of the rearing history, followed by barley. Oviposition and progeny production were the highest on maize, regardless of the rearing history. Since NHPI theory was not proven in our study, we can speculate that food attractiveness was most probably result of nutritive value and the presence of certain volatiles, and therefore was the main factor influencing host, food and oviposition preference of *S. oryzae* to maize. Additionally, we can speculate that kernel size plays very important role in host preference since in both wheat- and maize-reared population, adult weevils chose larger maize kernels before smaller wheat and barley kernels as the most preferable food source.

Future research should be directed towards identification of different volatiles in host-plants which influence the behavior of *S. oryzae* and conduct behavioral studies with identified volatiles. Also, population parameters of *S. oryzae* should be conducted on different hosts in a large number of generations in order to evaluate the adaptive response of *S. oryzae* to the change of host.

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INFERENCE OF HETEROTIC PROSPECTS FOR ASSORTED MORPHOPHYSIOLOGICAL TRAITS OF WHEAT (*TRITICUM AESTIVUM* L.) AFTER CROSSING WITH LOCAL LAND RACES

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Abstract. Manipulation of heterotic potential is considered to be a vital approach for enhancing yield potential of wheat to overcome food security threats. Heterotic potential of ten F1 bread wheat hybrids was estimated for grain yield and contributing traits. Hybrids were developed by crossing five genetically diverse elite wheat lines/varieties with two local land races following line × tester mating fashion at the regional Agricultural Research Institute, Bahawalpur (Pakistan). The resultant hybrid progenies were grown along with parental genotypes during rabi season 2014-15. Highly significant genetic variability was present in the experimental material for the traits under study. Most of the crosses showed significant heterosis over mid, better and standard parents. Maximum significant commercial heterosis was observed in plant height (27.66%), followed by tillers per plant (16.67%), peduncle length (13.59%) and grain yield per plant (10.68%).The highest increase in grain yield per plant over the commercial variety was observed for the cross CB-35 × LR1 which may be considered for selection as hybrid or pure line wheat varieties for increasing grain yield.
Keywords: *food security, hybrids, crosses, genetic variation, heterosis*

Introduction

Wheat (*Tritium aestivum* L.), is the most important crop and among the major three cereal crops that provide staple nutrient source for 40% of world population (Giraldo et al., 2019) and 20 percent of the total energy requirement in human food (Shewry, 2009). Wheat belongs to the *poaceae* family, originating from the Levant region of the Near East and Ethiopian Highlands, but now cultivated worldwide (Belderok, 2000). Major cultivated species of wheat include: *Triticum aestivum*, which is a hexaploid species and is widely cultivated in the world; *Triticum durum*, the only tetraploid form of wheat widely used today, and the second most widely cultivated wheat; *Triticum monococcum*, a diploid species with wild and cultivated variants; *Triticum dicoccum*, a tetraploid species, cultivated in ancient times but no longer has widespread use; *Triticum spelta*, another hexaploid species, which is cultivated in limited quantities (Moon, 2008). It is used to make flour for leavened, flat and steamed breads and most of the baked foods (Hrivna et al., 2018) and for fermentation to make beer and alcohol (Tsenov et al., 2008).

In Pakistan, wheat is averagely used for about 60 percent of daily diet of common men with average per capita consumption of 125 kg (Khan et al., 2003). Pakistan is among top

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ten wheat producing countries of the world (Ihsanullah et al., 2002). The contribution of wheat to value addition in agriculture is 8.9 percent, while its contribution to GDP is 1.6 percent (GOP, 2019). Breeding efforts have resulted in various varieties of hexaploid wheat, having improved yield and grain characters. Varieties and advanced lines with different morphological and economic characteristics are now available as breeding stock.

To feed flourishing population of Pakistan; the genetic improvement of wheat genotypes for high yield potential is a dire need. For this purpose, the exploitation of maximum genetic potential from available genetic resources of wheat is a prerequisite. F_1 hybrid carrying heterotic effects, which are featured in all crop species, the yield gains are limited to the F_1 generation. Heterosis is considered as the superiority of the hybrids in comparisons to either of its parents. It is the allelic or non-allelic interaction of genes under the influence of specific environment. Heterosis has been estimated in a range of cultivated crops and has been the purpose of considerable importance to study as mean of increasing productivity of crop plant. It is now well established that heterosis does occur with proper combination of parents. Formerly, utilization of heterotic effects for grain yield was mainly ascribed to cross-pollinated crops. However, later it was reported in wheat as being predominantly self-pollinated for the first time by Freeman (1919), who well-versed the supremacies of F_1 crosses over their parents (Özgen, 1989). Briggle (1963) described existence of heterosis in substantial quantity for grain yield components in different F_1 wheat crosses. Keeping in view the above facts, the current research was designed to create genetic diversity and to estimate heterotic potential of crosses among elite lines/varieties with local races of wheat.

Materials and Methods

The plant materials for study consisted of 7 wheat (*Triticum aestivum*) genotypes including four strains (CB-35, CB-212, CB-214 and CB-219), one commercial variety (Mairaj-08) and two land races (LR-1 and LR-2) obtained from germplasm resources, Regional Agricultural Research Institute, Bahawalpur (Pakistan). All the genotypes were 99% pure and true breeding. The selection criteria of crossing lines (dwarf) was based on inducing semi dwarfness in next generation for enhanced yield. Sowing was done in *Rabi* (Winter crop growing months) season 2013-14, the seeds of each genotype was sown in two rows of 5 m length each separated by 30 cm. Recommended cultural practices (like fertilization, irrigation, weeding) were carried out during the whole experimental duration. At heading stage, crossing was made following line × tester fashion by keeping advance strains and commercial variety as lines (female parents) and land races as testers (male parents). Usual method for emasculation and pollination was adopted to prevent self pollination which a major limitation in hybrid production. 10-15 pairs of spikes were crossed to get optimum quantity of F_0 seed for each cross. At maturity, crossed spikes were cut, threshed manually, packed and labeled individually for each family.

Ten adjacent plants were tagged from the two central rows (5 plants from each row) prior to heading stage from each experimental plot. Data for various morpho-physiological traits including plant height, tillers per plant, days to heading, days to anthesis, days to maturity, grain filling period, peduncle length, spike length, grains per spike, grain weight per spike, 1000-grain weight and grain yield per plant were measured from tagged plants at appropriate stage.

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Statistical Analysis

The analysis of variance was carried out following Steel and Torrie (1980). Significance of differences among genotypes for various plant variables was tested by least significant difference (LSD). Heterosis as compared to mid parent (MP Het.), better parent (BP Het.) and standard parent (SP Het.) as over the commercial variety i.e. Mairaj-08, was estimated using the formulae as

$$\text{Mid parent heterosis (MP Het.)} = (F_1 - \text{Mid parent})/\text{Mid parent}\rceil \times 100 \quad \text{(Eq.1)}$$
(Matzinger et al., 1962)

$$\text{Better parent heterosis (BP Het.)} = (F_1 - \text{Better parent})/\text{Better parent}\rceil \times 100 \quad \text{(Eq.2)}$$
(Fonseca and Patterson, 1968)

$$\text{Standard parent heterosis (MP Het.)} = (F_1 - \text{Standard parent})/\text{Standard parent}\rceil \times 100 \quad \text{(Eq.3)}$$
(Wynne et al., 1970)

The significance of heterosis was tested by applying t-test according to Wynne et al. (1970).

Results and Discussion

Analysis of variance revealed significant differences among all the genotypes for various traits under consideration. Variability due to genotypes was spliced into parents and hybrids, both the components also revealed significant variability with respect to all the characters. Parental genotypes were further split into lines and testers, both of which showed significant differences in performance for various morpho-physiological traits under study (*Table 1*). Mean performance of parents all the genotypes (parents and F_1 hybrids) and heterotic potential for various traits under consideration were as is as under.

Table 1. *Analysis of variance (mean square values) for various morphophysiological traits in wheat*

S.O.V	d.f.	Plant height	Tillers/ plant	Days to heading	Days to anthesis	Days to maturity	Grain filling period	Peduncle length	Spike length	Grains/ spike	Grain weight/ spike	1000-Grain weight	Grain yield/ plant
Reps.	2	4.137	0.137	4.941	5.353	11.353	1.118	3.843	3.078	5.706	0.129	2.431	5.471
Genotypes	16	1710.755	48.436	203.625	145.853	315.125	42.978	163.461	31.282	57.044	0.143	38.093	59.230
Parents	6	3828.714	86.413	327.857	229.714	483.191	60.429	324.714	47.079	104.857	0.030	29.714	95.937
Crosses	9	458.300	26.078	142.700	105.633	232.015	33.070	59.644	10.504	28.300	0.201	19.467	38.848
Lines	4	992.383	53.550	316.200	230.550	507.950	68.450	121.283	19.967	59.550	0.310	39.133	77.950
Testers	1	140.833	17.633	2.700	2.700	4.800	0.300	34.133	13.333	2.700	0.179	2.133	7.500
L × T	4	3.583	0.717	4.200	6.450	12.883	5.883	4.383	0.333	3.450	0.097	4.133	7.583
Error	32	4.262	0.929	3.941	5.228	7.603	2.868	4.343	1.245	4.268	0.027	4.931	3.991

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Greater plant height is considered an undesirable character due to its positive correlation with lodging and negative relation with earliness. Thus taller plants tend to lodge more resulting in low grain yield (Mazurek and Sabat, 1984). Introduction of *Rht* dwarfing genes in tall stature wheat varieties resulted semi-dwarf varieties responsive to inputs which brought green revolution in 1960's. In present studies, parental genotypes which were used as females were short to semi dwarf statured ranging from 42 cm (CB-212) to 94 cm (Mairaj-08) as compared to males (LR-1 122.00 cm and LR-2 128.00 cm tall) (*Table 2*, *Fig. 1*).

Table 2. *Mean performance with LSD of parents and crosses for various morpho-physiological traits in wheat*

Genotype	Plant height		Tillers/plant		Days to heading		Days to anthesis		Days to maturity		Grain filling period	
CB-35	56.00	M	15.67	BC	99.00	HI	113.00	GH	136.00	JK	23.00	EF
CB-212	42.00	O	17.33	A	126.00	A	136.00	A	168.00	A	32.00	AB
CB-214	48.00	N	16.00	AB	110.00	EF	120.00	EF	150.00	FGH	30.00	BCD
CB-219	61.00	L	14.33	CD	106.00	G	121.00	EF	144.33	I	23.33	EF
Mairaj-08	94.00	E	12.00	FGH	96.00	I	110.00	H	132.00	K	22.00	F
LR-1	122.00	B	5.67	J	112.00	DE	121.00	EF	151.00	EFG	30.00	BCD
LR-2	128.00	A	3.67	K	118.00	BC	128.00	BC	160.00	BC	32.00	AB
CB-35 × LR1	74.00	IJ	13.00	DEF	110.00	EF	120.00	EF	151.00	EFG	31.00	ABC
CB-35 × LR2	78.00	H	12.00	FGH	113.00	DE	123.00	DE	155.00	DE	32.00	AB
CB-212 × LR1	68.00	K	14.00	D	119.00	B	129.00	B	162.00	B	33.00	A
CB-212 × LR2	71.00	JK	12.33	EFG	118.00	BC	130.00	B	161.00	B	31.00	ABC
CB-214 × LR1	75.00	HI	13.67	DE	114.00	D	125.00	CD	154.00	DEF	29.00	CD
CB-214 × LR2	82.00	G	11.33	GH	115.00	CD	127.00	BC	156.33	CD	29.33	BCD
CB-219 × LR1	90.00	F	10.67	H	108.00	FG	120.00	EF	149.33	GH	29.33	BCD
CB-219 × LR2	94.00	E	8.67	I	107.00	FG	118.00	F	146.00	HI	28.00	D
Mairaj-08 × LR1	99.33	D	6.33	J	99.00	HI	114.00	G	136.00	JK	22.00	F
Mairaj-08 × LR2	103.00	C	5.67	J	100.00	H	113.00	GH	138.00	J	25.00	E

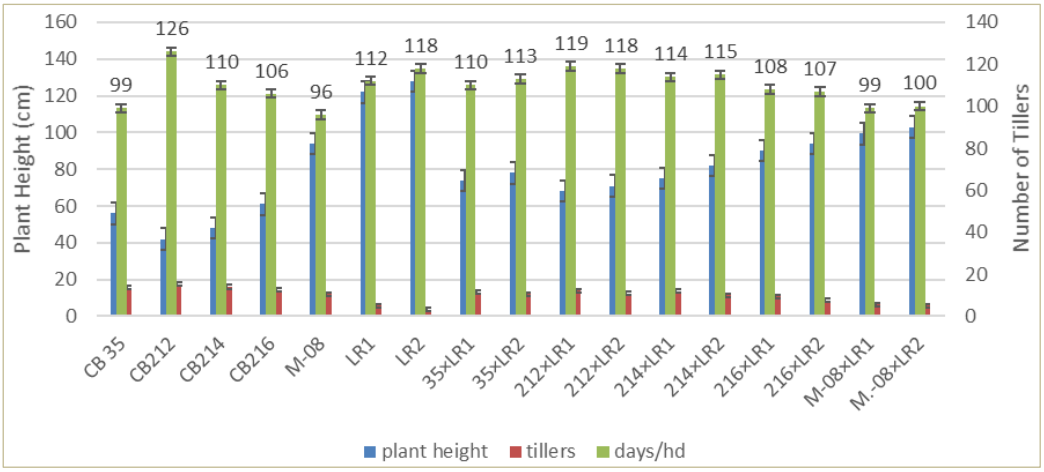

Figure 1. *Mean performance of parents and crosses for plant height, tiller per plant and days to heading*

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All the F_1 crosses lied in between the range of parental genotypes with respect to plant height. CB-212 × LR-1, among F1 crosses, was at the lowest position regarding height while maximum height was recorded for the cross Mairaj-08 × LR-2 (103.00 cm) (*Table 2*, *Fig. 1*).

Lower value of number of days taken to 50% heading is directly related to earliness and vice versa. In present studies, parental genotypes which were used as females were ranging between 96.00 cm (Mairaj-08) to 126.00 (CB-212). While the male parents LR-1 and LR-2 were 112.00 cm and 118.00 cm, respectively (*Table 2* and *Fig. 1*).

All the F_1 crosses lied in between the range of parental genotypes with respect to number of days to 50% heading. Mairaj-08 × LR-1, among F_1 crosses, showed the minimum number of days for 50% heading (99) and hence was considered as the most favorable cross regarding earliness, closely followed by Mairaj-08 × LR-2 (100) (*Table 2*). While maximum days to heading were observed for the cross CB-212 × LR-1 (119.00) (*Table 2* and *Fig. 1*).

Days to anthesis are also a parameter related to short or long duration for maturity. It is also most affected by the environmental conditions. Maximum days to anthesis were taken by the line CB-212 i.e. 136 days while minimum days to anthesis were recorded for Mairaj-08 (110 days) (*Table 2*, *Fig. 2*). The results indicated Mairaj-08 as the favorable parent regarding earliness with respect to number of days taken for anthesis. Among male parents, LR-1 showed early anthesis (121 days) as compared to LR-2 which took 128 days (*Table 2* and *Fig. 2*).

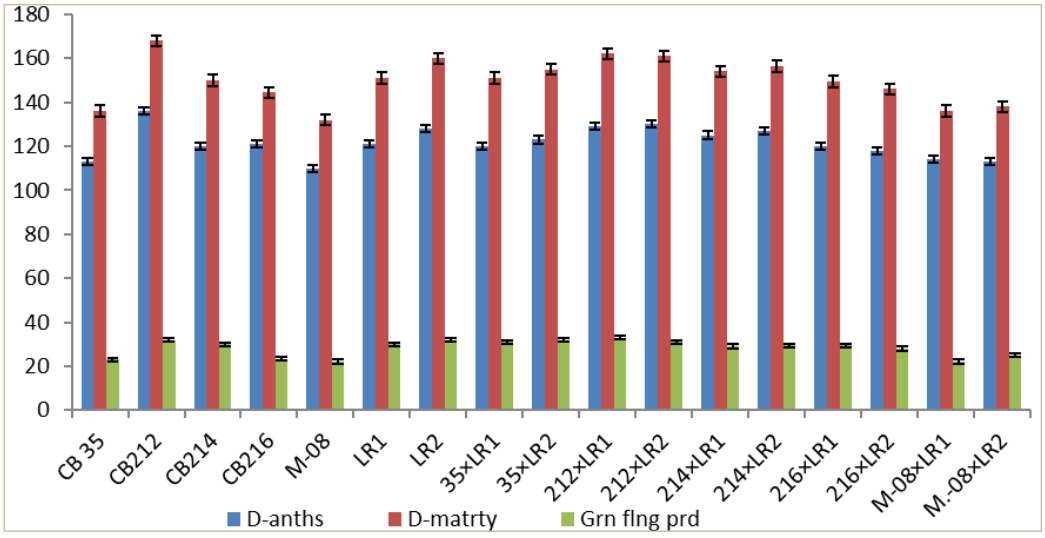

Figure 2. *Mean performance of parents and crosses for days to anthesis, days to maturity and grain filling period*

In some crosses where heterobeltiosis was observed over dominance might be involved and it may be concluded that effective selection of desirable recombinants from this material is possible. These results are in accordance with the previous findings (Sadeque et al., 1991; Ullah et al., 2006; Akbar et al., 2007) who also reported negative heterosis for plant height.

More tillers per plant results in more spikes per plant which increases the grain yield per plant. More tillering capacity was observed in lines ranging from 12.00 to 17.33 tillers

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as compared to testers (3.67 - 5.67 tillers per plant) (*Table 2*, *Fig. 1*). CB-212 produced the highest number of tillers per plant followed by CB-214 with 16.00 tillers while Mairaj-08 produced relatively lower number of tillers per plant (12.00) (*Table 2* and *Fig. 1*).

All the F_1 crosses lied in between the range of parental genotypes with respect to tillers per plant, Mairaj-08 × LR-2, among F_1 crosses, was at the lowest position regarding tillers per plant while maximum tillers per plant was recorded for the cross CB-214 × LR-1 (14.00) (*Table 2* and *Fig. 1*).

All the F_1 crosses lied in between the range of parental genotypes with respect to days to anthesis. Mairaj-08 × LR-2, took the lowest number of days regarding the trait (113.00), closely followed by Mairaj-08 × LR-1 (114.00 cm). CB-212 × LR-2 took the maximum number of days to anthesis (130 days), closely followed by CB-212 × LR-1 with 129 days to anthesis (*Table 2* and *Fig. 2*).

Days to maturity is the parameter directly related to crop duration whether early or late. A genotype taking more number of days to maturity is long duration and vice versa. Less number of days taken to maturity is desirable character. In current studies, among parental genotypes which were used as females, Mairaj-08 was the most early maturing with minimum days to maturity (132) followed by CB-35 which took 136 days to reach maturity (*Table 2*, *Fig. 2*). While CB-212 was recorded as the longest durational genotype taking 168 days to reach maturity (*Table 2*, *Fig. 2*). CB-214 and CB-219 took 150 and 144.33 days respectively, to reach their maturity stage (*Table 2*, *Fig. 2*). Among testers, LR-1 took 151 days while LR-2 took 160 days for maturity (*Table 2* and *Fig. 2*).

Range of F_1 crosses regarding number of days to maturity was recorded as 136 to 162 which are in between the two extremes of the parents for the concerned trait. Cross of Mairaj-08 with LR-1 and LR-2 were considered as the early maturing hybrids with 136 and 138 days taken to maturity, respectively (*Table 2* and *Fig. 2*). On the other hand, CB-212 × LR-1 took the maximum number of days to maturity with a value of 162 days, closely followed by CB-212 × LR-2 (161 days) (*Table 2* and *Fig. 2*).

Days taken for grain filling are also an important determinant of earliness or lateness of a variety or genotype. However it is also largely influenced by the environmental conditions. Minimum grain filling period was observed in Mairaj-08 (22 days), closely followed by CB-35 and CB-219 with values 23.0 and 23.33, respectively (*Table 2*, *Fig. 2*). CB-212 took the maximum days for grain filling i.e. 32 days, followed by CB-214 which required 30 days for filling of grains. While the testers LR-1 and LR-2 took 30 and 32 days period for grain filling (*Table 2* and *Fig. 2*).

In present studies, parental genotypes which were used as females were short statured for peduncle length ranging from 20 cm (CB-212) to 36 cm (Mairaj-08) as compared to males (LR-1 44 cm and LR-2 47 cm tall) (*Table 3* and *Fig. 3*).

All the F_1 crosses lied in between the range of parental genotypes with respect to peduncle length. CB-212 × LR-1, among F_1 crosses, was at the lowest position regarding peduncle length while maximum peduncle length was recorded for the cross Mairaj-08 × LR-1 (41.00) (*Table 3* and *Fig. 3*).

Almost half of the F1 hybrids showed increase in peduncle length over mid parental value but this increase was non-significant. All the crosses revealed heterobeltosis towards negative (lower) side, minimal decrease in peduncle length was recorded for the cross Mairaj-08 × LR-1 (*Table 4*). Above mentioned cross also gave the highest heterotic value of 13.89% for peduncle length over the standard parent (*Table 4*). These outcomes are in agreement with those of Farooque et al. (2005), Masood et al. (2005) and Ilker et

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al. (2010) who advocated that long peduncle has positive direct effect on grain yield due to its photosynthetic activity.

Table 3. *Mean performance with LSD of parents and crosses for various morpho-physiologicaltraits in wheat*

Genotype	Peduncle length		Spike length		Grains/spike		Grain weight/spike		1000-Grain weight		Grain yield/plant	
CB-35	26.00	JK	16.33	A	44.00	BCDE	2.42	A	41.00	A	35.33	ABCD
CB-212	20.00	L	17.00	A	42.00	DEFG	2.35	AB	38.00	ABCD	37.67	A
CB-214	23.00	KL	11.67	B	46.00	ABC	2.26	ABC	35.00	DEFG	36.00	ABC
CB-219	30.00	HI	9.33	CDE	47.00	AB	2.40	AB	40.00	AB	35.33	ABCD
Mairaj-08	36.00	DEF	11.00	BC	49.00	A	2.36	AB	39.00	ABC	34.33	BCDE
LR-1	44.00	AB	8.00	EFG	36.00	JK	2.27	ABC	36.00	CDEF	27.67	G
LR-2	47.00	A	6.67	FGH	33.00	K	2.13	BC	32.00	GH	22.00	H
CB-35 × LR1	30.33	HI	10.00	BCD	42.00	DEFG	2.30	ABC	37.00	BCDE	38.00	A
CB-35 × LR2	34.00	FG	9.00	DE	40.00	FGHI	2.29	ABC	35.00	DEFG	36.00	ABC
CB-212 × LR1	28.00	IJ	11.67	B	38.00	HIJ	2.26	ABC	34.00	EFGH	37.00	AB
CB-212 × LR2	32.00	GH	10.00	BCD	39.00	GHIJ	2.30	ABC	33.00	FGH	36.33	ABC
CB-214 × LR1	35.00	EFG	7.67	EFGH	46.00	ABC	1.46	D	28.00	I	37.67	A
CB-214 × LR2	37.00	DEF	6.67	FGH	45.00	BCD	2.06	C	30.33	HI	33.33	CDE
CB-219 × LR1	38.00	CDE	8.33	DEF	43.00	CDEF	2.20	ABC	34.00	EFGH	31.67	EF
CB-219 × LR2	39.00	CD	6.33	GH	41.00	EFGH	2.22	ABC	33.00	FGH	32.67	DE
Mairaj-08 × LR1	41.00	BC	7.00	FGH	37.00	IJ	2.19	ABC	32.00	GH	28.00	G
Mairaj-08 × LR2	41.00	BC	6.00	H	38.00	HIJ	2.32	ABC	31.00	HI	29.00	FG

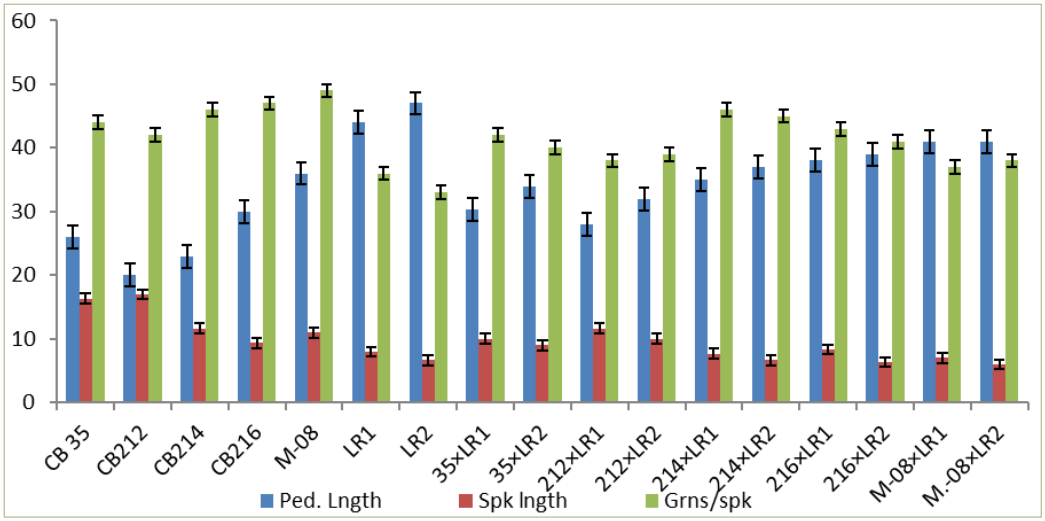

Figure 3. *Mean performance of parents and crosses for peduncle length, spike length and grains per spike*

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Table 4. *Heterosis percentage over mid parent, better parent and standard variety (Mairaj-08) for days to maturity, grain filling period, peduncle length and spike length in wheat*

CROSS	Days to maturity			Grain filling period			Peduncle length			Spike length		
	MP Het.	BP Het.	SP Het.	MP Het.	BP Het.	SP Het.	MP Het.	BP Het.	SP Het.	MP Het.	BP Het.	SP Het.
CB-35 × LR1	5.23**	0.00^{NS}	14.39**	16.98**	3.33^{NS}	40.91**	-13.33**	-31.06**	-15.74**	-17.81**	-38.78**	-9.09^{NS}
CB-35 × LR2	4.73**	-3.13*	17.42**	16.36**	0.00^{NS}	45.45**	-6.85^{NS}	-27.66**	-5.56^{NS}	-21.74**	-44.90**	-18.18*
CB-212 × LR1	1.57^{NS}	-3.57*	22.73**	6.45^{NS}	3.13^{NS}	50.00**	-12.50**	-36.36**	-22.22**	-6.67^{NS}	-31.37**	6.06^{NS}
CB-212 × LR2	-1.83^{NS}	-4.17**	21.97**	-3.13^{NS}	-3.13^{NS}	40.91**	-4.48^{NS}	-31.91**	-11.11*	-15.49*	-41.18**	-9.09^{NS}
CB-214 × LR1	2.33^{NS}	1.99^{NS}	16.67**	-3.33^{NS}	-3.33^{NS}	31.82**	4.48^{NS}	-20.45**	-2.78^{NS}	-22.03**	-34.29**	-30.30**
CB-214 × LR2	0.86^{NS}	-2.29^{NS}	18.43**	-5.38^{NS}	-8.33^{NS}	33.33**	5.71^{NS}	-21.28**	2.78^{NS}	-27.27**	-42.86**	-39.39**
CB-219 × LR1	1.13^{NS}	-1.10^{NS}	13.13**	10.00*	-2.22^{NS}	33.33**	2.70^{NS}	-13.64**	5.56^{NS}	-3.85^{NS}	-10.71^{NS}	-24.24**
CB-219 × LR2	-4.05**	-8.75**	10.61**	1.20^{NS}	-12.50**	27.27**	1.30^{NS}	-17.02**	8.33^{NS}	-20.83*	-32.14**	-42.42**
Mairaj-08 × LR1	-3.89**	-9.93**	3.03^{NS}	-15.38**	-26.67**	0.00^{NS}	2.50^{NS}	-6.82^{NS}	13.89**	-26.32**	-36.36**	-36.36**
Mairaj-08 × LR2	-5.48**	-13.75**	4.55*	-7.41^{NS}	-21.88**	13.64*	-1.20^{NS}	-12.77**	13.89**	-32.08**	-45.45**	-45.45**

**,* is equal to significant at 0.01, 0.001 respectively and NS showing non significant

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Longer spike having more spikelets is usually, not always, considered associated with higher seed number per spike and ultimately the yield. The highest spike length, among lines, was observed in CB-212 (17.00 cm), followed by CB-35 with 16.33 cm average spike length (*Table 3*). Minimum spike length was recorded for the genotype CB-219 (9.33 cm) (*Table 3*). CB-214 and Mairaj-08 produced spikes of 11.67 and 11.00 cm length respectively. Both the testers, LR-1 and LR-2 produced shorter spikes with average length of 8.00 and 6.67, respectively (*Table 3* and *Fig. 3*).

Character of grains per spike is considered one of the major directly related yield components. Greater the number of grains per spike, greater will be the yield and vice versa. In present studies, parental genotypes which were used as females were showed a range of 42 to 49. Mairaj-08 gave the highest number of grains per spike followed by CB-219 (47). On the other hand CB-212 gave the least number of grains per spike (42). Among male parents LR-1 produced 36 grains per spike while LR-2 showed 33 grains per spike (*Table 3* and *Fig. 3*).

All the F_1 crosses lied in between the range of parental genotypes with respect to grains per spike. CB-214 × LR-1 was the most desirable cross as it produced the maximum grains per spike (46), followed by CB-214 × LR-2 (45) (*Table 3*, *Fig. 3*). While Mairaj-08 × LR-1 performed poor with respect to the character as it produced the lowest grains per spike (37), followed by Mairaj-08 × LR-2 and CB-212 × LR-1 which produced equal number of grains per spike (38) (*Table 3* and *Fig. 3*).

A range of 6.00 cm to 11.67 cm regarding spike length was recorded in F_1 population. CB-212 × LR-1 was considered the most favorable cross for higher spike length which produced spikes of 11.67 cm length (*Table 3*, *Fig. 3*). CB-212 × LR-2 and CB-35 × LR-1 produced spike of equal length of 10.00 cm. While the lowest spike length was observed in Mairaj-08 × LR-2 (6.00 cm), followed by CB-214 × LR-2 with 6.67 cm spike length (*Table 3*).

Maximum grain weight per spike was recorded for the line CB-35 (2.42 g), followed by CB-219 showed 2.40 g weight of grains per spike. While the lines Mairaj-08, CB-212 and CB-219 gave 2.36 g, 2.35 g and 2.26 g weight of grains per spike (*Table 3*, *Fig. 4*). While LR-1 and LR-2 produced 2.27 g and 2.13 g grain weight per spike (*Table 3* and *Fig. 4*).

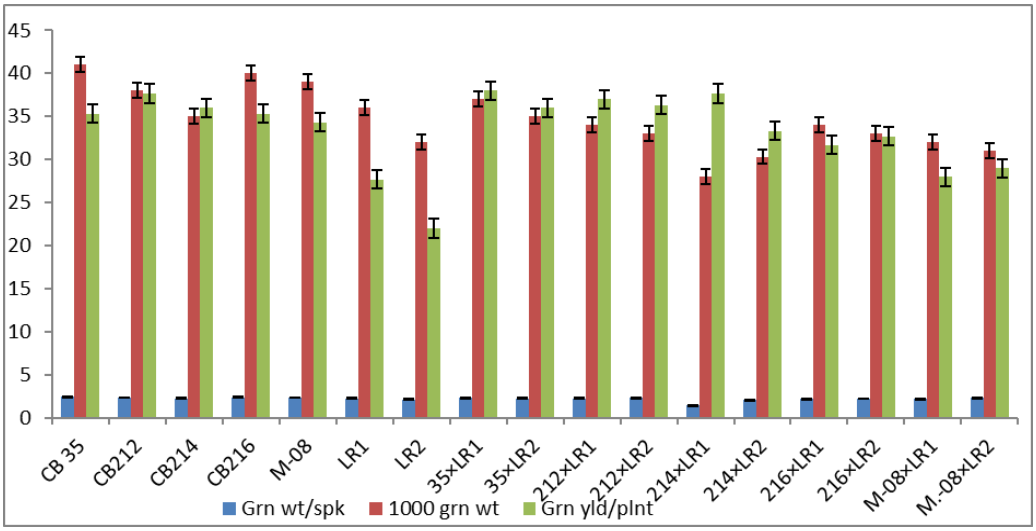

Figure 4. *Mean performance of parents and crosses for grain weight per spike, 1000 grain weight and grain yield per plant*

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Critical analysis of the data of mean performance of F_1 hybrids regarding grain weight per spike revealed that the cross Mairaj-08 × LR-2 produced the maximum grain weight per spike (2.32 g) (*Table 3*, *Fig. 3*). CB-35 × LR-1 and CB-212 × LR-2 showed equal value of 2.30 g for grain weight per spike. While CB-214 × LR-1 produced the lowest weight for grains per spike (1.46 g) (*Table 3* and *Fig. 3*).

All the F_1 crosses lied in between the range of parental genotypes with respect to 1000-grain weight. CB-214 × LR-1, among F_1 crosses, was at the lowest position regarding 1000-grain weight while maximum 1000-grain weight was recorded for the cross CB-35 × LR-1 (37.00) (*Table 3*, *Fig. 4*). All the crosses showed negative values for all three types of heterosis i.e. mid, better and standard parent. However CB-35 × LR-1 exhibited minimum decrease in 1000-grain weight in relation with mid (3.90%), better (9.76%) and standard parent (5.13%) (*Table 5*). Similar observations have been reported by Saleem and Hussain (1988), Abdullah et al. (2003), Hassan et al. (2007), Kumar et al. (2013) and Mahpara et al. (2015).

More tillers per plant results in more spikes per plant which increases the grain yield per plant. More grain yield per plant was observed in lines ranging from 37.67 to 34.33 as compared to testers (27.67 – 22.00). CB-212 produced the highest grain yield per plant followed by CB-214 with 36.00 grain yield per plant while Mairaj-08 produced relatively lower number of grain yield per plant (34.33) (*Table 3* and *Fig. 4*).

All the F_1 crosses lied in between the range of parental genotypes with respect grain yield per plant. Mairaj-08 × LR-1, among F_1 crosses, was at the lowest position regarding grain yield per plant while maximum grain yield per plant was recorded for the cross CB-214 × LR-1 (37.67) (*Table 3* and *Fig. 4*).

In present studies, parental genotypes which were used as females, having range of 35.00 (CB-214) to 41.00 (Mairaj-08) of 1000-grain weight as compared to males (LR-1, 36 and LR-2, 32 thousand grain weight) (*Table 3*, *Fig. 4*).

The negative estimates of heterosis and heterobeltiosis for plant height are preferred over their mid and better parent in wheat breeding because dwarfness is a desirable character (Budak and Yildirim, 1996). Almost all the hybrids showed reduction in plant height, most of which were significant too (*Table 6*). Maximum decrease in plant height as compared to mid parental value was observed in hybrid CB-35× LR-1 closely followed by CB-212 × LR-2, the later cross also showed the highest reduction in plant height over the better parent and standard parent (*Table 6*).

Maximum increase in tillers per plant over mid parent was observed in the cross CB-214 × LR-1 (26.15%), closely followed by Cb-35 × LR-2 (24.14%) (*Table 6*). While none of the crosses out yielded the better parent with respect to the higher parent. CB-212 × LR-1 showed 16.67% increase in tillers per plant as compared to the commercial parent (Mairaj-08), followed by CB-214 × LR-1 which showed 13.89% more tillers than the standard parent (*Table 6*). Heterosis in tiller plant per plant in wheat was also reported by many earlier researchers (Sadeque et al., 1991; Walia et al., 1993; Yu et al., 1997; Abdullah et al., 2002). So, these hybrids verified the earlier reviews.

Maximum decrease in days to heading over mid and better parent was observed in the cross Mairaj-08 × LR-2 (-6.54% and -15.25%, respectively) (*Table 6*). The above mentioned cross also showed minimum significant positive increase in days to heading (4.17%) over the standard parent (*Table 6*).

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Table 5. *Heterosis percentage over mid parent, better parent and standard variety (Mairaj-08) for grains per spike, grain weight per spike, 1000-grain weight and grain yield per plant in wheat*

CROSS	Grains/spike			Grain weight/spike			1000-Grains weight			Grain yield/plant		
	MP Het.	BP Het.	SP Het.	MP Het.	BP Het.	SP Het.	MP Het.	BP Het.	SP Het.	MP Het.	BP Het.	SP Het.
CB-35 × LR1	5.00^{NS}	-4.55^{NS}	-14.29**	-1.71^{NS}	-4.82^{NS}	-2.26^{NS}	-3.90^{NS}	-9.76*	-5.13^{NS}	20.63**	7.55^{NS}	10.68*
CB-35 × LR2	3.90^{NS}	-9.09*	-18.37**	0.44^{NS}	-5.51^{NS}	-2.97^{NS}	-4.11^{NS}	-14.63**	-10.26*	25.58**	1.89^{NS}	4.85^{NS}
CB-212 × LR1	-2.56^{NS}	-9.52*	-22.45**	-2.16^{NS}	-3.97^{NS}	-4.10^{NS}	-8.11^{NS}	-10.53*	-12.82**	13.27**	-1.77^{NS}	7.77^{NS}
CB-212 × LR2	4.00^{NS}	-7.14^{NS}	-20.41**	2.38^{NS}	-2.41^{NS}	-2.55^{NS}	-5.71^{NS}	-13.16**	-15.38**	21.79**	-3.54^{NS}	5.83^{NS}
CB-214 × LR1	12.20**	0.00^{NS}	-6.12^{NS}	-35.69**	-35.74**	-38.19**	-21.13**	-22.22**	-28.21**	18.32**	4.63^{NS}	9.71*
CB-214 × LR2	13.92**	-2.17^{NS}	-8.16*	-6.44^{NS}	-9.13^{NS}	-12.73*	-9.45*	-13.33*	-22.22**	14.94**	-7.41^{NS}	-2.91^{NS}
CB-219 × LR1	3.61^{NS}	-8.51*	-12.24**	-5.78^{NS}	-8.46^{NS}	-6.65^{NS}	-10.53*	-15.00**	-12.82**	0.53^{NS}	-10.38*	-7.77^{NS}
CB-219 × LR2	2.50^{NS}	-12.77**	-16.33**	-1.98^{NS}	-7.49^{NS}	-5.66^{NS}	-8.33^{NS}	-17.50**	-15.38**	13.95**	-7.55^{NS}	-4.85^{NS}
Mairaj-08 × LR1	-12.94**	-24.49**	-24.49**	-5.26^{NS}	-7.07^{NS}	-7.07^{NS}	-14.67**	-17.95**	-17.95**	-9.68*	-18.45**	-18.45**
Mairaj-08 × LR2	-7.32*	-22.45**	-22.45**	3.34^{NS}	-1.56^{NS}	-1.56^{NS}	-12.68**	-20.51**	-20.51**	2.96^{NS}	-15.53**	-15.53**

Table 6. *Heterosis percentage over mid parent, better parent and standard variety (Mairaj-08) for plant height, tillers per plant, days to heading and days to anthesis in wheat*

CROSS	Plant height			Tillers/plant			Days to heading			Days to anthesis		
	MP Het.	BP Het.	SP Het.	MP Het.	BP Het.	SP Het.	MP Het.	BP Het.	SP Het.	MP Het.	BP Het.	SP Het.
CB-35 × LR1	-16.85**	-39.34**	-21.28**	21.87**	-17.02**	8.33^{NS}	4.27**	-1.79^{NS}	14.58**	2.56^{NS}	-0.83^{NS}	9.09**
CB-35 × LR2	-15.22**	-39.06**	-17.02**	24.14**	-23.40**	0.00^{NS}	4.15**	-4.24**	17.71**	2.07^{NS}	-3.91*	11.82**
CB-212 × LR1	-17.07**	-44.26**	-27.66**	21.74**	-19.23**	16.67*	0.00^{NS}	-5.56**	23.96**	0.39^{NS}	-5.15**	17.27**
CB-212 × LR2	-16.47**	-44.53**	-24.47**	17.46**	-28.85**	2.78^{NS}	-3.28**	-6.35**	22.92**	-1.52^{NS}	-4.41**	18.18**
CB-214 × LR1	-11.76**	-38.52**	-20.21**	26.15**	-14.58**	13.89*	2.70*	1.79^{NS}	18.75**	3.73**	3.31*	13.64**
CB-214 × LR2	-6.82**	-35.94**	-12.77**	15.25*	-29.17**	-5.56^{NS}	0.88^{NS}	-2.54^{NS}	19.79**	2.42^{NS}	-0.78^{NS}	15.45**
CB-219 × LR1	-1.64^{NS}	-26.23**	-4.26*	6.67^{NS}	-25.58**	-11.11^{NS}	-0.92^{NS}	-3.57*	12.50**	-0.83^{NS}	-0.83^{NS}	9.09**
CB-219 × LR2	-0.53^{NS}	-26.56**	0.00^{NS}	-3.70^{NS}	-39.53**	-27.78**	-4.46**	-9.32**	11.46**	-5.22**	-7.81**	7.27**
Mairaj-08 × LR1	-8.02**	-18.58**	5.67**	-28.30**	-47.22**	-47.22**	-4.81**	-11.61**	3.13^{NS}	-1.30^{NS}	-5.79**	3.64*
Mairaj-08 × LR2	-7.21**	-19.53**	9.57**	-27.66**	-52.78**	-52.78**	-6.54**	-15.25**	4.17*	-5.04**	-11.72**	2.73^{NS}

**,* is equal to significant at 0.01, 0.001 respectively and NS showing non significant

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The outcome of the present study are in accordance with the results of Sadeque et al. (1991), Murai (1998), Wu et al. (2001) and Baric et al. (2004) who described that negative heterosis in days to heading may be an effective selection criteria for development of early maturing short durational cultivars in wheat.

The highest reduction in days to anthesis with respect to mid and better parent was recorded for the cross Mairaj-08 × LR-2 (*Table 6*). All the crosses showed increase in days to anthesis over the standard parent, while minimum increase was revealed from the above mentioned cross (*Table 6*). These findings are in accordance with previous reports by Chowdhry et al. (2005), Farooque et al. (2005), Ullah et al. (2006) and Boche (2013).

All the crosses showed negative heterosis over mid, better and standard parental value for spike length, however minimal decrease, also non-significant, in spike length as compared to mid and better parental value was recorded for the cross CB-219 × LR-1 (*Table 4*). While CB-212 × LR-1 showed minimum non-significant decrease in spike length as compared to the standard parent (*Table 4*). These significant outcomes are in agreement with those of Masood et al. (2005), Ilker et al. (2010) and Mahpara et al. (2015). Similar findings had been reported by researchers like, Walia et al. (1993), Li et al. (1997) and Hassan et al. (2007).

The genotypes produced as F_1 showed a range of 22 to 33 days for grain filling including Mairaj-08 × LR-1 at the lower extreme and CB-212 × LR-1 at the higher extreme (*Table 2* and *Fig. 2*). Grain filling period was considerably reduced as compared to mid and better parental value for the cross Mairaj-08 × LR-1 i.e. -15.38% and -26.67%, respectively (*Table 4*). While the same cross was at par the standard parent for the trait. These results were got supported by earlier reports of Murai (1998), Wu et al. (2001) and Baric et al. (2004), Ullah et al. (2006) and Beche et al. (2013).

The single cross Mairaj-08 × LR-2 was considered the most desirable genotype towards early maturity as it showed the highest heterotic effects over mid and better parent (-5.48% and -13.75%, respectively (*Table 4*). While none of the crosses showed reduction in maturity period as compared to standard parent, however the above mentioned cross showed minimum significant increase in maturity days over the commercial variety i.e. 4.55% (*Table 4*). These results of current study were supported by Farooque et al. (2005), Ullah et al. (2006), Hassan et al. (2007) and Boche (2013) who concluded that genotypes taking less number of days for maturity might possess the potential of yielding short durational pure lines.

The highest heterotic effects over mid parental value were observed for the cross CB-214 × LR-2 (13.92%), followed by CB-214 × LR-1 (12.20%) (*Table 5*). None of the crosses showed increase in grains per spike over better and standard parent, however the later mentioned cross exhibited minimum and non-significant decrease in grains per spike as compared to better parent and standard variety. Tiwari and Chakraborty (1992), Larik et al. (1999), and Hassan et al. (2007) observed similar results. These is agreement in earlier studies (Çifci and Yaðdi, 2007) but less than that of Fonseca and Patterson (1968) who found 100% heterobeltiosis in the crosses obtained from genetically different parents. On the other hand, Baric et al. (2004) found negative heterosis values in terms of number of grains spike in bread wheat crosses.

All the crosses exhibited negative values of heterosis over mid, better and standard parent except Mairaj-08 × LR-2 which presented positive yet non-significant heterosis over mid parental value (3.34%) (*Table 5*). The above mentioned cross also showed minimum and non-significant decrease as compared to better and standard parent with respect to grain weight per spike. These results were got supported by earlier reports of

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Murai (1998), Wu et al. (2001), Abdullah et al. (2002), Farooque et al. (2005), Akbar et al. (2007), Ullah et al. (2006) and Boche, (2013).

Maximum significant increase in grain yield per plant, with respect to mid parental value, was recorded for the cross CB-35 × LR-2 (25.58%), followed by the cross CB-35 × LR-1 (20.63%) (*Table 5*). The later cross also showed maximum yet non-significant value for heterobeltiosis and the highest significant value (10.68%) for standard heterosis (*Table 5*). Results of heterosis in grain yield per plant were found in agreement with those of Wu et al. (2001), Abduulah et al. (2002), Faroque et al. (2005), Ullah et al. (2006), Akbar et al. (2007), Kumar et al. (2013) and Mahpara et al. (2015).

Conclusion and Recommendations

The research made use of semi dwarf lines to cross with land races in order to increase grain yield and to obtain noval germplasm resistant to lodging, which to good extent. It is recommended that since this research provides a baseline that could attract researchers to follow such and different crossing combinations with local land races to obtain improved varieties with significant yield for increased farmer premiums.

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TEMPORAL AND SPATIAL VARIATION OF SOIL EROSION IN CENTRAL YUNNAN PROVINCE, CHINA

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Abstract. Soil erosion is one of the major global environmental problems. Understanding the spatiotemporal changes in soil erosion is of great significance to its prevention and control of soil erosion and the optimization of ecological environments. Taking Central Yunnan Province (CYP), China as the study area, multitemporal remotely sensed images, GIS technology and the RUSLE model were used to estimate the amount of soil erosion in the CYP during 1980-2018. The results show the following: (1) During the study period, the soil erosion situation in the CYP showed a growing development trend. With 2005 as a turning point, the area of micro-erosion first decreased and then increased, and the area of soil erosion with mild and above intensity was effectively controlled. (2) Through the global spatial autocorrelation, the spatial distribution of soil erosion intensity in the CYP has significant aggregation, and the global Moran I index is between 0.48 and 0.66. The areas with moderate and above erosion intensity were mostly distributed in the mountains, northern valleys, and the northwestern and southwestern regions of the CYP. Through the analysis of local spatial autocorrelation, it is also proved that the distribution of intensity soil erosion is mostly related to regional topography.
Keywords: *soil erosion, RUSLE, temporal and spatial variation, spatial autocorrelation, Moran I index*

Introduction

Central Yunnan Province (CYP) is located on the western Yunnan-Guizhou Plateau and the eastern Yunnan Plateau. Nearly half of the mountainous plains (dams) in Yunnan Province are concentrated in the region, which has abundant soil and good water resources. According to the Bulletin on Soil and Water Conservation of the First National Water Conservancy Census, the type of soil erosion in Yunnan Province is hydraulic erosion. By the end of 2011, the total area of soil erosion was 109,588 km^2, accounting for approximately 7% of the total area of water erosion in China. The area of mild erosion is 44,876 km^2, accounting for 40.95% of the total area of hydraulic erosion; the area of moderate erosion is 3,476 km^2, accounting for 31.72%; the area of intense erosion is 15,860 km^2, accounting for 14.47%; the area of extremely strong erosion is 8,963 km^2, accounting for 8.18%; and the area of intense erosion is 515 km^2, accounting for 4.68%. As the core area of Yunnan Province, the soil erosion situation in central Yunnan is not optimistic; thus far, there is still insufficient research on soil erosion in the region. (Ministry of Water Resources of the People's Republic of China, 2013). With sustainable development in mind, soil erosion will lead to the destruction of soil resources, reductions

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in arable land area, and decreases in soil fertility, all of which will affect productivity, deteriorate the ecological environment, destroy infrastructure, increase flood risk by silting up riverbeds, silt up rivers, lakes and reservoirs, and threaten urban and rural safety. Furthermore, soil erosion restricts the sustainability of agricultural production and seriously threatens the survival and development of human beings.

In the field of soil erosion research, the USLE (Universal Soil Loss Equation) model is the most widely used empirical model of soil erosion. According to their own soil erosion situation, many countries and regions have continued to improve and localize the USLE model. For example, the RUSLE (Revised Universal Soil Loss Equation) model was published in the United States in 1997 (Renard et al., 1997); the RUSLE2 model was obtained in 2002 (Gogichaishvili et al., 2014); and in 2008, Kirkby et al. (2010) considered soil infiltration and the impact of climate and climate drivers, and the PESERA (Pan European Soil Erosion Risk Assessment) model was improved by USLE; in 2015, Panagos et al. (2015) used European data for the estimation and scientific evaluation of the localized RUSLE model. From this attempt, the RUSLE2015 model was obtained. Additionally, the G2 model proposed in 2018 (Karydas et al., 2018) is based on the main principles and calculation formulas of the USLE and RUSLE models, and the monthly soil erosion rate model was constructed. The empirical model of soil erosion prediction has gradually been improved, and the localization research and treatment of relevant parameter factors of the model have also deepened; additionally, the obtained soil erosion simulation results are increasingly accurate.

Research on soil erosion in Yunnan Province has been carried out for a long time. Chen (1990) discussed the main influencing factors of soil erosion in the Jinsha River basin in Yunnan Province and proposed corresponding prevention and control measures for existing problems. Yue et al. (2003) used remote sensing and GIS technology to divide the soil erosion index system and use the overlay analysis method to investigate soil erosion in the Zhaotong area. Yao et al. (2005, 2006) combined the GIS spatial analysis with traditional statistical analysis with the Lancang River basin in Yunnan Province to study the soil erosion status of the region. Zhou et al. (2005, 2009) and Xu and Zhou (2009) studied the process from a single factor of soil erosion to a later model based on a soil erosion prediction model. Hong et al. (2016) used the indoor artificial simulated rainfall experiment and theoretical analysis to reveal the effect of rainfall intensity, slope and slope length on soil erosion on the Yunnan red soil slope and provided a reference for soil erosion control in the Yunnan red soil area. Yang (2002a,b) and Yang et al. (2002) carried out systematic and in-depth research on soil erosion in the Jinsha River basin in Yunnan Province, and they discussed the relationship between soil erosion control and the sustainable use of land resources (Yang et al., 2004, 2005). Zhang et al. (2004) and Huang et al. (2009) carried out a study on the correlation between soil erosion and soil erosion in the Fuxian Lake watershed. Wang et al. (2016) studied the damage caused by soil erosion in the form of ecological destruction by using the Taojiaxiao River in the Dongchuan District of Kunming city, Yunnan Province, as an example, and proposed conducting research on ecological environment construction in small watersheds, which can improve the local ecological environment. Resilience from disasters can have dividends in terms of economic development and people's lives. Ma et al. (2016) carried out remote sensing monitoring of soil erosion in the Fuxian Lake watershed and determined rainfall was the main influencing factor. The erosion amount of the watershed fluctuated, and it was found that the areas with serious soil erosion were concentrated on the eastern and western sides of Fuxian Lake and on some areas of the southern bank.

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Yang et al. (2016) used soil erosion as one of the basic indicators for constructing a basin-wide land degradation assessment model. The land degradation assessment study of the Fuxian Lake basin was conducted, and it was concluded that the inter-annual variation of land degradation and soil erosion tended to be the same. The correlation between the two was obvious. Zhu et al. (2016) used the case of Lishui County in Yunnan Province as an example to study the soil erosion in the alpine valley area. Based on the spatial differentiation law of regional soil erosion, it was concluded that the unreasonable land use pattern was the main cause of erosion. Ding et al. (2018) estimated the soil erosion in Yunnan Province in 2015 and compared it with the 2004 remote sensing survey data; additionally, the authors carried out dynamic change analysis and concluded that the soil erosion area of the province tended to decrease, among which the areas of mild and moderate erosion reduced significantly.

In terms of erosion models, the RUSLE model is the most commonly used and has a good estimation effect. Scholars have conducted soil erosion studies at the regional, county, watershed and provincial scales in Yunnan Province, but studies have not been conducted in specific administrative and economic regions. In addition, the distribution of soil erosion intensity is used as the research object to discuss. Research on the spatial distribution and agglomeration is also scarce. Therefore, through the study of soil erosion in the CYP, the RUSLE model is used to estimate the regional soil erosion situation from 1980 to 2018.

The objective of the present work was to assess the spatiotemporal variation of soil erosion in the CYP during the 1980-2018 period, using multitemporal remotely sensed images, GIS technology and the RUSLE model, which can provide certain decision support for regional land use planning and eco-environmental engineering management policy formulation.

Materials and methods

Location and description of the study area

The CYP includes the 4 cities of Kunming, Chuxiong, Yuxi and Qujing (*Fig. 1*), and there are 42 counties and urban areas in total. The area is located between 100˚43'-104˚50' E and 23˚19'-27˚03' N. It is adjacent to Guizhou Province and Guangxi Zhuang Autonomous Region in the east, Dali Bai Autonomous Prefecture and Puer city in the west, Honghe Hani Yi Autonomous Prefecture, Wenshan Zhuang and Miao Autonomous Prefecture in the south, and Sichuan Province, Zhaotong city and Lijiang city in the north. The total area is 94,558 km^2, accounting for 24% of the total land area of Yunnan Province. Located in the eastern Yunnan Plateau basin, the terrain is gentle and undulating. The overall terrain is high in the northwest, low in the southeast, and downgraded from the northwest to the southeast. The morphology of each region is widely distributed, and the overall morphology is relatively fragmented (Liu et al., 2017). Located at low latitude and high altitude, the region is controlled by the southwest monsoon and southeast monsoon and is dominated by the mid-subtropical monsoon climate. The region is rich in light energy resources, divided into dry and wet seasons, and rich in heat resources; it has an annual average temperature of 16-22˚C, a summer without heat, and a winter without severe cold. The annual average precipitation ranges from 684.31 mm to 1777.03 mm, and the overall distribution is higher in the southeast and lower in the rest of the region. Red soils and purple soils are the most widely distributed areas in the region. There are many types of soils in the eastern region, and

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they are scattered and irregular. Red soils are dominant in the central and eastern regions, while purple soils are distributed in patches in the western regions. Vertically, red soils, yellow soils, yellow brown soils, brown soils and sub-soils are distributed successively from low to high in the mountainous region, and there is also alpine meadow soil. The study area with alpine meadow soil belongs to the subtropical evergreen broad-leaved forest area, mainly in the mid-subtropical evergreen broad-leaved forest zone, and is distributed in a small area of the south subtropical monsoon evergreen broad-leaved forest zone, which is rich in forest resources, with an overall coverage rate of approximately 53.8%.

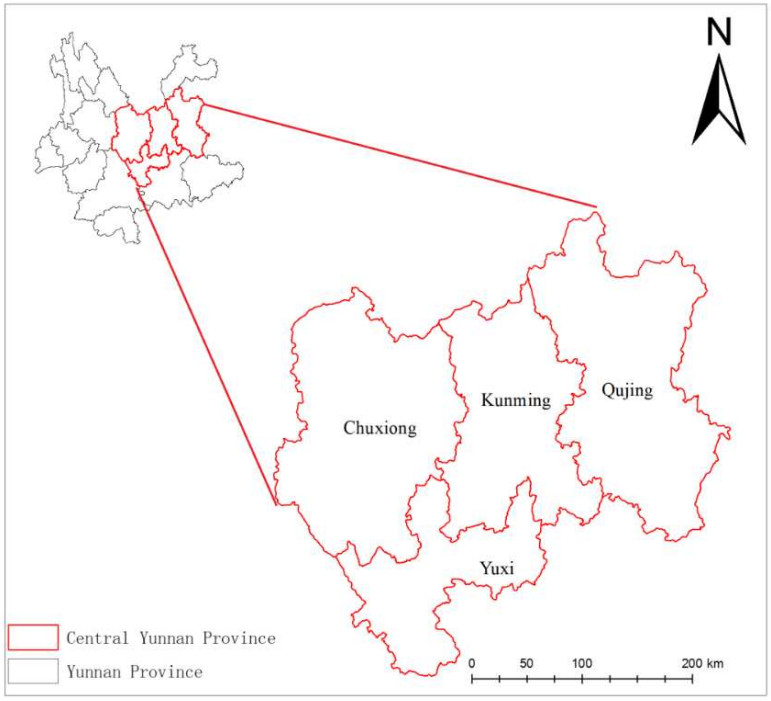

Figure 1. *Research Location Map*

In recent years, with the continuous development of the social economy and the acceleration of urbanization, various environmental protection measures, such as returning grain plots to forest, have been introduced and implemented. Soil erosion in the CYP has also changed, which has a certain impact on the regional ecological environment. By estimating the long time series of soil erosion, there is important ecological and economic value in studying the changing trend of its quantity and spatial distribution.

Data and preprocessing

The data sources mainly include (1) basic geographic information data; (2) historical remote sensing image data; and (3) thematic data.

The basic geographic information data are ASTER GDEM (Advanced Spaceborne Thermal Emission and Reflection Radiometer Global Digital Elevation Model) V2 30 m resolution elevation data, which are freely obtained from the geospatial data cloud (http://www.gscloud.cn/). Historical remote sensing image data mainly come from the

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official website of the National Aeronautics and Space Administration (NASA) (https://search.earthdata.nasa.gov/) and the geospatial data cloud (http://www.gscloud.cn/); most free data were downloaded, and some data, such as Landsat OLI, TM, and MSS, were purchased by the instructor team in advance; the satellite images were taken at 1980, 1990, 1995, 2000, 2005, 2010, 2015 and 2018. The thematic data mainly include meteorological, soil, field survey data and remote sensing survey reports on the soil erosion status in the CYP.

The projection coordinates of the source data of soil erosion factors are set as WGS_1984_UTM_Zone_47N, and the geographic coordinates are set as WGS_1984. According to the research needs and the characteristics of the research area, ENVI 5.3 was used to pre-process the remote sensing images, and the land cover remote sensing classification system and the interpretation mark library were established. The image data of the CYP are classified by the maximum likelihood classification method, and the classification results are superimposed with terrain gradient data to provide the terrain characteristics.

Model description

The Revised Universal Soil Loss Equation (RUSLE), which was an improved form of the Universal Soil Loss Equation (USLE), was used to estimate soil erosion in the CYP. Compared with the USLE, the RUSLE model has a simple structure, a clear physical meaning of parameters, simple calculations, and strong practicability and comprehensiveness.

The mathematical formula of RUSLE is as follows:

$$A = R \times K \times LS \times C \times P \quad \text{(Eq.1)}$$

In the formula, A is the annual average amount of soil erosion per unit area, i.e., the modulus of soil erosion, which is t/(hm^2·a); R is the rainfall erosivity factor, and the international general unit is (MJ·mm)/(hm^2·h·a); and K is the soil erodibility factor, and the international general unit is (t·h)/(MJ·mm). The other factors are dimensionless: among the LS factors of terrain, L is the slope length factor, S is the slope factor, C is the vegetation cover management factor, and P is the soil and water conservation measure factor.

Soil erosion factors

Rainfall erosivity (R) factor

Rainfall erosivity refers to the potential ability of rainfall to cause soil erosion and is the main dynamic factor leading to soil erosion (Fang et al., 2015). Wischmeier and Smith (1958) proposed a classical method for calculating the rainfall erosivity EI model based on the measured data of rainfall erosion. However, this method is limited by the difficulty of completely obtaining the measured data and cannot be popularized. According to the available monthly meteorological data and the applicability of the model in the CYP, the final calculation formula used in this study is as follows (Silva, 2004):

$$R = \sum_{i=1}^{12} 73.989 \times \left(\frac{P_i^2}{P_a} \right)^{0.7387} \quad \text{(Eq.2)}$$

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In the formula, R is the annual rainfall erosivity (MJ·mm)/(hm^2·h·a); P_i is the rainfall (mm) of the i-month; and P_a is the average annual rainfall (mm), which is easy to obtain and has high accuracy in complex terrain areas. Based on the data of 39 meteorological stations in the study area provided by the Kunming, Chuxiong, Yuxi and Qujing meteorological bureaus and 21 supplementary station data obtained from the China Meteorological Data Network (http://data.cma.cn/site/index.html) (*Table 1*, *Fig.2*), the monthly precipitation data of 65 meteorological stations were analyzed by Kring's spherical hemispherical variation function. The model is interpolated, and the annual rainfall erosivity in the CYP is calculated according to the R factor formula. The annual rainfall erosivity in typical years is shown in figures (*Fig. 3*).

Table 1. *Meteorological stations around the CYP*

No.	Site Name	No.	Site Name	No.	Site Name	No.	Site Name	No.	Site Name
1	Chu Xiong	13	Kun Ming	25	Hui Ze	37	E Shan	49	Meng Zi
2	Shuang Bai	14	Yi Liang	26	Fu Yuan	38	Xin Ping	50	Ping Bian
3	Mo Ding	15	Song Ming	27	Ma Long	39	Yuan Jiang	51	Geng Ma
4	Nan Hua	16	An Ning	28	Lu Liang	40	Zhao Yang	52	Lin Cang
5	Yao An	17	Dong Chuan	29	Shi Zong	41	Li Jiang	53	Yan Shan
6	Da Yao	18	Jin Ning	30	Luo Ping	42	Hua Ping	54	Guang Nan
7	Yong Ren	19	Tai Hua Shan	31	Hong Ta Qu	43	Da Li	55	Pan Zhi Hua
8	Yuan Mou	20	Lu Quan	32	Jiang Chuan	44	Jing Dong	56	Hui Li
9	Wu Ding	21	Xun Dian	33	Cheng Jiang	45	Lan Cang	57	Wei Ning
10	Lu Feng	22	Qi Lin Qu	34	Tong Hai	46	Si Mao	58	Shui Cheng
11	Fu Min	23	Zhan Yi	35	Hua Ning	47	Jiang Cheng	59	Pu An
12	Shi Lin	24	Xuan Wei	36	Yi Men	48	Lu Xi	60	Xing Ren

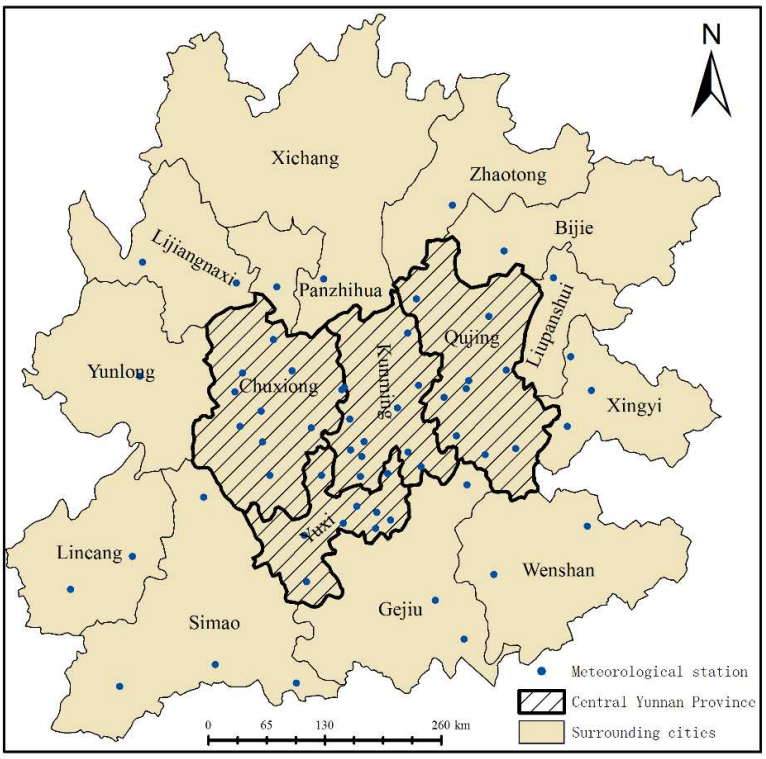

Figure 2. *Meteorological stations around the CYP*

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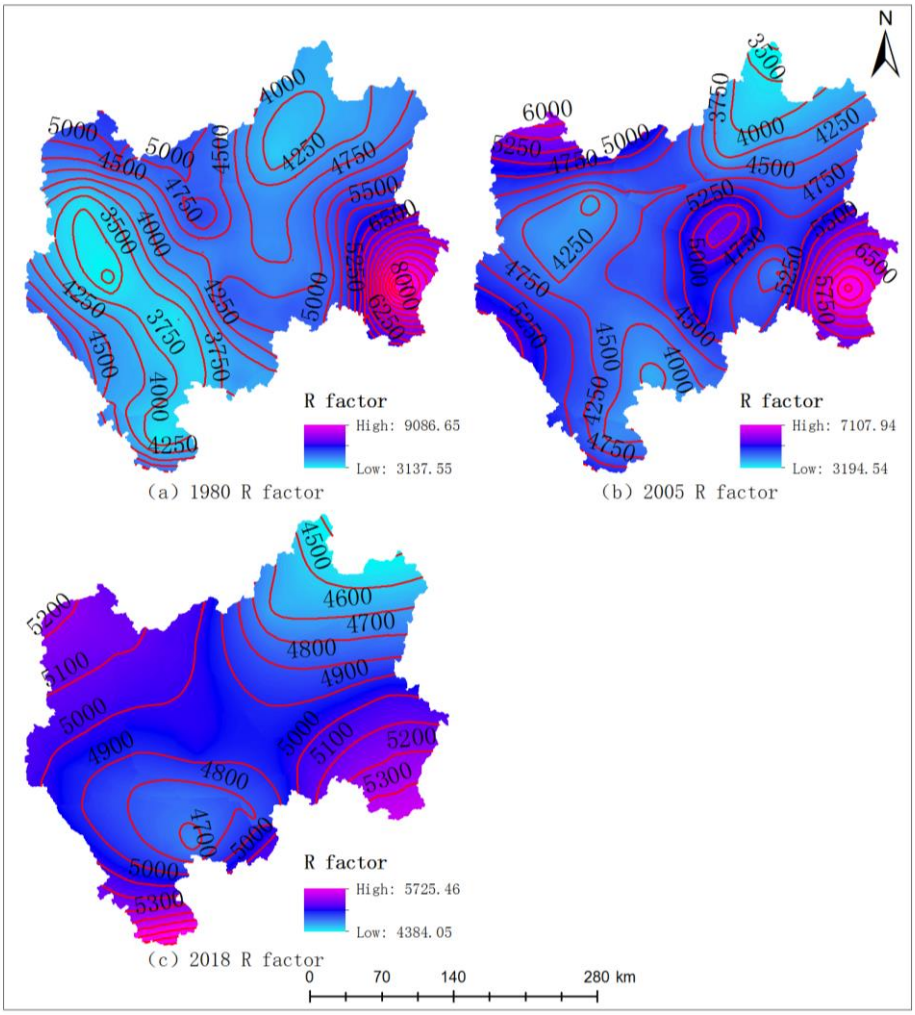

Figure 3. *Spatial distribution of rainfall erosivity R factor in the CYP ((MJ·mm)/(hm^2·h·a))*

Soil erodibility (K) factor

Among the methods for estimating the *K* factor of soil erodibility, the Norit diagram and the EPIC (Erosion-Productivity Impact Calculator) method developed by Williams et al. (1983) are represented. The soil data were provided by the Heihe Planning Data Management Center (http://westdc.westgis.ac.cn) based on the World Soil Database (HWSD). The distribution characteristics of soil texture and organic carbon content are detailed in the study area. Therefore, the productivity model EPIC was used to quantitatively calculate the soil erodibility *K* factor, and the results are revised (Chen et al., 2018) to make the model more appropriate for Chinese soil properties. The formula for calculating the soil erodible factor *K* by the EPIC model is as follows:

$$K_{EPIC} = \left\{0.2 + 0.3\exp\left[0.0256SAN\left(1 - \frac{SIL}{100}\right)\right]\right\}\left(\frac{SIL}{CLA + SIL}\right)^{0.3} \times \left(1.0 - \frac{0.25OC}{OC + \exp\left(3.72 - 2.95OC\right)}\right)\left(1.0 - \frac{0.7SN}{SN + \exp\left(22.9SN - 5.51\right)}\right) \quad \text{(Eq.3)}$$

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The revised formula is as follows:

$$K = -0.01383 + 0.51575K_{EPIC} \quad \text{(Eq.4)}$$

In the formula, K_{EPIC} and K are soil erodibility factors before and after amendment, respectively; *SAN*, *SIL*, *CLA* and *OC* are the percentage of sand, silt, clay and organic carbon in soil, respectively; and *SN = 1-SAN/100.*

The *K* value calculated by this model is reported in US metric units, and the value is multiplied by 0.1317 rpm to convert it into an international unit (t·h)/(MJ·mm), and the soil erodibility *K* factor in the CYP region is obtained (*Fig. 4*).

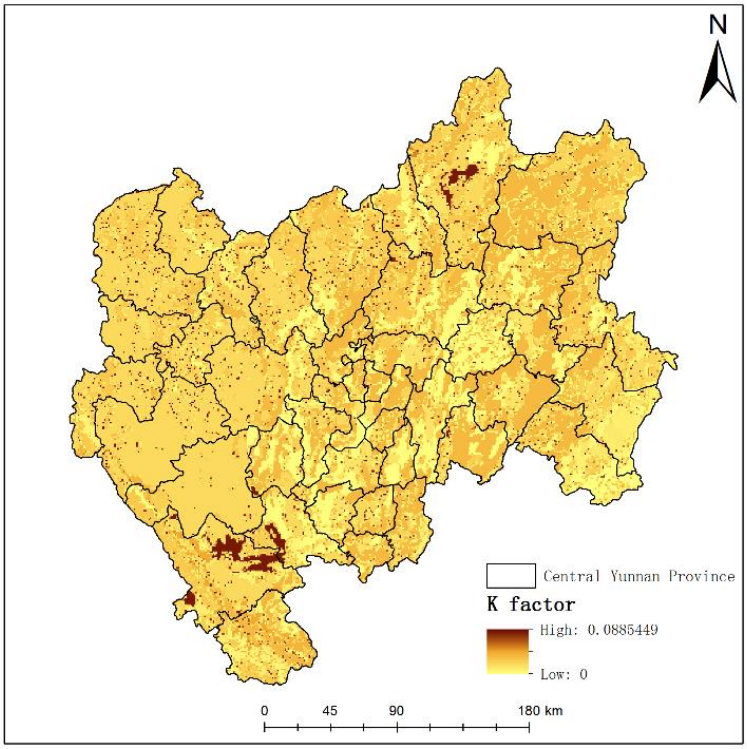

Figure 4. *Spatial distribution of soil erosion K factor in the CYP ((t·h)/(MJ·mm))*

Slope length and steepness (LS) factor

In the topographic *LS* factor, the slope and slope length directly affect the formation and development of the surface soil and the spatial distribution of vegetation, which in turn affects the flow direction, velocity and intensity of surface runoff in the process of surface runoff formation; ultimately, these factors affect the intensity of surface soil erosion. With regard to the calculation of the slope length factor, the method proposed by Wischmeier is more applicable and has higher accuracy; thus, this method is widely used in the field of soil erosion research. Therefore, the formula proposed by Wischmeier and Smith (1965) is used to calculate the *L* factor of slope length in the CYP (*Fig. 5*).

$$L = \left(\frac{\lambda}{22.13}\right)^{\alpha} \quad \text{(Eq.5)}$$

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$$\alpha = \beta / (1 + \beta) \quad \text{(Eq.6)}$$

$$\beta = (\sin\theta / 0.089) / [3.0(\sin\theta)^{0.8} + 0.56] \quad \text{(Eq.7)}$$

In the formula, L is the slope length factor, λ is the slope length, 22.13 is the standard plant slope length (m), α and β are the slope length factor indexes, and θ is the slope.

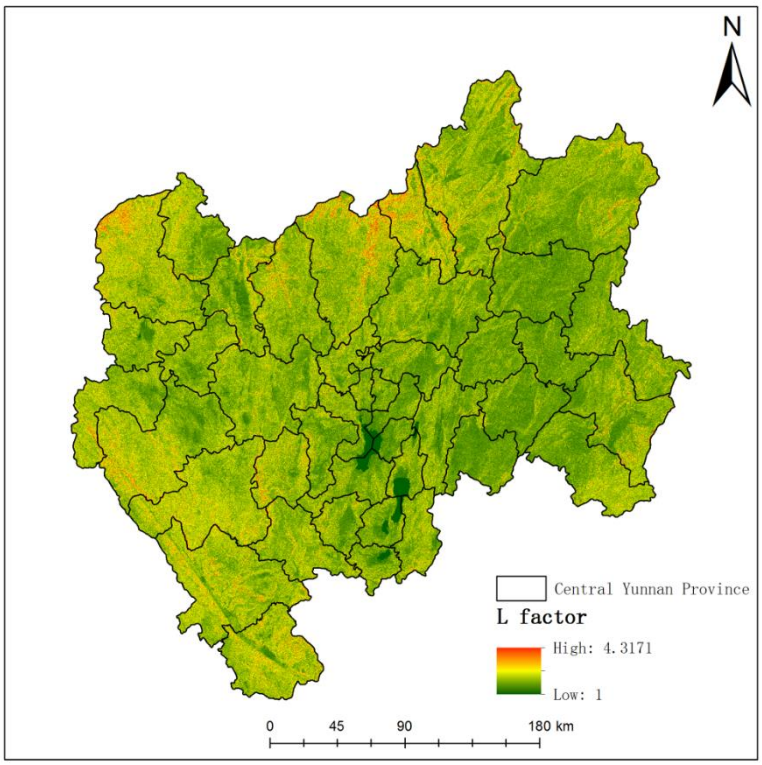

Figure 5. *Spatial distribution of slope length L factor in the CYP*

In a study of the relationship between soil erosion and slope, McCool et al. (1989) established the linear relationship between gentle slope and soil erosion in 1987, and Liu et al. (2000) added the formula for calculating soil erosion in areas with steep slopes. According to the geomorphological characteristics of the study area and combining these characteristics with the results of McCool et al. and Liu et al., the *S* factor of the slope in the CYP is calculated in stages (*Fig. 6*). The formula is as follows:

$$\begin{cases} S = 10.8\sin\theta + 0.03, \theta < 5° \\ S = 16.8\sin\theta - 0.05, 5° \le \theta < 10° \\ S = 21.91\sin\theta - 0.96, \theta \ge 10° \end{cases} \quad \text{(Eq.8)}$$

In the formula, S is the slope factor, and θ is the slope.

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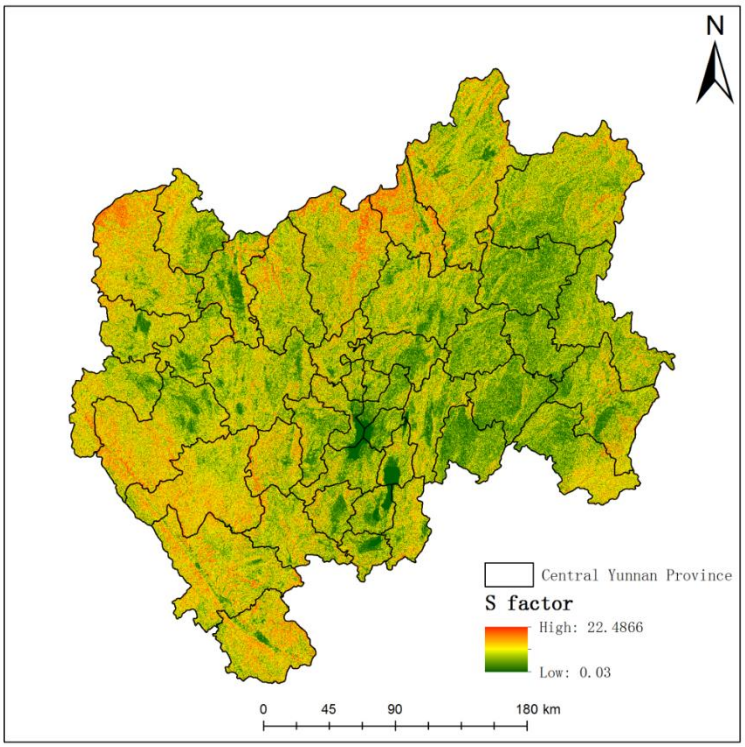

Figure 6. *Spatial distribution of slope S factor in the CYP*

Vegetation cover and management (C) factor

The vegetation cover management *C* factor refers to the standard plot with and without vegetation cover when other conditions are identical, which can reflect the soil erosion caused by different vegetation coverage types. Influence is dimensionless, its value range is [0,1], and its numerical value is directly proportional to the severity of soil erosion. The larger the *C* factor is, the more serious the soil erosion is. In the RUSLE model, factor *C* is susceptible to other factors and has a high sensitivity and variable characteristics.

The calculation of the vegetation cover management *C* factor is divided into three steps: the first step is to extract the normalized difference vegetation index NDVI, the second step is to calculate the vegetation coverage by the NDVI, and the third step is to calculate the *C* factor value of the vegetation cover management from the vegetation coverage.

We used the pixel dichotomy method to obtain the vegetation coverage of the study area and then used the calculation formula established by Cai et al. (2016) to obtain the *C* factor of vegetation cover management in the CYP:

$$F_c = \frac{NDVI - NDVI_{soil}}{NDVI_{veg} - NDVI_{soil}} \quad \text{(Eq.9)}$$

$$C = \begin{cases} 1, 0 \le F_c < 0.096 \\ 0.6508 - 0.3436 \lg(F_c), 0.096 \le F_c \le 78.3 \\ 0, F_c > 78.3 \end{cases} \quad \text{(Eq.10)}$$

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In the formula, F_c is the vegetation coverage (%), *NDVI* is the normalized difference vegetation index, $NDVI_{soil}$ represents the *NDVI* value of no vegetation coverage or bare soil area, $NDVI_{veg}$ represents the *NDVI* value of high vegetation coverage area, and *C* is the vegetation coverage management factor. Through the ArcGIS 10.2 raster calculator, the *C* factor values of the seven years from 1980 to 2018 were calculated in the CYP. The *C* factor values for typical years are shown in figures (*Fig. 7*).

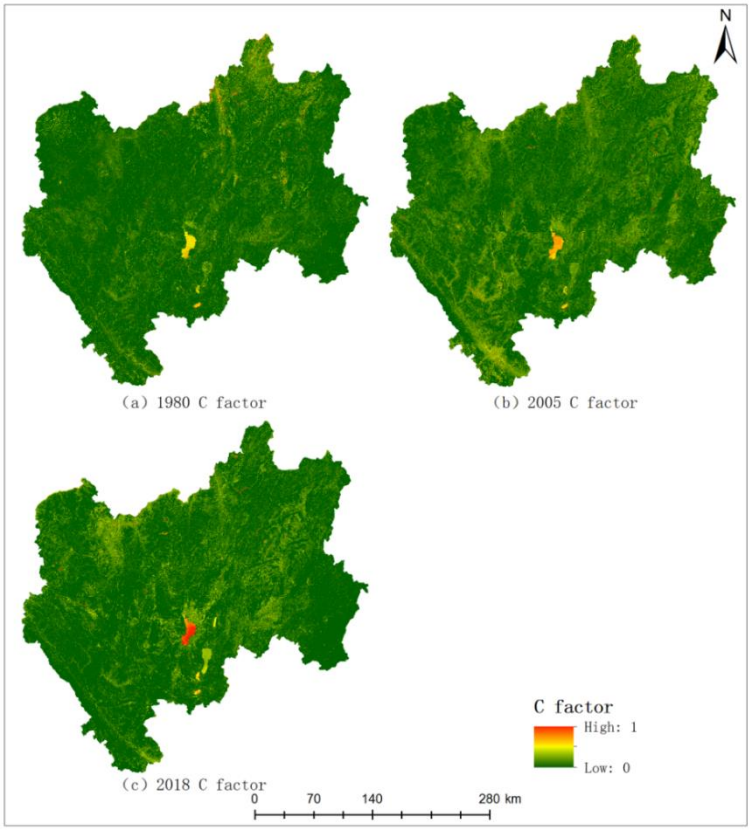

Figure 7. *Spatial distribution of vegetation cover management C factors in the CYP*

Conservation practice (P) factor

The soil and water conservation *P* factor reflects the ratio of soil loss in a certain period of time when the water conservation measures and the standard plots that have not used corresponding measures are in the same situation; the dimension is non-dimensional, and its value is between 0 and 1. Its value is positively correlated with the possibility of soil erosion. A value of 0 indicates that the probability of soil erosion in this area is almost zero and the soil and water conservation measures are good. A value of 1 indicates that soil erosion is very likely in this area and there are no effective soil and water conservation measures that can be taken.

In China, the *P* values of different soil and water conservation measures under certain land use types are generally obtained by comparison methods. However, the *P* values in different regions have large errors, and the values are difficult to determine. Therefore, based on the survey of soil and water conservation status in the CYP, reference is made to the *P* value table of different soil and water conservation engineering measures in China (Wang et al., 1996), the Handbook of the United States Department of Agriculture,

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the land use type map and vegetation cover map and the research results of predecessors. The corresponding *P* values are assigned to the local classes (*Table 2*). The *P* factors for soil and water conservation measures in typical years are shown in figures (*Fig. 8*).

Table 2. *Soil and water conservation measures P factor value*

Land use types	Code	*P* factor value
Paddy field	11	0.15
Dry land	12	slope<5, 0.11; 5≤slope<10, 0.22; 10≤slope<15, 0.31; 15≤slope<20, 0.58; 20≤slope<25, 0.71; slope≥25,0.8
Shrub wood	21	1.00
Arbor forest	22	1.00
Grassland	3	1.00
Water area	4	0.00
Construction land	5	0.00
Bare land	61	1.00
Bare rock	62	0.00

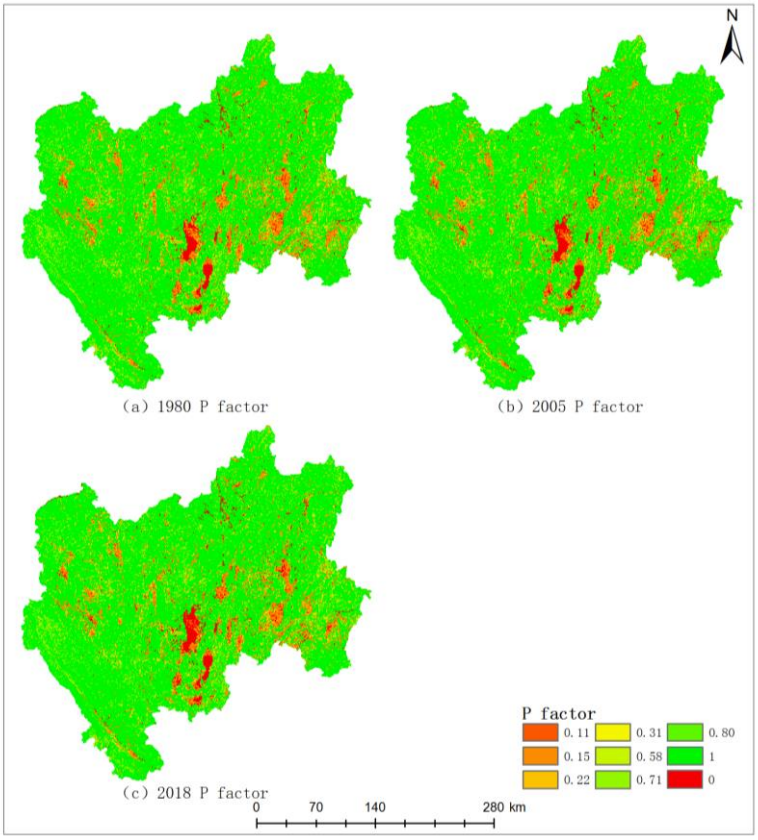

Figure 8. *Spatial distribution of soil and water conservation measures P factor in the CYP*

Creation of soil erosion hazard map

The raster layers of each factor are multiplied in the ArcGIS 10.2 raster calculator by the constant 100 to convert the unit t/(hm^2·a) to t/(km^2·a) to obtain the values in the study area. The generated soil erosion raster data (*Fig. 9*) had a resolution of 30 m.

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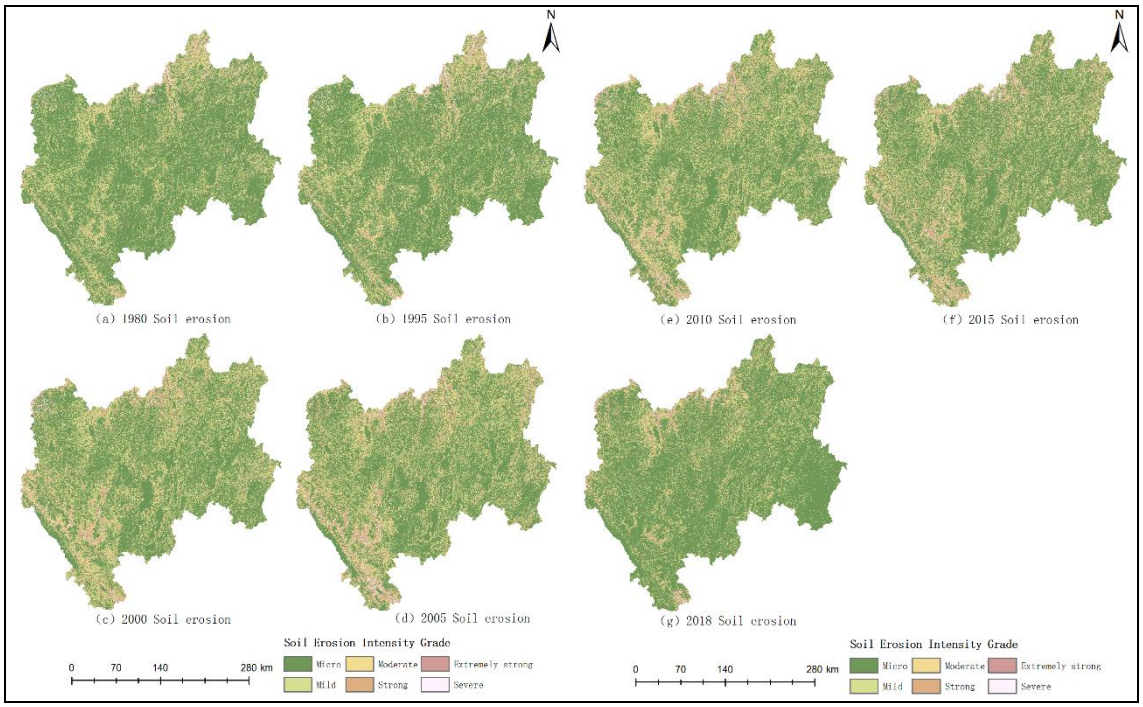

Figure 9. *Spatial distribution of soil erosion intensity in the CYP*

Comparing the estimated soil erosion results of the study area with the remote sensing survey report of the soil erosion status in Yunnan Province in 1999, it is concluded that the soil erosion modulus values in 2000 are larger than those in 1999; additionally, the proportion of each intensity area was more consistent, the total area of soil erosion increased, and the changes that were presented were associated with the objective law.

According to SL190-2007 "soil erosion classification and classification standard" issued by the Ministry of water resources of the people's Republic of China, the soil erosion in the CYP is classified into six levels (*Table 3*).

Table 3. *Classification standard of soil erosion intensity*

Level	Average modulus of soil erosion (t/(km^2·a))	Average loss thickness (mm/a)
Micro	<500	0.37
Mild	500 ~ 2500	0.37 ~ 1.9
Moderate	2500 ~ 5000	1.9 ~ 3.7
Strong	5000 ~ 8000	3.7 ~ 5.9
Extremely strong	8000 ~ 15000	5.9 ~ 11.1
Severe	>15000	>11.1

Spatial autocorrelation of soil erosion intensity distribution

According to the basic law of geographical analysis (Tobler, 1970): "there are corresponding relations between geographical things, among which the closer things are closer than those far away." As a spatial statistical method, spatial autocorrelation can better describe the relationship between geographical objects and measure the degree of aggregation or dispersion among spatial attributes of things.

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General spatial autocorrelation is used to describe the spatial characteristics of spatial element attribute values in the whole area, and reflects the similarity of its neighbor attribute values. It is of great significance for the analysis and description of the distribution characteristics of a specific spatial attribute in the entire area, it is commonly used Moran *I* index, its expression is (Zhang et al., 2007a):

$$I = \frac{n\sum_{i=1}^{n}\sum_{j=1}^{n} W_{ij}\left(x_i - \overline{x}\right)\left(x_j - \overline{x}\right)}{\sum_{i=1}^{n}\left(x_i - \overline{x}\right)^2 \sum_{i=1}^{n}\sum_{j=1}^{n} W_{ij}} \quad \text{(Eq.11)}$$

In the formula, n represents the number of spatial objects, $\overline{x}$ is the attribute mean, x_i and x_j are the attribute values of objects i and j, respectively, W_{ij} is the spatial adjacency matrix of objects i and j, W_{ij}=1, the two are adjacent, W_{ij}=0, the two are not adjacent.

The value range of Moran *I* index is [-1,1], the closer the value is to 1, the closer the attributes between objects are, showing a high value aggregation or a low value aggregation relationship; the closer the value is to -1, it means that the greater difference in attributes between objects; its value is 0, which means that there is no spatial autocorrelation between objects and they are randomly distributed.

Compared with the general spatial autocorrelation, the local spatial autocorrelation is to calculate the spatial correlation between each spatial object in the analysis area and its neighboring objects, calculate the local feature differences in the distribution of spatial objects in the analysis and analysis, and reflect the space in the local area Heterogeneity and instability, the calculation formula is (Zhang et al., 2007b):

$$I_i = \frac{\left(x_i - \overline{x}\right)\sum_{j=1}^{n} W_{ij}\left(x_j - \overline{x}\right)}{\frac{1}{n}\sum_{i=1}^{n}\left(x_i - \overline{x}\right)^2} \quad \text{(Eq.12)}$$

I_i is positive, indicating that the spatial object has similar properties to its neighbors; I_i is negative, indicating that it is different from the neighboring objects.

According to the spatial distribution data of soil erosion intensity grade in the CYP from 1980 to 2018, a 1000 m × 1000 m grid cell was created by using ArcGIS10.2, and the area proportion of each erosion intensity grade in each grid was counted as the data source of spatial autocorrelation analysis.

Results and discussion

Soil erosion factors distribution

The average *R* factor in the CYP is between 4,210.96 and 519.53 (MJ·mm)/(hm^2·h·a). Due to the influence of climate and topography, the spatial distribution is basically bounded by the northeastern and southwestern directions, showing higher *R* values in the southeast and northwest and lower *R* values in the northeast and southwest. Most of the

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maximum values are located in the southeastern region, and the maximum value is 9,086.65 (MJ·mm)/(hm^2·h·a), which appeared in 1980; the minimum values are mostly located in the central and western regions. The minimum value was 2,353.77 (MJ·mm)/(hm^2·h·a), which occurred in 2010.

The *K* factor ranges from 0 to 0.0885449, with an average value of 0.0390224, the numerical range is accurate and reliable. In general, the distribution of *K* value is relatively uniform.

The spatial distribution of the *L* and *S* factors of topographic slope length in the CYP is basically consistent with its overall topography, i.e., northwest to southeast inclination, which shows that the adopted calculation method of the *L* and *S* factors can better and quantitatively describe the effect of topography on soil and water loss in the study area.

Soil erosion intensity

Over the past 38 years in the CYP, soil erosion has generally shown a growing development trend. The average soil erosion modulus ranged from 1000.25 t/(km^2·a) to 2064.79 t/(km^2·a), and the multi-year average soil erosion modulus was 1624.47 t/(km^2·a), which represents a mildly erosive state. Overall, soil erosion shows a fluctuating trend. The two turning years were 2005 and 2015. The area of soil erosion first increased and then decreased, with 2005 as the only turning point. The area of soil erosion in 2005 was the largest, reaching 41,056.04 km^2. In 2018, the modulus of soil erosion and the area of soil erosion reached the lowest values. This result is due to the vigorous implementation of the policy of returning farmland to forest and grassland. People's awareness of environmental protection has also increased. Under the guidance of the government, soil and water conservation measures have been continuously improved and implemented, activities that causes ecological destruction have been effectively curbed, and overall, soil erosion has tended to be good.

From the perspective of the relationship between the average area of erosion intensity and the total area of the region in each period, the CYP is dominated by mild soil erosion, with an average annual area accounting for 48.67%, of which the largest proportion was 55.01% in 1980, and the largest area was 18,529.75 km^2 in 2000. The change trend of the area of moderate erosion is consistent with the overall change, and 2005 was the turning point in which the area of erosion transitioned from increasing to decreasing. Except for severe erosion, there is a good correlation between the other grade areas and the total soil erosion area. The correlation coefficients are as follows: mild 0.930; moderate 0.983, which the largest correlation coefficient; strong 0.924; extremely strong 0.929; and severe 0.398, which was the smallest correlation coefficient. From these results, it is possible to estimate the interannual variation of the overall soil erosion status by calculating and analyzing the interannual variation trend of the medium erosion intensity (*Table 4*).

Temporal change in soil erosion

To quantitatively describe the change in the erosion grade in central Yunnan from 1980 to 2018, the transfer matrices of erosion grade between adjacent years and initial years were calculated separately.

From 1980 to 1995, the soil erosion situation deteriorated, and the areas of erosion greater than moderate increased. The moderate erosion area increased most obviously, with an area of 648.47 km^2; the micro-degree and mildness experienced a net decrease, among which the micro-erosion transfer was the largest, with a net decrease of 1,445.32 km^2, and the area converted to mild erosion reached 2,651.27 km^2. The

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proportion of unchanged area of strong erosion was the smallest, with 61.72% unchanged, and 26.03% of the original area was converted into extremely strong erosion.

Table 4. *Statistics of area and proportion of different soil erosion intensity grades in the CYP (km^2)*

Year	Mild	%	Moderate	%	Strong	%	Extremely strong	%	Severe	%	A_{mean} ($t/(km^2 \cdot a)$)
1980	15771.02	55.01	6945.71	24.23	2682.65	9.36	1739.45	6.07	1531.25	5.34	1354.95
1995	15324.89	50.89	7593.87	25.22	3234.93	10.74	2085.40	6.93	1874.91	6.23	1574.83
2000	18529.75	48.09	10538.13	27.35	5254.52	13.64	2963.69	7.69	1248.73	3.24	1750.40
2005	17911.07	43.63	11191.28	27.26	6409.78	15.61	4058.24	9.88	1485.67	3.62	2064.79
2010	18112.15	47.33	10347.84	27.04	5267.43	13.76	3176.64	8.30	1362.88	3.56	1769.54
2015	16716.65	45.58	9454.21	25.78	5458.70	14.88	3492.82	9.52	1556.95	4.24	1884.56
2018	12461.16	50.16	6748.20	27.16	3382.80	13.62	1832.27	7.38	417.78	1.68	1000.25
Average	16403.81	48.67	8974.18	26.29	4527.26	13.09	2764.07	7.97	1354.02	3.99	1628.47

From 1995 to 2000, soil erosion continued to deteriorate, but the overall rate of erosion enhancement slowed. The area of severe erosion decreased 626.93 km^2, and the micro-erosion decreased to 8,406.05 km^2. The areas of moderate, strong and extremely strong erosion continued to increase, with net increases of 2,941.10 km^2, 2017.18 km^2 and 876.38 km^2, respectively.

From 2000 to 2005, the soil erosion situation was the most severe, and the erosion intensity was mostly converted into strong and extremely strong intensities, with net increases of 1,154.47 km^2 and 1,093.81 km^2, respectively. Under the background of the increase in the area of severe erosion and the continuous reduction in the areas of micro-degree and light erosion, the soil erosion in the CYP is deteriorating. The increased area of strong erosion is mainly from micro-degree and light erosion, which contributed to 1,049.78 km^2 and 1,035.07 km^2, respectively.

From 2005 to 2010, the soil erosion situation improved, and the areas of moderate, strong and extremely strong erosion changed from increasing to decreasing, with net decreases of 841.35 km^2, 1,140.76 km^2 and 879.96 km^2, respectively. The area of mild erosion had the largest increase, reaching 2,780.02 km^2, and its main source of growth was mild erosion, accounting for 38.19% of its original area.

From 2010 to 2015, with the overall situation of soil erosion improving, the area of mild erosion maintained a net growth trend, while the areas of strong, extremely strong and severe erosion rebounded, increasing by 191.26 km^2, 316.19 km^2 and 194.36 km^2, respectively.

From 2015 to 2018, the overall situation of soil erosion was good, and the area of erosion intensity above the micro-degree had a net decrease; thus, the soil erosion status was effectively contained.

Overall, soil erosion in central Yunnan was effectively controlled from 1980 to 2018. The area of mild erosion in the CYP increased to 3,827.88 km^2, and the proportion of the total area of soil erosion increased from 69.68% in 1980 to 73.73% in 2018, representing an increase of 4.05%. The areas of mild, moderate and severe erosion had net reductions of 3,309.87 km^2, 197.51 km^2 and 1,113.47 km^2, respectively. The proportion of these three factors in total soil erosion area changed from 55.01% to 50.16%, with a decrease of 4.85%, from 24.23% to 27.16%, with an increase of 2.93%, and from 5.34% to 1.68%, with a decrease of 3.66%, respectively. The results show that in the process of reducing the areas of mild, moderate and severe erosion, the rate of decreasing the area of mild

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erosion was the fastest, followed by that of severe erosion, and the deceleration of moderate erosion was slower than that of the former two.

In summary, during the 38 years, the erosion intensity in the CYP showed a weakening trend, but the changes in erosion grade were more complex. The area of mild erosion increased greatly, the intensity of severe erosion decreased significantly, the growth rate of strong and extremely strong erosion slowed, and the mild and moderate erosion decreased each year, which indicated that the intensity of soil erosion in the region decreased significantly. Soil erosion has been effectively curbed, and the overall erosion situation has improved.

Spatial distribution of soil erosion

By comparing and analyzing the soil erosion intensity maps of seven periods in the CYP, it can be seen that the distribution of the soil erosion grades in the mountains and valleys in the north and southwest of the CYP is complex and most grades represent extremely strong and severe erosion; however, in the middle and eastern regions with flatter land, mild erosion is dominant, of which Kunming Basin, Songming Basin, Yuxi Basin and Chengjiang Basin have mild erosion. It can be concluded that the spatial distribution of soil erosion intensity is significantly correlated with the topography in the region.

The spatial distribution of soil erosion intensity changes with time, showing a tendency to first deteriorate and then improve. Before 2005, the area of micro-erosion decreased each year. Starting with a uniform distribution, the area with relatively low terrain and relatively small terrain fluctuations in the southeast direction gradually decreased. In the western high terrain and high terrain fluctuation areas, the degree of soil erosion increased, and the intensity distribution became increasingly complex. After 2005, the micro-erosion surface became increasingly complex. The total erosion area above the mild level showed that the erosion area changed from increasing to decreasing, and the erosion could be effectively curbed.

According to the spatial distribution data of soil erosion intensity in the CYP from 1980 to 2018, the global spatial autocorrelation Moran's I index analysis was carried out based on the erosion intensity grade, and Monte Carlo simulation was used to test the spatial distribution of each erosion intensity. It was concluded that the spatial distribution of each erosion intensity showed a significant positive spatial autocorrelation, and spatial object clustering was obvious.

From 1980 to 2018, the numerical range and annual mean value of the overall Moran's I index of the soil erosion grades in central Yunnan were as follows: micro-erosion 0.62~0.69, 0.66; mild erosion 0.53~0.61, 0.56; moderate erosion 0.54~0.63, 0.58; strong erosion 0.58~ 0.64, 0.61; extremely strong erosion 0.57~0.65, 0.60; and severe erosion 0.43~0.52, 0.48. The Moran's I index of micro-erosion was larger in general, and its spatial aggregation degree was the highest, which indicated that the micro-erosion in the CYP showed a general aggregated distribution during the 38 years; however, the Moran's I index of severe erosion was smaller in general, its spatial distribution was the most dispersed, and it had higher fragmentation.

In the global Moran's I index of the spatial distribution of each grade (*Fig. 10*), except for the fluctuation of extremely strong erosion, the other five grades showed a fluctuating downward trend. This result indicates that the spatial distribution of soil erosion intensity in the CYP has gradually weakened in the past 38 years, and the degree of fragmentation has become increasingly serious. However, the extremely strong erosion shows that the

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spatial distribution of soil erosion intensity is becoming increasingly concentrated. Based on the spatial distribution of regional soil erosion area and its intensity, it can be seen that under the background of a decreasing area of total erosion, the spatial distribution of soil erosion is becoming increasingly dispersed, and the distribution centers are shifting; however, the distribution centers of extremely strong erosion have been located in the north for a long time because of the high elevation and high land level in the region. The large fluctuation of the potential and the high mountains and valleys are conducive to the occurrence of extremely strong erosion, and the prevention and control of soil erosion has not achieved significant results because of the topographic effects. Therefore, the extremely strong erosion shows an increasing trend in terms of the fluctuation in the Moran's I index.

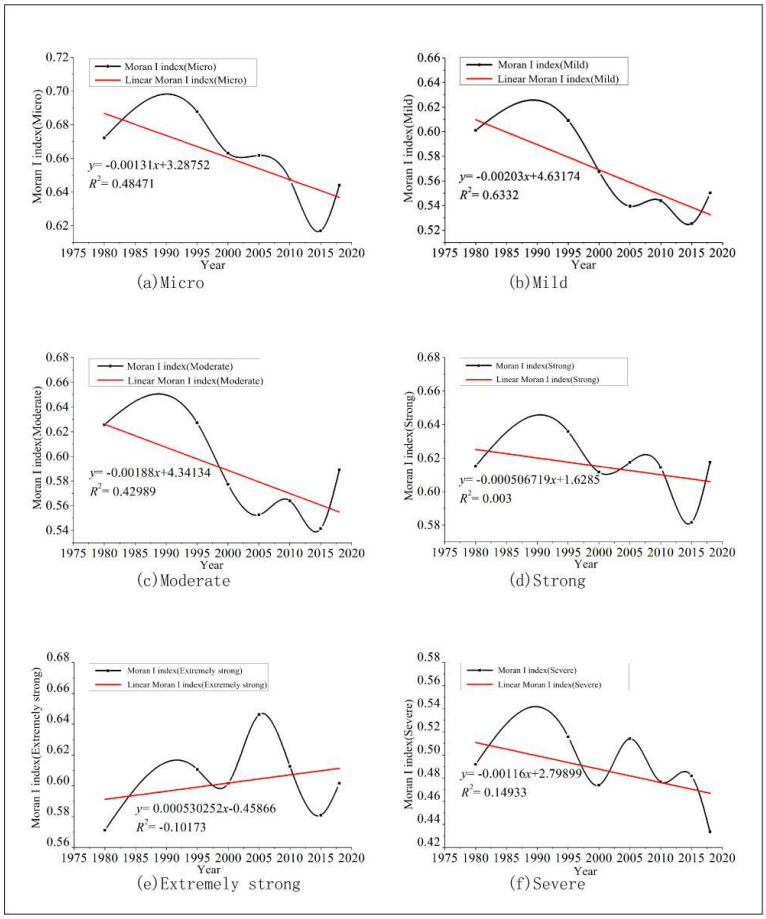

Figure 10. *Soil erosion overall Moran's I index in the CYP*

In general, the CYP region is affected by its overall topography and morphological distribution, there are different degrees of soil erosion in each region, and the spatial distribution is complex. In 2018, the entire region was dominated by micro-erosion, and extremely strong and severe erosion were still high in the western part of the region; however, the erosion area has been suppressed and reduced, and the overall soil erosion situation has improved significantly.

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Conclusion

Based on the comprehensive application of remote sensing and GIS technology, the RUSLE model was used to quantitatively characterize the soil erosion factors and the soil erosion status in seven periods for the CYP from 1980 to 2018, and the temporal and spatial changes were analyzed.

(1) Localized soil erosion factors in the CYP. The rainfall erosivity R factor and the topographic LS factor spatial distribution characteristics were more significant. The average R factor was between 4,120.96 (MJ·mm)/(hm^2·h·a) and 5,109.53 (MJ·mm)/(hm^2·h·a), and these values were affected by climate and topography. The spatial distribution changed correspondingly with the regional precipitation, and the overall distribution was more complicated. The spatial distribution of the topographic LS factor was basically consistent with the overall regional topography, e.g., it is inclined from the northwest to the southeast, showing a high numerical distribution in the northwest and a low numerical distribution in the southeast. Its value range is 0.03~97.68.

(2) From 1980 to 2018, the situation of soil erosion in the CYP showed an improving trend. From the aspect of erosion area, using 2005 as the turning point, the area of mild erosion first decreased and then increased, and the area of mild erosion was effectively controlled; from the aspect of the average erosion modulus, the average annual soil erosion modulus was 1,628.47 t/(km^2·a), which was categorized as mild erosion. This pattern experienced two turning points; specifically, the value increased each year from 1980 to 2005, declined from 2005 to 2010, rebounded from 2010 to 2015, and returned to a declining trend from 2015 to 2018. By 2018, the net increase in micro-erosion was three times higher than the increased areas of strong and extremely strong erosion, and these areas accounted for more than 73% of the total area. The overall erosion situation has been effectively contained.

(3) The spatial distribution of soil erosion intensity in the CYP shows a regular change, and its distribution location has a good correlation with regional topographic fluctuations. The erosion intensity above the moderate level is mainly distributed in the mountains and valleys in the north, northwest and southwest of the region. By calculating and analyzing the global Moran's I index of the spatial distribution of the soil erosion intensity from 1980 to 2018, it is further illustrated that the spatial distribution of the soil erosion intensity presents significant positive spatial autocorrelation overall, and spatial object aggregation is obvious. The spatial distribution law of each erosion intensity in the CYP is highly reliable.

In conclusion, from 1980 to 2018, although there were some fluctuations in the area occupied by each intensity grade of soil erosion in the CYP, the overall trend of development had improved. The area of mild erosion continued to increase, and the average soil erosion modulus remained at a low level. The spatial distribution of the soil erosion intensity had significant aggregation and distribution characteristics, and the location was closely related to the topography. Reviewing the whole research process, the delimitation of the range of the R factor of rainfall erosivity and the improvement of the accuracy of land use classification will be the main research topics to focus on in follow-up research; these approaches will enable us to obtain more accurate results of regional soil erosion and provide a more powerful scientific basis for the formulation of regional soil erosion control measures.

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SOYBEAN (*GLYCINE MAX* (L.) MERR.) RESPONSE TO COMMERCIAL INOCULATION WITH *BRADYRHIZOBIUM JAPONICUM*

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Abstract. The symbiotic nitrogen fixation is used to maximum advantage in the case of leguminous crops. It is therefore important to inoculate seeds with relevant strains of bacteria before sowing especially if the crop is to be grown for the first time on the land. A field experiment was done to assess the effect of inoculation with *Bradyrhizobium japonicum* on the growth and yield performance of soybean. Soybean cultivars inoculated with Nitrazon had significantly greater nodule numbers and dry weight of nodules compared to un-inoculated control. The bacterial preparation increased plant lodging and extended their vegetation period. Nitrazon had a positive effect on the number of pods per plant and thousand seed weight. The obtained increase in seed yield was 0.54 $t \cdot ha^{-1}$ compared to the control. Measurements of soil plant analysis development (SPAD) and leaf area index (LAI) showed that the plants were better nourished and had a higher green mass after Nitrazon application. Seed inoculation did not modify the leaf stomatal conductance (Gs). Nitrazon increased total protein content in seeds. The tested parameters were significantly diversified between cultivars and in the years of the study.
Keywords: *nitrogen fixation, soil plant analysis development, leaf stomatal conductance, leaf area index, yield*

Introduction

Soybean is one of the most economically important crops in the world, which results from the high value of its seeds and their versatile use. Soybean production is low in the European Union, but interest in growing this valuable plant has increased in recent years. Watson et al. (2017) reported that this was due to the growing demand for plant protein and the introduction of new high-yielding cultivars with good food and feed seed value. In soybean cultivation, inoculation of sowing material with bacteria fixing free nitrogen from the air is an especially important procedure (Gwata et al., 2004; Dwivedi et al., 2015; Vargas-Díaz et al., 2019). This treatment is particularly recommended when native strains of symbiotic bacteria are not present in the soil or there are too few of them (Solomon et al., 2012; Marinković et al., 2017; Jarecki, 2020). A good solution in this case may be the purchase for sowing material industrially inoculated with symbiotic bacteria (Flajšman et al., 2019) or the purchase of a commercial inoculant and its application to seeds (Kozieł et al., 2013; Pannecoucque et al., 2018). According to Deaker et al. (2004), increasing the amount of inoculant above the commercially recommended dose did not pose a threat and even caused a linear increase in nodulation and yield. Coskan and Dogan (2011) showed that the shape and size of nodules was characteristic of individual legumes. Nodules on soybean roots are usually round and the most effective are the large ones reddish in color inside (*Fig. 1*).

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a b

Figure 1. *Cross-section of bacterial nodules on soybean root, a - active, b - inactive (W. Jarecki)*

Numerous studies (Althabegoiti et al., 2008; López-García et al., 2009; Abou-Shanab et al., 2017) have demonstrated that the occurrence of Rhizobium bacteria in soil is common in some regions. In such case, inoculation with commercial preparations may be less effective, because native Rhizobium strains are competitive in establishing symbiosis (Wongphatcharachai et al., 2015; Onishchuk et al., 2017; Iturralde et al., 2019). Torres et al. (2012) also emphasized the need for periodic evaluation of commercial bacterial cultures to guarantee effective symbiosis after seed inoculation. Zhang et al. (2011) indicated in this aspect a high biodiversity and often a unique distribution of Rhizobium communities in individual regions. Giongo et al. (2008) demonstrated a high level for genetic diversity of Bradyrhizobium bacteria, which nodulate soybean in the fields. This information may be useful in experiments on new commercial inoculants better adapted to local environmental conditions. Shiro et al. (2013) observed that *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii*, but also others, are the main Rhizobia nodulating soybeans. Studies by Thilakarathna and Raizad (2017) have shown that local strains of nitrogen-fixing bacteria are often better adapted to local conditions of environmental stress, suggesting great potential for their commercialization. With proper nodulation, soybean plant demand for nitrogen is met at 40-57% (Zimmer et al., 2016) and even up to 60% (Salvagiotti et al., 2008) by biological N_2 fixing. In the discussed case, the need for soybean mineral nitrogen fertilization is ambiguous, especially with proper nodulation (Salvagiotti et al., 2008). Some studies indicated that soybean plants responded favorably to small doses of mineral nitrogen, e.g. in worse environmental conditions (Aboutalebian and Malmir, 2017; Cafaro La Menza et al., 2017; Gai et al., 2017). In turn, Albareda et al. (2009) and Kaschuk et al. (2016) reported that mineral soybean nitrogen fertilization was superfluous in case of proper symbiosis. This also applied to new high-yielding cultivars. Many reports (Zimmer et al., 2016; Leggett et al., 2017; Adjetey and Mbotho, 2019) showed that the use of seed inoculation significantly increased soybean yield compared to controls. In contrast, Abou-Shanab et al. (2017) and Ambrosini et al. (2019) reported that inoculation did not always increase soybean seed yield. The effect of using commercial biological preparations depends on many factors (Abou-Shanab et al., 2017; Kühling et al., 2018; Adjetey and Mbotho, 2019). Suzuki et al. (2014) examined *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* and showed that the former were more effective at lower temperatures. Interesting results were presented by López-García et al. (2009), which showed that the improvement of soybean nodulation resulted in a

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low, insignificant increase in yield, and nitrogen content in seeds did not change. Albareda et al. (2009) and Narożna et al. (2015) presented imortant studies on the survival of Rhizobium strains in soil in the successive years following soybean cultivation.

The purpose of this research was to determine the influence of nitrogen fixing bacteria on the growth and grain yield of some soybean cultivars, from different maturity groups, in the field conditions of South-East Poland.

Materials and methods

This study was conducted an individual farm in Makowisko (50º3'N 22º47'E), Podkarpackie Voivodeship, Poland (*Fig. 2*), in the years 2017-2019.

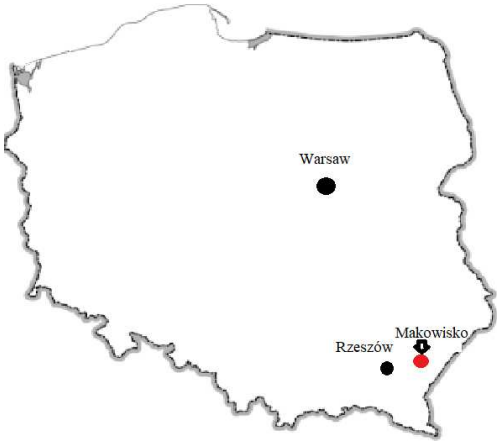

Figure 2. *Location of field experiment*

The first factor in the experiment was the Nitrazon biological preparation containing *Bradyrhizobium japonicum* and a control object - without inoculation. The second factor was soybean cultivars (main plots): Annushka, Lajma, Madlen, Violetta, Atlanta and Smuglyanka (AgeSoya Sp. z o.o., Poland). The cultivars are a high-yieldings recommended for cultivation in the area of the study. The experiment was carried out in four replications in a split-plot design. No soybean was previously grown in the experimental field. Seeds were inoculated according to the manufacturer recommendations (Farma Žiro Ltd., Czech Republic). Inoculation was done just prior to sowing.

The experiment was established on sandy loam soil, Haplic Luvisol (Food and Agriculture Organization of the United Nations, 2015). It was good wheat soil complex. Detailed descriptions of the soil characteristics are given in *Table 1*. Soil sample analysis was carried out at the District Chemical-Agricultural Station in Rzeszów.

Table 1. *Chemical soil properties before the experiment (0-60 cm)*

Years	Humus %	pH 1 mol/L KCl	Nmin kg ha^{-1} DM	P_2O_5	K_2O	Mg
				mg 100g^{-1} of soil		
2017	1.78	6.19	59.4	27.2	35.1	3.7
2018	1.71	5.92	55.1	23.8	24.1	2.8
2019	2.34	5.89	63.0	31.6	28.6	4.9

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Weather conditions are given according to meteorological data of the Experimental Stations for Variety Testing in Skołoszów. Distance from the experimental field about 12 km.

Sowing of seeds was carried out in the first days of May. The single plot area was 15 m^2. Soybeans were sown with 60 kernels capableof germination per m^2 and a space between rows of 45 cm. Winter wheat served as the forecrop.

NPK fertilization and plant protection treatments are described in detail in the publication Jarecki (2020).

Plant development stages are given according to the BBCH scale (Biologische Bundesanstalt, Bundessortenamtund CHemische Industrie). The full maturity stage of plants (BBCH) was given in days from the date of sowing. Plant lodging assessed before harvest on a scale of 1-9° (1° – the highest plant lodging, 9° – no lodging). The measurement of stomatal conductance of leaves (Gs) was performed with a Meter Porometr SC-1 apparatus (Pullman, USA). Soil plant analysis development (SPAD) was measured with a SPAD 502P chlorophylometer (Konica Minolta, Inc. Japan). Leaf area index (LAI) measurements was performed using a Meter LP-80 AccuPAR apparatus (Pullman, USA). Gs, SPAD and LAI were measured in the BBCH 70 stage.

To assess nodulation parameters, in the flowering stage (BBCH 65), 20 roots were randomly collected from one plot. Then in laboratory the number and dry weight of the nodules were determined.

At physiological maturity stage, 25 plants were collected for measuring the number of pods per plant and thousand seed weight (g). Seed yield obtained from the plots (BBCH 89) was converted into a yield per 1 ha with a moisture content of 15%.

The chemical composition of seeds was determined with the near-infrared spectroscopy (NIRS) method using an FT NIR MPA spectrometer (Bruker, Billerica, USA).

The significance of differences between the characteristic values was found based on Tuckey's halfconfidence intervals, with the significance level $p = 0.05$. Calculations were made using the software Statistica 10 PL (Stat Soft, Inc., Tulsa, USA).

Results and discussion

The weather conditions in 2017-2019 differed from the long-term average. In region, mean annual precipitation is 673 mm and mean annual temperature is 8.4°C. The detailed description of the climatic conditions is given in the *Table 2*.

Table 2. *Weather conditions in the years 2017-2019*

Months	Mean temperature (°C)				Sum of precipitation (mm)			
	2017	**2018**	**2019**	1978-2015	**2017**	**2018**	**2019**	1978-2015
April	6.4	10.9	7.3	9.0	42.7	24.3	46.7	46.0
May	12.6	14.9	11.8	14.1	68.4	47.0	158.6	71.6
June	17.3	17.0	19.5	16.6	48.7	104.7	25.4	79.2
July	18.0	18.5	17.9	18.5	43.0	98.1	60.2	94.3
August	18.4	18.4	17.7	18.1	21.2	84.3	101.9	63.0
September	12.5	12.9	12.7	13.4	102.5	34.6	33.7	62.5
October	8.3	7.3	8.1	8.8	58.4	40.1	37.9	47.7

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The number of nodules and the dry weight of nodules were significantly higher after the use of bacterial inoculant compared with the control (*Table 3*). On average, 19.9 nodules were found on a single soy root after inoculation Nitrazon, and 0.52 nodules on a un-inoculated control. The highest number of nodules and their highest dry weight were obtained in cultivars Lajma (*Fig. 3*) and Atlanta. The weather conditions had a significant impact on soybean nodulation. In 2018, an average of 14.6 nodules were recorded per root. The fewest nodules and their lowest dry weight were obtained in 2017.

Table 3. *Impact of seed inoculation on nodulation, lodging and maturity of plants*

Specification	The number of nodules	The dry weight of nodules (g)	Lodging (1-9°)	Maturity (number of days since sowing)
Inoculation				
Control	0.52^{b}	0.04^{b}	8.4^{a}	136^{a}
Nitrazon	19.9^{a}	0.71^{a}	7.9^{b}	138^{a}
Cultivar				
Annushka	10.0^{ab}	0.41^{ab}	8.3^{ab}	123^{c}
Lajma	12.8^{a}	0.54^{a}	8.6^{a}	128^{c}
Madlen	8.7^{bc}	0.23^{c}	7.9^{bc}	$138b^{c}$
Violetta	7.5^{c}	0.21^{c}	8.6^{a}	$137b^{c}$
Atlanta	12.7^{a}	0.52^{a}	8.0^{bc}	145^{ab}
Smuglanka	9.5^{bc}	0.34^{bc}	7.6^{c}	151^{a}
Year				
2017	6.36^{c}	0.21^{c}	8.7^{a}	128^{b}
2018	14.6^{a}	0.54^{a}	7.7^{c}	145^{a}
2019	9.65^{b}	0.37^{b}	8.1^{ab}	138^{ab}

Average values for each factor marked with different letters in a column differ significantly ($P < 0.05$)

a b

Figure 3. *Lajma cultivar, a – without inoculation, b - with inoculation (W. Jarecki)*

Althabegoiti et al. (2008) reported that commercial soybean inoculant could be used on seeds or in the soil, in which they would be sown. These authors obtained better results in the latter case. Zerpa et al. (2013) obtained the highest number of nodules on soybean roots (17.5 on average) after combined use of two commercial bacterial strain. Nodulation was not found in the control object. Adjetey and Mbotho (2019) demonstrated the efficacy of commercial inoculates, and observed few nodules in control plants. Abou-Shanab et al. (2017) concluded that the presence of native strains of Rhizobium bacteria in soil also

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caused nodulation on non-inoculated roots (control). The obtained results of soybean seed inoculation by the above-mentioned authors varied depending on the bacterial strain, soil nitrogen content, cultivar and study region. Thuita et al. (2012) report that both the number of nodules on the roots and their weight are significantly dependent on the inoculant used. At the same time, the obtained results were also heavily influenced by the cultivar. Marinković et al. (2018) obtained a varied number and dry weight of nodules on soybean roots depending on the strain of symbiotic bacteria. This provided the basis for selecting the best one for the production of commercial preparations.

In the present study, the application of Nitrazon significantly increased lodging of the plants compared to control. The difference obtained was 0.5°. Plants of the cultivar Smuglanka were characterized by the greatest lodging. Annushka, Lajma and Violetta cultivars were significantly less affected by lodging. In the years of research, the discussed parameter significantly differed. In 2018, plant lodging was the greatest and amounted to 7.7°. The bacterial strain extended the vegetation period by an average of two days compared to control, but this has not been statistically proven. The vegetation period of the studied cultivars ranged from 123 days in the cultivar Annushka to 151 days in the cultivar Smuglanka. Differences in the studied parameter were recorded during the years of the study. Soybean plants reached full maturity after 128 days in 2017 and 145 days in 2018.

The bacterial preparation has significantly increased the number of pods per plant and thousand seed weight. The obtained increase in seed yield was 16.9% compared with the control (*Table 4*). Madlen, Atlanta and Smuglanka were high yielding cultivars. The cultivar Lajma produced a significantly lower seed yield. Seed yield in 2018 was 3.65 t·ha^{-1}, while in 2017 – 3.27 t·ha^{-1}. Therefore, the obtained difference amounted to 11.6%.

Table 4. *Yield and yield components*

Specification	Yield (t·ha^{-1})	The number of pods per plant	Thousand seed weight (g)
Inoculation			
Control	3.19^{b}	29.6^{b}	147.9^{b}
Nitrazon	3.73^{a}	34.1^{a}	153.1^{a}
Cultivar			
Annushka	3.39^{ab}	31.9^{ab}	145.6^{b}
Lajma	3.35^{b}	30.8^{b}	147.5^{b}
Madlen	3.56^{a}	31.2^{b}	161.8^{a}
Violetta	3.39^{ab}	31.1^{b}	145.9^{b}
Atlanta	3.54^{a}	32.9^{a}	155.4^{ab}
Smuglanka	3.53^{a}	33.2^{a}	146.8^{b}
Year			
2017	3.27^{b}	32.8^{b}	141,9^{b}
2018	3.65^{a}	37.9^{a}	156.4^{a}
2019	3.46^{ab}	27.8^{c}	153.2^{a}

Average values for each factor marked with different letters in a column differ significantly ($P < 0.05$)

Macák and Candráková (2013) reported that the number of pods per plant, thousand seed weight and seed yield of soybean were variable in years, while the number of seeds in the pod was stable. Căpățână et al. (2017) obtained an increase in soybean seed yield

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after inoculation, but only by 3.76% compared to control. Solomon et al. (2012) and Thuita et al. (2012) confirmed that not every strain of *Bradyrhizobium japonicum* provided the expected results. They also added that in the case of soybean, yielding was mainly determined by the selection of proper cultivar. Abou-Shanab et al. (2017) concluded that inoculation of legumes with nitrogen-fixing bacteria did not always result in a significant increase in seed yield. Therefore, recommendations for the use of biological preparations should be related to regional habitat conditions. This was also confirmed by the experiments carried out by Leggett et al. (2017), who showed that the effects of soybean seed inoculation with *Bradyrhizobium japonicum* varied depending on the years of research and the specificity of a given area.

On average, soybean seeds contained 37.5% protein and 19.5% fat. The applied Nitrazon significantly increased protein content in seeds. Crude fat content was not changed compared to control (*Table 5*). The tested cultivars had significantly different content of protein and fat in seeds. The highest total protein content was determined in seeds of the cultivar Violetta and crude fat in the seeds of the cultivars Lajma and Madlen. The chemical composition of seeds was varied during the years of the study.

Table 5. *Chemical seed composition*

Specification	Protein total (% DM)	Crude fat (% DM)
Inoculation		
Control	36.3^{b}	19.6^{a}
Nitrazon	38.7^{a}	19.4^{a}
Cultivar		
Annushka	37.2^{ab}	19.5^{ab}
Lajma	36.7^{b}	20.2^{a}
Madlen	37.7^{ab}	20.0^{a}
Violetta	38.6^{a}	19.4^{ab}
Atlanta	37.6^{ab}	18.9^{b}
Smuglanka	37.2^{ab}	19.0^{b}
Year		
2017	36.8^{b}	20.5^{a}
2018	38.2^{a}	18.2^{b}
2019	37.5^{ab}	19.8^{ab}

Average values for each factor marked with different letters in a column differ significantly ($P < 0.05$)

Zimmer et al. (2016) stated that the use of commercial bacterial strain in soybean cultivation was justified. At the same time, they obtained various effects in the form of an increase in protein yield depending on the study area. Pannecoucque et al. (2018) and Flajšman et al. (2019) reported that commercial preparations effectively increased soybean nodulation, which resulted in an increase in protein content in seeds and protein yield compared to control. Cafaro La Menza et al. (2017) concluded that obtaining high soybean yields, including protein yields, required adequate nitrogen supply. However, with high nitrogen availability, they noted a slight increase in protein content and a decrease in fat in soybean seeds.

Plant nutritional status assessed by the SPAD index was higher after Nitrazon application compared to control. Among the tested cultivars, Annushka was characterized by a high SPAD index, while Atlanta and Smuglanka the lowest. Significant differences in the SPAD index have been shown in plants between 2017 and 2018 (*Fig. 4*).

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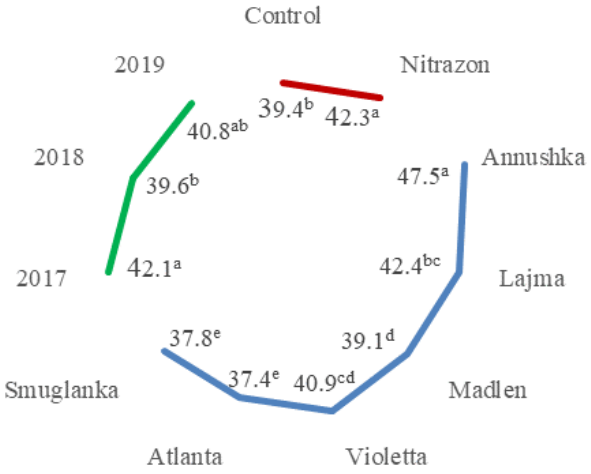

Figure 4. *Soil plant analysis development (SPAD value)*

Thompson et al. (1996) confirmed the usefulness of SPAD measurements for the assessment of chlorophyll content in soybean leaves, but the results were modified by environmental conditions. Fritschi and Ray (2007) believed that SPAD readings were useful, but should have also been supplemented with other measurement techniques. Kühling et al. (2018) reported that the SPAD value was higher after applying the inoculation procedure, but only at the beginning of soybean seed filling phase. In the research of Jarecki et al. (2016), better-fed plants (higher SPAD) were characterized by higher yields, which was confirmed by a strong correlation (r = 0.83). SPAD measurements can therefore be useful for predicting soybean seed yield. Vollmann et al. (2011) added that the assessment of chlorophyll content in leaves also allows to predict the quality parameters of soybean seeds.

Nitrazon significantly increased the LAI index compared to controls. Early cultivars (Annushka and Laima) were characterized by lower LAI values and late cultivars (Atlanta and Smuglanka) by its higher values. Differences in LAI were recorded during the years of the study. The highest LAI was recorded in 2017, while significantly lower in 2018 (*Fig. 5*).

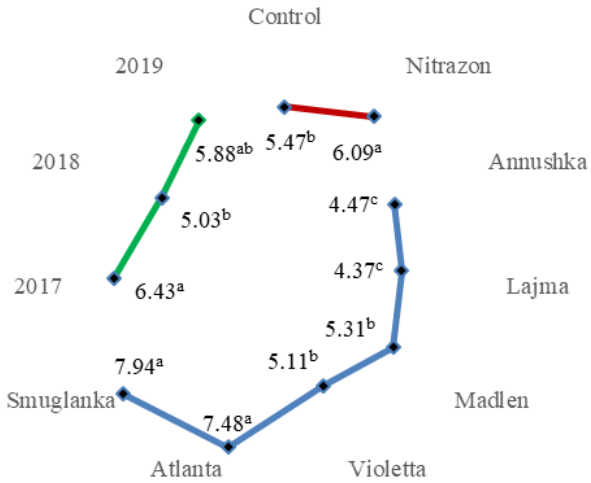

Figure 5. *Leaf area index (LAI)*

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Salvagiotti et al. (2008) have argued that the availability of nitrogen, which affects the growth and development of a single plant, largely determines soybean yield. Abou-Shanab et al. (2017) obtained a significantly higher dry weight of soybean plants after inoculation compared to control. However, seed yield did not increase significantly. Zerpa et al. (2013) showed an increase in soy leaf surface after the combined application of two commercial inoculant containing *Bradyrhizobium japonicum*. Single use of the test preparations was less effective. Adjetey and Mbotho (2019) obtained a greater number of leaves in the experiment with soybean after inoculation compared to control; nutrient uptake has also increased. Jarecki et al. (2016) reported that nitrogen availability for soybean plants increased the assimilation surface. However, they did not show a relationship between LAI measurements and seed yield.

The bacterial strain had no effect on leaf stomatal conductance (Gs) compared to control plants. The highest Gs had the cultivar Lajma and Violetta the lowest. In the years of the study, Gs ranged from 561 in 2019 to 836 in 2018 (*Fig. 6*).

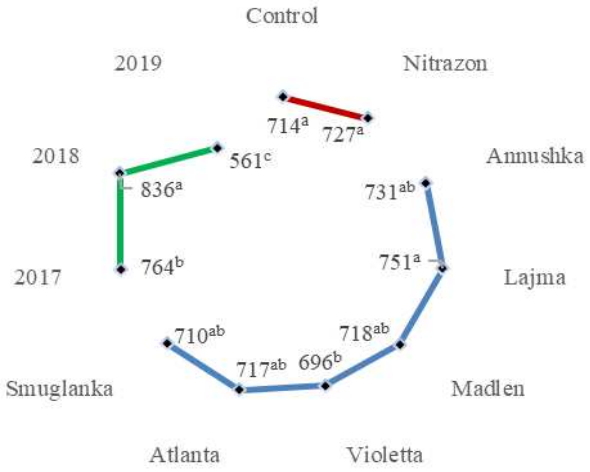

Figure 6. *Leaf stomatal conductance (Gs)*

Yu et al. (2018) believed that the results of leaf stomatal conductance were more accurate when carried out under controlled meteorological conditions compared to field conditions.

Conclusion

In this study, the use of commercial preparation - Nitrazon increased the number and dry weight of nodules on soybean roots compared to control. SPAD and LAI measurements showed that the use of *Bradyrhizobium japonicum* resulted in better nutrition and higher green mass of plants. The inoculation resulted in an increase in the number of pods per plant, thousand seed weight, seed yield, protein content in seeds compared to control. It was shown that the examined traits and parameters varied between cultivars and changed over the years of the study. Low seed yields were obtained in 2017 and high in 2018. Generally, inoculation of soybean seeds with nitrogen-fixing bacteria, can lead to a reduction of the consumption of nitrogen fertilizer in soybean cultivation. In next experiment, it is possible to assess the effectiveness of the commercial soybean seed coating with *Bradyrhizobium japonicum*.

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EFFECTS OF LEAD PHYTOTOXICITY ON DIFFERENT PEANUT VARIETIES GERMINATION AND SEEDLING GROWTH

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Abstract. Lead is a highly toxic environmental pollutant for plants and other living organisms, including humans. To determine the effects of lead phytotoxicity on germination and seedling growth of 10 peanut (*Arachis hypogaea* L.) varieties, a study was conducted to screen the influence of different lead nitrate ($PbNO_3)_2$ doses in May 2016 at 25 °C (±2) in the Department of Field Crops Laboratory, Faculty of Agriculture, Kahramanmaras Sutcu İmam University. The treatment was carried out according to the completely randomized plot experimental design with three replications. In the experiment, two pieces of filter paper were placed in each of the 120 sterile petri dishes and then, 25 seeds of each varieties were placed to germinate. After 14 days, some main germination and seedling growth parameters were observed. All the characteristics studied were affected differently by lead doses according to of varieties. Peanut varieties were observed to react differently to lead concentrations due to their different genotypic structure, and some did not have a significant effect on lead doses. Overall, it can be concluded that lead is privative, but plants employ mechanisms to resist it. Thus, further research should be carried out to select and develop cultivars that have superior tolerance to Pb.
Keywords: *germination, lead nitrate ($Pb(NO_3)_2$), peanut (Arachis hypogaea L.), seed, seedling vigor index, toxicity*

Introduction

Peanut (earthnut, groundnut, ground bean) belongs to the Arachis genus, an annual, soil enriching, self-pollinated legume, it is an oilseed, and a member of the Leguminosae family and the Papilionaceae subfamily. It is widely grown in the tropics and temperate regions in six continents between latitudes 40°N and 40°S, from warm temperate to equatorial climates (Konlan et al., 2013; Sharma and Bhatnagar-Mathur, 2006). There are numerous confinements to the productivity of the peanut crop that result in enormous economic losses. Traditional plant breeding practices and methodologies have not been successful in conferring resistance against different biotic and abiotic stresses owing to species restrictions in the natural system. Although it is not sufficiently successful, there is always a need to determine the resistance to abiotic stresses, especially in varieties developed as yield and resistant to certain diseases. Negative effects of non-living factors on living organisms in a given environment can be defined as abiotic stress. There are various types of abiotic stresses such as drought, over-watering, extreme temperatures (cold, frost and heat), salinity and mineral toxicity. Abiotic stresses are the main causes of poor plant growth and reduced plant production in the world (Onaga and Wydra, 2016). Minerals consist of heavy metals that are toxic even to a very low concentrations for both plants and animals. Some heavy metals, such as arsenic, cadmium, mercury, lead and selenium, do not perform any physiological functions known in plants and are called non-essential metals. These metals generally have negative effects on seed germination, plant growth, development, yield and seed quality depending on the plant species. Other minerals such as ferrum, zinc, cobalt,

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copper, manganese, molybdenum and nickel are essential elements for the normal growth and metabolism of plants. When the concentrations of these essential elements exceed the optimum level, it may cause poisoning (Mani and Sankaranarayanan, 2018). Environmental conditions' changes influence the biological and physiological reactions of plants. For general growth and development, plants need a variety of mineral elements. These minerals are existing in the soil and absorbed by the roots, a great many of them are transferred to the shoots and then distributed to various organs and tissues of the plant depending on their requirements (Mani and Sankaranarayanan, 2018).

Lead ranks second among all the hazardous heavy metals (Kumar et al., 2012) and is the most common heavy metal pollutant in the environment, according to the Environmental Protection Agency (EPA) (Lamhamdi et al., 2011). Lead inhibits the activity of many enzymes, disrupts mineral nutrition and water balance, alters hormonal status and affects membrane structure and permeability. Lead reduces the photosynthetic rate of plants, thereby limiting plant growth by disrupting the chloroplast infrastructure, reducing chlorophyll synthesis, inhibiting electron transfer, and inhibiting the activities of the Calvin cycle enzymes (Sengar et al., 2008). Lead toxicity causes inhibition of ATP production, lipid peroxidation and DNA damage. In addition, lead strongly inhibits seed germination, root growth, seedling growth, plant growth, sweating, chlorophyll production and water and protein content (Pourrut et al., 2011).

This study deals with the effects abiotic stress due to lead nitrate toxicity concentrations on germination and seedling growth parameters of *Arachis hypogaea* L., and to demonstrate the differences among these 10 varieties.

Material and methods

The study was carried out at 25 °C (±2) in the laboratory of Department of Field Crops, Faculty of Agriculture, Kahramanmaras Sutcu Imam University in May 2016 in Turkey.

In this study, which investigated how lead's phytotoxicity affects germination and subsequent seedling development, 10 (Arıoğlu-2003, Batem-5025, Florispan, Georgia Green, Halisbey, NC-V-II, NC-7, Osmaniye-2005, Sultan and Wilson) varieties of peanuts obtained from Çukurova University Faculty of Agriculture, Department of Field Crops and Osmaniye Agricultural Research Institute were used as materials. The cultivars used in this study, are the major peanut varieties have been registered in Turkey. In addition, all of the varieties planted in Turkey confectionary Virginia type varieties. The most common planted variety is NC-7. Also; There are also local seeds in the form of unregistered farmer populations such as Anamur, Osmaniye, Aydın (Kadiroğlu, 2008).

In this study, healthy and homogenous seeds of 10 peanut varieties were sterilized with 5% NaOCI (sodium hypochlorite) solution for 5 min and then rinsed with tap water. In the study, lead nitrate $Pb(NO_3)_2$ which was used as stress factor was prepared in four different doses as 0, 100, 200 and 400 mg L^{-1}, respectively. In the study, 25 seeds of each variety were sown on to germinate for 14 days in 120 sterile petri dishes, each with two filter papers. During sowing, 20 ml of all concentrations prepared separately were added to each petri dish. The same amount of tap water was added to the petri dishes, which were evaluated as controls. After two days, 15 ml of these solutions were added to each petri dish six times at two-day intervals. The petri dish's

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lids were closed and placed in growth booths, setting germination rates at the same time each day. In order to assess germination of the seed, seeds with a root formation of 1 mm were considered germinated seeds (Munzuroglu and Geckil, 2002). Germination test was terminated at the end of 14 days when the cotyledon leaves emerged completely. In the study, some basic germination and seedling parameters such as germination percentage, germination index, radicle length, plumule length, seedling length, radicle fresh weight, plumule fresh weight, seedling fresh weight, radicle dry weight, plumule dry weight, seedling dry weight, seedling vigor index and germinated seed number were observed. The germination rate was found by dividing the germinated seeds by the total number of seeds and then multiplying by 100 (Maquire, 1962).

$$Germination\ percentage = \frac{\text{Germinated seeds number}}{\text{Total seeds number}} x100$$

Germination index = ∑Gt/Dt, Gt is the number of germinated seeds in t days; Dt is the number of corresponding germination days (Ertekin et al., 2011).

The length of the seedling was found by measuring the length of the radicle and plumule separately and then adding both lengths (AOSA, 1984).

After measurements of the fresh samples, the samples were kept for 24 h in an oven set at 78 °C, and then analyses of the dry samples were performed (AOSA, 1984).

Seedling vigor index was found by multiplying seedling length by germination percentage (Abdul-Baki and Anderson, 1973; AOSA, 1984).

$$Seedling\ vigor\ index = \text{Seedling length } x \text{ Germination percentage}$$

Statistical analysis of data

All data obtained from the study were processed by SAS (v. 9.0, 2002) statistical package. The analysis of variance (ANOVA) was performed according to the Completely Randomized Experimental Design procedures at significant levels of $p < 0.01$ or $P < 0.05$. To detect the significant differences ($p < 0.01$ or $p < 0.05$) of variables, Least Significant Difference (LSD), a multiple comparison test, was performed. Additionally, the Pearson Correlation analysis was processed to determine the relationship among the observed parameters.

Results and discussion

Germination percentage (%)

In terms of tolerance to increasing lead doses, when the varieties were examined, statistically significant ($P < 0.01$) differences were found among the varieties. The highest germination rate (100%) was obtained from Batem-5025 variety, while the lowest (87.67%) was obtained from Osmaniye-2005 variety (*Table 1*). Differences among the lead doses in terms of their effects on germination rates were found to be insignificant ($P > 0.05$) and it was determined that germination rates ranged between 94.67% and 98.27% (*Table 1*). As shown in *Table 1,* lead doses had different effects on seed germination rates of peanut varieties. It was found that peanut varieties react differently to lead levels due to genotypic differences.

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Table 1. *The means and LSD groups of GP, GI, RL, PL, SL, RFW, PFW properties of Arachis hypogaea L. varieties*

Varieties	Doses (g L^{-1})	GP (%)	**	GI	**	RL (cm)	*;**	PL (cm)	**	SL (cm)	*;**	RFW (g)	*;**	PFW (g)	*;**
Arıoğlu-2003	Control	97.3		11.11		3.95	b-g	3.09		7.04	a-d	2.58	b-g	24.74	a-c
	100	100.0		11.67		4.14	b-f	2.96		7.10	a-d	2.56	b-g	24.77	a-c
	200	97.3		11.22		3.80	b-g	3.12		6.92	a-d	2.72	b-f	24.47	a-c
	400	94.7		10.64		3.35	c-g	3.36		6.71	a-e	1.72	e-h	22.23	b-d
	Mean	**97.3**	**AB**	**11.16**	**AB**	**3.81**	**C**	**3.13**	**AB**	**6.94**	**CDE**	**2.40**	**B**	**24.05**	**B**
Batem-5025	Control	100.0		12.00		5.61	a-c	3.71		9.32	a	1.75	e-h	15.62	e-h
	100	100.0		11.97		6.08	ab	3.47		9.55	a	1.87	e-h	16.99	d-f
	200	100.0		11.19		5.23	a-c	3.07		8.30	ab	2.13	d-h	21.14	cd
	400	100.0		11.89		4.75	a-d	3.11		7.86	a-c	2.28	c-h	19.29	c-e
	Mean	**100.0**	**A**	**11.76**	**A**	**5.42**	**A**	**3.34**	**A**	**8.75**	**A**	**2.01**	**BCD**	**18.26**	**D**
Florispan	Control	96.0		10.92		3.72	c-g	2.13		5.85	c-e	1.61	f-h	11.60	h
	100	100.0		11.93		5.31	a-c	2.70		8.01	ab	2.00	e-h	13.01	f-h
	200	98.7		12.11		5.66	a-c	2.97		8.64	a	2.08	e-h	12.70	gh
	400	98.7		11.83		5.45	a-c	3.45		8.90	a	1.93	e-h	13.39	f-h
	Mean	**98.3**	**AB**	**11.70**	**A**	**5.04**	**A**	**2.81**	**BC**	**7.85**	**ABC**	**1.91**	**CD**	**12.67**	**E**
Georgia Green	Control	94.7		11.22		4.27	b-e	2.50		6.77	a-d	1.65	f-h	15.02	f-h
	100	100.0		12.00		6.52	a	2.34		8.86	a	1.69	f-h	13.64	e-g
	200	100.0		12.08		3.83	b-g	2.31		6.15	c-e	1.40	h	13.39	f-h
	400	98.7		11.94		5.09	a-c	3.07		8.17	ab	1.68	f-h	15.87	f-h
	Mean	**98.3**	**AB**	**11.81**	**A**	**4.93**	**AB**	**2.56**	**CD**	**7.49**	**BCD**	**1.61**	**D**	**14.48**	**E**
Halisbey	Control	98.7		10.39		2.11	fg	2.22		4.33	e-g	1.80	e-h	24.31	bc
	100	98.7		10.22		2.23	fg	2.37		4.60	d-g	2.34	c-g	25.39	ab
	200	100.0		10.42		1.78	g	2.00		3.77	fg	1.58	gh	23.21	bc
	400	100.0		10.47		2.77	fg	2.07		4.85	d-g	1.83	e-h	24.19	bc
	Mean	**99.3**	**AB**	**10.38**	**C**	**2.22**	**E**	**2.16**	**DE**	**4.39**	**F**	**1.89**	**CD**	**24.27**	**B**
NC-V-II	Control	93.3		8.92		2.99	e-g	2.61		5.61	de	1.83	e-h	25.29	ab
	100	98.7		10.17		3.42	c-g	3.82		7.24	a-c	1.85	e-h	28.33	a
	200	94.7		9.22		3.01	e-g	3.66		6.67	b-e	1.99	e-h	26.61	ab
	400	100.0		10.50		3.87	b-g	3.25		7.12	a-c	3.11	bc	26.28	ab
	Mean	**96.7**	**AB**	**9.70**	**DE**	**3.32**	**CD**	**3.34**	**A**	**6.66**	**DE**	**2.19**	**BC**	**26.63**	**A**
NC-7	Control	86.7		9.53		2.06	fg	2.93		4.99	d-f	2.15	d-h	20.04	cd
	100	97.3		10.72		3.60	c-g	3.11		6.71	b-e	3.60	b	12.01	h
	200	100.0		10.75		4.65	b-d	2.71		7.36	a-c	2.99	bc	25.52	ab
	400	96.0		10.50		4.83	a-c	3.17		8.00	ab	2.91	b-d	26.65	ab
	Mean	**95.0**	**B**	**10.38**	**C**	**3.78**	**C**	**2.98**	**ABC**	**6.77**	**DE**	**2.91**	**A**	**21.06**	**C**
Osmaniye-2005	Control	80.0		8.67		3.62	c-g	2.20		5.82	de	2.70	b-f	25.37	ab
	100	92.0		9.72		3.45	c-g	1.73		5.18	de	2.52	c-g	26.12	ab
	200	90.7		9.68		4.28	b-d	2.12		6.40	c-e	2.98	bc	26.44	ab
	400	88.0		9.92		5.11	a-c	2.03		7.15	a-c	4.18	a	26.63	ab
	Mean	**87.7**	**C**	**9.50**	**E**	**4.11**	**BC**	**2.02**	**E**	**6.14**	**E**	**3.10**	**A**	**26.14**	**A**
Sultan	Control	100.0		10.47		2.98	fg	2.29		5.27	de	2.02	e-h	26.10	ab
	100	100.0		10.11		1.83	g	1.86		3.69	g	1.41	h	25.39	ab
	200	96.0		9.86		3.39	c-g	2.24		5.62	de	2.02	e-h	26.94	ab
	400	98.7		10.69		3.15	d-g	2.59		5.74	de	2.05	e-h	28.26	a
	Mean	**98.7**	**AB**	**10.28**	**CD**	**2.83**	**DE**	**2.25**	**DE**	**5.08**	**F**	**1.87**	**CD**	**26.67**	**A**
Wilson	Control	100.0		11.67		5.64	a-c	2.85		8.49	a	2.24	d-h	22.30	bc
	100	96.0		9.22		4.20	b-e	2.66		6.87	a-d	1.95	e-h	21.07	cd
	200	97.3		11.22		6.37	a	3.13		9.50	a	2.71	b-f	25.21	ab
	400	98.7		11.06		4.27	b-e	3.35		7.63	a-c	2.73	b-e	28.32	a
	Mean	**98.0**	**AB**	**10.79**	**BC**	**5.12**	**A**	**3.00**	**ABC**	**8.12**	**ABC**	**2.41**	**B**	**24.22**	**B**
LSD (0.05) for V		**4.46**		**0.66**		**0.85**		**0.45**		**0.96**		**0.421**		**1.849**	
LSD (0.05) for LD		**2.82**		**0.42**		**0.54**		**0.29**		**0.61**		**0.266**		**1.170**	
LSD (0.05) for V x LD		**15.46**		**2.29**		**2.96**		**1.57**		**3.33**		**1.457**		**6.406**	
CV (%)		**5.66**		**7.55**		**25.87**		**20.25**		**17.34**		**23.212**		**10.415**	

GP: Germination Percentage, GI: Gemination Index, RL: Radicle Length, PL: Plumule Length, SL: Seedling Length, RFW: Radicle Fresh Weight, PFW: Plumule Fresh Weight, V: Varieties, LD: Lead doses

*The mean in the same column, expressed in lowercase and indicated with different letters, is statistically different from each other within the $P \leq 0.05$ error limits according to LSD test

**The mean in the same column, expressed in capital and indicated with different letters, is statistically different from each other within the $P \leq 0.05$ error limits according to LSD test

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Germination index

It was seen that there were statistically significant ($P < 0.01$) variations among the varieties in terms of germination indexes. The highest (11.81, 11.77 and 11.70, respectively) germination indexes were determined from Georgia Green, Batem-5025 and Florispan, while the lowest (9.50) was found in Osmaniye-2005. No significant ($P > 0.05$) differences were found among the lead doses in terms of their effects on germination indexes (*Table 1*).

Radicle length (cm)

As can be seen from *Table 1,* in terms of radicle lengths, statistically significant differences ($P < 0.01$) were found among the varieties. The highest radicle lengths were measured from Batem-5025, Wilson and Florispan (5.42, 5.12, and 5.04 cm) varieties, respectively, while the lowest (2.22 cm) was observed from Halisbey varieties. The differences between lead doses were found to be insignificant ($p > 0.05$) in terms of their effect on radicle lengths and it was determined that the radicle lengths obtained from different doses ranged between 3.69 and 4.27 cm (*Table 1*). Due to the different reactions of the varieties to the increased lead doses, the effect of variety and dose interaction on radicle lengths was found to be statistically significant ($p < 0.05$). The highest radicle lengths (6.52 and 6.37 cm) were measured from Georgia Green and Wilson varieties at 100 and 200 mg $Pb(NO_3)_2$ L^{-1} doses, while the lowest (1.78 and 1.83 cm) were observed in Halisbey and Sultan varieties at doses of 200 and 100 mg $Pb(NO_3)_2$ L^{-1} (*Table 1; Fig. 1*).

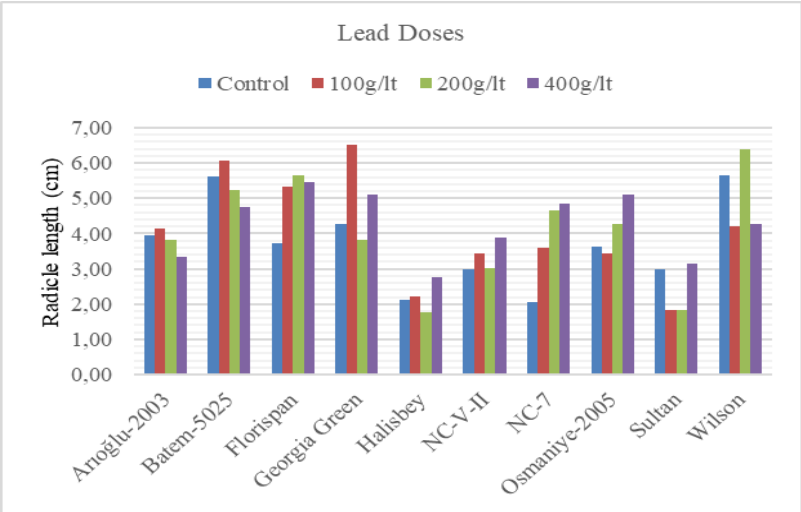

Figure 1. *The interaction of Arachis hypogaea L. varieties' radicle length*

Plumule length (cm)

In the experiment, it was found that there were statistically significant differences ($P < 0.01$) among the varieties in terms of plumule lengths. The highest plumule lengths were seen in Batem-5025 (3.34 cm) and NC-V-II (3.34 cm) varieties, while the lowest was measured from Osmaniye-2005 variety as 2.02 cm. Differences among lead doses were found to be insignificant ($P > 0.05$) in terms of their effects on plumule lengths, and plumule lengths were determined to vary between 2.65 and 2.95 cm (*Table 1*).

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Seedling length (cm)

From *Table 1,* it was found that there was a statistically significant ($P < 0.01$) variation among the varieties in terms of seedling lengths. The highest seedling length was observed in Batem-5025 variety as 8.76 cm, while the lowest seedling lengths were seen in Halisbey (4.39 cm) and Sultan (5.08 cm) varieties. As a result of the study, it was found that there were statistically significant ($P < 0.05$) differences among lead doses in terms of their effects on seedling lengths. The highest seedling length was measured as 7.21 cm at 400 mg $Pb(NO_3)_2$ L^{-1} dose, while the lowest was determined as 6.35 cm at the control dose (*Table 1*). The effect of variety and dose interaction on seedling lengths was statistically significant ($P < 0.05$) due to the different responses of varieties to lead doses. The lowest seedling length of 3.69 cm was observed in Sultan variety at 100 mg $PB(NO_3)_2$ L^{-1} doses (*Table 1; Fig. 2*).

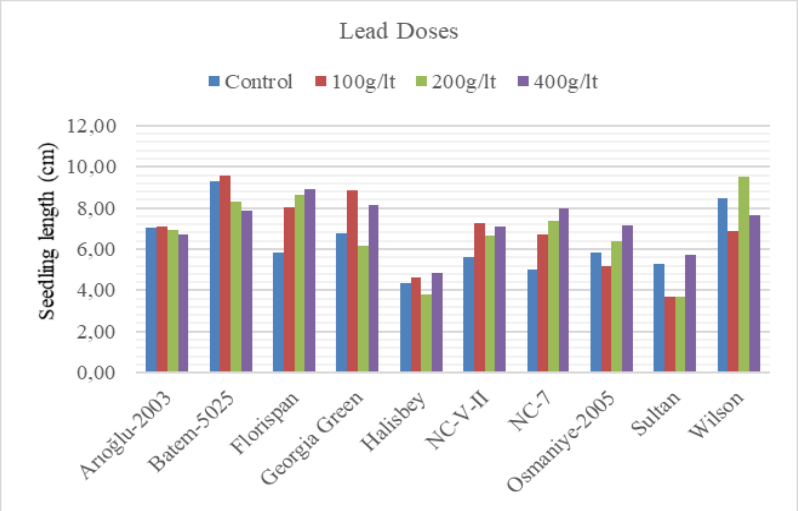

Figure 2. *The interaction of Arachis hypogaea L. varieties' seedling length*

Radicle fresh weight (g)

As indicated in *Table 1,* it was seen that there was a statistically significant ($P < 0.01$) variation among varieties in terms of fresh weights of radicles. The highest radicle fresh weights were observed in Osmaniye-2005 (3.096 g) and NC-7 (2.913 g) varieties, while the lowest was seen in Georgia Green as 1.61 g. Statistically ($P < 0.05$) there were significant variations among lead doses in terms of their effect of radicle on age weights. The highest radicle fresh weight was seen at 400 mg $Pb(NO_3)_2$ L^{-1} dose as 2.442 g, while the lowest (2.032 g) was seen at the control dose (*Table 1*). It was determined that the effects of variety and lead dose interaction on radicle fresh weights were statistically significant ($P < 0.01$). The highest radicle fresh weight (4.18 g) was obtained from Osmaniye-2005 cultivar and 400 mg $Pb(NO_3)_2$ L^{-1} lead dose, while the lowest weights (1.408 and 1.400 g) were detected from Georgia Green and Sultan at 200 and 100 mg $Pb(NO_3)_2$ L^{-1} doses (*Table 1; Fig. 3*).

Plumule fresh weight (g)

In terms of plumule fresh weight, when the varieties were examined, statistically significant ($P < 0.01$) differences were found among the cultivars. The highest radicle

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fresh weight was observed in Sultan, NC-V-II and Osmaniye-2005 (26.672, 26.627 and 26.141 g) varieties, respectively, while the lowest weights were observed in Florispan (12.675 g) and Georgia Green (14.480 g) varieties (*Table 1*). As seen in *Table 1,* it was determined that increasing lead doses produced significant ($P < 0.01$) different effects on plumule fresh weight. The highest plumula fresh weights (23.112 and 22.563 g) were observed at 400 and 200 mg $Pb(NO_3)_2$ L^{-1} lead doses, while the lowest weights (2.67 and 21.04 g) were determined at 100 mg $Pb(NO_3)_2$ L^{-1} and control treatments (*Table 1*). When the variety and lead dose interactions were examined in *Table 1,* it was observed that the effect interactions on plumula fresh weights were statistically very significant ($p < 0.01$). The highest plumule fresh weights (28.33, 28.26, and 28.32 g, respectively) were observed from NC-V-II, Sultan and Wilson at 100 and 400 mg of $Pb(NO_3)_2$ L^{-1} doses, while the lowest weights (11.601 and 12.011 g) were weighed in Fluorescent and NC-7 varieties from control and 100 mg $Pb(NO_3)_2$ L^{-1} applications (*Table 1; Fig. 4*).

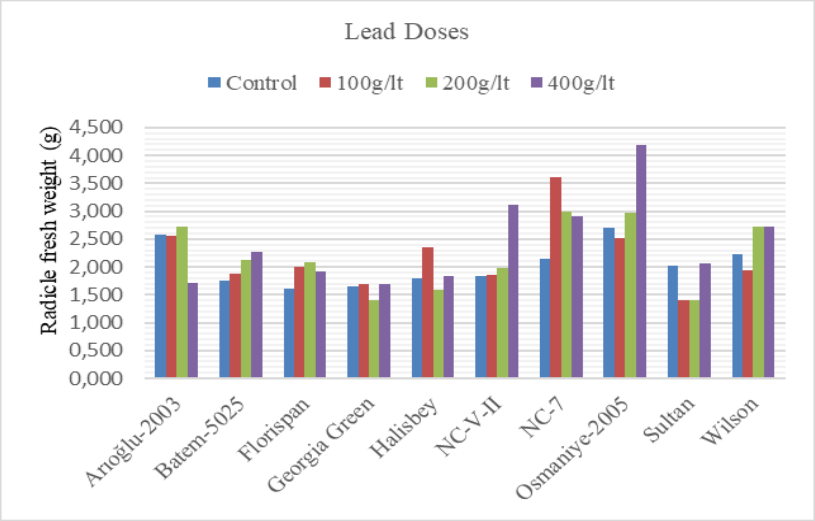

Figure 3. *The interaction of Arachis hypogaea L. varieties' radicle fresh weight*

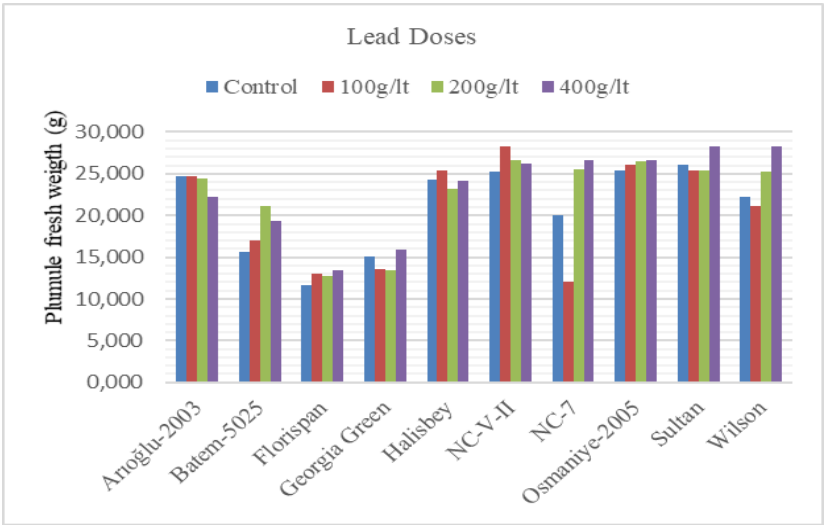

Figure 4. *The interaction of Arachis hypogaea L. varieties' plumule fresh weight*

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Seedling fresh weight (g)

As shown in *Table 2,* the differences among the varieties were found to be statistically significant ($P < 0.01$). The highest seedling fresh weight (29.237 and 28.821 g) was measured in Osmaniye-2005 and NC-V-II cultivars, while the lowest seedling fresh weights (14.581, 16.086, and 20.267 g, respectively) were seen in Florispan, Georgia Green and Batem-5025 cultivars, respectively. There was a statistically significant ($P < 0.01$) variation among the lead doses in terms of their effects on seedling age weights. The highest seedling fresh weights (25.554 and 24.824 g) were obtained from 400 and 200 mg $Pb(NO_3)_2$ L^{-1} lead doses, while the lowest seedling fresh weights (22.850 and 23.071 g) were being observed in 100 mg $Pb(NO_3)_2$ L^{-1} and control applications (*Table 2*). In terms of seedling fresh weights, interactions of varieties and doses have been observed to produce statistically ($p < 0.01$) significant variations. The average fresh weight value of seedlings most affected by stress conditions (13.212 g) was obtained from the control dose of Florispan variety (*Table 2; Fig. 5*).

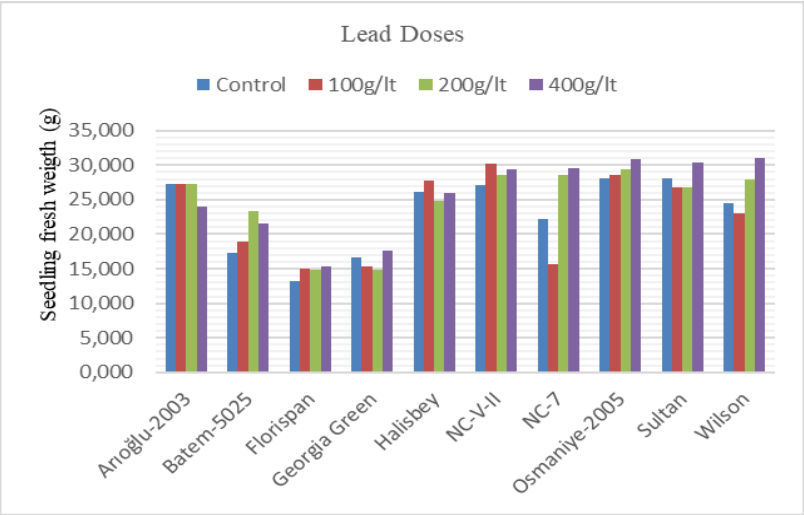

Figure 5. *The interaction of Arachis hypogaea L. varieties' seedling fresh weight*

Radicle dry weight (g)

When the averages of the dry weight of the radicles were examined from the results presented in *Table 2,* it was seen that there were statistically significant ($P < 0.01$) variations among the varieties. The highest radicle dry weight was observed in Osmaniye-2005 variety as 0.549 g, while the lowest dry weight was observed in Batem-5025 variety as 0.373 g. As a result of the analysis of variance, it was determined that lead doses were statistically significant ($p < 0.05$) in terms of their effect on the dry weight of the radicle. The highest radicle dry weights (0.476 and 0.468 g) were obtained from 400 and 200 mg $Pb(NO_3)_2$ L^{-1} doses, while the lowest weights (0.420 and 0.424 g) were determined in the control and 100 mg $Pb(NO_3)_2$ L^{-1} applications (*Table 2*). When the binary interactions were examined in terms of the dry weight of the radicles, it was found that the variety x dose interactions showed statistically significant ($P < 0.01$) variations. The highest radicle dry weight value was obtained from the dual combination of Osmaniye-2005 and 400 mg $Pb(NO_3)_2$ L^{-1} as 0.710 g (*Table 2; Fig. 6*).

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Table 2. *The means and the LSD groups of SFW, RDW, PDW, SDW, SVI, GSN properties of Arachis hypogaea L. varieties*

Varieties	Doses (g L^{-1})	SFW (g)	*;**	RDW (g)	*;**	PDW (g)	**	SDW (g)	**	SVI	*;**	GSN (number)	**
Arıoğlu-2003	Control	27.32	a-e	0.451	c-f	10.85		11.30		688.8	kl	24.33	
	100	27.33	a-e	0.430	c-f	10.11		10.54		710.0	jk	25.00	
	200	27.19	a-e	0.488	b-f	10.19		10.68		681.0	kl	24.33	
	400	23.95	d-f	0.307	f	8.84		9.15		636.1	l-n	23.67	
	Mean	**26.45**	**C**	**0.419**	**CDE**	**10.00**	**C**	**10.42**	**C**	**679.0**	**E**	**24.33**	**AB**
Batem-5025	Control	17.37	g-i	0.381	d-f	6.42		6.80		931.5	a	25.00	
	100	18.86	f-h	0.359	ef	6.43		6.79		953.0	a	25.00	
	200	23.27	ef	0.389	d-f	8.03		8.42		830.0	de	25.00	
	400	21.57	e-g	0.362	ef	6.64		7.00		785.7	fg	25.00	
	Mean	**20.27**	**E**	**0.373**	**E**	**6.88**	**D**	**7.25**	**D**	**875.0**	**A**	**25.00**	**A**
Florispan	Control	13.21	I	0.356	f	5.39		5.75		561.0	op	24.00	
	100	15.01	hi	0.504	b-e	5.22		5.73		806.3	ef	25.00	
	200	14.78	hi	0.486	c-f	4.95		5.44		850.6	cd	24.67	
	400	15.32	hi	0.499	b-f	5.16		5.66		877.9	bc	24.67	
	Mean	**14.58**	**F**	**0.461**	**BC**	**5.18**	**E**	**5.64**	**E**	**774.0**	**C**	**24.58**	**AB**
Georgia Green	Control	16.67	hi	0.412	d-f	7.32		7.73		647.8	l-n	23.67	
	100	15.33	hi	0.390	d-f	6.71		7.10		885.8	b	25.00	
	200	14.79	hi	0.320	f	6.28		6.60		621.5	n	25.00	
	400	17.56	gh	0.404	d-f	6.85		7.26		804.2	ef	24.67	
	Mean	**16.09**	**F**	**0.382**	**DE**	**6.79**	**D**	**7.17**	**D**	**739.8**	**D**	**24.58**	**AB**
Halisbey	Control	26.11	de	0.511	b-d	12.26		12.77		426.9	u	24.67	
	100	27.73	a-d	0.463	c-f	11.04		11.50		452.5	st	24.67	
	200	24.80	de	0.437	c-f	11.63		12.07		377.3	v	25.00	
	400	26.02	de	0.515	b-d	11.97		12.48		484.7	r	25.00	
	Mean	**26.16**	**C**	**0.482**	**ABC**	**11.72**	**B**	**12.21**	**B**	**435.4**	**I**	**24.83**	**AB**
NC-V-II	Control	27.11	b-e	0.379	ef	11.54		11.92		518.6	q	23.33	
	100	30.18	a	0.493	b-f	11.86		12.35		715.5	jk	24.67	
	200	28.60	a-d	0.461	c-f	11.88		12.34		633.6	mn	23.67	
	400	29.39	a-c	0.657	ab	11.59		12.25		712.1	jk	25.00	
	Mean	**28.82**	**A**	**0.498**	**AB**	**11.72**	**B**	**12.22**	**B**	**645.0**	**F**	**24.17**	**AB**
NC-7	Control	22.19	ef	0.332	f	11.34		11.67		431.7	tu	21.67	
	100	15.61	hi	0.437	c-f	11.38		11.82		651.7	lm	24.33	
	200	28.51	ab	0.564	bc	12.10		12.66		736.0	ij	25.00	
	400	29.56	a-d	0.433	c-f	10.69		11.12		770.2	gh	24.00	
	Mean	**23.97**	**D**	**0.442**	**BCD**	**11.38**	**B**	**11.82**	**B**	**647.4**	**F**	**23.75**	**B**
Osmaniye-2005	Control	28.07	a-d	0.461	c-f	12.29		12.75		460.7	s	20.00	
	100	28.64	a-d	0.453	c-f	13.09		13.55		471.4	rs	23.00	
	200	29.42	a	0.572	bc	13.04		13.61		579.9	o	22.67	
	400	30.81	ab	0.710	a	12.19		12.90		627.2	n	22.00	
	Mean	**29.24**	**A**	**0.549**	**A**	**12.65**	**A**	**13.20**	**A**	**534.8**	**gh**	**21.92**	**C**
Sultan	Control	28.12	a-d	0.442	c-f	13.11		13.55		526.5	q	25.00	
	100	26.79	c-e	0.308	f	12.59		12.90		369.0	v	25.00	
	200	28.96	a-d	0.404	d-f	14.75		15.16		537.4	pq	24.00	
	400	30.31	a	0.412	d-f	12.94		13.36		562.3	o	24.67	
	Mean	**28.55**	**AB**	**0.391**	**DE**	**13.35**	**A**	**13.74**	**A**	**498.8**	**H**	**24.67**	**AB**
Wilson	Control	24.53	de	0.474	c-f	10.78		11.26		848.6	d	25.00	
	100	23.01	ef	0.404	d-f	10.08		10.49		664.5	l	24.00	
	200	27.93	a-d	0.557	bc	11.76		12.31		924.3	a	24.33	
	400	31.05	a	0.463	c-f	11.60		12.06		752.0	hi	24.67	
	Mean	**26.63**	**BC**	**0.475**	**BC**	**11.06**	**B**	**11.53**	**B**	**797.4**	**B**	**24.50**	**AB**
LSD (0.05) for V		**2.032**		**0.069**		**0.808**		**0.821**		**16.05**		**1.12**	
LSD (0.05) for LD		**1.285**		**0.043**		**0.511**		**0.519**		**10.15**		**0.71**	
LSD (0.05) for V x LD		**7.040**		**0.238**		**2.800**		**2.844**		**55.61**		**3.86**	
CV (%)		**10.386**		**18.874**		**9.871**		**9.602**		**2.98**		**5.67**	

SFW: Seed Fresh Weight, RDW: Radicle Dry Weight, PDW: Plumule Dry Weight, SDW: Seed Dry Weight, SVI: Seedling Vigor Index, GSN: Germinated Seed Number, V: Varieties, LD: Lead doses

*The mean in the same column, expressed in lowercase and indicated with different letters, is statistically different from each other within the $P \leq 0.05$ error limits according to LSD test

**The mean in the same column, expressed in capital and indicated with different letters, is statistically different from each other within the $P \leq 0.05$ error limits according to LSD test

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Plumule dry weight (g)

As can be seen from *Table 2,* when the averages of plumule dry weights were examined, it was determined that there were very significant variations among the varieties as statistical ($P < 0.01$), while differences between lead doses were found to be insignificant ($p > 0.05$) in terms of their effects on plumule dry weights. The highest plumule dry weights were seen in Sultan (13.349 g) and Osmaniye-2005 (12.653 g) cultivars, while the lowest plumule dry weight was seen in Florispan variety as 5.182 g.

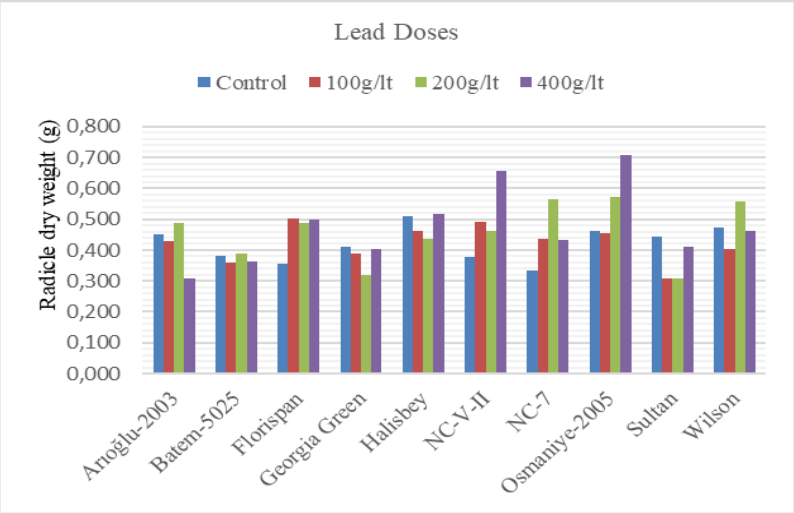

Figure 6. *The interaction of Arachis hypogaea L. varieties' radicle dry weight*

Seedling dry weight (g)

When the averages of seedling dry weights were examined from *Table 2,* it was found that there were statistically significant differences ($P < 0.01$) among the varieties, whereas the differences in lead doses were statistically insignificant ($P > 0.05$). The highest seedling dry weights were seen in Sultan (13.740 g) and Osmaniye-2005 (13.202 g) varieties, while the lowest seedling dry weight was seen in Florispan variety as 5.644 g.

Seedling vigor index

When the averages of seedling vigor indices were examined from *Table 2,* it was found that the differences among the varieties and the differences among the lead doses were statistically significant ($P < 0.01$). According to the results, Batem-5025 varieties had the highest seedling vigor index (875.05), while Halisbey varieties had the lowest seedling vigor index (435.37). The highest seedling vigor index (701.24) was detected at 400 mg $Pb(NO_3)_2$ L^{-1} lead dose, while the lowest seedling vigor index (604.27) was determined from the control application (*Table 2*). Due to the different reactions of the varieties to the lead doses, the effect of variety x dose interaction on seedling vigor indices was found to be statistically significant ($P < 0.01$). It was determined that Sultan variety with the lowest tolerance to lead doses had lowest seedling vigor index (369.05) at 100 mg $Pb(NO_3)_2$ L^{-1}, while Halisbey variety (377.33) was produced lowest seedling vigor index at 200 mg $Pb(NO_3)_2$ L-1 dose (*Table 2; Fig. 7*).

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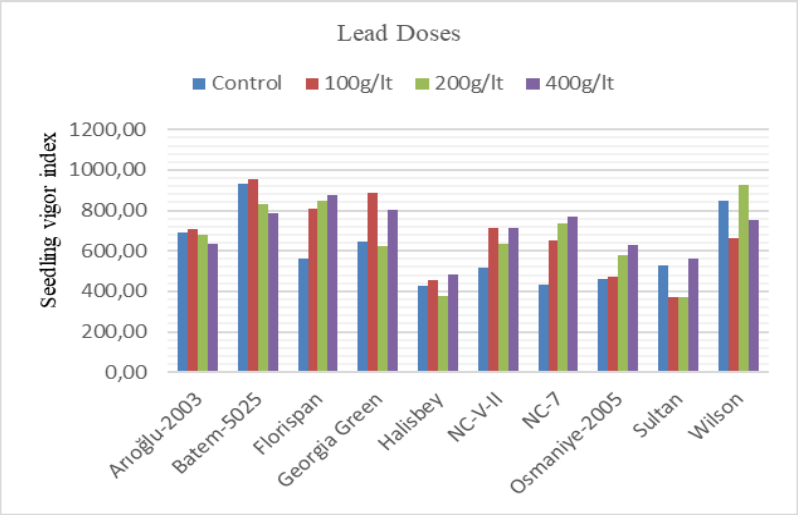

Figure 7. *The interaction of Arachis hypogaea L. varieties' seedling vigor index*

Germinated seed number (number)

In *Table 2,* when the average of germinated seed number, it was seen that there were significant variations ($P < 0.01$) among the varieties and it was determined that the differences between lead doses were insignificant ($P > 0.05$). The highest germinated seed number was observed in Batem-5025 cultivar as 25.00, while the lowest number was obtained in Osmaniye-2005 cultivar as 21.92.

The relationships among the observed characteristics

In the study, a correlation analysis was conducted to reveal the relationships between all investigated properties of peanut varieties subjected to lead concentrations, an abiotic stress factor. The correlations among the all investigated properties are thought to be significant ($P < 0.01$ and $P < 0.05$) due to the widespread and complex lead interactions occurring in the plant organs of different cultivars (*Table 3*). As presented in *Table 3,* it was found that germination percentage, one of the most important attribute, had significant positive correlations ($r = 0.689$ and $r = 0.273$) with the germination index and germinated seed number, but had significant negative correlations ($r = -0.233$ and $r = -0.232$) with germination seed dry weight and seedling vigor index. *Table 3* can be examined for the positive and negative relationship among other characteristics.

Discussion

Germination is the first event in a plant's life and is started by the regulation of enzymatic reactions that activate catabolic and anabolic processes in storage tissues and on the embryonic axis, respectively (Lamhamdi et al., 2011). Germination will be prevented even if a single component of these processes is influenced. Therefore, it is of great concern because of the heavy metal toxicity in the surrounding area and the effects of the abiotic stress conditions on plant growth. Plants under stress conditions are likely to be negatively affected by high concentrations of heavy metals. One known explanation of the effect of heavy metals on plant physiology is that this results in a variety of nutritional disorders (Lamhamdi et al., 2011; Sengar et al., 2008). A high

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concentration of lead, one of the most abundant and ubiquitously distributed toxic pollutants (Sharma and Dubey, 2005), may lead to an inhibition in germination, root elongation, seedling development, plant growth, and more than (Sethy and Ghosh, 2013). Although the effect of Pb varies depending on concentration, soil type, soil properties, and plant species (Lakshmi, 2014), Pb toxicity causes a reduction of germination percentage, length and dry mass of roots and shoots (Munzuroglu and Geckil, 2002).

Table 3. *Correlation table for observed parameters of Arachis hypogaea L. samples*

	GI	RL	PL	SL	RFW	PFW	SFW	RDW	PDW	SDW	SVI	GSN
GP	0.689 **	-0.172	0.163	0.072	0.164	-0.171	-0.130	-0.145	-0.024	-0.233 *	-0.232 *	0.273 **
GI		-0.820 **	0.450 **	0.173	0.442 **	-0.126	-0.471 **	-0.465 **	-0.080	-0.576 **	-0.573 **	0.568 **
RL			-0.479 **	-0.192 *	-0.474 **	0.022	0.510 **	0.489 **	0.078	0.578 **	0.575 **	-0.537 **
PL				0.234 *	0.925 **	0.282 **	-0.343 **	-0.293 **	0.321 **	-0.449 **	-0.432 **	0.764 **
SL					0.585 **	0.081	-0.005	0.006	0.062	-0.242 **	-0.237 **	0.523 **
RFW						0.267 **	-0.288 **	-0.242 **	0.292 **	-0.469 **	-0.453 **	0.841 **
PFW							0.324 **	0.432 **	0.703 **	0.328 **	0.352 **	0.082
SFW								0.993 **	0.289 **	0.827 **	0.829 **	-0.373 **
RDW									0.362 **	0.829 **	0.834 **	-0.345 **
PDW										0.260 **	0.296 **	0.091
SDW											0.999 **	-0.568 **
SVI												-0.559 **

**: Correlation is significant at the 0.01 level. Pearson Correlation

* : Correlation is significant at the 0.05 level.

In this study, the effect of increased lead doses on germination and seedling growth parameters were investigated. As seen in *Tables 1, 2* and *Figures 1-7,* lead concentrations had statistically significant different effects on some observed attributes such as radicle length, seedling length, radicle fresh weight, plumule fresh weight, seedling fresh weight, radicle dry weight, and seedling vigor index in peanut varieties. Increased lead doses have been found to cause an increase in the properties studied in some varieties, while a decrease in others. It was determined that peanut varieties react differently to lead concentrations due to their different genotypic structures and that lead doses in some of them do not have any significant effect. In a few varieties' germinations and growth parameters observed were suppressed differentially at all lead treatments. In some varieties, many features have been found to be promoted differently in some lead treatments. Some previous studies have also reported ineffective or positive effects of lead doses up to a certain level on germination parameters in some plants Zaier et al. (2010), however, Pb has often been reported to have negative effects on most plant species (Hussain et al., 2013; Mishra and Choudhuri, 1998; Pourrut et al., 2011; Sethy and Ghosh, 2013). In the present study, as a result of the use of 10 different genotypic varieties of peanut, both positive and negative effects of Pb were determined on the varieties. Similar to the results determined in this study, Islam et al. (2007) reported that at higher concentrations, lead accelerates germination and also causes adverse effects on the length of the radicle and hypocotyl in *Elsholtzia argyi*. Additionally, the results were similar to those of (Xiong, 1998), who studied lead uptake and tolerance of *Brassica pekinensis*. Plant species vary in the level of tolerance to elements for growth but may cause excessive mineral toxicity. These differences can be attributed to variable ion translocation to plant organs. The results of the present

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findings appear to be a useful indicator for determining the toxic nature and tolerance indices of Pb in *Arachis hypogaea*. Plants differ in their tolerance level to metal stresses. This information can be considered to be a step that contributes to discovering and finding the tolerance limit of *Arachis hypogaea* in lead treatments at different concentrations.

Conclusion

Traditional breeding practices and methodologies of plants have not been successful in providing resistance to different biotic and abiotic stresses due to species restrictions in the natural system. As a result, considering that the germination percentages of some peanut varieties give the highest values, especially at 200 mg L^{-1} lead dose. But, Batem-5025 variety was found to be the most tolerant variety against lead poisoning in terms of germination rate, germination index and importantly seedling vigor index. It is thought that peanut varieties have different mechanisms at the cell level and that these plants work simultaneously with multiple mechanisms, and that further investigation is needed to reveal clearer Pb tolerance and hyperaccumulation. Likewise, among the peanut varieties that have been taken into the test, other breeding studies should be carried out for varieties that show the most resistance to lead toxicity.

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EFFECTS OF PRESCRIBED BURNING ON PINE WOOD NEMATODE (*BURSAPHELENCHUS XYLOPHILUS)*

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Abstract. *Bursaphelenchus xylophilus* became one of the most damaging forest pests in recent years in Hunan province, and caused remarkable losses of pine tree. In order to explore the effect of fire disturbance on the prevention of *Monochamus alternatus* and *B. xylophilus.* forests dominated by *Pinus massoniana* were taken as the object of the study in Daolin town, Ningxiang city, Hunan province, China. Three kinds of forest were selected, such as healthy forest of *Pinus massoniana* (Control Ⅰ), infected forest without prevention(Ⅱ), and infected forest with prevention(Ⅲ). Then, 6 plots were set in every object forest. Three were the control plot, and the other three were treated with prescribed burning. We continuously investigated the number of *M. alternatus* and *B. xylophilus* before and after low intensity prescribed burning. The results indicated that the quantity of female *M. alternatus* in the three kinds of fire disturbance plots were reduced by 88.24%, 75.00% and 94.74%, respectively, while the males were reduced by 77.78%, 81.82% and 88.89% before and after the fire. There were significant differences for the quantity of *M. alternatus* before and after the prescribed burning (P<0.05). The results showed that the prevention efficacy of Ⅱ-2 was the best. Within 10 to 20 days after the fire, the quantity of *B. xylophilus* carried by *M. alternatus* changed significantly. The average quantity of *B. xylophilus* carried by female and male *M. alternatus* dropped from 1135 to 6 and from 397 to 35, respectively. It showed that prescribed burning could reduce the population density *of M. alternatu*s and carrying quantity of *B. xylophilus* of each *M. alternatus*. So prescribed burning could control the spread of pine wilt disease and prevent its outbreak. Prescribed burning has an important theoretical and practical significance in pine wilt disease prevention.

Keywords: *Monochamus alternatus, Bursaphelenchus xylophilus, pine wilt disease, fire disturbance, Pinus massoniana*

Introduction

Pine wood nematode disease, also known as pine wilt disease, is a devastating disease of pine. It can damage more than 50 species of pinus and a few non-pinus species and create huge economic losses (Yang et al., 2003; Gu et al., 2005; Pan, 2011; Lee et al., 2013). *M. alternatus* is the main vector of *B. xylophilus*. Nematodes invade healthy pine trees by feeding of adult beetles and by laying eggs of female beetles. Once the pine tree was infected, it died in a short time (Pajares et al., 2010; Teale et al., 2011; Chen et al., 2012; Shi et al., 2019). Studies had shown that *B. xylophilus* moves to the surface of *M. alternatus* before they fly out of the diseased trees, then it enters the body of *M. alternatus* through the ventral valve (Aikawa, 2008; Chen and Tu, 2012). Therefore, it is very important to control the transmission of pine wood nematode disease.

At present, physical, chemical and biological methods, such as trapping device, black light, wood trapping and killing were mostly used to control the population density of *M. alternatus* and *B. xylophilus* (Sanchez-Husillos et al., 2015; Zhu et al., 2017). Contact stomach toxicity, internal absorption, and agents with strong permeability, such as

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thiophosphorus thiaziamprid, chloramine, phosphorescent milk, etc. were generally used in chemical control like artificial and aircraft spray or trunk injection agent (Zhang et al., 2010; Zhan, 2014).

Biological control was dominated by the release of *Metarhizium anisoplia* and *Beauveria bassiana* as well as natural enemies (such as *Dastarcus helophoroides* and *Sclerodermus* SPP.) (Liu et al., 2007; Yang et al., 2013; Luo et al., 2015). Although scholars had done a lot of research and made great progress in the control of *M. alternatus* and pine wilt disease, there were some limitations in various control theories and technologies. It was difficult to popularize physical control in large areas. Chemical control caused secondary pollution and had a high cost. Biological control was less effective (Chou, 2015). Therefore, in order to prevent the occurrence and spread of pine wilt disease, exploring effective control technologies is necessary. This experiment attempted to control the population density of the vector insects of pine wilt disease by prescribed burning.

Prescribed burning refers to using low-intensity fire to remove undergrowth fuel at certain ecological conditions by planned and purposeful artificial control. It is a technique that achieves certain desired results for one or more goals, such as preventing forest fire, promoting natural regeneration, improving wildlife feed sources and reducing forest pests and diseases. Prescribed burning is an important measure for the healthy management of plantation, which is to take advantage of forest fire and reduce the source of diseases and pests in fuels and dead leaves.

Studies in home and abroad had shown that prescribed burning can reduce large-scale outbreaks of insect pests, and it is now used as a strategy to prevent the occurrence of trunk borer pests (Cui and Tian, 2010). Wang found that it was suitable for *Dendrolimus superans* and *Clostera anastomosis* prevention by low intensity prescribed burning (Wang, 2005). The population density of *D. superans* decreased by 1.793 times in prescribed burning plot compared to control plot, and the rate of trees infected by *C. anastomosis* decreased by 78.56 percentage. Cha et al. (2019) researched the control of *Mycosphaerellalaric-leptolepis* Ito et al. by prescribed burning. Prescribed burning reduced the fallen leaves which were infected by diseases. It can achieve the purpose of reducing and controlling the disease, and the effect of prevention was obvious, the cost of prevention was significantly reduced. Futai (2013) used the technology of prescribed burning to control grey spot, poplar leaf rust and poplar rot, and the average control effect were 76.9%, 83.7% and 79.02, respectively. It showed that the effect was remarkable, and the cost of prescribed burning was 20 times lower than that of chemical control. Han et al. (2003) used prescribed burning to prevent forest diseases, pests and mice in Xiaoxing'an Mountains. The results showed that prescribed burning had a good effect on the pest and leaf disease.

More and more attention had been paid to the research on the effects of prescribed burning on forest pests and diseases. It is well known that high intensity fire wreaks forest ecosystems, while low-intensity fire reduces litter, the combustibility of stand and the source of diseases and insects in the forest. Low-intensity fire promotes the sustainable development of forest ecosystems.

In this study, the technology of low-intensity prescribed burning was set up in the experimental stands. In order to find a new prevention and control technology, we want to study the effect of fire disturbance on the population density of *M. alternatus* and pine wilt disease. It has great significance in the theoretical and technical innovation of pine wilt disease prevention and control.

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Materials and methods

Study area

The experiment plot was located in Ningxiang County, Hunan province, with a latitude of 27°55′N to 28°06′N and a longitude of 112°39′E to 112°47′E. It belongs to Hilly landform, the terrain is higher in the northwest and southeast, while there is an alluvial plain in the middle (Huang, 2014). It is characterized by mainland monsoon climate. The alternation of warm and cold air are more obvious, with four distinct seasons. The average annual temperature and the average annual precipitation are about 16.6℃ and 1384.2 mm, respectively. The area is dominated by subtropical evergreen broadleaved forests with a forested cover of 42%. The coniferous forests consist of *Chinese fir* (*Cunninghamia lanceolata* [Lamb.]) and *Pinus massoniana* primarily, and broadleaved forests are dominated by *Phyllostachys heterocycla*, *Schima superba* and *Liquidambar formosana*. These were grouped into pure coniferous, pure broadleaved, and mixed forests. The economic forests are *Citrus reticulata*, *Camellia oleifera*, *Vernicia fordii* and *Sapium sebiferum*.

Experimental design

The trapper consists of an umbrella cover, a circular funnel, a cross baffle and a bug barrel. Attractants (APF-Ⅰ) and the trapper were produced by Fujian Chenkang agricultural and forestry technology co. LTD. In addition, various instruments and materials, such as anatomic tools, gauze, distilled water, hydro extractor, dropper, microscope slide, cover slide and microscope were used in the experiment.

All the plots were located in the middle slope, with a latitude of 28°1′N and a longitude of 112°44′E. The average height of the stand was 7 to 8.5 m, the average diameter at breast height of the stand was 18 to 24 cm, and the average age was 28 to 30 years and the canopy closure was 0.8. There were about 55 *Pinus massoniana* trees in each plot. Three kinds of stands, such as healthy forest (Ⅰ), infected forest without prevention (Ⅱ) and infected forest with prevention (Ⅲ), were selected in the experimental area on 11th April , 2019. Infected forest without prevention meant that there were infected trees in the stands but the infected trees were not removed. Infected forest with prevention meant that there were infected trees, which were cut down and burned. Medicine was applied on the cut hill.

Six plots were set in each experimental stand (*Figure S1*). The control plot and prescribed burning plot were repeated 3 times, so 18 plots were set up and individually numbered in *Figure 1*.

The distance between every two experimental stands was more than a 1000 m, and the distance between each group of plots was at least 50 m. The size of each plot was 30 m×30 m. The stand was a residual stand of *Pinus Massoniana* plantation after being destroyed. There were few trees in understory and shrub layer. A few other broad-leaved tree species were scattered in forest gaps.

The dead tree of pine wilt disease in infected forest was diagnosed with forest symptom. The needles of the whole crown become reddish brown, hanging upside down on the branches, and it was like fire from distant view. Then, we numbered all the dead trees. The prescribed burning plots (Ⅰ-2, Ⅱ-2 and Ⅲ-2) were cleaned up (*Figure S2*). In order to prevent the formation of forest fire weeds, shrubs and branches were cut down (branches of the trunk less than 2.5 m) and a control line with at least 10 m width was set around the prescribed burning plot.

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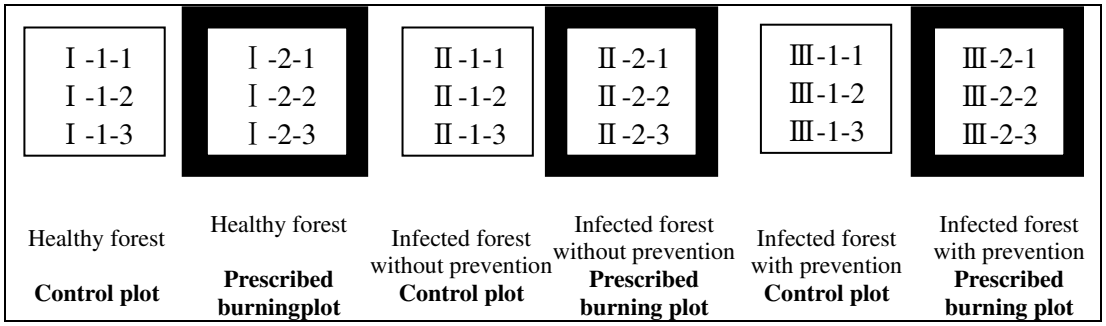

Figure 1. *Experimental design. Note: The black frame was the control line of prescribed burning plot, all of which were 10 m width*

Trappers set up and adult insect observation

In May, 2019, we began trap experiments in 18 plots. The trappers were numbered and hung in the middle of each plot (*Figure S3*). In order to maintain air circulation, weeds, shrubs and branches (which were less than 2.5 m) around the trapper were removed. The trapper was suspended from side branch of the pine tree and the bottom of the trapper was 1.5 m above the ground. To avoid collecting water of the insect bucket, 8 drain holes about 2 mm were drilled at the bottom of the insect bucket. The number of adult beetles of *M. alternatus* were recorded every 10 days during the trapping period, it included the number of females, males and the total *M. alternatus* (*Figure S4*). Numbers of *M. alternatus* which carried *B. xylophilus* were recorded. The number of *B. xylophilus* were recorded, it included the average number of *B. xylophilus* carried by females, males and the total. The attractant was replaced every 20 days.

Experiment of prescribed burning

When it was sunny continuously for five days, and there was no strong wind, we carried out prescribed burning in the non-control plots during the emergence period of *M. alternatus*. Prescribed burning was carried out in the early morning or late afternoon on the 28th June, 2019 (*Figure S5*). The wind direction and wind speed were measured before ignition, then the portable oil-drop igniter was used to light the fire every 1 m in the plot. The fire line was perpendicular to the wind direction, and the combustion effect was checked after the fire.

Identifying the female and male M. alternatus, B. xylophilus isolation

(1) Male and female *M. alternatus* were identified based on the tentacles.

(2) Isolation procedure of *B. xylophilus*: According to the principle of Baermann funnel method, an experiment was designed to isolate nematodes (Cai and Jiang, 2003). We cut up the adult beetle and wrapped debris with 2 layers of gauze, then placed it in a 10 ml centrifuge tube to which 7 ml distilled water was added. The gauze covering the fragments of *M. alternatus* was completely suspended in water (*Figure 2*). It was put aside for 24 hours, then the gauze was removed from the centrifuge tube by forceps. We placed the centrifuge tube in a centrifuge. The supernatant was centrifuged at 3000 R/Min for 3 min, then the supernatant was discarded.

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Figure 2. *Bursaphelenchus xylophilus isolation. Note: Laboratory site: Laboratory of Forestry College in Central South University of Forestry and Technology (112°59'35"E, 28°8'15"N)*

(3) Counting *B. xylophilus*: The supernatant was stabillized to 1.5 ml with distilled water and fully shaken to absorb 0.1 ml with a pipette, the liquid was put on a microscope slide, then it was covered by a cover slide. *B. xylophilus* was observed under microscope and counted, the procedure was repeated 5 times. The number of *B. xylophilus* carried by each *M. alternatus* were calculated.

$$A = b \times 15 \qquad \text{(Eq.1)}$$

where, A is the numbers of B. xylophilus (per adult) and b is the average of five observations (per 0.1 ml).

M. alternatus were collected every 10 days from the 23rd May to the 18th July, 2019. *B. xylophilus* carried by *M. alternatus* were isolated and counted according to the above methods.

Statistical analysis

Professional software of Excel 2010 and SPSS 23 were used for data processing. One-way ANOVA of SPSS was used for variance analysis and Duncan's new complex range test was used to compare the difference levels between each treatment.

Results and discussion

Quantity of M. alternatus at different time nodes

The quantity of *M. alternatus* adults in 18 plots before and after the fire were analyzed. The quantity of *M. alternatus* in all kinds of plots before and after the fire disturbance were few, so *M. alternatus* in three kinds of plots were combined for statistical analysis. As time went on after the fire disturbance, *M. alternatus* decreased significantly. It showed that the quantity of *M. alternatus* in 20 days after the fire were lower than that of 10 days after the fire, and the quantity of *M. alternatus* in 10 days after the fire were lower than that of before the fire. There were significant differences of *M. alternatus* in stand at different time nodes ($P<0.05$) (*Figure 3a*).

The results indicated that the quantity of female *M. alternatus* in three kinds of fire disturbance plots were reduced by 88.24%, 75.00% and 94.74%, respectively, while the male reduced by 77.78%, 81.82% and 88.89% before and after the fire. The difference in numbers between males and females were unanimous. The quantity of female *M. alternatus* were larger than male at different time nodes, but there were no significant differences (*Figure 3b*).

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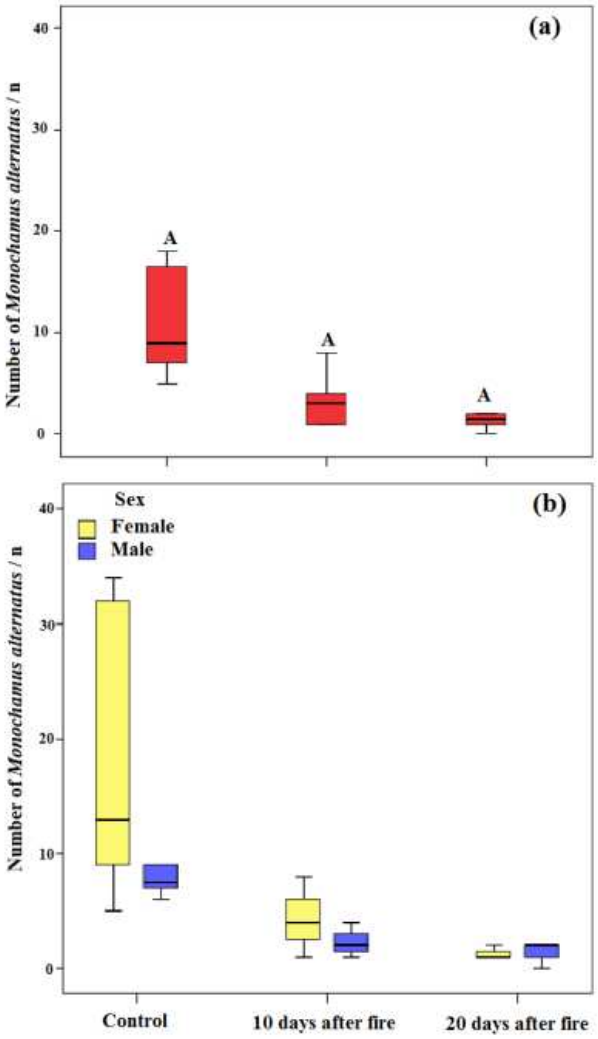

Figure 3. *Changing trend of quantity of M. alternatus at different time nodes. Note: The same letters represent significant differences (One-Way ANOVA of variance, Fish LSD of test, p<0.05). The notes in following figures were the same as in Figure 3*

B. xylophilus at different time nodes in healthy forest

In stands (I), the quantity of *B. xylophilus* at different time nodes decreased with the passage of time after the fire (10 days and 20 days) (*Figure 4a*). Within 10 to 20 days after the fire, *B. xylophilus* changed significantly (P=0.03). However, there were no significant differences of *B. xylophilus*in 10 days after the fire comparing with before and 20 days after the fire. *B. xylophilus* carried by female and male *M. alternatus* decreased with the passage of time after the fire. *B. xylophilus* carried by male *M. alternatus* changed significantly before and after the fire (P<0.05), but there were no significant differences between 10 days after the fire and 20 days after the fire. *B. xylophilus* carried by female *M. alternatus* showed significant differences between 20 days after the fire (6.33±1.97) and before the fire (1209.14±58.07) (P=0.01), and *B. xylophilus* 20 days after the fire were significantly lower than 10 days after the fire (1031.14±50.75, P=0.04) (*Figure 4b*).

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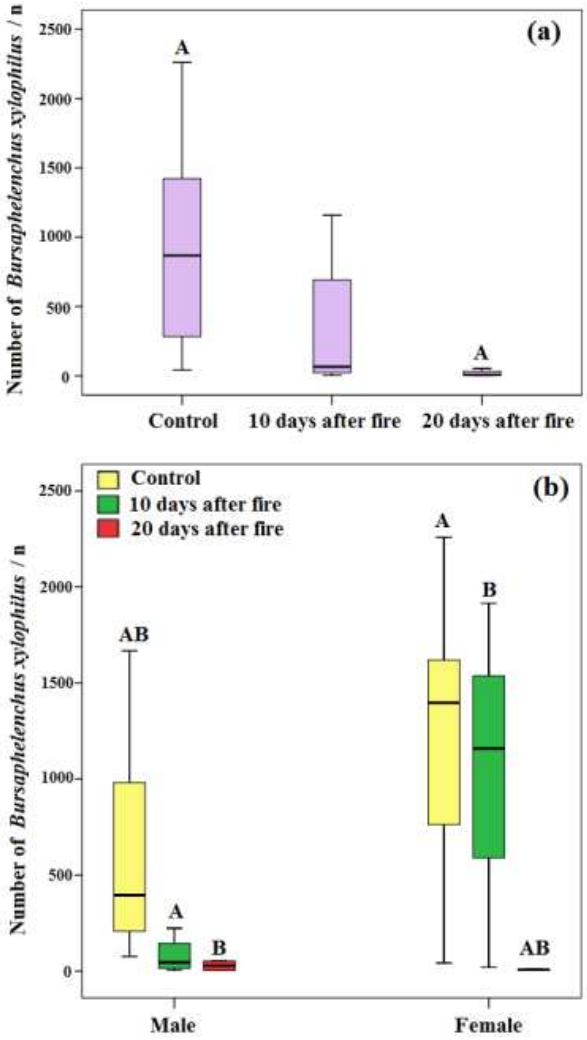

Figure 4. *Significance of B. xylophilus at different time nodes in healthy forest*

B. xylophilus at different time nodes in infected forest without prevention

In stands (Ⅱ), the quantity of *B. xylophilus* 10 days after the fire were significantly higher than in other time nodes (P<0.05), while there was no significant difference between 20 days after and before the fire (*Figure 5a*). *B. xylophilus* carried by female and male *M. alternatus* had the same change trend at different time nodes. *B. xylophilus* carried by male *M. alternatus* were significantly different at different time nodes. 10 days after the fire (316.00±74.17) the number of *B. xylophilus* were significantly larger than that before the fire (119.23±6.77), and 20 days after the fire (54.50±16.53) *B. xylophilus* decreased significantly. *B. xylophilus* carried by female *M. alternatus* were significantly different at different time nodes consistently with male *M. alternatus*. Compared with before the fire, *B. xylophilus* increased significantly 10 days after the fire (P=0.007) and decreased significantly 20 days after the fire (P=0.003). Within 10 to 20 days after the fire, *B. xylophilus* carried by female and male *M. alternatus* in prescribed burning plots were significantly lower than before the fire. The results indicated that prescribed burning could control the spread of pine wilt disease, but the effects do not appear immediately after the fire (*Figure 5b*).

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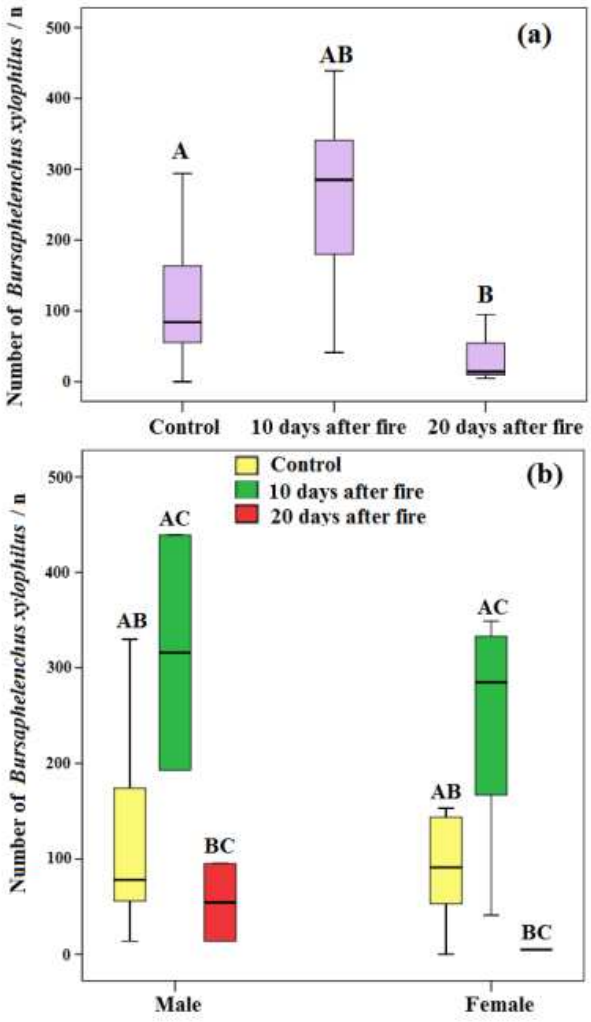

Figure 5. *Significance of B. xylophilus at different time nodes in infected forest without prevention*

B. xylophilus at different time nodes in infected forest with prevention

In stands (Ⅲ), changes of *B. xylophilus* were basically the same as in other stands (Ⅰ) (*Figure 6a*). *B. xylophilus* decreased with the passage of time after the fire in both female and male *M. alternatus*. *B. xylophilus* carried by male *M. alternatus* were significantly lower 20 days after the fire than 10 days after (P=0.012), and before the fire (P=0.000), while *B. xylophilus* did not decrease significantly 10 days after the fire. *B. xylophilus* carried by female *M. alternatus* before the fire were significantly larger than that of 10 days after the fire (P=0.031), and *B. xylophilus* carried by female *M. alternatus* 10 days after the fire were significantly larger than that of 20 days after the fire (P=0.000). There were significant differences among different time nodes (P<0.05) (*Figure 6b*).

Effectiveness of B. xylophilus prevention by prescribed burning

In three kinds of stand, within 10 to 20 days after the fire, *B. xylophilus* carried by female and male *M. alternatus* reduced significantly (carried by female: P=0.01; carried

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by male: *P*=0.022). The average quantity of *B. xylophilus* carried by female and male *M. alternatus* dropped from 1135 to 6 and from 397 to 35, respectively. It showed that prescribed burning could reduce the population density *of M. alternatu*s and quantity of *B. xylophilus* carried by *M. alternatus*. So it is beneficial to use prescribed burning to control the spread of pine wilt disease and prevent its outbreak (*Table 1*). This was consistent with the findings of Wang et al. (2019) that the amount of *B. xylophilus* carried by female *M. alternatus* were about 1.29 times of male adults.

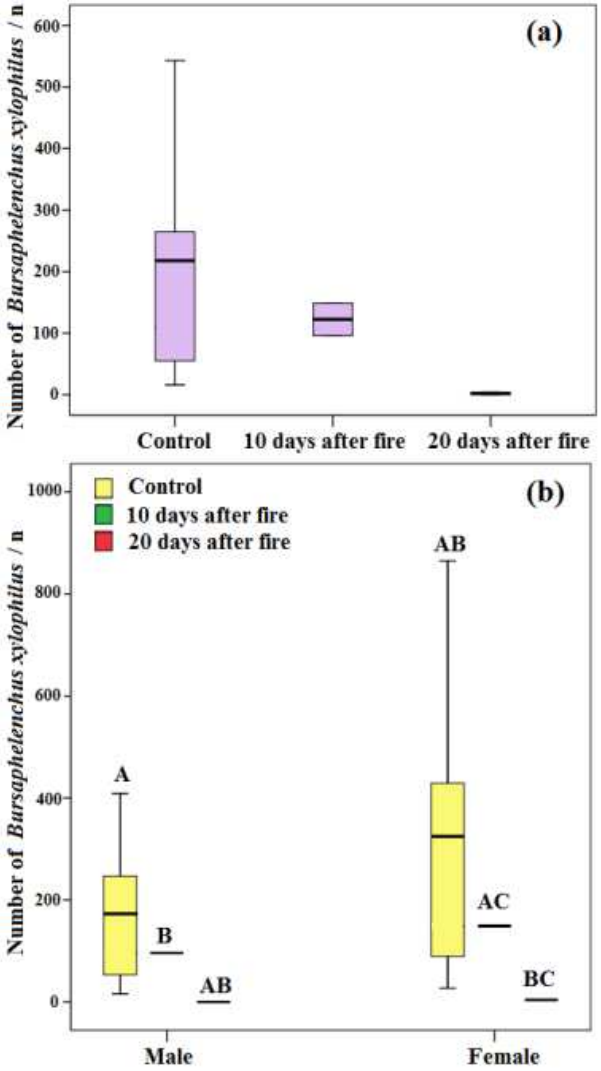

Figure 6. *Significance of B. xylophilus at different time nodes in infected forest with prevention*

Comparison of prevention effectiveness by prescribed burning

The mean number of *M. alternatus* in three kinds of stand were analyzed before and after the fire, the results showed that the quantity of *M. alternatus* in three kinds of fire disturbance plots were reduced, but only stands Ⅱ-2 and Ⅲ-2 reduced significantly (Ⅱ-2: *P*=0.012, Ⅲ-2: *P*=0.026). So it indicated that the prevention efficacy of fire disturbance in plots Ⅱ and Ⅲ were significantly better than in plot Ⅰ, and the prevention efficacy of Ⅱ-2 was the best (*Figure 7*). Whether it was the high

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temperature that killed the pupa in the xylem of *Pinus massoniana* or that killed the emergence of adult beetle, or other mechanisms and the comprehensive effect of various factors need further research.

Table 1. *B. xylophilus carried by female and male M. alternatus before and after the fire*

Plots serial	Trapping time	Female *M.alternatus* (n)	*B.xylophilus* carried by female *M. alternatus* (n)	Male *M.alternatus* (n)	*B.xylophilus* carried by male *M.alternatus* (n)
Ⅰ-2	Before the fire	20	1073	9	420
Ⅱ-2		28	740	8	556
Ⅲ-2		19	1593	8	216
Ⅰ-2	10 ~20 days after the fire	2	7	2	29
Ⅱ-2		1	7	2	55
Ⅲ-2		1	4	2	20

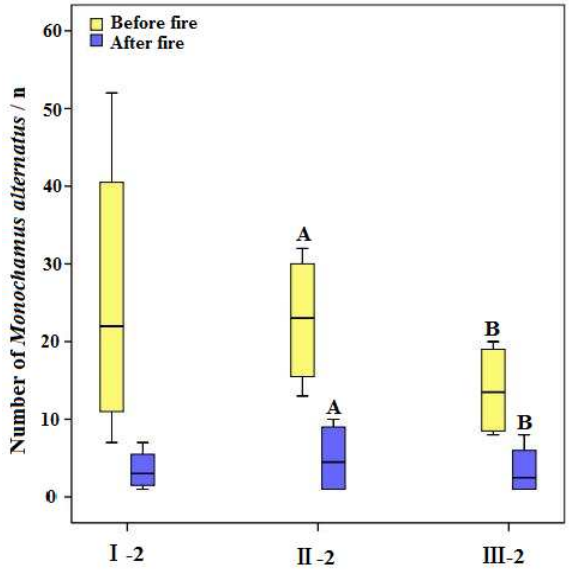

Figure 7. *Significance of M. alternatus in different plots*

Conclusion

In this study, there were no significant changes of *B. xylophilus* carried by female and male *M. alternatus* within 10 days after prescribed burning. However, within 10 to 20 days after prescribed burning, *B. xylophilus* carried by *M. alternatus* decreased significantly. This may be due to that *M.alternatus* in the upper part of the tree were not affected by fire and smoke, so neither *B. xylophilus* carried by *M. alternatus* were affected by fire. However, *M. alternatus* adult beetle, chrysalis and *B. xylophilus* stayed on the lower part of the trunk, thus these were inevitably affected by fire. This explains the sharp drop in the number of *M. alternatus* after prescribed burning, while the reduction in the number of *B. xylophilus* was delayed. In this study, *B. xylophilus* carried by female *M. alternatus* were significantly higher than that by male. The results showed that prescribed burning significantly reduced the population density of *M. alternatus* in *pinus massoniana* forest.

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The control efficacy of prescribed burning in plot (Ⅲ-2) was not as good as in plot (Ⅱ-2), the main reason is as follows: Firstly, it may be due to the occurrence of pine wilt disease. The infected trees had a strong attraction to *M. alternatus*, and the early stage of dead trees of pine wilt disease is often distributed in small groups, so *M. alternatus* are often distributed intensively (Parker et al., 2006). Secondly, the dead trees in the plot (Ⅲ-2) had been cut down and burned on the spot, so the distribution of *M. alternatus* scattered on the plot. The process of burning dead trees released heat and smoke, so *M. alternatus* spread to the surrounding.

It was an innovative research to control pine wilt disease by prescribed burning. The control efficacy is obvious, the cost of control technology is significantly reduced, and the method is simple and easy to operate. It can not only prevent the outbreak of pine wilt disease, but also promotes the transformation of ground litter, reduces combustion of stand and enhances soil fertility. In addition, prescribed burning has low cost, which makes up for the deficiency of existing prevention and control technologies. The technology has broad prospects in application. However, the question of whether high temperature killed the pupa in the xylem of *Pinus massoniana* or it prevented the emergence of adult beetle needs further research along with other questions (such as mechanism and the comprehensive effect of various factors).

Acknowledgements. This study was financially supported by the forestry department of Hunan province research project (No: XLK201923) and the National Natural Science Foundation of China (No: 31470659).

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APPENDIX
SUPPORTING INFORMATION

Figure S1. *Pictures of plot design*

Figure S2. *Pictures of cutting down the weeds, shrubs and branches*

Figure S3. *Pictures of trap experiments*

Figure S4. *Pictures of adult insect observation*

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Figure S5. *Pictures of experiment of prescribed burning*

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AQUEOUS EXTRACT OF MORINGA (*MORINGA OLEIFERA)* LEAF (AEMOL) ON THE GROWTH, SENSORY AND HISTOLOGY PARAMETERS OF BROILER CHICKENS

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Abstract. A completely randomized design experiment was used to determine the effects of aqueous extract of *Moringa oleifera* leaf (AEMOL) on growth, sensory and histology parameters of broiler chickens. Treatment 1 served as the control (antibiotics), Treatment two was given ordinary water ($AEMOL_0$). Treatments 3, 4, 5 and 6 contained 30, 60, 90 and 120 ml of AEMOL per litre of water per day, respectively. Data obtained were analyzed using one-way analysis of variance and mean separation was done using Duncan's test for multiple comparisons. Results showed that the extract influenced ($P < 0.05$) the feed intake and water intake at the broiler starter phase WHILE birds on the control diet had higher values. Finisher phase results showed that final weight, weight gain, feed intake, FCR and water intake were influenced ($P < 0.05$) by the extract with birds treated with 60 ml/l of AEMOL doing better for most parameters except the FCR. All the digestibility and sensory parameters measured were also influenced ($P < 0.05$) by the extract. However, histological parameters measured were not affected ($P > 0.05$) by the extract. It could be concluded that the extract inclusion levels up to 90 ml/l can be used to replace antibiotic growth promoter without compromising the advantages of antibiotic growth promoter.
Keywords: *antibiotics, performance, replace, phytochemical*

Introduction

Broilers are a good source of protein and income to many households and therefore their production attracts the attention of many people. Poultry production remains the most extensive and widely practiced of all livestock enterprises and an important component of socio-cultural and economic development in most countries as well as in food security improvement (Alders, 2005; Dieye et al., 2010). However, the industry in the developing countries is facing some challenges which amongst others include, high feed consumption to gain ratio and high cost of feed due to high prices of feed ingredients (Abbas, 2013). Numerous attempts have been made to overcome these challenges. One of such is the use of antibiotics as growth promoters and to prevent outbreak of diseases (Manyi-Loh et al., 2018), though not without attendant problems such as drug toxicity, residual effects and development of bacterial resistance (Finley et al., 2013). This has led to the ban on the use of antibiotics as growth promoters since

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2006 by the European Union, thereby shifted attention of researchers to safe alternatives such as the use plants/herbs (phytobiotic) in place of antibiotics. Some plants, such as *Moringa oleifera*, has been found to contain phytonutrients and phytochemicals as secondary metabolites which are physiologically active agents with therapeutic properties as antibiotics. Whereas the human medicinal uses of *Moringa oleifera* has been studied for many years its use in livestock production has received little attention (Nouman et al., 2013). Recently, research is focusing on its possible use to enhance growth, and nutrient utilization as a livestock fodder crop (Nouman et al., 2013). Incorporation of this herb and its products in livestock feeds and water instead of synthetic products have resulted in more rapid gain, higher production and better feed efficiency (Portugaliza and Fernandez, 2012). Other reports have indicated that Moringa oleifera leaves have potential prebiotic and antioxidant effects (Siddhuraju and Becker, 2003; Teixeira et al., 2014; Alabi et al., 2017). The underlying effects of the bioactive compounds in M. oleifera leaves are believed to induce prebiotic effects, bacterial and immune-stimulant activities (Ghazalah and Ali, 2008) resulting in increased productivity of broiler chickens.

Although there are several studies on the use of *Moringa oleifera,* however, there are scanty reports on the use of the aqueous *Moringa oleifera* leaf extract in drinking water to determine its impact on growth performance of broilers in poultry production in Minna, Middle Belt Nigeria. The aqueous extract of *Moringa oleifera* leaf is readily available, accessible and cost effective for use by the farmers and households. The present study was therefore designed to determine the effect of graded doses of aqueous *Moringa oleifera* leaf extract on growth performance, sensory and histopathology of broiler chickens and compare such with antibiotic treated broilers.

Materials and methods

This experiment was conducted at the Teaching and Research Farm of the School of Agriculture and Agricultural Technology, Federal University of Technology, Minna, Niger State. Minna lies within latitude 9° 37′ North and longitude 6° 32′ East. The average temperature in Minna is between 19 to 37 °C with an annual rainfall of 1312 mm. The mean annual relative humidity is between 21 and 73% (Climatetemp, 2016).

Sample preparation

The leaves of *Moringa oleifera* were harvested from Minna town and environs. They were air-dried in order to ease pounding and were subsequently ground to powder using a blender. The leaf powder was soaked in water after which the solution was drained using a 1 mm mesh. The chemical composition of the extract was determined.

Experimental design, treatments and procedures

A total number of 240-day old Hubbard broiler chickens were purchased from B-not Harel hatchery Ibadan, Nigeria. The birds were kept under intensive management for eight weeks. The birds were randomly allocated to six aqueous extract of *Moringa oleifera* leaf (AEMOL) treatments. Treatment 1 served as the control (having the antibiotics), while treatment 2 was given ordinary water to serve as $AEMOL_0$. Treatments 3, 4, 5 and 6 contained 30, 60, 90 and 120 ml of aqueous extract of *Moringa oleifera* leaf (AEMOL) per litre per day of water, respectively (*Table 1*). The treatments

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were replicated four times and each replicate had 10 birds. All necessary management requirements were strictly followed. Throughout the study, feed was given *ad-libitum*. Each treatment was fed broiler starter diet between day 1 and 21; and broiler finisher diet between days 22 and 56. Water was provided for 20 h and deprived of water for 4 h while placed on treatment dosages (so that the birds can take the treatment dosages).

Table 1. *Aqueous Moringa oleifera leaf extracts (AEMOL) inclusion levels*

Treatments	Inclusion level
AEMOL $_{0+}$	Control (Gendox® 1.25 mg/l)
AEMOL $_{0}$	0 ml/l
AEMOL $_{30}$	30 ml/l
AEMOL $_{60}$	60 ml/l
AEMOL $_{90}$	90 ml/l
AEMOL $_{120}$	120 ml/l

Growth performance

A known quantity of the feed was given to the chickens daily for a period of four weeks with the left over collected every morning (weighed and recorded) throughout the experimental period. The daily feed intake was obtained by subtracting the left-over quantity from the initial quantity offered the previous days. The feed was weighed using weighing balance (RADWAG). The feed conversion ratio (FCR) was calculated as dry matter intake per unit weight gain.

On day 21, digestibility study was carried out. This involves feeding the animals daily with known quantity of feed. A three-day acclimatization period was allowed prior to a four-day collection period. Droppings voided by each bird were collected on a daily basis at 09.00 am. Care was taken to avoid contamination from feathers, scales, debris and feeds. Total faeces voided by each replicate were collected using a collection tray. The faeces was weighed (wet basis) and oven dried at 85˚C until a constant weight was obtained. At the end of the faecal collection the total dry faeces for each replicate was bulked, mixed and 30% of it was weighed and ground to a size that could pass through a 2-mm sieve for proximate analysis. The difference between the nutrients in the feed consumed and the faeces or nutrients in the faeces voided multiply by 100 gives the apparent digestibility coefficient of the feed, i.e. FC (feed consumed) – FV (faeces voided) X100 = apparent digestibility coefficient of the feed.

Sensory evaluation

Meat samples from the breast meat were taken from two birds randomly selected from each of the replicates of the experimental birds and 5 g of salt was added to the meat before being subjected to boiling and frying. Samples of meat from each treatment were collected after removing the flesh from the bone (manually), cut into chops of an average weight of 40 g and labelled for identification. The meat was cooked in a pot with water at a temperature of 65 °C for 30 min using a gas cooker as described by Vasanthakumar et al. (1999). Twenty trained panelists were used in the assessment procedure. They were instructed to chew a sample from each treatment. The scoring was based on parameters stated on the scoring sheet: for appearance, juiciness, flavour, tenderness and overall acceptability. Water was served to the panelists to rinse their

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mouth after scoring each sample to reduce flavour carryover. The panelists scored each sample on a nine-point hedonic rating scale adopted from Vasanthakumar et al. (1999).

Histopathology

After the completion of the experimental period, the histological analysis was carried out at the histology laboratory of the Faculty of Veterinary medicine, Usman Danfodio University, Sokoto, Nigeria. Two birds per replicate were randomly selected, slaughtered, weighed and examined for respiratory tract gross lesions. Both lungs were removed and weighed. Tissue samples of the liver, lungs, kidney, intestine, spleen and heart were taken and fixed in 10% formalin. The tissues were dehydrated through a graded concentration of ethanol (70, 95 and 100%), cleared in xylene and embedded in paraffin wax. The embedded tissues were stained with hematxylene and eosine for light microscopic examination. Lesions observed were photographed using the Vanox T Olympus photographing microscope as described by Richard et al. (2008).

Lung weight expressed as a ratio of body weight was called the lung: body weight ratio. The thoracic air sacs were assigned gross lesion scores, where 0 = clear, 1 = cloudy, 2 = cloudy with minimal caseous exudate accumulation and 3 = severe caseous exudate accumulation. A 2.5-cm longitudinal hemi-sectioned portion of trachea from each chicken was immersion-fixed in a coded container that was three-quarters full of 10% formalin saline routinely processed, stained with haematoxylin and eosin and examined under light microscope. The severity and distribution of mucosal morphological lesions (loss of cilia, epithelial cell hypertrophy and hyperplasia, inflammation and necrosis) in sections of trachea were recorded. A score was assigned to each lesion and its distribution, which was the sum of A + B, where A represents the severity of the lesion within the section and B represents the distribution of the lesion across the section. For injury distribution, scores were either 0 = no injury, 1 = focal injury, 2 = multi focal injury or 3 = diffuse injury. For each of loss of cilia, epithelial cell hypertrophy/hyperplasia, inflammatory infiltrate and necrosis, a severity score was assigned where 0 = no cells (0%) affected, 1 = < 25% of cells affected, 2 = 25 to 50% of cells affected, 3 = 50 to 75% of cells affected and 4 = > 75% of cells affected. For inflammation, 0 = none, 1 = minimal: if inflammatory cells were directed toward the lumenal epithelium; 2 = mild: if inflammatory cells predominated within the epithelium, and fewer inflammatory cells were found in the lamina propria; 3 = moderate: if inflammatory cells were found scattered throughout the mucosa; and 4 = marked: if sheets of inflammatory cells filled the mucosa.

Data analysis

All data obtained were subjected to analysis of variance (ANOVA) for a completely randomized design. Where differences occurred, they were separated using Duncan's new multiple range test (SAS, 2013).

Results

Proximate and phytochemical composition of Moringa oleifera

The proximate composition of *Moringa oleifera* leaf meal analysis is presented in *Tables 2* and *3*. The dry matter, ether extract, crude protein, crude fibre, ash, and nitrogen free extract contents

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Moringa oleifera leaf meal contained 0.11 µg/ml of total flavonoids, 9.97 µg/ml of total phenols, 0.26 mg/ml of alkaloids, 1.17 µg/ml of tannins and 245.3 µg/ml of saponins. The aqueous extracts of *Moringa oleifera* leaf contained 0.11 µg/ml of total flavonoids, 10.78 µg/ml of total phenol, 0.21 mg/ml of alkaloids, 5.33 µg/ml of tannins and 22.55 µg/ml of saponins. While the aqueous *Moringa oleifera* residues had 1.22 µg/ml of total flavonoids, 9.70 µg/ml of total phenols, 0.27 mg/ml of alkaloids, 3.83 µg/ml of tannins and 195.3 µg/ml of saponins.

Table 2. *Proximate composition of Moringa oleifera*

Parameters	Percentage composition (%)
Dry matter	94.25
Ether extra	5.50
Crude protein	23.80
Crude fibre	16.57
Ash content	9.75
Nitrogen free extracts	38.63

Table 3. *Phytochemical composition of Moringa oleifera leaf meal, aqueous Moringa oleifera leaf extracts and Moringa oleifera residues*

Parameter	*Moringa* preparations		
	Ground *Moringa oleifera* leaf meal	Aqueous *Moringa oleifera* leaf extracts	*Moring oleifera* residues
Total flavonoids (µg/ml)	0.11	0.11	1.22
Total phenols (µg/ml)	9.97	10.78	9.70
Alkaloids (mg/ml)	0.26	0.21	0.27
Tannins (µg/ml)	1.17	5.33	3.83
Saponins (µg/ml)	245.30	22.55	195.30

Effect of aqueous extract of Moringa oleifera leaf on growth performance of broiler chickens at both starter and finisher phases

Table 4 shows the results of the effects of aqueous extract of *Moringa oleifera* leaf on growth performance of broiler chickens during the starter and finisher phases. The initial, final, growth rate, feed conversion ratio and mortality were not influenced ($P > 0.05$) by AEMOL treatments at the starter phase. However, feed intake and water intake were influenced ($P < 0.05$) by AEMOL treatments.

Birds on the $AEMOL_0$ treatment had the highest feed intake and was significantly ($P < 0.05$) higher than all other treatments which had similar ($P > 0.05$) feed intake values.

Water intake results showed that birds on control and $AEMOL_0$ treatments had the highest water intake, which were significantly higher ($P > 0.05$) than those birds on $AEMOL_{30}$ treatment.

The results of the growth performance at the finisher phase showed that all parameters measured were influenced ($P < 0.05$) by AEMOL treatments except the initial weight and the water intake which were not affected. Birds on AEMOL 60 ml/l had highest final weight and feed intake and were significantly ($P < 0.05$) higher than

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all the other treatments. Similarly, the weight gain results showed that birds on AEMOL 60 ml/l had significantly ($P < 0.05$) higher value than all the other treatment except for birds on the control treatment. The feed conversion ratio results showed that birds on the control treatment had the best value and was significantly ($P < 0.05$) better than all the other treatments.

Table 4. *Effect of aqueous extract of Moringa oleifera leaf on performance of Hubbard broiler chickens at both starter and finisher phases*

Parameters	Aqueous leaf extract treatments						SEM
	Control	$AEMOL_0$	$AEMOL_{30}$	$AEMOL_{60}$	$AEMOL_{90}$	$AEMOL_{120}$	
Starter phases							
Initial weight (g)	138.75	140.00	138.75	136.25	141.25	136.25	1.58
Final weight (g)	1241.25	1311.25	1242.50	1202.50	1283.75	1300.00	23.74
Growth rate (g)	39.37	41.83	39.42	38.08	40.80	41.56	0.82
Feed intake (g)	286.76^{b}	329.69^{a}	289.26^{b}	283.14^{b}	286.18^{b}	280.68^{b}	4.19
FCR	0.26	0.28	0.26	0.27	0.26	0.25	0.01
Water intake (ml)	254.54^{a}	258.04^{a}	215.44^{b}	249.02^{ab}	245.19^{ab}	246.14^{ab}	5.82
Finisher phases							
Initial weight (g)	1304.72	1264.72	1311.95	1348.47	1387.85	1332.78	23.74
Final weight (g)	2350.0^{c}	2242.00^{d}	2200.00^{e}	2392.00^{a}	2367.00^{b}	2042.00^{f}	25.28
Weight gain (g)	1045.3^{a}	983.95^{b}	888.05^{c}	1043.5^{a}	979.15^{b}	708.98^{d}	25.04
Feed intake (g)	3212.47^{bc}	3082.50^{c}	3300.42^{b}	3549.45^{a}	3351.29^{b}	3215.42^{bc}	7.16
FCR	3.07^{a}	3.15^{b}	3.71^{e}	3.40^{c}	3.42^{d}	4.53^{f}	0.04
Water intake (ml)	1304.72	1264.72	1311.95	1348.47	1387.85	1332.78	11.63

^{a,b,c,d,e,f}Means within the same row with different superscripts are significantly different at $P < 0.05$; $AEMOL_{0+}$ contained Gendox® 1.25 mg/l of water, $AEMOL_0$ contained 0 ml of moringa extract/l of water, $AEMOL_{30}$ contained 30 ml of moringa extract/l of water, $AEMOL_{60}$ contained 60 ml of moringa extract/l of water, $AEMOL_{90}$ contained 90 ml of moringa extract/l of water, $AEMOL_{120}$ contained 120 ml of moringa extract/l of water

Effect of aqueous extracts of Moringa oleifera leaf on apparent nutrient digestibility of Hubbard broiler chickens

Apparent nutrient digestibility results are shown in *Table 5*. The apparent nutrient digestibility results showed significant difference in all the treatments in the following order of highest to lowest: $AEMOL_0$, $AEMOL_{90}$, $AEMOL_{60}$, control, $AEMOL_{120}$ and $AEMOL_{30}$ respectively. Similar to the dry matter, the crude fibre digestibility differed significantly in all the treatments in the following order of highest to lowest: $AEMOL_0$, $AEMOL_{90}$, $AEMOL_{120}$, $AEMOL_{30}$, $AEMOL_{60}$ and control, respectively.

The ether extract digestibility results showed that the birds on $AEMOL_0$ treatment had the highest digestibility and their values were significantly ($P < 0.05$) higher than all the other treatments. Birds on $AEMOL_{30}$ and $AEMOL_{90}$ treatments had similar ($P > 0.05$) ether extract values. Similarly, birds on $AMOLE_{60}$ and $AEMOL_{120}$ treatments had similar ($P > 0.05$) ether extract digestibility. Their digestibility values were, however, lower ($P < 0.05$) than those on $AEMOL_{30}$ and $AEMOL_{90}$ treatments. Bird on the control treatment had the least ether extract digestibility and they were significantly lower ($P < 0.05$) than all the other treatments.

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The crude protein (CP) digestibility results showed that birds on $AEMOL_0$ and $AEMOL_{60}$ treatments had the highest CP digestibility and their digestibility were higher ($P < 0.05$) than the ones in other treatments. Birds on $AEMOL_{90}$ had significantly higher ($P < 0.05$) CP digestibility than those on control, $AEMOL_{120}$ and $AEMOL_{30}$ which were all significantly different ($P < 0.05$) from one another.

Birds on $AEMOL_0$ and $AEMOL_{60}$ had similar ($P > 0.05$) NFE (nitrogen free ether) digestibility. Their digestibility values were significantly ($P < 0.05$) lower than those of birds on $AEMOL_{90,}$ control, $AEMOL_{120,}$ and $AEMOL_{30}$ treatments, which were significantly ($P < 0.05$) lower than one another in descending order.

Table 5. *Effect of aqueous extract of Moringa oleifera leaf on apparent nutrient digestibility (%) of Hubbard broiler chickens*

	Aqueous leaf extract treatments						
Parameters	**Control**	**$AEMOL_0$**	**$AEMOL_{30}$**	**$AEMOL_{60}$**	**$AEMOL_{90}$**	**$AEMOL_{120}$**	**SEM**
Dry matter	79.17^{d}	83.57^{a}	65.94^{f}	82.69^{c}	82.88^{b}	70.92^{e}	1.43
Crude fibre	92.43^{f}	93.66^{a}	92.85^{d}	92.59^{e}	93.20^{b}	92.97^{c}	0.13
Ether extract	98.26^{d}	98.89^{a}	98.53^{b}	98.44^{c}	98.58^{b}	98.41^{c}	0.09
Crude protein	80.80^{c}	85.33^{a}	70.43^{e}	84.90^{a}	84.24^{b}	73.14^{d}	1.43
Nitrogen free extract	14.34^{c}	10.76^{e}	28.98^{a}	10.69^{e}	11.07^{d}	23.72^{b}	1.74

^{a,b,c,d,e,f}Means within the same row with different superscripts are significantly different at $P < 0.05$; $AEMOL_{0+}$ contained Gendox® 1.25 mg/l of water, $AEMOL_0$ contained 0 ml of moringa extract/l of water, $AEMOL_{30}$ contained 30 ml of moringa extract/l of water, $AEMOL_{60}$ contained 60 ml of moringa extract/l of water, $AEMOL_{90}$ contained 90 ml of moringa extract/l of water, $AEMOL_{120}$ contained 120 ml of moringa extract/l of water

Effect of aqueous extract of Moringa oleifera leaf on sensory evaluation of Hubbard broiler chicken meat

Presented in *Table 6* are the results of the effect of AEMOL treatments on appearance, flavour, juiciness, tenderness and general acceptability. All parameters measured were influenced ($P < 0.05$) by the treatments.

The meat appearance results showed that birds on the $AEMOL_0$, $AEMOL_{30,}$ and $AEMOL_{60}$ treatments had the best appearance ($P < 0.05$), but similar ($P > 0.05$) to meat from birds in $AEMOL_0$ and $AEMOL_{90}$ treatments. Likewise, meat from birds on the control, $AEMOL_{90}$ and $AMOLE_{120}$ treatments had similar ($P > 0.05$) appearance. However, the meat from birds on $AEMOL_{120}$ treatment had least value of appearance.

The results of the meat from birds on $AEMOL_0$, control, $AEMOL_{30,}$ and $AEMOL_{60}$ treatments, their values were similar ($P > 0.05$) in flavour but those on $AEMOL_0$ treatment had the best flavour, and were significantly better than those on $AMOLE_{90}$ and $AEMOL_{120}$ treatments. Meat of birds on control, $AEMOL_{30}$, $AEMOL_{60,}$ $AEMOL_{90}$ treatments had similar ($P > 0.05$) flavour. Meat juiciness results showed that meat form birds on control, $AEMOL_0$, $AEMOL_{30,}$ $AEMOL_{60}$ and $AEMOL_{90}$ treatments were similar ($P > 0.05$) in juiciness values. However, meat of birds from control, $AEMOL_{30}$, treatments and $AEMOL_{90}$ had more ($P < 0.05$) juiciness than those of meat of birds from $AEMOL_{120}$ treatment.

Meat from birds on the $AMOLE_0$ treatment were more tender ($P > 0.05$) than those of birds on $AEMOL_{120}$. However, their tenderness value was similar to those of birds from control, $AEMOL_{30}$, $AEMOL_{60}$ and $AEMOL_{90}$ treatments.

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The general acceptability results showed that meat from birds on AEMOL $_0$ treatment were better and more acceptable ($P < 0.05$) than those from other treatments except meat from $AEMOL_{30}$ treatment which had similar values. Birds on control, $AEMOL_{30}$, $AEMOL_{60}$ and AEMOL $_{90}$ treatments had similar ($P > 0.05$) acceptability values and they were however significantly more acceptable ($P < 0.05$) than meat from birds on $AEMOL_{120}$ treatment.

Table 6. *Effect of aqueous extract of Moringa oleifera leaf on sensory evaluation of Hubbard broiler meat*

	Aqueous leaf extract treatments						
Parameters	**Control**	**$AEMOL_0$**	**$AEMOL_{30}$**	**$AEMOL_{60}$**	**$AEMOL_{90}$**	**AEMOL $_{120}$**	**SEM**
Appearance	7.05^{ab}	7.60^{a}	7.35^{a}	7.45^{a}	7.10^{ab}	6.55^{b}	0.11
Flavour	7.00^{ab}	7.50^{a}	7.25^{ab}	7.25^{ab}	6.65^{bc}	6.16^{c}	0.11
Juiciness	6.75^{ab}	7.45^{a}	7.15^{a}	6.65^{ab}	7.00^{a}	6.25^{b}	0.11
Tenderness	7.20^{ab}	7.85^{a}	7.70^{ab}	6.95^{ab}	7.15^{abc}	6.40^{c}	0.12
General acceptability	7.25^{b}	8.30^{a}	7.65^{ab}	7.20^{b}	7.25^{b}	6.30^{c}	0.11

^{a,b,c}Means within the same row with different superscripts are significantly different at $P < 0.05$; AEMOL $_{0+}$ contained Gendox® 1.25 mg/l of water, AEMOL $_0$ contained 0 ml of moringa extract/l of water, AEMOL $_{30}$ contained 30 ml of moringa extract/l of water, AEMOL $_{60}$ contained 60 ml of moringa extract/l of water, AEMOL $_{90}$ contained 90 ml of moringa extract/l of water, AEMOL $_{120}$ contained 120 ml of moringa extract/l of water

Effect of aqueous extract of Moringa oleifera leaf on histological parameters of Hubbard broiler chickens at finisher phase

Table 7 shows the effect of aqueous extract of *Moringa oleifera* leaf on histological parameters of Liver, Lung, Kidney, Intestine, Spleen and Heart of Hubbard broiler chickens. All organs measured showed no sign ($P > 0.05$) of hypertrophy, hyperplasia, inflammation, necrosis and injury.

Discussion

The Proximate composition of *Moringa oleifera* leaf meal analysis results of dry matter (94.25), ether extract (5.50), crude protein (23.80), crude fibre (16.57), ash (9.75), and nitrogen free extract (38.63) in the present study were contrary to the values obtained by Makkar and Becker (1997) who reported the values as 2.15% of fat content, 27.29% of crude protein, 10.67% of crude fibre, 15% of ash content and 34.08% N.F.E. These differences in the value may be due to the processing methods, the period of harvesting of the plants and the climatic condition as reported by Fuglie (2001). The results also showed that *Moringa oleifera* leaf is considerably rich in protein (23.80%), crude fiber (16.57%) which is similar to the findings of Mabruk et al. (2010) and Zaku et al. (2015). However, the dry matter (94.25%) and ash (9.75%), the fat content (5.50%) and nitrogen free extract content (38.82%) contents in the present study are similar to those reported obtained by Mabruk et al. (2010) and Ogbe and John (2012).

The results also showed that aqueous processing of *Moringa oleifera* leaves do not reduce some the phytochemical properties except for saponin which were reduced. Nityanand (1997) and Akinmutimi (2004) observed similar results and reported that most processing methods employed in improving the food value of non-conventional feedstuffs do not eliminate the anti-nutritional factor substances completely.

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Table 7. *Effect of aqueous extract of Moringa oleifera leaf on histological parameters of Liver, Lung, Kidney, intestine, spleen and heart of Hubbard broiler chickens*

		Treatments					
Organs	**Parameters**	**Control**	**AEMOL$_0$**	**AMOLE$_{30}$**	**AMOLE$_{60}$**	**AMOLE$_{90}$**	**AMOLE$_{120}$**
		Scores	**Scores**	**Scores**	**Scores**	**Scores**	**Scores**
Liver	Hypertrophy	None	None	None	None	None	None
Liver	Hyperplasia	None	None	None	None	None	None
Liver	Inflammation	None	None	None	None	None	None
Liver	Necrosis	None	None	None	None	None	None
Liver	Injury	None	None	None	None	None	None
Lungs	Hypertrophy	None	None	None	None	None	None
Lungs	Hyperplasia	None	None	None	None	None	None
Lungs	Inflammation	None	None	None	None	None	None
Lungs	Necrosis	None	None	None	None	None	None
Lungs	Injury	None	None	None	None	None	None
Kidney	Hypertrophy	None	None	None	None	None	None
Kidney	Hyperplasia	None	None	None	None	None	None
Kidney	Inflammation	None	None	None	None	None	None
Kidney	Necrosis	None	None	None	None	None	None
Kidney	Injury	None	None	None	None	None	None
Intestine	Cilia	None	None	None	None	None	None
Intestine	Hypertrophy	None	None	None	None	None	None
Intestine	Hyperplasia	None	None	None	None	None	None
Intestine	Inflammation	None	None	None	None	None	None
Intestine	Necrosis	None	None	None	None	None	None
Intestine	Injury	None	None	None	None	None	None
Spleen	Hypertrophy	None	None	None	None	None	None
Spleen	Hyperplasia	None	None	None	None	None	None
Spleen	Inflammation	None	None	None	None	None	None
Spleen	Necrosis	None	None	None	None	None	None
Spleen	Injury	None	None	None	None	None	None
Heart	Hypertrophy	None	None	None	None	None	None
Heart	Hyperplasia	None	None	None	None	None	None
Heart	Inflammation	None	None	None	None	None	None
Heart	Necrosis	None	None	None	None	None	None
Heart	Injury	None	None	None	None	None	None

AEMOL $_{0+}$ contained Gendox® 1.25 mg/l of water, AEMOL $_0$ contained 0 ml of moringa extract/l of water, AEMOL $_{30}$ contained 30 ml of moringa extract/l of water, AEMOL $_{60}$ contained 60 ml of moringa extract/l of water, AEMOL $_{90}$ contained 90 ml of moringa extract/l of water, AEMOL $_{120}$ contained 120 ml of moringa extract/l of water

The growth performance results in this study is in agreement with the results reported by Portugaliza and Fernandez (2012) which indicated that AEMOL inclusion levels resulted in lower feed and water intake at the starter phase which is also consistent with less water intake in all AEMOL treated groups compare to control and AEMOL$_0$. The reduction in water and feed intake in all AEMOL treated groups compare to control and

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$AEMOL_{0,}$ may have translated to reduced growth performance as recorded in the present study. This is consistent with the findings of Portugaliza and Fernandez (2012). This may be due to the presence of antinutrient substance in the extract as reported by Ramchandra et al. (2019). Ramchandra et al. (2019) reported that chemical such as antinutrients substances present in the diet by themselves or their metabolic products arising in the system, reduce feed intake and thus, interfere with the feed utilization. The present results however, are contrast to the report of Oludoyi and Toye (2012) who reported a significant difference in bodyweight at week 4, between broiler chickens fed diet containing 0, 10 and > 15% MOLM, whereas no significant difference in body weight was observed between pullet groups.

The better final weight, weight gain and feed intake recorded AEMOL60 than in the control group suggests that 60 ml of AEMOL might be an optimal dose of aqueous extract of Moringa oleifera for better broiler growth performance at finisher phase. This is in line with the reports of Kakengi et al. (2003) and Olugbemi et al. (2010) who showed that Moringa oleifera inclusion levels ($AEMOL_{60}$) resulted in an increased body weight gain and feed intake due to high protein content in it. On the contrary, birds on aqueous extract of 120 ml per litre of water had the lowest weight gain despite the high protein content in the extract, suggesting an inverse growth relationship- the higher the dose, the higher the antinutrient contents and less the growth possible.

The higher feed intake at the finisher phase compared to the starter phase might be an indication that the older the birds, has better capacity to manage antinutrient contents. The improved FCR of birds on control could be as a result of the low feed intake recorded by the birds on this treatment group.

The results of sensory properties of broiler meat for the different treatments showed significant difference (appearance, flavour, tenderness, juiciness and general acceptance) among the experimental meat samples. No definite trend was in all the parameters assessed. Aqueous extract of *Moringa oleifera* leaf inclusion levels up to 90 ml/l were similar in the appearance, flavour, tenderness and general acceptability to control and recorded least flavour for $AEMOL_{120}$ treated group suggesting that AEMOL inclusion levels up to 90 ml/l can be used to replace growth promoters in terms of sensory attributes. Additionally, this might mean that poor flavour and tenderness of the meat are produced at inclusion levels above 90 ml/l. The present result is inconsistent with those of Safa et al. (2012) who reported that flavour and juiciness were not significantly influenced by *Moringa oleifera* leaf meal on broiler chickens.

The results of the histological parameters of tissue samples from the liver, lung, kidney, intestine, spleen, and heart of the broiler chickens did not show any adverse effects of administering aqueous extract of *Moringa oleifera* leaf to the broiler birds.

Conclusion

The results of this study showed that aqueous extract of Moringa oleifera leaf inclusion levels up to 60 ml/l can be used to replace antibiotic growth promoter without compromising the advantages of antibiotic growth promoter. Since it is easily available and can be source cheaply, the use may improve cost effectiveness, reduction in feed consumption with improved growth performance. It is recommended that other methods of extraction could be used to see if higher inclusion level will result in better performance.

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ENHANCING BANANA (*Musa* spp.) GROWTH AND PRODUCTIVITY BY BIO-FERTILIZERS IN SANDY SOIL

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Abstract. This investigation was carried out during the two successive seasons of 2016 and 2017 at the private orchard of the modern agriculture company (PICO), El Behera governorate, Egypt, to study the effect of Nitrogen Fixing Bacteria (NFB) and Phosphate Solubilizing Bacteria (PSB) and different rates of N and P as mineral fertilizers (*i.e.* 50% and 75% of recommended dose), compared to the standard recommended mineral fertilization on the growth, yield, fruit quality and leaf mineral contents of Williams banana plants. Results showed that T_8 (50% bio N + 50% bio. P + 50% mineral N and P) and T_7 (100% bio N + 50% bio. P + 50% mineral N and P) enhanced plant growth *i.e.* pseudostem length and perimeter also, it increased bunch weight and yield and improved most physical parameters (*i.e.* finger no./hand, finger length, finger weight, pulp weight, pulp weight and pulp ratio) and chemical characters (T.S.S, total sugar % and reducing sugar %), leaf mineral content (N, P, and K) and chlorophyll a and b were also significantly affected by treatments compared to the control during two season of study. The results suggest that, the utilized biofertilizers can partially substitute the amount of mineral fertilizers, leading to cleaner environment.
Keywords: *P dissolving bacteria, N fixing bacteria, fruit quality, yield, Williams, sustainable agriculture*

Introduction

Banana (*Musa* sp.) is a major crop in tropical and subtropical regions of the world. In Egypt, it represents most important fruit crop after citrus and grapes. It coveres an area of 28667 hectares with production of 1228458 tons in 2017 (FAO STAT). Among Crop management practices, plant nutrition in particular represents one of the major factors influencing banana yield. Moreover, all soils are deficient in N, P and K (Akhtar et al., 2003), besides, soil organic matter contents are low (Abbas et al., 2012) and this is true in new reclaimed lands in Egypt. Fertilization is very important not only to meet the crop requirements but also to improve soil fertility.

Soil microorganisms have enormous role in increasing the availability of accumulated phosphates in the soil for plant by solubilization (Goldstien, 1986; Gyaneshwar et al., 2002; Hamim et al., 2019). Moreover, the microorganisms involved P solubilization as well as better scavenging of soluble P can enhance plant growth by increasing the efficiency of biological nitrogen fixation, enhancing the availability of other trace elements and by production of plant growth promoting substances (Goldstien, 1986). This assumes more important for banana, which is a heavy feeder crop requiring large amount of nutrients (Ganapathi and Dharmatti, 2018).

Williams is one of the most widely grown banana varieties in the world (Xu et al., 2005; FAO, 2018). In Egypt, it is cultivated successfully in newly reclaimed soils for its excellent performance, the large bunch with longer fingers, the excellent taste and high tolerance to transportation (Barakat et al., 2011). Very little information is available on the effect of biofertilizers (Azotobacter chroococcum and Bacillus megatherium var

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phospahaticum) on this variety under sandy soil condition. Thus, the present investigation was undertaken to study the effect of biofertilizers as ecofriendly, and low-cost alternative fertilizers on growth and yield attributes of Williams banana.

Materials and methods

The present investigation was conducted during two successive seasons (2016 and 2017) in a private orchard of the modern agriculture company (PICO) located at Badr city (30˚36′36.5″N and 30˚45′45.5″ E), El Behera governorate, Egypt (*Fig. 1*). Monthly average of some metrological data during study period of the experimental site is illustrated in *Figure 2*.

Figure 1. *Experimental site location*

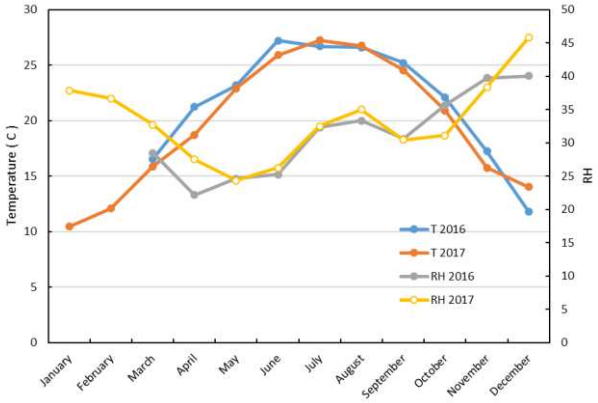

Figure 2. *Metrological data during study period of the experimental site*

At the beginning of the treatments, three soil samples were collected from different sites at 90 cm depth and analyzed as a composite sample for physical and chemical properties according to (Wilde et al., 1985). The analysis of orchard soil is presented in *Table 1*.

Table 1. *Physical and chemical analysis of soil*

Chemical properties							
pH	**E.C (mmhos/cm)**	**Organic matter %**	**Organic carbon %**	**P(available) Meq/L**	**SAR**	**K mg/100g**	**Ca Meq/L**
8.3	0.18	0.68	0.39	24	0.52	0.15	0.8
Physical properties							
Sand (%)		**Loam (%)**		**Clay (%)**		**Density (g/cm3)**	
75		8.75		16.25		1.21	

The mother plants were planted in mid-March at 3.5*1.5 m apart, received 30 m^3 organic matter/Feddan/year. Eight treatments were used; Control treatment fertilized by recommended dose of mineral fertilizers (250, 80 and 480 unite of NPK /4200 m^2/year) in forms of ammonium nitrate (33.5% N), phosphoric acid (80% P_2O_5) and potassium sulfate (48% K_2O), respectively. Two levels of each N and P (100% and 50% of recommended dose) were used in combination with two biofertilizers namely;

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Azotobacter chroococcum (Az14) as nonsymbiotic nitrogen fixation bactria (N.F.B) and Bacillus megatherium var phospahaticum (B6) as phosphate dissolving bacteria (P.D.B). The strains were obtained from Bacteriology Lab, Sakha. Agric. Res. Station. Three kg/ 4200 m^2 from each inoculum was used after mixed with suitable amount of sandy soil and added once at first week of April of each season in crescentic trenches around each plant. The chemical fertilizers were added through drip irrigation system from April to October. The recommended dose of K was applied to all treatments.

The applied treatments were as follows:

T1- Control (100% mineral N, P fertilizers)
T2- 100 % Bio P (P.D.B) +100% mineral N
T3- 100% Bio P + 50% mineral P +100% mineral N
T4- 100% Bio N (N.F.B) + 100% mineral P
T5- 100% Bio N + 50% mineral N +100% mineral P
T6- 100% Bio N +100% Bio P
T7- 100 % Bio N +100% Bio P + 50% mineral N, P
T8- 50% Bio P + 50 % Bio N + 50 % mineral N, P

Vegetative growth measurements

At the beginning of the florescence emergence the following parameters were recorded; Pseudostem length (cm) which was measured from the soil surface up to the petiole of the last emerged leaf. Circumference of pseudostem (cm) at 25 cm above soil surface.

Yield and fruit quality

In mid-February of each season, bunches were harvested at the green maturity stage. At harvest, bunch weight (kg), bunch length (cm), and finger number/hand were recorded, three hands were taken randomly/ bunch/ replicate to estimate fruit physical characters including finger weigh (g), length and perimeter (cm), pulp and peel weight / finger (g) and pulp ratio. Fruit chemical characters were estimated in ripe fingers as total soluble solids in pulp juice (TSS) using hand refractometer as Brix, total titratable acidity percentage as malic acid was estimated using phenolphthalein as indicator according to (AOAC, 1985) and total and reducing sugars (Dubois et al., 1956).

Leaf chlorophyll and mineral contents

Leaf samples were taken from the middle part of third upper leaf blade. Chlorophyll (a and b) content expressed as mg/g fresh weight was determined according to Moran and Porath (1980). Leaf samples were washed and oven dried at 70c to a constant weight. Total nitrogen was determined by using micro-kjedehl (AOAC, 1985). Potassium was measured using a flame-photometer and phosphorus was determined colorimetrically (Chapman and Pratt, 1961).

Statistical analysis

The obtained data in both seasons were statistically analyzed using analysis of variance method as simple experiment in randomized complete block design (Snedecor and Cochran, 1980) using CoStat 6.303, CoHort Software, 798 Lighthouse Ave. PMB 320, Monterey, CA, 93940, USA. Duncan' multiple range test were used for means comparison (Duncan, 1955).

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Results and discussion

Growth characters

Considering to height and circumference of pseudostem, data presented in *Table 2* indicate that planta treated with T_2 (100% Bio P (P.D.B) +100% N) recorded the lowest significant pseudostem length in the first season and so in the second season but with no significant difference with T3, T4 and T6. Whereas, the highest pseudostem length was recorded at T_8 (50% Bio P + 50% Bio N + 50% mineral NP), especially in the second season.

Table 2. *Effect of Biofertilizers in combination with different levels of N, P mineral fertilizers on vegetative growth characters*

Treatments	Pseudostem length (cm)		Pseudostem Circumference (cm)	
	2016	2017	2016	2017
T1	200.0a	194.8b	47.25b	49.5bcd
T2	172.5c	187.5d	40.25c	46.25e
T3	184.5b	189.5cd	49.0b	48.5cde
T4	188.3ab	191.3bcd	45.75b	47.25de
T5	199.3a	193.0 bc	49.5b	49.5 bcd
T6	195.0ab	189.5 cd	49.25b	50.25abcd
T7	197.0ab	193.8 b	53. 5 a	50.5 abc
T8	200.5a	198.5 a	55.5a	54.0 a

Means within each column followed by the same letter are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test. T1: Control (100% mineral NP), T2: 100% Bio P (P.D.B) +100% N, T3: 100% Bio P + 50% mineral P +100% N, T4: 100% Bio N (N.F.B) + 100% mineral P, T5: 100% Bio N + 50% mineral N +100% mineral P, T6: 100% Bio N +100% Bio P, T7: 100% Bio N +100% Bio P + 50% mineral NP, T8: 50% Bio P + 50% Bio N + 50% mineral NP

Pseudostem circumference showed significant higher values at T7, T8 in the first season and T6, T7 and T8 in the second season. These results came in line with Abdel Gawad et al. (2017), who reported that height and circumferences of Grande Naine banana pseudostem increased as consequence of biofertilization and feldspar application.

Data in *Table 3* show that, plants treated with T_8 (50% Bio P + 50% Bio N + 50% mineral N, P) gave the highest bunch weight in both seasons, while T_2 (100% Bio P (P.D.B) +100% N) presented the significant lowest value than all tested treatments. In addition, T8 recorded the maximum bunch length and the lowest one registered by T2. These results are in agreement with those reported by Abd El-Naby and Gomaa (2000) and Barakat et al. (2011) who recorded that banana plants supplied with mineral fertilization combined with organic manure improved bunch weight. Also, the results are confirmed by those obtained by Baiea et al. (2015) and Baiea and El-Gioushy (2015) on banana plants.

In both seasons, T_8 (50% Bio P + 50% Bio N + 50% mineral N, P) increased the yield by 2%, 4.1% than the control. Moreover, the rest treatments had intermediate yield among the highest value at T8 (20, 20.4 T) and the lowest one (14.8 ,12.4 T) at T2 (100% Bio P (P.D.B) +100% N) treatment during 1st and 2nd seasons, respectively. In this concern, Mai et al. (2005) observed that number of hands/bunch increased with 50% or 33% recommended dose of N plus Azospirillum phosphate solubilizing

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bacteria. Dave et al. (1991), Zake et al. (2000), and Abd el Moniem et al. (2008) found that biofertilization with algal extract significantly improved yield, bunch and hand weight.

Table 3. *Effect of Biofertilizers in combination with different levels of N, P mineral fertilizers on Bunch weight and length and yield*

Treatments	Bunch weight (Kg)		Bunch length (cm)		Yield Ton/ 4200 m^2	
	2016	2017	2016	2017	2016	2017
T1	24.5ab	24.5abc	95.75ab	97.0 a	19.6 ab	19.6 abc
T2	18.5 d	15.5 d	81.75c	74.75c	14.8 d	12.4 d
T3	21.75 c	22.5c	91.5 abc	89.25b	17.4 c	18.0 c
T4	21.5c	22.75 c	91.25abc	87.0b	17.2 c	18.2 c
T5	22.25c	23.25bc	98.0a	89.25b	17.8 c	18.6 bc
T6	21.25c	25.25ab	86.0 bc	96.75a	17.0c	20.2 ab
T7	23.0 bc	25.25 ab	95.75ab	95.5a	18.4 bc	20.2 ab
T8	25.0 a	25.5 a	98.25 a	98.5a	20.0 a	20.4 a

Means within each column followed by the same letter are not significantly different at P≤ 0.05 according to Duncan's multiple range test. T1: Control (100% mineral NP), T2: 100% Bio P (P.D.B) +100% N, T3: 100% Bio P + 50% mineral P +100% N, T4: 100% Bio N (N.F.B) + 100% mineral P, T5: 100% Bio N + 50% mineral N +100% mineral P, T6: 100% Bio N +100% Bio P, T7: 100% Bio N +100% Bio P + 50% mineral NP, T8: 50% Bio P + 50% Bio N + 50% mineral NP

With respect to fingers No./hand. the differences among the treatments did not reached the limit of significance in the 1st season (*Table 4*). However, in the 2nd season, T_8 (50% Bio P + 50% Bio N + 50% mineral NP) resulted in increasing the finger No/hand than T3 and T2, the latter presented the significant lowest value than all treatments.

Table 4. *Effect of Biofertilizers in combination with different levels of N, P mineral fertilizers on finger characters of Williams Banana fruits*

Treatments	Finger No./hand		Finger length(cm)		Finger perimeter(cm)	
	2016	2017	2016	2017	2016	2017
T1	16.75a	15.0ab	21.3 bc	21.0 ab	12.24 a	11.74a
T2	15.25a	12.0 c	20.5 cd	19.25b	11.29 bc	9.5b
T3	16.0a	14.5b	21.0 cd	21.5a	12.24ab	12.24a
T4	15.75a	16.0ab	19.63 d	20.88ab	10.5 c	11.0a
T5	16.5a	16.0ab	22.0abc	21.0ab	12 ab	12.24a
T6	16.0a	15.75ab	21.0 cd	21.5a	12.0 ab	12.24a
T7	16.50a	16.0 ab	23.0 a	22.0a	12.5 a	12.24 a
T8	16.75a	16.5a	23.3 a	22.5a	12.8 a	12.4 a

Means within each column followed by the same letter are not significantly different at P≤ 0.05 according to Duncan's multiple range test. T1: Control (100% mineral NP), T2: 100% Bio P (P.D.B) +100% N, T3: 100% Bio P + 50% mineral P +100% N, T4: 100% Bio N (N.F.B) + 100% mineral P, T5: 100% Bio N + 50% mineral N +100% mineral P, T6: 100% Bio N +100% Bio P, T7: 100% Bio N +100% Bio P + 50% mineral NP, T8: 50% Bio P + 50% Bio N + 50% mineral NP

The present data revealed that length and perimeter of finger augment as consequence of T_8 (50% Bio P + 50% Bio N + 50% mineral NP) and T_7 (100% Bio N

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+100% Bio P + 50% mineral NP) throughout the two growing seasons. on the contrary the lowest value obtained by T4 (100% Bio N (N.F.B) + 100% mineral P) and T2 for each of first and second season.

Data in *Table 5* revealed that, in both seasons, the highest significant finger weight was obtained by T7 followed by T8, while T6 recorded the lowest value. The results are in line with El-Shenawi and Hassouna (2004) who found that supplied Williams banana plants with N at 600 g ammonium nitrate /plant plus 5l HALEX biofertilizer presented the best finger weight. The highest pulp percentage was recorded at T8 treatment in both seasons.

Table 5. *Effect of Biofertilizers in combination with different levels of N, P mineral fertilizers on peel weight, pulp weight and pulp %*

Treatments	Finger weight (g)		Peel weight (g)		Pulp weight (g)		Pulp (%)	
	2016	2017	2016	2017	2016	2017	2016	2017
T1	138.3 c	150.0 b	41.8 b	57.25 bc	96.5 c	92.75 bc	69.75 ab	61.83bc
T2	115.0 e	134.5 c	39.25 b	48.10 de	75.25 e	86.4 c	65.43 c	64.23b
T3	135.5 c	144.8 b	42.75 b	53.05 cd	92.75 cd	91.75 c	68.45ab	63.36b
T4	125.3 d	142.8 b	40.55 b	53.55 c	84.75 cd	89.25 c	68.0 ab	62.5bc
T5	146.8 b	148.0 b	44.3 b	59.05 ab	102.50 bc	88.5 c	67.3 bc	59.67c
T6	114.8 e	115.5 d	40.56 b	45.00 e	74.25 e	70.50 d	64.67 c	61.3bc
T7	165.5 a	159.0 a	53.00 a	57.07 ab	112.00 a	101.30 a	67.97ab	63.71b
T8	160.3 a	158.0 a	51.80 a	63.00 a	108.00 ab	95.0 ab	70.32a	70.12a

Means within each column followed by the same letter are not significantly different at P≤ 0.05 according to Duncan's multiple range test. T1: Control (100% mineral NP), T2: 100% Bio P (P.D.B) +100% N, T3: 100% Bio P + 50% mineral P +100% N, T4: 100% Bio N (N.F.B) + 100% mineral P, T5: 100% Bio N + 50% mineral N +100% mineral P, T6: 100% Bio N +100% Bio P, T7: 100% Bio N +100% Bio P + 50% mineral NP, T8: 50% Bio P + 50% Bio N + 50% mineral NP

Data presented in *Table 6* show that, in the 1st season acidity percentage was similar in the fruit of T5 (100% Bio N + 50% mineral N +100% mineral P), T6 (100% Bio N +100% Bio P) beside the control plants, while the lowest acidity percentage obtained by T2 (100% Bio P (P.D.B) +100% N). Moreover, in the 2nd season the highest and the lowest value recorded when plants treated with T5 (100% Bio N + 50% mineral N +100% mineral P) and T2 (100% Bio P (P.D.B) +100% N), respectively.

In both seasons the highest fruit TSS as Brix was recorded at T6 (100% Bio N +100% Bio P) and the lowest value presented with T2 (100% Bio P (P.D.B) +100% N). Considering total sugars content, in the 1st season, T6 (100% Bio N +100% Bio P), T7 (100% Bio N +100% Bio P + 50% mineral NP) and T8 (50% Bio P + 50% Bio N + 50% mineral NP) gave the highest significant fruit total sugar content, while plants treated with T2 (100% Bio P (P.D.B) +100% N) had the lowest significant value. Moreover, in the second season T7 (100% Bio N +100% Bio P + 50% mineral NP) and T2 (100% Bio P (P.D.B) +100% N) had the highest and lowest values, respectively.

Fruit reducing sugar percent was significantly improved in response to T8 (50% Bio P + 50% Bio N + 50% mineral NP) in the 1st season as well as, T7 (100% Bio N +100% Bio P + 50% mineral NP) and T8 (50% Bio P + 50% Bio N + 50% mineral NP) in the 2nd season led to significant reduction in fruit reducing sugar value.

Attia et al. (2009) on banana observed that biofertilization increased TSS and decreased acidity.

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Table 6. *Effect of Biofertilizers in combination with different levels of N, P mineral fertilizers on finger chemical contents of Williams Banana fruits*

Treatments	Acidity (%)		TSS (Brix)		Total sugar (%)		Reducing sugar (%)	
	2015	2016	2015	2016	2015	2016	2015	2016
T1	0.25 a	0.21 bc	20.27 bc	22.12 cd	16.49 bc	17.66 a	15.00b	15.39d
T2	0.20 c	0.19 c	19.37 d	19.37d	13.18 e	13.27 c	11.16 d	11.51d
T3	0.21 bc	0.22 ab	24.5 ab	25.17 ab	16.04 cd	15.82b	14.39bc	13.09c
T4	0.23 ab	0.21 bc	22.5 c	21.5 cd	15.29 d	15.52 b	13.86 c	13.65 c
T5	0.25 a	0.24 a	23 bc	23 bc	16.33 bcd	17.94 a	14.34 bc	15.57b
T6	0.25 a	0.20 bc	25.25 a	26.75 a	17.17 a	17.95 a	15.09 b	16.52 ab
T7	0.22 bc	0.22 ab	23.12 bc	26.75a	17.72 a	18.72 a	15.14 b	17.36 a
T8	0.21 bc	0.22 ab	24.12 ab	25.5 ab	17.92 a	18.4 a	16.14 a	17.26 a

Means within each column followed by the same letter are not significantly different at P≤ 0.05 according to Duncan's multiple range test. T1: Control (100% mineral NP), T2: 100% Bio P (P.D.B) +100% N, T3: 100% Bio P + 50% mineral P +100% N, T4: 100% Bio N (N.F.B) + 100% mineral P, T5: 100% Bio N + 50% mineral N +100% mineral P, T6: 100% Bio N +100% Bio P, T7: 100% Bio N +100% Bio P + 50% mineral NP, T8: 50% Bio P + 50% Bio N + 50% mineral NP

Leaf chemical composition

Data in *Table 7* show that leaf N, P and K contents were affected by tested combination of mineral and biofertilizer treatments. In both seasons, T7 (100% Bio N +100% Bio P + 50% mineral NP) and T8 (50% Bio P + 50% Bio N + 50% mineral NP) showed to be most effective for inducing the greatest values of leaf N, P and K. Meanwhile, T2 (100% Bio P (P.D.B) +100% N) for leaf N%, T5 (100% Bio N + 50% mineral N +100% mineral P) for P content and T2 (100% Bio P (P.D.B) +100% N), T3 (100% Bio P + 50% mineral P +100% N) for leaf K% presented the lowest values for N, P and K, respectively in both seasons. Different workers have proposed different critical levels of N, P, K which ranged from 1.8- 4, 0.17- 0.29 and 1.66 - 6.4%, respectively (Angeles et al., 1993; Memon et al., 2010) and their levels were increased by biofertilizer.

Table 7. *Effect of Biofertilizers in combination with different levels of N, P mineral fertilizers on chlorophyll a and b content and N, P and K % content*

Treatments	Chlorophyll a (mg g-^{1})		Chlorophyll b (mg g-^{1})		N%		P%		K%	
	2016	2017	2016	2017	2016	2017	2016	2017	2016	2017
T1	2.592 ab	2.310 de	1.207 bc	1.063 c	2.90 b	3.11 b	0.23b	0.24ab	2.6 b	2.66b
T2	2.003 c	2.213 e	1.015 de	0.910 d	2.72 d	2.85 d	0.24ab	0.24ab	2.2 d	2.40 d
T3	2.162 c	2.310 de	0.930 e	0.952 cd	2.84 c	2.92 c	0.23b	0.24ab	2.2 d	2.40d
T4	2.375 b	2.273 e	1.020 de	1.035 cd	2.79c	2.90cd	0.23b	0.23ab	2.46 c	2.50 c
T5	2.750 a	2.615bc	1.300 ab	1.255 b	2.79 c	2.92c	0.20c	0.22b	2.50c	2.60b
T6	2.425 b	2.470 cd	1.072 cde	1.330 ab	2.79 c	2.92c	0.25ab	0.25a	2.60 b	2.62b
T7	2.752 a	2.815 a	1.415a	1.390a	3.25 a	3.35a	0.26 a	0.25a	2.8a	2.80a
T8	2.500b	2.660 ab	1.102 cd	1.450a	3.22a	3.32 a	0.25 ab	0.25a	2.82a	2.80 a

Means within each column followed by the same letter are not significantly different at P≤ 0.05 according to Duncan's multiple range test. T1: Control (100% mineral NP), T2: 100% Bio P (P.D.B) +100% N, T3: 100% Bio P + 50% mineral P +100% N, T4: 100% Bio N (N.F.B) + 100% mineral P, T5: 100% Bio N + 50% mineral N +100% mineral P, T6: 100% Bio N +100% Bio P, T7: 100% Bio N +100% Bio P + 50% mineral NP, T8: 50% Bio P + 50% Bio N + 50% mineral NP

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Chlorophyll content showed no clear trend, however, T7 recorded the highest significant content of chlorophyll a and b of in both seasons.

Conclusion

From the obtained results it can be concluded that integrating biofertilizers (Az14 as nitrogen fixation and B6 as phosphate dissolving bacteria) in fertilization program of Williams banana was beneficial in enhancing the most estimated parameters of banana in terms of yield and fruit quality, moreover T7 and T8 treatments are recommended due to their help in minimizing the application of mineral fertilizer to 50%, which is cost-effective and reducing environmental pollution leading to sustainable agriculture production.

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WATER AND SOIL PHYSICOCHEMICAL CHARACTERISTICS OF DIFFERENT RICE CULTIVATION AREAS

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Abstract. In this study, the physicochemical characteristics of water and soil in conventional and organic rice cultivations are determined. Physicochemical parameters for water and soil samples are assessed by laboratory analysis. Chemical and physical parameters for water quality include temperature, pH, dissolved oxygen (DO), chemical oxygen demand (COD), biochemical oxygen demand (BOD_5), total suspended solids, and heavy metals. Parameters for the physicochemical analysis of soil include pH, particle size distribution, organic carbon, electrical conductivity (EC), cation exchange capacity, and heavy metals. Based on the water quality index, water qualities of conventional and organic rice fields are categorized into Class III. Based on the independent sample t-test, significant differences are observed between conventional rice and organic rice for pH, DO, BOD_5, COD, ammoniacal nitrogen (NH_3-N), arsenic (As), chromium (Cr), iron (Fe), and nickel (Ni) ($p < 0.05$). For soil samples, significant differences are observed between conventional rice and organic rice for pH, organic matter (OM), EC, As, Cr, Fe, and Ni ($p > 0.05$). The results of this study can serve as a guideline and basis for future studies on irrigation water and soil quality in sustainable agricultural management in Malaysia.
Keywords: *Malaysia, physicochemical parameters, rice production, crop management, sustainable agriculture*

Introduction

Malaysia has 688,770 hectares of land for rice cultivation, which produced 2,739,606 metric tons of rice in 2016 (DOSM, 2018). Recently, the tremendous growth in the population has led to an increase in the rice productivity. The need for high rice production has led to the expansion of irrigated areas. As a result, the rice cultivation area has increased, while rice production has decreased from 2014 to 2017. Although the use of hybrid rice varieties introduced by the Malaysian Agricultural Research and Development Institute (MARDI) has overcome some of the issues, the yield is still a factor that limits the increased production level due to bad weather as well as pests and diseases (USDA, 2018). To drive the modernization of Malaysia's agro-food sector, the

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rice sector was included in one of the seven specialized industries listed by the National Agro-Food Policy (DAN) 2011–2020. DAN 2011–2020 targets to increase productivity and yields to ensure a sufficient food supply, high-value and sustainable agricultural development. By 2020, the Malaysian government also aims to achieve 80% self-sufficiency in rice (MOA, 2016). Hence, the government has provided several incentives such as subsidized seeds and fertilizers to encourage rice production.

The expansion of irrigated areas has become one of the major challenges in ensuring food security due to the high input used in rice cultivation such as water, fertilizers, and pesticides (Bouman, 2007; Hanafiah et al., 2019). With respect to these issues in rice cultivation, several methods have been explored for the application of less input in rice production, such as aerobic rice cultivation, system of rice intensification, ground-cover rice production system, raising beds, and alternate wetting and drying (Farooq et al., 2009). Moreover, organic cultivation practices have become an alternative to sustainable rice production as these practices avoid the use of synthetic fertilizers and chemical pesticides and exhibit benefits in terms of food security (Champagne et al., 2007; Keawpeng and Meenune, 2012).

Previous studies have independently reported that non-sustainable agriculture techniques can cause water quality problems (Reche et al., 2016; Banch et al., 2020; Bong et al., 2020). Water contamination in rice fields is caused by the chemical substances used for soil fertilization and pest control (Rhee et al., 2011). Iqbal (2011) has reported that pesticides do not exhibit adverse effects due to their low application amount; however, the continuous use of pesticides can slowly disturb the ecosystem and living organisms. Rice cultivation under flooded conditions causes a change in microbial activities from the aerobic to anaerobic fermentation of organic matter in rice fields. Hence, anaerobic respiration can produce substances that can cause the chemical reduction of soil components (Ethan, 2015). However, Haefele et al. (2014) have reported that some negative soil characteristics, including low nutrient reserves and an extremely low cation exchange capacity (CEC), are not considerably affected by flooding.

Water quality index (WQI) is a scale related to a group of parameters that are combined into a single number to determine the overall water quality status at a certain time and location (Yisa and Jimoh, 2010; Leščešen et al., 2015; Ashraf and Hanafiah, 2017; Hanafiah et al., 2018a, 2019). However, the water quality parameters used were varied. Meher et al. (2015) have reportedly utilized 14 parameters for determining the WQI of the Ganges River, including pH, total dissolved solids (TDS), alkalinity, dissolved oxygen (DO), conductivity, and turbidity. On the other hand, Al-Shujairi (2013) has developed WQI to evaluate the water quality of two rivers in Iraq by utilizing seven water quality parameters of pH, DO, TDS, total hardness, biochemical oxygen demand (BOD_5), nitrate (NO_3), and phosphate, respectively.

The physicochemical analysis of soil is another important factor in the agriculture sector for plant growth, crop nutrient management, and soil management. This assessment can aid farmers in managing the nutrient input for crops during cultivation. Several

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parameters can be employed for the monitoring of agricultural soil, including electrical conductivity (EC), soil organic matter (SOM), and heavy metal analysis. Aimrun et al. (2011) have reported that EC can provide information regarding soil texture, thereby permitting the estimation of the water content. In addition, EC can serve as a proxy for the physicochemical properties of soil, such as SOM, cation content, and CEC (Liu et al., 2011; Li et al., 2013; Wang et al., 2016, 2018). Several studies of soil in paddy fields have been conducted in Malaysia. Aimrun et al. (2011) have conducted a study regarding paddy soil properties and yield characteristics based on EC, while Khairiah et al. (2009) have conducted a study focusing on the heavy-metal content in paddy soil in Kedah. Physicochemical analysis of soil properties in rice fields can serve as a basis and reference to improve the rice cultivation process and reduce the environmental pollution in rice fields.

In recent years, as a result of increasing concerns of environmental pollution due to the application of chemical fertilizers, several efforts have been made to replace chemical fertilizers by organic fertilizers (Zhao et al., 2015; Lenka et al., 2016). Previously, Jat et al. (2015), Singh et al. (2017), and Thakur et al. (2016) have independently reported that organic rice cultivation increases rice production. However, the environmental impact also should be emphasized while simultaneously aiming for high rice productivity to ensure environmental sustainability. Currently, only a few studies on the impact of conventional and organic rice cultivations on the environment in Malaysia have been conducted. Accordingly, it is imperative to conduct a performance evaluation study on the physicochemical characteristics of water and soil for the purpose of increasing the rice yield and sustaining its production to meet the requirements in Malaysia. Hence, this paper aims to determine the physicochemical properties of water and soil for conventional and organic rice cultivations.

Materials and Methods

Samples collection and preservation

The water and soil samples were collected at Sabak Bernam, Selangor, Malaysia, from conventional and organic rice fields (3.684153°N, 101.022841°E) (*Figure 1*). Soil and water samples were collected from six sampling stations consists of conventional and organic rice fields. In total, 18 sampling points were included in the analysis. Water and soil samples were placed in polyethylene bottles and plastic bags, respectively. Water samples were preserved at 4–5°C to minimize biological activity and chemical changes. Soil samples were dried at room temperature in the laboratory before analysis was conducted. For BOD_5 tests, samples were collected using glass bottles wrapped with an aluminum foil to avoid the penetration of sunlight into the bottles. Samples for heavy metal tests were placed in different 50-mL polyethylene bottles. Samples were acidified to a pH less than 2 by the addition of HNO_3 to the samples. All samples that could not be analyzed immediately in the field were preserved following the method recommended by

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GEMS (1978) and were transported to the laboratory within 24 h of sampling. *Table 1* summarizes the description of each sampling station.

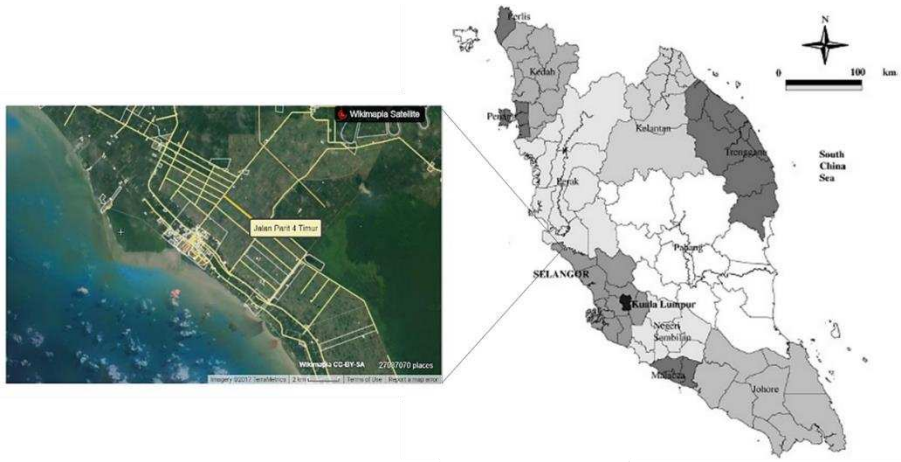

Figure 1. *Location of conventional and organic rice fields for water and soil sampling*

Table 1. *Description of each sample based on the sampling station*

Sampling station	Description
S1; P3	Located at the inlet of conventional rice field.
S2; P2	Located at the conventional rice field. There are paddy planting activities occurred such as fertilizing and pesticide application.
S3; P3	Sampling was done at the outlet of conventional rice field.
T1; Q1	Located at the inlet of organic rice field.
T2; Q2	Located at the middle of organic rice field. There are organic paddy planting activities occurred such as application of effective microbes.
T3; Q3	Located at the outlet of organic rice field.

S = represent water samples in the conventional rice field, T = represent water samples in the organic rice field, P = represent soil samples in the conventional rice field, Q = represent soil samples in the organic rice field

Water quality was analyzed according to temperature, pH, DO, BOD_5, COD, ammoniacal nitrogen, total suspended solid (TSS), and heavy metals. DO values, water temperature, and pH values were measured in situ using a YSI Model 556 Multi Probe system. COD, TSS, BOD_5, and ammoniacal nitrogen were analyzed in the laboratory following American Public Health Associations Standard (APHA, 1998) standard procedures. *Table 2* summarizes the parameters, methodology, and instruments used in this study.

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Table 2. *Parameters, applied methodologies, and instruments used in this study for the physicochemical analysis of water and soil*

Parameters	Methodology	Instrument
Water analysis		
Temperature	-	YSI Pro2030 DO Meter
pH	-	pH meter HI8424
Dissolved oxygen (DO)	-	YSI Pro2030 DO Meter
Bio-chemical oxygen demand (BOD_5)	BOD_5	YSI 5000 Dissolved Oxygen meter
Chemical oxygen demand (COD)	Closed reflux	Hach DR 6000 spectrophotometer
Ammoniacal nitrogen	Direct Nesslerization	
Total suspended solid (TSS)	Photometric	
Heavy metals	EPA Method 200.8	Inductively Coupled Plasma Mass Spectrometry (ICP-MS)
Soil analysis		
pH	Was determined in distilled water with ratio 1:2.5 of soil: distilled water	pH meter (DELTA 320 model)
Organic matter	Gravimetric method based on loss on ignition	Oven, furnace, weighting scale
Electrical conductivity	Was determined in saturated extract of gypsum	EC meter Model H 18819 Hanna
Particle size distribution	Pipette method together with dry sieving	Sieve (ASTM)
Cation exchange capacity	EPA Method 9081: Sum of basic cations with acidic cations through summation method	Centrifuge tube and stopper, mechanical shaker, volumetric flask 100mL, Inductively coupled plasma atomic emission spectroscopy (ICP-AES)
Heavy metals	Acid digestion Method 3050B	Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

Water quality analysis

The use of water is classified based on six classes (i.e., class I, IIA, IIB, III, IV, and V) by the Department of Environment (DOE, 2006). To determine the quality of water samples under the National Water Quality Standards for Malaysia (NWQS), WQI was calculated by the following equation *Eq. 1*:

$$WQI = (0.22 \times SIDO) + (0.19 \times SIBOD) + (0.16 \times SICOD) + (0.15 \times SIAN) + (0.16 \times SISS) + (0.12 \times SIpH) \quad \text{(Eq.1)}$$

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where,
SIDO = SubIndex DO (% saturation),
SIBOD = SubIndex BOD,
SICOD = SubIndex COD,
SIAN = SubIndex SS,
SIpH = SubIndex pH,
$0 \leq WQI \leq 100$.

Statistical analysis

Statistical analysis was done using the statistical package for social science software (SPSS). Data for physicochemical parameters of water and soil samples were represented as mean values. Independent sample t-tests were conducted to compare the results obtained for the water quality and physicochemical analysis of soil in different rice cultivation systems.

Results and discussion

Physicochemical properties of water in rice fields

Water quality is one of the key factors in the agriculture sector that ensures the healthy growth of crops (Harun and Hanafiah, 2018a, 2018b; Hanafiah et al., 2020; Nizam et al., 2020). The Department of Environment (DOE), Malaysia, has set six parameters to calculate the National Water Quality Index (Alssgeer et al., 2018; Hanafiah et al., 2018b; Ariffin et al., 2019): pH, DO, BOD_5, COD, ammoniacal nitrogen, and suspended solids. *Table 3* summarizes the physicochemical characteristics of water in conventional and organic rice fields.

pH is important for determining not only if a chemical or biological reaction can occur but also the degree of toxicity of some pollutants in water (Basheer et al., 2015). All samples were slightly acidic in the range from pH 5.41 ± 0.12 to 5.86 ± 0.18 for conventional rice fields and from pH 4.24 ± 0.13 to 4.50 ± 0.12 for organic rice fields (*Table 3*). Based on the NWQS for Malaysia, the average pH for conventional rice was categorized into class III, while the average pH for organic rice was categorized into class IV. Hence, the average pH values for conventional and organic rice are within the permissible range for irrigated agriculture. Results obtained from the independent sample t-test revealed a significant difference between conventional and organic rice ($p < 0.001$).

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Table 3. *Water analysis of conventional and organic rice fields*

Parameter	Conventional rice				Organic rice			
	S1	S2	S3	$S_{average}$	T1	T2	T3	$T_{average}$
pH	5.86±0.18	5.41±0.12	5.58±0.10	5.62	4.24±0.13	4.50±0.12	4.39±0.25	4.38
DO (mg/L)	5.70±0.22	6.57±0.29	4.15±0.14	5.47	4.28±0.07	5.46±0.41	3.45±0.45	4.40
BOD_5 (mg/L)	1.16±0.32	2.48±0.11	1.27±0.20	1.64	0.90±0.30	1.44±0.19	0.62±0.35	0.99
COD (mg/L)	11.45±3.48	29.88±0.32	27.68±1.45	23.00	27.16±2.02	47.83±2.84	38.09±1.62	37.69
NH_3-N (mg/L)	0.57±0.03	2.44±0.04	1.38±0.02	1.46	0.32±0.03	1.23±0.02	0.85±0.04	0.80
TSS (mg/L)	43.26±0.35	73.17±0.82	41.65±0.46	52.69	53.86±1.68	85.03±3.63	45.98±0.65	61.62
As (µg/L)	2.42±6.13E-05	3.38±3.30E-05	3.74±6.24E-05	3.18	2.57±2.55E-05	6.94±4.85E-05	6.54±3.69E-05	5.35
Cd (µg/L)	1.30±1.63E-05	8.81E-01±1.21E-05	1.50±6.11E-05	294.60	1.16±2.77E-05	7.70±1.84E-04	3.51E-01±1.73E-06	119.95
Cr (µg/L)	2.85±3.51E-05	2.84±2.95E-05	2.28±3.15E-05	2.66	5.93E-01±4.33E-05	1.24±1.67E-05	1.09±2.52E-05	198.44
Cu (µg/L)	11.70±6.11E-05	7.86±4.61E-05	11.80±1.30E-05	10.456	11.40±2.57E-05	15.70±1.10E-04	8.77±1.50E-05	11.96
Fe (µg/L)	7.23E02±4.60E-04	1.18E02±8.94E-04	1.40E02±4.24E-04	1101.00	7.46E02±9.45E-03	5.36E02±2.25E-03	5.14E02±7.07E-04	3748.67
Mn (µg/L)	31.30±4.11E-04	77.80±1.73E-02	87.90±5.13E-05	65.67	31.60±2.14E-05	65.90±1.11E-04	56.00±9.42E-05	51.17
Ni (µg/L)	3.46±1.06E-04	4.66±1.80E-05	4.10±2.95E-05	4.07	3.27±1.40E-05	3.59±2.65E-05	3.32±5.13E-06	3.39
Pb (µg/L)	9.57±3.04E-04	7.11±3.21E-05	12.30±1.91E-05	9.66	9.60±1.90E-05	9.94±3.95E-05	10.2±2.36E-05	9.91
Zn (µg/L)	4.10E02±1.02E-04	1.47E02±1.97E-04	2.19E02±1.57E-04	258.67	3.85E02±5.17E-03	1.53E02±2.49E-04	1.60E02±1.66E-04	232.67

*dissolved oxygen (DO), chemical oxygen demand (COD), biochemical oxygen demand (BOD_5), total suspended solids (TSS), ammoniacal nitrogen (NH_3-N), arsenic (As), cadmium (Cd), chromium (Cr), iron (Fe), manganese (Mn), lead (Pb), zinc (Zn) and nickel (Ni)

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On average, the conventional rice value was 1.24 greater than the organic rice value. pH values for conventional rice fields were greater than those observed for the water samples in organic rice fields due to the application of urea in the conventional rice field. The application of urea in agriculture leads to increased pH (Whalen et al., 2000; Liu et al., 2010). Among conventional rice samples, sample S2 exhibited the lowest pH as the sampling point was located in the rice field, which was related to the decay by stagnant water in the rice field (Lee et al., 2015). pH values reported herein were comparable with those (pH 5.51–5.99) reported previously by Lee et al., (2015). The pH values recorded for water were within the permissible range for irrigation purposes as specified by the DOE.

Average ranges of DO recorded for conventional and organic rice were 4.15 ± 0.14 mg/L to 6.57 ± 0.29 mg/L and 3.45 ± 0.45 mg/L to 5.46 ± 0.41 mg/L, respectively (*Table 3*). Based on the NWQS for Malaysia, the average DO for conventional rice was categorized into class II, while the average DO for organic rice was categorized into class III. Hence, the recorded DO values of water are within the permissible range for irrigation and fishery purposes as specified by the DOE, Malaysia. Results obtained from the independent sample t-test revealed that significant differences are observed between conventional and organic rice ($p < 0.05$). On average, the conventional rice value was 1.08 greater than the organic rice value. Among conventional rice samples, sample S2 exhibited the highest mean DO value, while among organic rice samples, sample T2 exhibited the highest mean DO value due to the location of both samples S2 and T2 in the rice field. Adequate DO is a major indicator for good water quality as DO is crucial for the survival of aquatic organisms. Ariffin et al. (2019) have reported that aquatic life experiences stress if the water oxygen levels drop to less than 5.00 mg/L, and some fish species (e.g., catfish and tilapia) require a minimum DO of 3.00 mg/L for survival. The DO value for conventional rice was greater than that for organic rice due to the depletion of oxygen water by organic materials used in rice farming (Xu et al., 2017). Al-Shami et al. (2010) have reported that DO values for rice fields are greater due to the photosynthesis by algal populations. Moreover, the strong wind and shallow water of rice fields lead to high water turbulence, leading to rich DO (Frei and Becker, 2005; Nugraheni, 2017; Sule et al., 2018).

Table 3 shows the average ranges of BOD_5 recorded for conventional and organic rice were 1.16 ± 0.32 mg/L to 2.48 ± 0.11 mg/L and 0.62 ± 0.35 mg/L to 1.44 ± 0.19 mg/L, respectively. Based on the NWQS, the average BOD_5 for conventional rice was categorized into class II, while the average BOD_5 for organic rice was categorized into class I. Hence, the recorded BOD_5 values of water are within the permissible range for irrigation and fishery purposes as specified by the DOE, Malaysia. Results obtained from the independent sample t-test revealed a significant difference between conventional and organic rice ($p < 0.05$). On average, the conventional rice value was 0.65 greater than the organic rice value. The BOD_5 values for conventional rice were greater than those for organic rice due to the fertilization activity. The highest BOD_5 values were observed for

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sample S2 (conventional rice) and sample T2 (organic rice) due to their sampling location in the rice field. During the cultivation phase, the BOD_5 value increased due to the decay process as well as other contributors such as the fertilizer application, which increased the organic content of water bodies (Hanafiah et al., 2018c). Ariffin et al. (2019) have reported that the nutrient content of chemical fertilizers can lead to the increase in the microorganisms in water, thereby contributing to the high BOD_5 values.

Average ranges of COD recorded for conventional and organic rice were 11.45 ± 3.48 mg/L to 29.88 ± 0.32 mg/L and 27.16 ± 2.02 mg/L to 47.83 ± 2.84 mg/L, respectively (*Table 3*). Based on the NWQS, the average COD for conventional rice was categorized into class II, while the average COD for organic rice was categorized into class III. Hence, the recorded COD values of water are within the permissible range for irrigation and fishery purposes as specified by the DOE, Malaysia. Results obtained from the independent sample t-test revealed a significant difference between conventional and organic rice ($p < 0.01$). On average, the organic rice value was 14.69 greater than the conventional rice value. The COD values for organic rice were greater than those for conventional rice due to the level of organic matter resulting from the organic fertilization of the organic rice field (Ahmad et al., 2014).

Average ranges of NH_3-N recorded for conventional and organic rice were 0.57 ± 0.03 mg/L to 2.44 ± 0.04 mg/L and 0.32 ± 0.03 mg/L to 1.23 ± 0.02 mg/L, respectively *(Table 3*). Based on the NWQS, the average NH_3-N for conventional rice was categorized into class IV, while the average NH_3-N for organic rice was categorized into class I. Hence, the recorded NH_3-N values of water are within the permissible range for irrigation and fishery purposes as specified by the DOE, Malaysia. Results obtained from the independent sample t-test revealed a significant difference between conventional and organic rice ($p < 0.05$). On average, the conventional rice value was greater than the organic rice value by 0.66. NH_3-N values for conventional rice were greater than those for organic rice due to the high application of urea in the conventional rice field. The decay of discharged organic waste in water can lead to the increase in the ammonia concentration (Li et al., 2008; Asman et al., 2017). Yang et al. (2017) have reported that a high pH value for the conventional rice field leads to a high ammoniacal nitrogen value as pH can increase the rate of dissolved ammonia available for volatilization.

Average ranges of TSS recorded for conventional and organic rice were 41.65 ± 0.46 mg/L to 73.17 ± 0.82 mg/L and 45.98 ± 0.65 mg/L to 85.03 ± 3.63 mg/L, respectively as shown in *Table 3*. Based on the NWQS, the average TSS results for conventional rice and organic rice were categorized into class III. Hence, the recorded TSS values of water are within the permissible range for irrigation and fishery purposes as specified by the DOE, Malaysia. Results obtained from the independent sample t-test revealed no significant differences between conventional and organic rice ($p > 0.05$). On average, the organic rice value was 8.93 greater than the conventional rice value. The nature of the muddy condition of rice fields as well as cultivation activities such as ploughing have contributed to high TSS (Al-Shami et al., 2010). Moreover, the

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application of organic fertilizers in the organic rice field has increased the density of phytoplankton, thereby contributing to the increase in the TSS (Ahmed et al., 2013).

Results obtained from the independent sample t-test revealed a significant difference between conventional and organic rice for As, Fe, and Ni ($p < 0.01$) and Cr was $p < 0.001$. On average, the organic rice values for As and Fe were greater than the conventional rice values by 0.02 and 2.65, respectively. On the other hand, the average conventional rice values for Ni and Fe were greater than the organic rice values by 0.01 and 0.02, respectively. Harun and Hanafiah (2018a) have reported that the application of chemical fertilizers in the rice field leads to the increase in the heavy metal concentration. Notably, the organic rice field in this study was previously implementing conventional rice cultivation practices. Hence, similar results for heavy metals are observed for conventional and organic rice fields. The highest concentration of Fe was observed in the conventional and organic rice fields. Iron toxicity in lowland rice fields was related to the flooded condition and pesticide application during cultivation (Fageria, 2007).

Nutrients lost from the rice cultivation phase contaminate water bodies. Hence, concerns over the water quality of rice fields have increased in recent decades. The obtained results revealed that the WQI for conventional and organic rice is categorized into class III based on the WQI classification by the DOE, Malaysia. Hence, the WQI is suitable for irrigation and fishery activities. However, for water supply purposes, extensive water treatment needs to be conducted (Halim et al., 2017; Manikam et al., 2019). The results obtained herein were similar to those previously obtained by Ahmad et al. (2014) and Haque et al. (2010). In conclusion, the conventional rice cultivation in the examined rice field follows the permissible rate of fertilizer and pesticide applications. The results obtained herein can serve as a guideline for the future water quality management in rice field studies.

Physicochemical analysis of soil

Physicochemical analysis of soil for conventional and organic rice fields was performed according to parameters such as pH, SOM, EC, particle size distribution, and heavy metal analysis. *Table 4* summarizes the results obtained.

Average ranges of pH recorded for conventional and organic rice were 5.13 ± 0.02 to 5.30 ± 0.01 and 4.20 ± 0.10 to 4.43 ± 0.06, respectively (*Table 4*). Results obtained from the independent sample t-test revealed a significant difference between conventional and organic rice ($p < 0.001$). On average, the conventional rice value was 0.86 greater than the organic rice value. The results obtained herein were comparable with those obtained by Aishah et al. (2010) and Khairiah et al. (2009), with the soil pH ranges within 4.63-5.14 and 4.5–5.0, respectively. In addition, the soil pH values for rice cultivation recommended by MARDI were within the pH range of 5.5–6.5 (Aishah et al., 2010).

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Table 4. *Physicochemical analysis of soil*

Parameter	Conventional rice				Organic rice			
	P1	P2	P3	$P_{average}$	Q1	Q2	Q3	$Q_{average}$
pH	5.14±0.05	5.30±0.01	5.13±0.02	5.19	4.43±0.06	4.20±0.10	4.37±0.06	4.33
SOM (%)	1.90±0.02	3.33±0.15	2.66±0.12	2.63	2.77±0.15	3.68±0.16	3.15±0.05	3.20
EC (µS/m)	0.25±0.02	0.35±0.03	0.32±0.01	0.31	0.22±0.01	0.26±0.01	0.22±0.01	0.23
Silt (%)	65.00±2.00	62.33±0.58	56.00±3.61	61.11	59.67±2.72	68.67±3.51	63.00±4.00	63.78
Clay (%)	29.00±1.30	32.00±4.04	36.67±5.69	32.56	28.81±1.92	23.00±4.36	29.00±6.08	26.94
Sand (%)	6.00±0.44	5.67±2.00	7.33±2.65	6.33	11.52±0.52	8.33±3.21	8.00±2.18	9.28
As (µg/L)	31.0±4.48E-05	13.95±0.01	16.63±1.30E-05	20.5	27.49±1.93E-05	40.09±2.24E-05	75.09±1.08E-05	47.57
Cd (µg/L)	0.94±6.25E-05	1.45±5.19E-05	0.59±5.13E-06	20.57	0.81±1.31E-05	1.39±0.00	0.21±8.54E-06	0.81
Cr (µg/L)	1.7E02±7.69E-05	12.53E01±4.20E-05	1.41E02±2.02E-04	145.65	1.40E02±0.01	1.30E02±0.00	1.39E02±5.27E-04	136.72
Cu (µg/L)	5.4E01±6.54E-05	47.12±5.43E-05	46.427±6.52E-05	49.18	50.99±0.01	46.56±0.01	37.08±2.48E-05	44.88
Fe (µg/L)	11.7E04±0.01	71.75E03±0.01	84.99E03±3.31E-04	91.33E03	68.67E03±51.39	15.27E04±0.01	25.66E04±0.01	15.33E04
Mn (µg/L)	5.55E02±0.002	5.47E02±8.31E-05	3.47E02±4.67E-05	483.00	4.90E02±5.75E-05	2.78E02±9.29E-06	2.55E02±5.77E-05	341.20
Ni (µg/L)	36.99±6.29E-05	29.09±4.47E-05	28.39±9.51E-05	31.49	30.08±5.44E-05	22.86±4.74E-05	24.64±1.25E-05	25.86
Pb (µg/L)	2.24E02±5.9E-05	1.92E02±0.01	2.07E02±3.68E-05	207.67	1.99E02±8.18E-05	1.99E02±2.44E-05	2.05E02±2.13E-05	201.33
Zn (µg/L)	1.57E02±4.66E-05	1.05E02±0.01	1.50E02±5.38E-05	137.00	1.33E02±2.55E-05	1.33E02±6.87E-05	1.13E02±5.88E-05	126.51

* soil organic matter (SOM), electrical conductivity (EC), arsenic (As), cadmium (Cd), chromium (Cr), iron (Fe), manganese (Mn), lead (Pb), zinc (Zn) and nickel (Ni)

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In addition, the use of chemical fertilizers has led to the increase in the soil humus, thereby affecting the pH values (Ahmad et al., 2014). Previously, Angelova et al. (2013), Sarwar et al. (2008) and Smiciklas et al. (2008) have reported that the application of organic fertilizers in rice fields leads to the decrease in the pH values as the application of organic fertilizers in organic rice field contributes to the production of organic acids such as amino acids and humid acid during the mineralization of organic materials by heterotrophs and nitrification by autotrophs; hence, a low pH is obtained (Sarwar et al., 2008).

Table 4 shows the average ranges of SOM recorded for conventional and organic rice were 1.90 ± 0.02% to 3.33 ± 0.15% and 2.77 ± 0.15% to 2.77 ± 0.15%, respectively. Results obtained from the independent sample t-test revealed a significant difference between conventional and organic rice ($p < 0.05$). On average, the SOM of organic rice was 0.57 greater than that of conventional rice. The application of an organic fertilizer leads to a high content of organic matter in the soil (Kushwaha et al., 2001; Selvakumari et al., 2001; Smiciklas et al., 2008). Organic matter plays a key role in binding soil particles; hence, soil strength is enhanced. In addition, high organic matter can contribute to the high productivity (Hasan et al., 2020).

Soil EC is the ability of the soil to transmit an electrical charge (Chan et al., 2008). Average ranges of EC recorded for conventional and organic rice were 0.25 ± 0.02 mS/m to 0.35 ± 0.03 mS/m and 0.22 ± 0.01 mS/m to 0.26 ± 0.01 mS/m, respectively (*Table 4*). In this study, EC values were within the range of the suggested EC value (<2.70 mS/m) for rice cultivation in tropical Asia (MAFF, 1970). Results obtained from the independent sample t-test revealed a significant difference between conventional and organic rice ($p < 0.01$). On average, the conventional rice value was 0.073 greater than the organic rice value. In this study, urea was applied in the conventional rice field. The combination of urea and NPK fertilizers can afford a high EC value (Han et al., 2016). Selvakumari et al. (2001), and Smiciklas et al. (2008) have reported that the reading of EC can increase under acidic and alkaline conditions by the application of organic materials to the soil.

Table 4 shows the particle size distribution percentage. Based on the obtained results, the soil samples contain a high percentage of the silt and clay fraction (grain size < 63 μm) for conventional and organic rice fields. Based on the United States Department of Agriculture (USDA), the type of soil for conventional rice and organic rice herein was classified as silty clay loam and silty clay, respectively. Results obtained from the independent sample t-test revealed no significant difference between silt, clay, and soil for conventional and organic rice ($p > 0.05$). Dou et al. (2016) have reported that soil texture as well as the interaction between the water regime and cultivar affect the rice yield. In addition, the high clay soil can contribute to the high yield due to the finer particles of clay soil, which can retain water and nutrients better than sandy soil (Tsubo et al., 2007; Dou et al., 2016; Aboudi Mana et al., 2017).

Results obtained from the independent sample t-test revealed a significant difference ($p < 0.01$) between conventional and organic rice for As, Fe, Ni, and Cr ($p < 0.0001$).

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Previously, the organic field in this study was practicing conventional rice farming; hence, the difference between the metal results is nearly the same. The use of chemical pesticides can lead to the increase in the heavy metal content of the rice field (Aimrun et al., 2011; Jamil et al., 2011). Results revealed that among the metals tested, the total Fe content was the highest. However, the value was considered to be less than that reported previously by Khairiah et al. (2009), where Fe values ranged from 254 to 379 mg/kg. Jamil et al. (2011) have reported that flooded conditions in rice fields lead to the precipitation of dissolved Fe. Hence, the oxidized condition of Fe can occur as finely grained hydrous oxides ($Fe(OH)_3$) with a disordered structure; hence, mixing with clays is possible (Khairiah et al., 2009).

Conclusions

In this study, the water quality and soil physicochemical characteristics were assessed by laboratory analysis. We found that water quality measured from the organic and conventional rice fields was classified as Class III. Although similar water and soil quality obtained for all sampling stations from both organic and conventional rice fields, organic farming is a better alternative to overcome the environmental contamination that is caused from the constant utilization of chemical fertilizers. However, future study can be done by considering a longer temporal duration of the water and soil quality measurements for a better understanding of the physicochemical characteristics of water and soil in conventional and organic rice fields in Malaysia.

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DISTRIBUTION OF HEAVY METALS IN THE TOPSOIL OF AGRICULTURAL LAND IN NAM DINH PROVINCE, VIETNAM

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Abstract. Surface soil samples were collected from different land use types of the agricultural land in Nam Dinh province, Vietnam to determine the level of heavy metal contamination and identifying potential sources. The farming areas of rice and cereal show similar heavy metal contents, however, the index shows that the content of heavy metals in the aquacultural area is lower compared to the other types. The mean enrichment factor (EF) values of Cr, Cu, Pb, and Zn are less than 1.5, suggesting that these are not a major concern in the research area; whereas, the mean EF values for Cd is higher than 2 and As varies from 5-20 suggest that the pollution of these heavy metals is present in agricultural soil. The data analysis indicates that Cr mainly originates from a natural source; Cd and As have a significant anthropogenic input; Cd, Pb and Zn have a mixed source.
Keywords: *different land use, paddy, cereal, aquaculture, environment*

Introduction

Soil is widely recognized as a pool of nutrients and pollutants, and plays a critical role in socio-ecological stability and national safety (Wu et al., 2018). Human-induced increase of soil contamination with heavy metal input is noteworthy, including atmospheric deposition, irrigation, application of sewage sludge, organic manures, fertilizers, and other soil amendments (Cheng, 2003; Vodyanitskii, 2013; Hou et al., 2014). When heavy metals enter the agricultural soil, they not only degrade environmental quality but they also influence the health of people and other organisms through the food chain (Nabulo et al., 2010). Therefore, it is of great importance to study the characteristics and sources of heavy metal contamination in agricultural soil to protect the environment and human health (Huang et al., 2018; Li et al., 2018).

Agriculture plays a prominent role in Nam Dinh province, with approximately 67% of the land used for agricultural production. It is usually used for 2 paddy rice crops per year equipped with a developed irrigation system (in total 3% of the province area are covered by rivers and channels (Dao et al., 2005). Traditionally, the biggest industry is textile and garment which has been developed since 1889 with French support. The craft production in Nam Dinh, especially bronze and silver casting, has a long tradition. For example, Tong Xa village has been well known for bronze and iron casting in the Red River delta since 1200, Xuan Tien commune with bronze casting and mechanical engineering since 1535, and Van Chang village with iron, steel and aluminum foundry since 1868. According to the data provided by provincial

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government, there are a total of 71 villages that are specialized on handicraft (e.g. metalworking, food processing, salt production) up to 2018 in Nam Dinh province (Joern et al., 2013). Such production places are most often based on family enterprises located all over the village; therefore, it can be stated that living and working place are rarely distinguished (Dao and Nguyen, 2000). However, all untreated wastewater from industrial and living usages discharges directly open channels or infiltrate into the soil. Previous research shows that water channels in handicraft villages are loaded with heavy metals (Zn, Pb, Cu, Ni, Cd, Cr, and Fe) and cyanides, exceeding the limits by up to 50 times (Le et al., 2003). In recent years, with the increase in population, the industrial sector has achieved prominence. This has resulted in environmental pollution caused by manufacturing activities in industrial zones. Furthermore, the Red and Day rivers receive pollutants from upstream before running across the province. Agricultural activities in the province utilize approximately 300 tons of insecticides per year (Dao et al., 2005), which causes the water to be polluted, that is then returned to the soil via the irrigation network.

Thus far, there has been very little published information on their contaminant level and sources in Nam Dinh province. To better investigate local soil quality, we have used a combined method of enrichment factor (EF) and multivariate statistical analyses with the objectives of analyzing pollution level, characteristics of spatial distribution and identifying potential sources of heavy metals in agricultural soil.

Materials and methods

Study area

Nam Dinh (19°52’ - 20°30’N, 105°55’ - 106°35’E) is a coastal province located to the south of Red river delta with an area of 1,652.6 km² and population of 1,825,771 people; population density is 1,196 people per km². The administrative unit of this province is composed of 9 districts and one city. Nam Dinh is a tropical area experiencing monsoon with hot and humid rain. The average annual temperature is ca. 23 – 24 °C, and the average annual rainfall is ca. 1,700 - 1,800 mm. On average, up to 250 days a year are sunny, with total sunshine hours from 1650 to 1700 h. The province is affected by storms or tropical depressions 4 to 6 times per year.

The river and estuary systems of the Red river and its tributaries (Dao, Ninh Co, Day rivers) have greatly impacted morphological and hydrological characteristics of the region. The terrain is quite flat, gradually dipping to the sea in Northwest - Southeast direction. The formation of this region was historically associated with and the evolution of the Red River Delta. It can be divided into two zones: (1) the low-lying plains with elevations of 0.2 to 3 m above sea level with some exceptions in the northwest (7-100 m above sea level) (Vu Ban, Y Yen, Nam Truc, Truc Ninh and Xuan Truong districts). This region has a high level of agriculture, textile and manufacturing industries; (2) the lowland coastal region including Giao Thuy, Hai Hau and Nghia Hung districts. Running from north to south, Dao river seperates the two regions. In the northern region of the province (My Loc, Y Yen, Vu Ban districts and Nam Dinh city), the water is pumped directly from stagnant canals which is not well circulated and used for irrigation purposes. In the southern region (Nam Truc, Truc Ninh, Xuan Truong, Hai Hau, Giao Thuy and Nghia Hung districts), irrigation water is taken from rivers, which is well circulated because water from upstream flows into the sea.

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Samples and methods

Samples

A total of 38 surface agricultural soil samples from different land use types were collected in May, 2018 by grid method (using 10 × 10 km grid). They included 20 samples from paddy field, 12 samples from cereals soil, and 6 samples from aquaculture farm (*Fig. 1* and *Table 1*). The samples were taken at depth of 0-20 cm with volume of 0.5 kg soil by using hand steel drill. Per site, two samples were taken. Samples were packed in plastic bags to send to the laboratory. All samples were dried at 40 °C, then desegregated prior to analysis.

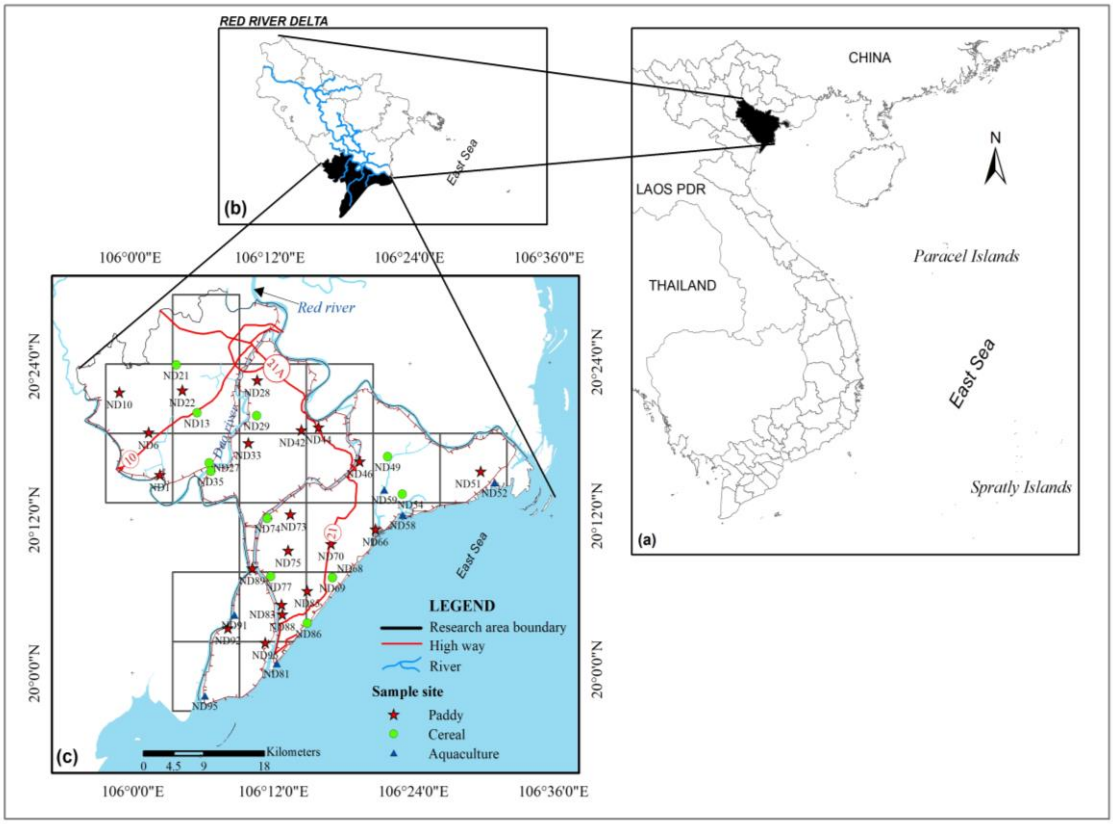

Figure 1. *Map of the study area (a), Red river delta (b), with the sampling sites detailed in (c)*

Methods

For heavy metal analysis, ~ 0.1 g dry soil was weighed into Teflon beakers, in which a mixture of concentrated 2 ml HF – 6 ml HCl – 2 ml HNO_3 were added (Elsorogy et al., 2016), a Teflon watch cover was put in place, and the sample was left at room temperature overnight. On the following day, the sample was digested using a scientific microwave system (Mars 6, USA) with the following heating program: the temperature rises from room temperature to 180 °C in 15 min, keep at 180 °C for decomposition for 30 min, then let solutions cool down to room temperature again. The solution is transferred to the plastic volumetric tube and filled up to the mark by using deionized water. Heavy metals (As, Cd, Cr, Cu, Pb, and Zn) concentration were determined by inductive couple plasma - mass spectrometry (ICP-MS, Agilent 7900). European

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commission community bureau of reference material Estuarine sediment sample identification No0087 was included for quality control. The analytical precision and error of the analysis are within 10%.

Table 1. *The global positioning system coordinates of the sampling points*

CODE	Yho	Ymi	Yse	Ydec	Xho	Xmi	Xse	Xdec	Land use type
ND1	20	15	30	20.25833	106	2	27	106.04083	Paddy
ND6	20	18	39	20.31083	106	1	26	106.02389	Paddy
ND10	20	21	57	20.36583	105	59	1	105.98361	Paddy
ND13	20	20	18	20.3383	106	5	40	106.0944	Cereal
ND21	20	24	3	20.4008	106	3	55	106.0653	Cereal
ND22	20	22	5	20.36806	106	4	22	106.07278	Paddy
ND27	20	16	19	20.2719	106	6	37	106.1103	Cereal
ND28	20	22	49	20.38028	106	10	51	106.18083	Paddy
ND29	20	20	3	20.3342	106	10	48	106.1800	Cereal
ND33	20	17	56	20.29889	106	10	4	106.16778	Paddy
ND35	20	15	49	20.2636	106	6	53	106.1147	Cereal
ND42	20	18	54	20.315	106	14	37	106.24361	Paddy
ND44	20	19	7	20.31861	106	15	66	106.26833	Paddy
ND46	20	16	30	20.275	106	19	30	106.325	Paddy
ND49	20	16	46	20.2794	106	22	1	106.3669	Cereal
ND51	20	15	33	20.25917	106	30	0	106.5	Paddy
ND52	20	14	39	20.2442	106	31	11	106.5197	Aquaculture
ND54	20	13	50	20.2306	106	23	15	106.3875	Cereal
ND58	20	12	11	20.2031	106	23	17	106.3881	Aquaculture
ND59	20	14	10	20.2361	106	21	42	106.3617	Aquaculture
ND66	20	11	6	20.185	106	20	55	106.34861	Paddy
ND68	20	7	21	20.1225	106	17	12	106.2867	Cereal
ND69	20	7	21	20.1225	106	17	12	106.2867	Cereal
ND70	20	10	3	20.1675	106	17	1	106.28361	Paddy
ND73	20	12	12	20.20333	106	13	38	106.22722	Paddy
ND74	20	12	2	20.2006	106	11	39	106.1942	Cereal
ND75	20	9	30	20.15833	106	13	25	106.22361	Paddy
ND77	20	7	29	20.1247	106	11	55	106.1986	Cereal
ND81	20	0	40	20.0111	106	12	23	106.2064	Aquaculture
ND83	20	5	17	20.08806	106	12	49	106.21361	Paddy
ND85	20	6	21	20.10583	106	15	2	106.25056	Paddy
ND86	20	3	48	20.0633	106	14	60	106.2500	Cereal
ND88	20	4	31	20.07528	106	12	52	106.21444	Paddy
ND89	20	8	9	20.13583	106	10	20	106.17222	Paddy
ND91	20	4	29	20.0747	106	8	46	106.1461	Cereal
ND92	20	3	29	20.05806	106	8	12	106.13667	Paddy
ND95	19	57	0	19.9500	106	6	24	106.1067	Aquaculture
ND96	20	2	19	20.03861	106	11	24	106.19	Paddy

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Total organic carbon was determined by titration method using Mohr salt $(NH_4)_2Fe(SO_4)_2.6H_2O$ after digestion of the sample by mixture of $K_2Cr_2O_7$-H_2SO_4, which followed the method of ISO 14235:1998. Grain-size distribution of desalted sediments was determined by wet sieving of sand and gravel and by the pipette technique for silt and clay fractions according to Vietnam Standards: Soil quality – Method for determination of particle size distribution (TCVN 8567:2010).

Enrichment factor (EF) is commonly used to discern metal contamination. EF is calculated as follows (*Eq. 1;* Salomons and Forstner, 1984; Sinex and Wright, 1988):

$$A = \frac{\left(\frac{Me}{Al}\right)_{Sample}}{\left(\frac{Me}{Al}\right)_{Background}} \quad \text{(Eq.1)}$$

where $(Me/Al)_{sample}$ is the metal to aluminum (Al) ratio in the samples; $(Me/Al)_{background}$ is the metal to Al ratio in background. Because Al is one of the most abundant elements on the earth and its concentration is generally not influenced by anthropogenic sources, it is commonly used for normalization purpose (Schropp and Windom, 1988). There is little information about heavy metal background values in the Nam Dinh province. Therefore, we adopted the values of upper continental crust (Taylor and McLennan, 1995) as the background values, which are (in $mg.kg^{-1}$): 80,400 for Al; 0.098 for Cd; 25 for Cu; 20 for Pb, and 71 for Zn. For As and Cr, the updated values of 5.7 $mg.kg^{-1}$ and 73 $mg.kg^{1}$, respectively were used (Hu and Gao, 2008). This approach has been widely used to determine the source heavy metal pollution in soil environment (José et al., 2017; Dragović and Mihailović, 2009; Loska et al., 2004).

Multivariate statistical methods such as cluster analysis (CA), principal component analysis (PCA) provide a classification tool based on the relationship between different metals in different sampling points, which can be used to distinguish between the natural and anthropogenic sources of heavy metal (Wang et al., 2019b; Han et al., 2006; Wu and Zhang, 2010; Nguyen et al., 2016a).

Cluster analysis was performed on the heavy metal concentrations and soil properties using SPSS 20.0 software (IBM, USA) using nearest neighbor linkage method based on correlation coefficients. The distance cluster represents the degree of association between elements, the smaller the value on the distance cluster, the more significant the association (Luo et al., 2007).

Principal component analysis using SPSS 20.0 software (IBM, USA) was used in the data set to determine the relationships and common origins between metals. PCA was performed with Varimax rotation with Kaiser Normalization, which facilitated the interpretation of output by minimizing the number of variables that loaded high loads on each component. In this study, all principal factors extracted from the variables were retained with eigenvalues > 1.0, as suggested by the Kaiser criterion (Kaiser, 1960). According to the results obtained from PCA, possible sources of chemical elements were interpreted (Lu et al., 2012).

In this paper, Inverse Distance Weighted which one of tool on ArcGIS 10.5 software is used for interpolating map of heavy metal content. The reclassify the results following the method of equal values to show the distribution of each heavy metal.

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Results and discussion

Soil properties in different land use types

Soil properties that could influence the accumulation ability of heavy metals include total organic carbon (TOC), pH, soil grain size, and concentration of aluminum (Al). The variation of these properties in the 38 samples is presented in *Table 2*. From *Table 2* we can recognize that TOC concentration varied among the land use types in Nam Dinh province. Highest TOC content occurred in the paddy soil (TOC_{mean} = 2.99%), which might be related to the tradition of applying organic fertilizers in rice production in this area (Nguyen et al., 2018). The second highest TOC content occurred in the cereals soil (TOC_{mean} = 1.09%), and the least in the aquaculture (TOC_{mean} = 0.58%).

The mean pH value in the studied area was 6.08. The pH values of the sampling sites ranged from 3.87 to 8.05, from extremely acidic to moderately alkaline. According to land use type, the mean pH of paddy soil was 5.6 ± 1.06, close to moderately acidic; the mean pH of cereal soil was 6.8 ± 1.08, near neutral; the mean pH of aquaculture soil was 7.82 ± 0.15, close to slightly alkaline.

Soil grain size varies in each land use type, with sand always being dominant and the mean clay percentage in different types of land use decreases in the order of paddy soil > cereal soil > aquaculture soil in the study area.

Table 2. *Soil properties of samples from different land use types*

Land use type	Parameter	pH	TOC (%)	Grain size			Al ($mg.kg^{-1}$)
				Sand (%)	Silt (%)	Clay (%)	
Paddy n = 20	Mean	5.6	2.99	34.76	36.6	28.64	46524.48
	Max	8.05	7.75	92.78	57.8	48.34	118010.8
	Min	3.87	0.22	6.16	2.52	4.12	2463
	SD	1.06	1.37	23.48	13.81	11.34	25161.74
Cereal n = 12	Mean	6.8	1.09	66.78	18.23	14.98	34433.53
	Max	7.83	3.08	98.18	41.5	35.98	77992.67
	Min	4.04	0.12	23.86	0	1.82	5710.1
	SD	1.08	0.71	25.59	14.43	11.64	22384.12
Aquaculture n = 6	Mean	7.82	0.58	72.32	14.19	13.49	49716.46
	Max	8.05	1	93.52	38	22.26	85773.7
	Min	7.59	0.06	41.78	0	6.48	12825
	SD	0.15	0.39	18.87	13.12	6.32	28360.69
All samples n = 38	Mean	6.08	2.34	45.45	30.38	24.17	44181.78
	Max	8.05	7.75	98.18	57.8	48.34	118010.8
	Min	3.87	0.06	6.16	0	1.82	2463
	SD	1.21	1.53	28.18	16.54	12.95	25149.49

Heavy metal concentration in agricultural soil in Nam Dinh province

Heavy metal concentrations in agricultural soil in Nam Dinh province are summarized in *Table 3* and *Figures 2-9*. The mean concentrations of heavy metals in agricultural soil are in decreasing order of Zn > Cr > Cu > Pb > As > Cd. A comparison of the concentrations of heavy metals in the studied soil with the documented data from

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agricultural soil areas in Vietnam show that the contents of As, Cr and Zn are close to previously reported result in the Ba Lat estuary of the Red River (located in Giao Thuy and Xuan Truong districts of Nam Dinh province), while the contents of Cu and Pb are lower 2 to 3 times. In comparison with heavy metal concentration in agricultural soil of Duy Tien district, Ha Nam province, which is also located in Red River delta, we found that the contents of As and Pb in this study are higher 2 and 2.5 times, respectively, while the contents of Cu and Zn are lower. The concentration of As, Cd is higher 5.3 and 1.2 times, respectively while the concentration of Cr, Cu, Pb and Zn is lower in comparison to their counterparts in the upper continental crust.

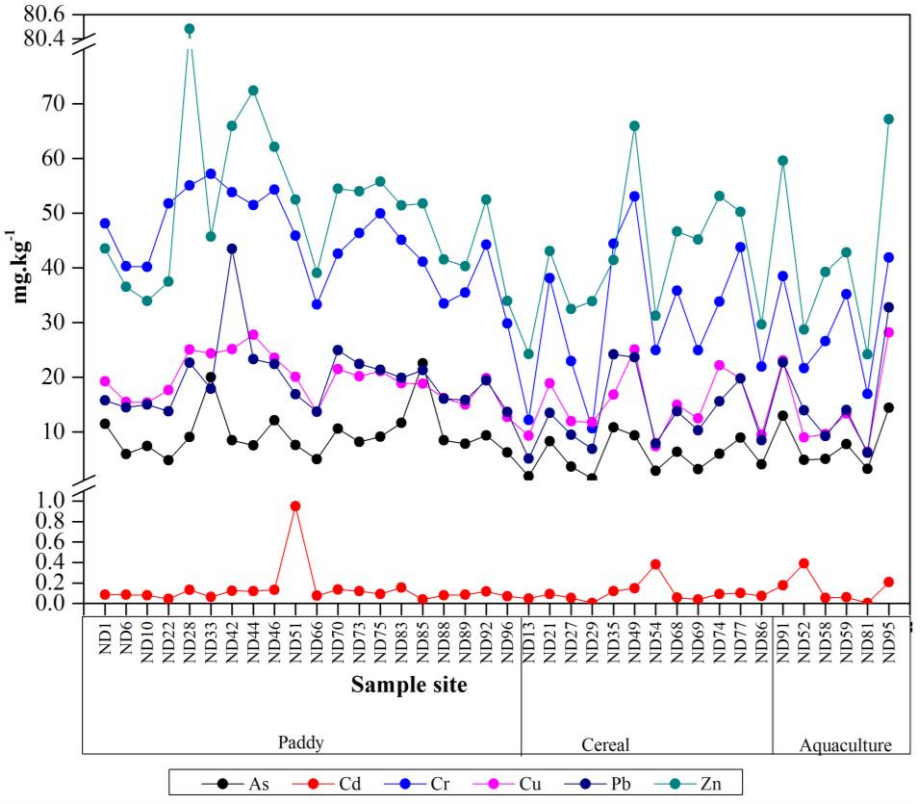

Figure 2. *Spatial variations of heavy metals of the agricultural soil in Nam Dinh province*

Concentrations of metals in soil from different land uses are also presented in *Table 3* and *Figure 3*. In general, paddy soil samples have the highest heavy metal concentration, which was followed by cereal soil samples and aquaculture soil samples. The concentrations of Cd do not exhibit a clear trend. The hotpots of higher Cd contents occurred in Giao Thuy district.

Assessment of heavy metal pollution

The enrichment factor (EF) values can give an insight into differentiating an anthropogenic source from a natural origin. Typically, an EF value of < 1.5 suggests a dominance of natural sources (Zhang and Liu, 2002). Further, EF values can also assist the determination of the degree of metal contamination. Five contamination categories are recognized on the basis of the enrichment factor (Sutherland, 2000; Loska and Wiechula, 2003) (*Table 4*).

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Table 3. *Heavy metal concentrations in the topsoil of the agricultural soil in Nam Dinh province, Vietnam*

	As	Cd	Cr	Cu	Pb	Zn	Mn	Al	Reference
	(mg.kg^{-1})						%		
Nam Dinh									
Mean±SD	7.99±4.36	0.12±0.16	37.82±12.33	16.96±5.99	16.78±7.53	45.74±13.49	0.06±0.03	6.01±2.79	This study
Min-Max	1.47-22.56	0.01-0.95	10.68-57.20	6.37-28.19	5.13-43.51	24.17-80.48	0.01-0.17	1.60-11.80	
Ba Lat									
Mean	14.5				43.4	59.5			Nguyen et al., 2016b
Min-Max	6.9-31.0	0.05-0.43	26.9-63.1	14.9-67.2	24.2-78.3	32.1-92.4			
Duy Tien, Ha Nam									
Mean	3.27	0.52		39.48	8.52	93.06			Phan and Tran, 2016
Min-Max	2.56-5.15	0.28-0.86		27.61-55.28	8.11-8.61	65.81-123.51			
Upper continental crust (UCC)	1.5	0.098	85	25	20	71			Taylor and McLennan, 1995

Table 4. *Contamination categories based on EF values*

EF < 2	Deficiency to minimal enrichment
EF = 2-5	Moderate enrichment
EF = 5-20	Significant enrichment
EF = 20-40	Very high enrichment
EF > 40	Extremely high enrichment

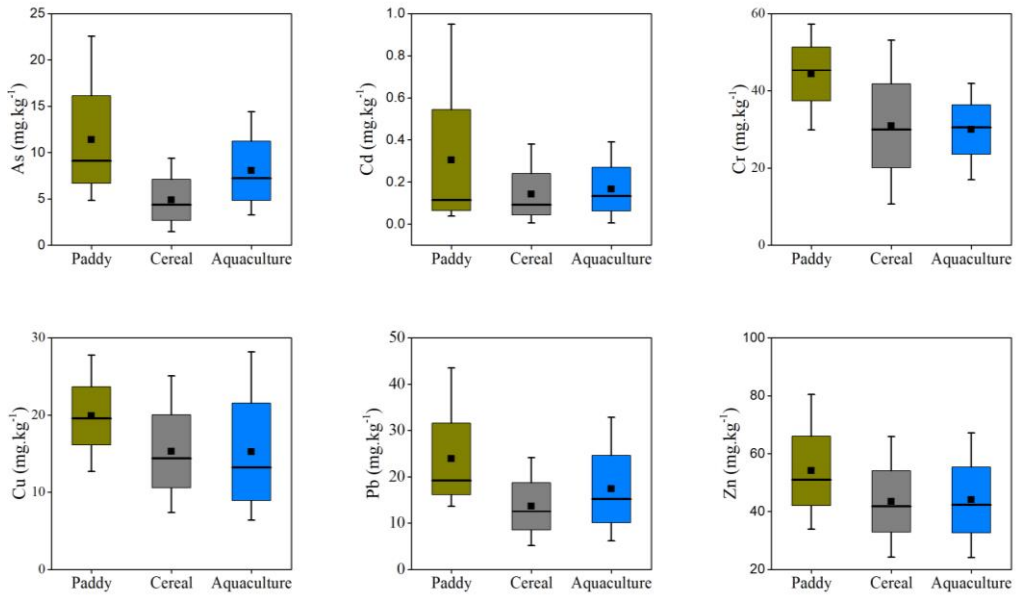

Figure 3. *Box plot of the heavy metal concentrations in soil samples of the three land use types. The black solid line inside the box is the median value, the black rhombus is the mean value, and the black vertical line is individual samples*

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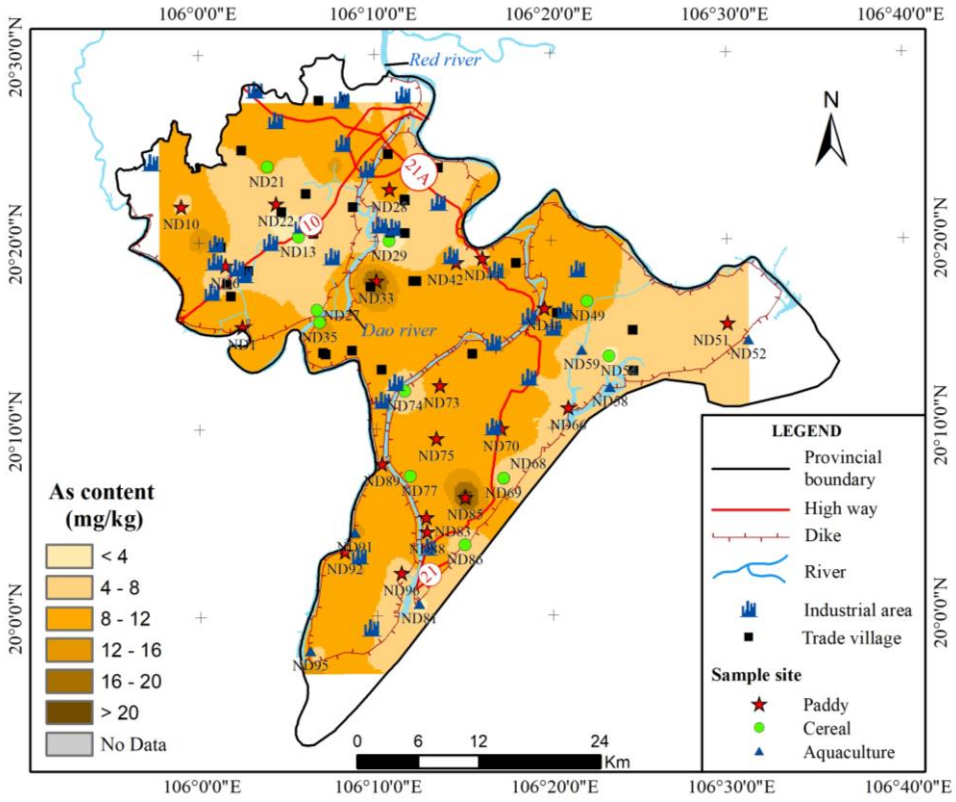

Figure 4. *Distribution of As in the topsoil of the agricultural soil in Nam Dinh province*

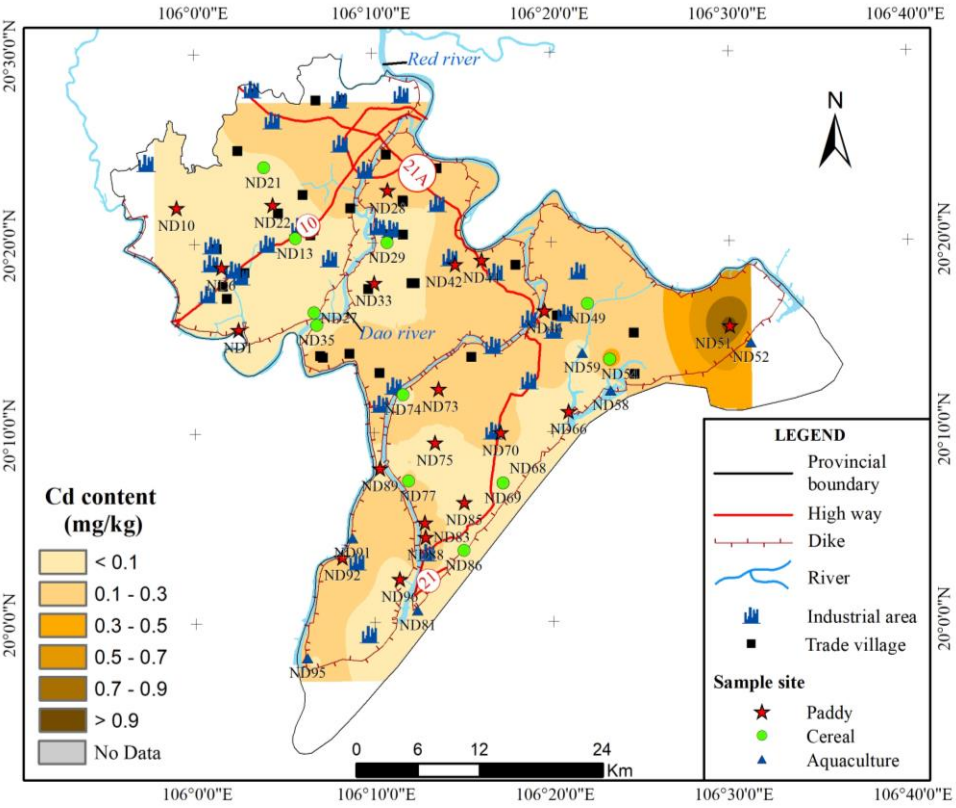

Figure 5. *Distribution of Cd in the topsoil of the agricultural soil in Nam Dinh province*

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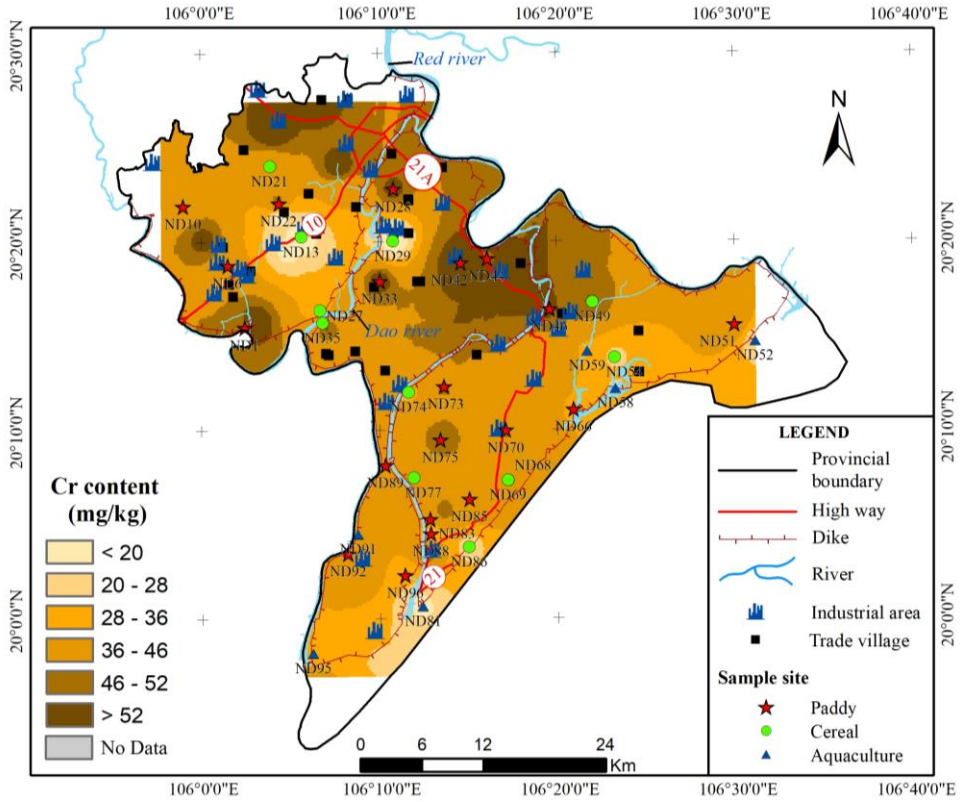

Figure 6. *Distribution of Cr in the topsoil of the agricultural soil in Nam Dinh province*

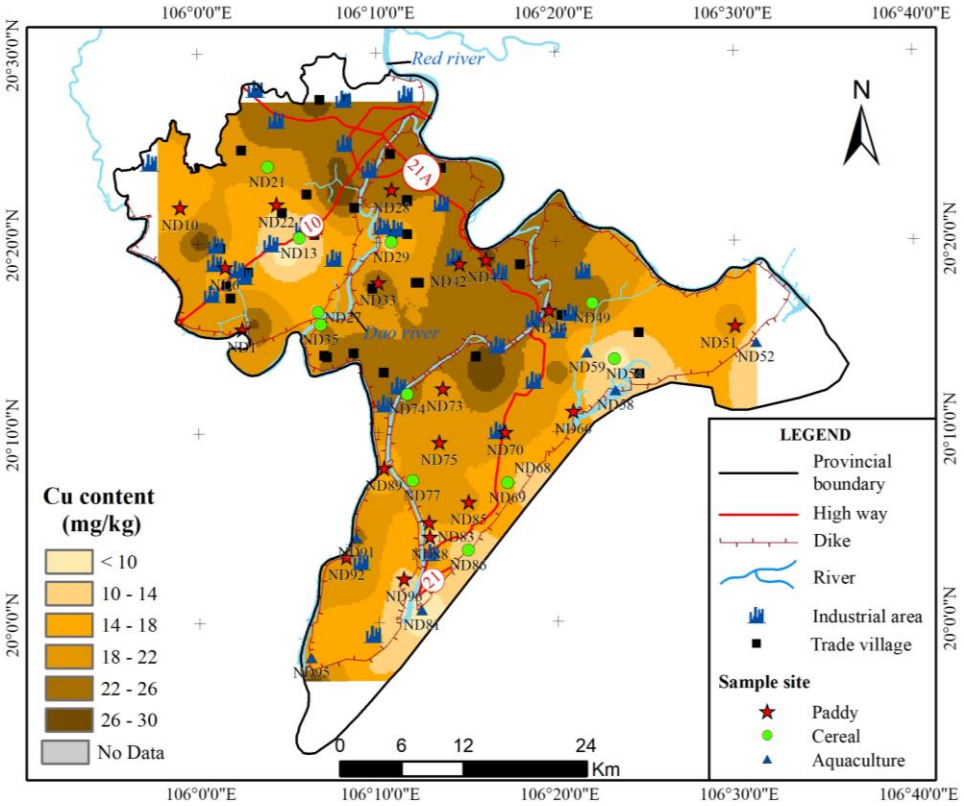

Figure 7. *Distribution of Cu in the topsoil of the agricultural soil in Nam Dinh province*

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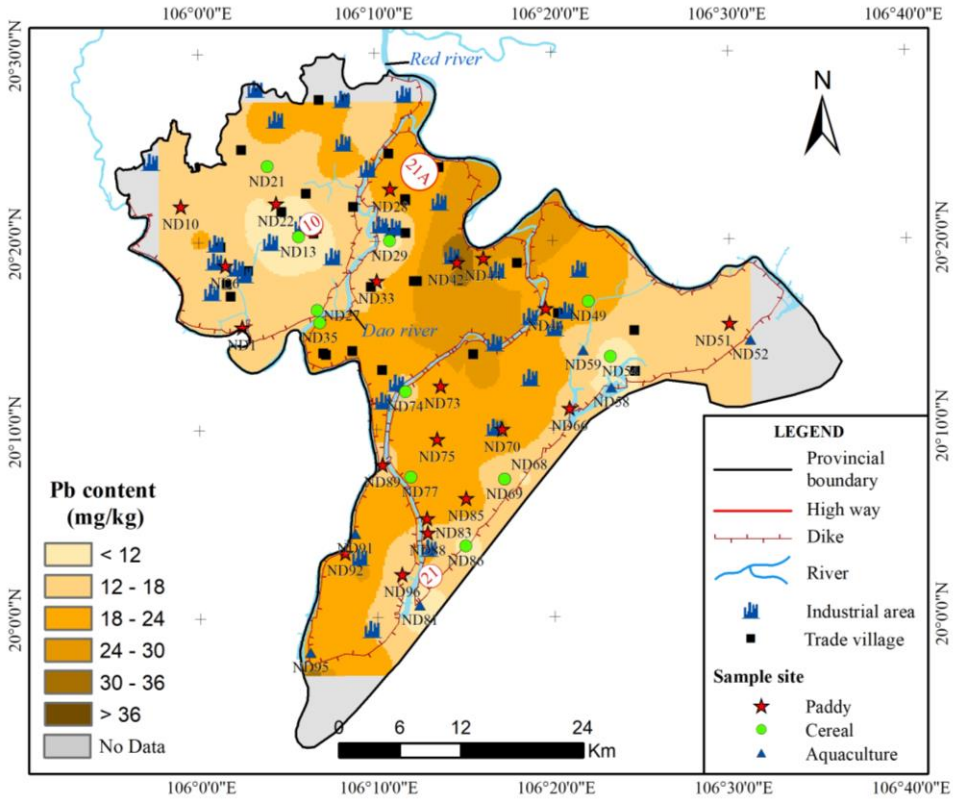

Figure 8. *Distribution of Pb in the topsoil of the agricultural soil in Nam Dinh province*

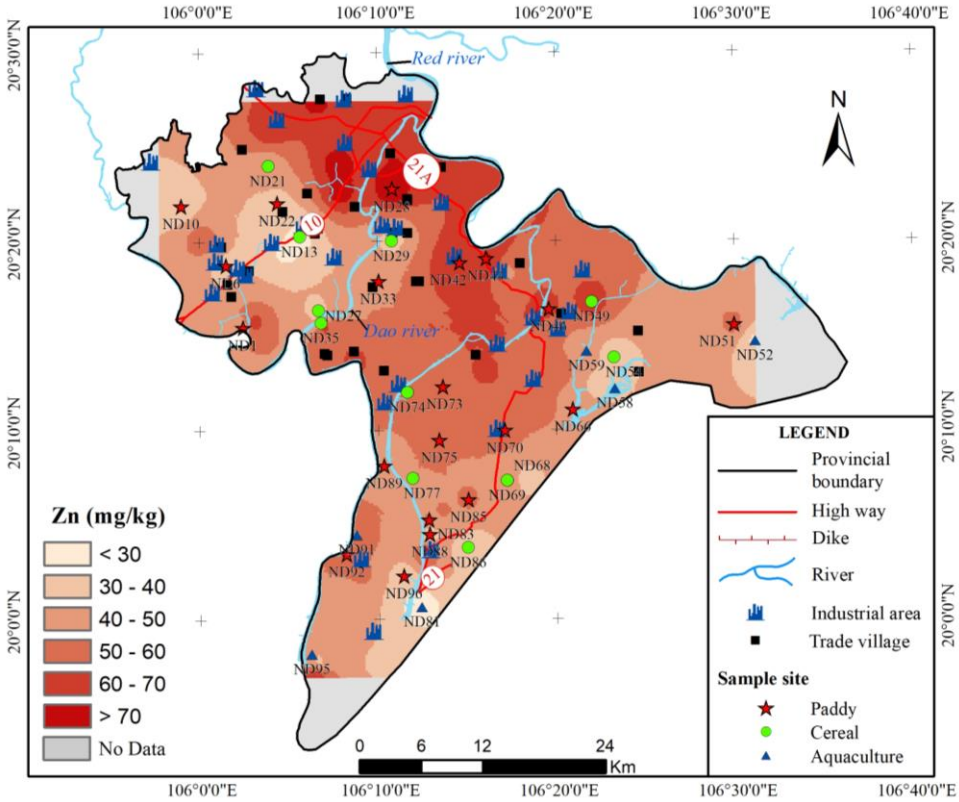

Figure 9. *Distribution of Zn in the topsoil of the agricultural soil in Nam Dinh province*

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The enrichment factors in agricultural soils in Nam Dinh province are shown in *Figure 10* and *Table* 5. It is clear that Cr, Cu, Pb, and Zn have a mean EF less than 1.5, confirming their mainly natural sources except some sites have EF values higher than 1.5 (e.g. ND29, ND35, ND73, ND96). On the other hand, the mean EF values of As and Cd are higher than 2, indicating existence of heavy metal contamination in agricultural soil of Nam Dinh province (Han et al., 2006). In this study, the mean EF values were ranked in the order of As > Cd > Pb > Cu > Zn > Cr. The element Cd has mean EF value between 2 and 5, which was classified as moderately contaminated. The mean EF values of As lie between 5 and 20, which means a significant contamination in this study area.

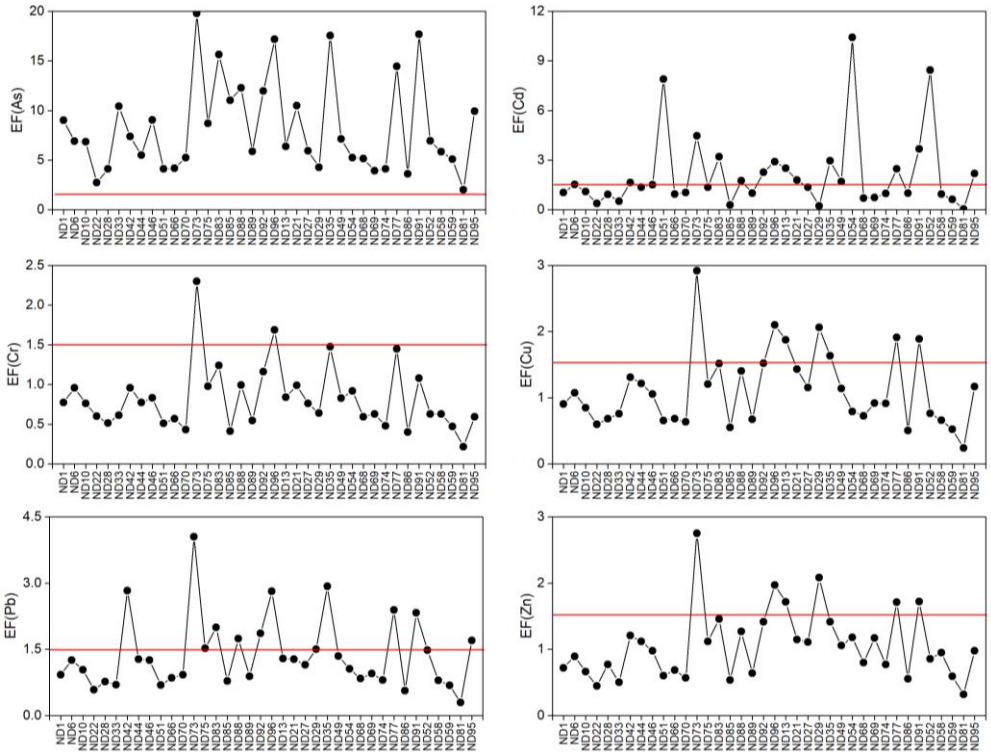

Figure 10. *Spatial variations of EF values of the agricultural soil in Nam Dinh province*

According to land use type and spatial distribution, the smallest mean EF values occurred in aquaculture soil samples, lower 1.4 to 2 times than its in paddy and cereal soil. There is no significant difference in the degree of metal contamination between paddy soil and cereal soil samples. The EF values of As show pronounced higher values in Nghia Hung, Hai Hau, and Nam Truc districts, these sites are localed near industrial zone and traditional handicraft village (e.g. ND88 near to the Thinh Long industrial zone; ND91 and ND92 near to Quy Nhat industrial zone, ND33 near to Dong Quy metal smelting village). The EFs (Cd) show higher in Hai Hau, Nghia Hung and Giao Thuy districts (ND51, ND52, ND54, ND73, ND83, ND92, ND96).

Sources of heavy metal in agricultural soil

Correlation analysis is not only an effective approach to reveal the relationships between heavy metals and soil physicochemical properties, but also an operative way to understand the controlling factors of heavy metals as well as their possible sources

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(Chai et al., 2015). Correlation analysis reveals diverse relationships between particle size, TOC and pH of soil and heavy metal contents (*Table 6*).

Table 5. *Enrichment factor (EF) of heavy metals for agricultural soil in Nam Dinh province*

Land use type	Parameter	EF(As)	EF(Cd)	EF(Cr)	EF(Cu)	EF(Pb)	EF(Zn)
Paddy soil n = 20	Mean	8.89	1.86	0.88	1.12	1.44	1.02
	Max	19.77	7.90	2.30	2.92	4.05	2.75
	Min	2.74	0.28	0.41	0.55	0.58	0.45
	SD	4.65	1.75	0.46	0.59	0.90	0.57
Cereal soil n = 12	Mean	8.15	2.35	0.85	1.30	1.42	1.26
	Max	17.68	10.41	1.47	2.06	2.93	2.08
	Min	3.63	0.22	0.40	0.50	0.56	0.55
	SD	5.17	2.62	0.33	0.53	0.71	0.44
Aquaculture soil n = 6	Mean	5.96	2.45	0.51	0.67	0.99	0.74
	Max	9.93	8.44	0.63	1.17	1.70	0.98
	Min	2.03	0.05	0.22	0.24	0.29	0.32
	SD	2.87	3.44	0.17	0.34	0.58	0.28
All samples n = 38	Mean	8.09	2.03	0.82	1.09	1.34	1.04
	Max	19.77	10.41	2.30	2.92	4.05	2.75
	Min	2.03	0.05	0.22	0.22	0.29	0.32
	SD	4.62	2.24	0.41	0.57	0.80	0.52

Table 6. *Statistical results from principal component analysis (PCA)*

	As	Cd	Cr	Cu	Pb	Zn	Mn	Al	pH	TOC	Sand	Silt	Clay
As	1.00	0.02	**0.64**	**0.65**	**0.59**	**0.52**	-0.02	**0.41**	-0.26	**0.45**	-0.27	0.33	0.17
Cd		1.00	0.14	0.14	0.14	0.16	0.08	0.11	0.15	-0.01	0.25	-0.24	-0.24
Cr			1.00	**0.82**	**0.73**	**0.74**	-0.05	**0.40**	**-0.46**	**0.52**	-0.34	**0.35**	0.30
Cu				1.00	**0.83**	**0.91**	-0.18	**0.34**	**-0.48**	**0.58**	**-0.40**	**0.43**	**0.34**
Pb					1.00	**0.80**	-0.13	0.24	-0.24	**0.46**	**-0.42**	**0.45**	**0.36**
Zn						1.00	-0.01	0.37	-0.32	**0.54**	-0.33	0.35	0.28
Rotated loading matrix (VARIMAX Gamma = 1.000)													
	PC1	***PC2***	***PC3***										
Cu	**0.91**	0.16	-0.19										
Zn	**0.90**	0.12	-0.03										
Cr	**0.87**	0.10	-0.14										
Pb	**0.84**	0.24	0.00										
As	**0.75**	0.06	-0.07										
Al	**0.55**	-0.53	-0.13										
Sand	-0.27	**-0.94**	0.14										
Clay	0.20	**0.93**	-0.11										
Silt	0.31	**0.91**	-0.15										
Cd	0.27	-0.35	**0.28**										
pH	-0.33	-0.09	**0.86**										
Mn	0.06	-0.11	**0.80**										
TOC	**0.57**	0.17	-0.58										
Eigenvalue	*5.64*	*2.64*	*1.48*										
% total variance	*43.40*	*20.33*	*11.34*										
% cumulative	*43.40*	*63.74*	*75.08*										

Bold type indicates significance at $p < 0.05$

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Based on Pearson's correlation coefficients Cr, Cu, Pb, and Zn were found significantly positively correlated with each other ($P < 0.01$). A highly positive correlation ($P < 0.01$) was found between Cu and Zn ($r = 0.91$), Cu and Pb ($r = 0.83$), Cu and Cr ($r = 0.82$), Pb and Zn ($r = 0.80$), Cr and Pb ($r = 0.73$), Cr and Zn ($r = 0.74$), indicating their similar sources. Arsenic has a positive correlation ($P < 0.01$) with Cr, Cu, Pb, and Zn, but these correlation coefficients were relatively weak, i.e., As and Cr ($r = 0.64$), As and Cu ($r = 0.65$), As and Pb ($r = 0.59$), As and Zn ($r = 0.52$). Cadmium does not show correlations with any heavy metals in agricultural soil, suggesting Cd perhaps has different sources or geochemical behaviour.

Soil pH does not show correlations with As, Cd and Pb, however, it shows a negative correlation with Cr, Cu, and Zn (*Table 6*), indicating that neutral soil contains less heavy metals (Chai et al., 2015; Kua et al., 1983; Manta et al., 2002). TOC, Al and silt percentage show a weakly positive correlation with As, Cr, Cu, Pb, and Zn ($p < 0.01$), indicating that these heavy metals are controlled by the contents of organic carbon as well as proportions of finer grain size fractions. Since Cd is not correlated with TOC, Al, and grain size, there exits other factor influencing its contents, which most probably is anthropogenic input.

The results are illustrated in the dendrogram in *Figure 11*, two distinct clusters are identified. The first cluster includes Cu, Zn, Pb, Cr, As and TOC, while Cd cluster into the second group.

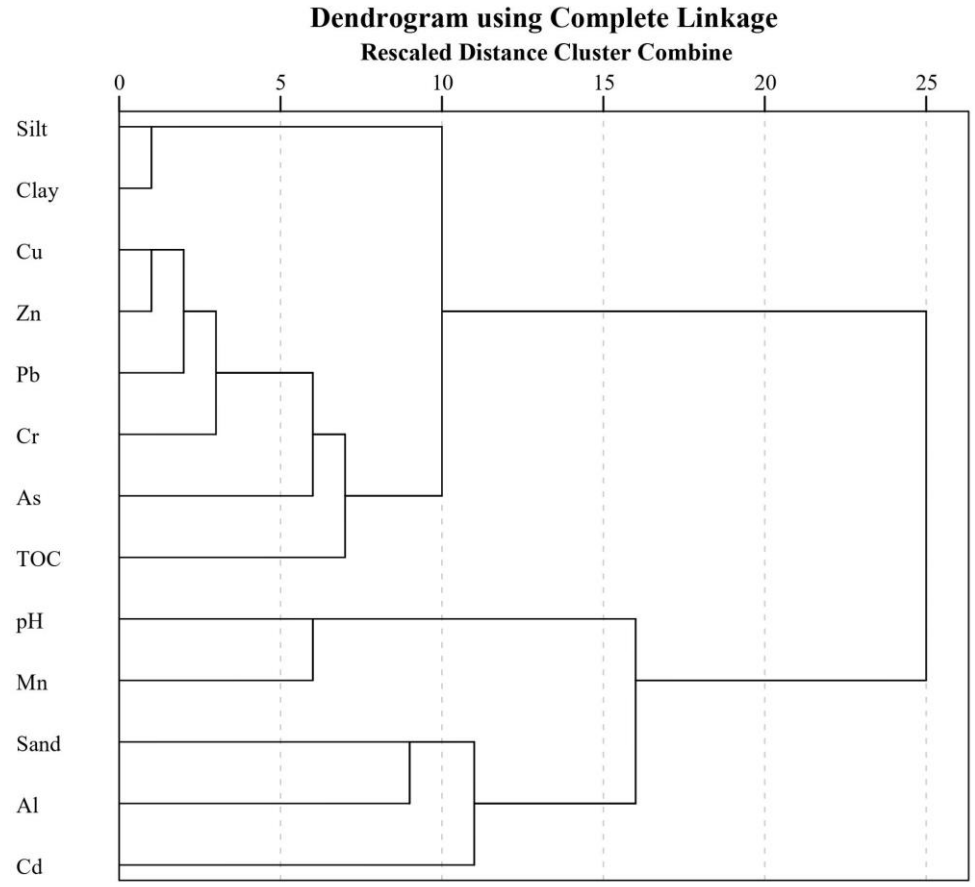

Figure 11. *Hierarchical cluster analysis among particle size and geochemical compositions in topsoil of agricultural soil in Nam Dinh province*

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The results of PCA are presented in *Table 6* and *Figure 12*. The extracted four components with eigen values > 1 explain 75.08% of total variance. The first principal component (PC1) accounts for 43.40% of total variance, showing high positive loadings of Cu (0.91), Zn (0.90), Cr (0.87), Pb (0.85), As (0.75), and medium loading of TOC (0.57) and Al (0.55). The second principal component (PC2) with a variance loading of 20.33% is dominated by the loading clay (0.94) and silt (0.91). The third principal component (PC3) is a less important component, accounting for 11.43 of the total variances, showing positive loading of Mn (0.80), pH (0.86) and Cd (0.28).

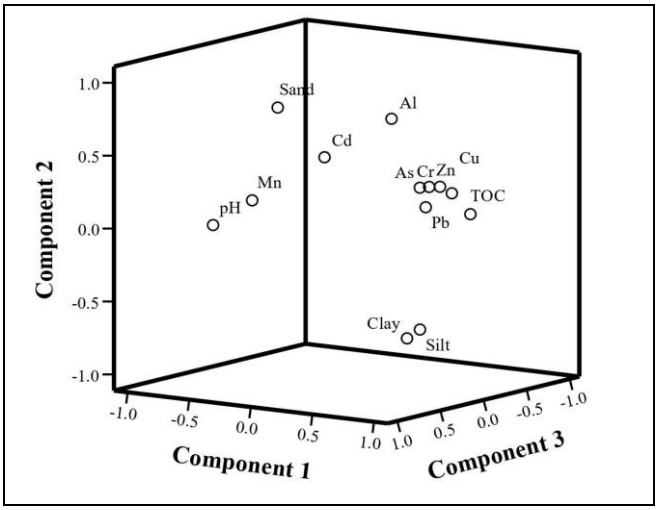

Figure 12. *Principal component analysis loading plots for rotated components of heavy metals and soil properties in Nam Dinh province*

According to the results from cluster and PCA analyses, two groups of elements can be identified: group 1 includes As, Cu, Zn, Pb and Cr, which show affinity with TOC and clay minerals; group 2 include Cd only.

Group 1 can be further subdivided into three sub-clusters. The first one sub-cluster includes Cr element. Since the concentration of Cr in the soil (10.68-57.20 mg/kg) was less than its concentration in UCC (85 mg/kg) and enrichment factor values of Cr were less than 1.5, it can state that Cr originated from natural sources. In addition, a previous study suggested that natural weathering processes are a major factor dominating the amount and distribution of Cr in surface sediments of the Red River, Vietnam, and it has a non-anthropogenic origin in Ba Lat estuary (Nguyen et al., 2016b).

The second sub-cluster includes As. The element As has a weak correlation with other heavy metals in this group, and it has enrichment factor values higher than 2. It indicated that this element has industrial and domestic sources. The main reason for the high enrichment factor values of As could be that the groundwater at these sites contains higher As level, which is commonly used to irrigate agricultural soil. The geology of Red delta shows similarity with the Ganges-Brahmaputra, where high As levels in the groundwater have been reported (Agusa et al., 2005, 2006; Berg et al., 2001, 2007; Chander et al., 2004; Nguyen et al., 2008). The same result was reported in agricultural soils in the Pear River, China (Chai et al., 2004; Huang et al., 2011). Element As was also found in pig and chicken manure, which are often used on the soil as a fertilizer (Zarcinas et al., 2005; Duan et al., 2016; Wang et al., 2019a). In contrast,

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increased industrial activities, such as chemical and fertilizer production as well as manufacturing in traditional handicraft villages (e.g. metal smelting and processing and painting), might have become additional sources of pollution in recent years.

The third sub-cluster includes Cu, Pb and Zn. These heavy metals have mean enrichment factor values less than 1.5 and they have a close correlation with TOC, silt and Al. These factors indicate a natural origin of Cu, Pb and Zn.

The second group indicates for Cd. Element Cd pollution was possibly caused by anthropogenic wastes, including sewage sludge, wastewater and/or fertilizers and pesticides.

As mentioned above, the EF values of Cd show higher values in Nghia Hung and Giao Thuy, these two districts are coastal districts of Nam Dinh province. These sites are located near the dike, high way, ecotourism zone, and industrial zone (e.g. ND95 near to Thinh Long ecotourism zone, ND51, ND52, ND54 near to the dike). Trinh and Shin (2004) also pointed out that, possible sources of Cd in Red River delta were irrigation water contaminated by inflow of improperly disposed wastewater, sewage sludge that was incorporated in the agricultural soil as an amendment, and vehicle tires.

Conclusions

Heavy metal contents are higher in soils of paddy fields and cereal fields, but lower in aquaculture soil.

According to cluster and PCA analyses, heavy metals can be grouped into two groups. Elements Cu, Pb, Cd, As and Zn have similar geochemical behaviours while Cd is different from them.

According to enrichment factor, elements Cr are mainly from natural source, while Cd and As have a significant anthropogenic input. Elements Cu, Pb and Zn have a mixed source.

Hot spots with high levels of As and Cd pollution are normally located next to industrial zones and traditional handicraft villages. These hotspots should be managed properly in terms of human health.

The effect of land use type on heavy metal concentrations will be discussed deeply in future studies.

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EFFECT OF ORGANIC FERTILIZER FORMS AND DOSES ON THE SEED GERMINATION AND SEEDLING DEVELOPMENT OF RAPESEED (*Brassica napus* L.)

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Abstract. In this study, the effects of organic fertilizer forms and doses on the seed germination and seedling growth of rapeseed were investigated. A study was carried out according to the Completely Randomized Experimental Design with three replications. Under liquid seaweed, germination index, radicle fresh weight, and radicle, plumule and seedling dry weights were found to be highest, while germination percentage, radicle length, plumule length, seedling length and germinated seed number proved to be the lowest. In liquid vermicompost; radicle length, plumule length, seedling length, radicle fresh weight, radicle dry weight and seedling vigor index were the highest. The liquid organic fertilizer with plant-derived amino acids proved to be the highest germination percentage value and the lowest radicle length, radicle fresh weigth, plumule fresh weight, seedling fresh weight, and radicle, plumule and seedling dry weight and seedling vigor index. The liquid vermicompost had an effect on increasing the seedling length by encouraging radicle and plumule length of the rapeseed, while liquid seaweed had negative effects on radicle and plumule length. It has been shown an effect to increase seedling fresh weight by promoting seedling growth. It was determined that the liquid organic fertilizer with plant-derived amino acids form encouraged the germination percentage of rapeseed. All tested fertilizers had an inhibitory effect on germination, on the other hand, the fertilizers promoted the seedling growth.
Keywords: *liquid seaweed, liquid vermicompost, organic fertilizer, rapeseed, seedling vigor index*

Introduction

Rapeseed, which can be grown sustainably in two different periods, summer and winter, contains 38-50% fat and 16-24% protein. Rapeseed is an important oil plant. In Turkey; it is also called rapiska, rapitsa and canola (Algan, 1990). Rapeseed oil with a neutral pH level is used as canned and frying oil in the food industry, and its nutritional properties and high boiling point ofconstitute some of its most important features (Tosun and Özkal, 2000). Rapeseed is also used as a raw material in the production of biodiesel with approximately 40% crude oil it contains, and it provides an important contribution to animal nutrition with the remaining 60% pulp.

Rapeseed is very useful in the beekeeping industry as well as in the food industry. It is a good source of food for bees that ensure dust intake from yellow flowers of rapeseed in March-April, when the flowers are scarce (Şeker, 2015). Rapeseed production with these benefits and uses; It ranks third in the world with 75001457 tons of oilseed plants such as soybean, cotton, peanut, sunflower, safflower and sesame. According to 2018 data, the world rapeseed cultivation is 37579575 ha and the yield is 1995.8 kg ha^{-1} (FAOSTAT, 2020).

According to the 2019 data in Turkey in rapeseed cultivation; 52515 ha cultivation, 3430.0 kg ha^{-1} yield and 180000 tons' production, and 61.87% of 111358 tons were

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produced in the West Marmara Region. The highest production is in Tekirdağ province with a ratio of 33.61% with 60497 tons (TURKSTAT, 2020).

Today, urbanization and industrialization have increased, fertile agricultural lands are decreasing, so it is necessary to obtain more products from the unit area in order to meet the nutritional needs of the increasing population. Here, fertilization has become mandatory to regain nutrients lost from the soil.

According to Adolf Mayer, "fertilization is the process of feeding the plant nutrients to the soil in order to increase the yield power of the culture soils and increase the quality and quantity of the product to be obtained. The substances used for this purpose are called "fertilizer" (Kacar, 2013).

Here, in order to increase the production quality of rapeseed, it should not disrupt the structure of the soil, crop rotation should be applied, organic fertilizer should be used, and biological control method which does not spoil the structure of the soil should be used. Human beings have to reestablish the natural balance lost in this ecological system as a result of wrong practices. Organic farming is a production system that is friendly to nature and the environment. It ensures that the soil has a sustainable yield, increases plant resistance, promotes biological methods without using chemicals in plant protection, and aims to increase the quality of the product in production.

Organic fertilization, on the other hand, improves soil yield, sustainability and water-holding capacity, improves soil and accelerates microbial activities (Yüksek et al., 2019). Another important feature of organic fertilizers is that they are a good soil conditioner (Aygün and Acar, 2004). The use of organic extracts (fertilization, plant growth regulators, etc.) has proven effective in improving seed germination and early seedling growth in abiotic stress conditions (Sharma et al., 2014). Seaweed and vermicompost extracts from these organic extracts have found use in seed applications (Demir et al., 2006; Ma et al., 2017; Masondo et al., 2018).

One of these extracts is vermicompost, also known as vermicompost, which is among the soil improvers and nutrients used in organic agriculture. Vermicompost is the process of composting organic waste by worms. The worm fertilizer produced as a result of the vermicompost process consists of completely organic materials, contains plant nutrients and some plant growth hormones. Therefore, it makes it possible to buy more products from the unit area.

Solid worm manure is a product obtained by composting. Liquid worm manure is called processed state of composted manure. So, it is obtained from solid worm manure. Liquid worm fertilizer is an odorless, brown and slightly alkaline (7-9 pH) organic liquid. In liquid worm manure; it contains humates, fulvic acids, amino acids, vitamins, natural hormones, micro and macro elements (N, P, K, O, Ca, Mg, S) found in solid earthworm fertilizer in a concentrated form of soil microorganism spores (Benitez et al., 2000).

Liquid worm fertilizer accelerates the growth and growth of the plant, has no harm on humans, animals and soil, stimulates the root formation of plants and allows them to form strong roots Protects the soil and the plant from harmful pests and pathogens that may occur in the soil. By providing natural immunity to plants, it eliminates the occurrence of disease in parts such as leaves and stems, accelerates flowering in pot plants, and provides faster crop formation in greenhouse plants. When applied to the leaves of the plant, it gives vitality to the leaf and supports the formation of more photosynthesis, accelerates the metabolism of the plant, prolongs the waiting period in storage of plants or nutrients, delays the decay of the plant, it is a form of liquid fertilizer which is highly recommended for use in agriculture. Liquid worm manure is dark colored, high viscosity and has a long

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shelf life. It is obtained using various technologies, and and the organic substance is rich in humic and fulvic acid amounts (Yıldırım, 2019). The second of these extracts, seaweed (maxicrop), was obtained from the seaweed called *Ascophyllum nodosum* on the Norwegian coast. There is auxin (indole and derivatives) and cytokinin (zeatin, kinetin, adenine, purine and adenosine) as growth regulators in seaweed. Seaweeds contain macro (0.75% N, 1% P_2O_5, 16% K_2O, 0.20% Mg, 2.90% S) and micro (30 ppm B, 290 ppm Fe, 12 ppm Cu, 56 ppm Zn,) elements. It is also an excellent source of bioactive compounds by growth promoting substances such as seaweeds, essential fatty acids, vitamins, amino acids, minerals, organic osmolites (e.g. betainees) and gibberellins (Spinelli et al., 2010).

Seaweed extracts and suspensions from brown algae are studied in the cultivation of garden plants. Marmarin (seaweed extract) is a biostimulant that increases the production and quality of agricultural products and is widely used in vegetable production (Amanpoor et al., 2011). Seaweed extracts have beneficial effects on plants such as early seed germination, increasing plant performance and yield, high resistance to biotic and abiotic stress and extending the shelf life of seeds. Seaweed extract as an organic biostimulant is one of the applications quickly accepted in horticultural crops due to its beneficial effects. In experimental studies, it has been observed that seedling growth is stimulated by using seaweed extracts (Demir et al., 2006). However, since the high concentration of seaweed can cause loss of yield in the plant, it is necessary to pay attention to the method and duration of application. (Spinelli et al., 2010).

The third of these extracts is a liquid organic fertilizer with plant-derived amino acids. It has a high content of organic matter and contains nitrogen. Therefore, it enables plants to grow faster, bushy and healthier. The liquid organic fertilizer with plant-derived amino acids provide a smooth flowering, promote the number and quality of fruit, shorten the harvest time, provide early crop, increase the resistance of plants against adverse soil and climate factors. Therefore, it is an important liquid organic fertilizer.

In the study, liquid worm manure, liquid seaweed manure, and liquid organic fertilizer forms with plant-derived amino acids were used. It is aimed to determine the encouraging or preventing effects of organic fertilizer forms and doses on the rapeseed (*Brassica napus* L.) seed germination and seedling growth. and liquid organic fertilizer with plant-derived amino acids

Material and Methods

This study was carried out in Kahramanmaraş Sütçü İmam University Faculty of Agriculture, Department of Field Crops, Industrial Plants Laboratory in the climate cabinet in January 2020 in Turkey. In the experiment, healthy and homogenous rapeseed (ES Hydromel variety) seeds were used as material.

The research aimed to investigate the effects of different organic fertilizer forms and doses on the germination and seedling growth of rapeseed using a completely randomized experimental design with three replications, in order to find the most optimal combination.

Three fertilizer forms (OF1: liquid seaweed, OF2: liquid organic fertilizer with plant-derived amino acids and OF3: liquid vermicompost) and six doses (FD1: control, FD2: 1000 ppm L^{-1}, FD3: 2000 ppm L^{-1} FD4: 4000 L^{-1}, FD5: 8000 ppm L^{-1}, FD6: 16000 ppm L^{-1}) were applied. Solutions were prepared by diluting fertilizer doses with tap water. Seeds were placed in petri dishes. After leaving two layers of drying paper on the bottom of each petri dish (90 mm), seven ml of fertilizer doses prepared were added

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to the drying papers in the petri dish. Surface sterilization was performed in the seeds for five minutes in the solution created with 5% NaOCl (sodium hypochlorite) (Yılmaz, 2015). 25 healthy and similar sized seeds were planted. It is coated with parafilm (PM-992) to prevent water loss in petri dishes. It was allowed to germinate for 14 days in an incubator with a temperature of 25 ± 2 °C. On the 15th day, the seeds were measured for germination and seedling growth (*Figure 1*). The germinated seeds were counted and divided by the total number of seeds and then multiplied by 100 to get the germination rate. Radicle and plumule lengths were measured with calipers. Seedling length was determined by sum of the radicle lenght and plumule lenght. Radicle and plumule were weighed as fresh, and radicle fresh weight and plumule fresh weight were summed and seedling fresh weight was obtained. The samples were then kept for 24 hours at 78 ℃ in the etuv and the dry weight of the radicle and dry weight of the plumula were weighed and the sum of the seedling dry weights were found. As a result of the multiplication of seedling length and germination rate, seedling vigor index was found.

Figure 1. *In the laboratory with climate cabinet, an oven, precision scales, etc., on the 15th day after placing; while seeds are measured for germination and seedling development*

Statistical analysis of data

All data obtained from the study were processed by SAS (v. 9.0, 2002) statistical package. The data were analyzed using analysis of variance (ANOVA) according to the Completely Randomized Experimental Design. Averages were compared by Least Significant Difference multiple comparison test (Stell and Toor, 1980).

Results and Discussion

Averages of the effects of organic fertilizer forms and doses on the seed germination and seedling growth of rapeseed are given in *Table 1* and *Table 2*. According to these tables, fertilizer doses has significantly affected all attributes of germination and seedling. The differences between fertilizer doses, organic fertilezer forms, and their interaction averages were found to be statistically significant for all observed attributes. However, since there was no germination in the OF1-FD6 application, the comparison of the fertilizer x dose interactions averages was performed over 17 values.

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Table 1. *The means of organic fertilizer forms and doses for GP, GI, RL, PL, SL, RFW properties of Brassica napus L. seeds, and LSD groups*

Fertilizer	Fertilizer Doses (ppm L^{-1})	GP (%)	*;**	GI	*;**	RL (mm)	*;**	PL (mm)	*;**	SL (mm)	*;**	RFW (mg)	*;**
OF1	FD1	95.11	a	8.22	a	112.72	a	43.21	b	155.93	a	7.74	d
	FD2	90.67	a	6.19	b	95.88	c	45.72	ab	141.60	b	4.87	f
	FD3	34.67	f	1.76	d	3.01	jk	7.22	h	10.23	h	7.60	d
	FD4	17.33	j	1.03	h	3.37	jk	6.24	h	9.61	h	7.88	d
	FD5	25.33	hi	1.37	fg	5.72	ij	14.30	f	20.03	g	17.40	a
	FD6	-		-		-		-		-		-	
	Mean	**43.85**	**C**	**3.09**	**A**	**36.78**	**B**	**19.45**	**C**	**56.23**	**C**	**7.58**	**A**
OF2	FD1	95.11	a	8.22	a	112.72	a	43.21	b	155.93	a	7.74	d
	FD2	57.33	c	3.55	c	36.64	fg	27.19	cd	63.83	d	8.60	c
	FD3	34.67	f	1.36	fg	10.88	i	11.84	g	22.72	g	9.17	c
	FD4	45.33	d	1.78	d	4.17	jk	7.68	h	11.85	h	3.02	i
	FD5	29.33	g	0.70	i	41.56	f	43.81	b	85.37	c	1.90	j
	FD6	58.67	c	1.66	de	19.38	h	18.50	e	37.88	f	1.03	k
	Mean	**53.41**	**A**	**2.88**	**B**	**37.56**	**B**	**25.37**	**B**	**62.93**	**B**	**5.24**	**B**
OF3	FD1	95.11	a	8.22	a	112.72	a	43.21	b	155.93	a	7.74	d
	FD2	62.67	b	3.83	c	106.87	ab	47.55	a	154.41	a	6.70	e
	FD3	26.67	gh	1.25	gh	47.83	e	7.09	h	54.92	e	15.50	b
	FD4	41.33	e	1.09	h	102.50	bc	43.64	b	146.13	b	6.90	e
	FD5	22.67	i	0.70	i	58.28	d	28.15	c	86.42	c	4.10	g
	FD6	26.67	gh	1.50	ef	34.82	g	25.71	d	60.53	d	3.50	h
	Mean	**45.85**	**B**	**2.76**	**C**	**77.17**	**A**	**32.56**	**A**	**109.72**	**A**	**7.41**	**A**
Mean		**47.70**		**2.91**		**50.50**		**25.79**		**76.30**		**6.74**	
LSD (0.05) for OF		**1.53**		**0.10**		**2.57**		**0.84**		**2.16**		**0.22**	
LSD (0.05) for FD		**2.16**		**0.15**		**3.64**		**1.19**		**3.05**		**0.31**	
LSD (0.05) for OF x FD		**6.49**		**0.44**		**10.92**		**0.58**		**9.15**		**0.93**	
CV (%)		**4.74**		**5.23**		**7.52**		**4.83**		**4.17**		**4.80**	

GP: Germination Percentage, GI: Germination Index, RL: Radicle Length, PL: Plumule Length, SL: Seedling Length, RFW: Radicle Fresh Weight, Fertilizer Doses: FD1: control, FD2: 1000 ppm L^{-1}, FD3: 2000 ppm L^{-1}, FD4: 4000 L^{-1}, FD5: 8000 ppm L^{-1}, FD6: 16000 ppm L^{-1} OF1: Liquid Seaweed, OF2: Liquid Organic Fertilizer with Plant-Derived Amino Acids, OF3: Liquid Worm Fertilizer

*: The mean in the same column, expressed in lowercase and indicated with different letters, is statistically different from each other within the P≤0.05 error limits according to LSD test

**: The mean in the same column, expressed in capital and indicated with different letters, is statistically different from each other within the P≤0.05 error limits according to LSD test

Germination Percentage (%)

It seems that OF2 is more encouraging in terms of germination percentage. The highest germination percentage among fertilizer forms was observed in OF2 with 53.41%, while the lowest germination percentage was obtained from OF1 fertilizer with 43.85%. In fertilizer x dose interaction, the highest germination percentage was observed in OF3-FD1, OF2-FD1, OF1-FD1 and OF1-FD2 (95.11, 95.11, 95.11, and 90.67%, respectively). When the control doses were ignored, the highest germination percentage was seen in OF1-FD2 with 90.67%. Therefore, in addition to seedling growth, liquid seaweed has been determined as a promoter in seed germination. However, the trend of germination percentage at increasing doses in liquid seaweed showed a downward decline. The lowest germination percentage was seen in OF1-FD4 with 17.33% (*Table 1* and *Figure 2*).

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Table 2. *The means of organic fertilizer forms and doses on PFW, SFW, RDW, PDW, SDW, FVI, GSN properties of Brassica napus L. seeds, and LSD groups*

Fertilizer	Fertilizer Doses (ppm L^{-1})	PFW (mg)	*;**	SFW (mg)	*;**	RDW (mg)	*;**	PDW (mg)	*;**	SDW (mg)	*;**	FVI	*;**	GSN (number)	*;**
OF1	FD1	47.228	c	54.972	d	0.852	d	5.195	c	6.047	d	14848.52	a	23.78	a
	FD2	41.733	d	46.600	e	0.535	f	4.591	d	5.126	e	12832.02	b	22.67	a
	FD3	71.633	a	79.233	a	0.836	d	7.880	a	8.716	a	354.74	ijk	8.67	f
	FD4	59.500	b	67.383	b	0.867	d	6.545	b	7.412	b	166.84	jk	4.33	j
	FD5	42.900	d	60.300	c	1.914	a	4.719	d	6.633	c	507.31	ij	6.33	hi
	FD6	-		-		-		-		-		-		-	
	Mean	**43.832**	**A**	**51.415**	**A**	**0.834**	**A**	**4.822**	**A**	**5.656**	**A**	**4784.90**	**B**	**10.96**	**C**
OF2	FD1	47.228	c	54.972	d	0.852	d	5.195	c	6.047	d	14848.52	a	23.78	a
	FD2	43.367	cd	51.967	d	0.946	c	4.770	cd	5.716	d	3658.81	e	14.33	c
	FD3	25.300	e	34.473	f	1.009	c	2.783	e	3.792	f	787.89	i	8.67	f
	FD4	18.692	fg	21.708	h	0.332	i	2.056	fg	2.388	h	537.63	ij	11.33	d
	FD5	18.467	fg	20.367	h	0.209	j	2.031	fg	2.240	h	2504.41	f	7.33	g
	FD6	15.100	h	16.133	i	0.114	k	1.661	h	1.775	i	2222.36	fg	14.67	c
	Mean	**28.025**	**C**	**33.270**	**C**	**0.577**	**B**	**3.083**	**C**	**3.660**	**C**	**4093.27**	**C**	**13.35**	**A**
OF3	FD1	47.228	c	54.972	d	0.852	d	5.195	c	6.047	d	14848.52	a	23.78	a
	FD2	47.067	c	53.767	d	0.737	e	5.177	c	5.914	d	9671.49	c	15.67	b
	FD3	44.500	cd	60.000	c	1.705	b	4.895	cd	6.600	c	1460.83	h	6.67	gh
	FD4	42.033	d	48.933	e	0.759	e	4.624	d	5.383	e	6043.51	d	10.33	e
	FD5	19.877	f	23.977	g	0.451	g	2.186	f	2.637	g	1963.63	gh	5.67	i
	FD6	17.333	g	20.833	h	0.385	h	1.907	g	2.292	h	1612.86	h	6.67	gh
	Mean	**36.340**	**B**	**43.747**	**B**	**0.815**	**A**	**3.997**	**B**	**4.812**	**B**	**5933.47**	**A**	**11.46**	**B**
Mean		**36.07**		**42.81**		**0.74**		**3.97**		**4.71**		**4937.21**		**11.93**	
LSD (0.05) for OF		**1.106**		**1.10**		**0.02**		**0.12**		**0.12**		**264.58**		**0.38**	
LSD (0.05) for FD		**1.564**		**1.55**		**0.03**		**0.17**		**0.17**		**374.17**		**0.54**	
LSD (0.05) for OF x FD		**4.693**		**4.66**		**0.10**		**0.52**		**0.51**		**1122.51**		**1.62**	
CV (%)		**4.528**		**3.79**		**4.76**		**4.53**		**3.80**		**7.91**		**4.74**	

PFW: Plumule Fresh Weight, SFW: Seedling Fresh Weight, RDW: Radicle Dry Weight, PDW: Plumule Dry Weight, SDW: Seedling Dry Weight, SVI: Seedling Vigor Index, GSN: Germinated Seed Number, Fertilizer Doses: FD1: control, FD2: 1000 ppm L^{-1}, FD3: 2000 ppm L^{-1}, FD4: 4000 L^{-1}, FD5: 8000 ppm L^{-1}, FD6: 16000 ppm L^{-1} OF1: Liquid Seaweed, OF2: Liquid Organic Fertilizer with Plant-Derived Amino Acids, OF3: Liquid Worm Fertilizer

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**: The mean in the same column, expressed in capital and indicated with different letters, is statistically different from each other within the P≤0.05 error limits according to LSD test

Figure 2. *The interaction of organic fertilizers' germination percentage*

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Demirkaya (2010), in his study on the effects of seaweed (*Ascophyllum nodosum*) extract applications on the viability and strength of pepper and onion seeds; found that OC (Osmotic Conditioning: Pre-Germination) applications with seaweed extract increase the germination rate of onion and pepper seeds according to the control application. The results are consistent with the highest finding of germination percentage in OF1-FD2 in fertilizer x dose interaction.

Demirkaya (2012), in his another study on the effects of seaweed (*Ascophyllum nodosum*) extract applications on the viability and strength of tomato seeds; found that OK applications with seaweed extract increased the germination rates of the seeds of three tomato varieties compared to the control application. The results are similar to the highest finding of germination percentage in OF1-FD2 in fertilizer x dose interaction.

Yıldırım and Güvenç (2005), in their study on the effect of seaweed extract practices on seed germination in leeks; They found that with the application of seaweed extract to leek seeds, the rate and speed of seed germination increased significantly compared to the control. The results are consistent with the highest finding of germination percentage in OF1-FD2 in fertilizer x dose interaction.

Matysiak et al. (2011), in their study on the effect of seaweed extracts and the mixture of humic and fulvic acid on germination and growth of corn; reported that extracts from seaweeds (Kelpak SL and AlgaminoPlant) stimulate corn seed germination more than humic and fulvic acids (HumiPlant) and increase by 16-19%. The results are consistent with the highest finding of germination percentage in OF1-FD2.

Germination Index

It is seen that OF1 is higher in terms of Germination index average. The highest germination index among the fertilizer forms was seen in OF1 with 3.09, while the lowest germination index was obtained from the OF3 fertilizer form with 2.76. In fertilizer x dose interaction, the highest germination index was found in 8.22 with OF3-FD1, OF2-FD1 and OF1-FD1. The lowest germination index was observed in OF2-FD5 and OF3-FD5 with 0.70 (*Table 1* and *Figure 3*).

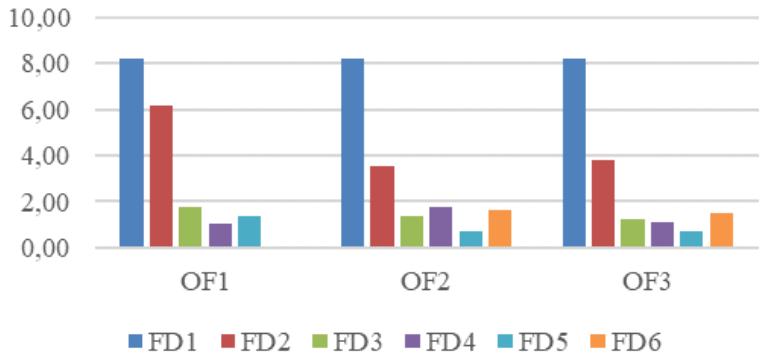

Figure 3. *The interaction of organic fertilizers' germination index*

Yıldırım and Güvenç (2005), in their study on the effect of seaweed extract applications on seed germination in leeks; they found that with the application of seaweed extract to leek seeds, the rate and speed of seed germination increased significantly compared to control. The results are in line with the highest germination index finding in OF1.

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Radicle Length (mm)

It is seen that OF3 is more encouraging in terms of the average of the radicle length. Among the fertilizer forms, the highest radicle length was observed in OF3 with 77.17 mm, while the lowest radicle length was obtained from OF2 (37.56 mm) and OF1 (36.78 mm) fertilizer forms. In fertilizer x dose interaction, the highest radicle length was 112.72 mm with OF3-FD1, OF2-FD1 and OF1-FD1. The lowest radicle length was observed in OF1-FD3, OF1-FD4 and OF2-FD4 (3.01, 3.37, and 4.17 mm, respectively) (*Table 1* and *Figure 4*).

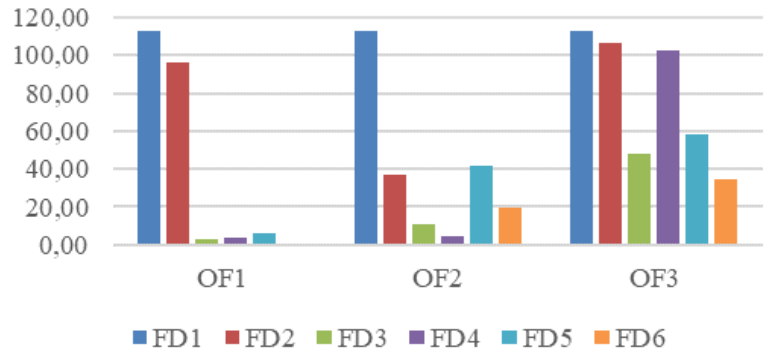

Figure 4. *The interaction of organic fertilizers' radicle length*

Plumule Length (mm)

When plumule length averages are examined, it is seen that OF3 has a more positive effect on plumule length. Among the fertilizer forms, the highest plumule length was 32.56 mm with OF3 fertilizer form. The lowest plumule length was obtained from OF1 fertilizer form with 19.45 mm. In fertilizer x dose interaction, the highest plumule length was observed in OF3-FD2 with 47.55 mm. The lowest plumule length was observed in OF1-FD4, OF3-FD3, OF1-FD3 and OF2-FD4 (6.24, 7.09, 7.22, and 7.68 mm, respectively) (*Table 1* and *Figure 5*).

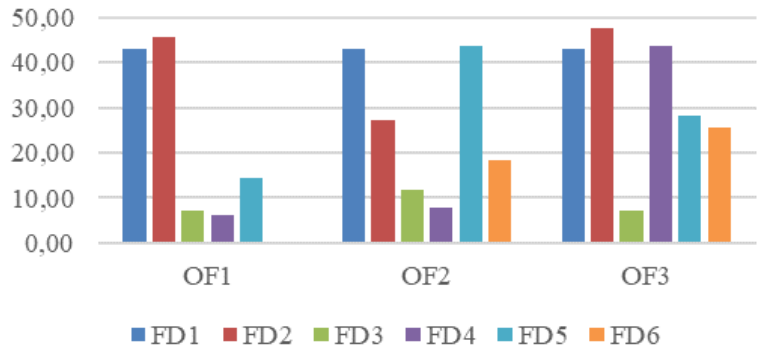

Figure 5. *The interaction of organic fertilizers' plumule length*

Seedling Length (mm)

It is seen that OF3 is more encouraging in terms of Seedling length average. While the highest seedling length was seen in OF3 with 109.72 mm in fertilizer forms, the lowest

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seedling length was obtained from OF1 fertilizer form with 56.23 mm. In fertilizer x dose interaction, the highest seedling length was observed in OF3-FD1, OF2-FD1, OF1-FD1 and OF3-FD2 (155.93, 155.93, 155.93, and 154.41 mm, respectively). The lowest seedling length was observed in OF1-FD4 OF1-FD3 and OF2-FD4 (9.61, 10.23, and 11.85 mm, respectively) (*Table 1* and *Figure 6*).

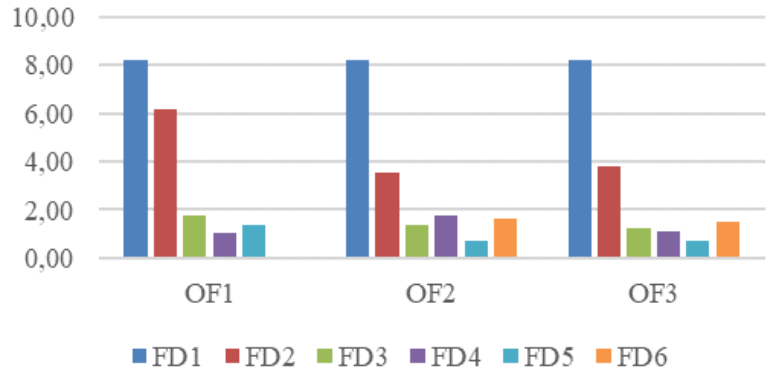

Figure 6. *The interaction of organic fertilizers' seedling length*

Alam et al. (2007), reported that vermicompost and N P K S fertilizers increased the effect of red amaranth on the growth, yield and yield components of red amaranth, and vermicompost application increased the control of vermicompost compared to control applications. The findings support the highest seedling length in OF3.

Radicle Fresh Weight (mg)

It is seen that OF1 and OF3 are higher in terms of radicle fresh weight average. While the highest radicle fresh weights were observed with OF1 (7.58 mg) and OF3 (7.41 mg) among fertilizer forms, the lowest radicle fresh weight was obtained from OF2 fertilizer form with 5.24 mg. In fertilizer x dose interaction, the highest radicle fresh weight was observed in OF1-FD5 with 17.40 mg. The lowest radicle fresh weight was observed in OF2-FD6 with 1.03 mg (*Table 1* and *Figure 7*).

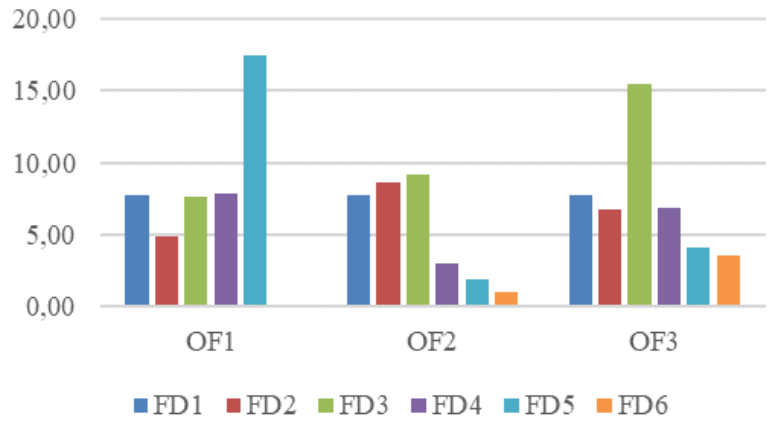

Figure 7. *The interaction of organic fertilizers' radicle fresh weigth*

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Plumule Fresh Weight (mg)

It is seen that OF1 is higher in terms of plumule fresh weight average. While the highest plumule fresh weight was observed in OF1 with 43.832 mg among fertilizer forms, the lowest plumule fresh weight was obtained from OF2 fertilizer form with 28.025 mg. In fertilizer x dose interaction, the highest plumule fresh weight was observed in OF1-FD3 with 71.633 mg. The lowest plumule fresh weight was seen in OF2-FD6 with 15.100 mg (*Table 2* and *Figure 8*).

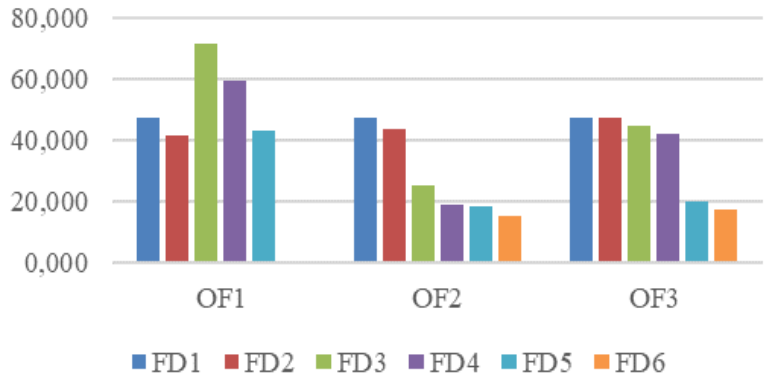

Figure 8. *The interaction of organic fertilizers' plumule fresh weigth*

Matysiak et al. (2011), in their study on the effect of seaweed extracts and the mixture of humic and fulvic acid on germination and growth of corn; reported that extracts from seaweeds (Kelpak SL and AlgaminoPlant) increased corn plumule fresh weight. The results are consistent with the highest plumule wet weight finding in OF1-FD3.

Seedling Fresh Weight (mg)

It is seen that OF1 is higher in terms of seedling fresh weight average. While the highest seedling fresh weight was seen in OF1 with 51.415 mg among fertilizer forms, the lowest seedling fresh weight was obtained from OF2 fertilizer form with 33.270 mg. In fertilizer x dose interaction, the highest seedling fresh weight was seen in OF1-FD3 with 79.233 mg. The lowest seedling fresh weight was observed in OF2-FD6 with 16.133 mg (*Table 2* and *Figure 9*).

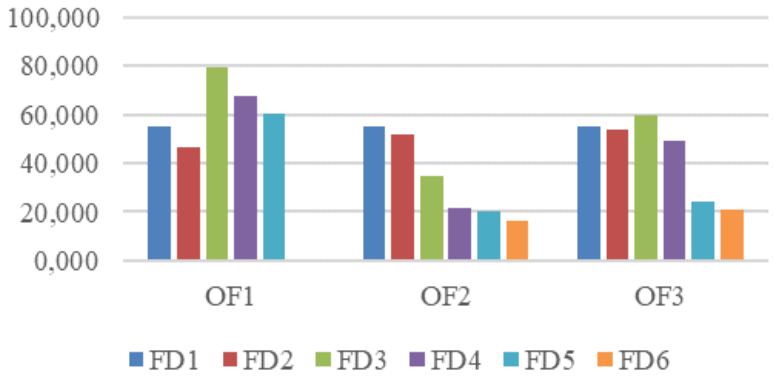

Figure 9. *The interaction of organic fertilizers' seedling fresh weigth*

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Radicle Dry Weight (mg)

It is seen that OF1 and OF3 are higher in terms of the average of radicle dry weight. Among the fertilizer forms, the highest radicle dry weights were seen in OF1 (0.834 mg) and OF3 (0.815 mg), while the lowest radicle dry weight was obtained from OF2 fertilizer form with 0.577 mg. In fertilizer x dose interaction, the highest radicle dry weight was found in OF1-FD5 with 1.914 mg. The lowest radicle dry weight was found in OF2-FD6 with 0.114 mg (*Table 2* and *Figure 10*).

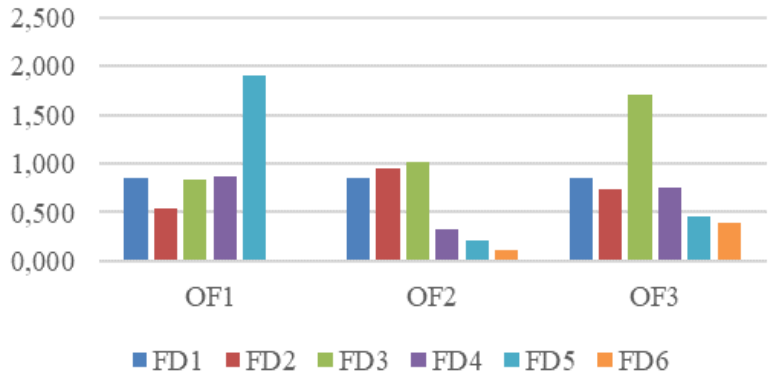

Figure 10. *The interaction of organic fertilizers' radicle dry weigth*

Plumule Dry Weight (mg)

OF1 is higher in terms of plumule dry weight average. While the highest plumule dry weight was observed in OF1 with 4.822 mg, the lowest plumule dry weight was obtained from OF2 fertilizer form with 3.083 mg. In fertilizer x dose interaction, the highest plumule dry weight was observed in OF1-FD3 with 7.880 mg. The lowest plumule dry weight was seen in OF2-FD6 with 1.661 mg (*Table 2* and *Figure 11*).

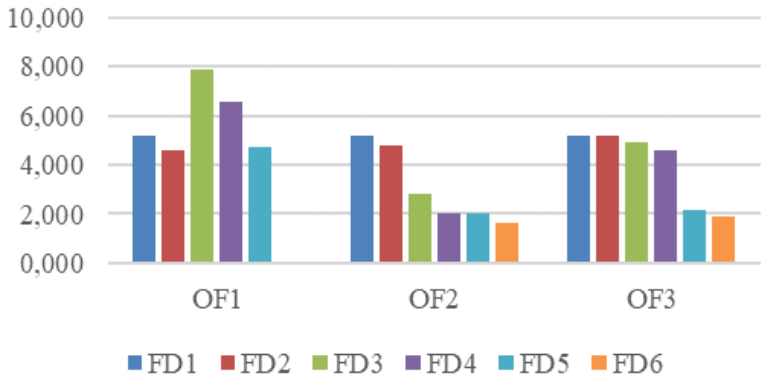

Figure 11. *The interaction of organic fertilizers' plumule dry weigth*

Seedling Dry Weight (mg)

As seen in *Table 2*, OF1 is higher in terms of seedling dry weight average. While the highest seedling dry weight was found in OF1 with 5.656 mg among fertilizer forms, the lowest seedling dry weight was obtained from OF2 fertilizer with 3.660 mg. In fertilizer

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x dose interaction, the highest seedling dry weight was found in OF1-FD3 with 8.716 mg. The lowest seedling dry weight was observed in OF2-FD6 with 1.775 mg (*Table 2* and *Figure 12*).

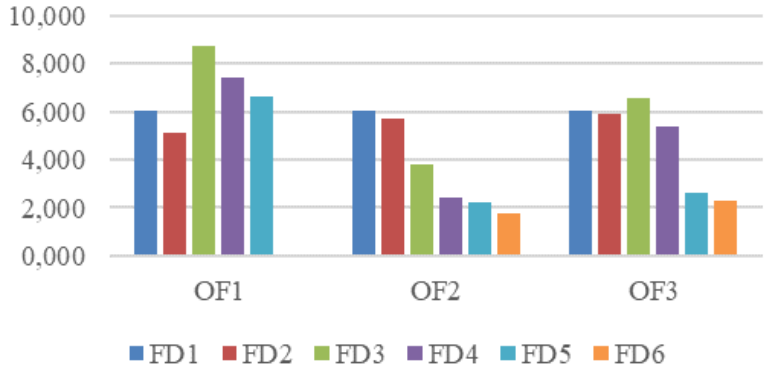

Figure 12. *The interaction of organic fertilizers' seedling dry weigth*

Seedling Vigor Index

It is seen that OF3 is higher in terms of seedling vigor index average. Among the fertilizer forms, the highest seedling vigor index was seen in OF3 with 5933.47, while the lowest seedling vigor index was obtained from OF2 fertilizer form with 4093.27. In fertilizer x dose interaction, the highest seedling vigor index 14848.52 was seen in OF3-FD1, OF2-FD1 and OF1-FD1. The lowest seedling vigor index was seen in OF1-FD4 with 166.84 (*Table 2* and *Figure 13*).

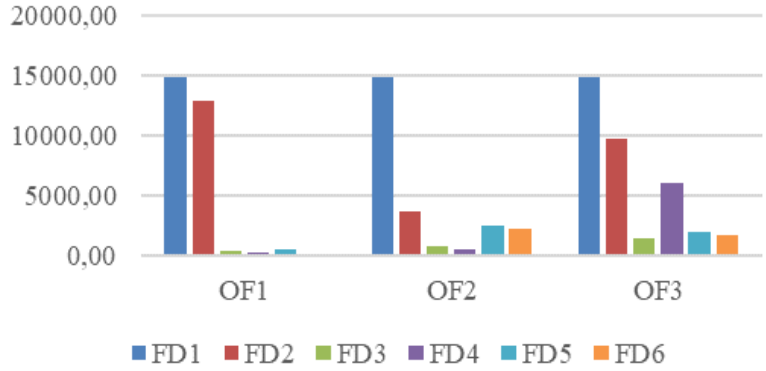

Figure 13. *The interaction of organic fertilizers' seedling vigor index*

Germinated Seed Number (number)

It is seen that OF2 is higher in terms of average of germinated seed number. The highest germinated seed number among the fertilizer forms was seen in OF2 with 13.35, while the lowest germinated seed number was obtained from the OF1 fertilizer form with 10.96. In fertilizer x dose interaction, the highest germinated seed number was observed in OF3-FD1, OF2-FD1, OF1-FD1 and OF1-FD2 (23.78, 23.78, 23.78, and 22.67, respectively). The lowest germinated seed number was found in OF1-FD4 with 4.33 (*Table 2* and *Figure 14*).

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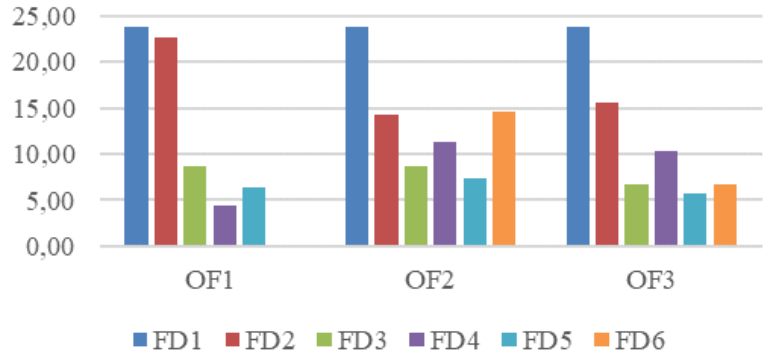

Figure 14. *The interaction of organic fertilizers' germinated seed number*

The relationships among the observed characteristics

In the study, a correlation analysis was conducted to reveal the relationships between all investigated properties of *Brassica napus* L. seeds subjected to organic fertilizer forms and doses. The many of correlations between all the examined properties are considered to be significant (P<0.01 and P<0.05) with different organic fertilizer form doses (*Table 3*). As presented in *Table 3*, one of the most important features is the germination percentage. It was found to have significant positive correlations with GI, RL, PL, SL, PFW, SFW, PDW, SDW, SVI, and GSN (r=0.942, r=0.764, r=0.701, r=0.765, r=0.359, r=0.315, r=0.358, r=0.315, r=0.916, and r=1.000, respectively). *Table 3* can be examined for the positive and negative relationship among other characteristics.

Table 3. *Correlation table for observed parameters of Brassica napus L. samples for fertizer forms and fertilizer doses*

	GI	RL	PL	SL	RFW	PFW	SFW	RDW	PDW	SDW	SVI	GSN
GP	0.942 **	0.764 **	0.701 **	0.765 **	0.030	0.359 **	0.315 *	0.031	0.358 **	0.315 *	0.916 **	1.000 **
GI		0.757 **	0.637 **	0.742 **	0.116	0.409 **	0.376 **	0.116	0.409 **	0.376 **	0.942 **	0.942 **
RL			0.883 **	0.991 **	0.046	0.294 *	0.262	0.047	0.294 *	0.262	0.914 **	0.764 **
PL				0.937 **	-0.116	0.169	0.121	-0.115	0.169	0.121	0.802 **	0.701 **
SL					0.002	0.266	0.229	0.002	0.266	0.229	0.904 **	0.765 **
RFW						0.597 **	0.724 **	0.999 **	0.597 **	0.724 **	0.027	0.030
PFW							0.986 **	0.597 **	1.000 **	0.986 **	0.338 *	0.359
SFW								0.724 **	0.986 **	1.000 **	0.296 *	0.315 *
RDW									0.597 **	0.724 **	0.027	0.031
PDW										0.986 **	0.338 *	0.358 **
SDW											0.296 *	0.315 *
SVI												0.916 **

**: Correlation is significant at the 0.01 level. Pearson Correlation

* : Correlation is significant at the 0.05 level.

Conclusion

According to the results of the research, it was observed that organic fertilizer forms significantly affect the parameters of rapeseed germination and seedling growth.

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The findings of the study showed that fertilizer x dose interactions were beneficial in determining the inhibitory properties of rapeseed seeds as well as in promoting germination and seedling growth. Although germination percentage, germination index, radicle length, plumule length, seedling length and plumula fresh weight values are highest in control doses; radicle fresh weight, plumule fresh weight, seedling fresh weight, radicle dry weight, plumule dry weight and seedling dry weight values were found to be highest, especially in 2000 ppm L^{-1} doses.

As a summary, while the liquid seaweed 2000 ppm L^{-1} dose application, plumule fresh weight, seedling fresh weight, radicle dry weight and seedling dry weight were highest, plumule fresh weight seedling fresh weight, plumule dry weight and seedling dry weight values proved to be second high values in 4000 ppm L^{-1} dosing application. The liquid vermicompost had an effect of increasing seedling length by promoting both root and shoot length of rapeseed. The liquid seaweed had a negative effect on both root and shoot length of rapeseed, while promoting seedling growth, and had an effect increase seedling fresh weight. The form of liquid organic fertilizer with plant-derived amino acids had a stimulating effect on the germination rate of rapeseed.

There was no germination at the highest applied dose (16000 ppm L^{-1}) of the liquid seaweed. In fertilizer doses; in 16000 ppm L^{-1} application, it was determined that it gave the lowest values in germination index, radicle length, radicle fresh weight, plumule fresh weight, seedling fresh weight, radicle dry weight, plumule dry weight and seedling dry weight, and the application of 16000 ppm L^{-1} fertilizer dose had a negative effect on the germination and seedling growth of rapeseed in organic fertilizer forms investigated.

According to the data obtained in the study, it was observed that the fertilizers of organic origin applied did not have a positive effect on the germination rate and index, but after the germination, it was observed that the seaweed manure had positive and significant effects on seedling growth, while the worm manure had the same effect on the seedling lengths.

In future studies, it is necessary to diversify the studies on organic fertilizers, especially liquid seaweed, which promotes seedling growth, and liquid worm manure, which promotes seedling length. In order to emphasize the importance of these fertilizer forms, it will be important to carry out more studies in different plants, as well as to use other organic fertilizers in these studies to be conducted, to obtain healthier and more accurate data.

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STIMULATING COMPOUNDS AFFECT THE GRAIN QUALITY CHARACTERISTICS AND THE NUTRITIONAL VALUE OF RICE (*ORYZA SATIVA*)

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Abstract. This investigation was carried out in the experimental farm and the grain quality laboratory of the Rice Research and Training Center, Kafr El-Sheikh Governorate, Egypt to determine the grain quality characteristics as well as the nutritional value after milling of three Egyptian rice genotypes (*Oryza sativa)* under foliar treatment with different stimulating compounds. A tow-year field experiment was laid out in split-plot design with four replications in the 2017 and 2018 rice growing seasons. Rice genotypes were allocated in the main plot and stimulating compounds in the subplot. After harvesting, 150-gram from each plot was taken to study the following characteristics: hulling, milling, head rice, gelatinous temperature, kernel elongation, amylose content, protein content, carbohydrate content, lipids content, ash, phosphorus, potassium and energy composition. There were significant differences for most of the studied characteristics caused by genotype, stimulating compound and the interaction between the two factors. The results revealed that the grain quality characteristics and the nutritional value of Egyptian rice genotypes can be enhanced by the application of some stimulating compounds such as amino acids and NPK compound fertilizer.
Keywords: *milling recovery, cooking quality, amino acid, ascorbic acid, NPK*

Introduction

Rice (*Oryza sativa*, L.) is considered to be one of the most important stable food crops in Egypt. It plays a critical role in Egyptian food security. Rice is mostly considered a starchy food, but since animal products can be scarce or expensive in Egypt, it is often the most important source of protein in Egyptians diet as well. The Egyptian rice varieties vary in grain quality characteristics as well as nutritional value after milling (Metwally et al., 2016). Those characteristics are not important only to the consumers but, they are important to the marketer and miller. The genotypic variations in grain quality and nutritional value of milled rice are mainly caused by the genetic, environmental and agronomical factors which have been reported as the main responsible for those variations. Singh et al. (2011) found genotypic variations among three Japonica rice cultivars on physicochemical, cooking and textural properties of milled grains. They found that amylose and protein contents of milled rice grains affected significantly the cooked grain quality. Anjum et al. (2007) indicated significant variation in cooking, chemical characteristics and mineral contents among four Pakistani rice cultivars therefore; differences can be exploited by the rice breeders in their hybridization.

Mohapatra and Bal (2006) studied the cooking quality and instrumental textural attributes of cooked rice for different milling fractions of three rice varieties. They

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found that cooking qualities as well as textural attributes were found to be varied among the varieties. Frei and Becker (2005) reported that rice protein quality is determined by the amino acid composition and its digestibility. Rice protein quality is very high when compared to other crops. Rice has favorable amino acid compositions, a high amount of lysine and a high protein digestibility which makes it a fairly good source of protein in diets where animal protein is limited.

Several researchers have reported that the use of stimulating substances is one of the effective means of improving rice grain quality and enhancing the milled rice nutritional value. Pan et al. (2013) indicated that foliar application plant growth regulators (gibberellic acid, paclobutrazol, 6-Benzylaminopurine) enhanced yield, grain quality characteristics and antioxidant enzyme activities in super hybrid rice. Kamboj and Mathpal (2019) found that foliar application of plant growth regulators i.e. gibberellic acid cytokinin enhanced the translocation of zinc from vegetative parts to the grains of the rice. They also reported a favorable effect of plant growth regulators on protein content in milled grains due to the translocation of synthesized proteins towards grain by increasing longevity of leaves thus resulted in higher grain protein content.

Therefore, the main objective of this study is to test the grain quality characteristics and the nutritional value of three rice genotypes under foliar application different stimulating compounds.

Materials and methods

At the Experimental Farm of RRTC (Rice Research & Training Center), Sakha, Kafrelsheikh Governorate, Egypt (31°5′17″N, 30°56′44″E altitude), a two-year field experiment was conducted to study the behavior of three rice genotypes treated with foliar application of different stimulating compounds. The selected tested genotypes were Sakha108, GZ9399 and GZ10154. Sakha108 is a newly registered variety while GZ9399 and GZ10154 are new promising genotypes. The parentage, type and origin of the studied genotypes are presented in *Table 1*.

Table 1. *Parentage, type and origin of the rice genotypes*

Genotype	Parentage	Type	Origin
Sakha108	Sakha 101/ HR5824-B-3-2-3 //Sakha 101	Japonica	Egypt
GZ9399	Giza178/IR65844-29-1-3-2-1	Indica/Japonica	Egypt
GZ10154	Sakha 105/Sakha 101	Japonica	Egypt

The soil of the experiment was clayey in texture, 8.15 and 8.12 pH, 1.06 and 1.04 dS/m EC, 1.46 and 1.39% organic matter, 23.91 and 24.51 mg kg^{-1} available nitrogen, 15.45 and 14.12 mg kg^{-1} available phosphorus, 346 and 357 mg kg^{-1} available potassium in first and second season respectively.

The different stimulating compounds were control (distilled water), amino acids, NPK 20:20:20, ascobin, vulvic acid, humic acids, and potassium sulphate. Stimulating compounds were applied twice; 20 and 40 days after transplanting (DAT). Amino acid (27.38% mixed amino acids + 9% micronutrients + 2% magnesium), ascobin (13% citric acid, 25% ascorbic acid plus 62% organic materials) and humic acid (65% humic acid + 10% K_2O) were applied at the rate of 1 g L^{-1}. Vulvic acid (50% vulvic acid + 20% organic acid) was applied at 1 ml L^{-1}. NPK (20-20-20) and potassium sulphate

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(50% K_2O) were applied at 2%. 500 letters per hectare was used as spray solution amount for all compounds.

Split-Plot Design with four replicates was used. Rice genotype and stimulating compound treatment were arranged as main plot and sub-plot, respectively. The plot size was 12 m^2. Rice seeds were sown in nursery 2nd and 4th of May for 2017 and 2018, respectively. Transplanted was done after 28 days of sowing nurseries with three to four seedlings per hill, spaced 20 X 20 cm within and between rows. All plots received optimum culture practices.

Grain quality characteristics: milling recovery, gelatinization temperature, kernel elongation and amylose content was estimated according to Cruz and Khush (2000): 150 (g) cleaned rough rice at 14% moisture content was dehulled using an Experimental Huller Machine (Satake - Japan). The brown rice was separated and weighed then the hulling percentage was calculated. The brown rice was milled using MC GILL Rice Miller No.2. (S.K. Appliances – India). The total milled rice was weighed and milled rice percentage was calculated. Whole milled grains were separated from the total milled rice using a rice sizing device SKU: 61-220-50 (Seedburo – USA). The percentage of head rice was calculated.

Six grains of whole milled rice were placed in boxes containing 1.7% KOH and arranged so that the kernels do not touch each. The boxes were covered and incubated for 23 h at 30 °C. The appearance and disintegration of endosperm were graded visually according to the numerical scale of the gelatinization temperature. Kernel elongation was measured using the Micrometer, the length of five milled grains was measured (mm) and their average was determined for each treatment (before cooking). Grains were left in a test tube filled with 30 ml of distilled water for 30 min, then for another 10 min in 98 °C a water tub. After that, the tubes were placed in cold water until reaching room temperature. Grains were lifted from the distilled water, dried (by filter paper), and measured again by graph papers (after cooking). Kernel elongation percentage was calculated as the percentage of grain expanding before and after cooking.

Amylose content was determined by weighing accurately 100 mg of sample into 100 ml volumetric flask then carefully adding 1 ml of 95% ethanol and 9 ml 1 N Na OH. The mixture was heated for 10 min in a boiling water bath to gelatinize the starch; then cooled, and the content made up to volume 100 ml with water. Pipette 5 ml portion of the gelatinization starch solution, 1 ml of 1 N acetic acid, and 2 ml of iodine solution were added and made up to a volume of 100 ml with distilled water. The content was shaken and stands for 20 min before reading the transmission at 620 nm by Spectronic 1201 Spectrophotometer (Milton Roy, USA)**.**

Protein, carbohydrate, lipids, ash, phosphorus and potassium determination in rice grain: Plant samples were taken from the grain after milling (50 g of milled rice). All plant samples were placed in paper bags and oven-dry at 70 °C for 48 h. Grain samples were ground to powder and digested according to the method of Chapman and Pratt (1961) before chemical analysis as follows: the nitrogen content of milled grains was determined by using the Microkieldahl method (Jackson, 1967) to calculate protein content. Total carbohydrate was calculated by difference as mentioned by Fraser and Holmes (1959). Lipids was determined according to A.O.A.C. (2000). The phosphorus content of milled grain was determined using Spectronic 1201 Spectrophotometer (Milton Roy, USA) following the procedures of Watanabe and Olsen (1965). The Potassium content of grain was determined using Elico CL378 Flame Photometer

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(RHYS international LTD, India) according to Peterpurgski (1968) method. The energy composition was calculated according to A.O.A.C. (2000) and Singh and Singh (2019).

The data was subjected to analysis of variance (ANOVA), and the differences among treatments' means were compared by Duncan's Multiple Range Test ($P < 0.05$) and multiple F. test according to Duncan (1955). The Simple correlation coefficient (r) among the nutritional properties of milled rice grains was generated using pooled values of two seasons. All statistical analyses were done using Costat Statistical Software - CoHort Software.

Results and discussion

There were marked differences in milling recovery characteristics due to genotype, stimulating compound and the interaction between the two factors (*Tables 2* and *3*). Sakha108 recorded the highest values of hulling percentage and head rice percentage. The highest percentage of milling was observed by the rice genotype GZ10154. The differences among rice genotypes may be due to the genetically inherited variants. Zhao et al. (2018) identified GS9 (Grain Shape Gene on Chromosome 9) gene by map-based cloning. GS9 regulates grain shape and hull thickness by altering cell division.

The application of different stimulating compounds enhanced significantly milling recovery characteristics. The highest percentages of hulling, milling and head rice were noted with foliar application of amino acid followed by NPK 20,20,20. Regarding the interaction effect between genotype and stimulating compound, the studied rice genotypes responded differently to the stimulating compound application. Sakha108 recorded the highest percentages of hulling and head rice under the amino acid foliar application. While the highest percentage of milling was recorded by GZ10154 rice genotype treated with amino acid. Pan et al. (2013) found that foliar application plant growth regulators improved the rice milling recovery characteristics. Gharieb et al. (2016) indicated that application of Ascobin (13% citric acid, 25% ascorbic acid and 62%organic materials) three times as foliar application increased hulling, milling and head rice percentages.

Table 2. *Hulling %, milling % and head rice % of the tested rice genotypes under foliar application of stimulating compounds*

Treatment	Hulling %		Milling %		Head rice %	
	2017	2018	2017	2018	2017	2018
Genotype:						
Sakha108	80.11a	80.05a	70.20b	69.73c	66.22a	65.41a
GZ9399	79.34c	79.13c	69.39c	70.21b	64.69b	63.97b
GZ10154	79.41b	79.34b	70.49a	71.43a	64.63c	63.27c
F test	*	**	*	*	*	*
Stimulating compound:						
Amino acid	80.13a	79.99a	70.62a	70.96a	65.66a	64.74a
NPK 20:20:20	79.97b	79.86b	70.42b	70.77b	65.50b	64.59b
Ascobin	79.82c	79.62c	70.26c	70.62c	65.33c	64.38c
Vulvic acid	79.61d	79.47d	70.04d	70.44d	65.19d	64.20d
Humic acid	79.45e	79.35e	69.85e	70.31e	65.04e	64.06e
Potassium sulphate	79.28f	79.19f	69.62f	70.14f	64.86f	63.87f
Control	79.10g	79.06g	69.40g	69.96g	64.70g	63.69g
F test	**	**	**	**	**	**
Interaction effect	**	**	**	**	**	**

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Table 3. *Hulling %, milling % and head rice % as affected by the interaction between rice genotype and stimulating compound*

Stimulating compound	Rice genotypes					
	Sakha108	**GZ9399**	**GZ10154**	**Sakha108**	**GZ9399**	**GZ10154**
	2017			**2018**		
	Hulling %					
Amino acid	80.69 a	79.81 f	79.90 e	80.50 a	79.70 g	79.79 f
NPK 20:20:20	80.47 b	79.69 g	79.76 fg	80.38 b	79.58 i	79.63 h
Ascobin	80.29 c	79.50 i	79.69 g	80.16 c	79.20 l	79.51 j
Vulvic acid	80.11 d	79.34 j	79.40 j	80.03 d	79.02 m	79.38 l
Humic acid	79.93 e	79.22 k	79.21 k	79.95 e	78.93 n	79.17 l
Potassium sulphate	79.75 fg	79.01 l	79.09 l	79.70 g	78.81 o	79.06 m
Control	79.59 h	78.86 m	78.86 m	79.63 h	78.70 p	78.85 o
	Milling %					
Amino acid	70.80 b	70.00 j	71.07 a	70.21 k	70.70 h	71.98 a
NPK 20:20:20	70.61 d	69.83 l	70.82 b	70.02 m	70.55 i	71.76 b
Ascobin	70.45 f	69.66 m	70.68 c	69.89 n	70.39 j	71.60 c
Vulvic acid	70.20 h	69.44 o	70.50 e	69.70 o	70.20 k	71.42 d
Humic acid	70.02 j	69.19 p	70.34 g	69.57 p	70.07 l	71.29 e
Potassium sulphate	69.80 l	68.91 q	70.15 i	69.44 q	69.89 n	71.10 f
Control	69.58 n	68.70 r	69.92 k	69.30 r	69.68 o	70.90 g
	Head rice %					
Amino acid	66.70 a	65.19 h	65.11i	64.50 h	65.90 a	63.83 l
NPK 20:20:20	66.55 b	64.96 k	65.00 j	64.38 i	65.74 b	63.65 m
Ascobin	66.41 c	64.80 l	64.80 l	64.11 j	65.60 c	63.44 n
Vulvic acid	66.25 d	64.70 m	64.63 n	63.95 k	65.42 d	63.25 o
Humic acid	66.10 e	64.53 o	64.49 p	63.80 l	65.27 e	63.13 p
Potassium sulphate	65.89 f	64.40 q	64.30 r	63.63 m	65.09 f	62.90 q
Control	65.70 g	64.30 r	64.10 s	63.47 n	64.91 g	62.70 r

The Gelatinous temperature, kernel elongation and amylose content were varied the rice genotypes by stimulating compounds application (*Tables 4* and *5*). GZ9399 recorded the highest values of gelatinous temperature and the lowest values of kernel elongation and amylose content. The highest amylose content and kernel elongation were observed by the rice genotype GZ10154. The variations among the studied genotypes in cooking qualities may be due to the differences in their shape, thickness and surface area. Mohapatra and Bal (2006) suggested that surface area and thickness of the rice grain are important factors in deciding the diffusion of water during cooking. GZ9399 has lower amylose content and higher gelatinous temperature and kernel elongation which suggests that amylose content plays a significant role in deciding the cooking quality of rice.

Starch is the main component of milled rice grain. It is made up of two starchy fractions: amylose and amylopectin. After cooking, rice grains with high amylose content are dry, fluffy, separate and hard, while those with low amylose content are glossy, soft and sticky. So, Amylose content is considered to be one of the most important predictors of the eating quality of cooked rice. Milled rice cultivars may be generally classified based on their apparent amylose content into waxy (1-2%), very low (2-12%), low (12-20%), intermediate (20-25%) or high (>25%) (Bao, 2012). Based on the results, the studied genotypes under the current study belong to low amylose content rice types that meet the Egyptian consumers' preferences. The significant variation in amylose content among the studied genotypes could be attributed to the genetic background of those varieties.

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Table 4. *Gelatinous temperature, kernel elongation and amylose % of the tested rice genotypes under foliar application of stimulating compounds*

Treatment	Gelatinous temperature		Kernel elongation %		Amylose %	
	2017	2018	2017	2018	2017	2018
Genotype:						
Sakha108	6.35c	6.19b	58.01 b	57.95 b	18.10b	17.89b
GZ9399	7.42a	6.87a	78.47 a	78.26 a	16.80c	16.72c
GZ10154	6.47b	5.86c	57.80 c	57.72 c	18.59a	18.53a
F test	*	*	*	*	*	*
Stimulating compound:						
Amino acid	7.06a	6.74a	65.10 a	65.39 a	18.10a	18.00a
NPK 20:20:20	6.95b	6.57b	65.12 a	64.76 b	18.02b	17.90b
Ascobin	6.80c	6.56b	64.91 b	64.69 c	17.93c	17.82c
Vulvic acid	6.68d	6.29c	64.77 c	64.58 d	17.83d	17.71d
Humic acid	6.50e	6.15d	64.60 d	64.51 e	17.76e	17.63e
Potassium sulphate	6.19f	5.98e	64.49 d	64.38 f	17.64f	17.51f
Control	6.04g	5.86f	64.34 e	64.20 g	17.53g	17.44g
F test	**	**	**	**	**	**
Interaction effect	**	**	**	**	**	**

Table 5. *Gelatinous temperature, kernel elongation and amylose % as affected by the interaction between rice genotype and stimulating compound*

Stimulating compound	Rice Genotypes					
	Sakha108	GZ9399	GZ10154	Sakha108	GZ9399	GZ10154
	2017			2018		
	Gelatinous temperature					
Amino acid	6.80 g	7.47 a	6.92 f	6.67 cd	7.39 a	6.17 g
NPK 20:20:20	6.65 h	7.38 b	6.84 g	6.50 de	7.20 ab	6.03 gh
Ascobin	6.50 j	7.30 c	6.60 i	6.39 ef	7.07 b	6.23 fg
Vulvic acid	6.38 k	7.18 d	6.49 j	6.20 fg	6.85 c	5.84 hi
Humic acid	6.20 mn	7.02 e	6.30 l	6.03 gh	6.70 cd	5.72 ij
Potassium sulphate	6.01 o	6.41 k	6.17 n	5.84 hi	6.52 de	5.60 jk
Control	5.93 p	6.23 m	5.98 o	5.70 ij	6.41 ef	5.48 k
	Kernel elongation %					
Amino acid	58.33 ef	78.76 a	58.23 f	58.90 h	78.59 a	58.70 i
NPK 20:20:20	58.53 e	78.70 a	58.14 fg	58.00 j	78.50 b	57.80 m
Ascobin	58.13 fg	78.60 ab	58.00 gi	57.94 k	78.43 c	57.71 n
Vulvic acid	58.01 gh	78.49 b	57.81 hj	57.85 l	78.30 d	57.60 o
Humic acid	57.80 ik	78.40 bc	57.62 jl	57.80 m	78.21 e	57.53 p
Potassium sulphate	57.72 jk	78.26 cd	57.50 lm	57.70 n	78.00 f	57.45 q
Control	57.60 kl	78.08 d	57.35 m	57.50 p	77.80 g	57.30 r
	Amylose					
Amino acid	18.37 f	17.09 l	18.85 a	18.19 h	17.03 o	18.79 a
NPK 20:20:20	18.30 g	17.00 m	18.77 b	18.10 i	16.90 p	18.70 b
Ascobin	18.21 h	16.89 n	18.70 c	18.01 j	16.84 q	18.62 c
Vulvic acid	18.10 i	16.80 o	18.60 d	17.88 k	16.72 r	18.54 d
Humic acid	18.07 i	16.71 p	18.51 e	17.80 l	16.63 s	18.46 e
Potassium sulphate	17.89 j	16.63 q	18.42 f	17.70 m	16.50 t	18.35 f
Control	17.78 k	16.52 r	18.30 g	17.61 n	16.43 u	18.28 g

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Foliar application of different stimulating compounds increased significantly gelatinous temperature, kernel elongation and amylose content. The highest values of gelatinous temperature, kernel elongation and amylose content were observed with the application of amino acid followed by NPK 20,20,20. Concerning the interaction effect between genotype and stimulating compound, GZ9399 recorded the highest values of gelatinous temperature and kernel elongation under the amino acid foliar application. While the highest percentage of amylose content was recorded by GZ10154 rice genotype treated with amino acid.

Data in *Tables 6* and *7* revealed significant differences among rice genotypes, stimulating compound and the interaction for protein, carbohydrate and lipids content percentage in both seasons. GZ10154 recorded the highest values of protein content and the lowest values of carbohydrate and lipids. The highest values of carbohydrate content were recorded by GZ9399. Lipids content was significantly higher in Sakha 108 than the other genotypes. Zhao et al. (2018) indicated that GS9 Gene on Chromosome 9 acts as a transcriptional activator to regulate rice grain protein content and appearance quality. Anjum et al. (2007) and Singh and Singh (2019) found genotypic variation in protein, carbohydrate and lipids content percentage among different rice varieties.

The application of different stimulating compounds increased significantly the contents of protein and lipids over control treatment. Foliar application of NPK recorded the highest values of protein content while amino acid foliar application produced the highest values of lipids content. There are highly significant differences in protein, carbohydrate and lipids content percentage with respect to genotype X stimulating compound interaction suggesting that the three rice genotypes responded differently to stimulating compound application. Foliar application of NPK to GZ10154 gave the highest content of protein. Application of amino acid to Gz9399 and Sakha108 recorded the highest values of carbohydrate and lipids respectively. Khan et al. (2016) found that application of plant growth regulators (gibberellic acid, indole acetic acid and kinetin) significant increase in soluble protein contents of two rice cultivars. These results found in accordance of Kamboj and Mathpal (2019).

Table 6. *Protein, carbohydrate and lipids content % of the tested rice genotypes under foliar application of stimulating compounds*

Treatment	Protein %		Carbohydrate %		Lipids %	
	2017	**2018**	**2017**	**2018**	**2017**	**2018**
Genotype:						
Sakha108	8.27b	7.41b	76.90b	77.92b	0.591a	0.557a
GZ9399	7.33c	6.49c	77.93a	78.94a	0.545b	0.516b
GZ10154	9.52a	7.81a	75.77c	77.72c	0.503c	0.491c
F test	*	*	*	*	*	*
Stimulating compound:						
Amino acid	8.91b	7.92b	77.05a	78.24ab	0.572a	0.552a
NPK 20:20:20	9.10a	8.32a	76.59d	77.60c	0.564b	0.539b
Ascobin	8.60c	7.50c	76.82bc	78.20b	0.556c	0.530c
Vulvic acid	8.39d	7.10d	76.80c	78.31ab	0.547d	0.521d
Humic acid	8.07e	6.89e	76.91a-c	78.34ab	0.539e	0.512e
Potassium sulphate	7.88f	6.58f	76.98ab	78.33ab	0.530f	0.503f
Control	7.68g	6.35g	76.91a-c	78.40a	0.518g	0.493g
F test	**	**	**	**	**	**
Interaction effect	**	**	**	**	**	**

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Table 7. *Protein, carbohydrate and lipids content % as affected by the interaction between rice genotype and stimulating compound*

Stimulating compound	Rice genotypes					
	Sakha108	GZ9399	GZ10154	Sakha108	GZ9399	GZ10154
	2017			2018		
	Protein %					
Amino acid	8.72 g	7.76 l	10.25 b	8.03 e	6.94 l	8.80 b
NPK 20:20:20	8.91 f	7.99 k	10.41 a	8.28 c	7.24 j	9.44 a
Ascobin	8.50 i	7.47 n	9.83 c	7.68 f	6.68 o	8.13 d
Vulvic acid	8.27 j	7.38 o	9.51 d	7.30 i	6.50 q	7.51 g
Humic acid	8.05 k	7.06 p	9.09 e	7.12 k	6.13 r	7.41 h
Potassium sulphate	7.82 l	6.90 q	8.94 f	6.89 m	6.08 s	6.78 n
Control	7.64 m	6.78 r	8.62 h	6.61 p	5.84 t	6.60 p
	Carbohydrate %					
Amino acid	77.04 de	78.29 a	75.82 hi	77.96 e-g	79.30 a	77.47 i
NPK 20:20:20	76.58 f	77.83 bc	75.36 k	77.46 i	78.70 c	76.63 j
Ascobin	76.94 e	77.97 bc	75.56jk	77.90 f-h	78.98 b	77.71 h
Vulvic acid	76.89 e	77.83 bc	75.67ij	78.02 d-g	78.87 bc	78.05 d-f
Humic acid	76.85 e	77.98 b	75.89gh	78.19 d	79.01 b	77.82 gh
Potassium sulphate	77.19 d	77.77 c	75.99gh	77.96 e-g	78.83 bc	78.22 d
Control	76.84 e	77.82 bc	76.08 g	78.13 de	78.89 c	78.18 d
	Lipids %					
Amino acid	0.617 a	0.569 g	0.530 l	0.599 a	0.539 ef	0.518 h
NPK 20:20:20	0.610 b	0.560 h	0.524 m	0.578 b	0.530 g	0.510 i
Ascobin	0.600 c	0.553 i	0.515 o	0.570 c	0.521 h	0.500 j
Vulvic acid	0.591 d	0.546 j	0.504 p	0.556 d	0.515 hi	0.493 k
Humic acid	0.583 e	0.538 k	0.496 q	0.543 e	0.510 i	0.485 l
Potassium sulphate	0.575 f	0.530 l	0.485 r	0.533 fg	0.503 j	0.474 m
Control	0.563 h	0.520 n	0.471 s	0.521 h	0.497 jk	0.462 n

The ash, phosphorus and potassium contents in milled rice grains differed significantly among genotypes and stimulating compounds, with a significant interaction (*Tables 8* and *9*). Ash content was higher in GZ9399 followed by GZ10154 without any significant difference between them in the second season. Sakha 108 recorded the highest content of phosphorus and potassium. Similar variations pattern has been reported earlier for rice genotypes by Anjum et al. (2007) and Singh et al. (2011). Singh and Singh (2019) found a genetic diversity of five rice varieties in mineral contents of rice grains. Stimulating compounds foliar application increased the content of ash, phosphorus and potassium in milled rice grains compared to control. Amino acid foliar application produced the highest values of ash content. Phosphorus content was higher in NPK treatment compared with other treatments. Potassium sulphate treatment increased significantly potassium content over the other treatments.

Regarding the interaction effect, ash content was higher in GZ9399 under the application of amino acid in both seasons. Phosphorus content in Sakha 108 rice milled grain was higher with the foliar application of NPK. The application of potassium sulphate to Sakha 108 produced the highest content of potassium. Pan et al. (2013) reported that foliar application of different stimulating compounds could enhance the lodging resistance and increase root biomass and root activity to improve phosphorus and potassium accumulation in milled grains of two rice cultivars.

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Table 8. *Ash, phosphorus and potassium content % in milled rice grain of the tested rice genotypes under foliar application of stimulating compounds*

Treatment	Ash %		Phosphorus %		Potassium %	
	2017	2018	2017	2018	2017	2018
Genotype:						
Sakha108	0.60 c	0.58 b	0.265 a	0.276 a	0.244 a	0.263 a
GZ9399	0.65 a	0.63 a	0.237 b	0.243 b	0.182 b	0.201 b
GZ10154	0.63 b	0.61 a	0.220 c	0.231 c	0.161 c	0.170 c
F test	*	*	*	*	*	*
Stimulating comp.:						
Amino acid	0.74 a	0.70 a	0.255 b	0.267 b	0.179 f	0.193 f
NPK 20:20:20	0.70 b	0.67 ab	0.262 a	0.274 a	0.213b 0.186	0.230 b
Ascobin	0.67 c	0.65 b	0.248 c	0.259 c	e	0.201 e
Vulvic acid	0.63 d	0.60 c	0.240 d	0.250 d	0.197 d	0.213 d
Humic acid	0.59 e	0.57 cd	0.233 e	0.243 e	0.205 c	0.221 c
Potassium sulphate	0.57 f	0.55 d	0.227 f	0.235 f	0.221 a	0.238 a
Control	0.51 g	0.51 e	0.219 g	0.225 g	0.168 g	0.184 g
F test	**	**	**	**	**	**
Interaction effect	**	**	**	**	**	**

Table 9. *Ash, phosphorus and potassium content % in milled rice grain as affected by the interaction between rice genotype and stimulating compound*

Stimulating compound	Rice genotypes					
	Sakha108	GZ9399	GZ10154	Sakha108	GZ9399	GZ10154
	2017			2018		
	Ash %					
Amino acid	0.72 c	0.77 a	0.75 b	0.69 ab	0.71 a	0.71 a
NPK 20:20:20	0.69 d	0.72 c	0.70 d	0.67 a-c	0.69 ab	0.66 a-d
Ascobin	0.65 ef	0.70 d	0.66 e	0.62 c-f	0.69 ab	0.64 b-e
Vulvic acid	0.64 fg	0.63 gh	0.62 hi	0.61 e-f	0.60 d-g	0.60 d-g
Humic acid	0.56 lm	0.61 ij	0.61 ij	0.55 gh	0.59 e-g	0.58 f-h
Potassium sulphate	0.54 n	0.60 j	0.58 k	0.53 h	0.58 f-h	0.55 gh
Control	0.42 o	0.57 kl	0.55 mn	0.42 i	0.55 gh	0.55 gh
	Phosphorus %					
Amino acid	0.281 b	0.251 g	0.235 j	0.291 b	0.263 g	0.248 k
NPK 20:20:20	0.289 a	0.258 e	0.241 i	0.298 a	0.271 e	0.253 i
Ascobin	0.274 c	0.243 h	0.229 k	0.285 c	0.254 h	0.240 m
Vulvic acid	0.260 d	0.236 j	0.220 m	0.278 d	0.243 l	0.230 o
Humic acid	0.255 f	0.230 k	0.214 o	0.270 f	0.235 n	0.224 q
Potassium sulphate	0.250 g	0.225 l	0.206 p	0.263 g	0.225 p	0.217 r
Control	0.241 i	0.217 n	0.200 q	0.251 j	0.216 s	0.210 t
	Potassium %					
Amino acid	0.230 f	0.162 o	0.145 q	0.245 f	0.185 n	0.150 s
NPK 20:20:20	0.260 b	0.201 i	0.180 l	0.281 b	0.220 i	0.190 m
Ascobin	0.235 e	0.174 m	0.150 p	0.254 e	0.190 m	0.159 r
Vulvic acid	0.243 d	0.185 k	0.163 o	0.268 d	0.200 k	0.171 q
Humic acid	0.252 c	0.193 j	0.171 n	0.275 c	0.209 j	0.179 o
Potassium sulphate	0.269 a	0.210 h	0.185 k	0.290 a	0.229 h	0.197 l
Control	0.221 g	0.150 p	0.134 r	0.234 g	0.174 p	0.145 t

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Figure 1 shows that the amounts of energy vary significantly among the different genotypes. The amounts in GZ9399 and GZ10154 were slightly higher than Sakha108. These variations are mainly due to genetic diversity. The application of different stimulating compounds increased energy amount in milled rice grains. The application of amino acid recorded the highest composition of energy for the three rice genotypes. Data in *Table 10* indicated that energy composition in milled rice grain had significant and positive correlations with each of lipids ($p = 0.05$), ash ($p = 0.01$) and phosphorus ($p = 0.01$) concentrations in milled grains. These results explain the superiority of amino acid application.

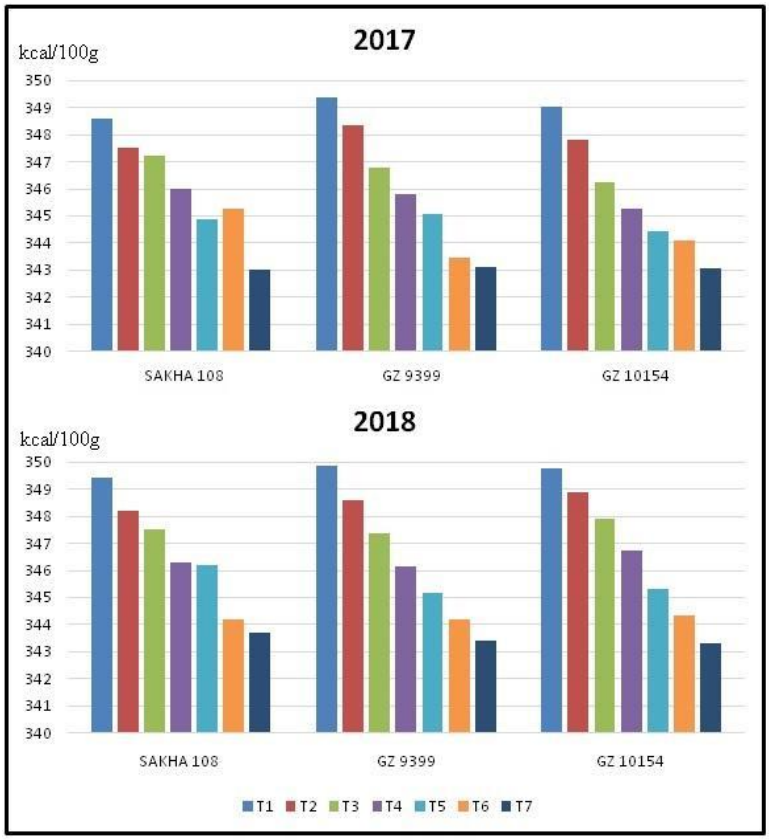

Figure 1. *Energy composition kcal/100 g of milled rice grains of the tested rice genotypes under foliar application of stimulating compounds. T1: Amino acid, T2: NPK 20:20:20, T3: Ascobin, T4: Vulvic acid, T5: Humic acid, T6: Potassium sulphate, T8: Control*

Table 10. *Simple correlation coefficient (r) among the nutritional properties of milled rice grains (Pooled data of two seasons)*

	Protein %	**Carbohydrate %**	**Lipids %**	**Ash %**	**P %**	**K %**	**Energy**
Protein %	1.000	-0.884**	0.497*	0.599**	0.153	-0.253	0.416
Carbohydrate %	-0.884**	1.000	-0.337	-0.194	0.194	0.262	0.055
Lipids %	0.497*	-0.337	1.000	0.563**	0.673**	0.344	0.505*
Ash %	0.599**	-0.194	0.563**	1.000	0.552*	-0.158	0.906**
P %	0.153	0.194	0.673**	0.552*	1.000	0.598**	0.765**
K %	-0.253	0.262	0.344	-0.158	0.598**	1.000	0.042
Energy	0.416	0.055	0.505*	0.906**	0.765**	0.042	1.000

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Conclusion

The foliar application of different stimulating compounds enhanced rice grain quality characteristics as well as the nutritional value of milled grains. The application of different stimulating compounds enhanced the translocation of assimilates to milled grains. Amino acid foliar application showed marked increases in the studied characteristics compared to other treatments. There were noticeable differences in grain quality characteristics and the nutritional value of milled grains among the studied genotypes. Finally, greater attention should be paid to grain quality characteristics in Egyptian rice production to meet Egyptian consumer preferences. Thus, more studies should be done to improve the grain quality characteristics of the Egyptian rice varieties.

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AFRICAN SWINE FEVER IN POLAND – PREVALENCE, PATHWAYS AND POTENTIAL SPREAD AND REDUCTION POSSIBILITIES

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Abstract. The paper presents the epizootic situation of African swine fever in Polish territory from the official confirmation of the first case to the end of 2019. In Poland, there is a genotype II of the virus, the transmission of which is associated with its appearance in Georgia in 2007, followed by its spread to the northwest and subsequent appearance in European countries. During the virus period, an annual increase in the number of cases found in feral domestic pigs and outbreaks in pigs was observed. It was not until 2019, when 2,477 cases were diagnosed, that the virus prevalence stabilized in wild boars and the number of outbreaks in pigs more than doubled compared to 2018. Depopulation of boars carried out during this period, indicated as the primary source of danger, did not bring the expected results. Administrative preventive measures have also failed. The current epizootic situation as well as the appearance of the virus even in places more than 300 km away from its earlier statements is a confirmation that wild boars are not the basic vector in the spread of ASFV. At the same time, during the disease occurrence, in samples of wild boars shot from regions where the virus appeared, the presence of antibodies increased in the following years. Seropositivity of wild boars indicates the initial phase of viral endemicity in these regions. This creates another epizootic threat, as these animals may become asymptomatic carriers that may infect other individuals in the wild. In the current situation, it seems necessary to develop clear biosecurity principles for pig farms. These principles should also cover all individuals who may become viral mechanical vectors in the environment. Enforcing compliance with biosecurity rules developed in all areas of activity related to pigs and wild boars should be a priority in limiting the spread of ASFV and its possible eradication in Poland.
Keywords: *wild boar, domestic pig, virus, prevention, biosecurity*

Introduction

The African swine fever virus (ASFV) is a DNA virus that belongs to the *Asfavirus* species of the family *Asfarviridae*. It causes acute viral haemorrhagic disease of pigs and wild boars, usually ending in death, therefore it poses a serious threat to pig production all over the world. Its properties provide it with very high resistance to inactivation by physical and chemical agents, which means that under optimal conditions it can be pathogenic for up to 6 years. The disease is characterized by clinical symptoms and sectional changes similar to the acute form of classical swine fever, in particular high fever, significant splenomegaly, high degree of ecchymosis and up to 100% mortality. In connection with the ban on the treatment of sick animals and the

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lack of vaccines against ASF, the disease is only combated by administrative methods, by killing infected herds and introducing strict biosecurity rules and by limiting, by shooting, the wild boar population as the primary virus reservoir in the natural environment (Markowska-Daniel and Pejsak, 2014; Pejsak and Truszczyński, 2017b; Fila and Woźniakowski, 2020).

The African swine fever virus in Poland was first found on February 14, 2014, based on samples from a wild boar that was found dead in the Sokole County, about 1 km from the border with Belarus. This case was confirmed by the European Reference Laboratory (EURL) for ASF in Valdeolmos, Spain, on February 18, 2014. On February 17, 2014, a second fallen boar was found near the border with Belarus (Pejsak et al., 2014a). In the following months of 2014, further cases of the virus were confirmed. They occurred only in the border belt of the Podlaskie Voivodeship reaching up to 10 km inland, along a length of about 50 km along the border with Belarus (Flis and Nestorowicz, 2019; Woźniakowski et al., 2016). The conducted phylogenetic analyzes showed that in all cases the virus was found in genotype II, i.e. identical as in other Eastern European countries, and showed 99.95% identity of nucleotides with the Georgia 2007/1 strain (Mazur-Panasiuk et al., 2019a; Śmietanka et al., 2016). Genotype II has also been confirmed by ASFV (African swine fever virus) studies conducted in Belgium in 2018. However, the entire genome sequence of ASFV Belgium 2018/1 showed 15 differences for overall sequence identity at the nucleotide level. A comparison of all available sequences of the entire genome of the virus occurring in Eastern European countries as well as in ASFV in China indicates that these genomes are almost identical with a probability of 99.9% (Forth et al., 2019; Gilliaux et al., 2019). These data confirm previous reports on the possible direction of virus transmission to Eastern European countries through Georgia and the Caucasus since 2007 and further towards north-eastern Europe (Flis and Nestorowicz, 2019; Markowska-Daniel and Pejsak, 2014; Pejsak et al., 2014a, b; Pejsak and Piekut, 2018).

Since the appearance of the first cases of virus occurrence, the Polish authorities together with veterinary services have undertaken a number of administrative activities introducing further provisions and guidelines to contribute to limiting the possibility of its transmission to new areas (Flis, 2019). This was mainly due to the lack of experience in the scope of the possibility of the virus spreading and the role of wild boars in its transmission to new areas, with varying levels of density of this species and the impact of hunting pressure. Therefore, initially two main hypotheses were adopted. The first of them said that the disease would rapidly spread westwards, while the second indicated the possibility of very rapid extinction of the disease due to the high virulence of the virus, which will naturally lead to depopulation of wild boars. It soon turned out that these hypotheses in Poland's environmental and social conditions did not work. Therefore, actions were taken to exterminate wild boars by hunting. It was related to the fact that the wild boar was indicated as the basic vector of virus spread to new areas (Flis and Kołodziejski, 2019; Flis, 2020; Pejsak et al., 2018b; Probst et al., 2017).

Undoubtedly, in Poland's environmental conditions, wild boars constitute the primary reservoir of the virus (Flis and Nestorowicz, 2019; Flis and Kołodziejski, 2019; Pejsak et al., 2018b). Therefore, high population density rates have an impact on the increase in the possibility of the virus traveling in the environment, where it spreads in a diffusive manner, i.e. without human intervention (Śmietanka et al., 2016; Pejsak and Truszczyński, 2017b). In addition, in border zones, the spread may also be partly dependent on the occurrence of the virus in neighboring countries (Flis and

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Nestorowicz, 2019; Flis, 2020; Markowska-Daniel et al., 2011). Assessment of the geographical pattern of virus spread indicates that wild boars can be a virus vector to areas not far away from the sites of its previous findings. This is due to the fact that the virus in the natural environment has clear connectivity with habitats, without a tendency to dynamically move over time. In the natural environment, regardless of the season, it spreads with an average pace of 1-2 km per month (Abrahantes et al., 2017; Depner et al., 2016; Podgórski and Śmietanka, 2018).

The aim of the study was to analyze the epizootic situation of African swine fever in Poland in terms of the preventive measures taken so far, including measures related to the depopulation of wild boars and its impact on the spread of the virus from its appearance, i.e. from February 2014 to the end of 2019. The analysis included virus cases found in feral pigs and outbreaks in pigs in individual years of the study period, as well as a geographical model of virus spread and indication of possible vectors related to transmission to diverse environments, including pig-keeping farms, which will allow for the development of further groups of activities preventive measures in the scope of limiting the possibilities of ASFV transmission to new areas.

The presented data include only officially reported cases in which the presence of ASFV has been confirmed in laboratory tests. Certainly, some of the fallen boars are not found and remain in the natural environment, constituting a reservoir for the virus. In addition, there may also be situations where a dead boar is found and the case has not been reported. The number of these wild boars is difficult, and even impossible to determine precisely. Information from conducted search campaigns, during which the boar's dwelling places are meticulously penetrated, indicates that these are not common cases. Thus, the number of such cases does not affect the results presented.

Materials and methods

Material

The research material was the data of the Main Veterinary Inspectorate. They concerned officially identified cases of African swine fever virus in feral pigs since 2014, when the virus was first detected until the end of 2019. These data come from monitoring studies from every wild boar shot in the danger area (blue zone), restricted area (red zone) and protection area (yellow zone). These data also include the results of tests for the presence of ASF from every dead boar, regardless of the cause of death from all over Poland. Samples are taken from all wild boars from car collisions. In addition, they are also collected from fallen boars found occasionally, by persons staying in the wild boar's habitat, as well as from those found as part of organized search for fallen animals. Depending on the method of obtaining biological material, different samples are taken. Blood is collected from the boars shot. This is done by hunters or other persons authorized by district veterinarians. On the other hand, if dead pigs are also able to take blood samples, and if the carcasses are already in a state of decay, samples are taken from internal organs, mainly spleen, possibly kidneys or lungs. In cases of highly advanced degradation, samples are taken from the bone marrow.

Analyses

The collected material was tested on an ongoing basis in the national reference laboratories for ASFV, to which samples collected in the field were delivered. Two

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diagnostic methods are used in these tests. The first of these involves an ELISA test (Enzyme-Linked Immunosorbent Assay) to detect anti-ASFV antibodies. The second study is based on the molecular method of qPCR (real-time PCR - Ingezim PPA COMPAC, Ingenasa, Spain), i.e. a polymerase chain reaction to detect and identify ASFV genetic material. Soft tissues or bone marrow are also examined by real-time PCR. Each positive or doubtful sample in the ELISA was tested by a confirmatory indirect immunoperoxidase (ITP) technique that shows higher sensitivity than the ELISA. This method is recommended by EURL for ASF (CISA-INIA Valdeolmos).

Material development

On the basis of the obtained data, the dynamics of virus transmission and the geographical pattern of its spatial distribution in individual years were compiled. The prevalence index in wild boars was also calculated. It represented the percentage of positive cases found to the total number of wild boars tested. Also presented are data obtained from the National Veterinary Institute in Pulawy regarding the number of seropositive wild boars, which allowed determining the percentage of such animals in relation to the number of tested animals. Also presented is a geographical model of the possibility of the virus spreading to new areas through environmental vectors such as wild boars and indirect vectors. mechanical, related to multidirectional human activity. An analysis was also made of the dynamics of the number of wild boars in terms of the level of hunting exploitation of the population of this species, which is directly related to the occurrence and spread of the virus in the natural environment and potential possibilities of reaching pig farms. Data on numbers and hunting of wild boars were obtained from the Polish Hunting Association.

Results

Occurrence of ASF virus in Poland

In the period from 2014, when the virus was found in Poland for the first time, the number of cases found in wild boars tended to increase (*Fig. 1*). However, a very dynamic increase of over 9 times occurred in 2017 when 741 cases were found. In the following year, a further more than 3-fold increase in cases was observed, while in 2019 there was a relative stabilization compared to the previous year. Slightly different tendency of outbreaks was observed in pigs. In the first two years, the number of outbreaks was small. There was a dynamic increase in the number of outbreaks in 2016-2018. In turn, in 2019 there was more than a 2-fold decrease compared to the previous year. The spatial distribution of the virus occurrence in individual years indicates a clear tendency of its spread towards the west and a bit slower, though successive towards the north and south (*Fig. 2a-f*). In the subsequent years of virus incidence, the number of wild boars covered by the ASF testing increased (*Table 1*). In 2014, 15,881 samples from wild boars were tested, and in 2019 77812 samples. In the initial 5 years, the prevalence index increased every year from 0.19 in 2014 to 5.56 in 2018 (*Fig. 3*). In 2019, despite the largest number of samples tested, this indicator dropped to 3.18.

Almost from the very beginning of the virus in Poland, ASFV antibodies were found during laboratory tests (*Table 1*). This indicates that these individuals have been in contact with the virus. The first such case was already recorded in December 2014. The percentage of seropositive wild boars increased with the increase in the number of

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samples and the virus cases found. It is difficult to clearly indicate whether these animals had contact with a small dose of the virus and the immune system generated immunoglobulins, which meant that the virus did not lead to death, or whether these individuals contracted the virus. The possibility of acquiring antibodies from the mother in utero or with colostrum is also not fully known, and the results of the study also show their occurrence in young wild boars.

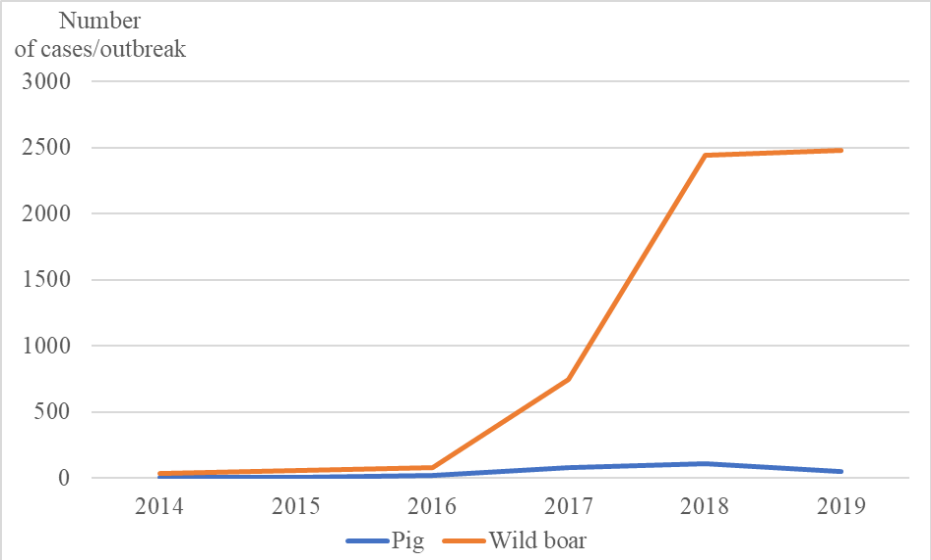

Figure 1. *The number of ASF virus outbreaks in pigs and cases found in wild boars in Poland in 2014-2019*

Table 1. *Presence of antibodies in tested wild boars*

Item	Years					
	2014	**2015**	**2016**	**2017**	**2018**	**2019**
Number of wild boars examined	15881	13356	14965	24698	43911	77812
Number of cases	30	53	80	741	2443	2477
Number of wild boars tested for antibodies*	4218	7694	8575	16303	35007	30395
Number of wild boars seropositive*	1	8	18	95	274	391
Percentage of wild boars with antibodies compared to tested	0.02	0.10	0.21	0.58	0.78	1.29

*Data from the State Veterinary Institute in Pulawy

Routes and directions of ASFV spread in wild boar

From the very beginning of the appearance of the virus in Poland, it spread mainly in the natural environment, which was confirmed by subsequent cases of the virus in boars in this region. The main areas were related to the border belt, where the virus spread to subsequent areas along the eastern border of Poland and successively occupied further areas to the west. In this case, it is undeniable that the main vector of the virus was certainly infected with wild boars or their corpses. In border areas, it could certainly also be cross-border migrations of infected wild boars (*Fig. 4*).

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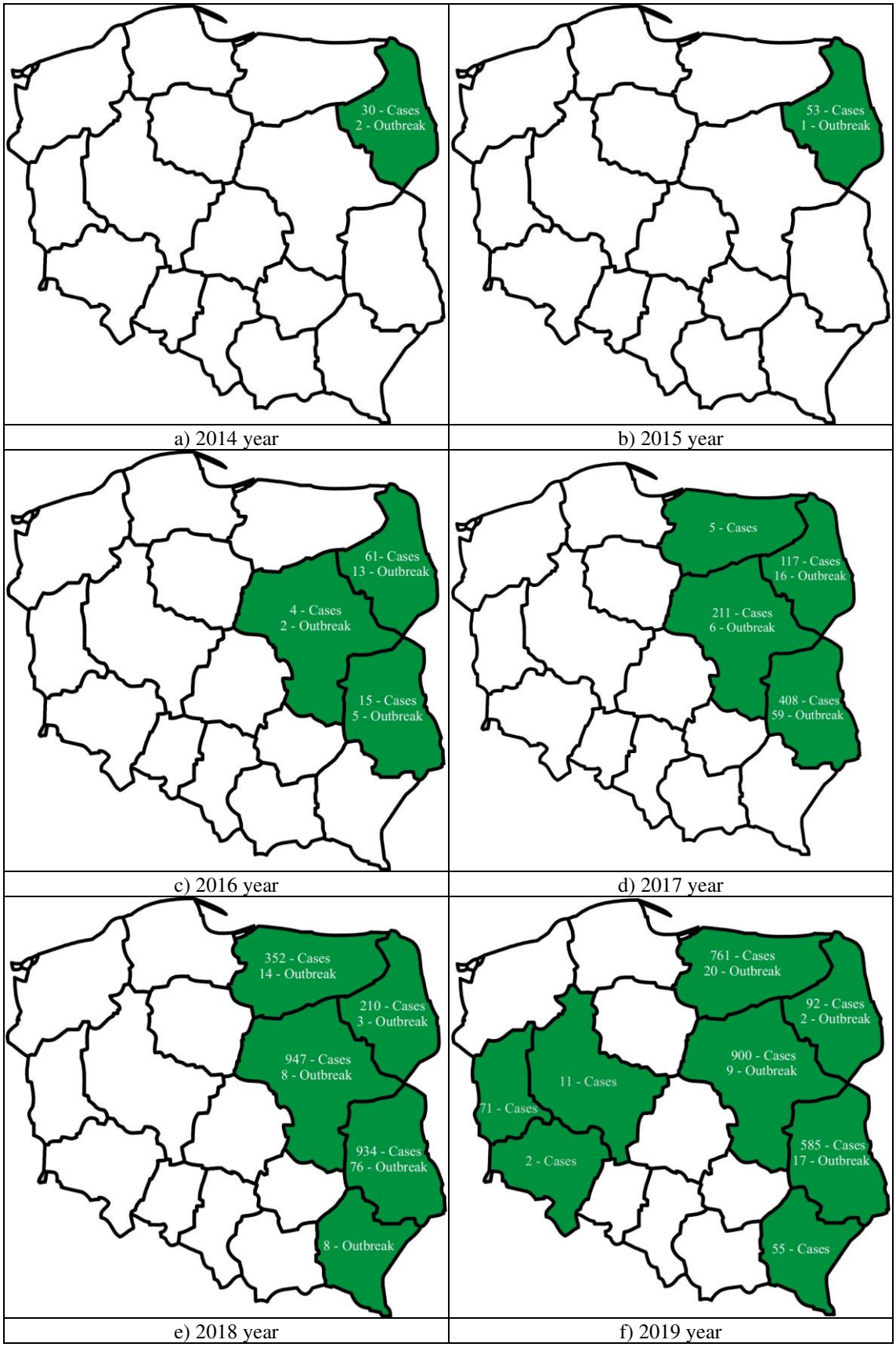

Figure 2. *(a-f) Geographical pattern of ASF virus in Poland in the years 2014-2019*

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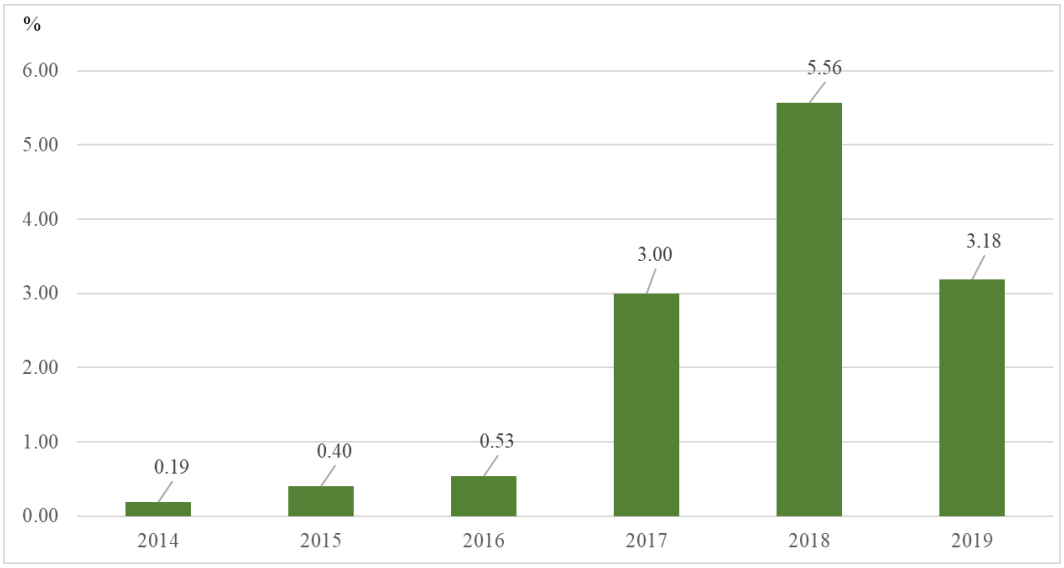

Figure 3. *Prevalence index in wild boars in Poland in 2014-2019*

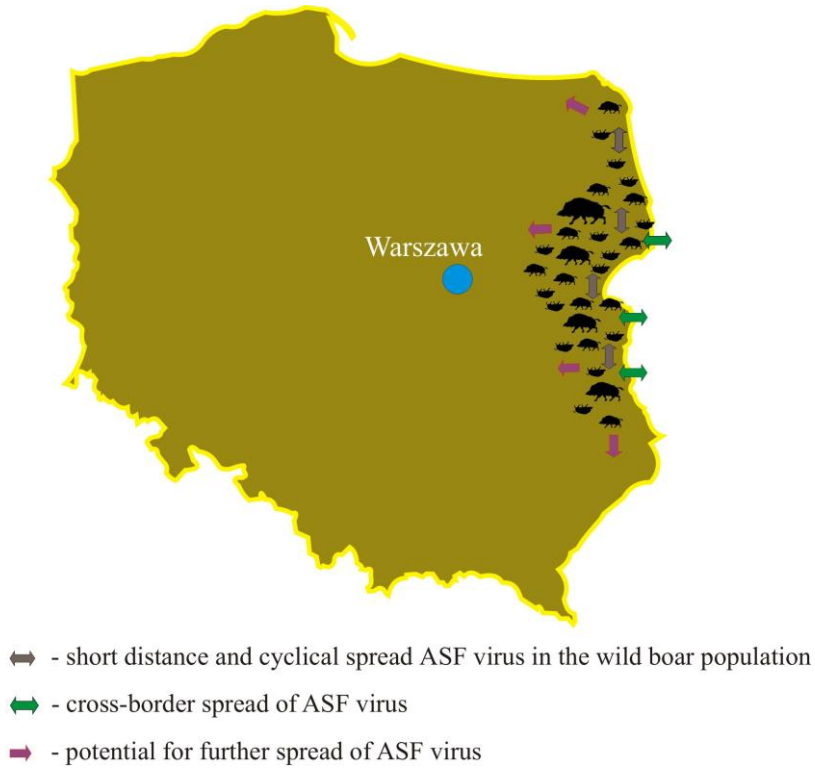

Figure 4. *Geographic pattern of short-distance ASF virus movement in determined wild boars by environmental factors in Poland in the years 2014-2016*

However, starting from 2017, there was an unexpected spread of the virus to new areas, quite distant from the sites of earlier statements. The first of them were around Warsaw, and the next regions of northern Poland, just off the Kaliningrad region. Cross-border migration of feral pigs was indicated as the main reason for the emergence of a new region of virus occurrence, just off the northern border, quite far away from its

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current sites. However, the appearance of the virus near Warsaw cannot be equated with any wild boar migration. At most, two hypotheses were mentioned. The first of these was about transporting the unexplored carcass of a wild boar from areas where the virus had not yet been officially diagnosed and could have been present in the environment and the boar was infected. The second, more likely, said that unconscious human activity and the introduction of the virus into new areas contributed to this by failing to follow biosecurity principles when moving from areas where the virus was already present in the natural environment (*Fig. 5*).

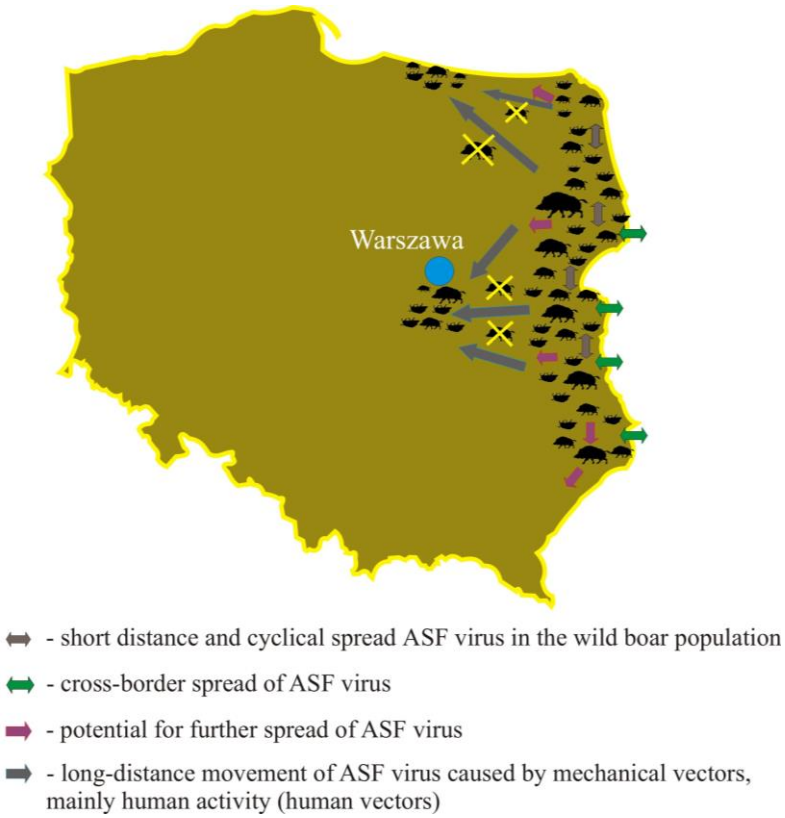

Figure 5. *Geographic pattern of short and long-distance ASF virus movement in determined wild boars by environmental factors and human vectors in Poland in the year 2017*

Another case of virus transmission to new, very remote areas was recorded in Poland in 2019, when the virus was diagnosed in a dead boar, near the town of Slawa on Tarnowskie Lake in southwestern Poland (*Fig. 6*). The determined distance in a straight line from the most westerly case in places of earlier virus occurrence in boars and pigs in the eastern and central part of Poland was slightly over 300 km. Thus, there is no epidemiological and, above all, geographical link with existing virus cases. This is another confirmation that mechanical vectors, mainly humans, play a key role in virus transmission.

Routes of ASF virus entry into pig-keeping farms

In Poland, 246 outbreaks in pigs have been reported since the appearance of the ASF virus. In the initial period, the outbreaks were few and were found in small farms,

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holding a few pigs each. This was mainly due to the way pigs were kept and fed, and thus the lack of any biosecurity. Along with the extension of the range of occurrence, outbreaks also appeared on larger farms. The successively increasing losses reached hundreds or even thousands of pigs, which were slaughtered and disposed of. In total, during the period 2014-2019, 67415 pigs were slaughtered and disposed of, which generated huge economic losses. Regardless of the type of farms in which there were outbreaks, although the paths of virus penetration were not always recognized and described in detail, in most cases they were associated with a mechanical vector associated with human activity. There is no reason to conclude, which is also logical for the wild boar to drag the virus into the farm, including the piggeries. In addition, the aspect related to virus neutralization in facilities where sick pigs were kept was not fully understood. Thus, in areas where the virus was present, the infection could have a two-way nature, and the virus could circulate between farms and the environment.

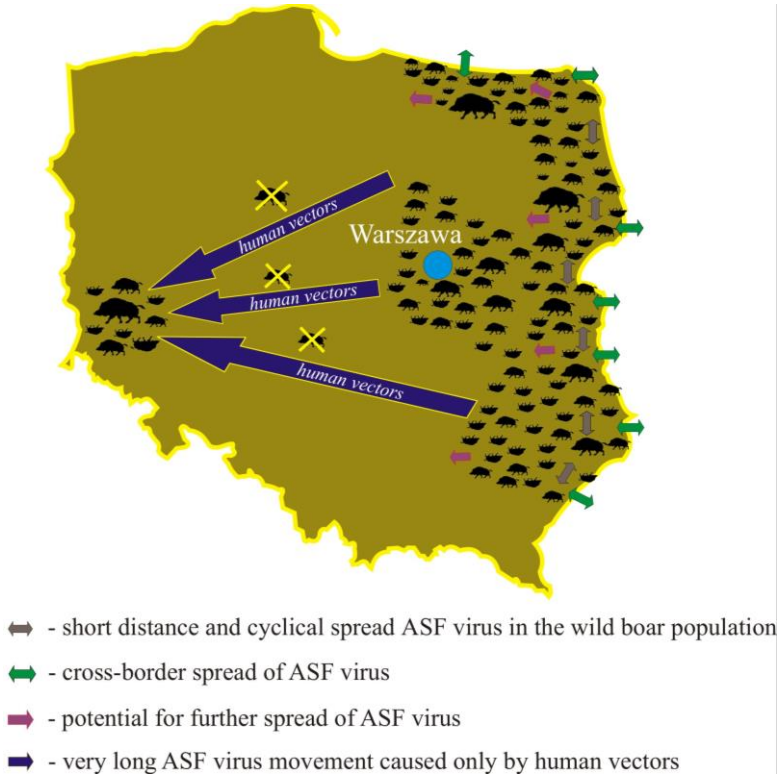

Figure 6. *Geographic pattern of short and very long-distance ASF virus movement in determined wild boars by environmental factors and human vectors in Poland in the year 2019*

Preventive actions

Even before the virus was officially confirmed in Poland, the first administrative and preventive initiatives were undertaken. In January 2014, guidelines were issued regarding the rules of conduct aimed at preventing the virus from entering the territory of the Republic of Poland. However, less than a month after this, the virus was officially confirmed and these guidelines became pointless. The next package of rules of conduct was released in March 2014 and contained a number of guidelines for pig farmers. It included guidelines on farm biosecurity rules and the possibilities for pigs to move. At that time, the wild boar was defined as the main virus vector in terms of its

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transferability to pig-keeping farms. It is worth emphasizing that these guidelines in the initial period of implementation introduced a total ban on hunting boars.

In the following years, further programs were introduced to detect early infections by the African swine fever virus and to increase knowledge about this disease. In these programs, the approach to wild boars changed radically, and the guidelines contained in them pointed to the need for depopulation of this species. What is more, in addition to hunting, compulsory (sanitary) shots were implemented based on the decisions of the Veterinary Inspection, as well as cash bonuses for shots carried out. These guidelines also introduced the mandatory search for fallen wild boars and the rules of conduct of hunters during hunting and after shooting in the field of biosecurity. Although they introduced clear guidelines for the biosecurity of pig farms, in many cases they were not followed, as confirmed by the inspections carried out. Such activities contributed to reducing the number of wild boar populations, which were indicated as the main source of virus transmission to farms. Further guidelines on the depopulation of wild boars, due to massive hunting conducted since 2017, caused a drastic decrease in the number of wild boars (*Fig. 7*). Arguments regarding wild boars as the primary source of threat to pig farming were not entirely correct, as the depopulation carried out in no way affected the number of cases and the possibility of the virus spreading to new areas as well as the number of outbreaks on pigs on farms.

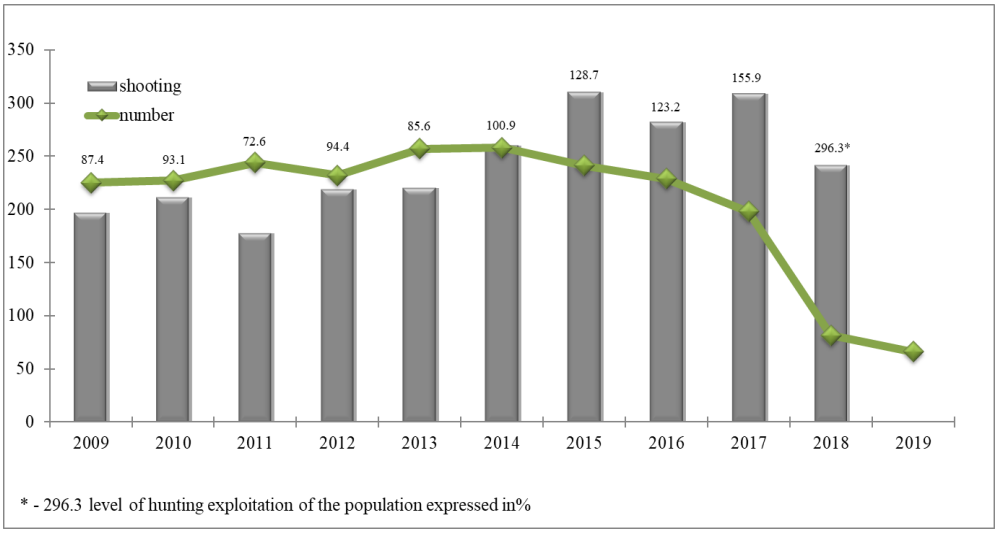

Figure 7. *Numbers and hunting of wild boars (thousand individuals) in districts leased by the Polish Hunting Association in the last decade*

Discussion

African swine fever as an infectious disease, causing almost 100% mortality in porcine animals (Suidae), poses a serious threat to the European and global pig market. Starting from 2007, he expanded the range of his presence to include Eastern European countries, and in subsequent years his successive expansion towards Western Europe occurs. Due to the fact that currently no vaccine is available, the only way to combat and thus limit further spread is to follow the principles of biosecurity (Markowska-Daniel and Pejsak, 2014; Pejsak et al., 2018b; Mazur-Panasiuk et al., 2019b).

The presented epizootic situation in Poland indicates that the boar remains the primary reservoir of the virus in the natural environment, but it is not the main vector of

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its spread. At the same time, all administrative measures taken so far to combat and limit the transmission to new areas have not yielded any results (Flis, 2019; Pejsak et al., 2018b). It was determined by the speed of virus transmission in the natural environment. Research in this area indicates that the virus, regardless of the season of the year and environmental conditions, spreads at an average rate of about 1-2 km per month (Abrahantes et al., 2017; Depner et al., 2016; Podgórski and Śmietanka, 2018). This is also confirmed by the results of Russian studies, which highlighted the lack of dependence of the epidemic in pigs and wild boars, as they were independent of each other. At the same time, the authors of these studies indicate a high probability of infecting wild boars through various by-products associated with breeding and keeping pigs (Vergne et al., 2017).

The increase in the spatial development of the virus in Poland in the years 2014-2019 has shown that there are certainly different directions of infection in both wild boars and pigs. The presented results indicate almost unambiguously that the key role is played by wild boars, however, other transmission routes should not be underestimated, especially to pig-keeping farms, including a number of factors referred to as mechanical vectors. Some tick species from the *Ornithodorus* genus may play an important role in transmitting the pathogen. Although in European conditions these ticks do not occur in the natural environment, this route of transmission should not be underestimated, even by importing accompanying animals susceptible to this group of arachnids. As a rule, they are asymptomatic carriers of the virus, however, ASFV can replicate in their body and survive up to five years, being a potential source of infection for a sensitive species (Arias et al., 2018; De Carvalho Ferreira et al., 2014; Galindo and Alonso, 2017; Fila and Woźniakowski, 2020; Plowright et al., 1969). Some species of flies may also be involved in the transmission of the virus, which may be mechanical vectors of the virus, transferring it within 24 h through blood infected with the virus, as well as infection may occur orally by ingestion by animals infected with the virus (Galindo and Alonso, 2017; Baldacchino et al., 2013; Mellor et al., 1987; Olesen et al., 2018b). The possibility of transmission of the virus through mammals and birds of prey, as well as scavengers, which can be mechanical vectors of the spread of the virus is not fully understood. German studies conducted on carcasses of dead boars have shown that both birds and mammals, including domestic dogs, have had contact with carrion, and thus these animals may be mechanical viral vectors (Probst et al., 2017). In the case of virus transmission to pig-keeping farms, a key role may also be played by feed contaminated with pathogen, coming from fields and meadows, not quarantined (Guinat et al., 2016; Thomson, 1985).

The development of the epizootic situation in Poland during the 6 years of the presence of the ASF virus shows that the virus occurs in the natural environment, where its only reservoir is wild, but the basic vector of virus spread is the human factor. This is confirmed by its transmission to the areas around Warsaw (Mazur-Panasiuk and Woźniakowski, 2019; Pejsak et al., 2018a), or the appearance in Western Poland at a distance of 300 km, from earlier places of its finding (Flis, 2020). Also cases of its appearance in other European countries (Czech Republic, Belgium) distant hundreds of kilometers from places of its occurrence in this period (Forth et al., 2019; Gilliaux et al., 2019; Flis and Kołodziejski, 2019). Confirmation of the thesis that mechanical vectors play a key role in virus transmission in Poland are data from other European countries. In the case of the Czech Republic, the virus vector was workers from Eastern Europe,

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while the Belgian case was associated with the transport of carcass of an infected wild boar by hunters from Lithuania (Flis and Nestorowicz, 2019).

There is also no doubt that wild boars remain the primary reservoir of the virus. However, taking into account the behavior of this species and the fact of the emergence of new cases of the virus in areas quite distant by places of earlier statements, it is impossible to agree with the thesis that it is boars that are the main factor associated with the spread of the virus to new areas (Flis, 2020; Flis and Nestorowicz, 2019; Keuling et al., 2009; Schultz et al., 2019b). Therefore, one of the priority tasks in the field of prevention in the spread of the virus is to comply with the principles of biosecurity resulting from the introduced veterinary regimes (Flis and Kołodziejski, 2019; Pejsak et al., 2018a). This is confirmed by the analysis of the spatial spread of the virus in Poland. They point to the need for more effective prevention and control measures in areas of permanent virus prevalence during the epidemic season, in pigs in summer and in wild boars in winter. It is also necessary to permanently assess the level of risk of spreading the virus (Lu et al., 2019).

In ASFV transmission, some role may be played by wild boars, which have been in contact with the virus and have infected it. The number of such animals during the period of virus occurrence in Poland is gradually increasing, and this is confirmed by the presence of antibodies in the tissues of these animals. These animals mainly come from areas where the virus has been around for a long time. Thus, they can be asymptomatic carriers of the virus. However, there is no evidence that every seropositive animal is a long-term virus carrier (Ståhl et al., 2019). A similar trend in the growing number of seropositive wild boars occurs in Estonia, where their incidence is definitely higher in those regions where the virus was the first to appear (Schulz et al., 2019a). Research results from Poland and the Baltic States show that there is a positive correlation between the time of infection and the occurrence of seropositive individuals (Martínez-Avilés et al., 2020). Such a course of the disease may be the beginning of its endemic in these regions (Schulz et al., 2019a). Also studies in pigs who have recovered from an acute ASF infection in the Netherlands have shown that these pigs can transmit the disease to other individuals through direct contact (Eblé et al., 2019). Research on the possibility of virus transmission through the environment in which virus infected pigs euthanized, conducted in Poland, Podlasie, showed that the introduction of healthy pigs to the same pens the next day led to the development of the disease in one week. Only at least 3 days quarantine eliminated virus transmission to subsequent individuals through a contaminated environment (Olesen et al., 2018a). Thus, in the current conditions of multi-directional possibilities of virus transmission to new areas, both in Poland and other European countries, farms keeping pigs should be almost isolated from the outside world. This is due to the fact that the virus is introduced to farms via indirect transmission paths, with a lack of biological security (Nurmoja et al., 2018; Pejsak and Truszczyński, 2018).

In the scope of preventive actions, elements related to the utilization of all remains of dead animals by specialized rendering companies with the principles of biosecurity are extremely important. This applies to both pigs and wild boars or their remains remaining in the wild. Therefore, one of the most important preventive elements in wild boars should be searching for dead animals, their utilization and proper disinfection of places where carrion was found (Flis, 2020; Flis and Kołodziejski, 2019; Halasa et al., 2016; Pejsak and Truszczyński, 2017a; Pejsak and Woźniakowski, 2017).

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Conclusion

The presented data regarding the epizootic situation associated with the occurrence of the ASFV virus on the territory of Poland clearly indicate that despite the administrative and preventive measures taken in the field of virus eradication, they did not bring the expected results. During the period of six years, the virus spread to almost half of Poland's territory. Analysis of the geographical pattern of virus spread indicates that mechanical vectors, and mainly multidirectional human activity, play a key role in its transmission to new areas. Previous efforts in the field of depopulation of feral pigs considered both as a reservoir and as a virus vector have failed. Although the density of wild boar population is an important element in virus transmission within neighboring groups, it does not play a key role in its transmission to new, quite often remote areas from the sites of previous statements. Wild boar is also not directly related to the transmission of the virus to pig farms. Nevertheless, in Polish conditions, regardless of population density, wild boars remain the only reservoir of the virus in the natural environment.

Due to describing the next possibilities of virus transmission to new areas through the so-called mechanical vectors should be focused on searching for dead animals, as well as their remains at various stages of decomposition, as well as their removal from the environment and thorough disinfection of their locations. All such activities reduce the germ reservoir in the environment, and thus the possibility of its further transmission. The knowledge and observance of biosecurity rules by pig farmers, as well as hunters and other people staying in places of potential occurrence of boars or their periodic concentration should be absolutely binding. The possibility of limiting the use of the environment should also be considered, especially in places susceptible to the occurrence of wild boars. The effect of this initiative should be to limit further possibilities of spreading the virus among wild boars and result in limiting its further transmission to new areas. New fast and comprehensive solutions should also be sought to combat the virus, especially in areas where it appears for the first time. The Czech case is the best confirmation that decisive action may limit transmission possibilities and even lead to virus eradication.

It is also necessary to conduct further research on the possibilities of virus transmission to new areas through diverse vectors, both environmental and those associated with human activities. These studies should focus on determining the magnitude of the impact of human-dependent factors in the spread of the virus, and on this basis indicate the possibility of developing methods to minimize anthropogenic factors that have a significant impact on further transmission of the virus. The research aimed at determining the role of wild boars in the spread of the virus, as well as determining the level of density of the wild boar population guaranteeing the inhibition of virus transmission from the environment to farms keeping pigs are also significant.

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Conflicts of interests. The sponsors had no role in the design, execution, interpretation, or writing of the study.

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COMPARISON OF SOIL PROPERTIES OF AN ADJACENT CLAY MINE SPOIL, A MINING SITE RECLAIMED WITH STONE PINE (*Pinus pinea* L.) PLANTATION AND A NATURAL FOREST

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Abstract. The purpose of this study was to compare the soil properties of an adjacent natural forest, clay mine spoil and mine site reclaimed with Stone pine (*Pinus pinea* L.). Bulk density, sand, silt, clay, pH, electrical conductivity (EC), nitrogen (N), carbon (C) and C/N ratios of soil samples taken from three different soil depths (0-5, 5-15 and 15-30 cm) were determined and compared. Furthermore, the mass, N and C contents and C/N ratios of the forest floor were determined in the Stone pine plantation and natural forest. Almost 24 years after the establishment, 6.79 ton/ha forest floor mass was found in the Stone pine plantation, while it was found to contain 2.71 ton C/ha and 0.04 ton N/ha, which were significantly lower than those in the natural forest. In general, with some exceptions (EC in 0-5 and 5-15 cm, and bulk density in 15-30 cm), there were significant differences on the soil properties. Sand and clay contents varied substantially depending on the material and mixture. The soil C (1.5-2.1%), N (0.05-0.06%) and pH (5.34-5.69) in the Stone pine plantation significantly increased. Despite these properties, this shows that rehabilitation with plantation will take a much longer time compared to the natural forest area.
Keywords: *afforestation, carbon, nitrogen, pH, restoration*

Introduction

Mine production and mining operations have been exponentially increasing across the entire world. Open pit sites in particular lead to drastic degradation of soil and land. Mining activities have disruptive effects on the ecosystem, and affect the physical, chemical and biological soil properties of mining sites. First the plant cover is destroyed, then fertile top soil layers are removed from the site through major excavations, and the soil is compacted and destroyed due to the sue of heavy duty vehicles to open large pits created as a result of deep slopes. Moreover, soil organic matter disappears substantially and the material becomes too poor in terms of plant nutrients including primarily nitrogen. This is especially important for the restoration of sites after mining operations (Shrestha and Lal, 2011; Frouz et al., 2015; Gu et al., 2019).

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Most of the mineral and natural resources are located in forestlands around the globe (Burton and Macdonald, 2011). Mineral extraction and processing in forestlands lead to their destruction at a large extent (Burton and Macdonald, 2011) and turning forestlands into mining sites lead to extremely harmful changes in the ecosystem and soil (Ahirwal and Maiti, 2016). Such practices increase the pressure on forest areas (Gençay et al., 2018).

It is essential to rapidly restore severely destroyed areas after mining activities (Frouz et al., 2015). Plantation is the most commonly used method to restore abandoned mining sites. However, mining tailings-spoils that have very poor physical, chemical and biological properties and levelled mine soils usually inhibit plant existence and growth (Asensio et al., 2013; Rodríguez-Vila et al., 2016). The use of forest tree species among the plant species is a common way while it requires selecting the appropriate species (Mukhopadhyay et al., 2013).

Moreover, different tree species have different restorative properties (Mukhopadhyay et al., 2013). Therefore, it is crucial to determine the improvements and changing properties after establishing plantations for the restoration and reclamation of mining sites. Comparison with adjacent control sites is a preferred way to demonstrate the rehabilitative power of the treatments conducted (Shrestha and Lal, 2011; Liu et al., 2017; Gu et al., 2019). There are several studies regarding the rehabilitative features of tree species for mine sites (Juwarkar et al., 2010; Mukhopadhyay and Maiti, 2011; Gu et al., 2019).

The main physical and chemical soil properties played a determining role in these studies. Bulk density and soil texture type (sand, silt and clay contents), which are among the main soil properties identified, have an important impact on hydro-dynamic properties of the soil, are also important parts of soil quality parameters (Kantarcı, 2000; Yuan et al., 2017) and important for plant development (Asensio et al., 2013). In addition to organic soil and forest floor mass, carbon and nitrogen contents of forest floor (Walmsley et al., 2019) in the restored mining sites, presence of soil carbon and nitrogen are important criteria for biological activity and soil fertility (Frouz et al., 2013; Ahirwal and Maiti, 2016). Besides, soil acidity (pH) and electrical conductivity (EC) have an impact in many soil chemical processes while they also have biological effects (Mukhopadhyay et al., 2013; Mosseler and Major, 2017).

This study was conducted in order to determine the change in soil properties caused by clay mining activity and the effect of Stone pine (*Pinus pinea* L.) plantations in mine spoils after the termination of clay mining. To this end, the study was designed to compare the soil properties at different depths (0-5, 5-15 and 15-30 cm) and the existing forest floor of three different sites, which included the abandoned clay mine spoil, the adjacent clay mine reclaimed with 24-year old Stone pine (*Pinus pinea* L.) plantation and the adjacent natural forest.

Material and method

Research area

The research area was located in Şile-Istanbul in the northwest of Turkey (N41°08'07"-41°08'16"; E29°27'11"-29°27'21"). The clay mine site studied in the research and its surroundings are in the south of Istanbul-Şile Highway, while it is 53 km to Istanbul and 17 km to Şile. The distance of the research area to the sea (Black Sea) is around 6.5 km in air distance. As regards the topography of the research area, it is situated on peneplain, its elevation is 115-150 m (*Fig. 1*).

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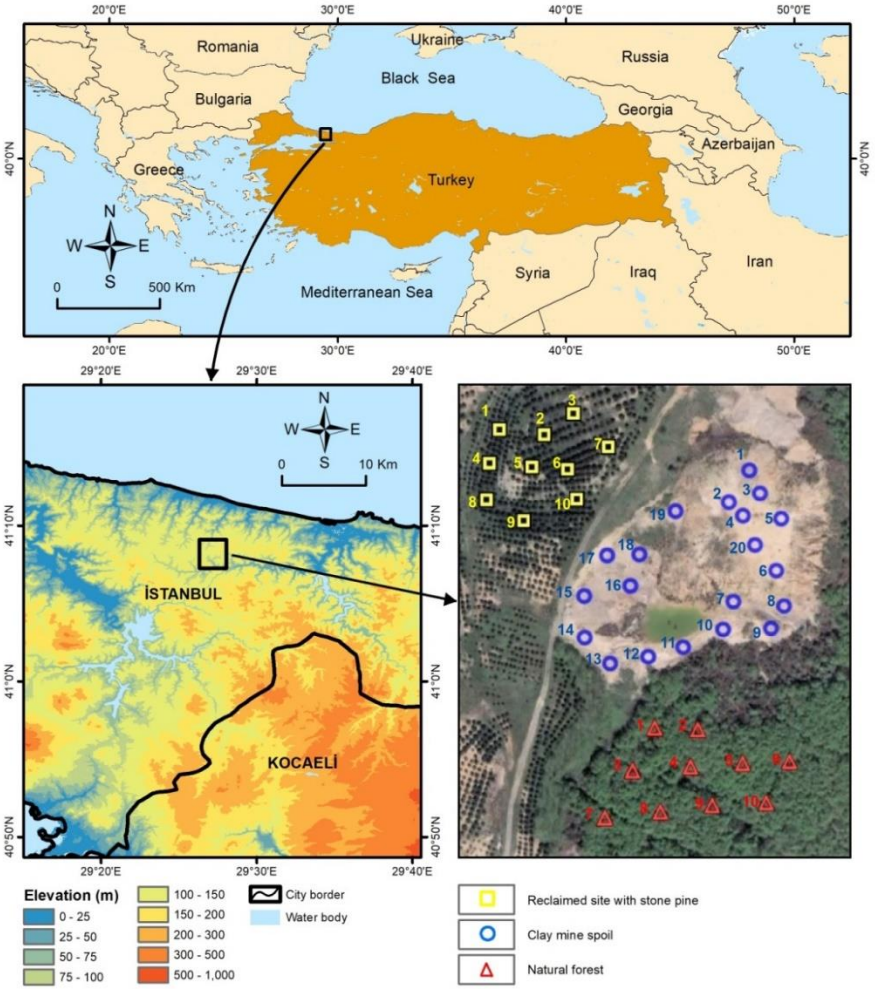

Figure 1. *Location of study area*

As for its geological structure, Neogene sediments are common in the site, while mainly sandy layers are located on the surface and clay is found in the lower layers (Ertek et al., 1998; Demir et al., 2016). In the geological literature of Turkey, Şile (Istanbul) Neogene Basin is referred to as a storage of natural industrial raw materials, which supplies 90% of industrial clay demands in Turkey (Sezer, 2006). Brown forest soil is the common soil type in the natural forests where land degradation did not occur.

Transition climate between the Mediterranean climate and Black Sea Climate is dominant in the research area. According to the data of Şile Meteorology Station, its mean annual precipitation is 929 mm. Its mean annual temperature is 13.6 °C, mean monthly minimum temperature is -3.8 °C in February, mean monthly maximum temperature is 31.8 °C in September and relative humidity is 80.5% (Sezer, 2006). The natural forest from which sample plots were selected is an oak coppice forest and includes mainly *Quercus petraea* as the dominant species, while *Fagus orientalis, Castenae sativa* and *Sorbus torminalis* are the other tree species included in the mixture in some of the sample plots. In addition to these species, *Quercus robur, Quercus frainetto, Alnus glutinosa, Acer campestre, Fraxinus excelsior, Carpinus betulus, Tilia tomentosa, Populus tremula, Corylus avellana, Cornus mas, Mespilus germanica,*

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Buxus sempervirens are the other tree or shrub species distributed in the forests in this region (Dönmez, 1979).

Sampling and methods

This study was conducted to determine the effect of Stone pine (*Pinus pinea* L.) plantation introduced for the rehabilitation of the clay mine spoils after clay extraction operations on soil properties in three different adjacent sites including 1) the clay mine spoil 2) clay mine site reclaimed with Stone pine plantation and 3) natural forest. The Stone pine plantation was established by direct planting method without any rehabilitation or laying topsoil almost 24 years ago (determined according to the annual growth ring samples taken from the trees) on top of the mining tailings-spoils. The huge pits in the clay mine tailings site were filled and the site was leveled and abandoned. In the adjacent natural forest land, *Quercus petraea* of coppice origin which protects its natural structure is the dominant species. Adjacent sites were selected for common site characteristics (*Fig. 2*).

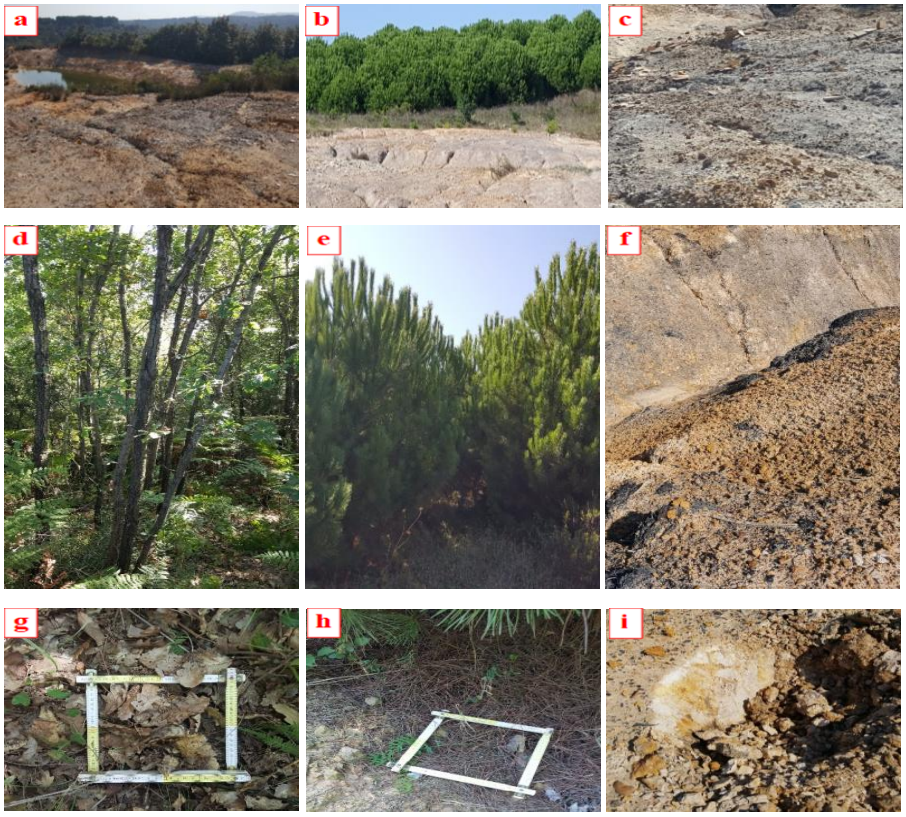

Figure 2. *Pictures from research sites, a) transect view on clay mine spoil and natural forest, b) transect view on clay mine spoil and Stone pine plantation, c) a part from clay mine spoil, d) natural forest, e) Stone pine plantation, f) different surface layers of clay mine spoil, g) forest floor of natural forest, h) forest floor of Stone pine plantation, i) heterogeneity structure of clay mine spoil*

The number, diameter (dbh) and height of the trees were determined in 10 sample plots of 5x5=25 m^2 in the natural forest and of 4x3=12 m^2 from the Stone pine plantation because plantation has same planting distance (4x3 m). The density, average

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diameter (dbh) and height of the trees in these sites are presented in *Table 1*. The mean age of the Stone pine plantation was found to be 24 years according to the annual ring samples collected from a tree in each sample plot of the plantation.

Table 1. *Density, average diameter (dbh) and height of trees on sample plots*

Characteristics	Natural forest	Stone pine plantation
Tree number (ha)	3760 ± 1053	3250 ± 264
Diameter (dbh) (cm)	8.08 ± 1.39	9.39 ± 1.18
Height (m)	6.98 ± 1.09	4.45 ± 0.63

± standard deviation, Stone pine (*Pinus pinea* L.) plantation age: 24 ± 2, main species in natural forest is oak (*Quercus petraea* L.)

The soil samples were collected from 10 plots in the natural forest and Stone pine plantation and 20 plots in the clay mine spoils site (*Table 2*). We selected more sampling points from mine spoils site to represent the area because the mine site is more heterogeneous (*Figs. 1-2*). Soil samples for bulk density were collected from these three sites at depths of 0-5 cm, 5-15 and 15-30 cm in the selected plots that could represent the overall site during the field studies. Moreover, forest floor samples were collected from areas of 1/4 m^2 in from each sample plot in the natural forest and Stone pine plantation with 5 repetitions (*Fig. 2*). As the forest floor did not cover the entire soil surface contrary to the one in the natural forest, the percentage of forest floor coverage was determined in plantation. This value was found to be 51.3% on average, while the coverage percentage of each site was averaged to their total forest floor mass.

Table 2. *The coordinates of sample plots*

Site	Point number	Longitude (E)	Latitude (N)	Site	Point number	Longitude (E)	Latitude (N)
Stone pine plantation	1	29°27′12.33″	41°08′14.75″	Clay mine spoil	1	29°27′18.16″	41°08′14.31″
	2	29°27′13.39″	41°08′14.70″		2	29°27′17.72″	41°08′13.72″
	3	29°27′14.04″	41°08′15.10″		3	29°2718.44″	41°08′13.92″
	4	29°27′12.13″	41°08′14.15″		4	29°27′18.07″	41°08′13.50″
	5	29°27′13.13″	41°08′14.13″		5	29°27′18.97″	41°08′13.50″
	6	29°27′13.97″	41°08′14.12″		6	29°27′18.92″	41°08′12.57″
	7	29°27′14.89″	41°08′14.56″		7	29°27′17.95″	41°08′11.97″
	8	29°27′12.09″	41°08′13.52″		8	29°27′19.15″	41°08′11.95″
	9	29°27′12.99″	41°08′13.19″		9	29°27′18.87″	41°08′11.53″
	10	29°27′14.21″	41°08′13.62″		10	29°27′17.74″	41°08′11.45″
Natural forest	1	29°27′16.23″	41°08′09.62″		11	29°27′16.81″	41°08′11.10″
	2	29°27′17.25″	41°08′09.63″		12	29°27′16.01″	41°08′10.89″
	3	29°27′15.75″	41°08′08.82″		13	29°27′15.12″	41°08′10.73″
	4	29°27′17.12″	41°08′08.93″		14	29°27′14.51″	41°08′11.17″
	5	29°27′18.36″	41°08′09.03″		15	29°27′14.46″	41°08′11.90″
	6	29°27′19.50″	41°08′09.10″		16	29°27′15.53″	41°08′12.13″
	7	29°27′15.15″	41°08′07.94″		17	29°27′14.96″	41°08′12.64″
	8	29°27′16.45″	41°08′08.08″		18	29°27′15.71″	41°08′12.69″
	9	29°27′17.68″	41°08′08.23″		19	29°27′16.50″	41°08′13.49″
	10	29°27′18.98″	41°08′08.31″		20	29°27′18.39″	41°08′13.00″

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The soil samples collected from the area for bulk density were air dried, ground in a mortar, screened through a 2-mm sieve and fine soil fraction (<2 mm) and stones were removed. The samples were oven dried at 105 °C till constant weight and their weight in unit bulk was found. Then, based on these weight values, the amount of fine soil (<2 mm) per hectare was calculated for each depth layer (0-5, 5-15 and 15-30 cm). The particle diameter (sand, silt and clay contents) in the soil samples prepared for analysis was analyzed with Bouyoucos hydrometer, soil reaction (pH) was analyzed with pH-meter containing glass electrode in a solution mixed with distilled water at a ratio of 1/2.5; salinity (electrical conductivity, EC) with salinity meter in a solution mixed with distilled water at a ratio of 1/5; organic carbon with Walkley-Black wet oxidation method and the total nitrogen with semi-micro Kjeldahl method (Karaöz, 1989a,b). To prepare the forest floor samples for analysis, they were dried at 70 C° till constant weight. The dried samples were weighed and the amount of forest floor mass in areas of 1/4 m^2 was calculated. Based on these values, forest floor masses per hectare was found. Then, the forest floor samples were ground to be prepared for analysis while their carbon and nitrogen contents were determined with Leco Truspec 2000 CN analysis device by dry combustion method.

Amounts per hectare were determined on the basis of the carbon and nitrogen concentrations of forest floor and soil found through analyses. Then the similarities and differences between the sites were compared with Analysis of Variance (ANOVA) for each data obtained through analysis results and calculations while the features found to be significant according to the analysis of variance were evaluated with Duncan post-hoc test due to inequality of the numbers of sample plots and sampling points among sites. As there was no forest floor on the mine spoil, the forest floor characteristics of the Stone pine plantation and the natural forest were compared with 2-independent samples t test. IBM SPSS STATISTIC 20 for Windows computer software package was used for the statistical evaluation of the data. Base ANOVA results were presented in *Appendix Tables 1-3*.

Results

Forest floor characteristics

The mass of forest floor in the Stone pine plantation (6.79 ton/ha) was much lower than the one in the natural forest (28.07 ton/h). The carbon concentration of the forest floor was almost the same in both sites (around 40%), while the nitrogen concentration was higher in the oak-dominated natural forest (0.81%) compared to the Stone pine plantation (0.51%). The carbon content per hectare was almost 4 times higher in the natural forest than in the Stone pine plantation due to the high amount of forest floor mass in the natural forest. The nitrogen content was found to be 0.04 ton/ha and 0.22 ton/ha, respectively, for the Stone pine plantation and the natural forest. C/N ratio was determined to be higher (94.21) in the Stone pine plantation where the nitrogen content was lower although both sites had a very similar carbon content (*Table 3*).

Soil properties

The soil sand content ranged from 81.25% to 88.25% in the natural forest, which was markedly higher compared to the other two sites. The clay mine spoil was found to have the lowest soil sand content, which was 38.05-43.25%. The soil clay content was

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much lower in the natural forest compared to the other two sites and ranged from 7.23% to 11.35%. The clay mine spoil had the highest soil clay content, ranging from 41.50% to 43.13% (*Table 4*). When the sites were compared as regards the texture type of soil, the main soil texture type was loamy sand in the natural forest while it was heavy clay in the clay mine spoil. The soil texture in the Stone pine plantation was mainly sandy clay.

Table 3. *Comparison of forest floor variables between natural forest and stone pine plantation*

Plots	Forest floor mass (ton/ha)	Carbon (C, %)	Nitrogen (N, %)	Carbon mass (ton/ha)	Nitrogen mass (ton/ha)	C/N
Natural forest	28.07 ± 3.81^{b}	40.91 ± 6.17^{a}	0.81 ± 0.26^{b}	11.47 ± 2.24^{b}	0.22 ± 0.07^{b}	55.93 ± 20.48^{a}
Stone pine plantation	6.79 ± 0.58^{a}	40.06 ± 7.96^{a}	0.51 ± 0.22^{a}	2.71 ± 0.52^{a}	0.04 ± 0.01^{a}	94.21 ± 45.56^{b}
Sig. (2-tailed)	0.000	0.647	0.000	0.000	0.000	0.000

± standard deviation, Means within columns following by the same letter are not statistically different at 0.05 significance level in two independent samples t-test

Table 4. *Soil properties*

Soil depth	Properties	Natural forest	Stone pine plantation	Clay mine spoil	*P*
0-5 cm	Sand (%)	81.25 ± 10.02^{c}	50.75 ± 8.20^{b}	41.75 ± 8.64^{a}	*0.000*
	Silt (%)	7.40 ± 7.47^{a}	8.40 ± 6.74^{a}	16.75 ± 7.10^{b}	*0.001*
	Clay (%)	11.35 ± 6.57^{a}	40.85 ± 5.73^{b}	41.50 ± 6.58^{b}	*0.000*
	Bulk density (<2mm, g/l)	752.07 ± 153.98^{a}	1190.15 ± 108.23^{b}	1242.76 ± 161.49^{b}	*0.000*
	pH	5.33 ± 0.43^{b}	5.69 ± 0.64^{b}	4.07 ± 0.76^{a}	*0.000*
	EC (µs/cm)	130.69 ± 99.76^{a}	97.01 ± 42.94^{a}	133.80 ± 144.02^{a}	*0.702*
	C (%)	4.092 ± 0.750^{c}	2.060 ± 0.519^{b}	1.292 ± 0.350^{a}	*0.000*
	N (%)	0.390 ± 0.060^{c}	0.056 ± 0.012^{b}	0.029 ± 0.009^{a}	*0.000*
	C/N	10.59 ± 1.82^{a}	36.91 ± 9.59^{b}	45.51 ± 14.70^{b}	*0.000*
	C (ton/ha)	15.078 ± 2.517^{c}	12.222 ± 3.215^{b}	7.927 ± 2.041^{a}	*0.000*
	N (ton/ha)	1.464 ± 0.366^{b}	0.338 ± 0.079^{a}	0.182 ± 0.053^{a}	*0.000*
5-15 cm	Sand (%)	83.05 ± 10.11^{b}	50.05 ± 8.13^{a}	43.25 ± 10.54^{a}	*0.000*
	Silt (%)	6.08 ± 2.73^{a}	8.42 ± 6.57^{a}	13.90 ± 6.25^{b}	*0.002*
	Clay (%)	10.87 ± 8.36^{a}	41.53 ± 3.56^{b}	42.85 ± 7.51^{b}	*0.000*
	Bulk density (<2mm, g/l)	1075.34 ± 133.01^{a}	1258.12 ± 169.90^{b}	1316.28 ± 159.91^{b}	*0.000*
	pH	5.10 ± 0.39^{b}	5.38 ± 0.38^{b}	4.11 ± 0.79^{a}	*0.000*
	EC (µs/cm)	46.70 ± 19.83^{a}	82.69 ± 39.27^{a}	128.20 ± 131.57^{a}	*0.096*
	C (%)	2.767 ± 0.271^{c}	1.604 ± 0.412^{b}	1.139 ± 0.293^{a}	*0.000*
	N (%)	0.229 ± 0.045^{c}	0.051 ± 0.009^{b}	0.028 ± 0.008^{a}	*0.000*
	C/N	12.51 ± 3.14^{a}	32.47 ± 10.88^{b}	41.81 ± 13.86^{b}	*0.000*
	C (ton/ha)	29.876 ± 5.411^{c}	20.371 ± 6.823^{b}	14.906 ± 3.914^{a}	*0.000*
	N (ton/ha)	2.498 ± 0.696^{b}	0.650 ± 0.157^{a}	0.377 ± 0.112^{a}	*0.000*
15-30 cm	Sand (%)	88.05 ± 4.15^{c}	48.65 ± 9.19^{b}	38.05 ± 10.81^{a}	*0.000*
	Silt (%)	4.72 ± 2.66^{a}	9.74 ± 8.34^{a}	18.82 ± 7.59^{b}	*0.000*
	Clay (%)	7.23 ± 1.70^{a}	41.61 ± 5.59^{b}	43.13 ± 8.98^{b}	*0.000*
	Bulk density (<2mm, g/l)	1157.82 ±43.55^{a}	1221.00 ± 94.82^{a}	1161.08 ± 216.52^{a}	*0.597*

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	pH	5.30 ± 0.32[b]	5.34 ± 0.77[b]	3.42 ± 0.95[a]	*0.000*
	EC (µs/cm)	29.97 ± 10.21[a]	77.76 ± 39.76[a]	389.28 ± 460.56[b]	*0.010*
	C (%)	2.356 ± 0.220[c]	1.485 ± 0.361[b]	0.993 ± 0.369[a]	*0.000*
	N (%)	0.148 ± 0.034[c]	0.054 ± 0.013[b]	0.025 ± 0.009[a]	*0.000*
	C/N	16.56 ± 3.92[a]	29.49 ± 12.83[b]	42.09 ± 19.42[b]	*0.000*
	C (ton/ha)	40.860 ± 3.303[c]	27.351 ± 7.578[b]	17.533 ± 7.592[a]	*0.000*
	N (ton/ha)	2.593 ± 0.649[c]	1.002 ± 2.249[b]	0.431 ± 0.137[a]	*0.000*

± standard deviation, means within rows following by the same letter are not statistically different at 0.05 significance level in Duncan Post-Hoc Test

The mean soil bulk density was found to be statistically significantly different and lower in the natural forest compared to the other two sites at depths of 0-5 and 5-15 cm. At these two depths, the highest mean soil bulk density was found in the clay mine spoil whereas the lowest was in the natural forest. At the deepest layer which was 15-30 cm, there was no statistically significant difference between the sites as regards mean soil bulk density (*Table 4*).

As for soil pH; it was statistically significantly different in the clay mine spoil compared to the other sites at three depths, which was lower compared to the soil pH in the Stone pine plantation and natural forest (*Table 4*). There was no statistically significant difference as regards mean soil EC between the three sites at depths of 0-5 and 5-15 cm. The soil EC was higher in the clay mine spoil (389.28 µs/cm) at the depth of 15-30 cm compared to the other two sites, which was statistically significantly different (*Table 4*).

There was a statistically significant difference in soil carbon and nitrogen concentrations between three sites at all depths. The highest carbon and nitrogen concentrations at all depths were found in the natural forest while the lowest values were found in the clay mine spoil. The soil carbon and nitrogen concentrations in the Stone pine plantation were in the range between the values of the other two sites. The carbon and nitrogen concentrations at the depth of 0-5 cm where the values are the highest are ranked from the highest to the lowest as follows: 0.390 - 4.092%, in the natural forest; 0.056 - 2.060% in the Stone pine plantation, and 0.029 - 1.292% in the clay mine spoil (*Table 4*). As for C/N ratio, there were statistical differences at all three depths between the natural forest, Stone pine plantation and mine spoil. The lowest C/N ratio for all depths was found in the natural forest while the highest was found in the mine spoil (*Table 4*). The total carbon storage per hectare was statistically significantly different in three different sites at all three depths in parallel to soil carbon content. At the depth of 0-30 cm in all sites, the total carbon storage per hectare was ranked from the highest to the lowest as follows: 85.814 ton/ha (15.078 + 29.876 + 40.860) in the natural forest, 59.944 ton/ha (12.222 + 20.371 + 27.351) in the Stone pine plantation, and 40.366 ton/ha (7.927 + 14.906 + 17.533) in the clay mine spoil. The highest nitrogen storage per hectare at the depth of 0-30 cm was found as 6.555 ton/ha (1.464 + 2.498 + 2.593) in the natural forest followed by the Stone pine plantation with 1.990 ton/ha (0.338 + 0.650 + 1.002), while the lowest was found to be 0.990 ton/ha (0.182 + 0.377 + 0.431) in the clay mine spoil, which was similar to total carbon storage (*Table 4*).

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Discussion

Forest floor

The forest floor from the tree species planted in mine sites provide important benefits including primarily nutrients such as carbon and nitrogen (Frouz et al., 2013). Similar results were obtained in this study as regards forest floor carbon and nitrogen concentrations, which were consistent with the previous studies. Polat (2010) found that the forest floor carbon concentration in Stone pine plantations ranged from 39% to 55% at different layers. Keskin and Makineci (2009) reported that the mean forest floor organic carbon content ranged from 38% to 51% and nitrogen content from 0.5% to 0.8% in coal mine sites reclaimed with Stone pine plantation trials in Istanbul-Ağaçlı. The forest floor carbon concentration in the natural oak forest was also similar to the ones reported by other studies. For example, the forest floor carbon ranges from 39% to 44% across different oak stands in Turkey and their forest floor nitrogen concentration is approximately 1% (Akburak, 2013; Çakır, 2013; Makineci et al., 2015). Walmsley et al. (2019) reported that the nitrogen concentration was 0.53% and 0.36%, respectively, in the forest floor of oak (*Quercus robur*) and pine (*Pinus* spp.) planted in mine sites and biological activity was lower in the forest floor with high C/N ratio like as for pine species.

Forest floor accumulated in 24 years in the Stone pine plantation, which was found to cover around 50% of the soil due to planting density. Our observations and findings revealed that decomposition of Stone pine forest floor was not fast on clay mine spoils. Poor decomposition may be the result of poor physical soil properties, high bulk density and high clay content leading to poor water and air drainage conditions as well as slower biological activity of the severely acidic substrate. Moreover, high C/N ratio of Stone pine forest floor lead to slow decomposition. Similarly, previous studies reported that the forest floor of Stone pine plantations decomposed slowly under limited conditions such as mine sites and dunes (Keskin and Makineci, 2009; Polat, 2010; Abdalmoula et al., 2019). Forest floor decomposition and carbon to nitrogen ratio vary depending on several factors such as habitat and the chemical properties of the forest floor (Kantarcı, 2000; Treschevskaya et al., 2019).

Soil properties

Soil texture, sand, silt, clay ratios

As a significant difference between the sites as regards soil type as one of the physical soil properties, the soils in the natural forest had a very high sand content while the other sites had a high clay content. The significant difference between sand contents was because the natural structure of the geological material in the forest was sandy. Large pits are created as this material is removed during surface mining. After the mine is abandoned, the excavated clay mine tailings-spoils mix with the material from different depths having different physical properties to use this very heterogeneous material to level the site. Natural soil mixes due to excavation, stockpiling, and replacement operations because of the reclamation activities during and after mining. Therefore, a material that has very different properties than the natural forest structure is formed.

In a similar study, Mosseler and Major (2017) found that one of two adjacent mining tailings sites had a high soil clay content while the another was an abandoned shale overburden site. Two different Salix species (*Salix discolor* and *Salix eriocephala*) were

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tried in those sites. The clay content was found to be higher than 42% in the site with high soil clay content while the shale overburden site had a higher sand content (67.2%) (Mosseler and Major, 2017). Contrary to our results, Shrestha and Lal (2011) and Yuan et al. (2017) reported that the reclaimed mine site had a higher sand content and they associated it with the high sand content of the material. However, they also reported that clay content increased depending on weathering in the reclaimed sites in the following years compared to the initial years. These findings show that different results can be obtained depending on the structural characteristics of the material. The research area in our study was a clay mine spoil which had a high clay content. On the contrary, the soil in the natural forest had a higher sand content.

Bulk density

The bulk density at all three depths in the study was significantly higher in the Stone pine plantation and clay mine spoil compared to the natural forest. The highest mean bulk density (<2 mm) was found to be 1316 g/l. There was a significant difference between the Stone pine plantation and mine site spoil. High bulk density is an expected result in reclaimed mine sites, which is increased due to the compaction of the soil by heavy duty vehicles during the leveling of the soil (Ussiri et al., 2006). Moreover, the material is highly mixed in mine sites and areas reclaimed on mine spoils and has different physical properties. In our study, it can be suggested that increased organic carbon content especially at the topsoil layer (0-5 cm) in the plantation did not reach the level that could change the soil bulk density. In this case, the heterogeneous physical properties of the mixed material are the determining factor. Increased bulk density an important physical soil property for plant growth because it affects water retention capacity and drainage conditions by decreasing soil pore volume (Asensio et al., 2013).

Several studies have reported that organic carbon content is low in mine sites and soil bulk density increases due to compaction and properties of the material (Keskin and Makineci, 2009; Sever and Makineci, 2009; Ahirwal and Maiti, 2016; Gu et al., 2019). Shrestha and Lal (2011) reported that soil bulk density at the depth of 0-15 cm in reclaimed mine sites was 54% higher than the one in the protected areas. Ussiri et al. (2006) stated that soil bulk density can be as high as 1.82 Mg m^{-3} in reclaimed mine sites due to the compaction of the soil by heavy duty vehicles, Maiti (2007) reported that bulk density was 2040 g/l at the topsoil layer of non-reclaimed mine sites and 1760 g/l in reclaimed mine soil. Besides, Ganjegunte et al. (2009) did not find any difference in bulk density between the reclaimed site and protected area at the depth of 0-30 cm.

pH and EC

Soil chemical properties which include pH, EC, C, N and C/N ratio are affected substantially by mining and reclamation activities.

Acidic soil reaction was usually observed in all sites whereas very low pH values were measured in the mine spoil. Soil pH was higher in the Stone pine plantation at each depth compared to the other sites. Because the mixed material has a completely different character in mine sites, pH might vary significantly due to the heterogeneous structure. In fact, Rodríguez-Vila et al. (2016) reported that pH varied in a wide range from 2.7 to 9.03. Changing pH is an expected result after plantation while soil pH tends to increase in reclaimed sites (Ussiri et al., 2006; Chatterjee et al., 2009; Liu et al., 2017; Gu et al., 2019).

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Depending on soil pH values in acidic soils, soil EC is usually low and salinity problem is not expected for plant growth. Besides, difference between the sites only at the depth of 15-30 cm was significant. Soils with low pH usually have low salinity (Mosseler and Major, 2017).

Soil pH and EC variations are affected by the properties of the geological materials of the sites and those of the material that is formed after mining operations. Carbonates, lime, different dissoluble chemical compounds contained in these material and chemical contamination with the material have an important impact on pH (Shrestha and Lal, 2011; Mukhopadhyay et al., 2013). Additionally, Yuan et al. (2017) reported that substances contained in the decomposed litter (humic acid, organic acid and fulvic acid) also affected soil pH.

C and N

Mine spoils usually have a very low organic matter-carbon content. Treschevskaya et al. (2019) reported that almost the whole soil organic matter disappeared due to mining operations, while Akala and Lal (2001) stated that 70% of soil organic matter disappeared. Soil carbon is very important as the main source of soil nutrients, for soil ecology and its biological properties, climate change and carbon balance as well as improvement of water retention and drainage conditions. It is indeed the main subject of several similar studies. Moreover, nitrogen is a primary nutrient for all living organisms and organic matter is the main source of nitrogen in terrestrial ecosystems. In this scope, low organic carbon content in mine sites to be reclaimed is important as it limits plant growth and nutrition. However, increased carbon content in a reclaimed site is a crucial indicator of the improvement of the site.

Organic carbon and nitrogen contents at three depths were significantly different in the sites studied in this research, which increased in mine spoil, Stone pine plantation and natural forest, respectively. The main reason for increased nitrogen content must be the high organic carbon content. This result demonstrated that Stone pine could adapt to the poor environment of the mine spoil and can grow after plantation, which supplied organic matter to the soil. However, the organic carbon and nitrogen concentrations were nearly half of the values in the natural forest even in 24 years after plantation, which indicated that a long time was needed for development. Because of huge degradations on soil and land via surface mining activities, the natural forest soil differs in nature from the one being remediated. Also, it is not possible to reach the same level of soil health status under the plantation at all. Liu et al. (2017) reported that although soil organic matter increased through reclamation after mining operations, it required a long time even decades for it to reach the levels under natural conditions. Cui et al. (2012) stated that it required more than 40 years. In some circumstances, the species of trees planted in mine reclamation sites have important effects on the soil but the question if these effects are the result of the properties of the material or the characteristics specific to the tree species could not be answered (Józefowska et al., 2016).

Slow decomposition due to high C/N ratio as a result of forest floor properties is another possible reason for low organic carbon content in Stone pine plantations. Similarly, Yuan et al. (2017) reported that soil organic carbon and nitrogen increased in reclaimed mine sites, C/N ratio changed very differently but reclaimed mine sites had a higher C/N ratio.

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Similar results have been demonstrated by similar studies on Stone pine plantations. For example, organic carbon concentration in Stone pine plantations was reported to be 0.07-0.56% (Atmaca and Yılmaz, 2006), 0.30% (Kizildag et al., 2012) and 0.6 – 7.0% (Kılcı et al., 2000). In addition, organic carbon content was found to be 0.13-2.74% and nitrogen content to be 0.02-0.407% in reclaimed coal mine sites with Stone pine plantations (Keskin and Makineci, 2009).

Soil organic carbon and nitrogen may vary highly in natural oak forests. In fact, various studies revealed that soil carbon content was 0.02-10.5% in natural oak forests, and thus nitrogen and C/N ratio might differ substantially (Akburak, 2013; Çakır, 2013; Makineci et al., 2015).

Conclusion

The most important conclusion of the study is that Stone pine could survive and growth on mine spoils that have very poor physical and chemical properties under the ecological conditions of the research area. Moreover, 24 years after plantation, soil organic carbon sequestration has been limited despite the forest floor accumulation under Stone pine. Soil carbon and nitrogen contents in the plantation are still half of the natural forest and it requires a long time for restoration.

The extent of such changes and differences may vary depending on the type of mineral, mining method, substrate properties, selected tree species, habitat conditions and etc. Furthermore, reclamation technique may also result in differences. The land was just leveled; soil laying, nutrient supplementation, fertilization or any other soil rehabilitation were not performed. The limitation of the study was that only a limited number of sampling was performed and it was sampled just once. There is a need for further studies to collect more information about the reclamation of these sites through sampling a larger area with higher repetitions.

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APPENDIX

Table A1. *Base ANOVA results for 0-5 cm soil depth*

Properties		Sum of Squares	df	Mean Square	F	Significance
Sand (%)	Between Groups	10531.875	2	5265.938	66.519	0.000
	Within Groups	2929.100	37	79.165		
	Total	13460.975	39			
Silt (%)	Between Groups	788.225	2	394.113	7.798	0.001
	Within Groups	1870.070	37	50.542		
	Total	2658.295	39			
Clay (%)	Between Groups	6722.850	2	3361.425	82.379	0.000
	Within Groups	1509.770	37	40.805		
	Total	8232.620	39			
Bulk density (<2mm, g/l)	Between Groups	1697507.657	2	848753.829	38.563	0.000
	Within Groups	814345.214	37	22009.330		
	Total	2511852.871	39			
pH	Between Groups	21.273	2	10.636	23.928	0.000
	Within Groups	16.447	37	0.445		
	Total	37.719	39			
EC (µs/cm)	Between Groups	9651.737	2	4825.869	0.357	0.702
	Within Groups	500324.658	37	13522.288		
	Total	509976.395	39			
C (%)	Between Groups	52.473	2	26.236	98.761	0.000
	Within Groups	9.829	37	0.266		
	Total	62.302	39			

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N (%)	Between Groups	0.933	2	0.466	479.856	0.000
	Within Groups	0.036	37	0.001		
	Total	0.969	39			
C/N	Between Groups	8201.948	2	4100.974	30.539	0.000
	Within Groups	4968.519	37	134.284		
	Total	13170.467	39			
C (ton/ha)	Between Groups	368.338	2	184.169	29.720	0.000
	Within Groups	229.283	37	6.197		
	Total	597.620	39			
N (ton/ha)	Between Groups	11.499	2	5.750	160.894	0.000
	Within Groups	1.322	37	0.036		
	Total	12.822	39			

Table A2. *Base ANOVA results for 5-15 cm soil depth*

Properties		**Sum of Squares**	**df**	**Mean Square**	**F**	**Significance**
Sand (%)	Between Groups	10873.900	2	5436.950	55.418	0.000
	Within Groups	3630.000	37	98.108		
	Total	14503.900	39			
Silt (%)	Between Groups	469.603	2	234.802	7.244	0.002
	Within Groups	1199.372	37	32.415		
	Total	1668.975	39			
Clay (%)	Between Groups	7472.403	2	3736.202	76.116	0.000
	Within Groups	1816.172	37	49.086		
	Total	9288.575	39			
Bulk density (<2mm, g/l)	Between Groups	390709.622	2	195354.811	7.987	0.001
	Within Groups	904947.905	37	24458.051		
	Total	1295657.528	39			
pH	Between Groups	13.079	2	6.540	16.460	0.000
	Within Groups	14.700	37	0.397		
	Total	27.780	39			
EC (µs/cm)	Between Groups	46811.601	2	23405.801	2.500	0.096
	Within Groups	346350.739	37	9360.831		
	Total	393162.340	39			
C (%)	Between Groups	17.715	2	8.858	85.406	0.000
	Within Groups	3.837	37	0.104		
	Total	21.552	39			
N (%)	Between Groups	0.284	2	0.142	249.838	0.000
	Within Groups	0.021	37	0.001		

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	Total	0.305	39			
C/N	Between Groups	5725.598	2	2862.799	22.020	0.000
	Within Groups	4810.323	37	130.009		
	Total	10535.921	39			
C (ton/ha)	Between Groups	1495.635	2	747.817	28.417	0.000
	Within Groups	973.672	37	26.315		
	Total	2469.307	39			
N (ton/ha)	Between Groups	31.391	2	15.696	120.254	0.000
	Within Groups	4.829	37	0.131		
	Total	36.221	39			

Table A3. *Base ANOVA results for 15-30 cm soil depth*

Properties		**Sum of Squares**	**df**	**Mean Square**	**F**	**Significance**
Sand (%)	Between Groups	16942.700	2	8471.350	99.809	0.000
	Within Groups	3140.400	37	84.876		
	Total	20083.100	39			
Silt (%)	Between Groups	1469.283	2	734.642	15.212	0.000
	Within Groups	1786.892	37	48.294		
	Total	3256.175	39			
Clay (%)	Between Groups	9410.563	2	4705.281	94.596	0.000
	Within Groups	1840.412	37	49.741		
	Total	11250.975	39			
Bulk density (<2mm, g/l)	Between Groups	27981.618	2	13990.809	0.524	0.597
	Within Groups	988749.182	37	26722.951		
	Total	1016730.800	39			
pH	Between Groups	36.149	2	18.075	28.378	0.000
	Within Groups	23.566	37	0.637		
	Total	59.715	39			
EC (µs/cm)	Between Groups	1136475.122	2	568237.561	5.197	0.010
	Within Groups	4045474.116	37	109337.138		
	Total	5181949.238	39			
C (%)	Between Groups	12.389	2	6.195	54.524	0.000
	Within Groups	4.204	37	0.114		
	Total	16.593	39			
N (%)	Between Groups	0.103	2	0.051	138.121	0.000
	Within Groups	0.014	37	0.000		
	Total	0.117	39			
C/N	Between Groups	4470.592	2	2235.296	9.408	0.000
	Within Groups	8790.676	37	237.586		

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	Total	13261.268	39			
C (ton/ha)	Between Groups	3658.978	2	1829.489	39.573	0.000
	Within Groups	1710.535	37	46.231		
	Total	5369.513	39			
N (ton/ha)	Between Groups	31.323	2	15.661	122.891	0.000
	Within Groups	4.715	37	0.127		
	Total	36.038	39			

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PHYTOREMEDIATION OF ORGANIC AND INORGANIC COMPOUNDS IN A NATURAL AND AN AGRICULTURAL ENVIRONMENT: A REVIEW

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Abstract. Phytoremediation is currently an area of trending research due to its huge potential as a sustainable substitute for traditional methods of restoring contaminated sites. It is a profitable and ecological alternative to mechanical and chemical remediation techniques used worldwide. An increase in soil, water, and air pollution has severely disturbed an ecosystem functions and poses a huge threat to the natural and agricultural environment as well as public health. Remediation of the contaminated environment is one of the paramount concerns of the world. Hence this article deliberates on the general problems of pollutants linked to phytoremediation techniques of organic and inorganic contaminants, especially agrochemicals, petroleum, and explosive compounds. The paper also reviews a systematic assessment of the recent progress in the phytoremediation of contaminants in a natural and agricultural environment. Additionally, we highlight the benefits and limitations of phytoremediation along with a brief clarification of the resilient mechanistic removal of contaminants by a three-phase method. Finally, the perspective of biotechnological approaches in remediation is also suggested; taking into consideration the future of synergistic remediation approaches and genetically improved plants to enhance phytoremediation.
Keywords: *hyperaccumulation, pollutant, phytoextraction, phytotransformation, phytovolatilization*

Introduction

Phytoremediation is a term related to ecological restoration technology that takes plants as the main source. Therefore, phytoremediation can be explained as using plants (shrubs, trees, aquatic plants, and grasses) and associated microorganisms for the elimination, degradation, or separation of contaminated sites in an environment (Ossai, 2019; Chirakkara, 2015; Bruneel, 2019). Different approaches have been employed to remediate contaminated sites, but phytoremediation is well-thought-out as a possible alternative, effective, ecologically friendly, and best likened to other outmoded physicochemical methods (Burges et al., 2018; Rajput et al., 2019).

During the process of phytoremediation, compounds that can be remediated include (i) organic waste (ii) metals (iii) inorganic substances (iv) agrochemicals (v) metalloids (vi) radiochemical elements (vii) petroleum hydrocarbons (viii) explosives and (ix) chlorinated solvents (Cristaldi et al., 2017; Misra, 2019; Abdel-Shafy and

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Mansour, 2018). The key sources of organic and inorganic pollutants occur naturally but others are a result of the use of fuels, solvents, and pesticides. Several organic and inorganic compounds are harmful and connected to health issues worldwide (Dixit et al., 2015; Li et al., 2017). These pollutants are on the rise within the environment through several activities in agriculture (pesticides, herbicides), industry (chemicals, petrochemicals), spillage (fuel, solvents), wood processing, and military activities (explosives, chemical weapons), etc. (Tripathi et al., 2020). Many contaminants within the agricultural soils can cause a substantial amount of damage to plant growth, soil ecological functions, and human health (Cai et al., 2019; Ngole-Jeme and Fantke, 2017). For instance, nitrobenzene impedes soybean seed growth and initiate genotoxicity in the cells of its root tip (Guo et al., 2010). There are also varied contaminants generally in soils or areas to be remediated, which contain trace elements and organic chemicals. Even though this is still a herculean task, many groups have faced action recently on contaminated soil phytoremediation (Gutiérrez-Ginés et al., 2014; Xu et al., 2019).

The extreme pressures from organic and inorganic waste and the magnitude of contamination have caused widespread concern as intentional or unintentional exposure of these materials poses a major threat to the environment and public health (Cristaldi et al., 2017; Rajput et al., 2019). Also, the events and mishaps of these contaminations worldwide have drawn the attention of the general public. Progressively, more people are alarmed and as a result, researchers are in search of the most effective remedies to curb these pollutions. The Soil Pollution Prevention and Control action plan by countries like China in 2016 was estimated to cost US$90 billion, aiming to utilize and develop effective remediation methods (Hou and Li, 2017). Conversely, these approaches do have some disadvantages such as high cost in operation and mobilizing contaminated material to the site of treatment which intensifies the risk of secondary contaminations.

Many techniques have evolved in the past decades intending to restore sanity to our environment and a wide acknowledgment of these methods is on the basis that it is an environmentally friendly method and a cheaper approach to get rid of contaminants. Currently, the extent of studies that have employed phytoremediation techniques to clean and reduce environmental pollution is increasing (Dixit et al., 2015; Rodriguez-Narvaez et al., 2017). However, despite several reports by many authors on the issue of remediation, there is still missing information regarding materials on the possible antagonistic or synergistic effects of diverse contaminants and their cross-accumulation or detoxification in both natural and agricultural environment.

Here, we:

- Provide an overview of phytoremediation of contaminants from agrochemicals (pesticides/fertilizers), petroleum compounds, explosive compounds, and inorganic compounds.
- Discuss the challenges in phytoremediation methods and also try to suggest an improvement in the biotechnological approaches for future research perspectives to enhance the phytoremediation of organic and inorganic pollutants.

This paper, we believe will provide a critical and extensive review of studies in natural and agricultural environment specifically in addressing the recent literature regarding remediation of organic and inorganic compounds.

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Phytoremediation techniques

Phytoremediation has great promising as a natural on-site treatment with solar energy capable of treating the land and a vast area of moderately contaminated sites. Contrasting other kinds of remediation, plants grown for ornamental purposes in gardens and landscaping projects have become an important tool in recent years (Liu et al., 2018; Dodangeh et al., 2018; Cheng et al., 2017; Ranieri et al., 2016). Besides being attractive to the environment, some of these ornamental plants accumulate or decompose pollutants when they grow in soil polluted by heavy metals and organic pollutants (Liu et al., 2018). Just like heavy metals, an organic pollutant in plants is remediated by several natural biophysical and biochemical processes. Trees with large roots and high transpiration rates, such as *Populus* (*Populus spp.*) or *Willow* (*Salix spp.*), are well-known in the phytoremediation process (Srivastav et al., 2018). Such plants have a multiplicity of effects on these toxic compounds. They can be fixed, stored, volatilized, and converted to varying degrees (or even mineralized) or a combination of the processes which are conditional on the particular compound, environmental conditions, and plant genotypes. Phytoremediation remains a widely used method; however, if a cautious selection of the best appropriate plants and proper agronomic methods are not used appropriately, there could be a lack of control and plant availability. The mechanism that plants use to promote remediation (*Fig. 1*) includes plant extraction, plant degradation, plant stabilization, plant volatilization, and rhizosphere decomposition.

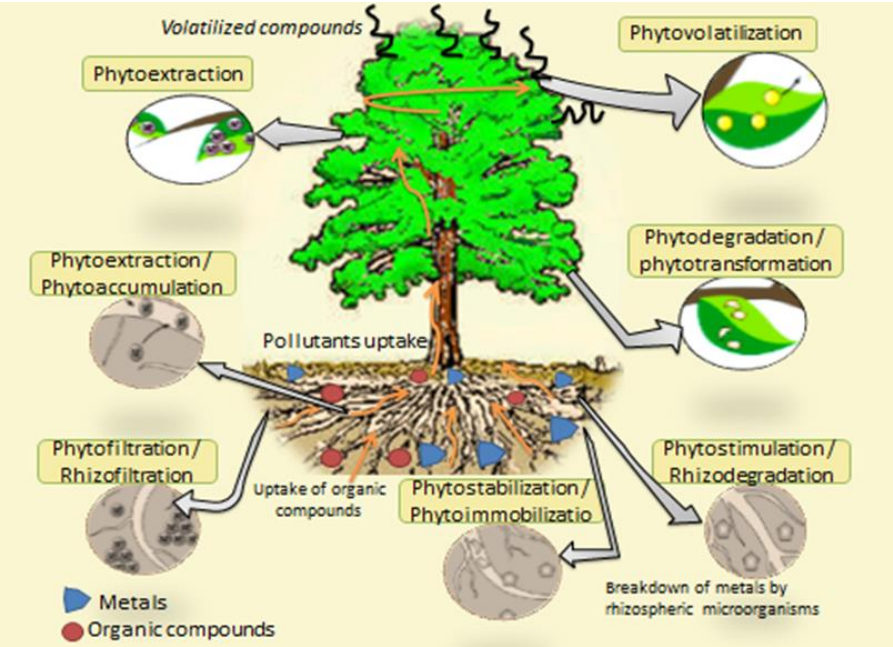

Figure 1. *Presentation of phytoremediation of organic and inorganic pollutants from a contaminated environment (adapted from Favas et al., 2014; Avensblog, 2020)*

Phytodegradation (phytotransformation)

Phytodegradation (also known as phytotransformation) involves the uptake of organic contaminants to be decomposed (metabolized) or mineralized in plant cells by certain enzymes. These include nitroreductase (through the decomposition of

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nitroaromatic compounds), dehalogenase (through the decomposition of chlorinated solvents and pesticides), and volatile enzymes (involved in the decomposition of aniline) (Kumar et al., 2018). Plant species such as Populus and Sage are illustrations of plants with these enzyme systems (Leung et al., 2019; Feng et al., 2017). For instance, Chen et al. (2016) reported that the Armoracia rusticana possess the ability to degrade benzophenone within its tissues. These green plants are classified as the biosphere 'green liver'. Also, a current study employed phytotransformation for organic compounds like insecticides, chlorinated solvents, herbicides, inorganic nutrients, and munitions (Kumar et al., 2018).

Phytostabilization (phytoimmobilization)

Phytostabilization or phytoimmobilization is an in situ technology whereby toxic compounds are integrated into the root cell wall lignin to form non-toxic substances, hence decreasing the existence of pollutants in the natural and agricultural environment (Khalid et al., 2017; Mahar et al., 2016). Metals are precipitated in insoluble forms under the direct action of root exudate and subsequently enter the soil medium. The main challenge relating to this technique is the avoidance of the concentration of pollutants and suppressing their dispersion in soil (Favas et al., 2014; Ali et al., 2013; Fan et al., 2017). Studies conducted by Khalid and coworkers (Khalid et al., 2017) have referenced a great number of examples of plants grown for this purpose which are but not limited to species of the genera *Haumaniastrum*, *Eragrostis*, *Ascoliepis, Gladiolus*, and *Alyssum*. These plant species can also contribute to phytostabilization owing to their capability to release a greater number of chelating agents. Other research studies (Lebrun et al., 2018) showed that in phytoimmobilization, unclean soil is covered with vegetation that is resistant to high applications of harmful elements, thereby limiting soil erosion and the leaching of pollutants within groundwater. These substances immobilize pollutants, inhibiting their uptake and reducing their mobility in soil. As a result, plants with stabilizing potential play an essential role in polluted areas of agricultural production and vegetation restoration (Eskander and Saleh, 2017; Saha et al., 2017). It is reported (Eskander and Saleh, 2017), that these substances were known to immobilize pollutants, prevent absorption, and thereby decrease their movement in the soil. This accounts for why plants with phytostabilization potential are very valuable for mining, waste, and vegetation restoration in contaminated sites. Similarly, Yadav and colleagues (Yadav et al., 2018) have also revealed that phytostabilization is very useful in removing inorganic contaminants, from soil contaminated with Arsenic (As), Copper (Cu), Lead (Pb), Zinc (Zn), Cadmium (Cd), Chromium (Cr) and other metals in their sediments.

Phytovolatilization

Phytovolatilization is the capability of some green plants to engross certain metals/metalloids and later release them through vaporization. Some ions groups like IIB, VA, and VIA in the periodic table (especially mercury (Hg), selenium (Se), and As) are being taken by the roots, transformed into non-toxic forms and later volatilized into the atmosphere (Limmer and Burken, 2016). Conversely, the disadvantage of phytovolatilization lies in the fact that toxic compounds discharged into the atmosphere can undergo precipitation and re-deposit back into the environment, as this will give rise to other toxic substances (Nikolić and Stevović, 2015). Examples of plant species from

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studies (Ali et al., 2013; Mani and Kumar, 2014) include *Stanleya pinnata* and *Astragalus bisulcatus* or transgenic plants (with bacterial genes) such as *Liriodendron tulipifera, Brassica napus, Arabidopsis thaliana*, or *Nicotiana tabacum*. This method can also be applied to tackle organic compounds and other heavy metals like Se and Hg.

Phytoextraction (phytoaccumulation, phytoabsorption or phytosequestration)

This mechanism refers to the absorption of pollutants by roots, followed by displacement and accumulation in air particles. Phytoextraction technology as reviewed by Parmar et al. (2015) involves the eradication of contaminants from soil, groundwater, or surface water by living plants. This technique according to research by Sarwar et al. (2017) is mainly engaged in the remediation of soils polluted by metals (Cd, Nickel (Ni), Cu, Zn, Pb), however, it could also be used for other elements (such as Se, As) and organic compounds. This method favors hyperaccumulator species (which will be discussed in this paper) that can maintain a great absorption of exact metals in their air portions (0.01-1% dry weight, depending on the metal) (Parmar, 2015). Phytoaccumulation, also called phytoextraction or hyperaccumulation, reported by Xiao et al. (2017) uses cationic pumps and sorption to remove metals, salts, and organic compounds from the soil by absorbing water available to plants. The process commences by sowing the metal-accumulating plant in metal-polluted soil and coupled with extensive agricultural practices. Many authors (Van der Ent et al., 2013; Xiao et al., 2017; Reeves et al., 2018) have proposed *Elsholtzia splendens, Alyssum bertolonii, Thlaspi caerulescens* and *Pteris vittata* as known examples of hyperaccumulating plants of Cu, Ni, Zn/Cd, and As respectively. Several plant species near metal mining areas as stated by Saxena et al. (2020) thrived in soils heavily contaminated with metals, hence for remediation of contaminated areas, it is necessary to select the kind of plants which can absorb and transport metals in various conditions. However, some soils are highly contaminated hence the elimination of metals through this method will save an uncertain amount of time.

Phytofiltration

This technique uses plants to absorb distillate and/or precipitate contaminants, especially radioactive elements, and heavy metals, from the environment through the root structure or further submerged organs. In this process, Parmer and Singh (Parmar, 2015) reviewed that plants are stored in a hydroponic system through which wastewater passes and is "filtered" by the roots (rhizofiltration) or other structures that can absorb and concentrate pollutants (hyperaccumulators) or tolerate pollutants for the best results. Phytofiltration remediates metallic substances like Cu, Ni, vanadium (V), Cr, Pb, including other radionuclides such as caesium (Cs), strontium (Sr) and uranium (U). Nevena et al. (Cule et al., 2016) in an experiment used the ornamental plant (*Canabis indica*) to remediate Pb in wastewater. Their findings showed that the removal rate was 81.16% higher. The authors (Cule et al., 2016) supported the idea that terrestrial plants are most appropriate for rhizofiltration than aquatic plants and that *C. indica* is best used in rhizofiltration methods or floating islands for treatment of water polluted with Pb. Zhang et al. (2005) researched on the efficiency of Cu removal from contaminated water by *Elsholtzia argyi* and *Elsholtzi splendens* in hydroponics. Their results demonstrate that *Elsholtzia argyi* showed better Cu phytofiltration (removal rate of 50-90%) than *Elsholtzi splendens* (removal rate of 45-80%), which was linked with better

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capability to higher Cu concentrations and translocation to shoots. One could infer that various physicochemical properties of plant species are factors in the choice of plant for phytofiltration. This technology is capable of dealing with industrial discharge, agricultural runoff, and mine drainage. Several authors have indicated some promising examples of such plants as *Brassica juncea*, *Helianthus annus*, *Fontinalis antipyretica*, *Phragmites australis*, and some species of *Salix*, *Populus*, *Lemna,* and *Callitriche* (Van der Ent et al., 2013; Bonanno and Cirelli, 2017; Favas and Pratas, 2016; Tatar et al., 2019).

Rhizodegradation (phytostimulation)

Rhizodegradation or phytostimulation is the breakdown of organic compounds in the soil through the microbial activity of the root (rhizoshere) (Echereme, 2018). This is improved biodegradation of contaminants by a particular plant species root-associated fungi and bacteria (Ali et al., 2013; Khalid et al., 2017). There are free-living, symbiont mycorrhizal fungi that are associated with plant roots and are significantly more beneficial for the production and biochemical availability of nutrients such as cobalt (Co), Cu, Zn, Ni, nitrogen (N), phosphorus (P), potassium (K), sulphur (S) and Calcium (Ca) through the extensive hyphal network (Sarwar et al., 2017). In research by Jiang et al. (2017), it was demonstrated that bacterial species, for instance, *Pseudomonas*, *Acinetobacter*, *Bacillus*, and *Cupriavidus* improved their environmental adaptability and was resistant to Pb, Cd, and Cu in rhizospheric soil around the plant. The authors deduced that *Boehmeria nivea* L. (known to be evolving around chemical refineries) was improved by increased concentrations of the particular microorganisms (Jiang et al., 2017). Also, the plants themselves can release biodegradable enzymes. The microbial association in the rhizosphere is heterogeneous due to the changing spatial distribution of nutrients, but species of the genus *Pseudomonas* are the organisms largely connected with the root (Ali et al., 2013; Singh and Singh, 2016; Salem et al., 2018). The death of plants ought to be considered and agronomic methods must be used to reduce it by timely planting in the growing period, digging a hole in diameter, and feasibly filling it with unpolluted plant soil, for better survival of trees, thus bringing a greater efficiency of phytoremediation.

Phytodesalination

According to literature, this is a recently informed remediation approach that removes saline from internal salts. Studies have reveals that the efficacy of *Suaeda maritima* and *Sesuvium portulacastrum* in eradicating and accumulating sodium chloride (NaCl) from highly salty soil, has demonstrated to be very effective (Ali et al., 2013; Kumar et al., 2019b). Phytodesalination may possibly occur in parallel with phytoremediation of heavy metal contaminated soils in arid regions, increasing the prospect of this process. Halophytes (salt-tolerant plants) have been suggested (Padmavathiamma, 2014) to naturally adjust to cope with environmental stresses, such as heavy metals and other organic contaminants (Padmavathiamma, 2014). Iniyalakshimi (Iniyalakshimi, 2019) also found that *S. portulacastrum*, which has high sodium and chloride absorption ability may be a possible candidate for reducing secondary salinization due to irrigation with sodium-rich industrial wastewater (Fan et al., 2019). Taken together, this indicates that *S. portulacastrum* (SpSOS1 and SpAHA1) coordinates to alleviate salt toxicity by cumulating the efficiency of Na + extrusion to

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maintain K + homeostasis and defend the plasma membrane from oxidative harm induced by salt stress. In a current study (Lastiri-Hernández et al., 2020), the capacity of halophytes species namely; *Bacopa monnieri* (L.), *Sesuvium verrucosum* Raf. and *Wettst* was assessed to increase their chemical properties in saline soil for 240 days in a field. It was also reported that the association of these plants has a phytodesalination ability of 1.21 t Naþ ha1 and this served to prepare the conditions for the crop growth (Lastiri-Hernández et al., 2020). This technology involves an enzymatic breakdown of pollutants, relevant for soil, groundwater, or surface water, and sediment sludges. This process is an impressive kind from phytoextraction, even though it has its drawbacks. Some of these drawbacks could include the duration of the process, the properties of the soil (salinity, sodicity and porosity), the number and the initial weight of the plant species.

Phytoremediation of organic contaminants

Currently, phytoremediation is offered as a cost-effective method to remove many kinds of organic contaminants such as petroleum products, aromatic hydrocarbons (BTEX), chlorinated solvents, explosives, and cyanides as aforementioned (Hattab-Hambli et al., 2020).

Furthermore, one of the largest environmental disasters was in the Gulf of Mexico, which had a major impact on the ecosystem and human health (Sandifer et al., 2017; Singleton et al., 2016; Schaum et al., 2010; Han and Clement, 2018; Babcock-Adams et al., 2017; Eklund et al., 2019). Crude oil kills small invertebrates, destroying soil biological life thereby rendering such polluted soils habitable to only anaerobic bacteria. Phytoremediation, as a natural, solar energy-driven in situ method, has great potential for treating soils and large areas of moderately contaminated areas. Plants are carefully selected and appropriate agronomic methods are used to properly manage phytoremediation of organic pollutants (Schwitzguébel, 2017). Phytoremediation can restore, balance a stressful environment due to the natural, synergistic relationships between plants, microorganisms, and the environment. Further, during the in-situ phytoremediation process, organic matter, nutrients, and oxygen are added to the soil through the metabolic processes of plants and microorganisms, thereby improving the quality and texture of the clean area (Schwitzguébel, 2017; Wiszniewska et al., 2016).

Phytoremediation of agrochemicals

The predominant misuse of agrochemicals over several years has polluted the agricultural and natural environment. These chemicals cause various damages to agricultural lands, various water bodies, and other land-dwelling organisms.

Various organic compounds (OCs), for example, organochlorine pesticides (OCPs) and other agrochemicals are extremely toxic and persistent in the environment (Sun et al., 2018).

The potential use of alfalfa, tomato, sunflower, and soybean species to remove endosulfan from the soil was studied by Mitton et al. (2016). Their research results showed that, except soybean, the phytoextraction rate of all other species at 60 days increased with the decrease of soil pesticide levels. Sunflower plants had the highest phytoextraction rate (2.23%), followed by tomato (1.18%), soybean (0.43%), and alfalfa (0.11%). In additional research (Zhao, 2018), the dissemination of dichloro diphenyl trichloroethane (DDT) residues in agricultural soils in southwestern Ontario, Canada,

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was also studied to determine the degree of degradation of tomato plants. The DDT concentration in tomato fruits was 211.75 ug/kg, which was lower than the prescribed value. Consequently, tomato fruits are eligible and valued, and may be a potential choice for future field research. In a study (Qu et al., 2017) involving atrazine-contaminated lake sediments, spiked watermilfoil (*Myriophyllum spicatum*), and curled algae (*Potamogeton crispus*) were opened to 0.10 mg atrazine kg-1 soil at a decreased rate of 76.15% and 75.65% respectively in comparison to Atrazine's decline of 46.3% non-cultivated soil.

To correct these contaminations, researchers have essentially used consortia of various bacterial strains, such as atrazine and deisopropylrazine (Fan and Song, 2014) via rhizodegradation/phytostimulation of agrochemicals from agricultural environment. From a biotechnological point of view, bacterial strains can also be used to decompose certain pesticides, such as the one Myresiotis et al. (2012) used to remediate the soil contaminated with hydrochloride, metribuzin, acidobenzolar-S-methyl, propamocarb, thiamethoxam and napropamide. Phytoremediation of pesticides is affected by some factors. Their low bioavailability in soils might confine the attainment of this technology.

Large numbers of plants have been tested to proficiently accumulate pesticides and some of these plants are listed in *Table 1*. The capacity of these plants to uptake pesticide residues varies greatly between plant species (Handford et al., 2015; Romeh, 2015). These plants are widely used because of their significant role in agriculture and gardening and as a result of these good prospects for the accumulation of innumerable organic pollutants, they are therefore generally considered phytoremediation plants (Singh and Singh, 2017).

Significant interactions can occur between different pollutants in a polluted environment. When there is soil-to-plants absorption, the accumulation potential is affected by various plant properties such as water absorption potential and root depth/structure (Eevers et al., 2017). When pesticides are trapped in plant root tissues, they can be immobilized in the roots or transferred to an aerial portion of the plant where the analytes can be stored, metabolized, or evaporated (Eevers et al., 2017). In general, the objective of effective phytoremediation is not only phytoaccumulation but also the degradation of pollutants in plant tissues as this can be done with or without endophytic bacteria.

Phytoremediation of explosive compounds

After World War II, the commissioning and removal of weapons at military factories brought about the pollution of the agricultural environment and other biological systems with explosives like 2,4,6-trinitrotoluene (TNT), hexahydro-1,3,5-trinitro-1, 3,5 triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetraazocin (HMX) (Taylor, 2017; Srivastava, 2015; Sheehan et al., 2020; Aguero and Terreux, 2019). These compounds are the most widely distributed organic explosive contaminates found in nature. The use of explosives during military exercises and operations leads to large-scale environmental pollution, in which case the ecological balance is disturbed (Ndibe et al., 2018). As a consequence of their manufacturing and disposal practices, these explosives with their transformation products are major pollutants in soils, ground and surface waters worldwide. For instance, ammunition contamination in the 1920s in Verdun, France, still posed a danger to public health (Rylott and Bruce, 2019; Gorecki et al., 2017).

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Table 1. *Phytoremediation of pesticide by certain plant species from previous studies*

Plant species	Agrochemical	Formula	Molecular weight (g/mol)	References
Castor bean/castor oil plant (*Ricinus communis*)	Aldrin,	$C_{12}H_5Cl_6$	364.92	Rissato et al., 2015
	Chlordane, Chlorpyrifos,	$C_{10}H_6Cl_8$	409.78	
	Dichlorodiphenyldichloroethylene (DDE)	$C_9H_{11}Cl_3NO_3PS$	350.59	
	Diclofop methyl, Dllieldrin	$C_{14}H_5Cl_4$	318.03	
	Endrin	$C_{16}H_{14}Cl_2O_4$	341.2	
	Hexachloro-cycohexane (HCH)	$C_{12}H_5Cl_6O$	380.91	
	Heptachlor, methoxychlor	Cl2H, Cl_6O	380.91	
		$C_6H_6Cl_6$	290.83	
		$C_{1O}H_5Cl_7$	373.32	
	DDT	$C_{14}H_9C_{15}$	354.49	Huang et al., 2011; Rissato et al., 2015
Maize/corn (*Zea mays*)	Dichloro diphenyl dichloroethane (DDD)	$C_{14}H_{l0}C_{14}$	320.05	Bogdevich and Cadocinicov, 2010
	DDE	$C_{14}H_5Cl_4$	318.03	
	DDT	$C_{14}H_9C_{15}$	354.49	
	Endosulfan	$C_9H_6Cl_6O_5S$	406.92	Mukherjee and Kumar, 2012
	Endosulfan sulphate	$C_9H_6Cl_6O_5S$	406.92	Somtrakoon et al., 2014; Somtrakoon, 2014
	Hexachloro-cycohexane (HCHs)	$C_6H_6Cl_6$	290.83	Alvarez et al., 2015; Bogdevich and Cadocinicov, 2010
Chinese violet cress (*Orychophragmus violaceus* L. O. E. Schulz)	DDD	$C_{14}H_{l0}C_{14}$	320.05	Sun et al., 2015
	DDE	$C_{14}H_5Cl_4$	318.03	
	DDT	$C_{14}H_9C_{15}$	354.49	
	HCHs	$C_6H_6Cl_6$	290.83	
Common sunflower (*Helianthus annus*)	Azoxystrobin	$C_{22}H_{17}N_3O_5$	403.4	Romeh, 2015
	DDD, DDE, DDT	$C_{14}H_{l0}C_{14}$ $C_{14}H_5Cl_4$ $C_{14}H_9C_{15}$	320.05 318.03 354.49	Mitton et al., 2014
Common yarrow *(Achillea millefolium)*	DDT	$C_{14}H_9C_{15}$	354.49	Moklyachuk et al., 2012
Sweet flag – Calamus (*Acorus calamus*)	Atrazine	$C_8H_{14}ClN_5$	215.68	Wang et al., 2012
Welsh onion/bunching onion/long green onion/Japanese bunching onion/spring onion (*Allium fistulosum*)	Phoxim	$C_{12}H_{15}N_2O_3PS$	298.3	Wang et al., 2011
Love-lies-bleeding (*Amaranthus caudate*)	Glyphosate	$C_3H_8NO_5P$	169.07	Al-Arfaj et al., 2013
Field mustard/Turnip rape/bird rape/Keblock (*Brassica campestris*)	Endosulfan	$C_9H_6Cl_6O_3S$	406.92	Mukherjee and Kumar, 2012
Broadleaf plantain/white man's foot/Greater plantain (*Plantago major*)	Azoxystrobin	$C_{22}H_{17}N_3O_5$	403.4	Romeh, 2015
	Chlorpyrifos	$C_9H_{11}Cl_3NO_3PS$	350.59	Romeh and Hendawi, 2013
	Cyanophos	$C_9H_{10}NO_3PS$	243.22	Romeh, 2014a,b

Source (common English name): https://en.wikipedia.org/wiki/ (accessed on April 7, 2020)

Explosives are sensitive to a xenobiotic, and their survival in an environment is dangerous for living organisms; as they can migrate through aboveground soils,

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polluting groundwater (Taylor, 2017). Despite the devastating effects of pollution on environmental and human health, the global market for explosives was forecast to grow from $ 23.8 billion in 2017 to $ 31.2 billion by 2022, therefore lucrative, clean-up methods are required instantly (BCC, 2018). There are innumerable practices connected with the conversion of explosive pollutants by plants. RDX has been described (Hannink et al., 2002) to have minor toxicity than TNT. Additionally, it can translocate within plants, and consequently, these compounds can be stored in various parts of the plants.

In the USA (Virginia Commonwealth University), a study by Via et al. (2016) was conducted to investigate the impacts of explosives polluted soils using ecological metrics in an experimental minefield on vegetative communities. Their results showed that RDX and TNT contaminated plots had shifted in dominant functional traits, suggesting an influx of more tolerant species as a result of new tolerant species filling open niches in contaminated plots (Via et al., 2016). In another study (Kiiskila et al., 2015), many agricultural and ornamental important plants were examined for their capability to remediate TNT and RDX in barley (*Hordeum sativum*), Soybean (*Glycine max*), alfalfa (*M. sativa*), chickpea (*Cicer arietinum*), maize (*Zea mays*), sunflower (*Helianthus annuus*), pea (*Pisum sativum*), and ryegrass (*Lolium multiflorum*) species. Furthermore, Das (2017) reported the two most effective species for TNT uptake- *Eurasian watermilfoil*, *Myriophyllum spicatum*, and vetiver grass as well as *Chrysopogon zizanioides*. For RDX phytoremediation, reed canary grass, rice, and fox sedge showed good promise, although degradation of RDX in the plant tissue is limited (Kiiskila et al., 2015). Conversely, there are limitations to this new technology. It is only effective when treating shallow soils, ground, and surface waters. Plants can also efficiently remove pollutants only close to the root zone since phytotoxicity is also a drawback to this methodology.

Phytoremediation of petroleum compounds

Economic growth and industrialization have led to increased emissions of petroleum hydrocarbons (PHC) and trace elements (TE). Due to their tenacity in an environment and various toxicological effects on living things, they are well-thought-out to be the greatest toxic pollutants in the world (Marchand, 2018). Most chemical contaminated soils are linked with the release of petroleum products into the environment. The intensification of global oil and gas activities, including oil exploration, drilling, production, land storage, and transportation has also increased the menace of crude oil spills and outflows (Okotie et al., 2018). Due to health problems, these water bodies and land contaminated with crude oil are often not suitable for domestic and agricultural uses (Tang and Angela, 2019a).

However, the exploration of crude oil has brought economic development, especially in developing countries (like Ghana, Nigeria, Niger etc.), but the natural and agricultural environment in these countries has also been damaged by the negative effects of these oil industries (Ngene et al., 2016). For instance, water resources in the Niger Delta are no longer fit for human drinking, but oil exploration in Nigeria cannot be stopped as 90% of Nigerian foreign currency earnings come from crude oil exploration (Okotie et al., 2018; Adekola et al., 2017; Siakwah, 2018). In addition to the detrimental effects of the oil industry in our environment, various reports (Ramirez et al., 2017) points out its negative impacts on human health as well. These are psychological problems initiated by crude oil leakage, respiratory tract irritation, and

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blood disorders (Ramirez et al., 2017; Taheri et al., 2018). Sylvia reported that contaminated sites pose a risk to human life owing to severe health complications caused by unreceptive health effects from introduction to oil-soil contamination (Adipah, 2019).

Phytoremediation is said to be a slowly developing process that can clean the environment for a long time. This method is also influenced by external parameters, including type and leaching of pollutants, soil chemistry, and photosynthesis (Lim et al., 2016) and water-related effects on conservation and weather conditions. Exposure of plants to organisms and pollutants for a longer period reduces their ability to uptake contaminants (Zabbey et al., 2017). In current research by Viesser et al. (2020), three bacteria were isolated namely; *Bacillus thurigiensis*, *Bacillus pumilus*, and *Rhodococcus hoagie*. These were able to use petroleum hydrocarbons as the sole carbon during vitrodegradation assays. The authors found that *R. hoagii* had the highest efficacy of petroleum consumption, attaining 87% of degradation after only 24 h of cultivation.

Another experiment (Heidari et al., 2018) was conducted to study the effect of oil-contaminated soil on *Echinacea purpurea* with four concentrations of crude oil. The results depicted that this plant has a possibility for removing TPHs, up to 45.5% at 1% crude oil contamination. The authors further reported that *E. purpurea* is a widely - spread species that can be commendably used for phytoremediation of ≤ 10000 mg kg^{-1} crude oil-contaminated soil. Other studies suggested that the faster-growing flora (e.g. grass species) are plants that effectively restore polycyclic aromatic hydrocarbons (PAH) in contaminated soil (Srivastav et al., 2018; Kumar et al., 2019a). The adsorption properties of TPH ought to be investigated as it aids to reduce the concentration of organic material in the soil. Thorough remedial studies have to be carried out not only to assess the effectiveness of the repair but also to investigate and implement the potential for secondary pollutants.

These studies demonstrate that agricultural lands with low rates of oil contamination allow the growth of plants. Furthermore, previous studies in phytoremediation of contaminants have not focused so much on petroleum hydrocarbons rather all attention has been on heavy metals remediation (Tang and Angela, 2019b), hence further studies must be focused on this area.

Inorganic contaminants

Inorganic materials belong to man-made environmental activities, for instance, smelting, mine drainage, chemical, and metallurgical processes, and also natural processes. These sources release inorganic pollutants usually in the form of minerals such as metals, salts, and natural substances (Masindi and Muedi, 2018; Fayiga et al., 2018). Heavy metal contaminants are usually brought about by human activities, but natural and biological contaminations are also common such as erosion and volcanic activity, mining pollution over time; which causes toxic particles release in vegetation nutrients and forest (Banunle, 2018). These contaminants are poisonous since they sometimes accumulate in the food chain (Masindi and Muedi, 2018). Pb, Co, Cd and other toxic heavy metals cannot be biodegraded, so they can be distinguished from other pollutants, but they can accumulate in plants, even if the concentration is relatively low, it can cause various diseases and diseases. Higher levels of essential and non-essential heavy metals in the soil may inhibit plant growth and cause toxic symptoms in most

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plants (Ochonogor, 2014). Several remediation techniques for inorganic compounds are known in literature but phytoremediation stands out.

Phytoremediation of inorganic contamination

Phytoremediation of inorganic contaminants comprises three technologies, plant extraction (similarly known as phytoextraction/phytoaccumulation), root filtration (rhizofiltration), and plant stabilization (phytostabilization) (Chirakkara et al., 2016; DalCorso et al., 2019). In phytoextraction as aforementioned, heavy metals within the soil are absorbed by the roots of the plants, transferred to the ground part, and accumulated in the soil. The contaminants do not decompose, but remain in roots and/or plant tissue (Chandra et al., 2017). After harvesting plant parts containing inorganic contaminants, it is advisable to keep them in a safe place for discarding. It is reported according to some authors (Banunle, 2018; Sharma, 2018) that the amount of contaminated plant material treated is relatively small compared to the amount of contaminated soil treated with in situ remediation techniques. There are differences in rhizofiltration from the phytoaccumulation, as this is applied to contaminants dissolved in shallow, underground, or wastewater (Chirakkara et al., 2016).

In the process of rhizofiltration of plant root, inorganic contaminants will be adsorbed in the roots or deposited on the roots (Makombe, 2018; Makombe and Gwisai, 2018). The fixation of pollutants in the root zone by the effects of soil chemistry, microbiology, and physics are also referred to as phytostabilization (Touceda-González et al., 2017). Some soils are so severely contaminated hence metals removal using plants would take an impractical expanse of time. Therefore, the normal practice is to select drought-resistant, fast-growing fodder or plants which will be able to grow in metal-contaminated and nutrient-deficient soils.

Hyperaccumulation process by some plant species

The remediation techniques noted here is plant extraction (phytoextraction), identified as phytoaccumulation, where residues of pollutants are taking by plant roots and transferred to other parts of the plant. Some metals are more deadly than others (e.g. arsenic, cadmium is less toxic) for instance cobalt, nickel, chromium, mercury, and selenium are very toxic even in small quantities (Masindi and Muedi, 2018). Plant extraction according to Rascio and Navari-Izzo (2011) can be accomplished by removing high concentrated contaminants from the soil, such as using hyperaccumulators; uptake of low concentrations of extracts while maintaining high growth status as in *Populas sp*. (Nissim et al., 2018). Subsequently, phytostabilization removes contaminants and reduces their leaching from the soil by reducing crop roots. Roots can also be an important material for transforming harmful metals into low-toxic forms (Ali et al., 2013). Additionally, damage to plants depends on the microorganisms that come with the root system and the enzymes that are secreted by the root to clear the germs and then remove them by absorption and transpiration.

The roots that accumulate higher concentrations of iron in each tissue are higher; and so, their habitat is acknowledged as hyperaccumulators (Srivastav et al., 2018). Qatari plants reported in recent studies by Al-Thani (2019) are the most candidates for active phytoremediation of contaminated compounds of such nature. Considering that monitoring is employed, *Phragmites australis*, *Typha domingensis*, *Amaranthus spp.*, *Nerium oleander*, and *Ricinus communis* are noted as important species of plant for

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successful ecological remediating and conserving a healthy environment (Al-Thani, 2019). *Table 2* summarizes some examples of plant species and their metal accumulation capabilities that have been tested for phytoremediation studies. In general, different methods of phytoremediation have potent characteristics which make them suitable for diverse soil and water pollution remediation. These methods of study are delimited by duration, nonetheless it supports the capability of selected phytoremediation plants to be considered (Tang and Angela, 2019a). For phytoremediation through phytoaccumulation, the shoot must be obtained after uptake. The shoot can be burned or otherwise destroyed (Valipour et al., 2015).

Table 2. *Some plant species used in the hyperaccumulation of metals and their accumulation capacity*

Plant species	Family	Polluted medium	Metals	Metal accumulation capacity (mg kg-^{1}DW)	Phytoremediation mechanism and metal accumulation compartment	References
Blue stool (*Noccaea caerulescens*)	Brassicaceae	Water	Pb	1,700–2,300	Rhizofiltration (aerial parts/root)	Dinh et al., 2018
Hemp (*Cannabis sativa L.*)	Cannabaceae	Soil	Cd	151	Phytoextraction (aboveground plant parts	Ahmad et al., 2016
Madwort (*Alyssum markgrafii*)	Brassicaceae	Soil	Ni	4,038	Phytoextraction (aboveground plant parts)	Salihaj et al., 2018
Hemp (*Cannabis sativa L.*)	Cannabaceae	Soil	Cu	1,530	Phytoextraction (aboveground plant parts)	Ahmad et al., 2016
Southern cone marigold (*Tagetes minuta*)	Asteraceae	Water	As	380.5	Phytoextraction (shoots)	Salazar, 2014
Johnson grass (*Sorghum halepense L.*)	Poaceae	Soil	Pb	1,406.80	Phytostabilization (reduction in rhizosphere)	Salazar, 2014
Water/red birch (*Betula occidentalis*)	Betulaceae	Soil	Pb	1,000	Phytoextraction (shoots)	Koptsik, 2014
Alpine pennygrass (*Thlaspi caerulescens*)	Brassicaceae	Soil	Cd	5,000	Phytoextraction (shoots)	Koptsik, 2014
Sunflower (*Helianthus annuus*)	Asteraceae	Soil	Pb	5,600	Phytoextraction (shoots)	Koptsik, 2014
Alpine pennygrass (*Thlaspi caerulescens*)	Brassicaceae	Soil	Ni	16,200	Phytoextraction (shoots)	Koptsik, 2014
Black mustard (*Brassica nigra*)	Brassicaceae	Soil	Pb	9,400	Phytoextraction (shoots)	Koptsik, 2014
Alfalfa/lucerne (*Medicago sativa*)	Fabaceae	Soil	Pb	43,300	Phytoextraction (shoots)	Koptsik, 2014

Benefits and limitations of phytoremediation technology

Phytoremediation does not only have advantages but also disadvantages that need to be considered when using this technique. Economically it is cost advantageous, but tracking results can be time-consuming and more of these are elaborated in *Table 3*. Choosing the technique that can recover multiple pollutants at once is tough (Srivastav et al., 2018). The concentration of contaminants and the occurrence of extra toxins should not exceed the tolerance of the plants used. It is not easy to choose plants that effectively remove various impurities. In applying this technique, these limitations and the likelihood of these pollutants entering the food chain should be considered.

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Table 3. *Benefits and limitations of the phytoremediation technology of compounds (Dhanwal et al., 2017; Ashraf et al., 2019)*

Benefits	Limitations
Application	
It can be employed in situ, i.e., on-site removal of contaminants, whether within the soil, water, or groundwater	It is mainly applicable to the top layer of the soil and mine tailings
It is very effective at sites where low amount/toxic contaminants are present	It offers limited applicability to diverse kinds of wastes, especially with high-level toxicity wastes
Cost factor time factor	
It offers lower labor expenditures and reduced cost in operations	Plant deaths may occur in highly toxic sites which could increase the cost of the process
It comes with low investment cost and minimal equipment requirement (constitutes substantial savings)	Mostly there is incomplete removal of contaminants with long-term low performance
Contaminants can be recovered from the plant tissues and offer an opportunity for commercialization	Good cultivation practices and maintenance is required to avoid accidents
Performance	
It can be used for remediating soils that are nonproductive for agricultural purposes	The effectiveness of this remediation process is affected by seasonal factors
It has the potential to treat sites polluted with more than one type of pollutant	A good considerate of the performance and physiological changes of plants in response to different varieties of wastes is needed
Impact on the environment and population	
It is aesthetically pleasing and widely accepted by the public community	There is the possibility of bioaccumulation of pollutants in the food chain
It can reduce erosion of soils, especially thinner inorganic soils	There is the possibility of introduction and spreading of undesirable invasive species of plants
It is nondestructive, nonintrusive, highly biologically active, therefore have a very low environmental impact on soil and water	Proper discarding of plant matter is required with proper risk assessment
It reduces leaching of particulate substance and spreading of toxicants	

Interaction of phytotransformation, phytoextraction, and phytovolatilization technologies

Phytotransformation, otherwise known as phytodegradation, as aforementioned is the decomposition of organic contaminants released by plants: the action of compounds (like enzymes) is produced by plants (Saleem, 2016). There is the degradation of organic pollutants into simple compounds that integrate within plant tissues to encourage plant growth. Restoration of sites by phytotransformation relies on the direct uptake of contaminants via a medium and buildup in the plant (Abdullah et al., 2020). Plants absorb hydrocarbons and extra complex organic molecules and then metabolize or mineralize them through sunlight-driven chemical reactions (Schwitzguébel, 2017). For instance, some enzymes can break down waste from ammunition (explosives), chlorinated solvents, or herbicides. This technology can similarly be used to get rid of noxious waste from petrochemical and storage sites, fuel leakages, leachate stacking,

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and agrochemicals (Kumar et al., 2018). To successfully implement this technique, the transformed compounds accumulated in the plant must be non-toxic or less harmful than the parent compound. Hybrid *Poplars* are proven in research to convert TCE into trichloroethanol, dichloroacetic acid, and trichloroacetic acid which is partially mineralized into CO_2 (Leung et al., 2019; Feng et al., 2017). Here, organic pollutants and heavy metals are being investigated too. Soil conditioning, including chelating agents, may be needed to break the compounds that connect pollutants with soil particles to encourage their uptake by plants (Verma, 2017; Franchi and Petruzzelli, 2017). In some cases, plant transformation is used in cooperation with other restoration techniques or as an improving technique (Mustapha and Lens, 2018).

The discharge of volatile contaminants into the atmosphere via plant leaves refers to phytovolatilization as previously described and is a form of phytotransformation (Limmer and Burken, 2016). Even if the discharge of contaminants into the atmosphere might not attain the full clean-up goal, phytovolatilization treatment may be ideal merits to the long-term properties of the soil and the danger of groundwater contamination (Limmer and Burken, 2016). In literature, phytovolatilization is generally considered beneficial because it usually dilutes atmospheric pollutants and undergoes photochemical degradation. In this technology plants is not just a research area of traditional organic pollutants but also an important research area for other contaminants that occur naturally in the soil and roots of plants. Phytovolatilization of many inorganic and organic contaminations in the natural and agricultural environment is usually observed. Studies by Arya et al. (2017) on the phytovolatilization of pollutants have shed light on ways by which many pollutants are evaporated from plants. In phytovolatilization, certain organic contaminants become volatile in plants and then become evaporated. Compounds having a low octanol-air partition coefficient (log Koa b 5) are reported as been more volatile in plants (Limmer and Burken, 2016). Research conducted by Nwaichi et al. (2015) showed that plant can promote the migration and/or degradation of carbon monoxide in the soil; just like *Fibrristylis littoralis* which is employed for the biological regeneration of agricultural soil polluted by crude oil (up to 92% PAH, shelf life 90 days). Moreover, compounds that cannot be transferred to plants owing to being hydrophobic can still be absolved in plant tissues during particle deposition or air distribution over the soil (Limmer and Burken, 2016). But, the relative prominence of various remediation techniques remains unclear, especially for less studied connections (Limmer and Burken, 2016).

Phytoextraction interactions with phytovolatilization are two methods of removing organic contaminants and detoxification in agricultural soils. It has been reviewed in this paper that phytoextraction removes soil contaminants by concentrating them on parts of the harvested plant. Previous research studies (Izinyon and Seghosime, 2013; Sun et al., 2018) have unraveled *Zucchini* (*Cucurbita pepo*) as a good organic compound storage medium for agricultural land. The mechanistic interaction of phytoremediation involving phytotransformation, phytoextraction, and phytovolatilization technologies is illustrated in *Figure 2.*

Future perspectives in biotechnological enhancement for phytoremediation

Recent studies have offered the physiological and molecular technique of remediation to improve remediation and the cleanup of the agricultural and natural environment. Food waste being used has also helped to control the proper management

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of food/vegetables/fruit. This gives plants the natural ability to absorb, decompose, or concentrate pollutants from soil, water, and air. Contaminants and toxic metals are the core objectives in phytoremediation

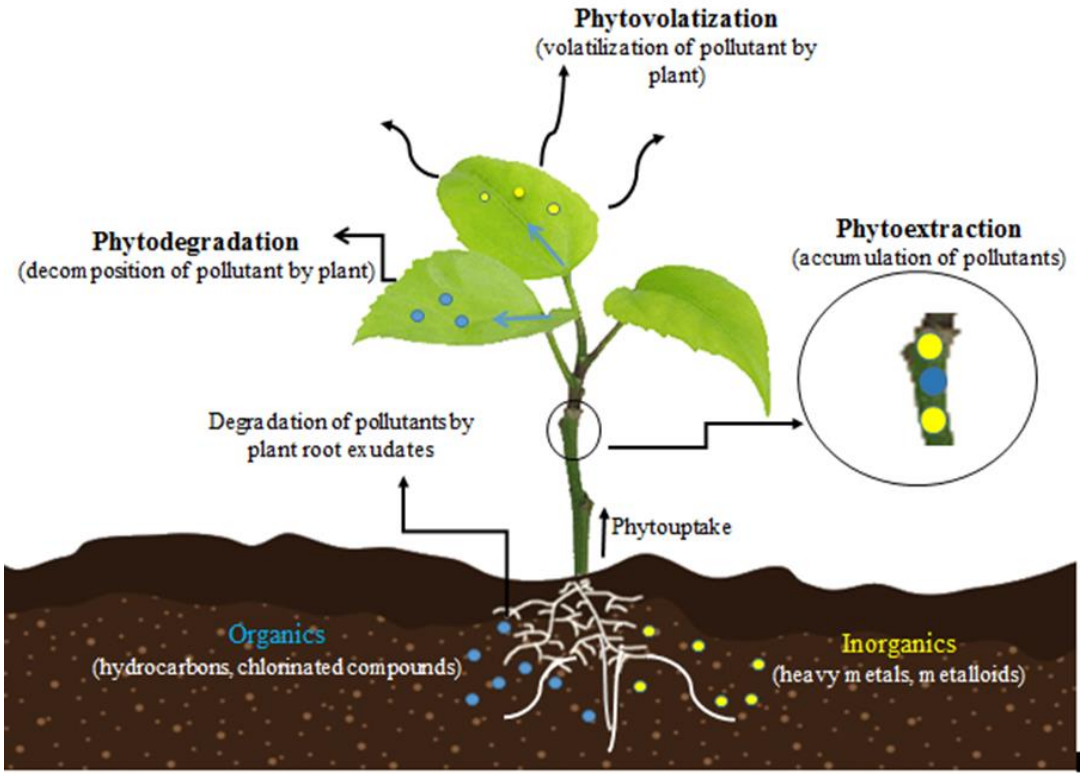

Figure 2. *Mechanistic overview of 3-phase phytoremediation process*

Plant-microbial assisted interaction

Plant and rhizosphere microorganisms are generally employed to remediate contaminated land. Under this mechanism, plants work together to promote soil regeneration through the action of roots and soil microorganisms. Microorganisms utilize root metabolites (secretions), which in turn allow plants to benefit from microbial processing/mineral dissolution (Asemoloye et al., 2019). Plants can absorb pollutants from the soil and transfer them to themselves, while microorganisms mainly decompose pollutants. With the introduction of transgenic techniques, microorganisms and plants can progress to better degrade pollutant. Nonetheless, the use of inherently improved organisms in many countries, different political and moral issues, and related legal power limits the effectiveness of the application. It is well known that microorganisms play key roles in the decomposition of toxic substances as they help in the elimination of undesirable molecules in the environment (Maier and Gentry, 2015). As microbiology evolves, biologists hire the smallest living things on earth to clean and remediate highly oil-contaminated ecosystems. Undoubtedly, microbes are a viable and unused resource for novel environmental biotechnologies (Gaur et al., 2018). Plant growth in a contaminated field often improves soil quality by increasing microbial populations and organism diversity, plus adding organic matter to the soil. Biological

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treatment (mainly microbial decomposition) is a natural way of eradicating contaminants by decomposing contaminated nutrients. Soil microorganisms can be tested aerobically (aerobic biodegradation) or anaerobically (aerobic biodegradation). Organic compounds through biological treatment have been effectively performed on agricultural land under natural conditions (Girma, 2015; Odukkathil and Vasudevan, 2016). Plant in combination with microbes efficiency can be enhanced by mixing, aerating, and adding nutrients, for example, heterotrophic bacteria are employed to clean agricultural soils contaminated with crude oil (Odukkathil and Vasudevan, 2016). An important condition is the presence of microbes with sufficient metabolic potential. Microbes can be native to the infected area (biostimulation) or insulated from the subsequent area (bio-augmentation) (Varjani and Upasani, 2019; Salimizadeh et al., 2018). If these microorganisms are present (Nishiwaki et al., 2018), the biodegradation of contaminants can be sustained by providing sufficient nutrients, moisture, and oxygen. Other variables, for example, salinity are usually out of control. The destructive potential of microorganisms for various pollutants has been previously reported (Mishra et al., 2020; Chen et al., 2019), namely; hydrocarbons, pesticides, and furans for many compounds, including PCBs, PAHs, DDT, and others. The application of the pesticide in the soil can cause problems because according to Abatenh et al. (2017), biological methods are appropriate for conditions where the pesticide is not harmful to microorganisms and plants employed for remediation. Compared to other biological methods, microbiological re-purification is considered to be the most effective method, but high molecular weight hydrocarbons, with low adsorption and solubility, limit their availability to microorganisms (Koshlaf and Ball, 2017). Microbe-assisted phytoremediation can be also carried out by stimulation via inoculation with pesticides degrading microorganisms (Mitton et al., 2012). It is reported that microorganisms can secrete enormous amounts of surfactants and enzymes, hence it is possible for pesticides to undergo extracellular degradation due to these secreted enzymes (Sharma et al., 2018; el Zahar Haichar et al., 2014).

A study by Balcom et al. (2016) reported the potency of microorganisms to metabolize micropollutants such as xenobiotics during the process of wastewater treatment.

These include the concentration of contaminants and chemical nature, the physicochemical properties of the surroundings, and their availability to microorganisms (Koshlaf and Ball, 2017; Bharathi et al., 2017). Due to various factors, monitoring and improving the biological treatment procedure is a challenging system. These factors consist of the microbial populace that can decompose the contaminant and environmental factors (temperature, soil type, pH, nutrient uptake, and oxygen or other electrons) present (Abatenh et al., 2017; Kothe, 2015). The non-existence of information concerning the effects of several environmental factors on the proportion and the extent of biodegradation creates uncertainty.

Nano-phytoremediation enhancement

Nano-phytoremediation involves a combined application of nanotechnology and phytoremediation to decontaminate the environment. Nanotechnology improves the efficacy of phytoremediation. Nanotechnology can provide an environmentally friendly alternative to remediation and management without harming nature. Nanoparticles are used to remediate soil and water contaminated by heavy metals, organic and inorganic pollutants (Srivastav et al., 2018). A variety of plants, bacteria, and fungi in studies

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(Mallikarjunaiah et al., 2020) also enhance their ability to accumulate very high concentrations of metals, which are also recognized as hyperaccumulators. Plant species used in nano-plant engineering cleaning (nano-phytoremediation) technology to decontaminate contaminated soil include the application of phytoremediation techniques (for instance, phytodegradation, phytoextraction, phytostabilization) and nanoparticle.

Due to the high efficiency of pollutants remediated by plants using this technology, numerous reports (Srivastav et al., 2018) have shown that various nanoparticles/nanomaterials significantly detoxify or restore contaminated soil from organic, inorganic, and heavy metal pollutants. Nano zerovalent iron (nZVI), bimetallic nanoparticles (Pd/Fe) and magnetite nanoparticles (nFe3O4), according to the literature (Alonso et al., 2018) may rapidly decompose organic pollutants such as atrazine, chlorpyrifos, trichloroethylene (TCE), pentachlorophenol, pyrene, polychlorinated biphenyls, lindane, 2,4-Dinitrotoluene and ibuprofen from the contaminated soil environment.

Studies by Pillai and Kottekottil (2016) reveals that plant species (*Cymbopogon citratus*) and nanoparticle (nZVIs) can be used to remediate Endosulfan pollutant at an efficiency rate of 86.16 ± 0.09 (%). Souri and coworkers (Souri et al., 2017) also reported that Arsenic (As) pollutant was removed using plant (*Isatis cappadocica*) and nanoparticle (SANPs) at 705 ppm and 1188 ppm accumulate in roots and shoots, respectively. A recent study by Ma and Wang (Ma and Wang, 2018) proves that 82% of Trichloroethylene pollutants can be remediated by a combination of Fullerene (nC60) and *Populus deltoids* from the environment. The success of phytoremediation, or more precisely the phytoextraction, depends on the hyperaccumulator specific to the particular pollutant. Preferably, researchers (Srivastav et al., 2018) suggest that the nano-phytoremediation must be able to accumulate excess contaminants (organic, inorganic, and heavy metals), specifically in the aboveground part (sinking potential). It ought to be a fast-growing plant with high biomass (growth and productivity). The use of selected nanoparticles significantly increased plant growth and treatment with nano augmentations improves the efficiency of phytoremediation and significantly removed pollutants from the soil (Pillai and Kottekottil, 2016).

Despite the many benefits, we obtain from this combination of technology; research on phytoremediation using nanomaterials is very scanty. So far, only microcosm studies (Simonin and Richaume, 2015) have been carried out, so future studies need to use more realistic studies and a better understanding of field practicals is needed.

Genetic engineering enhancement

Genetically improved technology creates effective means of plants and microbes combined with systems of bacterial-plant remediation. Combined cultivation of contaminant sources and individual microbes, whole cells, living plants and plant wastes, food, agriculture, and forestry wastes plays key roles in reducing the content of heavy metals in mining locations to bring a good return to agriculture or the natural environment. Similarly, genetic manipulations of plant hosts in microbial communities can improve phytoremediation capabilities (Tripathi et al., 2020). This includes isolation of bacteria and gene integration that direct the production of specific enzymes thus resulting in degradation of pollutants using the transport of modified organisms and an increase in the number of responsible microbial species in the host system. Biotechnology (genetic engineering) from a previous research study (Dhanwal et al.,

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2017) can be engaged to attach one or more active accumulator genes from higher plants into smaller plants, thereby increasing the final biomass. Novel catalytic enzymes can be used in present biotechnology for soil remediation; new microorganisms can be a replica with precise degrading genes for active hydrocarbon degradation (Asemoloye et al., 2019).

Currently, genetically improved plant species are produced using genetic engineering techniques and are used for a plant in remediating soils contaminated with methyl mercury, a neurotoxic agent (Dhanwal et al., 2017). Conversely, modern biotechnological advances, for instance, the new omic revolution, the emergence of nanotechnology, and the innovation of new catabolic genes, may improve the competence of bioremediation and its applicability in removing soil pollution. To identify plant traits that depend on a particular combination of several genes; for instance, a quantitative map of the trait loci between zinc-tolerant (*Arabidopsis helleri)* and non-zinc-tolerant (*Arabidopsis lyrate)* hybrids identified several genomic regions, and combining them explains why more than 42% of the plant was tolerance to zinc (Nahar et al., 2017). Arabidopsis and Transgenic tobacco are some examples of the genes of transgenic bacteria merB and merA (Dhanwal et al., 2017) that have the potential to eliminate mercury from the soil. Studies have shown that transgenic plants combined with bacterial genes can transform the herbicide Simazine into different non-toxic forms (Azab et al., 2016). Nahar et al. (2017) also reported the *Arabidopsis thaliana* AtACR2 gene (encoding arsenic reductase 2) which was cloned and transformed into the tobacco genome (*Nicotiana tabacum*). The results obtained showed that transgenic tobacco has a higher tolerance to arsenic than wild tobacco.

For genetic manipulation, the use of bacteria is employed to assist plant remediation. Therefore, several strains of bacteria and fungi are known to degrade these compounds (Gilani et al., 2016). In another study, Gilani et al. (2016) identified 14 species of *Pseudomonas* and isolated them from the soil as the organisms that could degrade chlorpyrifos. Many of these soils have been contaminated with a mixture of pollutants let us say pesticides (Barchanska et al., 2019). Therefore, preserving the steadiness of genes transferred to a host is a very difficult task (Tripathi et al., 2020), and there are signs that recombinant organisms with certain characters often lose their efficacy to degrade some contaminants.

Improvements in omics technology provide opportunities for isolating and cultivating such useful microorganisms and studying non-cultivable organisms. The next-generation of sequencing technology can provide an ideal method for analyzing microbial communities (Van Dorst et al., 2016). Although the higher nutrient content in the artificial medium usually restricts oligotrophic bacteria (i.e microorganisms that require only a small amount of nutrients) and prefers copiotrophs bacteria (i.e. microorganisms that grow under nutrient-rich conditions), therefore, it is similar to the new cultivation technology of the natural environment is closing the gap between culturally dependent methods and culturally independent techniques (Van Dorst et al., 2016; Ferrari et al., 2011).

Conclusion

Biotechnological integration may provide a crucial step towards the development viability of the remediation of the ecosystem. However, there is a need for more research into understanding the mechanisms underlying plant-microbial interactions of

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the rhizosphere. Further exploration is also required to develop the kinetics and simulations for synergistic degradation procedures and their field applications. Additionally, developments in bioremediation are expected to focus on ways to provide conditions that promote plant growth and microbial activity in the soil to enable bioavailability and degradation of organic and inorganic compounds.

Since phytoremediation research is actually interdisciplinary, we recommend the following:

a) Plant breeders, biotechnologists, physiologists, agronomists, soil scientists, biochemists, and environmentalists must work together to produce robust methods to develop genetically modified plants and enhance the potential of existing plants for better contaminant control.
b) In transgenic research on phytoremediation, future research should be feasible to solve the problem of mixed pollution that occurs in many contaminated locations to remove mixed or complex pollutants.
c) Future research should also focus on making better use of metabolic diversity, not only for plants, but also for a better understanding of the complex interactions between pollutants in the rhizosphere, plant roots, soil, and microorganisms (bacteria and mycorrhizae).

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IMPROVEMENT OF QUALITY AND YIELD OF GREENHOUSE TOMATO (*SOLANUM LYCOPERSICUM* L.) PLANTS BY MICRO-SPRINKLER IRRIGATION UNDER PLASTIC FILM

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Abstract. This study clarified the applicability of micro-sprinkler irrigation under plastic film (MSPF) in a greenhouse and finding the optimal micropores group spacing and capillary arrangement density. The effects of different micropores group spacing (L1: 30 cm micropores group spacing, and L2: 50 cm micropores group spacing) and capillary arrangement density (C1: one pipe for one row, C2: one pipe for two rows, and C3: one pipe for three rows) with the MSPF on the growth, quality, yield (Y), and water use efficiency (WUE) of tomato were studied. A completely randomized trial design was used, and drip irrigation under plastic film and micro-sprinkler irrigation were used as controls. The results showed that MSPF in a greenhouse can lead to saving water, increase yield, and improve crop quality. Compared to 50 cm micropores group spacing, 30 cm was better for tomato growth and also increased fruit quality, yield, and WUE. Considering our results comprehensively, the combination of one pipe for two rows under a micropores group spacing of 30 cm is recommended as technical parameters for greenhouse tomato cultivation under MSPF in arid and semi-arid sandy loam soils.

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Keywords: *micropores group spacing, capillary arrangement density, quality, water use efficiency, comprehensive benefit evaluation*

Abberviations:

No	Abbreviations	Full name
1	L	Micropores group spacing
2	C	Capillary arrangement density
3	PH	Plant height
4	SD	Stem diameter
5	LAI	Leaf area indices
6	SFW	Weight of single fruit
7	VD	Vertical diameters
8	TD	Transverse diameters
9	TSS	Total soluble solids content
10	TSU	Total soluble sugar
11	SSC	Soluble sugar content
12	TA	Titratable acids
13	SAR	Sugar/acid ratio
14	SP	Soluble protein
15	VC	Vitamin C
16	LY	Lycopene
17	Y	Yield
18	WUE	Water use efficiency
19	CRITIC	Criteria importance though intercrieria correlation
20	TOPSIS	Technique for order preference by similarity to an ideal solution

Introduction

The development of facility agriculture has provided a powerful guarantee for vegetable production in arid and semi-arid sandy loam soils of northwest China. However, the irrigation water used for facilitating agriculture in this region mainly originates from groundwater, and the resulting groundwater resource exploitation has exacerbated the water crisis in these arid and semi-arid areas; therefore, it is urgently required to mitigate the excessive use of irrigation water in this region (Du et al., 2014). Therefore, the realization of a more efficient utilization of water resources has become the current research hotspot in this region. As a common irrigation method for the greenhouse tomato (*Solanum Lycopersicum* L.) in this area, drip irrigation offers the advantages of water-saving, fertilizer-saving, and labor-saving. It has been widely used on tomato, pepper, melon, and other crops (Liu et al., 2012; Zhang et al., 2017; Wang et al., 2018a). Because of the existence of sediment, chemical precipitates, or biomass in the irrigation water body, the emitters in the drip irrigation system clog easily, resulting in decreased irrigation uniformity, accompanied by decreased crop yield and an increased cost (Yu et al., 2010; Feng et al., 2018). Drip irrigation belongs to local irrigation, and the soil wetting body per unit area of tillage layer is limited, which greatly hinders the development of the crop root system (Michelakis et al., 1993; Zhou et al., 2017).

As one of the many development forms of drip irrigation, micro-sprinkler irrigation is an irrigation form, where sprinkler (micro) pores are arranged in groups on the wall of a thin-walled drip irrigation plastic pipe (flat strip after coiling) (Zhang et al., 2009). The emitter energy dissipation structure is removed. Under identical working pressures, the flow velocity of micro-sprinkler irrigation is about 15 times larger than that of labyrinth drip irrigation. This results in strong sediment-carrying capacity and anti-clogging performance of the micro-sprinkler irrigation. This technique can solve the clogging

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problem of drip irrigation emitters (Yu et al., 2010; Feng et al., 2018). At the same time, the flow rate of a single group of micro-sprinklers is much higher than that of drip irrigation, which easily increases the ratio of the horizontal and vertical migration distance of soil moisture peaks, and improves the uniformity of the soil wetting body and the water per unit area of tillage layer (Ould et al., 2001; Del et al., 2020). Micro-sprinkler irrigation offers the advantages of decreasing the restriction of horizontal root growth and short irrigation duration (Man et al., 2014; Li et al., 2019a). Therefore, micro-sprinkler irrigation has been used for winter wheat, summer corn, lawn, seedlings, and other crops, where it has achieved good results (Man et al., 2014; Baram et al., 2018; Fletcher et al., 2018; Li et al., 2018a). However, the shape, area, and uniformity of soil wetting in a micro-sprinkler irrigated area are affected by the wind speed. In addition, problems, such as difficulty to control weeds and high damage rate of micro sprinklers currently obstructs micro-sprinkler irrigation treatment (Man et al., 2014, 2017; Li et al., 2019b).

The development of facility agriculture provides a good environment for the application of micro-sprinkler irrigation, since it offers flat land, no wind indoors, and short capillary laying distance (Tsitsimpelis et al., 2016). However, the space of facility agriculture is relatively closed, and micro-sprinkler irrigation water spray atomization easily increases air humidity. High temperature and high humidity have been shown to facilitate crop diseases and insect pests (Gómez-Rodríguez et al., 2003; Er and Gökçe, 2004; Camara et al., 2017), resulting in less use of micro-sprinkler irrigation in facility agriculture. Plastic film mulching provides a solution for the application of micro-sprinkler irrigation in facility agriculture. Plastic film mulching can restrain the water jet of micro-sprinkler irrigation, decrease spray water atomization and ineffective water evaporation, and help to improve irrigation water use efficiency (WUE) (Massatbayev et al., 2016; Wang et al., 2019a). Therefore, the combination of micro-sprinkling irrigation and film mulching technology, and the use of multiple groups of small micro-pores beneath the film can compensate for the shortcomings of the micro-spray pipes used in the greenhouse. This technique is called micro-sprinkler irrigation under plastic film (MSPF, *Fig. 1*). The exploration of MSPF is of great significance to enrich the greenhouse micro-irrigation technology system, expanded the scope of the application of micro-sprinkler irrigation, and decrease the water needed for crops, and increases both the yield and quality of crops.

Figure 1. *Micro-sprinkler irrigation under plastic film (MSPF)*

The tomato is one of the main vegetables grown in facility agriculture in the arid to semi-arid areas of northwest China. The tomato has rich nutritional value and is the main cash crop with which local farmers increase their income (Malherbe and Marais, 2015;

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Liu et al., 2019). The greenhouse tomato in this area belongs to sparse planting crops. Based on its unique planting structure pattern, the selection and arrangement of orifice flow directly changes the soil water distribution and indirectly affects the WUE and investment cost of tomato plants. The orifice flow position on the capillary pipe (i.e., dripper spacing) and the orifice flow position between the capillary pipe (i.e., capillary arrangement density) are two aspects of the orifice flow position. Therefore, for the sustainable development of the greenhouse tomato industry in this region, it is important to determine the optimal position of the orifice flow position on the capillary pipe and the orifice flow position between the capillary pipe. These values will enable the best balance between increasing production and improving quality while reducing the use of irrigation water. Previous studies on drip irrigation showed that the confluence time of the wetting peaks at two adjacent dippers was shorter at identical dipper flow rate. Moreover, the surface wetting shape of soil moisture under two adjacent emitters was approximately rectangular when the emitter spacing decreased from 80 to 30 cm (Ould et al., 2001; Elmaloglou and Diamantopoulos, 2010). The more uniform the wetting body was, the more irrigation water use could be decreased and irrigation WUE increased (Xu et al., 2012; Sui et al., 2018). When the dipper spacing increased from 15 to 30 cm, the yield of onions first increased and then decreased (Enciso et al., 2007). The smaller the capillary arrangement density is, the more uniform the horizontal water content distribution will be, and the higher the crop dry matter accumulation and yield will be. However, the higher capillary arrangement density easily increases the investment cost. This is also not conducive for the improvement of vegetable flavor, nutrition, and WUE (Bozkurt et al., 2006; Chen et al., 2015; Zhou et al., 2017; Liu et al., 2019; Lv et al., 2019; Yang et al., 2019).

At present, relevant research mainly focused on the orifice flow position on the capillary pipe (i.e., dripper spacing) and the orifice flow position between the capillary pipe (i.e., capillary arrangement density) in the drip irrigation capillary with small flow. However, few studies investigated the influence of the change of the orifice flow position on the capillary pipe (i.e., micropores group spacing) and orifice flow position between the capillary pipe (i.e., capillary arrangement density) on greenhouse crops using MSPF. In production practice, yield is the goal pursued by farmers, quality is the demand of users, and WUE is the core of the effective utilization of agricultural water resources (Wang et al., 2019b). Sufficient soil moisture can increase yield, but fruit quality and crop WUE will both decrease, while profit loss will increase when too much water is used (Luo and Li, 2018). It remains difficult to optimize quality, yield, and WUE. The technique for order preference by similarity to an ideal solution (TOPSIS) method provides a solution for multi-objective decision-making optimization. It has been widely used to comprehensively evaluate both the advantages and disadvantages of the tomato irrigation system. Luo and Li (2018) takes tomato yield, quality and water use efficiency as evaluation indexes, uses principal component standard deviation method to obtain each index weight, and establishes a comprehensive evaluation model based on weighted TOPSIS combination to select the best irrigation mode and irrigation quantity. Liu et al. (2019) used the yield, quality, and WUE of tomato as evaluation index, where the weight of each index was made equal to 1 by an artificial method. A comprehensive evaluation model based on weighted TOPSIS combination was established to optimize the best irrigation frequency and irrigation quantity. At present, the TOPSIS method is relatively rarely applied for comprehensive benefit evaluations of MSPF.

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Therefore, compared with drip irrigation and micro-sprinkler irrigation, this study explored the applicability of MSPF in the greenhouse. The responses of tomato growth, quality, yield, and WUE to different micropores group spacing and capillary arrangement density were investigated. In addition, a comprehensive benefit evaluation model of greenhouse tomato quality, yield, and WUE was established by the TOPSIS method to optimize different treatments. The combination model of micropore spacing and capillary arrangement density was obtained for the best quality, yield, and WUE of tomato plants under MSPF. This paper enriched the data on tomato micro-irrigation technology systems in arid and semi-arid sandy loam soil of facility agriculture. Data support and a theoretical basis are provided for water-saving, yield increases, and improved quality of facility agricultural crops in this area by using greenhouse experiments and multi-objective optimization data analysis.

Materials and methods

Experimental site and management

The experiment was conducted from March 27, 2019, to July 23, 2019, in a greenhouse at the Modern Agricultural Science and Technology Exhibition Center of Xi'an City, Shaanxi Province, China (108°52°E, 34°03°N). It has a warm temperate semi-humid continental monsoon climate. The soil is sandy loam, and the mass fractions of sand, silt, and clay are 63.9%, 29.63%, and 6.47%, respectively. The average bulk density of the 1.0 m soil layer was 1.48 g/cm^3, the water holding capacity of field weight was 27.40%, and the depth of groundwater table on the site exceeded 30 m. The content of organic matter, total phosphorus (P), total potassium (K), total nitrogen, available nitrogen, available P, and available K in the plough layer before sowing were 15.53 g/kg, 10.12 g/kg, 2.01 g/kg, 1.36 g/kg, 70.45 mg/kg, 112 mg/kg, and 85.23 mg/kg, respectively. The irrigation water originated from groundwater, the pH of which was 6.8, the chemical oxygen demand (COD) was 53.2 mg/L, the anionic surfactant content was 3.2 mg/L, and the chloride content was 0.48 mg/L.

The greenhouse (85 m long and 15 m wide) was oriented from north to south. The tomato variety 'Jingfan 401' (Jingyan Yinong Seed Sci-tech Co. Ltd., Beijing, China), with a 50 cm row spacing and a 40 cm plant spacing, was planted on a ridge. The length of the ridge was 3.4 m and the width was 1.2 m. The irrigation plot is shown in *Fig. 2*. The distance between each plot was 4 m; one 1.0-m deep building waterproof film made up of styrene-butadiene-styrene block copolymer was buried in the middle to prevent the horizontal infiltration and movement of soil moisture, thus avoiding their effect on other plot experiments. Tomato plants were topped when the four-eared fruit were retained and the field management measures, such as fertilisation, irrigation, and pesticides, were kept similar in all treatments. The source of irrigation water in the region was groundwater. To ensure the survival of seedlings on the day of planting, the irrigation was unified with reference to the local tomato planting experience. Spring tomatoes were planted on 27 March 2019 and irrigation treatment was initiated on 4 April 2019. The irrigation treatment was continued until 15 July 2019 and tomatoes were completely harvested on 25 July 2019. Autumn tomatoes were planted on 23 August 2019, irrigation treatment was continued between 30 August 2019 and 17 January 2020, and tomatoes were complete harvested on 30 January 2020. The micro-sprinkler pipe of MSPF (Hebei Plentirain Irrigation Equipment Technology Co., Ltd., Hebei, China) adopts three thin-walled oblique micropores with a diameter of 32 mm and a micropore diameter of 0.8 mm.

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The micropores group spacing is shown in the experimental design (*Table 1* and *Fig. 3*). Drip irrigation under mulch (CK1, Hebei Plentirain Irrigation Equipment Technology Co., Ltd., Hebei, China) with thin-walled labyrinth tooth channel was selected as control. The geometric parameters of the channel were 54.3 × 1.1 × 0.83 mm, the distance between emitters was 30 cm, and the emitter flow rate was 2 L/h. Micro-sprinkler irrigation (CK2, Hebei Plentirain Irrigation Equipment Technology Co. Ltd, Hebei, China) adopted three thin-walled oblique micropore pipes with a diameter of 32 mm and a micropore diameter of 0.8 mm an was also used as control. The micropores group spacing was 10 cm.

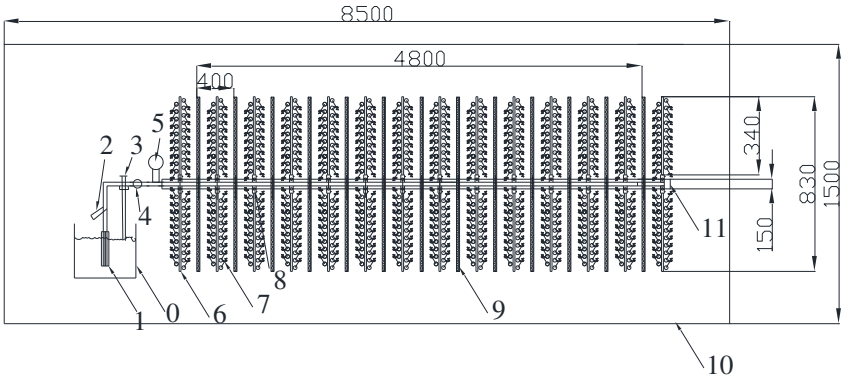

Figure 2. *Schematic diagram of greenhouse layout(cm). Note: 0 - water tank; 1 - the pump (WQD10-12-0.75S, PEOPLE PUMB, Crop., Shanghai, China); 2 - filter; 3 - backwater valve; 4 - electromagnetic flowmeter; 5 - pressure gauge; 6 - capillary; 7 - tomato; 8 - capillary valve; 9 - plastic screens; 10 - greenhouse boundaries; 11 - pathway*

Table 1. *Experimental factor and design*

No.	Treatment	Irrigation method	Micropores group spacing cm	Capillary arrangement density	Irrigation amount mm
1	L1C1	MSPF	30	one pipe for one row	353.30
2	L1C2		30	one pipe for two rows	
3	L1C3		30	one pipe for three rows	
4	L2C1		50	one pipe for one row	
5	L2C2		50	one pipe for two rows	
6	L2C3		50	one pipe for three rows	
7	CK1	Drip irrigation under plastic film	30	one pipe for two rows	
8	CK2	Micro-sprinkler irrigation	10	one pipe for two rows	

Experimental design

Two factors were set up in this study: micropores group spacing L (*Fig. 3*) and capillary arrangement density C (*Fig. 4*). Of these, the micropores group spacing (L) used two levels: 30 cm (L1) and 50 cm (L2); the capillary arrangement density (C) used three levels: one pipe for one row (one capillary pipe irrigated one crop, C1), one pipe for two rows (one capillary pipe irrigated two rows of crops, C2), one pipe for three rows (one capillary pipe irrigated three rows of crops, C3). One pipe for two rows were used for

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both CK1 and CK2 control treatments. A total of eight treatments were implemented, each of which was repeated three times, for total of 24 test areas (*Table 1*).

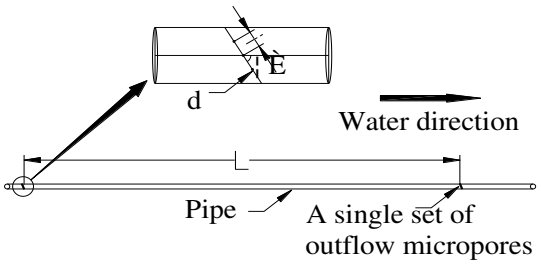

Figure 3. *Schematic diagram of micropores group (inside) spacing structure parameters. Note: diameter of micropore is d=0.7 mm; The internal spacing of the micropores group spacing was i =0.4 cm; The Angle of micropores is =68°; The micropores group spacing is L*

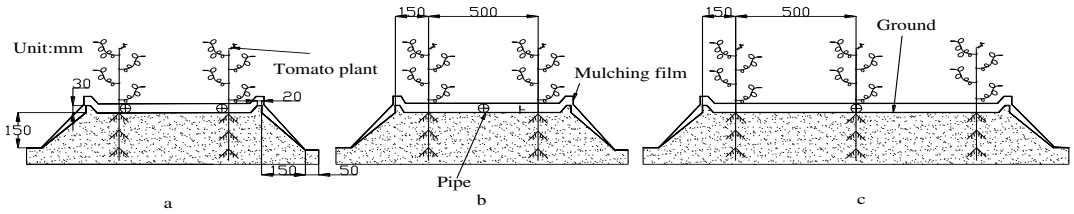

Figure 4. *Schematic diagram of capillary arrangement density (a: one pipe for one row; b: one pipe for two rows; c: one pipe for three rows)*

The irrigation amount was controlled on the basis of the cumulative evaporation from a 20-cm diameter standard pan (E_{pan}, DY.AM3, Weifang Dayu Hydrology Technology Co., Ltd., Shandong, China) following Dinc et al. (2018) and Liu et al. (2013). The evaporation amount was measured at 08:00 am every 5 d. The irrigation amount was evaluated after the measurement. The W of irrigation quota was calculated according to Formula (*Eq.1*), and the irrigation times and amounts were recorded (*Fig. 5*).

$$W = A \times E_{pan} \times k_{cp} \quad \text{(Eq.1)}$$

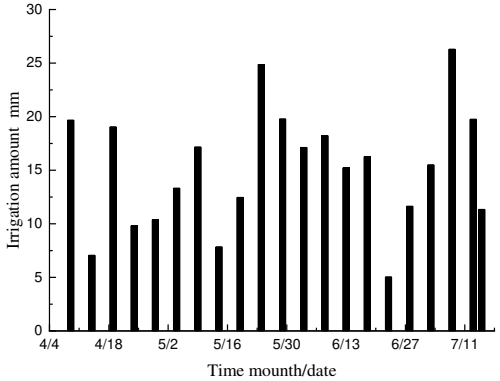

Figure 5. *Irrigation records*

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In the formula: E_{pan} represents the evaporation within the interval of two irrigation, basing on the cumulative evaporation from a 20 cm diameter pan (mm); A represents the capillary control area (mm), and k_{cp} represents the crop- pan coefficient. In this paper, adopting adequate irrigation mode, the crop- pan coefficient of k_{cp} is 1.0 (Zhu et al., 2020).

Measurements and computational methods

Growth

The growth-related indexes were measured at 26, 51, 73, and 110 days after planting, and three plants were randomly selected from each plot. Plant height (PH) was measured with a ruler (Ma and Upadhyaya, 2016); the stem diameter (SD) was measured with electronic Vernier calipers at the base stem (Ma and Upadhyaya, 2016), and the leaf area index (LAI) was measured by AccuPARLP-80 canopy analyzer (Decagon Devices, Inc., Pullman, Wash. USA).

Three tomato plants were randomly selected in each plot, the stem of the plant was assumed as the center, and a hole was dug with a straight diameter of about 0.2 m and a depth of about 0.4 m to obtain the root system of the plant. Rhizosphere soil was carefully shaken off and the residual root system was slowly washed to remove the soil, using a weak water flow. Then, the root system and soil were placed on a 100-mesh steel screen during flushing to minimize root loss. After washing, the root system was dried in an oven at 105 °C for 15 min, followed by drying at 75 °C to constant weight. Finally, the dry matter mass of the root system was obtained.

Quality

Fruit shape, flavor, and nutritional indexes are the main three factors of tomato quality. Three tomato fruits were randomly selected in each plot when the tomatoes were ripe at the first stage. Individual fruits were homogenized to determine the fruit flavor and nutritional indexes.

Shape indices: The weight of single fruit (SFW) was measured using precision 0.01 g electronic scale. Vertical diameters (VD) and transverse diameters (TD) of the fruit were measured using vernier calipers.

Flavor indices: The total soluble solids (TSS) was measured using a hand-held refractometer with automatic temperature compensation (PR-32α Atago, Tokyo, Japan); the total soluble sugar (TSU) was measured using anthrone method (Decruyenaere et al., 2012). The soluble solid content (SSC) was measured using anthrone method (Liu et al., 2019). Titratable acids (TA) were determined by diluting an aliquot of the blended fruit and titrating against 0.1 mol/L NaOH using phenolpthalein as an indicator. Titratable acids (TA) content was determined by diluting an aliquot of the blended fruit and titrating against 0.1 mol/L NaOH by using phenolphthalein as an indicator. TAs were estimated by mL aliquot of blended fruit (Gould, 1992). Sugar/acid ratio (SAR) was determined by dividing the soluble sugar concentration by titratable acid.

Nutritional indices: the soluble protein (SP) content was measured using Coomassie Brilliant Blue assay (Liu et al., 2019). The vitamin C (VC) was calculated by the classical titration method with 2,6-dichlorophenol indophenols sodium salt solutions (Liu et al., 2019). Lycopene (LY) was extracted with 2% dichloromethane and petroleum as solvents to enhance its solubility and the absorption at 502 nm was measured using ultraviolet spectrophotometer (Cefali et al., 2015).

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Yield and water use efficiency

During the maturation period, 4 tomatoes were randomly selected from each plot and the quality of mature tomatoes was measured using an electronic scale. After obtaining yield per plant, the yield per hectare was derived.

Time-domain reflectometry soil moisture sensor (TRIME-PICO-IPH, IMKO, Inc., Ettlingen, Germany) was used to measure the soil volume moisture content of 0–10, 10–20, 20–30, 30–40, 40–50, 50–60, 60–70, and 70–80-cm soil layers. It was measured once before and after each growth period. Two monitoring points were selected in each district as shown in *Figure 6* (monitoring point 1 was arranged at the outflow micropore; monitoring points 2 was arranged at distance m between the two groups of micropore in the vertical flow direction, where m = 25 cm). Water consumption (ET_a) and crop water use efficiency (WUE) were calculated using formula (*Eq.2*) and (*Eq.3*), respectively (Du et al., 2017).

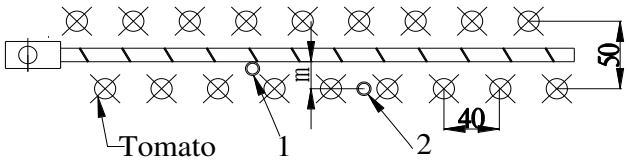

Figure 6. *Schematic diagram of capillary and TRIME pipe arrangement (unit: cm)*

$$ET_a = I \pm 1000 \times H \times (\theta_{t1} - \theta_{t2}) \quad \text{(Eq.2)}$$

In the formula, ET_a represents crop water consumption during growth period (mm); I represents the irrigation quota of crop growth period (mm); H represents the depth of the wetting layer with plan (H = 0.8 m); θ_{t1} and θ_{t2} represent 80-cm average soil volumetric water content at times $t1$ and $t2$ (cm^3/cm^3), respectively.

$$WUE = Y/ET_a \quad \text{(Eq.3)}$$

In the formula, WUE indicates crop water use efficiency (kg/m^3); Y indicates crop grain yield (kg/hm^2).

Standardization and homogenization of raw data

In order to eliminate the influence of different evaluation indices dimensions, first standardize the data, and at the same time, in order to ensure that the evaluation indices has the same direction, it is also necessary to carry out the same chemotactic treatment of the data, reference to "Effect of planting row spacing and irrigation amount on comprehensive quality of short-season cultivation tomato in solar greenhouse in northwest china" (Wu et al., 2018), which is convenient for the selection of reference vectors when constructing the comprehensive benefit evaluation model by TOPSIS method.

Data analysis

Data was tested for normal distribution, which was followed by evaluation of the differences between groups, using SPSS22.0 (IBM Crop., Armonk, NY, USA). F test was

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used and the significant level was set to $P < 0.05$. OriginPro2019 (Origin Lab Corporation, Northampton, MA, USA) was used to draw plots. Except for special annotations, the data are visualized by showing averages ± standard deviations.

Results

Effects of different treatments on plant height, stem diameter, and leaf area indexes on plant growth of tomato

Table 2 shows the effects of different treatments on PH, SD, and LAI of tomato. With progressing tomato growth, PH and LAI increased first and then decreased, while SD increased. The PH, SD, and LAI of L1C2 were higher than those of CK1 and CK2 by 7.34% and 0.13%, 8.32% and 4.69%, 22.95% and 2.76%, respectively. With decreasing capillary arrangement density, PH, SD, and LAI of tomato decreased, and no significant difference was found between C1 and C2 ($P > 0.05$). C1 and C2 were significantly higher than C3 by about 10.64% and 7.15%, 8.35% and 7.10%, 24.97% and 17.14% ($P < 0.05$), respectively. The PH, SD, and LAI of 30 cm tomato were 1.04, 1.03, and 1.14 times that of the 50 cm micropores group spacing.

Table 2. *Experimental factor and design Effects of different treatments on PH, SD and LAI*

Indices	Treatment	26 days after planting	51 days after planting	73 days after planting	110 days after planting
PH	L1C1	41.96±3.62a	105.12±12.5a	137.2±7.03a	137.13±9.01a
	L1C2	41.72±2.05a	102.47±12.84a	135.17±13.79ab	135.47±14.85a
	L1C3	37.18±2.98bc	95.54±25.77ab	127.08±18.08ab	128.53±14.58ab
	L2C1	40.54±2.24abc	103.09±14.44a	135.28±14.51ab	135.88±12.12ab
	L2C2	38.39±4.38abc	98.13±8.89ab	128.62±8.47ab	129.86±6.36ab
	L2C3	36.43±4.79c	84.49±10.61b	123.79±13.8b	122.73±13.73b
	CK1	37.74±6.66abc	87.09±13.41b	130.88±4.8ab	130.74±3.63ab
	CK2	41.42±3.94ab	103.27±8.84a	134.51±8.44ab	135.08±5.94a
SD	L1C1	8.71±1.13a	10.25±1.28a	12.02±1.21a	12.5±1.18ab
	L1C2	8.93±1.22a	10.65±1.05a	12.32±0.25a	12.88±0.91a
	L1C3	8.29±1.67a	9.9±1.75a	11.78±1.03a	11.54±1.4b
	L2C1	8.84±1.74a	10.5±1.14a	12.46±0.75a	12.31±0.89ab
	L2C2	8.69±1.19a	9.82±1.11a	11.66±0.55a	11.64±0.59b
	L2C3	8.16±1.84a	9.41±1.47a	11.49±0.99a	10.28±0.93b
	CK1	8.4±1.75a	9.42±0.86a	11.68±1.43a	11.83±0.48b
	CK2	8.48±1.58a	10.12±0.85a	11.89±0.87a	12.29±0.81ab
LAI	L1C1	0.97±0.09ab	2.89±0.26a	8.05±0.69a	7.5±0.53a
	L1C2	1.0±0.13a	2.85±0.25ab	8.07±1.04a	7.46±0.54a
	L1C3	0.93±0.12ab	2.58±0.29cd	6.59±2.12cd	6.43±1.57bc
	L2C1	0.89±0.1bc	2.66±0.25bc	7.61±0.67abc	7.36±1.1a
	L2C2	0.82±0.05c	2.4±0.12d	7±1.38abc	5.97±0.76c
	L2C3	0.84±0.02c	2.34±0.06e	5.66±0.82d	4.98±0.27d
	CK1	0.81±0.05c	2.33±0.17e	6.72±1.3bcd	5.9±0.55c
	CK2	0.95±0.08ab	2.89±0.21a	7.92±1.09ab	7.09±1.17ab

Note: PH, Plant height; SD, Stem diameter; LAI, Leaf area indexes, the data are all average ±standard deviation in the chart, different letters in the same column meant significant difference at 0.05 level, the same as blow

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Effects of different treatments on tomato quality

In terms of fruit shape indexes (*Table 3*), compared with CK1 and CK2, SFW, VD, and TD of L1C2 increased by 28.38% and 5.66%, 11.70% and 4.69%, 14.43% and 1.67%, respectively. Under the same irrigation amount, with decreasing capillary arrangement density, the SFW, VD, and TD of tomato followed a decreasing trend, and decreased by about 34.10%, 16.38%, and 9.98%, respectively. The SFW, VD, and TD in the 30 cm micropores group spacing were 1.20, 1.04, and 1.10 times higher than that of the 50 cm micropores group spacing.

In terms of fruit flavor indexes (*Table 3*), compared with CK1, the TSS, TSU, and SAR of L1C2 decreased by 0.32%, 3.30%, and 0.31%, respectively. Compared with CK2, the TSS, TSU, and SAR of L1C2 increased by 20.00%, 12.35%, and 26.63%, respectively. The spacing of micropores significantly affected the TSS of tomato ($P < 0.05$), and the capillary arrangement density significantly affected both TSS and SAR ($P < 0.05$). In the micropores group with 30 cm spacing, TSS, TSU, and SAR were 1.06, 1.04, and 1.02 times higher than that of 50 cm spacing. Under the same irrigation amount, TSS, TSU, and SAR increased by 15.38%, 10.30%, and 23.75%, respectively, with decreasing capillary arrangement density.

In terms of fruit nutritional indexes (*Table 3*), compared with CK1, L1C2 tomato flavor indices SP, VC, and LY decreased by 0.12%, 0.08%, and 2.67%, respectively; compared with CK2, these increased by 0.02%, 18.39%, and 9.40%, respectively. The micropores group spacing significantly affected the VC of tomato ($P < 0.05$), and the capillary arrangement density significantly affected the SP, VC, and LY ($P < 0.05$). In the 30 cm micropores group spacing, SP, VC, and LY were 1.05, 1.13, and 0.99 times as much as that of the 50 cm spacing. Under the same irrigation amount, the SP, VC, and LY increased by 8.72%, 16.07%, and 13.28%, respectively, with decreasing capillary arrangement density.

Effects of different treatments on water consumption, yield, and water use efficiency of tomato

Compared with CK1, the water consumption and yield of L1C2 was significantly higher than 8.46%, 19.39% ($P < 0.05$), and there was no significant difference in the improvement of WUE (10.03%, $P > 0.05$; *Fig. 7*). Compared with CK2, there was no significant difference in the increase of L1C2 yield (4.69%, $P > 0.05$); however, the water consumption was significantly decreased by 8.01% ($P < 0.05$) and WUE was significantly increased by 13.87% ($P < 0.05$). Compared with L1C1, L1C3, L2C1, L2C2, and L2C3, the yield and WUE of L1C2 increased by 0.99% and 0.99%, 47.18% and 47.18%, 13.69% and 13.69%, 24.74% and 24.74%, as well as 52.08% and 52.08%, respectively. At the same time, the micropores group spacing and capillary arrangement density significantly affected yield and WUE ($P < 0.05$), and the interaction of both factors had significant effects on WUE ($P < 0.05$). With decreasing capillary arrangement density, the water consumption, yield, and WUE of tomato decreased. The yield and WUE of C1 and C2 were significantly higher than those of C3 by about 39.85% and 34.76%, 32.10% and 31.94% ($P < 0.05$); however, there was no significant difference in yield and WUE between C1 and C2 ($P > 0.05$). The water consumption, yield, and WUE of the 30 cm micropores group spacing were 1.01, 1.14, and 1.13 times higher than that of the 50 cm micropores group spacing.

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Table 3. *Effects of different treatments on the quality of greenhouse tomato*

Treatment	Shape indices			Flavor indices			Nutritional indices		
	SFW g	VD mm	TD mm	TSS %	TSU%	SAR	SP mg/g	VC mg/g	LY %
L1C1	138.56±21.99a	56.7±7.24a	64.28±8.36a	4.14±0.39d	6.97±1.34a	6.62±0.96bc	5.19±0.67ab	15.98±2.28bc	54.36±10.32b
L1C2	139.42±25.75a	57.86±4.17a	66.28±5.06a	5.17±0.66a	7.75±1.6a	8.2±1.61ab	5.39±0.39ab	19.19±2.44a	62.48±8.15ab
L1C3	106.59±43.91ab	52.09±3.88ab	58.93±10.33ab	4.92±0.47ab	7.87±1.47a	8.54±2.03a	5.63±0.48a	19.1±2.63a	65.58±11.39a
L2C1	119.65±31.97ab	58.88±7.19a	59.28±5.14ab	4.07±0.53d	6.84±1.32a	6.34±2.15c	4.89±0.56b	14.63±1.46c	58.75±10.13ab
L2C2	113.92±45.17ab	53.21±7.72ab	59.58±7.86ab	4.57±0.13bc	7.39±1.25a	8.19±1.71ab	5.15±0.47ab	16.22±1.55bc	60.48±8.59ab
L2C3	85.96±55.67b	47.23±11.69b	53.52±11.13ab	4.79±0.17ab	7.53±1.06a	8.45±1.2a	5.42±0.51ab	17.38±2.89ab	64.85±9.05a
CK1	108.6±49.35ab	51.8±10.09ab	57.92±11.04b	5.19±0.56a	8.01±1.7a	8.23±0.47ab	5.39±0.51aab	19.21±2.07a	64.15±9.75ab
CK2	131.95±33.22a	55.26±6.05a	65.19±7.21a	4.31±0.38cd	6.9±1.7a	6.48±1.8c	5.39±0.25ab	16.21±1.91bc	57.11±7.26ab
F-value									
L	1.4353ns（2.9）	6.368*（11.7）	0.618ns（1.3）	5.112*(9.6)	0.569ns(1.2)	0.079ns(0.2)	3.022ns(5.9)	10.651**(18.2)	0.044ns(0.1)
C	5.722**（19.3）	3.355*（12.3）	15.539**（39.3）	17.943**(42.8)	1.787ns(6.9)	7.698**(24.3)	3.833*(13.8)	8.513**(26.2)	3.633*(13.1)
L×C	1.301ns（5.1）	0.950ns（0.2）	11.741**（32.9）	2.044ns(7.8)	0.036ns(0.2)	0.031ns(0.1)	0.033ns(0.1)	0.628ns(2.5)	0.551ns(2.2)

Notes: SFW, Single fruit weight; VH, Vertical height; TD, Transverse diameter; TSS, Total soluble solids; TSU, Total soluble sugar; SAR, Sugar / acid content ratio; SP, Soluble protein; Vc, Vitamin C; LY, Lycopene; the bracketed number is total variance relative contribution %, *: $P < 0.05$; **: $P < 0.01$; ns: $P > 0.05$

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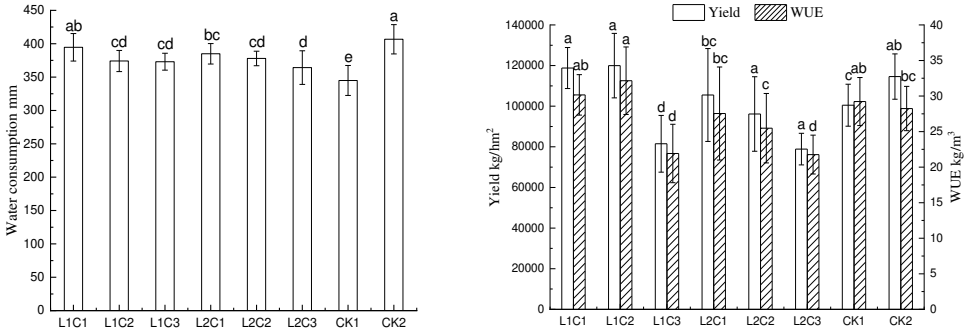

Figure 7. *Effects of different treatments on tomato water consumption, yield and WUE. Note: the data are all average ±standard deviation in the figure, different letters in the same column meant significant difference at 0.05 level, the same as blow*

Optimal evaluation of comprehensive benefit of tomato quality, yield and water use efficiency based on the technique for order preference by similarity to an ideal solution method

The SFW, VD, TD, TSS, SP, TSU, VC, LY, SAR, yield, and WUE of tomato were selected as evaluation variables in *Table 4*. Firstly, these 11 indexes were weighted by Criteria Importance Though Intercrieria Correlation (CRITIC) method (*Table 4*). The calculation method is referred to the "effect of planting row spacing and irrigation amount on comprehensive quality of short-season cultivation tomato in solar greenhouse in northwest China" (Wu et al., 2018). The comprehensive benefit evaluation model is constructed by the TOPSIS method (*Table 5*), and the calculation method is referred to "optimizing irrigation frequency and amount to balance yield, fruit quality and WUE of greenhouse tomato" (Liu et al., 2019). The order of weight obtained by the CRITIC method was as follows: Yield > SFW > SAR > WUE > VD > LY > VC > TSS > TD > TSU > SP, among which, the yield weight was highest (0.150). The evaluation results of the TOPSIS comprehensive benefit evaluation model showed that the comprehensive benefit of L1C2 was best (C*i* = 0.939), followed by L1C1. The comprehensive benefit of drip irrigation under mulch ranked 4th, and micro-sprinkler irrigation ranked 3rd.

Table 4. *Weighting values of each evaluation indices*

Indices	SFW	VD	TD	TSS	TSU	SAR	SP	VC	LY	Yield	WUE
Weight	0.144	0.081	0.067	0.074	0.054	0.124	0.040	0.076	0.070	0.150	0.120

Table 5. *The ranking of irrigation schedule calculated using TOPSIS for all the treatments*

Treatment	D^+	D^-	C_i	Ranking
L1C1	0.015	0.034	0.696	2
L1C2	0.003	0.039	0.939	1
L1C3	0.029	0.019	0.388	7
L2C1	0.021	0.023	0.518	5
L2C2	0.021	0.020	0.490	6
L2C3	0.036	0.014	0.282	8
CK1	0.018	0.024	0.575	4
CK2	0.016	0.030	0.647	3

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Discussion

Effects of irrigation methods on quality and yield of greenhouse tomato

The yield and WUE of tomato plants under MSPF were 19.39% and 10.03% higher compared with drip irrigation under plastic film (*Fig. 7*). This may be because the flow rate of MSPF was about 45 times higher than that of the single group with drip irrigation under plastic film and identical working pressure. Under identical irrigation amount, the flow rate of the single group of MSPF exceeded that of drip irrigation with smaller orifice flow, and the irrigation time was shorter, so that the ratio of soil water horizontal to vertical migration distance increased. The larger surface wetting area increases the wetting volume and irrigation uniformity per unit area of the tillage layer, and decreases the deep transport of soil water (Topp, 1969; Zotarelli et al., 2009; Elmaloglou and Diamantopoulos, 2010; Selim et al., 2013). The average soil volume moisture content of the 0-40 cm soil layer under MSPF was 9.34% higher than that of drip irrigation under plastic film (*Fig. 8*) and root dry matter accumulation (*Fig. 9*). This provided a strong guarantee for the stable yield of greenhouse tomato (Ould et al., 2001), resulting in higher yield of tomato under MSPF. However, because of the large surface wetting area of MSPF and the vigorous growth of tomato plants, soil water evaporation was further intensified. Compared with drip irrigation under plastic film, the water consumption of tomato under MSPF increased by 8.46% (*Fig. 7*). The yield increase of MSPF (19.39%) was about 2.29 times that of its water consumption (8.46%); therefore, the WUE of crops under MSPF was higher than that of drip irrigation under plastic film. This study found that TSS, TSU, SAR, SP, VC, and LY of MSPF decreased by 0.32%, 3.30%, 0.31%, 0.12%, 0.08%, and 2.67% compared with drip irrigation under plastic film. This may be due to the higher moisture content of tomato fruits under MSPF and excessive water dilution of fruit flavor and nutrition index content per unit mass. This is consistent with previous studies, which showed that with increasing irrigation water, the soil volume and water content increased, which reduce the tomato fruit flavor and nutritional content (Topp, 1969; Meek et al., 1992; Li et al., 2020).

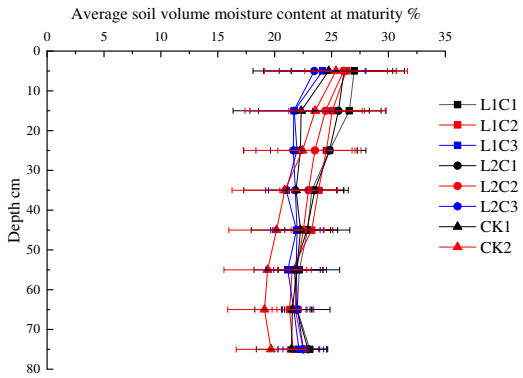

Figure 8. *Average soil volume moisture content in different treatments at mature stage (70 days after transplant). Note: the data are all average ±standard deviation in the figure*

Compared with micro-sprinkler irrigation, the yield of tomato under MSPF increased by 4.69%, because the micro-sprinkling irrigation water flows through the capillary pipe and is sprayed out through the micropore water flow on the capillary pipe wall. The spray height reached more than 1 m under normal working pressure. The spray water flow could

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easily be atomized and increase air humidity, thus resulting in tomato canopy humidity as high as 70%, which is 1.56 times higher than that of MSPF (*Fig. 10*). Previous studies have shown that high-humidity environments can easily cause leaf moisture condensation, leaf cell rupture, decreased leaf photosynthesis, limited dry matter accumulation, and delayed fruit morphological development (Mortensen, 1992; Panchal et al., 2016). It is also possible that under the same amount of irrigation, part of the water is used to increase air humidity, which decreases soil water infiltration. The average volumetric moisture content of the 0-40 cm soil during the mature period was 7.48% lower than that of MSPF (*Fig. 8*). The lower soil volumetric moisture content decreased the increase of tomato yield. This study found that the WUE of tomato under MSPF was significantly higher (by 13.87%) than that of micro-sprinkler irrigation. This may be because the yield of micro-sprinkler irrigation was lower than that of MSPF, and the water consumption of tomato under MSPF was significantly lower than that of micro-sprinkler irrigation (by 8.01%; *Fig. 7*). This study also showed that the related indexes of tomato flavor and nutrition in micro-sprinkler irrigation were lower than those of MSPF. This may be due to the atomization of water droplets in micro-sprinkler irrigation, which increases air humidity and decreases the temperature difference between day and night in the greenhouse; these effects are not conducive to the accumulation of tomato flavor and nutritional indexes (Max et al., 2009; Luo et al., 2016; Sun et al., 2016).

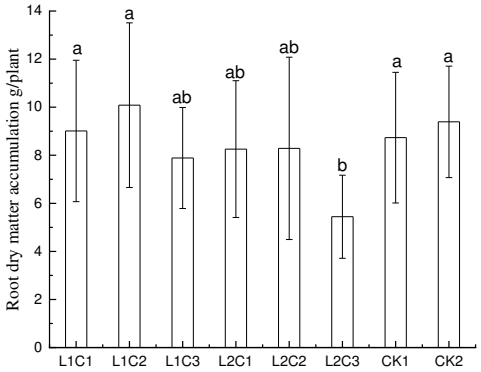

Figure 9. *Effects of MSPF on dry matter accumulation of tomato root during mature stage. Note: the data are all average ±standard deviation in the figure, different letters in the same column meant significant difference at 0.05 level, the same as blow*

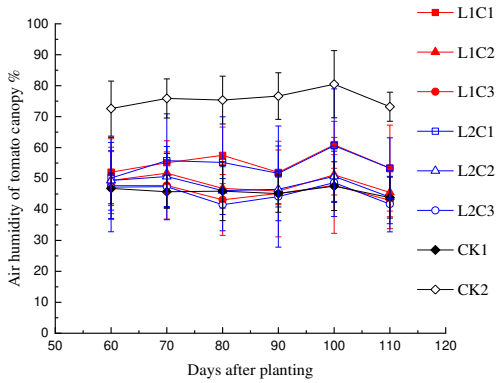

Figure 10. *Effects of different treatments on air humidity in tomato canopy. Note: the data are all average ±standard deviation in the figure*

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Effects of micropores group spacing on quality and yield of greenhouse tomato

This study found that the PH, SD, LAI, and yield of tomato plants with the 30 cm micropores group spacing were better than that of those with 50 cm (*Table 2*). Maybe, the influence of the change of micropores group spacing with MSPF on the soil wetting body is similar to that of dripper spacing with drip irrigation under plastic film, and a phenomenon of intersection of wetting peaks between two groups of adjacent micropores on the capillary pipe was identified. The difference is that under the same working pressure and amount of irrigation, the single group flow of MSPF is higher than that of drip irrigation. Moreover, a larger flow rate easily increases the ratio of horizontal to vertical migration distance of soil water wetting peak. Shortening the confluence time of adjacent wetting peaks is beneficial to the overall deep transport of soil water between the two groups of micropores in the capillary, and improves the soil volume moisture content and irrigation uniformity in the tillage layer per unit area (Ould et al., 2001; Del et al., 2020). Diameter measurement showed that the 30 cm micropores group achieved better tomato maturity than that of the 50 cm micropore group, the average soil volume moisture content of the 0-40 cm soil layer increased by 1.60% (*Fig. 8*), and the higher soil volume moisture content of the tillage layer provided a strong guarantee for tomato growth (Abdelhafeez et al., 1975; Silveira et al., 2020). This is consistent with the conclusion that PH, SD, LAI, and yield of pomegranate, as studied by Meshram et al. (2019), were higher than that of 50 cm.

The present study showed that micropores group spacing had a significant effect on tomato yield. Wang et al. (2005), did not find a significant difference in cucumber yield between 50 and 30 cm drippers spacing when drip irrigation was used, which may be due to the different amounts of irrigation in their experiment. In this study, the irrigation amount was controlled by evaporation pan, and the cumulative amount of irrigation was 353 mm, while Wang controlled the lower limit of soil irrigation and irrigated 385 mm during the growth period. Enciso et al. (2007) found no significant difference in onion yield under different dripper spacings, and the conclusion that the micropores group spacing had a significant effect on tomato yield, as found in this study, is also inconsistent. Because the maximum spacing of dripper set by Enciso was 30 cm (which was much smaller than 50 cm in this study), the soil water distribution was uniform, and could not easily cause yield differences because of drought stress on crops (Ould et al., 2001; Jiménez et al., 2010). Furthermore, the WUE of micropores group spacing at 30 cm was 1.13 times higher than that of 50 cm (*Fig. 7*). It is possible that the soil volume of the tillage layer with a micropores group spacing of 30 cm is moist and uniform, and there is no obvious high water and bottom water area, which can accurately and in time meet the water demand of plants (Li et al., 2018b), resulting in a significant increase in tomato yield. At the same time, the water consumption of the 30 cm micropores group spacing did not significantly increase (*Fig. 7*), and finally showed a significant improvement in WUE (Wang et al., 2005; Li et al., 2019c). Elmaloglou and Diamantopoulos (2010) suggested that decreased dripper spacing can shorten irrigation duration and improve irrigation efficiency, which is consistent with the conclusion that reducing the micropores group spacing to 30 cm can improve WUE.

Previous studies found that too high or too low soil moisture can decrease the contents of soluble solids, VC, and lycopene (Wu et al., 2018). This study showed that the flavor and nutrition of tomato with the 30 cm micropores group spacing were better than that of 50 cm, indicating that the soil moisture distribution at 30 cm micropores group spacing was more suitable spacing for greenhouse tomato under MSPF. This study also found that

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micropores group spacing had a significant effect on tomato TSS, which was inconsistent with the conclusion of Enciso et al. (2007) who reported that dripper spacing had no significant effect on onion solids. The reason for this difference is mainly because all water required by tomato plants originated from irrigation water, while Enciso's onion water requirements were also met by rainfall, which could provide similar irrigation water and optimal soil moisture conditions in all treatments.

Effects of capillary arrangement density on tomato quality and yield in greenhouse

The study of the capillary arrangement density of tomato in the greenhouse showed that the PH, SD, LAI, yield, and water consumption of tomato plants decreased with decreasing capillary arrangement density. Because the same single group flow rate, micropores group spacing, and irrigation amount were used, the higher the capillary arrangement density, the higher the amount of flow per unit area. This makes it easy to increase the surface wetting area, which is conducive to the overall decreasing movement of surface soil moisture and decreases the deep migration of soil moisture. The average soil volume moisture content of one tube and two rows of tillage layer (0-40 cm) was significantly higher than that of one tube and three rows (*Fig. 8*). The higher soil volume moisture content in the higher tillage layer provides a strong guarantee for the growth and stable yield of tomato (Tracy et al., 2013).

With the decrease of capillary arrangement density, a lower surface wetting rate can easily reduce soil ineffective water evapotranspiration, and the lower LAI decreases leaf transpiration, resulting in decreased tomato water consumption. Wang et al. (2018a) found that the yield of tomato under greenhouse drip irrigation increased first and then decreased with decreasing capillary arrangement density. This was inconsistent with the decreasing trend the tomato yield showed in this study, which was mainly because of the different of soil types and irrigators used, and possibly also because of differences of capillary arrangement density used for drip irrigation. In this study, the arrangement of one tube and two rows was the maximum spacing of Wang's drip irrigation. With regard to the differences of tomato yield between three tubes for four rows and one tube and two rows with MSPF, further experiments are needed. The bermudagrass yield of Cantrell et al. (2009), the spring wheat yield of Lv et al. (2019), and the maize yield of Bozkurt et al. (2006) for drip irrigation decreased with decreasing capillary arrangement density. This conclusion is consistent with the conclusion of tomato yield with MSPF in this study.

Furthermore, TSS, TSU, SP, VC, and LY increased with decreasing capillary arrangement density (*Table 3*), which may be because under the same irrigation amount, the smaller the capillary arrangement density was, the longer the irrigation duration was. This aggravated the deep soil water transport and decreased the soil volume moisture content per unit area, thus resulting in a decrease of tomato fruit moisture content and increases of TSS, TSU, SP, VC, and LY concentration per unit weight tomato fruit (Patanè and Cosentino, 2010; Wang et al., 2018a,b). Wang et al. (2018a) found that the SAR of muskmelon in northwest China increased with decreasing capillary arrangement density under drip irrigation, which was consistent with the conclusion of SAR of tomato fruit in this study. This indicates that the effect of capillary arrangement density of micro-sprinkler irrigation on tomato taste was consistent with that of drip irrigation in this area.

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Conclusions

Under the same amount of irrigation, the spring and autumn tomato growth, fruit morphology, yield, and WUE were better with MSPF than with drip irrigation and micro-sprinkler irrigation. Improvements were about 7.34% and 0.13%, 8.32% and 4.69%, 22.95% and 2.76%, 28.38% and 5.66%, 11.70% and 4.69%, as well as 14.43% and 1.67%, respectively. Compared with drip irrigation under plastic film, no significant decreases in fruit flavor and nutrition were found. TSS and VC were significantly higher than that of micro-sprinkler irrigation by about 20.00% and 19.39%, indicating that MSPF was suitable for greenhouse tomato irrigation. With decreasing capillary arrangement density, tomato growth, fruit shape, yield, and WUE decreased, while tomato fruit flavor and nutrition increased. Compared with 50 cm micropores group spacing, 30 cm was beneficial for tomato growth, fruit quality, yield, and WUE. The comprehensive benefit evaluation model of tomato quality, yield, and WUE by the TOPSIS method and the comprehensive evaluation results of yield and WUE identified the L1C2 treatment as better. With the goal of improving WUE and low-cost investment without significantly reducing yield, it is recommended to use a combination of one pipe for two rows with a micropores group spacing of 30 cm in arid and semi-arid sandy loam soils. This study provides a theoretical basis and data support for the large-scale promotion of MSPF. While the presented results describe the optimum irrigation if the row spacing is 50 cm, but it remains an open question that the further experiments are needed to investigate other row spacings.

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ANALYSIS OF HOST PREFERENCE AND RELATIONSHIP OF APHID SPECIES AND THEIR PARASITOIDS IN WHEAT FIELDS AND SURROUNDING AREAS, IN DIYARBAKIR AND ŞANLIURFA PROVINCES, TURKEY

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Abstract. This study was carried out to investigate the relationship and host preference of aphids and their parasitoids on different host plants in Diyarbakır and Şanlıurfa provinces in 2014-2015. In this study, 24 aphid species belonging to 18 genera, 10 parasitoid species belonging to 6 genera and 20 plant species belonging to 8 families were evaluated. Biodiversity Pro V7 package program was used for diversity cluster analysis and to determine similarity between host plant species, aphids and their parasitoids. *Lysiphlebus fabarum* (Marshall) and *Praon volucre* (Haliday) were observed as the most active parasitoids, with preference of five different aphid species, while the remaining aphid species were parasitized by the other parasitoid species. However, *Aphelinus paramali* (Zehavi & Rosen) was found to be specifically parasitizing only one aphid species. The highly parasitism percentage of 10 parasitoid species over 24 different aphid species and their complex relationship in wheat fields and surrounding areas, provided a wonderful natural balanced of biodiversity and possibility to control of aphids in Southeast Anatolia Region. The results of this study will provide useful knowledge in order to introduce biological control possibilities under the framework of integrated pest management.
Keywords: *crops, weeds, diversity, aphids, natural enemies, parasitism, biological control, IPM*

Introduction

Winter wheat, (*Triticum aestivum* L.) is main crop in Turkey and constitutes 3.5% of the world wheat area (Anonymous, 2018a). This area also accounts for 20% of total cultivated farmland in Turkey (Anonymous, 2018b).

Aphids among other insects are the main pests causing the highest damage to wheat production at approximately 30% yield loss per year (Webster and Kenkel, 1999). There are nearly 4,400 aphid species belonging to 599 genera in the Aphidomorpha (Hemiptera) superfamily in the world, 3,706 species of which live in the Palearctic region (Remaudière et al., 2006; Blackman and Eastop, 2020; Favret, 2020). Many studies have been conducted to determine the number of aphid species in Turkey and according to these studies, 558 aphid species belonging to 8 subfamilies of Aphidoidea were determined in Turkey (Düzgüneş et al., 1982; Görür et al., 2012; Şenol et al., 2014; Kök et al., 2016; Akyürek et al., 2019; Özdemir, 2020).

The locomotive sector of the Southeast Anatolia Region is agriculture. 3.2 million ha of 7.5 million ha area in the region is suitable for agricultural activities. Currently, 93.6% of red lentils, 96.3% of pistachios and 35.3% of wheat, are supplied from this region (Anonymous, 2020). Various studies related to aphids and natural enemies on wheat have also been carried out in this region (Bodenheimer and Swirski, 1957; Tuatay and Remaudiere, 1964; Tuatay, 1988; Kıran, 1994; Elmalı and Toros, 1994; Ölmez, 2000; Yüksel, 2003; Şimşek et al., 2005; Remaudiere et al., 2006; Ölmez Bayhan et al., 2012, 2013; Aslan, 2013; Bayram and Bayhan, 2013, 2016; Bayram et al., 2018).

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Aphids are able to transmit plant viruses directly or indirectly from secondary host plants to main crops (Kennedy et al., 1962). Therefore, it should be well known to understand the presence secondary host plants such as early growing plants, weeds or uncultivated plants as much as main crops for estimating aphid damage and their ability by carrying viruses from one plant to another (Bayram et al., 2018).

Aphid species generally live on their host plant species in colonies. Aphids change their host plants as facultative or obligate and this change includes two (dioeciously species) or more (heteroecious species) host plants. Although there is no clear relationship between main crops and secondary host plants, there is a clear explanation that main crops and secondary host plant species are classified under the same genus and the same families (Kristoffersen, 2003). Host preferences of aphid individuals are affected by numerous factors such as the structure of plant surface, as well as plant color and odor. Some specific substances such as phenols, alkaloids and oils affect aphid feeding and host plant preference (Özdemir, 2013).

As it is difficult to control aphids and chemical control is not a sustainable or environmentally friendly method, studies generally have been focused on alternative control strategies such as biological control, biotechnical control and cultural measures. There are many studies about aphid parasitoids (Kıran, 1994; Ölmez, 2000; Kavallieratos and Lykouressis, 2000; Kavallieratos et al., 2001; Praslićka et al., 2003; Legrand et al., 2004; Aslan, 2013; Bayram and Bayhan, 2013, 2016; Bayram et al., 2018). Investigation on host preference and relationship between aphids and parasitoids is one of the most important points for establishing a comprehensive control strategy. Therefore, this study was carried out to investigate host preference and relationship of aphids and parasitoids on wheat and neighboring habitats.

Materials and Methods

Field studies

Studies were carried out in wheat growing areas and neighboring habitats randomly, from different fields in 2014 and 2015 in Diyarbakır (Yenişehir and Kayapınar central counties) and Şanlıurfa (Akçakale, Siverek counties) provinces (*Figure 1*). Aphid colonies consisting of both live and mummified aphids were collected together with their host plants. Field studies were conducted during April, May and June by examining and sampling shoots, plants, leaves, branches and trunk of each plant once a week, while in November and February months by sampling once a month, with irregular controlling of 70 different locations. Each sample was placed in a plastic container and brought to the laboratory for rearing.

Laboratory studies

Studies were established under controlled conditions (25±3°C, 70% ± 10% RH and 16:8 L:D) in climatic rooms. Samples were examined daily for emerged aphids and parasitoids. Both adult aphids and parasitoids were preserved in 70% ethanol for subsequent identification. The slide mounting technique was mainly based on the method of Hille Ris Lambers (1950). The specimens were studied using a LEICA DM LB2 compound light microscope and morphological characters were measured using LAS 4.1 version software. Measurements of morphological characters were made according to Blackman and Eastop (2020).

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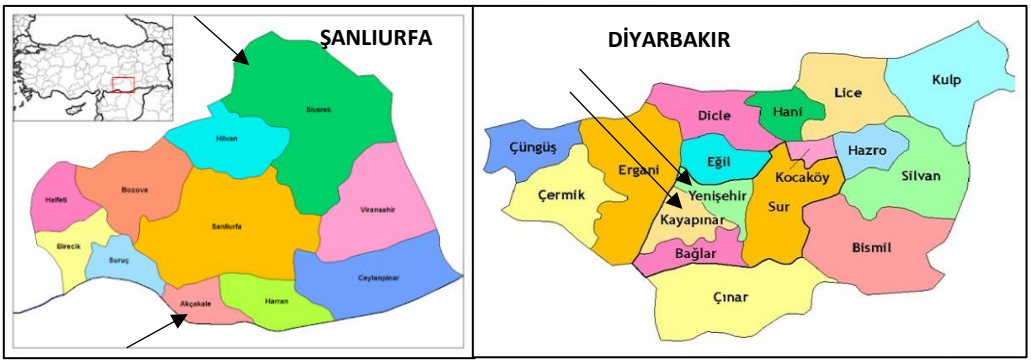

Figure 1. *Map of Diyarbakır and Şanlıurfa provinces in which field studies were carried out*

Host plant species

Wheat fields and the nearest area around these fields were examined carefully and plant samples were taken together with mummified aphids to laboratory for identification. Secondary host plants such as uncultivated plants and weeds around wheat fields were identified by Prof. Dr. Bekir BÜKÜN (Dicle University, Faculty of Agriculture, Plant protection Division) and Erdal ATEŞ (Plant Protection Research Institute, Diyarbakır). Identification studies of host plants, aphids and their parasitoids were published by Bayram et al. (2018).

Preparation and identification of aphids and parasitoid specimens

Aphids and parasitoids were examined and separated under a binocular and were placed into small bottles or tubes with 70% alcohol. The tubes and bottles with aphids and parasitoids were recorded with required information and prepared for identification. The colors of aphid mummies were also recorded, considering that they could be useful for identification and classification. Selected fresh specimens of aphids were immersed in 75% ethanol and preserved for future identification. Adult parasitoids were preserved in 96% ethanol. Some specimens were mounted on slides. Aphids were removed from their host plant with a small soft brush and put into a tube which contained 70% alcohol. The preservation techniques were mainly based on the method of Hille Ris Lambers (1950). The morphological terminology used as key for parasitoid species identification was based on (Sharkey and Wharton, 1997; Kavallieratos and Lykouressis, 2000; Kavallieratos et al., 2001) literatures. Aphid species were identified by specialist Dr. Işıl Özdemir (Central Plant Protection Research Institute, Ankara). Parasitoid species were identified by specialist Zeljko Tomanoviç (University of Belgrade, Faculty of Biology, Serbia).

Cluster analysis of host plants, aphids and parasitoids

Jaccard similarity index (Magurran, 2004) was used for determining the faunal similarity of host plant families from which aphids were obtained and Biodiversity Pro V7 package program (Biodiversity Pro. 1997), which is a statistical package program for Windows PC, enabling many measures of diversity to be calculated for a dataset of taxa by samples, was used for determining cluster analysis of aphid similarity from which parasitoids were obtained.

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Determination of the amount of emerging parasitoid individuals and parasitized aphid species

Mummified aphids and plant samples were collected from different locations randomly. These samples were recorded and each of the samples was separated in the laboratory. Plant materials with mummified aphids were taken into plastic boxes and required information was recorded on the boxes. The cover of the plastic boxes was cut as widely round and closed with tight textured nylon muslin to provide ventilation. The samples were kept for at least 14 days and controlled daily until parasitoid adult emerged. Each sample was followed separately and parasitoid exit recorded according to parasitoid and aphid species. The obtained data determined the total number of parasitoid exit for each aphid species and the number of aphid species parasitized by the same parasitoid species.

Results and Discussion

The cluster similarity analysis of main crops (wheat) and surrounding host plants and uncultivated plants in terms of hosting aphids was shown on *Figure 2*. *Alopecurus myosuroides* Hudson*, Carduus crispus* L.*, Cirsium vulgare* (Savi) Airy-Shaw.*, Daucus carota* L. var. *carota, Galium aparine* L.*, Orobanche* sp. and *Sonchus oleraceus* L., host plants were determined as specific host plants for obtaining aphid species. However, other host plant species such as; *Amaranthus retroflexus* L.*, Avena fatua* L.*, A. sterilis* L.*, Centaurea solstitialis* L.*, Lolium perenne* L.*, Onopordum acanthium* L.*, Papaver rhoeas* L.*, Silybum marianum* (L.) Gaertn, *Triticum aestivum* L. Emd., *T. durum* Desf., and *Vicia sativa* L., were determined as unspecific host plants for obtained aphid species and these aphid species could feed on other host plants at a certain rate. *Lens culinaris* Medicus and *Lupinus albus* L., plant species hosted the same aphid species (*Figure 2*).

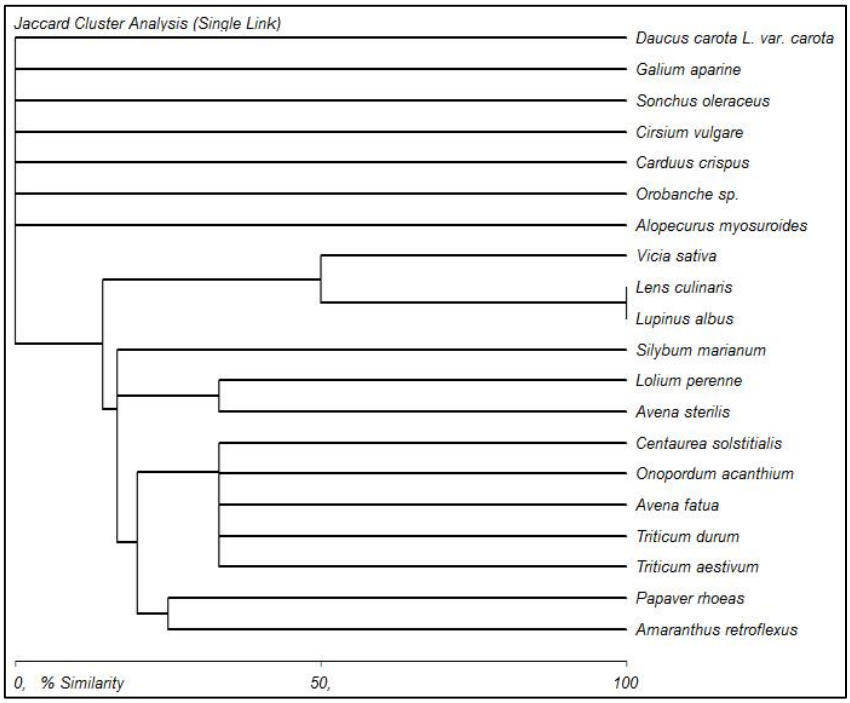

Figure 2. *Similarity of cereals and uncultivated plant species in terms of hosting aphid species*

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The cluster similarity analysis of aphids in terms of host plant preference was shown on *Figure 3*. *Aphis gossypii* Glover, *A. galiiscabri* Schrank, *Diuraphis noxia* (Kurdjumov), *Dysaphis foeniculus* (Theobald), *Hyperomyzus lactucae* (L.), *Smynthurodes betae* Westwood and *Uroleucon (Uromelan) jaceae* (L.) aphid species fed on certain host plant groups and those host plant species weren't preferred by the other aphid species. *Uroleucon cichorii* (Koch) and *Capitophorus elaeagni* (deI Guercio) aphid species preferred the same host plant. However, the other aphid species could tend to prefer the same host plant species in different rates. The percentage of similarity of host plant preference of *Anoecia corni* (Fabricius), *Aphis fabae* Scopoli, *Aulacorthum solani* (Kaltenbach), *Lipaphis erysimi* (Kaltenbach), *Macrosiphum euphorbiae* (Thomas), *Metopolophium dirhodum* (Walker), *Myzus* (*Nectarosiphon*) *persicae* (Sulzer), *Sipha* (*Rungsia*) *maydis* Passenger, and *Uroleucon* sp., was 50%. The percentage of similarity of host plant preference for other aphid species such as; *Aphis craccivora* Koch, *Brachycaudus (Acaudus) cardui* (L.), *B. helichrysi* (Kaltenbach), *Rhopalosiphum maidis* (Fitch), *R. padi* (L.) and *Sitobion avenae* (Fabricus) was less than 50% (*Figure 3*).

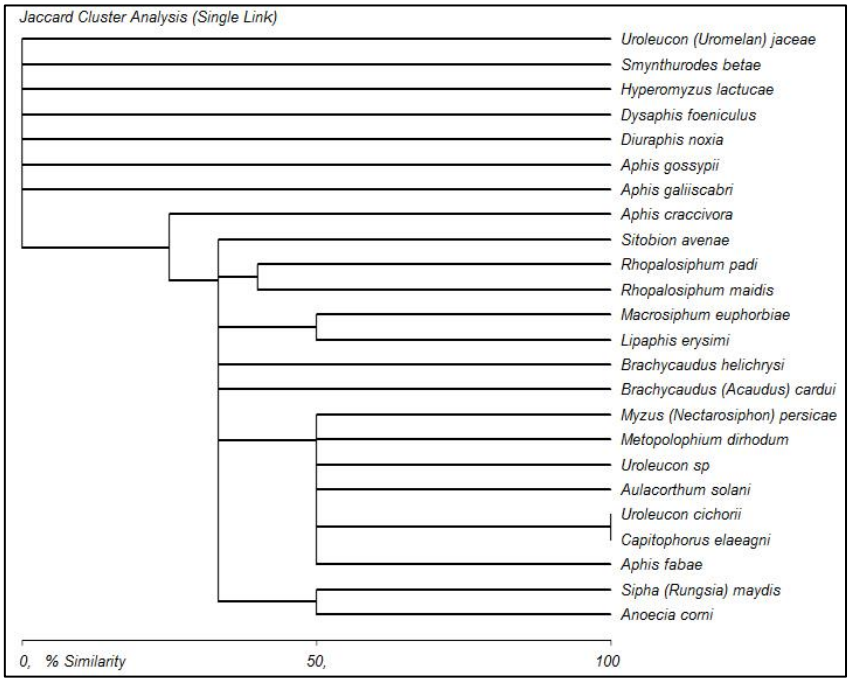

Figure 3. *Similarity of aphid species in terms of host plant preferences*

The cluster similarity analysis of parasitoids in terms of aphid preference was shown on *Figure 4*. Parasitoid species that parasitizing *Rhopalosiphum maidis, Hyperomyzus lactucae, Brachycaudus helichrysi* and *Aphis gossypii* aphid species were tending to prefer only one aphid species. However, the other parasitoid species could tend to prefer different aphid species at a certain rate. *Lipaphis erysimi, Diuraphis noxia* and *Aulacorthum solani* aphid species were parasitized by the same parasitoid. *Uroleucon cichorii, U. (Uromelan) jaceae, Rhopalosiphum padi, Macrosiphum euphorbiae* and *Aphis galiiscabri* aphid species were parasitized by one parasitoid (*Praon volucre* Haliday). *Myzus (Nectarosiphon) persicae* and *Dysaphis foeniculus* were parasitized by the same parasitoid (*Lysiphlebus fabarum*). *Brachycaudus (Acaudus) cardui, Aphis fabae,*

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and *A. craccivora,* aphid species were also parasitized by the same common parasitoid species (*Figure 4*).

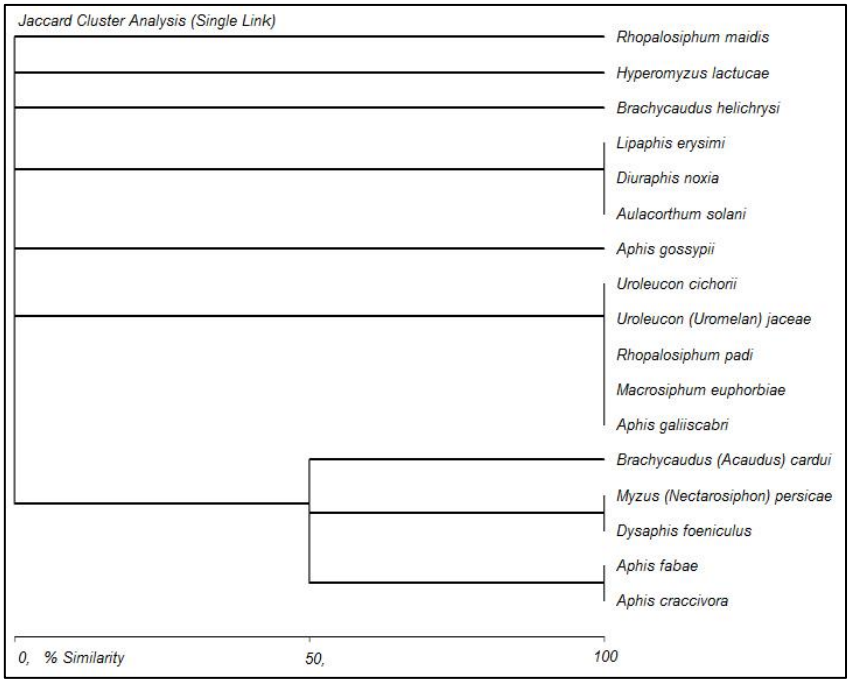

Figure 4. *Similarity of parasitoid species on aphid species*

The cluster similarity analysis of parasitoid species and the number of emerging parasitoid individuals and parasitized aphid species were shown on *Figure 5* and *Figure 6*. It was observed that certain parasitoid species such as *Praon volucre, Diaeretiella rapae* (M'Intosh)*, Aphidius rhopalosiphi* de Stefani-Perez*, Aphidius matricariae* Haliday and *Aphidius colemani* Viereck were specialized on certain aphid species. *Binodoxys acalephae* (Marshall)*, Lysiphlebus fabarum* (Marshall) and *Aphidius ervi* Haliday tended to prefer common aphid species. The other parasitoid species *Lysiphlebus testaceipes* (Cresson) and *Aphelinus paramali* Zehavi & Rosen were parasitizing the same aphid species (*Figure 5*). It was revealed that *L. fabarum* is parasitizing *Brachycaudus (Acaudus) cardui*; *Praon volucre* is parasitizing *Uroleucon cichorii, U. (Uromelan) jaceae, Rhopalosiphum padi, Macrosiphum euphorbiae* and *Aphis galiiscabri*; *Aphidius ervi* is parasitizing *Myzus (Nectarosiphon) persicae*; *Aphidius rhopalosiphi* is parasitizing *Rhopalosiphum maidis*; *Aphidius matricariae* is parasitizing *Brachycaudus helichrysi*; *Lysiphlebus testaceipes*; and *Aphelinus paramali* is parasitizing *Aphis gossypii*; and *Diaeretiella rapae* is parasitizing *Diuraphis noxia* (*Figure 5*).

Praon volucre was found the most effective parasitoid both in terms of obtaining parasitoid individuals (147 individuals) and having the ability of parasitizing 5 different aphid species. *Lysiphlebus fabarum* also has a high parasitizing tendency towards many different aphid species (5 species) and it has a high parasitizing capacity (110 individuals) (*Figure 6*). Although those five aphid species were parasitized by the other parasitoid species (*Binodoxis acalephae*, *Aphidius ervi*), *Lysiphlebus fabarum* has a high parasitizing capacity and tendency of so many different aphid species including ability of highly competitive. *Diaeretiella rapae* parasitized three different aphid species, while

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Aphidius rhopalosiphi parasitized two different aphid species. *Aphidius ervi, A. colemani, A. matricariae, Aphelinus paramali and Lysiphlebus testaceipes* parasitoid species were obtained only from one separate aphid species (*Figure 6*).

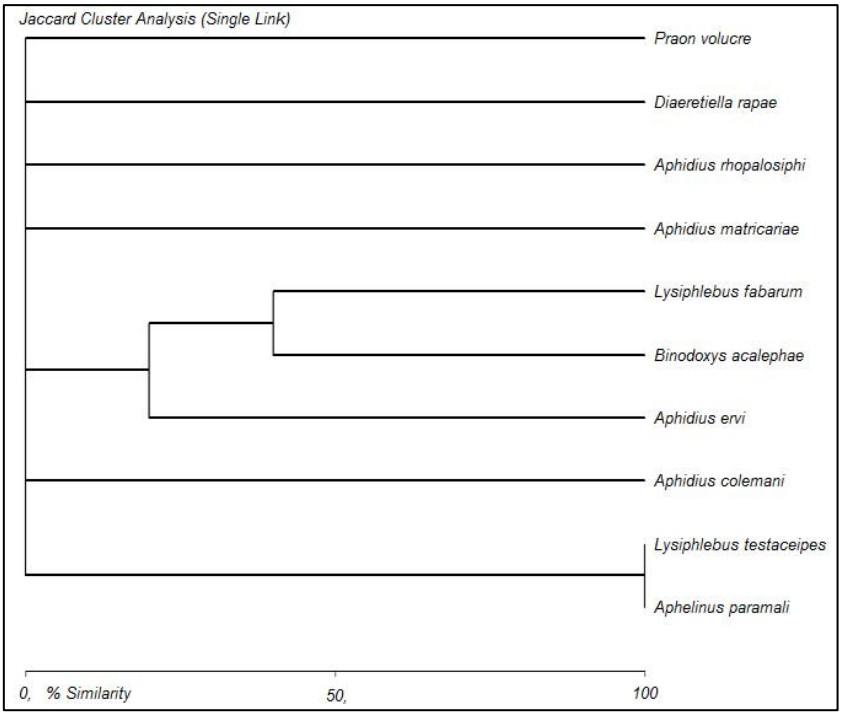

Figure 5. *Similarity of parasitoid species in terms of aphid preferences*

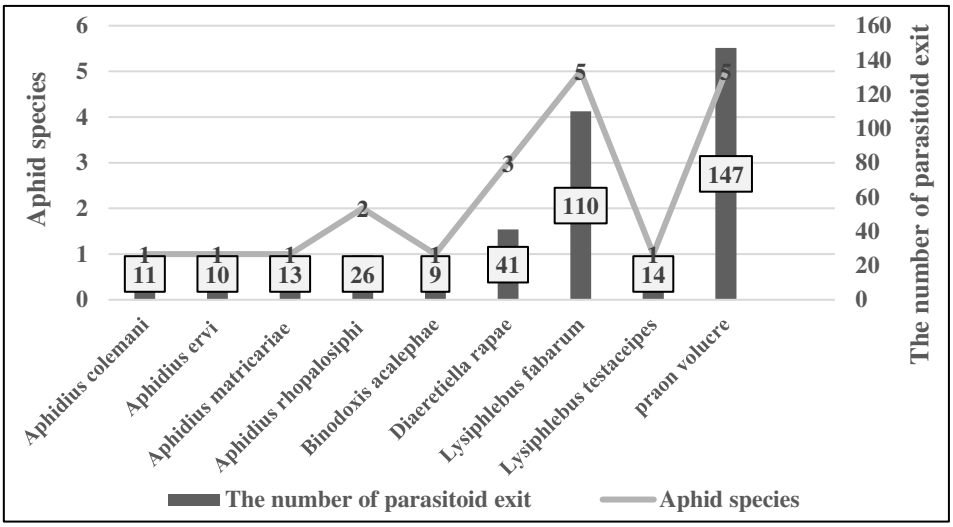

Figure 6. *The number of emerged parasitoid individuals and the number of aphid species parasitized by the same parasitoids*

According to former studies conducted in the world related to host plants, aphids and their parasitoids; Nine aphid species [*D. noxia, M. dirhodum, R. maidis, R. padi*, *S. graminum* Rond., *Sitobion avenae, S. fragariae* (Walker), *Sipha elegans* del Guercio and

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S. maydis] were determined in wheat fields of Yugoslavia (Petrović, 1996). In Southeastern Europe 422 host plant species, 208 aphid species and 122 parasitoid species, 115 of which belonged to Aphidiinae genus were identified and 561 parasitoid-aphid relations were described (Kavallieratos et al., 2004). The relationship of *S. avenae* and *Aphidius rhopalosiphi* in spring, autumn and winter months in Belgium revealed that the fields where the parasitoid were available, the aphid population decreased rapidly and lasted at a low level (Legrand et al., 2004). Four aphid parasitoid species belonging to Aphidiidae family in the Angola region were determined. While *D. rapae* and *Ephedrus persicae* Froggatt parasitoid species were determined as cosmopolitan species. *Aphidius platensis* Brethes was obtained from some domestic aphids or some materials that collected from out and *A. camerunensis* Mack was only determined on some *Sitobion* species (Stary and Van Harten, 1972). The most dominant aphid species were *Aphis fabae* and *Cirsii acanthoidis* Börner, while the most related parasitoids were *L. cardui* (Marshall), and *L. fabarum* on *Cirsium arvense* (L.) in Czechoslovakia (Stary, 1986). Four parasitoid species (*A. ervi, A. eadyi* StarýFebGonzález & Hall, *A. picipes* (Nees) and *P. barbatum* Mackauer) were found on pea aphid in Yugoslavia, and *A. ervi* was found as the most dominant species (Tomanović et al., 1996). Eight new aphid species with their host plants, including 12 Aphidiinae parasitoids, four of which new record was reported in Serbia and Montenegro (Tomanović, 2000). *A. ervi*, *A. uzbekistanicus* Luzhetzki, *A. rhopalosiphi* and *P. gallicum* Starý were found as the most intense parasitoid species in the wheat fields of Southern agro-eco systems of the Pannonia region (Tomanović and Brajkovic, 2000). A new aphid parasitoid species *Praon uroleucon* Tomanović & Kavallieratos, identified in Yugoslavia, and this parasitoid species parasitize *Uroleucon* aphid species on *Carduus acanthoides* L., host plant (Tomanović et al., 2003). Seven aphid parasitoid species (*Aphidius uzbekistanicus*, *A. ervi*, *A. picipes, A. rhopalosiphi*, *Ephedrus plagiator* Nees, *Praon volucre* and *P. gallicum*) were determined in wheat fields of Slovakia (Praslićka et al., 2003).

Aphid parasitoids generally hibernate in diapause in winter as prepupae in mummified aphids in the regions where the temperature is mild (climate, weather). By the migration of aphids from one host to another host plant habitat with summer or in the warmer season conditions, seasonal diapause of parasitoids also could be observed (Minks and Harrewijn, 1988). Stary (1964) reported that the relation between dioecious aphids and their parasitoid is even more complex. These aphid species always change their habitats throughout the season from primary host plants to secondary host plants due to their migration behavior. Therefore, their parasitoids habitat also changes throughout the season depending on the migration of aphids. In this case, any dioecious aphid could be parasitized by different parasitoid species complex according to its habitat type. For example, *Brachycaudus cardui* aphid species could be found on the edges of wooded areas and in the parks on *Prunus spinosa* L., and *P. domestica* L. host plants in spring or in winter and parasitized by *Ephedrus plagiator*, while this species migrates towards the end of spring or at the beginning of summer on weeds such as *Carduus* sp. and *Arctium* sp., and parasitized by *Lysiphlebus fabarum* and *Lipolexis gracilis* Forster. However, Monoecious aphids even if migrating to other plants for feeding purposes, they do not change their habitat types throughout the season. Therefore, studies revealed that these aphid species generally parasitized by the same parasitoid species.

According to the former studies conducted in Turkey about host plants, aphids and their parasitoids; 13 aphid species, 5 parasitoid species and 21 predator species were determined in the wheat fields of Konya province (Elmalı, 1993). 10 aphid parasitoids

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belonging to 6 genera were determined on weeds areas of Ankara province (Güz and Kılınçer, 2005).

According to the former studies conducted in Southeastern Anatolia Region, five aphid species *Sitobion avenae*, *Rhopalosiphum padi*, *R. maidis*, *Schizaphis graminum* and *Myzus persicae* and two parasitoid species *Lysiflebus faborum* and *Ephedrus plagiator* were determined in the wheat fields of Southeastern Anatolia Region (Kıran, 1994). In addition, 24 aphid species belonging to 18 genera, 10 parasitoid species belonging to 6 genera were determined in wheat and surrounded areas on 20 host plants belonging to 8 families in Southeastern Anatolia Region (Bayram et al., 2018).

By this study, the cluster analysis of similarities and differences of host plants, aphids and parasitoids was evaluated. Since there are many host plant samples, aphids and parasitoid species, it is hard to exactly differ their relation with each other, or to evaluate each element separately. While some aphid species preferred one host plant species, the other aphid species preferred more host plant species and two or more aphid species could be seen over the same host plant species as well.

The aphid species are generally a significant food source both for parasitoids and predator. While some aphid parasitoids which are so specific and representing the isolated complexes have no relation in the food chain, the other aphid parasitoids have a more complex relationship. *Lysiphlebus fabarum* and *Praon volucre* were found very active having both abilities of parasitizing five different aphid species and reproductive capacity. However, *Aphelinus paramali* was found specified only one aphid species. The difference of the host plant change or the presence of main crops and uncultivated host plant species, in connection with aphid host series determine the composition of parasitoid species and their percentage into this composition. The parasitizing activity of 10 parasitoid species over 24 different aphid species and their complex relationship with the highly parasitizing rate is a wonderful natural balance in wheat fields and surrounded areas of Southeastern Anatolia Region (Bayram et al., 2018). Sometimes main crops are not available in the environment, so the presence of uncultivated plants or weeds around the main crops is a good opportunity for reproduction of first generations of aphid colonies on these host plants and also an integrative element for first generation of parasitoids which is emerging at the beginning of spring and lying eggs to aphid colonies. The richness of biodiversity guarantees the sustainability of not only the aphid living, but also the beneficial insects living. Although Monoecious aphids migrate to other plants for feeding purposes, they do not change their habitat types throughout the season, so Monoecious aphids are generally parasitized by the same parasitoid species.

The same as harmful organisms, beneficial insects also could not survive in the pesticide used areas. However, the environment that has not been treated with pesticides such as weeds or uncultivated areas are suitable both for pests and for beneficial insects to survive. These areas are good reservoirs and provide a perfect biodiversity. The reason for numerous cereal aphid species kept below the threshold of economic damage depends on these herbaceous plants in the vicinity of grain fields. The relationship of aphids and their parasitoids depend on vegetation diversity of the same or different areas. The availability of these flowering weeds before the vegetables, cottons or other cultivated crops planted in early spring, provides an opportunity for parasitoids to feed on these flowering weeds together with aphids and then migrate to other cultivated areas (Bayram et al., 2018).

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Conclusion

This study has aimed to investigate the host preference and relationship between aphids and their parasitoids on different host plants growing on wheat fields and surrounding areas in Turkey, in order to help develop biological control methods.

Although chemical control is primary resort for pest control in main crops, uncultivated plants, or weeds around main crops are not treated with chemicals, so these plants are reserving so many pests and beneficial insects as well. In Southeastern Anatolia Region wheat production is getting mature at the beginning of summer, so aphids could give damage to only some local points with some limited colonies which are formed at the edge of the fields. Except for any extra situation there is no needs for chemical control. Producers also do not use chemicals for aphid control in wheat fields, and the use of some chemicals against Sunnpest suppresses aphid population as well.

The presence of many aphid species, the richness of parasitoid species and their effectiveness, tendency and complex relationship over many aphid species in main crops, and uncultivated host plants and weeds are valuable and helpful resources for suppressing aphid population by using these beneficial insects. The survival of these parasitoids is useful not only for the wheat production, but also for suppressing the aphid populations in other secondary products. This resource should be well protected by avoiding unnecessary pesticide usage and by educating growers for increasing their awareness about the importance of beneficial insects. The analysis of interaction of host plants, similarities and differences among host plants, aphids and parasitoids, and determining the most active and common parasitoid species will be a useful knowledge in the framework of biological control and integrated pest management.

Future studies should focus on the protection as well as the production, reproduction, preservation of these beneficial insects and their transition to secondary host plants following wheat main crop. The possibilities of using these beneficial insects for controlling aphid species in corn, vegetables, fruits and industrial plants which are cultivated in the summer should be investigated. Opportunities should be sought to preserve this natural enemy in the Southeastern Anatolia Region, to survive and to benefit throughout the year. Usually weeds are not liked, whereas it should be taken into consideration that these weeds are a good habitat for beneficial insects to survive, and these plants that grow by themselves around wheat fields should not be destroyed.

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INVESTIGATION OF THE CHEMICAL COMPOSITION OF IVY (*HEDERA HELIX* L.) SPECIES GROWING IN ÇAYCUMA, TURKEY

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Abstract. In this study, the extractive substances of *Hedera helix* L. (forest ivy) wood and bark, naturally found around Filyos Stream in the Çaycuma district of Zonguldak, were investigated, for both ground-creeping individuals and for those clinging to Oriental plane (*Platanus orientalis* L.). The samples taken from the wood and bark of the plants were compared. According to the results of wood hexane experiments, linoleic acid (26.98%) was the most abundant in normal wood samples. In the same experiments on bark, while linoleic acid (25.40%) was determined to have the highest content in normal *H. helix*, palmiteloic acid (17.09%) was the most abundant compound in plants clinging to trees. In acetone:water (95:5,v:v) extract experiments on wood, the penetrating ivy wood contained the greatest amount of α-D-glucopyranoside (58.47%). Based on acetone:water (95:5,v:v) experiments on bark samples, sucrose (35.94%) had the highest content in normal bark.
Keywords: *ivy, oriental plane, lipophilic extractives, hydrophilic extractives, fatty acids*

Introduction

The morphology and anatomy of plants change with enviromental factors. The physiological events that enable the growth, reproduction, survival behaviors, metabolism, and geographical distribution of plants are significantly influenced by the physical, chemical, and biotic factors of the surrounding environment (Lambers et al., 2008).

Hedera helix L. (forest ivy), an evergreen and clinging plant belongs to the *Araliaceae* family. It is located in forests and bushes with its fast polyploid capacity Although, it has a fast height increment the diameter increase slowly (Mandade et al., 2010). It can climb up to 30 m high by adhering strongly on solid surfaces with the help of adventitious roots where its root diameter can reach 20 cm (Brendler et al., 2003; Horz and Reichling, 2003; Schnitzler and Heuze, 2006; Stavretovic, 2007). The plant's ability to adhere to surfaces depends on the secretion of an adhesive composing of 60-85 nm diameter spherical nanocomposite polysaccharide and nanoparticles (Xia et al., 2010; Burris et al., 2012).

The leaves of *Hedera helix* L. are used in medical treatments. The main compounds found in the leaves are α-hederin and hedefracoside C with strong antioxidant activity (Gülçin et al., 2004). These compounds belong to the saponin group. Saponins have steroid or triterpenoid structure, and are usually used in disease treatment (Küçükkurt and Fidan, 2008; Mingjun et al., 2008; Fazio et al., 2009; Yollu, 2015; Muşmula et al., 2017). Also, *Hedera helix* L. is used extensively in traditional medicine due to its analgesic and anti-inflammatory properties (Mandade et al., 2010; Uddin et al., 2011).

Because of its climbing properties, it is also used for landscaping in walls, fences.

In its natural habitat this plant covers the ground area and it is described as “normal ivy” in this study (*Figure 1*), also, it embraces many tree species. Oriental plane (*Platanus orientalis* L.) is one of the tree species embraced by ivy (*Figure 2*).

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Figure 1. *Normal ivy (Hedera helix L.) in its natural habitat*

Figure 2. *Penetrated ivy (Hedera helix L.) to Oriental plane (Platanus orientalis L.)*

Oriental plane (*Platanus orientalis* L.), a member of the *Platanaceae* family and *Platanus* L. genus, can live up to 500-2000 years. *Platanus orientalis* L. natural distribution areas include Southern Europe and Southwest Asia, particularly the forests of Turkey and Iran (Davis, 1982; Anşin and Özkan, 2006; Zencirkiran and Erken, 2012). Naturally, they are typically found near the streams of forest and riversides; unnaturally, they are often used as ornamental plants and shade trees in cities and villages (Doğu, 2002).

In the chemical composition of the wood, the main components of the cell wall are polymer compounds, such as cellulose, hemicelluloses, and lignin. At the same time, the wood also contains extractives with lower molecular weights (Gindl and Teischinger, 2003; Papadopoulos, 2005; Dönmez and Dönmez, 2013). Most of the chemical groups found in wood are also seen in the bark with varying amounts. Generally, the content of

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the extractive substance in the bark is higher than that in the wood of the same tree (Sjöström et al., 1981). The extractives affect several properties of the tree, such as texture, odor, taste, and color. Extractives are lipophilic and hydrophilic substances that are soluble in neutral solvents (Fengel and Wegener, 1989; Sjöström, 1993; Holmbom, 1999). Lipophilic extractives, which are soluble in dichloromethane, ether and some hydrocarbon solvents, consist mostly of fats, fatty acids, resin acids and sterols. Comparatively, hydrophilic extractives are substances like carbohydrates, lignans, phenols, stilbens and chalkones that are soluble in polar solvents like water, alcohol and acetone (Holmbom, 1999). These chemicals are now being extracted for different industries, such as pharmaceutical, cosmetic, and food industry (Dönmez, 2018).

According to Yaman (2009), *Hedera helix* L.'s effect on the anatomical structure of *Platanus orientalis* L. is incidental. After a certain period of time on the tree, it was concluded that the invader cannot cause detrimental damage to the tree. In this context, compounds in the tree's wood support normal growth and both species can actually benefit (*Hedera helix* and *Platanus orientalis*). On the other hand, the literature regarding the chemical composition of *P. orientalis* on *H. helix* is out of our knowledge.

In this study, it was aimed to investigate chemical composition, especially extractive substances, of *Hedera helix.* Both normal and penetrating on *Platanus oreintalis* was analysed for the first time, thus, this work provides a novel source in literature.

Materials and Methods

Materials

Both normal grown ivy and the ivy penetrating to the oriental plane were used as study material. Both wood and bark samples were taken, with three replicates, from Filyos Stream in the Çaycuma district of Zonguldak in April 2019, where the altitude was 40 m (41°22'56"N, 32°5'13"E). The sampling area can be seen in *Figure 3*. Cross-sections of the samples were prepared according to TAPPI standards. All samples were stored at -24 °C until analyses. Wood was first debarked, and the bark was cut into small pieces. All samples were freeze-dried and ground by a Wiley mill into 1-mm (Ekman, 1983).

Figure 3. *The sampling area*

Extraction

Approximately 10 g of grounded wood and bark from each sample was successively extracted first with n-hexane and then with acetone:water (95:5, v:v) in a soxhlet. Samples were weighed before and after extraction to determine the extractive yield. 100

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ml aliquots of extractives were removed using rotary evaporator, followed by gravimetric analyses (Dönmez et al., 2016). For gravimetric analyses, 10 ml aliquot was evaporated to dryness, i.e, constant weight, leaving a film of extractives in the solvent container. All results, given in mg/g, were calculated from freeze-dried samples. The appropriate amount of mixture was evaporated under nitrogen prior to silylation. The extracts obtained from hexane and acetone:water (95:5, v:v) were injected into GC-MS to identify the lipophilic and hydrophilic components.

Chromatographic analyses of the extracts

Chromatographic analysis of the extracts was carried out using a Shimadzu GC-2010 gas chromatograph equipped with an MS-QP 2010 mass spectrometer (Shimadzu Corporation, Kyoto, Japan). The instrument was equipped with a column, Rxi-5Sil MS (30 m × 0.25 mm i.d. × 0.25 μm film thickness; Restek, Bellefonte, PA, USA); the temperature program was as follows, from 60 °C (1 min) to 280 °C at 2 °C/min; injection temperature, 280 °C; the carrier gas was He with a flow rate of 1 mL/min; the injection mode was split (10:1); the MS temperature was 280 °C.

Results and Discussion

Wood and bark of the normal ivy and ivy penetrating to the oriental plane were analyzed by GC-MS to determine lipophilic and hydrophilic components after extraction with different solvents. In both normal and penetrated ivy wood, hexane-soluble extracts were found to be lower than acetone:water (95:5, v:v) extracts. In comparison, the amount of hexane-soluble extracts in penetrated ivy wood was almost 50% higher than that in normal wood. Specifically, hexane extracts were determined to be 37.46 mg/g in penetrated ivy bark, 28.31 mg/g in normal, 3.22 mg/g in penetrated ivy wood, and 2.30 mg/g in normal wood. The amount of acetone:water-soluble extracts was found to be 31.31 mg/g in normal bark, 28.44 mg/g in penetrated ivy bark, 5.79 mg/g in normal wood, and 6.32 mg/g in penetrated ivy wood. In hexane extracts, the increase of 40% in wood and 32% in bark samples of *H. helix* penetrating to the oriental plane tree were determined compared to normal *H. helix*. In addition, a 9% increase in wood samples of *H. helix* penetrating to the oriental plane tree compared to normal grown wood, on the contrary, a 10% decrease in the penetrated wood compared to normal grown wood was observed in acetone-water extracts.

After isolating hexane-soluble extracts, the lipophilic components in wood and bark samples were obtained via a wiping process, then transmitted to vials and analyzed by GC-MS. The amounts of soluble substances were calculated gravimetrically and are shown in *Table 1*; lipophilic and hydrophilic components are displayed in *Table 2* and *Table 3*, respectively.

Table 1. *Gravimetric analysis of extracts from normal ivy and ivy penetrating to the oriental plane wood and bark (mg/g of dry weight)*

Sample Tree	Specimen	Hexane extract	Acetone:water extract
Normal *Hedera helix*	**Wood**	2.30	5.79
	Bark	28.31	31.31
***Hedera helix* penetrated to oriental plane**	**Wood**	3.22	6.32
	Bark	37.46	28.44

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Table 2. *The hexane-soluble extractive components determined by chromatographic analysis (%)*

Name of Component	Normal *Hedera helix*		Penetrated *Hedera helix* to oriental plane	
	Wood (%)	Bark (%)	Wood (%)	Bark (%)
Palmitic Acid	9.57	18.67	9.78	3.46
Propanoic Acid	3.46	0.74	2.24	0.85
Stearic Acid	3.47	3.14	2,07	4.65
Oleic Acid	7.71	3.45	1.22	16.03
Linoleic Acid	26.98	25.40	22.49	6.18
Linolenic Acid	-	-	6.12	6.06
Miristic Acid	-	-	6.42	3.78
Palmitoleic Acid	-	12,26	-	17.09
Tetracosanoic Acid	4.25	6.94	-	6.09
Dimethylmalonic Acid	-	-	0,68	-
Octadecanol	-	-	4.65	2.35
Hexadecanol	-	-	2.49	3.15
Phtyol	-	4.50	-	-
Ethanol	-	5.55	-	0.76
Trans-Farnesol	-	4.62	-	2.48
2-Methylpentanol	-	-	1.18	0.45
2-Phenyl-1,2-propanediol	-	-	-	0.57
Scyllo-Inositol	-	1.77	-	1.58
Stigmasterol	8.04	2.27	4.73	5.65
Stigmastenol	-	-	2,10	-
Linalool	1.00	-	-	-
Hexacosane	-	-	5.03	-
Octacosane	-	-	6.45	3.78
Eicosane	-	-	-	2.89
Docosane	-	-	3.69	1.23
Diethylacetamide	9.39	2.32	6.09	2.58
N-Ethylacetamide	18.08	4.54	11.73	5.17
Decanediamide	-	2.29	-	0.52
Phosphoric Acid	3.91	-	0.84	0.86
Oxalic Acid	-	0.74	-	-
O-Menthone	1.11	-	-	-
Succinate	-	2.80	-	1,79
D-Threo-2,5-Hexodiulose	3.03	-	-	-

Wood and bark samples were taken from the same tree due to concepts of the value of environmental factors such as the area where plants are located in their growing areas. So, the extractive substances in normal ivy and in ivy penetrating to oriental plane, wood and bark were compared.

In hexane extracts, the highest component in normal and penetrating wood samples was linoleic acid (26.98%, 22.49%, respectively). Moreover, in bark samples, while linoleic acid (25.40%) was determined as the highest in normal *H. helix*, palmiteloic acid (17.09%) was the most abundant compound in penetrating tree. When observed in terms of components, the highest amount of the components in both normal and

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penetrating *Hedera helix* was the same while different components come to the fore in bark samples.

Table 3. *The acetone: water-soluble extractive components determined by chromatographic analysis (%)*

Name of Component	Normal *Hedera helix*		Penetrating Hedera helix to oriental plane	
	Wood (%)	Bark (%)	Wood (%)	Bark (%)
Palmitic Acid	1.17	-	0.51	1.09
Linoleic Acid	1.94	1.32	1.87	6.22
Oleic Acid	-	1.10	-	1.54
Glycerol	3.33	1.68	3.09	2.30
Arabinitol	7.51	1.50	0.72	-
Galactitol	0.85	1.07	-	-
Myo-Inositol	-	-	1.60	-
Erythritol	-	2.04	-	-
D-Glucitol	-	-	-	1.39
2-Methyl-4-Keto-Pentan-2-Ol	-	3.70	5.38	-
2-Keto-D-Gluconic Acid	0.61	2.03	1.96	1.50
α-D-Gluco-pyranoside	-	-	58.47	-
Arabinopyranose	-	1.07	-	-
α-D-Manno-pyranose	-	6.58	-	4.18
β-D-Glucopyranose	-	-	-	2.81
D-Fructose	2.21	-	4.32	3.55
D-Turanose	-	3.38	-	2.86
Maltose	-	-	-	8.51
β-D-Glucose	1.81	-	2.92	-
α-D-Galactose	-	-	0.51	-
D-Galactose	-	-	-	0.73
Glucopyranose	2.09	-	3.76	0.98
Sucrose	47.10	35.94	-	43.15
Maltose	0.91	5.11	3.68	-
Oxalic Acid	0.85	-	0.74	-
Benzoic Acid	-	5.39	-	0.75
β-Hydroquinone	3.56	6.99	0.69	2.56
6,7Dihydroxycoumarin	6.74	12.74	0.86	7.94
Diethylacetamide	2.91	1.65	2.57	1.54
Ethylacetamide	5.68	3.15	5.08	3.00
Uridine	6.02	3.56	1.25	-
Pentane	4.71	-	-	3.40

In the wood samples, acetone:water (95: 5, v: v) extract experiments to determine the structure and amount of hydrophilic components, sucrose (47.10%) was determined as the highest in normal *Hedera helix* wood while α-D-Glucopyranoside (58.47%) was the dominant compound in penetrating wood. In addition, sucrose had the highest amount both in normal and penetrating bark of *H. helix*, i.e. 35.94%, 43.15%, respectively.

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Conclusion

Lipophilic and hydrophilic components of normally grown ivy and ivy penetrating to oriental plane tree in Turkey were obtained by extraction with hexane and acetone:water solvents. Analysis of the extractives were performed by GC-MS. Herein, the wood and bark of normal and penetrating ivy plants grown around Filyos Stream in the Çaycuma district of Zonguldak (Turkey) have been examined for the first time. It was determined that the amount of fatty acids in the bark of ivy is high. In order to evaluate this plant in industrial areas, more comprehensive studies should be done. On the other hand, this study provides a source for future research on the chemical analysis and influence of ivy penetration on plane tree wood and bark, providing a basis for the comparison and evaluation of similar conditions.

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Minerals

	Root							
	N	P	Ca	Mg	K	Na	K/Na	Pb
Control	2.81±0.03^{a}	0.31±0.02^{b}	0.35±0.00^{b}	0.49±0.00^{e}	1.64±0.01^{b}	0.62±0.02^{b}	2.63±0.10^{bc}	0.00
Pb	1.71±0.06^{c}	0.22±0.01^{c}	0.34±0.00^{c}	0.66±0.00^{c}	1.16±0.06^{d}	0.71±0.02^{a}	1.64±0.12^{e}	1.25
2.5% MLE	2.78±0.05^{a}	0.31±0.01^{b}	0.35±0.00^{b}	0.71±0.01^{a}	1.49±0.02^{c}	0.60±0.00^{b}	2.48±0.03^{c}	0.00
Pb+2.5% MLE	2.64±0.07^{b}	0.38±0.07^{a}	0.33±0.00^{d}	0.55±0.00^{d}	1.16±0.06^{d}	0.62±0.00^{b}	1.87±0.09^{d}	0.34
5% MLE	2.73±0.03^{a}	0.34±0.06^{ab}	0.30±0.00^{e}	0.68±0.01^{b}	1.64±0.04^{b}	0.60±0.00^{b}	2.73±0.05^{b}	0.00
Pb+5% MLE	2.64±0.02^{b}	0.39±0.02^{a}	0.36±0.00^{a}	0.70±0.01^{a}	2.00±0.01^{a}	0.62±0.01^{b}	3.25±0.01^{a}	0.18
Statistics								
F	230.47	8.60	361.64	266.16	146.31	32.70	117.93	18872.6
P	0.0000 ***	0.0012 **	0.0000 ***	0.0000 ***	0.0000 ***	0.0003 ***	0.0000 ***	0.0000 ***
LSD at 0.05	0.0847	0.0657	0.0042	0.0193	0.0921	0.0249	0.1884	0.0122

	Shoot							
	N	P	Ca	Mg	K	Na	K/Na	Pb
Control	2.47±0.11^{bc}	0.32±0.02^{cd}	1.05±0.00^{b}	0.34±0.01^{d}	2.05±0.08^{b}	0.56±0.02^{c}	3.63±0.26^{b}	0.00
Pb	1.68±0.01^{e}	0.24±0.02^{d}	0.92±0.01^{d}	0.42±0.00^{c}	1.67±0.03^{c}	0.63±0.00^{a}	2.65±0.05^{c}	0.28
2.5% MLE	2.61±0.12^{ab}	0.49±0.04^{b}	1.04±0.00^{b}	0.60±0.01^{b}	2.49±0.01^{a}	0.60±0.02^{ab}	4.15±0.13^{a}	0.00
Pb+2.5% MLE	2.35±0.12^{c}	0.54±0.06^{b}	0.93±0.00^{cd}	0.41±0.01^{c}	2.01±0.01^{b}	0.62±0.01^{a}	3.27±0.04^{b}	0.12
5% MLE	2.71±0.03^{a}	0.88±0.16^{a}	1.18±0.03^{a}	0.67±0.01^{a}	2.40±0.01^{a}	0.59±0.02^{abc}	4.04±0.12^{a}	0.00
Pb+5% MLE	2.08±0.06^{d}	0.42±0.04^{bc}	0.96±0.00^{c}	0.32±0.04^{d}	1.94±0.09^{b}	0.58±0.01^{bc}	3.34±0.24^{b}	0.08
Statistics								
F	59.89	27.47	90.22	126.96	70.83	5.34	23.03	40.75
P	0.0000 ***	0.0000 ***	0.0000 ***	0.0000 ***	0.0000 ***	0.0325 *	0.0008 ***	0.0000 ***
LSD at 0.05	0.1518	0.1307	0.0355	0.0438	0.1253	0.0359	0.3974	0.0299

PRIMING WITH MORINGA (*MORINGA OLEIFERA* LAM.) LEAF EXTRACT BOOSTS THE GROWTH AND PHYSIO-BIOCHEMICAL ATTRIBUTES OF LEAD-STRESSED FENUGREEK (*TRIGONELLA FOENUM-GRAECUM* L.) SEEDLINGS

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Abstract. Lead (Pb) is a potentially toxic environmental concern, affecting all forms of life on the planet. However, moringaleaf extract (MLE) attained enormous attention owing to its high phytohormones, antioxidants and essential nutrient contents. To evaluate the potential of MLE in alleviating the phytotoxicity of Pb on fenugreek seedlings, a pot experimentwas conducted at the laboratory of Biological Sciences Department, Jeddah University. The uptake of Pb by fenugreek seedlings negatively affected growth rate, chlorophyll content, soluble sugars, enzymatic and non-enzymatic antioxidants, availability of the macronutrients (N, P, Ca, K), as well as K/Na ratio. Contrastingly, H_2O_2, MDA, soluble proteins and Mg content were increased. Seed priming in the aqueous MLE significantly ameliorated the ionic and osmotic deteriorations resulting fromPb uptake by fenugreek seedlings.MLE potentially retrieved seedling growth, balanced carbon and nitrogen metabolism, decreased the ROS-induced oxidative damage, in addition to sustaining ionic and osmotic homeostasis in Pb-stressed fenugreek seedlings. To sum up, the present study proved MLE as a potent eco-friendly approach to counteract Pb-phytotoxicity.
Keywords:*metalpollution, Moringaoleifera, stress markers,antioxidantpotential, ionic homoeostasis*

Introduction

Lead (Pb) is one of the most hazardous non-essential heavy metals in any ecosystem, it accumulates in excessive concentrations due to its non-degradable nature(Mahaffey, 1990).Lead pollution can originate from many sources like leaded gasoline, agro-fertilizers, mining processes, stoneware, boat constructing, paints, pipes manufacturing, batteries, artificial limbs, colorants, ink industry and traffic exhausts(Karri et al., 2008). Exposure of plants to lead stress results in damaging effectstotheir morphology, growth, biochemistry, ultrastructure, photosynthesis,productivityand quality. The deteriorations caused by lead stress are time, dose and growth-stagedependent. Several reports revealed that lead exposure had resulted in reduced germination ratio, biomass production, protein content (Hussain et al., 2013), photosynthetic activity, lamellar organization of the chloroplast (Hu et al., 2007) and yield criteria (Sobhy et al., 2019). Moreover, Pb stress triggered the increase in the level of some stress markers like membrane leakage, lipid peroxidation (MDA), H_2O_2 and $OH^{\bullet}$ radical in wheat (*Triticum aestivum* L.) seedlings (Sobhy et al., 2019). The same authors reported that the activities of particular antioxidant enzymes (SOD, CAT and POD) were increased in wheat seedlings due to up-regulation of the decoding genes.

On the organelles level, lead toxicity had been proven through the damage of cristae, enlargement of mitochondria, distortion of endoplasmic reticulum, damageddictyosomesand irregular darknuclei in different plant cells (Sandalio et al., 2001).

Fenugreek (*Trigonellafoenum-graecum* L.) is a self-pollinating annual forage leguminous plant (Family: *Fabaceae*) native to the Eastern Mediterranean region but is widely distributed in various parts of the world (Abdel Latef et al., 2017). The plant

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possesses a long taproot, long erect cylindrical stem and trifoliate leaves with inversely ovate leaflets. After blossoming, the plant produces long pods with 10 to 20 angular-flattened seeds. Many factors, like farming practices, climatic conditions, use of herbicides and fertilizers, irrigation systems and genotypes, affect seed yield and quality of fenugreek (Pavlista and Santra, 2016). Choudhary et al. (2012) reported that number of pods/plant and number of seeds/pod in fenugreek subjected to Pb-stress were decreased in a dose-dependant manner.

Traditionally, fenugreek leaves and seeds have been used extensively in the treatment of various ailments (Basch et al., 2003). It has been used for centuries in folkloric medicine as a hypocholesterolemic, antidiabetic, antineoplastic, antitumor, antioxidative, anti-inflammatory, antiulcerogenic, antihypotriglyceridemic and antipyretic substance (Dixit et al., 2010; Xue et al., 2011). The therapeutical activity of fenugreek has been attributed to a variety of metabolites such as vitamins, amino acids, fatty acids, saponins (neogitogenin, disogenin, gitogenin, saponaretin, homorientin, neogigogenin and trigogenin) polysaccharides, fibers, fixed oils, flavonoids, and alkaloids (trigonelline and choline) (Yoshikawa et al., 1997).

The natural phyto-extracts of some algae, weeds, and plant species have been ascribed to be effective tools in ameliorating the deteriorations caused by various biotic and abiotic stressors.Saad-Allah and Nessim (2016) reported that the aqueous extract of the seaweed *Halimedaopuntia* potentially augmented cadmium toxicity in rocket plant (Eruca sativa Mill.) via improving the water content, photosynthesis efficiency and water-soluble osmolytes. Also, Hegazi et al. (2015)concluded that the extract of *Ascophyllumnodosum* seaweed functioned as an anti-salinity agent through the upregulation of some antioxidant enzymes level and enhancing the uptake of some essential macronutrients.In the meantime, Rady et al. (2013)affirmed that salt imposed common bean(*Phaseolus vulgaris*L.) seedlings exhibited an improved growth rate by seeds pre-soaking in the leaf extract of *Moringaoleifera*.The extract had increased the antioxidative, osmoprotective and ion uptake potentials of NaCl-stressed seedlings. Likewise,Abdel Latef et al. (2017) studied the response of salt-stressed fenugreek seedlings to the foliar application of fresh moringa leaf extract. They concluded that the interruption in the growth rate of stressed fenugreek had been resumed through the enhanced metabolic activity, minerals uptake and up-regulation of some salt tolerance genes. In the present study, we aimed at assessing the morpho-physiological, antioxidant and nutritional status responses of lead-stressed fenugreek seedlings to seed priming in the powdered moringa leaf extract.

Materials and Methods

Preparation of aqueous moringa leaf extract

The fully expanded and healthy fresh moringa(*Moringaoleifera* Lam.) leaves were collected from the streets of Jeddah city, KSA during the summer season (August 2019). The collected leaves were washed several times with tap water then once with distilled water and dried in an electrical oven at 60°C till constant weight. The dried leaves were ground using an electric mixer and sieved through a 0.2 mm sieve. The aqueous extract of moringa leaves was prepared by soaking of 50 g of the powdered leaves in 1L of the distilled water in a 2L Erelnimerflask. The flask was placed on a rotary shaker (180 rpm) for 24 hours then the extract was filtered through Whatman No1 filter paper to get rid of leaf tissues and the supernatant was taken and used in the

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preparation of two different concentrations (2.5 and 5.0%) of the aqueous extract. The prepared extracts were placed in the fridge at 4°C until the time of use.

Growth conditions and treatments

Seeds of fenugreek (*Trigonellafoenum-graecum*L.) were obtained from the Ministry of Environment, Water and Agriculture, Mecca branch, Kingdom of Saudi Arabia (KSA) and chosen for superficial homogeneity of size and shape. The seeds were disinfected in 5% Clorox for 4 minutes with stirring then washed with distilled water. The seeds then were separated into 2 groups; the first was soaked in distilled water for 24 hours as a control and the second was primed by soaking either in2.5 or 5.0% of aqueous moringa leaf extract (MLE) solutions for 24hours. Thereafter,10 seeds of every treatment were sown in plastic pots (25 cm diameter and 18 cm depth) filled with 8 kg of clay-sandy soil (2:1 w/w) and four pots were used as a replica for each treatment. Seeds were irrigated with tap water and left to grow for 5 days, before the treatment with Pb stress, under normal environmental conditions (10 hours photoperiod at 28/16°C±2 day/night and 62% relative humidity) at the laboratory of Biological Science Department, Faculty of Science, Jeddah University, KSA. The Pb stress was applied as lead acetate (100 mMPb$(CH_3COO)_2$ solution), while the 5-days old seedlings were irrigated once at 70% field capacity with tap water (the sub-lethal concentration of Pb$(CH_3COO)_2$ (100 mM) was previously determined in a preliminary experiment). The freshly harvested 25-days old seedlings were separated into roots and shoots, washed thoroughly with tap water several times followed by deionized water,and stored at -80°C. For dry matter analysis, the plant samples were oven-dried at 60°C till constant weight. Three replicates were utilized for each treatment of all the experiments.

Growth parameters

The collected 25-days old seedlings were used to assess Pb toxicity through measuring growth criteria (lengths, fresh and dry weights of both roots and shoots).

Photosynthetic pigments

The third fully-expanded leaf in each seedling was selected to estimate chlorophyll a and b by Arnon (1949) method and carotenoids by Horvath et al. (1972) method. Concisely, 0.1 g of fresh leaves were extracted in 85% cold acetone, whereas the absorbance was measured using a UV/visible spectrophotometer and expressed as mg/g fw.

Soluble sugars and protein estimation

The total free sugars fraction in dry leaves and roots of fenugreek was estimated using the phenol-sulfuric acid method (Dubois et al., 1965) with glucose as a standard sugar. Protein fraction was assessed using Bovine serum albumin (BSA) as a standard protein by the method of Bradford(1976). The quantified sugars and proteins were expressed as mg/g dw.

Hydrogen peroxide and lipid peroxidation quantification

Hydrogen peroxide(H_2O_2) content of fresh fenugreek leaves was estimated by the method given byVelikova et al. (2000). TheH_2O_2content of leaves was calculated using the extinction coefficient (0.28 $\mu M^{-1}cm^{-1}$) and expressed as µmol/gfw.Malondialdehyde (MDA) in fresh leaf homogenates was estimated according to the method ofHeath and

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Packer (1968) by the aid of thiobarbituricacid(TBA) using the extinction coefficient (155 mM^{-1} cm^{-1}) and expressed as nmol/gfw.

Enzymatic activity assay

Fresh leaves (0.5 g) were homogenized in pre-chilled 0.1MK-phosphate buffer (pH 6.8) containing 0.1 mM EDTA. Homogenates were centrifuged at10000 rpm for 15 min and the resultant supernatant was used for enzymes assay.

The activity of CAT(catalase) wasmeasured by the method of Havir and McHale (1987). The decline in H_2O_2 was monitored spectrophotometricallyat 240 nm and the activity was calculated using the molar extinction coefficient 36 x 10^3 mM^{-1} m^{-1} and expressed as µM/g fw. min^{-1}.

POD (peroxidase) activity as assessed in the leaf extracts using 7.2 mMguiacol and the reddish-brown color development was monitored using a UV/vis spectrophotometer at 470 nm. POD activity was expressed asµM/g fw min^{-1} based on the molar extinction coefficient of26.6 mM^{-1} cm^{-1}(Kato and Shimizu, 1987).

APX(ascorbate peroxidase) activity was determined using a mixture of 0.5 mM ascorbic acid, 0.1 mM H_2O_2, K-phosphate buffer and 1 mM EDTA-sodium salt (Nakano and Asada, 1981). The decrease inascorbateabsorbance at 290 nm and APX activity was calculated as µM/g fw. min^{-1}using the extinction coefficient of 2.8 mM^{-1} cm^{-1}.

Polyphenol oxidase (PPO) activity of leaf homogenates was determined as described by Kumar and Khan (1982)in a mixture of 100 mM K-phosphate buffer and 100 mMcatechol. Sulfuric acid (2.5 N) was used to stop the reaction and the absorbance of the developed purpurogallin was measured at 495 nm. PPO activity was expressed as µM/g fw min^{-1}using the extinction coefficient of catechol (22.6$mM^{-1}cm^{-1}$).

Radical scavenging and total antioxidant capacity

The free radicals scavenging activity of the root and shoot ethanolic extracts was evaluated using the technique adopted by Hatano et al. (1988). A 0.1 ml aliquot of each extract was well vortexed with 3 ml of 36 x 10^{-2}% 1,1-diphenyl-2-picrylhydrazyl (DPPH˚) radical in methanol and kept for 30 min in dark. The bleaching in DPPH color by the extracts was monitored at 417 nm using UV/vis. spectrophotometer and the activity was expressed as a percentage.

The total antioxidant capacity (TAC) of the extracts was estimated using 3 ml of phosphomolybdate reagent (0.6 mol. $L^{-1}H_2SO_4$, 28 mmol L^{-1} sodium phosphate and 4 mmol L^{-1}amm.molybdate) and 300 µL of the extracts. The contents were boiled at 95°C for 90 min, cooled and the absorbance was recorded at 765 nm. The TAC was reported in µg/g dwusing ascorbic acid as a standard (Ahmed et al., 2012).

Mineral ions content

The dry plant root and shoot samples were wetly digested by a mixture of 70% $HNO_3$and 30% H_2O_2(5:2 v/v). The concentrations of Ca, Mg, K, Na and Pbin the digested samples were quantified using an inductively coupled plasma-opticalspectrophotometer(Polyscan 61E, Thermo Jarrell-Ash Corp., Franklin, MA, USA) at the Central Lab of Jeddah University.Nitrogen and phosphorus were calorimetricallyestimated in the samples digest by the assay of Allen et al. (1974) using Rochelle reagent for N and molybdenum blue method for P against their standard

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calibration curves. The mineral content of root and shoot samples was expressed as mg/g dw.

Statistical analysis

The experimental results were expressed as a mean of three replicates ± the standard error (SE). The obtained data were statistically analyzed(ANOVA test) using SPSS software (V20) and the variations between means were evaluated using the LSD test at 5% level. All the experimental values were compared to the control treatment.

Results

Growth parameters

In the current study, fenugreek seedlings treated with 100 mMlead acetate exhibited evident toxicity symptoms reflected as a reduction in growth parameters as shown in *Fig. 1*. Compared with the control, Pb stress resulted in a significant reduction in the fresh and dry weights of both shoot and root. The presoaking of fenugreek seeds in aqueous moringa leaf extracts (MLEs) was considerably effective in increasing the growth parameters of non-stressed fenugreek seedlings, but non-significantly alleviated the growth reduction mediated by Pb; whereas the degree of alleviation was dose-dependent. Compared to Pb treatment, priming in 5% MLE was the most valuable and slightly increased the growth parameters offenugreek seedlings.

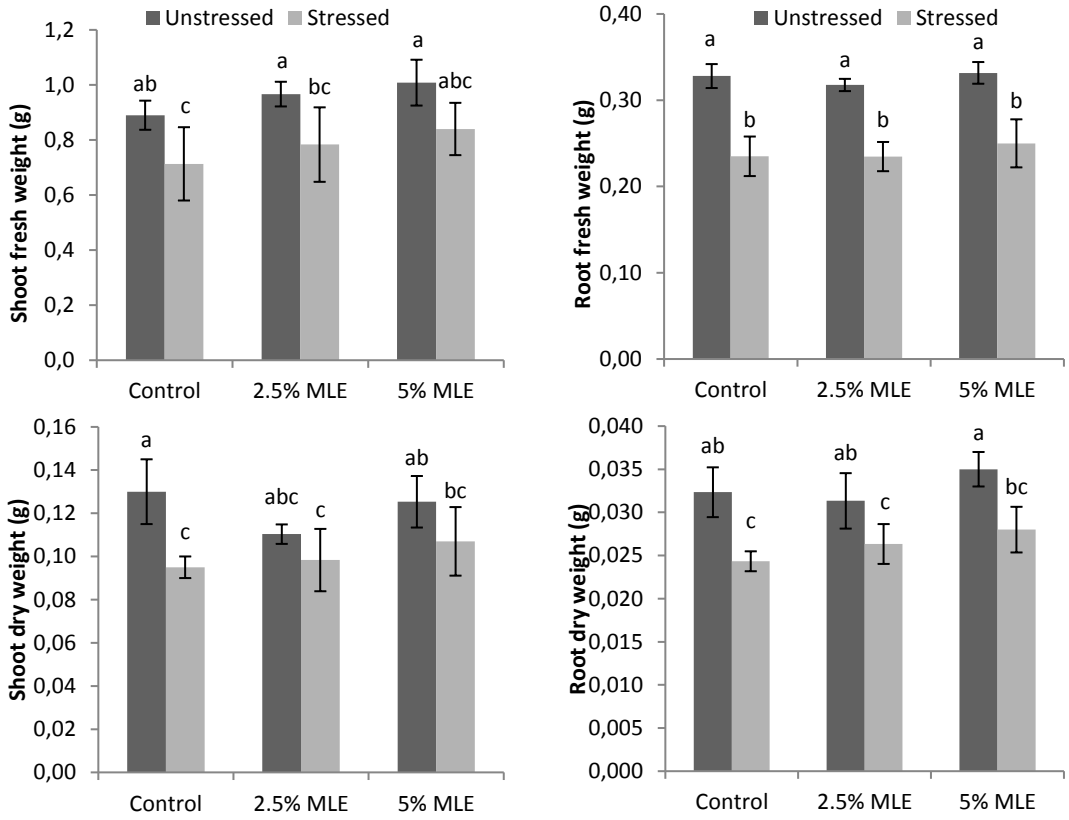

Figure 1. *Growth parameters of Pb-stressed and pre-soaked in MLE (0.0, 2.5 and 5%) fenugreek seedlings. Different letters indicate significant differences at 5% level and the error bars represent SD*

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Photosynthetic pigments

The application of Pb was extremely destructive concerning photosynthetic pigments as observed in *Fig. 2*. The leaves content of Chla andChl b were significantly declined with percentages of 26.47and 49.50%, respectively compared to the control. Meanwhile, carotenoids content was significantly increased following lead treatment, the percentage of increase was 27.11% as compared to the control. The priming with MLE triggered an enhanced response and ameliorated Pb-stress by raising the level of chlorophyll content and lowering that of carotenoids to a significant level as compared to Pb treatment. Compared to Pb stress, 5% MLE had a more pronounced impact by increasing chlorophyll a and b contents and lowering carotenoids content.

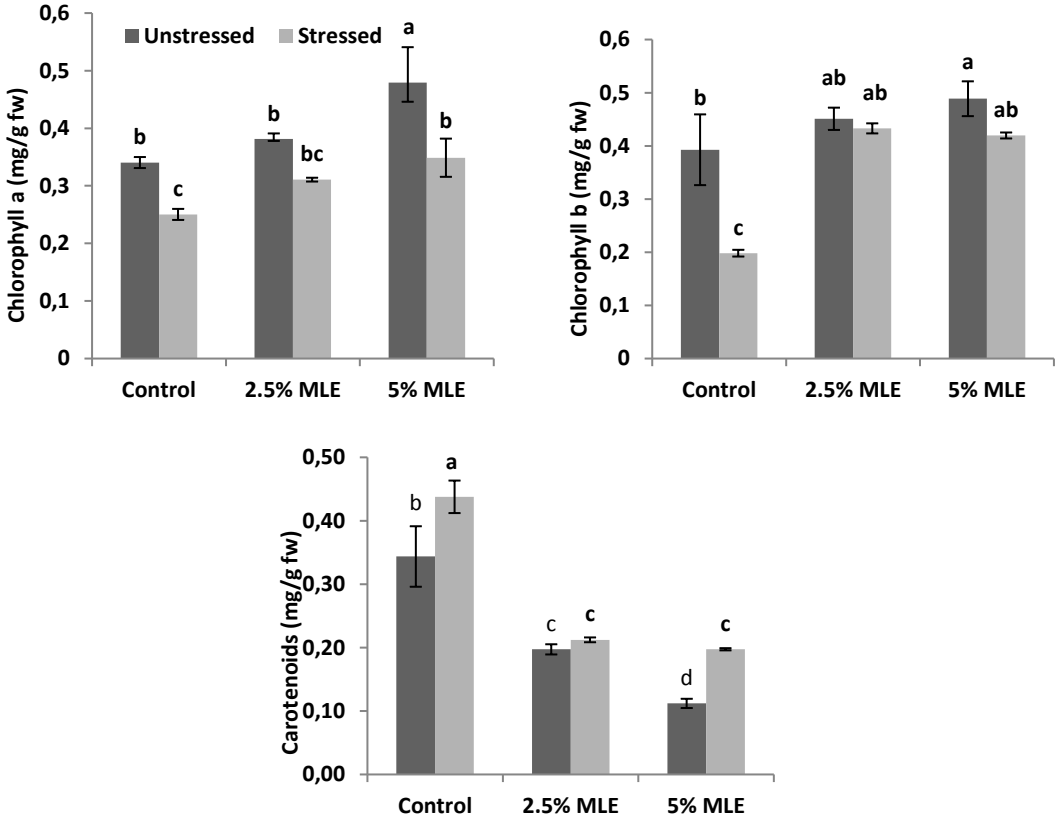

Figure 2. *Photosynthetic pigments of Pb-stressed and pre-soaked in MLE (0.0, 2.5 and 5%)fenugreek seedlings. Different letters indicate significant differences at 5% level and the error bars represent SD*

Soluble sugars

The total soluble sugars content of thefenugreek seedlings root and shoot was significantly affected by Pb-stress as represented in *Fig. 3*. The exposure to 100 mM lead acetate had lowered fenugreek root and shoot sugar content, but the effect was more pronounced on the root, as the reduction was with percentages of 43.12 and 11.90% in root and shoot, respectively, compared with the control. However, priming in MLE had declined the sugar content in fenugreek root and shoot. The most evident decline was observed at 5% MLE in the root and 2.5% MLE in the shoot. The combined

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interactions of Pb and MLE non-significantly affected soluble sugar contents of both root and shoot, comparable to Pb-stress.

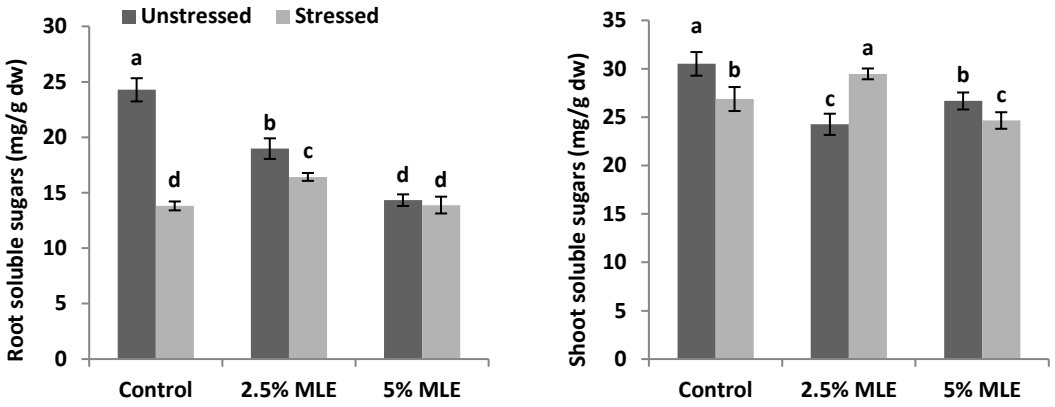

Figure 3. *Soluble sugars content in roots and shoots of Pb-stressed and pre-soaked in MLE (0.0, 2.5 and 5%) fenugreek seedlings. Different letters indicate significant differences at 5% level and the error bars represent SD*

Soluble proteins

Contrasting to soluble sugars, the soluble protein content of fenugreek seedlings was significantly increased as affected by lead treatment (*Fig. 4*). The achieved increment in protein level was 46.24 and 113.75% in root and shoot, respectively, relative to the control's content. Likewise, MLE treatments have increased the content of proteins in fenugreek seedlings compared with the control, especially 2.5% dose in roots and 5% in shoots. Meanwhile, the priming in MLEs was effective in decreasing the soluble protein content of Pb-stressed fenugreek seedlings, but the level still close to or slightly higher than that of the control.

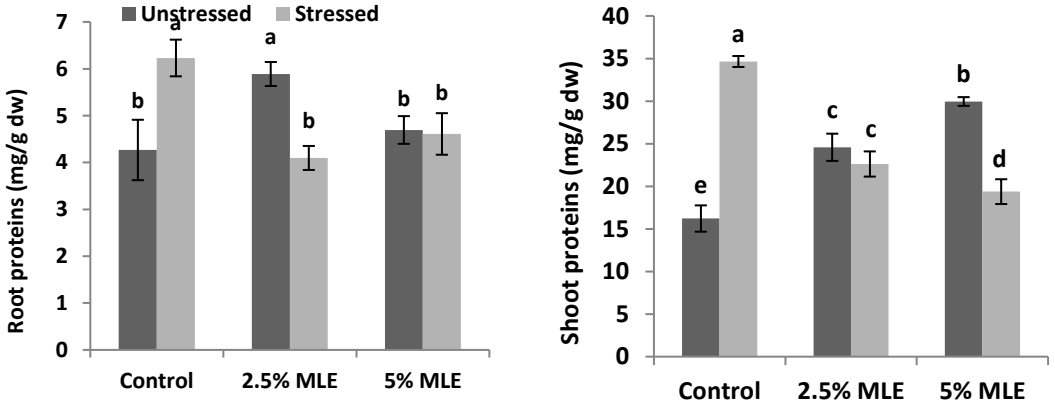

Figure 4. *Soluble proteins content in roots and shoots of Pb-stressed and pre-soaked in MLE (0.0, 2.5 and 5%) fenugreek seedlings. Different letters indicate significant differences at 5% level and the error bars represent SD*

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Stress indices

Hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) contents in the leaves of fenugreek seedlings were significantly affected by the applied dose of lead stress (*Fig. 5*). Irrigating fenugreek seedlings with 100 mMPb-acetate triggereda reasonable increment in the oxidative stress markers;H_2O_2 and MDA, in the leaf tissues, compared to the control. The applied dose of Pb acetate showed 43.24 and 47.90% increase in the level of H_2O_2 and MDA, respectively, relative to the control levels. Nevertheless, seed pre-soaking in MLE effectively lowered the leaf content of both stress markers;H_2O_2 and MDA, in fenugreek leaves, particularly 2.5% MLE. Likewise, the pre-treatment with MLE efficiently ameliorated the deleterious impact of Pb-stress on fenugreek seedlings.

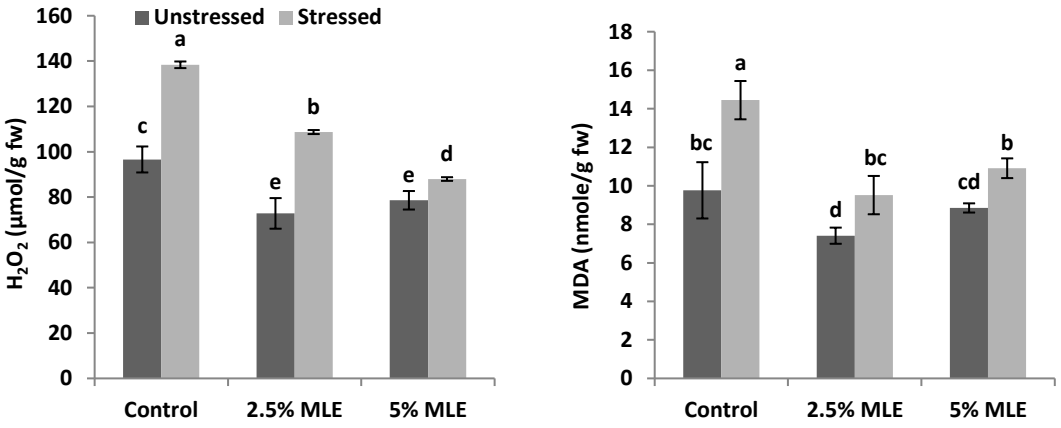

Figure 5. *Stress markers (H_2O_2 and MDA) of Pb-stressed and pre-soaked in MLE (0.0, 2.5 and 5%) fenugreek seedlings. Different letters indicate significant differences at 5% level and the error bars represent SD*

Enzymatic activity

The activities of catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX) and polyphenol oxidase (PPO) in fenugreek leaves were significantly affected by lead and aqueous moringa leaf extract (MLE) treatments (*Fig. 6*). As for CAT, the exposure of fenugreek seedlings to 100 mMPb resulted in 47.32% decline in CAT activity, compared with the control. Additionally, both MLE treatments caused a sharp decline in CAT activity(53.36 and 53.68% with 2.5 and 5% MLE, respectively). The combined interactions betweenPb and MLE showed the least CAT activity throughout the experiment.

Nonetheless, POD activity showed a 20.84% rise following Pb exposure, but non-significantly affected by both MLE treatments, either as single treatments or combined with Pb stress, compared with the untreated control. Likewise, Pb stress significantly affected APX activity, as it caused a 31.59% increase compared to the control. Both, 2.5 and 5.0%MLE treatments non-significantly diminished APX activity relative to the control activity, 5.95 and 13.69%, respectively. Combined interaction of Pb and MLE was concentration dependant. The low dose (2.5%) slightly declined APX activity, but the higher dose (5.0%) reasonably increased its activity, compared to their stress counterparts.

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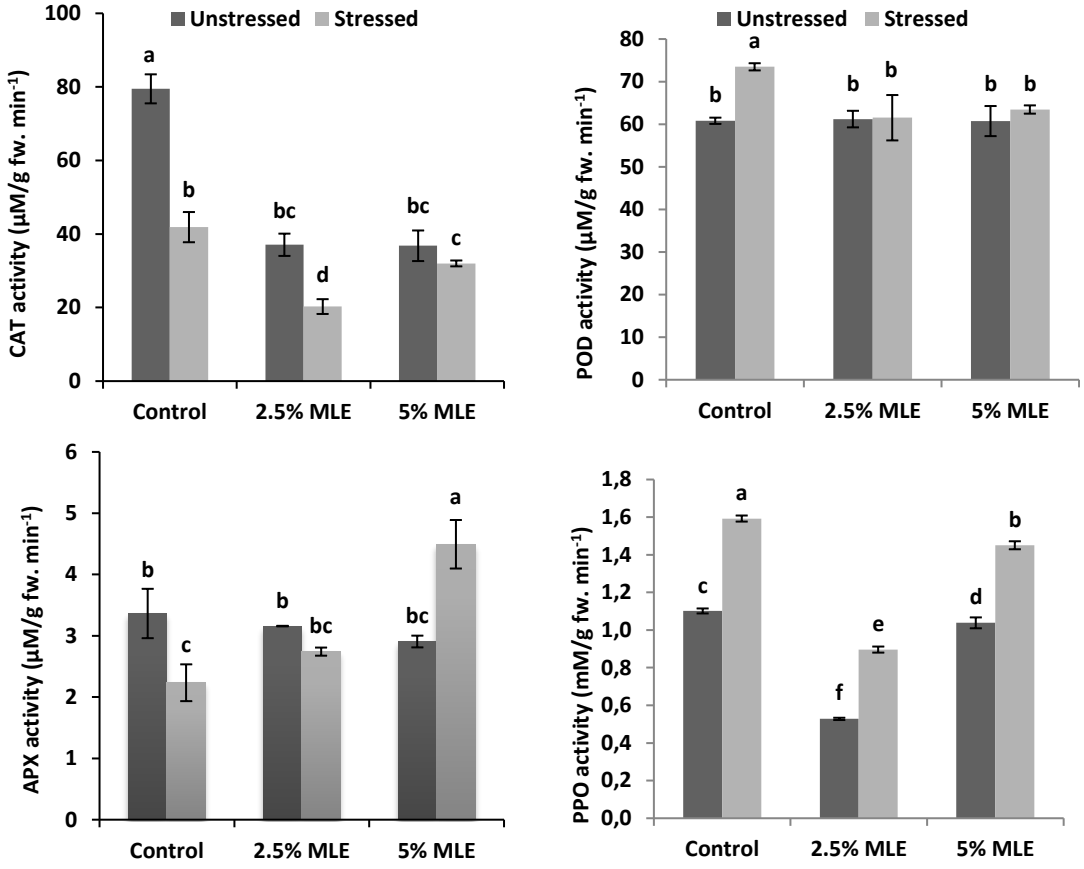

Figure 6. *Enzymatic activities (CAT, POD, APX and PPO) of Pb-stressed and pre-soaked in MLE (0.0, 2.5 and 5%) fenugreek seedlings. Different letters indicate significant differences at 5% level and the error bars represent SD*

Like POD, PPO activity was markedly induced (44.54%) by Pb treatment. However, both MLE treatments; 2.5 and 5.0%,showed a pronounced decrease in PPO activity in comparison with the untreated control, particularly the low dose (49.09 and 5.45%, respectively). Combined treatments of Pb and MLE significantly enhanced PPO activity, compared to single MLE treatments, but the activity still lower than that of Pb sole treatment.

Free radical scavenging activity

The free radical (DPPH) scavenging activity of fenugreek root and shoot ethanolic extracts was significantly affected by Pb and MLE treatments (*Fig. 7*). Treatment of fenugreek seedlings with 100 mMPb-acetate non-significantly lowered the DPPH activity of the root and the shoot extracts (3.79 and 11.33%, respectively). However, the effect of MLE on DPPH activity was dose-dependent. Priming in 2.5% MLE significantly increased the DPPH activity in both root and shoot of fenugreek extracts (33.67 and 18.83%, respectively), but priming in 5.0% MLE resulted in a 16.15 and 19.26% decrease in the DPPH activity of root and shoot extracts, respectively.

The interactive combinations of MLE and Pb treatments decreased the scavenging activity of fenugreek ethanolic root and shoot extracts, except for the mutual treatment with both Pb-acetate and 5.0% MLE in case of the shoot, this treatment remarkably

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decreased the DPPH activity of the ethanolic extract, compared to the sole Pb treatment. All in all, the DPPH scavenging activity of fenugreek shoot extracts was higher than those of the root under all treatments.

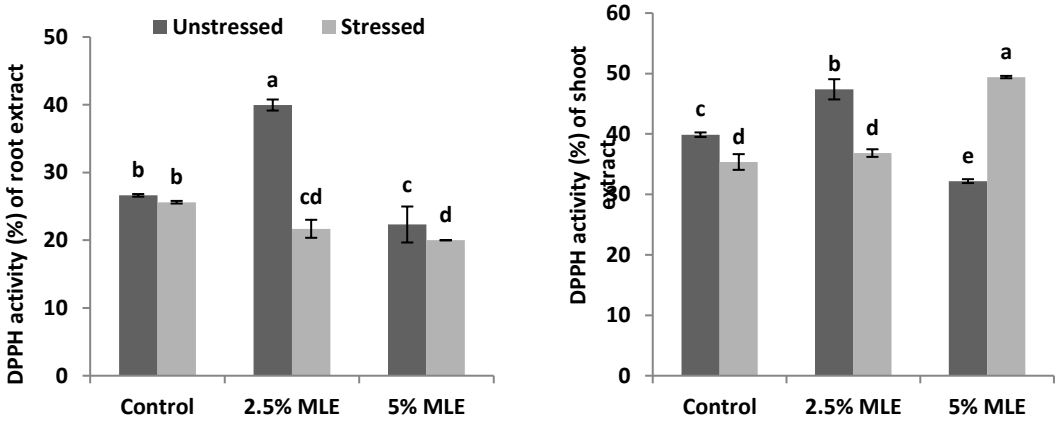

Figure 7. *DPPH free radical scavenging activity of root and shoot ethanolic extracts of Pb-stressed and pre-soaked in MLE (0.0, 2.5 and 5%) fenugreek seedlings. Different letters indicate significant differences at 5% level and the error bars represent SD*

Total antioxidant capacity

The phosphomolybdateassay is used as a criterion for total antioxidant capacity (TAC) in fenugreek root and shoot ethanolic extracts (*Fig. 8*). The TAC of root extract was not affected by Pb treatment, however, that of the shoot extract was slightly decreased, compared to the non-stressed counterparts. The TAC of the root extract was significantly raised by priming in 2.5% MLE, however priming in 5.0% MLEdid not affect the TAC, compared to the control treatment. As for shoot ethanolic extract, 2.5% MLE did not affect TAC but 5.0% MLE addressed a slight decrease inTAC, comparable to the control treatment.

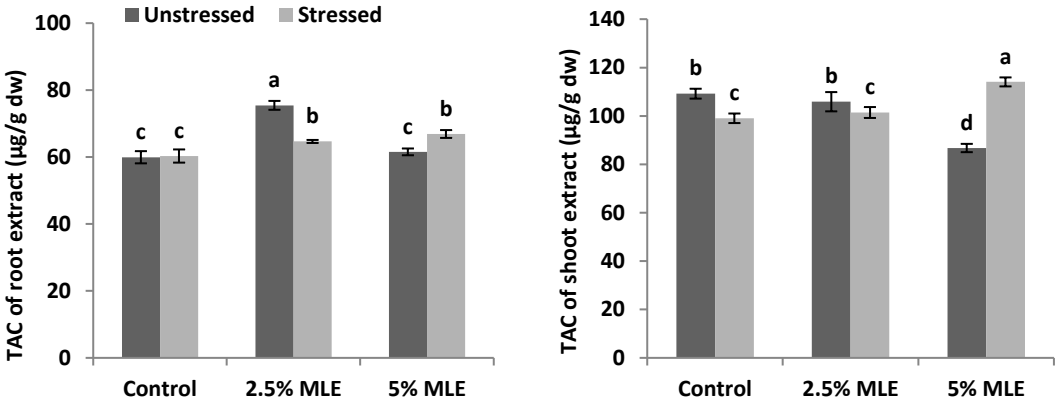

Figure 8. *Total antioxidant capacity (TAC) of root and shoot ethanolic extracts of Pb-stressed and pre-soaked in MLE (0.0, 2.5 and 5%) fenugreek seedlings. Different letters indicate significant differences at 5% level and the error bars represent SD*

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The interactive treatments of Pb-acetate and MLE were concentration dependant. The low concentration of MLE (2.5%) decreased the TAC, but the high concentration (5.0%) increased it in the root and the shoot extracts, relative to the individual lead-exposed treatments.

Elemental content of fenugreek root

The contents of N, P, Ca, Mg, K, Na and Pb, as well as K/Na ratio in the roots of fenugreek grown under Pb-stress and pre-soaked in MLE were determined and tabulated as mg g^{-1}dw (*Table 1*). Treatment with Pb-acetate resulted in a pronouncedaccumulation of Pb ions in theroots of stressed-untreated fenugreek seedlings as compared with the control. Comparable withPb treatment, priming with2.5 and 5.0%MLE distinctly declined the Pb content of fenugreek. Likewise, the levels of Mg and Na were increased as affected by lead stress, but the priming with MLE lowered their accumulation to values relatively near to the control. However, N, P, and K contents, as well as the ratio of K/Na, were decreased by the exposure of fenugreek to Pb stress, but Cacontent relatively remained unaffected by Pb treatment. Fenugreek priming with MLE significantly increased the root content of N, P, and K ions of Pb-stressed fenugreek, supporting improved ionic homeostasis and enhanced root growth.

Table 1. *Ion concentrations (N, P, Ca, Mg, K, Na, and Pb) in the root of Pb-stressed fenugreek seedlings pre-treated with 2.5 and 5.0% MLE. Different letters in the same column represent significant variations at 0.05 level*

Treatments	Mineral ions (mg g^{-1}dw)							
	N	**P**	**Ca**	**Mg**	**K**	**Na**	**K/Na**	**Pb**
Control	2.81±0.03^{a}	0.31±0.02^{b}	0.35±0.00^{b}	0.49±0.00^{e}	1.64±0.01^{b}	0.62±0.02^{b}	2.63±0.10^{bc}	0.00
Pb	1.71±0.06^{c}	0.22±0.01^{c}	0.34±0.00^{c}	0.66±0.00^{c}	1.16±0.06^{d}	0.71±0.02^{a}	1.64±0.12^{e}	1.25
2.5% MLE	2.78±0.05^{a}	0.31±0.01^{b}	0.35±0.00^{b}	0.71±0.01^{a}	1.49±0.02^{c}	0.60±0.00^{b}	2.48±0.03^{c}	0.00
5.0% MLE	2.73±0.03^{a}	0.34±0.06^{ab}	0.30±0.00^{e}	0.68±0.01^{b}	1.64±0.04^{b}	0.60±0.00^{b}	2.73±0.05^{b}	0.00
Pb+2.5% MLE	2.64±0.07^{b}	0.38±0.07^{a}	0.33±0.00^{d}	0.55±0.00^{d}	1.16±0.06^{d}	0.62±0.00^{b}	1.87±0.09^{d}	0.34
Pb+5.0% MLE	2.64±0.02^{b}	0.39±0.02^{a}	0.36±0.00^{a}	0.70±0.01^{a}	2.00±0.01^{a}	0.62±0.01^{b}	3.25±0.01^{a}	0.18
	Statistics							
F	230.47	8.60	361.64	266.16	146.31	32.70	117.93	18872.6
P	0.0000 ***	0.0012 **	0.0000 ***	0.0000 ***	0.0000 ***	0.0003 ***	0.0000 ***	0.0000 ***
LSD at 0.05	0.0847	0.0657	0.0042	0.0193	0.0921	0.0249	0.1884	0.0122

*** = very highly significant, ** = highly significant, * = significant at 0.05 level

Elemental content of fenugreek shoot

The contents of N, P, Ca, Mg, K, Na and Pb,in addition to K/Na ratio in the shoots of fenugreek grown under Pb-stress and presoaked in MLE were estimated and represented as mg g^{-1}dw (*Table 2*). Treatment with Pb-acetate resulted in an evident uptake of Pb ions into thefenugreek shoot system as compared with the control. Comparable with Pb-stress, priming with2.5 and 5.0%MLE distinctly declined Pbcontent (57.14 and 71.42%, respectively)in fenugreek shoot. Similarly, Na level increased as affected by Pb-stress, nevertheless,pre-soaking in MLEslightly decreasedNauptake into fenugreek shoot. On the other hand, other minerals (N, P, Ca, K, andK/Na ratio) were observably decreased by fenugreek exposure to Pb-stress, but Mg content relatively increased as

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affected byPb treatment. Fenugreek priming with MLE significantly increased the shoot content of N, P, Ca, Mg, and K ions of Pb-stressed fenugreek, contributing to ionic homeostasis in the stressed fenugreek seedlings.

Table 2. *Ion concentrations (N, P, Ca, Mg, K, Na, and Pb) in the shoot of Pb-stressed fenugreek seedlings pre-treated with 2.5 and 5.0% MLE. Different letters in the same column represent significant variations at 0.05 level*

Treatments	Mineral ions (mg g^{-1}dw)							
	N	P	Ca	Mg	K	Na	K/Na	Pb
Control	2.47±0.11^{bc}	0.32±0.02^{cd}	1.05±0.00^{b}	0.34±0.01^{d}	2.05±0.08^{b}	0.56±0.02^{c}	3.63±0.26^{b}	0.00
Pb	1.68±0.01^{e}	0.24±0.02^{d}	0.92±0.01^{d}	0.42±0.00^{c}	1.67±0.03^{c}	0.63±0.00^{a}	2.65±0.05^{c}	0.28
2.5% MLE	2.61±0.12^{ab}	0.49±0.04^{b}	1.04±0.00^{b}	0.60±0.01^{b}	2.49±0.01^{a}	0.60±0.02^{ab}	4.15±0.13^{a}	0.00
5.0% MLE	2.71±0.03^{a}	0.88±0.16^{a}	1.18±0.03^{a}	0.67±0.01^{a}	2.40±0.01^{a}	0.59±0.02^{abc}	4.04±0.12^{a}	0.00
Pb+2.5% MLE	2.35±0.12^{c}	0.54±0.06^{b}	0.93±0.00^{cd}	0.41±0.01^{c}	2.01±0.01^{b}	0.62±0.01^{a}	3.27±0.04^{b}	0.12
Pb+5.0% MLE	2.08±0.06^{d}	0.42±0.04^{bc}	0.96±0.00^{c}	0.32±0.04^{d}	1.94±0.09^{b}	0.58±0.01^{bc}	3.34±0.24^{b}	0.08
	Statistics							
F	59.89	27.47	90.22	126.96	70.83	5.34	23.03	40.75
P	0.0000 ***	0.0000 ***	0.0000 ***	0.0000 ***	0.0000 ***	0.0325 *	0.0008 ***	0.0000 ***
LSD at 0.05	0.1518	0.1307	0.0355	0.0438	0.1253	0.0359	0.3974	0.0299

*** = very highly significant, * = significant at 0.05 level

Discussion

Globally, plants are endangered by the increased human, industrial and agricultural activities, particularly after the industrial revolution in the past century. One of the most challenges faced by plants is heavy metals pollution. Lead (Pb) was reported as a potential heavy metal, neither possess any role in cellular metabolism nor essential for plant growth and development (Nas and Ali, 2018), although it is simply uptaken and accumulated within various plant parts. The declined fenugreek growth by lead exposure in the current study could be ascribed to the extensive phytotoxicity represented in the distortion of cellular structures, ionic imbalance, reduced chlorophyll synthesis, induction of ROS over-accumulation and hormonal imbalance(Kumar et al., 2012). As one of the heavy metals, Pb impairs the hydrolysis and the translocation of carbohydrates leading to reduced plant growth (Kuriakose and Prasad, 2008).

On the other hand, the study results demonstrated that moringa leaf extract (MLE) pretreatment significantly improved the growth rate of Pb-stressed fenugreek seedlings. These results are in agreement with those of Latif and Mohamed (2016) in salt-stressed common bean and Abdel-Latef et al. (2017) in salt-stressed fenugreek. These results affirmed thatMLE could be considered growth-promoting due to its high content of growth-regulating substances (zeatin), antioxidants (ascorbate, phenols and carotenoids) and essential nutrients (Yasmeen et al., 2013). Those authors concluded that MLE induces the endogenous hormonal level causing improved plant growth in the presence and/or absence of any other stressful factor. Additionally, Taiz and Zeiger (2010) reported that MLE is an excellent source of cytokininswhich promote many physiological processes in the growing plants as chlorophyll biosynthesis, cell division and cell elongation.

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The induced reduction in chlorophyll content by Pb is considered as a common consequence of ROS mediated chlorosisdue to the impairment of chlorophyll biosynthesis and induction of chlorophyll degradation (Desoky et al., 2018). The study results showed a significant reduction in chlorophyll (a+b) content in Pb-stressed fenugreek leaves. Furthermore, chlorophyll b was reported to be more sensitive to lead stress than chlorophyll a. The powerful inhibition in the activity of α-amino levulinate dehydrogenase; a key enzyme in chlorophyll biosynthesis, by lead ions was reported to be the main cause of chlorophyll decline as a result of lead exposure (Prasad, 1996). Other reports showed that lead restrainschlorophyll biosynthesis by decreasing the uptake of some essential ions like Mg and Fe (Nas and Ali, 2018). Lead destroys the photosynthetic apparatus because it has high affinity towards protein nitrogen and sulfur ligands (Ahmed and Tajmir-Riahi, 1993).Sofy et al. (2020)reported that lead stress reduced the total chlorophyll content and disrupted the chloroplast ultrastructure of maize leaves. They attributed that to the increased levels of lipid peroxidation and membrane leakage, in addition to the interaction of lead with-SH group in chlorophyll biosynthetic enzymes.

Priming of fenugreek seeds in MLE provoked a protective mechanism for the growing seedlings against lead stress. This was apparent during this study through the improved chlorophyll content of lead-stressed fenugreek seedlings. Yasmeen et al. (2013) also previously reported that MLE improved the total chlorophyll content of wheat plants subjected to salt stress. Moreover, Latif and Mohamed (2016) reported that the foliar spray of common bean plants with MLE significantly improved the chlorophyll content of salt-stressed plants. They attributed this effect to the high content of macronutrients in MLE such as magnesium, which is a main constituent of chlorophyll. Other reports showed that the high content of cytokinins, particularly zeatin, in MLE prevent early senescence, producing large leaf area with high chlorophyll content (Elzaawely et al., 2017) through upregulatingcytokinindependantisopentenyltransferase, which is a key enzyme in the biosynthetic pathway of chlorophyll.

Carotenoids, as main antioxidant molecules, were significantly boosted by lead stress. Similar results were obtained by Lim et al. (2012) in buckwheat(*Fagopyrum esculentum*Moench.) subjected to salt stress, they attributed this increase to the induction of themevalonate pathway to induce the biosynthesis of abscisicacid (ABA) to improve the plant tolerance to salt stress. Also, Zaid et al. (2020)reported that carotenoids content highly increased by the exposure of menthol plants (*Mentha piperita* L.) to cadmium stress. The excessive accumulation of carotenoids under abiotic stress conditions was attributed to their capability to scavenge ROS, in addition to providing photoprotection against photooxidation of photosynthetic reaction centers (Gururani et al., 2015). Moreover, carotenoids were reported to offer protection to thylakoid membrane lipids, quenching singlet oxygen and chlorophyll triplet excited state (Li et al., 2012).Consequently, the increased carotenoids provide protection to the photoassimilating apparatus against Pb-stress.

Pre-treatment of fenugreek with MLE significantly reduced the level of carotenoids in the leaves of Pb-stressed fenugreek seedlings. Our results are in harmony with those obtained by Abd El-Mageed et al. (2017) in squash. The declined level of carotenoids in fenugreek subjected to Pb-stress might be due to the accompanying antioxidant properties in the aqueous MLE, as it has been reported to be a rich source of natural antioxidants such as flavonoids, ascorbic acid, carotenoids and phenolics(Siddhuraju

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and Becker, 2003). These antioxidant molecules could offer protection to the stressed fenugreek seedlings, allowing it to harness the mevalonate pathway in the production of other essential molecules sustaining plant growth under stress conditions.

The results of this study revealed that Pb-stress down-regulated the accumulation of soluble sugars in the root and the shoot of fenugreek seedlings. This reduction was ascribed to the loss of chlorophyll, inhibition of photosynthetic enzymes (like RUBP carboxylase/ oxygenase) and reduced leaf area in stressed plants (Pandey et al., 2001), in addition to the increased respiration rate to adapt with the high energy demand (John et al., 2008).

The single treatment with MLE resulted in decreasing the soluble sugar content of Pb-stressed fenugreek seedlings, but the combined interaction of Pb and MLE slightly affected their content, compared to Pb treatment. Free soluble sugars function as anti-stress osmoregulators and their decrease in response to priming with MLE could be attributed to the fact that this extract containsvariousosmoregulators, like proline and soluble sugars (Abd El-Mageed et al., 2017), which improve the vigor, cellular turgidity, and water use efficiency of stressed plants. Accordingly, these plants direct the photosynthates into the production of other imperative compounds like structural sugars and essential secondary metabolites.

In comparison with the control, soluble proteins content of fenugreek seedlings root and shoot was significantly increased in response to Pb and MLE treatments. The increased protein accumulation in response to lead toxicity has been previously reported in wheat (Lamhamdi et al., 2011) and grass pea (*Lathyrus sativus* L.) (Brunet et al., 2009). Such accumulation of proteins may protect plants against Pb-stress through sustaining cellular redox like ascorbate, glutathione and phytochelatins make (Jiang and Liu, 2010). Lamhamdi et al. (2011)postulated that increased protein accumulation under Pb-stress could be a direct consequence of stimulating specific stress proteins. Increasing of total soluble protein content in stressed plants by using MLE has been previously approved in snap bean (*Phaseolus vulgaris* L.)(Elzaawely et al., 2017). Those authors accredited the increase in protein to the increased nitrogen concentration due to MLE treatment. Moreover, the increased protein biosynthesis could be explained by the availability of antioxidant compounds in the extract, providing protection to the metabolic machinery involved in protein biosynthetic pathway.

The current investigation revealed a prominent increment in lipid peroxidation (MDA) and H_2O_2 accumulation in the leaves of fenugreek seedlings exposed to Pb-stress, substantiating Pb-induced oxidative damage in fenugreek seedlings.Wang et al. (2012) found that eelgrass(*Vallisnerianatans*L.) showed an increased production of MDA and H_2O_2 as a result of Pb treatment. They explained this effect by the deteriorationscaused by the excessive generation of ROS through the frequent creation of fatty aldehydes and short-chained alkanes caused by Pb ions. Likewise, Ahamed and Siddiqui (2007) confirmed ROS generation due to lead toxicity, causing oxidant/antioxidant-balance disturbances, and lipid metabolism alternation. However, MLE-treated fenugreek plants counteracted oxidative damage through the decreased generation ofMDA and the accumulation of H_2O_2. This could be caused by the high content of antioxidant compounds, vitamins and cytokinins in MLE, in addition to the induction of antioxidants, like ascorbate, phenols and anthocyanins, in the stressed plant tissues (Batool et al., 2016).

Lead is well-known to be a redox inert-metal, causing excessive ROS generation via disrupting cellular oxidant/antioxidant balance (Zulfiqar et al., 2019). To meet oxidative injury, plant have developed several enzymatic and non-enzymatic molecules. In the

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present investigation, catalase (CAT) and ascorbate peroxidase (APX) activities decreased in fenugreek leaves by receiving 100 mMPb-acetate. This might be a consequence of the severe oxidative damage imposed by Pb(Erdei et al., 2002). Verma and Dubey (2003) reported that the Pb-induced decline in CAT and APX activities arise from ROS-mediated inactivation of these enzymes, enzymes-synthesis decline, or enzyme subunits assembly modification. On the contrast, peroxidase (POD) and polyphenol oxidase (PPO) activities were raised following Pb-treatment. The probable explanation of the increased POD activity is the dependence of fenugreek in eliminating the rapidly accumulated H_2O_2on this enzyme, to compensate for the diminishedCAT activity. Similar results were obtained by Sobhy et al. (2019) in wheat and Hou et al. (2019) in bamboo grass(*Pogonatherumcrinitum*Thunb.).PPO has been established as a key enzyme in the photosynthetic apparatus, and it can provide and evidence for heavy metals accumulation in various plant species (Lavid and Tel-Or, 2001). The exposure of plants to Pb-stress has been correlated with the increased activity of PPO, due to its crucial role in respiration, oxidation-reduction reactions, and synthesis of phenol-containing molecules like lignin(El-Beltagi et al., 2016).The declined activity of antioxidant enzymes, except for POD which remained unchanged, as consequence of pre-treating fenugreek with MLEpull the awareness to the ability of this extract, owing to its elevated antioxidative capacity, to eliminate the generated ROS, so there is no need for the upregulation of enzymatic antioxidant machinery, and the plant could sustain its normal growth based on the high content of antioxidants in MLE (Yasmeen et al., 2013).

Under normal growth conditions, plants produce specific secondary metabolites, some of which can scavenge ROS. However, the scavenging potential of these metabolites has been reported to decrease under stress conditions (Ahmad et al., 2016). In the current study, Pb-stress resulted in decreased radical scavenging (DPPH) activity and total antioxidant capacity (TAC) in fenugreek roots and shoots.Tripathi et al. (2016)attributed that the increased Pb-stress mediated accumulation of $H_2O_2$and MDA is the main cause of the antioxidant potential decreasing in the stressed plants. MLE presoaked fenugreek seedlings showed a mitigating response to Pb-stress in terms of DPPH and TAC at the low concentration (2.5%), but the higher concentration (5.0%) non-significantly affected the non-enzymatic antioxidant status of fenugreek.Yasmeen et al. (2013) stated that the high content of cytokinins in MLE was behind its effectiveness in ameliorating environmental stresses via quenching ROS. Moreover, the reason may be due to the reduced absorption and transfer of lead tofenugreek tissues.

Pb-stress was demonstrated to increase the uptake of Pb and Na ions, disturbing the uptake and mobility of essential minerals (N, P, Ca and K), reducing K/Na ratio and interrupting ionic and osmotic homeostasis. Transport of Pb to the plant cells occurs through the cation channels on plasma membranes, especially Ca channels, causing other nutrients deficiency, hindering water uptake and deterioration of membrane integrity (Lamhamdi et al., 2011). Therefore, the decreased content of essential nutrients could be considered as a consequence of intensive membrane deteriorations as a direct effect of Pb ions or the generated ROS. The decreased level of essential macronutrients after Pb-exposure results in the decreased growth rate of fenugreek seedlings, which may be correlated with reduced cell division and elongation, as well as metabolic redox imbalances.

The high content of essential nutrients in MLE promoted high ionic homeostasis in tissues leading to higher levels of essential macronutrients (N, P, K and C) supporting the normal growth of Pb-stressedfenugreek. The balanced ionic status of MLE-treated plants

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promoted fenugreek seedlings to sustain membrane integrity, ionic homeostasis, water use efficiency and antioxidant capacity, consequently improved growth. Several authors adjudged enhanced ionic status of stressed plants following MLE treatment (Yasmeen et al., 2013; Abdel-Latef et al., 2017).

Conclusion

Being a heavy metal, lead negatively affected growth rate, photosynthetic pigments, nitrogen metabolism, antioxidant status and ionic homeostasis in fenugreek seedlings. Seed priming with moringa leaf extract (MLE) is a cost-effective and eco-friendly approachthat could be used to improve Pb-resistance in fenugreek by modulating nitrogen metabolism, antioxidative machinery, ionic homeostasis with decreased Pb and Na concentrations, thereby promoting enhanced growth rate under stress conditions. However, further studiesare recommended to elucidate the phytochemical constituents of MLEand the mode of action of this extract in modulating metal-stressed plants growth.

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ELECTRONIC APPENDIX

This manuscript has 13 electronic appendices with basic data.

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THE EFFECT OF SEED COATING THICKNESS ON SUGAR BEET (*Beta vulgaris* L.) YIELD AND QUALITY UNDER DIFFERENT IRRIGATION CONDITIONS

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Abstract. Sowing naked seeds is generally preferred in arid and semiarid regions. This research aimed to find the optimal dimensions for both pelleted and naked seeds based on seed distance in rows and field emergence irrigation setting in sugar beet (*Beta vulgaris* L.) cultivation under the environmental conditions of Central Anatolia in Turkey. For comparison, the research considered two field emergence irrigation conditions, namely, irrigated and non-irrigated, at 8 and 17 cm seed distances within rows for naked seeds of two dimensions: N3.25-Ø4.50 and N3.00-Ø3.50 mm and for pelleted seeds of five dimensions: P3.50-Ø3.75, P3.75-Ø4.00, P4.00-Ø4.25, P4.25-Ø4.50, and P4.50-Ø4.75 mm. The results showed that the highest beet yield and refined sugar content were 66.67 and 10.31 t ha^{-1}, respectively, obtained in the plot that was irrigated and drilled with N3.00-Ø3.50 mm seeds at 8 cm sowing distance. The lowest values were obtained from the non-irrigated plot that was drilled with P4.50-Ø4.75 mm seeds at 17 cm sowing distance in all conditions. In field emergence irrigation conditions, planting pelleted seeds from 3.50 to 4.25 mm in dimension or 0.25-0.75 mm in thickness at row distances of 8 and 17 cm can be concluded to be the optimum application.
Keywords: *sugar beet, seed pelleting, germination, field emergence irrigation, row space, quality indicators*

Introduction

Decreasing the manual labor and increasing machinery operations by as much as possible are among the goals of sugar beet (*Beta vulgaris* L.) farming. In practice, considerable progress has been achieved with machinery operations except for thinning and singling. Reducing manual labor generally depends on three factors: uniform and healthy seedling rows, uniform seedling distribution, and adequate weed control (Draycott, 2006). Achieving a uniform seedling distribution and the optimum plant density depends on the seed specifications, proper cultivation, and sowing technique. The application of suitable mechanical and chemical weed control is also important to ensure a high yield and quality (SBGG, 2016).

Sugar beet is generally planted at 8 cm seed distance in a row and at 45 cm distance between rows by using precision drilling machines; a singling distance of 20-25 cm is applied in Turkey. In addition, planting at 45 cm row spacing and at the desired seed spacing within a row can be carried out with the use of pneumatic drilling machines. The optimum plant frequency to obtain the highest yield and quality has been determined to be between 70000 and 90000 plants ha^{-1} (Hozayn et al., 2013; SBGG, 2016). The forward speed of the drilling machine is one of the major factors affecting the sowing quality; a forward speed of 4.5-5 km h^{-1} seems to provide the most appropriate seed distribution. A high speed causes an irregular seed distribution, changing the depth settings and leaving

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the seeds on the soil surface. The planting depth is also a major factor in yield and quality. Sugar beet seeds must be sown at a depth of 2-4 cm and should be checked continuously during sowing. Further, proper planting facilitates the subsequent hoeing and harvesting process (Hozayn et al., 2013).

Spacing is the distance between two successive seeds in a row. Misses (voids) refer to the absence of a seed where there should be one theoretically, that is, where the spacing is more than 1.5 times the theoretical seed spacing. Multiples (doubles) refer to the presence of two or more seeds where there should only be one, that is, where the spacing is less than 0.5 times the theoretical seed spacing (Lammers et al., 2015). One seed within targeted rows and single seedling germination in each cell are desired in sugar beet cultivation. To achieve these goals, it is important to use precisely calibrated pelleted and naked seeds. The vast majority of sugar beet seeds sown in Turkey consist of naked genetic monogerm seeds. Genetically monogerm sugar beet seeds belonging to different varieties are processed and calibrated according to sizes of 3.25-Ø4.50 mm at the Turkish Sugar Factories Corporation seed processing plant. They are then delivered to farmers after being treated with one insecticide (imidacloprid or thiamethoxam), two fungicides (hymexazol and thiram) against disease and pests. The variety of seeds produced by private seed companies can be bought from the market and sown according to the preference of farmers (TSFAS, 2019).

In recent years, an increase has been observed in the number of producers sowing pelleted genetically monogerm seeds, called thin pelleted seeds, 3.50 to 4.00 mm in dimension. The smoother surface and more spherical structure of pelleted seeds result in a more precise seed distribution in a row, especially when precision sowing machines are used. Also, larger amounts of pesticide may be mixed into the coating material depending on the naked seeds, thus increasing the efficacy of pesticides against both pests and diseases. On the other hand, seed pelleting increases the costs and decreases the amount of field emergence in some regions where arid and semi-arid climate conditions prevail and during years of arid climate and with insufficient rainfall periods, especially just after drilling in spring. The germination rate of naked sugar beet seeds delivered to farmers by the Turkish Sugar is required to be at least 85% (TSFAS, 2019). Although the standard naked seeds satisfy this requirement, the rate may decrease to 68% after pelleting (Duan and Burris, 1997).

Pelleting of sugar beet seeds is done after polishing, dimension measurement, and separation by weight. To prepare the seeds for pelleting, one or all of these processes are carried out depending on the physical characteristics of the seed lot to be used. The pre-cleaning dimension of harvested seeds is generally between 3.25 and 6.00 mm. The seed size is decreased to 3.25-Ø3.50 mm by polishing before the pelleting process (Draycott, 2006). Polishing is the process of correcting the disk-shaped, jagged outer surface of beet seeds and obtaining the desired dimension by chipping the pre-pelleting of the seed outer surface (pericarp). Thinning of the pericarp by polishing increases the water intake and germination speed. After polishing, the seed units are passed through a multilevel sieve with round holes; seeds that are too small to be pelleted are removed, whereas those that are too large are polished again. Next, the seed units are passed through a sieve with oval holes, and those with multiple embryos are separated (Draycott, 2006). The remaining seeds are separated by weight and assessed by an X-ray test; those found to be 100% full are then sent to the pelleting units. Pellet sizes of 3.50-Ø4.75 and 3.75-Ø4.75 mm are widely preferred in Europe; only Finland and Sweden use pelleted seeds in the range of 4.00 to 5.00 mm. The thickness of the pelleting material increases

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the seed weight by an average of 180% (between 150 and 200%) or by 0.50-0.75 mm (Draycott, 2006). In Turkey, a pellet thickness of 0.50 mm is generally applied.

The pelleting process consists of four stages (*Fig. 1*). During seed pelleting, naked seeds are pelleted with a mixture of clay, wood flour, and adhesive. The process is done in rapidly rotating cylindrical boilers; inside the drums, water and the pelleting powder are sprayed onto the seeds. After that, the seeds are dried, and the water in the pelleting material is removed by evaporation, thus turning the pelleted seed into a hard pellet (KWS, 2020). Turkey has no domestic production of the sugar beet seed pelleting material, which is currently sourced from abroad.

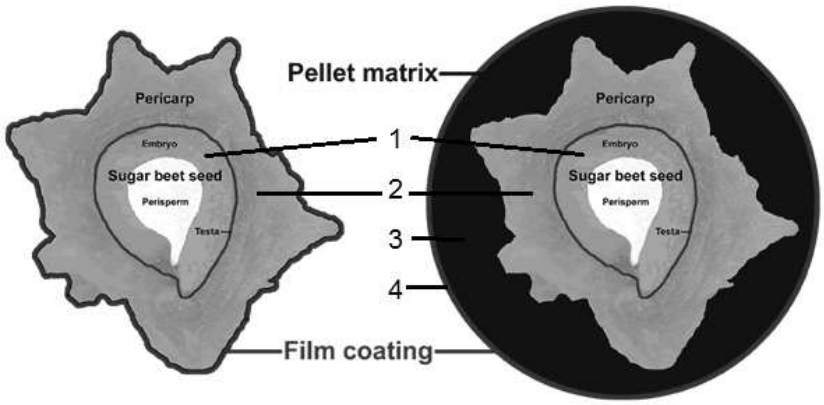

Figure 1. *Sectional view of the pelleted seed*

In this study, it was aimed to determine the most suitable seed type and seed coating thickness for high yield and quality by comparing uncoated and coated sugar beet seeds under different of sowing distances and with and without emergence irrigation conditions.

Materials and methods

This research was carried out at the Sugar Institute in the Eskişehir (39°47′N, 30°31′E), Etimesgut (39°56′45″N, 32°40′10″E), and Ilgın (38°16′45″N, 31°54′50″E) experimental stations over a three-year period from 2012 and 2014 in Turkey. The soil textures in the three locations were silty-clayey and clayey, and the regional climatic characteristics were arid and semi-arid. During the vegetation period of sugar beet, the total annual precipitation in April-August period is given in *Fig. 2* and the average temperature values by months are given in *Table 1*. Climate data (temperature, precipitation) in the test fields were taken hourly by the automatic climate station in the test regions. In the three-years period, the three regions had similar mean rainfall rates despite their different geographic locations. Although there are minor differences in regions, the temperature between June-August periods was 15-25°C that were desired for the development of sugar beet.

The field used for sowing was prepared first by using a subsoiler, after which a disc harrow was used to clean the preplant wheat stubble in preparation for planting in autumn. The field was ploughed after the application of all the potassium fertilizer and two thirds of the phosphorus fertilizer according to the soil analysis. The seedbed was prepared with the combined use of a cultivator, harrow, and rotary harrow, which supplied the remaining one third of the phosphorus fertilizer and half of the nitrogen fertilizer in spring. The remaining half of the nitrogen fertilizer was applied before the first hoeing. According to

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the soil analysis, 120-170 kg ha^{-1} of nitrogen, 50-100 kg ha^{-1} of phosphorus, and 70-100 kg ha^{-1} of potassium were present in the soil as pure substances at varying rates by region and year.

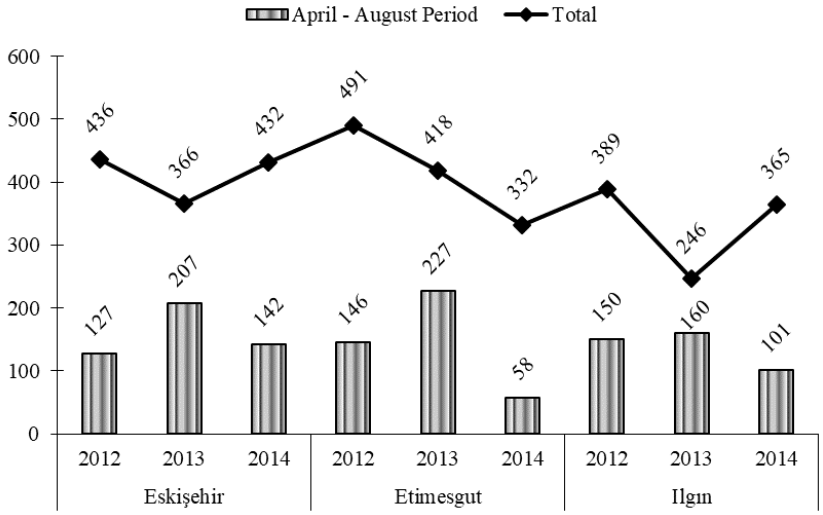

Figure 2. *Precipitation status (mm)*

Table 1. *Average temperature values of the regions by months*

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Dec	Nov
Etimesgut												
2012	2.6	4.1	6.6	9.9	15.1	21.2	24.8	27.0	20.5	13.0	11.0	4.1
2013	0.9	1.5	6.3	9.1	13.2	17.3	22.6	21.8	17.4	9.7	1.5	2.2
2014	-3.0	-5.9	1.2	12.0	15.2	20.5	23.7	21.7	19.9	14.7	7.8	2.4
Eskişehir												
2012	2.7	6.1	7.0	10.0	15.4	19.4	22.1	25.0	19.2	11.1	10.5	5.6
2013	1.3	1.5	5.1	8.3	13.7	15.1	22.5	20.5	18.0	10.1	2.5	2.2
2014	-2.1	-4.2	2.6	12.6	15.9	21.1	23.5	21.2	19.1	15.7	8.3	3.6
Ilgın												
2012	4.5	6.7	8.5	10.6	16.1	19.7	24.1	25.6	20.0	12.8	12.0	7.2
2013	3.6	-1.4	5.8	9.8	13.7	18.3	23.6	21.7	18.0	10.5	2.6	3.1
2014	-3.7	-6.1	1.0	11.1	13.7	18.9	21.5	19.2	16.8	13.5	7.1	3.9

Field tests were carried out by applying a randomized split plot design in four replications. The experimental plots were 10 m long and 4.5 m wide, and the seeds were planted in the plots at 10 rows each with 45 cm row spacing. The total trial area was 4977 m^2, the sowing plot was 45 m^2, and the harvest plot was 20 m^2. The main plots consisted of areas under field emergence irrigated and non-irrigated conditions, and the subplots were planted at 8 and 17 cm planting distances with naked seeds of two different dimensions: N3.25-Ø4.50 and N3.00-Ø3.50 mm pelleted seeds of five different dimensions: P3.50-Ø3.75, P3.75-Ø4.00, P4.00-Ø4.25, P4.25-Ø4.50 and P4.50-Ø4.75 mm. In the plots with a seed drilling distance of 8 cm, the beets were manually thinned to 20-25 cm at the 4-6 leaf stage. In the plots with a drilling distance of 17 cm, the beets were singled and but not thinned even if there were more than one seedling in the queue (*Fig. 3*).

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Figure 3. *General view of the parcels and plants in the trial area*

In the study, 15 mm water was applied to the plots which will be applied emergence irrigation by sprinkler irrigation method immediately after sowing. According to the needs of sugar beet 6-7 irrigation was made and approximately 100-150 mm water applied in each irrigation during the vegetation period.

Genetic monogerm sugar beet seeds, called "Giraf," from SESVanderhave (Belgium) were used in the study. A mechanical precision drilling machine manufactured by Turkish Sugar was used for sowing in the trial plots. The seed pelleting and classification processes were done at the BETA Agriculture and Trade Co. seed processing plant in Merzifon (*Fig. 4*). First, the raw seed dimension of 3.00-6.00 mm was decreased to 3.00-3.50 mm by polishing, after which the seeds were pelleted. Then, the pelleted seeds were passed through sieves with round holes of 4.50, 4.25, 4.00, 3.75, and 3.50 mm and divided into five groups. The pelleted seeds in these groups and the naked seeds were then sprayed with pesticide, painted with the company's promotional color, and packaged. *Table 2* shows the average mass of 1000 seeds according to the seed dimension.

Figure 4. *Classification of seeds according to seed sizes in Beta Agriculture and Trade Co. seed processing plant*

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Table 2. *Laboratory emergence values according to the seed dimension used in the research*

Seed dimension	Coated thickness (mm)	Thousands of seed mass (g)	14th day laboratory germination values		
			2012	2013	2014
U 3.25 - Ø 4.50 mm	-	11.4	98	99	99
U 3.00 - Ø 3.50 mm	-	12.6	96	96	97
C 3.50 - Ø 3.75 mm	0.25	17.9	93	99	99
C 3.75 - Ø 4.00 mm	0.50	21.1	99	99	100
C 4.00 - Ø 4.25 mm	0.75	22.6	97	99	100
C 4.25 - Ø 4.50 mm	1.00	23.5	99	100	99
C 4.50 - Ø 4.75 mm	1.25	33.4	99	100	100

U: uncoated seed, C: coated seed

Both pelleted and naked seeds were treated with thiram (3.2 g active ingredient/1 kg seed) and hymexazol (3.5 g active ingredient/1 kg seed) to protect against fungal disease and imidacloprid (9 g active ingredient/1 kg seed) to protect against underground pests (wireworms, springtails, and millipedes) and flea beetles (Kaya and Gürkan, 2011). Seed germination tests of all seed plots used in the trial were carried out in a laboratory (*Table 2*).

For the germination tests, at least 100 seeds from each test subject were placed in germination containers laid on a flat folded filter paper. Each container was sprayed with 40 ml of water in the application with four replications. The number of seeds germinated after 4^{th} and 14^{th} days was counted in a germination room kept at a constant temperature of 20-22°C under a 16 h light/8 h dark cycle (ISTA, 2019).

The duration of field emergence is usually 14 days from the date of sowing on. However, the counts were done weekly from the beginning to the completion of the field emergence. The plant emergence rate was determined as the proportion of the total number of plants counted after complete plant germination to the amount of seed thrown per unit area based on the sowing distance in a row and between rows (Lammers et al., 2015).

After the harvesting, the beets were washed, weighed, and sampled for laboratory analysis with the use of a fraise hob. The amount of dry matter was measured with the Anton Paar Abbemat 500 refractometer manufactured in Germany. The sugar content was considered as the percentage of polar sugar (P). The amount of polar sugar was determined by extracting 26 g of beet pulp with 178.2 ml of aluminum sulfate ($Al_2 (SO_4)_3$) liquor, after which polar meter readings were taken. The amounts of sodium (Na), potassium (K), and α-N were determined by the Anton Paar Betalyser system applying flame photometry principles. The refined sugar content (RSC) and refined sugar yield (RSY) was obtained by calculations (Reinefeld et al., 1974).

Variance analysis and F-tests were applied to the results. No comparisons were made when the F-value was found to be non-significant. Duncan's multiple comparison method was used when the F-values were significant.

Results and discussion

Field emergence

The germination power of the seed varieties was found to be 99% in laboratory conditions (*Table 2*). Water is one of the most important factors in the germination of all

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plants. After sowing, achieving rapid seed germination and plant emergence depends on having appropriate soil weathering during sowing and maintaining adequate moisture in the soil. Sugar beet is a plant which is extremely sensitive to water. It should be considered to obtain a high yield and quality of sugar beet during the sowing period from April to mid-September.

Based on the combined results over the three-year study period, higher field emergence was obtained from the irrigated plots than from the non-irrigated ones (*Table 3*). In particular, pelleted seeds of P3.50-Ø3.75, P3.75-Ø4.00, and P4.00-Ø4.25 mm showed a considerable advantage in field emergence under both irrigated and non-irrigated conditions. The total annual rainfall and the amount of precipitation showed a variation during the vegetation period (from April to August). The highest average rainfall in the period from April to August was measured in 2013 and the lowest in 2014 (*Fig. 2*). Therefore, field emergence irrigation may be necessary to ensure germination and field emergence exactly from year to year after sowing even if adequate rainfall is not being taken before sowing.

Table 3. *Seedlings in the parcels after the field emergence is completed (means of 3 years and 3 locations, 10^3 ha^{-1})*

Subjects	2012		2013		2014	
	Irr	**Non-irr**	**Irr**	**Non-irr**	**Irr**	**Non-irr**
8cm, U3.25-4.50 mm	143.4±19.2	109.8±7.1	148.7±44.2	132.5±30.8	150.8±11.8	101.5±6.7
8cm, U3.00-3.50 mm	174.4±18.5	137.7±10.0	169.0±30.1	150.5±26.4	183.4±9.5	140.4±15.6
8cm, C3.50-3.75 mm	203.4±22.2	183.7±4.5	185.6±36.1	142.5±23.7	224.5±17.9	160.7±24.8
8cm, C3.75-4.00 mm	199.9±18.6	173.0±4.1	172.2±42.1	165.0±35.4	214.2±20.5	165.2±16.5
8cm, C4.00-4.25 mm	190.7±16.7	150.7±10.3	174.6±40.0	163.3±30.3	205.2±17.0	138.7±22.9
8cm, C4.25-4.50 mm	148.5±16.0	145.4±5.2	165.9±27.2	147.4±27.1	167.7±13.4	129.3±8.6
8cm, C4.50-4.75 mm	65.6±7.9	63.5±9.4	85.7±19.9	69.1±14.7	63.8±7.2	67.0±10.8
Subjects	2012		2013		2014	
	Irr	Non-irr	Irr	Non-irr	Irr	Non-irr
17cm, U3.25-4.50 mm	54.5±5.2	48.7±3.1	76.3±22.3	84.3±23.2	57.2±5.6	44.5±3.2
17cm, U3.00-3.50 mm	58.6±6.8	52.8±1.3	76.6±27.4	75.8±19.2	63.3±2.7	50.3±2.1
17cm, C3.50-3.75 mm	93.0±8.1	80.9±10.5	96.0±21.3	116.6±24.0	93.6±6.2	64.6±5.2
17cm, C3.75-4.00 mm	89.7±10.4	75.1±12.3	97.3±8.1	102.1±22.5	84.8±9.4	66.4±7.7
17cm, C4.00-4.25 mm	77.5±8.8	73.1±11.5	99.4±25.1	108.1±20.3	84.2±3.7	61.2±5.1
17cm, C4.25-4.50 mm	73.5±12.2	54.0±5.6	88.4±19.6	96.4±20.3	68.6±11.7	46.8±6.9
17cm, C4.50-4.75 mm	26.7±2.1	21.1±1.9	66.5±34.6	58.1±23.1	26.9±4.6	28.8±8.4

Beet yield

Considering the mean values, a higher beet yield was obtained in the plots in which field emergence irrigation was applied. In the irrigated plots, better results were achieved at 8 cm sowing distance than at 17 cm; in the non-irrigated plots, similar yield values were obtained for both sowing distances. The average beet yield was 61.71 t ha^{-1} in the irrigated plots, compared to 57.85 t ha^{-1} in the non-irrigated ones (*Table 4*, *Fig. 5*). The difference between these yields was statistically significant ($P<0.01$).

Regarding the beet yield based on sowing distance in a row, a value of 60.67 t ha^{-1} was obtained at 8 cm and 58.89 t ha^{-1} at 17 cm. The difference between these yields was also statistically significant ($P<0.01$) (*Table 4*).

The differences in yield according to the dimension of the pelleted seeds, except for those P4.50-Ø4.75 mm, were not significant. When considering average values; the

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highest value was obtained as 61.22 t ha^{-1} with P3.50-Ø3.75 mm seeds. However, the highest yield of 66.67 t ha^{-1} was obtained in the irrigated plot sown with N3.00-3.50 mm seeds at 8 cm distance; the lowest value of 44.77 t ha^{-1} was obtained in the non-irrigated plot sown with 4.50-4.75 mm seeds at 17 cm distance (*Fig. 5*).

Table 4. *Statistical evaluation according to the data's*

Subjects*	Beet yield (t ha^{-1})	Sugar content (%)	Refined sugar yield (t ha^{-1})
Non-irrigated	57.85	17.49	8.64
Irrigated	61.71**	17.72**	9.38**
8 cm	60.67	17.63	9.15
17 cm	58.89**	17.58	8.87**
U3.25 - Ø 4.50 mm	61.11 A	17.50 C	9.01 B
U3.00 - Ø 3.50 mm	60.93 A	17.63 C	9.19 AB
C3.50 - Ø 3.75 mm	61.22 A	17.86 AB	9.49 A
C3.75 - Ø 4.00 mm	60.49 A	17.98 A	9.44 A
C4.00 - Ø 4.25 mm	60.86 A	17.85 AB	9.40 AB
C4.25 - Ø 4.50 mm	61.07 A	17.68 BC	9.26 AB
C4.50 - Ø 4.75 mm	52.78 B	16.73 D	7.27 C
SEM	59.78±8.890	17.61±0.12	9.01±1.729

* U: uncoated seed, C: coated seed

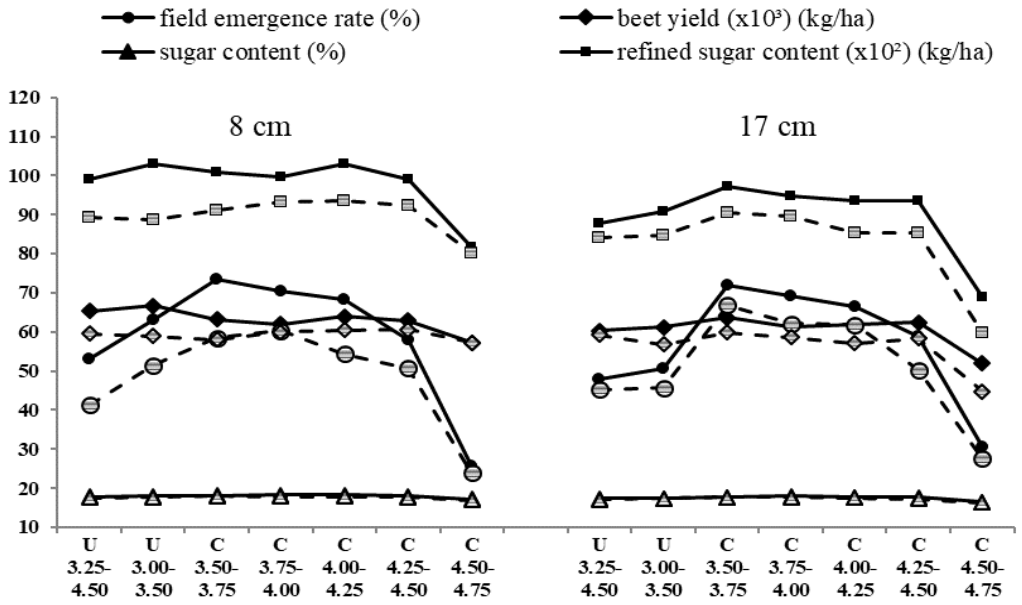

Figure 5. *Quality data's obtained from plots (means of 3 years and 3 locations, dashed is non irrigated values)*

Sugar content

Higher sugar content was obtained in the irrigated plots and at a sowing distance of 8 cm compared with the other plots (*Fig. 5*). The average sugar contents were determined as 17.72% in the irrigated plots and 17.49% in the non-irrigated ones. The difference between these values was found to be statistically significant (P<0.01).

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Regarding the sugar content by sowing distance in a row, 17.63% was obtained at 8 cm and 17.58% at 17 cm. The difference between these values was not statistically significant (*Table 4*).

The highest sugar content obtained based on seed dimension was 17.98% for P3.75-Ø4.00 mm seeds. Although the differences in sugar content between the seeds P3.50-Ø3.75, P3.75-Ø4.00, and P4.00-Ø4.25 mm in dimension were not significant, the differences between these subjects and the others were significant ($P<0.01$). The highest sugar content of 18.31% was obtained in the irrigated plot sown with P3.75-Ø4.00 mm seeds at 8 cm distance, whereas the lowest value of 16.32% was achieved in the non-irrigated plot sown with P4.50-4.75 mm seeds at 17 cm distance (*Table 4*, *Fig. 5*).

Refined sugar content

Similarly to the beet yield and sugar content, a higher refined sugar content was obtained in the irrigated plots and at 8 cm sowing distance compared to the other plots (*Table 4*, *Fig. 5*). The average refined sugar content in the irrigated plots was 9.38 t ha^{-1}, compared to 8.64 t ha^{-1} in the non-irrigated ones. The difference between these values was statistically significant ($P<0.01$).

Regarding the refined sugar content by sowing distance in a row, a value of 9.15 t ha^{-1} was obtained at 8 cm and 8.87 t ha^{-1} at 17 cm. The difference between these values was statistically significant ($P<0.01$).

The highest refined sugar contents were 9.49 and 9.44 t ha^{-1}, respectively, for the seeds P3.50-Ø3.75 and P3.75-Ø4.00 mm in dimension. The differences between seeds based on pelleting thickness were not significant. The highest refined sugar content was 10.31 t ha^{-1}, obtained in the irrigated plot sown with N3.25-3.50 mm seeds at 8 cm distance; the lowest value was 5.98 t ha^{-1}, achieved in the non-irrigated plot sown with P4.50-4.75 mm seeds at 17 cm distance (*Table 4*, *Fig. 5*).

In the statistical evaluations carried out in the three years of the research, location and year interactions were found. In essence, it is normal to have this type of interaction because the location and years differ in properties. However, since the results obtained in three years and three locations are parallel to the applied methods, the methods were evaluated over the averages.

Although it is not statistically significant in these three years, better results were observed comparing the irrigated plots to the non-irrigated ones with 8 cm sowing distance to 17 cm all of the three locations and years. A significant difference between irrigated and non-irrigated plots was found in the beet yield at Ilgın in 2014 ($P<0.05$), the sugar content at Etimesgut in 2012 ($P<0.05$), and the refined sugar content at Ilgın in 2012 ($P<0.05$) and 2014 ($P<0.01$) (*Table 5*). The difference between sowing distances of 8 and 17 cm was not significant in all the years and locations.

Regarding the difference between seed pelleting thicknesses by year and location, seeds P3.50-Ø3.75, P3.75-Ø4.00, and P4.00-Ø4.25 mm in dimension showed better results in all locations. The difference between the seeds P3.50-Ø3.75, P4.00-Ø4.25 mm in dimension and the other treatments were significant in terms of beet yield, sugar content, and refined sugar content ($P<0.05$, $P<0.01$) (*Table 5*).

Seed germination is controlled by environmental factors (light, temperature, water). The field emergence rates of the seed variety and in the specific region have to be known to determine the optimal sowing distance in a row. In this research, the average field germination rates at three different experimental stations was determined as 58% in irrigated plots and 50% in non-irrigated plots. These values are representative of the

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Central Anatolian Region and show the average levels in Turkey. This rate is lower than the level of 76% in the United Kingdom (BBRO, 2020). The data indicate that sowing at a distance of up to 17 cm can be done in similar circumstances. Larger sowing distances are discouraged because they present a higher risk under climate conditions of arid and semi-arid countries as well as Turkey. On the other hand, sowing sugar beet seeds at 8 cm distance in a row and adjusting to 20-25 cm by thinning and singling ensure a high yield and quality despite the lower field emergence in Central Anatolia, where sugar beet is cultivated intensively (Çakmakçı and Oral, 1995; Tuğrul et al., 2012). The 8 cm sowing distance also provided the best results in this study. However, in many areas, farmers do not carry out the necessary thinning and singling because they think it will decrease the number of plants in the field. Eventually, more frequent seedlings prevent the development of sugar beets (Draycott, 2006).

Table 5. *Interaction table according to location, year and subject*

Location / Subject	2012			2013			2014		
	Beet yield	**Sugar content**	**Refined sugar content**	**Beet yield**	**Sugar content**	**Refined sugar content**	**Beet yield**	**Sugar content**	**Refined sugar content**
Etimesgut									
A1 - A2	ns	+	ns	ns	ns	ns	ns	++	ns
B1 - B2	ns	ns	ns	ns	ns	ns	ns	ns	ns
C1 - C5	++	ns	++	++	++	++	++	++	++
Eskişehir									
A1 - A2	ns	ns	ns	ns	ns	ns	ns	ns	ns
B1 - B2	ns	ns	ns	ns	ns	ns	ns	ns	ns
C1 - C5	++	++	++	++	++	++	++	++	++
Ilgın									
A1 - A2	ns	ns	+	ns	ns	ns	+	ns	++
B1 - B2	ns	ns	ns	ns	ns	ns	ns	ns	ns
C1 - C5	++	++	++	++	++	++	++	++	ns

ns: no significant, +: P<0.05, ++: P<0.01

Conclusions

In this study, field emergence irrigation was shown to significantly increase the beet yield and quality in the conditions of Central Anatolian Region which has arid and semi-arid climate in Turkey. Thus, taking into account the climatic conditions after sowing, proper irrigation scheduling would be beneficial to the level of 15-20 mm in the soil condition of the absence of enough moisture. In particular, field emergence irrigation is of great importance in ensuring sufficient emergence when sowing pelleted seeds.

In conclusion, frequent beet rows fail to achieve adequate growth and cause yield loss to the farmers. On the other hand, labor costs also seem to be a growing problem every year. Considering these reasons, if the field and seedbed preparation is carried out at the right time by the appropriate technical quality, and the climatic conditions are suitable, beets can be sown at 12 and 17 cm distances. Increasing seed pelleting thickness reduces remarkably the field emergence, yield and quality. In this case, the seeds P4.50-Ø4.75 mm in dimension, which have the thickest pellet, are not suitable in similar arid and semi-arid climatic conditions. The P4.25-Ø4.50 mm seeds, which have the second thickest pellet, provided better results for C5; however, pelleting with this thickness is considered to be risky. Besides their high field emergence, the seeds pelleted

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P3.50-Ø3.75, P3.75-Ø4.00 and P4.00-Ø4.25 mm in dimension provided a high yield and quality due to their advantages of allowing uniform and high-dose pesticide application. The seeds pelleted 3.50 to 4.25 mm in size were considered to be of the appropriate thickness to obtain the best results. The results indicate that, given the regional climatic conditions, besides planting naked seeds at 8 and 17 cm sowing distances, the best results are obtained by sowing seeds pelleted 3.50 to 4.25 mm in dimension and 0.25-0.75 mm in thickness under irrigated conditions.

Nowadays due to global warming, many regions where sugar beet is grown are under the problem of drought in the spring season or the problem of obtaining sufficient water with rainfall or both. For this reason, continuing trials with thinly coated or uncoated seeds are among the important issues, especially in areas where the water required for germination of seeds and sprouts of the plants to reach the soil surface cannot be adequately met with rains.

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ESTIMATION OF THE CARRYING CAPACITY OF YANGTZE FINLESS PORPOISE *(Neophocaena asiaeorientalis asiaeorientalis*) IN TIAN-E-ZHOU OXBOW BASED ON LINEAR FOOD NETWORK MODEL

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Abstract. A food network model of the Tian-E-Zhou Oxbow ecosystem in the Yangtze River was established by combining fishery resource data with other aquatic biological data. The ecosystem can be divided into seven discrete trophic levels, and nutrient flow occurs mainly between the trophic levels I-V. The average material transfer efficiency of each trophic level was 10.5%, there were no obvious key species in the system, and large aquatic plants had the largest key index (-0.173). The matter and energy recirculation ratio in the system was low, while the values of other indicators such as system connection index (CI), system omnivore index (SOI), Finn's cycle index and Finn's mean path length were all at a low level, indicating that the Tian-E-Zhou Oxbow ecosystem was at an early stage of development. Finally, based on the food network model, the environmental carrying capacity of the Yangtze finless porpoise in the Tian-E-Zhou Reserve was calculated as 0.207 t/km^2, counting about 89 individuals.
Keywords: *ECOPATH with ECOSIM, trophic modeling, energy flow, food web, ecosystem*

Introduction

Capacity originates from the logistic equation of population ecology. It was originally a term in animal husbandry management and has now become an important concept in wildlife management. Leopold (1933) was the first to propose the definition of capacity to be the maximum number of wild animals allowed to survive in an environmental condition. Later, a number of scholars successively proposed the definition of tolerance. For example, Edwards et al. (2013) defined the capacity as the maximum population quantity that can be obtained in the habitat under the environmental conditions favored by the animals within a specific time interval (usually 1 year) without the occurrence of ecosystem degradation and damage to animal quality. Over the years, the carrying capacity concept has been used to describe the growth limits of natural populations, account for resource use by rising human populations and make environmental management decisions (Chapman and Byron, 2018). Animals exist in specific habitats, and changes in habitat conditions will directly or indirectly affect the survival and reproduction of these animals and the sizes of the populations. Therefore, the carrying capacity is not a long-term stable value and will change with changes in various

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ecological factors in the habitat. It becomes a relative constant only for a specified period (Storch and Okie, 2019).

ECOPATH is a mass-based, static, and ecosystem-based model that takes all trophic levels of the ecosystem into account and is mainly used to simulate ecosystem status and internal energy flows (Zhu et al., 2020). Researchers have evaluated carrying capacity through the ECOPATH model based on the assumption that the carrying capacity value is reached when the population increase made the model unbalanced (Byron et al., 2011; Ferreira et al., 2018). This approach represents an advance in that it places the organism at an ecosystem level to assess the carrying capacity. ECOSIM allows for the temporal exploration of potential impacts of a further increase in population based on dynamic species interaction, and has been used for carrying capacity evaluation (Gao et al., 2018; Outeiro et al., 2020).

The Tian-E-Zhou Oxbow is an ex-situ reservation. Five Yangtze finless porpoises (*Neophocaena asiaeorientalis asiaeorientalis*) were moved there in 1990. After years of development, a population of about 60 finless porpoises was present in the site in 2015 (2015 census data). The water area of the Tian-E-Zhou Oxbow has shrunk in recent years due to climate change and the development of the surrounding shoals. In addition, the water quality in the oxbow has been deteriorating. It is, therefore, necessary to calculate whether the population of the Yangtze finless porpoise has reached the saturation state. If the population density is too high, measures should be taken to improve the habitat. Therefore, the assessment of carrying capacity is of vital importance for both species protection and habitat protection in the reserve.

In order to achieve this goal, we built a nutrition ecosystem network channel model (ECOPATH) in Tian-E-Zhou Oxbow with Yangtze finless porpoise population, fishery resources survey database, and other aquatic biological resources survey data. The ecosystem characteristics and energy flow regularity of the Tian-E-Zhou Oxbow were systematically analyzed, the carrying capacity of the Yangtze finless porpoise was estimated and the main factors affecting the carrying capacity were analyzed.

Materials and Methods

Study area

The Tian-E-Zhou Oxbow (112°31'-112°37' E, 29°46'-29°51' N) is located on the northern bank of the Yangtze River about 20 km downstream of Shishou City, Hubei Province, central China (*Fig. 1*). It is oxbow shaped and was originally the main channel of the Yangtze River. It was formed by the natural cutting and straightening of the Yangtze River in 1972. The oxbow is 20.9 km long, with a water surface width of 0.2-1.5 km, average water depth of 4.5 m, and a total area of about 20 km^2.

Model description

In this study, the ECOPATH method was used to construct the nutrition network model in the Tian-E-Zhou Oxbow ecosystem. This method was originally proposed by Polovina (1984) of the Hawaii Institute of Oceanography in the United States and was used to describe the biological production and food digestion processes of aquatic ecosystems in a stable state. Subsequently, combined with the ecological theory of energy analysis of Ulanowicz (1986), it gradually developed into a method of quantitative analysis of ecosystem structure and function.

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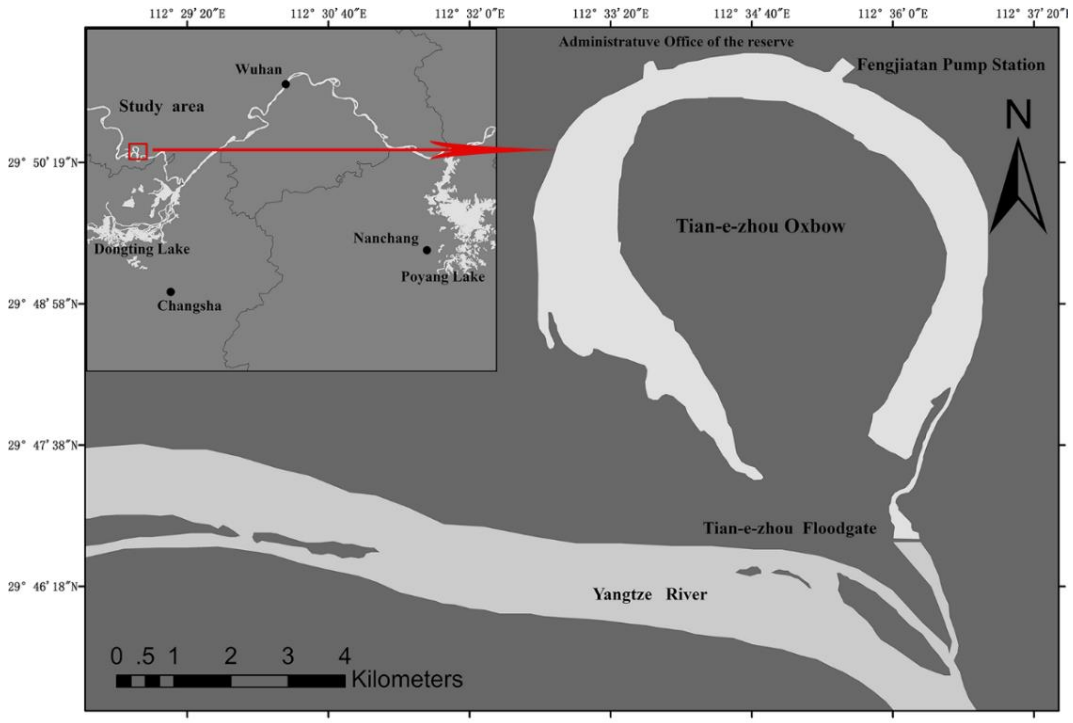

Figure 1. *Location of the study area*

The ECOPATH model defines that an ecosystem is composed of a series of ecologically related functional groups, all of which can combine to cover the process of energy flow in an ecosystem. The model assumes that the energy input and output balance of each functional group can be described by the following formula (Christensen et al., 2008):

$$\boldsymbol{B_i \times (P/B)_i \times EE_i - \sum_{j=1}^{n} B_j \times DC_{ji} - Y_i - B_i = 0} \qquad \text{(Eq.1)}$$

In type, the $\boldsymbol{(P/B)_i}$ is the ratio of functional group i production and biomass, $\boldsymbol{(Q/B)_j}$ for predation function group j consumption and the ratio of biomass, $\boldsymbol{DC_{ji}}$ prey for functional groups of the predator functional group j I always prey on quantity proportion, $\boldsymbol{\sum_{j=1}^{n} B_j \times (Q/B)_j \times DC_{ji}}$ on behalf of the functional groups were all in the amount of functional groups feeding.

The ECOPATH model needs to input the basic parameters including biomass $\boldsymbol{B_i}$, biological turnover ratio $\boldsymbol{(P/B)_i}$, consumption and biomass ratio $\boldsymbol{(Q/B)_i}$, the ecological efficiency of $\boldsymbol{EE_i}$, food nutrition matrix $\boldsymbol{DC_{ji}}$ and total catches a $\boldsymbol{Y_i}$. Any one of the first four parameters can be unknown. The other parameters are calculated by the model, but $\boldsymbol{EE_i}$ is the best to have as an unknown quantity, because the parameters are difficult to measure directly. The latter two parameters, namely food composition $\boldsymbol{DC_{ji}}$, and catch quantity $\boldsymbol{Y_i}$, require input.

The results of the biomass and ecological network analysis indicators total system throughput (TST) (Ulanowicz, 2012), biomass, primary production, respiration, transfer efficiency (Christensen and Walters, 2004), Connectance index, Finn's cycling index

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(Finn, 1976), system omnivory index and Finn's mean path length were downloaded from ECOSIM and ECOSIM network analysis in ECOPATH (Christensen et al., 2005; Behera et al., 2020).

The data source

In this study, the biomass data of finless porpoises were obtained from the population census in 2015. Fish data were obtained from the fish catch and hydroacoustic survey in October 2014, and the other data were obtained from the biological resources survey of the reserve in 2011 as well as published literature and scientific investigation reports on the Tian-E-Zhou Oxbow (e.g., Wang et al., 2012; Sun et al., 2013; Ma et al., 2014).

Functional group division

First, the main biological groups or species in the Tian-E-Zhou Oxbow ecosystem were divided into 18 functional groups according to their biological and ecological characteristics, namely, classification status, nutritional type, individual size, and the water layer they occupied (Christensen et al., 2005; Guo et al., 2013; Li et al., 2018). Species of significant ecological or economic value that needed to be analyzed separately were treated as functional groups for easy study. See *Table 1* for the names of each functional group and the main categories it includes. Among them, the detrital functional group was necessary to construct the nutrition network model. The two functional groups of large aquatic plants and phytoplankton belong to the primary producers, while the remaining 15 functional groups are all consumers, covering the primary consumers, secondary consumers, and top consumers. The defined 18 functional groups can cover the entire process of energy flow in the oxbow ecosystem.

Table 1. *Classification of functional groups within Tian-E-Zhou Oxbow ecosystem*

Number	Functional group	Species composition
1	Finless porpoise	*Neophocaena asiaeorientalis asiaeorientalis*
2	Snakehead fish	*Channa argus*, *Siniperca chuati*
3	Topmouth culter	*Culter alburnus*, *Culter dabryi*, *Culter mongolicus*
4	Catfish	*Pelteobagrus fulvidraco*, *Pelteobagrus eupogon*, *Silurus asotus*, etc.
5	Common carp	*Cyprinus carpio*
6	Crucian carp	*Carassius auratus*
7	Small pelagic fish	*Hemiculter leucisculus*, *Hemiculter bleekeri*, *Toxabramis swinhonis*, *Hyporhamphus intermedius*, *Coilia brachygnathus*, etc.
8	Small bottom fish	*Pseudorasbora parva*, *Rhinogobius giurinus*, *Abbottina rivularis*, *Sarcocheilichtys nigripinnis*, *Rhodeus ocellatus*, *Pseudorasbora parva*, etc.
9	Silver carp	*Hypophthalmichthys molitrix*
10	Bighead carp	*Aristichthys nobilis*
11	Bluntnose bream	*Megalobrama amblycephala*, *Parabramis pekinensis*, *Ctenopharyngodon idellus*
12	Other fish	*Xenocypris davidi*, *Squaliobarbus curriculus*, etc.
13	Shrimp and Crabs	*Macrobrachium nipponense*, etc.
14	Zoobenthos	Mollusca, Insecta, Oligochaeta, etc.
15	Zooplankton	*Rotifer*, *Cladocera, Copeoda*, etc.
16	Phytoplankton	*Phytoplankton*
17	Macrophyte	plants
18	Detritus	Decayed animal and plant, etc.

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Parameter settings

Yangtze finless porpoise

The biomass of Yangtze finless porpoise was obtained according to the population census data in the reserve in 2015. The P/B coefficient and Q/B coefficient refer to the setting coefficient of other ECOPATH models and the harbor porpoise of the same family and close weight, respectively 0.02 and 15 (Nurhakim, 1979; Plaganyi and Butterworth, 2005).

Fish

The total biomass of fish was obtained from 2014 hydroacoustic and catch survey data. According to the results of the hydroacoustic survey (unpublished data), the volume density of fish in the Tian-E-Zhou Oxbow was 0.18 Ind./m^2, which was converted to 0.81 Ind./m^2 of biomass. The P/B value of fish is derived from the following formula (Beverton and Holt, 1957; Pauly, 1980):

$$F = Y / B \quad \text{(Eq.2)}$$

$$P / B = Z = F + M \quad \text{(Eq.3)}$$

$$M = K^{0.65} \times L_{\infty}{}^{-0.279} \times T_c{}^{0.463} \quad \text{(Eq.4)}$$

Y and B represent catch and fish biomass, respectively. While F and M stand for fishing mortality and natural mortality, respectively, K is the parameter of the Von Bertalanffy growth equation, $\boldsymbol{L}_{\infty}$ and is the asymptotic value of the body length of the Von Bertalanffy growth equation, K and $\boldsymbol{L}_{\infty}$ are obtained by a FishBase database query. T is the average annual water temperature (℃).

The Q/B coefficient of fish is obtained by the empirical formula of Palomares and Pauly (1998).

Shrimp and crab

The biomass P/B and P/Q values of shrimp and crab were referenced to freshwater lakes or reservoirs in the Yangtze River basin at the same latitude (Halfon et al., 1996; He et al., 2000; Christensen et al., 2000; Deng et al., 2014).

Benthic and plankton

The biomass of benthic fauna, zooplankton and phytoplankton was based on the 2011 survey data of aquatic organisms, averaging 14.03 t/km^2, 16.2 t/km^2 and 32.4 t/km^2, respectively. The P/B and Q/B coefficients were obtained by referring to results from other lakes at the same latitude or by estimating the P/Q coefficients (Yan and Liang, 2003; Liu et al., 2007; Zeng et al., 2011).

Detritus

Detritus includes both bacterial and organic detritus, and the bacterial biomass is estimated at 17.5% of the phytoplankton biomass according to Heymans et al. (2004), and this result can be used generally. The biomass of organic debris was estimated by referring to the linear model proposed by Pauly et al. (1993).

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Model debugging

Because the ECOPATH model builds a steady-state system model, the energy flow budget for each functional group must be balanced. First, ecological nutrition efficiency EE = (feed intake + catch)/ production. The sum of predation and catch in a functional group should be greater than 0 and less than its production, so 0<EE<1. The EE value of the functional group under high predation or fishing pressure in the system can be close to 1. Some underutilized functional groups usually have low EE values. Second, the respiration rate of each functional group must be greater than 0, and the R/B coefficient (the ratio of respiration rate to biomass) of fish should be between 1 and 10. Finally, the P/Q coefficient of the functional group represents the ratio of production to consumption, which is generally distributed between 0.1 and 0.3 in ecology, but may be less than 0.1 for some vegetative functional groups. When running the ECOPATH model, if these conditions are not met, the model cannot achieve balance or will lack the necessary biological significance. The researcher can then repeatedly adjust other input parameters in an appropriate range of settings, such as B, P/B, or Q/B, and the composition of food, to find the equilibrium that has the actual meaning of the optimized parameter values while debugging steps of the specific reference (Christensen et al., 2008).

Result

Model output parameters and ecological significance

After the ECOPATH food web model of the Tian-E-Zhou Oxbow ecosystem had been balanced from 2011 to 2014, its basic parameters were summarized in *Table 2*. Since the biomass accumulation and migration of each functional group were all 0 in this study, the ecological nutrient conversion efficiency EE reflects the degree to which the production of each functional group is utilized by predation and fishing. It can be seen from *Table 2* that the EE values of channa Argus, silver carp, and bighead ranged from 0.769 to 0.831, indicating that they were highly utilized, which was consistent with the annual catch of the oxbow. The EE value of small pelagic fish was 0.823, mainly due to the influence of the feeding pressure of the Yangtze Finless porpoise. The EE value of zooplankton was as high as 0.918, which was related to silver carp and bighead. Phytoplankton, large aquatic plants, and detritus were not utilized to a high degree (EE values were 0.21, 0.35, and 0.035, respectively).

Overall features of the Tian-E-Zhou Oxbow ecosystem

The total system throughput (TST) of the aquatic ecosystem of Tian-E-Zhou Oxbow was 19271.490 t/km^2/year, the total production (TP) of the system was 8977 t/km^2/year, and the total primary productivity (TPP) was 8486.625 t/km^2/year, accounting for 94.53% of the total production (*Table 3*).

The ratio of total primary productivity (TPP) to total respiration (TR) of the system (TPP/TR) is an important indicator of system maturity. Generally, the value of TPP/TR in a mature ecosystem is close to 1, while TPP/TR>1 in a developing system. TPP/TR<1 for systems exposed to organic pollution (Odum, 1971). The TPP/TR=18.131 (*Table 3*) indicates that the Tian-E-Zhou Oxbow ecosystem is still a developing system.

The connection index (CI) and System omnivory index (SOI) are indicators of the complexity of the connections within an ecosystem, and the CI and SOI are higher, meaning that the system within the greater the chance of a variety of nutrients can be

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reused. The ecosystem is a more stable and mature system of CI index between the functional groups, and the SOI index value is close to 1 (Odum, 1971; Christensen, 1995). The CI and SOI indices of the Tian-E-Zhou Oxbow were 0.267 and 0.262 (*Table 3*), respectively, indicating that the degree of aggregation and stability among the functional groups of the ecosystem was low.

Table 2. *Input and output parameters for the ECOPATH model of Tian-E-Zhou Oxbow*

Functional group	Trophic level	Biomass (t/ km^2)	Yield (t km^2)	P/B (year^{-1})	Q/B (year^{-1})	EE	P/Q
Finless porpoise	3.627	0.138		0.020	15.000		0.001
Snakehead fish	3.619	0.320	0.200	0.760	4.180	0.831	0.182
Topmouth culter	3.610	0.870	0.530	0.990	4.470	0.623	0.221
Catfish	3.548	0.440	0.310	1.990	9.030	0.501	0.220
Common carp	2.833	0.740	0.180	1.520	9.540	0.556	0.159
Crucian carp	2.308	0.680	0.250	2.100	7.000	0.729	0.300
Small pelagic fish	2.596	2.840		1.600	15.000	0.823	0.107
Small bottom fish	2.649	2.100	0.200	2.300	17.390	0.763	0.132
Silver carp	2.500	5.200	4.500	1.090	13.600	0.805	0.080
Bighead carp	3.000	7.500	7.400	1.630	8.970	0.769	0.182
Bluntnose bream	2.006	0.190	0.110	1.530	10.830	0.449	0.141
Other fish	2.130	0.140	0.090	2.100	10.600	0.404	0.198
Shrimp and Crabs	2.537	1.510	0.15	3.500	20.000	0.785	0.175
Zoobenthos	2.481	14.030		3.400	8.000	0.680	0.425
Zooplankton	2.111	16.200		25.000	130.000	0.918	0.192
Phytoplankton	1.000	32.400		260.000		0.210	
Macrophyte	1.000	50.100		1.250		0.350	
Detritus	1.000	7.670				0.035	

Note: Estimated parameters in bold by model, P-production, B-biomass, Q-consumption

Table 3. *Global ecosystem properties in Tian-E-Zhou Oxbow*

Parameter	Value 1	Value 2	Unit
Sum of all consumption, TC	2492.185	2493.220	t/km^2/year
Sum of all exports, TEX	8018.562	8018.355	t/km^2/year
Sum of all respiratory flows, TR	468.064	468.270	t/km^2/year
Sum of all flows into detritus, TDET	8292.676	8292.470	t/km^2/year
Total system throughput, TST	19271.490	19272.311	t/km^2/year
Sum of all production, TP	8977	8977.001	t/km^2/year
Total net primary production, TPP	8486.625	8486.625	
Total primary production/total biomass, TPP/TB	62.679	62.647	
Total primary production/total respiration, TPP/TR	18.131	18.123	
Total biomass, TB	135.398	135.467	t/km^2/year
Connectance index, CI	0.267	0.267	
System omnivory index, SOI	0.262	0.260	
Finn's cycling index, FCI	2.48	2.48	%
Finn's mean path length, FML	2.271	2.271	

Note: V1 is means the current status of the system (2011-2015); V2 is means the status at ecological carrying capacity for the Yangtze finless porpoise. The calculation method of all parameters is based on Christensen et al. (2005)

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Finn's circulation index (FCI) is the ratio of the circulation flow to the total flow in the system, and Finn's average path length (FML) is the average length of each cycle through the food chain. The higher the proportion of material recycling, and the longer the food chain through which the nutrient flow passes, the higher the system maturity (Christensen, 1995). The FCI and FML of the Tian-E-Zhou Oxbow ecosystem were 2.488% and 2.271 (*Table 3*), respectively, indicating that the water area had low material and energy recirculation ratio, short flow path, and the system was in the early stage of development.

Nutrient-level structure and material energy flow of the Tian-E-Zhou Oxbow ecosystem

The trophic levels of each functional group in the Ecosystem of Tian-E-Zhou Oxbow were divided into 7 discrete trophic levels (*Table 4*). It can be seen from the table that detritus and two primary producers completely occupy the nutritive grade I. The vegetative or omnivorous consumer functional group mainly occupied the trophic level II and III, among which zooplankton completely occupied the trophic levels II. Carnivorous fish and the functional group of the Yangtze finless porpoise mainly occupied the III and IV nutritional grades, and a small amount occupied the V and VI nutritional grades; the trophic level was completely dominated by the Yangtze finless porpoise. According to the specific flow distribution of each discrete trophic level, the nutrients in the Ecosystem of Tian-E-Zhou Oxbow mainly flowed between the first five discrete trophic levels. The traffic for the Ⅵ and traffic Ⅶ nutrition levels was very low, less than 1%, and can be ignored.

Table 4. *Trophic level decomposition of the functional groups in Tian-E-Zhou Oxbow ecosystem*

Functional group	Trophic level						
	Ⅰ	Ⅱ	Ⅲ	Ⅳ	Ⅴ	Ⅵ	Ⅶ
Finless porpoise			0.484	0.474	0.0387	0.00280	0.000022
Snakehead fish			0.526	0.383	0.0861	0.00491	0.000019
Topmouth culter			0.506	0.440	0.0529	0.00183	
Catfish			0.590	0.338	0.0723	0.000375	
Common carp		0.400	0.447	0.137	0.0167		
Crucian carp		0.780	0.163	0.0565	0.000833		
Small pelagic fish		0.480	0.503	0.0167			
Small bottom fish		0.500	0.416	0.0832	0.000833		
Silver carp		0.550	0.450				
Bighead carp		0.100	0.900				
Bluntnose bream		0.995	0.00500				
Other fish		0.900	0.0833	0.0167			
Shrimp and Crabs		0.600	0.317	0.0833			
Zoobenthos		0.667	0.333				
Zooplankton		1.000					
Phytoplankton	1.000						
Macrophyte	1.000						
Detritus	1.000						

The total flow of a trophic level is the sum of the flux of all substances flowing through it per unit time. The total capacity of each trophic level is affected by respiration, arrests, food intake, catches, and the amount of debris. The transfer efficiency of each trophic

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level is equal to the ratio of the sum of its food intake and catch to the total flow, indicating the efficiency of utilization of the trophic level in the ecosystem. The energy flow process among different trophic levels in the Tian-E-Zhou Oxbow is shown in *Fig. 2*. Trophic level I includes phytoplankton and detrital functional groups, so the energy flow process of the Tian-E-Zhou Oxbow ecosystem can be divided into the grazing food chain and the detrital food chain. After the two food chains were merged, the transfer efficiency of trophic levels II, III, IV, V, VI, and the two food chains in the Tian-E-Zhou Oxbow ecosystem, in turn, were 9.0%, 11.9%, 11%, 10.9%, 10.2%, and 5.3%, respectively, with a total average conversion efficiency of 10.5% (*Table 5*).

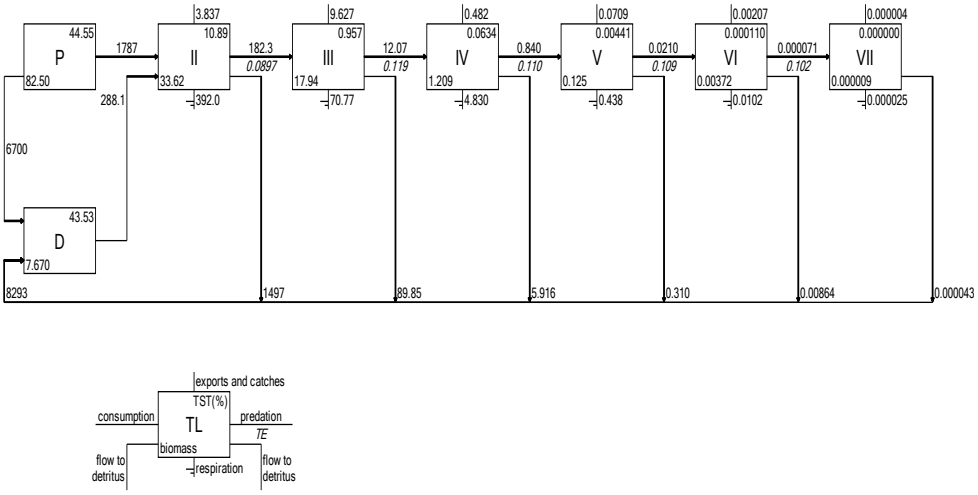

Figure 2. *Trophic flows transferred along trophic levels in Tian-E-Zhou Oxbow ecosystem*

Table 5. *Transfer efficiencies among trophic levels in Tian-E-Zhou Oxbow ecosystem (%)*

Source	Trophic level					
	II	III	IV	V	VI	VII
Producer	8.7	11.9	11.0	11.0	10.2	
Detritus	10.8	11.7	11.0	10.8	10.1	
All flows	9.0	11.9	11.0	10.9	10.2	5.3
The energy from the debris shows a flow proportion: 0.45						
Primary producer approach to conversion efficiency: 10.4%						
Detrital pathway conversion efficiency: 11.2%						
Total conversion efficiency: 10.5%						

The carrying capacity of theYangtze finless porpoise in the Tian-E-Zhou Oxbow

The Yangtze finless porpoise is the top predator of the Tian-E-Zhou Oxbow ecosystem and a major conservation target of the Reserve. As the population continues to grow, it may cause a significant change in the energy flow of the Tian-E-Zhou Oxbow ecosystem. According to the definition of the carrying capacity in this study, when the population of the Yangtze finless porpoise in the ecosystem gradually increases, the predation pressure on its food resources is bound to increase, resulting in EE greater than 1. Then, the ecosystem is destroyed, and the model is out of balance. At present, the population is the carrying capacity of the Tian-E-Zhou Oxbow. In the iterative process, the biomass

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(population number) of the finless porpoise continuously increased. When it increased to 0.207 t/km^2, the model was not balanced. At this time, EE >1 in the upper-middle and upper small fish functional groups was >1 (*Table 6*). Therefore, it is estimated that the average biomass of the finless porpoise supported by the Tian-E-Zhou Oxbow ecosystem is 0.207 t/km^2 (about 89 individuals).

Table 6. *Changes in Tian-E-Zhou Oxbow ECOPATH model while estimating the carrying capacity of Yangtze finless porpoise*

Frequency	The biomass of the Yangtze finless porpoise(t/km^2)	EE of Small pelagic fish	Changes in the model
1	0.138	0.823	Balance
2	0.148	0.838	Balance
3	0.158	0.874	Balance
4	0.168	0.900	Balance
5	0.178	0.926	Balance
6	0.188	0.952	Balance
7	0.198	0.977	Balance
8	0.208	1.003	No balance
9	0.207	1.001	No balance
10	0.206	0.998	Balance

Discussion

The Tian-E-Zhou Oxbow was originally the channel of the Yangtze River, which naturally curved and straightened. It was seasonally connected to the Yangtze River; however, after the completion of the Tian-E-Zhou Oxbow floodgate in 1998 (*Fig. 1*), it was cut off from the Yangtze River, so its ecosystem features are similar to lakes or small reservoirs. Due to the lack of large aquatic plants, the total flow was only 62.62 t/km^2 year^{-1}, which was much lower than the total flow of phytoplankton (8424 t/km^2 year^{-1}) (*Fig. 2*). Because the biomass of herbivorous fish is very small, the primary producer of the grazing food chain in the Tian-E-Zhou Oxbow ecosystem is the phytoplankton. However, the detrital food chain is also a significant energy flow channel, and the detrital energy flow accounts for 45% of the total flow of the system. In *Fig. 2*, we found that the total amount of organisms flowing into the debris of the biological chain finally reached 8290 t/km^2 year^{-1}, indicating that there was still a large quantity of nutrients left in the Tian-E-Zhou Oxbow ecosystem. This nutrient load has caused the deposition of a large amount of unused debris at the bottom of the water body, increasing the endogenous pollution and resulting in a nutrient excess and the emergence of a large amount of phytoplankton. The main fish species, including free-range silver carp, bighead, and a large number of silver carp, are caught every year. It can also be seen from *Fig. 2* that the output of nutrient grades II (3.837 t/km^2 year^{-1}) and III (9.627 t/km^{2}year^{-1}) is the largest, and they are the main exporters.

According to the ecosystem development described by Odum (1971) and Christensen (1995), TPP/TR is >1 in the immature ecosystem and will approach 1 when the ecosystem becomes mature. The Ecopath model has been constructed for many lakes to assess the maturity of aquatic ecosystems. The TPP/TR ratio was 18.13 in the Tian-E-Zhou Oxbow ecosystem (*Table 3*). This ratio was higher than that of most lakes in China, including Shangshe, Gehu, Dianchi, Chaohu, Taihu (Jia et al., 2012; Li et al., 2013, 2019; Shan et

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al., 2014; Kong et al., 2016). That may because the Tian-E-Zhou Oxbow was cut off with Yangtze River in 1998 and belongs to a relatively young lake.

The average conversion efficiency of the general ecosystem was approximately 10% (Lindeman, 1942), while the conversion efficiency between the trophic levels of the Tian-E-Zhou Oxbow ecosystem was 10.5% (*Table 5*), which is relatively close. However, the conversion efficiency between grade I and grade II in primary producers is low (8.7%) (*Table 5*), which may be due to a suboptimal proportion of silver carp and bigheads, resulting in a loose connection between functional groups. On the one hand, the filter pressure of silver carp on phytoplankton is low; on the other hand, the pressure of bighead, which is large, is high, which leads to less feeding of phytoplankton by the bighead, which will lead to the overgrowth of phytoplankton which cannot be fully utilized, and there is the risk of "bloom". Therefore, it is suggested that the stocking ratio of silver carp and bighead in the Tian-E-Zhou Oxbow should be adjusted to a level that is beneficial to the stability of the ecosystem.

By improving the conversion efficiency of residual nutrients and the ecosystem characteristics of the Tian-E-Zhou Oxbow, the aim is to improve the habitat quality of the Yangtze finless porpoise and expand the carrying capacity of the oxbow to accommodate the porpoise and protect the population more effectively. In this study, the carrying capacity of the Tian-E-Zhou Oxbow for the finless porpoise was 0.207 t/km^2, accounting for about 89 individuals (*Table 6*). According to this study (*Table 2*), the biomass of the small fish is the main factor affecting the carrying capacity of the habitat for the finless porpoise.

The carrying capacity is a fundamental feature of wildlife conservation biology that influenced by many factors. For example, in aquatic ecosystems, the primary productivity of the animal habitat determines the amount of plant growth available, and plant growth is under the influence of various physical and chemical factors in the water (Hobbs et al., 1985). The availability of plants is closely related to the limitation of the mobility of aquatic animals (Hobbs et al., 1982). The efficiency determines the primary consumers of resources, layer upon layer, top determines the number of consumers. The purpose of estimating the carrying capacity of the Tian-E-Zhou Oxbow for the Yangtze finless porpoise is to determine the capacity of the system from the perspective of energy balance. The ECOPATH model itself does consider the biological growth changes at each trophic level; it only takes fixed parameter values and does not take into account the spatial changes. Therefore, there is still a shortage of biological variables, such as life history. The carrying capacity is a process of dynamic change that can be influenced by both population and habitat changes of species. Therefore, dynamic changes that cannot be described by the static model need to be considered when calculating the carrying capacity. However, the estimation method of carrying capacity used in this study can provide a referential calculation method for the selection of future protected areas, which can help determine the carrying capacity of ex-situ reserves for the Yangtze finless porpoise.

Conclusion

In this study, a food web model of the Tian-E-Zhou oxbow ecosystem was developed based on the EwE modeling framework. The aim is to use the model to estimate the ecological carrying capacity of the Tian-E-Zhou oxbow ecosystem for the Yangtze finless porpoise. The model shows that the material and energy recycling was low, together with

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a low connectance index (CI), system omnivory index (SOI), Finn's cycled index, and Finn's mean path length, indicating that the Tian-E-Zhou ecosystem is an immature ecosystem and still at its development stage. Finally, based on the ECOPATH model, the carrying capacity of the Tian-E-Zhou reserve for Yangtze finless porpoise was 0.207 t/km^2, accounting for about 89 individuals. The ecological capacity of target species in the Tian-E-Zhou ecosystem is determined on the basis of mass balance, without further consideration for spatial changes and life history. The set of parameters used to determine the ecological carrying capacity is one of the possible parameter combinations that can achieve similar results. This model is open for further improvement. In addition, DNA barcoding for prey identification or a combination of DNA-barcoding and stable isotope analyses may further elucidate the energy flow of the Tian-E-Zhou ecosystem as well as enable proper evaluation of the carrying capacity of Tian-E-Zhou reserve for the Yangtze finless porpoise.

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EFFECTS OF GRAZING EXCLUSION BY FENCE ON VEGETATION CHARACTERISTICS AND COMMUNITY DIVERSITY OF MONGOLIAN GRASSLANDS

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Abstract. Grazing exclusion by fence was often used in the management of grassland ecosystems. However, the direction and the strength of effects of grazing exclusion on grassland vegetation characteristics and community diversity are currently disputable. In addition, little research was conducted on the effects of grazing exclusion on vegetation phenology. In this study, we aim to determine the effects of grazing exclusion on vegetation characteristics, community diversity and vegetation phenological periods. For this, we performed a fencing experiment in three grassland types in Mongolia. Each grassland type was set by two treatments: grazing exclusion and freely grazing. We found that grazing exclusion generally increased vegetation height regardless of grassland type. In contrast, variations of vegetation cover caused by grazing exclusion were not consistent in three grassland types. Grazing exclusion decreased species richness and Shannon diversity. However, community evenness varied little without grazing disturbances. Grazing exclusion advanced the vegetation phenological periods in three grassland types. This study fills the knowledge gap of the effects of grazing exclusion on vegetation characteristics, community diversity and vegetation phenological development in Mongolian grasslands. Other management strategies like rotational grazing and reseeding are encouraged to be used in tandem with grazing exclusion to restore Mongolian rangelands.
Keywords: *vegetation cover, species richness, Shannon-Wiener diversity, phenological periods, grassland type*

Introduction

Grasslands are an important component of terrestrial ecosystems and greatly influence provisions of ecosystem services like food supplies, biodiversity maintenance and climate regulation (Solen et al., 2019). Grasslands sustain nomadic peoples and facilitate the production of livestock (Kemp et al., 2013). However, increasing anthropogenic activities, in particular livestock volume and the resulting overgrazing often cause grassland degradation, especially in arid and semi-arid regions (Ahlborn et al., 2020; Bosch, 1989). Overgrazing significantly alters the community structure through decreasing vegetation cover and species diversity (Mysterud, 2006). Simultaneously, overgrazing has important management implications, since it leads to great losses of economic resources and threatens plant diversity (Stein et al., 2016).

To restore degraded grassland ecosystems, grazing exclusion by fence is a useful management practice (Xiong et al., 2016). Fencing can be regarded as a direct reference to determine the benefits of grazing removal on ecosystem services and functioning in grasslands (Davies and Boyd, 2019). Nevertheless, grazing gradient experiments focus

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on ecological consequences of livestock grazing intensity (Stein et al., 2016). Degraded grasslands enter self-recovery state in case grazing activities are forbidden for a suitable period (Golodets et al., 2010). A large number of researches have been conducted to examine effects of grazing exclusion on vegetation characteristics and diversity under different grazing regimes (Augustine et al., 2017; Koch et al., 2017; Sagar et al., 2019; Yao et al., 2019). Grazing exclusion by fence often increases vegetation height and cover (Li et al., 2014; Zhu et al., 2016a). However, the findings are not consistent in terms of plant diversity. Palatable plant species may benefit from fencing owing to their fast tissue regrowth (Guo et al., 2016). Grazing exclusion may decrease richness of grazing avoided species since slow growing tissue constrains their competition capacity (Rota et al., 2016). In addition, the response of community diversity to grazing exclusion is largely determined by fencing time. Grazing history strongly affects the forage quality of grasses (Adler et al., 2004). Long-term grazing exclusion by fence generally has negative effects on the community diversity owing to density effects (Wu et al., 2009; Yao et al., 2019). Short-term grazing exclusion is beneficial for the community vegetation diversification since most of plant species rapidly grow without grazing (Zhang and Zhao, 2015). Oppositely, grassland species richness and diversity were also found to increase under the condition of long-term grazing exclusion (Sagar et al., 2019; Zhu et al., 2016a). Several studies show that grazing exclusion by fence plays little role in maintaining grassland plant diversity (Koch et al., 2017; Xiong et al., 2016). These fencing experiments were implemented in different grassland types characterized by specific combinations of plant species. Diverse responses of plant species to grazing exclusion largely shape the structure and the diversity of grassland communities. However, it needs to be strengthened for effects of grassland type and their interaction with grazing removal time on characteristics and the diversity of vegetation communities. Nevertheless, several long-term grazing exclusion experiments have been carried out to explore these effects (Goheen et al., 2018; Irisarri et al., 2016).

Besides vegetation characteristics and community diversity, grazing exclusion also strongly affects vegetation phenological development. Key aspects of vegetation phenology such as green up, seeding and flowering have profound implications for restoration of degraded grasslands. Short-term grazing exclusion advances the phenology of *Kobresia pygmaea* (Zhu et al., 2016b). Moreover, grazing alters the flowering probability of the short-lived perennial *Cirsium vulgare* (Marco and Silvertown, 2014). Annual grass species in the ungrazed plots flower earlier than in the grazed ones (Bergmeier, 1998). However, different species have distinctive sensitivities and subsequent phenological response to grazing exclusion. In addition, the consequences of grazing for the same species are likely to vary across different grassland types, which are characterized by site-specific grazing history and vegetation community properties (Stahlheber and D'Antonio, 2013). Therefore, currently available work needs to be strengthened towards clarifying the effects of grazing exclusion on the vegetation phenology.

To fill the above-mentioned knowledge gap, the study was conducted in three grassland types of Mongolia, namely meadow, mountain steppe and dry steppe. Grasslands occupy 83.4% of the land area in Mongolia, while the nomadic people are the majority among population (Li et al., 2005). Livestock grazing is a traditional land use form of the Mongolian grasslands (Ahlborn et al., 2020). With an increase of the population, most grasslands in Mongolia are experiencing overgrazing and an increased threat of degradation (Wang et al., 2020). Restoring the degraded grasslands is

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necessary for a long-term utilization of natural resources in the vast grassland region. Although a grazing restriction has been implemented for a period in this area, little is known about effects of grazing exclusion on vegetation characteristics, community diversity and vegetation phenology. We examined the response of these factors to grazing exclusion through performing a 6-10 years enclosure trial and addressed the following questions: (1) How does grazing exclusion by fence affect vegetation characteristics and community diversity in Mongolian grasslands? (2) Is the impact of grazing exclusion dependent on grassland type? (3) How does vegetation phenology respond to grazing exclusion?

Materials and methods

Study site

Three grassland types, namely dry steppe (47°12' N, 108°44' E), mountain steppe (48°17' N, 108°43' E) and meadow (48°11' N, 108°26' E), are located in the southeast of the Ulaanbaatar on the Mongolian Plateau (*Fig. 1*). According to historical climatological data from 1993 to 2002, mean annual temperature is 1.2 ℃, with the highest mean daily temperature in July (21.4 ℃) and the lowest temperature in January (-22.9 ℃). Mean annual precipitation is less than 200 mm. More than 50% of annual precipitation occurs during the vegetation growing season (June to September). The soil of the area is mostly composed of burozem and of a high calcium content. The vegetation communities in three grassland types are characterized by various combinations of dominant species: *Potentilla anserine*, *Carex duriuscula*, *Carex pediformis*, *Carex coriophora* and *Taraxacum cerataphorum* in the meadow; *Potentilla acaulis*, *Aster alpinus, Festuca lenensis, Carex pediformis* and *Stipa baicalensis* in the mountain steppe; *Artemisia frigida*, *Allium bidentatum*, *Artemisia adamsii*, *Kochia prostrata* and *Astragalus galacitites* in the dry steppe. Most plant species in the area green up in late April and senesce in late October. The average grazing intensity is 7 large stock units per 100 ha in the meadow and the mountain steppe, while a heavier grazing intensity occurs in the dry steppe with a value of 11 large stock units per 100 ha. One large stock unit could be simply deemed as 1 cow.

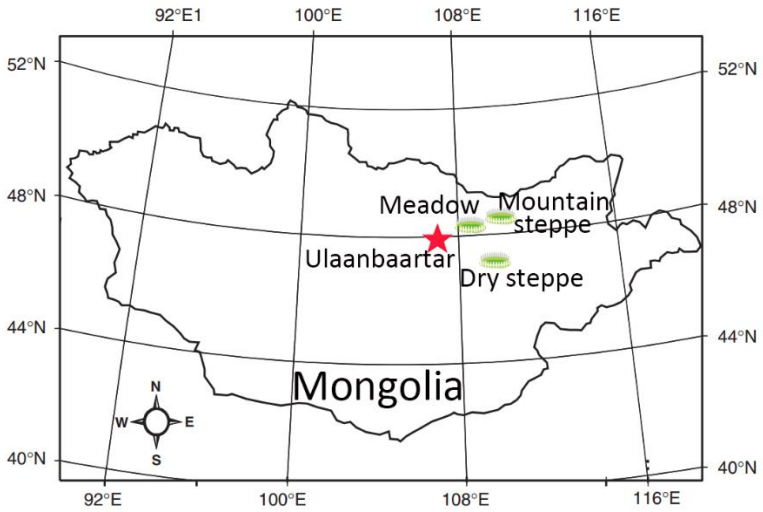

Figure 1. *Geographical locations of three Mongolian grassland types*

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Experimental design

To examine effects of grazing exclusion on vegetation characteristics, community diversity and vegetation phenology of the Mongolian grasslands, the enclosure was set up to completely exclude livestock grazing in the meadow from 2009, the mountain steppe and the dry steppe from 2013 (*Fig. 2*). We set one enclosure in each grassland type. Each enclosure covered an area of 0.1 ha (50 m × 20 m). Three sites of the enclosure were selected over a homogeneous area and had similar geographical conditions like slope, elevation and soil types. A freely grazed area was selected as the control treatment nearby the enclosure of each typical grassland. That is, each grassland was carried out by two treatments: grazing exclusion by fence (GE) and freely grazing (FG). In 2013, we randomly sampled ten quadrats (1 m × 1 m) of fenced plots in the mountain steppe and the dry steppe respectively. Similarly, we chose ten quadrats (0.5 m × 0.5 m) of fenced plots in the meadow, where grasses were much denser. The quadrats in the fence had a distance of 5 m to fence margins to minimize edge effects. Simultaneously, ten quadrats were separately sampled in grazed plots for three grassland types. The quadrats in grazed plots had the same distance of 20 m to the corresponding ones in fenced plots. We used a bootstrapping method to sample 3 quadrats from 10 for both fenced and grazed plots in each grassland. We performed the implementation 10 times in three grassland types respectively. We found that a small number of quadrats depicted well general changes in vegetation cover regardless of treatments and grassland type (see *Table A1* in the Appendix). Thus, we separately determined three quadrats in the dry steppe, the mountain steppe and the meadow. Then we sampled the same quadrats in the following years. We used the same quadrats to weaken confounding effects of abiotic factors like soil properties on the vegetation communities. Effects of grazing exclusion on grassland communities were often studied from a taxonomic perspective. Thus, the number, the height, vegetation cover and phenology of plant species, as well as the number of species individual were investigated in each quadrat at the end of August (late in the growing season) of every year. We measured vegetation cover through visual estimates. Vegetation height was determined from the surface of ground to reproductive leaves or branches. The number of plant species was recorded according their vegetation cover in one quadrat. Specifically, we set a standard of the cover more than 0.05 to identify whether the species should be counted. We elaborately investigated 108 quadrats in a 6 consecutive years experiment (3 quadrats × 2 treatments × 6 years × 3 grassland types). Moreover, we tried to examine the response of vegetation palatability to grazing exclusion. We classified all the plant species into two categories according to their palatability for livestock: palatable species (grass species and sedge species) and non-palatable species (forb species, Artemisia species and leguminous species) (Yao et al., 2019). We showed palatability-related results of the vegetation characteristics and the community diversity under treatments of grazing exclusion and freely grazing (see *Table A2* in the Appendix). This aims to offer a mechanistic and supplementary interpretation of grazing exclusion effects on grassland communities.

We examined the response of vegetation to grazing exclusion in terms of phenology. Vegetation phenological periods were divided into five stages: germination, growth, bud, flower and seeds. The five periods were assigned with sequential numbers from 1 to 5 respectively. Categorization of the phenological stages followed the system of Moore et al. (1991). In case certain species had different values of phenological periods for one enclosure, the phenological period corresponding to the largest cover ratio

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(cover of one species divided by total cover of all species in one quadrat) among quadrats was determined as the phenology of the species. Then we evaluated the phenological period of the same species inside (ungrazed) and outside (grazed) the enclosure for three grassland types. To quantify effects of grazing exclusion on the vegetation phenological period, we established an evaluation index of relationship change of phenological stages (*EIRCP*) to describe phenological differences between fenced plots and grazed plots. The index was calculated according to *Equations 1* and *2*.

$$APS = \frac{\sum_{i=i}^{n} PS_i}{n} \quad \text{(Eq.1)}$$

$$EIRCP = \frac{APS_{inside} - APS_{outside}}{4} \quad \text{(Eq.2)}$$

PS_i denotes the phenological period of species i; APS denotes the average phenological period of all species inside (ungrazed) or outside (grazed) the enclosure; The number 4 represents the maximum phenological differences between inside and outside the enclosure. We assumed that it is impossible for the case that the phenological stage is 5 (seeds) in the fence plot, while the stage is 1 (germination) in the grazed plot. If EIRCP is larger than 0, then it means the vegetation phenology advances; If EIRCP is smaller than 0, then it means the vegetation phenology delays. The value of ERCIP ranges from -1 to 1.

We thereafter calculated the distribution of EIRCP relative to 0 in each grassland type. In addition, we provided species proportions in terms of phenological changes for three grassland types (see *Fig. A1* in the Appendix).

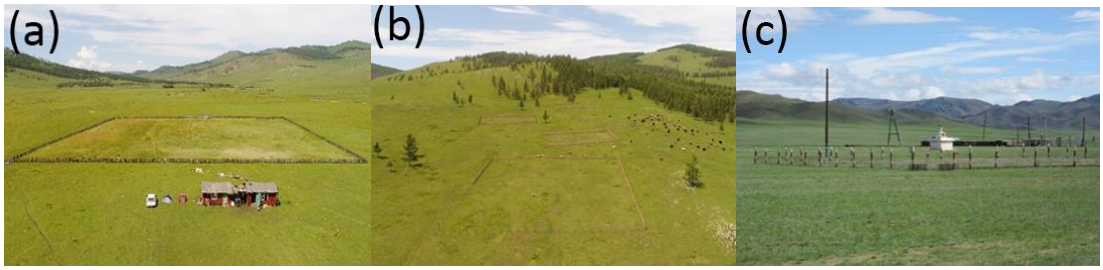

Figure 2. *The picture of experimental sites: (a) meadow (b) mountain steppe (c) dry steppe*

Data analysis

Vegetation characteristics of grassland communities were evaluated in terms of plant height and vegetation cover. Richness index (the number of plant species), Shannon-Wiener diversity index (*H'*) and Pielou evenness index (*E*) were calculated for each quadrat.

$$Richness = S \quad \text{(Eq.3)}$$

$$H' = -\sum_{i=1}^{S} P_i(lnP_i) \quad \text{(Eq.4)}$$

$$E = \frac{H'}{lnS} \quad \text{(Eq.5)}$$

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P_i denotes the cover ratio of species i to total species; H' denotes Shannon-Wiener index; E denotes Pielou evenness index.

We used Wilcoxon signed rank test to assess differences of the vegetation characteristics and the community diversity between grazing exclusion and grazed treatments within each year for three grassland types. Statistical analyses were performed using R software (R-core-Team, 2017).

Results

Response of vegetation characteristics to grazing exclusion

Grazing exclusion by fence significantly increased vegetation height for all three grassland types (*Fig. 3*). However, the height varied differently with grassland type. It was with a substantially higher height without grazing than the grazing scenario for the meadow, and a comparatively lower height than that of the other two grassland types under grazing treatment. Grazing exclusion generally did not facilitate the increment of vegetation cover. The response of vegetation cover to grazing also varied with grassland type. Grazing exclusion increased the cover in the dry steppe while decreased the cover in the meadow and the mountain steppe. In addition, the effect was only significant in the dry steppe. There was no clear inter-annual tendency of grazing exclusion induced variations of vegetation height and cover.

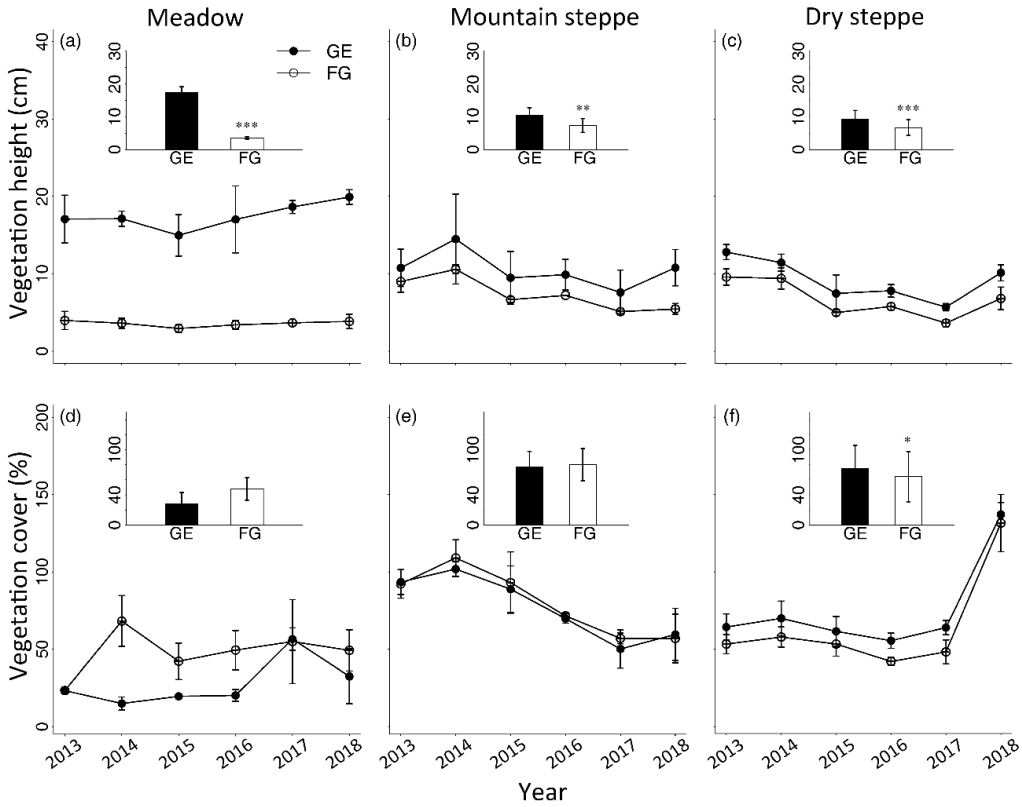

Figure 3. *Vegetation height (a, b, c) and cover (d, e, f) under two treatments: grazing exclusion by fence (GE) and freely grazing (FG) of three grassland types from 2013 to 2018. Scatter plots show time-series variations of vegetation characteristics. Bar plots attached show the average value of six years. Error bars are shown by standard deviation. Letters within bar plots indicate differences with a significant level ($P < 0.05$) between two treatments*

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Response of community diversity to grazing exclusion

Grazing exclusion generally had negative effects on the species richness and the Shannon diversity in three grassland types (*Fig. 4*). Species richness in fenced plots significantly differed from that in grazed plots. Similarly, the effect of grazing exclusion suited well in terms of Shannon diversity in the meadow and the dry steppe, but performed weakly in the mountain steppe. No obvious differences of the community evenness were found between fenced plots and grazed plots. The inter-annual effects of grazing exclusion on the community diversity were lack of regularity for three grassland types.

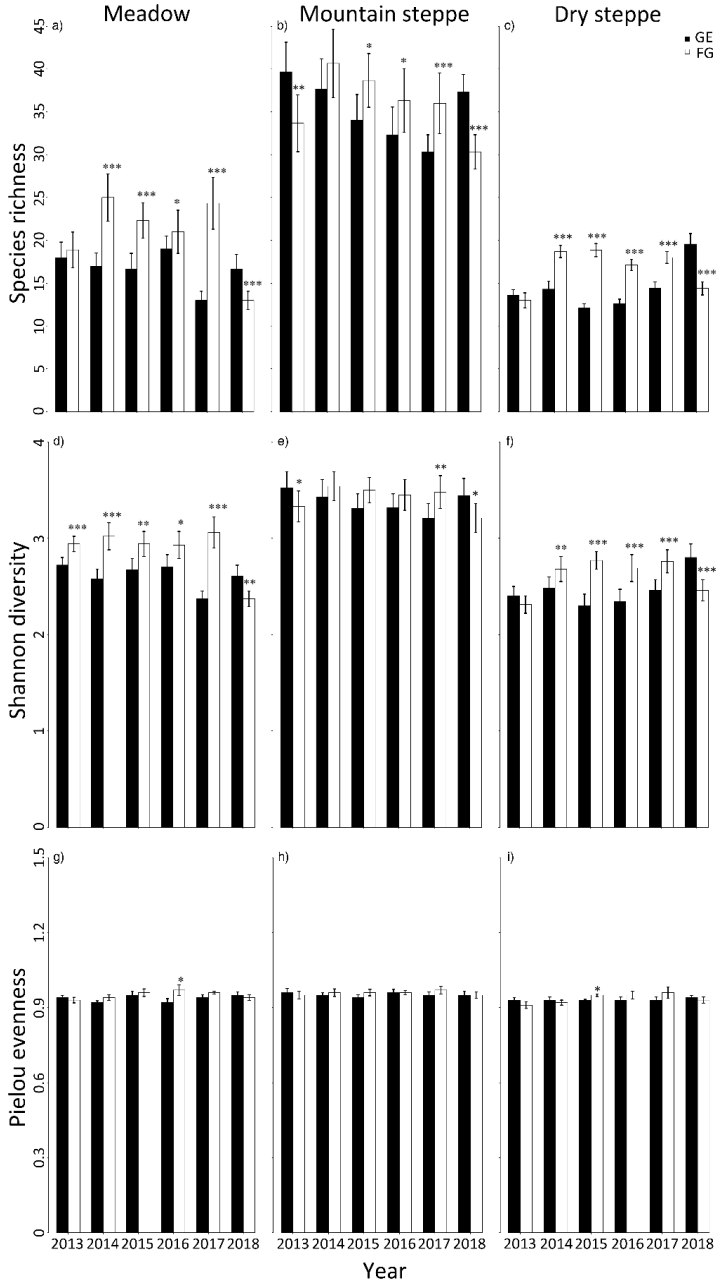

Figure 4. *Species richness (a, b, c), Shannon-Wiener diversity (d, e, f) and Pielou evenness (g, h, i) under treatments of grazing exclusion by fence (GE) and freely grazing (FG) in three grassland types from 2013 to 2018. Asterisks indicate a significance level of the statistical test (***<0.001; **<0.01; *<0.05)*

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Response of phenological characteristics to grazing exclusion

Grazing exclusion by fence generally advanced vegetation phenological stages in three grassland types (*Fig. 5*). However, the phenology of the meadow and the mountain steppe vegetation postponed by 0.02 and 0.19 respectively in 2014. In addition, the positive phenological variations tended to be greater in the meadow than other two grasslands. Dry steppe vegetation often had earlier phenological stages than that of mountain steppe.

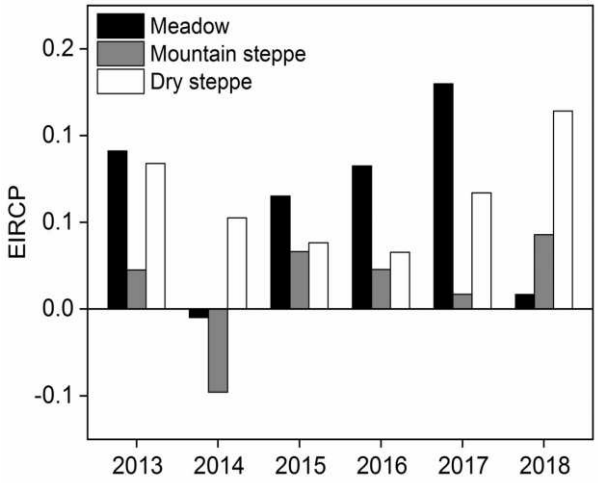

Figure 5. *Vegetation phenological changes (fenced plots vs. grazed plots) in three grassland types from 2013 to 2018*

Discussion

Livestock grazing is an important and direct way influencing grassland communities (Stein et al., 2016; Weiss and Jeltsch, 2015). Fencing is often considered as a useful tool to explore grazing exclusion effects on vegetation communities (Zhu et al., 2016b). Understanding the direction, the magnitude and mechanisms of grazing exclusion effects on vegetation communities greatly improves grassland management practices (Wang et al., 2014). In this study we focused on the response of aboveground vegetation indices to grazing exclusion by fence. Even though belowground parameters like soil properties and nutrients are also sensitive to grazing exclusion in Mongolian grasslands (Wang et al., 2019). Vegetation characteristics and diversity have been widely used to assess effects of grazing exclusion on grassland communities. However, the assessment is missing in Mongolian grasslands. Our study showed that grazing exclusion increased vegetation height and advanced vegetation phenological stages, but decreased community diversity. The effect of grazing exclusion varied with grassland type.

Vegetation characteristics response to grazing exclusion in three grassland types

Grazing exclusion by fence generally facilitated an increment of vegetation height for three Mongolian grasslands. This finding is in line with other fencing experiments in grasslands (Deng et al., 2014; Li et al., 2014), since tall plants are sensitive to livestock grazing (Díaz et al., 2007). In addition, positive changes in vegetation height induced by grazing exclusion did not exhibit a clear regularity of inter-annual variability. Grazing exclusion did not increase vegetation cover in the meadow and the mountain steppe, and

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significantly increased the cover in the dry steppe. The finding verifies different responses of vegetation cover to grazing exclusion across various steppes in Mongolian rangelands (Khishigbayar et al., 2015). Vegetation cover was more vulnerable to harsher environments. Fencing might stimulate a greater increment of the cover in the dry steppe. In addition, the meadow had a longer time since enclosure. This might explain a larger difference of vegetation cover between fenced and grazed plots. Recovery of vegetation cover is a lasting procedure. Li (2014) found vegetation needed 6 years to recover after grazing exclusion by fence in a degraded grassland in northeast China. For some savanna ecosystems these shifts can take many years (Goheen et al., 2018). Other management strategies like reseeding and fertilization may be performed to increase survival rates of plant species, thereby leading to an enhancement of vegetation cover. Moreover, variations of vegetation cover may be largely constrained by local climatic conditions. General arid environments in Mongolia hinder the restoration of vegetation cover. We further found that grazing exclusion positively affected vegetation cover of palatable species in the meadow and the mountain steppe, while the effect was not available in the dry steppe (see *Table A2* in the *Appendix*). This indicates that analyses from a perspective of functional groups aid us to comprehensively understand effects of grazing exclusion on vegetation characteristics. The communities were further found to be generally composed of unpalatable species across years in three grassland types (see *Table A2* in the *Appendix*). Palatable species are preferably selected by livestock (Guo et al., 2016). The result suggests that overgrazing in historical periods led to a decreased quality of forage grass and the dominance of unpalatable species in Mongolian grasslands. Thus, palatable species grow rapidly. Grazing history can impact the response of rangeland community cover and diversity to grazing management (Vermeire et al., 2018).

Community diversity response to grazing exclusion in three grassland types

We found that grazing exclusion had negative effects on the species richness and Shannon-Wiener diversity in three Mongolian grasslands. This is consistent with the findings in other grassland ecosystems (Wu et al., 2009; Yao et al., 2019; Zou et al., 2016). However, other studies showed contradictory results compared with ours (Sagar et al., 2019; Zhang and Zhao, 2015; Zhu et al., 2016a). Grazing leads to an occurrence of empty space, which allows more species to live on it. Grazing may also create the heterogeneity of habitats and thus benefits species coexistence (Guo et al., 2018). Another possible reason for the lower diversity in fenced plots is that grazing exclusion prevents seeds in freely grazed areas into the small fenced area (only 0.1 ha). Pielou evenness was found to be little controlled by grazing exclusion. Several studies have indicated that short-term grazing exclusion may be essential and important for improving species diversity (Jing et al., 2014; Xiong et al., 2016). Long-term fenced plots are likely to form climax species. This intensifies species competition for light and water resource, and thus decreases plant diversity (Zhu et al., 2016a). However, our results showed that the community diversity decreased in fenced plots regardless of years. This is not in line with typical discussions of fencing time impact on the community diversity. From a perspective of grassland management, grazing exclusion by fence is not an effective approach for restoring the diversity of the whole community in the degraded Mongolian grasslands. Moderate or rotational grazing rather than grazing exclusion by fence may be a better choice, since grazing weakens the dominance of certain undesirable species, allows the persistence of grazing-adapted

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species and thus promote species coexistence (Stahlheber and D'Antonio, 2013). Fencing treatment could be regarded as a scenario without disturbances, while freely grazing scenario was full of disturbances. Both scenarios may correspond to a lower species diversity level based on intermediate disturbance hypothesis (Connell, 1979). We found that grazing exclusion had positive effects on the richness of palatable species in the meadow and the mountain steppe, while the effect did not exist in the dry steppe (see *Table A2* in the Appendix). This indicated that grazing exclusion improved the palatability of vegetation communities, but was constrained by water availability. Empirical and simulation studies show that the diversity of grasses is susceptible to interactions of livestock grazing and aridity (Guo et al., 2018; Rota et al., 2016).

Vegetation phenology response to grazing exclusion in three grassland types

Vegetation phenological stages tended to be earlier in fenced plots of three Mongolian grasslands in terms of the whole community. Grazing exclusion treatment stimulates the growth of shallow-rooted plants (Zhu et al., 2016b). Plants in Mongolian grasslands are assumed to have short roots to utilize water resource in the upper soil layer, since local arid environment does not allow much water to infiltrate into deep soil layers. Grazing exclusion can also lead to soil desiccation induced by an increase of water required by non-grazed plant species (Bergmeier, 1998). Moreover, the phenological development of taller plant species is less constrained by water availability than that of lower ones (Dorji et al., 2013). We further found that phenological periods of species differed in their response to grazing exclusion (see *Fig. A1* in the *Appendix*). Specifically, certain number of species had no changes or even negatively altered for phenological stages in fenced plots. This provides species-specific evidence for restoring degraded grasslands. In future, it is necessary to investigate soil moisture and temperature under grazing exclusion and grazed treatments in Mongolian grasslands, to obtain a general and mechanistic explanation of grazing exclusion effects on vegetation phenology. We are aware that the measurement of vegetation phenology in several days is not enough to assess its response to grazing exclusion, since we cannot identify how many times of grasses were eaten by livestock. Moreover, the regrowth of grazed plants was not adequately considered.

Conclusion

Our study shows that grazing exclusion by fence significantly affects vegetation characteristics and community diversity. The effects are strongly modulated by grassland type. Grazing exclusion by fence is not an effective way to restore the vegetation cover and the diversity of plant communities in degraded Mongolian grasslands. However, grazing exclusion may play a positive role in promoting the growth of palatable species. Appropriate alternative management practices, such as rotational grazing, reseeding and fertilization, should be considered for the grassland restoration. In addition, factors influencing vegetation phenology need a further study in Mongolian rangelands.

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APPENDIX

Determining the number of quadrats

We found that mean vegetation cover of selected three quadrats was generally similar with that of ten quadrats regardless of treatments and grassland type. In addition, resampling based on the bootstrapping method little influenced mean vegetation cover of randomly combined three quadrats. Thus, three quadrats well depicted general changes in vegetation cover. We finally determined fixed three quadrats based on a rule of minimum differences between mean vegetation cover of three quadrats and that of total ten quadrats for each treatment and each grassland.

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Table A1. *Mean vegetation cover (%) of randomly sampled three quadrats from ten respectively in fenced and grazed plots for three grassland types in 2013*

	Meadow		Mountain steppe		Dry steppe	
Treatment / Times	GE	FG	GE	FG	GE	FG
1	24.56	22.79	92.43	93.23	61.03	56.12
2	23.13	24.13	95.34	92.87	63.44	50.34
3	22.87	22.31	91.17	94.39	61.38	51.32
4	24.02	23.87	96.24	90.21	67.10	52.28
5	23.46	24.04	94.46	91.32	65.88	51.46
6	22.68	22.25	91.25	93.52	6.14	55.75
7	22.30	23.12	93.45	92.80	62.34	54.32
8	23.78	24.65	95.75	95.21	64.43	53.47
9	24.37	23.84	92.19	93.47	65.26	55.89
10	23.54	24.13	94.98	92.45	62.43	52.37
Mean value of ten quadrats	23.29	23.34	93.41	92.16	64.29	53.30

Response of vegetation community palatability to grazing exclusion

Vegetation height of palatable species was higher than that of unpalatable species in the meadow and the mountain steppe. In contrast, this showed an opposite trend in the dry steppe. The richness of unpalatable species was much greater than that of palatable species. Vegetation cover indicated a similar trend. However, the cover dominance of unpalatable species did not exist in the meadow. In addition, grazing exclusion by fence generally increased the cover ratio and the richness of palatable species in the meadow and the mountain steppe. The effect was not available for the dry steppe. The richness of unpalatable species decreased in fenced plots compared to grazed ones in three grassland types.

Table A2. *Vegetation height (H) and richness (R) of palatable species (P) and non-palatable species (NP), as well as cover ratio (CR) of palatable species to the community under treatments of grazing exclusion by fence (GE) and freely grazing (FG) in three grassland types from 2013 to 2018*

Grasslands	Year	Treatment	H_P (cm)	H_{NP} (cm)	R_P	R_{NP}	CR_P
Meadow	2013	GE	20.08	14.99	5.67	12.89	0.45
		FG	5.46	3.04	6.22	13.56	0.49
	2014	GE	23.60	13.09	6.33	12.33	0.35
		FG	5.09	2.86	7.00	19.00	0.39
	2015	GE	15.41	7.26	6.50	10.50	0.57
		FG	4.03	2.30	6.00	17.33	0.38
	2016	GE	21.21	13.21	7.00	9.67	0.57
		FG	4.24	2.95	5.00	16.33	0.37
	2017	GE	28.75	13.21	7.67	11.33	0.79
		FG	4.66	3.11	5.33	20.00	0.45
	2018	GE	23.72	12.94	6.67	6.33	0.78
		FG	4.57	3.00	5.00	12.00	0.58

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Grasslands	Year	Treatment	H_P (cm)	H_{NP} (cm)	R_P	R_{NP}	CR_P
Mountain steppe	2013	GE	12.31	9.92	7.33	29.22	0.34
		FG	11.00	7.90	6.00	27.67	0.28
	2014	GE	18.21	12.48	8.67	31.00	0.32
		FG	13.51	9.20	7.67	33.00	0.28
	2015	GE	12.23	7.98	7.67	30.00	0.32
		FG	9.83	5.05	7.33	31.33	0.27
	2016	GE	11.75	8.93	6.67	27.33	0.32
		FG	8.80	6.34	7.33	29.00	0.28
	2017	GE	9.80	6.88	7.33	25.00	0.24
		FG	6.25	4.64	7.00	29.33	0.21
	2018	GE	13.77	9.17	7.00	23.33	0.29
		FG	7.34	4.47	7.00	30.67	0.27
Dry steppe	2013	GE	7.27	13.29	1.20	11.10	0.01
		FG	4.18	11.26	1.33	11.67	0.02
	2014	GE	7.73	11.51	1.63	12.40	0.01
		FG	3.00	9.86	1.86	16.86	0.01
	2015	GE	3.29	7.79	2.10	12.20	0.02
		FG	2.57	5.43	2.00	16.86	0.04
	2016	GE	6.63	7.77	1.50	10.70	0.02
		FG	5.39	5.75	2.00	15.43	0.04
	2017	GE	6.67	6.87	1.78	11.10	0.03
		FG	3.31	4.26	2.71	15.29	0.06
	2018	GE	10.20	10.01	2.30	12.20	0.04
		FG	5.68	7.08	2.86	16.71	0.12

Species distributions of phenological changes

Species with negative phenological variations were generally the minority of grassland communities. Grazing exclusion advanced species phenological stages. However, the effects did not show a clear inter-annual regularity.

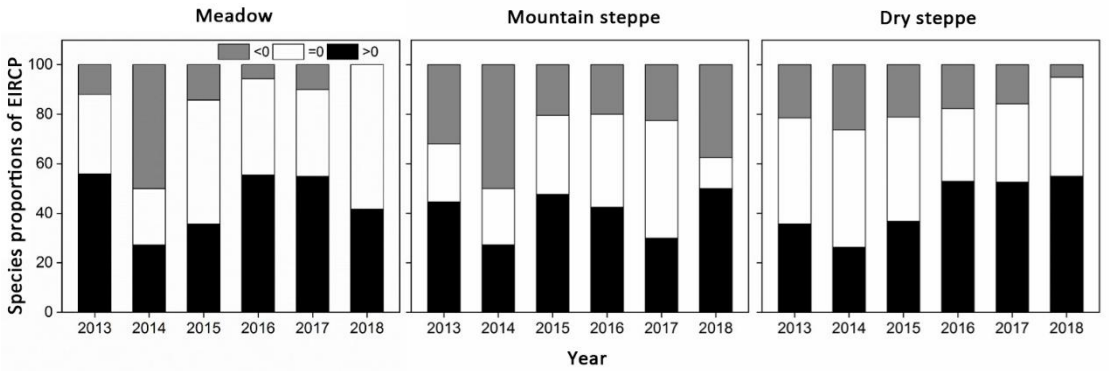

Figure A1. *Species proportions of phenological changes (fenced plots vs. grazed plots) in three grassland types from 2013 to 2018. The expression "<0" denotes negative phenological variations; The expression "=0" denotes no phenological variations; The expression ">0" denotes positive phenological variations*

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ASSESSMENT OF THE GENETIC DIVERSITY IN *AEGILOPS TAUSCHII* (COSS.) BY USING SSR MARKERS AND MORPHYSIOLOGICAL TRAITS

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Abstract. *Aegilops tauschii* Coss. (2n=2x=14, DD) is a problematic weed and has contributed in the D genome of common wheat. The genetic diversity among 40 Chinese populations were evaluated by microsatellites marker and morphysiological traits. Dry weight biomass showed variation while in the case of plant height high variation was found between populations. We determined 27 alleles by using eight primers with an average of 3.37 allele per locus. The maximum polymorphism information content (PIC) was 0.63 with an average of 0.20 and maximum allele frequency was 1.00 with an average of 0.88. Cluster analysis divided *Aegilops tauschii* into different groups, which showed obvious genetic difference between these populations. This study will be helpful for weed management and wheat crop breeding program.
Keywords: *Aegilops tauschii, molecular marker, genetic diversity, morphysiological trait, microsatellites*

Abbreviations: PIC: Polymorphism Information Content

Introduction

(*Aegilops tauschii* Coss.) (*Ae. tauschii*) has a troublesome effect on wheat crop growing areas of China (Dudnikov, 2000; Zhang et al., 2007). It is native to tropical Asia to temperate Asia and Europe (Wei et al., 2008). It has infested about 330,000 hectares (Zhang et al., 2007) of winter wheat in different provinces including Shanxi, Shandong, Henan, Shaanxi, Inner Mongolia, Jiangsu and Hebei in China. Moreover, due to insufficient prevention and limitation of control strategies, the spreading pace of this weed tremendously damage winter wheat productivity, particularly in the main wheat producing regions. *Ae. tauschii* distributed from the Mediterranean region; present in Syria, Russia, Kazakhstan, Afghanistan, Pakistan, Turkey, all the way to Iran and extending to the eastwards of Yili Valley of Xinjiang in China (Li, 2005). Iran and Yili valley of Xinjiang were usually familiar with the center of the origin of *Ae. tauschii*. *Ae. tauschii* is recognized in natural habitats for its world distribution, whereas subspecies (strangulate) of *Aegilops* is native to south-western Caspian Iran and Afghanistan (Ogbonnaya et al., 2005; Wang et al., 2013; Kalia et al., 2016). Some scientists also proposed that *Ae. tauschii* presented in the Yellow river region in China was introduced with common wheat through trade along the Silk Road (Li and Mo, 2004; Zhao, 2007).

Ae. tauschii involved in the origin of the hexaploid wheat. Moreover, crop breeding has resulted in genetic diversity among the hexaploid wheat and *Ae. tauschii* populations. On the base of the morphological parameter, decidedly less genetic differentiation in the D genome of the wheat crop exists (Zahra et al., 2010). Due to the

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genetic similarity of *Ae. tauschii* with wheat, it may have an essential role in wheat crop improvement (Knaggs et al., 2000). Through hybridization, many useful biotic and abiotic stress resistance genes of *Ae. tauschii* can be utilized in wheat variety improvement programs (Hsam et al., 2001).

Genetic diversity can assessed by phenotypic (physiological and morphological parameters) and microsatellite markers (AFLP, RFLP, and SSR). Morphological parameters of crops are useful for introductory assessment because of their rapid range for diversity (Majidi et al., 2009; Sun et al., 2014). Molecular markers used to evaluate the genetic diversity because of their polymorphism, reproducibility, co-dominance, and simplicity (Roder et al., 1995). In molecular markers, simple sequence repeats (SSR) are universal and commonly used for genetic diversity evaluation (Vieira et al., 2016). Furthermore, SSR markers are more critical for crop improvement (Mian et al., 2005). *Aegilops* species possessing the D genome could be rich abiotic sources. Molecular markers have been used in several studies to assess genetic diversity among different populations (Roy et al., 2006). Genes from *Ae. tauschii* can be utilized in wheat variety improvement programs (Hsam et al., 2001).

In China, *Ae. tauschii* is a problematic weed and competes for resources from the early stage until maturity stage in wheat. It is scattered in more than ten provinces all across the country. Due to morphological similarities with the wheat crop, it is difficult to be controlled with herbicides. It is known that DD genome of *Ae. tauschii* has a rich source of potential variability. For genetic diversity of *Ae. tauschii* populations collected from five different provinces of China were assessed by using morphysiological traits and fluorescent dye-labeled SSR markers. The results of this study will be beneficial to both weed management and wheat breeding.

Materials and methods

Forty populations of *Ae. tauschii* seeds were used from five different provinces (Henan, Shaanxi, Shandong, Hebei, and Shanxi) of China (*Table 1*, *Fig. 1*). The experiment was conducted at the greenhouse of the Institute of Plant Protection, Chinese Academy of Agriculture Sciences, Beijing, China. Each population was replicated four times following a completely randomized design for pot experiment. Plants floated over half-strength Hoagland's nutrient solution (Hoagland and Arnon, 1950). Sixty days after sowing, plant height (cm) and dry weight biomass (g) were recorded. For SSR analysis, plant leaves were taken and stored at -80ºC for DNA extraction. DNA was isolated from leaves by plant kit (TIANGEN). DNA purification and quantification were carried out following Shah et al. (2009). SSR primers were designed by BATCHPRIMER3 (*Table 2*).

Table 1. *Aegilops tauschii populations collected from different provinces of China*

Name of province	Population Collection
Henan	10
Shaanxi	3
Shandong	10
Hebei	12
Shanxi	5
Total	40

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Figure 1. *Collection of Aegilops tauschii population's seed from different provinces of China. Circles indicated provinces for seed collection*

Table 2. *Details of forward and reverse SSR primers*

Primers	Forward Primer (5'-3')	Reverse Primer (5'-3')
AG-5	AGAACATCTGGCGTAACATAG	GGTTTTGTCGCAGAATTAGTA
AG-7	AGCTTCATACGGCTTCTCTAT	AGCGCTTTTTCTTATTCTAGC
AG-12	TGCAGAAACTACCCAAATCTA	GCCACAAGGGACTATCTAAAC
AG-14	AGAGCAAATATAGGACCCAAG	CTCTCGTATTCGTCCTCTGA
AG-15	ATTATCGCTTAGCTTTCGACT	GTTGCAAAAATAAGAGCTTGA
AG-18	TTGACACGAGGAACTACTCAC	CTGTCTCGCAATACCTTCTAC
AG-19	CTTTGCCACCTACTGCTACTA	CGGATACTGCCATACAATTAC
AG-20	CCAGTTAAGGTGGGATATGAT	GATTGGCGGATTTCTAATAGT

Polymerase chain reactions (PCRs) were carried out in 20 μL reactions comprising 40 ng of genomic DNA 1 μL, 0.6 μM of each forward and reverse primer, 7.8 μM of ddH_2O and 10 μM of PCR master mix. The PCR profile consisted of denaturation at 95°C for 10 min, followed by 35 cycles of 95°C for 30 sec, 45°C-60°C (depending on primers) for 30 sec and 72°C for 2 min, with a final extension at 72°C for 10 min. The genetic diversity of these populations were analyzed by using eight SSR fluorescent markers by capillary electropherogram.

Statistical analysis

Means, variances and coefficient of variances for plant height and dry weight biomass were measured by Statistix-8.0. For molecular data, allelic size was determined by using GeneMarker version 2.2.0 (Applied Biosystems). PowerMarker 3.1 was used to determine the allele amplification, allele frequency and polymorphism information content. Phylogenetic tree was drawn by PowerMarker MEGA 3.5.

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Results

Means, variances and coefficient of variance in 40 *Ae. tauschii* populations showed variation in dry weight biomass and plant height (*Table 3*). Different populations have a different coefficient of variance in plant height, with maximum 12% and minimum 1.8%. In dry weight biomass, maximum and minimum coefficient of variance were recorded 25% and 3% in different populations. Analysis of variance in plant height and dry weight biomass showed a relationship between populations, but some populations showed variation as compared with others in these two traits. Populations 6, 9, and 23 showed variation in plant height (*Fig. 2*), and populations 23 and 26 showed variation in dry weight biomass (*Fig. 3*). Amplification of eight primers in *Ae. tauschii* showed 27 alleles with an average of 3.37, maximum polymorphism information content (PIC) was 0.63 with the average of 0.20, and allele frequency ranges 0.41 to 1 with an average of 0.88 (*Table 4*). D genome microsatellite markers revealed the allelic range in the Chinese *Ae. tauschii* populations and has extended and showed polymorphism.

Table 3. *Mean standard deviation (STDEV) and coefficient of variance (CV %) of plant height (cm) and dry weight biomass (g) of 40 Aegilops tauschii populations*

Populations	Plant Height (cm)			Dry Weight Biomass (g)		
	Mean	STDEV	CV%	Mean	STDEV	CV%
PI	11.7	0.75	8.5	3.94	0.29	7
P2	15.0	1.47	9.8	3.24	0.45	14
P3	17.9	1.03	5.8	3.29	0.38	11
P4	13.6	0.30	2.2	3.57	0.44	12
P5	14.6	0.43	2.9	3.51	0.53	15
P6	12.1	0.45	3.8	3.00	0.49	16
P7	13.8	0.57	4.1	3.50	0.41	12
P8	13.8	0.24	1.8	3.29	0.26	8
P9	13.9	0.63	4.5	3.87	0.12	3
P10	14.4	0.48	3.3	4.02	0.11	3
P11	14.3	0.65	4.5	2.85	0.25	9
P12	14.4	0.51	3.5	3.10	0.33	11
P13	13.8	0.65	4.7	2.71	0.38	14
P14	15.8	0.73	4.6	3.14	0.42	13
P15	13.8	0.74	5.4	3.07	0.38	13
P16	12.6	0.48	3.8	2.92	0.23	8
P17	11.6	0.75	6.5	2.91	0.16	6
P18	14.4	0.48	3.3	2.98	0.27	9
P19	10.9	1.07	9.8	2.80	0.08	3
P20	15.5	0.41	2.7	2.72	0.17	6
P21	15.3	0.39	2.6	3.38	0.31	9
P22	12.6	0.75	5.9	3.81	0.13	4
P23	13.4	0.48	3.6	5.02	0.50	10
P24	11.4	1.37	12.0	3.89	0.21	5
P25	11.7	0.53	4.5	3.13	0.13	4
P26	13.2	0.40	3.0	2.49	0.52	21
P27	12.6	0.43	3.5	2.69	0.30	11
P28	14.9	0.84	5.6	2.80	0.24	9
P29	13.2	0.29	2.2	3.78	0.23	6
P30	14.2	0.86	6.0	3.56	0.31	9
P31	14.9	0.52	3.5	3.91	0.38	10
P32	12.5	0.71	5.7	3.84	0.10	3
P33	13.1	0.59	4.5	3.82	0.66	17
P34	12.3	0.86	7.0	3.00	0.35	12
P35	15.4	0.48	3.1	3.46	0.88	25
P36	14.4	0.48	3.3	2.79	0.39	14
P37	14.2	0.26	1.8	3.17	0.46	15
P38	14.6	0.42	2.9	3.20	0.26	8
P39	12.2	0.93	7.6	2.94	0.34	12
P40	15.0	1.42	9.4	3.10	0.26	9

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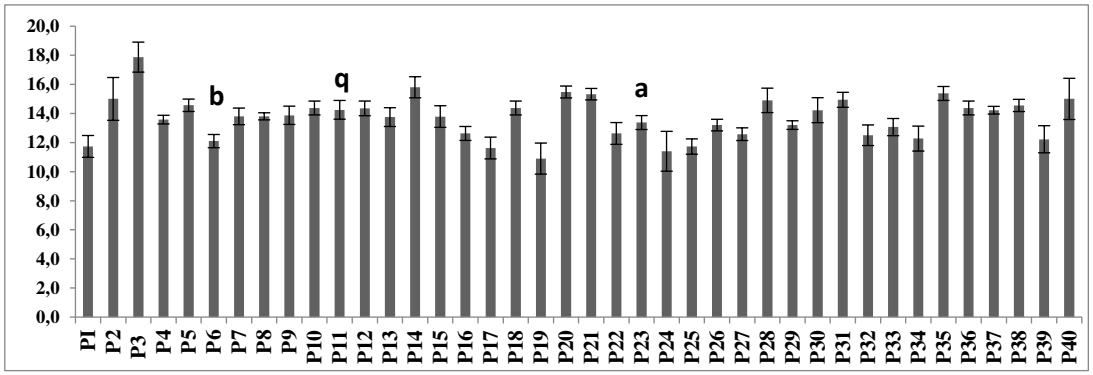

Figure 2. *Mean value of plant height of 40 populations of Aegilops tauschii. Bar indicating with letters showed variation. While other populations showed a close relationship with each other*

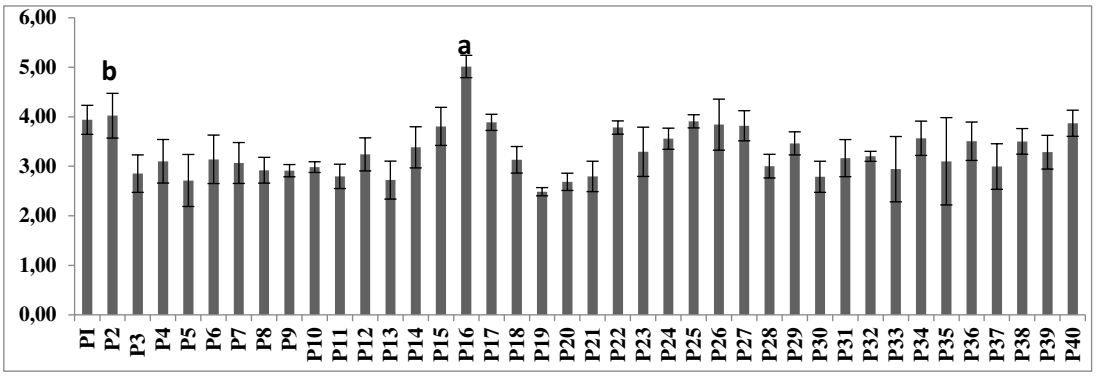

Figure 3. *Mean value of dry weight biomass of 40 populations of Aegilops tauschii. Bar indicating with letters showed variation. While other populations showed a close relationship with each other*

Table 4. *Allele amplification, allele frequency, and polymorphism information content for used SSR primers in 40 Aegilops tauschii populations*

Primers	Allele Amplification	Allele Frequency	PIC Value
AG-15	7	0.82	0.30
AG-7	4	0.41	0.63
AG-18	2	1.00	0.00
AG-14	4	1.00	0.53
AG-20	2	1.00	0.00
AG-19	4	0.87	0.21
AG-12	2	1.00	0.00
AG-5	2	1.00	0.00
Mean	3.37	0.88	0.20

On the base of cluster analysis (*Fig. 4*), 40 populations of *Ae. tauschii* were divided into three groups (I-III). Furthermore, the genetic relationships between populations elucidated by a dendrogram. Group I has six populations from Shanxi, Henan, and

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Shandong, showing their similar genetic background. Group II clusters have 26 populations of *Ae. tauschii* from Shaanxi, Shandong, Hebei, Henan, and Shandong provinces. The populations collected from Shaanxi, Henan, Shanxi, and Shandong were present in-group III. These results illustrated that the populations in the same group have a similar genetic background.

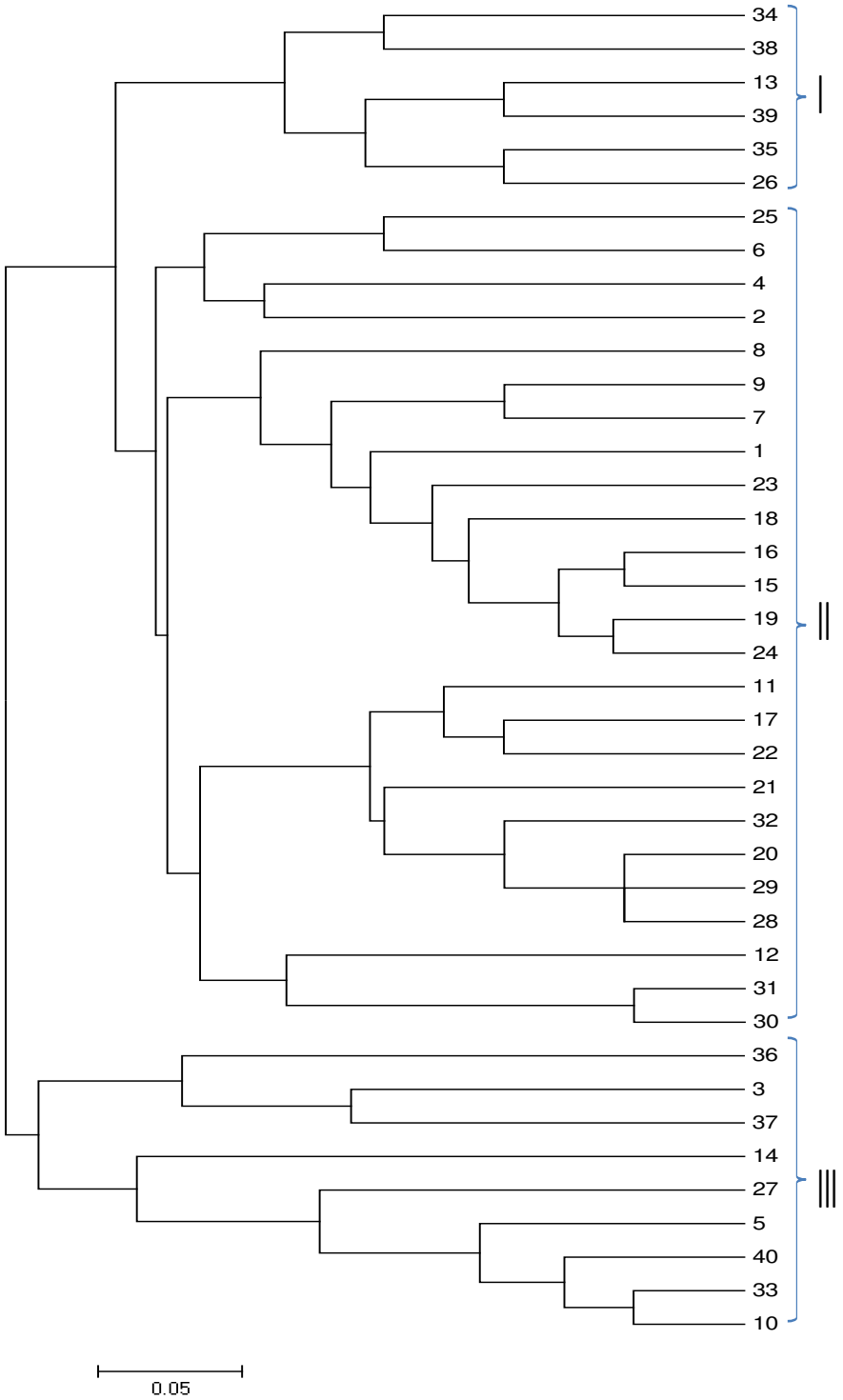

Figure 4. *Genetic relationship determined by PowerMarker (MEGA 3.5) cluster analysis based on the similarity coefficient of 40 populations of Ae. tauschii.*

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Discussions

The results showed the significant effect of *Ae. tauschii* on the genetic diversity by using morphysiological traits and SSR markers. Fifty-five populations of *Ae. tauschii* showed variances on the base of the morphysiological characteristics (Naghavi et al., 2007). Based on data recording plant height, some populations showed a close relationship with each other while populations 3 and 15 showed variation. Zara et al. (2010) and Ojaghi et al. (2010) reported high diversity in *Ae. tauschii* on the base of plant height, which is a little larger than ours. In our results, dry weight biomass showed close relationships between populations while in some populations showed variations. Zaharieva et al. (2003) reported higher genetic diversity in three species of *Aegilops* on the base of different morphological traits. Agronomic traits are available for wheat improvement and showed a high level of genetic diversity (Giuliani et al., 2009). Knaggs et al. (2000) reported that morphysiological characteristics of *Ae. tauschii* could be used in crop improvement varieties. Similarly, in the case of many studies, they believe that seed collection belongs to geographic regions in morphological diversity. Many studies showed that morphological and physiological parameters are not in compliance with the genetic diversity, while molecular markers cover a large part of the genome including coding and noncoding parts (Semagn, 2002). Our results showed that on the base of the molecular marker relationship between *Ae. tauschii* was obvious, while the genetic difference is present.

Genetic diversity based on morphological and physiological parameters were not confirming like SSR markers. No relationship was present between morphological and physiological parameters with genetic diversity (Tahernezhad et al., 2010). In microsatellite markers, simple sequence repeats (SSR) revealed higher genetic diversity in populations. A high level of polymorphisms and average allelic richness showed a high level of genetic diversity in crops. SSR markers also confirmed the phenotypic evaluation of populations (Zaharieva et al., 2003). The result on the base of eight SSR primers and morphological traits showed genetic diversity between populations. Obtained results were confirmed by Naghavi et al. (2007) with a PIC value of 9.21 and an allelic range of 6-15 that was achieved by the SSR marker and it could be helpful for a breeder in a crop breeding program. Results on the base of eight primers showed genetic diversity between populations. In the evaluation of the genetic diversity in Iranian *Ae. tauschii* populations using 13 microsatellites, a total of 66 alleles were amplified with PIC value of 0.65 (Saeidi et al., 2006). In our study, maximum (PIC) was 0.63 (*Table 4*) with the mean of 0.20, that was lower than 0.82 (Naghavi et al., 2007). Similarly, the genetic diversity of 46 *Commelina communis* populations using 12 SSR markers were assessed with an average PIC value of 0.20 (Yang et al., 2018). PIC values showed variation because they depended on (GT) content, number allele per locus, and type of motifs (Roder et al., 1995). Amplification of wheat SSR primers in *Ae. tauschii* (DD) showed similar regions between *Ae. tauschii* and wheat genome (Zhang et al., 2004). Microsatellite markers from the D genome revealed allelic amplification which showed polymorphism in *Ae. tauschii* populations of China. Allelic amplification showed a high level of genetic diversity in *Ae. tauschii* populations of Iran (Saeidi et al., 2006). This showed the relationship between populations and subdivisions in groups showed diversity. Amplification of universal wheat primers in *Ae. tauschii* of China showed the close relationship between wheat and *Ae. tauschii* (Pestsova et al., 2000).

Bibi et al. (2009) and Akhundova (2010) reported genetic variation among wheat populations using microsatellite markers with an allelic range of 2-4. SSR markers in

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wheat populations were reported with 3.6 alleles per primer (Ahmad, 2002; Almanza-Pinzon et al., 2003). Genetic diversity among wheat populations were studied with 21 SSR markers, which had an allelic range of 2-6, genetic similarity coefficient variation ranged from 0.45-0.90, and an average PIC value was 0.47 (Singh et al., 2006). Cluster analysis showed that populations from the same geographical region were present in the same group and their division in groups showed diversity. Populations from the same region have a great chance of being descended from a similar ancestor. On the basis of the genetic similarity coefficient, *Ae. tauschii* was divided into different groups. Cluster analysis divided *Ae. tauschii* (Iranian populations) into different groups and reported a high level of genetic variation between populations (Zahra et al., 2010).

Conclusion

Studying of *Ae. tauschii* populations of China by using molecular markers and morphysiological parameters has an essential role in wheat crop improvement. It is evident through molecular marker that *Ae. tauschii* will be helpful for crop breeding programs. Cluster analysis divided *Ae. tauschii* populations into three groups, which showed diversity and its different origins. High PIC value, allele amplifications and major allele frequency in some primers showed that specific parts of *Ae. tauschii* could be a valuable source for wheat crop improvement. Due to a rich source of potential variability, *Ae. tauschii* may perform vary when herbicides are applied in different wheat-growing areas. The results obtained from the study will help to weed management strategies in these regions.

Ethics and conflict of interests. The authors declare compliance with ethical standards and that they have no conflict of interests.

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EVALUATING THE UPTAKE OF TEN HEAVY METALS BY KIDNEY BEAN (*PHASEOLUS VULGARIS* L.) GROWN IN A SOIL-SLUDGE MIXTURE USING A REGRESSION MODEL

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Abstract. Severe human health risks can be caused by consuming vegetables contaminated by heavy metals (HMs); thus, assessing the HM uptake by these plants is important. The current work was performed to construct a regression model for predicting the concentration of ten HMs in four tissues of *Phaseolus vulgaris* (roots, stems, leaves and pods) based on their concentration in a soil-sludge mixture, soil organic matter (OM) and soil pH. For pods, the regression equation with the highest coefficient of determination ($R^2 = 0.99$) and model efficiency ($ME = 1.00$) but the lowest mean normalized bias ($MNB = 0.01$) was that of cobalt. For leaves, the equation with the highest R^2 (0.90) and ME (0.92) but the lowest MNB (0.001) was that of molybdenum. Comparable findings were obtained for molybdenum in the stems and manganese in the roots. All t values that assessed the difference between the actual and predicted values of the ten HMs in the four tissues were nonsignificant. Thus, these models could be used as a risk assessment tool for *P. vulgaris* cultivated in soil-sludge combinations.
Keywords: *bioconcentration factor, prediction modelling, sewage sludge, translocation factor, vegetable crops*

Introduction

Contamination of cultivated lands by heavy metals (HMs) due to severe human impacts (such as industrial effluents, sewage sludge, chemical fertilizers and pesticides, untreated irrigation water contaminated by wastewater and mining activities) represents a hazardous global problem (Ramadan and Al-Ashkar, 2007). Some HMs are essential to plants at low concentrations (e.g., Zn, Mn, Fe and Cu), but others pose a toxicity risk to living organisms (e.g., Ni, Cd, Pb, As, Hg and Cr). Therefore, many studies have evaluated the risks associated with the accumulation of HMs in soil-crop systems (Belaid et al., 2012; Galal, 2016; Farahat et al., 2017; Jamali et al., 2007, 2009; Zhao et al., 2010).

Although HMs occur in different forms in the soil, only the dissolved forms are available for uptake by plants (Aydinalp and Marinova, 2003). The adsorption and desorption properties of soil are among the main factors that limit the bioavailability of HMs for plants (Krishnamurti et al., 1999). Some soil factors, such as pH, organic matter (OM), and Fe and Mn oxides, influence the adsorption and desorption of HMs in

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soil (Usman et al., 2008). In view of this, soil pH has a significant negative correlation with the mobility and availability of HMs in soil solution, with acidity increasing the availability of HMs and alkalinity having a decreasing effect (Du Laing et al., 2008; Novotná et al., 2015; Zeng et al., 2011).

Consequent to the treatment of drainage water resulting from municipal and industrial activities, sewage sludge (SS) is produced as an organic byproduct that has excess macro- and micro-nutrients (Eid and Shaltout, 2016). Approximately 1.3 billion tons of SS is produced by cities globally per annum, and this is predicted to expand to 2.2 billion tons globally per annum by 2050 worldwide (Hoornweg and Bhada-Tata, 2012). Thus, there is consideration for how such quantities of SS can be disposed of safely to ensure it does not impact the environment (Eid et al., 2017) with the best means indicated as landfills and land application (Li et al., 2018). Some studies have indicated that the mixing of cultivated soil with certain amounts of SS has an enhancing effect on soil fertility and plant nutrients (Aráujo et al., 2007). Many studies (Singh and Agrawal, 2008, 2010; Grotto et al., 2015; Rehman et al., 2018; Asgari Lajayer et al., 2019) reported that SS could be added as a valuable fertilizer that improves the growth and yield of plant crops. However, this practice may lead to a risk to humans and the environment because of the presence of HMs that could contaminate soil and water, cause phytotoxicity, and the entry of such contaminants into the food supply (Mamais et al., 2000; Dolgen et al., 2007; Singh and Agrawal, 2007). The degree of danger is dependent on the quantity of SS applied, its structure, the crop species used and the conventional controls employed (Latare et al., 2014).

To reduce the toxicity levels of HMs and their movement through the food chain, it is important to assess the impact of soil variables on the availability and uptake of HMs by plants (Zeng et al., 2011). Regression models are useful mathematical tools for predicting the concentration of HMs in crop plants using certain soil factors such as HMs, pH and OM (Waegeneers et al., 2011). Comparable equations were constructed for barley (Adams et al., 2004; Novotná et al., 2015; Eid et al., 2020a), broad bean (Eid et al., 2019), carrot (Legind and Trapp, 2010; Bešter et al., 2013), cauliflower (Kumar et al., 2019), celery (Wang et al., 2004), chicory (Bešter et al., 2013), Chinese cabbage (Zhang et al., 2016), cucumber (Eid et al., 2018a), endive (Bešter et al., 2013), *Eruca sativa* (Eid et al., 2020b), garden pea (Eid et al., 2020c), hop (Novotná et al., 2015), lettuce (Legind and Trapp, 2010), maize (Tudoreanu and Phillips, 2004; Yang et al., 2013; Novotná et al., 2015), onion (Bešter et al., 2013), potato (Bešter et al., 2013; Novotná et al., 2015), rapeseeds (Novotná et al., 2015), red beet (Bešter et al., 2013), rice (Zeng et al., 2011; Rafiq et al., 2014), rye grass (Tudoreanu and Phillips, 2004), soy bean (Tudoreanu and Phillips, 2004), spinach (Wang et al., 2004; Eid et al., 2018b), tomato (Ramadan and Al-Ashkar, 2007; Bešter et al., 2013), wheat (Adams et al., 2004; Chaudri et al., 2007; Novotná et al., 2015; Eid et al., 2020d) and zucchini (Bešter et al., 2013). However, differences in soil, climate, SS composition and management factors require more specific estimates for different climatic regions or different cropping systems (Binder et al., 2002). Therefore, for safe crop production, it is important to evaluate the toxic levels of the HMs in the soil by establishing regression equations between the HMs in the plants on the one hand and those in the soil supporting the plants on the other hand (Eid et al., 2020d).

The kidney bean (*Phaseolus vulgaris* L.) is a herbaceous annual leguminous plant that has a long history of cultivation (Gentry, 1969). *P. vulgaris* is an annual plant belonging to family *Fabaceae* that originated in Central-South America. It is an erect or

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twining plant with compound leaves (each with three leaflets), and its flowers are usually white to purple in colour. Its pods are long, narrow, and straight or slightly curved, with pointed tips. The size and colour of its seeds are variable. The pods are cooked in immature stages, leaves are edible, and dried seeds are cooked and sometimes fried in oils as a salad (Huxley, 1999; Tindall, 1978). *P. vulgaris* is the most important food legume for direct consumption in the world (Jones, 1999). It was therefore selected for assessment in this research study. To the best of the authors' knowledge, no regression models are available for the uptake of HMs by *P. vulgaris* grown in soil amended with SS. Therefore, the present study was carried out to develop a mathematical model for predicting the potential uptake of HMs by *P. vulgaris* after growing it in an agricultural soil with different rates of SS addition under greenhouse conditions. Such models are suitable for assessing the severe impact of cultivating some vegetable crops in soil mixed with different amounts of SS.

Materials and methods

Materials and experimental design

White seeds of *P. vulgaris* (Strike, Seminis Vegetable Seeds, Inc., St. Louis, USA) were obtained from a local market in Abha City, Saudi Arabia. The soil used in the experiment was collected from nearby cultivated fields at a depth of 0-20 cm (Lat.: 18° 14′ 36.37″ N, Long.: 42° 33′ 58.25″ E), while the SS was obtained from the municipal sewage treatment station in Abha (Lat.: 18° 13′ 59.19″ N, Long.: 42° 31′ 16.35″ E). This station treats some 41,275 m^3 day^{-1} wastewater and produces 90 tons day^{-1} dry SS (Eid et al., 2017). Soil and SS samples were air dried for 2 weeks, ground and passed through a 2-mm sieve.

The experiment was performed in the greenhouse of the Biology Department, King Khalid University, Abha. Based on a preliminary experiment, the SS was mixed with agricultural soil at concentrations of 0, 10, 20, 30, 40 and 50 g kg^{-1}. Each treatment consisted of six plastic pots (each with a 6-L volume, filled with four kg of the respective treatment, and planted with ten equally sized seeds). The experimental units were arranged following a completely randomized design (CRD). The seeds were sown (directly after mixing the soil with SS) on the 4th of January 2018 for a period of 57 days under a natural day/night regime, irrigated using tap water as required with the soil water holding capacity (SWHC) in each pot varying between 40-50% and weeded manually as needed. The SWHC was determined and maintained on a volume basis using the following formula (*Eq. 1*):

$$\text{Volumetric SWHC (\%)} = (\text{depth of water in cm} / \text{depth of soil in cm}) \times 100 \quad \text{(Eq.1)}$$

Fifteen days after sowing, the emerged plants were manually thinned to one individual in each pot.

Sample analysis

Plant materials were harvested on 1 March 2018, then cleaned under running tap water, separated into four tissues (roots, pods, stems and leaves), dried for one week at 60 °C, powdered in a plastic mill and stored for later use. For physicochemical analyses, post-harvest soil samples were air-dried for two weeks, ground and passed through a 2-

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mm sieve. Dried powders (pre- and post-harvest soil and SS samples) were analysed for OM using the loss-on-ignition method at 550 °C for 2 h (Wilke, 2005), while pH was measured in 1:5 soil:water extracts (Allen, 1989).

For HM estimation, 0.5-1.0 g of each dried powders and plant sample was digested following the mixed-acid digestion method (HNO_3 and $HClO_4$; 3:1, v/v). A microwave sample preparation system was used for digestion (PerkinElmer Titan MPS, PerkinElmer Inc., USA). Blank samples were used to verify the accuracy of the digestion procedure and the subsequent analyses. Ten HMs (Cd, Co, Cr, Cu, Fe, Mn, Mo, Ni, Pb and Zn) were estimated by inductively coupled plasma optical emission spectrometry (Thermo Scientific iCAP 7000 Plus Series; Thermo Fisher Scientific, USA) as outlined by Allen (1989). The detection limits of the HMs (in $\mu g\ l^{-1}$) were as follows: 6.0 for Ni; 2.0 for Co, Cr and Cu; 1.0 for Fe, Pb and Zn; 0.3 for Mn; and 0.1 for Cd and Mo. The settings of the instrument and the conditions of operation were as specified by the manufacturer. Standard solutions with known concentrations of the ten HMs were prepared for system standardization.

Quality assurance and control

Certified reference material (tomato leaves, SRM 1573a) was used to verify the accuracy of HM estimates. The reference material was digested and analysed using the methods applied to the *P. vulgaris* samples. Digestion and measurement of the ten HMs were performed in triplicate. Accuracy was assessed by comparing the actual concentration with the certified value and then expressed as a percentage. Recovery rates had a range of 95-104% for SRM 1573a.

Data analysis

Before performing the one-way analysis of variance (ANOVA-1), the distribution of the HM, bioconcentration factor (BCF) and translocation factor (TF) data was tested for normality using the Shapiro-Wilk W test and for homogeneity of variance using Levene's test; when necessary, the data were log-transformed. The HM data for *P. vulgaris* tissues were subjected to an ANOVA-1 to evaluate the differences among *P. vulgaris* tissues. Significant difference between means among the four tissues were identified using the Tukey's HSD test at $p < 0.05$. The BCF determines the capacity of a plant, such as *P. vulgaris*, to accumulate an HM in its roots (Galal et al., 2017) and is calculated using the following formula (*Eq. 2*):

$$BCF = C_{Root} / C_{Soil} \quad \text{(Eq.2)}$$

where C_{Root} is the concentration of an HM in the root ($mg\ kg^{-1}$) and C_{Soil} is its concentration in the soil ($mg\ kg^{-1}$). In addition, the TF determines the ability of *P. vulgaris* to translocate an HM from the root to each of the stem, leaf and pod (Eid and Shaltout, 2016) and is calculated using the following formulas (*Eqs. 3, 4* and *5*):

$$TF_{Stem} = C_{Stem} / C_{Root} \quad \text{(Eq.3)}$$

$$TF_{Leaf} = C_{Leaf} / C_{Root} \quad \text{(Eq.4)}$$

$$TF_{Pod} = C_{Pod} / C_{Root} \quad \text{(Eq.5)}$$

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where C_{Root}, C_{Stem}, C_{Leaf} and C_{Pod} are the concentrations of an HM in these tissues (mg kg^{-1}). The BCFs and TFs data were subjected to an ANOVA-1 to evaluate the differences among the ten HMs. Significant difference between means among the ten HMs were identified using the Tukey's HSD test at $p < 0.05$. Pearson's simple linear correlation coefficient (r) was estimated for the BCF of a certain HM in the plant tissues with soil pH and soil OM. The same coefficient was determined to assess the relationship between the plant HMs and soil HMs, pH and OM.

Eighteen observations from the dataset for each of the root, stem and leaf were selected randomly as a validation dataset. The other eighteen observations were used to establish the regression models for predicting the HM contents in *P. vulgaris* tissues, based on some soil variables used as independent variables (pH, OM, Cd, Co, Cr, Cu, Fe, Mn, Mo, Ni, Pb and Zn). The number of individuals that produced pods was twelve (in treatments with concentrations of 10 and 20 g kg^{-1} only); thus, six observations were used as a validation dataset, while the other six observations were used in the regression procedure. Following Novotná et al. (2015) and Yang et al. (2013), the OM content, pH value and HM content of the soil are considered the most important factors with which to explain plant HM concentrations. The general equation of the model was established using the following formula (*Eq. 6*):

$$C_{Plant} = a + b \times C_{Soil} + c \times pH + d \times OM \quad \text{(Eq.6)}$$

where C_{Plant} is the concentration of a given HM in any *P. vulgaris* tissue; C_{Soil} is its concentration in the soil; OM is the soil organic matter content (%); and a, b, c and d are the regression coefficients.

Model quality was evaluated using the coefficient of determination (R^2), model efficiency (*ME*), model strength (*MNAE*) and model bias (*MNB*) using the formulas by Novotná et al. (2015) (*Eqs. 7, 8* and *9*):

$$ME = 1 - (\sum (C_{Model} - C_{Measured})^2 / \sum (C_{Measured} - C_{Mean})^2) \quad \text{(Eq.7)}$$

$$MNAE = (\sum (|C_{Model} - C_{Measured}| / C_{Measured}))/n \quad \text{(Eq.8)}$$

$$MNB = \sum (C_{Model} - C_{Measured}) / \sum C_{Measured} \quad \text{(Eq.9)}$$

where C_{Model} is the predicted concentration of an HM given by the model, $C_{Measured}$ is the actual HM concentration, C_{Mean} is the mean of the actual HM concentrations, and n is the number of observations. Student's t-test was applied to assess the significance of the difference between the predicted concentration of an HM in a tissue and its actual value in the same tissue. All these analyses were carried out in SPSS 15.0 (SPSS, 2006).

Results

For prior testing of the quality of the used agricultural soil and SS, the OM, pH value, and concentrations of ten common HMs were analyzed. The data presented in *Appendix 1* show that the agricultural soil from the study region is poor in fertility because it contains less than 1% OM. The pH of this soil is 8.7 (basic), which reduces the availability of most nutrients (Sharma et al., 2017). The HM concentrations in the agricultural soil ranged between a minimum of 1.1 mg kg^{-1} for Mo and a maximum of

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42,400 mg kg^{-1} for Fe; while in the SS they have a range of 0.9 mg kg^{-1} for Mo and 24,100 mg kg^{-1} for Fe (*Appendix 1*). In addition, the concentration of 5 HMs (Cd, Co, Fe, Mn and Mo) are higher in the agricultural soil than in the SS, while the reverse is true regarding the other 5 HMs.

After plant harvesting, the pH values of the soil-sludge mixture were alkaline, with a range of 6.8 - 8.5, a mean of 7.5 and a coefficient of variance (CV) of 7%; the OM ranged between 1 and 9%, with a mean of 5% and a CV of 47% (*Table 1*). The concentrations of the ten HMs in the soil descended as follows: Fe > Mn > Cr > Zn > Ni > Co > Cu > Pb > Cd > Mo. Iron had the highest mean concentration of 41,878 mg kg^{-1}, while Mo had the lowest mean concentration of 1 mg kg^{-1}. Regarding the plant tissues, most of the HM concentrations were lower in the pods than in the other tissues, except Co, Mo and Ni (*Table 2*). In contrast, the HMs were more abundant in the roots than in all the areal tissues, except Mo. In descending order, the ten HMs in the three areal tissues were Fe > Mn > Zn > Mo > Ni > Cu > Cr > Co > Pb > Cd, while those in the roots were Fe > Mn > Zn > Cr > Cu > Ni > Co > Mo > Pb > Cd.

Table 1. *Chemical characteristics of the soil (n = 36) amended with sewage sludge after harvesting Phaseolus vulgaris plants grown for 57 days*

Value	pH	OM (%)	Heavy metal concentration (mg kg^{-1})									
			Cd	Co	Cr	Cu	Fe	Mn	Mo	Ni	Pb	Zn
Minimum	6.8	1	2	24	123	15	23,488	557	1	29	3	64
Maximum	8.5	9	4	49	159	39	67,273	796	2	44	5	120
Mean	**7.5**	**5**	**3**	**29**	**141**	**28**	**41,878**	**623**	**1**	**34**	**4**	**95**
CV (%)	7	47	18	17	7	28	16	8	14	10	20	21

CV: coefficient of variance, OM: organic matter content

Table 2. *Heavy metal concentrations in pods, leaves, stems and roots of Phaseolus vulgaris plants harvested after 57 days*

Tissue	Value	Heavy metal concentration (mg kg^{-1})									
		Cd	Co	Cr	Cu	Fe	Mn	Mo	Ni	Pb	Zn
Pod	Minimum	0.1	1	1	7	118	25	12	7	0.1	21
	Maximum	0.3	2	2	11	165	38	17	14	1	29
	Mean (*n* = 12)	**0.2**a	**2**a	**2**a	**9**a	**147**a	**32**a	**14**b	**11**b	**0.3**a	**26**a
	CV (%)	30	9	21	17	12	15	13	29	83	13
Leaf	Minimum	0.1	1	2	7	183	138	7	3	0.2	20
	Maximum	0.4	2	5	13	1,230	228	20	9	2	33
	Mean (*n* = 36)	**0.2**a	**1**a	**3**a	**10**a	**389**a	**187**b	**15**b	**5**a	**1**a	**27**a
	CV (%)	30	38	35	17	78	16	26	30	53	15
Stem	Minimum	0.2	1	1	8	85	24	10	3	1	19
	Maximum	1	2	6	18	734	191	48	8	4	60
	Mean (*n* = 36)	**0.4**b	**1**a	**3**a	**12**a	**272**a	**76**a	**29**c	**5**a	**1**a	**34**a
	CV (%)	40	40	55	19	78	73	41	24	57	34
Root	Minimum	0.2	6	19	2	4,078	258	2	11	2	53
	Maximum	2	16	67	46	17,760	1190	15	27	9	222
	Mean (*n* = 36)	**1**c	**11**b	**39**b	**27**b	**10,283**b	**673**c	**7**a	**18**c	**4**b	**126**b
	CV (%)	52	24	38	47	41	42	50	26	53	50
F-value		47.4***	346.9***	167.9***	44.7***	155.5***	105.1***	55.9***	146.7***	56.5***	64.3***

F-values represent one-way ANOVA, degrees of freedom = 3. Means in the same column followed by different letters are significantly different at $p < 0.05$ according to Tukey's HSD test. ***: $p < 0.001$, CV: coefficient of variance

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The BCFs and TFs were < 1 for most HMs, except the BCFs of Mn (1.07), Mo (6.12), Pb (1.03) and Zn (1.25) and the TFs of Mo (2.53-4.57) in the three areal tissues (*Table 3*). In addition, the BCFs and TFs varied among the studied HMs (*Table 3*). Correlations were significant and negative between the BCFs of all ten HMs and soil pH, with the highest *r*-value of 0.89 for Mn and the lowest of 0.37 for Pb (*Fig. 1*), but significant and positive between the BCFs and soil OM, with a maximum *r*-value of 0.84 for Mn and a minimum of 0.36 for Pb (*Fig. 2*).

Table 3. *Descriptive statistical results (mean ± standard error) of the bioconcentration factors (BCFs) of heavy metals from soil to Phaseolus vulgaris roots and the translocation factors of heavy metals from Phaseolus vulgaris roots to stems (TF_{stem}), leaves (TF_{leaf}) and pods (TF_{pod})*

Heavy metal	BCF (*n* = 36)	TF_{stem} (*n* = 36)	TF_{leaf} (*n* = 36)	TF_{pod} (*n* = 12)
Cd	0.29 ± 0.02ab	0.49 ± 0.03ab	0.27 ± 0.02a	0.27 ± 0.03a
Co	0.38 ± 0.01ab	0.11 ± 0.01a	0.12 ± 0.01a	0.17 ± 0.01a
Cr	0.27 ± 0.02ab	0.07 ± 0.00a	0.07 ± 0.00a	0.05 ± 0.00a
Cu	0.92 ± 0.05bcd	0.83 ± 0.21b	0.63 ± 0.14b	0.42 ± 0.02a
Fe	0.25 ± 0.02a	0.03 ± 0.00a	0.04 ± 0.00a	0.02 ± 0.00a
Mn	1.07 ± 0.07cd	0.10 ± 0.01a	0.32 ± 0.02a	0.06 ± 0.00a
Mo	6.12 ± 0.47e	4.57 ± 0.37c	2.53 ± 0.21c	3.84 ± 0.73b
Ni	0.53 ± 0.02abc	0.31 ± 0.01ab	0.29 ± 0.01a	0.77 ± 0.05a
Pb	1.03 ± 0.08cd	0.32 ± 0.03ab	0.21 ± 0.02a	0.11 ± 0.02a
Zn	1.25 ± 0.07d	0.30 ± 0.01ab	0.26 ± 0.02a	0.35 ± 0.02a
F-value	128.7***	101.8***	83.4***	24.7***

F-values represent one-way ANOVA, degrees of freedom = 9. Means in the same column followed by different letters are significantly different at $p < 0.05$ according to Tukey's HSD test. ***: $p < 0.001$

With a few exceptions, the correlation between an HM in the four tissues and the same HM in the soil was significant and positive (*Table 4*). In addition, significant negative correlations were detected between soil pH and only some HMs in the pods ($r = -0.76$ with Co, -0.67 with Mo, -0.89 with Ni and -0.81 with Zn, $p < 0.001$) but between soil pH and all HMs in the other three tissues. In contrast, soil OM showed the reverse trend with some HMs in the pods and all HMs in the other three tissues.

Correlations between the measured and predicted HM values, with high R^2, high *MEs* and low mean *MNAEs*, reflected the goodness of fit of the model. Additionally, the presence of a non-significant difference (*p*-value) between the measured and predicted concentrations of the HMs indicated good performance of the developed model. For the pods, the regression equation that had the highest R^2 (0.99) and highest *ME* (1.00) but the lowest *MNB* (0.01) was that of Co. For the leaves, the equation with the highest R^2 (0.90) and highest *ME* (0.92) but the lowest *MNB* (0.001) was that of Mo. Similar results were obtained for Mo in the stems and Mn in the roots (*Table 5*). Applying a *t* test that assessed the significance of the differences between the actual and predicted values of the ten HMs in the four tissues revealed that the differences were nonsignificant ($p > 0.05$).

Discussion

The soil-sludge combination supporting *P. vulgaris* was slightly alkaline (pH = 7.5). High soil pH is known to enhance the adsorption of many HMs, thus decreasing their solubility in soil solutions (Chaudri et al., 2007; Zeng et al., 2011). The adsorption and

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desorption properties of soil are among the factors affecting the mobility and availability of HMs (Krishnamurti et al., 1999). Both properties are associated with soil pH, OM, clay minerals and oxidation-reduction status (Antoniadis et al., 2008; Usman et al., 2008). Significant negative correlations were detected between soil pH and most of the estimated HM concentrations in the four tissues of *P. vulgaris*. Zhao et al. (2010) reported that this soil factor plays a vital role in determining the solubility and availability of soil HMs.

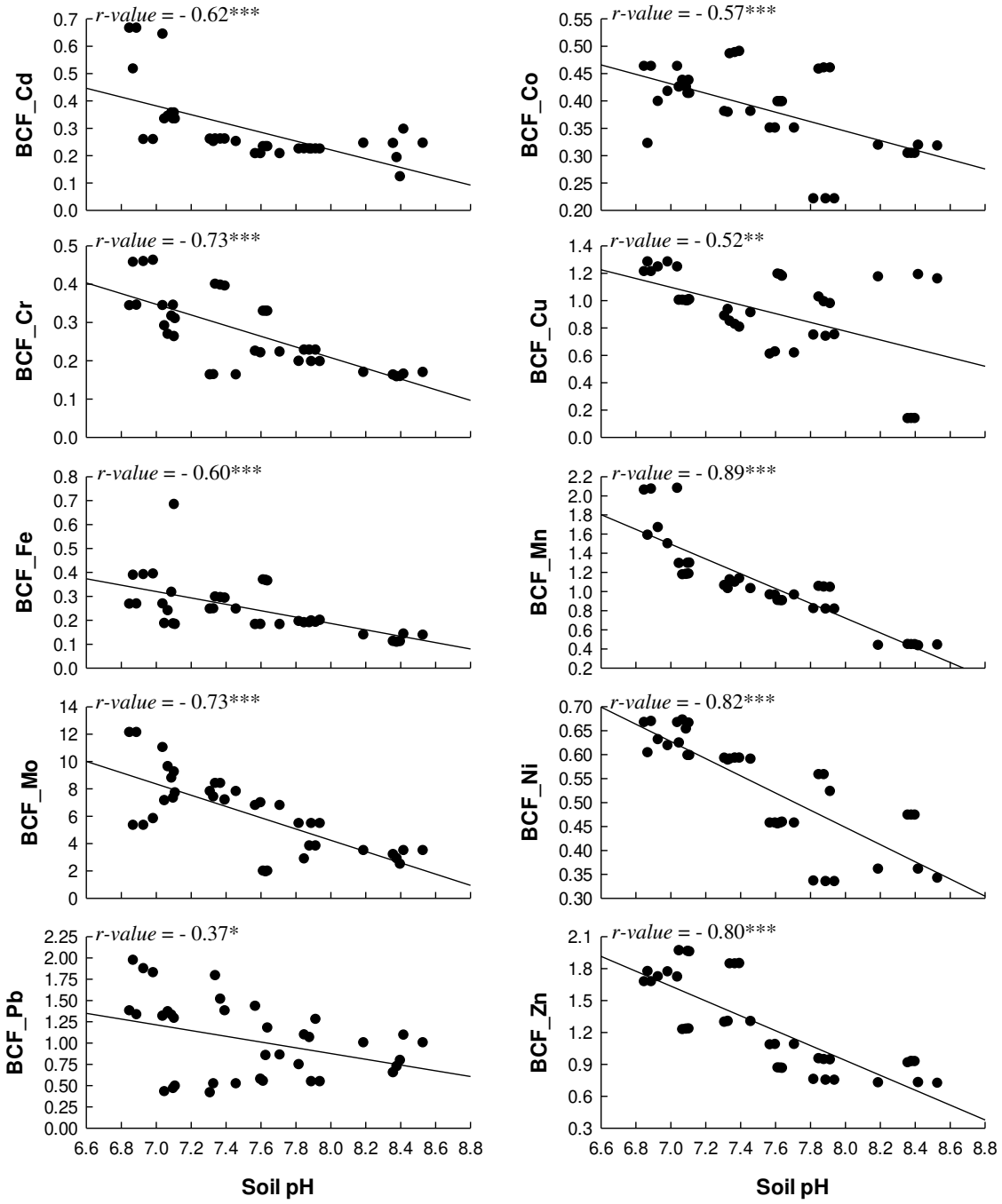

Figure 1. *Linear relationships (r-values, n = 36) between bioconcentration factors (BCFs) of heavy metals in Phaseolus vulgaris roots and soil pH. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$*

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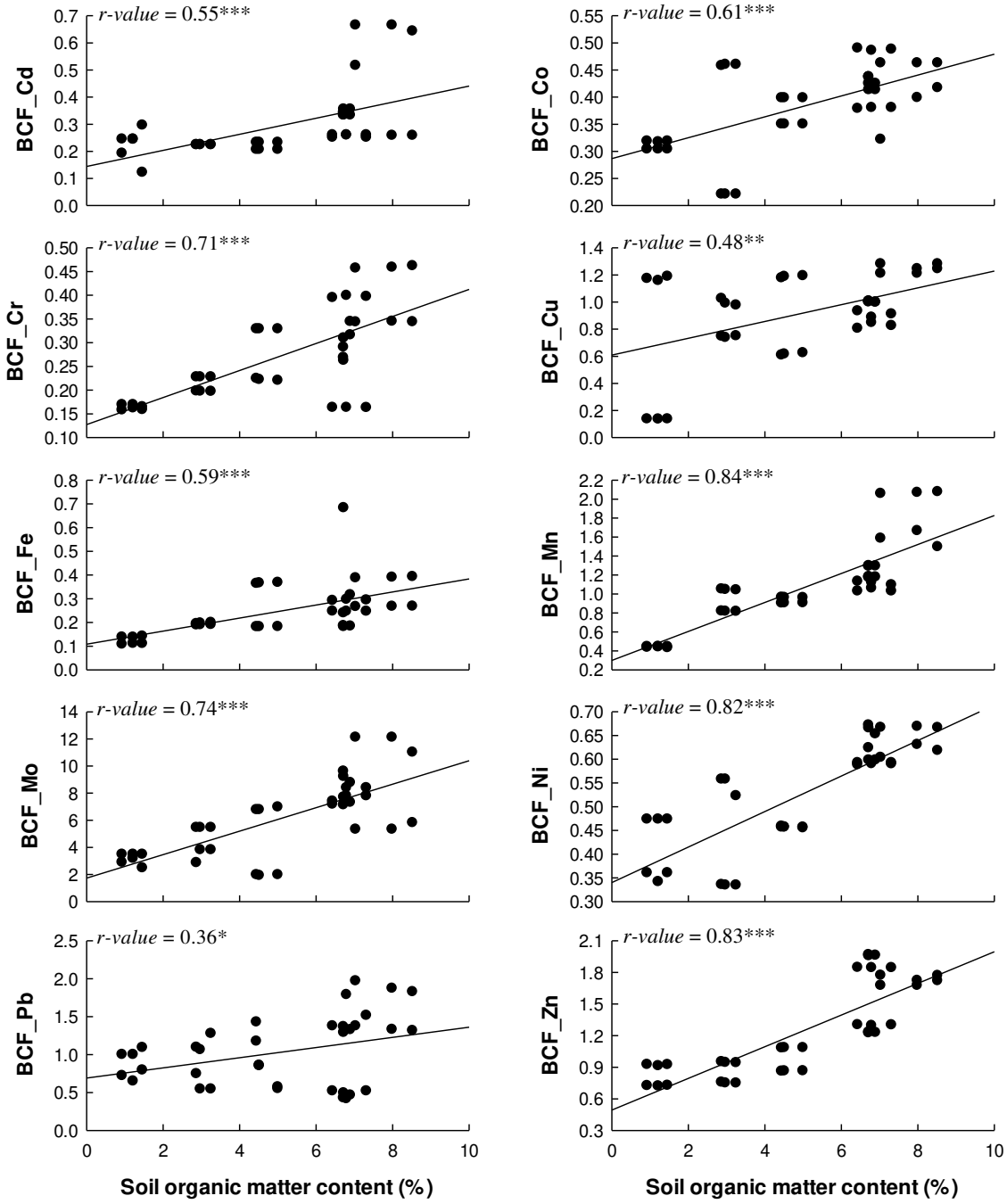

Figure 2. *Linear relationships (r-values, n = 36) between bioconcentration factors (BCFs) of heavy metals in Phaseolus vulgaris roots and soil organic matter content. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$*

In contrast, soil OM had significant positive correlations with most of the estimated HM concentrations in the tissues of the studied species. OM content plays an important role in determining the availability and mobility of HMs in soils, and it helps provide organic chemicals to the soil solution that act as chelates to increase HM availability to plants (Zeng et al., 2011). Decomposition of soil OM forms soluble organic-HM complexes and consequently more bioavailable HMs (Antoniadis and

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Alloway, 2002). Therefore, SS must be applied carefully under appropriate conditions to reduce the accumulation of HMs in soil and consequently their transfer to plant tissues.

Table 4. *Pearson correlation coefficient (r-value) between heavy metals in Phaseolus vulgaris tissues and their concentrations in the soil*

P. vulgaris **heavy metals**	**Soil heavy metals**										**Soil pH**	**Soil OM**
	Cd	**Co**	**Cr**	**Cu**	**Fe**	**Mn**	**Mo**	**Ni**	**Pb**	**Zn**		
Pod (n = 12)												
Cd	−0.33	−0.36	0.09	0.10	−0.36	−0.29	−0.10	−0.11	−0.09	−0.16	−0.19	0.43
Co	0.28	0.83	0.08	0.83	0.72	0.25	0.89	0.02	0.96	0.94	−0.76	0.77
Cr	−0.48	0.50	−0.53	0.29	0.67	−0.51	0.53	−0.66	0.47	0.40	−0.51	0.54
Cu	0.15	−0.52	0.74	0.48	−0.68	0.19	−0.09	0.51	0.03	0.01	−0.54	0.55
Fe	−0.52	0.52	−0.59	0.26	0.70	−0.55	0.56	−0.71	0.49	0.38	−0.51	0.52
Mn	0.83	−0.20	0.96	0.43	−0.53	0.86	−0.07	0.99	0.10	0.21	−0.11	0.11
Mo	0.28	−0.31	0.80	0.67	−0.52	0.32	0.11	0.58	0.25	0.24	−0.67	0.69
Ni	−0.23	0.32	0.00	0.68	0.33	−0.24	0.60	−0.23	0.64	0.54	−0.89	0.90
Pb	−0.20	−0.32	0.25	0.29	−0.32	−0.16	0.11	0.03	−0.05	−0.03	−0.33	0.38
Zn	0.58	0.28	0.76	0.97	−0.01	0.59	0.56	0.63	0.73	0.75	−0.81	0.84
Leaf (n = 36)												
Cd	0.54	0.70	0.22	0.48	0.24	0.53	0.54	0.57	0.45	0.45	−0.67	0.63
Co	0.66	0.78	0.44	0.75	0.36	0.64	0.59	0.77	0.81	0.83	−0.85	0.81
Cr	0.56	0.71	0.35	0.56	0.16	0.61	0.69	0.80	0.64	0.62	−0.76	0.74
Cu	0.66	0.31	0.73	0.82	0.46	0.33	0.47	0.54	0.74	0.61	−0.89	0.86
Fe	0.46	0.74	0.22	0.53	0.23	0.64	0.65	0.80	0.67	0.55	−0.64	0.60
Mn	0.76	0.55	0.40	0.75	0.21	0.46	0.62	0.63	0.72	0.80	−0.85	0.86
Mo	0.72	0.47	0.72	0.80	0.36	0.42	0.75	0.62	0.83	0.82	−0.87	0.88
Ni	0.57	0.69	0.45	0.78	0.47	0.48	0.59	0.62	0.82	0.76	−0.85	0.80
Pb	0.42	0.11	0.68	0.66	0.44	0.16	0.35	0.38	0.69	0.47	−0.72	0.66
Zn	0.75	0.53	0.53	0.80	0.32	0.48	0.57	0.63	0.88	0.78	−0.94	0.88
Stem (n = 36)												
Cd	0.51	0.24	0.54	0.50	0.21	0.28	0.29	0.40	0.44	0.59	−0.70	0.69
Co	0.60	0.46	0.35	0.65	0.15	0.42	0.52	0.60	0.78	0.71	−0.81	0.78
Cr	0.55	0.58	0.52	0.76	0.44	0.48	0.61	0.69	0.77	0.64	−0.80	0.78
Cu	0.61	0.80	0.41	0.70	0.34	0.70	0.42	0.81	0.55	0.68	−0.81	0.81
Fe	0.45	0.48	0.46	0.68	0.42	0.36	0.55	0.59	0.74	0.58	−0.74	0.70
Mn	0.49	0.54	0.56	0.76	0.47	0.43	0.55	0.65	0.75	0.65	−0.79	0.78
Mo	0.81	0.67	0.47	0.83	0.28	0.61	0.63	0.75	0.81	0.83	−0.90	0.91
Ni	0.37	0.45	0.53	0.62	0.39	0.38	0.19	0.59	0.50	0.50	−0.75	0.75
Pb	0.48	0.76	0.28	0.56	0.25	0.71	0.71	0.82	0.65	0.53	−0.64	0.63
Zn	0.68	0.82	0.37	0.71	0.27	0.74	0.60	0.84	0.72	0.72	−0.82	0.81
Root (n = 36)												
Cd	0.54	0.31	0.64	0.69	0.42	0.16	0.51	0.40	0.79	0.67	−0.77	0.70
Co	0.46	0.64	0.26	0.56	0.29	0.35	0.49	0.51	0.56	0.64	−0.78	0.78
Cr	0.58	0.49	0.48	0.54	0.21	0.46	0.52	0.68	0.49	0.59	−0.78	0.76
Cu	0.66	0.42	0.72	0.86	0.43	0.43	0.72	0.62	0.84	0.66	−0.83	0.81
Fe	0.56	0.44	0.70	0.69	0.40	0.48	0.38	0.63	0.45	0.50	−0.76	0.76
Mn	0.71	0.57	0.54	0.79	0.42	0.47	0.59	0.65	0.82	0.77	−0.92	0.87
Mo	0.64	0.28	0.67	0.76	0.42	0.18	0.48	0.35	0.80	0.83	−0.79	0.80
Ni	0.55	0.64	0.48	0.73	0.39	0.48	0.47	0.68	0.70	0.70	−0.89	0.89
Pb	0.33	0.56	0.39	0.44	0.35	0.39	0.50	0.56	0.43	0.50	−0.59	0.56
Zn	0.66	0.56	0.44	0.73	0.27	0.42	0.58	0.61	0.80	0.87	−0.86	0.87

OM: organic matter content. n = 12: $p < 0.05$ ($r \geq 0.58$), $p < 0.01$ ($r \geq 0.72$), $p < 0.001$ ($r \geq 0.86$). n = 36: $p < 0.05$ ($r \geq 0.33$), $p < 0.01$ ($r \geq 0.43$), $p < 0.001$ ($r \geq 0.55$). Nonsignificant r values ($p > 0.05$) are underlined

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Table 5. *Regression models between heavy metal concentrations in Phaseolus vulgaris tissues (mg kg^{-1}) and soil heavy metals (mg kg^{-1}), pH and organic matter (OM) content (%)*

Equation	R^2	*ME*	*MNAE*	*MNB*	Student's *t*-test	
					t-value	*p*
Pod						
$Cd_{pod} = -3.576 - 0.054 \times Cd_{soil} + 0.460 \times pH + 0.099 \times OM$	0.54	0.98	0.21	0.00	0.02	0.98
$Co_{pod} = 2.317 + 0.025 \times Co_{soil} - 0.217 \times pH + 0.052 \times OM$	0.99	1.00	0.01	0.01	1.56	0.18
$Cr_{pod} = 8.114 - 0.036 \times Cr_{soil} - 0.347 \times pH + 0.274 \times OM$	0.89	0.99	0.07	0.07	2.59	0.12
$Cu_{pod} = 23.505 - 0.010 \times Cu_{soil} - 2.686 \times pH + 0.611 \times OM$	0.32	0.89	0.18	0.12	1.67	0.16
$Fe_{pod} = -191.906 + 0.008 \times Fe_{soil} - 0.254 \times pH + 8.584 \times OM$	0.66	0.99	0.13	0.12	1.69	0.23
$Mn_{pod} = -21.811 + 0.107 \times Mn_{soil} - 1.444 \times pH - 0.176 \times OM$	0.74	0.99	0.07	0.01	0.30	0.78
$Mo_{pod} = 99.371 - 34.628 \times Mo_{soil} - 6.639 \times pH - 1.001 \times OM$	0.62	0.99	0.07	0.00	0.08	0.94
$Ni_{pod} = 92.323 - 0.558 \times Ni_{soil} - 9.183 \times pH + 2.105 \times OM$	0.97	0.99	0.05	0.01	0.21	0.85
$Pb_{pod} = 1.808 - 0.629 \times Pb_{soil} - 0.023 \times pH + 0.261 \times OM$	0.39	0.87	0.54	0.10	0.40	0.71
$Zn_{pod} = 55.093 + 0.050 \times Zn_{soil} - 5.102 \times pH + 1.648 \times OM$	0.77	0.99	0.06	0.01	0.22	0.84
Leaf						
$Cd_{leaf} = 0.930 + 0.004 \times Cd_{soil} - 0.093 \times pH - 0.002 \times OM$	0.44	0.39	0.17	0.03	0.69	0.50
$Co_{leaf} = 3.561 + 0.048 \times Co_{soil} - 0.500 \times pH + 0.024 \times OM$	0.87	0.88	0.10	0.00	0.10	0.93
$Cr_{leaf} = 16.501 - 0.029 \times Cr_{soil} - 1.356 \times pH + 0.095 \times OM$	0.63	0.64	0.15	0.01	0.20	0.85
$Cu_{leaf} = 26.640 - 0.045 \times Cu_{soil} - 2.391 \times pH - 0.009 \times OM$	0.80	0.77	0.07	0.01	0.34	0.74
$Fe_{leaf} = 4480.959 - 0.002 \times Fe_{soil} - 514.335 \times pH - 24.985 \times OM$	0.42	0.52	0.45	0.04	0.29	0.77
$Mn_{leaf} = 276.061 + 0.050 \times Mn_{soil} - 19.950 \times pH + 5.949 \times OM$	0.75	0.75	0.06	0.00	0.12	0.91
$Mo_{leaf} = 17.856 + 8.829 \times Mo_{soil} - 2.170 \times pH + 0.632 \times OM$	0.90	0.92	0.07	0.00	0.00	1.00
$Ni_{leaf} = 24.194 + 0.070 \times Ni_{soil} - 2.771 \times pH - 0.078 \times OM$	0.73	0.73	0.14	0.00	0.10	0.92
$Pb_{leaf} = 5.113 + 0.170 \times Pb_{soil} - 0.633 \times pH - 0.051 \times OM$	0.56	0.50	0.58	0.04	0.41	0.68
$Zn_{leaf} = 97.118 + 0.009 \times Zn_{soil} - 9.185 \times pH - 0.423 \times OM$	0.89	0.90	0.04	0.00	0.29	0.78
Stem						
$Cd_{stem} = 1.770 - 0.037 \times Cd_{soil} - 0.174 \times pH + 0.019 \times OM$	0.51	0.32	0.31	0.08	1.03	0.32
$Co_{stem} = 6.229 + 0.005 \times Co_{soil} - 0.694 \times pH + 0.014 \times OM$	0.66	0.67	0.19	0.02	0.33	0.75
$Cr_{stem} = 16.967 - 0.005 \times Cr_{soil} - 1.877 \times pH + 0.110 \times OM$	0.65	0.68	0.26	0.03	0.37	0.71
$Cu_{stem} = 28.016 - 0.029 \times Cu_{soil} - 2.288 \times pH + 0.385 \times OM$	0.67	0.66	0.07	0.02	0.79	0.44
$Fe_{stem} = 2386.891 - 0.004 \times Fe_{soil} - 302.592 \times pH - 2.840 \times OM$	0.56	0.62	0.46	0.06	0.50	0.62
$Mn_{stem} = 466.786 + 0.084 \times Mn_{soil} - 61.969 \times pH + 4.757 \times OM$	0.64	0.62	0.48	0.02	0.19	0.86
$Mo_{stem} = 53.364 + 14.133 \times Mo_{soil} - 7.149 \times pH + 2.519 \times OM$	0.87	0.85	0.16	0.00	0.02	0.99
$Ni_{stem} = 9.916 + 0.066 \times Ni_{soil} - 0.986 \times pH + 0.143 \times OM$	0.60	0.59	0.14	0.04	0.95	0.35
$Pb_{stem} = 1.533 + 0.340 \times Pb_{soil} - 0.255 \times pH + 0.041 \times OM$	0.46	0.48	0.29	0.03	0.41	0.69
$Zn_{stem} = 122.663 + 0.056 \times Zn_{soil} - 13.081 \times pH + 0.847 \times OM$	0.69	0.69	0.13	0.01	0.33	0.75
Root						
$Cd_{root} = 11.361 - 0.138 \times Cd_{soil} - 1.263 \times pH - 0.073 \times OM$	0.61	0.63	0.20	0.08	1.06	0.30
$Co_{root} = 5.812 + 0.191 \times Co_{soil} - 0.434 \times pH + 0.616 \times OM$	0.70	0.68	0.12	0.03	0.96	0.35
$Cr_{root} = 170.335 - 0.131 \times Cr_{soil} - 16.199 \times pH + 1.772 \times OM$	0.61	0.55	0.20	0.04	0.69	0.50
$Cu_{root} = 87.997 + 0.915 \times Cu_{soil} - 11.069 \times pH - 0.629 \times OM$	0.77	0.74	0.38	0.01	0.15	0.88
$Fe_{root} = 28452.324 + 0.064 \times Fe_{soil} - 3187.528 \times pH + 628.992 \times OM$	0.61	0.48	0.20	0.05	0.68	0.51
$Mn_{root} = 4344.638 + 0.337 \times Mn_{soil} - 513.371 \times pH - 1.470 \times OM$	0.84	0.84	0.13	0.00	0.04	0.97
$Mo_{root} = 16.434 + 1.769 \times Mo_{soil} - 1.980 \times pH + 0.725 \times OM$	0.65	0.70	0.26	0.06	0.98	0.34
$Ni_{root} = 32.315 + 0.250 \times Ni_{soil} - 3.556 \times pH + 0.822 \times OM$	0.82	0.84	0.09	0.02	0.88	0.39
$Pb_{root} = 33.307 - 0.452 \times Pb_{soil} - 3.559 \times pH - 0.082 \times OM$	0.36	0.26	0.48	0.07	0.63	0.54
$Zn_{root} = 48.039 + 1.463 \times Zn_{soil} - 14.568 \times pH + 9.521 \times OM$	0.83	0.83	0.19	0.00	0.05	0.96

R^2: coefficient of determination, *ME*: model efficiency, *MNAE*: mean normalized average error, *MNB*: mean normalized bias

The build-up of HMs in plants is a complex process that depends on various soil properties, SS composition and application rate, species phenology and physiology, rhizosphere biochemistry, climatic factors, chelating effects and chemical speciation of

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HMs (Dolgen et al., 2007). The root system is considered the main accumulator of HMs and filter for HM uptake; it slowly accumulates HMs (particularly if present in high concentrations), thus preventing or diminishing their transport to the above-ground biomass (Eid and Shaltout, 2016; Sawidis et al., 2011). The findings of the present study confirm this observation, as almost all the estimated HMs were higher in the roots of *P. vulgaris* than in its stems, leaves and pods.

Singh et al. (2004) reported that the hyper-accumulation of HMs in a root system may be ascribed to complexation of HMs with sulfhydryl groups, resulting in less HM translocation to the shoot system. Furthermore, higher accumulation of an HM in the root of a certain plant than in its above-ground tissues may occur because the root is the first tissue to come into contact with HMs; therefore, greater accumulation occurs there (Eid and Shaltout, 2014). In a plant such as *P. vulgaris*, this is a preferential behaviour that prevents toxic levels of HMs from occurring in the edible shoot parts, particularly the pods, which are cooked in immature and dried stages (Huxley, 1999; Tindall, 1978).

The three HMs with the highest concentrations in the four tissues of *P. vulgaris* and the supporting soil-sludge mixture were Fe > Mn > Zn, suggesting their easy uptake as a result of their higher levels in the soils and because they are essential for the growth of the studied plant (Dolgen et al., 2007; Lopes et al., 2012). In contrast, Pb and Cd tend to accumulate poorly in plant tissues and, if their uptake occurs, they accumulate in the root system (Latare et al., 2014).

The addition of SS to cultivated soil often leads to soil pollution by HMs and HM accumulation and phytotoxicity in the food supply (Yilmaz and Temizgül, 2012). The HM contents in the soil mixed with SS in the current work, except the Cd and Fe contents, were in the normal range reported by Allen (1989) and Kabata-Pendias (2011). In addition, most of the HM concentrations in *P. vulgaris* tissues were not in the critical ranges proposed by Kabata-Pendias (2011); however, some of them were within the normal range reported by Allen (1989) (e.g., concentrations of Cu, Fe, Mn, Pb and Zn). Thus, although the mixing of cultivated soil with SS may be practical for some crops, it might not be practical for others, such as *P. vulgaris*. Nevertheless, regular checking of HM levels in crop products that are cultivated in soil mixed with SS is recommended to avoid toxic effects of HMs in the food chain.

The BCF (root:soil ratio) is a rough estimate of HM uptake but reflects fewer details about site conditions (Zeng et al., 2011). In the current work, soil pH had a negative effect on BCF values, while soil OM had a positive effect. Significant negative *r* coefficients between soil pH and BCF of HMs in the root system of *P. vulgaris* could be interpreted in the view that alkalinity decreases the availability of HMs in soil solutions while acidity increases their availability (Du Laing et al., 2008; Novotná et al., 2015; Zeng et al., 2011). In addition, the presence of significant positive *r* coefficients between soil OM and BCF of HMs in the root system of the studied plant may be interpreted in the view that OM increases HM mobility in soil and HM uptake by plants (Singh and Agrawal, 2010). This finding is comparable to that of some previous related studies (Boshoff et al., 2014; Chaudri et al., 2007; Zeng et al., 2011). The BCF was < 1 for some metals (e.g., Fe, Cr, Ni, Cd, Co and Cu), which means that either the accumulation capacity of the root system was low for these HMs or they were transferred in low concentrations (see Novotná et al., 2015). In contrast, the BCF was > 1 for Mn, Mo, Pb and Zn, which means that *P. vulgaris* is a hyper-accumulator and may be used as a phytoremediator for these metals. Regarding the TF (aerial

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tissue:root ratio), all the values were < 1, except that for Mo, which means that the root is an effective bio-filter for these elements, while it is less effective for Mo.

Regression models are complex approaches with which we can predict the HM contents in plant tissues using some soil variables, e.g., HMs, pH and OM (Chaudri et al., 2007; Waegeneers et al., 2011). The results of the studied species indicated that the constructed models performed well for most of the estimated HM concentrations in the tissues of *P. vulgaris*, taking into account the parameters that indicate the high performance of the models (R^2, *ME*, *MNAE* and *p*-value). All the measured soil factors (HM, pH and OM) consistently contributed to HM concentrations in the plant tissues. Similar findings were reported by other investigators (e.g., Bešter et al., 2013; Boshoff et al., 2014; dos Santos-Araujo et al., 2017; Gan et al., 2017).

To the best of the researchers knowledge, to this day, no study has been performed where regression models have been developed to forecast of HMs uptake by *P. vulgaris* from the soil amended with SS. The mathematical models for the uptake of HMs by the roots, stems, leaves and pods of *P. vulgaris* constructed here are comparable to those produced for some other crops cultivated in soil amended with SS. For example, the variability (R^2) in Cd in the tissues of cucumber was 49-76% (Eid et al., 2018a), in garden pea was 25-67% (Eid et al., 2020c), in *Eruca sativa* was 49-81% (Eid et al., 2020b), in spinach was 83-88% (Eid et al., 2018b), compared with 44-61% for the *P. vulgaris* tissues. In their study on the Cd models of some vegetables, Bešter et al. (2013) reported R^2 values of 41% for tomato and 90% for endive. Regarding the same element, Boshoff et al. (2014) reported an R^2 range of 10-47% for *Urtica dioica* and 31-38% for *Agrostis* and *Poa* species. In Brazil, dos Santos-Araujo et al. (2017) recorded an R^2 of 45% for lettuce and 47% for carrot. A possible reason for the lower R^2 values of some HM models in previous studies (*Table 6*) may be the large number of replicates used in model construction which results in a large amount of noise in the datasets that may influence the predictive ability of the models (Römkens et al., 2004). The pollution levels, physico-chemical characteristics of the soil at the sampling sites, soil texture, soil microbial activity, soil types with different origins and mineralogy, distinct land management practices and analytical methods used in digestion of sample materials (Du Laing et al., 2003, 2009; Kabata-Pendias, 2011) could be other reasons for the different R^2 values between HM models.

Table 6. *Regression models for predicting the concentration of Cd in plants based on the concentration of Cd in soil and soil properties*

Plant	*n*	Model	R^2	Reference
Kidney bean	18	$Cd_{pod} = -3.576 - 0.054 \times Cd_{soil} + 0.460 \times pH + 0.099 \times OM\ (\%)$	54%	Present study
	18	$Cd_{leaf} = 0.930 + 0.004 \times Cd_{soil} - 0.093 \times pH - 0.002 \times OM\ (\%)$	44%	
	18	$Cd_{stem} = 1.770 - 0.037 \times Cd_{soil} - 0.174 \times pH + 0.019 \times OM\ (\%)$	51%	
	18	$Cd_{root} = 11.361 - 0.138 \times Cd_{soil} - 1.263 \times pH - 0.073 \times OM\ (\%)$	61%	
Agrostis and *Poa* species	37	$\log Cd_{plant} = -0.56 + (0.58 \times \log Cd_{soil})$	38%	Boshoff et al. (2014)
	37	$\log Cd_{plant} = 0.08 + (0.27 \times \log CaCl_2\ [Cd]_{soil})$	31%	
Barley	90	$\log Cd_{grain} = 0.04 + 0.21 \times \log Cd_{soil} - 0.23 \times pH$	22%	Adams et al. (2004)
Cabbage	16	$Cd_{plant} = 0.007 + 0.002 \times Cd_{soil}$	44%	Bešter et al. (2013)
Carrot	54	$Cd_{plant} = 0.107 + 0.017 \times Cd_{soil} - 0.00007 \times Mn_{soil}$	47%	Bešter et al. (2013)
Carrot	238	$Cd_{plant} = -0.19 + 0.46 \times Cd_{soil}$	33%	dos Santos-Araujo et al. (2017)
	238	$Cd_{plant} = 0.89 + 0.42 \times Cd_{soil} - 0.17 \times pH$	45%	
	238	$Cd_{plant} = 0.90 + 0.42 \times Cd_{soil} - 0.17 \times pH - 0.01 \times OM\ (\%)$	45%	
	238	$Cd_{plant} = 0.92 + 0.43 \times Cd_{soil} - 0.18 \times pH - 0.01 \times OM\ (\%) - 0.04 \times Clay\ (\%)$	45%	

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Chicory	29	$Cd_{plant} = 0.016 + 0.017 \times Cd_{soil}$	60%	Bešter et al. (2013)
Cucumber	18	$Cd_{root} = 0.11 + 0.23 \times Cd_{soil} - 0.04 \times pH + 0.07 \times OM$ (%)	49%	Eid et al. (2018a)
	18	$Cd_{stem} = -0.56 + 0.02 \times Cd_{soil} + 0.07 \times pH + 0.03 \times OM$ (%)	55%	
	18	$Cd_{leaf} = -0.14–0.002 \times Cd_{soil} + 0.02 \times pH + 0.03 \times OM$ (%)	56%	
	18	$Cd_{fruit} = 0.74 + 0.50 \times Cd_{soil} - 0.15 \times pH + 0.17 \times OM$ (%)	76%	
Endive	26	$Cd_{plant} = 0.089 + 0.032 \times Cd_{soil} - 0.014 \times OM$ (%)	90%	Bešter et al. (2013)
Eruca sativa	18	$Cd_{leaf} = 0.326 + 0.204 \times Cd_{soil} - 0.070 \times pH + 0.136 \times OM$ (%)	81%	Eid et al. (2020b)
	18	$Cd_{root} = 1.518 - 0.298 \times Cd_{soil} - 0.088 \times pH + 0.067 \times OM$ (%)	49%	
Garden pea	15	$Cd_{Pod} = 4.373 - 0.052 \times Cd_{Soil} - 0.480 \times pH - 0.051 \times OM$ (%)	60%	Eid et al. (2020c)
	15	$Cd_{Shoot} = 1.366 + 0.003 \times Cd_{Soil} - 0.133 \times pH - 0.020 \times OM$ (%)	25%	
	15	$Cd_{Root} = -1.455 + 0.249 \times Cd_{Soil} + 0.144 \times pH + 0.042 \times OM$ (%)	67%	
Hop	13	$Cd_{plant} = 0.061 \times Cd_{soil} - 0.28 \times OM$ (%)	51%	Novotná et al. (2015)
Lettuce	293	$Cd_{plant} = -0.06 + 0.39 \times Cd_{soil}$	35%	dos Santos-Araujo et al. (2017)
	293	$Cd_{plant} = 1.10 + 0.44 \times Cd_{soil} - 0.18 \times pH$	42%	
	293	$Cd_{plant} = 1.35 + 0.48 \times Cd_{soil} - 0.18 \times pH - 0.28 \times OM$ (%)	44%	
	293	$Cd_{plant} = 1.11 + 0.39 \times Cd_{soil} - 0.14 \times pH - 0.22 \times OM$ (%) $- 0.14 \times$ Clay (%)	47%	
Maize	79	$Cd_{shoot} = 90.1 + 0.24 \times Cd_{Soil} - 12.9 \times pH$	17%	Tudoreanu and Phillips (2004)
Onion	35	$Cd_{plant} = 0.208 + 0.005 \times Cd_{soil} - 0.002 \times OM$ (%) $- 0.027 \times pH$	85%	Bešter et al. (2013)
Potato	29	$Cd_{plant} = 0.042 + 0.007 \times Cd_{soil}$	76%	Bešter et al. (2013)
Potato	17	$Cd_{plant} = -0.018 + 2.46 \times Cd_{soil} - 0.0041 \times$ Clay (%) $+ 0.036 \times Zn_{soil} + 0.021 \times$ pH:OM (%) $- 0.0056 \times Zn_{soil}$:pH $- 0.37 \times Cd_{soil}$:pH	60%	Novotná et al. (2015)
Red beet	20	$Cd_{plant} = 0.017 + 0.026 \times Cd_{soil}$	67%	Bešter et al. (2013)
Rice	33	$\log Cd_{grain} = 0.473 \times \log Cd_{soil} - 0.157 \times pH + 0.445 \times \log$ OM (g/kg) $- 0.984$	66%	Mu et al. (2020)
Rye grass	156	$Cd_{shoot} = 35.3 + 0.37 \times Cd_{Soil} - 4.9 \times pH$	13%	Tudoreanu and Phillips (2004)
Spinach	12	$Cd_{leaf} = 0.402 + 0.014 \times Cd_{soil} - 0.047 \times pH + 0.043 \times OM$ (%)	88%	Eid et al. (2018b)
	12	$Cd_{root} = 2.144 + 0.060 \times Cd_{soil} - 0.294 \times pH + 0.130 \times OM$ (%)	83%	
Tomato	51	$Cd_{plant} = 0.020 + 0.002 \times Cd_{soil} - 0.000008 \times Mn_{soil}$	41%	Bešter et al. (2013)
Urtica dioica	66	$\log Cd_{plant} = 0.26 + (0.24 \times \log CaCl_2\ [Cd]_{soil})$	10%	Boshoff et al. (2014)
	68	$\log Cd_{plant} = -0.13 + (0.69 \times \log Cd_{soil}) - (0.87 \times \log$ clay %)	47%	
Wheat	162	$\log Cd_{grain} = 0.28 + 0.44 \times \log Cd_{soil} - 0.18 \times pH$	49%	Adams et al. (2004)
Wheat	100	$\log Cd_{plant} = -1.75 + 0.59 \times Cd_{soil} - 0.23 \times OM$ (%)	64%	Novotná et al. (2015)
Wheat	14	$\log Cd_{grain} = 1.386 + \log Cd_{soil} - 0.279 \times pH$	85%	Liu et al. (2015)

n: number of samples, R^2: coefficient of determination

Conclusion

The HM concentrations in the different tissues of *P. vulgaris* showed that most of the investigated HMs were accumulated in the plant roots rather than in the other tissues. The SS is a rich source of OM, can increase solubility of soil HMs, and therefore the use of SS as amendment must be applied carefully to avoid the accumulation of these HMs in the soil and their transfer to plants. All the TFs values were less than unity, except that for Mo, which means that the root is an effective bio-filter for these HMs, while it is less effective for Mo. Prediction models for HMs concentrations proved that pH, OM and HMs concentrations of the soil were good predictors for the uptake of HMs by *P. vulgaris*. These models functioned well and demonstrated great levels of efficacy and low rates of error, and can be employed in examining *P. vulgaris* plants grown in soil amended with SS. Applying the *t* test, there are no significant differences between the actual and predicted values of the ten HMs in *P. vulgaris* tissues, which reflect the best-fitting of these equations for predicting the uptake of these HMs. An

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extended field study for several growth seasons using the same SS may be required to confirm whether the repeated application of SS causes environmental problems in the future. Additionally, in future work, this study could be extended to investigate the microbial effects of SS application on soil quality and plant growth. Also, investigations of suitable methods for SS treatments are needed before the application in the agricultural system to avoid the health and environmental hazards.

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APPENDIX

Appendix 1. *Selected chemical properties of sewage sludge and agricultural soil used in the pot experiment (means ± standard error, n = 3)*

Properties	Agricultural soil	Sewage sludge
pH	8.7 ± 0.02	7.0 ± 0.02
Organic matter (%)	0.9 ± 0.2	65.0 ± 0.9
Cd (mg kg^{-1})	2.9 ± 0.1	1.2 ± 0.1
Co (mg kg^{-1})	35.5 ± 1.1	25.9 ± 1.3
Cr (mg kg^{-1})	134.3 ± 0.7	176.2 ± 1.9
Cu (mg kg^{-1})	15.0 ± 0.6	162.6 ± 2.3
Fe (mg g^{-1})	42.4 ± 0.5	24.1 ± 0.5
Mn (mg kg^{-1})	677.3 ± 3.2	560.7 ± 9.8
Mo (mg kg^{-1})	1.1 ± 0.0	0.9 ± 0.0
Ni (mg kg^{-1})	68.1 ± 3.7	138.7 ± 3.7
Pb (mg kg^{-1})	3.5 ± 0.4	671.1 ± 6.2
Zn (mg kg^{-1})	77.2 ± 1.9	667.6 ± 13.4

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RESPONSES OF SULFUR AND PHOSPHORUS DOSES ON THE YIELD AND QUALITY OF FENUGREEK (*Trigonella foenum-graecum* L.)

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Abstract. This study was conducted to determine the effects of sulfur and phosphorus doses on the yield and quality of fenugreek in Siirt, Turkey, in the 2016-2017 and 2017-2018 growing seasons. Field trials were designed in a factorial complete block design with three replications at the experimental fields of the Agricultural Faculty of Siirt University. In the study, plant height (cm), first pod height (cm), the number of pods (pod plant^{-1}), the number of seeds in the pod (seed pod^{-1}), pod length (cm), thousand-seed weight (g), seed yield (kg ha^{-1}), protein content (%) and trigonelline content (%) were determined. All of the growth and yield parameters, except for plant height and pod length, were significantly affected by sulfur fertilization. All the parameters were affected by phosphorus fertilization. The highest seed yield (2224 kg ha^{-1}) and trigonelline content (1.26%) were obtained under 30 kg S ha^{-1} and 90 kg P ha^{-1} applications.
Keywords: *Trigonella foenum-graecum, fertilizer, medicinal plants, seed yield, trigonelline*

Introduction

Nowadays, due to the many side effects of synthetic and chemical drugs, trending towards alternative medicine or complementary medicine is gradually increasing. Medicinal and aromatic plants used for this purpose can be found in Turkey among many other countries, mainly by gathering from the natural flora. Therefore, medicinal plants of the desired quantity and quality cannot be obtained. This situation brings up the production of medicinal and aromatic plants with increasing the usage area.

Fenugreek (*Trigonella foenum-graecum* L.) which is grown for its seeds, fresh shoots, and leaves and is an important multi-purpose plant, one of the oldest known to have medicinal and aromatic properties. It has Mediterranean and Asian origin, and is an annual plant belonging to the Leguminosae family (Baldemir and İlgün, 2015; Bienkowski et al., 2016), containing about 50 species, and 45 of these species are found in the natural flora of Turkey (Davis, 1982; Beyzi et al., 2010). India is the world's largest producer of fenugreek, and it is also grown in South Asia, the Middle East, Far East, China, Iran, Pakistan, Turkey, the Mediterranean region, Europe, North Africa, Australia, Canada, the USA, and Argentina (Basu et al., 2019).

Fenugreek is a plant that has economic value in food, feed, medicine, and cosmetics. Fenugreek seeds are used in traditional medicine in the treatment of diabetes and cancer, as a natural dyestuff in the cosmetics industry, in animal feeding, as a coating material of some products in the food industry, and in making spices (Boeker, 1963; Baytop, 1984; Arslan et al., 1989; Hornok, 1992; Akgül, 1993; Küçük and Gürbüz, 1999; Soylu et al., 2000; Kızıl and Arslan, 2003; Tunçtürk et al., 2011; Abd Elhamid et al., 2016). It is also used as a green manure plant since it is a legume plant, and it has been reported to play a role in improving the physical and chemical properties of the soil (Abdelgani et al., 1999).

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Phosphorus (P), which fulfills many functions related to plant growth, development, and metabolism and is essential for young tissues and cannot be renewed, is the most crucial macronutrient element after nitrogen. It is also called the key to life because it regulates many metabolic activities of plant life. If sufficient P is given to the plant, it causes rapid growth and increases plant resistance. In the case of P deficiency, plants stop and slow down the growth of the above-ground organs and accelerate the root growth. P, which also plays an essential role in increasing the yield of legumes by increasing biological activities such as nodulation, nitrogen fixation, nutrient uptake in the soil and in the rhizosphere environment, alleviates adverse effects of drought on physiological parameters in plants (Kacar and Katkat, 2007; Turan and Horuz, 2012; Yadav et al., 2014; Singh and Singh, 2016; Gezgin, 2018).

Generally, the properties of soils in Turkey affect the uptake of soil phosphorus by plants negatively (high pH, high lime content, low organic matter content, high clay content). It is very important to apply appropriate amounts of P to legume plants or to increase the availability of soil phosphorus, according to the results of soil analysis (Gezgin, 2018). Legumes' benefiting from soil phosphorus also differs according to its species and varieties (Gökmen Yılmaz et al., 2017).

Elemental sulfur (S) is a natural material and can be applied to increase the availability of plant nutrients and to reduce deficiencies in calcareous and alkaline soils (Manesh et al., 2013). Sulfur has vital importance in the activation of the process of photosynthesis, carbohydrate metabolism, and certain enzyme systems in plants; it can increase plants' seed and oil yields and protein contents. It is considered to be an essential nutrient in the vitamin and amino acids synthesis of legume plants. Sulfur is found in cysteine, cystine, and methionine, among the amino acids, and in the composition of proteins. It is necessary for chlorophyll formation (Lal et al., 2015). It accelerates root growth and nodule formation (Tonguç et al., 2017; Bolat and Kara, 2017). It has been reported that the amount of nitrogen fixed by legumes increases with the treatment of sulfur, and as a result of this, soil fertility improves (Mohamed El-Sayed Ali, 2018).

The reduced use of fossil fuels in the world and in Turkey and the widespread use of sulfur-free fertilizers have caused sulfur deficiency to emerge as a factor limiting the yield in plant production. Sulfur affects nitrogen utilization efficiency, and adverse effects of sulfur deficiency are observed on the growth of plants, chlorophyll amount, photosynthesis capacities, yield, and yield parameters (Tonguç et al., 2017). Sulfur positively affects not only the above-ground organs of plants but also the root growth, just like phosphorus. As a matter of fact, in the soybean plant given sulfur, the number and weight of side roots, the number and weight of nodules in which nitrogen fixation occurred were reported to increase significantly (Zhao et al., 2008). In beans, plant weight, the number of branches, the number of pods, the number of grains per plant, thousand-seed weight, harvest index, and yield were indicated to significantly increase in comparison with unfertilized plants (Tonguç et al., 2017).

This study was conducted to determine the effects of sulfur and phosphorus doses on the yield and quality characteristics of fenugreek (*T. foenum-graecum* L.).

Materials and methods

The study was carried out between 2016-2018 under the ecological conditions of Siirt province located in the Southeastern Anatolia Region of Turkey, which has a

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semi-arid climate. Siirt is located at 37° 58' 7.37" N and 41° 51' 3.87" E coordinates with 894 m altitude (*Figure 1, Figure 2*).

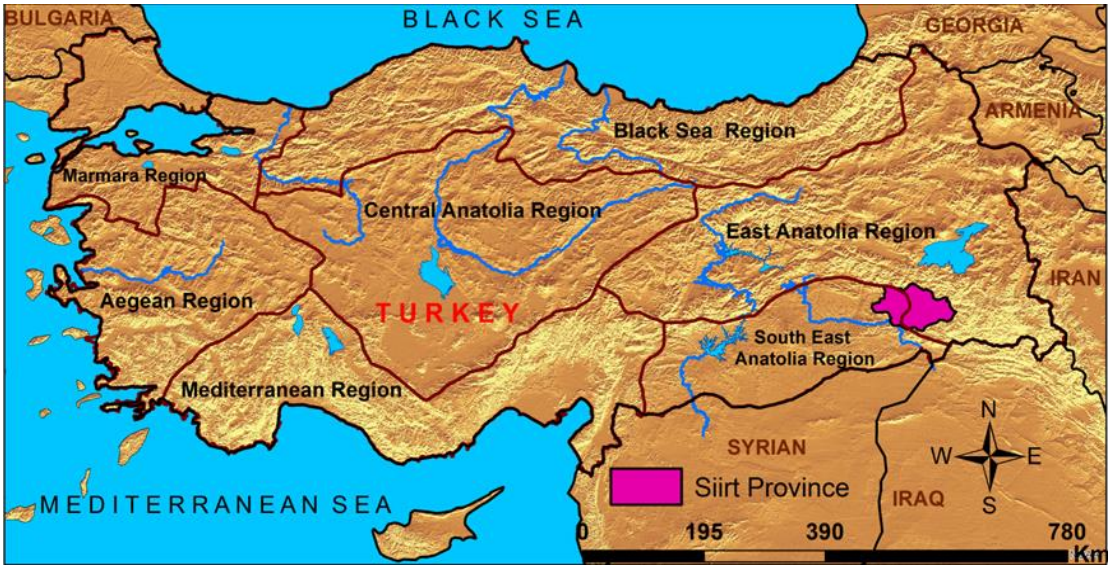

Figure 1. *Location of the study area in Turkey*

Figure 2. *A photo of the research area*

In the second year of the study, less precipitation (522.8 mm) occurred in comparison with the first year (574.2 mm), while the long-term annual precipitation was 634.1 mm. While the highest precipitation occurred in March and April during the 2016-2017 vegetation period, the highest precipitation was recorded in May during the 2017-2018 vegetation period (*Table 1*). The mean temperature during the study years and the long-term mean temperature (38 years) were 11.2 °C, 13.8 °C, and 11.4 °C, respectively *Table 1* (Anonymous, 2018).

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Table 1. *Climate characteristics of trial area*

Climate parameters	Research years	Months								
		Nov	**Dec**	**Jan**	**Feb**	**Mar**	**Apr**	**May**	**June**	**Mean/Sum**
Average temparature (°C)	2016-2017	10.4	3.3	3.0	2.7	9.6	14.0	19.5	26.9	11.2
	2017-2018	11.2	8.0	5.7	8.2	13.7	16.8	19.8	27.4	13.8
	Long term*	10.3	4.9	3.0	4.5	8.8	14.3	19.5	26.2	11.4
Monthly precipitation (mm)	2016-2017	55.4	116.6	46.4	29.2	119.2	132.8	74.6	0.0	574.2
	2017-2018	86.0	47.4	56.4	74.2	47.6	61.6	139.6	10.0	522.8
	Long term*	85.1	91.1	82.2	96.6	108.7	96.3	64.3	9.8	634.1

*: 1980-2018

In the study, some physical and chemical analysis results of the soils taken before establishing the field trial were presented in *Table 2*. In the first year (2016-2017), the trial area soils were loamy textured, and they were clay-loam textured in the second year (2017-2018); both trial area soils were slightly alkaline, salt-free, their lime content was "medium calcareous," the organic matter content was "low," and the available potassium (K) content was "sufficient". The available P content of the soils in the first year was determined to be "very little," and the available P content of the soils in the second year was determined to be "low" (*Table 2*).

Table 2. *Some physical and chemical properties of the study area soils (0-20 cm)**

Properties	Value	
	2016-2017	**2017-2018**
Clay, %	47.56	34.16
Silt, %	12.11	26.00
Sand, %	40.33	39.84
pH	7.72	7.53
Electrical conductivity (EC), mS cm^{-1}	0.363	0.150
Lime ($CaCO_3$), %	12.0	8.2
Organic matter, %	1.31	1.78
Available phosphorus, kg P_2O_5 ha^{-1}	24	49
Available potassium, kg K_2O ha^{-1}	1430	1250

*: Analyses were carried out in Siirt University, Science and Technology Application and Research Center Laboratory

As the plant material in the study, fenugreek (*T. foenum-graecum* L.) seeds, belonging to the "Konya population" and seeded locally in the Central Anatolia Region of Turkey, were used.

In this study, the field trial was established as three replications in randomized blocks according to the factorial trial design. In the study, 4 different sulfur doses (S_0= 0, S_{10}= 10, S_{20}= 20, and S_{30}= 30 kg S ha^{-1}) and 4 different phosphorus doses (P_0= 0, P_{30}= 30, P_{60}= 60, and P_{90}= 90 kg P_2O_5 ha^{-1}) constituted the subject of the study. Elemental sulfur was used as the source of sulfur fertilizer, and triple superphosphate (43-44% P_2O_5) was used as the source of phosphorus fertilizer. According to the

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research subjects, both fertilizer forms were mixed by applying them to the soil before seeding.

The seeding process was performed manually on 14 November 2016 in the first year and on 17 November 2017 in the second year on the grooves opened with the help of a marker. In the study, row distance was 30 cm and parcel distance was 100 cm. Each parcel constituted from four rows, while length and width of the parcels were 3 meter by 1.2 meter each with a total area of 3.6 m^2 per parcel. Sowing norm was 30 kg ha^{-1}, and sowing was done manually in rows opened with the help of a marker. Weed control was performed mechanically by hand several times in both years. At the harvest, two border rows and 50 cm from each side were excluded to eliminate border effects.

Plant height, first pod height, pod length, the number of pods per plant, and the number of seeds per pod were determined in 10 plants randomly selected in each parcel before harvest. The harvest was carried in the entire plot area, excluding borders in the first week of July in both years. The harvested plants were dried in the shade for 3-4 days, and seed yields were calculated per hectare. Crude protein determination in seeds was performed by the Kjeldahl nitrogen determination method, and trigonelline analysis was performed by the HPLC method. In trigonelline analysis was used Agilent TC-C18 (ODS 25 cm * 4.6 mm) column. Column temperature was maintained at 27 °C and the flow rate of the mobile phase was kept at 1 ml per min. The changes in absorbance at wavelength 210 nm were recorded with UV detector. The peak area was calibrated to trigonellin content with a standard.

The data were analyzed by JMP statistical software. A homogeneity test was applied to the data obtained from the study. According to the results of the homogeneity test, they were subjected to combined variance analysis (ANOVA) according to the factorial trial design in randomized blocks. According to the F-test results, differences between the groups were determined by the LSD multiple comparison test. In the study, the correlation coefficients of the pairwise relationships between the examined properties were calculated (Yurtsever, 1984; Düzgüneş et al., 1987).

Results and discussion

Plant height

In the study, the effect of S doses on the plant height of fenugreek was statistically insignificant, and according to S doses, plant height varied between 62.4-65.2 cm as the mean of P doses and years. The effect of phosphorus doses on plant height was determined to be statistically significant at the $p<0.01$ level. This difference occurred between the P_0 and P_{30} doses of phosphorus and its P_{60} and P_{90} doses. In this study, in which plant height increased according to P doses, the highest plant height was determined in P_{60} (65.2 cm) and P_{90} (66.3 cm) P doses. When the interaction of SxP was examined, the highest plant height was determined in the S_0P_{90} application as 70.1 cm, but no statistically significant difference was found between it and S_0P_{60}, $S_{10}P_{60}$, $S_{10}P_{90}$, $S_{30}P_0$, and $S_{30}P_{90}$ applications. The lowest plant height was measured in unfertilized parcels (control). SxP interaction was determined to be statistically significant ($p<0.01$) (*Table 3*). When the studies on this subject were reviewed, increasing P applications were reported to increase plant height, similarly to the results of our study (Halesh et al., 2000; Khiriya et al., 2001; Nehara et al., 2006; Meena et al., 2012; Mehta et al., 2012; Verma et al., 2014; Srivastava et al., 2015; Ahmad, 2017; Basu et al., 2019).

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Table 3. *Means of yield components at different sulfur and phosphorus doses in fenugreek*

S doses	P doses	Plant height (cm)			First pod height (cm)			Pod length (cm)		
		2017	**2018**	**Mean[1]**	**2017**	**2018**	**Mean[1]**	**2017**	**2018**	**Mean[1]**
S_0	P_0	59.8	57.8	58.8 e	36.4	46.2	41.3	14.4	13.3	13.8
	P_{30}	63.8	61.4	62.6 cde	44.5	50.1	47.3	14.5	14.6	14.6
	P_{60}	69.9	68.8	69.4 ab	43.5	48.9	46.2	14.8	15.3	15.0
	P_{90}	69.0	71.1	70.1 a	42.3	52.7	47.5	15.4	16.4	15.9
S_0 Mean		65.6	64.8	65.2	41.7	49.5	45.6 A	14.8	14.9	14.8
S_{10}	P_0	60.7	56.9	58.8 e	41.3	38.2	39.8	14.1	14.5	14.3
	P_{30}	60.8	62.4	61.6 de	40.3	42.6	41.4	14.7	15.4	15.1
	P_{60}	65.1	66.5	65.8 a-d	43.1	42.7	42.9	15.2	15.7	15.4
	P_{90}	66.3	68.53	67.4 abc	40.4	44.7	42.5	17.7	16.3	17.0
S_{10} Mean		63.2	63.6	63.4	41.3	42.1	41.7 B	15.5	15.5	15.5
S_{20}	P_0	61.7	64.5	63.1 cde	39.9	36.1	38.1	14.2	14.2	14.2
	P_{30}	62.6	60.1	61.4 de	39.1	40.0	39.6	15.9	15.8	15.8
	P_{60}	61.7	65.7	63.7 cde	43.1	43.2	43.1	15.2	15.2	15.2
	P_{90}	62.6	60.1	61.4 de	40.9	42.4	41.7	16.2	16.3	16.3
S_{20} Mean		62.2	62.6	62.4	40.7	40.4	40.6 B	15.4	15.4	15.4
S_{30}	P_0	64.4	67.1	65.8 a-d	35.2	35.5	35.4	14.2	14.5	14.4
	P_{30}	64.1	64.3	64.2 b-d	43.4	40.3	41.8	15.3	15.5	15.4
	P_{60}	61.3	62.5	61.9 de	46.3	48.3	47.3	15.9	16.1	16.0
	P_{90}	66.4	66.1	66.3 a-d	41.8	44.6	43.2	15.2	15.6	15.4
S_{30} Mean		64.1	65.0	64.5	41.7	42.2	41.9 B	15.2	15.4	15.3
Phosphorus mean										
P_0		61.7	61.6	61.6 b	38.2	39.0	38.6 b	14.3	14.2	14.2 c
P_{30}		62.8	62.1	62.5 b	41.8	43.3	42.6 a	15.1	15.3	15.2 b
P_{60}		64.5	65.9	65.2 a	43.9	45.8	44.9 a	15.3	15.6	15.4 b
P_{90}		66.1	66.5	66.3 a	41.3	46.1	43.7 a	16.1	16.2	16.1 a
Means		63.8	64.0		41.3	43.5		15.2	15.3	
CV (%)		7.2			10.8			6.5		
Year (Y)		ns			*			ns		
Sulfur (S)		ns			**			ns		
Phosphorus (P)		**			**			**		
SxP		**			ns			ns		
SxPxY		ns			ns			ns		

S_0= Control, S_{10}= 10 kg ha^{-1} sulfur, S_{20}= 20 kg ha^{-1} sulfur, S_3= 30 kg ha^{-1} sulfur, P_0= Control, P_{30}= 30 kg ha^{-1} phosphorus, P_{60}= 60 kg ha^{-1} phosphorus, P_{90}= 90 kg ha^{-1} phosphorus, [1]: The difference between the means indicated by the same letter in the same column and group is not significant, CV: Coefficient of variation, ns: Not significant, *: $p<0.05$, **: $p<0.01$

First pod height

In the fenugreek plant, S and P doses had a statistically significant effect on the first pod height at the $p<0.01$ level. The highest first pod height was measured in plants in parcels not treated with S, and no statistically significant difference was found between the other S doses. In other words, increased S doses did not affect the first pod height (*Table 3*). Tunçtürk et al. (2011) reported that the highest first pod height was obtained in the dose of 40 kg S ha^{-1} and that contrary to the results of our study, the first pod height increased with increasing S doses. It is thought that seeding time and genotype differences may be effective in this difference in the literature. As a matter of fact, Babagil (2010) stated that the first pod height is a property that is significantly affected by genotype and environmental factors. When the results of phosphorus doses were examined, as the mean of years and S doses, the lowest first pod height (38.6 cm) was

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measured in parcels not treated with phosphorus fertilizer, while the P_{30}, P_{60}, and P_{90} doses of phosphorus were statistically in the same group and yielded the highest values (*Table 3*). In the studies conducted on fenugreek, the highest first pod height was determined in 40 kg P ha^{-1} by Khiriya and Singh (2003) and in 30 kg P ha^{-1} doses by Tunçtürk (2011), Nehara et al. (2006) reported that increased phosphorus doses increased yield properties.

Pod length

While the effects of S doses on pod length were statistically insignificant in the fenugreek plant, the effects of P doses were significant at the $p<0.01$ level. The pod length varied between 14.8-15.5 cm according to sulfur doses. The pod length was observed to increase in parallel with the increasing P doses. The highest pod length was measured to be 16.1 cm at P_{90} dose, and the lowest value was measured in the control parcel (14.2 cm) (*Table 3*). Similarly to our results, Khiriya et al. (2001), Khiriya and Singh (2003), Bhunia et al. (2006), and Meena et al. (2012) reported that increased P doses increased the pod length.

Number of pods

The statistical analysis results demonstrated that S and P doses and SxP interaction had significant effects on the number of pods per plant at the $p<0.01$ level. In the study, the number of pods was determined to increase in the fenugreek plant depending on the increase in S and P doses. In both fertilizer applications, the highest values were determined at the highest fertilizer doses (*Table 4*). In the studies conducted by Kumar (2011) on pea and by Nawange et al. (2011) on chickpeas, it was reported that increasing phosphorus and sulfur applications increased the number of pods.

The number of pods per plant was reported to vary between 7.7-8.5 by Tunçtürk (2011), between 30.6-33.0 by Meena et al. (2012), between 46.73-50.72 by Lal et al. (2015), between 6.4-8.6 by Srivastava et al. (2015), and between 11.6-23.1 by Mitoo et al. (2018). The reason for the number of pods per plant obtained in this study to be higher than these values in the literature may be differences in genotype, soil, climate, and cultural practices.

When the SxP interaction was examined, the highest number of pods was obtained from $S_{30}P_{60}$ and $S_{30}P_{90}$ applications, while the lowest number of pods was obtained from the S_0P_0 application (*Table 4*).

Number of seeds per pod

When sulfur applications were examined, it was observed that the number of seeds per pod increased up to S_{20} dose (15.59 seeds) depending on the increase in S doses, and after this dose, it was observed that it decreased statistically significantly. This difference between sulfur doses was statistically significant at the $p<0.01$ level. In terms of phosphorus doses, the number of seeds per pod increased with increasing P doses. Although the highest value was obtained to be 15.85 seeds at P_{90} dose, the difference between P_{90} and P_{60} doses (15.62 seeds) was statistically insignificant. This difference between phosphorus doses was determined to be statistically significant at the $p<0.01$ level. According to the sulfur x phosphorus interaction, the lowest number of seeds per pod was in S_0P_0 interaction (12.40), and the highest values were determined in $S_{20}P_{90}$, $S_{20}P_{30}$, $S_{20}P_{60}$, $S_{30}P_{60}$, $S_{30}P_{90}$, S_0P_{90}, $S_{10}P_{90}$, $S_{10}P_{60}$, and S_0P_{60} interactions. SxP

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interaction was determined to be very important in terms of the number of seeds per pod ($p<0.01$) (*Table 4*). Srivastava et al. (2015) reported that the number of seeds per pod varied between 13.20-16.60 and that the application of phosphorus increased the number of seeds per pod in the fenugreek plant. Mitoo et al. (2018) stated that the number of seeds per pod varied between 11.67-15.07 and increased with increasing phosphorus and sulfur applications.

Table 4. *Means of yield components at different sulfur and phosphorus doses in fenugreek*

S doses	P doses	Number of pods (pods/plant)			Number of seeds in pod (seeds/pod)			Thousand-seed weight (g)		
		2017	**2018**	**Mean[1]**	**2017**	**2018**	**Mean[1]**	**2017**	**2018**	**Mean[1]**
S_0	P_0	37.33	36.30	36.82 g	12.89	11.90	12.40 d	12.24	13.10	12.67
	P_{30}	45.70	42.57	44.13 c-f	14.57	15.07	14.82 bc	13.23	13.20	13.21
	P_{60}	43.70	45.67	44.68 cde	15.46	15.63	15.55 ab	13.07	13.42	13.25
	P_{90}	45.60	49.30	47.45 bc	15.76	16.13	15.95 a	13.52	13.30	13.41
S_0 Mean		43.08	43.68	43.27 B	14.67	14.68	14.68 C	13.02	13.25	13.14 B
S_{10}	P_0	39.33	39.73	39.53 fg	14.07	14.87	14.47 c	12.58	13.54	13.06
	P_{30}	39.93	43.13	41.53 d-g	14.73	14.77	14.75 c	13.73	13.89	13.81
	P_{60}	45.57	45.73	45.65 cd	15.40	15.73	15.57 ab	14.27	13.62	13.95
	P_{90}	44.90	48.30	46.60 bcd	15.70	15.73	15.72 a	13.42	14.12	13.77
S_{10} Mean		42.43	43.97	43.33 B	14.98	15.28	15.13 B	13.50	13.79	13.65 AB
S_{20}	P_0	39.70	38.47	39.08 fg	14.63	14.60	14.62 c	12.64	13.21	12.93
	P_{30}	43.57	45.00	44.28 cde	16.17	15.87	16.02 a	13.38	13.22	13.30
	P_{60}	48.27	46.90	47.58 bc	15.63	15.63	15.63 a	13.93	13.72	13.82
	P_{90}	51.90	50.03	50.97 b	16.00	16.20	16.10 a	14.60	15.91	15.26
S_{20} Mean		45.86	45.10	45.48 B	15.61	15.58	15.59 A	13.64	14.02	13.83 A
S_{30}	P_0	37.93	38.93	38.43 g	14.23	14.57	14.40 c	12.67	13.18	12.93
	P_{30}	39.13	41.93	40.53 efg	14.83	14.80	14.82 bc	12.88	13.32	13.10
	P_{60}	56.33	63.20	59.77 a	15.70	15.73	15.72 a	14.03	14.00	14.02
	P_{90}	56.77	55.97	56.37 a	15.63	15.67	15.65 a	15.24	15.47	15.36
S_{30} Mean		47.54	50.01	48.78 A	15.10	15.19	15.15 B	13.70	13.99	13.84 A
Phosphorus mean										
P_0		38.58	38.61	38.59 c	13.95	13.98	13.97 c	12.54	13.31	12.90 c
P_{30}		42.08	43.16	42.62 b	15.08	15.12	15.10 b	13.83	14.20	13.36 bc
P_{60}		48.47	50.38	49.42 a	15.55	15.68	15.62 a	13.26	13.41	13.76 b
P_{90}		49.80	50.90	50.34 a	15.78	15.93	15.85 a	13.69	14.70	14.45 a
Means		44.73	45.76		15.09	15.18		13.47	13.76	
CV (%)			8.9			4.6			6.6	
Year (Y)			ns			ns			ns	
Sulfur (S)			**			**			*	
Phosphorus (P)			**			**			**	
SxP			**			**			ns	
SxPxY			ns			ns			ns	

S_0= Control, S_{10}= 10 kg ha^{-1} sulfur, S_{20}= 20 kg ha^{-1} sulfur, S_{30}= 30 kg ha^{-1} sulfur, P_0= Control, P_{30}= 30 kg ha^{-1} phosphorus, P_{60}= 60 kg ha^{-1} phosphorus, P_{90}= 90 kg ha^{-1} phosphorus, [1]: The difference between the means indicated by the same letter in the same column and group is not significant, CV: Coefficient of variation, ns: Not significant, *: $p<0.05$, **: $p<0.01$

Thousand-seed weight

The results of the statistical analysis showed that S doses had a significant effect on the thousand-seed weight at the $p<0.05$ level and P doses at the $p<0.01$ level. When *Table 4* was examined, the highest thousand-seed weight among S doses was

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determined at S_{20} (13.83 g) and S_{30} (13.84 g) doses. However, no statistically significant difference was determined between them and S_{10} (13.65 g) S dose (*Table 4*). Ramkishor and Kumawat (2015) stated that different doses of S applied to the fenugreek plant had significant effects on the thousand-seed weight, and that the application of 40 and 60 kg S per hectare increased the thousand-seed weight compared to control subjects. Srivastava et al. (2015) obtained the highest thousand-seed weight to be 13.75 and 13.76 g at the 40 and 80 kg ha^{-1} P doses, respectively. Tunçtürk et al. (2011) stated that the thousand-seed weight was significantly affected by S application in the first year and that the highest thousand-seed weight was obtained to be 18.8 g at dose of 40 kg ha^{-1}. The increased P doses increased the thousand-seed weight in the fenugreek plant. While the lowest thousand-seed weight was determined at P_0 dose (12.90 g), the highest thousand-seed weight was determined at P_{90} dose (14.45 g) (*Table 4*). Tunçtürk (2011) reported that P doses increased the thousand-seed weight, and the highest thousand-seed weight was obtained from 60 and 90 kg ha^{-1} P (17.8 g and 18.0 g) applications. The highest thousand-seed weight was determined by Sammuria and Yadav (2008) from 40 and 60 kg ha^{-1} P application (11.62 g and 11.74 g, respectively), and by Mitoo et al. (2018) to be 9.98 g from 40 kg ha^{-1} P application, which was the highest dose.

Seed yield

The variance analysis results showed that S and P doses and SxP interaction had significant effects on seed yield at the $p<0.01$ level (*Table 5*). When *Table 5* was examined, it was determined that the highest seed yield was obtained to be 2224 kg ha^{-1} from the $S_{30}P_{90}$ application, and the lowest seed yield was obtained to be 1038 kg ha^{-1} from the S_0P_0 application. As P and S doses increased, seed yield was determined to increase in fenugreek. In both fertilizer applications, the highest seed yields were determined at the highest fertilizer doses. As the mean of years and P doses, the highest fenugreek seed yield with the sulfur application was obtained to be 1822 kg/ha (S_{30}), and as the mean of years and S doses, the highest fenugreek seed yield in phosphorus fertilizer applications was obtained to be 1810 kg ha^{-1} (P_{90}) (*Table 5*). Many researchers stated that the phosphorus element is necessary for energy transfer in plants and that the yield, and above ground and root development of the plant are adversely affected in P deficiency or excess (Kacar and Katkat, 2007; Singh and Singh, 2016). Tunçtürk et al. (2011), Lal et al. (2015), Ramkishor and Kumawat (2015), Singh Manohar et al. (2017), and Verma et al. (2017) reported that increasing sulfur doses increase seed yield, and Halesh et al. (2000), Tunçtürk (2011), and Meena et al. (2012) reported that increasing phosphorus doses increase seed yield in the fenugreek plant.

Protein content

In the study, the effects of S and P doses on the protein content of fenugreek seed were found to be statistically significant ($p<0.01$). In terms of sulfur doses, as the mean of years and P doses, the highest protein ratio was determined at S_{30} (26.0%) dose and the lowest at S_0 (25.3%) dose. In terms of phosphorus doses, increasing P doses increased protein content in fenugreek, but statistically, P doses, except for P_0, were in the same group (*Table 5*). Baldaneeya Nitesh (2018) and Tunçtürk et al. (2011) stated that they determined the highest protein content at a dose of 40 kg ha^{-1} S. Mehta et al. (2012) reported that although increasing P doses increased protein content, there was no

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difference between 20 and 40 kg ha^{-1} P doses. Tunçtürk (2011) also reported similar results.

Table 5. *Means of yield, protein and trigonelline ratio at different sulfur and phosphorus doses in fenugreek*

S doses	P doses	Seed yield (kg ha^{-1})			Protein ratio (%)			Trigonelline ratio (%)		
		2017	2018	Mean*	2017	2018	Mean*	2017	2018	Mean*
S_0	P_0	1005	1071	1038 ı	24.6	25.1	24.9	0.78	0.83	0.81 ı
	P_{30}	1121	1241	1182 h	24.9	25.5	25.2	0.88	0.91	0.90 hı
	P_{60}	1323	1432	1378 fg	25.2	25.6	25.4	0.91	0.94	0.93 gh
	P_{90}	1520	1527	1523 de	25.5	25.8	25.6	0.93	0.95	0.94 efg
S_0 Mean		1242	1318	1280 D	25.1	25.5	25.3 C	0.89	0.88	0.89 D
S_{10}	P_0	1199	1246	1223 h	25.6	24.9	25.3	0.84	0.89	0.87 h
	P_{30}	1270	1327	1299 gh	25.8	25.2	25.5	0.87	0.89	0.88 h
	P_{60}	1462	1416	1439 ef	25.7	25.5	25.6	0.92	0.94	0.93 gh
	P_{90}	1501	1559	1530 de	25.8	25.7	25.8	0.94	0.96	0.95 ef
S_{10} Mean		1358	1387	1373 C	25.7	25.4	25.5 B	0.91	0.89	0.90 C
S_{20}	P_0	1234	1283	1259 gh	25.7	25.3	25.5	0.98	0.99	0.99 e
	P_{30}	1329	1374	1351 fg	25.9	25.6	25.8	1.09	1.12	1.11 c
	P_{60}	1661	1584	1622 cd	26.0	25.8	25.9	1.10	1.14	1.12 c
	P_{90}	1902	2027	1964 b	25.7	25.6	25.9	1.19	1.22	1.21 ab
S_{20} Mean		1531	1567	1549 B	25.8	25.7	25.8 B	1.09	1.12	1.11 B
S_{30}	P_0	1428	1430	1429 ef	25.7	26.1	25.9	1.04	1.06	1.05 d
	P_{30}	1692	1687	1690 c	26.0	26.2	26.1	1.16	1.19	1.18 b
	P_{60}	1898	1989	1944 b	26.0	26.4	26.2	1.20	1.23	1.22 ab
	P_{90}	2043	2405	2224 a	25.3	26.5	25.9	1.25	1.26	1.26 a
S_{30} Mean		1765	1879	1822 A	25.8	26.3	26.0 A	1.17	1.19	1.18 A
Phosphorus mean										
P_0		1217	1258	1237 d	25.4	25.4	25.4 b	0.91	0.94	0.93 c
P_{30}		1353	1408	1380 c	25.7	25.6	25.6 a	1.00	1.03	1.02 b
P_{60}		1586	1605	1596 b	25.8	25.8	25.8 a	1.03	1.06	1.05 b
P_{90}		1741	1879	1810 a	25.6	26.0	25.8 a	1.08	1.10	1.09 a
Means		1474	1538		25.6	25.7		1.02	1.04	
CV (%)			7.0			1.6			4.1	
Year (Y)			**			ns			ns	
Sulfur (S)			**			**			**	
Phosphorus (P)			**			**			**	
SxP			**			ns			**	
SxPxY			ns			ns			ns	

S_0= Control, S_{10}= 10 kg ha^{-1} sulfur, S_{20}= 20 kg ha^{-1} sulfur, S_3= 30 kg ha^{-1} sulfur, P_0= Control, P_{30}= 30 kg ha^{-1} phosphorus, P_{60}= 60 kg ha^{-1} phosphorus, P_{90}= 90 kg ha^{-1} phosphorus, *: The difference between the means indicated by the same letter in the same column and group is not significant, CV: Coefficient of variation, ns: Not significant, **: $p<0.01$

Trigonelline content

The effects of S and P doses and SxP interaction on the trigonelline content of fenugreek seeds were found to be significant at the $p<0.01$ level (*Table 5*). When *Table 5* was examined, the highest trigonelline content was determined in $S_{30}P_{90}$ (1.26%), but there was no statistically significant difference between $S_{30}P_{60}$ (1.22%) and $S_{20}P_{90}$ (1.21%). On the other hand, as both S and P doses increased, trigonelline content was observed to increase (*Table 5*). Kan et al. (2007) stated that applied different phosphorous fertilizer sources did not change the trigonelline content, that trigonelline

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content varied between 0.86-1.26%, and that ecological factors might be effective on the trigonelline content. Dar et al. (2015) reported that a 40 kg P ha^{-1} application increased the trigonelline content. Mutlu (2011) reported in the study conducted in fenugreek of different origins that the trigonelline content varied between 0.66-1.40%. The trigonelline content obtained in this study was higher than the values reported by Akgül (1993) (0.36%), Mehrafarin et al. (2010) (0.20-0.36%), and Mathur and Yadav (2011) (0.27%).

Relationships between the ınvestigated properties

The simple correlation coefficients showing linear relationships between the seed yield per plant and the investigated properties were presented in *Table 6*. The correlation analysis revealed that the seed yield was positively and significantly correlated with the number of pods per plant (r= 0.703**), pod length (r= 0.334**), the number of seeds per pod (r= 0.509**), and thousand-seed weight (r= 0.571**). Increases that occurred in these properties caused significant increases in the plant seed yield. The highest correlation coefficients in terms of seed yield were determined in the relationships between the number of pods per plant, the number of seeds per pod, and thousand-seed weight. Parchin et al. (2019) stated that there was a positive relationship between the number of pods per plant and seed yield.

Table 6. *Correlation coefficients related to pairwise relations between the seed yield and other properties*

	2	**3**	**4**	**5**	**6**	**7**
1. Plant height	0.274**	0.094	0.218*	0.243*	0.104	0.154
2. First pod height	-	0.280**	0.080	0.068	0.110	0.078
3. Number of pods per $plant^{-1}$		-	0.391**	0.478**	0.436**	0.703**
4. Pod length			-	0.343**	0.321**	0.334**
5. Number of seeds per pod^{-1}				-	0.299**	0.509**
6. Thousand-seed weight					-	0.571**
7. Seed yield						-

*: Significant at the p<0.05 level, **: Significant at the p<0.01 level

A positive and significant relationship was determined between plant height and the first pod height. Positive and significant relationships were detected between the number of pods per plant and pod length and between the number of seeds per pod and thousand-seed weight. Positive and significant relationships were found between the thousand-seed weight and the number of pods per plant and between pod length and the number of seeds per pod. The number of pods per plant was stated to be the most important factor affecting seed yield in many studies (Şehirali, 1980; Pooran-Chand, 1999; Amini et al., 2002; Kumar Singh et al., 2019, Singh et al., 2019), and finding the number of pods per plant as the most leading factor in this study supports the other literature on this subject.

Conclusions

This study, which was carried out under the ecological conditions of Siirt province located in the Southeastern Anatolia Region of Turkey, which has a semi-arid climate, revealed the importance of sulfur and phosphorus fertilizers when an evaluation was

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made in terms of yield and some quality criteria. Increased sulfur and phosphorus doses positively affected all of the investigated properties. When an evaluation was made in terms of yield and yield components, 30 kg S and 90 kg P doses could be stated to be suitable for per hectare. However, it was revealed that studies involving further increasing doses of both elements should be performed due to the linear increase determined in yield and quality properties in parallel to the increasing sulfur and phosphorus doses.

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YIELD AND QUALITY OF BLACK CUMIN (*Nigella sativa* L.) ACCORDING TO LEONARDITE AND NITROGEN DOSES

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Abstract. This study aimed to reveal the effect of different levels of leonardite and nitrogen fertilizer applied to the soil on seed yield and some quality parameters of black cumin (*Nigella sativa* L.). The study was carried out in the Southeastern Anatolia Region of Turkey between the years 2016-2018. In the study, 4 different doses of leonardite and nitrogen fertilizer were administered. In the study, the highest plant height and number of branches were found in L_2N_{90}, the highest number of capsules was found in L_2N_{60}, and the highest number of seed per capsule was found in L_2N_{60} and L_3N_{60}. As a result of the study, the highest seed yield was obtained from L_2 and L_3 doses of leonardite and from N_{60} dose of nitrogen. It was determined that the fixed oil ratio, essential oil ratio contained in the black cumin seeds, and essential oil components parallel with the increase in leonardite and nitrogen doses. It was concluded that leonardite, a soil conditioner, could be used in black cumin cultivation under semi-arid climate conditions and that 2000 kg ha^{-1} leonardite and 60 kg ha^{-1} nitrogen could be applied to improve the yield and quality of black cumin.
Keywords: *soil conditioner, thymoquinone, seed yield, Nigella sativa, essential oil, fixed oil*

Introduction

The blackcumin is a annual herbaceous plant from the Ranunculaceae family. It is distributed over a wide area from southern and eastern shores of Indian continent to mediterranan basin (Egypt and Turkey) (Seyyedi et al., 2015; Kılıç and Arabacı, 2016). It has 12 species distributed in Turkey. *Nigella sativa* and *Nigella damascena* species are commonly cultivated (Baydar, 2013). One of the most famous books in the history of medicine by İbn-i Sina, the author of "El Kanun Fi‟ ttıb", states that black cumin stimulates metabolism, prevents drowsiness and fatigue, regulates body energy and restores vitality lost by disease (Nasr, 2008). The seeds of black cumin have high economic value and contain fixed oil, essential oil, protein and carbohydrates. It also contains nigellidin, nigellisin and nigellimin in alkaloids in seeds (Baydar, 2013).

Black cumin is an important spice species and have high production especially in East and Southeast Asia. There is no specific report of production area and amount for black cumin, but for spice production India, China and Turkey leads world production (Dessie et al., 2020).

Humic acids, which are among the most important components of humic substances, are heterogeneous natural resources with high molecular weight and colors varying from yellow to black (Akıncı, 2011), and they have significant benefits in terms of both improving soil properties and agricultural production such as increasing the cation exchange capacity of the soils and neutralizing the pH of the soil (Stevenson, 1994), making plant nutrients bound to soil colloids available (Yılmaz, 2007), slowing down the evaporation of water in the soil (Akıncı, 2011; Sesveren and Taş, 2018), improving soil microflora (Larcher, 2003; Calvo et al., 2014; Li et al., 2019), improving plant growth, yield, and quality (Sharif et al., 2002; Esringü et al., 2015; Selladurai and Purakayastha, 2016; Çöl and Akınerdem, 2017; Ahmad et al., 2018), and increasing the

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uptake of mineral elements in plants (Mackowiak et al., 2001; Eyheraguibel et al., 2008; Khaled and Fawy, 2011). Leonardite constitutes the raw material of humic acids that are classified as soil conditioners (Pekcan et al., 2018). Leonardite, which is a sedimentary rock formed by the changes of plant and animal remains during a period of millions of years (Pekcan et al., 2018), is an all-natural organic material that has not reached the level of coal (Sesveren and Taş, 2018). The use of materials such as leonardite to increase the organic matter content of the soil, especially in agricultural soils containing low organic matter with an intense polyculture production pattern, takes an important place nowadays.

It was reported that humic acid applications in *Brassica napus* ssp. *oleifera* L. plant had positive effects on plant height, the number of sub-branches attached to the main stem, the number of capsules in the main stem, the number of seeds per capsule, thousand grain weight, seed yield, oil ratio, and oil yield properties (Gürsoy and Kolsarıcı, 2017), that better tuber yields were obtained with humic acid and fulvic acid applications in the potato plant especially in dry and cool periods (Wadas and Dziugieł, 2019), and that potassium fertilizer and humic acid doses applied in increasing amounts in *Helianthus annuus* L. plant had significant effects on the yield and yield components and mineral content of the plant (Yağmur and Okur, 2017). Similarly, it was determined that the tuber yield in potato (Şanlı et al., 2013), wheat grain yield (Kolay et al., 2016), rye dry matter yield (Adiloğlu et al., 2017) increased in parallel with the increase in leonardite doses.

Obtaining high yields and quality products in agricultural production depends on giving the nutrients needed by the plant to plants in the appropriate period and dose, along with the accurate and timely implementation of many other cultural practices. In this sense, nitrogen (N) is the primary element among nutrients that are absolutely essential for many cultivated plants. The unconscious and excessive use of fertilizers, which are the most important inputs of agricultural activities, is shown to be one of the most important causes of soil pollution nowadays, and high doses of fertilizer applications also lead to toxic accumulation in plants, the inhibition of the intake of some other nutrients, and of course, economic losses. Therefore, the determination of nutrient needs of cultivated plants in different ecologies and the preparation of fertilization programs according to the soil analysis results constitute the most important branches of sustainable agriculture techniques.

Moreover, it is necessary to carry out cultural application techniques that minimize agricultural inputs such as fertilizer. For this purpose, it is essential to use soil conditioners such as leonardite in plant production, especially in agricultural soils where soil organic matter is low and, consequently, the intake of nutrients is prevented, and to reveal the interaction with other nutrients, and to show its effects on product yield and quality. In this study, it was aimed to reveal the effect of leonardite and nitrogen fertilizer applied to the soil at different levels on seed yield and some quality parameters of black cumin.

Materials and methods

The study was carried out between 2016-2018 under the ecological conditions of Siirt province located in the Southeastern Anatolia Region of Turkey, which has a semi-arid climate. Siirt is located at 37° 58' 7.37" N and 41° 51' 3.87" E coordinates with 894 m altitude (*Figure 1, Figure 2*).

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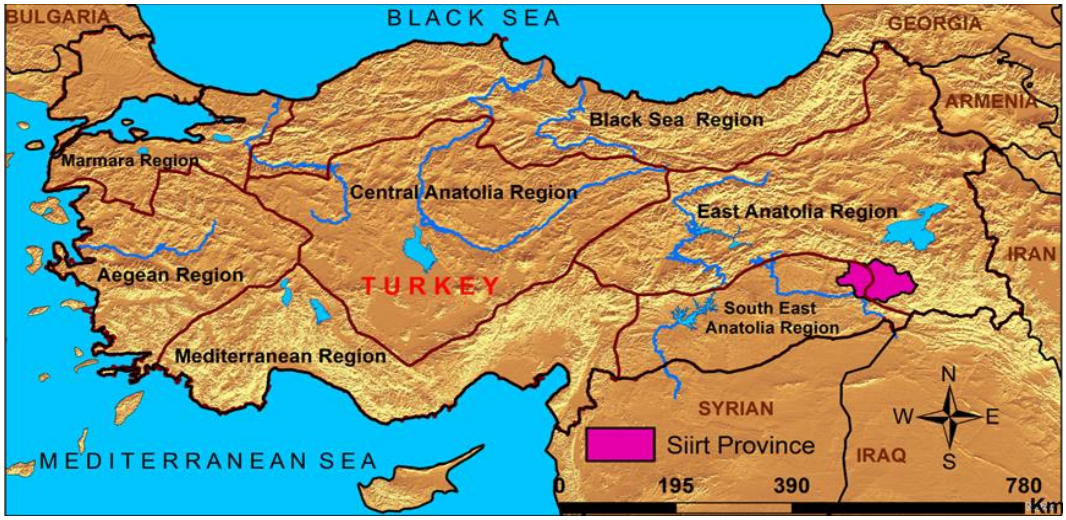

Figure 1. *Location of the study area in Turkey*

Figure 2. *A photo of the research area*

In the second year of the study, less precipitation (522.8 mm) occurred in comparison with the first year (574.2 mm), while the long-term annual precipitation was 634.1 mm. While the highest precipitation occurred in March and April during the 2016-2017 vegetation period, the highest precipitation was recorded in May during the 2017-2018 vegetation period (*Table 1*). The mean temperature during the study years and the long-term mean temperature (38 years) were 11.2 ºC, 13.8 ºC, and 11.4 ºC, respectively (*Table 1*) (Anonymous, 2018).

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Table 1. *Climate characteristics of trial area*

Climate parameters	Research years	Months								
		Nov	Dec	Jan	Feb	Mar	Apr	May	June	Mean/Sum
Average temparature (°C)	2016-2017	10.4	3.3	3.0	2.7	9.6	14.0	19.5	26.9	11.2
	2017-2018	11.2	8.0	5.7	8.2	13.7	16.8	19.8	27.4	13.8
	Long term*	10.3	4.9	3.0	4.5	8.8	14.3	19.5	26.2	11.4
Monthly precipitation (mm)	2016-2017	55.4	116.6	46.4	29.2	119.2	132.8	74.6	0.0	574.2
	2017-2018	86.0	47.4	56.4	74.2	47.6	61.6	139.6	10.0	522.8
	Long term*	85.1	91.1	82.2	96.6	108.7	96.3	64.3	9.8	634.1

*: 1980-2018

In the study, some physical and chemical analysis results of the soils taken before establishing the field trial were presented in *Table 2*. In the first year (2016-2017), the trial area soils were loamy textured, and they were clay-loam textured in the second year (2017-2018); both trial area soils were slightly alkaline, salt-free, their lime content was "medium calcareous," the organic matter content was "low," and the available potassium (K) content was "sufficient." The available P content of the soils in the first year was determined to be "very little," and the available P content of the soils in the second year was determined to be "low" (*Table 2*).

Table 2. *Some physical and chemical properties of the study area soils (0-20 cm)**

Properties	Value	
	2016-2017	2017-2018
Clay, %	47.56	34.16
Silt, %	12.11	26.00
Sand, %	40.33	39.84
pH	7.72	7.53
Electrical conductivity (EC), mS cm^{-1}	0.363	0.150
Lime ($CaCO_3$), %	12.0	8.2
Organic matter, %	1.31	1.78
Available phosphorus, kg P_2O_5 ha^{-1}	24	49
Available potassium, kg K_2O ha^{-1}	1430	1250

*: Analyses were carried out in Siirt University, Science and Technology Application and Research Center Laboratory

As the plant material in the study, black cumin (*N. sativa* L.) seeds, belonging to the "Isparta population" and seeded locally in the Mediterranean Region of Turkey, were used.

In this study, the field trial was established as three replications in randomized blocks according to the factorial trial design. In the study, 4 different leonardite doses (L_0= 0, L_1= 1000, L_2= 2000, and L_3= 3000 kg ha^{-1} leonardite) and 4 different nitrogen doses (N_0= 0, N_{30}= 30, N_{60}= 60, and N_{90}= 90 kg N ha^{-1}) constituted the subject of the study. “Leonagro” was used as the source of leonardite, and urea (46% N) was used as the source of nitrogen fertilizer. The content of the leonardite material is given in *Table 3*. According to the research subjects, both fertilizer forms were mixed by applying them to the soil before seeding.

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Table 3. *Some properties of leonardite using the trial*

pH	Humic + Fulvic acid (%)	Organic Matter (%)
6-8	40	40

The seeding process was performed manually on 15 November 2016 in the first year and on 17 November 2017 in the second year on the grooves opened with the help of a marker. In the study, row distance was 30 cm and parcel distance was 100 cm. Each parcel constituted from four rows, while length and width of the parcels were 3 meter by 1.2 meter each with a total area of 3.6 m^2 per parcel. Sowing norm was 30 kg ha^{-1}, and sowing was done manually in rows opened with the help of a marker.Weed control was performed mechanically by hand several times in both years. At the harvest, two border rows and 50 cm from each side were excluded to eliminate border effects.

Plant height, number of branches, number of capsules, number of seed per capsule, seed weight were determined in 10 plants randomly selected in each parcel before harvest. The harvest was carried in the entire plot area, excluding borders in the first week of July in both years. The harvested plants were dried in the shade for 3-4 days, and seed yields were calculated per decare. Thousand seed weight, fixed oil and essential oil analysis were performed. For the fixed oil percentage grinded samples were dried at 105 ºC for 3 hours and 10 g sample was fed into a Soxhlet apparatus (SOXTHERM® 2000). The extraction was performed on a water bath at 60 ºC for 4 h with 200 mL petroleum ether. For the essential analysis percentage; seeds dried at 35 ºC in room were crushed bu using grinder. Distillation process was carried out using the Clevenger apparatus. Distilled water (250 ml) was used, and 25 g crushed fruit were watered with 250 ml distilled water (1:10). Distillation lasted for approximately 5 h at boiling point. The essential oil collected at the end of distillation was measured in mL and calculated as % (v/w). The essential oil components of black cumin seed were determined using Headspace GC-MS. Crushed black seed samples was taken 1 g and placed in a 25 mL Chromacol Headspace vial. The vial was heated in a Triplus RSH Headspace oven for 90 minutes at 120 °C. The heated Headspace was sent from the vial to the GC-MS with an injection volume of 2.5 mL. The analysis was carried out in a Trace 1310 gas chromatograph equipped with an ISQ single quadrupole mass spectrometer (Thermo Fisher Scicentific, Austin, TX). The procedure was set to an initial temperament 60 °C for 10 min, then ramp at 1 °C/min to 140 °C, 1 min in 140 °C, then ramp at 15 °C/min to 230 °C and finally 5 min in 230 °C. The ion source and detector temperature was 220 °C and 220 °C, respectively. Separation of sample was performed on a Thermo TG-WAXMS GC column (60 m x 0.25 mm ID x 0.25 µm) using helium as carrier gas at 1.2 ml/min. Mass spectral scan range was set at the rate of 55–550 (amu). Peak identification was conducted by comparison of the known components stored in the NIST Demo, Wiley7, Wiley9, redlip, mainlip, WinRI.

The data were analyzed by JMP statistical software. A homogeneity test was applied to the data obtained from the study. According to the results of the homogeneity test, they were subjected to combined variance analysis (ANOVA) according to the factorial trial design in randomized blocks. According to the F-test results, differences between the groups were determined by the LSD multiple comparison test (Yurtsever, 1984).

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Results and discussion

Plant height

According to the results of a two-year study, the effects of leonardite and nitrogen fertilizer doses on the plant height of black cumin were found to be statistically significant at a level of $p<0.01$ (*Table 4*).

Table 4. *Means of yield components at different leonardite and nitrogen doses in black cumin*

L doses	N doses	Plant height (cm)			Number of branches (unit/plant)			Number of capsules (unit/plant)		
		2017	2018	Mean*	2017	2018	Mean*	2017	2018	Mean*
L_0	N_0	49.87	50.87	50.37 g	3.47	3.60	3.53 g	4.63	4.77	4.70 h
	N_{30}	53.27	56.20	54.73 efg	3.60	3.70	3.65 d-g	5.47	6.07	5.77 cd
	N_{60}	61.70	64.20	62.95 bc	3.80	3.83	3.82 ab	6.57	7.13	6.85 a
	N_{90}	59.23	65.97	62.60 bc	3.80	3.83	3.82 ab	5.07	5.57	5.32 d-g
L_0 Mean		56.02	59.31	57.66 C	3.67	3.74	3.70	5.43	5.88	5.66
L_1	N_0	51.50	54.09	52.80 g	3.63	3.73	3.68 b-f	4.93	5.27	5.10 fgh
	N_{30}	61.13	62.97	62.05 bc	3.57	3.63	3.60 fg	4.97	5.30	5.13 e-h
	N_{60}	62.80	61.25	62.02 bc	3.80	3.80	3.80 bc	6.27	5.67	5.97 bc
	N_{90}	58.53	58.80	58.67 cde	3.70	3.90	3.80 bc	5.33	6.07	5.70 cde
L_1 Mean		58.49	59.28	58.88 BC	3.68	3.77	3.72	5.38	5.58	5.48
L_2	N_0	52.73	55.71	54.22 fg	3.70	3.70	3.70 b-f	4.87	5.00	4.93 gh
	N_{30}	51.13	56.89	54.02 fg	3.47	3.60	3.53 g	4.70	4.80	4.75 gh
	N_{60}	64.46	62.31	63.39 b	3.53	3.70	3.62 efg	7.40	6.43	6.92 a
	N_{90}	65.30	71.47	68.38 a	3.97	3.93	3.95 a	5.40	5.90	5.65 c-f
L_2 Mean		58.41	61.60	60.00 AB	3.67	3.73	3.70	5.59	5.53	5.56
L_3	N_0	56.03	59.13	57.58 def	3.77	3.80	3.78 bcd	4.92	4.95	4.94 gh
	N_{30}	61.30	62.56	61.93 bcd	3.70	3.80	3.75 b-e	5.60	5.63	5.62 c-f
	N_{60}	58.40	63.87	61.13 bcd	3.70	3.80	3.75 b-e	6.48	6.53	6.51 ab
	N_{90}	62.50	66.97	64.74 ab	3.67	3.67	3.67 c-g	5.83	5.88	5.86 cd
L_3 Mean		59.56	63.13	61.35 A	3.71	3.77	3.74	5.71	5.75	5.73
N Mean										
N_0		52.53	54.95	53.74 c	3.64	3.71	3.68 b	4.84	4.99	4.92 d
N_{30}		56.71	59.66	58.18 b	3.58	3.68	3.63 b	5.19	5.45	5.32 c
N_{60}		61.84	62.91	62.37 a	3.71	3.78	3.75 a	6.68	6.44	6.56 a
N_{90}		61.39	65.80	63.60 a	3.78	3.83	3.81 a	5.41	5.86	5.64 b
Means		58.12 B	60.82 A		3.68 B	3.75 A		5.53	5.69	
CV (%)		6.40			3.23			8.99		
Year (Y)		**			**			ns		
Leonardite (L)		**			ns			ns		
Nitrogen (N)		**			**			**		
LxN		**			**			**		
LxNxY		ns			ns			ns		

L_0= Control, L_1= 1000 kg ha^{-1} leonardite, L_2= 2000 kg ha^{-1} leonardite, L_3= 3000 kg ha^{-1} leonardite, N_0= Control, N_{30}= 30 kg ha^{-1} nitrogen, N_{60}= 60 kg ha^{-1} nitrogen, N_{90}= 90 kg ha^{-1} nitrogen, *: The difference between the means indicated by the same letter in the same column and group is not significant, CV: Coefficient of variation, ns: Not significant, **: $p<0.01$

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The plant height values increased in parallel with the increase in leonardite and nitrogen doses, and the highest plant height was measured at the highest doses of both leonardite and nitrogen. In the study, when the interaction of organic material and nitrogen doses was evaluated together, according to two-year averages, the highest plant height value was obtained in L_2N_{90} (68.38 cm) subject, and the lowest values were found in L_0N_0 (50.37 cm) subject. LxN interaction was found to be statistically significant at a level of $p<0.01$ (*Table 4*).

Nitrogen is an essential plant nutrient that promotes vegetative growth in plants. Therefore, nitrogen fertilizer applications affect plant height. In their studies carried out with black cumin, Shah (2004), Özgüven and Şekeroğlu (2007), Tuncturk et al. (2012), Yimam et al. (2015), Muhammad et al. (2017), Kızılyıldırım (2019), and Sultana et al. (2019) reported that plant height values increased in parallel with the increase in nitrogen fertilizer doses and/or plant height was positively affected by nitrogen dose applications, similarly to the results of our study.

It has also been reported in the results of many studies that organic material applications such as humic acid and leonardite, which is a source of humic acid, improve the soil properties, and therefore, plant height is positively affected in plants (Laz, 2011; Demirkıran et al., 2012; Betül et al., 2016; Eleroğlu and Korkmaz, 2016; Çöl and Akınerdem, 2017; Yağmur and Okur, 2017).

In the study, the difference between the years was also found to be significant, and the highest plant height values were determined in the second year of the study (*Table 4*). These differences in plant height values between the years can be explained by the difference in precipitation regimes over the years.

Number of branches

With respect to the number of branches, the effects of N doses alone were found to be statistically significant ($p<0.01$), while the effects of leonardite doses were found to be insignificant. As an average of leonardite doses, the highest number of branches was statistically found in N_{60} (3.75 unit plant^{-1}) and N_{90} (3.81 unit plant^{-1}) nitrogen doses that constituted the first group, and the lowest values were found in other doses of nitrogen. In the study, when LxN interaction was examined, while the highest value for the number of branches was found in L_2N_{90} (3.95 unit plant^{-1}) subject, the lowest value was found in L_0N_0 (3.53 unit plant^{-1}) subject (*Table 4*).

According to the results of the two-year study carried out with black cumin by Tuncturk et al. (2012), it was reported that nitrogen fertilizer doses increased the number of branches and that the difference between 40-80 kg nitrogen applications per hectare in terms of the number of branches was insignificant. While Özgüven and Şekeroğlu (2007) reported that the highest number of branches was obtained from 90 kg N ha^{-1} application dose among nitrogen fertilizer dose applications, Kızılyıldırım (2019) and Sultana et al. (2019) reported that it was obtained from 100 kg N ha^{-1} and 60 kg N ha^{-1} application doses, respectively. These results in the literature were found to be relatively compatible with the results of our study.

Number of capsules

With respect to the number of capsules per plant, while the effects of N doses alone were found to be statistically significant ($p<0.01$), the effects of leonardite doses were insignificant. As an average of leonardite doses, the highest number of capsules was found in N_{60} (6.56 unit plant^{-1}), and the lowest number of capsules was found in the

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control subject in which nitrogen fertilization was not applied (N_0) with 4.92 unit plant^{-1}. In the study, when LxN interaction was examined, the highest values for the number of capsules were statistically found in L_0N_{60} (6.85 unit plant^{-1}) and L_2N_{60} (6.92 unit plant^{-1}) subjects that constituted the first group. The lowest number of capsules per plant was found in L_0N_0 subject with 4.70 units. In the study, LxN interaction was found to be statistically very significant ($p<0.01$) (*Table 4*).

The number of branches per plant affects the number of capsules obtained per plant (Tuncturk et al., 2012). Indeed, in our study, nitrogen doses increased the number of branches, and consequently, more capsules were produced by plants. In their study, Tuncturk et al. (2012) investigated the effects of different nitrogen doses (0, 20, 40, 60, and 80 kg N ha^{-1}) in black cumin and reported that the number of capsules per plant increased up to the nitrogen dose of 60 kg N ha^{-1}, that the highest number of capsules was achieved with 7.5 capsules at this dose according to the average of two years, and that there was a statistically significant decrease in the number of capsules after 60 kg N ha^{-1} nitrogen dose. These results were in parallel with the results of our study. It was also reported in some other studies (Özgüven and Şekeroğlu, 2007; Rana et al., 2012; Yimam et al., 2015; Kızılyıldırım, 2019) that the number of capsules in black cumin increased depending on the level of nitrogen fertilizer.

Number of seeds per capsule

According to the results of the two-year study, the effects of leonardite and nitrogen fertilizer doses on the number of seeds per capsule in black cumin were found to be statistically significant at a level of $p<0.01$. When the two-year data were considered, the number of seeds per capsule increased in parallel with the increase in leonardite doses, and as an average of nitrogen doses, the highest and lowest values were obtained at L3 dose (92.52 seeds) and L_0 dose (62.32 seeds), respectively (*Table 5*). The positive effect of leonardite on the number of seeds per capsule can be explained by its contribution by improving the soil structure. Namely, humic substances such as leonardite serve as a reserve in terms of elements such as carbon, nitrogen, Sulphur, and phosphorus, therefore, in the study soil where soil organic matter was quite low (*Table 2*), it was considered that leonardite, known as the soil conditioner, made some plant nutrients in the soil available to plants, and consequently, flower and seed formation increased. Gürsoy and Kolsarıcı (2017) reported that humic acid doses in the soil covered with leonardite increased the number of seeds per capsule in the colza plant.

When the effects of nitrogen levels were evaluated, it was determined that the number of seeds per capsule increased up to N_{60} nitrogen dose and decreased statistically significantly after this dose and that the highest value for the number of seeds per capsule, as an average of leonardite doses, was found at N_{60} nitrogen dose with 100.69 seeds. The lowest values in terms of the number of seeds per capsule were found at N_0 dose (53.81 seeds) (*Table 5*). As in the results of our study, Shah (2004) and Mollafilabi et al. (2010) reported in their study on black cumin that there were significant decreases in the number of seeds per capsule after a certain level of nitrogen. In their study carried out with the datura plant, Esendal et al. (2000) reported that the number of seeds per capsule increased in parallel with the increase in nitrogen doses. On the contrary, Tuncturk et al. (2012) and Kızılyıldırım (2019) determined that nitrogen fertilizer levels in black cumin did not affect the number of seeds per capsule and that the number of seeds per capsule varied between 52.4-55.1 and 110.70-126.73,

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respectively, depending on the levels of nitrogen fertilizer applied. Unlike these data in the literature, significant differences between nitrogen doses in terms of the number of seeds per capsule in our study can be explained by the fact that the soil-plant nutrition relationship occurred differently in terms of nitrogen and other plant nutrients depending on the soil and climatic conditions, in addition to different varieties used.

Table 5. *Means of yield components at different leonardite and N doses in black cumin*

L doses	N doses	Number of seeds per capsule (number)			Seed weight capsule (g)			Thousand grain weight (g)		
		2017	2018	Mean[1]	2017	2018	Mean[1]	2017	2018	Mean[1]
L_0	N_0	42.00	43.94	42.97 ı	0.44	0.47	0.46	2.10	2.26	2.18
	N_{30}	47.32	49.31	48.31 h	0.51	0.52	0.51	2.30	2.30	2.30
	N_{60}	80.68	85.07	82.88 d	0.54	0.55	0.54	2.35	2.28	2.32
	N_{90}	73.72	76.49	75.11 e	0.54	0.57	0.56	2.30	2.33	2.32
L_0 Mean		60.93	63.70	62.32 D	0.51	0.53	0.52 B	2.26	2.29	2.28 C
L_1	N_0	48.95	50.69	49.82 h	0.46	0.46	0.46	2.28	2.25	2.27
	N_{30}	54.91	58.00	56.45 g	0.49	0.50	0.49	2.37	2.41	2.39
	N_{60}	91.35	93.54	92.45 c	0.54	0.56	0.55	2.29	2.36	2.33
	N_{90}	72.09	77.65	74.86 e	0.56	0.56	0.56	2.27	2.26	2.26
L_1 Mean		66.82	69.97	68.40 C	0.51	0.52	0.52 B	2.30	2.32	2.31 BC
L_2	N_0	59.52	61.29	60.41 fg	0.50	0.51	0.50	2.34	2.33	2.34
	N_{30}	74.63	75.90	75.27 e	0.49	0.54	0.51	2.35	2.36	2.36
	N_{60}	111.37	114.13	112.75 a	0.54	0.57	0.56	2.34	2.36	2.35
	N_{90}	93.67	94.55	94.11 c	0.55	0.57	0.56	2.36	2.38	2.37
L_2 Mean		84.79	86.47	85.63 B	0.52	0.54	0.53 A	2.35	2.36	2.35 A
L_3	N_0	62.00	62.12	62.06 f	0.51	0.52	0.51	2.23	2.28	2.26
	N_{30}	91.10	91.34	91.27 c	0.53	0.53	0.53	2.27	2.29	2.28
	N_{60}	114.57	114.97	114.77 a	0.53	0.57	0.55	2.38	2.37	2.37
	N_{90}	102.00	102.06	102.03 b	0.55	0.60	0.58	2.35	2.37	2.36
L_3 Mean		92.42	92.62	92.52 A	0.53	0.55	0.54 A	2.31	2.32	2.32 AB
N Mean										
N_0		53.12	54.50	53.81 d	0.47	0.49	0.49 d	2.24	2.28	2.26 b
N_{30}		66.99	68.62	67.81 c	0.50	0.52	0.51 c	2.32	2.34	2.33 a
N_{60}		99.49	101.88	100.69 a	0.54	0.56	0.55 b	2.34	2.34	2.34 a
N_{90}		85.37	87.68	86.53 b	0.55	0.57	0.56 a	2.32	2.34	2.33 a
Means		76.24 B	78.17 A		0.52 B	0.54 A		2.31	2.32	
CV (%)			5.93			4.19			7.17	
Year (Y)			*			**			ns	
L			**			**			**	
N			**			**			**	
L x N			**			ns			ns	
L x N x Y			ns			ns			ns	

L_0= Control, L_1= 1000 kg ha^{-1} leonardite, L_2= 2000 kg ha^{-1} leonardite, L_3= 3000 kg ha^{-1} leonardite, N_0= Control, N_{30}= 30 kg ha^{-1} nitrogen, N_{60}= 60 kg ha^{-1} nitrogen, N_{90}= 90 kg ha^{-1} nitrogen, [1]: The difference between the means indicated by the same letter in the same column and group is not significant, CV: Coefficient of variation, ns: Not significant, *: $p<0.05$, **: $p<0.01$

When LxN interaction was examined, the highest value for the number of seeds per capsule was statistically obtained from L_3N_{60} (114.77 seeds) and L_2N_{60} (112.75 seeds) applications, which constituted the first group. The lowest value was found in L_0N_0 (42.97 seeds). In the study, the highest values for the number of seeds per capsule were found in the second year of the study as an average of leonardite and nitrogen doses.

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With respect to the number of seeds per capsule, the difference between the years ($p<0.05$) and LxN interaction ($p<0.01$) were found to be statistically significant (*Table 5*).

Seed weight capsule

The effects of leonardite and nitrogen fertilizer doses on the seed weight capsule in black cumin were found to be statistically significant at a level of $p<0.01$. When the two-year data were considered, seed weight capsule increased in parallel with the increase in leonardite doses, and as an average of nitrogen doses, the highest values were obtained at L_3 dose (0.54 g) and L_2 dose (0.53 g), respectively (*Table 5*). The highest seed weight capsule value was 0.56 with N_{90} nitrogen doses as an average of leonardite doses (*Table 5*).

Thousand-seed weight

In the study, nitrogen doses had a statistically significant effect at a $p<0.01$ level on the thousand grain weight of black cumin, and the highest thousand seed weight values were found at other nitrogen doses (N_{30}, N_{60}, and N_{90}), except for the control (N_0) subject. The effects of leonardite applications were found to be significant at a $p<0.01$ level (*Table 5*). The highest thousand seed weight value was obtaned from L_2 (2.35 g), followed by L_3 (2.32 g) leonardite doses. In the studies carried out by Başalma (1999) in the colza plant, by Esendal et al. (2000) in the datura plant, and by Ashraf et al. (2006) and Muhammad et al. (2017) in black cumin, the researchers determined that nitrogen fertilizer doses significantly increased thousand grain weight compared to the control. On the other hand, Tuncturk et al. (2012) and Kızılyıldırım (2019) reported that no significant differences were observed between nitrogen fertilizer doses in terms of thousand grain weight in black cumin.

Seed yield

In the study, when the effects of leonardite applications were examined, the highest seed yield, as an average of nitrogen doses, was statistically found at L_3 (1673.3 kg ha^{-1}) and L_2 (1645.8 kg ha^{-1}) leonardite doses included in the first group. In terms of seed yield, the lowest results were obtained at L_0 and L_1 doses. This difference between leonardite applications was found to be statistically very significant ($p<0.01$) (*Table 6*). It was considered that leonardite applied to the soil prevented the evaporation of water in the plant root zone and conserved the water in the soil, and consequently, drought and temperature stress occurred, and it increased the effectiveness of the existing water in the soil under the climate and soil conditions of Siirt province. Accordingly, the application of leonardite in these and similar soils, which are low in organic matter, plays a role in the conversion of plant nutrients in the soil into receivable form and increases the efficiency of the use of nutrients by plants. Therefore, humic substances such as leonardite have indirect effects on the yield increase in plants by increasing the uptake of minerals. In our study, it was considered to be effective on the increase in the seed yield of black cumin, depending on the increase in leonardite doses. Similar studies carried out in different plants on this subject support the results of our study. For example, it was reported that humic acid dose applications increased the seed yield in colza (Gürsoy et al., 2016) and tuber yield in the potato plant (Çöl and Akınerdem, 2017) compared to the control, that leonardite applications increased the tuber yield in

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potato (Şanlı and Karadoğan, 2011) compared to the control, and that leonardite applications increased the amount of dry matter and the levels of some macronutrients (Adiloğlu et al., 2017) in the rye (*Secale cerale* L.) plant compared to the control.

Table 6. *Means of seed yield, fixed oil and essential oil ratio at different leonardite and nitrogen doses in black cumin*

L doses	N doses	Seed yield (kg ha^{-1})			Fixed oil ratio (%)			Essential oil (%)		
		2017	2018	Mean^{1}	2017	2018	Mean^{1}	2017	2018	Mean^{1}
L_0	N_0	736.7	790.0	763.3	36.08	37.00	36.54	0.23	0.25	0.24
	N_{30}	1100.0	1120.0	1110.0	36.34	37.22	36.78	0.26	0.26	0.26
	N_{60}	1783.3	1837.7	1810.0	36.80	37.74	37.27	0.28	0.29	0.28
	N_{90}	1580.0	1710.0	1645.0	37.30	37.63	37.47	0.28	0.30	0.29
L_0 Mean		1300.0	1364.2	1332.0 B	36.63	37.40	37.02 B	0.26	0.28	0.27 B
L_1	N_0	1023.3	1053.3	1038.3	36.66	37.58	37.12	0.25	0.26	0.25
	N_{30}	1150.0	1170.0	1160.0	36.45	37.39	36.92	0.26	0.28	0.27
	N_{60}	1810.0	1863.3	1836.7	37.58	38.25	37.92	0.28	0.31	0.29
	N_{90}	1636.7	1756.7	1696.7	37.87	38.20	38.03	0.29	0.31	0.30
L_1 Mean		1405.0	1460.8	1432.9 B	37.14	37.86	37.50 A	0.27	0.29	0.28 B
L_2	N_0	1290.0	1356.7	1323.3	36.61	37.59	37.10	0.26	0.28	0.27
	N_{30}	1226.7	1493.3	1360.0	36.40	37.30	36.85	0.27	0.30	0.29
	N_{60}	1886.7	2046.7	1966.7	37.00	37.27	37.13	0.30	0.32	0.31
	N_{90}	1910.0	1956.7	1933.3	38.27	38.60	38.43	0.30	0.33	0.31
L_2 Mean		1578.3	1713.3	1645.8 A	37.07	37.69	37.38 A	0.28	0.31	0.30 A
L_3	N_0	1120.0	1206.7	1163.3	36.44	37.11	36.77	0.25	0.28	0.27
	N_{30}	1566.7	1606.7	1586.7	36.46	37.36	36.86	0.27	0.30	0.28
	N_{60}	2043.3	2110.0	2076.7	37.69	38.02	37.85	0.29	0.31	0.30
	N_{90}	1870.0	1863.3	1866.7	37.86	37.93	37.89	0.30	0.32	0.31
L_3 Mean		1650.0	1696.6	1673.3 A	37.11	37.61	37.36 A	0.27	0.30	0.29 A
N Mean										
N_0		1042.5	1101.6	1072.1 d	36.45	37.34	36.89 c	0.25	0.27	0.26 c
N_{30}		1260.8	1347.5	1304.2 c	36.41	37.35	36.88 c	0.26	0.29	0.27 b
N_{60}		1880.8	1964.2	1922.5 a	37.27	37.83	37.55 b	0.29	0.31	0.30 a
N_{90}		1749.2	1821.6	1785.4 b	37.83	38.09	37.96 a	0.29	0.32	0.31 a
Means		1483.3 B	1558.7 A		36.99 B	37.65 A		0.27 B	0.29 A	
CV (%)		11.85			1.33			4.60		
Year (Y)		*			**			**		
L		**			**			**		
N		**			**			**		
L x N		ns			ns			ns		
L x N x Y		ns			ns			ns		

L_0= Control, L_1= 1000 kg ha^{-1} leonardite, L_2= 2000 kg ha^{-1} leonardite, L_3= 3000 kg ha^{-1} leonardite, N_0= Control, N_{30}= 30 kg ha^{-1} nitrogen, N_{60}= 60 kg ha^{-1} nitrogen, N_{90}= 90 kg ha^{-1} nitrogen, ^{1}: The difference between the means indicated by the same letter in the same column and group is not significant, CV: Coefficient of variation, ns: Not significant, *: $p<0.05$, **: $p<0.01$

When the effects of nitrogen doses alone were examined, as an average of leonardite doses, the highest seed yield was found to be 1922.5 kg ha^{-1} at N_{60} dose, and the lowest seed yield was found to be 1072.1 kg ha^{-1} at N_0 dose, and seed yield decreased significantly at N_{90} dose. With respect to seed yield, this difference between nitrogen fertilizer doses was found to be statistically significant at a $p<0.01$ level (*Table 6*). It was also reported in the results of some studies that nitrogen fertilization in black cumin

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had significant and positive effects on seed yield. When these studies were reviewed, it was reported that the highest seed yield in black cumin was obtained from 60 kg N ha^{-1} nitrogen dose under the ecological conditions of India (Shah, 2004), Çukurova-Turkey (Özgüven and Şekeroğlu, 2007), Van-Turkey (Tuncturk et al., 2012), Ethiopia (Yimam et al., 2015), and Eskişehir-Turkey (Sağlam, 2018), from 30-60 kg N ha^{-1} nitrogen dose under the conditions of South Korea (Ashraf et al., 2006), from 30 kg N ha^{-1} nitrogen dose in the ecology of Iraq-Sulaymaniyah (Muhammad et al., 2017), and from 80 kg N ha^{-1} nitrogen dose under the climatic and soil conditions of Kahramanmaraş-Turkey (Kızılyıldırım, 2019).

It can be said that the results of our study are generally compatible with these data in the literature. The fact that nitrogen doses have different effects in different ecologies in terms of the seed yield of black cumin can be explained by different physical and chemical properties (especially organic matter) of soils where the study was carried out, along with the genotypic difference of the plant material used.

In the study, the difference between the years in terms of seed yield was also found to be statistically significant ($p<0.05$), and the highest values, as the average of leonardite and nitrogen doses, were found in the second year of the study (*Table 6*). It was considered that this difference between the years in terms of seed yield was due to differences in precipitation and temperature between the years.

In the study, although the LxN interaction was found to be statistically insignificant, it was remarkable that the seed yield was high in the treatments in which leonardite and nitrogen fertilizer were applied together at increasing ratios (e.g., such as L_2N_{60} and L_3N_{60}) (*Table 6*), which was considered as a significant result in that soil-conditioning organic materials such as leonardite increase the effectiveness of chemical fertilizers.

Fixed oil

One of the most critical factors that determine the seed quality in black cumin is the fixed oil ratio (Akgül, 1993). In the study, it was observed that leonardite applications increased the fixed oil ratio in the seeds of the black cumin plant. It was determined that this increase was statistically very significant at a ($p<0.01$) level compared to the subject without leonardite application (L_0) and that the highest values were obtained from other applications, except for L_0. The fixed oil ratio of black cumin varied between 37.02-37.50% along with leonardite applications (*Table 6*).

When nitrogen fertilizer doses were examined alone, it was determined that the fixed oil ratio of black cumin seeds increased in parallel with the increase in nitrogen doses and that the highest fixed oil ratio was obtained from N_{90} nitrogen dose with 37.96% as an average of leonardite doses. This difference between nitrogen fertilizer doses in terms of fixed oil ratio was found to be statistically significant at a $p<0.01$ level (*Table 6*).

While the fatty acid composition of seeds varies by species and genotypes (Karaca and Aytaç, 2007), different cultural applications in the planting-harvesting process (Küçükemre, 2009; Arslan et al., 2011; Kulan et al., 2012) and the ecological (Karaca and Aytaç, 2007) and topographic (Yüksek et al., 2016) differences also affect the oil ratio. In this sense, different results were obtained with respect to the effects of nitrogen fertilization on the fixed oil ratio consisting of saturated and unsaturated fatty acids. For example, it was reported that different doses of nitrogen fertilization did not generally affect the fixed oil ratio in black cumin (Shah, 2004; Özgüven and Şekeroğlu, 2007; Kızılyıldırım, 2019). On the contrary, it was reported that significant differences

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occurred between nitrogen fertilizer doses in terms of fixed oil ratio in the fennel (*Foeniculum vulgare* Mill.) plant (Tunçtürk et al., 2011) and oil ratio in the safflower (*Carthamus tinctorius* L.) plant (Katar et al., 2012).

It was considered that the emergence of significant differences between leonardite and nitrogen applications in terms of fixed oil ratio in the research soil, where soil organic matter, and accordingly, the amount of nitrogen in the soil were low, was affected by the increased availability of plant nutrients and the synergistic relationship between some nutrients. Nevertheless, it was reported that black cumin seeds contained fixed oil at ratios varying between 21.83-40.58% (Akgül, 1993; Türker and Bayrak, 1997; Kalçın, 2003; Kulan et al., 2012; Ertaş, 2016; Selicioğlu, 2018; Kızılyıldırım, 2019), and it was observed that the fixed oil ratio values obtained as a result of leonardite and nitrogen applications in our study were within these limits in the literature.

Essential oil

In the study, both leonardite and nitrogen fertilizer doses had statistically very significant ($p<0.01$) effects on the essential oil of black cumin seeds. While the highest essential oil in leonardite applications was found at L_2 and L_3 doses (0.30% and 0.29%, respectively) as an average of years and nitrogen doses, the highest essential oil in nitrogen fertilizer applications was found at N_{60} (0.30%) and N_{90} doses (0.31%) as an average of years and leonardite doses (*Table 6*).

According to the result of the study, it was observed that leonardite applications and nitrogen fertilizer applications, also known as organic fertilizer sources, increased the essential oil of black cumin seeds. Our results obtained by nitrogen fertilizer dose applications were found to be consistent with the results obtained by Türközü (2005), Özgüven and Şekeroğlu (2007), and Kızılyıldırım (2019). For example, in some other studies in which nitrogen fertilizer applications were performed, Yıldırım and Kan (2006) and Tunçtürk et al. (2011) reported that nitrogen fertilizer doses had no effect on essential oil in fennel, contrary to the results of our study.

Essential oil components

Essential oil components obtained by the Headspace GC-MS analysis of black cumin essential oil are presented in *Table 7*. A total of 6 components were found in black cumin essential oil. The main component of black cumin essential oil is thymoquinone. In our study, the effects of leonardite and nitrogen doses alone and together on thymoquinone content were found to be statistically significant ($p<0.01$). Accordingly, it was observed that the composition of thymoquinone of the essential oil of black cumin seeds increased in parallel with the increase in both leonardite and nitrogen doses and that the highest values were obtained at high doses. Indeed, this situation also manifested itself in the LxN interaction, and the highest thymoquinone content was found in L_3N_{90} subject by 47.09% (*Table 7*). Another major component of black cumin essential oil is P-cymene, and leonardite and nitrogen doses had a very significant ($p<0.01$) effect on P-cymene content. The highest P-cymene content and the lowest P-cymene content were found at L_3 dose by 41.66% and at L_0 (41.27%) leonardite dose, respectively, as an average of years and nitrogen doses. When the effects of nitrogen doses alone were reviewed, the highest P-cymene content and the lowest P-cymene content were found at N_{90} dose by 43.11% and at N_0 nitrogen dose by 38.67%, respectively, as an average of years and leonardite doses (*Table 7*).

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Table 7. *The effect of leonardite and nitrogen fertilizer doses on the components of black cumin essential oil*

LxN	Thymoquinone			Beta-pinene			Sabinene		
	2017	2018	Mean*	2017	2018	Mean*	2017	2018	Mean*
L_0N_0	44.52	44.09	44.31 d	5.28	5.30	5.29 f	2.56	2.64	2.60
L_0N_{30}	44.58	44.67	44.63 cd	5.31	5.35	5.33 ef	2.62	2.66	2.64
L_0N_{60}	45.65	45.60	45.63 b	5.34	5.38	5.36 de	2.68	2.74	2.71
L_0N_{90}	45.70	45.80	45.75 b	5.57	5.60	5.59 bc	2.84	2.88	2.86
L_1N_0	44.68	44.72	44.70 cd	5.31	5.33	5.32 ef	2.57	2.61	2.59
L_1N_{30}	44.75	44.73	44.74 cd	5.34	5.35	5.35 de	2.66	2.69	2.68
L_1N_{60}	45.73	45.73	45.73 b	5.52	5.55	5.54 c	2.72	2.76	2.74
L_1N_{90}	45.72	45.74	45.73 b	5.61	5.63	5.62 b	2.86	2.86	2.86
L_2N_0	44.74	44.71	44.72 cd	5.33	5.35	5.34 ef	2.59	2.66	2.62
L_2N_{30}	44.84	44.85	44.84 c	5.37	5.36	5.35 de	2.67	2.69	2.68
L_2N_{60}	45.81	45.89	45.85 b	5.55	5.60	5.58 bc	2.73	2.75	2.74
L_2N_{90}	47.50	47.34	47.42 a	5.61	5.61	5.61 b	2.87	2.90	2.89
L_3N_0	44.77	44.75	44.76 c	5.36	5.34	5.35 de	2.59	2.66	2.63
L_3N_{30}	44.85	44.86	44.86 c	5.37	5.42	5.40 d	2.66	2.71	2.68
L_3N_{60}	45.84	45.82	45.83 b	5.59	5.63	5.61 b	2.74	2.77	2.76
L_3N_{90}	47.03	47.15	47.09 a	5.65	5.72	5.69 a	2.87	2.89	2.88
Mean	45.42	45.40		5.43	5.47		2.70 b	2.74 a	
CV (%)	1.85			2.16			2.09		
L_0			45.08 b			5.39 c			2.70
L_1			45.23 b			5.46 b			2.72
L_2			45.71 a			5.47 b			2.73
L_3			45.63 a			5.51 a			2.74
N_0			44.62 c			5.33 d			2.61 d
N_{30}			44.77 c			5.36 c			2.67 c
N_{60}			45.76 b			5.49 b			2.74 b
N_{90}			46.50 a			5.63 a			2.87 a
	L^{**}, N^{**}, LxN^{**}			L^{**}, N^{**}, LxN^{**}			Y^{**}, N^{**}		
LxN	**Limonene**			**P-cymene**			**Linalool**		
	2017	2018	Mean*	2017	2018	Mean*	2017	2018	Mean*
L_0N_0	2.02	2.05	2.03	37.94	39.08	38.51	1.86	1.97	1.91
L_0N_{30}	2.24	2.17	2.21	41.55	42.35	41.95	2.22	2.25	2.24
L_0N_{60}	2.25	2.24	2.25	41.47	41.81	41.64	2.37	2.33	2.35
L_0N_{90}	2.32	2.27	2.30	42.67	43.27	42.97	2.48	2.39	2.44
L_1N_0	2.10	2.12	2.11	38.21	38.93	38.57	1.93	1.95	1.94
L_1N_{30}	2.27	2.25	2.26	41.57	42.25	41.91	2.21	2.25	2.23
L_1N_{60}	2.29	2.29	2.29	41.81	42.62	42.21	2.36	2.32	2.34
L_1N_{90}	2.32	2.34	2.33	42.76	43.35	43.05	2.48	2.47	2.48
L_2N_0	2.15	2.12	2.13	38.42	38.85	38.63	1.94	1.93	1.94
L_2N_{30}	2.29	2.28	2.29	41.57	41.96	41.76	2.27	2.28	2.27
L_2N_{60}	2.32	2.31	2.32	41.83	42.50	42.17	2.38	2.40	2.39
L_2N_{90}	2.37	2.37	2.37	42.81	43.67	43.24	2.50	2.52	2.51
L_3N_0	2.16	2.15	2.16	38.67	39.23	38.95	1.89	1.98	1.94
L_3N_{30}	2.32	2.27	2.29	41.85	42.41	42.13	2.29	2.33	2.31
L_3N_{60}	2.28	2.30	2.29	41.99	42.79	42.39	2.39	2.43	2.41
L_3N_{90}	2.35	2.39	2.37	42.89	43.46	43.17	2.51	2.50	2.50
Mean	2.25	2.24		41.12 b	41.78 a		2.25	2.27	
CV (%)	2.89			1.06			1.94		
L_0			2.20 b			41.27 c			2.23 b
L_1			2.25 a			41.44 bc			2.25 b
L_2			2.28 a			41.45 b			2.28 a
L_3			2.28 a			41.66 a			2.29 a
N_0			2.11 c			38.67 c			1.93 d
N_{30}			2.26 b			41.94 b			2.26 c
N_{60}			2.28 b			42.10 b			2.37 b
N_{90}			2.34 a			43.11 a			2.48 a
	L^{**}, N^{**}			Y^{**}, L^{**}, N^{**}			L^{**}, N^{**}		

L: Leonardite, N: Nitrogen, CV: Coefficient of variation, Y: Year, *: The difference between the means indicated by the same letter in the same column and group is not significant, **: $p<0.01$

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In the study, other components found in black cumin essential oil were beta-pinene, sabinene, limonene, and linalool. It was determined that the amounts of these components were affected by leonardite (except for sabinene) and nitrogen dose applications and that the components generally had the highest values at the highest leonardite and nitrogen doses (*Table 7*).

In other studies on black cumin, the main component was determined to be p-cymene (47.4%, 49.06%, and 43.58%, respectively) by Orchid et al. (2004), Ashraf et al. (2006), and Toma et al. (2010). Moretti et al. (2004), Orchid et al. (2004), Toma et al. (2010), and Harzallah et al. (2011) determined that thymoquinone ratios in black cumin essential oil were 3.8%, 20.8%, 1.65%, and 0.79%, respectively. On the other hand, Akgören Palabıyık and Aytaç (2018) reported that black cumin oil contained thymoquinone by 67.7% as essential oil components, followed by carvacrol by 8.4%, and junipene by 4.8%. In our study, the main component of black cumin essential oil was thymoquinone by 45.41%. The fact that the main components of black cumin essential oil were different from some studies may also be due to different climatic characteristics along with the different genotypic features of the plant material used. Namely, the fact that the temperature is high but the amount of precipitation is low especially in May and June (*Table 1*) was considered to lead to a high amount of thymoquinone. A similar situation was also indicated by Akgören Palabıyık and Aytaç (2018), and Herlina et al. (2017) reported that high temperature had an effect on high thymoquinone content.

Conclusions

According to the results of this study carried out under semi-arid climatic conditions, the applications of leonardite, known as a soil conditioner, and nitrogen fertilizer doses significantly affected the seed yield and yield components of black cumin. The highest seed yield was obtained from 2000 kg ha^{-1} leonardite and 60 kg ha^{-1} nitrogen applications. If some amount is waived in terms of essential oil and components of essential oil, the L_2N_{60} application can be recommended with respect to yield and quality.

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THE INFLUENCE OF IRRIGATION WITH INTENSIVE FISH FARM WATER ON THE QUALITY INDICATORS OF AEROBIC RICE (*Oryza sativa* L.)

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Abstract. This study examined the effect of effluent water from an intensive fish farm on rice (*Oryza sativa* L.) grown under aerobic condition in Hungary. During the experiment four treatments were used: T_1 - effluent water; T_2 - effluent water supplemented with gypsum; T_3 - effluent water diluted with surface water and supplemented with gypsum; T_C - control treatment. A number of quality parameters (TKW - thousand kernel weight, MQP - milling quality parameters, MC - mineral content) of the selected Hungarian rice variety (M 488) were studied. While, in the TKW test no statistically significant difference was found between the treatments, MQP test showed that there was a statistically significant difference between treatments in the percentage of whole polished (white) rice. The highest percentage was in T_C with 68.5%, T_1, T_2, and T_3 had 60%, 61.1% and 59.12%, respectively. The analytical analysis of MC highlighted, that the Ca and Na contents in rice seeds were not affected by the treatments, however, under T_3 a statistically significant decrease in the P, K and Mg contents of rice seeds was observed. Altogether, irrigation with fish farm water affects some quality parameters of the chosen rice variety in different ways, this effect can remain stable while reducing stress levels.
Keywords: *effluent water, environment, rice quality, water saving, water stress*

Introduction

One of the necessary conditions for obtaining a high quality crop is associated with water quality that meets the standards of irrigation (Suarez, 2011; Limjuco et al., 2016). Currently, climate change and water scarcity make it difficult for farmers to access quality irrigation water (Deressa et al., 2011; Chand and Kumar, 2018). These are undesirable limiting stress factors for the growth, development, and productivity of plants (Wang et al., 2006). Stresses, such as drought and high salinity in irrigation water can damage life cycle of plants, change cell size, disrupt gas exchange, and ultimately reduce yield (Bongi and Loreto, 1989; Rhodes and Nadolska-Orczyk, 2001; Tenhaken, 2015; Chowdhury, 2016; Joshi et al., 2016; Dresselhaus and Huckelhoven, 2018).

Many researchers have focused on alternative resources, such as wastewater in order to eliminate the existing shortage and to provide plants with the necessary water in a timely manner (Haruvy, 1997; Toze, 2006; Drechsel and Evans, 2010). Since they are rich in chemical composition, in some cases, these waters can be used as fertilizers (Rahimi et al., 2012; Ryu et al., 2012). It is also possible to make some progress in increasing productivity through the use of wastewater for irrigation (Khan et al., 2009). Another progressive aspect of this is the opportunity of reuse of wastewater, which in most cases is directly discharged into rivers, seas and oceans (Nair, 2008; Kamal et al.,

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2008). Eventually, this reuse will lead to environmental protection and minimization of potential damage. For instance, in recent decades, it was noted that an increase in the number of fish farms due to the disposal of waste into natural water resources has led to a disruption in the chemical and environmental balance of the water (Jones, 1990). According to a study by Ruiz-Zarzuela et al. (2009) in Northeast Spain, water from fish farms leads to a decrease in pH and dissolved oxygen in the river, which could affect water quality and aquatic life. However, the application of effluent water from fish farms in agriculture may be more suitable. According to Castro et al. (2006) the application of effluents from fish ponds greatly increased tomato yields in Northeast Brazil. Abdelraouf et al. (2014) reported in their study in Egypt, drainage water of fish ponds could be a good option for saving current water resources.

Changes at the global level, as well as existing problems also affect rice (*Oryza sativa* L.) cultivation. Rice is one of the most water-demanding crops among cereals and is the main agricultural crop that satisfies the nutritional needs of a large part of the world's population (Vergara, 1991; Muthayya et al., 2014). In addition, as the number of people increases, the demand for this product increases too (Khush, 2005). According to some researchers, the use of aerobic rice systems, which is considerably more water-efficient than the traditional method, can be an advantageous method in conditions of water scarcity (Bouman et al., 2002; Pinheiro et al., 2006). But another important factor in this event is related to the quality and yield of rice. Thus, in this study, our aim was to identify the effect of effluent water from fish farm on the development of aerobic rice by studying the qualitative characteristics and parameters of the mineral composition. In addition, we also focused on taking another step to the reduction of water demand and environmental pollution to improve a complex agricultural system.

Materials and methods

The experiment was conducted at the National Agricultural Research and Innovation Centre, Research Institute of Irrigation and Water Management (NAIK ÖVKI) Lysimeter Station in Szarvas, Hungary (46°51'48"N, 20°31'39"E). Possible changes of effluent water from the intensive catfish farm on rice was explored during the experiment. For that purpose Hungarian rice variety named M 488 was planted in 16 gravitation lysimeters under aerobic condition in May 2019. The treatments were applied by a micro sprinkler irrigation method and with four replications. The applied experimental design has been shown in *Figure 1*. Generally, lysimeters are devices used to study the dynamics of water, evapotranspiration and changes of other substances in the soil (Lanthaler, 2004). However, the reason for choosing gravitation lysimeter was to create aerobic conditions for the plants and at the same time to separate them from the vertical and horizontal effects of the surrounding environment and soil.

The non-weighted, backfilled gravitation lysimeters at the NAIK ÖVKI have the volume of 1 m^3 and each of them has a surface of 1 squaremeter. Four lysimeters were installed into one block together (one treatment in irrigation studies). The bottom 10 cm of the lysimeter is a layer of gravel to collect percolated water in case of heavy rain or high amount of irrigation. The plants have 80 cm of soil for the development. The type of soil in the lysimeters was vertisol (expansive clay). The plant density was set to 40 plants/m^2 (*Figure 2*).

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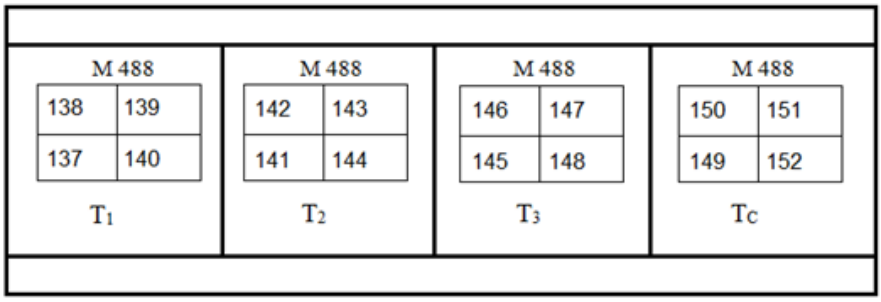

Figure 1. *The applied experimental design. T_1 - effluent water, T_2 - effluent water supplemented with gypsum, T_3 - effluent water mixed with surface water and supplemented with gypsum, T_C - river water (control). The numbers in the cells represent the identification number of the gravitational lysimeters. M 488 – Hungarian rice variety*

Figure 2. *Lysimeter experiment of rice developed with different quality of irrigation water*

Four types of treatments have been applied during the experiment: T_1 - effluent water; T_2 - effluent water supplemented with gypsum; T_3 - effluent water diluted with surface water and supplemented with gypsum; T_C - control treatment, water from oxbow, which is a section of the Körös River in eastern Hungary. The chemical parameters of treatments are listed in the *Table 1*.

Seeds were sown on May 22, 2019. On the first and second irrigation days (22nd of May and 7th of June) all plants in lysimeters were irrigated with river water, only after that they were irrigated on the basis of treatments. On July 4, 0.5 kg of fertilizer (NH_4NO_3 + $CaMg(CO_3)_2$) was applied (84.4 kg N*ha^{-1}), and pesticides were not used during the experiments. The total applied irrigation water was 200 mm. Irrigation time and amount of used water are shown in the following *Table 2*.

Meteorological data were measured using meteorological equipment (Agromet-Solar automatic weather station, Boreas Ltd., Hungary) that was installed next to the experimental field. Rainfall during the growing season was 303.7 mm (*Table 3*).

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Table 1. *The basic chemical parameters of treatments*

Chemical parameters	T_1	T_2	T_3	T_C
pH	7.77	7.71	7.70	7.55
Electrical Conductivity (EC) (µS/cm)	1180	1905.00	1033.75	371.86
m-alkalinity	13.77	14.65	8.23	3.00
Bicarbonate (mg/l)	838.67	894.00	502.00	182.67
Ammonium-N (mg/l)	20.40	23.45	10.39	0.37
Nitrate-N (mg/l)	0.03	-	0.47	0.43
Nitrite-N (mg/l)	0.02	0.13	0.13	0.06
Total inorganic N (mg/l)	20.45	23.58	10.60	0.64
Total organic N (mg/l)	5.86	4.98	2.51	-
Total N (mg/l)	26.3	28.55	13.10	1.19
P-orthophosphate (mg/l)	1.72	2.55	1.38	0.12
Total P (mg/l)	2.18	2.67	1.53	0.15
Chloride (mg/l)	29.90	33.15	27.15	22.54
Sulphate (mg/l)	32.65	448.75	164.18	34.58
Ca (mg/l)	23.23	187.50	90.83	39.04
Mg (mg/l)	10.08	11.02	10.69	9.80
Na (mg/l)	249.00	266.75	131.25	28.90
K (mg/l)	6.08	6.61	5.43	3.71

T_1 - effluent water, T_2 - effluent water supplemented with gypsum, T_3 - effluent water mixed with surface water and supplemented with gypsum, T_C - river water (control)

Table 2. *Irrigation dates and amount of applied water*

	Irrigation water applied (mm)										
Irrigation dates	**22. May**	**07. June**	**14. June**	**02. July**	**04. July**	**12. July**	**18. July**	**26. July**	**12. August**	**22. August**	**Total**
T_1	20	20	20	20	20	20	20	20	20	20	200
T_2	20	20	20	20	20	20	20	20	20	20	200
T_3	20	20	20	20	20	20	20	20	20	20	200
T_C	20	20	20	20	20	20	20	20	20	20	200

T_1 - effluent water, T_2 - effluent water supplemented with gypsum, T_3 - effluent water mixed with surface water and supplemented with gypsum, T_C - river water (control)

Table 3. *Monthly precipitations and temperatures (average, minimum and maximum) during the growing season*

	Precipitation (mm)	T avg. (°C)	T min. (°C)	T max. (°C)
May	48.7	17.8	8.0	26.0
June	162.4	23.5	14.2	34.0
July	68.1	22.2	10.3	34.1
August	24.5	24.1	10.8	36.3

After the harvest (24th of September, 2019) and standard post-harvest operation (cleaning, drying, and storing) basic tests – Thousand Kernel Weight (TKW), Milling Quality Parameters (MQP) and Mineral Content (MC) of rice seeds were analysed (*Table 4*).

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Table 4. *Conducted test and used equipments*

№	Conducted tests	Used equipments (methods)
1	Moisture content	Sartorius MA45
2	Thousand Kernel Weight (TKW)	1) Sartorius BP221S 2) Satake THU Laboratory Husker
3	Milling Quality Parameters (MQP)	Satake TM05 Test Mill laboratory
4	Analysis of Mineral Content (MC)	1) Thermo Scientific Solaar M6 atomic absorption spectrophotometer 2) Thermo Scientific ICAP 6000 ICP-OES

In order to begin those tests, moisture content of rice seeds from every sample was defined. At the beginning, the grains of each sample were divided into tiny particles, then by using Sartorius MA45 moisture analyser moisture content was found. The average moisture content was computed after the replications of four measurements.

For Thousand Kernel Weight (TKW) test, 100 paddy seeds were counted from each sample and weighed on Sartorius BP221S analytical balance. Afterwards, husk of seeds was removed by using Satake THU Laboratory Husker equipment and cargo (brown) rice weighed. The obtained results were multiplied by 10. After four replications of tests, the average TKW of paddy and cargo rice was determined.

100 g of rice from each sample was prepared for Milling Quality Parameter (MQP) analysis. First, a husk layer of seeds removed and cargo rice was weighed. Later, by using Satake TM05 Test Mill laboratory equipment brown rice was polished and the results were weighed. Subsequently, the percentage whole and broken white (polished) rice were calculated. The experiment was repeated five times and the average value is defined. According to the following formulas, the results were calculated (Lapis et al., 2019):

$$\%\, Cargo\ rice = \frac{weight\ of\ brown\ rice\ (g)}{weight\ of\ paddy\ rice\ (g)} * 100 \qquad \text{(Eq.1)}$$

$$\%\, Polished\ rice = \frac{weight\ of\ polished\ rice\ (g)}{weight\ of\ paddy\ rice\ (g)} * 100 \qquad \text{(Eq.2)}$$

$$\%\, Whole\ p.rice = \frac{weight\ of\ whole\ p.rice\ (g)}{weight\ of\ paddy\ rice\ (g)} * 100 \qquad \text{(Eq.3)}$$

Mineral Content (MC) test involves determination of amount basic minerals (Ca, Mg, K, Na, and P) in rice grains. In order to carry on analyses, paddy rice from each sample were hulled with Satake THU Laboratory Husker and brown rice received. After standard procedures, every sample was wet digested in 6 ml HNO_3 and 2 ml H_2O_2. One day later, the samples were kept in a microwave oven at a temperature of 180 °C for 1.5 hours. Afterwards, samples were analysed by using AAS and ICP-OES (NAIK ÖVKI Laboratory of Environmental Analytics, Szarvas, Hungary).

Based on standard methods, Ca, Mg, K and Na content were measured by Thermo Scientific Solaar M6 atomic absorption spectrophotometer. Determination of P was done with Thermo Scientific ICAP 6000 ICP-OES inductively coupled plasma atomic emission equipment, according to MSZ EN ISO 11885:2000 international and Hungarian standard.

The collected data were subjected to the analysis of variance (ANOVA) using IBM SPSS software (version 22). The significant differences among mean values were

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determined with the Tukey test at 5% level of probability. In condition of violation of homogeneity of variances (Levene's test, $p<0.05$), Games-Howell post-hoc test was set under the terms of Welch test ($p<0.05$).

Results and discussion

Moisture content

The general idea behind of controlling moisture content of paddy seeds to receive moisture content below 14% (International Rice Research Institute, 2013). For the experiments, samples were placed in storage room in unmonitored condition. Although, conducted experiments were done moisture free basis, in our experiments we also got results less than 14% in all samples. The moisture content of samples under T_1, T_2, T_3 and T_C was 7.97%, 7.46%, 7.69%, 7.28%, respectively.

Thosand Kernel Weight (TKW)

One of the main indicators of the vitality, quality, and productivity of rice seeds is associated with TKW (Wu et al., 2018). In many cases, the TKW of rice grains in cultivation under flooding conditions is greater than in aerobic rice systems, however, it may vary depending on the rice cultivar (Castaneda et al., 2003; Reddy et al., 2010). The results of our experiments (*Table 5*) show, in both paddy seeds and cargo seeds, the highest TKW between treatments was observed with T_1. Nevertheless, the result of statistical analysis indicated non-significant differences between treatments ($p>0.05$). The absence of such a statistically significant difference specifies a similar reaction of rice to all treatments.

Table 5. *Thousand kernel weight of paddy and cargo seeds of rice developed with different quality of irrigation (Szarvas, 2019)*

Treatment		TKW of paddy seed (g)	TKW of cargo seed (g)
T_1	Average	22.15a	17.88a
	CI	[21.54; 22.76]	[17.4; 18.35]
T_2	Average	22.0a	17.68a
	CI	[20.88; 23.13]	[16.65; 18.72]
T_3	Average	22.01a	17.76a
	CI	[21.65; 22.38]	[17.2; 18.32]
T_C	Average	21.48a	17.28a
	CI	[21.17; 21.78]	[17.19; 17.37]

T_1 - effluent water, T_2 - effluent water supplemented with gypsum, T_3 - effluent water mixed with surface water and supplemented with gypsum, T_C - river water (control). CI - confidence interval (lower and upper bound). Values with the same letter are not significantly different at $p<0.05$

Milling Quality Parameters (MQP)

The importance of rice milling is mainly related to the percentage of whole white rice (Dhankhar et al., 2014). On the one hand, if it is connected with the tradition of consumption, on the other hand, it is closely connected with marketing goals (Dela Cruz and Khush, 2000; Zhou et al., 2019). Usually, most consumers before buying a product,

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pay attention not only to the shape of the rice, but also to the colour and aroma of the rice (Rachmat et al., 2006). While in our experiment in the analysis of cargo rice percentage and polished (white) rice percentage, apparent differences were not found, in the percentage of whole polished rice there were statistically significant differences between treatments (*Table 6*). T_1, T_2, and T_3 had a statistically similar results ($p>0.05$), 60%, 61% and 59.12%, respectively. However, the highest percentage of whole polished rice was observed in the control treatment (68.5%) and this difference was statistically significant ($p<0.05$) between T_1, T_2, and T_3. According to the initial assumption, this difference between the results is due to the chemical composition between the control and the other treatments. One of the main indicators of the effluent water from intensive fish farm is that it contains a lot of sodium. Usually, rice cultivated under aerobic conditions and irrigated at regular intervals is subject to abiotic stresses (Jabran et al., 2017). This sensitivity to abiotic factors manifests itself as a reduction in a number of plant parameters (Singh et al., 2012; Kato and Katsura, 2014). The high salt content in the water prevents the plant from absorbing the water it needs (Ghosh et al., 2016). The greatest challenge is when the temperature is high, which causes the plant to become more stressed (Clermont-Dauphin et al., 2010; Mishra et al., 2015; Ali et al., 2019). Although the response to salinity may vary depending on the rice variety, increasing the salinity level of irrigation water at all stages of cultivation can significantly affect rice (Castillo et al., 2007; Fraga et al., 2010; Chang et al., 2019). This may ultimately affect the quality of the rice seeds.

Table 6. *Milling quality parameters of cargo and polished seeds of rice developed with different quality of irrigation (Szarvas, 2019)*

Treatment		Cargo (Brown) (%)	Polished (White) (%)	Whole polished rice (%)
T_1	Average	79.2a	72a	60a
	CI	[78.16; 80.24]	[71.12; 72.88]	[54.81; 65.19]
T_2	Average	78.2a	71.1a	61.1a
	CI	[77.65; 78.76]	[68.72; 73.48]	[58.14; 64.06
T_3	Average	78.8a	72.8a	59.12a
	CI	[78.19; 79.41]	[72.19; 73.41]	[57.93; 60.31]
T_C	Average	78.8a	72.9a	68.5b
	CI	[77.86; 79.74]	[71.29; 74.51]	[66.92; 70.08]

T_1 - effluent water, T_2 - effluent water supplemented with gypsum, T_3 - effluent water mixed with surface water and supplemented with gypsum, T_C - river water (control). CI - confidence interval (lower and upper bound). Values with the same letter are not significantly different at $p<0.05$

Salinity also has a direct effect on the protein content of rice grains, where protein loss can increase the rice seed breakage (Leesawatwong et al., 2004; Balindong et al., 2018). Since the decrease in protein content in salt-sensitive rice varieties is observed more distinctly (Billah et al., 2017). Moreover, according to Rao et al. (2013), salinity in the soil is another reason for the decline in head rice recovery.

Mineral Content (MC)

The content of Ca in all treatments had a statistically similar ($p>0.05$) result (*Table 7*). Analogous results were also noted in Na content. While there was no statistically significant difference in Mg content between the control and T_1, T_2, T_3, the difference

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between T_3 and T_1, T_2 was statistically significant ($p<0.05$). P and K content in T_3 was lower compared to the control and T_1, T_2, which was a statistically significant difference ($p<0.05$).

Table 7. *Average mineral content in M 488 rice seeds, (Szarvas, 2019)*

Treatment		Ca (mg/kg dry matter)	Mg (mg/kg dry matter)	P (mg/kg dry matter)	K (mg/kg dry matter)	Na (mg/kg dry matter)
T_1	Average	396.5a	1652.5b	4240b	3600b	200.25a
	CI	[306.71; 486.29]	[1573.07; 1731.93]	[3954.47; 4525.53]	[3327.78; 3872.22]	[165.4; 235.1]
T_2	Average	412.75a	1717.5b	4490b	3807.5b	211.25a
	CI	[338.41; 487.09]	[1657.43; 1777.57]	[4266.85; 4713.15]	[3469.86; 4145.14]	[181.02; 241.48]
T_3	Average	421a	1405a	3440a	3005a	226.75a
	CI	[361.7; 480.3]	[1364.96; 1445.04]	[3293.58; 3586.42]	[2807.75; 3202.25]	[208.92; 244.58]
T_C	Average	427.75a	1650ab	4297.5b	3665b	201.25a
	CI	[369.83; 485.67]	[1419.41; 1880.59]	[3469.62; 5125.38]	[3217.85; 4112.15]	[183.28; 219.22]

T_1 - effluent water, T_2 - effluent water supplemented with gypsum, T_3 - effluent water mixed with surface water and supplemented with gypsum, T_C - river water (control). CI - confidence interval (lower and upper bound). Values with the same letter are not significantly different at $p<0.05$

The high salt content in irrigation water creates stressful conditions and negatively affects the mineral metabolism in plants (Subekti et al., 2020). In our experiments, since the salinity level was normal in control, there were no obstacles to the absorption of P, K from water. Compared to T_1, T_2, the salt content in T_3 was lower, however, due to the low P, K in T_3, the content of P and K in the brown seeds may be lower. It should be noted that under stress, the development of the roots decreases, and the absorption of minerals is weakened (Hu and Schmidhalter, 2005; Hakim et al., 2014). In general, in each part of the plant a different amount of mineral accumulates (Sperotto et al., 2017). In other words, the low transportation and accumulation of minerals in T_3 can be associated with a high salt content and a low content of P, K. Furthermore, despite the low content of minerals in control, because of optimal regime, there was no interfere to the transportation of minerals.

Conclusion

Limited water resources, problems with the use of existing water resources, droughts and various global climatic phenomena require the use of alternative irrigation methods. In the current experiment, an analysis of the quality of rice irrigated with water discharged from an intensive fish farm was made. The study showed that both the direct use of intensive fish farm water (T_1) and supplemented with additives (T_2, T_3) does not adversely affect TKW and MC of rice grains (excluding P and K during T_3 irrigation), but reduces percentage of whole polished rice. Based on general ideas, the influence of direct use of intensive fish farm water (T_1) or supplemented with additives (T_2, T_3) on the quality indicators of the studied rice variety (M 488) under conditions of minimizing the harming effects of all the stress factors is stable. Although the most important determining factor

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is the amount of irrigation water and the total number of irrigation applications, to further clarify these conditions and the effect of effluent water on the development of aerobic rice, more quality parameters and more genotypes would be necessary in the upcoming experiments. But effluent water from the intensive African catfish farm could be utilized for irrigation purposes what can hinder the negative effects (mainly nutrient content) of this water for the natural water bodies. Finally, the use of effluent water in a more complex agriculture system, such as agroforestry, can also provide a natural solution for the utilization of effluents and also for the restoration and conservation of natural water resources.

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ALTERATION OF ORAL MICROBIOME IN CHILDREN AFTER USING MISWAK (*SALVADORA PERSICA* L.) MADE FROM ARAK AS A NATURAL TOOTHPASTE

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Abstract. The influence of Miswak (*Salvadora persica* L.) that is made from arak on the oral microbiome signatures of 6- to 12-year-old Saudi children was studied. Deep sequencing was done for the V3-V4 regions of bacterial 16S rRNA. Sequence tags were assigned to 291 OTUs (operational taxonomic units) across samples with ≥ 97% similarity. The results indicated that factors like ethnic background and/or geographic origin can influence differences in healthy oral microbiome signatures. The results indicated some changes in the overall structure and diversity of oral microbiome. At genera level, there are five abundant phyla. At the species level, there are two opportunistic pathogens and the unassigned species as well as bacteria of the Proteobacteria family significantly decreased, the unassigned species belonging to the two genera increased due to swaking. The high abundance of *Streptococcus* and *Megasphaera* genera and low abundance of *Veillonella* genus are biomarkers of good oral hygiene as the first two genera (producer) can catabolize carbohydrates to the useful short-chain organic acids in biofilm formation, while the third (consumer) relies mainly on the fermentation of organic acids. These results shed light on the possible anti-microbial and anti-inflammatory properties of Miswak in addition to the role in removing plaque. In summary, we claim that Miswak is an excellent natural toothpaste for maintaining good oral hygiene, especially for children.
Keywords: *oral microflora, dysbiosis, Streptococcus, Megasphaera, Veillonella, organic acids*

Introduction

The human body contains ~100 trillion bacterial cells representing 1000 bacterial species or more. This bacteriome influences important parameters in human health including immune response, nutrient absorption, body weight, etc. (Gill et al., 2006; Pflughoeft and Versalovic, 2012). Although most of these bacterial species promote human health, others contribute to human illness (Greenblum et al., 2012; Ley, 2010). Other reports indicate that

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the type of bacteria is not the only factor affecting human health, but the global microbiome balance was proved to have influence on human health (Huttenhower et al., 2012; Methé et al., 2012). Therefore, it is important to detect the normal symbiotic bacterial community that holds the healthy performance in human and how dysbiosis of this community encounters for diseases (Costello et al., 2009; Qin et al., 2010; Turnbaugh et al., 2009).

The mouth cavity is the major gateway to the human body, which influences the gastrointestinal microbiome and subsequent status of human health (Meurman, 2010). Dysbiosis of oral bacteriome has been linked with several life-threatening disorders including cardiovascular disease, stroke, pneumonia, etc. (Awano et al., 2008; Beck and Offenbacher, 2005; Joshipura et al., 1996, 2003; Offenbacher et al., 1998; Seymour et al., 2007). Simon-Soro et al. (2018) indicated that combined assessment of host response along with host oral microbiome can reveal clusters of health and disease. This indicates that oral microbiome can offer biomarkers of oral health (Alcaraz et al., 2012) and disease (Gomez and Nelson, 2017). Simon-Soro and colleagues also indicated that susceptibility to oral disease can be influenced by host immune factors, with emphasis to those existing in saliva. The latter contains a complex mixture of innate anti-microbial proteins and adaptive immune mediators with a significant impact on the microbial colonization of the oral cavity (Hancock et al., 2016; Mookherjee and Hancock, 2007). Previous reports indicate the high diversity among oral microbiomes of healthy individuals (inter-individual variation) (Aas et al., 2005; Nasidze et al., 2009), but little is known about the link between the structure and diversity of the oral microbiomes of healthy individuals in different ethnics/geographic regions (Mason et al., 2013). Interestingly, Nasidze and colleagues (Nasidze et al., 2009) indicated that normal saliva microbiome differs as we go further from the equator. In addition, saliva microbiome differs due to the human lifestyle and diet (Nasidze et al., 2011).

Salvadora persica L., also known as Miswak, is a tooth (or chewing) stick that is recommended by Prophet "Muhammad". *Salvadora persica* belongs to the family Salvadoraceae and order Brassicales (*Table 1*). This plant is a well-branched evergreen tree, with soft yellow wood, that is capable to tolerate severe abiotic stresses (Haque and Alsareii, 2015; Khatak et al., 2010). The World Health Organization (WHO) has recommended the use of this stick as a natural toothbrush for healthy oral hygiene (WHO, 1984). The plant is known to contain important chemical constituents such as vitamin C, alkaloids, trimethylamine, tannins, saponins, organic sulphur compounds, lignan glycosides, etc. (Ohtani et al., 1992). Besides, the plant possesses a number of bioactive compounds with important pharmacological properties (Aumeeruddy et al., 2018). These properties include anti-microbial, anti-oxidant, anti-ulcer, anti-inflammatory, and anti-tumor activities besides being recently feasible in several biotechnological applications (Lebda et al., 2018).

Table 1. *The taxonomic classification of Salvadora persica*

Kingdom	Plantae
Division	Magnoliphyta
Class	Magnoliopsida
Order	Brassicales
Family	Salvadoraceae
Genus	Salvadora
Species	*Persica oleoides*
Binomial name	*Salvadora persica*

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The present study aims at detecting the influence of swaking with *Salvadora persica* L. (Miswak) for one month on maintaining or restoring healthy oral microbiome in Saudi children and assessing the possible protective effects against human opportunistic pathogens.

Materials and methods

Recruitment of participants and sample collection

The study was approved by the Ethics Committee of King Abdulaziz University Hospital (KAUH, Jeddah, Saudi Arabia) under number 066-16 in 2018 and written informed consent was obtained from parents of all participant children. All participant children received oral examination and those with a history of immunosuppression or systemic diseases, use of medications that reduce saliva flow, or exposure to anti-microbials in the previous three months were excluded from the study. A total of 10 Saudi children were selected based on the pervious criteria with age ranging from 6 to 12 years old. Children have used Miswak once a day for one month. The saliva samples were collected before (BEF) and after (AF) swaking in Oragene-DNA (OG-500) Self-Collection kit (DNA Genotek Inc., Canada) and mixed with stabilizing reagent in the collection tubes per manufacturer's instructions and stored at 4 °C.

DNA extraction and partial 16S rRNA gene sequencing

Genomic DNA was extracted using the QIAamp® DNA Mini kit (Qiagen®51306; Hilden, North Rhine-Westphalia, Germany) according to the manufacturer's instructions. DNA purity was evaluated via A260/A280 ratio using NanoDrop 7000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and DNA integrity was checked by 1% agarose gel electrophoresis. PCR amplification of the V3-V4 regions of bacterial 16S rRNA was performed using the universal primers 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). PCR program was the following: initial denaturation at 95°C for 5 min; 25 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 40 s; and final extension of 72 °C for 10 min. Amplicons were run on agarose gel (1.2%), then gel-purified using DNA Gel Extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Amplicons were, then, shipped to Beijing Genome Institute (BGI) in China for library construction and deep sequencing on Illumina Miseq platform to recover ~300 bp pair-end reads of the V3 and V4 regions. The ends of each read were overlapped to generate high-quality, full-length reads. The resulted sequencing data has been deposited in the European Nucleotide Archive (ENA) (https://www.ebi.ac.uk/ena/submit/sra/#studies) under the project number PRJEB27276.

16S dataset processing and statistical analysis

Sample size estimation was performed to determine the probability that the samples are representative (Motulsky, 2010). The raw sequencing data were analyzed using the Quantitative Insights Into Microbial Ecology 2 (QIIME2) package v.2018.11; (https://qiime2.org) (Bokulich et al., 2018; Bolyen et al., 2019). V3-V4 16S rRNA sequence reads were trimmed using trimmomatic software (Version 0.33) and merged into single sequences using FLASH (Version 1.2.10). Merged sequences were filtered to remove the low-quality sequences. The latter are the reads shorter than 110

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nucleotides, reads truncated at any site with an average quality score of < 20 over a 50-bp sliding window, or the truncated reads that were shorter than 50 bp. Only sequences that overlapped for more than 10 bp were assembled. The unique sequence set was linked to tags and classified into operational taxonomic units (OTUs) with a cutoff of 97% identity using the *de novo* OTU selection strategy. We retained only OTUs with at least 0.01% mean relative abundance, as predominant. OTUs were ranked by the relative abundance values as x- and y-axis, then the rank curve was drawn by software R (Version 3.1.1). Taxonomies were assigned by the RDP classifier (Version 2.2) (Cole et al., 2013) against the Human Oral Microbiome Database (Chen et al., 2010) (HOMD RefSeq, Version 13.2) and the Greengenes database (version 13.8: 16S rDNA database, http://qiime.org/home_static/dataFiles.html) with a confidence threshold of 0.7. Chimeric sequences were removed using Usearch (Version 8.0).

Alpha diversity was assessed by Shannon and Simpson indices that were calculated by Mothur (v1.31.2), and the corresponding boxplot of alpha diversity and rarefaction curve were drawn by software R (Version 3.1.1). Drawing rarefaction curve was based on calculating OTU numbers of the extracted tags (in multiples of 500) and detecting the maximum depth (no. reads) permitted to retain all samples in the dataset. Sequences were extracted randomly according to the minimum sequence number for all samples, and the extracted sequences formed a new 'OTU table biom' file. To detect the beta diversity within and between groups, the weighted and unweighted UniFrac distances were calculated (Lozupone et al., 2011) and plotted *via* principal coordinate analysis (PCoA) using package 'ade4' of software R (Version 3.1.1). UniFrac uses the system evolution information to compare the composition of community species between samples. The results can be used as a measure of beta diversity. It takes into account the distance of evolution between species, and the bigger the index, the greater the differences between samples. The UniFrac is divided into weighted UniFrac and unweighted UniFrac of which the weighted UniFrac considers the abundance of sequences, while unweighted UniFrac gives more weight on species presence/absence.

Heat maps were generated using the package 'gplots' of software R (Version 3.0.3) (https://github.com/talgalili/gplots). At phylum level, all species were used to draw the heat map and taxa of which abundance was less than 0.5% in all samples were classified as 'others'. To minimize the differences degree of the relative abundance value, the values were all log transformed. The representative sequences were aligned against the Silva core set. Representative OTU phylogenetic tree was constructed using the QIIME 2 (v.2018.11) built-in scripts including the fast-tree method for tree construction. The tags with the highest abundance of each genus was chosen as the corresponding genus representative sequences, and genus level phylogenetic tree was obtained by the same way as the OTU phylogenetic tree. Then, the phylogeny tree was imaged by R phylogenetics packages. Venn diagram was drawn by Venny (http://bioinfogp.cnb.csic.es/tools/venny/), while differences in the relative abundances of taxa at the phylum, genus and species levels were analyzed using Metastats (Paulson et al., 2011). PERMANOVA was used to test significance among values. All statistical tests were two-sided, and P value ≤ 0.05 was considered significant. Benjamini-Hochberg false discovery rate (FDR) correction was used to correct for multiple hypothesis testing where applicable. A GitHub repository link (https://github.com/shandley/Microbiome-Analysis-Using-R.git) providing the applied codes was added.

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Results

Statistics of oral 16S rRNA sequence datasets

In the present study, the oral microbiome was detected for a group of 10 Saudi children (subjects) who used Miswak as a natural toothpaste. Illumina MiSeq was used in analyzing 20 salivary samples based on the 16S rRNA. Statistics of the raw data description and its processing is shown in *Table 2*. The average sequence length per read was 297 bp across different samples ranging from 293 to 300 bp. A total of 2,305,029 clean sequence reads were generated across subjects before and after swaking with average read numbers of 116,476 and 114,027 per subject, respectively (*Fig. 1*). A total of 2,241,369 tag-linked sequences were generated across subjects before and after swaking with average read numbers of 113,346 and 110,791 per subject, respectively. While, a total of 1,700,687 sequence tags were generated across subjects before and after swaking with average read numbers of 86,072 and 83,995 per subject, respectively (*Fig. 1*). These sequence tags were assigned to 291 OTUs (operational taxonomic units) across samples with ≥ 97% similarity. A summation of 4,147 OTUs for the 20 samples were generated with an average of 207 OTUs per sample ranging from 146 to 257 OTUs and averages of 206 and 208 OTUs per subject before and after swaking (*Figs. 2* and *A1*). The overall number of OTUs before swaking (BEF) was 289, while 282 after swaking (AF). The results for the number of observed species (number of OTUs) per subject indicated increases in four subjects, while resulted in decreases in five subjects and no change in one subject (*Fig. 2*).

Table 2. *Statistics of data generated from deep sequencing for 10 Saudi children before (BEF) and after (AF) swaking for one month*

Sample ID	Reads length (bp)	Raw data (Mbp)	N base (%)	Low quality (%)	Clean data (Mbp)	Data utilization (%)	Raw reads	Clean reads	Read utilization (%)
BEF2	300:295	89.24	0.002	7.765	73.06	81.87	149,985	130,663	87.12
BEF6	299:295	79.81	0.001	6.864	66.83	83.74	134,352	119,170	88.70
BEF8	298:295	73.22	0.001	7.257	60.23	82.26	123,481	108,164	87.60
BEF9	296:295	84.64	0.001	7.787	69.78	82.44	143,209	125,526	87.65
BEF10	297:294	70.10	0.001	7.719	57.33	81.79	118,613	103,360	87.14
BEF12	296:294	65.60	0.000	7.847	53.49	81.55	111,179	96,635	86.92
BEF14	294:294	79.26	0.001	7.752	64.36	81.21	134,790	117,250	86.99
BEF15	293:294	70.97	0.002	7.396	57.99	81.71	120,904	105,761	87.48
BEF19	300:294	92.49	0.001	7.376	76.47	82.69	155,701	136,473	87.65
BEF20	299:294	82.00	0.001	7.163	67.93	82.84	138,284	121,761	88.05
AF2	294:296	74.17	0.002	6.002	62.50	84.27	125,711	113,133	89.99
AF6	293:296	64.83	0.001	6.418	54.24	83.67	110,064	98,130	89.16
AF8	300:296	83.51	0.001	6.229	70.41	84.32	140,110	125,524	89.59
AF9	299:296	88.14	0.003	5.953	74.70	84.76	148,127	133,382	90.05
AF10	298:296	65.37	0.001	6.203	54.80	83.82	110,056	98,229	89.25
AF12	296:296	80.26	0.001	6.397	67.79	84.46	135,575	121,608	89.70
AF14	297:295	88.56	0.002	7.310	73.27	82.74	149,588	131,711	88.05
AF15	296:295	69.51	0.003	7.725	57.17	82.25	117,610	102,860	87.46
AF19	294:295	64.55	0.001	7.524	52.85	81.87	109,588	95,807	87.42
AF20	293:295	80.93	0.001	7.712	66.05	81.62	137,635	119,882	87.10

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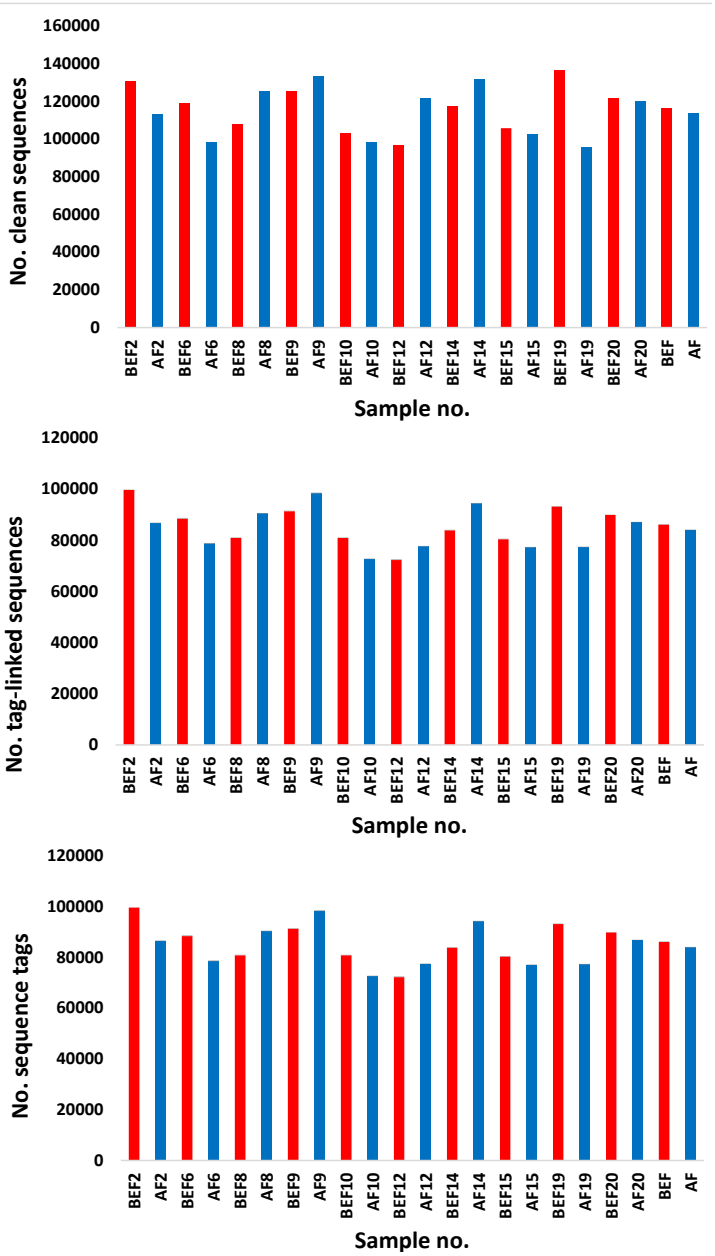

Figure 1. *Comparison among numbers of clean and tagged sequences along with the recovered sequence tags at the sample and group levels before (red) and after (blue) swaking for one month to sequences statistics. BEF = before swaking, AF = after swaking*

Diversity and rarefaction curve analysis

Description of observed species detected from OTU annotation is shown in *Table A1*. Alpha diversity was applied to analyze complexity of species. Shannon and Simpson indices as alpha diversity measures indicated no significance between BEF and AF groups (*Fig. 2*). Shannon and Simpson values reflect the species diversity of the community at both species' richness and evenness levels. But Shannon index comprises more weight on sequence richness, while Simpson index comprises more weight on evenness. With the same species richness, the greater the species evenness, the greater the community diversity. Alpha diversity per subject indicated increases in six subjects

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due to swaking for one month in terms of Shannon measures (*Fig. 2*). Almost opposite results were detected in terms of Simpson measures (*Fig. 2*). These results indicated that the use of Miswak has resulted in increased microbial species richness and decreased species evenness. In other words, Miswak likely changed the overall structure of oral microbiome during such a short period of treatment. Alpha diversity at the group level showed much higher diversity in AF group due to swaking than BEF group (*Fig. 3*). However, further prolonged studies might be required to prove whether salivary bacterial diversity is related to oral hygiene status, especially after swaking, or not.

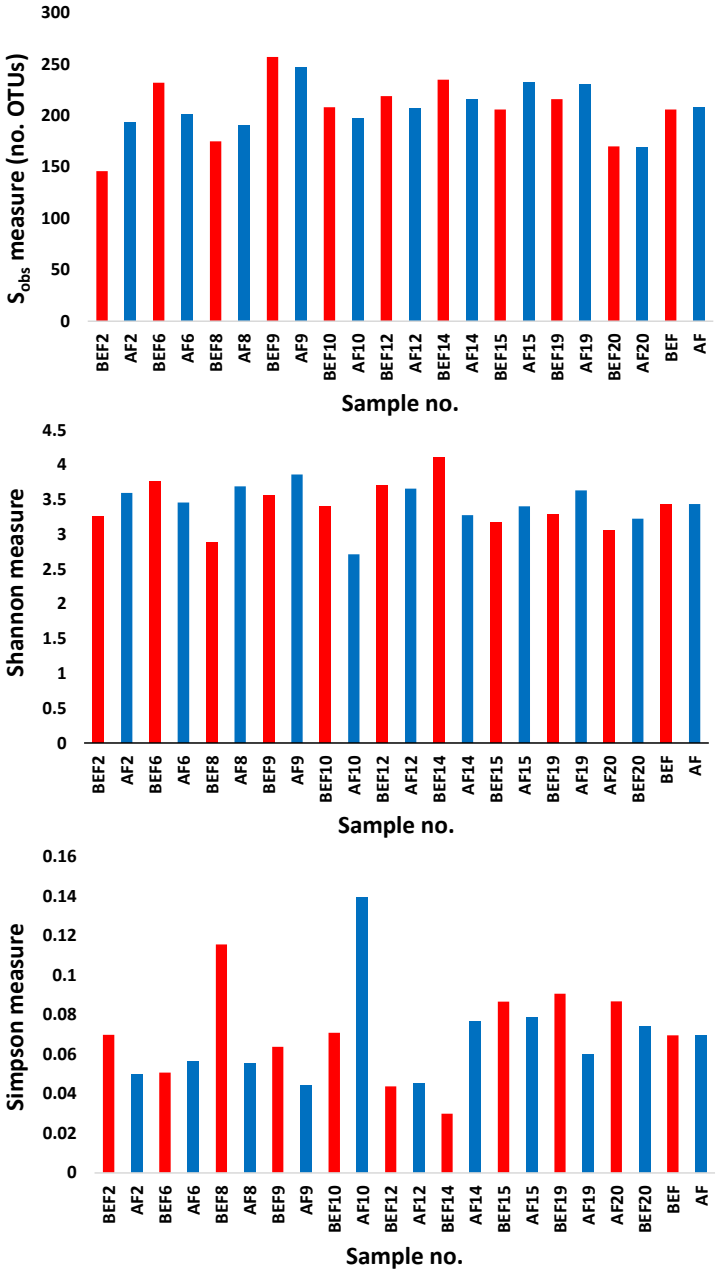

Figure 2. *Alpha diversity measures at the sample and group levels before (red) and after (blue) swaking for one month to describe number of species (OTUs) per sample or group, sample or group richness (Shannon index) and evenness (Simpson index). BEF = before swaking, AF = after swaking*

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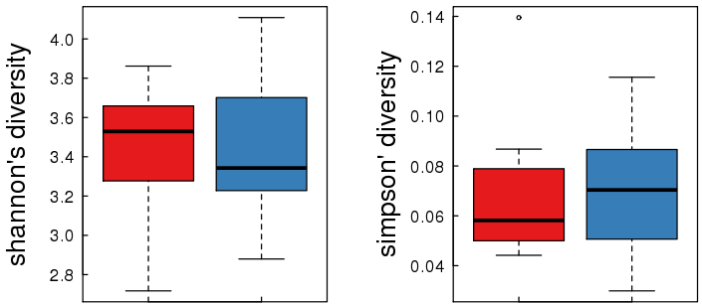

Figure 3. *Alpha diversity indices as boxplots to describe richness and evenness at the group level of the samples before (red) and after (blue) swaking for one month*

Rarefaction curves based on stacked number of OTUs (*Fig. 4*) were analyzed in order to describe the maximum depth permitted to retain all samples in the dataset for studying taxonomic relative abundance and to evaluate if produced data is enough to cover all species in the microbial community. When the curve tends to drop (*Fig. 4*) or no longer climbs, this indicates that the produced data is enough for further analysis. The more the curve continues to climb with increasing sequencing reads, the higher the complexity will be in samples, i.e., there will still be species uncovered by the sequencing data. The two rarefaction measures indicated that the maximum number of sequences reads to be used for further analysis of taxonomy abundance is 72,000 (*Fig. 4*).

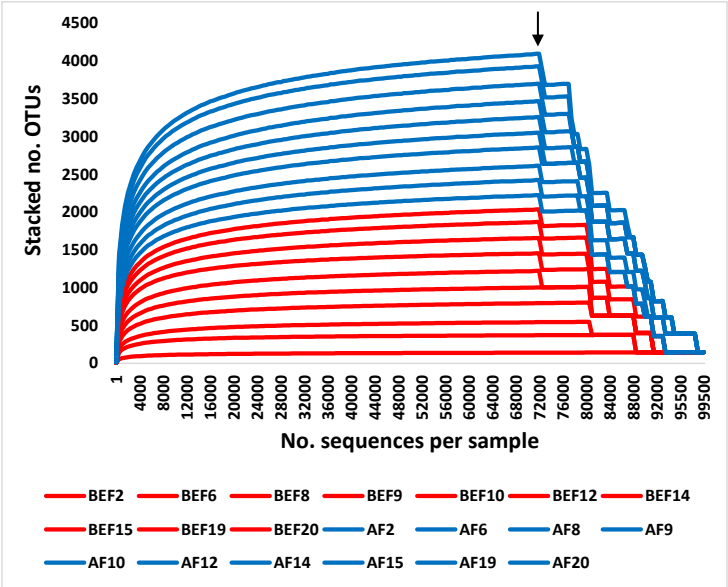

Figure 4. *Stacked number of OTUs as rarefaction measures to describe the maximum depth permitted to retain all samples in the dataset for studying taxonomic relative abundance. The arrows indicate the suitable sample size for analyzing taxonomy abundance (72,000 sequence reads). BEF = before swaking, AF = after swaking*

In order to display the diversity and differences of OTU composition in different samples and groups, principal coordinate analysis (PCoA) was used (*Fig. 5*). PCoA summarizes factors mainly responsible for this difference. When similarity is high, the two groups are closely located. Based on the OTU abundance information, the relative

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abundance of each OTU in each sample and group was calculated, thus the PCoA of OTUs was plotted (*Fig. 5*). The PCoA plot partially showed a similar tendency in the distances within and between groups. The diversity of BEF subjects was higher towards PCoA 2 direction (PC2), while diversity of AF subjects was higher towards PCoA 1 direction (PC1). Overall, the diagram showed that mean value of BEF group was localized in the positive directions of PCoA 1 and PCoA 2 (PC1 and PC2), while that of AF group was localized in the negative directions (*Fig. 5*). These results indicated that the microbiome signatures of the two groups differed due to swaking, while holding relatively similar microbiome compositions as the swaking time of the experiment was short.

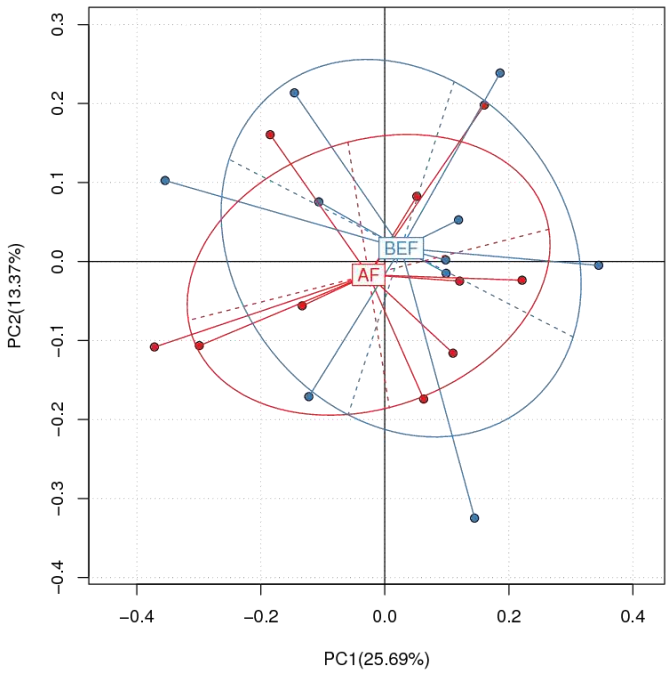

Figure 5. *PCoA based on OTU abundance of different samples. Blue box indicate sample mean before swaking. Red box indicate sample mean after swaking for one month. X-axis is the first principal coordinate and Y-axis is the second. Numbers in brackets represent contributions of PCoAs to differences among samples. A dot represents each sample, and different colors represent different groups*

Oral microbiomes at phylum and genus levels

Phylogenetic tree describing taxonomic groups of oral microbiomes at phylum and genus levels are shown in *Figure 6*. A phylogenetic tree is a branching diagram showing the inferred evolutionary relationships among various biological taxa based upon similarities and differences in their physical or genetic characteristics. The evolutionary distance between taxa is closer if the branch length is shorter. Besides the taxa composition and abundance analysis, phylogenetic tree could clarify the species evolutionary relationship further. The results indicated that the most common phyla are Actinobacteria (seven genera), Bacteroidetes (six genera), Firmicutes (28 genera), Fusobacteria (two genera), Proteobacteria (11 genera), Sphirochaetes (one genus), Synergistetes (two genera) and Tenericutes (two genera) (*Fig. 6*).

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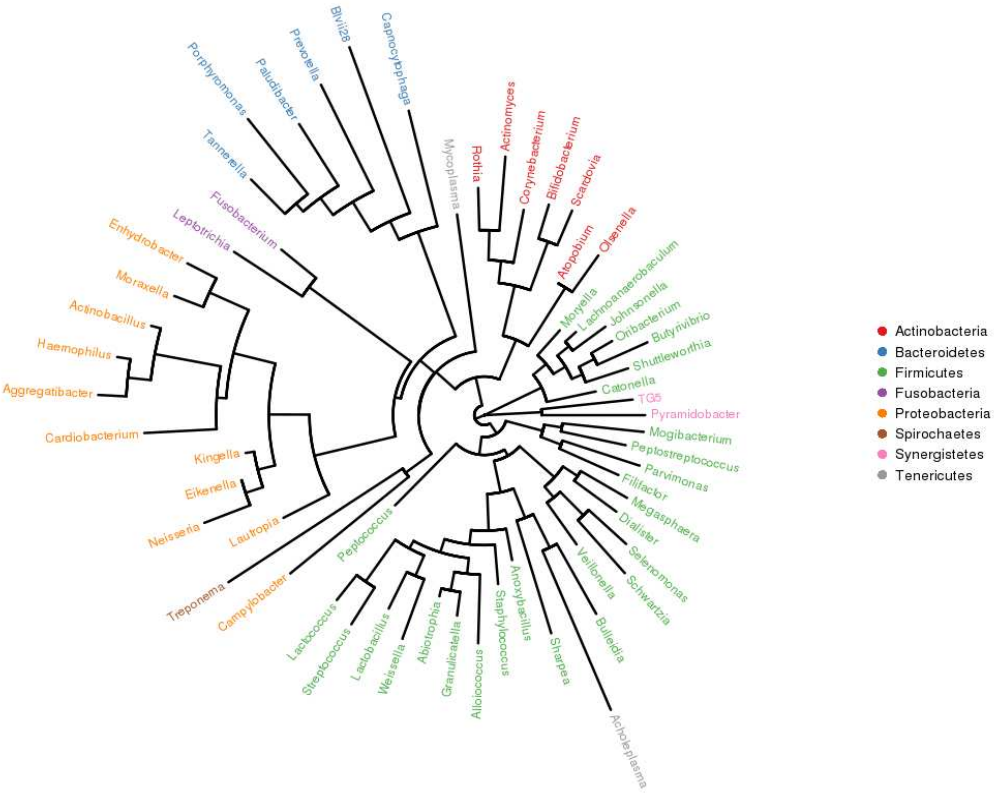

Figure 6. *Genus level phylogenetic tree of oral microbiome. Genera with the same color belong to the same phylum*

Abundance of individual OTUs across samples was also studied in which OTUs with number of sequences over 10,000 were considered highly abundant (*Fig. 7*). This criterion was met by a number of 29 out of the 219 OTUs (*Table A2*). These selected OTUs are OTU1-OTU12, OTU14, OTU15, OTU17, OTU20, OTU21, OTU23, OTU25-OTU29, OTU32, OTU33, OTU37, OTU40, OTU193 and OTU261. *Table A2* also indicates richness of these OTUs for different samples before and after swaking. Description of these selected highly abundant OTUs in terms of taxonomy of their phyla, genera and/or species is shown in *Table A3*. The results of *Table A3* indicate that the highly abundant OTUs belong to five of the previously mentioned phyla (e.g., Bacteroidetes, Firmicutes, Proteobacteria, Fusobacteria, Actinobacteria) in addition to the recently discovered phylum of Saccharibacteria, previously known as TM7. These results are consistent with those of log-scaled percentage heat map at the phylum level (*Fig. A2*). A heat map is a graphical representation of data where the individual values contained in a matrix are represented as colors.

The five highly abundant phyla included a number of 25 genera/species (*Table A3*). The latter include assigned and unassigned species of genera *Prevotella* and *Porphyromonas* of Bacteroidetes; *Streptococcus*, *Veillonella*, *Gemella*, *Megasphaera*, *Clostridium* and *Granulicatella* of Firmicutes; *Haemophilus*, *Campylobacter*, *Neisseria, Moraxella* and *Pasteurella* of Proteobacteria; *Fusobacterium* and *Leptotrichia* of Fusobacteria; while *Rothia* of Actinobacteria. However, no genus/species information is available for the new phyla Saccharibacteria TM7-3 because, up to date, these bacteria is culture-independent.

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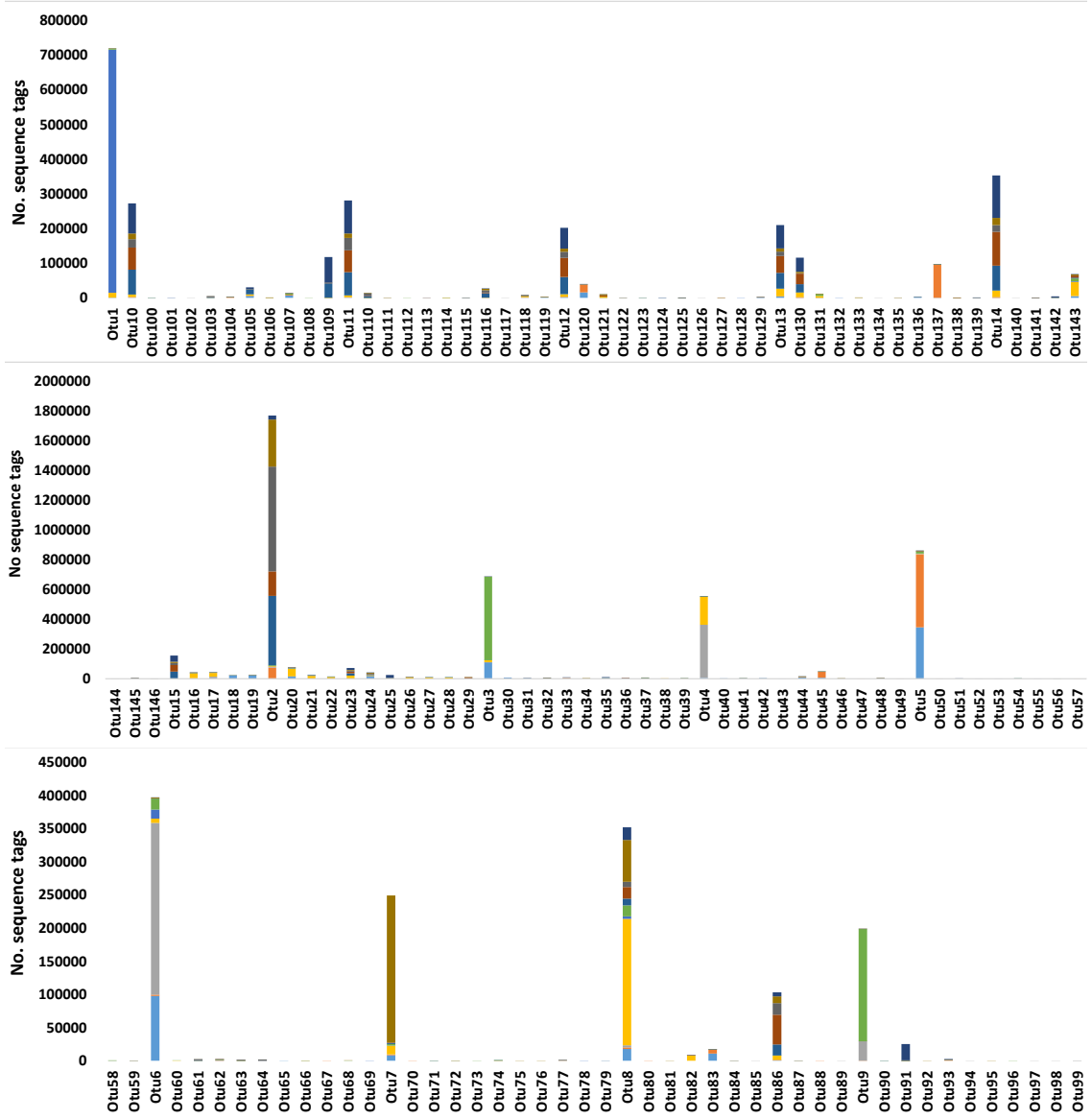

Figure 7. *OTU abundances across samples*

At the genus level, *Streptococcus* was the most abundant genus, with *S. infantis* as the most abundant species, followed by *Veillonella* (mainly *V. dispar*), *Prevotella* (mainly *P. melaninogenica*), *Haemophilus* (mainly *H. parainfluenzae*), *Rothia* (mainly *R. mucilaginosa*), *Neisseria* (mainly *N. subflava*), *Fusobacterium*, *Campylobacter* and the candidate phyla *Saccharibacteria* (*Table A3*). These results are consistent with those of log-scaled percentage heat maps at genus and species levels (*Figs. A3* and *A4*). Many of the highly abundant genera (e.g., *Veillonella, Campylobacter*, *Clostridium*, *Prevotella*, etc.) and species (e.g., *V. dispar*, *H. parainfluenzae*, *R. mucilaginosa*, etc.) are opportunistic pathogens and reflect the lack or the poor hygiene that requires attention, especially at childhood period.

Venn diagram indicated the existence of 280 common OTUs in both the BEF and AF groups (*Fig. 8*). The number of OTUs uniquely found in BEF group was nine representing the taxonomic groups *Streptococcus* spp., *Aggregatibacter*,

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Veillonellaceae, *Prevotella* spp., *Pyramidobacter piscolens*, *Bifidobacterium* spp., *Bulleidia* spp., *Prevotella* spp. and *Streptophyta I*), while two (*Actinobacillus* spp. and *Kingella* spp.) in AF group. Each of these taxonomic groups were present in only 1-2 samples, while only *Streptococcus* spp. was present in five BEF samples. However, this result was not considered affective because the number of sequences for the specific OTU of this taxa (e.g., OTU138) is as little as 72. Thus, the 11 group-specific OTUs were not considered effective in distinguishing between the two groups or in detecting the influence of swaking for one month on children oral microbiomes (*Fig. 8*).

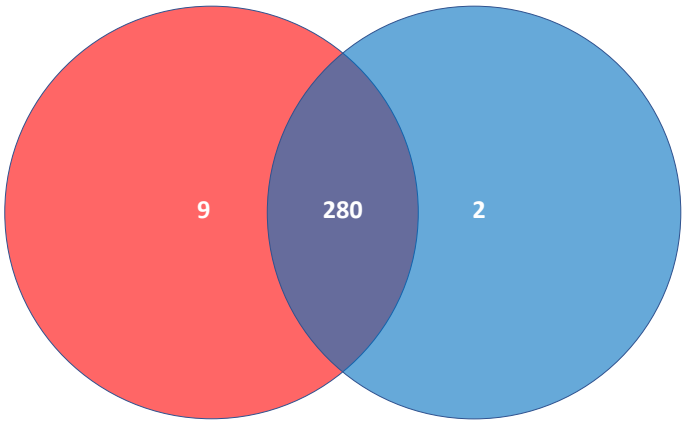

Figure 8. *Venn diagram describing the unique (nine for BEF and two for AF) and shared OTUs (280) between the two groups of samples before (red) and after (blue) swaking for one month. BEF = before swaking, AF = after swaking. The nine BEF OTUs involve OTU138, OTU181, OTU224, OTU232, OTU243, OTU257, OTU260, OTU266 and OTU 267. The two AF OTUs involve OTU127 and OTU269. The other OTUs (280) are shared between the two groups with different relative abundances*

Differential abundance of microbes due to swaking for one month

Differential abundance of microbes of different subjects before and after swaking for one month was studied at phylum, genus and species levels (*Figs. 9, 10* and *11,* respectively). Weighted Unifrac diversity distances within subjects indicated major changes in microbiome signature due to swaking in six out of the ten subjects, while unweighted Unifrac diversity distances within subjects indicated major changes in only two subjects. We did not consider the unweighted Unifrac results as the number of diverged subjects is less than four. As indicated earlier, prolonged swaking time might result in higher distances at the weighted and unweighted Unifrac levels. A number of 12 phyla, 27 genera and 21 species showed considerable changes within each subject's microbiome due to swaking (*Figs. 9, 10* and *11,* respectively). Overall, phyla Actinobacteria and Bacteroidetes decreased, while Firmicutes increased due to swaking for one month.

Statistical analysis for the highly abundant OTUs recovered from oral microbiomes with number of sequences over 10,000 indicated significant increases due to swaking across subjects in two OTUs referring to unassigned species of the genera *Streptococcus* and *Megasphaera* (*Fig. 12; Table A4*), while significant decreases in five OTUs referring to opportunistic pathogens *Veillonella dispar* and *Rothia mucilaginosa*; unassigned species of genera *Campylobacter* and *Prevotella* as well as bacteria of the family Pasteurellaceae (*Fig. 13; Table A4*).

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UniFrac diversity distance											
W	0.227	0.146	0.305	0.191	0.201	0.170	0.241	0.281	0.279	0.118	NA
UW	0.273	0.202	0.159	0.144	0.119	0.158	0.193	0.169	0.143	0.154	NA

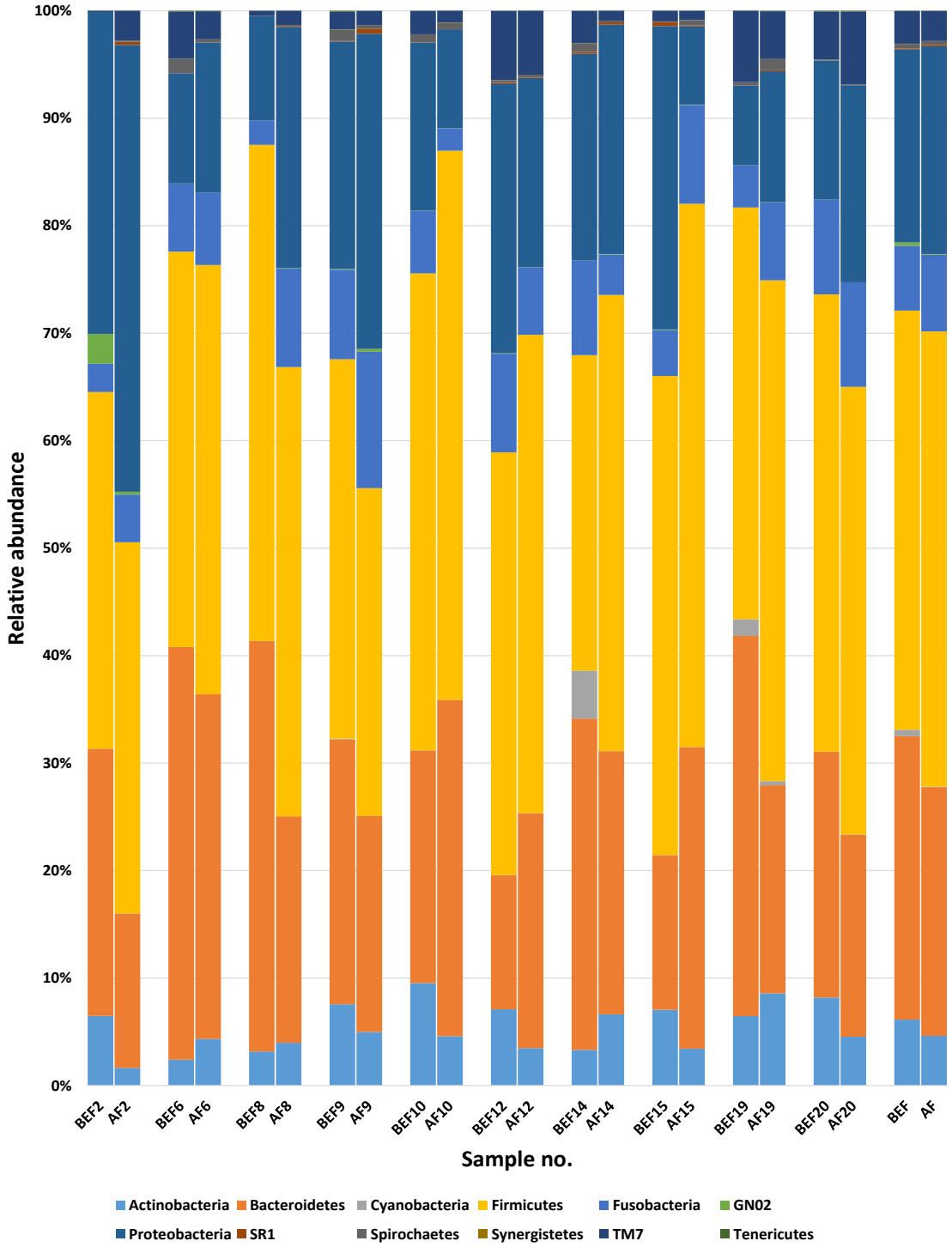

Figure 9. *Relative abundance at phylum level as measured by Metastats at sample and group levels before (red) and after (blue) swaking for one month. BEF = before swaking, AF = after swaking. On top of the figure: pink box = distance of ≥ 2, while blue box = distance of < 2 to describe both weighted_Unifrac (W) and unweighted_Unifrac (UW) diversity distances between sample pairs of each subject (before/after)*

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UW	0.273	0.202	0.159	0.144	0.119	0.158	0.193	0.169	0.143	0.154	NA

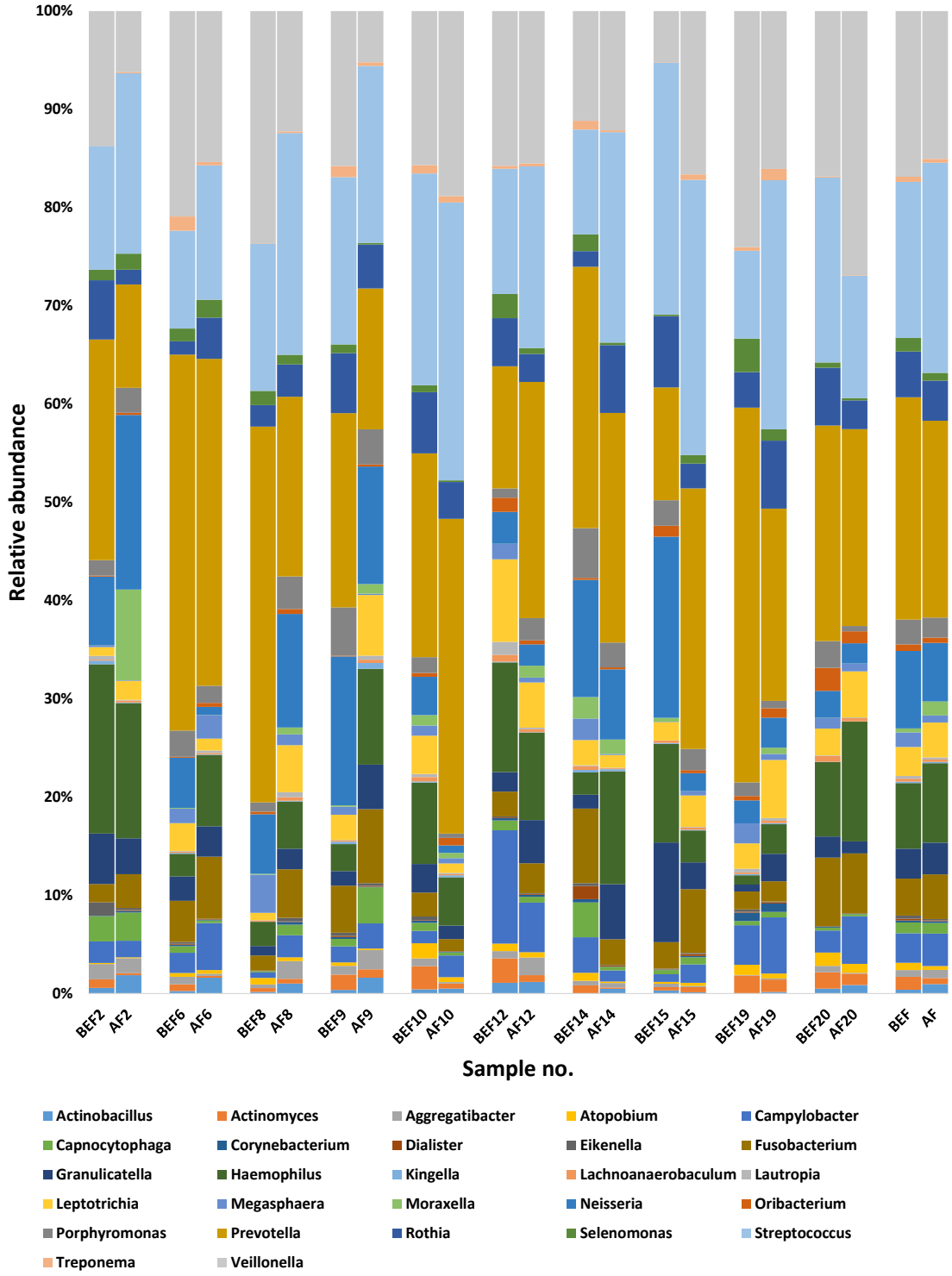

Figure 10. *Relative abundance at genus level as measured by Metastats at sample and group levels before (red) and after (blue) swaking for one month. BEF = before swaking, AF = after swaking. On top of the figure: pink box = distance of ≥ 2, while blue box = distance of < 2 to describe both weighted_Unifrac (W) and unweighted_Unifrac (UW) diversity distances between sample pairs of each subject (before/after)*

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UniFrac diversity distance											
W	0.227	0.146	0.305	0.191	0.201	0.170	0.241	0.281	0.279	0.118	NA
UW	0.273	0.202	0.159	0.144	0.119	0.158	0.193	0.169	0.143	0.154	NA

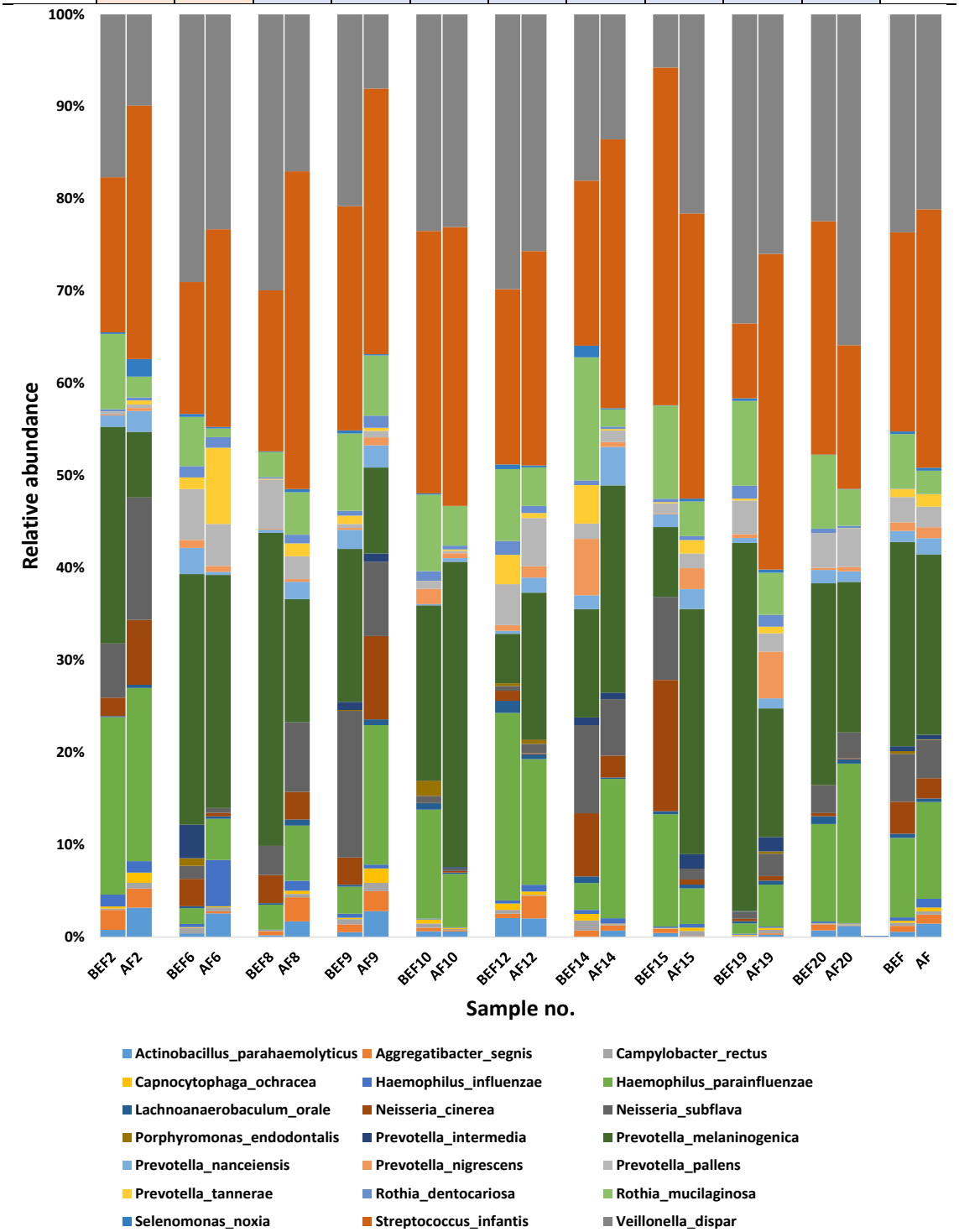

Figure 11. *Relative abundance at species level as measured by Metastats at sample and group levels before (red) and after (blue) swaking for one month. BEF = before swaking, AF = after swaking. On top of the figure: pink box = distance of ≥ 2, while blue box = distance of < 2 to describe both weighted_Unifrac (W) and unweighted_Unifrac (UW) diversity distances between sample pairs of each subject (before/after)*

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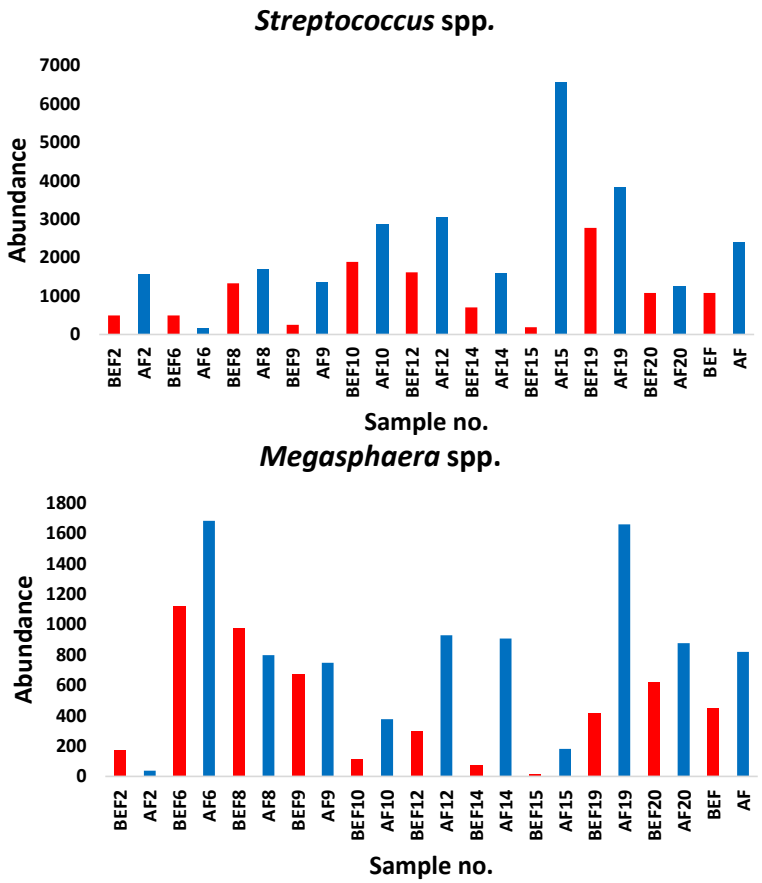

Figure 12. *High abundance of the unassigned species of the genera Streptococcus and Megasphaera due to swaking for one month. BEF = before swaking, AF = after swaking*

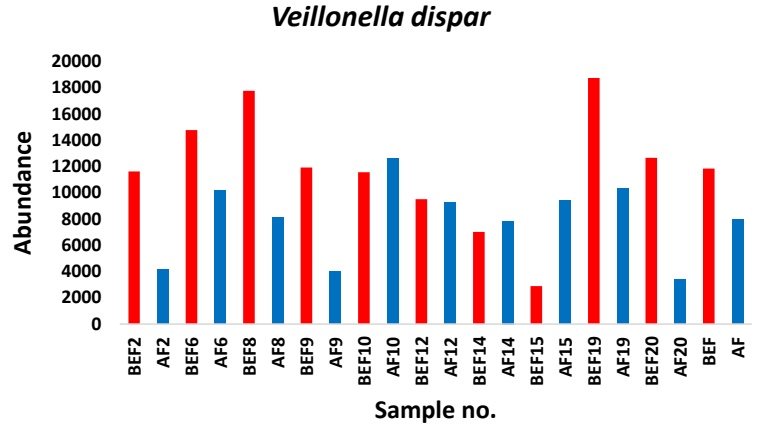

Figure 13. *Low abundance of Veillonella dispar, Rothia mucilaginosa, unassigned species of the genera Campylobacter and Prevotella as well as bacteria of the family Pasteurellaceae due to swaking for one month. BEF = before swaking, AF = after swaking*

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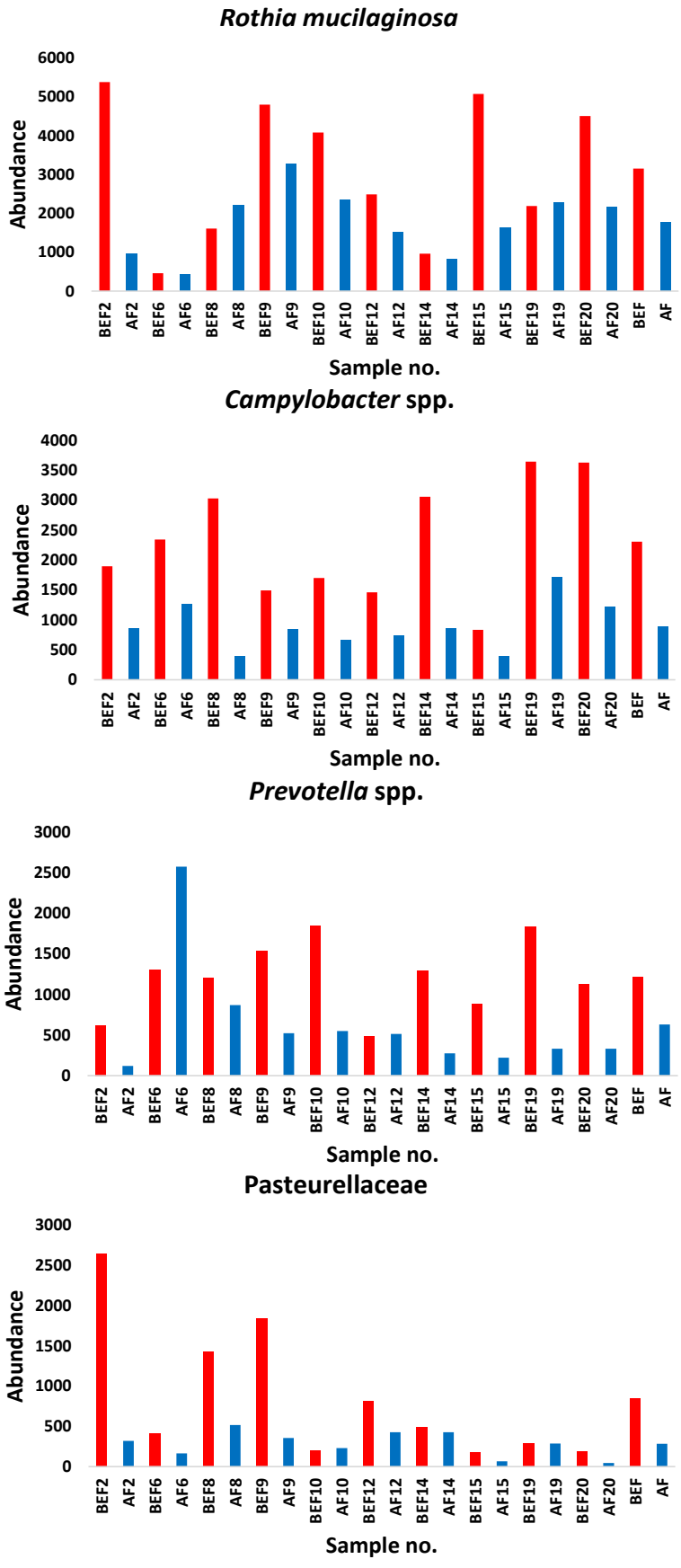

Figure 13. *Contiued*

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Discussion

Oral microbiome significantly affects overall human health. It is organized as biofilms adapted to every niche within the mouth. This biofilm has fundamental role in host homeostasis and the protection against oral as well as gastric diseases (Ismail et al., 2009; Kau et al., 2011).

Normally, the predominant bacteria in saliva are commensal and produce enzymes that degrade biofilm matrix polymers (Kaplan et al., 2004; Pereira et al., 2012) Poor oral hygiene can lead to changes in microbial communities and cause dental caries and periodontal disease that seriously affect children's overall life-time health status (Marsh, 2003; Prabhu and John, 2015). Therefore, it is important to detect oral microbe biofilm signatures in healthy and infected individuals in order to develop preventive strategies, especially in childhood.

Earlier studies indicated that changes in the microbiome balance might have a more important influence on human health than a distinct microbial species being either beneficial or harmful (Huttenhower et al., 2012; Methé et al., 2012). Although a distinct microbial species might not be harmful in its primary habitat, oral bacteria were linked with systemic, life-threatening disorders including cardiovascular disease, pneumonia and stroke (Awano et al., 2008; Beck and Offenbacher, 2005; Joshipura et al., 2003; Joshipura et al., 1996; Seymour et al., 2007). Oral microbiome is highly divergent among healthy individuals as several ecological factors, such as life style, external environment and oral hygiene, can contribute to this divergence (Aas et al., 2005; Crielaard et al., 2011; Nasidze et al., 2009; Sheiham and Watt, 2000).

Other factors include ethnic background and/or geographic origin (Aas et al., 2005; Ledder et al., 2007). Several bacterial genera/phyla fundamentally influence human oral health/disease status. They include *Actinomyces* spp., *Gemella* spp., *Granulicatella* spp., *Veillonella* spp., *Haemophilus* spp., *Capnocytophaga* spp. and TM7. For example, early colonization of *Veillonella* spp. and existence of phylum TM7 guide the development of microbial biofilm communities (Liu et al., 2012; Periasamy and Kolenbrander, 2010). As indicated earlier, *Streptococcus* was the most abundant genus in oral micrbiomes of Saudi children regardless of swaking, followed by *Veillonella*, *Prevotella*, *Haemophilus*, *Fusobacterium*, *Rothia*, *Neisseria*, *Campylobacter* and the candidate phyla *Saccharibacteria* (*Table A3*). These results are consistent with previous studies of the human oral microbiota (Hoffman et al., 2018; Li et al., 2014; Takeshita et al., 2014).

High-throughput technologies are employed in detecting microbiome signatures in different human organs and their relation to disease risk/status (Gao et al., 2017; Jo et al., 2017). The approach of the cost-efficient, high-throughput characterization of the human microbiome relies on the use of microbial 16S ribosomal RNA (rRNA) gene sequence that is considered as a barcode of microbes, either culturable or unculturable (Ahn et al., 2011). Such an approach can provide new insights into the diversity, normal microbial signatures and health/disease status (Ahn et al., 2011; Belda-Ferre et al., 2012; Johansson et al., 2016; Zhou et al., 2016). Many problems raised when isolating oral bacterial DNA from clinical samples due to the lack of bacterial lysis uniformity, particularly for gram-positive bacteria (Lazarevic et al., 2013). Although Vesty et al. (2017) recommended the use of enzymatic lysis method for bacterial DNA extractions, Garbieri et al. (2017) recommended the use of commercial DNA extraction kits. We have chosen the QIAamp® DNA Mini kit (Qiagen®51306; Hilden, North Rhine-Westphalia, Germany) for extracting high-quality oral bacterial DNA. Another problem lies in the choice of the hypervariable regions (V1-V9) of 16S rRNA gene that

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adequately and reliably detect oral microbiome signatures. Besides, it is important to know the proper database for OTU annotation. Based on the most recent studies, the 16S V3-V4 regions of oral microbiome were successful in detecting accurate microbial signatures and in providing adequate phylogenetic assignment (Furquim et al., 2017; Jiang et al., 2016; Yu et al., 2017). Accordingly, we have chosen the V3-V4 regions for analyzing the oral microbiome. We have also chosen the Greengenes (version 13_8: 16S rDNA database, http://qiime.org/home_static/dataFiles.html) (DeSantis et al., 2006) and Human Oral Microbiome (HOMD, http://www.homd.org/) (Chen et al., 2010) databases for annotating the oral microbiome sequences.

The influence of swaking on human oral microbiome was statistically studied on the 29 most abundant families, genera and species (*Table A4*). The overall results indicated that swaking for one month resulted in the significant increases of the unassigned species of the genera *Streptococcus* and *Megasphaera* (*Table A4; Fig. 12*) and the significant decreases of the bacteria *Veillonella dispar* and *Rothia mucilaginosa*; unassigned species of genera *Campylobacter* and *Prevotella* as well as bacteria of the family Pasteurellaceae (*Table A4; Fig. 13*). Although extremely abundant, *Streptococcus anginosus* (previously known as *Streptococcus milleri*) and *S. infantis* showed insignificant increases due to swaking (*Table A4*). Otherwise, the influence of Miswak was arbitrary for the other highly abundant taxa with no specific pattern across subjects. Although it resides in normal flora of the oral cavity, *Streptococcus anginosus* is considered as a strong virulent pathogen (Liu et al., 2018). It is speculated that the significant increase of oral *Streptococcus anginosus* associates with periodontal diseases (Drucker and Green, 1977; Liu et al., 2018; Rawlinson et al., 1993). *Streptococcus infantis* is a microbe that lives mainly in the human upper respiratory tract and usually found in healthy mouths, e.g., free of or are at low risk of tooth decay (http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2444020/, http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3472979/).

Miller et al. (2017) showed an increased evidence that the commensal acetaldehydogenic microorganism *Rothia mucilaginosa* can be an opportunistic pathogen in immune-compromised hosts, while Amer et al. (2017) indicated that the increased abundance of *R. mucilaginosa* is linked to oral leukoplakias from lingual sites. Elevated levels of *R. mucilaginosa* was also shown to be associated with human endocarditis, meningitis and peritonitis (https://en.wikipedia.org/wiki/Rothia). Amer et al. (2017) also indicated that the increased levels of *Campylobacter concisus* is associated with severe dysplasia. *Campylobacter* spp. is the main cause of food poisoning (campylobacteriosis) affecting up to 1% of the world (https://en.wikipedia.org/wiki/Campylobacter). Recently, Liu et al. (2018) detected high abundance of *Prevotella intermedia* in saliva of gout and hyperuricemia (HUA) patients and suggested that abundance of this bacterial species is linked with gum inflammation and the progression of periodontitis. This conclusion complements a previous one made by Kamma et al. (1994). As for the bacteria of the family Pasteurellaceae, it was recently reported to be associated with caries in young children living in a rural province in China (Xu et al., 2018). The significant decreases of *Rothia mucilaginosa*, *Campylobacter* spp., *Prevotella* and Pasteurellaceae due to swaking in the present study (*Fig. 13*) can be considered as biomarkers of good hygiene in children.

Most of the normal commensal oral bacteria benefit the host through the production of various metabolites. They mostly include short chain fatty acids, vitamins, co-factors and other metabolites (Nallabelli et al., 2016). However, there is a strong reverse action

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of the two colonizers, e.g., *Streptococcus* and *Veillonella* in oral cavity bacteria (Hoffman et al., 2018). Mashima et al. (2017) indicated that unassigned species of *Streptococcus* spp. decreased, while *Veillonella dispar*, *V. parvula* and unassigned species of *Veillonella* spp. increased with poor oral hygiene status. *Streptococcus*, a facultative anaerobe and an initial colonizer, is able to catabolize (and transport) carbohydrates to short-chain organic acids, e.g., lactic acid and pyruvic acid (Cotter and Hill, 2003). On the other hand, *Veillonella*, an obligate anaerobe and subsequent colonizer, is unable to catabolize sugars, thus, it relies mainly on the fermentation of organic acids to propionic and acetic acids, carbon dioxide, and hydrogen (Delwiche et al., 1985). It is reported that oral *Veillonella* depends on organic acids produced by oral *Streptococcus* (Mashima and Nakazawa, 2014; Palmer et al., 2006). The proximity of the producer/consumer shuttle is important in such metabolite transfers (Mashima and Nakazawa, 2014; Palmer et al., 2006). Organic acids participate mainly in biofilm formation due to the action of *Streptococcus* (Periasamy and Kolenbrander, 2010). Anaerobic environment promotes growth of *Veillonella* due to the prior growth of aerobic and facultative organisms that results in an increased plaque thickness to yield conditions suitable for anaerobic growth (Ritz, 1967). The growth of *Streptococcus* may be inhibited by anaerobic conditions that favor the growth of *Veillonella*. Therefore, the high abundance of *Veillonella* in salivary microbiome is considered as a biomarker of poor oral hygiene and occurrence of caries in children (*Fig. 14*). Pharmacological studies indicated that Miswak possesses anti-microbial, anti-plaque and anti-inflammatory activities that makes it useful in defeating plaque (Lebda et al., 2018). The latter potential activities align with our results in terms of the decrease of *Veillonella* spp., the bacteria that favors the accumulation of plaque. *Veillonella* was also found in higher proportion in caries or periodontal subjects (Zhang et al., 2015; Zhou et al., 2016).

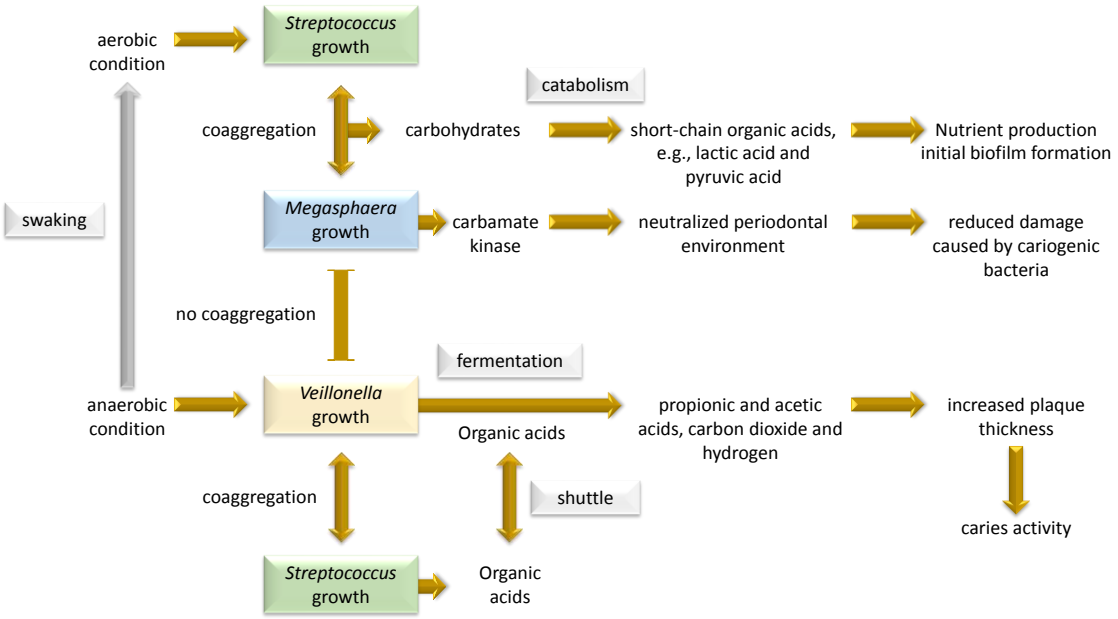

Figure 14. *Roles of the three genera Streptococcus, Veillonella and Megasphaera and possible influence of swaking on oral hygiene*

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Conclusion

In general, we claim that swaking hinders the anaerobic condition required for *Veillonella* growth, thus favors the aerobic condition that promote *Streptococcus* growth. Our results also indicate parallel significant increases of the unassigned species of the genera *Streptococcus* and *Megasphaera* (*Fig. 12; Table A4*). Interestingly, *Megasphaera* spp. strain DISK18, a nonpathogenic species, was recently reported to coaggregate with *Streptococcus* during oral early colonization but not with *Veillonella* (Nallabelli et al., 2016). It is speculated that genera *Megasphaera* and *Veillonella* compete for coaggregating with *Streptococcus* due to homology of surface receptors of these two genera (Kreth et al., 2009; Nallabelli et al., 2016). Whole genome sequencing of the *Megasphaera* strain showed a lack of virulence genes, that are associated with oral pathogenesis, or genes encoding collagenase or gelatinase. The latter indicates that *Megasphaera* unlikely participates in periodontal disease. Instead, this bacteria harbors carbamate kinase that participate in neutralizing the periodontal environment, thus, reduce the damage caused by cariogenic bacteria (*Fig. 14*). In summary, we consider Miswak as an excellent natural toothpaste with differential influence on oral microbiome that makes it feasibly useful for maintaining good oral hygiene, especially for children. Future studies on the new selected species are still required in order to support the results of the present research.

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APPENDIX

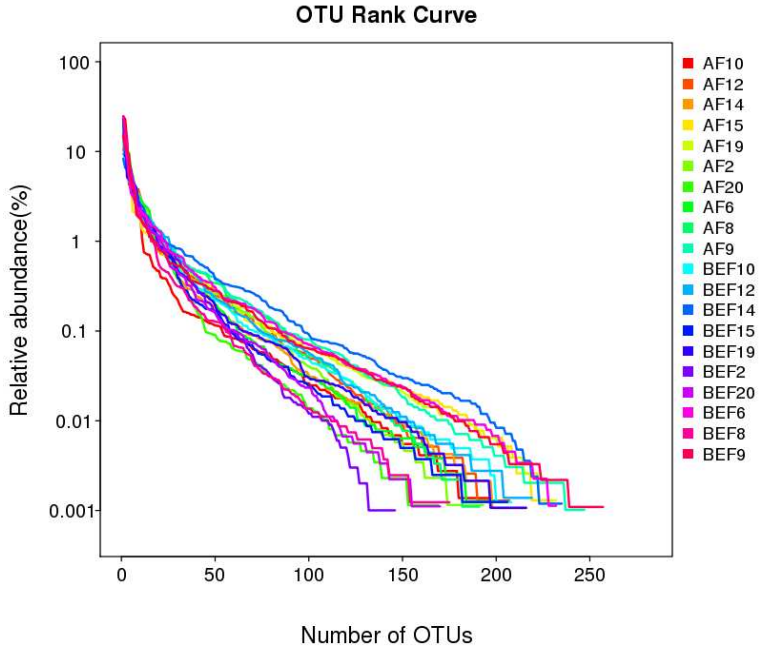

Figure A1. *Number and relative abundance of OTUs of different samples. BEF = before swaking, AF = after swaking*

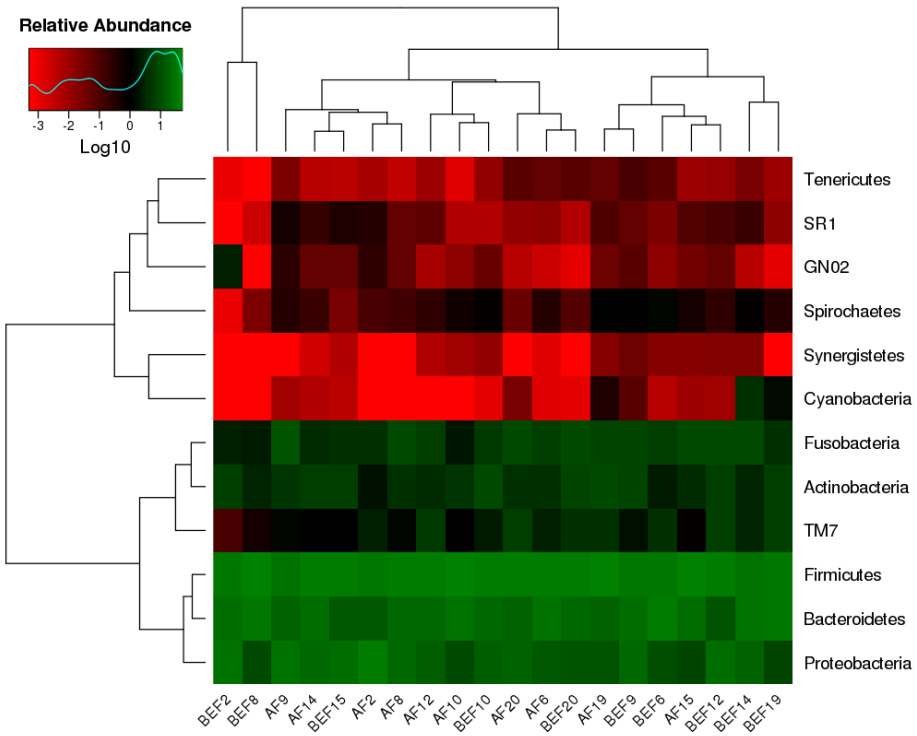

Figure A2. *Log-scaled percentage heat map at phylum level. BEF = before swaking, AF = after swaking*

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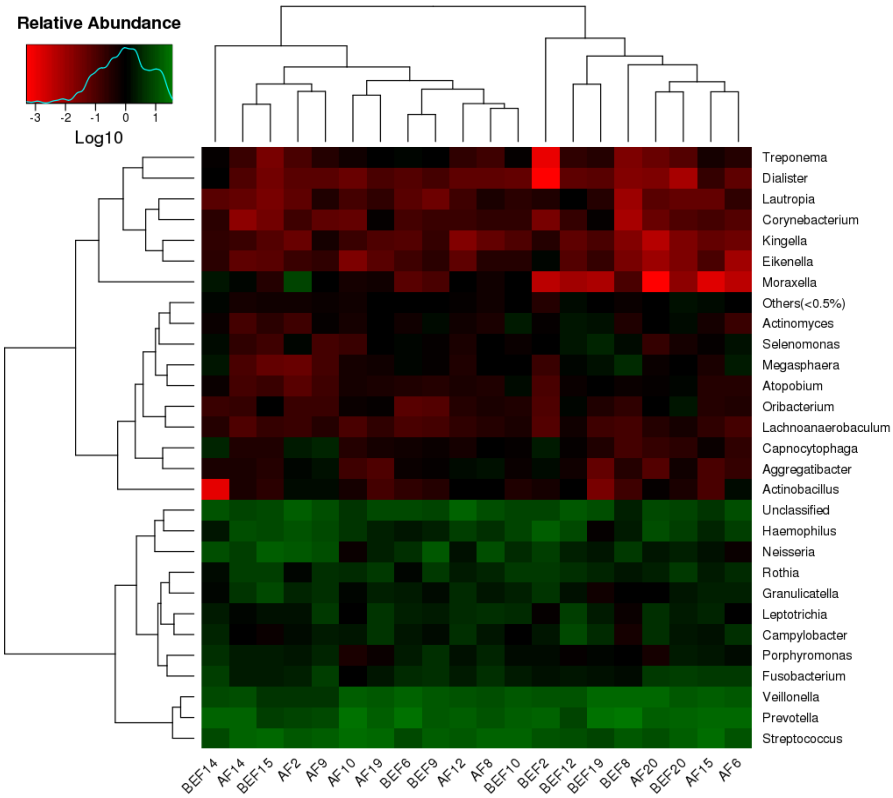

Figure A3. *Log-scaled percentage heat map at genus level. BEF = before swaking, AF = after swaking*

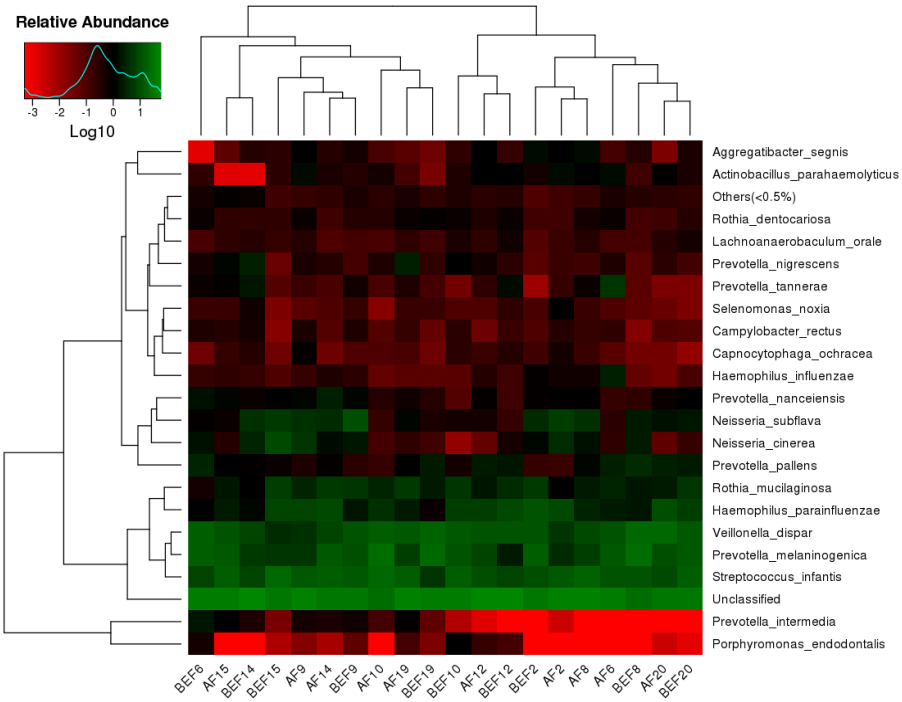

Figure A4. *Log-scaled percentage heat map at species level. BEF = before swaking, AF = after swaking*

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Table A1. *Description of observed species detected from OTU annotation across subjects and swaking*

OTU no.	OTU abundance	Taxonomy
Otu2	235993	Bacteria; Firmicutes; Bacilli; Lactobacillales; Streptococcaceae; Streptococcus; Streptococcus_infantis
Otu1	195394	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella; Prevotella_melaninogenica
Otu3	163677	Bacteria; Firmicutes; Clostridia; Clostridiales; Veillonellaceae; Veillonella; Veillonella_dispar
Otu4	92051	Bacteria; Proteobacteria; Gammaproteobacteria; Pasteurellales; Pasteurellaceae; Haemophilus; Haemophilus_parainfluenzae
Otu11	58531	Bacteria; Fusobacteria; Fusobacteriia; Fusobacteriales; Fusobacteriaceae; Fusobacterium
Otu8	58093	Bacteria; Actinobacteria; Actinobacteria; Actinomycetales; Micrococcaceae; Rothia; Rothia_mucilaginosa
Otu32	50183	Bacteria; Firmicutes; Clostridia; Clostridiales; Veillonellaceae; Veillonella; Veillonella_dispar
Otu14	46624	Bacteria; Firmicutes; Bacilli; Gemellales; Gemellaceae
Otu6	40872	Bacteria; Proteobacteria; Betaproteobacteria; Neisseriales; Neisseriaceae; Neisseria; Neisseria_subflava
Otu5	39815	Bacteria; Proteobacteria; Epsilonproteobacteria; Campylobacterales; Campylobacteraceae; Campylobacter
Otu12	34029	Bacteria; Firmicutes; Bacilli; Lactobacillales; Streptococcaceae; Streptococcus
Otu10	33068	Bacteria; TM7; TM7-3
Otu7	32641	Bacteria; Firmicutes; Bacilli; Lactobacillales; Carnobacteriaceae; Granulicatella
Otu29	29782	Bacteria; Proteobacteria; Betaproteobacteria; Neisseriales; Neisseriaceae; Neisseria
Otu261	27911	Bacteria; Proteobacteria; Betaproteobacteria; Neisseriales; Neisseriaceae; Neisseria; Neisseria_cinerea
Otu17	23783	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella; Prevotella_pallens
Otu15	22697	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella
Otu25	20994	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Porphyromonadaceae; Porphyromonas
Otu21	18426	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Paraprevotellaceae; Prevotella
Otu20	17155	Bacteria; Fusobacteria; Fusobacteriia; Fusobacteriales; Leptotrichiaceae; Leptotrichia
Otu37	14672	Bacteria; Firmicutes; Clostridia; Clostridiales; Veillonellaceae; Megasphaera
Otu27	13910	Bacteria; Firmicutes; Bacilli; Lactobacillales; Carnobacteriaceae; Granulicatella
Otu26	13468	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella; Prevotella_nanceiensis
Otu9	13242	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Moraxellaceae; Moraxella
Otu40	13051	Bacteria; Firmicutes; Clostridia; Clostridiales; Veillonellaceae; Veillonella
Otu33	12906	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Paraprevotellaceae; Prevotella
Otu193	12432	Bacteria; Proteobacteria; Gammaproteobacteria; Pasteurellales; Pasteurellaceae
Otu23	11707	Bacteria; Firmicutes; Clostridia; Clostridiales
Otu28	10024	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Paraprevotellaceae; Prevotella; Prevotella_tannerae
Otu109	9878	Bacteria; Proteobacteria; Gammaproteobacteria; Pasteurellales; Pasteurellaceae; Haemophilus
Otu49	9750	Bacteria; Proteobacteria; Gammaproteobacteria; Pasteurellales; Pasteurellaceae; Actinobacillus; Actinobacillus_parahaemolyticus
Otu18	9704	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella; Prevotella_nigrescens

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OTU no.	OTU abundance	Taxonomy
Otu55	8814	Bacteria; Fusobacteria; Fusobacteriia; Fusobacteriales; Leptotrichiaceae; Leptotrichia
Otu24	8592	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Oribacterium
Otu38	8380	Bacteria; Actinobacteria; Coriobacteriia; Coriobacteriales; Coriobacteriaceae; Atopobium
Otu42	8302	Bacteria; Firmicutes; Clostridia; Clostridiales; Veillonellaceae; Selenomonas
Otu68	8159	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella
Otu53	8127	Bacteria; Proteobacteria; Gammaproteobacteria; Pasteurellales; Pasteurellaceae; Aggregatibacter; Aggregatibacter_segnis
Otu245	7056	Bacteria; Firmicutes; Clostridia; Clostridiales; Veillonellaceae; Veillonella
Otu51	6869	Bacteria; Actinobacteria; Actinobacteria; Actinomycetales; Micrococcaceae; Rothia; Rothia_dentocariosa
Otu41	6741	Bacteria; Firmicutes; Clostridia; Clostridiales; Mogibacteriaceae
Otu50	6255	Bacteria; Actinobacteria; Actinobacteria; Actinomycetales; Actinomycetaceae; Actinomyces
Otu19	6183	Bacteria; Proteobacteria; Gammaproteobacteria; Pasteurellales; Pasteurellaceae; Haemophilus; Haemophilus_influenzae
Otu39	6124	Bacteria; TM7; TM7-3; CW040
Otu277	5959	Bacteria; Proteobacteria; Betaproteobacteria; Neisseriales; Neisseriaceae; Neisseria; Neisseria_subflava
Otu65	5820	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Porphyromonadaceae; Porphyromonas
Otu31	5140	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella
Otu35	5103	Bacteria; Fusobacteria; Fusobacteriia; Fusobacteriales; Leptotrichiaceae; Leptotrichia
Otu22	5023	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella; Prevotella_intermedia
Otu13	4905	Bacteria; Cyanobacteria; Chloroplast; Streptophyta
Otu88	4889	Bacteria; Fusobacteria; Fusobacteriia; Fusobacteriales; Leptotrichiaceae; Leptotrichia
Otu155	4814	Bacteria; Fusobacteria; Fusobacteriia; Fusobacteriales; Leptotrichiaceae; Leptotrichia
Otu61	4797	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella
Otu70	4788	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Porphyromonadaceae; Porphyromonas
Otu34	4397	Bacteria; Bacteroidetes; Flavobacteriia; Flavobacteriales; Flavobacteriaceae; Capnocytophaga
Otu52	4247	Bacteria; Firmicutes; Clostridia; Clostridiales
Otu66	4201	Bacteria; TM7; TM7-3; CW040; F16
Otu76	4044	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Lachnoanaerobaculum; Lachnoanaerobaculum_orale
Otu69	3903	Bacteria; Bacteroidetes; Flavobacteriia; Flavobacteriales; Flavobacteriaceae; Capnocytophaga
Otu57	3763	Bacteria; TM7; TM7-3
Otu36	3476	Bacteria; Proteobacteria; Betaproteobacteria; Neisseriales; Neisseriaceae; Eikenella
Otu48	3440	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Burkholderiaceae; Lautropia
Otu16	3440	Bacteria; GN02; BD1-5
Otu64	3415	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Paraprevotellaceae; Prevotella
Otu84	3406	Bacteria; Proteobacteria; Epsilonproteobacteria; Campylobacterales; Campylobacteraceae; Campylobacter; Campylobacter_rectus
Otu47	3381	Bacteria; Bacteroidetes; Flavobacteriia; Flavobacteriales; Weeksellaceae

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OTU no.	OTU abundance	Taxonomy
Otu93	3367	Bacteria; Proteobacteria; Gammaproteobacteria; Pasteurellales; Pasteurellaceae; Aggregatibacter
Otu126	3331	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella
Otu87	3074	Bacteria; Bacteroidetes; Flavobacteriia; Flavobacteriales; Flavobacteriaceae; Capnocytophaga
Otu77	2993	Bacteria; Firmicutes; Clostridia; Clostridiales; Veillonellaceae; Selenomonas; Selenomonas_noxia
Otu271	2986	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella; Prevotella_melaninogenica
Otu75	2939	Bacteria; Actinobacteria; Actinobacteria; Actinomycetales; Corynebacteriaceae; Corynebacterium
Otu98	2827	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella
Otu59	2750	Bacteria; Actinobacteria; Actinobacteria; Actinomycetales; Actinomycetaceae; Actinomyces
Otu80	2727	Bacteria; Fusobacteria; Fusobacteriia; Fusobacteriales; Leptotrichiaceae; Leptotrichia
Otu86	2462	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella
Otu81	2433	Bacteria; Actinobacteria; Actinobacteria; Actinomycetales; Actinomycetaceae; Actinomyces
Otu206	2425	Bacteria; Actinobacteria; Actinobacteria; Actinomycetales; Actinomycetaceae; Actinomyces
Otu137	2241	Bacteria; Firmicutes; Clostridia; Clostridiales; Veillonellaceae; Selenomonas
Otu62	2126	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Catonella
Otu67	2056	Bacteria; Fusobacteria; Fusobacteriia; Fusobacteriales; Leptotrichiaceae; Leptotrichia
Otu71	2001	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella
Otu45	1972	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella; Prevotella_melaninogenica
Otu280	1908	Bacteria; Proteobacteria; Gammaproteobacteria; Pasteurellales; Pasteurellaceae; Haemophilus
Otu274	1904	Bacteria; Proteobacteria; Betaproteobacteria; Neisseriales; Neisseriaceae
Otu108	1902	Bacteria; Fusobacteria; Fusobacteriia; Fusobacteriales; Fusobacteriaceae; Fusobacterium
Otu117	1867	Bacteria; Spirochaetes; Spirochaetes; Spirochaetales; Spirochaetaceae; Treponema
Otu90	1864	Bacteria; Firmicutes; Erysipelotrichi; Erysipelotrichales; Erysipelotrichaceae; Bulleidia; Bulleidia_moorei
Otu72	1834	Bacteria; Bacteroidetes; Flavobacteriia; Flavobacteriales; Weeksellaceae
Otu100	1827	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella
Otu78	1823	Bacteria; SR1
Otu146	1794	Bacteria; Proteobacteria; Epsilonproteobacteria; Campylobacterales; Campylobacteraceae; Campylobacter
Otu44	1788	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae
Otu46	1702	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Porphyromonadaceae; Porphyromonas; Porphyromonas_endodontalis
Otu105	1665	Bacteria; Fusobacteria; Fusobacteriia; Fusobacteriales; Leptotrichiaceae; Leptotrichia
Otu58	1645	Bacteria; Bacteroidetes; Flavobacteriia; Flavobacteriales; Flavobacteriaceae; Capnocytophaga; Capnocytophaga_ochracea
Otu30	1638	Bacteria; Fusobacteria; Fusobacteriia; Fusobacteriales; Leptotrichiaceae
Otu79	1577	Bacteria; Fusobacteria; Fusobacteriia; Fusobacteriales; Leptotrichiaceae
Otu56	1534	Bacteria; Firmicutes; Clostridia; Clostridiales; Veillonellaceae; Dialister
Otu124	1505	Bacteria; Firmicutes; Clostridia; Clostridiales; Veillonellaceae; Selenomonas

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OTU no.	OTU abundance	Taxonomy
Otu140	1498	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Paraprevotellaceae; Prevotella
Otu115	1295	Bacteria; Firmicutes; Bacilli; Lactobacillales; Aerococcaceae; Abiotrophia
Otu43	1184	Bacteria; Firmicutes; Clostridia; Clostridiales
Otu125	1134	Bacteria; Fusobacteria; Fusobacteriia; Fusobacteriales; Fusobacteriaceae; Fusobacterium
Otu114	1133	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella
Otu103	1108	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella; Prevotella_nanceiensis
Otu82	1104	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Butyrivibrio
Otu144	1087	Bacteria; Spirochaetes; Spirochaetes; Spirochaetales; Spirochaetaceae; Treponema
Otu134	1077	Bacteria; Firmicutes; Bacilli; Lactobacillales; Streptococcaceae; Streptococcus; Streptococcus_anginosus
Otu106	1067	Bacteria; Firmicutes; Clostridia; Clostridiales; Peptostreptococcaceae; Peptostreptococcus
Otu151	964	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Moryella
Otu85	953	Bacteria; Proteobacteria; Betaproteobacteria; Neisseriales; Neisseriaceae
Otu92	952	Bacteria; Proteobacteria; Betaproteobacteria; Neisseriales; Neisseriaceae; Kingella
Otu54	939	Bacteria; Firmicutes; Clostridia; Clostridiales; Veillonellaceae; Megasphaera
Otu97	904	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella
Otu63	884	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Porphyromonadaceae; Porphyromonas
Otu239	826	Bacteria; Proteobacteria; Gammaproteobacteria; Pasteurellales; Pasteurellaceae; Haemophilus; Haemophilus_parainfluenzae
Otu91	824	Bacteria; Firmicutes; Clostridia; Clostridiales; Mogibacteriaceae; Mogibacterium
Otu219	807	Bacteria; Bacteroidetes; Flavobacteriia; Flavobacteriales; Flavobacteriaceae; Capnocytophaga; Capnocytophaga_ochracea
Otu94	803	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales
Otu276	776	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella
Otu73	773	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella
Otu122	739	Bacteria; Bacteroidetes; Flavobacteriia; Flavobacteriales; Weeksellaceae
Otu129	711	Bacteria; Firmicutes; Bacilli; Lactobacillales; Streptococcaceae; Streptococcus
Otu169	705	Bacteria; Spirochaetes; Spirochaetes; Spirochaetales; Spirochaetaceae; Treponema
Otu145	698	Bacteria; TM7; TM7-3
Otu131	688	Bacteria; Proteobacteria; Betaproteobacteria; Neisseriales; Neisseriaceae; Kingella
Otu99	619	Bacteria; Cyanobacteria; Chloroplast; Streptophyta
Otu120	611	Bacteria; Firmicutes; Clostridia; Clostridiales; Tissierellaceae; Parvimonas
Otu287	608	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella; Prevotella_melaninogenica
Otu154	607	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Porphyromonadaceae; Tannerella
Otu143	604	Bacteria; Bacteroidetes; Flavobacteriia; Flavobacteriales; Flavobacteriaceae; Capnocytophaga; Capnocytophaga_ochracea
Otu217	556	Bacteria; Bacteroidetes; Flavobacteriia; Flavobacteriales; Flavobacteriaceae; Capnocytophaga
Otu268	556	Bacteria; Firmicutes; Clostridia; Clostridiales; Veillonellaceae; Selenomonas

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OTU no.	OTU abundance	Taxonomy
Otu102	551	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella
Otu107	548	Bacteria; Spirochaetes; Spirochaetes; Spirochaetales; Spirochaetaceae; Treponema; Treponema_amylovorum
Otu139	546	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Porphyromonadaceae; Paludibacter
Otu192	540	Bacteria; Spirochaetes; Spirochaetes; Spirochaetales; Spirochaetaceae; Treponema; Treponema_socranskii
Otu60	539	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Moraxellaceae; Moraxella
Otu133	502	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella
Otu74	491	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales
Otu111	474	Bacteria; TM7; TM7-3; I025; Rs-045
Otu89	474	Bacteria; Spirochaetes; Spirochaetes; Spirochaetales; Spirochaetaceae; Treponema
Otu136	467	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella
Otu194	452	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Paraprevotellaceae; Prevotella
Otu186	435	Bacteria; Spirochaetes; Spirochaetes; Spirochaetales; Spirochaetaceae; Treponema
Otu119	433	Bacteria; Bacteroidetes; Flavobacteriia; Flavobacteriales; Weeksellaceae
Otu104	419	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales
Otu159	412	Bacteria; Bacteroidetes; Flavobacteriia; Flavobacteriales; Flavobacteriaceae; Capnocytophaga
Otu285	406	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella
Otu189	398	Bacteria; Firmicutes; Clostridia; Clostridiales; Veillonellaceae
Otu96	387	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales
Otu156	380	Bacteria; Bacteroidetes; Flavobacteriia; Flavobacteriales; Flavobacteriaceae; Capnocytophaga
Otu101	373	Bacteria; Firmicutes; Clostridia; Clostridiales; Veillonellaceae; Dialister
Otu168	368	Bacteria; Proteobacteria; Betaproteobacteria; Neisseriales; Neisseriaceae; Neisseria; Neisseria_oralis
Otu113	348	Bacteria; Bacteroidetes; Flavobacteriia; Flavobacteriales; Flavobacteriaceae; Capnocytophaga
Otu123	347	Bacteria; Firmicutes; Clostridia; Clostridiales; Peptostreptococcaceae; Filifactor
Otu118	323	Bacteria; Proteobacteria; Betaproteobacteria; Neisseriales; Neisseriaceae; Kingella
Otu110	321	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Moraxellaceae; Enhydrobacter
Otu121	309	Bacteria; Spirochaetes; Spirochaetes; Spirochaetales; Spirochaetaceae; Treponema
Otu83	307	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Moryella
Otu184	305	Bacteria; Proteobacteria; Gammaproteobacteria; Cardiobacteriales; Cardiobacteriaceae; Cardiobacterium
Otu132	288	Bacteria; Firmicutes; Clostridia; Clostridiales; Peptococcaceae; Peptococcus
Otu112	275	Bacteria; Firmicutes; Clostridia
Otu177	264	Bacteria; TM7; TM7-3
Otu95	261	Bacteria; Bacteroidetes; Flavobacteriia; Flavobacteriales; Flavobacteriaceae; Capnocytophaga
Otu164	241	Bacteria; Firmicutes; Clostridia; Clostridiales; Veillonellaceae; Schwartzia
Otu176	233	Bacteria; Firmicutes; Clostridia; Clostridiales; Peptostreptococcaceae

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OTU no.	OTU abundance	Taxonomy
Otu183	231	Bacteria; Firmicutes; Clostridia; Clostridiales; Veillonellaceae; Megasphaera
Otu153	225	Bacteria; Firmicutes; Clostridia; Clostridiales; Mogibacteriaceae
Otu221	224	Bacteria; Firmicutes; Clostridia; Clostridiales; Veillonellaceae; Selenomonas
Otu170	218	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella
Otu165	215	Bacteria; Firmicutes; Clostridia; Clostridiales; Veillonellaceae; Selenomonas
Otu172	213	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Porphyromonadaceae; Tannerella
Otu162	211	Bacteria; Actinobacteria; Actinobacteria; Actinomycetales; Propionibacteriaceae
Otu147	207	Bacteria; Firmicutes; Clostridia; Clostridiales; Veillonellaceae
Otu202	207	Bacteria; Firmicutes; Clostridia; Clostridiales; Veillonellaceae; Veillonella
Otu180	202	Bacteria; Actinobacteria; Actinobacteria; Actinomycetales; Actinomycetaceae; Actinomyces
Otu148	198	Bacteria; Spirochaetes; Spirochaetes; Spirochaetales; Spirochaetaceae; Treponema; Treponema_amylovorum
Otu187	189	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae
Otu182	188	Bacteria; Spirochaetes; Spirochaetes; Spirochaetales; Spirochaetaceae; Treponema; Treponema_amylovorum
Otu163	187	Bacteria; Actinobacteria; Actinobacteria; Actinomycetales; Actinomycetaceae; Actinomyces
Otu141	184	Bacteria; Actinobacteria; Actinobacteria; Bifidobacteriales; Bifidobacteriaceae; Bifidobacterium
Otu130	183	Bacteria; TM7; TM7-3
Otu149	178	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Shuttleworthia; Shuttleworthia_satelles
Otu281	174	Bacteria; Proteobacteria; Betaproteobacteria; Neisseriales; Neisseriaceae; Kingella
Otu142	169	Bacteria; Spirochaetes; Spirochaetes; Spirochaetales; Spirochaetaceae; Treponema
Otu116	155	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Porphyromonadaceae; Porphyromonas
Otu174	148	Bacteria; Bacteroidetes; Flavobacteriia; Flavobacteriales; Flavobacteriaceae; Capnocytophaga
Otu191	144	Bacteria; Spirochaetes; Spirochaetes; Spirochaetales; Spirochaetaceae; Treponema
Otu152	143	Bacteria; Fusobacteria; Fusobacteriia; Fusobacteriales; Leptotrichiaceae; Leptotrichia
Otu150	143	Bacteria; Tenericutes; Mollicutes; Mycoplasmatales; Mycoplasmataceae; Mycoplasma
Otu179	140	Bacteria; Fusobacteria; Fusobacteriia; Fusobacteriales; Leptotrichiaceae; Leptotrichia
Otu171	139	Bacteria; Tenericutes; Mollicutes; Mycoplasmatales; Mycoplasmataceae; Mycoplasma
Otu185	133	Bacteria; Firmicutes; Clostridia; Clostridiales; Veillonellaceae; Selenomonas
Otu160	131	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella
Otu196	128	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella
Otu216	126	Bacteria; Proteobacteria; Betaproteobacteria; Neisseriales; Neisseriaceae; Neisseria; Neisseria_bacilliformis
Otu251	125	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales
Otu128	122	Bacteria; Tenericutes; Mollicutes; RF39
Otu228	122	Bacteria; GN02; BD1-5
Otu238	111	Bacteria; TM7; TM7-3; CW040; F16

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OTU no.	OTU abundance	Taxonomy
Otu250	111	Bacteria; Spirochaetes; Spirochaetes; Spirochaetales; Spirochaetaceae; Treponema
Otu188	103	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella
Otu209	101	Bacteria; Proteobacteria; Betaproteobacteria; Neisseriales; Neisseriaceae
Otu190	100	Bacteria; Actinobacteria; Actinobacteria; Actinomycetales; Corynebacteriaceae; Corynebacterium
Otu201	100	Bacteria; Fusobacteria; Fusobacteriia; Fusobacteriales; Leptotrichiaceae
Otu207	99	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella
Otu204	96	Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus; Lactobacillus_helveticus
Otu205	96	Bacteria; Actinobacteria; Actinobacteria; Actinomycetales; Propionibacteriaceae
Otu135	95	Bacteria; TM7; TM7-3; CW040
Otu227	95	Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus; Lactobacillus_salivarius
Otu279	93	Bacteria; Proteobacteria; Gammaproteobacteria; Pasteurellales; Pasteurellaceae; Haemophilus; Haemophilus_parainfluenzae
Otu167	93	Bacteria; Proteobacteria; Alphaproteobacteria; Rickettsiales; mitochondria
Otu225	93	Bacteria; Firmicutes; Erysipelotrichi; Erysipelotrichales; Erysipelotrichaceae; Bulleidia
Otu197	92	Bacteria; Firmicutes; Clostridia; Clostridiales; Peptostreptococcaceae
Otu214	89	Bacteria; Actinobacteria; Actinobacteria; Bifidobacteriales; Bifidobacteriaceae; Scardovia
Otu240	86	Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus; Lactobacillus_vaginalis
Otu158	85	Bacteria; Actinobacteria; Coriobacteriia; Coriobacteriales; Coriobacteriaceae
Otu237	85	Bacteria; Actinobacteria; Coriobacteriia; Coriobacteriales; Coriobacteriaceae
Otu291	85	Bacteria; Proteobacteria; Betaproteobacteria; Neisseriales; Neisseriaceae; Neisseria; Neisseria_subflava
Otu223	84	Bacteria; Firmicutes; Clostridia; Clostridiales; Veillonellaceae; Schwartzia
Otu127	82	Bacteria; Proteobacteria; Gammaproteobacteria; Pasteurellales; Pasteurellaceae; Actinobacillus
Otu203	81	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella
Otu226	80	Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus
Otu233	75	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella
Otu215	75	Bacteria; Cyanobacteria; Chloroplast; Streptophyta
Otu157	73	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella; Prevotella_nanceiensis
Otu138	72	Bacteria; Firmicutes; Bacilli; Lactobacillales; Streptococcaceae; Streptococcus
Otu242	71	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella
Otu210	69	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella
Otu270	67	Bacteria; Synergistetes; Synergistia; Synergistales; Dethiosulfovibrionaceae; TG5
Otu222	67	Bacteria; Actinobacteria; Coriobacteriia; Coriobacteriales; Coriobacteriaceae; Olsenella; Olsenella_profusa
Otu256	66	Bacteria; Firmicutes; Bacilli; Bacillales; Staphylococcaceae; Staphylococcus
Otu272	66	Bacteria; Firmicutes; Clostridia; Clostridiales; Veillonellaceae; Dialister
Otu289	65	Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus; Lactobacillus_delbrueckii

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OTU no.	OTU abundance	Taxonomy
Otu195	63	Bacteria; Firmicutes; Erysipelotrichi; Erysipelotrichales; Erysipelotrichaceae; Sharpea
Otu235	61	Bacteria; Proteobacteria; Gammaproteobacteria; Cardiobacteriales; Cardiobacteriaceae; Cardiobacterium
Otu263	61	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Oribacterium
Otu255	60	Bacteria; Synergistetes; Synergistia; Synergistales; Dethiosulfovibrionaceae; TG5
Otu234	59	Bacteria; Firmicutes; Clostridia; Clostridiales
Otu166	59	Bacteria; Firmicutes; Clostridia; Clostridiales; Mogibacteriaceae
Otu246	58	Bacteria; Bacteroidetes; Flavobacteriia; Flavobacteriales; Flavobacteriaceae; Capnocytophaga
Otu175	55	Bacteria; Firmicutes; Bacilli; Lactobacillales; Aerococcaceae; Alloiococcus
Otu231	53	Bacteria; Actinobacteria; Actinobacteria; Actinomycetales; Actinomycetaceae; Actinomyces
Otu252	53	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella
Otu253	52	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae
Otu218	50	Bacteria; Firmicutes; Bacilli; Lactobacillales; Streptococcaceae; Lactococcus
Otu181	50	Bacteria; Proteobacteria; Gammaproteobacteria; Pasteurellales; Pasteurellaceae; Aggregatibacter
Otu244	49	Bacteria; Actinobacteria; Actinobacteria; Actinomycetales; Actinomycetaceae
Otu229	48	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella
Otu248	46	Bacteria; Actinobacteria; Actinobacteria; Actinomycetales; Actinomycetaceae
Otu273	46	Bacteria; Proteobacteria; Betaproteobacteria; Neisseriales; Neisseriaceae; Kingella
Otu200	46	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; BS11
Otu224	44	Bacteria; Firmicutes; Clostridia; Clostridiales; Veillonellaceae
Otu264	42	Bacteria; Firmicutes; Clostridia; Clostridiales; Veillonellaceae; Dialister
Otu208	42	Bacteria; Firmicutes; Clostridia; Clostridiales; Peptostreptococcaceae
Otu247	41	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella
Otu236	40	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Johnsonella; Johnsonella_ignava
Otu290	38	Bacteria; Firmicutes; Clostridia; Clostridiales; Mogibacteriaceae
Otu211	38	Bacteria; Firmicutes; Bacilli; Lactobacillales; Streptococcaceae; Streptococcus; Streptococcus_sobrinus
Otu220	35	Bacteria; Firmicutes; Clostridia; Clostridiales; Acidaminobacteraceae
Otu265	35	Bacteria; Firmicutes; Clostridia; Clostridiales; Mogibacteriaceae
Otu178	35	Bacteria; Proteobacteria; Gammaproteobacteria; Cardiobacteriales; Cardiobacteriaceae
Otu212	35	Bacteria; Tenericutes; Mollicutes; RF39
Otu230	34	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales
Otu199	32	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales
Otu198	31	Bacteria; Proteobacteria; Gammaproteobacteria; Cardiobacteriales; Cardiobacteriaceae
Otu241	31	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae
Otu288	30	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Paraprevotellaceae; Prevotella

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OTU no.	OTU abundance	Taxonomy
Otu249	29	Bacteria; GN02; BD1-5
Otu232	28	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Rikenellaceae; Blvii28
Otu173	28	Bacteria; Tenericutes; Mollicutes; Acholeplasmatales; Acholeplasmataceae; Acholeplasma
Otu213	26	Bacteria; TM7; TM7-3
Otu286	25	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Catonella
Otu258	21	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella
Otu161	21	Bacteria; Spirochaetes; Spirochaetes; Spirochaetales; Spirochaetaceae; Treponema
Otu269	20	Bacteria; Proteobacteria; Betaproteobacteria; Neisseriales; Neisseriaceae; Kingella
Otu262	17	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Johnsonella; Johnsonella_ignava
Otu284	15	Bacteria; Actinobacteria; Actinobacteria; Actinomycetales; Corynebacteriaceae; Corynebacterium
Otu278	11	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Anoxybacillus; Anoxybacillus_kestanbolensis
Otu259	11	Bacteria; Firmicutes; Bacilli; Lactobacillales; Leuconostocaceae; Weissella; Weissella_cibaria
Otu266	10	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella
Otu243	9	Bacteria; Synergistetes; Synergistia; Synergistales; Dethiosulfovibrionaceae; Pyramidobacter; Pyramidobacter_piscolens
Otu283	9	Bacteria; Fusobacteria; Fusobacteriia; Fusobacteriales; Leptotrichiaceae; Leptotrichia
Otu282	9	Bacteria; Fusobacteria; Fusobacteriia; Fusobacteriales; Leptotrichiaceae; Leptotrichia
Otu254	8	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales
Otu275	7	Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae
Otu260	5	Bacteria; Firmicutes; Erysipelotrichi; Erysipelotrichales; Erysipelotrichaceae; Bulleidia
Otu267	5	Bacteria; Cyanobacteria; Chloroplast; Streptophyta
Otu257	4	Bacteria; Actinobacteria; Actinobacteria; Bifidobacteriales; Bifidobacteriaceae; Bifidobacterium

Table A2. *Richness of OTUs of different samples before and after swaking*

	BEF2	AF2	BEF6	AF6	BEF8	AF8	BEF9	AF9	BEF10	AF10	BEF12	AF12	BEF14	AF14	BEF15	AF15	BEF19	AF19	BEF20	AF20	Total
Otu2	11062	11570	7278	9371	10372	16437	13938	14394	14000	16547	6064	8382	6956	16884	18405	13464	4544	13569	14256	8500	235993
Otu1	15251	2972	12398	10906	19902	6250	8512	4404	9177	17991	1640	5479	4465	12883	3700	11478	21756	5495	12085	8650	195394
Otu3	8044	3356	9825	5142	13444	5971	10524	3200	9441	9744	5498	6325	5589	6824	2186	8438	13926	7560	10655	1798	147490
Otu4	12642	7845	842	1941	1587	2831	1634	7295	5771	3147	6416	4897	1116	8691	6067	1646	619	1804	5901	9359	92051
Otu11	1690	2370	3060	4357	1089	3688	3934	6327	1752	793	1498	1760	5147	1874	1641	4427	1395	1329	5658	4742	58531
Otu32	3563	802	4934	5040	4315	2150	1399	816	2116	2866	3997	2932	1420	1023	704	965	4818	2740	1990	1593	50183
Otu8	5378	948	458	419	1608	2198	4793	3265	4079	2346	2484	1503	959	830	5074	1636	2190	2268	4500	2157	49093
Otu14	2425	5232	2153	2774	803	3783	951	1463	2915	2086	2246	5832	1170	4094	3432	1546	236	1448	1352	683	46624
Otu6	3818	4766	708	208	1158	3386	8793	2683	371	110	27	362	3432	2877	3156	501	392	946	1669	1509	40872

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	BEF2	AF2	BEF6	AF6	BEF8	AF8	BEF9	AF9	BEF10	AF10	BEF12	AF12	BEF14	AF14	BEF15	AF15	BEF19	AF19	BEF20	AF20	Total
Otu12	487	1567	468	130	1323	1673	194	1306	1862	2852	1573	2972	570	1585	179	6526	2685	3759	1072	1246	34029
Otu10	1	640	2043	969	284	488	1059	495	1233	517	3846	3684	679	222	515	397	4118	2307	3827	5744	33068
Otu7	3968	2096	1733	1851	393	1201	548	2721	284	438	385	2072	769	4233	6953	1337	133	844	534	148	32641
Otu5	1896	864	2344	1265	3027	405	1494	853	1702	662	1462	739	3057	873	835	396	3641	1719	3626	1232	32092
Otu261	1318	2960	1512	173	1817	1428	1695	4513	10	94	348	40	2661	1374	7146	241	141	193	196	51	27911
Otu29	1204	4196	1753	151	291	1799	1931	2459	276	1394	938	2197	1258	1718	495	1368	933	351	27	154	24893
Otu17	209	158	2811	1991	3170	1185	253	344	390	143	1413	1889	642	727	553	699	2042	790	2090	2284	23783
Otu15	792	455	2528	1294	3798	773	483	272	1072	1604	1043	2485	695	355	198	523	2114	674	255	1284	22697
Otu25	785	1237	597	596	331	1719	3075	1734	61	116	386	878	2679	1383	1222	760	952	311	1780	392	20994
Otu21	622	119	1301	2576	1203	870	1530	523	1843	548	485	513	1291	273	882	220	1835	330	1130	332	18426
Otu20	91	128	1132	460	105	1377	454	2226	941	247	2634	1290	584	277	166	373	1213	2217	595	645	17155
Otu27	745	527	229	314	364	487	694	1237	1869	513	809	661	251	619	413	638	442	1081	1215	802	13910
Otu26	800	947	1297	155	177	898	1172	1185	54	173	104	544	457	2199	556	751	212	338	822	627	13468
Otu9	1	6634	0	60	79	562	106	849	778	362	5	750	1575	1182	263	1	0	21	14	0	13242
Otu40	797	78	1525	386	445	1210	745	462	27	281	17	326	793	2069	718	1551	747	723	108	43	13051
Otu33	825	25	2041	579	244	1491	699	2294	0	43	63	283	390	574	1564	594	403	566	176	52	12906
Otu37	171	37	1119	1683	977	798	672	748	111	376	299	929	73	907	16	180	417	1659	623	877	12672
Otu23	201	2117	328	924	24	606	214	2345	232	49	538	1131	744	209	241	83	798	673	149	302	11908
Otu193	2650	318	416	163	1426	515	1844	354	197	230	811	427	482	428	178	65	292	285	189	45	11315
Otu28	10	185	639	3606	53	663	515	174	27	84	1015	193	1624	97	70	627	115	286	21	20	10024
Otu109	1870	1259	696	845	244	442	294	639	215	162	132	276	317	678	899	456	92	207	102	53	9878
Otu49	523	1345	204	1129	125	813	306	1411	314	340	660	731	1	413	227	1	26	105	406	670	9750
Otu18	65	140	436	277	55	136	120	437	820	281	198	440	2378	302	37	981	229	2004	120	248	9704
Otu55	264	153	309	101	178	476	1056	707	461	60	243	119	300	277	304	916	91	378	1065	1356	8814
Otu24	92	149	64	288	201	387	69	169	282	530	883	249	149	180	801	225	368	648	1902	956	8592
Otu38	106	63	321	261	527	315	297	140	1134	341	460	334	580	118	132	230	845	349	1110	717	8380
Otu42	266	35	419	953	808	287	184	29	242	71	1156	198	259	54	50	132	2355	396	288	120	8302
Otu68	738	501	886	618	89	369	408	351	131	103	556	876	377	191	96	197	700	414	194	364	8159
Otu53	1419	871	1	96	265	1246	487	1075	183	80	147	892	277	325	226	47	36	57	375	22	8127
Otu245	195	212	240	200	252	515	492	79	3	61	17	61	248	583	157	1055	225	60	986	1415	7056
Otu51	159	137	619	509	101	450	305	656	526	227	480	279	199	146	178	188	782	505	292	131	6869
Otu41	97	16	186	744	368	220	63	91	230	12	1292	545	506	532	134	421	255	108	452	469	6741
Otu50	41	39	194	45	149	265	905	423	386	119	938	161	261	2	65	150	574	252	779	507	6255
Otu19	836	520	181	2196	44	510	240	209	55	35	107	259	164	339	79	170	64	52	93	30	6183

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Otu39	62	1011	892	320	21	178	97	525	14	64	268	477	26	200	32	63	1343	448	60	23	6124
Otu277	37	801	10	16	713	208	319	1298	8	41	130	9	276	652	1339	15	7	22	34	24	5959
Otu65	629	276	158	392	311	187	324	890	92	130	21	122	283	754	587	531	7	42	79	5	5820
Otu31	18	287	163	176	131	164	173	442	76	83	30	49	2064	678	65	337	85	86	24	9	5140
Otu35	0	4	8	69	138	854	9	395	0	0	1190	401	14	11	25	16	78	213	199	1479	5103
Otu22	0	2	1854	0	0	0	508	462	4	89	0	1	317	411	21	680	56	618	0	0	5023
Otu13	0	0	2	1	0	0	11	4	0	0	0	0	3543	4	3	2	1329	3	1	2	4905
Otu88	322	351	375	61	33	400	174	441	683	181	83	228	265	110	65	302	251	472	64	28	4889
Otu155	5	440	87	38	75	416	272	467	585	96	330	310	461	113	45	440	110	251	188	85	4814
Otu61	208	226	349	333	103	310	94	172	218	115	36	156	935	274	94	440	144	456	68	66	4797
Otu70	26	320	564	236	102	774	254	398	221	57	67	243	572	39	64	247	158	66	363	17	4788
Otu34	1344	485	86	12	9	178	112	548	35	8	53	72	737	163	139	56	180	57	87	36	4397
Otu52	57	174	85	323	105	404	48	239	25	13	326	1007	208	21	32	71	800	186	97	26	4247
Otu66	15	321	626	587	45	141	122	105	138	103	298	196	696	92	149	64	227	141	56	79	4201
Otu76	79	144	101	109	100	310	117	305	336	83	423	185	281	83	167	176	140	173	471	261	4044
Otu69	595	668	192	34	18	152	155	403	54	19	143	44	867	78	56	161	61	71	69	63	3903
Otu57	10	219	178	92	25	336	129	111	299	55	110	155	980	86	42	94	412	322	66	42	3763
Otu36	1262	142	142	7	23	301	269	247	257	18	56	49	217	58	55	88	199	55	22	9	3476
Otu16	2754	203	9	0	0	46	60	216	27	7	25	0	0	33	36	16	0	7	1	0	3440
Otu48	359	54	62	187	8	395	37	351	228	91	798	108	61	51	22	38	300	180	47	63	3440
Otu64	4	33	355	3	0	7	71	64	156	8	185	774	232	20	15	94	107	271	26	990	3415
Otu84	98	261	309	189	18	178	330	456	235	60	131	30	426	75	15	247	46	153	68	81	3406
Otu47	78	301	645	224	105	415	203	124	0	0	35	51	110	120	728	100	65	50	21	6	3381
Otu93	20	233	597	53	13	210	194	640	409	24	306	226	64	75	37	41	12	16	145	52	3367
Otu126	263	432	277	210	63	308	62	90	77	46	58	167	520	84	33	241	88	226	31	55	3331
Otu87	260	296	126	31	33	222	189	851	67	53	151	74	307	37	56	95	47	82	61	36	3074
Otu77	112	798	163	76	47	156	192	66	72	13	159	75	485	84	19	131	153	134	21	37	2993
Otu271	205	13	191	134	254	120	271	145	151	119	49	115	80	121	93	83	515	19	241	67	2986
Otu75	29	125	112	64	6	216	153	61	163	32	136	129	234	15	15	94	678	555	82	40	2939
Otu98	82	33	179	26	381	302	15	32	185	86	103	96	20	13	2	33	375	245	2	17	2227
Otu59	722	43	178	36	9	44	70	93	311	63	176	128	179	30	64	44	342	140	66	12	2750
Otu80	0	3	188	72	10	135	80	233	80	42	427	336	58	62	73	120	332	335	72	69	2727
Otu86	18	228	112	33	10	123	53	130	68	27	112	71	441	103	17	200	71	127	436	82	2462
Otu81	10	6	33	8	113	32	173	135	627	105	277	45	47	15	21	72	205	258	119	132	2433

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Otu206	15	6	51	30	37	38	150	83	397	59	92	70	64	62	77	66	314	186	387	241	2425
Otu137	397	224	248	53	78	166	312	25	101	18	43	38	141	33	32	131	96	86	17	2	2241
Otu62	41	50	150	70	52	76	38	67	102	88	172	74	98	53	32	103	116	142	438	164	2126
Otu67	1	0	16	0	9	48	71	800	0	0	8	44	80	234	638	63	23	21	0	0	2056
Otu71	0	9	203	0	6	0	34	35	440	290	162	125	207	146	38	195	4	107	0	0	2001
Otu45	0	1	1189	1	0	0	710	71	0	0	0	0	0	0	0	0	0	0	0	0	1972
Otu280	475	213	72	97	30	69	64	148	34	35	41	89	28	201	188	42	10	14	28	30	1908
Otu274	459	125	33	3	43	184	252	194	93	5	66	4	269	29	19	29	52	25	15	5	1904
Otu108	1	94	204	65	28	82	58	184	51	41	27	80	181	241	158	188	83	78	33	25	1902
Otu117	0	40	308	111	11	70	88	106	174	109	63	46	233	29	12	140	111	140	57	19	1867
Otu90	28	7	30	213	217	53	98	41	84	45	68	86	66	71	65	127	88	65	199	213	1864
Otu72	145	386	30	59	15	144	49	227	71	62	49	75	62	69	142	55	9	55	92	38	1834
Otu100	18	223	81	28	19	99	14	79	53	26	31	24	701	164	22	131	28	75	5	6	1827
Otu78	0	260	21	12	2	48	49	475	4	4	80	48	144	200	315	60	14	71	5	11	1823
Otu146	13	84	55	137	7	134	192	78	29	6	105	59	228	33	19	283	67	140	46	79	1794
Otu44	0	6	9	23	54	169	5	80	19	14	806	249	15	4	22	48	74	112	12	67	1788
Otu46	0	0	421	0	0	0	52	19	815	0	97	166	0	7	5	0	23	94	1	2	1702
Otu105	135	311	82	27	25	114	12	56	121	27	155	117	98	28	16	77	25	186	41	12	1665
Otu58	38	328	22	18	15	70	58	709	160	53	57	26	24	11	9	26	1	19	0	1	1645
Otu30	0	1	0	0	0	0	1416	221	0	0	0	0	0	0	0	0	0	0	0	0	1638
Otu79	104	1	52	23	27	451	31	235	1	2	18	122	40	136	120	64	60	80	6	4	1577
Otu56	0	63	32	21	14	52	88	66	35	29	30	38	770	75	23	72	60	55	1	10	1534
Otu124	98	53	157	123	125	123	35	9	83	12	52	23	223	35	6	148	70	111	18	1	1505
Otu140	301	115	205	5	17	108	78	144	32	5	45	13	263	14	3	100	24	20	5	1	1498
Otu115	131	187	62	38	4	60	21	209	79	45	32	113	61	35	104	21	20	31	36	6	1295
Otu43	0	156	0	0	1	0	1	0	0	0	3	117	5	88	727	1	33	0	38	14	1184
Otu125	17	1	65	20	96	201	9	91	1	3	6	47	154	143	142	87	17	19	9	6	1134
Otu114	77	75	131	15	26	163	59	46	34	18	47	38	44	180	49	31	71	19	10	0	1133
Otu103	0	3	144	0	23	2	9	3	1	9	0	46	123	222	141	192	83	102	1	4	1108
Otu82	0	16	22	53	150	15	1	13	9	11	71	36	10	12	39	47	288	84	155	72	1104
Otu144	0	0	273	23	0	0	151	53	149	111	18	28	64	7	0	76	26	94	9	5	1087
Otu134	27	52	75	57	27	44	57	24	45	27	32	72	48	74	25	253	25	91	15	7	1077
Otu106	3	13	57	53	7	19	35	58	28	25	56	62	77	66	36	116	77	44	134	101	1067
Otu151	11	9	10	33	24	35	34	48	56	18	195	31	42	4	9	56	71	66	159	53	964

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Otu85	2	3	0	1	0	0	474	311	0	2	57	37	10	41	12	2	0	0	0	1	953
Otu92	326	33	58	4	11	36	17	14	54	15	36	11	138	3	8	29	83	51	21	4	952
Otu54	0	1	35	6	55	94	22	7	5	0	17	5	568	20	15	89	0	0	0	0	939
Otu97	41	10	15	3	0	14	59	55	0	0	8	20	66	105	44	59	10	9	94	292	904
Otu63	0	0	381	0	0	0	407	96	0	0	0	0	0	0	0	0	0	0	0	0	884
Otu239	3	43	24	5	16	26	57	261	40	4	55	20	18	49	45	38	0	0	56	66	826
Otu91	6	1	46	76	13	21	15	13	72	33	193	108	2	13	8	22	14	28	84	56	824
Otu219	10	81	7	22	5	48	15	30	6	12	119	55	184	10	12	96	22	36	10	27	807
Otu94	0	0	14	19	0	0	24	24	174	55	76	31	44	18	6	51	26	200	26	15	803
Otu276	31	85	80	6	16	112	25	25	60	41	4	22	95	6	5	32	22	68	23	18	776
Otu73	0	0	109	0	0	0	8	195	11	19	0	0	368	0	0	63	0	0	0	0	773
Otu122	39	223	13	15	6	90	22	163	24	5	37	25	4	15	9	26	2	12	6	3	739
Otu129	5	15	26	36	9	31	50	64	24	30	40	72	68	15	8	50	92	67	4	5	711
Otu169	0	3	112	18	0	0	331	24	31	31	10	13	0	26	0	0	17	89	0	0	705
Otu145	11	125	71	31	1	0	6	6	40	42	36	52	61	77	32	10	26	60	2	9	698
Otu131	0	0	0	20	0	0	138	393	19	100	2	0	3	8	5	0	0	0	0	0	688
Otu99	0	0	2	0	0	0	33	4	1	0	6	0	153	1	0	6	98	293	0	22	619
Otu120	0	1	108	18	7	12	81	13	1	4	15	11	60	22	6	123	2	3	41	83	611
Otu287	2	1	47	4	0	8	8	31	21	0	20	146	25	10	9	19	50	12	6	189	608
Otu154	16	21	57	30	6	47	100	40	25	2	59	12	71	10	4	33	27	13	27	7	607
Otu143	103	56	6	11	8	49	13	40	40	6	48	52	68	17	11	32	14	28	2	0	604
Otu217	12	20	3	6	2	50	10	93	39	13	3	0	256	0	3	7	10	22	6	1	556
Otu268	11	6	9	66	53	26	3	3	17	6	65	16	23	4	7	30	69	33	92	17	556
Otu102	0	0	0	0	0	0	371	89	83	0	8	0	0	0	0	0	0	0	0	0	551
Otu107	0	0	86	0	1	11	98	28	32	16	2	18	145	21	4	34	18	32	2	0	548
Otu139	0	62	9	47	10	40	46	49	49	13	39	39	51	5	0	18	5	23	22	19	546
Otu192	1	34	34	17	7	49	44	10	35	16	31	15	108	27	2	27	27	49	3	4	540
Otu60	3	1	0	3	4	0	0	2	4	0	1	2	0	85	12	0	6	416	0	0	539
Otu133	0	115	37	14	8	27	48	26	20	12	10	15	30	33	5	42	10	37	7	6	502
Otu74	0	0	9	0	0	0	260	41	0	0	11	7	0	0	0	0	2	161	0	0	491
Otu111	0	74	39	25	10	43	7	3	29	7	89	20	13	14	1	5	44	51	0	0	474
Otu89	0	0	0	0	0	0	10	29	13	63	0	0	36	0	0	47	42	234	0	0	474
Otu136	12	93	6	7	8	116	21	28	7	4	43	15	45	9	3	13	11	17	4	5	467
Otu194	16	1	50	0	18	20	54	110	16	1	3	0	83	15	15	25	10	11	4	0	452

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Otu186	0	3	91	8	0	10	63	18	42	12	14	3	27	9	3	28	26	67	6	5	435
Otu119	26	9	33	26	1	19	27	35	10	10	7	12	4	25	130	13	11	22	11	2	433
Otu104	0	0	162	0	0	0	186	61	0	0	0	0	0	3	7	0	0	0	0	0	419
Otu159	15	30	10	25	2	31	24	83	92	2	0	8	48	7	1	14	9	9	1	1	412
Otu285	18	6	19	11	106	25	1	1	17	19	1	22	8	16	0	4	112	8	1	11	406
Otu189	0	25	51	25	9	25	2	10	65	17	74	8	8	0	0	18	24	37	0	0	398
Otu96	0	0	52	7	0	0	199	57	0	0	0	0	21	13	6	31	0	1	0	0	387
Otu156	0	62	40	5	4	15	16	57	101	11	13	11	9	0	0	9	1	26	0	0	380
Otu101	0	0	30	26	1	0	24	2	0	0	15	10	143	10	0	62	2	33	5	10	373
Otu168	5	17	50	11	0	0	51	34	20	2	58	2	19	13	6	51	12	16	0	1	368
Otu113	0	31	26	0	0	2	7	229	0	24	0	3	0	0	0	11	0	11	1	3	348
Otu123	0	0	78	7	0	0	25	14	56	26	15	5	25	9	2	33	0	52	0	0	347
Otu118	11	7	3	1	5	1	0	5	0	4	4	5	47	154	44	13	8	10	1	0	323
Otu110	35	41	0	0	31	27	4	2	0	0	4	2	1	58	114	2	0	0	0	0	321
Otu121	0	8	28	35	0	0	9	5	78	42	18	32	0	19	3	0	5	27	0	0	309
Otu83	2	2	0	5	4	3	22	13	0	0	10	4	5	0	2	19	10	3	152	51	307
Otu184	9	13	34	8	0	15	44	41	4	3	14	2	46	2	0	15	29	18	2	6	305
Otu132	0	1	3	5	3	10	6	5	23	5	19	4	32	4	1	14	3	23	89	38	288
Otu112	0	0	49	0	0	0	13	3	124	38	8	0	0	0	0	0	2	38	0	0	275
Otu177	14	5	14	1	0	1	26	15	2	0	4	11	7	135	11	5	4	5	2	2	264
Otu95	0	0	0	0	1	34	20	165	0	0	0	4	7	4	26	0	0	0	0	0	261
Otu164	0	39	9	22	5	28	5	0	3	0	7	4	54	16	3	19	9	17	0	1	241
Otu176	0	2	0	6	5	2	6	10	23	7	3	12	14	29	8	41	4	14	27	20	233
Otu183	0	0	22	0	0	0	11	0	15	1	0	0	107	11	8	44	3	9	0	0	231
Otu153	0	0	65	1	1	9	1	6	13	11	18	19	10	21	5	24	1	15	4	1	225
Otu221	54	14	14	1	11	2	13	0	0	0	4	4	54	2	0	29	20	2	0	0	224
Otu170	1	14	17	3	2	15	26	53	0	0	2	1	42	11	1	16	9	5	0	0	218
Otu165	47	36	3	3	2	22	2	0	3	0	7	9	21	9	7	29	7	8	0	0	215
Otu172	0	0	29	24	0	0	21	2	37	15	16	5	22	6	1	19	2	11	3	0	213
Otu162	1	2	11	8	0	4	32	20	3	0	48	18	8	1	1	32	9	6	1	6	211
Otu147	0	0	23	7	5	7	2	7	8	1	1	0	107	14	0	12	6	7	0	0	207
Otu202	6	2	15	2	6	21	30	6	0	8	0	3	26	17	4	38	15	3	2	3	207
Otu180	1	10	29	5	0	3	6	4	4	4	9	7	22	1	3	41	12	31	6	4	202
Otu148	0	0	0	0	0	0	13	22	42	52	11	12	0	0	0	0	16	30	0	0	198

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	BEF2	AF2	BEF6	AF6	BEF8	AF8	BEF9	AF9	BEF10	AF10	BEF12	AF12	BEF14	AF14	BEF15	AF15	BEF19	AF19	BEF20	AF20	Total
Otu187	1	7	2	1	3	9	26	63	11	0	3	3	14	1	0	9	4	4	17	11	189
Otu182	0	5	111	28	1	2	0	1	2	3	3	3	0	14	0	3	1	11	0	0	188
Otu163	0	27	36	5	1	4	2	6	12	4	6	12	17	3	4	5	23	11	5	4	187
Otu141	0	1	3	5	0	12	3	2	15	16	0	1	64	0	6	49	2	5	0	0	184
Otu130	0	0	0	16	0	0	1	0	0	0	0	0	25	25	15	10	10	81	0	0	183
Otu149	0	6	3	0	5	5	0	7	6	0	3	6	97	4	5	27	0	4	0	0	178
Otu281	0	1	3	0	2	7	43	80	4	12	0	0	1	6	5	1	1	7	1	0	174
Otu142	0	0	71	0	0	0	33	7	0	0	0	0	14	4	0	15	6	19	0	0	169
Otu116	0	0	0	0	0	0	0	0	0	0	0	0	126	4	4	21	0	0	0	0	155
Otu174	0	8	4	11	1	7	1	1	8	14	15	17	33	0	1	7	3	17	0	0	148
Otu191	0	0	5	0	0	0	97	9	28	0	2	3	0	0	0	0	0	0	0	0	144
Otu150	0	0	34	0	0	0	40	4	0	0	6	5	1	1	2	0	1	39	10	0	143
Otu152	0	0	22	2	0	0	14	73	0	0	4	1	0	0	0	14	2	9	0	2	143
Otu179	0	4	2	14	2	37	3	11	11	5	6	14	0	1	18	3	1	7	0	1	140
Otu171	1	1	26	6	0	2	29	5	5	1	1	2	15	2	1	6	7	2	5	22	139
Otu185	8	0	18	0	1	6	2	0	3	1	2	0	26	2	0	10	29	24	0	1	133
Otu160	0	0	37	0	0	0	0	0	47	22	0	0	0	1	1	0	4	19	0	0	131
Otu196	0	22	3	6	3	38	1	1	5	4	0	0	22	2	0	6	3	9	0	3	128
Otu216	33	8	9	2	0	2	7	10	9	0	1	3	28	0	2	9	1	2	0	0	126
Otu251	14	0	12	1	11	7	11	6	0	1	0	1	0	18	8	18	13	0	2	2	125
Otu128	0	5	0	4	0	1	2	11	5	0	0	0	6	0	0	1	0	0	45	42	122
Otu228	0	16	4	0	0	1	3	25	4	3	14	6	4	15	3	8	1	13	0	2	122
Otu238	0	4	5	4	1	6	5	6	0	0	3	15	28	0	1	5	1	5	10	12	111
Otu250	0	2	12	1	0	0	7	12	3	0	5	5	25	1	0	24	2	8	0	4	111
Otu188	0	0	20	0	5	23	1	0	0	0	10	11	1	5	0	0	3	24	0	0	103
Otu209	1	4	22	0	0	12	26	12	0	0	2	7	1	0	2	1	1	9	1	0	101
Otu190	0	0	2	0	0	5	2	0	10	1	9	1	0	0	11	0	27	32	0	0	100
Otu201	0	1	0	0	0	0	6	10	0	1	0	0	14	20	22	11	0	3	5	7	100
Otu207	5	14	8	4	0	0	7	2	0	0	12	1	17	0	0	22	2	5	0	0	99
Otu204	0	0	10	5	1	8	0	0	5	3	0	0	15	3	0	29	17	0	0	0	96
Otu205	0	2	9	1	4	3	5	6	7	2	1	2	24	8	2	4	11	4	0	1	96
Otu135	0	0	36	0	0	0	41	5	0	0	0	0	12	0	0	1	0	0	0	0	95
Otu227	0	0	15	3	0	2	20	3	6	13	4	0	1	1	0	23	0	0	4	0	95
Otu167	0	0	0	0	0	0	3	0	0	0	0	1	58	0	0	0	20	9	0	2	93

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	BEF2	AF2	BEF6	AF6	BEF8	AF8	BEF9	AF9	BEF10	AF10	BEF12	AF12	BEF14	AF14	BEF15	AF15	BEF19	AF19	BEF20	AF20	Total
Otu225	0	2	8	1	0	0	6	1	0	0	5	3	47	1	0	19	0	0	0	0	93
Otu279	11	14	1	4	1	2	1	7	4	5	9	1	0	8	4	1	1	3	4	12	93
Otu197	0	0	4	0	5	4	17	7	5	3	1	0	6	0	0	7	3	30	0	0	92
Otu214	7	6	0	2	2	5	2	4	8	3	0	5	18	8	4	5	0	8	2	0	89
Otu240	0	0	21	5	7	10	4	5	6	3	2	0	1	8	1	13	0	0	0	0	86
Otu158	0	2	3	0	1	1	0	2	1	1	1	0	59	0	2	11	0	1	0	0	85
Otu237	0	0	43	3	9	11	0	1	7	7	0	0	1	2	0	1	0	0	0	0	85
Otu291	0	26	0	0	4	4	2	15	0	1	0	0	10	13	10	0	0	0	0	0	85
Otu223	2	4	8	0	2	8	2	0	3	0	15	4	17	2	1	2	2	12	0	0	84
Otu127	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	82	0	0	0	0	82
Otu203	0	0	0	0	0	0	51	24	0	0	0	0	0	0	0	6	0	0	0	0	81
Otu226	2	0	4	6	1	2	16	8	8	6	2	1	2	1	0	5	0	0	1	0	65
Otu215	0	0	0	0	0	0	23	0	0	0	0	0	31	0	0	0	16	5	0	0	75
Otu233	0	0	1	0	0	0	3	2	0	0	2	3	41	0	0	23	0	0	0	0	75
Otu157	0	0	0	0	0	0	3	17	12	35	3	3	0	0	0	0	0	0	0	0	73
Otu138	1	0	0	0	1	0	2	0	1	0	0	0	67	0	0	0	0	0	0	0	72
Otu242	0	13	7	2	0	0	0	0	0	0	4	0	20	3	1	15	0	6	0	0	71
Otu210	0	0	15	4	1	2	3	3	4	0	2	1	14	1	1	4	0	7	2	5	69
Otu222	0	0	2	1	0	1	1	2	1	2	0	0	34	1	1	13	0	7	1	0	67
Otu270	0	0	7	0	0	0	13	0	5	4	12	1	1	2	2	9	0	11	0	0	67
Otu256	9	11	0	2	0	1	1	0	7	1	2	2	1	8	1	2	2	11	3	2	66
Otu272	0	0	10	0	2	0	0	2	3	1	0	1	31	4	0	12	0	0	0	0	66
Otu289	0	0	6	0	0	0	3	10	0	3	0	0	19	0	0	0	24	0	0	0	65
Otu195	0	0	0	0	0	0	1	0	0	0	2	4	21	0	2	33	0	0	0	0	63
Otu235	1	0	0	2	0	8	5	1	4	1	9	1	12	0	0	11	3	3	0	0	61
Otu263	3	4	0	1	1	5	1	2	5	1	1	1	7	2	1	11	3	8	2	2	61
Otu255	0	0	8	1	0	0	11	0	5	2	4	3	17	0	2	4	0	3	0	0	60
Otu166	0	0	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	1	36	19	59
Otu234	0	0	5	2	0	0	11	2	10	7	0	0	0	2	1	0	0	15	2	2	59
Otu246	0	5	0	7	0	4	3	1	0	2	1	0	21	2	2	1	4	4	1	0	58
Otu175	3	1	0	0	0	0	2	0	4	0	0	0	0	0	1	0	0	43	1	0	55
Otu231	1	5	13	3	0	1	1	2	1	1	2	0	7	3	2	4	4	2	0	1	53
Otu252	0	0	8	1	0	3	1	2	12	1	5	0	17	0	1	0	0	2	0	0	53
Otu253	0	1	28	0	0	1	9	11	0	0	0	0	2	0	0	0	0	0	0	0	52

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	BEF2	AF2	BEF6	AF6	BEF8	AF8	BEF9	AF9	BEF10	AF10	BEF12	AF12	BEF14	AF14	BEF15	AF15	BEF19	AF19	BEF20	AF20	Total
Otu181	0	0	0	0	0	0	50	0	0	0	0	0	0	0	0	0	0	0	0	0	50
Otu218	0	2	0	0	1	0	0	2	0	16	2	0	1	1	0	2	21	1	0	1	50
Otu244	0	1	2	6	1	0	0	0	7	2	4	5	7	0	0	2	2	5	3	2	49
Otu229	0	0	0	2	0	0	0	0	0	2	0	1	39	1	0	2	0	1	0	0	48
Otu200	0	0	0	0	0	0	44	2	0	0	0	0	0	0	0	0	0	0	0	0	46
Otu248	0	2	15	1	0	0	3	2	5	0	5	3	0	0	1	1	1	7	0	0	46
Otu273	0	0	9	0	0	5	0	0	5	0	1	0	0	5	5	0	11	5	0	0	46
Otu224	0	0	44	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	44
Otu208	1	0	0	0	0	0	0	0	0	0	0	0	13	0	0	1	4	23	0	0	42
Otu264	0	0	2	0	0	0	3	0	2	0	0	0	21	4	2	5	1	2	0	0	42
Otu247	0	0	0	0	0	0	2	2	0	0	0	0	16	0	0	17	0	0	4	0	41
Otu236	0	1	0	5	0	0	2	3	0	0	1	1	2	1	2	9	1	8	3	1	40
Otu211	0	0	22	3	0	0	0	2	0	9	1	0	0	1	0	0	0	0	0	0	38
Otu290	0	0	19	0	0	0	3	3	0	0	0	0	3	0	5	5	0	0	0	0	38
Otu178	0	0	0	0	0	0	5	30	0	0	0	0	0	0	0	0	0	0	0	0	35
Otu212	0	0	0	30	0	0	0	0	0	0	1	0	0	1	0	0	1	2	0	0	35
Otu220	0	0	11	0	0	0	1	0	3	0	2	4	6	1	0	6	0	1	0	0	35
Otu265	0	0	0	0	0	0	3	1	7	5	5	7	0	0	0	0	3	4	0	0	35
Otu230	0	0	0	0	0	0	14	18	0	0	0	0	0	2	0	0	0	0	0	0	34
Otu199	0	0	0	0	0	0	30	1	0	0	0	0	0	0	1	0	0	0	0	0	32
Otu198	0	0	0	0	0	0	5	26	0	0	0	0	0	0	0	0	0	0	0	0	31
Otu241	0	2	1	0	0	0	3	7	8	0	0	0	0	0	0	1	0	1	8	0	31
Otu288	0	0	0	1	0	0	4	2	0	0	0	0	17	0	1	5	0	0	0	0	30
Otu249	0	0	0	2	0	0	0	10	3	1	0	0	0	0	0	0	0	11	0	2	29
Otu173	0	0	0	0	0	0	24	4	0	0	0	0	0	0	0	0	0	0	0	0	28
Otu232	0	0	0	0	0	0	0	0	0	0	7	0	13	0	0	0	8	0	0	0	28
Otu213	0	1	0	0	1	0	3	15	0	0	0	0	0	0	0	1	1	4	0	0	26
Otu286	0	0	17	0	0	0	0	0	0	0	2	3	0	2	1	0	0	0	0	0	25
Otu161	0	0	16	0	0	0	2	3	0	0	0	0	0	0	0	0	0	0	0	0	21
Otu258	0	0	0	0	0	0	1	1	0	0	0	0	0	1	4	14	0	0	0	0	21
Otu269	0	0	0	6	0	0	0	14	0	0	0	0	0	0	0	0	0	0	0	0	20
Otu262	0	0	0	0	0	0	3	0	0	0	0	0	3	0	0	5	1	5	0	0	17
Otu284	0	0	0	0	0	0	0	0	11	3	0	0	0	0	0	0	1	0	0	0	15
Otu259	0	0	0	0	0	0	0	2	0	0	0	0	6	0	0	0	3	0	0	0	11

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	BEF2	AF2	BEF6	AF6	BEF8	AF8	BEF9	AF9	BEF10	AF10	BEF12	AF12	BEF14	AF14	BEF15	AF15	BEF19	AF19	BEF20	AF20	Total
Otu278	0	0	0	0	0	0	0	7	0	1	0	0	0	0	0	0	1	2	0	0	11
Otu266	0	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10
Otu243	0	0	0	0	0	0	9	0	0	0	0	0	0	0	0	0	0	0	0	0	9
Otu282	0	0	4	2	0	0	1	2	0	0	0	0	0	0	0	0	0	0	0	0	9
Otu283	0	0	0	0	2	0	0	3	0	0	2	1	0	0	0	0	0	0	0	1	9
Otu254	0	0	0	0	0	0	0	0	0	0	1	0	3	0	0	0	2	1	0	1	8
Otu275	0	0	6	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7
Otu260	0	0	4	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	5
Otu267	0	0	0	0	0	0	1	0	0	0	0	0	4	0	0	0	0	0	0	0	5
Otu257	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	4

Table A3. *Taxonomy and description of the highly abundant OTUs recovered from oral microbiomes of children regardless of swaking*

Phylum	Genus/Species	Description	OTUs
Bacteroidetes	*Prevotella* spp.	A genus of gram negative anaerobic bacteria. Some strains are present in humans, including *Prevotella denticola*. They are predominantly oral and can be opportunistic pathogens [1].	15,21,33
	Prevotella melaninogenica	A species of bacterium in the normal flora of the upper respiratory tract. It is formerly called *Bacteroides melaninogenicus*, is a gram-negative, anaerobic, rod-shaped bacterium that inhabits the oral cavity. It is an important human pathogen in various anaerobic infections [2].	1
	Prevotella pallens	A genus of gram-negative bacteria. *Prevotella* spp. are members of the oral, vaginal, and gut microbiota and are often recovered from anaerobic infections of the respiratory tract [3].	17
	Prevotella tannerae	An obligately anaerobic, non-spore-forming, nonmotile, gram-negative, rod-shaped bacterium that was isolated from the human gingival crevice [4].	28
	Prevotella nanceiensis	A novel species isolated from human clinical samples, e.g., blood cultures, lung abscess pus, broncho-alveolar lavage fluid, obligately anaerobic, non-spore-forming, non-motile, gram-negative coccoid and short rods [5].	26
	Porphyromonas spp.	Associated with severe and chronic periodontal (tissues surrounding and supporting the tooth) diseases. Progression of the disease is caused by colonization by this organism in an anaerobic environment in host tissues and severe progression results in loss of the tissues supporting the tooth and eventually loss of the tooth itself [6].	25
Firmicutes	*Streptococcus* spp.	First isolated in 1924 from human carious (cavities) lesions and is the main cause of tooth decay. This organism thrives in a bacterial community known as a dental plaque which forms on the surface of teeth. This organism has also been implicated in cases of infective endocarditis [7].	12
	Streptococcus infantis	A species of alpha-haemolytic streptococci. It has been isolated from human tooth surfaces and pharynx [8].	2
	Veillonella spp.	Well known for its lactate fermenting abilities. They are a normal bacterium in the intestines and oral mucosa of mammals. In humans they have been rarely implicated in cases of osteomyelitis and endocarditis [9].	40

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Phylum	Genus/Species	Description	OTUs
	Veillonella dispar	A member of the normal human oral microbial community [10].	3,32
	Gemella morbillorum	Previously known as *Streptococcus morbillorum*, is an anaerobic, gram-positive coccus which is a component of the normal flora of the human gastrointestinal tract and the human oral microflora [11].	14
	Megasphaera spp.	A commensal genus of Firmicutes classified within the class Negativicutes as a member of the Clostridia. *Megasphaera* spp. reside in the human genitourinary tract. It has not yet been characterized using traditional methods, or the species name has not yet been validly published [12].	37
	Clostridium spp.	Distinguished from the Bacilli by lacking aerobic respiration. They are obligate anaerobes and oxygen is toxic to them [13].	23
	Granulicatella spp.	A facultatively anaerobic gram-positive, non-motile, non-sporulating bacterium. It is part of normal human oral flora and is thought to be a cause of endocarditis [14].	7
	Granulicatella adjacens	Formerly called *Streptococcus adjacens* or *Abiotrophia adiacens*, a species of gram-positive, non-motile, non-sporulating cocci isolated from the throat flora, urine and blood of patients with endocarditis [15].	27
Proteobacteria	*Haemophilus parainfluenzae*	A slow-growing gram negative bacteria that is a normal part of the human oropharyngeal flora. It is a cause of endocarditis in children [16].	4
	Campylobacter spp.	The leading cause of bacterial food poisoning (campylobacteriosis) in the world, and is more prevalent than *Salmonella enteritis* (salmonellosis). Severe health and economic problems are a result of widespread infections that affect up to 1% of the population [17].	5
	Neisseria spp.	A nonpathogenic, commensal bacterium closely related to the pathogenic *Neisseria meningitidis*. Of the 11 species that colonize humans, only two are pathogens, *N. meningitidis* and *N. gonorrhoeae* [18].	29
	Neisseria subflava	Commonly isolated from the oral and respiratory tract of humans. This bacterium can be an opportunistic pathogen and has occasionally been isolated from cases of endocarditis or bacteremia [19].	6
	Neisseria cinerea	Isolated in Germany from the nasopharyngeal mucosa of a healthy human [20].	261
	Moraxella spp.	Part of the commensal flora of the upper respiratory tract. It is also recognized as the cause of a variety of human infectious diseases including acute otitis media and sinusitis, which occur primarily in infants and young children [21].	9
	Pasteurella spp.	One of the first pathogens ever studied, and is named after Louis Pasteur, who used it in his vaccination studies in the 1880s. This organism usually resides in the mucous membranes of the intestinal, genital, and respiratory tissues and is an opportunistic pathogen [22].	193
Fusobacteria	*Fusobacterium* spp.	Belongs to the normal microflora of the human oral and gastrointestinal tracts. It is a very long and slender spindle-shaped bacillus with sharply pointed ends that is characterized by the ability to invade soft tissues. Although not considered a major dental pathogen on its own, this anaerobe facilitates the aggregation and establishment of several other species including the dental pathogens *Porphyromonas gingivalis* and *Bacteroides forsythus* [23].	11
	Leptotrichia spp.	An anaerobic, gram-negative rod bacteria. It is a constituent of normal oral flora. Leptotrichiabuccalis can be clearly identified using live blood analysis in dark field. They have a distinct form, which separates them from other rod forms [24].	20
Actinobacteria	*Rothia mucilaginosa*	A gram-positive, cocci-shaped bacterium that inhabits the oral cavity. The bacterium is considered an opportunistic pathogen and has been associated with endocarditis, meningitis, and peritonitis [25].	8
Saccharibacteria (TM7-3)	N/A	A major lineage of Bacteria, or a candidate phylum known solely through environmental 16S rRNA sequences as no species had been grown in the lab [26].	10

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Table A4. *Statistical analysis across subjects of the highly abundant OTUs recovered from oral microbiomes of children due to swaking*

OTU no.	OTU abundance	Taxonomy	BEF2	AF2	BEF6	AF6	BEF8	AF8	BEF9	AF9	BEF10	AF10	BEF12	AF12	BEF14	AF14	BEF15	AF15	BEF19	AF19	BEF20	AF20	Mean BEF	Mean AF	P value
Otu2	235993	Bacteria; Firmicutes; Bacilli; Lactobacillales; Streptococcaceae; Streptococcus; Streptococcus_infantis	11062	11570	7278	9371	10372	16437	13938	14394	14000	16547	6064	8382	6956	16884	18405	13464	4544	13569	14256	8500	10687.5	12911.8	0.208
Otu1	195394	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella; Prevotella_melaninogenica	15251	2972	12398	10906	19902	6250	8512	4404	9177	17991	1640	5479	4465	12883	3700	11478	21756	5495	12085	8650	10888.6	8659.8	0.474
Otu3	147490	Bacteria; Firmicutes; Clostridia; Clostridiales; Veillonellaceae; Veillonella; Veillonella_dispar	8044	3356	9825	5142	13444	5971	10524	3200	9441	9744	5498	6325	5589	6824	2186	8438	13926	7560	10655	1798	8913.2	5835.8	0.08
Otu4	92051	Bacteria; Proteobacteria; Gammaproteobacteria; Pasteurellales; Pasteurellaceae; Haemophilus; Haemophilus_parainfluenzae	12642	7845	842	1941	1587	2831	1634	7295	5771	3147	6416	4897	1116	8691	6067	1646	619	1804	5901	9359	4259.5	4945.6	0.611
Otu11	58531	Bacteria; Fusobacteria; Fusobacteriia; Fusobacteriales; Fusobacteriaceae; Fusobacterium	1690	2370	3060	4357	1089	3688	3934	6327	1752	793	1498	1760	5147	1874	1641	4427	1395	1329	5658	4742	2686.4	3166.7	0.446
Otu32	50183	Bacteria; Firmicutes; Clostridia; Clostridiales; Veillonellaceae; Veillonella; Veillonella_dispar	3563	802	4934	5040	4315	2150	1399	816	2116	2866	3997	2932	1420	1023	704	965	4818	2740	1990	1593	2925.6	2092.7	0.049
Otu8	49093	Bacteria; Actinobacteria; Actinobacteria; Actinomycetales; Micrococcaceae; Rothia; Rothia_mucilaginosa	5378	948	458	419	1608	2198	4793	3265	4079	2346	2484	1503	959	830	5074	1636	2190	2268	4500	2157	3152.3	1757	0.025
Otu14	46624	Bacteria; Firmicutes; Bacilli; Gemellales; Gemellaceae	2425	5232	2153	2774	803	3783	951	1463	2915	2086	2246	5832	1170	4094	3432	1546	236	1448	1352	683	1768.3	2894.1	0.093
Otu6	40872	Bacteria; Proteobacteria; Betaproteobacteria; Neisseriales; Neisseriaceae; Neisseria; Neisseria_subflava	3818	4766	708	208	1158	3386	8793	2683	371	110	27	362	3432	2877	3156	501	392	946	1669	1509	2352.4	1734.8	0.417
Otu12	34029	Bacteria; Firmicutes; Bacilli; Lactobacillales; Streptococcaceae; Streptococcus	487	1567	468	130	1323	1673	194	1306	1862	2852	1573	2972	570	1585	179	6526	2685	3759	1072	1246	1041.3	2361.6	0.05
Otu10	33068	Bacteria; TM7; TM7-3	1	640	2043	969	284	488	1059	495	1233	517	3846	3684	679	222	515	397	4118	2307	3827	5744	1760.5	1546.3	0.519
Otu7	32641	Bacteria; Firmicutes; Bacilli; Lactobacillales; Carnobacteriaceae; Granulicatella	3968	2096	1733	1851	393	1201	548	2721	284	438	385	2072	769	4233	6953	1337	133	844	534	148	1570	1694.1	0.878
Otu5	32092	Bacteria; Proteobacteria;	1896	864	2344	1265	3027	405	1494	853	1702	662	1462	739	3057	873	835	396	3641	1719	3626	1232	2308.4	900.8	0

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OTU no.	OTU abundance	Taxonomy	BEF2	AF2	BEF6	AF6	BEF8	AF8	BEF9	AF9	BEF10	AF10	BEF12	AF12	BEF14	AF14	BEF15	AF15	BEF19	AF19	BEF20	AF20	Mean BEF	Mean AF	P value
		Epsilonproteobacteria; Campylobacterales; Campylobacteraceae; Campylobacter																							
Otu261	27911	Bacteria; Proteobacteria; Betaproteobacteria; Neisseriales; Neisseriaceae; Neisseria; Neisseria_cinerea	1318	2960	1512	173	1817	1428	1695	4513	10	94	348	40	2661	1374	7146	241	141	193	196	51	1684.4	1106.7	0.493
Otu29	24893	Bacteria; Proteobacteria; Betaproteobacteria; Neisseriales; Neisseriaceae; Neisseria	1204	4196	1753	151	291	1799	1931	2459	276	1394	938	2197	1258	1718	495	1368	933	351	27	154	910.6	1578.7	0.122
Otu17	23783	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella; Prevotella_pallens	209	158	2811	1991	3170	1185	253	344	390	143	1413	1889	642	727	553	699	2042	790	2090	2284	1357.3	1021	0.204
Otu15	22697	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella	792	455	2528	1294	3798	773	483	272	1072	1604	1043	2485	695	355	198	523	2114	674	255	1284	1297.8	971.9	0.452
Otu25	20994	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Porphyromonadaceae; Porphyromonas	785	1237	597	596	331	1719	3075	1734	61	116	386	878	2679	1383	1222	760	952	311	1780	392	1186.8	912.9	0.372
Otu21	18426	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Paraprevotellaceae; Prevotella	622	119	1301	2576	1203	870	1530	523	1843	548	485	513	1291	273	882	220	1835	330	1130	332	1212.2	630.4	0.046
Otu20	17155	Bacteria; Fusobacteria; Fusobacteriia; Fusobacteriales; Leptotrichiaceae; Leptotrichia	91	128	1132	460	105	1377	454	2226	941	247	2634	1290	584	277	166	373	1213	2217	595	645	791.5	924	0.676
Otu27	13910	Bacteria; Firmicutes; Bacilli; Lactobacillales; Carnobacteriaceae; Granulicatella	745	527	229	314	364	487	694	1237	1869	513	809	661	251	619	413	638	442	1081	1215	802	703.1	687.9	0.935
Otu26	13468	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Prevotellaceae; Prevotella; Prevotella_nanceiensis	800	947	1297	155	177	898	1172	1185	54	173	104	544	457	2199	556	751	212	338	822	627	565.1	781.7	0.368
Otu9	13242	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Moraxellaceae; Moraxella	1	6634	0	60	79	562	106	849	778	362	5	750	1575	1182	263	1	0	21	14	0	282.1	1042.1	0.283
Otu40	13051	Bacteria; Firmicutes; Clostridia; Clostridiales; Veillonellaceae; Veillonella	797	78	1525	386	445	1210	745	462	27	281	17	326	793	2069	718	1551	747	723	108	43	592.2	712.9	0.615
Otu33	12906	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales;	825	25	2041	579	244	1491	699	2294	0	43	63	283	390	574	1564	594	403	566	176	52	640.5	650.1	0.975

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OTU no.	OTU abundance	Taxonomy	BEF2	AF2	BEF6	AF6	BEF8	AF8	BEF9	AF9	BEF10	AF10	BEF12	AF12	BEF14	AF14	BEF15	AF15	BEF19	AF19	BEF20	AF20	Mean BEF	Mean AF	P value
		Paraprevotellaceae; Prevotella																							
Otu37	12672	Bacteria; Firmicutes; Clostridia; Clostridiales; Veillonellaceae; Megasphaera	171	37	1119	1683	977	798	672	748	111	376	299	929	73	907	16	180	417	1659	623	877	447.8	819.4	0.027
Otu23	11908	Bacteria; Firmicutes; Clostridia; Clostridiales	201	2117	328	924	24	606	214	2345	232	49	538	1131	744	209	241	83	798	673	149	302	346.9	843.9	0.112
Otu193	11315	Bacteria; Proteobacteria; Gammaproteobacteria; Pasteurellales; Pasteurellaceae	2650	318	416	163	1426	515	1844	354	197	230	811	427	482	428	178	65	292	285	189	45	848.5	283	0.049
Otu28	10024	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Paraprevotellaceae; Prevotella; Prevotella_tannerae	10	185	639	3606	53	663	515	174	27	84	1015	193	1624	97	70	627	115	286	21	20	408.9	593.5	0.63

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A NOVEL ABA FUNCTIONAL ANALOGUE B2 ENHANCES SALINITY TOLERANCE IN WHEAT

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Abstract. Salinity stress is considered to be the most damaging type of abiotic stress, with devastating effects on wheat production. Abscisic acid (ABA) is a plant hormone that plays an important role in inducing the plant's response to abiotic stresses. Based on the structures of ABA, pyrabactin (PYR) and coronatine (COR), a target chemical compound, B2, was designed in this study. In a previous study, B2 enhanced drought tolerance in wheat by enhancing the ROS capacity, which was achieved by improving the antioxidant enzymes activity and the osmotic adjustment ability. Furthermore, it had a significant effect on improving photosynthesis and increasing the endogenous hormones in wheat under stress conditions. Two wheat cultivars (*Triticum aestivum L.*), JIMAI22 (JM22) and SHANNONG12 (SH12), were grown under hydroponic conditions to explore the effect of B2 in mitigating salt stress. The results showed that B2 had a function analogous to ABA, especially at a concentration of 0.1 μmol·L-1. Under a salinity stress of 150 mM NaCl, treatment with 0.1 μmol·L-1 B2 increased the leaf water content in both wheat cultivars, improving their photosynthetic efficiency and the antioxidant enzyme activity. Moreover, B2 enhanced the expression level of ABA-responsive genes (*TaABAOH2*, *TaNCED1*, and *TaAREB3*). Furthermore, it enhanced the expression level of salt-responsive genes (*TaMYB3R1* and *TaERF3*). Moreover, B2 also induced the endogenous plant hormone (ABA) signaling pathway, which led to an improvement of the salt tolerance in the wheat cultivars.
Keywords: *abscisic acid alternatives, salt stress, ABA and salt-responsive genes, hormonal signaling antioxidant, Triticum aestivum*

Introduction

Wheat is recognized as one of the most commonly cultivated food crops throughout the world and the primary source of proteins and carbohydrates. Current wheat production does not meet the demands of a rapidly growing global population. While plant breeders are working hard to improve wheat production, it is difficult to achieve the required production level due to complex issues, especially abiotic stresses (Jatoi et al., 2011; Moaveni, 2011).

Currently, soil salinity and drought are considered the most damaging abiotic stresses, affecting overall agricultural production. Salinity plays a crucial role in limiting plant production, especially in barren and semi-barren regions (Ashraf et al., 2004; Hussain et al., 2009). The main cause of salt stress in crops could be the use of saline water for irrigation purposes, due to the excessive amount salt in the ground water. Plants have internal mechanisms that help them realize the incoming stresses and regulate their physiological and metabolic processes accordingly to face the calamities (Zhang et al., 2006).

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The plant root system rapidly senses the salt stress, which reduces the plant growth. In the short term, the water availability to the plant is hindered by increasing osmotic stress due to a high salt content, while in the long term, it is hampered by the raised ion toxicity, causing a nutrient deficit in the cytosol (Munns, 2005). A high salt concentration leads to a shortage in plant water uptake, even in well-watered soil, due to a reduction in the osmotic potential of the soil solutes, which makes it difficult for the plant roots to absorb water from the soil solution (Rengasamy, 2006). A high level of salinity also substantially reduces the pigment content of the leaves (Al-Sobhi et al., 2006).

High concentrations of NaCl, such as 125 mM and 150 mM, have a completely inhibitory, rather devastating, effect on most of the wheat cultivars (Amor et al., 2005), as most of the growth processes are badly affected. Growth rates and biomass production are efficient indicators to evaluate the level of the stress caused by salt and a plant's ability to resist it (Amor et al., 2005).

The higher salinity concentrations show clear effects on root and shoot lengths, since the roots are directly exposed to salt. A high level of NaCl causes a reduction in root and shoot development due to its toxic effects and an imbalanced nutrient uptake by plants. It also inhibits root elongation and development by reducing the water uptake by plants.

High salt concentrations decrease the water potential of plants and affect the water availability for the plant physiological functions (Jakab et al., 2005). The osmotic stress, as a result of salt accumulation, leads to a reduction of water absorption through roots and hence creates a water deficit in wheat leaf (Rahnama et al., 2010).

One of the main defense strategies in plants is their antioxidant system. This includes enzymatic and non-enzymatic compounds, which act as detoxification agents, scavenging toxic free radicals and helping the plants overcome destructive oxidative stress (Parida et al., 2004; Li et al., 2011). The major antioxidant defense enzymes are superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD). SOD can scavenge the toxic O2- radicals, and its enzymatic action results in the formation of H_2O_2, which is subsequently converted to H2O by POD (Li et al., 2011).

ABA is one of the plant hormones that has the ability to regulate the plant physiological responses. It is known as a stress hormone because of its rapid accumulation in response to stresses and its mediation of stress responses, which helps plants to overcome stresses (Zhang et al., 2006). Exogenous ABA application accumulates proline by activating its biosynthesis and inhibiting its degradation pathways, which in turn plays a protective role in salt-stressed plants through scavenging free radicals (Wang et al., 2017). Recent studies show that plant growth responses to abiotic and biotic stresses can be regulated by plant hormones. These hormones have interrelated growth, inducing or inhibiting activities, which leads to a modulation of different plant biosynthesis and physiological responses (Peleg and Blumwald, 2011).

High plants have developed strategies to understand stress signals and modulate the specific stress-responsive gene expressions to acclimate to the stress conditions. Many genes induced by stress have been identified, such as key enzymes of the ABA biosynthesis pathway, osmotic adaptation proteins, cell protective enzymes and transcription factors (TFs). TFs, including AP2/ERF proteins, ABA responsive element (ABRE)-binding proteins and MYB, which play an important role in controlling stress-related gene expressions. Previous studies declare that different ERF members play various functions in plant responses to abiotic stress (Rong et al., 2014).

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To synthesize the B2 compound, the sub active structure splicing method of integrating ABA, Pyrabactin (PYR), and coronatine (COR) was used, based on the similarities of the amide structure of COR and the sulfonamide-structure of PYR (Zhou et al., 2019) (*Fig. A1* in the *Appendix*).

In a previous study, B2 enhanced drought tolerance in wheat by enhancing the ROS capacity, which was achieved by improving the antioxidant enzymes activity and the osmotic adjustment ability. Furthermore, it had a significant effect on improving photosynthesis and increasing the endogenous hormones in wheat under stress conditions (Zhou et al., 2019).

Jimai22 is widely known as salt tolerant genotype (Dugasa et al., 2019) however Shannong12 was used as test material as it is not investigated for its salinity tolerance previously.

In the present study, the efficiency of compound B2 in enhancing wheat tolerance to salinity stress was investigated. This was because the function of this compound is analogous to that of ABA. The study was conducted to observe the morphological and physiological changes, occurring in different wheat cultivars. Moreover, the variation of the expression level of the related genes, with the use of B2 under high saline conditions, was also investigated.

Materials and methods

Wheat seedlings preparation and salt stress treatment

The experiment was conducted in Research Institute China Agricultural University, Beijing, China. Wheat seeds were sterilized by soaking them in 5% sodium hypochlorite solution for 10 min. The seeds were then washed 3–4 times with distilled water and planted in a plastic net, placed in a hydroponic box. At the one-leaf stage, after germination, the seedlings were transferred into plastic hydroponic boxes, containing Hoagland solution (Hoagland and Arnon, 1950). All the growth-boxes were placed in a greenhouse, set at 22–25 °C, 50% humidity and 14 h light and 10 h dark conditions, with a light intensity of 400 $mol \cdot m^2 \cdot s^{-1}$. Two wheat cultivars, Jimai22 (JM22) and Shannong12 (SH12), were grown to study their performance. After 48 h, when the seedlings were at the two-leaf stage, the chemical compound B2 and ABA treatments were applied by soaking the roots in the treatment solutions for 48 h. The ABA concentration was 0.01 $\mu mol \cdot L^{-1}$. B2 was added to the concentrations of 0.1 $\mu mol \cdot L^{-1}$, 0.01 $\mu mol \cdot L^{-1}$ and 0.001 $\mu mol \cdot L^{-1}$. After the treatment, the seedlings were transplanted into Hoagland nutrient solution. Five days later, seedlings were transplanted into Hoagland nutrient solution, containing 150 mM NaCl for salt stress treatment. For the purpose of conducting different analyses, leaf samples were taken from the seedlings, including those under the normal conditions (NC) and NaCl treatments. The treatments were replicated three times, average values of five seedlings were used for each replicate, and samples were collected for physiological measurements or RNA extraction.

Plant growth and biomass accumulation

For the measurement of fresh weight and dry matter accumulation, shoot, and root were separated and weighed for their fresh weight. The shoot and root dry weight was

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recorded after drying in hot air oven at 65 °C for 72 h. All the related traits were recorded after eight days of planting.

Root characteristics

Three plants from each treatment were selected, shoot and roots were separated. The roots of each plant were scanned, (the roots of the same plant were submerged in a water tray to ensure separation of roots and to reduce their overlap, and the roots were scanned by using Epson photo scanner (Epson, Long Beach, CA). Scanned images of roots of respected treatments were analyzed using WinRHIZO Pro image analysis software (Regent Instruments, Inc., Quebec City, QC) to calculate the total root length (sum of the lengths of all roots from the same plant), root volume and total root surface area.

Relative water content (RWC)

Three leaves from each treatment were taken for relative water content (RWC) measurement. The fresh leaf weight (FW) was recorded. The leaves were then dipped in distilled water, for 24 h. The leaves were taken out of the water to record the turgid weight (TW). To record the dry leaf weight (DW), the leaves were dried in an electric oven, set at 65 °C for 72 h. The relative water content (RWC) of the leaves was calculated as per the formula given by Cornic (1994).

Antioxidant enzymes extraction and assays

The supernatant, used for the detection of SOD, POD and CAT activities, was according to the protocol described by Parida et al. (2004), where 0.5 g of leaf tissue was homogenized in 4 mL of 50 mM sodium phosphate buffer (pH = 7.0), containing 1% (w/v) PVP, 0.1 mM EDTA-Na_2, 1 $mM \cdot L^{-1}$ iso ascorbic acid and 0.05% (w/v) Triton X-100 in an ice bath. The homogenate was centrifuged at 12000 rpm and 4 °C for 15 min.

The soluble proteins content was quantified using the Bradford (1976) method, absorbance values, obtained by a standard curve was prepared with bovine serum albumin.

ABA was extracted and purified using the methods of Bollmark et al. (1988) and Yang et al. (2001) with some modifications. Quantifications were conducted using the ELISA approach. The mouse monoclonal antigens and antibodies, used in ELISA, were produced at the Phytohormones Research Institute China Agricultural University (checked and proven).

Malondialdehyde (MDA) and hydrogen peroxide (H2O2) content

For the determination of lipid peroxidation in shoots was done by, thiobarbituric acid (TBA) test, which determines Malondialdehyde (MDA) as an end product of lipid peroxidation (Heath and Packer, 1968). Fresh leaf material (500 mg) was homogenized in 5 ml 0.1% (w/v) TCA solution. The homogenate was centrifuged at 10000× g for 20 min, and 0.75 ml of the supernatant was added to 1.5 ml of 0.5% (w/v) TBA in 20% TCA. The mixture was incubated in boiling water for 30 min, and then reaction stopped by placing the reaction tubes in an ice bath. Then the samples were centrifuged at 10000×g for 5 min, and the absorbance of the supernatant was recorded at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The amount of MDA–TBA complex (red pigment) was calculated from the extinction coefficient 155 mM^{-1} cm^{-1}.

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$$\text{MDA (nmol g}^{-1}\text{ fresh weight)} = (\text{Abs532} - \text{Abs600})/155 \quad \text{(Eq.1)}$$

H_2O_2 levels were determined according to Alexieva et al. (2001). The absorbency of the supernatant was recorded at 390 nm. The content of H_2O_2 was given on a standard curve.

Proline content

Free proline content in shoots of wheat seedling was extracted with 3% sulfosalicylic acid and centrifuged at 12 000×g. An aliquot was mixed with glacial acetic acid and acidic ninhydrin for 30 min at 100 °C. The reaction was terminated in an ice bath, and chromophore was extracted with five mL of toluene. The chromophore-containing toluene was warmed to room temperature, and absorbance was recorded at 520 nm (Bates et al., 1973).

Determination of photosynthetic parameters

Photosynthetic speed using a portable photosynthetic apparatus LI-6400 (LI-Cor Inc, Lincoln, NE, USA) to measure the photosynthesis rate, stomatal conductance, intercellular CO_2 concentration and transpiration rate. Measurement conditions: leaf surface temperature: 25-28 °C, light intensity 1000 μmol/m^2 s, flow rate 500 mL/s. Each treatment was repeated 3 times, and the newly developed leaves of the seedling leaves were selected at the measurement site.

Statistical analysis

Two separate experiments were done each one contains one cultivar using complete randomized design (CRD) was used with three replications for each treatment. Analysis of variance (ANOVA) was carried out using Proc Mixed of SAS package version 9.2 (SAS 2008) and means were compared by Duncan's multiple range test at 5% level of probability (Steel and Torrie, 1980).

Extraction of RNA and quantitative RT-PCR

Total RNAs were extracted from the leaves using an Easy Pure Plant RNA Kit (Trans Gene Biotech, Beijing). A Nano-Drop2000 spectrophotometer was used to determine the concentrations. Two μg of the total RNA was used to synthesize cDNA, as per the method described in detail by Zhou et al. (2019). Each sample had three independent replicates, and each replicate had three biological repetitions. The $2-\Delta\Delta Ct$ formula of Livak and Schmittgen (2001) was used to calculate the relative expression level, where reference gene was β-actin gene (Gene Bank ID: AB181991.1) (*Table A1* in the *Appendix*).

Results

B2 effect on plant growth and biomass accumulation, root characteristics and relative water content (RWC) under salt stress

Significant differences were found among different treatments, according to statistical analyses, in terms of the plant dry weight and root/shoot ratio of wheat seedlings under salinity stress conditions.

The data regarding the total plant dry weight of seedlings pretreated with B2 showed that cv. JM22 had a 36.8% increase, and cv. SH12 had a 22.7% increase, in plant dry

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weight (compared with the control) under salt stress conditions. An increase in the root dry weight was also observed due to the pretreatment of B2. Compared with the control, an increase of 56.7% and 35.2% in the root weight was found for cv. JM22 and cv. SH12, respectively, under high saline conditions. Moreover, a significant increase of 20.8% for cv. JM22 and 14.8% for cv. SH12, in terms of the root/shoot ratio, was also observed in the seedlings pretreated with 0.1 μmol·L-1 B2 under salt stress conditions (*Table 1*). On the other hand, the lower concentrations of B2 were not effective under salinity conditions for both wheat cultivars, while the 0.001 μmol·L-1 B2 was effective, compared with the control, in cv. JM22 under normal conditions (NC) in terms of the total dry weight and the root dry weight traits.

The data clearly showed that the seedlings treated with ABA or B2, with different concentrations, performed better under salt stress, compared with the untreated ones. It was also observed that 0.1 $\mu mol \cdot L^{-1}$ B2 not only increased the total root length but also enhanced the total root volume and root total surface in JM22 and SH12 (*Table 1*).

The plant physiological state can be reflected by the relative water content (RWC) of the leaves under salt-stressed conditions. *Table 1* shows that the cv. SH12 leaves of seedlings treated with B2 0.1 and 0.001 $\mu mol \cdot L^{-1}$ had a substantially higher RWC than the control plants, while the cv. JM22 leaves of seedlings treated with B2 0.1, 0.01 and 0.001 $\mu mol \cdot L^{-1}$ had a significant improvement in RWC under the same concentration of salt. Additionally, ABA-treated seedlings had a significant improvement in both cultivars. ABA and B2 enhanced the RWC of both wheat cultivars, SH12 and JM22, under both saline and non-saline conditions.

Based on the preliminary presented data 0.1 $\mu mol \cdot L^{-1}$ of B2 was the best effective concentration among the three investigated concentrations. Therefore, the other two concentrations of B2 were excluded from the following experemints.

Influence of B2 on wheat leaf endogenous ABA level

Under the salinity stress conditions, the ABA endogenous hormone level increased significantly with B2 treatment. The ABA content in leaves, for the treated seedlings (with 0.1 μM B2 and ABA), increased, compared with the control, and the endogenous ABA content increased by 88% and 22% in JM22, respectively, and increased by 106% and 76% in SH12, respectively (*Fig. 1*).

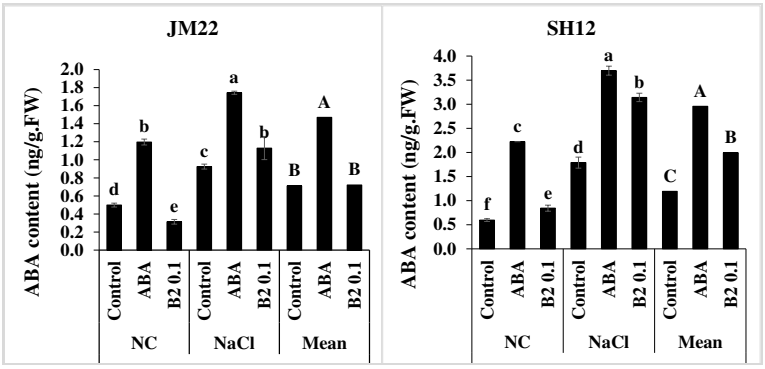

Figure 1. *Effect of B2 on the content of endogenous hormone ABA in both wheat cultivars Jimai22 and Shannong12. Normal conditions (NC), under salt stress (NaCl) and the mean of each treatment under both conditions (Mean). The data are expressed as the mean ± SE of three replicates. Means are compared using Duncan's test at 5% level of significant. Bars labeled with same letters are not significantly differ from each other*

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Table 1. *Effect of B2 on Plant growth and biomass accumulation, root characteristics and relative water content (RWC) in both wheat cultivars Jimai22 and Shannong 12. Normal conditions (NC) and under salt stress (NaCl). The data are expressed as the mean ± SE of three replicates. Means are compared using Duncan's test at 5% level of significant. Values labeled with same letters are not significantly differ from each other*

Treatment			Plant dry weight (mg/plant)	Root dry weight (mg/plant)	Root/shoot ratio	Total root length (cm)	Root surface (cm^2)	Root volume (cm^3)	RWC %
Jimai 22	NC	Control	421.8 ^{c}	118.5 ^{d}	0.39 ^{b}	2087.8 ^{d}	189.3 ^{e}	1.4 ^{f}	90.0 ^{b}
		ABA	553.3 ^{a}	165.8 ^{a}	0.43 ^{a}	2268.7 ^{c}	213.5 ^{d}	1.7 ^{de}	93.3 ^{a}
		B2 0.1	495.8 ^{ab}	148.2 ^{b}	0.43 ^{a}	2487.9 ^{b}	249.5 ^{b}	1.9 ^{c}	91.7 ^{ab}
		B2 0.01	338.8 ^{d}	98.7 ^{e}	0.4 ^{ab}	1901.1 ^{ef}	231.3 ^{c}	2.3 ^{a}	92.8 ^{ab}
		B2 0.001	510.2 ^{a}	144.8 ^{b}	0.4 ^{ab}	2988.0 ^{a}	284.0 ^{a}	2.2 ^{b}	92.2 ^{a}
	NaCl	Control	315.7 ^{d}	82.7 ^{f}	0.4 ^{c}	1136.1 ^{g}	121.0 ^{f}	1.1 ^{g}	81.0 ^{d}
		ABA	444.7 ^{bc}	132.2 ^{c}	0.4 ^{a}	2051.0 ^{de}	203.8^{de}	1.6 ^{e}	82.6 ^{cd}
		B2 0.1	432.0 ^{c}	129.5 ^{c}	0.4 ^{a}	2007.0 ^{de}	205.1^{de}	1.7 ^{de}	83.7 ^{c}
		B2 0.01	314.7 ^{d}	90.5 ^{ef}	0.4 ^{ab}	1939.9 ^{def}	208.0 ^{d}	1.8 ^{d}	84.1 ^{c}
		B2 0.001	331.8 ^{d}	96.2 ^{e}	0.4 ^{ab}	1815.2 ^{f}	203.1^{de}	1.8 ^{de}	83.4 ^{c}
	Mean	Control	368.8 ^{C}	100.6 ^{D}	0.37 ^{C}	1611.9 ^{D}	155.2^{D}	1.2 ^{E}	85.5 ^{B}
		ABA	499.0 ^{A}	149.0 ^{A}	0.43 ^{A}	2159.9 ^{B}	208.6 ^{C}	1.7 ^{D}	87.9 ^{A}
		B2 0.1	463.9 ^{A}	138.8 ^{B}	0.43 ^{A}	2247.5 ^{B}	227.3 ^{B}	1.8 ^{C}	87.7 ^{A}
		B2 0.01	326.8 ^{B}	94.6 ^{D}	0.40 ^{B}	1920.5 ^{C}	219.6 ^{B}	2.1 ^{A}	88.5 ^{A}
		B2 0.001	421.0 ^{D}	120.5 ^{C}	0.40 ^{B}	2401.6 ^{A}	243.5^{A}	2.0 ^{B}	87.8 ^{A}
Shannong 12	NC	Control	326.7 ^{b}	95.2 ^{c}	0.41^{cd}	854.7 ^{d}	101.3 ^{d}	1.0 ^{c}	89.3 ^{b}
		ABA	417.5 ^{a}	132.0 ^{a}	0.46 ^{a}	1971.3 ^{a}	193.8 ^{a}	1.6 ^{a}	92.0 ^{a}
		B2 0.1	357.5 ^{b}	109.0 ^{b}	0.44^{ab}	1057.3 ^{c}	116.9 ^{c}	1.0 ^{c}	92.0 ^{a}
		B2 0.01	350.7 ^{b}	104.0 ^{bc}	0.42^{bc}	1542.8 ^{b}	149.0 ^{b}	1.2 ^{b}	92.4 ^{a}
		B2 0.001	284.8 ^{c}	61.5 ^{e}	0.28 ^{e}	563.9 ^{e}	60.1 ^{g}	0.5 ^{f}	91.9 ^{a}
	NaCl	Control	263.3 ^{cd}	74.8 ^{d}	0.40^{cd}	385.5 ^{f}	50.2 ^{g}	0.5 ^{f}	83.6 ^{cd}
		ABA	325.3 ^{b}	101.7 ^{bc}	0.45 ^{a}	845.3 ^{d}	87.9 ^{e}	0.7 ^{d}	88.3 ^{b}
		B2 0.1	323.2 ^{b}	101.2 ^{bc}	0.46 ^{a}	788.3 ^{d}	77.5 ^{ef}	0.6 ^{ef}	85.3 ^{c}
		B2 0.01	247.0 ^{d}	70.3 ^{d}	0.40^{cd}	633.5 ^{e}	73.6 ^{f}	0.7 ^{de}	81.5 ^{d}
		B2 0.001	207.3 ^{e}	58.3 ^{e}	0.39 ^{d}	837.3 ^{d}	109.7^{cd}	1.2 ^{b}	85.2 ^{c}
	Mean	Control	295.0 ^{C}	85.0 ^{C}	0.40 ^{B}	620.1 ^{E}	75.7 ^{E}	0.7 ^{D}	86.4 ^{C}
		ABA	371.3 ^{A}	116.8 ^{A}	0.46 ^{A}	1408.3 ^{A}	140.8^{A}	1.1 ^{A}	90.1 ^{A}
		B2 0.1	340.2 ^{B}	105.1 ^{B}	0.45 ^{A}	922.8 ^{C}	97.2 ^{C}	0.8 ^{C}	88.7 ^{AB}
		B2 0.01	298.8 ^{C}	87.2 ^{C}	0.41 ^{B}	1088.2 ^{B}	111.3 ^{B}	0.9 ^{B}	86.9 ^{C}
		B2 0.001	246.1 ^{D}	59.9 ^{D}	0.33 ^{C}	700.1 ^{D}	84.9 ^{D}	0.8 ^{C}	88.5 ^{B}

B2 effect on the wheat leaves soluble protein content under high salinity levels

After going through the salt stress, the recorded plant content of soluble protein was higher in B2- and ABA-treated leaves, compared with the control. It increased significantly, by 17% in cv. JM22 and 31% in cv. SH12 B2-treated seedlings, in comparison with the control seedlings, while it increased by 9 and 15% in JM22 and SH12, respectively, in ABA-treated seedlings (*Fig. 2*). Pre-treatment with both

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0.1 μmol·L^{-1} B2 and ABA efficiently enhanced the wheat leaf content of soluble protein under high saline conditions.

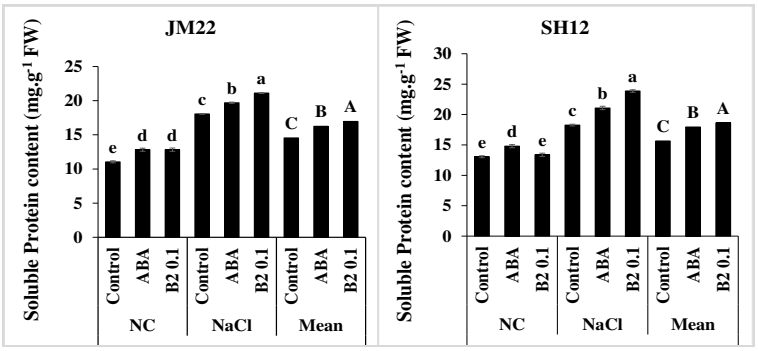

Figure 2. *Effect of B2 on the soluble protein content in both wheat cultivars Jimai22 and Shannong12. Normal conditions (NC), under salt stress (NaCl) and the mean of each treatment under both conditions (Mean). The data are expressed as the mean ± SE of three replicates. Means are compared using Duncan's test at 5% level of significant. Bars labeled with same letters are not significantly differ from each other*

B2 influence on the antioxidant enzymes activity under saline conditions

Three days after the salt application, the antioxidant enzyme activity was analyzed. The antioxidant activity of CAT, SOD and POD enzymes was obviously affected by salt stress. The SOD activity significantly increased due to B2 in the leaves of both wheat cultivars. The rate of increase was 17 and 18% for cv. JM22 and cv. SH12, respectively. Furthermore, there was a significant enhancement in the POD activity, where the rate of increase reached 16% in cv. JM22 and 23% in cv. SH12. Additionally, the antioxidant enzyme CAT activity increased significantly in both cv. JM22 and cv. SH12, at 15 and 10%, respectively. In addition, ABA significantly enhanced the antioxidant enzyme activity of SOD, POD and CAT in both cultivars, JM22 and SH12, and the rates of increase were 23 and 16% for the SOD activity, 17 and 8% for the POD activity and 34 and 30% for the CAT activity, respectively, compared with the untreated seedlings under the saline condition (*Fig. 3a, b, c*).

Proline content

After application of salt stress, it was notable that the plant content of proline become higher in leaves. It increased significantly by 14% and 15% in JM22 and SH12 treated seedlings with B2 and 19% and 26% with ABA compared with the non-treated seedlings in the same concentration of salt (*Fig. 4*). Pre-treatment with both 0.1 μmol·L^{-1} B2 and ABA could efficiency enhance leaves content of proline in both cultivars of wheat crop under the high-level salt conditions.

Lipid peroxidation and ROS accumulation

Under salt stress conditions, data clearly presented that ABA and the chemical compound B2 have improving effect on MDA in both wheat cultivars JM22 and SH12, both treatments successfully decreased the MDA level by 16% and 33% with ABA treatment and 14% and 30% with B2 treatment in both JM22 and SH12 respectively (*Fig. 5a*).

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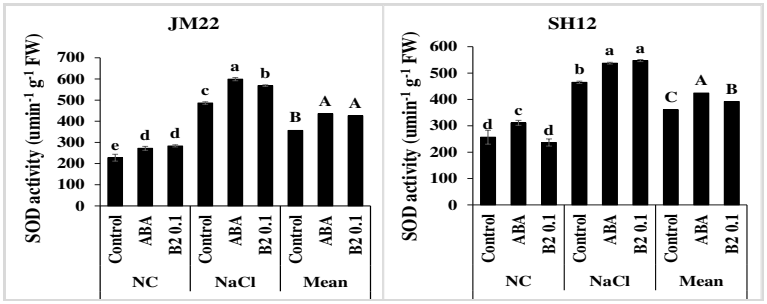
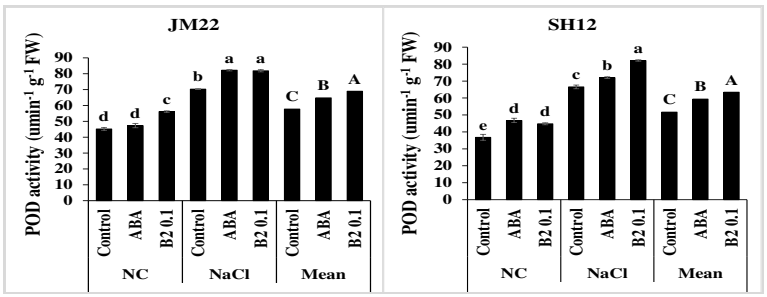


(a)



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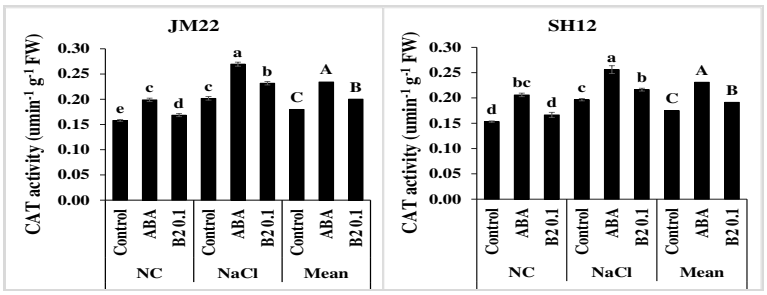

(c)

Figure 3. *Effect of B2 on the antioxidant enzyme activity. (a) Superoxide dismutase (SOD). (b) Peroxidase (POD). (c) Catalase (CAT) in both wheat cultivars Jimai22 and Shannong12. Normal conditions (NC), under salt stress (NaCl) and the mean of each treatment under both conditions (Mean). The data are expressed as the mean ± SE of three replicates. Means are compared using Duncan's test at 5% level of significant. Bars labeled with same letters are not significantly differ from each other*

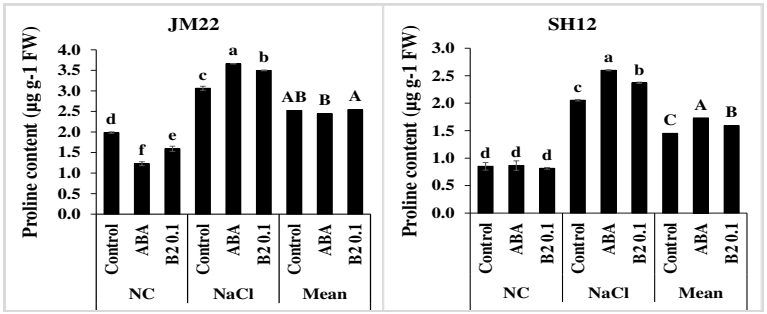

Figure 4. *Effect of B2 on proline content in both wheat cultivars Jimai22 and Shannong12. Normal conditions (NC), under salt stress (NaCl) and the mean of each treatment under both conditions (Mean). The data are expressed as the mean ± SE of three replicates. Means are compared using Duncan's test at 5% level of significant. Bars labeled with same letters are not significantly differ from each other*

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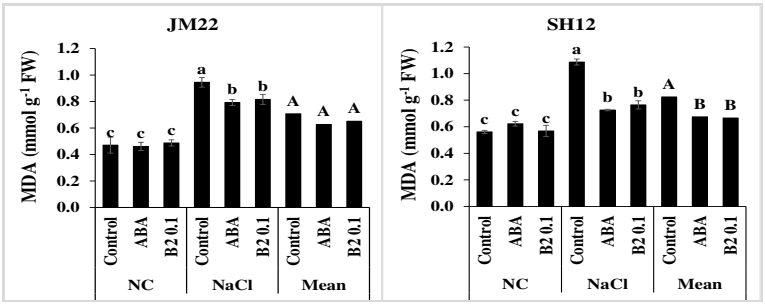
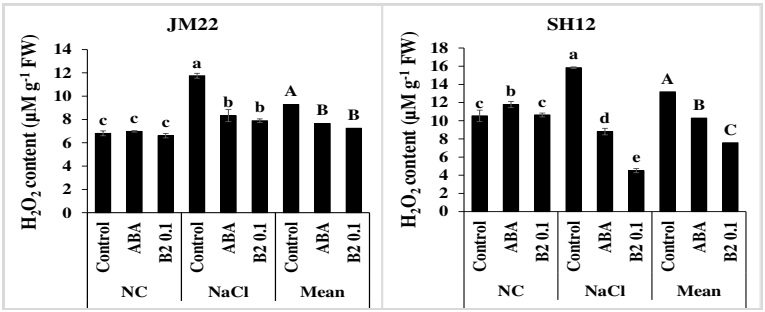
Both ABA and B2 treatments had significantly showed lower accumulation of H_2O_2 in salt conditions. Where, ABA and B2 reduced the level of H_2O_2 in both wheat cultivars leaves, the decreasing rate were 29% and 44% with ABA and 32% and 71% with B2 in JM22 and SN12 respectively (*Fig. 5b*).



(a)



(b)

Figure 5. *Effect of B2 on MDA (a) and H_2O_2 (b) content in both wheat cultivars Jimai22 and Shannong12. Normal conditions (NC), under salt stress (NaCl) and the mean of each treatment under both conditions (Mean). The data are expressed as the mean ± SE of three replicates. Means are compared using Duncan's test at 5% level of significant. Bars labeled with same letters are not significantly differ from each other*

Effect of B2 on wheat leaf photosynthetic rate under salinity stress

The data showed that seedlings treated with ABA or B2 had a high photosynthesis rate, as compared with the control treatment, while growing under salt stress conditions. The photosynthesis rate of ABA- and B2-pretreated seedlings increased by 43 and 39% in cv. JM22 and by 37 and 28% in cv. SH12, respectively, compared with the non-treated seedlings under a high salinity. Additionally, there was a notable effect of ABA and B2 on wheat leaf stomatal conductance and transpiration rate under salt stress in both the cultivars. Moreover, there was a significant increase in B2-treated seedlings in both indicators, Stomatal conductance (Sc) and Transpiration rate (Tr), in the wheat cultivar, SH12, under salt stress conditions, and it increased by 84 and 68%, respectively, while the effect was not notable in JM22 under the stress conditions. On the other hand, the increase rate was not significant for ABA-treated seedlings in both indicators, Stomatal conductance (Sc) and Transpiration rate (Tr), in both SH12 and JM22 (*Fig. 6a, b, c*).

Effect of ABA and B2 on the expression of salt and ABA-responsive genes

With the B2 and ABA pre-treatment of wheat seedlings, the expression level of salt-responsive genes (i.e., *TaERF3* and *TaMYB3R1*), along with the ABA-responsive genes

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(i.e., *TaABAOH2, TaNCED1*, and *TaAREB3*), increased significantly under salt stress. However, the time for each gene to reach the highest expression level was different, depending on the treatment and cultivar. The expression level of *TaERF3* increased with B2 and ABA treatments, compared with the control, under salt stress, based on time. Here, B2 was comparable with ABA in terms of the increase in the expression level of the mentioned gene. In cv. JM22, B2 increased *TaERF3* expression at 12, 24, 48 and 72 h, and its effect was higher than that of ABA from 24 to 72 h (*Fig. 7a, b*). However, in cv. SH12, the significant increase started at 6 h for the treatment with both B2 and ABA (*Fig. 7d*). On the other hand, an initial increase in the expression level of *TaMYB3R1* was seen in cv. JM22 at 3 h due to ABA treatment. However, the expression dropped down with time (*Fig. 7c*). In the case of cv. SH12, *TaMYB3R1* expression increased linearly at 24, 48 and 72 h due to the effect of B2, unlike the nonlinear influence of ABA (*Fig. 7d*). Under salt stress, B2 improved the expression level of *TaABAOH2* in cv. JM22 and cv. SH12, especially at 72 and 24 h, respectively (*Fig. 8e, f*). Furthermore, like ABA, B2 enhanced the expression level of *TaNCED1* in cv. JM22 at 72 h and significantly enhanced the expression of the same gene in cv. SH12 at 24 h (*Fig. 7g, h*). The effect of B2 and ABA was almost similar on the expression of *TaAREB3* in cv. JM22, which reached its peak at 72 h. However, in cv. SH12, only ABA affected the gene expression at 12 h, with no significant effect of B2, compared with the control (*Fig. 7i, j*).

Discussion

Soil salinity and drought are considered to be the most damaging abiotic stresses affecting agricultural production. It has previously been reported that ABA affects plant growth and development and has a role in plant responses to abiotic stresses, including salt, cold and drought stresses (Zhu, 2002).

Under saline conditions, applying a proper concentration of ABA enhances the salinity tolerance and increases the growth rate in sorghum and rice crops (Gurmani and Bano, 2007). Shoot fresh and dry weight decreases with an increase in salinity, but the application of ABA decreases the inhibitory effect of salt on shoot weight to some extent. The root weight of wheat also increases, if the seed is pretreated with ABA (Gurmani and Bano, 2007). These previous studies are in accordance with the present study. Here, both the treatments of ABA and B2 enhanced the total dry weight, root/shoot ratio, and root growth rate.

The plant physiological state is reflected by the relative water content (RWC) of its leaves. The reduction of the leaf water potential (LWP) and relative water content (RWC) were significantly moderated by ABA treatment under saline conditions. Additionally, ABA priming enhanced the RWC and LWP of salt-acclimated plants under subsequent salt stress. This indicates that ABA helps in maintaining a better water status of wheat plants under salinity stress (Davies and Zhang, 1991). The positive effect of ABA-induced antioxidant activity and the reduction in oxidative stress was further manifested by an increase in RWC, plant growth and membrane stability index (Gong and Li, 1998).

In this study, both B2 and ABA promoted the RWC of both wheat cultivars. In accordance with these observations. B2 enhanced the root growth, and plant total fresh and dry weight, leading to an improved water absorption capacity of wheat seedlings and hence an improved RWC (Karcher et al., 2008).

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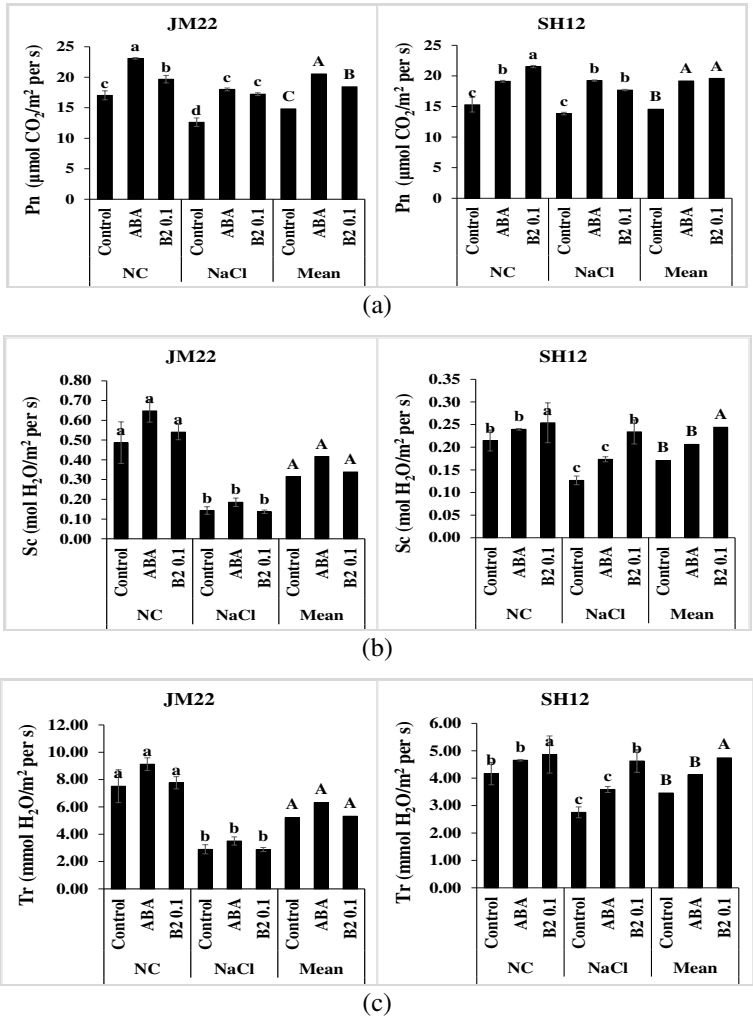

Figure 6. *Effect of B2 on the (a) Photosynthesis rate (Pn) (b) Stomatal conductance (Sc). (c) Transpiration rate (Tr) in both wheat cultivars Jimai22 and Shannong12. Normal conditions (NC), under salt stress (NaCl) and the mean of each treatment under both conditions (mean). The data are expressed as the mean ± SE of three replicates. Means are compared using Duncan's test at 5% level of significant. Bars labeled with same letters are not significantly differ from each other*

ABA accumulation in plant cell decreased the level of Na^+, regardless of whether these plants were salt acclimated. The endogenous ABA content notably increased with the salt treatment. A reduced Na^+ concentration and increased ABA level were caused by the combination of salt acclimation and ABA priming. ABA wheat plant priming improves the endogenous ABA content, which leads to a reduction in Na^+ uptake and accumulation (Davies and Zhang, 1991).

In the present study, the endogenous ABA level increased under salinity conditions. This indicates that B2 induced ABA accumulation in wheat plants. It also contributes to antioxidant enzyme's resisting system regulation and osmotic stress adjustment and enhancement, thus reducing oxidative damage.

One of the main defense strategies in plants is their antioxidant system. This includes enzymatic and non-enzymatic compounds, which act as detoxification agents of toxic-free radicals and therefore help the plant to overcome the destructive oxidative process

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(Li et al., 2011; Zhang et al., 2016). The major antioxidant defense enzymes are SOD, CAT, and POD. POD decomposes H_2O_2 through the oxidation of co-substrates, such as phenolic compounds and/or other antioxidants, whereas CAT breaks down H_2O_2 into H2O and O2 (Mittler, 2002; Li et al., 2011). An increase in SOD activity decreases free radicals, especially superoxide radicals that harm cell membranes, increase plant tolerance to oxidative stress and scavenges O_2^- radicals (Manjili et al., 2012).

Many studies indicate that the antioxidant system has a critical role in plant resistance to oxidative damage caused by salinity stress (Mittler, 2002; Xie et al., 2008; Qiu et al., 2014). Therefore, improving the antioxidant enzyme activities in plants results in an enhancement of the plant tolerance to salt stress. ABA has a positive effect in inducing the POD, SOD, and CAT activity in wheat plants exposed to a high salt stress (Davies and Zhang, 1991). ABA has a positive effect on adaptation responses in wheat (Agarwal et al., 2005).

The present results show that B2 mimics ABA and can enhance the scavenging capacity of reactive oxygen species (ROS) by improving the antioxidant enzymatic activities in wheat seedlings under saline conditions. Therefore, B2 could diminish the oxidative damage, which was also confirmed by the improvement in the soluble protein level, SOD, POD, and CAT, which led to the enhancement in the osmotic adjustment potential of the wheat seedlings.

Plants show many physiological and biochemical responses to cope the environmental abiotic stresses such as the accumulation of proline (Delauney and Verma,1993). In fact, free proline accumulates in a wide variety of higher plants, such as tobacco, soybean, barley, wheat and rice (Yoshiba et al.,1999). Proline play a major role in free radical scavenging, adjusting cell osmotic and a compatible solute that protects enzymes (Verbruggen et al., 1993). The metabolism of proline is essential for the plant stress resistance (Hare et al.,1999). Furthermore, it has been confirmed that accumulation of proline in cells improve osmotic and salt stress tolerance in transgenic plants (Kavi-Kishor et al., 1995; Zhu, 2002), although there also existed the regards that proline was an alternative result from adaptive or detrimental processes responding to osmotic stress (Larher et al., 2003). Proline also has a main function as protecting the enzymes from dehydration and salt accumulation (Thomas, 1990), and because of increasing abscisic acid concentration in response to various stresses (Unyayar et al., 2004), it seems that abscisic acid spraying increases proline content as a defense mechanism. ABA increased the proline content of flag leaf in the high salt level conditions (Flower and Yeo, 1989).

One of the ABA functions is enabling the plant to rapidly respond to salt stress by inducing stomatal closure. This is achieved through the modulation of the cytosolic $CA2^+$ concentration, which leads to the depolarization of the guard cell, as a result of an improved ABA level (Wang et al., 2017). It has been proved that salt causes the inhibition of photosynthesis, which could be due to stomatal or non-stomatal limitations. Gas stomata conductance (gs) decreases significantly due to salt stress, while ABA application improves the gs level in wheat plants under saline conditions (Davies and Zhang, 1991).

ABA has a photosynthesis regulation function in wheat plants adapted to salt through the regulation of non-stomatal parameters. ABA plays an effective role in the plant's resistance to salt stress by decreasing the water loss and adjusting the osmotic stress through stomatal control. ABA priming treatment improved the carbon absorbance and water use efficiency of the adapted wheat plants under salt stress (Davies and Zhang, 1991).

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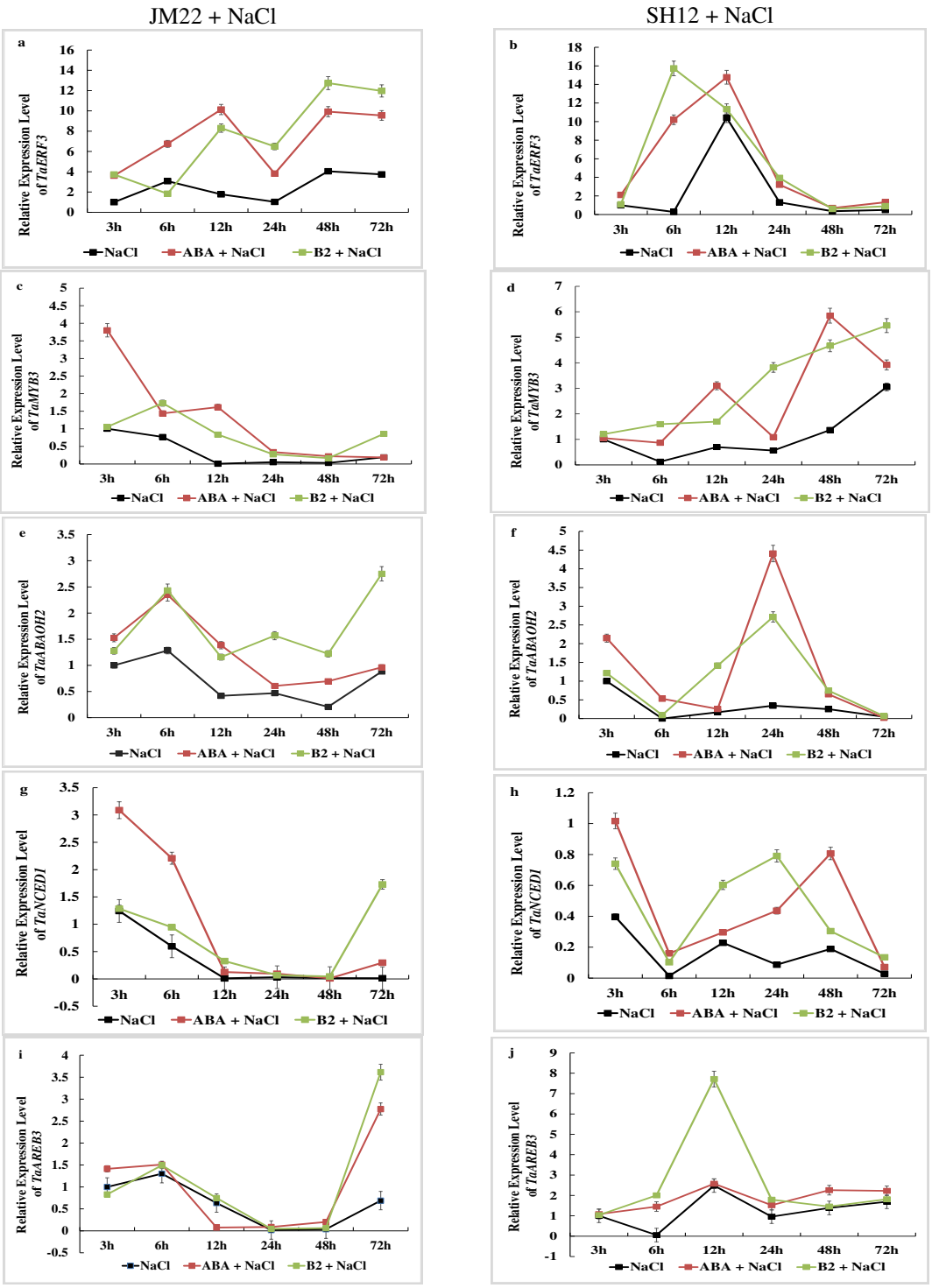

Figure 7. *Expression of salt- and ABA-responsive genes in wheat seedlings at 3 h, 6 h, 12 h, 24 h, 48 h, and 72 h under salt stress conditions. Control under salt conditions (NaCl); ABA treatment of wheat seedlings under salt conditions (ABA + NaCl); B2 treatment of wheat seedlings under salt conditions (B2 + NaCl). (a) TaERF3 gene expression level in JM22, (b) TaERF3 gene expression level in SH12, (c) TaMYB3R1 gene expression level in JM22, (d) TaMYB3R1 gene expression level in SH12, (e) TaABAOH2 gene expression level in JM22, (f) TaABAOH2 gene expression level in SH12, (g) TaNCED1 gene expression level in JM22, (h) TaNCED1 gene expression level in SH12, (i) TaAREB3 gene expression level in JM22, and (j) TaAREB3 gene expression level in SH12*

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It is well known that the salt-induced inhibition of photosynthesis is attributed to stomatal and non-stomatal limitations (Wang et al., 2017). It might be that ABA improves the photosynthesis of wheat plants under salt stress due to the regulation of some other non-stomatal parameters. Nevertheless, stomatal control by ABA appears to be an efficient strategy for decreasing water loss and the negative effects of osmotic stress, caused by salinity (Wang et al., 2017).

A comparison of the influence of both ABA and B2 analogues on the expression of some genes related to salt stress and the ABA response was also performed. The effect of B2 on the expressions of these genes was found to be similar and sometimes superior to that of ABA, which reflected its efficiency as a stress regulator. However, the activation of gene expression was mainly based on the gene, time, and cultivar. The results showed that B2, like ABA, simulated the expression level of all the studied genes. In addition, among the tested genes, some were involved in drought and salt stress, such as transcription factor *TaERF3* (Zhang et al., 2007a), which was previously isolated and characterized in wheat. This transcription factor may directly regulate the expressions of different stress-related genes by interacting either with their promoters or with other TFs (Buttner and Singh, 1997; Zhang et al., 2007b). Additionally, *TaERF3* may indirectly regulate the expression of other stress-related genes involved in proline and chlorophyll accumulation, redox homeostasis (H_2O_2 reduction), and stomatal closure, which consequently become the induction of abiotic stress tolerance (Rong et al., 2014). It is well known that ERF proteins possess a vital role in regulating the expression of specific stress-related genes. Moreover, ERF subfamily members were primarily involved in biotic stresses as well (Berrocal et al., 2002; Onate et al., 2007; Chen et al., 2008). On the other hand, recent studies show that ERF members are also involved in diverse functions in plant responses to abiotic and biotic stresses (Rong et al., 2014). MYB proteins play multifunctional roles in the regulation of gene expressions (Rosinski and Atchley, 1998; Jin and Martin, 1999). For instance, they control the cell shape and morphogenesis involvement in abiotic and biotic stress responses to ABA (Cai et al., 2011). The *TaMYB3R1* gene expression was up-regulated, and its expression remained high under both cold and salt stresses. This suggested that *TaMYB3R1* may take part in abiotic stress regulation in wheat. In the present study, the *TaMYB3R1* gene was affected by ABA and B2, based on the time and plant genotype. However, B2 was more efficient than ABA in enhancing the gene expression in cv. SH12 in a linear mode through time. This result proved that the novel analogue (B2) has a good influence in enhancing stress tolerance in wheat.

Conclusions

From this study, it is apparent that B2 and ABA have a similar performance in improving the active oxygen radical scavenging efficiency by improving the antioxidant enzyme activity and enhancing the osmotic adjustment ability in wheat under salt stress. Moreover, the photosynthesis rate and the plant endogenous ABA content also increased, which confirms that B2 has a special regulating physiological mechanism. Suggestion that the findings could be beneficial in eradicating the salt stress problem in agricultural field crops is premature. Further studies should be performed to explain the effect of B2 on other crop plants such as rice since it considered as the main food crop in China, and investigate the effect of B2 based on the molecular analysis.

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APPENDIX

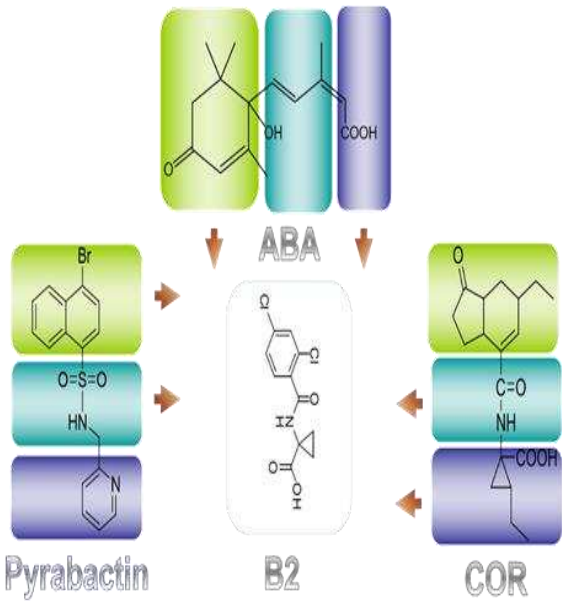

Figure A1. *The synthesis process of B2. We adopted sub active structure splicing method, integrating ABA, Pyrabactin and Coronatine, and then synthesized compound B2*

Table A1. *Sequences of primers used in this study*

Gene name	Primer	Sequence (5'-3')
Actin	F	GACCCAGACAACTCGCAACT
	R	CTCGCATATGTGGCTCTTGA
TaNCED1	F	CGACGGGTACATTCTCACCT
	R	CCTGAGATTCGAGCTCCTTG
TaABAOH2	F	TACAGGTGGGAGGTTGTTGG
	R	TCGTAGTCGTCATCAGTCGG
TaAREB3	F	CTCAAGGACACGAGCGATGCTG
	R	CATTCAGCGGCTGAGGGACAAAC
TaMYB3R1	F	ACGAGAAAGACCGACACCTGC
	R	AACCCAGTGACAGAAAGGAAGCA
TaERF3	F	AGCAATCAGGCAAAGCAACC
	R	ACGACTCAGAAGGAACCACGAC

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ASSOCIATION BETWEEN PARKINSON'S DISEASE AND DENTAL AMALGAM FILLING: A STUDY OF NATIONWIDE POPULATION-BASED CASE CONTROL IN TAIWAN

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Abstract. Parkinson's disease (PD) is a common associated motor dysfunction nervous system disease. Low-level occupational mercury vapor exposure is a commonly known critical factor that increases the risk of PD. Dental amalgams, used for dental restoration, are a major source of mercury exposure for people. However, the relationship between PD and the use of amalgam fillings (AMFs) has yet to be clarified. This paper of retrospective case–control aimed to examine the relationship between AMF use and the risk of PD; it analyzed Taiwan's NHIRD (National Health Insurance Research Database) based on population administrative database, for the 2000-2013 period. For the case and control groups, Charlson Comorbidity Index, age, urbanization level, sex and monthly income were all matched by a propensity score method for a 1:1 ratio; 5712 cases and 5712 controls participated in this study. This results of case–control based on nationwide population revealed no significant association between PD and AMF in Taiwanese people. This result provides meaningful implications despite being statistically not significant.
Keywords: *dental amalgam, mercury, NHIRD, nationwide population, case-control study*

Introduction

Parkinson's disease (PD) is a neurodegenerative disease. The incidence rate among women aged younger than 60 years is 8.6 per 100,000 people; this figure increases to 29 and 78.4 per 100,000 people among women aged 60–69 and 70–79 years old, respectively. The incidence rate among men aged younger than 60 years is 11.1 per 100,000 people; this figure increases to 49.5 and 140.7 among those aged 60–69 and 70-79 years old, respectively (Van Den Eeden et al., 2003). The risk and prevalence rate of PD increases with age (Bower et al., 1999; Baldereschi et al., 2000). With respect to prevalence rate, PD, as a progressive neurodegenerative disease, is second only to Alzheimer's disease (Wirdefeldt et al., 2011). The main clinical symptoms of PD are mostly associated with motor dysfunction—including rigidity, resting tremor, bradykinesia, and postural instability (Frank et al., 2006; Davie, 2008). Generic and environmental risk factors may cause the dopaminergic neurons death in the brain, where these dead neurons, in turn, inhibit the neural pathways from the basal ganglia to the motor cortex (Davie, 2008).

Dental amalgams are a common dental filling material with a 50% mercury content. Traditional low-copper amalgams have a copper content lower than 6%. The high-copper dental amalgams in the 1960s were developed with a copper content of 8%–10% (Haque et al., 2019). In high-copper dental amalgams, mercury is more soluble under acidic conditions relative to its low-copper counterpart (Okabe et al., 2003). High-copper amalgams, because of the amount of mercury vapor released from it (Bengtsson and Hylander, 2017), has a higher corrosion rate and longer corrosion time relative to early amalgams that contain metal composites.

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Autopsy reports have demonstrated that dental amalgam fillings (AMFs) cause mercury accumulation in the human body (Mutter, 2011). AMF use has been found to correlate with mercury content in the brain and kidney tissues (Eggleston and Nylander, 1987; Nylander et al., 1987; Barregård et al., 1999; Guzzi et al., 2006) and to positively correlate with mercury content in the blood and urine (Kingman et al., 1998; Zimmer et al., 2002; Levy et al., 2004). A recent epidemiological study with 2137 participants revealed that relative to those who had not, people who had had 7 or more of their teeth filled with AMF had a 30%–50% higher mercury content in their urine (Dutton et al., 2013).

The pathogenesis of PD has yet to be elucidated. Several case reports and epidemiological studies have indicated that exposure to certain metals long-term (e.g., aluminum, manganese, copper, iron, mercury, lead, zin, and those in amalgams) is a potential factor of risk for PD. The PD development may be associated with the dose of mercury exposure (Ngim and Devathasan, 1989). However, studies have also determined that working in jobs involving long-term exposure to manganese, mercury, or aluminum has no effect on PD development (Semchuk et al., 1993; Vieregge et al., 1995). Accordingly, to control the incidence rate of PD, the study used the Database of National Health Insurance Research Database (NHIRD) to investigate the relationship between PD development and amalgam use.

Materials and Methods

Study Design and Data Source

The data used in this paper were collected from the 2010 Longitudinal Health Insurance Database (2010LHID), a publicly accessible database compiled by Taiwan's National Health Research Institutes. LHID2010 contains raw insurance claims data and registration documents for 2000–2013, including the information of 1 million people selected randomly from beneficiaries registered compulsory National Health Insurance (NHI) procedure in Taiwan, which covered 99.9% of the Taiwanese population in 2010 (Charlson et al., 1987). The present study defined diagnoses corresponding to the ICD-9-CM (International Classification of Diseases, Ninth Revision, Clinical Modification). Because data from the LHID 2010 were used for research and were deidentified secondary data, therefore the consent of written informed was not required.

Control Groups and Selection of Case

This study limiting is the adult population, the present case–control study excluded those aged younger than 20 years, those who had withdrawn from the NHI procedure, or those with missing data in the 2010LHID. Additionally, only individuals who had received three or more consensus diagnoses of PD were included in the sample to improve the diagnostic effectiveness for PD in the LHID2010. Propensity score matching by a 1:1 ratio was performed to conditionally select participants in the population for comparison according to urbanization level, the Charlson Comorbidity Index (CCI) (Charlson et al., 1994), age, socioeconomic status, and sex. The CCI ranges from 0 to 3, with a high CCI indicating a high risk of mortality for a particular patient (diabetes, hypertension, etc.) (Charlson et al., 1987). *Figure 1* illustrates the research process.

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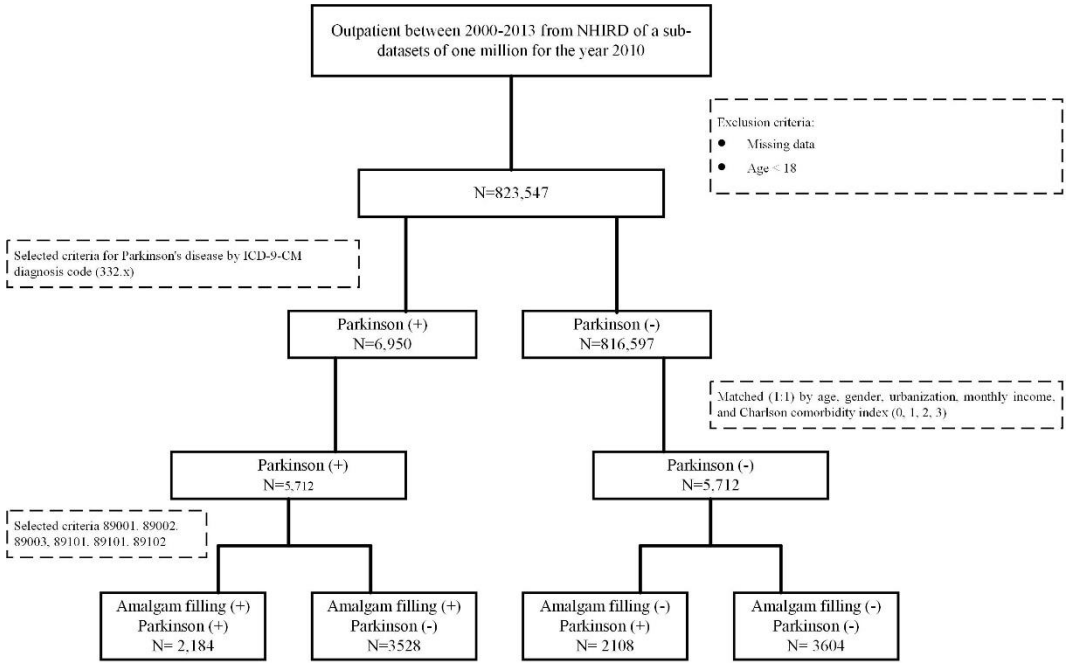

Figure 1. *The flowchart of the study*

Exposure Assessment

This study identified patients, from the LHID2010, who had received AMF-related treatments according to the following procedure codes: Dental amalgam fillings (89001C, 89002C, 89003C) and other dental treatments (89101C, 89102C, 89103C) (*Table 1*). Confounding factors—namely age, urbanization level, CCI, socioeconomic factors, and sex—were included in the analysis (*Figure 1*).

Table 1. *The amalgam filling codes*

Dental amalgam fillings		Other dental treatments	
Code	**Treatment**	**Code**	**Treatment**
89001C	Amalgam restoration for single-face.	89101C	Amalgam restoration for single-surface in specific cases.
89002C	Amalgam restoration for two-face.	89102C	Amalgam restoration for two- surface in specific cases.
89003C	Amalgam restoration for three-face.	89103C	Amalgam restoration three-surface in specific cases.

Statistical Analysis

SPSS 18 (International Business Machines Corporation, Chicago, IL, United States) was used for data analyses. For the continuous variables, this study used Student's *t*-test; for categorical variables, this study used a chi-square test, and the odds ratio (OR) for calculating between the control and case groups; and multiple logistic regression was used in a stratified analysis. All results are presented in terms of the OR and 95% confidence interval (95% CI). Sex, region of residence, age, CCI, and income were adjusted for in the analyses. Statistical significance was defined when the p-value was <0.05.

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Result

This study recruited 5712 cases and 5712 controls. As listed in *Table 2*, the average age was 76.87 ± 11.73 years old; 5806 (50.82%) and 5618 (49.18%) of them were women and men, respectively. The case and control groups did not significantly differ with respect to their demographic characteristics, specifically their sex, region of residence, CCI, age, urbanization level, and monthly income ($P > 0.05$).

Table 2. *The individual's demographic characteristics in this research*

	Total (N=11,424)		**Parkinson (n=5,712)**		**Non- Parkinson (n=5,712)**		**P-value**
	Population	**%**	**Population**	**%**	**Population**	**%**	
Age	76.87±11.73		76.92±11.67		76.83±11.80		**0.659**
Age groups							**0.573**
18-29	62	0.54%	33	0.58%	29	0.51%	
30-39	96	0.84%	47	0.82%	49	0.86%	
40-49	173	1.51%	77	1.35%	96	1.68%	
50-59	605	5.30%	297	5.20%	308	5.39%	
60-69	1395	12.21%	697	12.20%	698	12.22%	
70-79	3565	31.21%	1798	31.48%	1767	30.93%	
>80	5528	48.39%	2763	48.37%	2765	48.41%	
Gender							**0.605**
Female	5806	50.82%	2885	50.51%	2921	51.14%	
Male	5618	49.18%	2827	49.49%	2791	48.86%	
Urbanization							**0.672**
Urban	6378	55.83%	3204	56.09%	3174	55.57%	
Suburban	3596	31.48%	1784	31.23%	1812	31.72%	
Rural	1450	12.69%	724	12.68%	726	12.71%	
CCI†							**0.254**
0	515	4.51%	269	4.71%	246	4.31%	
1	1106	9.68%	572	10.01%	534	9.35%	
2	1642	14.37%	800	14.01%	842	14.74%	
≥3	8161	71.44%	4071	71.27%	4090	71.60%	
Monthly income							**0.598**
＜NT$ 20,000	9481	82.99%	4751	83.18%	4730	82.81%	
NT$ 20,000~40,000	1235	10.81%	612	10.71%	623	10.91%	
＞NT$ 40,000	708	6.20%	349	6.11%	359	6.29%	

† Charlson comorbidity index

As presented in *Table 3*, PD and AMF were not directly related (adjusted OR = 1.067, THE CI of 95%: 0.99–1.15).

Table 4 illustrates the gender -stratified OR for PD and AMF. With respect to sex, no PD and AMF were not associated. The adjusted OR for AMF and PD for men and women were 1.096 (The CI of 95%: 0.984–1.211) and 1.023 (The CI of 95%: 0.920–1.138), respectively.

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Table 3. *The odds ratio between the amalgam filling and Parkinson's disease*

	With Parkinson (n =5,712)		Without Parkinson (n=5,712)	
	Number of patients	**%**	**Number of patients**	**%**
AMF	2184	38.24%	2108	36.90%
Non-AMF	3528	61.76%	3604	63.10%
OR (95% CI)	1.058 (0.981-1.142)		1.00	
Adjusted OR (95% CI)	1.067 (0.987-1.153)		1.00	

Abbreviations: PD—Parkinson's disease; OR—odds ratio; AMF—amalgam filling; CI—confidence interval. Adjustment by gender, CCI, urbanization, monthly income, and age; * $p < 0.05$

Table 4. *Odds ratio for gender of those with a diagnosis of Parkinson's disease with an amalgam filling*

Sex	With PD Number of AMF	Without PD Number of AMF	OR (95% CI)	Adjusted OR (95% CI)
Female	1092	1793	1.023 (0.920–1.138)	1.043 (0.936–1.163)
Male	1092	1735	1.096 (0.984–1.211)	1.098 (0.984–1.225)

Abbreviations: PD—Parkinson's disease; OR—odds ratio; AMF—amalgam filling; CI—confidence interval. Adjustment by urbanization, CCI, age, and monthly income; * $p < 0.05$

Discussion

As far as authors' know, the present study is the first to examine the association between PD and AMF based on a large-scale population-based database and tracking time 13 years. The results revealed no positive correlation between AMF and PD. To ensure accurate PD diagnosis, this study recruited only patients with at least three PD diagnoses. The effectiveness of the PD diagnoses was validated through the NHI system of conducting regular verification of diagnoses; such verification is part of the NHI's treatment guidelines to ensure that health insurance claims are legitimate. The findings of this registry-based study are significant and meaningful.

Dental amalgams of all types contain mercury, which is released in the form of mercury vapor. Mercury's toxicity can cause cell damage through the increased production of free radicals (Olivieri et al., 2000). Heavy metals are suspected to cause the generation of free radicals, which increases oxidative stress in neurons, which, in turn, causes PD. Usually, nervous system diseases are the body accumulation of heavy metals or associated with environmental exposure. Heavy metals can affect the basal ganglia in the brain, which causes physical derangement through damaging neurons in the substantia nigra pars compacta. PD is a highly common motor disorder (Montgomery, 1995). Case reports and epidemiological studies have reported long-term exposure to mercury to be a potential risk factor for PD (Ngim and Devathasan, 1989), whereas some studies have revealed no association between the two (Semchuk et al., 1993; Vieregge et al., 1995). In the present study, PD and AMF use were not positively correlated. This is attributable to possibly differing levels of mercury exposure in occupational exposure than in exposure from AMF use. Mercury can leach out from the surface of AMFs when chewing and tooth brushing. However, this study did not assess the extent of exposure to mercury vapor in patients using AMFs.

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This study has some limitations. First, the NHIRD provides no data on family medical history, symptoms, PD severity, dietary habits, and the brand and formula of amalgam used; such missing information makes the analysis robust, especially with regard to controlling for confounders. Second, the procedure codes in the NHI did not indicate the positions of AMFs. Third, this study did not collect information on the use of other metal dental restoration implements (e.g., dental inlays or crowns) from the NHIRD. Fourth, propensity score matching cannot cope with unobservable confounders, which limits the analysis of this study.

Conclusions

On the basis of results for a nationwide population, the present study revealed no association between PD and AMF in Taiwanese people. Dental amalgams are a potential source of environmental mercury exposure, and it must be investigated as a possible contributor to mercury-induced neurotoxicity. An in-depth risk–benefit analysis on the use of AMF must include the effects, service life, and performance of dental amalgams. Overall, despite dental amalgam fillings use very widespread, the safety data of dental amalgam fillings safety are inadequate. Most reassurance is provided for Parkinson's disease in this study. The further, most in need investigation are effects and neurodegenerative diseases on children and infants.

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EFFECT OF SEEDING RATE ON SOIL WATER CONSUMPTION, YIELD AND QUALITY UNDER WIDE SPACE SOWING OF DRYLAND WINTER WHEAT ON THE LOESS PLATEAU IN CHINA

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Abstract. A field experiments was conducted at the Wenxi experimental site of Shanxi Agriculture University, China. A study was designed to evaluate the effect of different planting density and tillage conditions, spike, protein content, protein yield and grain yield of dryland wheat cultivar (Liangxing-99). The five doses of nitrogen application included were the following: 150 kg·hm^{-2}, 225 kg·hm^{-2}, 300 kg·hm^{-2}, 375 kg·hm^{-2}, and 450 kg·hm^{-2}. The nitrogen rate/amount has extremely significant effects on the number of ears, thousand-grain weight and yield and water use efficiency of winter wheat. Nitrogen absorption and fertilizer productive efficiency significantly were increase by 50% and 51%, respectively, while nitrogen harvest index, the number of ears and yield significantly increased by 22%, 46% and the yield by 25%, 55%. The water use efficiency significantly increased by 33%. The treatment significantly improved grain albumin 13%, gliadin 14%, gluten protein 17%, total protein 14%, and grain/alcohol ratio 3%; wet gluten content increased by 23%, water absorption by 14% and, the softening degree was reduced. Improving nitrogen uptake at different growing stages, ultimately increased yield and improved quality. GS activity of flag leaf was closely related with the total grain protein, glutenin, and gliadin content at the middle filling stage. Wide space sowing in Jinnan area was beneficial and increased water consumption during the growth period, also tiller dynamics, promoted nutrient operation, and increased yield and grain protein content.
Keywords: *plant height, leaf ratio, protein content, tiller dynamics, water consumption, yield of dryland wheat*

Introduction

Wheat (*Triticum aestivum* L.) is one of the most important food crops in the world and it is also an important food crop in China. Wheat production has an important role in the national economic production of China. Improving yield and quality in production has always been an important task for wheat cultivation workers. The yield and quality of wheat are affected by varieties, environmental factors and cultivation measures (Mao et al., 2015). The arid and semi-arid area of the Loess Plateau is typically rainfed and 60% of the dryland area is under wheat cultivation (Jin et al., 2007). The cropping system of the Loess Plateau is mainly a mono-cropping of winter wheat with a 3-month fallow during the rainy summer season (Deng *et al.,* 2006). Most studies in the field of water and N (nitrogen) have only focused on soil water and Nitrogen fertilization (Li et al., 2009). High yield of good quality is based on the used agro-technical measures. One of the most important agro-technical measures is optimal nutrient provision. Insufficient precipitation during the growth stages of wheat and shortages of irrigation water in dryland wheat producing areas are major constraints to high yield (Lei et al., 2017). Nitrogen is a nutrient of high importance for plant growth, development and grain quality assurance, but it is also one of the most mobile plant nutrients in the soil

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(Myrbeck et al., 2014). Nitrogen fertilization is a common practice to increase food production, but its performance depends on soil water status (Turner et al., 2005).

Despite many years of research effort there is still no accurate method available for determining how much fertilizer N can be applied to intensively managed cereal crops to obtain high target yields, maintain adequate soil fertility and minimize environmental risk (Zhu et al., 2006; Robertson et al., 2009). Although emerging evidence indicates that integrated use of manure and chemical Nitrogen fertilizer is a feasible approach to improve winter wheat production and water use efficiency (WUE) in the Loess Plateau (Liu et al., 2012). Winter wheat yield and biomass are dependent on water availability (Kang et al., 2002).

Crop yield and soil nitrate-N residue (NR) have been shown to increase after long-term N fertilizer application, and at the same time nitrate residue (NR) and its accumulation in soil have been attracting increasing attention. The results from a typical rain fed area of southern Spain showed that the NR in soil increased with time, mostly it accumulated in the 30-60 cm soil layer (López et al., 2013). In a wheat maize system in North China, the NR at harvest was as high as 221-275 kg N ha^{-1} in the 0-90 cm soil layer and 213-242 kg ha^{-1} in the 90-180 cm soil layer (Ju et al., 2006). Available water and nitrogen are considered the most limiting factors in wheat production in most parts of the world, especially in arid and semi-arid region (Gonzalez et al., 2010).

Therefore, precipitation stored in the soil during the summer fallow period after wheat harvest is utilized by the subsequent crop and crucial for the success of cropping in the Loess Plateau (Zhang et al., 2007; Schlegel et al., 2017).

Water storage in soil has been affected by the different management practices such as tillage and fertilizer application (Grigoras et al., 2012). Winter wheat yield has been increased by the application of fertilizer but it also resulted in increasing soil water depletion and formation of dry subsoil layer especially in the high land areas (Yan et al., 2015). Hence, for sustainable wheat production, it is crucial to seek management practices for improving water-and N-use efficiency (Fu et al., 2014).

Supplemental irrigation and Nitrogen fertilizer application are required to match soil water stress and stabilize yields (Tavakkoli et al., 2004). It was also found that utilization of more nitrogen decreased breaking strength of the second internode significantly (Berry et al., 2000). Reducing spring nitrogen could reduce the height of gravity and increased stem strength by increasing both stem diameter and wall width (Crook et al., 1995). The highest Nitrogen uptake in the growth period occurred from reviving stage to anthesis stage. The proportion of Nitrogen accumulated in leaf and stem was high before the anthesis stage and the accumulated Nitrogen rate in stem reached peak at the anthesis stage (Zhao et al., 2006). Recent technological advances have focused on the simultaneous and synergistic improvement of several factors including water use, nitrogen efficiency, yield and grain quality (Parry et al., 2011).

Nitrogen (N) was a key element for plant nutrition. Applying N and P (phosphorus) fertilizers and other management practices increased the yield of wheat but in some cases these show adverse effects due to severely limiting irrigation (El Mejahed and Aouragh, 2005). Nitrogen use efficiency can be increased by combining fertilizer, soil, and water management. Two main approaches can be undertaken: increasing the use of N during crop growing season and decreasing the losses of N by applying optimum doses (Cui et al., 2010). At the early filling stage, GS activity of flag leaf was closely related with the total grain protein, glutenin, and gliadin content, while at the middle filling stage, GS activity of flag leaf was closely related with glutenin/gliadin (glu/gli) (Yu et al., 2009).

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The objective of this study were to find the best sowing method and optimize doses of nitrogen level to increase the yield and quality of winter wheat crop. Wide space sowing (WSS) with 300 kg·hm-^{2} in Wenxi Shanxi area was beneficial to the growth and development of winter wheat, improved nitrogen uptake at different growing stage, ultimately increased yield and improved quality. The WSS was beneficial and increased water consumption during the growth period, also tiller dynamics, promoted nutrient operation, and increased yield and grain protein content.

Materials and methods

Description of experimental site

Field experiment was performed at the Agriculture Research Station of Shanxi Agriculture University (35°200N, 111°170E), in Wenxi county, Shanxi Province of China. The experimental site was located in the southeast of the Loess Plateau. The southeast of the Loess Plateau is a typical semi-arid region with an average annual rainfall of 450-630 mm, of which 60-70% precipitation falls in July-September, the altitude is 450-700 m and the annual average temperature is 11-13 °C. This region is characterized by a semiarid climate which receives 491 mm of average annual precipitation, 12.9 °C annual mean temperature, and 2242 h of annual sunshine. The straw was returned to the field after the previous stubble corn was harvested and planted on the 25th October, 2017 and harvested in early June the next year. The soil in the test site adopts the Chinese soil classification standard and belongs to calcareous cinnamon soil. See *Table 1* for the soil fertility parameters of the 0-20 cm soil layer.

Table 1. *Soil nutrient properties from experimental location*

Soil nutrients	2016-2017	2017-2018
Organic matter (g kg^{-1})	12.07	12.61
Total nitrogen (g kg^{-1})	0.86	0.88
Alkali-hydrolysis nitrogen (mg kg^{-1})	36.42	44.07
Available phosphorous (mg kg^{-1})	16.26	10.71
Available potassium (mg kg^{-1})	218.76	188.87

Precipitation distribution

Natural precipitation was the main source of water for crop cultivation in this area, with precipitation mainly concentrating in July-September, which was the fallow period of wheat. Precipitation during experimental years and long-term average of 13 years are given in *Table 2*. Total precipitation in 2016-2017 was 406.3 mm during winter wheat growth period and it was 165.4 mm in the fallow periods. In 2017, precipitation before flowering was more than average in 2017. In 2017-2018, total precipitation was 416.6 mm, and precipitation during growth was 198.3 mm. Precipitation from sowing to wintering and jointing to maturity was abundant.

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Table 2. *Precipitation during study years (2016-2018) and difference in precipitation from average precipitation in the last 13 years (2005-2018) in different growth stages of wheat at the experimental site in Wenxi, China. (Source: Meteorological Observation of Wenxi County, Shanxi Province, China)*

Growth stages	2005-2016 (means)	2014-2015	2015-2016	2016-2017	2017-2018
S-W	53.2 ± 35.9	21.5	101.2	95.5	152.4
W-J	30.2 ± 13.6	50.8	11.0	66.8	00
J-A	37.8 ± 15.8	61.2	57.1	27.2	49.8
A-M	64.4 ± 29.7	17.6	122.8	51.4	16.1
Fallow period	265.0 ± 107.5	365.6	94.7	165.4	198.3
Total	450.5 ± 96.7	516.7	386.8	406.3	416.6

Fallow period: S-W, W-J, J-A, A-M indicate Jun 20 to Sep 30; S-W (sowing-wintering): Oct 01 to Nov 30: W-J (wintering-jointing): Dec 1 to Apr 10, J-A (jointing-anthesis): Apr 11 to May 11; A-M (anthesis-maturity): May 12 to Jun 19, total growth period and total precipitation, respectively

Experimental design and treatments

The experiment, a typical winter wheat-summer fallow, was started with the winter wheat season, covering 2 successive wheat crops at the same experimental plot. The seeds of winter wheat (*Triticum aestivum* L.) cultivar 'Liang xing-99' were obtained from Wenxi Agriculture Jinnan. Wheat stubble of 20–30 cm from the previous wheat crop was left in field to reduce evaporation and to increase organic carbon content in soil. The two factors split-plot design was adopted, with nitrogen rate as the main factor and nitrogen density as sub-plot factor. The sowing methods were wide space sowing (WSS). The details of the machinery and sowing techniques are given in *Figure 1* in with five nitrogen level (150 kg·hm^{-2}, 225 kg·hm^{-2}, 300 kg·hm^{-2}, 375 kg·hm^{-2}, and 450 kg·hm^{-2}). The plot area was 75 m^2 (2.5 m × 30 m), and it was repeated three times. The straw was returned to the field after the previous stubble corn was harvested and planted on the 25th October, 2017. Before planting, 150 kg·N·hm-^{2}, 150 kg·P_2O_5·hm-^{2}, 90 kg·K_2O·hm-^{2}, jointing topdressing 90 kg·N·hm-^{2} were applied, harvested on the 10th June, 2018 (*Fig. 2*).

Measurement: determination of agronomic traits

Plant height and leaf area

Twenty (20) plants with uniform growth and representativeness were taken at each growth stage and the plant height was measured; at the same time the length, width and number of green leaves of the second leaf were measured.

For the determination of leaf area, the length and width of the second leaf and total number of leaves were calculated. Leaf area was measured using the following formula:

$$\text{Leaf area} = \text{length} \times \text{width} \times \text{number of green leaves} \times 0.85 \quad \text{(Eq.1)}$$

where 0.85 was the adjustment factor. Then leaf area index (LAI) was calculated by dividing the leaf area (cm^2) by the ground surface area.

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Sowing method	Sowing technique	Line spacing	Tillage
Wide space sowing (WSS)	2BMF-12/6, tillage, auto-fertilization	Line space: 22-25 cm	Sub-soiling, rotary tillage

Figure 1. *Field preparation at experimental site of Shanxi Agricultural University*

Dry matter quality

Twenty (20) plants with uniform growth and representativeness were taken at each growth stage and placed in an 80 °C oven for 30 min and then dried at 80 °C to constant weight.

Determination and calculation of plant nitrogen content

After drying and pulverizing each organ of the plants the nitrogen content was measured by H_2SO_4-H_2O_2-indigo blue colorimetry. Nitrogen accumulation, running volume and nitrogen efficiency were calculated with reference to Xue et al. (2017). Twenty (20) plants from each plot were collected randomly for the measurement of plant nitrogen concentration at jointing, anthesis and maturity. Plant samples and grains were oven-dried at 105 °C for 30 min and then at 75 °C for 48 h for dry weight. Dry plant samples were cut to 4-5 cm length and ground using plant ball mill (FZ102, Beijing, China). Dry grains were also ground to powder using FZ102 mill. Ground samples of 0.25 g were digested with H_2SO_4-H_2O_2, and then total nitrogen concentration was determined using the standard indophenol-blue colorimetric method (Meyer et al., 1983). Calculation of nitrogen accumulation and translation and nitrogen efficiency were carried out as follows (Przurj et al., 2003):

Plant nitrogen accumulation amount = plant dry weight × plant nitrogen concentration;

Contribution of pre-anthesis organs nitrogen translation amount to grain = pre-anthesis organs nitrogen translation amount / grain nitrogen accumulation × 100%;

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Nitrogen uptake efficiency (kg' kg^{-1}) = plant nitrogen accumulation amount / nitrogen fertilizer amount;
Nitrogen use efficiency (kg' kg^{-1}) = grain yield / plant nitrogen accumulation amount;
Nitrogen productive efficiency (kg' kg^{-1}) = grain yield / nitrogen fertilizer amount.

Figure 2. *Field preparation use fertilizer machine site of Wenxi*

Determination of total tiller and effective tillers rate

The total number of tillers was investigated at the jointing stage and the number of fertile tillers were counted at late grain filling stage. The number of tillers was calculated from the area of 1 m^2 at 3 random points from each plot and average at each plot was the number of stem and tillers. The effective tiller rate was calculated as the proportion of main stem and tiller at jointing stage to the number of effective panicles at maturity.

Grain to leaf area ratio

Grain to leaf ratio was calculated according to Feng et al. (1999), using *Equations 2* and *3*:

$$\text{Grain number to leaf area ratio} = \frac{\text{total number of grain per unit area}}{\text{total leaf area on the same plot at booting stage}} \quad \text{(Eq.2)}$$

$$\text{Grain weight to leaf ratio} = \frac{\text{grain weight (mg) per unit area}}{\text{total leaf area on the same plot at booting stage}} \quad \text{(Eq.3)}$$

Total leaf area was taken from the same plot at booting stage (cm^2).

Determination of soil moisture

Soil was drilled from 0-300 cm depth with soil drill at sowing, wintering, jointing, flowering and maturity stages. The soil was divided into 20 cm layers and soil water content and soil water storage were determined.

The soil water storage was calculated by methods as described by He et al. (2009):

$$SWSi = Wi \times Di \times Hi \times \frac{10}{100} \quad \text{(Eq.4)}$$

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where SWS_i is the soil water storage capacity (mm) of the i-th soil layer; W_i is the soil water content (%) of the i-th soil layer; D_i is the soil bulk density (g cm^{-3}) of the i-th soil layer; H_i is the thickness of soil layer (cm). The soil water content and soil bulk density were both measured using the oven-drying method Gardner et al. (1986):

Water consumption

Water consumption in wheat fields was measured using a simplified formula as described by Noor et al. (2020):

$$ET = P - \Delta S \quad \text{(Eq.5)}$$

where P is the effective precipitation (mm) during that stage and ΔS is the reduction of soil water storage at each stage and was measured as $\Delta S = S_1 - S_2$, where S_1 and S_2 were the soil water content at the beginning and at end of the stage, respectively. Whereas, runoff and drainage were considered negligible.

The water consumption intensity (CWR, mm d^{-1}) was calculated as:

$$CwR = \frac{ET_i}{d} \quad \text{(Eq.6)}$$

where ETi is the water consumption (mm) of wheat in each growth stage and d is the number of days in the growth stage.

Determination of spike number

Comparison of the spike of interest with the model spike occurs in an n-dimensional vector space, which dimensions are defined by the total spikelet number of the spike of interest. The geometrical difference in GYDAS between the two spikes is based on the scalar product of these two vectors:

$$\cos\measuredangle\left(\vec{a}, \vec{b}\right) = \frac{\vec{a} \cdot \vec{b}}{|\vec{a}| \cdot |\vec{b}|}, \quad \text{(Eq.7)}$$

Plant density

Seeds were sown at a density of 225 × 104 ha^{-1} in rows 20 cm apart between September 28 and October 30 from 2016 to 2018.

Biomass and yield

At maturity, the number of panicles per unit area, the average number of grains per panicle and weight of 1000 grains were investigated from 50 plants per plot, and plants from 20 m^2 area were harvested to calculate economic yield. For determining the aboveground dry biomass, plant samples were kept at 105 °C for 30 min, and then oven dried at 75 °C for 12 h until constant weight.

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Determination of grain protein and component content

At maturity period 30 ears were burned at 105 °C for 30 min, dried at 80 °C to constant weight, crushed with a DE-100 g mini high-speed universal grinder produced by Zhejiang Hongjingtian Industry and Trade Co., Ltd and a continuous extraction method was used to determine the protein content of the grains.

Determination of kernel quality

The MJZ-II type gluten index tester was used to determine the wet gluten content and the micro dough LAB 2800 micro flour analyzer was used to determine the flour characteristics.

Statistical analysis

Data subjected to analysis of variance (ANOVA) and SAS 9.0. Charts were constructed using Microsoft Excel 2007. Mean values were calculated and significance of the difference between treatments was tested by LSD (least significant difference) method at the significance level of $P = 0.05$.

Results

Soil water consumption

Table 3 shows that with the increase of the seeding amount, the water consumption during the growing period and the proportion of water storage water consumption tend to increase and then decrease. The seeding amount was 300 kg·hm^{-2}, which is the highest, and the water consumption during the growing period was 369.48 mm. The water consumption ratio of water storage was 40.72%, and the water consumption ratio of precipitation and irrigation water showed a trend of decline and then increased, and reached the lowest value when the seeding amount was 300 kg·hm^{-2}. It can be seen that the wide sowing amount of 300 kg·hm-^{2} was conducive to increasing the water consumption during the growth period and the proportion of stored water consumption, and reducing the proportion of water consumption and irrigation water consumption. The water consumption ratio of precipitation and irrigation water was significantly reduced. Compared with sowing, the water consumption during growing stage and water storage under even sowing the proportion of water increased and the proportion of water consumption in precipitation and irrigation decreased. Wide space sowing and increased the water consumption during the growth period and soil water consumption ratio.

Table 3. *Effects of seeding rate on soil water consumption ratio at 0-200 cm depth from different water sources of winter wheat*

Seeding rate (kg·hm^{-2})	Soil water consumption in the growing stage (mm)	Soil water consumption ratio (%)	Precipitation consumption ratio (%)	Irrigation consumption ratio (%)
150	447.71 ^{c}	37.84 ^{c}	48.76 ^{a}	13.40 ^{a}
225	451.13 ^{b}	38.31 ^{c}	48.39 ^{a}	13.30 ^{a}
300	469.48 ^{a}	40.72 ^{a}	46.50 ^{c}	12.78 ^{c}
375	459.96 ^{b}	39.49 ^{b}	47.46 ^{b}	13.04 ^{b}
450	447.68 ^{c}	37.83 ^{c}	48.76 ^{a}	13.40 ^{a}

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Effects of seeding rate on soil water consumption plant height and leaf area of dryland wheat

The effects of nitrogen rate on soil water consumption on plant height and leaf area of winter wheat from 2016 to 2017 were as follows, the plant height was significantly the highest when the sowing amount was 375 kg·hm^{-2} in the wintering period from 2017 to 2018, the plant height was significantly the highest when the sowing amount was 450 kg·hm^{-2} in the wintering period; in the two years, when the sowing amount was 300 kg·hm^{-2}, the plant height was significantly the highest from the elongation stage to the maturity stage and the plant height was significantly the lowest when the sowing amount was 150 kg·hm^{-2} (*Fig. 3*). In those two years, when the sowing amount was 300 kg·hm^{-2} the leaf area was significantly the highest in each growth period and the leaf area was significantly the lowest when the sowing amount was 150 kg·hm^{-2}. It can be seen that the increase of sowing volume (150-300 kg·hm^{-2}) was beneficial to the increase of plant height and leaf area of wheat.

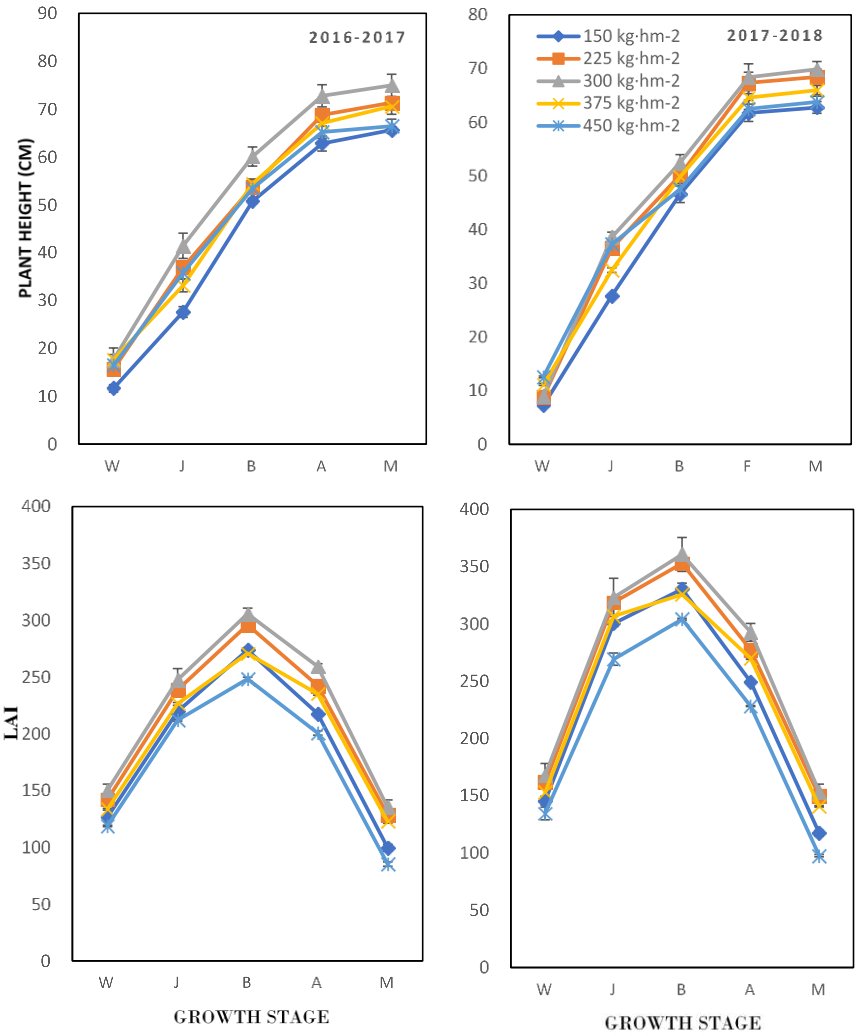

Figure 3. *Effects of seeding rate on plant height and leaf area index (LAI) at different growth stages of dryland wheat. W, J, B, F, A, and M indicate Wintering, Jointing, Booting, Flowering, Anthesis and maturity W-J: Wintering to jointing, Oct 1 to Apr 10; J-B: Jointing to Booting, Apr 11 to May 10; B-F: Booting to Flowering, May 11 to May 25 B-A: Booting stage to anthesis, May 26 to Jun 10; A-M: Anthesis to maturity, Jun 12 to Jun 19*

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Effects of seeding rate on soil water consumption amount and on population dynamics of WSS dryland wheat

Effects of nitrogen rate on soil water consumption in wintering stage examined throughout two years can be seen in *Figure 4*. Group with 375 kg·hm^{-2} sowing amount showed the highest tailoring and had a significant jointing stage, mature stage and sowing quantity. Group 300 kg·hm^{-2} showed the highest tiller number in the growth period (2016 to 2017), and the flowering period and mature period reached significant level (2017 to 2018), the sowing quantity of different levels the nitrogen level of 150 kg·hm^{-2} tiller number was lowest significantly. Lower sowing quantity of the wide space sowing (WSS) was 300 kg·hm^{-2}, which was conducive to the formation of tiller number and yield.

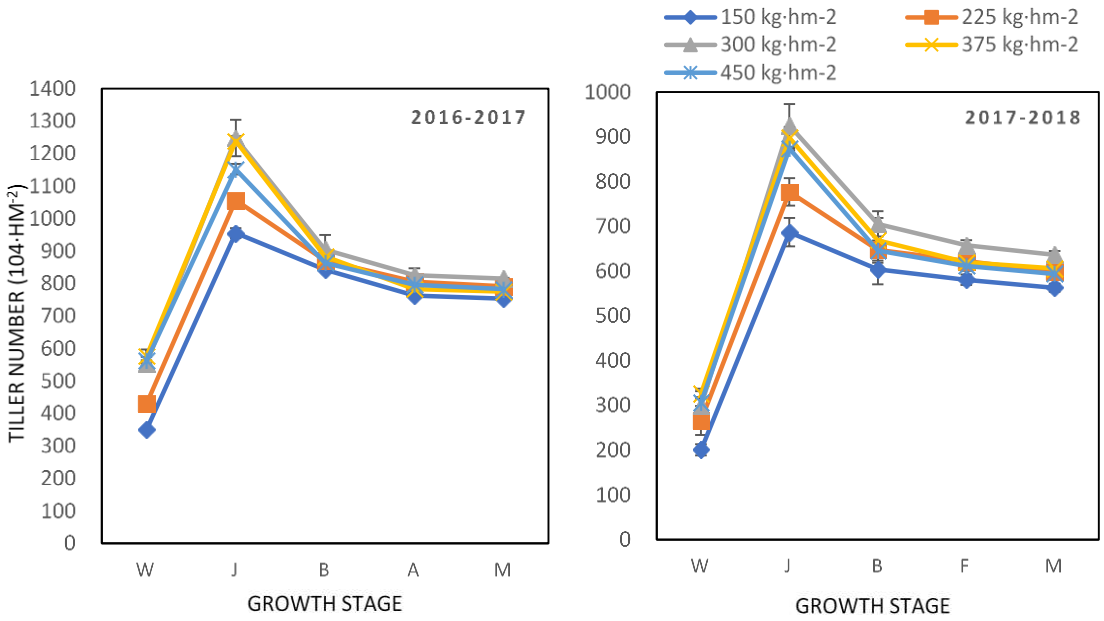

Figure 4. *Effects of seeding rates on water consumption of tiller dynamics at different growth stages of dryland wheat. W, J, B, F, A, and M indicate Wintering, Jointing, Booting, Flowering, Anthesis and maturity W-J: Wintering to jointing, Oct 1 to Apr 10; J-B: Jointing to Booting, Apr 11 to May 10; B-F: Booting to Flowering, May 11 to May 25 B-A: Booting stage to anthesis, May 26 to Jun 10; A-M: Anthesis to maturity, Jun 12 to Jun 19. Different letters indicate significant difference among treatments at the significance level of $p \leq 0.05$*

Effects of seeding rate on dry matter accumulation characteristics of winter wheat

Effects of nitrogen rate on dry matter accumulation characteristics are shown in *Table 4*. The results show that with the increase of sowing amount the dry matter mass of the plants increased first and then decreased in each growth period and the highest sowing amount was 300 kg·hm^{-2}. During the wintering period and booting period the dry matter quality of the plants was the highest when the sowing amount was 300 kg·hm^{-2}, but there was no significant difference from 225 kg·hm^{-2}, when the sowing amount was 450 kg·hm^{-2}. During the flowering period and maturity period the dry matter quality of the plants was the highest significantly when the sowing amount was 300 kg·hm^{-2} and the difference was not significant when the sowing amount was 225 kg·hm^{-2}. It can be seen in *Table 4*, that it is beneficial to improve the dry matter quality of the plants especially in the late growth period, when the down sowing quantity of the WSS is 300 kg·hm^{-2}.

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Table 4. *Effects of seeding rate on dry weight at different growth stages (kg·hm^{-2})*

Seeding rate (kg·hm^{-2})	Wintering	Jointing	Booting	Anthesis	Maturity
150	3605.25 ^{b}	4624.41 ^{d}	7487.85 ^{c}	9397.70 ^{d}	11304.81 ^{c}
225	3786.32 ^{ab}	4906.33 ^{bc}	8506.25 ^{b}	10727.24 ^{b}	13759.48 ^{b}
300	4022.77 ^{a}	5213.31 ^{a}	9246.29 ^{a}	11429.16 ^{a}	14589.44 ^{a}
375	3804.43 ^{ab}	5054.21 ^{ab}	8642.98 ^{ab}	10890.31 ^{b}	13657.66 ^{b}
450	3729.79 ^{b}	4785.13 ^{cd}	8104.08 ^{bc}	9886.77 ^{c}	11250.73 ^{c}

Effects on agronomic characters at maturity stage

Agronomic characters (*Table 5*) show that the Nitrogen rate was 300 kg·hm^{-2}, with the longest ear length (7.51 cm), which was not significantly different from 375 kg·hm^{-2}. The number of pregnable spikelet was the highest (13.87), and the number of non-pregnable spikelet was the lowest (1.17). The number of pregnable spikelets can be increased and the number of non-pregnable spikelets can be reduced when the wide space sowing amount is 300 kg·hm^{-2}, which is ultimately beneficial to the formation of yield.

Table 5. *Effects of seeding rate on agronomic characters at maturity*

Seeding rate (kg·hm^{-2})	Spike length (cm)	Bearing spikelet number	Sterility spikelet number
150	7.20 ^{b}	13.17 ^{b}	1.87 ^{bc}
225	7.21 ^{b}	13.30 ^{b}	1.72 ^{c}
300	7.51 ^{a}	13.87 ^{a}	1.17 ^{d}
375	7.32 ^{ab}	12.20 ^{c}	2.17 ^{b}
450	6.15 ^{c}	10.43 ^{d}	2.80 ^{a}

Effects of seeding rate on nitrogen uptake at different growing stages

The effect of seeding rate on nitrogen uptake at different growing stages of winter wheat is presented in *Table 6.* The results show that with the increase of sowing amount the nitrogen accumulation of plants in each growth period showed a trend of increasing first and then decreased and reached the maximum at 300 kg·hm^{-2} and it was also observed that with the increase of sowing amount the contribution rates of nitrogen accumulation before flowering and nitrogen accumulation before flowering on grains increased first and then decreased reaching the highest at 300 kg·hm^{-2} and the nitrogen accumulation after flowering and nitrogen accumulation after flowering had no significant effect on the contribution rates of grains.

Table 6. *Effects of seeding rate on nitrogen uptake at different growing stage*

Seeding rate (kg·hm^{-2})	Wintering	Jointing	Booting	Flowering	Maturity
150	30.17^{d}	39.29^{e}	85.65^{e}	104.06^{e}	132.00^{e}
225	35.21^{c}	48.10^{c}	116.53^{c}	129.25^{c}	160.50^{c}
300	40.21^{a}	54.05^{a}	124.14^{a}	143.38^{a}	171.00^{a}
375	37.65^{b}	51.39^{b}	118.79^{b}	136.11^{b}	165.00^{b}
450	29.66^{d}	40.94^{d}	102.21^{d}	110.78^{d}	141.00^{d}

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Effects of seeding rate on pre-anthesis accumulated nitrogen translocation and nitrogen accumulation amount after anthesis of winter wheat

Effects of seeding rate on pre-anthesis accumulated nitrogen translocation and nitrogen accumulation amount are presented in *Table 7.* With the increase of sowing amount, the accumulation of nitrogen in each growth stage showed a trend of first increasing and then decreasing, reaching the highest at 300 kg·hm^{-2}. The sowing methods mainly promote the operation of nitrogen accumulation before flowering to increase the contribution rate to grains, among which WSS was better.

Table 7. *Effects of seeding rate on pre-anthesis accumulated nitrogen translocation and nitrogen accumulation amount after anthesis of winter wheat*

Seeding rate (kg·hm^{-2})	PANT		NAAA	
	TA (kg·hm^{-2})	CN in grains (%)	TA (kg·hm^{-2})	CN in grains (%)
150	81.96^{c}	74.58^{b}	27.94^{b}	25.42^{ab}
225	101.53^{b}	76.46a^{b}	31.25^{a}	23.54^{ab}
300	112.14^{a}	80.24^{a}	27.62^{b}	19.76^{b}
375	106.04^{b}	78.59^{ab}	28.89^{ab}	21.41^{ab}
450	85.93^{c}	73.98^{b}	30.22^{ab}	26.02^{a}

PANT: Pre-anthesis accumulated nitrogen translocation amount from vegetative organs to grains after anthesis; NAAA: Nitrogen accumulation amount after anthesis; TA: Translation amount; CN: Contribution nitrogen

Effects of seeding rate on nitrogen use efficiency (NUE) of winter wheat

The nitrogen use efficiency (*Table 8*) shows that with the increase of sowing amount the nitrogen absorption efficiency and nitrogen fertilizer production efficiency first increased and then decreased and reached the highest at 300 kg·hm^{-2}, while the nitrogen utilization efficiency first decreased and then increased with no significant effect on the nitrogen harvest index. It can be seen that when the lower sowing amount was 300 kg·hm^{-2}(WSS), it was beneficial and the accumulation of nitrogen in each growth period increased along with the contribution rate of nitrogen running amount with grains before flowering and the nitrogen absorption efficiency and nitrogen fertilizer production efficiency improved.

Table 8. *Effects of seeding rate on nitrogen use efficiency (NUE) of dryland wheat*

Seeding rate (kg·hm^{-2})	N uptake efficiency (kg·kg^{-1})	N use efficiency (kg·kg-^{1})	N productive efficiency (kg·kg^{-1})	Nitrogen harvest index
150	0.88^{d}	45.44^{a}	39.98^{c}	0.83^{a}
225	1.07^{b}	40.14^{d}	42.95^{b}	0.83^{a}
300	1.14^{a}	41.86^{c}	47.72^{a}	0.82^{b}
375	1.10^{ab}	41.85^{c}	46.04^{a}	0.82^{ab}
450	0.94^{c}	43.98^{b}	41.34^{bc}	0.82^{ab}

Effects of seeding rate yield and composition of dryland wheat

The effects of seeding rate yield and composition are presented in *Table 9*. The results show that with the increase of sowing amount, the panicle-number and yield show a trend

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of increasing first and then decreasing. When the sowing amount is 300 kg·hm^{-2}, the panel-number is the highest significantly, reaching 636.00×104·hm^{-2} and the highest yield is 7158.30 kg·hm^{-2}, but there is no significant difference from 375 kg·hm^{-2}. The number of panicle grains was the highest when the sowing amount was 300 kg·hm^{-2}, but there was no significant difference from 150 kg·hm^{-2} and 375 kg·hm^{-2}. The difference of 1000-grain weight in the range of 300-450 kg·hm^{-2} was not significant. It can be seen that the appropriate sowing amount is the key to increase the wheat yield, and the excessive or low sowing amount is not conducive to the formation of yield, and the number of ears is the key factor affecting the formation of yield. The appropriate sowing amount under wide space sowing is 300 kg·hm^{-2}.

Table 9. *Effects of seeding rate and N input on yield components of dryland wheat*

Seeding rate (kg·hm^{-2})	Ear number (10^4·hm^{-2})	Grain number per spike	1000-grain weight (g)	Yield (kg·hm^{-2})
150	562.33 ^{c}	32.95 ^{ab}	39.12 ^{c}	5997.57 ^{c}
225	597.67 ^{b}	31.99 ^{b}	39.90 ^{bc}	6442.10 ^{b}
300	636.00 ^{a}	33.85 ^{a}	41.20 ^{a}	7158.30 ^{a}
375	605.33 ^{b}	32.72 ^{ab}	41.06 ^{a}	6905.33 ^{a}
450	593.33 ^{b}	29.50 ^{c}	40.68 ^{ab}	6201.40 ^{bc}

Effects of seeding rate on protein and component contents of mature grains

The protein and component contents of mature grains are presented in *Table 10*. The results show that with the increase of sowing amount the contents of grain protein components and protein yield first increased and then decreased. The yield of grain albumin, globulin and protein was significantly the highest when the sowing volume was 300 kg·hm^{-2} and the content of altoprotein, glutenin, grain/alcohol ratio and protein was the highest but there was no significant difference from that of 225 kg·hm^{-2}. It can be seen that the seed size of 300 kg·hm^{-2} was beneficial and increased the content of protein and components in grains.

Table 10. *The protein components and protein yield of wheat under different nitrogen rate*

Seeding rate (kg·hm^{-2})	Albumin (%)	Globulin (%)	Gliadin (%)	Glutenin (%)	Glu/Gli	Protein content (%)	Protein yield (kg·hm^{-2})
150	2.44 ^{b}	1.57 ^{b}	4.29 ^{b}	4.28 ^{c}	1.00 ^{b}	13.80 ^{c}	827.56 ^{c}
225	2.53 ^{b}	1.62 ^{b}	4.36 ^{a}	4.51 ^{a}	1.03 ^{a}	14.66 ^{a}	944.13 ^{b}
300	2.74 ^{a}	1.81 ^{a}	4.40 ^{a}	4.59 ^{a}	1.04 ^{a}	15.04 ^{a}	1076.81 ^{a}
375	2.50 ^{b}	1.61 ^{b}	4.23 ^{c}	4.37 ^{b}	1.03 ^{a}	14.22 ^{b}	982.16 ^{b}
450	2.28 ^{c}	1.31 ^{c}	3.91 ^{d}	3.90 ^{d}	1.00 ^{b}	13.26 ^{d}	822.40 c

Correlation analysis of water consumption from soil at different growth stages and yield and yield related components of wheat

Correlation coefficients of water and plant nitrogen accumulation with yield and quality traits are presented in *Table 11*. The results show that water consumption during growth period showed a significant or extremely significant positive correlation with the

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amount of nitrogen accumulated before flowering yield panicle number, 1000-grain weight, protein content and grain/alcohol ratio. The yield was positively correlated with panicle number and positively correlated with panicle grain number and 1000-grain weight. The protein content was positively correlated with the amount of nitrogen accumulated before flowering and the grain/alcohol ratio.

Table 11. *The correlation index between grain yield and quality traits in different seeding rate*

Grain yield quality	Soil water consumption in the growing stage	Yield	Protein content
PANT amount	0.8887**	0.9580**	0.8610*
Yield	0.9640**	——	——
Ear number	0.8850**	0.9236**	——
Grain number per spike	0.6676	0.8447*	——
1000-grain weight	0.7299*	0.8200*	——
Protein content	0.9640**	——	——
Glu/Gli	0.8013*	——	0.9246**

*Significant at 0.05 level. **Significant at 0.01 level

Discussion

Wheat production in Hugong Township, Wenxi region are facing great challenges due to scant water supply and nutrient deficit. Due to the sparse and deep groundwater resources, rainfall is the sole water source for wheat production in the Loess Plateau, which is limited (200-600 mm) and unevenly distributed. 30%-40% of annual rainfall occurs during winter wheat growing season, whereas most of the rain falls between July and September, which is concurrent with the summer fallow between two growing seasons of winter wheat (Li et al., 2015). Water stress is the main limiting factor for wheat production in Loess Plateau and other arid and semiarid regions (Liu et al., 2007). The growth of winter wheat plants depends on the soil water stored by rainfall during summer fallow season (60-70%) and growing season (30-40%). The roots of rain fed winter wheat are not able to utilize water from deeper soil layers and ground water (Li et al., 2017). Hence, the limiting precipitation during growth season means that winter wheat must utilize soil water stored from precipitation during fallow season (Xue et al., 2019).

Effect of seeding rate on Soil water consumption

In addition, nitrogen also significantly affects crop yield under water deficient conditions. Winter wheat under water deficient conditions, supplying post-anthesis nitrogen fertilizer increases grain yield by decreasing the sink limitation and not by increasing source strength (Madani et al., 2010). Therefore, nitrogen fertilizer has no relation with sesame phonological traits under higher rainfall areas, whereas in drought-prone areas, zero fertilization might flower earlier than the fertilized one. Taller plants (116 cm), higher LAI (0.78), higher branches per plant (8.7), and TDM per plant (32.7 g) were obtained with the application of 100 kg·N/ha in Nigeria (Haruna et al., 2011). Nitrogen could influence leaf area, active life span, chlorophyll content, tuber size and tuber bulking time to affect yield (Goffart et al., 2008). The use of wide range precision sowing method under the condition of corn straw mulch significantly improved water use

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efficiency and reduced water consumption (Liu et al., 2015). The soil water storage capacity of 0-300 cm soil layer with full membrane soil hole sowing increased during sowing jointing and grouting and the water consumption intensity increased during sowing jointing water use efficiency increased. (Hou et al., 2017). Compared with conventional sowing, the total water consumption under wide space sowing increased and water use efficiency increased (Li et al., 2015). The yield and yield parameters were significantly affected due to application of nitrogen and irrigation water. For higher yield, it is better to apply nitrogen at the stem elongation and heading unless it is not irrigated with minimum amount of water (Cao et al., 2008a).

The effect is better and the percentage of soil water consumption in the 0-200 cm soil layer under wide space sowing is significantly higher than in other sowing methods and the water consumption during the growth period and water use efficiency under (WSS) are increased. The increase of 17% and 33% may be due to the uneven distribution of the roots in the upper layer of conventional seeding which caused the root density between individual plants to be much lower than the density of the roots in the row. This caused the soil moisture to be lost in the manner of soil evaporation. The individual growth and nutrient movement of wheat during the whole growth period has a very important influence on the formation of grain yield (Chu et al., 2018), there was a significant negative correlation between nitrogen accumulation and nitrogen use efficiency and a significant positive correlation between nitrogen absorption efficiency and nitrogen use efficiency. This study shows that wide space sowing can significantly increase plant nitrogen accumulation at various growth stages and the contribution rate to grains is mainly increased by the amount of nitrogen before flowering, which is consistent with previous research results (Xue et al., 2017). Nitrogen absorption efficiency, nitrogen fertilizer production efficiency and nitrogen harvest index significantly improved, but the mechanism that affects nitrogen operation by wide space sowing needs further study. The number of spike grains per spike and thousand grain weight are the elements of yield formation. Dang et al., 2015 showed that compared with the traditional sowing method, the use of wide precision sowing increased the number of ears by 5%, the number of ears by 5%, the weight per thousand by 3%, and the yield increased by 12%. The number of ears and yield showed a very significant positive correlation. There was a significant positive correlation between ear number and thousand-grain weight and yield. It can be seen that wide space sowing (WSS) mainly increased yield by increasing ear number which is the same as that of (Li et al., 2012). The yield is significantly positively related to the water consumption during the growth period and the nitrogen accumulation before flowering. Wide space sowing has high water consumption during the growth period and high nitrogen before bloom which promotes nutrient absorption and operation and increases yield. In addition the broad soiled sowing population structure is reasonable, significantly increasing the number of tillers in the middle and late stages of reproductive growth, along with ear length, and the number of fertile spikelet's, while it significantly reduces the number of infertile spikelet's, increases the leaf area in the later stages of growth and increases growth stages. The accumulation of dry matter is conducive to the increase of yield.

Effect of seeding rate on the regulation of quality

The technical quality of wheat is a very complex character, applied technologies and agro-ecological conditions. The high content of mineral nutrition, especially nitrogen, affects the quality of the process. These results showed that higher N rate has a positive

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effect on wheat grain quality. Higher density provides a greater number of primary tillers per square meter, which causes the formation of grains with larger size and mass. These grains have a more favorable ratio of storage proteins and starch, which requires better quality. In crops with a lower density, a greater number of secondary tillers are created, which produce small grains with less weight and lower quality. These results agree with those of Geleta et al. (2002). From our results, we conclude that the application of slurry had small but significant influences on yield and quality compared to mineral fertilization. This effect was also found by Sieling et al. (1997).

Quality parameters investigated in this paper significantly depended on applied nitrogen doses. The application of an increased nitrogen fertilization dose caused an increase of quality traits. The highest increase of sedimentation value and wet gluten content established in N3 variant when applied 120 kg ha^{-1} of nitrogen, which agree with our previous results (Zecevic et al., 2005). By analysis of variance, it was established that both of the analyzed quality traits significantly depended on genotypes and investigated years. Interactions between genotypes, applied nitrogen doses and years were also highly significant, which means that new genotypes positively reacted to nitrogen application (Guarda et al., 2004). Research showed that the use of wide stubble sowing can make wheat individuals grow robustly which can significantly increase the bulk density and hardness of wheat grains, significantly improve water absorption wet gluten content and sedimentation value and significantly increase the maximum resistance of dough, the standard value of power and flour quality, but has no significant effect on flour protein content and dough ductility (Wang et al., 2012). Some studies have shown that the amount of nitrogen before flowering affects wheat grain protein content. It has a large regulatory effect (Desai et al., 1978) and some studies have shown that the contribution of nitrogen accumulation and operation amount before and after flowering to grain protein varies from species to species. The protein content has a regulating effect and the nitrogen accumulation after flowering mainly has a regulating effect on the grain protein content in medium protein varieties. There was a significant positive correlation between protein content in grains and nitrogen accumulation before flowering, and a significant or very significant positive correlation with grain/alcohol ratio, wet gluten content, water absorption, and quality of powder maps. The grain/alcohol ratio is an important index for evaluating the quality of wheat (Zou et al., 2006). The grain/alcohol ratio is related to water consumption during growth, nitrogen accumulation before flowering, wet gluten content, water absorption, dough formation time, and flour. There is a significant or very significant positive correlation between the mass numbers in the prime image. It can be seen that increasing the water consumption during the growth period and the amount of nitrogen accumulation before flowering are conducive to increasing the protein content of the grain, increasing the grain/alcohol ratio and ultimately facilitating the formation of quality. Under this test condition, the use of (WSS) is the most beneficial to improve the grain quality indicators such as protein and component content, grain/alcohol ratio, wet gluten content, water absorption, dough formation time and stabilization time.

Effect of seeding rate on the regulation of yield amount

Dry land wheat that sowing amount in open field was 354 × 104 hm^{-2}, and 245 × 104 hm^{-2} on the film was good for improving grain yield (Feng et al., 2013). Suitable seeding rate for wide space sowing is 105 kg·hm^{-2}. At this time, the highest yield of wheat is 13% higher than that of conventional precision sowing and the highest yield is 8643 kg·hm^{-2}.

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A research work by Cao et al. (2008b) showed that the suitable nitrogen rate under trench sowing was 150 kg·hm^{-2} and the yield at this time was 8644 kg·hm^{-2}. Under the conditions of this experiment the interaction effects of the sowing method and the sowing amount were analyzed: the sowing method, sowing amount, sowing method × the sowing amount had a significant effect on the number of ears, grain number, thousand-grain weight yield, and water use efficiency of winter wheat. Among them the most important influence on yield was the sowing method followed by the sowing amount; the most important effect on water use efficiency was the sowing amount, followed by the sowing method. The suitable seeding capacity for (WSS) was 300 kg·hm^{-2}, the yield at this nitrogen amount was 7158 kg·hm^{-2} and the water use efficiency was 14.2%; the suitable seeding capacity for trenching was 300 kg·hm^{-2}. The yield at this sowing rate was 6343 kg·hm^{-2} and the water use efficiency was 13.4%; the suitable nitrogen rate for uniform sowing was 375 kg·hm^{-2} and the yield at this sowing rate is 6780 kg·hm^{-2}, the suitable sowing capacity for conventional sowing is 225 kg·hm^{-2}, the yield at this sowing capacity was 5832 kg·hm^{-2} and the water use efficiency was 13.7%. It can be seen that the appropriate nitrogen amount should be selected according to different nitrogen rate. Under this experimental condition, the matching nitrogen amount of (WSS) was 300 kg·hm^{-2}, which is conducive to the improvement of grain yield and water use efficiency. It is suitable for the local sowing method and volume.

Effect of seeding rate on N input yield components and accumulation characteristics of dryland wheat

Constructing a reasonable population structure, that is individuals can fully absorb water, light, heat and nutrient resources, promote the healthy growth of individuals, coordinate the contradictions between individual groups and it is extremely important for the coordination of wheat yield and quality (Bhatta et al., 2017; Lin et al., 1996). Nitrogen rate can affect the nitrogen accumulation and translation of winter wheat. Rational close planting of wheat is beneficial to increase nitrogen accumulation amount, so as to realize synergistic improvement of grain yield and nitrogen utilization efficiency. Enhanced nitrogen accumulation in winter wheat requires seeding rate at an appropriate density. Previous study indicated that with irrigation the winter wheat plant density increased from 270 to 405 m^{-2}, nitrogen use efficiency (NUE) increased significantly mainly due to increasing root length density and enhanced nitrogen uptake (Dai et al., 2014).

Wheat grain nitrogen mainly comes from the redistribution of pre-anthesis nitrogen translocation amount from various organs, accounting for about 53.0%, 80.8% of grain nitrogen (Geleta et al., 2002). According to them the increase of nitrogen volume, the leaf area index (LAI) of Guomai 301 in the flowering stage gradually increased, the dry matter accumulation in the flowering stage increased first and then decreased and the number of ears and yield increased first and then decreased. Under the conditions of this experiment, the relevant analysis of factors related to yield formation shows that the water consumption during the growth period, the number of ears, the 1000 grain weight and the yield have a significant or very significant correlation and the nitrogen rate was 300 kg·hm^{-2}. In the period the water consumption and the proportion of stored water consumption increased and the water consumption and irrigation water consumption decreased and the output gradually increased with the increase of the nitrogen amount. Increasing the sowing volume within a certain range increased the nitrogen accumulation in the above ground, the nitrogen utilization efficiency decreased and the nitrogen utilization increased first and then decreased (Zhu et al., 2018).

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A research by Zhang et al. (2015) showed that increasing planting density within a certain range increased nitrogen accumulation and absorption efficiency in the above-ground area and reduced nitrogen use efficiency. Wang et al. (2012) showed that with the increase of planting density, the nitrogen harvest index, grain accumulation and nitrogen content gradually decreased, and the contribution rate of nitrogen transport from vegetative organs to the grain during the flowering period showed an upward trend. Plant nitrogen accumulation wave, nitrogen absorption and utilization rate and nitrogen fertilizer partial production efficiency showed a trend of rising first and then decreasing. Xue et al. (2017) showed that increasing the sowing amount increased the amount of nitrogen accumulation in various organs before flowering and the contribution of nitrogen accumulation in leaves glumes and cobs to flowering increased before flowering. This study showed that with the increase of nitrogen rate the nitrogen accumulation of plants in each growth stage showed a trend of first increase and then decline and the contribution rate of nitrogen accumulation before flowering and the amount of nitrogen accumulation before flowering showed first contributions to grains. It reached the highest at 300 $kg \cdot hm^{-2}$. It can be seen that when the wide space sowing rate is 300 $kg \cdot hm^{-2}$, it is beneficial to improve accumulation of nitrogen in each growth period, increasing the contribution rate of nitrogen operation before flowering with grain, improving nitrogen absorption efficiency and nitrogen fertilizer production efficiency, are beneficial to plant nutrient operation. Adjusting the nitrogen rate to build a reasonable population structure, promoting wheat's absorption of soil moisture and nitrogen affect plant nutrient operation and thereby form protein in the grain. In the range of 245 ~ 330 × 104 hm^{-2}, the grain protein content increased first and then decreased with increasing planting density. Under the conditions of this test a correlation analysis was performed and the protein content was extremely significantly positively correlated with the nitrogen accumulation and the grain/alcohol ratio before flowering. The change trend of first increase and then decrease reached the highest at 300 $kg \cdot hm^{-2}$, which may be due to the plant population under the nitrogen amount, low intercellular CO2 concentration, strong nitrogen metabolism, flag leaf GS and GOGAT activity, and grain GS activity. The GOGAT activity of the grain during the early grain filling period is high, the nitrogen accumulation is high before the flowering operation and finally the grain protein content increases (Wang et al., 2014).

Conclusion

Under this experimental condition, the matching nitrogen amount of WSS was 300 $kg \cdot hm^{-2}$, which is conducive to the improvement of grain yield and water use efficiency. It is suitable for the local sowing method and volume. Sowing methods are beneficial to the increase of winter wheat grain yield. Correlation analysis showed that there was a very significant positive correlation between yield and water consumption during the growing period, and nitrogen accumulation before flowering. Wide sowing increased water consumption during the growing period, and nitrogen transportation before flowering increased tiller number, leaf area in late growth period, dry matter accumulation in each growth period, and ear length. The water consumption during the growing period increases, the nitrogen accumulation and operation before flowering increases, the grain protein and component content increases, the grain/alcohol ratio increases, The effect of wide space sowing with 300 $kg \cdot hm^{-2}$ is the most obvious, and it is conducive to increasing water consumption in the growing period, increasing nitrogen accumulation in each growing period, and improving flowering. The amount of pre-nitrogen operation increases with the

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contribution of grains, improves nitrogen absorption efficiency and nitrogen fertilizer production efficiency, and promotes plant nutrient operation.

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EVALUATION OF THE EMERGENCE OF IMIDAZOLINONE RESISTANT WEEDY RICE (*ORYZA SATIVA* L.) IN MALAYSIA

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Abstract. The Clearfield® Rice Production System (CPS) technology successfully manages weedy rice infestation and increases rice production in Malaysia. However, there were concerns on the recurring presence of weedy rice in the CPS fields in recent years, suspecting that weedy rice has become resistant to the imidazolinone (IMI) herbicide. This research was designed to evaluate the occurrence of herbicide resistant weedy rice in the CPS fields. A total of 17 weedy rice biotypes were collected and assessed for resistance to IMI (imazapic + imazapyr) during germination and vegetative stages using different herbicide concentrations. Different dosages of IMI herbicide only reduced germination rate by ~30% but significantly decreased the weedy rice seedlings' viability. Low viability rate for commercial (13.9±13.8%) and double (7.3±4.1%) herbicide dose indicated application of IMI herbicide as pre-emergence herbicide was effective to control weedy rice. The application of IMI herbicide at later stage after the recommended period increased weedy rice escape potential in the CPS fields by 64.7% to 76.5% for one- and two-dose applications, respectively. There is a possibility that weedy rice in Malaysia has developed certain levels of resistance towards IMI herbicide. Stringent ecological risk evaluation of CPS is needed to mitigate the development of herbicide resistant weedy rice in the future.
Keywords: *Clearfield® rice, direct-seeding, herbicide application, herbicide resistant, weed management*

Introduction

Weedy rice (*Oryza sativa* L.) is one of the notorious weed species in rice granaries all over the world. In most rice agroecosystem, the spread of weedy rice becomes significant mainly after the shift from rice transplanting to direct seeding (Azmi and Karim, 2008; Chauhan, 2013). The herbicide-tolerant Clearfield® rice technology (Croughan, 2003) provides an option to control weedy rice in rice agroecosystems using imidazolinone herbicides. An herbicide-tolerant rice cultivar from Clearfield® Rice Production System (CPS) was introduced to Malaysian rice farmers in 2010 as the current best solution to combat weedy rice especially in direct-seeding system (Azmi et al., 2012). This imidazolinone tolerant variety (IMI-TR) rice was developed by crossing United States IMI-TR Line No. 1770 with local cultivar, MR220, using a conventional breeding technique (Azmi et al., 2012). Introduction of CPS as a pilot study in Seberang Perak rice granaries has become popular to other rice growing states (i.e., Selangor and Kedah) because of the success of this system to control many grasses weed species including weedy rice while boosting rice production (Sudianto et al., 2013).

This system used imidazolinone (IMI) herbicide (a.i. imazapic + imazapyr) which is a selective herbicide that inhibits the ALS enzyme and the three amino acid branched

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chains: isoleucine, leucine, and valine. It stops protein synthesis, and eventually, kills any susceptible plants (weeds) including weedy rice (Azmi et al., 2012; Sudianto et al., 2013). This system reported to increase yield production by 5 to 8 times (Azmi et al., 2012). Despite the current popularity of CPS in most major rice granaries in Malaysia, Sudianto et al. (2013) has listed four major challenges for this system to become sustainable in Malaysia including the evolution potential of the weedy rice to be resistant to the herbicide application.

Low levels of natural hybridization were known to occur between the rice cultivars and weedy rice with gene flow generally ranges from 0.003% to 0.25% (Gealy, 2005; Shivrain et al., 2008). Engku et al. (2016) also observed that Clearfield®-weedy rice hybrids can occur within 5 m distance. IMI resistant weedy rice populations were reported in the United States after two cropping seasons (Burgos et al., 2008, 2014) and similar events were reported in other countries adopting similar CPS technology (Gressel and Valverde, 2009; Busconi et al., 2012; Scarabel et al., 2012; Rosas et al., 2014). Unfortunately, Malaysia has high potential risks of gene flow and evolution of resistant weedy rice populations because of multiple cropping of rice in a year and freezing temperatures, which would reduce the density of volunteer plants, do not occur (Shivrain et al., 2008; Burgos et al., 2014).

Despite various reports of gene flow and IMI-tolerance weedy rice in the world, there are still limited reports or evidences on weedy rice IMI resistant status in Malaysia. A preliminary study at three townships in Kedah reported that there is high likely that weedy rice has developed resistance to IMI herbicide at various level (Hamdani et al., 2015) based on the weedy rice escapes in CPS rice fields in these areas (Jaafar et al., 2014). Dilipkumar et al. (2018) reported the resistant biotype of weedy rice in Malaysia can be 2.1 to 2.8 times more resistant than the Clearfield® rice itself. Harun et al. (2018) also reported the farmers concern on the recurring weedy rice existence in the CPS fields and the potential of weedy rice resistance to IMI herbicide.

Herbicide resistance in weed population is heritable and in fact is evolution in action (Baki, 2010). This attribute indicates that weed exposure to herbicides is less likely to cause mutation but rather, an herbicide resistance weed (HRW) arises from natural selection of genetic variants possessed among weed populations (Barret and Schluter, 2007; Neve et al., 2009). Weeds in ecosystems always retain a certain level of genetic diversity to survive in a balance ecosystem until a new stress imposing to the population (Novoplansky, 2009). This instituting extra pressures to nature to inevitably response with the landscape shifts. Weeds did not change to become resistant; instead the fittest genotype was selected to survive in a changed environment (Ammann, 2000; Gressel, 2000; Gienapp et al., 2008; Heap, 2019). This study was designed to evaluate the resistant status of weedy rice from various populations collected from CPS rice fields.

Materials and Methods

Weedy rice collection

Seeds from a total of 17 different weedy rice biotypes were sampled during harvesting period in October 2016 at rice fields in Sawah Sempadan (3°25'35.0724" N, 101°10'36.1704" E) in the state of Selangor, Malaysia. Weedy rice samples were randomly selected from high weedy rice infestation fields. These weedy rice biotypes were selected due to observation of weedy rice presence despite the Clearfield® Rice Production System (CPS) has been practiced in the fields for the past six years.

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Communication with field owners indicated that selected fields during sample collection were planted with Clearfield® rice MR220CL2 for two consecutive seasons. Weedy rice seeds were hand-threshed and placed into an individual paper bag. Seed samples were air-dried at room temperature for 3 days (d) before placed in a -4ºC refrigerator for further experiments (to test for resistance to IMI herbicide). Conventional local rice cultivars MR220 and Clearfield® rice MR220CL2 certified seeds were obtained from the Muda Agricultural Development Authority (MADA).

Seed bioassay with imidazolinone (imazapic + imazapyr) herbicide treatments

The resistant of weedy rice seeds to IMI (a.i. imazapic + imazapyr) herbicide was tested by standard germination method using a half (75 g a.i. ha^{-1}), single (150 g a.i. ha^{-1}), and double (300 g a.i. ha^{-1}) doses of herbicide application. A sample of ~30 seeds from each weedy rice biotype was distributed in standard petri dishes (9-cm petri dish diameter) with a Whitman no. 1 filter paper in an incubator set at 40ºC overnight to break the dormancy. This will eliminate the possibility of false negative germination data.

Seeds on each petri dish with three replications were then wetted with ~5 ml concentrated of IMI herbicide using 2.2 g/L concentration for two doses, commercially concentrated 1.1 g/L for single-dose application and 0.56 g/L herbicide concentration for a half-dose application. The seeds for control treatment was applied with distilled water. Samples were placed in an incubator set at 30ºC and 100% relative humidity in the light. Germinated seeds were determined by the emergence of radical or coleoptiles. The viability of germinated seedlings was determined based on the seedling appearances as viable (green in colour) and non-viable (white/non-pigmented). The germination rate and number of the viable seedlings were counted at 1, 3, 5, 7, 10 and 14 d after imbibition.

Weedy rice growth response to imidazolinone (imazapic+imazapyr) herbicide

After-ripened (dormancy released) seeds from all collected weedy rice biotypes and two cultivated rice were germinated in a 30ºC incubator for 7 d. The seedlings were transplanted into pots (40 cm x 40 cm x 25 cm dimension), with 30 plants per pot. The pots were filled with clay soil mixed with growth medium and placed in water containers (60 x 120 x 15 cm dimension) in a greenhouse at Rimba Ilmu, Institute of Biological Sciences, University of Malaya (3°13'N, 101°66'E). The experimental lay out was arranged in a Complete Randomised Design (CRD) with three replications for every weedy rice population and treatment. The seedlings were carefully thinned to 25 per pot during the 2-leaf stage.

The IMI herbicide at 1.1 g/L (150 g a.i ha^{-1}) concentration replicating the commercial dose (one-dose) was applied to the plants at 2-leaf stage (3 d after transplanting) using hand-held spray. The plant response (injury and death) to the treatment was recorded for every three days after the herbicide application. Plants with >50% of the total leaves retained green pigmentation were considered survive from herbicide application. The second application of herbicide was applied to the plants at 21 days after first application with an increased concentration dosage to 2.2 g/L (double dose from commercial recommended dose or 300 g a.i. ha^{-1}) and the plant response was recorded for the subsequent three days. No application of herbicide for control treatment. A total of nine (9) morphological traits were recorded for all weedy rice populations from the control treatment (*Table 1*). All materials were grown with natural temperature, humidity, and day length in the greenhouse during the experiment. The water level in the pots were kept at 5 cm depth throughout the experiment.

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Table 1. *Traits evaluated for all weedy rice biotypes collected from the Clearfield® Rice Production (CPS) fields*

Biotype ID	Traits[a]								
	PH	**SH**	**PT**	**PC**	**FL**	**HC**	**HD**	**AN**	**SW**
MWR_Pop01	100.5	64	Compact	Red	Intermediate	straw	92	Awn	14.3
MWR_Pop02	112	54	Intermediate	Red	Non-erect	straw	92	Awnless	16.1
MWR_Pop03	97	38	Open	Red	Intermediate	straw	94	Awn	17.41
MWR_Pop04	122	48	Intermediate	White	Non-erect	straw	93	Awn	17.4
MWR_Pop05	88.5	60	Compact	White	Non-erect	straw	92	Awnless	14.4
MWR_Pop06	95.5	35	Intermediate	Red	Non-erect	straw	93	Awnless	18.5
MWR_Pop07	77.5	67	Intermediate	Red	Intermediate	straw	93	Awnless	13.4
MWR_Pop08	98	35.5	Intermediate	White	Non-erect	straw	90	Awnless	17.54
MWR_Pop09	101.5	46.5	Compact	White	Non-erect	straw	90	Awnless	13.7
MWR_Pop10	112	64	Intermediate	White	Erect	straw	88	Awn	15.55
MWR_Pop11	109	64	Intermediate	Red	Intermediate	Furrowed	88	Awnless	14.1
MWR_Pop12	151	81.5	Intermediate	White	Non-erect	straw	84	Awnless	14.9
MWR_Pop13	144	93.6	Intermediate	Red	Non-erect	straw	89	Awnless	17.4
MWR_Pop14	139.5	75	Intermediate	Red	Non-erect	straw	88	Awnless	14.2
MWR_Pop15	145	91.5	Open	Red	Non-erect	straw	86	Awnless	18.8
MWR_Pop16	151.5	77.6	Open	Red	Non-erect	straw	87	Awnless	18.33
MWR_Pop17	147	91.3	Intermediate	Red	Non-erect	straw	82	Awn	19.41

[a] Traits evaluated; PH, plant height measured in cm; SH, seed shattering percentage (%); PT, panicle type; PC, color of the pericarp; FE, flag leaf type; HC, hull color; HD, days to heading; AN, presence of awn; SW, 1000 seed weight (g)

Data analysis

The germination rate and growth response data were analysed as a one-way factorial, with different dosage of herbicide treatments as the factor. The variation was tested with ANOVA to detect difference between treatments and means were compared by Tukey's test using the SAS GLM procedure and Tukey option (SAS Institute, 2011). The level of significance was set at $p < 0.05$.

Results

Effects of imidazolinone (imazapic + imazapyr) herbicide application during germination stage

The average germination rate for different dosages of IMI-herbicide (imazapic+imazapyr) application after 7 d of herbicide treatments showed that half- (93.14±6.46%) and one- (90.97±7.52%) dose applications were significantly higher than the double-dose (72.57±9.43%) application (*Fig. 1*). Daily record of germination rate also displayed that weedy rice in the half- and one-dose treatments reached nearly full germination capacity at 4 d after imbibition while the double-dose treatment showed slower rate at 5 d after imbibition. At this pre-emergence treatment (germination test), imazapic+imazapyr only reduced ~10% and ~30% germination percentage under commercial- and double-dose herbicide applications, respectively indicating that this herbicide has limited effect on the germinability of weedy rice. Under controlled condition (germination with distilled water), all weedy and cultivated rice recorded near 100% germination rate (*data not shown*). However, this germination data cannot be an indication of resistant level in weedy rice because the fate of survival of the seedlings to

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maturity are yet to be determined. Therefore, the pigmentation of the seedlings was observed to determine the seedlings potential viability.

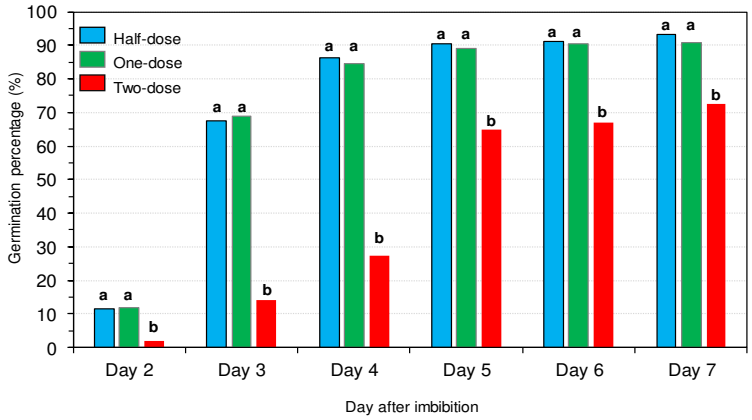

Figure 1. *Cumulative germination percentage recorded for 7 d after imidazolinone herbicide treatment at half (1.1 g/L), single (2.2 g/L), and double (4.4 g/L) herbicide concentration represented by blue, green and red column bars, respectively. Bars with the same letter show no significant difference ($p<0.05$) within the treatments*

Viability of seedlings post germination after IMI-herbicide treatments at different dosage was determined by the green pigmentation of the seedlings 14 d after imbibition (*Fig. 2*). Seedlings developed normal (green) pigmentation after treatments were counted as 'survived' from herbicide treatment and considered resistant towards the herbicide treatment while unpigmented (white color) seedlings were considered not be able to endure through maturity. The seedlings viability percentage was significantly reduced over the increment of herbicide dosage (*Fig. 3*). Increment from half-dose to one-dose significantly ($p<0.001$) decreased seedlings survival by 45.8% while two-dose herbicide treatment decreased seedlings survivability by 64.8% ($p=0.0017$).

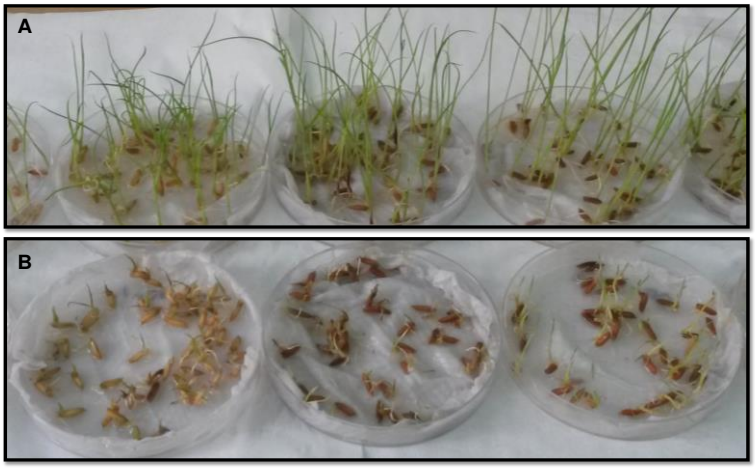

Figure 2. *Weedy rice seeds' response towards pre-emergence imidazolinone herbicide treatments. The seedlings' viability was determined based on green (A) and white (B) appreances to indicate viable and non-viable seedlings, respectively*

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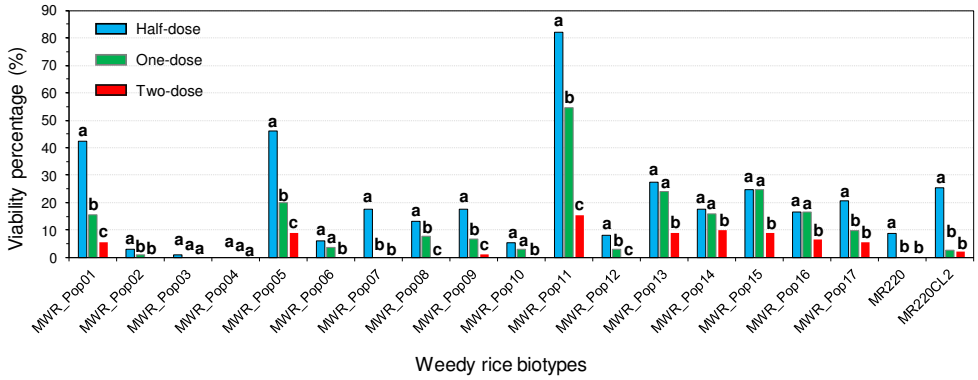

Figure 3. *Percentage of seedlings viability for each weedy rice biotypes after 14 d of imidazolinine herbicide treatments at half (1.1 g/L), single (2.2 g/L), and double (4.4 g/L) herbicide concentration represented by blue, green and red column bars, respectively. Bars with the same letter show no significant difference ($p<0.05$) within the treatments*

At half dosage of IMI-herbicide, average percentage of viable seedlings ranged from 1% to 82% while weedy rice MWR_Pop04 displayed no seedlings developing green pigmentation (*Fig. 3*). This indicates that this weedy rice population was the most susceptible population towards IMI herbicide. Application of IMI-herbicide at one-dose displayed three weedy rice populations were fully controlled (susceptible) at this application rate. Additional five weedy rice biotypes displayed susceptibility towards the herbicide when the dosage was increased to double the recommended dosage (*Fig. 3*).

Current recommended dosage of IMI herbicide with herbicide concentration of 150 g a.i. ha^{-1} only successful to control 23.5% of weedy rice biotype samples as pre-emergence herbicide. Despite relatively low percentage of viable seedlings (13.9±13.8%) for the remaining 13 populations, weedy rice has developed a degree of resistance to potentially escape current herbicide dosage. At an increased dosage, IMI herbicide can control half of the weedy rice population but the remaining still maintaining low level of resistance ranging from 1.1% to 15.6%. Weedy rice MWR-Pop11 displayed the most resistant population with 54.44 and 15.56% of its seedlings survived the herbicide treatments at one- and two-dose application, respectively. Clearfield® rice MR220CL2, an imidazolinone tolerant rice, displayed 25.56%, 2.67%, and 2.22% seedlings viability percentage after herbicide application at half-, one- and two-doses, respectively.

Weedy rice growth response on imidazolinone herbicide application

The effects of IMI herbicide to weedy rice growth varied between biotypes (*Fig. 4*). A total of 11 (64.71%) weedy rice biotyeps responded to one-dose herbicide application after 3 d with mortality ranging from 1.4% to 6.4%. The mortality rate was gradually increased over time for the majority of the populations except for four biotypes (MWR_Pop03, MWR_Pop12, MWR_Pop13, and MWR_Pop17) which showed stagnant mortality below 10% starting at day 15 and two populations (MWR_Pop15 and MWR_Pop16) have all plants survived commercial dose of IMI herbicide. At 21 d after one-dose herbicide application, weedy rice populations MWR_Pop06, MWR_Pop07, MWR_Pop08, and MWR_Pop11 showed high susceptibility to the herbicide treatment where all individuals died from the treatment.

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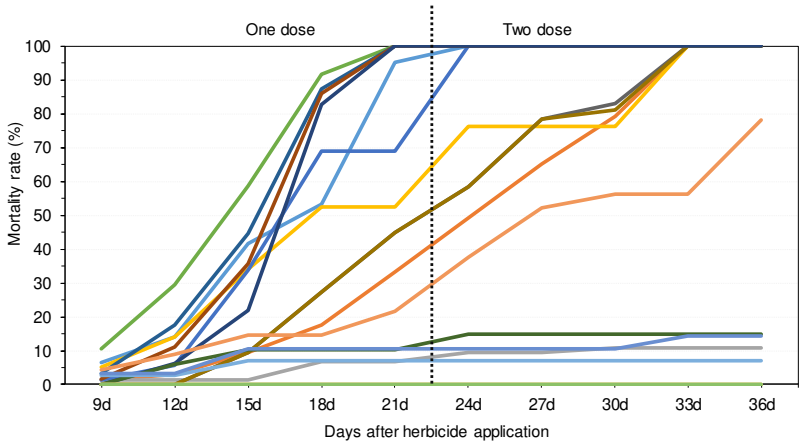

Figure 4. *Effects of imidazolinone herbicide on mortality rate to 17 weedy rice biotypes over time. Vertical dotted line indicates the time of the two-dose herbicide concentration application*

The increase dosage of herbicide after 21 d to two-dose affected the other six weedy rice biotypes where MWR_Pop01 and MWR_Pop05 reached 100% mortality seven days after the 2-dose treatment while MWR_Pop02, MWR_Pop04, MWR_Pop09, and MWR_Pop10 died at day 37 (*Fig. 4*). The increased mortality rate of MWR_Pop14 however stopped at 78.3%. The remaining weedy rice biotypes showed no effect (survived) or stagnant mortality rate for both application dosages.

Clearfield® rice variety MR220CL2 showed complete resistant towards IMI herbicide of both dosages while commercial variety MR220 displayed total susceptible to the herbicide (*Fig. 5*). A total of two weedy rice biotypes (MWR_Pop15 and MWR_Pop16) showed similar resistant pattern with Clearfield® rice. *Figure 4* also showed that IMI herbicide can have total control to only 23.52% and 35.29% of the weedy rice biotypes for one- and two-dose applications, respectively. The remaining 41.18% of the weedy rice biotypes showed certain degrees of resistance towards herbicide application ranging from 4.74 to 100%.

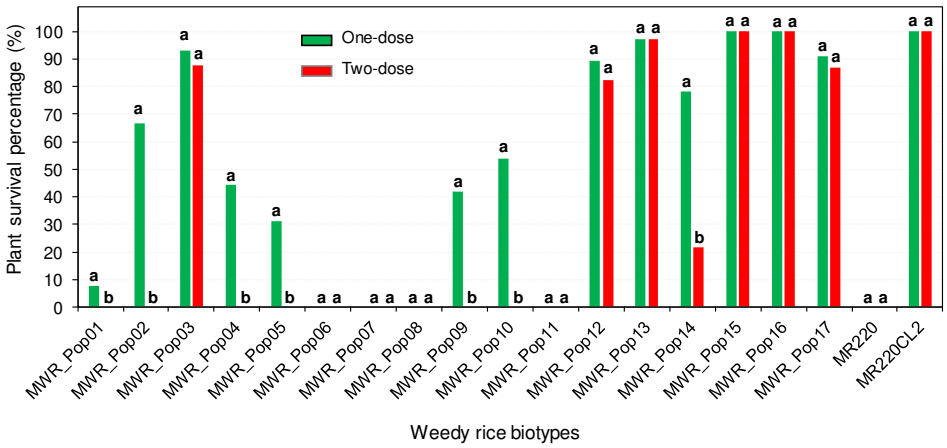

Figure 5. *The growth response (survival rate) of weedy and cultivated (MR220 and MR220CL2) rice after imidazolinine herbicide treatments at commercial (150 g a.i. ha^{-1}), and double (300 g a.i. ha^{-1}) herbicide concentration represented by green and red column bars, respectively. Bars with the same letter show no significant difference ($p<0.05$) within the treatments*

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Discussion

It is high likely that several weedy rice biotypes in Malaysia have already 'evolved' to be resistant to imidazolinone herbicide (a.i. imazapic + imazapyr) possibly from consequential conferment of resistant genes from Clearfield® rice to weedy rice (Shivrain et al., 2008; Jaafar et al., 2014; Dilipkumar et al., 2018). Different weedy rice biotypes from this study showed wide variation of IMI herbicide responses to the weed during pre-emergence (*Figs. 1,3*) and post-emergence (*Figs. 4,5*) herbicide applications at various dosage. This confirmed that sampled weedy rice biotypes have various degrees of resistant towards IMI herbicide. This variation might be caused by accidental and/or voluntary hybridization between IMI tolerant cultivated rice with weedy rice in the Clearfield® rice fields.

Engku et al. (2016) reported the potential gene flow between Malaysian Clearfield® rice (MR220CL1 and MR220CL2) to various weedy rice biotypes producing resistant progenies in the F_1 population. The hybridization introduces gene flow and subsequently increases genetic diversity and heterogeneity of the hybrid weedy rice populations (Chang, 2003), initiating hybridization-differentiation cycles of next generations (Gu et al., 2004; Mispan et al., 2013). This increases genotypic selection for adaptive/survival traits (Mispan et al., 2013; Zhang et al., 2017; Qiu et al., 2017) which later expand the probability of survival potential from selection pressure due to continuous IMI herbicide application (Kuk et al., 2008; Dilipkumar et al., 2018).

Viability percentage of the seedlings for herbicide treatment as a pre-emergence application at half-dose (21.4±19.6%), commercial dose (13.9±13.8%) and double-dose (7.3±4.1%) rate (*Fig. 3*) indicates the application of IMI herbicide as pre-emergence herbicide might help in managing weedy rice at the early stage. Imidazolinone herbicide reportedly to have a slight effect on the percentage of seed germination in chickpea but a significant shift down in the speed of germination (Hoseiny-Rad and Jagannath, 2011). This is in line with the stewardship guideline for the Clearfield® Production System (CPS) to apply the recommended herbicide only between 0 to 7 d after sowing (DAS). High percentage of imazapic (52.5%) - one of active ingredients in IMI herbicide - acting as pre-emergence herbicide might contribute to this action (Dilipkumar et al., 2018).

However, this study showed that MR220CL2 (imidazolinone tolerance variety) seeds also affected by the pre-emergence application (*Fig. 5*) if the seeds were directly sowed before pre-germination. The usage of IMI herbicide as pre-emergence herbicide could be more effective in transplanting method especially with an increased dosage from the commercial rate. However, the environmental impact of regular usage and high dosage of this herbicide need to be properly assessed because the current CPS practice showed potential herbicide leachate and carryover in the rice field soil (Bzour et al., 2019).

The application of IMI herbicide as a post-emergence herbicide can increase the potential of weedy rice to escape the CPS. Only 23.5% of weedy rice sampled populations can be fully controlled (susceptible) by commercial dosage and additional 35.3% were controlled by 2-dose. The low formulation rate of imazapyr (17.5% or equivalent of 38.5 g a.i. ha^{-1}) as a post-emergence herbicide in the herbicide will increase the probability for diverse weedy rice populations to survive. Dilipkumar et al. (2018) reported that imazapyr can control resistant biotype of weedy rice at 4,995 g a.i. ha^{-1}. This wide margin creates ample window for weedy rice to adapt in the CPS environment and consequently become resistant to the herbicide through spontaneous mutation over time (Tan et al., 2005; Sales et al., 2008; Kuk et al., 2008).

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Unfortunately, disobedience of some farmers to follow the CPS guidelines and stewardships has been reported (Dilipkumar et al., 2018; Harun et al., 2018) and personally observed. This include practicing CPS for more than three consecutive seasons in the same field, late application of recommended herbicide at 10 to 15 DAS, and reducing the herbicide dosage to cut input cost.

Continous application of same herbicide will enhance selection pressure for resistant weedy rice. Previous experience in Malaysia already reported that continuous use of phenoxy herbicides since late 1980s has caused the weed species shift to graminaceous species including weedy rice in Malaysia rice granaries (Baki, 2006; Baki and Shakirin, 2010; Mispan et al., 2019). Late application of IMI herbicide will reduce herbicide efficacy which leads to weedy rice survival (Dilipkumar et al., 2018). This study also showed that reducing herbicide dosage will significantly increase seedlings survivability (*Fig. 3*), consequently facilitate weedy rice escape in the rice fields.

Conclusion

This research demonstrated diverse weedy rice biotypes in one of the rice granaries in Malaysia have developed various degrees of resistant towards IMI herbicide (a.i. imazapic + imazapyr). Intervention strategies are required to ensure the sustainability of the CPS technology in the country (Dilipkumar et al., 2018). Understanding the escape mechanism of weedy rice from herbicide application and their adaptation strategies from dynamic changes of agronomic practices in rice cultivation is needed to offer valuable insights into weedy rice management strategies in the future. Malaysia will face ecological risks of continuous weedy rice adaptation in favor to its survival if no stringent ecological risk evaluation including the screening and mitigation strategies to break the selection of herbicide resistant weedy rice in the CPS system (Sudianto et al., 2013; Mispan et al., 2019).

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TEMPORAL AND SPATIAL DISTRIBUTION CHARACTERISTICS OF RHIZOSPHERE ORGANIC ACIDS UNDER WATER LEVEL FLUCTUATIONS IN THREE TYPES OF LAKES IN CHINA

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Abstract. Organic acids are influenced by many factors, such as season variations, water levels, vegetation and others. In the study, the rhizosphere soils of lakeshore plants in Poyang Lake, Chaohu Lake and Wuchang Lake in China were sampled under different water level fluctuations, and the amounts of organic acids were measured. Water level fluctuations affect the temporal and spatial characteristics of rhizosphere organic acids. The annual average of the total amount of organic acids in the rhizosphere soil of lakeshore plants in Wuchang Lake was higher than that in the other lakes. The seasonal variations in the amounts of organic acids were the same in the three lakes. The total amounts of organic acids in the rhizosphere soils at high water level sites were higher than those in the low water level sites of the three lakes. The effects of the interactions of season variations and water level fluctuations on the amount of organic acids in the rhizosphere were significant. The results revealed that the lake types changed in response to anthropogenic influence; Wuchang Lake, as a quasi-natural lake, suffered from much more human pollutants than Chaohu Lake as a reservoir-like lake and Poyang Lake as an intermittent lake.
Keywords: *rhizosphere soils, lake type, lakeshore, season variations*

Abbreviations: ANOVA, Analysis of variance; CA, Citric acid; CHL, Chaohu lake; MA, Malic acid; MC, Moisture content; OA, Oxalic acid; PYL, Poyanghu lake; SA, Succinic acid; SOM, Soil organic matter; TA, Tartaric acid; TN, Total nitrogen; TP, Total phosphorus; ToA, Total amount of organic acids; WCL, Wuchanghu lake

Introduction

A suitable water level is an important factor for maintaining the structure, function and biological integrity of lake ecosystems (Coops et al., 2003; Wantzen et al., 2008; Leira and Cantonati, 2008). Water level fluctuations are defined as the differences or changes in the water level within a period of time and are influenced by many factors, such as the water storage capacity, rainfall, and human factors involved in dam, highway and railway construction (Beckmann et al., 2005; Wang et al., 2007). Water level fluctuations have an important influence on the diversity of aquatic organisms; many studies have shown the effect of water level fluctuations on the digestion and retention of pollutants, the stabilization of the lakeshore, the maintenance of the water self-purification capacity, and the support of regional biological communities (Naiman and Décamps, 1997; Venkatachalam et al., 2005). For instance, water level fluctuations were found to influence the mobilization and transformation of nutrients such as nitrogen, phosphorus

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and chlorophyll a, as well as the diversity of phytoplankton and zooplankton (Sherr et al., 2003; Zhang et al., 2013; Liu et al., 2018). However, few studies have focused on the relationship between water level fluctuations and the root-soil environment, especially the effect of water level fluctuations on organic acids in rhizosphere soil.

Low molecular weight organic acids, such as citric acid, oxalic acid, and malic acid, are three of the most active forms of carbon in the plant-rhizosphere-soil interface and have great importance in physiological and biochemical processes, such as microbiological activity, nutrient acquisition, and the detoxification of heavy metals (Bais et al., 2006; Keiluweit et al., 2015; Chen et al., 2017). Numerous studies have shown that the allocation, transformation and exudation characteristics of organic acids are important mechanisms in response to environmental stresses. For instance, the roots of dicotyledonous species such as lupine (*Lupinus albus* L.), rape (*Brassica napus* L.) and alfalfa (*Medicago sativa* L.) exuded large amounts of organic acids under phosphorus deficiency; this organic acid mobilizes the insoluble phosphorus compound in rhizosphere soils, which increases the release of inorganic phosphorus and promotes phosphorus uptake (Haichar et al., 2014; Zhao and Wu, 2014; Baetz and Martinoia, 2014). Lakeshore plant species such as umbrella sedge and *Pistia stratiotes* adapt to phosphorus deficient environments via the regulation of the composition and amount of root-exuded organic acids (Zhang et al., 2019a,b). The roots of three lakeshore plants, *Canna indica*, *Zizania aquatic* and *S. warburgii Seemen* exude many organic compounds, mainly low molecular weight organic acids and aromatic proteins in root exudations; furthermore, the amount of such exudates was found to have a positive correlation with the biomass of the three plants (Lu et al., 2009). The mangrove *Kandelia candel* exudes high amounts of formic acid, butyric acid, malic acid, citric acid and lactic acid, and these low molecular weight organic acids increased the release of available heavy metals in wetland sediments (Lu et al., 2007). The composition and amount of organic acids in rhizosphere soils via root exudation can directly influence many biological processes in the rhizosphere-soil-plant interface under environmental stresses (López-Bucio et al., 2000; Zhao and Wu, 2018). Therefore, investigation of the composition and amount of organic acids in rhizosphere soils of lakeshore plants will provide important information on nutrient acquisition, heavy metal detoxification and plant growth.

In the present study, the composition and amount of organic acids under different water level fluctuations in different seasons were investigated in the rhizosphere soils of lakeshore plants in three different lakes in the lower-middle reaches of the Yangtze River basin of China. Furthermore, the effects of the physicochemical parameters of the rhizosphere soils on the amounts of organic acids were analyzed, and the responses of organic acids in different lakes under different water level fluctuations were investigated.

Materials and Methods

Study areas

Three lakes, Poyang Lake (PYL), Chaohu Lake (CHL) and Wuchang Lake (WCL), in the middle and lower reaches of the Yangtze River that are affected by human disturbance were investigated (*Fig. 1*). PYL is located in Jiangxi Province, and CHL and WCL are located in Anhui Province. The three lakes are at relatively low elevations. CHL and WCL are regulated by the Yuxi and Wanhe sluices, which were constructed as a consequence of human disturbance, and the water surface areas of PYL have been greatly reduced. The climate of three lakes is a typical subtropical monsoon climate characterized by hot and

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rainy summers and cold and dry winters. The high temperature period is concentrated in July and August, the rainy season is from April to June, and the cold season occurs in January and February. The annual precipitation and annual air temperature at PYL, CHL and WCL are 1600.0 mm, 995.7 mm and 1299.6 mm, and 17.2 °C, 16.1 °C and 16.5 °C, respectively (Zhang, 2013; Zhang et al., 2018).

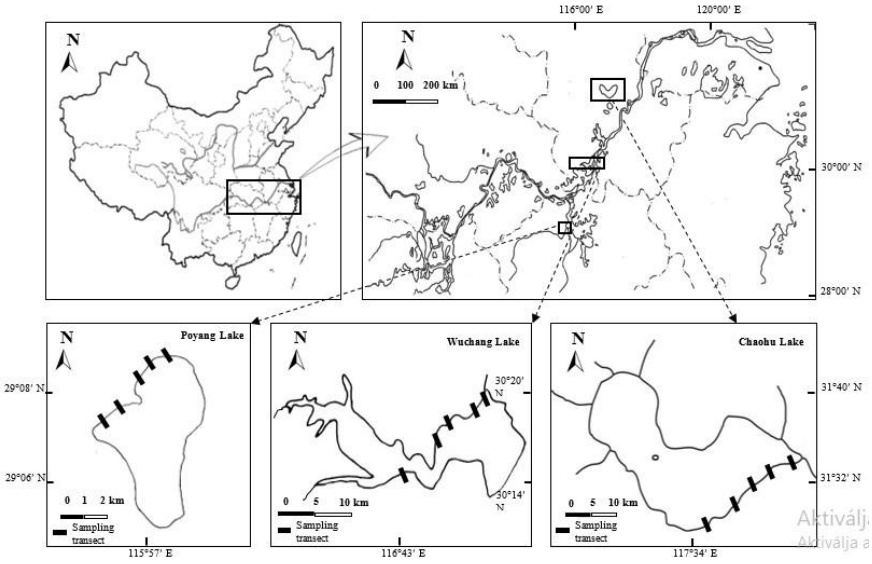

Figure 1. *Study area and soil sampling sites of three lakes*

PYL with a mean bottom elevation is 14.67 m amsl, and the water levels are low and stable with a mean water depth of less than 1.0 m. The mean bottom elevation of CHL is 5.0-6.0 m above mean sea level (amsl), and the water level is often maintained at 7.5-8.0 m amsl. The annual average water level of CHL was raised about 1.5 m after the building of the sluice (Zhang et al., 2014). The mean bottom elevation of WCL is 10.0 m amsl, and the water level is often controlled at 11.5 m amsl. The main uses of WCL are for aquaculture and flood control (Zhang et al., 2016). The other limnological parameters of the three lakes are shown in *Table 1*.

Table 1. *Limnological parameters of the three lakes. Note: Data are from Wang and Dou (1998), Zhang (2013), and Zhang et al. (2018)*

	Poyang Lake	**Chaohu Lake**	**Wuchang Lake**
Area (km^2)	30	769.6	100.5
Mean water depth (m)	0.70	2.69	3.43
Maximum water depth (m)	4.43	3.77	4.31
Annual precipitation (mm)	1600	995.7	1299.6
Annual air temperature (°C)	17.2	16.1	16.5
Recharge coefficient of lake	-	12.0	10.8
Catchment area (km^2)	-	9258	1083.7
Volume (m^3)	-	20.7×10^8	3.45×10^8

The floristic composition along the shores of the three lakes are significantly different (Zhang et al., 2015, 2016). The vegetations along the shores of CHL and PYL are relatively simple, and the dominant species of the lakeside zone near CHL is mainly

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Cynodon dactylon, while *Carex sp*. is the dominant species in PYL. The floristic composition along the shore of WCL are relatively diverse, with *Cynodon dactylon* and *Carex sp.* covering some of the area, and the wild emergent aquatic plant species *Zizania latifolia* having a large distribution area.

Rhizosphere soil sampling

The rhizosphere soils of the lakeshore plants around lakes that exhibited three different types of fluctuations were investigated in the summer of 2016 (August, Summer), the autumn of 2016 (November, Autumn), the winter of 2017 (February, Winter) and the spring of 2017 (May, Spring). The main vegetation that grew in the soil samples were *Cynodon dactylon* in CHL, *Carex sp.* in PYL, and *Zizania latifolia* in WCL. The sections with limited artificial interference were selected in the three lakes, the five typical sample points were evenly distributed throughout this section, ranging from the lowest perennial average water level to the highest perennial average water level of the lake, which were called 1, 2, 3, 4 and 5 successively. The distance between two adjacent sample points remained equal. According to the water level fluctuations of the three lake types in the normal hydrological year 2015, water levels in these lakes are higher in winter and spring, and low in late spring to early summer, which is opposite to the natural condition (Zhang et al., 2018). Therefore, the elevations of the sample points in PYL were 14.75 m (PYL1), 15.56 m (PYL2), 16.38 m (PYL3), 17.19 m (PYL4) and 18.00 m (PYL5). The elevations of the five sample points in CHL were 8.15 m (CHL1), 8.61 m (CHL2), 9.07 m (CHL3), 9.52 m (CHL4) and 9.98 m (CHL5). The elevations of the sample points in WCL were 10.82 m (WCL1), 11.52 m (WCL2), 12.22 m (WCL3), 12.91 m (WCL4) and 13.61 m (WCL5).

The rhizosphere soil samples were randomly collected in triplicate in the three different lakes owing to the obvious lakeshore plant layer. The soil samples were collected always at the same site, the sampling sites of WCL, CHL and PYL were shown in *Figure 1*.

The soils within 0.5 cm from the root system were collected after excavating the roots and the aboveground parts of plants following the method described by Gollany et al. (1997), and the rhizosphere soil samples were divided into two parts.

One part was stored at -24 °C as fresh soil. The other part was dried and sieved through 2 mm mesh, and the soil physicochemical parameters, such as pH, moisture content (MC), soil organic matter (SOM), total nitrogen (TN) and total phosphorus (TP), were determined. The pH and MC were measured by the potentiometric method and the oven-drying method, respectively; SOM was measured by the $K_2Cr_2O_7$ titration method; the TN and TP contents were measured using the Kjeldahl method and molybdenum blue colorimetry, respectively (Lu, 2000).

Extraction and purification of organic acids in rhizosphere soils

A total of 5 g of fresh rhizosphere soil samples with the root chips, residues and others large particulate matter removed were placed in a 50 mL Erlenmeyer flask, and 25 mL 0.1% H_3PO_4 extracted solution was added. The soils and the extracted solution were mixed with a glass rod, the mixed soil solution was oscillated for 2 h; then, the mixture was transferred to a 50 mL centrifuge tube. The tube underwent centrifugation at 10000 r min^{-1} for 10 min; then, the supernatant was collected and passed through a 0.22 μm water phase filtration membrane.

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The solutions containing organic acids were collected and passed through a cation exchange column (12 mm × 15 mm) filled with 5 g of Amberlite IR-120B resin (H^+ form, Alfa Co.). Afterward, these solutions were passed through an anion exchange column (12 mm × 15 mm) filled with 3 g of Dowex 1 × 8 resin (100 mesh to 200 mesh; OH^- form; Acros Co.). The solutions were evaporated under rotary conditions of 40 °C after steaming to nearly dry, and the residue was obtained in 1 mL water for a quick rhizosphere soil sample test.

Determination of the amounts of organic acids in rhizosphere soils

The amounts of organic acids, including oxalic acid (OA), citric acid (CA), malic acid (MA), tartaric acid (TA) and succinic acid (SA), in the rhizosphere soils of the three lakes were analyzed by Waters Acquity H-Class ultra-performance liquid chromatography coupled with a four element pump system, automatic injection system, ultraviolet detector system, solvent system and chromatography column system (Zhao et al., 2019). The standard samples of OA, CA, MA, TA and SA were obtained from Sigma-Aldrich-Fluka (ref. 02288, 27488, 75688, 251380, and S7501, respectively). The concrete analytical conditions were the following: chromatography column, BEH C18 column (2.1 mm i.d. × 500 mm, 1.7 μm); eluent solution, 10 mM KH_2PO_4 solution (adjust pH to 2.20±0.10 by 1 mol L^{-1} H_3PO_4 solution); flow rate, 0.30 mL min^{-1}; temperature of the chromatography column, 30 °C and injection volume, 1 μL. The eluent solution and analyte were filtered using a 0.22 μm membrane and then ultrasonically degassed before use.

Statistical analysis

All experiments were performed in triplicate with the same treatment independently replicated. Statistical analyses of data were carried out by one-way ANOVA, two-way ANOVA and bivariate correlations. Significance was assigned at the $p < 0.05$ level with Duncan's test. All analyses were conducted using SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

Results

Soil physicochemical parameters

The pH values were significantly different among the three lakes in the same season; the difference in MC was not significant in winter but exhibited significant differences in the other three seasons; SOM, TN and TP were not significantly different, except for SOM and TN, which were higher in PYL than in WCL and CHL in spring (*Table 2*). MC was not significantly different in the four seasons in WCL, while the other soil parameters were different (*Table 2*). The pH in autumn and winter was lower than that in spring and summer, while MC, SOM, TN and TP were not significantly different in CHL and PYL (*Table 2*).

The total amount of organic acids in the rhizosphere soil of lakeshore plants in different seasons

The total amount of organic acids (ToA), including OA, CA, MA TA and SA, in the rhizosphere soil of the lakeshore plants in winter was lower than that in the other three seasons in the three lakes, which was higher in summer than in the other three seasons in

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CHL and PYL and was highest in WCL (*Fig. 2*). The ToA in WCL was significantly higher than that in CHL and PYL during the same season (*Fig. 2*). The annual averages of the total amounts of organic acids in WCL, CHL and PYL were 2.783 mg kg^{-1}, 1.668 mg kg^{-1}, and 1.954 mg kg^{-1}, respectively.

Table 2. *The soil parameters at the sampling sites in Wuchanghu Lake, Chaohu Lake and Poyang Lake*

Lake name	Soil parameters	Season			
		Spring	Summer	Autumn	Winter
Wuchang Lake	pH	7.22±0.80Aa	7.37±0.30Aa	6.05±0.18Ab	5.800±0.30Ab
	MC (%)	22.13±4.88Aa	24.41±4.60Aa	26.06±5.04Aa	29.47±18.29Aa
	SOM (mg kg^{-1})	1.03±0.22Aa	1.01±0.48Aa	1.63±0.58Aab	2.57±1.93Ab
	TN (mg kg^{-1})	0.72±0.11Aa	0.62±0.26Aa	1.00±0.30Aab	1.65±0.88Ab
	TP (mg kg^{-1})	0.44±0.09Aa	0.33±0.09Ab	0.32±0.05Ab	0.42±0.08Aab
Chaohu Lake	pH	8.68±0.20Ba	8.29±0.15Bb	7.58±0.05Bc	7.68±0.11Bc
	MC (%)	18.67±4.16Aa	21.13±4.07Aa	18.31±5.46Ba	16.60±2.84Aa
	SOM (mg kg^{-1})	0.84±0.34Aa	1.96±1.54Aa	1.08±0.81Aa	1.44±1.28Aa
	TN (mg kg^{-1})	0.54±0.16Aa	1.14±0.72Aa	0.80±0.48Aa	0.97±0.84Aa
	TP (mg kg^{-1})	0.58±0.24Aa	1.12±1.19Aa	0.69±0.51Aa	1.16±1.18Aa
Poyang Lake	pH	5.65±0.12Ca	6.06±0.62Ca	4.90±0.14Cb	4.68±0.19Cb
	MC (%)	28.56±4.52Ba	30.73±4.07Ba	28.57±3.55Aa	27.55±4.98Aa
	SOM (mg kg^{-1})	1.46±0.40Ba	1.65±0.44Aa	1.74±0.62Aa	1.88±0.12Aa
	TN (mg kg^{-1})	1.05±0.26Ba	1.17±0.22Aa	1.13±0.38Aa	1.3±0.17Aa
	TP (mg kg^{-1})	0.39±0.09Aa	0.37±0.06Aa	0.38±0.06Aa	0.38±0.06Aa

Values are means ± SE (n=3). The different capital letters indicate a significant difference in the three lakes in the same season at $p<0.05$. The different lowercase letters indicate a significant difference in the four seasons at the same lake at $p<0.05$. MC: moisture content; SOM: soil organic matter; TN: total nitrogen; TP: total phosphorus

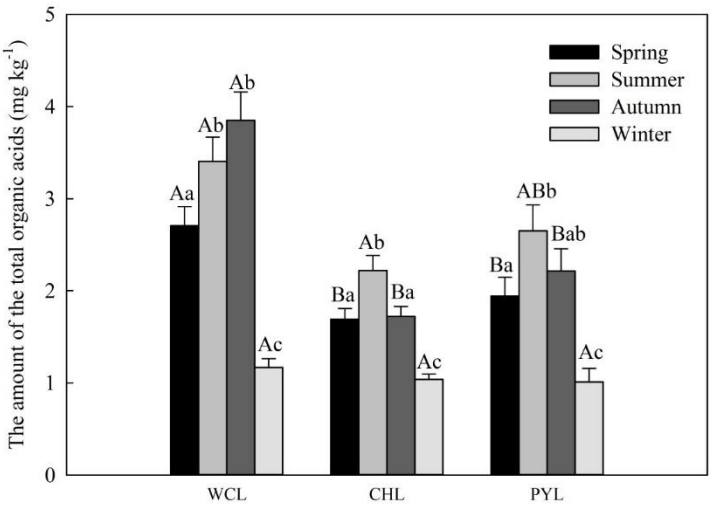

Figure 2. *The variations in the total amount of organic acids in the rhizosphere soil of lakeshore plants in three lakes in different seasons. Note: Columns with bars indicate means ± SD (n= 12). The different capital letters indicate a significant difference in the three lakes in the same season at $p<0.05$. The different lowercase letters indicate a significant difference in the four seasons under the same lake at $p<0.05$. PYL: Poyanghu Lake; CHL: Chaohu Lake; WCL: Wuchanghu Lake*

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The effect of water level fluctuations on the characteristics of the ToA in the rhizosphere soil in different seasons

The seasonal variations in organic acids in the rhizosphere soil were significant at the different water levels in the three lakes, as shown in *Fig. 3*. At the different water level fluctuations, the ToA was between 0. 607 mg kg^{-1} and 6.817 mg kg^{-1} in WCL, which was highest in summer, and lowest in winter, and the ToA at high water levels, such as at WCL3, WCL4, and WCL5, was higher than that at low water levels, such as WCL1 and WCL2 (*Fig. 3*). At the different water level fluctuations, the ToA was between 0.712 mg kg^{-1} and 3.798 mg kg^{-1} in CHL. The ToA had was not significantly different in the sites with low water level fluctuations, including CHL1 and CHL2, in the different seasons; however, the ToA values were higher at the sites with high water level fluctuations, including CHL4 and CHL5. The ToA was the maximum at CHL4, which was two-fold higher than that at CHL1 in the all seasons except winter (*Fig. 3*). The ToA in the sites with different water level fluctuations in PYL was between 0.410 mg kg^{-1} and 3.909 mg kg^{-1}. The seasonal variations in the amount of organic acids were the following: summer > autumn > spring > winter. The ToA at PYL4 was higher than that at the other sites.

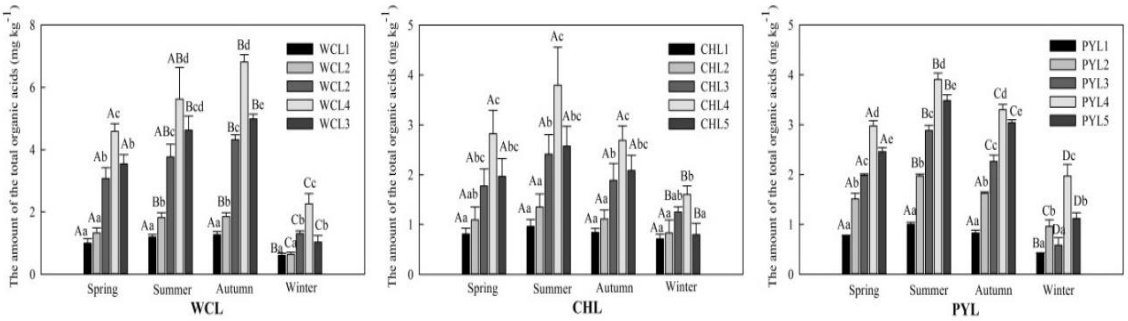

Figure 3. *The effect of water level fluctuations on the characteristics of the total amounts of organic acids in rhizosphere soil in different seasons in the three lakes. Note: Columns with bars indicate means ± SD (n= 3). The different capital letters indicate a significant difference in the four seasons under the same water level fluctuations in the same lakes at p<0.05. The different lowercase letters indicate a significant difference in the water level fluctuations in the same season in the same lakes at p<0.05. PYL: Poyanghu Lake; CHL: Chaohu Lake; WCL: Wuchanghu Lake*

The proportions of the five organic acids in the rhizosphere in different seasons in the three lakes

The contributions of OA, CA, MA, TA and SA to the ToA in the three lakes differed in the different seasons. The proportions of the five organic acids varied with season in WCL, and the proportion of CA increased, which accounted for 40% in winter (*Fig. 4*). The dominant organic acids were CA and MA in CHL, and the proportion of CA and MA was over 60% in all four seasons (*Fig. 4*). CA, MA and SA were the dominant organic acids in the rhizosphere soils of PYL, accounting for more than 75% of the ToA, especially more than 90% in winter (*Fig. 4*).

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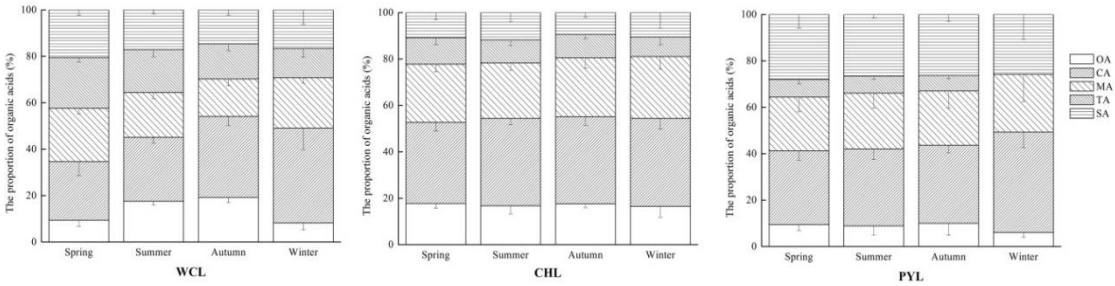

Figure 4. *The proportions of the five organic acids in rhizosphere soil in different seasons in the three lakes. Note: Columns with bars indicate means ± SD (n= 3). PYL: Poyanghu Lake; CHL: Chaohu Lake; WCL: Wuchanghu Lake; OA: oxalic acid; CA: citric acid; MA: malic acid; TA: tartaric acid; SA: succinic acid*

The interaction of seasonal variation and water level fluctuations with the characteristics of organic acids in rhizosphere soil

The effect of the interaction of the seasonal variation and water level fluctuations on the characteristics of the amounts of five organic acids and the ToA in rhizosphere soil of lakeshore plants in three lakes was significantly different based on the two-way ANOVA. The mean square value was between 0.002 and 4.839, and the F value was between 5.602 and 123.355 in the three lakes (p<0.001) (*Table 3*). The mean square value or F value of ToA in WCL was higher than that in PYL and CHL, which indicated the effect of interaction of seasonal variations and water level fluctuations on WCL was stronger than that on other two lakes (*Table 3*).

Table 3. *The interaction of seasonal variations and water level fluctuations on the characteristics of organic acids in rhizosphere soil of lakeshore plants based on two-way ANOVA*

Lake name	Organic acids	Degree	Mean square (MS)	F	Significance (p value)
Wuchanghu Lake	OA	12	0.263	123.355	<0.001
	CA	12	0.649	29.793	<0.001
	MA	12	0.115	23.254	<0.001
	TA	12	0.276	16.617	<0.001
	SA	12	0.180	15.361	<0.001
	ToA	12	4.839	47.289	<0.001
Chaohu Lake	OA	12	0.069	5.800	<0.001
	CA	12	0.175	5.129	<0.001
	MA	12	0.073	14.791	<0.001
	TA	12	0.013	5.863	<0.001
	SA	12	0.019	5.602	<0.001
	ToA	12	1.090	10.744	<0.001
Poyanghu Lake	OA	8	0.002	13.366	<0.001
	CA	8	0.035	17.620	<0.001
	MA	8	0.065	30.201	<0.001
	TA	8	0.002	7.860	<0.001
	SA	8	0.022	10.523	<0.001
	ToA	8	0.100	13.622	<0.001

OA: oxalic acid; CA: citric acid; MA: malic acid; TA: tartaric acid; SA: succinic acid; ToA: total amount of organic acids

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The correlations between the amounts of organic acids and environmental factors in the three lakes

Table 4 shows the correlations between the amounts of organic acids and physicochemical parameters of rhizosphere soils such as pH, MC, SOM, TN and TP in the three lakes. The amount of one organic acid exhibited a linear correlation with the other organic acids and the ToA. The Pearson correlation coefficient (R) ranged from 0.502 to 0.963 in the rhizosphere soils of lakeshore plants in the three lakes (p <0.01). The soil parameters had different influences on the amount of organic acids in the rhizosphere soils of lakeshore plants in the three different lakes. There were no significant differences between the amounts of five organic acids or the ToA and MC, SOM, TN and TP, except for the significant difference between TN and OA (p<0.05). There were highly negative linear correlations between the individual organic acids or the ToA and pH (p<0.01), except for the nonsignificant difference between TA and pH (p>0.05) in PYL. In CHL, the soil physicochemical parameters included pH, MC and SOM, and the individual organic acids or the ToA exhibited significant differences. The differences between the individual organic acids or the ToA and TN were highly significant (p<0.01), except for the nonsignificant difference between TA or SA and TN (p>0.05). Meanwhile, there was no significant difference between the individual organic acids or the ToA and TP (p>0.05), except for the highly significant differences between OA and TP (p<0.01). In WCL, the soil physicochemical parameters, including MC, SOM, TN and TP, exhibited a negative linear correlation with the individual organic acids or the ToA (p<0.01), while pH had an influence on the amounts of MA, TA and SA.

Table 4. *The Pearson correlation coefficients between the amounts of organic acids and soil physicochemical parameters of rhizosphere soils in Poyang Lake, Chaohu Lake and Wuchang Lake*

		CA	MA	TA	SA	ToA	pH	MC	SOM	TN	TP
Poyang Lake	OA	0.739**	0.722**	0.904**	0.769**	0.827**	-0.502**	0.075	-0.219	-0.290*	-0.106
	CA	1	0.864**	0.837**	0.892**	0.959**	-0.381**	-0.015	0.086	-0.096	-0.014
	MA		1	0.818**	0.841**	0.942**	-0.372**	-0.012	-0.059	-0.242	0.020
	TA			1	0.749**	0.877**	-0.270	0.006	0.111	-0.017	-0.031
	SA				1	0.951**	-0.513**	0.068	0.009	-0.164	-0.046
	ToA					1	-0.465**	0.026	-0.017	-0.195	-0.019
Chaohu Lake	OA	0.822**	0.757**	0.636**	0.526**	0.895**	-0.278**	0.230**	0.458**	0.375**	0.205**
	CA	1	0.853**	0.619**	0.578**	0.963**	-0.287**	0.341**	0.427**	0.314**	0.124
	MA		1	0.620**	0.502**	0.923**	-0.269**	0.386**	0.460**	0.331**	0.096
	TA			1	0.508**	0.720**	-0.403**	0.452**	0.208**	0.127	-0.109
	SA				1	0.649**	-0.337**	0.430**	0.154*	0.028	-0.106
	ToA					1	-0.330**	0.390**	0.449**	0.325**	0.105
Wuchang Lake	OA	0.827**	0.693**	0.788**	0.760**	0.881**	0.013	-0.209**	-0.228**	-0.332**	-0.557**
	CA	1	0.800**	0.827**	0.822**	0.947**	-0.135	-0.357**	-0.180*	-0.235**	-0.477**
	MA		1	0.913**	0.878**	0.913**	-0.196**	-0.457**	-0.342**	-0.415**	-0.337**
	TA			1	0.890**	0.946**	-0.185*	-0.367**	-0.317**	-0.416**	-0.388**
	SA				1	0.929**	-0.157*	-0.373**	-0.295**	-0.376**	-0.372**
	ToA					1	0.053	-0.379**	-0.278**	-0.363**	-0.469**

OA: oxalic acid; CA: citric acid; MA: malic acid; TA: tartaric acid; SA: succinic acid; ToA: total amount of organic acids; MC, soil moisture content; SOM, soil organic matter; TN, total nitrogen; TP, total phosphorus. ** indicates a significant difference at p<0.01; * indicates a significant difference at p < 0.05

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Discussion

The influence of lake types on the amount of organic acids

According to the different water level fluctuation patterns, the lakes in this study area could be divided into three types (Zhang, 2013). WCL is a quasi-natural fluctuating lake, which includes most lakes and some barrier lakes, and the water level fluctuations are similar to those of the Yangtze River. CHL is a reservoir-like fluctuating lake, which is mainly obstructed by other lakes and maintains a high water level all year round. The water level decreases due to irrigation or flood storage from April to June. PYL is an intermittent fluctuating lake, which is similar to floodplains and maintains stable low water levels most of the time, except in the high water level period at the Yangtze River trunk stream.

The composition and content of organic acids in the rhizosphere soil of the different lakes were different, and the annual average values of the ToA varied greatly. The annual average ToA in WCL was higher than that in PYL and CHL, which indicated that the carbon storage capacity in the rhizosphere soil of the quasi-natural lake wetland was higher than that in the intermittent lake and reservoir-like lake. The amount of organic acids in rhizosphere soils responded to the carbon storage and transformation capacities (Jones et al., 2009; Kuzyakov and Razavi, 2019). Due to the limited variations in the water level fluctuations and limited anthropogenic impacts, the carbon storage capacity in the rhizosphere soils of the WCL was higher than that in the CHL and PYL. CHL, as a reservoir-like lake, has a large amount of runoff, and the water that is stored in the lake is not enough to meet the demands because of the large amount of industrial and agricultural water consumption in the region, leading to the loss of carbon in rhizosphere soils. Hence, the annual average organic acid values were low in this lake. Furthermore, the dominant plant species and their distribution in the three lakes influenced the amount of organic acid in the rhizosphere. *Zizania latifolia*, as the dominant species in WCL, had the maximum biomass; *Carex sp*., as the dominant species in PYL had intermediate biomass, and the biomass of *Cynodon dactylon*, as the dominant species in CHL, was minimal (Zhang et al., 2018). The photosynthetically fixed carbon increased as the biomasses of these annual or biennial herbaceous plants increased. The carbon transformation increased from the aboveground parts (leaf, stem) to the belowground parts (root), resulting in an increase in root-exuded organic acids in the rhizosphere soils, which explained the higher ToA in the rhizosphere of quasi-natural lakes such as WCL than in the other two types of lakes.

The variational characteristics of the amount of organic acids under water level fluctuations in four seasons

The effects of seasonal variations on the amount of organic acids in the rhizosphere soil were evident, and the effects were irrelevant of the lake type. The amounts of organic acids were higher in summer and autumn than in spring and winter in the three lakes, resulting from the rapid growth and strong photosynthesis of wetland plants in summer and autumn, when plant roots exuded much more organic acids to rhizosphere soils. Due to the weak photosynthesis and low water level, the organic acid storage in the rhizosphere soil was low in winter (Wu and Zhao, 2013).

The amounts of organic acids at the sites with different water level fluctuations were different, and these were not related to the lake type. The amounts of ToA were higher at the sites with high water level fluctuations than that with low water level fluctuations.

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These differences not only were relevant to the above-mentioned factors, such as photosynthesis and biomass, but also may be related to other factors, such as the soil sample location and root morphology. Previous studies have shown that the root morphological parameters such as root length, root density, root volume, and root surface area at the sites with low water fluctuations were lower than those at sites with moderate and high water fluctuation levels (Zhang et al., 2018). These findings were consistent with the variation characteristics of organic acids in rhizosphere soils in this study. The root morphological parameters were positively correlated with the amount of organic acids in the rhizosphere soils of lakeshore plants. When the root morphological parameters were high, much more root organic acids were exuded into the rhizosphere soils.

The differences in dominant organic acids

The dominant types of organic acid were different in the three lakes. These were CA, MA and SA in PYL and CA and MA in CHL. In WCL, the distributions of the five organic acid contents were average, and only the CA content was higher in the high water level area. CA is the dominant organic acid among the three lakes in our study, which is the main organic acid in the Krebs cycle, not only accumulates in plant organs and tissue, but also is released into the rhizosphere via root exudates (Sweetlove et al., 2010; Araújo et al., 2012; Yu et al., 2017).

The correlation between the amount of organic acids and the soil physicochemical parameters

There were significant positive correlations among the amounts of organic acids in the three lakes, which indicated that a single organic acid was not exuded into soils, while multiple organic acids exuded simultaneously into the rhizosphere soil through the plasma membrane via diffusion and anion channels (Ryan et al., 2001; Adeleke et al., 2017; Silva and Lambers, 2020). There was a negative correlation between the amount of organic acids and pH, which indicated that an increase in the amount of organic acids led to an increase in H^+ release, decreasing the pH; however, this condition did not change the adsorption sites. MC and SOM had the same influences on the amount of organic acids, which included no influence on the amount of organic acids in PYL, a positive influence in CHL, and a negative influence in CHL because of the different heights of the water level fluctuations. TN and TP, as indicators of pollution sources, had limited impacts on the release of organic acids in PYL and CHL because the two lakes were greatly affected by human activities and exhibited low release of organic acids. TN and TP exhibited significant negative correlations with the amount of organic acids in the rhizosphere soil of WCL, which indicated that an increase in the exogenous input of nitrogen and phosphorus decreased the amount of organic acids in the rhizosphere soil. Thus, WCL, as a quasi-natural lake, was greatly influenced by human activities, and this lake may be converted to another type of lake and lose the function of the natural wetlands.

Conclusion

The organic acids of rhizosphere soil in different types of lakes were influenced by many factors, such as season variations, water levels, vegetations and the soil

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environment. The annual average amount of the total organic acids in the rhizosphere soil in the quasi-natural WCL was higher than that in the intermittent PYL and reservoir-like CHL, which was related to the vegetation density, photosynthesis and root morphological parameters of WCL. The seasonal differences in the amounts of organic acids were independent of lake type, and the amount of organic acids in the three lakes in winter was lower than that in the other three seasons. The dominant organic acids were different, and CA was the dominant organic acid in the three lakes. The CA accumulation in plant tissue can promote the release into rhizosphere soil. There were positive correlations among the amounts of organic acids in the three lakes. pH exhibited a negative correlation with the amount of organic acids. MC and SOM had no effect on the amount of organic acids in PYL, a positive effect in CHL and a negative effect in WCL. TN and TP had a greater impact on the amount of organic acids in WCL than in PYL and CHL, which would lead to changes to WCL as a result of anthropogenic influence.

In future studies, it will be necessary to consider the following two points: first, quantitatively analyze the organic acids characteristics in plant-rhizosphere soil- aquatic environment, the carbon distribution and transfer of organic acids should be focused via stable carbon isotope tracer technology. Second, in order to better understand the function of organic acid in plants especially root-exuded organic acids, the heavy metal concentration, nutrition such as phosphorus and nitrogen, and microbial activities should be investigated in rhizosphere soil-aquatic environment, and discuss the influence of anthropogenic intake pollution source.

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APPLICATION OF SPATIAL REGRESSION MODELS FOR FOREST BIOMASS ESTIMATION IN GUIZHOU PROVINCE, SOUTHWEST CHINA

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Abstract. At the regional scale, many studies have been devoted to the construction of biomass models to improve the accuracy of regional biomass estimation, while only a few studies were carried out to investigate spatial influence. Therefore, the current research examined the spatial autocorrelations as well as variations between forest biomass and forest variables using 419 forest biomass plots sampled in the Guizhou Province in the year 2010. Besides, 4 global models, including the ordinary least squares model (OLS), linear mixed model (LMM), spatial lag model (SLM), spatial error models (SEM), together with geographically weighted regression model (GWR, the local model), were fitted to the associations of forest biomass with basal area, height and age of the stand. As suggested by our findings, distinct spatial autocorrelations as well as variations exist between forest biomass and these variables. OLS is not appropriate for modeling. SLM and SEM efficiently accounted for the spatial autocorrelations within model residual; however, they were unable to manage spatial heterogeneities. However, LMM and GWR, which had combined spatial variations as well as dependence during the modeling process, performed well in data fitting and response variable predicting. Of them, GWR reduced spatial heterogeneity to a greater extent than LMM.
Keywords: *linear mixed model, spatial lag model, spatial error model, geographically weighted regression*

Introduction

It is of crucial importance to estimate the forest biomass to understand source dynamics and atmospheric carbon sinks (Brown and Lugo, 1984). To be specific, forest biomass, particularly that is located in the subtropics, accounts for a leading global carbon emission source. In order to evaluate the ecological quality and the effectiveness of forestry construction and to provide decision-making for promoting rational use of energy and forest management, the large-scale study of forest biomass and its spatial distribution is necessary (Rodríguez-Veiga et al., 2016).

Forest biomass has been frequently predicted based on the timber volume data extracted via the forest inventories, for which, field plots were used for statistical sample collection to directly measure the forest parameters (such as breast height diameter, height, as well as tree species). Forest biomass estimation methods are continuously improving with the advancement of research. Thereafter, timber volume is converted into the above-ground biomass through the application of the biomass expansion factor (BEF) where timber volume information is obtained (Brown and Lugo, 1984). Fang et al. (2001) improved the BEF method for forest biomass estimation overcoming the shortcoming of static conversion parameters of the BEF method improving the biomass-volume model and providing a more accurate calculation of the parameters for various dominant tree species. Remote sensing methods, often used to

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estimate large-scale forest parameters (Patenaude et al., 2005; Ploton et al., 2017), can hardly accurately estimate spatial distributions due to the limited sensitivity of the selected satellite sensors to forest parameters (Benson et al., 2016; Ghosh et al., 2018). Different technical means and auxiliary data were employed to improve the estimation precision. Remote sensing methods also use process models, which have a perfect theoretical foundation and clear physical meaning; however, they require more complex data sources as input parameters (Chiesi et al., 2011).

As we all know, forest biomass information is usually extracted across the huge geographic areas, which leads to distinct differences of topographic characteristic, species abundance and compositions, as well as vegetation coverage; as a result, the carbon storage and forest biomass among the different locations are also different (Liu et al., 2014). The neighbor locations are similar to each other, but the remote locations are not similar. This can be explained by two aspects of spatial effects, spatial heterogeneity (non-stationarity of space), together with spatial autocorrelation (namely, spatial dependence) (Anselin and Griffith, 1988). Of them, spatial heterogeneity represents the instability of structure that manifests as the systemically altering model variables or the diverse response functions. Meanwhile, spatial autocorrelations are those correlations of the random variable value in a region with identical variable values in adjacent regions. Ignoring spatial heterogeneity and spatial autocorrelation lead to false significance test results, inferior predictions (Anselin and Griffith, 1988), as well as damaging influence on modeling and data analytic results (Páez and Scott, 2005). In recent years, the indexes of global and local spatial autocorrelations, such as Geary's C, Moran's I, Getis'G*, and Getis'G, are extensively utilized for measuring spatial autocorrelation degrees across different regions (Boots, 2002; Páez and Scott, 2005; Fu et al., 2014). Several statistical regression approaches have been adopted in incorporating the spatial autocorrelations to model the associations across variables, including the spatial error model (Lichstein et al., 2002), spatial lag model (Anselin, 1993), spatial Durbin model (Overmars et al., 2003), spatial filter model (Borcard and Legendre, 2002), and linear mixed model (Zhang et al., 2009), and others (Dormann et al., 2010). Spatial heterogeneity represents the function of spatial scales that are referred to as the measuring units. A variety of regression approaches are developed for modeling the local alterations in the complicated associations of spatial random variables, including random coefficient model (Fotheringham and Brunsdon, 1999), spatial expansion approach (Anselin, 1992), multilevel modeling approach (Duncan, 1997; Jones1997), and spatial adaptive filtering approach (Gorr and Olligschlaeger, 1994). Recently, the geographically weighted regression (GWR) is increasingly used for exploring spatial heterogeneities (Fotheringham et al., 2002; Zhang et al., 2004; Liu et al., 2014).

The GWR is the local regression model that can effectively solve spatial nonstationarity. The parameters of each sample are estimated by using the locally weighted least square method and the spatial location is taken into account in the estimation process. In other words, GWR offers diverse regression modeling parameters of a localized model in every region to incorporate the locoregional spatial variation into the modeling processes. Therefore, it is becoming an important method to detect the non-stationarity of space and has been showing better performance than ordinary models when applied to many fields such as social economics (Öcal and Yildirim, 2010; Wang et al., 2019), geography (Erdoğan, 2010), soil (Song et al., 2019; Li et al., 2020), environment (Qin et al., 2019) and forestry (Propastin, 2012; Lin et al., 2018; Monjarás-Vega et al., 2020). Consequently, in the forestry fields, the influences of vegetation

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competition, micro-environment, and growth potential; besides, effects of managing activities on carbon storage and forest biomass are assessed, analyzed, simulated and visualized (Liu et al., 2014; Zhen et al., 2013), while the study about spatial influence in modeling forest parameters is still rare, especially in regional scale (Liu et al., 2014).

The current research analyzed forest biomass for its spatial distribution in the Guizhou Province of southwest China. Typically, this study aimed to achieve the following objectives: (1) to use five models, including the ordinary least squares (OLS) model, the spatial lag model (SLM), the spatial error model (SEM), the linear mixed model (LMM), together with the geographically weighted regression model (GWR) fitting forest biomass information; (2) to analyze and compare these five models for their performances; and (3) to evaluate model residues for spatial autocorrelation as well as spatial heterogeneity.

Materials and methods

Study area

Guizhou Province (103°36′E to 109°35′E and 24°37′N to 29°13′N) is located in southwest China (*Fig. 1*) with a total land area of 176128 km^2 and 57% forest coverage rate. 92.5% of the whole province is mountainous and hilly, with an average altitude of 1100 m. This area is of subtropical monsoon climate. Affected by mountainous terrain, the weather conditions are variable. The average annual temperature is 10~18 ℃, the annual precipitation is 1,000~1,500 mm, the relative humidity is above 70%, the annual sunshine hours are 1300 h, and the frost-free period is about 270 d (Editorial Committee of Guizhou Forest, 1991). Affected by climate, soil and mountainous terrain, the vegetation types in the province are diverse. The central and northern parts of the country are dominated by mid-subtropical evergreen broad-leaved forests, while the southern part is a south-subtropical evergreen broad-leaved forest. The middle-eastern part is humid forest and the western part is semi-humid forest. Cold and warm sub alpine coniferous forests are distributed in high altitude areas, while intrazonal karst evergreen deciduous broad-leaved mixed forest and secondary deciduous broad-leaved forest are distributed in limestone and dolomite mountains (Editorial Committee of Guizhou Forest, 1991).

According to the forest management plan of Guizhou province (2016-2050) and our study aims, the study area was divided into six ecological geographical sub-regions. The details are presented in *Table 1* and *Figure 1*.

Table 1. *The six ecological geographical sub-regions of Guizhou province*

Code of sub-regions	Name of sub-regions
A	The low-middle mountain sub-region of *Pinus massoniana*, bamboo and broad-leaved mixed forest for soil and water conservation
B	The low-middle mountain sub-region of *Pinus massoniana* and broad-leaved forest for dual-purpose of precious large-diameter timber
C	The high-middle mountain sub-region of *Pinus yunnanensis, Pinus armandii, Cryptomeria fortune* and broad-leaved mixed forest for soil and water conservation
D	The middle mountain sub-region of *Pinus massoniana*, broad-leaved mixed forest for special purpose
E	The low mountain sub-region of *Cryptomeria fortune* forest for fast-growing and high-yielding timber
F	The middle mountain sub-region of *Cryptomeria fortune, Pinus yunnanensis* and broad-leaved forest for dual-purpose of precious large-diameter timber

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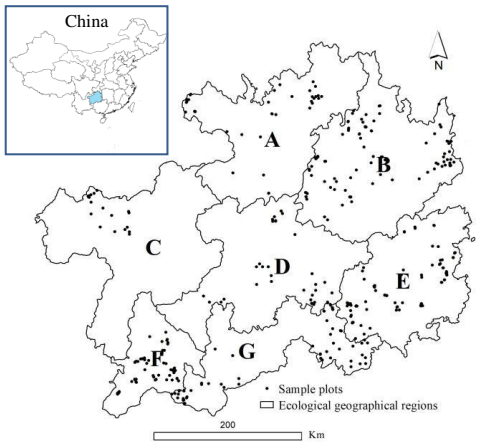

Figure 1. *Geographical location of the six regions with the number of sample plots of the study area in Guizhou, China. A, B, C, D, E and F are the code of sub-regions corresponding to name of sub-regions respectively described in Table 1*

Data

For the current research, the utilized stand as well as tree data were extracted based on 6 locations in the year 2010 from 419 permanent sample plots, including 283 Arbor plots (666.67 m^2 per plot), 51 bamboo plots (100 m^2 per plot), and 85 shrub plots (16 m^2 per plot), observed via the Chinese National Forest Inventory (CNFI), together with the Guizhou Provincial Forestry Department. The topographical descriptors and geographical locations were also collected for every plot. In addition, the stand as well as tree parameters were determined and analyzed for describing the slope (°), elevation (m), numbers of tree species and trees of every species, as well as diameter at breast height in cm (DBH, > 2 cm for bamboo and shrub plots, > 5 cm for Arbor plots), and living tree height in m (HT). In addition, additional stands as well as tree variables were subsequently calculated based on unit hectare, which included the tree number per hectare (TPH) (trees/hm^2), the living tree volume per hectare (m^3/hm^2), the mean living tree age (year), the basal area of trees per hectare (BA, the accumulated area in cross-section determined based on the breast height (1.3 m) for each tree within one stand) (m^3/hm^2), the average height of living trees (H), and vegetation coverage (%). The 1-year meteorological data were extracted based on 77 weather stations Guizhou Province in the year 2010 and Kriging interpolation was conducted with the data for obtaining the precipitation and temperature information for each sample plot. In each arbor plot, the biomass models based on the Guizhou province universal biomass equation were used (Zeng et al., 2011). The tree species, without a clear corresponding model, were referred to as the approximate dominant tree species (group) parameters. In each Bamboo plot, the biomass models were calculated according to Tian (2011) and that of the shrub plot was calculated according to Liu et al. (2009).

Theoretical background

Ordinary least squares (OLS)

Assume there are diverse *n* observations regarding the response variable y, together with *p* predictor variables x. The association of y with x is regressed based on OLS (*Eq. 1*):

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$$y = x\beta + \varepsilon \quad \text{(Eq.1)}$$

where, β stands for the unclear fixed-effects parameter vector, whereas ε indicates the model error term following a normal distribution N (0, σ^2). Thus, the OLS estimator is acquired through (*Eq. 2;* Littell et al., 2006)

$$\hat{\beta} = (X'X)^{-1}X'Y \quad \text{(Eq.2)}$$

It is supposed that, the association given based on *Equation 1* is constant or universal within the geographical area.

Linear mixed model (LMM)

The LMM represents a special case of generalized linear models, which is presented in the form of *Equation 3:*

$$y = X\beta + Z\gamma + \varepsilon \quad \text{(Eq.3)}$$

in the formula, y stands for the response variable vector, x represents the fixed-effects predictor matrix, β indicates the unclear fixed-effects model coefficient vector, whereas Z suggests the given random-effects design matrix, γ represents the unclear random-effects parameter vector, while ε stands for a random error term. The following assumptions are given: (1) E (γ) = 0 and Var (γ) = G represents the random-effects covariance matrix; (2) E (ε) = 0 and Var (ε) = R suggests the model residual covariance matrix; (3) Cov (γ, ε) = 0; meanwhile, (4) γ and ε show normal distribution. Besides, variance y is calculated based on V = ZGZ′ + R, which is predicted through establishing the design matrix Z of random-effects and indicating the G and R covariance structures. Generally speaking, OLS has not been deemed as the optimal parameter prediction approach, whereas the maximum likelihood approaches are frequently adopted for obtaining γ and β (Littell et al., 2006).

Spatial lag model (SLM)

The SLM represents the formal spatial diffusion procedure, which obtains great spatial data dependences (Anselin, 1993, 2001). Generally, SLM is appropriate for assessing the spatial dependence presence and strength. To be specific, SLM is completed through incorporating one spatial lag term for dependent variable y to that OLS model (*Eq. 1*) mentioned above, as shown below (*Eq. 4*):

$$y = X\beta + \rho Wy + \varepsilon = (1 - \rho W)^{-1}X\beta + (1 - \rho W)^{-1}\varepsilon \quad \text{(Eq.4)}$$

where *W* represents the row-sum weight matrix after standardization processing, *W*y stands for the response variable that is lagged spatially, ρ indicates the spatial autocorrelation variable with normal distribution N (0, $\sigma^2 I$), and *I* denotes the identity matrix. For response y, its value in every region depends on the x in a specific region and in adjacent regions based on spatial multiplier $(1-\rho W)^{-1}$. It should be noted that, according to *Equation 1,* ε becomes correlated with predictor variable (namely, the spatially lagged *W*y). As OLS is not an appropriate parameter prediction method any

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more, since it will produce ineffective and biased predictions, while the maximum likelihood approach is adopted.

Spatial error model (SEM)

It is assumed in SEM, that spatial autoregressive procedure takes place within error term only, rather than within response or predictor variables (Anselin, 1993, 2001). This is because that, the variables with spatial correlation or spatial region boundaries that do not coincide with the practical behavior units are eliminated (Graaff et al., 2001). SEM is recognized to be the special regression using one non-spherical error term, and this approach is suitable to examine the possible spatial autocorrelation effect induced by using the spatial data, regardless of the model spatiality. SEM combines OLS regression model with the spatial autoregression model within an error term ε, as shown below (*Eq. 5*):

$$y = X\beta + \varepsilon = X\beta + \lambda W\varepsilon + \xi = X\beta + (1 - \lambda W)^{-1}\xi \quad \text{(Eq.5)}$$

In the formula, W stands for the row-sum weight matrix after standardization, $W\varepsilon$ represents the error term with spatial lag, λ indicates the spatial autocorrelation variable, whereas ξ suggests the ordered error term with normal distribution N (0, $\sigma^2 I$), and I denotes the identity matrix. For y, its value in every region is subjected to influences of error in every region based on spatial multiplier $(1-\lambda W)^{-1}$. Nonetheless, different from β, λ has been identified to be the nuisance variable, and this factor itself is not the focus, yet it is of necessity for correcting spatial dependencies. For y, its average value is independent from error spatial dependence, and that maximum likelihood approach has been used to estimate the parameters.

Geographically weighted regression (GWR)

The GWR is extended based on the conventional regression that allows variations, in this way, the regression coefficient is location-specific, and does not represent a universal estimate (Fotheringham and Brunsdon, 1999). The possible GWR model is shown below (*Eq. 6*):

$$y = \beta_0(u_i, v_i) + \sum_{k=1}^{p} \beta_k(u_i, v_i) X_k + \varepsilon \quad \text{(Eq.6)}$$

In the formula, y stands for a response variable, whereas X_k represents diverse p predictor variables (k = 1, 2, …, p), and β_0 (ui, vi), β_1 (ui, vi), …, β_p (ui, vi) suggests those regression coefficients for the kth predictor variable and ith location. εi stands for a random error term with normal distribution N (0,$\sigma^2 I$), and I denotes the identity matrix. Generally, GWR aims to estimate the above-mentioned coefficients for every independent variable x in every geographical region i using neighbors within a given bandwidth and weighted least-squares regression. Those GWR model parameters in every region i in the matrix are predicted according to the following formula (*Eq. 7*):

$$\hat{\beta} = (X'W_iX)^{-1}X'W_iY \quad \text{(Eq.7)}$$

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where W_i stands for the (m × m) diagonal matrix of spatial weights; X indicates the [m × (n + 1)] matrix of independent parameters, in which n is the explanatory variable number; whereas Y represents the (m × 1) matrix of dependent variables.

The weighted function is used to determine the weighted approach, and it is adaptive or fixed (Mcmillen, 2004). With regard to the size of adaptive kernel, every point weight is determined based on the Gaussian function (*Eq. 8*):

$$W_{ij} = \exp(-\frac{1}{2}(\frac{d_{ij}}{r})^2) \quad \text{(Eq.8)}$$

In the formula, d_{ij} stands for the Euclidean distance of an estimated site i compared with a sampling site j, r stands for the parameter of bandwidth. Notably, the selection of bandwidth within the as-mentioned GWR model represents a critical factor that affects the results of regression analysis. At present, both the corrected Akaike information criterion (AICc) and cross-validation approaches have been extensively utilized for determining bandwidth.

Model specification

The current research aimed was to examine forest biomass for its spatial distributions as well as patterns using the regression models. In every plot, forest biomass was used as the dependent response parameter (ton/hm^2). The stand as well as tree parameter number was analyzed and screened through gradual regression to be model predictors, including the tree basal area per hectare (BA), stand age (Year) and stand height (m). *Table 2* shows the baseline variable data.

Table 2. *Baseline variable data adopted in the current research*

Variable	Plot number	Average	Maximum	Minimum	Std
Biomass (t/hm^2)	419	51.25	261.57	0.01	47.25
Basal area (BA, m^2/hm^2)	419	161962.70	772947.11	477.59	126107.37
Age (Year)	419	20.83	98.00	0.00	15.06
Height (m)	419	8.79	25.00	0.24	5.44

The multiple linear model shown below was adopted for regressing the forest biomass relative to 3 estimators (Basal area (BA)), stand age and stand height) using OLS, SLM, SEM, LMM as well as GWR models:

$$Biomass = \beta_0 + \beta_1 \cdot BA + \beta_2 \cdot Age + \beta_3 \cdot Height + \varepsilon \quad \text{(Eq.9)}$$

where β_0, β_1, β_2, and β_3 stand for the regression coefficients predicted based on statistics, whereas ε is model residual that represents the heterogeneity of observed forest biomass compared with the predicted one. OLS model was adopted to be the model comparison benchmark in the current research. In LMM model, seven regions were used to be fixed effects, and covariance matrix R was used to model those spatial autocorrelations across various sample plots in those seven regions. For both SLM and SEM models, distance bandwidths performed better than k-nearest neighbors as spatial weights. For the GWR model, the best bandwidth size was 30 km determined according to the golden section

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search (automated) and adaptive bisque kernel function, because it has a higher coefficient of determination (R^2) and lower residuals than other methods. Meanwhile, for those five models, model residuals were determined based on global Moran's Z- and I-values, with the bandwidths range of 5-45 km at an interval of 5 km, which described the pooled spatial autocorrelations within those model residuals at a variety of spatial scales. Specifically, local Moran's I-value, an index for local spatial autocorrelation (LISA), was calculated according to the best bandwidth (30 km) in every model residual for OLS, LMM, as well as GWR (Boots, 2002). Meanwhile, Z-value is used as the standard deviation (SD). At a significant level of $\alpha = 0.05$, $Z > 1.96$ and $Z < -1.96$ indicate that the model residual has significant spatial autocorrelation, and $-1.96 < Z < 1.96$ indicates otherwise. Intra-block spatial variances, illustrating the local spatial variability, were computed for the above-mentioned five model residuals, and the block size was 5-30 km at an interval of 5 km (Zhang et al., 2009). It is assessed based on the fact that 1/2 GWR coefficients should fall in the range of Q1 (25% quartile) to Q3 (75% quartile), whereas approximately 68% normally distributed LMM or OLS coefficients must fall in the range of 1 SD. The studied association might be not fixed spatially when inter-quartile range was > 1 SD of an equal global variable for the GWR local coefficients (Fotheringham et al., 2002; Zhang et al., 2004).

In this research, we aimed to examine model errors for their spatial heterogeneity and autocorrelation using the above 5 regression methods to fit the associations of biomass with relevant variables, but not to develop a predictive regional forest biomass model. Then, Akaike's Information Criterion (AIC), R^2, and mean squared error (MSE) summation were adopted to evaluate the pooled model fitting effect. Moreover, the LMM and OLS models were fitted using SAS 9.3 (SAS Institute Inc., 2011). In addition, SLM and SEM were fitted using GeoDa software (Anselin et al., 2006; GeoDa Center for Geospatial Analysis and Computing, 2009), while GWR 4.0 software (Nakaya et al., 2009; Department of Geography, Ritsumeikan University, Kyoto, Japan) was used to fit GWR model.

Results

Model fitting

The model fitting data of those five regression models adopted in this study are displayed in *Table 3*. As observed, OLS model had good data fitting effect (R^2 was 0.91) when not considering the independence assumption violation. With regard to SLM model, it adopted those spatially lagged dependent variables to be the predicting variables, so as to calculate spatial autocorrelation; meanwhile, SEM model managed spatial autocorrelation within a model error term (residuals). Besides, SLM and SEM performed better in data fitting (larger R^2, smaller MSE, smaller AIC, and lower global Moran's I (Z)) than the OLS models. For SME model, those tested values [Lagrange multiplier (LM) (error), as well as robust LM (error)] were relatively high relative to those for SLM model, indicating that the SEM model was better (*Table 4*).

According to *Table 3*, LMM performed well in data fitting (higher R^2, lower MSE, lower AIC, and lower global Moran's I (Z)) than OLS, SLM, and SEM. However, the best fit was observed for the GWR model that had greater ^{R2}, smaller MSE, lower AIC, and lower global Moran's I (Z) and SH%. As suggested by Z-values, for SLM and OLS models, their model residuals displayed distinct spatial autocorrelations ($Z > 1.96$, $\alpha = 0.05$). Nonetheless, for SEM, LMM and GWR models, their model residuals

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displayed no distinct autocorrelations ($-1.96 < Z < 1.96$, at $\alpha = 0.05$) indicating a reduction in the spatial autocorrelation.

Table 5 lists the model coefficient estimates, standard errors (SE), as well as p-values for three predicting factors. Each model coefficient was deemed to be of statistical significance upon $\alpha = 0.05$ in all the five models. For local models, their means for 2 regression coefficients were similar to those for global counterparts (*Table 5*). In addition, the inter-quartile range for GWR model, local intercepts, BA coefficients, age coefficients and height coefficients did not fall within the scope of ± 1 SD of those other four models, indicating the non-stationary and/or different association of forest biomass with those 5 predicting factors. However, the GWR model generated the geographically different model coefficients (a model coefficient set was generated in every region). *Figure 2* illustrates the spatial variations for GWR model coefficients, which shows that the association of forest biomass with those three predicting factors are different at different study locations. Generally speaking, local intercepts were negative over the study area, Age coefficients were positive across our study locations, whereas age and height coefficients were either positive or negative based on different regions. Local intercepts were smaller within the eastern study locations while larger across the northern and southern study locations. BA coefficient was opposite to that of local intercepts, larger in the east, but smaller in north and west. The values of age coefficients were smaller in west and south, and larger in north and east, while age coefficients were smaller in north and south, and larger in the central region. As demonstrated from *Figure 2*, those magnitudes and signs for such regression coefficients depended on the geographical location, suggesting the region-specific influences of those three tree predictors on the forest biomass. The coefficient shows a positive value indicating a positive impact on forest aboveground biomass, otherwise it is a negative contribution. The trend between coefficients distribution was similar indicating their impacts on estimating forest aboveground biomass have regional similarity, and vice versa.

Spatial autocorrelation and heterogeneity in model residuals

Compared with OLS model, the rest four models generated markedly lower global Moran's *I*- values (*Table 5*). Generally, GWR model generated negative values, while SLM, LMM and SEM generated positive values. *Figure 3* shows spatial correlation diagram for model residuals of those five models, with the lag distance ranging from 5 to 45 km. For OLS model, its residuals displayed great spatial autocorrelation compared with those for other models at every tested lag distance. For SLM model, its residuals behaved similar to that of the OLS model but had smaller values across the lag distances (*Fig. 3*). The LMM and SEM models generated similar Moran's *I* values (<0.05) among various lag distances. For GWR model, its model residual showed no significant spatial autocorrelation (Z-value < 1.96) across the range of lag distances and approached zero especially at larger spatial scales (15–45 km).

Figure 3 also shows the intra-block within model residuals representing mean local spatial variation of a known block size. As observed, OLS, SLM, as well as SEM models had remarkably greater and relatively the same intra-block sizes. The GWR had the smallest intra-block local spatial variance among the various block sizes, and then LMM model ranked the second place. Nonetheless, the different intra-block variability across those five models were low at the block size interval of 5 km. It became greater and approached one stable value with the increase in block size.

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Table 3. *Model fitting statistics for the five regression models and measures of spatial autocorrelation and heterogeneity for the model errors*

Models	AIC	MSE	R^2	Moran's I (Z)
OLS	3244.32	55486.60	0.91	0.21(5.33278)
SLM	3235.55	54063.50	0.94	0.15(3.768887)
SEM	3215.54	50833.67	0.95	0.037(1.018091)
LMM	3169.11	43422.45	0.95	0.064(1.720469)
GWR	3103.43	28780.41	0.96	-0.076(-1.908456)

Table 4. *Spatial dependence diagnostics for the OLS model residuals*

Test statistics	Value	P
Moran's I	0.106588	0
Lagrange Multiplier (lag)	1	0.0005686
Robust LM (lag)	1	0.0215412
Lagrange Multiplier (error)	1	0
Robust LM (error)	1	0

Table 5. *Model coefficient estimates, standard error (SE), and p-values of the five models*

Model	Statistics	$\widehat{\beta}_0$	$\widehat{\beta}_1$	$\widehat{\beta}_2$	$\widehat{\beta}_3$	Spatial parameter
OLS	Estimate (SE)	-15.8505(1.1568)	$3.24\times10^{-4}(5.7\times10^{-6})$	0.3102(0.0439)	0.9348(0.1486)	
	β−1·S.D~β + 1·S.D	-16.6371~-15.0639	3.20×10^{-4}~3.28×10^{-4}	0.2803~0.3400	0.8338~1.0358	
SLM	Estimate (SE)	-17.9163(1.3020)	$3.15\times10^{-4}(6.2\times10^{-6})$	0.3320(0.0440)	0.8063(0.1529)	$\hat{\rho}$ = 0.0813
	β−1·S.D~β + 1·S.D	-18.8017~-17.0309	3.11×10^{-4}~3.19×10^{-4}	0.3020~0.3619	0.7023~0.9103	
SEM	Estimate (SE)	-14.7527(1.6506)	$3.17\times10^{-4}(5.9\times10^{-6})$	0.2290(0.0480)	1.1458(0.1593)	$\hat{\lambda}$ = 0.5569
	β−1·S.D~β + 1·S.D	-15.8751~-13.6303	3.13×10^{-4}~3.21×10^{-4}	0.1964~0.2616	1.0375~1.2541	
LMM	Estimate (SE)	-14.0511(2.0003)	$3.10\times10^{-4}(1.27\times10^{-5})$	0.2865(0.0656)	0.9334(0.1769)	
	β−1·S.D~β + 1·S.D	-15.4113~-12.6909	3.01×10^{-4}~3.19×10^{-4}	0.2419~0.3311	0.8131~1.0537	
GWR	Estimate (SE)	-14.3394(-29.6306~-5.1777)	$3.01\times10^{-4}(1.91\times10^{-4}$~$4.23\times10^{-4})$	0.2928(0.0054~0.8564)	1.003(-0.4457~2.6462)	
	Q1~Q3	-19.6553~-8.5991	2.66×10^{-4}~3.33×10^{-4}	0.1541~0.3795	0.4845~1.344	

Numbers in parentheses are the standard errors of the regression coefficients for OLS, SLM, SEM, and LMM and the ranges of the coefficients for GWR models. $\widehat{\beta}_0$ for Intercept, $\widehat{\beta}_1$ for Basel area, $\widehat{\beta}_2$ for Average age, $\widehat{\beta}_3$ for Average height, the same as follow. Q1 and Q3 represents 25% quartile and 75% quartile of GWR coefficients, respectively

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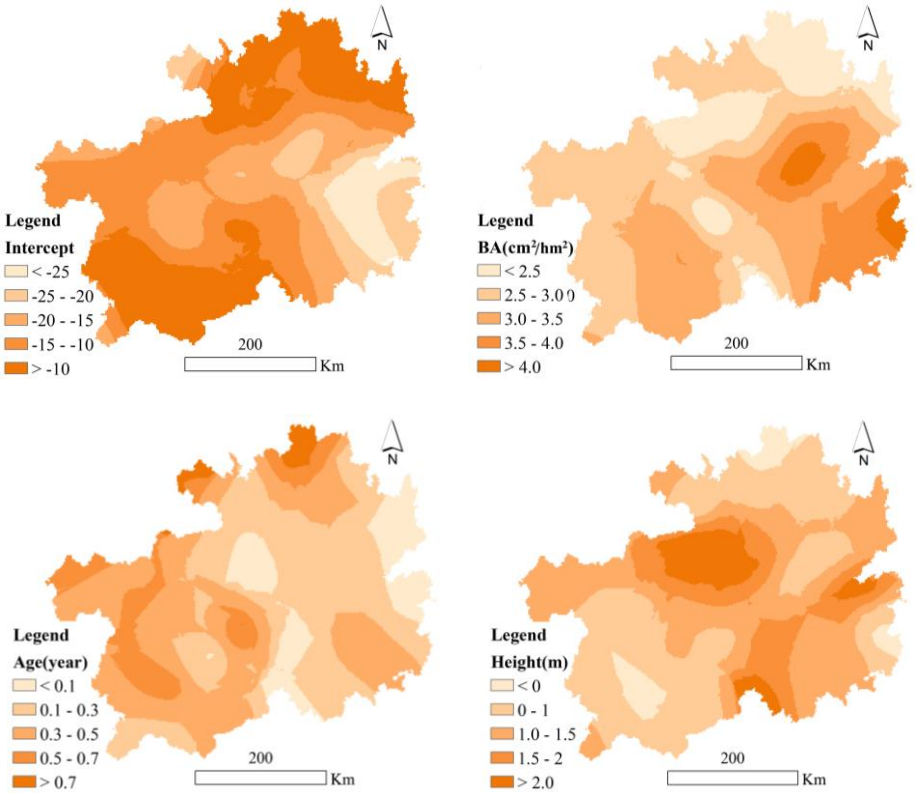

Figure 2. *Contour maps of the model coefficient estimates from the GWR model at the bandwidth 30 km.* $\hat{\beta}_0$ *for Intercept,* $\hat{\beta}_1$ *for BA,* $\hat{\beta}_2$ *for Age,* $\hat{\beta}_3$ *for height*

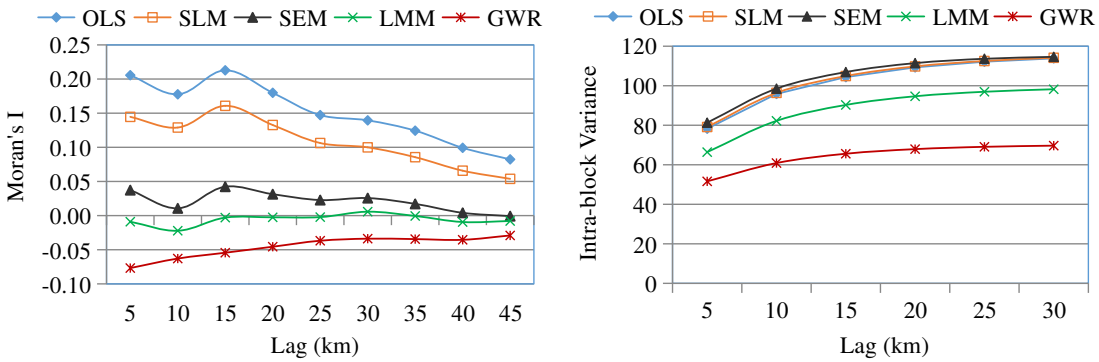

Figure 3. *Correlogram and intrablock variance of the five model residuals*

To illustrate the spatial details of residual autocorrelations, distributions of the Local Moran's *I* at 30 km spatial scales were computed for residuals from each of the five models (*Fig. 4*). It was observed that the local Moran's *I* values of OLS model residual were greater, which were positive (black circles) among the study region, which indicated that OLS model under-predicted or over-predicted the clusters (hotspots). SLM had similar spatial patterns for those 3 plots, and the local Moran's *I* values were greater and more positive (black dots), which indicated that the negative or positive model residuals were clustered. SEM produced few hot spots compared with those of SLM and OLS models. In contrast, the LMM and GWR models produced lower values,

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which were more negative (white circles), which indicated the opposite signs of model residuals were clustered (cold spots).

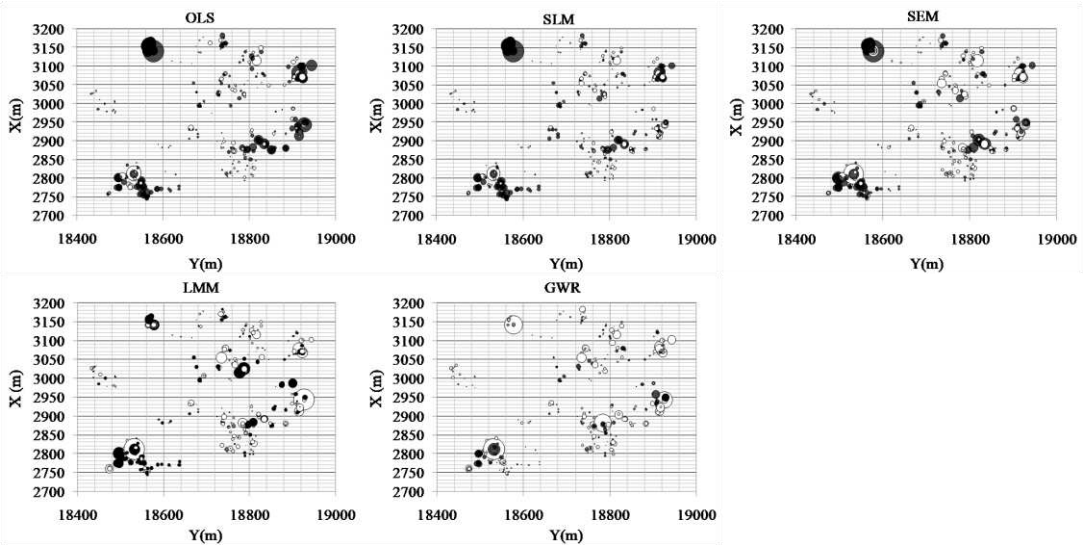

Figure 4. *Local Moran's I value distributions at 30 km spatial scales of the five model residuals. Black circles indicate positive values of local Moran's I with cluster "hot spots", while white circles indicate negative values of the local Moran's I with cluster "cold spots".*

Discussion

Model variables and coefficients

In our study, three variables, basal area, stand age, stand height were selected to estimate forest biomass. They all had statistically significant effects on the amount and distribution of forest biomass. In both global and local models, the three variables were the most important stand variables for our study area. The model coefficients of the three variables were all positive (*Table 4*), indicating that larger basal area, stand age and stand height would sequester more biomass in the forests, which is consistent with most studies (Mani and Parthasarthy, 2007; Cannell, 1984; Fang, 2001).

Other factors, including the temperature and precipitation data, were also considered as variables, but their influence was not significant. Our study also showed that the relationship between basal area and forest biomass was better than the relationship between DBH (diameter at breast height) and forest biomass. That is basal area explained 85% of the model, age explained 3%, height explained 3% (data not shown). Brown (2002) found that DBH alone explained more than 95% of the variation in above ground carbon content in tropic forests. Liu et al. (2014) also selected DBH as the main variable when estimating forest carbon stock of north forests in Heilongjiang province. However, Basal area has been used more frequently as a surrogate for biomass and carbon in tropical forests (Mani and Parthasarthy, 2007; Sagar and Singh, 2006), and often has been found to be the best predictor of biomass in combination with the mean tree height at the stand level (Cannell, 1984; Masera et al., 1997). Fang (2001) improved BEF for forest biomass estimation, which using only a simple linear relationship between biomass and volume when taking into account the influence of forest age, because other factors, site conditions, climate factors, etc., are already included in the volume. Basal area, stand age, stand height used in our study is the

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explanation of this information including density, forest age, and site conditions. In many studies, shrubbery and bamboo plots often not be included or be estimated using other methods instead of BEF when forest biomass is estimated (Guo et al., 2013). Owing to the investigation standards of shrubbery and bamboo plots are different from those of arbor forests, for their starting DBH usually lower. And forest types and tree species in Guizhou province are diverse. At present, the volume equation of over 30 tree species including *Pinus massoniana*, *Cunninghamia lanceolata*, *Pinus yunnanensis* and *Pinus armandii* and so on have been established, while there is no corresponding volume equation for other tree species whose volume equation have not been established. And the volume equation also varies with the region. To avoid the impact of volume calculation errors on biomass modeling, this study established the relationship between three variables, namely basal area, stand age and stand height, with forest biomass, which not only avoided the trouble of establishing biomass according to different tree species, but these three variables also reflected the factors of volume, environment and other factors of the sample plot. In addition, the purpose of this study is to attach more importance to reveal the influence of spatial correlation and heterogeneity of biomass distribution on model fitting than the biomass model itself. Facts have proved that the three variables, basal area, stand age, and stand height are feasible for the estimation of regional forest biomass, and the model has a high explanatory degree with R^2 of the least square model reaches 91%.

OLS, SLM, SEM, and LMM global models remained unchanged among the studied regions, however, their coefficients were insufficient for the accurate description of three predicting factor effects on the forest biomass across various local regions. To be specific, GWR model offered the local model coefficients, and they were observed by the GIS technique to observe more details about on the association of forest biomass with the predicting factors (*Fig. 2*). Clearly, those predicting factors had different influences on forest biomass, depending on the different locations. For GWR model, its local model coefficients offered more details about the effects of micro-site variation and managing measures on forest biomass and tree growth. It would help to plan the management and make a decision (Lu and Zhang, 2012; Zhen et al*.,* 2013). In view of the above analysis, GWR can be combined with remote sensing data to improve the accuracy of traditional models for estimating forest parameters, and in addition, the forest parameters can be further predicted and validated through each Reflectivity Pixels from remote sensing model and remote sensing data can be used to predict the spatial distribution of and a relatively more accurate spatial distribution map can be draw (Propastin, 2012; Chen, 2012). However, the distance between sampling points, the number of samples could influence the estimation of GWR model, and even the influence of outliers, weak data problem and even lack of independence weaken and limit its application (Zhang et al., 2004; Zhang and Shi, 2004; Shi et al*.,* 2006).

Spatial autocorrelations as well as heterogeneities within the model residuals

Our results indicated that when considering spatial autocorrelations within model residuals, SLM, SEM, LMM, and GWR obtained a significant improvement over the traditional OLS model, which resulted in a biased hypothesis tested according to model coefficient. Particularly in that figure of local Moran's *I*, those spatial autocorrelations in residuals showed obvious characters. Across the entire study area, for OLS model, the local Moran's *I* values for its residuals were mostly positive (presented as "hot spots") while that of other models, especially the GWR model residuals were mostly negative

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(presented as "cold spots"). Zhang et al. (2009) obtained similar conclusions while studying the relationship between DBH and tree height using these spatial regression models. As suggested by Moran's *I* and LM tests (*Table 4*) for the model residuals (*Fig. 3*), SEM model showed higher suitability to manage spatial autocorrelations than SLM. Therefore, spatial autocorrelations are the troublesome things due to model misspecification rather than the dependent variable being influenced by the values of the neibouring dependent variables (Luo et al., 2016). Though SLM and SEM models allowed for direct and effective correction for spatial autocorrelations among data corrected spatially, they were unable to manage spatial heterogeneity, as shown by those intra-block variance patterns among various block sizes like those for OLS model (*Table 3*; *Fig. 4*). Data fitted by LMM model were superior to those fitted by SLM and SEM, which offered a larger number of spatial autocorrelations in the expected model residuals (*Table 4*; *Fig. 3*), like in other reported studies (Zhang et al., 2009). LMM model, one of the global models, is also used to manage spatial correlation through 2 manners, including adjustment and characterization. Of them, the adjustment manner obtains the rate estimates for response variables using EBLUP, the global kriging in Geostatistics, whereas the characterization manner estimates the spatial covariance variables, such as semivariogram range, partial still, and nugget (Ozdenerol, 2006). LMM used the exponential spatial covariance structure, like geographical weight function in GWR model, to calculate those spatial weights for adjacent forest plots. As a result, LMM model emphasized the "local" data determined based on those semivariogram variables, so as to provide a large number of expected spatial autocorrelations and heterogeneities in model residuals relative to other global models.

According to other research (e.g., Zhang et al., 2009; Kupfer and Farris, 2007), GWR model accommodates the spatial heterogeneity in the meantime of markedly reducing the spatial autocorrelation within model residuals (*Fig. 3*). GWR, the local spatial model, adopts the moving window across an observation set distributed spatially to produce a model coefficient set based on data subsamples surrounding certain points spatially (Páez and Scott, 2005). Although GWR does not merge the spatial autocorrelation during the process of modeling, since it assumes a normal distribution N $(0, \sigma^2 I)$ in model error term. Besides, the GMR model definitely considers the spatial locations and emphasizes the local variations regarding the associations among variables. It is precisely due to the above characteristics of GWR, it represents an efficient approach in the mountainous regions with complicated terrains (Wang et al., 2020). The GWR model can be improved according to the need of the research. For example, Propastin (2012) extended the GWR model to develop a geographically altitudinal weighted regression (GAWR) model for managing altitudinal (vertical) as well as spatial (horizontal) instabilities in estimating the aboveground biomass for a rainforest region in tropics. Therefore, considering the diversity and complexity of forest ecosystems, GWR offers the highly precise visual data for afforestation planning and management measures with cost- and labor-effectiveness as long as it is properly applied (Zhang et al., 2009; Liu et al., 2014).

Conclusion

It is concluded that basal area, stand age, stand height were closely associated forest biomass in our study. They were the explanation of density, forestage, and site conditions. The distinct spatial autocorrelations as well as variations exist between

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forest biomass and the variables, thus the OLS was not appropriate for modeling. SLM and SEM efficiently accounted for the spatial autocorrelations within model residual, but insufficient to deal with the problem of spatial heterogeneity. In contrast, the LMM and GWR incorporated the spatial dependence and variation into modeling processes, and consequently, fitted the data better and predicted the response variable more accurately. Therefore, in the presence of obvious spatial variations and autocorrelations between dependent and independent variables, and spatial regression models such as LMM, especially GWR should be considered.

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FORAGE COMPOSITION, BIOMASS AND CARRYING CAPACITY DYNAMICS IN YABELLO RANGELAND, SOUTHERN ETHIOPIA USING DIFFERENT GRAZING SITES

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Abstract. Forage species composition, biomass production and carrying capacity potential are primary indicators of rangeland conditions. The article aimed to assess the changes occurring in forage species composition, biomass production and carrying capacity of different grazing sites with relation to seasonal variation by using the benchmark and biomass methods in Yabello rangeland, Southern, Ethiopia. Results indicated that the species abundance and height showed significant variation across the sampling site in relation to seasonal difference. *Chloris roxburghiana, Cenchrus ciliaris* and *Chrysopogon aucheri* grass species were shown to be dominant and registered the highest average single species coverage and biomass yield for all grazing sites. The grazing vegetation index value of conserved grazing site were shown higher value 62.8% and 64.4% during rainy season and 48% and 54.7% during dry season from open communal and woody plant infested grazing site respectively. 76.7% and 90.9% higher biomass production was observed from ranch grazing site as compared to open-communal and woody covered grazing area, respectively. The carrying capacity variation within ranch grazing site was 77%, 76% and 76.7% greater from open-communal grazing site and 92.7%, 86.4% and 90.9% greater from woody plant infested grazing site compared during rainy season, dry season and yearly average value respectively.
Keywords: *abundance, stoking rate, production, palatability value, height*

Introduction

Forage species play an important role in livestock feeding in arid and semi-arid regions (Arzani et al., 2006), and also improve ecosystem services for the welfare of pastoral societies. Biomass of forage in rangelands is mainly determined by the amount of, distribution and duration of rainfall, effects of invasive alien species, livestock grazing intensity and other anthropogenic factors (Kassahun, 2008; Lemus, 2010). Recently, most pastoral areas of Ethiopia, including the Yabello rangelands, have been exhibiting a shift from herbaceous species to woody plants, a feature that is accompanied with some degree of degradation resulting from overgrazing, expansion of cultivation, also frequent drought and settlement resulted in decline in forage biomass and carrying capacity (Gemedo-Dalle et al., 2006; Oba et al., 2008; Angassa and Oba, 2010; Angassa, 2014). The Yabello pastoralists have been practicing transhumance to counter seasonal fluctuations in forage and water availability (Angassa and Oba, 2010; Habtamu, 2013; Takele et al., 2014). The factors that have been reported to affect the forage production and carrying capacity of

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rangelands are caused due to seasonal variability (Snyman, 1998), species variation (Arzani et al., 2008), soil nutrient status of production location (Tessema et al., 2011), grazing pressure (Adisu, 2009) and mismanagement aspects (Van der et al., 2005). In the semi-arid Yabello rangeland, this translates into seasonal shortages of forage and low carrying capacity of rangelands (Alemayehu, 2006), further hindering sustainable livestock production and affecting the socio- economic of the pastoralist livelihood (Herlocker, 1999; Gemedo-Dalle et al., 2005; Bikila et al., 2014).

Assessment of rangeland based on forage biomass productivity was used to sustainable rangeland management through balancing the livestock population with an amount of forage production, that used to reduce further degradation (Ganskopp and Bohnert, 2001; Arzani et al., 2006; Keno and Suryabhagavan, 2018). The Yabello pastoralists have been known to exist since before the thirteenth century (Oba and Kotile, 2001) and have adapted the local knowledge to manage their range land from different threating factors like drought, invasive plant species, overgrazing and other anthropogenic factors (Oba and Kotile, 2001; Teshome et al., 2012). Assessing forage productivity and carrying capacity are key factors of rangeland inventory and monitoring programs which are highly required for the sustainability of natural resources (Galt et al., 2000; Tsegaye et al., 2010; Lemus, 2010; Abdella, 2010; Bikila et al., 2014; Hailu, 2017; Cheng et al., 2017; Meshesha et al., 2019). Land use evaluation is an important tool in making decisions in planning type of animals to be used and land suitable to them accordingly based on their specified requirements, preference and predictors of specific activates (Mligo, 2009; Lin et al., 2010; Tamrat and Stein, 2015; Bikila et al., 2016; Siraj and Abdella, 2018). The productivity and carrying capacity of Yabello rangeland is degrading and decreasing ultimately (Habtamu, 2013). Since, Yabello rangeland is a depleted range area, the assessment of present potential of the range resource is important in order to plan its sustainable development. However, there is not enough information available on the effect of invasive plant infestation on native grass species, forage biomass production and therefore carrying capacity of the study area. Therefore, in this study we examined the change of herbaceous species ground cover, forage production and carrying capacity in the Yabello rangelands of southern Ethiopia.

Materials and methods

Study area

The study was conducted at Yabello district in the Borana zone of Southern Ethiopia using both ranch, communal and woody grazing site (*Fig. 1*). The site was selected because it is one of the most arid parts of Borana zone and, therefore, the pastoral communities of this district are the most vulnerable to the rangeland degradation due to overgrazing because of the large number of livestock population and bush encroachment. Yabello is located at 566 km south of Addis Ababa along Addis – Moyale road. The total area of district is 15,430 km^2 of which 68% (10,492 km^2) is rangeland and it is located between 4°30′55.81″ and 5°24′36.39″ north latitudes and between 7°44′14.70″ and 38°36′05.35″ east longitudes (Gemedo-Dalle et al., 2015). The altitude is about 1000-1500 m, with a maximum altitude of 2000 m. The rainfall of the area is characterized as bi-modal. Most (73%) of the rainfall occurs in March to May, which is called the long (gaana) rainy season, and the remainder (27%) occurs in September to November, which is called the short rainy (hagaya) season (Gemedo-Dalle et al., 2015).

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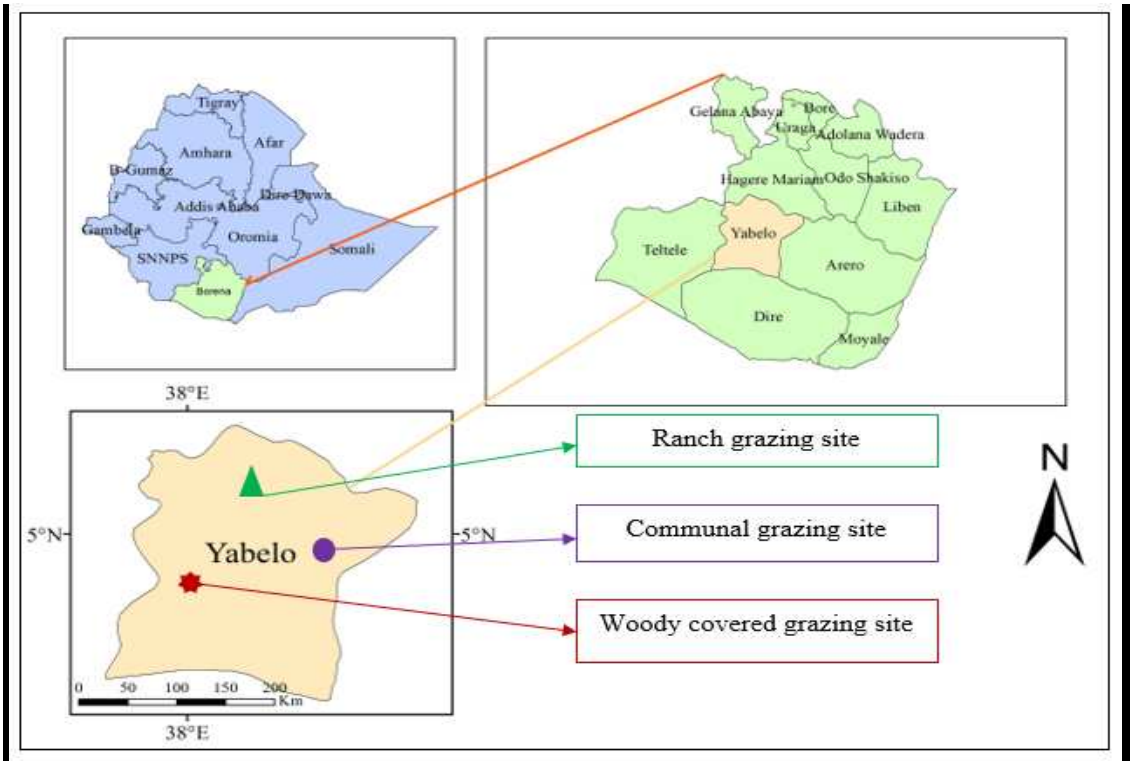

Figure 1. *Location of the study area*

The mean annual rainfall is recorded between 450-700 mm (Angassa, 2014) while the mean annual temperature varies from 19-24°C with little seasonal variation (*Fig. 2*). The potential evapotranspiration is 700-3000 mm (Billi et al., 2015). The soil in the study area includes, 53% red sandy loam soil, 30% black clay, and volcanic light-colored silt clay and 17% silt and the vegetation is mainly dominated by encroaching woody species, and those that frequently thinned out including *Senegalia mellifera*, *Vachellia reficiens* and *Vachellia oerfota* (Coppock, 1994). According to the latest census conducted in 2015, the national census reported that the total population for this district was 70,501, of whom 36,246 were men and 34,255 were women; 4,874 or 6.91% of this population includes urban dwellers. Cattle, goats, sheep, camel, mule, donkey and horse are the main reared livestock species.

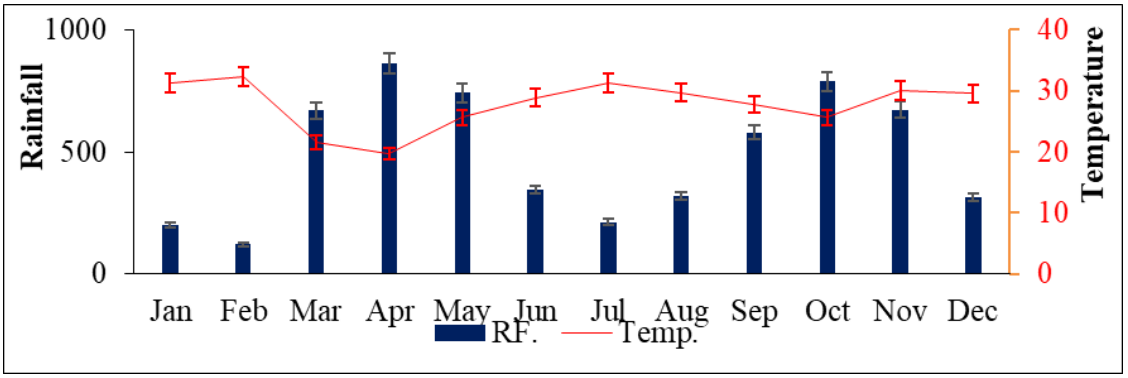

Figure 2. *Average monthly rainfall and temperature (±SE) for year 2019 in the Yabello rangeland site. RF = rainfall, Temp = temperature. (source: - EMA, 2019)*

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Data collection methods

Sampling species composition and forage biomass production

In order to quantify the forage composition and biomass dynamics in different grazing land types, above ground herbaceous was collected and biomass measurement was conducted. Within each rangeland grazing type, one linear 5 km transect was assigned and six 25 * 25 m^2 sampling plots were systematically placed at 500 m interval at each grazing site in total 3*3= 9 plots. And within each plot three were (3) 5 * 5 m^2 sub plots randomly assigned in total 3*3*3= 27 subplots. Finally, five (5) 1 * 1 m^2 quadrants in total 3*3*3*5= 135 quadrants were assigned by throwing randomly to the back side in order to minimize any biases resulting from selective placement within each sub plot for herbaceous and grass species sample collection. All the above ground forage samples were harvested by using cutter and collected in paper bags. The fresh weight of forage sample was measured in the field with a scale.

Samples were taken to Yaballo Pastoral and Dryland Agriculture Research Center soil laboratory and oven dried for 24 h at 105°C to determine the biomass. Then the dry matter was measured after 24 h of drying and converted into kilogram per hectare (kg/ ha), and the proper use factor (PUF) have been taken as 30% to calculate available forage (Sintayehu, 2006; Meshesha et al., 2019). Thereafter, dry matter (DM) biomass and livestock carrying capacity were determined by the following procedures described by Niguse, 2008 and grazing vegetation index percentage (GVI) methods. Identification of the grass species were done in the field with the help of field identification keys and plates, using Flora of Ethiopia books, and Addis Ababa University national herbarium (Elmore et al., 2000; Gemedo-Dalle et al., 2005). Data collection on grass species sampling was commenced twice per year, in the dry season (January-February 2019) and in the rainy season (March-May 2019) at the time when grass species were identified easily and peak biomass were recorded. Field data was collected with three replications for each season.

Grazing site vegetation index, height and palatability of grass species

The grazing site vegetation index (GVI) valuing each study site (*Fig. 3*) was analyzed using both percentage coverage (PC) of each species within each sampling plot and in combination of ecological index values (EIV) (Vorster, 1982; Solomon et al., 2006). The EIV and palatability value (PV) of grass species was recorded by direct observation of the grazing livestock, semi-structured focus group discussion and field on site explanation of the local pastoralists with a total of 110 participants (71 males and 39 females) and identification was carried out with the help of elder pastoralist and district experts. The participants were selected based on their experience, direct linkage of livestock rearing, age, year spent on the study site and based on recommendation of experts in addition with their voluntarism. For this study the EIV was grouped into four (4) classes with its value, namely: decreasers = 10; increaser I=7; increaser II = 4; Increaser III = 1 (Solomon et al., 2006). PC value of each species was calculated.

The PV was also grouped in to four (4) classes and values were assigned, like: highly palatable = 8; Moderately palatable = 6; less palatable = 4 and unpalatable = 2. The percentage composition of grass species in each class was summed up, after which the sum for each class was multiplied by EIV and PC value. These amounts were then totaled to give the vegetation index. For our case we also calculated the grand total score of our benchmark grazing site and the most probable maximum assuming that all species were grouped under decreases. The average height of each individual grass species at each grazing site were measured both

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during February and May for dry and rainy season respectively when the two seasonal features clearly and at the time where grass species were identified easily and peak biomass were recorded. And the field data collected with three replications for each season.

Figure 3. *Picture representation of sampling site*

Carrying capacity determination used ranch grazing site as the benchmark

To determine grazing capacity with the benchmark method, the GVI percentage, obtained from either the ecological index or grazing value index, was used. It also incorporated the average annual rainfall for the grazing site. The equation used for this method was described below at *Eq. 1* (Niguse, 2008; Habtamu, 2013).

$$CC\ (AU/ha) = \{[-0.03 + 0.00289 \times GVI\%] + [(RF - 419.7) \times (0.000633)]\} \quad (Eq.1)$$

where, CC = Carrying capacity in animal unit per hectare (AU)/ha, GVI = grazing vegetation index in % of the benchmark rangeland, RF = Average annual rainfall for the grazing site.

Carrying capacity determination using the biomass method

This method described by (Moore and Jung, 2001), uses the grass biomass per hectare to determine the grazing capacity for animal units (AU) for one year. It assumes that one AU consumes 11.25 kg grass per day (2.5% of its body mass of 450 kg). The method also includes a utilization factor 0.30 (30%) depending on the recommendation for savanna rangelands including Borana and calculated using *Eq. 2* given below (Habtamu, 2013).

$$CC = \frac{d \div [DM \times Uf]}{r} \quad (Eq.2)$$

where, **CC** = Carrying capacity in AU/ha, **d** = number of days in the year (or period to be grazed), **DM** = dry matter (biomass) in kg/ha, **Uf** = utilization factor (0.3), **r** = daily dry matter required by one grazing animal (2.5% of bodyweight), which is 11.25 kg for an AU (450 kg grazing animal (cattle).

Statistical analysis

The rangeland productivity, livestock, carrying capacity, palatability value, percentage cover, height data were analyzed by means of Microsoft Excel program and descriptive statistics in the Statistical Package for Social Sciences (SPSS) to generate descriptive statistics. Significant differences evaluation at $P < 0.05$ were done by using analysis of

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variance (ANOVA) used to analyse difference with regards to different grazing site and seasonal variation and Principal Component Analysis (PCA) was performed on the complete dataset to reduce its complexity and get a better understanding of the underlying vegetation structure.

Results and Discussion

Grass species composition of rangeland

A total of 23 grass species were identified and recorded using both their scientific and local name. The species and their average coverage for the different grazing site are presented in *Table 1*. *Chloris roxburghiana*, *Cenchrus ciliaris* and *Chrysopogon aucheri* grass species showed dominance and registered the highest average single species coverage in both season across all grazing sites and highly abundance at communal and woody grazing site. This is because of high resistance capacity of species during overgrazing and competent features with woody plant species, as a result this species are highly recommended for rehabilitation of degraded rangeland. Results showed that there was a significant difference ($P<0.05$) in most grass species coverage across different grazing sites both during rainy and dry season. And among the total 23 grass species that were found in the study area, in ranch grazing site, coverage of 20 and 19 species was above 1%, abundance of 2 and 1 species was below 1% and the remaining 1 and 3 species did not exist in the grazing site during rainy and dry season respectively (*Table 1*).

Table 1. *Grass species cover (%) across different grazing land site and season (Values showed the average percentages based on 1 m^2 quadrats in which a given species was recorded)*

No.	List of Species		Grazing site					
	Scientific name	Local name	Ranch		Communal		Woody	
			Rs	Ds	Rs	Ds	Rs	Ds
1.	*Chrysopogon aucheri*	Alaloo	45^{**}	34^{**}	19^{**}	14^{**}	56^{**}	43^{**}
2.	*Dactyloctenium aegyptium*	Ardaa	8^{*}	6^{*}	7^{*}	3*	$+^{*}$	$-^{*}$
3.	*Xerophyta humilis*	Areedoo	5	4	14^{*}	12^{*}	1^{*}	$+^{*}$
4.	*Aristida kenyensis*	Biilaa	39^{**}	21^{**}	$+^{*}$	$-^{*}$	5^{*}	2^{*}
5.	*Eragrostis capitulifera*	Biilaa	3	2	+	+	2	+
6.	*Harpachne schimperi*	Biilaa	8^{*}	6^{*}	18^{*}	21^{*}	24^{*}	29^{*}
7.	*Leptothrium senegalense*	Biilaa diidaa	28^{**}	13^{**}	-	-	-	-
8.	*Melinis repens*	Buuyyoo xirooftuu	15^{**}	9^{**}	11	10	4^{*}	3^{*}
9.	*Themeda triandra*	Gaaguroo	19^{*}	17^{*}	4	4	+	+
10.	*Digitaria milanjiana*	Hiddoo	7^{*}	2^{*}	24^{**}	20^{**}	10^{**}	$+^{**}$
11.	*Chloris roxburghiana*	Hiddoo luucolee	54^{**}	33^{**}	49^{**}	40^{**}	34^{**}	27^{**}
12.	*Digitaria naghellensis*	Ilmogorii	45^{**}	33^{**}	$+^{*}$	$-^{*}$	-	-
13.	*Panicum maximum*	Loloqaa	18^{*}	12^{*}	-	-	$+^{*}$	$-^{*}$
14.	*Bothriochloa insculpta*	Luucolee	1^{**}	$-^{**}$	7	5	2^{*}	$+^{*}$
15.	*Cenchrus ciliaris*	Mata guddeessa	51^{**}	37^{**}	26^{*}	21^{*}	23^{**}	15^{**}
16.	*Pennisetum mezianum*	Ogoondhichoo	41^{**}	32^{**}	$+^{**}$	$-^{**}$	-	-
17.	*Eragrostis papposa*	Saamphillee	-	-	$-^{**}$	$+^{**}$	$+^{*}$	1^{*}
18.	*Sporobolus discosporus*	kootichaa	21^{**}	8^{**}	2^{*}	$+^{*}$	1^{**}	$-^{**}$
19.	*Grewia tenax*	Saarkama	1^{*}	$+^{*}$	17^{**}	5^{**}	9^{*}	2^{*}
20.	*Cynodon dactylon*	Sardoo	$+^{*}$	$-^{*}$	3	2	1^{**}	$-^{**}$
21.	*Cyperus sp.*	Saattuu	41^{**}	27^{**}	$+^{*}$	$-^{*}$	-	-
22.	*Cyperus bulbosus*	Saattuu arbaa	17^{*}	10^{*}	8^{*}	14^{*}	3	4
23.	*Sporobolus pellucidus*	Salaqoo	$+^{**}$	8^{**}	9^{*}	11^{*}	5^{*}	8^{*}

Note: * = significant, ** = highly significant, + = indicates grass species present with cover <1%, - = indicates grass species absent, Rs = rainy season, Ds = dry season

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At the communal grazing site, abundance of 15 and 14 species was above 1%, 5 and 3 species were below 1% and the reaming 3 and 6 species did not exist in the grazing site both rainy and dry season, respectively. On woody covered grazing site, 15 and 10 species were found with a coverage above 1%, 4 and 5 species were below 1% and the reaming 4 and 8 species did not exist in the grazing site both at rainy and dry seasons, respectively. As we have seen from *Table 1*, even if the species was found in all grazing site, its abundance showed declined pattern from conserved ranch grazing site to open communal and woody covered rangeland site.

And also, seasonal variation had a great impact on the grass species occurrence and abundance based on our result and past reported data (Han et al., 2013). Almost all, grass species showed increasing trend during rainy season and decreasing trend during dry season, with the exception of *Cyperus bulbosus, Sporobolus pellucidus, Harpachne schimperi* and *Eragrostis papposagrass* species that showed increasing trend of their abundance during dry season as compared to rainy season. From this we can understand that this grass species had the capacity to resist rainfall security and will be recommended for rangeland rehabilitation in the aera where more frequent drought occurred, and this result is in agreement with the data reported by Han et al., 2013.

In general, from this study we can observe that grass species existence and abundance had a direct influence and it is linked with grazing land type. That is grass coverage was higher in well managed ranch rangeland site and showed great decreasing rate both in abundance and total absence in the non-managed open communal and woody plant infested grazing site. This told us random grazing trend and bush infestation of rangeland had a great impact on both the composition and mass of herbage species and this had a direct influence on the livelihood of livestock pastoralists as well as the economy of both the local people and the whole country. This finding on the pattern of species composition showed an opposite trend that data reported by (Lanta et al., 2009), whose studies indicated that excluding herbage from livestock grazing decreases species richness and increases it under conditions of grazing pressure. The spatial distribution and composition of grass species in relation to seasonal variation as assessed by the component analysis of PCA is provided (*Fig. 4*). The first two PCA component axes cumulatively explained 97.7% of the total variance in species, which shows that the two components have accounted for most of the species types composition in the rotated space.

Grazing site vegetation index of rangeland

Concerning the grazing system, we used a well-managed and conserved government ranch site as our benchmark in order to analyze the ecological status of grass species. We used two variations, one relies on the ecological index value classification (decreasers and increasers) of grasses and the other percentage occurrence value of each species are calculated above (*Table 1*). The recorded multiplicative value is in the 'Score' columns. In most studies a grand total was assumed as 900-1000 for the benchmark site (excellent rangeland condition) (Marake et al., 2019), but in our case the grand total value was obtained by collecting and analyzing all the grass species existed on the site that was a highly conserved and well managed government ranch site. The percentage difference between these two sites (i.e. the well managed ranch and non-managed open communal and woody covered grazing site) represents the ecological index value (EIV).

In *Table 2* above the EIV indicated the assigned values rather than classified groups. That is, 10= for decreasers; 7= for increaser I; 4= for increaser II and 1= for increaser III. In this case, grouping of grass species under decreasers and increasers was conducted based on their

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basal area coverage percentage conducted in *Table 1* above in combination with direct field observation of their occurrence and abundance in each grazing site and past trends of the species based on elder pastoralist information obtained during focus group discussion and field assistance and grouped as follows: abundance greater than 20% under decreasers, 15-20% under increaser I, 10-14% under increaser II and with abundance less than 10% under increaser III (the same technique used by Baars (2002)).

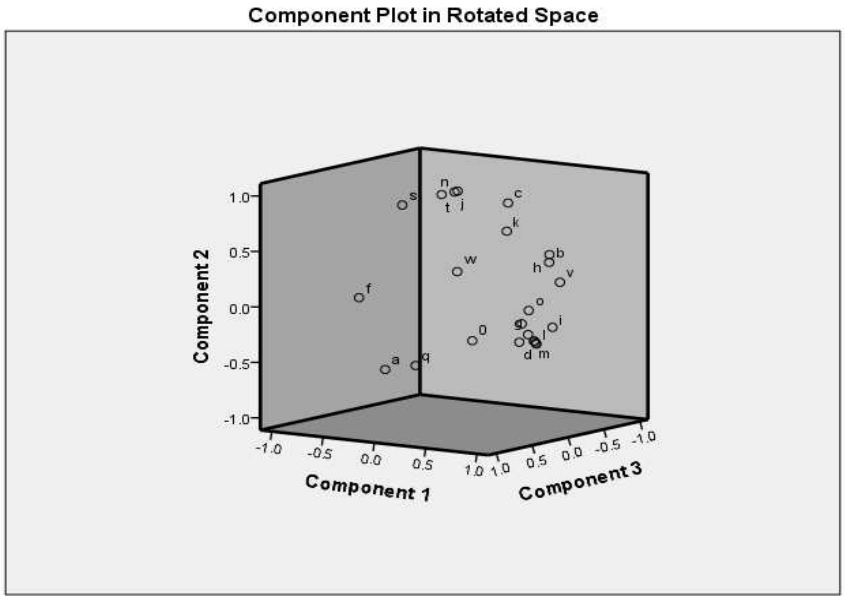

Figure 4. *PCA component analysis of grass species based on occurrence frequency with in each grazing site in relation to seasonal variation. (a=Chrysopogon aucheri, b=Dactyloctenium aegyptium, c=Xerophyta humilis, d=Aristida kenyensis, e=Eragrostis capitulifera, f=Harpachne schimperi, g=Leptothrium senegalense, h=Melinis repens, i=Themeda triandra, j=Digitaria milanjiana, k=Chloris roxburghiana, l=Digitaria naghellensis, m=Panicum maximum, n=Bothriochloa insculpta o=Cenchrus ciliaris, p=Pennisetum mezianum, q=Eragrostis papposa, r=Sporobolus discosporus, s=Grewia tenax, t=Grewia tenax, u=Cyperus sporobouls, v=Cyperus bulbosus, w=Sporobolus pellucidus)*

In the ranch grazing site, 39.1% and 30.4% of grass species were grouped under decreasers, 17.4% and 4.3% of grass species under increaser I, 0% and 13% of grass species under increaser II and 43.5% and 39.1% of grass species under increaser III during rainy and dry grazing season, respectively. In the open-communal grazing site, 13% and 17.4% of grass species were grouped under decreasers, 13% and 0% of grass species under increaser I, 8.7% and 21.7% of grass under increaser II and 52.2% and 34.8% of grass species under increaser III during rainy and dry grazing season, respectively. At woody plant infested grazing site, 17.4% and 13% of grass species were grouped under decreasers, 0% and 4.3% of grass species under increaser I, 4.3% and 0% under increaser II and 60.9% and 47.8% of grass species under increaser III during rainy and dry grazing season, respectively. And the average ratio of each grass species category was: in ranch grazing site, 34.8%, 13%, 8.7% and 43.8% of grass species were grouped under decreasers, increaser I, increaser II and increaser III, respectively.

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Table 2. *Ecological status of species analyzed by the benchmark method, using ecological index groups and percentage coverage (%) of grass species for each grazing sites*

No.	List of Species		Grazing site																	
	Scientific name	Local name	Ranch						Communal						Woody					
			Rs			DS			Rs			Ds			Rs			Ds		
			PC	EIV	SC	PC	EIV	SC	PC	EIV	SC	PC	EIV	SC	PC	EIV	SC	PC	EIV	SC
1.	*Chrysopogon aucheri*	Alaloo	45	10	**450****	34	10	**340****	19	7	**133****	14	4	**56****	56	10	**560****	43	10	**430****
2.	*Dactyloctenium aegyptium*	Ardaa	8	1	**8**	6	1	**6**	7	1	**7**	3	1	**3**	+	1	**1***	-	-	**-***
3.	*Xerophyta humilis*	Areedoo	5	1	**5**	4	1	**4**	14	4	**56***	12	4	**48***	1	1	**1**	+	1	**1**
4.	*Aristida kenyensis*	Biilaa	39	10	**390****	21	10	**210****	+	1	**1***	-	-	**-***	5	1	**5**	2	1	**2**
5.	*Eragrostis capitulifera*	Biilaa	3	1	**3**	2	1	**2**	+	1	**1**	+	1	**1**	2	1	**2**	+	1	**1**
6.	*Harpachne schimperi*	Biilaa	8	1	**8**	6	1	**6**	18	7	**126****	21	10	**210****	24	10	**240****	29	10	**290****
7.	*Leptothrium senegalense*	Biilaa diidaa	28	10	**280****	13	4	**52****	-	-	**-**	-	-	**-**	-	-	**-**	-	-	**-**
8.	*Melinis repens*	Buuyyo	15	7	**105****	9	1	**9****	11	4	**44**	10	4	**40**	4	1	**4**	3	1	**3**
9.	*Themeda triandra*	Gaaguroo	19	7	**133***	17	7	**119***	4	1	**4**	4	1	**4**	+	1	**1**	+	1	**1**
10.	*Digitaria milanjiana*	Hiddoo	7	1	**7***	2	1	**2***	24	10	**240****	20	10	**200****	10	4	**40****	+	1	**1****
11.	*Chloris roxburghiana*	Hiddoo luucolee	44	10	**440****	33	10	**330****	49	10	**490****	40	10	**400****	34	10	**340****	27	10	**270****
12.	*Digitaria naghellensis*	Ilmogorii	45	10	**450****	33	10	**330****	+	1	**1***	-	-	**-***	-	-	**-**	-	-	**-**
13.	*Panicum maximum*	Loloqaa	18	7	**126****	12	4	**48****	-	-	**-**	-	-	**-**	+	1	**1***	-	-	**-***
14.	*Bothriochloa insculpta*	Luucolee	1	1	**1***	-	-	**-***	7	1	**7**	5	1	**5**	2	1	**2**	+	1	**1**
15.	*Cenchrus ciliaris*	Mata guddeessa	51	10	**510****	37	10	**370****	26	10	**260****	21	10	**210****	23	10	**230****	15	7	**105****
16.	*Pennisetum mezianum*	Ogoondhichoo	41	10	**410****	32	10	**320****	+	1	**1***	-	-	**-***	-	-	**-**	-	-	**-**
17.	*Eragrostis papposa*	Saamphillee	-	-	**-**	-	-	**-**	-	-	**-***	+	1	**1***	+	1	**1**	1	1	**1**
18.	*Sporobolus discosporus*	*kootichaa*	21	10	**210****	8	1	**8****	2	1	**2**	+	1	**1**	1	1	**1***	-	-	**-***
19.	*Grewia tenax*	Saarkama	1	1	**1**	+	1	**1**	17	7	**119****	5	1	**5****	9	1	**9***	2	1	**2***

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No.	List of Species		Grazing site																
	Scientific name	Local name	Ranch						Communal						Woody				
			Rs			DS			Rs			Ds			Rs			Ds		
			PC	EIV	SC	PC	EIV	SC	PC	EIV	SC	PC	EIV	SC	PC	EIV	SC	PC	EIV	SC
20.	Cynodon dactylon	Sardoo	+	1	**1***	-	-	**-***	3	1	**3**	2	1	**2**	1	1	**1***	-	-	**-***
21.	*Cyperus sporobolus*	Saattuu	41	10	**410****	27	10	**270****	+	1	**1***	-	-	**-***	-	-	**-**	-	-	**-**
22.	*Cyperus bulbosus*	Saattuu arbaa	17	7	**119****	10	4	**40****	8	1	**8****	14	4	**56****	3	1	**3**	4	1	**4**
23.	*Sporobolus pellucidus*	Salaqoo	+	1	**1***	8	1	**8***	9	1	**9****	11	4	**44****	5	1	**5**	8	1	**8**
Grand total score means of each site			**176.9**^{a}			**107.6**^{b}			**65.8**^{b}			**55.9**^{c}			**62.9**^{b}			**48.7**^{c}		
Seasonal percentage difference (grazing site vegetation index with related to ranch site) calculated as: GVI%= (GTR-GT each site)/GTR X 100									**62.8**^{a}			**48.0**^{b}			**64.4**^{a}			**54.7**^{b}		
Yearly average grazing site Vegetation index, YGVI %= (YGTR-YGT each site) * 100/ YGTR, (YGT= GTRs+ GTDs /2)									**57.2**						**60.8**					

Note: * = significant, ** = highly significant, GVI= grazing site vegetation index, GTR= grand total of ranch site, GT= grand total, YGVI = yearly grand vegetation index, YGT= yearly grand total, Rs= rainy season, Ds= dry season, GTRs= grand total of rainy season, GTDs= grand total of dry season, YGTR= yearly grand total of ranch site, -= grass species absent, Pc=percentage coverage, EIV= ecological index value, Sc= Score (PC∗ EIV). GT and GVI value with different letter at the same row indicate there is a significant difference related to benchmark site across each season

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In open-communal grazing site, 17.4%, 8.7%, 17.4% and 43.8% of grass species were grouped under decreasers, increaser I, increaser II and increaser III, respectively. At wood plant infested grazing site, 17.4%, 4.3%, 4.3% and 56.5% of grass species were grouped under decreasers, increaser I, increaser II and increaser III, respectively. From this ratio, we can understand that decreasers grasses were highly abundant in rangelands in a good condition for grazing (ranch), but decreased in number when the rangeland was over-grazed or infested by woody plant species. With regard to GVI value, the conserved grazing site (ranch) showed higher value such as 62.8% and 64.4% during rainy season and 48% and 54.7% during dry season compared with unmanaged open-communal grazing site and woody plant infested grazing site respectively (*Table 2*). And also, ranch grazing site showed 57.2% and 60.8% higher grass species composition from communal and woody grazing site during assessing the yearly GVI value.

Palatability classification of rangeland grass species

Classification of grass species based on palatability value (PV) at different grazing site was recorded by directly observing the grazing livestock in the field for two different seasons. These field observations were further confirmed from knowledge gathered from pastoralist and local experts. In order to calculate the degree of palatability, we simply focus on grazing site variation. There were no visible differences based on the morphological grass parts preferred by livestock. This is may be because of all grass species in our study area have showed almost similar morphological and phenological palatability features. And this observational result is in agreement with the data reported by Marake et al. (2019). Results regarding palatability value of existing grass species in our study rangelands revealed great variation in Palatability rate (*Table 3*).

In ranch grazing site, among the total 22 identified grass species (*Table 1*), 3 (14%) species grouped under highly palatable (Hp), 5 (23%) under moderately palatable (Mp), 8 (36%) under less palatable (Lp) and 6 (27%) under unpalatable (Up). At ranch grazing site most species grouped under less palatable grass species, this is due to the well managed grazing area that the grazers have the chance to access different forage source grass species, as a result their preference of grazing become diverse and not focused on a certain grass species. In the open-communal grazing site, among the total 21 identified grass species (*Table 1*), 7 (33%) species were grouped under highly palatable (Hp), 9 (43) under moderately palatable (Mp), 5 (24%) under less palatable (Lp) and no species were grouped under unpalatable (Up) grouped. In the woody infested grazing site, from the 19 grass species identified (*Table 1*), 6 (31%) species were grouped under highly palatable (Hp), 10 (53%) under moderately palatable (Mp), 3 (16%) under less palatable (Lp) and no grass species remain as unpalatable. From this we can understand that, in the degraded grazing site all species that can be preferable for grazing, because there is scarcity of forage source and no species remain unpalatable if the rangeland faced degradation.

In general, the palatability rating score (highly palatable and moderately palatable) were found to be the highest in the woody area (84%), followed by the open- communal grazing site (76%) and lastly on the ranch (37%) and the rating scores of the other two species categories (less palatable and unpalatable) were found the highest in the ranch area (63%), followed by open-communal grazing site (24%) and lastly woody infested grazing site (16%). This is because of accessibility of variety of species and no scarcity on the properly utilized and managed grazing site (ranch) and the current study is directly in line with the data reported by Solomon et al., 2006.

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Table 3. *Palatability classification of grass species based on palatability value (PV)*

No.	List of *Species*		Grazing site											
	Scientific name	Local name	Ranch				Communal				Woody			
			Hp	Mp	Lp	Up	Hp	Mp	Lp	Up	Hp	Mp	Lp	Up
1.	*Chrysopogon aucheri*	Alaloo	8	-	-	-	8	-	-	-	8	-	-	-
2.	*Dactyloctenium a.*	Ardaa	-	-	4	-	-	6	-	-	-	6	-	-
3.	*Xerophyta humilis*	Areedoo	-	6	-	-	8	-	-	-	-	-	4	-
4.	*Aristida kenyensis*	Biilaa	-	-	-	2	-	-	4	-	-	6	-	-
5.	*Eragrostis capitulifera*	Biilaa	-	6	-	-	-	-	4	-	-	6	-	-
6.	*Harpachne schimperi*	Biilaa	-	6	-	-	8	-	-	-	8	-	-	-
7.	*Leptothrium senegalense*	Biilaa diidaa	-	-	-	2	-	-	-	-	-	-	-	-
8.	*Melinis repens*	Buuyyoo	-	6	-	-	-	6	-	-	-	6	-	-
9.	*Themeda triandra*	Gaaguroo	-	6	-	-	-	6	-	-	-	6	-	-
10.	*Digitaria milanjiana*	Hiddoo	-	-	4	-	-	6	-	-	-	-	4	-
11	*Chloris roxburghiana*	Hiddoo	8	-	-	-	8	-	-	-	8	-	-	-
12.	*Digitaria naghellensis*	Ilmogorii	-	-	-	2	-	-	4	-	-	-	4	-
13.	*Panicum maximum*	Loloqaa	-	-	4	-	-	-	-	-	-	6	-	-
14.	*Bothriochloa insculpta*	Luucolee	-	-	-	2	-	6	-	-	-	6	-	-
15.	*Cenchrus ciliaris*	Mata	8	-	-	-	8	-	-	-	8	-	-	-
16.	*Pennisetum mezianum*	Ogoondhichoo	-	-	4	-	-	6	-	-	-	-	-	-
17.	*Eragrostis papposa*	Saamphillee	-	-	-	-	-	-	4	-	-	6	-	-
18.	*Sporobolus discosporus*	kootichaa	-	-	4	-	-	6	-	-	-	6	-	-
19.	*Grewia tenax*	Saarkama	-	-	-	2	-	-	4	-				
20.	*Cynodon dactylon*	*Sardoo*	-	-	4	-	-	6	-	-	-	6	-	-
21.	*Cyperus sp.*	Saattuu	-	-	4	-	8	-	-	-	-	-	-	-
22.	*Cyperus bulbosus*	Saattuu arbaa	-	-	4	-	8	-	-	-	8	-	-	-
23.	*Sporobolus pellucidus*	Salaqoo	-	-	-	2	-	6	-	-	8	-	-	-

Note: Hp= highly palatable, Mp= moderately palatable, Lp= less palatable, Up = unpalatable, -= no vale under that PV, 8 = indicates Hp, 6 = indicate Mp, 4 = indicate = Lp, 2 = indicate Up

Grass species height of rangeland

The height of grass species recorded from the study sites also showed a significant variation across the grazing site and presented below in *Table 4*.

The grass species collected from the sampling site showed height variation across the grazing site difference with relation to seasonal influence. Grass species height in woody covered grazing site was found the shortest as compared to ranch and communal grazing site and in the ranch grazing site all grass species showed a better height among other sites. This difference in height could possibly be due to less vigour associated with herbage under woody vegetation cover as a result of light competition effect. This may cause species to easily break in case of environmental disturbances like grazing and wind, hence not able to grow tall to the heights comparable to those with no light shade effect in open grass land area.

In general, the impact of unmanaged livestock grazing and infestation of woody plant species showed significant ($P<0.05$) variation with regards to livestock forge species growth on the grazing site, so that species heights in a well-managed ranch site significantly showed a better growth performance than those in over-grazed and woody infested range site. However, as we have seen from *Table 4*, the height of herbage species

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Chrysopogon aucheri, Chloris roxburghiana and *Cenchrus ciliaris* showed great resistance across all sampling sites and served as a main source for forage for livestock throughout the year. According to the data obtained directly from our field investigation and also from both direct interview and focal group discussion of the local communities those grass species had high resistance capacity and recommended for further degraded range land rehabilitation method either through reseeding or direct planting of it. Our data were directly supported by the study conducted by Yeneayehu et al. (2020), on effects of vegetation cover, grazing and season on herbage species composition and biomass in the case of Yabello rangeland, Southern Ethiopia.

Table 4. *Grass species height (m) per different grazing site and season (Values from 1 m^2 quadrats in which a given species was recorded)*

No.	List of *Species*		Grazing site					
	Scientific name	Local name	Ranch		Communal		Woody	
			Rs	Ds	Rs	Ds	Rs	Ds
1.	*Chrysopogon aucheri*	Alaloo	0.74**	0.39**	0.61**	0.35**	0.52**	0.18**
2.	*Dactyloctenium a.*	Ardaa	0.35**	0.13**	0.28*	0.21*	0.11**	-**
3.	*Xerophyta humilis*	Areedoo	0.48**	0.20**	0.28*	0.31*	0.21**	-**
4.	*Aristida kenyensis*	Biilaa	0.57**	0.36**	0.06	-	0.19*	0.10*
5.	*Eragrostis capitulifera*	Biilaa	0.39**	0.17**	0.24*	0.10*	0.17*	0.10*
6.	*Harpachne schimperi*	Biilaa	0.39**	0.09**	0.47**	0.27**	0.27*	0.17*
7.	*Leptothrium senegalense*	Biilaa diidaa	0.59**	0.29**	-	-	-	-
8.	*Melinis repens*	Buuyyoo	0.55**	0.07**	0.38*	0.31*	0.06	0.02
9.	*Themeda triandra*	Gaaguroo	0.17**	0.01**	0.19*	0.21*	0.19*	0.12*
10.	*Digitaria milanjiana*	Hiddoo	0.49**	0.14**	0.48**	0.16**	0.33**	0.03**
11	*Chloris roxburghiana*	Hiddoo	0.68**	0.33**	0.49*	0.32*	0.37**	0.23**
12.	*Digitaria naghellensis*	Ilmogorii	0.21*	0.10*	0.32**	-**	-	-
13.	*Panicum maximum*	Loloqaa	0.03	0.07	-	-	0.2	-
14.	*Bothriochloa insculpta*	Luucolee	0.02	-	0.31*	0.22*	0.29**	0.11**
15.	*Cenchrus ciliaris*	Mata	0.88**	0.43**	0.59**	0.31**	0.45**	0.22**
16.	*Pennisetum mezianum*	Ogoondhichoo	0.19*	0.10*	0.36**	-**	-	-
17.	*Eragrostis papposa*	Saamphillee	-	-	-	0.02	0.21*	0.15*
18.	*Sporobolus discosporus*	kootichaa	0.49**	0.27**	0.25**	0.03**	0.25**	-**
19.	*Grewia tenax*	Saarkama	0.36**	0.11**	0.35*	0.23*	0.25*	0.17*
20.	*Cynodon dactylon*	*Sardoo*	0.09*	-*	0.18**	0.04**	0.41**	-**
21.	*Cyperus sp.*	Saattuu	0.24*	0.14*	0.22**	-**	-	-
22.	*Cyperus bulbosus*	Saattuu arbaa	0.32**	0.07**	0.39*	0.30*	0.31*	0.24*
23.	*Sporobolus pellucidus*	Salaqoo	0.22	0.21	0.15**	0.01**	0.07	0.03

Note. * = significant, ** = highly significant, - =indicates species absent, Rs= rainy season, Ds= dry season

Grass species biomass production of rangeland

In the current study, only grass species that are identified and available to animals for grazing are classified as forage. Data were collected in rainy (May) and dry season (February). Comparison of means for forage biomass production (kg/ha) and grazing status of the three (3) grazing sites (ranch, open-communal and woody covered) during the study period was given in *Fig. 5* below. According to the results, total biomass production from ranch grazing site were 4,584 and 1,890 kg/ha, from open-communal

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grazing site were 1,053 and 453 kg/ha and from woody covered grazing site were 336 and 256.2 kg/ha were recorded during rainy and dry season, respectively. Seasonal variation showed significant impact on the biomass production of rangeland across all grazing sites. 58.8%, 57% and 23.8% higher biomass production was observed during rainy season at ranch, open-communal and woody covered grazing site, respectively. This is due to rainfall is the primary determinant factor for forage production both in the conserved and degraded rangeland area.

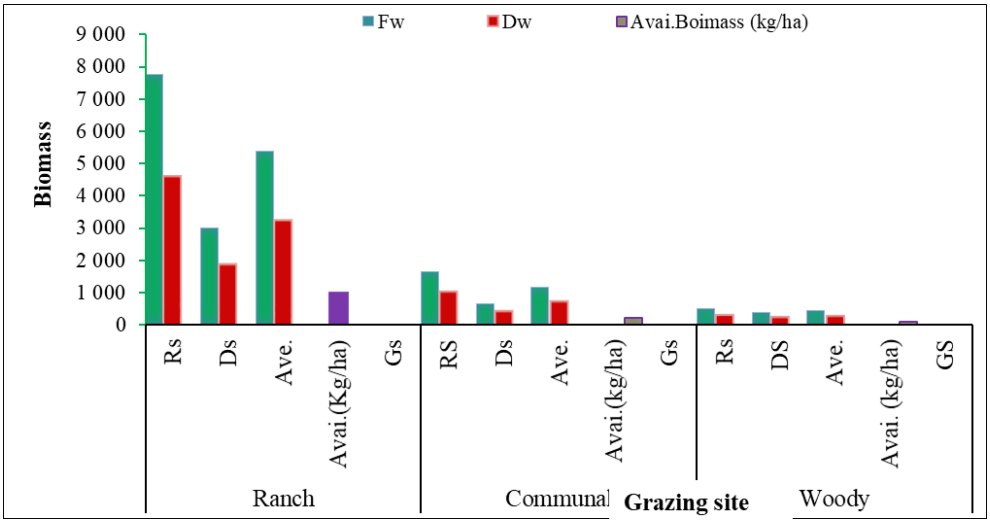

Figure 5. *Seasonal forage biomass production (kg/ha) and grazing status of range sites of the Yabello rangeland. (Note: Fw= fresh weight(kg), Dw= dry weight (kg) Rs = rainy season, Ds= dry season, Ave= average biomass (kg/ha),Avai= availbale biomass (averge dry mass* Uasble factor (0.3)) , Gs = grazing status of the grazing site, 10= repersentes slightly grazing, 4 = represents overgrazing)*

The biomass production rate during dry season also showed the same reduction trend across all grazing site. Overall forage productivity was also high during rainy season across the three grazing sites. Average biomass was higher at well managed grazing site (ranch) as compared to others, that is 3,237 kg/ha, 753 kg/ha and 296.1 kg/ha at ranch, open-communal and woody covered grazing site, respectively. That means, 76.7% and 90.9% higher biomass production was observed from open- communal and woody covered grazing area, respectively. The available forage biomass with a useable factor of 30% recorded from each grazing site was 971.1 kg/ha, 225.9kg/ha and 88.8 kg/ha from ranch, open-communal and woody covered rangeland grazing site, respectively.

Based on the total available biomass production and field observational results the grazing status (Gs) of ranch grazing site was well managed and livestock grazing was done through planned and programed way and the status was under very good condition. It used as a demonstration sample site for different rangeland management practice and awareness creation was conducted at the district and classified as slight grazing category. Whereas, the rest were grouped under overgrazing category (*Fig. 4*) above. Since, rainfall is the major determinant factor for rangeland biomass production, the productivity measured and reported here in our result should be interpreted with caution and it is expected to be valid in an average annual rainfall amount of around 605 mm (which is the case of the data obtained from the Ethiopian metrological authority, for the year 2019

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in our study area) and our conclusions agree with the data reported by Alemayehu (2006), Sisay (2006) and Elias (2007).

Carrying capacity of the rangeland

In this study, carrying capacity was calculated by two methods: (I) using GVI value calculated for each season and yearly for each grazing site (*Table 2*) and the average rainfall for the year 2019 used ranch grazing site as our benchmark and (II) by using biomass method.

Carrying capacity of rangeland using ranch grazing site as benchmark

According to our results showed at *Table 5* and *Fig. 6* below the seasonal and overall carrying capacity of degraded rangelands such as open-communal and woody covered grazing site was low as compared to the ranch grazing site. The carrying capacity during rainy season was 11.22 ha/AU/Y and 11.2 ha/AU/Y, in dry season was 11.27 ha/AU/Y and 11.24 ha/AU/Y with an average overall carrying capacity was 11.24 ha/Au/Y and 11.23 ha/AU/Y for open-communal and woody covered grazing site, respectively.

Table 5. *Carrying capacity of Yabello rangeland at different grazing sites (as benchmark method)*

Grazing site	season	GVI value (%)	Average RF (mm)	CC (ha/AU/y)	CC (AU/ha/Y
Communal	Rs	62.8	605	(-)11.22	0.08919
	Ds	48	605	(-)11.27	0.08872
	Ave.	55.4	605	(-)11.24	0.08894
Woody	Rs	64.4	605	(-)11.2	0.08921
	Ds	54.7	605	(-)11.24	0.08892
	Ave.	59.6	605	(-)11.23	0.08907

Note. Rs= rainy season, Ds= dry season, Ave.= average, GCI= grazing condition index, ha/AU/Y= hectare per animal unit per year, AU/ha/Y= animal unit per hectare per year (Note. To convert AU/ha to ha/AU, divide 1 by AU/ha value (Abdullah et al., 2017)

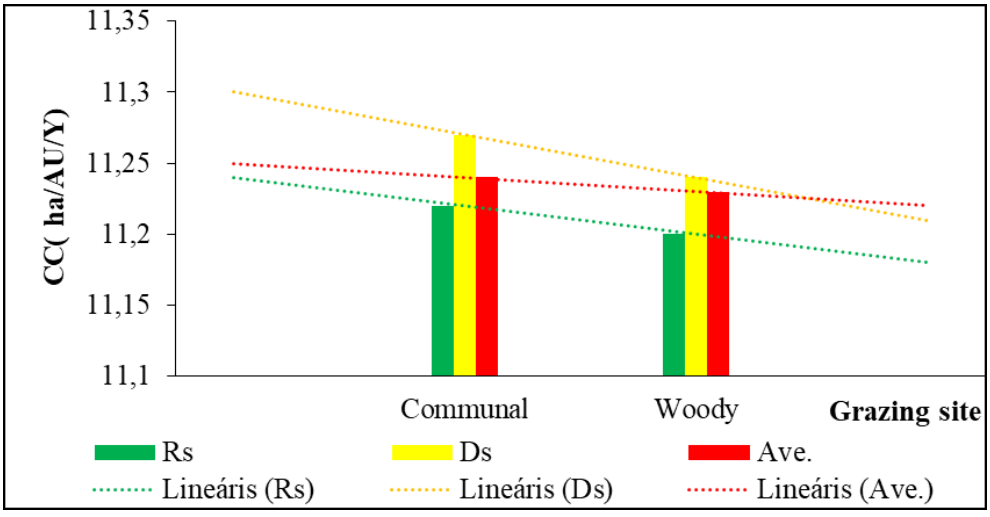

Figure 6. *Carrying capacity comparison of grazing sites (communal and woody) during different seasons*

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The negative (-) sign at *Table 5* indicated the carrying capacity difference as compared with the ranch site. That means, the carrying capacity of the degraded grazing sites were less by 11 ha/AU/Y when compared with the conserved ranch grazing site. In other words, in order to support equal number of livestock overgrazing rate would have been zero or no overgrazing would have been observed in ranch grazing site, while in the case of open-communal and woody grazing site the overgrazing rate would have been 11 ha/AU/Y. That means the degraded grazing area was exposed to overgrazing 11 times higher than the normal or recommended capacity that can support. From this we can understand that in order to support equal number and type of livestock it would be needed additional 11 times grazing site area in case of degraded (open-communal and woody infested) grazing site.

In general, from this result does not mean that the carrying capacity of grazing site both at rainy and dry season were equal. Instead, the dry biomass production at rainy season was higher and it is clear that during rainy season the carrying capacity of Yabello rangeland was better across all grazing sites. Therefore, biomass productivity ultimately decreased due to overgrazing and infestation of woody invasive plant species leading to poor carrying capacity. Our result is in agreement with the data reported by Solomon et al. (2007) for the same study site using different method.

Carrying capacity by biomass method

This technique clearly provided visible and clear information about the impact of seasonal variation and overgrazing on biomass productivity potential and carrying capacity across the grazing site.

Carrying capacity value ranged between the interval 2.99 to 53.40 ha/AU/Y or 0.33 to 0.019 AU/ha/Y with the average yearly value of 4.23 to 46.24 ha/AU/Y or 0.24 to 0.022AU/ha/Y (*Table 6*). When we have seen seasonal carrying capacity difference across the grazing site 58.7%, 57% and 23.7% better carrying capacity potential was observed from ranch, open-communal and woody covered grazing site, respectively during rainy season as compared with dry season. Based on this we can concluded that, it is crucial to analyze the vulnerability of pastoral livelihoods to combined threats within a risk-prone environment and developed adaptation strategies to reduce the impact of drought on their livestock and our result is highly constituent with the data reported by Habtamu (2013), Angassa and Oba (2010) and Bat et al. (2016).

Table 6. *Carrying capacity of Yabello rangeland at different grazing site (Biomass method)*

Grazing site	Season	Biomass Kg/ha	Available biomass Kg/ha	CC ha/AU/Y	CC AU/ha/Y	Grazing status value
Ranch	Rs	4,584	1,375.2	2.99**	0.33	10
	Ds	1,890	567.0	7.24**	0.14	
	Ave.	3,237	971.1	4.23**	0.24	
Communal	Rs	1,053	315.9	13.00**	0.077	4
	Ds	453	135.9	30.22**	0.033	
	Ave.	753	225.9	18.18**	0.055	
Woody	Rs	336	100.8	40.73**	0.025	4
	Ds	256.2	76.9	53.40**	0.019	
	Ave.	296.1	88.8	46.24**	0.022	

Note. ** = highly significant, CC= carrying capacity, Rs= rainy season, Ds= dry season, Ave.= average, GCI= grazing condition index, ha/AU/Y= hectare per animal unit per year, AU/ha/Y= animal unit per hectare per year. And under grazing status value column,10 = is value of slightly grazing, 4= is value of over grazing

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The maximum carrying capacity was observed at well managed (ranch) grazing site throughout the year (both in rainy and dry season). That was 2.99 ha/AU/Y, 7.24 ha/AU/Y and 4.23 ha/AU/Y at rainy season, dry season and in yearly, respectively. The carrying capacity potential of ranch grazing site showed higher values of 77%, 76% and 76.7% compared to open-communal grazing site, and 92.7%, 86.4% and 90.9% compared to woody-plant infested grazing site during at rainy season, dry season and yearly respectively. The reason for the low carrying capacity potential of non-conserved grazing site (communal and woody) is that degradation resulted from overgrazing and infestation of woody plant species in combination with both climatic and anthropogenic factors. And the current status of open-communal and woody grazing site is under degradation because of overgrazing (*Table 6*). Overall, the rangeland of Yabello showed overgrazing and changed to non-usable stage because of serious degradation, which challenges the survival rate of livestock, livelihood of the pastoral community and the economy of the country.

Conclusion

Based on our overall results, we clearly understood that the seasonal variation and grazing site management difference had a great influence on the available grass basal area coverage, height, biomass production and carrying capacity potential of rangeland. It helps to put a baseline data for decision how it is valuable to conserve and manage grazing site based on the data recorded from our benchmark site, since plant cover, dry mass and carrying capacity are the primary indicators for how a certain range land site would be used sustainably without further deterioration. In Yabello rangeland the pastoralists community are more challenged because of the factors like overgrazing, invasive plant species infestation, frequent drought and other climatic and human factors caused for rapid decline of biomass production and carrying capacity rate. The main target to quantify the change of optimal grazing site with relation to grass basal cover, biomass production, growth height performance and carrying capacity was to create awareness with in the community and the scholars with regards to how to conserve further degradation by incorporating scientific techniques and applying practical immediate managerial decision. The forage biomass production in Yabello area is primarily determined by the inconsistence variability of rainfall and rapid infestation rate of woody plant. The overall biomass production in the rangeland was not adequate to meet the requirements of the tropical livestock unit in the area. There is a big difference between the conserved and non- conserved grazing site due to the ultimate influence of overgrazing and woody plants. However, this study was preliminary, based on the result we highly recommend that subsequent ecological studies should be conducted on spatial and temporal variations of forage production. The productive potential of rangelands is not the same across the study site and carrying capacities are needed to be periodically reviewed to accommodate any changes in land resources, or environment. There is a severe problem of overgrazing that leads to year-round stress on grazed species. Therefore, the adjustment of stocking rate is compulsory and planned grazing should be introduced and implemented to release the stress over grazed species.

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

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ANALYSIS OF ECO-ENVIRONMENTAL VULNERABILITY: IMPLICATION FOR BUSH ENCROACHMENT AND LIVESTOCK POPULATION DYNAMICS OF THE TELTELE RANGELAND, SOUTHERN, ETHIOPIA

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Abstract. Eco-environmental vulnerability is one of the challenges of Teltele rangeland Southern, Ethiopia. This research aimed to analyzing change of grassland area to bush covered area via remote sensing method using NDVI values, temperature, perception, land use change and the local community background knowledge from 1990-2015. The eco-environmental Vulnerability Integrated Index (EVSI) for each class,1990-1995, 1995-2000, 2000-2005, 2005-2010 and 2010-2015 shows an upward trend. That is 1.59, 2.61, 2.91, 2.67 and 2.8 respectively for each interval. The higher EVSI value, is the more serious impact of eco-environmental vulnerability or encroachment of the bush plant coverage. Based on the NDVI data, the net change in open bare grazing area to bush covered vegetation over the past 25 years was 43.2%. The key factor in the rapid encroachment of bush vegetation was the frequency of drought (El Niño), causing a decline in the population of pastoralist community of Teltele were faced with poverty due to their livelihood. As a result, trend of livestock population dynamics shows a decreasing in the pattern. Based on the current results, the design of the scientific management techniques for eco-environmental vulnerability and raising awareness in the local community will be the priority area for further studies.
Keywords: *NDVI, invasive species, degradation, pastoralist, remote sensing*

Introduction

Environmental vulnerability has been defined as a significant impact observed due to the variation of the mean state of climate relevant variables such as temperature, precipitation and wind over a period of time (IPCC, 2007) and exposure to climate change, sensitivity and adaptive capacity (IPCC, 2001) and became a major threat to humanity (Watson et al., 1998; O'Brien and Levchenko, 2000). Mainly in Africa, the impact of vulnerability to climate change is difficult compared to other developed countries, because of the lack of economic, development and institutional capacity (IPCC, 2001; Sithole and Murewi, 2009). Poverty, human diseases and high population density with high demand for food, water, and livestock forage are among the major impact due to climate change (Davidson et al., 2003; Thomas et al., 2000; Masike and Urich, 2008). Arid and semi-arid environments of most part of the world including Ethiopia, mainly characterized by high inter-annual rainfall variability and reoccurring

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droughts (Ellis and Swift, 1988; Mogotsi et al., 2012) which are likely to be exacerbated by climate change (IPCC, 2013). And also, the Teltele rangeland, Southern, Ethiopia highly faced such climate impact like drought and flooding. In the rangeland areas livestock production sector as a livelihood option is one key sector which will bear the brunt of these climatic conditions fluctuation (Stige et al., 2006; Sithole and Murewi, 2009).

Livestock rearing and farming are the most important source of income for the people lived in arid and semi-arid rangeland area in most parts of the world. In most rangeland area of Ethiopia, including Teltele rangeland livestock rearing is the direct source of income for local pastoralist communities (Bongers and Tennigkeit, 2010). It is a home for the livestock production sector as a woreda and national level with 95% of the family income source rely on livestock in the area (Tache and Oba, 2010). According to report of different studies (Li et al., 2012; Shapiro et al., 2017), estimated direct contribution of livestock production in lowland pastoral systems of Teltele in combination with other part of Ethiopia to agricultural growth domestic product (GDP) and national GDP to be 39 and 17%, respectively. The area supports more than 70,500 families with an annual population growth rate of 2.5–3% (Dalle et al., 2015). Livestock production dominated by the Borana breed and it was been the major source of livelihood for Teltele pastoralists. This breed is one of the most productive, fast-growing and fertile as compared with other indigenous cattle breeds in Ethiopia (Elias et al., 2015). Cattle, goats, sheep, camel, mule, donkey and horse are the main livestock species reared on the study site. According to central statically agency report in, 2014 to 2015 there were a total of 499,719 (197,876 cattle, 185,846 goat, 105,158 sheep, 4 horses, 65 mule, 9,704 donkey and 1,062 camel) livestock population were recorded in Teltele rangeland. Livestock play a crucial role in the subsistence economy, culture and religion of pastoralists in Teltele rangeland, and represent both social capital and an insurance against disaster (Angassa and Oba, 2008). Teltele pastoralists are used their indigenous knowledge for rangeland and water resource management strategies.

Now adays, threatening factors mainly due to environmental and socio-economic changes may severely affect ecosystem services of the rangelands, such as forage supply and carrying capacity potential in worldwide and also highly happened in Ethiopia rangeland area, particularly in Teltele rangeland (UNEP, 2009; Augustine, 2010; Brink et al., 2014). Climatic factors like drought and unpredictable rainfall have a huge impact on the rangeland's vegetation status and its productivity (Neely et al., 2009; UN, 2011). In several part of Ethiopia, including Teltele, mean annual precipitation is projected to decrease by 10 to 20% (Haile et al., 2010; Elias et al., 2015) and climate change is therefore, expected to threaten the pastoralist livelihoods. Under which local circumstances, changing rainfall characteristics may limit the ability of pastoralists to secure their livelihood sustainably if they depend only on local forage resources is an open question (Angassa and Oba, 2008). Drought also one of the major causes for rapid encroachment of Bush plant species, since this species can easily adaptive potential with environmental change as compared with native plant species. The vulnerability of pastoralists is becoming severe due to the recurrent occurrence of climate change, due to both climatic and anthropogenic factors and caused the declining of carrying capacity of the rangelands (Sulieman and Elagib, 2012). Therefore, it is crucial to monitor and analyze the vulnerability of pastoral livelihoods of the pastoralists and develop adaptation strategies to reduce its impact both economic and

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social aspects (Funk and Browny, 2006; Fensholt et al., 2012). Traditionally, many pastoralists have used the installation and movement of their herds near the trading center, areas accessible to forage and water points as an adaptive method when climate change like drought occurred (Morton, 2010; Thornton et al., 2009; Sulieman and Elagib, 2012).

Furthermore, the issues like government policies, frequent impact of climate change, the increase of both livestock and human population and other political issue from time to time limits this traditional movement of the pastoralists and this make the degradation of Teltele rangeland severe in combination with climate change factors. As a result, the Teltele livestock practice, quantity and quality practice were affected by chronic interactions stocking rate and environmental variables like rainfall, soil erosion and invasive plant species encroachment and its impact is not understood and quantified yet. In order to monitor the change in vegetation, NDVI data (focused on pixel change) in combination with climate data (rainfall and temperature) evaluation was mandatory (Gu et al., 2008). However, to date, there is no any documented study data about the impact of environmental vulnerability of Teltele rangeland and associated communities entirely dependent on the rangeland. This become one of a major gap for substantiable rangeland management through balancing grazing capacity and maintain livestock performance. So, the main objectives of this study were to: (1) analyzing spatial-temporal patterns of vegetation change due to eco-environmental vulnerability by using normalized difference vegetation index (NDVI) satellite data and climatic data, (2) evaluating the effect of change in climate parameters on livestock population dynamics and (3) assessing the strategies employed by the Teltele pastoralists to reduce impact of climate cahnge. The basic question of the study was, how does environmental change become a major cause for bush encroachment in the rangeland area? How encroachment of bush plant species effect on the rangeland productivity? Simply stated, the null hypotheses of this study were: (1) environmental change does not any role for rapid encroachment of bush plant, (2) Push plant species does not have any effect on rangeland forage productivity and (3) there is no any effect on livestock population due to environmental variability.

Materials and methods

Study site

The study was conducted in Teltele Woreda of Borana zone, Southern, Ethiopia (*Fig. 1*) which covered an area of 15,430 km^2 of which 68% (10,492 km^2) is rangeland. The site was selected because it is one of the most arid parts of Borana zone and therefore, pastoral communities in this region are the most vulnerable to climatic variability. It is located 666 km south of Addis Ababa, the capital city of Ethiopia. It lies approximately between 04° 56’ 23”N latitude and 37° 41’ 51”E longitude and the altitude are about 496 m to 1500 m, the maximum altitude of 2059 m. The annual mean temperatures vary from 28 °C to33 °C with little seasonal variation. The rainfall of the site is characterized as bi-modal. Which is the 60% of rainfall occurs from March to May, and 27% from September to November with high temporal and spatial fluctuations (*Fig. 2*) (Dalle et al., 2015). The potential evapotranspiration is 700-3000 mm (Billi et al., 2015). The soil in the study site includes, 53% red sandy loam soil, 30% black clay, and volcanic light-colored silt clay and 17% silt and the vegetation mainly dominated by encroaching woody species, and those that frequently thinned,

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include *Senegalia mellifera*, *Vachellia reficiens* and *Vachellia oerfota* (Gemedo et al., 2005; Coppock, 1994). According to the latest 2015 national census report a total population of the study site was 339,460 in total (22/km^2) and of whom 179,518 men and 159,942 women. And 50,944 (15%) of its population are urban dwellers and the remaining 288,516 (85%) of the population are living in the rural area mainly on livestock rearing activity.

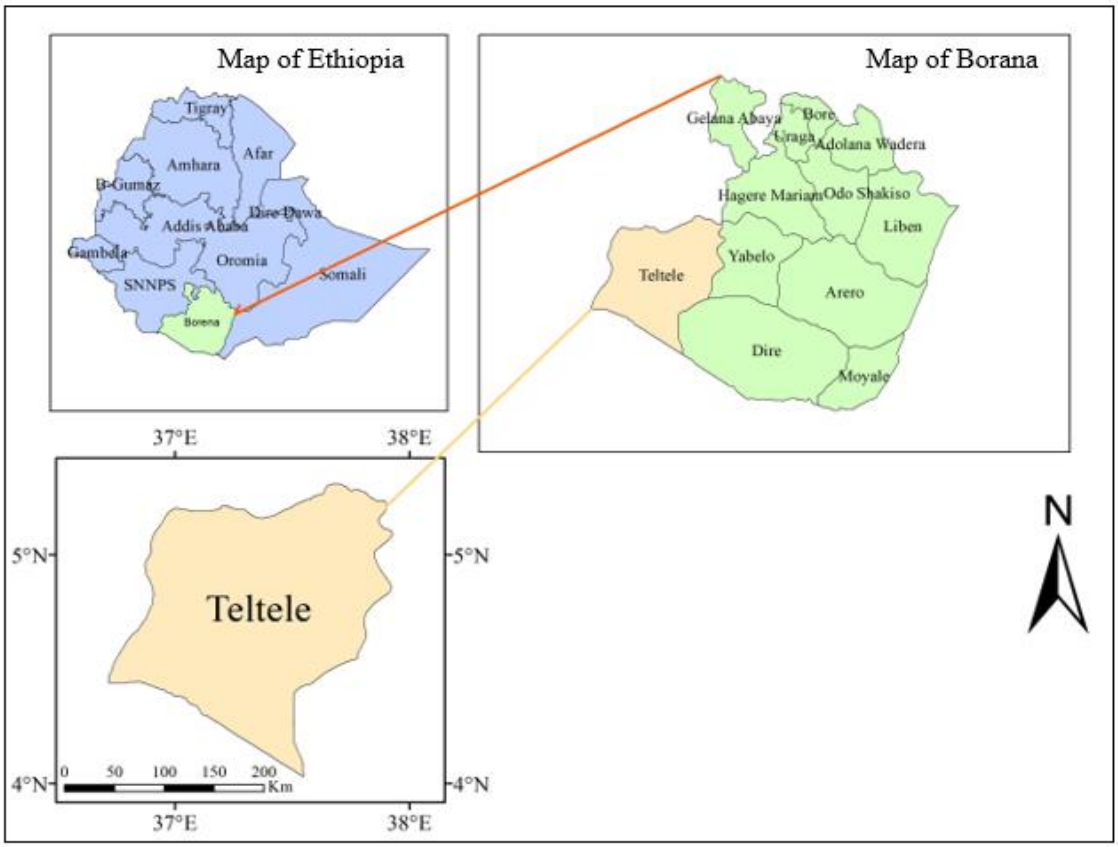

Figure 1. *Location map of the study area*

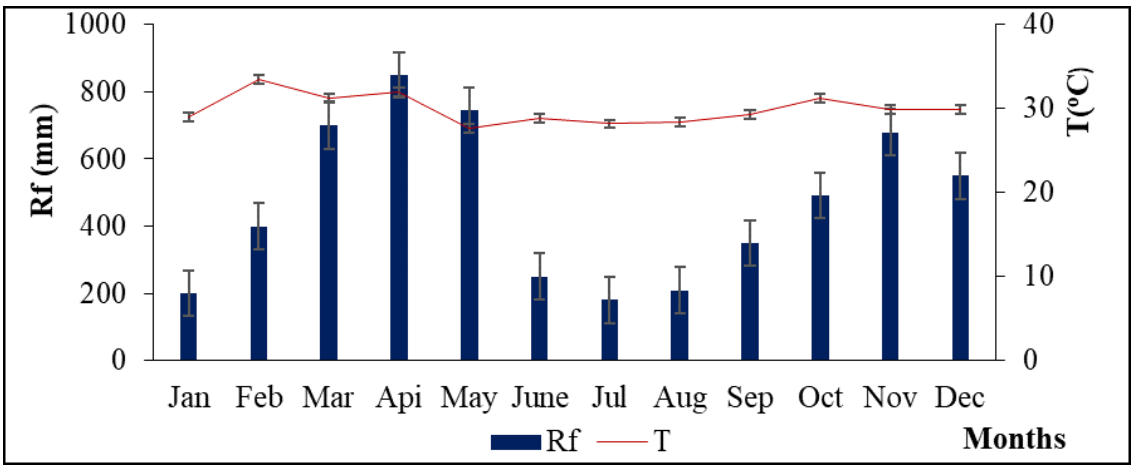

Figure 2. *Monthly mean (±SE) temperature and rainfall pattern for Teltele from 1990-2015. T = temperature, Rf = rainfall. (Source: EMA, 2015)*

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Dataset

This study combined multispectral satellite remote sensing data, in-depth fieldwork surveys and rangeland use policy analysis linked with rangeland vegetation change source. It seeks to compile some of the perceptions and experiences of local communities and individuals who are on the frontlines of climate change. The traditional weather patterns of the area, historical trends (1990-2015) in climatic variables (temperature and rainfall) collected from Ethiopian meteorological authority (EMA, 2015) and using geographical information system (GIS) and remote sensing (RS) data in order to analyze eco-environmental vulnerability. To monitoring the spatial and temporal variation in vegetation in the study area, we used the annual average of third Generation Standard Difference Vegetation Index (NDVI3g) data (1990-2015). Then from the general precipitation, temperature and NDVI data derived from the Global Inventory Modeling and Mapping Studies (GIMMS) with 8 km grid resolution of Ethiopia, we resample our study area to digital elevation model of 300 m resolution in order to get our focusing site data with high resolution, because we only need the satellite data of our study (Teltele) rangeland site (*Fig. 3*). For NDVI grid cell values we simply took the maximum, minimum and an average annual mean value in order to reduce disturbance in the trends, such as those attributable to bare soil and sparsely vegetated areas (Slayback et al., 2003; Wang et al., 2011). Vegetation maps of the Teltele district in 1990-2015 were obtained from the remote sensing data with spatial scale 1:100,000. The Landsat TM imageries acquired in five-year interval 1990-1995, 1995-2000, 2000-2005, 2005-2010, 2010-2015 and also changes from 1990-2015 were used for range land vegetation pattern and degradation assessment and the characteristic of Landsat used for analysis was described in *Table 1*. These years were chosen because of the availability of data, the quality of the images, and in order to compare the changes with in equal time intervals. And also, further, verification was done through interviews and semi-structured focal group discussions with the local pastoral community and stakeholders for the accuracy of the rangeland current status and vegetation degradation images analyzed by using ArcMap 10 software.

Socio-demographic profile of the respondents

To understand the level of pastoralists awareness on the changing climate, the types and nature of the impacts, the changing climate variability has had on the livestock production, the coping strategies that pastoralists have employed in reaction, as well as the existence of external support to help the pastoralists adapt to the climate change impacts was collected through both informal and formal group discussion with pastoral community, individual and key informant. Interview questionnaire were composed of both structured and unstructured questions and basically focused on the issues such as: How climate change trend looks like on the study area, its influence on the grazing lands and local communities, the possible causes, the main problems of climate change on the range land, traditional coping mechanisms and extension service provided against these condition were explored. The discussion was used as a means of creating awareness in the pastoralist's community, generating ideas regarding issues related to rangeland management and coping mechanisms. In order to obtain a representative sample data for the study site, both stratified and clustered sampling methods were applied for all pastoralists and stakeholder selection. Since, the pastoralists commonly live in scattered way, rather than nuclear clustering of households was necessary.

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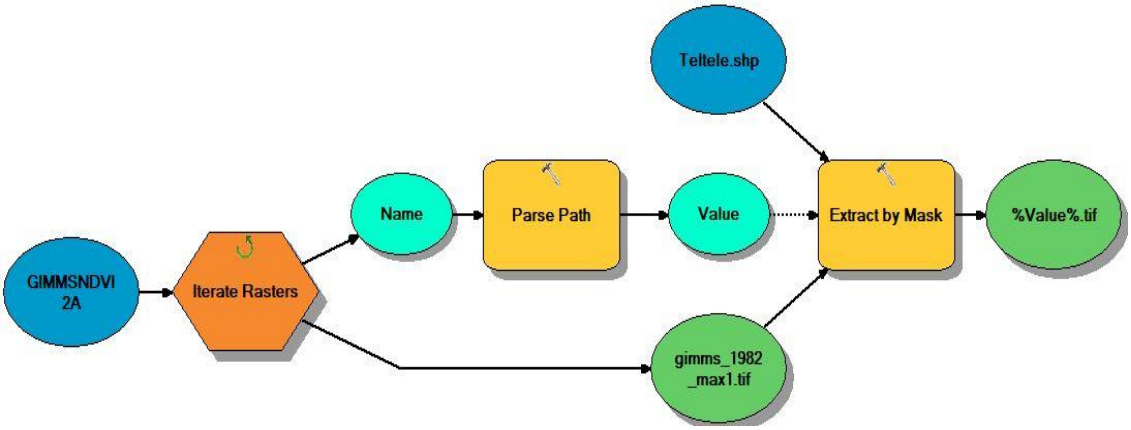

Figure 3. *Schematic representation of the model we used to extract and resample our study area data*

Table 1. *Characteristic of Landsat used for LULC change analysis*

Data	Year of acquisition	Bands/color	Resolution (m)	Spectral resolution/bands
Landsat Thematic Mapper (TM)	1992, 1995, 2000, 2005, 2010, 2015, 2019	Multi-spectral	300	Band 1-5: 0.45-1.75 Band 6: 10.4-12.5 Band 7: 2.08 – 2.35

A total of 104 (69 males and 35 females) head of households were interviewed. From this key informant purposefully selected based on age (>35), experience, training participation and way of better life based on the recommendation of the local people and stakeholders (n = 20 in total, 14 males and 6 females) with addition of 4 government official worker stakeholders (3 males and 1 females) from different sectors (livestock, agriculture, tourism and meteorology), and the remaining 80 (52 males and 28 females) were selected randomly based on the our clustering. The total number of sampling population was mainly depending on our time and budget, but we tried to made it representative of the study site. Such approaches have proven effective with Ethiopian pastoralists like Teltele because these people place very high cultural value on livestock and have well developed mental skills to track animal inventories (Solomon and Coppock, 2002). The trends of the priority livestock (cattle, goat, sheep and camel) population dynamics and production performance indicators, such as birth rates, death rates and off-take rates for each livestock species, was collected from statistical abstracts of Teltele district, southern Ethiopia for the last twenty-five (25) years. The general framework methodology we adopted to identified the change of precipitation, temperature and the vegetation index change using NDVI was summarized in *Figure 4*.

Analysis of eco-environmental vulnerability change trend

In order to understand the cause of vegetation change in the study area, the general trend of environmental viability was assessed through both interview and group discussion with the local communities in addition to the satellite data. Spatial analyses of vegetation, precipitation, temperature and NDVI change were done using ArcMap

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10.3.1. Study site statistics function was used to extract NDVI values of the Regions of Interest (ROIs) from corresponding vegetation types for all months from January to December for the years 1990-2015. The data were coded, tabulated and analyzed using Microsoft excel and SPSS. The data collected from the officials and key informants were analyzed through descriptive narration and also using SPSS based on the research questions. Then, the Eco-environmental Vulnerability Integrated Index (EVSI) for each category was calculated using *Equation 1*.

$$EVSI_j = \sum_{i=1}^{n} P_i \times \frac{A_i}{S_j} \quad \text{(Eq.1)}$$

In this formula, n = is the number of degradation level (in our case n = 4, that was described under result section in *Table 3* and *Fig. 9*), EVSIj = the EVSI in unit j, A_i = the occupied area of degradation level i in analysis unit j (area of pixel for each level or assign number), Sj = the area of analysis unit j (total area of analysis), and P_i = is the leveled or assign number value of level i (in our case i = 1, 2, 3, 4) (Gunnula et al., 2011; Yang et al., 2016; Xu et al., 2010; Ainong et al., 2006).

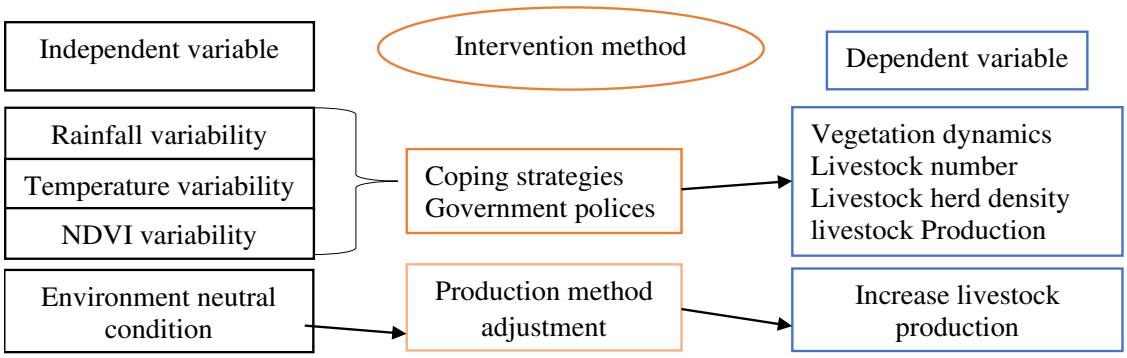

Figure 4. *Conceptual framework for this methodology (developed by Author, 2020)*

Results and discussion

Socio-demographic characteristics of the respondents

The respondents' gender, occupation and level of education were some of the main demographic feature's that the authors considered for this study to remove the biased from our data.

During data collection we also tried to considered the gender proportion into account within the participants. But, in the area and in the country in general, the activity of field work including farming, livestock rearing and other outside activity mainly performed by male household, whereas, females are mostly focused on housework including keeping children, prepared food and others. As a result, the experience and knowledge about rangeland status mostly obtained from male households. However, we tried to included and balance gender involvement during our data collection i.e. 66% of males and 34% of females of the total number of participants. This distribution made it possible to understand the perception and coping method of adaptation to climate vulnerability of male and female pastoralists. Then, the level of education is one of the

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basic factors on the socio-economic practice within a family and as we have seen from *Figure 5,* the majority of pastoralists (78%) were illiterate followed by primary education (11%) level. This is because of the educational system was still not well exercised and adapted, due to lack of awareness about the value of being educated and these leads the community did not enforce to accesses the infrastructure with in the pastoral community based on the data obtained from the respondents. And also, to some extent, the situation has occurred in some way to other parts of the country. As a result, most of the livelihood community that depends on livestock occupation has been dispossessed, and this was the major factor that caused most of the pastoralists source of income to depend on livestock rearing (66%) followed by running their own business beside it (19%). Considering, their educational level, our questioners were focused on their experience observation and impacts based on their expectation, rather than scientific explanation.

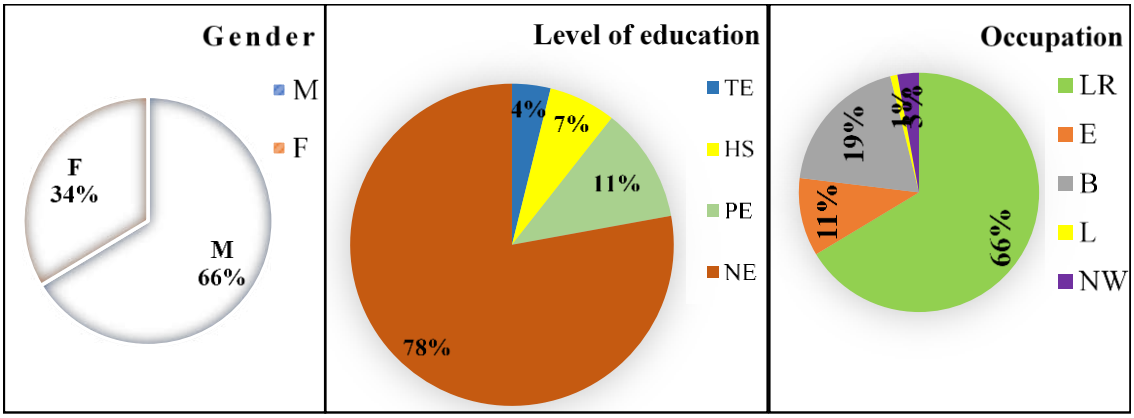

Figure 5. *Distributions of respondent's demographic characteristics. (M = male, F = female, TE = tertiary education (college and university), HS = high school, PE = primary school, NE = not educated, LR = livestock rearing, E = employment, B = business, L = labor work, NW = Not known work)*

Analysis of eco-environmental vulnerability

Trend, pattern and impact of environmental vulnerability based on respondent's Perception

In order to understand the trend, pattern and followed impact of climate change (mainly rainfall and temperature) from their experience and daily activities, respondents were asked the following basic questions: (1) Is there any change observed in the recent year? (2) What was the nature of the change? (3) what was the impact of the change? And (4) What is the possible reason that cause the change? Their ideas and response were described in *Figure 6* and *Table 2*.

From *Figure 6* we clearly understand that majority of the respondents confirmed the occurrence of climate change (93.3 and 89.4%), trends of change (97.1 and 92.3%) and its associated impact (98.1% and 84.6%) for both rainfall and temperature respectively. Respondents mentioned that climate change was significant impact on forage production and water availability in the rangeland, that affected their livestock health and caused death and a decrease in the number and of productivity, which had a direct effect on their livelihoods.

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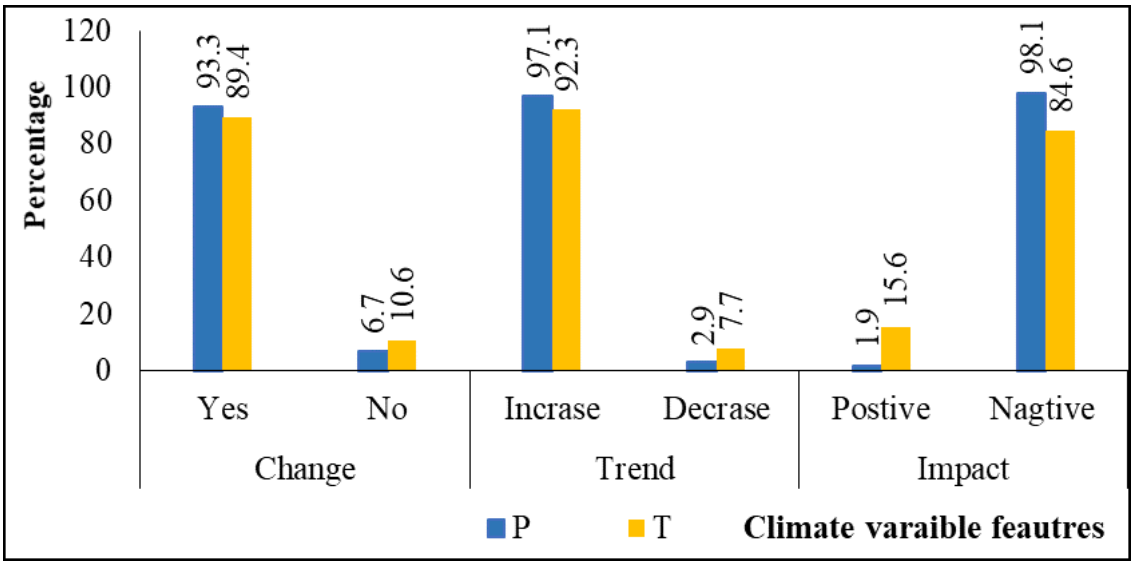

Figure 6. *Respondents data with trend, pattern and impact of eco-environmental vulnerability. P = precipitation, T = temperature*

Table 2. *Drivers that cause climate change in Teltele rangeland*

No.	Possibilities	Number of respondents (n)	Percentage (%)
1.	Drought	24	23.0
2.	Increase population number	16	15.4
3.	Poor social- interaction	7	6.7
4.	Bush encroachment	35	33.7
5.	Gods plan and nature	6	5.8
6.	Government policies	11	10.6
7.	Insects and disease	5	4.8
	Total	**104**	**100**

With regards to the major drivers of impact of climate change on vegetation in Teltele rangeland, the bush encroachment ranked as the primary reason (33.7%), followed by drought (23%) and increase of the population both human and livestock in the district (15.4%) without additional land provided (*Table 1*). Government policies have also had their own impact on the livelihood of pastoralists in Teltele, which promote transformation of rangeland into cultivated land and restrict the movement of pastoralists who were traditionally used to coping climate change impact. Not only this but also government policies also caused for the introduction and expansion of invasive plant species on the rangeland area due to a reason for watershed activates before scientifically assessed its long-term impact. Most of the invasive bush species on Teltele rangeland introduced and expanded due to the above-mentioned reason. And also, the policies restricted from eradicated all invasive plant species from the rangeland by the local communities in the name of forest deforestation activates and punished the one who participated on such activity. Further, free movement of pastoralists for searching food for their livestock from one area to another area was restricted and enforced them to spent in specific grazing area for a long period of time and caused both degradation

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and scarcity of forage for their livestock. Both productivity of rangeland and their livestock decline and faced for poverty and scarcity of food. As a result, such government policies on the study area became caused for climate change and degradation of rangeland because of rapid encroachment of non-native spices. But there is still a big gap under the term of climate change, rather the pastoral community thought that it was due to God's plan and to the nature which could be intended to punish us. This was an indicator of community's low awareness of the climate change with which the whole world is grappling with and our data are consisted with the data reported by Han et al. (2008).

Effect of climate change on vegetation pattern

Among the major driving factors of vegetation degradation in arid and semi-arid rangeland area like Teltele, a frequently occurrence of climate change, mainly a decrease in rainfall pattern and increase of temperature. The remote sensing data from these results indicated that the annual temperature was going to increase on an alarming scale and on the contrary annual rainfall rate shows a decline trend (*Figs. 7* and *8*). This told us in the vegetation of arid and semi-arid rangeland that the eco-environmental vulnerability was high and resulted in vegetation degradation and followed the decline of livestock population dynamics and faced a change in the pastoral livelihoods. Then, our result directly related to the data reported by Lin et al. (2013). In the pasture rangeland of Teltele, the frequency of eco-environmental vulnerability creates favorable conditions for rapid encroachment of invasive bush plant species which compete with the native grass species. And the grazing rangeland area was becoming changed to bush covered non-grazing area (*Fig. 7*) and leads to scarcity of forage for livestock. Comparing the level of degradation of vegetation dynamics from 1990-2015, the significant open degraded area was observed in during the study period from 1995-2000, which major part of area were bare due to high drought occurrence especially in 1999 what is called El Niño occurred (1.05 °C increasing was recorded), but in 2010-2015, this open degraded area was becoming encroached by bush plant species and change to green area. The vegetation greenness changed from 1990-2015 showed appositive value. From this we can understand that in Teltele rangeland encroachment rate of invasive bush plant species was significantly rapid due to eco-environmental vulnerability of the area. Currently, this is the big challenge for pastoralist community to covered all rangeland site and also their farming area.

From *Figures 7* and *8* we can clearly understand that both precipitation and temperature variability had significant impact on the rangeland vegetation index in Teltele. The trend of temperature change from 1990-2015 showed a positive value both the highest (1.32 °C) and lowest (0.83 °C) recorded value. The rainfall pattern also showed both positive highest value (206.14 mm) and negative lowest value (-190.10 mm) and this told to us the maximum amount of rainfall showed an increased pattern and that cased for flooding and caused for rangeland degradation. Whereas, the lowest yearly recorded rainfall showed a decline trend and since lowest rainfall is very essential for vegetation growth and also for recharge ground water table that is important for increasing both water and forage availability for livestock. But the normal rainfall distribution became decline and this is the real cause for degradation in Teltele rangeland happened currently. In the period between 2000 and 2005 the rainfall distribution was recorded highest both yearly maximum (863.15 mm) and minimum (189.58 mm) and the highest flooding that highly degraded the rangeland area was

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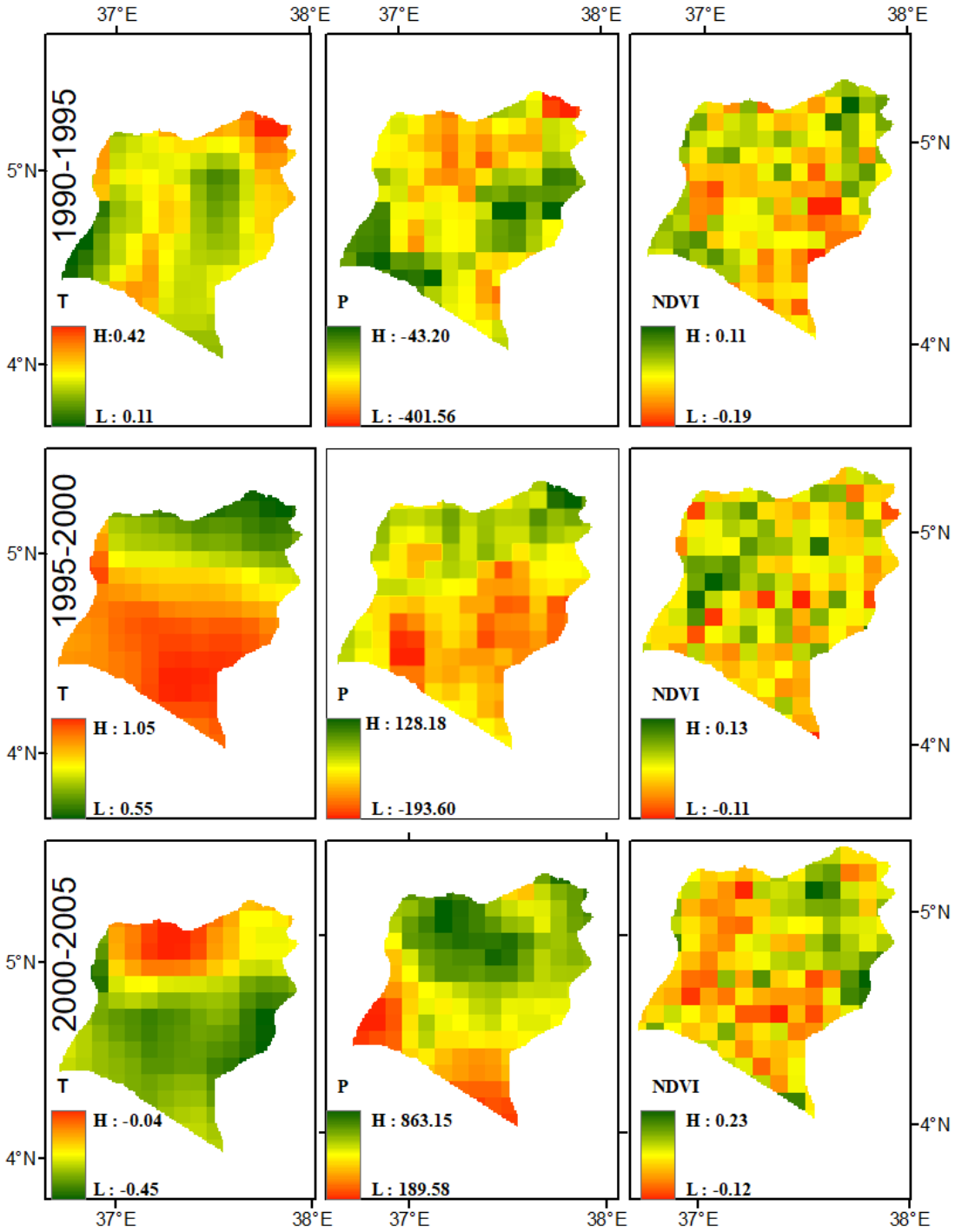
recorded and at the sometime encroachment of bush plant species got favorable reached highly occupied most part of the rangeland (highest NDVI value = 0.23) and less competition from native plant species (*Fig. 7*). The net change of both highest and lowest recorded temperature value in Teltele rangeland from 1990-2015 was showed significantly increasing pattern. The mean highest rainfall value trend also showed an increasing and this up normal maximum rainfall amount caused for rapid degradation of rangeland soil and other resource, but the normal or minimum rainfall distribution showed a decreasing trend. Therefore, net changing trend of rangeland vegetation greenness also showed variation with related to highest and lowest value.



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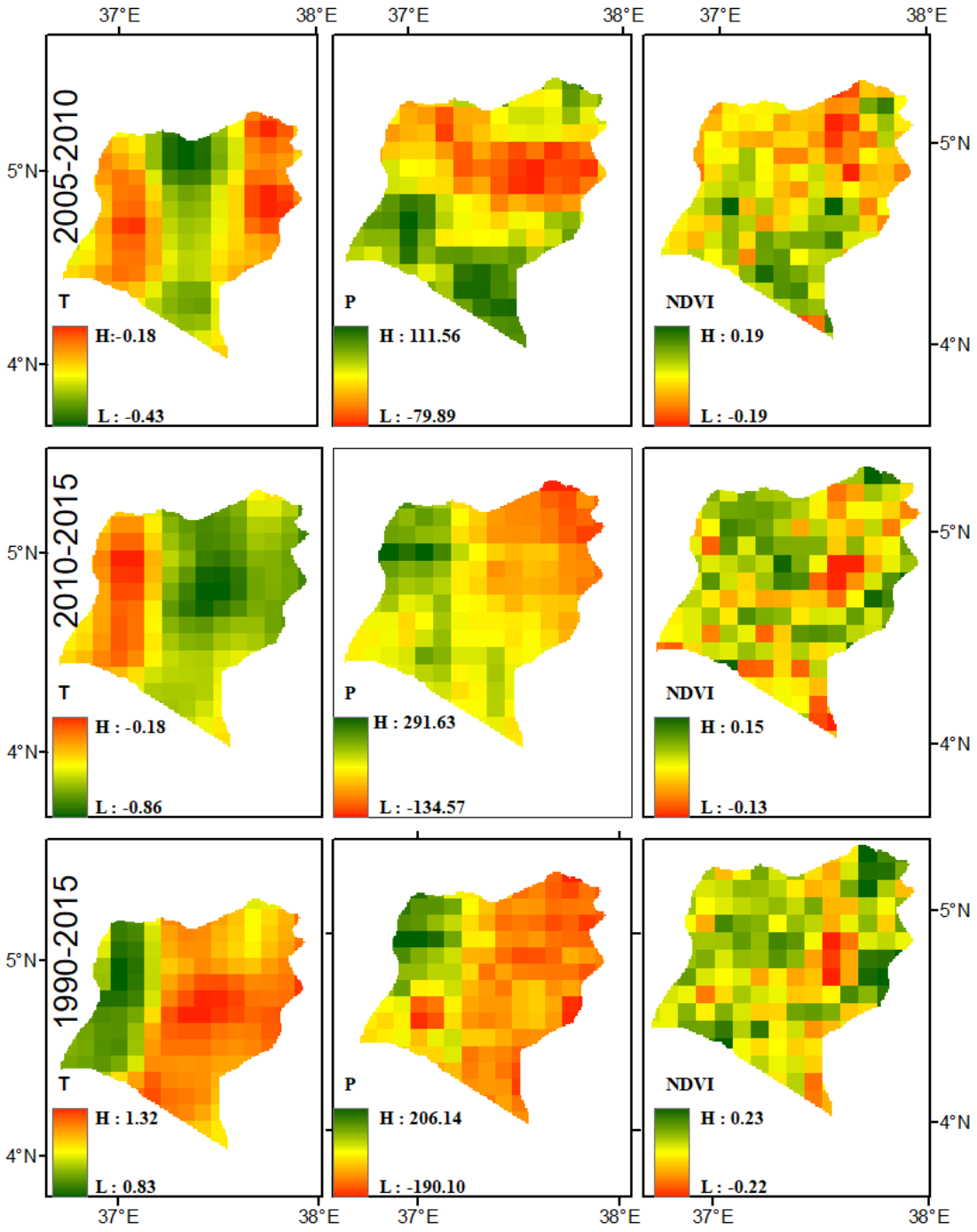

Figure 7. *Temperature, precipitation and NDVI change trends for Teltele rangeland from 1990-2015, T = temperature, P = precipitation, H = high value, L = low value*

The highest NDVI greenness value highly related with encroached bush plant species that occupied major area and easily detected by satellite data, showed appositive value (0.23) indicated that rapid encroachment of non-native spices on the Teltele rangeland. Whereas, the lowest NDVI value showed a decreasing trend (-0.22) and this highly related with the grazing grass species and showed a decline due to the mentioned eco-

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environmental factors. This variation depends on the fluctuation patterns of climate change (rainfall and temperature). Mostly, if the temperature was low and rainfall was high (2000-2005), the rangeland vegetation index (NDVI) value was high. Since, the rangeland vegetation phenology was high during the periods of heavy rainfall. The general trend of the vegetation index dynamics of the Teltele rangeland with respect to the slope analysis, was calculated based on *Equation 1* given by Yang et al. (2016) and Xu et al. (2010) using the mean NDVI value. Based on the calculated result, the Teltele rangeland had a positive (0.0055) slope value. Rangeland with a positive slope value indicate an increasing trend, while those with a negative slope value indicate a decrease trend of vegetation greenness (Cai et al., 2014). Climate change is the main driving force both either directly and indirectly for change of vegetation index, especially in the arid and semi-arid rangeland like Teltele. For instance, for rapid encroachment rate of bush plant species climate impact is the major root cases.

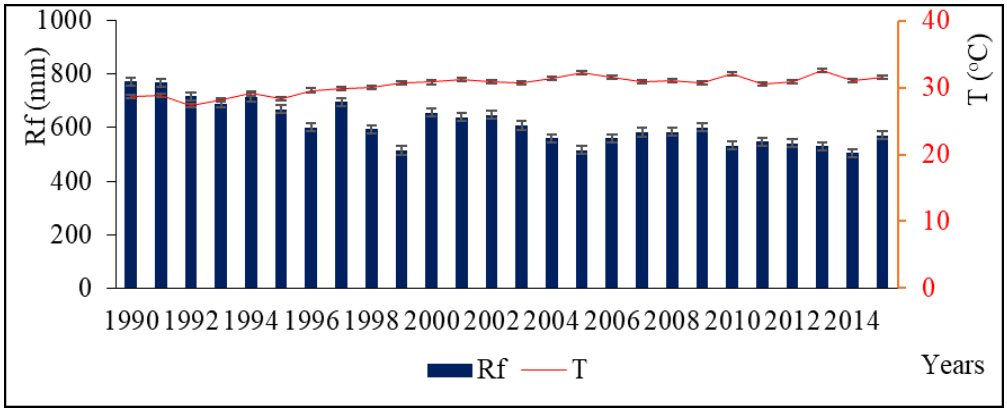

Figure 8. *Annual mean (±SE) rainfall and temperature for Teltele rangeland from 1990-2015, Rf = rainfall, T = temperature*

However, most of eco-environmental vulnerability studies focused only on the numerical change value (Amting, 1997) without considering the spatial variation of the rangeland. From our result, we can understand that the eco-environmental venerability of vegetation dynamics varies according to the slope variation. A rangeland sites with higher slope or elevation value and sites with flat were not equally impact on climate change. This means that slope steepness influences on the disturbance of livestock and human activity and the disturbance was highly observed at flat rangeland site. In addition, a combination effect of climate change was less at the highest slope, that is why the vegetation dynamics was shown a better rate at highest elevation site. The human activity and livestock disturbance in combination with climate change significantly increased the rate of rangeland degradation and showed high vegetation index change. Our result is entirely in agreement with the data reported by Potapov et al. (2008), Yi et al. (2014) and Herrmann et al. (2005).

Vulnerability grade

To scale up the observation of degradation level of our study, we used the histogram as a graphical tool to explore the statistical distribution of the classes and clusters in the attribute space (Apan, 1997) and the axes represents our categorical interval NDVI values. Taking the year from 1990-1995 as our baseline for classification since during

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this year interval, we observed the maximum level of degradation as a result, we used it as our reference to classify our interval scale (*Fig. 9*).

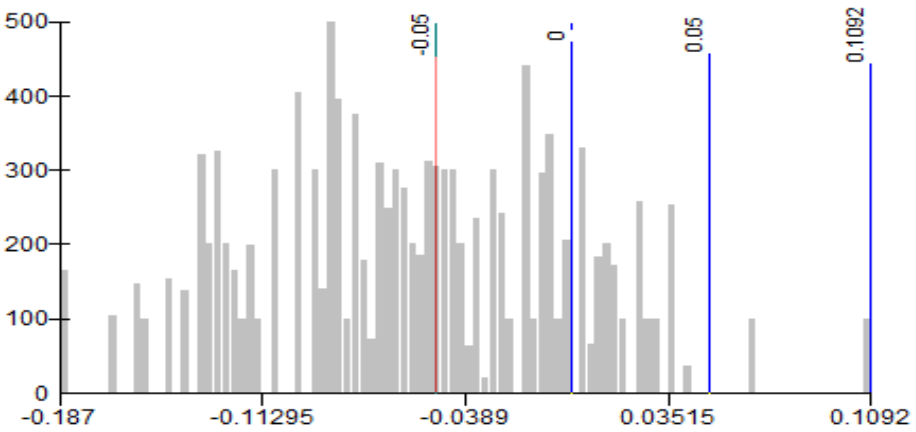

Figure 9. *Degradation distribution histogram of the integrated index of eco-environmental vulnerability from 1990-1995*

Based on the NDVI value classified in *Table 3,* the eco-environmental vulnerability integrated index for each year interval (combined in five years) the degradation level of Teltele rangeland was clearly analyzed and generate based on remote sensing data (*Fig. 10*).

In order to understand the eco-environmental vulnerability change trend in Teltele rangeland area, the value of EVSI was calculated based on *Equation 1* and described in *Table 4*.

Table 3. *Eco-environmental vulnerability classification interval in Teltele rangeland (based on Fig. 9)*

Degradation level	Assign number	NDVI value	Level characteristics
Over degradation	1	< -0.05	Open bare degraded rangeland area
Moderate degradation	2	0-(-0.05)	Relatively the bare degraded rangeland area sparsely covered by bush plant species
Moderate improvement	3	0-0.05	almost 50% of the degraded bare rangeland area covered by bush plant
High improvement	4	> 0.1092	More than 75% of the degraded bare rangeland area covered by bush plant

Table 4. *The degradation proportion data of each level from the study site based on Equation 1*

Dl	1990-1995			1995-2000			2000-2005			2005-2010			2010-2015		
	Pn	%	EVSI	Pn	%	EVSI	Pn	%	EVSI	Pn	%	EVSI	Pn	%	EVSI
1	7,225	57.0	1.59	1,358	10.7	2.61	1,500	11.8	2.91	2,460	19.6	2.67	1,818	14.4	2.8
2	3,451	27.2		4,524	35.7		3,049	24.1		3,203	25.5		2,642	20.9	
3	1,793	14.2		4,240	33.5		2,962	23.4		2,615	20.8		4,151	32.9	
4	200	1.6		2,547	20.1		5,158	40.7		4,291	34		4,019	31.8	

Dl = degradation level, Pn = pixel number

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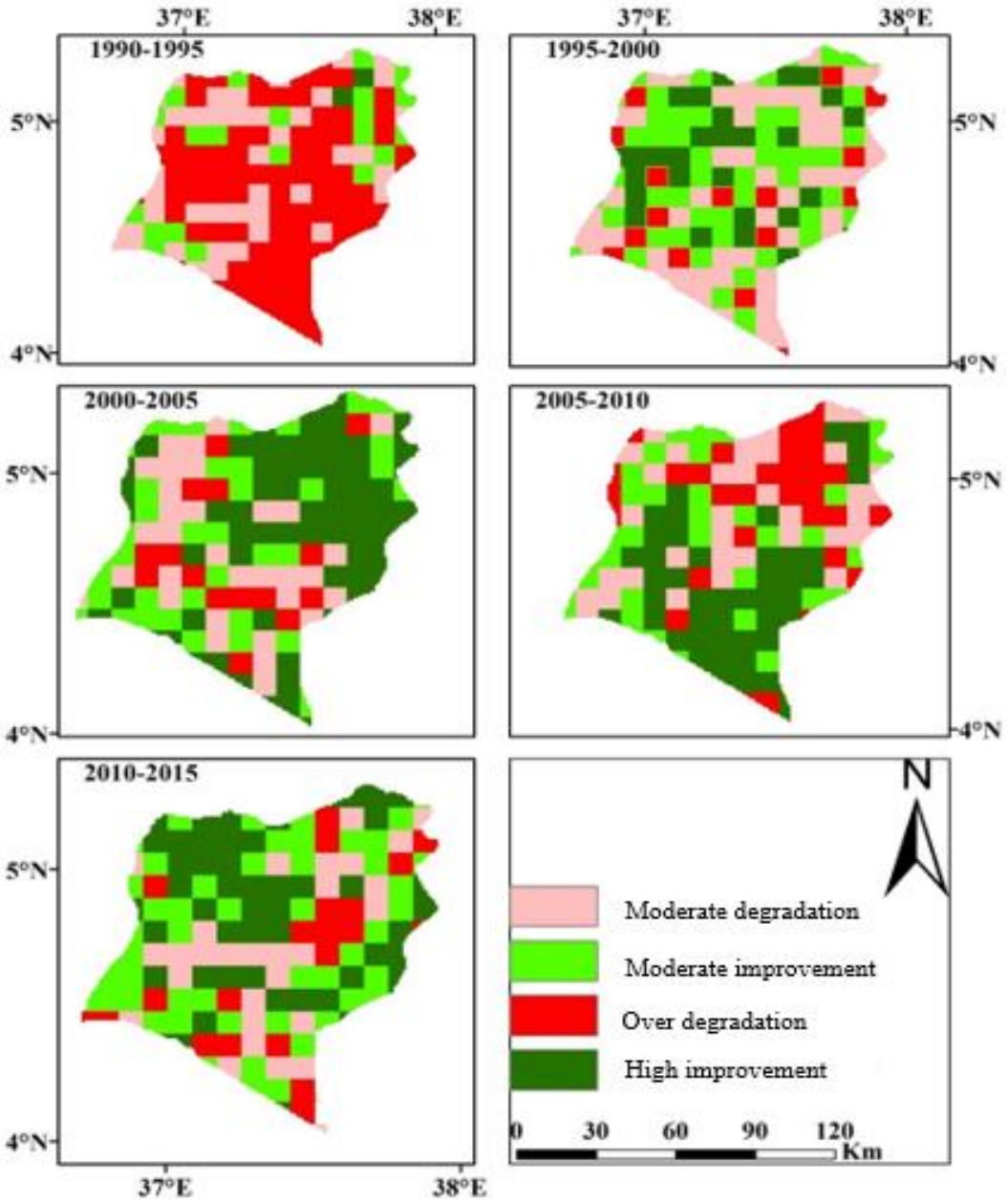

Figure 10. *The degradation distribution of eco-environmental vulnerability level in Teltele rangeland from 1990-2015*

When we have seen the general trend of eco-environmental vulnerability from *Table 4,* the rangeland situation between the time interval from 1990-1995 with an EVSI value 1.59 showed a better status from encroachment of bush plant species than the vulnerability status from 1995-2000, 2000-2005, 2005-2010 and 2010-2015 which have an EVSI value 2.61, 2.91, 2.67 and 2.8 respectively. The higher EVSI value indicated the more serious impact of eco-environmental vulnerability and the lower EVSI value indicated that impact of eco-environmental change is not that much significant (Ainong et al., 2005). The majority of rangeland area occupied by non-native bush plant species that is highly competent with the native grass species and caused rapid decline of forage biomass in our Teltele rangeland.

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The pattern of degradation level or the rangeland area occupied by the invasive bush plant species looks like: (1) from 1990-1995, level 1 degradation was highest (57%) followed by level 2 (27.2%), level 3 (14.2%) and level 4 (1.6%). This indicated that the major degraded Teltele rangeland area during from 1990-1995 was bare area and was not occupied by the current rapid encroached bush plant species, (2) from 1995-2000 the major bare degraded area of the rangeland encroached by the bush plant species and the degradation level goes from level 1 to level 2, i.e. level 1(10.7%), level 2 (35.7%), level 3 (33.5%) and level 4 (20.1%) and this indicated that majority of bare area replaced by encroached non-palatable plant species, (3) from 2000-2005 the major encroachment rate (level 4) of bush plant species was observed (40.7%) followed by level 2 (24.1%) and level 3 (23.4%). This is because of the occurrence of a severe drought called El Niño in 1999 which facilitates the encroachment rate of bush plant species while the native grass species was highly degraded, due to effect of drought, high competition with those encroached plant species and overgrazing, (4) from 2005-2010, the encroachment rate showed an increasing trend and the local community tried to adopt the intervention techniques to reduce the degradation impact of bush plant species like mechanical cutting and others. That is why the level 1 bare area showed an improvement as compared to 1995-2000 and 2000-2015. The area coverage (in %) of each degradation level was in level 1(19.6%), level 2 (25.5%), level 3 (20.8%) and level 4 (34%), (5) from 2010-2015 the level of encroachment trend of bush plant also showed an increasing trend, level 4 (31.8%), level 3 (32.9%), level 2 (20.9) and level 1 (14.4%). Thus, this was due to the drought (El Niño) occurrence of in the Teltele rangeland during 2014 that offered an opportunity for the rapid encroachment rate of bush plant species and high degradation of the native grass species in the study area.

Livestock population dynamics

The livestock population dynamic data was collected from the district livestock office and from the respondents based on type, breed, age, sex and purpose possessed by the local communities from 1990-2015. In order to easily manage the data during analysis we, simply took five (5) year interval data (i.e. 1990, 1995, 2000, 2005, 2010 and 2015 data) and tried to see how the change looks like.

The livestock population trend from 1990-2015, showed a decline rate across in almost all livestock species found in the study area, with the exceptional of the donkey (+ sign indicates that population increment from year to year) as we have seen from *Table 5*. Then, the reduction rate was high between 1995-2000 and 2000-2005 as compared to data from other years for all species. Because of the high incidence of drought (El Niño) in the study area in 1999 and high disease occurrence during 2004 in the study site respectively, there was a loss of more livestock population through die, due to lack of forage source, water availability and diseases. The expansion of farming practices is also another major cause of declining numbers of livestock, because of loss of pasture area and this also supported by government policies which state that every livelihood should keep their own livestock in his/her own grazing site near to his/her settlement area, and not allocate more communal grazing land and mobilization from one area to another for grazing. Thus, the government has divided the communal rangeland area for independent young community for farming practice, therefore, the number of livestock per household level should be reduced. Donkey species became increase in a unique manner compared to other species found in the study area, which is also due to the impact of climate change. During the drought period the pastoral

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community collected forage and water from far distance for their livestock and for themselves. And for this purpose, donkey used as the main transportation method which is main cause for the increment of their number. From 1990-2015, the net change of livestock number was 29%, indicating that a 29% reduction of livestock number was observed as compared to the livestock population number of 2015 with 1990.

Table 5. *Total number of each livestock species from 1990-2015 in Teltele*

Study period	Livestock type							Total
	Cattle	Goat	Sheep	Horses	Mule	Donkey	Camel	
1990	270,332	290,475	132,900	67	900	6,078	3,560	704,312
1995	268,867	288,245	109,345	61	843	6,247	3,089	676,697
2000	209,123	228,389	131,000	42	612	6,978	1,978	578,122
2005	207,004	211,790	108,556	29	289	7,039	1,467	536,174
2010	199,356	193,890	106,009	15	149	8,125	1,290	508,834
2015	197,876	185,846	105,158	4	65	9,704	1,062	499,719
Population dynamics from 1990-2015 (%)								
1990-95	0.5	0.8	17.7	8.9	6.3	(+)2.9	13.2	3.9
1995-00	22.2	20.8	(+)19.8	31.1	27.4	(+) 11.2	36.0	14.6
2000-05	1.0	7.3	17.1	31.0	52.7	(+) 0.9	25.8	7.3
2005-10	3.7	8.5	2.3	48.3	48.4	(+) 15.4	12.1	5.1
2010-15	0.7	4.1	0.8	73.3	56.4	(+) 19.4	17.7	1.8
1990-15	26.8	30.0	20.9	94	92.8	(+) 59.6	70.2	29.0

Livestock population dynamics across household

Livestock population dynamics in average, across the total households found in Teltele district was shown in *Figure 11*. In order to calculate the livestock number per household, we collected the total human population data from the national census reported of Teltele district for the mean value of each 5-year interval 1990-1995 (30,156), 1995-2000 (38, 412), 2000-2005 (49,480), 2005-2010 (57,609), 2010-2015 (70,501) and divided the total number of livestock population corresponding with each year. Both the total livestock number per household level and pattern of total population number within the district was showed sharp decline trend. Livestock holdings were laid with an average of 23.44, 17.6, 11.7, 9.3 and 8 head per household from 1990-2015 each five-year interval respectively, with a net decline of 65.9% overall. This is due to direct and indirect impact of climate change, like the drought that faced the livestock for forage and water scarcity and caused for death and un planned selling of the pastoralist to their livestock to cope up from the impact. Our data directly linked with the data reported by Solomon and Coppock (2002) stated that cattle deaths were mainly due to malnutrition and starvation, with only a few due to disease, predation or other factors because of climate change impact in Borana rangeland with part of our study site.

Association of rainfall variability with livestock dynamics

The variability of rainfall in combination with other factors had a direct impact on the livestock population in the study area (Bao and Wang, 2000). The frequent fluctuation in both temperature and rainfall has also become an emergency cause of insects and other related factors that cause disease and loss of livestock in Teltele district.

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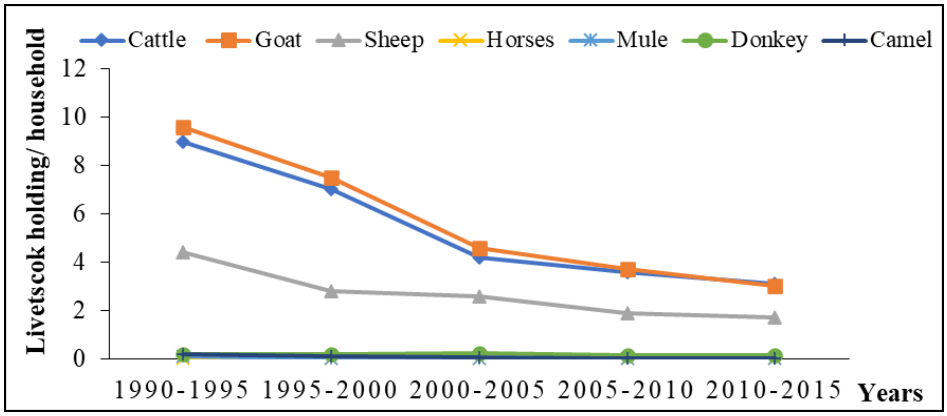

Figure 11. *Mean value of livestock population dynamics (average number of head/household)*

As we see in *Figure 12,* livestock population decreased with the decrease in rainfall, but failed to show a significant relationship with mean annual rainfall, because of the coping strategies taken by the pastoralist community. Our result was found to be consistent with the data reported by Stige et al. (2006), Angassa and Oba (2007) and Alemayehu and Fantahun (2012). Since livestock population in the Teltele district depends mainly on the natural resource (both forage and water) found in the communal grazing area, this makes the impact of rainfall significant in combination with other factors.

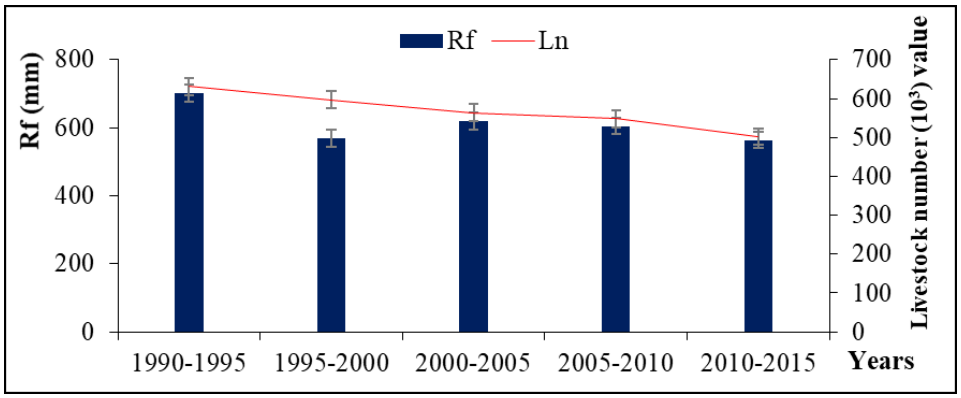

Figure 12. *Association of mean (±SE) annual rainfall and livestock population number, Rf = rainfall, Ln = livestock number*

Correlation analysis of precipitation with grazing livestock population number

Amount of precipitation was a major impact on the productivity and general vegetation status of rangeland and highly effect on the health of the grazing site. And also, the number of livestock highly rely on the amount and distribution of precipitation both directly in indirectly. As a result, perception and livestock number had showed a significant positive correlation to each other (*Fig. 13*).

The linear regression correlation trend showed that precipitation and livestock population number had significantly ($R^2 = 0.5934$) positive relationship. Further, we can understand that, higher rangeland rainfall value confirmed better biomass production and used to satisfy the forage demand of grazing animal and resulted high number of grazing livestock population.

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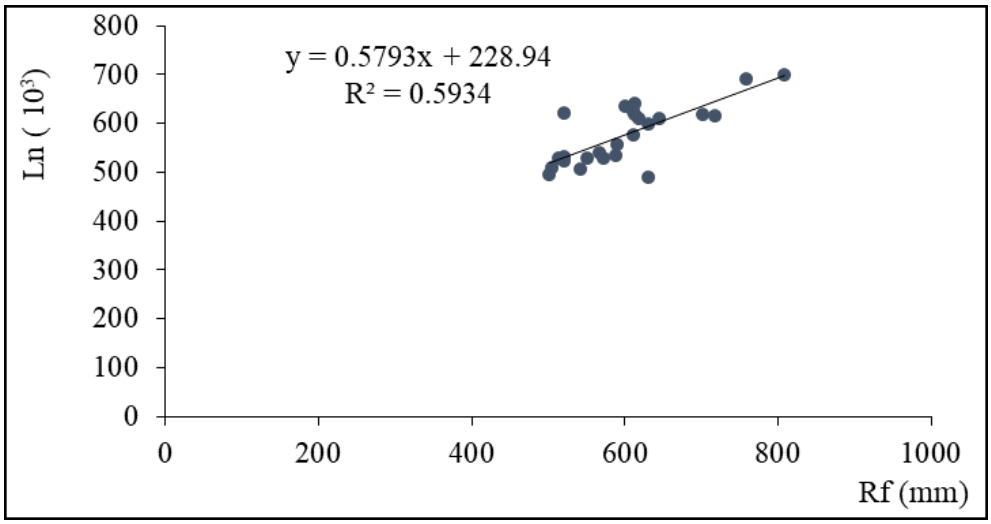

Figure 13. *Correlations between rainfall and livestock number at Teltele rangeland, Rf = rainfall, Ln = livestock number*

Coping strategies to climatic variation on the study site

The techniques used by the local community to adapt to climate change, survive or reduce its impacts and overcome this difficult condition depend mainly on factors like: rangeland condition, livelihood income background, educational level, institutional and social network, capacity and accessibility of infrastructures such as market, road and other services (IPCC, 2007). Due to the lack of accessibility and awareness in the Teltele pastoralists community, the implementation of coping strategies was weak compared to other parts of the country. The major coping techniques listed by the local community for each possibility of climate change and how many of them applied that technique based on the respondent's data were presented in *Table 6*.

The respondents mentioned the above coping strategies listed in *Table 6*, that were implemented during climate change, mainly when strong drought (El Niño) occurred, that caused for a great impact on both the livestock and human life. During drought time the priority action taken by the pastoralists were migration or mobility to search better water and forage (77%), Sale their livestock (69%) and transport water and forage from remote area (60%) respectively. To rehabilitate rangelands infested by bush encouragement clearing or thinning the area (87%), diversified the livestock that have the capacity to eat the bush plant (69%) and used as a source of energy and income (57%) were among the priority action taken by the local communities based on the respondents data. Peace building practice through the local elders (96%) was also the primary solution used by the community to solve conflict or poor social-interactions when happened. Livestock diseases were also another challenged issue that was happened linked to climate change. The use of traditional medicine in combined with the modern (94%), was the priority action taken by the local community followed by isolating the infected animal from the herd and made it stay at home for follow-up. Moreover, to avoid further infection of the disease, selling livestock was prohibited and taken as the next strategies that was implemented in Teltele district pastoralist during climate change. Our result was highly in line with the data reported by Kgosikoma and Batisani (2014).

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Table 6. *Coping strategies used by the local communities to reduce climate impact*

No.	Drivers	Coping techniques used by the community	Frequency (%)
1	Drought	Migration or mobility to search better water and forage	77
		Conserving water through wells	38
		Transport water and forage from remote area	60
		Enclosing pasture land	39
		Sale their livestock	69
		Using water supplied by NGO Diversified their livestock to tolerate drought	29
2	Bush infestation	Clearing or thinning	87
		Burning the rangeland	24
		Used as source of income (charcoal)	57
		Herd diversification	69
3	Poor social interaction	Engaging peace building through local elders	96
		Assign local guards and take immediate action	29
		Developed traditional assets	73
		Apply government policies	75
4	Insects and disease	Using both traditional and modern medicine	94
		Isolating sick animal from the herd	76
		Reporting the case to the government	34
		Dealing with market bans and sell the livestock	48

Conclusion

Our study focuses on the impact of the eco-environmental vulnerability of the Teltele rangeland and evaluates the current situation of the encroachment of bush plant species. The eco-environmental vulnerability was analyzed using NDVI3g time series data, rainfall and temperature records. As revealed in this analysis, the variation of vegetation greenness, changes through NDVI value showed a direct correlation with the variability of rainfall and temperature. This means, that if the mean value of temperature was high, the NDVI value showed a decline, whereas if the mean rainfall value was high, the NDVI value showed high value. Thus, this tell us there is a significant relationship NDVI positively with rainfall and negatively with temperature. However, during the time when high rainfall was occurred and flooding, rangeland area became degraded and NDVI value also not significantly high related with amount of rainfall, due to consequence of degradation instead of greenness the rangeland site. Further, the rangeland vegetation showed a better status, when the rainfall was at normal standard and not caused for further degradation in the form of flooding compared with dry season. The degradation level of Teltele rangeland showed an upward trend between 1990 and 2015 mainly due to the encroachment of invasive bush plant species that almost occupied the entire rangeland area. The frequent fluctuation of both rainfall and temperature creates an appropriate condition for a rapid encroachment of invasive bush plants and, on the contrary the native grass species degraded at an alarming rate and this is the major challenge for semi-arid rangeland in Africa including Ethiopia now days. Further, the livestock population in Teltele area decreased from time to time, for this encroachment of bush plant species due to frequent drought as the primary factor based

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on the data obtained from both NDVI and the respondents. The local pastoral communities have enough information on the level of impact of eco-environmental vulnerability and practice different coping strategies to further reduce the influence on their livelihoods, but the scientific understanding still shown a gap and this is due to the lack of awareness. Therefore, based on our results, we highly recommend that the design the scientific management techniques for eco- environmental vulnerability and awareness within the local community be the priority for further studies.

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Author contributions. Available data collection, writing up and gap assessment and design was done by Yeneayehu Fenetahun, while editing and proofin: as well as supervision of the whole work during this project were performed by Professor XU-Xin-wen and Dr. Wang Yong-dong.

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COPROLOGICAL STUDY OF GASTROINTESTINAL PARASITES IN DAIRY CATTLE IN SULAYMANIYAH PROVINCE, KURDISTAN REGION, IRAQ

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Abstract. Gastrointestinal (GI) parasitic diseases are a serious problem in cattle management, and one of the most significant reasons for economic losses incattle for developing countries. This study aimed to assessthe prevalence of helminth and protozoan infection of gastrointestinal tract in local dairy cattle in Sulaymaniyah province of Iraq.A total of 1,376rectal fecal specimens of local dairy cattle were randomly collected in different regions of Sulaymaniya province. Direct fecal smear, saturated sugar flotation technique, and simple sedimentation method, were used to detect the parasitic stages in the coprological specimens.The overall prevalence rate was 60.46%.The single and mixed parasite infection rates were 37.21% and 23.25%, respectively. The infection rates of protozoa, nematodes, trematodes, and cestodes were 58.14%, 18.60%, 15.11%, and 3.48%, respectively. Among all parasites, *Eimeria* spp. (29.07%) was the most abundant one, followed by *Buxtonella sulcata* (18.60%) and *Strongyle* nematodes (17.44%). There was significant variation in the prevalence rates of nematodes and protozoa between various age categories and distributional regions. The results indicated that improved management system and proper anthelmintic treatment strategies should be applied in the regions to diminish the high incidence of GI parasitismin local dairy cattle.
Keywords:*prevalence, helminthes, protozoa, livestock,fecal analysis*

Introduction

Parasitosis is one of the biggest issues affecting livestock especially cattle of all age and breed categories (Rafiullah et al., 2011;Awraris et al., 2012). The endoparasites intervene with dietary status, growing process and byproductsof the cattle population (Pilarczyket al., 2009; Awraris et al., 2012; Khan et al., 2013).

The animals' gastrointestinal tract holds a broad range of parasitic protozoa and helminthes, which cause sub clinical and clinical parasitic infestation. These internal parasites inauspiciously affect the health conditions of animals and cause great economic impacts on the livestock industry (Wimmer et al., 2004; Bilal et al., 2009).

In the case of rearingfarm animals for food production, investigations that aid in evaluating the economic impacts caused by parasitic infestation are significant, particularly in smallholder farming structures in developing countries (Perry and Randolph, 1999; Sahoo et al., 2002). As parasites may cause clinical and nonclinical parasitism leading to economic losses, the purpose of veterinarians and producers is to avoid parasitism via goodhusbandry,adequate nutrition, control of epidemiological factors, and effective therapeutic treatment (McDermottet al., 1999; Kaewthamasornand Wongsamee, 2006).

Although the numbers of studies havereported the prevalence of gastrointestinal (GI) parasite of ruminants inIraq (Al-Taee et al., 2011; Nassrullah, 2011; Nasrullah et al., 2014; Minnat, 2014; Al-Zandee et al., 2016; Hassan et al., 2018; Al-Robaiee et al., 2019), there is no published study available related to GI parasites of cattle in the province of Iraq. Thus, the objective of the current studywas to record the prevalence of

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GI parasites among local dairy cattle in different regions of Sulaymaniyah province.The recorded data will provide fundamental knowledge of GI parasite infection levels in the province and it could be supportive for control strategies.

Materials and Methods

Study area and sample population

This study was conducted in Sulaymaniyah province, Kurdistan Region, north-east of Iraq. It is located between 35°04′- 36°30′ latitude and 44°50′- 46°16′ longitude. The area is characterized by seasonal rainfall from October to May, and inadequate farmer awareness about the husbandry and control of the endoparasites in the ruminants. Cattle population in the study regions (Penjwen, Chwarta, Said Sadq, and Piramagroon) is estimated to40,355 according to the data recorded by Sulaymaniyah Veterinary Directorate in December 2015.

A total of 1,376 rectal fecal specimens of local dairy cattle, involving560 calvesand 816 cows, were randomly collected from different regions of Sulaymaniyah province. Among these samples, 200 were collected from Penjwen, 330 from Chwarta, 470 from Said Sadq, and 376 from Piramagroon. The samples were stored at 4 °Cuntil parasitological assessment. The age groups of cattle were sorted as calves (under1 year old) and cows (above 1 year old).The sampling period was begins from March to August 2018.

Parasitological analysis

Fecal specimens were analyzed for the existence of helminth eggs, protozoan oocysts and cysts,applying the simple flotation technique using saturated sugar solution (Sheather's sucrose solution). A simple sedimentation procedure was utilized to find the ova of flukes and some other nematodes and cestodes, whose ova do not recover in a saturated sugar solution. For diagnosis of protozoan trophozoites, the direct fecal smears were performed. The parasitic stages were identified based on morphological keys (Urquhart et al., 1994; Zajac and Conboy, 2012; Kandasamy et al., 2013).

Statistical interpretation

The data calculated by Chi-square (X^2) test using SPSS® software V.25. The probability values below 0.05 were considered statistically significant.

Results

Out of 1,376 fecal samples examined, the overall infestation rate of GI parasite was60.46%. The single and mixed parasite infection rates were 37.21% and 23.25%, respectively with statistically significant differences. *Eimeria* spp. was the most dominant parasite that recognized in majority of multiple infections (*Table 1*).

This study found that the highest prevalence ofprotozoan infection was (58.14%), followed by nematodes (18.60%), trematodes (15.11%), and cestodes (3.48%).There was significant variation in the prevalence rates of nematodes and protozoa between various age categories and distributional regions (*Table 2; Fig. 1; Fig. 2*).

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Table 1.*Prevalence of gastrointestinal parasites in local dairy cattle (N^a = 1,376) in Sulaymaniyah province*

Species of parasite	No.[b] (%)	*P*-value
Single parasitic infection		< 0.001
Eimeria spp.	144(10.46)	
Buxtonella sulcata	176(12.80)	
Strongyle type	96(6.97)	
Cryptosporidium spp.	48(3.49)	
Dicrocoelium dendriticum	48(3.49)	
Subtotal	**512(37.21)**	
Multiple parasitic infection		
Eimeria spp. + *Strongyle* type	64(4.65)	
Eimeria spp. + *Cryptosporidium* spp.	32 (2.32)	
Eimeria spp. + *Buxtonella sulcata*	16(1.16)	
Eimeria spp. +*Moniezia* spp.	16(1.16)	
Eimeria spp. + *Toxocara vitulorum*	16(1.16)	
Eimeria spp. + *Fasciola* spp.	16(1.16)	
Eimeria spp. + *Paramphistomum cervi* + *Fasciola* spp.	16(1.16)	
Eimeria spp. + *Cryptosporidium* spp. + *Moniezia* spp.	16(1.16)	
Eimeria spp. + *Paramphistomum cervi* + *Buxtonella sulcata*	16(1.16)	
Eimeria spp. + *Paramphistomum cervi* + *Buxotenella sulcata* + *Strongyle* type	16(1.16)	
Eimeria spp. + *Cryptosporidium* spp. + *Paramphistomum cervi* + *Fasciola* spp.	16(1.16)	
Eimeria spp. + *Cryptosporidium* spp. +*Moniezia* spp. + *Fasciola* spp. + *Buxtonella sulcata*	16(1.16)	
Strongyle type + *Cryptosporidium* spp.	16(1.16)	
Strongyle type + *Paramphistomum cervi*	16(1.16)	
Strongyle type + *Fasciola* spp.	16(1.16)	
Strongyle type + *Buxtonella sulcata*	16(1.16)	
Subtotal	**320(23.25)**	
Total (Overall)	**832(60.46)**	

[a]No. = total number of examined cattle, [b]No.= number of infected cattle

It was recorded three kinds of protozoan cysts/oocysts or trophozoites in the examined fecal samples, namely *Eimeria* spp. (29.07%), *Buxtonella sulcata* (18.60%), and *Cryptosporidium* spp. (10.46%). The prevalence of *Eimeria* spp. in calves (35.89%) was statistically higher than in cows (24.38%), while *Cryptosporidium* spp. was significantly lower in calves (6.96%) than cows (12.86%). The increasing prevalence of *B. sulcata* associated with an expansion in age.

In the current investigation, the recognized nematode eggs included *Strongyle* nematodes and *Toxocara vitulorum. Strongyle* nematode (17.44%) was the most predominant species detected in local dairy cattle, and the commonness in Piramagroon was fundamentally higher than those in other regions. *T. vitulorum* was recovered in 16 cattle.

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Table 2.*Risk factors associated with prevalence of gastrointestinal parasites in local dairy cattle(N[a] = 1,376) in Sulaymaniyah province*

Species of parasites	Prevalence	Age category			Region				
	No.[b] (%)	Calf	Cow	*P*-value	Penjwen	Chwarta	Said Sadq	Piramagroon	*P*-value
		(n[c]=560)	(n[c]=816)		(n[c]=200)	(n[c]=330)	(n[c]=470)	(n[c]=376)	
Protozoa	**800(58.14)**	**303(54.10)**	**497(60.90)**	**< 0.01**	**110(55.00)**	**170(51.51)**	**295(62.76)**	**225(59.84)**	**< 0.01**
***Eimeria* spp.**	400(29.07)	201(35.89)	199(24.38)	< 0.001	56(28.00)	76(23.03)	145(30.85)	123(32.71)	< 0.02
Buxtonella sulcata	256(18.60)	63(11.25)	193(23.65)	< 0.001	39(19.50)	72(21.81)	117(24.89)	28(7.44)	<0.001
***Cryptosporidium* spp.**	144(10.46)	39(6.96)	105(12.86)	< 0.001	15(7.50)	22(6.66)	33(7.02)	74(19.68)	<0.001
Nematodes	**256(18.60)**	**143(25.53)**	**113(13.84)**	**< 0.001**	**32(16.00)**	**44(13.33)**	**92(19.57)**	**88(23.40)**	**<0.001**
Strongyles	240(17.44)	137(24.46)	103(12.62)	< 0.001	31(15.50)	40(12.12)	82(17.44)	87(23.13)	<0.001
Toxocara vitulorum	16(1.16)	6(1.07)	10(1.22)	0.79	1(0.50)	4(1.21)	10(2.12)	1(0.26)	0.06
Trematodes	**208(15.11)**	**43(7.67)**	**165(20.22)**	**< 0.001**	**37(18.50)**	**51(15.45)**	**78(16.59)**	**42(11.17)**	**0.06**
Paramphistomum cervi	80(5.81)	10(1.78)	70(8.57)	< 0.001	35(17.50)	20(6.06)	17(3.61)	8(2.12)	<0.001
***Fasciola* spp.**	80(5.81)	24(4.28)	56(6.86)	< 0.04	1(0.50)	30(9.09)	25(5.31)	24(6.38)	<0.001
Dicrocoelium dendriticum	48(3.48)	9(1.60)	39(4.77)	< 0.001	1(0.50)	1(0.30)	36(7.65)	10(2.65)	<0.001
Cestodes	**48(3.48)**	**18(3.21)**	**30(3.67)**	**0.75**	**1(0.50)**	**2(0.60)**	**17(3.61)**	**28(7.44)**	**<0.001**
***Moniezia* spp.**	48(3.48)	18(3.21)	30(3.67)	0.290	1(0.50)	2(0.60)	17(3.61)	28(7.44)	<0.001

[a]N= total number of examined cattle, [b]No. = number of infected cattle, [c]n = number of examined cattle

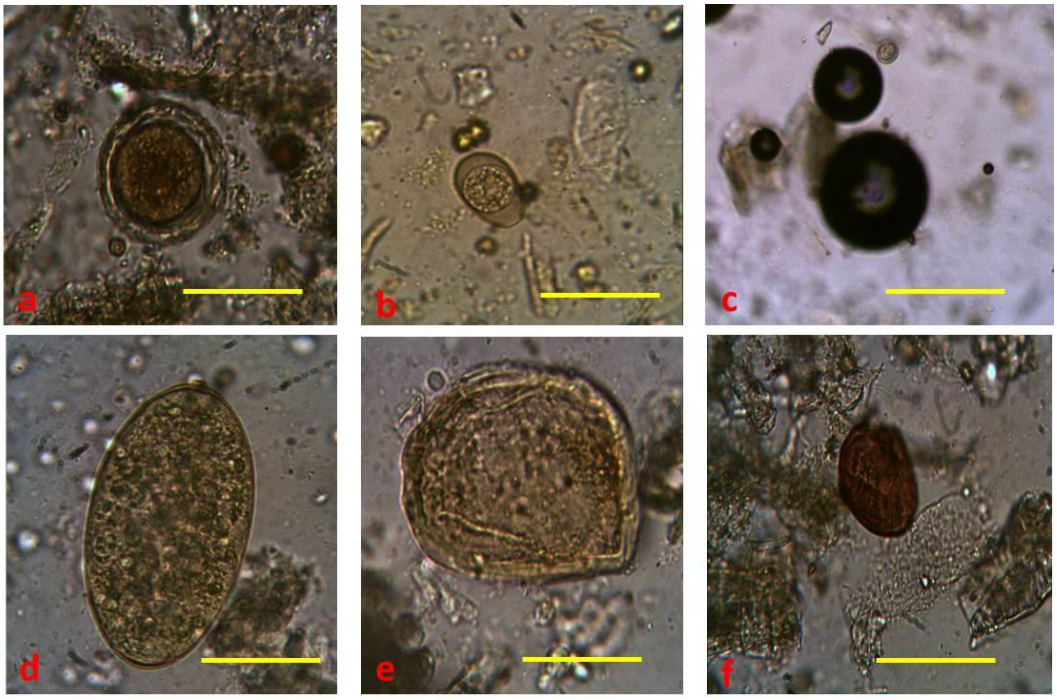

Figure 1.*Fecal smears of cattle under the X40 objective microscope, scale bars are 100 µm:a. Toxocara vitulorum (egg);b. Eimeria spp. (oocyst); c. Cryptosporidium spp. (oocyst);d. Fasciola spp. (egg); e. Moniezia spp. (egg); f. Dicrocoelium dendriticum (egg)*

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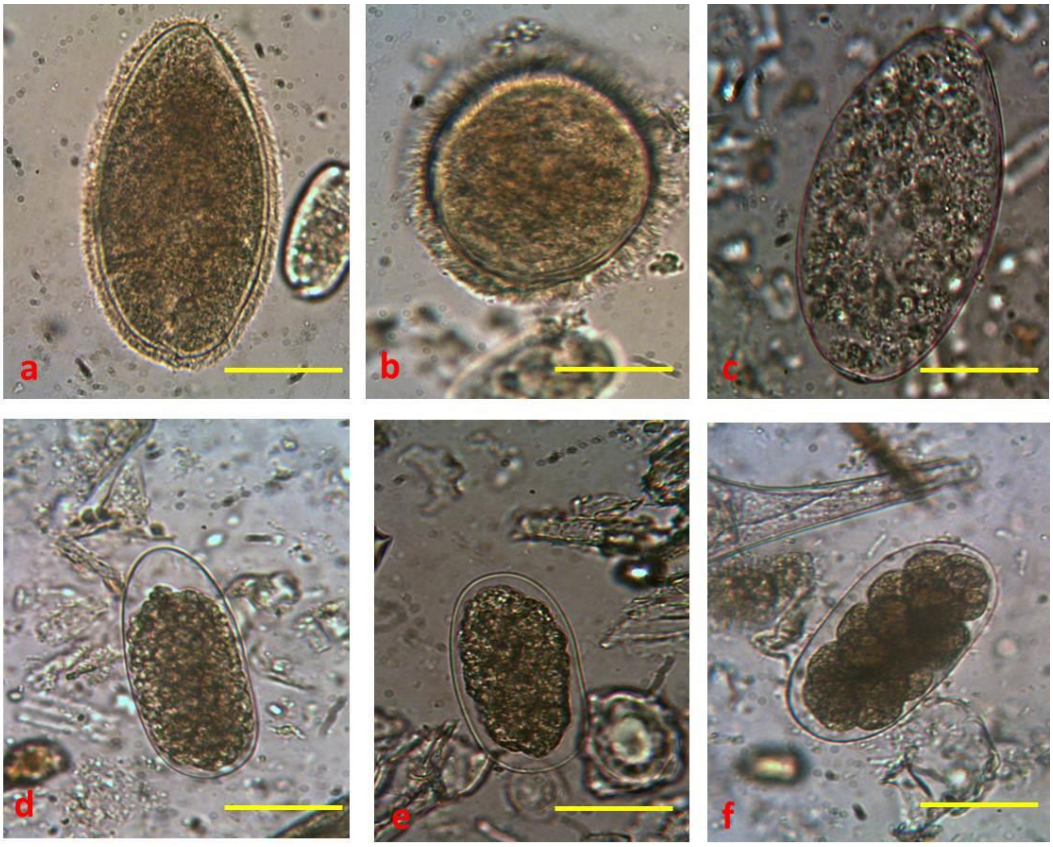

Figure 2.*Fecal smears of cattle under the X40 objective microscope, scale bars are 100 µm:a. Buxtonella sulcata (trophozoite);b.Buxtonella sulcata (cyst); c. Paramphistomum cervi (egg); d.;e.;f. Strongyle type(eggs)*

The infection rate of trematodes (15.11%) was comparatively lower than that of nematodes and protozoa. The identified trematodes involved *Paramphistomum cervi* (5.81%), *Fasciola* spp. (5.81%), and *Dicrocoelium dendriticum* (3.48%). There was apparent variation between different age groups and different regions for these flukes infection.

The prevalence of tapeworms (3.48%) was markedly lower than that of other parasites. *Moniezia* spp. eggs only recovered among infected cattle. There was no obvious difference between age categories of cattle; however, the prevalence rates in Piramagroon and Said Sadq regions were statistically higher than those in Chwarta and Penjwen areas.

Discussion

Control of GI parasitic diseases in livestock requires comprehensive information about the epidemiology, field management, and environmental conditions such as rainfall and temperature. The numbers of protozoan oocysts and helminth eggs developed inside the host animals vary according to the parasite species, degree of host susceptibility,the health,and immune status of the animals (Sharma and Busang, 2013).

Gastrointestinal nematode diseases of cattle continue to be a limitation on the proficient raising of cattle all through the world. In much less developed agricultural

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systems, parasitic infections may also cause serious clinical signs, such as stunted growth, tissue edema, and diarrhea (Gasbarre et al., 2001).Indeed in well-managed herds with no signs and symptoms of parasitism, the existences of GI parasites restrict the growth inyoung animals and diminish milk production in growing-up bovines (Hawkins, 1993).In Sulaymaniyah province of Iraq, infections triggered by GI parasites are predominant in small ruminants since of the helpful local weather for the transmission of infection (Nassrullah et al., 2014).

With this study, the findings demonstrated highly GI parasitic infections in cattle (60.46%) in the Sulaymaniyah province of Iraq. The results are consistent with other reports from a number of countries (Regassa et al., 2006; Tung et al., 2012; Huang et al., 2014; Hussain et al., 2014; Hamid etal., 2017).These high levels of infection rate have reflected the lack of success from the de-worming program and highlighted ineffective husbandry.

In present research recorded a high prevalence of protozoan infection (58.14%). This finding was similar to the consequence of the studies mentioned by Tung et al. (2012) and Huang et al. (2014).During the current study, a significantly higher proportion of calves (35.89%) were infectedwith *Eimeria* spp. than cows (24.38%). A recent investigation carried out in Sri Lanka has also recorded the similar findings (Gunathilaka et al., 2018). This might be due to the excessive humidity and reasonable temperature encourages the survival and sporulation of the oocysts. As their immunity is additionally lower than the adult cattle, calves might bemore susceptible to coccidian diseases (Bilal et al., 2009).*B. sulcata* infection was the second most dominant protozoan infection found in 18.60% of the local dairy cattle in the present study, and the increasing prevalence correlated with the rising in age. This finding was lower than the results of a previous research performed in Iraq (Al-Bakri et al., 2010).Fox and Jacobs (1986) showed that the quantity of carbohydrate in the food would affect the population growth or decrease of *B. sulcata*. The variation in the prevalence of infection may be due to differentfactors, such as environment, farm management practices and stress factors.*Cryptosporidium* spp. was the third protozoa recorded 10.46% of cattle with a significantly higher prevalence of infection in cows compared to calves. However,Roy et al. (2006) indicated calves to be most susceptible to infection and they also actas reservoirs.

According to the results of the current investigation, nematodes infected 18.60% of local dairycattle. The infection rate was markedly lower than a recentstudy conducted by Hamid et al. (2017).Improper antihelmintic administration, poor husbandry, or the evasion of immune responses might accelerate the risk of nematode infection.

In this study, *P. cervi* (5.81%)and *Fasciola* spp. (5.81%) were the most abundant trematodes followed by *D. dendriticum* (3.48%).On the contrary, this result was higher than that registered by Tung et al. (2012). Environmental pollution may be one of the factors that reduce the number of snail population in the areas.Additionally, feeding fresh grass contaminated with metacercaria to their cattle from farmers could increase the risk of trematode infection.

During this research, *Moniezia* spp. was infected a very low proportion of cattle. A similar observation was reported by Jittapalapong et al. (2011). The variation in the prevalence rate of monieziasis between distributional regions may be due to the distribution of intermediate host, the free-living soil mites on pasture, in these regions.

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Conclusion

To the best knowledge, this is the first report on prevalence of GI parasites in Cattle in Sulaymaniyah province, Iraq.This study showed a high rate of GI parasites in cattle and it was concluded that GI parasite was common and endemic in the study areas. In addition to that, *Eimeria* spp. and *Strongyle* nematodes were the most abundant parasites recovered in the cattle. The farmers should apply improved management systems and regular de-worming treatments for controlling and prevention of parasitic diseases. Future investigations are essential to evaluate the economic impact of GI parasites in the study regions.

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DIVERSITY AND ECOLOGICAL ANALYSIS OF SERPENTINE FLORA IN THE KOSOVO SECTION OF THE IBAR RIVER VALLEY - COMPARISION WITH THE FLORA OF NEARBY REGIONS

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Abstract. Formation of serpentine flora and vegetation is primarily influenced by local geology and they represent an extraordinary area for botanical research studies (from both taxonomical and ecological perspective). During a sixteen-year research of serpentine terrain flora of the Ibar river middle-stream valley in northern Kosovo and Metohija, the existence of 882 taxa grouped into 83 families and 386 genera has been proven. The collected serpentine flora was analysed on taxonomical, ecological and phytogeographical basis. Out of the total number of identified taxa, 73 (8.27%) are endemic, sub-endemic, relict and endomorelic. 31 taxa belong to a group of internationally significant vascular plants. Of these, 14 taxa have been protected by CITES Convention. The taxonomic structure of the serpentine flora of the Ibar river middle-stream valley is compared to the serpentine flora of Mt. Studena and Goleš Mt. (parts of Ibar serpentine massive).
Keywords: *floristic composition, Ibar serpentine massif, endemism, floristic similarity, Serbia*

Introduction

Serpentine is technically a mineral, but the same word is often used for all ultramafic rocks, the soils that form from them, and the unique ecosystems that form on them (Harrison and Rajakaruna, 2011). Serpentine is a ferromagnesian silicate mineral which contains high concentrations of nickel (Ni) and often chromium (Cr) and cobalt (Co) in a form available for plants (Westerbergh and Saura, 1992). Serpentine soils are often deficient in plant essential nutrients such as nitrogen (N), phosphorus (P), potassium (K), and sulfur (S) (Rajakaruna and Boyd, 2014), while ratio of calcium (Ca): magnesium (Mg) is less than 1.0, and pH values range from basic to ultrabasic (Brooks, 1987; Selvi, 2007; Pavlova, 2007; Bani et al., 2013). Owing to this chemical composition, Novák (1926) described serpentine ranges as "dead rocks". Serpentine soils are typically recognized on the landscape as patchily distributed rock outcrops with stunted vegetation (Anacker, 2014). Serpentine outcrops are often steep and comparatively rocky, making them particularly vulnerable to erosion, which results in shallow soils (Brady et al., 2005) and stony structure (Kurt et al., 2013).

The serpentinite is rather unfavorable to plant growth, and its physical conditions also are inhospitable for many plants (Bani et al., 2013). However, plants would not be

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what they are, if they weren't able to conquer any tolerably suitable substratum by adapting to it (Vasić and Diklić, 2001). Plants adapted to grow under these constraints are called serpentinophytes (Selvi, 2007), distinguished by characteristic structural-morphological adaptations (Stevanović and Jakovljević, 2014); they posses capacity for removal and accumulation of metals (Branković et al., 2017). Many serpentine species are xeromorphic, and water deficiency has been suggested as another stress factor of many serpentine soils (Tumi, 2013). The serpentine flora contains both basophilous and acidophilous plants (Marin and Tatić, 2001). Vegetation growing on serpentinised rocks is often reduced in height, biomass, and ground cover (Gavrilović et al., 2017).

Ultramafic outcrops (also called "serpentine"), formed during tectonic movements, are widespread but sparse, covering roughly 3% of the Earth's surface (Guillot and Hattori, 2013). The largest serpentine areas in Europe are in the Balkans (Stevanović et al., 2003), with an estimated size of over 1,300 km^2 (Pustahija, 2011).

The serpentine areas on the Balkan Peninsula traverse discontinuously from the northwest to central Bosnia, over western and central Serbia, Metohija, Albania, Epirus and northern Thessaly all the way to the Island of Evia, Greece (Stevanović and Jakovljević, 2014). In Bulgaria serpentine areas are smaller and scattered (Pavlova, 2010), distributed in Soutwestern & Central Bulgaria (Stevanović et al., 2003). It should be emphasized that the floristic opulence of these habitats increases with the serpentinites of western and central Serbia, over Albania to northern Pindo and the Evia Island, Greece (Stevanović and Jakovljević, 2014).

In the territory of Serbia the largest serpentine surfaces are located in its western and central regions, as well as in Kosovo and Metohija territory, covering about 250000 ha (Stevanović et al., 2003). These serpentine zones are distinctly delimited from the adjacent surrounding regions with a different geological substratum (e.g., limestone) (Dudić et al., 2007). Kosovo, as a part of the Balkan, hosts an ultramafic area of 487 km^2 within its territory (Salihaj et al., 2018). The largest complexes of serpentine in Kosovo region are found in the valley of the Ibar River and the same are continued in a discontinuous chain through Koznica Mt. and Goleš Mt. to the southwest of Kosovo (Krasniqi and Millaku, 2007). The Ibar serpentine massif stretches along the middle course of the Ibar River and represents a link in the extension chain of the serpentines in Bosnia-Zlatibor and the Ibar gorge-Albania direction (Prodanović et al., 2008). The identified age ranges from post-trias to lower jurassic.

The serpentine terrains of northeastern Kosovo, i.e. the middle stream of the Ibar river, have been rarely visited and floristically researched, which resulted in scarce scientific literature (Pavlović, 1967; Rexhepi, 1979, 1992; Krasniqi et al., 1981; Ranđelović et al., 1982; Krivošej et al., 1993, 1995-1998; Tatić and Krivošej, 1997). A more thorough exploration of this area was started in 2003 by Prodanović, whose work resulted in publishing new chorological data on the flora of Kosovo and Metohija, and entire Serbia (Prodanović, 2007; Prodanović et al., 2004, 2008, 2010, 2012, 2013, 2018; Krivošej et al., 2003, 2011, 2013). However, a comprehensive study involving all vascular serpentine flora in the Ibar valley has not been published so far.

This paper presents a synthesis of all research studies performed in these area, both literature data and intensive field study data, starting from 2003 up to the present days. Its aim is to compile a complete checklist of floristic diversity of this area, analyse it on taxonomical, ecological and phytogeographical base and to compare the flora of two serpentinite regions in central Kosovo and central Serbia. It would be a significant scientific contribution to taxonomic and ecological studies of flora in whole Serbia.

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Only a good knowledge of floristic diversity may initiate the procedures that may lead to preserving and protecting rare and endangered and internationally important species in these areas.

Materials and Methods

Study area

The intensive research studies of serpentine terrain in middle stream of the Ibar river started in March 2003 and continued till today. The researched area covers terrains in the Ibar river canyon north of Kosovska Mitrovica towards the administrative boundary of Kosovo and Metohija and central Serbia and a 50 km long village area of Donje Jarinje (*Fig. 1*). It should be emphasized that this highway more or less follows the Ibar river course. The actual length of the investigated area, due to naturally winding river course, is much bigger.

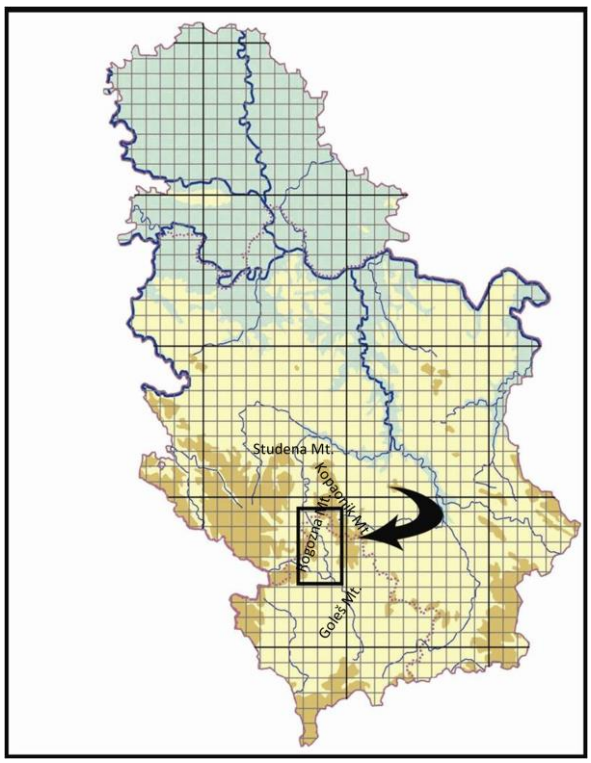

Figure 1. *Geographical position of investigated area in Serbia (UTM 10x10 sq km)*

The geographic relief of the Ibar river middle stream valley may be characterized as mountainous region – the Mountains of Rogozna, Kopaonik and Mokra raise up in the direction of the Ibar river canyon and they provide the main landscape characteristics to Kosovska Mitrovica basin. The altitude on the researched terrain varies between 500 and 900 meters above sea level. The climate of the Ibar river middle steam is defined as moderate –continental. The average air temperature is 10.2 ^{0}C. The researched area belongs to the zone with limited precipitation rate, around 614 mm.

The researched area in north Kosovo and Metohija covers the position between the Mesian and Illyrian provinces, since the Ibar river represents the farthest eastern

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boundary of the Illyrian province. Due to this herbal-geographic position complex floristic and vegetation relations occur.

Data collection

The floral material was collected in the wider area on both sides of the river, through the gorge. The collected material was processed on usual ways for herbariums and stored in the Herbarium of the Institute for Natural Conservation of Serbia (a department in Belgrade) and some specimens are stored in the Herbarium of the Institute of Botany and Botanical Garden "Jevremovac", University of Belgrade (BEOU). The contemporary literature has been used for plant determination. The nomenclature used for all the registered species in the area under investigation was adjusted to comply with Euro+Med Plantbase (2006) and The Plant List (2013). The floristic catalogue is arranged in alphabetical order of families and genera.

The floristic elements for phytogeographic analyses have been processed and analyzed in line with herbal-geographic classification of Stevanović (1992a). The well-known Raunkiaer system (1934) amended by Stevanović (1992b) for Serbian conditions, has been used for the classification of life forms. Checklist of Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), as well as International Union for Conservation of Nature (IUCN) were used to determine the category of internationally significant and endangered species.

For the analysis of the similarity of flora in the researched area in the Ibar river valley and Studena planina/Mt. Studena and Goleš Mt., as parts of the Ibar serpentine massive, Sørensen's (1948) similarity index was used.

Results and Discussion

Taxonomic analysis of the flora

Biologically, serpentine sites frequently host a depauperate flora compared to the surrounding regions (Obratov-Petković et al., 2006). Serpentine habitats are important centres for floristic differentiation and speciation (Stevanović et al., 2003) distinguished by high number of endemics. Considering the fact that the serpentine terrains with the insufficiently developed soil and unfavourable water and mineral regime, and high levels of magnesium, iron, nickel and chrome are "inhospitable" for floral development, the total number of 882 identified taxa in the investigated area still show floral treasure (Prodanović et al., 2008).

During a 16-year research of serpentine terrain flora of the Ibar river middle-stream valley, 882 taxa (species, subspecies) has been identified, which represent about 25% of total flora of Serbia. They have been grouped into 83 families and 386 genera (*Table 1*). Class *Polypodiopsida* is presented by 8 families and 13 taxa. Only two species of *Pinopsida* can be found. Floristically speaking, the richest class is *Magnoliopsida* with 867 taxa. The greatest number of taxa is noted in family *Compositae* (105), followed by *Leguminosae* (86), *Poaceae* (66), *Lamiaceae* (51), *Brassicaceae* (50), *Caryophyllaceae* (49) which coincides with the most numerous families in the flora of entire Serbia (Stevanović et al., 1995).

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Table 1. *Catalogue of the vascular plants observed in the serpentine terrains of Kosovo's section of the Ibar river valley*

CLASS/ FAMILY/*Species*	**Source/Literature**	**Common English names**
POLYPODIOPSIDA		
ASPLENIACEAE		
Asplenium adiantum-nigrum subsp. *serpentini* (Tausch) Heuf	Prodanović, 2007	black spleenwort
Asplenium ceterach L. subsp. *ceterach*	Prodanović, 2007	rustyback
Asplenium ruta-muraria L.	Prodanović, 2007	wall-rue
Asplenium trichomanes L.	Prodanović, 2007	maidenhair spleenwort
CYSTOPTERIDACEAE		
Cystopteris fragilis (L.) Bernh.	Prodanović, 2007	brittle bladder-fern
DENNSTAEDTIACEAE		
Pteridum aquilinum (L.) Kuhn	Prodanović, 2007	bracken
DRYOPTERIDACEAE		
Dryopteris filix-mas (L.) Schott	Prodanović, 2007	
EQUISETACEAE		male-fern
Equisetum arvense L.	Prodanović, 2007; Krivošej et al., 2013	field horsetail
Equisetum palustre L.	Prodanović, 2007	marsh horsetail
OPHIOGLOSSACEAE		
Ophioglossum vulgatum L.	Krivošej et al., 2013	adder's tongue
POLYPODIACEAE		
Polypodium vulgare L.	Prodanović, 2007	polypody
PTERIDACEAE		
Paraceterach marantae (L.) R.M. Tryon	Rexhepi, 1979; Prodanović, 2007	
Cheilanthes persica (Bory) Mett. ex Kuhn	Krivošej et al., 2003; Prodanović, 2007	
PINOPSIDA		
CUPRESSACEAE		
Juniperus communis L.	Prodanović, 2007	common juniper
Juniperus oxycedrus L.	Pavlović, 1967; Rexhepi, 1979; Rexhepi 1992; Prodanović, 2007	western prickly juniper, cade juniper
MAGNOLIOPSIDA		
ADOXACEAE		
Adoxa moschatellina L.	Prodanović, 2007	moschatel
Viburnum lantana L.	Prodanović, 2007	wayfaring tree
AMARANTHACEAE		
Amaranthus albus L.	Prodanović, 2007	tumble pigweed
Amaranthus blitoides S. Watson	Prodanović, 2007	mat amaranth
Amaranthus retroflexus L.	Prodanović, 2007	redroot pigweed
Chenopodium bonus-henricus L.	Prodanović, 2007	poor-man's asparagus
Chenopodium hybridum L.	Prodanović, 2007	goosefoot
Chenopodium opulifolium Schrad. ex W.D.J. Koch & Ziz	Prodanović, 2007	grey goosefoot
Chenopodium polyspermum L.	Prodanović, 2007	manyseed goosefoot
Dysphania botrys (L.) Mosyakin & Clemants	Prodanović, 2007	jerusalem oak goosefoot
Polycnemum majus A. Braun	Prodanović, 2007	giant needleleaf
AMARYLLIDACEAE		
Allium carinatum subsp. pulchellum (G.Don.) Bonnier & Layens	Prodanović, 2007	witch's garlic
Allium flavum L.	Pavlović, 1967; Prodanović et al., 2004; Prodanović, 2007	small yellow onion
Allium moschatum L.	Prodanović, 2007	wild onion
Allium pendulinum Ten.	Prodanović, 2007	italian garlic

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Allium scorodoprasum L.	Pavlović, 1967; Prodanović, 2007	rocambole
Allium sphaerocephalum L.	Prodanović, 2007	round-headed leek
Galanthus nivalis L.	Prodanović, 2007	snowdrop
ANACARDIACEAE		
Cotinus coggygria Scop.	Pavlović, 1967; Rexhepi, 1979; Prodanović, 2007	smoke tree
APIACEAE		
Aegopodium podagraria L.	Prodanović, 2007; Krivošej et al., 2013	goutweed
Anthriscus caucalis M.Bieb.	Prodanović, 2007	bur-chervil
Anthriscus cerefolium (L.) Hoffm.	Prodanović, 2007	garden chervil
Anthriscus sylvestris (L.) Hoffm.	Prodanović, 2007	cow parsley, wild chervil
Bifora radians M. Bieb.	Prodanović, 2007	wild bishop
Bupleurum praealtum L.	Prodanović, 2007	hare's-ears
Bupleurum rotundifolium L.	Prodanović, 2007	round-leaved thoroughwax
Bupleurum tenuissimum L.	Prodanović et al., 2013	slender hare's-ear
Chaerophyllum aureum L.	Prodanović, 2007	golden chervil
Chaerophyllum bulbosum L.	Prodanović, 2007	turnip rooted chervil
Chaerophyllum temulum L.	Prodanović, 2007	rough chervil,
Conium maculatum L.	Prodanović, 2007	poison hemlock
Daucus carota L.	Prodanović, 2007	wild carrot, bird's nest
Eryngium campestre L.	Rexhepi, 1979; Prodanović, 2007	field eryngo
Eryngium palmatum Pančić & Vis.	Prodanović, 2007	blue eryngo, flat sea holly
Eryngium serbicum Pančić	Prodanović, 2007; Prodanović et al., 2008	serbian sea holly
Falcaria vulgaris Bernh.	Prodanović, 2007	sickleweed
Foeniculum vulgare Mill.	Prodanović, 2007	sweet fennel
Heracleum sphondylium L.	Prodanović, 2007	common hogweed
Laser trilobum (L.) Borkh.	Prodanović, 2007	gladich
Laserpitium siler L.	Prodanović, 2007	laserwort
Laserpitium siler L. subsp. *siler*	Prodanović, 2007	laserwort
Myrrhoides nodosa (L.) Cannon	Prodanović, 2007	sweet cicely
Oenanthe silaifolia M.Bieb.	Prodanović, 2007; Krivošej et al., 2013	narrow-leaved water-dropwort
Orlaya grandiflora (L.) Hoffm.	Prodanović, 2007	white laceflower
Pastinaca sativa L.	Prodanović, 2007	parsnip
Pastinaca sativa L. subsp. *urens* (Godr.)	Prodanović, 2007	wild parsnip
Peucedanum alsaticum L.	Prodanović, 2007	hog’s fennel
Peucedanum austriacum (Jacq.) W.D.J. Koch	Prodanović, 2007	giant hog’s fennel
Peucedanum cervaria (L.) Cusson ex Lapeyr.	Prodanović, 2007	hart’s word
Peucedanum officinale L.	Prodanović, 2007	marsh hog's fennel
Peucedanum oreoselinum (L.) Moench	Prodanović, 2007	mountain hog's Fennel
Pimpinella saxifraga L.	Prodanović, 2007	burnet-saxifrage,
Physospermum cornubiense (L.) DC.	Prodanović, 2007	bladderseed
Scandix pecten-veneris L.	Prodanović, 2007	Venus' comb
Seseli pallasii Besser	Prodanović, 2007	
Seseli peucedanoides (M.Bieb.) Koso.-Pol.	Prodanović, 2007	
Seseli rigidum Waldst. & Kit.	Prodanović, 2007	
Seseli rigidum Waldst. & Kit. subsp. *rigidum*	Prodanović, 2007	
Sium latifolium L.	Prodanović, 2007	water hemlock
Smyrnium perfoliatum L.	Prodanović, 2007	perfoliate alexanders
Torilis japonica (Houtt.) DC.	Prodanović, 2007	erect hedgeparsley
Trinia glauca (L.) Dumort.	Prodanović, 2007	honewort

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CLASS/ FAMILY/*Species*	Source/Literature	Common English names
subsp. *glauca*		
APOCYNACEAE		
Vinca major L.	Prodanović, 2007	greater periwinkle
Vincetoxicum hirundinaria Medik.	Prodanović, 2007	white swallow-wort
ARACEAE		
Arum maculatum L.	Prodanović, 2007	cuckoo pint
ARALIACEAE		
Hedera helix L.	Prodanović, 2007	english ivy
ARISTOLOCHIACEAE		
Aristolochia clematitis L.	Prodanović, 2007	birthwort
Asarum europaeum L.	Prodanović, 2007	wild ginger
ASPARAGACEAE		
Anthericum liliago L.	Pavlović, 1967; Prodanović, 2007	lily
Asparagus officinalis L.	Prodanović, 2007	sparrow grass
Asparagus tenuifolius Lam.	Rexhepi, 1979	
Convallaria majalis L.	Prodanović, 2007	lily of the valleys
Leopoldia comosa (L.) Parl.	Prodanović, 2007	tassel hyacinth
Muscari racemosum Mill.	Prodanović, 2007	starch grape hyacinth
Ornithogalum gussonei Ten.	Prodanović, 2007	star-of-Bethlehem
Ornithogalum pyramidale L.	Prodanović, 2007	pyramidal star-of-Bethlehem
Ornithogalum refractum Kit. ex Schltdl.	Prodanović, 2007	star-of-Bethlehem,
Ornithogalum umbellatum L.	Prodanović, 2007	garden star-of-Bethlehem
Polygonatum hirtum (Bosc ex Poir) Pursh	Prodanović, 2007	king Solomon's-seal
Polygonatum odoratum (Mill.) Druce	Prodanović, 2007	fragrant Solomon's seal
Scilla bifolia L.	Prodanović, 2007	twin leaf squill
BERBERIDACEAE		
Berberis vulgaris L. f. *vulgaris*	Prodanović, 2007	common barberry
Epimedium alpinum L.	Prodanović et al., 2013	alpine barrenwort
BETULACEAE		
Alnus glutinosa (L.) Gaertn.	Prodanović, 2007; Krivošej et al., 2013	european black alder
Carpinus betulus L.	Prodanović, 2007	common hornbeam
Carpinus orientalis Mill.	Pavlović, 1967; Prodanović et al., 2004; Prodanović, 2007	oriental hornbeam
Corylus avellana L.	Prodanović, 2007; Krivošej et al., 2013	hazel
Ostrya carpinifolia Scop.	Prodanović et al., 2004; Prodanović, 2007	hop-hornbeam
BORAGINACEAE		
Anchusa azurea Mill.	Prodanović, 2007	bugloss
Anchusa officinalis L.	Prodanović, 2007	common bugloss, alkanet
Anchusa officinalis L. subsp. *officinalis*	Prodanović, 2007	common bugloss, alkanet
Asperugo procumbens L.	Prodanović, 2007	german-madwort
Buglossoides arvensis (L.) I.M. Johnst.	Prodanović, 2007	corn gromwell
Buglossoides purpurocaerulea (L.) I.M. Johnst.	Prodanović, 2007	purple gromwell
Cerinthe minor L.	Prodanović, 2007	honeyworts
Cynoglossum creticum Mill.	Prodanović, 2007	blue houndstonge
Cynoglossum officinale L.	Prodanović, 2007	houndstongue
Echium rubrum Forssk.	Rexhepi, 1979; Krivošej et al., 1993; Prodanović, 2007	viper's bugloss rubrum
Echium italicum L.	Prodanović, 2007	italian bugloss
Echium vulgare L.	Prodanović, 2007	common vipersbugloss
Halacsya sendtneri (Boiss.) Dörfl.	Pavlović, 1967; Rexhepi, 1979; Ranđelović et al., 1982; Prodanović, 2007; Prodanović et al., 2008	halacsya
Heliotropium europaeum L.	Prodanović, 2007	european turn-sole

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Lappula squarrosa (Retz.) Dumort.	Prodanović, 2007	bur forget-me-not
Myosotis arvensis (L.) Hill.	Prodanović, 2007	field forget-me-not
Myosotis discolor Pers.	Prodanović, 2007	changing forget-me-not
Myosotis sparsiflora J.K.Mikan ex Pohl.	Prodanović, 2007	forget-me-not
Nonnea pulla DC.	Prodanović, 2007	monkswort
Onosma montana Sm.	Prodanović, 2007	onosma
Pulmonaria mollissima Wulfen ex Hornem.	Prodanović, 2007	lungwort
Pulmonaria officinalis L.	Prodanović, 2007	common lungwort
Symphytum tuberosum L.	Prodanović, 2007	tuberous comfrey
BRASSICACEAE		
Aethionema saxatile subsp. *graecum* (Boiss. & Spruner) Hayek	Rexhepi, 1979; Prodanović, 2007	burnt candytuft
Aethionema saxatile (L.) R.Br. subsp. *saxatile*	Prodanović, 2007	burnt candytuft
Alliaria petiolata (M.Bieb.) Cavara & Grande	Prodanović, 2007	garlic mustard
Alyssum alyssoides (L.) L.	Prodanović, 2007	yellow alyssum
Alyssum markgrafii O.E.Schulz.	Pavlović, 1967; Rexhepi, 1979; Krivošej et al., 1993; Prodanović, 2007; Prodanović et al., 2008;	
Alyssum montanum L. subsp. *serbicum* Novák f. *macrophyllum* Novák	Pavlović, 1967; Prodanović, 2007; Prodanović et al., 2008	mountain alyssum
Alyssum montanum L. subsp. *serbicum* Novák f. *microphyllum* Novák	Pavlović, 1967; Prodanović, 2007	mountain alyssum
Alyssum montanum L. subsp. *serbicum* Novák f. *serbicum*	Pavlović, 1967; Prodanović, 2007	mountain alyssum
Alyssum wierzbickii Heuff.?	Prodanović, 2007	alpine alyssum
Arabidopsis arenosa (L.) Lawalrée	Prodanović, (unpublished, field observation)	rockcres
Arabidopsis thaliana (L.) Heynh.	Prodanović, 2007	thale cress, mouse-ear cress
Arabis hirsuta (L.) Scop.	Prodanović, 2007	hairy rock-cress
Arabis turrita L.	Prodanović, 2007	rockcress,
Berteroa incana (L.) DC.	Prodanović, 2007	hoary alyssum
Calepina irregularis (Asso) Thell.	Prodanović, 2007	white ballmustard
Camelina sativa (L.) Crantz.	Prodanović, 2007	camelina, false flax
Capsella bursa-pastoris (L.) Medik	Prodanović, 2007	shepherd's purse
Cardamine bulbifera (L.) Crantz.	Prodanović, 2007	coralroot
Cardamine graeca L.	Krivošej & Prodanović, 2011	southern bitter-cress
Cardamine impatiens L.	Prodanović, 2007	narrowleaf bittercress
Cardamine pratensis L.	Prodanović, 2007	cuckoo flower, lady's smock
Descurainia sophia (L.) Webb. ex Prantl	Prodanović, 2007	flixweed
Diplotaxis muralis (L.) DC.	Prodanović, 2007	wall rocket
Draba lasiocarpa Rochel	Prodanović, 2007	whitlow-grasses
Draba muralis L.	Prodanović, 2007	whitlowgrass
Erysimum cuspidatum (M. Bieb.) DC.	Prodanović, 2007	wallflower
Erysimum diffusum Ehrh.	Pavlović, 1967; Prodanović, 2007	diffuse wallflowe
Erysimum odoratum Ehrh.	Prodanović, (unpublished, field observation)	smelly wallflower
Erysimum kuemmerlei Jáv.	Prodanović, 2007	wallflower
Erysimum sylvestre (Crantz) Scop.	Prodanović, 2007	wallflower
Fibigia clypeata (L.) Medik	Prodanović et al., 2004; Prodanović, 2007	false-gypsophila ankyropetalum
Hesperis matronalis L.	Prodanović, 2007	mother-of-the-evening
Isatis tinctoria L.	Prodanović, 2007	dyer's woad, glastum

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Lepidium campestre (L.) R.Br.	Prodanović, 2007	field pepperwort , field cress
Lepidium draba L.	Prodanović, 2007	whitetop, hoary cress
Lepidium ruderale L.	Prodanović, 2007	roadside pepperweed
Myagrum perfoliatum L.	Prodanović, 2007	bird's-eye cress, muskweed
Odontarrhena bertolonii subsp. *scutarina* (Nyár) Španiel & al.	Prodanović, 2007	
Rorippa amphibia (L.) Besser	Prodanović, 2007	great yellowcress
Rorippa austriaca (Crantz) Spach.	Prodanović, 2007	austrian yellow-cress
Rorippa lippizensis (Wulfen) Rchb.	Prodanović, 2007	yellowcress
Rorippa sylvestris (L.) Besser	Prodanović, 2007	creeping yellowcress, yellow fieldcress
Sisymbrium altissimum L.	Prodanović, 2007	tall hedge-mustard, tumblemustard
Sisymbrium loeselii L.	Prodanović, 2007	small tumbleweed mustard
Sisymbrium officinale (L.) Scop.	Prodanović, 2007	hedge mustard
Sisymbrium orientale L.	Prodanović, 2007	indian hedgemustard
Sisymbrium strictissimum L.	Prodanović, 2007	perennial rocket
Thlaspi arvense L.	Prodanović, 2007	stinkweed, bastard cress, fanweed
Thlaspi kovatsii Heuff.	Prodanović, 2007	pennycress
Turritis glabra L.	Prodanović, 2007	tower rockcress, tower mustard
CAMPANULACEAE		
Asyneuma canescens (Waldst. & Kit.) Griseb. & Schenk	Prodanović, 2007	harebells
Asyneuma limonifolium (L.) Janch.	Pavlović, 1967; Ranđelović et al., 1982; Prodanović, 2007	harebells
Campanula cervicaria L.	Prodanović, 2007	bristly bellflower
Campanula lingulata Waldst. & Kit.	Rexhepi, 1979; Prodanović et al., 2004; Prodanović, 2007	bellflower
Campanula persicifolia L.	Prodanović, 2007	peach-leaved bellflower
Campanula rapunculoides L.	Prodanović, 2007	creeping bellflower
Campanula rapunculus L.	Prodanović, 2007	rampion bellflower
Campanula rapunculus L. f. *montana* Pančić	Prodanović, 2007	rampion bellflower, rampion
Campanula trachelium L.	Prodanović, 2007	nettle-leaved bellflower
Legousia speculum-veneris (L.) Durande ex Vill.	Prodanović, 2007	looking glass, large Venus's-looking-glass
CANNABACEAE		
Humulus lupulus L.	Prodanović, 2007	common hop
CAPRIFOLIACEAE		
Cephalaria leucantha (L.) Scharad. ex Roem. & Schult.	Rexhepi, 1979; Prodanović, 2007	giant scabious
Knautia arvensis (L.) Coult.	Prodanović, 2007	field scabious
Knautia integrifolia (Honck. ex L.) Bertol	Prodanović, 2007	whole-leaved scabious
Lonicera caprifolium L.	Prodanović, 2007	italian woodbine, perfoliate honeysuckle
Scabiosa argentea L.	Prodanović, (unpublished, field observation)	silver scabious
Scabiosa columbaria L.	Rexhepi, 1979; Prodanović, 2007	pigeon scabious, pincushion flower
Scabiosa fumaroides Vis. & Pančić	Prodanović, 2007; Prodanović et al., 2008	
Valeriana officinalis L.	Prodanović, 2007	garden heliotrope, valerian
Valeriana tuberosa L.	Krivošej et al., 1995-1998; Prodanović, 2007	tuberous valerian
Valerianella coronata (L.) DC.	Prodanović, 2007	corn lettuce
Valerianella dentata (L.) Pollich	Ranđelović et al., 1982; Prodanović, 2007	corn salad
Valerianella locusta (L.) Laterr.	Prodanović, 2007	corn salad

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CLASS/ FAMILY/*Species*	Source/Literature	Common English names
Valerianella rimosa Bastard	Prodanović, 2007	beaked corn salad
CARYOPHYLLACEAE		
Agrostemma githago L.	Prodanović, 2007	corncockle
Arenaria serpyllifolia subsp. *leptoclados* (Rchb.) Nyman	Prodanović, 2007	thyme-leaf sandwort
Arenaria serpyllifolia L.	Prodanović, 2007	thyme-leaf sandwort
Cerastium brachypetalum Desp. ex Pers	Prodanović, 2007	gray chickweed
Cerastium decalvans Schloss. & Vuk.	Prodanović, 2007	balkan rozhets
Cerastium fontanum Baumg.	Prodanović, 2007	mouse-ear chickweed
Cerastium pumilum Curtis	Prodanović, 2007	dwarf mouse-ear, european chickweed
Cerastium pumilum var. *glutinosum* (Čelak) E.Rico	Prodanović, 2007	dwarf mouse-ear
Cerastium semidecandrum L.	Prodanović, 2007	fivestamen chickweed
Dianthus carthusianorum L.	Prodanović, 2007	carthusian pink
Dianthus giganteus d'Urv	Prodanović, 2007	pink
Dianthus pinifolius Sm.	Prodanović, 2007	immediate children
Dianthus pinifolius subsp. *serbicus* Wettst.	Pavlović, 1967; Prodanović, 2007	Immediate children
Dianthus sylvestris Wulfen subsp. *sylvestris*	Rexhepi, 1979; Prodanović, 2007	wood pink
Herniaria glabra L.	Prodanović, 2007	smooth rupturewort
Herniaria hirsuta L.	Prodanović, 2007	hairy rupturewort.
Herniaria incana Lam.	Prodanović, 2007	grey rupturewort
Holosteum umbellatum L.	Prodanović, 2007	jagged chickweed
Minuartia glomerata (M.Bieb.) Degen.	Prodanović et al., 2004; Prodanović, 2007	stitchwort
Minuartia hamata (Hausskn.) Mattf.	Prodanović, 2007	sandwort
Minuartia hirsuta (M.Bieb) Hand.-Mazz.	Rexhepi, 1979; Prodanović, 2007	hairy sandwort
Minuartia setacea (Thuill.) Hayek	Prodanović, 2007	sandwort
Minuartia verna (L.) Hiern	Prodanović, 2007	leadwort
Moehringia trinervia (L.) Clairv.	Prodanović, 2007	apetalous sandwort , three-nerved sandwort
Petrorhagia illyrica subsp. *haynaldiana* (Janka) P.W. Ball & Heywood	Pavlović, 1967; Prodanović, 2007	
Petrorhagia illyrica (Ard.) P.W.Ball & Heyood.	Pavlović, 1967; Prodanović, 2007	
Petrorhagia prolifera (L.) P.W. Ball. & Heywood	Rexhepi, 1979; Prodanović, 2007	proliferous pink
Petrorhagia saxifraga (L.) Link.	Pavlović, 1967; Rexhepi, 1979; Prodanović et al., 2004; Prodanović, 2007	tunic flower, coat flower
Saponaria glutinosa M. Bieb.	Prodanović, 2007	soapwort
Saponaria officinalis L.	Prodanović, 2007	common soapwort, bouncing-bet
Scleranthus annuus subsp. *polycarpos* (L.) Bonnier & Layens	Prodanović, 2007	annual knawel
Scleranthus perennis subsp. *dichotomus* (Schur) Nyman	Prodanović, 2007	perennial knawel
Silene latifolia subsp. *alba* (Mill.) Greuter & Burdet	Prodanović, 2007	bladder campion
Silene armeria L.	Prodanović, 2007	sweet William catchfly
Silene baccifera (L.) Roth.	Prodanović, 2007	campion
Silene bupleuroides L.	Prodanović, 2007	campion
Silene conica L.	Prodanović, 2007	striped corn catchfly
Silene coronaria (Desr.) Clairv. ex Rchb.	Prodanović, 2007	rose campion
Silene flos-cuculi (L.) Greuter & Burdet	Prodanović, 2007	ragged-robin
Silene italica (L.) Pers.	Prodanović, 2007	italian catchfly

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Silene nutans L.	Prodanović, 2007	nottingham catchfly
Silene otites (L.) Wibel.	Prodanović, 2007, Ranđelović et al., 1982	spanish catchfly
Silene viscaria (L.) Jess.	Prodanović, 2007	sticky catchfly
Silene vulgaris (Moench) Garcke	Prodanović, 2007	bladder campion
Stellaria aquaticua (L.) Scop.	Prodanović, 2007	giant chickweed
Stellaria graminea L.	Prodanović, 2007; Krivošej et al., 2013	grassleaved stichwort
Stellaria holostea L.	Prodanović, 2007	greater stitchwort
Stellaria media (L.) Vill.	Prodanović, 2007	common chickweed
Stellaria nemorum L.	Prodanović, 2007	nodding chickweed
CELASTRACEAE		
Euonymus europaeus L. var. *europaeus* f. *angustifolia* (Schultz) Rony	Prodanović, 2007	european spindle
Euonymus europaeus L. var. *grandifolia* Form. f. *scaberula* (Beck) Jovanović	Prodanović, 2007	european spindle
Euonymus latifolius (L.) Mill.	Prodanović, 2007	broadleaf spindle
Euonymus verrucosus Scop.	Prodanović, 2007	spindle tree
CERATOPHYLLACEAE		
Ceratophyllum submersum L.	Prodanović, 2007	soft hornwort
CISTACEAE		
Fumana bonapartei Maire et Petitm.	Pavlović, 1967; Rexhepi, 1979; Ranđelović et al., 1982; Prodanović, 2007; Prodanović et al., 2008	needle sunrose
Fumana procumbens (Dunal) Gren. & Godr.	Prodanović et al., 2004; Prodanović, 2007	sprawling needle sunrose
Helianthemum nummularium (L.) Mill. subsp. *nummularium*	Prodanović et al., 2004; Prodanović, 2007	common rock-rose
Helianthemum salicifolium (L.) Mill.	Prodanović, 2007	rock rose, sunrose
CLUSIACEAE		
Hypericum barbatum Jacq.	Prodanović, 2007	bearded St. John’s wort
Hypericum hirsutum L.	Prodanović, 2007	hairy St John's-wort
Hypericum perforatum L.	Rexhepi, 1979; Prodanović, 2007	perforate St. John’s wort
Hypericum rumeliacum Boiss.	Prodanović, 2007	St. John’s wort
COLCHICACEAE		
Colchicum autumnale L.	Prodanović et al., 2004; Prodanović, 2007; Krivošej et al., 2013	autumn crocus, meadow saffron
COMPOSITAE		
Achillea coarctata Poir.	Prodanović, 2007	yarrow
Achillea collina (Becker ex Rchb.f.) Heimerl.	Prodanović, 2007	yarrow
Achillea critmifolia Waldst. & Kit.	Prodanović, 2007	yarrow
Achillea millefolium L.	Prodanović, 2007	milfoil, common yarrow
Achillea pseudopectinata Janka	Prodanović, 2007	yarrow
Ambrosia artemisiifolia L.	Prodanović, 2007	common ragweed, annual ragweed
Anthemis arvensis L.	Prodanović, 2007	corn chamomile, mayweed
Arctium lappa L.	Prodanović, 2007	greater burdock
Artemisia absinthium L.	Prodanović, 2007	absinth wormwood
Artemisia alba Turra	Prodanović, 2007	white wormwood
Artemisia campestris L.	Prodanović, 2007	common sagewort
Artemisia scoparia Waldst. & Kitam.	Prodanović, 2007	virgate wormwood
Artemisia vulgaris L.	Prodanović, 2007	common mugwort
Bellis perennis L.	Prodanović, 2007; Krivošej et al., 2013	common daisy
Carduus candicans Waldst. & Kit.	Prodanović, 2007	thistle
Carduus nutans L.	Prodanović, 2007	musk thistle

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Carlina acaulis L.	Prodanović, 2007	stemless carline thistle, dwarf carline thistle
Carthamus lanatus L.	Prodanović, 2007	woolly carthamus, woolly safflower
Centaurea jacea L.	Prodanović, 2007	brown knapweed
Centaurea orientalis L.	Prodanović, 2007	knapweed
Centaurea phrygia L.	Rexhepi, 1979; Prodanović, 2007	wig knapweed
Centaurea scabiosa L. subsp. *fritschii* (Hayek) Hayek	Prodanović, 2007	greater knapweed
Centaurea scabiosa L. subsp. *spinulosa* (Spreng.) Arcang	Prodanović, 2007	scabious knapweed, greater knapweed
Centaurea solstitialis L.	Prodanović, 2007	yellow star-thistle
Centaurea stoebe L. subsp. *austrialis* (Pančić ex A.Kern) Greuter	Pavlović, 1967; Rexhepi, 1979; Prodanović, 2007	spotted knapweed
Centaurea stoebe L.	Prodanović, 2007	panicled knapweed, spotted knapweed
Chondrilla juncea L.	Prodanović et al., 2004; Prodanović, 2007	rush skeletonweed, nakedweed
Cichorium intybus L.	Prodanović, 2007	chicory
Cirsium arvense (L.) Scop.	Prodanović, 2007	creeping thistle
Cirsium creticum (Lam.) Urv.	Prodanović, 2007	bull thistle
Cirsium lanceolatum (L.) Hill.	Prodanović, 2007	bull thistle, common thistle
Cota austriaca (Jacq.) Sch.Bip.	Prodanović, 2007	austrian chamomile
Cota tinctoria (L.) J.Gay.	Prodanović, 2007	golden marguerite, yellow chamomile
Crepis biennis Lapeyr.	Prodanović, 2007	rough Hawksbeard
Crepis foetida subsp. *rhoeadifolia* (M.Bieb.) Čelak	Prodanović, 2007	stinking hawksbeard
Crepis sancta (L.) Bornm.	Pavlović, 1967; Prodanović, 2007	holy hawksbeard
Crepis setosa Haller f.	Prodanović, 2007	bristly hawksbeard
Crupina vulgaris Pers ex Cass.	Ranđelović et al., 1982; Prodanović, 2007	bearded creeper
Cyanus segetum Hill	Prodanović, 2007	cornflower, bachelor's button
Cyanus triumfettii (All.) Dostál ex Á. Löve & D.Löve	Prodanović, 2007	mountain cornflower
Cyanus triumfettii (All.) Dostál ex Á. Löve & D.Löve subsp. *axillaries* (Čelak.) Štepánek	Prodanović, 2007	mountain cornflower
Doronicum columnae Ten.	Prodanović, (unpublished, field observation)	leopard's bane
Doronicum hungaricum (Sadler) Rchb.f	Prodanović, 2007; Krivošej & Prodanović, 2011; Prodanović et al.,2012	
Doronicum orientale Hoffm.	Prodanović, (unpublished, field observation)	leopard's bane
Echinops ritro subsp. *ruthenicus* (M.Bieb.) Nyman	Prodanović, 2007	southern globethistle
Echinops sphaerocephalus L.	Prodanović, 2007	glandular globe-thistle
Erigeron acer L.	Prodanović, 2007	bitter fleabane
Erigeron canadensis L.	Prodanović, 2007	horseweed, canadian horseweed
Eupatorium cannabinum L.	Prodanović, 2007	hemp-agrimony, holy rope
Filago arvensis L.	Prodanović, 2007	field cottonrose
Gallatella albanica Degen	Rexhepi 1992; Krivošej et al., 1993; Prodanović, 2007; Prodanović et al., 2008	
Gallatela linosyris (L.) Rchb.	Prodanović, 2007	goldilocks aster
Hieracium bauhini Besser subsp. *besserianum* Spreng	Prodanović, 2007	hawkweed
Hieracium bauhini Besser subsp. *filiferum* (Tausch) Zahn	Prodanović, 2007	hawkweed

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Hieracium bauhini Besser subsp. *heathinum* (N.P.) Zahn	Prodanović, 2007	hawkweed
Hieracium bauhini Besser subsp. *pseudo-kerneri* Zahn	Prodanović, 2007	hawkweed
Hieracium bauhini Besser subsp. *pseudosparsum* Zahn	Prodanović, 2007	hawkweed
Hieracium cymosum Vill.	Prodanović, 2007	hawkweed
Hieracium hoppeanum Wallr. ex Nyman	Prodanović, 2007	Hoppe's hawkweed
Hieracium lachenalii Suter subsp. *festinum* (Boreau) Zahn.	Prodanović, 2007	common hawkweed, yellow hawkweed
Hieracium murorum L. subsp. *gentile* (Boreau) Sudre	Prodanović, 2007	wall hawkweed
Hieracium schmidtii subsp.*pallidum* (Biv.) O.Bolòs & Vigo	Prodanović, 2007	Schmidt's hawkweed
Hieracium sabaudum L. subsp. *vagum* Zahn	Prodanović, 2007	new england hawkweed, european hawkweed
Hypochaeris maculata L.	Prodanović, 2007	spotted cat's ear
Hypochaeris radicata L.	Prodanović, 2007	catsear, hairy cat's ear, false dandelion
Inula britannica L.	Prodanović, 2007	british elecampane, british yellowhead
Inula conyza (Griess.) DC.	Prodanović, 2007	ploughman's spikenard
Inula ensifolia L.	Rexhepi, 1979; Prodanović, 2007	swordleaf inula
Inula hirta L.	Prodanović, 2007	hairiness inula
Inula oculus-christi L.	Prodanović, 2007	Christ's eye hoary fleabane
Inula salicina L.	Prodanović, 2007	irish fleabane
Jurinea mollis (L.) Rchb.	Rexhepi, 1979; Prodanović, 2007	
Klasea radiata (Waldst. & Kit.) A.Löve & D. Löve	Prodanović, 2007	radiating outwords
Lactuca muralis (L.) Gaertn.	Prodanović, 2007	wall lettuce
Lactuca perennis L.	Prodanović, 2007	mountain lettuce, blue lettuce
Lactuca serriola L.	Prodanović, 2007	prickly lettuce
Lactuca viminea (L.) J. Presl.& C. Presl.	Prodanović, 2007	pliant lettuce
Leontodon hispidus L.	Prodanović, 2007	bristly hawkbit
Leucanthemum vulgare (Vaill) Lam.	Prodanović, 2007	ox-eye daisy
Matricaria chamomilla L.	Prodanović, 2007	chamomile
Onopordum acanthium L.	Prodanović, 2007	cotton thistle
Petasites hybridus (L.) G.Gaertn. &al.	Prodanović, 2007	butterbur
Pilosella bauhini (Schult.) Arv.-Touv.	Prodanović, 2007	
Pilosella piloselloides (Vill.) Soják	Prodanović, 2007	hawkweed
Podospermum laciniatum (L.) DC.	Prodanović, 2007	divided -eaved viper's grass
Pulicaria dysenterica (L.) Gaertn.	Prodanović, 2007	common fleabane
Scolymus hispanicus L.	Krasniqi et al., 1981	golden thistle, spanish oyster thistle
Scorzonera austriaca Willd. f. *latifolia* Vis.	Pavlović, 1967; Rexhepi, 1979; Prodanović, 2007	
Scorzonera hispanica L. var. *glastifolia* (Willd.) Wallr.	Prodanović, 2007	black salsify, spanish salsify
Senecio erucifolius L.	Prodanović, 2007	hoary ragwort
Senecio leucanthemifolius subsp. *vernalis* (Waldst. & Kit.) Greuter	Prodanović, 2007	eastern groundsel
Serratula tinctoria L.	Prodanović, 2007	dyer's plumeless saw-wort, saw-wort
Solidago virgaurea L.	Prodanović, 2007	goldenrod, woundwort
Sonchus asper (L.) Hill subsp. *glaucescens* (Jord.) Ball ex Ball	Prodanović, 2007	prickly sow-thistle
Tanacetum corymbosum (L.) Sch.-Bip.	Prodanović, 2007	corymbflower tansy
Tanacetum vulgare L.	Prodanović, 2007	common tansy, garden tansy
Taraxacum campyloides G.E.Haglund	Prodanović, 2007	dandelion

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Tephroseris papposa (Rchb.) Schur.	Prodanović, 2007	
Tragopogon balcanicus Velen.	Prodanović, 2007	
Tragopogon dubius Scop. subsp. *major* (Jacq.) Vollm	Prodanović, 2007	yellow salsify
Tragopogon dubius Scop.	Prodanović et al., 2004; Prodanović, 2007	yellow salsify
Tragopogon pratensis subsp. *orientalis* (L.) Čelak.	Prodanović, 2007	ack-go-to-bed-at-noon, meadow salsify
Tragopogon pterodes Petrović	Pavlović, 1967;	
Tussilago farfara L.	Prodanović, 2007; Krivošej et al., 2013	coltsfoot
Xanthium orientale subsp. *italicum* (Moretti) Greuter	Prodanović, 2007	rough cocklebur, clotbur
Xeranthemum annuum L.	Pavlović, 1967; Rexhepi, 1979; Ranđelović et al., 1982; Prodanović, 2007	annual everlasting, immortelle
CONVOLVULACEAE		
Calystegia sepium (L.) R.Br.	Prodanović, 2007	hedge bindweed
Convolvulus arvensis L.	Prodanović, 2007	field bindweed
Convolvulus cantabrica L.	Pavlović, 1967; Rexhepi, 1979; Ranđelović et al., 1982; Prodanović et al., 2004; Prodanović, 2007	cantabrican morning glory
Cuscuta epithymum (L.) L.	Prodanović, 2007	dodder, lesser dodder
Cuscuta europaea L.	Prodanović, 2007	greater dodder, european dodder
CORNACEAE		
Cornus mas L.	Prodanović, 2007	cornelian cherry dogwood
Cornus sanguinea L.	Prodanović, 2007	common dogwood
CRASSULACEAE		
Hylotelephium telephium (L.) H. Ohba.	Prodanović, 2007	orpine, livelong, life-everlasting
Sedum acre L.	Rexhepi, 1979; Ranđelović et al., 1982; Prodanović et al., 2004; Prodanović, 2007	goldmoss stonecrop
Sedum album L.	Pavlović, 1967; Rexhepi, 1979; Prodanović et al., 2004; Prodanović, 2007	white stonecrop
Sedum dasyphyllum L.	Rexhepi, 1979; Prodanović, 2007	corsican stonecrop
Sedum hispanicum L.	Prodanović, 2007	spanish stonecrop
Sedum ochroleucum Chaix	Prodanović, 2007	european stonecrop
Sedum sexangulare L.	Prodanović, 2007	tasteless stonecrop
Sempervivum heuffelii Schott	Pavlović, 1967; Prodanović, 2007	houseleeks
Sempervivum marmoreum Griseb.	Prodanović, 2007	house leek (houseleek)
CUCURBITACEAE		
Bryonia cretica subsp. *dioica* (Jacq.) Tutin	Prodanović, 2007	english mandrake
Echinocystis lobata (Michx) Torr. & A. Gray	Prodanović, 2007	wild cucumber, prickly cucumber
CYPERACEAE		
Carex acuta L.	Prodanović, 2007	acute sedge
Carex appropinquata Schumach.	Prodanović, 2007	fibrous tussock-sedge
Carex caryophyllea Latour.	Prodanović, 2007	vernal sedge
Carex distans L.	Prodanović, 2007	distant sedge
Carex divulsa Stokes.	Prodanović, 2007	grey sedge
Carex filiformis L.	Prodanović, 2007	downy-fruited sedge
Carex hirta L.	Prodanović, 2007	hairy sedge
Carex leporina L.	Prodanović, 2007	eggbract sedge, oval sedge
Carex montana L.	Prodanović, 2007	mountain, soft-leaved sedge
Carex muricata subsp. *pairae* (F.W.Schultz.) Čelak.	Prodanović, 2007	rough sedge, prickly sedge
Carex ornithopoda Willd.	Prodanović et al., 2004; Prodanović,	bird-foot sedge

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	2007	
Carex panicea L.	Prodanović, 2007	carnation sedge
Carex pendula Huds.	Prodanović, 2007	pendulous sedge, hanging sedge
Carex riparia Curtis	Prodanović, 2007	greater pond sedge
Carex spicata Huds.	Prodanović, 2007	spiked sedge
Carex vulpina L.	Prodanović, 2007	true fox sedge
Schoenoplectus lacustris (L.) Palla	Prodanović, 2007	lakeshore bulrush
DIPSACACEAE		
Lomelosia palaestina (L.) Raf.	Prodanović et al.,2010	palestine scabious
EUPHORBIACEAE		
Euphorbia amygdaloides L.	Prodanović, 2007; Krivošej et al., 2013	wood spurge
Euphorbia barrelieri Savi	Prodanović, 2007	spurge
Euphorbia cyparissias L.	Prodanović et al., 2004; Prodanović, 2007	cypress spurge
Euphorbia esula subsp. *tommasniana* (Bertol.) Kuzmanov	Prodanović, 2007	green spurge, leafy spurge
Euphorbia falcata L.	Prodanović, 2007	sickle spurge
Euphorbia glabriflora Vis.	Pavlović, 1967; Rexhepi, 1979; Ranđelović et al., 1982; Krivošej et al., 1993; Prodanović, 2007	spurge
Euphorbia glareosa Pall. ex M.Bieb.	Prodanović, 2007	spurge
Euphorbia helioscopia L.	Prodanović, 2007	sun spurge
Euphorbia ilirica Lam.	Prodanović, 2007	illyrian spurge
Euphorbia myrsinites L.	Rexhepi, 1979; Ranđelović et al., 1982; Prodanović, 2007	myrtle spurge, blue spurge
Euphorbia platyphyllos L.	Prodanović, 2007	broadleaved spurge
Euphorbia salicifolia Host.	Prodanović, 2007	spurge
Euphorbia stricta L.	Prodanović, 2007	tintern spurge
Euphorbia taurinensis All.	Prodanović, 2007	spurge
Mercurialis ovata Sternb. & Hoppe	Prodanović, 2007	
Mercurialis perennis L.	Prodanović, 2007	dog's mercury
FAGACEAE		
Quercus cerris L.	Prodanović, et al., 2004; Prodanović, 2007	turkey oak
Quercus frainetto Ten.	Prodanović et al., 2004; Prodanović, 2007	hungarian oak
Quercus petraea (Matt.) Liebl.	Prodanović, 2007	sessile oak
Quercus pubescens Willd.	Pavlović, 1967; Prodanović et al., 2004; Prodanović, 2007	downy oak, pubescent oak
GENTIANACEAE		
Centaurium erythraea Rafn	Prodanović, 2007	european centaury
Gentiana cruciata L.	Prodanović, 2007	cross gentian
GERANIACEAE		
Erodium ciconium (L.) L' Hér.	Prodanović, 2007	redstem filaree, redstem stork's bill
Erodium cicutarium (L.) L'Hér.	Prodanović, 2007	redstem filaree, redstem stork's bill
Geranium columbinum L.	Prodanović, 2007	long-stalked crane's-bill, longstalk cranesbill
Geranium dissectum Jusl.	Prodanović, 2007	cut-leaved crane's-bill
Geranium lucidum L.	Prodanović, 2007	shining cranesbill, shiny geranium
Geranium molle L.	Prodanović, 2007	dove's-foot crane's-bill
Geranium phaeum L.	Prodanović, 2007	dusky crane's-bill
Geranium pyrenaicum Burm.f.	Prodanović, 2007	hedgerow cranesbill
Geranium robertianum L.	Prodanović, 2007	herb-Robert, red robin
Geranium sanguineum L.	Prodanović, 2007	bloody crane's-bill
IRIDACEAE		

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Crocus chrysanthus (Herb.) Herb.	Prodanović, 2007	golden crocus
Iris graminea L.	Prodanović, 2007; Krivošej et al., 2013	grass leaved Iris
Iris pseudacorus L.	Prodanović, 2007	yellow iris, water flag
Iris reichenbachii Heuff.	Prodanović, 2007	
JUNCACEAE		
Juncus bufonius L.	Prodanović, 2007	toad rush
Juncus compressus Jacq.	Prodanović, 2007	compressed rush
Juncus inflexus L.	Prodanović, 2007	hard rush, blue rush
Luzula campestris (L.) DC.	Prodanović, 2007	field wood-rush
Luzula forsteri (Sm.) DC.	Prodanović, 2007	southern wood-rush
LAMIACEAE		
Ajuga chamaepitys (L.) Schreb.	Prodanović et al., 2004; Prodanović, 2007	yellow bugle, ground-pine
Ajuga chamaepitys subsp.*chia* (Schreb.) Arcang.	Prodanović, 2007	yellow bugle, ground-pine
Ajuga genevensis L.	Prodanović, 2007; Krivošej et al., 2013	blue bugle, geneva bugleweed
Ajuga laxmannii (Murray) Benth.	Prodanović, 2007	bugleweed, ground pine
Ballota nigra L.	Prodanović, 2007	black horehound
Clinopodium acinos (L.) Kuntze	Prodanović et al., 2004; Prodanović, 2007	basil thyme, spring savory
Clinopodiuma alpinum (L.) Kuntze	Prodanović, 2007	alpine calamint
Clinopodium nepeta subsp. *glandulosum* (Req.) Govaerts	Prodanović, 2007	lesser calamint
Clinopodium thymifolium (Scop.) Kuntze	Prodanović, 2007	clinopodium thymifolium from the wild
Clinopodium vulgare L.	Prodanović, 2007	wild basil
Galeopsis speciosa Mill.	Prodanović, 2007	arge-flowered hempnettle
Glechoma hederacea L.	Prodanović, 2007	creeping charlie
Glechoma hirsuta Waldst.& Kit.	Prodanović, 2007	ground-ivy
Lamium amplexicaule L.	Prodanović, 2007	common henbit
Lamium bifidum subsp. *balcanicum* Velen.	Prodanović, 2007	dead-nettles
Lamium galeobdolon (L.) L.	Prodanović, 2007	yellow archangel, yellow deadnettle
Lamium garganicum L. subsp. *garganicum*	Prodanović, 2007	large dead nettle
Lamium garganicum L. subsp. *glabratum* (Griseb.) Briq.	Prodanović, 2007	large dead nettle
Lamium purpureum L.	Prodanović, 2007	red dead-nettle
Leonurus cardiaca L.	Prodanović, 2007	motherwort in english
Lycopus europaeus L.	Prodanović, 2007	gypsywort
Marrubium peregrinum L.	Prodanović, 2007	horehound
Marrubium vulgare L.	Prodanović, 2007	white horehound, common horehound
Melittis melissophyllum L.	Prodanović, 2007	bastard balm
Mentha aquatica L.	Prodanović, 2007	water mint
Mentha longifolia (L.) L.	Prodanović, 2007; Krivošej et al., 2013	asian mint
Nepeta cataria L.	Prodanović, 2007	catnip, catswort
Origanum vulgare L.	Prodanović, 2007	origanum, wild marjoram
Phlomis tuberosa L.	Prodanović et al.,2018	tuberous jerusalem sage
Prunella laciniata (L.) L.	Ranđelović et al., 1982; Prodanović, 2007	cutleaf selfheal
Prunella vulgaris L.	Prodanović, 2007	common self-heal, heal-all
Salvia amplexicaulis Lam.	Prodanović, 2007	stem-clasping sage
Salvia nemorosa L.	Prodanović, 2007	woodland sage, balkan clary
Salvia pratensis L. subsp. *pozegensis* (Watzl) Diklic	Prodanović, 2007; Prodanović et al., 2008	meadow sage
Salvia sclarea L.	Prodanović et al., 2004; Prodanović,	salvia romana, clary sage

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	2007	
Salvia verticillata L.	Prodanović, 2007	lilac sage
Scutellaria altissima L.	Prodanović, 2007	tall skullcap
Scutellaria galericulata L.	Prodanović, 2007	marsh skullcap, hooded skullcap
Sideritis montana L.	Prodanović et al., 2004; Prodanović, 2007	mountain ironwor
Stachys germanica L.	Prodanović, 2007	downy woundwort
Stachys officinalis (L.) Trevis	Prodanović, 2007	common hedgenettle, betony
Stachys palustris L.	Prodanović, 2007	marsh woundwort
Stachys recta subsp. *baldacii* (K. Malý) Hayek	Ranđelović et al., 1982; Rexhepi, 1979; Prodanović, 2007	stiff hedgenettle
Stachys recta L. subsp. *recta* var. *chrysophaea* (Pančić) Hayek f. *chrysophae*	Prodanović, 2007	stiff hedgenettle
Stachys scardica (Gris.) Hayek	Rexhepi, 1979; Prodanović, 2007	
Teucrium chamaedrys L.	Prodanović et al., 2004; Prodanović, 2007	wall germander
Teucrium montanum L.	Pavlović, 1967; Ranđelović et al, 1982; Prodanović, 2007	mountain germander
Teucrium montanum L. subsp. *montanum*	Prodanović, 2007	mountain germander
Thymus longicaulis C. Presl.	Pavlović, 1967	thyme
Thymus pulegioides subsp. *montanus* (Benth.) Ronniger.	Prodanović, 2007	broad-leaved thyme
Ziziphora capitata L.	Prodanović, 2007	oriental zizifora
LEGUMINOSAE		
Amorpha fruticosa L.	Prodanović, 2007	false indigo bush
Astragalus cicer L.	Prodanović, 2007	chickpea milkvetch
Astragalus dasyanthus Pall.	Prodanović, 2007; Prodanović et al., 2008; Prodanović et al.,2012	milkvetch
Astragalus glycyphyllos L.	Prodanović, 2007	liquorice milkvetch
Astragalus hamosus L.	Prodanović, 2007	southern milk vetch
Astragalus onobrychis L.	Pavlović, 1967; Ranđelović et al., 1982; Prodanović, 2007	sainfoin milk vetch
Chamaecytisus ciliatus var. *alpestris* (Schur.) Diklić	Prodanović, 2007	
Colutea arborescens L.	Prodanović, 2007	bladder senna
Coronilla scorpioides (L.) Koch.	Prodanović, 2007	yellow crown vetch, annual scorpion-vetch
Cytisus austriacus subsp. *heuffelii* (Wierzb.) Asch. & Graebn	Prodanović, 2007	broom
Cytisus decumbens (Durande) Spach.	Ranđelović et al., 1982; Prodanović, 2007	scotch broom
Cytisus hirsutus L.	Prodanović, 2007	clustered broom
Cytisus procumbens (Willd.) Spreng.	Prodanović, 2007	broom
Dorycnium pentaphyllum subsp. *germanicum* (Gremli) Gams	Pavlović, 1967; Rexhepi, 1979; Prodanović, 2007	prostrate canary clover, badassi
Dorycnium pentaphyllum subsp. *herbaceum* (Vill.) Rouy	Prodanović et al., 2004; Prodanović, 2007	prostrate canary clover, badassi
Genista januensis Viv.	Prodanović, 2007	broom
Genista sagittalis L.	Prodanović, 2007; Krivošej et al., 2013	winged broom
Genista tinctoria L.	Prodanović, 2007	dyer's greenweed, dyer's broom
Hippocrepis comosa L.	Prodanović, 2007	horseshoe vetch
Hippocrepis emerus (L.) Lassen	Ranđelović et al., 1982; Prodanović, 2007	scorpion senna
Lathyrus hallersteinii Baumg.	Prodanović, 2007	yellow pea
Lathyrus hirsutus L.	Prodanović, 2007	caley pea, singletary pea, hairy vetchling

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Lathyrus latifolius L.	Prodanović, 2007	everlasting-pea, perennial pea
Lathyrus niger (L.) Bernh.	Prodanović, 2007	black pea
Lathyrus nissolia L.	Prodanović, 2007	grass vetchling, grass pea
Lathyrus pannonicus (Jacq.) Garcke	Prodanović, 2007	felted vetch
Lathyrus pratensis L.	Prodanović, 2007; Krivošej et al., 2013	meadow vetchling
Lathyrus setifolius L.	Prodanović, 2007	narrow-leaved red vetchling
Lathyrus sphaericus Retz.	Prodanović, 2007	grass pea, round-seeded vetchling
Lathyrus tuberosus L.	Prodanović, 2007	tuberous pea, tuberous vetchling
Lathyrus venetus (Mill.) Wohlf.	Prodanović et al., 2004; Prodanović, 2007	bushy vetchling
Lathyrus vernus (L.) Bernh.	Prodanović, 2007	spring vetchling, spring pea
Lembotropis nigricans (L.) Griseb.	Ranđelović et al., 1982; Prodanović, 2007	black broom
Lens nigricans (M. Bieb.) Godr.	Prodanović, 2007	lens
Lotus corniculatus L.	Prodanović, 2007	birdsfoot trefoil
Medicago arabica (L.) Huds.	Prodanović, 2007	spotted medick
Medicago falcata L.	Ranđelović et al., 1982; Prodanović, 2007	yellow lucerne, sickle alfalfa
Medicago lupulina L.	Prodanović et al., 2004; Prodanović, 2007	black medick, nonesuch
Medicago minima (L.) L.	Prodanović, 2007	little burclover
Medicago orbicularis (L.) Bartal.	Prodanović, 2007	blackdisk medick, button clover
Medicago prostrata Jacq.	Pavlović, 1967; Rexhepi, 1979; Prodanović, 2007	alfalfa, wild
Medicago rigidula (L.) All.	Prodanović, 2007	tifton burclover, rigid medick
Medicago sativa L.	Prodanović, 2007	lucerne
Melilotus albus Medik.	Prodanović, 2007	honey clover, white melilot
Melilotus officinalis (L.) Pall.	Prodanović, 2007	yellow sweet clover
Onobrychis alba (Waldst. & Kit.) Desv.	Prodanović, 2007	sainfoin
Onobrychis viciifolia Scop.	Prodanović, 2007	common sainfoin
Ononis spinosa subsp. *hircina* (Jacq.) Gams	Prodanović, 2007	spiny restharrow
Ononis pusilla L.	Prodanović, 2007	
Oxytropis pilosa (L.) DC.	Prodanović, 2007; Prodanović et al.,2012	hairy milk vetch
Pisum sativum subsp. *elatius* (M.Bieb.) Asch.& Graebn.	Prodanović, 2007	dun pea
Robinia pseudoacacia L.	Prodanović, 2004; Prodanović, 2007	black locust
*Securigera elegans (*Pancic) Lassen	Prodanović, 2007	crownvetch
Securigera varia (L.) Lassen	Prodanović et al., 2004; Prodanović, 2007	purple crownvetch
Trifolium angustifolium L.	Prodanović, 2007	narrowleaf crimson clover
Trifolium arvense L.	Prodanović, 2007	hare's-foot clover
Trifolium dalmaticum Vis.	Prodanović, 2007	dalmatian clover
Trifolium fragiferum L.	Prodanović, 2007	strawberry clover
Trifolium glomeratum L.	Krivošej & Prodanović, 2011	clustered clover
Trifolium hirtum All.	Prodanović, 2007	rose clover
Trifolium incarnatum L.	Prodanović, 2007	crimson clover, italian clover
Trifolium medium L.	Prodanović, 2007	zigzag clover
Trifolium montanum L.	Prodanović, 2007; Krivošej et al., 2013	mountain clover
Trifolium ochroleucon Huds.	Prodanović, 2007	sulphur clover
Trifolium patens Schreb.	Prodanović, 2007; Krivošej et al., 2013	clover
Trifolium pignantii Fauchė & Chaub.	Prodanović, 2007	clover
Trifolium pratense L.	Prodanović, 2007	red clover
Trifolium repens L.	Prodanović, 2007	white clover, landino clover
Trifolium striatum L.	Prodanović, 2007	knotted clover

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Trifolium strictum Jusl	Prodanović, 2007; Krivošej & Prodanović, 2011	upright clover
Trifolium trichopterum Pancic	Pavlović, 1967; Prodanović, 2007	
Trigonella esculenta Willd.	Prodanović, 2007	fenugreek
Trigonella gladiata M. Bieb.	Prodanović et al.,2010	fenugreek
Vicia cracca L.	Prodanović, 2007	tufted vetch, cow vetch, bird vetch
Vicia cracca subsp. *incana* (Gouan) Rouy.	Prodanović, 2007	tufted vetch, cow vetch, bird vetch
Vicia grandiflora Scop.	Prodanović, 2007	large-flowered vetch, large yellow vetch
Vicia hirsuta (L.) Gray	Prodanović, 2007	hairy tare, hairy vetch
Vicia lathyroides L.	Prodanović, 2007	spring vetch
Vicia pannonica Crantz	Prodanović, 2007	hungarian vetch
Vicia peregrina L.	Prodanović, 2007	wandering vetch
Vicia pisiformis L.	Prodanović, 2007	pea vetch
Vicia sativa L.	Prodanović, 2007	common vetch
Vicia sativa subsp. *nigra* (L.) Ehrh.	Prodanović, 2007	common vetch, garden vetch
Vicia sparsiflora Ten.	Prodanović, 2007	vetch
Vicia tenuifolia Roth.	Prodanović, 2007	fine-leaved vetch, cow vetch
Vicia villosa Roth.	Prodanović, 2007	winter vetch, hairy vetch
LILIACEAE		
Erythronium dens-canis L.	Prodanović, 2007	dog's tooth violet
Fritillaria montana Hoppe ex W.D.J. Koch	Prodanović, 2007	missionbells
Gagea lutea (L.) Ker Gawl.	Prodanović, 2007	yellow star-of-bethlehem
Gagea pusilla (F.W.Schmidt) Sweet	Krivošej et al., 1995-1998, Prodanović, 2007	yellow star-of-bethlehem
Lilium martagon L.	Prodanović, 2007	martagon lily, turk's cap lily
Tulipa sylvestris L.	Prodanović, 2007	wild tulip, woodland tulip
Tulipa serbica Tatić & Krivošej	Tatić & Krivošej, 1997; Prodanović, 2007; Prodanović et al., 2008	serbian tulip
LINACEAE		
Linum bienne Mill.	Prodanović, 2007	pale, narrowleaf flax
Linum austriacum L.	Prodanović, 2007	austrian flax
Linum flavum L.	Prodanović, 2007	golden flax, yellow flax
Linum nervosum Waldst.& Kit.	Krivošej & Prodanović, 2011	
Linum tenuifolium L.	Pavlović, 1967; Prodanović, 2007	narrow-leaved flax
LYTHRACEAE		
Lythrum salicaria L.	Prodanović, 2007	purple loosestrife
MALVACEAE		
Althaea hirsuta L.	Prodanović, 2007	hairy marshmallow
Althaea officinalis L.	Prodanović, 2007	marsh-mallow
Lavatera thuringiaca L.	Krasniqi et al., 1981; Prodanović, 2007	garden tree-mallow
Malva sylvestris L.	Prodanović, 2007	high mallow
Tilia cordata Mill.	Prodanović, 2007	small-leaved lime
Tilia tomentosa Moench	Prodanović, 2007	silver lime
MELANTHIACEAE		
Veratrum nigrum L.	Prodanović, 2007	black false hellebore
NYMPHAEACEAE		
Nuphar lutea (L.) Sm.	Prodanović et al.,2010	yellow pond-lily
OLEACEAE		
Fraxinus ornus L.	Prodanović et al.,2004; Prodanović, 2007	manna ash
Ligustrum vulgare L.	Prodanović, 2007	wild privet
ONAGRACEAE		
Epilobium angustifolium L.	Prodanović, 2007	fireweed

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Epilobium hirsutum L.	Prodanović, 2007	great willowherb
Epilobium parviflorum Schreb.	Prodanović, 2007	hoary willowherb
Oenothera biennis L.	Prodanović, 2007	common evening-primrose
ORCHIDACEAE		
Anacamptis morio (L.) R.M.Bateman, Pridgeon & M.W.Chase	Prodanović, 2007; Prodanović et al., 2008	green-winged orchid
Anacamptis papilionaceae (L.) R.M.Bateman, Pridgeon & M.W.Chase	Prodanović, 2007; Prodanović et al., 2008	butterfly orchid
Anacamptis pyramidalis (L.) Rich.	Prodanović, 2007	pyramidal orchid
*Cephalanthera damasonium (*Mill.) Druce	Prodanović, 2007	white helleborine
Cephalanthera rubra (L.) Rich.	Prodanović, 2007; Prodanović et al., 2008	red helleborine
Epipactis helleborine (L.) Crantz.	Prodanović, 2007	broad-leaved helleborine
Epipactis helleborine (L.) Crantz. subsp. *helleborine*	Prodanović, 2007	broad-leaved helleborine
Epipactis microphylla (Ehrh.) Sw.,	Prodanović et al., 2004; Prodanović, 2007; Prodanović et al., 2008;	small-leaved helleborine
Himantoglossum caprinum (M. Bieb.) Spreng.	Prodanović et al.,2018	
Limodorum abortivum (L.) Sw.	Prodanović, 2007; Prodanović et al., 2008	violet limodore
Neottia nidus-avis (L.) Rich.	Prodanović, 2007; Prodanović et al., 2008;	bird's-nest orchid
Neotinea tridentata (Scop.) R.M.Bateman, Pridgeon & M.W.Chase	Prodanović, 2007; Prodanović et al., 2008; Krivošej et al., 2013	three-toothed orchid
Ophrys apifera Huds.	Prodanović, 2007	bee orchid
Orchis mascula (L.) L.	Prodanović, 2007; Prodanović et al., 2008;	early-purple orchid, early spring orchis
Orchis purpurea Huds.	Prodanović, 2007; Prodanović et al., 2008;	lady orchid
Platanthera bifolia (L.) Rich	Prodanović, 2007; Prodanović et al., 2008	lesser butterfly-orchid
OROBANCHACEAE		
Lathraea squamaria L.	Prodanović, 2007	common toothwort
Melampyrum arvense L.	Prodanović, 2007	field cow-wheat
Melampyrum cristatum L.	Prodanović, 2007	crested cow-wheat
Melampyrum heracleoticum Boiss. & Orph.	Prodanović, 2007; Prodanović et al., 2008	cow wheat
Orobanche alba Stephan ex Willd.	Prodanović, 2007	scalloped broomrape
Orobanche caryophyllacea Sm.	Prodanović, 2007	clove-scented broomrape
Parentucellia latifolia Caruel	Prodanović, 2007	broadleaf glandweed
Pedicularis comosa subsp. *campestris* (Griseb. & Schenk) Soó	Prodanović, 2007	glandweed
Pedicularis comosa L.	Prodanović, 2007	glandweed
Pedicularis friderici-augusti Tomm.	Prodanović, 2007	Frederick Augustus' lousewort
Rhinanthus rumelicus Velen.	Prodanović, 2007	rattle
PAPAVERACEAE		
Chelidonium majus L.	Prodanović, 2007	reater celandine, nipplewort
Corydalis cava (L.) Schweigg. & Körte	Prodanović, 2007	hollow root
Corydalis solida (L.) Clairv.	Prodanović, 2007	fumewort
Papaver dubium L.	Prodanović, 2007	long-headed poppy
Papaver rhoeas L.	Prodanović, 2007	common poppy, corn poppy
PLANTAGINACEAE		
Digitalis laevigata Waldst. & Kit.	Prodanović, 2007	grecian foxglove
Digitalis lanata Ehrh.	Prodanović et al., 2004; Prodanović, 2007	woolly foxglove
Linaria genistifolia (L.) Mill.	Pavlović, 1967; Prodanović, 2007	toadflax
Linaria genistifolia (L.) Mill. subsp. *genistifolia*	Prodanović, 2007	broomleaf toadflax

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Linaria vulgaris Mill.	Prodanović, 2007	yellow toadflax
Plantago argentea Chaix	Rexhepi, 1979; Prodanović, 2007	silver plantain
Plantago lanceolata L.	Prodanović, 2007	ribwort plantain
Plantago major L.	Prodanović, 2007	roadleaf plantain, white man's foot, greater plantain
Plantago major L. subsp. *major*	Prodanović, 2007	roadleaf plantain, white man's foot, greater plantain
Plantago media L.	Prodanović, 2007	hoary plantain
Plantago subulata L.		plantain
Veronica anagallis-aquatica L.	Prodanović, 2007	water speedwell
Veronica arvensis L.	Prodanović, 2007	wall speedwell, corn speedwell
Veronica austiaca L.	Prodanović, 2007	broadleaf speedwell, large speedwell
Veronica beccabunga L.	Prodanović, 2007	european speedwell, brooklime
Veronica chamaedrys L.	Prodanović, 2007	germander speedwell, bird's-eye speedwell
Veronica hederifolia L.	Prodanović, 2007	ivy-leaved speedwell
Veronica officinalis L.	Prodanović, 2007	heath speedwell
Veronica persica Poir.	Prodanović, 2007	persian speedwel
Veronica polita Fr.	Prodanović, 2007	grey field-speedwell
Veronica praecox All.	Prodanović, 2007	breckland speedwell
Veronica prostrata L.	Prodanović, 2007	rock speedwell
Veronica serpyllifolia L.	Prodanović, 2007	thyme-leaved speedwell
Veronica serpyllifolia L. subsp. *serpyllifolia*	Prodanović, 2007	thyme-leaved speedwell
Veronica spicata L.	Prodanović, (unpublished, field observation)	spike speedwell
PLUMBAGINACEAE		
Goniolimon incanum (L.) Hepper	Prodanović, 2007	lavender statice, sea lavender
Goniolimon collinum (Griseb.) Boiss.	Pavlović, 1967; Prodanović, 2007	sea lavender
Goniolimon tataricum (L.) Boiss.	Prodanović, 2007	german statice, tatarian statice
POACEAE		
Aegilops cylindrica Host	Prodanović, 2007	jointed goatgrass
Aegilops geniculata Roth.	Prodanović, 2007	goat grass, ovate goatgrass
Agropyron cristatum (L.) Gaertn.	Rexhepi, 1979; Prodanović, 2007	crested wheatgrass
Agrostis stolonifera L.	Prodanović, 2007	creeping bentgrass, fiorin
Alopecurus myosuroides Huds.	Prodanović, 2007	mousetail grass, black grass
Alopecurus pratensis L.	Prodanović, 2007; Krivošej et al., 2013	meadow foxtail
Anthoxanthum odoratum L.	Prodanović, 2007; Krivošej et al., 2013	sweet vernal grass
Apera spica-venti (L.) P. Beauv.	Prodanović, 2007	common windgrass
Arrhenatherum elatius (L.) P. Beauv.ex J.Presl & C.Presl	Prodanović, 2007	false oat-grass
Brachypodium silvaticum (Huds.) P. Beauv.	Prodanović, 2007	slender false-brome
Bothriochloa ischaemum (L.)	Prodanović, 2007	yellow bluestem
Briza media L. Keng	Prodanović, 2007; Krivošej et al., 2013	quaking grass
Bromus arvensis L.	Prodanović, 2007	field brome
Bromus commutatus Schrad.	Prodanović, 2007	meadow brome
Bromus erectus Huds.	Rexhepi, 1979; Prodanović, 2007	erect brome, upright brome
Bromus hordeaceus L.	Prodanović, 2007	soft brome
Bromus inermis Leyss.	Prodanović, 2007	smooth brome
Bromus racemosus L.	Prodanović, 2007	smooth brome
Bromus squarrosus L.	Prodanović, 2007	corn brome
Bromus sterilis L.	Rexhepi, 1979; Prodanović, 2007	poverty brome
Bromus tectorum L.	Prodanović, 2007	drooping brome, cheatgrass
Chrysopogon gryllus (L.) Trin	Rexhepi, 1979; Prodanović, 2007	
Cynodon dactylon (L.) Pers.	Prodanović, 2007	bermuda grass, couch grass

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Cynosurus cristatus L.	Prodanović, 2007; Krivošej et al., 2013	crested dogstail grass
Dactylis glomerata L.	Prodanović et al., 2004; Prodanović, 2007; Krivošej et al., 2013	cock's-foot, orchard grass
Danthonia alpina Vest.	Prodanović, 2007; Krivošej et al., 2013	heathgrass, wallaby grass
Dasypyrum villosum (L.) Borbás	Prodanović, 2007	mosquitograss
Eragrostis minor Host	Prodanović, 2007	ovegrass, canegrass
Elymus hispidus (Opiz) Melderis	Prodanović, 2007	hairy couch grass
Elytrigia repens (L.) Nevski.	Prodanović, 2007	couch grass
Festuca arundinacea Schreb.	Prodanović, 2007	tall fescue
Festuca pratensis Huds.	Prodanović, 2007	meadow fescue
Festuca rubra L.	Prodanović, 2007	red fescue
Festuca valesiaca Schleich. ex Gaudin	Prodanović, 2007	volga fescue
Glyceria fluitans (L.) R.Br.	Prodanović, 2007	floating sweet-grass, water mannagrass
Glyceria notata Chevall.	Prodanović, 2007	plicate sweet grass
Helictotrichon compressum (Heuff.) Henrard	Prodanović, 2007	
Holcus lanatus L.	Prodanović, 2007	yorkshire fog, tufted grass
Hordeum murinum subsp. *leporinum* (Link) Arcang.	Prodanović, 2007	wall barley
Koeleria macrantha (Ledeb.) Schult.	Prodanović, (unpublished, field observation)	prairie junegrass
Koeleria pyramidata (Lam.) P. Beauv.	Prodanović, 2007	
Lolium perenne L.	Prodanović, 2007	perennial ryegrass, english ryegrass
Melica ciliata L.	Pavlović, 1967; Prodanović et al., 2004; Prodanović, 2007	hairy melic, silky spike melic
Melica nutans L.	Prodanović, 2007	mountain melick
Melica uniflora Retz.	Prodanović, 2007	wood melick
Milium effusum L.	Prodanović, 2007	american milletgrass, wood millet
Phalaris arundinacea L.	Prodanović, 2007	reed canary grass
Phleum montanum K.Koch	Prodanović, 2007	Timothy grass
Phleum phleoides (L.) H. Karst.	Prodanović, 2007	boehmer's cat's-tail, purple-stem cat's-tail
Phleum pratense L.	Prodanović, 2007	Timothy grass, meadow cat's-tail
Phragmites australis (Cav.) Trin. ex Steud.	Prodanović, 2007	common reed
Poa annua L.	Prodanović, 2007	annual meadow grass
Poa bulbosa L.	Prodanović, 2007	bulbous bluegrass
Poa compressa L.	Prodanović, 2007	canada bluegrass
Poa nemoralis L.	Prodanović, 2007	wood bluegrass
Poa pratensis L.	Prodanović, 2007; Krivošej et al., 2013	kentucky bluegrass
Poa perconcinna J. R. Edm.	Prodanović, 2007	
Poa trivialis L.	Prodanović, 2007	rough bluegrass
Sclerochloa dura (L.) P. Beauv.	Prodanović, 2007	common hardgrass
Sesleria rigida Heuff. ex Rchb.	Prodanović, 2007	
Stipa capillata L.	Prodanović, 2007	needle grass
Stipa joannis Celak	Prodanović, 2007	feather grass
Taeniatherum caput-medusae (L.) Nevski	Prodanović, 2007	medusahead wildrye
Tragus racemosus (L.) All.	Krivošej et al. 1995-1998; Prodanović, 2007	stalked bur grass
Vulpia myuros (L.) C.C.Gmel.	Prodanović, 2007	annual fescue
POLYGALACEAE		
Polygala major Jacq.	Prodanović, 2007	milkworts, snakeroots
Polygala supina Schreb.	Pavlović, 1967; Prodanović, 2007	milkwort
POLYGONACEAE		

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Fallopia convolvulus (L.) Á. Löve	Prodanović, 2007	black-bindweed, wild buckwheat
Fallopia dumetorum (L.) Holub	Prodanović, 2007	european climbing buckwheat
Persicaria lapathifolia (L.) Delarbre	Prodanović, 2007	pale persicaria
Polygonum aviculare L.	Prodanović, 2007	common knotgrass
Rumex acetosa L.	Prodanović, 2007; Krivošej et al., 2013	sorrel
Rumex acetosella L.	Prodanović, 2007	red sorrel, sheep's sorrel
Rumex conglomeratus Murray	Prodanović, 2007	clustered dock, sharp dock
Rumex crispus L.	Prodanović, 2007	curled dock, yellow dock
Rumex obtusifolius L.	Prodanović, 2007	bitter dock
Rumex pulcher L.	Prodanović, 2007	fiddle dock
PRIMULACEAE		
Anagallis arvensis L.	Prodanović, 2007	scarlet pimpernel
Anagallis foemina Mill.	Prodanović, 2007	blue pimpernel
Androsace elongata L.	Prodanović et al.,2012	rock jasmine, elongate ilisha
Lysimachia nummularia L.	Prodanović, 2007; Krivošej et al., 2013	moneywort, creeping jenny
Primula veris Huds.	Prodanović, 2007	ommon cowslip
Primula acaulis (L.) L.	Krivošej et al., 2013	common primrose
RANUNCULACEAE		
Anemone apennina L.	Prodanović, 2007	blue anemone
Anemone nemorosa L.	Prodanović, 2007	wood anemone
Clematis recta L.	Prodanović, 2007	erect clematis, ground virginsbower
Consolida regalis Gray	Prodanović, 2007	forking larkspur, rocket-larkspur
Ficaria verna Huds.	Prodanović (unpublished, field observation)	fig buttercup
Helleborus odorus Waldst. & Kit. ex Willd.	Prodanović et al., 2004; Prodanović, 2007	fragrant hellebore
Helleborus multifidus subsp. *serbicus* (Adamovic) Merxm.& Podlech	Prodanović, 2007	hellebore
Hepatica nobilis Mill.	Prodanović, 2007	common hepatica, liverwort
Nigella arvensis L.	Prodanović, 2007	love-in-a-mist, black bread-weed
Pulsatilla vulgaris subsp. *grandis* (Wender) Zämelis	Prodanović, 2007	pasqueflower
Ranunculus arvensis L.	Prodanović, 2007	corn buttercup
Ranunculus bulbosus L.	Prodanović, 2007	bulbous buttercup
Ranunculus millefoliatus Vahl	Prodanović, 2007; Krivošej et al., 2013	erusalem butercup
Ranunculus polyanthemos L.	Prodanović, 2007	multiflowered buttercup
Ranunculus psilostachys Griseb.	Prodanović, 2007	buttercup
Ranunculus repens L.	Prodanović, 2007	the creeping buttercup
Ranunculus sceleratus L.	Prodanović, 2007	celery-leaved buttercup
Ranunculus serbicus Vis.	Prodanović, 2007	
Thalictrum flavum L.	Prodanović, 2007	common meadow-rue
Thalictrum lucidum L.	Prodanović, 2007	shaning meadow rue
Thalictrum minus L.	Prodanović, 2007	lesser meadow-rue
RESEDACEAE		
Reseda lutea L.	Prodanović, 2007	wild mignonette
Reseda luteola L.	Prodanović, 2007	dyer's weed, weld, woold, yellow weed
Reseda phyteuma L.	Prodanović, 2007	rampion mignonette
RHAMNACEAE		
Frangula alnus Mill.	Prodanović, 2007	alder buckthorn
Paliurus spina-christi Mill.	Prodanović, 2007	Jerusalem thorn
Rhamnus saxatilis subsp. *tinctoria* Nyman	Prodanović, 2007	rock buckthorn
ROSACEAE		
Agrimonia eupatoria L.	Prodanović, 2007	agrimony, cocklebur

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Amelanchier ovalis Medik.	Prodanović, 2007	snowy mespilus
Aremonia agrimonioides (L.) DC.	Krivošej et al., 2013	bastard-agrimony
Cotoneaster integerrimus Medik.	Prodanović, 2007	cotoneaster
Crataegus monogyna Jacq.	Prodanović et al., 2004; Prodanović, 2007	ommon hawthorn
Filipendula vulgaris Moench.	Prodanović, 2007; Krivošej et al., 2013	dropwort,fern-leaf dropwort
Fragaria vesca L.	Prodanović et al., 2004; Prodanović, 2007; Krivošej et al., 2013	wild strawberry
Geum urbanum L.	Prodanović, 2007; Krivošej et al., 2013	wood avens
Malus florentina (Zuccagni) C.K. Schneid.	Prodanović et al., 2013	florentine crabapple
Malus sylvestris (L.) Mill.	Prodanović, 2007	european crab apple
Potentilla argentea L.	Prodanović et al., 2004; Prodanović, 2007	hoary cinquefoil, silver cinquefoil
Potentilla heptaphylla subsp. *australis* (Nyman) Gams	Prodanović et al., 2004; Prodanović, 2007	
Potentilla hirta L. var. *zlatiborensis* Novak	Rexhepi, 1979; Prodanović, 2007	hairy cinquefoil
Potentilla micrantha Ramond ex DC	Prodanović, 2007	pink barren strawberry
Potentilla neglecta Baumg.	Prodanović, 2007	
Potentilla recta L.	Prodanović, 2007	sulfur cinquefoil
Potentilla tommassiniana F. W. Schultz.	Pavlović, 1967	
Potentilla visianii Pančić	Rexhepi, 1979; Prodanović, 2007; Prodanović et al., 2008	
Prunus avium (L.) L.	Prodanović et al., 2004; Prodanović, 2007	wild cherry
Prunus mahaleb L.	Prodanović, 2007	mahaleb cherry
Prunus spinosa subsp. *dasyphylla* (Schur) Domin	Prodanović, 2007	blackthorn, sloe
Prunus spinosa L. subsp. *spinos*a	Prodanović et al., 2004; Prodanović, 2007	blackthorn, sloe
Pyrus spinosa Forssk.	Prodanović, 2007	almond-leaved pear
Rosa arvensis Huds.	Prodanović, 2007	field rose
Rosa canina L.	Prodanović, 2007	dog rose
Rosa corymbifera Borkh.	Prodanović, 2007	rose
Rosa x dumetorum Thuill	Prodanović, 2007	corymb rose
Rosa gallica L.	Prodanović, 2007; Krivošej et al., 2013	french rose
Rosa micrantha Borrer ex Sm.	Prodanović, 2007	rugosa rose
Rosa rubiginosa L.	Prodanović, 2007	sweetbriar rose
Rosa spinosissima L.	Prodanović, 2007	scotch rose
Rubus caesius L.	Prodanović, 2007	european dewberry
Rubus ulmifolius Schott	Prodanović, 2007	elm-leaf blackberry
Sanguisorba minor Scop	Prodanović, 2007; Krivošej et al., 2013	salad burnet
Sanguisorba officinalis L.	Prodanović, 2007	great burnet
Spiraea media Schmidt	Prodanović, 2007	spirea
Sorbus aucuparia L.	Prodanović, 2007	mountain ash
Sorbus torminalis (L.) Crantz	Prodanović, 2004; Prodanović, 2007	wild service tree, chequers
Waldsteinia geoides Willd.	Prodanović et al.,2010	barren strawberries
RUBIACEAE		
Asperula cynanchica L.	Rexhepi, 1979; Prodanović, 2007	squinancywort, squincywort
Asperula purpurea (L.) Ehrend.	Prodanović, 2007	purple squinancywort
Cruciata glabra (L.) Opiz.	Prodanović, 2007	
Cruciata laevipes Opiz.	Prodanović, 2007; Krivošej et al., 2013	crosswort smooth bedstraw
Cruciata pedemontana (Bellardi) Ehrend., All.	Prodanović, 2007	Piedmont bedstraw
Galium aparine L.	Prodanović, 2007	catchweed bedstraw
Galium mollugo L.	Prodanović, 2007	hedge bedstraw

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Galium pseudoaristatum Schur	Prodanović, 2007	bedstraw
Galium verum L.	Prodanović, 2007	yellow bedstraw
Sherardia arvensis L.	Prodanović, 2007	blue fieldmadder
RUTACEAE		
Dictamnus albus L.	Prodanović, 2007	burning bush, dittany
Haplophyllum boissierianum Vis. et Pančić	Pavlović, 1967; Ranđelović et al., 1982; Prodanović, 2007; Prodanović et al., 2008	
SALICACEAE		
Populus alba L.	Prodanović, 2007	silver poplar, silverleaf poplar
Populus nigra L.	Prodanović, 2007	black poplar
Populus tremula L.	Prodanović, 2007; Krivošej et al., 2013	aspen, common aspen
Salix alba L.	Prodanović, 2007	golden willow, white willow
Salix caprea L.	Prodanović, 2007	goat willow, pussy willow
Salix eleagnos Scop.	Prodanović, 2007	bitter willow, olive willow
Salix x fragilis L.	Prodanović, 2007	crack willow
Salix purpurea L.	Prodanović, 2007	purple willow
SANTALACEAE		
Arceuthobium oxycedri (DC.) M. Bieb.	Ranđelović et al., 1982; Prodanović 2007	juniper dwarf mistletoe
Comandra umbellata subsp. *elegans* (Rochel ex Rchb.) Piehl	Prodanović, 2007	bastard toadflax
Thesium arvense Horv.	Rexhepi, 1979; Prodanović, 2007	
SAPINDACEA		
Acer campestre L.	Prodanović et al., 2004; Prodanović, 2007	field maple
Acer campestre subsp. *marsicum* (Guss.) Hayek	Prodanović, 2007	field maple
Acer platanoides L.	Prodanović, 2007	emerald queen maple, norway maple
Acer pseudoplatanus L.	Prodanović, 2007	sycamore
Acer tataricum L.	Prodanović, 2007	tatarian maple
SAXIFRAGACEAE		
Saxifraga paniculata Mill.	Rexhepi, 1979; Prodanović, 2007	alpine saxifrage
Saxifraga bulbifera L.	Prodanović, 2007	bulbous saxifrage
Saxifraga rotundifolia L.	Prodanović, 2007	round-leaved saxifrage
Saxifraga tridactylites L.	Prodanović, 2007	rue-leaved saxifrage
SCROPHULARIACEAE		
Scrophularia canina subsp. *bicolor* (Sm.) Greuter	Prodanović, 2007	dog figwort
Scrophularia canina L.	Ranđelović et al., 1982; Prodanović, 2007	dog figwort
Scrophularia canina L. subsp. *tristis* (K. Malý) V. Nikolic	Pavlović, 1967; Prodanović, 2007	dog figwort
Scrophularia nodosa L.	Prodanović, 2007	figwort, woodland figwort
Scrophularia umbrosa Dumort.	Prodanović, 2007	green figwort
Verbascum banaticum Schrad.	Prodanović, 2007	mullein
Verbascum chaixii Vill.	Prodanović, 2007	narrow-leaved mullein
Verbascum nigrum L.	Prodanović, 2007	black mullein, dark mullein
Verbascum phlomoides L.	Prodanović, 2007	orange mullein
Verbascum phoeniceum L.	Prodanović, 2007	purple mullein
SOLANACEAE		
Datura stramonium L.	Prodanović, 2007	thorn apple, jimsonweed
Hyoscyamus niger L.	Prodanović, 2007	henbane, black henbane
Lycium barbarum L.	Prodanović, 2007	chinese boxthorn, Himalayan goji
Solanum dulcamara L.	Prodanović, 2007	bittersweet, bittersweet nightshade

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THYMELAEACEAE		
Thymelaea passerina (L.) Coss. & Germ.	Prodanović et al.,2010	sparrow-wort
ULMACEAE		
Ulmus carpinifolia Gled.	Prodanović, 2007	elm
Ulmus laevis Pall.	Prodanović, 2007	european white elm, fluttering elm
Ulmus minor Mill.	Prodanović et al., 2004; Prodanović, 2007	elm
Ulmus minor Mill var. *tortuosa* (Host) Hayek	Prodanović, 2007	elm
URTICACEAE		
Parietaria officinalis L.	Prodanović, 2007	eastern pellitory-of-the-wall
Urtica dioica L.	Prodanović, 2007	common nettle, stinging nettle
VERBENACEAE		
Verbena officinalis L.	Prodanović, 2007	common vervain
VIBURNACEAE		
Sambucus ebulus L.	Prodanović, 2007	danewort, dane weed
Sambucus nigra L.	Prodanović, 2007	elderberry, black elder
VIOLACEAE		
Viola arvensis Murray	Ranđelović et al., 1982; Prodanović, 2007	field pansy
Viola hirta L.	Prodanović, 2007	sweet violet, english violet
Viola kitaibeliana Schult.	Prodanović, 2007	Kitaibel's violet
Viola reichenbachiana Boreau	Prodanović, 2007	early dog-violet, pale wood violet

A vast majority of genera belong to the familiy *Compositae* (51), followed by *Poaceae* (38), *Apiaceae* (26), *Brassicaceae* (24), *Leguminosae* (24), *Lamiaceae* (22). Among the genera the most species rich are *Trifolium* (17) followed by *Carex* (16), *Euphorbia* (14), *Veronica* (14), *Vicia* (13), *Lathyrus* (12), *Hieracium* (11), *Centaurea* (8), *Medicago* (8), *Rosa* (7) etc. The dominance of the genera *Trifolium, Vicia* and *Lathyrus* (*Leguminosae* family) is most likely conditioned by the higher presence of areas under xerothermic meadows and pastures as well as thermophilic rocks in the study area. Genus *Carex* is on second position. This is explained by the fact that species within this genus require moist habitats, which were abundant in the investigated terrains, on the banks of river Ibar, as well as streams flowing into the river. Genera *Trifolium* and *Carex* are also dominant in serpentine complexes in Eastern Rhodopes (Bulgaria) (Pavlova et al., 2004).

31 taxa in total have been recorded in this area for the first time and they present a novelty for the flora of Kosovo and Metohija and entire Serbia. The records for most of these were first published in Prodanović et al. (2004, 2008, 2010, 2012, 2013, 2018).

Life-form spectrum and phytogeographical analysis

The analysis of life forms indicates domination of hemicryptophytes (48.29%) (*Fig. 2*). The significant representation of therophytes (21.99%), is typical for the serpentine floras, where the plants are adapted to ensure reproduction within a short period of time under stress conditions (Brooks, 1987). Other herbal life forms are present in smaller numbers, geophytes (10.54%) phanerophytes (8.27%), chamaephytes (6.91%), scandentophytes (1.81%) etc. Such biological spectrum is characteristic for the Balkan Peninsula, as well as for the territory of Serbia (Diklić, 1984).

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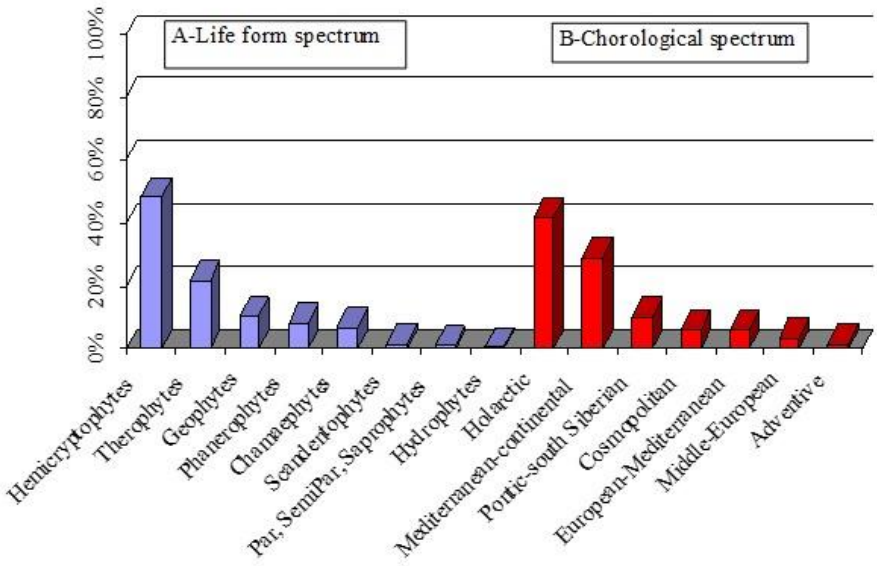

Figure 2. *Life form spectrum (A) and chorological spectrum (B), by basic area types, of the serpentinite flora in the middle course of the Ibar river valley*

The chorological floristic analysis indicates that this is an area with interim holarctic-Mediterranean character (*Fig. 2*). Holarctic areal type with 369 taxa (41.83%) is the most presented on the terrain, followed by Mediterranean – continental areal type (29.18%) and pontic – south Siberian areal type (10.31%), cosmopolitan areal type (6.45%) as well as central European –Mediterranean (6.23%). The least reported species in the serpentine terrains of the Ibar river valley are Middle European dispersion areal type and Adventive areal type. The presence of plants belonging to Adventive areal type indicates anthropogenic influence, which is present in settlements around river banks, as well as around main roads and local rural roads. Xeromorphic species are predominant due to conditions for their growth on serpentine soil.

Endemism and internationally significant vascular plants

Serpentine habitats are the most important endemic regions in the world and also called "geological islands" (Kurt et al., 2013). Despite the shallow active layer of serpentine soil, biodiversity is high with a great number of interesting local and regional endemics (Shuka and Hallaçi, 2010). It is estimated that the total number of endemic plants in the Balkans is bigger than 2200 taxa. 300-350 (15-16%) taxa of this number have been identified on the serpentine background; 123 of them are obligate serpentine endemics (Stevanović et al., 2003).

Due to diverse physical and geographical characteristics, the Republic of Serbia has a considerable number of endemic plant taxa. Centres of endemism are located in the southern and eastern regions of Serbia and on the territory of Kosovo and Metohija (Gavrilović et al., 2017). The serpentine mountains of Central Serbia along the river Ibar represent a relatively large core of ophiolithic flora in the Central Balkans. It is the centre of distribution of old trans-regional endemics such as: *Halacsya sendtneri, Haplophyllum boisserianum, Potentilla visianii* and *Eryngium serbicum*. Local endemics include *Tulipa serbica* (Stevanović et al., 2003).

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Out of the total taxon number in the investigated area, 73 (8.27%) belong to group of endemic, sub-endemic, relict and endomorelic taxa. There are 40 of endemic and sub-endemic species (*Eryngium serbicum, Helleborus multifidus* subsp. *serbicus, Tulipa serbica, Odontarrhena bertolonii* subsp. *scutarina (syn. Alyssum janchenii), Alyssum markgrafii, Galatella albanica, Dianthus pinifolius, Euphorbia glabriflora, Fumana bonapartei, Halacsya sendtneri, Haplophyllum boissierianum, Hieracium bauhini* subsp. *pseudosparsum, Hypericum rumeliacum, Lamium bifidum* subsp. *balcanicum, Potentilla visianii, Ranunculus psilostachys, Scabiosa fumaroides, Scrophularia canina* subsp. *tristis, Sedum album* (syn. *S. serpentini), Stachys scardica, Linum flavum, Trifolium trichopterum, Rorippa lippizensis, Salvia amplexicaulis, Digitalis laevigata, Melampyrum heracleoticum, Eryngium palmatum, Achillea coarctata, Alyssum montanum* subsp. *serbicum, Campanula lingulata, Cytisus austriacus* subsp. *heuffelii, Galium pseudoaristatum, Iris reichenbachii, Lamium garganicum* L. subsp. *garganicum, Lamium garganicum* L. subsp. *glabratum, Lathyrus hallersteinii, Petrorhagia illyrica* subsp. *haynaldiana, Poa perconcinna, Ranunculus serbicus, Rhinanthus rumelicus, Trifolium pignantii*).

Based on the distributional range of obligate serpentine endemics, Stevanović et al. (2003) presented the following general classification: (1) trans-Balkan or trans-regional Balkan endemics (taxa distributed in the greater part of serpentine areas in the Balkans); (2) regional endemics (taxa restricted to a single floristic subregion or province); and (3) local or steno-endemics (taxa distributed in a single floristic district or narrow geographical area such as a single mountain or island).

On the investigated terrains in the middle course of the Ibar river valley, 11 taxa from obligate serpentine endemics are in the trans-regional endemic (TRE) category : *Alyssum markgrafii*, *Sedum album* (syn. *S. serpentini*), *Potentilla visianii, Potentilla heptaphylla subsp. australis, Euphorbia glabriflora, Haplophyllum boissierianum, Fumana bonapartei, Eryngium serbicum, Halacsya sendtneri, Stachys recta* subsp. *baldacii* and *Scrophularia canina* subsp. *tristis.*

4 taxa are obligate serpentine endemics from the regional endemics (RE) category: *Helleborus multifidus* subsp. *serbicus, Alyssum montanum* subsp. *serbicum, Galatella albanica* and *Tulipa serbica* (stenoendemic according to Millaku et al., 2018). It was right here, on the Ibar Valley serpentines, Beli Laz locality (locus classicus), that this endemic *Tulip* was identified (Tatić and Krivošej, 1997). Unfortunately, in the last three years, due to the regional dumping site development, the primary habitat has been devastated, without any possibility to be protected.

Out of the total taxon number, 31 taxa belong to a group of internationally significant vascular plants (IUCN, Stojanović et al., 2015). Out of that, 14 taxa have been protected by CITES Convention. These are the followings: *Anacamptis pyramidalis, Cephalanthera rubra, Epipactis helleborine, Epipactis microphylla, Galanthus nivalis, Limodorum abortivum, Neottia nidus-avis, Orchis mascula, Orchis morio*, *Orchis papilionaceae, Orchis purpurea, Orchis tridentata, Ophrys apifera* and *Platanthera bifolia.*

Comparative floristic analysis

In order to gain a better insight into the flora diversity in these habitats, as well as the level of floristic closeness, a comparative analysis of the flora from Studena mountain, near the city of Kraljevo, has been done by Tatić (1969) in his multi-year research as well as with flora of Goleš mountain, researched by Krasniqi et al. (2019).

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The Ibar serpentine massive with the valley in the lower course of the Ibar river traverses though central Serbia and extend west from the mountain Studena (Lukić et al., 2015) where it covers the largest space on Studena mountain; the river itself flows 17.1 km along the mountain. In his studies Tatić reported 390 taxa, the largest number of which belonged to *Compositae, Poaceae, Leguminosae, Caryophyllaceae, Rosaceae* and *Lamiaceae* families, which is similar to the spectrum of the most numerous families of flora in the Kosovo part of the Ibar serpentine range. The number of two mutual taxa in two compared areas is 196, so the index of similarity by Sørensen is 30.66%, which is unexpectedly low since Studena mountain represents the continuation of the Ibar serpentine massive that starts in the middle course of the river in Kosovo.

An assumption of floristic dissimilarities in the Kosovo part of the Ibar valley and Studena mountain, has been based on the differences between the altitudes of these two areas (the Ibar gorge 500-900 ma, Studena mountain above 1000 ma, with the highest peak of Kavgalija 1356). Furthermore, the composition of the geological background has probably influenced floral development. The geological background of Studena mountain exclusively consists of serpentinite; in the Kosovo part of the Ibar valley apart from serpentinite, there are also peridotite and unmodified ultramaphyte rocks. While Tatić emphasized phytocenologic research studies on Studena mountain, our attention was turned to purely floristic research projects in the Kosovo's part of the Ibar valley (which may be corroborated by a total number of 882 identified taxa), taking also into account the size of the investigated area. Since the serpentine is considered as a vulnerable habitat, it is important to emphasize that no Studena mountain species recorded by Tatić has been classified as extinct in Serbian flora. Our assumption is that the described species can be found there nowadays, but we also recommend future field research to prove this assumption.

Mountain Goleš, in the central part of Kosovo and Metohija, represents the beginning of the serpentine-peridotite massif, which covers the left bank of the Sitnica river, goes towards the Čičavica Mountain, enters the valley of the upper stream of the Ibar river in Ibarski Kolašin, and continues along the middle Ibar river course. Krasniqi et al. (2019) recorded 295 taxa on this mountain (with a surface area of 22.2 km^2) over a three-year study (2015-2019). Considering the size of the mountain range covered by the conducted survey, a larger number of taxa would be expected. However, such floristic "poverty" confirmed the fact that serpentinite habitats are floristically poorer than habitats with other types of geological substrates. The number of two mutual taxa in the two compared areas is 218, so the index of similarity by Sørensen is 37.04%. It should be noted that this value is not large, although the number of common species is as high as 2/3 of all taxa found on Goleš Mountain. The reason could certainly be a disproportion to the number of identified species and the size of territories explored.

Families with the highest number of taxa on Goleš mountain were *Compositae* (34), *Leguminosae* (25), *Rosaceae* (25), *Poaceae* (18), and *Caryophyllaceae* (18). In taxonomic spectrum in the Ibar river valley, *Compositae* (105) and *Leguminosae* (86) are also dominant, followed with *Poaceae* (66), *Lamiaceae* (51), *Brassicaceae* (50), *Caryophyllaceae* (49). *Rosaceae* family in taxonomic spectrum of most dominant plant families in the Ibar river valley occupies eight position. Significant involment of the *Lamiaceae* and *Caryophyllaceae* families indicate the expression of stronger Mediterranean floristic influences, as well as a greater presence of bare rocky habitats.

Krasniqi et al. (2019) on Goleš Mountain state that out of the total number of identified taxa, 8 of them are endemic, of which 5 endemic species occur on the Ibar

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valley serpentinite (*Galatella albanica, Halacsya sendtneri, Linum flavum, Potentilla visianii* and *Haplophyllum boissierianum*). Various floristic elements meet and overlap in mountain ranges in the central part of Kosovo, dominated by European floral element, followed by Euro-Asiatic and Sub-Mediterranean, Balcan, Mediterranean and Pontic elements similar to the interim holarctic- Mediterranean character of flora in the Ibar river valley.

Due to the background characteristics, the serpentine areas of the Ibar valley and their flora have not been exposed to negative anthropogenic influences so far. Yet, in the previous 3-4 years, the negative human influences have been detected in the areas along the highway. We consider that establishing a better connection between scientific and professional public and the people who are in charge of planning and infrastructural construction along the Ibar valley could contribute to the preservation of the habitats of some significant and endangered species, which unfortunately was not the case with locus classicus of the *Tulipa serbica* species.

Conclusion

The presence of 882 taxa grouped into 83 families and 386 genera has been identified in the course of the research of serpentinite flora performed in the middle stream Ibar river valley, for the period of 16 years. Taxonomic spectrum of families is dominated by *Compositae* (105), followed by *Leguminosae* (86), *Poaceae* (66), *Lamiaceae* (51), *Brassicaceae* (50), *Caryophyllaceae* (49), *Apiaceae* (43) etc. The greatest number of genera belong to families *Compositae* (51), followed by *Poaceae* (38), *Apiaceae* (26), *Brassicaceae* (24), *Leguminosae* (24), *Lamiaceae* (22). Among the genera the most species rich are *Trifolium* (17) followed by *Carex* (16), *Euphorbia* (14), *Veronica* (14), *Vicia* (13), *Lathyrus* (12), *Hieracium* (11), *Centaurea* (8), *Medicago* (8), *Rosa* (7) etc. An analysis of life forms showed that the investigated area has hemicryptophyte characters, with significant participation of therophytes (21.99%). The chorological spectrum is dominated by Holarctic species (41.83%). The presence of 73 taxa from a group of endemic, sub-endemic, relict and endomorelic taxa was determined. Out of the total taxon number, 31 taxa belong to a group of internationally significant vascular plants. Out of that, 14 taxa have been protected by CITES Convention. A comparative analysis of the serpentinite flora in the Ibar river valley and the Studena mountain as well as in the Goleš mountain showed a relatively small floristic similarity, which was unexpected, since these two mountains represent the beginning and continuation of the Ibar serpentine massive that starts in the middle course of the river in Kosovo. Only a good knowledge of floristic diversity may initiate the procedures that may lead to preserving and protecting rare and endangered and internationally important species in these areas. Since serpentinite habitat is considered as vulnerable we are proposing continual habitat monitoring as well as monitoring of all endangered and rare species in the Ibar river valley in future research projects. For some species seed collecting for a seed bank could be one of the conservation measures.

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EFFECTS OF NITROGEN FERTILIZATION ON CORIANDER (*CORIANDRUM SATIVUM* L.): YIELD AND QUALITY CHARACTERISTICS

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Abstract. Coriander (*Coriandrum sativum* L.) is a spice plant belonging to the Apiaceae family. In this study, the aim was to specify the effects of different nitrogen doses on the yield and quality properties of coriander. In this research; plant height, number of branches, number of umbels, thousand seed weight, seed yield, oil content, fatty acid composition, essential oil rate and essential oil composition were examined. The essential oil contents in coriander fruits was determined by Clevenger apparatus. Fatty oil was isolated by cold press. Essential oil and fatty oil compositions were determined by Gas chromatography–mass spectrometry (GC/MS-QP2020) device. While nitrogen doses did not positively affect fruit yield, they had a significantly positive effect on the herbal characteristics of coriander (plant height, number of umbels and thousand fruit weight). It was revealed that nitrogen doses had positive significant effect essential oil rate. Increasing nitrogen doses affected rate of linalool. Nitrogen doses had a significant effect only on butyric acid, and for fatty oil rate and other major fatty acids their effect was not significant. It has been determined that high fruit yields are obtained at 60 and 80 kg ha^{-1} nitrogen applications and that the rate of essential oil and linalool is the highest at 100 kg ha^{-1} nitrogen doses especially in the second year.
Keywords: *Coriandrum sativum, essential oil, fatty oil, linalool, nitrogen doses*

Introduction

Coriandrum sativum L. is a medicinal and spice plant belonging to the Apiaceae family. It is named as “kişniş” in Turkish and “coriander" in English and is one of the oldest spices in the world. Its matured fruits and fresh leaves are used as spices and traditional medicine. Matured fruits contain between 0.03-2.7% of essential oil. The major component of the oil is linalool, accounts for about two-thirds of the oil (Shahwar et al., 2012). Essential oil of coriander is used in cosmetic industry because of the valuable components such as linalool.

The variation of essential oil content and composition in essential oil plant depends on their genetic structure, climatic conditions and agronomic applications (Telci et al., 2006a,b, 2010). The commercial value of coriander fruit is determined by its physical properties, chemical composition and bioactive effects (Furan and Geboloğlu, 2017). The essential oil and main component linalool are important raw materials in the perfume, cosmetic and pharmaceutical industries. It is also used in food and preventive medicine due to its bactericidal and fungicidal effects.

As in other medicinal and spice plants, in coriander, quality is as important as yield. The effects of cultural practices and environmental factors on these plants are more common than all other cultivated plants. Nitrogen is a plant nutrient that is effective not only in terms of growth and yield, but also in terms of seed quality, it plays an important role in the synthesis of plant components with the effect of different enzymes (Jr. Jones et al., 1991; Marschner, 2011). Nitrogen fertilization increases plant growth, essential oil, fixed oil and total carbohydrate and soluble sugar. Nitrogen is involved in photosynthesis, respiration and protein synthesis. It gives the leaves their dark green color, promotes hard vegetative

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growth and leads to more efficient use of existing inputs and ultimately to higher productivity (Pawar et al., 2007). Khalid (2013) stated that in the nitrogen dose applications, the values obtained were significantly higher than those of the control group and that they found significant results in plant growth characteristics.

Coriander production has been increasing day by day in Mardin province, as in different regions of Turkey. However, in the region where the experiment is carried out, there are not enough studies related to coriander cultivation, especially the need for nitrogen and the relationship between nitrogen and yield and quality. The yield of coriander is low in Mardin and its vicinity compared to the actual yield potential due to unsuitable fertilization and agricultural practices. For this reason, the research was carried out in order to evaluate the nitrogen demand of the registered Gamze cultivar in Mardin region of Turkey under the plain conditions of Mardin province and to find the effect of different nitrogen levels on yield and quality.

Material and Methods

Coriander (*Coriandrum sativum* L.) is a spice plant belonging to the Apiaceae family, which is used. As plant material; Gamze variety, which adapts to the plain conditions of Mardin province and is preferred by farmers, was used. This study was carried out for two years under plain conditions of Mardin Province in Turkey during the 2014-2015 and 2015-2016 vegetation periods.

Experiment area has a hot and dry weather in summers and rainy and warm in winters. *Table 1* shows the climatic data for the application area. While the total rainfall data of January in the first year that the study was conducted and of April in the second year, were low compared to the average of long years, the total amount of precipitation in the other months was parallel to the average of long years. In the first and second years, monthly average temperature and humidity values were similar to the temperature average values of long years.

Table 1. *Some meteorological data for long years (2004-2016) * and 2014-2015-2016 growing periods in Mardin province* **

	Rainfall (mm)			Temperature (℃)			Relative humidity (%)		
Months	**Long Years**	**2014-2015**	**2015-2016**	**Long Years**	**2014-2015**	**2015-2016**	**Long Years**	**2014-2015**	**2015-2016**
November	33.3	76.8	46.0	14	12.7	12.4	51.6	53.1	50.3
December	54.8	100.4	34.8	9.1	5.8	7.3	54.3	72.2	51.7
January	42.8	8.3	73.2	7.1	6.8	5.2	60.3	66.6	75.2
February	47.6	76.0	35.8	8.8	8.2	11.0	60.0	68.7	65.8
March	34.2	89.9	59.9	13.1	10.8	12.0	52.0	60.3	59.0
April	37.7	25.4	9.3	17.5	14.0	17.4	49.3	53.0	41.3
May	17.3	11.1	12.3	23.7	21.2	21.0	37.0	37.3	42.0
June	2.4	0.2	0.5	30.5	26.9	29.1	22.8	29.0	28.2
July	0.4	0.0	0.0	34.1	33.1	32.5	22.0	19.6	22.4

*Long Years Means: Long years average: It is the average of the data of at least 10 years.
**Sources: Turkish State Meteorological Service

The soil has a clay-loam structure and is poor in organic matter (1.18%). It is slightly alkaline (pH = 8.05), lime content is very high (36.65%), and no salinity problems (0.010%). Phosphorus (31.50 kg), potassium (104.16 mg kg^{-1}) is sufficient for soil uptake.

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Magnesium (292.6 mg kg^{-1}), copper (19.134 mg ha^{-1}), zinc (0.8112 mg kg^{-1}) and iron (7.9050 mg kg^{-1}) are sufficient in the soil structure, but manganese (3.390 mg kg^{-1}) is very low (*Table 2*).

Table 2. *Soil properties of the experimental area**

Analyzes (0-30 cm)	Limit Values	Analysis results	Analysis Method
Phosphor (P)	< 3 Trace	29.2 mg kg^{-1}	TS ISO 11263
Potassium (K)	>30 Sufficient	111.44 mg kg^{-1}	TS 8341
Lime (%)	>25 Excessive calcic	33.39%	TS EN ISO 10693
pH	7.5-8.5 Light Alkaline	8.08	TS ISO 10390
Organic substance (%)	1-2 Little	1.15%	TS8336
Salinity (%)	<2 Salt-free	0.010%	TS ISO 11265
Mangan (Mn)	4-14 Insufficient	5.150 mg kg^{-1}	TUZUNER 1990
Iron (Fe)	>4.5 Sufficient	11.121 mg kg^{-1}	TUZUNER 1990
Copper (Cu)	>0.2 Sufficient	33.000 mg kg^{-1}	TUZUNER 1990
Zinc (Zn)	> 8 Excessive	11.314 mg kg^{-1}	TUZUNER 1990
Calcium (Ca)	1150-3500 Sufficient	1216.6 mg kg^{-1}	TUZUNER 1990
Magnesium (Mg)	160-480 Sufficient	250.6 mg kg^{-1}	TUZUNER 1990
Sodium (Na)	--	64.68mg kg^{-1}	TUZUNER 1990
Organic Carbon	--	0.67%	TS8336
Carbon/Nitrogen (C/N)	--	0.55%	Calculation method
Structure	Sand 39 .2%- Silt 28.0% Clay %32.7	CL (Clayey loamy)	TUZUNER A.1990

Source: MARTEST analysis laboratories

The experiment was set up according to the Randomized Blocks Experiment Design with three replications. In the trial area, 5 different doses (0, 40, 60, 80 and 100 kg ha^{-1}) of nitrogen (Ammonium sulphate) were applied on the soil. The plot area has an area of totally 4.5 m^2 as 5 rows on each plot with 3 m length, 1.5 m width and 30 cm planting distance. In the plantation, 1 m distance between plots and 2 m between blocks were arranged. The number of plants in the study was arranged as 66000 plant ha^{-1}. Sowings were performed manually on 15 October 2014 in the first year and 16 October 2015 in the second year. After the sowing, weeding was performed three times and irrigation was made 5 times in the summer months. On June 22, the harvest performed manually; calculations were made on the current area by removing 25 cm as edge effect from the row tops and one row on the sides.

Essential oil and fatty oil analysis

Volumetric method was used to determine the extraction and quantity of essential oils for distillation in ripe fruits; Coriander fruits are ground and exposed to distillation with Clevenger apparatus for 2.5 hours (Clevenger, 1928). The essential oil samples obtained were kept in the refrigerator at 4 ℃ until analysis.

In the fatty oil extraction method, fatty acids were converted to methyl ester derivatives, 0.1 g oil was taken in a 15 ml plastic centrifuge with a lid and 10 ml n-hexane was added. It was strongly shaken after closing its lid and 2N KOH solution with methanol (0.5 ml) was appended. It was strongly shaken again after closing its lid and left

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in a dark environment for 2 hours until the supernate clarified. For analysis, the supernate was exposed to GC method and after that the sample was prepared for the analysis.

Fatty acids were dissolved in 40 mg of oil n-heptane for methylation prior to analysis. The tube was rinsed off with 2 M KOH (2 mL) and then for phase formation was waited. The supernatant containing the fatty acids was taken in vials and diluted in n-hexane. Fatty oil and essential oil component analysis performed with the GC/MS-QP2020. GC/MS conditions were given in *Table 3*.

Table 3. *GC/MS conditions*

System	GC/MS-QP2020
GC capillary column	For Essential Oil Analysis: Rtx-2330 RESTEK (60 m x 0.25 mm x 0.2 µm) For Fatty Oil Analysis: Rtx-2330 RESTEK (60 m x 0.25 mm x 0.2 µm)
Injection Mode	Split
Pressure	For Essential Oil Analysis: 80 kPa For Fatty Oil Analysis: 100 kPa
Split Rate	For Essential Oil Component Analysis: 25 For Fatty Oil Component Analysis: 100
GC oven initial temperature	For Essential Oil Component Analysis: Initial 40 ℃ 2-min. holding period 4 ℃ $min.^{-1}$ until 240 ℃ Final temperature 240 ℃. 3 min. holding period For Fatty Oil Component Analysis: Initial 140 ℃ 5-min. holding period 4 ℃ $min.^{-1}$ until 240 ℃ Final temperature 240 ℃. 15 min. holding period
Injection block temperature	For Essential Oil Component Analysis: 240 ℃ For Fatty Oil Component Analysis: 250 ℃
FID Temperature	250 ℃
Injection Volume:	1 µ l

Data analysis

Agronomic characteristics of the study were analyzed by using the JMP 5.0.1 statistical program (SAS Institute Inc., 2002), and the differences between means of nitrogen doses were compared using Student's t-test at the 0.05 probability level (Gosset, 1908).

For essential oil and fatty oil, the analysis was applied using the IBM SPSS Statistics for Windows (IBM Corp., 2017). The significance of year differences of essential oil and fatty acids between fruit samples was tested by analysis of variance (ANOVA) and represented by critical value from an F-test (F) and statistical significance (p). Essential oil means, fatty oil means, major components of essential oil and fatty acids with significant variation were compared by using Duncan Multiple Range Test (Duncan, 1955). Year difference significances were compared by T-test.

Results and Discussion

For coriander (*Coriandrum sativum* L.), data on the effect of different nitrogen doses on plant characteristics were given in *Table 4* and on quality characteristics were given in *Table 5, Table 6, Table 7* and *Table 8*.

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Agronomic characteristics and yield

Plant height (cm)

When the different nitrogen doses in coriander were examined in terms of plant height, the difference between years was insignificant and the difference between nitrogen doses was statistically significant ($p<0.05$) and interaction factor was insignificant. Regarding the average value of two years, the highest value was found to be 62.8 cm in 60 kg ha^{-1} nitrogen application group and the lowest value was found to be 39.2 cm in the control group. After the application of 60 kg of nitrogen per hectare, decreases were observed in plant height (61.6 and 57.0 cm) (*Table 4*). Nitrogen fertilization was found to have a negative effect on plant height when the optimum dose given to the plant was exceeded.

Table 4. *Some agronomical characteristics of different nitrogen dose applications in coriander (Coriandrum sativum L.)* *

Nitrogen Doses (kg ha^{-1})	Plant Height (cm)			Number of Branches (piece $plant^{-1}$)			Number of umbels (piece $plant^{-1}$)			1000 fruit weight (g)			Fruit yield (kg ha^{-1})		
	2015	**2016**	**Mean**	**2015**	**2016**	**Mean**	**2015**	**2016**	**Mean**	**2015**	**2016**	**Mean**	**2015**	**2016**	**Mean**
0	38.2	40.1	**39.2d**	4.8	7.7	**6.2**	10.2	13.4	**11.8bc**	12.3	11.7	**12.0b**	1686	2267	**1976**
40	53.6	56.9	**55.3c**	4.5	5.8	**5.2**	12.2	16.3	**14.3ab**	13.0	12.7	**12.9ab**	1883	2529	**2206**
60	62.0	63.6	**62.8a**	4.3	5.1	**4.7**	10.8	9.5	**10.1c**	12.7	12.0	**12.4b**	2136	2266	**2201**
80	61.1	62.2	**61.6ab**	4.9	5.4	**5.2**	12.8	13.5	**13.1ab**	12.8	12.7	**12.8ab**	2027	2721	**2374**
100	55.3	58.7	**57.0bc**	5.4	6.1	**5.8**	15.1	14.5	**14.8a**	13.5	13.3	**13.4a**	2017	2282	**2150**
Mean	**54.0**	**56.3**		**4.8**	**6.0**		**12.2**	**13.4**		**12.9**	**12.5**		**1950**	**2413**	

* Different letters after the same column data indicate significant difference at the 0.05 level or similar

In his study about effect of different fertilizers effect on coriander, Kan (2007) found effect of DAP (include 18% nitrogen) fertilizer on plant height as insignificant. Erdoğdu and Esendal (2018), in their study about cultivar and nitrogen doses, specified significant ($p<0.01$) positive relation between nitrogen doses and plant height. They determined that plant height varies between 90.20- 109.40 cm regarding to mean data of doses. According to Okut and Yıldırım (2005) effect of nitrogen doses on plant height is insignificant. However, Karadoğan et al. (1997) and Kırıcı et al. (1997) stated a positive significant relation between nitrogen doses and plant height.

Number of branches per plant

The difference between the number of branches by years, the difference between average of years in terms of fertilizer doses and interaction factors were found to be insignificant. The mean number of branches was found to be 4.8 in the first year and 6.0 in the second year. Regarding the average of two years, the highest number of branches was obtained in the control group (6.2 piece $plant^{-1}$), and the lowest number of branches (4.7 piece $plant^{-1}$) was obtained with 60 kg ha^{-1} nitrogen application. It was observed that increased nitrogen doses did not affect the number of branches in the plant (*Table 4*).

Kan (2007) found effect of DAP fertilization and year interaction on number of branches as significant ($p<0.05$) and according to mean data, he obtained that number of

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branches varies between 3.76- 5.09 with DAP fertilization and year interaction. Khalid (2013) obtained significant relation of number of branches and nitrogen doses and measured values between 2.8- 7.1 cm for coriander. In literature statistically significant increase was observed parallel to increasing nitrogen doses (Karadoğan et al., 1997; Kırıcı et al., 1997; Nayak et al., 2009).

Number of umbels per plant

The difference in the number of umbels by years was found to be insignificant and the difference between nitrogen dose applications was found to be significant ($p<0.05$). It was calculated that there were 12.2 umbels per plant in 2015 and 13.4 umbels in 2016. It was found that the highest number of umbels by the average of years is 100 kg ha^{-1} in nitrogen application and the lowest number of umbels is (10.1 plants) in 60 kg ha^{-1} nitrogen application (*Table 4*). Applications over 60 kg of nitrogen per hectare resulted in a decrease in the number of umbels in the plant. In different nitrogen dose applications (up to 60 kg ha^{-1}), the number of umbels decreased as the plant height increased. The values obtained regarding the number of umbels: Some researchers do not find any significant effect of nitrogen doses on number of umbels per plant (Karadoğan et al., 1997; Okut and Yıldırım, 2005; Erdoğdu and Esendal, 2018). However, Kırıcı et al. (1997) stated that nitrogen application has positive significant effect on number of umbels per plant and found values between 13.15-16.80 umbels per plant.

1000 fruit weight (g)

Regarding thousand fruit weight, difference by years was insignificant and the difference between nitrogen dose averages was significant ($p<0.05$). In the first year a thousand fruit weight was found to be 12.9 g and in the second year it was found to be 12.5 g. The highest average value (13.4 g) was obtained in 100 kg ha^{-1} nitrogen application, and the lowest value (12.0 g) was obtained in the control group (*Table 4*).

In previous studies conducted on 1000 seeds, Kan (2007) did not find any significant effect of nitrogen doses on thousand seed weight, Kandemir (2010); Karadoğan et al. (1997) and Okut and Yıldırım (2005) obtained significant positive effect of nitrogen doses on thousand seed weight and they found values between; 7.01g - 8.25g; 9.74 g - 11.06 g and 8.01 g - 8.77 g, respectively.

The highest weight of 1000 fruits at the highest nitrogen dose (100 kg ha^{-1}) indicates that nitrogen increases protein accumulation in fruits (Pawar et al., 2007). It can be said that the differences in thousand fruit weight in coriander stem from different agricultural practices as well as environmental differences as in other herbal characteristics.

Fruit yield (kg ha^{-1})

The effect of different nitrogen dose applications on seed yield and the difference between the year averages were found to be insignificant. Yield per hectare in the first year was determined as 1950 kg in the first year and as 2413 kg in the second year. Regarding the different nitrogen doses average, the highest value (2374 kg ha^{-1}) was obtained in the 80 kg ha^{-1} nitrogen application and the lowest value (1976 kg ha^{-1}) was obtained from the control group (*Table 4*).

Kan (2007) and Reddy and Rolston (1997) did not found any significant effect for nitrogen application on fruit yield, Patel et al. (2013) found significant relation and obtained 1203 kg from the application of 80 kg of nitrogen per hectare. Karadoğan et al.

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(1997) and Okut and Yıldırım (2005) stated that increasing nitrogen doses cause an increase in seed yield. Kırıcı et al. (1997) found significant effect and measured highest value as 1780 kg ha^{-1} at 60 kg ha^{-1} nitrogen application. Erdoğdu and Esendal (2018) found significant positive effect and obtained values between 982.0 - 1467.0 kg ha^{-1} in coriander. Akbarinia et al. (2007), in their nitrogen amount experiment, found that there was a significant increase in seed yield at 60 kg ha^{-1} nitrogen application.

In fruit yield, genetic control was found to be more dominant than nitrogen fertilization.

Quality characteristics

Essential oil rate

Variation in essential oil rate was given in *Tables 5 and 6*. Variation of mean data of years in essential oil content was not significant statistically and means are the same for both years as 0.18%. While main factor of year was not significant, doses and interaction factors were significant ($p<0.01$). Regarding to mean data of doses, statistically ($p<0.05$) highest value was obtained at 80 kg nitrogen per hectare application as 0.20%; statistically ($p<0.05$) lowest value was obtained at control group as 0.17% (*Table 6*). While the same value was obtained in both years at 80 kg ha^{-1} nitrogen application, relatively higher value was obtained at 40 and 60 kg ha^{-1} nitrogen application in second year, at control group and 100 ha^{-1} nitrogen application in first year.

Table 5. *Variation of mean and standard deviation in coriander essential oil content and components cultivated in two different years*

Components (%)	RT	1st Year	2nd Year
		Mean+SD	Mean+SD
α-pinene	6.803	6.28±1.65	6.58±1.78
Camphene	8.115	0.73±0.18	0.72±0.21
β-pinene	9.502	0.62±0.17	0.61±0.16
Sabinene	10.003	0.45±0.12	0.44±0.12
Myrcene	11.554	0.92±0.29	0.92±0.24
Limonene	12.788	1.9±0.60	1.86±0.48
γ-terpinene	14.565	7.24±2.23	7.39±1.65
p-cymene	15.501	3.59±1.10	3.69±0.98
Terpinolene	15.878	0.34±0.19	0.36±0.09
Camphor	24.285	3.96±1.20	3.77±0.57
Linalool	25.254	57.71±6.22	63.89±7.37
Terpinen-4-ol	26.956	0.32±0.16	0.26±0.08
α-Terpineol	29.850	0.34±0.14	0.31±0.06
Isoborneol	30.027	0.15±0.09	0.33±0.40
Geranylacetate	31.601	5.56±1.94	4.8±0.680
L-Citronellol	31.753	0.13±0.12	0.15±0.03
Geraniol	34.147	2.74±1.02	2.44±0.36
Dodec-2(E)-enal	34.701	0.4±0.31a	0.16±0.05b
Essential Oil		0.18±0.02	0.18±0.02

In the study about coriander, İzgi et al. (2017) stated that essential oil content of coriander varied from 0.2% to 0.6%. Erdoğdu (2012), Kan (2007), Khalid, (2013) and Kırıcı et al. (1997) reported that the effect of nitrogen dose on the proportion of essential

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oil is statistically insignificant. However, in another study, Khalid (2015) found effect of fertilizer (nitrogen and phosphorus) on essential oil rate as positively significant ($p<0.05$) and values between 0.2% - 0.3%. Patel et al. (2013) found significant positive relation and values between 0.41 - 0.51%. In study about response of coriander to nitrogen treatment, Lenardis et al. (2000) found that essential oil rate was increasing statistically with increasing nitrogen doses in coriander. Gil et al. (2002) stated that fertilizer (nitrogen) factor was not significant for essential oil, but year factor is significant for European coriander and interaction (year x fertilizer) factor is significant for Argentinean coriander ($p<0.05$). In their study about nitrogen doses and density effect on coriander quality, Moosavi et al. (2013) found that nitrogen doses have positive significant effect on essential oil rate of coriander and obtained values between 0.15 - 0.33% and tested highest value from 120 kg ha^{-1} nitrogen application. Akbarinia et al. (2007) found that the highest essential oil rate was obtained with 90 kg N per hectare application.

Table 6. *Variation in essential oil content and major components of essential oil of coriander (Coriandrum sativum L.) according to Year (Y) and Nitrogen doses (N)*[1]

	Years	Nitrogen Doses (kg ha^{-1})					Mean^{Y}
		0	**40**	**60**	**80**	**100**	
	1st	0.17	0.16	0.16	0.20	0.19	**0.18a**
Essential Oil (%)	**2nd**	0.16	0.21	0.20	0.20	0.15	**0.18a**
	Mean^{N}	**0.17d**	**0.19b**	**0.18bc**	**0.20a**	**0.18cd**	
Factors		**Y^{NS}**		**N****		**YxN****	
	1st	53.44	59.37	57.42	59.2	59.13	**57.71b**
Linalool (%)	**2nd**	61.25	57.26	63.51	63.12	74.29	**63.89a**
	Mean^{N}	**57.35b**	**58.32b**	**60.47b**	**61.16b**	**66.71a**	
Factors		**Y****		**N***		**YxN***	
	1st	3.65	6.77	8.33	7.77	9.68	**7.24a**
γ-terpinene (%)	**2nd**	8.04	8.33	9.01	7.04	4.55	**7.39a**
	Mean^{N}	**5.84c**	**7.55b**	**8.67a**	**7.4b**	**7.12b**	
Factors		**Y^{NS}**		**N****		**YxN****	
	1st	3.84	6.5	6.21	6.36	8.49	**6.28a**
α-pinene (%)	**2nd**	7.75	7.53	7.84	6.46	3.34	**6.58a**
	Mean^{N}	**5.8c**	**7.02a**	**7.03a**	**6.41b**	**5.92bc**	
Factors		**Y^{NS}**		**N****		**YxN****	
	1st	2.3	6.53	7.12	6.83	5.03	**5.56a**
Geranyl acetate (%)	**2nd**	5.06	5.77	4.38	4.75	4.02	**4.80b**
	Mean^{N}	**3.68c**	**6.15a**	**5.75a**	**5.79a**	**4.53b**	
Factors		**Y****		**N****		**YxN****	
	1st	2.38	5.07	4.87	4.52	2.95	**3.96a**
Camphor (%)	**2nd**	3.86	4.49	3.19	4.12	3.21	**3.77a**
	Mean^{N}	**3.12c**	**4.78a**	**4.03b**	**4.32b**	**3.08c**	
Factors		**Y^{NS}**		**N****		**YxN****	

*:$p<0.05$; **:$p<0.01$; NS: not significant; [1]: Different letters after the same column data indicate significant difference at the 0.05 level or similar

Results in the study is suitable with Gil et al. (2002), Lenardis et al. (2000), Moosavi et al. (2013) and Patel et al. (2013). However, values are lower. It can be caused from difference of essential oil between cultivars. According to these results, it can be said that,

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ecological conditions, cultural applications and cultivars can cause differences for nitrogen effect on essential oil rate.

Essential oil composition

In the study where the effect of different doses of nitrogen in the coriander on essential oil components was determined, 18 components in total were detected (*Table 5*). The major components of coriander were found to be linalool, γ-terpinene, α-pinene, geranyl acetate and camphor. It was observed significant ($p<0.05$) differences between main factor years for linalool and geranyl acetate. Mean data between nitrogen doses were statistically significant for linalool ($p<0.05$), γ-terpinene, α-pinene, geranyl acetate and camphor ($p<0.01$). Significant interaction was tested for linalool ($p<0.05$), γ-terpinene, α-pinene, geranyl acetate and camphor ($p<0.01$). Highest mean value for linalool was obtained at 100 kg ha^{-1} nitrogen application (66.71%). Lowest value was measured at control group (57.35%). Linalool rate was increased by increasing nitrogen doses. Highest value (8.67%) for γ-terpinene was obtained at 60 kg ha^{-1} nitrogen application and lowest value (5.84%) was obtained at control group. Highest value (7.03%) for α-pinene was measured at 60 kg ha^{-1} nitrogen application and lowest value (5.80%) was obtained at control group. For geranyl acetate, highest value (6.15%) was obtained at 40 kg ha^{-1} nitrogen application and lowest value (3.68%) was obtained at control group. For camphor, highest value (4.78%) was obtained at 40 kg ha^{-1} nitrogen application and lowest value (3.08%) was obtained at 100 kg ha^{-1} nitrogen application. Regarding to components of essential oil, linalool was increase with increasing nitrogen doses, γ-terpinene was increase until 60 kg ha^{-1} nitrogen application; α-pinene, geranyl acetate and camphor were increase until 40 kg ha^{-1} nitrogen application.

In their study about cultivar and location, İzgi et al. (2017) found 12 total components and 4 major components in Gamze cultivar as linalool (74.7% - 82.2%), α-pinene (0.3% - 4.1%), neryl acetate (3.6% - 6.5%) and γ-terpinene (1.9% - 4.2%). In his study of nitrogen treatment effect on essential oil composition of coriander, Khalid (2014) obtained 15 different contents of essential oil, and found linalool (75.5% - 76.9%), limonene (6.8% - 7.4%) and camphor (3.7% - 3.9%) as major components of coriander and stated that values of major component differ from control group significantly. In another study about effect of micro and macro nutrients on essential oil of coriander, Khalid (2015), found that major components of essential oil in coriander (linalool, limonene and camphor) increase with increasing fertilizer (NP) doses but effect is not significant statistically. However, he found that α-pinene increase significantly ($p<0.05$) with fertilizer application.

The results that obtained from this study was lower than literature, but effect of fertilizer factor was suitable with literature. It can be caused from different ecological conditions, cultivars and cultural applications.

Fatty oil content (%)

Variation in fatty oil content was given in *Tables 7 and 8*. While fatty oil content was higher at second year, year differences were not statistically significant. Although highest value (23.64%) was obtained at 80 kg ha^{-1} nitrogen application and lowest value (23.14%) at 40 kg ha^{-1} nitrogen application, differences between doses were not statistically significant.

In their study about cultivar effect on coriander, Gökduman and Telci (2018) reported that mean fatty oil rate is 18.64% for Gamze cultivar. Keskin and Baydar (2016)

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determined the mean fatty oil rate of the Gamze type as 24.62% under the ecological conditions of Isparta. Khalid (2012) found significant effect of fertilizer (NP) on fatty oil rate and values between 2.5% - 6.8%. Regarding to mean data of fatty oil, Khalid (2013) found difference of nitrogen doses significant ($p<0.05$) and values between 5.5% - 8.2% in coriander. Values found in this study is higher than Khalid (2012, 2013)'s findings and same with Keskin and Baydar (2016) and Gökduman and Telci (2018).

Table 7. *Variation of mean and standard deviation in coriander fatty oil content and fatty acids cultivated in two different years*

Components	RT	1st Year	2nd Year
		Means+SD	Means+SD
Butyric Acid C4:0	5.643	23.78 ± 2.77	25.15 ± 2.37
Caproic Acid C6:0	6.188	0.08 ± 0.06	0.15 ± 0.03
Caprylic Acid C8:0	7.435	0.01 ± 0.03	0.02 ± 0.04
Palmitic Acid C16:0	18.47	2.60 ± 0.27	2.59 ± 0.17
Palmitoleic Acid C16:1	19.642	0.14 ± 0.01	0.14 ± 0.01
Stearic Acid C18:0	22.146	0.66 ± 0.07	0.65 ± 0.05
Petroselinic Acid C18:1n9c	23.129	61.77 ± 6.33	60.43 ± 4.19
Linolelaidic Acid C18:2	24.134	0.09 ± 0.01	0.08 ± 0.01
Linolenic Acid C18:2n	24.641	10.23 ± 1.06	10.1 ± 0.72
Arachidic Acid C20:0	25.604	0.02 ± 0.03	0.02 ± 0.03
α-Linolenic Acid C	26.403	0.06 ± 0.03	0.05 ± 0.01
Eicosadienoic Acid C20	28.316	0.07 ± 0.11	0.11 ± 0.05
Fatty Oil		23.09 ± 2.33	23.43 ± 1.52

Table 8. *Variation in fatty oil content and major fatty acids of coriander (Coriandrum sativum L.) according to Year (Y) and Nitrogen doses (N)*

	Years	Nitrogen Doses (kg ha^{-1})					Mean^{Y}
		0	40	60	80	100	
Fatty Oil (%)	**1st**	23.15	23.03	23.21	23.33	22.74	**23.09a**
	2nd	23.19	23.24	23.18	23.94	23.59	**23.43a**
	Mean^{N}	**23.17**	**23.14**	**23.20**	**23.64**	**23.17**	
Factors		**Y^{NS}**		**N^{NS}**		**YxN^{NS}**	
Petroselinic Acid C18:1n9c (%)	**1st**	59.89	62.96	62.42	61.39	62.19	**61.77a**
	2nd	60.90	60.17	61.12	57.79	62.17	**60.43a**
	Mean^{N}	**60.40**	**61.57**	**61.77**	**59.59**	**62.18**	
Factors		**Y^{NS}**		**N^{NS}**		**YxN^{NS}**	
Butyric Acid C4:0 (%)	**1st**	26.13	22.10	22.96	24.32	23.38	**23.78a**
	2nd	24.37	25.52	24.13	28.37	23.38	**25.15a**
	Mean^{N}	**25.25ab**	**23.81b**	**23.55b**	**26.35a**	**23.38b**	
Factors		**Y^{NS}**		**N***		**YxN***	
Linolenic Acid C18:2n (%)	**1st**	9.86	10.53	10.36	10.09	10.3	**10.23a**
	2nd	10.37	10.10	10.29	9.51	10.25	**10.10a**
	Mean^{N}	**10.12**	**10.32**	**10.33**	**9.80**	**10.28**	
Factors		**Y^{NS}**		**N^{NS}**		**YxN^{NS}**	

*: $p<0.05$; **:$p<0.01$; NS: not significant

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It has been found that the effect of genetic control on the fatty oil rate in the coriander is stronger than nitrogen fertilization.

Fatty acids composition (%)

A total of 12 fatty acids were determined in the fatty oil samples obtained from different nitrogen applications in coriander. The components of the major fatty acids were found as petroselinic acid, butyric acid and linolenic acid. Doses and interaction factor were not statistically significant for petroselinic acid and linolenic acid. Year differences were not statistically significant for major components of fatty acids. Regarding to dose differences, while highest mean value (62.18%) for petroselinic acid was observed at 100 kg ha^{-1} nitrogen application, mean differences between dose groups were not statistically significant. For linolenic acid, highest value (10.33) was obtained at 60 kg ha^{-1} nitrogen application; however dose differences were not statistically significant. Doses and interaction factor were statistically significant ($p<0.05$) for butyric acid, but year factor was not significant. Statistically ($p<0.05$) highest value of butyric acid (26.35%) was observed at 60 kg ha^{-1} nitrogen application and lowest value was observed at 100 kg ha^{-1} nitrogen application.

Information on the fatty acid content of coriander in order to nitrogen application in literature is very limited. Akbarinia et al. (2007) measured that highest fatty acid compositions were obtained with 90 kg per hectare nitrogen application. Keskin and Baydar (2016) found petroselinic acid, major component of fatty oil in coriander, at level of 79.16%. Msaada et al. (2010), in their studies, where they examined the fatty oil content of coriander in different maturation periods determined 12 components, and among them, identified petroselinic (80.36 ± 8.45), linoleic (14.12 ± 1.35), palmitic (4.09 ± 0.42) and stearic (0.66 ± 0.05) acid as the major components. Ramadan and Mörsel (2002) found 12 components and reported that the main oil components and rates in coriander fruit were petroselinic acid (65.7%) and linoleic acid (16.7%). In the study of oil rate in different part of Tunisian coriander, Sriti et al. (2009) obtained 9 components and found petroselinic acid (74.07%), linoleic acid (13.41%) and oleic acid (5.91%) as major components in fatty acids of fruit. In their study of salinity effect on coriander, Neffati and Marzouk (2008) obtained 9 different components of fatty acids and found α-linolenic acid (24.14%) as a major component.

According to this study, it can be concluded in literature that nitrogen fertilization has a positive effect on butyric acid in coriander fruits. The limited change in the nitrogen applications of the primary metabolite fatty acids can be explained by the fact that the control of the genetic structure in the plant is more dominant.

Conclusion

This research aims to determine the effect of nitrogen fertilization in coriander on fruit yield and quality in plain conditions of Mardin province. The results are given below as items.

1. It has been observed that nitrogen doses have an insignificant effect on fruit yield.
2. It has been tested that nitrogen doses have an insignificant effect on essential oil rate.
3. Linalool increased in parallel with increasing nitrogen doses.
4. Nitrogen fertilization did not affect fatty oil rate and other major components statistically.

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5. Year differences were not statistically significant for essential oil and fatty oil rates.
6. In coriander seeds; It has been determined that genetic control is stronger in the fruit yield, fatty oil content and fatty acids composition compared to nitrogen dose applications.
7. Since nitrogen fertilization increases the linalool ratio in the fruit and thus the essential oil quality, the use of the appropriate dose will increase the market value of the coriander product.
8. At the end of the study; In addition to the application of nitrogen dosage, additional studies on quality relationships with some other important plant nutrients, sowing time and harvest time have emerged.

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GENETIC DIVERSITY AMONG DURIAN (*DURIO ZIBETHINUS* MURR.) POPULATIONS FROM NIAS ISLAND, INDONESIA USING RAPD MARKERS

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Abstract. Indonesian durian (*Durio zibethinus* Murr.) germplasms cultivated on Nias Island were assessed for their genetic diversity and relationships using Random Amplified Polymorphic DNA (RAPD) markers. Fifty accessions were collected from five populations or zones in Nias Island, Indonesia. All RAPD fragments were polymorphic as revealed by the use of OPA-01, OPA-03, OPA-07, OPA-13, OPN-06, and RAPD-05 primers within and among the durian germplasms. Primer OPA-03 was evaluated as a highly informative marker. Similarity values (Jaccard) ranged from 0.216 to 0.423 between different populations and 0.00 to 0.89 within the populations. The genetic structure between populations ranged from 1.71 to 1.92 for mean number of alleles (n_a), and from 1.35 to 1.59 for effective number of alleles (ne). Shannon's diversity index (I) ranged from 0.34 to 0.51, and the Nei's gene diversity index (h) ranged from 0.21 to 0.34. Result of cluster analysis based on Unweighted Pair Group Method with Arithmetic Mean (UPGMA) grouped all germplasms into thirteen clusters, some were admixtures between different populations. Result of Principal Coordinate Analysis (PCoA) confirmed the UPGMA dendrogram with the results of five main clusters among durian accessions from Nias Island, Indonesia.
Keywords: *durian, germplasm, polymorphism, principal coordinate analysis, molecular diversity*

Introduction

Durian (*Durio zibethinus* Murr.) is one of the edible *Durio* species with an approximate number of 30 species dispersed throughout the world (Brown, 1997). This popular tropical fruit species has been cultivated mainly in Southeast Asia, including Malaysia, Thailand, and Indonesia as a potential export commodity. Indonesia's durian production is thought to increase following an average annual increment of 4.86% in 2013 which also coherently increased its selling price from year to year due to its popularity and high market demands (CDISA, 2014). The popularity basis of this fruit is due to its sweet and strong-pungent aroma with a fleshy texture of its edible pulps (Belgis et al., 2017). In addition, the fruits are rich in essential nutrients which may provide beneficial health properties to the consumers as well as supporting the local business of many durian farmers to improve their socio-economic life (Ho and Bhat, 2015).

There are currently two mostly known and favored durian species from Indonesia, namely durian (*D. zibethinus* Murr.) and lai (*D. kutejensis* Hassk. & Becc.), both were reported originate from and harvested mainly in Kalimantan (Borneo) (Reksodihardjo, 1962). Moreover, Borneo is described as one of the durian germplasm centers in the world with a total of 18 species, followed by Malaya (11 species), and Sumatra (7 species) while several Indonesian durian species has also been regarded as endemic germplasms (Kostermans, 1953; Nyffeler and Baum, 2000; Uji, 2005). Until 2015, the

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Government of Indonesia has listed 93 superior local durian cultivars compiled from three officially-commercialized durian species, the *D. zibethinus, D. kutejensis,* and *D. oxleyanus* with future possibilities on identifying other wild cultivars to be promoted and listed in Indonesian collection of indigenous durian cultivars (Santoso et al., 2016).

Information on the diverse local cultivars will also support the plant breeding sector, especially the crop breeder to produce progenies with adaptive and varying phenotypes or to select parental lines with improved productivity responding to future changes (Moreno and Trujillo, 2005). Among three durian species in Indonesia, *D. zibethinus* cultivars are mostly known and consumed especially in Sumatra and Java due to its abundance and availability regardless of harvesting period. Nias Island is one of the regions in North Sumatra, located in the eastern Indian Ocean, known for its intense trade of many local *D. zibethinus* cultivars compared to other provinces in Indonesia. The island is inhabited by local tribe or the Nias people who are practically cultivating *D. zibethinus* in many agricultural fields or harvesting the wild cultivar directly from the forest (Hannum et al., 2019). Moreover, Gunungsitoli as the central city of Nias Island, has been declared by the Government of Indonesia as an iconic city for durian tourism to increase the socio-economic life of the locals. However, there is no information and comprehensive study on their *D. zibethinus* germplasm originating from the island.

To date, studies on identifying or grouping the *D. zibethinus* populations (cultivars) based on their genetic characteristics using genetic markers have been reported. Genetic markers and polymerase chain reaction (PCR), produced a more informative result in characterizing the genetic diversity than morphological features, along with its advantages and limitations (Weising et al., 1995; Ibrahim et al., 2010). Genomic profiling using RAPD markers, has been reported as a preliminary investigation on the classification of durian cultivars in countries with abundant durian germplasms, including Indonesia (Ruwaida et al., 2009; Vanijajiva, 2011; Mursyidin and Daryono, 2016; Prihatini et al., 2016; Rosmaina et al., 2016). RAPD analysis was favored as the initial method in genetic fingerprinting due to its simplicity, low-cost, tiny amount of target DNA requirement, and non-requirement of target genetic sequences (Welsh and Mc-Clelland, 1990; Williams et al., 1990). Practicality of these markers increased their performance as attractive guidance in studying the overall genetic variation and population genetic structure (Lynch and Milligan, 1994). To our knowledge, RAPD-based profiling of local durian cultivars has been reported for some regions in Indonesia, but still no information for the cultivars originating from Nias Island in North Sumatra. In this study, six RAPD markers were utilized to evaluate the genetic variation among 50 durian accessions with an emphasize on the relationship between and within durian populations from Nias Island, Indonesia.

Methodology

Plant materials

Durian (*Durio zibethinus* Murr.) accessions from five different populations comprising Nias City, Central Nias, West Nias, North Nias, and South Nias were collected randomly from Nias Island in 2019 (*Fig. 1*). The list of 50 durian accessions and their vernacular names is listed in *Table 1*. The foliar parts were sampled and preserved in a dried zip-lock bag filled with silica beads prior experimentation in the laboratory (*Fig. 2*).

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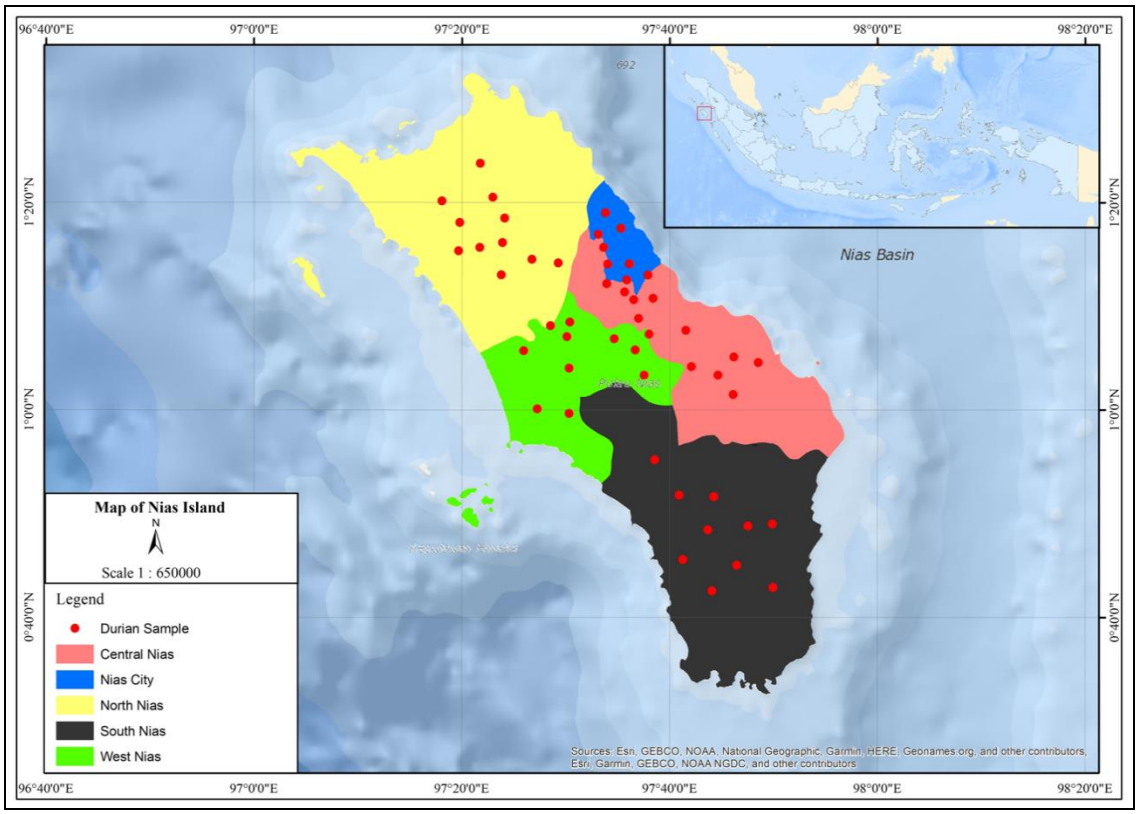

Figure 1. *Map showing sampling sites of Nias Island for study of durian cultivar diversity*

Figure 2. *A typical durian (D. zibethinus) tree and its fruit in Nias Island. Scale bar = 5 cm*

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Table 1. *List of 50 durian germplasms from Nias Island, Indonesia used in this study*

Code	Local name	Zone of collection	GPS Coord.	Code	Local name	Zone of collection	GPS Coord.
N1-1	Afia	Nias City	1°22’35”N 97°32’51”E	N3-6	Hilimbowo Ma’u	West Nias	1°3’47”N 97°37’47”E
N1-2	Bawodesolo	Nias City	1°20’4”N 97°31’47”E	N3-7	Hilimbuasi_1	West Nias	1°2’43”N 97°35’25”E
N1-3	Hilihao_1	Nias City	1°15’3”N 97°35’23”E	N3-8	Hilimbuasi_2	West Nias	1°3’12”N 97°35’3”E
N1-4	Hilihao_2	Nias City	1°15’3”N 97°36’21”E	N3-9	Sisobawino	West Nias	1°2’12”N 97°36’43”E
N1-5	Lasaratarakhaini_1	Nias City	1°18’56”N 97°30’30”E	N3-10	Tuwuna	West Nias	1°5’14”N 97°34’18”E
N1-6	Lasaratarakhaini_2	Nias City	1°20’18”N 97°31’48”E	N4-1	Alo’oa	North Nias	1°27’31”N 97°29’16”E
N1-7	Lasaratarakhaini_3	Nias City	1°19’49”N 97°32’25”E	N4-2	Botolakha_1	North Nias	1°26’3”N 97°29’56”E
N1-8	Sisobahili_1	Nias City	1°19’59”N 97°35’17”E	N4-3	Botolakha_2	North Nias	1°26’16”N 97°30’24”E
N1-9	Sisobahili_2	Nias City	1°19’26”N 97°35’0”E	N4-4	Hiligodu	North Nias	1°23’53”N 97°23’18”E
N1-10	Teluk Belukar	Nias City	1°23’54”N 97°31’26”E	N4-5	Hilimaziaya	North Nias	1°23’31”N 97°25’39”E
N2-1	Botombawo_1	Central Nias	1°12’31”N 97°34’21”E	N4-6	Hilimbosi_1	North Nias	1°24’2”N 97°29’30”E
N2-2	Botombawo_2	Central Nias	1°12’14”N 97°34’10”E	N4-7	Hilimbosi_2	North Nias	1°23’57”N 97°29’39”E
N2-3	Fadoro Lai’o	Central Nias	1°11’46”N 97°34’7”E	N4-8	Hilinduria	North Nias	1°23’19”N 97°23’51”E
N2-4	Hilimbowo	Central Nias	1°10’49”N 97°32’48”E	N4-9	Umbu Balodano	North Nias	1°24’6”N 97°29’27”E
N2-5	Hiliwaele	Central Nias	1°10’6”N 97°35’27”E	N4-10	Ambukha 1	North Nias	0°54’5”N 97°39’56”E
N2-6	Lalai_1	Central Nias	1°11’22”N 97°36’42”E	N5-1	Ambukha 2	South Nias	0°53’31”N 97°40’34”E
N2-7	Lalai_2	Central Nias	1°9’46”N 97°35’36”E	N5-2	Amorosa	South Nias	0°54’53”N 97°40’1”E
N2-8	Lawe-lawe	Central Nias	1°12’29”N 97°33’21”E	N5-3	Caritas Sogawunasi_1	South Nias	0°56’55”N 97°38’14”E
N2-9	Lolofaoso Lalai	Central Nias	1°9’51”N 97°37’2”E	N5-4	Caritas Sogawunasi_2	South Nias	0°57’10”N 97°38’21”E
N2-10	Lolowua	Central Nias	1°11’16”N 97°34’38”E	N5-5	Ehosakhozi	South Nias	1°1’16”N 97°36’25”E
N3-1	Ambukha_1	West Nias	1°2’8”N 97°35’28”E	N5-6	Hilimbosi_3	South Nias	1°24’11”N 97°29’54”E
N3-2	Ambukha_2	West Nias	1°2’24”N 97°35’11”E	N5-7	Hilisangowola	South Nias	0°57’15”N 97°36’52”E
N3-3	Duria	West Nias	1°2’14”N 97°36’56”E	N5-8	Koendrafo	South Nias	0°52’51”N 97°40’3”E
N3-4	Hili’uso_1	West Nias	1°5’12”N 97°34’58”E	N5-9	Lawa-lawa luo	South Nias	0°57’0”N 97°37’40”E
N3-5	Hili’uso_2	West Nias	1°4’9”N 97°35’11”E	N5-10	Suka Maju	South Nias	0°54’39”N 97°39’36”E

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DNA isolation and PCR amplification

Extraction of DNA genomes was carried out from foliar parts of selected accessions following the principle of hexadecyltrimethylammonium bromide (CTAB) with modification on using 4% CTAB solution and additional 2 mg of polyvinylpyrrolidone (PVP) during extraction (Murray et al., 1980). The isolated DNA was quantified at $A_{260/280}$ using NanoPhotometer P-Class® (Implen, US) and was visually checked on 1% agarose. Six decamer RAPD primers namely OPA-01, OPA-03, OPA-07, OPA-13, OPN-06, RAPD-05 were screened for amplification of scorable and reproducible DNA fragments. PCR reaction of a 20 µL reaction mixture contains: 10 µL (0.5 unit) of DreamTaq Green PCR Master Mix (2×), 2 µL (100 ng) of DNA template, 1 µL (0.6 mM) of primer, and 7 µL nuclease-free water. Thermal cycler (SensoQuest GmbH, Germany) was used for PCR-RAPD reactions with specification as follows: 95 °C (30 s), 34 °C (30 s), 72 °C (1 min), and 72 °C (5 min). The reaction products (5 µL) were mixed thoroughly with 2 µL loading dye prior loading. Both PCR products and DNA ladder (1 kb) were separated in 1.5% agarose with 1× TAE buffer. The PCR products were resolved by running gel at 70 V for 45 min. The gels were stained in 1 L of distilled water added with 10 µL of ethidium bromide for 10 min. The gels were observed under UV light and documented inside of a gel imaging device (G:Box Syngene, India). PCR-RAPD was repeated twice to ensure the reproducibility of reactions.

Data analysis

DNA polymorphism was evaluated and scored manually based on the presence (1) and absence (0) for each scoreable DNA fragment to generate a binary data set. The number of alleles and polymorphic bands were calculated for each primer as the percentage of polymorphisms (%). Determination of the marker suitability was evaluated through three parameters:

The polymorphic information content (PIC) value of each primer was the average of all loci in a primer (Powell et al., 1996):

$$Polymorphic\ Information\ Content\ (PIC) = 2fi(1 - fi) \quad \text{(Eq.1)}$$

The marker index (MI) value which explains the usefulness of a marker to discriminate the polymorphism. The effective multiplex ration (EMR) was obtained from a multiplication of the number of loci polymorphic in the germplasm set of interest (Varshney et al., 2007):

$$Marker\ Index\ (MI) = EMR\ x\ PIC \quad \text{(Eq.2)}$$

The resolving power (RP) value shows the ability of the most informative primers to discriminate genotypes of interest, where i_b is the band informativeness (Prevost and Wilkinson, 1999):

$$Resolving\ Power\ (RP) = \sum i_b \quad \text{(Eq.3)}$$

Genetic diversity among populations was estimated by following parameters: the average number of alleles (n_a), effective number of alleles (n_e), heterozygosity (h) (Nei,

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1973), Shannon's diversity index (*I*) (Shannon, 1949), and percentage of polymorphic loci (P%) using POPGENE ver. 1.32 (Yeh and Boyle, 1997). The binary data of each population and accession were calculated for the similarity values (Jaccard, 1908) using NTSYS-pc ver. 2.1 by applying Similarity for Qualitative Data, Simple Matching (SimQUAL-SM) feature (Rohlf, 2000). The genetic relatedness or cluster analysis among populations and within 50 durian accessions was visualized in a UPGMA dendrogram by applying the Sequential Agglomerative Hierarchical and Nested (SAHN) module in NTSYS-pc. In addition, a Principal Coordinate Analysis (PCoA) was used to plot the ordination matrices using EIGEN and PROJ features (NTSYS-pc).

Results

The assessment of genetic diversity among 50 durian germplasms used six RAPD primers to characterize their genetic information based on the presence/absence of polymorphic bands (*Fig. 3*). We observed that all primers produced legible and reproducible polymorphic patterns in two repetitions. Two factors in determining the genetic diversity using molecular markers are marker informativeness and marker performance with the results listed in *Table 2*. Each RAPD primer was evaluated for its informativeness based on the following parameters (*Table 2*). Sixty-six clear and reproducible RAPD fragments (200–2800 bp) was generated in this study. Interestingly, all RAPD bands showed a high level of polymorphism (100%) which also meant that no conserved pattern or monomorphic bands were observed. The average of polymorphic bands was eleven with the range from nine (OPA-01, RAPD-05) to fourteen (OPN-06) with an average of eleven. The polymorphic RAPD fragments were carefully scored and utilized for clustering and ordination analysis herein.

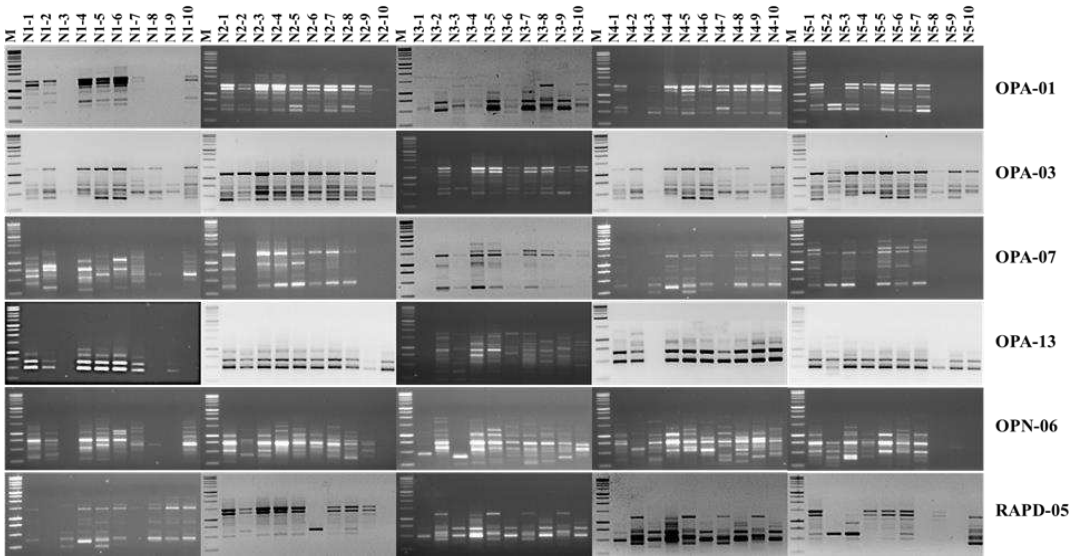

Figure 3. *Genetic profile of the 50 Durio zibethinus Murr. accessions (N1-1–N5-10) generated by five RAPD primers. Marker used was 1 kb (250-10,000 bp)*

Each accession was analyzed for the evaluation of its marker performance based on the following parameters (*Table 2*). The average PIC value was 0.42 for 66 polymorphic bands with the range between 0.29 to 0.48. Forty-three RAPD fragments were regarded

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as greatly informative (PIC ≥ 0.4), four fragments were low (0.0–0.2), while the remaining nineteen fragments were medium (0.2–0.4) (*Fig. 4*). OPA-01 was observed as with the highest PIC value (0.48) while the lowest (0.29) was obtained from RAPD-05. The highest EMR (7.36) was observed for primer OPA-03 with the mean per primer was 4.46 (*Table 2*). The overall performance from each RAPD marker was evaluated from its marker index (MI). The MI for the six primers ranged from 0.50 (RAPD-05) to 3.46 (OPA-03) with an average of 1.94. The resolving power (RP) of RAPD primers is a parameter which indicates a discriminatory properties, ranged from 3.48 (RAPD-05) to 10.24 (RAPD-03) with an average of 7.47. There were significant correlations for PIC–MI ($r = 0.85$, $P = 0.032$), EMR–MI ($r = 0.99$, $P = 0.000$), EMR–RP ($r = 0.94$, $P = 0.006$), and MI–RP ($r = 0.90$, $P = 0.015$) which showed that the parameters of marker performance resulted in our study were considerably related each other.

Table 2. *Polymorphism and marker characteristics of RAPD primers*

Primer name	Sequence (5′- 3′)	Number of fragments			% Polymorphism	PIC	EMR	Marker index	Resolving power
		Total	Mono-	Poly-					
OPA-01	CAGGCCCTTC	9	0	9	100	0.48	4.80	2.30	7.28
OPA-03	AGTCAGCCAC	13	0	13	100	0.47	7.36	3.46	10.24
OPA-07	GAAACGGGTG	11	0	11	100	0.44	4.54	2.00	7.96
OPA-13	CAGCACCCAC	10	0	10	100	0.44	3.86	1.70	7.32
OPN-06	GAGACGCACA	14	0	14	100	0.38	4.44	1.69	8.56
RAPD-05	AACGCGCAAC	9	0	9	100	0.29	1.74	0.50	3.48
Total	-	66	-	66	-	-	-	-	-
Average	-	11	-	11	100	0.42	4.46	1.94	7.47

Genetic diversity of five durian populations from Nias Island was estimated based on the RAPD amplification results under following range of values: effective number of alleles (n_e) from 1.35 to 1.59, mean number of alleles (n_a) from 1.71 to 1.92, Nei's gene diversity index (h) from 0.21 to 0.34, and Shannon's diversity index (I) from 0.34 to 0.51 (*Table 3*). Based on the polymorphic loci (P%), West Nias and North Nias displayed the highest variation of durian cultivars with the percentage of 92.42 for both zones. According to the overall variation resulted from P%, the ranking among Nias durian population was West Nias/North Nias > South Nias > Central Nias > Nias City. Our findings then strongly suggested that the durian germplasms cultivated in five different regions were highly varying as evidenced effectively through RAPD genetic analysis.

Table 3. *Estimates of genetic diversity within five populations (zones) using RAPD markers*

Index	Nias City	Central Nias	West Nias	North Nias	South Nias	All
n_a	1.71	1.88	1.92	1.92	1.89	2.00
n_e	1.35	1.55	1.52	1.59	1.56	1.77
h	0.21	0.32	0.31	0.34	0.33	0.42
I	0.34	0.47	0.47	0.51	0.49	0.60
P%	71.21	87.88	92.42	92.42	89.39	100.0

n_a = Average number of alleles; n_e = Effective number of alleles; h = Heterozygosity; I = Shannon's diversity index; P% = Percentage of polymorphic loci

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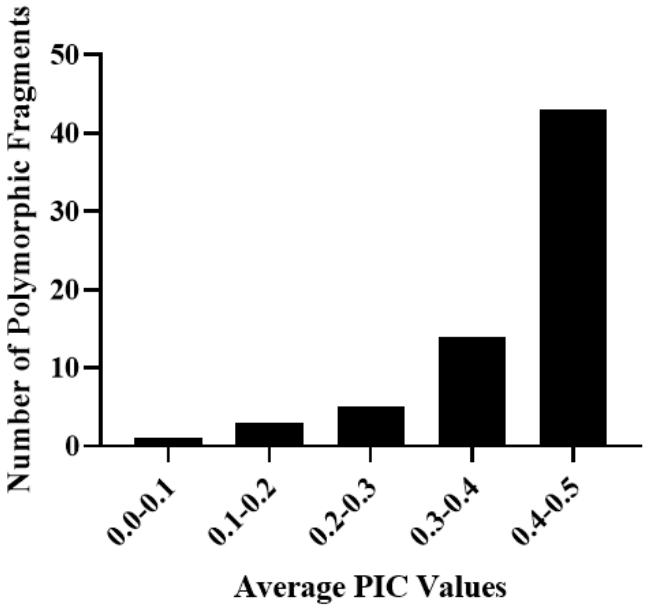

Figure 4. *Average PIC values for polymorphic bands generated by RAPD primers in 50 durian accessions*

The similarity values among populations were estimated using Jaccard's similarity matrices retrieved from RAPD binary data. The similarity values ranged from 0.216 to 0.423 with an average of 0.31 (*Table 4*). A UPGMA dendrogram was constructed to depict the populations grouping (*Fig. 5*). The result showed the ingrouping of Nias City, West Nias, and North/South Nias populations with Central Nias being an outlier. In addition, results showed that populations of North Nias and South Nias were more similar (42%) than others while the population of North Nias was separated indicating its high genetic dissimilarity.

Table 4. *Similarity matrix based on Jaccard's coefficient from five populations (zones)*

Index	Nias City	Central Nias	West Nias	North Nias	South Nias
Nias City	1.000	-	-	-	-
Central Nias	0.263	1.000	-	-	-
West Nias	0.333	0.294	1.000	-	-
North Nias	0.216	0.233	0.367	1.000	-
South Nias	0.410	0.175	0.342	0.423	1.000

Genetic diversity among 50 durian individuals or accessions were based on the Jaccard's similarity values. The similarity value of overall accessions ranged from 0.00 to 0.89 with an average of 0.44 (data not shown) indicating a close genetic relationship within the accessions. A UPGMA dendrogram revealed the eight main clusters and four outliers (cluster 6,7,8,10), with cluster (1) comprises the largest members with fourteen germplasms (*Fig. 6*). In order to visualize a better degree of differentiation among accessions, the spatial analysis using PCoA was utilized to represent the relative genetic similarities among individuals. The result was plotted into a two-dimensional plot generated from PCoA based on the calculated eigenvalues and eigenvectors.

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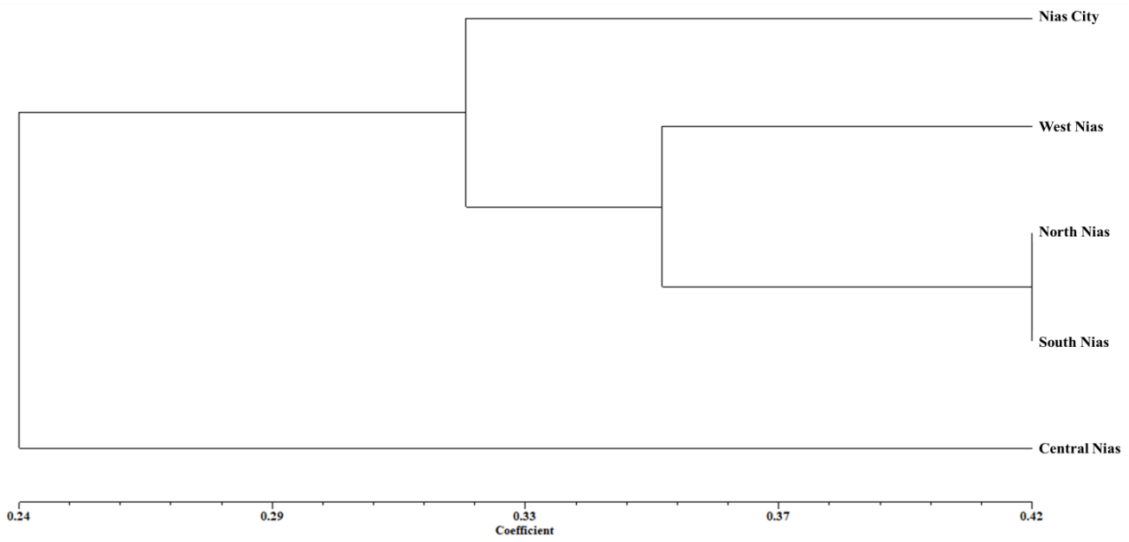

Figure 5. *A UPGMA dendrogram among five populations (zones) based on Jaccard's similarity coefficient calculated from RAPD data set*

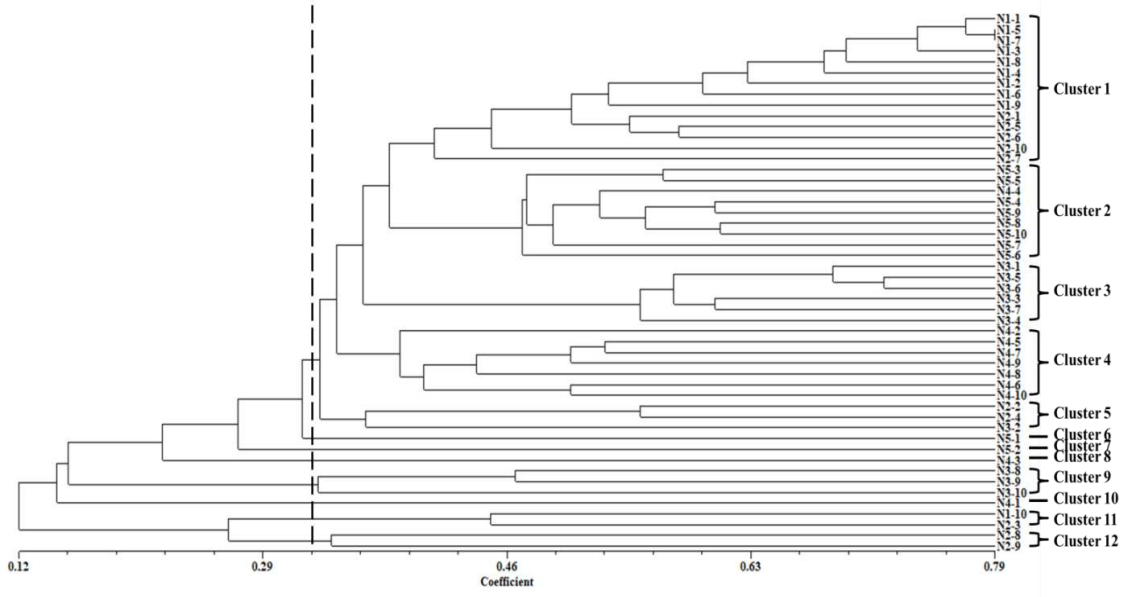

Figure 6. *A UPGMA dendrogram among 50 durian cultivars (N1-1–N5-10) based on Jaccard's similarity coefficient calculated from RAPD data set*

Results showed that the first two principal coordinates contributed for 53.9% of the total variation, with lesser contribution of other coordinates (<4%) each (*Fig. 7*). The accessions were later grouped into five clusters based on their coordinates; cluster (1) comprises four germplasms, cluster (2) with one germplasm, N4-1, cluster (3) with three germplasms, cluster (4) with sixteen germplasms, and cluster (5) with twenty-six germplasms. Moreover, the three-dimensional plot was generated and confirmed the coordinative trend with the 2D plot indicating a clear distinction among the 50 accessions (*Fig. 8*).

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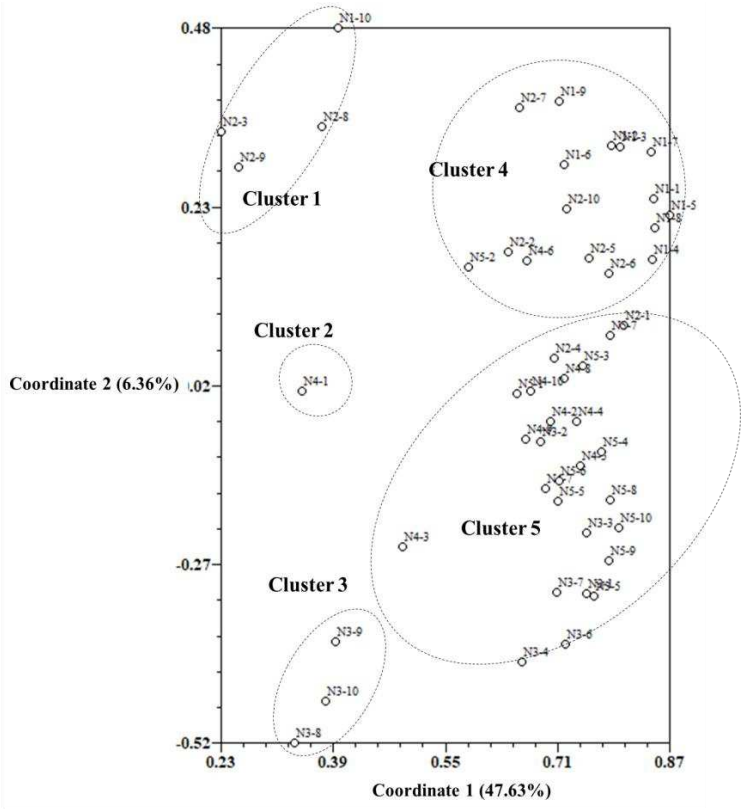

Figure 7. *Two-dimensional plot generated from Principal Coordinate Analysis (PCoA) of 50 durian cultivars using RAPD markers*

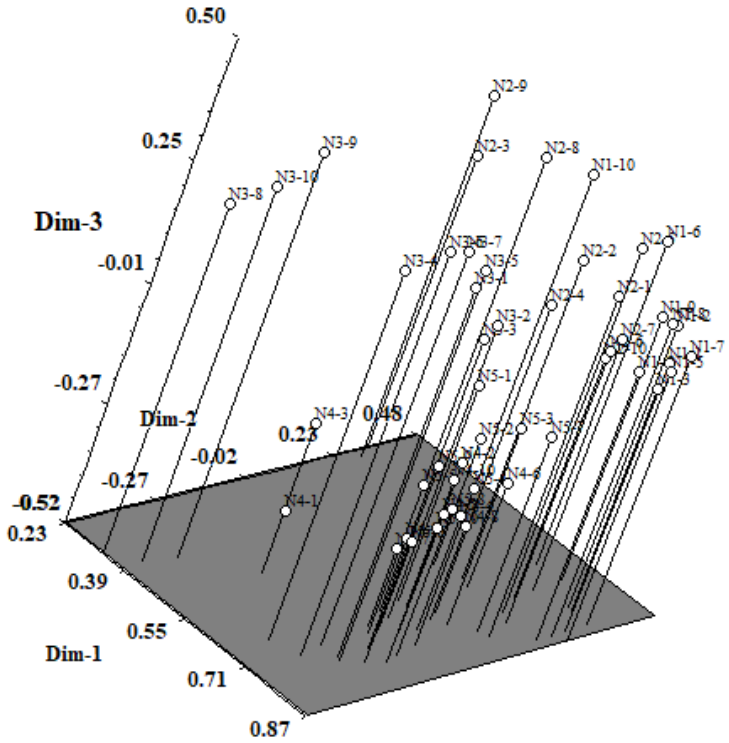

Figure 8. *Three-dimensional plot generated from Principal Coordinate Analysis (PCoA) of 50 durian cultivars using RAPD markers*

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Discussion

Identification of new crop germplasms by using DNA fingerprint is important prior the release and introduction of new varieties/cultivars to the agricultural fields and the differentiation of wild accessions bearing beneficial phenotypic traits. Genetic diversity and structure among 50 durian (*D. zibethinus* Murr.) accessions cultivated in different zones (regions) of Nias Island, Indonesia were analyzed using RAPD markers in this study. The PCR-RAPD has been utilized to employ the genetic characterization and profiling among germplasms for the major reason of its simplicity and ease of application. However, RAPD technique had some limitations, such as dominant type of inheritance, poor reproducibility, and co-migration issue (Munthali et al., 1992; Lowe et al., 1996). In this study, we optimized the reaction conditions and found out that during two repetitions, good and scorable DNA fragments were generated and they were consistent hereafter. Six RAPD primers (OPA-01, OPA-3, OPA-07, OPA-13, OPN-06, RAPD-05) produced an absolute polymorphism percentage (100%) among 50 accessions based on the absence/presence or binary data of DNA fragments. Therefore, the six RAPD markers used in this study were regarded as equally effective in determining polymorphisms as revealed from its PIC value in the level from moderate to high informative (Hildebrand et al., 1992).

RAPD primer with high PIC value also indicated the more usefulness of its utilization among primers, following other parameters such as EMR, MI, and RP in determining the genetic distance within a population (Kalinowski, 2002). Variety of RAPD primers have been reported to determine the genetic diversity of other local durian cultivars. Ruwaida et al. (2009) also employed six RAPD primers (OPA-01, OPA-02, OPA-07, OPA-16, OPA-18, OPA-19) in assessing the diversity of five durian cultivars from Java, Indonesia and reported them to be sufficient in separating between cultivars. Mursyidin and Daryono (2016) utilized five RAPD primers (OPA-01, OPA-07, OPA-16, OPA-18, OPA-19) to differentiate 11 durian accessions from South Kalimantan, Indonesia. Li and Midmore (1999) suggested that the use of few primers will be satisfactory in differentiating genotypes with high variations.

The high level of diversity in durian cultivars may due to the existence of wild hybrids within populations since durian is known as open pollinating plants in which pollination being facilitated by mostly fruit or nectarivorous bats (Bumrungsri et al., 2009). In addition, utilization of different DNA markers and other profilling techniques may produce different results in estimating the durian genetic diversity. Rosmaina et al. (2016) reported the high variation among five durian cultivars from Indonesian germplasm with a low genetic relationship or Nei and Li's genetic distance (0.27 to 0.47) based on RAPD analysis. Prihatini et al. (2016) reported a high variation among 32 durian F_1 hybrids from Indonesia based on RAPD analysis (0.14 to 0.77). Vanijajiva (2012) applied the inter simple sequence repeat (ISSR) markers to detect genetic variation among 14 durian accessions from Thailand and reported the less genetic relationship (0.63 to 1.00). Sundari et al. (2017) reported a medium to high level of genetic diversity (0.41 to 0.93) 15 durian cultivars from Ternate Island, Indonesia based on RAPD analysis. Nurlaila et al. (2019) reported the medium level of variation among 18 local durian varieties from South Sulawesi, Indonesia by using the morphological traits (0.08 to 0.50).

Based on our results, the fifty durian accessions collected from five different zones or populations in Nias Island were highly diverse (0.21 to 0.42) among populations. Hence, the result again supported the fact that the genetic diversity of Nias cultivars

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were slightly higher than previous reports and worth investigated. Based on the cluster analysis, the dendrogram showed a close relationship between North and South durian populations, while Central Nias and Nias City were different despites its adjacent location. Furthermore, the dendrogram among 50 accessions showed that some accessions with different geographical origins or zones were clustered together in the same group. We assumed that the phenomenon may due to the mobility of local farmers from the Central Nias/Nias city or any initial geographical points to other regions cultivating the durian seeds in the new agricultural locations. The similar finding was also reported from sesame (*Sesamum indicum* L.) populations in West Bengal, India as revealed from the RAPD analysis (Saha et al., 2019). Human factor may then be responsible for the geographical detachment of associated cultivars in some cases (Stankiewicz et al., 2001).

Deeper analysis among accessions was done by plotting the converted binary data into eigenvalues and projected using principal coordinate analysis (PCoA). Based on the 2D plot, we assigned five clusters out of the thirteen durian clusters constructed previously from UPGMA dendrogram analysis, with some admixtures among populations. Confirmatory analysis using the 3D plot projection using three coordinates supported our grouping although some accessions between cluster (4) and (5) were indiscriminative to some coordinates. N4-1 was the only cultivar discriminated and far distantly from other accessions hence grouped into own cluster (2). The existence of N4-1 cultivar in Nias Island revealed its unique genetic identity which may be explored thoroughly for any beneficial and tolerant traits to environmental changes.

In practical term, N4-1 may divert even more further than its members in the long term which can later be categorized as a new species or sub-species. Further investigations are needed to uncover this phenomenon since no comprehensive study on the botanical backgrounds or information of durian cultivars from Nias Island so far. The PCoA method has been used to discriminate many crop cultivars based on the RAPD database, such as Brazilian coffee plants (Sera et al., 2003), Indian coconut accessions (Upadhypay et al., 2004), Turkish marijuana accessions (Pinarkara et al., 2009), Thailand durian populations (Vanijajiva, 2011), durian populations from Indonesia (Santoso et al., 2016), and our study. Interestingly, Santoso et al. (2016) found that the Sumatra and Kalimantan durian cultivars were closely related based on the PCoA projection through the use of microsatellites as molecular markers. However, the study only used the accessions from the mainland of North Sumatra and still not incorporated the accessions from Nias Island. We assumed that more distinctive genotypes may be observed by studying the durian cultivars from Nias Island as evidenced from RAPD analysis in this study, but this will need a more comparative studies in the future.

Conclusion

Based on the genetic similarity and population genetic structure analysis, the study concluded that the local durian cultivars originating from Nias Island were highly diverse. The result of clustering analysis produced thirteen clusters with some admixtures between different geographical populations. Under population level, the North Nias and South Nias were closely related revealing a possible human factor in durian diversification in the level of varieties or cultivars. Based on PCoA projection, we further grouped the durian populations into five clusters with major accessions

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grouped into cluster (4) and (5). Moreover, N4-1 is the only cultivar placed in its own cluster (2) indicated a distinct genetic identity among cultivars. As a recommendation related to this study, we suggest that N4-1 may be possibly harnessed and studied for its beneficial genotypic and phenotypic agricultural traits such as its fruit quality and productivity with other characteristics in agronomical aspect. Therefore, a correlation study between agro-morphological and genetical traits from each cultivar must be assessed for a rapid identification of cultivars under practical terms in the future.

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SPATIAL STAND STRUCTURE ANALYSIS OF ULUDAĞ FIR FORESTS IN THE NORTHWEST OF TURKEY

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Abstract. Forest structural characteristics, which are one of the key determinants of species and habitat diversity, have also been used as a constitutive factor in sustainable forest management. This study aimed to analyse the spatial structure (Clark-Evans, segregation and species mingling) of pure fir (7 plots, 1000-1600 m a.s.l.), fir dominated (5 plots, 1000-1400 m a.s.l.) and fir-beech co-dominated (7 plots, 1000-1300 m a.s.l.) stands at the center and edge distribution of *Abies nordmanniana* ssp. *bornmuelleriana* in Sub-euxine zone of Euro-Siberian region of Turkey. The Shannon diversity of tree species was found significantly ($P < 0.01$) lower in the edge distribution (Bolu-Aladağ) (0.418 ± 0.081) compared to Yenice (0.824 ± 066). *Fagus orientalis* showed a clumped distribution in fir dominated stands where it is not competitive compared to *Abies nordmanniana* ssp. *bornmuelleriana* but it had a spatial association with other species in co-dominated stands. Light demanding deciduous trees such as *Quercus hartwissiana*, *Carpinus betulus* and *Populus tremula* were observed in pure groups, whereas *Pinus sylvestris* and *Pinus nigra* did not show a tendency towards clumping and had a spatial association with other species since they were located in upper stand layer. As shown in this study, spatial data in stand profiles provide new opportunities for understanding ecosystem structure and silvicultural treatments.
Keywords: *Euro-Siberian, mingling, aggregation index, beech, species segregation index, stand profile*

Introduction

Forest tree composition is shaped by competitive interactions among individuals in natural forests which determine the size and position of individual trees. These interactions among species are reflected in complex stand structures and highly diverse mixture of species (Hui et al., 2018). Specific structures generate particular processes of growth and regeneration, thus tree growth and the interactions between trees depend, to a large degree, on the structure of the forest (Forrester, 2019; Bastias et al., 2020). On the other hand, forest structure is not only the outcome of natural processes but is also determined to a considerable extent by silvicultural interventions (Gadow et al., 2012). Aguirre et al. (2003) define stand structure as the spatial distribution of the tree positions, the spatial mingling of the different tree species and the spatial arrangement of the tree dimensions. Spatial stand structure, which is an important facet of habitat diversity, is associated with ecological stability and species diversity (Neumann and Starlinger, 2001; Pommerening, 2002; Kint et al., 2003; Szmyt, 2012; Gao et al., 2014; Bastias et al., 2020). Therefore, forest ecosystem diversity does not only refer to species richness but to a range of phenomena that determine the heterogeneity within a community of trees, including the diversity of tree sizes and tree locations (Ni et al., 2014).

Forest stand structure is essential inputs for understanding ecosystem structure and functioning, and hence for sustainable forest management (Kint et al., 2003; Schall et al., 2018). For this reason, forest structural characteristics have been a key determinant in silvicultural methods (i.e. thinning methods). Therefore, the description of stand structural characteristics has been commonly undertaken in silvicultural researches.

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Forest stand structural characteristics have been traditionally illustrated using hand-drawn profiles based on actual measurements which show the horizontal and vertical projection of the stand (hereafter stand profile) (Çoban et al., 2016, 2018). Stand profiles, which are a depiction of a section through the forest, offer good opportunities for illustrating and analyzing horizontal and spatial forest structures (Nielsen and Nielsen, 2005). In Turkey, the earliest use of stand profiles in silvicultural researches dated back to 1970's (Aksoy, 1978; Bozkuş, 1986; Yönelli, 1986; Özalp, 1989; Ertaş, 1996; Çoban, 2013). Especially, stand profile measurements were carried out in phytosociological studies. These profiles were used to characterize vertical stand structure and tree size distributions. However, the spatial pattern of trees recorded and marked in the horizontal profile has not been evaluated so far. This spatial data provide a comprehensive description of the spatial structure of a forest using indices which have been developed in the past few decades in order to quantify neighborhood competition (Aguirre et al., 2003; Hui et al., 2018). In addition, determination of the spatial pattern of tree species in mixed stands is important in gaining a better understanding of the underlying ecological processes which will provide critical information about community structure and species coexistence (Ni et al., 2014).

In this study, it was aimed to determine spatial patterns of pure and mixed stands of fir in different site conditions. It was hypothesized that (1) tree species diversity of fir forests do not differ in the center and edge distributions in Sub-euxine zone of Euro-Siberian phytogeographic region (2) *Abies nordmanniana* (Stev.) Spach. subsp. *bornmuelleriana* (Mattf.) Coode & Cullen and *Fagus orientalis* Lipsky., which have similar shade tolerance, will have the same spatial pattern in all stand types.

Materials and methods

Site description

The study area is located in the northwest of Turkey, which is the main distribution area of *Abies nordmanniana* (Stev.) Spach. subsp. *bornmuelleriana*, hereafter will be referred as *Abies bornmuelleriana*. Stand profiles were selected from Karabük-Yenice (Çitdere) (Özalp, 1989) and Bolu-Aladağ forests (Aksoy et al., 2012) representing both center and edge distributions in Sub-euxine zone of Euro-Siberian phytogeographic region. Çitdere (6091 ha) is located in Yenice district of Karabük province which is in the western part of the Blacksea Region. It lies between 41°00'14" and 41°05'06"northern latitudes and 32°21'06" and 32°27'45" eastern longitudes. The altitude of the region ranges from 640 m a.s.l. to 1810 m a.s.l. Aladağ mountain (4502 ha) is situated between 31°33′30″ - 31°38′00″ eastern longitudes and 40°37′30″ - 40°41′30″ northern latitudes in the southern part of the Bolu province. The altitude of the mountain ranges from 750 m a.s.l. to 1840 m a.s.l. (*Fig. 1*).

The meteorological stations representing climate of the study areas are Karabük-Büyükdüz (1962-1970) and Bolu Şerif Yüksel Research Forests (Aladağ Mt.) (1975-1995). Mean annual precipitation and temperature were measured as 1371 mm and 6.2 °C at Büyükdüz station (1560 m), 882 mm and 5.7 °C at Bolu-Aladağ (1550 m).

Vegetation

The Euro-Siberian phytogeographical region was subdivided into Euxine, Sub-euxine and Xero-euxine zones from north to south (Zohary, 1973) since mountain

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ranges create varied climate characteristics. The Euxine zone is characterized by deciduous and mixed forests from the colline to submontane zone and various mixture combinations of *Abies bornmülleriana-Fagus orientalis* forest in the montane zone. The Sub-euxine zone is characterized by mixed *Carpinus-Quercus* and *Pinus nigra* forests and pure coniferous forest consist of *A. bornmülleriana* on northern slopes and *Pinus sylvestris* on southern slopes in the montane belt (above 1200/1300 m) (Akman, 1995; Mayer and Aksoy, 1998). The Xero-euxine zone surrounds Central Anatolia and characterized by forest-steppe vegetation with *Quercus pubescens* as the main tree (Zohary, 1973; Mayer and Aksoy, 1998; Kurt et al., 2006).

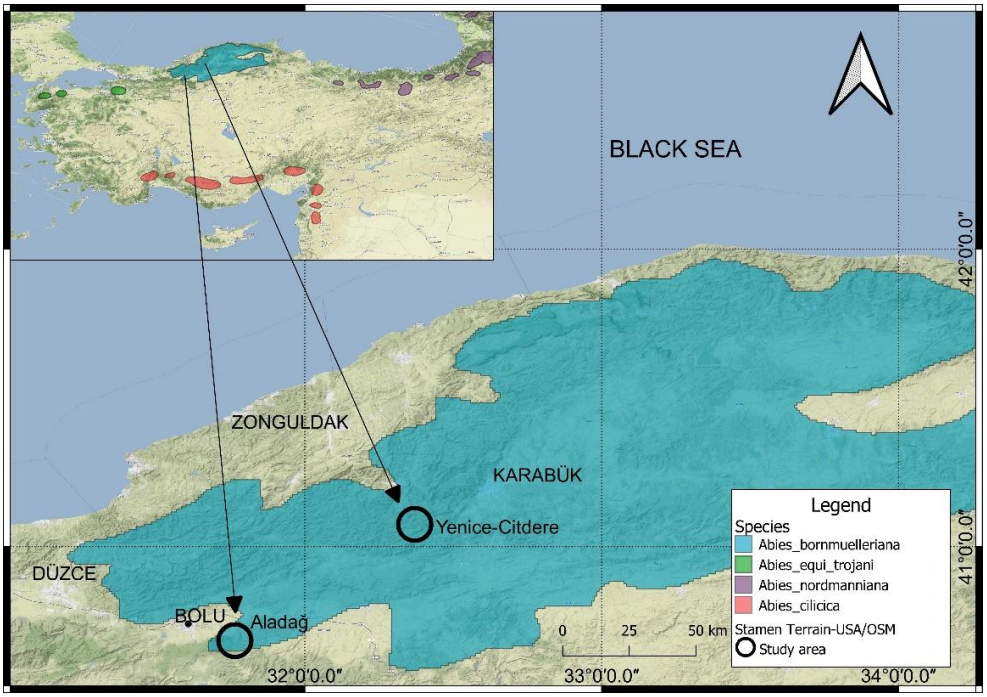

Figure 1. *Location of the study area (species distributions were taken from Alizoti et al., 2011)*

According to Çoban and Willner (2019), *Abies bornmuelleriana* dominated forests were classified under the alliance of *Lonicero caucasicae-Piceion orientalis* Çoban et Willner 2009, the order *Abieti nordmannianae-Piceetalia orientalis* Çoban et Willner 2019 and the class *Vaccinio-Piceetea* Br.-Bl. in Br.-Bl. et al. 1939. Mesic beech and fir-beech forests under the alliance *Fagion orientalis* Soó 1964, the order *Rhododendro pontici-Fagetalia orientalis* Passarge 1981 and the class *Carpino-Fagetea sylvaticae* Jakucs ex Passarge 1968.

Data preparation

Tree spatial data was taken from the original stand profiles of Özalp (1989) and Aksoy et al. (2012) which were used to characterize the stand structure of forest communities. Field sampling of the latter study was carried out in 1978 and vegetation data was published in 2012. Stand profiles are based on actual measurements of the positions, heights, heights of the lowest branches, and the diameters at breast height of trees over 5 m (Aksoy, 1978). In each diagram, tree positions, which were shown on millimeter papers at scale 1:200 for the whole plot, were measured with respect to a

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Cartesian coordinate system. Stand profiles where Abies bornmuelleriana had a relative species per hectare (SPH) of at least 20% were included in the analysis. As a result, 19 stand profiles were obtained which were further grouped into pure fir (>85% fir), fir dominated (70-85% fir) and fir-beech co-dominated (20-70% fir) stands. The classification of the available data resulted in 7 stand profiles in both pure fir and fir-beech co-dominated stands and 5 stand profiles in fir dominated stands (*Table 1*).

Table 1. *Details of sample plots and their classification (species less than 10% of relative species per hectar (SPH) in any of the plots were not shown)*

Plot	Group	Altitude (m)	Aspect	Slope (°)	*Abies bornmuelleriana*		*Fagus orientalis*		*Carpinus betulus*		
					SPH	Rel. freq.	SPH	Rel. freq.	SPH	Rel. freq.	Total SPH
A1	Pure fir	1120	NNW	22	500	86	80	14			580
A4		1030	WNW	17	820	93					820
A5		1600	NNW	6	700	100					760
A6		1200	N	19	460	88	60	12			520
A7		980	SSE	7	660	92					720
A8		1510	NNE	23	580	97	20	3			600
A9		1060	S	8	840	95					880
C6	Fir dominated	1120	NNW	13	680	83	140	17			820
C7		1350	SSW	32	1220	72	160	9	180	11	1700
A2		1300	NNW	27	460	74	160	26			620
A3		980	NNW	31	620	72	120	14	60	7	860
A10		1400	NNE	40	840	81	160	15			1040
C1	Fir-beech co-dominated	1120	ENE	13	240	48	220	44			500
C2		1030	SSE	21	340	31	640	58	40	4	1100
C3		1180	NNW	17	360	44	460	56			820
C4		1400	NNW	20	180	20	580	74			780
C5		1250	NE	27	320	52	260	41			620
C8		1090	NNE	6	500	54	400	43			920
C9		1300	NNW	10	529	35	784	51			1529

Pure forests occurred between 1000 and 1600 m a.s.l., fir dominated forests 1000-1400 m a.s.l. and fir beech co-dominated forest between 1000 and 1300 m a.s.l. Pure forests were also found on steeper slopes compared to other units (*Fig. 2*).

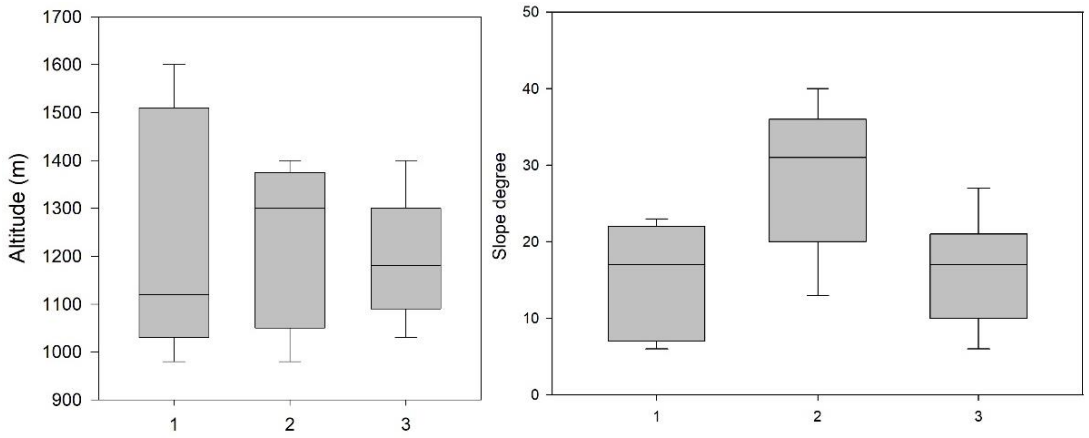

Figure 2. *Altitude and slope ranges of forest types (1: Pure fir, 2: Fir dominated, 3: Fir beech co-dominated)*

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Computed indices

In order to analysis spatial structure of the stands, following distance-dependent indices, also known as spatially explicit indices were used.

Species mingling (M_i)

Mingling (M_i) is defined as the proportion of neighbours which do not belong to the same species as the reference tree (Gadow, 1993; Füldner, 1995). The distribution of the M_i values, in conjunction with the species proportions within a given tree population, allows a detailed study of the spatial diversity within a forest. With four neighbours, indices have five possible values: 0.00, 0.25, 0.50, 0.75 and 1.00. While higher values show different species intermingled, small values indicate pure groups. For instance, a value of Mi = 0 is obtained if the neighbouring species belong to the same species, whereas Mi = 1 when all neighbouring trees are different than reference tree (*Eq. 1*).

$$M_i = \frac{1}{n}\sum_{j=1}^{n} v_{ij} \qquad \text{(Eq.1)}$$

where n is the number of the nearest neighbours, $v_{ij} = 1$ if the $species_j \neq species_i$ and $v_{ij} = 0$ otherwise.

Aggregation index of Clark-Evans (CE)

Clark-Evans index (CE) expresses the extent to which a forest deviates from the stands with a complete randomised spatial distribution of trees (*Eq. 2*). It uses distances between nearest neighbours and it is measured for all individuals located on the plot (Clark and Evans, 1954). The values are restricted to the range between 0 and 2.15 and values lower than 1 indicate clumped distribution, while regularity is assumed if the values are over 1. While complete regular hexagonal distribution results in the highest index of 2.15, random distribution equals to 1 (Neumann and Starlinger, 2001).

$$CE = \frac{1}{n}\sum_{i}^{n} r_i 2\sqrt{p} \qquad \text{(Eq.2)}$$

where r_i is the distance from one tree to his next neighbour and r is density of tree per square meter.

Species segregation index (S)

The Pielou Index (S) (Pielou, 1977) compares the observed number of mixed pairs with the one expected under random conditions, independently of their spatial pattern (*Eq. 3*). Values of the S index vary between −1 and 1. If $0 < S < 1$ indicates that the nearest neighbours are always same species (spatial separation or segregation of species) and if $-1 < S < 0$, spatial association between two species is observed (Kint et al., 2003). In the plots more than two species, segregation index is calculated by comparing one species against all others.

$$S = 1 - \frac{N.(b+c)}{m.s+n.r} \qquad \text{(Eq.3)}$$

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N is the numbers of all pairs of trees, m and n are the number of individuals of A and B respectively, r and s are the number of times species A and B are found as the nearest neighbours of a reference tree (Pommerening, 2002).

Species related Shannon index

The Shannon index (Shannon and Weaver, 1949) which describes species diversity was calculated based on SPH. The Shannon index takes the relative abundance of different species into account rather than simply expressing species richness. The Shannon index is affected by both the number of species and their evenness.

$$H = -\sum_{j=1}^{n} p_j . \ln\left(p_j\right) \qquad \text{(Eq.4)}$$

where p_j is relative frequency of tree species and n is number of tree species.

All of the values mentioned above were calculated using CRANCOD 1.4 (Pommerening, 2012) and stand profile figures were produced using the package ggplot2 (Wickham, 2009). Statistical significiance of groups in terms of measured parameters was tested using Systat SigmaPlot (SigmaPlot 14 trial version, www.systatsoftware.com). When the Shapiro-Wilk test showed the homogeneity and Brown-Forsythe test Equal Variances ($p > 0.05$), Student's t-test was applied to the data. Pearson Product Moment Correlation was used to measure the strength of association between pairs of variables.

Results

Tree species diversity

Fir and beech mixed forests of Yenice were found to have significantly ($P < 0.01$) higher diversity of tree species (0.824 ± 066) compared to Aladağ (0.418 ± 0.081). There was not a statistically significant difference between fir dominated (0.702 ± 0.103) and fir-beech co-dominated stands (0.798 ± 0.065). *Fagus orientalis* was the most common tree species in the mixture in both regions. Species such as *Carpinus betulus, Pinus sylvestris, Pinus nigra, Taxus baccata, Sorbus torminalis, Populus tremula* and *Quercus hartwissiana* had a scattering occurrence in the mixtures (less than 10% relative SPH). *Quercus hartwissiana* joined stand mixture only in Yenice region. Also, tree species diversity decreased with the ratio of fir ($r(18) = -0.69$, $p < 0.01$) (*Table 2*).

Spatial structure of stands

Mean distance to the first neighbour of all trees was 2.06 ± 0.16, 1.88 ± 0.19 and 2.07 ± 0.16 for pure fir, fir dominated and fir-beech co-dominated stands. When segregation of species was compared among stand types, there was a segregation ($S > 0$) between accompanying species in fir dominated stands (*Fig. 4c*). This means low numbers of mix pairs of the nearest neighbours. On the other hand, low spatial associations both on plot and species level were found in the co-dominated and pure stands except for plots C3 and C9 (*Figs. 3* and *4b, d*). Unlikely, *Abies bornmuelleriana* spatially associated with other species whereas *Fagus orientalis* segregated in the plot C2 (*Fig. 4a*) where *Carpinus betulus* and *Quercus hartwissiana* admix. In that plot,

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Fagus orientalis form pure groups. In general, light demanding coniferous trees (*Pinus nigra* and *Pinus sylvestris*) had spatial association with other species, whereas light demanding deciduous trees (*Quercus hartwissiana*, *Carpinus betulus* and *Populus tremula*) highly segregated and clumped. A positive correlation was found between altitude and segregation index ($r(19) = 0.58$, $p < 0.01$) means that a spatial association and low mixtures occur among different species at higher altitudes.

Clark-Evans Aggregation index at plot level showed a regular distribution (CE > 1) of trees except for plots A8, C2 and C9 (*Figs. 3* and *4a, d, e*) which have a low clumping trend. However, when species were evaluated separately, all of the species showed a clumping distribution except *Abies bornmuelleriana.* It had a low clumping only in fir-beech co-dominated stands where *Fagus orientalis* had also similar pattern. Other trees were much more clumped compared to main dominant species. Increasing fir ratio caused a regular distribution for *Abies bornmuelleriana* and a strong clumping distribution of *Fagus orientalis* in fir dominated stands except for plot A10 (located on a steep slope at 1400 m) where *Abies bornmuelleriana* also had a low clumping degree (*Fig. 3*). A negative correlation was found between fir ratio and CE index of Beech ($r(15) = -0.70$, $p < 0.01$) showing clumping distribution pattern in fir dominated stand. Besides, increasing altitude caused a significant decrease in beech CE index ($r(15) = -0.52$, $p < 0.05$).

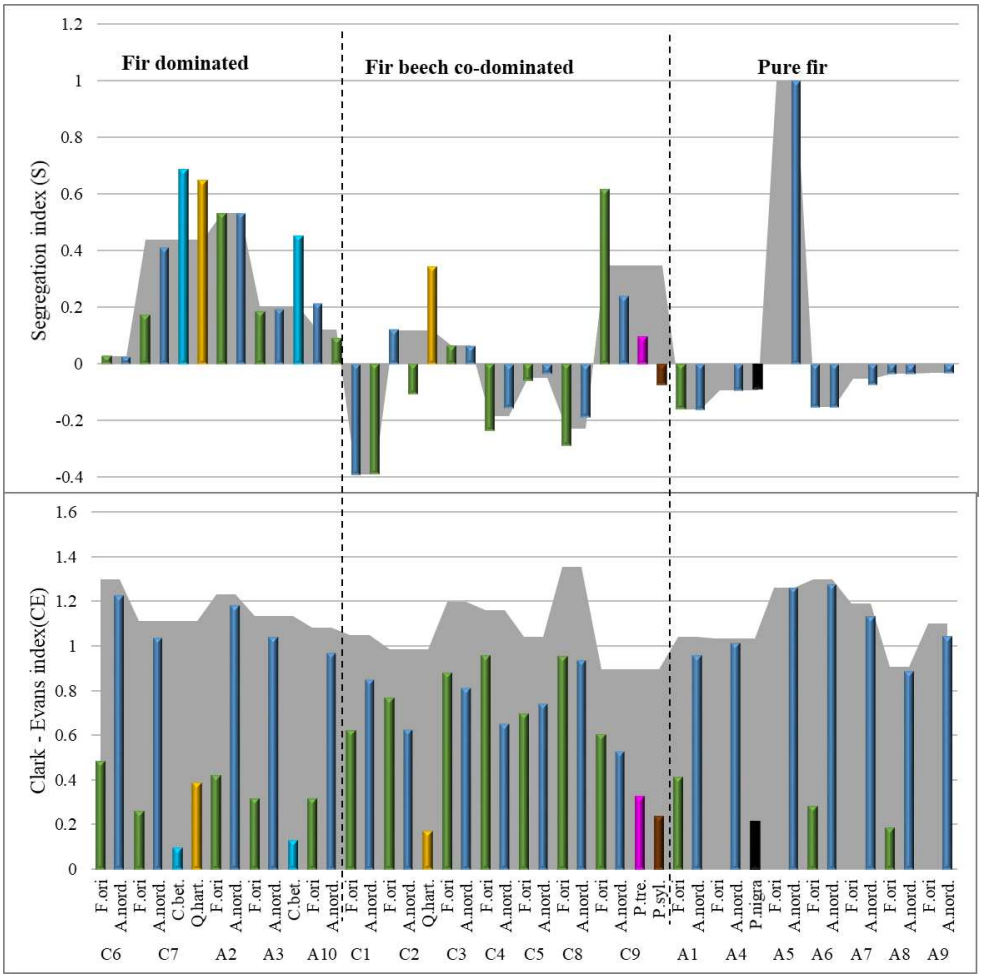

Figure 3. *Species segregation (S) and Clark-Evans aggregation (CE) index in plots (gray area represent plot mean; F.ori: Fagus orientalis, A.nord.: Abies bornmuelleriana, C.bet.: Carpinus betulus, Q.hart: Quercus hartwissiana, P.trem.: Populus tremula, P.syl: Pinus sylvestris)*

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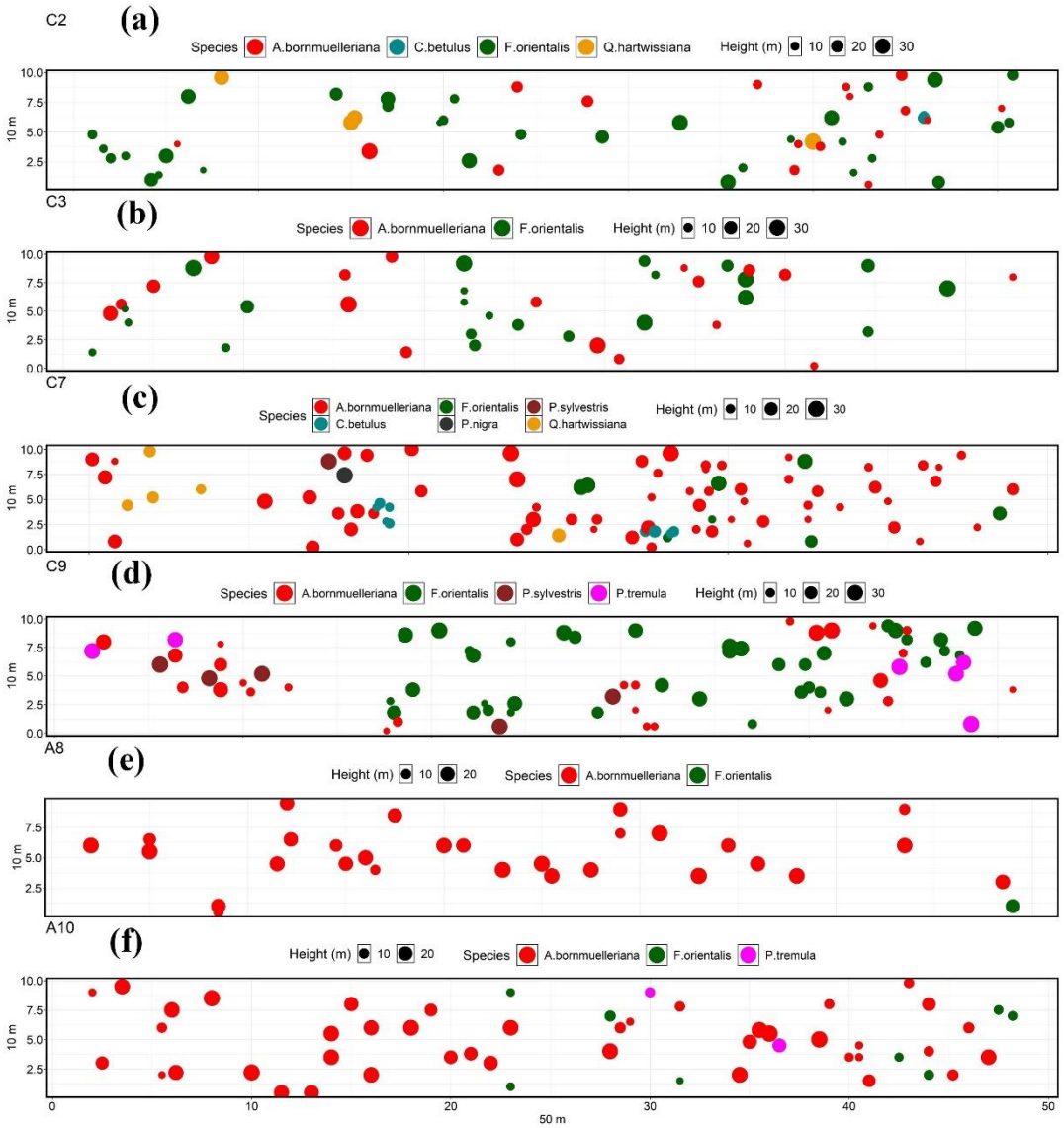

Figure 4. *Some of the horizontal stand profiles mentioned in the text (sizes of the circles represent tree heights)*

The Mi distribution clearly reveals that *Abies bornmuelleriana* and *Fagus orientalis* have an opposite behaviour in fir dominated and co-dominated stands. *Abies bornmuelleriana* does not form pure groups in fir-beech co-dominated stands where it occurs from low to complete mixtures, but *Fagus orientalis* can also occur in pure groups (17-40 %) (*Figs. 4a, b, d* and *5*). On the other hand, *Abies bornmuelleriana* predominantly occurs either in pure groups (20-60%) or low to medium mixtures in fir dominated stands. Here, *Fagus orientalis* occurs from medium to complete mixtures with other deciduous trees but not in pure groups (*Figs. 4c, f* and *5*). In addition, correlation analysis showed a negative correlation between fir ratio and species Mi ($r(18) = -0.80$, $p < 0.001$) and Shannon diversity ($r(18) = -0.69$, $p < 0.01$) which means that abundant fir ratio decrease mixture and tree species diversity (*Table 2*). However, increasing beech ratio causes a regular distribution of beech ($r(15) = 0.69$, $p < 0.01$) and high mingling of species ($r(15) = 0.66$, $p < 0.01$) in the stand.

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Pinus sylvestris is surrounded by mostly different species in line with CE and S indexes. Since small clusters or individuals of species (i.e. *Carpinus betulus* and *Quercus hartwissiana*) were always surrounded by different species, Mi index does not reveal pure groups of species, instead a low mingling value (*Figs. 4c* and *5*). However, using CE, S and Mi indexes give detailed information about the spatial distribution of all species.

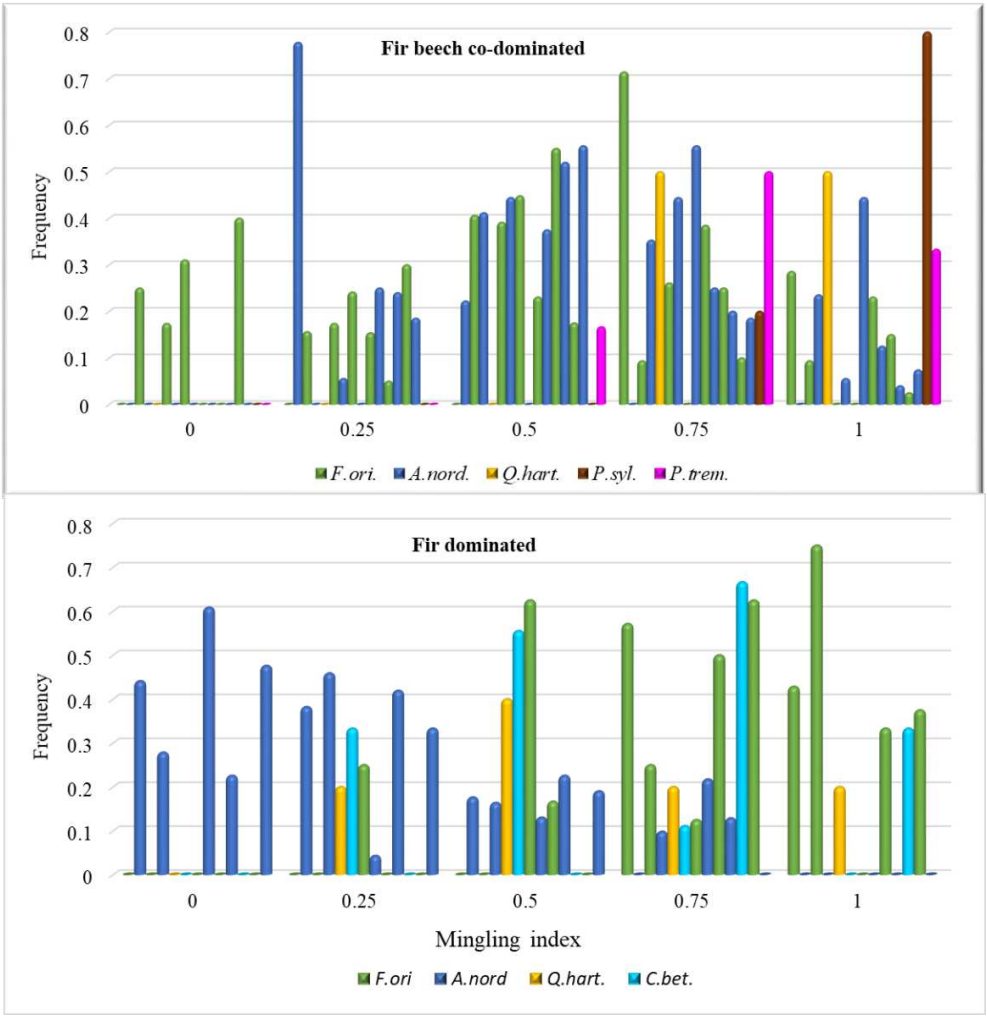

Figure 5. *Species mingling (Mi) index of species in fir beech co-dominated and fir dominated stands (F.ori: Fagus orientalis, A.nord.: Abies bornmuelleriana, P.syl: Pinus sylvestris, P.trem.: Populus tremula, Q.hart: Quercus hartwissiana, C.bet.: Carpinus betulus)*

Table 2. *Pearson product moment correlation between indices and other parameters (*P < 0.05, **p < 0.01, ***P < 0.001; CE: aggregation index of Clark-Evans, S: species segregation index, Mi: species mingling)*

	Fir ratio	Beech ratio	CE)	S	Mi	Beech CE	Fir CE	Beech Mi	Fir Mi	Shannon index
Altitude	0.06	-0.13	-0.09	0.58**	-0.027	-0.52*	0.13	-0.21	0.21	0.09
Slope (°)	-.035	-0.452	-0.265	0.042	0.083	-0.267	-0.247	0.389	-0.016	0.19
Fir ratio			0.18	0.31	-.80***	-0.70**	0.63**	0.86***	-.97***	-.69**
Beech ratio			-0.05	-0.34	0.66**	0.69**	-0.41	-0.87***	0.93***	0.40

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Discussion and conclusions

Northern Anatolia exhibits a great variety of forest types composed of many tree species owing to differing edaphic, climatic and topographical factors (Çoban and Willner, 2019). Mountain ranges extending east-west direction cause both altitudinal and horizontal differences in forest communities. For instance, the isolation effect of high mountains increases continental effect which is reflected in the dominance of coniferous forest at the expense of deciduous trees towards inland (e.g. Aladağ Mountain). For this reason, deciduous mixed forests are mostly replaced by pure or fir dominated montane coniferous forests on north facing slopes, while southern slopes are dominated by *Pinus sylvestris* dominated forests in Sub-euxine zone (Aydın et al., 2008). The current study showed a significantly lower tree species richness in fir and beech forests of Aladağ mountain (0.418 ± 0.081) compared to Yenice (0.824 ± 066). Accordingly, tree species richness of Yenice forest was given as 24 containing 20 deciduous trees (Özalp, 1989) and 15 containing 12 deciduous trees on Aladağ (Aksoy et al., 2012). This reveals a decrease in the numbers of tree species in forest composition from north to south due to decreasing annual temperature and precipitation. Thus, the first hypothesis was not supported by the results of this study since tree species diversity was significantly lower in fir stands of Aladağ mountain located in the edge of sub-euxine zone. However, species richness is only one component of forest biodiversity which comprises genetic and ecosystem diversity (Simberloff, 1999). According to Pommerening (2002), spatial stand structure, which is an important factor in determining habitat and species diversity, is linked to the ecological stability of forests. For this reason, determination of forest structural attributes are suggested for an effective forest management (Latham et al., 1998; Ehbrecht et al., 2017; Vilà-Cabrera et al., 2018; Põldveer et al., 2020).

Spatial pattern is a matter of concern within silviculture since regular distribution provides maximal space and optimal growing conditions, while a clustered distibution causes increment loss (Pretzsch, 1995; Neumann and Starlinger, 2001; Del Río et al., 2016). Spatial distribution of tree species in mixed forest is determined by many factors related to habitat heterogeneity and environmental requirements of species. Besides, species with differing tolerances to environmental factors are confined to their own niche which causes clumped distribution (Pielou, 1961). Segregation between two species occurs when one species is more likely to be found near its own species. According to Pielou (1961), if both species are aggregated it does not necessarily follow that they will be segregated from each other. Thus, one of the species may have clumped pattern whereas other has regular pattern also means segregated distribution. Therefore, segregated distribution reveals that at least one of the species has been effected from habitat patchiness or familial clumping. Segregated and aggregated stands present group mixture of two species (Pielou, 1961). This situation was revealed with the comparison of CE and S indexes in the fir dominated forest where clumped pattern of *Fagus orientalis* (Beech $CE < 1$) and regular pattern of *Abies bornmuelleriana* (Fir $CE > 1$) revealed a segregated pattern between two species (*Figs. 3* and *4f*). Similarly, *Quercus hartwissiana, Populus tremula* and *Carpinus betulus* had clumping pattern with segregated and clumping distribution ($S > 1$, $CE < 1$). On the other hand, clumped and unsegregated distribution of both species in fir-beech co-dominated forest means more or less single mixtures of both species. According to Neumann and Starlinger (2001), both CE and S are describing microstructure by focusing on the distances between single trees or sample points to trees, respectively. Besides, mingling index

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describes mutual positioning of different species or intermingling (Szmyt, 2012; Põldveer et al., 2020). In this study, differing spatial patterns were determined among species. *Fagus orientalis*, which is the most common tree species co-occuring with *Abies bornmuelleriana*, had both regular and clumping distribution depending on the stand type. In contrast, a regular distribution of *Abies bornmuelleriana* was found in pure fir and fir dominated stands which were also supported with the correlation between fir ratio and fir CE ($r(18) = 0.63$, $p < 0.01$). Such variations might be caused by both habitat heterogeneity and silvicultural characteristics of the species. Petritan et al. (2015) explained the regular distribution of fir overstorey trees by their smaller crown radii compared to beech and the different crown shape, which allow them to grow closer together. On the other hand, *Abies bornmuelleriana* and *Fagus orientalis* are characterised as shade-tolerant species and can thrive under sheltered conditions for a long time. However, fir has a lower assimilation capacity which reflects its greater shade tolerance and ability to grow assimilation tissue under lower lights conditions than beech. For this reason, beech reacts much faster to changes in light intensity and adapts better in gap openings (Čater and Levanič, 2013). Besides, the behaviour is not totally fixed and shade tolerance within species may be affected by site quality (Carter and Klinka, 1992). Shade tolerance of both dominant species provided a high degree of spatial mingling in fir-beech co-dominated forest. *Fagus orientalis* showed segregation and low mingling values in fir dominated forest but the spatial association with other species in fir-beech co-dominated stands. This might be caused by habitat heterogeneity or beech are not so competitive in fir dominated sites where it can only thrive in groups. Özel and Ertekin (2012) indicate that the mixture rates of fir increase depending on altitude in NW Anatolia. Hence, *Fagus orientalis* is confined to the most suitable niches as determined by the correlation between altitude with CE of beech ($r(15) = -0.52$, $p < 0.05$) and with S ($r(19) = 0.58$, $p < 0.01$) which indicate increasing clumping pattern at higher altitudes. Bulušek et al. (2016) indicated that clumped spatial pattern of beech was positively influenced by increasing altitude, extreme conditions of the site as well as by stand density in European beech (*Fagus sylvatica* L.) stands. Thus, the second hypothesis was not supported by the results of this study since the spatial pattern of *Fagus orientalis* does not solely depend on shade tolerance but site condition or stand type which effect its competition capacity.

The main differences in the spatial pattern were determined between light demanding deciduous trees and shade tolerant species. In stands dominated by both shade tolerant species, light demanding deciduous trees such as *Quercus hartwissiana*, *Carpinus betulus* and *Populus tremula* were observed in pure groups. This finding was also supported by Petritan et al. (2012) who highlighted that oak in beech dominated stand has a greater chance of survival when co-occuring in groups. In contrast, light demanding *Pinus sylvestris* and *Pinus nigra* did not show a tendency towards clumping and had a spatial association with other species. Because, these species always situated within the overstorey layer and excluded from suppress of shade tolerant species such as *Abies bornmuelleriana* and *Fagus orientalis*. Indeed, *Pinus sylvestris* and *Pinus nigra* situated in the upper stand layer in all stand types (Özalp, 1989). In addition, pioneer characters of these species allowed early establishment in the successional stage. As a result, light demanding coniferous trees which showed a random distribution and spatial association with other species. Whereas other light demanding deciduous trees showed segregated and clumped distribution.

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In conclusion, the diversity of spatial patterns in pure and mixed stands arise as a result of the species identity of dominant species (Petritan et al., 2015) and habitat heterogeneity. However, species with identical characteristics can create varied patterns depending on the dominated species and site quality. In this respect, spatial and structural patterns of diverse forests must be determined for developing appropriate silvicultural methods. Stand profiles, which are based on actual field measurement, are useful tools for analysing both structural and spatial characteristics of the forest using spatial and structural indices. Especially, if the sizes (diameter and height) of each tree were given in the profile, competition between tree species can be calculated with different indices. Thus, as indicated by Kint et al. (2003), the impact of management and/or competition on stand structure can be evaluated detaily. Stand profiles, which were sampled from different forest types, provide new opportunities using spatial pattern analysis. According to Yılmaz et al. (2019), analysing the spatial structure of forests reveals competition relationships among trees which make valuable contributions to silvicultural treatments. Especially, detection of tree spatial patterns and structural attributes in a forest stand can provide critical information on occurring dynamics, and steer management decisions (Carrer et al., 2018). Therefore, old or new stand profiles can be used to develop appropriate silvicultural methods for various stand types using spatial pattern analysis. At this point, terrestrial laser scanner technology, which provides many individual tree attributes with high accuracy, can be used to obtain stand profile data (Yurtseven et al., 2019).

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DIFFERENT RESPONSE PATTERNS OF FISH FOREGUT AND HINDGUT MICROBIOTA TO HOST HABITATS AND GENOTYPES

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Abstract. Fish gut microbiota can be affected by factors such as diet, habitat, and genotype. However, whether the foregut and hindgut microbiota respond similarly to these factors is still ambiguous. Given the fact that foregut has more communication with fish habitat and food intake, we hypothesized that the foregut microbiota is possibly more affected by external factors (e.g., habitat), while the hindgut microbiota tends to be mainly influenced by the host itself (e.g., genotype). To test our hypothesis, the V4 region of the gut bacterial 16S rRNA gene was amplified and sequenced by the MiSeq platform. A total of 1,139,703 high-quality sequences and 7,698 OTUs (without singlets) were obtained. Results indicated that the major factors that affect the fish gut microbiota patterns were the gut sections and habitats, rather than fish species. The foregut microbiota was indeed more similar to the water microbiota than hindgut microbiota, whereas the hindgut microbiota between fish species showed relatively more differences than that of foregut microbiota. Therefore, foregut and hindgut microbiota exhibited different response patterns to the habitat environments and host genotypes. This finding extended our understanding of the maintenance mechanism of fish gut microbial biodiversity.
Keywords: *freshwater lake, gut microbiome, high-throughput sequencing, silver carp, bighead carp*

Introduction

Gut microbiota not only assist their host in digesting food, but also help host to resist the invasion of pathogens (Stevens and Hume, 1998; Mountfort et al., 2002; Saha et al., 2006; Nicholson et al., 2005, 2012; Mardinoglu et al., 2015; Macpherson et al., 2018; Martens et al., 2018). Increasing evidence indicated that different gut positions were colonized by different microbiota (Eckburg et al., 2005; Ni et al., 2014a). Although it has been confirmed that many factors, such as diet, genotype, geography, lifestyle, and development of host (Ni et al., 2014b; Lloyd-Price et al., 2016; Yadav et al., 2016; Yan et al., 2016; Li et al., 2017; Ding et al., 2018), cast the composition and metabolism of gut microbiota, the influence degrees of these factors on gut microbiota at different gut positions are still ambiguous. Considering gut dysbiosis closely relates to host diseases

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(Martin et al., 2009; Nicholson et al., 2012; Lloyd-Price et al., 2016; Xiang et al., 2018; Huang et al., 2018; Ni et al., 2019), elucidating the influence of external and internal factors on the composition of gut microbiota will promote artificial regulation to the composition of gut microbiota and also can help the host to prevent some diseases.

Silver carp (*Hypophthalmichthys molitrix*, M.) and bighead carp (*Hypophthalmichthys nobilis*, N.) are important players in the biological network of freshwater ecosystems (Ni and Jiang, 1954; Xie, 2003). They are also major targets of aquaculture and are key protein resources in China. Previous studies showed that these species have different feeding preferences, i.e., silver carp and bighead carp are prefer to filter-feeding the phytoplankton and zooplankton, respectively (Ni and Jiang, 1954; Chen, 1982). In addition, comparing with hindgut, the foregut has more connection with the water environment, and foregut microbiota are more likely to be influenced by habitat.

Therefore, we hypothesized that the fish foregut microbiota was more possibly affected by external factors such as host habitats and diets, while the hindgut microbiota tend to be mainly influenced by host genotypes. To test the hypothesis, 16S rRNA genes of the foregut and hindgut microbiota of silver carp and bighead carp collected from different habitats (lake and pond) were analyzed using high-throughput sequencing. The finding of the present study would extended our understanding of the maintenance mechanism of fish gut microbial biodiversity.

Materials and methods

Experimental design and sampling procedures

Silver carp and bighead carp were collected from three freshwater lakes, i.e., Shangshe Lake (S, 30°7′-30°9′ N, 114°11.5′-114°16.5′ E), Wuhu Lake (W, 30°47′-30°50′ N, 114°28′-114°33′ E), and Niushan Lake (N, 30°16′-30°22′ N, 114°27′-114°38′ E), and two freshwater ponds in China under different environmental conditions (*Fig. 1*). The experimental lakes are all shallow-water freshwater lakes in the middle reaches of the Yangtze River, with complex freshwater biological communities and high biological productivity. The lake is less polluted, and the utilization method is mainly fishery development. Niushan Lake belongs to the Liangzi Lake water system, with a water area of 40 km^2. The existing area of Shangshe Lake and Wuhu Lake is 11.9 km^2 and 21.2 km^2, respectively. The two ponds are artificially stocked and managed aquaculture water bodies which located on the south bank of Liangzi Lake (30°04'-30°20' N, 114°31'-114°42' E). Sampling was performed in May 2013. Three individuals of each species were randomly selected in each site with the exception of Niushan Lake, in which only one silver carp and three bighead carp were catched, and then immediately transported to the laboratory with in situ water. The fish body was cleaned with 70% alcohol and anatomized according to a previously described method (Ni et al., 2014b). About 0.5 g of the gut contents from the foregut and hindgut were aseptically extracted and then stored at -20 °C for downstream DNA extraction. Water samples from the three lakes and two ponds (i.e., Shangshe Lake-SW, Wuhu Lake-WW, Niushan Lake-NW, Pond I-PIW, and Pond II-PIIW) were also collected for environmental microbiota analysis. About 500 mL of each water was first filtered with a 1.2-μm glass-fiber (GF/C) filter and then with a 0.22-μm filter to collect microbes for DNA extraction.

All experiments involving animals were performed under the protocols approved by the Institutional Animal Care and Use Committee of Institute of Hydrobiology, Chinese Academy of Sciences (Approval ID: keshuizhuan 08529).

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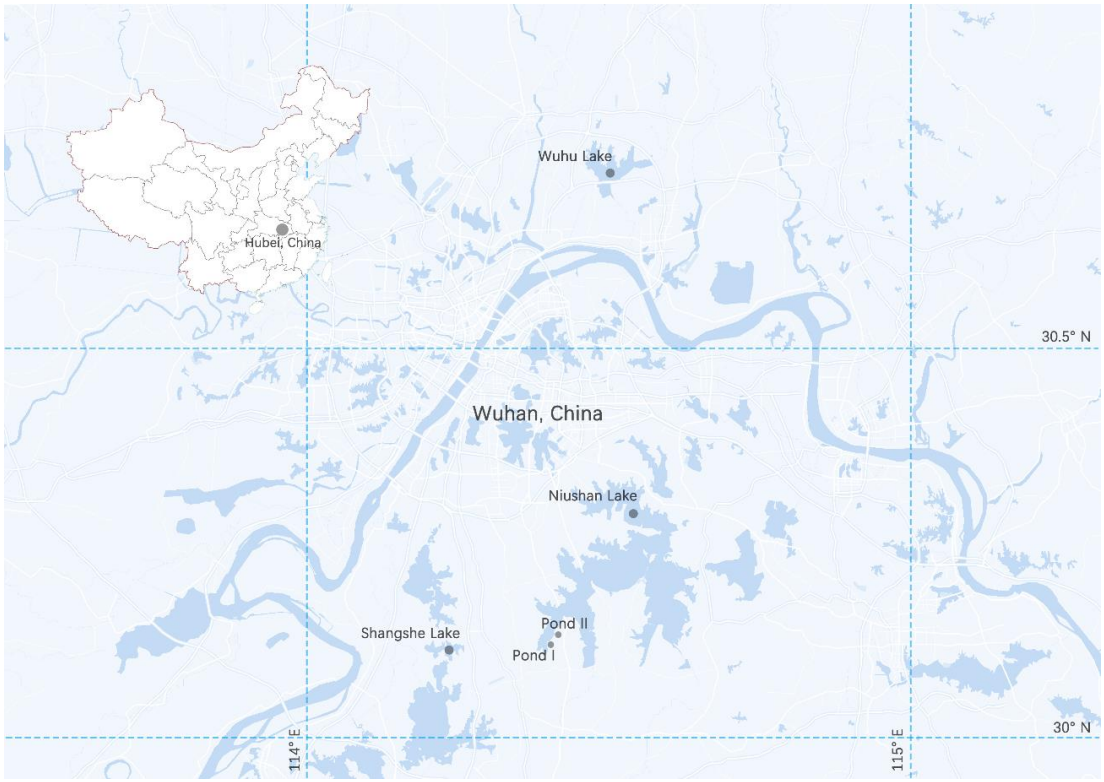

Figure 1. *Map shows the locations of sampling lakes and ponds*

DNA extraction and high-throughput sequencing

Genomic DNA of the gut microbiota was extracted from approximately 0.5 g of gut contents using a PowerFecal® DNA isolation kit (MoBio, CA, USA) according to the manufacturer's instructions. Genomic DNA of the habitat water microbiota was extracted from the filter membranes using the PowerFecal ® DNA isolation kit (MoBio, CA, USA) too. DNA concentration was determined by a NanoDrop ND-1000 and then diluted to the same concentration (2 ng/μL) for subsequent PCR amplification. The V4 region of the bacterial 16S rRNA gene was amplified using 515F and 806R primers to compare the gut microbiota as our previous description (Yan et al., 2016). The PCR products were visualized using 1% agarose gels stained with ethidium bromide. The successfully amplified PCR products were quantified using a PicoGreen dsDNA assay kit (Invitrogen, CA, USA). The products of each sample were equally combined and then subjected to gel purification. The purified DNA was re-quantified using the PicoGreen dsDNA assay kit, and the DNA library was applied for sequencing on the MiSeq platform according to the manufacturer's instructions.

The MiSeq reads were qualitatively filtered and processed with the Galaxy pipeline (http://zhoulab5.rccc.ou.edu:8080/root). After trimming the primer and deleting the sequences that contain N, the sequences with lengths of 245-260 bp were retained for subsequent analysis. The OTUs were generated by UCLUST clustering method with 97% sequence similarity. Then singlets were removed from further analysis. To exclude the interference of sequencing depth on the absolute abundance of each OTU, the relative abundance of each OTU was used in further analysis.

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Data analysis

The UPGMA clustering was performed using the software XLSTAT 7.5.2. Redundancy analysis (RDA), principal component analysis (PCA), and non-parametric multivariate analysis of variance (PERMANOVA) were conducted using vegan package (Dixon, 2003) in R version 3.0.1 (R Core Team, 2017).

Results

A total of 1,139,703 (18684 ± 3720, mean ± S.E.) high-quality sequences and 7,698 OTUs (619 ± 360) without singlets were obtained from the foregut and hindgut samples of silver carp and bighead carp, and the habitat water. Excepting a small part (2.86 ± 3.29%) of sequences could not be classified into any phylum, a total of 7,667 bacteria OTUs were categorized into 23 phyla, whereas 9 OTUs were attributed to archaea (Euryarchaeota or Crenarchaeota). Bacteroidetes primarily appeared in hindgut microbiota of lake fishes. Cyanobacteria primarily appeared in the foregut microbiota of lake fishes and freshwater. Fusobacteria primarily appeared in gut microbiota of pond fishes and hindgut microbiota of lake fishes (*Fig. 2*). UPGMA clustering showed that freshwater microbiota embedded the branch of foregut microbiota (*Fig. 2*). This result showed that the freshwater microbiota were more similar with foregut microbiota of the fishes.

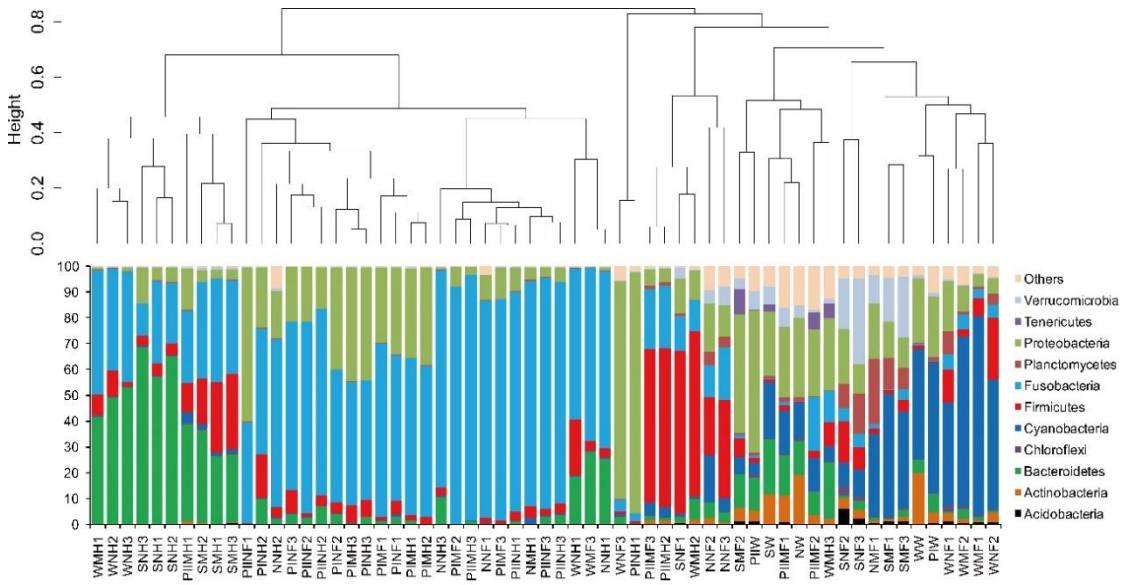

Figure 2. *Dominant phyla of gut and pond water microbiota. SM: the gut microbiota of silver carp collected from Shangshe Lake; SN: the gut microbiota of bighead carp collected from Shangshe Lake; WM: the gut microbiota of silver carp collected from Wuhu Lake; WN: the gut microbiota of bighead carp sampled from Wuhu Lake; NM: the gut microbiota of silver carp sampled from Niushan Lake; NN: the gut microbiota of bighead carp sampled from Niushan Lake. PIM: the gut microbiota of silver carp sampled from pond I; PIN: the gut microbiota of bighead carp sampled from pond I; PIIM: the gut microbiota of silver carp sampled from pond II; PIIN: the gut microbiota of bighead carp sampled from pond II. In the last lowercases of the sampled names, F denotes that the samples were obtained from the foreguts and H represents the samples from the hindguts*

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At OTU level, the result of RDA revealed that the gut microbiota clustered according to gut sections (i.e., foregut or hindgut) and habitats (i.e. lake or pond) rather than fish species (*Fig. 3A*). Although neither foregut nor hindgut microbiota of fishes were separated according to the fish species (MNAOVA, p = 0.24 for foregut; and p = 0.09 for hindgut), both foregut microbiota (MNAOVA, p = 0.005) and hindgut microbiota (MNAOVA, p = 0.005) were separated from each lake and each pond (*Fig. 3B,C*). Excluding the influence of habitat, only the foregut microbiota of Pond II fishes (MNAOVA, p = 0.005) and the hindgut microbiota of Shangshe Lake fishes (MNAOVA, p = 0.005) were significantly separated from silver carp and bighead carp. In addition, the hindgut microbiota of Wuhu Lake (MNAOVA, p = 0.06) and Pond I fishes (MNAOVA, p = 0.08) were significant differences between silver carp and bighead carp. These results implied that the hindgut microbiota easier separated according to fish species. For the fishes living in the lakes, their foregut tend to cluster together, whereas their hindgut tend to separated according to the fish species and lake (*Fig. 3D*). However, those trends was not true in the gut microbiota from pond fishes (*Fig. 3E*).

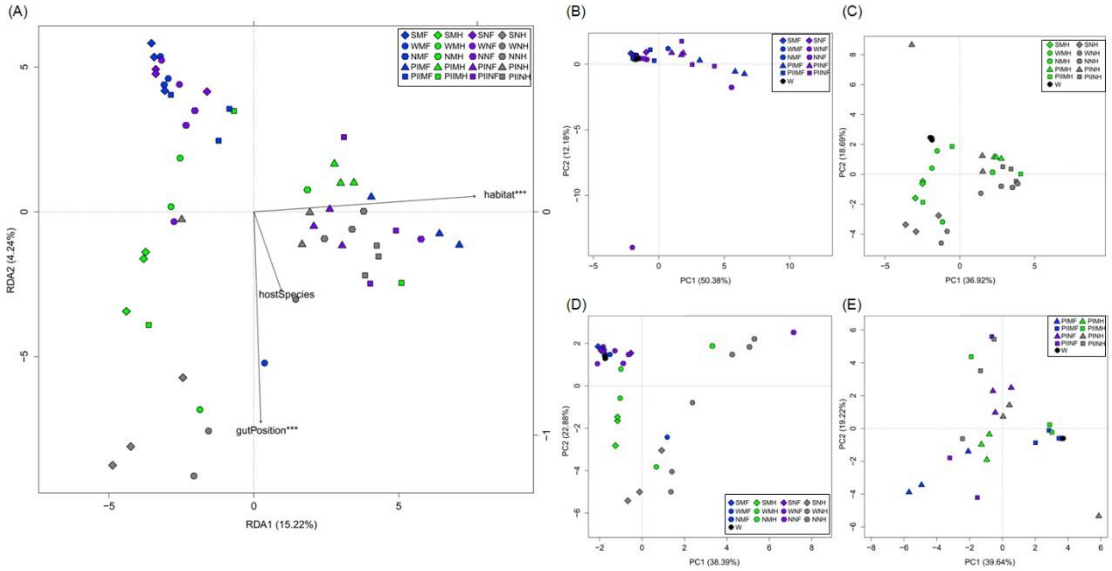

Figure 3. *RDA profiles of microbiota from fish guts and freshwater. SM: the gut microbiota of silver carp sample from Shangshe Lake; SN: the gut microbiota of bighead carp sample from Shangshe Lake; WM: the gut microbiota of silver carp sample from Wuhu Lake; WN: the gut microbiota of bighead carp sample from Wuhu Lake; NM: the gut microbiota of silver carp sample from Niushan Lake; NN: the gut microbiota of bighead carp sample from Niushan Lake. PIM: the gut microbiota of silver carp sampled from pond I; PIN: the gut microbiota of bighead carp sampled from pond I; PIIM: the gut microbiota of silver carp sampled from pond II; PIIN: the gut microbiota of bighead carp sampled from pond II. In the last lowercases of the sample names, F denotes that the samples were obtained from the foreguts and H represents the samples from the hindguts. W: the water microbiota of lakes and ponds. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$*

Heatmap and clustering based on the dominant OTUs compositions of microbiota revealed that almost all of the gut microbiota of pond fishes were clustered into a clade. However, the foregut microbiota of lake fishes were separated from hindgut microbiota, only with individual outliers (*Fig. 4*). This indicated that the gut microbiota differentiation

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of silver carp and bighead carp was decreased under artificial pond culturing condition. Similar to the UPGMA clustering based on the dominant phyla of the microbiota, water microbiota embedded the branch of foregut microbiota (*Fig. 4*). An OTU of Cetobacterium was enriched in the gut microbiota of pond fishes. However, other OTUs of Cetobacterium was enriched in the hindgut microbiota of lake fishes (*Fig. 4*).

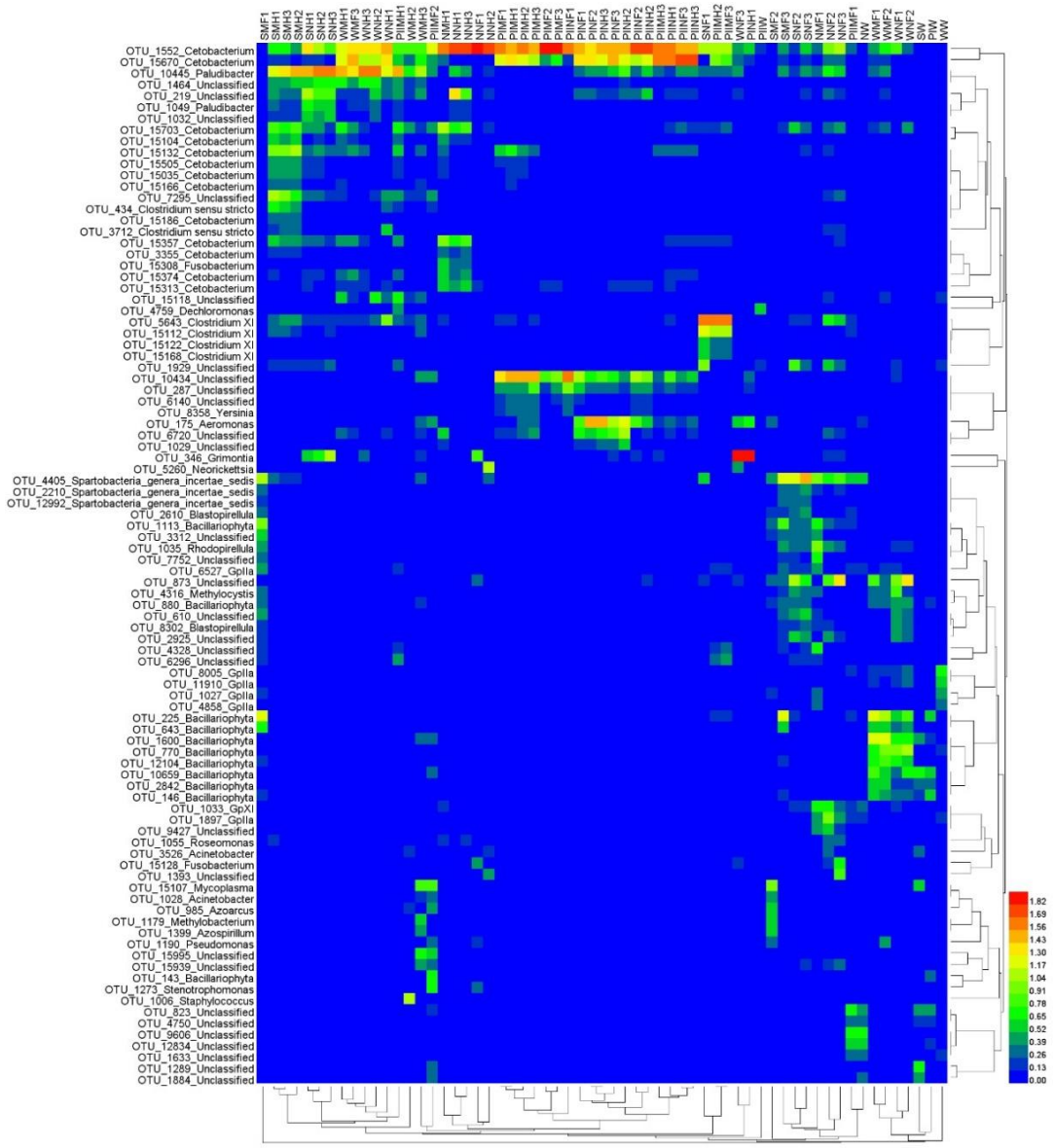

Figure 4. *Heatmap profile showed the changes of dominant OTUs. SM: the gut microbiota of silver carp sample from Shangshe Lake; SN: the gut microbiota of bighead carp sample from Shangshe Lake; WM: the gut microbiota of silver carp sample from Wuhu Lake; WN: the gut microbiota of bighead carp sample from Wuhu Lake; NM: the gut microbiota of silver carp sample from Niushan Lake; NN: the gut microbiota of bighead carp sample from Niushan Lake. PIM: the gut microbiota of silver carp sampled from pond I; PIN: the gut microbiota of bighead carp sampled from pond I; PIIM: the gut microbiota of silver carp sampled from pond II; PIIN: the gut microbiota of bighead carp sampled from pond II. In the last lowercases of the sample names, F denotes that the samples were obtained from the foreguts and H represents the samples from the hindguts*

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Discussion

Fish gut microbiota is significantly affected by various factors, such as gut position (Eckburg et al., 2005; Ni et al., 2014a), diet (Ley et al., 2008; Muegge et al., 2011), and habitat (Ni et al., 2014b). In the present study, we found the gut positions (foregut or hindgut) and host habitats (lake or pond) significantly affected the gut microbiota of silver carp and bighead carp (*Fig. 3A*). This indicated that microbiota formed independent communities in the foregut and hindgut of silver carp and bighead carp, it is consistent with previous studies (Eckburg et al., 2005; Ni et al., 2014a). The differences of microbiota at different gut positions probably caused by the differences of external and internal environments at different gut positions. The foregut is closer to fish mouth and its microbiota was probably easier impacted by external environmental factors of host, such as diet compositions, habitat environments, and some other unexpected factors. Simultaneously, the foregut microbiota would more similarly with habitat water microbiota, as shown in the present study (*Fig. 2* and *Fig. 4*). However, the hindgut is far from fish mouth and its microbiota was probably affected by internal factors, such as host health, and host genotype. Therefore, the hindgut microbiota was generally separated according to the fish species as showed in the present study (*Fig. 3B,C*).

Geographic isolation is the major factor that restricts the spread of microorganisms and causes the distance-decay relationship (Green et al., 2004; Green and Bohannan, 2006; Ni et al., 2014a). Geographic differences in gut microbiota were reported in human (Escobar et al., 2014; Shin et al., 2016; He et al., 2018) and fish (Ni et al., 2014b). Our results showed that the gut microbiota of the fishes differed from their living habitats. This result indicated that the effect of geographic isolation not only affect the freshwater microbiota, but also showed important impacts on the gut microbiota of fishes living in the freshwater.

Previous studies that used classical morphological methods to identify food contents indicated silver carp mainly feeds on phytoplankton, and bighead carp prefer to the zooplankton (Ni and Jiang, 1954; Chen, 1982). Host diet is an important factor that affects the compositions of gut microbiota (Heavey and Rowland, 1999; Savas et al., 2005; Ley et al., 2008; Ward et al., 2009; Muegge et al., 2011; Tims et al., 2011; Walker et al., 2011). Wu et al. (2012) found that the core bacteria of the herbivorous grass carp include Proteobacteria, Firmicutes, and Actinobacteria. Wu et al. (2010) indicated that the dominant phyla of the carnivorous yellow carp are Proteobacteria, Fusobacteria, and Bacteroidetes. Li et al. (2012) reported that the dominant phyla of omnivorous common carp are Proteobacteria and Fusobacteria. These studies implied that the dominant microbiota phyla are affected by host diet. However, the present study did not show feeding habit resulted in different hindgut microbiota between silver carp and bighead carp. This inconsistency may be caused by one or more factors, such as substantial overlapping of their feeding, and disturbance of other unexpected factors on gut microbiota.

Conclusion

In conclusion, gut sections (i.e., foregut or hindgut) and habitats (i.e., lake or pond) rather than fish species were the major factors that can significantly affeced the gut microbitoa of silver carp and bighead carp. The foregut microbiota would more similarly with habitat water microbiota, and the hindgut microbiota was easer separated according to the fish species than foregut microbiota. Therefore, there have different response

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patterns of foregut and hindgut microbiota to habitat environments and host genotypes. Although the finding extended our understanding of the maintenance mechanism of fish gut microbial diversity, the function of the microbial diversity and the function and inflencing factors of each component of the gut microbiota still need to further study.

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ALTERATION OF THE GUT MICROBIOME FOR PATIENTS WITH INFLAMMATORY BOWEL DISEASE: A REVIEW

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Abstract. Inflammatory bowel disease (IBD) is a set of multifactorial gut inflammatory conditions. The most common types of IBD are ulcerative colitis (UC) and Crohn's disease (CD), which are attributed to a deregulated immune response to an imbalance in the gut microbiome. The occurrence of IBD is increasing worldwide, with over one million people in the USA and 2.5 million in Europe estimated to have one form of the disease. Furthermore, an increase in IBD has recently been reported in industrialized countries in Asia, South America, Africa and the Middle East, which suggests that it has developed into a global disease with rising prevalence in each continent that may incur substantial healthcare costs in the future. Studying the gut microbiome of patients with IBD can provide a deeper understanding of the role that gut microbiota plays in the development of disease. This will further help in therapeutic microbiome manipulation of patients with IBD.
Keywords: *ulcerative colitis, Crohn's disease, immune response, intestinal microbiota, dysbiosis*

Introduction

Bacteria, viruses, fungi, protozoa, and many other microorganisms can live together as a community, but can also live in close association with humans, plants, and other complex organisms. This is possible through relationships of parasitism, commensalism, and mutualism between microorganisms and their hosts (Lederberg and McCray, 2001). The interacting community of microorganisms within or in proximity to multicellular organisms is referred to as a microbiome. The microbiome forms an ecological community of pathogenic, commensal, and symbiotic microorganisms in a biological environment, such as the human body. There is a strong mutual relationship between microbes and humans as the human body can benefit from microbe's ability to support the conditions necessary for human healthy life, while microbes benefit from

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obtaining needed resources of the human body. The nasal, skin, oral, urogenital, and gastrointestinal linings of an individual comprise unique and varying microbial communities. The human microbiome is a significant contributor to balanced immunogenicity through symbiosis. Alteration of the microbiome can result in the emergence of diseases. Thus, the condition of microbiomes is an essential effector on human health. Understanding the microbial compositions of healthy individuals is key to determining the influence of the microbiome on the occurrence of human disease.

Inflammatory bowel disease (IBD) and a number of other diseases have exhibited differences in the function and structure of gut microbiota. This can be observed in healthy humans, where changes in gut microbiota can result in a disturbance of the immune system. Over one million Americans and two million Europeans have been reported to suffer from relapsing-remitting forms of IBD (Kaplan, 2015). The data suggests an increase in IBD globally, but with the highest incidence and prevalence reported in Western countries. Nonetheless, the newly industrializing countries of Asia, South America, Africa, and the Middle East, have reported a rapid increase in IBD (Kaplan, 2015).

Lozupone et al. (2012) have characterized the pathophysiology of IBD as a reluctance to regulate immune response to imbalance in the gut microbiome. Therefore, compared to healthy individuals, patients with IBD have lower numbers of bacteria with anti-inflammatory capability and an increased number of bacteria with pro-inflammatory capability. Dysbiosis is currently considered the most appealing target for scientific research on IBD as it gives the opportunity for clinicians to intervene and alter the natural course of the disease. Given the large variation in gut microbiota across populations, the complex task of mapping the microbiota of healthy populations must be carried out. The microbiota of the diseased population was recently mapped though the Human Microbiome Project (HMP) run by NIH.

The Human Gut Microbiome

There are about a hundred trillion microbial organisms that comprise the human gut microbiota (Lozupone et al., 2012). These diverse organisms include viruses, protozoa, fungi and bacteria, which comprise more than 1000 different types of bacterial species (Honda and Littman, 2012) (*Fig. 1*). These bacterial species are made up of more than three million non-redundant microbial genes (Qin et al., 2010). A number of factors, including diet (Collado et al., 2010; Goldsmith and Sartor, 2014), age (Hopkins et al., 2001), gender, genetic composition (Khachatryan et al., 2008), geographic location (Sonnenburg et al., 2004), and health or disease status (Collado et al., 2010) of the individual influence the gut microbiota after birth. Four phyla (e.g., Bacteroidetes, Actinobacteria, Proteobacteria, Firmicutes) were found to dominate over 99% of human intestinal bacteria (Ley et al., 2008), of which two phyla (Firmicutes and Bacteroidetes) are most common in the intestinal bacteriome of healthy adults (Andoh, 2016). These microbes are densely populated in the colon and the distal ileum (Eckburg et al., 2005). The human gut hosts a large microbial community whose genetic content is described as a metagenome. The colonizers consist of a metagenome made up of 100 times the population of genes in the human genome. Dominguez-Bello et al. (2010) indicated that this metagenome is usually investigated by targeted sequencing of marker genes, including 16S ribosomal RNA.

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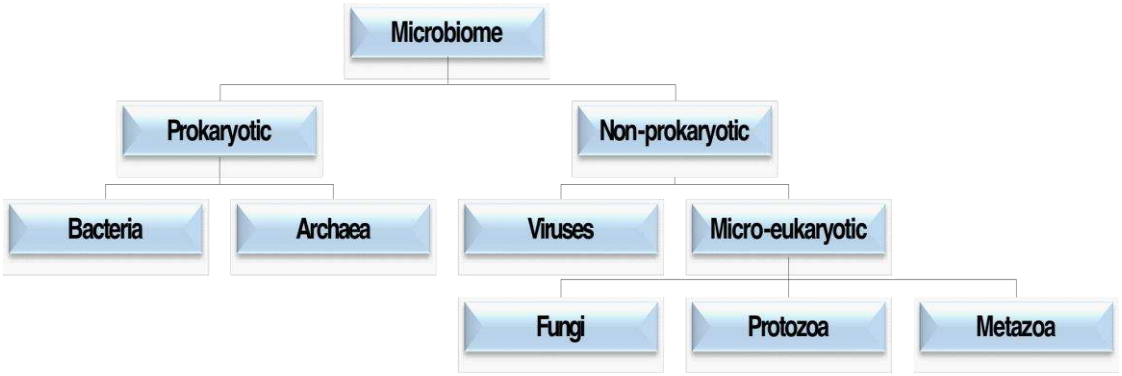

Figure 1. *Microorganisms of the human gut microbiome*

Development Process of the Human Microbiota

As a person ages, the microbiota varies in stability and diversity. Interactions between the gut microbiome and the host begin from birth and continue throughout life. In the early years of an individual, the density and stability of the microbiome are usually low (Claesson et al., 2011). By early adulthood, the microbiome begins to demonstrate significant diversity and stability (Lozupone et al., 2012), while the microbiome reaches maximum complexity and remains relatively stable throughout most of an adult's life. However, in later years and in the elderly, Biagi et al. (2010) note that the stability and diversity of the microbiome decreases.

The mode of birth highly determines the microbiota of an infant. In the case of vaginal birth, the microbiota of the infant takes after that of the mother. In a Caesarian birth, as Dominguez-Bello et al. (2010) pointed out that the microbiota in the skin of the newborn takes the characteristics of the mother. Feeding methods also affect the establishment of the microbiome; the microbiota of formula- and breast-fed newborns differ both in diversity and structure (Guaraldi and Salvatori, 2012). While the microbiota of breast-fed babies basically comprises Bifidobacteria, Penders et al. (2006) noted that microbiota of bottle-fed with formula babies basically consists of *Clostridium difficile, lactobacilli predominate, Bacteroides fragilis, and Escherichia coli*. At the age of three, a child's microbiome resembles that of an adult, and stabilizes with time. At this age, the number of species approximates 100, although this continues to increase and may exceed 1000 in adulthood. The ecological system of the gut also stabilizes and becomes more complex in adult life owing to the predominant composition of Firmicutes and Bacteroidetes (*Fig. 2*). However, old age causes a reduction in the stability of the ecosystem. Notably, the dominating phyla at this stage shifts from Firmicutes to Bacteroides, and the number of Bifidobacteria reduces while that of Proteobacteria rises. As an individual reaches old age, the microbiota shifts towards a *Clostridium*- dominated community, and the population of Bacteroidetes rises. This explains the significant difference between the microbiota of a young adult and that of an older person (Penders et al., 2006). Pregnancy enhances the development of Proteobacteria and Actinobacteria, as well as enhancing diversity. However, the gut microbiota gradually regains its original formulation after delivery.

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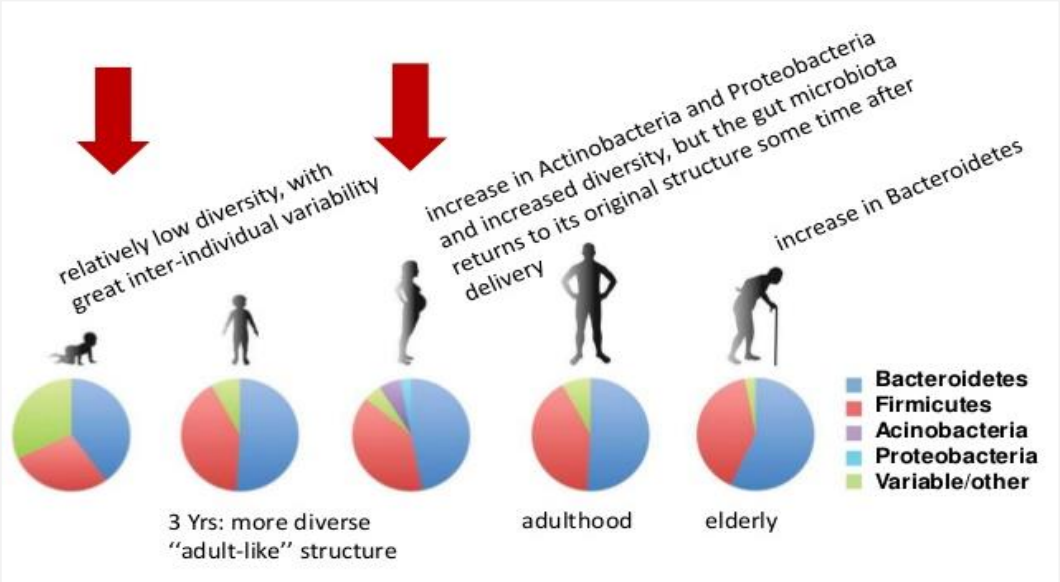

Figure 2. *The structure of the human intestinal microbiota across the life cycle. Modified from Kostic et al. (2013)*

Effects of Geology on Gut Microbial Assorted Variety and Stability

A geographical region can be associated with various ways of life that correspond with a variety of dietary practices (including such factors as country, city, countryside, religion, etc.). In the microbiota creation in volunteers from Venezuela, Malawi, and the US (0-70 years old), it was found that regardless of age, the microbiota structure could be categorized by country (Yatsunenko et al., 2012). The least microbially diverse group in that investigation was grown-up Americans of which the bacteria *Prevotella*, in particular, was underrepresented in this group. When comparing African and European children, De Filippo et al. (2010) discovered a greater number of *Prevotella* in African children. The fecal microbiota of African children is rich in Actinobacteria and Bacteroidetes, but has lower quantities of Firmicutes; European children have microbiota that are rich in Proteobacteria and over double the normal quantity of Firmicutes compared to Bacteroidetes. Moreover, Ou et al. (2013) noted a greater quantity of *Prevotella* in Africans in contrast to African Americans. A comparative study was carried out between the Tanzanian Hadza hunter-gatherers, and Italians (Schnorr et al., 2014). In a few African populaces in this examination, high levels of *Succinivibrio* and *Treponema* exist. These two microscopic organisms have a high fiber-debasing potential. This high potential may be predictable owing to a vigorously plant-based diet. Additionally, the Hadza gut microbial environment has relatively low levels of *Bifidobacterium.* This is thought to be the effect of the absence of dairy foods in the diet and lack of contact with domesticated animals.

Inflammatory Bowel Disease (IBD)

IBD is defined as a chronic intestinal inflammatory condition caused by host-microbe interactions in genetically susceptible individuals. The most common types of IBD are ulcerative colitis (UC) and Crohn's disease (CD), and the basic distinctions between them are location and severity. CD can affect any part of the digestive tract, but

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UC develops only in the colon and rectum. Patients with IBD often suffer from ongoing symptoms of abdominal pain, diarrhea, gastrointestinal bleeding, and malnutrition. Persistent disease activity has been linked to repeated hospitalizations and development of complications, such as the need for bowel resection, colorectal cancer, and mortality (Fakhoury et al., 2014). Both exogenous factors (abnormal microbiota) and host factors (intestinal epithelial cell barrier function, and innate and adaptive immune function) cause a chronic state of dysregulated mucosal immunity.

Global Incidence and Prevalence of Inflammatory Bowel Disease

The occurrence of IBD is increasing worldwide in the 21st Century. The prevalence of IBD varies with geographical region. The highest reported prevalence rates of the IBD are in Europe (UC, 505 per 1,000,000 persons/year; CD, 322 per 1,000,000 persons/year) (Ng et al., 2017). Regions with the highest annual incidence of IBD burden are Europe (UC, 24.3 per 100,000 persons/year; CD, 12.7 per 100,000 persons/year) and North America (UC, 19.2 per 100,000 persons/year; CD, 20.2 per 100,000 persons/year). The regions with the lowest reported IBD incidence are Asia and the Middle East (UC, 6.3 per 100,000 persons/year; CD, 5.0 per 100,000 persons/year) (Ng et al., 2017).

Urban areas may show a higher prevalence of CD than rural areas, and CD may also be higher in areas of higher socio-economic classes (Soon et al., 2012). The incidence of the disease begins to increase mostly among economically stable individuals. However, the ailment becomes more complex with time. An individual who initially belongs to low-incidence populations and migrates to a developed country before adolescence may show an increased prevalence of IBD. This is often the case with the first generation setting in a nation with increased IBD prevalence.

The "hygiene hypothesis" aims to explain the variation in incidence levels between developing and developed countries. According to this theory, an individual less exposed to childhood infections loses potentially beneficial organisms that enhance the development of a regulatory T cell. Additionally, such a person may not develop a strong immune repertoire because he or she has not encountered the noxious organisms (Pugazhendhi et al., 2011; Sood et al., 2014). Thus, such an individual might be more likely to develop IBD and other chronic immune diseases. It is often surmised that the emergence of IBD in Third World nations is a result of adoption of a Western lifestyle, including diet and the reliance on Western approaches to vaccination and medication. The significance of such developments in early life may be of particular significance. Though UC has been documented to have emerged before CD, the prevalence of CD in developed nations has overtaken that of UC in the past 20 years. However, developing nations have recorded an increasing incidence level of UC. India, for example, has recorded a drop in UC/CD ratio from 10:1 to 8:1. One notable trend is that the prevalence of CD rises once the disease has been present in a population for some time. Hong Kong has observed a UC/CD ratio that has declined to 1:1 from 8:1 (Ng et al., 2015). In general, the incidence of CD reaches its optimum in early adulthood, and the incidence rate declines in the elderly. The incidence of UC remains stable from early adulthood to retirement age.

East Asia and other parts of Asia have reported a trend of rising prevalence and incidence of IBD. Despite this trend being more acute in developing nations, some developed countries, such as Japan, have been shown to be affected. Also, the CD incidence rate has been more pronounced in female adults over male adults, although

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the past decade has recorded a higher incidence of CD in boys than in girls in developing countries. Over time, it may transpire that an equal CD prevalence for both sexes is reached. For example, studies from East Asia are suggesting a male predominance of CD and an equal sex ratio of UC (Ng et al., 2016).

Alterations in Intestinal Microbiota Implicated in the Development of IBD

The gut microbiota is believed to play a central role in the pathogenesis of IBD. Many studies give corroborated evidence for gut microbiota dysbiosis in IBD patients compared to healthy individuals. The gut microbiota of healthy people is made principally of microorganisms from the phyla Bacteroidetes (for the most part *Bacteroides* or *Prevotella* species), which are gram-negative, or Firmicutes (for the most part *Clostridium* and *Lactobacillus* species), which are gram-positive. Actinobacteria (that incorporate *Bifidobacterium* sp.), Proteobacteria (including *Escherichia coli*), and Verrucomicrobia (including *Akkermansia mucinophila*) are commonly present in small numbers in the gut microbiota of healthy individuals. The creation of gut microbiota varies in people with respect to age and advancement of disease (Huttenhower et al., 2012) (*Table 1*).

Table 1. *Composition of intestinal microbiota in healthy humans*

Reference	Sample	Microbial signature
(King et al., 2019)	50 healthy samples sequenced at GWU and 49 healthy samples taken from The Human Microbiome Project	• 155 bacterial species were identified, Bacteroidetes (31 phylotypes), Actinobacteria (32 phylotypes) and Firmicutes (63 phylotypes) had the highest abundance. • More than half of Firmicutes sequences were members of the Clostridia (20%) class, which was the highest abundant class, followed by Bacteroidia (18.5%), Bifidobacteriales (16.6%), Enterobacterales (14%), and Lactobacillales (14%). • Clostridiales and Bacteroidales orders had the highest abundance. • There were 26 members of the Bifidobacteriaceae family, belonging to *Bifidobacterium longum*, which were the most abundant species.
(Gill et al., 2006)	2 healthy adult subjects	• 133 bacterial phylotypes were identified; Actinobacteria (10 phylotypes) and Firmicutes (62 phylotypes) had the highest abundance. • 60% of the Firmicute sequences were members of Clostridia class (including Clostridia cluster XIV and Faecalibacteria.).
(Eckburg et al., 2005)	3 healthy adult subjects	• 395 bacterial phylotypes were identified, Bacteroidetes (65 phylotypes) and Firmicutes (301 phylotypes) had the greatest quantities. • Most (95%) of the Firmicutes sequences were members of the Clostridia class. • Low abundance of Proteobacteria sequences (including *Escherichia coli*), Actinobacteria, Fusobacteria, and Verrucomicrobia phyla. • *Bacteroides thetaiotaomicron* were detected in each subject.

GWU = George Washington University

A change in microflora synthesis, as in IBD, can contribute to intestinal damage. A few examinations have exhibited the Firmicutes phylum were less well represented in BD patients compared to healthy controls (Huttenhower et al., 2012; Rajilić-Stojanović et al., 2013), whereas members of Gammaproteobacteria were relatively light (Rajilić-Stojanović et al., 2013; Sokol et al., 2017) (*Table 2*). In CD patients, the Clostridia cluster IV group, in particular *Faecalibacterium* sp., has been shown to relatively low

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(Gill et al., 2006). Members of the Clostridia group XIVa, belonging to the *Roseburia* genus, also seem to be low in IBD patients (Rajilić-Stojanović et al., 2013). Data on Bacteroidetes report a general decrease in biodiversity in IBD patients (Mar et al., 2016; Sokol et al., 2017), also, significantly lower levels of putative beneficial OTUs; including *Prevotella copri* and the butyrate-producing bacterium *Faecalibacterium prauznitzii* (Willing et al., 2010).

Table 2. *Changes in gut bacteriome composition in IBD patients*

Reference	Sample	Microbial signature			
		Phylum	**Class**	**Family**	**Genus & Species**
Sokol et al. (2017)	Adult (235 patients with IBD, 38 control) fecal sample.	↓Bacteroidetes	Bacteroidia	↓Bacteroidaceae	-------
				↓Prevotellaceae	-------
		↓Firmicutes	Bacilli	↑Streptococcaceae	-------
				↑Erysipelotrichaceae	-------
			Clostridia	↓Lachnospiraceae	-------
				↓Ruminococcaceae	-------
			Negativicutes	↓Veillonellaceae	-------
		↑Proteobacteria	Gamma proteobacteria	↑Pseudomonadaceae	-------
				↑Enterobacteriaceae	-------
		↑Fusobacteria	Fusobacteria	↑Fusobacteriaceae	-------
Eun et al. (2016)	Adult (15 CD, 15 control), fecal sample	↓Bacteroidetes	Bacteroidia	↓ Bacteroidaceae	-------
				↓ Prevotellaceae	-------
			Clostridia	↓Lachnospiraceae	-------
				↓Ruminococcaceae	-------
			Negativicutes	↓Veillonellaceae	-------
		↑Proteobacteria	Gamma proteobacteria	Pseudomonadaceae	↑*Pseudomonas sp.*
				Enterobacteriaceae	↑*Escherichia sp.*
					↑*Shigella sp.*
		↑Fusobacteria	Fusobacteria	Fusobacteriaceae	↑*Fusobacterium sp.*
Mar et al. (2016)	Adult (30 UC, 13 control), fecal sample	↓Bacteroidetes	Bacteroidia	Prevotellaceae	↓ *Prevotella sp.*
				Bacteroidaceae	↓*Bacteroides sp.*
		↓Firmicutes	Clostridia	Lachnospiraceae	↓ A number of unclassified species
				Ruminococcaceae	
			Bacilli	Streptococcaceae	↑*Streptococcus sp.*
				Enterococcaceae	↑*Enterococcus sp.*
		↑Actinobacteria	Actinobacteria	Bifidobacteriaceae	↑*Bifidobacterium sp.*
Quince et al. (2015)	23 CD,21 control), fecal sample	Firmicutes	Clostridia	Ruminococcaceae	↓*Subdoligranulum sp.*
					↓*Feacalibacterium sp*
				Peptostreptococcaceae	↑*Peptostreptococcus sp.*
				↓Lachnospiraceae	-------
			Bacilli	↑Enterococcaceae	-------
		Actinobacteria	Actinobacteria	Coriobacteriaceae	↑*Atopobium sp.*
Rajilić-Stojanović et al. (2013)	Adult (15 UC, 15 control), fecal sample	Firmicutes	Clostridia	Ruminoccaceae	↓*Ruminococcus bromii*
				Eubacteriaceae	↓*Eubacterium rectale*
				lachnospiraceae	↓*Roseburia sp.*
				Peptostreptococcaceae	↑*Peptostreptococcus*
					↑*Clostridium difficile*
		Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiaceae	↓*Akkermansia sp.*
		Fusobacteria	Fusobacteria	Fusobacteriaceae	↑*Fusobacterium sp.*
		Proteobacteria	Epsilonproteobac-teria	Helicobacteraceae	↑*Helibacter sp.*
				Campylbacteraceae	↑ *Campylobacter sp.*

IBD, Inflammatory bowel disease; CD, Crohn's disease; UC, Ulcerative colitis; ↑, Increased; ↓, Decrease

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The Physiological Functions of the Gut Microbiota

The physiological benefits that gut microbiota have on the host are: 1) to supply of nutrients and energy (sustenance), 2) improvement of the immune system, and 3) functioning as host gatekeeper (*Fig. 3*) (Nishida et al., 2018). Details of these benefits are the following.

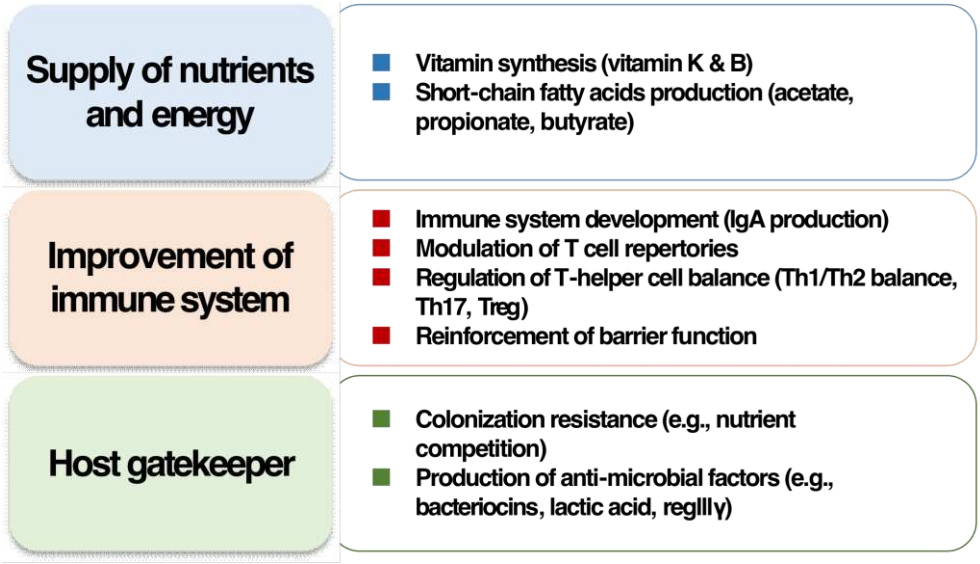

Figure 3. *Physiological functions of gut microbiota. Modified from Nishida et al. (2018)*

Supply of Nutrients and Energy

The gut microbiota supply energy and nutrients to the host (O'Hara and Shanahan, 2006). Commensal microorganisms in humans, for instance Bifidobacterium, can coordinate and provide supplements including water-dissolvable B vitamins and vitamin K (LeBlanc et al., 2011). In addition, intestinal bacteria provide short-chain fatty acids (SCFAs; C2-C6) by fermenting resistant starch or indigestible carbohydrates (dietary fiber). The Bacteroidetes and Firmicutes produce SCFAs from indigestible carbohydrates via participation together with species that have some ability to ferment oligosaccharide (e.g., Bifidobacterial). Anions in the colon are SCFAs, overwhelmingly, acetate, butyrate, and propionate (Marchesi et al., 2016). The latter are vital for a number of aspects of the host's physiology. For example, they are important for supplementing procurement, immune capacity, cell signaling, expansion control, and pathogen protection. SCFA levels are important for their anti-inflammatory effects and imperative for the upkeep of the mucosal barrier; for example, butyrate positively affects cell multiplication, separation, and development after epithelial damage (Lopetuso et al., 2016). The levels of SCFAs are significantly decreased in IBD, which may be a key factor in the degeneration of intestinal and immune homeostasis. The SCFA-producing bacteria are decreased in IBD patients, which include *Faecalibacterium* (Frank et al., 2007), Odoribacter, Leuconostocaceae, *Phascolarctobacterium*, and *Roseburia* (Ahuja, 2015).

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Immune System Improvement

Microbiota that live in the intestinal tract play a vital role in the improvement of the host immune system. The host immune system, in turn, shapes the structure and function of gut microbiota (Kamada and Núñez, 2014). Germ-free (GF) mice (insufficient in the gut microbiota) show impaired immune development, which is distinguished by immature lymphoid tissues (Bouskra et al., 2008), reduced amounts of intestinal lymphocytes, and diminished elements of antimicrobial peptides (Cash et al., 2006) and immunoglobulin A (IgA) (Hapfelmeier et al., 2010). Reconstitution of the gut microbiota of mice is sufficient to restore these abnormalities of the immune system (Umesaki et al., 1995). One of these microorganisms is *Candidatus Arthromitis*, known as a segmented filamentous bacteria (SFB). The colonization of SFB alone advances the improvement of the mucosal immune system (Ivanov et al., 2009). The improvement of the host immune system is dependent on host-unequivocal microbiota, as the immune system becomes underdeveloped in GF mice colonized with human microbiota. The gut microbiota, furthermore, regulates T-cell repertoires and controls the T helper (Th) cell profile (Shanahan, 2002). Authoritative white platelets (in like manner called Tregs) are CD4+ lymphocytes, which coordinate or smother distinctive cells in the immune system (Littman and Rudensky, 2010). It has been shown that SCFA-producing strain in *Clostridium* clusters IV, XIVa, and XVIII from a sound human fecal precedent started the partition and improvement of colonic Tregs through butyrate production (Atarashi et al., 2013). This result supports part of the clinical data. The degree of *Clostridium* bunches XIVa and IV in the fecal models is lower in IBD patients compared to healthy individuals (Frank et al., 2007). The low number of *Faecalibacterium prausnitzii*, which belong to *Clostridium* cluster IV, is related to a high risk of recurrence of CD after surgery (Walker et al., 2011). The headway of Th17 cells, which are a subpopulation of effector T cells, is defined by their production of interleukin (IL)-17A, IL-17F, IL-21, and IL-22 (Littman and Rudensky, 2010), that are modulated by the gut microbiota. In GF or antibiotic-treated mice, the abundance of Th17 cells in the intestinal mucosa is decreased. One study has shown that the gut microbiota plays a part in the improvement of Th17 cells (Wu et al., 2016). A past report has demonstrated that microbes with adhesive properties to intestinal epithelial cells, for instance, *Citrobacter rodentium* and *Escherichia coli* (EHEC) O157, advance the enrollment of Th17 cells (Atarashi et al., 2015).

Host Gatekeeper

The gut microbiota also combats pathogens. Animals in GF condition are powerless to sullying by intestinal pathogens. An anomaly in the mucosal immune system may add to this lack of protection. Another instrument against pathogens that deform the physical and dietary of the gastrointestinal tract is the colonization of commensal microbiota, which neutralizes the colonization of pathogens (O'Hara and Shanahan, 2006; Sekirov et al., 2010). This framework that actively hinders the interruption of pathogens by commensal microorganisms is called a 'colonization obstacle' (Buffie and Pamer, 2013). The gut microbiota improves colonization assurance from intestinal pathogens by direct and indirect mechanisms of action. Some commensal microorganisms truly stifle intestinal pathogens by competing for nutrients or by starting the production of inhibitory substances. *Bacteroides thetaiotaomicron*, which is an abundant colonic anaerobe, utilizes starch used by *Citrobacter rodentium*, which

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adds to the dismissal of pathogens from the intestinal lumen (Kamada et al., 2012). *B. thuringiensis* secretes bacteriocins that target spore-molding *Bacilli* and *Clostridia*, for instance, *Clostridium difficile* (Huang et al., 2016).

Commensal microbiota and microbial products protect against pathogens indirectly by activating immune responses. For instance, lipopolysaccharides and flagellin derived from the gut microbiota enhance the expression of antimicrobial peptide and RegIIIγ, from epithelial cells by stimulating Toll-like receptor 4^+ stromal cells and $TLR5^+CD103^+$ dendritic cells (Brandl et al., 2008; Kinnebrew et al., 2010). Segmented filamentous bacteria (SFB) promote the secretion of IgA from B cells, the production of antimicrobial peptides, and the development of Th17 cells in the intestinal mucosa (Talham et al., 1999; Ivanov et al., 2009). The gut microbiota plays a central role in the pathogenesis of IBD. A number of studies provide varied evidence for gut microbiota dysbiosis in IBD patients diverging from healthy controls (Sartor and Wu, 2017; Nishino et al., 2018).

Conclusion

The population of useful bacteria is reduced in IBD, while the population of pathogenetic bacteria appear to grow. While microbiota differ at the phylogenetic level, the presence of inflammation is considered the major engine for microbiome changes in such diseases. Studies provide the platform for investigating microbiome diversity perturbations in diseases with varying etiology as well as plan for therapeutic microbiome manipulation, in populations which are unique, displaying a variable number of enteric infections and gut autoimmune diseases.

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DEVELOPING AN OPTIMAL METHOD FOR THE ROOTING AND SEEDLING GROWTH OF MEDICINAL SPECIES *BUDDLEJA POLYSTACHYA* FRESEN: A STEP TOWARDS MASS PRODUCTION

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Abstract. For the improving regeneration and conservation of medicinal *B. polystachya*, stem cuttings were obtained from trees in Aseer area, Saudi Arabia, and two experiments were conducted to investigate factors affecting rooting (season and auxin type and concentration) and assessing growth performance of rooted cuttings. To test rooting factors, cuttings were collected during autumn, winter, and spring and treated with 500, 1000, and 2000 ppm indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), and naphthyl acetic acid (NAA) during each season. Following the evaluation of rooting, the second experiment was conducted using the best rooted cuttings, to test growth performance of seedlings under the effect of different auxins and four types of growth media. Cuttings collected during spring and treated with IBA had the highest rooting percentage (91%–98%), number of roots (23–29 roots cutting $^{-1}$), and root length (26–39 cm). Regarding seedling growth, planting cuttings in growth substrate containing clay, sand, and peat moss (1:1:1 v/v) resulted in highest quality seedlings in terms of leaf number, stem height and relative growth rate. We concluded that spring cuttings collection treatment with 1000 ppm IBA, and transplantation to fertile growth media can be a useful technique for mass production of *B. polystachya*
Keywords: *propagation, auxins, cuttings, growth media, season*

Introduction

The development of vegetative propagation methods is a useful tool for the conservation of genetic resources, domestication of tree species, and national and international forest conservation plans. Hence, vegetative techniques are used as an essential tool for the mass multiplication of superior phenotypes/genotypes and production of true to type uniform plants (Thakur et al., 2008). Rooting of stem cuttings is a simple and economically feasible method of vegetative propagation usually utilized for many tree species (Mewar and Naithani, 2016). Several factors affect rooting such as the mother plant age, season, and application of root exogenous promoting hormones. Therefore, it is essential to understand the critical factors influencing rooting (Shekhawat and Manokari, 2016; Siddiqui and Hussain, 2007).

Roots development of cuttings differs from species to species. A variety of pre-treatments are performed to facilitate the successful rooting of cuttings such as, application of auxins which is the most common pretreatment in the vegetative propagation of plants. Many studies suggested that auxins a play detrimental role in rooting capacity by facilitating the earlier production of rooted cuttings that will necessary for vegetative propagation (Fogaca and Fett-Neto, 2005; Topacoglu et al., 2016). Adekola and Akpan (2012) and Sardoei et al. (2013) concluded that these auxins

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included indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), and naphthyl acetic acid (NAA). The rooting period of plants following auxin application varies from species to species. In addition to other factors, auxins play a vital role in controlling plant growth and development influence the production of primary, secondary, and adventitious roots; they are the most frequently used plant growth hormones for rooting of cuttings in nursery practice. The commonly used auxin is IBA is synthetic auxin commonly used as an effective hormone to promote adventitious roots initiation compared to natural IAA (Pop et al., 2011). Successful propagation with the use of cuttings needs to select the right time cuttings collection, and this is intimately related to the natural phytohormones contents (Cristofori et al., 2010). In general, the growth medium is the most important factor determining seedling quality in the nursery (Baiyerin and Mbah, 2006).

Buddleja is a genus of flowering plants comprising approximately 100 species native to the tropics in America, Asia, and Africa (Corte's et al., 2006; El-Sayed et al., 2008). *B. polystachya* Fresen. is a multi-branched shrub that grow up to five meters; however, it may irregularly reached twelve meters height under favorable environment. This tree is widespread in the semi-arid highlands near the Red Sea coastal and distributed naturally in some countries like Eritrea, Ethiopia, Saudi Arabia, and Yemen where grown around forest, usually beside watercourse, at elevations 2200-3600 m above sea level (Chaudhary, 2001; Mohamedkassm et al., 2013). Several *Buddleja* species have been used in traditional medicine in many parts of the world, and the roots, leaves, and flowers of various Buddleja species have been used in folk medicine (Corte's et al., 2006; El-Sayed et al., 2008; Mohammed et al., 2016) reported that crude extracts of *B. polystachya* leaves contain certain chemical constituents that could possibly lead to antimalarial drug development and further more Al Ati et al. (2015) isolated and evaluated fifteen compounds from *B. polystachya* growing wild in Asir, one of these compound ethyl acetate fraction showed the most significant anti-inflammatory activity. Unfortunately, this valuable species is now present in a list of seriously endangered species according to International Union for Conservation of Nature and Natural Resources (IUCN, 2016).

This research work represented step to develop efficient and easy methods for the rehabilitation and conservation of *B. polystachya*, an ecologically and medicinally valuable species, by identifying the appropriate season for collection of cuttings and determining the types and concentrations of auxins for vegetative propagation and factors improving seedling growth such as growth media and auxin types.

Material and methods

First experiment

Site and seasons of cuttings collection: Healthy and uniform woody stem cuttings (average length 15 ± 3.5 cm) were collected during specific months of each season, autumn (September), winter (December), and spring (March), from stock trees of *B. polystachya* growing wild as scattered trees among others tree species in the Aseer region (18°12′63″ N, 42°31′01″ E; 2093.4 m above sea level), in the southwest of Saudi Arabia. This region is characterized by an average annual rainfall of 373.4 mm; monthly mean, minimum, and maximum temperature of 19.2 °C, 7.56 °C, and 31.0 °C, respectively; and relative humidity between 14.5% to 91.6%. All stem cuttings were collected in the early dawn (07:00 am) and kept in a plastic sealed ice container to

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minimize drying. This experiment was designed to test the interaction effects of auxin type, their concentrations, and season of collecting cuttings on vegetative propagation of *B. polystachya* using stem cuttings. This experiment was repeated thrice during autumn, winter, and spring.

Experimental site

The study was conducted in a greenhouse at the College of Food and Agricultural Science, King Saud University, Saudi Arabia under controlled temperature (25 ± 1 °C) conditions with 12/12 h of light/darkness, and irrigated whenever needed during the period of the experiment. The vegetative propagation unit inside the greenhouse was covered using transparent plastic and equipped with a mist device.

Description and application of treatments

Three phases of the first experiment were conducted using three auxin types and concentrations, during autumn (September, October and November 2017), winter (December 2017, January and February 2018), and spring (March, April and May 2018) in order to test major factors affecting rooting ability of this species. Thirty treatments were applied, comprising three growth auxins, three concentrations of each auxin, and three seasons of cuttings collection (3 auxins×3 concentrations ×3 seasons + 3 controls) (*Table 1*). The plant growth auxins (IAA, IBA, and NAA) were imported from Sigma. Stock solutions of the three auxins were prepared at concentrations of 500, 1000, and 2000 ppm by weighing the respective fresh weight (g) of each auxin and dissolving in distilled water. Cuttings were immersed in each auxin concentration for 5 min before planting in nursery beds (3×0.5×2 m) with sand rooting media and cuttings were inserted 5–6 cm deep using a wooden stick.

Measurements

After 12 weeks, the assessment of rooting success was performed by harvesting five replicates from each treatment to evaluate the root formation of stem cuttings collected for the period of the first collection season (autumn) by manually counting the number of rooted cuttings (for rooting percentage computation) and number of roots per cutting, whereas root length was measured from the point of emergence to the tip using a linear meter. The evaluation of rooting was performed in the same way for the remaining two seasons (winter and spring).

Second experiment

Following screening for the factors improving rooting from the results of the first experiment, the second experiment was performed to test the effect of auxin type and seedling growth media on the growth of seedlings. Results of the first experiment showed approximately higher rooting percentage, number of roots and root length occurring in cuttings treated with 1000 ppm of the three auxins and collected during spring than in those of the other treatments and seasons. Therefore, three replicates of cuttings from each treatment of 1000 ppm of IAA, IBA, and NAA were transplanted into four growth media (clay, sand, combination of clay + sand (1:1 v/v), and clay + sand + peat moss (1:1:1 v/v). *Table 2* showed the growth media properties, such as soil organic carbon percentage (OC %) was obtained as described by Nelson and

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Sommers (1982), soil texture was determined using the micropipette method (Miller and Miller, 1987), and water-holding capacity of soil was determined by the pressure plate method as described by Topp et al. (1993). After 12 weeks, for the evaluation of seedling growth, number of leaves per cutting were counted manually and relative growth rate of seedling height RGR(H) was estimated according to *Equation1* (Ostos et al., 2008).

$$RGR(H) = (LnH2 – LnH1) / t2 – t1 \quad \text{(Eq.1)}$$

where: RGR(H) = seedling height relative growth rate; H1 and H2 = initial and final seedling height, respectively; t2 and t1 = initial time and final time in month respectively.

Table 1. *Treatment descriptions*

Treatments	Treatment description		
	Auxin	Auxin concentration (ppm)	Season
IAA*500*Autumn	IAA	500	Autumn
IAA*500*Winter	IAA	500	Winter
IAA*500*Spring	IAA	500	Summer
IAA*1000* Autumn	IAA	1000	Autumn
IAA*1000*Winter	IAA	1000	Winter
IAA*1000*Spring	IAA	1000	Summer
IAA*2000* Autumn	IAA	2000	Autumn
IAA*2000*Winter	IAA	2000	Winter
IAA*2000*Spring	IAA	2000	Summer
IBA*500*Autumn	IBA	500	Autumn
IBA*500*Winter	IBA	500	Winter
IBA*500*Spring	IBA	500	Summer
IBA*1000*Autumn	IBA	1000	Autumn
IBA*1000*Winter	IBA	1000	Winter
IBA*1000*Spring	IBA	1000	Summer
IBA*2000*Autumn	IBA	2000	Autumn
IBA*2000*Winter	IBA	2000	Winter
IBA*2000*Spring	IBA	2000	Summer
NAA*500*Autumn	NAA	500	Autumn
NAA*500*Winter	NAA	500	Winter
NAA*500*Spring	NAA	500	Summer
NAA*1000*Autumn	NAA	1000	Autumn
NAA*1000*Winter	NAA	1000	Winter
NAA*1000*Spring	NAA	1000	Summer
NAA*2000*Autumn	NAA	2000	Autumn
NAA*2000*Winter	NAA	2000	Winter
NAA*2000*Spring	NAA	2000	Summer
DW*0*Autumn	Distilled water	0	Autumn
DW*0*Winter	Distilled water	0	Winter
DW*0*Spring	Distilled water	0	Summer

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Table 2. *Growth media properties*

Growth media	Property		
	Grain size (mm)	WHC (mm/m2)	OC (%)
Sand	0.1-2.0	60-113	0.10
Clay	< 0.01	122-195	1.20
Sand + Clay (1:1)	0.075-0.10	106-148	1.32
Sand + clay + peat moss (1:1:1)	0.90-0.14	125-181	20.50

Experimental design

For the first experiment a factorial experiment (3 × 3 × 3) was set up using three different factors (three plant growth auxins, three concentrations of each auxin, and three seasons of cutting collection). The experiment was laid out in a completely randomized manner with five replicates per treatment. Then, the second experiment was designed as a factorial (3 × 4) of three auxins and four growth media.

Statistical analysis

The following statistical analyses were used: ANOVA was used to test the effect of treatments, the least significant difference multiple range-tests were used to identify differences between means, and the general linear model was used to test the interaction between the three tested factors (auxins, concentrations, and seasons of collecting cuttings). All statistical analyses were achieved by using SAS version 9.3.

Results

Rooting percentage

The interaction of auxin types, auxin concentrations, and time of collection of cuttings had highly significant ($p < 0.05$) effects on rooting percentage. All cuttings treated with the different concentrations of auxins showed a high mean rooting percentage (72%) regardless of collection season. However, although there is no significant difference between means of 1000, 2000 and 500 ppm IBA treatments, but the highest rooting percentages (98, 95% and 91%) were obtained in cuttings collected in spring when treated with the three levels of IBA (1000, 2000 and 500 ppm receptively), followed by cuttings treated with 2000 ppm of IAA and NAA (89.5%, and 86.4%) The minimum rooting percentages (59%, 63%, and 65%) were obtained in the control cuttings collected during the three season's autumn, winter and spring, respectively (*Fig. 1.*)

Number of roots per cutting

The interaction among auxin types, auxin concentrations, and collection season of cuttings on number of roots per cutting was highly significant. However, the combination of IBA, its concentration, and collection season resulted in differences in the mean number of roots per cutting, and cuttings collected during spring and treated with 500 or 1000 ppm of IBA, had the maximum roots per cutting (28.6 and 26.4 roots, respectively), followed by treatment with 2000 ppm IAA (24.6 roots) in comparison to the other treatments. In contrast, the minimum number of roots per cutting (1.0–2.8 roots) was recorded in the control cuttings during all seasons (*Fig. 2*).

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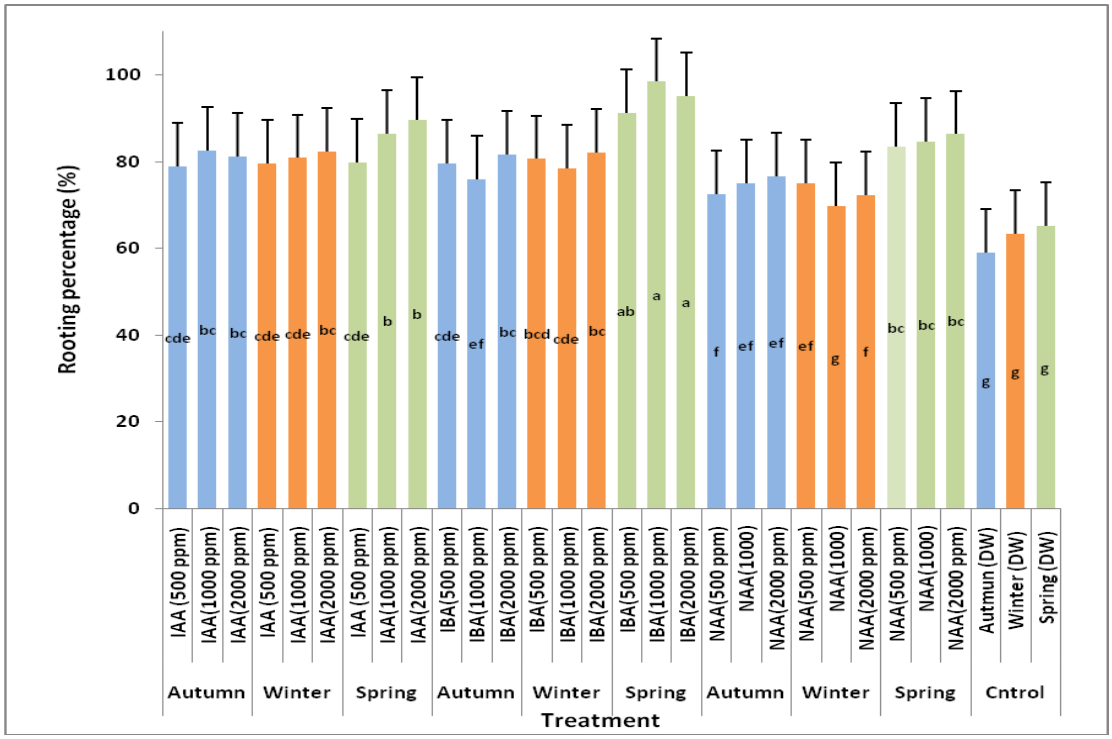

Figure 1. *Effect of treatments (three concentrations of IAA, IBA, and NAA and three seasons of cuttings collection) on rooting percentage of B. polystachya after 120 days. Columns with different letters indicate least significant differences (P < 0.05). DW = Distilled water*

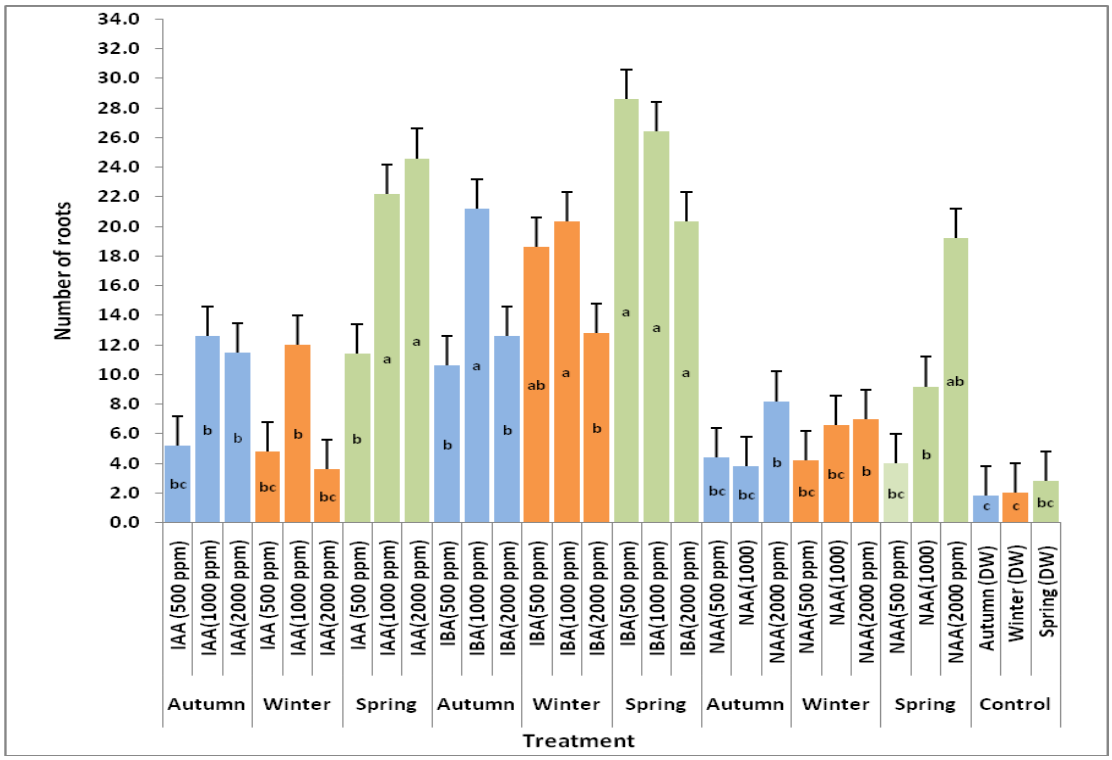

Figure 2. *Effect of treatments (three concentrations of IAA, IBA, and NAA and three seasons of cuttings collection) on number of roots per cutting of B. polystachya after 120 days. Columns with different letters indicate least significant differences (P < 0.05). DW = Distilled water*

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Root length (cm)

The interaction effects of factors significantly improved root length in cuttings. The longest roots were 39.8, 27.5, 26.3, and 24.9 cm, recorded in cuttings collected during spring and treated with 500, 1000, and 2000 ppm of IBA and 2000 ppm of IAA, respectively. In addition, some cuttings collected during autumn had longer roots, especially under treatments with 1000 and 2000 ppm IBA. All control cuttings had the least root lengths (*Fig. 3*).

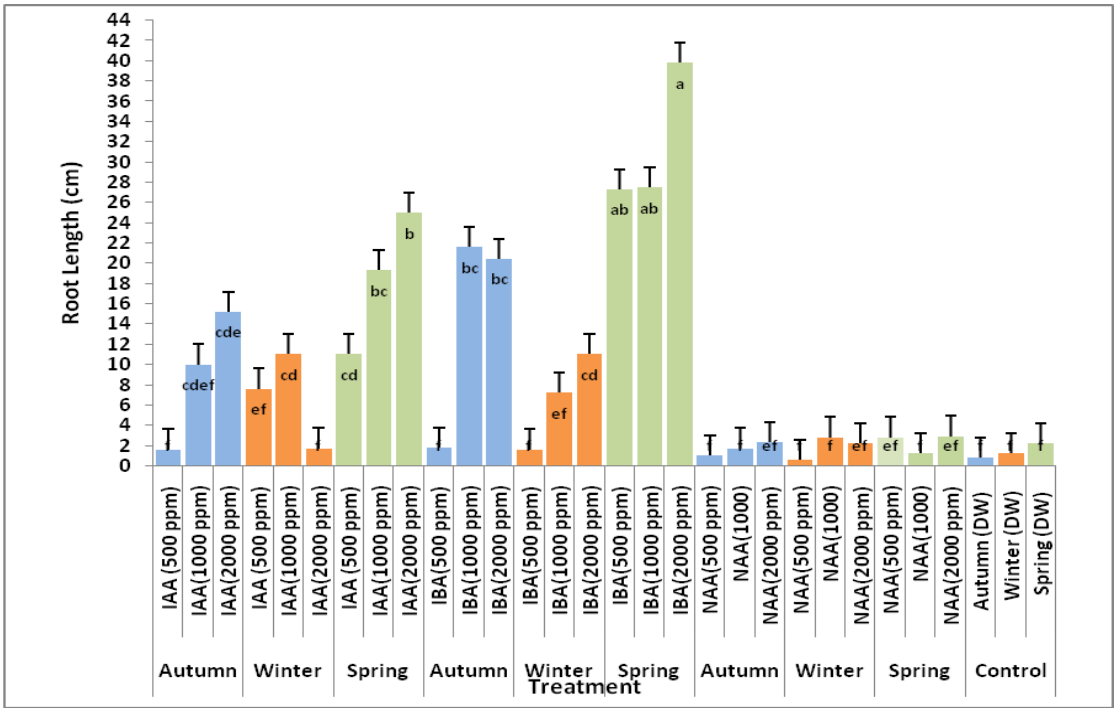

Figure 3. *Effect of treatment (three concentrations of IAA, IBA, and NAA and three seasons of cuttings collection) on root length of B. polystachya after 120 days. Columns with different letters indicate least significant differences ($P < 0.05$). DW = Distilled water*

Factors enhancing seedlings growth

Type of auxins and growth media significantly increased leaves per seedling. However, cuttings treated with IAA, IBA, and NAA grown in a mixture comprising peat moss with clay and sand (1:1:1 v/v) had the highest number of leaves (number of leaves increased by 144, 135, and 130%, respectively) compared to that in the control cuttings grown in the same media. In addition, cuttings treated with IBA grown in clay + sand (1:1v/v) had a higher percentage (115%) of number of leaves per cutting than the control seedlings grown in the same media. RGR(H) had the same trend as number of leaves per seedling, i.e., seedlings grown in media comprising a mixture of clay, sand, and peat moss had the highest rate of growth, followed by those grown in clay + sand media (*Table 3*).

Discussion

This study found that vegetative propagation of *B. polystachya* is considerably easy with the use of stem cuttings. However, for mass vegetative propagation aimed at

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obtaining a higher rooting percentage and vigorous seedlings with the highest number of long roots within the shortest time, the best time for collection of cuttings is spring and 1000 ppm IBA and 2000 ppm IAA and NAA are the best concentrations for treatment with auxins. The vegetative propagation of *B. polystachya* from stem cuttings is to some extent easy with or without the application of auxins, but cuttings treated with auxins had significantly higher number of roots and root length, especially the treatment with IBA. All three auxins promoted adventitious roots formation in the stem cuttings (*Fig. 4*), which is necessary for the vegetative propagation of many plant species (Druege et al., 2019). Auxins or plant growth hormones were used effectively in many plant species to promote the rooting of cuttings (Soundy et al., 2008; Singh et al., 2011; Sağlam et al., 2014).

Table 3. *Interaction effect of Auxin and growth media on leaves per cutting and RGR(H)*

Treatment	Leaves per seedling	RGR(H) (cm cm^{-2} month^{-1})
IAA*clay	14.85 ± 1.30 ^{bc}	0.395 ± 0.035 ^{bc}
IAA*sand	18.67 ± 2.01 ^{b}	0.624 ± 0.015 ^{ab}
IAA*clay-sand (1:1v/v)	11.94 ± 0.96 ^{dc}	0.177 ± 0.011 ^{cd}
IAA*clay + sand + peat moss (1:1:1v/v)	28.41 ± 3.41 ^{a}	1.044 ± 0.118 ^{a}
IBA*clay	15.66 ± 2.07 ^{bc}	0.449 ± 0.084 ^{bc}
IBA*sand	15.66 ± 1.93 ^{bc}	0.468 ± 0.074 ^{bc}
IBA*clay-sand (1:1v/v)	25.10 ± 3.05 ^{ab}	0.9200.070 ^{ab}
IBA*clay + sand + peat moss (1:1:1v/v)	27.36 ± 2.65 ^{ab}	0.982 ± 0.03 ^{ab}
NAA*clay	14.47 ± 1.98 ^{cd}	0.369 ± 0.002 ^{bc}
NAA*sand	17.25 ± 2.11 ^{bc}	0.545 ± 0.012 ^{bc}
NAA*clay-sand (1:1)	13.00 ± 1.74 ^{bc}	0.262 ± 0.004 ^{bc}
NAA*clay + sand + peat moss (1:1:1v/v/	26.70 ± 3.07 ^{ab}	1.006 ± 0.027 ^{a}
DW*clay	10.50 ± 2.50 ^{cd}	0.049 ± 0.002 ^{d}
DW*sand	10.96 ± 3.22 ^{cd}	0.092 ± 0.041 ^{d}
DW*clay-sand (1:1v/v)	11.63 ± 1.87 ^{cd}	0.151 ± 0.023 ^{cd}
DW*clay + sand + peat moss (1:1:1v/v)	11.63 ± 1.74 ^{cd}	0.163 ± 0.024 ^{cd}
Level of significant (p-value)	< 0.0141	< 0.0001

a **b** **c** **d**

Figure 4. *Root density of stem cuttings collected during spring and treated with 1000 ppm of (a) IAA, (b) IBA, (c) NAA and (d) control*

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Various studies investigated the effects of different auxins on vegetative propagation via cuttings rooting and plant growth. Sevik and Guney (2013) study the effects of IAA, IBA, NAA, and gibberallic acid (GA3) in *Melissa officinalis*; Stefancic et al. (2005) investigated the effects of IAA and IBA in *Prunus spp*.; and Chhun et al. (2003) study the effects of IAA, IBA, and NAA in *Oryza sat*iva. These studies generally confirmed that the auxins or hormones have a significant effect on rooting, and consistent with these findings, the present study confirms that auxins can be a useful substance for increasing rooting in cuttings (Salmi and Hesami, 2016). The results revealed that increasing the level of the IAA, IBA, and NAA enhanced rooting percentage, number of roots per cutting, and root length. The role of increasing levels of auxin is well documented. An increased auxin concentration in the stem cutting produced by active auxin transferred from the leaves activated adventitious root creation from the cambium (Justamante et al., 2019). We found that the appropriate time for *B. polystachy*a cuttings collection is spring, and our results further confirmed that the season of cuttings collection is a critical factor for successful rooting. Our results confirmed the findings of other studies, the time of cuttings collection to be a major factor influence propagation of plant (Klein et al., 2000; Swamy et al., 2002; Haile et al., 2011). Ling and Zhong (2012) reported enhancing in rooting percentage of the Tetraploid Locust during May. Seasonal differences in successful rooting are common in woody plants, and the optimal season for rooting must be established independently for each species (Hussain et al., 2014). The rooting medium considered as proper atmosphere for rooting of cuttings (Tchinda et al., 2013), we used sand as the rooting medium in the first experiment, following Adugna et al. (2015) who used fine sand as rooting medium for *V. planifolia* stem cuttings and obtained 99.3% percentage of rooting. Although all seedlings were treated with the same concentrations of auxins and collected in the same season (spring), the interaction between IBA and combination of clay, sand, and peat moss was found to be more effective compared to that of the other two auxins and media (*Table 3* and *Fig. 5*).

Figure 5. *Growth performance of seedlings under the effect of media (clay, sand, and peat moss (1:1:1 v/v) treated with 1000 ppm of (a) IAA, (b) IBA, (c) NAA, and (d) control*

The combination of clay, sand, and peat moss was the best growth medium in comparison to the other media. This interaction enhanced seedling growth in terms of increasing number of leaves and RGR(H). This can be due to the higher content of organic matter and water holding capacity of the medium. These results are previously conformed by findings of by Neelam et al. (2001) who reported that media with improved soil physical

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and chemical traits and aeration leaded to superior plant growth and consistence with previously findings of Ahmed et al. (2017) who reported that media consisting of clay, sand and peat moss in equal ratios enhanced seedlings growth of *B. aegyptiaca*. Furthermore, there are several studies indicating the effect of organic matter on soil fertility (Martinez et al., 2003), survival and growth (Larchevêque et al., 2006), biomass (Moreno-Peñaranda et al., 2004), and seedling quality (Mañas et al., 2009).

Conclusions

This study developed an optimal method for the vegetative propagation of *B. polystachya* via woody stem cuttings. The results confirm that woody stem cuttings collected during the spring season and treated with 1000 ppm of IBA or 2000 ppm IAA or NAA had a high rooting percentage with high quality rooted cuttings, i.e., dense and longer roots. Rooted cuttings planted in a combination of clay, sand, and peat moss in equal proportion had dense, long roots. However, in general, seedlings resulting from stem cuttings treated with IBA were superior to seedlings from the other treatments in terms of shoot development (the highest number of leaves and stem height relative to the growth rate) after 6 months. Rooting of stem cuttings of *B. polystachya* is a feasible propagation method for producing high quality seedlings, which can be used as an adequate stock for rehabilitation, conservation, and future studies on the medicinal properties of this valuable tree species. Further research work should be needed to investigate mass propagation of this species using other methods of propagation as micro- propagation or crafting. More research will be needed to evaluate growth performance and or success of seedlings resulting from vegetative propagation under field or nursery conditions.

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ISOLATION AND CHARACTERIZATION OF TWO IMPORTANT HONEYBEE-KILLING VIRUS SPECIES, DEFORMED WING VIRUS (DWV) AND BLACK QUEEN CELL VIRUS (BQCV) FROM *MESSOR CONCOLOR* ANTS (HYMENOPTERA: FORMICIDAE)

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Abstract. Pathogenic organisms such as viruses can infect multiple host species from different trophic levels. Honeybee, *A. mellifera* is naturally infected by many viruses. Understanding the mechanism underlying virus spread in honeybee colonies highly depends on the pathogenic ability of honeybee killing viruses in other insects such as ants, *Messor concolor* (Hymenoptera: Formicidae). The purpose of this study was to investigate the existence and prevalence of two significant viruses, deformed wing virus (DWV) and black queen cell virus (BQCV) of honeybees in ants from the nests around different honeybee apiaries in Southeast Turkey by using reverse transcription polymerase chain reaction (RT-PCR). Both viruses were present in two nests while the former existed in another hive. Some ant nests were uninfected. This study is the first to report the molecular detection of the honeybee infecting DWV and BQCV in ants. Further studies should be devoted to the mechanisms underlying virus spread between honeybee and other interacting insects especially the Hymenopterans.
Keywords: *honeybee virus, new host, foodborne transmission, Southeast Turkey*

Introduction

Insects are host to a wide range of viruses that have significant effects on their nature. Honeybee viruses are probably the best-known insect viruses due to important economic losses (Smith et al., 2013; McMenamin and Genersch, 2015). Presence of more than twenty different virus species infecting honeybee, most of which are single-stranded positive RNA viruses, have been reported so far (de Miranda et al., 2013). Understanding the transmission mechanism of honeybee viruses in a hive and apiary is necessary to reveal population dynamics of bee viruses as an important threat. The deformed wing virus (DWV) (Iflaviridae) and black queen cell virus (BQCV) (Dicistroviridae) are the most commonly found virus species (de Miranda et al., 2013).

Deformed wing virus (DWV) is probably the most commonly encountered virus species infecting honeybees (Bailey and Ball, 1991; Lanzi et al., 2006; Berényi et al., 2007). Deformed wing, shortened abdomens, discoloring, and a decrease in the life span of bees are the clinical indicators noticed under heavy DWV infection. Deformed wing virus infection mostly does not result in clear symptoms when transmitted by other vectors than the ectoparasitic mite, *Varroa destructor* (Mesostigmata: Varroidae) (de Miranda and Genersch, 2010). DWV transmits both vertically and horizontally and also isolated from all cast (Queen, Drone, Worker) and life stage (egg, larva, pupae, and adult) of honeybee. Commercial and wild bumble bees can be infected by DWV. *Bombus terrestris* and *Bombus pascorum* shows wing deformities, (Genersch et al., 2006), and serology in *Apis cerana* and *Apis florea* (Allen and Ball, 1996; Ellis and Munn, 2005). DWV was also recorded in other insects such as small hive beetle, *Aethina tumida* (Coleoptera;

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Nitidulidae) and the wax moth, *Galleria mellonella* (Lepidoptera: Pyralidae) which are in interaction with honeybee (Eyer et al., 2009; Traiyasut et al., 2016).

BQCV, one of the most common honeybee virus, multiplies in adult bees, particularly when ingested with the microsporidian parasite spores *Nosema apis* (Bailey et al., 1983; Tapaszti et al., 2009). The infection ability of these viruses is not restricted to *A. mellifera*. Previous reports indicated that wild species of bees, *Apis dorsata* and *A. florea* (Zhang et al., 2012), and *B. terrestris* (Choi et al., 2015) were also infected by DWV and BQCV. Understanding and monitoring the global dispersal of DWV and BQCV are crucial for predicting their epidemy and successfully controlling the aforementioned viruses (Freiberg et al., 2012). Previous studies revealed presence of some honeybee viruses infecting other Arthropoda and, especially, ant species. For example, the earliest bee virus detected in an ant species (*Camponotus vagus*) is the chronic bee paralysis virus (CBPV) which is both viral and replicative (Celle et al., 2008). In another study, beside BQCV and DWV, some other viruses such that Israeli acute paralysis virus (IAPV), Kashmir bee virus (KBV), and sacbrood virus (SBV) were detected in the species of 11 non-Apis hymenoptera and pollen pellets of forager bees (Singh et al., 2010). Further research approved the existence of honeybee viruses in some ant species such as Camponotus sp. and *Tetramorium caespitum* (Levitt et al., 2013). In addition, the Argentine ant (*Linepithema humile*) was also reported being infected by DWV, BQCV, and KBV (Gruber et al., 2017). Moreover, Lake Sinai viruses (LSVs) were detected in *Messor concolor*, *M. capitatus* and *M. barbarous* (Bigot et al., 2017). Caged ants (*Myrmica rubra*) fed with infected *A. mellifera* pupae can be infected by DWV (Schläppi et al., 2019). In total, 57 samples belonging to 13 different ant genera collected in apiaries were analyzed and 51 (89%) of the samples were found infected at least by one of the following honeybee viruses; DWV, BQCV, IAPV, SBV, KBV, and Acute bee paralysis virus (ABPV) (Payne et al., 2020).

During routine surveys, I found that infected honeybee individuals were taken by ants to their nests which could be a possible route of transmission for honeybee infecting viruses from bees to ants. Thus, the aim of this study was first to demonstrate whether two important honeybee infecting viruses, BQCV and DWV exist in *M. concolor*. Further, the genome variability of a partial sequence of DWV and BQCV were evaluated.

Material and Methods

Survey

Samples from 7 ant nests were collected from Southeast Turkey (Hakkari and Şırnak provinces), in June 2016 (*Figure 1*). From each nest, four ant samples were collected. This samples were placed in a freezer at -80 ºC till the laboratory analysis. The collected samples were transported to the laboratory for diagnostic studies and RNA isolations. Samples were stored in 70% ethyl alcohol for diagnosis at -80 for RNA isolation.

RNA extraction

For preparing homogenates from ant samples, liquid nitrogen was used in a sterile mortar. All homogenized ant samples were placed into the sterile Eppendorf tubes. The RNA was prepared using a modified silica-capture method (Foissac et al., 2001). Four ants (*M. concolor*) were extracted for each ant nest. Total RNA was resuspended in free RNases and DNases water and kept at 20 ºC temperature. Total RNAs were placed in a freezer at –20 ºC till for laboratory analysis.

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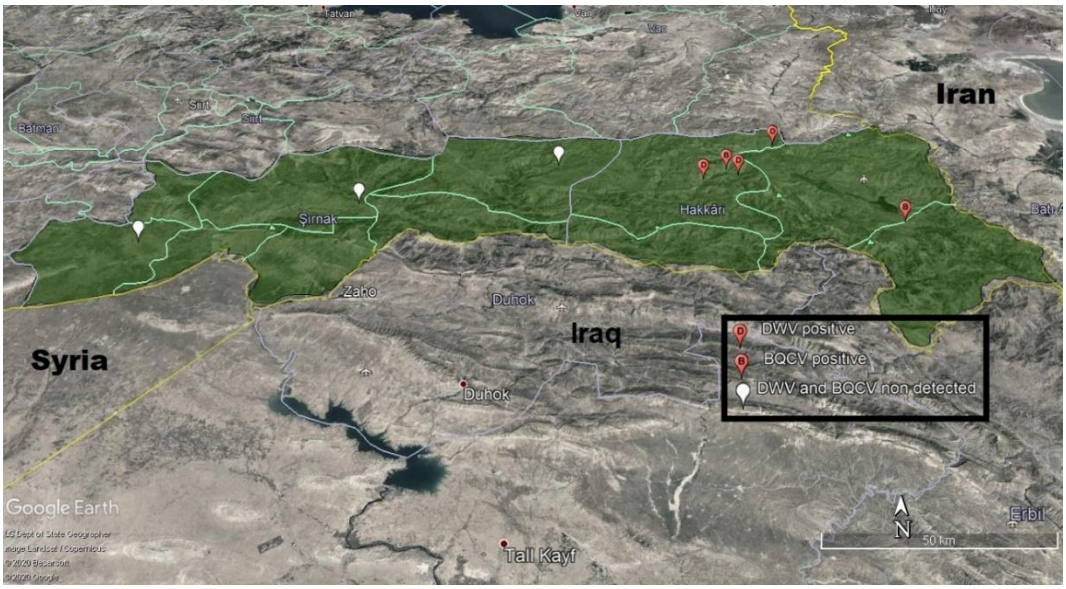

Figure 1. *The map of the collected ant sample is the green area. Each symbol shows the initial letter of the virus isolated from the collected ant sample*

RT-PCR of CP and RdRp genes

The DWV and BQCV viruses were detected using the procedure described in Rüstemoğlu and Sipahioğlu (2019). Genome-specific primers amplifying the 488 bp and 567 bp fragments of RNA depended RNA polymerase (RdRp) and partial coat protein (CP) genes were characterized by molecular cloning and sequencing for DWV and BQCV, respectively. The reverse transcription polymerase chain reaction was performed using a purified RNA from the RevertAid First Strand cDNA kit in accordance with the instruction of the producer company (Vilnius, Thermo-Fermentas Lithuania). The final volume was completed to 25 µl for PCR reaction. The solution comprised of 2.5 µl of 10 × reaction buffer (200 mM Tris–HCl pH of 8.4 and 500 mM KCl), 1 µl of cDNA, 0.5 µl of dNTPs (20 mM each), 1.5 µl of $MgCl_2$ (25 mM), 0.5 µL of each primer (100 pmol), 0.2 µL of Taq DNA polymerase and 18.3 µl of nuclease free water. Following, the partial CP and RdRp gene were amplified using thermal cycling diagram in a RT-PCR. The thermal cycle diagram was as follow; 2 minutes 35 cycles in a minute at 94 ºC temperature, 30 seconds at 57 ºC temperature, 45 seconds at 72 ºC temperature and finally incubated for 10 minutes at 72 ºC temperature. The amplified products at the end of the thermal cycling were separated using 1.5% agarose gel and stained with ethidium bromide to visualize (Sambrook et al., 1989).

Molecular cloning and sequencing

The virus isolates identified were cloned and sequenced separately. The isolation of amplified pieces was carried out in 1% agarose gel and recovered by using a GeneJET Gel Extraction Kit based on the prescription provided by the producer company (Thermo Scientific). The pGEM®-T Easy vector (Promega) was used to purify the DNA fragments. The plasmid was employed to turn competent cells of Escherichia coli JM 109 to ampicillin resistance using electroporation (BioRad, USA). Blue-white selection on X-gal medium plate was used to choose the transformants harboring the DNA of

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DWV-Ant-1 and BQCV-Ant-1 isolate and a colony PCR was employed to screen as positive clones. One clone, named DNA of DWV-Ant-1 and BQCV-Ant-1 was selected for the sequencing of DNA. Automated DNA sequences of Refgen Research and Biotechnology Company (İstanbul, Turkey) was used in sequencing the clones of cDNA.

Phylogenetic analysis

The sequences of DWV and BQCV used in comparisons were procured from the database of Genbank. The unreleased isolates for Turkish sequences of DWV and BQCV isolates were also used in the phylogenetic analysis. Multiple sequence alignments and phylogenetic reconstructions were performed for sequence similarity and phylogenetic analysis using the software of CLC Main Workbench and MEGA-X (Kumar et al., 2018). The stability of phylogenies was deduced by 1000 bootstrap replication using the method of maximum likelihood described in Tamura and Nei (1993).

Results

PCR results indicated that DWV (3/7) and BQCV (2/7) were found in the *M. concolor* nests (*Figure 2*). All positive samples were detected within the borders of Hakkari province (*Figure 1*). The sequence of DWV (GenBank Accession No. MT831948) isolates obtained from surveys had %99-95 similarity when compared with other DWV isolate records from different countries. The DWV sequence obtained from this study shows the most nucleotide similarity with KX373900.1 (France) (99.02%), MH267695.1 (Sweden), KT215905.1 (United Kingdom), KF840795.1 (Lithuania), HM067438.1 (United Kingdom) (98.77%) and KP835214.1 (Turkey), KF840794.1 (Lithuania), (98.53%) isolates, respectively. The BQCV sequence (GenBank Accession No. MT338252) obtained indicated the highest similarity to four isolates MK431882.1 (99.78%), MK431880.1 (99.10%), MK431881.1 (98.65%) and KP835213.1 (98.24%), respectively. These four BQCV isolates were obtained from *A. mellifera* in Turkey (Hakkari and Muğla). Besides these four isolates in the phylogenetic tree, BQCV (*M. concolor*) was clustered in a common branch with South Korean isolates JQ434132.1 (Ac), KP835213.1 (Am) and JQ434135.1 (Am). Phylogenetic trees were used to illustrate the relationships between the sequences. The phylogenetic trees were constructed with the confidence values for each node based on 1000 bootstrap replicates (*Figure 3* and *Figure 4*).

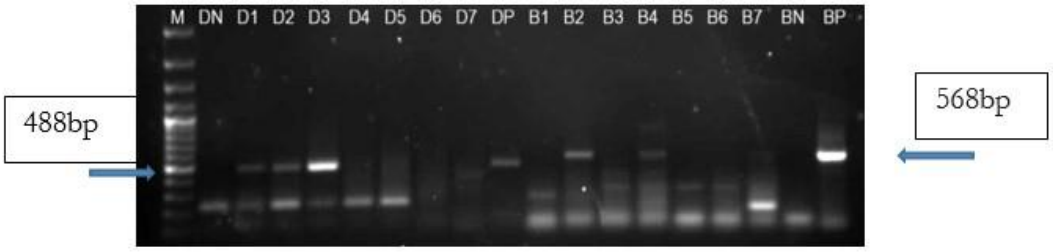

Figure 2. *Electrophoresis PCR products of DWV(D) and BQCV(B) in ants (M. concolor). Lane M: 100bp plus (thermo). Lane DN: DWV negative control. Lane DP: DWV positive control (488 bp). Lane D1-D3: DWV Positive amplification DWV (488 bp). Lane D4-D7: none-amplification DWV. Lane B2, B4: Positive amplification of BQCV (568 bp). Lane B1, B3, B5, B6, B7: none-amplification BQCV. Lane BN: BQCV negative control. Lane BP: BQCV positive control (568 bp)*

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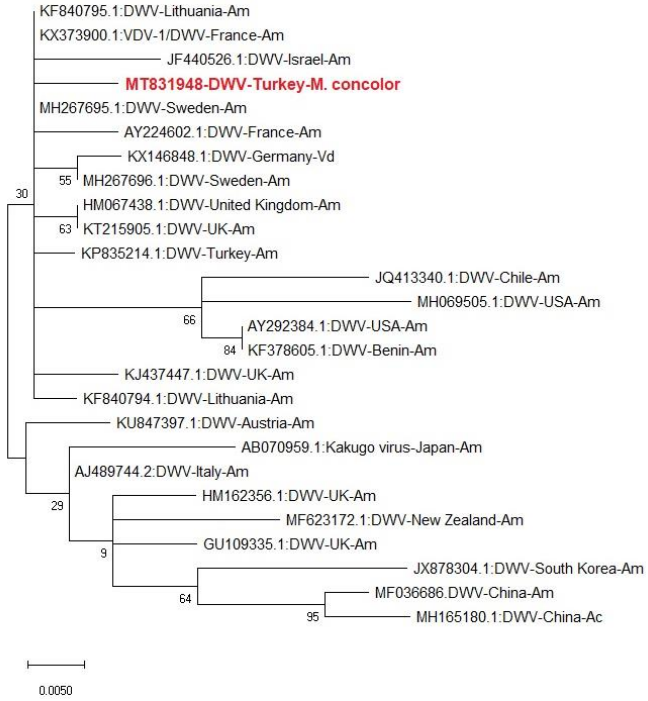

Figure 3. *Phylogram of the MT831948-DWV- Turkey-M. concolor isolated based on sequences of polyprotein gene created using the maximum likelihood method in MEGA X. The ratio of correct partition in a 1000-replicate bootstrap analysis can be observed in the statistical base of the nodes. Am = Apis mellifera, Ac = Apis cerana, Vd= Varroa destructor*

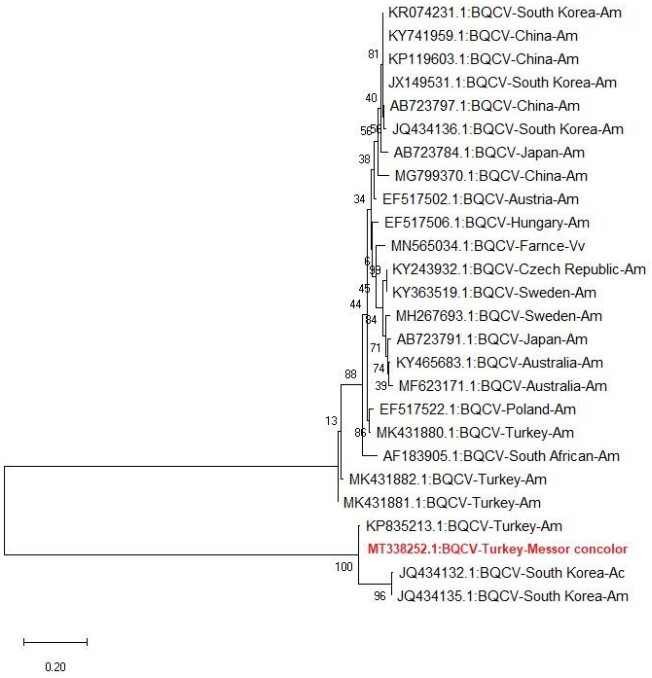

Figure 4. *Phylogram of the MT338252.1. BQCV- Turkey-M. concolor isolated based on sequences of Capsid Protein (CP) created using the Maximum likelihood method in MEGA X. The ratio of correct partition in a 1000-replicate bootstrap analysis can be observed in the statistical base of the nodes. Am = Apis mellifera, Ac = Apis cerana, Vv= Vespa velutina*

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Discussion

This study clearly revealed that the isolates of both viruses, BQCV and DWV extracted from same hosts do not cluster (*Figures 3 and 4*). This may indicate that these two honeybee viruses have not undergone any specific changes to their hosts. The DWV and BQCV sequences obtained from *M. concolor* were mostly identical with nucleotide sequences found *A. mellifera* from same geographic origin.

The earliest bee viruses identified in ants (*Camponotus vagus* and *Formica rufa*) was the Chronic bee paralysis virus (CBPV) (Celle et al., 2008), followed by BQCV, DWV, IAPV, and SBV (Levitt et al., 2013). The DWV, BQCV, and KBV were detected in in Argentine ant (*Linepithema humile*) (Gruber et al., 2017). Lake Sinai viruses (LSV) as a honeybee virus detected in *Messor concolor*, *M. capitatus* and *M. barbarous* (Bigot et al., 2017). ABPV and DWV were detected from the ants (*Lasius platythorax*) collected from the apiary (Schläppi et al., 2020). Further the efforts on honeybee virus in ants revealed the presence of at least one of the DWV, BQCV, IAPV, ABPV, KBV, and SBV in Brachymyrmex, Forelius, Linepithema, Solenopsis, Nylanderia, Pheidole, Camponotus, Aphaenogaster, Crematogaster, Pogonomyrmex, and Pseudomyrmex ant genera (Payne et al., 2020). BQCV and DWV can infect other Apis species (*Apis florea* and *Apis dorsata*) (Zhang et al., 2012), some of bumble bee species *(Bombus terrestris, B. pascuorum, B. huntii, B. impatiens, B. vagans*, *B. ternarius*) (Genersch et al., 2006; Singh et al., 2010; Li et al., 2011; Peng et al., 2011), and isolated from the eastern carpenter bee (*Xylocopa virginica*), mining bees (*Andrena* sp.), yellow jackets (*Vespula vulgaris*), and sand wasp (*Bembix* sp.), pollen pellets from forager bees (Singh et al., 2010). Herein, the existence of DWV and BQCV in *M. concolor*, a carnivore ant species is reported for the first time.

The phylogenetic analysis showed that viruses isolated from the different host can cluster each other, for both BQCV and DWV, the sequences isolated from the ants clustered together with previous sequences from the *A. mellifera*. According to the phylogenetic tree, the hosts from the same geographical origin likely cluster each other, and besides during this the survey, it was observed that ants attack the honeybees, especially which have disabilities and cannot enter the hive because of wing deformations. These results suggest that BQCV and DWV might have transmitted from *A. mellifera* to *M. concolor*. *Messor concolor* could be another vector of these viruses as a possible spread route. In apiaries, any virus infected organism could pose a danger for honeybee populations especially, the hymenopteran insects sharing same sources with honeybees. On the other hand, since the infected honeybee individuals are dismissed from the hives and preyed by ants, the virus density in hives may reduce. Otherwise, infected honeybee individuals could be a greater threat for hives via conataminating healthy individuals by topical contact (Amiri et al., 2014, 2019; Coulon et al., 2018). Further, the genome variability of a partial sequence of DWV and BQCV were evaluated and it does not appear to be separated by the host, but rather moving between species.

Conclusion

In summary, this study reports the presence of two significant honeybee infecting virus species, DWV and BQCV from *M. concolor*, which is carnivore ant species, under natural conditions. These findings suggest that other trophic levels could be involved in spread of honeybee viruses. However, it is not clear how such an involvement could affect honeybee population dynamics. The presence of the honeybee infecting viruses should

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be checked across other arthropod communities around apiaries which could help a better understanding of virus transmission routes.

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THE EFFECTS OF DROUGHT ON THE GRAIN YIELD OF SOME WHEAT GENOTYPES (*TRITICUM AESTIVUM* L.) UNDER THE AGROECOLOGICAL CONDITIONS OF SOUTH SERBIA

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Abstract. The paper aimed at identifying wheat genotypes tolerant to drought stress. The study repeated in three consecutive years was done on the alluvial soil of the Southern Morava river valley in South Serbia. The average wheat grain yield was higher by 124.5% under irrigation conditions than without irrigation. Over the experimental period, the average water consumed on wheat evapotranspiration under irrigation conditions was higher by 38.9% than without irrigation. Based on correlation statistical results and principal component analysis (PCA), in the case of stress resistant wheat genotypes, stress susceptibility index (SSI), tolerance index (TOL), mean productivity (MP), geometric mean productivity (GMP), stress tolerance index (STI), yield index (YI), yield stability index (YSI) as well as the LSD test was proved to be invariably efficient for determining the stress-tolerant genotypes. The result of tolerance index concerning drought and LSD test denoted that the Pobeda variety had a superior tolerance to stress due to drought than the other genotypes. Considering a comparatively low percentage of irrigated wheat fields in Serbia, it seemed to be outstandingly significant to identify the wheat genotypes tolerant to drought stress so that stable yields could be obtained.
Keywords: *winter wheat, drought stress, irrigation, water utilization efficiency, evapotranspiration*

Introduction

Crop production has been exposed to stress recently due to climate changes which have brought about extremely high temperatures with rather long dry periods. Drought stress takes place when soil and atmospheric humidity is low and the ambient air temperature is high. This condition is the result of an imbalance between the evapotranspiration flux and water intake from the soil (Lipiec et al., 2013). Namely, the stress caused by drought has a significantly negative effect on the crop yields. Implementation of crop management practices can potentially alleviate the harmful effects of drought and heat stresses and includes: soil management and culture practices, irrigation, crop residues and mulching and selection of more appropriate crop varieties (Lamaoui et al., 2018).

Wheat (*Triticum aestivum* L.) is said to be the most important crop in human nutrition with 728.1 million tons produced worldwide (FAO, 2018). However, Lizumi et al. (2018) found out that climate changes from 1981 to 2010 had decreased the wheat grain yield globally by 1.8%. In their research, Liu et al. (2016) foresaw that the wheat yield would

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fall by 4-6% per each degree of temperature rise. Having analysed the temperatures and rainfalls in Europe over the last three decades, Moore and Lobell (2015) established the wheat yield to have dropped by 2.5%.

Thus, the extreme drought in Spain and Portugal in 2005 led to a lower yield by 50-60% (Isendahl and Schmidt, 2006) while in Serbia, according to Dragović and Maksimović (2000), it caused yield to drop even by 81%. Jovanović et al. (2013) also reported decreased crop yield by 10-50% depending on the intensity of drought.

Further, Hall (1993) defined drought as a relative yield of a genotype when compared to that under stress conditions. Thus, researchers had to select the genotypes prone to stress in order to use genetic variation for improving stress-tolerant varieties (Clarke et al., 1984). The authors who suggested the indices for establishing stress-tolerant genotypes, were numerous (Fischer and Maurer, 1978; Rosielle and Hamblin, 1981; Bouslama and Schapaugh Jr, 1984; Fernandez, 1992; Gavuzzi et al., 1997). In contrast, the wheat varieties of a high genetic potential for grain yield selected under optimal growing conditions were reported as non-resistant to drought (Blum, 1979; Ceccarelli and Grando, 1991). Richards (1996) also stressed that wheat selection should be made under stress and optimal growing conditions.

The research aimed to determine drought stress resistant wheat varieties grown in Serbia, based on the effect of drought stress on their grain yield, in order to provide information on which varieties are less susceptible to climate change.

Materials and methods

Experimental location

The study lasted for three years in the experimental field, in Batušinac (43º15´24″ N and 21º49´13″ E, altitude: 201 m asl), municipality of Merošina, not far from the city of Niš in South Serbia (*Fig. 1*). The study location is approximately 240 km away from Belgrade.

Experimental design

The trials were set up in a random block system in three repetitions. Drought tolerance of the seven varieties Pobeda, Zvezdana, Rapsodija, Renesansa, Evropa 90, Simonida and NS Rana 5, created at the Institute of Field and Vegetable Crops in Novi Sad (the National Institute of the Republic of Serbia) and that of the two varieties (KG 56 and Takovčanka), selected at the Institute for Small Grains – Kragujevac, were studied. In the experimental field, winter wheat sowing was carried out from the 10^{th} to 25^{th} October. Seeding rate was 500 germinative seeds per m^2. The areas of elementary plots were 6 m^2 and, during vegetation, usual agrotechnical measures for wheat were used. The total amount of nutrients deposited to soil was: N - 130 kg ha^{-1}, P_2O_5 - 85 kg ha^{-1}, K_2O - 110 kg ha^{-1}. Irrigation was done by drip irrigation method and its term was determined by observing dynamics of soil moisture down to 60 cm of depth, pre-irrigation soil moisture amounted to 70% of the field water capacity (FWC). Soil moisture content was measured by thermogravimetric analysis in the oven at 105-110ºC.

Rainfall was measured at the experimental field by rain gauge. The mean monthly air temperatures and monthly amount of rainfall for the study period and multi-annual average for the city of Niš (*Table 1*) were taken from the website of RHS (2019).

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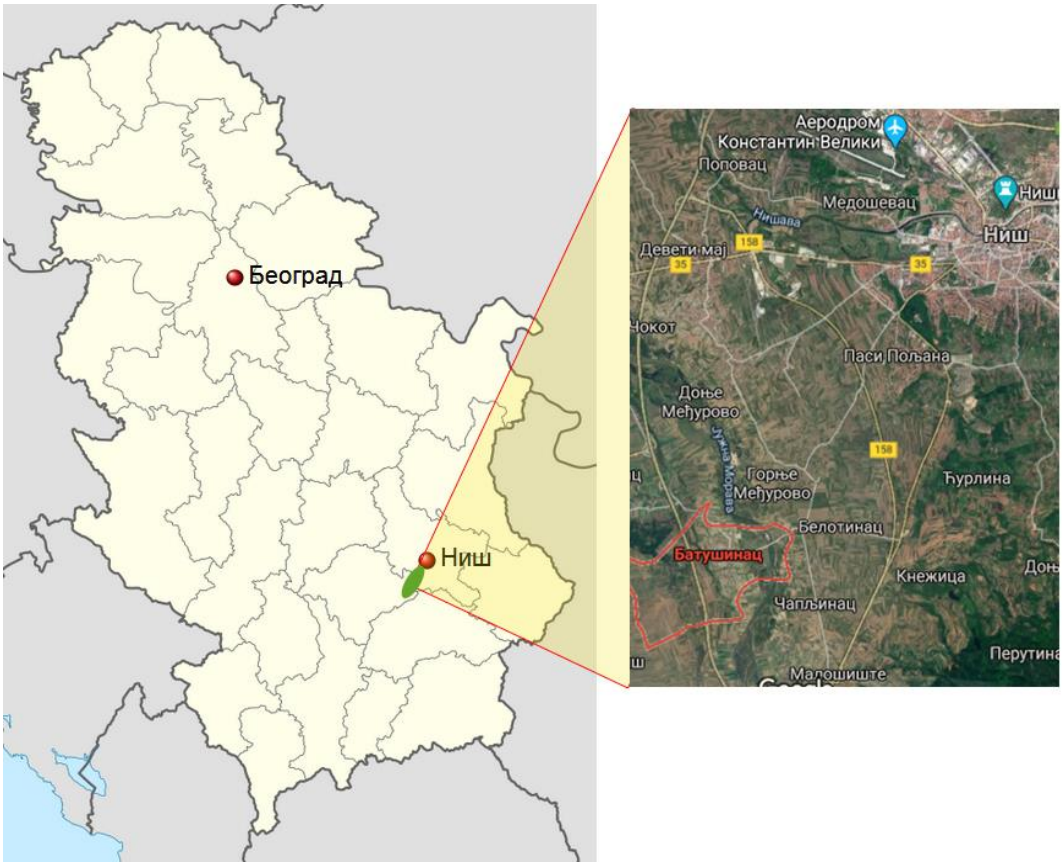

Figure 1. *Study location map (Niš, South Serbia)*

Table 1. *Mean monthly air temperatures (ºC) and monthly amount of rainfall (mm)*

Year	2014/15		2015/16		2016/17		1961-1990		1981-2010	
Month	T_{mean} (ºC)	Rainfall (mm)	T_{mean} (ºC)	Rainfall (mm)	T_{mean} (ºC)	Rainfall (mm)	T_{mean} (ºC)	Rainfall (mm)	T_{mean} (ºC)	Rainfall (mm)
X	12.3	56.8	12.1	81.3	11.1	89.5	11.9	34.1	12.3	45.5
XI	9.1	47.2	7.7	60.2	7.1	129.6	6.4	56.8	2.6	54.8
XII	2.8	87.3	2.9	1.7	-0.5	9.1	1.7	53.6	-0.8	51.5
I	2.1	60.0	0.5	101.3	-4.3	16.6	-0.2	41.3	0.6	38.8
II	3.6	45.2	9.1	45.1	4.9	32.9	2.5	40.3	2.4	36.8
III	6.9	79.5	8.3	62.5	10.8	37.5	6.7	45.3	7.0	42.5
IV	11.5	33.8	14.9	31.2	11.5	69.4	11.9	51.3	12.2	56.6
V	18.4	40.0	15.9	65.6	17.0	68.0	16.6	66.7	17.1	58.0
VI	20.2	53.4	22.5	37.3	22.9	26.0	19.5	69.7	20.4	57.3
VII	25.3	7.5	23.4	64.2	24.7	21.8	21.3	43.6	22.5	44.0
X-VII	11.2	510.7	11.7	550.4	10.5	500.4	9.8	502.7	9.6	485.8
IV-VII	18.8	134.7	19.2	198.3	19.0	185.2	17.3	231.3	18.0	215.9

Winter wheat evapotranspiration (ET) was calculated using the water balance method (*Eq. 1a, b, c*; Simsek et al., 2005):

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$$ETm = R + I \pm \Delta S - D - Ro \quad \text{(Eq.1a)}$$

$$ETa = R \pm \Delta S - D - Ro \quad \text{(Eq.1b)}$$

$$\pm \Delta S = R + I - D - Ro - ET \text{ , (ETm or ETa)} \quad \text{(Eq.1c)}$$

where:
ETm = evapotranspiration determined in irrigation treatment for the growing season;
ETa = evapotranspiration determined in treatment without irrigation for the growing season;
±ΔS = change in root zone water storage over a given time interval;
R = rainfall;
I = irrigation water applied;
D = drainage water (percolation);
Ro = surface runoff which was set to zero.

Water utilization efficiency of winter wheat (WUE) has been calculated as the observed wheat grain yield divided by water consumption for evapotranspiration (*Eq. 2*):

$$WUE = \frac{GY}{ET} \quad \text{(Eq.2)}$$

where:
WUE = water utilization efficiency (kg ha^{-1} mm^{-1});
GY = wheat grain yield (kg ha^{-1});
ET = evapotranspiration (mm).

Drought resistance indices were calculated as below:

- Stress Susceptibility Index (*Eq. 3*; Fischer and Maurer, 1978):

$$SSI = \frac{1 - \left(\frac{Y_s}{Y_p}\right)}{1 - \left(\frac{\overline{Y_s}}{\overline{Y_p}}\right)} \quad \text{(Eq.3)}$$

- Tolerance Index (*Eq. 4*; Rosielle and Hamblin, 1981):

$$TOL = Y_p - Y_s \quad \text{(Eq.4)}$$

- Mean Productivity (*Eq. 5*; Rosielle and Hamblin, 1981):

$$MP = \frac{(Y_s + Y_p)}{2} \quad \text{(Eq.5)}$$

- Geometric Mean Productivity (*Eq. 6*; Fernandez, 1992):

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$$GMP = \sqrt{Y_p \times Y_s} \quad \text{(Eq.6)}$$

- Stress Tolerance Index (*Eq. 7*; Fernandez, 1992):

$$STI = \frac{(Y_s + Y_p)}{(\overline{Y_p})^2} \quad \text{(Eq.7)}$$

- Yield Index (*Eq. 8*; Gavuzzi et al., 1997):

$$YI = \frac{Y_s}{\overline{Y_s}} \quad \text{(Eq.8)}$$

- Yield Stability Index (*Eq. 9*; Bouslama and Schapaugh Jr, 1984):

$$YSI = \frac{Y_s}{Y_p} \quad \text{(Eq.9)}$$

where:
Y_s - yield of cultivar under stress condition;
Y_p - yield of cultivar under irrigation condition;
$\bar{Y}_s$ - total yield mean under stress condition;
$\bar{Y}_p$ - total yield mean under irrigation condition.

Statistical analysis

Data reported for the wheat yield were assessed by the analyses of variance (ANOVA) and Fisher's LSD test was used for all the significant differences at the $P < 0.05$ levels between the means. The relationship between crop yield and water used by evapotranspiration was evaluated using regression analysis. All the statistical analyses were made using software package JMP 15, Copyright SAS Institute Inc.

Results and discussion

The average wheat grain yield was higher by 124.5% with irrigation than without it (*Table 2*). Similarly, the effect of yield increased by 118.3% was established by Dutta et al. (2017) for the approximate values calculated for wheat ET. The highest irrigation during the season of 2014/15 exerted yield increase by 133.3%. The highest percentage of yield increase over the production year probably resulted from the rainfall deficiency in the period from April to July (134 mm) with longer dry periods.

The water used for wheat evapotranspiration with irrigation over the three years of the experiment amounted to 356.9 mm whereas ET amounted to 256.8 mm, being lower by 38.9% under stress conditions. The highest ET value (377.7 mm) with irrigation was recorded in 2015/16 when the highest mean monthly temperature of 19.2ºC was reported for the period from April to July (*Table 1*). The lowest ET value (227.4 mm) was recorded in the production year of 2014/15, during which the lowest amount of 134.7 mm rain fell.

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Table 2. *Grain yield, evapotranspiration and WUE of winter wheat under stress and irrigation conditions*

Year	Conditions	Soil water supplies (mm)	Rainfall (mm)	Irrigation (mm)	Grain yield (t ha^{-1})	ET (mm)	WUE (kg ha^{-1} mm^{-1})
2014/15	Irrigation	63.6	134.7	150	5.89	348.3	16.9
	Stress	92.7	134.7	-	2.68	227.4	11.8
2015/16	Irrigation	59.4	198.3	120	6.25	377.7	16.5
	Stress	77.9	198.3	-	2.85	276.2	10.3
2016/17	Irrigation	69.4	185.2	90	6.37	344.6	18.5
	Stress	81.8	185.2	-	2.73	267.0	10.2
Average	Irrigation	64.2	172.7	120	6.17	356.9	17.3
	Stress	84.1	172.7	-	2.75	256.8	10.8

Note: ET - evapotranspiration, WUE - water utilization efficiency

The ET values calculated for wheat throughout the research with irrigation (344.6-377.7 mm) were similar to those cited by Vučić (1976) and Bošnjak (1999) in the environmental conditions of South Serbia. The ET value determined with irrigation was similar to those from 345 to 385 mm evidenced by Luchiari et al. (1997). In addition, Balwinder-Singh et al. (2011) reported the plant requirements for water to range from 345 to 404 mm. Cui et al. (2018) reported wheat ET to be 402 mm at the invariable value of 70% of FWC being the same as that in the current research. Different ET values of wheat were due to differences in wheat varieties, irrigation regime and pedo-climatic conditions pertained to the area studied.

The WUE of winter wheat irrigation along with optimal soil humidity averaged 17.3 kg ha^{-1} mm^{-1} whereas it averaged 10.8 kg ha^{-1} mm^{-1} without irrigation (*Table 2*). The rational irrigation regime used resulted in significantly higher WUE values, complying with results of numerous authors (Kang et al., 2002; Huang et al., 2005; Sun et al., 2006; Mahamed et al., 2011; M'hamed et al., 2015).

The inter-relation of the linear regression of wheat yield with water consumption on ET (*Fig. 2*) was determined with a positive high significant correlation (r = 0.95**) displayed between the yield and the total water consumption on ET over the three years of the experiment.

All the nine wheat genotypes showed considerably lower mean values (86.6-204.7%) under stress conditions than when irrigated (*Table 3*). Thus, the average wheat yield with irrigation proved to be the highest with Renesansa (6.55 t ha^{-1}) and the lowest with Simonida (5.75 t ha^{-1}). The genotype Pobeda achieved the highest (3.95 t ha^{-1}) and NS Rana 5 the lowest yield (1.92 t ha^{-1}) under stress conditions without irrigation.

Further, the linear regression (*Fig. 3*) confirmed a high percentage of variation between Yp and Ys whereas the correlation coefficient between the wheat yield with irrigation and stress was insignificant (r = 0.61).

The variance analysis showed a noticeable difference in wheat yield as an interaction between the genotype and agroecological conditions with or without irrigation. LSD test also revealed a significant difference in the yield between the genotypes with irrigation conditions (*Table 4*). However, no significant difference in yield could be reported between the following genotypes: Pobeda - Zvezdana, Rapsodija - Renesansa, Renesansa - Evropa 90, KG 56 - Evropa 90, Takovčanka - Simonida and Simonida - NS Rana 5, respectively (*Table 4*).

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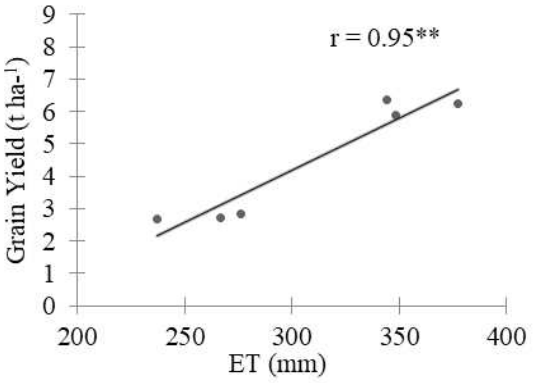

Figure 2. *Regression of grain yield by ET*

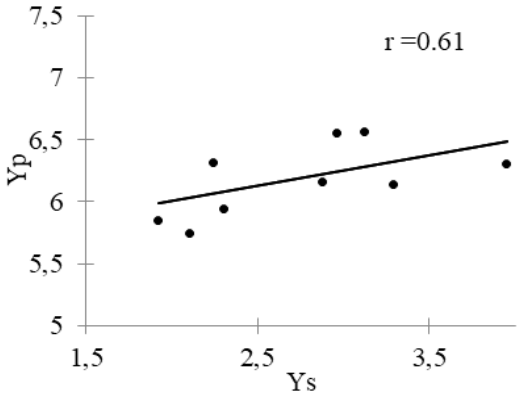

Figure 3. *Regression of Y_p by Y_s*

Table 3. *Mean grain yield of 9 winter wheat genotypes under stress and irrigation conditions (t ha^{-1})*

Genotypes (A)	Conditions (B)							
	2014/15		2015/16		2016/17		Average (A)	
	Irrigation	Stress	Irrigation	Stress	Irrigation	Stress	Irrigation	Stress
Evropa 90	6.31	2.81	5.97	3.26	6.19	2.56	6.16	2.88
KG 56	5.83	2.20	5.64	2.42	6.34	2.28	5.94	2.30
NS Rana 5	5.58	1.83	6.06	1.92	5.92	2.02	5.85	1.92
Pobeda	5.66	3.94	6.51	3.88	6.73	4.02	6.30	3.95
Renesansa	5.96	2.95	6.73	2.77	6.95	3.16	6.55	2.96
Simonida	5.34	1.99	6.06	2.16	5.85	2.14	5.75	2.10
Zvezdana	5.79	2.97	6.16	3.56	6.47	3.36	6.14	3.29
Takovčanka	6.06	2.14	6.25	2.54	6.55	2.03	6.32	2.24
Rapsodija	6.45	3.26	6.87	3.14	6.35	2.97	6.56	3.12
Average (B)	5.89	2.68	6.25	2.85	6.37	2.73	6.17	2.75

Table 4. *Irrigation by genotypes - Fisher's LSD test (2014/15-2016/17)*

Genotypes	Pobeda	Zvezdana	Rapsodija	Renesansa	Evropa 90	KG 56	Takovčanka	Simonida	NS Rana 5
Pobeda	-0.124	-0.112	0.135*	0.147*	0.270*	0.295*	0.496*	0.577*	0.683*
Zvezdana	-0.112	-0.124	0.123*	0.135*	0.257*	0.283*	0.484*	0.565*	0.671*
Rapsodija	0.135*	0.123*	-0.124	-0.112	0.010*	0.035*	0.236*	0.317*	0.423*
Renesansa	0.147*	0.135*	-0.112	-0.124	-0.002	0.023*	0.224*	0.305*	0.411*
Evropa 90	0.270*	0.257*	0.010*	-0.002	-0.124	-0.098	0.102*	0.183*	0.289*
KG 56	0.295*	0.283*	0.035*	0.023*	-0.098	-0.124	0.076*	0.157*	0.263*
Takovčanka	0.496*	0.484*	0.236*	0.224*	0.102*	0.076*	-0.124	-0.043	0.062*
Simonida	0.577*	0.565*	0.317*	0.305*	0.183*	0.157*	-0.043	-0.124	-0.018
NS Rana 5	0.683*	0.671*	0.423*	0.411*	0.289*	0.263*	0.062*	-0.018	-0.124

Note: *significantly different at LSD 0.05

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The variety Pobeda exposed to stress achieved a positive significant difference in its yield compared to the remaining varieties, meaning its best tolerance to stress due to water deficit (*Table 5*). In addition to Pobeda, Zvezdana and Evropa 90 also exhibited a significant tolerance to stress with no significant variations found in yield between the following wheat genotypes: Zvezdana - Rapsodija, Rapsodija - Renesansa, Renesansa - Evropa 90, KG 56 - Takovčanka, Takovčanka - Simonida, Simonida - NS Rana 5.

Table 5. *Genotypes under stress conditions - Fisher's LSD test (2014/15-2016/17)*

Genotyps	Pobeda	Zvezdana	Rapsodija	Renesansa	Evropa 90	KG 56	Takovčanka	Simonida	NS Rana 5
Pobeda	-0.175	0.472*	0.645*	0.812*	0.856*	1.456*	1.533*	1.670*	1.844*
Zvezdana	0.472*	-0.175	-0.002	0.164*	0.208*	0.808*	0.885*	1.023*	1.196*
Rapsodija	0.645*	-0.002	-0.175	-0.009	0.035*	0.635*	0.712*	0.849*	1.023*
Renesansa	0.812*	0.164*	-0.009	-0.175	-0.131	0.468*	0.545*	0.683*	0.856*
Evropa 90	0.856*	0.208*	0.035*	-0.131	-0.175	0.424*	0.500*	0.638*	0.812*
KG 56	1.456*	0.808*	0.635*	0.468*	0.424*	-0.175	-0.099	0.038*	0.212*
Takovčanka	1.533*	0.885*	0.712*	0.545*	0.500*	-0.099	-0.175	-0.038	0.135*
Simonida	1.670*	1.023*	0.849*	0.683*	0.638*	0.038*	-0.038	-0.175	-0.002
NS Rana 5	1.844*	1.196*	1.023*	0.856*	0.812*	0.212*	0.135*	-0.002	-0.175

Note: *significantly different at LSD 0.05

The lower values of SSI and TOL showed susceptibility and tolerance of the genotypes affected by stress. Based on the values calculated for SSI (0.67) and TOL (2.35), Pobeda expressed a more superior tolerance to stress than the other genotypes did (*Table 6*). It is worth mentioning that Zvezdana (SSI-0.84; TOL-2.85), Rapsodija (SSI-0.94; TOL-3.44) and Evropa 90 (SSI-0.95; TOL-3.28) expressed a better tolerance to drought than the remaining wheat genotypes did (Clarke et al., 1992; Golabadi et al., 2006; Talebi et al., 2009; Ilker et al., 2011). Guttieri et al. (2001) stressed that when the SSI values were higher than one, wheat varieties were manifested as extremely susceptible to stress caused by drought.

Table 6. *Drought tolerance indices and mean yield of 9 wheat genotypes under stress and irrigation conditions*

Genotypes	Y_p	Y_s	SSI	TOL	MP	GMP	STI	YI	YSI
Evropa 90	6.16	2.88	0.95	3.28	4.52	4.21	0.46	1.04	0.47
KG 56	5.94	2.30	1.09	3.64	4.12	3.69	0.36	0.84	0.39
NS Rana	5.85	1.92	1.20	3.93	3.89	3.35	0.30	0.70	0.33
Pobeda	6.30	3.95	0.67	2.35	5.12	4.98	0.65	1.44	0.63
Renesansa	6.55	2.96	0.98	3.59	4.75	4.40	0.51	1.08	0.46
Simonida	5.75	2.10	1.14	3.65	3.92	3.47	0.32	0.76	0.36
Zvezdana	6.14	3.29	0.84	2.85	4.75	4.49	0.53	1.20	0.53
Takovčanka	6.32	2.24	1.16	4.08	4.28	3.76	0.37	0.81	0.35
Rapsodija	6.56	3.12	0.94	3.44	4.84	4.52	0.54	1.13	0.46

Note: Y_p - yield of cultivar under irrigation condition, Y_s - yield of cultivar under stress condition, SSI - stress susceptibility index, TOL - tolerance index, MP - mean productivity, GMP - geometric mean productivity, STI - stress tolerance index, YI - yield index, YSI - yield stability index

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The values of indices MP-5.12, GMP-4.98, STI-0.65 calculated for Pobeda favoured stress-tolerance of this genotype along with the similar values found for the yield under irrigation and stress conditions of MP-4.84, GMP-4.52, STI-0.54 for Rapsodija, MP-4.75, GMP-4.49, STI-0.53 for Zvezdana and MP-4.75, GMP-4.40, STI-0.51 for Renesansa, respectively, thereby expressing their high tolerance to stress due to water deficit. Fernandez (1992) claimed STI index to have probably altered the genotypes having a high yield and withstanding stress due to drought. Numerous authors (Talebi et al., 2009; Dadbakhsh et al., 2011; Khodarahmpour et al., 2011; Mohammadi et al., 2011; Sareen et al., 2012) pointed out STI, GMP and MP efficiency with the stress-tolerant genotypes. Khayatnezhad et al. (2010) pointed out that none of these indices could distinctly determine high yielding genotypes under the optimal and stress conditions.

The analysis of calculated results YI (1.44) and YSI (0.63) denoted that Pobeda exhibited a higher stress tolerance than the remaining eight wheat genotypes did. In addition to Pobeda as stress-tolerant to drought, the values of YI and YSI reported for Zvezdana (1.20; 0.53), Rapsodija (1.13; 0.46), Renesansa (1.08; 0.46) and Evropa 90 (1.04; 0.47), respectively, also confirmed their good stress tolerance to drought.

In addition, wheat yield attained with irrigation showed no significant correlation with SSI and TOL indices but it did with MP, GMP and STI (*Table 7*). Thus, when irrigated, the wheat yield achieved a negative with SSI and TOL but a positive significant correlation with MP, GMP and STI. These results comply with those reached by: Fernandez (1992), Shafazadeh et al. (2004), Golabadi et al. (2006), Talebi et al. (2009), Boussen et al. (2010), Anwar et al. (2011), Sarren et al. (2012) and Abdolshahi et al. (2013).

Table 7. *Correlation between drought tolerance indices with yield under irrigation and drought stress conditions*

Variables	Yp	Ys	SSI	TOL	MP	GMP	STI	YI	YSI
Yp	**1**	0.60	-0.47	-0.20	**0.78***	**0.72***	**0.70***	0.49*	0.46
Ys	0.60	**1**	**-0.98***	**-0.90***	**0.96***	**0.98***	**0.97***	0.28*	**0.98***
SSI	-0.47	**-0.98***	**1**	**0.95***	**-0.91***	**-0.94***	**-0.95***	-0.22*	**-0.99***
TOL	-0.20	**-0.90***	**0.95***	**1**	**-0.76***	**-0.82***	**-0.84***	-0.08	**-0.96***
MP	**0.78***	**0.96***	**-0.91***	**-0.76***	**1**	**0.99***	**0.99***	0.39	**0.90***
GMP	**0.72***	**0.98***	**-0.94***	**-0.82***	**0.99***	**1**	**0.99***	0.37	**0.94***
STI	**0.70***	**0.99***	**-0.95***	**-0.84***	**0.99***	**0.99***	**1**	0.32	**0.95***
YI	0.49	0.28*	-0.22	-0.08*	0.39	0.37	0.32*	**1**	0.20
YSI	0.46	**0.98***	**-0.99***	**-0.96***	**0.90***	**0.94***	**0.95***	0.20	**1**

Note: *significantly different at $p < 0.05$; Y_p - yield of cultivar under irrigation condition, Y_s - yield of cultivar under stress condition, SSI - stress susceptibility index, TOL - tolerance index, MP - mean productivity, GMP - geometric mean productivity, STI - stress tolerance index, YI - yield index, YSI - yield stability index

Based on PCA made for the three years of studies, the first two factors accounting for 93.9% data deviation were differentiated (*Table 8*). The first component accounted for 79.3% variation of the calculated indices. This component had a highly negative correlation with SSI and TOL, but a highly positive with Yp, Ys, MP, GMP, STI and YSI and a barely positive one with YI. The second component accounted for 14.6% index

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deviation and was positively correlated with Yp, SSI, TOL and YI but barely correlated with the remaining indices embraced by the current studies.

Table 8. *Principal Component Analysis (PCA) - Correlations between variables and components*

Factor	Total	% of variance	Yp	Ys	SSI	TOL	MP	GMP	STI	YI	YSI
1	7.14	79.35	0.66	0.99	-0.97	-0.87	0.98	0.99	0.99	0.35	0.96
2	1.31	14.58	0.63	-0.08	0.21	0.44	0.14	0.06	0.03	0.77	-0.23

Note: Y_p - yield of cultivar under irrigation condition, Y_s - yield of cultivar under stress condition, SSI - stress susceptibility index, TOL - tolerance index, MP - mean productivity, GMP - geometric mean productivity, STI - stress tolerance index, YI - yield index, YSI - yield stability index

The biplot, resulting from the two basic factors for the genotypes and indices, is presented in *Fig. 4.* Fernandez (1992) denoted to the angles and directions of the vectors indicating the strength and correlation between either one of the two attributes. Thus, a significantly positive correlation was revealed between YI and Yp, Yp and MP, MP, GMP and STI. A significantly positive correlation was also noticed between STI and TOL but solely due to the impact of the second component whereas a significantly negative correlation resulted from that of the first component, which is in agreement with the correlation results outlined in *Table 7*. Therefore, in order to efficiently identify the stress-tolerant genotypes, STI, GMP and MP should be used, which is corroborated by the findings of Fernandez (1992), Talebi et al. (2009), Nouri et al. (2011) as well as by those of Mollasadeghi et al. (2011), Sareen et al. (2012).

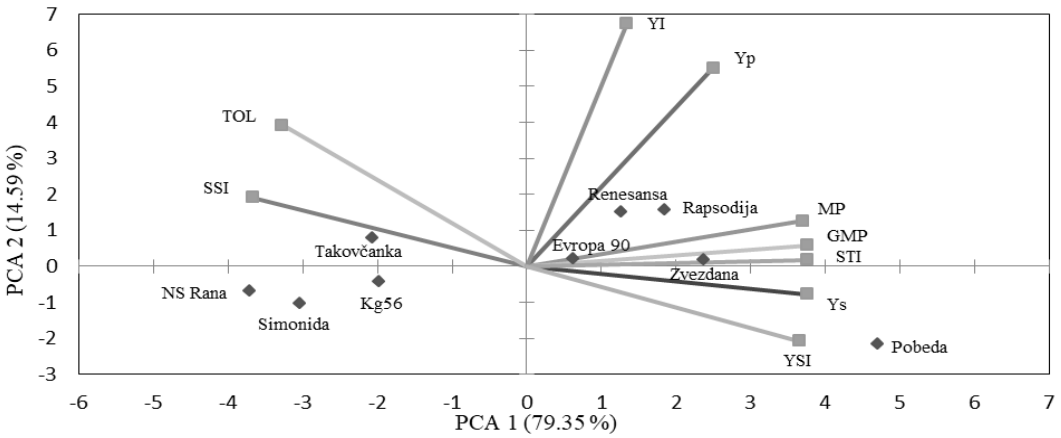

Figure 4. *PCA of drought tolerance indices*

Based on everything mentioned above as well as on the observations about the wheat genotypes within biplot, it may be inferred that Pobeda had a noticeably high tolerance to stress caused by drought along with Renesansa, Rapsodija, Evropa 90 and Zvezdana resisting the stress conditions quite satisfactorily, too.

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Conclusion

Climate changes were the major reason for identifying stress-tolerant wheat genotypes, which would simultaneously lead to their stable yields. Drought led to a significant decrease in the average yield (124.5%). The average water used for wheat evapotranspiration under irrigation conditions over the study period amounted to 356.9 mm and that used for ET under stress conditions to 256.8 mm. The stress tolerance indices of STI, MP and GMP helped to differentiate adequately and efficiently the wheat genotypes of a high yield, both in optimal and in stress conditions. YSI, TOL and SSI values clearly indicated the stress-tolerant genotypes. As confirmed by Fisher's LSD test, the YSI, TOL and SSI proved to be invariably efficient for revealing the stress-tolerant wheat genotypes. The variety Pobeda exhibited a more superior tolerance to stress than it did with the remaining genotypes. Renesansa, Rapsodija, Evropa 90 and Zvezdana also displayed a considerably good tolerance to stress. The study results will, therefore, pave the way for high and stable wheat yields provided that modern farming techniques and adequate agroecological management are used.

Overall, climate changes will pose a grave threat to agricultural production in the future and that´s all the more reason to keep on making long-term experiments of the crop stress tolerance caused by climate extremes so that stable yields can be achieved.

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THE DEVELOPMENT OF WEED VEGETATION IN THE PANNONIAN BASIN AS SEEN IN THE ARCHAEOBOTANICAL RECORDS

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Abstract. Thanks to archaeobotanical investigations, we can now treat it as a fact that the territory of Hungary is one of the longest inhabited parts of Europe. The Pannonian Basin plays a bridging role in the spread of plant cultivation knowledge along the path leading from the Middle East to the Balkans. The relationship between cultivated plants as well as weeds has been determined by the lifestyles of the populations that have lived in the Pannonian Basin and local climatic conditions. The cultivation of several species of plants is only associated with a specific archaeological era or a specific culture. The populations that have lived here have always brought and grown their own cultivated plants with them when they moved into the area. The archaeobotanical research in Hungary reaches back more than 140 years. During this long period of time 50 researchers were active in this topic and about 400 archaeological sites have been processed. Near 10 million seeds of 700 plant taxa (mostly species) were identified in the Pannonian Basin. Our catalogue of the weed remains from Hungarian excavations, indicates species and number of items, is classified on the basis of periods and sites, and ranges from the Neolithic to the Late Middle Ages.
Keywords: *excavation, archaeophyton, Carpathian Basin, ecology, plant cultivation*

Introduction

The study of prehistoric and historic weeds is an important topic of archaeobotany. Many attempts are made to match existing archaeobotanical data with the known agricultural systems of different archaeological eras. Küster (1985) was one of the forerunners who dealt with the distribution and origin of cereal weeds (Secalietea). Based on Rademacher (1968), and Willerding (1986) distinguished five different phases of weed flora development. Almost at the same time Knörzer (1984) described the prehistoric weed association of the North Rhein region under the botanical term *Bromo-Lapsanetum praehistoricum*. Recently, Frumin (2013, 2015, 2017) – partly based on Pysek's results (2002) – proposed an evaluation criterion for archaeobotanical weed records.

The ecological evaluation of weed species is based on the adaptation of ecological indicator values published in Raunkiaer's lifestyle system (1934). This was later re-introduced by Ellenberger et al. (1991). The so-called area classification evaluation system was developed by Ehrendorfer (1973) and further improved later on by Oberdorfer (1983).

The terms *thanatocoenology* and *thanatocoenosys* were created by Willerding (1983) based on the analysis of the ecological properties of weed remains recovered from

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various excavations. Later on Jacomet et al. (1989) recommended a metric evaluation system of the possible habitats instead of attempting to reconstruct once existing plant associations. The so called *'comprehensive weed history method'* turned out to be an important and significant approach to understanding the agricultural development of archaeological cultures (Willerding, 1986; Jacomet et al., 1989; Kreuz et al., 2005; Kreuz and Schäfer, 2011).

In Hungary, the history of weed association development studies was written by herbologists and ecologist. Identification guides that were compiled in the last century also dealt with the dispersion of weed species. The works of Schermann (1966), Hunyadi (1988), Radics (1998), and Hunyadi et al. (2000) have to be mentioned among others. The ecological history and recent distribution of weeds within the Pannonian Basin has always been a significant issue and was dealt by numerous scholars such as Ubrizsy (1955), Ujvárosi (1957, 1973), Czimber (1987), Bartha (2000), Csontos (2001), Priszter (1997), Dancza (2011), and Lehoczky et al. (2013).

Weed associations were identified and described by Soó (1964-1985) and Borhidi et al. (2012). Based on the works of Ujvárosi (1952), Kárpáti et al. (1968), Soó (1973), Horváth et al. (1995), and Borhidi (1995) the so called life-form classification system and scale of Hungarian weed flora was also developed; a system aiming at the numerical and ecological analysis of weed associations.

The problems of apophytes (native species), archaeophytes (non-native, arriving before the 15th century) and neophytes (non-native, arriving after the 15th century) within the Pannonian Basin was first addressed by Terpó et al. (1999), Pinke and Pál (2005), Botta-Dukát and Balogh (2008), Balogh and Gyulai (2014) as well as by Henn et al. (2014) later on.

The study of invasive plants falls also within the issues of weed studies (Mihályi and Botta-Dukát, 2004). Processes of *synanthropisation* and anthropogenic effects influencing landscape changes were studied by Terpó (2000) and Pinke et al. (2011). Grouping of synanthropic plant species of different archaeological eras was done by Berzsényi (2000). Due to the effects of intensive agriculture many of the archaeophyton species are endangered by extinction in the Pannonian Basin today. For this reason, their examination also falls within the interest of historical agro-biodiversity research. A few of the adventive weeds are already on the list of protected and endangered plant species of Hungary (Udvardy, 2000).

In comparison to the above mentioned works, only very few studies dealt with the history, dispersion and development of weed species from an archaeobotanical perspective (Füzes, 1990; Gyulai et al., 1992; Berzsényi, 2000; Gyulai et al., 2013; Gyulai and Lakatos, 2013; Kenéz, 2014; Pósa et al., 2015). The basis of these is a catalogue of the weed finds sorted according to cultures and taxa, which was first compiled by Hartyányi et al. (1968, 1974), and later on improved by Gyulai (2010).

Materials and methods

The archaeobotanical record

Since the beginning of archaeological research (1860) in Hungary, nearly 50,000 sites were found. Out of these, 414 sites (1%) have been studied from an archaeobotanical aspect (*Fig. 1*). Most settlements are known to be from the Middle Neolithic (55 sites), the Roman Age (53 sites), and the Late Middle Ages (37 sites) (*Fig. 2*). Our research is based on the supplemented and updated archaeobotanical

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dataset compiled by Gyulai (2010). This is a catalogue consisting of the seed, fruit, food and beverage remains from Hungarian excavations, indicating species and number of items, classified on the basis of periods and sites, ranging from the Neolithic to the Modern Ages, from the beginning of archaeobotanical research, up to the present, and in a chart form.

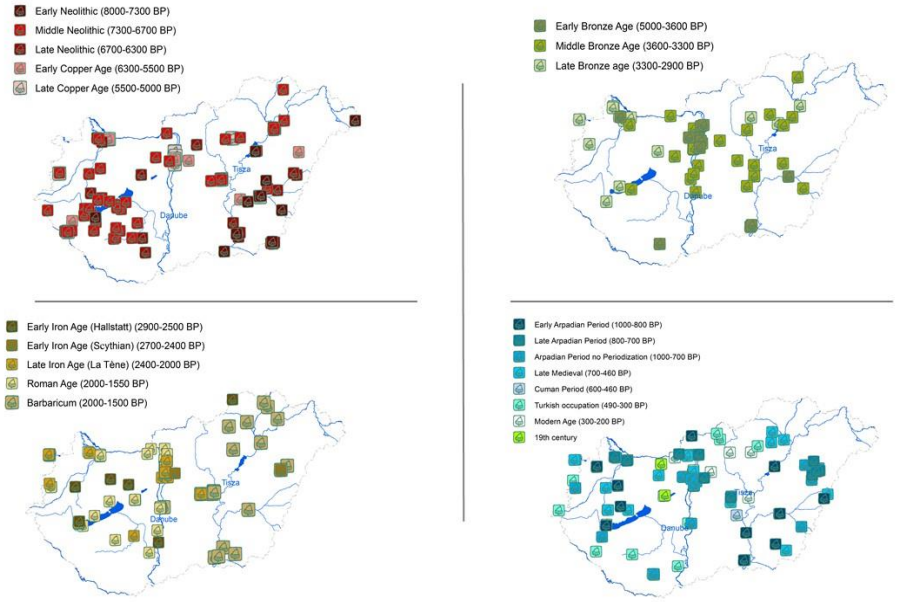

Figure 1. *Map of the archaeological sites in Hungary*

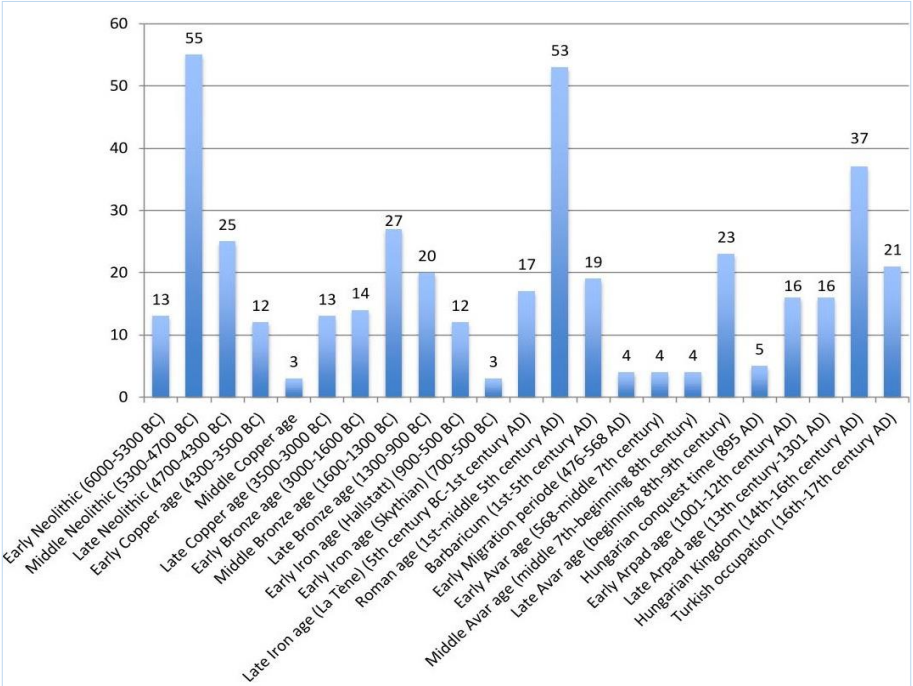

Figure 2. *Number of archaeological sites in every archaeological era, at which archaeobotanical analysis was carried out*

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Methodological considerations

The research dataset was completed by the selection of weeds, based on the identification criteria published earlier by Jacomet et al. (1989). This resulted in the list of weed species according to archaeological periods. The weed species have been classified as follows: cereal weeds, root- or summer crop weeds and ruderal weeds. The database has been expanded with additional data: type of area, life-form, residence time status (apophyte, archaeophyte, neophyte) and height (low, medium, high). In the list of weed species, a value of '1' was assigned to species which were present in the archaeological period, whereas '0' means they weren't present. In case a weed was present in multiple periods, all instances were assigned a '1', since if we didn't, our results would've been deformed. Analysing the huge data set was conducted using the IBM SPSS Statistics 22.0 software. The statistical analysis encompassed the following:

- Relationship between cereals and cereal weeds.
- Fluctuation of cereal weed species.
- Distribution of cereal weed species, depending on if they are present in the latest age or not, regarding first appearance.
- Distribution of cereal weed species by plant height, first appearance.
- Distribution of cereal weed species by life form, first appearance.
- Distribution of cereal weed species by area.

Results

The distribution of the plant species broken down to the archaeological eras of the Pannonian Basin gives us an overview of when the number of weeds increased throughout the history of the geographical area. The periods of increase of weed species: Middle Neolithic, Middle Bronze age, Roman age, Late Middle Ages (*Fig. 3*).

It is interesting to note that the number of weed species of the settled nomadic cultures with steppe origins coming from the east (Middle Copper age, Scythians, Sarmatians, Avars, Hungarians) was always higher than in the previous period. Some segetal weeds (e.g. *Agrostemma githago*, *Bromus sp.*) arrived with Neolithic farmers from the south-east, who later migrated slowly towards the west accompanied by indigenous species of Central and Eastern Europe.

Distribution of cultivated and weed species

Together with the rising number of cultivated species the diversity of weeds grew as well (*Fig. 4*).

However, there is no significant difference in the proportion of cereal weed, root- or summer crop weed and ruderal weed species during the different periods. Although the typical process is growth, yet in some age to fall: Copper Age, Early Bronze Age, Migration Period. In our opinion, the separation of winter- and summer crop weeds was not realised in the Pannonian Basin; they arrived into this separated geographical area in with their original ecological 'faith'.

The increasing number of ruderal species may be closely related to the rising number of settlements in certain periods. At the beginning of the Neolithic era (e.g. Körös culture), new, foreign species associated with plant cultivation appeared in the landscape. At the beginning of cereal cultivation, einkorn wheat, emmer wheat and barley were the characteristic cereals with shorter growth-cycle common millet was

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added in the Bronze Age. Both cultivated and wild ones also arrived, primarily from Asia Minor and the Mediterranean, and to a lesser extent from Asia. These had lived in association with domesticated plants, often as wild relatives of domesticates in their places of origin. But, in a cultivated context, they were simply weeds. The first segetal associations of the Neolithic and the Bronze Age had relatively high number of species, much higher than expected. According to archaeological plant material, white goosefoot (*Chenopodium album*) was present in very large quantities, while *Avena fatua*, *Bromus arvensis*, *B. secalinus*, *Chenopodium hybridum*, *Fallopia convolvulus*, *Galium spurium*, *Vicia angustifolia* were also common. In the Roman age several new segetal species appeared: *Abutilon theophrasti*, *Anthemis cotula*, *Bifora radians*, *Centaurea cyanus*, *Cynodon dactilon*, *Diplotaxis muralis*, *Lathyrus hirsutus*, *Lepidium draba*, *Myagrum perfoliatum*, *Myosotis arvensis*, *Torilis arvensis*, *Vicia villosa*. In the Middle and in the early Modern Age the weed flora enriched with new species, in which grain trade also played a role: *Alopecurus myosuroides*, *Amaranthus retroflexus*, *Euphorbia exigua*, *Galeopsis tetrahit*, *Lepidium perfoliatum*, *Ranunculus sardous*, *Silene noctiflora*, *Vaccaria pyramidata*, *Valerianella carinata*.

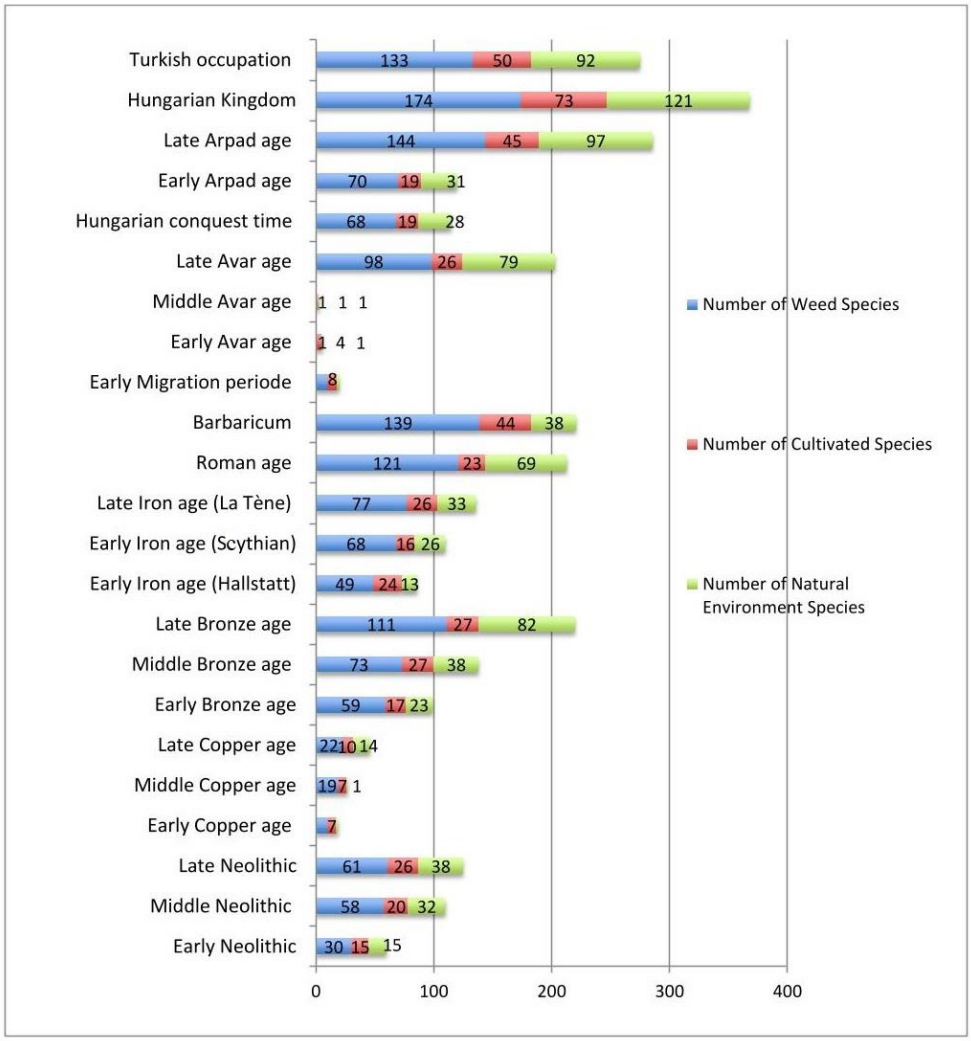

Figure 3. *The number of weed, cultivated and natural environment plant species throughout the archaeological eras of the Carpathian Basin*

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	Early Neolithic (6000–5300 BC)	Middle Neolithic (5300–4700 BC)	Late Neolithic (4700–4300 BC)	Eneol /Copper age (4300–3000 BC)	Early Bronze age (3000–1600 BC)	Middle Bronze age (1600–1300 BC)	Late Bronze age (1300–900 BC)	Early Iron age (900–500 BC)	Late Iron age (La Tène) (5th century BC–1st century AD)	Roman age (1st–middle 5th century AD)	Barbaricum (1st–5th century AD)	Migration periode (5th–9th century)	Hung. conq. - Early Arpad age (895–12th century)	Late Arpad age (13th century–1301 AD)	Late medieval/Hung. Kingdom (14th–16th century AD)	Early New Age/Turkish occupation (1526–17th century AD)
Number of cultivated species	15	20	26	13	17	27	27	24	26	23	44	32	28	45	73	50
Number of cultivated seeds	655	394918	476944	9573	2816	278687	159441	101339	10644	225212	232938	118180	367965	38835	3632970	241954
Number of cereal species	12	12	12	9	10	13	12	11	12	13	13	10	10	13	13	10
Number of cereal grains	644	394549	457496	9563	2806	253622	156806	101102	10402	218719	231847	115578	332292	37444	1728354	171661
Number of cereal weed species	21	42	43	26	45	58	77	64	63	90	97	73	80	96	123	92
Number of cereal weed seeds	203	3622	751	232320	272	39394	4480	5494	1327	5169	4792	141808	8774	80887	156471	89006
Number of root-or summer crop species	3	8	12	4	7	13	14	10	11	6	15	11	13	17	30	21
Number of root- or summer crop weed species	10	17	11	6	10	13	27	24	18	31	27	24	23	33	36	27
Number of root- or summer crop weed seeds	64	3346	460	160	65	36988	3756	3816	942	3955	2276	140517	52874	75825	89864	5493
Number of ruderal species	10	22	26	14	25	25	47	35	26	47	60	38	42	68	78	68
Number of ruderal seeds	247	3234	470	179	57	37609	3695	3381	1058	24586	2338	140986	53385	90195	50526	241011
Number of natural environment species	15	32	38	16	23	38	82	48	33	69	38	86	82	97	121	92
Number of natural environment seeds	395	175	1589	1786	116	185	3660	107	811	928	476	2401	987	2693	1538224	6568

Figure 4. *The most important data of the Hungarian Archaeobotanical Database*

Distribution of weed species by residence time status

There is no significant difference between the proportion of apophytes and archaeophytes during the different archaeological eras (*Fig. 5*).

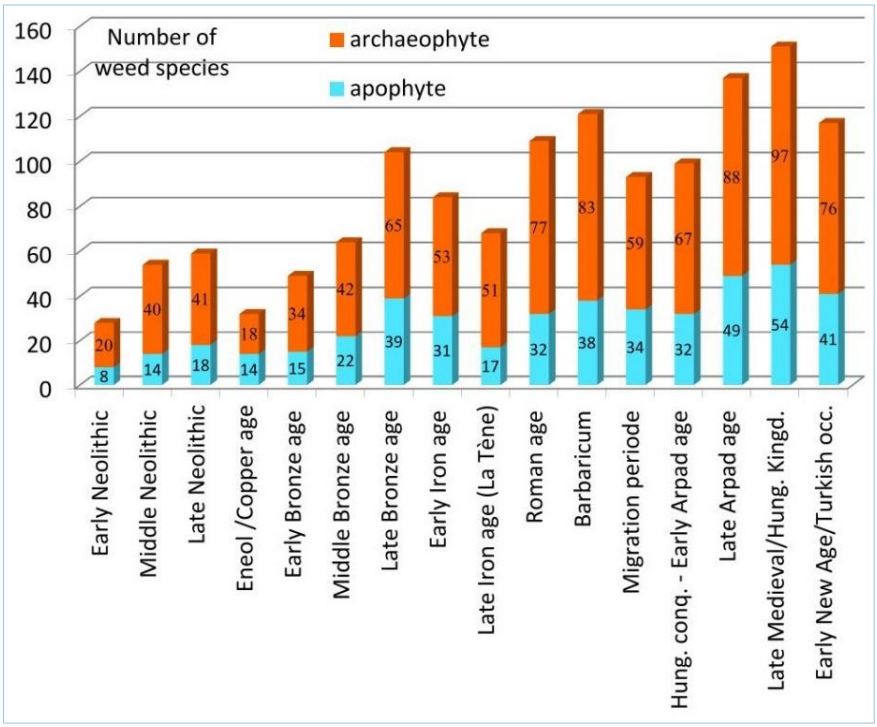

Figure 5. *The number of archaeophyte and apophyte weed species throughout the archaeological era of the Carpathian Basin*

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Until the Early Modern Age apophyte and archaeophyte species were dominant. However, there are terminological errors of their interpretation. When compared to western Europe, Bulgarian and Hungarian LBK species, five of them cannot be Hungarian apohytes because they also occurred in Bulgaria. So, the current division in other ages should also be reviewed. In addition to weeds introduced with sowing-seeds, elements of the previous flora were also present for some time. Domesticated species cultivated on arable land meant competition for the components of natural vegetation. Other plant species were less able to adapt to the changed conditions resulting from cultivation and subsequently disappeared. During the Middle and Late Neolithic period, a whole range of foreign weed species migrated to the central regions of Europe. Before AD 1500, the landscape was dominated by associations of archaeophytes and apophytes: archaeophytes: e.g. *Agrostemma githago*, *Centaurea cyanus*, *Echinocloa crus-galli*, *Papaver rhoeas*, *Setaria pumila*, *Sinapis arvensis*, *Stachys annua*, apophytes: *Artemisia vulgaris*, *Agropyron repens*, *Centaurea cyanus*, *Consolida regalis*, *Digitaria sanguinea*, *Portulaca oleracea*, *Raphanus raphanistrum*.

Relationship among cereals and cereal weeds

The number of cereal species in the Early Neolithic was higher than in later periods (*Fig. 6*). The existing cereal species were supplemented with new ones. In the latter two the high number of weeds may be related to manuring. In the beginning hulled wheats (einkorn wheat, emmer wheat) and naked barley, but later, after the Roman Age common wheat and rye were dominant. Until the Late Iron age, the number of cereal weed seeds were low compared to cereal grains. (Seed treatment was more effective perhaps?) The trend changed in the Roman Age. The number of cereal weed remains are many times higher than that of cereal grains. The number of weed species by winter cereals (einkorn wheat, emmer wheat, spelt, common wheat, dwarf wheat) and rye was proportionally higher than that of hulled wheat.

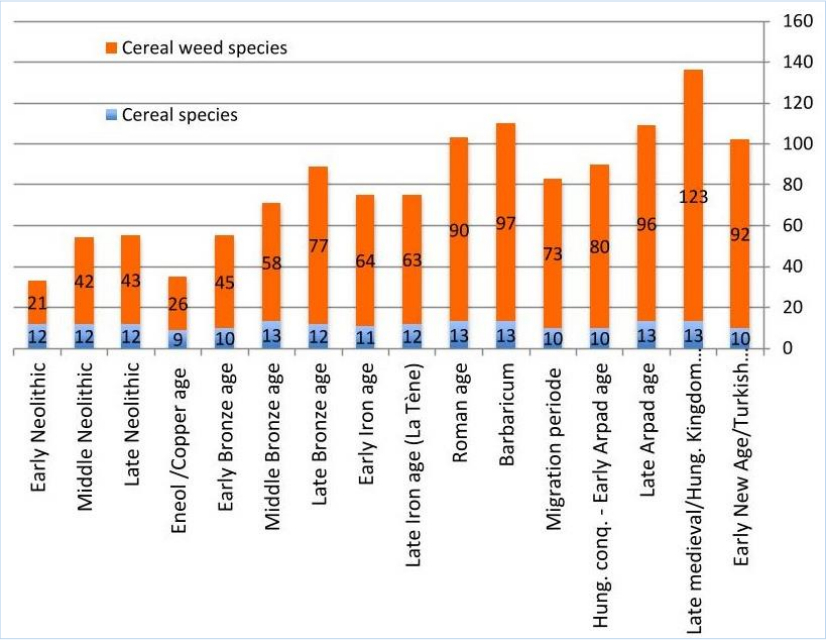

Figure 6. *The relation between cereal and cereal weed species throughout archaeological eras of the Carpathian Basin*

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The Neolithic, Bronze Age, Roman Age/Barbaricum and Late Middle Ages were the periods when most cereal weed species appeared (*Fig. 7*). Many cereal weed species disappeared in the Roman period (7) and even more in late Middle Ages (50!). In the Roman Age and late Medieval period, the weed flora changed thoroughly. Why? Both periods saw the use of ploughshare and manuring (*Fig. 8*).

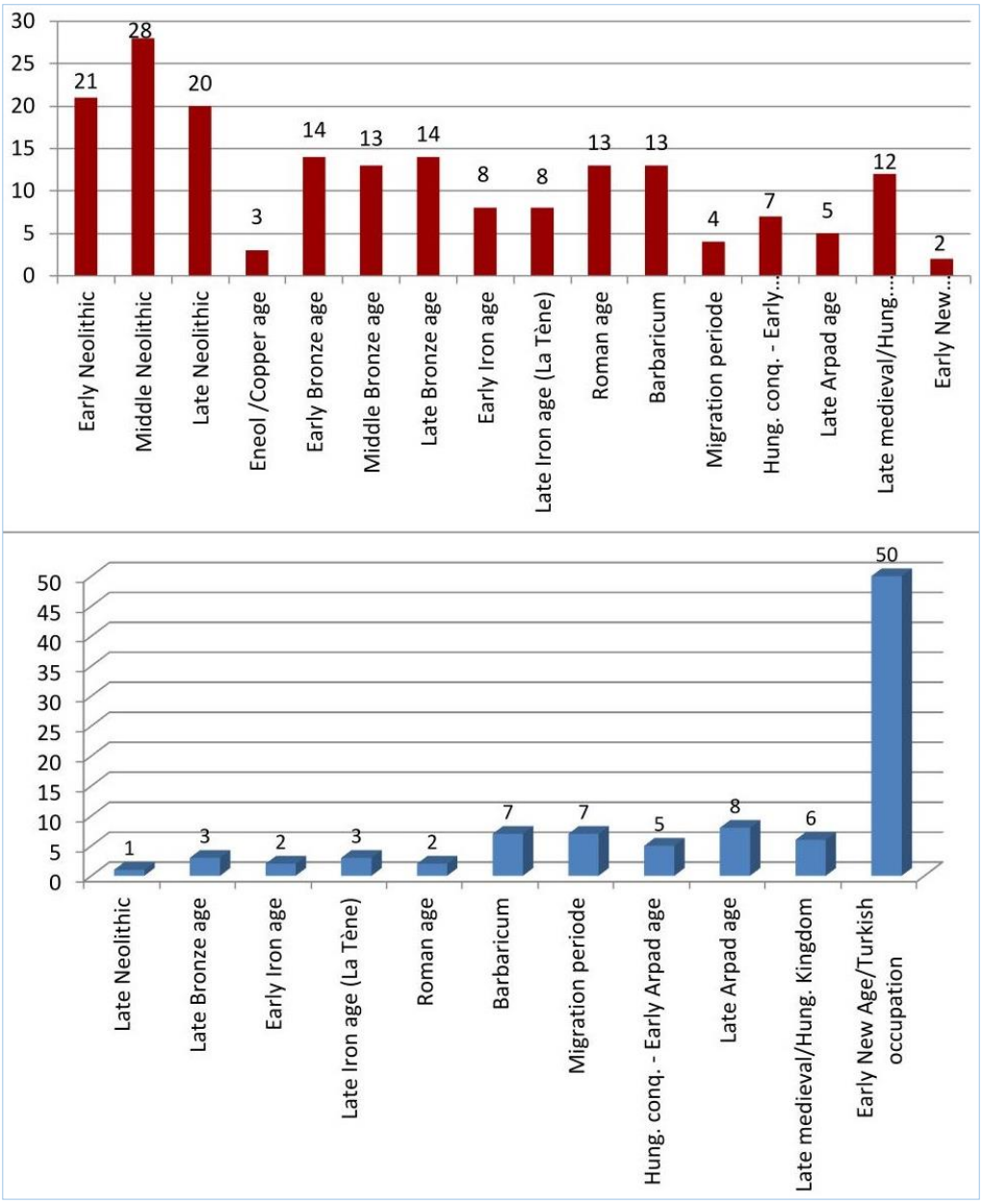

Figure 7. *The number of appearing (above) and disappearing (below) weed species within the archaeological eras of the Carpathian Basin*

Distribution of cereal weed species by plant height in first appearance

The presence of cereal weeds allows us to infer the time of sowing and the method of cultivation of cereals and the method of harvesting (high or low, with a sickle or with a scythe). Distribution of cereal weeds by height suggests that from the beginning until

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Late Middle Ages the use of sickle for harvesting was common. Dominant are the medium, medium/high and high species (*Fig. 9*). They are mostly high weed plants, which indicates that cereals were harvested using a sickle, at about two-thirds of the height of stalks. In the Modern Age the new weeds are higher, therefore the harvesting method starts to change: from sickle to scythe. Of course, do not forget that the cereals were previously higher than they are today.

	Early Neolithic (6000–5300 BC)	Middle Neolithic (5300–4700 BC)	Late Neolithic (4700–4300 BC)	Eneol /Copper age (4300–3000 BC)	Early Bronze age (3000–1600 BC)	Middle Bronze age (1600–1300 BC)	Late Bronze age (1300–900 BC)	Early Iron age (900–500 BC)	Late Iron age (La Tène) (5th century BC–1st century AD)	Roman age (1st–middle 5th century AD)	Barbaricum (1st–5th century AD)	Migration periode (5th–9th century)	Hung. conq. - Early Arpad age (895–12th century)	Late Arpad age (13th century–1301 AD)	Late medieval/Hung. Kingdom (14th–16th century AD)	Early New Age/Turkish occupation (1526–17th century AD)
Climate phase	Boreal/Atlantic	Atlantic	Atlantic	Atlantic	Subboreal	Subboreal	Subboreal	Subboreal	Subatlantic	Subatlantic	Subatlantic	Subatlantic	Subatlantic	Subatlantic	Subatlantic	Subatlantic
Number of settlements	13	55	25	28	14	27	20	15	17	53	19	35	19	16	37	21
Number of Culture	1	6	3	6	3	7	4	2	1	1	1	5	1	1	1	2
Culture	Körös-Starčevo	LBK Great Hungarian Plain, LBK Transdanubia group, LBK Notenkopf and Sopot-Bicske cultural phases, Sopot, Szakálhát-Szilmeg group, Tiszadob group	Tisza, Herpály, Lengyel	Bodrogkeresztúr, Balaton-Lasinja, Ludanice, Protoboleráz, Boleráz, Baden	Bell Beaker, Somogyvár-Vinkovci, Makó	Füzesabony,N ágyrév, Vatya, Magyarád, Hatvan, Pécel, Otomány	Gáva, Urnfield, Tumulus, Kyjatice	Hallstatt, Skythian	La Tène (Celtic)	Roman	Sarmatian	Gepid, Longobard, German, Avar, Slav	Hungarian	Hungarian	Hungarian	Hungarian, Turks
Mode of life	settled	settled	settled	wandering livestock/settled	settled	settled	settled	settled, settled nomad	settled	settled	settled nomad	nomad/settled nomad	settled	settled	settled	settled
Land use	slash-and-burn agriculture	slash-and-burn agriculture	slash-and-burn agriculture	slash-and-burn agriculture	slash-and-burn agriculture	not controlled fallow change	not controlled fallow change	not controlled fallow change	not controlled fallow change	controlled fallow change + manuring	not controlled fallow change	not controlled fallow change	controlled fallow change	controlled fallow change	controlled fallow change	two-field rotation + manuring
Soil tillage equipment	digging stick	digging plow (ralo)?	digging plow (ralo)?	digging plow (ralo)?	digging plow (ralo)	digging plow (ralo)	digging plow (ralo)	digging plow (ralo)	digging plow (ralo) with iron slippers	single-sided plow with iron slippers	digging plow (ralo)	digging plow (ralo) with iron slippers	single-sided plow with iron slippers	single-sided plow with iron slippers	single-sided plow with ploughshare	single-sided plow with ploughshare
Harvest	with hand, primitive sickle/harvest knife	primitive sickle or harvest knife	primitive sickle/harvest knife	primitive sickle or harvest knife	primitive sickle or harvest knife	bronze sickle	bronze sickle	sickle	iron sickle	serrated or "toothed" iron sickle	sickle	curved and hooked sickle	curved and hooked sickle	curved and hooked sickle	curved and hooked sickle	curved and hooked sickle + scythe

Figure 8. *Land use, harvest and other important data of the Hungarian Archaebotanical Database*

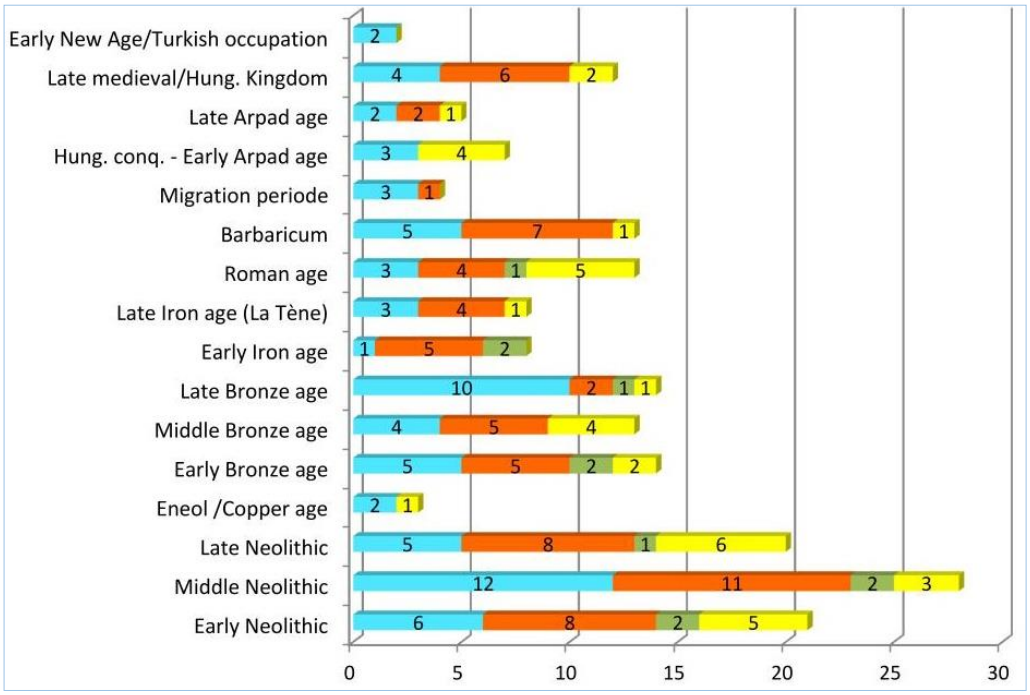

Figure 9. *Distribution of cereal weed species by plant height and first appearance*

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Discussion and conclusions

The up-to-date archaeobotanical database of Hungary contains the results of each known archaeological site, which was studied from an archaeobotanical point of view. Regardless of their publication the database contains all accessible data. This database, which provides an overview of the archaeobotanical results from 186 onwards was the basis of the present study, in which we analysed the development of weeds in the Pannonian Basin. Not only the list, but the changes in the appearance of apophyte and archaeophyte species was also compiled. The weed species were sorted according to height, by life form and by distribution area.

The appearance of weeds is connected to certain archaeological ears and phases: Early and Middle Neolithic, Middle Bronze age, Roman age, Late Medieval. This overlaps with the expansion of harvested species and a connection between the harvested and weed flora is emphasised.

The earliest dominant weed species in the Neolithic are: *Agrostemma githago, Avane fatua, Bromus arvensis, Bromus secalinus, Chenopodium album, Fallopia convolvulus, Galium spurium*. The most widespread and dominant seven weed species of the Pannonian Basin were studied.

The examination of the weeds according to their height shows that the ratio of medium and high species does not change from the beginning (Neolithic), which suggests that sickle was used until the Early Middle Ages to harvest cereals. Scythe was probably only used for harvesting later on.

No significant change can be detected in the ratio of the apophytes and the archaeophytes over time.

The fluctuation in the area-based distribution of the weed species is high. The strong Mediterranean effect in the Neolithic decrease by time and the number of Eurasian and Circumpolar species increases.

The species composition and the change of the number of the cereal weeds is related to land management practices (e.g. use of plow), soil fertilising techniques (e.g. manuring) and with the increase of the level of arable crop production.

The weeds of autumn- and spring-sown cereals already separated at the beginning of the Neolithic. This ecological separation already appeared outside the Pannonian Basin. At the beginning the presence of winter crop weed species was higher, but with the appearance of the leguminous plants in the Bronze Age and the garden species in the Late Middle Ages, the significance of summer crop weeds or row crop weeds increased.

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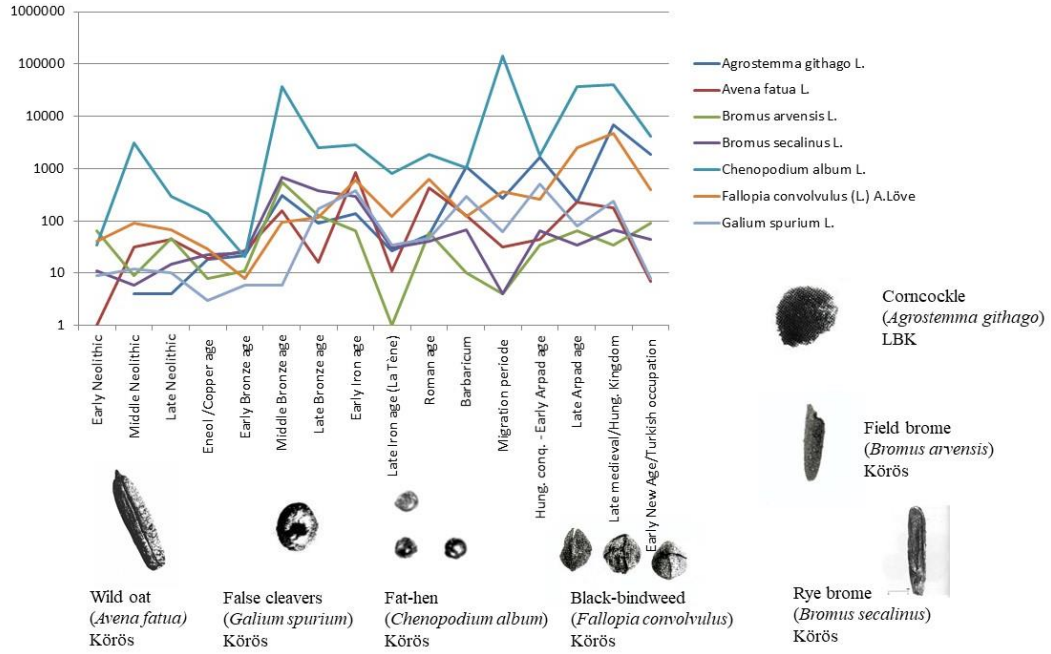
APPENDIX



Appendix 1. *Career of the "seven great evil". Number of grains in logarithmic distribution*

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PLANT STRESS INDUCED BY EXCESSIVE SUCROSE AND AGAR CONCENTRATION ON *IN VITRO* GERMINATION AND PLANTLET GROWTH OF *LAURUS NOBILIS* L. (LAURACEAE)

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Abstract. *Laurus nobilis* L. a member of Lauraceae family, is a beautiful medicinal and aromatic, evergreen plant. In this study shortening of *in vitro* germination time and the appropriate sucrose and agar concentration for this approach were investigated. Excessive sucrose concentration negatively affected germination percentage, root length, shooting percentage and shoot length in Murashige and Skoog Medium (MS). Medium without sucrose (0 g/L sucrose) having the highest germination rate (96.3%), the highest root length per plant (3.84 cm), the highest shooting rate (96.3%) and the highest shoot length per plant (1.68 cm) in all concentrations of sucrose used (0, 10, 20 and 30 g/L) after 4 weeks of culturing. Although sucrose and agar interactions exerted effects on the germination percentage, additionally all concentrations of agar used (3, 6 and 9 g/L) showed that adding excessive amounts to the growth medium was not necessary to induce germination and shooting in general. 0 mg/L sucrose and 3 g/L agar found optimum composition of the media for both rooting and shooting in all used media. This protocol will be useful for rapid and economic large-scale *in vitro* cultivation of *Laurus nobilis* to obtain aseptic seedlings.
Keywords: *recalcitrant seed culture, tissue culture, organic additives, medium specification, rooting, shooting*

Introduction

Developing an efficient and rapid system for *in vitro* tissue and organ culture is highly dependent on plant genus, species, physical and chemical culture environment. The content of culture media for germination, shooting, rooting and multiplication has an enourmous efficacy on costs in large-scale commercial manufacturers producing ornamental plants, vegetable seedling, fruit sapling and secondary metabolites as well as governmental or private scientific research and development centers.

During the past decades different media have been developed for *in vitro* plant culture. One of the known important components are carbon sources such as sucrose. Sucrose is mostly used 2-4% to bring in the young explant ready to use carbon (Gamborg et al., 1976). Especially 3% concentration is choosen in lots of studies as known recommendation of (Murashige and Skoog, 1962). In some cases high level of sucrose can be caused decreasing *in vitro* germination or the other growth parameters (Jo et al., 2009; Huh et al., 2016). The second known components are gelling agents such as agar. Generally, if solid media is aimed to use, agar is added 0.6-0.8% ratio to increase media viscosity (Debergh, 1983). In some cases increasing ratio of the gelling agent led to a progressive decrease in

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adventitious shoot per explant (Owens and Wozniak, 1991; Casanova et al., 2008). On the contrary, decreasing ratio of the agent cause turning media solid to liquid is not suitable in some conditions such as germination, rooting or shoot multiplication in some plants. Because of inability of the explant to hold on the surface and subside to the bottom cause undesired physical condition as non-stability of the explant and oxygen deficiency for the plant parts. The model plants for the study, bay laurel (*Laurus nobilis* L.) belongs to the family Lauraceae family which include valuable genus (Werff and Richter, 1996; Judd et al., 1999; Marques, 2001). Bay laurel are dioecious, evergreen tree or shrub known as laurel, bay laurel or sweet bay (Marzouki et al., 2009). Nearly all plant parts have been used as medicinal-aromatic, ornamental, plant-animal health and environmental purpose for a long time (Patrakar et al., 2012; Chalal et al., 2017). Active chemicals of most of these usage obtained from traditionally grown or naturally existing plants. The high economic value of the species in Lauraceae family has caused these to be destroyed in the natural habitats over years as our long term observations. Optimized tissue culture techniques can provide protection of natural habitats. There are a few studies for different purposes given some information the *in vitro* propagation of *Laurus nobilis* L. (Rady et al., 1999; Souayah et al., 2002; Al-Gabbiesh et al., 2014; Nadarajan and Pritchard, 2014; Royandazagh, 2019). The objective of the present study was to evaluate the effect of sucrose and agar concentrations on *in vitro* germination, primary root and primary shoot growth of bay laurel (*L. nobilis*) in *in vitro* seedlings to achieve scientific and economic gain.

Materials and Methods

The research was carried out at Kocaeli University, Plant Tissue Culture and Biotechnology Laboratory during 2018-2019. The fruits of laurel were picked up from only one tree that have been observing for many years, at natural habitats of Kocaeli City in Turkey. As soon as the bay laurel fruits were collected at 2018 November, 24; they were brought to the laboratory and the fruit flesh was peeled with the help of paper towel. One day after this procedure, the seed coats were removed with fingernail to obtain naked seed. The naked seed (the average weight of 100 naked seeds was 0.574 g/seed) kept in refrigerator (8 ºC) until the next day to use. Seeds were washed with running tap water for 1 hour and soaked for 40 min. in 20% commercial bleach (Na-hypochloride 5≤) solution and rinsed in two changes of sterile distilled water. Seeds were cultured on MS medium (Murashige and Skoog, 1962) with full micro, macro elements and vitamins on four trade mark sucrose ($C_{12}H_{22}O_{11}$-MW:342.29) (0, 10, 20 and 30 g/L) and three trade mark agar-agar concentration (3, 6 and 9 g/L). The pH of the medium was adjusted to 5.6 with 1 M KOH or 1 M HCl prior to autoclaving for 15 min. at 121 ºC. The media were filled in sterile glass petri plates in 6 cm diameter subsequently sterile-naked seeds were placed on the surface of media and kept in culture room at 23 ºC under dark condition for four weeks. Each experimental treatment combination consisted of two factors; sucroseXagar (4X3). The experiment laid out in completely randomised design with 3 replications. In only one repeat which was carried out with 3 glass petri dishes each of which contained only one seed. Results were evaluated weekly after cultures along four weeks. The recorded parameters were germination percentages (%), root length (cm), shooting percentages (%) and shoot length (cm) of the newly germinating seeds. The data were subjected to analysis of variance and significant differences among the treatments were tested using two-way ANOVA and means were separated by Duncan Multiple Range Test at $P \leq 0.05$.

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Results and Discussion

Germination started as of the first week and negative effect of high sucrose concentration observed and statistically detected by the time. There was no positive or negative effect of agar concentration on germination percentage along 4 weeks and at the end of study (*Tables 1, 2, 3, 4; Fig. 1*). The combination of sucrose and agar in used MS medium significantly affected *in vitro* seed germination percentage by the second week. At the same time root length was also statistically and negatively affected by high sucrose concentration at the end of the experiment.

Table 1. *Laurus nobilis L. germination percentage and root length at the end of the 1st week in the modified MS medium*

Germination Percentage (%) at the end of the 1st week					**Root Length (cm) at the end of the 1st week**				
	3 g/L Agar	**6 g/L Agar**	**9 g/L Agar**	**Mean of Sucrose*** **		**3 g/L Agar**	**6 g/L Agar**	**9 g/L Agar**	**Mean of Sucrose*** **
0 g/L Sucrose	33.3*	22.2	33.3	**29.6 A**	**0 g/L Sucrose**	0.17a****	0.10ab	0.10ab	**0.12 A**
10 g/L Sucrose	22.2	11.1	33.3	**22.2 AB**	**10 g/L Sucrose**	0.13ab	0.03b	0.13ab	**0.10 AB**
20 g/L Sucrose	22.2	11.1	0.0	**11.1 AB**	**20 g/L Sucrose**	0.06b	0.03b	0.00b	**0.03 AB**
30 g/L Sucrose	0.0	0.0	0.0	**0.0 B**	**30 g/L Sucrose**	0.00b	0.00b	0.00b	**0.00 B**
Mean of Agar**	**19.5**	**11.1**	**16.7**		**Mean of Agar****	**0.09**	**0.04**	**0.06**	

*N.S.; No significant difference in sucroseXagar concentration interaction in germination percentage, **N.S.; No significant difference in agar concentration in germination percentage and in root length, ***Capital letters denote significantly differences in sucrose concentration in germination percentage and in root length, ****Lower-case letters denote significantly differences in sucroseXagar concentration interaction in root length

Table 2. *Laurus nobilis L. germination percentage and root length at the end of the 2nd week in the modified MS medium*

Germination Percentage (%) at the end of the 2nd week					**Root Length (cm) at the end of the 2nd week**				
	3 g/L Agar	**6 g/L Agar**	**9 g/L Agar**	**Mean of Sucrose****		**3 g/L Agar**	**6 g/L Agar**	**9 g/L Agar**	**Mean of Sucrose****
0 g/L Sucrose	88.9a***	100.0a	66.6abc	**85.2 A**	**0 g/L Sucrose**	1.23a***	0.80bc	1.25a	**1.09 A**
10 g/L Sucrose	88.9a	66.6abc	88.9a	**81.5 A**	**10 g/L Sucrose**	0.88ab	0.46bc	1.03ab	**0.79 AB**
20 g/L Sucrose	66.6abc	66.6abc	77.7ab	**70.3 A**	**20 g/L Sucrose**	0.37bc	0.56bc	0.79bc	**0.57 BC**
30 g/L Sucrose	22.2c	55.5bc	33.3bc	**37.0 B**	**30 g/L Sucrose**	0.13c	0.85bc	0.67bc	**0.55 C**
Mean of Agar*	**66.7**	**72.2**	**66.6**		**Mean of Agar******	**0.65 B**	**0.67 B**	**0.94A**	

*N.S.; No significant difference in agar concentration in germination percentage, **Capital letters denote significantly differences in sucrose concentration in germination percentage and in root length, ***Lower-case letters denote significantly differences in sucroseXagar concentration interaction in germination percentage and in root length, ****Capital letters denote significantly differences in agar concentration in root length

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Table 3. *Laurus nobilis L. germination percentage and root length at the end of the 3rd week in the modified MS medium*

	Germination Percentage (%) at the end of the 3rd week					**Root Length (cm) at the end of the 3rd week**			
	3 g/L Agar	**6 g/L Agar**	**9 g/L Agar**	**Mean of Sucrose****		**3g/L Agar**	**6 g/L Agar**	**9 g/L Agar**	**Mean of Sucrose******
0 g/L Sucrose	100.0a***	100.0a	88.9a	**96.3 A**	**0 g/L Sucrose**	2.96	2.24	1.9	**2.37**
10 g/L Sucrose	88.9a	66.6ab	100.0a	**85.2 AB**	**10 g/L Sucrose**	2.08	1.49	2.45	**2.01**
20 g/L Sucrose	66.6ab	66.6ab	77.7ab	**70.3 BC**	**20 g/L Sucrose**	1.52	2.44	2.28	**2.08**
30 g/L Sucrose	33.3b	66.6ab	66.6ab	**55.5 C**	**30 g/L Sucrose**	1.80	1.50	1.49	**1.59**
Mean of Agar*	**72.2**	**74.9**	**83.3**		**Mean of Agar***	**2.09**	**1.92**	**2.03**	

*N.S.; No significant difference in agar concentration in germination percentage and in root length or sucroseXagar concentration interaction in root length, **Capital letters denote significantly differences in sucrose concentration in germination percentage, ***Lower-case letters denote significantly differences in sucroseXagar concentration interaction in germination percentage, **** N.S.; No significant difference in sucrose concentration in root length

Table 4. *Laurus nobilis L. germination percentage and root length at the end of the 4th week in the modified MS medium*

	Germination Percentage (%) at the end of the 4th week					**Root Length (cm) at the end of the 4th week**			
	3 g/L Agar	**6 g/L Agar**	**9 g/L Agar**	**Mean of Sucrose******		**3 g/L Agar**	**6g/L Agar**	**9g/L Agar**	**Mean of Sucrose******
0 g/L Sucrose	100.0a***	100.0a	88.9 ab	**96.3 A**	**0 g/L Sucrose**	4.22**	3.93	3.37	**3.84 A**
10 g/L Sucrose	88.9ab	66.6ab	100.0 a	**85.2 AB**	**10 g/L Sucrose**	3.70	2.91	3.76	**3.46 AB**
20 g/L Sucrose	66.6ab	88.9ab	77.7 ab	**77.7 AB**	**20 g/L Sucrose**	2.72	3.02	3.76	**3.17 AB**
30 g/L Sucrose	44.4b	77.7ab	66.6 ab	**62.9 B**	**30 g/L Sucrose**	2.63	2.14	2.93	**2.57 B**
Mean of Agar*	**72.2**	**83.3**	**83.3**		**Mean of Agar***	**3.32**	**3.00**	**3.46**	

*N.S.; No significant difference in agar concentration in germination percentage and in root length, **N.S.; No significant difference in sucroseXagar concentration interaction in root length, *** Lower-case letters denote significantly differences in sucroseXagar concentration interaction in germination percentage, **** Capital letters denote significantly differences in sucrose concentration in germination percentage and in root length

Shooting started after two weeks of culture and similarly shooting percentage and shoot length statistically affected from high sucrose concentration negatively and sucroseXagar interaction after 4 weeks (*Tables 5, 6 7, 8; Fig. 2*). Similarly, there were no positive effect of agar concentrations on shooting percentage along 4 weeks of study. Agar doses showed effectiveness on shoot length in 2nd and 3rd week but at the end of the study the effect has disappeared and agar concentrations showed similarity in statistics.

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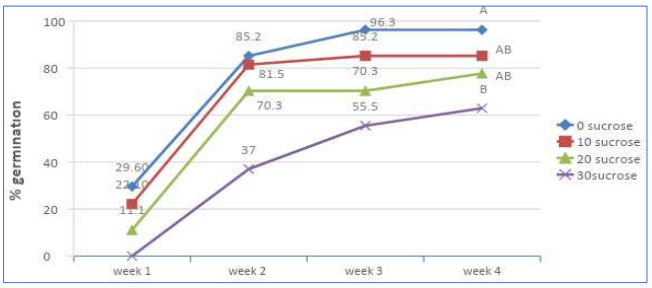

Figure 1. *Germination percentage in all sucrose concentrations along 4 weeks*

Table 5. *Laurus nobilis L. shooting percentage and shoot length at the end of the 1st week in the modified MS medium*

	Shooting Percentage (%) at the end of the 1st week					Shoot Length (cm) at the end of the 1st week			
	3 g/L Agar	**6 g/L Agar**	**9 g/L Agar**	**Mean of Sucrose*** **		**3 g/L Agar**	**6 g/L Agar**	**9 g/L Agar**	**Mean of Sucrose*** **
0 g/L Sucrose	0*	0	0	**0**	**0 g/L Sucrose**	0*	0	0	**0**
10 g/L Sucrose	0	0	0	**0**	**10 g/L Sucrose**	0	0	0	**0**
20 g/L Sucrose	0	0	0	**0**	**20 g/L Sucrose**	0	0	0	**0**
30 g/L Sucrose	0	0	0	**0**	**30 g/L Sucrose**	0	0	0	**0**
Mean of Agar**	**0**	**0**	**0**		**Mean of Agar****	**0**	**0**	**0**	

*N.S.; No significant difference in sucroseXagar concentration interaction in shooting percentage and shoot length, **N.S.; No significant difference in agar concentration in shooting percentage and shoot length, ***N.S.; No significant difference in sucrose concentration in shooting percentage and shoot length

Table 6. *Laurus nobilis L. shooting percentage and shoot length at the end of the 2nd week in the modified MS medium*

	Shooting Percentage (%) at the end of the 2nd week					Shoot Length (cm) at the end of the 2nd week			
	3g/L Agar	**6 g/L Agar**	**9 g/L Agar**	**Mean of Sucrose ****		**3 g/L Agar**	**6 g/L Agar**	**9g/L Agar**	**Mean of Sucrose ** **
0 g/L Sucrose	88.9a*** *	100a	66.6abc	**85.2 A**	**0 g/L Sucrose**	0.28ab*** **	0.26ab	0.37a	**0.30**
10 g/L Sucrose	77.8ab	33.3bc	88.9a	**66.7 A**	**10 g/L Sucrose**	0.27ab	0.25ab	0.32a	**0.28**
20 g/L Sucrose	66.6abc	55.5abc	66.6abc	**62.9 AB**	**20 g/L Sucrose**	0.18ab	0.21ab	0.24 ab	**0.21**
30 g/L Sucrose	22.2c	55.5abc	33.3bc	**37.0 B**	**30 g/L Sucrose**	0.10b	0.18ab	0.30 ab	**0.19**
Mean of Agar*	**63.9**	**61.1**	**63.9**		**Mean of Agar *****	**0.21C**	**0.23B**	**0.31 A**	

*N.S.; No significant difference in agar concentration in shooting percentage, **N.S.; No significant difference in sucrose concentration in shoot length, *** Lower-case letters denote significantly differences in sucroseXagar concentration interaction in shooting percentage and shoot length, **** Capital letters denote significantly differences in sucrose concentration in shooting percentage, *****Capital letters denote significantly differences in agar concentration in shoot length

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Table 7. *Laurus nobilis L. shooting percentage and shoot length at the end of the 3rd week in the modified MS medium*

	Shooting Percentage (%) at the end of the 3rd week					Shoot Length (cm) at the end of the 3rd week			
	3 g/L Agar	**6 g/L Agar**	**9 g/L Agar**	**Mean of Sucrose *****		**3 g/L Agar**	**6 g/L Agar**	**9 g/L Agar**	**Mean of Sucrose *****
0 g/L Sucrose	100a*	100a	66.6abc	**88.9 A**	**0 g/L Sucrose**	0.92abc*	0.67bc	1.18a	**0.92 A**
10 g/L Sucrose	88.9ab	55.5bc	88.9ab	**77.8 AB**	**10 g/L Sucrose**	0.58c	0.67bc	1.12ab	**0.79 AB**
20 g/L Sucrose	66.6abc	55.5bc	77.7ab	**66.6 BC**	**20 g/L Sucrose**	0.43c	0.59c	0.81abc	**0.61 B**
30 g/L Sucrose	33.3c	66.6abc	55.5bc	**51.8 C**	**30 g/L Sucrose**	0.50c	0.53c	0.60c	**0.54 B**
Mean of Agar**	**72.2**	**69.4**	**72.2**		**Mean of Agar******	**0.61 B**	**0.62 B**	**0.93 A**	

* Lower-case letters denote significantly differences in sucroseXagar concentration interaction in shooting percentage and shoot length, ** N.S.; No significant difference in agar concentration in shooting percentage, *** Capital letters denote significantly differences in sucrose concentration in shooting percentage and shoot length, **** Capital letters denote significantly differences in agar concentration in shoot length

Table 8. *Laurus nobilis L. shooting percentage and shoot length at the end of the 4th week in the modified MS medium*

	Shooting Percentage (%) at the end of the 4th week					Shoot Length (cm) at the end of the 4th week			
	3 g/L Agar	**6 g/L Agar**	**9 g/L Agar**	**Mean of Sucrose *****		**3 g/L Agar**	**6 g/L Agar**	**9 g/L Agar**	**Mean of Sucrose *****
0 g/L Sucrose	100a*	100a	88.9ab	**96.3 A**	**0 g/L Sucrose**	1.87a*	1.40abcd	1.77ab	**1.68 A**
10 g/L Sucrose	88.9ab	55.5bc	88.9ab	**77.8 AB**	**10 g/L Sucrose**	1.28abcd	1.60abc	1.65ab	**1.51 A**
20 g/L Sucrose	66.6abc	77.7ab	77.7ab	**74.0 BC**	**20 g/L Sucrose**	0.77c	0.72c	1.37abcd	**0.95 B**
30 g/L Sucrose	33.3c	66.6abc	66.6abc	**55.5 C**	**30 g/L Sucrose**	0.83c	0.89cd	1.05bcd	**0.93 B**
Mean of Agar**	**72.2**	**75.0**	**80.5**		**Mean of Agar****	**1.19**	**1.15**	**1.46**	

* Lower-case letters denote significantly differences in sucroseXagar concentration interaction in shooting percentage and shoot length, ** N.S.; No significant difference in agar concentration in shooting percentage and shoot length, *** Capital letters denote significantly differences in sucrose concentration in shooting percentage and shoot length

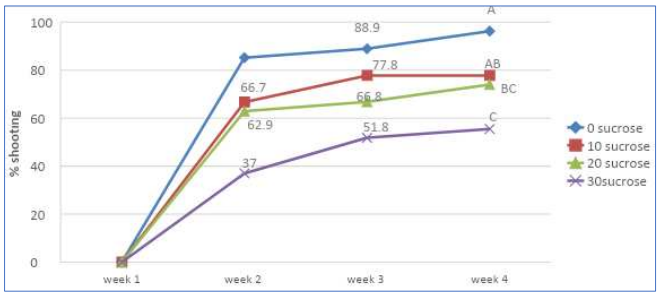

Figure 2. *Shooting percentage in all sucrose concentrations along 4 weeks*

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Studies are available outlining the ineffectiveness of excessive agar or sucrose doses in *in vitro* cultures. For example; Rabaiolli et al. (2017) demonstrated better *in vitro* rhizogenesis was achieved when ½ WPM medium supplemented with 30 cm^3 vermiculite without agar than 3.5 g/L or 7 g/L used agar for primary and secondary root percentages in *in vitro* and survival rate and leaf number in *ex vitro* in *Handroanthus chrysotrichus*. Suthar et al. (2011), in their work on *Boswellia serrata* in *in vitro*, studied agar at the rate of 0.0; 0.2; 0.4; 0.6; 0.8% w/v in MS+0.5 mg/L BAP+0.05 mg/L NAA for shoot multiplication from shoot clusters and 0.0 to 1% (w/v) agar in rooting medium containing 0.5 mg/L IBA+ 0.25 mg/L NAA+ antioxidants solution from rooting from shoots. According to their results 0.2% agar concentration was the best in number of shoots and 0.0% agar gave the highest rate of shoot length, number of leaves, fresh and dry weight, chlorophyll a, b and total. Similarly, they found that 0.0 % agar gave the highest rooting percentage, number of roots, root length and shoot length. Casanova et al. (2008) studied on agar concentration (0, 2, 4, 6, 8, 10 and 12 g dm^3) and vessel closure for *Dianthus caryophyllus in vitro* culture. They emphasized that the highest organogenic response was obtained in MS medium solidified with 2 g dm^3 agar (17.7 shoots per petal) and the least response was obtained in MS medium with 12 g dm^3 (4.3 shoots per petal) after 30 days of culture. Cortés-Olmos et al. (2018) studied on different sucrose (20 and 30 gL^{-1}) and agar (8 and 10 gL^{-1}) in full and half strength MS medium for *Lophophora williamsii*. They found that neither agar and sugar concentrations nor MS strength changed seed germination percentage after 49 days. But seedling size and areoles per seedling found higher in lower sucrose (20 gL^{-1}) and lower agar (8 gL^{-1}) than higher ones (30 gL^{-1} sucrose and 10 gL^{-1} agar). In another study, Gürel and Gülşen (1998) studied on two cultivars of *Prunus amygdalus* shoot tip *in vitro* culture in MS media. Sucrose concentrations were 2, 3, 4, 5 and 6% at 0.7% agar and agar were examined at the rate of 0.5; 0.6; 0.7; 0.8 and 0.9% at 3% sucrose. They emphasized that not in initiation stage but both multiplication and transplantation stage highest and lowest sucrose levels negatively affected shoot production and growth rate of developing shoots. 3 and 4% sucrose level found better in this stages. In addition, they found that the increasing concentration of agar caused a decrease in the growth of shoot tip explants in initiation stage 0.5; 0.6 and 0.7% agar were found significantly better than 0.8 and 0.9 agar on shoot development. Schulze et al. (2017) studied on *in vitro* germination of *Prunus lusitanica*. They used MS media with or without GA_3 and with different BA with two sucrose level (30 and 60 gL^{-1}). Their findings showed that radical and shoot emergence were greater on media with 30 gL^{-1} sucrose than with 60 gL^{-1}. In *Hancornia speciosa* cultured *in vitro*, dos Santos et al. (2017) observed that increasing sucrose concentration up to 60 g L^{-1} reduced germination speed and seedling height when they use (15, 30, 45 and 60 g L^{-1} sucrose) after 60 days of *in vitro* culturing of the naked embryos.

Conclusion

In conclusion, the results of this study revealed that increasing sucrose concentration as carbon source negatively influence germination efficiency of *Laurus nobilis* from naked seeds derived from mature female tree (*Fig. 3*). Among all the treatments, all parameters recorded to evaluate seedling growth of *Laurus nobilis* showed better performance in 0 g/L of sucrose than 10, 20, and 30 g/L in MS medium. Higher concentration of sucrose caused lateness in germination and shooting with reduced germination and shooting rate, root and shoot length (*Figs. 4, 5, 6, 7*). When sucrose doses

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are ignored and only agar doses are considered, there were no differencies in all parameters at the end of the experiment excepting middle stage of experiment. When sucrose and agar interaction were examined, 0 g/L sucrose with 3 g/L agar and 0 g/L sucrose with 6 g/L agar were found 100% in both germination and shooting percentage than 9 g/L agar. This results supports the hypothesis that agar as viscosity source and sucrose as carbon source reduces the availability of nutrients in media to the plants. Moreover, root and shoot length found the highest in 0 g/L sucrose with 3 g/L agar among all the treatments (0, 10, 20, and 30 g/L sucrose with 3, 6 and 9 g/L agar). The decreased germination and seedling capacity in seed explants may result from a decrease in the amount of water available in the medium that have high rate of sucrose and agar. In this perspective, it must be underlined sucrose and agar compositions should be experienced in all plant genus and explant types for commercially or academically valuable application to lower the cost and to achieve the right results from other tested treatments.

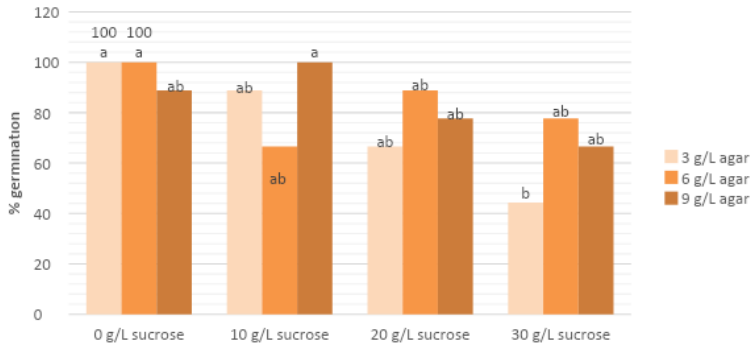

Figure 3. *Germination percentage in all sucrose and agar concentrations at the end of the 4th week*

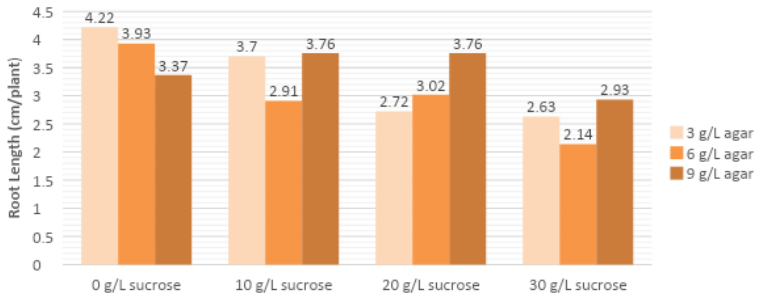

Figure 4. *Root length per plant in all sucrose and agar concentrations at the end of the 4th week*

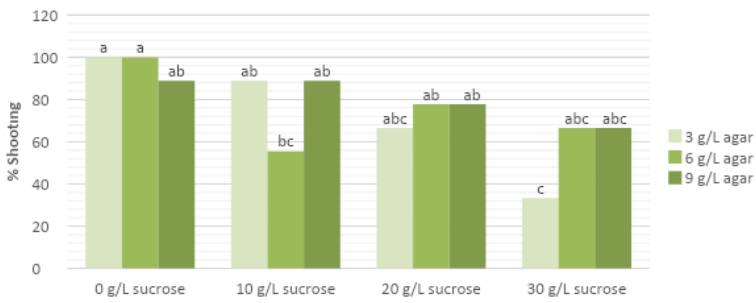

Figure 5. *Shooting percentage in all sucrose and agar concentrations at the end of the 4th week*

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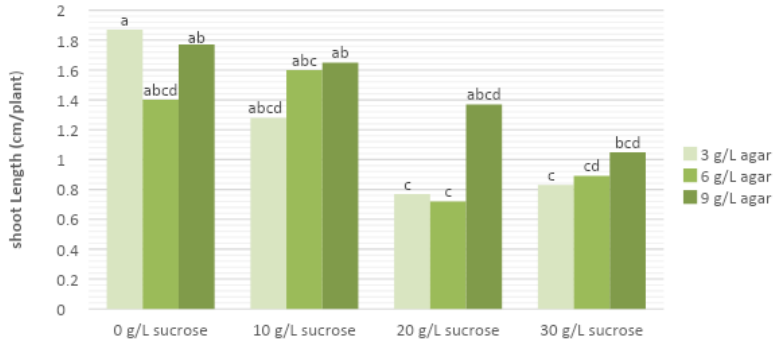

Figure 6. *Shoot length per plant in all sucrose and agar concentrations at the end of the 4th week*

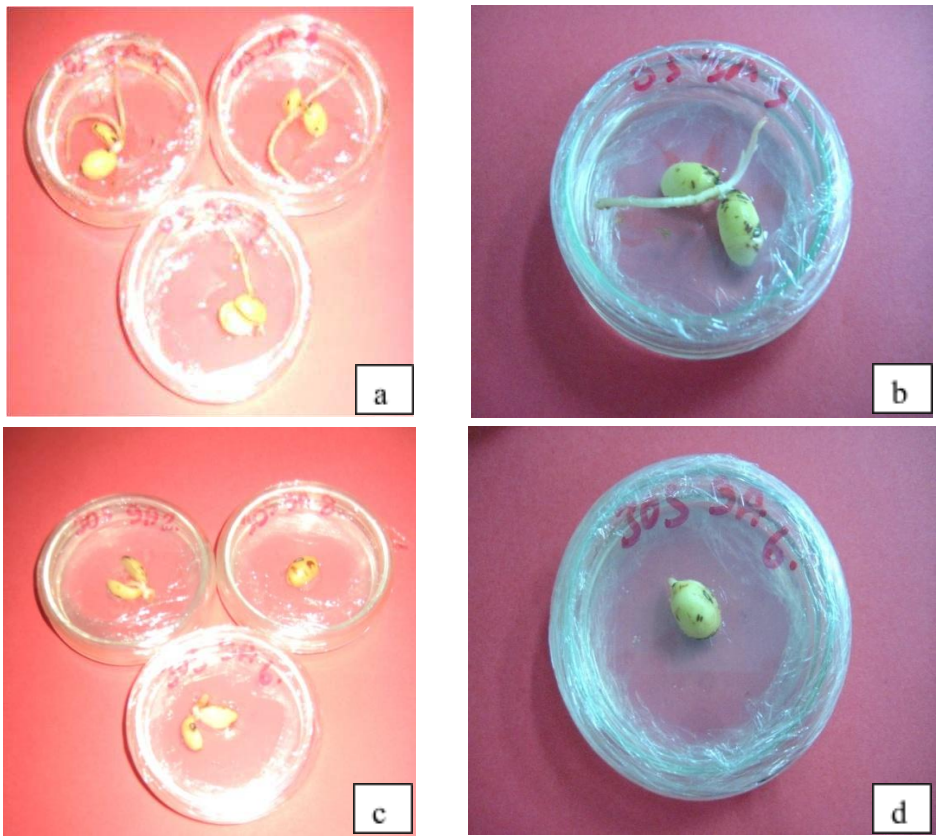

Figure 7. *Gemination and shooting of Laurus nobilis L. in different sucrose and agar concentration after 4 weeks; (a, b) successful germination and shooting at 0 g/L sucrose and 3 g/L agar, (c, d); poor germination and shooting at 30 g/L sucrose and 3 g/L agar*

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CHARACTERISTICS OF ZOOPLANKTON FUNCTIONAL GROUPS AND THEIR ENVIRONMENTAL FACTORS IN THE HARBIN SECTION OF THE SONGHUA RIVER, CHINA

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Abstract. In order to determine the spatial and temporal relationship between environmental factors and zooplankton functional groups in the Harbin section of the Songhua River, China, we collected water samples of zooplankton separately at 6 sampling sites in spring, summer and autumn in 2012. In this study, a total of 26 species of zooplankton were collected from Harbin section of the Songhua River, belonging to 6 functional groups. Both environmental factors and the biomass of zooplankton functional groups exhibited spatial and seasonal differences. Water temperature (WT), chemical oxygen demand (CODcr), total phosphorus (TP), nitrate (NO^{3-}) and dissolved iron (Fe^{3+}) were significantly higher in summer. On the contrary, pH, dissolved oxygen (DO) and 5 days' biochemical oxygen demand (BOD_5) were significantly lower in summer. The biomass of zooplankton functional groups was higher in spring and lower in summer. According to the results of Redundancy analysis (RDA): water temperature (WT), dissolved oxygen (DO), pH, chemical oxygen demand (CODcr), 5 days biochemical oxygen demand (BOD_5), total phosphorus (TP), N: P ratio (N:P) and dissolved iron (Fe^{3+}) were the major factors influencing zooplankton functions.
Keywords: *functional traits, biomass, environmental factors, Songhua River, Redundancy analysis (RDA)*

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Introduction

Zooplankton functional groups play an important ecological role in aquatic environments, such as the energy and material flow links between phytoplankton from small primary producers and fish of larger secondary consumers. In the meantime, these aquatic organisms perform important functions in the biogeochemical cycle by participating in alternative food webs, such as consumers (e.g., microbial and detritus) (Leoni, 2016; Lira et al., 2018). Zooplankton has become increasingly important in biological monitoring projects because of their rapid response to natural and anthropogenic environmental changes (Vieira et al., 2011; Mano and Tanaka, 2016). Traditional systems divided zooplankton into different communities to reflect water quality condition and biodiversity, but it had trouble reflecting the ecological function of aquatic organisms (Hood et al., 2006). Therefore, in order to study the functional role of zooplankton in the ecosystem, ecologists proposed the concept of functional groups. The study of functional groups, is more directly reflected the interaction between environmental factors and aquatic communities, and is also helpful in understanding aquatic ecosystem and biodiversity (Hoeinghaus et al., 2007). The species characteristics of the functional groups were more closely related to the environment, which can better understand the indicative role of zooplankton in environmental changes (Le et al., 2005).

Functional traits are the characteristics of the interaction between an organisms and their ecosystem (Tilman, 2001; Petchey and Gaston, 2002). Including taxonomic and functional groups analysis may increase the evaluation of biological responses to environmental changes (Petchey and Gaston, 2006; Cianciaruso et al., 2009). In zooplankton communities, Litchman et al. (2013) pointed out that the functional groups of organisms may include three ecological functions of feeding, growth/reproduction and survival. According to Obertegger et al. (2011) and Rizo et al. (2017), zooplankton functional groups related to raptorial or microphage organisms in zooplankton feeding guilds association were used for assessment. At present, functional groups studies have been used more and more in many fields of ecology. For instance, ecological succession (Raevel et al., 2012), meta-community (Gianuca et al., 2018) and beta diversity (Pool et al., 2014).

The zooplankton was classified into different functional groups, which can effectively simplify the food webs, and simulate the ecological processes of zooplankton communities comprehensively and accurately. However, the designation and measurement of zooplankton functional groups is still a difficult task, especially for small organisms (Martiny et al., 2013). In order to study the plankton in Sanjiang plain in China's freshwater ecosystem, according to the size, feeding habits and nutrition level (Zhao, 2005; Benedetti et al., 2018), zooplankton was divided into ten functional groups: protozoa filter feeders (PF), protozoa carnivore (PC), rotifer filter feeders (RF), rotifer carnivore (RC), small copepods and cladocerans filter feeders (SCF), small copepods and cladocerans carnivore (SCC), middle copepods and cladocerans filter feeders (MCF), middle copepods and cladocerans carnivore (MCC), large copepods and cladocerans filter feeders (LCF) and large copepods and cladocerans carnivore (LCC).

Phytoplankton composition and structure have been researched by different scientists in the Songhua River in China (Bao et al., 1987; Wei, 2018). These authors have reported: (i) dominance of *Diatoms* and *Chlorophyta* and (ii) the most influential environmental parameters of pH, water temperature, specific conductance and total phosphorus in the Harbin section of the Songhua River, China. However, despite

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phytoplankton and zooplankton forms essential component of aquatic foodweb, no studies about zooplankton community structure or functional groups has been conducted in the Songhua River, China. Songhua River is one of the seven major rivers in China, which is of great significance. The main objectives of this study are: (i) to assess the seasonal variation of zooplankton functional groups and their biomass and (ii) determine the environmental variables influencing the biomass of the seasonal variation of zooplankton functional groups in the Harbin section of the Songhua River. The results in this research are important for management of the Songhua River and other aquatic systems with similar characteristics in our nation.

Materials and methods

Research area

The study was conducted in the Harbin section of the Songhua River located at 45°N 126°W in Heilongjiang Province, Northeast China (*Fig. 1, Table 1*). Songhua River, is one of the seven major rivers in China. Harbin section of the Songhua River runs from Sanjiazi Village to Dadingzi Mountain with a total length of 66 km (Li et al., 2014). The annual precipitation was 300-1200 mm, and the overall trend was decreasing from southeast to northwest. The average annual runoff was 632.0×10^8 m^3 (1955-2010), and the average annual sediment transport was $1{,}259 \times 10^4$ t (1955-2010) (Songliao Water Resources Commission, 2012).

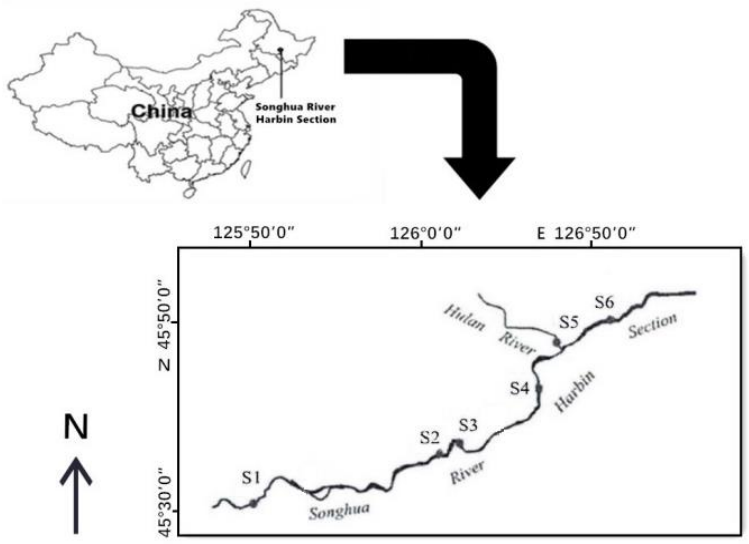

Figure 1. *Distribution map of sampling sites (S1-S6) in the Harbin section of the Songhua River, China*

Table 1. *Six sampling site coordinates in the Harbin section of the Songhua River, China*

Sampling sites	Latitude	Longitude
S1	N45°32'21"	E125°53'"12
S2	N45°46'22"	E126°29'32"
S3	N45°45'20"	E126°33'5"
S4	N45°51'35"	E126°42'11"
S5	N45°55'31"	E126°46'31"
S6	N45°57'36"	E126°50'48"

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The survey of zooplankton groups was carried out in 6 sampling sites in spring (April 20, 2012), summer (July 10, 2012) and autumn (October 10, 2012), respectively. Each sampling site was sampled once on both sides of the Songhua River. Affected by monsoon climate, the surface water of the river was covered by ice from the end of November to the beginning of next April. The sampling sites of S1 and S2 located in the upstream, S3 and S4 located in the midstream and S5 and S6 located in the downstream.

Field sampling and analysis

Sampling collection and analysis

Water temperature (WT) was measured on field using water thermometer (Mercury-in-glass thermometer, Dezhou Runxin Experimental Instrument Co. LTD, China). PH, chemical oxygen demand (CODcr), permanganate index (COD_{Mn}), biological oxygen demand (BOD_5), total nitrogen (TN), total phosphorus (TP), dissolved oxygen (DO), ammonia-nitrogen (NH_4^+-N), nitrate (NO^{3-}) and ferric ion (Fe^{3+}) were determined using the standard methods on surface water (GB/T5750-2006) of the Chinese standard methods proposed by Ministry of Environmental Protection of People's Republic of China (MEP, 2006). We used a zooplankton net of 25 cm diameter on boat, 55 cm length and 65 μm mesh to zooplankton samples from subsurface (0.5-1 m depth) when flow velocity was under the range of 0.01-2.40 m/s, and then fixed in labelled bottles of 30 ml for further analysis. In the laboratory, zooplankton samples were identified using a microscope at 400×magnification (Motic BA210, Motic Inc., China). In this study, we considered copepod nauplii as a taxon (Wang, 1961; Crustacean Research Group, 1979; Jiang and Du, 1979; Han and Shu, 1995). The biomass of the zooplankton functional groups was evaluated using wet weight method of Zhang and Huang (1991).

Classification of zooplankton functional group

According to the researchers, the basic principle for classification of zooplankton functional groups is their size. The sampled zooplankton species in the Songhua River were classified into six functional groups according to their body size/length and mode of feeding (Zhao, 2005; Benedetti et al., 2018). The six functional groups are group PF (protozoa filter feeders), group PC (protozoa carnivore), group RF (rotifer filter feeders), group RC (rotifer carnivore), group SCF (small copepods and cladocerans filter feeders) and group MCF (middle copepods and cladocerans filter feeders). Group PF and RF are passive filter feeders feeding on phytoplankton, bacteria and organic detritus. Group RC and PC are active ambush feeders that target small motile prey or other zooplanktons. Functional group SCF consists of small copepods and cladocerans of body size less than 0.7 mm that mostly feed through filter-feeding on phytoplankton, bacteria, organic detritus and protozoa. Functional group MCF consists of those zooplankton species with body size in the range of 0.7-1.5 mm. MCF, is a filter-feeder feeding on phytoplankton, bacteria, organic detritus and protozoa, same as group SCF. Ecologically, MCF plays a major role as a food source for fish and also can control algal blooms (Meerhoff et al., 2007).

Data analysis

One-way analysis of variance (ANOVA), and Tukey's honestly were used in IBM SPSS STATISTICS 19.0 to determine significant difference between the seasonal changes of water environmental variables and zooplankton functional groups biomass

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(Sharma et al., 2017). Water environmental factors (except pH) and biomass of zooplankton functional groups used log (x+1) transformation before conducting analysis in order to satisfy the normality and variance assumption. Relationship between the biomass of zooplankton functional groups and environmental factors was computed using CANOCO 4.5 software (Shen et al., 2014). We used Detrended Correspondence Analysis (DCA) before multivariate ordination analysis. This result of DCA showed that the largest gradient length of the axis was 1.584 less than 3. So the species data was linearly distributed and were more suitable for Redundancy analysis (RDA).

Results

Seasonal variations in environmental factors

The mean values of seasonal environmental variables recorded at the 6 sampling sites in the Harbin section of the Songhua River were presented in *Table 2* and *Fig. 2*. Most of the environmental factors presented statistical differences between seasons (One-way ANOVA and Tukey HSD test, $P< 0.05$). However, the mean values of COD_{Mn}, NH_4^+-N and TN did not vary significantly between seasons ($P> 0.05$). While WT, pH, DO, COD_{Cr}, BOD_5, TP, N:P, NO^{3-} and Fe^{3+}, were significantly different with seasonal variations ($P< 0.05$). The maximum mean values of WT, COD_{Cr}, TP, NO^{3-} and Fe^{3+} were observed in summer and their minimum mean values were observed in autumn, except TP and Fe^{3+}. On the contrary, the minimum mean values of pH, DO and BOD_5, were observed in summer and their maximum in spring, except DO. Unlike other significantly seasonal variations, the maximum mean value of N:P was observed in spring and its minimum mean value was observed in autumn.

Table 2. *One-way ANOVA and Tukey HSD methods were used to detect that the mean value (± standard error) of environmental factors varies with seasonal changes in this chart*

	Spring	Summer	Autumn	P-value
WT (℃)	20.33±1.51a	26.83±0.98b	11.5±0.84c	0.000
pH	8.66±0.46a	7.62±0.15b	8.02±0.15b	0.000
DO (mg/L)	8.67±2.56a	5.50±1.33b	10.13±0.27a	0.001
COD_{Mn} (mg/L)	6.60±1.81a	7.21±0.49a	5.49±0.99a	0.078
COD_{Cr} (mg/L)	21.67±5.5a	98.83±5.12b	14.00±1.79c	0.000
BOD_5 (mg/L)	5.67±1.57a	1.93±1.6b	2.10±0.52b	0.000
NH_4^+-N (mg/L)	0.36±0.31a	0.53±0.31a	0.23±0.16a	0.187
TP (mg/L)	0.06±0.02a	0.15±0.02b	0.14±0.05b	0.001
TN (mg/L)	1.45±0.27a	1.93±0.41a	1.58±0.42a	0.102
N:P	27.94±10.41a	13.04±2.49b	12.32±2.8b	0.001
NO^{3-} (mg/L)	0.96±0.46a	2.61±0.22b	0.84±0.09a	0.000
Fe^{3+} (mg/L)	0.32±0.15a	2.71±1.38b	0.78±0.46a	0.000

P value was a reference for determining test result using the method of One-way ANOVA. Environmental factors include: water temperature (WT), pH, dissolved oxygen (DO), potassium permanganate index (COD_{Mn}), 5 days biochemical oxygen demand (BOD_5), chemical oxygen demand (COD_{Cr}), ammonia nitrogen (NH_4^+-*N*), total phosphorus (TP), total nitrogen (TN), N: P ratio (N:P), nitrate (NO^{3-}) and dissolved iron (Fe^{3+}). By Tukey HSD ANOVA method, the mean value in each row with letters of a, b and c reflected the difference of seasons

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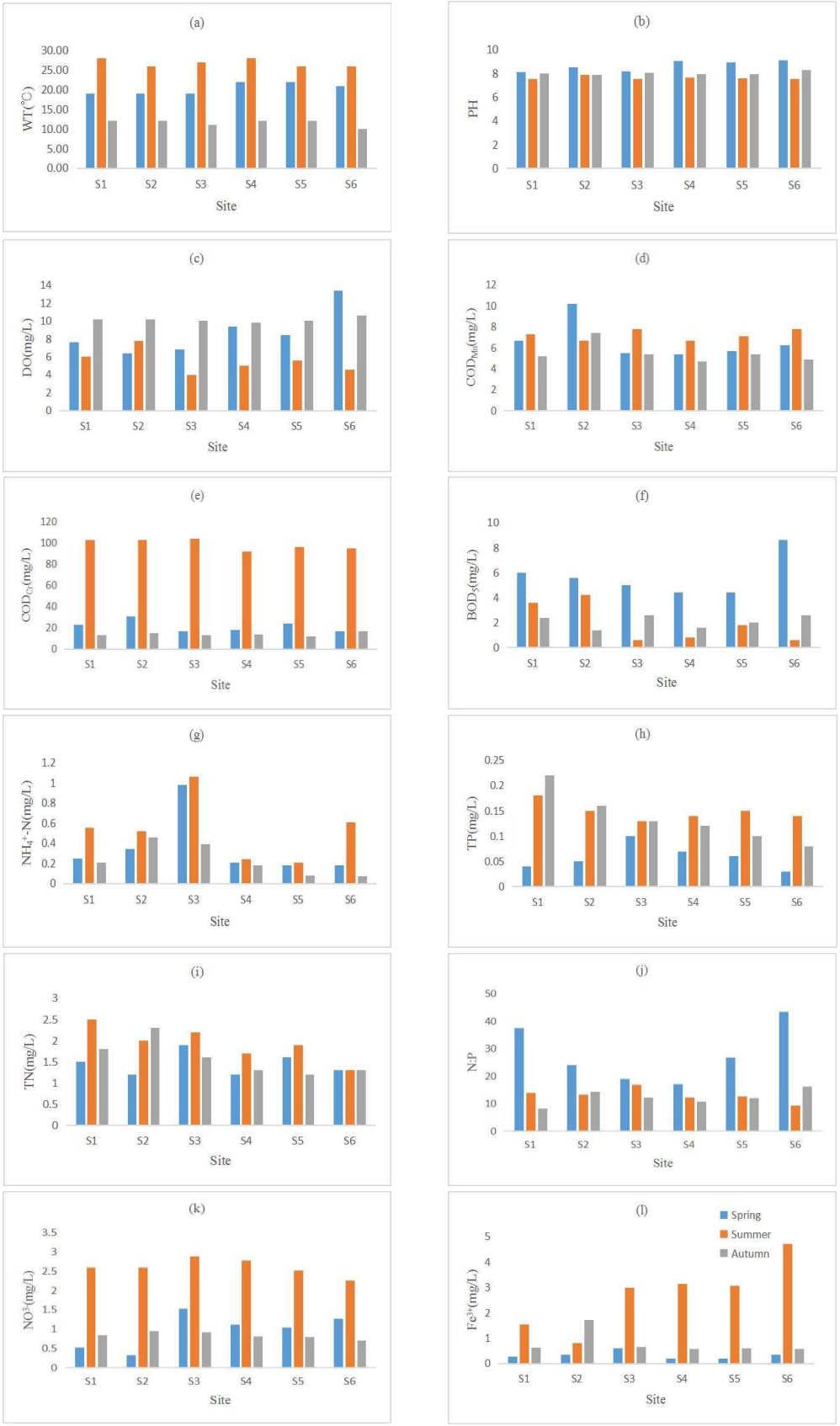

Figure 2. *Spatial variations of selected environmental variables of the different seasons in the Harbin section of the Songhua River, China*

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Seasonal and spatial variation of zooplankton functional groups

During the study period, 26 zooplankton species belonging to 20 genera were observed in spring, summer and autumn in 2012 in the Harbin section of the Songhua River, China (*Table 3*), where rotifers were composed of 17 species accounted about 65.38%, followed by protozoans with 6 (23.08%), cladocerans 2 (7.69%) and copepods 1 (3.85%). There were seasonal and spatial variation in biomass of zooplankton functional groups (*Figs. 3 and 4*). Our results showed that the largest number of zooplankton species was 17 in autumn, followed by 15 in spring and 14 in summer. The values of zooplankton functional group biomass were higher in spring at 0.48 mg/L, followed by 0.42 mg/L in autumn and 0.17 mg/L in summer. In spring and summer, RF functional group had the highest biomass contribution of about 41.10% and 49.52%, respectively. In autumn, approximately 62.58% of the total biomass contribution was of PF (*Fig. 3*). MCF was the second most contributors of the total biomass in summer, even if it was only collected in summer. The total biomass contribution of the PC was about 4.31%, and no PC was collected in spring and summer. Spatially, with exception of sampling sites S1 and S6 were dominated by SCF, all the sampling sites were dominated by group RF in spring. In summer, groups PF, RF and RC were presented in almost all sampling sites. However, MCF, only present in S2 and S3, and were dominated in S2 and S3. Group PF in all the sampling sites were dominated in autumn.

Table 3. *Zooplankton listed by functional groups and their biomass percentage ratio in the Harbin section of the Songhua River (*) present*

Taxonomic group	Species	Functional groups	Spring	Summer	Autumn	Percentage of total biomass(%)
Protozoa	*Difflugia acuminata*	PF		*		0.42
	Strobilidium velox	PF	*	*	*	3.77
	Strombidium viride	PF	*		*	13.82
	Tintinnopsis wangi	PF	*	*	*	2.51
	Vorticella microstoma	PF	*	*	*	14.07
	Askenasia volvox	PC			*	1.67
Rotifera	*Brachionus angularis*	RF	*		*	1.51
	Brachionus quadridentatus	RF	*	*	*	25.47
	Brachionus urceus	RF	*			0.07
	Filinia longiseta	RF	*		*	0.40
	Filinia maior	RF		*		0.04
	Gastropus hyptopus	RF		*		0.02
	Keratella cochlearis	RF	*	*	*	0.23
	Keratella quadrata	RF	*			0.38
	Keratella valga	RF		*	*	0.08
	Lecane ludwigii	RF	*			0.02
	Lecane luna	RF		*		0.01
	Monostyla quadridentata	RF			*	0.01
	Ploesoma hudsoni	RF		*		6.98
	Trichotria tetractis	RF			*	0.01
	Polyarthra trigla	RC	*	*	*	13.51
	Synchaeta stylata	RC	*		*	0.27
	Trichocerca lophoessa	RC		*	*	0.07
Cladoceran	*Diaphanosoma sp*	MCF		*		4.19
	Bosmina sp	SCF	*		*	4.19
Copepoda	*microcyclops javanus*	SCF	*		*	6.28

Protozoa filter feeders (PF), protozoa carnivore (PC), rotifera filter feeders (RF), rotifer carnivore (RC), small copepods and cladocerans filter feeders (SCF) and middle copepods and cladocerans filter feeders (MCF)

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Figure 3. *Distribution of zooplankton functional group biomass of spring, summer and autumn in the Harbin section of the Songhua River, China*

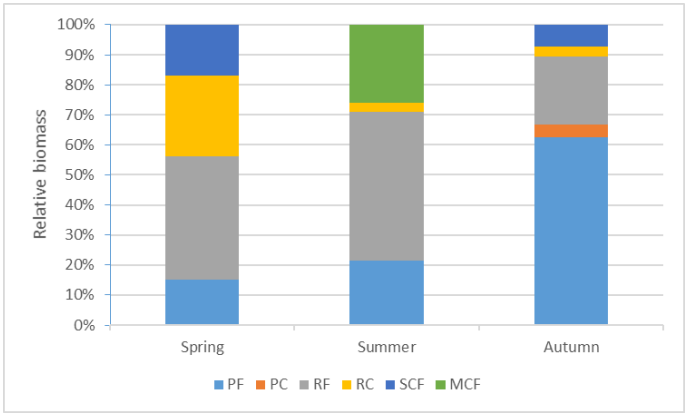

Figure 4. *Seasonal variation of relative mean biomass of zooplankton functional groups in the Harbin section of the Songhua River, China*

Redundancy analysis (RDA) of zooplankton functional groups with environmental factors variables

It was carried out to determine the relationship between the biomass of zooplankton functional groups and environmental factors variables in the RDA ordination in *Table 4*. The Monte Carlo test showed that the first axis and all typical axes were significant (F-ratio = 3.894, p-value = 0.0580; F-ratio = 3.974, p-value = 0.0040; 499 random permutations under simplified model). The first two of RDA eigenvalue axes together explained 75.3% (axis 1: 43.8%; axis 2: 31.5%) zooplankton functional group biomass and 83.2% (axis 1: 48.4%; axis 2: 34.8%) relationship of the zooplankton functional group biomass and environmental factors. The relationship between zooplankton

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functional groups and environmental factors can be referred to arrow directions in *Fig. 5*. Axis 1 was mainly positive correlated with WT (r = 0.4939) and NH_4^+-N (r = 0.4442) and negatively related with TP (r = -0.3256). While Axis 2 was mainly positive correlated with WT (r = 0.7158) and COD_{Cr} (r = 0.7097) and negatively related with DO (r = -0.4201). Groups of PF and PC were positively related with DO, and negatively correlated with WT, COD_{Mn}, COD_{Cr} and NO^{3-}. Groups of RF, RC and SCF were positively correlated with pH, N:P and BOD_5, and negatively related with Fe^{3+} and TP. Conversely, Group MCF were positively correlated with Fe^{3+} and negatively correlated with pH.

Table 4. *Redundancy analysis (RDA) results of zooplankton functional groups in the Harbin section of the Songhua River, China*

	Eigenvalues	Species-environment correlations	Cumulative percentage variance of species data	Cumulative percentage variance of species-environment relation
Axes 1	0.438	0.960	43.8	48.4
Axes 2	0.316	0.961	75.3	83.2
Axes 3	0.088	0.923	84.1	92.9
Axes 4	0.038	0.914	87.9	97.1
Sum of all canonical eigenvalues	0.905			

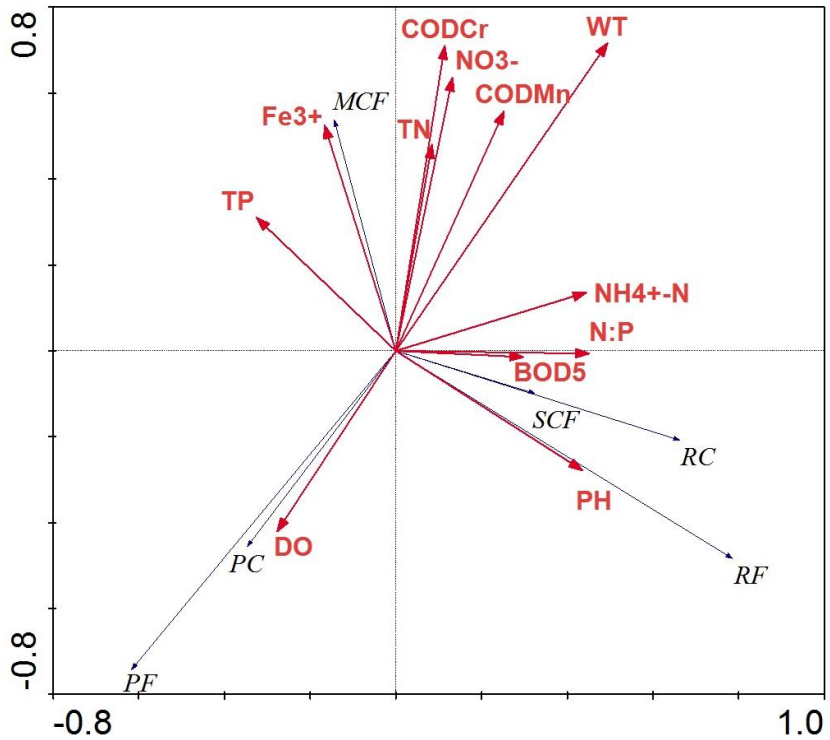

Figure 5. *Biological ordination diagram of Redundancy analysis (RDA) of zooplankton functional groups (blue lines with black letters) and environmental factor variables (red lines with red letters) in the Harbin section of the Songhua River, China*

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Discussion

Seasonal variation of zooplankton functional groups biomass

Biomass is one of the most important variables to describe the spatial and temporal variation of zooplankton functional groups in aquatic ecosystems (Shi et al., 2015), which can reduce the disturbance of simulated ecosystem caused by large differences in zooplankton. The present study provided novel insights into the seasonal changes of the zooplankton biomass in the Harbin section of the Songhua River, China. Comparison with other waters of regions showed that the zooplankton biomass in the Harbin section of the Songhua River was significantly lower. In the recently published literature, the zooplankton biomass of Tuanjie Reservoir in Mudanjiang city (Sun et al., 2019) and Small Xingkai Wetland in Jixi City (Ma et al., 2019) showed were higher than thoese in the same province of the Songhua River in each quarter. The result of Shi et al. (2016) showed that the rivers with high velocity and sediment content were not suitable for the habitat and reproduction of zooplankton (Fang et al., 2012). The study of Mwagona et al. (2018) had also shown that zooplankton growth was affected when their nutrient levels were low, reducing their reproductive rate and prolonging their growth cycles. In this study, the biomass of zooplankton functional groups had significant differences between different seasons. Differences in functional groups and life history traits of the dominant species have to be taken into account to explain these seasonal biomass patterns. In spring and summer, the biomass of zooplankton was mainly RF, of which 86.79% was composed of *Brachionus quadridentatus* and 87.29% was composed of *ploesoma hudsoni*. In autumn, PF was dominant, mainly composed of *Strombidium viride* and *Vorticella microstoma*, which accounted about 46.50% and 46.61% of the total biomass, respectively. Studies have found a strong positive correlation between rotifer biomass and temperature (Galkovskaja, 1987). Therefore, when the water temperature turned cold, the RF who fed on bacteria, phytoplankton and organic matter gets less, and the PF which also fed on these things got less grazing pressure and became the dominant specie due to the decrease of RF. In spatial distribution, seasonal dominant functional groups changed most obviously in the sampling sites of S2 and S3: RF→MCF→PF. Summer temperature and nutrients provided the best conditions for MCF to be the dominant functional group. These factors greatly enrich the source of food resources for MCF. MCF which fed on bacteria, phytoplankton and organics inhibits the growth of RF by predation (An, 2016).

The driving factors of zooplankton functional groups in the Harbin section of Songhua Rive, China

The interaction between zooplankton functional groups and its relationship with water environmental factors, such as temperature, nutrients, bottom-up effect of phytoplankton, top-down effect of fish feeding, interspecies competition, were the main factors affecting the growth of zooplankton (Yang, 2006). At the same time, these physiological processes of zooplankton function groups showed a highly sensitive response to changes in the environment, and zooplankton function groups can amplify subtle changes in water environment and made it a very good indicator for freshwater ecosystems (Richardson, 2008). In temperate regions, water temperature and nutrients were the main environmental factors (Wen and Xie, 2013). In this study, RDA results indicated that water temperature was the main factor affecting zooplankton. The big difference in temperature of the Songhua River water along the year round considered

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as a controlling factor related to range of tolerance of species. From the result of RDA, some function groups reacted positively to water temperature as group MCF, contrary to groups of PC and PF. We believe that the reproduction of these groups is related to temperature preference. Related researches showed that higher water temperature can promote the metabolism of zooplankton in the meanwhile they also increase the consumption of nutrients and proteins, and body mortality (Zheng, 1992). *Brachionus quadridentatus* quickly produced resting eggs at higher or lower temperatures (Xiang et al., 2018). However, MCF, which was composed of *Diaphanosoma*, was positively correlated with water temperature. Experiments had shown that *Diaphanosoma* often appeared in summer and prefer to live in warm water environment (Brendonck and Meester, 2003). The growth rate of *Diaphanosoma* was accelerated when water temperature rose, and the growth rate of *Diaphanosoma* was slower when water temperature was lower. In addition, RDA analysis indicated that group PF was positively related with DO and negatively related with NH_4^+-N and COD_{Cr}, and groups of RC and SCF were negatively correlated with TP. Similarly, with the research of Shen (1999) confirming that most protozoans were aerobic biological. The diversity of protozoans in the abundant water environment would be higher than that of the anaerobic environment. Lu et al. (2007) in the study of wastewater found that, although *Vorticella microstoma* was the indicator species of mesosaprobic zone. When increasing the DO in aeration activated sludge, the density of *Vorticella microstoma* in activated sludge was significantly higher than the control group. In the study of protozoans, it was found that the sessile ciliates diversity index (such as *Epistylisplicatilis*, *Vorticella convallaria*, *Pseudocarchesium aselli* and *Opercularia cylindrata*) and crawling ciliates (*Aspidisca costata* and *Aspidisca sulcate*) were negatively correlated with NH_4^+-N (Chen et al., 2003). In the study of zooplankton in Small Xingkai Lake Wetlands, it was found that COD was the main negative correlation factors affecting the functional groups of zooplankton (An, 2016). The author thought that the pollutants mainly came from agricultural wastewater around the river, which were directly affected the bottom-up effects of phytoplankton and indirectly affected the growth rate of zooplankton. In the study of Zhao and Yu (2019) of on zooplankton functional groups in Tai Lake, RDA results showed that zooplankton was positively related with COD_{Cr}. The results of Ma et al. (2019) revealed that TP were the main factors responsible of zooplankton functional groups biomass dynamics. The results of Meng et al. (2014) found that chlorophyta grew in phosphorus-restricted environments made thicker cell wall to makes less edible. The research of Siebielec et al. (2015), da Silva Cerozi and Fitzsimmons (2016) clearly proved that dissolved calcium ion should react with the phosphate in water forming insoluble calcium phosphate and other compounds that were not conducive to phytoplankton growth and propagation when the water environment was slight alkaline. In fact, Marzolf (1990) and Adamczuk et al. (2015) thought phytoplankton itself didn't meet all of the nutritional requirements of zooplankton. It further confirms that filter feeders of the protozoas and rotifera provided an important food supplement to carnivore functional groups under the condition of limited amounts of bacterial phytoplankton. So we should be undertaken to follow the changes in the ecosystem continuous monitoring of water characteristics and biota in the Harbin section of the Songhua River, China.

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Conclusions

In this current study, a total of 26 species of zooplankton species were observed in 2012 in the Harbin section of the Songhua River, China. Zooplankton was divided into 6 functional groups: protozoa filter feeders (PF), protozoa carnivore (PC), rotifer filter feeders (RF), rotifer carnivore (RC), small copepods and cladocerans filter feeders (SCF) and middle copepods and cladocerans filter feeders (MCF). The biomass of zooplankton functional groups ranged from large to small in spring, autumn, and summer. One-way ANOVA and Redundancy analysis (RDA) showed that the environmental parameters as water temperature (WT), pH, dissolved oxygen (DO), chemical oxygen demand (COD_{cr}), 5 days biochemical oxygen demand (BOD_5), total phosphorus (TP), N: P ratio (N:P), nitrate (NO^{3-}) and dissolved iron (Fe^{3+}) influenced on the change of the biomass of zooplankton functional groups. Groups of PF and PC had strong relationships with dissolved oxygen (DO), water temperature (WT) and chemical oxygen demand (COD_{cr}) of environmental parameters. Groups of RF, RC and SCF had strong relationships with pH and total phosphorus (TP). Group MCF had strong relationships with dissolved iron (Fe^{3+}) and total phosphorus (TP). This study revealed that the zooplankton functional groups followed certain predictable pattern in the seasonal gradient. It could be useful for assessing and predicting the growth and development of zooplankton in the future. At present, functional-approach research studies for zooplankton communities in continental aquatic environments are scarce. Therefore, the scientific community must take into account the environmental and spatial dynamics of these organisms and conduct more research on the traits related to their different ecological functions.

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