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Ovitek: Oblika plodov preučevanih sort pistacije (črke označujejo sorto glede na preglednico 1; merilo 5 mm); (foto: Fariba Sharifnia, 1–10)
Cover: Fruit shape of the investigated pistachio cultivars (the letters indicate cultivars name according to Table 1, scale bar 5 mm); (photo: Fariba Sharifnia, 1–10)

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Analysis of genetic diversity in selected sugarcane (*Saccharum officinarum* L.) accessions using inter simple sequence repeat (ISSR) markers

Faith Eweluegim EMEGHA [1, 2], David Adedayo ANIMASAUN [3], Folusho BANKOLE [1], Gbadebo OLAOYE [1]

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Analysis of genetic diversity in selected sugarcane (*Saccharum officinarum* L.) accessions using inter simple sequence repeat (ISSR) markers

Abstract: Genetic diversity information among a population is important in exploiting heterozygosity for the improvement of crop species through breeding programmes. This study was therefore, conducted to assess genetic diversity and establish molecular relationships among 20 selected exotic sugarcane accessions from the Unilorin Sugar Research Institute germplasm using Inter Simple Sequence Repeat (ISSR) molecular markers. Genomic DNA was extracted from the sugarcane leaf. Fragments amplification was then performed by polymerase chain reaction (PCR) with ISSR markers and the data obtained were analyzed using MEGA 4 software. Analysis of the electropherogram showed a total of 39 loci consisting of 369 bands, out of which 95.8% were polymorphic. The biplot analysis showed all the markers contributed to the observed diversity with the least achieved with ISSR6. The principal co-ordinate analysis grouped the accessions into four clusters, comprising mixtures of all the six collection sites. The polymorphism obtained in the present study showed that the ISSR markers are effective for assessment of genetic diversity of the sugarcane accessions as it reveals the genetic similarity or divergence of the accessions regardless their place of origin or cultivation.

Key words: Dendrogram; genetic diversity; germplasm resources; ISSR marker; sugarcane

Analiza genetske raznolikosti izbranih akcesij sladkornega trsa (*Saccharum officinarum* L.) z uporabo označevalcev na osnovi enostavnih ponavljajočih se zaporedij (ISSR)

Izvleček: Informacija o genetski raznolikosti znotraj populacij je pomembna za uporabo heterozigotičnosti za izboljšanje gojenih rastlin v žlahtniteljskih programih. Ta raziskava je bila narejena za oceno genetske raznolikosti in vzpostavitev molekularnih povezav med 20 izbranimi ekzotičnimi akcesijami sladkornega trsa na Inštitutu za preučevanje genetskih resursov sladkornega trsa v Unilorinu (Sugar Research Institute germplasm) z uporabo molekularnih markerjev na osnovi enostavno ponavljajočih se zaporedij. Genomska DNK je bila ekstrahirana iz listov sladkornega trsa s pomočjo mini kita (DNeasy Mini Kit,Qiagen). Namnožitev fragmentov je bila izvedena s polimerazno verižno reakcijo (PCR) z ISSR označevalci, pridobljeni podatki so bili analizirani s programom MEGA 4. Analiza elektroferogramov je pokazala, da je celokupno število 39 lokusov sestavljalo 369 trakov, od katerih je bilo 95,8 % polimorfnih. Biplot analiza je pokazala, da so vsi označevalci prispevali k opaženi raznolikosti, z najmanjšim deležem označevalca ISSR6. Analiza glavnih komponent je združila akcesije v štiri skupine, ki so bile mešanica vzorcev iz vseh šestih vzorčenih mest. Polimorfizem, ugotovljen v tej raziskavi je pokazal, da so vsi ISSR označevalci učinkoviti za ugotavljanje genetske raznolikosti akcesij sladkornega trsa ne glede na njihovo mesto izvora in načina gojenja.

Ključne besede: dendrogram; genetska raznolikost; genetski viri; ISSR označevalci; sladkorni trs

1 Department of Agronomy, Faculty of Agriculture, University of Ilorin, Kwara State, Nigeria
2 Corresponding author, e-mail: faith.emegha84@gmail.com
3 Department of Plant Biology, Faculty of Life Sciences, University of Ilorin, Kwara State, Nigeria

1 INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) is one of the most important economic crops. In Nigeria, sugarcane is mainly grown for its sugar juice, as a raw material for manufacturing sugar (Wayagari et al., 2003a,b), molasses and bagass, and recently ethanol and renewable energy. As an industrial crop, sugarcane production has contributed to the nation's GDP and provided the opportunities for job creation. Other use of sugarcane include; fertilizer, bio-plastics, paper, sugarcane wax (Khushk and Pathan, 2006).

Commercial cultivation is mainly through planting of vegetative cutting (setts) of mature stalks. During sugarcane breeding programs, exchange and shipment of elite clones and breeding lines in the form of stalk cuttings across different test locations occur regularly for the purpose of verifying parental source or desired use of a clone in an experiment (Pan, 2010). To increase sugar yield within the Nigerian sugar industry, it is important to optimize varietal trials and breeding activities of sugarcane (NSDC, 2015).

In Sugarcane breeding, mislabeling errors could occur during the process of planting and selection, due to the use of large number of accessions in the varietal development program, which can only be revealed later in the selection program. Mislabeling could alter breeding goals by using the wrong variety for breeding activities. Molecular tools ensure that breeders have the correct clones involved in their crosses as well as varietal trials (Pan, 2010). Diversity analysis based on morphological attributes may not be sufficient or may be inflated due to environmental influences, particularly for new varieties or those that are new to a region (Animasaun et al., 2015). In these situations, a more accurate and clear means of identification is required to avoid downstream consequences such as risk of disease outbreak and/or poor productivity.

The use of molecular marker techniques such as RFLP (Restriction fragment length polymorphism), SSLP (Simple sequence length polymorphism), AFLP (Amplified fragment length polymorphism), RAPD (Random amplification of polymorphic DNA), ISSR (Inter simple sequence repeat), SSR Microsatellite polymorphism (Simple sequence repeat), SNP (Single nucleotide polymorphism), RAD markers (Restriction site associated DNA makers) etc, in the analysis of genetic variation among genetic materials, has facilitated correct determination of the nature of association among economic traits. This is because the techniques ensure development of accurate genetic maps since they are devoid of environmental influences, thereby helping to achieve set objectives within breeding programs leading to the achievement of significant yield increases in breeding programmes (Dilon et al., 2007).

The use of Inter Simple Sequence Repeat (ISSR) marker in crops plant diversity study and fingerprinting is advantageous (Animasaun et al., 2015). The markers can detect a range of loci and allelic diversity among the genetic materials (Ajibade et al., 2000, Pfeiffer et al., 2011) as well as provide information on their unique identity that deserves conservation attention and improvement programmes (Da Costa et al., 2011, Animasaun et al., 2021). Furthermore, molecular-based information obtained would be a reliable basis for developing a template and workable strategy for germplasm conservation and future improvement through the selection of appropriate parents to maximize yield, establishment of proper identity of the genotypes and maintain genetic diversity. The approach also has the capacity to provide useful information on the extent of genetic diversity among the germplasm accessions, and prevent possible misidentification, which may render the work previously done during selection unreliable.

The ultimate goal in sugarcane breeding is to develop genetically improved varieties with high sugar yield (cane yield and sucrose content) that is economically sustained over several ratoon crops. Therefore, germplasm materials are usually assessed for their breeding behaviour with the objective of utilizing them either for direct cultivation on the sugar estates or as parents in hybridization for evolving new and superior progenies intended as replacement to the existing cultivars (Kwajaffa & Olaoye, 2014). In an effort to identify productive sugarcane varieties for the rainforest and savanna ecologies of Nigeria, a detailed evaluation of 40 selected sugarcane varieties from six (6) breeding stations was conducted under the auspices of the West Africa sugarcane Development (WASD) Project (Olaoye et al. 2017), which provided some information on morphology, cane yield and yield components.

However, in order to have a proper diagnostic assessment of the genetic attributes of these varieties, twenty accessions were further selected for genetic diversity and allelic polymorphism assessment using the molecular approach. The objectives were to characterize the selected sugarcane accessions using ISSR marker and provide detailed information on the nature of genetic diversity among them for further varietal improvement activities.

2 MATERIALS AND METHODS

2.1 PLANT MATERIAL

Twenty (20) exotic sugarcane accessions were selected from the pool of germplasm materials from six sugarcane breeding stations currently being maintained at the Unilorin Sugar Research Institute (USRI) Farm. The accessions were selected base on the morphological superiority reported elsewhere (Olaoye et al., 2017). Details of their origin, parentage (where available) and yield attributes are contained in Table 1.

2.2 DNA ISOLATION

Genomic DNA (gDNA) was isolated from young unfolding leaf tissues. About 1 g of fresh leaf tissue was grounded into a fine powder in prechilled mortar and genomic DNA from individual accession was extracted using, DNAeasy Plant Mini Kit (QIAGEN, USA). The DNA extraction was performed in accordance with the manufacturer's instruction. DNA concentration were determined comparatively by electrophoresis at current of 100 amps, 80 volts, for 40 minutes using agarose gel (0.8 %) electrophoresis, by applying 5 µl gDNA loaded after mixing with 3 µl 6X loading dye (Promega, USA) to check the quality of the DNA by comparing the intensity of the bands with a 1kb standard (Thermo Scientific, USA). The gel was visualized under a UV transilluminator and the gel was imaged with a gel documentation system (Ingenius-3, Syngene, USA) to confirm the quality of the genomic DNA.

Table 1: List of selected 20 Sugarcane varieties and their attributes

					Parental identification	
S/N	Variety	Point of collection	°Brix content (°Bx):	Cane yield (t ha^{-1})	Female	Male
1	B97375	Barbados	NA	NA		
2	B96812	Barbados	NA	NA		
3	B96723	Barbados	NA	NA		
4	B93757	Barbados	23.71	58.52		
5	B47419+	Barbados	20.13	72.89		
6	DB8134	Demarara	NA	NA		
7	M1176/77	Mauritius	22.31	81.91	N55805	CP5530
8	M1246/84	Mauritius	21.2	93.40	M555/60	R570
9	M1334/84	Mauritius	19.58	95.55	M555/60	
10	M1954/91	Mauritius	21.89	69.38	M2077/78	M1030/71
11	RB72/454	Brazil	21.26	58.89	CP53-76	
12	RB86/3129	Brazil	21.63	608.09	RB763411	
13	SP81-3250	Brazil	20.53	72.09	CP70-1547	SP71-1279
14	RB94/2991	Brazil	22.89	66.42		
15	Co88025	Coimbatore	20.92	59.00		
16	Co91017	Coimbatore	21.27	64.64		
17	Co997+	Coimbatore	21.48	79.29		
18	CoC671	Coimbatore	22.63	72.80		
19	KNB9288	Sudan	NA	NA	B871245	POLY
20	KNB9253	Sudan	NA	NA		

Brix content (°Bx): This is the proportion of sucrose in a solution, therefore its correlates with density of liquid. One degree brix is 1 g of sucrose in 100 g of solution
Sources: G. Olaoye, Y. A Abayomi and F.O .Takim (2017). NSDC 2015
NA: Information not available

2.3 ISSR PRIMER SELECTION AND PCR CONDITION

Eight reproducible and informative ISSR primers of 14-19 bp were selected from a total of 10 tested ISSR primers. The primers were selected based on published experimental results on sugarcane and related *Saccharum* species (Da Costa *et al.*, 2011). The selected primers synthesized by a commercial molecular biology company (Inqaba Biotec West Africa Ltd. Ibadan, Nigeria) were used for the Polymerase Chain Reaction (PCR) procedure. The primers were optimized and PCR conditions for experiment were set-up. For an efficient molecular characterization of sugarcane genotypes, initially six individuals were randomly selected to screen the primers for their polymorphism and reproducibility at 52, 54, 57, 61, 62 and 65 °C annealing temperature (Table 2). Band intensity and reproducibility of all conditions were compared and optimized. The selection includes a spectrum of primers with different repeat motifs.

The PCR reaction was carried out with 20 µl final reaction volume in 200 µl thin wall PCR tube in a Thermocycler (Applied Biosystems, Foster city, USA), containing 10.5 µl reaction mixture, 1.5 µl of 10 pmole primer 1 µl of 50 ng genomic DNA and 7 µl of ultra-pure nuclease free water (Ambion, USA). The PCR condition was an initial denaturation at 94 °C for 5 minutes, followed by 35 cycles of denaturation at 94 °C for 1 sec, annealing at 59 °C (annealing temperature was different for each primers) for 30 sec and extension at 72 °C for 1 min followed by a single cycle of final extension at 72 °C for 10 min. The reaction was put on hold at 4 °C. The PCR products were visualized to confirm amplification by the method of Animasaun et al., 2018.

The targeted PCR amplification was confirmed by mixing 5 µl of PCR product with 2 µl of 6X gel 6X gel loading dye (Promega, USA) and electrophoresed on 1.5 % agarose gel stained with 0.75 µl EN-Vision blue eye DNA dye for 40 min at 100 volts in 1X TBE buffer. Fragment sizes of the amplicons were determined from the gel by comparison with standard molecular weight marker ladder-low range Generuler1 Kb DNA Ladder (Thermofisher, USA). The amplified loci were visualized and photographed in a gel documentation system (Ingenius-3, Syngene, USA).

2.4 BAND SCORING AND DATA ANALYSIS

The PCR fragments were scored for the presence (1) or absence (0) of equally sized bands and two matrices of the different ISSR phenotypes were assembled and used in the statistical analysis. The fragments were only considered on ability to detect clearly resolved and polymorphic amplified loci among the populations studied for the eight ISSR primers selected for analysis. The data were entered in to binary matrix for analysis. Module analysis was performed with NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System) and Cluster Analysis was performed using the unweighted pair group method with arithmetic averages (UPGMA), genetic differentiation and Shannon's index (I) was determined using PAST software.

In addition, to compare genotypes and evaluate patterns of genotype clustering, neighbour-joining (NJ) was used with Free Tree 0.9.1.50 (Saitou & Nei, 1987; Pavliček et al., 1999). To further examine patterns of genetic relationship among individual genotypes principal coordinate analysis (PCoA) was performed using agglomerative technique using the Un-weighted Pair Group Method with Arithmetic Mean (UPGMA) Method and dendrogram was constructed as the output for the genetic relationship.

Table 2: List and properties of ISSR primers selected for the molecular study of the molecular characterization of 20 exotic sugarcane varieties (Ilorin, Nigeria)

S/N	Oligo ID	Sequence (5' – 3')	*Tm (°C)	No of base pairs	GC- contents (%)
1	ISSR 1	GAGAGAGAGAGACC	52.61	14	57.14
2	ISSR 2	CTCTCTCTCTCTCTCTAC	57.62	18	50.00
3	ISSR 3	CACACACACACAAG	49.69	14	50.00
4	ISSR 4	CAGCACACACACACACA	60.16	19	52.63
5	ISSR 5	GTGTGTGTGTGTCC	52.61	14	57.14
6	ISSR 6	CTCTCTCTCTCTCTCTGC	59.9	18	55.56
7	ISSR 8	AGCACGAGCAGCAGCGG	64.43	17	70.59
8	ISSR 10	AGCACGAGCAGCAGCGT	62.02	17	64.71

*Tm: melting temperature of the primers

3 RESULT AND DISCUSSION

Genetic diversity in sugarcane provides breeders with the necessary materials and opportunity to develop improved and new varieties possessing desirable characteristics (Govindaraj et al., 2015). Molecular analysis is important to protect genetic identity/purity because, morphological traits could be environmentally influenced (Animasaun et al., 2018). This is also necessary for authentication of accession prior to multiplication of setts for planting. Data obtained from molecular techniques can be analysed prior to application in diversity studies by analyzing the genetic relationship among samples (Govindaraj et al., 2015).

3.1 LEVEL OF POLYMORPHISM

The ISSR analysis, carried out on 20 varieties produced 369 bands/alleles with an average of 46.13 alleles per primer. Eight primers produced distinct and reproducible bands among the primers tested and the amplified PCR products showed arrays of monomorphic and polymorphic bands, 349 were polymorphic alleles and 20 monomorphic alleles with an average of 43.25 ISSR polymorphic alleles per primer**.** In other words, the ISSR markers detected high level of polymorphism. A total of 39 loci were amplified by eight ISSR primers, out of which 38 (91.66 %) were polymorphic. Loci amplification per primer ranged from 3 to 7 with an average of 4.88 loci per primer, and a mean allelic richness of 46.1 alleles/primers (Table 3), number of polymorphic alleles ranged from 2 (ISSR 6) to 7 (ISSR8). The code, sequence and other properties of the primers used are presented in Table 2. Though, seven out of eight of the primers were highly polymorphic, ISSR6 resulted in the least polymorphic loci with 33.3 % polymorphism, ability of primer ISSR 8 and ISSR 10 to produce higher allele frequencies and polymorphic loci in this study indicated that the two primers are most informative and suitable for diversity study in sugarcane accessions.

ISSR markers have been showed to possess high resolution ability in sugarcane fingerprinting and diversity analysis (Da Costa et al., 2011), they are effective and efficient in the identification of polymorphisms within and among populations and/or species. This current study is the first investigation to assess molecular genetic diversity within and among introduced sugarcane accessions in the USRI germplasm. Again, since polymorphic information is related to expected heterozygosity and is usually determined from allele frequency (Animasaun et al., 2015), the existing variation in the studied accession could be selected for the crop improvement.

The present study reveals the existence of high level of genetic diversity and relatedness among and within the investigated sugarcane accessions, which were introduced into Nigeria as part of University of Ilorin Sugar Research Institute (USRI) germplasm. Similar findings were reported for some sugarcane accessions by Srivastava and Guota (2008) who recorded 78.48 % polymorphism in a diversity screening among sugarcane varieties in India using ISSR markers. Smiullah et al. (2013) also detected 85.25 % polymorphism with ISSR markers on Sugarcane accessions from Pakistan. Thus, the studied genotypes showed considerable heterologous amplification of the alleles, whereby 91.66 % were polymorphic and only 8.34 % were monomorphic. High polymorphism and higher number of alleles are very important for correct estimation of genetic diversity of a germplasm (Animasaun et al., 2015). The degree of polymorphism

Table 3: Amplification information of 8 ISSR markers used in the diversity study of the 20 exotic sugarcane accessions in the germplasm of USRI

S/N	Marker Code	TNA	TNL	NML	NPL	P %	M %
1	ISSR1	32	5	0	5	100	-
2	ISSR 2	34	5	0	5	100	-
3	ISSR 3	16	4	0	4	100	-
4	ISSR4	50	5	0	5	100	-
5	ISSR 5	49	4	0	4	100	-
6	ISSR 6	30	3	1	2	33.3	66.6
7	ISSR 8	81	7	0	7	100	-
8	ISSR 10	77	6	0	6	100	-
	TOTAL	369	39	1	38	91.66 %	8.3%
	Average	46.1	4.88				

TNA: Total number of alleles; TNL: total number of loci; NML: Number of monomorphic loci; NPL: Number of polymorphic loci; P %: percentage polymorphism; M %: Percentage monomorphism

showed the extent of diversity and effectiveness of the markers (Pfeifer et al., 2011) and allele phenotype are use as reference to interpret microsatellite profile in diversity studies (Esselink et al., 2004).

3.2 GENETIC SIMILARITY AND DISTANCE

Estimate of similarity coefficient was determined using Jaccard similarity coefficient-based pairwise comparisons, based on the DNA amplification of the 20 accessions of sugarcane (supplementary table 1), similarity ranged from 0.78 to 0.13 with mean of 0.455. Accessions DB8134 and M1246/84 showed the highest genetic similarity having similarity coefficient of 0.78 and were adjudged to be closely related. However, the maximum genetic distance was observed between accessions DB8134 and SP81-3250 with 0.13, M1246/84 and SP81-3250, B74541 and SP81-3250 had a genetic similarity coefficient of 0.136 each, followed by M1334/4 and SP81-3250 with genetic distance of 0.148.

3.3 BIPLOT ANALYSIS

The distribution of the accessions into different spatial plane and co-ordinates by biplot analysis (Fig 1) showed the involvement of markers in separating accessions into quadrants. Co-occurrence of B74541 and M1246/84 in quadrant three and B80689 and Co88025 in quadrant four on the same plane suggested a common ancestor. The overlapping of B85266 and B93757, which are close to RB72/454, indicated the accessions are genetically similar. Samples in quadrant I, III and IV are more closely related, except for M1954/91, while those in quadrant II are diffused i.e. they are relations separated by geographical isolation for a long time (Animasaun et al., 2015).

The spatial closeness of the accessions in biplot analysis indicate their genetic similarity. In addition, the dispersion of the markers from the centroid reflects their effectiveness in delimiting the accessions (Animasaun et al., 2015). However, the distant location of M1954/91, SP81-3250 and DB8134 in quadrant I, II and IV implies the existence of genetic distance. This may be due to accumulation of some genes through selection resulting from domestication of the accessions by local farmers (Animasaun et al., 2015). Meanwhile the obtained marker efficiency as revealed by biplot analysis supported ISSR makers as a useful tool for the initial assessment of intraspecific genetic variation (Devarumath et al., 2012).

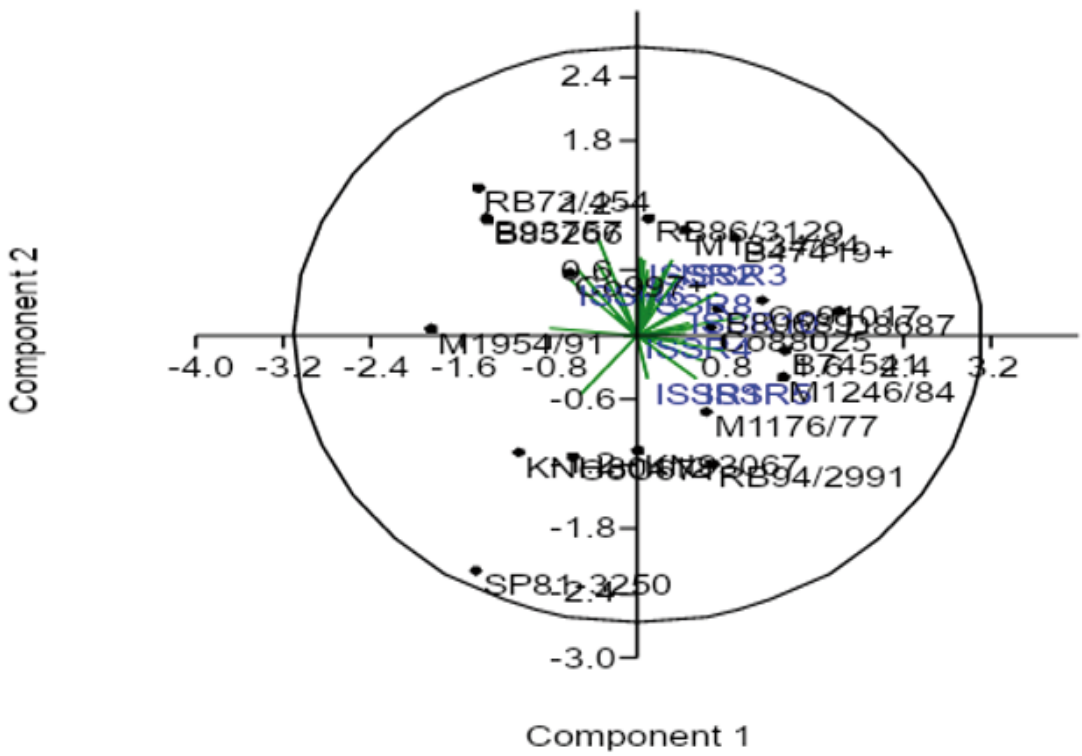

Figure 1: Bootstrapped Biplot of twenty accessions of sugarcane accessions characterized by eight ISSR primers for diversity and genotyping analysis (at $p < 0.05$)

3.4 CLUSTER ANALYSIS

Neighbor Joining-based and UPGMA tree construction methods on the basis of Jaccard's similarity coefficient was used to construct dendrogram to examine the relationship among sugarcane accessions based on 369 ISSR bands amplified by eight primers. The dendrogram derived from UPGMA-based cluster analysis of the whole ISSR data with 20 sugarcane accessions shows all the accessions in the first cluster were clustered closely together which shows high genetic similarity consisting of nine (9) accessions (group 2). Cluster analysis by dendrogram depicting the genetic relationship classified the accessions into to group (1 and 2). On the other hand, the second group (group 2) sub-divided into 2a and 2b (Fig 3). The 2b group split into two clusters, 2b(i) and 2b(ii) (Fig 3). Cluster 2a is comprised of four genotypes, cluster 2b(i) and 2b(ii) is made up of three and four accessions respectively. The different accessions formed clusters irrespective of their different geographical origins. The neighbor joining (Fig 2) showed that B97375 is a distance neighbors from the other accessions. In addition, RB86/3129 and Co997+ were joined together as neighbours, M134/84 was a close neighbor to Co88025. Also, B96723 was the closest neighbour to RB72/454.

Clustering parameters such as, Principal coordinate analysis, UPGMA, Neighbour joining showed close clustering of individuals and intermixing in clusters irrespective of their origin. Interestingly, according to Ullah et al. (2013) accessions grouped in same cluster are more similar to each other but less similar to the accessions in other clusters. This means that accessions in the same cluster are genetically similar or related. This knowledge is important because, based on their genetic relationship, recognizing and classifying different individuals in homogeneous groups will help breeders select parents and improve efficiency in preparing crosses for breeding programs (Zeni Neto et al., 2020).

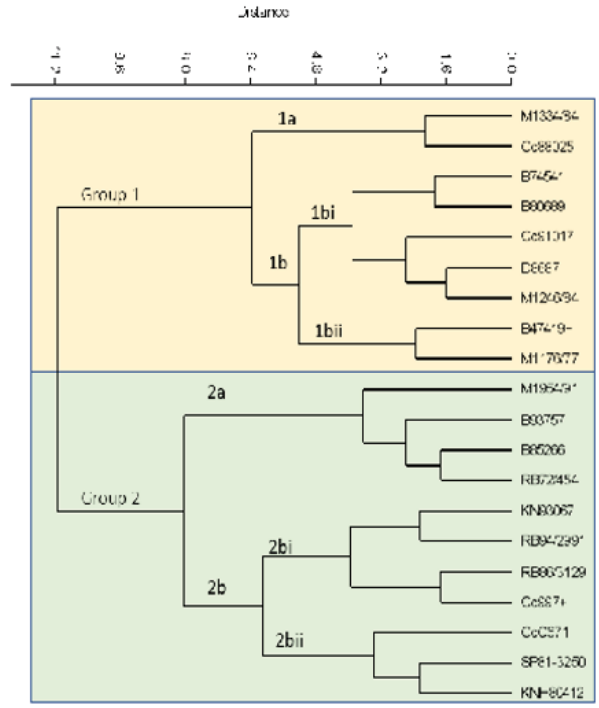

Figure 3: Genetic relatedness of the sugarcane genotypes based on UPGMA cluster analysis

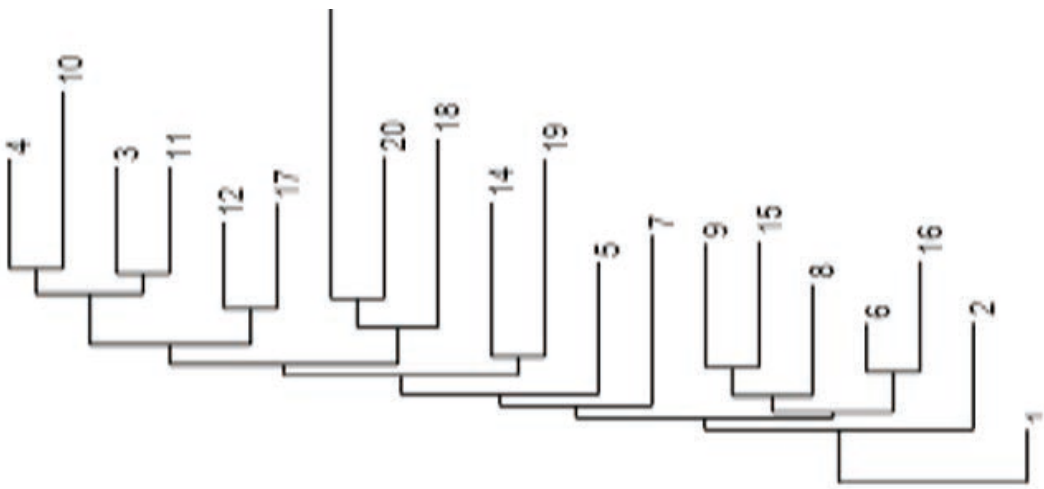

Figure 2: Neighbor joining diagram of genotypes based on ISSR marker analysis. 1 = B97375, 2 = B96812, 3 = B96723, 4 = B93757, 5 = B47419+, 6 = DB8134, 7 = M1176/77, 8 = M1246/84, 9 = M1334/84, 10 = M1954/91, 11 = RB72/454, 12 = RB86/3129, 13 = SP81-3250, 14 = RB94/2991 15 = Co88025, 16 = Co91017, 17 = Co997+, 18 = CoC671, 19 = KNB9288, 20 = KNB9253

3.5 PRINCIPAL COORDINATE ANALYSIS

Pattern of variation among the sugarcane accessions was also observed using Principal coordinate analysis based on Jaccard's similarity coefficient using PAST Statistical software package. The sugarcane accessions clustered in the four quadrants irrespective of their place of origin. The ordination of the accessions on principal component axes PCo1 versus PCo2 based on cluster analysis of ISSR allelic data (Fig 4). It provided distinct, groupings that established sub-groups within the quadrants, which illustrate the degree of relatedness and diversity within the quadrants. In quadrant 1 accessions B96723, B93757, RB72/454 appeared to be closely related, but formed a sub group with accessions Co997+ and M1954/91 located a far distance away from the other accessions in quadrant 1. The occurrence of accessions B96723 and B93757 and the closeness of RB72/454 in quadrant 1 showed that they are closely related and indicates a common ancestor. In quadrant II, accessions SP81-3250, KNB9253, CoC671 formed a sub group while KNB9288 stands alone located close to the centroid. Also clustering of accessions SP81-3250, CoC671, KNB-9288, SP81-3250, KNB-9253 are far apart in quadrant II suggesting different origin. The diffuse pattern shows genetic divergence among the populations with accession SP81-3250 being at the farther end of the quadrant revealing utmost genetic variation and distance from other populations. In quadrant III, two subgroups were formed one with accessions M1176/77 and RB94/2991 and another with B97375 and M1246/84 showing similarity. Quadrant IV had the highest number of accessions (7) among other quadrants. Accessions RB86/3129, M1334/84, B47419+ formed a sub-group within the quadrant. The clustering of accessions B97375, B96812 and Co88025 in quadrant IV indicates a common ancestor, while accession DB8134 is far away from other accessions in quadrant IV.

Principal coordinate analysis revealed the important components contributing to the observed variation among the 20 sugarcane accessions. This form of admixture may result from the participation of sugarcane genotypes in breeding programs to enhance some of the characteristics of commercially exploited varieties, so that parental breeding lines may be exchanged across the world's sugarcane growing regions to achieve these goals (Tazeb et al., 2017). Another explanation for the high levels of similarity between subgroups and groups is

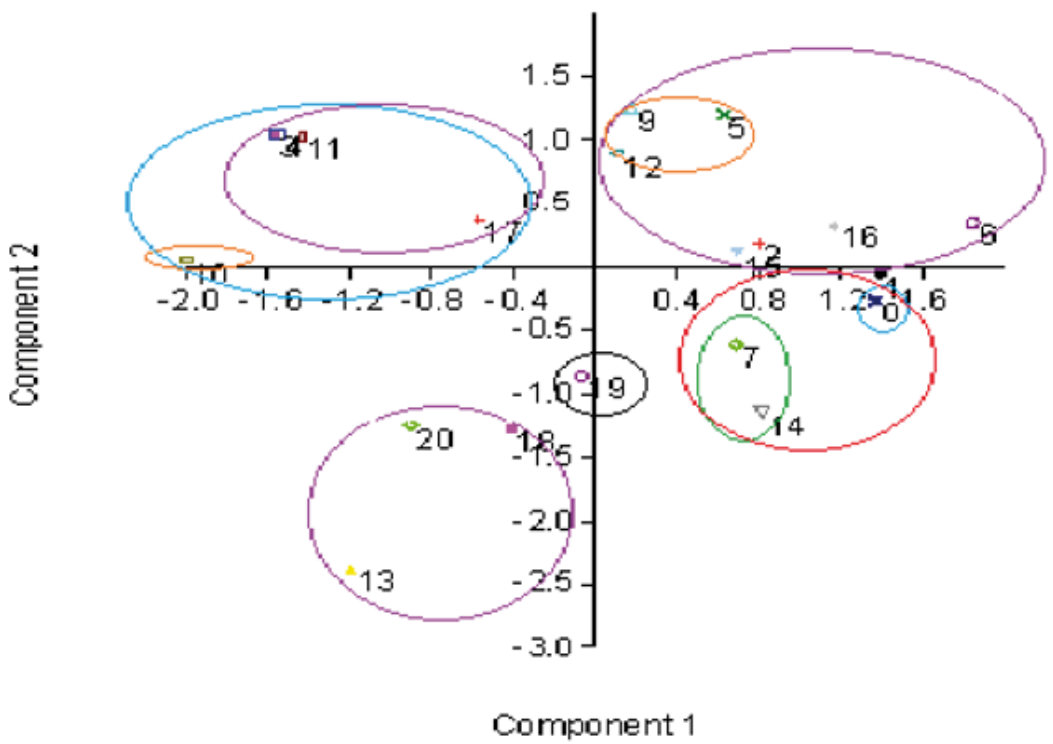

Figure 4: The ordination of twenty Sugarcane (*Saccharum officinarum*) accessions on principal component axes PCo 1 versus PCo 2 based on cluster analysis of ISSR allelic data. 1 = B97375, 2 = B96812, 3 = B96723, 4 = B93757, 5 = B47419+, 6 = DB8134, 7 = M1176/77, 8 = M1246/84, 9 = M1334/84, 10 = M1954/91, 11 = RB72/454, 12 = RB86/3129, 13 = SP81-3250, 14 = RB94/2991, 15 = Co88025, 16 = Co91017, 17 = Co997+, 18 = CoC671, 19 = KNB9288, 20 = KNB9253

that during the sugarcane breeding program, the lineages were exposed to a higher degree of intercultivar gene flow (Rodriguez et al., 2005). Comprehensively, it is possible for geographically different population to form a cluster with other population, because the majority of commercial sugar cane cultivars bred after the turn of the 20th century are interspecific hybrids between *Saccharrum officinarum* and *Saccharrum spontaneum* L. (D'Hont et al., 1996*)*. Thus, the cross progeny may have clustered from other regions with their progenitors or parents or the parents may have clustered from distantly related populations with their cross progeny *(*Tazeb et al., 2017*)*. Clustering of all sugarcane accessions together irrespective of their sources showed their remarkable genetic similarity and reinforced the postulate of a common progenitor for the accessions (Jauhar & Hanna, 1998).

4 CONCLUSION

Adequate genetic information is prerequisite to identify potential parental combinations required in hybridization programme aimed to create segregating progenies with maximum genetic variability for further selection. The information from this study could provide accurate information to sugarcane breeding in Nigeria for strategic conservation of the germplasm resources and future improvement work of the sugarcane by selecting suitable parents for breeding programs aimed at optimizing sugar yield, establishing the proper identity of the accessions and preventing duplication of accessions.

In addition, ISSR markers showed reliability and efficiency in detecting polymorphisms within and among the sugarcane accessions studied. It has also been shown that ISSR markers have high-resolution ability in sugarcane fingerprinting and diversity analysis and are therefore effective and efficient in the identification of polymorphisms within and among populations and/or species. However, seven out of the eight primers were polymorphic, ability of primer ISSR 8 and ISSR 10 to produce higher allele frequencies and polymorphic loci in this study indicated that the two primers are the most informative and suitable for diversity study in sugarcane. It is, therefore recommended that molecular marker approach be deployed in investigating the remaining germplasm for diversity and breeding programs.

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Vpliv sezone na in vitro razgradljivost in fermentabilnost krmil v vampovem soku navadnega jelena (*Cervus elaphus* L.)

Andrej LAVRENČIČ [1, 2], Darko VETERNIK [3]

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Vpliv sezone na in vitro razgradljivost in fermentabilnost krmil v vampovem soku navadnega jelena (*Cervus elaphus* L.)

Izvleček: Prehod iz poletja v zimo povzroči spremembe v fiziologiji prebavnega trakta navadnega jelena in v tam prisotnih prebavnih procesih. Zato je bil namen raziskave ugotoviti, kako sezona vpliva na in vitro navidezno (*iv*NRSS) in pravo razgradljivost suhe snovi (*iv*PRSS), na kazalnike in vitro tvorbe plina in na sproščanje hlapnih maščobnih kislin (HMK) iz enajstih krmil, ki jih zauživajo ali dokrmljujemo košutam navadnega jelena v Sloveniji (plodovi kostanja in gradna ter želod in dva vzorca sveže trave, dva vzorca mrve in travne silaže, jabolčne tropine in koreni sladkorne pese). *Iv*NRSS in *iv*PRSS, določeni z inkubacijo krmil v puferiranem vampovem soku, se med sezonama nista razlikovali, prav tako tudi nismo ugotovili velikih razlik pri večini kazalnikov produkcije plina. Le kazalnik »C« (specifična hitrost tvorbe plina) je bil večji ($p < 0,05$) v zimski sezoni. Tudi količine HMK so bile med sezonama zelo podobne. Vendar pa je bil delež ocetne kisline pogosto nekoliko večji pozimi kot jeseni ($0,05 < p < 0,10$), medtem ko sta bila deleža propionske in maslene kisline pri krmilih, ki so vsebovala več vlaknine, večja ($0,05 < p < 0,10$) jeseni kot pozimi. Nasprotno pa smo ob inkubaciji krmil, ki vsebujejo veliko škroba (želod in kostanj), določili večji ($p < 0,05$) delež propionske kisline pozimi, medtem ko je bil pri teh krmilih delež maslene kisline večji ($p < 0,05$) jeseni. Čeprav sta bila tako število uporabljenih substratov (n = 11) kot število živali (n = 6), darovalk vampovega soka, majhna, pa ti rezultati kažejo na spremembo presnove vampovih mikroorganizmov med jesenjo in zimo.

Ključne besede: navadni jelen; *Cervus elaphus* L.; prehrana živali; sezona; krmila; in vitro prebavljivost; in vitro produkcija plina; hlapne maščobne kisline; vamp

The effect of season on in vitro degradability and fermentability of feeds in red deers' (*Cervus elaphus* L.) rumen fluid

Abstract: Transition from summer to winter changes red deer digestive tract physiology and digestive processes. The objective of the trial was to determine the effects of season on in vitro apparent (*iv*ADMD) and true dry matter (*iv*TDMD) digestibility, in vitro gas production parameters and short-chain fatty acid synthesis (SCFA) in red deer hinds of eleven substrates naturally occurring in Slovenia (chestnut fruits, acorns of common and sessile oak, two fresh grasses) and those frequently used in supplemental red deer feeding (two grass hays and two grass silages, apple pomace and sugar beet roots). There were no differences in *iv*ADMD, *iv*TDMD, determined by incubation of feeds in buffered rumen fluid, as there were no differences in majority of gas production parameters between autumn and winter season. Only the parameter "C" (specific gas production rate) was frequently higher ($p < 0.05$) in winter season than in autumn season. The amounts of SCFA were similar between two seasons. However, the proportion of acetic acid tended to be higher in winter, while the proportions of propionic and butyric acid tended to be higher in autumn than in winter especially in high fibre feeds. On contrary, in high starch feeds such as oak acorns and chestnut fruits, the proportion of propionic acid was higher ($p < 0.05$) in winter, while of butyric acid in autumn ($p < 0.05$). Despite the fact that the number of used substrates (n = 11) and animal rumen fluid donors (n = 6) were small, these results indicate a shift in rumen microbial metabolism between autumn and winter season.

Key words: red deer; *Cervus elaphus* L.; animal nutrition; season; feed; in vitro digestibility; in vitro gas production; short-chain fatty acids; rumen

1 Univerza v Ljubljani, Biotehniška fakulteta, Oddelek za zootehniko, Ljubljana, Slovenija
2 Korespondenčni avtor, e-naslov: andrej.lavrencic@bf.uni-lj.si
3 LPN Kozorog Kamnik, Kamnik, Slovenija

1 UVOD

Uspešno upravljanje s populacijo navadnega jelena (*Cervus elaphus* L.) in s tem povezano preprečevanje škod na gozdnem drevju, še posebej na območjih, kjer je populacija jelenov zelo številčna, je v veliki meri odvisno od poznavanja navad jelenov in od njihove učinkovitosti izkoriščanja naravnih virov. Pri tem pogosto navajamo razlike v obnašanju jelenov med hranjenjem, ki so povezane s prisotnostjo plenilcev in obstojem kakovostnega kritja, ter razlike, povezane z oblikovanjem skupin, ki pa so odvisne od socialnih povezav, gostote živali na posameznem območju in stopnje vznemirjanja živali (Hafner in Černe, 2015).

Podobno kot druge živali so tudi prežvekovalci podvrženi naravnim ritmom pridobivanja in izgubljanja telesne mase, zauživanja krme, porabe energije, presnove in razmnoževanja. Dva pomembna mehanizma, ki vplivata na navedene kazalnike, sta razpoložljivost prehranskih virov in dolžina dneva (Clauss in sod., 2010), za katera pa ni povsem jasno, ali delujeta povsem ločeno ali v različnih kombinacijah. Zauživanje krme in prirast telesne mase sta najmanjša v zimski in največja v poletni sezoni (Milne in sod., 1978, Stevens in sod., 2003; Arnold in sod., 2004). Sibbald in Milne (1993), Arnold in sod. (2015) ter Weckerly in sod. (2018) navajajo, da se v poletni sezoni pri jelenih povečata tudi napolnjenost in masa prebavil (vamp in siriščnik). Weckerly in sod. (2018) so pri belorepem jelenu (*Odocoileus virginianus*) ugotovili, da je masa prebavil povezana predvsem z napolnjenostjo prebavil (digesta load) in ne s hranilno vrednostjo krme. Ker je napolnjenost prebavil dobro povezana s prostornino vampa, je logično, da je v zimski sezoni manjša tudi prostornina predželodcev in drugih jelenovih prebavnih organov (Hofmann, 1985; Sibbald in Milne, 1993; Arnold in sod., 2015). Z manjšo prostornino predželodcev pa je povezana tudi dnevno zaužita količina energije, ki je v pozni jeseni in zgodnji zimi najmanjša, svoj vrh pa doseže med junijem in oktobrom (Arnold in sod., 2015).

S prehranskega vidika so v prehrani navadnega jelena pomembni predvsem obnašanje med zauživanjem krme, sposobnost jelenov za prebavo večjih količin krme, učinkovito izkoriščanje hranljivih snovi in končnih produktov prebave ter zmožnost živali, da zadostijo potrebam po hranljivih snoveh (Milne in sod., 1978). V splošnem velja, da so razlike v fermentaciji in razgradnji krme in hranljivih snovi v predželodcih povezane z razlikami v kemični sestavi zaužitih obrokov. Palmer in sod. (1976) navajajo, da lahko pričakujemo veliko variabilnost v aktivnosti vampovega soka med jeleni iz naravnega okolja, saj ti jeleni zauživajo zelo raznoliko krmo, od dobro prebavljivih sadežev in semen do slabo prebavljive voluminozne krme. Prav tako pa lahko pričakujemo veliko variabilnost v aktivnosti vampovega soka med sezonami, saj se razpoložljivost naravnih prehranskih virov med sezonami zelo spreminja, spreminja pa se lahko tudi zaradi dokrmljevanja jelenov v obdobju pomanjkanja naravnih virov krme.

Namen naše raziskave je bil ugotoviti, ali sezona vpliva na aktivnost vampovega soka navadnega jelena. Kriteriji, ki smo jim sledili, so bili in vitro navidezna in prava razgradljivost suhe snovi, kazalniki in vitro tvorbe plina in količina in vitro tvorjenih hlapnih maščobnih kislin ter razmerja med njimi. Pri tem smo uporabili krmo, ki jo jeleni običajno najdejo v naravi ali pa so sestavni del obroka pri zimskem dokrmljevanju.

2 MATERIAL IN METODE

V študiji smo uporabili 11 krmil, ki jih pogosto najdemo bodisi v naravnem okolju bodisi jih pogosto uporabljamo pri zimskem dokrmljevanju navadnega jelena. Vsa ta krmila smo uporabili že v prejšnjih poskusih (Lavrenčič in Veternik, 2018a in b). Krmila iz naravnega okolja jelena so bila: dva vzorca sveže trave-paše (z območja Jelendola in Kokre), plodovi divjega kostanja (*Aesculus hippocastanum*) ter želod doba (*Quercus robur*) in gradna (*Quercus petraea*). Vzorci krmil, ki so bila namenjena dokrmljevanju, so bili: dva vzorca mrve in travne silaže (z območja Jelendola in Kokre), koreni sladkorne pese in jabolčne tropine. Vzorce paše, korenov sladkorne pese in jabolčnih tropin smo pred kemijskimi analizami in inkubacijo v vampovem soku v 48 urah posušili pri 60 °C. Suhe vzorce smo zmleli skozi 1 mm sito in analizirali na vsebnost surovih beljakovin (SB), surovega pepela (SP), surovih maščob (SM) in surove vlaknine (SV) z metodami po Neumann in Bassler (1986), medtem ko smo vsebnosti v nevtralnem detergentu netopne vlakinen analizirali z aparatom ANKOM^{220} Fibre Analyser (Ankom Technology, Macedon, NY) z metodo po avtorjih Goering in Van Soest (1970), pri čemer pa smo uporabili natrijev sulfit. Kemična sestava uporabljenih krmil je podana v preglednici 1.

Za primerjavo značilnosti vampovega soka smo izbrali dve sezoni. Za jesensko sezono smo vzeli mesece september, oktober in november, medtem ko so zimsko sezono predstavljali meseci december, januar in februar. V lovni sezoni smo uplenili šest košut, od tega štiri jeseni, med sredino septembra in sredino oktobra 2011, dve pa pozimi, prvo v sredini decembra 2011, drugo pa v januarju 2012, za kar smo dobili posebno dovoljenje Ministrstva za kmetijstvo, gozdarstvo in prehrano (št. 341-1/2012/6 z dne 18. 1. 2012). Da-

tumi uplenitev so bili v skladu z začetkom zimskega dokrmljevanja jelenov, ki ga v Lovišču s posebnim namenom (LPN) Kozorog Kamnik začnejo izvajati konec novembra ne glede na vremenske in snežne razmere. V celotni zimi 2011-2012 je bilo jelenjadi ponujeno skupno približno 30 ton sena, travne silaže in sladkorne pese, vendar pa živali krmišča niso redno obiskovale. Ocenjujemo, da je bila številčnost jelenjadi na krmiščih le okoli 30 % v primerjavi s številčnostjo v zimah, ki so bile bogate s snežno odejo. Košute smo v tem LPN uplenili tako na območju Jelendola (46°24'27.79" N in 14°24'30.65" E; 850 m nadmorske višine; občina Tržič) kot na območju lovskega revirja Kokra (46°21'57.37" N in 14°25'25.36" E; 925 m nadmorske višine; občina Preddvor). Takoj po uplenitvi smo iz živali odstranili vamp in ga v največ 45 minutah v zaprti, toplotno izolirani posodi prepeljali v laboratorij. Vsako inkubacijo, ki je predstavljala eno serijo, smo opravili za vsako žival posebej.

In vitro navidezno in pravo razgradljivost suhe snovi (*iv*NRSS in *iv*PRSS) smo določili po postopkih, ki sta jih opisala Lavrenčič in Veternik (2018a). Iz vsebine vampa vsake košute posebej smo pripravili inokulum tako, da smo vampovo vsebino ročno oželi skozi štiri pasti bombažne gaze in jo razredčili z raztopino pufra v razmerju 1 : 2 (v/v). Okoli 450 mg zmletega krmila smo zatehtali v filtrske vrečke ANKOM F57 (ANKOM Technology, Macedon, NY, USA) in jih toplotno zavarili. Za vsako krmilo smo pripravili štiri vrečke in jih po 2 vstavili v 2 inkubacijski posodi (2 vrečki/posodo). V vsako posodo smo vstavili 24 vrečk F57 (2 vrečki/krmilo + dve prazni vrečki (slepi vzorec)) in dodali dva litra puferiranega vampovega soka, ki smo ga predhodno prepihovali z ogljikovim dioksidom. Posode smo vstavili v inkubator na 39 °C za 24 h. V času inkubacije je bilo zagotovljeno mešanje puferiranega vampovega soka v inkubacijskih posodah. Po končani inkubaciji smo vrečke intenzivno sprali pod tekočo vodo, posušili in stehtali ter izračunali *iv*NRSS. Vrečke smo v nadaljevanju tretirali še 1 uro v raztopini nevtralnega detergenta (ND) pri 100 °C v aparaturi ANKOM^{220} fibre analyser (ANKOM Technology, Macedon, NY, USA), jih sprali v vodi, posušili in stehtali ter izračunali in vitro pravo razgradljivost suhe snovi (*iv*PRSS). Obe, tako *iv*NRSS kot *iv*PRSS, sta bili izračunani kot delež med razliko zatehte suhe snovi vzorca in ostankom suhe snovi v vrečki po inkubaciji oz. obdelavi vzorca z ND. Dobljene *iv*NRSS in *iv*PRSS smo nato korigirali na izgubo mase slepega vzorca (prazne vrečke) med inkubacijo oz. obdelavo z ND (količnik med maso oprane in posušene prazne vrečke po inkubaciji oz. obdelavi z ND in maso neinkubirane oz. z ND neobdelane prazne vrečke).

Inokulum za izvedbo plinskega testa smo pripravi na enak način kot inokulum za določanje *iv*NRSS (Menke in Steingass, 1988; Lavrenčič in Veternik, 2018b). Okoli 200 mg zmletega krmila smo inkubirali v anaerobnih pogojih pri 39 °C v 100 ml steklenih brizgalkah, ki so vsebovale 30 ml puferiranega vampovega soka (inokuluma). Količino sproščenega plina smo odčitali po 0, 2, 4, 6, 8, 10, 12, 24, 36, 48, 72 in 96 urah. Po 24 urah smo tekočino dveh od štirih brizgalk prenesli v 50 ml centrifugirne epruvete in jih do analize na vsebnost hlapnih maščobnih kislin (HMK) zamrznili na −20 °C. V vsaki seriji smo inkubirali tri brizgalke s slepim vzorcem (vsebovale so samo inokulum brez substrata) in tri brizgalke s standardnim vzorcem.

Vse postopke od uplenitve živali do začetka inkubacije smo izvedli v manj kot 2 urah, v času, ki ga še kot primernega navajata tudi Schwartz and Nagy (1972).

Ekstrakte za analizo HMK smo pripravili iz puferiranega vampovega soka po 24 urah inkubacije po modificirani metodi Holdeman in sod. (1977). HMK smo določili s plinskim kromatografom Hewlett Packard 5890 A (Hewlett Packard, Bellefonte, Pennsylvania, USA), opremljenim s split/splitless injektorjem in FID detektorjem. Za ločevanje HMK smo uporabili 30 m NUKOL TM, FUSED SILICA kapilarno kolono (SUPELCO, Bellefonte, Pennsylvania, USA).

Dobljene količine plina smo korigirali na 1 *g* suhe snovi krmila in tudi na količino plina, sproščenega iz slepega vzorca. Kazalnike plinskega testa smo za vsak substrat ocenili s pomočjo Gompertzove enačbe (Lavrenčič in sod., 1997): $Y_t = B \times \exp(-C \times \exp(-D \times t))$, kjer je Y_t količina sproščenega plina (ml/g DM) v času 't', 'B' največja količina sproščenega plina (skupna potencialna količina plina; (ml/g SS)); 'C' specifična hitrost sproščanja plina, na katero vpliva konstantni faktor 'D', s katerim opisujemo zmanjševanje specifične hitrosti tvorbe plina (ki je posledica zmanjševanja hitrosti rasti mikroorganizmov in zmanjševanja količine fermentabilnega substrata) in 't' čas v urah. Poleg kazalnikov tvorbe plina smo izračunali tudi količino plina, sproščenega po 24 urah inkubacije (GAS24; ml/g suhe snovi), s pomočjo prvega in drugega odvoda Gompertzove enačbe po času pa še največjo hitrost tvorbe plina (MFR) in čas, ko je bila dosežena največja hitrost tvorbe plina (TMFR; Lavrenčič in sod., 1997).

Neto količino HMK, sproščeno v 24 urah inkubacije, smo izračunali tako, da smo od bruto količine posameznih HMK odšteli posamezne HMK, ki so se sprostile v tem času v slepem vzorcu. Dobljene količine smo nato korigirali na 1 g inkubirane suhe snovi krmila.

Rezultate smo nato analizirali z enofaktorsko (one-way analysis of variance) analizo variance s proceduro splošnega linearnega modela (GLM) statističnega paketa SAS/STAT version 9.4 (SAS,

2015). Primerjali smo kazalnike med sezonama (jesen vs zima) za vsako krmilo posebej. Rezultate predstavljamo kot povprečne razlike, dobljene s testom najmanjših kvadratov (least square means). Kot statistično značilne smo sprejeli razlike pri $p \leq 0{,}05$, medtem ko smo kot trende obravnavali razlike pri $0{,}05 < p < 0{,}10$.

3 REZULTATI

Vzorce, katerih sestavo navajamo v preglednici 1, sta v svoji raziskavi uporabila že Lavrenčič in Veternik (2018a). Med njimi je obstajala velika raznolikost v kemični sestavi in bi jih lahko v grobem razdelili v dve skupini: na voluminozno krmo, kamor uvrščamo svežo travo-pašo, mrvo in travne silaže ter glede na vsebnost surove vlaknine in NDV tudi jabolčne tropine, ki pa jih lahko, zaradi fizikalne strukture, uvrščamo tudi v skupino močne krme, za katero je značilna majhna vsebnost vlaknine (tako surove vlaknine kot NDV) in relativno velika vsebnost BDI in NVOH (preglednica 1).

Podatke o in vitro navidezni (*iv*NRSS) in pravi razgradljivosti suhe snovi (*iv*PRSS) obravnavanih krmil pri košutah v jesenski in zimski sezoni prikazujemo v preglednici 2. Tako *iv*NRSS in *iv*PRSS sta bili številčno večinoma vedno večji v jesenski sezoni kot pozimi, a smo le za svežo travo iz Jelendola in za korene sladkorne pese ugotovili trende ($0{,}05 < p < 0{,}10$) pri *iv*NRSS. Nasprotno pa so imeli plodovi divjega kostanja ter želod doba večje *iv*NRSS in *iv*PRSS pozimi kot jeseni, a je bila le *iv*PRSS želoda doba statistično večja ($p < 0{,}05$).

Kazalnike plinskega testa, ocenjene s pomočjo Gompertzovega modela, prikazujemo v preglednici 3. Skupna potencialna tvorba plina (kazalnik "B") je bila večinoma večja v zimski sezoni, čeprav so bile razlike statistično značilne ($p < 0{,}05$) le pri sveži travi iz Jelendola, jabolčnih tropinah, korenih sladkorne pese ter želodu gradna in doba. Velik vpliv sezone pa smo ugo-

Preglednica 1: Kemična sestava krmil, uporabljenih v poskusu
Table 1: Chemical composition of feeds used in the experiment

	SS DM	SB CP	SM EE	SV CF	SP Ash	BDI NFE	NDV NDF	NVOH NFC
Krmilo / Feed	(g/kg)	(g/kg SS – g/kg DM)						
Sveža trava (Jelendol) Fresh grass (Jelendol)	202	203	29	197	64	506	440	171
Sveža trava (Kokra) Fresh grass (Kokra)	282	134	24	278	70	494	600	91
Travna silaža (Jelendol) Grass silage (Jelendol)	387	128	31	340	97	404	640	42
Travna silaža (Kokra) Grass silage (Kokra)	586	194	28	237	107	434	517	72
Mrva (Jelendol) Grass hay (Jelendol)	867	107	18	304	97	472	543	178
Mrva (Kokra) Grass hay (Kokra)	843	93	19	282	70	537	563	192
Jabolčne tropine Apple pomace	153	58	28	218	27	669	482	338
Koreni sladkorne pese Sugarbeet roots	202	68	5	61	27	840	141	695
Plodovi divjega kostanja Chestnut fruits	372	85	16	143	25	730	389	422
Želod gradna Sessile oak acorns	579	52	36	130	22	760	278	542
Želod doba Common oak acorns	508	53	37	134	22	753	291	532

SS = suha snov – DM = dry matter; SB = surove beljakovine – CP = crude protein; SM = surove maščobe – EE = ether extract; SV = surova vlaknina – CF = crude fiber; SP = surovi pepel – Ash = crude ash; BDI = brezdušični izvleček (BDI = SS – (SP + SB + SM + SV)) – NFE = nitrogen-free extract (NFE = DM – (Ash + CP + EE + CF)); NDV = v nevtralnem detergentu netopna vlaknina – NDF = neutral detergent fiber, NVOH = nevlakninasti ogljikovi hidrati (NVOH = SS – (SP + SB + SM + NDV)) – NFC = non-fiber carbohydrates (NFC = DM – (Ash + CP + EE + NDF))

tovili pri kazalniku »C« (specifični hitrosti razgradnje). Ta je bil večji ($p < 0,05$) pri večini krmil, inkubiranih v vampovem soku košut, uplenjenih v zimski sezoni. Izjema je bila samo mrva iz Jelendola, medtem ko smo pri korenih sladkorne pese in želodu gradna ugotovili le trend ($0,05 < p < 0,10$). Nasprotno pa se kazalnik »D« pri posameznih substratih med sezonama ni razlikoval ($p > 0,05$), čeprav je bil pri številnih substratih številčno manjši v jesenski sezoni. Statistično značilno ($p < 0,05$) različna kazalnika »D« smo dobili pri inkubaciji mrve iz Kokre in pri inkubaciji želoda gradna.

Pri primerjavi izračunanih kazalnikov tvorbe plina (preglednica 4) smo ugotovili, da se je v 24 urah sprostilo več plina (GAS24) ob fermentaciji substratov, ki smo jih inkubirali v vampovem soku košut, uplenjenih v zimski sezoni, pri tem pa so bile razlike statistično značilne ($p < 0,05$) le za svežo travo iz Jelendola, za mrvo iz Kokre in korene sladkorne pese, medtem ko smo za mrvo iz Jelendola ugotovili le trend. Čas, ko je bila hitrost tvorbe plina največja (TMFR), je bil številčno večinoma daljši pri inkubaciji vzorcev v inokulumu, pripravljenim iz vampove vsebine košut, uplenjenih pozimi. Vendar pa smo statistično značilne razlike ($p < 0,05$) izračunali samo za nekatera krmila, pri inkubaciji sveže trave iz Jelendola, jabolčnih tropin ter za želode gradna in doba, medtem ko smo pri inkubaciji sveže trave iz Kokre, korenov sladkorne pese in plodov divjega kostanja izračunali zgolj trend ($0,05 < p < 0,10$). Tudi največja hitrost tvorbe plina (MFR) je bila večinoma večja ob inkubaciji vzorcev v inokulumu, pripra-

Preglednica 2: In vitro navidezna razgradljivost suhe snovi (*iv*NRSS; g/kg) in in vitro prava razgradljivost suhe snovi (*iv*PRSS) krmil, inkubiranih v inokulumih, pripravljenih iz vampove vsebine jeseni in pozimi uplenjenih košut
Table 2: In vitro apparent dry matter degradability (*iv*ADMD; g/kg) and in vitro true dry matter digestibility (*iv*TDMD; g/kg) of feeds, incubated in inocula, prepared from rumen contents of hinds shot in autumn and winter

	*iv*NRSS *iv*ADMD			*iv*PRSS *iv*TDMD		
Krmilo Feed	jesen autumn	zima winter	SED	jesen autumn	zima winter	SED
Sveža trava (Jelendol) Fresh grass (Jelendol)	646*	564*	44,8	801	750	34,4
Sveža trava (Kokra) Fresh grass (Kokra)	454	437	33,1	611	595	25,2
Travna silaža (Jelendol) Grass silage (Jelendol)	491	460	41,8	606	587	34,3
Travna silaža (Kokra) Grass silage (Kokra)	560	526	68,5	742	702	32,0
Mrva (Jelendol) Grass hay (Jelendol)	540	517	32,7	670	652	37,2
Mrva (Kokra) Grass hay (Kokra)	465	423	32,8	624	586	28,4
Jabolčne tropine Apple pomace	497	489	16,8	704	699	11,5
Koreni sladkorne pese Sugarbeet roots	934*	881*	24,7	960	939	13,1
Plodovi divjega kostanja Chestnut fruits	408	436	25,4	725	738	12,7
Želod gradna Sessile oak acorns	523	517	20,3	767	775	7,7
Želod doba Common oak acorns	506	542	39,4	755[b]	791[a]	13,2

*iv*NRSS = in vitro navidezna razgradljivost suhe snovi (mg/g SS) – *iv*ADMD = in vitro apparent dry matter degradability (mg/g DM); *iv*PRSS = in vitro prava prebavljivost suhe snovi (mg/g SS) – *iv*TDMD = in vitro true dry matter digestibility (mg/g DM); SED = standardna napaka razlike – SED = standard error of the differenc

[a,b] povprečja, označena z različnimi črkami znotraj parametra se statistično razlikujejo pri $p < 0,05$ – [a,b] means with different superscripts within parameter differ significantly ($p < 0.05$)

* povprečja znotraj parametra kažejo trende ($0,05 < p < 0,10$) – means within the parameter show trends ($0.05 < p < 0.10$)

vljenem iz vampove vsebine košut, uplenjenih v zimski sezoni, pri čemer pa smo statistično značilne razlike ($p < 0,05$) izračunali pri obeh mrvah (iz Jelendola in iz Kokre), pri korenih iz sladkorne pese in želodu gradna, medtem ko smo trend ($0,05 < p < 0,10$) izračunali le pri inkubaciji travne silaže iz Kokre.

V preglednici 5 prikazujemo podatke o količini sproščenih hlapnih maščobnih kislin (HMK) in njihovih deležih. Ob inkubaciji vzorcev v inokulumu, pripravljenem iz vampovega soka košut, uplenjenih pozimi, se je pri večini vzorcev sprostilo več HMK kot iz tistega, pripravljenega iz vampovega soka jeseni uplenjenih košut. Kljub temu pa smo statistično značilne razlike ($p < 0,05$) izračunali le pri mrvi iz Jelendola, medtem ko smo pri jabolčnih tropinah in želodu gradna izračunali le trend ($0,05 < p < 0,10$). Nasprotno pa smo pri inkubaciji sveže trave iz Kokre, travne silaže iz Jelendola in želoda doba ugotovili, da je bila produkcija HMK večja, če smo te vzorce inkubirali v inokulumu pripravljenem iz vampa jeseni uplenjenih košut, pri tem pa je bila razlika statistično značilna ($p < 0,05$) le pri inkubaciji sveže trave iz Kokre. Pri 24-urni inkubaciji v vampovem soku košut, uplenjenih pozimi, smo v vseh vzorcih določili številčno večji delež ocetne kisline, čeprav smo statistično značilno odstopanje ($p < 0,05$) zabeležili samo pri korenih sladkorne pese, trende ($0,05 < p < 0,10$) pa pri sveži travi iz Kokre, travni silaži iz Kokre, obeh vzorcih mrve in plodovih divjega kostanja. Nasprotno pa so bili deleži maslene kisline pri vseh vzorcih številčno večji v vampovem soku košut, uplenjenih jeseni, a smo statistično značilne razlike ($p < 0,05$) izračunali le pri vzorcih, uvrščenih v skupino

Preglednica 3: Ocenjeni kazalniki in vitro tvorbe plina krmil, inkubiranih v inokulumih, pripravljenih iz vampove vsebine jeseni in pozimi uplenjenih košut
Table 3: Estimated in vitro gas production parameters of feeds, incubated in inocula, prepared from rumen contents of hinds shot in autumn and winter

	B (ml/g SS – ml/g DM)			C			D		
Krmilo Feed	jesen autumn	zima winter	SED	jesen autumn	zima winter	SED	jesen autumn	zima winter	SED
Sveža trava (Jelendol) Fresh grass (Jelendol)	260[a]	277[b]	5,4	2,00[b]	2,23[a]	0,052	0,159	0,162	0,0064
Sveža trava (Kokra) Fresh grass (Kokra)	222	230	8,3	2,01[b]	2,22[a]	0,071	0,092	0,091	0,0087
Travna silaža (Jelendol) Grass silage (Jelendol)	226	225	10,1	2,51[b]	2,79[a]	0,149	0,109	0,113	0,0431
Travna silaža (Kokra) Grass silage (Kokra)	223	223	8,0	2,24[b]	2,51[a]	0,118	0,111	0,127	0,0151
Mrva (Jelendol) Grass hay (Jelendol)	229	235	7,8	2,04	2,24	0,152	0,132	0,150	0,0100
Mrva (Kokra) Grass hay (Kokra)	219	216	6,3	1,81[b]	2,12[a]	0,091	0,096[b]	0,124[a]	0,0107
Jabolčne tropine Apple pomace	307[b]	342[a]	18,1	2,32[b]	2,57[a]	0,111	0,216	0,170	0,2107
Koreni slad. pese Sugarbeet roots	339[b]	380[a]	6,6	2,15*	2,43*	0,144	0,305	0,293	0,0176
Plodovi div. kostanja Chestnut fruits	260	264	28,2	2,16[b]	2,59[a]	0,108	0,120	0,120	0,0125
Želod gradna Sessile oak acorns	278[b]	293[a]	5,7	2,48*	2,75*	0,135	0,160[a]	0,131[b]	0,0056
Želod doba Common oak acorns	279[b]	294[a]	5,4	2,51[b]	3,09[a]	0,106	0,131	0,126	0,0067

B = skupna potencialna tvorba plina – B = total potential gas production; C = relativna hitrost tvorbe plina, na katero vpliva konstantni kazalnik mikrobne učinkovitosti D – C = relative gas production rate as affected by a constant factor of microbial efficiency D
SED = standardna napaka razlike – SED = standard error of the difference
[a,b] povprečja, označena z različnimi črkami znotraj parametra se statistično razlikujejo pri $p < 0,05$ – [a,b] means with different superscripts within parameter differ significantly ($p < 0.05$)
* povprečja znotraj parametra kažejo trende ($0,05 < p < 0,10$) – means within the parameter show trends ($0.05 < p < 0.10$)

močnih krmil (koreni sladkorne pese, plodovi divjega kostanja ter želod doba in gradna), pri voluminozni krmi pa samo pri inkubaciji sveže trave iz Kokre. Ob inkubaciji vzorcev voluminozne krme in korenov sladkorne pese v vampovem soku košut, uplenjenih jeseni, smo zabeležili tudi številčno večji delež propionske kisline, medtem ko je bil pri inkubaciji plodov divjega kostanja ter želodu doba in gradna, delež propionske kisline večji, če smo jih inkubirali v vampovem soku košut, uplenjenih pozimi. Pri tem smo statistično značilne razlike določili pri plodovih divjega kostanja in želodu gradna. Pri mrvi iz Kokre, jabolčnih tropinah, korenih sladkorne pese in želodu doba pa smo ugotovili samo trend ($0,05 < p < 0,10$).

4 RAZPRAVA

Vpliv sezone na prebavljivost, razgradljivost in fermentabilnost hranljivih snovi je le redko obravnavan pri jelenih. V redkih virih avtorji (npr. Milne in sod., 1978; Sibbald in Milne, 1993; Freudenberger in sod., 1994; Domingue in sod., 1991) ugotavljajo, da v in vivo prebavljivosti organske, suhe snovi oz. dušika pri jelenih ni bilo velikih razlik med poletno in zimsko sezono. Nasprotno pa sta Jiang in Hudson (1996) ugotovila, da je bila in vivo prebavljivost paše veliko večja poleti kot pozimi, kar je povsem razumljivo, saj sta slednja v poskusu uporabila svežo travo oz. pašo, katere hranilna vrednost se je znotraj in med sezonami neprestano spreminjala, medtem ko so prej našteti avtorji vedno

Preglednica 4: Ocenjeni kazalniki in vitro tvorbe plina krmil, inkubiranih v inokulumih, pripravljenih iz vampove vsebine jeseni in pozimi uplenjenih košut
Table 4: Calculated in vitro gas production parameters of feeds, incubated in inocula, prepared from rumen contents of hinds shot in autumn and winter

	GAS24 (ml/g SS – ml/g DM)			TMFR (h)			MFR (ml/h)		
Krmilo Feed	jesen autumn	zima winter	SED	jesen autumn	zima winter	SED	jesen autumn	zima winter	SED
Sveža trava (Jelendol) Fresh grass (Jelendol)	249[a]	264[b]	4,7	4,4[b]	5,0[a]	0,17	15,2	16,3	0,53
Sveža trava (Kokra) Fresh grass (Kokra)	174	177	2,9	7,3*	8,9*	0,57	7,3	7,6	0,45
Travna silaža (Jelendol) Grass silage (Jelendol)	183	186	10,6	8,4	9,1	1,38	10,7	9,3	3,33
Travna silaža (Kokra) Grass silage (Kokra)	190	197	8,1	7,1	7,3	0,69	9,5*	10,3*	1,14
Mrva (Jelendol) Grass hay (Jelendol)	209*	220*	5,4	5,3	5,4	0,40	11,1[b]	13,0[a]	0,69
Mrva (Kokra) Grass hay (Kokra)	179[b]	192[a]	4,1	6,4	6,2	0,50	7,5[b]	9,7[a]	0,66
Jabolčne tropine Apple pomace	301	327	16,9	3,3[b]	5,6[a]	0,97	23,7	21,4	10,76
Koreni slad. pese Sugarbeet roots	338[b]	379[a]	6,5	2,5*	3,0*	0,25	37,8[b]	40,9[a]	1,92
Plodovi div. kostanja Chestnut fruits	226	226	5,1	6,6*	8,0*	0,71	11,3	11,6	0,67
Želod gradna Sessile oak acorns	263	258	5,1	5,7[b]	7,9[a]	0,24	16,1[a]	13,7[b]	0,64
Želod doba Common oak acorns	249	252	4,0	7,0[b]	9,0[a]	0,22	13,4	13,6	0,59

GAS24 = prostornina plina, proizvedena v 24 urah inkubacije – Gas24 = gas volumen produced in 24 hours of incubation; MFR= največja hitrost tvorbe plina – MFR = maximum fermentation rate; TMFR = čas, ko je dosežena največja hitrost tvorbe plina – TMFR = time of maximum fermentation rate; SED = standardna napaka razlike – SED = standard error of the difference

[a,b] povprečja, označena z različnimi črkami znotraj parametra se statistično razlikujejo pri $p < 0,05$ – [a,b] means with different superscripts within parameter differ significantly ($p < 0.05$)

* povprečja znotraj parametra kažejo trende ($0,05 < p < 0,10$) – means within the parameter show trends ($0.05 < p < 0.10$)

Preglednica 5: Količine in deleži hlapnih maščobnih kislin, ki so se sprostile iz krmil, inkubiranih v inokulumih, pripravljenih iz vampove vsebine v jeseni in pozimi uplenjenih košut
Table 5: Total amount of short-chain fatty acids and molar proportions of acetate, propionate and butyrate released from feeds, incubated in inocula, prepared from rumen contents of hinds shot in autumn and winter

	HMK – SCFA (mmol/g SS – mmol/g DM)			Ac (g/g HMK – g/g SCFA)			Pr (g/g HMK – g/g SCFA)			Bu (g/g HMK – g/g SCFA)		
Krmilo Feed	jesen autumn	zima winter	SED	jesen autumn	zima winter	SED	jesen autumn	zima winter	SED	jesen autumn	zima winter	SED
Sveža trava (Jelendol) Fresh grass (Jelendol)	6,76	6,84	1,260	0,618	0,676	0,0680	0,269	0,237	0,0484	0,113	0,087	0,0205
Sveža trava (Kokra) Fresh grass (Kokra)	5,05[a]	3,83[b]	0,513	0,669*	0,729*	0,0287	0,222	0,192	0,0227	0,109[a]	0,079[b]	0,0121
Travna silaža (Jelendol) Grass silage (Jelendol)	4,79	6,13	1,622	0,593	0,628	0,0475	0,300	0,264	0,0410	0,107	0,108	0,0131
Travna silaža (Kokra) Grass silage (Kokra)	5,84	4,74	1,387	0,650*	0,712*	0,0139	0,270	0,220	0,0410	0,079	0,067	0,0095
Mrva (Jelendol) Grass hay (Jelendol)	5,86[b]	9,49[a]	1,310	0,654*	0,716*	0,0398	0,241	0,203	0,0302	0,105	0,081	0,0147
Mrva (Kokra) Grass hay (Kokra)	4,44	4,83	0,861	0,669*	0,744*	0,0418	0,240*	0,188*	0,0324	0,091*	0,068*	0,0164
Jabolčne tropine Apple pomace	7,31*	11,24*	1,782	0,667	0,721	0,0243	0,204*	0,180*	0,0127	0,129	0,099	0,0341
Koreni slad. pese Sugarbeet roots	10,51	13,19	1,816	0,526[b]	0,618[a]	0,0384	0,320*	0,262*	0,3100	0,154[a]	0,120[b]	0,1305
Plodovi div. kostanja Chestnut fruits	6,40	7,67	1,947	0,612*	0,663*	0,0230	0,135[b]	0,163[a]	0,0089	0,253[a]	0,174[b]	0,0350
Želod gradna Sessile oak acorns	7,15*	9,62*	1,111	0,661	0,692	0,0210	0,106[b]	0,157[a]	0,0176	0,234[a]	0,151[b]	0,0271
Želod doba Common oak acorns	7,77	7,61	1,401	0,610	0,646	0,0390	0,149*	0,198*	0,0271	0,241[a]	0,156[b]	0,0217

HMK = hlapne maščobne kisline – SCFA = short-chain fatty acids; Ac = ocetna kislina – Ac = acetic acid; Pr = propionska kislina – Pr = propionic acid; Bu = maslena kislina – Bu = butyric acid
SED = standardna napaka razlike – SED = standard error of the difference
[a, b] povprečja, označena z različnimi črkami znotraj parametra se statistično razlikujejo pri $p < 0,05$ – [a, b] means with different superscripts within parameter differ significantly ($p < 0.05$)
* povprečja znotraj parametra kažejo trende ($0,05 < p < 0,10$) – means within the parameter show trends ($0.05 < p < 0.10$)

uporabili predhodno konzervirano voluminozno krmo (mrvo) in krmila.

Za razliko od in vitro pogojev, ki smo jih imeli v našem poskusu, so vsi zgoraj omenjeni avtorji ugotavljali prebavljivost oz. razgradljivost z in vivo metodami. V in vivo pogojih sta prebavljivost in razgradljivost hranljivih snovi pogojena s trajanjem zadrževanja krme v prebavilih, predvsem v predželodcih (MRT). Milne in sod. (1978), Domingue in sod. (1991), Sibbald in Milne (1993) in Freudenberger in sod. (1994) so ugotovili, da jeleni poleti zaužijejo več krme kot pozimi, kar bi ob nespremenjeni prebavljivosti hranljivih snovi pomenilo, da je poleti MRT krajši kot pozimi, kar je skladno z rezultati Domingue in sod. (1991), ki so ugotovili, da se poleti poveča iztok tekoče faze. Nasprotno pa Freudenberger in sod. (1994) ugotavljajo, da se poleti, kljub nespremenjenem zauživanju krme, upočasni iztok lignina (marker) iz predželodcev, kar nakazuje na podaljšan MRT pri jelenih v poletni sezoni. Slednje je lahko posledica visokih temperatur okolja, povsem možno pa je tudi, da je to posledica večje prostornine (in mase) predželodcev pri jelenih v poletni sezoni (Sibbald in Milne, 1993; Arnold in sod., 2015).

Na *iv*NRSS in *iv*PRSS posameznih krmil vpliva tudi obrok, ki ga košute zauživajo (Gordon in sod., 2002). V pričujočem poskusu nismo mogli spremljati količin in vrste sestavin, ki so jih košute dejansko zaužile z obroki. Menimo, da so bili obroki po sestavi, kakovosti in hranilni vrednosti v jeseni in pozimi zelo podobne, saj košute poselijo predvsem območja, na katerih rastejo rastline z večjo hranilno vrednostjo (Barboza and Bowyer, 2000) in da košute izbirajo obroke tako, da je v njih najmanj 30 % trav (Adamič, 1990, cit. po Jerina, 2007). V sezoni 2011–2012 so imele košute jeseni na voljo tudi velike količine plodov kostanja, žira in želoda, ki so bili takrat dostopni v velikih količinah. Kljub temu, da so imele košute pozimi poleg plodov plodonosnega drevja na voljo tudi mrvo, travno silažo, jabolčne tropine in korene sladkorne pese, s katerimi delavci LPN redno dokrmljujejo jelenjad na obeh območjih (D. Veternik, ustni vir, 25. januar 2012), slednjih niso zauživale v velikih količinah. Zato predvidevamo, da so bili jesenski in zimski obroki po svojih fizikalnih in kemijskih lastnostih zelo podobni.

V nam dostopni literaturi nismo uspeli dobiti nobenih podatkov o vplivu sezone na kazalnike tvorbe plina pri navadnem jelenu. Dobljeni kazalniki tvorbe plina, predvsem skupna potencialna tvorba plina in produkcija plina v prvih 24 urah inkubacije, se niso razlikovali od tistih, ki sta jih Lavrenčič in Veternik (2018b) določila ob fermentaciji teh krmil v puferiranem vampovem soku ovac. Skupna potencialna tvorba plina v zimski sezoni je bila večja predvsem pri vzorcih močne krme, medtem ko pri inkubaciji vzorcev voluminozne krme nismo zaznali večjih razlik. Prav tako večjih razlik nismo zaznali pri kazalniku D, medtem ko je bil kazalnik C v zimski sezoni pri večini krmil večji kot jeseni (preglednica 3). Posledica teh sprememb v kazalnikih tvorbe plina je podaljšan čas, v katerem tvorba plina doseže največjo hitrost, kar je še posebej izrazito pri močni krmi (preglednica 4). Vendar pa se prostornina v 24 urah proizvedenega plina ni bistveno razlikovala med sezonama, prav tako pa med sezonama ni bilo bistvenih razlik v *iv*PRSS. Tudi količine sproščenih hlapnih maščobnih kislin se med sezonama niso razlikovale, čeprav smo nekoliko večje količine HMK določili pri skoraj vseh substratih v zimski sezoni. Tudi Domingue in sod. (1991) pri navadnem jelenu niso ugotovili statistično značilnih razlik v količini sproščenih HMK, medtem ko Freunderberger in sod. (1994) in DeLiberto in sod. (1989) pri belorepem jelenu (*Odocoileus virginnianus*) navajajo večjo sintezo HMK v poletni kot v zimski sezoni, kar utemeljujejo z večjo prostornino predželodcev v poletni sezoni, zaradi česar se MRT podaljša, s tem pa se podaljšata tako čas delovanja kot aktivnost mikroorganizmov. Nasprotno pa Arnold in sod. (2015) navajajo, da je učinkovitost prebave največja pozimi, saj se zaradi podaljšanega MRT, ki je posledica manjšega zauživanja krme in manjše velikosti prebavnih organov, poveča sinteza HMK. Vendar pa DeLiberto in sod. (1989) niso ugotovili nobenih razlik v sproščanju HMK med jesensko in zimsko sezono.

So se pa v preučevanih sezonah razlikovali deleži tvorjenih posameznih HMK. Tako se je pri fermentaciji v predželodcih košut jeseni tvoril manjši delež ocetne kisline (trend) kot pozimi, medtem ko sta bila jeseni deleža propionske in maslene kisline običajno večja kot pozimi. Le deleži propionske kisline, ki so nastale ob fermentaciji plodov kostanja in želoda, so bili večji pozimi kot jeseni. Razlike v deležih propionske in maslene kisline med preučevanima sezonama so posebej izrazite pri krmi z majhno vsebnostjo vlaknine, ne pa tudi pri voluminozni krmi. Ozka razmerja med ocetno in propionsko kislino so značilna za obroke, v katerih prevladuje močna krma in vsebujejo veliko fermentabilnih ogljikovih hidratov. V našem primeru je takšne obroke jelenjad zauživala v obeh sezonah, saj je bil takrat obrod plodonosnih vrst dreves, ko sta hrast in bukev, izjemno velik, obenem pa jelenjad ni množično obiskovala njej namenjenih krmišč. Vendar pa smo pri večini krmil izračunali (podatki niso prikazani) nekoliko širše razmerje med ocetno in propionsko kislino ob njihovi fermentaciji v inokulumu, pripravljenem iz vampove vsebine košut, uplenjenih pozimi. Samo pri krmilih, ki vsebujejo veliko škroba (plodovi kostanja in želod obeh hrastov), so bila razmerja med ocetno in propionsko

kislino širša, ko smo jih določili v inokulumu, pripravljenim z vampovim sokom košut, uplenjenih jeseni. Iz propionske kisline se v procesu glukoneogeneze tvori glukoza, zato povečan delež propionske kisline v jesenski sezoni pomeni tudi boljšo oskrbo živali z energijo, kar sovpada z jelenjim rukom in potrebami po energiji za uspešno osemenitev pri samicah. Ocetna kislina je prekurzor za sintezo telesnih maščob. Za nalaganje telesnih maščob pa mora biti na razpolago dovolj NADPH, ki se tvori iz glukoze. Če glukoze oziroma NADPH primanjkuje, večina ocetne kisline oksidira, zaradi česar se poveča tvorba presnovne toplote (Domingue in sod., 1991), ki pa jelenjadi omogoča večje možnosti za preživetje v mrzlih zimah.

5 SKLEPI

Pri inkubaciji vzorcev krmil v inokulumu, pripravljenem iz vampovega soka košut, uplenjenih v jesenski in zimski sezoni, nismo ugotovili večjih razlik v in vitro navidezni razgradljivosti (*iv*NRSS) in pravi razgradljivosti (*iv*PRSS) suhe snovi, kakor tudi ne v kazalnikih in vitro tvorbe plina, razen v kazalniku »C« (specifična hitrost tvorbe plina), ki je bil v zimski sezoni večji kot jeseni. Tudi razlike v količini sproščenih hlapnih maščobnih kislin (HMK) med sezonama niso bile velike. Zaradi velikega obroda plodonosnega drevja v jeseni 2011 in zaradi dokrmljevanja košut s travno silažo, mrvo, jabolčnimi tropinami in koreni sladkorne pese predvidevamo, da se aktivnost mikroorganizmov v vampu košut ni bistveno spremenila. Predvidevamo, da bi bili tudi rezultati in vivo podobni, saj se srednji čas zadrževanja krme v prebavilih med sezonami ne spreminja bistveno, poleti zaradi večje prostornine prebavil, pozimi pa zaradi manjše količine zaužitih sestavin obroka.

Ugotovili pa smo, da so vampovi mikroorganizmi košut med sezonama nekoliko spremenili presnovo, kar se kaže v spremenjenih razmerjih med posameznimi HMK. Iz večje količine ocetne kisline lahko košute dobijo več toplote, kar je pomembno predvsem v hudih zimah, iz večje količine propionske kisline jeseni pa potrebno energijo za uspešno reprodukcijo. Tudi deleži maslene kisline so bili večji jeseni, torej v času intenzivnega nalaganja telesnih rezerv. Te domneve bi morali še potrditi z dodatnimi in vitro raziskavami, predvsem pa bi morali v poskus vključiti večje število živali. Prav tako pa bi morali te domneve potrditi ali ovreči tudi z in vivo raziskavami na samih živalih ter v naravnem okolju.

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Influence of ozonised irrigation water on the morphological, bacteriological and sensory characteristics of 'Saint-Pierre' tomatoes grown in Algeria

Fouzia BENALI [1, 2], Nadia RAMDANI [1]

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Influence of ozonised irrigation water on the morphological, bacteriological and sensory characteristics of 'Saint-Pierre' tomatoes grown in Algeria

Abstract: This article focuses on the study of the influence of ozonised water irrigation on the morphological, bacteriological and sensory characteristics of 'Saint-Pierre' tomatoes grown in Algeria. The results were compared with those irrigated with non-ozonised tap water called control of the same varietal type and grown under the same conditions. The work was carried out on seedlings of tomatoes grown and irrigated with ozonised water at different ozonisation times: 10- seconds, 20- seconds and 30-seconds, corresponding to lot I (tomato at 10-s), lot II (tomato at 20-s) and lot III (tomato at 30-s), respectively. Irrigation with ozonised water does not cause defects in shape, skin or colour of the fruits. They are, distinguished by a round shape, very red in colour, consistent and slightly acidic in taste. With good microbiological stability in accordance with the standard and good organoleptic quality except for the taste character of tomatoes at 30-seconds where a majority of evaluators estimated that 'they were bland and no big difference for the other criteria analysed. In general, our results showed that the ozonisation of irrigation water improves the growth, development, vigour and yield of tomato plants without altering the marketability of the fruits. This process encourages the use of ozonised water in agriculture since it has a high added value from an environmental and economic point of view and it can be generalized to other crops.

Key words: ozonised water; irrigation; 'Saint-Pierre' tomatoes; morphological; bacteriological and sensory characteristics

Vpliv ozonirane vode za namakanje na morfološke, bakteriološke in senzorične lastnosti paradižnika 'Saint-Pierre', rastočega v Alžiriji

Izvleček: Članek se osredotoča na vpliv ozoniranja vode za namakanje na morfološke, bakteriološke in senzorične lastnosti paradižnika 'Saint-Pierre' rastočega v Alžiriji. Rezultati so primerjani s tistimi, kjer je bila voda za namakanje iz vodovoda in ni bila ozonirana, kar je služilo kot kontrola, pri isti sorti paradižnika, gojenega v enakih razmerah. Sadike paradižnika za bile zalivane z vodo, ozonirano različno dolgo in sicer: 10 s, 20 s in 30 s, kar je ustrezalo naborom paradižnikov v poskusu: I (paradižnik pri 10 s), II (paradižnik pri 20 s) III (paradižnik pri 30 s). Zalivanje z ozonirano vodo ni povzročilo poškodb v obliki, kožici in barvi plodov. Ti so bili značilno okrogle oblike, zelo rdeči, čvrsti in z rahlo kislim okusom. V primerjavi s standardom so imeli dobro mikrobiološko stabilnost in dobro organoleptično kakovost, razen okusa tistih, ki so bili zalivani z vodo ozonirano 30 s, za katere je večina ocenjevalcev ocenila, da okus ni značilen, med ostalimi preučevanimi lastnostmi pa ni bilo velikih razlik. Na splošno so rezultati pokazali, da je ozoniranje vode za zalivanje izboljšalo rast, razvoj, vitalnost in pridelek paradižnika brez sprememb tržnih lastnosti plodov. Postopek vzpodbuja uporabo ozonirane vode v kmetijstvu, saj ima veliko dodano vrednost z vidika okolja in ekonomičnosti in bi se lahko splošno uporabljal tudi pri drugih kulturnih rastlinah.

Ključne besede: ozonirana voda; zalivanje; 'Saint-Pierre' paradižnik; morfološke, bakteriološke in senzorične lastnosti

1 Djillali Liabès University, Faculty of Exact Sciences, Department of Chemistry, Sidi Bel-Abbès, Algeria
2 Corresponding author, e-mail: fouzia.benali@univ-sba.dz

1 INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is one of the most consumed market garden crops in the world after potatoes (FAOSTAT, s. d.), of variable shape (spherical, oblong, elongated), and various colours (white, pink, red, yellow, orange, green, purple and black) depending on the variety (Joseph et al., 2017; Renaud, 2003). In general, tomato occupies an important place in the human diet; where it is consumed fresh, whole, dried, paste, puree, juice, sauce or tomato powder (Bhat et al., 2020; Nethaji et al., 2020; Siti Fadlilah et al., 2020). It is low in calories and very rich in water, vitamins, antioxidants and macrominerals such as iron, calcium, sulphur and potassium. It is rich in sugars (fructose and glucose), essential amino acids, organic acids and dietary fibres (Ali et al., 2020; García-Alonso et al., 2020). From the medicinal point of view, consumption of tomato fruits and their derivative products has been associated with the prevention of cardiovascular disease (Cámara et al., 2020; Cheng et al., 2017; Saini et al., 2020), several types of cancer (Rowles et al., 2018; Wu et al., 2021; Yang et al., 2013), as well as the maintenance of bone health (Walallawita et al., 2020). Tomato production is increasing over the years, where its annual production in Algeria rises from 107 million tonnes in 2014 to 148 million tonnes in 2019 (FAOSTAT, s. d.), cultivated across various regions of the country, in particular, wilayas of Tlemcen, Mostaganem, Ain-Defla, Chlef, Tipaza, Jijel, Skikda, Guelma, Annaba, Adrar and Biskra (Algeria's Ministry of Agriculture and Rural Development, 2020). Plant diseases and pests affect tomato production, causing considerable yield drops and significant economic losses.

Ozone has been applied for irrigation water disinfection and to control nematodes that reduce crop yield of tomato (Guo et al., 2019; Landa Fernández et al., 2019). It is also used to residual pesticides decomposition following the overuse of chemicals in agricultural fields (Mitsugi et al., 2017). It is a multifunctional reagent; it breaks down quickly into oxygen without leaving any chemical residues, In addition to its toxicity against a wide range of microorganisms (Pandiselvam et al., 2017) it is advantageous for many other applications, such as purification and disinfection treatment of waste and drinking water, preservation and extension of food shelf life, sterilization of equipment, and elimination of unwanted aromas produced by bacteria during storage and shipping. It is also used to inactivate microorganisms on fresh produce, such as fruits, vegetables, meat, poultry, fish and eggs, and dry produce, like cereals, pulses and spices. It is used in the gaseous form to disinfect the air in cold rooms by removing ethylene to slow down the ripening process of fruits and vegetables without altering the quality characteristics, and in aqueous form using the ozonized water for washing food and ensure product safety (Horvitz & Cantalejo, 2014; Pandiselvam et al., 2017).

The goal of our research is to study the influence of irrigation using ozonised water on tomato seedlings by varying the ozonisation times of water: 10-seconds, 20-seconds and 30-seconds while comparing the results with the control (tomato seedlings irrigated with tap water without ozonisation), in order to assess the effects of irrigation with ozonised water on the morphological, bacteriological and sensory characteristics of 'Saint-Pierre' tomatoes grown in Algeria. To the best of our knowledge this study has not been reported before, and it offers high added value for environmental, scientific and economic research fields.

2 MATERIAL AND METHODS

2.1 EXPERIMENTAL DESIGN

The study was carried out during the year 2020 in the north-western region of Algeria, exactly in the region of Oran, on tomato seedlings (seeds) of the same varietal type with the 'Saint-Pierre' appellation, of indeterminate growth. Sown in spring season in individual pots filled with potting soil, divided into four lots referenced as follows: lot I (tomato at 10-s); lot II (tomato at 20-s); lot III (tomato at 30-s) and finally lot IV (control tomato), where each lot contains five pots.

The irrigation water source used was tap water, then it was divided into four variants A, B, C and D. The first three (A, B and C) were exposed to constant flows of gaseous ozone produced by ozone generator FM-C900 (BEYOK ozone, China), as shown in Figure1, during 10, 20 and 30-seconds, respectively. Variant D consists only of non-ozonised tap water as a control. The plants of batch I, II and III were then irrigated, regularly and respectively, with variants A, B, C and subsequently compared with the control plants.

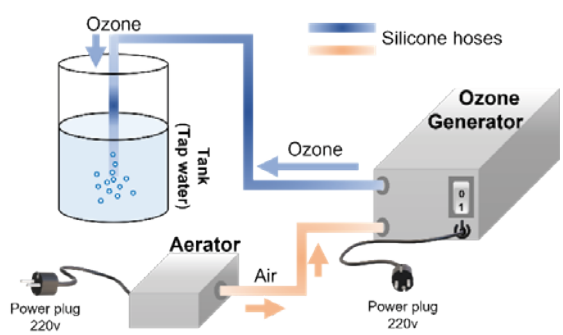

Figure 1: Schematic of the experimental setup

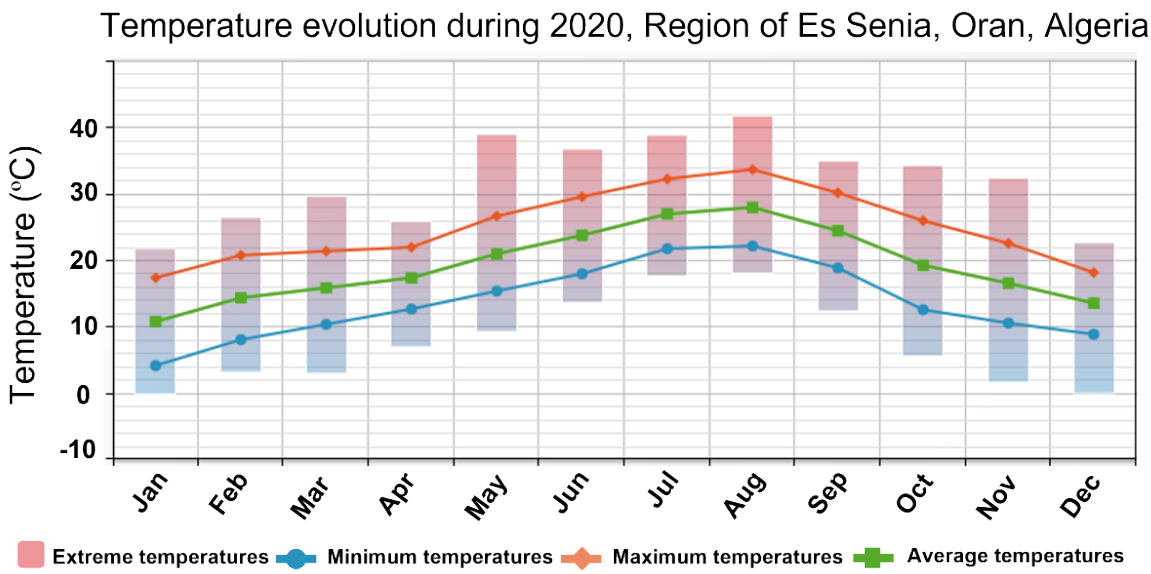

Figure 2: Temperature evolution during the test period of the year 2020 (Nomades, s. d.)

After five weeks of cultivation, the seedlings are transplanted into large pots and staked, put outdoors, in a well-lit and sunny place. During the test period, the monthly average temperature was variable ranging from 20 °C to 30 °C, see Figure 2, with an average monthly humidity ranging from 76 % to 67 % (Nomades, s. d.). The irrigation was performed regularly twice a week during the grow cycle.

2.2 OZONE GENERATOR

The cost, complexity, hazard potential, and generation of residual ozone were arguments that prevented investment in ozonation of irrigation water. The recent ozone generators have been manufactured with automated ozonation systems, more compact infrastructure, low cost and simplified maintenance which reduce investment and operating costs and makes renovations more feasible.

When operating an ozone water treatment system, the main concern in terms of failure is leakage in the ozone lines (feed gas and off gas). Serious ozone leaks represent a significant health hazard for farmers and crops, hence the integration of ozone gas sensors in all ozonation systems. A fail-safe system can represent a significant percentage of the overall cost but generally offers a moderate level of maintenance to ensure proper operation (Graham et al., 2011).

We should note that, it will be better for our future research to integrate ozone gas sensors into the ozonation system, in order to better control of any unexpected issues related to gas leakages.

2.3 QUALITY ANALYSIS

Fruit quality analyses were carried out with the aim of determining the morphological, bacteriological and organoleptic characteristics of each batch of tomatoes (I, II, III and IV). These analyses were carried out on samples of whole tomatoes, fresh, ripe, firm, healthy and of uniform red colour for the four batches, in order to evaluate the effects of ozonised water on the quality of the tomatoes by comparing them with the control samples.

2.4 MORPHOLOGICAL ANALYSIS

The analysis consists of determining the morphological characteristics of tomatoes by calculating the shape coefficient (C_f), the number of cells (N_ℓ), the mass volume (ρ) and the average fruit mass (P_m) (Agassounon Djikpo Tchibozo et al., 2012).

The other physical parameters were determined using a calliper such as height and diameter. These analyses make it possible to identify and characterize the variety of the tomato studied.

The shape coefficient is given by the following equation:

$$C_f = \frac{Average\ fruit\ height}{Average\ fruit\ diameter} \qquad \text{(Eq. 1)}$$

It allows varieties to be classified into three categories:

$C_f < 0.8$: it is a flattened shape;
$C_f > 1$: it is an elongated shape;
$0.8 < C_f < 1$: it is a round shape.

2.5 MICROBIOLOGICAL ANALYSIS

The purposes of these analyses were to assess the microbiological quality of the four batches of tomatoes (I, II, III and IV). They allow checking the possible presence or the absence of microorganisms in the samples to be analysed which is summarized in the search and the enumeration of the total coliforms and the faecal coliforms according to the standard ISO 7251:2005, as well as the determined yeasts and moulds by the ISO 21527-1:2008 method. A quantity of 10 ± 2 g of each sample of tomatoes was weighed and added to 90 ml of Ringer's solution, each placed in sterile sachets, crushed and homogenized in the Stomacher, thus constituting the stock solution.

The analysis was carried out by seeding the stock suspension of 1 ml aliquots and its 10^{-1}, 10^{-2} and 10^{-3} decimal dilutions on suitable culture media and under aseptic conditions.

Yeasts and moulds testify to the appearance of phenomena of deterioration of tomatoes, discoloration and modification of the flavour, on the other hand the presence of total coliforms and faecal coliforms reflect the hygienic level of the fruits. Table 1 groups the culture media used.

2.6 APPRAISAL OF SENSORY CHARACTERISTICS

The objective of this analysis is, first to evaluate the organoleptic characteristics of the experimental and control tomatoes according the parameters of standard ISO 5492: 2008 which is summarized in: the appearance, colour, consistency, smell, acidity, aroma and taste. Second, rank the tomato samples offered for tasting noting their acceptability or their preferences over each other.

The evaluation was carried out on fresh, mature, healthy, firm and red tomatoes by a panel of tasters. Tomato samples and pre-established questionnaires were distributed to each taster who agreed to collaborate in the study and no information was provided on the different batches of tomatoes in order to objectively assess the perceived sensory characteristics.

2.7 UNCERTAINTIES AND SHORTCOMINGS

It should be noted that our seedlings have been placed in outdoor conditions, while greenhouse and commercial conditions are to be expected in our future research in order to collect additional information such as gas exchange parameters and economic feasibility. Soil analyses before and after treatment with ozonised water will be recommended to assess any impact that could modify the quality of the latter. In addition, irrigation with aqueous ozone at 30-seconds gave a better yield but the tomatoes are of lower taste quality, this concentration should be taken into account and should be considered as a threshold value and study the possibility of improving the sensory qualities of fruits.

Finally, increasing the number of panel of tasters will lead to a better appraisal of the organoleptic characteristics.

3 RESULTS AND DISCUSSION

3.1 MORPHOLOGICAL CHARACTERISTICS OF THE TOMATOES STUDIED

The four lots of tomatoes studied are distinguished by smooth-looking fruits, of a pronounced red colour, strongly lobed (6 to 8 lobes) and round ($C_f > 0.8$). The width of the fruits for the four lots is slightly more extended, which gives it the appearance of a large fruit, the height, the diameters and the average mass of the fruits are variable (Figure 3).

The highest average mass was observed in the tomato at 30-s, followed by the tomato at 20-s then the tomato at 10-s and finally the control tomato with 81 g, 77 g, 72 g and 63 g respectively; the same for the density which is 0.97 g cm^{-3}, 0.94 g cm^{-3}, 0.93 g cm^{-3} for the tomatoes at 30-s, 20-s and 10-s and 0.91 g cm^{-3} for the control tomatoes. (Table 2).

The tomatoes responded differently despite the same growing conditions and the same varietal type, the highest number of tomatoes picked during the whole period of the trial was observed in the tomatoes at 30-s with 31.86 % followed by the tomatoes at 20-s with 28.32 % then the tomatoes at 10-s with 22.12 % and finally 17.70 % for the control tomatoes.

The difference in the quantity of tomatoes picked for the three lots (I, II, III) is proportional to the concentration of the ozonised water.

In July, the batches (I, II, III) show an early harvest in relation to the control batch. (Figure 4).

During the hottest months the four lots recorded a large number of tomatoes picked with different quanti-

Table 1: The culture media used for the enumeration and the quantitative enumeration of the germs investigated in the tomatoes studied

Designation	Culture centre	Cultivation conditions	Norm
Total coliforms	VRBL Agar (Violet Red Bile Lactose Agar).	Incubation : 37 °C for 24 ± 2 h	ISO 7251:2005
Faecal coliforms	VRBL Agar (Violet Red Bile Lactose Agar).	Incubation : 44 °C for 24/48 ± 2 h	ISO 7251:2005
Yeasts and moulds	DRBC Agar (Dichloran-rose bengalchloramphènicol)	Incubation : 25 °C ± 1 °C for 5 days	ISO 21527-1:2008

ties and as the seasons progressed the quantities of fruit picked decreased.

The largest quantities of the picked tomatoes were recorded during the month of August for the four lots. (Figure 4).

Tomatoes at 30-s were among the best performing tomatoes compared to other lots in terms of production density, average mass and density (Figure 3) and (Table 2).

No defect in development, shape, epidermis or colouring was observed in comparison with the control tomatoes, total absence on all the samples studied of protuberance or crevice and no presence of corky bruising of umbilical form or elongated epistolary scar. These fruits have good resistance to cracking, a very developed placenta and a large number of seeds. Table 2 shows the main morphological characteristics of the four batches of tomatoes at 10-s, 20-s, 30-s and control.

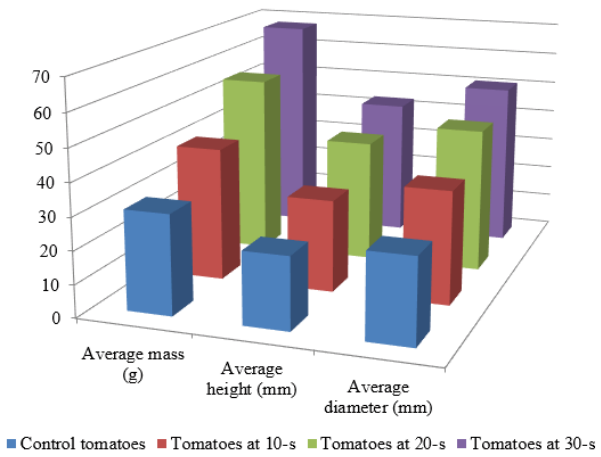

Figure 3: The average values of mass, height and diameter of experimental and control tomatoes

3.2 RESULTS OF MICROBIOLOGICAL ANALYSIS

The results obtained for the enumeration of total and faecal coliforms as well as for the quantitative enumeration of yeasts and moulds, show a total absence of all the germs sought both for tomatoes irrigated with ozonised water as for control tomatoes, while the standard tolerates 10^3 cfu g^{-1} for total coliforms, which means that the samples studied show stability and a good hygienic level. This coincides with the work of (Heß & Gallert, 2015; (Güzel-Seydim et al., 2004) where ozone has been used as an antimicrobial agent on a variety of pathogenic organisms, such as strains *Escherichia coli*, *Enterococcus*, *Staphylococcus*, fungi and viruses on the other hand (Guo & Wang, 2017) reveal that *E. coli* treated with moderate concentrations of ozonised water (0.5 mg l^{-1} ozone at 28 °C) were completely inactivated. Irrigation with ozonised water has no harmful influence on the experimental tomatoes compared to the control tomatoes and the results obtained show compliance with the standard (Inter ministerial decree, Official Journal No. 39, 2017). These tomatoes pose no health risk, and have good marketability. These results are shown in Table 3.

Table 2: Morphological characteristics of experimental and control tomatoes

Designation	Coefficient of form C_f	Number of lobes N_ℓ	Average fruit mass P_m (g)	Volumic mass in ρ (g cm^{-3})	Protuberance crevasse, Green collar	Seeds number
Tomato at 10-s	0.83	6-8	72.27	0.93	absence	> 200
Tomato at 20-s	0.84	6-8	77.18	0.94	absence	> 200
Tomato at 30-s	0.85	6-8	80.87	0.97	absence	> 200
Control tomato	0.81	6-8	63.22	0.91	absence	> 200

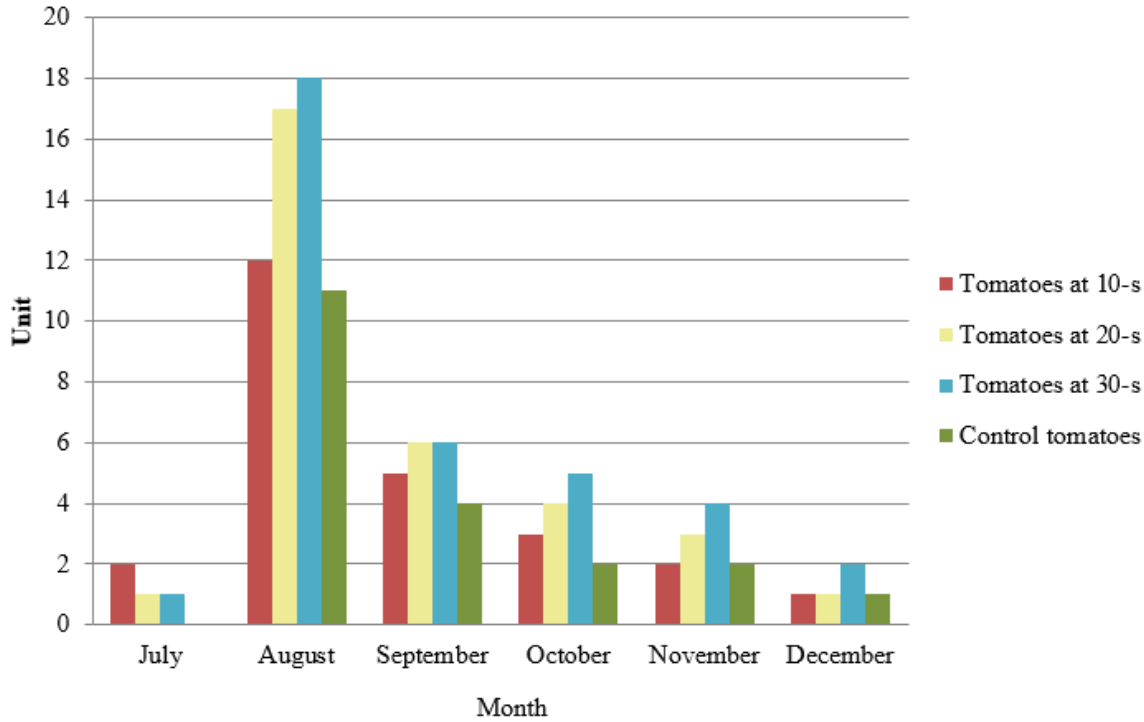

Figure 4: The number of tomatoes picked of the four lots during the test period

3.3 SENSORY CHARACTERISTICS OF TOMATOES

The test was carried out by a panel of tasters composed of twelve (12) people, men and women aged 27 to 58 years. It made it possible to evaluate the organoleptic qualities of the experimental tomatoes compared to the control tomatoes. The criteria used are colour (very red to red), taste (salty, sweet), acidity, consistency, texture (melting, crunchy and floury), aroma and odour.

The sensory assessment was carried out on freshly harvested tomatoes for the four lots (I, II, III and IV). It emerges that twelve of the evaluators find that the three experimental batches are very red, identical to the controls. Recalling that colour of the fruit is linked to the abundant content of carotenoids in the peel and flesh of the fruit. As for the taste, the tomatoes at 10-s, 20-s and the control tomatoes were judged to be lightly salted by eight of the tasters, while for the tomatoes at 30-s the assessment is in favour of bland with seven evaluators (Table 4b). On the other hand, eight people believed that the four batches of tomatoes were not very acidic while one taster did not find them at all acidic. The other most sought-after criterion is the scent character, nine of the evaluators believed that tomatoes had a strong odour, qualified as a strong tomato odour to little pronounced. The consistency of tomato fruits is also an important quality criterion as appreciated as the colour and the taste; it determines the resistance of the fruits to handling and their behaviour in the marketing circuit. The firmness was estimated manually, and ten evaluators considered the four lots of tomatoes to be very firm to firm, while one taster felt they were not very firm (Table 4a).

In order to assess (the salinity, acidity, sweetness,

Table 3: Bacteriological characteristics of tomatoes irrigated with ozonised water and controls

	E. coli			Yeasts and moulds		
Designation	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}
Tomato at 10-s	absence	absence	absence	absence	absence	absence
Tomato at 20-s	absence	absence	absence	absence	absence	absence
Tomato at 30-s	absence	absence	absence	absence	absence	absence
Control tomato	absence	absence	absence	absence	absence	absence

juiciness), and to translate the more or less intense characteristics of each of the criteria, we use the evaluation of the hedonic quality of the four batches (I, II, III, IV) tomatoes.

The results show that all the lots show tomatoes of red colour, of round shapes, smooth and shiny, fleshy, tender, slightly acidic and less salty, of firm and tasty flesh, of easily removable cuticle.

As a result, irrigating with ozonised water has no significant effect on the perception of the organoleptic characteristics of tomatoes compared to controls except for the taste character of tomatoes at 30-s where seven people of the panel estimated that 'they were bland and there is no difference for the other criteria.

The panellists' results were reported in Table 4 (a,b).

3.4 COMPARATIVE ANALYSIS

In our study, the agronomic characteristics of tomatoes irrigated with ozonised water such as seedling development, early germination, growth, vigour, plant size and yield were greater compared to control plants. Which is consistent with the work of Martínez-Sánchez & Aguayo(2019) where irrigation with aqueous ozone improved the development of greenhouse-grown pepper seedlings and the microbiological quality of the water. The plants developed a higher number of leaves and secondary roots, which could improve the adaptability and yield of the seedlings when transplanted into the fields. Studies by Ohashi-Kaneko et al. (2009) reported that treatment with ozonised water improves root respiration and increases nutrient uptake and biomass production.

The yield of the experimental tomatoes (10-s, 20-s and 30-s) was significantly higher than in the control plants. Guo & Wang (2017) reported that direct spraying of ozonised water on growing crops in fields increases their antioxidant content and photosynthetic activity, which enhances crop protection and prevents infection with plant pathogens. Plants subjected to ozonised water treatments below 10 mg l^{-1} had slightly increased plant diameter and height. The fresh mass of the leaf treated with 6 mg l^{-1} of ozonised water was remarkably increased by 40.6% compared to the control. Rozpądek et al. (2015) reported that plants subjected to aqueous ozone treatments during vegetation showed accelerated growth and reached marketable quality more quickly.

The effects of irrigation of tomatoes with ozonised water at 10-s, 20-s and 30-s showed no morphological damage compared to the control, this is supported by the work of Guo & Wang (2017) reporting that no negative effects were observed after treatment with ozonised water spray at concentrations below 8 mg l^{-1} carried out on Chinese cabbage, on the other hand, visible damage to

Table 4a: Summarizes sensoria's parameters of the four batches of the tomatoes as perceived by the tasters

Sensory parameters of the four batches of tomatoes (I, II, III, IV)							
Colour		Acidity		Consistency		Aroma and odour	
Level	Tasters number	Level	Tasters number	Level	Tasters number	Level	Tasters number
Very red	12	Very acidic	0	Very consistent	1	Very pronounced	0
Red	0	Acid	1	Consistent	9	Pronounced	3
Less red	0	Less acidic	8	Less consistent	1	Less pronounced	6
Not red	0	Not at all acidic	1	Not consistent	0	Not pronounced	1
Indifferent	0	Indifferent	2	Indifferent	1	Indifferent	2

Table 4b: Summarizes sensoria's parameters of the four batches of the tomatoes as perceived by the tasters

Other flavour Tomatoes at 10-s, 20-s and controls		Other flavour for the tomatoes at 30-s	
Level	Tasters number	Level	Tasters number
Very salty	0	Very salty	0
Salty	1	Salty	1
Slightly salty	8	Slightly salty	1
Not at all salty	1	Not at all salty	2
Fade	0	Fade	7
Indifferent	2	Indifferent	1

the leaves was observed after the plants were exposed to 10 mg l^{-1} of ozonised water spray for 15 days during the reproductive phase of the plants.

Aqueous ozone is a germination activator in limited quantities; excessive doses may affect the quality of the seeds. These results vary according to the varieties of species in question (Pandiselvam et al., 2020).

An additional positive effect of ozonisation is the inactivation of pathogenic bacteria, such as *Escherichia coli*, *Enterococcus* and *Staphylococcus* strains (Heß & Gallert, 2015) which raise crop protection and prevents infection with plant pathogens, It is also used to disinfect irrigation water and as an alternative to pesticides (Guo et al., 2019; Landa Fernández et al., 2019).

Finally, as future research, we can consider the irrigation with ozonised water at different concentrations at a large scale either in greenhouse or in open fields. Determining the threshold values of aqueous ozone concentrations that can be applied into the different parts of the plants and at all growth stages, and duration and frequency of irrigation beyond which damage could occur affecting crops would be interesting to be investigated.

The effect of ozonised water spraying onto the tomato fruits and leaves could also be taken into account for the rest of our work.

4 CONCLUSION

The objective of this work was the experimental study of the effect of irrigation with ozonised water on the morphological, bacteriological and sensory characteristics of 'Saint-Pierre' tomatoes grown in Algeria. On the basis of our study and taking into consideration all the observations on the experimental part, the results of the analysis reveal that the experimental fruits do not present any defect in development, shape, skin or colouring, all the batches show satisfactory firmness with good resistance to cracking. These characteristics constitute an advantage in the marketing of tomatoes; moreover, the latter have a good hygienic level, and present no health risk for consumers.

The greater quantities of tomatoes picked as well as the size of the fruit is proportional to the concentration of the ozonized water. This observation proves the positive influence of irrigation with ozonised water as well as the different concentrations of ozone on the tomato plants. Despite this, seven tasters felt that 30-s tomatoes tasted bland unlike other lot I and II. The yield of 20-s tomatoes was lower than that of tomatoes at 30-s but with better taste quality than the latter. Therefore, this concentration is highly recommended for cultivation under the climatic conditions of the study region.

Irrigation with ozonised water can be used to increase the yield without negative impact on the environment and the quality of the product, moreover the ozonised water generator does not require large investments, nor specific infrastructure of low energy consumption and maintenance, it is easy to use, which means that it can be implemented anywhere.

However, studies must be continued for a better application of this technique in other growing conditions and on different varietal types or other market garden products. We have carried out this research on a small number of samples and on a single varietal type; we plan in the future to repeat this study on different plant species and to study them on a larger number of specimens.

Our study will serve as a basis for introducing ozonised water into crop irrigation in the region of Oran (Western Mediterranean region) to increase yield, size and to accelerate germination, flowering and tomatoes fruit production. As perspectives, similar studies must be carried out in other regions of Algeria such as the Tellian, arid, semi-arid, Atlas and Saharan regions and determining the impact of different concentrations of aqueous ozone on the characteristics agronomic, morphological, physicochemical, bacteriological, organoleptic and nutritional, for a better understanding of the climate effect in parallel to the application of ozonised water at the large scale. A soil analysis would also be recommended for tomatoes produced with an environmentally friendly process.

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Effects of soil nutrient amendments on growth and grain yield performances of quality protein maize grown under water deficit stress in Ibadan, Nigeria

Folake ANJORIN [1, 2], Adeyinka ADEBAYO [1], Taiwo OMODELE [1], and Adewale ADETAYO [1], James ADEDIRAN

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Effects of soil nutrient amendments on growth and grain yield performances of quality protein maize grown under water deficit stress in Ibadan, Nigeria

Abstract: Drought and poor soil fertility are major limitations to crop production, globally. To investigate the impacts of water deficit stress (WS) and soil nutrient amendment (SA) on growth and yield performances of maize. A two years factorial field study was carried out, using a quality protein maize (QPM) (ILE-1-OB) and a non QPM–drought tolerant check (TZPBSR-W) varieties in Ibadan. Treatments include; six fertilizer application rates; 50 and 100 (kg N ha^{-1}) of NPK-20-10-10, 10.7 kg N ha^{-1} of Tithonia Poultry Compost (TPC), 50 N + 10.7TPC and 100 N + 10.7TPC (kg N ha^{-1}), three WS; the control (FW), WS at vegetative stage (STR1), and WS at reproductive stage (STR2). Leaf area (LA) and grain yield (GY) were measured using standard procedures. From the results, across WS, LA ranged from STR1 (458.90 ± 12.4) to FW (598.81 ± 13.1 cm^2), GY varied from STR2 (2.94 ± 0.2 t ha^{-1}) to FW (6.59 ± 0.2 t ha^{-1}), across fertilizers, LA varied from 0 N (397.65 cm^2) to 100N + 10.7TPC (622.71 cm^2) and 50 N + 10.7TPC (611.03 cm^2), respectively. The GY varied from 0 N (2.37 t ha^{-1}) to 100 N + 10.7TPC (5.82 t ha^{-1}) and 50N + 10.7TPC (5.26 t ha^{-1}). Drought stress reduced growth and GY performances of QPM, while SA with 50 kg N ha^{-1} of inorganic fertilizer and 10.7 kg N ha^{-1} of TPC enhanced growth and grain yield of maize under WS.

Key words: fertilizer application rates; grain yield; growth and yield performances;quality protein maize; soil nutrient amendments; water deficit stress

Učinki gnojenja na rast in pridelek zrnja na proteinih obogatene koruze v razmerah sušnega stresa, Ibadan, Nigeria

Izvleček: Suša in slaba rodovitnost tal sta v globalnem obsegu glavna dejavnika, ki omejujeta produktivnost gojenih rastlin. Za preučevanje vpliva vodnega deficita (WS) in dodajanja hranil v tla (SA) na rast in pridelek koruze je bil izveden dvoletni faktorski poljski poskus na sorti ILE-1-OB, bogati na proteinih (QPM) in na sušo odporni sorti TZPBSR-W kot kontroli, ki ni obogatena s proteini (non QPM), v Ibidanu, Nigerija. Obravnavanja so obsegala: šest načinov gnojenja (50 in 100 (kg N ha^{-1}) z NPK-20-10-10, 10,7 kg N ha^{-1} komposta iz vrste *Tithonia* pomešanega s kokošjim gnojem (TPC), 50 N + 10,7 TPC in 100 N + 10,7 TPC (kg N ha^{-1}), tri stopnje vodnega deficita (WS) v vegetativni (STR1) in reproduktivni fazi (STR2) in kontrolo s polnim namakanjem. Listna površina (LA) in pridelek zrnja (GY) sta bila izmerjena s standardnimi metodami. Listna površina je v vegetativni fazi ob pomanjkanju vode znašala 458,90 ± 12,4 cm^2, ob polnem zalivanju pa 598,81 ± 13,1 cm^2. Pridelek zrnja je ob vodnem deficitu v reproduktivni fazi znašal 2,94 ± 0,2 t ha^{-1}, pri polnem zalivanju pa 6,59 ± 0,2 t ha^{-1}. Listna površina je bila glede na načine gnojenja sledeča: 0 N (397,65 cm^2), 100 N + 10,7 TPC (622,71 cm^2) in 50 N + 10,7 TPC (611,03 cm^2). Pridelek zrnja je glede na načine gnojenja dosegel naslednje vrednosti: 0 N (2,37 t ha^{-1}), 100 N + 10,7 TPC (5,82 t ha^{-1}) in 50 N + 10,7 TPC (5,26 t ha^{-1}). Sušni stres je zmanjšal rast in pridelek sorte QPM, gnojenje s 50 kg N ha^{-1} kot anorganskim gnojilom dopolnjeno z 10,7 kg N ha^{-1} v organski obliki je pospešilo rast in pridelek zrnja koruze v razmerah vodnega deficita.

Ključne besede: na proteinih obogatena koruza**;** odmerki in vrste gnojil; rast; pridelek zrnja; sušni stress

1 Institute of Agricultural Institute Research and Training, Obafemi Awolowo University, Ibadan, Nigeria
2 Corresponding author, e-mail: folakeawoeyo@yahoo.com

1 INTRODUCTION

Maize is an important cereal crop with wide range of utilization in several countries of the world. Apart from been a major staple crop, maize is an important ingredient in livestock feed formulation for the rising poultry business in the sub Saharan Africa. Maize has remained a unique crop with great potentials to survive across different agro-ecology and vegetations, globally. However, the detrimental impacts of drought and poor soil fertility on profitable maize production in the tropics cannot be overemphasized (Goldblatt, 2010; Ammani et al., 2012). Unpredictable weather conditions, erratic rainfall patterns, and incidences of occasional pockets of drought even at the peak of rains are characteristics attributes of Nigeria's climate, lately. The consequences of climate change are gradually having its turn on the nation's vegetation and cropping system.

An estimated value of about 15 % reductions in global maize production has been attributed to drought alone (Edmeades, 2013). Inadequate water availability affects virtually all physiological and metabolic processes in maize development. Processes such as germination, seedling growth, leaf formation, stem elongation, and overall crop development (Anjorin et al., 2017; Anjorin et al., 2018). The severity of damage resulting from drought stress depends on the duration of drought and the phenological stage of plant development as at time of stress (Chaves et al., 2002; Jongdee et al., 2002). The reproductive developmental stage has been shown to be the most critical stage for maize sensitivity to drought. Monneveeux et al. (2006) in a similar view, reported that grain yield in maize could be drastically reduced by drought prolonged beyond 12 days during grain filling and flowering stages.

Apart from drought, uncontrolled soil nutrient mining due to continuous cropping without supplementary replacement has been a common and regular practice in most countries of sub-Saharan Africa (Ngetich et al., 2012). An estimated average annual nutrient depletion ranged from 20 kg to 50 kg NPK $ha^{-1}yr^{-1}$ in majority of developing countries to more than 100 kg NPK $ha^{-1}yr^{-1}$ in the least developed countries of Africa (Tan et al., 2005).

Crops appear more devastated especially when both drought and nutrient stresses occur simultaneously. However, the use of drought tolerant crop genotypes and fertilizers has the potentials to enhance crop growth and yield in the face of prevailing climatic challenges. Over time, several integrated soil fertility management strategies (ISFM) that could enhance soil fertility potentials and productivity in Africa had been advocated (Scoones & Toulmin, 1998). These include the use of fertilizers, organic inputs and improved germplasm in addition to the technicalities of adapting these practices to local environments (Vanlauwe et al., 2010; Sanginga & Woomer, 2009).

Therefore, there is a need for a balance in moisture and nutrient availability in the crop environment with regards to stages of plant development for optimum crop yield. As at present much work has not been carried out in this part of the world on soil fertility management strategies with regards to occurrences of drought during various phenological growth stages in maize. Hence, this study aimed at assessing the impact of inorganic and organic fertilizers (using *Tithonia* poultry compost) soil amendment interventions at ameliorating the impact of water deficit stress (drought) on maize phenology.

2 MATERIALS AND METHODS

2.1 EXPERIMENTAL SITE, LOCATION AND DESIGN

The study was conducted on the research field (Longitude 3°50'56.1"E and latitude 7°22' 20" N) during the dry seasons between Decembers – March in 2014/ 2015 and 2015/2016 at the Institute of Agricultural Research and Training (I.A.R&T), Moor Plantation in Ibadan. The I.A.R&T is located in the derived savanna agro ecology of Nigeria (Figure 1).

2.2 TREATMENTS

2.2.1 Water Deficit Stress

(i) No water stress (FW): plots receive water up to field capacity till plant maturity

(ii) Water stress for 14 days (withdrawn of watering) at three weeks after seedling emergence, while normal watering resumed till plant maturity (STR1)

(iii)Water stressed imposed in maize plots by water withdrawer for 14 days at 6 weeks after seedling emergence after which normal watering resumed till plant maturity (STR2).

2.2.2 Fertilizer rates

(i) Three rates of N fertilizer (NPK-20-10-10); 0 N, 50 N, 100 N (kg ha^{-1})

(ii) One rate of *Tithonia* - Poultry Compost (TPC): 10 TPC (t ha^{-1}) (10.7 kg N ha^{-1}),

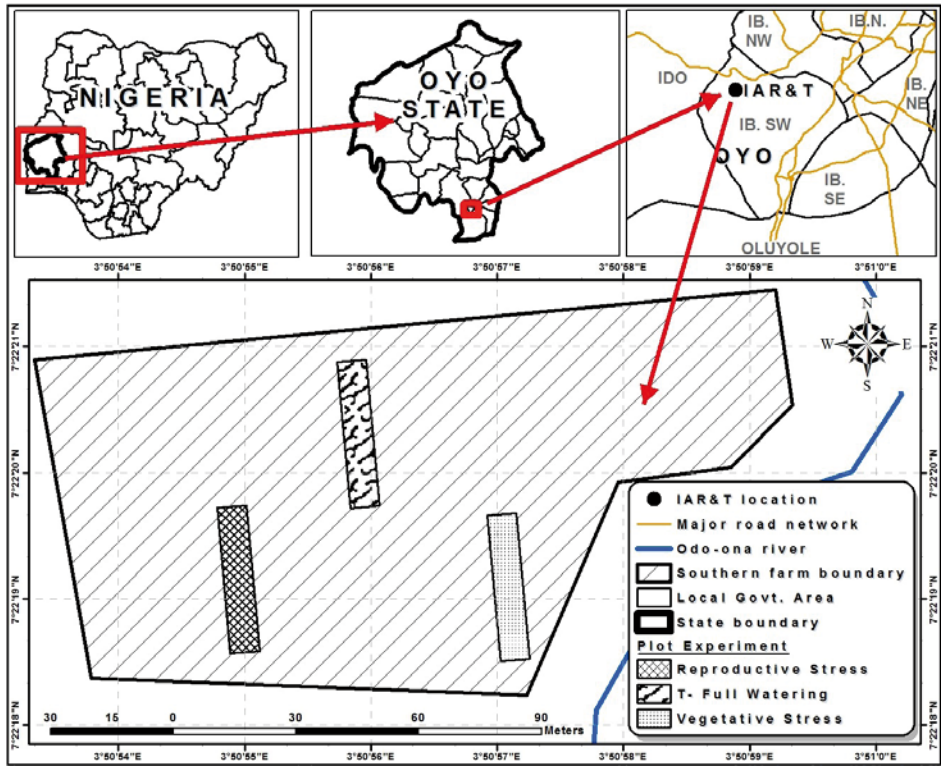

Figure 1: Map showing the experimental plots and location of the experiment at the Institute of Agricultural Research and Training in Ibadan, Oyo state, Nigeria

(iii) Two rates of N fertilizer and TPC combinations; 50 N + 10 TPC and 100 N +10 TPC.

2.2.3 Varieties

Two maize varieties consisting of one quality protein maize variety (ILE-1-OB) and a drought tolerant maize (TZPBSR-W) (Smale et al., 2011) are both open pollinated (intermediate maturing) high yielding characterized by flint texture and white colour seeds, were collected from the seed store of I.A.R & T, Ibadan.

2.2.4 Experimental design

The maize field was planted in 3 x 6 x 2 factorial arrangements using randomized complete block design (r = 3). Each of the three main plots was 27.5 m by 14 m in size were separated by 5 m apart to prevent water seepage across the main plot during irrigation processes, the sub-plot was 4 m x 7.5 m while the sub-sub plot was 4 m x 3.75 m. There were thirty - six plots in the each main plot, each of the sub - sub plot consisted of six (6) rows of two plants per hill at a planting distance of 75 cm x 50 cm inter rows and intra rows spacing, respectively.

2.3 LAND PREPARATION, PLANTING AND CROP MANAGEMENT

The pre crop for both first and second year is maize. The land was prepared mechanically by ploughing and harrowing. Initial wetting was done before each of the operations to ease the operations because the land was very dry and compacted as expected during the dry season. After land preparations, maize seeds were sown at three seeds per hill. The young maize seedlings down to two vigorous healthy seedlings per stand. Pre emergence herbicides (Atrazin® 4 kg ha^{-1} and Glyphosate) were applied to control weeds, while subsequent weeding was done with local hoes.

2.4 COMPOST PREPARATION AND FERTILIZER APPLICATION

The compost was prepared from fresh cuttings of Mexican sunflower (*Tithonia diversifolia* (Hemsl.) us-

ing the heap method described by Fernhill, (2011). Nine (9) kilogram of Mexican sun flower (*Tithonia diversifolia*) plant cuttings of about 10 centimeters long were weighed, chopped and spread on the earth surface. The spread plant cuttings were alternated in layers with the spreading of three kilogram (3 kg) of cured fecal poultry droppings to form heap of 1.3 m height. Several heaps made were sprinkled with water before covering with black polythene sheet to increase temperature, moisture maintenance and escape of gases. The heaps were over turned fortnightly with the aid of long garden fork and moisturized adequately to enhance effective microbial growth and activities. Adequate aeration was achieved using 1 m diameter pipes inserted vertically and horizontally into the heaps to ensure adequate ventilation. The pH and temperature were monitored until the compost matured (AAFRD, 2005). The compost heaps were allowed to stay for a period of $2^{1}/_{2}$ months after which the compost materials were ready for use. The compost material was spread thinly on a drying surface under shade and allowed to dry very well before storing in bags. Sample of the matured compost were analysed for chemical properties (Anjorin, 2018). Compost was applied a-week before planting to each of the designated plots to initiate early mineralization of nutrients. Inorganic fertilizer (urea) was applied to the designated plots in splits at two weeks and five weeks after emergence based on the pre - determined rate.

2.5 IRRIGATION

Irrigation was done using sprinklers while tensiometer (Eijkelkamp.co) was used to monitor the soil water potential.

2.6 DATA COLLECTION

- Plant height (using meter rule and measured in centimeter from the base of the plant to the base of the last emerged leaf).

- Leaf area (obtained by measuring in cm^2 using the meter rule to measure the length of a fully expanded tagged leaf and the breadth at mid leaf. The product of the length and the width was multiplied by 0.75 which is the calibration factor for maize leaf (Francis et al., 1969).

- Number of ears per plant (by visual counting)

- Number of rows per cob, number of kernels per row (by visual counting), number of kernels per cob (obtained by multiplying the number of kernels per rows with number of rows per cob), cob length (measuring the length of a cob using the meter rule)

- Grain yield was taken from total ear harvest per plot.

- Mass of 1000 grains and total grain yield (after

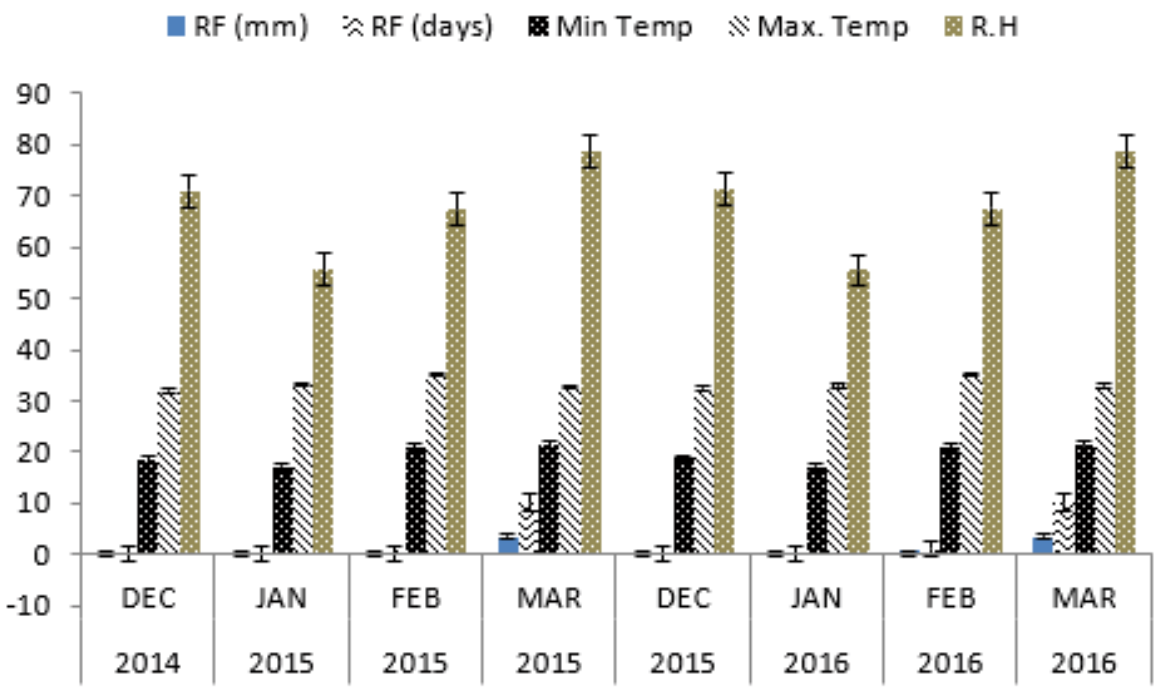

Figure 2: Mean monthly temperature (°C), humidity and precipitation (mm) during 2014, 2015 and 2016 planting seasons. Source: Nigerian Meteorological Agency, Ibadan (NIMET)

adjusting to 12 % moisture content) using weighing balance.

2.7 DATA ANALYSIS

The data collected were pooled across the two years and subjected to analysis of variance (ANOVA) for split - split - split plot in RCBD using Statistical Tool For Agricultural Research (STAR, version 2.0.1 2014). Significant means were separated using Tukey Honest Significant Difference at 5% probability level.

3 RESULTS

3.1 WEATHER INFORMATION

The mean monthly temperature (°C), humidity and precipitation (mm) during the experimental studies were shown in Figure 2. No rainfall was recorded for the months of December, January, February (actual periods when water deficit stress was imposed). About 3.43 and 3.49 mm total number of rainfall were recorded in March in 2015 and 2016, respectively. Maximum temperatures were recorded in February while relative humidity values were significantly reduced in January and February of the years of the trials.

3.2 SOIL PHYSICO-CHEMICAL PROPERTIES

The soil obtained from the experimental field was a loamy-sandy soil of classification series "Typic Kanhaplustalf". Result of the chemical analyses showed that there were slight variations in the soil chemical properties in the two years of the experimental studies (Table 1). The pH value of the soil samples appeared slightly acidic in 2015 (6.00) and slightly basic in 2016 (7.25). Soil total nitrogen (0.06 %, 0.05 %), available phosphorus (13.16 mg kg^{-1}, 6.84 mg kg^{-1}), organic carbon (0.44 %, 0.86 %), potassium and the micronutrients were very low in 2015 and 2016 compared with recommended soil requirement for Nigerian soils.

3.3 CHEMICAL PROPERTIES OF COMPOST USED

The compost was slightly basic with pH value of 8.30, total nitrogen content was 0.70 %, while the values of phosphorus and potassium were 0.91 mg kg^{-1} and 0.61 cmol kg^{-1}, respectively (Table 2). The compost had high carbon to nitrogen ratio value (7.47), and very high micronutrients (Iron (9587), Zinc (436) and manganese (597) mg kg^{-1}).

3.4 PLANT HEIGHT (PHT)

Water deficit stress significantly influenced plant height and fertilizer application rates ($p < 0.001$) (Table 3). Significant reduction in plant heights were observed in maize subjected to water deficit stress at three weeks after emergence compared with maize grown under FW and STR2, plant heights ranged from 119.79 cm (STR1) to 150.76 cm (FW) (Table 4). Across the fertilizer rates, maize heights ranged between 116.88 cm (0 N) to 141.18 cm (100 N + 10 TPC), there was no significant difference in the plant heights observed across the fertilizer application rates, except for the control which had relatively shorter plants. Maize variety TZPBSR-W (136.43 cm) are taller than ILE-1-OB (132.01 cm).

Table 1: Pre-planting physico - chemical properties of soil used for the experiments

	2015	2016
Parameter		
pH (H_20)	6.00	7.25
Organic carbon (%)	0.44	0.86
Total nitrogen (%)	0.06	0.05
Available P (mg kg^{-1})	13.16	11.84
Bulk density (Mg m^{-3})	1.31	1.31
ECEC(cmol)	7.11	5.56
Base saturation (%)	99.02	99.28
Exchangeable cation (cmol kg^{-1})		
K	0.22	0.37
Na	0.39	0.63
Ca	5.53	3.80
Al+H	0.07	0.04
Exchangeable micronutrient (mg kg^{-1})		
Fe	7.10	0.06
Zn	3.60	0.65
Cu	1.10	0.15
Mn	22.8	44.10
Soil particle analysis		
Sand g kg^{-1}	854	842
Silt g kg^{-1}	82	86
Clay g kg^{-1}	64	72
Textural class	loamy -Sandy	loamy -Sandy

Table 2: Chemical properties of the *Tithonia* poultry compost used as soil amendment

Parameter	Values
pH (H_20)	8.30
Organic carbon (%)	5.25
Total nitrogen (%)	0.70
Available P (mg kg^{-1})	0.91
C/N ratio	7.47
Exchangeable cation (cmol kg^{-1})	
K	0.61
Na	0.62
Ca	4.95
Mg	0.92
Exchangeable micronutrient(mg kg^{-1})	
Fe	9587
Zn	436
Cu	31.0
Mn	597

Water deficit stress and fertilizer interaction (WS x F) effect on plant height was significant ($p < 0.05$). Plant height ranged from 0 N (99.99 cm) (STR1) to 160.40 cm 10 TPC (FW) (Figure 3a). Plant heights at STR1 across the various fertilizers application rates were not significantly different but higher than 0N (99.99 cm) ($p < 0.05$). Highest plant height was observed at 10 TPC (160.40 cm) (FW) but lowest at 0 N (STR1).

Water deficit stress and variety interaction (WS x V) interaction effect on plant heights was significant ($p < 0.05$) (Table 5). Maize variety TZPBSR-W (160.26 cm) had taller stems under full watering than ILE-1-OB (149.18 cm), however no differences observed in the heights at STR1 and STR2, respectively.

3.5 LEAF AREA (LA)

Leaf area differed significantly across water deficit stress and fertilizer application rates ($p < 0.001$) (Table 3). The leaf areas varied from 458.90 cm^2 (STR1) to 598.81 cm^2 (FW) (Table 4). Across F rates, the largest leaf area size was observed when 100 N + 10 TPC was applied (622.71 cm^2), this LA value was however not significantly different from LA's obtained when 50 N + 10 TPC (611.03 cm^2) and 10 TPC (581.57 cm^2) were applied, while the control (0 N) had least LA size of 397.65 cm^2. The leaf areas of the two maize varieties were not significantly different.

Water deficit stress and fertilizer interaction (WS x F) effect on LA was significant ($p < 0.001$) (Figure 3b). Large leaf area (LA) sizes of maize plant were observed at 100 N + 10 TPC (645.31 cm^2) and 50 N + 10 TPC (647.47 cm^2) under FW. The leaf areas obtained were not significantly different from LA's obtained under 50 N and 100 N and 10 TPC fertilizer applications rates except 0 N (465.11 cm^2). Similar trend was observed in STR2 across the fertilizers application rates. Significant reduction in leaf sizes were observed in STR1 across the fertilizer rates, however considerably larger leaf area sizes were observed with applications of 100 N + 10 TPC (541.47 cm^2) and 50 N + 10 TPC (528.19 cm^2), respectively.

3.6 NUMBER OF EAR PER PLANT (E/P)

The number of ear per plant was not significantly influenced by WS ($p < 0.05$) (Table 3), however the E/P varied significantly across fertilizer application rates ($p < 0.01$). Applications of 100 N + 10 TPC (1.57) and 50 N + 10 TPC (1.60) produced more ear per plant than other F-application rates and the control which had the least value of 1.27 of ear per plant.

Fertilizer and variety interaction (F x V) effects on number of ear per plant of two maize was significant ($p < 0.05$). Maize variety ILE-1-OB (1.39) had fewer numbers of ears than TZPBSR-W (1.15) under the control, maize variety TZPBSR-W (1.55) had more ears than ILE-1-OB (1.35) under 100 N (Table 6).

3.7 COB LENGTH (CBT)

The cob length was significantly influenced by water deficit stress and fertilizer application rates ($p < 0.001$) (Table 3), across WS, the cob length ranged from 12.46 cm (STR2) to 18.02 cm (FW) (Table 4). Cobs length ranged between 0 N (12.02 cm) to 100 N + 10 TPC (16.44 cm). However, no significant difference was observed between cob lengths of 100 N (15.45 cm) and 10 TPC (15.28 cm).

3.8 NUMBERS OF ROWS PER COB (R/C)

Water deficit stress and fertilizer significantly influenced number of rows per cob ($p < 0.001$) (Table 3). The effect of WS on R/C, varied between STR2 (11.83) to FW (13.72), while applications of 100 N + 10 TPC and 50 N + 10 TPC and 100 N had the highest number of rows per cob compared with R/C of other fertilizer applications rates but lowest in the control (11.03) (Table

Table 3: Mean square of ANOVA of the effects of water, fertilizer, variety and result of f-interaction on growth and yield components of two maize varieties evaluated in Ibadan

Source o variation	D.F	PHT (cm)	LA (cm^2)	E/P	CBT (cm)	R/C	K/R	K/C	1000-KM (g)	GY ($t\ ha^{-1}$)
Rep	2	20.76^{ns}	1056.68^{ns}	0.02^{ns}	0.92^{ns}	0.28^{ns}	29.13*	5225.06*	33.68^{ns}	0.88^{ns}
Water Deficit Stress (WS)	2	8751.46***	189971.98***	0.09^{ns}	278.99***	32.93***	1408.25***	383053.62***	7671.47**	128.15***
Error(a)	4	30.48	1193.08	0.18	2.26	0.39	2.20	472.02	96.68	0.66
Fertilizer (F)	5	1549.10***	129468.95***	0.25**	53.88***	16.09***	435.31***	115200.02***	4336.72***	26.16***
WS x F	10	170.81*	7490.99***	0.04^{ns}	2.83^{ns}	0.99^{ns}	16.65**	4302.01**	538.55*	1.50**
Error (b)	30	73.09	1492.64	0.05	1.55	0.50	3.44	1041.12	201.57	0.40
Variety (V)	1	524.92**	4572.76^{ns}	0.00^{ns}	1.45^{ns}	1.05^{ns}	3.35^{ns}	1493.16^{ns}	255.38^{ns}	0.41^{ns}
WS x V	2	217.86*	5150.80^{ns}	0.01^{ns}	1.34^{ns}	0.38^{ns}	6.39^{ns}	1412.44^{ns}	115.18^{ns}	0.51^{ns}
F x V	5	30.39^{ns}	1131.86^{ns}	0.08*	1.76^{ns}	0.39^{ns}	4.91^{ns}	1065.81^{ns}	107.53^{ns}	0.57^{ns}
WS x F x V	10	30.23^{ns}	1337.78^{ns}	0.021^{ns}	0.67^{ns}	0.74^{ns}	4.14^{ns}	1136.89^{ns}	55.21^{ns}	0.37^{ns}
Error (c)	36	54.61	2072.01	0.025	1.30	0.79	4.19	1315.16	115.31	0.34
Total	107									

* ** *** Significant at $p < 0.05, 0.01, 0.001$, ^{ns} = not significant. D.F = Degree of freedom † Means not followed by the same.
Letters within a column are significantly different at P = 0.05 according to Tukey HSD. PHT = Plant height, LA = Leaf area, E/P = Ear per plant, CBT = Cob length R/C = Row per cob, K/R = Kernel per row, K/C = Kernel per cob, 1000-KM = Mass of 1000 kernels and GY = Grain yield

Table 4: Main effect of Water Deficit Stress, fertilizer and variety effect on growth and yield components of two maize varieties evaluated in Ibadan

	PHT (cm)	LA (cm^2)	E/P	CBT (cm)	R/C	K/R	K/C	1000-KM (g)	GY ($t\ ha^{-1}$)
Water regime									
FW	150.76a	598.81a	1.54a	18.02a	13.72a	30.34a	419.68a	244.73a	6.59a
STR1	119.79c	458.90c	1.46a	14.90b	12.50b	23.05b	292.98b	231.43b	3.92b
STR2	132.11b	562.77b	1.44a	12.46c	11.83c	17.90c	215.33c	215.57c	2.94c
F.rate (F)									
0 N	116.88b	397.65c	1.27b	12.02d	11.03c	15.57c	177.34c	203.78c	2.37c
50 N	132.58a	494.84b	1.51ab	14.64c	12.18b	21.58b	266.72b	222.23ab	3.98b
100 N	133.75a	533.15ab	1.45ab	15.45bc	13.11a	25.57a	337.58ab	233.16ab	4.85ab
10 TPC	140.13a	581.57a	1.49ab	15.28bc	12.82ab	23.33ab	306.96ab	236.01a	4.61ab
50 N + 10 TPC	140.79a	611.03a	1.60a	16.44ab	13.50a	26.69a	362.46ab	246.68a	5.26a
100 N+ 10 TPC	141.18a	622.71a	1.57a	16.91a	13.46a	29.84a	404.92a	241.60a	5.82a
Variety	15.01	67.85	0.27	1.26	0.72	3.26	56.66	15.95	0.78
TZPBSR-W	136.43a	546.18a	1.48a	15.01a	13.14a	23.58a	305.61a	232.13a	4.58a
ILE-1-OB	132.01b	533.65a	1.48a	15.24a	12.77a	23.94a	313.31a	229.05a	4.51a
s_e	1.69	10.45	0.02	0.29	0.14	0.69	11.38	2.17	0.19
Mean	134.22	540.16	1.48	15.12	12.68	23.76	309.33	230.58	4.48

† Means not followed by the same letter within a column are significantly different at $p < 0.05$ according to Tukey Honest Significant Difference. STR 1 = Water stress at vegetative growth stage, STR 2 = Water stress at reproductive growth stage and FW = Full watering, PHT = Plant height, LA = Leaf area, E/P = Ear per plant, CBT = Cob length R/C = Row per cob, K/R = Kernel per row, K/C = Kernel per cob, 1000 KM = Mass of 1000-kernels and GY = Grain yield

4). Interaction effects on number of rows per cob were not significant

3.9 NUMBERS OF KERNELS PER ROW (K/R)

Number of kernels per row varied across the replicates ($p < 0.05$), WS and F ($p < 0.001$) and WS x F (0.01) (Table 3). Effect of WS on K/R was the lowest at STR2 (17.90) but the highest at FW (30.34), across fertilizer application rates (Table 4). Across SA rates, the number of kernel per row also ranged between 0 N (15.57) to 100 N + 10 TPC (29.84), though number of K/R at 100 N + 10 TPC was not significantly different from K/R recorded for 50 N + 10 TPC (26.69) and 100 N (25.57). The number of kernels per row ranged from 0 N (22.64) to 100 N + 10 TPC (34.37) under FW, while no significant difference among K/R formed by the applications of 100 N + 10 TPC (34.37), 50 N + 10 TPC (28.79) and 10 TPC (32.99) (Figure 3c). High significant reductions in number K/R was observed at STR2 and K/R ranged from 0 N (9.76) to 100 N + 10 TPC (24.37) followed by 50 N + 10 TPC (21.59).

3.10 NUMBERS OF KERNELS PER COB (K/C)

The numbers of kernels per cob varied significantly across the replicates ($p < 0.05$), WS and F ($p < 0.001$) and WS x F ($p < 0.01$) (Table 3). Effect of WS on K/C varied from STR2 (215.33) to FW (419.68) (Table 4). Across F-rates, the highest number of kernel per cob was recorded at 100 N + 10 TPC (404.92) and the lowest in the control (177.34), number of kernels per cob at 100 N, 10 TPC and 50 N + 10 TPC were not significantly different. Water deficit stress and fertilizer interaction effect was significant on K/C, least number of K/C was obtained at STR2 (98.86) while application of 100 N + 10 TPC (514.94) gave highest number of K/C at FW.

3.11 MASS OF1000-KERNELS (1000-KM)

The mass of 1000-kernels was significantly influenced by WS ($p < 0.01$), F ($p < 0.001$) and WS x F ($p < 0.05$) (Table 3). The effect of WS on 1000-kernel mass varied from STR2 (215.57 g) to FW (244.73 g) (Table 4). Across the fertilizer application rates, the mass of 1000-kernel was the highest at 50 N + 10 TPC (246.68 g), though not significantly different from 1000-KM of 100 N+ 10 TPC (241.60 g) and 10 TPC (236.01 g), while the control had the least value of 203.78 g.

Water deficit stress, fertilizer interaction effect shows that the highest 1000-kernel mass was obtained at 50 N + 10 TPC under FW (266.13 g), this value was not significantly different from 1000-kernel mass observed at 100 N + 10 TPC (261.38 g), 10 TPC (257.78 g) and 10 TPC (257.50 g), while the smallest value of 1000-kernel mass was observed under STR2 at 0 N (179.10 g). Across STR1, the mass of 1000-kernels were not significantly different.

3.12 GRAIN YIELD (GY)

Grain yield varied significantly at WS, F ($p < 0.001$) and WS x F ($p < 0.01$) (Table 3). The effect of WS on GY ranged between STR2 (2.94 t ha^{-1}) and FW (6.59 t ha^{-1}) (Table 4). Across F- applications, 100 N + 10 TPC (5.82 t ha^{-1}) and 50 N + 10 TPC (5.26 t ha^{-1}) produced the highest GY, while the control showed the least GY (2.37 t ha^{-1}). Application of 8.33 t ha^{-1} (100 N + 10 TPC) under FW

Table 5: Water deficit stress and Variety interaction effect on plant heights of two maize varieties in Ibadan

Water stress	Variety	Plant height
FW	ILE-1-OB	149.18 ± 5.18
	TZPBSR-W	160.26 ± 4.06
STR1	ILE-1-OB	126.03 ± 3.27
	TZPBSR-W	128.90 ± 2.54
STR2	ILE-1-OB	141.80 ± 4.96
	TZPBSR-W	139.76 ± 4.42

STR 1 = Water stress at vegetative growth stage, STR 2 = Water stress at reproductive growth stage and FW = Full watering

Table 6: Variety and fertilizer application rates interaction effects on number of ear per plant of two maize varieties in Ibadan

Fertilizer	Variety	Number of earper plant
0 N	ILE-1-OB	1.39 ± 0.08
	TZPBSR-W	1.15 ± 0.06
50 N	ILE-1-OB	1.52 ± 0.06
	TZPBSR-W	1.49 ± 0.11
100 N	ILE-1-OB	1.35 ± 0.05
	TZPBSR-W	1.55 ± 0.06
10 TPC	ILE-1-OB	1.43 ± 0.07
	TZPBSR-W	1.54 ± 0.08
50 N + 10 TPC	ILE-1-OB	1.64 ± 0.11
	TZPBSR-W	1.55 ± 0.07
100 N + 10 TPC	ILE-1-OB	1.58 ± 0.06
	TZPBSR-W	1.57 ± 0.07

produced the highest GY, while GY was the lowest at 0 N under STR2 (0.88 t ha^{-1}) (Figure 3f). The GY of the two maize varieties were not significantly different ($p < 0.05$).

4 DISCUSSION

Drought and low soil fertility are major abiotic factors militating against profitable maize production in the tropics. The use of drought tolerant crop genotypes and soil amendment has potential to enhance growth and yield performances of crops grown under drought condition. To investigate the role of soil nutrient amendment on the growth and yield responses of crop to water deficit stress, field experiment was established in Ibadan, Nigeria.

Results obtained show that 14 days withdrawal of watering during the vegetative growth stage (STR1) resulted in maize plants with reduced heights and leaf areas. The reduction in leaf area as a result of water deficit stress may be attributed to decrease in rate of leaf initiation and expansion and or accelerated rate of leaf senescence and leaf shedding which consequently reduce grain yield compared with grain yield obtained under well watered condition (Bolaños & Edmeades, 1996; Nam et al., 1998; Anjum et al., 2011). As leaves with reduced leaf area do not fully intercept solar radiation which in turn strikes the ground, and consequently increased the evaporation - transpiration ratio (Araus, 2002). Reduction in plant height from water deficit stress interferes with over all crop photosynthetic efficiency (Imadi et al., 2016). Hence, plants with greater heights are often larger in overall plant size, intercept more light and use water faster by transpiration.

In this study, water deficit stress at vegetative stage (STR1) accounted for 41 % loss in grain yield, this finding agreed with the report of Rufino et al. (2018). Water deficit stress affects all the various metabolic processes and yield components in plant and in turn reduced crop yield potential. Borra's et al. (2003), inferred that the overall indirect impact of water stress during vegetative stage on grain yield is source limiting as water stress decreased the source potential and available assimilates level and decreases grain weight. For instance, the kernel rows in maize are determined between V7 to V8 maize growth phase, while the number of kernels on each ear and size of ear in maize is determined at V12 of the maize growth stage (Ritchie & Hanway, 1993; Annonymous, 2013). Therefore, occurrence of water deficit stress during vegetative growth phases becomes detrimental to

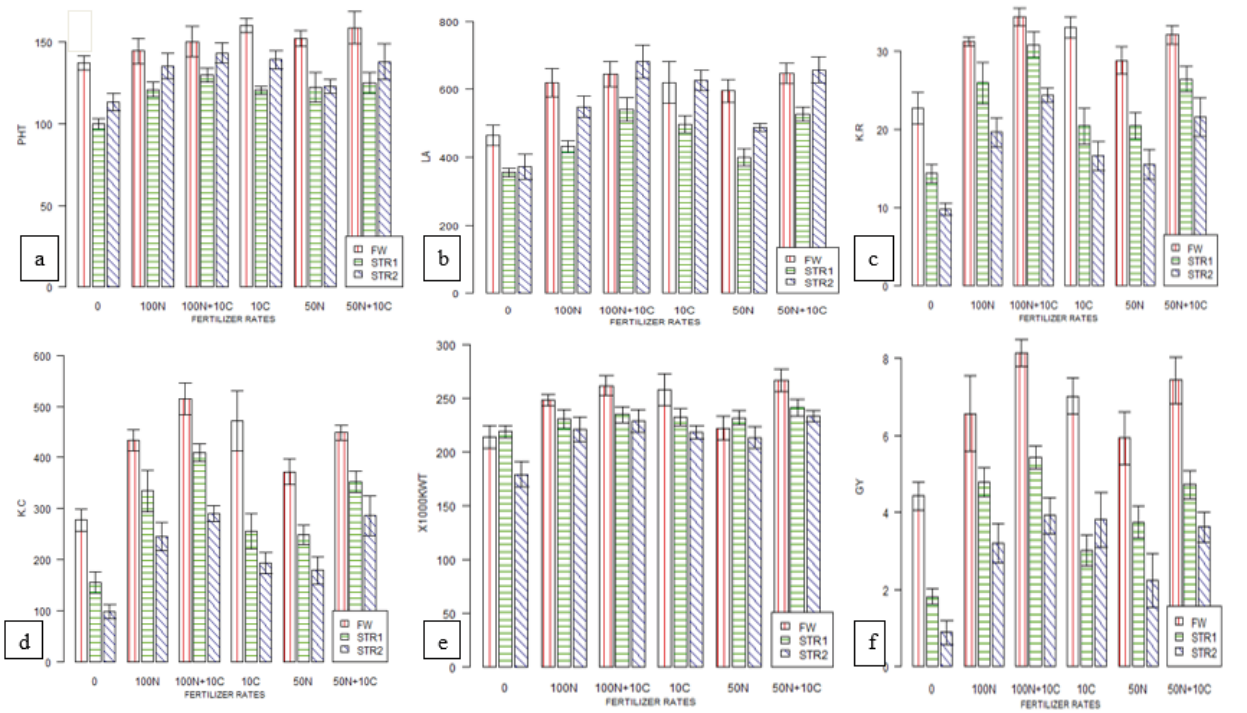

Figure 3: Water deficit stress and fertilizer interaction effects on (a) PHT (Plant Height) (b) LA (Leaf Area) (c) Number of K/R (Kernels/Row), (d) K/C (Kernels/cob), (e) 1000-Kernel weight and (f) GY(Grain Yield) of two maize varieties planted under three water deficit stress and six fertilizer application rates in Ibadan. FW = Full watering, STR1 = Water stress at vegetative growth stage STR 2 = Water stress at reproductive growth stage

the final crop grain yield (Ritchie & Hanway, 1993). This is because, water deficit stress during vegetative growth stage decreases plant source potential and assimilates level thereby decreasing grain weights (Borra's et al., 2003; Fatemi et al., 2006 and Khalili et al. 2010)

The impact of preanthesis water deficit stress (STR2) in this study resulted in 55.37 % loss in grain yield, this finding agreed with the reports of Denmead & Shaw, (1960) and Sah et al. (2020). Farre & Faci, (2009), and Mansouri, et al. (2010), which earlier inferred that grain yield of maize is highly determined by the amount of irrigation water. The number of ear formed per plant were not significantly different across the water deficit stress regime, but ears obtained from plants subjected to preanthesis water deficit stress (STR2) were smaller in size with few grains while some were even barren. The significant reduction in the number of grain per row and 1000-kernel weight under the water deficit stress observed in this study agreed with the earlier reports of Carpici (2009) and Kuscu (2010). In the view of Grant et al. (1989) and Hargurdeep & Westgate (2010) water deficit stress during pre anthesis stage of maize development could be implicated for abnormal development of embryo sac, grain sterility and decreased fertile grain number. While imposition of water deficit stress during preanthesis growth stage resulted in reduced number of kernels per cob, kernel set per row and the total grain yield (sink limiting).

Increased fertilizer applications significantly enhance R/C, K/R, K/C, Weight of 1000-kernels and GY across the water deficit stress regime in this study. Deficiencies in N supply have been reported to impair pollination synchronization, increased kernel abortion (Uribelarrea et al., 2002; Uhart & Andrade, 1995), resulting in reduced kernel number per plant and decrease grain yield observed in the fertilizer control (Carcova et al., 2000; Paponov et al., 2005). Apart from water, soil nutrient especially nitrogen also had significant impact on the yield components and grain yield of maize in this study. Increased maize growth and yield responses were obtained under increased fertilizer application rates especially when 10 t ha^{-1} of compost was added to each inorganic fertilizer rates of 50 and 100 kg ha^{-1} respectively. Application of inorganic fertilizer with compost to crop has been reported to have the advantage of providing nutrients to meet crop nutrition requirements and maintain soil health (Abedi et al., 2010; Kazemeini et al., 2010; Efthimiadou et al., 2010). High level of micronutrient in the compost (Table 2) may have helped to improve general plant performance. Apart from water, soil nutrient especially nitrogen also had significant impact on the growth and yield components of maize in this study.

Application of nitrogen fertilizer have been shown to increased the uptake of other nutrients, this is because nitrogen enhances growth and development of small roots and root hairs which in turn facilitate the absorbing ability per unit of dry weight (Gheysari et al., 2009; Hammad et al., 2011). Nitrogen is also needed to establish and maintain the enzymatic processes essential for carbon utilization and growth, and is also a major constituent of endosperm storage protein (Cazetta et al., 1999; Duvnjak et al., 2021). The use of 10 t ha^{-1} of Tithonia poultry compost in combination to each of 100 kg N ha^{-1} and 50 kg N ha^{-1} of nitrogen fertilizer significantly enhanced grain yield than sole applications of each of inorganic fertilizer rate in this study. The compost (Table 2) has a very high carbon to nitrogen ratio, also very rich in essential micronutrients needed for maize production. Application of inorganic fertilizer with compost to crop has been reported to have the advantage of providing nutrients to meet crop nutrition demands and maintain soil health (Efthimiadou et al., 2010). Compost had been reported to improve soil water holding capacity as well as buffering rapid changes in soil pH (Tambone et al., 2007; Zemánek, 2011).

The significant water regime by fertilizer interaction effects on the various growth and yield components in this study indicated that growth and yield increased resulting from fertilizer application depended on the availability of water (Pandey et al., 2000). Hence, adequate moisture availability is vital to nutrient mineralization, growth and grain yield of maize (Hokmalipour et al., 2010). Water deficit stress at the vegetative stage of growth not only deprived the plant of adequate moistures supply needed for cellular meristematic activities but also hinder nutrient supply which are needed for the development of yield component potential. Despite the impact of the water stress on the various yield components of maize, increased application of fertilizer was seen to enhanced grain yield of the two maize varieties. Increased nitrogen application has been shown to have the capability of improving drought tolerance and enhancing grain yield in maize (Boutras, 2001; Xu et al., 2005). Variety TZPBSR-W appeared to performed better than ILE-1-OB most especially under well watered condition but such superiority could not be maintain under the first and second water stress conditions as observed in the number of kernels per row and number of kernels per cob. The effect of water stress on seed formation, kernel set and grain yield was most severe during the reproductive growth stage and under reduced nutrient availability. Water stress and low nutrient availability might have reduced the sink strength and capacity of the maize plants which are determined by genetic and environmental factors (Alvarez Prado et al., 2014).

Moisture availability and nutrient availability to a large extent, determines seed formation, kernel set, and

the final grain yield in this study. Nitrogen fertilizer effect on the various yield components and grain yield improved as the N application increases. Maize plant performed best when inorganic fertilizer was used along with organic fertilizer than when organic or inorganic fertilizer was applied alone. The result of the present finding on water regime nitrogen interaction also revealed that growth and yield components and grain yield performed better under adequate moisture availability. Nitrogen had been reported to improve water use efficiency in maize (Ogola et al., 2002). Growth and yield components were improved with increase N application even under water stress conditions. Therefore, optimization of N and water management could be an efficient way to attain sustainable agriculture. The two maize varieties were similar in yield responses to the varying stress periods and fertilizer application rates in the two years of the experimental studies. Similar report of variability in crop genotypic response under water stress had earlier been reported by Hufsteler et al., (2007); Abayomi & Abidoye, (2009).

Application of 10 t ha^{-1} of *Tithonia* poultry compost alone to the maize field produced taller maize plants and broader leaves better than maize plants obtained when 100 kg N ha^{-1} inorganic fertilizer were applied, but this alone could not sustain the plant adequately beyond the pollination process. The evidence of this was the rapid appearance of yellow lower leaves in treatment with 10 t ha^{-1} (*Tithonia* poultry compost alone). Explanations for this could be that the N supply by the compost alone at the transition stage from vegetative to reproductive was not adequate enough for N demand for post pollination activities. Hence the need for remobilisation of N from the lower leaves for grain filling was inevitable.

5 CONCLUSION

Climate change and its associated attributes have impacted negatively on general crop development across the world. Drought emanating from erratic rainfall pattern has constituted serious menace to profitable maize production in the sub Saharan Africa. From this study it was obvious that water deficit stress reduced growth and yield performances of the two maize varieties resulting into grain yield losses of 41.0 % and 55.37 % under vegetative and reproductive stages water deficit stresses, respectively. However, this study has been able to explore soil fertility management at enhancing growth and yield performances of maize subjected to water deficit stress. Different rates of nitrogen fertilizer from inorganic, organic sources and their combinations were applied to the two maize varieties at different phenological growth –water deficit stages. From the result, it is obvious that 50 kg N of inorganic fertilizer and 10.7 kg N of *Tithonia* Poultry Compost significantly enhance growth and yield performances of the two maize varieties across water stresses in this study. The 50 kg N of inorganic fertilizer represents half dose of recommended 100 kg N of nitrogen fertilizer (inorganic) application rate for the agro ecological zone of the country. Minimal use of inorganic fertilizer rate will help reduce environmental issues associated with the increase use of chemical fertilizers and cost of production. The maize varieties grown under 50 kg N ha^{-1} NPK-20-10-10 and 10.7 kg N ha^{-1} TPC subjected to water deficit stress must have benefited immensely from fast release of plant nutrient (inorganic fertilizer) with high; micronutrients, organic carbon content and moisture retention of compost. Augmenting reduced rate of inorganic fertilizer with *Tithonia* compost is hereby recommended for profitable maize production in derived savanna ecology of Nigeria. In spite of the numerous benefits associated with the use of compost, the bulkiness and availability of enough quantities for large scale maize production remains a great challenge. Farmers should be adequately trained on compost preparation techniques and the importance of combine use of inorganic and organic fertilizers to boost maize production in the face of the prevailing climate change. Government should support and empower unemployed youth to embrace commercial compost production so as to cater for the anticipated high compost demand by commercial farmers. More funding should be made available for soil fertility management and climate change adaptability studies.

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Phytotoxic effects of essential oils from *Nepeta glocephalata* Rech.f. and *N. ispahanica* Boiss. on selected weed species

Marjan DYANAT [1, 2], Farzad ASGARI [1]

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Phytotoxic effects of essential oils from *Nepeta glocephalata* Rech.f. and *N. ispahanica* Boiss. on selected weed species

Abstract: In the present study the bioherbicidal activity of essential oils hydrodistilled from *Nepeta glocephalata* Rech.f and *N. ispahanica* Boiss were investigated on four weed species (barnyard grass (*Echinochloa crus-galli* (L.) Beauv), redroot pigweed (*Amaranthus retroflexus* L.), lambsquarters (*Chenopodium album* L.) and canary grass (*Phalaris canariensis* L.)). A total of 37 components were identified from the essential oils of *N. glocephalata and N. ispahanica* constituting approximately 98.61 % and 96.1 % of the oils, respectively. In laboratory bioassay different concentrations (0, 1, 2, 4 and 8 µl ml^{-1}) of two *Nepeta* essential oils on germination, root and shoot length were studied. Results showed by increasing the concentration of oils, all studied traits of the weeds were decreased compared with control. In a glass house bioassay post-emergence application of *Nepeta* essential oils (1.25 %, 2.5 %, 5 % and 10 %, v/v) on 3-week-old weed plants caused visible injury (7-days after spray) ranging from chlorosis to necrosis of plant weeds. In foliar application under glasshouse conditions, both *Nepeta* essential oils reduced the seedling dry mass and concentrations of chlorophyll a chlorophyll b. The study concludes that *Nepeta* essential oils have phytotoxic effects and could be used as bioherbicides but the selectivity of these compounds should be considered also.

Key words: *Nepeta glocephalata* Rech.f.; *N. ispahanica* Boiss.; bioherbicide; 1, 8-cineole; chlorophyll a; weed seed germination; root length

Fitotoksični učinki eteričnih olj iz dveh vrst mačje mete (*Nepeta glocephalata* Rech.f. in *N. ispahanica* Boiss.) na izbrane vrste plevelov

Izvleček: V raziskavi je bila preučevana bioherbicidna aktivnost vodnih destilatov eteričnih olj iz dveh vrst mačje mete (*Nepeta glocephalata* Rech.f in *N. ispahanica* Boiss.) na štiri plevelne vrste (navadna kostreba (*Echinochloa crus-galli* (L.) Beauv), navadni (srhkodlakavi) ščir (*Amaranthus retroflexus* L.), bela metlika (*Chenopodium album* L.) in kanarska čužka (*Phalaris canariensis* L.)). Celokupno je bilo v eteričnih oljih obeh vrstah določenih 37 sestavin, ki so predstavljale 98,61 % oziroma 96,1 % olja. V laboratorijskem poskusu so bili preučevani učinki različnih koncentracij (0, 1, 2, 4 in 8 µl ml^{-1}) eteričnih olj iz obeh vrst mačje mete na kalitev, dolžino korenin in poganjkov izbranih plevelov. Rezultati so pokazali, da so se vrednosti vseh merjenih parametrov plevelov zmanjševale s povečevanjem koncentracije eteričnih olj. V poskusu v rastlinjaku so bile preučevane vidne poškodbe uporabe eteričnih olj iz obeh vrst mačje mete (1,25 %, 2,5 %, 5 % and 10 %, v/v) na tri tedne starih sejankah plevelov, sedem dni po škropljenju z eteričnimi olji, ki so se pojavile kot kloroze in nekroze. Pri foliarni uporabi eteričnih olj obeh vrst mačje mete v rastlinjaku se je zmanjšala suha masa sejank plevelov, zmajšale so se tudi vsebnosti klorofila a in b. Na osnovi raziskave lahko zaključimo, da imajo eterična olja obeh vrst mačje mete fitotoksične učinke in bi lahko bile uporabljene kot bioherbicidi vendar je pri tem potrebno upoštevati selektivne učinke njihovih sestavin.

Ključne besede: *Nepeta glocephalata* Rech. f.; *N. ispahanica* Boiss.; bioherbicid; 1, 8-cineol; klorofil a; kalitev semen plevelov; dolžina korenin

1 Department of Agricultural Sciences and Food Industries, Science and Research Branch, Islamic Azad University, Tehran, Iran
2 Corresponding author, e-mail: Ma_dyanat@yahoo.com

1 INTRODUCTION

Herbicide-resistant weeds and environmental concerns have led researchers to consider using alternative ways to manage weeds (Vyvyan, 2002; Ashraf et al., 2017). Allelopathy is one of these ways (Weston, 1996). Allelopathic compounds can reduce the use of synthetic herbicides and thus reduce environmental pollution and lead to more safe crops (Singh et al., 2002, 2003, 2005a, b). Among the natural plant products, essential oils constitute an important group of that provide a characteristic odor to the aromatic plants (Singh et al*.,* 2002). Earlier studies have documented that essential oils and their constituents inhibited seed germination and retard plant growth (Barney et al., 2005; Batish et al., 2006; Ens et al., 2009). The allelopathic activities of some essential oils and their monoterpenes on seeds germination or seedling growth at several species have been shown in previous studies (Dudai et al*.,* 1999; Abrahim et al*.,* 2000; Tworkoski, 2002; Singh et al*.,* 2004; Dudai et al., 2004; Armirante et al., 2006; Kordali et al., 2006; Kordali et al., 2007). Allelopathic properties of essential oils from different aromatic plants belonging to Lamiaceae, Compositae, Myrtaceae, Cupressaceae, Rutaceae and Verbenaceae families have been reported (Dudai et al., 1999; Angelini et al*.,* 2003; Kaur et al*.,* 2010; Amri et al*.,* 2013 ; Verdeguer et al*.,* 2011). Also allelopathic potential of the essential oil of many plants from family Lamiaceae such as *Salvia apiana* Jeps. and *Salvia leucophylla* Greene (Muller et al., 1964), *Satureja hortensis* L. and *Thymus vulgaris* L. (Tworkoski 2002), *Rosmarinus officinalis* L., *Satureja montana* L. (Angelini et al., 2003), *Lavandula* spp. and peppermint (*Mentha* × *piperita* 'Mitcham') (Campiglia et al., 2007*;* Mahdavikia and Saharkhiz, 2015), *Zataria multiflora* Boiss and its different chemotypes (Saharkhiz et al., 2010), *Satureja khuzestanica* Jamzad, *Satureja bachtiarica* Bunge, *Satureja rechingeri* Jamzad and *Satureja spicigera* (K.Koch) Boiss. (Taban et al., 2013) have been previously reported.

Genus *Nepeta* is one of the largest genera of the Lamiaceae family that comprises about 300 herbaceous perennial and annual species (Formisano et al., 2011). The greatest diversity and richness of species is found in Southwestern Asia, (especially Iran and Turkey), and the Western Himalayas. There are seventy-nine species of *Nepeta* in Iran and about 39 of them are endemics (Jamzad, 2012). Much research was done on diversity, species richness and chemical properties of *Nepeta* species. Most *Nepeta* species are rich in essential oils. Diverse biological activities of *Nepeta* oil such as feline attractant, canine attractant, insect repellant, arthropod defense (Tucker and Tucker, 1988, Wagner and wolf, 1977), antibacterial, antifungal and antiviral activities (Tucker and Tucker, 1988) have been reported previously. There are several reports on the chemical composition of the essential oils of the genus *Nepeta* found in Iran (Sefidkon, 2004, 2005; Sajjadi, 2005; Sonboli et al., 2005; Jamzad, 2012). Allelopathic potential of this genus was revealed. Phytotoxicity of *Nepeta* essential oils has been mainly tested (Kobaisy et al., 2005, Eom et al., 2006, Mancini et al., 2009, Mutlu et al., 2011, Kekec et al., 2012, Bozari et al., 2013, Živković, 2013). Allelopathy of water extracts has been studied by Mutlu and Atici (2009) and Babaahmadi et al. (2013). No bioassays or field experiments had been done to study the allelopathic potential of *Nepeta glocephalata* Rech.f and *N.ispahanica* Boiss., Endemic plants of Iran.

The aim of the present study was to study the essential oil composition of *N. glocephalata* and *N. ispahanica* in order to know if these compositions have phytotoxic effects on germination, seedling growth injury and photosynthesis of barnyard grass (*Echinochloa crus-galli* (L.) Beauv), a most important weed in rice (*Oryza sativa* L.), redroot pigweed (*Amaranthus retroflexus* L.) and lambsquarters (*Chenopodium album* L.), annual plants seriously influencing summer crops and canary grass (*Phalaris canariensis* L.), serious weed of wheat (*Triticum aestivum* L.) fields in Iran.

2 MATERIALS AND METHODS

2.1 PLANT MATERIAL

Above ground parts (leaves and flowers/inflorescences) of *N. glocephalata* rech.f. were collected from natural sites of Kashan, Esfahan Province, at an altitude of 1600 m and the above ground parts of *N. ispahanica* Boiss. were collected from north-west of Tehran, at an altitude of 1800 m during the flowering period in July 2015 in Iran. The air-dried of the plant were powdered and hydrodistillated in a Clevenger-type apparatus for 3 h. The essential oils were dried over anhydrous sodium sulphate and stored at 3 °C in a dark before analysis.

2.2 GC AND GC/MS ANALYSES

The oils were analyzed by GC and GC/MS. The GC analyses were performed using a Perkin-Elmer (UK) 8500 gas chromatograph equipped with Flame Ionization Detector (FID) and a DB-5 fused silica column (30 m × 0.25 mm, film thickness 0.25 µm .Oven temperature was held at 60 °C for 3 min and programmed to 275 °C at a rate of 3 °C/min; injector temperature (split: 1: 25) 250 °C; detector temperature, 280 °C; carrier gas, N2 at 12 psi**.** Varian 3700 chromatography equipped with a CP-

Sil5CB column (25 m×0.25 mm i.d., film thickness 0.39 µm) combined with a Varian MAT 44S, ionization energy 70ev. The carrier gas was He and injector temperature was 270 ^{0}C. Approximately, 0.1 µl of neat oil was injected under split condition (100:1) and the oven temperature was held at 60 ^{o}C for 5min., programmed at 5 ^{o}C min^{-1}. to 220 C and then holds at this temperature for 20 min.

2.3 IDENTIFICATION OF COMPONENTS

The compounds in the oil were identified by comparison of their retention indices (RI, HP-5) with those reported in the literature as well as by comparing their mass spectra with the Wiley GC–MS Library, Adams Library, Mass Finder 2.1 Library data, and published mass spectra data (McLafferty and Stauffer, 1989; Adams, 2007).

2.4 GERMINATION AND SEEDLING GROWTH BIOASSAY

Seeds of two monocotyledon weeds (barnyard grass (*Echinochloa crus-galli* (L.) Beauv) and canary grass (*Phalaris canariensis* L.)) and two dicotyledon weeds (redroot pigweed (*Amaranthus retroflexus* L.) and lambsquarters (*Chenopodium album* L.)) were collected from weeds growing in the summer crops. The germination tests were done in petri dishes (9 cm dia) in a germination chamber at 30 °C (day) and 20 °C (night) for barnyard grass, canary grass and redroot pigweed and at 20 °C and 10 °C for canary grass, respectively. For each essential oil, an oil-in-water emulsion was prepared at 1, 2, 4 and 8 µg ml^{-1} concentrations. Distillated water used as control. Each Petri dish contained 25 weed seeds placed on two layers of filter paper (Whatman® No.5) wetted with 6 ml oil-in-water emulsion. To prevent evaporation, petri dishes were sealed with parafilm (16). After 14 days, all germinated seeds were counted. Seeds showing root emergence (2 mm) were recorded as germinated. After 14 days no seed germinations were observed. The germination percentages were determined. Root and shoot length were measured by scientific ruler.

2.5 GLASS HOUSE STUDIES

In another experiments, the effects of *Nepeta* species oils on 3-week-old weed plants raised under controlled conditions in experimental glass house were studied. Plants of the four weed species were raised from the seeds in plastic pots 12-cm in diameter. Pots were filled with 730 g garden soil (soil: sand: manure: 3:1:1, w/w) and ten seeds of each weed species were sown per pot. Pots were thinned to 5 equal-sized healthy plants per pot at one-week after sowing. Plants were watered every other day. Studied treatments in this experiment were 1.25, 2.5, 5 and 10 % (v/v) solution of essential oil or distilled water (control) at 3-week-old plants. A hand pressure sprayer filled with flooding nozzle was used for spraying at a rate of 400 l ha−1. The weed plants were examined for visible injury levels in terms of percent chlorotic and necrotic areas at 7-days after spray (DAS). Fresh leaves of all weed species (100 mg fresh leaf samples) were homogenized in 80 % aqueous acetone (5 ml). The homogenate was filtered through Whatman filter paper no. 1. The final volume was adjusted to 5 ml by acetone (80 %). Chlorophyll a and chlorophyll b contents were determined spectrophotometrically using Unico 1200-Spectrophotometer at 663 nm for chlorophyll a and 647 nm for chlorophyll b. Calculations were completed using Lichtenthaler's equation (Lichtenthaler, 1987) and expressed as mg g^{-1} dry mass. Also dry mass of plant were measured after were oven-drying at 750 ^{0}C for 48 h.

2.6 STATISTICAL ANALYSIS

All the experiments were repeated and the presented data are average of the two experiments. The experimental design used for both experiments was completely randomized in a 5 x 2 factorial scheme (5 concentrations × 2 *Nepeta* essential oils), with four replications. ANOVA was used to test for significant differences between the means of each *Nepeta* species and each essential oil concentration. For all statistical analysis, the SAS ver 9.1 program was used. The means were compared by Tukey's HSD post hoc test ($p < 0.05$).

3 RESULTS

3.1 CHEMICAL COMPOSITIONS OF THE EXAMINED ESSENTIAL

The chemical compositions of the two *Nepeta* essential oils compounds were listed in Table 1. Total of 35 compounds were identified in *N. ispahanica* and *N. glocephalata* essential oils by GC/MS analysis. Eighteen components were identified, representing more than 96.1 % of the total oil components of *N. ispahanica* essential oil detected. The major components of *N. ispahanica* oil were 1,8-cineole (66.4 %), β-pinene (10.7 %) and α-pinene (3.1 %). Twenty-nine compounds reached 98.6 % of the total *N. glocephalata* es-

sential oils. The main components of *N. glocephalata* oil were 1,8-cineole (34.1 %), β-pinene (21.5 %), α-pinene (8.1 %) sabinene (7.8 %), (Z)-β-ocimene (7.6 %) and (E)-β-ocimene (6.9 %). Other components were present in amounts less than 3 %.

Table 1: Percentage composition of the essential oils of *Nepeta* species

			%	
No	Compound	IR	N. ispahanica	N. glocephalata
1	α-Thujene	935	-	0.8
2	α-Pinene	940	3.1	8.1
3	Camphene	954	-	0.2
4	Sabinene	981	1.9	6.6
5	β-Pinene	986	10.7	21.5
6	Myrcene	998	-	1.7
7	δ-3-Carene	1011	-	0.5
8	α-Terpinene	1024	-	0.2
9	*p*-Cymene	1034	-	0.8
10	1,8-Cineole	1041	66.4	34.1
11	(Z)-β-Ocimene	1046	-	7.1
12	(E)-β-Ocimene	1056	-	6.5
13	γ-Terpinene	1066	-	0.3
14	*trans*-Sabinene-hydrate	1075	0.4	0.8
15	*cis*-Sabinene hydrate	1088	0.4	-
16	Tepinolene	1095	-	0.3
17	Linalool	1107	-	0.4
18	*trans*-Pinocarveole	1129	1.1	-
19	*cis*-p-menth-2-en-1-ol	1131	-	0.2
20	Verbenol	1134	0.6	-
21	Allo-ocimene	1137	-	0.2
22	*trans*-Sabinole	1149	-	0.5
23	Pinovarvone	1172	0.9	0.2
24	Myrtenal	1175	1.0	-
25	δ-Terpineole	1177	1.1	0.5
26	Myrtenol	1184	1.0	-
27	Terpinen-4-ol	1187	1.0	1.8
28	Cryptone	1196	-	0.2
29	α-Terpineole	1200	2.0	2.9
30	Myrthanol	1207	-	0.5
31	4aα,7α,7aα-Nepetalactone	1422	0.1	-
32	β-caryophyllene	1434	0.2	0.1
33	Germacrene D	1496	-	1.2
34	Bicyclogermacrene	1512	-	0.3
35	4aβ,7α,7aα-Nepetalactone	1575	2.1	-
36	β-caryophyllene oxide	1585	2.1	-
37	Spathulenole	1595	-	0.1
Total		-	96.1	98.6

Retention Indices (The retention indices were determined on CPSil5CB column)

3.2 GERMINATION AND SEEEDLING GROWTH BIOASSAY

The effect of *Nepeta* species essential oils against seed germination, root length and shoot length of barnyard grass, canary grass, redroot pigweed and lambsquarters is shown in Figs. 1–3. Significant differences were found among control and all concentrations of *Nepeta* species essential oil tested. The essential oils of two *Nepeta* species reduced the germination of all studied weeds. Furthermore the difference between the control and the lowest concentration was significant for all weed species. At 1 µg ml^{-1} of *N. ispahanica* germination reduction compare to control was 25 %, 11 % and 44 % and 49 % for barnyard grass, canary grass, redroot pigweed and lambsqaurters, respectively. Also germination reduction of barnyard grass, canary grass, redroot pigweed and lambsqaurters was 21.5 %, 8 % and 29 % and 44.75 % at 1 µg ml^{-1} of *N. glocephalata*, respectively (Fig. 1). Redroot pigweed and lambsquarters were most sensitive to *N. ispahanica* essential oil, their germination was completely inhibited by it at concentration 4 µg ml^{-1}. At highest concentration 8 µg ml^{-1} germination percentages were 6.5 % and 10.20 % for canary grass by *N. ispahanica* and *N. glocephalata*, respectively. Barnyard grass seeds germinated 3 % and 11.75 % at highest concentration of *N. ispahanica* and *N. glocephalata* essential oils, respectively. The regression lines between seed germination and essential oil concentrations confirm the different susceptibility of weed species (Fig. 1). There were significant differences among control and all concentrations, and between two *Nepeta* species essential oils for each weed species. The regression analysis between oil concentrations and root length showed that increasing concentration of essential oil increased the inhibitory effects on weed root length till a lethal dose (Fig. 2). When the root length of redroot pigweed and lambsqaurters was completely inhibited by essential oils of *N. ispahanica* at 4 µg ml^{-1}, the root length was reduced respectively to 70 % for barnyard grass and to 73 % for canary grass, which explain that monocots weeds were more resistant than dicots. No significant differences among control and all concentrations, and between two *Nepeta* essential oils observed for shoot length of barnyard grass and canary grass. The shoot reduction of barnyard grass compared with control was the 29 % and 28 % with *N. glocephalata* and *N. ispahanica* essential oils at the highest concentration, respectively. Canary grass showed a reduction of the 28 % with *N. glocephalata* and 25 % with *N. ispahanica* essential oils at the same concentration. Shoot length of redroot pigweed and lambsqaurters reached to zero at 4 and 8 µg ml^{-1} of *N. ispahanica* at 8 µg ml^{-1} of *N. glocephalata*.

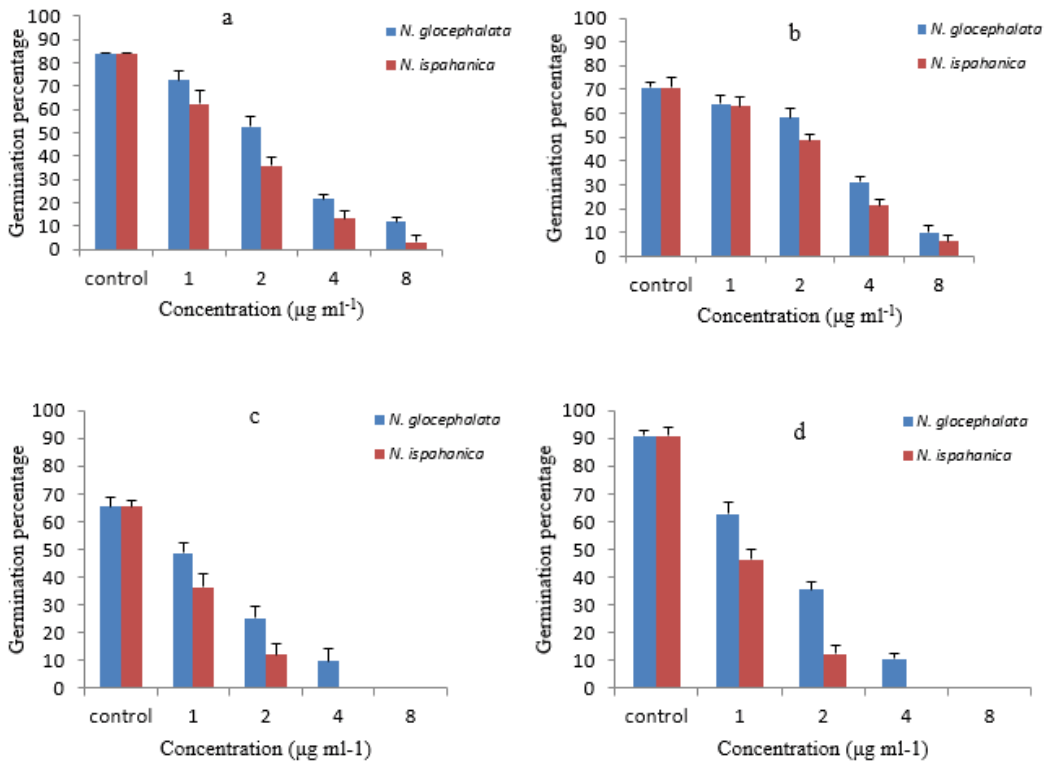

Figure 1: Effect of *N. ispahanica* and *N. glocephalata* essential oils on (a) barnyard grass, canary grass (b) redroot pigweed (c) and lambsqaurters (d) germination measured after 2 weeks. Vertical bars along each data point represent the standard error

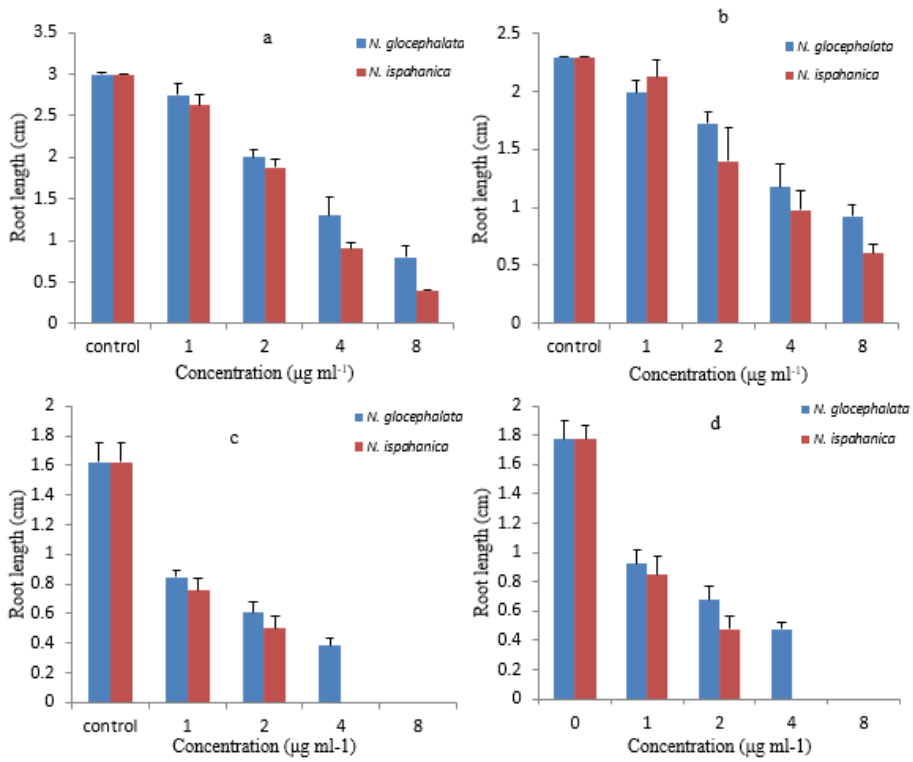

Figure 2: Effect of *N. ispahanica* and *N. glocephalata* essential oils on (a) barnyard grass, canary grass (b) redroot pigweed (c) and lambsqaurters (d) root length measured after 2weeks. Vertical bars along each data point represent the standard error

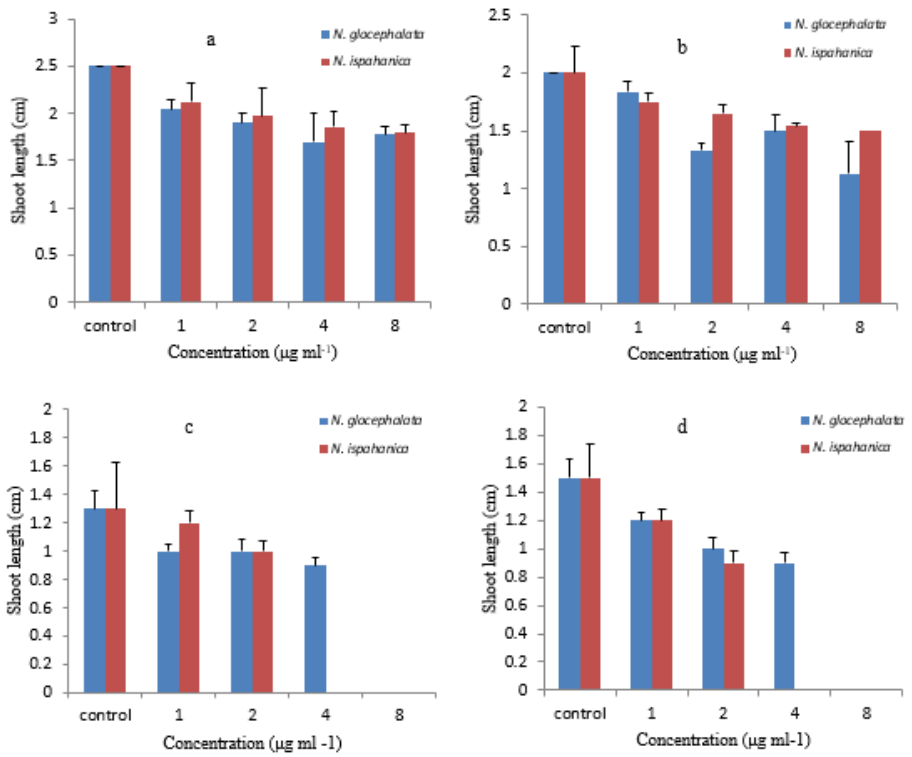

Figure 3: Effect of *N. ispahanica* and *N. glocephalata* essential oils on (a) barnyard grass, canary grass (b) redroot pigweed (c) and lambsqaurters (d) shoot length measured after 2 weeks. Vertical bars along each data point represent the standard error

3.3 GLASS HOUSE STUDIES

For more investigation of herbicidal activity of *Nepeta* essential oils, an experiment was done on 3-week-old weeds. The mature plants of test weeds were damaged upon spray of *Nepeta* essential oils and showed visible injury ranging from chlorosis to necrosis of plants. In general, the visible injury symptoms observed 7 days after spraying increased with increasing concentrations of both *Nepeta* essential oils (Table 2). At the lowest concentration 1.25 % of both *Nepeta* essential oils, all the test weeds showed sign of injury. At the highest concentration (10 v/v) visible injury by *N. ispahanica* essential oil were 44 % 45.5 % 59.25 % and 51.62 % for barnyard grass, canary grass, redroot pigweed and lambsquarters, respectively. While visible injury of barnyard grass, canary grass, redroot pigweed and lambsquarters caused by *N. glocephalata* essential oil were 39 % 36.62 % 44 % and 41.5 %, respectively at 7 days after spraying that did not have significant difference each other's (Table 2).

Increasing *Nepeta* essential oils concentration decreased dry mass of all weed species. The inhibition rates of barnyard grass dry mass ranged from 24.89 % to 75.21 %, and from 16.75 to 61.17 % in *N. ispahanica and N. glocephalata*, respectively. In canary grass the inhibition rates of dry mass, ranged from 22.49 to 63.26 %, and from 13.95 to 56.56 % at concentrations for *N. ispahanica and N. glocephalata*, respectively. Essential oil of *N. ispahanica* caused dry mass inhibition of redroot pigweed from 37.5 % to 90 % while *N. glocephalata* reduced it from 23.75 % to 81.5 %. The inhibition rates of lambsquarters dry mass ranged from 19.75 % to 86 %, and from 14.75 to 76 % in *N. ispahanica and N. glocephalata*, respectively. There was a significant difference in the inhibition of dry mass among concentrations and the the highest inhibition of dry mass caused by *N. ispahanica* at concentration of 10 v/v that was significantly different from other treatments.

Increasing essential oil concentration decreased chlorophyll a and chlorophyll b at all studied weeds. For example contents of chlorophyll a in barnyard grass were reduced 12.77 %, 25.40 %, 44.38 % and 49.62 % by *N. ispahanica* at concentrations of 1.25 %, 2.5 %, 5 % and 10 % v/v, respectively. *N. glocephalata* concentrations of 1.25 %, 2.5 %, 5 % and 10 % inhibited contents chlorophyll a in barnyard grass, 11.65 %, 15.56 %, 29.1 % and 43.56 %, respectively that the difference between the concentrations of 1.25 v/v and 2.5 v/v was not significant. The highest inhibition of chlorophyll a in canary grass was caused by *N. ispahanica* at concentration of 10 v/v which was not significantly different from *N. glocephalata.* In redroot pigweed, *N. ispahanica* at the concentration of 1.25 v/v decreased chlorophyll a by 22.05 % and decreased further by increasing concentration. In lambsquarters, no significant difference was observed between *N. ispahanica* and *N. glocephalata* at a concentration of 2.5 v/v (Table 4).

N. ispahanica essential oil inhibited chlorophyll b of barnyard grass by 11.30 %, 21.59 %, 29.13 % and 36.79 % at concentrations of 1.25 %, 2.5 %, 5 % and 10 %, respectively. At concentrations of 1.25 %, 2.5 %, 5 % and 10 % *N. glocephalata* essential oil reduced chlorophyll b by 7.86 %, 12.09 %, 22.21 % and 33.35 %, respectively in barnyard grass. The highest of inhibition of chlorophyll b in canary grass and redroot pigweed was caused by *N. ispahanica* at a concentration of 10 v/v which was not significantly different from *N. glocephalata* in redroot pigweed. In lambsqarters, there was no significant difference between *N. ispahanica* and *N. glocephalata* at a concentration of 1.25 v/v, but at the highest concentration, a significant difference was observed between these two species (Table 5). In this studies chlorophyll a decreased more than chlorophyll b in all species weeds (Tables 4 and 5).

4 DISCUSSION

Many researchers reported the presence of nepetalactones in several *Nepeta* species in relatively high concentrations (Sefidkon and Shaabani, 2004; Rustaiyan et al., 2000, Rustaiyan and Nadji, 1999, Sajjadi and Khatamsaz, 2000) but no nepetalactones were detected in *N. glocephalata* essential oil. 1, 8-cineole, which was the first major component of the studied oils, has been reported in the oil of some *Nepeta* species from Iran (Rustaiyan et al., 2000; Rustaiyan and Nadji, 1999; Sajjadi and Khatamsaz, 2000). 1, 8-Cineole was also reported previously to be the main compound of *N. ispahanica* oil (Sefidkon et al., 2005). β-pinene has also been found in the oils of some *Nepeta* species (Thappa et al., 2001; Baser et al., 2000; Rustaiyan et al., 2000; Rustaiyan and Nadji, 1999; Sefidkon et al., 2002) but the concentrations of it found in this study was the most in comparison with previous studies. β-pinene and α-pinene are typical in most *Nepeta* species (Gkinis et al., 2003; Thappa et al., 2001, Baser et al., 2000; Rustaiyan and Nadji, 1999; Sefidkon et al., 2002).

The herbicidal activity of both *Nepeta* essential oils were due to the high percentage of 1,8-Cineole. This is in agreement with Zunino and Zygadlo (2004) who reported that monoterpenes such as 1,8- cineole, thymol, geraniol and camphor have been reported to inhibit root growth in maize (*Zea mays* L.). In a study with 27 monoterpenes, against seed germination and primary root growth of radish (*Raphanus sativus* L.) and garden cress (*Lepidium sativum* L.), only 1, 8-cineole, inhibited

Table 2: Effects of *Nepeta* essential oils on visible injury of barnyard grass, canary grass, redroot pigweed and lambsquarters at 7 days after spraying

	Barnyard grass		Canary grass		Redroot pigweed		Lambsquarters	
Concentration (v/v)	*N. ispahanica*	*N. glocephalata*	*N. ispahanica*	*N. glocephalata*	*N. ispahanica*	*N. glocephalata*	*N. ispahanica*	*N. glocephalata*
1.25	19 ± 1.63 d	13.75 ± 1.89 e	17.87 ± 2.01 d	10.97 ± 0.41 e	21.5 ± 3.76 e	11.125 ± 1.10 f	20.25 ± 2.28 d	12.43 ± 0.47 e
2.5	32 ± 2.16 c	19.5 ± 0.57 d	28.01 ± 1.31 c	20.01 ± 1.07 d	31.5 ± 1.08 cd	25.875 ± 2.78 de	31.75 ± 1.48 c	22.68 ± 1.21 d
5	40.75 ± 0.9 ab	29.25 ± 0.95 c	37.28 ± 0.4 b	29.01 ± 0.81 c	43.75 ± 1.04 b	36.5 ± 1.22 c	42.25 ± 0.45 b	32.87 ± 0.93 c
10	44 ± 3.36 a	39 ± 1.82 b	45.55 ± 2.65 a	36.6 ± 2.47 b	59.25 ± 6.7 a	44 ± 2.61 b	51.62 ± 3.01 a	41.5 ± 0.54 b

Values are means ±standard error of four replicates. Within each species, different letters indicate that means are different at the 95 % level of probability (Tukey's HSD post hoc test)s

Table 3: Effects of *Nepeta* essential oils on dry weights inhibition % of barnyard grass, canary grass, redroot pigweed and lambsquarters at 7 days after spraying

	Barnyard grass		Canary grass		Redroot pigweed		Lambsquarters	
Concentration (v/v)	*N.ispahanica*	*N.glocephalata*	*N. ispahanica*	*N.glocephalata*	*N.ispahanica*	*N.glocephalata*	*N.ispahanica*	*N.glocephalata*
1.25	24.89 ± 1.67 g	16.74 ± 0.56 h	22.49 ± 4.53 f	13.95 ± 2.18 g	37.5 ± 1.73 f	23.75 ± 2.21 g	19.75 ± 2.5 f	14.75 ± 2.21 f
2.5	39.55 ± 1.84 e	28.58 ± 0.96 f	32.55 ± 2.23 d	22.15 ± 0.80 e	53 ± 2.16 e	39.25 ± 0.95 f	41 ± 2.58 d	29.5 ± 2.64 e
5	69.13 ± 1.70 c	51.74 ± 1.84 d	52.60 ± 7.06 b	43.95 ± 3.30 c	77.5 ± 2.08 c	63 ± 2.44 d	61.5 ± 3 c	59 ± 4.6 c
10	75.21 ± 0.61 a	67.17 ± 0.24 b	63.26 ± 4.52 a	56.56 ± 2.8 ab	90 ±01 a	81.5 ± 1.29 b	86 ± 1.41 a	76 ± 1.82 b

Values are means ±standard error of four replicates. Within each species, different letters indicate that means are different at the 95 % level of probability (Tukey's HSD post hoc test)

Table 4: Effects of *Nepeta* essential oils on Chlorophyll a inhibition% of barnyard grass, canary grass, redroot pigweed and lambsquarters at 7 days after sprayingg

	Barnyard grass		Canary grass		Redroot pigweed		Lambsquarters	
Concentration (v/v)	*N. ispahanica*	*N.glocephalata*	*N. ispahanica*	*N. glocephalata*	*N. ispahanica*	*N. glocephalata*	*N. ispahanica*	*N.glocephalata*
1.25	12.77 ± 6.79c	11.65 ± 0.55c	13.80 ± 3g	8.71 ± 1.36g	22.05 ± 1.02d	14.47 ± 1.45f	20.27 ± 5.85cd	11.65 ± 0.51e
2.5	25.40 ± 1.31b	15.56 ± 3.57c	31.59 ± 5.92ef	19.09 ± 2.43e	37.80 ± 1.97d	26.47 ± 5.13e	32.90 ± 5.74 c	27.56 ± 0.69 c
5	44.38 ± 5.0a	29.1 ± 5.74b	39.13 ± 4.41bc	32.21 ± 1.33cd	52.47 ± 2.17b	44.69 ± 3.78c	54.38 ± 5.72 a	41.6 ± 4.53 b
10	49.62 ± 2.5a	43.56 ± 2.43a	46.79 ± 3.57a	41.60 ± 1.77ab	59.32 ± 1.0a	53.07 ± 1.85b	59.62 ± 2.54 a	54.81 ± 0.56 a

Values are means ±standard error of four replicates. Within each species, different letters indicate that means are different at the 95 % level of probability (Tukey's HSD post hoc test)

Table 5: Effects of *Nepeta* essential oils on Chlorophyll b inhibition % of barnyard grass, canary grass, redroot pigweed and lambsquarters at 7 days after spraying

	Barnyard grass		Canary grass		Redroot pigweed		Lambsquarters	
Concentration (v/v)	*N. ispahanica*	*N.glocephalata*	*N. ispahanica*	*N. locephalata*	*N. ispahanica*	*N. glocephalata*	*N.ispahanica*	*N.glocephalata*
1.25	11.30 ± 2.20c	7.86 ± 2.12cd	17 ± 2.37d	8.937 ± 1.46e	19.54 ± 3.42ef	15.11 ± 4.87f	15.43 ± 1.25d	11.04 ± 1.20e
2.5	21.59 ± 5.93b	12.09 ± 5.99c	28.75 ± 1.49c	20.68 ± 1.91d	29.63 ± 2.71cd	23.52 ± 2.52de	25.80 ± 1.38c	18.91 ± 3.19d
5	29.13 ± 4.41ab	22.21 ± 1.34b	39.25 ± 0.46ab	25.87 ± 2.13c	38.27 ± 2.99ab	33.18 ± 1.11bc	32.90 ± 1.73b	26.21 ± 0.48c
10	36.79 ± 3.58a	33.35 ± 2.88a	42.12 ± 2.21a	37 ± 3.36b	45.11 ± 4.2a	40 ± 2.37ab	40.50 ± 2.60a	37.34 ± 2.35a

Values are means ±standard error of four replicates. Within each species, different letters indicate that means are different at the 95 % level of probability (Tukey's HSD post hoc test)

their root elongation at the lowest concentrations (10^{-5} M, 10^{-6} M) applied (De Martino et al., 2010). Romagni et al. (2000) have shown that 1, 8-cineole, and its natural analogue 1, 4-cineole, both suppress the growth of several weeds. 1, 8-cineole inhibits the germination, speed of germination, seedling growth, chlorophyll content and respiratory activity of *Ageratum conyzoides* L. 1753 not Hieron. 1895 nor Sieber ex Steud. 1840. Singh et al. (2002) and De Feo et al. (2002) have investigated the herbicidal activity of 10 volatiles compounds from *Ruta graveolens* L. essential oils and showed that 1,8-cineole significantly inhibits the germination and radical elongation of radish.

The effects of the allelochemicals in studied traits directly dependent on the concentration and *Nepeta* species. The germination and root length decreased with increasing concentrations of essential oils. These results are in agreement with that of Ibáñez and Blázquez (2017) who reported that there are significant effects in shoot and/or shoot + root length of weeds depending on the weed and dose. *N. ispahanica* oil exerted the more inhibitory effect than *N. glocephalata* for all weed species. It can be due to higher concentration of 1,8-cineole in *N. ispahanica*. While the inhibition is not similar between the *Nepeta* species oils, weed species differed in their response to the toxic effect of each oil. It was reported that the degree of allelopathic interference can even vary within species (Li et al. 2009).

My observations in glass house indicated that both *Nepeta* oils can act as contact herbicides. These observations are in agreement with previous studies showing that volatile oils and even their monoterpenes exhibit herbicidal activity (Tworkoski, 2002; Singh et al., 2005, 2006). Batish et al. (2004, 2007) concluded that the 5 % essential oil from *E. citriodora* caused 50–80 % visible injury in *A. viridis*, *P. minor* and *E. crus-galli*. In addition Poonpaiboonpipat et al. (2013) reported that the essential oil lemon grass (*Cymbopogon citratus* (DC ex Nees) Stopf) applied on barnyardgrass in greenhouse caused leaf wilting. The reduction in seedling dry mass, chlorophyll a and chlorophyll b content observed in my study is in agreement with previous reports indicating that the monoterpenes had a potential to reduce chlorophyll content (Chowhan et al. 2011; Kaur et al. 2010; Gouda et al., 2016). It may be due to inhibition of biosynthesis of chlorophyll and/or degradation of chlorophyll.

5 CONCLUSION

From the present study, it could be concluded that *Nepeta* essential oils strongly inhibited the germination and root length of all weeds. Dicot weeds (lambsquarters and redroot pigweed) were significantly more sensitive than monocot weeds (barnyard grass and canary grass). Indeed, at the dose of 4 μg ml^{-1}, germination of lambsquarters and redroot pigweed was totally inhibited by *N. ispahanica* essential oil. Further studies are required to investigate the herbicidal potential of *Nepeta* essential oils under field conditions and determine the effects on crop species and other weed species. This study is considered the first study regarding of herbicidal effects of *N. glocephalata and N. ispahanica* but the selectivity of these compounds should be considered.

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Evaluation of biochemical treatments applied in polluted soils irrigated with low quality water for long periods of time through the CO_2 efflux

Mohamed SABER [1], Alaa ZAGHLOUL [2, 3]

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Evaluation of biochemical treatments applied in polluted soils irrigated with low quality water for long periods of time through the CO_2 efflux

Abstract: To sightsee the bearings of the certain remediation amendments, usually applied in the bioremediation of soils irrigated with low quality water for extended periods on the indigenous microbial population, a greenhouse experiment was conducted at National Research Centre (NRC) where the soil ecosystem was supplied with varied mineral remediation amendments and the carbon dioxide (CO_2) refluxes were followed up. In this study, microbial activity through CO_2 efflux was taken as an indicator to evaluate the effectiveness of eight soil amendments in minimizing the hazards of inorganic pollutants in soil ecosystem irrigated with low quality water s for more than 40 years. Results showed that Ni and Zn were the most dominant contaminants that adversely influenced indigenous microbial activities in untreated soil, while Cu was the most persuasive. All trailed remediation amendments significantly minimized the hazards of inorganic pollutants in treated soil ecosystems. In addition, modified bentonite (Probentonite) was the best persuasive one. Mechanisms take place between trailed remediation amendments and inorganic pollutants in the studied soil ecosystems were discussed. In conclusion application of certain raw or modified clay minerals especially Probentonite could be a good tool in decreasing the rate of the studied inorganic pollutants in a contaminated soil ecosystem irrigated with low quality water for extended periods.

Key words: soil; low quality water; bioremediation; potential toxic elements; soil indigenous microbial Activities; modified clay minerals

Ovrednotenje biokemičnih obravnavanj onesnaženih tal, ki so bila dalj časa namakana z vodo slabe kakovosti z meritvijo sproščanja CO_2

Izvleček: Za prepoznavanje obremenitev, ki jih povzročajo nekateri remediacijski dodatki, ki se navadno uporabljajo pri bioremediaciji tal namakanih dalj časa z vodo slabe kakovosti na samoniklo mikrobno populacijo, je bil izveden poskus v rastlinjaku v nacionalnem raziskovalnem centru (NRC). Tlem so dodajali različne mineralne remediacijske dodatke in spremljali sproščanje ogljikovega dioksida (CO_2). Mikrobna aktivnost, izražena kot iztok CO_2, je služila kot indikator ovrednotenja učinkovitosti osmih talnih dodatkov, ki naj bi zmanjšali škodo, ki jo v talnem ekosistemu povzročajo anorganska onesnaževala iz vode za namakanje slabe kakovosti v obdobju več kot 40 let. Rezultati so pokazali, da sta bila Ni in Zn dominantna kontaminanta, ki sta negativno vplivala na aktivnost samoniklih mikrobov v obravnavanih tleh medtem, ko je bil učinek Cu največji. Vsi poskusi dodatkov v remediaciji so značilno zmanjšali tveganja poškodbe tal zaradi anorganskih polutantov v obravnavanih talnih ekosistemih. Pri tem je bil spremenjeni bentonit (Probentonite) najučinkovitejši. V raziskavi so interpretirani mehanizmi, ki potekajo med dodatki v remediaciji in anorganskimi onesnaževali v preučevanih talnih ekosistemih. Zaključek je, da je dodatek nekaterih osnovnih ali spremenjenih glinenih mineralov, še posebej probentonita lahko dobro sredstvo za zmanševanje onesnaženja z nekaterimi anorganskimi onesnaževali v onesnaženih ekosistemih, ki so bili namakani z vodo slabe kakovosti v daljšem obdobju.

Ključne besede: tla; voda slabe kakovosti; bioremediacija; potencialno toksični elementi; aktivnost samoniklih talnih mikrobov; spremenjeni glineni minerali

1 Department of Agricultural Soil Microbiology, National Research Centre, Dokki, Cairo, Egypt
2 Soils and Water Use Department, National Research Centre, Dokki, Cairo, Egypt
3 Corresponding author, e-mail: alaazaghloul2008@gmail.com

1 INTRODUCTION

Through low quality water farming varied organic and inorganic contaminates reach the soil together with enteric pathogens, and cause vital adverse agricultural, environmental and public health troubles**.** Biological pollutants include bacteria, viruses, and parasites that are responsible for waterborne diseases, such as typhoid fever, cholera, dysentery, polio, hepatitis, and schistosomiasis. The presence of *coliform* bacteria is indicator of recent fecal pollution, this type of contamination is exclusively attributed to human and animal waste. Also, in these materials inorganic pollutants include cations and anions, most of them naturally occurring in soils, sediments, and rocks. Cations include heavy metals, such as cadmium (Cd), chromium (Cr VI), lead (Pb), manganese (Mn), mercury (Hg), and nickel (Ni). These highly toxic chemicals may reach soil after mineral dissolution with low quality waters applied or from industrial activities or after industrial emissions (Saber et al., 2015).Although low quality water farming was always associated with potential benefits as well as with problems, yet appropriate practices to ensure its safe and effective sustainable use are not well developed (Doaa Ali et al, 2020). The role of microorganism on the health of soil ecosystem is incontestable. Since 1930 huge amounts of agrochemicals were applied to soil ecosystems and adversely impacted their biome activities, Khan and Scullion (2000) recorded a shift in bacterial to fungal population in soil ecosystems as a result of contamination with inorganic pollutants. Recently, one of the narrative concepts of soil health is bioremediation of contaminated soil ecosystems using varied remediation amendments, some of which with negative impacts on soil microbial activities (Kelly and Tate III, 1998). It is worthy to state that the influence of the inorganic pollutants on the respiration intensity in a soil ecosystem irrigated with low quality water for extended periods was somewhat inconsistent, Yonebayashi and Hattori (1989) and Doelman and Haanstra (1984) verified significant decreases in CO_2 evolution from such soils, while Bardgett and Saggar (1994) and Welp (1999), on the other hand, recorded high increases. Such phenomenon is linked with varied interferences.

The current study aimed at evaluating the delayed effect of a natural modified clay mineral fortified with certain remediation amendments on a soil irrigated with low quality water s for extended periods through their microbial activities represented by CO_2 evolution.

2 MATERIAL AND METHODS

2.1 STUDY SITE

A surface soil sample (0-30 cm), irrigated with low quality water for 40 years was collected from El-Rahawy farm, Giza governorate. The chemical characterization of the soil showed it to have pH, 6.83; EC, 0.2 dSm^{-1}; OM (organic matter), 0.2 %; clay content, 4.4 % and with a sandy texture. Determined inorganic pollutants in the tested soil were 18 ppm Ni; 35.65 ppm Cu; 400.6 ppm Zn; 596 ppm Fe; 45.19 Mn 57.7ppm Pb and the Zn equivalent parameter was 633.9.

2.2 EXPERIMENTAL DESIGN

In a completely randomized pot experiment with four replicates, single and combined mixtures of varied remediation inorganic amendments were trailed to retain PTE's from a contaminated soil ecosystem irrigated with low quality water for extended periods. The soil was treated with either 2 % probentonite (T1), 2 % kaolinite (T2), 1 % probentonite + 1 % Kaolinite (T3), 1 % probentonite + 1 % rock phosphate (RP) (T4), 1 % kaolinite + 1 % RP (T5), 1 % Bentonite + 0.5 % kaolinite + 0.5 % RP (T6), 2 % iron oxide (T7) and 1 % iron oxide + 1 % RP (T8). Treated and untreated control soils were moistened to 60 % of the soil water holding capacity and incubated for 60 days at 25 °C. At the end of incubation time (60 days), a kinetic study was carried out on treated and untreated soil ecosystems followed by a distribution study of inorganic pollutants studied.

2.3 INORGANIC POLLUTANTS INSTRUMENTATION AND ANALYSIS

A Perkin–Elmer flame atomic absorption spectrometer (FAAS) and HACH DR890 colorimeter was used in inorganic pollutants instrumentation and analyses. Atomic absorption measurements were carried out using air: acetylene flame while HACH colorimeter measurement with the provided test kits. The operating parameters for working elements were set of as recommended by the manufacturer.

2.4 POTENTIAL TOXIC ELEMENTS DISTRIBUTION ANALYSIS

Inorganic pollutants were fractionated to water soluble, exchangeable, (readily available form RA),

carbonate-bound, Fe-Mn oxides-bound ad organic-bound which was considered to be the residual fraction (Zaghloul, 2002). The soil quality criterion index (Zn equivalent model) was numerically expressed for the levels of PTE's toxicity as described by Saber et al. (2012). A quality criterion index for zinc equivalent over 250 units indicated a risky situation. Kinetic studies were carried out using the Electrical Stirred Flow Unit (ESFU) method.

2.5 CO_2 EFFLUX AND KINETIC EQUATION

CO_2 evolved during the incubation was trapped in 1 M NaOH, and the excess NaOH was titrated with 0.1 M HCl after the addition of $BaCl_2$. Total CO_2 mineralized was calculated as cumulative CO_2 evolution (Leifeld et al., 2002). The specific respiration activity (qCO_2) was expressed as the production of CO_2-C per unit biomass C and time.

Kinetic equations**:** The following four kinetic equations representing both empirical and theoretical equations were used to test the conformity of the CO_2 release data to each of them.

2.5.1 First order equation

$$Log\ (q_j - q_t) = Log\ q_0 - k_1\ t$$

where:

q_j = the maximum amount of CO_2 release

q_0 = the initial amount of CO_2 at the time of added the resin.

q_t = the amount of CO_2 release at time t.

t = time in minute

k_1 = the rate constant of reaction n in sec^{-1}

2.5.2 Elovich équation

$$q = (1/b) \ln (ab) + (1/b) \ln t$$

where:

q = amount of CO_2 desorbed at time t

a = constant in ppm CO_2 min^{-1}

b = constant in (ppm CO_2) $^{-1}$

t = time in minute

2.5.3 Modified Freundlich equation

$$q = k_d\ t^{b\backslash}$$

where:

q = amount of CO_2 desorbed in time t

k_d = desorption rate coefficient in mg CO_2 kg^{-1} soil min^{-1}

$b\backslash$ = constant in mg CO_2 kg^{-1} soil

2.6 SOIL MICROBIAL BIOMASS

Soil microbial biomas was measured by the fumigation–extraction method after 24, 72, 96, 168, 240 and 336 hours. Three replicates of each treatment were fumigated with ethanol-free chloroform for 24 h at 25 °C. The soil samples were then extracted with 0.5 M K_2SO_4 for 30 min. Three replicates of non-fumigated soil samples were extracted similarly. The extracted PTE's were determined by dichromate oxidation at 100 °C (2 ml of extract, 1.5 ml of 15 M H_2SO_4 and¶ 1.5 ml of saturated aqueous solution of $K_2Cr_2O_7$). The residual $K_2Cr_2O_7$ was determined by photometrical at 565 nm (Kuzyakov, 1997). The calibration of the extracted C (carbon) measurements was carried using glucose. CO_2 evolved during incubation was trapped in 1 M NaOH, and the excess NaOH was titrated with 0.1 M HCl after addition of $BaCl_2$ (USEPA. 2001). Total mineralized C was calculated as cumulative CO_2 evolution (Leifeld et al., 2002). The specific respiration activity (qCO_2) was expressed as the production of CO_2-C per unit biomass C and time (Anvar and Oliver Dilly, 2002).

2.7 STATISTICAL ANALYSIS

Multiple linear regressions, discriminant analysis and the fitting of curves to the data were performed using separate two-way ANOVAs. The data of biomass inorganic pollutants and soil organic C were analyzed by discriminate analysis. Statistical analyses aimed to examine the succession of the applied remediation amendments in returning the studied contaminated soil ecosystem to its normal settings. SAS software was used to evaluate the kinetic models that describe CO_2 efflux under the action of the used different remediation amendments.

3 RESULTS AND DISCUSSION

3.1 KINETICS OF CO_2 EFFLUX IN BIO-REMEDIATED SOIL ECOSYSTEM

Results drawn in Figure 1 demonstrate the kinetics of CO_2 efflux from both contaminated and bio-remediated soil ecosystems. All trailed remediation amendments

led to marked decreases in the rate of CO_2 efflux compared to control contaminated soil ecosystem. In control contaminated soil ecosystem, maximum CO_2 efflux reached 14.11 mg kg^{-1} soil; while being 10.45 mg kg^{-1} soil in T3 (soil fortified with a mixture of bentonite + kaolinite) and decreased to 8.2 in T8 (iron oxide + RP). Other trailed remediation amendments, thereafter decreased CO_2 efflux with values higher than in abovementioned treatments was recorded.

Chander and Brookes (1993), stated that when Zn and Cu are present together in the soil ecosystem the increase in Zn and Cu bioavailability above 123 or 3.0 ppm causes marked decreases in the intensity of soil microbial biomass.

Results given in Figure 2 show that the impacts of the trailed remediation amendments in minimizing the hazards of PTE's varied according to their type. Modified bentonite, iron oxide, rock phosphate as well as the mixture of these remediation amendments decreased significantly the evolution of CO_2 or restored the soil ecosystem to its normal conditions. Of all treatments T8, T3 and T7 were the best that condensed normal conditions, in some cases; however, certain remediation amendments increased the CO_2 to a non-significant level compared to control especially when fortified with kaolinite (T5) even in the absence of RP.

3.2 RATE CONSTANTS OF BEST FITTED MODELS DESCRIBE CO_2 EFFLUX AS AFFECTED BY REMEDIATION AMENDMENTS APPLIED TO SOIL ECOSYSTEM

Kinetic approach was used to evaluate the effectiveness of the trailed remediation amendments on CO_2 efflux that express the biomass activity as well as the depressing action of PTE's associated with the trailed remediation amendments.

As given in Tables 1-3, the rate constants of CO_2 efflux from the soil irrigated with low quality water for extended periods, as an indicator for microbial activity, varied according to type of the trailed remediation amendment. For MFE, the best fitted model, the application of kaolinite and RP (T5) decrease CO_2 efflux to 0.71 mg g^{-1} soil, in control the value was 0.77 mg g^{-1} min^{-1}. Fortification of the soil ecosystem irrigated with low quality water with iron oxide also decreased CO_2 efflux to 0.68 mg g^{-1} min^{-1}, and hence might be used to cure such contaminated soil ecosystem. Also, using bentonite in T1 decreased the CO_2 flux to 0.68 mg g^{-1} min^{-1}. All other trailed remediation amendments significantly decreased CO_2 efflux compared to control. The negative values recorded for the capacity factor in the same model means returning the soil ecosystem to optimum conditions for microbial activity.

3.3 MICROBIAL BIOMASS IN PTE'S CONTAMINATED AND BIO-REMEDIATED SOIL ECOSYSTEMS

Results given in Table 4 show the amount of contaminants absorbed by the microbial biomass in the soil ecosystem. In control microorganism's absorbed significant amounts of inorganic pollutants reached 0.46 ppm Zn, 0.03 Cu and 0.10 Ni. The existence and uptake of all inorganic pollutants directly led to an increase in the microbial activity in the soil ecosystem. For example, in T1 only Ni was absorbed by microorganisms, meanwhile other studied inorganic pollutants did not presented in side microorganisms.

The same trend was re-exhibited in T3, T5 and T7 treatments. Bentonite + kaolinite + rock phosphate (RP) treatment (T6) and iron oxide + RP treatment (T8) also increased the microbial activity through increasing CO_2 efflux of rate constants of Elovich kinetic model (Table 3). Results in the same table point to that bentonite as remediation amendment decreased inorganic pollutants uptake at different degrees with some exception observed in case of Ni.

Although the application of oxides as remediation amendment was extensively mentioned in literature, the application of iron oxides in this study enhanced Zn and Ni by microorganisms found in the soil ecosystem. It is worth to state that Cu was the only PTE that was depressed by the trailed remediation amendments though the increasing of non fumigated values compared to fumigated ones.

3.4 CONTRIBUTION OF TRAILED REMEDIATION AMENDMENTS IN SOIL ECOSYSTEMS ON MINIMIZING THE HAZARDS OF PTE'S

The effect of trailed remediation amendments on minimizing the hazards of Ni, Cu and Zn is drawn in Figure 3 that show all trailed treatments significantly decreased the available forms of the studied inorganic pollutants compared to control. The comparison between the different treatments indicated that bentonite significantly decreased the available form of PTE's. For instances, the application of modified bentonite to soil (T4) led to a decrease reaching 34 % of available of Cu, 79 % of available Zn and 77 % of available Ni.

The mixture of bentonite and kaolinite decreased 68 % of available Cu, 66 % of available Zn and 59 % of

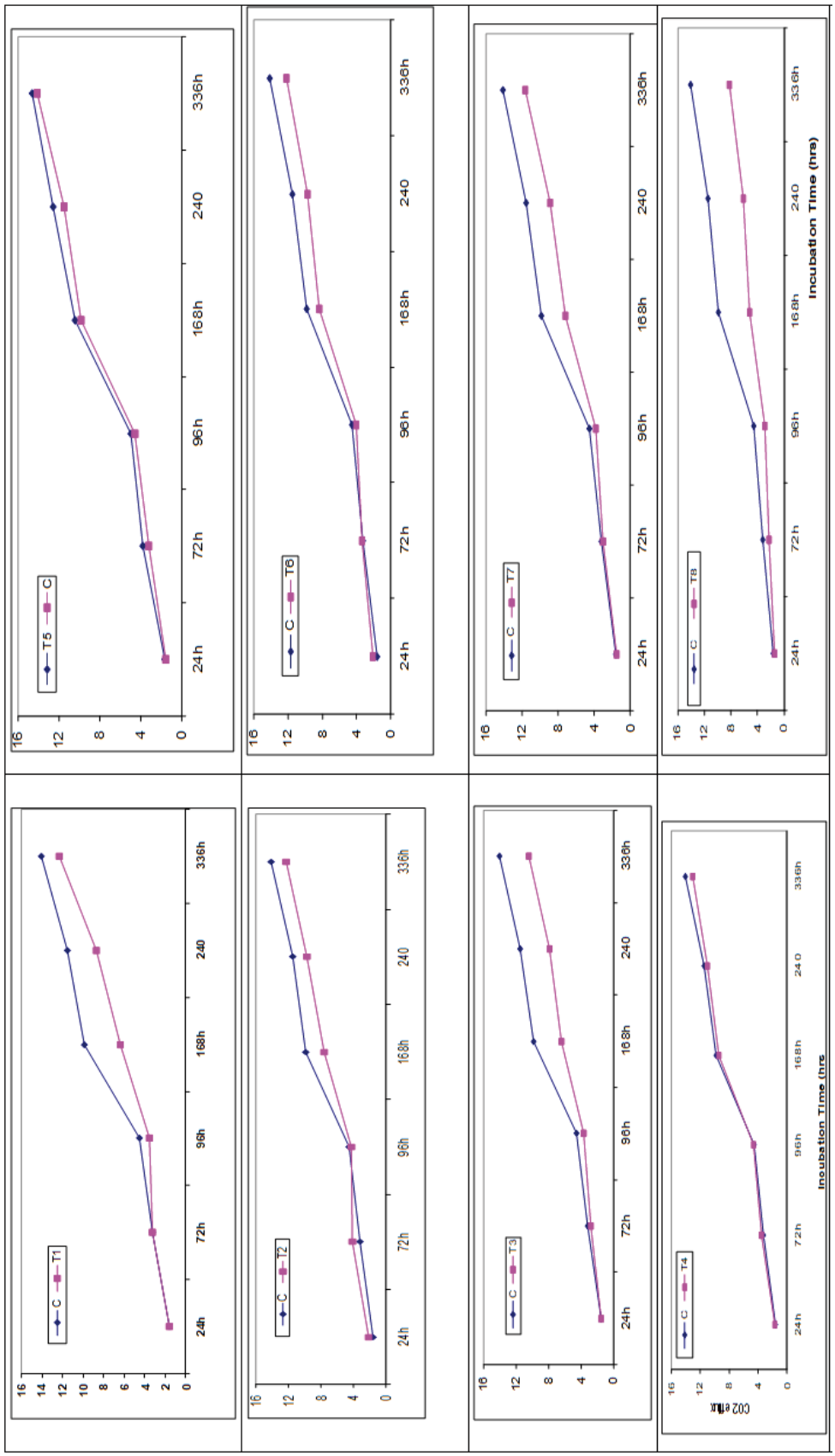

Figure 1: Kinetics of CO_2 efflux from contaminated soil as affected by remediation materials compared to control treatment. Where: (T1) 2 % probentonite, (T2) 2 % kaolinite, (T3) 1 % probentonite + 1 % Kaolinite, (T4) 1 % probentonite + 1 % rock phosphate (RP), (T5) 1 %kaolinite + 1 % RP, (T6) 1 % bentonite + 0.5 % kaolinite + 0.5 % RP, (T7) 2 % iron oxide and (T8) 1 % iron oxide + 1 % R

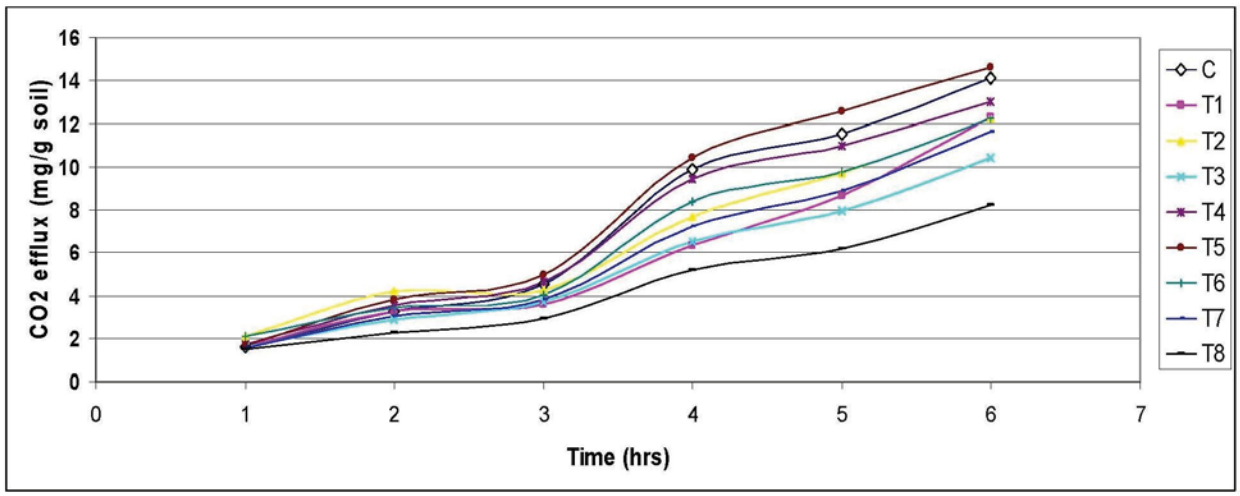

Figure 2: Map showing the experimental plots and location of the experiment at the Institute of Agricultural Research and Training in Ibadan, Oyo state, Nigeria. (T1) 2 % probentonite, (T2) 2 % kaolinite, (T3) 1 % probentonite + 1 % kaolinite, (T4) 1 % probentonite + 1 % rock phosphate (RP), (T5) 1 % kaolinite + 1 % RP, (T6) 1 % bentonite + 0.5 % kaolinite + 0.5 % RP, (T7) 2 % iron oxide and (T8) 1 % iron oxide + 1 % RP

Table 1: Rate constants of 1st order model describe CO_2 efflux from contaminated soil as affected by remediation treatments

Treatments	a*10^4	*b*	R^2	SE
cont	-5.51	11.58	0.99**	0.21
T1	-5.96	10.95	0.99**	0.40
T2	-5.12	9.62	0.99**	0.35
T3	-7.71	12.53	0.97**	0.81
T4	-8.95	14.36	0.99**	0.80
T5	-6.43	11.33	0.96**	0.73
T6	-5.94	11.00	0.99**	0.43
T7	-3.88	7.40	0.99**	0.36
T8	-5.51	11.58	0.98**	0.21

Table 2: Rate constants of MFE describe CO_2 efflux from contaminated soil as affected by remediation treatments

Treatments	a	b	R^2	SE
cont	0.77	2.29	0.99**	0.05
T1	0.68	-1.86	0.99**	0.04
T2	0.74	-2.17	0.99**	0.04
T3	0.72	-2.39	0.99**	0.05
T4	0.68	-2.56	0.99**	0.05
T5	0.71	-1.99	0.98**	0.07
T6	0.75	-2.29	0.99**	0.05
T7	0.68	-1.86	0.99**	0.04
T8	0.74	-2.17	0.99**	0.04

available Ni, this trend perhaps represents the selectivity of used clay minerals in retain PTE's. Results pointed to that increasing of Cu and Zn was retained by kaolinite over bentonite, meanwhile a reverse trend was observed in Ni. The modification of kaolinite with RP increased the retention of Ni by 64 % in the soil ecosystem; meanwhile it did not exceed 18 % under sole kaolinite application.

The same treatment, however, did not influence the retention level of Cu and Zn in the soil ecosystem. In contrast, fortification of the soil ecosystem with sole iron oxide decreased the retention of inorganic pollutants to 65, 72 and 45 % of Cu Zn and Ni respectively, while the mixture of PR with iron oxide decreased these values respectively to 50, 30 and 36 % of total form in soils. Results drawn in the same figure indicated that the mixture of all treatment (T6) did not exhibit the predicted trend since the decreasing orders of Inorganic pollutants did not exceed 34, 62 and 55 % of total Cu, Zn and Ni compared to control in order to be a valued treatment but not the best

3.5 DISTRIBUTION OF THE STUDIED PTE'S IN REMEDIATED SOIL ECOSYSTEM

Results drawn in Figure 4 exhibit the distribution of Ni, Cu and Zn in soil ecosystem irrigated with low qual-

ity water for long periods as affected by the trailed remediation amendments applied to minimize the hazards of PTE's and to optimize microbial activities through remediation of soil ecosystem. Generally, as shown in the Figure 4, trailing the different remediation amendments decreased the readily available form and increased the residual one with rates varied according to amendment used. Three main categories of the trailed remediation amendments are distinguished, the 1st category included the best ones, i.e., pro-bentonite (T4), pro-kaolinite (T5) and iron oxide (T7), which minimized the readily available form to zero in Ni and from 98 to 100 % for Cu and Zn, i.e., increased the residual form in these treatments. The 2nd category included the remediation amendments able to minimize the available form of one of the tested inorganic pollutants to zero such as T3 (the mixture between bentonite and kaolinite) in case of Cu. The 3rd category included the rest of treatments that significantly minimized inorganic pollutants at different rates according to the studied inorganic pollutants.

Table 3: Rate constants of Elovich equation describe CO_2 efflux from contaminated soil as affected by remediation treatments

Treatments	a	*b*	R2	SE
cont	3.85	-28.08	0.92**	1.77
T1	3.82	-27.09	0.95**	1.40
T2	3.34	-24.00	0.94**	1.25
T3	4.57	-33.12	0.95**	1.54
T4	5.24	-38.31	0.95**	1.76
T5	3.97	-28.46	0.94**	1.59
T6	3.82	-27.77	0.94**	1.44
T7	2.54	-18.10	0.93**	1.04
T8	3.85	-28.08	0.92**	1.77

It should be mention that all applied remediation amendments are locally available in Egypt and are considered with economic low coast. Various methods such as hydrometallurgical technologies, ion exchange, electro dialysis, reverse osmosis, precipitation and adsorption had been trailed to remove inorganic pollutants from aqueous solution phase in aquatic ecosystems (La Grega et al., 1994). It is well known that the reduction in the readily available forms of inorganic pollutants' in contaminated soil ecosystems is a commonly technique used to reduce the negative impacts of inorganic pollutants on environment and improve the quality of contaminated soil ecosystems (Zaghloul, 2006). It is well known that clay minerals interact with almost all soil contaminants (Prost and Yaron, 2001). The adsorption of Ni, Cd, Zn, and Pb by the clay mineral montmorillonite was reported by Schulthess and Huang (1990). Sorption technique is one of the most efficient methods of cleaning the environment from Inorganic pollutants.

In this study microbial activity through CO_2 efflux was used as an indicator to evaluate the effectiveness of eight remediation amendments in minimizing the hazards of PTE's in soil ecosystem. Bentonite is a well-known as one of the most effective remediation amendments

Table 4: Microbial biomass for Ni, Cu and Zn in both contaminated and remediated soils

Treatments	NF Zn	Fum. Zn	NF Cu	Fum Cu	NF Ni	Fum Ni
			ppm			
Control	2.65	3.11	1.24	1.27	1.80	10.9
T1	0.86	0.42	1.69	1.34	1.89	2.36
T2	2.00	0.99	0.82	0.71	2.3	1.61
T3	0.54	0.31	0.58	0.41	1.88	2.23
T4	6.53	4.85	1.21	1.08	2.04	2.22
T5	3.13	2.71	0.85	0.77	1.44	2.02
T6	0.3	0.44	0.96	0.52	1.66	2.36
T7	1.21	0.78	0.89	0.89	1.75	2.05
T8	3.29	4.39	0.88	0.78	1.89	2.71

NF: non-fumigated, Fum: Fumigated

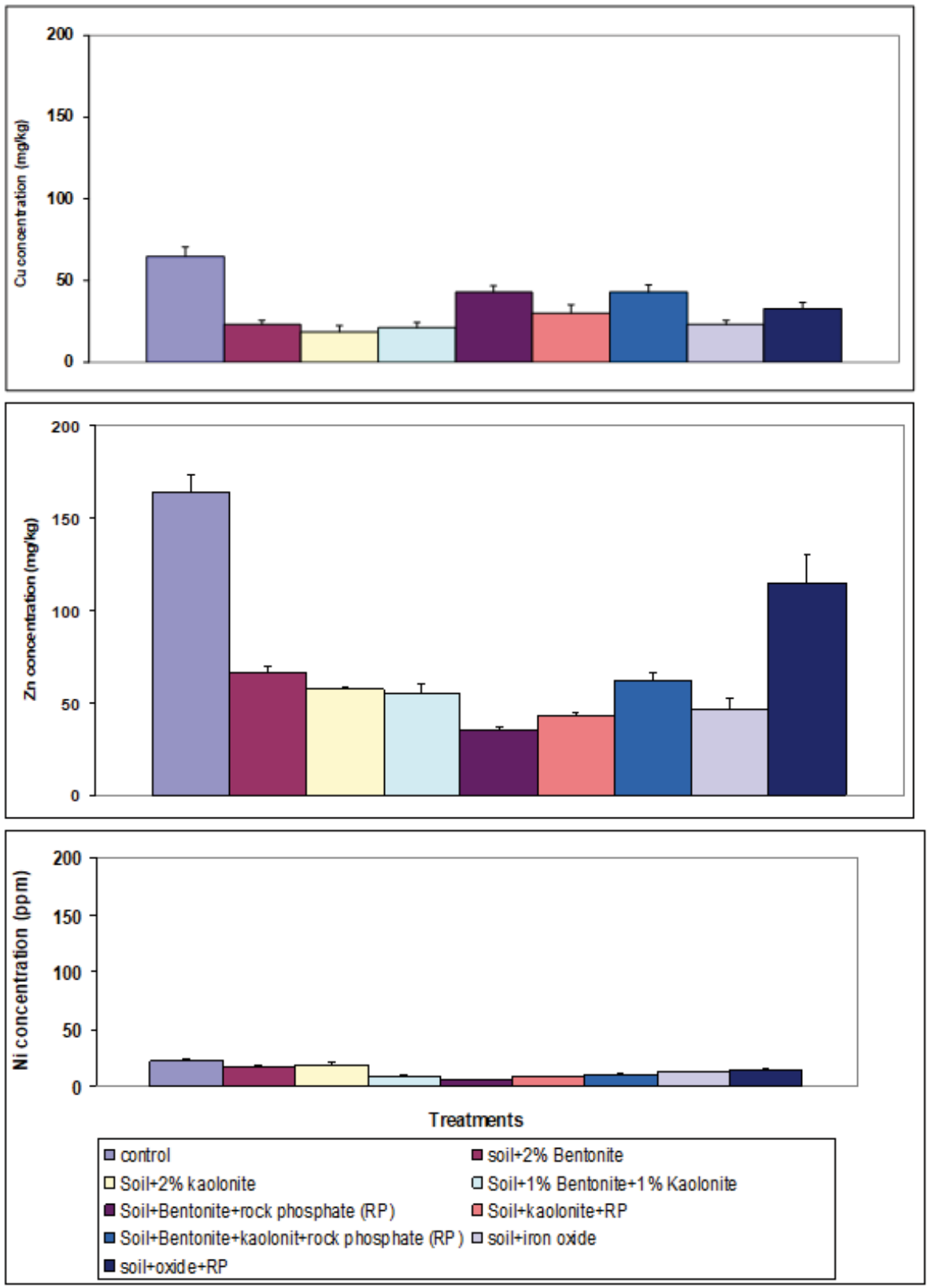

Figure 3: Potential toxic elements availability in contaminated as affected by remediation materials compared to control treatments

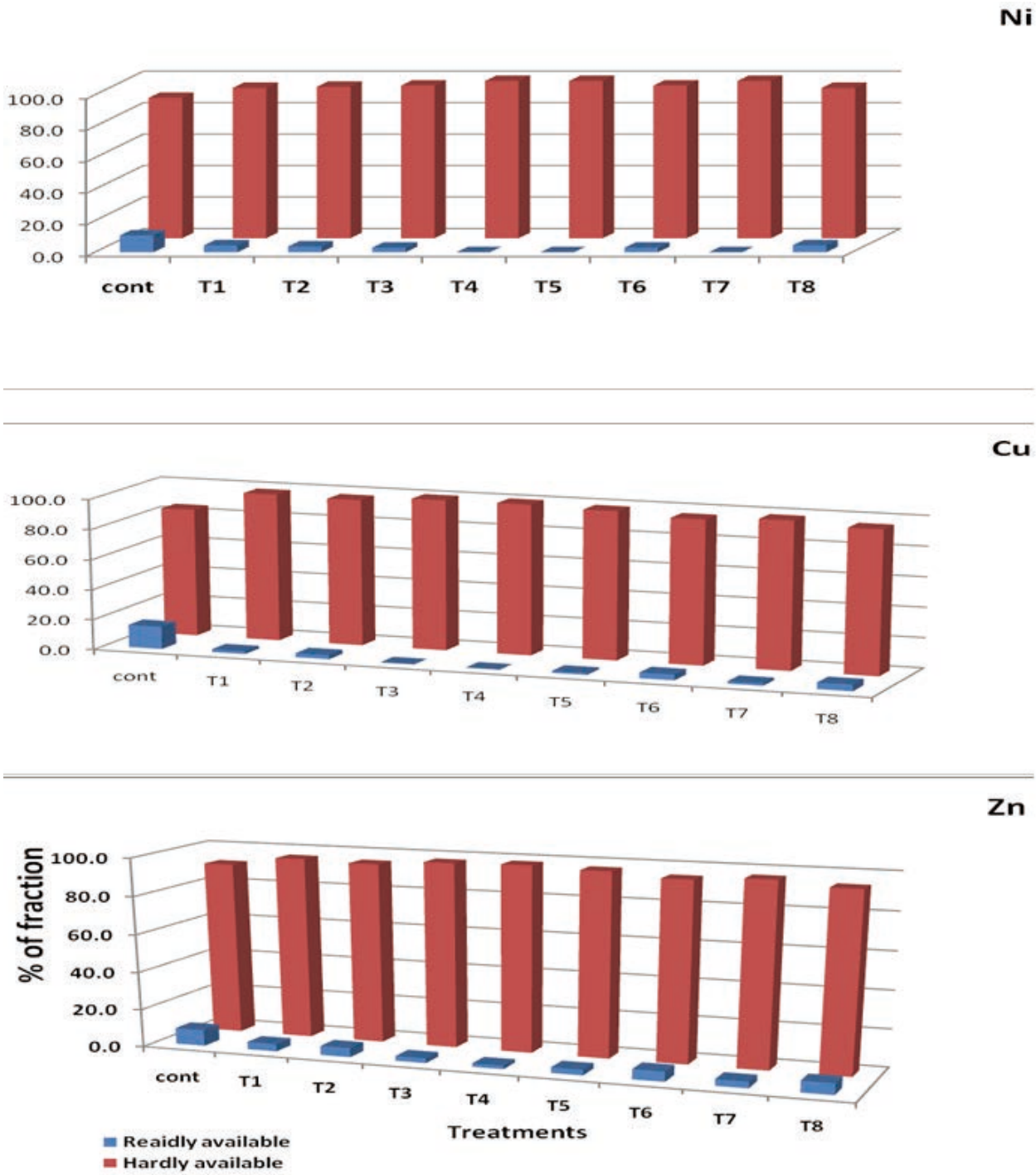

Figure 4: Distribution of PTE's in the studied low quality water soils as affcted by certain remediative amendments. (T1) 2 % probentonite, (T2) 2 % kaolinite, (T3) 1 % probentonite + 1 % kaolinite, (T4) 1 % probentonite + 1 % rock phosphate (RP), (T5) 1 % kaolinite + 1 % RP, (T6) 1 % bentonite + 0.5 % kaolinite + 0.5 % RP, (T7) 2 % iron oxide and (T8) 1 % iron oxide + 1 % RP

used in curing soil ecosystems contaminated with certain inorganic pollutants such as Ni, Cu, Zn and others (Andini et al., 2006). In addition, bentonite has been shown to improve the overall soil quality (Phillips, 1998). Application of bentonite to soils irrigated with low quality water s significantly retained PTE's in different mechanisms such as sorption and ion exchange mechanisms. The optimum conditions of soil ecosystem to decrease the availability of PTE's might obviously monitored through the microbial activity of decreasing the CO_2 efflux.

Although both bentonite and pro-bentonite showed priority in minimizing the hazards of PTE's in the studied soil ecosystem, yet, kaolinite exhibited the least exchangeability among bentonite clay mineral group, as several studies confirmed the potential of natural kaolinite in metal ion adsorption from solution. O'Day et al. (1994) mentioned that CO_2 is always binding to kaolinite as co-complexes at both inner and outer sphere complexes using X-ray absorption spectroscopy (XAS). Boron adsorption on kaolinite was studied by Singh and Mattigod (1992) using phenomenological equations and surface complication reactions. Samaneh and Jalali (2016) evidenced strong preference for the ion exchanged form of kaolinite for Cu ions. Exchange capacity of both cation as well as anion of kaolinite and their relation with homo ionic counterparts with Na+ was critically examined by Ferris and Jepson (1975).

Results indicated that the succession of more than one model in describing the kinetic results having high R^2 values ranged between 96-99** in used models. This means that the different mechanisms that took place in the sorption of studied PTE's by the trailed remediation amendments improved CO_2 efflux by decreasing the available form of PTE's.

The outer groups were situated along the unshared plane of the alumina hydroxyl sheet, while the inner groups were located along the plane that is shared with and borders on the silica oxide sheet. The movement of the inner hydroxyl plane is restricted as a result of chemical bonding between the silica and alumina sheets. The pro- clay mineral treatment in all cases directly increased the retention of PTE's, this trend decreased the microbial biomass of these treatment. This result might be due to the mode of phosphate reaction with all PTE's in having complex compounds (Ma and Harris, 1997). Worth to mention that iron oxide exhibited the same trend when RP was applied to the soil ecosystem.

Sorption and immobilization of inorganic pollutants in soil ecosystem is an effective detoxification process and thus it is an essential part of the buffering capacity of the soil ecosystem (Welp 1999).

Immobilization of inorganic pollutants caused an increase in basal respiration rate, litter decomposition and microbial activity. There are several methods for immobilization of inorganic pollutants in soil, through either adding natural and synthetic chemical additives such as alkaline materials, phosphate minerals, iron and manganese oxides, alumino-silicates or coal fly ashes (Mench et al., 1998). Clay minerals are among the major materials that interact with almost all soil contaminants (Prost and Yaron, 2001). The adsorption of Ni, Cd, Zn, and Pb by montmorillonite was reported by Schulthess and Huang (1990). Immobilization of inorganic pollutants by natural zeolite (clinoptilolite) and six synthetic zeolites was studied by Oste et al. (2002), who found that the synthetic zeolites had an effect on immobilization of Cd and Zn. The improvement of the quality of the microbe's media through is mainly due to ameliorative action of PTE's in soil ecosystem irrigated with low quality water for extended periods.

4 CONCLUSIONS AND RECOMMENDATIONS

The application of microbial activity through CO2 flux in evaluation soil remediation technology(ies) can be a viable and innovative best way. In this work, the use of some clay minerals, crude or modified with some microbes, significantly reduced the content of harmful heavy metals in a soil ecosystem irrigated with low-quality water for long periods. The treatments used led to a significant decrease in the available forms of the studied heavy elements in parallel with the increase in the residual unavailable forms of pollutants. According to the obtained results, heavy metal ions showed a tendency to accumulate on clay minerals, which shows the importance of this method of treatment and suitability for improving soil quality by restoring appropriate ecosystem conditions and flourishing microbial activity. This method is also characterized by low costs and an economical way that encourage the farmers to use it for having safe food. It is worth noting the importance and necessity of using this technique more in other studies with other polluted metals that did not fall within the scope of this research.

5 ABBREVIATIONS

NRC: National Research Centre
PR: Rock phosphate
CO2: Carbon dioxide
Cu: copper
Ni: Nickel
Zn: Zinc
PR: Rock phosphate

$K_2Cr_2O_7$: Potassium dichromate
ANOVA: Analysis of variance
R2: Coefficient of determination
SE: Standard Error
NF: Non-fumigated
Fum: Fumigated

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Influence of plant growth regulators and salicylic acid on the production of some secondary metabolites in callus and cell suspension culture of *Satureja sahendica* Bornm.

Sarieh TARIGHOLIZADEH [1], Rouhollah MOTAFAKKERAZAD [1, 2], Morteza KOSARI-NASAB [1, 3], Ali MOVAFEGHI [1], Sakineh MOHAMMADI [1], Mohsen SABZI [4], Amir-Hossein TALEBPOUR [5]

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Influence of plant growth regulators and salicylic acid on the production of some secondary metabolites in callus and cell suspension culture of *Satureja sahendica* Bornm.

Abstract: The impact of combinations of plant growth regulators (PGRs) on callus culture of *Satureja sahendica* Bornm. was investigated. In nodal explants, the response of secondary metabolite production to different concentrations of PGRs was analyzed regarding the presence and absence of polyvinylpyrrolidone (PVP). The explants were cultured on MS media in presence of auxins (2,4-dichlorophenoxyacetic acid and naphthylacetic acid) and cytokinins (thidiazuron and kinetin); which were used in equal concentrations of 0.5, 1, and 2 mg l^{-1}. The treatment of 2 mg l^{-1} 2,4-D + 2 mg l^{-1} Kin (MD3) led to the highest production of total phenolics (4.303 ± 0.449 mg GAE g^{-1}) and flavonoids (24.903 ± 7.016 mg QE g^{-1}). Moreover, the effect of salicylic acid (SA) on the production of secondary metabolites in cell suspension culture of *Satureja sahendica* was evaluated. The cell suspension culture was established by culturing the nodal-derived friable callus in the liquid medium containing different concentrations of SA (0, 100, 150, 200 μM). An inverse relationship exists between the fresh mass and secondary metabolites contents. In addition, there was a significant difference among concentrations of SA in the production of total phenolics and flavonoid compounds. SA enhances secondary metabolites production and decreases cell fresh mass.

Key words: *Satureja sahendica*; callus induction; cell suspension culture; secondary metabolites; growth regulators; salicylic acid

Vpliv rastlinskih rastnih regulatorjev in salicilne kisline na tvorbo nekaterih sekundarnih metabolitov v kalusu in suspenziji celične kulture vrste šetraja *Satureja sahendica* Bornm.

Izvleček: Preučevan je bil vpliv kombinacije rastlinskih rastnih regulatorjev (PGRs) na kulturo kalusa vrste šetraja *Satureja sahendica* Bornm. V nodijskih izsečkih je bil preučevan odziv tvorbe sekundarnih metabolitov na različne koncentracije PGRs glede na prisotnost in odsotnost polivinilpirolidona (PVP). Izsečki so bili gojeni v MS gojišču v prisotnosti auksinov (2,4-diklorofenoksiocetna kislina in naftilocetna kislina) in citokininov (tidiazuron in kinetin), ki so bili uporabljeni v enakih koncentracijah 0,5; 1, in 2 mg l^{-1}. Obravnavanje 2 mg l^{-1} 2,4-D + 2 mg l^{-1} Kin (MD3) je vodilo k največji tvorbi celokupnih fenolov (4,303 ± 0,449 mg GAE g^{-1}) in flavonoidov (2,903 ± 7,016 mg QE g^{-1}). Dodatno je bil ovrednoten učinek salicilne kisline (SA) na tvorbo sekundarnih metabolitov v kulturi suspenzije celic šetraja. Kultura suspenzije celic je bila vzpostavljena z gojenjem rahlega kalusa, pridobljenga iz nodijskih izsečkov v tekočem mediju, ki je vseboval različne koncentracije SA (0, 100, 150, 200 μM). Pojavilo se je obratno sorazmerje med svežo maso in vsebnostjo sekundarnih metabolitov. Med različnimi koncentracijami salicilne kisline ni bilo opaziti značilnih razlik na tvorbo celokupnih fenolov in flavonoidov. Salicilna kislina vzpodbuja tvorbo sekundarnih metabolitov in zmajšuje svežo maso celične kulture.

Ključne besede: *Satureja sahendica*; indukcija kalusa; kultura suspenzije celic; sekundarni metaboliti; rastni regulatorji; salicilna kislina

1 Department of Plant Sciences, Faculty of Natural Sciences, University of Tabriz, Tabriz, Iran
2 Corresponding author, email: rmotafakker@tabrizu.ac.ir
3 Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
4 Ahar Faculty of Agriculture and Natural Resources, University of Tabriz, Iran
5 East Azerbaijan Research Center for Agriculture and Natural Resources, Tabriz, Iran

1 INTRODUCTION

Satureja is an extra-large genus of Lamiaceae: Nepetoideae and comprises about 200 species of often aromatic shrubs and herbs distributed in Asia, the Mediterranean region, and North America. The flora of Iran possesses 14 species of this genus, 9 of which are endemic. The members of this genus is widely found in mountainous areas in Iran (Rechinger, 1982; Jamzad, 1996; Mozaffarian, 1996). Many species of the genus are traditionally used for curing diseases such as wounds, muscle pains, diarrhea, nausea, infections, and gastroenteritis (Hadian et al., 2012). Like many members of Lamiaceae, *Satureja* species are very rich in secondary metabolites such as flavonoids, phenolics diterpenes, and phenolic acids, and therefore they have gained attention from researchers for their applications in various fields (Ghotbabadi et al., 2012).

Satureja sahendica Bornm., locally known as "Marze-Sahandi", is an endemic species of *Satureja* in Iran and is distributed in Northwestern and Western regions in East Azerbaijan, Zanjan, and Kurdistan provinces (Mozaffarian, 1993). This plant is a perennial, branched, bushy, aromatic, and late flowering herb (Ghahreman, 1988, 1993). Literature reviews show that 39 components were identified in the oil of *S. sahendica* and the main constituents of the essential oils were thymol (19.6 - 41.7 %), *p*-cymene (32.5 - 54.9 %), and γ-terpinene (1.0 - 12.8 %) (Sefidkon et al., 2004; Hassanpouraghdam et al., 2009). In recent years, the presence of several antioxidant compounds in this plant was reported. Furthermore, some flavonoids such as derivatives of diosmetin, quercetin, luteolin, and apigenin were found in this species (Saeidnia et al., 2011; Hadjmohammadi et al., 2012).

Although a significant amount of research has been done on the composition of the oil and the chemical and physical characteristics as well as medicinal properties of this species, tissue culture-related activities have been limited to some species of *Satureja*. Actually, major work has not been done to analyze *Satureja sahendica* metabolites. For instance, a study evaluated antimicrobial and antioxidant activities of the essential oil from aerial parts of *Satureja hortensis* L. via callus culture (Güllüce, 2003). Moreover, the production and optimization of rosmarinic acid (an important phenolic acid) were investigated in the callus culture of *S. hortensis* (Tepe & Sokmen, 2007).

Development of appropriate conditions and techniques for the production of phenolic compounds is required due to their medical and commercial values including a range of biological activities such as antibacterial, anti-viral, and anti-cancer (Taveira et al., 2010). Previous researches illustrated that plant *in vitro* culture is a suitable technique for the production of valuable metabolites in most plants, and the stimulants could be applied as physical and chemical stress for the production of these metabolites as one of the most successful strategies (Ravishandera et al., 1999; Dörnenburg & Knorr, 1995). The production and accumulation of secondary metabolites in plants is known as a part of the defense response against pathogenic attacks, which could be triggered and activated by growth regulators and elicitors (Zhong et al., 1996; Wang et al., 2004; Al-Sane et al., 2005; Shilpashree & Rai, 2009). Elicitors, either biological or non-biological compounds, increase the secondary metabolites production by activating the genes involved in the biosynthesis of these compounds (Neumann et al., 2009). Salicylic acid (SA) or 2-hydroxybenzoic acid, a type of plant phenolics, is an effective inducer of genes involved in plants' defense system. Therefore, SA can effectively induce the enhancement of secondary metabolites production such as alkaloids, terpenoids, phenolics, and phytoalexins (Vlot, 2008). It has been reported that the production of taxol in the suspension culture of *Taxus baccata* L. was considerably increased by SA compared to the control (Khosroushahi, 2006). Salicylic acid caused also a significant increase in the production of alkaloids in the hairy root culture of *Brugmansia x candida* Pers. (Alvarez et al., 2000) and it likewise enhanced the synthesis of total flavonoids in the suspension culture of *Andrographis paniculata* (Burm.f.) Nees (Mendhulkar, 2013). The application of SA for the production of curcumin in the *Catharanthus roseus* (L.) G.D on cell culture has been also reported (Matkowski, 2008).

Due to lack of a basic investigation on plant tissue culture in *S. sahendica*, and after our last research on the *in vitro* production of secondary metabolites of *Lallemantia iberica* (M.Bieb.) Fisch. & C.A.Mey. as a member of Lamiaceae (Pourebad et al., 2015), we became interested to work on tissue and cell culture of this plant species. Correspondingly, based on our preliminary accomplished work (Tarigholizadeh et al., 2015), we aimed to study callus and cell suspension culture conditions and determination of total phenolic and flavonoid compounds in *S. sahendica* using different concentrations and combinations of growth regulators and SA. The present work focuses on the secondary metabolites production of *S. sahendica* via callus and cell suspension cultures and highlights the potential of this plant species for further pharmaceutical researches.

2 MATERIALS AND METHOD

2.1 PLANT MATERIAL

Seeds of *Satureja sahendica* Bornm. were obtained from the Botanical garden of East- Azerbaijan province in Iran, and were surface-sterilized with 70 % ethanol for 1 minute, rinsed once with water, followed by 10 minutes immersion in 1 % formaldehyde plus a few drops of 80 % Tween and interspersed with washings in sterile distilled water. After that, seeds were sterilized with commercial hypochlorite solution (20 %) for 10 minutes. After 3 rinses with sterile distilled water, the seeds were treated with giberellic acid (200 mg l^{-1} for 15 min) for the elimination of the dormancy problem (Tarigholizadeh et al., 2015). Then, the seeds were cultivated on a hormone-free MS basal medium (Murashige & Skoog, 1962).

2.2 CALLUS INDUCTION

Callus culture was started by nodal explants of *in vitro* germinated 30-days-old seedlings of *S. sahendica* and were cultured on MS medium supplemented with different combinations of 2,4-D (2,4-dichlorophenoxyacetic acid) and kin (kinetin) as well as NAA (naphthylacetic acid) and TDZ (thidiazuron) in different concentrations as presented in Table 1. In order to examine the effect of polyvinylpyrrolidone (PVP) on callus growth and secondary metabolites production, we aimed to apply a distinct set of experiments with the same PGRs and their combinations mentioned above and 400 µl PVP. Therefore, there were two types and series of media (each medium: 250 ml) for callus induction: with the presence of PVP (PVP+) and absence of PVP (PVP-). The pH value of all media were adjusted at 5.6 - 5.8 with 1 N NaOH prior to adding of agar (8.0 g l^{-1}) and subsequently they were autoclaved for 15 minutes (121 ˚C, 104 kPa) and dispensed into glass jars (each containing 250 ml). Three glass jars containing seven explants and each were cultured per treatment. For control groups, we used MS with and without PVP. All cultures were maintained in the growth room 16 h light (40 µmol m^{-2} s^{-1} white cool fluorescence) and 8 h dark at 23 ± 2˚C for four weeks. Then, callus samples were subcultured once and four weeks later, were collected for evaluation of the following parameters: callogenesis percentage, morphological traits and total phenolic and flavonoid contents.

Table 1: List of MS media supplemented with different growth regulators for callus induction

Growth regulators (mg l^{-1})	Media (-PVP or + PVP)
NAA 0.5 + TDZ 0.5	MN1
NAA 1.0 + TDZ 1.0	MN2
NAA 2.0 + TDZ 2.0	MN3
2,4-D 0.5 + Kin 0.5	MD1
2,4-D 1.0 + Kin 1.0	MD2
2,4-D 2.0 + Kin 2.0	MD3

2.3 ESTABLISHMENT AND MAINTENANCE OF CELL SUSPENSION CULTURE

In order to callogenesis for establishing cell suspension culture, callus induction was carried out in the same above-mentioned way. However, the exerted PGRs were only 2,4-D and Kin with two different concentrations of each (0.5, 1 mg l^{-1}). To establish suspension culture, 0.5 g of healthy and two-month-age callus tissues were transferred to 50 ml of MS medium with mentioned PGRs compounds without agar and were kept on shakers at 110 rpm (revolutions per minute) and 25 ˚C in the dark. Cultured samples were subcultured once per three weeks to establish cell lines. In each subculture, 5 ml of medium containing cells was added to 45 ml of new medium with the same PGRs. Based on previous studies and our preliminary researches, different concentrations of SA were chosen and their effects on cultured cells and tissues were examined. For this propose, 5 ml of cell culture medium was transferred to 45 ml MS liquid medium containing 0 (control), 100, 150 and 200 µM concentrations of sterilized SA. Samples were then placed on a shaker (110 rpm, 25 ˚C) in darkness for three weeks. Finally, the samples were collected to examine the influence of different concentrations of SA on growth and secondary metabolites production.

2.4 DETERMINATION OF TOTAL PHENOLIC AND FLAVONOID CONTENTS

Callus cultures derived from nodal segments using the different combinations of growth regulators were dried in oven at 35 ˚C for 30 h. Then, 0.5 g of dried callus from each sample was mixed with methanol in a small glass tube and were put at 25 ˚C for 40 h. This procedure was done for each treatment and extracts and they were centrifuged at 16300 x g for 15 minutes. The supernatant was separated for measurement of

total phenolics and flavonoids content. In addition, in cell suspension culture, cell growth was determined by measuring the fresh mass (due to low dry mass in cell suspension culture) and total cell extract was prepared to measure total phenolics and flavonoids contents.

For the determination of total phenolics content in callus and cell culture, Folin-Ciocalteu reagent was used (Singleton et al., 1999). In brief, 100 µl of each extract was combined with 2.5 ml of distilled water, and then mixed thoroughly with 100 µl of Folin-Ciocalteu reagent. After 6 minutes, 150 µl of 20 % (w/v) sodium carbonate was added and the solution was left at room temperature for 30 minutes in the dark. The absorbance of the reaction mixtures was measured at 650 nm. The results were expressed in the form of gallic acid equivalents (GAE) per gram of dry mass.

The total flavonoids content in callus and cell culture was estimated by using the aluminum chloride colorimetric method (Chang et al., 2002). A quantity of 500 µl of each sample solution was combined with 2.5 ml of distilled water and subsequently with 150 µl of 5 % sodium nitrite ($NaNO_2$) solution, after 6 min of mixing, 300 µl of 10 % aluminum chloride ($AlCl_3$) was added and then allowed to stand 5 minutes, followed by adding 1000 µl of 1 M NaOH solution to the mixture. The obtained solution was thoroughly mixed, after which the absorbance was determined at 510 nm. The results were expressed as mg quercetin equivalents (QE) per gram of dry mass.

2.5 STATISTICAL ANALYSIS

All experiments were performed in a completely randomized design. Each treatment was comprised of three replicates. A one-way analysis of variance (ANOVA) was applied to statistically analyze the data that was obtained from callus tissues and the means were compared by Duncan's Multiple Range Tests (DMRT). IBM SPSS statistic ver. 22 was used to determine the significance at $p \leq 0.05$.

3 RESULTS AND DISCUSSION

3.1 CALLUS INDUCTION AND MORPHOLOGY

Table 2 shows the effects of PGRs on *S. sahendica* nodal explants cultured on MS medium, displaying the callogenetic and morphologic properties of callus tissues. Shoot formation was also observed during callus growth, and therefore the percentage of produced organs by nodal explants was reported (in relation to PVP). First callus tissues appeared on nodal explants after 10 days of culture. However, callogenesis in the combination of 2, 4-D + Kin occurred one week later. Callus tissues covered almost all the explants surface within 30 days. Green-compact and yellowish green-friable callus tissues were formed by NAA+TDZ and 2, 4-D + Kin groups, respectively. In treatments with 2, 4-D, and Kin, shoot formation was not observed and high concentrations of these PGRs resulted in a low callogenesis efficiency (Table 2). As expected, the absence of PGRs in MS and MS + PVP media (control groups) showed a strong shooting response without production of any callus tissues, and therefore this group was excluded from further examination (data not shown). The PVP application with growth regulators induced a general increase in callogenesis (Table 2). Actually, the addition of PVP to the medium was effective in overcoming the browning of the culture medium and promoted callogenesis. PVP hydrogen bonds are able to absorb phenolic substances, and thus reduce their amount in medium. These substances are released from tissues and cells into the surrounding medium and their accumulation may result in decreased rates of growth and development of cultured materials (Saxena et al., 1986; Leyser et al., 2003). Similar effects of PVP were also reported by Saxena et al. (1999) and Ogita et al. (2001) in a bamboo (*Dendrocalamus strictus* (Roxb.) Nees) tissue culture. In the current study, *S. sahendica* nodal material was used to test PVP and PGRs effects on *in vitro* cultured tissues. As it is shown, NAA + TDZ promoted callus production with significant shoot formation. Previous reports revealed that TDZ alone and in combination with NAA strongly promoted compact-green nodular callus and shoot formation in shrubs (Tarigholizadeh et al., 2015, Al-juboory et al., 1998; Murthy, 1998; Thiruvengadam & Chung, 2015).

3.2 THE EFFECT OF PGRS ON CALLUS TISSUES GROWTH

Our experiments revealed that callus growth of *S. sahendica* was strongly affected by type, combination, and different concentrations of PGRs (Table 3). Statistically significant differences were found between 2,4-D + Kin and NAA + TDZ treatments. Moreover, callus growth was also affected by the presence of PVP. PVP exerted its positive effects on both fresh and dry mass of callus tissues in PVP+ media (Fig. 1 and Table 3). As shown in Figure 1 and Table 3, we obtained the highest fresh mass from MN3 and MN1 in PVP- and PVP+ media, respectively. However, there were no significant differences among the different concentrations

Table 2: Effect of different combinations of PGRs on callus induction and morphology in presence (PVP+) or absence (PVP-) of polyvinylpyrrolidone

	Callogenesis %		Callus tissues morphology	Shoot formation %	
Media	PVP+	PVP-	PVP±	PVP+	PVP-
MN1	100 ± 0.00 [a]	90.48 ± 9.52 [ab]	Green, Compact	42.86 ± 0.00 [b]	76.19 ± 4.76 [b]
MN2	95.24 ± 4.763 [b]	100 ± 0.00 [a]	Green, Compact	71.43 ± 0.00 [a]	90.47 ± 4.76 [a]
MN3	100 ± 0.00 [a]	100 ± 0.00 [a]	Green, Compact	52.38 ± 12.60 [b]	71.43 ± 8.25 [b]
MD1	100 ± 0.00 [a]	100 ± 0.00 [a]	Yellowish-Green, Friable	0.00 ± 0.00 [c]	0.00 ± 0.00 [c]
MD2	71.43 ± 0.00 [c]	90.48 ± 9.52 [ab]	Yellowish-Green, Friable	0.00 ± 0.00 [c]	0.00 ± 0.00 [c]
MD3	90.47 ± 4.763 [b]	66.67 ± 17.17 [b]	Yellowish-Green, Friable	0.00 ± 0.00 [c]	0.00 ± 0.00 [c]

Data within the two columns (PVP-/PVP+) of each growth parameter, followed by different letters are significantly different at $p \leq 0.05$. The data are presented as means ± SE (n = 3)

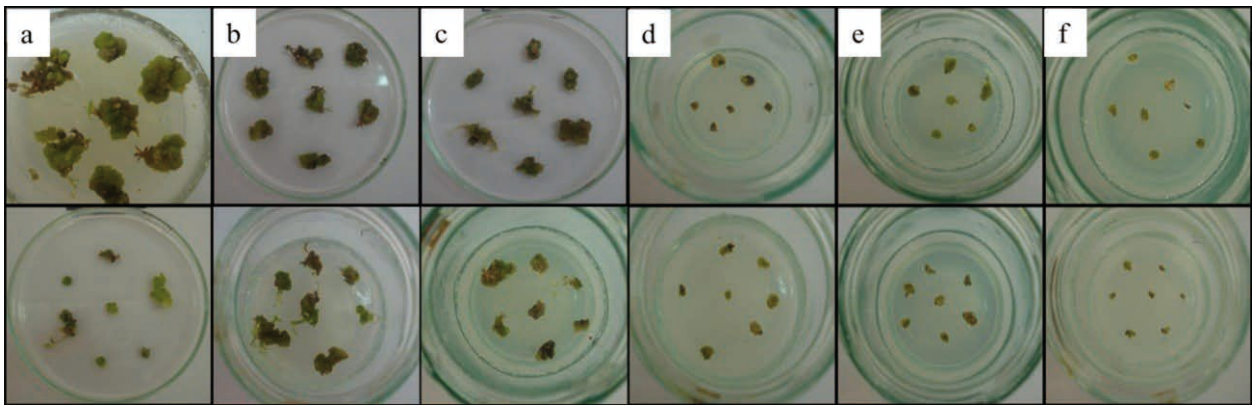

Figure 1: *S. sahendica* callus tissues grown on MS medium in the presence (upper row) and in the absence (lower row) of PVP and with the addition of PGRs. a (MN1), b (MN2), c (MN3), d (MD1), e (MD2), f (MD3)

Table 3: Effect of different combinations of PGRs on callus tissues growth in MS PVP- and PVP+ medium

	Fresh mass (mg)		Dry mass (mg)	
Media	PVP+	PVP-	PVP+	PVP-
MN1	1.969 ± 0.535 [a]	0.848 ± 0.162 [ab]	0.118 ± 0.023 [a]	0.066 ± 0.014 [ab]
MN2	1.966 ± 0.393 [a]	1.354 ± 0.435 [a]	0.130 ± 0.022 [a]	0.096 ± 0.031 [a]
MN3	1.460 ± 0.197 [a]	1.384 ± 0.155 [a]	0.099 ± 0.017 [a]	0.094 ± 0.011 [a]
MD1	0.287 ± 0.028 [b]	0.477 ± 0.086 [bc]	0.026 ± 0.001 [b]	0.047 ± 0.014 [abc]
MD2	0.334 ± 0.026 [b]	0.256 ± 0.056 [bc]	0.026 ± 0.002 [b]	0.022 ± 0.003 [bc]
MD3	0.160 ± 0.02 [b]	0.140 ± 0.028 [c]	0.020 ± 0.004 [b]	0.011 ± 0.004 [c]

Different letters within two columns (PVP-/PVP+) of each growth parameter represent significant differences among treatments at $p \leq 0.05$. The data are presented as means ± SE (n = 3)

of growth regulators within a group. The highest dry mass was achieved by MN2 in both PVP- and PVP+ media. Once again, there were no significant differences within a group. As an obtained result, the presence of NAA and TDZ in the medium improved the growth of callus tissues. These result regarding all other examined growth parameters reinforced the findings of our previous work where the effects of the PGRs and PVP were observed on callus relative growth rate (RGR) (Tarigholizadeh et al., 2015). Correspondingly, Ali et al. (2013) reported that NAA in combination with TDZ was more effective for callus formation of *Artemisia absinthium* L. than other combinations of PGR. They achieved the highest callogenesis frequency (83.3 %) and maximum

callus biomass (FW: 132 gl^{-1}) on MS medium supplemented with 1 mg l^{-1} NAA + 1 mg l^{-1} TDZ. Similarly, the dry mass showed the highest amount in 1 mg l^{-1} of both NAA and TDZ in callus of *S. sahendica*, although varying this concentration resulted in the reduction of dry mass. Previously, decrease in callogenic responses or mass production has been observed using 2,4-D in combination with Kin (Jeong et al., 2007; Hakkim et al., 2007; Johnson et al., 2011; Walla Abdelazeez et al., 2017). Thus, combination of 2, 4-D and Kin is not appropriate for the induction of callus tissues in nodal explants of *S. sahendica* compared with NAA + TDZ group.

3.3 THE EFFECT OF PVP AND PGRS ON PHENOLIC COMPOUNDS PRODUCTION IN CALLUS TISSUES

The present study shows that total phenolics concentration depends on the type and combination of PGRs in the culture medium. Accumulation of phenolic compounds indicated differences between two groups of growth regulators used in this study. As can be seen in Figures 2 and 3, media containing 2, 4-D + Kin not only stimulated a high accumulation of total phenolics, but also enhanced flavonoid contents. Based on the results, MD3 medium maximized the amount of these compounds in both PVP- (the highest amount for flavonoids: 24.903 ± 7.016 mg QE g^{-1}) and PVP+ (the highest amount for total phenolics: 4.303 ± 0.449 mg GAE. g^{-1}) media. It should be noted that along with PGRs, PVP may play a significant role in production of total phenolics. Based on the obtained results, PVP nearly improved the amounts of total phenolics and flavonoids, except for 2,4-D + Kin treatment, which accumulation of flavonoids decreased along with the presence of PVP (Fig. 2 and 3). Similarly, Rani and Nair (2006) reported that PVP has an effect on *Vitix negundo* L. callus organogenesis. They suggested that this might be due to PVP ability to bind phenolics and some toxic substances. In addition, high production of isoflavones and growth index were achieved in callus culture of *Genista* plants after addition of PVP to MS medium (Luczkeiwicz & Glod, 2003). Previously, enhanced phenolic compounds accumulation by the combination of 2, 4-D, and Kin was also observed in *Ocimum sanctum* L. (Hakkim et al., 2011). Furthermore, the highest accumulation of withanolide A was showed in MS medium containing 2,4-D (9.05 µM) and Kin (2.32 µM) in cell suspension cultures of *Withania somnifera* (L.) Dunal (Sivanandhan et al., 2013). Also, Han et al. (2012) have obtained the highest amounts of rutin and GABA via incubation of the immobilized *Morus bombycis* Koidzumi cells in a full-strength MS liquid medium containing 1 mg l^{-1} of 2,4-D and 0.1 mg l^{-1} Kin. Based on former reports, maximum production of phenolic compounds was obtained from the active growing cells (Fu et al., 2005; Antonigni et al., 2007). In most cases, secondary products accumulation was promoted at the end of rapid cell division in the growth cycle. However, in some of the cell culture systems, the production of secondary metabolites did not follow a parallel way with the cell growth (James et al., 2008) and the production occurred along with low growth. In the present study, as shown in Figures 1 and 2 and Table 3, an opposite relationship between growth and secondary products formation was found in NAA + TDZ and 2,4-D + Kin groups. According to our results, it can be deduced that the type of PGRs was more effective than other parameters for the production of total pheno-

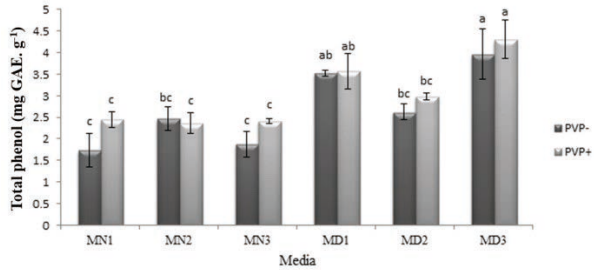

Figure 2: Content of total phenolics (mg GAE. g^{-1}) in callus tissues of *S. sahendica* affected by different combinations of PGRs in MS medium (in absence and presence of PVP: PVP- and PVP +). The results are presented as means ± SE (n = 3)

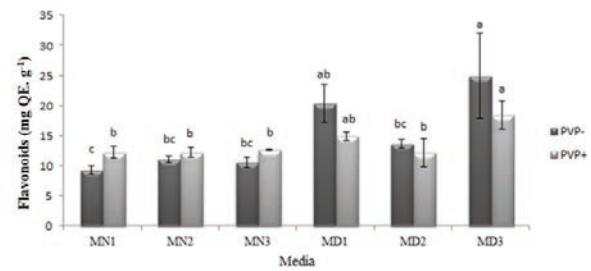

Figure 3: Content of flavonoids (mg QE. g^{-1}) in callus tissues of *S. sahendica* affected by different combination of PGRs in MS medium (in absence and presence of PVP: PVP- and PVP +). The results are are presented as means ± SE (n = 3)

lic compounds. Similarly, a reverse correlation between rosmarinic acid (RA) accumulation and callus growth was reported in the callus culture of *Satureja hortensis* L. (Tepe & Sokmen, 2007). Based on these results, RA accumulation and growth relationship is anthocyanin type and phenolic compound accumulation enhanced in the stationary phase of cell growth.

We suggest that the presence of 2,4-D in media is most likely more responsible for the above-mentioned opposite relationship. As it can be seen in Table 2, application of 0.5 mg l^{-1} of both 2,4-D and Kin in medium resulted in a 100 % callogenesis in presence and absence of PVP and callus formation percentage showed a significant reduction in high concentrations of these two PGRs. Actually, 2, 4-D as an auxin is commonly used as an herbicide, especially for broadleaf weeds control (WHO, 1989; US EPA, 2005b; Tomlin, 2006). It owns herbicidal and lethal effects at high concentrations, and this function probably causes the production of higher concentrations of secondary metabolites by explants in culture media.

3.4 THE EFFECT OF SA AND PGRS ON CELL GROWTH

The mean comparison of cells' fresh mass indicated a significant difference among the different concentrations of SA and control samples. Higher concentrations of SA reduced cell fresh mass. According to the data presented in Table 4, the highest (304.67 ± 3.48[a]) and the lowest (148 ± 3.76[c]) amount of fresh mass in the liquid media were obtained by 100 µM (1 mg l^{-1} 2,4-D + 1 mg l^{-1} Kin) and 200 µM of SA (0.5 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kin), respectively. The treated cells with SA showed a higher amount of fresh mass in 1 mg l^{-1} 2,4-D + 1 mg l^{-1} Kin compared to 0.5 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kin (Table 4). It should be noted that due to proper and adequate growth of callus tissues derived from MS media containing 0.5 and 1 mg l^{-1} of 2,4-D and Kin; these treatments have been chosen for further examination.

3.5 THE EFFECT OF SA AND PGRS ON PHENOLIC COMPOUNDS IN CELL SUSPENSION CULTURE

According to the variance analysis and the results of phenolics and flavonoids assessment in control and treated samples with different concentrations of SA in cell suspension culture, there was a significant difference among treatments at both concentrations of 2,4-D + Kin in nodal explants of *S. sahendica*. Total phenolics and flavonoids content was enhanced by increasing the concentrations of SA, but this increase was not linear and the amount of total phenolics was reduced by high concentrations of SA. The highest and the lowest total phenolics content were obtained by 150 µM SA (2.1 ± 0.22 mg GAE g^{-1}) with 0.5 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kin and control treatment (0.78 ± 0.007 mg GAE g^{-1}) with 1 mg l^{-1} 2,4-D + 1 mg l^{-1} Kin, respectively (Fig. 4 and 5). On the other hand, there was no significant difference among different concentrations of SA, except for the control treatment (Fig. 4). According to Table 3, the highest (358.6 ± 0.00 mg QE g^{-1}) and the lowest (70.88 ± 0.47 mg QE g^{-1}) production of flavonoids were obtained by 150

Table 4: Effect of different combination of PGRs and SA on cell fresh mass in suspension cell culture of *Satureja sahendica*

PGRs treatments	Elicitor concentrations	
2,4-D + Kin (mg l^{-1})	Salicylic acid (µM)	Fresh mass (mg)
0.5 : 0.5	Control	175 ± 1.76[b]
	100	266 ± 4.62[a]
	150	186.67 ± 6.96[b]
	200	148 ± 1.73c
1 : 1	Control	185.33 ± 2.96[c]
	100	304.67 ± 3.48[a]
	150	283.67 ± 3.18[b]
	200	158 ± 4.04[d]

Different letters within the same column represent statistically significant differences among treatments at $p \leq 0.05$

μM of SA and control treatment with 0.5 mg l^{-1} (2,4-D + Kin), respectively. No significant difference was found among different concentrations of SA in both concentrations (0.5 and 1 mg l^{-1}) of 2,4-D + Kin (Fig. 6).

Investigation of SA effects on total phenolics and flavonoids contents in suspension culture of nodal-derived callus tissues of *S. sahendica* showed that these parameters were increased by 100 to 150 μM of SA and decreased by enhancement in SA concentration to 200 μM. Presumably, reduction in total phenolics and flavonoids contents in 200 μM of SA can be caused by the limited ability of cells in response to stresses or reduced enzymes activity. The maximum amount of phenolics and flavonoids content was obtained by 150 μM of SA and the flavonoids and total phenolics were 2 and 5 times more than the control group, respectively (with 0.5 Kin + 0.5 2,4-D). Interestingly, with a further increase in the SA concentration, not only the increasing in accumulation of total phenolics and flavonoids was not achieved, but also the accumulation process of these parameters was declined.

Similar to our results, Esmaeilzadeh Bahabadi and Rezaei (2014) using cell culture of *Trigonella foenum-graecum* L. showed a significant reduction in cell growth with the increasing concentrations of SA. Thus, higher concentrations of SA have a detrimental effect on plant's oxidative condition and eventually cause plant death

Figure 4: Comparison of total phenolics and flavonoids content after treatment with different concentrations of SA (0, 100, 150, and 200 μM, left to right) in cell suspension of *S. sahendica*. Upper row: Callus tissues grown on MS medium containing 1 mg l^{-1} 2,4-D + 1 mg l^{-1} Kin. Lower row: callus tissues grown on MS medium containing 0.5 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kin

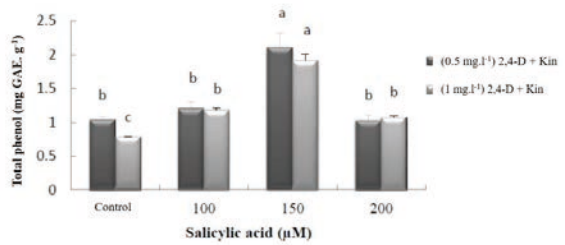

Figure 5: Content of total phenolics (mg GAE. g^{-1}) in cell suspension culture of *S. sahendica* after treatment with different concentrations of SA in MS medium containing different combinations of 2, 4-D + Kin. The results are presented as means ± SE (n = 3)

(Kovacik, 2009). They also reported that total phenolics content was increased by 50 mg l^{-1} SA. Due to an inverse relationship between growth and accumulation of secondary metabolites, the inhibition of cell growth by SA might induce the synthesis of secondary metabolites. Because precursors of secondary metabolite biosynthesis originate from the primary metabolism, under severe stress the primary metabolism changes to the secondary metabolism and the necessary resources are diverted from development to defense (Harfouche et al., 2008). Moreover, total phenolics and flavonoids contents were increased by different concentrations (0, 50, 100, 200, and 250 μM) of SA in *Cynara scolymus* L. (Samadi et al., 2014) and by the increasing concentration of SA up to 100 μM, total phenolics and flavonoids contents showed

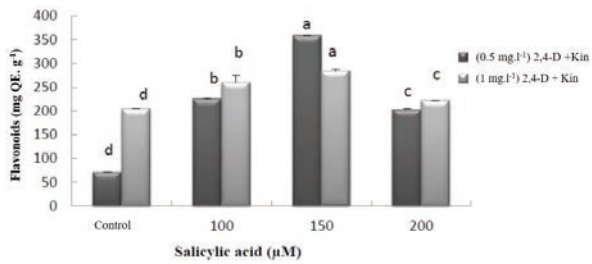

Figure 6: Content of flavonoids (mg QE. g^{-1}) in cell suspension culture of *S. sahendica* after treatment with different concentrations of SA in MS medium containing different combinations of 2, 4-D + Kin. The results are presented as means ± SE (n = 3)

a significant increase. These results are in agreement with the findings of the present work. They also reported that the changes of phenylpropanoid compounds were positively correlated with phenylalanine ammonia lyase (PAL) activity, and total phenolics and flavonoids content was also increased by PAL increased activity (Samadi et al., 2014). The effects of different elicitors on polyphenols content and activity of other polyphenol-related enzymes were also studied. For instance, shikimate dehydrogenase (SDH), tyrosine ammonia lyase TAL, cinnamate-4-hydroxylase (C4H), 4-coumarate/coenzyme A ligase (4-CL), and dihydroflavonol 4-reductase (DFR) were activated by elicitors such as SA and methyl jasmonate (Ruiz-García and Encarna Gómez-Plaza, 2013; Kim et al., 2020). Interestingly, the activity of PAL was directly related to the concentration of SA, so that the PAL activity changes were similar to total phenolics and flavonoids accumulation via increasing the amount of SA. The previous reports have suggested that the amount of phenolics and flavonoids was strongly influenced by PAL enzyme activity (Sun et al., 2012; Ruiz-García and Encarna Gómez-Plaza, 2013). SA, as a stress-inducing compound, activates the signaling pathway of PAL, subsequently, PAL results in activation of the phenylpropanoid pathway and increasing the production of phenolic compounds by increasing the transcription of specific mRNA, in which these compounds counteract the induced stress. Elicitors, such as SA and methyl jasmonate, play an important role in the signaling process which induces the biosynthesis of phenolic compounds and expression of plant defense genes (Wen et al., 2004; Wang et al., 2009). Exposure to elicitors may lead to an increase in the content of defense related compounds such as total phenolics, flavonoids and phytoalexins. This might be due to an increase in the expression levels of responsible genes for the biosynthesis of these metabolites. Similar to our results, Sadeghian et al. (2013) reported that in *Satureja khuzistanica* Jamzad polyphenol oxidase and superoxide dismutase enzymes activity as well as total protein contents of SA (0, 50, 100, 200, and 400 mg l^{-1}). Moghadam et al. (2013) found that the application of SA (125, 250, 500 mmol) in suspension culture of *Portulaca oleracea* L. hairy roots increased dopamine production with the highest amount at the concentration of 250 mM. Moreover, flavonoid content was enhanced by the application of SA (0.05, 0.5, 1, and 1.5 mM) in the suspension culture of *Andrographis paniculata* (Matkowski, 2008). The studies about the effect of SA concentration (1, 1.5, 2 mM) on stimulating of *Cicer arietinum* L. immune system showed that this plant quickly responds to 1.5 mM of SA, and polyphenol oxidase activity increases in this concentration (Rajjou et al., 2006). Despite decreasing total phenolic contents at higher concentrations, increasing above mentioned concentration (1.5 mM) was reported. These results show that exposure to 1.5 mM of SA is harmless for plants and this concentration may induce the chemical defense response. However, treatment of samples with 2 mM SA caused phytotoxicity, which in turn might lead to low production of phenolic compounds (War et al., 2011). These findings are in fair agreement with the findings of current work. Also, it has been reported that the high concentration of SA induces hypersensitivity response that leads to cell death, while low concentrations of SA induces immune response (Namadeo, 2007). Therefore, the increased production and accumulation of phenolic compounds and flavonoids which found in *S. sahendica* suspension culture can be attributed to the induceded defense responses with SA.

4 CONCLUSION

In the present study, the culture medium for callus induction from nodal explants of *Satureja sahendica* was optimized. Furthermore, the production of some secondary metabolites in the obtained callus tissues were determined. It was found that callus growth and secondary metabolites production of *S. sahendica* was strongly affected by type, combination, and different concentrations of PGRs and statistically significant differences were found between 2,4-D + Kin and NAA + TDZ treatments. Moreover, the media containing 2, 4-D + Kin stimulated the production of both total phenolics and flavonoid compounds. Along with PGRs, PVP was generally an effective component in improvement of the studied parameters. In addition, the impact of SA on the accumulation and biosynthesis of secondary metabolites by cell suspension cultures of *S. sahendica* was also investigated. Based on the obtained results, SA can be used as a stimulant to improve the amount of phenolic compounds in the cell suspension cultures of this plant under controlled conditions.

5 AUTHOR CONTRIBUTIONS

R.M., M.K.-N. and A.M. planned the research. All authors have read and agreed to the published version of the manuscript.

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In vitro antifungal potential of surfactin isolated from rhizospheric *Bacillus thuringiensis* Berliner 1915 against maize (*Zea mays* L.) fungal phytopathogen *Fusarium graminearum* Schwabe

Muddasir KHAN [1, 2], Muhammad SALMAN [1], Abdullah [1], Syed Hussain SHAH [1]; Muhammad ISRAR [3]

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In vitro antifungal potential of surfactin isolated from rhizospheric *Bacillus thuringiensis* Berliner 1915 against maize (*Zea mays* L.) fungal phytopathogen *Fusarium graminearum* Schwabe

Abstract: *Fusarium graminearum* fungus cause significant loss in maize (*Zea mays* L.) and other cereal crops all over the world. The usage of chemical agents cause severe environmental problems. *Bacillus* species and other plant growth-promoting bacteria (PGPR) play key role in biopesticide development. A wide range of environmentally safe antimicrobial agents are already being manufactured. The current investigation was focused on exploring the antifungal activity of *Bacillus thuringiensis* lipopeptide surfactin against fungal phytopathogen *Fusarium graminearum*. *B. thuringensis* was isolated from the rhizosphere of maize crop and cultivated to produce lipopeptides. Surfactin was identified by high-performance liquid chromatography (HPLC) from the extract at 210 nm, retention time 3-5 minutes and the obtained peaks area was 3.990. The growth of *F. graminearum* was successfully inhibited by surfactin at different concentrations. Among these, 80 % concentration showed the highest zone of inhibition in comparison to 60 %, 40 % and 20 % concentrations ($p < 0.005$), respectively. The current study concludes *B. thuringensis* lipopeptide surfactin has a high potential to inhibit the growth of *F. graminearum*.

Key words: surfactin; *Bacillus;* biological control; HPLC; *Fusarium graminearum*

In vitro protiglivni potencial surfaktina, izoliranega iz bakterije *Bacillus thuringiensis* Berliner 1915 iz rizosfere koruze (*Zea mays* L.) proti patogeni glivi *Fusarium graminearum* Schwabe

Izvleček: Gliva *Fusarium graminearum* povzroča znantne izgube v pridelku koruze in drugih žit širom po svetu. Uporaba kemičnih sredstev za zatiranje povzroča resne okoljske probleme. Vrste iz rodu *Bacillus* in druge rast vzpodbujajoče bakterije (PGPR) igrajo ključno vlogo pri razvoju biopesticidov. Proizveden je bil že širok spekter okolju prijaznih antimikrobnih agensov. Raziskava se osredotoča na uporabo protiglivne aktivnosti lipopeptidnih surfaktinov iz bakterije *Bacillus thuringiensis* proti patogeni glivi *Fusarium graminearum*. Bakterija *B. thuringensis* je bila izolirana iz rizosfere posevka koruze in gojena za proizvodno lipopeptidov. Surfaktin je bil določen s tekočinsko kromatografijo visoke ločljivosti (HPLC) iz izvlečka pri 210 nm, retencijskim časom 3-5 minut, dobljeni višek je bil 3.990. Rast patogene glive je bila uspešno zavrta pri različnih koncentracijah surfaktina. 80 % koncentracija surfaktina je pokazala največjo sposobnost zaviranja v primerjavi s koncentracijami 60 %, 40 % in 20 % ($p < 0{,}005$). Na osnovi te raziskave lahko zaključimo, da ima lipopeptidni surfaktin iz bakterije *B. thuringensis* velik potencial za zaviranje rasti glive *F. graminearum*.

Ključne besede: surfaktin; *Bacillus;* biološka kontrola; HPLC; *Fusarium graminearum*

1 Department of Health and Biological Sciences, Abasyn University Peshawar, Khyber Pakhtunkhwa, Pakistan
2 Corresponding author, e-mail: mk03025678947@gmail.com, muddasir.khan0302@gmail.com
3 Department of Microbiology, Abbottabad University of Science and Technology, Khyber Pakhtunkhwa, Pakistan

1 INTRODUCTION

Globally *Bacillus thurenginisis* is considered to be the most predominant soil-dwelling bacterium found in the plants rhizosphere known for their antimicrobial properties. Aforementioned, *Bacillus* strains are known as plant growth promoting rhizobacteria (PGPR) that are associated with plants tolerance against biotic, and abiotic stresses caused by certain fungal phytopathogens (Saxena et al., 2019). In this context the worldwide major loss of maize and other cereal crops is due to fungal phytopathogens. The repertoire of fungal phytopathogens including *Acremonium alternatum* Link (Pal and Gardener, 2006), *Ustilago maydis* (DC.) Corda[(Kwon *et al.,* 2021), *Aspergillus niger* van Tieghem, *Aspergillus flavus* Link, *Puccinia sorghi* Schwein., *Fusarium* species (Rehman et al., 2021), *Helminthosporium*, *Alternaria*, *Rhizopu*s, *Penicillium*, *Drechslera* (Snetselaar and McCann, 2017), *Macrophomina phaseolina* (Tassi) Goid., and *Colletotrichum graminicola* D.J. Politis (Saleem et al., 2012), cause varieties of disease in maize.

Maize (*Zea mays* L.) is the most important cereal crop in the world, covering 75 % of the food requirements all over the world (Hussain et al., 2013). In Pakistan, among the cereal crops, maize is the third most important crop, after wheat and rice. Among these, globally the most important and significant phytopathogen is *Fusarium graminearum*, which causes significant loss of grain crops (Rauwane et al., 2020). The wide range of diseases caused by this plant pathogen includes; fruit rots, *Fusarium* head blight (FHB), wilts, and root rots (Kant et al., 2011).

Chemical compounds have been used to manage these fungal phytopathogens for many decades. They have a potential to generate major environmental problems. Alternative and less environmentally detrimental measures are required to control these plant diseases. *Bacillus* species and other PGPR play a key role among biopesticides. They produce various antimicrobial compounds such as enzymes, lipopeptides, and antibiotics that stimulate plant development while inhibiting pathogenic microbes (Shafi et al., 2017). For *B. thurenginisis* cyclic peptides including, surfactin, mycobacillin, mycosubtilin, subtilin, bacilysin, fengycin, bacillomycin, and iturin are reported that exhibit both antibacterial, and antifungal properties (Khan et al., 2021; Ntushelo et al., 2019).

Surfactin is a lipopeptide composed of cyclic depsipeptides of *β-hydoxy* hepta with possible amino acid combinations of alanine, valine, leucine, or isoleucine at positions 2, 4, and 7 in the cyclic depsipeptide moiety and *β-hydoxy* fatty acid chain variants of C_{13} to C_{16} in the cyclic depsipeptide moiety and *β-hydoxy* fatty acid chain variant (Hue et al., 2001). According to the investigations surfactin has natural antifungal properties produced by *Bacillus* spp. that could inhibit the growth of certain fungal species including, *F. graminearum* (Khan et al., 2021), *Fusarium oxysporum* Schlecht. emend. Snyder & Hansen (Kim et al., 2010), *Colletotrichum gloeosporiodes* (Penz.) Penz. & Sacc. (Snook et al., 2009), *Fusarium verticillioides* (Sacc.) Nirenberg (Dunlap et al., 2011), and *Fusarium moniliforme* (Sacc.) Nirenberg (Vitullo et al., 2012).

Therefore, the current study was designed to isolate and characterize *B. thuringiensis* lipopeptide from rhizospheric soil and also to assess its antifungal efficacy against the fungal phytopathogen *F. graminearum* of maize.

2 MATERIALS AND METHODS

2.1 BACTERIAL AND FUNGAL ISOLATION

A total of 20 maize rhizospheric soil samples were collected from various locations in Peshawar, Pakistan, for the isolation of *B. thuringiensis* (Figure 1). *B. thuringiensis* was identified using colony morphology, gram staining, and biochemical-tests such as citrate hydrolysis, catalase, indole production, nitrate reduction, Voges-Proskauer (VP), motility, H_2S production, and crystal formation (Amin et al., 2015). *F. graminearum* was isolated using a sample acquired from a diseased maize plant in Peshawar, Pakistan (Figure 1), and identified using colony morphology and microscopic analysis (Uddin et al., 2019; John et al., 2006).

2.2 LIPOPEPTIDE EXTRACTION AND IDENTIFICATION

In a shaking flask containing nutrient broth medium (Oxoid™), all morphological and biochemical based confirmed isolated colonies of *B. thuringiensis* were injected. The flask was incubated for 16 hours at 30 °C with shaking incubator at 200 rpm. Afterward the culture was transferred to an Erlenmeyer flask containing 99 ml of Tryptic Soy Broth (TSB) medium (Oxoid™) and incubated overnight at 30 °C with shaking incubator at 200 rpm. The optical density (OD) of the *B. thuringiensis* growth curve was measured at 600 nm using a spectrophotometer (Shimadzu, UV-1800). After the decline phase of *B. thuringiensis* growth, the culture was removed and centrifuged at 6000 rpm for 30 minutes. The supernatant was filtered using a sterile 0.22 µm filter (Mater et al., 2009). The extract was then centrifuged for 10 minutes at 1000 rpm and 20 °C. The deposit was dissolved in a solution

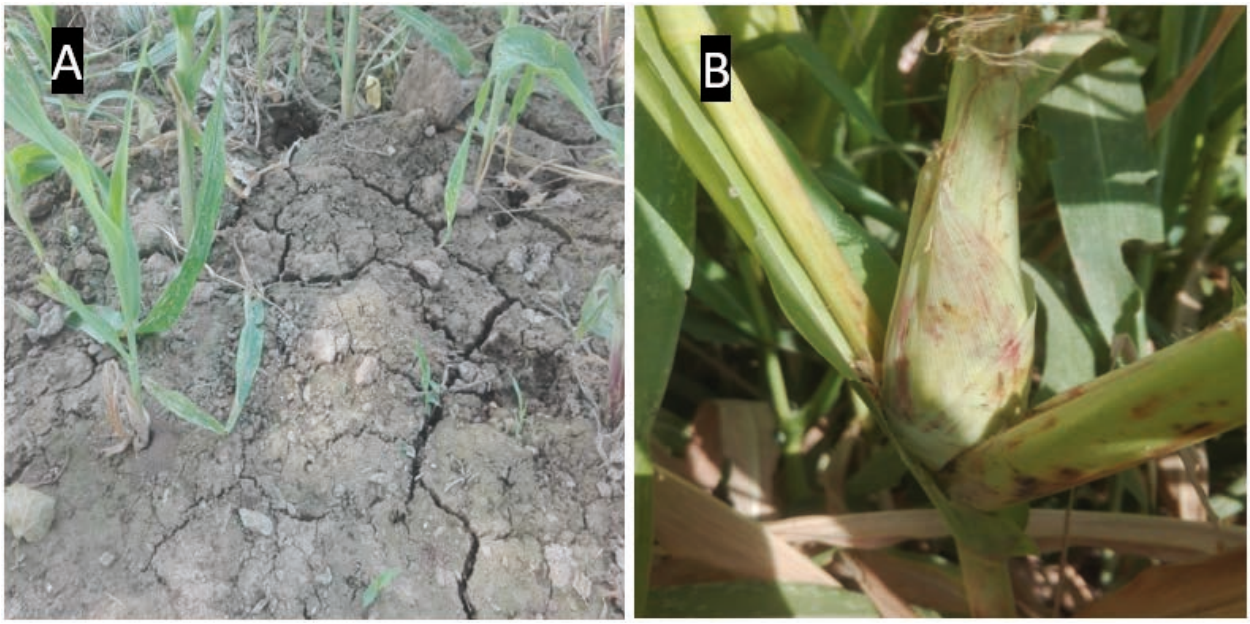

Figure 1: (A) Sampling site of maize rhizospheric soil for the isolation of *B. thuringiensis,* **(B)** Diseased maize for the isolation of *F. graminearum*

of methanol (Analytical grade, VWR Chemicals BDH®) and water (50:50, v/v) and filtered again using a 0.22 µm filter membrane. For purification, the sample was treated three times with 20 ml chloroform (VWR Chemicals BDH®). The bottom layer was collected and chloroform was evaporated at 50 °C temperature by using a hotplate stirrer. Methanol was used to dissolve the residue. Surfactin from the extract were identified by introducing 50 µl of the extract into a Shimadzu 20A UV-Vis HPLC at a wavelength range of 200–250 nm. The isocratic HPLC method was employed, along with a 4.6 × 150 mm C-18 normal phase column (Mater et al., 2009). For the identification of surfactin by HPLC experiment, acetonitrile was utilized as a mobile phase. Surfactin were discovered after comparing the observed peak to previously published data (Meena et al., 2014).

2.3 ANTIFUNGAL ACTIVITY OF LIPOPEPTIDE EXTRACT

To test the antifungal activity of surfactin, four 5 mm wells were created on potato dextrose agar (PDA) (Oxoid™) using a sterilized cork borer. The methanol was used as a control and also used to create concentrations of the lipopeptide extract of 20 %, 40 %, 60 %, and 80 %, respectively. The wells were filled with 200 *µl* of methanol (control), 20 %, 40 %, 60 %, and 80 % concentrations of lipopeptide extract, respectively. A colony of active growing *F. graminearum* was placed in the middle of media plates using sterile forceps and incubated at 30 °C for 3-7 days. Five repetitive antifungal analysis of the extracted lipopeptde was done by the same method described above. The inhibitory zones were measured and recorded (Mater et al., 2009). The obtained mean zone of inhibitions was analyzed using a one-way ANOVA test using the Statistical Packages for Social Sciences (SPSS) version 23.0 software and Microsoft Excel.

3 RESULTS AND DISCUSSION

3.1 BACTERIAL ISOLATE

In 20 rhizospheric soil samples *B. thuringiensis* 12 isolates were confirmed by various criteria such as, colony morphology, gram staining, and biochemical assays (Table 1). Previous results revealed that *Bacillus* species are primarily found in rhizospheric soil and that their metabolites have antibiotic characteristics as they can inhibit or restrict the development of other microorganisms (Amin et al., 2015).

3.2 FUNGAL ISOLATE

In context to this study, *F. graminearum* was isolated from infected maize plants and identified using colony morphology (white to pinkish), and microscopic assessment (Hyaline septate hyphae, two to multi-celled and

Table 1: Morphological and Biochemical characteristics of *B. thuringiensis*

Tests	Results
Colony Morphology	Circular, rough, opaque, fuzzy white or slightly yellow
Gram Staining	Gram Positive
Shape	Rod shaped
Motility	Positive
Catalase	Positive
Indole production	Negative
Citrate utilization	Positive
H2S production	Negative
Crystals formation	Positive
Identified Strains	B. thuringiensis

sickle-shaped) in the current investigation. *Fusarium* head blight (FHB) disease is caused by *F. graminearum* in maize. This fungus exhibit certain sign of early bleaching during infection which could reduce grain production and quality (Ntushelo et al., 2019).

3.3 LIPOPEPTIDE IDENTIFICATION

According to the current study findings, *B. thuringiensis* was grown to produce lipopeptides, and the optical density (OD) of the growth curve was measured (Figure 2). Lipopeptides isolated from *B. thuringiensis* were analyzed by HPLC using acetonitrile as the mobile phase. At 210 nm and retention period 3-5 minutes, the observed peak area was 3.990 (Figure 3), which is similar to the peaks found earlier in surfactin literature data (Mubarak et al., 2015). Previous studies are in agreement with our findings. According to the Deepak and Jayapradha (2015), they identified lipopeptide surfactin by HPLC which are produced by *B. thuringiensis*. In another study, the lipopeptde fengycin produced by *B. thuringiensis* was identified by HPLC techniques (Kim et al., 2004).

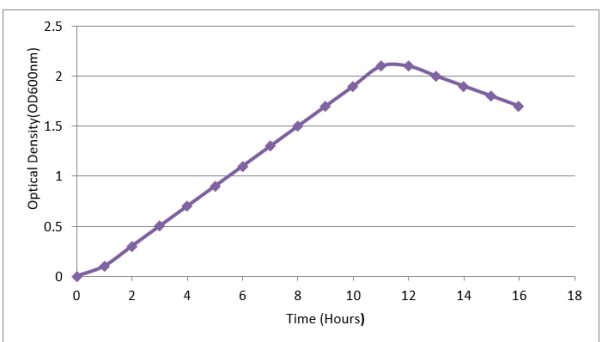

Figure 2: Optical density (OD) of the growth curve of *B. thuringiensis* at 600 nm wavelength

3.4 ANTIFUNGAL ACTIVITY OF LIPOPEPTIDE

B. thuringiensis lipopeptide surfactin against the development of *F. graminearum* was investigated in this work. The surfactin lipopeptide efficiently suppressed the growth of *F. graminearum* (Figure 4). According to earlier research, isolated *Bacillus* spp. from the rhizosphere, particularly *B. subtilis*, reduced the growth of *F. graminearum*. *Bacillus* spp. is also effective in the prevention of *Fusarium* head blight (FHB) and root rot; they stimulate plant development and inhibit the mycelial growth of fungal infections through antagonistic action (Herba et al., 2020; Madhi et al., 2020; Dukare et al., 2020). In this study, lipopeptide surfactin from *B. thuringiensis* was tested against *F. graminearum* at 20 %, 40 %, 60 %, and 80 % concentrations (Figure 4). The zone of inhibition was the greatest at the 80 % concentration, followed by the 60 %, 40 %, and 20 % concentrations ($p < 0.005$), respectively. These findings are in agreement with previous report, in which the surfactin action against *F. oxysporum* (Deepak and Jayapradha, 2015) was screened. According to a recent study, microorganisms were isolated from plant anthers and wheat kernels to test their antagonistic activity against *F. graminearum*, the causative agent of *Fusarium* head blight (FHB). *B. subtilis* has a strong antifungal impact on *F. graminearum* mycelium, sporulation, and DON formation, with inhibition values of 87.9 %, 95.6 %, and 100 %, respectively (Zhao et al., 2014).

4 CONCLUSION

Lipopeptides obtained from *Bacillus* species have less negative environmental effects as compared to chemical compounds. The current study concluded that *B. thuringiensis* isolated from the rhizosphere of maize crop may produce lipopeptide surfactin, which has a high potential to inhibit the growth of *F. graminearum*. The study is also emphasizing surfactin as potential biological control agent with widespread usage. We are also encouraging other researchers to take advantage of newly invented techniques to explore mechanism of action of various *Bacillus* strains against phytopathogens.

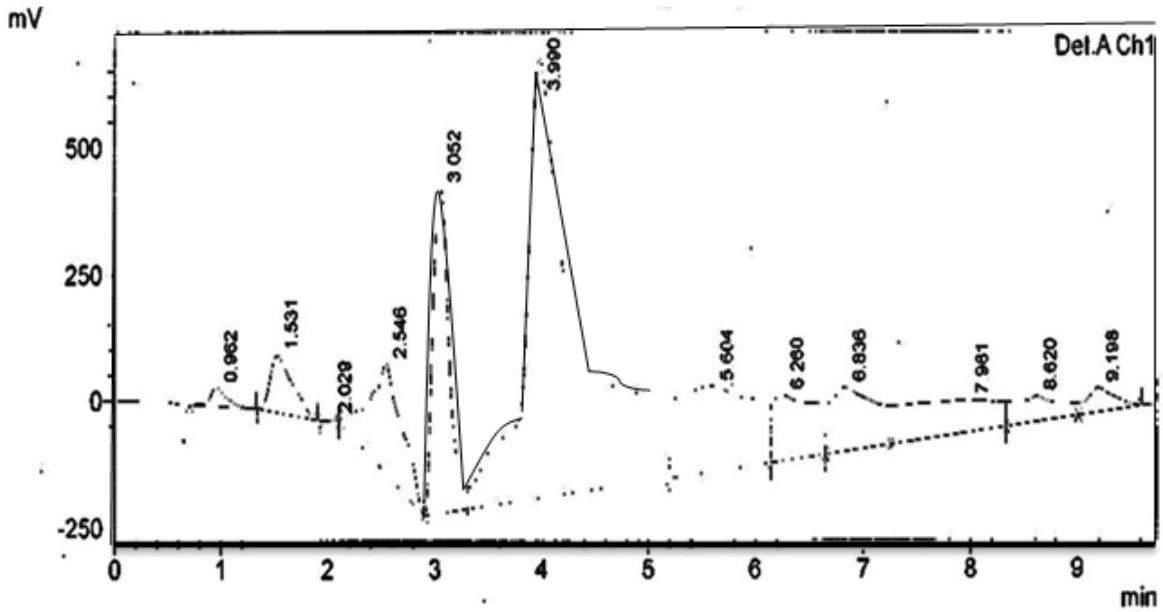

Figure 3: HPLC Chromatogram of *B. thuringiensis* lipopeptide surfactin obtained at 210nm, retention time between 3-5 minutes and peak area 3.990

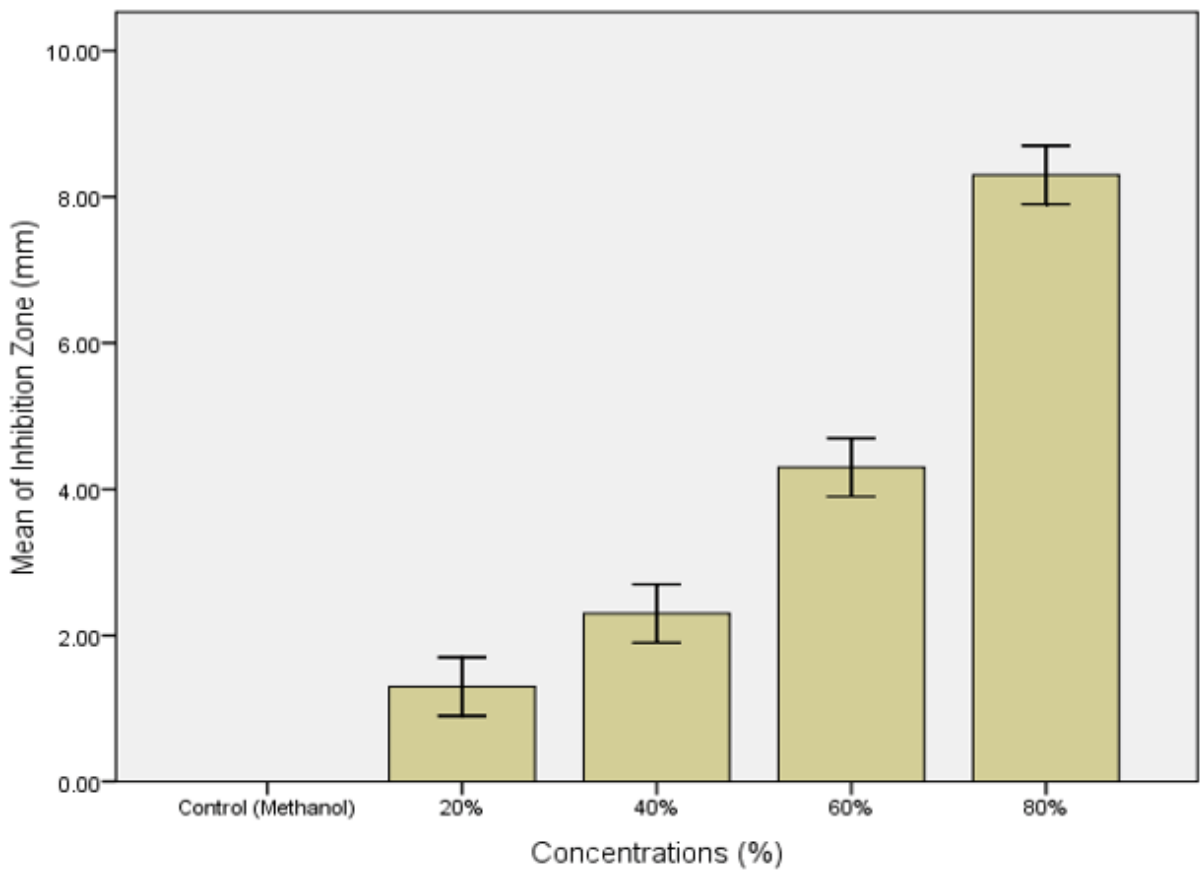

Figure 4: *B. thuringiensis* lipopeptide surfactin zone of inhibition (mean) against *F. graminearum* at various concentrations ($p < 0.005$)

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Expression of *IRT1* gene in barley seedlings under zinc deficiency at optimal and low temperatures

Natalia KAZNINA [1, 2], Natalia REPKINA [1], Yulia BATOVA [1], Alexandr TITOV [1]

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Expression of *IRT1* gene in barley seedlings under zinc deficiency at optimal and low temperatures

Abstract: The deficiency or excess of zinc (Zn) cause negative effect on plant metabolism and development. Therefore, plants have established a tightly controlled system, including protein transporters to balance the uptake and utilization of metal ions. In this study, the relative expression of *HvIRT1* gene, encoding the transmembrane protein IRT1 was analyzed in shoots and roots of barley (*Hordeum vulgare* 'Nur') under zinc deficiency at optimal (22 °C) or low (4 °C) temperatures. The Zn deficiency (0 μmol) caused an increase in *HvIRT1* gene expression under both optimal temperature condition and cold. Although, the difference in mRNA content of *HvIRT1* gene in roots of barley under optimal and low temperature was not observe. However, the *HvIRT1* expression in leaves was higher at optimal temperature compare with cold condition. Moreover, long-term (7 days) of low temperature influence along with zinc deficiency leads to a significant decrease in the amount of *HvIRT1*transcripts in leaves, that corresponds to a decrease of photosynthesis rate and biomass accumulation. Overall, these findings suggest that *HvIRT1* gene play an important role in plant's response to zinc deficiency under optimal temperatures condition as well as at cold.

Key words: *IRT1*; *Hordeum vulgare*; zinc deficiency; low temperatures

Izražanje *IRT1* gena v sejankah ječmena ob pomanjkanju cinka pri optimalnih in nizkih temperaturah

Izvleček: Pomanjkanje ali prebitek cinka (Zn) povzročata negativne učinke na presnovo in razvoj rasttlin. Zaradi tega so rastline razvile dobro nadzorovan sistem, vključno s proteinskimi transporterji za uravnavanje privzema in porabe kovinskih ionov. V raziskavi je bilo analizirano izražanje *HvIRT1* gena, ki kodira transmembranski protein *IRT1* v poganjkih in koreninah ječmena (*Hordeum vulgare* 'Nur') ob pomankanju zinka pri optimalni (22 °C) in nizki (4 °C) temperaturi. Pomanjkanje cinka (0 μmol) je povzročilo povečano izražanje *HvIRT1* gena pri optimalni kot pri nizki temperaturi. Razlika v vsebnosti mRNK *HvIRT1* gena v koreninah ječmena v optimalnih razmerah in pri nizki temperaturi ni bila opažena, a kljub temu je bilo izražanje gena *HvIRT1* v listih večje pri optimalni temperaturi v primerjavi s hladnimi rastnimi razmerami. Daljša izpostavitev (7 dni) nizki temperature je ob pomanjkanju cinka povzročila značilno zmanšanje transkriptov *HvIRT1* v listih, kar ustreza upadu fotosinteze in akumulacije biomase. Ta odkritja nakazujejo, da igra *HvIRT1* gen pomembno vlogo pri odzivu rastlin na pomanjkanje cinka tako v optimalnih razmerah kot pri nizkih temperaturah.

Ključne besede: *IRT1*; *Hordeum vulgare*; pomanjkanje cinka; nizke temperature

1 Institute of Biology, Karelian Research Centre, Russian Academy of Sciences, Petrozavodsk, Russia
2 Corresponding author, e-mail: kaznina@krc.karelia.ru

1 INTRODUCTION

Zn deficiency has been recognized as an important factor affecting crop production. Cell transmembrane proteins from ZIP family (*zinc-iron-regulated transporter*) play an important role in providing plants of the necessary amount of zinc (Pedas et al., 2008; Lee and An, 2009; Yamunarani et al., 2013). The IRT1 (*iron-regulated transporter1*) proteins, belonging to the ZIP family, were firstly discovered in cereals. ZIP proteins are able to transport various divalent cations, such as Fe^{2+}, Zn^{2+}, Cu^{2+}, $Mn^{2+}$from the rhizosphere through the plasma membrane into the cytoplasm of root cells, as well as from xylem vessels in leaf mesophyll cells (Palmer and Guerinot, 2009). It has been reported that zinc deficiency leads to increase in the activity of IRT1 protein and *IRT1* gene expression in parallel with high accumulation of Zn in roots and shoots of rice, maize and *Arabidopsis* (Ishimaru, 2006; Pedas et al., 2008; Yamunarani et al., 2013; Kabir et al., 2017 etc.). Therefore, IRT1 protein play essential role in Zn uptake, translocation and storage of Zn in plant cells especially under Zn deficiency. Although, most of the evidence from these studies was performed on plants under optimal temperature conditions, however, in nature plants are often exposed to low temperatures during the growing season, that caused in decrease in supply of nutrients to root cells, that result in their deficiency in plants (Hacisalihoglu et al., 2001). Perhaps this effect may be associated with a decrease in the activity of transport proteins (Guerinot, 2000; Hacisalihoglu et al., 2001). Despite this data, the effect of cold on *IRT1* gene expression and IRT1 protein activity is still unclear. Some reports demonstrated that the IRT1 protein activity regulated at the both translation and transcription level (Shin et al., 2013; Brumbarova et al., 2015). According these findings, we studied the expression of the *IRT1* gene in the roots and leaves of barley under zinc deficiency at optimal and low temperatures.

2 MATERIALS AND METHODS

2.1 PLANT MATERIAL AND GROWTH CONDITIONS

Seeds of barley (*Hordeum vulgare* 'Nur')were purchased from the Tula Research Institute of Agriculture, Tula, Russia. Seedlings were cultivated in a growth chamber with 14 h photoperiod, a photo-synthetic photon flux density of 180 μmol m^{-2} s^{-1}, a temperature of 22 °C and a relative humidity of 60 - 70 % on Hoagland-Arnon nutrient solution (pH 6.2 to 6.4) with optimal (variant Zn 2 μmol + 22 °C) zinc content or its deficiency (variant Zn 0 μmol + 22 °C). Seven-day-old seedlings (initial level) were separated. One part of the plants of both variants was exposed to low temperature (4 ° C) (variants Zn 2 μmol + 4 °C and Zn 0 μmol + 4 °C), and the other part was left under the optimal temperature during 7 days. All parameters were measured at day 0 (initial level) and 1, 3, 7 days after treatments.

2.2 BIOMASS AND NET PHOTOSYNTHETIC RATE DETERMINATION

For biomass determination plants were collected, their shoots and roots separated and dried in an oven at 85 °C for 24 h. The net photosynthetic rate (P_N) was measured during a day using portable photosynthesis system HCM-1000 (Walz, Effeltrich, Germany).

2.3 GENE EXPRESSION

The expression pattern of *HvIRT1* gene in leaves and roots was monitored by real-time PCR. Frozen roots and leaf tissues were homogenized with liquid nitrogen. Total RNA was extracted using a TRizol reagent (*Evrogen*, Moscow, Russia) as instructed by them anufacturer. The total RNA was treated with RNase free DNase (*Syntol*, Moscow, Russia) to remove genomic DNA. The purity of RNA samples and their concentrations were determined spectrophotometrically (*SmartSpecPlus*, *Bio-Rad*, Hercules, USA): samples withA260/A280 ratios within 1.8 - 2.0 were used for further analysis. The total RNA (1 μg) was reverse-transcribed using a *MMLV* RT kit (*Evrogen*) following the supplier's recommendations. Real-time quantitative PCR was performed using the *iCycler iQ* detection system (*Bio-Rad*). Analyzes were performed using a *SYBR Green* PCR kit (*Evrogen*). The PCR conditions consisted of denaturation at 95 °C for 5 min followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 56 °C for 30s, and extension at 72 °C for 45 s. A dissociation curve was generated at the end of each PCR cycle to verify that a single product was amplified using *iCycler iQ*. To minimize sample variations, mRNA expression of a target gene was normalized relative to the expression of a housekeeping gene *actin*. The mRNA content of target gene (*HvIRT1)* were quantified in comparison to the *actin* by the ΔΔCt method (Livak and Schmittgen, 2001). Primers were designed (using the *Primer Design* program): *HvActin* (U21907) ATGTTTTTTTCCAGACG (direct) and ATCCAAGCCAACCCAAGT (reverse), *HvIRT1 (*EU54802) GTGCTTCCACCAGATGTTTGAG (direct) andGGATGCCGACGACGATGA (reverse).

2.4 STATISTICAL ANALYSIS

All data are presented as means ± standard errors (SEs) from at least three independent replicates. Significant differences between variants and relative to the initial level were calculated by two-way analysis of variance (ANOVA) using Microsoft Excel 2010. Student's *t*-test was applied to compare statistical significance at level of $p < 0.05$.

3 RESULTS AND DISCUSSION

Table 1 shows the effect of Zn deficiency under optimal and low temperatures on plant dry mass (DM) accumulation and net photosynthetic rate (P_N). Under optimal temperature conditions Zn deficiency did not significant affect the DM and P_N parameters compare with plants grown with optimal Zn concentration. However, after 7 days Zn deficiency (Zn 0 µmol + 22 °C) caused slightly reduction root DM compare with variant Zn 2 µmol + 22 °C.

Despite the Zn concentration the low temperature leads to reduce DM accumulation and P_N parameter. Although, after 7 days Zn deficiency in combination with low temperature (Zn 0 µmol + 4 °C) leads to significant decrease in root DM and photosynthesis activity compare with variant Zn 2 µmol + 4 °C.

Under optimal growth conditions (variant Zn 2 µmol + 22 °C) the transcript level of *HvIRT1* gene gradually increase in roots and leaves of barley during 7 days (Fig.).While, at the initial level, the *HvIRT1* gene expression was 3-fold higher in roots seedlings grown with Zn deficiency (Zn 0 µmol + 22 °C). Further *HvIRT1* gene mRNA content in variant Zn 0 µmol + 22°C slightly increased in compare with variant Zn 2 µmol+ 22 °C. At the initial level there was no significant difference in mRNA content of *HvIRT1* gene in leaves between Zn 2 µmol + 22 °C and variant Zn 0 µmol + 22 °C variants (Fig.). However, leaves of barley exposed to Zn deficiency (Zn 0 µmol + 22 °C) showed higher the *HvIRT1*mRNA content within 1 day and slightly decrease on seventh day in compare with Zn 2 µmol + 22 °C variant.

Low temperature caused an increase in *HvIRT1* gene expression in roots along with time of exposure in both variants (Zn 2 µmol + 4 °C and Zn 0 µmol + 4 °C) (Fig.). However, on 7th day of experiment the amount of *HvIRT1* gene transcripts in roots of barley variant Zn 0 µmol + 4 °C were greater than in variant Zn 2 µmol + 4 °C. Low temperature resulted in *HvIRT1* gene transcript accumulation in leaves of barley variant Zn 2 µmol + 4 °C. After 1 day of cold impact the *HvIRT1* gene expression was 4-fold higher in variant Zn 2 µmol + 4 °C compare with initial level and 10-fold higher on seven day of experiment. In variant Zn 0 µmol + 4 °C the *HvIRT1* gene mRNA content increased after 3 days of exposure to low temperature and significant decreased on seven day of experiment.

In general, our results demonstrated a high tolerance of barley 'Nur' to Zn deficiency. It was shown that under conditions of zinc deficiency, the accumulation of biomass and photosynthetic activity remained stable until the end of the experiment. Similar data were described previously (Hajiboland and Beiramzadeh. 2008; Kabir et al., 2017). The capability of plants to grow under Zn deficiency mostly depend on metal transporters activity, including protein IRT1 (Suzuki et al., 2012; Yamunarani et al., 2013; Kabir et al., 2017). Zn deficiency under optimal temperature leads to increase in *HvIRT1* transcript amount in roots and leaves of barley that resulted in activation of transport metal ions in cells and kept growth and photosynthetic activity. This also support the fact that after 7 days the gene expression of *HvIRT1* decreased along with photosynthetic activity. The negative effect of Zn deficiency on photosynthesis process was described previously in *Oryza sativa* L. (Hajiboland and Beiramzadeh, 2008), *Zea mays* L. (Liu et al., 2016), *Sorgum bicolor* (L.) Moench (Li et al., 2013), however the transport protein activity was not studied.

There are fragmentary data about influence of low temperature on metal transporters activity. The negative effect of 0°C temperature on gene expression, encoding protein transporters ZIP1 and ZIP3 was described previously (Grotz et al., 1998). We have also shown the increase in *HvIRT1* gene expression in barley under chilling (Kaznina et al., 2019). It is supposed that due to negative influence of chilling on nutrient transport into cells the increase in *HvIRT1* gene expression in roots that we reported can be a result of requirement of mesophyll cells of leaves in nutrients that are necessary for photosynthesis process. There are no data about activity of transporter proteins under chilling and Zn deficiency condition. Thus considering the results described above for barley under low temperature and Zn deficiency during 7 days in leaves the *HvIRT1* mRNA content significantly decreased. It seems to be a result of decrease in requirement in nutrients caused by slowdown of photosynthetic activity and growth of seedlings under stress conditions. Additionally, it can be a result of interruption in signal transduction from leaves to roots that was demonstrated previously in plants under chilling and optimal nutrient level (Giehl et al., 2009; Romera et al., 2011).

Table 1: The effect of zinc deficiency on the root and shoot dry biomass and photosynthesis rate of barley plants 'Nur' at optimum (22 °C) and low (4 °C) temperatures

	Time, days			
Variant	0 (initial point)	1	3	7
		Dry root biomass, mg		
Zn 2+22°C	5.51 ± 0.55 aA	6.72 ± 0.48 aA	7.29 ± 0.42 bA	8.21 ± 0.60 bA
Zn 0 +22°C	5.10 ± 0.45 aA	6.51 ± 0.41 aA	6.92 ± 0.38 bA	7.96 ± 0.46 bA
Zn 2+4°C	5.51 ± 0.55 aA	6.20 ± 0.47 aA	6.26 ± 0.48 aB	7.36 ± 0.54 bAB
Zn 0+4°C	5.10 ± 0.45 aA	6.13 ± 0.29 aA	6.23 ± 0.35 aB	6.36 ± 0.46 aB
		Dry shoot biomass, mg		
Zn 2+ 22°C	20.13 ± 1.88 aA	20.89 ± 1.21 aA	27.21 ± 1.48 bA	31.32 ± 2.41 bA
Zn 0 +22°C	18.99 ± 1.89 aA	19.51 ± 1.09 aA	24.06 ± 1.62 bA	32.13 ± 1.32 bA
Zn2+4°C	20.13 ± 1.88 aA	20.00 ± 1.51 aA	20.87 ± 1.50 aB	24.77 ± 1.39 bB
Zn 0 +4°C	18.99 ± 1.89 aA	19.63 ± 1.16 aB	18.42 ± 0.95 aC	19.28 ± 1.21 aC
		Net photosynthetic rate, μmol CO_2 $m^2 \cdot s^{-1}$		
Zn 2+ 22°C	6.82 ± 0.16 aA	7.77 ± 0.17 bA	6.54 ± 0.50 aA	6.53 ± 0.10 aA
Zn 0 +22°C	6.95 ± 0.14 aA	7.69 ± 0.14 bA	6.46 ± 0.49 aA	6.27 ± 0.12 aA
Zn2+4°C	6.82 ± 0.16 aA	4.65 ± 0.20 bB	4.86 ± 0.12 bB	4.16 ± 0.09 cB
Zn 0 +4°C	6.95 ± 0.14 aA	4.83 ± 0.12 bB	4.35 ± 0.15 bB	3.82 ± 0.17 cC

Different lowercase letters indicate significant differences in columns (between variants), uppercase letters - in rows (relative to the initial level) ($p < 0.05$).Values perform mean ± SE (n = 10)

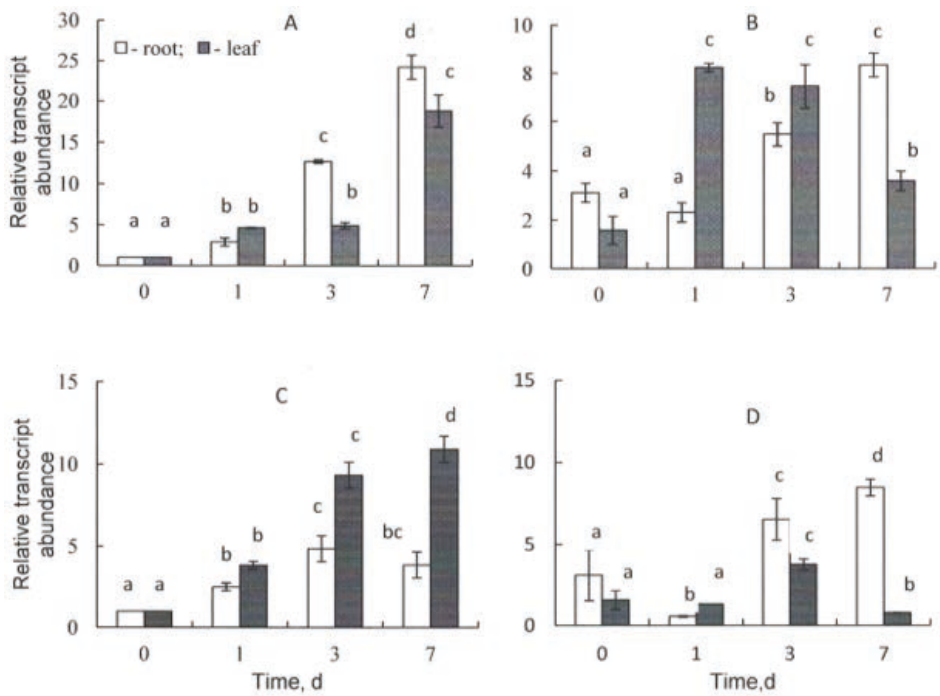

Figure 1: The effect of zinc optimum (a, c) and zinc deficiency (b, d) on *HvIRT1* gene transcription in the roots and leaves of barley plants 'Nur' at 22 °C (a, b) and 4 °C (c, d). Different lowercase letters indicate significant differences relative to the initial level ($p < 0.05$)

4 CONCLUSIONS

According to our results it was shown that Zn deficiency caused in increase in *HvIRT1* gene expression in leaves of barley not only under optimal temperature as was shown in another reports but also under cold. For the first time we demonstrated that long-term exposure (7 days) to low temperature leads to significant decrease in *HvIRT1* mRNA amount in leaves in parallel to slow down in photosynthetic activity and growth. Taken together, the results presented here illustrate the participation of the *HvIRT1* gene in adaptation to Zn deficiency under optimal and low temperatures conditions.

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Evaluation of traits related to bread wheat (*Triticum aestivum* L.) root in drought tolerance applied at the beginning of vegetative and reproductive stages

Arman ESKANDARI SHAHRAKI [1, 2], Mohammad-Mehdi SOHANI [1], Sadollah HOOSHMAND [3], Ali AALAMI [1], Habibullah SAMIZADEH [1]

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Evaluation of traits related to bread wheat (*Triticum aestivum* L.) root in drought tolerance applied at the beginning of vegetative and reproductive stages

Abstract: Roots play an important role in wheat grain yield, especially under drought stress conditions. To investigate root characteristics under drought stress conditions in bread wheat, 90 lines F10 obtained from the crossing ('Yecora Rojo' × 'Chinese Spring') randomly with the parents of the population were examined. The study was conducted in the form of a split-plot design with a randomized complete block base in three conditions including: 1. no stress, 2. application of drought stress at the beginning of the vegetative stage, and 3. application of drought stress at the beginning of the reproductive stage. The results showed, interaction between genotype and condition of drought was significant for all root-related traits, except shallow root dry mass, at the level of 1 % probability. The response of root-related traits to different types of drought stress was very complex. The longest root length, decrease for 13.3 % was during stress at the beginning of the vegetative stage in comparison to non-stress conditions, while the same trait increased for 4.9 % during stress at the beginning of the reproductive stage, comparison to non-stress conditions. The results of principal component analysis under non-stress conditions showed that by considering the distribution of genotypes compared to the first two components, genotypes can be identified that have more yield with the proper root condition and vice versa.

Key words: deep root; drought tress; main components; shallow root; tolerance index

Ovrednotenje lastnosti korenin krušne pšenice (*Triticum aestivum* L.) povezanih s sušnim stresom v začetku vegetativne in reproduktivne faze razvoja

Izvleček: Korenine imajo pomembno vlogo za pridelek zrnja pšenice, še posebej v razmerah suše. Za preučevanje značilnosti korenin krušne pšenice v razmerah sušnega stresa je bilo pridobljenih 90 linij F10 iz naključnih križanj med starševskima sortama Yecora Rojo in Chinese Spring. Raziskava je bila izvedena kot poskus z deljenkami kot popolni naključni bločni poskus v treh stresnih razmerah: 1. brez stresa, 2. sušni stres na začetku vegetativne faze razvoja in 3. sušni stres na začetku reproduktivne faze razvoja. Rezultati so pokazali, da je bila interakcija med genotipom in razmerami stresa značilna za vse s koreninami povezane lastnosti na ravni 1 % verjetnosti, razen za suho maso plitvih korenin. Odziv s koreninami povezanih lastnosti na različne vrste sušnega stresa je bil zelo kompleksen. Zmanšanje dolžine najdaljših korenin za 13,3 % v primerjavi s kontrolo je bilo, ko je sušni stres nastopil na začetku vegetativne faze razvoja med tem, ko se je isti parameter povečal za 4,9 % ob nastopu sušnega stresa na začetku reproduktivne faze razvoja v primerjavi z nestresnimi razmerami. Rezultati analize glavnih component iz poskusa v nestresnih razmerah so pokazali, da bi z upoštevanjem razvrstitve genotipov glede na dve prvi komponenti te lahko razdelili na tiste, ki imajo več pridelka in primeren koreninski sistem in obratno.

Ključne besede: globoke korenine; sušni stres; glavne komponenete; plitve korenine; tolerančni indeks

1 Department of Agriculture and Plant Breeding, Guilan University, Rasht, Iran
2 Corresponding author, e-mail: ph.d.eskandari@gmail.com
3 Department of Agriculture and Plant Breeding, Shahrekord University, Shahr-e-kord, Iran

1 INTRODUCTION

Water scarcity is a serious challenge to survival, especially in arid and semi-arid regions. Different climatic models predict that drought stress will increase in frequency and intensity and this will confirm the shortage of available water in the future (Nadeem, 2019). Therefore, it seems necessary to understand the consequences of these changes on the production of different types of crops (Shanker, 2014). To date, many traits such as: number of seeds per plant, plant biomass, thousand-seed mass (Ghassemi-Golezani, 2018), grain yield, harvest index (Kadam, 2012), relative leaf water content, amount of tissue lost water, leaf wax content, leaf thickness, stomatal characteristics (Heidari, 2012), number of days to spike, number of days to physiological maturity, plant shading temperature, green content of the plant (Hasani, 2016), to study how crops react to drought stress in the field and laboratory has been studied. Most of the collected information about drought stress is related to traits that consider conditions above the soil surface and limited attention has been paid to plant root traits. Ignoring the selection of root traits in wheat is mainly due to problems in measuring the traits of the root system and how the roots are distributed (Richards, 2008). Under water restriction conditions, plant growth is directly determined by the ability to absorb and convert water into plant biomass (Jin et al., 2018), therefore the ability of roots to grow under drought stress conditions is an adaptive feature for plants, especially in rainfed conditions and with limited irrigation (Dalal, 2018). Root architecture is one of the most promising features for drought stress and can be used positively in drought resistance breeding programs. This feature enables the plant to extract water more efficiently from deeper soil layers, under very dry environments (Nadeem, 2019). Therefore, genetic modification of plants to have an effective root system, with all its problems, is very important for optimal production in rainfed conditions and with limited water (Kadam, 2012).

Hammer et al. (2009) showed that root architecture and its associated water uptake are more important than canopy architecture and plant light uptake for biomass and plant performance in high-density vessels. Advances in water uptake from subsoil by rainfed wheat can make a significant contribution to its yield (Jin, 2015b). In order for wheat to be highly productive, it is necessary to remove barriers to plant growth by supporting the root system that is effective in absorbing water and nutrients (Jin, 2015a). Drought tolerance depends on the plant's ability to avoid water leakage from plant tissues, which is affected by root architecture, including: increased root length, root density, and deep rooting, because the plant can much more than the soil to search for water absorption (Sofi, 2018).

Gao and Lynch (2016) reported that they are very effective in tolerating drought stress, increasing rooting depth, and subsequently improving water uptake from deeper soil levels. Also, traits such as root thickness, root dry mass, root volume and root density have high heritability that can have a positive effect on drought stress tolerance (Kadam, 2012). Axial roots are a key element in the plant root phenotype. Axial roots are the main structure of root biomass and form a framework for lateral root growth and therefore have a significant effect on lateral root penetration into deeper soil slopes (Gao and Lynch, 2016). On the other hand, the usefulness of a strong root system to increase yield in environments without water stress is much more effective than the same type of root system in drought stress conditions, because a strong root system may reduce the risk of depletion of soil water before completion, increase the grain filling stage (Sofi, 2018).

In general, according to different reports for the root system, the root reaction under drought stress conditions is very complex and it is very difficult to maintain a balance between traits to modify its characteristics. However, roots are a semi-latent plant that is difficult to ignore their importance in yield (Koolachart et al., 2013; Bardgett et al., 2014). At present, the study of root systems and their importance in water and nutrient uptake and their role in drought stress resistance has been considered by agricultural researchers. The study of plant root systems is very limited due to the difficulties associated with root studies, including observation, measurement of related traits and their manipulation in the field and the use of destructive methods (Sharma et al., 2011; Thangthong et al., 2016).

Carrying out agronomic and physiological studies related to root systems in wheat and their results can be very useful in breeding programs to promote, adapt and stabilize grain yield of new wheat cultivars. As a result, identifying and understanding root characteristics for crop development is essential in stressful conditions (Jin et al., 2018). Due to the scarcity of water resources in the country and facing a drought crisis, it is necessary to pay attention to valuable gene resources for use in the wheat breeding program. Identifying beneficial gene sources and genes that control drought tolerance and improving some traits and creating ideal types will play an important role in the development of wheat breeding programs. Therefore, the purpose of this study was to investigate the important root-related traits in recombinant (RIL) layers of bread wheat in tolerance to drought stress applied at the beginning of the vegetative and reproductive stages.

2 MATERIALS AND METHODS

From 156 recombinant F10 inbred lines from the cross between two Chinese Spring cultivars as female parents and 'Yecora Rojo' as male parents, 90 lines were randomly selected and named with RIL letters and genotype number. This population was prepared by single seed selection. The two parents of the population differed significantly in some drought tolerance characteristics such as carbon isotope discrimination and other agronomic and morphological traits (Ehdaie and Waines, 1994). In addition to 90 recombinant inbred lines, the parents of the population and four cultivars named Sorkhatakhm, Pishtaz, Kalhidari and Aflak were also examined in this research. This study was conducted in the form of a split-plot design with a randomized complete block base in three conditions including: 1. no stress, 2. application of drought stress at the beginning of the vegetative stage (code 30 of Zadoks growth scale), and 3. application of drought stress at the beginning of the reproductive stage or boots swollen (code 45 of Zadoks growth scale). Since accurate evaluation of root characteristics in field conditions is almost impossible, an attempt was made to use near-field and controllable conditions in this field.

First, a part of the field was selected, then the surface soil of that area was collected to a depth of 35 cm from the ground. In the same area, a canal 1 m deep, 2 m wide and 25 m long was dug to place the planting tubes. In order to implement the experimental design, plastic pipes with a diameter of 12 cm and a length of one meter were prepared and the pipes with a homogeneous combination of soil containing 35 % of arable soil collected from the field and 65 % of sand (in order to facilitate root tissue separation), was filled and used as an experimental unit. At the end of the plastic pipes, there were holes for water to drain. Also, the soil prepared for filling the culture tubes was sampled and sent to the laboratory to determine the field capacity (FC), wilting point (PWP) and other characteristics, and the soil characteristics used are given in Table 1.

In each experimental unit, three seeds of one genotype were planted and after confirming the establishment of plants, one plant was maintained and the rest were removed. Drought stress was applied in two phenological stages including: beginning of the vegetative stage (code 30 of Zadoks growth scale), and beginning of the reproductive stage or boots swollen (code 45 of Zadoks growth scale) with complete cessation of irrigation, after each line reached the desired phenological stage. Under conditions without drought or normal stress, irrigation was done when the volume percentage of moisture reached about 70 % of the soil field capacity within the cultivation pipes. Volumetric moisture content was measured by PMS-714 humidity meter every three days in stress-free treatment. In order to properly feed the wheat, the fertilizer regime used for each cultivation tube, under stress and non-stress conditions, included 500 ml of Hoogland 50 % fertilizer solution, which was added to each tube in two stages and 250 ml in each stage, before reaching Added to the desired phenological stages for stress application and irrigation time. After physiological examination of most recombinant inbred lines and before harvest, the height of the last leaf from the soil surface was measured. To obtain healthy roots and prevent damage to them, the planting tubes were gently removed from the pit and placed on a horizontal platform, then the tubes were cut and the soil mass inside them was carefully removed. The tubes containing the remaining soil that had not been removed from the roots were then gently inserted into the tub to remove the sand around the roots. The washed roots were placed on a plastic surface and the traits related to the roots included: the size of the longest root, the mass of the shallow roots (zero to 30 cm depth), the mass of the deep roots (deeper depth). (30 cm), root biomass, root to biomass ratio of total plant, root to shoot ratio, plant height, stem dry mass, panicle length, panicle mass, number of seeds per panicle, seed mass per plant, plant biomass, mass thousands of seeds and biological yield were measured. For more detailed information on the tolerance or susceptibility of genotypes to drought stress, stress tolerance indices, which in many studies had a significant correlation with yield, including mean productivity (MP) indices, geometric mean productivity (GMP) and stress tolerance index (STI) were calculated and evaluated.

Table 1: The soil profile used to fill the planting tubes

Sand (%)	Silt (%)	Clay (%)	EC ($ds.m^{-1}$)	FC (%)	P.W.P (%)	pH
70	13.5	16.5	3.39	37.1	15.4	7.4

$$MP = \frac{Ys+Yp}{2} \qquad GMP = \sqrt{(Ys)(Yp)} \qquad STI = \left(\frac{Yp}{\overline{Yp}}\right)\left(\frac{Ys}{\overline{Ys}}\right)\left(\frac{\overline{Ys}}{\overline{Yp}}\right) = \frac{(Yp)(Ys)}{(\overline{Yp})^2}$$

Yp and Ys yield genotypes under normal and stress conditions, respectively.

Descriptive statistics, analysis of variance, evaluation of genotype response based on tolerance or drought sensitivity indices, ranking of genotypes based on tolerance indices at all levels of stress, grouping of genotypes based on average rank index and checking the accuracy of grouping by independent comparison test, was done. Euclidean distance was used to measure the dissimilarity between genotypes by hierarchical clustering method (average linkage method). The main components for

each of the stress-free and stress-free environments were analyzed.

3 RESULTS AND DISCUSSION

The results of analysis of variance from the study of traits are presented in Table 2. According to the results, it was found that there is a significant difference between genotypes in terms of all root-related traits at the level of one percent probability. Also, the interaction of genotype under environmental conditions was significant for all root traits, except shallow root dry mass, at the level of 1 % probability. Bardgett et al. (2014) reported that diversity in root traits not only exists between different species and cultivars, but also the diversity of root traits within the species is very high, and this has the potential for plants. It helps to show different abilities in absorbing water and nutrients, and there is a lot of evidence that some root-related traits respond quickly to environmental changes. In another study of drought stress on wheat, the difference between maximum root length, total root biomass, root biomass up to a depth of 30 cm and root biomass greater than 30 cm depth, the difference have shown significance (Kadam et al., 2012).

Also Sofi et al. (2018) showed that drought stress significantly affects root biomass, rooting depth, total root length, root volume. The results of analysis of variance of yield-related traits and yield components showed a significant difference in the level of 1 % probability between genotypes and also the existence of genotype interaction at drought stress levels for all these traits (Table 2).

This indicates genetic diversity as well as different trends in the response of genotypes to drought stress conditions. Sinha et al. (2019) reported that there is an inverse relationship between drought stress levels and yield.

Among the studied traits, the response of root-related traits to different types of drought stress was of particular importance (Table 3). The longest root decrease by 13.3 % compared to non-stress conditions during stress was determined at the beginning of the vegetative stage, while the same trait increased by 4.9 % compared to non-stress conditions during stress at the beginning of the reproductive stage. Root system-related studies that emphasize grain yield in wheat have reported a variety of positive, negative, and neutral relationships (Sofi et al., 2018).

In 2018, study on wheat roots under drought stress, drought stress significantly increased the root length of some genotypes (Dalal et al., 2018). During the application of stress at the beginning of the vegetative stage, the dry mass of shallow roots (depth between 0 to 30 cm) decreased by 2.8 % compared to the non-stress conditions. The main feature of shallow roots, which are located at upper soil levels, is mainly the absorption of water-soluble nutrients, and the performance of roots with a penetration depth of more than 30 cm, in most cases water absorption from the deeper soil levels. (Ehdaie et al., 2016). Root biomass decreased by 13.1 % compared to non-stress conditions during stress application at the beginning of vegetative stage, while the same trait shows a 3.4 % increase compared to non-stress conditions during stress application at the beginning of reproductive stage (Table 3).

Since root biomass was the result of the total dry mass of shallow and deep roots and the dry mass response of shallow roots to both drought stress conditions was decreasing, so the increase of root biomass during stress application at the beginning of reproductive stage. It can be caused by the increase in dry mass of deep roots. Biomass is a root trait that has been proposed as an important feature in drought stress resistance and yield stability in bread wheat in places with variable moisture regimes (Ehdaie et al., 2012). In the ratio of root biomass to total biomass of wheat plant, a 51.9 % increase in this proportion was observed with the application of stress at the beginning of the vegetative stage and 29.6 % with the application of stress at the beginning of the reproductive stage compared to non-stress conditions (Table 3). The increase in the amount of this proportion during drought stress is due to the destructive effects of stress on plant biomass (biomass above the soil surface) which is part of the total plant biomass and is at the denominator of this proportion. The root-to-shoot ratio showed an increase of 94.7 % in terms of stress at the beginning of the vegetative stage and 60.5 % in terms of stress at the beginning of the reproductive stage compared to non-stress conditions. Stunting of plant shoots including shoots and stems is one of the primary effects of drought stress, which occurs indirectly through chemical signaling from root to stem (Jin et al., 2015a). Some reports suggest that the root-to-shoot ratio can be an important feature for drought tolerance, so that the higher the root-to-shoot ratio, the higher the plant's tolerance to drought stress. A study on shoot and root characteristics of maize hybrids in drought tolerance showed that irrigated diets had less effect on root dry mass compared to stem dry mass (Jin et al., 2018). In the study of root-related traits, the response of the wheat plant was very different and even contradictory in some cases due to the stressful stage of growth.

Table 2: Results of analysis of variance

		Mean Squares													
S.O.V.	Df	Longest root (cm)	Shallow root mass (g)	Root biomass (g)	Ratio of root biomass to Total plant biomass	Ratio of root to shoot	Plant height (cm)	Stem dry mass (g)	Spike length (cm)	Spike mass (g)	Number of grain per-spike	Grain mass per-plant (g)	Total plant biomass (g)	Thousand grain mass (g)	Biological yeild (g)
Block	2	1060.4^{ns}	6.65**	38.02*	0.218^{ns}	1.67^{ns}	26.3^{ns}	0.14^{ns}	0.46^{ns}	3.75^{ns}	1306^{ns}	1.28^{ns}	63.55*	62.9^{ns}	4.14^{ns}
Condition of Drought	2	2322.6^{ns}	0.18^{ns}	12.23^{ns}	1.451**	9.22**	923.3**	1.55*	32.3**	792.29**	16220**	139.49**	1020.9**	3861**	863.9**
Block × Condition of Drought	2	521.2	0.23	5.08	0.047	0.47	10.5	0.11	0.19	1.19	417	0.295	4.42	15.8	0.72
Genotype	95	108.9**	0.12**	0.94**	0.030**	0.27**	211.7**	0.44**	4.34**	7.23**	3080**	1.241**	11.58**	296**	9.55**
Condition of Drought × Genotype	190	76.9**	0.07^{ns}	0.82**	0.008**	0.81**	73**	0.18**	1.55**	1.76**	409**	0.279**	3.49**	32.6**	2.09**
Subsidiary error	570	38.54	0.07	0.49	0.004	0.03	16.3	0.07	0.17	0.38	116	0.088	1.25	7.45	0.51
C.V. (%)		21.2	15.5	30.5	18.1	29.8	10.4	14.2	7.3	23.2	24.4	27.4	16.2	11.2	15.5

Ns, * and **: Indicates no significant difference, significant difference at 5 % and significant difference at 1 %, respectively

Table 3: The average of traits in three conditions including: 1. no stress, 2. application of drought stress at the beginning of the vegetative stage, and 3. application of drought stress at the beginning of the reproductive stage..

Characteristics	Without drought stress	Stress at the beginning of the vegetative stage	Percentage change compared to the without drought stress	Stress at the beginning of the reproductive stage	Percentage change compared to the drought stress
Longest root (cm)	30.17	26.15	-13.3	31.64	+04.9
Shallow root mass (g)	1.77	1.72	-02.8	1.75	-01.1
Root biomass (g)	2.37	2.06	-13.1	2.45	+03.4
Ratio of root biomass to Total plant biomass	0.27	0.41	+51.9	0.35	+29.6
Ratio of root to shoot	0.38	0.74	+94.7	0.61	+60.5
Plant height (cm)	40.50	37.04	-08.5	38.30	-05.4
Stem drymass (g)	2.04	1.90	-06.8	1.96	-03.9
Spike length (cm)	6.13	5.46	-10.9	5.76	-06.0
Spike mass (g)	4.38	1.07	-75.5	2.52	-42.4
Number of grain per-spike	68.59	20.57	-70.0	43.18	-37.0
Number of spikelets per-spike	82.20	27.40	-66.6	55.20	-32.8
Grain mass per-plant (g)	1.80	0.41	-77.2	1.02	-43.3
Total plant biomass (g)	8.81	5.04	-42.8	6.94	-21.2
Thousand grain mass (g)	27.32	20.19	-26.1	25.21	-07.7
Biological yeild (g)	6.43	2.98	-53.7	4.48	-30.3

In order to investigate the relationship between the measured traits, the correlation coefficient between the traits was calculated and interpreted (Table 4). According to the results, a positive and very significant relationship at the level of 1 % probability was between the yield (grain mass per plant) with plant biomass (0.89**). Also, a very significant negative relationship was observed between yield (grain mass per plant) with the ratio of root biomass to total plant biomass (0.71**) and ratio root to shoot biomass (0.65 **). Correlation table coefficients show a positive and very significant relationship between the longest root with root biomass (0.75**), total deep root length (0.55**) at the level of 1 % probability.

Contrary to the results of this study, Kadam et al.(2012) showed that root biomass had a significant correlation with all measured traits except the longest root and no correlation was observed between root biomass and the longest root. In another study to locate quantitative traits related to root and shoot characteristics in a population of recombinant inbred lines of spring wheat, it was reported that between plant biomass and number of seeds per spikelet, dry mass of shallow roots, dry mass of deep roots and root biomass, there is a correlation at the level of 1 % probability (Ehdaie et al., 2016).

Since grain yield in genotypes may be independent of each other under normal and stress conditions, the tolerance or susceptibility index to stress was used to distinguish genotypes that responded better to stress conditions. The use of these indices was the identification and selection of genotypes that have relatively high yields under both normal and stress conditions. Studies show that the average productivity indices (MP), geometric mean productivity (GMP) and stress tolerance index (STI), due to high correlation with grain yield, are suitable for identifying high yield genotypes under normal conditions, mild stress and severe drought stress (Ali and El-Sadek, 2016; Khosravi et al., 2020). In this study, the genotypes that showed the highest MP, GMP and STI and also had high yield, drought tolerant genotypes were considered and vice versa. After ranking the genotypes based on all three indices of tolerance or sensitivity to stress, the average ratings obtained for each genotype were also calculated as the mean rank index (RM). Correlation coefficients between the three indices of drought tolerance and yield under stress conditions at the beginning of the vegetative and non-stress stages showed that there is a positive and significant correlation at the level of one percent probability between all indices of stress tolerance and yield. Therefore, it seems that the indicators used in this study can be effective in identifying and differentiating high-yield genotypes under drought and non-stress conditions. Since the accuracy of independent comparison test is higher than independent comparison test and practical analysis test of observations or division of effects of treatments, so in order to group genotypes based on average rank index and measure the validity of grouping accuracy, the comparison test were used independently. First, the genotypes were ranked from 1 to 96 based on the mean RM (T) rank index. Then genotypes with ranks between one and 48 were placed in the first group and the rest of the genotypes were classified from 49 to 96 in the second group. For independent comparison test, the first group was given a coefficient of +1 and the second group was given a coefficient of -1. Then, the independent comparison test between the first and second groups was performed according to the measured

Table 4: Correlation between the measured traits in all three stress conditions including: 1. no stress, 2. application of drought stress at the beginning of the vegetative stage, and 3. application of drought stress at the beginning of the reproductive stage

Characteristics	Longest root (cm)	Root biomass (g)	Total deep roots length (cm)	Ratio of root biomass to Total plant biomass	Grain mass per-plant (gr)	Total plant biomass (g)	Ratio of root to shoot
Longest root (cm)	1.00	0.75**	0.55**	0.29ns	0.19ns	0.49*	0.28ns
Root biomass (g)		1.00	0.48*	0.47*	0.18ns	0.56**	0.47*
Total deep roots length (cm)			1.00	0.13ns	0.03ns	0.17ns	0.12ns
Ratio of root biomass to Total plant biomass				1.00	-0.71**	-0.42*	0.96**
Grain mass per-plant (g)					1.00	0.89**	-0.65**
Total plant biomass (g)						1.00	-0.37ns
Ratio of root to shoot							1.00

Ns, * and **: Indicates no significant difference, significant difference at 5 % and significant difference at 1 %, respectively

data related to grain yield in plant genotypes. The results of the independent comparison test between the two groups are given in Table 5. The results showed a significant difference in the level of 1 % probability between the two groups based on the mean rank index.

Mohammadi and Abdullahi (2017) also reported the use of a stress tolerance index could not lead breeders to the best option and genotypes should be selected based on a combination of several tolerance indices or sensitivity to provide a more practical criterion for improving stress tolerance traits.

Determining the size of the inbred lines and proximity to each other, as well as increasing productivity in effective parenting, can reduce breeding volume, costs, and time. To separate the genotypes, cluster analysis was used by Euclidean distance to measure the dissimilarity between genotypes by hierarchical clustering method (average linkage method), and finally the genotypes were divided into four main groups (Figure 1). The first group consists of two subgroups, which subgroup (a) includes eight genotypes with the names of RIL19, RIL46, RIL51, RIL62, RIL64, RIL89, RIL114 and RIL164 and subgroup (b) contains 63 genotypes. It seems that the reason for the divergence of the two subgroups from each other is how the genotypes in the group react to drought stress for grain yield per plant. So that the genotypes of subgroup (a) in all three conditions of the experiment were weak in terms of grain yield and most of them in terms of yield rank under non-stress conditions, drought stress at the beginning of vegetative stage and drought stress at the beginning of vegetative stage. Are at the bottom of the table. On the other hand, although in subgroup (b) grain yield per plant was very diverse and in some cases to some extent, but in general grain yield per plant genotypes in this subgroup, in all three conditions without stress and application of two The type of drought stress at the beginning of the vegetative and reproductive stages was favorable and was higher than the average population of the studied genotypes. The second group consists of eighteen genotypes named Aflak, RIL8, RIL9, RIL10, RIL16, RIL23, RIL48, RIL67, RIL76, RIL79, RIL82, RIL87, RIL112, RIL113, RIL122, RIL137, RIL150 and RIL151. The mean rank index of most genotypes in this group was in the upper half of the ranking table during the stress application conditions at the beginning of the vegetative stage and during the stress application conditions at the beginning of the reproductive stage.

This indicates that the genotypes in this group did not respond well to drought stress in terms of tolerance or susceptibility to stress. The genotypes of this group in terms of the average of some measured traits such as: longest root size, root biomass and plant biomass, in all three conditions of the experiment, had higher ranks than the average of all studied genotypes. In other words, these genotypes had a favorable response to drought stress in terms of the average of the mentioned traits. In the third group, only one genotype named RIL35 was included. The fourth group consisted of six ruby egg genotypes, RIL117, RIL118, RIL141, RIL146 and RIL163. Seed yield per plant in this group was very low and weak compared to other genotypes under non-stress conditions, drought stress at the beginning of the vegetative stage and drought stress at the beginning of the reproductive stage. However, there were good potentials among the genotypes of this group in terms of shallow root dry mass, root biomass, plant biomass and longest root.

To determine the share of each measured trait in the variance of the study population, as well as reducing the number of studied traits by considering the correlation matrix between the measured traits and indices, the principal component analysis method was used. Under drought stress conditions at the beginning of the vegetative stage, the results showed that the first four main components had specific values greater than one and together accounted for 86.19 % of the diversity in the study population (Table 6, Figure 2a). Based on the high and positive specific values related to biological yield (BY), spike mass (SM), grain mass per plant (GMP) and total plant biomass (TPB) in the first component, the first component can be considered related to the yield of genotypes. In the second component, the highest eigenvalues were related to MP, GMP and STI indices, genotype yield under non-stress conditions (YP), 1000-grain mass (TGW) and genotype yield during drought stress conditions at the beginning of vegetative stage (YS1). The second component can be introduced as a component related to the response to sensitivity or tolerance to drought stress. The third component had the highest coefficients for deep root mass (DRM), total deep root length (TDRL), root biomass (RBio) and longest root (LR). According to these

Table 5: Independent comparison test between two groups of genotypes based on total rank of mean RM(T)

Independent comparison based on grain yield trait per-plant	contrast	Df	Contrast ss	Mean Square	F value	P r> F
	One	1	2369.8	2369.8	11.77	0.0007

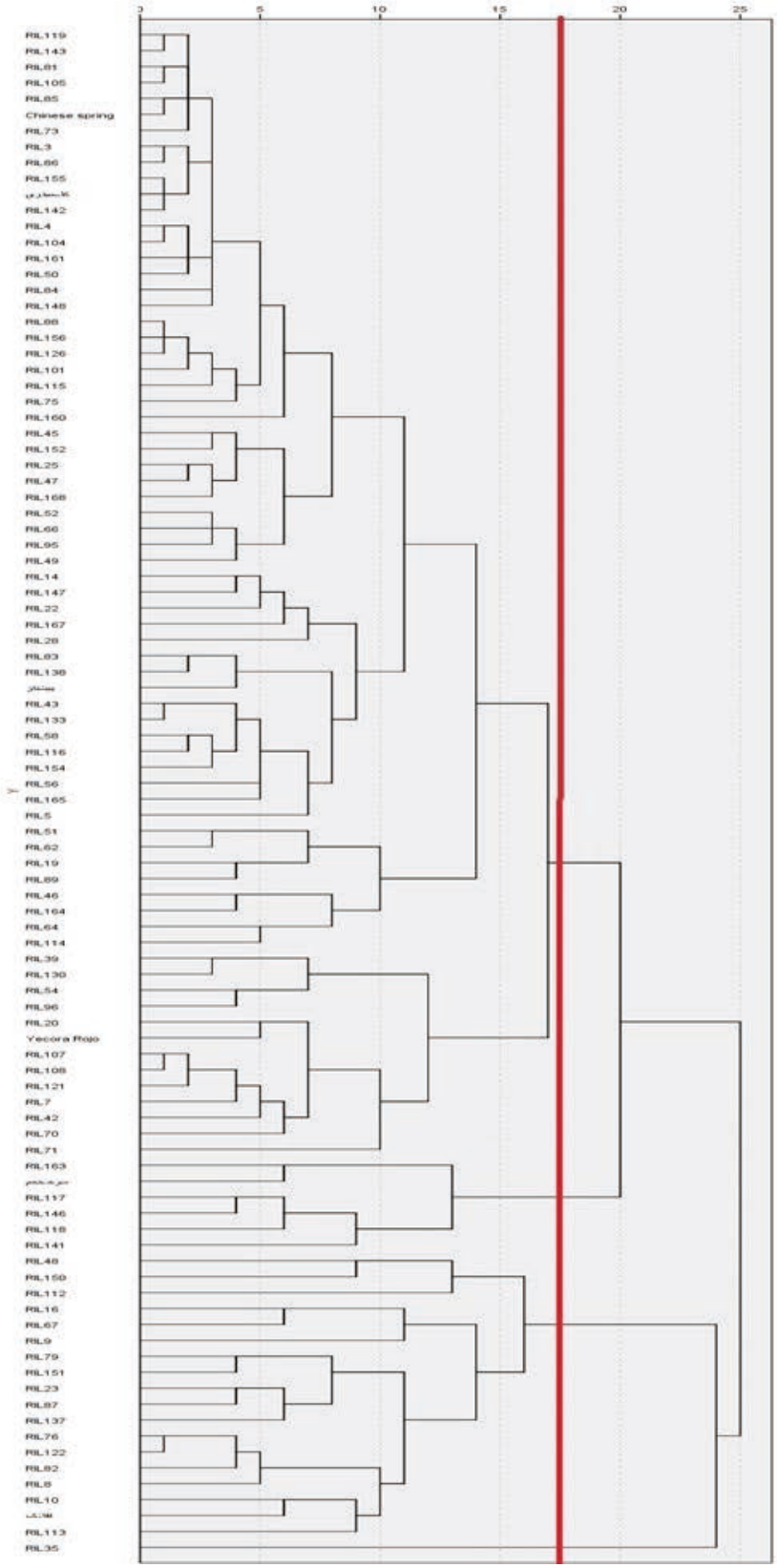

Figure 1: Cluster analysis of 96 genotypes based on data obtained from measuring all traits

Table 6: Principal component analysis using evaluation traits under drought stress conditions at the beginning of the vegetative stage

Characteristics	Abbreviation	First component	Second component	Third component	Fourth component
Biological yeild (g)	BY	0.96	-0.20	-0.10	
Spike mass (g)	SW	0.88	-0.21	-0.24	0.28
Grain mass per-plant (g)	GWP	0.88	-0.24	-0.23	0.30
Total plant biomass (g)	TPB	0.87	-0.36	0.25	0.10
Ratio of root biomass to total plant biomass	RRT	-0.74		0.59	0.21
Number of grain per-spike	NGS	0.68	-0.58	-0.29	0.26
Ratio of root to shoot	RRS	-0.65	-0.10	0.64	0.26
Mean productivity	MP(1)	0.37	0.87	0.24	0.10
Geometric mean productivity	GMP(1)	0.41	0.86	0.23	0.14
Stress tolerance index	STI(1)	0.44	0.85	0.20	0.15
Yield genotypes under normal conditions (g)	YP	0.11	0.83	0.28	
Thousand grain mass (g)	TGW	0.54	0.74	0.15	0.22
Number of spikelets per-spike	NSS	0.59	-0.60	-0.33	0.28
Deep root dry mass (g)	DRW	0.15	-0.41	0.81	0.23
Total deep roots length (cm)	TDRL	0.15	-0.39	0.80	0.15
Root biomass (g)	Rbio	0.21	-0.44	0.80	0.24
longest root (cm)	LR		-0.29	0.71	
Spike length (cm)	SP	0.55	-0.11	0.31	-0.72
Yield genotypes under stress conditions at the beginning of the vegetative stage (g)	YS(1)	0.54	0.74	0.15	0.22
Stem dry mass (g)	SDW	0.56		0.30	-0.72
Plant height (cm)	PH	0.55		0.33	-0.69
Shallow root mass (g)	SRW	0.22	-0.22	0.23	0.12
Ratio of number of grain to number of spikelets per spike	RNN	0.48			
Eigen value		7.51	5.73	4.26	2.13
Variance (%)		32.69	24.95	18.54	10.00
Cumulative variance (%)		32.69	57.64	76.19	86.19

Table 7: Principal component analysis using evaluation traits under drought stress conditions at the beginning of the reproductive stage

Characteristics	Abbreviation	First component	Second component	Third component	Fourth component
Biological yeild (g)	BY	0.95	0.22		0.16
Spike mass (g)	SW	0.91	0.18		0.33
Grain mass per-plant (g)	GWP	0.91	0.19		0.33
Total plant biomass (g)	TPB	0.86	0.21	0.39	0.18
Ratio of root biomass to total plant biomass	RRT	-0.81	-0.16	0.53	
Number of grain per-spike	NGS	0.89	-0.36		0.21

Continued					
Ratio of root to shoot	RRS	-0.79	-0.17	0.47	
Mean productivity	MP(2)	-0.18	0.97		
Geometric mean productivity	GMP(2)	-0.16	0.98		
Stress tolerance index	STI(2)	-0.15	0.97		
Yield genotypes under normal conditions (g)	YP	-0.25	0.94		
Thousand grain mass (g)	TGW	-0.10	0.97		
Number of spikelets per-spike	NSS	0.80	-0.45		0.19
Deep root dry mass (g)	DRW			0.95	0.17
Total deep roots length (cm)	TDRL	-0.11	0.11	0.89	0.14
Root biomass (g)	Rbio			0.96	
longest root (cm)	LR		0.16	0.84	
Spike length (cm)	SP	0.71	0.18	0.22	-0.56
Yield genotypes under stress at the beginning of the reproductive stage (g)	YS(2)	-0.10	0.97		
Stem dry mass (g)	SDW	0.66	0.28	0.22	-0.62
Plant height (cm)	PH	0.62	0.28	0.24	-0.63
Shallow root mass (g)	SRW	0.12		0.25	-0.25
Ratio of number of grain to number of spikelets per spike	RNN	0.53	0.20	-0.15	
Eigen value		7.87	6.52	4.31	1.65
Variance (%)		34.24	28.35	18.74	07.20
Cumulative variance (%)		34.24	62.59	81.33	88.54

Table 8: Principal component analysis using evaluation traits under no drought stress conditions

Characteristics	Abbreviation	First component	Second component	Third component	Fourth component
Biological yeild (g)	BY	0.93	-0.25	0.19	
Spike mass (g)	SW	0.87	-0.34	0.21	0.24
Grain mass per-plant (g)	GWP	0.87	-0.33	0.22	0.24
Total plant biomass (g)	TPB	0.96		0.15	0.13
Ratio of root biomass to Total plant biomass	RRT	-0.43	0.87	-0.14	
Number of grain per-spike	NGS	0.73	-0.32	-0.55	0.19
Ratio of root to shoot	RRS	-0.46	0.83	-0.13	
Thousand grain mass (g)	TGW			0.99	
Number of spikelets per-spike	NSS	0.70	-0.29	-0.58	0.14
Deep root dry mass (g)	DRW	0.48	0.79		0.14
Total deep roots length (cm)	TDRL	0.54	0.75		
Root biomass (g)	Rbio	0.51	0.82		0.16
longest root (cm)	LR	0.54	0.70		0.12
Spike length (cm)	SP	0.74			-0.54
Stem dry mass (g)	SDW	0.72	0.25		-0.56
Plant height (cm)	PH	0.72	0.11		-0.58

Continued					
Shallow rootmass(g)	SRW	0.30	0.46		0.13
Ratio of number of grain to number of spikelets per spike	RNN	0.37	-0.25		0.26
Eigen value		7.70	4.67	2.83	1.32
Variance (%)		40.57	24.59	14.93	06.94
Cumulative variance (%)		40.57	65.16	80.10	87.05

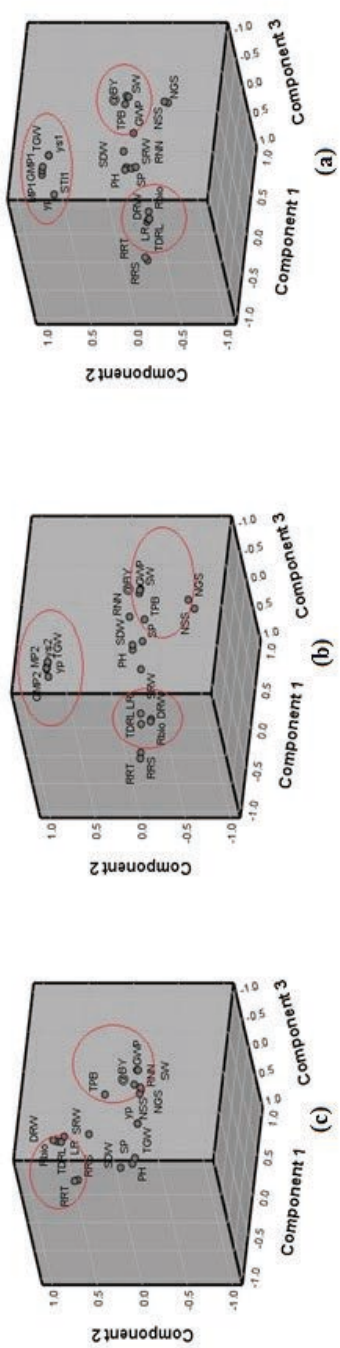

Figure 2: Three-dimensional diagram of the distribution of measured traits and indices relative to the principal components a) in terms of stress at the beginning of the vegetative stage, b) in terms of stress at the beginning of the reproductive stage, c) in conditions without stress

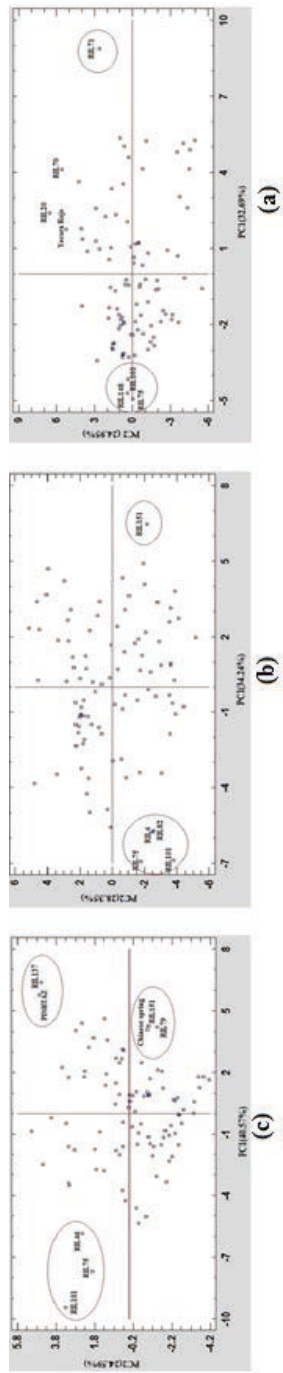

Figure 3: Two-dimensional diagram of the distribution of genotypes relative to the first of two principal components a) under drought stress at the beginning of the vegetative stage, b) under drought stress at the beginning of the reproductive stage, c) without stress

results, the third component related to root traits is considered.

Based on the output of principal components during drought stress conditions at the beginning of the vegetative stage, it can be concluded that based on the first and second components and considering the distribution of genotypes relative to the first two components, genotypes that in terms of the first component, they have the highest value, have more performance during stress conditions, and also according to the second component, the reaction of these genotypes to drought stress at the beginning of the vegetative stage will be visible. Accordingly, the genotypes at the far right of the graph (Figure 3a) have a high yield and are more tolerant to drought stress at the beginning of the growing stage. These are low-yield, drought-sensitive genotypes when drought stress was applied at the beginning of the growing stage.

The results of principal component analysis during drought stress conditions at the beginning of the reproductive stage are shown in Figures (2b) and (3b). As in the case of stress at the beginning of the vegetative stage, here too the first four components had specific values greater than one, describing a total of 88.54 % of the diversity in the study population (Table 7). Due to the higher and positive eigenvalues for biological yield (BY), grain mass per plant (GMP), spike mass (SM), number of grains per spike (NGS) and total plant biomass (TPB) in the first component, can be The first component was related to the performance of the population. The highest eigenvalues in the second component are related to GMP, MP, STI, 1000-grain mass (TGM) indices, genotype yield during drought stress conditions at the beginning of reproductive stage (YS2) and genotype yield under non-stress conditions (YP). Conditions also, we define the second component as the component related to the response of susceptibility or tolerance of genotypes to drought stress at the beginning of the reproductive stage. In the third component, the highest coefficients were related to root biomass (RBio), deep root mass (DRM), total deep root length (TDRL) and longest root (LR). As a result, the third component related to the diversity of root traits was considered.

Under stress-free conditions, the results of principal component analysis showed that the first four principal components with eigenvalues greater than one, in total, accounted for 87.05 % of the variance in the population of the recombinant inbred lines studied (Table 8). Accordingly, the first component with total plant biomass (TPB), biological yield (BY), grain mass per plant (GMP) and spike mass (SM), justified 40.57 % of the diversity in the population (Figure 2c). Therefore, even in stress-free conditions, the first component can be introduced related to the performance of genotypes. The second component with a justification of 24.59 % of the diversity of the study population and the highest specific values related to the traits of root to plant biomass ratio (RRT), root to shoot ratio (RRS), root biomass (RBio), deep root mass (DRM) and the sum of deep root lengths (TDRL), could be introduced as a component of root traits.

The result of principal component analysis output under stress-free conditions showed that based on the first and second components and considering the distribution of genotypes relative to the first two components, it is possible to identify genotypes that have the highest yield under conditions. No stress in terms of root traits had the best reaction and vice versa (Figure 3c).

4 CONCLUSION

Despite the significant difference in the level of 1 % probability between genotypes and also the interaction of genotype with environmental conditions, for all root traits except shallow root dry mass, understanding the response of these traits to the types of drought stress was high complicated. For example, the longest root trait decreased by 13.3 % compared to stress-free conditions at the beginning of the vegetative stage, while the same trait increased by 4.9 % compared to non-stress conditions at the beginning of the reproductive stage. Also, in the case of root biomass trait during stress application at the beginning of the vegetative stage compared to the non-stress state decreased by 13.1 %, while the same trait increased by 3.4 % during stress application at the reproductive stage. However, finding a successful combination of shoot and root traits that can be used in breeding to improve further growth and productivity is a big challenge, because in the present study, the response of the wheat plant to many stages It is different from stressful growth and even in some cases contradictory.

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Classification of determinant factors of irrigated vegetable problems using exploratory factor analysis in Swaida governorate, Syria

Maya AL-ABDALA [1, 2], Afraa SALLOWM [3], Safwan ABOUASSAF [1]

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Classification of determinant factors of irrigated vegetable problems using exploratory factor analysis in Swaida governorate, Syria

Abstract: The objective of this research was to classify the determinant factors of irrigated vegetable problems and the amount of variance that is explained by each factor in Swaida Governorate/ Syria by using the Exploratory Factor Analysis. The research is based on the data which were collected through questionnaires that were obtained according to the opinions of farmers. It included questions about some of the social and economic characteristics of farmers, and the concerning problems related to irrigated agriculture by using multiple-choice questions (on a 3-point scale) during the 2019-2020 Based on a sample size of 92 farmers, representing 54.9 % of the studied statistical community, and distributed randomly within the areas of spread of irrigated vegetable cultivation.. The results showed the success of using the exploratory factor analysis technique, using the Principal components methodology and Varimax in classifying six factors with an initial eigenvalues greater than one for each, and these factors are: agricultural technological progress, agricultural employment, sale outlets, natural conditions, prices, production requirements. These factors explained (13.21 %, 12.65 %, 12.55 %, 11.12 %, 10.94 %, and 9.85 %) of the total variance respectively, and together explained 70.33 %.

Key words: exploratory factor analysis; principal component; factors; Varimax; irrigated vegetables

Razvrstitev odločitvenih dejavnikov povezanih s problemi namakanja zelenjave s faktorsko analizo na območju upravne enote Swaida, Sirija

Izvleček: Namen raziskave je bil s faktorsko analizo razvrstiti odločitvene dejavnike, povezane s problemom namakanja zelenjave, in določiti vpliv posameznega dejavnika na območju upravne enote Swaida v Siriji. Raziskava temelji na mnenjih kmetov, ki so bila pridobljena s pomočjo anketnih vprašalnikov. Ti so vključevali vprašanja o nekaterih socialnih in ekonomskih značilnostih kmetov in problemih z namakanjem, s katerimi se srečujejo. Anketirani so imeli možnost odgovoriti na vprašanja na 3-točkovni skali. Anketa, ki je potekala v obdobju 2019-2020, je temeljila na vzorcu 92 kmetov, kar je predstavljalo 54,9 % kmetov preučevane statistične regije. Anketiranci so bili izbrani naključno znotraj območja, kjer se pri gojenju zelenjave uporablja tudi namakanje. Rezultati so pokazali smiselnost uporabe faktorske analize. Z uporabo metode glavnih komponent in metode Varimax je bilo šest dejavnikov, z začetno lastno vrednostjo večjo od ena, razvrščenih glede na delež variabilnosti, ki jo pojasnjujejo. Ti dejavniki so: razvoj agrotehnike, zaposlitev v kmetijstvu, možnost prodaje, naravne danosti, cene, proizvodni stroški, pojasnjujejo pa13,21 %, 12,65 %, 12,55 %, 11,12 %, 10,94 %, in 9,85 % celotne variabilnosti oz. vsi skupaj pojasnjuejo 70,33 % celotne variabilnosti.

Ključne besede: faktorska analiza; metoda glavnih komponent; dejavniki; Varimax; namakanje zelenjave

1 General Commission for Scientific Agricultural Research (GCSAR), Syria
2 Corresponding author, e-mail: mayaabdala6@gmail.com
3 Agricultural Economics, Faculty of Agriculture, University of Damascus, Syria

1 INTRODUCTION

Agriculture is one of the important sectors in the Syrian economy, through its contribution to employing the labor force and covering the increasing food needs, especially in the current economic crisis and economic blockade. This study applies to the Swaida Governorate, where agriculture is the main production base and its ability to absorb the workforce in all steps of production until marketing, and thus contribute to solving the unemployment problem in the governor's countryside in particular. In spite of the fluctuation in its contribution to the local product, and the difficult economic conditions amid insecurity and the lack of available resources and weak implementation of rural development programs, the minimum level of self-sufficiency in agricultural products is sought.

Factor analysis is one of the important statistical methods that enabled researchers to classify scientific phenomena in multiple fields. It is used to find out the different correlations between data and to summarize it by identifying common characteristics. Therefore, factor analysis is one of the applications of the inductive approach, whereby a set of relationships can be traced back to common factors that describe and explain these relationships (Zeina, 2015). There are many studies that dealt with the exploratory factor analysis methodology in various fields such as agriculture, marketing, psychological sciences, and others, as it depends on the ordinal variables and listed according to a certain scale. Pavel and Moldovan (2019) in the study exploring the role of external factors determining local economic development in rural areas in Romania, based on data collected for the 398 communes from the North-West development region of Romania between 2007 and 2014, using exploratory factor analysis of principal components, where the results showed the location from urbanization and the presence of direct contact with European roads affect the level of local economic development and there is no impact of non-refundable investment programs in infrastructure in accelerating economic development.

Discovering the different dimensions of food security in relation with urban agriculture based on a sample of 360 families. The results of exploratory factor analysis identified three latent factors from 31 statements to which respondents indicated their level of agreement on a 5-point scale: the food availability and accessibility, adequate nutritional intake and reduction in fresh food expenditure. Contribution of each factor to the total variance were the following: 25.12 %, 24.29 %, and 20.2 %) respectively, as together interpreted 69.61 % (Rezai et al., 2016).

In order to exploratory factor analysis of barriers and problems affecting the development of nanotechnology in agriculture, four factors with eigenvalues greater than 1, were extracted after orthogonal rotation using the varimax technique. These factors explained 74.40 % of the total variance. According to extracted results, the financial support factor with the variance of 24.18 had the highest importance in the explanatory variables. After that, the Communication – Management, the cognitive learning and operating infrastructure factors were following it respectively (Ahmadi et al., 2013).

To assess the performance of Agri Clinic entrepreneurs promoted under the scheme on Agri Clinic and Agri Business Centers in India. Thus, an attempt has been made to evolve a set of factors influencing the entrepreneurial behavior through a data reduction process of factor analysis using Varimax. The factors include planning orientation, work orientation, personal efficacy, market orientation, location, business acumen, dynamism, service orientation, in-depth knowledge, achievement motivation, social networks, interest, internal control, marketing strategy and innovativeness. These factors collectively explained 86.91 % of the total variance (Chandrashekar et al., 2012). There are many problems and constraints facing irrigated vegetable agriculture that pose great risks. These constraints lead to unstable yields. The most important of them are: the prevailing and fluctuating weather conditions, the spread of diseases and insects and the epidemically, the ineffectiveness of pesticides and fertilizers and their high prices, in addition to the problems related to marketing such as the lack of sales outlets or oversupply, and the monopoly of brokers. Therefore, it is necessary to identify the most important factors responsible for explaining the largest proportion of the total variation in the production of irrigated vegetable projects in Swaida Governorate by using the exploratory factor analysis methodology. The main goal is to classify the determining factors of the problems of irrigated vegetable cultivation into identifying the most important factors responsible for explaining the largest proportion of the total variation in the production of irrigated vegetable projects.

2 MATERIALS AND METHODS

2.1 STUDY AREA

The study was conducted in Swaida governorate, southern Syria during the 2020 agricultural season, in the places where irrigated crops spread. Knowing that the irrigated cropping patterns in Swaida governorate are divided between summer and winter crops and fruit trees. Where the area average of the irrigated pattern,

excluding the fruit trees during 2016-2018 period was about 11008.33 Dunums (area unit = 1000 m^2). The summer cropping pattern accounted 69.86 % by about 7690 Dunums, including tomatoes, watermelons, melons, cucumbers, eggplant, pepper, etc., and the winter cropping pattern represented 30.14 % by about 3318.33 Dunums, including wheat, peas, cauliflower, cabbage, onions, garlic and others (Ministry of Agriculture and Agrarian Reform Statistics, 2016-2018).

2.2 DATABASE

The study based on preliminary data through field visits the irrigated vegetable farmers who own wells. For the interviews, a structured questionnaire was designed with some of the social and economic characteristics of farmers and the concerning problems related to irrigated agriculture with multiple-choice questions using a three-point scale ranging from (1 = there is no problem), (2 = medium problem), (3 = strong problem) during 2019-2020 season in Swaida Governorate. Where the sample size is consisted of 92 observations represented 54.9 % of the studied statistical community, based on a formula (Glenn, 1992) (Yamane, 1967):

$$n = \frac{N}{1 + N(e)^2}$$

Where: N_ The studied community (168 wells) worked for at least three consecutive years in the irrigation of vegetable crops (Agricultural Extension Department, 2020), e- Precision Level ± 7% Where Confidence Level is 95 %. were randomly distributed in the study area.

Data was processed using IBM Spss Statistics 26.

2.3 STATISTICAL METHODS

The study relied on descriptive analysis methods to describe the study variables such as means, percentage, charts, and exploratory factor analysis.

2.3.1 Related concepts and terms

Factor Analysis (FA): Is an interdependence technique whose primary purpose is to define the underlying structure among the variables in the analysis. Recently was developed originally for the analysis of scores on mental tests; however, the methods are useful in a much wider range of situations, for example, analyzing sets of tests of attitudes, sets of physical measurements, and sets of economic quantities (Anderson, 2003).

Principal Components Method (PCA): Is one of the most important methods of factor analysis. It can be used to analyze interrelationships among a large number of variables, and explain them in terms of their common underlying dimensions to find a way of condensing the information contained in a number of original variables into a smaller set of factors with a minimal loss of information by providing an empirical estimate of the structure of the variables considered (Hair et al., 2009). As long as PCA is used descriptively as convenient ways to summarize the relationships in a large set of observed variables, assumptions regarding the distributions of variables are not in Force (Tabachink & Fidell, 2013).

Exploratory Factor Analysis (EPA): Is to discover the underlying structure of observed variables and identifies latent factors that explain the covariation among a set of variables. Ideally, the derived factors should consist of relatively homogenous variables, where each item loads strongly onto one factor and minimally on the other factor(s). It is assumed that each common factor affects every observed variable and that the common factors are either all correlated or uncorrelated (McDonald, 1985).

2.3.2 Mathematical Models of EPA

In the EPA model, (p) is the number of variables ($X_1, X_2, \dots, X_p$) and (m) denotes the number of underlying factors ($F_1, F_2, \dots, F_m$). X_j is the variable represented in Eigenvalue (latent) factors. Hence, this model assumes that there are (m) underlying factors whereby each observed variable is a linear function of these factors together. This model intends to reproduce the maximum correlations:

$$Xj = \alpha j1F1 + \alpha j2F2 \dots\dots\dots\dots \alpha jmFm + ej$$

Where: j = 1, 2, ... , p

The factor loadings are $a_{j1}, a_{j2}, \dots, a_{jm}$ which denotes that a_{j1} is the factor loading of (jth) variable on the (1st) factor. The specific or unique factor is denoted by ej. The factor loadings give us an idea about how much the variable has contributed to the factor; the larger the factor loading the more the variable has contributed to that factor (Harman, 1976).

2.3.3 Procedures in exploratory factor analysis

Measure of sampling adequacy (MSA)**:** Calculates both for the entire correlation matrix and for each individual variable evaluation the appropriateness of apply-

ing factor analysis. Values above 0.5 for either the entire matrix or an individual variable indicate appropriateness (Hair et al., 2009).

Correlation matrix: When the data are appropriate, it is possible to create a correlation matrix by calculating the correlations between each pair of variables. As stated important information for the analysis in the correlation matrix are (Field, 2009): the variables have to be intercorrelated, but they should not correlate too highly (extreme multicollinearity and singularity). The coefficients less than 0.90 and suggested removing one of a pair of items with bivariate correlation scores greater than 0.8), the level of significance, the determinant (which should be greater than zero), and KMO and Bartlett's tests.

Tests of Bartlett sphericity and Kaiser-Meyer-Olkin (KMO): were used to determine the level of confidence that can be expected when using EFA on data (Hair et al., 2009). The Bartlett test of sphericity is based on the statistical distribution of Chi-square and tests the null hypothesis for the overall significance of all correlation within a correlation matrix, (i.e., no correlation between the variables). Levels of significance greater than 0.1 indicate that the data are not suitable for the treatment with the method in question; in this case, the null hypothesis can not be rejected. The KMO test presents normalized values (between 0 and 1) and shows the proportion of common variance of the variables, or what percentage of the variables is accounted for by common factors. To interpret the results, values close to 1 indicate that the factor analysis method is perfectly suited for data processing. On the other hand, values below 0.5 indicate the inadequacy of the method (Pituch & Stevens, 2016).

Orthogonal Rotation**:** Is ordinarily used after extraction to maximize high correlations between factors and variables and minimize low ones. Numerous methods of rotation are available, but the most commonly used is varimax. Varimax is a variance-maximizing procedure. The goal of varimax rotation is to maximize the variance of factor loadings by making high loadings higher and lower ones lower for each factor. (Tabachink & Fidell, 2013).

2.3.4 Criteria for the number of factors to extract

Eigenvalue (Latent root) Criterion: The most commonly used technique is the Eigenvalue criterion. Is simply to apply, the rationale the latent criterion is that any individual factor should account for the variance of at least as single variable if it is to be retained for interpretation. With PCA each variable contributes a value 1 root considered significant, all factors with roots less than 1 are insignificant and are disagreed (Thomapson, 2004).

Communalities**:** are the measure of the proportion of variance explained by the extracted factors, representing the amount of variance accounted by the factor solution for each variable, to assess whether the variables meet acceptable levels of explanation. The communalities should be more than 0.50 for each variable and more than 0.60 in average (Hair et al., 2009).

Scree Test: As (Cattell, 1966) proposed a graphical test for determining the number of factors. A scree plot graphs eigenvalue magnitudes on the vertical access, with eigenvalue numbers constituting the horizontal axis. Showing how to simplify the scree plot through dynamic graphic procedures when successive factor analyses are performed (Ledesma et al., 2015).

There are four steps for applying EPA which are involved briefly by Tighza (2012):

- Preparation of a correlation matrix between the measured variables.
- Extracting the initial factors and exploration of possible data reduction.
- Rotation to a terminal solution (find the interpretable factors).
- Naming the identifying factors.

3 RESULTS AND DISCUSSION

3.1 CHARACTERISTICS OF THE STUDY SAMPLE POPULATION

3.1.1 Natural properties

Geographical: The sample covered into three agricultural settlement zones according to the amount annual rainfall, the largest percentage was in the second zone with about 86.96 %, and covered three administrative regions, Swaida region was the largest - about 60.78 % Table (1).

Rainfall ratio: It ranged annually between a minimum of 125 mm and a maximum of 380 mm, and an average amounted about 287.2 mm.

Altitude: It ranged from a minimum of 650 m to a maximum of 1470 m, and an average about 1004.5 m.

3.1.2 Economic characteristics

Main job: An irrigated vegetable farming is 47.7 % of the farmers. It is followed by 28.3 % who are self-employed, 15.2 % are employees in the public sector and 8.7 % have their own business.

Contribution to income: The cultivation of irrigated vegetables contributes about 53.21 % of the family income, while other kinds of agriculture contribute about

Table 1: Distribution of farmers according to the agricultural settlement zones and administrative regions in Swaida governorate during the 2020 agricultural season

		Settlement Zones				
First		First	Second	Third	Total	%
Administrative Regions	Salkad	6	22	2	30	32.61
	Swaida	4	52	0	56	60.87
	Shahba	0	6	0	6	6.52
Total		10	80	2	92	100
%		10.87	86.96	2.17	100	

Source: Survey results

20.33 %, and only 26.47 % income came from non-agriculture.

3.1.3 Social Characteristics

Age of the farmers: It ranged between 26 and 70 years as a min and max respectively, and about 46.4 years in average.

Experience: There was a variation in the farmers' experience regarding producing of irrigated vegetables, which ranged from 1 year to 40 years, and reached an average of about 13.55 Years.

Educational levels: It ranged between the highest percentages of those with primary education (about 64 %), then about 20 % of them with university education, and 14 % with secondary education and only 2 % were illiterate. Figure (1).

3.2 THE PROBLEMS FACING FARMERS

The results of the responses about relative frequency were used to analyze the main problems that effect on cultivation of irrigated vegetable where it was found that 98.9 % of farmers emphasized the problem of high costs inputs, 83.7 % State support for production requirements and 69.6 % Infection with diseases and insects respectively are strong problems, Table (2).

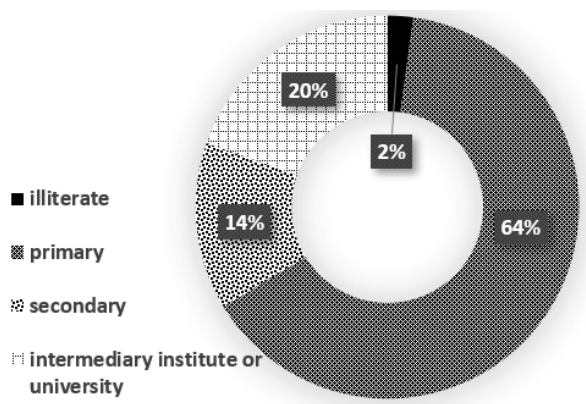

Figure 1: Percentage distribution of farmers according to educational level

3.3 DETERMINE THE FACTORS RESPONSIBLE FOR THE LARGEST PROPORTION OF THE TOTAL VARIANCE IN THE PRODUCTION OF IRRIGATED VEGETABLES PROJECTS, THE RESULTS OF (EPA)

3.3.1 The first Step: Analysis of the correlation matrix between the measured variables

According to Table (1) in the Appendix, the value of (MSA) appears in the diagonal cells of the variable (X8) equal to 0.341, which is less than 0.5, therefore it is deleted and re-analyzed. The Re-analysis excluding the variable (x8) and showed the following results:

Correlation coefficients: they should not exceed 0.9, as it is impossible to estimate the percentage of variance that the variables contribute to forming the extracted factors. Table (3) shows the inter-correlations matrix in the upper half, and the statistical significance in the lower half, which fulfills the condition.

The determinant of the matrix: which requires that the absolute value of the determinant must be greater than 0.0000, and it appears at the bottom of Table (3), Determinant = .028, meaning that the second condition is also fulfilled, and therefore the matrix does not involve the problem of exaggerated correlation between the variables.

Bartlett's Test of Sphericity: Table (4) shows the significance of the Bartlett test (Sig < 0.00) and the acceptance of the alternative hypothesis and thus the matrix is not neutral (Identity Matrix), and there are

some relationships between the variables that can be analyzed.

Kaiser-Meyer-Olkin (KMO(: Table (4) shows that the KMO value is equal to 0.586 and is greater than 0.5, thus the reliability of the factors that will be obtained from the analysis, and that the sample size is sufficient.

Measure of Sampling Adequacy (MSA): The MSA values in the diagonal cells show that all correlation coefficients equal or exceed the value 0.5, indicating that the level of correlation between each variable with other variables in the correlation matrix is sufficient for analyzing.

Table 2: Relative frequency of the responses to evaluate the characteristics related to the problems of irrigated vegetable production

		Ranking (%)		
Variables	Problems	There Is No (1)	Medium (2)	Strong (3)
X1	Availability Of Manpower	17.4	35.9	46.7
X2	Labor Efficiency	12.0	53.3	34.8
X3	Infection With Diseases And Insects	7.6	22.8	69.6
X4	Natural Disasters	55.4	20.7	23.9
X5	Availability Of Inputs	48.9	17.4	33.7
X6	High Costs Of Inputs	1.1	0	98.9
X7	Effectiveness Of Inputs	8.7	29.3	62.0
X8	State Support For Production Requirements	2.2	14.1	83.7
X9	Vegetable Price Fluctuations	6.5	30.4	63.0
X10	Brokers' Control	10.9	27.2	62.0
X11	Low Selling Price	7.6	13.0	79.3
X12	Disposing Of The Product	52.2	21.7	26.1
X13	Availability Of Internal And External Markets	50.0	22.8	27.2
X14	Providing Farmers With The Necessary Expertise	57.6	18.5	23.9
X15	Availability Of Information About New Technologies	63.0	16.3	20.7

Source: Survey results

Table 3: Correlation Matrix between the measured variables (The Problems of Irrigated Vegetable in Swaida governorate during the 2020 agricultural season)

		x1	x2	x3	x4	x5	x6	x7	x9	x10	x11	x12	x13	x14	x15
Correlation	x1	1.00													
	x2	0.63	1.00												
	x3	0.15	0.19	1.00											
	x4	0.04	0.13	0.31	1.00										
	x5	0.16	0.00	0.03	0.15	1.00									
	x6	0.04	0.20	0.10	-0.04	-0.13	1.00								
	x7	0.15	0.20	0.10	0.09	0.08	0.09	1.00							
	x9	0.02	0.11	-0.01	0.14	0.28	0.27	0.17	1.00						
	x10	0.22	0.13	0.02	0.09	0.18	0.08	0.07	0.30	1.00					
	x11	0.24	0.22	0.24	0.19	-0.12	0.30	0.05	0.05	0.14	1.00				
	x12	0.14	0.23	0.33	0.21	0.19	0.09	0.29	0.03	-0.03	0.20	1.00			
	x13	0.21	0.31	0.23	0.22	0.15	0.10	0.16	0.12	0.13	0.19	0.58	1.00		
	x14	-0.12	-0.06	0.09	0.17	0.16	-0.17	0.17	0.03	0.09	-0.04	0.23	0.14	1.00	
	x15	-0.08	-0.02	0.20	0.12	0.12	-0.18	0.16	0.00	0.21	-0.09	0.24	0.27	0.75	1.00

Continued

Sig. (1-tailed)														
	x1													
	x2	0.00												
	x3	0.08	0.04											
	x4	0.34	0.10	0.00										
	x5	0.06	0.49	0.38	0.07									
	x6	0.35	0.03	0.16	0.35	0.10								
	x7	0.08	0.03	0.18	0.20	0.22	0.21							
	x9	0.43	0.14	0.48	0.10	0.00	0.00	0.05						
	x10	0.02	0.11	0.42	0.19	0.04	0.23	0.25	0.00					
	x11	0.01	0.02	0.01	0.03	0.12	0.00	0.31	0.32	0.09				
	x12	0.09	0.01	0.00	0.02	0.03	0.19	0.00	0.38	0.38	0.03			
	x13	0.02	0.00	0.01	0.02	0.07	0.18	0.06	0.12	0.12	0.03	0.00		
	x14	0.13	0.29	0.20	0.05	0.06	0.06	0.05	0.38	0.19	0.36	0.01	0.10	
	x15	0.22	0.41	0.03	0.12	0.13	0.04	0.06	0.50	0.02	0.20	0.01	0.00	0.00

a. Determinant = .028

Source: IBM Spss Statistics 26 Output /survey results

Table 4: KMO and Bartlett's Test

Kaiser-Meyer-Olkin Measure of Sampling Adequacy		.586
Bartlett's Test of Sphericity	Approx. Chi-Square	305.846
	Df	91
	Sig.	.000

Source: IBM Spss Statistics 26 Output /survey results

T**able 5**: Measure of sampling adequacy (MSA) for the measured variables (The problems of irrigated vegetable)

	Anti-image Matrices													
		x1	x2	x3	x4	x5	x6	x7	x9	x10	x11	x12	x13	x14	x15
Anti-image Covariance	x1	0.50	-0.31	-0.04	0.07	-0.15	0.08	-0.05	0.09	-0.12	-0.10	0.02	-0.01	0.03	0.03
	x2	-0.31	0.51	-0.02	-0.06	0.11	-0.09	-0.05	-0.06	0.03	0.03	-0.02	-0.08	0.00	0.00
	x3	-0.04	-0.02	0.75	-0.21	0.00	-0.07	0.02	0.03	0.05	-0.10	-0.13	0.05	0.09	-0.12
	x4	0.07	-0.06	-0.21	0.79	-0.07	0.12	-0.01	-0.07	-0.03	-0.11	0.01	-0.07	-0.08	0.05
	x5	-0.15	0.11	0.00	-0.07	0.74	0.10	0.03	-0.21	-0.07	0.15	-0.11	-0.02	-0.06	0.03
	x6	0.08	-0.09	-0.07	0.12	0.10	0.74	-0.03	-0.20	-0.04	-0.17	-0.04	-0.01	0.02	0.06
	x7	-0.05	-0.05	0.02	-0.01	0.03	-0.03	0.85	-0.12	0.00	0.03	-0.15	0.05	-0.03	-0.03
	x9	0.09	-0.06	0.03	-0.07	-0.21	-0.20	-0.12	0.73	-0.18	0.02	0.06	-0.05	-0.02	0.04
	x10	-0.12	0.03	0.05	-0.03	-0.07	-0.04	0.00	-0.18	0.75	-0.11	0.10	-0.02	0.06	-0.14
	x11	-0.10	0.03	-0.10	-0.11	0.15	-0.17	0.03	0.02	-0.11	0.74	-0.06	-0.05	-0.06	0.08
	x12	0.02	-0.02	-0.13	0.01	-0.11	-0.04	-0.15	0.06	0.10	-0.06	0.53	-0.27	-0.06	0.02
	x13	-0.01	-0.08	0.05	-0.07	-0.02	-0.01	0.05	-0.05	-0.02	-0.05	-0.27	0.56	0.09	-0.11
	x14	0.03	0.00	0.09	-0.08	-0.06	0.02	-0.03	-0.02	0.06	-0.06	-0.06	0.09	0.39	-0.27
	x15	0.03	0.00	-0.12	0.05	0.03	0.06	-0.03	0.04	-0.14	0.08	0.02	-0.11	-0.27	0.34

Continued

Anti-image Correlation	x1	.536[a]	-0.61	-0.07	0.12	-0.25	0.14	-0.08	0.15	-0.19	-0.17	0.04	-0.02	0.06	0.07
	x2	-0.61	.617[a]	-0.03	-0.10	0.18	-0.15	-0.08	-0.10	0.04	0.04	-0.04	-0.15	-0.01	0.01
	x3	-0.07	-0.03	.646[a]	-0.27	0.00	-0.10	0.03	0.04	0.07	-0.13	-0.20	0.07	0.16	-0.24
	x4	0.12	-0.10	-0.27	.637[a]	-0.10	0.15	-0.01	-0.09	-0.04	-0.15	0.01	-0.10	-0.14	0.10
	x5	-0.25	0.18	0.00	-0.10	.499[a]	0.14	0.04	-0.29	-0.10	0.20	-0.17	-0.03	-0.11	0.07
	x6	0.14	-0.15	-0.10	0.15	0.14	.578[a]	-0.04	-0.27	-0.05	-0.23	-0.06	-0.02	0.03	0.11
	x7	-0.08	-0.08	0.03	-0.01	0.04	-0.04	.743[a]	-0.15	0.00	0.04	-0.23	0.08	-0.06	-0.05
	x9	0.15	-0.10	0.04	-0.09	-0.29	-0.27	-0.15	.509[a]	-0.25	0.03	0.10	-0.08	-0.04	0.08
	x10	-0.19	0.04	0.07	-0.04	-0.10	-0.05	0.00	-0.25	.541[a]	-0.15	0.15	-0.03	0.12	-0.27
	x11	-0.17	0.04	-0.13	-0.15	0.20	-0.23	0.04	0.03	-0.15	.632[a]	-0.10	-0.07	-0.11	0.17
	x12	0.04	-0.04	-0.20	0.01	-0.17	-0.06	-0.23	0.10	0.15	-0.10	.655[a]	-0.50	-0.13	0.04
	x13	-0.02	-0.15	0.07	-0.10	-0.03	-0.02	0.08	-0.08	-0.03	-0.07	-0.50	.660[a]	0.18	-0.26
	x14	0.06	-0.01	0.16	-0.14	-0.11	0.03	-0.06	-0.04	0.12	-0.11	-0.13	0.18	.536[a]	-0.73
	x15	0.07	0.01	-0.24	0.10	0.07	0.11	-0.05	0.08	-0.27	0.17	0.04	-0.26	-0.73	.522[a]

a. Measures of sampling adequacy (MSA)

Source: IBM Spss Statistics 26 Output /survey results

3.3.2 The second step: Extraction analysis

Method: One of the statistical approaches in extracting factors is principal components analysis; the factors with eigenvalues (own values) > 1.0 and factorial loads > 0.4 were used as consideration criteria. The results were also combined with the orthogonal methods of rotation Varimax.

Detraining the extracted factors: Table (6) shows the number of each extracted factor with Eigenvalues, and percentage of variance and cumulative variance of each of the factors._Six factors with eigenvalues greater than 1 were extracted. Factors that influence the irrigated vegetable farming, according to factors loadings after orthogonal rotation using the Varimax were classified. These factors explained 70.33 % of the total variance, and only less than 29.67 % of variance were due to factors that were not identified through factor analysis. It is noticed that the rotation distributes the variance ratios among the factors in a relatively balanced manner and does not make it concentrated in the first factor or second factor, and this is evident by comparing column (9) in Table (6) where the ratios of interpretation of variances were reached from the total variance of each factor. For example; the first factor has the highest Eigenvalue equals 1.85 and the total explained variance equals 13.21 %.

The total explained variance for the first factor = (Eigenvalue / the number of eigenvalues) * 100 = = (1.85/14) * 100 = 13.21 %.

Commonalities: Table (7) shows the values of the communalities which is greater than 0.05 for each variable, and the average for all variables is 0.703, greater than 0.60. Thus, we have obtained the values of the explained variance for each variable, for example; as the extracted value of the variable x_1 (availability of manpower) equals 0.848 of the variances in the variable values are explained by the common factors.

The Scree test: Is a heuristic graphic method that consists of:

a) Plotting the eigenvalues (y-axis) against the components (x-axis), and

b) Inspecting the shape of the resulting curve in order to detect the point at which the curve changes drastically.

The eigenvalues are plotted as a bold point within the graph, and successive values are connected by a line. Factor extraction should be stopped at the point where there is an "elbow", or leveling of the plot (Thomapson, 2004). This plot suggests that six factors should be extracted (Figure 2).

3.3.3 The third step, rotation

Table (8) shows the loading of the variables on the six factors before rotation and after orthogonal rotation by the Varimax method.

It is noticed that most of the items in the component matrix before rotation loaded on most of the factors. It is also showing a common loading in most of the items on

Table 6: Total explained variance of each extracted factors with eigenvalues

Total Variance Explained									
Component	Initial Eigenvalues			Extraction Sums of Squared Loadings			Rotation Sums of Squared Loadings		
	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %
1	2.95	21.07	21.07	2.95	21.07	21.07	1.85	13.21	13.21
2	2.10	14.98	36.05	2.10	14.98	36.05	1.77	12.65	25.87
3	1.44	10.29	46.34	1.44	10.29	46.34	1.76	12.55	38.41
4	1.23	8.81	55.14	1.23	8.81	55.14	1.56	11.12	49.53
5	1.06	7.60	62.74	1.06	7.60	62.74	1.53	10.94	60.48
6	1.06	7.59	70.33	1.06	7.59	70.33	1.38	9.85	70.33
7	0.86	6.12	76.45						
8	0.70	5.01	81.45						
9	0.67	4.79	86.25						
10	0.63	4.48	90.73						
11	0.48	3.40	94.13						
12	0.35	2.50	96.64						
13	0.28	2.01	98.65						
14	0.19	1.36	100.00						
Extraction Method: Principal Component Analysis									

Source: IBM Spss Statistics 26 Output /survey result

Table 7: Communalities (extracted value of variance) for the measured variables (The problems of irrigated vegetable)

	Initial	Extraction
x1	1.000	.848
x2	1.000	.730
x3	1.000	.548
x4	1.000	.645
x5	1.000	.750
x6	1.000	.734
x7	1.000	.507
x9	1.000	.762
x10	1.000	.721
x11	1.000	.614
x12	1.000	.754
x13	1.000	.562
x14	1.000	.798
x15	1.000	.872
Extraction Method: Principal Component Analysis		

Source: IBM Spss Statistics 26 Output /survey results

the six factors, as most of the variables were of a high load on the first factor, meaning there is a clear absence in the balance of the loading on the extracted factors, which shows the difficulty in interpretation.

Whereas, after the rotation the variance explained by each factor was redistributed. As a change in the pattern of loading, up and down is observed on each factor, and a change in the percentage of explained variance, and it is noticed that the load values that are smaller than 0.6 are hidden and the variables are arranged according to the load, which makes the interpretation easier.

The factor matrix after rotation, which includes six factors. Where all the variables were loaded after rotation on the six factors, except the variable x11. Thus, according to extracted results, the six extracted factors will be named. Table (8).

Figure (3) shows a schematic representation of the rotation of the axes, an orthogonal rotation, meaning that the factors were rotated while maintaining them independent. Before rotation, all the factors were independent (not completely related), and the orthogonal rotation ensures that all the factors remain unrelated.

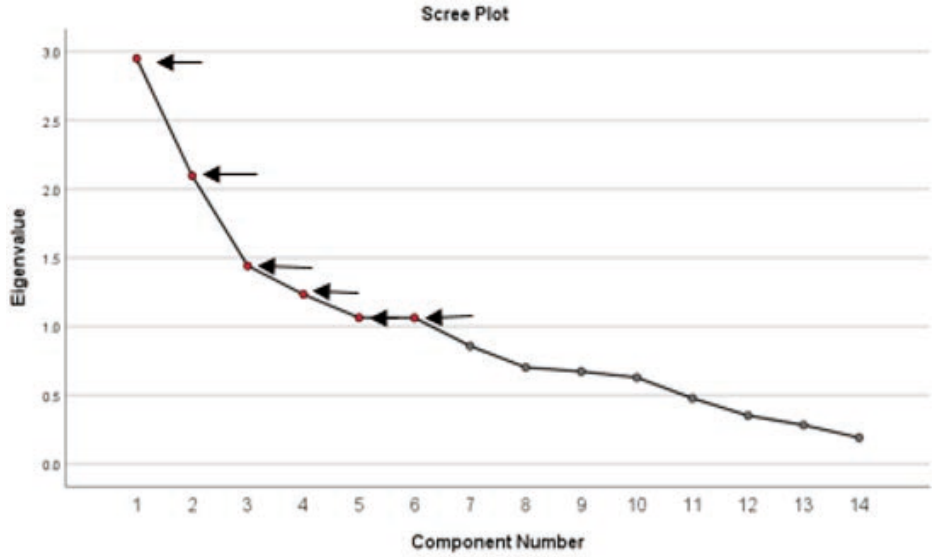

Figure 2: Scree plot Test suggests that six factors should be extracted

Table 8: Components matrix and loadings before rotation and after orthogonal rotation by the Varimax method, for the measured variables (The problems of irrigated vegetable)

	Component Matrix[a]						Rotated Component Matrix[a]					
	Component						Component					
	1	2	3	4	5	6	1	2	3	4	5	6
x1	0.462	0.479		.597-				0.907				
x2	0.556	0.487						0.789				
x3	0.506									0.669		
x4	0.459				.610-					0.772		
x5			0.503		.405-							.676-
x6		0.512		0.586								0.701
x7	0.438				0.452				0.605			
x9			0.679								0.854	
x10			0.6			0.49					0.601	
x11		0.431										
x12	0.681					.415-			0.791			
x13	0.691								0.624			
x14		.745-					0.858					
x15	0.434	.734-					0.909					
- Extraction Method: Principal Component Analysis.							- Extraction Method: Principal Component Analysis.					
- a. 6 components extracted.							- Rotation Method: Varimax with Kaiser Normalization.					
							- a. Rotation converged in 9 iterations.					

Source: IBM Spss Statistics 26 Output /survey result

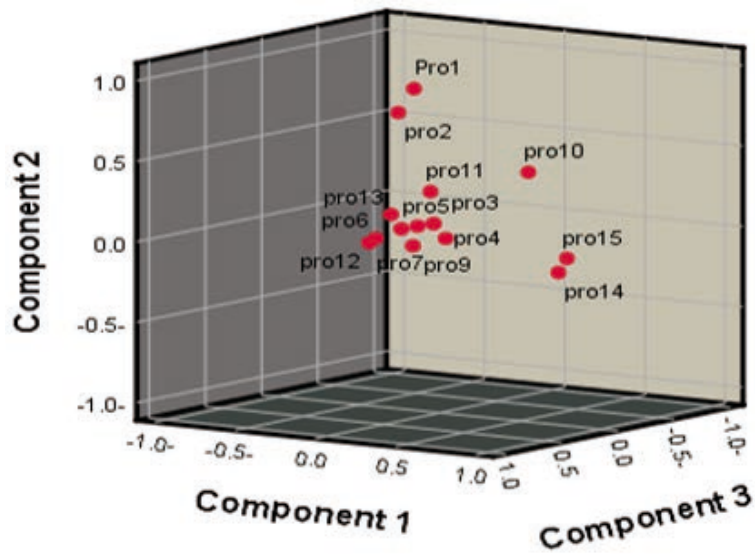

Figure 3: Component plot in rotated space

3.3.4 The fourth step, naming the factors

According to the results extracted from Table (8) above, the six identifying factors were named depending on the percentage of each load factor. All six factors included two determinants except for the third factor (the problem of sale outlets) which included three determinants. Table (9). The exploratory factor analysis technique was used to correspond to the assumptions in the concerning problems with the irrigated vegetable problems in Swaida Governorate, Syria during 2019-2020 Season, which directly effect on the production. The use of the exploratory factor analysis technique enabled the understanding of how variables are interrelated. It allowed the adjustment of the assessment instrument after the removal of variables with low indicators,

Table 9: The factors responsible for the largest proportion of the total variance in the production of irrigated vegetables projects

Factor NO.	Name assigned to factor	Determinants included in Factor Analysis	Factor loadings %
The first	The problem of agricultural technological progress.	(X14): Providing farmers with the necessary expertise	0.858
		(15): Availability of information about new technologies	0.909
The second	The problem of agricultural employment.	(X1) Availability of manpower	0.907
		(X2) Labor efficiency	0.789
		(X7) Effectiveness of Inputs	0.605
The third	The problem of sale outlets	(X12) disposing of the product	0.791
		(X13) Availability of internal and external markets	0.624
The fourth	The problem of natural conditions	(X3) Infection with diseases and insects	0.669
		(X4) natural disasters	0.772
The fifth	The problem of prices	(X9) Vegetable price fluctuations	0.854
		(X10) Brokers' control	0.601
The sixth	The problem of production requirements	(X5) Availability of Inputs	0.676
		(X6) High costs of Inputs	0.701

Source: Depending on Table (8), survey results

namely: [State support for production requirements X_8] and [Low selling price X_{11}]. Therefore, according to the results extracted from the exploratory factor analysis technique, using the Principal components methodology and Varimax rotation the six identifying factors with an initial Eigenvalues greater than one for each and depending on the percentage of each load factor were named: agricultural technological progress, agricultural employment, sale outlets, natural conditions, prices, production requirements. These factors explained (13.21%, 12.65%, 12.55%, 11.12%, 1.94%, and 9.85%) of the total variance respectively, and together explained 70.33%.

4 CONCLUSIONS

This research is unique because it showed the effectiveness of using the exploratory factor analysis methodology in identifying the most important factors responsible for explaining the largest percentage of the total variation in the production of irrigated vegetable projects in As-Swaida Governorate. The study recommends policy makers to addressing all the obstacles facing irrigated vegetable farming in Swaida Governorate in order to reduce their negative effects on the production process, like: marketing management through: preparing to purchase quantities of production directly from farmers, especially the surplus, establishing formal marketing offices to limit the control of brokers, concluding export deals with friendly neighboring countries, and setting minimum prices. Direct and indirect supervision of the production process through: supporting agricultural extension, intensifying agricultural courses related to modern agricultural technologies and marketing methods. Managing strict control, especially the quality of production requirements (seeds, fertilizers, irrigation water, pesticides....).

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Appendix 1

	Anti-image Matrices														
		x1	x2	x3	x4	x5	x6	x7	x8	x9	x10	x11	x12	x13	x14	x15
Anti-image Correlation	x1	.532[a]	-0.61	-0.09	0.10	-0.24	0.15	-0.10	0.12	0.15	-0.14	-0.18	0.01	0.03	0.09	0.03
	x2	-0.61	.566[a]	0.03	-0.05	0.17	-0.18	-0.01	-0.30	-0.08	-0.05	0.08	0.03	-0.26	-0.09	0.10
	x3	-0.09	0.03	.734[a]	-0.24	0.00	-0.11	0.06	-0.18	0.05	0.01	-0.11	-0.16	-0.01	0.10	-0.17
	x4	0.10	-0.05	-0.24	.638[a]	-0.10	0.13	0.01	-0.13	-0.09	-0.08	-0.13	0.04	-0.15	-0.17	0.14
	x5	-0.24	0.17	0.00	-0.10	.515[a]	0.14	0.03	0.02	-0.29	-0.09	0.19	-0.17	-0.02	-0.10	0.06
	x6	0.15	-0.18	-0.11	0.13	0.14	.548[a]	-0.06	0.12	-0.27	-0.01	-0.24	-0.08	0.03	0.06	0.07
	x7	-0.10	-0.01	0.06	0.01	0.03	-0.06	.738[a]	-0.19	-0.14	-0.06	0.06	-0.18	-0.01	-0.10	0.01
	x8	0.12	-0.30	-0.18	-0.13	0.02	0.12	-0.19	.341[a]	-0.04	0.30	-0.13	-0.21	0.41	0.25	-0.32
	x9	0.15	-0.08	0.05	-0.09	-0.29	-0.27	-0.14	-0.04	.511[a]	-0.25	0.03	0.11	-0.09	-0.05	0.09
	x10	-0.14	-0.05	0.01	-0.08	-0.09	-0.01	-0.06	0.30	-0.25	.469[a]	-0.18	0.08	0.10	0.18	-0.34
	x11	-0.18	0.08	-0.11	-0.13	0.19	-0.24	0.06	-0.13	0.03	-0.18	.589[a]	-0.07	-0.12	-0.14	0.20
	x12	0.01	0.03	-0.16	0.04	-0.17	-0.08	-0.18	-0.21	0.11	0.08	-0.07	.641[a]	-0.54	-0.18	0.11
	x13	0.03	-0.26	-0.01	-0.15	-0.02	0.03	-0.01	0.41	-0.09	0.10	-0.12	-0.54	.512[a]	0.27	-0.36
	x14	0.09	-0.09	0.10	-0.17	-0.10	0.06	-0.10	0.25	-0.05	0.18	-0.14	-0.18	0.27	.475[a]	-0.75
	x15	0.03	0.10	-0.17	0.14	0.06	0.07	0.01	-0.32	0.09	-0.34	0.20	0.11	-0.36	-0.75	.465[a]

a. Measures of Sampling Adequacy (MSA)

Source: IBM Spss Statistics 26 Output /survey result

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Introduction of the best criterion for evaluation of tolerance to drought stress in sorghum's genotypes

Leyla NAZARI [1, 2], Ebrahim DEHGHANIAN [3], Afshar ESTAKHR [1], Azim KHAZAEI [4], Behzad SORKH-ILALEHLOO [4], Mohammad Reza ABBASI [5]

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Introduction of the best criterion for evaluation of tolerance to drought stress in sorghum's genotypes

Abstract: Sorghum (*Sorghum bicolor* (L.) Moench) is the fifth important cereal considered a drought-tolerant crop. However, its reduction of grain yield considerably occurs in a shortage of water. In the current study, 10 sorghum genotypes were assessed for their grain yield under normal irrigation and water deficit irrigation. As well, the efficacy of several drought indices was evaluated for the selection of high-yield and drought-tolerant genotypes. The experiment was conducted as a split-plot considering three irrigation levels as main-plot and 10 genotypes as sub-plot. Correlation among the indices, clustering of the genotypes along with principal component analysis was employed. Yield production was significantly and positively correlated with indices MP (mean productivity), STI (stress tolerance index), GMP (geometric productivity), HM (harmonic mean), and YI (yield index) in all the irrigation levels. Therefore, these indices are more effective in the selection of high-yielding genotypes under different water conditions. Rank means of stress indices for each genotype revealed that genotype TN-04-79 in mild deficit irrigation and genotypes KGS23 and TN-04-79 in severe deficit irrigation were the most tolerant.

Key words: sorgum; drought stress; grain yield; water productivity; drought response indices

Uvajanje najboljših kriterijev za ovrednotenje tolerance na sušo pri genotipih sirka

Izvleček: Navadni sirek (*Sorghum bicolor* (L.) Moench) je peto najpomembnejše na sušo odporno žito, a se kljub temu njegov pridelek zrnja znantno zmanjša ob pomanjkanju vode. V tej raziskavi je bilo ocenjenih 10 genotipov navadnega sirka glede na pridelek zrnja ob normalnem namakanju in v razmerah vodnega deficita. Ocenjeni so bili tudi različni indeksi tolerance na sušo pri izboru genotipov z velikimi pridelki zrnja in dobre tolerance na sušo. Poskus je bil izveden kot puskus z deljenkami, kjer so bila obravnavanja z namakanjem na glavnih ploskvah in 10 genotipov na podploskvah. Uporabljene so bile korelacije med indeksi in združevanje genotipov glede na glavno komponento. Velikost pridelka je bila značilno pozitivno povezana z indeksi MP (poprečna produktivnost), STI (indeks tolerance na stres), GMP (geometrična produktivnost), HM (harmonično poprečje) in YI (indeks pridelka) pri vseh načinih namakanja. Ti indeksi so torej bolj učinkoviti pri izboru visoko donosnih genotipov v razmerah različne preskrbe z vodo. Poprečje rangov stresnih indeksov za vsak genotip je odkrilo, da je genotip TN-04-79 najučinkoviteši ob blagem pomanjkanju vode, genotipa KGS23 in TN-04-79 pa sta bila najbolj odporna na sušo.

Ključne besede: navadni sirek; sušni stres; pridelek zrnja; učinkovitost izrabe vode; indeksi odziva na sušni stres

1 Crop and Horticultural Science Research Department, Fars Agricultural and Natural Resources Research and Education Center, Agricultural Research, Education and Extension Organization (AREEO), Shiraz, Iran
2 Corresponding author, e-mail: l.nazari@areeo.ac.ir
3 Agricultural Engineering Research Department, Fars Agricultural and Natural Resources Research and Education Center, Agricultural Research, Education and Extension Organization (AREEO), Shiraz, Iran
4 Seed and Plant Improvement Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran
5 Crop and Horticultural Science Research Department, Khorasan Razavi Agricultural and Natural Resources Research and Education Center, Agricultural Research, Education and Extension Organization (AREEO), Mashhad, Iran

1 INTRODUCTION

Sorghum (*Sorghum bicolor* (L.) Moench) is a C_4 and drought-tolerant crop used for food, feed, and fiber (Ludlow et al., 1990). Its tolerance to drought can be attributed to morphological characteristics (e.g. deep root system and thick leaf wax), physiological responses (e.g. stay green and osmotic adjustment), and adaptive mechanisms allowing tolerance under extreme drought conditions (reviewed in Tari et al., 2013). In the dry region of Asia and the Middle East, drought is one of the most important abiotic stresses, leading to the limitation of plant growth and yield productivity (Zhang et al., 2018). Therefore, improving yield production per unit of water (water productivity) is an efficient strategy in dry regions (Ali and Talukder, 2008).

Blum (2005) suggested that the selection of genotypes should mainly focus on high yield under non-stress conditions and secondly under water stress conditions. The selection of genotypes that have tolerant genes is difficult as drought tolerance is a quantitative trait with intricate heritability. Therefore, despite the lack of information on drought tolerance mechanisms, researchers have proposed the utility of different selection indices to screen drought-tolerant genotypes (Anwaar et al., 2019). Hence, we have employed the following selection criteria for screening drought-tolerant genotypes and introducing the best indices.

Several indices based on the yield under control (Y_p) and stress (Y_s) have been introduced for the selection of drought-tolerant genotypes. Among these, the indices employed in various stress conditions are stress tolerance (TOL) and mean productivity (MP) introduced by Rosielle and Hamblin (1981), Stress susceptibility index (SSI) by (Fischer and Maurer, 1978), stress tolerance index (STI) and geometric mean productivity (GMP) by Fernandez (1992), Harmonic mean of yield (HM) by Jafari et al. (2009), yield index (YI) by Gavuzzi et al. (1997), yield stability index (YSI) by Bouslama and Schapaugh (1984), yield reduction ratio (YRR) by Golestani-Araghi and Assad (1998). Selection of high-yield genotypes in both normal and deficit irrigation using a combination of these indices is preferred. Therefore, different statistical analyses including analysis of variance (ANOVA), correlation, principal component analysis (PCA), and cluster analysis were performed. The study aimed to investigate the efficiency of the mentioned indices for screening tolerant genotypes of sorghum to drought stress.

2 MATERIALS AND METHODS

2.1 EXPERIMENTAL SITE

The experiment was conducted at the Research Farm of Fars Agricultural and Natural Resources Research and Education Center, Shiraz, Iran (52°42' E, 29°46' N, 1.604 m elevation) with a semi-arid environment (Fig. 1a). It is characterized by mean annual precipitation of 345 mm

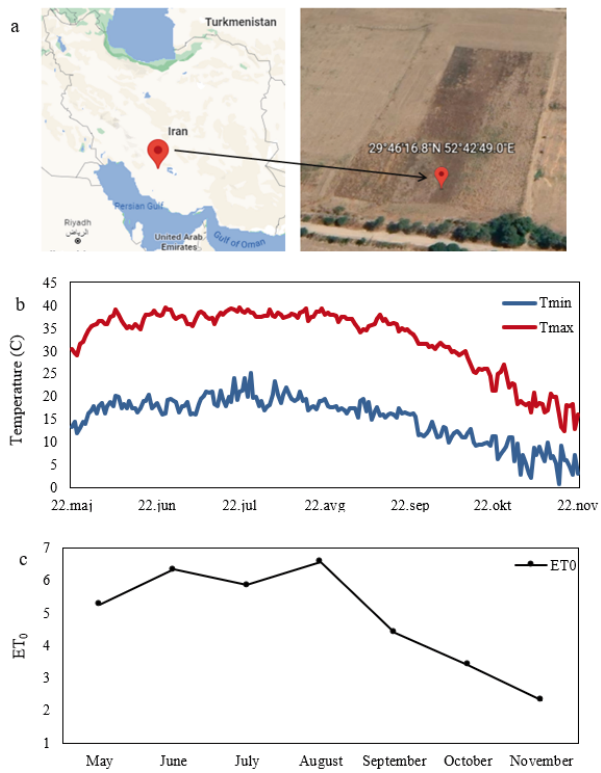

Fig. 1: The spatial position of the experimental site captured on 07/11/2021 (a); minimum and maximum of temperature (b) and ET_0 (c) during the growth season (2018) conducted at Zargan, Iran (52°42'E, 29°46'N)

Table 1: The chemical properties of the soil in the experimental area

soil depths cm	pH	EC*10³	O.C %	phosphorus ppm	potassium ppm	sand %	silt %	clay %	zinc ppm	magnesium ppm	copper ppm
0-30	8.0	0.97	0.95	11.2	434	20.4	46.2	33.4	0.80	7.60	0.90
30-60	7.9	2.15	0.81	4.2	310	20.4	42.8	36.8	0.96	8.50	0.96

EC: electrical conductivity; O.C: organic carbon; extractable phosphorus was measured according to Olson method

and an annual temperature of 15.8 °C. Minimum and maximum temperature and ET_0 during growth season are presented in Fig. 1b,c. The soil is characterized by fine, carbonatic, active, thermic Typic Calcixerepts (soil taxonomy, 2014) and Cambic Calcisol (Lomic, Ochric) (WRB, 2015). The fertilizers were distributed based on soil test results (Table 1).

2.2 EXPERIMENTAL DESIGN

The plants (10 sorghum genotypes, supplementary Table 1) were cultivated manually as split-plot in a randomized complete block design with three replicates on 6 June 2018. Water deficit treatment was considered as the main factor and genotype as the sub-factor. Subplots were 12 m^2 including 4 rows of 5 m long with a row distance of 0.6 m. Tinning was performed 4 weeks after sowing with a target of 10 plants per linear meter. Weed control was performed manually during the season.

Irrigation treatments were applied to the main plots at three levels of normal irrigation, mild and severe water-deficit irrigation defined as irrigation when the evaporation rates from pan class A exceeded 60, 120, and 180 mm, respectively. Water stress was started from the 5 leaves stage and continued during the season.

Irrigation was applied using a tape drip and the irrigation volume was recorded by using a volumetric counter. FAO-CROPWAT 8.0 as a decision support system (DSS) was used to calculate the reference crop evapotranspiration (ET_o) (Clarke, 2001) and schedule different levels of irrigation. The accuracy of this method was demonstrated by comparing it to original crop water requirements (Surendran et al., 2019). Meteorological data were taken daily from the Zargan Meteorological Station near the experimental field. The irrigation requirement was calculated according to Doorenbos and Pruitt (1977) (Table 2).

2.3 MEASUREMENTS AND DROUGHT INDICES

Agronomic characteristics including plant height (PH), panicle length (PL), stem diameter (SD), and the number of leaves per plant (NoL) were recorded for 10 plants per plot from the middle two-row of each plot. As well, 1000 seed mass (1000 SM), dry matter yield (DMY), and harvest index (HI) were recorded. Water productivity (WP) was calculated as Ali and Talukder (2008) (Table 2).

Drought tolerance indices were calculated according to the equations in Table 2. Ranking of the genotypes

Table 2: Description, equation and reference of crop water requirement, water productivity, and drought tolerance indices

Index	description	equation	Reference
Crop Water Requirement (mm/day)	K_c: water requirement coefficient changing with the growth stages of sorghum; ET_o: the reference evapotranspiration of plant under specified conditions measured by pan evaporation.	$ET_c = K_c \times ET_o$	Doorenbos and Pruitt, 1977; Doorenbos and Kassam, 1986
Water productivity (WP)	crop production per unit volume of water; high values are more desirable	$WP = \frac{Grain\ or\ seed\ yield}{water\ applied\ to\ the\ field}\ (kg\ m^{-3})$	Ali and Talukder, 2008
Tolerance index (TOL)	Low values indicate more stability under deficit irrigation	$TOL = Y_p - Y_s$	Rosielle and Hamblin (1981)
Mean productivity (MP)	High values are more desirable	$MP = \frac{Yp+Ys}{2}$	Rosielle and Hamblin (1981)
Stress susceptibility index (SSI)	Values < 1 are more tolerant	$SSI = \frac{1-\frac{Ys}{Yp}}{1-\frac{\bar{Y}s}{\bar{Y}p}}$	Fischer and Maurer (1978)
Stress tolerance index (STI)	High values indicate more tolerant	$STI = \frac{(\bar{Y}p \times Ys)}{(\bar{Y}p)^2}$	Fernandez (1992)
Geometric productivity (GMP)	High values are more desirable	$GMP = \sqrt{Y_p \times Y_s}$	Kristin et al. (1997)
Harmonic mean of yield (HM)	High values are more desirable	$HM = \frac{2Y_p \times Y_s}{Y_p + Y_s}$	Jafari et al. (2009)
Yield index (YI)	High values indicate more tolerant	$YI = \frac{Y_s}{\bar{Y}_s}$	Gavuzzi et al. (1997)
Yields stability index (YSI)	High values indicate more stability under normal and deficit irrigation	$YSI = \frac{Y_s}{Y_p}$	Bouslama and Schapaugh (1984)
Yield reduction ratio (YRR)	Low values indicate more suitable for deficit irrigation	$YRR = 1 - \frac{Y_s}{Y_p}$	Golestani-Araghi and Assad (1998)

based on the indices was performed according to the method of Mickky et al. (2019).

The means of grain yield and the indices were ranked considering that indices with higher values are more desirable except TOL, SSI, and YRR. Afterward, rank mean (R') and standard deviation of rank (SDR) were calculated. Rank mean is defined as the average of ranking values across all drought tolerance indices of each genotype. Rank sum (RS) of each genotype was then determined by the addition of rank mean (R') and standard deviation of rank (SDR).

2.4 STATISTICAL ANALYSIS

Analysis of variance (ANOVA) and mean comparison were performed using SAS release 9.2 (SAS Institute, Cary, NC, USA). Before doing ANOVA, normality tests were conducted. Provided that *F*-values were significant, a mean comparison was done (Duncan's test, $p \leq 0.05$). Drought stress indices, principal component analysis (PCA) and Pearson's correlation between the indices were performed using iPASTIC that is an online tool kit for the estimation of plant abiotic stress indices (Khalili et al., 2016). Genotypes were clustered using Ward's hierarchical clustering.

3 RESULTS AND DISCUSSION

Here, we evaluated 10 sorghum genotypes for drought tolerance collected from different parts of Iran and kept at The National Plant Gene-Bank of Iran, SPII. Natural genetic diversity may play an important role in food security through pre-breeding programs or the introduction of important traits or genes into existing cultivars (Priyanka et al., 2021). As well, the efficacy of drought stress indices for screening of these genotypes was scrutinized. Using the yield values of Y_p and Y_s, various indices were calculated (Table 4) and the genotypes were ranked for each index (Table 5).

3.1 MORPHOLOGICAL TRAITS

Significant differences were observed between irrigation regimes for all traits ($p < 0.01$ for PH, SD, and DMY; $p < 0.05$ for PL and 1000 SM) except NoL. There were significant differences between genotypes for all traits ($p < 0.01$) indicating significant variation among the genotypes. The interaction effect of deficit irrigation × genotype was significant ($p < 0.01$) except for NoL and 1000 SM.

Table 3: The main effects of irrigation level and genotype on morphological traits, yield, and water productivity of 10 sorghum genotypes

Treatments	PH cm	PL cm	SD mm	NoL	1000 SM g	Yield kg ha^{-1}	DMY kg m^{-3}	HI %	WP kg m^{-3}
Irrigation level									
normal irrigation	175.2^{a}	29.8^{a}	26.4^{a}	16.9^{a}	29.4^{a}	5847.2^{a}	27874.1^{a}	23.2^{a}	0.65^{a}
mild deficit irrigation	152.0^{b}	27.6^{ab}	24.5^{b}	14.0^{a}	28.5^{ab}	4026.4^{b}	22260.9^{b}	21.3^{b}	0.58^{b}
severe deficit irrigation	138.7^{c}	25.3^{b}	23.6^{c}	14.6^{a}	26.5^{b}	2759.2^{c}	21480.3^{b}	16.5^{c}	0.43^{c}
Genotype									
MGS2	105.3^{g}	30.7^{c}	25.9^{c}	12.8^{bc}	2.9^{f}	2910.1^{f}	16188.9^{e}	17.7^{e}	0.37^{e}
KGS23	94.0^{h}	18.9^{c}	24.0^{d}	10.9^{bc}	4.7^{bc}	4683^{bc}	16466.6^{e}	29.2^{b}	0.63^{b}
TN-04-78	120.0^{f}	24.0^{d}	30.1^{b}	16.7^{b}	3.5^{e}	3491.1^{e}	24276.2^{c}	14.4^{e}	0.45^{d}
TN-04-79	221.1^{a}	10.0^{g}	24.9^{cd}	15.2^{bc}	6.5^{a}	6517.6^{a}	30906.7^{b}	21.3^{c}	0.88^{a}
TN-04-129	91.9^{h}	22.3^{d}	33.4^{a}	13.4^{bc}	4.3^{c}	4304.2^{cd}	20498.8^{d}	20.8^{d}	0.57^{c}
TN-04-134	201.6^{b}	14.6^{f}	21.5^{e}	22.7^{a}	6.2^{b}	4974.3^{b}	28825.6^{b}	16.7^{e}	0.64^{b}
TN-04-142	227.0^{a}	7.7^{g}	21.7^{e}	28.4^{a}	1.6^{g}	1558.7^{g}	48770.0^{a}	3.2^{f}	0.19^{f}
TN-04-59	159.3^{d}	53.4^{a}	24.5^{cd}	10.4^{bc}	5.0^{b}	5047.8^{b}	18799.6^{de}	26.8^{b}	0.67^{b}
TN-04-86	151.2^{e}	40.9^{b}	21.2^{e}	9.8^{c}	4.7^{bc}	4685.4^{bc}	15641.6^{e}	30.3^{a}	0.63^{b}
TN-04-90	180.6^{c}	53.2^{a}	21.1^{e}	11.4^{bc}	3.9^{d}	3937.2^{d}	18343.6^{de}	22.6^{c}	0.538^{c}

PH: plant height; PL: panicle length; SD: stem diameter; NoL: number of leaves; 1000 SM: 1000 seed mass; DMY: dry matter yield; HI: harvest index; WP: water productivity.
Means followed by the same letter in a column do not differ by Duncan's test at 5 % probability

The average PH reduced 13.2 % and 20.8 % under mild and severe deficit irrigation, respectively (Table 3). The reduction ratios for PL were 7 % and 15 % under mild and severe deficit irrigation, respectively (Table 3). Ashraf and Foolad (2007) indicated a reduction in turgidity and cell growth and development under water shortage observed as a reduction in PH or panicle size. A significant effect of drought stress on the PH of forage sorghum has also been demonstrated (Mutava et al., 2011).

Stem diameter (SD) decreased 7.2 % and 10.6 % under mild and severe stress, respectively (Table 3). Our results were in line with Almodares et al. (2013) demonstrating that the stem diameter of sorghum decreased proportionally to water deficit intensity. The reduction of SD in sugarcane under water deficit has been proven (Silva et al., 2008). Controversial results were reported by other studies pointing out no reduction in SD under drought stress (Almodares et al., 2013; Fracasso et al., 2016; Ottman et al., 2001). The 1000 SM reduction was 9.8 % from normal irrigation to severe deficit irrigation (Table 3). Deficit irrigation resulted in a notable fall in DMY equal to 79.9 % and 77.1 % under mild and severe stress, respectively (Table 3).

Genotypes TN-04-79 and TN-04-142 exhibited the highest PH value and genotypes KGS23 and TN-04-129 were the lowest in PH (Table 3). Generally, taller sorghum genotypes are favored for small-scale farms that mechanical harvests are not employed (Devnarain et al., 2016). Genotypes TN-04-90 and TN-04-59 had the highest PL and genotypes TN-04-79 and TN-04-142 ranked the last (Table 3). Different values of SD were obtained with the highest value for the genotype TN-04-129 (Table 3). The highest NoL belonged to TN-04-134 and TN-04-142. Genotypes TN-04-79 and TN-04-90 ranked the highest and the lowest 1000 SM, respectively (Table 3). The genotype TN-04-142 produced the highest DMY. There were no significant differences between MGS2, KGS23, TN-04-59, TN-04-86, and TN-04-90 in DMY as the lowest rank (Table 3).

3.2 GRAIN YIELD, HARVEST INDEX, AND WATER PRODUCTIVITY (WP)

The effects of deficit irrigation, genotype, and their interaction on yield, HI, and WP were significant ($p < 0.01$). Grain yield and HI decreased significantly in response to water deficit, resulting in lower values equal to 52.8 % and 28.9 %, respectively (Table 3). The mean of WP under severe deficit irrigation was reduced by 31.7 % compared to normal irrigation (Table 3). Chimonyo et al. (2016) reported no significant reduction in sorghum yield under deficit irrigation in comparison to full irrigation (3160 kg ha^{-1} vs. 3240 kg ha^{-1}), indicating sorghum as drought tolerant, which is suitable for marginal lands. However, our results noted that sub-optimal irrigation resulted in sub-optimal WP. Hence, an important point to farmers is the benefit of irrigating sorghum considering the water supply.

The highest grain yield and WP under normal irrigation belonged to genotype TN-04-79 followed by TN-04-134 (Fig. 2). Under mild deficit irrigation, genotype TN-04-79 had the highest grain yield and WP, because WP of this genotype under mild deficit irrigation was slightly higher (not statistically significant) than that value under normal irrigation. The highest values of grain yield and WP, when severe deficit irrigation was imposed, were related to genotype KGS23 followed by TN-04-79 and TN-04-86. Moreover, the highest WP obtained for genotype TN-04-79 under mild deficit irrigation and the lowest value of WP belonged to genotype TN-04-142 under severe stress (Fig. 2).

It has been reported that sorghum WP was in a range of 1.24-1.34 kg m^{-3} in Nebraska under normal irrigation (Maman et al. 2003). Grain WP in the trial of Hadebe et al. (2020) was relatively lower in a range of 0.75-1.1 kg m^{-3} for three different genotypes. Moreover, they attributed high WP under irrigation to high yield proportional to water applied in the field. The effect of genotype, duration, and extent of water stress may account for the variation of results in this study with those of other studies.

3.3 VALUES AND RANKS OF DROUGHT INDICES

Mean comparison of ranking values (R), ranking mean values (R') and rank sum (RS) under mild deficit irrigation showed that genotype TN-04-79 performed superiorly except for TOL (ranked 3) (Table 5). The superior genotype based on the TOL index was genotype TN-04-129. On the other hand, genotype TN-04-142 performed inferiorly based on Y_p, Y_s, and all drought tolerance indices except for TOL ranked 8 (Table 5). A different trend in the response of the genotypes to severe deficit irrigation was observed. While genotype TN-04-79 performed superiorly based on Y_p, MP, STI, GMP, and HM, genotype KGS23 was superior when considering Y_s, TOL, SSI, YSI, YRR, and YI indices (Table 5).

It could be concluded that different drought-tolerance indices presented herein introduced different genotypes as drought tolerant. Similar results have been reported for the screening of drought-tolerant genotypes based on various indices (Nikneshan et al., 2019; Abd El-Mohsen et al. 2015). Therefore, the selection of tolerant

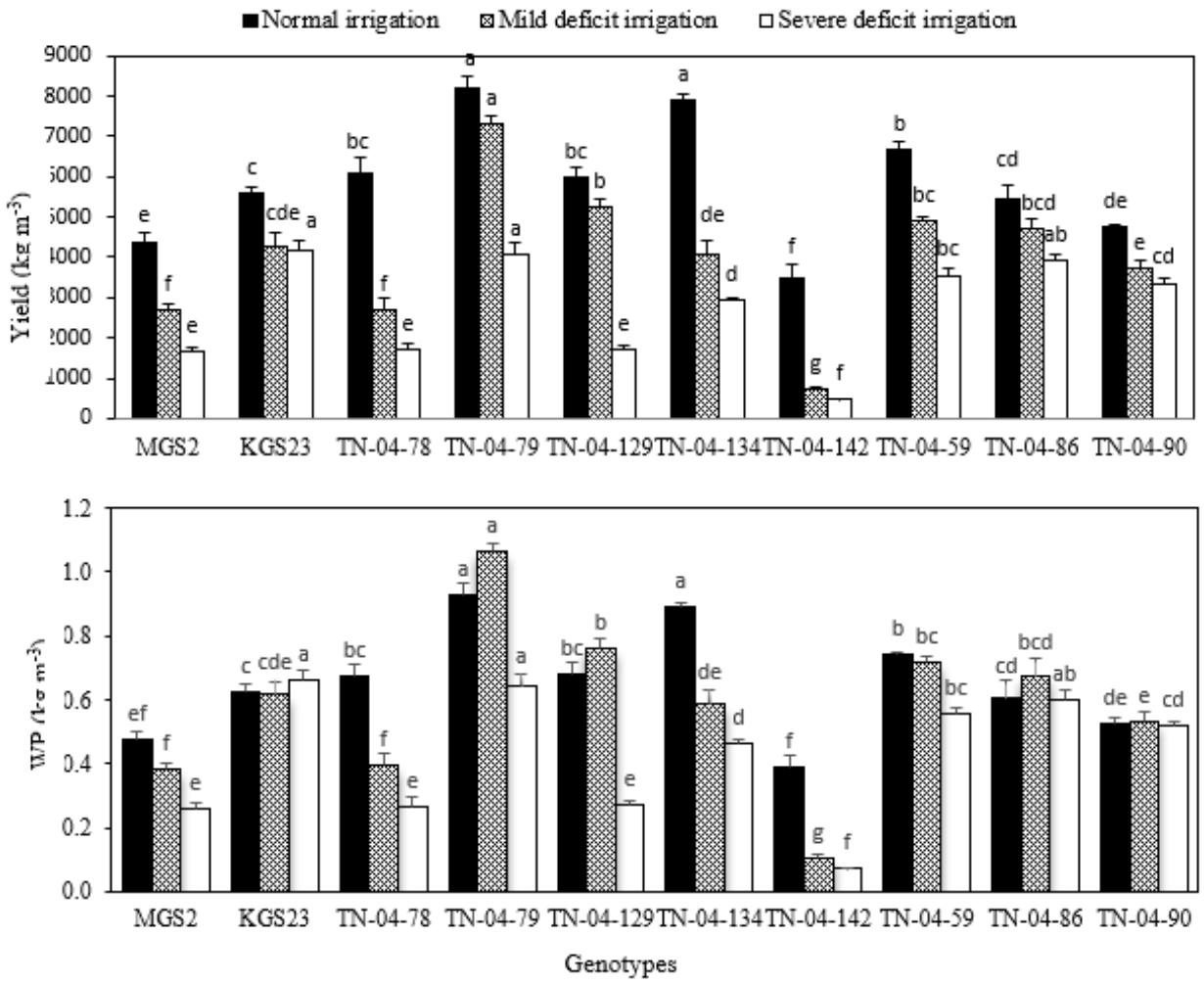

Figure 2: Grain yield and water productivity (WP) of 10 grain sorghum genotypes under normal irrigation, mild and severe deficit irrigation. Means followed by the same letter are not significantly different in each level of irrigation treatment (Duncan's test, $p < 0.05$)

genotypes was adopted by the ranking method based on ranking mean values (R'), standard deviation of ranks (SDR), and rank sum (RS).

According to values of R' and RS calculated based on the yield under normal irrigation and mild deficit irrigation, genotype TN-04-79 exhibited the first mean rank value and sum rank value followed by genotype TN-04-129 indicating that these genotypes can be primarily categorized as the most tolerant to mild deficit irrigation. Whilst, genotype TN-04-142, MGS2, and TN-04-78 exhibited the worst mean rank and rank sum, respectively, that can be considered as the most susceptible to mild water deficit irrigation (Table 5). On the other hand, R' and RS calculated based on yield in normal irrigation and severe deficit irrigation presented different results. The first mean rank and sum rank value belonged to genotype KGS23, while genotype TN-04-142 was inferior in R' and RS (Table 5).

3.4 CORRELATIONS AMONG DROUGHT INDICES

Pearson's correlation coefficients (r) between Y_p, Y_s, and the indices were determined to select the best indices for the screening of drought-tolerant genotypes (Fig. 3). A positive significant correlation between Y_p and Y_s under mild and severe deficit irrigation was recorded (Fig. 3). This may imply that high yielding potential under normal irrigation is necessarily accompanied by reasonable yield under mild and severe deficit irrigation. Similar

Table 4: Mean values ± standard deviation of grain yield (ton/ha) and drought tolerance indices of ten sorghum genotypes under normal irrigation, mild and severe water deficit irrigation

	Calculated based on yield under normal and mild deficit irrigation										
genotype	Y_p	Y_S	TOL	MP	SSI	STI	GMP	HM	YSI	YRR	YI
MGS2	4.39±0.36	2.67±0.28	1.71±0.32	3.53±0.28	0.74±0.10	0.34±0.06	3.42±0.28	3.32±0.29	0.61±0.06	0.39±0.06	0.97±0.10
KGS23	5.58±0.27	4.28±0.54	1.31±0.28	4.93±0.40	0.45±0.12	0.70±0.12	4.88±0.43	4.84±0.45	0.76±0.06	0.24±0.06	1.55±0.20
TN-04-78	6.07±0.69	2.70±0.5	3.38±0.73	4.38±0.48	1.04±0.15	0.48±0.12	4.03±0.49	3.72±0.53	0.45±0.08	0.55±0.08	0.98±0.18
TN-04-79	8.18±0.57	7.30±0.32	0.88±0.39	7.74±0.42	0.20±0.08	1.75±0.18	7.73±0.41	7.71±0.41	0.89±0.04	0.11±0.04	2.65±0.12
TN-04-129	5.98±0.45	5.23±0.36	0.75±0.78	5.60±0.12	0.23±0.23	0.91±0.03	5.58±0.11	5.56±0.10	0.88±0.12	0.12±0.12	1.89±0.13
TN-04-134	7.91±0.23	4.06±0.62	3.85±0.47	5.99±0.40	0.92±0.13	0.94±0.17	5.66±0.49	5.35±0.58	0.51±0.07	0.49±0.07	1.47±0.22
TN-04-142	3.47±0.60	0.72±0.11	2.76±0.50	2.10±0.35	1.50±0.03	0.07±0.02	1.58±0.25	1.19±0.19	0.21±0.01	0.79±0.01	0.26±0.04
TN-04-59	6.68±0.29	4.91±0.19	1.78±0.42	5.79±0.12	0.50±0.10	0.96±0.04	5.72±0.12	5.65±0.12	0.74±0.05	0.26±0.05	1.78±0.07
TN-04-86	5.45±0.63	4.69±0.43	0.76±0.23	5.07±0.52	0.26±0.05	0.75±0.16	5.06±0.52	5.04±0.51	0.86±0.03	0.14±0.03	1.70±0.16
TN-04-90	4.75±0.11	3.71±0.37	1.03±0.28	4.23±0.23	0.41±0.12	0.52±0.06	4.20±0.25	4.16±0.26	0.78±0.06	0.22±0.06	1.35±0.13
	Calculated based on yield under normal and severe deficit irrigation										
genotype	Y_p	Y_s	TOL	MP	SSI	STI	GMP	HM	YSI	YRR	YI
MGS2	4.39±0.36	1.67±0.19	2.72±0.55	3.03±0.09	1.16±0.15	0.21±0.01	2.70±0.05	2.41±0.14	0.39±0.08	0.61±0.08	0.61±0.07
KGS23	5.58±0.27	4.19±0.37	1.39±0.52	4.89±0.19	0.47±0.16	0.68±0.06	4.83±0.21	4.78±0.24	0.75±0.08	0.25±0.08	1.52±0.13
TN-04-78	6.07±0.69	1.70±0.28	4.37±0.72	3.89±0.39	1.35±0.10	0.30±0.07	3.21±0.34	2.65±0.36	0.28±0.05	0.72±0.05	0.62±0.10
TN-04-79	8.18±0.57	4.07±0.47	4.11±0.62	6.13±0.42	0.95±0.11	0.97±0.15	5.76±0.44	5.42±0.48	0.50±0.06	0.50±0.06	1.47±0.17
TN-04-129	5.98±0.45	1.71±0.18	4.27±0.61	3.84±0.16	1.34±0.09	0.30±0.02	3.19±0.09	2.65±0.18	0.29±0.05	0.71±0.05	0.62±0.06
TN-04-134	7.91±0.23	2.95±0.10	4.96±0.23	5.43±0.13	1.18±0.03	0.68±0.03	4.83±0.12	4.30±0.12	0.37±0.01	0.63±0.01	1.07±0.04
TN-04-142	3.47±0.60	0.48±0.02	2.99±0.59	1.98±0.31	1.62±0.04	0.05±0.01	1.29±0.13	0.85±0.04	0.14±0.02	0.86±0.02	0.17±0.01
TN-04-59	6.68±0.29	3.55±0.28	3.13±0.50	5.12±0.13	0.88±0.11	0.69±0.05	4.87±0.16	4.63±0.21	0.53±0.06	0.47±0.06	1.29±0.10
TN-04-86	5.45±0.63	3.91±0.31	1.54±0.66	4.68±0.37	0.52±0.18	0.62±0.09	4.61±0.34	4.54±0.32	0.72±0.10	0.28±0.10	1.42±0.11
TN-04-90	4.75±0.11	3.35±0.22	1.39±0.30	4.05±0.09	0.55±0.11	0.46±0.03	3.99±0.11	3.93±0.13	0.71±0.06	0.29±0.06	1.21±0.08

Yp = grain yield under normal irrigation, Ys = grain yield under deficit irrigation, TOL = tolerance index, MP = mean productivity, SSI = stress susceptibility index, STI = stress tolerance index, GMP = geometric productivity, HM = harmonic mean of yield, YSI = yield stability index, YRR = yield reduction ratio, YI = yield index

Table 5: Ranking values (R), ranking mean values (R'), standard deviation of ranks (SDR) and rank sum (RS) of grain yield of ten sorghum genotypes under normal irrigation and water deficit irrigation after 120 mm evaporation from Pan class A

Mild deficit irrigation														
	R											R'	SDR	RS
Genotype	Y_p	Y_s	TOL	MP	SSI	STI	GMP	HM	YSI	YRR	YI			
MGS2	9	9	6	9	7	9	9	9	7	7	9	8.18	1.17	9.35
KGS23	6	5	5	6	5	6	6	6	5	5	5	5.45	0.52	5.98
TN-04-78	4	8	9	7	9	8	8	8	9	9	8	7.91	1.45	9.36
TN-04-79	1	1	3	1	1	1	1	1	1	1	1	1.27	0.65	1.92
TN-04-129	5	2	1	4	2	4	4	3	2	2	2	2.55	1.29	3.84
TN-04-134	2	6	10	2	8	3	3	4	8	8	6	5.55	2.73	8.28
TN-04-142	10	10	8	10	10	10	10	10	10	10	10	9.82	0.60	10.42
TN-04-59	3	3	7	3	6	2	2	2	6	6	3	4.00	1.84	5.84
TN-04-86	7	4	2	5	3	5	5	5	3	3	4	4.18	1.40	5.58
TN-04-90	8	7	4	8	4	7	7	7	4	4	7	6.09	1.70	7.79
Severe deficit irrigation														
	R											R'	SDR	RS
Genotype	Y_p	Y_s	TOL	MP	SSI	STI	GMP	HM	YSI	YRR	YI			
MGS2	9	9	4	9	6	9	9	9	6	6	9	7.73	1.85	9.58
KGS23	6	1	1	4	1	3	4	2	1	1	1	2.27	1.74	4.01
TN-04-78	4	8	9	7	9	7	7	7	9	9	8	7.64	1.50	9.14
TN-04-79	1	2	7	1	5	1	1	1	5	5	2	2.82	2.23	5.05
TN-04-129	5	7	8	8	8	8	8	8	8	8	7	7.55	0.93	8.48
TN-04-134	2	6	10	2	7	4	3	5	7	7	6	5.36	2.46	7.82
TN-04-142	10	10	5	10	10	10	10	10	10	10	10	9.55	1.51	11.05
TN-04-59	3	4	6	3	4	2	2	3	4	4	4	3.55	1.13	4.67
TN-04-86	7	3	3	5	2	5	5	4	2	2	3	3.73	1.62	5.35
TN-04-90	8	5	2	6	3	6	6	6	3	3	5	4.82	1.83	6.65

Yp = grain yield under normal irrigation, Ys = grain yield under deficit irrigation, TOL = tolerance index, MP = mean productivity, SSI = stress susceptibility index, STI = stress tolerance index, GMP = geometric productivity, HM = harmonic mean of yield, YSI = yield stability index, YRR = yield reduction ratio, YI = yield index

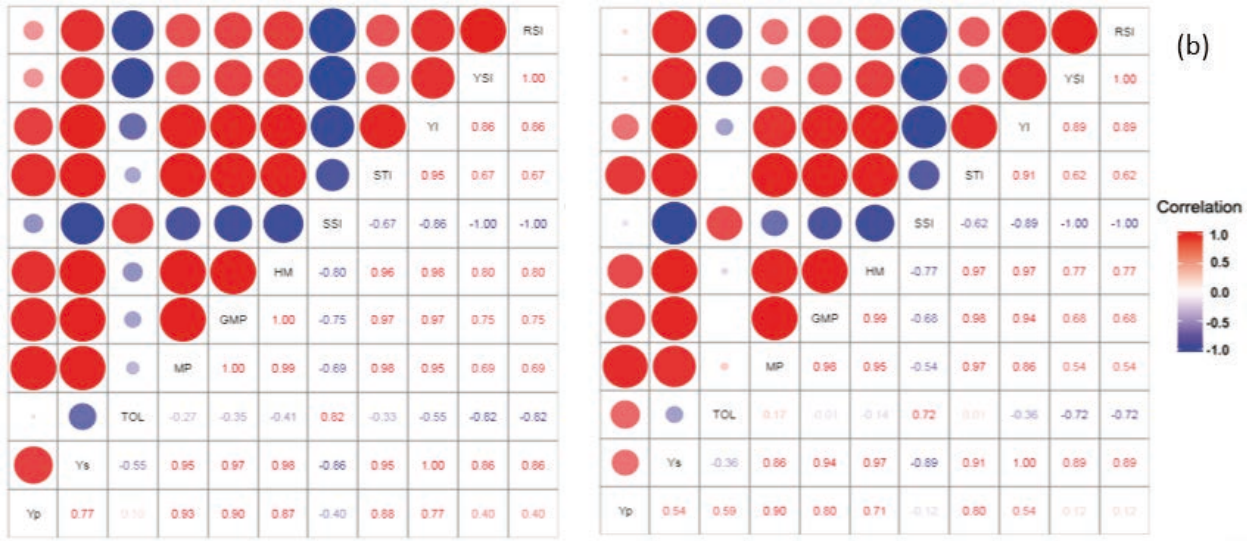

Fig. 3: Heat map based on the actual values of indices (Pearson's correlation analysis) across 10 sorghum genotypes produced using iPASTIC online tool kit. Y_p, yield under normal irrigation; Y_s, yield under mild deficit irrigation for (a) and under severe deficit irrigation for (b); TOL, tolerance index; MP, mean productivity, GMP, geometric mean probability; HM, Harmonic mean; SSI, stress susceptibility index; STI, stress tolerance index; YI, yield index; YSI, yield stability index; RSI, relative stress index

results of the wheat response to drought were previously recorded by Abebe et al. (2020).

While there was a significant correlation between Y_s (mild and severe deficit irrigation) and all the indices, there was no correlation between Y_p and SSI, YSI, and YRR. There was no correlation between Y_p and TOL under mild deficit irrigation. On the other hand, a positive significant correlation was obtained between Y_p and TOL calculated based on yield in severe deficit irrigation suggesting that selection based on the low score of TOL may lead to enhanced yield under severe deficit irrigation but reduced yield under normal irrigation (Fig. 3). Also, yield in all irrigation treatments was significantly and positively correlated with MP, STI, GMP, HM, and YI (Fig. 3). Thus, it can be concluded that these indices were more efficient in the selection of genotypes with high yield potential under different water conditions.

Indices being significantly correlated with grain yield under both normal irrigation and water deficit irrigation are suitable for the screening of genotypes (Mitra, 2001). Therefore, indices MP, STI, GMP, HM, and YI which were positively correlated with both Y_p and Y_s at $p \leq 0.01$ (Fig. 3) may be considered as better predictors of yield in different irrigation. As well, sorghum genotypes with high values of MP, STI, GMP, HM, and YI can be thus regarded as drought tolerant. Our results are somewhat in agreement with those findings of Nouri et al. (2011) and Golabadi et al. (2006) who found a correlation between either Y_s or Y_p and MP, GMP, and STI.

A perfect positive correlation (r = 1) was noted between Y_s and YI and between SSI and YRR. On the other hand, a perfect negative correlation (r = -1) was noted between SSI and YSI, YSI, and YRR in both mild and severe water deficit irrigation (Fig. 3). A similar finding was recorded by Mickky et al. (2019) who evaluated 10 wheat cultivars based on drought tolerance indices under normal irrigation (Y_p) and deficit irrigation (Y_s).

3.5 CLUSTER ANALYSIS

Classification of genotypes according to Y_p, Y_s, and various indices under normal irrigation and mild deficit irrigation categorized 10 sorghum into three groups; group 1 including MGS2, TN-04-90, TN-04-78 and TN-04-14; group 2 including KGS23, TN-04-86, TN-04-12, TN-04-99 and TN-04-13; and group 3 including genotype TN-04-79 (Fig. 4a). Clustering based on yield and drought tolerance indices under normal irrigation and mild deficit irrigation grouped the genotypes into tolerant, semi-tolerant/susceptible, and susceptible. The first group with the lowest value of R' and RS (TN-04-79) can be distinguished as tolerant to mild deficit irrigation. The second group had mean values of R' (2.75-5.67) and RS (3.97-8.44) considered as semi-sensitive/tolerant and the third group with higher R' and RS was the most susceptible genotypes to mild deficit irrigation (Fig. 4 a).

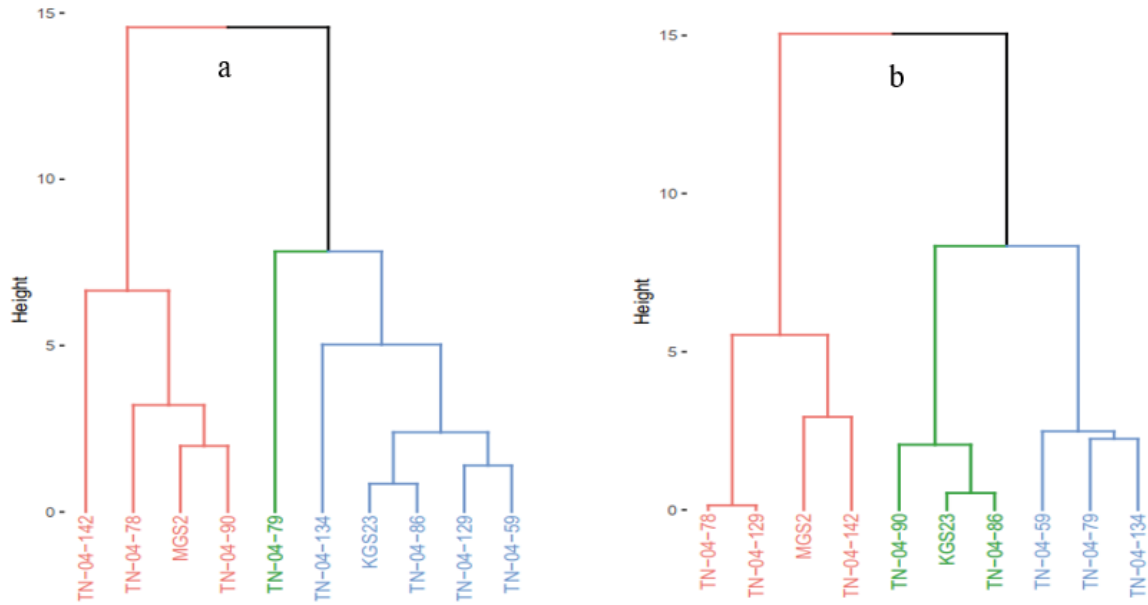

Fig. 4: Dendrograms of the cluster analysis and similarity coefficients among 10 sorghum genotypes based on Y_p, Y_s, and the drought tolerance indices under normal irrigation and mild deficit irrigation (a) and under normal irrigation and severe deficit irrigation (b)

On the other hand, three different clusters were observed based on Y_p, Y_s, and drought tolerance indices under normal irrigation and severe deficit irrigation (Fig. 4b). Genotypes MGS2, TN-04-14, TN-04-78, and TN-04-12 were classified into group 1; KGS23, TN-04-86, and TN-04-90 into group 2; and TN-04-79, TN-04-13, and TN-04-59 into group 3. The first and second groups included the genotypes with the lowest to medium values of R' and RS and thus were considered to be tolerant or semi-tolerant. The genotypes of the third group had the highest values of R' and RS indicating the most susceptible to severe deficit irrigation. Cluster analysis has been extensively employed for the determination of genetic diversity and classification of genotypes under various abiotic stresses (Golabadi et al. 2006; Mohammadi et al. 2011).

3.6 PRINCIPAL COMPONENT ANALYSIS (PCA) AND BIPLOT

The PCA results revealed that the first two principal components accounted for 98.51 % (PC1:81.03 %, PC2:4.63 %) of the total variation in yield performance and nine yield-based indices calculated under normal irrigation and mild deficit irrigation. Biplot showed that the PC_1 was positively correlated with yield (Y_p and Y_s under mild stress) and all indices except TOL and SSI, whereas PC_2 was positively correlated with yield (Y_p and Y_s under mild deficit irrigation) and all indices excluding RSI and YSI (Fig. 5a).

On the other hand, the PCA biplot for yield (Y_p and Y_s under severe deficit irrigation) and drought tolerance indices of sorghum genotypes was reflecting 99.28 % (PC_1:74.6 %, PC_2:24.63 %) of the total variability in data (Fig. 5b). The biplot categorized the indices into three groups (Fig. 5). The first group was those with high PC_1 and PC_2 (Y_p, MP, STI, GMP, HM, YI, and Y_s in Fig. 5a and Y_p, MP, STI, GMP, and HM in Fig 5b). The second group was indices with low PC_1 but high PC_2 (SSI and TOL) (Fig. 5a,b) and the third group were those with high PC_1 but low PC_2 including RSI (Fig. 5a,b) and Y_s and YI (Fig. 5b).

The cosine of the angle between the vectors of any two indices in a biplot is an indicator of the correlation coefficient. Therefore, we can note that those indices whose vector has been placed between the vectors of Y_p and Y_s are appropriate for the selection of drought-tolerant genotypes. It can be implied that MP, GMP, STI, HM, and YI allocating between Y_p and Y_s are the best indices to distinguish tolerant from susceptible genotypes. Herein, the results obtained from PCA (Fig. 5) confirmed those obtained from correlation coefficients (Fig. 3).

The results of our study showed that TN-04-79 and TN-04-59 are tolerant genotypes with the highest values for the MP, GMP, STI, and HM indices, while genotypes KGS23, TN-04-129, TN-04-86, and TN-04-90 under mild stress and genotypes KGS23, TN-04-86, TN-04-90 with the highest values for YSI and RSI were the most

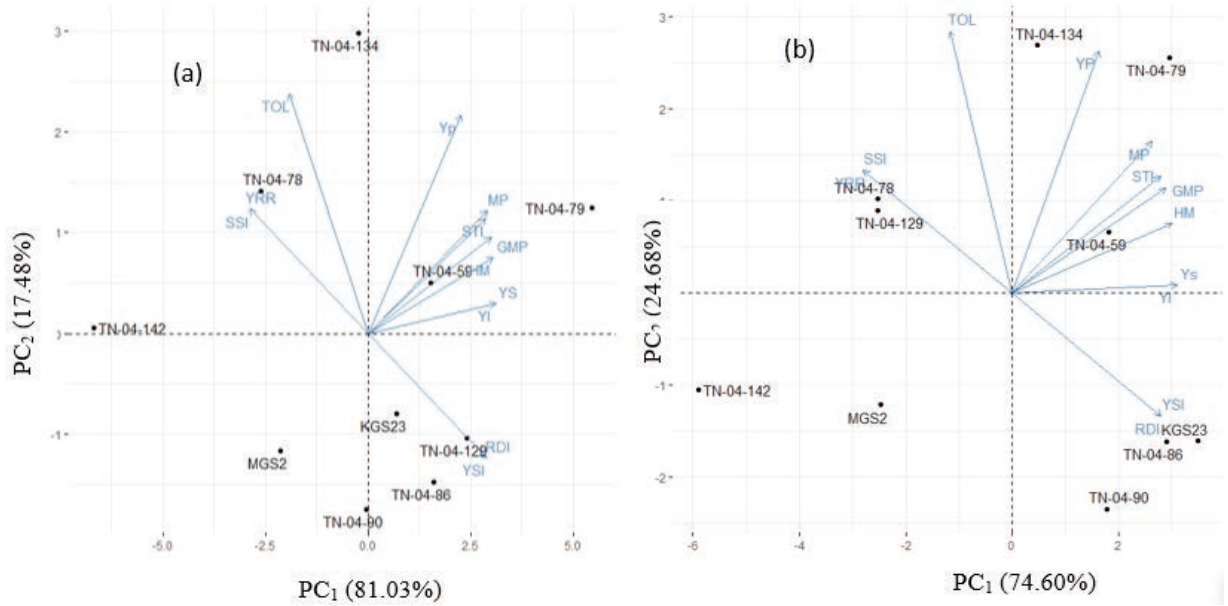

Fig. 5: Principal components (PC) analysis based on the correlation matrix of yield under normal irrigation (Y_p) and yield under mild deficit irrigation (a) and severe deficit irrigation (b) (Y_s) and nine tolerance and susceptibility indices calculated using iPASTIC online tool kit. Y_p, yield under normal irrigation; Y_s, yield under mild deficit irrigation for (a) and under severe deficit irrigation for (b); TOL, tolerance index; MP, mean productivity, GMP, geometric mean probability; HM, Harmonic mean; SSI, stress susceptibility index; STI, stress tolerance index; YI, yield index; YSI, yield stability index; RSI, relative stress index.

stable genotypes (Table 4). Introduction of these tolerant genotypes into the sorghum breeding programs may be suggested to policymakers to release new cultivars tolerant to drought stress. It has been noted that increasing harvest index can improve yield stability (Kashiwagi et al., 2015). The reduction of grain yield under deficit irrigation could lead to a lower harvest index. We also found that indices including MP, GMP, HM, STI, YI, and YSI are strongly correlated with sorghum yield. Thus, these drought-tolerant indices should benefit the breeders in breeding programs.

4 CONCLUSIONS

In the current study, eight sorghum genotypes collected from different parts of Iran along with two promising lines reported as drought-tolerant were compared in terms of response to deficit irrigation. The grain yield and water productivity of the genotypes were significantly influenced by water deficit irrigation. The relative efficacy of selection indices could be an advantage using two or more traits simultaneously than using single traits independently. Thus, indices including MP, STI, GMP, HM, and YI, highly correlated with Y_p and Y_s, may be more suitable for the selection of drought-tolerant genotypes. Screening of tolerant genotypes to water deficit irrigation using the ranking method and cluster analysis discriminated genotypes as the most tolerant, semi-tolerant/sensitive, and susceptible. Therefore they are recommended to be used in breeding programs as parents for improvement of drought tolerance in commercial cultivars. Further evaluation of these genotypes based on drought indices across multiple locations and years is still demanded to validate their stability for developing sorghum cultivars.

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6 ABBREVIATIONS

ANOVA: Analysis of variance; PCA: principal component analysis; PH: plant height; PL: panicle length; SD: stem diameter; NoL: number of leaves per plant; 1000 SM: 1000 seed mass; DMY: dry matter yield; HI: harvest index; WP: Water productivity; Y_p: grain yield under normal irrigation; Y_s: grain yield under deficit irrigation; MP: mean productivity; TOL: tolerance index; SSI: stress susceptibility index; STI: stress tolerance index; GMP: geometric productivity; HM: harmonic mean of yield;

YSI: yield stability index; YRR: yield reduction ratio; YI: yield index.

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Influence of crop load on the yield and grape quality of Merlot and Vranac (*Vitis vinifera* L.) varieties in Trebinje vineyard

Mersija DELIĆ [1], Fikreta BEHMEN [2], Saša MATIJAŠEVIĆ [3], Šaćira MANDAL [4], Saud HAMIDOVIĆ [2], Senad MURTIĆ [5, 6]

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Influence of crop load on the yield and grape quality of Merlot and Vranac (*Vitis vinifera* L.) varieties in Trebinje vineyard

Abstract: The aim of this study was to study the impact of crop load on the yield and grape quality of 'Merlot' and 'Vranac' (*Vitis vinifera* L.) in Trebinje vineyard. The crop load levels studied in this trial were 9 buds (V1) and 12 buds (V2) per vine at each variety trained on Lenz-Moser bilateral cordon system. The impact was determined by measurements of yield per vine and grape quality characterized by the contents of total soluble solids, titratable acidity, total phenolics, total flavonoids and by total antioxidant capacity. The measured parameters of grape quality of 'Merlot' and 'Vranac' were not influenced significantly by crop load levels. V2, compared to the V1, showed the potential for increasing grape yield only for Merlot variety under experimental conditions. The results of this study also showed a positive correlation between total phenolics/flavonoids and total antioxidant capacity of grape berries in both varieties, regardless of crop loads applied.

Key words: flavonoids; total phenolics; pruning; yield

Vpliv obtežitve na pridelek in kakovost grozdja sort Merlot in Vranac (*Vitis vinifera* L.) v vinogradu v Trebinju

Izvleček: Namen raziskave je bil preučiti vpliv obtežitve trt na pridelek in kakovost sort Merlot in Vranac (*Vitis vinifera* L.) v vinogradih Trebinja. Velikost obremenitve trt v tem poskusu so bile trte z 9 (V1) in 12 brsti (V2) na vsako sorto, gojeno na Lenz-Moser bilateralnem kordonu. Vpliv je bil določen z meritvami pridelka na trto, kakovost grozdja je bila določena z vsebnostjo celokupne suhe snovi, titrabilne kislosti, vsebnosti celokupnih fenolov in flavonoidov in celokupne antioksidacijske sposobnosti. Izmerjeni parametri kakovosti grozdja sort Merlot in Vranac niso bili znalično vplivani z obremenitvijo trt. V2 je v primerjavi z V1 pokazala zmožnost povečanja pridelka grozdja samo pri sorti Merlot v razmerah te raziskave. Rezultati te raziskave so pokazali še pozitivno korelacijo med vsebnostjo celokupnih fenolov/flavonoidov in celokupno antioksidacijsko kapaciteto grozdnih jagod pri obeh sortah ne glede na obremenitev trt.

Ključne besede: flavonoidi; celokupni fenoli; rez; pridelek

1 University of Sarajevo, Faculty of Agriculture and Food Sciences, Department of Viticulture, Sarajevo, Bosnia and Herzegovina
2 University of Sarajevo, Faculty of Agriculture and Food Sciences, Department of Horticulture, Sarajevo, Bosnia and Herzegovina
3 University of Belgrade, Faculty of Agriculture, Department of Viticulture, Belgrade, Serbia
4 University of Sarajevo, Faculty of Pharmacy, Department of Natural Sciences in Pharmacy, Sarajevo, Bosnia and Herzegovina
5 University of Sarajevo, Faculty of Agriculture and Food Sciences, Department of Plant Physiology, Sarajevo, Bosnia and Herzegovina
6 Corresponding author, e-mail: murticsenad@hotmail.com

1 INTRODUCTION

The grape yield and quality are influenced by many factors such as climatic conditions, soil chemical and physical properties variety, viticulture management practices, crop load, leaf removal, irrigation, and rootstock-scion relationship (Pachnowska and Ochmian, 2018). Among the factors affecting vine growth and development, winter pruning has the greatest impact on the grape yield and quality of the growing season due to its impact on bud fertility and nutrient reserves in grapevine (Qiu et al., 2019). Winter pruning refers to the removal of non-beneficial plant parts during the late dormant season, and retaining only selected buds for the next season's fruiting. In some wine regions, such as France, the exact number of buds for each variety is outlined by the France's Appellation d'Origine Contrôlée regulations (Gangjee, 2012). However, if to many buds are left at winter pruning, the vine will produce many shoots leading to a dense canopy and opposite, if a few buds left, the shoots may grow too vigorously leading to low yield (Collins et al., 2020). In order to achieve the right balance between vegetative growth and grape production it is essential to determine an optimal crop load for each grapevine variety (Pellegrino et al., 2014). Optimal crop load varies from one variety to another, depending mainly on variety itself, vineyard location, soil type, climatic conditions, etc. (Raj Kumar et al., 2017). Crop loads also impacts chemical grape composition and thus the wine quality. From an economic point of view, grape growers must find the correct balance between quantity, fruit quality and long-term vine health when determining appropriate crop levels on their vines (Čuš, 2004; Schamel and Schubert, 2016). Accordingly, permanent necessity for achieving optimal crop loads is always present, especially in regions where the connections among pruning practices and grape production are poorly understood.

Lenz-Moser bilateral cordon is the most commonly used vine training system for 'Merlot' and 'Vranac' (*Vitis vinifera* L.) in Trebinje region in Bosnia and Herzegovina. Crop load levels commonly used within this training system in Trebinje region are 9 and 12 buds per vine. The effect of crop load treatments on yield of 'Merlot' and 'Vranac' has been extensively investigated. Bogičević et al. (2015) examined the effect of early leaf removal and cluster thinning treatments on berry growth and grape composition of Vranac variety. Results showed that early leaf removal followed by cluster thinning resulted in a lower berry mass and number of berries per cluster, and thus a lower yield. Peppi et al. (2017) noted that an adjustment in mechanical pruning with regard to cutting height is a feasible alternative to obtain regular and sustainable yields in 'Merlot' with considerably lower labour inputs in the vineyard. On the other hand, the effect of crop load treatments on the content of bioactive compounds such as total phenolics and flavonoids in berries of Merlot and Vranac varieties has been less studied.

Therefore, this study is primarily intended to evaluate the impact of crop load on total phenolics, total flavonoids and total antioxidant capacity of grapevine varieties Merlot and Vranac in Trebinje vineyard. The study also included the measurements of the following grape quality parameters: total soluble solids, titratable acidity as well as yield. The hypotheses tested were: (1) lower crop load can effectively increase total phenolic and flavonoid contents and total antioxidant capacity in berries of Merlot and Vranac varieties as compared to higher crop load; (2) total soluble solids, titratable acidity as well as yield in both varieties Merlot and Vranac will differ depending on the crop loads. Understanding the relationship between the crop loads and the grape production in 'Merlot' and 'Vranac' can provide valuable information for management decisions that need to be made in the vineyard.

2 MATERIALS AND METHODS

2.1 EXPERIMENTAL SITE AND PLANT MATERIAL

This study was conducted during 2018 at a commercial vineyard planted with 'Merlot' and 'Vranac' vines grafted onto Richter 110 rootstock. The vineyard was located in a village Zagradinje (42°38′N 18°14′E), 10 kilometers away from Trebinje (Herzegovina region), in a zone of altered Mediterranean climate. In contrast to the narrow coastal area, Trebinje region is characterized by warmer and drier summers, while winters are more humid. According to Köppen and Geiger, climate in Trebinje region is classified as Csa (hot-summer Mediterranean climate). The average annual temperature in studied area is 14.2 °C, and the precipitations average is 1,338 mm. The rain in Trebinje falls mostly in the winter, with relatively little rain in the summer.

Vineyard was planted in 2008 on a sandy-loam soil, with planting distance of 2.5 m between rows and 1.0 m between vines in the row. Training system at both Merlot and Vranac was a Lenz-Moser bilateral cordon system. The crop load treatments applied at the study were as follows: (V1) - 9 buds/vine (spurs with 2-3 buds; 3 spurs/cordon) and (V2) - 12 buds/vine (spurs with 2-3 buds; 5 spurs/cordon). Each crop load treatment consisted of 4 plots located in two different rows (12 vines each; 48 vines per treatment) with one guard row between treatments. Crop loads used in this study are the most preva-

lent for Merlot and Vranac in Trebinje vineyards and are therefore selected for this study.

Vranac is an autochthonous grapevine variety of Montenegro. The clusters are medium in size (cluster mass is in the range of 180 to 220 g) and are well filled with large, thin-skinned berries. The wine of this variety has a pleasant taste, velvety sweetness and intensive dark red colour (Šuković et al., 2020). Merlot is a red grapevine variety from Bordeaux, France. It produces medium to large clusters (cluster mass is in the range of 220 to 300 g) with berries medium large in size and round with blue colour. The Merlot wine is velvety-red, fruity and pleasant taste and very refreshing (Renouf et al., 2010).

2.2 GRAPE YIELD AND QUALITY ANALYSIS

Grape yield was weighted at harvest and expressed as kg per vine. Total soluble solids (TSS) were measured using an Atago PAL-1 digital refractometer and expressed in degrees Brix (ISO, 2003). Titratable acidity (TA) was measured by titrating 10 ml of grape juice with 0.1 mol l^{-1} NaOH, using phenolphthalein as indicator (AOAC, 2000) and expressed as grams of tartaric acid per litre of grape juice (g l^{-1}).

2.2.1 Extraction of phenolics from grape berries

The extraction of phenolic compounds from the fully ripened and matured grape berries was performed using a 30 % aqueous ethanol solution (Canals et al., 2005). Before extraction, a fresh grape berries were oven dried at 50 °C until constant mass, ground and then sieved to pass a 2-mm sieve. The average moisture content was 77.8 % for Vranac and 79.0 % for Merlot. Detailed extraction procedures were as follows: 1 g of air-dried grape sample was placed into 100 ml Erlenmeyer flask and mixed with 40 ml 30 % aqueous ethanol solution. The flask was heated in a water bath at 35-37 °C for 1 h. After heating, the flask was cool down to room temperature, and then the mixture was filtered through filter paper into 50 ml flask and diluted to the mark with 30 % aqueous ethanol solution. The extract thus obtained was used for the analysis of total phenolic content, total flavonoid content and total antioxidant capacity.

2.2.2 Total phenolic content

The total phenolic content (TPC) of the extract was measured by the Folin-Ciocalteu assay (Ough and Amerine, 1988) with slight modifications. The test sample (0.25 ml of extract) was mixed into 25 ml flask with 15 ml of distilled water and 1.25 ml of Folin Ciocalteu reagent (diluted by distilled water in the ratio 1:2). After 5 min, 3.75 ml of saturated sodium carbonate solution (8 % w/v in water) was added. The flask was filled to the mark with a 30 % aqueous ethanol solution and heated in a water bath at 50 °C for 30 min. After heating, the flask was cool down to room temperature, and the absorbance of blue colour was measured using a UV Spectrophotometer (Amersham, Ultrospec 2100 pro) at 765 nm. TPC was calculated from a standard curve of gallic acid (5 - 500 mg l^{-1}) and the results were expressed as mg of gallic acid equivalent per 100 g fresh mass (mg GAE 100 g^{-1} FM).

2.2.3 Total flavonoid content

The total flavonoid content (TFC) of the extract was measured by the aluminium chloride colorimetric assay (Zhishen et al., 1999). The test sample (1 ml of extract) was mixed into 10 ml flask with 4 ml of distilled water and 0.3 ml 5 % $NaNO_2$. After 5 min, 0.3 ml 10 % $AlCl_3$ was added. The flask was incubated at room temperature for 6 min, and thereafter 2 ml of 1 mol l^{-1} NaOH was added. The flask was filled to the mark with distilled water and after 15 min the absorbance of red colour was measured at 510 nm. TFC was calculated from a standard curve of catechin (0-100 mg l^{-1}) and the results were expressed as mg of catechin equivalent per 100 g of fresh mass (mg CE 100 g^{-1} FM).

2.2.4 Total antioxidant capacity

The total antioxidant capacity (TAC) of the extract was determined by ferric reducing antioxidant power (FRAP) assay (Benzie and Strain, 1996). The test sample (80 µl of extract), 240 µl of distilled water, and 2080 µl of FRAP reagent (reagent was obtained by mixing 0.3 mol l^{-1} acetate buffer (pH = 3.6), 10 mmol l^{-1} TPTZ (2,4,6-tripyridyl-s-triazine) and 20 mmol l^{-1} $FeCl_3$ x 6 H_2O in ratio 10 : 1 : 1) were added into 10 ml Erlenmeyer flask and heated in a water bath at 37 °C for 5 min. After heating, the flask was cool down to room temperature, and the absorbance of blue colour was measured at 595 nm. TAC was calculated from a standard curve of $FeSO_4$ x $7H_2O$ (0 - 2 mmol l^{-1}) and the results were expressed as mmol Fe^{2+} per 100 g fresh mass (mmol Fe^{2+} 100 g^{-1} FM).

2.3 STATISTICAL ANALYSIS

All the chemical measurements of TPC, TFC and TAC were conducted in triplicates and the results were expressed as the mean ± standard deviation. Experimental data were subjected to analysis of variance (ANOVA) using Microsoft Excel 2013 statistical program. In order to interpret the relationships between total TPC / TFC and TAC, Pearson's correlation coefficient analysis were conducted using the Microsoft Excel 2013 software.

3 RESULTS AND DISCUSSION

The results of the analysis of yield and grape quality parameters of 'Merlot' and 'Vranac', depending on crop load treatments are presented in Table 1 and Table 2, respectively.

The results of this study showed that the yield of the Vranac variety in the crop load treatment with nine buds/vine was significantly lower compared with the higher crop load treatment (twelve buds/vine). This result is expected, since the lower crop load reduces the number of fruitful buds and consequently the number of grape clusters per vine, and thus the yield (Rubio and Yuste, 2002). Interestingly, this hypothesis for the 'Merlot' has not been confirmed in this study. The probably reason for that is less sensitivity of the 'Merlot' to differences in the number of buds in relation to the 'Vranac'. Contrarily, a higher crop load levels allows the vine to produce many grape clusters, which often results in a higher yield (Aipperspach et al., 2020). However, if to many buds left, then vine may produce many shoots that are outwardly observable as a large, leafy canopy leading to poor fruit quality and a weakening of the vine (Keller, 2010). Accordingly, achieving optimal crop load for each variety is essential for vine growth and development and thus for grape production.

In this study, there was no observed change in the quality of the grapes in the both varieties: Merlot and Vranac, regarding to the crop loads. These results are inconsistent with most other studies (Petri and Clingeleffer, 2006; Reynolds et al., 2007; Brighenti et al., 2017; Drenjančević et al., 2017). Khamis et al. (2017) noted the grape quality parameters have inverse correlation with the number of buds per vine, that is, the lower crop loads increase the total soluble solids and phenolic content. Positive correlation between lower crop load levels and total soluble solids or phenolic contents in grape has been reported in many other studies (Peña-Neira et al., 2007; Gil-Muñoz et al., 2009; Bubola et al., 2011). However, some of the studies failed to find an association between crop removal treatment and some of the above-mentioned grape quality parameters (Karoglan et al., 2014; Mawdsley et al., 2018). In our study, there is no evidence that lower crop load level increases total soluble solids, total phenolics and total flavonoids in grape berries. Unfortunately, the drawback of the present study is that only two crop load levels were used in the experiment and it is very difficult to draw conclusions from. Our hypothesis was that lower crop load level (9 buds/vine) would significantly increase total soluble solids, total phenolics and total flavonoids in grape berries of both cultivars as compared to higher crop load level (12 buds/vine), however, the study results did not confirm it.

The results of the present study have also shown

Table 1: Yield, total soluble solids (TSS), titratable acidity (TA), total phenolic content (TPC), total flavonoid content (TFC) and total antioxidant capacity (TAC) of 'Merlot'

Crop load treatments	Yield (kg per vine)	TSS (°Brix)	TA (mg l^{-1})	TPC (mg GAE 100 g^{-1} FM)	TFC (mg CE 100 g^{-1} FM)	TAC (mmol Fe^{2+} 100 g^{-1} FM)
9 buds/vine	3.32 ± 0.51	20.83 ± 0.8	7.14 ± 0.06	147.4 ± 9.1	80.7 ± 7.3	2.10 ± 0.21
12 buds/vine	3.57 ± 0.76	21.30 ± 1.2	7.16 ± 0.04	154.2 ± 9.7	84.3 ± 5.1	2.27 ± 0.37

Values expressed as an average ± standard deviation

Table 2: Yield, total soluble solids (TSS), titratable acidity (TA), total phenolic content (TPC), total flavonoid content and total antioxidant capacity (TAC) of 'Vranac'

Crop load treatments	Yield (kg per vine)	TSS (°Brix)	TA (mg l^{-1})	TPC (mg GAE 100 g^{-1} FM)	TFC (mg CE 100 g^{-1} FM)	TAC (mmol Fe^{2+} 100 g^{-1} FM)
9 buds/vine	3.32 ± 0.44b	22.13 ± 1.1	7.07 ± 0.11	166.8 ± 14.1	75.3 ± 6.3	2.26 ± 0.33
12 buds/vine	4.18 ± 0.53a	21.80 ± 0.9	7.16 ± 0.14	154.1 ± 19.6	74.3 ±8.1	2.08 ± 0.29

Values expressed as an average ± standard deviation.
Different letters in each column represent significant difference among variants

Table 3: Pearson's correlation coefficient between total phenolics (TPC)/total flavonoids (TFC) contents and total antioxidant capacity (TAC)

	Merlot		Vranac	
	9 buds/vine	12 buds/vine	9 buds/vine	12 buds/vine
TPC vs. TAC	0.935	0.922	0.911	0.915
TFC vs. TAC	0.944	0.951	0.932	0.926

that the total soluble solids, titratable acidity and total phenolics of grape berries fell within the range expected for 'Vranac' and 'Merlot'. For example, in the study conducted by Mitić et al. (2012) total phenolics in grape of 'Vranac' and 'Merlot' were 158.6 ± 1.9 and 169.2 ± 2.7 mg GAE 100 g^{-1} FM, respectively. However, numerous studies reported much higher contents of total phenolics in berries of 'Vranac' and 'Merlot' than those determined in the present research (Pajović et al., 2014; Franco-Bañuelos et al., 2017). Scientists generally agree that total phenolic contents as well as other grape quality parameters depends on many factors such as cultivar, viticulture practices and environmental conditions in the vine-growing regions. Toscano et al. (2019) reported that sub-optimal environmental conditions can influence the biosynthesis and accumulation of many secondary metabolites in plants, including phenolic compounds.

The results of this study also showed a positive correlation between total phenolics / flavonoids and total antioxidant capacity of grape berries in both varieties, regardless of crop loads applied (Table 3). Similar correlations were also determined in other published studies (Garrido et al., 2016; Cosme et al., 2018).

These results suggest that the phenolic compounds are carriers of antioxidant activity in the plant, and this hypothesis has, in fact, been confirmed by many scientists (Borges et al., 2010; Stanković et al., 2012; Balea et al., 2018).

4 CONCLUSIONS

Higher crop load level (12 buds/vine) as compared to lower crop load level (9 buds/vine) within the Lenz-Moser bilateral cordon system showed potential for increasing grape yield only for 'Merlot' under experimental conditions. However, the quality grape parameters of 'Merlot' and 'Vranac' were not influenced significantly by crop loads. Unfortunately, this study has some limitations, which have to be pointed out. The main limitation of the present study is (1) one year was included in the study and (2) small differences among crop load treatments. Therefore, further investigations involving more crop load treatments should be done to confirm the conclusions of this study.

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Inheritance of plant height, straw yield and flag leaf area in MBB x Gaviota durum wheat (*Triticum durum* Desf.) cross

Manel SALMI [1, 2], Zine El Abidine FELLAHI [3], Abdelkader BENBELKACEM [4], Amar BENMAHAMMED [5], Hamenna BOUZERZOUR [5]

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Inheritance of plant height, straw yield and flag leaf area in MBB x Gaviota durum wheat (*Triticum durum* Desf.) cross

Abstract: Plant height, straw mass and flag leaf area are recognized by physiologists as morphological markers of drought stress tolerance. Developing varieties intended for arid and semi-arid zones need to select for these traits. Understanding the genetic control of a given trait helps breeder to handle the segregating populations under study in a more efficient and consistent manner by choosing the best breeding method available to realize significant genetic advance. For this purpose, six generations: parents, F1, F2, BC1, BC2, derived from MBB x 'Gaviota' durum wheat (*Triticum durum* Desf.) cross were grown to investigate the nature of gene action involved in the inheritance pattern of the three traits. The results indicated that the six-parameter model fitted the best the data related to the variability present in the generation means of the studied traits. Generation mean analysis indicated that non-allelic interactions were important factors controlling the expression of these characters with complementary type of gene action governing FLA and STW inheritance. High heritability estimates, moderate to high expected responses to selection, significant genetic correlations with grain yield and greater role of non-additive effects in controlling the inheritance of the three studied traits suggested that breeding methods exploiting both fixable and non-fixable components be applied to break unfavorable linkage and to accumulate useful genes in the base population, followed by mono-trait or index based selection in late advanced generations.

Key words: gene effects; non-allelic interaction; durum wheat; plant height; straw yield; flag leaf area; heritability

Višina rastlin, masa slame in površina lista zastavičarja so od fiziologov prepoznane morfološke lastnosti, ki nakazujejo toleranco na sušni stres

Izvleček: Višina rastlin, masa slame in površina lista zastavičarja so od fiziologov prepoznane morfološke lastnosti, ki nakazujejo toleranco na sušni stres. Sorte, vzgojene za sušna območja morajo biti izbrane glede na lastnosti, ki omogočajo prenašanje suše. Razumevanje genetske kontrole za določeno lastnost pomaga žlahtniteljem uravnavati različne populacije v raziskavi na bolj učinkovit in verodostojen način pri izbiri najboljše metode žlahtnenja za dosego pomembne genetske prednosti. V ta namen je bilo gojeno šest generacij rastlin iz križanja starševske generacije, F1, F2, BC1, BC2 z MBB x 'Gaviota' trde pšenice (*Triticum durum* Desf.) kot osnova za preučevanje delovanja genov, ki so vključeni v vzorec dedovanja treh lastnosti. Rezulatati so pokazali, da se je model šestih parametrov najbolje prilegal podatkom, povezanih z variabilnostjo preučevanih lastnosti kot povprečje v generaciji. Analiza generacijskih povprečij je pokazala, da so bile nealelske interakcije pomemben dejavnik za nadzor izražanja tistih lastnosti, ki so komplementarne delovanju genov in ki vodijo FLA in STW dedovanje. Velike vrednosti dednosti, zmerne glede na pričakovan odziv selekcije, značilna genetska korelacija s pridelkom zrnja in večja vloga neaditivnih učinkov pri kontroli dednosti treh preučevanih lastnosti nakazujejo, da bi žlahtniteljske metode, ki uporabljajo vezane in nevezane komponente lahko bile uporabljene za prekinitev nezaželjenih povezav genov in pospešitev uporabnih genov v osnovnih populacijah, ki bi jim sledil izbor posameznih ali indeksiranih lastnosti v kasnejših izboljšanih generacijah.

Ključne besede: učinki genov; nealelske interakcije; trda pšenica; višina rastlin; pridelek slame; površina lista zastavičarja; dednost

1 University of Mostefa Ben Boulaid, Life and Natural Sciences Faculty, Ecology and Environment Department, Batna, Algeria
2 Corresponding author, e-mail: salmi.mnl@gmail.com
3 University of Mohamed El Bachir El Ibrahimi, Natural, Life and Earth Sciences and the Universe Faculty, Agronomy Department, Bordj Bou Arréridj, Algeria
4 Algerian National Institute of Agronomic Research (INRAA), Research Unit of Constantine, Algeria
5 University of Ferhat Abbas, Life and Natural Sciences Faculty, Ecology and Plant Biology Department, Setif, Algeria

1 INTRODUCTION

In the arid and semi-arid regions, rainfed grown durum wheat (*Triticum durum* Desf.) crop suffers from the combined effects of drought and heat stresses, undergoing substantial grain yield losses (Royo et al., 2014; Lui et al., 2015). To minimize yield decline, drought tolerance improvement is then seen as a key breeding component for the development of cultivars devoted to these environments, where reducing wind erosion and meeting livestock feeding requirement have heightened the importance of increasing straw production along with grain yield. In fact, crop residues are usually not burned but either maintained to protect soil from wind erosion or grazed and straw is balled, stored and fed to livestock during the winter months (Annichiarico et al., 2005; Chenaffi et al., 2011). In this context plant height, a straw yield correlated trait, is seen as an important characteristic influencing cultivars adoption under such growing conditions (Rabti et al., 2020; Jatayev et al., 2020; Haddad et al., 2021). Because of their straw yield advantage, tall varieties, derived from land-races, are still cultivated and not replaced by newly released reduced height cultivars (Rabti et al., 2020). For a full expression of their potential, dwarf wheat varieties need to be grown in favorable, well-watered conditions which permit application of relatively high levels of nitrogen fertilizer which is usually not the case in arid and semi-arid environments. This type of plant material becomes, under drought stress conditions, too short, yielding less than tall varieties, showing reduced flag leaf area, kernel size, kernel Zn, Fe, Mg and Mn concentration, coleoptiles, and roots length (Aziz et al., 2017; Velu et al., 2017; Jatayev et al., 2020; Rabti et al., 2020). Positive relationship between plant height and grain yield, under drought stress growing conditions, implied that there is a minimum height below which grain yield limitation becomes evident (Slafer et al., 2005; Royo et al., 2014). in this context, Yani and Rashidi (2012) reported that straw yield and plant height had positive and significant role in the expression of grain yield under drought conditions. Asadi et al. (2019) noted that straw yield explained 65 % of grain yield variation and exerted substantial direct effect on grain yield under water deficit conditions. Belagrouz et al. (2018) reported significant correlation between grain yield water use efficiency, harvest index and plant height, suggesting that selection for plant height and harvest index could improve both water use efficiency and grain yield under drought prone environments. Flag leaf is the main photosynthetic organ providing the major assimilate required for spike growth. It senses environmental signals and consequently adapts to surrounding environment by minimizing area reduction and delaying senescence, caused by terminal drought stress (Farook et al., 2009; Belkherchouche et al., 2015). Joshi et al. (1984) found that flag leaf size was positively correlated with grain yield, suggesting that optimal flag leaf dimensions could be an important breeding target under drought prone environments. Information on straw yield, plant height and flag leaf area inheritance can assist developing adapted cultivars for areas practicing cereal-livestock farming systems and conservation agriculture (Chenaffi et al., 2011; Jatayev et al., 2020). Genetic variation for quantitative characters in segregating population is of prime concern to breeders. It determines selection efficiency which depends upon the nature and magnitude of genetic variability available. Genetic models devoted to the estimation of different genetic effects have been developed. Among these models, generation means analysis provides information on the relative importance of gene effects due to additive, dominance deviations and non-allelic interactions, in determining generation means (Mather and Jinks, 1982; Shayan et al., 2018; Salmi et al., 2019). Plant height was found to be controlled mainly by over dominance while flag leaf area was reported to be mostly under additive control combined with partial dominance and epistatic gene actions (Saleem et al., 2005; Shabbir et al., 2012; Yang et al., 2016). Salmi et al. (2019) found that dominance acted in the direction of increased plant height. Joshi and Sharma (1984) reported that genes affecting smaller leaves are partially dominant over genes affecting larger leaves. Evidence for non-allelic interactions was reported and linkage among loci appeared to be an important component of flag leaf dimensions heredity (De Pace et al., 2001). The present study aimed to investigate the inheritance pattern of straw yield, plant height and flag leaf area in a durum wheat cross involving a tall and a semi-dwarf varieties.

2 MATERIALS AND METHODS

2.1 SITE, PLANT MATERIAL, AND EXPERIMENTAL DESIGN

Two durum wheat (*Triticum durum* Desf.) varieties, namely Gaviota (GTA), a semi-dwarf cultivar derived from 'Crane' /4/ 'Polonicum PI 185309'//*Triticum glutinosum enano*/2 * 'Tehuacan 60'/3/'Grulla' cross (http://wgb.cimmyt.org/gringlobal/AccessionDetail.aspx?id=1783), and a tall cultivar, Mohammed Ben Bachir (MBB), a head-row selection derived from a land race adapted to Setif's high plateaus region (Algeria), were hybridized during the 2015-2016 cropping season at the Field Crop Institute, Agricultural Experimental Station of Setif (ITGC, AES, 36°12'N 05°24'E, 1080 m above sea level,

Setif, Algeria). During the 2016-2017 cropping season, F_1 was selfed to produce F_2, and crossed to the parents to obtain back cross generations (BC_1 and BC_2). The parents were crossed again to get the first filial generation (F_1). The following cropping season (2017-2018), the six basic generations were grown in a randomized completed block design, with five replications. Parents, F_1, and BC's generations were sown in one row, 2 m long, 20 cm inter-row spacing and 10 cm plant-plant spacing in the row. F_2 generation was sown in thirty rows 2 m long. Recommended cultural practices for the area were followed as reported in Salmi et al. (2019).

2.2 DATA COLLECTION AND ANALYSIS

Data were collected from 5, 5, 10, and 30 plants per replication for the parents, F_1, BC's, and F_2 generations, respectively. Prior to harvest, length of the main stem of each plant was measured from the ground level to the base of the spike and recorded as plant height estimate in cm (PHT, cm). Straw mass (STM, g plant^{-1}) was determined as the difference between above ground plant biomass (BIO, g plant^{-1}) and plant grain yield (GY, g plant^{-1}). Flag leaf area (FLA, cm²) was estimated by the product of leaf length x leaf width x 0.749 (Spagnoletti-Zeuli and Qualset, 1990). Collected data were subjected to an analysis of variance using Cropstat software (2007) to test generation effect. Whenever this effect, tested against the residual mean square, was significant, genetic analysis for the specific trait was undertaken. To test the presence of additive vs. dominance genes effects, contrast method (Steel and Torrie, 1982) was applied to check the significance of the following comparisons: F_1 vs. mid-parent, P_1 vs. P_2, F_2 vs. average BC's, and BC_1 vs. BC_2. The notations adopted for gene effects were [m], [d], [h], [i], [j], and [l] representing main, additive and dominance gene effects, and additive × additive, additive × dominance, and dominance × dominance epistatic interactions, respectively. The appropriate genetic model (three vs. six parameters) was also determined using both ABCD and joint scaling tests. These tests provide information regarding the absence or the presence of gene interactions (Mather and Jinks, 1982). Significance of any one or both scaling tests implies inadequacy of the additive–dominance model. The C and D scaling tests provide check for dominance x dominance (l) and additive x additive (i) types of epistasis, respectively. The genetic parameters [m], [d], [h], [i], [j], and [l] were estimated by weighted least square method. The purpose of using weights was to account for differential precision with which means of different generations were estimated based on varying sample size. Gene effects were tested for significance using the t-test (Kearsey and Pooni, 1996). Three vs six-parameter models testing ware performed using GENMEANS subroutine implemented in Tnaustat software (Manivannan, 2014). Genotypic and environmental variance components, of the measured traits, were estimated by equating the observed values of the different generations, according to Mather and Jinks, (1982) as follows: $\sigma^2_E = ¼ (\sigma^2_{P1} + \sigma^2_{P2} + 2\sigma^2_{F1})$, $\sigma^2_D = (2\sigma^2_{F2} - \sigma^2_{BC1} - \sigma^2_{BC2})$ and $\sigma^2_H = 4 (\sigma^2_{F2} - ½\sigma^2_D - \sigma^2_E)$. The significance of the mean value of a particular parameter was tested against its corresponding standard error, via a Student's t-test, as suggested by Mather and Jinks (1982). Broad-sense heritability (H^2 bs) was calculated according to Kearsey and Pooni, (1996), as follow: $H^2bs = (\sigma^2_D + \sigma^2_H) / (\sigma^2_D + \sigma^2_H + \sigma^2_E) = (\sigma^2_G) / (\sigma^2_P)$, where σ^2_D, σ^2_H, σ^2_E, σ^2_G, and σ^2_P stand for the additive, dominance, environmental variance components, genetic, and phenotypic variances, respectively. Narrow sense heritability (h^2ns) was estimated according to Hallauer and Miranda Filho (1989) as follow: $h^2_{ns} = \sigma^2_D / (\sigma^2_D + \sigma^2_H + \sigma^2_E) = (\sigma^2_D) / (\sigma^2_P)$, Standard errors (SE) of these estimates were calculated as: $SE_{(h2bs)} = [SE (\sigma^2_G)] / (\sigma^2_P)$ and $SE_{(h2ns)} = [SE (\sigma^2_D)] / (\sigma^2_P)$. Significance of these parameters, h^2_{bs} and h^2_{ns} was tested using a t-test equals to the ratio of heritability over its standard error (Halloran et al., 1979). The expected response to selection (ERS) was derived according to Sing and Chaudhary (1999) as follows: $ERS = 2.06 * h^2_{bs} * \sqrt{\sigma^2_{F2,}}$ and expressed as percent of the over mean (X_{bar}) of the given trait: ERS (%) = (100 * ERS) / X_{bar}. Relationship between studied traits and grain yield was inspected through genotypic correlation coefficient (rg), which was derived as the ratio of covariance to the square root of the product of the corresponding variance of the two traits considered. Genotypic covariance was determined using the property of the analysis of variance of the sum of two variables as suggested by Kwon and Torrie (1964) and described in Mansouri et al. (2018), using Past software (Hammer et al., 2001). The standard error of rg was derived using the formulae of Reeves (1955), reported by Koots and Gibson (1996), as follows: $SE_{rg} = [(1- rg²) / \sqrt{2}] * [(\sqrt{SE_{h^2i}} * SE_{h^2j}) / (\sqrt{h^2i} * \sqrt{h^2j})]$, where h^2i and h^2j are the traits heritability's. Student's t-test was used to determinate the significance of the correlation coefficient.

3 RESULTS AND DISCUSSION

3.1 VARIATION AND MEAN PERFORMANCES

Significant generation effect was revealed for flag leaf area (FLA), plant height (PHT) and straw mass (STM) by the analysis of variance, indicating the presence of substantial genetic variability and allowing to

carry out in deep bio-metrical analysis (Table 1). A significant generation effect is a prerequisite to perform the inheritance study of the targeted traits applying generation means analysis model. Among generations traits mean estimates, varied from 19.67 to 29.70 cm² for FLA, from 82.00 to 136.25 cm for PHT, and from 12.32 to 31.98 g plant^{-1} for STM (Figures 1, 2 and 3). Contrast analysis indicated that deviations between parental mean values for the studied traits were significant (Table 1). 'Gaviota' had significantly greater FLA (29.70 cm²) than MBB (26.08 cm²), while MBB showed significantly greater PHT (136.25 cm) and STM (31.55 g plant^{-1}) than 'Gaviota' whose mean values for both traits were 86.87 cm and 15.24 g/plant, respectively (Figure 1, 2 and 3).

These results suggested that the crossed parents carry different allelic combinations involved in the genetic control of the studied traits. On average, F1 exhibited equal FLA (29.35 cm²) to the best parent GTA (29.70 cm²), equal PHT (82.00 cm) to the semi-dwarf parent GTA (86.87 cm) and equal STM (31.98 g plant^{-1}) to the tall parent MBB (31.55 g plant^{-1}), within the limits of their standard errors (Figures 1, 2 and 3). Furthermore, F1 means differed significantly from mid-parent average for PHT and STM but not for FLA, suggesting that dominance was predominantly involved in the genetic control of PHT and STM; while additive genetic control was predominantly expressed for FLA (Table 1, Figures 1, 2 and 3). Dominance acted in the direction of

Table 1: Mean square deviations of the analysis of variance for flag leaf area, plant height and straw mass in Gaviota x MBB durum wheat cross

Sources of variation	DF	FLA (cm²)	PHT (cm)	STM (g/plant)
Generations	5	89.56**	2091.65**	398.08**
Replications	4	9.56	8.81	25.00
Homogeneous (Homo)	2	21.56**	4504.12**	455.19**
P1 vs P2	1	8.10**	1190.50**	18.10**
F1 vs ½(P1+P2)	1	2.55^{ns}	568.92**	6.69*
Heterogeneous (Het)	2	20.38^{ns}	579.97**	164.99^{ns}
F2 vs ½(BC1+BC2)	1	4.95*	177.96**	8.88**
BC1 vs BC2	1	16.57**	9.17**	1.11^{ns}
Homo vs Het	1	89.86**	56.58**	20.43**
Residual	20	4.05	5.12	36.72

$^{Ns, *, **}$: non-significant and significant effects at 5 % and 1 % probability level, respectively. FLA: Flag leaf area, PHT: Plant height; STM : Straw mass

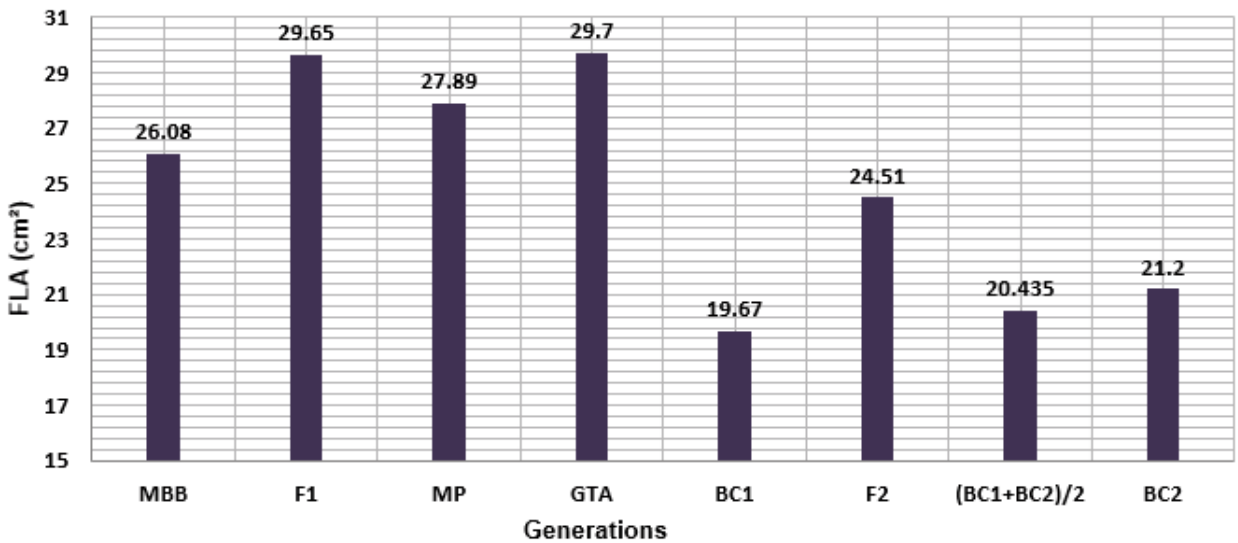

Figure 1: Mean of flag leaf area of the basic generations

reduced PHT and increased STM, suggesting that GTA carries more dominant genes controlling PHT, while MBB carries more dominant genes controlling STM. Based F1 data analysis, these results agreed with findings of several authors who reported that non-additive genetic effects appeared as an important component of the genetic architecture of PHT and STM, while additive gene effects were prevalent for FLA (Saleem et al., 2005; Shabbir et al., 2012; Yang et al., 2016). Mean values of the F2 generation deviated significantly from the average of BCs generations for the three studied traits, being significantly higher and laying within the parental range for PHT and STM and outside of this range for FLA (Table 1, Figures 1, 2 and 3).

3.2 GENE EFFECTS

ABCD and joint scaling tests, applied to appraise presence of epistasis, were found significant, invaliding the additive–dominance model adequacy for explaining the inheritance pattern of PHT, STM and FLA, and suggesting the adoption of higher than three-parameter model (Table 2). These results indicate that higher order

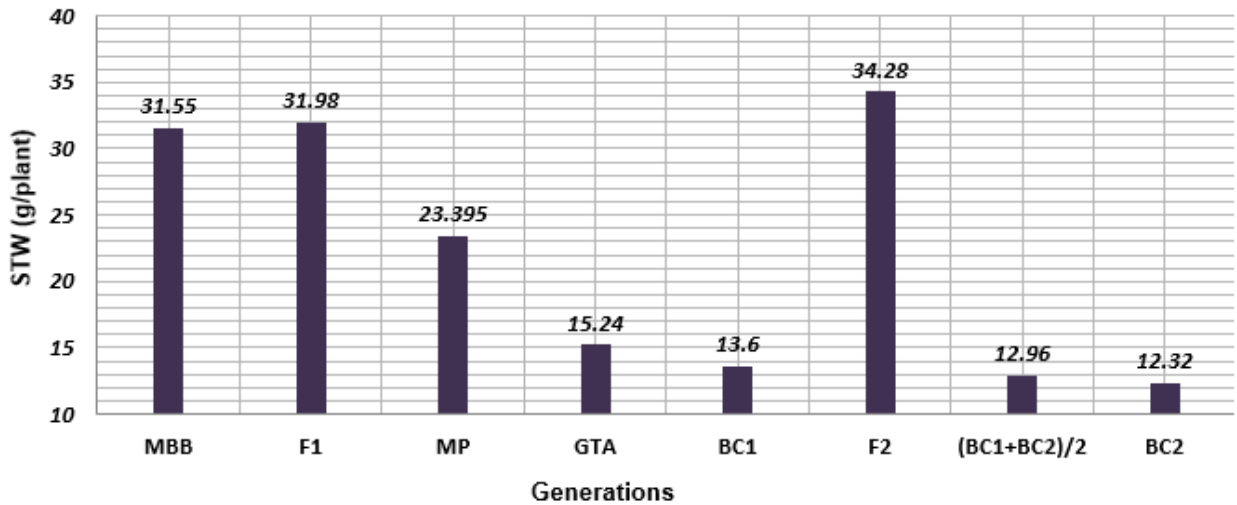

Figure 2: Mean of straw yield of basic generations

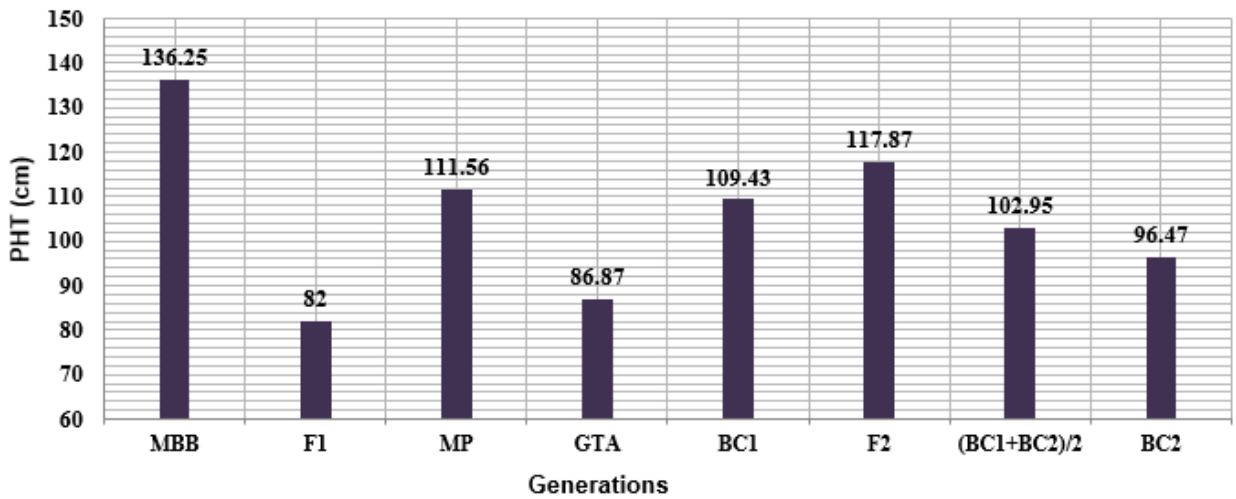

Figure 3: Mean of plant height of the basic generations

inter-allelic interactions played an important role in the expression of the measured traits, and additive–dominance model alone will not be sufficient to deal with the inheritance pattern of such traits. Estimates of the gene effects derived from this six-parameter model are given in Table 2. Gene main effect [m] was significant for all three analyzed traits, indicating that these traits are controlled by minor genes and quantitatively inherited. For PHT, additive [d] and dominance [h] gene affects, and additive * additive [i] and additive * dominance [j] non-allelic interactions were significantly involved in the inheritance of this trait. Dominance [h] gene effects and additive * additive [i] non-allelic interactions came out as the salient features of the genetic control of this character as this is indicated by the high absolute values of the genetic parameters. The negative sign of the additive * dominance [j] component indicated that genes involved in the control of this trait were dispersed the parents.

These results agreed with Novoselović et al. (2004), Ojaghi and Akhundova (2010), Mohamed et al. (2013), Dorri et al. (2014) and Fellahi et al. (2016) who reported that non-additive gene effects played an important role in the inheritance of PHT. Salmi et al. (2019) found that dominance acted in the direction of increased plant height But Akhtar and Chowdhry (2006) as well as Hannachi et al. (2013) reported that additive gene effects were predominant in the genetic control of this character. For STM, the additive [d] gene effects were not significant while dominance [h] gene effects, additive * additive [i], additive * dominance [j] and dominance * dominance [l] allelic interactions were significant. The gene effects [h], additive * additive [i], and dominance * dominance [l] allelic interactions exhibited the largest effects. Being significant and of the same sign, dominance [h] gene effects and dominance * dominance [l] non-allelic interactions suggested the implication of complementary type of epistasis in the genetic control of this trait. A greater magnitude of dominance [h] compared to additive [d] gene effects, as this is the case in the present study, for this trait, arises, according to Kearsey and Pooni (1996), when genes are dispersed in the parents. For FLA, the additive * dominance [j] epistatic component was not significant while the additive [d] and dominance [h] gene effects, the additive * additive [i], and dominance * dominance [l] allelic interactions were significant. The dominance * dominance [l] component exhibited the largest effect. The implication of complementary type of epistasis in the genetic control

Table 2: Scaling tests, gene action types, heritability (H^2bs, h^2ns), expected response to selection (ERS) and genotypic correlation (rg_{GY}/…) estimates for plant height, straw yield and flag leaf area in MBB x GTA durum wheat cross

Traits		PHT	STM	FLA
Scaling test	A	9.65 ± 4.96^{ns}	$37.14 \pm 1.11^{**}$	$17.72 \pm 1.71^{**}$
	B	$31.25 \pm 2.56^{**}$	$20.44 \pm 2.18^{**}$	$15.86 \pm 0.66^{**}$
	C	$84.03 \pm 4.41^{**}$	$26.38 \pm 2.65^{**}$	$17.04 \pm 1.35^{**}$
	D	$21.55 \pm 3.54^{**}$	$41.99 \pm 1.69^{**}$	$8.12 \pm 1.11^{**}$
Joint scaling test	X^2	860.27^{**}	569.90^{**}	745.72^{**}
Genetic parameters	m	$117.79 \pm 1.09^{**}$	$34.25 \pm 0.62^{**}$	$24.51 \pm 0.32^{**}$
	[d]	$13.87 \pm 2.78^{**}$	0.19 ± 1.14^{ns}	$2.89 \pm 0.90^{**}$
	[h]	$- 72.67 \pm 7.09^{**}$	$75.39 \pm 3.42^{**}$	$14.49 \pm 2.23^{**}$
	[i]	$- 43.11 \pm 7.09^{**}$	$83.98 \pm 3.39^{**}$	$16.24 \pm 2.22^{**}$
	[j]	$- 10.84 \pm 2.79^{*}$	$8.35 \pm 1.21^{**}$	1.08 ± 0.91^{ns}
	[l]	2.18 ± 11.99^{ns}	$141 \pm 5.27^{**}$	$49.54 \pm 3.85^{**}$
Type of gene actions		-----	Complementary	Complementary
$H^2_{bs} \pm SE$		0,98 ± 0.40*	0,99 ± 0.41*	0,98 ± 0.40*
$h^2_{ns} \pm SE$		0,57 ± 0.23*	0.76 ± 0.31*	0,39 ± 0.16*
$rg_{GY/}$		-0.57 ± 0.15*	1.21 ± 0.17*	0.91 ± 0.07*
ERS		8.25	22.72	7.03
ERS%		6.99	99.44	29.69

PHT = Plant height, STM = straw mass, FLA = Flag leaf area. m = mean main effect, [d] = assistive effect, [h] = dominance effect, [i] = additive x additive effect, [j] = additive x dominance effect, [1] = dominance x dominance effect. Ns, * and ** = non-significant and significant effects at 5 % and 1 % probability levels, respectively

of this trait is suggested by the dominance gene effects [h] and the dominance * dominance [l] allelic interaction which were significant and of the same sign. Dominance [h] component was greater than additive [d] gene effects suggesting that genes controlling FLA are dispersed in the parents. In this context Saleem et al. (2005); Inamullah et al. (2006); Munir et al. (2007); Ijaz et al. (2013) and Yang et al. (2016) found that FLA was mostly under additive genetic control combined to partial dominance and epistasis type of gene actions. Joshi and Sharma (1984) mentioned that dominance acted in the direction of reduced FLA.

Shayan et al. (2019) reported that the additive-dominance model fitted best the variation present among generation means for FLA, while for PHT and STM, the six-parameter model was adequate implying the presence of non-allelic interactions in the inheritance of these two traits. Divergence in the result among various studies seems to indicate that genetic model adequacy as well as the preponderance of significant gene effects and non-allelic interactions are dependent upon the cross combination genetic background and the experimental growth conditions experienced. The complementary epistasis type implicated in the inheritance of STM and FLA suggested the possibility of heterosis expression for these two traits. In fact, Punia et al. (2011), referring to Jinks and Jones (1958), mentioned that heterosis is likely to be expressed with greater magnitude in crosses where complementary type of interaction is expressed. The fact that generation means variation fitted a digenic epistatic model suggested that improvement of PHT, STM and FLA would be fairly difficulty compared to the situation where the additive-dominance model was the most adequate. Furthermore, Sirohi and Gupta (1993) suggested that traits showing high magnitude of dominance [h] than additive [d] gene effects, as this is the case for PHT, STM and FLA, in the present study, can be improved through conventional breeding approaches. But selection need to be delayed until later generations when the dominance effects would have diminished and desirable segregants become available.

3.3 HERITABILITY, EXPECTED RESPONSE TO SELECTION, AND GENOTYPIC CORRELATIONS

Being significant, broad sense heritability estimates for FLA, PHT and STM were appreciably high, taking values of 98.00, 98.00 and 99.00 %, respectively. Their corresponding narrow sense counterparts were also significant, but of lower magnitude, being still high for STM (76.00 %), moderate for PHT (57.00 %) and low for FLA (39.00 %). High h^2ns values indicate that the environment influences less the expression of the given character. In fact, estimates of heritability are useful for a breeder to weigh the proportion of variation which is inheritable from that which is non-inheritable. Heritability values observed in the present study were in the range of those reported in similar studies. Fellahi et al. (2020) reported h^2bs values of 86.50 % for PHT and 77.40 % for FLA. Novoselovic et al. (2004) reported h^2bs values ranging from 0.54 to 0.81 for PHT of several crosses. These high H^2bs values suggested that these traits are less impacted by environmental variation, and then are easily amenable to improvement. In this context, Johnson et al. (1966) mentioned that since plant height and straw mass heritability were sufficiently high then selection in the F_2 for these traits could be effective. So, based on the heritability estimates observed in the present study, STM, FLA and PHT appeared amenable to significant improvement applying early selection.

Genotypic correlation coefficients, relating grain yield (GY) to FLA, PHT and STM, found in this study, were significant, taking values of 0.91, -0.57 and 1.21, respectively (Table 2). while PHT was posirivelly correlated with STM (r = 0.315**), but negatively correlated with FLA (r = -0.153*), and STM was positivelly correlated with FLA (r = 0.269**). These correlation coefficients indicated that selection of high values for FLA and STM will be accompanied by increased GY, but selection to increase PHT had a negative impact on GY and on FLA. The negative correlation relating PHT to GY, observed in the present study, contradicted Ataei et al. (2017) results which showed that GY was positively and highly correlated with PHT and peduncle under drought stress conditions, emphasizing the importance of PHT as selection criterion to improve drought tolerance. In this context and according to Davidson et al. (1992) and Belkherchouche et al. (2015) wheat peduncle is a transient source of water-soluble carbohydrates, playing a crucial role in minimizing grain yield decline under drought stress conditions. Similarly, Mohsin et al. (2009) found that grain yield correlated positively with FLA, PHT, biomass (BIO), under drought stress. Under rainfed growing conditions, Mansouri et al. (2018) reported that above ground plant biomass exhibited significant and positive correlation coefficients with GY, STM and PHT. The expected response to selection estimates were low, being less than 10 % for PHT (8.24 cm or 6.99 %, X_{barF2} = 117.87 cm) and high, above 20 %, for STM (22.74 g $plant^{-1}$ or 99.4 % X_{barF2} = 22.85 g $plant^{-1}$), FLA (7.03 cm^2 or 26.6 %, X_{barF2} = 23.67 cm^2) (Table 2). These results indicated that, based on the magnitude of the variability expressed by each trait, moderate to appreciable genetic gain could be made via mono trait selection.

Durum wheat production is often impacted by

drought stress, particularly in the arid and semi-arid regions. To overcome this situation, it is necessary to develop improved varieties devoted to these specific environments. Morphological characters like plant height, flag leaf area, and straw yield, had been identified and proposed as morphological markers for drought tolerance. Breeding procedure for drought tolerance depends upon the pattern of inheritance and the nature of actions of the genes involved in the genetic control of the drought related traits. A better understanding of the complexities of the genetic control of these traits will be useful for cultivar improvement. Globally, from the results of this study, it can be summarized that the additive–dominance model was inadequate, suggesting the adoption of a six-parameter genetic model. Additive [d] and dominance [h] gene effects, and non-allelic interactions ([i], [j] and [l]) were involved in the inheritance of PHT, with the predominance of [h] and [i] components. Complementary type of epistasis was implicated in the inheritance of STM and FLA. These results were in lines with findings of some studies (Novoselovic et al., 2004; Ojaghi and Akhundova, 2010; Mohamed et al., 2013; Dorri et al., 2014; Fellahi et al., 2016) and diverged from those of others studies (Inamullah et al., 2006, Munir et al., 2007; Ijaz et al., 2013; Yang et al., 2016; Shayan et al., 2019), suggesting that genetic model adequacy as well as the preponderance of significant gene effects and non-allelic interactions, governing the inheritance of a given trait, are dependent upon the cross combination genetic background and the experimental growth conditions experienced. Due to the presence of non-allelic interactions PHT, STM and FLA improvement would be fairly difficulty, requiring the implementation of conventional breeding approaches such as the inclusion of F2's showing high performances in multiple crosses for further improvement of the studied traits in order to synthesize a dynamic population accumulating most of the favorable genes. This mating procedure seems to be a good technique to disrupt linkage, to generate usefull recombination and to accumulate favorable genes in the base population. Selection need to be delayed until later generations when the dominance effects would have diminished and desirable segregants become available. This strategy is supported by the high heritability estimates, the moderate to high expected genetic gains and the significant genotypic correlation coeffcicients relating the studied traits to grain yield.

4 CONCLUSION

The results of the present study indicated that non-allelic interactions, in addition to additive and dominant gene effects are important factors controlling the expression of PHT, STM and FLA. Application of conventional selection procedure may not be rewarding for the improvement of these characters. But inter mating among the selected segregants followed by few generations of selfing could be useful to break the undesirable linkage and allow accumulation of favorable alleles for improvement of these traits.

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Endofitne glive v biotičnem varstvu rastlin pred škodljivimi organizmi in njihov posreden vpliv na rastline

Primož ŽIGON [1, 2], Franci Aco CELAR [3]

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Endophytic fungi as biological control agents and their indirect effects on plants

Abstract: The use of entomopathogenic fungi represents one of the most important non-chemical alternatives for pest control in crop production. In addition to their pathogenicity to arthropods, they have many other important effects that favor their use in biological control. They live in plants as endophytes and have an inhibitory effect on plant pathogens. They inhabit the rhizosphere of many plants in natural and agricultural ecosystems and have a stimulatory effect on their growth and development. These recently acquired ecological functions are not yet fully understood, but point to the broader potential of using entomopathogenic endophytic fungi in crop production, not only as biopesticides but also as mycofungicides and growth stimulants (biostimulants). To achieve the full potential of entomopathogenic endophytic fungi in daily agricultural practice, practical application should be considered in the development of commercial products and the application techniques of entomopathogenic endophytic fungi that allow successful colonization of plants should be considered.

Key words: entomopathogenic fungi; endophyte; beneficial organisms; bioinsecticides; *Beauveria; Metarhizium*

Endofitne glive v biotičnem varstvu rastlin pred škodljivimi organizmi in njihov posreden vpliv na rastline

Izvleček: Uporaba entomopatogenih gliv predstavlja enega temeljnih ukrepov nekemičnega varstva rastlin pred škodljivci. Poleg patogenosti za členonožce imajo te glive tudi druge lastnosti, zaradi katerih so širše uporabne v biotičnem varstvu rastlin. V rastlinah živijo kot endofiti in lahko delujejo zaviralno tudi na različne povzročitelje rastlinskih bolezni. Naseljujejo rizosfero številnih rastlin v naravnih in kmetijskih ekosistemih ter delujejo spodbujevalno na njihovo rast in razvoj. Te v zadnjem času dognane ekološke funkcije še niso podrobneje raziskane, vendar kljub temu nakazujejo na širši potencial uporabe entomopatogenih endofitnih gliv pri pridelavi rastlin, ne le kot sredstev za biotično zatiranje škodljivcev, pač pa tudi kot mikofungicidov in sredstev za krepitev rasti in razvoja (biostimulantov). Za umestitev uporabe entomopatogenih endofitnih gliv v vsakdanjo kmetijsko prakso je potrebno pri razvoju komercialnih pripravkov upoštevati praktičnost uporabe in preučiti tehnike nanosa entomopatogenih endofitnih gliv, ki omogočajo uspešno kolonizacijo rastlin.

Ključne besede: entomopatogene glive; endofiti, koristni organizmi, bioinsekticidi; *Beauveria*, *Metarhizium*

1 Kmetijski inštitut Slovenije, Oddelek za varstvo rastlin, Ljubljana
2 Korespondenčni avtor, e-naslov: primoz.zigon@kis.si
3 Univerza v Ljubljani, Biotehniška fakulteta, Oddelek za agronomijo, Ljubljana

1 UVOD

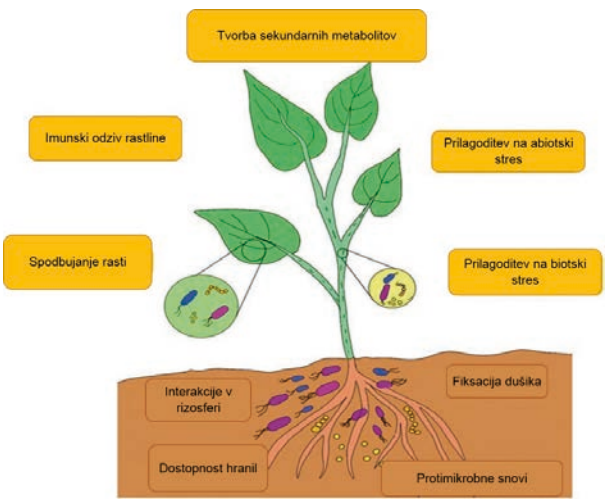
1.1 ENDOFITI

Endofiti so različne vrste gliv in bakterij, ki živijo v notranjosti rastlinskega tkiva ter so z rastlinami v mutualističnem odnosu (Wilson, 1995). Dokaze za obstoj endofitizma so odkrili v fosiliziranih ostankih rastlin, kar kaže na vzpostavitev tovrstnih odnosov že vse od pojava prvih kopenskih rastlin (Field in sod., 2015a; Field in sod., 2015b). Endofiti z gostiteljskimi rastlinami tvorijo kompleksne večplastne interakcije, ki imajo pozitivne učinke na rastline. Z namenom prilagoditve in preživetja znotraj rastlin mikroorganizmi vzpostavijo mutualistični odnos v okviru katerega imata oba organizma medsebojno korist (Kogel in sod., 2006; Hardoim in sod., 2015). Kolonizacija rastlinskega tkiva brez povzročanja vidnih posledic vzpostavitve tega odnosa, ki bi se odražal v obliki poškodb rastlinskega tkiva, poteka vsaj del življenjskega kroga endofitnega organizma. Gre torej za okužbo rastlin, ki se ne odraža v pojavu vidnih bolezenskih znamenj (Petrini, 1991). Endofiti so lahko prisotni v rastlinskih tkivih po celotni rastlini ali posameznih organih kot so korenine, steblo, listi, cvetovi, semena itd. Njihov življenjski prostor znotraj rastlin obsega notranjost celic (intercelularno) in/ali se nahajajo le v medceličnem prostoru (intracelularno) (Hardoim in sod., 2015). Endofitni mikroorganizmi pripomorejo k odzivu in prilagoditvi rastlin na biotske in abiotske strese ter z vzbujanjem različnih mehanizmov inducirane odpornosti omogočajo le tem premostitev stresnih situacij (Rodriguez in Redman, 2008; Rho in sod., 2018). Endofiti namreč stimulirajo tvorbo signalnih molekul kot so etilen, jasmonska kislina in salicilna kislina, s čimer vplivajo na aktivacijo mehanizmov inducirane odpornosti (Robert-Seilaniantz in sod., 2011). Eden izmed pomembnih odzivov rastlin na stres je tudi tvorba sekundarnih metabolitov. Endofiti posredno vplivajo na povečano tvorbo rastlinskih sekundarnih metabolitov z aktivacijo genov za njihovo sintezo, določeni endofiti pa sekundarne metabolite, ki so udeleženi v obrambnih odzivih rastlin, tvorijo tudi sami (Van Wees in sod., 2008; Zamioudis in Pieterse, 2012). Endofiti lahko vplivajo na večjo dostopnost v tleh vezanih hranil in mineralov ter sproščanje le teh v talno raztopino, s čimer rastlinam omogočajo njihov privzem in tako vplivajo na boljše fiziološko stanje ter prehranjenost gostiteljskih rastlin. Druge prilagoditve rastlin na abiotski stres, ki jih omogočajo endofiti, so povezane s tvorbo rastlinskih hormonov, stresnih proteinov, antioksidantov in encimov, ki povečujejo toleranco rastlin na stresne razmere (Rho in sod., 2018).

Po drugi strani je vzpostavitev mutualističnega odnosa z rastlinami ključna za preživetje in obstoj nekaterih endofitov, obligatnih heterotrofov, ki so odvisni od privzema hranil iz rastlinskega tkiva (Bamisile in sod., 2018). Rastline endofitnim organizmom nudijo ustrezno okolje za rast, zagotavljajo vir ogljika in drugih hranil ter v nekaterih primerih preko prenosa s semenom gostiteljskih rastlin omogoča njihov obstoj in ohranjanje (Hardoim in sod., 2015). Rastline s koreninskimi izločki v območje korenin privabljajo množico mikroorganizmov. Mikroorganizmi v tleh, patogeni ali nepatogeni, imajo enak potencial za okužbo rastlin. Ta je odvisna od razmer v rizosferi, vrste in odziva gostitelja ter sposobnosti mikroorganizma, da zaobide njegov imunski odziv (Philippot in sod., 2013). Endofiti iz rizosfere vstopajo v rastlinsko tkivo preko koreninskih laskov in naravnih odprtin. Vstop endifitov in kolonizacijo rastlinskega tkiva pogojuje tvorba specifičnih encimov, signalnih molekul (na primer flavonoidov) in drugih metabolitov, ki omogočajo premostitev obrambnih mehanizmov rastlin in razlikovanje koristnih endofitnih organizmov od drugih potencialno patogenih mikroorganizmov v rizsoferi (Kogel in sod., 2006; Hardoim in sod., 2015).

1.1.1 Endofitne glive

Izraz endofiti je prvič uporabil Heinrich Anton de Bary in z njim označil glive, katerih hife preraščajo celice in tkiva avtotrofnih organizmov (Bary, 1866). Prisotnost endofitnih gliv (EG) v rastlinskem tkivu je prvič dokazal E. M. Freeman (Freeman in Ward, 1904), ki je izoliral glivo iz rodu *Epichloë* iz semen omotne ljuljke (*Lolium temulentum* L.). Do danes so EG našli v številnih rastlinskih vrstah, ki rastejo praktično na vseh območjih in vrstah rastišč od severnega do južnega pola zemeljske



Slika 1: Vpliv endofitov na rast in odziv rastlin na biotske in abiotske stresne dejavnike (Sharma in sod., 2020)

oble (Saikkonen in sod., 2004; Arnold, 2007; Rodriguez in sod., 2009). EG tvorijo različne vrste sekundarnih metabolitov kot so alkaloidi, cikloheksani, flavonoidi, kinoni in terpeni, ki imajo protimikrobne, antioksidativne, antikancerogene in citotoksične učinke (Rana in sod., 2019; Mantzoukas in Eliopoulos, 2020). Imajo tudi pomembno vlogo pri odzivu rastlin na okužbo/napad rastlinskih patogenov in herbivorov, omogočajo boljšo prehranjenost rastlin, saj povečujejo dostopnost makro in mikro hranil ter omogočajo vezavo atmosferskega dušika (Bacon, 1993; Behie in sod., 2013). Za vzpostavitev simbiotskega odnosa EG tvorijo hidrolitične encime (celulaze, lipaze, proteaze, oksidaze), ki omogočajo premostitev obrambnih mehanizmov rastline in s tem uspešno kolonizacijo rastlinskih tkiv. Znotraj rastlin hife gliv rastejo skozi parenhim tudi vse do ksilema. Sistemična kolonizacija rastlin je značilna za akropetalno (od spodaj navzgor) rastoče hife gliv, torej predvsem v primeru, da gliva izvira iz okuženega semena ali pa vstopa v rastlino preko korenin (Mantzoukas in Eliopoulos, 2020). Uspešnost kolonizacije rastlin je odvisna od razmer v rizosferi (kompeticija mikroorganizmov), okoljskih razmer (vlaga, temperatura), rastlinske vrste in njenega fiziološkega stanja (Carroll, 1988). Številne vrste EG imajo pomembno vlogo pri odzivu rastlin na okužbo/napad rastlinskih patogenov in škodljivcev. Za EG velja, da imajo načeloma pozitiven vpliv na rastline, saj se okužba, ki jo povzročajo, ne odrazi v bolezenskih znamenjih. Kljub temu za nekatere vrste EG velja, da lahko latentna okužba pod določenimi pogoji, ki so lahko posledica okoljskih sprememb, stresa ali obdobja senescence rastlin, povzroči tudi pojav bolezni (Saikkonen in sod., 2004; Bamisile in sod., 2018).

Na podlagi do sedaj opravljenih raziskav ugotavljajo veliko vrstno pestrost EG, ki izhajajo iz debel zaprtotrosnic (Ascomycota) in prostotrosnic (Basidiomycota) ter poddebla Mucoromycotina. Asimptomatske okužbe so med drugim vzrok za slabo poznavanje in manjšo raziskanost interakcij med glivami in rastlinami, zato je bilo izoliranih in taksonomsko opisanih relativno malo vrst. Endofiti so lahko specializirani na ravni družine, ali pa okužujejo širši krog gostiteljev na različnih rastiščih. Taksonomsko lahko EG razvrščamo v dve skupini glede na družino, ki ji posamezne vrste pripadajo: vrste iz družine Clavicipitaceae (Ascomycota, Hypocreales) kolonizirajo relativno ozek krog gostiteljskih rastlin iz družine trav (Poaceae) kot so *Festuca* spp., *Lolium* spp. idr. V drugo skupino sodijo druge glive, ki ne izhajajo iz družine Clavicipitaceae in kolonizirajo širši krog gostiteljskih rastlin (Carroll, 1988; Rodriguez in sod., 2009; Hardoim in sod., 2015). Za enostavnejše razumevanje poznanih kompleksnih interakcij med EG in njihovimi gostiteljskimi rastlinami jih lahko razvrščamo glede na način prenosa med gostiteljskimi rastlinami (horizontalno ali vertikalno), glede na način kolonizacije in širjenja v po rastlini (sistemično ali omejeno na posamezne organe oziroma rastlinska tkiva) ali glede na način prehranjevanja (nekrotrofi in biotrofi) (Rodriguez in sod., 2009). Poleg morfološke identifikacije so za določevanje gliv vsekakor pomembne novejše molekulske tehnike na osnovi DNK, ki omogočajo natančnejšo klasifikacijo in filogenetsko opredelitev EG (Vega in sod., 2009; Bamisile in sod., 2018).

1.1.2 Entomopatogene glive

Glive so prevladujoči patogeni členonožcev, med njimi žuželk in pajkovcev, med katerimi so tudi pomembni rastlinski škodljivci. Entomopatogene glive (EPG) so namreč tekom evolucije razvile prefinjene mehanizme izkoriščanja žuželk za zadovoljevanje svojih prehranskih potreb. EPG so paraziti teh organizmov in pri njih povzročajo bolezenska stanja. Med EPG uvrščamo preko 700 različnih vrst, od katerih večina okužuje širok krog gostiteljev, njihovi posamezni sevi pa so bolj patogeni za določene vrste žuželk in pršic (Vega in sod., 2009; Sandhu in sod., 2012). Redke vrste EPG so bolj specializirane in okužujejo ožji krog gostiteljev, kot na primer *Aschersonia aleyrodes* Webber 1897, ki okužuje le kaparje (Coccoidea) in ščitkarje (Aleyrodidae) (Humber, 2008). Insekticidno učinkovanje EPG je specifično glede na gostiteljske organizme kot so npr. gosenice metuljev (Lepidoptera), uši (Aphidae), resarji (*Thrips* spp.) in druge kozmopolitske vrste, ki so znani škodljivci kmetijskih rastlin. Do okužbe pride po naključju, ko spore EPG z vetrom ali vodo pridejo v stik z gostiteljem in kalijo na površju njegovega telesa. Vanj prodrejo skozi naravne odprtine ali neposredno prek zunanje kutikule. Uspešnost in hitrost kalitve spor je odvisna od okoljskih dejavnikov kot sta vlaga in temperatura, dostopnosti hranil, kisika, pH ter vsebnosti protimikrobnih snovi na kutikuli gostitelja (Sandhu in sod., 2012). Prodor EPG skozi zunanjo epikutikulo poteka mehansko, v večini primerov na podlagi tvorbe specifičnih kaveljčkom podobnih struktur ali apresorijev. Sestava notranjega dela kutikule (prokutikula) je kompleksnejša, sestavljajo jo predvsem hitinske fibrile, beljakovine in lipidi (Hackman, 1953). Prodor glive je zato poleg mehanskega učinka odvisen tudi od biokemičnega vpliva in tvorbe hidrolitičnih encimov, ki razgrajujejo celične strukture v kutikuli (Pedrini in sod., 2007). Okužba je odvisna tudi od učinkovitosti različnih strategij, na podlagi katerih EPG zaobidejo imunski odziv gostitelja. Pri številnih vrstah pride do spremembe načina rasti in tvorbe blastospor, preko katerih pride do uspešne kolonizacije hemocela in privzema hranil iz hemolimfe. Posledica je postopno izčrpavanje,

podhranjenost in s tem onemogočanje vitalnih funkcij gostitelja (Sandhu in sod., 2012). Poleg tega pri številnih EPG pride do tvorbe toksičnih metabolitov, ki pospešijo pogin gostitelja, olajšajo prodor hif in omogočajo širjenje blastospor v hemolimfi (Vidal in Jaber, 2015). Hife nato prepredejo notranjost telesa in prehajajo na površje kadavra, kjer tvorijo nove spore, ki širijo okužbo naprej.

O insekticidnih lastnosti EPG in njihovem potencialu za zatiranje škodljivcev so govorili že v 19. stoletju, potem ko je italijanski entomolog Agostino Bassi dokazal, da je okužba z glivo *Beuveria bassiana* (Bals.-Criv.) Vuill. (1912) povzročila bolezensko stanje sviloprejke (Vega in sod., 2009). Z začetkom proizvodnje in množične uporabe kemičnih insekticidov sredi prejšnjega stoletja, prave potrebe po uporabi biotičnih agensov za varstvo rastlin ni bilo, zato do nadaljnjega razvoja tega področja ni prišlo. Danes je pri iskanju okoljsko sprejemljivejših načinov zatiranja rastlinskih škodljivcev uporaba EPG ena ključnih alternativ uporabi kemičnih insekticidov. Prednosti uporabe EPG v primerjavi s sintetičnimi insekticidi so predvsem v zmanjšanju neželenih učinkov na neciljne organizme in s tem ohranjanje biotske pestrosti agroekosistema ter zmanjšanju ostankov kemijskih spojin v okolju ter s tem manjši vpliv na zdravje ljudi in živali (Vega in sod., 2009; Lacey in sod., 2015). Uporaba entomopatogenih gliv v biotičnem varstvu večinoma temelji na t. i. pristopu preplavnega biotičnega varstva, pri katerem gre za ciljni vnos z namenom čimprejšnjega zatrtja škodljivca pri čemer nadaljnje razmnoževanje in ohranjanje glive v prostoru ni pomembno. EPG se uporabljajo v obliki tehničnih mikoinsekticidnih formulacij pri katerih je bistvena hitrost učinkovanja, da je dosežena čim prejšnja smrtnost tarčnega organizma (Faria in Wraight, 2007). Za namene masovne proizvodnje pripravkov mora biti produkcija EPG enostavna in cenovno sprejemljiva, pripravki pa morajo dosegati konstantno učinkovitost v poljskih razmerah. Kljub temu, da je več kot 700 vrst EPG iz približno 90 rodov, se v praksi v glavnem uporabljajo le glive iz rodov *Beauveria, Metarhizium, Lecanicillium* in *Isaria*, ki ustrezajo prej omenjenim kriterijem (Vega in sod., 2009). Več kot 60 % komercialnih mikoinsekticidov temelji na vrstah *Beauveria bassiana* in *Metarhizium anisopliae* (Metchnikoff) Sorokin (1883), ki okužujeta številne rastlinske škodljivce in sta najbolje preučeni vrsti EPG, saj sta se med prvimi uporabljali za namene biotičnega varstva (Sandhu in sod., 2012). Komercialne biopesticidne formulacije pretežno vsebujejo EPG v obliki blastokonidijev, ki se jih nanaša na površje rastlin. Učinkovitost EPG je v tem primeru v naravnih razmerah omejena, saj so spore občutljive na UV svetlobo in pomanjkanje vlage (Vega in sod., 2009).

1.1.3 Entomopatogene endofitne glive

Vloga EPG pa ni omejena zgolj na zatiranje škodljivcev, pač pa je njihova ekosistemska funkcija, ki posredno vpliva na rast rastlin, mnogo širša. V številnih nedavnih študijah namreč ugotavljajo, da nekatere vrste EPG z rastlinami tvorijo različne interakcije, kar nakazuje na njihovo endofitno naravo v smislu kolonizacije rastlinskih tkiv (Mantzoukas in Eliopoulos, 2020). Poleg sposobnosti okuževanja rastlinskih škodljivcev imajo entomopatogeni endofiti (EPGE) lahko tudi antagonistične lastnosti proti povzročiteljem rastlinskih bolezni in z naselitvijo v rizosferi posredno vplivajo na izboljšanje razmer za rast rastlin (Jaber in Enkerli, 2017; Jaber in Ownley, 2018). Ta njihova večstranska vloga in multiplikativni učinki na rastline kažejo na večji potencial EPGE v biotičnem varstvu rastlin, ne le kot sredstev za zatiranje rastlinskih škodljivcev, pač pa tudi v varstvu pred povzročitelji bolezni in posrednimi vplivi na spodbujanje rasti gostiteljskih rastlin (Vega, 2018). Iz rastlinskih tkiv so bile v preteklih raziskavah izolirane številne glive, ki so znani patogeni rastlinskih škodljivcev in na ta način dokazali njihovo naselitev v rastlinskem tkivu. Za znane pomembnejše vrste EPG kot so *Beauveria bassiana*, *Beauveria brongniartii* (Sacc.) Petch, *Clonostachys rosea* (Link) Schroers, Samuels, Seifert & W. Gams, *Cordyceps farinosa (*Holmsk.) Kepler, B. Shrestha & Spatafora, *Neotyphodium* spp., *Cladosporium* spp., *Acremonium* spp. in *Akanthomyces lecanii* (Zimm.) Spatafora, Kepler & B. Shrestha je bila na podlagi postopkov izolacije iz rastlinskih tkiv dokazana njihova v naravi prisotna endofitna vloga (Preglednica 1). Izmed naštetih je najbolj preučena *B. bassiana*, ki je splošno razširjena talna gliva in fakultativni endofit, ki okužuje preko 700 vrst žuželk in pršic. *B. bassiana* se v tleh širi kot saprofit, ki pa ima slabo tekmovalno sposobnost za odmrlo organsko snov, ki ji predstavlja osnovni vir hranil za nadaljnjo rast (Hajek, 1997). Vzpostavitev mutualistične interakcije z rastlinami je zato velikega pomena za obstoj in širjenje glive. Citološke študije kažejo, da nekatere EPGE pretežno rastejo na površju korenin in kolonizirajo le celice epiderma in koreninske skorje ter tako le v manjši meri vstopajo neposredno v globje tkivo gostitelja. Z različnimi postopki inokulacije je bila sposobnost kolonizacije rastlinskega tkiva uspešno dokazana tudi za nekatere druge vrste gliv, kot so *Metarhizium anisopliae* (Metschn.) Sorokī, *Fusarium oxysporum* Schltdl., *Trichoderma lixii* (Pat.) P. Chaverri, *Fusarium fujikuroi* Nirenberg in *Trichoderma asperellum* Samuels, Lieckf. & Nirenberg, ki so v različnih raziskavah izkazovale insekticidno delovanje (Akello in Sikora, 2012; Batta, 2013; Muvea in sod., 2014).

2 ENTOMOPATOGENE ENDOFITNE GLIVE KOT BIOTIČNI AGENSI ZA VARSTVO PRED RASTLINSKIMI ŠKODLJIVCI

EPGE po napadu rastlinskih škodljivcev na različne načine vplivajo na njihovo prehranjevanje in fiziološko stanje. Med pogostejšimi negativnimi posledicami prehranjevanja z rastlinami, ki so okužene z EPGE se kažejo v upočasnitvi rasti in razvoja, motnjah hranjenja in zmanjšani sposobnosti razmnoževanja in manjšem številu odloženih jajčec (Vidal in Jaber, 2015). Insekticidne učinke EPGE na škodljive žuželke in pršice so dokazali ob prisotnosti v različnih gostiteljskih vrstah (Preglednica 1). Opravljene so bile številne raziskave, ki pa nimajo enotnih zaključkov o vzrokih za negativne učinke na škodljivce. Hife gliv preraščajo rastlinska tkiva, vendar ni konkretnejših dokazov, da bi znotraj tkiv prihajalo tudi do sporulacije, zato ob prehranjevanju rastlinskih škodljivcev s koloniziranim tkivom, ki ga preraščajo hife EPGE ne pride do neposredne okužbe škodljivca in nastanka mikoz (Vega, 2008). EPG v vlogi endofitov na škodljivce posredno vplivajo preko drugih mehanizmov kot so antibioza, antiksenoza ali krepitev inducirane odpornosti rastlin. S povečano tvorbo signalnih molekul, zlasti jasmonske kisline, se sproži sinteza inhibitorjev in drugih snovi, na primer polifenol oksidaze, ki na škodljivce delujejo toksično. EPGE po napadu škodljivcev lahko vplivajo na hitrejšo aktivacijo rastlinskih obrambnih mehanizmov in močnejši obrambni odziv rastlin (Ownley in sod., 2010; Dara, 2019). V številnih raziskavah omenjajo tudi kopičenje mikotoksinov ali drugih sekundarnih metabolitov, ki jih EPGE izločajo v rastlinskem tkivu in povzročajo različne posledice na škodljivcih (Carroll, 1988; Vega, 2008; Gurulingappa in sod., 2011). Eden od obrambnih mehanizmov, ki ga lahko sprožajo EPGE je tudi sprememba v sestavi ali zmanjšanje tvorbe hlapnih komponent – kairomonov, ki so za škodljivce predstavljajo pomemben orientir pri iskanju gostitelja (Vega, 2018).

2.1 UPORABA ENTOMOPATOGENIH ENDOFITNIH GLIV V BIOTIČNEM VARSTVU RASTLIN PRED ŠKODLJIVCI

Sposobnost kolonizacije rastlin je bila dokazana za večino vrst EPG, zaradi česar je njihov potencial za uporabo v biotičnem varstvu še posebej izrazit (Vega, 2018). Življenje znotraj rastlinskega tkiva omogoča manjšo odvisnost od okoljskih razmer in daljše obdobje varstva pred škodljivci. Uspešnost okužbe in kolonizacije rastlinskega tkiva je odvisna od biotskih in abiotskih dejavnikov, kot sta vlaga in temperatura (Vega, 2008). Na okužbo vplivajo tudi interakcije z drugimi mikroorganizmi, lastnosti rastnega medija-substrata, vrsta in starost gostiteljskih rastlin ter velikost glivnega inokluluma (vcepka). Za namene načrtnega vnosa EPGE v rastline se uporabljajo različne metode inokulacije rastlin kot so foliarni nanos, zalivanje s suspenzijo EPGE, namakanje koreninskega sistema pred sajenjem, pomakanje semen v suspenzijo glive ali njeno neposredno injiciranje v rastlinsko tkivo (Bamisile in sod., 2018; Vega, 2018). Foliarni nanos suspenzije konidijev je enostavna in največkrat uporabljena metoda aplikacije v raziskavah in pri uporabi komercialnih bioinsekticidov. Pri takem načinu vnosa EPGE večinoma pride do lokalne kolonizacije tkiva, na katerega je bila suspenzija nanešena in se ne izrazi v sistemični okužbi rastline. Pri tem načinu je vstop EPGE skozi listno povrhnjico lahko omejen zaradi manjše zastopanosti naravnih odprtin, kot so na primer listne reže in drugih morfoloških lastnosti listov, ki onemogočajo prodor v rastlinsko tkivo (Tefera in Vidal, 2009). Inokulacija rastlin v začetnih fazah razvoja, bodisi z uporabo semen, okuženih z EPGE ali njenim nanosom na površje semen (seed coating), omogoča manjšo izpostavljenost neugodnim vremenskim razmeram in vzpostavitev ustreznih okoljskih razmer za okužbo v tleh (Vidal in Jaber, 2015). Pred sajenjem je inokulacijo sadik z EPGE možno izvesti tudi s pomakanjem sadik v glivno suspenzijo. Inokulacija rastlin ob začetku rasti, ima poleg potencialno dolgotrajnejšega varstva rastlin v občutljivejših fazah razvoja, tudi pozitiven vpliv na rast in hitrejši mladostni razvoj rastlin (Bamisile in sod., 2018). Okužba z EPGE pred sajenjem ali med rastjo z zalivanjem omogoča varstvo semena in podzemnih delov rastline pred talnimi škodljivci, proti katerim je s foliarnim nanosom EPGE skorajda nemogoče učinkovito ukrepati. Po drugi strani so EPGE, ki jih vnašamo v tla z zalivanjem ali neposredno inokulacijo podzemnih delov rastlin, podvržene tekmovanju z drugimi mikroorganizmi v rizosferi in izpostavljene potencialnim antagonistom. Prav tako ni nujno, da v primeru uspešne kolonizacije korenin pride kasneje tudi do sistemične okužbe nadzemnega dela rastline (Parsa in sod., 2013).

3 ENTOMOPATOGENI ENDOFITI KOT BIOTIČNI AGENSI ZA VARSTVO PRED RASTLINSKIMI PATOGENI

Za nekatere EPGE je bilo ugotovljeno, da poleg vpliva na škodljive žuželke in pršice, rastline varujejo tudi pred patogenimi organizmi. V ospredju nedavno opravljenih raziskav je bilo zlasti preučevanje vplivov vrst *B. bassiana* in *Lecanicillium* spp. za katere so v različnih raziskavah dokazali antagonistično delovanje predvsem proti različnim povzročiteljem glivičnih bolezni (Ownley

Preglednica 1: Entomopatogene endofitne glive, za katere je bila v preteklih raziskavah na podlagi različnih načinov inokulacije ugotovljena sposobnost kolonizacije različnih vrst gostiteljskih rastlin in učinkovitost proti njihovim škodljivcem

Entomopatogena gliva	Gostiteljska rastlina	Škodljivec	Način inokulacije	
Sarocladium strictum (W. Gams) Summerb.	paradižnik (*Solanum lycopersicum* L.)	južna plodovrtka (*Helicoverpa armigera* [Hübner, 1808])	Z	(Jallow in sod., 2008)
Aspergillus flavus Link	cvetača (*Brassica oleracea* var. *botrytis*)	*Spodoptera litura* Fabricius, 1775	F	(Kaur in sod., 2015)
Aspergillus brasiliensis Varga, Frisvad & Samson	cvetača (*Brassica oleracea* L. var. *botrytis*)	*Spodoptera litura*	F	(Kaur in sod., 2015)
Beauveria bassiana	bob (*Vicia faba* L.)	grahova uš (*Acyrthosiphon pisum* Harris, 1776)	ST	(Akello in Sikora, 2012)
		črna fižolova uš (*Aphis fabae* Scopoli, 1763)	ST	(Akello in Sikora, 2012)
		južna plodovrtka (*Helicoverpa armigera*)	F	(Vidal in Jaber, 2015)
	jagodnjak (*Fragaria x ananassa* Duchesne)	siva breskova uš (*Myzus persicae* [Sulzer, 1776])	Z , RI	(Dara in Dara, 2013)
	pšenica (*Triticum aestivum* L.)	strune (*Limonius californicus* [Mannerheim, 1843], *Hyponoidus bicolor* [Eschscholtz])	Z, SC	(Reddy, Zhao, in sod., 2014)
	bombaž (*Gossypium hirsutum* L.)	*Helicoverpa zea* Boddie, 1850	ST, F	(Lopez in Sword, 2015)
	paradižnik (*Solanum lycopersicum*)	južna plodovrtka (*Helicoverpa armigera*)	F, I, RI	(Qayyum in sod., 2015)
		paradižnikov molj (*Tuta absoluta* [Meyrick, 1917])	F	(Klieber in Reineke, 2016)
		tobakov ščitkar (*Bemisia tabaci* [Gennadius, 1889])	F, I	(El-Deeb in sod., 2012)
	paprika (*Capsicum annum* L.)	siva breskova uš (*Myzus persicae*)	Z	(Jaber in Araj, 2018)
	cvetača (*Brassica oleracea*var. *botrytis*)	kapusov molj (*Plutella xylostella* [Linnaeus, 1758])	F	(Gautam in sod., 2016)
		kapusova muha (*Delia radicum* [Linnaeus, 1758])	Z	(Razinger in sod., 2014)
	koruza (*Zea mays* L.)	koruzna vešča (*Ostrinia nubilalis*)	F, I, RI	(Lewis in Bing, 1991)
		ameriška koruzna sovka (*Spodoptera frugiperda* Smith & Abbot, 1797)	F, SC	(Ramirez-Rodriguez in Sánchez-Peña, 2016)
	melona (*Cucumis melo* L.)	tobakov ščitkar (*Bemisia tabaci*)	F	(Garrido-Jurado in sod., 2017)
	vinska trta (*Vitis vinifera* L.)	smokvin volnati kapar (*Planococcus ficus* [Signoret, 1875])	F	(Rondot in Reineke, 2018)
		zeleni škržatek (*Empoasca vitis* [Göthe, 1875])	F	(Rondot in Reineke, 2018)
	oljna ogrščica (*Brassica napus* L. var. *napus*)	južna plodovrtka (*Helicoverpa armigera*)	F	(Vidal in Jaber, 2015)
	pravi datljevec (*Phoenix dactylifera* L.)	palmov rilčkar (*Rhynchophorus ferrugineus* [A.G.Olivier, 1791])	I	(Gómez-Vidal in sod., 2006)
Beuveria brongiartii	fižol (*Phaseulus vulgaris* L.)	/	F	(Jaber in Enkerli, 2017)

Nadaljevanje				
Clonostachys rosea	čebula (*Allium cepa* L.)	tobakov resar (*Thrips tabaci* [Lindeman, 1889])	ST, RI	(Muvea in sod., 2014)
Fusarium oxysporum	fižol (*Phaseulus vulgaris*)	*Liriomyza huidobrensis* (Blanchard, 1926)	ST	(Akutse in sod., 2013)
Fusarium fujikuroi	bob (*Vicia faba*)	*Liriomyza huidobrensis*	ST	(Akutse in sod., 2013)
Trichoderma lixii	bob (*Vicia faba*)	*Liriomyza huidobrensis*	ST	(Akutse in sod., 2013)
Cordyceps fumosorosea	paprika (*Capsicum annum*)	siva breskova uš (*Myzus persicae*)	Z	(Mantzoukas in Lagogiannis, 2019)
Akanthomyces lecanii	navadna buča (*Curcurbita maxima* L.)	bombaževčeva uš (*Aphis gossypii* [Glover, 1877])	F	(Gurulingappa in sod., 2011)
Lecanicillium longisporum (Petch) Zare & W. Gams	kumara (*Cucumis sativus* L.)	bombaževčeva uš (*Aphis gossypii*)	F	(Kim in sod., 2008)
Metarhizium anisopliae	fižol (*Phaseulus vulgaris*)	grahova uš (*Acyrthosiphon pisum*)	ST	(Akello in Sikora, 2012)
		črna fižolova uš (*Aphis fabae*)	ST	(Akello in Sikora, 2012)
		Ophiomyia phaseoli (Tryon, 1895)	ST	(Mutune in sod., 2016)
	cvetača (*Brassica oleracea* var. *botrytis*)	kapusova muha (*Delia radicum)*	Z	(Razinger in sod., 2014)
	oljna ogrščica (*Brassica napus* L. var. *napus*)	kapusov molj (*Plutella xylostella*)	F	(Batta, 2013)
Metarhizium brunneum Petch	koruza (*Zea mays*)	strune (*Agriotes obscurus*)	ST	(Kabaluk in Ericsson, 2007)
	paprika (*Capsicum annum*)	siva breskova uš (*Myzus persicae*)	Z	(Jaber in Araj, 2018)
	cvetača (*Brassica oleracea* var. *botrytis*)	kapusova muha (*Delia radicum)*	Z	(Razinger in sod., 2014)
	melona (*Cucumis melo* L.)	tobakov ščitkar (*Bemisia tabaci*)	F	(Garrido-Jurado in sod., 2017)
	pšenica (Triticum aestivum)	strune (*Limonius californicus, Hyponoidus bicolor*)	Z, SC	(Reddy, Tangtrakulwanich, in sod., 2014)
Metarhizium robertsii J.F. Bisch., S.A. Rehner & Humber	pšenica (Triticum *aestivum*)	strune (*Limonius californicus, Hyponoidus bicolor*)	Z, SC	(Reddy, Tangtrakulwanich, in sod., 2014)
	sirek (*Sorghum bicolor* L.)	*Sesamia nonagrioides* Lefebvre, 1827	F	(Mantzoukas in sod., 2015)
	koruza (*Zea mays*)	/	SC	
Purpureocillium lilacinum (Thom Luangsa-ard, Houbraken, Hywel -Jones & Samson	bombaž (*Gossypium hirsutum*)	*Helicoverpa zea*	ST	(Lopez in Sword, 2015)
Trichoderma harzianum Rifai	čebula (*Allium cepa*)	tobakov resar (*Thrips tabaci*)	ST, RI	(Muvea in sod., 2014)

F = foliarni nanos, Z = zalivanje, SC = oplaščenje semena, ST = namakanje semena, I = injiciranje, RI = pomakanje korenin, / = ni bilo posebej preučeno za zatiranje ciljnega organizma

in sod., 2010; Jaber in Ownley, 2018). Kako EPGE vplivajo na zmanjšanje okužb in poškodb zaradi glivičnih in drugih povzročiteljev bolezni (še) ni povsem znano, najverjetneje pa gre za sočasen odziv na več ravneh. Načini delovanja antagonističnih gliv temeljijo na več mehanizmih, ki posamično ali sinergistično delujejo na povzročitelje rastlinskih bolezni preko tekmovanja (kompeticije) za življenjski prostor in hrano, mikoparazitizma, antibioze in vzpodbujanja inducirane sistemične odpornosti rastlin (Ownley in sod., 2010).

Za EPGE *Trichoderma* spp. in *Lecanicillium* spp. je značilno, da nekatere patogene glive ob neposrednem stiku parazitirajo (mikoparazitizem) ter jih na ta način slabijo in preprečujejo njihovo rast. Na zmanjšanje okužb z glivnimi patogeni vpliva že sama uspešnost kolonizacije rastlinskih tkiv z EPGE, ki s patogenimi glivami tekmujejo za prostor in hranila (kompeticija). Torej, če je EPGE predčasno prisoten v rastlini, je za patogeno glivo znotraj rastlinskega tkiva na voljo manj prostora in hranil, zato je možnost okužbe in širjenja manjša. Poleg tega predhodna kolonizacija v rastlinah sproži sintezo lignina in drugih komponent v celičnih stenah, ki krepijo mehansko odpornost in onemogočajo penetracijo glivnih hif (Jaber in Ownley, 2018). Antibioza temelji na tvorbi toksičnih hlapnih in nehlapnih organskih molekul in tvorbi encimov, ki so vključeni v razgradnjo celičnih struktur drugih mikroorganizmov. Poleg tega so EPGE tudi bogat vir sekundarnih metabolitov, ki delujejo protimikrobno in citotoksično proti povzročiteljem bolezni. *B. bassiana* na primer tvori beauvericin, ki ima široko učinkovanje proti številnim mikroorganizmov. Tvorijo ga tudi nekatere druge EPG (Ownley in sod., 2010). Sistemična inducirana odpornost označuje obrambni odziv rastlin na stresne razmere zaradi biotskih ali abiotskih dejavnikov, ki ga spodbudijo nepatogeni organizmi, tudi EPGE. Dokazano je, da predčasna kolonizacija rastline z EPGE poleg vpliva na zmanjšanje posledic napada škodljivcev, lahko vpliva tudi na zmanjšanje okužb in bolezenskih znamenj s strani povzročiteljev bolezni (Dara, 2019). Kolonizacija rastlin z EPGE namreč v rastlinah lahko poveča vsebnost salicilne kisline in s tem vpliva na izražanje genov za sintezo protimikrobnih encimov kot so hitinaze in glukozidaze (Jaber in Ownley, 2018). Na ta način so obrambni mehanizmi rastline predčasno v stanju pripravljenosti, kar omogoča rastlinam hitrejši in odločnejši odziv proti povzročiteljem bolezni.

Antagonistične učinke *Beauveria* spp. so ugotovili ob prisotnosti v različnih rastlinah proti različnim povzročiteljem glivičnih bolezni kot so *Botrytis cinerea* Pers., *Fusarium oxysporum*, *Gaeumannomyces graminis* (Sacc.) Arx & D.L. Olivier, *Phytium* sp., *Rhizoctonia solani* J.G. Kühn in *Septoria* sp. (Renwick in sod., 1991; Bark in sod., 1996; Sang Myeong in sod., 1999). Pri paradižniku in bombažu so ob tretiranju semen z *B. bassiana* preprečili okužbe kalečih rastlin z talnima patogenima glivama *Pythium myriotylum* Drechsler in *Rhizoctonia solani.* Uporaba istega seva *B. bassiana* je pri bombažu vplivala tudi na zmanjšanje virulentnosti bakterije *Xanthomonas axonopodis* pv. *malvacearum* (Smith 1901) Vauterin et al., 1995 (Ownley in sod., 2008). Podobno je *B. bassiana* omogočila varstvo čebulic čebule pred okužbami s talno glivo *Fussarium oxysporum*, povzročiteljico fuzarijske gnilobe (Flori in Roberti, 1993). Foliarni nanos suspenzije konidijev komercialnega seva ATCC 74040 glive *B. bassiana* je imel značilen vpliv na zmanjšanje pojavnosti simptomov okužb z virusom rumenega mozaika na bučkah (ZYMV) in peronospore na vinski trti (*Plasmopara viticola* (Berk. & M.A. Curtis) Berl. & De Toni) (Jaber in Salem, 2014; Jaber, 2015). V eni izmed študij so preučevali tudi vpliv glive *Lecanicillium leccanii* na zmanjšanje okužb z mokro gnilobo *Globisporangium ultimum* (Trow) Uzuhashi, Tojo & Kakish. in pepelovko *Podosphaera fuliginea* (Schltdl.) U. Braun & S. Takam. na bučah (Benhamou in Brodeur, 2001).

4 ENTOPOMATOGENI ENDOFITI KOT SPODBUJEVALCI RASTI

Vse večje število raziskav potrjuje, da lahko EPGE pomembno vplivajo na hitrejšo rast in razvoj rastlin. Okužba z EPGE namreč lahko stimulira rast koreninskega sistema, s čimer se izboljša privzem hranil in vode v rastline. Kolonizacija glavnatega zelja z *B. bassiana* je v lončnem poskusu vplivala na boljšo rast rastlin, ki se je kazala v večji biomasi rastlin in večjim pridelkom, zaradi boljšega privzema hranil (Dara in sod., 2017). Inokulacija krompirja z glivo *M. brunneum* je povzročila povečanje vsebnosti dušika in fosforja v rastlinah, kar se je odrazilo v povečanju biomase, površine listov in pridelka (Krell in sod., 2018). Aplikacija gliv *B. bassiana*, *M. brunneum* in *C. fumosorosea* z zalivanjem je vplivala na boljšo rast zelenih delov rastline ter značilno povečanje pridelka jagod (Dara, 2016). Z izboljšanjem mineralne prehrane EPGE vplivajo na boljšo vitalnost rastlin ter s tem pripomorejo k blaženju ali preprečevanju posledic abiotskih in biotskih stresnih dejavnikov, med drugim tudi povzročiteljev bolezni. Tako je kolonizacija buč z glivo *B. bassiana* zaradi vpliva na izboljšanje rasti zmanjšala posledice okužb z virusom rumenega mozaika bučke (ZYMV) (Jaber in Salem, 2014). Podobno je na zmanjšanje simptomov koreninske bolezni, ki jo povzroča gliva *Fusarium phaseoli* (Burkh.) T. Aoki & O'Donnell, vplivala tudi inokulacija fižola z glivo *Metarhizium robertsii* (Sasan in Bidochka, 2013). Posledica kolonizacije rastlin z EPGE je povečanje tvorbe beljakovin vključenih v fotosintetske reakcije

in presnovo (Raad in sod., 2019). Okrepljena rast rastlin je lahko tudi posledica povečanja tvorbe fitohormonov v rastlinah in sideroforov (molekul bogatih z Fe^{3+} ioni), ki jih tvorijo EPGE in tako vplivajo na boljšo oskrbo rastlin z železom (Rana in sod., 2019). Zanimivi so izsledki raziskav, ki kažejo, da *B. bassiana* in nekatere vrste *Metarhizium* spp. lahko oskrbujejo rastline z dušikom iz kadavrov parazitiranih žuželk in pršic (Behie in sod., 2012; Behie in Bidochka, 2014). Po okužbi in poginu gostiteljskih žuželk EPGE vzpostavijo endofitno interakcijo, na podlagi katere pride do privzema dušika v rastline. Na ta način lahko EPGE izdatno vplivajo na oskrbo rastlin s tem hranilom in kroženje dušika v ekosistemu. Domnevajo, da gre pri tej interakciji med EPGE in rastlinami za mutualističen odnos pri katerem rastline v zameno za dušik EPGE oskrbujejo z ogljikom. Vpliv EPGE na povečanje rasti je odvisen tudi od načina inokulacije. Vpliv *B. bassiana, B. brongniartii* in *M. brunneum* na boljšo rast rastlin je bil ugotovljen le pri inokulaciji semen, medtem ko pri foliarni aplikaciji EPGE ta odziv ni bil dosežen (Jaber in Enkerli, 2017).

5 ZAKLJUČEK

Uporaba EPG za zatiranje škodljivcev je bila preučena v številnih raziskavah. Med najpogosteje uporabljenimi EPG v biotičnem varstvu rastlin so vrste *Beauveria bassiana*, *Metarhizium* spp. in nekatere druge, ki so v višjih rastlinah prisotne tudi kot endofiti. Endofitizem EPG omogoča kolonizacijo rastlinskih tkiv in s tem sistemično varstvo rastlin pred škodljivci skozi daljše časovno obdobje. Poleg tega, da vplivajo na zmanjšanje poškodb zaradi napada škodljivcev, lahko EPGE v gostiteljskih rastlinah omogočijo tudi boljšo oskrbo le teh s hranili in s tem njihovo boljšo rast, lažjo premostitev stresnih razmer zaradi abiotskih dejavnikov ter zmanjšanje okužb s patogenimi organizmi. Med drugim se okužba z EPGE v rastlinah lahko odraža tudi v povečani tvorbi sekundarnih metabolitov, ki delujejo toksično na druge organizme, tudi na ljudi. Pri preučevanju načinov inokulacije in posrednih vplivov na gostiteljske rastline ostaja še veliko neznank, prav tako je za širšo uporabo EPGE ključno razumevanje abiotskih in biotskih dejavnikov, ki vplivajo na uspešnost kolonizacije rastlinskega tkiva. Pomemben kriterij pri razvoju in uporabi komercialnih biopesticidov na podlagi EPGE je nepredvidljiva učinkovitost in nekonsistentnost, ki je posledica vpliva številnih dejavnikov na vzpostavitev interakcije z gostiteljem. Do sedaj je bila večina raziskav v zvezi s preučevanjem EPGE kot biotičnih agensov opravljena v laboratorijskih razmerah, zato je za razvoj celovite strategije zatiranja škodljivcev potrebno več pozornosti nameniti tudi preučevanju ustreznih okoljskih razmer in ustreznih metod inokulacije, ki omogočajo razvoj endofitizma in dolgotrajnejše pozitivne učinke na nivoju celotne rastline.

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Development of efficient integrated management package against sweet potato weevil (*Cylas formicarius* [Fabricius, 1798])

Mohammad Monirul Hasan TIPU [1, 2], Raunak JAHAN [3], Jubaidur RAHMAN [4], Md Mizanur RAHMAN [5], Md Ariful ISLAM [6], Md Mosiur Rahman Bhuyin APU [7]

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Development of efficient integrated management package against sweet potato weevil (*Cylas formicarius* [Fabricius, 1798])

Abstract: The sweet potato weevil (*Cylas formicarius*, 1798) is one of the most damaging sweet potato pests. To prevent an economic crop loss, it is very important to develop a suitable and efficient integrated pest management strategy. A field experiment was set up with three replications at Jamalpur to select the best integrated management package from eight different treatments against sweet potato weevil. The results showed that the lowest percentage of infestation by number (2.94 %) and mass (3.22 %) was found when the crop was planted on November 01; earthing-up for two times, Carbofuran 5G was sprayed @ 15 kg ha^{-1} at 60 days after planting with irrigation and tuber was harvested at 130 days after planting. The marketable yield (23.75 kg) and percent increase of yield than control (50.86 %) performed the highest in the same combination. These findings suggested an effective integration of different management strategies to reduce sweet potato weevil infestation in Bangladesh successfully.

Key words: sweet potato; sweet potato weevil; integrated management; marketable tuber yield

Razvoj učinkovitega integriranega načina zatiranja hrošča *Cylas formicarius* (Fabricius, 1798) na sladkem krompirju

Izvleček: Hrošč *Cylas formicarius* (Fabricius, 1798) je najpomembnejši škodljivec sladkega krompirja. Za preprečitev izpada pridelka je potrebno razviti ustrezen in učinkovit način integriranega zatiranja škodljivca. V ta namen je bil v Jamalpurju izveden poljski poskus s tremi ponovitvami za izbor najustreznejšega načina integriranega zatiranja škodljivca med osmimi obravnavanji. Rezultati so pokazali, da je bil najmanjši odstotek napada, tako v številčnosti škodljivca (2,94 %) kot v masi pridelka (3,22 %) ugotovljen v obravnavanju, ko je bil sladki krompir posajen prvega novembra in dvakrat osipan, poškropljen s karbofuranom 5G 15 kg ha^{-1} 60 dni po saditvi, z namakanjem in spravilom gomoljev 130 dni po saditvi. Tržni pridelek (23,75 kg) in odstotek povečanja pridelka v primerjavi s kontrolo (50,86 %) sta bila največja v istem obravnavanju. Te ugotovitve nakazujejo učinkovito vključevanje različnih načinov zatiranja za učinkovito zmanjšanje napada sladkega krompirja od hrošča *Cylas formicarius* v Bangladešu.

Ključne besede: sladki krompir; škodljivec sladkega krompirja; integrirano varstvo; tržni pridelek gomoljev

1 Plant Pathology Division, Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur, Bangladesh
2 Corresponding author, email: tipubari2013@yahoo.com
3 Department of Aquaculture, Bangladesh Agricultural University (BAU), Mymensingh, Bangladesh
4 Agronomy Division, Regional Agricultural Research Station, Bangladesh Agricultural Research Institute (BARI), Jamalpur, Bangladesh
5 Tuber Crops Research Centre, Regional Agricultural Research Station, Bangladesh Agricultural Research Institute (BARI), Jamalpur, Bangladesh
6 Oilseed Research Centre, Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur, Bangladesh
7 Horticulture Research Centre, Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur, Bangladesh

1 INTRODUCTION

The sweet potato (*Ipomoea batatas* (L.) Lam.) has an important role in transforming the nutrition and food security for developing countries significantly in recent years (Korada et al., 2010). The scientific information developed in the sweet potato research has enabled the growers to boost productivity and quality. There are some fundamental needs facing farmers in all major sweet potato producing countries, but there are other significant needs specific to certain regions. The sweet potato weevil (SPW) (*Cylas formicarius* [Fabricius, 1798]) has become widely dispersed, mainly in tropical and subtropical areas of the world (Hue and Low, 2015), and it recently has been found in higher latitude areas as well. It is the most severe pest of sweet potato in Bangladesh. It causes damage both in the field and in storage. The larvae mine the sweet potato tuber and damage the inside tissue. The tuber becomes spongy in appearance, riddled with cavities, and dark in color (Uritaini et al., 1975; Kyereko et at., 2019). The sweet potato larvae make a tunnel inside the root tissue, which is the primary cause of inviting several soil-borne pathogens. Once these pathogens enter the tuber, they become responsible for causing further damage like secondary infection by different pathogenic bacteria and fungi (Onwueme and Charles, 1994). Besides, the sweet potato weevil larvae have an ability to cause damage to the vascular system of the plant. As a result, the number and size of tuber roots those are stored for the future become drastically reduced (Hue and Low, 2015). Sweet potato weevil is causing about 50 to 100 % yield loss in the field (Sorensen, 2009).

It is challenging to deal with sweet potato weevils when they are already in the crop. Cultural practices have proven to be effective control against the sweet potato weevil, but insecticide applications remain the primary basis of control (Muruvanda et al., 1986; Sutherland, 1986). Management of this pest through the shifting of the planting dates could be one of the best ways. The weevil population reaches a peak at the beginning of the dry season because of the high temperature and rainfall (Ladanyi and Hufnagel, 2006; Gomi et al., 2007). So, if it may be possible to harvest two weeks earlier, it may reduce the yield loss. Another way of reducing the sweet potato weevil infestation is to hail-up of soil by re-ridging around the plant-base to fill soil cracks (Beyene, 2015). Pheromone traps are usually used as monitoring, training, and management tools. Many effective traps have been designed by farmers using locally available materials. Different traps are so delicate that they fail to catch weevils make misleading information that the pest is not present (Beyene, 2015). Many insecticides control sweet potato weevil as a foliar spray or basal granular applications. The only chemical method cannot solve the weevil infestation, but good husbandry can control them by preventing spreading. So, management of this pest by using a suitable integrated management strategy is important to save the environment. Yet, no effective integrated management practice against sweet potato weevil has so far been developed or recommended. Therefore, we designed the present study to select the best integrated management package against sweet potato weevil for higher yield.

2 MATERIALS AND METHODS

The study was conducted during the winter season of 2015 at Regional Agricultural Research Station, BARI, Jamalpur, as it was reported to be the hot spot area. Jamalpur is located between 24°55′10″ North and 89°56′53″ East, and the soil is neutral in pH and silty loam in texture. The experiment comprising eight treatments were replicated thrice following RCBD. Eight treatments namely, T_1 (Earthing-up one time + Planting 01 Nov. + Pheromone trap + harvest 130 DAP), T_2 (Earthing-up one time + Planting 15 Nov. + Pheromone trap + harvest 120 DAP), T_3 (Earthing-up two times + Planting 01 Nov. + Carbofuran 5G @ 15kg ha^{-1} at 60 days after sowing with irrigation + harvest 130 DAP), T_4 (Earthing-up two times + Planting 15 Nov. + Carbofuran 5G @ 15kg ha^{-1} at 60 days after sowing with irrigation + harvest 120 DAP), T_5 (Earthing-up three times + Planting 01 Nov. + harvest 130 DAP), T_6 (Earthing-up three times + Planting 15 Nov. + harvest 120 DAP), T_7 (Farmer's practice) and T_8 (Control) were evaluated. BARI SP-8 sweet potato variety was used for this experiment. The spacing between plants was 30 cm and rows 60 cm. The plot size for each treatment was 3 m x 3 m. All plantings were from vine cuttings, and standard horticultural procedures were followed. The roots in each plot were counted and weighed, and evaluated for severity of weevil damage. A sampling of adult weevils using a sweeping-net was carried out six times at 30 days intervals starting from 30 days after planting. The stem and roots were taken as samples from different plants of respective plots and then they were dissected to count the number of adult weevils, pupae, and larvae. The data on the extent of damage on root tubers and stem (vines) was recorded according to the rating scale described by Rangi et al. (1994). The data on the infestation percentage on the stem (vine) and tuber were calculated. Data were statistically analyzed in the MStat program, and means were separated by DMRT.

3 RESULTS AND DISCUSSION

The different integrated treatments were tested for evaluating the efficacy in controlling sweet potato weevil. The effects of all treatment combination on root infestation by sweet potato weevil were presented in Table 1. Significant variation in controlling sweet potato weevil was observed in the combination of various management packages.

In the case of percent infestation of the root by number, the lowest percentage of infestation was found in T_3 (2.94%), which was statistically identical with T_6 (3.56 %) and followed by T_1 (5.53 %), T_2 (4.38 %), T_4 (6.71 %) and T_5 (4.42 %), respectively. Correspondingly, the lowest percentage of infestation of the root by mass was also found in T_3 (3.22 %), which was followed by T_1 (8.20 %), T_2 (7.52 %), T_4 (9.81 %), T_5 (5.78 %), and T_6 (5.32 %), respectively. Among all the treatments, marketable yield per plot was ensured significantly the highest in T_3 (23.75 kg), which was followed by T_2 (18.36 kg), T_4 (20.26 kg), T_5 (20.33 kg), and T_6 (21.51 kg), respectively. The lowest yield per plot was found in T_8 (11.67 kg), which was statistically identical with T_7 (13.17 kg) and followed by T_1 (15.04 kg) and T_2 (18.36 kg), respectively. Among all the treatments, the percent increase of yield over control was found the highest in T_3 (50.86 %) and the lowest in T_7 (11.39 %).

Our present study suggested that early planning on November 01, earthing up for two times, applying Carbofuran 5G @ 15kg ha^{-1} at 60 DAP with irrigation and harvesting after 130 days of planting worked the best to manage sweet potato weevil successfully. Bohinc et al. (2019) found combination of calcium cyanamide (1000 kg ha^{-1}), propolis (5 ml 1^{-1} H_2O) and limestone dust (345 kg ha^{-1}) was effective against different potato pests in summer. Hue and Low (2015) described earthing up as an excellent approach that prevented the entry of weevils into tuber and oviposition by female weevils. Palaniswami and Mohandas (1994) also observed that the weevil infestation was significantly reduced by this method. Timely harvesting also reduces weevil infestation at a significant level. Ebregt et al. (2005) found that harvesting 14 days earlier decrease the yield loss of sweet potato by weevil attack. The findings of the present study were strongly supported by Taye and Tadesse (2013), where they reported that carbofuran could efficiently manage sweet potato weevil infestation when this chemical was used with other pesticides.

Table 1: Effect of different integrated treatments against sweet potato weevil infestation at Jamalpur

Treatments	Infestation by number (%)	Infestation by mass (%)	Marketable yield/plot (kg)	Increase/decrease yield over control (%)
T_1 (Earthing-up (One time) + Planting 01 Nov. + Pheromone trap + harvest 130 DAP)	5.53 bc	8.20 bc	15.04 bc	22.41
	(2.32)	(2.78)		
T_2 (Earthing-up (One time) + Planting 15 Nov. + Pheromone trap + harvest 120 DAP)	4.38 bc	7.52 bc	18.36 abc	36.44
	(2.09)	(2.71)		
T_3 (Earthing-up (Two times) + Planting 01 Nov. + Carbofuran 5G @ 15kg/ha at 60 DAP with irrigation + harvest 130 DAP)	2.94 c	3.22 c	23.75 a	50.86
	(1.65)	(1.75)		
T_4 (Earthing-up (Two times) + Planting 15 Nov. + Carbofuran 5G @ 15kg/ha at 60 DAP with irrigation + harvest 120 DAP)	6.71 bc	9.81 bc	20.26 ab	42.40
	(2.46)	(3.01)		
T_5 (Earthing-up (Three times) + Planting 01 Nov. + harvest 130 DAP)	4.42 bc	5.78 bc	20.33 ab	42.60
	(2.09)	(2.40)		
T_6 (Earthing-up (Three times) + Planting 15 Nov. + harvest 120 DAP)	3.56 c	5.32 bc	21.51 ab	45.75
	(1.80)	(2.20)		
T_7 (Farmer's practice)	9.13 b	12.39 b	13.17 c	11.39
	(2.98)	(3.47)		
T_8 (Control)	15.12 a	19.82 a	11.67 c	
	(3.88)	(4.42)		

In a column, treatment means having a common letter(s) are statistically identical by LSD at 5 % level of significance. Figure in the parenthesis indicates square root transformation

4 CONCLUSION

Understanding the insights of sweet potato weevil and their infestation is crucial so that a precise preventive method could be designed. Integrating several cultural practices and chemicals like early planting, earthing up at the proper time, timely harvesting, and appropriate chemical insecticide can manage sweet potato weevil infestation in the crop field. The combination of various IPM strategies that we explained in the study could be an efficient package to prevent the weevil infestation for achieving the nation's fundamental demand of ensuring food and nutrition security.

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