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Intraspecific variation of major alkaloids in Afghanistan opium poppy (*Papaver somniferum* L.)

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Abstract

Objective: Opium poppy (*Papaver somniferum* L.) is an important source of physiologically active medicinal alkaloids. It is one of the world's best medicinal plants and the only commercial source for narcotic analgesics, morphine, codeine, the vasodilator papaverine, the cough suppressant, and the powerful anticancer drug noscapine. The current study aimed to examine the content of major alkaloids in the capsules of seven populations of poppies, collected from Balkh Province, Afghanistan.

Methods: Three locations (replications) were considered for each population. In each location, 15 plants with the uniform main capsules were randomly selected. A 1.5 to 2 g of capsule powder was obtained from each population/replication. Such amounts collected from three locations were then mixed to assess the major alkaloids between populations, 200 mg of which was used for extraction. Major alkaloids (morphine, codeine, thebaine, noscapine, and papaverine) were measured, using the HPLC method.

Results: The results indicated that the highest amount of noscapine (1398.4 µg/g DW), papaverine (802.6 µg/g DW), and thebaine (1129.2 µg/g DW) were detected in the P1 population, which appeared to be the most valuable medicinal-industrial population. For morphine and codeine, P6 (2947.7 µg/g DW) and P7 (5638.3 µg/g DW), respectively, had the highest amounts of these substances. Pharmacologically, the P4 population having the lowest morphine (narcotic drug) and the highest thebaine (non-narcotic drug) is prescribable for the medication of addicts. Monoploid genome size (2Cx DNA) was positively and significantly related to noscapine, papaverine, and codeine, indicating that a higher genome size population produced more of these alkaloids. The UPGMA phenogram constructed based on 10 variables showed four distinct cluster groups of the studied populations.

Conclusion: The P1 population stood out for its medicinal and industrial value due to its high contents of noscapine, papaverine, and thebaine. Conversely, The P4 population, while containing the lowest level of morphine (a narcotic drug), also had a relatively high amount of thebaine (a non-narcotic precursor to other alkaloids). These dual-compounds metabolites make it a potentially useful population to treat drug-affected patients.

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Introduction

Opium poppy (*Papaver somniferum* L.) is an annual plant (Bagheri *et al.* 2017), related to the Papaveraceae family and Papaveroideae subfamily. Papaveraceae is a large family of about 40 genera and 800 species that grows from tropical to alpine ecosystems (Labanca *et al.* 2018). The opium poppy is an important medicinal plant. It is a traditional source of isoquinoline alkaloids. Its dried latex contains more than 30 individual alkaloids (Kara and Baydar 2021), covering the most important commercial natural source of drugs and ornamental usage in some countries (Singh *et al.* 2014). Mainly, morphine and codeine have essential roles as analgesic drugs, thebaine as a starting material for industrial production of many pharmaceutical drugs, having non-narcotic and non-addictive nature as well as the ease of synthetic conversion to other highly-demanded drugs (Tarkesh Esfahani *et al.* 2021). Likewise, papaverine plays an important role as a coronary vasodilator, and noscapine as a potential anti-cancer and cough suppressant drug (Bagheri *et al.* 2017; Sharopov *et al.* 2018). The large diameter of its capsules is positively related to morphine's high contents (Labanca *et al.* 2018; Kara and Baydar 2018). The European Food Safety Authority introduced an acute reference dose of 10 µg morphine/kg of body weight as a safe rate for morphine in food products (Carlin *et al.* 2020). In the 21st century, the opium poppy has two main legal uses as a source of alkaloid compounds for the pharmaceutical industry and as a source of its seeds for the food industry (Gümüşçü *et al.* 2007).

Miller *et al.* (2005) reported negligible wind pollination and some insect pollination, while self-pollination was reported to be the dominant mode of opium poppy fertilization. Other reports also verified that the opium poppy is a predominantly self-pollinating species (Omidbaigi 2005; Singh *et al.* 2017; Solanki *et al.* 2017; Jesus *et al.* 2021).

The biosynthetic pathway of major alkaloids in *P. somniferum* begins with central intermediate (S)-reticuline (Ozber and Facchini 2022), leading to papaverine with two hypothetical pathways-NH pathway and NH3 pathway (Han *et al.* 2010). On the other hand, the noscapine biosynthetic route comprises the embodiment of benzylisoquinoline moiety, explained by Battersby *et al.* (1967), through the experiment with [14C] Tyr and [14C] norlaudanosoline. Furthermore, the common

pathway of three alkaloids (thebaine, codeine, and morphine) starts from tyrosine which converts to dopamine, and then to norlaudanosoline. Norlaudanosoline condenses with S-adenosyl methionine (SAM) to form reticuline. Reticuline undergoes a complex enzymatic cyclization to form salutaridine. Salutaridine is then modified through a series of steps to yield thebaine, including methylation, oxidation, and the rearrangement of the carbon skeleton. Thebaine can be converted to codeine by the enzyme thebaine 6-O-demethylase, which removes a methyl group from a specific position on the molecule (Unterlinner *et al.* 1999). Finally, codeine is converted to morphine by the enzyme codeine demethylase, which removes another methyl group (Hagel and Facchini 2010; Onoyovwe *et al.* 2013; Vadhel *et al.* 2023). In summary, morphine, thebaine, and codeine together share the most similar pathways, while papaverine and noscapine have somewhat separate routes with (S)-reticuline as the starting point.

There are several techniques available for the analytical study of *P. somniferum* alkaloids such as TLC (Pothier and Galand 2005), HPLC with massive diversity of detection methods (Sankar *et al.* 2019, 2020; Liang *et al.* 2022), LC-MS (Carlin *et al.* 2020), UV, DAD, or fluorescence methods, GC, GC-MS, HPLC coupled with MS (HPLC-MS), CE-MS, 2D LC, and IMS (Filipiak-Szok *et al.* 2018; Tajabadi *et al.* 2022). According to the research, there is no comprehensive study related applying the HILIC method for the separation and isolation of opium alkaloids (Bagheri *et al.* 2017). To select the best column for analytical purposes, bare silica, and zwitterionic stationary phases were recommended (Bagheri *et al.* 2017). In the present study, our goal was to use the HILIC method by the bare silica column to determine the major alkaloids of opium poppy.

Materials and Methods

The five major alkaloids (Figure 1) reference standards of morphine, codeine, thebaine, noscapine, and papaverine were purchased from Temad Co. Darou Pakhsh (Tehran, Iran). Acetonitrile (ACN) of HPLC gradient grade was obtained from Chem-Lab NV (Zedelgem, Belgium). The ultra-pure water was provided by the Millipore Milli-Q system (Billerica, MA, USA) and used throughout the experiment. All stock solutions were kept at 4 °C. Seven populations of *P. somniferum* L. were collected from seven different districts of Balkh Province, Afghanistan (Table 1, Figure 2). The shade drying method was applied to the collected ripped capsules and then the seeds were removed. Three locations (replications) were considered for each population. We reviewed previous similar studies and took into account their methodologies. Peer-reviewed evidence supports the use of single replicates in HPLC experiments for practical reasons like cost, time constraints, or sample availability. In such studies, all replications are mixed and ultimately one replicate is used for each

population (Kumar *et al.* 2016; Ganorkar and Shirkhedkar 2017; Abdollahi *et al.* 2020). Hence, in the current study, in each location, 15 plants with uniform main capsules were randomly selected. A 1.5 to 2 g of capsule powder was obtained from each replication per population and then, mixed (4.5-6 g) to assess the major alkaloids in the populations. A power analysis was performed to determine the number of replications required to achieve a significance level of 0.05 with a power greater than 0.8 (Garland-Campbell 2018). An amount of 200 mg of pooled capsule powder from each population was considered for extraction for injecting into HPLC, using three technical replications per population for statistical analysis. Twenty ml of methanol (MeOH, HPLC grade) solvent (Merk, Darmstadt, Germany) was added to dissolve all chemical and physicochemical compounds. It was placed in power sonic 505 (Seoul, Korea) for 2 h. The sample was then placed in the reflux oil bath at the MeOH boiling point temperature (65 °C) for the next 2 h. Finally, after the filtration of the sample, for evaporation of the solvent, the rotary was used and the extract was kept at 4 °C until the three injections at HPLC. Ammonium acetate buffer (60 mM) was prepared in the purified water and the pH was subsequently adjusted to 3, using glacial acetic acid. The mobile phase was implemented for the simultaneous separation of opium alkaloids on Bare Silica (250 × 4.6 nm dimensions) with 10 µm particle size, 100 pore size (A°), and 300 surface area (m^2/g). The HILIC column was comprised of ammonium acetate buffer (solvent A) and ACN (solvent B) (Acevska *et al.* 2014). The five major alkaloids were separated, using the gradient elution as follows: 0 to 5% of solvent A in 10 min, and 95% of solvent B at a same time. Reaching 15% within 25 min solvent A was equal to 85% solvent B. Finally, solvent A reached 85% in 75 min, and solvent B reached 15% at the same time. The 20 µl injection was performed with a flow rate of 0.7 ml/min (Bagheri *et al.* 2017). Stock solutions were

Table 1. Collected locations of *Papaver somniferum* populations of Afghanistan, Balkh Province.

Population codes	Locality	Latitude (N)	Longitude (E)	Altitude (m)
P1	Chahar Kint	36°20'3343"	67°31'23.38"	1822
P2	Balkh	36°45'54.62"	66°53'19.24"	341
P3	Kishindeh	35°48'11.83"	67°05'38.92"	1862
P4	Chimtal	36°28'27.42"	66°57'3.47"	553
P5	Khulm	36°42'50.09"	66°57'7.92"	365
P6	Sholgara	36°22'12.24"	66°53'29.67"	551
P7	Chahar Bolak	36°48'5.64"	66°57'3.68"	335

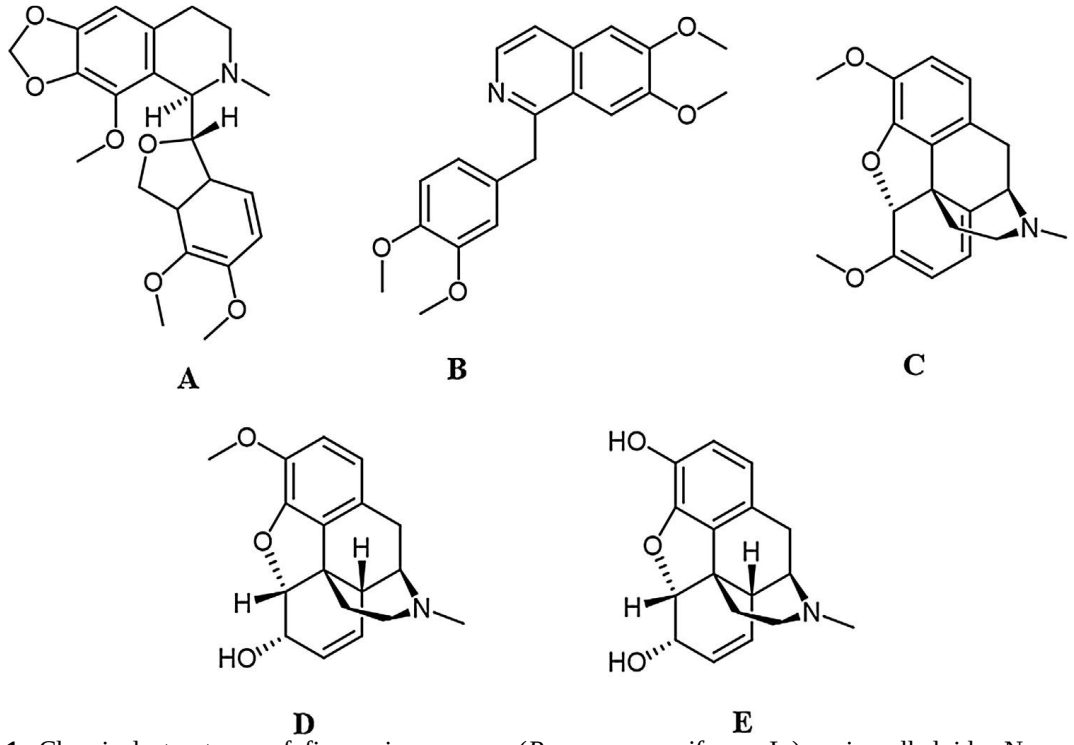


Figure 1. Chemical structures of five opium poppy (*Papaver somniferum* L.) major alkaloids, Noscapine (A), papaverine (B), thebaine (C), codeine (D), and morphine (E).

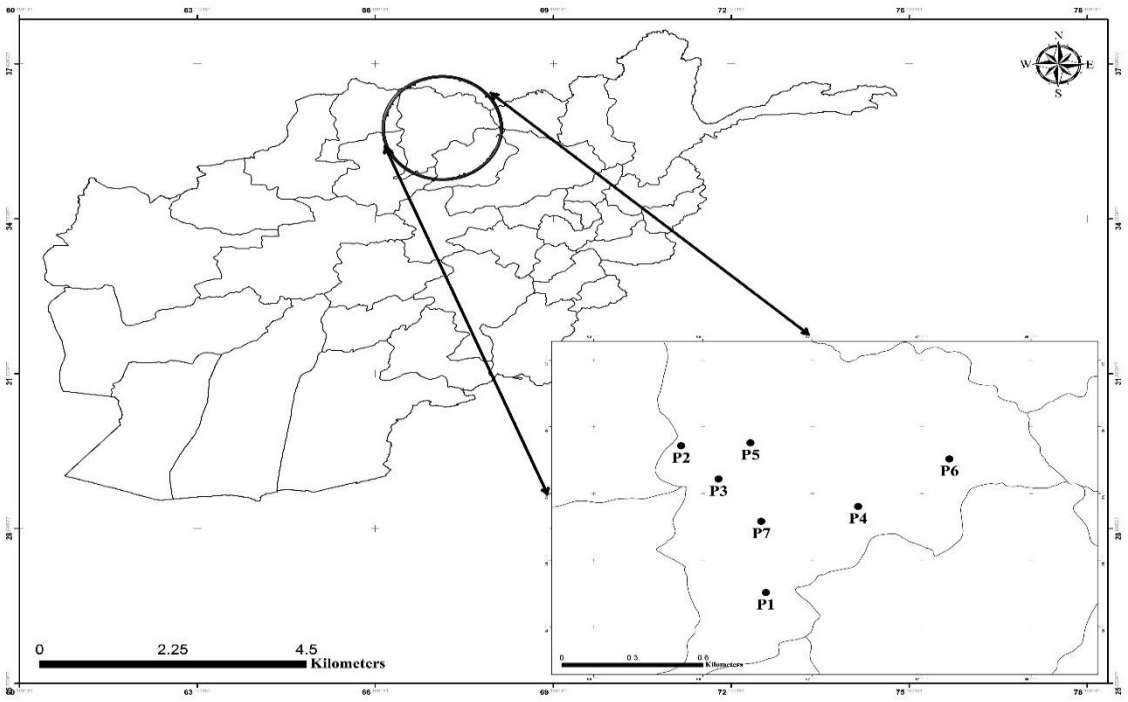


Figure 2. Geographic distribution of collected *Papaver somniferum* populations on the map of Afghanistan, using ArcGIS.

prepared at the concentrations of 5 mg/ml for morphine, codeine, thebaine, and papaverine, and 2.5 mg/ml for noscapine in the acetonitrile/deionized water solvent mixture (95:5, v/v). These stock solutions were stored at 4 °C. Working solutions for the analytical HPLC were prepared from a suitable amount of the preliminary stock solutions and were diluted by the acetonitrile/deionized water solvent mixture. A Knauer HPLC system (Berlin, Germany) with EZChromElite software was used to monitor and acquire the chromatographic data. The column was maintained at room temperature and sample compounds were detected at a wavelength of 280 nm.

The normality test was first carried out on the residuals, using Minitab 17. Log 10 transformation was used for the noscapine data, while NScor transformation was used for the other four alkaloids' data. Analysis of variance (ANOVA) was done based on a completely randomized design with three technical replicates, using the General Linear Models (GLM) in SAS 9.1 software. Mean comparisons were performed, using the least significant difference test. Moreover, a cluster analysis was carried out on cytogenetic characteristics (chromosome length and monoploid genome size; Rasekh and Karimzadeh 2023), geographic variables (longitude, latitude, and altitude), and major alkaloids' amounts (noscapine, papaverine, thebaine, codeine, and morphine), using Minitab 17 software, through the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method and the Euclidean distance (r = 0.985), using NTSYSpc ver. 2.10e to assess similarities and variations among the populations. Also, the Pearson correlation was calculated among the above-mentioned 10 variables. Considering the three different biosynthesis pathways in papaverine, noscapine, and the other three alkaloids (morphine, thebaine, and codeine), correlation coefficients were separately calculated. The amount of alkaloids was also regressed upon the genome size, using a linear regression equation.

Results

Different parts (capsule, stem, leaf, and root) of opium poppy plants were first considered for the morphinane alkaloids' content. Of those, the capsules appeared to be the best for accumulating the highest content of alkaloids. Hence, the capsules' alkaloids were considered in the current study. Sharopov *et al.* (2018) also emphasized a common source of morphinane alkaloids in the capsules of poppy plants. In the present report, ANOVA showed statistically significant ($p < 0.01$) differences among the opium poppy populations from the Balkh Province, Afghanistan (Table 2), indicating an intraspecific variation for all five major alkaloids. Between-population mean comparisons (Table 3) showed remarkable differences among populations for each major alkaloid. The means of major

Table 2. Analysis of variance of five major alkaloids of *Papaver somniferum* populations of Afghanistan, Balkh Province.

SOV	df	Mean squares				
		Noscapine	**Papaverine**	**Thebaine**	**Codeine**	**Morphine**
Populations	6	0.49512**	2.9778**	2.9771**	2.9778**	2.9778**
Error	14	0.00000061	0.0531	0.0509	0.0531	0.0531
CV%	-	0.03	7.68	7.52	7.68	7.68

** Significant difference ($p < 0.01$).

Table 3. Means (± SE) of five major alkaloids in *Papaver somniferum* populations of Afghanistan, Balkh Province.

Populations	Noscapine (µg/g DW)	Papaverine (µg/g DW)	Thebaine (µg/g DW)	Codeine (µg/g DW)	Morphine (µg/g DW)
P1	1398.40^{a} ± 1.06	802.60^{a} ± 4.890	1129.20^{a} ± 12.500	1243.00^{b} ± 0.979	1948.40^{b} ± 16.00
P2	707.24^{b} ± 1.13	49.44^{cd} ± 0.077	251.89^{e} ± 00.020	474.11^{cd} ± 0.034	1294.30^{cd} ± 00.05
P3	275.33^{e} ± 0.01	45.98^{ed} ± 0.117	283.24^{de} ± 02.450	98.23^{f} ± 0.076	293.18^{e} ± 00.44
P4	430.51^{d} ± 0.06	53.29^{bc} ± 0.054	1099.02^{b} ± 02.100	530.30^{bc} ± 0.260	278.15^{f} ± 00.05
P5	145.31^{f} ± 0.04	24.76^{f} ± 0.001	980.95^{bc} ± 00.000	443.70^{de} ± 0.004	697.75^{ed} ± 00.57
P6	98.42^{g} ± 0.20	24.31^{e} ± 0.001	338.48^{cd} ± 00.005	391.96^{e} ± 0.850	2947.70^{a} ± 01.00
P7	656.27^{c} ± 0.16	162.88^{b} ± 0.432	42.12^{f} ± 00.007	5638.3^{a} ± 0.180	1722.30^{bc} ± 04.30
Mean	530.21	166.47	589.31	1259.94	1311.68
Range	98.42-1398.40	24.76-802.60	42.122-1129.20	98.23-5638.3	278.15-2947.7
$LSD_{1\%}$	0.002	0.56	0.548	0.56	0.56

alkaloids for all seven opium poppy populations were as follows (Table 3): noscapine (530.21 µg/g DW), papaverine (166.47 µg/g DW), thebaine (589.31 µg/g DW), codeine (1259.94 µg/g DW), and morphine (1311.68 µg/g DW). The highest amounts of noscapine (1398.4 µg/g DW), papaverine (802.6 µg/g DW), and thebaine (1129.2 µg/g DW) were detected in the P1 population. For morphine and codeine, P6 (2947.7 µg/g DW) and P7 (5638.3 µg/g DW), respectively, appeared to have the highest amounts (For HPLC chromatograms refer to Supplementary Figure 1).

The UPGMA phenogram constructed based on similarities of cytogenetic traits, geographic variables, and major alkaloid amounts (Figure 3) showed four major clusters. P1 population was located in the 1st cluster, four populations (P2, P4-P6) were located in the 2nd cluster, while P3 and P7 were placed in the other two separate clusters.

Noscapine, papaverine, and codeine alkaloids were positively and significantly ($p < 0.01$) related to the monoploid genome size (Tables 4, 5; Figure 4). In other words, these three alkaloids

accumulated more in the capsules of all *P. somniferum* populations as their genome sizes increased. Altitude also positively and significantly ($p < 0.05$) correlated with thebaine (Table 5).

Table 4. Pearson correlation coefficients of noscapine and papaverine alkaloids with cytogenetical and geographical characteristics in *Papaver somniferum* populations of Afghanistan, Balkh Province

Alkaloids	CL (µm)	GS (pg)	Altitude	Latitude	Longitude
Noscapine	-0.139^{ns}	0.924**	0.301^{ns}	0.142^{ns}	0.591^{ns}
Papaverine	-0.009^{ns}	0.934**	0.367^{ns}	-0.005^{ns}	0.631^{ns}

$^{ns, **}$ Non-significant ($p > 0.05$) and significant difference ($p < 0.01$), respectively; CL: Chromosome length; GS: Genome size.

Table 5. Pearson correlation coefficients of thebaine, codeine, and morphine alkaloids with cytogenetical and geographic characteristics in *Papaver somniferum* populations of Afghanistan, Balkh Province

Alkaloids	CL (µm)	GS (pg)	Codeine	Morphine	Altitude	Latitude	Longitude
Thebaine	-0.751**	0.146^{ns}	-0.060^{ns}	-0.065^{ns}	0.443*	-0.300^{ns}	0.634^{ns}
Codeine	0.427^{ns}	0.760**	1	0.126^{ns}	-0.291^{ns}	0.648^{ns}	-0.216^{ns}
Morphine	0.269^{ns}	0.247^{ns}	--	1	0.011^{ns}	0.129^{ns}	0.187^{ns}

$^{ns, *, **}$ Non-significant ($p > 0.05$) and significant differences ($p < 0.05$, $p < 0.01$), respectively.

Discussion

During three years (2005-2007), 300 accessions of (*P. somniferum* L.) were examined for five major alkaloids (morphine, codeine, thebaine, papaverine, and noscapine), analyzed by HPLC in Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany (Dittbrenner *et al.* 2009), in which, the 3-year codeine content in the 40 selected accessions was 1800.6 µg/g DW in 2005, 1360.7 µg/g DW in 2006, and 1432.9 µg/g DW in 2007. In the present study, the average codeine amount of the seven populations of opium poppy of Balkh-Afghanistan was 1259.9 µg/g DW, ranging from 98.23 (P3) to 5638.3 µg/g DW (P7), showing 18% lower compared to that reported by Dittbrenner *et al.* (2009). Interestingly, the highest amount in our study was for the P7 population (5638.3 µg/g DW), showing 3.7-fold higher in codeine than that in the 3-year average of all accessions (1531.4 µg/g DW) studied by Dittbrenner *et al.* (2009). The same extent was not true of the other four (noscapine, papaverine, thebaine, and morphine) alkaloids in our study.

NCS and *SAT* were reported as two important genes with significant contributions in regulating the thebaine content of diploid and induced tetraploid *Papaver bracteatum* Lindl. (Tarkesh Esfahani *et al.* 2021). Hence, the *NCS* and *SAT* genes were enhanced remarkably in the leaves of the tetraploids (6.6-fold and 13.5-fold, respectively) at the transcription level compared to those of diploids that had a thebaine content of 6.9 µg/g DW (Tarkesh Esfahani *et al.* 2021). Compared to the thebaine content of the Iranian diploid *P. bracteatum* Lindl. (6.9 µg/g DW), the diploid Afghan *P. somniferum*

populations evaluated in the present study were 85-fold higher (589.31 µg/g DW) ranging from 42.12 µg/g DW (P7) to 1129.20 µg/g DW (P1), proving a great superiority of Afghan opium poppy.

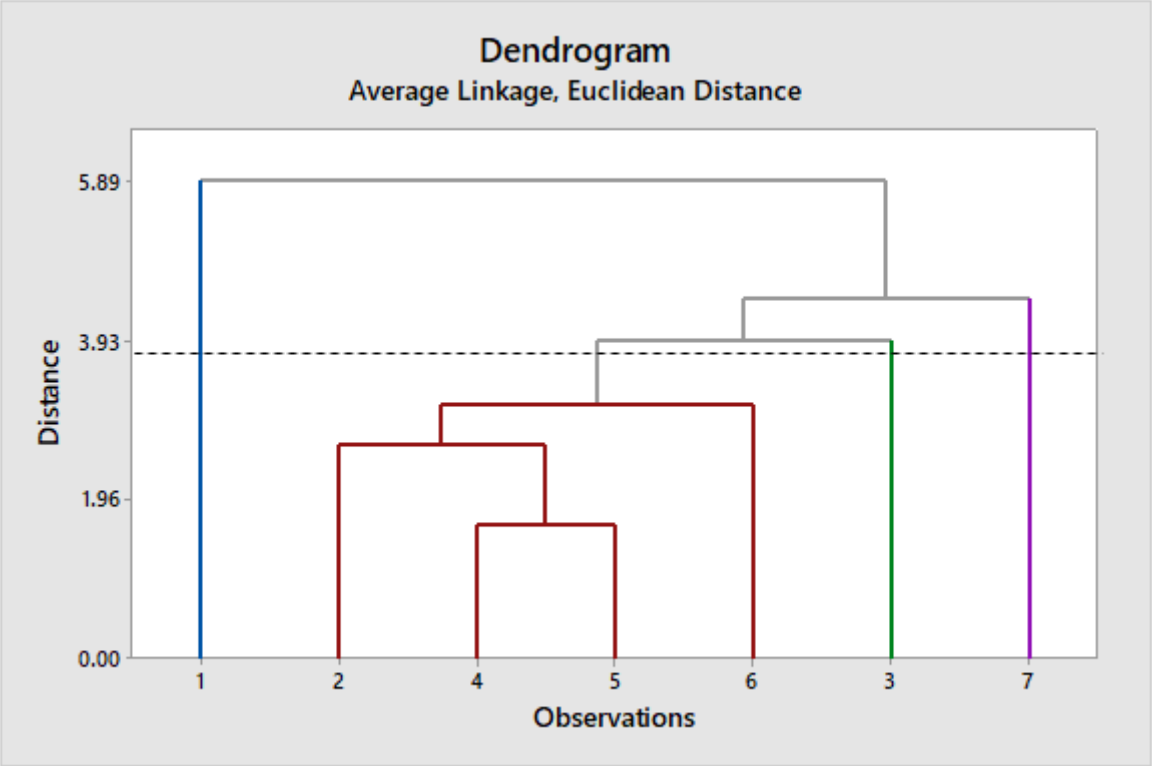


Figure 3. Dendrogram showing the phenetic relationships among seven *Papaver somniferum* populations (r = 0.985).

Gene expression analysis through semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) has exhibited that the transcription level of *SAT* gene (thebaine producer) of the benzylisoquinoline biosynthesis pathway increased 2.2-fold in the tetraploid plants in comparison to that in the controls (diploid) plants (Madani *et al.* 2019). Thus, *P. bracteatum* has enormous potential for synthesizing anti-addiction compounds such as thebaine (8.8 µg/g DW compared with 1.5 µg/g DW in the controls (5.86-fold higher) (Madani *et al.* 2019). Induced tetraploids have shown a substantial increase in thebaine in both reports of Madani *et al.* (2019) and Tarkesh Esfahani *et al.* (2021). However, shockingly we observed the mean content of 589.3 µg/g DW for thebaine from the seven populations of opium poppy of Balkh-Afghanistan in the current study without using any elicitors or inducing tetraploid. In our study, the thebaine content was 85.4-fold higher that of the Iranian diploid, *P. bracteatum*, reported by Tarkesh Esfahani *et al.* (2021) and 66.8-fold higher than in the induced tetraploids of Iranian poppy reported by Madani *et al.* (2019).

Different abiotic elicitors play a significant role in improving the production of different secondary metabolites. Among which, methyl jasmonate (MeJA) is capable of enhancing the amounts

of various alkaloids in different plants (Cho *et al.* 2007; Holková *et al.* 2010; Mishra *et al.* 2013; Akhgari *et al.* 2019; Tarkesh Esfahani *et al.* 2021; Santos *et al.* 2022). The effects of induced MeJA elicitation on the root production of thebaine in Iranian endemic *P. bracteatum* showed that the highest thebaine content (9.3 µg/g DW), compared to that in the non-treated diploid controls (6.8 µg/g DW;1.4-fold increase), identified in the plants treated by 0.5 mM MeJA (Tarkesh Esfahani *et al*. 2021). In the present study, the mean content of thebaine was 589.3 µg/g DW in all seven populations of opium poppy of Balkh-Afghanistan, indicating a 63.4-fold increase compared to that in the induced 0.5 mM MeJA elicitation (9.3 µg/g DW) of *P. bracteatum* report (Tarkesh Esfahani *et al*. 2021).

In the current study, among seven *P. somniferum* populations studied, the P1 population showed 1129.2 µg/g DW thebaine, proving a 121-fold increase rather than *P. bracteatum* report. Therefore, from an economic perspective, the P1 population seems to be strategically the most valuable, exceptional, and irreplaceable. Madani *et al.* (2019) confirmed that an induced tetraploid plant had 30.55-fold higher noscapine content (4677.4 µg/g DW) compared to the control (diploid) plants (153.8 µg/g DW). The same comparison was approved in gene expression analysis for *TYDC* (noscapine producer gene) with about a 2-fold increase.

The alkaloid composition of different parts of *P. somniferum* was investigated by Sharopov *et al.* (2018) and this report for capsule alkaloid content indicates morphine, codeine, and papaverine 929.3 µg/g DW, 1280.5 µg/g DW, and 2062.9 µg/g DW, respectively. The grand mean of morphine alkaloid of seven populations in the current study was obtained as 1311.7 µg/g DW (278.2-297.7 µg/g DW) which was 1.4-fold higher than the Sharopov *et al.* (2018) report. Following that, the grand mean of codeine of seven studied populations was 1259.9 µg/g DW, ranging from 98.23-5638.3 µg/g DW which shows the same story in both reports. The comparison of the current study with the above report indicates that the P7 population with 5638.3 µg/g DW codeine content had a 4.4-fold higher amount of this metabolite and the P6 population with 2947.7 µg/g DW morphine content possessed 3-fold higher amounts. However, in the case of papaverine, the P1 Afghan population showed a 2.5-fold lower amount. Furthermore, the study conducted by Abedini *et al.* (2018) regarding the transcriptome data of eight genera of the Papaver family (Papaveraceae), revealed that noscapine biosynthesis genes are present in the Iranian poppy (*P. bracteatum*), while they do not exist in other genera of this family. Thus, in agreement with Abedini *et al.* (2018), in the current report, the uppermost amount of noscapine in the P1 Afghan population (the most southern latitude, Table 1, Figure 1) was 1398.4 µg/g DW, indicating the activity of the genes of this alkaloid in this species.

In the study of Gümüşçü *et al.* (2007) 99 lines out of 1000 lines of the opium poppy plant

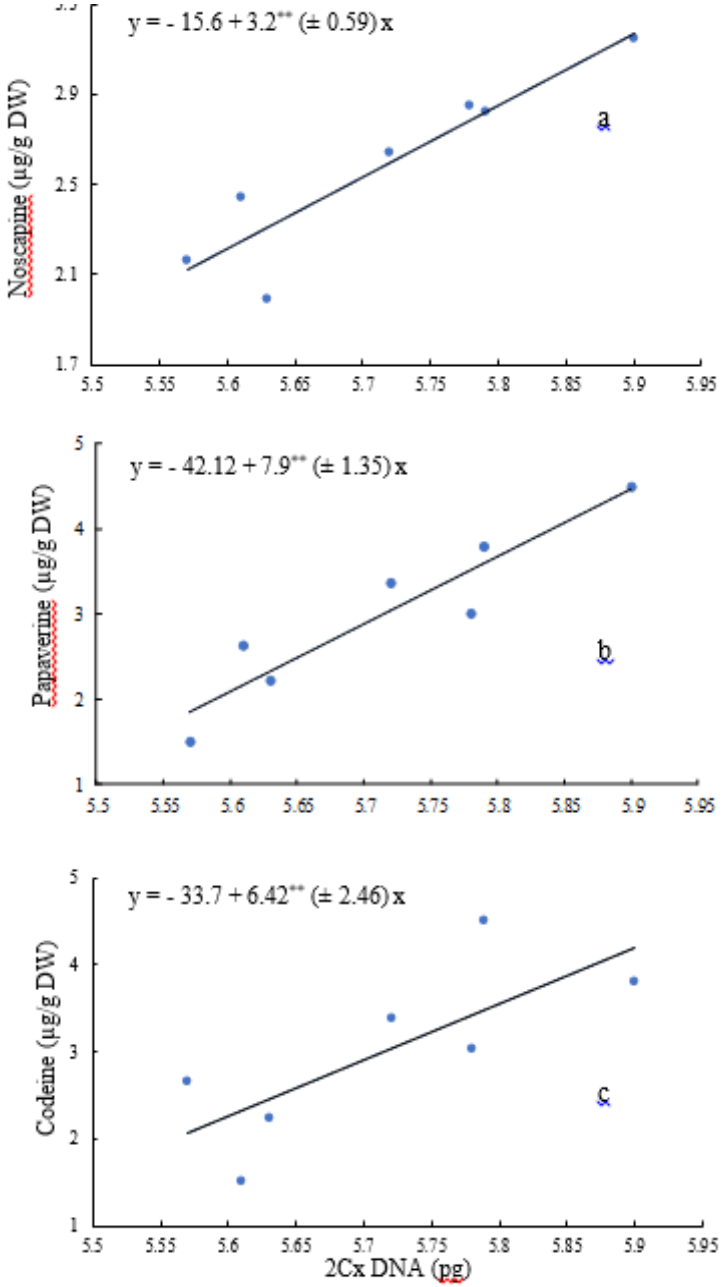


Figure 4. Linear regression equation of noscapine (a), papaverine (b), and codeine (c) with monoploid genome size (2Cx DNA, pg) in *Papaver somniferum.*

(*P. somniferum*) were selected in Turkey. The major alkaloids noscapine, papaverine, thebaine, codeine, and morphine were reported to be 0.099%, 0.029%, 0.041%, 0.049%, and 0.475%, respectively. Compared to Afghan opium, this study found a significantly lower content of codeine and morphine (252 and 28-fold lower, respectively). In other words, the major alkaloids of Balkh, Afghanistan opium show a good performance, most likely indicating the genetic and environmental potential of this country compared to the populations and the environment of Turkey, reported by Kara and Baydar (2021).

Conclusion

The P1 population of *P. somniferum* stands out for its medicinal and industrial value due to its high contents of the valuable alkaloids noscapine, papaverine, and thebaine. Conversely, the P4 population, while containing the lowest level of morphine (a narcotic drug), also has a relatively high amount of thebaine (a non-narcotic precursor to other alkaloids). These dual-compounds metabolites make it a potentially useful population to treat drug-affected patients.

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Ethical considerations

The authors avoided data fabrication and falsification.

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Conflict of interest

The authors declare that they have no conflict of interest with any organization concerning the subject of the manuscript.

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Genetic variability of spike and kernel traits in a collection of bread wheat landraces

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Abstract

Objective: Wheat landraces are a valuable source of allelic diversity for various traits that could be used to broaden the genetic basis of elite germplasms. Assessment of genetic variation of grain yield (GY) and correlated traits is essential for implications in wheat yield improvement.

Methods: In the present study, 140 spring wheat landrace accessions, collected from various geographical regions of Iran, were evaluated for GY and spike-related characters at two successive cropping seasons.

Results: Higher genetic variation was observed among the landraces for the studied traits as revealed by phenotypic (PCV) and genotypic (GCV) coefficient of variation. For all traits, PCV was higher than GCV indicating the influence of the growing season on the variation of the traits. Although the heritability estimate (h^2) for GY was low (0.35), the yield-related traits showed higher h^2 ranging from 0.63 to 0.93. The distribution patterns of landraces, as revealed by cluster analysis and principal component analysis, did not align closely with their geographical distribution. However, accessions with similar phenotypic characteristics were grouped together.

Conclusion: The population structure and genetic relationships of the Iranian bread wheat landraces presented here highlight their diverse genetic architecture. The results of this study provide valuable information for the utilization of landraces in the genetic improvement of bread wheat.

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Introduction

Wheat accounts for approximately 20% of global caloric consumption and protein. It is cultivated on more land area than any other commercial crop, thereby playing an indispensable role in ensuring

food security (Curtis and Halford 2014). To meet the demands of the world's growing population, wheat production should increase by about 50% by 2050 (Kettlewell *et al.* 2023). Urbanization, development, and climate constraints limit the expansion of wheat cultivation areas globally. Therefore, utilizing the potential yield of wheat through genetic improvement is a possible way to increase wheat production. Significant genetic variability in a source population, which increases the chances of selecting superior genotypes, is essential for the success of breeding programs (Tehseen *et al.* 2022). However, continuous breeding practices, such as artificial selection for quality and yield traits, and the presence of few genotypes in the background of most wheat varieties, have narrowed the genetic diversity in bread wheat over the past decades. This has resulted in the vulnerability of elite wheat germplasms to biotic and abiotic stresses, as well as climate changes (Novoselović *et al.* 2016; Tehseen *et al.* 2022).

Wheat landraces are genetically heterogeneous populations that have evolved over many generations under local farming systems and in a multitude of environments. Landraces consist of abundant patterns of variation with novel alleles for the genes controlling useful traits for adaptation and tolerance to biotic and abiotic stresses, which are not utilized in elite wheat breeding programs (Harlan 1992; Cseh *et al.* 2021). Dissecting the genetic diversity and variability in a collection of landraces from different geographical regions would be the first step in broadening the genetic base of elite wheat germplasm. Iran is one of the primary gene centers for *Triticum* species, especially *T. aestivum*, and a large collection of bread wheat landraces from wheat-growing areas in the country has been gathered (Vikram *et al.* 2021). Characterizing the genetic diversity of Iranian wheat landraces for yield-related traits can provide valuable information that can be used to broaden the narrow genetic base in the Iranian wheat breeding program.

Grain yield, as the primary candidate in wheat breeding programs, is a complex trait influenced by morphological and physiological characteristics, grain-related traits, and environmental factors. These factors make yield enhancement increasingly challenging (Liu *et al.* 2018). However, dissecting GY into its components, such as spike length (SL), the number of spikes per unit area, the number of spikelets per spike (NSS), the number of kernels per spike (NKS), and 1,000-kernel weight (TKW), which exhibit higher heritability than that of GY and are less influenced by environmental factors, could enhance the efficiency of GY improvement in wheat. The NKS, TKW, and the number of spikes per unit area are major components that collectively determine the final yield of wheat (Hai *et al.* 2008). Other spike-related traits, such as NSS, SL, and the number of spikes per plant, also play a significant role in wheat yield improvement (Guo *et al.* 2017). This study aimed to characterize a

collection of spring wheat landraces for spike and kernel traits and to identify superior landraces for utilization in breeding programs.

Materials and Methods

Plant materials and field trials

In the current study, we utilized 140 Iranian spring bread wheat landrace accessions collected from diverse geographical regions across the country. The accessions were evaluated at the East Azarbaijan Agricultural and Natural Resources Research Center in Iran during the 2019-2020 and 2020-2021 growing seasons, employing a randomized complete block design with two replications. Each accession was planted in a 1.50 m long two-row plot with an inter-row spacing of 20 cm and a 7 cm spacing between plants within the row. The trials were managed using recommended agronomic practices to ensure proper growth and development.

Nine spike and kernel traits, including GY, TKW, SL, NSS, NKS, spike weight (SW), grain weight per spike (GWS), and awn length (AL), were measured. Ten random spikes from each accession per replication in each year were used for trait measurements. TKW (gr) was determined by weighing 250 kernels from each accession and multiplying the result by four in two replicates for each year. SL (cm) was measured from the base of the rachis to the topmost spikelet, excluding the awns. NSS was counted from the basal sterile spikelet to the top fertile spikelet. Spikelet fertility (SF) was computed as the number of kernels per spike divided by the number of spikelets per spike. SL, NSS, GWS (gr), and AL (cm) were the means of 10 randomly selected spikes in each replication.

Statistical analysis

The mixed linear model based on restricted maximum likelihood (REML) was used to assess the effects of cropping seasons, accessions, and their interaction in explaining the phenotypic variance in the studied traits. This followed the Mixed Linear Model (MLM) variance component analysis procedure of SAS 9.2 (SAS Institute 2004), considering seasons as a fixed factor and genotypes as a random factor. META-R (Multi-Environment Trial Analysis with R) v6.04 (Alvarado *et al.* 2020) was used to estimate the best linear unbiased predictions (BLUPs) for all the traits. BLUPs over two cropping seasons (years) were obtained using the following model: $yijk = \mu + Yi + Rk(i) + Gj + YGij + eijk$, where *yijk* is the trait of interest, μ is the overall mean, *Yi* is the fixed effect of the i^{th} year, *Rk(j)* is the random effect of the k^{th} replicate nested within the i*th* year, *G*j is the random effect of the j^{th} genotype, *YG*ij is the random effect of the year × genotype interaction, and *eijk* is the random error term. To estimate the broad-sense heritability (h^2) across years, variance components were estimated

and h^2 was calculated as follows: $h^2 = \frac{\sigma_g^2}{\sigma_g^2+\sigma_{gy}^2/y+\sigma_e^2/ry}$, where σ_g^2, σ_{gy}^2, and σ_e^2 are genetic, genetic by year interaction, and residual variance components, respectively. The r term represents the number of replications within each year and y term represents the number of years in the analysis. The phenotypic (PCV) and genotypic (GCV) coefficient of variations expressed as percentages were calculated as The phenotypic (PCV) and genotypic (GCV) coefficient of variations expressed as percentages were calculated as $PCV = \frac{\sqrt{\sigma_p^2}}{\bar{X}} x100$ and $GCV = \frac{\sqrt{\sigma_g^2}}{\bar{X}} x100$, respectively, where σ_p^2, σ_g^2, and $\bar{X}$ are phenotypic and genotypic variances, and grand mean, respectively. Phenotypic and genetic correlations were calculated as follows: $r_{p(xy)=\frac{\sigma_{P(x,y)}}{\sqrt{\sigma_{Px}^2\sigma_{Py}^2}}}$, and $r_{g(xy)=\frac{\sigma_{G(x,y)}}{\sqrt{\sigma_{Gx}^2\sigma_{G\,y}^2}}}$, where $r_{p(x)y}$ and $r_{g(x)y}$ are the phenotypic and genotypic correlation coefficients between traits *x* and *y*, $\sigma_{P(x,y)}$ and $\sigma_{G(x,y)}$ are phenotypic and genotypic covariances between traits *x* and *y*, σ_{Px}^2 and σ_{Py}^2 are phenotypic variances for traits *x* and *y*, σ_{Gx}^2 and $\sigma_{G\,y}^2$ are genotypic variances for traits *x* and *y*, respectively. The structural equation modeling (SEM) option implemented in JMP Pro 17.0.0 (SAS Institute Inc, Cary, NC, USA, 2021) was used to further partition the correlations between traits into direct and indirect effects.

Principal component analysis (PCA) using adjusted means of all the traits across years (BLUPs) was executed using JMP Pro 16.0.0 (SAS Institute Inc, Cary, NC, USA, 2021). The number of principal components (PCs) was determined by inspecting the eigenvalues of PCs, and the first two PCs were subjected to a biplot to display shifts occurring in the morphological traits measured on the landraces across two years. A heatmap of 352 wheat landraces and 16 morpho-agronomics traits was constructed based on the hierarchical cluster analysis (Ward's methods) and squared Euclidian distance using JMP Pro 17.0.0 (SAS Institute Inc, Cary, NC, USA, 2021). Mahalanobis' D^2 statistic was used to calculate the pairwise distance between inferred clusters.

Results

Genetic variability

Phenotypic variation of the traits among genotypes, assessed by using mean, standard error (SE), PCV, (GCV, and h^2 values are presented in Table 1. The SE for the studied traits ranged from 0.01(SF) to 2.60 (GY). The PCV and GCV ranged from 7.84% to 50.77% and 5.69% to 46.19%, respectively. The highest PCV (50.77%) was observed for AL, followed by GY (32.04%), while the lowest PCV

Table 1. Mean squares from the combined analysis of variance, mean, phenotypic (PCV), and genotypic (GCV) coefficients of variation, and broad sense heritability (h2) for eight spike and kernel traits of 140 spring bread wheat accession tested in two successive years.

Traits	Mean Squares					Mean ± SE	PCV	GCV	h^2
	Year (Y) df = 1	Rep df = 2	Genotype (G) df = 139	G x Y df = 139	Error df = 278				
GY (gr/plot)	119661.77^{ns}	326746.37	30218.64***	19512.50***	12067.34	424.18± 2.60	32.04	12.20	0.35
(TKW (gr)	47554.93***	49.41	65.86***	24.17***	6.05	37.32 ± 0.22	13.54	8.66	0.63
SL (cm)	30.63*	0.77	10.93***	0.75*	0.60	11.39 ± 0.13	15.75	13.99	0.93
SW (gr)	40.78*	2.40	0.71***	0.19**	0.14	3.06 ± 0.04	17.90	11.78	0.73
NSS	147.95*	2.20	5.078***	1.12***	0.66	17.48 ± 0.07	7.84	5.69	0.78
NKS	682.67^{ns}	448.01	198.11***	54.16*	41.16	53.61 ± 0.43	17.06	11.19	0.73
GWS (gr)	52.00*	1.88	0.48***	0.17**	0.11	2.25 ± 0.02	20.37	12.57	0.69
AL (cm)	3.03^{ns}	4.67	16.54***	0.98**	0.65	4.27 ± 0.16	50.77	46.19	0.94
SF					0.11	3.07± 0.01	14.20	6.51	0.50

ns, *, **, and *** are non-significant, and significant at 0.05, 0.01, and 0.001 probability levels, respectively; GY: Grain yield; TKW: Thousand kernel weight; SL: Spike length; SW: Spike weight; NSS: Number of spikelets per spike; NKS: Number of kernels per spike; GWS: Grain weight per spike; AL: Awn length (cm); SF: Spikelet fertility.

(7.84%) was noted for NSS. Regarding GCV, AL exhibited the highest value (46.19%), followed by SL (13.99%), while NSS showed the lowest GCV (5.69%) value. The h^2 reached its highest value for AL (0.94), followed by SL (0.93), while GY (0.35) had the lowest h^2 value, followed by SF (0.50) (Table 1).

The analysis of variance for nine spike and kernel-related traits using the data combined over the two seasons (years) is presented in Table 1. Most of the traits (six out of the total nine) exhibited significant variation over the years. Mean square values for all the traits indicated highly significant variation among landrace accessions ($p < 0.0001$). The genotype x year interaction was very highly significant ($p < 0.0001$) for GY, TKW, and NSS, highly significant ($p < 0.01$) for SW, NSS, GWS, and AL, and significant ($p < 0.05$) for SL and NKS.

Correlation between traits

The phenotypic correlation coefficient between all pairs of traits was significant except those of Al with other traits and TKW with NSS and NKS (Table 2). A highly significant positive correlation ($p < 0.0001$) was observed between GY and TKW, SW, SL, NSS, and GWS. The correlation of GY with NKS and SF was also positive and significant ($p < 0.001$). A significant positive correlation was observed among SW, SL, NSS, NKS, GWS, and SF. GY showed a positive significant genetic correlation with all the traits studied except AL where the correlation was negative and non

significant. A significant positive genetic correlation was observed between TKW and GY, SW, SL, as well as GWS, and its correlation with SF was significant and negative. Unlike phenotypic correlation, AL had a significant positive genetic correlation with SW, NKS, and SF. Genetic correlations among SW, SL, NSS, NKS, GWS, and SF were positive and significant.

Table 2. Genetic (below diagonal) and phenotypic (above diagonal) correlation coefficient among nine spike and kernel related traits in 140 Iranian spring bread wheat landrace accessions.

Traits	GY	TKW	AL	SW	SL	NSS	NKS	GWS	SF
GY		0.43****	-0.01^{ns}	0.72****	0.72****	0.52****	0.44****	0.78****	0.29***
TKW	0.46****		-0.01^{ns}	0.48****	0.31***	-0.13^{ns}	-0.16^{ns}	0.56****	-0.17*
AL	0.01^{ns}	-0.06^{ns}		0.18*	0.01^{ns}	0.00^{ns}	0.17^{ns}	0.06^{ns}	0.28***
SW	0.62****	0.48****	0.15^{ns}		0.56****	0.65****	0.78****	0.98****	0.72****
SL	0.48****	0.26**	0.02^{ns}	0.55****		0.45****	0.43****	0.57****	0.33****
NSS	0.31***	-0.08^{ns}	0.00^{ns}	0.56****	0.44****		0.86****	0.64****	0.57****
NKS	0.38****	-0.13^{ns}	0.14^{ns}	0.74****	0.40****	0.70****		0.72****	0.91****
GWS	0.64****	0.54****	0.05^{ns}	0.97****	0.52****	0.52****	0.70****		0.63****
SF	0.31***	-0.13^{ns}	0.19*	0.62****	0.25**	0.28***	0.88****	0.59****	

ns, *, **, ***, and **** are non-significant, and significant at 0.05, 0.01, 0.001, and 0.0001 probability levels, respectively; GY: Grain yield, TKW: Thousand kernel weight, ALL: Awn length, SW: Spike weight, SL: Spike length, NSS: Number of spikelets per spike, NKS: Number of kernels per spike, GWS: Grain weight per spike, SF: Spikelet fertility.

Structural equation modeling

The correlations between traits were further partitioned into direct and indirect effects using SEM. Best-fit SEM showing the direct standardized effects of spike and kernel traits on GY, had $\chi^2 = 855.75$ (df = 14, p = < 0.0001), RMSEA = 0.66, CFI = 0.35, AIC = 3786.23, and -2 Log Likelihood = 3709.17. GY was considered as the dependent variable and TKW, SL, and GWS as the causal variables. Also, the NSS and SF were chosen as the casual variables for SL and GWS. AL and NKS were treated as casual variables for TKW (Figure 1). In the diagram, significant and non-significant effects were shown by solid and dashed lines, respectively. The highest significant direct effect on GY was shown by GWS (57.602) followed by SL (4.150). These two traits, however, exhibited a substantial indirect counter-balance effect via one another. TKW which had a strong positive correlation (r = - 0.46**) with GY showed a significant direct effect but smaller than the effects of GWS and SL. Its significant correlation with GY was largely due to the positive indirect effects *via* the GWS and SL. The direct effects of NSS on SL (0.689) and GWS (0.101) were positive and significant, while SF only showed

a positive significant direct effect on GWS (0.811). The direct effect of NKS on TKW was negative but negligible and Al had a positive and non-significant direct effect on TKW.

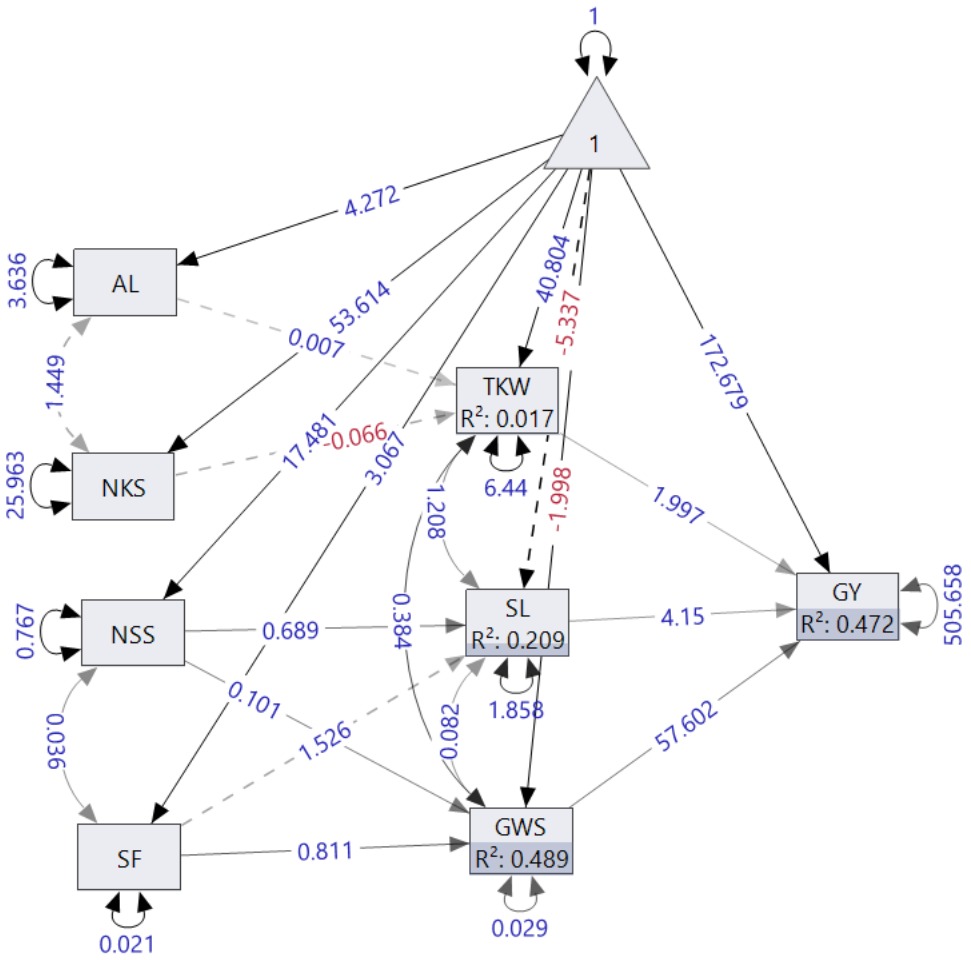


Figure 1. Best-fit structural equation model showing the direct standardized effects of spike and kernel traits on grain yield. The model-fitting parameters are: chi-square = 855.75 (df = 14, p = <0.0001), RMSEA = 0.66 and CFI = 0.35, AIC = 3786.23, and -2 Log Likelihood = 3709.17. GY: Grain yield, TKW: Thousand kernel weight, AL: Awn length, SW: Spike weight, SL: Spike length, NSS: Number of spikelets per spike, NKS: Number of kernels per spike, GWS: Grain weight per spike, SF: Spikelet fertility.

Genetic relationships and population structure

Principal component analysis using the BLUP values of nine spike and kernel-related traits in 140 spring wheat landraces across two seasons revealed that three components with eigenvalue > 1 explained 49.71, 18.38, and 11.35% of the total phenotypic variation of the traits studied. GY, SW, NKS, GWS, and SF showed a positive association with PC1, while TKW had a positive and NKS and SF negative association with PC2. AL showed a positive association with PC3, while NSS showed a negative association. A two-dimensional graphical representation (loading plot) of landraces and projection of nine spike and kernel traits on PCs are presented based on the first two

components (Figure 2). There was low correspondence between the distribution of landraces based on two PCs and their geographical origins.

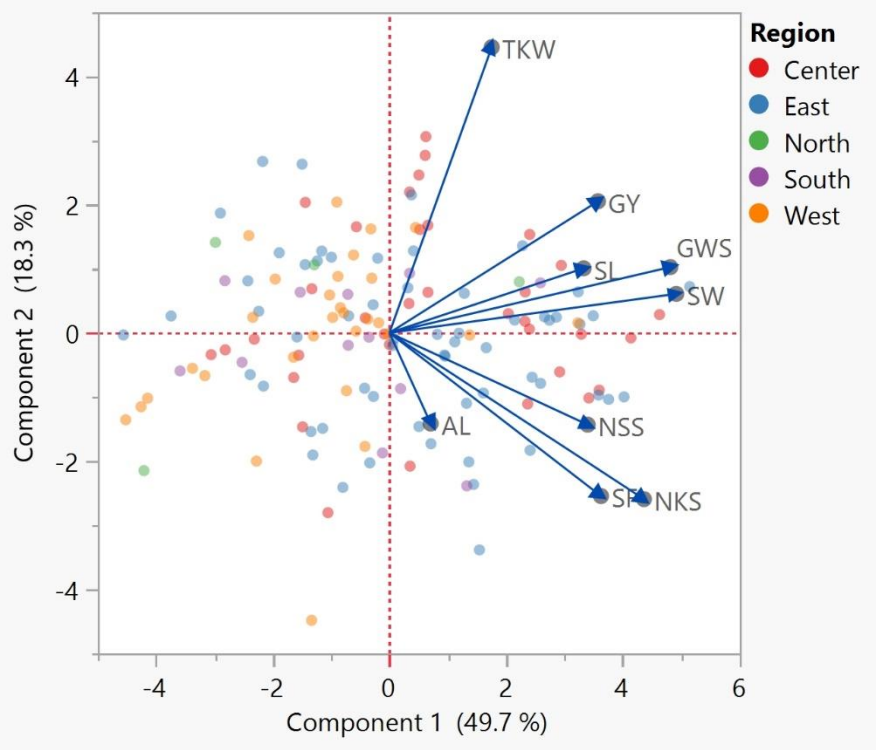


Figure 2. A biplot of principal component analysis of 140 Iranian bread wheat landraces with their contribution value based on nine spike and kernel traits. The genotypes are colored based on their geographical regions. Arrows indicate the loadings for each trait along the first two components, which comprised 68% of the total genetic variation for nine traits. GY: Grain yield, TKW: Thousand kernel weight, AL: Awn length, SW: Spike weight, SL: Spike length, NSS: Number of spikelets per spike, NKS: Number of kernels per spike, GWS: Grain weight per spike, SF: Spikelet fertility.

The resulting dendrogram from Ward's clustering algorithm, based on the squared Euclidean distance coefficient, was presented as a heatmap and constellation plot (Figures 3 and 4). The 140 spring wheat landraces were categorized into five clusters as revealed by the heatmap and constellation trees. Although these clusters did not necessarily consist of accessions from identical origins, naturally, the accessions with greater phenotypic similarity tended to group together. Clusters I, II, III, IV, and V consisted of 29 (20.71%), 30 (21.43%), 19 (13.57%), 21 (15.00%), and 41 (29.29%) accessions, respectively. Cluster I comprised accessions with a higher mean value than the grand mean for GY, TKW, SW, NSS, and GWS. In contrast, accessions in Cluster II showed a higher mean value for TKW, AL, and SL compared to the grand mean. The mean of accessions in Cluster III was higher than the grand mean for AL, NSS, NKS, and SF, while in Cluster IV, the accession mean for all traits was less than the grand mean. Cluster V comprised accessions with the highest mean values for all the traits studied except TKW and AL (Table 3). Pairwise Mahalanobis' distances (D^2 statistics) between the clusters are presented in Table 4. The maximum divergence was observed

between Clusters III and V (43.21), followed by Clusters III and IV (23.86), while Clusters I and II (8.16) showed the minimum between-cluster distance, followed by Clusters II and III (14.71). The intra-cluster variation of Clusters I to V was estimated as 3.15, 3.08, 3.99, 3.79, and 2.46, respectively.

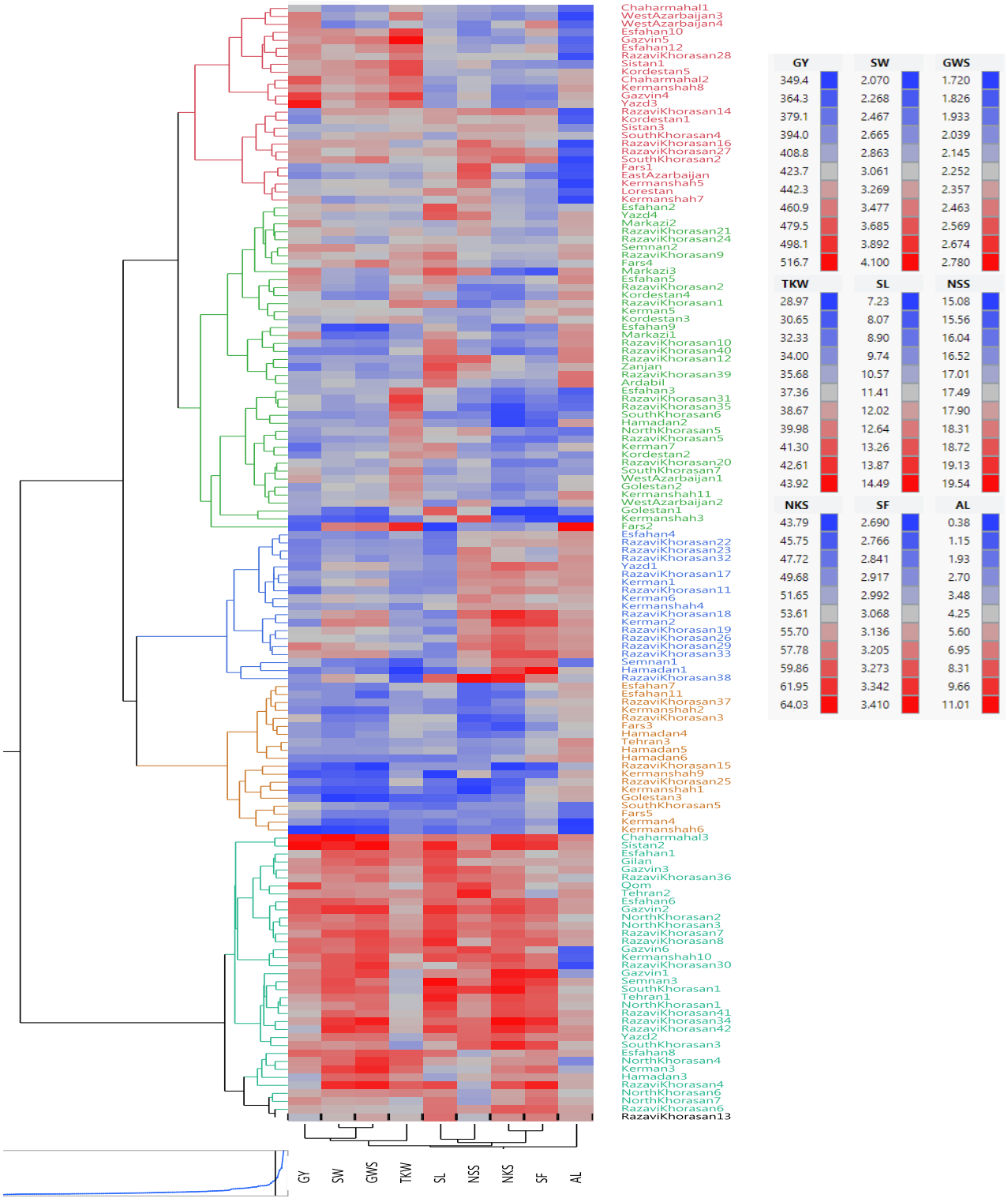


Figure 3. Heatmap of the spike and kernel traits measured on 140 Iranian spring bread wheat landraces; GY: Grain yield, TKW: Thousand kernel weight, AL: Awn length, SW: Spike weight, SL: Spike length, NSS: Number of spikelets per spike, NKS: Number of kernels per spike, GWS: Grain weight per spike, SF: Spikelet fertility.

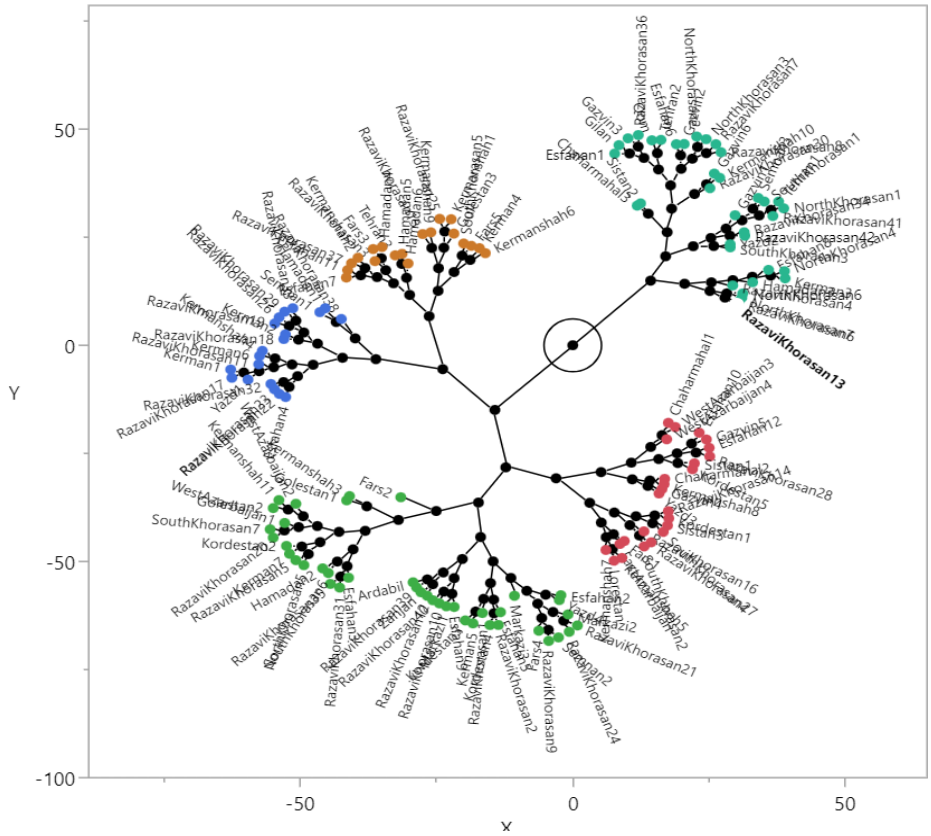


Figure 4. Constellation plot from hierarchical cluster analysis showing grouping of 140 Iranian bread wheat landraces in five clusters.

Table 3. Mean values of different spike and kernel traits of Iranian spring wheat landraces in cluster analysis.

Cluster	Count	GY	TKW	AL	SW	SL	NSS	NKS	GWS	SF
I	29	428.63	40.11	3.45	3.01	10.66	17.02	49.41	2.26	2.95
II	30	420.45	36.82	4.51	2.87	12.08	17.52	50.80	2.13	2.96
II	19	393.97	34.88	4.16	2.49	9.73	16.17	48.34	1.93	3.01
IV	21	405.47	34.64	5.14	3.01	10.08	18.02	57.77	2.19	3.17
V	41	447.34	38.21	4.28	3.53	12.84	18.11	58.96	2.52	3.20

GY: Grain yield, TKW: Thousand kernel weight, ALL awn length, SW: Spike weight, SL: Spike length, NSS: Number of spikelets per spike, NKS: Number of kernels per spike, GWS, Grain weight per spike, SF: Spikelet fertility.

Table 4. Inter- and intra-cluster distance for five clusters as revealed by Mahalanobis' D2 statistics in Iranian spring wheat landraces based on spike and kernel traits.

Cluster	I	II	III	IV	V
I	3.15				
II	8.16	3.08			
III	17.12	14.71	3.99		
IV	21.86	16.95	23.86	3.79	
V	19.90	14.88	43.21	15.88	2.46

Discussion

Bread wheat is the most widely grown staple crop which plays a crucial role in feeding 35% of the world's population by providing approximately 20% of proteins and calories (Tadesse *et al.* 2019). The current average annual increase in wheat production is 1%. However, to meet the demand of the world's growing population by 2050, the required increase rate of wheat production has been estimated to be 1.7% (Tadesse *et al.* 2019; Pal *et al.* 2022). Therefore, considering global climate changes and a gradual decrease in arable lands, yield improvement is the most important goal for global wheat breeding programs.

Compared to most other species, wheat has restricted genetic variability due to its allohexaploid nature, which is contributed by three interrelated diploid species and a few plants of ancestral species involved in the formation of wheat (Cox 1997; Charmet 2011). Although wheat is a young species, it was one of the first species to be domesticated. Therefore, domestication, centuries of cultivation, and modern breeding have further restricted the genetic variability of wheat (Dubcovsky and Dvorak 2007, El Baidouri *et al.* 2017). These processes resulted in the loss of genetic diversity in elite wheat breeding materials, and commercial wheat cultivars are prone to various biotic and abiotic stresses. It has been found that the current genetic diversity cannot meet the requirements of future wheat breeding. To combat climate change and to provide a sufficient quantity of food with better quality, there is a need to harness wheat landraces (Nadeem *et al.* 2021).

In the present study, a set of 140 Iranian spring bread wheat landraces from diverse geographical regions of the country were characterized using nine spike and kernel-related traits. PCV and GCV are two genetic parameters commonly used to detect the amount of variability present in the germplasm. PCV and GCV estimates were categorized as low (< 10%), moderate (10–20%), and high (> 20%) (Burton and DeVane 1953). In our study, high PCV was observed for AL, GY, and GWS, while other traits, except NKS, showed moderate PCV. The GCV was high for AL, moderate for SP, GWS, GY, SW, and KNS, and low for TKW, SF, and NSS (Table 1).

Deciphering the phenotypic and genetic relationships among the traits could provide insight into identifying the selection criteria for improving traits with low heritability. The correlation between examined traits ranged from non-significant to highly significant. Among the studied traits, the associations of AL with other traits were not significant, as revealed by the phenotypic correlation coefficient. However, at the genetic level, it showed a positive and significant correlation with SW, NKS, and SF. Jung *et al.* (2021) reported a positive correlation between AL and TKW. They related this to the awn contribution to total ear net photosynthesis which can reach 50% or more.

Ntakirutimana and Xie (2020) mentioned that higher grain yield in grasses could be achieved by optimizing awn length to provide a surface for cooling through transpiration efficiency.

Knowledge of genetic variation, heritability, and correlations across distinct traits is essential for the development of breeding strategies and will benefit multi-trait selection gains. In the present study, the estimates of h^2 ranged from 0.35 for GY to 0.94 for AL. All the yield components and related traits showed a higher level of heritability (> 60%), ranging from 63% for TKW to 93% for SL. The degree of heritability and genetic advancement generally determine the effectiveness of selection, while genetic variability is important for continuous genetic improvement (Ehdaie and Waines 1989). Wheat GY is a complex trait with low heritability, collectively determined by spike number, NKS, and TKW. SL, as well as the NSS, play an important role in improving wheat GY by affecting the NKS and the number of spikes per plant (Li *et al.* 2019). Therefore, direct selection for superior yield may not be adequately effective for yield improvement in wheat (Moghaddam *et al.* 1997). In our study, yield-contributing traits such as TKW, SL, NSS, and NKS showed high heritability and strong correlations with GY, therefore selection based on these components can be effective for GY improvement of wheat.

PCA and cluster analysis, two common multivariate methods, were used to analyze the genetic relationships and population structure of 140 Iranian spring bread wheat landraces from diverse geographical regions of the country. Grouping of landraces using PCA and cluster analysis did not correspond well with their geographical regions. Vikram *et al*. (2022), in an assessment of Iranian bread wheat landrace accessions' potential for genetic improvement using morphological and molecular data, also reported no concordance between genetic and geographical groupings. This could be explained by the frequent exchange of landraces between farmers and the long-term transportation of seeds between various regions of the country. We grouped the 140 wheat landraces into five clusters based on spike and kernel-related traits using agglomerative clustering. According to Mahalanobis distances (D^2 statistics), cluster V accessions with high mean values for most of the studied traits depicted the utmost divergence against cluster III. The estimation of inter-group distance, which reflects the relative divergence of accessions in the clusters, facilitates the selection of highly diverse parental lines with contrasting traits and provides better exploitation of landrace germplasm resources (Kant and Mani 1999). Therefore, crossing between accessions from clusters III and V may help develop heterotic combinations to derive desirable recombination and select new genotypes.

Conclusion

It has been claimed that plant breeding activities reduce the genetic base of elite wheat germplasm, which could seriously restrict further wheat improvement to cope with climate change. Therefore, broadening the genetic base of the cultivated wheat germplasm pool is essential for the development of high-yielding, input-responsive, and next-generation climate-smart varieties. Iranian bread wheat landraces with a wide genetic background for the most valuable traits have been utilized in breeding programs in countries such as Mexico, India, and Pakistan. In the present study, we characterized a set of 140 spring bread wheat landraces collected from diverse regions based on spike and kernel-related traits. Various statistical analyses revealed high genetic variation among the landraces for the traits studied, which can be utilized in breeding programs as well as a panel for association analysis to map new allelic diversity.

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Ethical considerations

The authors avoided data fabrication and falsification.

Conflict of interest

The authors declare that they have no conflict of interest with any organization concerning the subject of the manuscript.

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Selecting superior groundnut (*Arachis hypogaea L.*) genotypes using multi-trait selection indices

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Abstract

Objective: Groundnut (*Arachis hypogaea* L.) is an annual oilseed and proteinous crop whose production is mainly affected by genotype × environment interactions, making it hard to select superior genotypes. The multi-trait selection indices have been used to choose genotypes based on multiple traits.

Methods: In this study, 11 groundnut genotypes were evaluated based on a randomized complete block design with three replications in three locations, Talesh, Masal, and Rasht, Guilan province, Iran, during two growing seasons (2019-2020 and 2020-2021). Variance components were estimated using the restricted maximum likelihood method, and factor analysis was applied to grouping the traits. Multi-trait genotype-ideotype distance index (MGIDI) and ideal genotype selection index (IGSI) were calculated to select superior genotypes using 16 agronomical characteristics.

Results: Although by considering the 30% selection intensity, the genotypes selected by the MGIDI and IGSI indices in the three locations were somewhat different, the ICG192 groundnut genotype was selected as a superior genotype in all three areas based on both MGIDI and IGSI indices.

Conclusion: The results revealed relative compliance between the MGIDI and IGSI indices in the selection of superior genotypes, and they may be used for genotype selection based on multiple traits.

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Introduction

Groundnut (*Arachis hypogaea* L.) is an important annual oilseed and protein crop cultivated on about 31.57 million hectares with a production of ~53.64 million tons worldwide (FAO 2022). The peanut cultivation area in Iran covers approximately 1300 hectares. An estimated 90% of Iran's peanut production occurs in the Guilan province, particularly in the Astana Ashrafieh area (Nobahar *et al.* 2019). The prevalent variety of peanuts in this area is Goli or NC2 which is known for its low harvest index and extended growth cycle. Introducing new germplasm is a crucial approach to fostering the early development of high-yielding cultivars in the region.

Critical genotype by environment (G×E) interaction intuitively decreases the association between genotype and phenotype, making it troublesome to distinguish top genotypes and achieve breeding advancement (Delacy *et al.* 1996). One of the important methods introduced in the analysis of multi-environment trial data is the restricted maximum likelihood (REML) method, which is based on Henderson's theoretical model (Henderson 1984), estimating variance components by providing genetic correlations, and flexibility in linear models for analysis. Balanced and unbalanced data, high usefulness in bulked and alpha lattice tests plus reduction of the negative estimates of genetic parameters are among the advantages of using this method (Searle *et al*. 1992; Liu *et al*. 1997; Holland 2006).

Grain yield is a complex trait influenced by polygenes and environmental factors. Successful selection of superior genotypes based on grain yield and other agronomic characteristics has stood as a challenge for plant breeders. Multivariate information is common in biological experiments and utilizing the data on different traits is crucial to form better choices for treatment suggestions or genotype determination. In any case, recognizing genotypes/treatments that combine high performance over several characteristics has been a challenging assignment. Several linear selection indexes (Céron-Rojas and Crossa 2018) can help breeders select better genotypes. A simple linear phenotypic selection index that is easy to use (Bhering *et al.* 2012; Bizari *et al.* 2017; Burdon and Li 2019; Jahufer and Casler 2015) is the Smith-Hazel (SH) index (Hazel 1943; Smith *et al.* 1936). To calculate the SH index, breeders use the matrix of phenotypic and genotypic (co)variances and the economic weight vector to determine how to select the vector of index coefficients to establish the relationship between genetic and phenotypic values. Since the SH index requires transformation of the phenotypic covariance matrix (Smith 1936), the presence of multicollinearity (which can occur when measuring more than one trait) can result in poorly conditioned matrices and biased index coefficients, thus, affecting the estimates of genetic gains. In addition to multicollinearity issues,

breeders often face difficult choices about distributing economic value and translating it into true economic weights (Bizari *et al.* 2017). The MGIDI index was found to have many practical applications since it allows a unique and easy-to-interpret selection process. In addition to dealing with collinear traits, the MGIDI index doesn't require the use of economic weights such as in the SH index, in which one can predict genetic and economic gains for various possible combinations of genetic parameters and assumed economic weights (Bizari *et al.* 2017; Burdon and Li 2019). Olivoto and Nardino (2021) evaluated 44 wheat genotypes for 14 agronomic traits. In their study, the MGIDI index outperformed the FAI-BLUP and SH indexes in selecting traits with desired gains. Furthermore, the MGIDI index was computationally more efficient. Yue et al. (2022b) examined 12 agronomic traits of 28 maize genotypes in a 2-year field experiment across 24 environments. They compared MGIDI, FAI-BLUP, Smith-Hazel, and MTSI methods for genotype ranking. The study indicated MGIDI's superiority over the other methods, suggesting that it is an optimal tool for selecting genotypes based on multiple traits. Pour-Aboughadareh and Poczai (2021) studied 180 wild relatives of wheat and landraces from the *Triticum* and *Aegilops* genera under control and water-deficit stress. After 30 days of stress treatment, seedlings were sampled and 23 traits were assessed. MGIDI, Smith-Hazel (SH), and factor analysis and ideotype design (FAI) were employed to identify preferred accessions with favorable root and physiological traits. The findings underscored the effectiveness of selection indices, particularly MGIDI, for choosing superior plant genetic materials through multi-trait evaluation in the early growth phase. Also, the IGSI index has been used to select the best genotypes by integration of different drought tolerance indices (Zali *et al.* 2019), stability analysis parameters (Zali *et al.* 2015; Najafi Mirak *et al.* 2018), and different morphological and phenological traits (Abdollahi Hesar *et al.* 2020). These indices focus on the selection of superior genotypes using multiple traits. So, examining the overall response of genotypes and selecting superior genotypes becomes more efficient (Olivoto and Nardino 2020).

The lower the MGIDI value of a genotype, the smaller the distance from the ideal genotype, so it is considered a superior genotype. Concerning IGSI, the most superior genotypes have the lowest deviation from the positive perfect genotype and the highest distance from the negative one. The IGSI value is in the range of 0-1. If it is near 1, the genotype is near ideal; if it is near 0, the underlying genotype is near the non-ideal type. However, the application of these selection indices has not been detailed in groundnut breeding. This research aimed to select suitable peanut genotypes using the MGIDI and IGSI indices based on multi-trait considerations.

Materials and Methods

In this study, 11 groundnut (*A. hypogaea* L.) genotypes provided by the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) were evaluated during two cropping seasons (2019-2020 and 2020-2021) in three different locations, including Talesh, Masal, and Rasht, northern Iran. Descriptions of the experimental sites are given in Table 1. The experiment layout was a randomized complete block design with three replications. Each plot included three rows of five-meters long, a row space of 50 cm, and 20 cm between plants on the rows. The evaluated agronomic traits were plant height, number of secondary branches, pod number per plant, seed number per pod, 100-seed weight, pod length, pod width, seed length, seed width, dry forage yield, total pod yield, biomass, seed yield, harvest index, seed oil percentage, and oil yield.

The variance component estimates for each trait were obtained by REML, using the following model:

$$Y = Xr + Zg + Wi + e$$

Where Y is the data vector, Xr is the fixed effect, Zg is the random genotypic effect, Wi is the random effect of the G×E interaction, and e is the random residual.

The broad sense heritability for each trait was calculated based on variance component estimates as shown below:

$$h^2 = \frac{\hat{\sigma}_g^2}{\hat{\sigma}_p^2} = \frac{\hat{\sigma}_g^2}{\hat{\sigma}_g^2 + \hat{\sigma}_r^2 + \hat{\sigma}_{gr}^2}$$

Where $\hat{\sigma}_g^2$ is the estimated genetic variance; $\hat{\sigma}_r^2$ is the estimated environmental variance, and h^2 is heritability on the plot mean basis. CV_g, the genetic coefficient of variation, and CV_r, the environmental coefficient of variation, were also calculated for the evaluated traits:

$$CV_g = \frac{\sqrt{\hat{\sigma}_g^2}}{\bar{X}} \times 100$$

$$CV_r = \frac{\sqrt{\hat{\sigma}_r^2}}{\bar{X}} \times 100$$

The significance of genotype, environment, and G×E interaction effects was verified using the likelihood ratio test. The analysis was performed in the R (V.4.2.3) software with the "metan" package.

We used MGIDI, proposed by Olivoto and Nardino (2020), to rank genotypes based on multiple traits. First, the data were rescaled between 0 and 100 to generate an idiotypic matrix followed by factor analysis, according to Olivoto and Nardino (2020). The MGIDI was then calculated as the Euclidean distance between a genotype and the ideal genotype based on each factor score:

Table 1. The experimental sites and geographical characteristics of test environments.

Location	Year	Latitude, longitude	Altitude (m)	Monthly precipitation (mm)						Monthly temperature (°C)					
				March	April	May	June	July	August	March	April	May	June	July	August
Talesh	2019	37.80° N	80	145.00	68.60	7.40	51.30	23.90	117.70	12.00	17.80	23.90	25.90	26.00	22.80
	2020	48.90° E		180.80	109.00	7.10	30.70	148.80	190.20	11.40	16.60	23.90	26.00	25.40	23.80
Masal	2019	37.36° N	84	107.60	60.00	6.30	91.70	24.50	67.30	13.20	18.50	25.70	27.00	26.80	23.80
	2020	49.13° E		100.00	47.90	2.90	10.40	138.00	27.80	13.10	18.80	25.40	27.50	26.30	25.10
Rasht	2019	37.27° N	4	122.70	74.50	6.20	171.10	25.30	138.00	13.20	19.30	24.70	26.20	25.80	22.90
	2020	49.59° E		135.50	67.50	0.30	19.80	103.20	97.70	12.20	17.90	24.80	26.70	25.20	24.00

$$\mathrm{MGIDI_i} = \left[\sum_{j=1}^{f}\left(\gamma_{ij} - \gamma_j\right)^2\right]^{0.5}$$

Where $MGIDI_i$ is the multivariate distance index from the ideal genotype for the i_{th} genotype, and γ_{ij} and γ_j are the scores of the ith genotype and the ideal type for the jth factor, respectively. The genotypes with the lowest MGIDI are close to the ideal genotype and thus provide desired values for the measured traits. A selection intensity of 30% was considered for calculating the selection differential for the studied traits. Also, the contribution of each factor in the MGIDI index of the i_{th} genotype (ω_{ij}), was used to determine the genotype advantages and disadvantages and was calculated as:

$$\omega_{ij} = \frac{\sqrt{D_{ij}^2}}{\sum_{j=1}^{f}\sqrt{D_{ij}^2}}$$

where D_{ij} is the distance between the i_{th} genotype and the ideal genotype for the j_{th} factor (Olivoto and Nardino 2020).

Also, IGSI (Zali *et al.* 2015) was used to select desirable genotypes by considering multiple traits simultaneously. The normalized (r_{ij}) values of the j^{th} genotype (j = 1, 2, ..., n) for the i^{th} trait (i = 1, 2, ..., m) were calculated as:

$$r_{ij} = \frac{X_{ij}}{\sqrt{\sum_{ij}^{n} X_{ij}^2}}$$

Then, the r_i^+ and r_i^- values were determined, corresponding to the normalized values of the favorable and the unfavorable genotypes for each trait, respectively. The two distances denoted by d_i^+ and d_i^- were used to measure the distance of each genotype from either the favorable or unfavorable genotype:

$$d_i^+ = \sqrt{\sum_{i=1}^{n}\left(r_{ij} - r_j^+\right)^2}$$

$$d_i^- = \sqrt{\sum_{i=1}^{n}\left(r_{ij} - r_j^-\right)^2}$$

$$IGSI = \frac{d_i^-}{d_i^+ + d_i^-}$$

Additionally, a heatmap was generated to assess the interrelationships between various genotypes and corresponding traits. The heatmap was drawn in the R (V.4.2.3) software with the "gplots" package. Factor analysis based on principal component analysis was performed in the R (V.4.2.3) software with the "metan" package. In this method, factors with eigenvalues ≥ 1 were selected and factor loadings greater than 0.5, regardless of their sign, were considered significant coefficients for each independent factor. The largest factor coefficient among the coefficients of each factor represents the factor to which the said attribute is assigned.

Results and Discussion

A significant difference was observed through the likelihood-ratio test at a 1% probability among genotypes concerning various plant characteristics including plant height, number of secondary branches, 100-seed weight, pod length, pod diameter, dry forage yield, total pod yield, biomass, seed yield, harvest index, oil percent, and oil yield, as shown in Table 2. The environmental effect was notable across all characteristics examined, except for seed length. Furthermore, G×E interaction played a significant role in all traits as indicated in Table 2. The relative genotypic and environmental coefficients of variation were greater than 1.0 for certain traits such as plant height, pod diameter, dry forage yield, total pod yield, biomass, seed yield, seed oil percent, and oil yield, while they were below 1.0 for others like the number of secondary branches, pod number per plant, seed number per pod, 100-seed weight, pod length, seed length, seed diameter, and harvest index. The accuracy of genotype selection for 16 characteristics varied from 0 (seed length) to 0.975 (biomass), with biomass, total pod yield, and pod diameter exhibiting the highest selective accuracies. Notably, the most significant coefficients of genetic variance were observed as 15.51%, 15.1%, and 10.3%, for oil yield, seed yield, and fresh forage yield, respectively. Coefficients of determination for G×E interaction effects were high for seed length, seed diameter, and seed oil percent, underscoring the substantial contribution of the G×E interaction variance to the overall phenotypic variance, as shown in Table 2.

Heritability estimates based on entry mean suggested a predominant influence of genetic effects on most assessed traits.

The assessment of 12 morphological traits related to yield by Samadi Gorji *et al.* (2018) indicated significant differences between genotypes for all traits. The broad sense heritability in their study varied from 80.25% (seed width) to 99.54% (100-seed weight), with a value of 96.85% for seed yield. The highest phenotypic and genotypic variation coefficients were associated with pod weight. Fondra *et al.* (2000) identified pod number per plant, pod length, number of seeds per pod, seed weight, pod weight, and seed width as crucial traits in evaluating diversity among peanut genotypes. Additionally, Golaktya and Makneh (1991) pointed out that the main stem height and the ratio of seed to pod volume encompassed the most genetic diversity among 18 groundnut genotypes. Investigating 10

Table 2. Likelihood ratio test and estimates of genetic parameters for 16 agronomic traits of 11 groundnut genotypes.

Traits	Genetic parameters									
	LRT_g	**LRT_{ge}**	**E/F**	**δ^2_p**	**R^2_{ge}**	**h^2_g**	**As**	**CV_g**	**CV_r**	**CV_g/CV_r**
PH	10.953^{***}	100.429^{***}	80.70^{***}	82.7	0.517	0.757	0.87	8.01	6.24	1.28
NSB	17.1269^{***}	6.3179^{*}	25.20^{***}	2.42	0.152	0.823	0.907	9.75	14.4	0.679
NPP	16.313^{ns}	28.070^{***}	21.75^{***}	11.8	0.297	0.816	0.904	9.3	10.3	0.902
SNPP	0.66^{ns}	53.085^{***}	5.973^{***}	0.0075	0.547	0.317	0.563	1.21	3.33	0.363
SW	8.045^{**}	58.368^{***}	4.973^{***}	18.4	0.464	0.708	0.841	3.46	4.02	0.862
PL	8.006^{**}	44.683^{***}	18.93^{***}	6.49	0.42	0.707	0.841	3.6	4.65	0.774
PD	28.441^{***}	14.994^{***}	34.52^{***}	1.44	0.186	0.887	0.942	5.48	5.34	1.03
SL	0^{ns}	155.12^{***}	1.534^{ns}	3.06	0.834	0	0	0	4.77	0
SD	1.975^{ns}	132.597^{***}	9.419^{***}	0.375	0.714	0.474	0.689	3	3.63	0.825
DFY	25.911^{***}	78.992^{***}	5.764^{***}	306649	0.352	0.876	0.936	10.2	6.04	1.7
TPY	35.421^{***}	70.602^{***}	43.16^{***}	218689	0.282	0.911	0.954	9.95	5.16	1.93
BY	56.390^{***}	50.919^{***}	12.41^{***}	711163	0.172	0.951	0.975	9.66	4.19	2.31
SY	28.548^{***}	86.931^{***}	31.34^{***}	187925	0.342	0.888	0.942	15.1	8.03	1.88
HI	5.771^{*}	87.017^{***}	28.68^{***}	13.5	0.562	0.653	0.808	5.92	6.45	0.919
OP	9.057^{**}	276.966^{***}	25.73^{***}	2.74	0.662	0.727	0.853	1.76	0.626	2.81
OY	27.91^{***}	79.11^{***}	25.84^{***}	52612	0.3378	0.8851	0.9408	15.51	8.759	1.771

***Significant at $p \leq 0.001$; **Significant at $p \leq 0.01$; *Significant at $p \leq 0.05$; ^{ns}nonsignificant. LRT_g and LRT_{ge}: Likelihood ratio tests for genotype and genotype by environment interaction (GEI), respectively; E/F: The F value for environment effects; δ^2_p: Phenotypic variance; R^2_{ge}: The coefficient of determination for GEI effects; h^2_g: Heritability based on entry mean; As: The accuracy of genotype selection; CV_g and CV_r: The genotypic and environmental coefficients of variation, respectively; PH: Plant height; NSB: Number of secondary branches; NPP: Pod number per plant; SNPP: Seed number per pod; SW: 100-seed weight; PL: Pod length; PD: Pod diameter; SL: Seed length; SD: Seed diameter; DFY: Dry forage yield; TPY: Total pod yield; BY: Biomass; SY: Seed yield; HI: Harvest index; OP: Seed oil percent; OY: Oil yield.

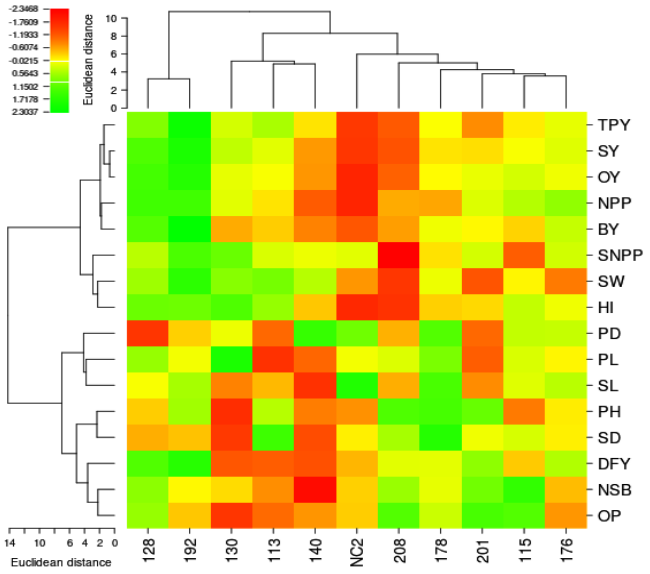
peanut genotypes, Vange and Maga (2014) discovered a substantial coefficient of genotypic variation and genetic advance for the number of pods per plant, the number of branches per plant, and seed yield.

Figure 1 shows the two-way heatmap clustering pattern to group the genotypes and analyze the measured traits based on the average of two years of data at each location. The genotypes were divided into two clusters in Talesh (Figure 1a). The first cluster included the genotypes 192 and 128. These genotypes had higher plant height, number of secondary branches, pod number per plant, seed number per pod, 100-seed weight, pod length, dry forage yield, total pod yield, biomass, seed yield, harvest index, seed oil percentage, and oil yield. The second cluster included other genotypes (115, 178, 176, 140, 201, 208, 130, NC2, and 113) that had lower values than the mean for all traits except pod diameter, seed length, and seed diameter (Figure 1a). The genotypes in Masal were divided into two clusters (Figure 1b). The first cluster included the genotype 192. This genotype had higher values than the mean for all traits except seed diameter and seed oil percentage. Other genotypes were included in the second cluster, which had lower values than the mean for all the traits except for seed diameter and seed oil percentage. In Rasht, genotypes were divided into two clusters (Figure 1c). The first cluster consisted of the genotypes 192 and 128. These genotypes had higher values than the mean for all traits. In contrast, genotypes in the second cluster (115, 178, 176, 140, 201, 208, 130, NC2, and 113) had lower values than the mean for all traits (Figure 1c). Therefore, the two-side dendrogram can show relationships between groundnut genotypes and measured traits. Likewise, Pour-Aboughadareh *et al.* (2021) used the two-way heatmap clustering pattern to find the relationships among the 20 investigated barley genotypes and 18 measured growth and physiological traits under salinity stress conditions. Considering the genetic diversity of 76 peanut genotypes from the National Plant Seed Bank of Iran, Aalami *et al.* (2007) observed relatively low diversity in morphological traits. Foundra *et al.* (2000) conducted cluster analysis and principal component analysis on 14 morphological traits in a population of 86 peanuts, highlighting pod length, number of seeds in the pod, pod weight, and seed weight as key characteristics for evaluating diversity. Cluster analysis using Ward's method classified these genotypes into nine groups.

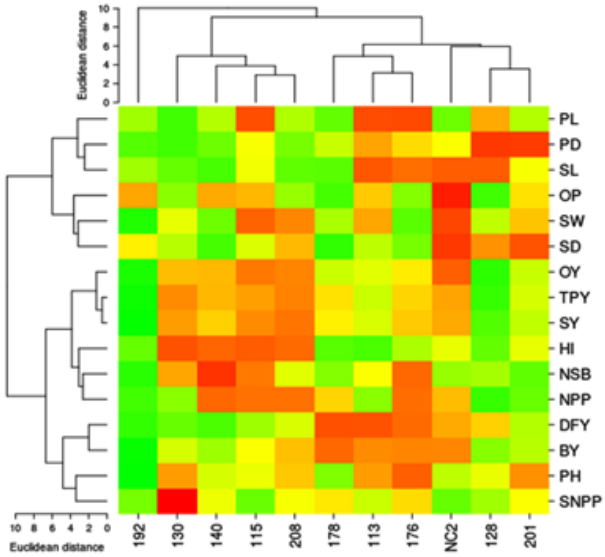
Based on the factor analysis in Talesh, four factors with eigenvalues greater than 1 were chosen, which determined 85.80% of the total variation (Table 3). Seed yield, oil yield, biomass, total pod yield, pod number per plant, harvest index, 100-seed weight, dry forage yield, and pod diameter were comprised in Factor 1. Factor 2 consisted of seed oil percentage, number of secondary branches, and seed number per pod. Factor 3 included pod length, seed width, and plant height, and Factor 4

included seed length and pod width. The average communality was 0.858 with a range of 0.506-0.997, which means that these factors explained a large part of the variables' variance (Table 3).

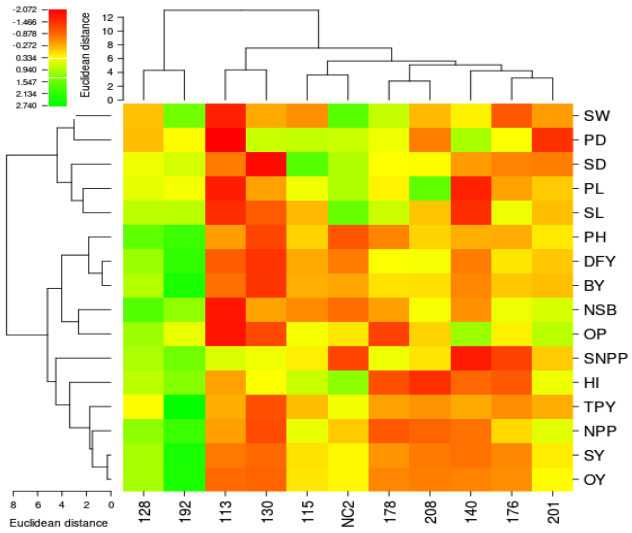
In Masal, five factors with eigenvalues greater than 1 were chosen, which explained 92.98% of the total variation (Table 3). Oil yield, seed yield, total pod yield, pod number per plant, biomass, number of secondary branches, 100-seed weight, and plant height were included in Factor 1. Factor 2 comprised of pod length, seed length, and pod diameter. Factor 3 included dry forage yield and harvest index, and Factor 4 included seed width. The remaining two traits, the number of seeds per



(a)



(b)



(c)

Figure 1. Heatmap and grouping of groundnut genotypes and traits in a) Talesh, b) Masal, and c) Rasht. Colors are representative of a relative scale (-2 to +2) derived after data standardization. The red indicates lower values, and the green indicates higher values.

SW: 100-seed weight; PD: Pod diameter; SD: Seed diameter; PL: Pod length; SL: Seed length; PH: Plant height; DFY: Dry forage yield; BY: Biomass; NSB: Number of secondary branches; OP: Seed oil percent; SNPP: Seed number per pod; HI: Harvest index; TPY: Total pod yield; NPP: Pod number per plant; SY: Seed yield; OY: Oil yield.

pod and seed oil percentage were included in Factor 5. The average communality was 0.93 with a range of 0.80-0.99, indicating that these factors explained a large part of the variables' variance (Table 3).

In Rasht, four factors with eigenvalues greater than 1 were chosen, which explained 84.95% of the total variation (Table 3). Factor 1 consisted of the number of secondary branches, plant height, dry forage yield, biomass, and seed oil percentage. Harvest index, seed yield, total pod yield, oil yield, and pod number per plant were included in Factor 2. Factor 3 comprised pod length, seed length, 100-seed weight, and seed diameter. Factor 4 included the seed number per pod and pod diameter. The average communality was 0.84 with a range of 0.52-0.99, which indicated that these selected factors explained a considerable portion of the variance of the variables (Table 3). Safari *et al.* (2012) found two significant canonical variables, with one concentrating on the weight of 100 seeds, oil yield, the weight of 100 pods, the ratio of seed volume to pod volume, and the number of pods per plant, playing a critical role in distinguishing cultivars.

Table 3. The eigenvalues and explained proportion of variance in the factor analysis, and the factor loadings after varimax rotation for groundnut traits in three regions.

	Talesh				**Masal**					**Rasht**			
Traits	**FA1**	**FA2**	**FA3**	**FA4**	**FA1**	**FA2**	**FA3**	**FA4**	**FA5**	**FA1**	**FA2**	**FA3**	**FA4**
BY	0.923	0.142	-0.185	0.165	0.729	0.110	0.661	-0.003	0.129	0.715	0.449	0.405	0.209
DFY	0.595	0.513	-0.313	0.279	0.203	0.292	0.927	0.007	0.074	0.795	0.264	0.424	0.236
HI	0.845	-0.152	0.326	-0.186	0.671	-0.183	-0.688	0.010	0.104	0.024	0.878	0.118	-0.056
NPP	0.893	0.365	0.099	-0.084	0.813	0.014	0.017	-0.424	-0.281	0.646	0.705	0.081	0.093
NSB	0.154	0.901	-0.001	0.199	0.671	0.378	-0.322	-0.454	0.183	0.889	0.054	0.238	-0.015
OP	-0.136	0.864	-0.298	-0.020	0.197	-0.052	-0.257	0.346	-0.758	0.710	0.185	0.048	-0.608
OY	0.978	0.133	0.025	-0.038	0.985	-0.084	-0.034	0.074	-0.098	0.575	0.754	0.301	0.062
PD	-0.492	-0.496	0.151	0.430	-0.189	0.642	0.386	0.479	0.101	-0.111	0.127	0.402	-0.676
PH	0.055	0.315	-0.874	0.036	0.622	0.456	0.114	0.200	0.552	0.847	0.431	0.057	0.239
PL	0.130	0.221	0.621	0.593	0.034	0.929	0.098	-0.171	-0.132	0.245	-0.071	0.863	0.077
SD	-0.130	0.158	-0.797	0.215	-0.103	0.063	-0.094	0.928	-0.220	0.182	0.311	0.621	-0.080
SL	-0.016	0.049	-0.257	0.909	-0.112	0.698	0.387	0.421	-0.174	0.263	0.225	0.827	-0.076
SNPP	0.608	-0.553	0.200	0.101	0.201	-0.345	-0.182	-0.100	0.837	0.208	0.333	0.111	0.820
SW	0.700	-0.544	0.157	0.022	0.645	0.071	0.173	0.634	-0.144	0.006	0.443	0.700	-0.311
SY	0.995	-0.009	0.084	0.002	0.981	-0.028	0.075	0.002	0.161	0.539	0.766	0.316	0.134
TPY	0.944	-0.250	-0.008	0.008	0.978	-0.131	0.038	-0.031	0.135	0.428	0.765	0.297	0.119
Eigenvalues	6.78	3.81	1.58	1.56	6.07	3.77	2.30	1.52	1.22	8.62	2.26	1.38	1.31
Variance (%)	42.37	23.78	9.88	9.77	37.95	23.55	14.40	9.48	7.60	53.87	14.14	8.64	8.20
Cumulative V. (%)	42.37	66.15	76.03	85.80	37.95	61.50	75.90	85.39	92.98	53.87	68.02	76.66	84.95

BY: Biomass; DFY: Dry forage yield; HI: Harvest index; NPP: Pod number per plant; NSB: Number of secondary branches; OP: Seed oil percent; OY: Oil yield; PD: Pod diameter; PH: Plant height; PL: Pod length; SD: Seed diameter; SL: Seed length; SNPP: Seed number per pod; SW: 100-seed weight; SY: Seed yield; TPY: Total pod yield.

Multi-trait genotype-ideotype distance index (MGIDI)

In Talesh, the selected genotypes using the MGIDI index were 192, 178, and 176 (Figure 2a), considering 30% selection intensity. The strengths and weaknesses of the genotypes showed that genotype 192 had the lowest values in Factor 1, Factor 3, and Factor 2. So, for traits that had the higher coefficients in these factors as seed yield, oil yield, biomass, total pod yield, pod number per plant, harvest index, 100 seed weight, dry forage yield, seed oil percentage, and number of secondary branches, this genotype was close to the ideal genotype (Figure 2b). Also, two selected genotypes 178 and 176 showed the lowest values for Factors 2 and 3 and were close to the ideal genotype regarding traits such as seed oil percentage, number of secondary branches, plant height, and pod length which had higher coefficients in these factors.

In Masal, the genotypes 192, 178, and 140 were selected based on the MGIDI index (Figure 2c). These genotypes were close to the ideal genotype for traits that had higher coefficients in Factor 1 (for genotype 192), Factor 2, Factor 3, and Factor 4 (for genotype 178), and Factor 3 and Factor 4 (for genotype 140). This shows that for oil yield, seed yield, total pod yield, pod number per plant, biomass, number of secondary branches, 100-seed weight, and plant height, the best-selected genotype (192) had the lowest difference from the ideal genotype. These traits were the most effective characteristics in selecting the superior genotypes (Figure 2d).

In Rasht, the genotypes 192, 128, and 115 were selected using the MGIDI index (Figure 2e). Also, based on the strengths and weaknesses of the genotypes, the best-selected genotype (192) had lower values concerning Factor 1, Factor 2, and Factor 3 and had a higher number of secondary branches, plant height, dry forage yield, biomass, seed oil percentage, harvest index, seed yield, total pod yield, oil yield, pod number per plant, pod length, seed length, 100-seed weight, and seed diameter (Figure 2f). MGIDI index has been used to select suitable genotypes in some crops. Maranna *et al.* (2021) evaluated sixty-eight advanced breeding lines of soybean (*Glycine max* L.) for yield and attributing traits. They showed that the presence of multicollinearity and difficulty in assigning economic weightage to the traits under consideration in the case of the SH index can affect genetic gain. Therefore, to overcome these weaknesses, the genotype-ideotype distance index (MGIDI) was developed which accounted for the multicollinearity issue and selected all the traits under consideration favorably. Pour-Aboughadareh *et al.* (2021) used the MGIDI index to choose the salt-tolerant barely genotypes considering all measured traits. Their results suggested that using the MGIDI index in the early growth stage can accelerate screening nurseries in barley breeding

programs. Also, Yue *et al.* (2022a) and Vahedi *et al.* (2023) used MGIDI based on multiple traits for selecting superior maize and cow cockle genotypes, respectively.

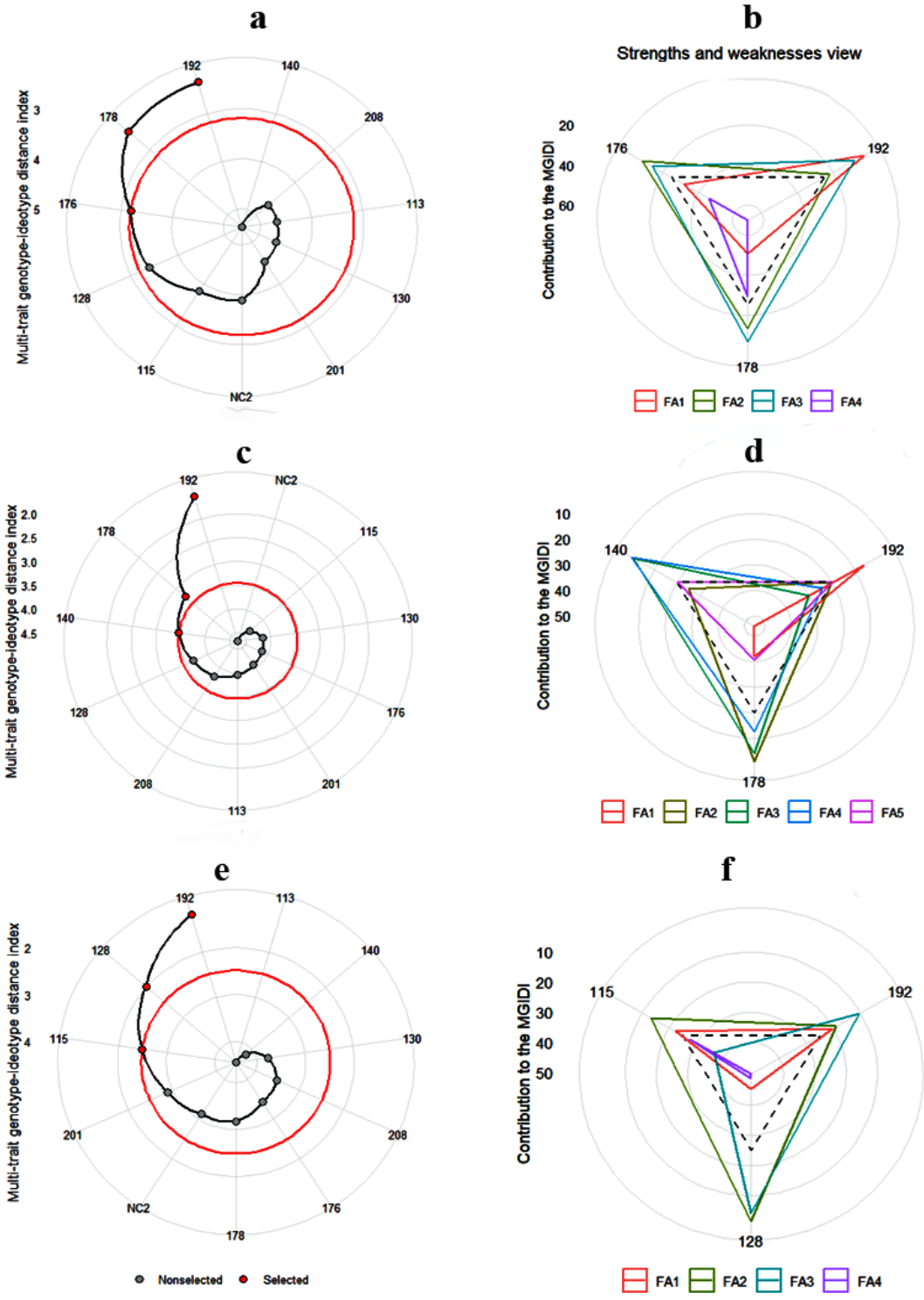


Figure 2. Genotype ranks obtained by the MGIDI index and the strengths and weaknesses of the selected genotypes based on the MGIDI index in Talesh (a, b), Masal (c, d), and Rasht (e, f). The selected genotypes are represented in red circles considering 30% selection intensity.

In Talesh, the selection differential was positive for all traits except for seed oil percentage and the number of secondary branches. Among the characteristics, oil yield (14.40%), seed yield (14.38%), and dry forage yield length (12.18%) showed the highest selection differential percentage (SD%) (Table 4). In Masal, the selection differential was positive for all traits except for pod number per plant. Seed yield (7.26%), seed length (7.49%), and seed diameter (6.55%) showed the highest SD% in this location (Table 4). In Rasht, selection differentials were positive for all traits. Oil yield (21.52%), seed yield (20.43%), and plant height (16.81%) showed the highest SD% (Table 4).

IGSI index

According to IGSI in Talesh, the genotypes 192 and 128 as evidenced by their highest IGSI values (0.80 and 0.72, respectively) showed the most favorable performance. Conversely, genotypes NC2, 140, and 208 displayed poorer performance, indicated by their low IGSI values of 0.22, 0.27, and 0.35, respectively (Table 5). In Masal, genotype 192 had the highest IGSI value (0.86), and genotypes

Table 4. Estimates of the grand mean (X_o), mean of the selected genotypes (X_s), and selection differential percentage (SD%) in three locations for groundnut.

Traits	Masal				Rasht				Talesh			
	Factor	X_o	X_s	%SD	Factor	X_o	X_s	SD%	Factor	X_o	X_s	SD%
BY	FA1	7425.30	7797.78	5.02	FA3	6816.67	7663.89	12.43	FA1	7615.30	8458.33	11.07
DFY	FA5	3788.64	3986.11	5.21	FA3	4037.88	4611.11	14.20	FA1	3419.70	3836.11	12.18
HI	FA5	28.94	29.52	2.00	FA1	21.31	22.95	7.71	FA1	33.13	34.20	3.23
NPP	FA1	21.64	21.59	-0.25	FA1	16.28	18.81	15.56	FA1	24.33	26.24	7.82
NSB	FA1	8.06	8.39	4.03	FA3	6.13	6.47	5.53	FA2	10.53	10.35	-1.74
OP	FA4	53.18	53.19	0.03	FA3	52.01	52.80	1.52	FA2	49.22	48.72	-1.01
OY	FA1	1139.32	1208.94	6.11	FA1	762.03	926.00	21.52	FA1	1242.56	1421.44	14.40
PD	FA2	13.88	14.75	6.21	FA2	15.19	15.46	1.84	FA2	13.33	13.67	2.50
PH	FA1	57.34	60.87	6.16	FA3	42.40	49.53	16.81	FA3	87.06	94.94	9.06
PL	FA2	31.07	32.48	4.54	FA4	34.77	35.49	2.09	FA3	33.27	33.68	1.24
SD	FA3	6.82	7.27	6.55	FA4	6.92	7.32	5.80	FA3	7.13	7.34	3.00
SL	FA2	14.56	15.65	7.49	FA4	15.16	15.83	4.38	FA4	14.74	15.36	4.25
SNPP	FA4	1.66	1.67	0.72	FA2	1.56	1.60	2.89	FA1	1.72	1.75	2.05
SW	FA1	59.13	61.79	4.50	FA4	57.48	58.42	1.63	FA1	60.85	62.04	1.96
SY	FA1	2144.62	2300.28	7.26	FA1	1466.74	1766.39	20.43	FA1	2549.55	2916.11	14.38
TPY	FA1	3631.06	3811.11	4.96	FA1	2778.79	3052.78	9.86	FA1	4195.61	4622.22	10.17

BY: Biomass; DFY: Dry forage yield; HI: Harvest index; NPP: Pod number per plant; NSB: Number of secondary branches; OP: Seed oil percent; OY: Oil yield; PD: Pod diameter; PH: Plant height; PL: Pod length; SD: Seed diameter; SL: Seed length; SNPP: Seed number per pod; SW: 100-seed weight; SY: Seed yield; TPY: Total pod yield.

176, 115, and NC2 exhibited inferior performance, indicated by their low IGSI values of 0.28, 0.30, and 0.38, respectively (Table 5). In Rasht, genotypes 192 and 128 with scores of 0.92 and 0.63 were superior. In contrast, the genotypes 113, 130, and 140 had the lowest IGSI values (0.16, 0.17, and 0.22, respectively), categorizing them as the weakest genotypes (Table 5).

The present study indicated that utilizing IGSI as a selection index can help identify suitable genotypes under varying environmental conditions. The IGSI index has been used to convert drought tolerance indices and various traits into a singular index or employ stability parameters to select superior genotypes. Zali *et al.* (2015) using the IGSI index based on various drought tolerance indices, assessed canola genotypes to identify tolerant genotypes. They indicated the efficacy of the IGSI index for the identification of drought-tolerant canola genotypes. They showed that the best genotype would be the one that has the least deviation from the positive ideal parameter and the highest deviation from the negative ideal parameter. Also, Hemadesh *et al.* (2021) employed this approach to identify the most superior barley cultivars. Our study based on the IGSI index identified genotypes 192 and 128 as superior groundnut genotypes across all three locations in northern Iran.

Conclusion

This study evaluated 16 traits of 11 groundnut genotypes across three locations in a two-year field experiment. The groundnut genotype 192 was selected as superior in all three locations based on MGIDI and genotypes 192 and 128 were superior groundnut genotypes based on the IGSI index.

Table 5. Ideal genotype selection index (IGSI) values, and the ranking of groundnut genotypes in the three studied locations.

Genotype	Code	Talesh		Masal		Rasht	
		IGSI	Rank	IGSI	Rank	IGSI	Rank
ICG130	130	0.47	8	0.42	5	0.17	10
ICG140	140	0.27	10	0.39	6	0.22	9
ICG113	113	0.50	7	0.38	8	0.16	11
ICG115	115	0.52	4	0.30	10	0.39	4
ICG128	128	0.72	2	0.55	2	0.63	2
ICG176	176	0.52	3	0.28	11	0.31	8
ICG178	178	0.51	5	0.50	4	0.32	6
ICG192	192	0.80	1	0.86	1	0.92	1
ICG201	201	0.50	6	0.51	3	0.39	5
ICG208	208	0.35	9	0.38	7	0.31	7
NC2	NC2	0.22	11	0.38	9	0.40	3

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Ethical considerations

The authors avoided data fabrication and falsification.

Conflict of interest

The authors declare that they have no conflict of interest with any organization concerning the manuscript's subject.

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Evaluation of grain yield stability of barley genotypes using additive main effects and multiplicative interaction model

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Abstract

Objective: Barley as one of the most important cereals is crucial to supplying the required energy for both humans and animals. Grain yield is strongly influenced by environmental conditions and breeders often determine the stability of high-yielding genotypes across various locations before recommending a cultivar for release.

Methods: For this research, 18 barley genotypes were tested at four different research stations including Gachsaran, Moghan, Khorramabad, and Gonbad in Iran. The additive main effects and multiplicative interaction (AMMI) model was used to identify the stable genotypes.

Results: Partitioning of the GE interaction indicated that the first interaction principal component axes (IPCA) captured 35.9% of the interaction sum of squares. However, the five IPCAs accounted for 87.4% of the total interaction. Moreover, 13 AMMI-based stability statistics were calculated. Using cluster analysis, AMMI stability indices were divided into four groups. This analysis showed that most indices do not correlate with the grain yield. AMMI indices were placed in separate groups with large distances from the grain yield. However, the yield stability index (YSI) showed the highest correlation with grain yield, and based on that, G13, G18, and G11 genotypes were recognized as superior genotypes. G13 and G18 genotypes had an acceptable performance in two years and four locations, so they may be regarded as genotypes with both high yield and stability in these environments. However, by using the AMMI2 mega-environmental analysis to select suitable cultivars for each mega-environment, the target region was divided into three sub-regions, and the winner genotypes of each sub-region were identified. The first mega-environment consisted of the areas Gonbad and Ghachsaran, where genotype G14 was the winner; the second mega-environment consisted of the Khorramabad area, where genotype G2 was the winner, and the third mega-environment included only the Moghan area, where genotype G13 was the winner.

Conclusion: In conclusion, the AMMI2 mega-environmental analysis identified three mega environments and the best genotypes specifically adapted to these environments. Since two test areas Gonbad and Ghachsaran were located in the first mega-environment, it seems that the performance ranking of the test genotypes in these areas will be almost the same, and in coming years, the test can be performed in only one of these areas.

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Introduction

Barley (*Hordeum vulgare* L.) is widely cultivated and used in Iran and the world. Barley is the fourth most important grain product in the world after wheat, rice, and maize, and its grains are used as animal feed and forage, malt beverage, human food, soil amendment, and have medicinal value (El-Hashash and El-Absy 2019). According to FAO (2022), barley cultivated area in Iran during the 2022 growing season was about 1.65 million hectares and harvested production was estimated at three million tons.

Achieving high-yielding and stable barley varieties is considered one of the most important goals for breeding this crop. The crop performance is strongly influenced by environmental conditions so broad changes in their performance are observed both between years in a location and between locations in a year (Pacheco *et al.* 2005). The different response of genotypes from one environment to another is called genotype × environment (GE) interaction. GE interaction makes it difficult to compare genotypes over a range of years or locations and plays a key role in developing strategies for crop improvement (Yan and Hunt 1998). Depending on whether GE interactions are controlled or not, it can lead to gains or losses in breeding programs (Yan and Kang 2003). To investigate the GE interaction, varieties should be grown in a wide range of environments and the ultimate goal of the plant breeding programs is the selection of high-yielding and high-compatible genotypes for these environments. However, it is difficult and almost impossible to find genotypes that can be highly adapted to all environmental conditions. Therefore, plant breeders manage GE interaction by selecting genotypes with specific adaptations.

Various statistical methods including univariate, multivariate, and nonparametric have been developed to determine the grain yield stability to explain the information included in the GE interaction data matrix (Finlay and Wilkinson 1963; Shukla 1972; Lin and Binns 1988; Fox *et al.* 1990; Kang and Pham 1991). Each of them reflected different aspects of stability and no single method can adequately explain cultivar performance across environments (Annicchiarico 1997). The response level of genotypes in a given environment can be better predicted using multiplicative

models such as the additive main effects and multiplicative interaction model (AMMI) (Gauch 1988; Gauch and Zobel 1988, 1989). Recently, due to higher efficiency, the tendency of most researchers to use multivariate methods has increased. AMMI is a popular extension of the analysis of variance and principal component analysis (PCA) for studying the GE interaction. This model is a hybrid analysis that incorporates both the additive and multiplicative components of the two-way data structure. AMMI extracts genotype and environment main effects and uses principal component axes to explain patterns in the GE interaction matrix (Romagosa and Fox 1993; Kanouni *et al.* 2023). Various stability indices have been introduced based on the AMMI model, including averages of the squared eigenvector values (Zobel 1994), AMMI's stability values (ASV) (Purchase 1997), the sum of the absolute values of the IPC scores (SIPC) (Sneller *et al.* 1997), AMMI stability index (ASI) (Jambhulkar *et al.* 2017), D index (Annicchiarico 1997), and AMMI based stability parameter (ASTAB) (Rao and Prabhakaran 2005). The use of the AMMI stability parameters permits the evaluation of yield stability after reducing the noise from the GE interaction effects (Sabaghnia *et al.* 2012). Also, AMMI allows the determination of mega-environments which are defined as a subset of locations with homogeneous environmental conditions, where the performance of certain genotypes is more similar through the years (Kandus *et al.* 2010). The identification of mega-environments is important for the following reasons. The first reason is that marginal environments show a high GE interaction effect because they are often affected by various stresses. Therefore, genotypes that win in highly favorable environments may rank poorly in these conditions. On the other hand, within favorable environments, conventional management procedures in the past have produced a rather coherent environment easily targeted by plant breeders (Gauch and Zobel 1997). In general, most plant breeders feel that instead of selecting one genotype for all environments, they should select genotypes suited to the conditions of mega-environments.

This study was conducted to understand the extent and causes of GE interactions using the AMMI methodology in barley production areas of Iran.

Materials and Methods

Experiments

The data in this study were obtained from the sets of barley yield trials conducted for three years (2017- 2019) in different areas of Iran. In each year, 16 genotypes along with two check cultivars (Mahoor and Khorram) were tested at four different research stations. All the selected research stations are located in warm climates including Gachsaran, Moghan, Khorramabad, and Gonbad. The names, pedigree, and origin of studied genotypes are given in Table 1. At each environment, a

randomized complete block design with four replications was used. The experimental plots consisted of six rows with 6 m long and 0.2 m spacing between rows, which resulted in a plot area of 7.2 m^2 and the seeding rate was 350 seeds per m^2 for each genotype. The seeds were planted with an experimental planter (Wintersteiger). Weeds were controlled by a chemical herbicide (Granstar). At the harvest time, grain yield was determined for each genotype at each plot after removing marginal plants in each test environment.

Table 1. Genotype code, name, or pedigree of 18 studied barley genotypes tested in four locations in Iran.

Genotype code	Name or pedigree of the genotype
G1	Mahoor as check
G2	Khorram as check
G3	HART-BAR/CANELA//MSEL CBSS01Y00777T-Z-0Y-10M-0M-1M-0Y(PRBYT2010-11-44)
G4	CANELA/CHERI CBSS01Y00007S-0Y-6M-0M-1M-0Y(PRBYT2010-11 -28)
G5	Moroc9-75//WI2291/WI2269 ICB93-1132-0AP-31AP-0AP-5TR-8AP-0AP(PRBYT2010-11-81)
G6	6B89.2027/5/ATACO/BERMEJO//HIGO/3/CLNB/80.5138//GLORIABAR/COPAL/4/CHEVRONBAR/6/LEGACY CBSS01Y00858T-B-0Y-9M-0M-2M-0Y(PRBYT2010-11-31)
G7	Moroc9-75//WI2291/WI2269 ICB93-1132-0AP-31AP-0AP-6TR-38AP-0AP(PRBYT2010-11-85)
G8	MNS1//CALI92/ROBUST CBSS01Y00154S-0Y-10M-0M-3M-0Y(PRBYT2010-11-33)
G9	Moroc9-75//WI2291/WI2269 ICB93-1132-0AP-31AP-0AP-5TR-20AP-0AP(PRBYT2010-11-82)
G10	MSEL//CLI18/E.QUEBRACHO CBSS01Y00023S-0Y-10M-0M-1M-0Y(PRBYT2010-11-26)
G11	Giza127/4/Gloria'S'/Saida//Mtn'S'/EH165/3/LBIran/Una80//Lignee640 ICB97-0488-0AP-21AP-6TR-0AP(PRBYT2010-11-20)
G12	MSEL//CLI18/E.QUEBRACHO CBSS01Y00023S-0Y-10M-0M-1M-0Y(PRBYT2010-11-42)
G13	WI2291//Apm/PI000046/3/Hml-02/4/Arda/Moroc9-75 ICB01-0006-0AP-28AP-0AP(PRBYT2010-11-99)
G14	WI2291/4/7028/2759/3/6982//Ds/Apro/5/Zanbaka/3/ER/Apm//Lignee131I CB94-0590-0AP-9A-0AP-0AP-14AP-0AP-9AP-0AP(PRBYT2010-11-93)
G15	Moroc9-75//WI2291/WI2269 ICB93-1132-0AP-33AP-0AP-18TR-41AP-0AP(PRBYT2010-11-88)
G16	Moroc9-75//WI2291/WI2269 ICB93-1132-0AP-31AP-0AP-6TR-46AP-0AP(PRBYT2010-11-86)
G17	Moroc9-75//WI2291/WI2269 ICB93-1132-0AP-13AP-0AP-19AP-0AP(PRBYT2010-11-122)
G18	Hml-02//WI2291/Bgs ICB83-1554-1AP-1AP-6AP-0AP-23AP-0AP-13AP-0AP(PRBYT2010-11-121)

Statistical analysis

Analysis of variance was carried out for each test environment. Homogeneity of residual variances was verified by Bartlett's homogeneity test, before carrying out the combined analysis of variance. The AMMI model was used to study the GE interaction as follows:

$$Y_{ij} = \mu + \alpha_i + \beta_j + \sum_N \lambda_n \gamma_{in} \delta_{jn} + \rho_{ij} + \varepsilon_{ij}$$

Where Y_{ij} is the observed mean yield of ith genotype in the environment j, μ the grand mean, α_i the genotype main effect, β_j the environment main effect, λ_n the eigenvalue of the interaction

principal component analysis (IPCA), γ_{in} and δ_{jn} are the genotype and environment scores for the IPCA axis, ρ_{ij} interaction residual, N the number of IPCA retained in the model, and ε_{ij} the random error term.

MATMODEL software (Gauch 2007) and the associated program, AMMIWINS, were used for the AMMI and mega-environment analyses. AMMIWINS identifies each mega-environment by its winning genotypes, counts its number of wins, and calculates the average expected yield over those environments included in that mega-environment.

Thirteen stability statistics derived from the AMMI analyses were used. The $SIPC_1$, $SIPC_v$, and $SIPC_f$ are sums of the absolute value of the IPC scores: $\sum_{n=1}^{N} \lambda_n^{0.5} \gamma_{in}$ for the *i*th genotype (for $SIPC_1$, N was one; for $SIPC_v$, N was the number of IPC that were retained in the AMMI model via cross-validation test; for $SIPC_f$, N was the number of IPCs that were retained in the AMMI model via Gollob's F test); EV_1, EV_v, and EV_f, which were suggested by Zobel (1994), are averages of the squared eigenvector values: $\sum_{n=1}^{N} \frac{\gamma_{in}^2}{N}$ (for EV_1, N was one; for EV_v, N was the number of IPC that were retained in the AMMI model via cross-validation test; for EV_f, N was the number of IPCs that were retained in the AMMI model via Gollob's F test). AMMI's stability values (ASV) were calculated using the following formula (Purchase 1997):

$$ASV = \left(\frac{SS_{IPCA1}}{SS_{IPCA2}}(IPCA1)^2(IPCA2)^2\right)^{0.5}$$

Where, $SSIPCA_1$ and $SSIPCA_2$ are the sums of squares of the interaction of first and second IPC, respectively. AMMI stability index (ASI) has been used to get a quantitative value for the stability analysis by Jambhulkar *et al.* (2017), which helps in the interpretation of the results:

$$ASI = ([PC_1^2 \times \theta_1^2] + [PC_2^2 \times \theta_2^2])^{0.5}$$

θ_1, θ_2, and θ_n are percentage sum of squares explained by the 1st, 2nd, and nth IPC, respectively.

Modified AMMI stability index (MASI) and Modified AMMI stability value (MASV) are the extensions of ASV and ASI as implied by Ajay *et al.* (2018, 2019):

$$MASV = \left(\sum_{n-1}^{N-1}\left(\frac{SSIPC_n}{SSIPC_{n+1}}\right) \times (PC_n)^2 + (PC_N)^2\right)^{0.5}$$

$$MASI = \left(\sum_{n=1}^{N} PC_n^2 \times \theta_n^2\right)^{0.5}$$

Rao and Prabhakaran (2005) used the ASTAB index with the following formula:

$$ASTAB = \sum_{n=1}^{N} \lambda_n \gamma_{in}^2$$

Annicchiarico (1997) used the D index that measured as the unsquared Euclidean distance from the n =1, 2, …, N PC axes included in the AMMI model:

$$D = \left(\sum_{n=1}^{N} (\lambda_n \gamma_{in})^2 \right)^{0.5}$$

Zali *et al.* (2012) used the sum across environments of the absolute value of GEI modeled by AMMI (AVAMGE):

$$AVAMGE = \sum_{i=1}^{N} \sum_{j=1}^{M} \left| \lambda_n \lambda \gamma_{in} \delta_{jn} \right|$$

Zali *et al.* (2012) also used the absolute value of the relative contribution of IPCs to the interaction (Za):

$$Za = \sum_{i=1}^{N} |\theta_n \gamma_{in}|$$

The yield stability index was calculated using the equation YSI = RASV + RY (Farshadfar 2008). The terms RASV and RY in this context stand for the genotype mean yield ranking across environments and AMMI stability value ranking, respectively.

Statistical packages for the combined analysis of variance, AMMI analysis, and twelve AMMI stability indices were calculated using RStudio, R version 4.3.2 by using 'Agricolae' and 'Metan' packages.

Results and Discussion

An annual mean yield of the 18 investigated barley genotypes across 12 environments (a combination of four locations and three years) is shown in Table 2. Bartlett's test which was performed to assess homogeneity of variances before combined analysis, showed non-significance ($p > 0.05$) Chi squares statistic, indicating homogeneity of variances. The combined analysis of variance for grain yield showed highly significant differences among genotypes, environments, and their interactions (Table 3). This analysis showed that 54% of the total sum of squares was attributable to environmental effects (E), 3.4% to genotypic effects (G), and 15.4% to GE interaction. This result shows that genotypes performance is greatly influenced by the environment. A large sum of squares for environments indicated that the environments were diverse. The significant GE interaction for yield confirms the

differential response of genotypes to environments. A significant large-scale GE interaction could mean that there are at least two target breeding environments with different climatic conditions in a region, instead of one (Pour-Aboughadareh *et al.* 2022). Since the GE interaction contributes more to the overall variance, there is a greater likelihood that certain cultivars performed better in a particular environment.

Two basic approaches have been used to determine the optimal number of multiplicative terms to be retained in the GE interaction component. The result of full cross-validation across 1000 randomizations of the data is shown in Table 3. The full cross-validation yielded minimum root mean square prediction differences (RMSPD) at the first component, although the RMSPD values for the other four components were very similar in size and any could be chosen to represent the optimum number of components for the model. Also, partitioning of GE interaction indicated that the AMMI-5 model described the GE interaction patterns for yield using the first five IPCA scores based on F Gollob's. Results from the AMMI analysis also showed that the first PC axis (IPCA1) of the interaction captured 35.9% of the interaction sum of squares. Similarly, the second PC axis (IPCA2) explained a further 19.4% of the GE interaction sum of squares. The five IPCAs accounted for 87.4% of the total interaction.

The results for average grain yield and the estimated AMMI-based stability parameters for 18 tested genotypes are shown in Table 4. Genotype G13 showed the best average grain yield in a total

Table 2. Annual mean yield (kg.ha^{-1}) of 18 barley genotypes tested in four locations in Iran.

Genotype code	Cropping season											
	2016-2017				2017-2018				2018-2019			
	E1	E2	E3	E4	E1	E2	E3	E4	E1	E2	E3	E4
G1	1062	2306	2830	2017	1611	2207	2309	3255	2161	2481	3213	2591
G2	1473	2548	3608	2031	1695	2289	2875	3194	1918	2416	2274	2201
G3	1105	2012	2653	2132	1479	1997	2566	2792	1854	2579	2611	2489
G4	1119	2078	2306	1927	1307	2374	2303	3289	1494	2676	2494	2681
G5	1321	2173	3378	1627	1629	1809	2713	3230	1938	2618	2560	2285
G6	1234	2135	2431	1562	1475	2114	2471	2508	1716	2124	2053	2083
G7	1174	2170	2153	1784	1416	2217	2925	3072	1658	2461	2372	2223
G8	1428	1833	2731	1623	1859	2016	2188	3087	2290	2318	2405	2397
G9	1334	2247	3166	1267	1916	2009	2529	3367	2499	2809	1627	2149
G10	1051	2223	2302	1865	1390	1868	2446	2502	1734	2560	2199	1806
G11	1200	1973	2807	1386	1826	2046	2838	2633	2451	2568	2873	2389
G12	1266	2243	2381	1802	1718	2067	2623	2986	2170	2461	2559	2792
G13	1312	2350	3171	2151	1897	2557	2733	3086	2481	2398	2466	2504
G14	1581	2081	1988	1902	1985	1709	2175	3228	2390	2543	2637	3241
G15	1104	2209	2689	2132	1637	1832	2390	2872	2171	2376	2321	2443
G16	1155	2437	2618	1964	1588	1974	2176	2553	2020	2366	2200	2045
G17	1309	2177	2670	1280	1786	2162	3014	2743	2264	2579	1735	1870
G18	1351	2108	2656	2097	1650	2056	2861	3395	1949	2649	2597	2413

E1: Gachsaran; E2: Moghan; E3: Khorramabad and E4: Gonbad.

of 12 test environments, followed by genotypes G2, G1, and G18, while G6, G10, and G16 showed the lowest performance. According to the minimum values of EV_1 and EV*v* parameters (these two parameters were similar and thus, only EV_1 was calculated), genotypes G8, G10, and G11 were the most stable genotypes (Table 4). Genotypes G12, G3, G6, and G13 were the most stable genotypes based on EV_f parameter. Genotypes that are introduced by EV as stable genotypes often indicate low mean yield across test environments. However, G10 and G6 had a high average performance among the investigated genotypes and they can be considered as the most favorable genotypes. According to the $SIPC_1$ statistic, G10, G8, and G11 were the most stable genotypes. Based on the $SIPC_f$ statistic, which uses the sum of the absolute value of eigenvalues of the remaining components, G15 was the most stable genotype, followed by G3, G12, and G6. Genotypes G14, G9, and G17 were ranked as the least stable based on both $SIPC_1$ and $SIPC_f$ scores. According to the ASV parameter, which measures the distance from the genotype coordinate point to the origin in a two-dimensional scatter diagram of IPCA2 against IPCA1 scores, genotypes with the lowest ASV values are considered the most stable. Based on the ASV, the most stable genotypes for grain yield were G15, G16, and G6 (Table 4). G14 and G9 were ranked the least stable because they had the highest ASV values. The

Table 3. Additive main effects and multiplicative interactions (AMMI) analysis of variance for grain yield (kg.ha^{-1}) of the 18 barley genotypes tested in 12 environments.

SOV	df	Mean squares	Gollob's F	RMSPD†	Explained (%)
Environment (E)	11	16.561 **			54.0
Year (Y)	2	11.170			12.3
Location (L)	3	32.710			53.9
L × Y	6	10.287 **			33.9
Replication/E	36	0.550			
Genotype (G)	17	0.675 **			3.4
G × E	187	0.278 **			15.4
IPCA 1	27	0.691	4.977 **	0.417††	35.9
IPCA 2	25	0.403	2.903 **	0.420	19.4
IPCA 3	23	0.297	2.141 **	0.424	13.1
IPCA 4	21	0.254	1.826 *	0.426	10.2
IPCA 5	19	0.240	1.729 *	0.425	8.8
G × Y	34	0.330 *			21.6
G × L	51	0.379 **			37.1
IPCA 1	19	0.153	3.041 **	0.269	44.9
IPCA 2	17	0.140	2.793 **	0.267††	36.9
G × L × Y	102	0.210 **			41.3
Pooled error	648	0.142			27.2

* and **: Significant at the 0.05 and 0.01 probability levels, respectively.
†RMSPD: The root mean square prediction differences, predicted by MATMODEL software with repeating 1000 times splitting data.
††: The selected model with a minimum root mean square predictive difference.

ranking of genotypes in terms of stability by the ASI index was almost similar to ASV. Therefore, genotypes G16, G15, and G6 were the most stable genotypes.

Genotype G6, followed by G13, G3, G12, and G18, showed higher stability compared to other tested genotypes based on the ASTAB. According to AVAMGE, G15, G12, G6, and G13 were the most stable genotypes. Based on the distance between the IPCA points and the origin in space (D index), genotypes G6, G15, G13, and G18 showed the greatest stability compared to the other genotypes. The MASV and MASI indices identified genotypes G11 and G6 as superior genotypes and G14, G9, and G17 were ranked the least stable because they had the highest MASV and MASI values. The YSI index was used to exploit both yield and stability at the same time, and the most stable genotypes with the highest grain yield were G13, G18, and G11, which had the lowest YSI values.

Table 4. Average yields ($kg.ha^{-1}$) and AMMI stability parameter estimates of 18 barley genotypes tested in 12 environments.

Genotype	Yield	ASTAB	ASI	ASV	YSI	AVAMGE	D	EV_1	EV_f	MASI	MASV	$SIPC_1$	$SIPC_f$	ZA
G1	2337	416.7	4.43	22.84	16	2283.7	818.1	0.067	0.055	4.86	30.70	12.04	38.76	0.19
G2	2377	519.7	6.00	30.96	17	2422.6	955.5	0.109	0.063	6.16	36.52	15.33	46.72	0.24
G3	2189	133.0	3.03	15.63	21	1310.5	484.2	0.025	0.016	3.07	18.50	7.29	22.06	0.12
G4	2171	556.0	5.27	27.20	27	2631.4	935.7	0.088	0.076	5.56	35.07	13.76	45.29	0.22
G5	2273	417.1	3.00	15.47	16	1935.3	756.2	0.031	0.064	3.68	27.02	8.24	42.04	0.18
G6	1992	120.6	1.47	7.57	21	1170.6	406.0	0.006	0.018	1.85	14.56	3.52	23.50	0.10
G7	2135	441.8	2.34	12.05	22	1987.1	774.3	0.007	0.067	3.25	27.57	3.99	41.35	0.17
G8	2181	216.0	2.41	12.42	21	1696.9	569.8	0.001	0.029	2.63	20.86	1.18	23.93	0.10
G9	2243	900.6	8.06	41.56	26.00	3437.5	1258.1	0.196	0.109	8.19	48.16	20.60	54.45	0.29
G10	1995	234.5	1.80	9.28	23	1653.1	562.3	0.001	0.036	2.29	20.01	1.16	30.99	0.12
G11	2249	705.2	1.69	8.73	12	2570.5	904.5	0.001	0.123	2.94	29.99	1.66	48.92	0.17
G12	2255	134.6	3.37	17.39	19.00	1168.5	500.5	0.037	0.015	3.43	19.39	8.99	22.46	0.12
G13	2425	127.6	1.87	9.66	8	1212.4	420.0	0.012	0.020	2.15	15.02	5.15	23.88	0.10
G14	2288	1020.3	9.06	46.74	23	3673.9	1371.5	0.233	0.115	9.09	52.84	22.43	56.58	0.32
G15	2181	146.6	1.16	6.01	13	1082.2	417.8	0.005	0.025	1.63	13.84	3.24	21.78	0.08
G16	2091	349.8	1.02	5.28	17	1780.3	627.0	0.002	0.063	1.96	20.21	1.98	31.80	0.11
G17	2132	689.8	7.04	36.33	31	3252.7	1106.4	0.172	0.083	7.35	42.50	19.27	49.81	0.26
G18	2315	146.4	1.76	9.09	9	1254.6	441.9	0.007	0.023	2.03	15.65	3.99	25.49	0.11

ASTAB: AMMI-based stability parameter; ASI: AMMI stability index; ASV: AMMI's stability values; YSI: Yield stability index; AVAMGE: Sum across environments of the absolute value of GEI modeled by AMMI; D: The index that was measured as the unsquared Euclidean distance; EV_1, EV_f: Averages of the squared eigenvector values; MASI: Modified AMMI stability index; MASV: Modified AMMI stability value; $SIPC_1$, $SIPC_f$: Sums of the absolute value of the IPC scores; Za: Absolute values of the relative contribution of IPCs to the interaction.

Relationship between AMMI-based stability indices and grain yield

To better understand the relationships of different AMMI indices with grain yield, a hierarchical cluster analysis based on unweighted values of 13 AMMI stability indices and average yield, was executed (Figure 1). Spearman's coefficient of correlation was used as a similarity measure required in the unweighted pair-group average method. The dendrogram showing the hierarchical classification of stability methods is illustrated in Figure 1. The 13 AMMI stability indices were classified into four groups (Figure 1). The grouping of indices showed that most of them do not correlate with the grain yield. AMMI indices were placed in separate groups with a large distance from the grain yield.

We found those EV_1 and $SIPC_1$ parameters that use only one significant PC, clustered together (Group 1), indicating that the two measures were similar in their power of classifying genotypes

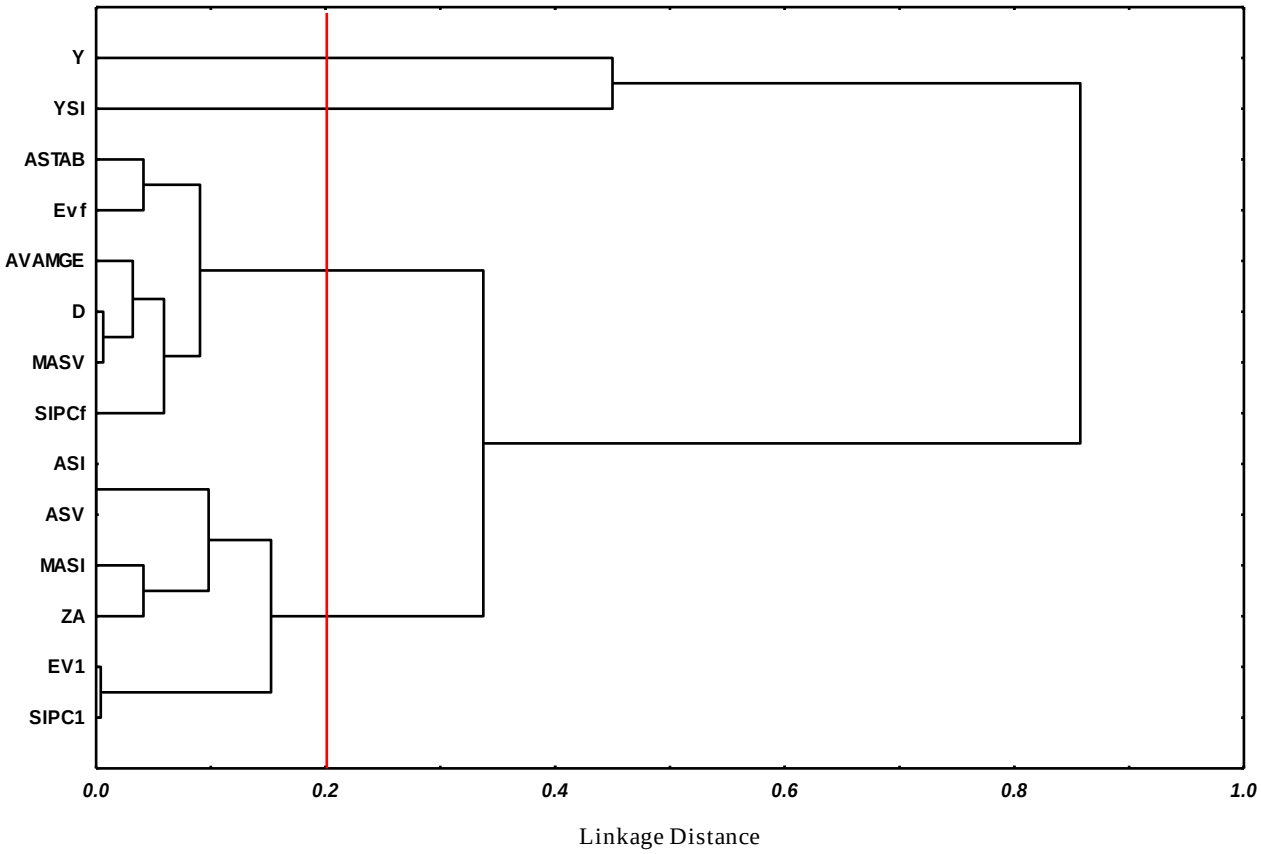


Figure 1. Dendrogram showing the hierarchical classification of average yield and 12 AMMI stability indices based on Spearman's correlation coefficients for 18 barley genotypes.
Y: Yield; YSI: Yield stability index; ASTAB: AMMI-based stability parameter; EV1, EVf: Averages of the squared eigenvector values; AVAMGE: Sum across environments of the absolute value of GEI modeled by AMMI; D: Index measured as the unsquared Euclidean distance; MASV: Modified AMMI stability value; SIPC1, SIPCf: Sums of the absolute value of the IPC scores; ASI: AMMI stability index; ASV: AMMI's stability values; MASI: Modified AMMI stability index; Za: Absolute values of the relative contribution of IPCs to the interaction.

according to their stability under different environmental conditions. According to these methods, the most stable genotypes were G8, G10, and G11.

Group 2 included ASI, ASV, MASI, and Za. The first two indices used the first two main components (IPCA1 and IPCA2) along with their sum of squares to estimate the stability of genotypes and had almost similar results in the grouping of genotypes. When more than two main components are significant in the ASI methods, part of the information in the data becomes unusable. To solve this problem, Ajai *et al.* (2018) used a modified version that would cover all available IPCAs. The MASI and Za use more significant components than ASV and ASI, and considering that 5 IPCA were significant in our experiment, their results can be more reliable. According to these methods, the most stable genotypes were G16, G15, and G6.

Group 3 included AVAMGE, D, MASV, $SIPC_f$, EV_f, and ASTAB which used maximum significant PCs. These indices which were not generally associated with yield, were evaluated independently for the grain yield. Based on these indices, the most stable genotypes for the grain yield were G15, G12, G13, G6, and G3.

Group 4 included YSI, which was the closest index to the grain yield, and based on this index, G13, G18, and G11 genotypes were chosen as the superior genotypes.

Mega-environment analysis

For a long time, most breeders used the term stability to denote a genotype that always showed consistent performance under all environmental conditions. This idea of stability corresponds to the concept of homeostasis, which is widely used in quantitative genetics and may be considered as biological (static) concept of stability (Becker and Leon 1988). Biological stability is not acceptable to most plant breeders who prefer the agronomic concept of stability (Becker 1981; Mortazavian and Azizi-nia 2014). In the static or biological concept, the performance of a genotype remains unchanged regardless of the environmental conditions and stable genotypes do not react to the improvement or environmental conditions (Lin *et al*. 1986). Therefore, according to these parameters, stable genotypes are recommended for locations where growing conditions are unfavorable. This concept of stability is useful for quality traits, disease resistance, or stress characteristics (Cheshkova *et al*. 2020). In developing countries such as Iran, due to limited access to agricultural inputs, the need for stable genotypes that can cope with environmental changes increases. In this situation where the availability of agricultural inputs is not guaranteed, the selection of genotypes for different locations should be done with greater precision. To meet the need of different areas for compatible cultivars, especially areas with high fertility, we used AMMI2 mega-environmental analysis to select suitable

cultivars for each mega-environment. Because the environmental factor in this analysis is a combination of locations and years, it is not helpful when recommendations of cultivars to specific locations are required. Therefore, we used the set of cultivar × location means averaged across years. Partitioning of the genotype by location (GL) interaction indicated that the AMMI2 model described the GL interaction pattern for yield using the first two IPCA scores based on Gollob's F test. The two principal components explained only 81.8% of the GL interaction sum of squares (Table 3). The first mega-environment consisted of the areas Gonbad and Ghachsaran, where the genotype G14 was the winner (in terms of yield and stability). The second mega-environment consisted of the Khorramabad area, where genotype G2 was the winner. The tertiary mega-environment represented the Moghan area, where genotype G13 was the winner (Table 5). If two or more test areas are located in the same mega-environment, it means that they have no crossover interactions with each other. Therefore, the performance ranking of the test genotypes in these areas will be almost the same, and in the coming years, the test can be performed in only one of these areas.

Table 5. AMMI2 mega-environments and their winning genotypes for the 18 barley genotypes grown in four locations at three years.

AMMI mega-environments	Winner genotypes	Expected values for yield (t.ha^{-1})
Mega-environment 1	G14	
Ghachsaran		1.986
Gonbad		2.791
Mega-environment 2	G2	
Khorramabad		2.919
Mega-environment 3	G13	
Moghan		2.435

Conclusion

The barley genotypes investigated in this study exhibited wide variability for grain yield. AMMI stability indices were classified into four different groups using cluster analysis, and each of them introduced their own superior genotypes. The distinguishing characteristic of each group was the number of components that the indices used to evaluate the genotypes. The AMMI procedure used in this study indicated a more complex interaction which required five PC axes to account for a considerable amount of variation due to GE interaction. Therefore, it was clear the parameters that use all five IPCAs, were better than those parameters that used only the first IPCA. Finally, based on grain yield and all AMMI stability indices, the genotypes G13 and 18 had good yield performance and stability in several environments and can be a candidate for introduction as a new cultivar. When

the contribution of the GE interaction to the total variance is higher, the genotypes are more likely to have evolved for a specific environment. Therefore, the target region was divided into different sub-regions (mega-environments) which were relatively uniform in terms of genotypic responses and the winner genotypes of each sub-region were identified. Based on the mega-environmental analysis, the genotype G13, which is recognized as the superior genotype based on the stability indicators, is only recommended for the Moghan area. While the genotype G2 was recommended for Khorramabad and G14 for Gonbad and Gachsaran.

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Ethical considerations

The authors avoided data fabrication and falsification.

Conflict of interest

The authors declare that they have no conflict of interest with any organization concerning the subject of the manuscript.

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In vitro propagation of *Physalis alkekengi* L. using axillary buds

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Abstract

Objective: Chinese lantern (*Physalis alkekengi* L.) is an important medical herb due to its physalin. This plant is widely used to treat various diseases. Therefore, developing a repeatable micropropagation method for this plant would be valuable.

Methods: In this study, shoot tips and axillary buds of Chinese lantern were treated with different growth regulators (IAA, NAA, BAP, GA3, and IBA) with various concentrations. In another experiment, the effect of salt concentration onthe MS medium was investigated.

Results: The results showed that using axillary buds as an explant in the full-strength MS, supplemented with 2 mg/L BAP and 0.5 mg/L IAA, resulted in a higher number of shoots per explant (3±1.22) and the highest number of nodes (13±1.22) and stem length (8.04±1.92). Compared with other strengths of MS salts, the full-strength MS medium, supplemented with 2 mg/L BAP and 0.5 mg/L IAA, or 0.5 mg/L BAP alone, produced the highest number of shoots per explant, number of nodes, and stem length. However, the MS salt supplemented with a low concentration of BAP (0.5 mg/L) alone looks economically feasible. Decreasing the salt concentration of the basal medium decreased all micropropagation characteristics and in most cases, the decrease was statistically significant. The addition of GA3 to the combination of 2 mg/L BAP and 0.5 mg/L IAA, significantly improved stem elongation at the concentration range of 0.5 – 1.5 mg/L The number of roots was higher (2±00) after 30 days on the MS basal medium when supplemented with 1 and 2 mg/L NAA or 2 mg/L IAA. The well-rooted plantlets were isolated and transplanted to paper cups for hardening and the well-established plants were transferred to the field for acclimatization.

Conclusion: Our results showed that the full strength of MS medium, supplemented with 2 mg/L BAP plus 0.5 mg/L IAA and 0.5–1.5 mg/L GA3 improved micropropagation characteristics of *P. alkekengi.*

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Introduction

Herbal medicine is gaining *more* popularity *in* today's medical profession. According to a report by the World Health Organization (WHO), 80% of people in developing countries use medicinal plants for primary health care (Behrens 2004). *Physalis* is of great commercial importance because it is rich in antioxidants, carotenoids, and some other important compounds (Lima *et al.* 2020). *Physalis alkekengi* L. (Ground cherry) belonging to Solanaceae is an indigenous plant in Iran and some other countries. The extracts of this plant may be used as a remedy for diseases such as inflammation, rheumatism, arthritis, etc. *P. alkekengi* contains citric acid, physalins, and vitamin C (Raj *et al.* 2015). *Physalis* fruits and shoots may treat intestinal and digestive issues and are applied as antimutagenic, anticoagulant, antispasmodic, and antileukemic agents (Shariff *et al.* 2006; Helvaci *et al.* 2010). Thus, this herb has gained the interest of the pharmaceutical industry.

Micropropagation is the process of vegetative growth and propagation from all types of plant tissues, even seeds (Zhou *et al.* 2006). This method vastly leads to fast propagation, conservation, and improved production of secondary metabolites in medicinal plants (Manzoor *et al.* 2019). Propagation of *P. alkekengi* through seed is difficult because of seed dormancy. Therefore, *in vitro* tissue culture may serve as an alternative for propagation of this plant (Zhang *et al.* 2019). Efforts for micropropagation by shoot regeneration have not been reported in *P. alkekengi* yet. However, some reports are available on *in vitro* shoot proliferation and plant regeneration of other *Physalis* species (Afroz *et al.* 2009; Ramar *et al.* 2014; Guney *et al.* 2016; de Jesús Romo-Paz *et al.* 2021). Thus, this study was conducted to efficiently develop an *in vitro* regeneration protocol for *P. alkekengi* L. through the internode culture.

Materials and Methods

Healthy seeds of *P. alkekengi* L. were collected from Arasbaran, East Azarbaijan Province, Iran. The sterilization of seeds was done by dipping them in %2.5 sodium hypochlorite for 15 minutes followed by continuous shaking. The seeds were stored in the refrigerator for 14 days to break the seed dormancy in the sterile packages. After 14 days, the seeds were transferred to the culture vials containing ½ MS medium for germination. Axillary buds of *P. alkekengi* L. were collected from one-month *in vitro* plants. They were put on the MS medium (Murashige and Skoog 1962) with various combinations and concentrations of plant growth regulators such as BAP, NAA, and IAA. The sucrose content of the media was 3% and the pH of the media was maintained at 5.8 before autoclaving at 15-pound pressure for 15 minutes. Gelling of the media was done with 0.8% agar

powder (tissue culture grade).

In the next step, the effect of medium salt strength (MS, ½MS, and ¼MS) with three selected combinations of plant growth regulators (2 mg. l-1 BAP, 0.5 mg. l-1 BAP, and 2 mg. l-1 BAP plus 0.5 mg. l-1 IAA) was investigated. Finally, in the third experiment, the effect of adding GA3 in seven concentrations (0, 0.25, 0.5, 0.75, 1, 1.5, and 2 mg/L) was tested in the chosen salt stringency and cytokine-auxin combination. In all experiments, the cultures were maintained under a cool fluorescent light intensity of 2000-3000 lux for a 16-8 h light-dark period and a temperature of 22 ± 2 °C. The number of shoots, stem length, and number of nods per shoot were recorded for each explant after one month for the two first experiments and after 20 days for the third experiment.

At the final stage, for root induction, the 45-day-old shoots were cut and cultured in the MS medium containing 1 and 2 mg/ml of different auxins (IAA, IBA, and NAA). After one month, the number of shoots with roots and the number of roots per shoot were recorded. After root development, the plantlets were adapted to the external environment and plants were transferred to the soil.

Also, an experiment with different concentrations of salt was conducted as factorial based on a completely randomized design. The other three experiments were conducted as a completely randomized design. All experiments were performed in five replications. After the test for assumptions, the data were subjected to a one-way analysis of variance followed by Duncan's multiple range test.

Results and Discussion

Multiple shoots were initiated within 15 days of inoculation in almost all media. Maximum number of shoot proliferation was observed within 25-30 days. The nodal explants were first grown on the MS basal medium supplemented with different concentrations of IAA, NAA, and BAP. A 2 mg/L BAP in combination with 0.5 mg/L IAA induced a significantly higher number of nodes (13±1.22) when compared to the combination of 2 mg/L BAP with 0.5 mg/L NAA (5.8±0.89). Among different combinations of BAP with IAA or NAA, the MS basal medium, supplemented with 2 mg/L BAP and 0.5 mg/L IAA, revealed a higher number of shoots (3±1.22) and the highest number of nodes (13±1.22) and stem length (8.04±1.92) (Table 1 and Figure 1). In a study conducted by Mascarenhas *et al.* (2019), the use of 12.50 µM BAP showed the highest direct shooting from explants. According to Ahmadi *et al.* (2022), the highest shoot induction was achieved in Oregano (*Origanum vulgare* L.) in the media supplemented with 1 mg/ l BAP.

Table 1. The effect of different concentrations of plant growth regulators on multiple shoot induction from nodal explants of *Physalis alkekengi* L. in the MS medium.

Plant growth regulators (mg/L)			No. of shoots per explant	No. of nodes per shoot	Stem length
BAP	NAA	IAA			
0	0	0	1±0.002g✳	2.4±0.55 c	2.07±0.51ce
0.5	0	0	4.8±1.64a	6.2±0.45b	7.64±0.37a
1	0	0	2.2±0.84ef	2.8±0.84c	2.07±0.50ce
2	0.5	0	4.2±0.45ab	5.8±0.89b	7.96±1.38a
0.5	0.5	0	1±0.005g	5.6±1.34b	4.44±0.73b
1	0.5	0	1.4±0.55f	1±0.006e	1.08±0.19e
2	1	0	1.6±0.55ef	1±0.89d	1.04±0.62f
0.5	1	0	1.2±0.45f	1.4±0.55de	0.64±0.23f
1	1	0	1.6±0.55ef	1±0.006e	0.94±.47ef
2	0	0	1.8±0.45def	1±0.008e	1.2±0.38e
0.5	0	0.5	1.8±0.85def	2.6±1.78c	5.42±1.78b
1	0	0.5	2.4±1.14dc	2.2±1.92cd	4.92±0.75b
2	0	0.5	3±1.22abc	13±1.22a	8.04±1.92a
0.5	0	1	2.4±0.55cf	2.4±0.89c	4.2±0.80bc
1	0	1	2±0.70ef	2.8±0.84c	3.54±0.62c
2	0	1	2.4±0.55ce	2.8±1.09c	2.48±0.49ce
2	1	0.5	2±0.007ef	1±0.002e	2.02±2.79ce

*Data are in the form of mean ± SE. Means with different letters in each column are significantly different at $p \leq 0.05$ according to Duncan's multiple range test.

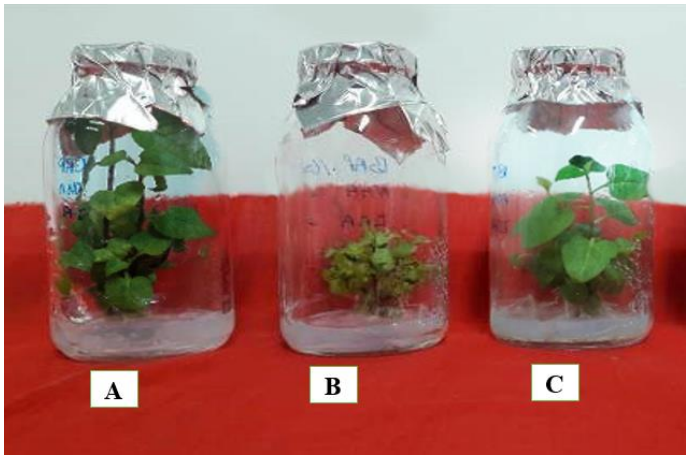


Figure 1. Multiple shoot induction of *Physalis alkekengi* L. from internode explants at the MS medium with selected different concentrations of A) 2 mg/L BAP + 0.5 mg/L IAA, B) 0.5 mg/L BAP + 0.5 mg/L IAA, and C) 0.5 mg/L BAP.

In the in-vitro propagation of plants, shoots are regenerated directly from nodal explants in the presence of meristematic tissue. This type of explant is used to produce plantlets free of bacteria or viruses and also mass propagation of plants. However, the type and concentration of plant growth regulators affect the shoot proliferation efficiency (Intzaar *et al.* 2013).

Shoot tips have always been preferred for *in vitro* studies because they can be handled easily and restore their regeneration potential over other explants. Some earlier findings showed that more shoots were produced from the nodal explants (Jain and Babbar 2003; Sheeba *et al.* 2010). The nodal explants showed an active site of positive morphogenetic response and readily developed multiple shoots.

Different types and concentrations of cytokinin led to differences in multiple shoot induction. Although many growth regulators are available for shoot multiplication, BAP is used more widely. In the present study, BAP was used to find its efficiency in shoot multiplication, because of its economic cost for commercial micropropagation. The presence of BAP in the culture medium led to an increase in the potential for shoot multiplication. Ramar *et al.* (2014) reported that BAP was highly effective in inducing bud break which led to the sprouting of a large number of shoots in *P. peruviana* L.

The MS medium containing BAP was reported more effective compared to Kin for the induction and proliferation of axillary buds in *Solanum nigrum* L and *Hybanthus enneaspermus* (Padmapriya *et al.* 2011; Velayatham *et al.* 2012). The superior effect of BAP on shoot bud induction, shoot tip multiplication, and *ex vitro* rooting of *Centella asiatica* (Alagumanian *et al.* 2015) and *Plumbago zeylanica*. L (Velayatham *et al.* 2005) has been reported.

Figure 2 and Table 2 show growth patterns of *P. alkekengi* L. regarding the number and length of shoots taken after four weeks of *in vitro* culture. Favored shoot bud initiation from nodal explants was observed within one month of inoculation. The highest number of shoots (3±0.005) and maximum stem length (7.91±0.96) was achieved in the full-strength MS medium. By decreasing the salt strength of the MS basal medium, shoot proliferation decreased. In addition, the quality of propagated plants was very low, which was indicated by abnormal light color and appearance (Figure 2).

Investigating the effects of GA3 on the proliferation of *P. alkekengi* indicated that the addition of GA3 to the proliferation medium improved shoot length, and 1 mg/L GA3 gave the highest shoot length (6.96±0.96) followed by the 0.75 and 1.5 mg/L GA3 (Table 3). The physiological effects of GA3 on plant tissue culture are well-known. This compound is used for shoot elongation of *in vitro* propagated plants. In a study to investigate the effect of different growth regulators on *P. angulate* L.

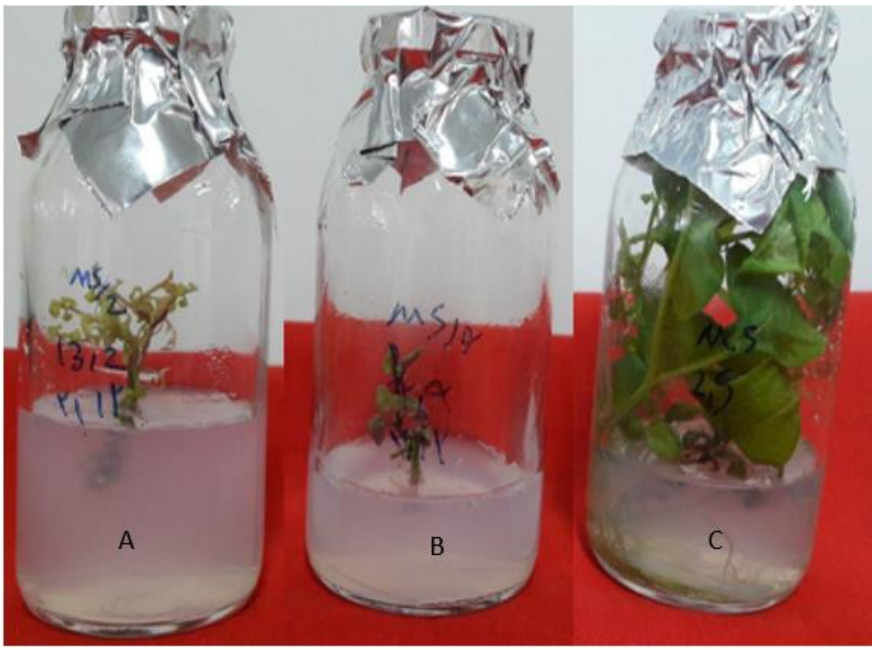


Figure 2. Effect of salt concentration of MS medium on the proliferation of *Physalis alkekengi* L. and the quality of regenerated plants: A) 1/2MS, B) 1/4MS, and C) MS.

Table 2. Effect of various concentrations of plant growth regulators and different concentrations of MS on multiple-shoot induction from nodal explants of *Physalis alkekengi* L.

Medium	BAP	IAA	No. of shoots	No. of nodes per shoot	Stem length
	2	0.5	2.6±0.55b*	4±1.22ab	4.1±0.92b
1/4MS	2	0	1.8±0.84bc	3.6±0.89bc	3.35±0.33bc
	0.5	0	1.6±0.55b	2.6±0.55c	2.1±0.74d
	2	0.5	2.4±0.55b	3.4±0.55bc	3.24±0.62c
1/2MS	2	0	1.4±0.55bc	0.2±0.70d	2.6±0.42d
	0.5	0	1.4±0.55bc	1.8±0.45c	1.7±0.27e
	2	0.5	3±0.005a	4.8±0.45a	4.97±0.77ab
MS	2	0	2.4±0.55a	4.2±0.84ab	3.4±0.74bc
	0.5	0	2.8±0.84a	5.4±1.34a	7.91±0.96a

*Data are in the form of mean ± SE. Means with different letters in each column are significantly different at $p \leq 0.05$ according to Duncan's multiple range test.

through meristem culture, BAP in combination with 1.5 mg/L IAA + 0.25 mg/L GA3 produced maximum shoots per explant (Kumar *et al.* 2015). In another study on *Acacia sinuate*, the shoot was not able to elongate in the proliferation medium; however, elongation was observed after it was transferred to a GA3-supplemented medium (Vengadesan *et al.* 2002). Accordingly, the role of GA3

combined with BAP enhanced the shoot multiplication and elongation in *Melia azedarach* (Vila *et al.* 2002) and *Vitis vinifera* (Tarinejad *et al.* 2019) cultures.

Table 3. Effect of various concentrations of GA3 on shoot elongation of *Physalis alkekengi* L. in the full-strength MS medium, supplemented with 2 mg/L BAP and 0.5 mg/L IAA.

Basal medium	GA3	Stem length
MS	0	4.1±0.91$_{b*}$
	0.25	5.006±1.65$_{ab}$
	0.5	6.44±0.95$_{a}$
	0.75	6.76±0.80$_{a}$
	1	6.96±0.96$_{a}$
	1.5	6.64±0.88$_{a}$
	2	5.9±1.2$_{ab}$

*Data are in the form of mean ± SE. Means with different letters in each column are significantly different at $p \leq 0.05$ according to Duncan's multiple range test.

In vitro rooting of micro-plantlets was successful in the full-strength basal MS medium with 8% agar within 30 days. Almost all used plant growth regulators induced root in all concentrations, except for 1 mg/L IAA. The maximum number of root initiation (2) was observed at 1 to 2 mg/L NAA and 2 mg/L IAA but IBA induced the weak rooting results. Similar results were reported in *P. peruviana* L. (Ramar *et al.* 2014). In another study, the optimal medium for rooting *P. angulata* was MS culture medium containing 1.07 μM NAA with an average of 30.51±0.94 roots (de Jesús Romo-Paz *et al.* 2021). Our observation showed that almost 95% of rooted plants adapted to soil conditions and continued their growth in the greenhouse (Figure 3).

Table 4. Effect of various concentrations of plant growth regulators on multiple root induction from internodal explants of *Physalis alkekengi* L.

Medium	IBA	IAA	NAA	No. of roots per shoot
MS	0	1	0	0±0c*
	0	2	0	2±0.003a
	0	0	1	2±0009a
	0	0	2	2±0.006a
	1	0	0	1.2±0.45b
	2	0	0	1.4±0.55b

*Data are in the form of mean ± SE. Means with different letters in each column are significantly different at $p \leq 0.05$ according to Duncan's multiple range test.



Figure 3. Hardening of the propagated shoots ***of*** *Physalis alkekengi* L. for transferring to the soil in the greenhouse.

Conclusion

A reproducible protocol was developed using nodal explants of four-week-old plants of *P. alkekengi*. To our knowledge, this is the first work on the micropropagation of this plant with a good rate of shoot multiplication. Results indicated the possibility of optimizing plant multiplication *in vitro* by using BAP, IAA, and GA3. Propagated plants were rooted in the MS medium supplemented with NAA or IAA, and almost 100% percent of plants were adapted to greenhouse conditions. The reported protocol can be adapted for *in vitro* conservation and mass production of other ecotypes of *P. alkekengi.*

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Ethical considerations

The authors avoided data fabrication and falsification.

Conflict of interest

The authors declare that they have no conflict of interest with any organization concerning the subject of the manuscript.

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The impact of silicon dioxide on bread wheat seedlings under saline stress

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Abstract

Objective: Silicon has beneficial effects on a wide range of plant species under abiotic stresses. For this reason, investigating the role of silicon in improving the growth of crops under stress has always been of interest.

Methods: This study aimed to investigate the effect of silicon dioxide on seedlings of bread wheat, Pishtaz cultivar. After sterilizing wheat seeds with 70% ethanol, the seeds were planted in pots filled with perlite. The experiment was arranged as factorial based on a randomized complete block design. At the two-leaf stage, silicon dioxide was applied at four levels (0, 15, 30, and 45 mg/l). After one week, salinity stress was applied at two levels of 0 and 100 mM. After one week of applying salt stress, different morphophysiological traits including root and shoot length, root and shoot fresh and dry weight, and content of chlorophyll a, b, carotenoids, sodium, potassium, and iron were measured.

Results: The results showed that salinity has a negative effect on morphophysiological traits, and on the contrary, silicon, especially at a concentration of 45 mg/liter, improves these traits under salt stress. Also, the sodium content in the presence of silicon decreased strongly in the wheat seedlings under salinity, and on the contrary, the K/Na increased. Silicon also had a positive effect on the content of iron and chlorophyll of seedlings under salt stress.

Conclusion: These results show that silicon improves the growth of bread wheat seedlings by facilitating the absorption of mineral elements, homeostasis of nutrients, and preventing the destruction of chlorophyll.

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Introduction

Wheat (*Triticum aestivum* L.) is one of the most important crops in the world, which is considered a sustainable food for about a third of the world's population (FAO 2020). After rice and maize, wheat is the main food of majority of the world's population (Abdelrhim *et al.* 2021). However, the growth and yield of wheat is influenced by various environmental stresses such as drought, salinity, and high temperature (Tahmasebi Shamansouri *et al.* 2018). Salt stress is an environmental threat to all crops worldwide. Currently, millions of hectares of arable land for agriculture are too salty, and hundreds of thousands of hectares of agricultural land are lost for food production due to salinization every year (Harper *et al.* 2021).

Salinity stress initially reduces the absorption of water by the plant by creating osmotic stress and in this way prevents the growth of the plant. Secondly, a large amount of ions enters the plant. When the concentration of ions inside the plant cells increases, the ion homeostasis is destroyed which causes toxicity. At this stage, the second negative effect of salinity on plants is shown, which is called ion toxicity or salt specific effects of salinity (Munns and Tester 2008; Parihar *et al.* 2015). Also, salinity stress reduces photosynthetic activity by increasing the activity of reactive active oxygen species (ROS) and reducing photosynthetic pigments, which ultimately leads to a decrease in the growth and yield of crops (Parida and Das 2005). ROSs are highly reactive and cause damage to lipids, protein, and nucleic acid and generally cause damage to plant metabolism (Ashraf and Harris 2004; Maleki *et al.* 2017).

Due to biochemical, biological, and physiological processes that occur in the plants, they are strongly affected by the presence of excessive salts in the rhizosphere, and consequently, the growth and yield of the plant are strongly affected (Tester and Davenport 2003). To overcome salt stress, the plant tries to reduce the harmful effects of salinity stress by creating defense mechanisms such as the production of various enzymatic and non-enzymatic antioxidants (Weisany *et al.* 2012). In addition to the mechanisms used by the plant, it is possible to improve plant growth under salinity by using supplements such as silicon (Si). Among all of the known trace elements, silicon, which is the most abundant element after oxygen, is the most important mineral element in the soil, and is also known as a useful nutrient that improves seed germination, plant growth, and crop yield (Liang *et al.* 2007; Sabaghnia and Janmohammadi 2015). In addition, the use of silicon increases plant water absorption and transport (Yavaş and Aydın 2017), antioxidant activities (Ma *et al.* 2016), and photosynthetic performance (Wang *et al.* 2019). In addition to improving plant growth, silicon stimulates the resistance mechanisms of plants against biotic (De Curtis *et al.* 2012; Wordell Filho *et al.* 2013) and

abiotic (Sabaghnia and Janmohammadi 2014; Sabaghnia and Janmohammadi 2015) stresses. Therefore, in this study, an attempt was made to investigate the effect of different concentrations of silicon on growth of bread wheat seedlings under salt stress conditions.

Materials and Methods

Plant materials and growth conditions

In this study, a bread wheat cultivar, Pishtaz, was used. The seeds were first sterilized using 70% ethanol for 30 seconds and then they were cultivated in pots (15 cm in diameter and 17 cm in height) and under greenhouse conditions. The experiment was conducted in the Graduate University of Advanced Technology's research greenhouse in 2022. Perlite was used as a culture medium. Wheat seedlings were grown in greenhouse conditions using a photoperiod of 16 hours of light/8 hours of darkness and at a temperature of 24 °C at day/18 °C at night. After planting wheat, irrigation was done using water until the seeds germinated. After germination and appearance of greenness, irrigation continued by using the Hoagland solution.

Salt stress treatment

This experiment was conducted as factorial based on the randomized complete block design. The were silicon dioxide at four levels (0, 15, 30, and 45 mg/l) and salt stress at two levels (0 and 100 mM). At two-leaf stage, SiO_2 treatment was applied by dissolving in the Hoagland solution. Hoagland's solution without SiO_2 was used as the control. Three replicates of each treatment were considered. After one week of treatment, salinity stress was applied for seven days.

Measurement of morphological and physiological traits

After seven days of applying salt stress, morpho-physiological traits including root and shoot length, root and shoot fresh weight, root and shoot dry weight, chlorophyll a, b, carotenoids, and sodium, potassium, and iron were measured. The content of chlorophyll and carotenoids was measured according to the method of Wellburn (1994). To measure ions, leaf samples were dried at 72 °C for 48 hours. Then, 0.03 g of completely powdered dry samples was put into the test tubes and after that, 5 ml of acid solution (mixture of 50 ml of HCL with 250 ml of nitric acid) was add. This solution was kept in the room temperature for 24 hours to dissolve the plant sample well in the acid. Then, the resulting solution was passed through a filter paper and made up to 50 ml with distilled water. This solution was used to measure ions in the Varian SpectrAA 220 Atomic Absorption Spectrometer.

Statistical analysis

The data were analyzed using Excel and SAS software. After analysis of variance, Duncan's multiple range tests was used to compare the means.

Results and Discussion

Analysis of variance

The variance analysis revealed that salinity stress affected all traits, except for potassium content and fresh and dry weight of roots, significantly at 0.01 probability level. Also, the effect of silicon dioxide and its interaction with salinity were significant for all traits at least at a 0.05 probability level (Table 1).

Morphological traits

Means of different morphophysiological traits of wheat seedlings grown in different concentrations of silicon dioxide under salt stress are shown in Figure 1. Salt stress (without Si) caused a significant decrease in all morphological traits compared to the control (without NaCl and Si). On the contrary, the addition of silicon dioxide improved the morphological traits under salinity. In the salt-free conditions, the addition of 15 mg/l silicon dioxide caused a 31.7% and 26.4% increase in root and shoot length, respectively. Under the 100 mM of NaCl, 45 mg/l of silicon dioxide caused an increase of 37.4 and 24.1% in root and shoot length, respectively, compared to the control (without Si), (Figure 1A, 1B). The exposure of 15 mg/l silicon dioxide in salt-free conditions and 45 mg/l silicon dioxide in 100 mM NaCl respectively raised root fresh weight by 36.6% and 93.7 %, and shoot fresh weight by 30.1% and 110% correspondingly (Figure 1C, 1D). The root dry weight increased by 37.8% and 98.3% in the presence of 45 mg/l silicon dioxide under 0 and 100 mM NaCl, respectively. Shoot dry weight raised by 14.8% and 25.8% % in the presence of 45 mg/l silicon dioxide under 0 and 100 mM NaCl correspondingly (Figure 1E, 1F).

Physiological traits

Our results showed that under salinity stress and in the absence of silicon, the content of chlorophyll and carotenoid decreases drastically. But by application of silicon, the content of chlorophyll improves (Figure 2). Chlorophyll a content increased by 9.10 and 43.3% in the presence of 45 mg/l silicon dioxide under 0- and 100- mM salinity, chlorophyll b by 7.6% and 87.1%, and total chlorophyll by 8.8% and 70.9%, respectively (Figure 2A, 2B, 2C). However, the carotenoid content increased by 24.8% in the presence of 45 mg/l silicon dioxide in salt-free conditions (Figure 2D).

Table 1. Analysis of variance of the impact of silicon dioxide on wheat seedlings under salt stress for various traits.

Source of variation	df	Mean squares						
		Chlorophyll a	Chlorophyll b	Total Chlorophyll	Carotenoids	K^+	Na^+	K^+/Na^+
Replication	2	0.34	2.61	4.79	0.014	31.70	20.5*	0.46
Salt stress (a)	1	311.5**	693.7**	1935.1**	15.33**	7.74	2879.5**	9.41**
Silicon dioxide (b)	3	5.4**	267.1**	291.4**	1.22**	1644.3**	1619.6**	7.18**
a × b	3	2.9*	82.5**	92.95**	0.819**	3476.5**	2389.0**	3.38**
Error	14	0.62	2.9	3.33	0.025	16.49	5.107	0.166

Table 1 Continued

Source of variation	df	Mean squares						
		Fe	Root length	Shoot length	Root fresh weight	Shoot fresh weight	Root dry weight	Shoot dry weight
Replication	2	0.0038*	0.34	0.38	0.0000002	0.00021	0.000035	0.000009
Salt stress (a)	1	2.78**	24.00**	47.14**	0.000041	0.0108**	0.000016	0.00054**
Silicon dioxide (b)	3	0.41**	13.19**	5.00**	0.00087**	0.00699**	0.00054**	0.00031**
a × b	3	0.0236**	17.41**	38.31**	0.00063**	0.00302**	0.000066*	0.00014**
Error	14	0.00073	0.62	0.371	0.0000147	0.000189	0.000013	0.00003

*,**Significant at 0.05 and.01 probability levels, respectively.

The results of this study showed that the elements' uptake by wheat seedlings is affected by both salt stress and silicon application. Under salt stress (without Si), the content of sodium and potassium ions increased compared to the control (without NaCl and Si), and the K/Na as well as the content of iron decreased sharply (Figure 3). Potassium content increased by 150% and 27.5% in the presence of 15 mg/l silicon dioxide in the salt-free conditions and 30 mg/l silicon dioxide in 100 mM NaCl, respectively (Figure 3A). The highest amount of sodium was observed under salt stress without the presence of silicon dioxide (Figure 3B). Also, the K/Na increased by 54.7% and 371% and Fe content by 44.5% and 90.3% with the use of 15 mg/l of silicon dioxide in the salt-free conditions and 45 mg/l of silicon dioxide in the presence of 100 mM NaCl (Figure 3C and 3D).

Salt stress disrupts cell activities due to increased osmotic stress and ion toxicity, which ultimately suppresses plant growth (Munns *et al.* 2006). Salinity causes changes in plants at the morphological, physiological, and metabolic levels by creating an imbalance in mineral absorption (Kumari *et al.* 2022; Shabala and Munns 2017) and reduced water absorption by plants (Lopez and

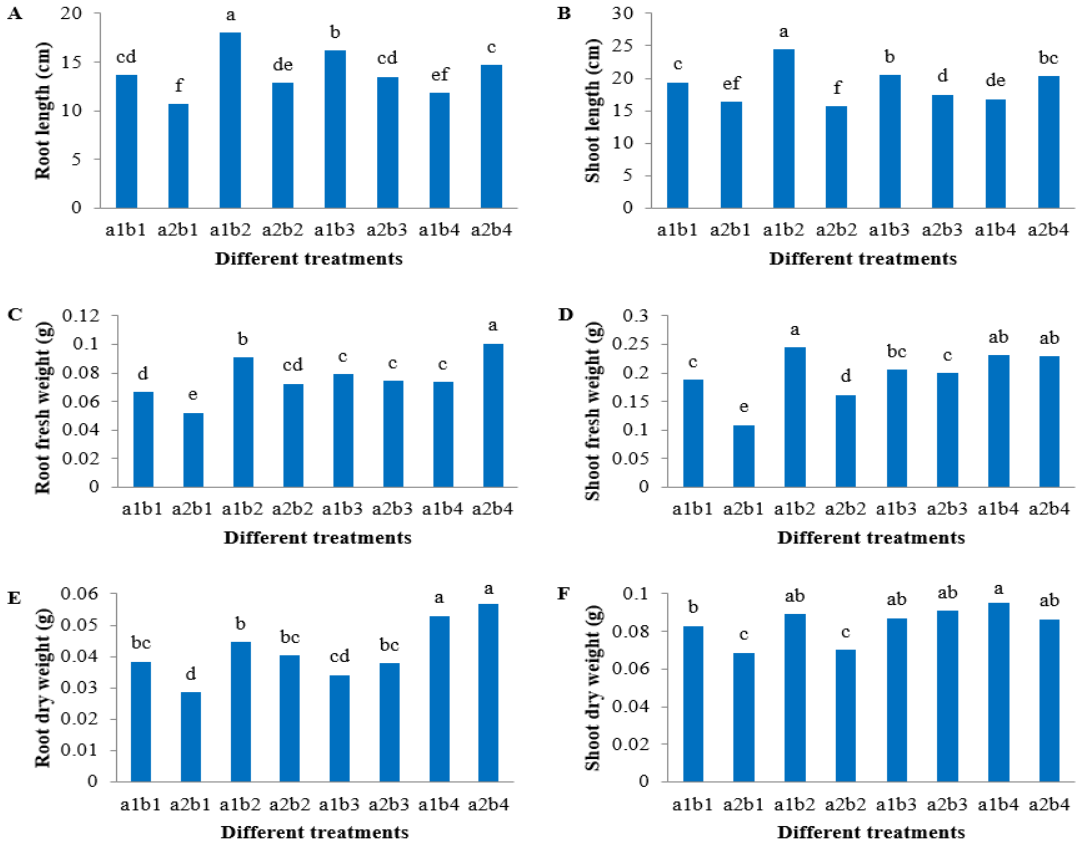


Figure 1. Mean comparisons for the effect of silicon dioxide under salt stress on bread wheat seedlings for root length (A), shoot length (B), fresh weight of root (C), fresh weight of shoot (D) , dry weight of root (E), and dry weight of shoot (F) (a1 and a2 indicate the salinity levels of 0 and 100 mM, respectively, and b1, b2, b3, and b4 indicate the silicon dioxide concentrations of 0, 15, 30, and 45 mg/l, respectively); Means with different letters are significantly different at 0.05 probability level.

Satti 1996). Also, salt stress has an inhibitory effect on plant growth through the reduction of chlorophyll content. Chlorophyll is one of the most important molecules related to photosynthesis, which is responsible for harvesting solar energy and transferring it to photosynthetic complexes (Wang and Grimm 2021). Chlorophyll a is the most abundant form of chlorophyll, which is synthesized from glutamic acid. Chlorophyll b (another form of chlorophyll) is then synthesized from chlorophyll a during the chlorophyll cycle (Tanaka and Tanaka 2006). In fact, chlorophyll content is one of the characteristics that are related to the photosynthetic capacity of plants, which is strongly affected by salinity stress.

Our study showed that the salt stress (without Si) caused a significant decrease in all morphological traits, including root and shoot length, fresh and dry weight at the seedling stage compared to the control (without NaCl and Si), which indicates inhibition of cell division and elongation. The observed reduction in morphological traits in wheat seedlings in response to 100 mM salt stress may be due to the increase in the absorption of sodium ions and the decrease in the K/Na under salt stress (Figure 3B and 3C), which leads to the production of ROS that cause disruption of

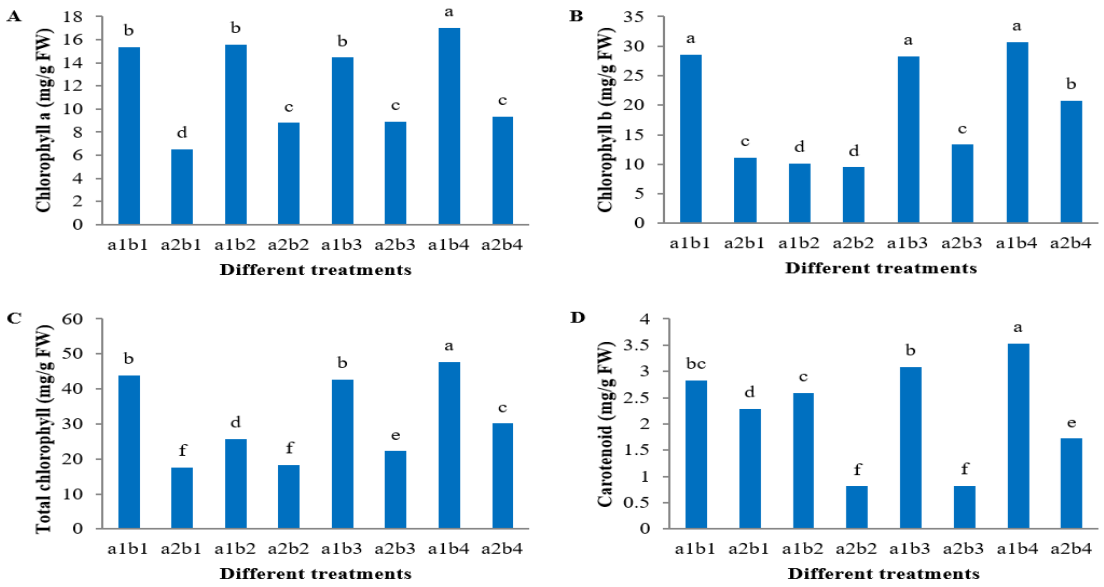


Figure 2. Mean comparisons for the effect of silicon dioxide under salt stress on bread wheat seedlings for chlorophyll a (A), chlorophyll b (B), total chlorophyll (C), and carotenoids (D) (a1 and a2 indicate the salinity levels of 0 and 100 mM, respectively, and b1, b2, b3, and b4 indicate the silicon dioxide concentrations of 0, 15, 30, and 45 mg/l, respectively); Means with different letters are significantly different at 0.05 probability level.

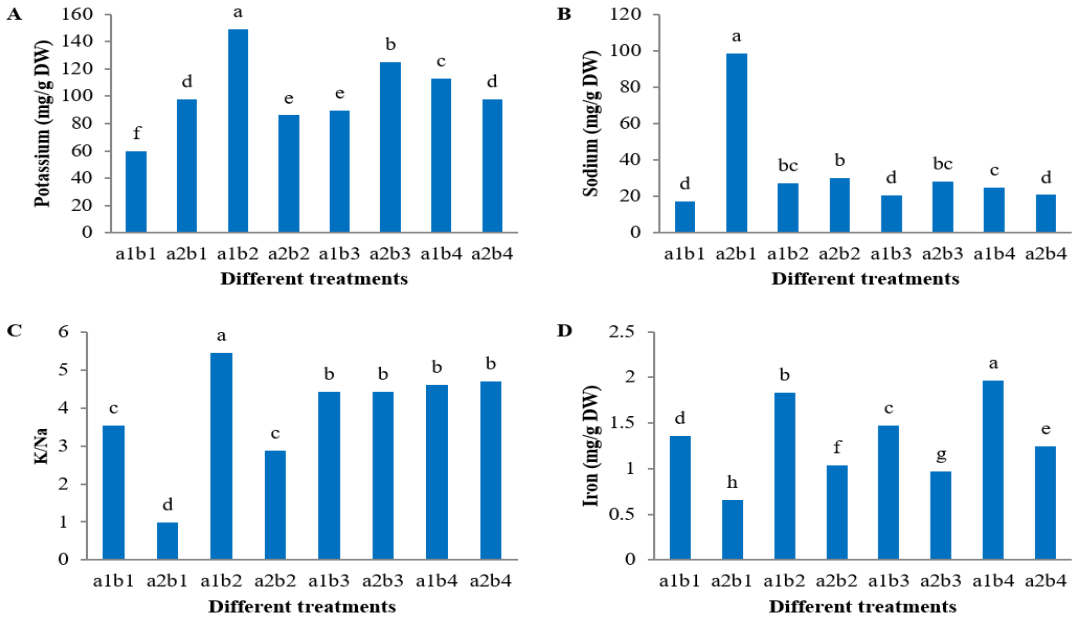


Figure 3. Mean comparisons for the effect of silicon dioxide under salt stress on bread wheat seedlings for Na content (A), K content (B), K/Na (C), and Fe content (D) (a1 and a2 indicate the salinity levels of 0 and 100 mM, respectively, and b1, b2, b3, and b4 indicate the silicon dioxide concentrations of 0, 15, 30, and 45 mg/l, respectively); Means with different letters are significantly different at 0.05 probability level.

the plasma membrane as well as ionic imbalance and thus suppression of metabolic and growth processes (Maleki *et al.* 2017). The results of present study showed that the content of Na^+ and K/Na in the absence of silicon under salt stress increases and decreases, respectively. These results are similar to several studies that have stated ionic imbalance due to the excessive accumulation of

sodium ions, which reduces the absorption of other mineral nutrients (Gupta and Huang 2014; Daoud *et al.* 2018). Also, the iron content under salt stress (without Si) decreased drastically compared to the control. In addition to being a part of some proteins and enzymes such as antioxidants, iron also plays an important role in some cellular processes such as photosynthesis. In fact, iron deficiency directly causes chloroplast degeneration and reduced chlorophyll synthesis (Li *et al.* 2021). In this study, the content of chlorophyll a, b, total chlorophyll, and carotenoids also decreased under salt stress (without Si). It seems that the significant decrease in iron content under salt stress can be related to the decrease in chlorophyll content. On the other hand, reducing the chlorophyll content reduces the efficiency of photosynthesis. For this reason, under salt stress, the studied morphological traits have significantly decreased compared to the control.

In our study, silicon treatment improved the morphological traits of wheat seedlings under salt stress compared to the seedlings treated with salt alone (Figure 1). Also, silicon application decreased sodium ion and increased K/Na under salt stress (Figure 3), which ultimately led to the improvement of wheat growth under salt stress. The role of silicon in preventing Na^+ uptake, maintains the homeostasis of nutrients and improves the physiological and morphological characteristics of wheat under salt stress (Javaid *et al.* 2019). Various studies have also shown that the addition of silicon can protect the growth and yield of crops against the harmful effects of salt stress (Watanabe *et al.* 2001; Gong *et al.* 2006; Sienkiewicz-Cholewa *et al.* 2018). The positive effect of silicon on plants is attributed to the effect of this element on the metabolism and transfer of nutrients in plants, which causes higher absorption of them (Linjuan *et al.* 1999; Watanabe et al. 2001). In our study, total chlorophyll increased by 8.8% and 70.9% in the presence of 45 mg/l silicon dioxide under 0 and 100 mM salinity, respectively (Figure 2). In the studies conducted on wheat, mung beans, maize, and grapes, the results show the harmful effects of salinity stress as well as the beneficial effects of silicon treatment on chlorophyll content (Sacala and Durbajlo 2012; Ghassemi-Golezani *et al.* 2015; Mahmood *et al.* 2016; Qin *et al.* 2016; Daoud *et al.* 2018; Sienkiewicz-Cholewa *et al.* 2018). The negative effect of salinity stress on chlorophyll content may be caused by the disruption in chlorophyll biosynthesis or its rapid destruction, while silicon may limit these deleterious changes (Sienkiewicz-Cholewa *et al.* 2018). In the present study, iron content also increased significantly with the addition of silicon dioxide in both control and salt stress conditions (Figure 3D). Considering the role of iron in the synthesis of chlorophyll and ultimately in photosynthesis, it seems that silicon can play an important role in improving the content of chlorophyll under salt stress by improving iron absorption. Finally, the improvement of chlorophyll content is directly related to the improvement of morphological traits.

Conclusion

The results showed that silicon dioxide treatment by reducing Na content and increasing the K/Na maintained the ionic balance and prevented the formation of ROS, and as a result improved various growth traits under salt stress. The increase in iron content and consequently the chlorophyll content in the presence of silicon, the growth of wheat seedlings improved under salt stress conditions. The results of this study show the importance of silicone application in reducing the harmful effects of salt stress in wheat seedlings.

Ethical considerations

The authors avoided data fabrication and falsification.

Conflict of interest

The authors declare that they have no conflict of interest.

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Comparative analysis of phenolic compounds and biological activities of four varieties of *Coffea arabica* L.

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Abstract

Objective: The consumption of coffee has increased globally because of its flavor and health benefits. The present study aimed to investigate the phenolic compounds and biological activity of four varieties of Coffea arabica L. (Heirloom 1-4) from the flora of Africa.

Methods: Ethanolic extracts of the green and roasted coffee beans were screened in vitro for their total phenols and flavonoids using Folin-Ciocalteu and AlCl3 assays, respectively. In addition, the antioxidant activity of the extracts was evaluated by three complementary tests DPPH free radical-scavenging, β-carotene/linoleic acid, and metal chelating activity. The antibacterial activity of the aqueous extracts of the samples against two gram-positive and two gram-negative bacteria was also studied by the disk diffusion method.

Results: Results showed that the ethanolic extracts from both green and roasted beans of the variety Heirloom 4 contain the highest amounts of phenols and flavonoids. In addition, the antioxidant activity assays indicated that the activity of the studied extracts was lower than ascorbic acid (a standard synthetic antioxidant) in the metal-chelating activity and the DPPH radical scavenging assay. However, the oxidation of linoleic acid in the β-carotene/linoleic acid system was inhibited by all studied extracts. Furthermore, the aqueous extracts of the green and roasted beans of all varieties showed proper antibacterial activity against the bacterial strains tested.

Conclusion: In conclusion, several factors including altitude, variety, extraction methods, and type of solvent used affected the bioactivity of the coffee extracts. All antioxidant activity assays showed a close relationship with the phenolic content of green and roasted samples. Antioxidant activity assays indicated that the green or roasted bean extracts of coffee have potent free radical scavenging activity. Moreover, the differences in the antioxidant activity of green and roasted bean extracts in the same variety could be attributed to the roasting process.

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Introduction

For years, plants have been used globally as food and medicine. They produce bioactive metabolites, which are used to prevent and treat diseases caused by free radicals (Gomaa *et al*. 2019). Excessive free radicals in cells are the cause of oxidative stress in humans which results in various diseases and early aging (Yahayu *et al*. 2020). The human body possesses antioxidant defensive systems that quench free radicals. They are found in enzymatic and non-enzymatic forms (Nimse and Pal 2015). Polyphenols are one of the most important antioxidants, which are found in plants (Priftis *et al*. 2015). In recent years, the antioxidant activity of phenolic compounds such as flavonoids has attracted the interest of many researchers, and some plants rich in phenolic compounds such as cocoa (Davinelli *et al.* 2018), coffee (Priftis *et al.* 2015; Sentkowska *et al*. 2016), and legumes (Kamalvand *et al.* 2021) are useful in the treatment of several diseases.

The *Coffea* L. genus of the Rubiaceaea family has over 500 species. Among these, *Coffea arabica* L. (arabica) and *Coffea canephora* L. (robusta) are the most important commercial species (Jeszka-Skowron *et al.* 2015). Arabica comes mostly from Brazil and Ethiopia, while canephora inhabits South Asia (mainly Vietnam) and the lowlands of Central and West Africa (Sentkowska *et al*. 2016). *C. arabica* L. is known for its low caffeine content and fine aroma and accounts for 65% of the world's coffee production (Cho *et al.* 2013). The chemical composition of coffee brews depends on the coffee species and varieties, as well as the preparation method, brew volume, and roasting degree (Alves *et al.* 2010). Coffee, both green and roasted beans, contains several bioactive compounds with antioxidant activity (Makiso *et al*. 2023). The antioxidant activity of coffee depends on some factors such as coffee bean type and variety, regional conditions, bean processing, and extracting methods (Jung *et al.* 2021; Yust *et al.* 2024). The roasting process is required to bring out coffee flavors (Jung *et al*. 2021). The association between coffee roasting and antioxidant activity has been investigated in some studies, but results were inconsistent. Studies have indicated that the antioxidant activity of coffee is associated with compounds such as caffeine, chlorogenic acids, and tocopherols (Parras *et al.* 2007). Phenolic compounds act as hydrogen donors, metal chelators, reducing agents, and singlet oxygen quenchers to enforce their antioxidant effects (Cho *et al*. 2013).

There is no knowledge about the inter-variety variation concerning the *in vivo* effects of the coffee bean extracts. The existence of any possible differences among coffee varieties may help us to unravel the distinct metabolic properties of the bioactive compounds. Hence, this study aimed to assess the total phenolic content, antioxidant properties, and antibacterial activities of the green and

roasted bean extracts from four *C. arabica* varieties, namely Heirloom 1, Heirloom 2, Heirloom 3, and Heirloom 4.

Materials and Methods

Chemicals

Gallic acid, ascorbic acid, aluminum chloride, butylated hydroxytoluene (BHT), chloroform, ethanol, Folin-Ciocalteu reagent, nutrient agar, potassium acetate, sodium bicarbonate, and Tween-40 were purchased from Merck (Darmstadt, Germany). β-carotene, 1, 1-diphenyl-2-picrylhydrazyl (DPPH), linoleic acid, and quercetin were obtained from Sigma (St. Louis, MO, USA).

Coffee sample preparation

The green and roasted beans of four *Coffea arabica* varieties were purchased from the SET Coffee Company (Tehran, Iran). They included Heirloom 1 (Ethiopia, Yirgacheffe, 1950 MASL, natural process), Heirloom 2 (Ethiopia, Yirgacheffe, 1800 MASL, washed process), Heirloom 3 (Ethiopia, Guji, 2100 MASL, washed process), and Heirloom 4 (Ethiopia, Limu, 1600 MASL, washed process).

Extract preparation

Both green and roasted beans were ground to powder. For extraction, 4 g of the powder was added to 100 mL ethanol 70% at room temperature followed by magnetic stirring for 1 h. The mixtures were kept in the dark under refrigeration (2-8 °C) for 18 h. The extracts were recovered by filtration on cellulose support under reduced pressure, and then, concentrated in a rotatory evaporator (60 °C, 82 rpm). The dried extracts were resuspended in 10 mL distilled-deionized water, centrifuged at 4000 rpm for 10 min, and the supernatants were collected for further analyses (Affonso *et al*. 2016).

Determination of total phenol and flavonoid contents

The Folin-Ciocalteu method was used to determine the total phenol content of the extracts (Singleton and Rossi 1965). Briefly, 100 µL of the ethanolic extract (1 mg/mL) was mixed with 0.75 mL Folin-Ciocalteu reagent which was previously diluted 10-fold with distilled water and kept at 22 °C for 5 min. Then, 0.75 mL sodium bicarbonate (60 g/L) solution was added and mixed thoroughly. The absorbance of samples was determined by the UV-Vis Array Photonix Ar 2017 spectrophotometer at 725 nm at 22 ºC after 90 min. The total phenols content was determined as gallic acid equivalent (GAE) and presented as mg GAE/g dry weight.

The aluminum trichloride method was applied to determine the total flavonoid content with quercetin as a reference compound (Chang *et al*. 2002). Based on this method, a flavonoid-aluminum complex is formed with the maximum absorptivity at 415 nm, after the remaining reaction at room temperature for 30 min. Briefly, 0.5 mL of each extract (1:10 g/mL) was separately mixed with 0.1 mL of 10% aluminum chloride, 1.5 mL of ethanol, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water. The calibration curve was obtained by preparing the quercetin solution in ethanol at different concentrations from 12.5 to 100 g/mL.

Antioxidant activity assays

***DPPH free radical scavenging activity*:** The hydrogen atoms of the corresponding extracts and several pure compounds were determined by bleaching of the purple-colored methanol solution of DPPH. The stable 1,1- diphenyl-2-picrylhydrazyl radical (DPPH) was used to determining of the free radical scavenging activity of the extracts. The method of Mensor *et al.* (2001) was applied to measure the radical scavenging ability. Briefly, the ethanolic solution of 0.5 mL DPPH (0.3 mM) was added to 2.5 mL of the different concentrations of coffee extracts (0.2, 0.4, 0.6, 0.8, and 1 mg/mL). The samples were first kept in the dark at room temperature. Then their absorbance was read at 517 nm after 30 min. The antiradical activity (I) was measured as follows:

$$I\% = 1- (A_s – A_b)/A_c \times 100$$

Blank samples (A_b) consisted of 1 mL ethanol and 2.5 mL of different concentrations of the extract and the control sample (A_c) contained 1 mL 0.3 mM DPPH and 2.5 mL ethanol. The optical density of the samples, the control, and the empty samples were determined by comparing them with ethanol. The amount of discoloration was plotted against the sample concentration to calculate the IC_{50} value, which is the amount of sample needed to decrease the absorbance of DPPH by 50% (Koleva *et al*. 2002).

***Metal chelating activity*:** The method of Dinis *et al.* (1994) was utilized to determine the chelating activity of ferrous ions. The reaction mixture consisted of 0.5 mL of different concentrations of test compounds, 0.05 mL (2 mM) of $FeCl^{2+}$ solution, and 1.6 mL of deionized water. After 30 s, 0.1 mL 5 mM ferrozine solution was added. The Fe^{2+}-ferrozine magenta complex was very soluble and stable in water. The absorbance was measured at 562 nm after 10 min at room temperature. The relative activities of test compounds to chelate ferrous ion were presented as a percentage of absorbance disappearance as the following formula:

$$\text{Metal chelating activity (\%)} = (A_{control} - A_{sample})/ A_{control} \times 100$$

Where, A_s is the reaction mixture absorbance in the presence of the coffee, and A_c is the absorbance of the reaction mixture in the absence of the coffee extract.

***β-Carotene/linoleic acid model system*:** The antioxidant capacity was determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation (Barrière *et al*. 2001). A stock solution of β-carotene/linoleic acid was prepared as follows: first, 0.5 mg β-carotene was dissolved in 1 mL chloroform, then 25 µL linoleic acid and 200 mg Tween-40 were added. The chloroform was evaporated using a vacuum evaporator. Then, 100 ml of distilled water, saturated with oxygen (30 min at 100 mL/min), was added with vigorous shaking. In brief, 350 µL portions of the extracts were added to 2.5 mL of the above mixture in test tubes. The test tubes were incubated in a hot water bath at 50 ºC for 2 h, together with two blanks, one consisted of the antioxidant BHT as a positive control, and the other comprised the same volume of ethanol instead of the extracts. The absorbance was measured at 470 nm by a spectrophotometer. The inhibition percentage (I%), representing the antioxidant activity of the samples was calculated as follows:

$$I\% = (A_{\beta\text{-carotene after 2h assay}} / A_{\text{initial } \beta\text{-carotene}}) \times 100$$

Where $A_{\beta\text{-carotene after 2h assay}}$ is the absorbance of the remaining β-carotene after 2 h assay in the samples and $A_{\text{initial } \beta\text{-carotene}}$ is the absorbance of β-carotene at the beginning of the experiment (Amiri and Ghiasvand 2016).

Antibacterial activity assay

The antibacterial activity of the aqueous extracts was evaluated against two gram-positive (*Bacillus cereus* PTCC 1247, *Staphylococcus aureus* PTCC 1337) and two gram-negative (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* PTCC 1707) bacteria by the disk diffusion method (Bauer *et al*. 1966) at three different concentrations (0.1, 0.2, and 0.4 g/mL of the extracts). The extracts were prepared by adding samples to 10 mL boiling distilled water and placing them on boiling water vapor for 20 min. Then, 25 µL of the extracts were applied to blank sterile paper discs (6 mm diameter). These discs were placed onto the nutrient agar medium, previously inoculated with a bacterial suspension (0.5 McFarland standard). The cultures were incubated at 37 ºC for 24 h. The antibacterial activity against each test organism was quantified by determining the mean zone of inhibition. Negative controls were made using discs consisting of 25 µL sterile distilled water, while positive controls were made by chloramphenicol, clindamycin, nitrofurantoin, and vancomycin.

Statistical analysis

The data were recorded as means ± standard deviations. One-way analysis of variance was performed at the p-value ≤ 0.05. Means were compared by Duncan's multiple range test.

Results and Discussion

Total phenol and flavonoid contents

The total phenol content of the green and bean extracts measured by Folin Ciocalteu reagent in terms of gallic acid equivalent (standard curve equation: y = 0.0041x - 0.022, R^2 = 0.9982) are shown in Table 1. The results showed the total phenol content of Heirloom 3 and Heirloom 4 varieties were significantly different from other varieties in green bean extracts. In the roasted coffee beans, Heirloom 4 had higher total phenol content than other varieties (Table 1). The roasting process significantly increased the total phenolic content, which could be due to thermal degradation of complex phenolic compounds such as chlorogenic acids into simpler compounds such as melanoidins (Fuller and Rao 2017; Acidri *et al*. 2019). The differences in phenolic content of the varieties can be affected by environmental factors. Altitude is an environmental factor that affects the coffee quality by optimizing temperature. Temperature has an important effect on coffee plants, especially in fruit development and ripening stages. Joët *et al.* (2010) showed that chlorogenic acids and fatty acids in the seed were controlled by the mean air temperature during seed development. The suitable temperature for the arabica coffee is between 18-21 ºC and other temperatures will result in incomplete fruit maturity. Therefore, when the temperature is high throughout the growing period, several chemical compounds accumulate and reduce the coffee quality (Adugna 2021). However, our results showed that the Heirloom 4 variety which grows in the low altitude represents the highest content of phenolic compounds in both green and roasted bean extracts. In another study, Link *et al.* (2014) reported that the chlorogenic acid content declined with altitude but the alkaloid content

Table 1. Total phenol content of the roasted and green beans in four *Coffea arabica* varieties studied.

Variety	Roasted beans (mg GAE/g DW[a])	Green beans (mg QE/g DW[a])
Heirloom 1	229.52 ± 6.06[b]	34.22 ± 1.83[b]
Heirloom 2	230.7 ± 12.31[b]	35.85 ± 2.76[b]
Heirloom 3	234.78 ± 4.53[b]	52.11 ± 4.76[a]
Heirloom 4	325.35 ± 14.31[a]	55.64 ± 2.82[a]

[a]DW: Dry weight; Data are expressed as mean ± standard deviation (n = 3); Means with different letters in each column denote significant differences according to Duncan's multiple range test (p ≤ 0.05).

increased. In addition, Sridevi and Parvatam (2014) showed the negative effect of altitude on caffeine content.

Results from total flavonoids assessment in terms of quercetin equivalent (the standard curve equation: $y = 0.0091x - 0.0206$, $R^2 = 0.995$) in green beans extracts showed that there were significant differences between the Heirloom 3 and Heirloom 4 and the other varieties in total flavonoid content, however, in the roasted beans extracts the total flavonoid content of Heirloom 4 was significantly higher than other varieties which represents its highest quality (Table 2). It is established that phenolic compounds contribute to the quality (Acidri *et al*. 2019) and nutritional value (Bondam *et al.* 2022) of coffee, and has health-beneficial effects (Bondam *et al.* 2022). They also participate in plant defense mechanisms to alleviate the harmful effects of several biotic and abiotic stresses (Machado *et al.* 2023).

Antioxidant activity

***DPPH radical scavenging activity*:** Coffee plants are indeed an important source of dietary antioxidants, and the antioxidant activity of coffee is primarily due to the accumulation of polyphenols, including phenolic compounds (Priftis *et al.* 2015; Sentkowska *et al.* 2016). Phenolic compounds act as free radical terminators (Kalpoutzakis *et al.* 2023), and their bioactivity properties may be associated with their abilities to chelate metals, inhibit lipoxygenase, and scavenge free radicals (Decker 1997; Yin *et al.* 2008). According to Sacchetti *et al*. (2005), among a large number of tests, the results of a single assay can provide only a reductive suggestion of the antioxidant properties of extracts toward food matrices and must be interpreted with some caution. Moreover, the chemical complexity of extracts, often a mixture of several compounds with different functional groups, chemical behavior, and polarity, could lead to scattered results, depending on the test type. Therefore, Sacchetti *et al.* (2005) recommended multiple

Table 2. Total flavonoid content of the roasted and green beans in four *Coffea arabica* varieties studied.

Variety	Roasted beans (mg GAE/g DW[a])	Green beans (mg QE/g DW[a])
Heirloom 1	154.68 ± 4.08^{c}	16.21 ± 0.39^{b}
Heirloom 2	162.07 ± 4.37^{b}	16.32 ± 2.39^{b}
Heirloom 3	163.69 ± 5.25^{b}	19.40 ± 0.76^{ab}
Heirloom 4	191.71 ± 7.88^{a}	22.19 ± 1.41^{a}

[a]DW: Dry weight; Data are expressed as mean ± standard deviation (n = 3); Means with different letters in each column denote significant differences according to Duncan's multiple range test ($p \leq 0.05$).

assays in screening work. They further stated that among the methods that evaluate the antioxidant activity, very few of them, including DPPH, are useful for determining the activity of both hydrophilic and lipophilic species, thus ensuring a better comparison of the results and covering a wider range of possible applications. The results of the free radical-scavenging capacities of the corresponding extracts are shown in Tables 3 and 4. The extracts from the green beans of all varieties at different concentrations exhibited more than 50% scavenging activity, whereas the roasted beans showed more than 60% scavenging activity. Ascorbic acid as the standard compound had the IC_{50} value lower than those of all studied extracts from both green and roasted beans. Hence, it was a more potent antioxidant than the tested samples, because the higher antioxidant activity is reflected in a lower IC_{50}. The radical scavenging activity of the green and roasted beans extracts respectively, decreased in the following order: Heirloom 4 (IC_{50} = 0.105 mg/mL, IC_{50} = 0.102 mg/mL) > Heirloom 3 (IC_{50}= 0.165 mg/mL, IC_{50} = 0.1156 mg/mL) > Heirloom 2 (IC_{50} = 0.195 mg/mL, IC_{50} = 0.141 mg/mL) > Heirloom 1 (IC_{50} = 0.196 mg/mL, IC_{50} = 0.150 mg/mL) (Figures 1 and 2). These differences might have been derived from local, climate, and seasonal factors related to the cultivation regions of the varieties. Phenolic compounds with a higher number of hydroxyl groups have enhanced ROS scavenging activity (Świętek *et al.* 2019). It was reported that the decrease in the absorbance of DPPH

Table 3. DPPH radical scavenging activity of the green beans in four *Coffea arabica* varieties studied.

	DPPH concentration (mg/mL)					
Variety	**0.2**	**0.4**	**0.6**	**0.8**	**1**	**Average**
Heirloom 1	52.62 ± 4.13^{m}	68.51 ± 2.45^{kl}	$78.42 \pm 4.40^{h-j}$	$85.97 \pm 2.06^{d-g}$	$89.90 \pm 4.44^{c-e}$	75.08
Heirloom 2	66.77 ± 5.40^{l}	73.51 ± 4.44^{jk}	$80.02 \pm 2.86^{g-i}$	88.66 ± 2.06^{de}	$91.24 \pm 3.49^{b-d}$	80.04
Heirloom 3	67.33 ± 2.06^{l}	75.30 ± 3.49^{ij}	$82.38 \pm 3.01^{f-h}$	88.66 ± 0.79^{de}	$94.84 \pm 1.27^{a-c}$	81.70
Heirloom 4	$74.18 \pm 0.63^{i-k}$	$82.04 \pm 0.32^{f-h}$	$84.51 \pm 0.32^{e-g}$	$86.53 \pm 2.54^{d-f}$	$89.90 \pm 1.59^{c-e}$	83.43
Ascorbic acid	96.43 ± 0.16^{ab}	96.85 ± 0.20^{ab}	98.05 ± 0.22^{a}	98.45 ± 0.62^{a}	99.06 ± 0.12^{a}	97.76

Data are expressed as mean ± standard deviation (n = 2); Means with different letters denote significant differences according to Duncan's multiple range test ($p \leq 0.05$).

Table 4. DPPH radical scavenging activity of the roasted beans in four *Coffea arabica* varieties studied.

	DPPH concentration (mg/mL)					
Variety	**0.2**	**0.4**	**0.6**	**0.8**	**1**	**Average**
Heirloom 1	62.64 ± 0.31^{k}	$79.10 \pm 11.00^{g-j}$	$85.43 \pm 1.72^{d-h}$	$91.43 \pm 2.99^{a-e}$	97.67 ± 1.42^{ab}	83.25
Heirloom 2	76.54 ± 1.73^{ij}	$81.65 \pm 4.56^{f-i}$	$91.66 \pm 3.93^{a-e}$	$92.88 \pm 1.26^{a-d}$	98.33 ± 0.47^{a}	88.21
Heirloom 3	76.76 ± 0.16^{ij}	$78.65 \pm 3.46^{h-j}$	$87.44 \pm 2.04^{c-g}$	$89.21 \pm 6.76^{b-f}$	$95.55 \pm 0.00^{a-c}$	85.52
Heirloom 4	71.31 ± 6.92^{j}	$79.32 \pm 5.03^{g-j}$	$80.54 \pm 1.10^{g-i}$	$83.77 \pm 3.15^{e-i}$	96.78 ± 2.67^{ab}	82.34
Ascorbic acid	96.43 ± 0.16^{ab}	96.85 ± 0.20^{ab}	98.05 ± 0.22^{a}	98.45 ± 0.62^{a}	99.06 ± 0.12^{a}	97.76

Data are expressed as mean ± standard deviation (n = 2); Means with different letters denote significant differences according to Duncan's multiple range test ($p \leq 0.05$).

radical caused by phenolic compounds is due to the reaction between antioxidant molecules and radicals, resulting in the scavenging of the radicals by hydrogen donation and is visualized as a discoloration from purple to yellow (Thirumalai *et al.* 2011), and flavonoids with their hydroxyl groups may be also responsible for the radical scavenging potential of the extracts. Moreover, a strong positive correlation between total phenol content and scavenging activity was reported here, while

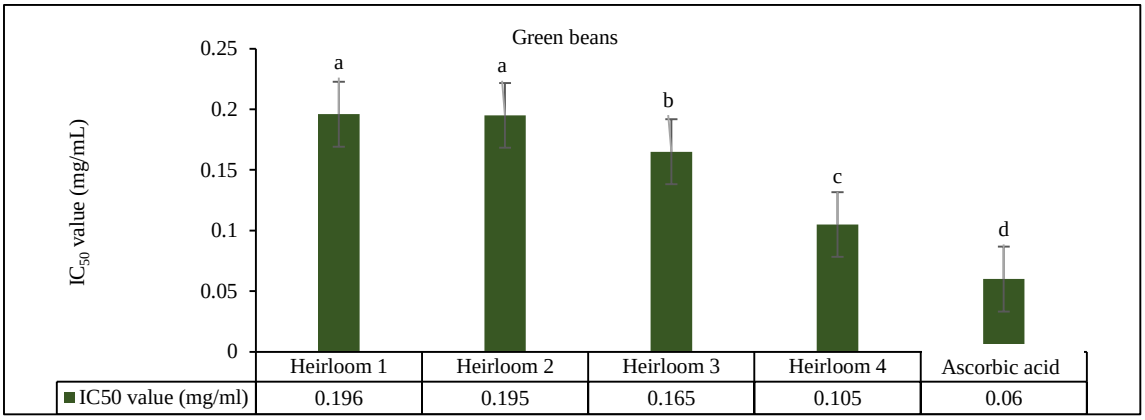


Figure 1. IC_{50} values of DPPH scavenging activity of the green beans in four *Coffea arabica* varieties studied and ascorbic acid; Means with different letters denote significant differences according to Duncan's multiple comparison test ($p \leq 0.05$).

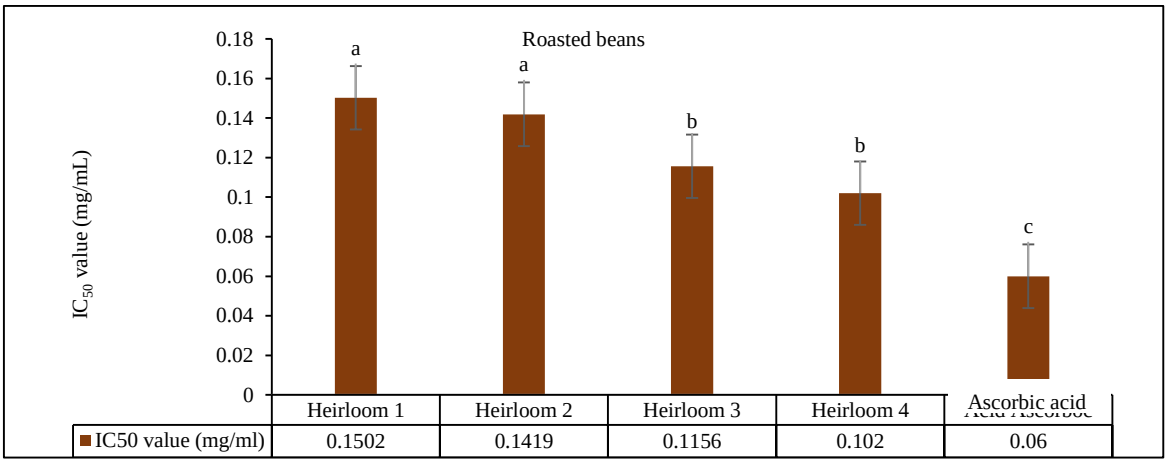


Figure 2. IC_{50} values of DPPH scavenging activity of the roasted beans in four *Coffea arabica* varieties studied and ascorbic acid; Means with different letters denote significant differences according to Duncan's multiple comparison test ($p \leq 0.05$).

these characteristics were strongly and negatively correlated with the DPPH IC_{50} (Table 5). Several experimental data in the literature support that the total phenolic content contributes to the antioxidant activity of some plants (Karamian and Ahmadi Khoei 2022). However, the antioxidant activity assessed by the DPPH assay did not correlate with the total phenolic content in different *Epilobium* species, contradicting that these compounds contribute to antioxidant activity (Abbasi Karin *et al.* 2023). The variation in phytochemical traits of medicinal plant species can be attributed to the effect

of various geographical conditions and also, developmental and genetic factors (Rahmati and Ghorbanpour 2023).

***Metal chelating activity*:** Lipid peroxidation can be described generally as the reaction of lipids with molecular oxygen. It involves hydrogen-atom abstraction by peroxyl radicals, with oxygen addition to carbon radicals resulting in lipid peroxyl radicals and hydroperoxides (Yin *et al.* 2011). Iron is an essential metal for the transport of oxygen, respiration, and activity of enzymes. Also, it is the most important pro-oxidant among transition metals because of its high reactivity (Cho *et al*. 2013). The ferrous iron reacts with hydrogen peroxide (Fenton reaction) and consequently produces reactive free radicals (Prescott and Bottle 2017). According to our results, the metal chelating activity was enhanced with increasing concentrations of both green and roasted bean extracts (Tables 6 and 7). The highest metal chelating average was obtained by Heirloom 4 and Heirloom 2 in green and roast beans, respectively. Also, all extracts had weaker metal chelating activity than ascorbic acid, representing a synthetic antioxidant. Moreover, the green beans had higher metal-chelating activity than the roasted beans. The low metal chelating activity after thermal processing indicates the low thermal stability of some antioxidants. Tannic acid, a polyphenolic in coffee beans, is regarded as a strong antioxidant because it can chelate metal ions such as Fe^{2+} and Cu^{2+} ions (Lopez *et al*. 1999).

***β-Carotene/linoleic acid assay*:** The oxidation of linoleic acid in the β-carotene/linoleic acid assay was effectively inhibited by Heirloom 4 green and roast bean extracts (73.99% ± 4.04 and 94.22% ± 1.41, respectively), followed by Heirloom 3 (73.20% ± 3.36; 92.71 ± 2.43), Heirloom 2 (63.48% ± 2.66; 81.54 ± 5.04), and Heirloom 1 (61.94% ± 1.48; 77.46 ± 3.62) (Table 8). Our results showed that ethanolic extracts of the green and roasted beans of Heirloom 3 and Heirloom 4 were significantly different from Heirloom 1 and Heirloom 2 in the inhibition of linoleic acid oxidation (Figure 3). The β-carotene/linoleic acid assay showed that all green bean extracts have lower activity than BHT as a synthetic antioxidant, whereas the Heirloom 4 and Heirloom 3 roasted bean extracts represented higher activity than BHT.

Antibacterial activity assay

In a preliminary antibacterial test conducted before this experiment, we found that the ethanolic extracts of green and roasted coffee samples did not have any antibacterial activity. However, their boiled-water extracts showed antibacterial activity. Additionally, the aqueous extracts of the roasted beans exhibited better results than the extracts from the green beans (mean of roasted beans: 8.16±

Table 5. Linear correlation between evaluated antioxidant characteristics of the roasted and green beans in four *Coffea arabica* varieties studied and ascorbic acid.

	AA_1 in roasted beans	**IC_{50} in roasted beans**	**AA_1 in green beans**	**IC_{50} in green beans**
Total phenol content	0.971	0.112	0.921	0.108
Total flavonoid content	0.963	0.121	0.911	0.142

AA_1 = Antioxidant activity (by scavenging DPPH free radical); IC_{50} = The amount of sample necessary to decrease DPPH. by 50%.

Table 6. The metal chelating activity of the green beans in four *Coffea arabica* varieties studied and ascorbic acid.

Variety	**Fe-Chelate concentration (mg/mL)**					
	0.2	**0.4**	**0.6**	**0.8**	**1**	**Average**
Heirloom 1	55.38 ± 3.10^{ij}	62.71 ± 3.29^{gh}	69.30 ± 1.53^{ef}	73.73 ± 1.15^{cd}	75.58 ± 2.96^{c}	67.34
Heirloom 2	57.45 ± 1.84^{i}	62.08 ± 0.92^{h}	66.43 ± 2.91^{fg}	68.49 ± 2.34^{ef}	75.23 ± 1.31^{c}	65.94
Heirloom 3	52.47 ± 3.82^{j}	57.45 ± 1.87^{i}	63.04 ± 2.20^{gh}	69.34 ± 2.85^{ef}	74.23 ± 2.03^{cd}	63.31
Heirloom 4	63.36 ± 2.61^{gh}	70.69 ± 3.14^{de}	77.54 ± 4.17^{c}	84.50 ± 0.88^{b}	87.43 ± 0.50^{b}	76.70
Ascorbic acid	95.44 ± 0.57^{a}	96.61 ± 0.35^{a}	97.19 ± 0.22^{a}	97.83 ± 0.11^{a}	98.27 ± 0.34^{a}	97.07

Data are expressed as mean ± standard deviation (n = 3); Means with different letters denote significant differences according to Duncan's multiple range test ($p \leq 0.05$).

Table 7. The metal chelating activity of the roasted beans in four *Coffea arabica* varieties studied and ascorbic acid.

Variety	**Fe-Chelate concentration (mg/mL)**					
	0.2	**0.4**	**0.6**	**0.8**	**1**	**Average**
Heirloom 1	29.99 ± 4.87^{k}	46.90 ± 1.32^{fgh}	60.67 ± 5.93^{cde}	64.01 ± 2.60^{bcd}	67.75 ± 0.49^{b}	53.86
Heirloom 2	43.18 ± 2.50^{hi}	57.90 ± 3.22^{e}	66.41 ± 2.77^{bc}	69.64 ± 2.70^{b}	70.02 ± 2.76^{b}	61.43
Heirloom 3	35.33 ± 1.93^{jk}	44.79 ± 0.92^{ghi}	49.75 ± 3.14^{fg}	57.73 ± 1.99^{e}	64.88 ± 1.52^{bc}	50.50
Heirloom 4	38.94 ± 8.94^{ij}	44.29 ± 2.75^{ghi}	51.58 ± 4.24^{f}	58.86 ± 2.39^{de}	65.88 ± 6.65^{bc}	51.91
Ascorbic acid	95.44 ± 0.57^{a}	96.61 ± 0.35^{a}	97.19 ± 0.22^{a}	97.83 ± 0.11^{a}	98.27 ± 0.34^{a}	97.07

Data are expressed as mean ± standard deviation (n = 3); Means with different letters denote significant differences according to Duncan's multiple range test ($p \leq 0.05$).

Table 8. Inhibition percentage of the linoleic acid oxidation by the roasted and green beans in four *Coffea arabica* varieties studied and BHT.

Variety	**Roasted beans (%)**	**Green beans (%)**
Heirloom 1	77.46 ± 3.62^{c}	61.94 ± 1.48^{c}
Heirloom 2	81.54 ± 5.04^{b}	63.48 ± 2.66^{c}
Heirloom 3	92.71 ± 2.43^{a}	73.20 ± 3.36^{b}
Heirloom 4	94.22 ± 1.40^{a}	73.99 ± 4.04^{b}
BHT	82.06 ± 3.82^{b}	82.06 ± 3.82^{a}
Control	21.99 ± 2.19^{d}	21.99 ± 2.19^{d}

Data are expressed as mean ± standard deviation (n = 3); Means with different letters in each column denote significant differences according to Duncan's multiple comparison test ($p \leq 0.05$).

0.61; mean of green beans: 3.20 ± 0.57). The roasted samples showed higher activity against all tested bacteria as compared with the negative control (Table 9), whereas some green bean extracts demonstrated the same antibacterial activity as the negative control (Table 10). Among the roasted bean extracts, Heirloom 3 showed the highest activity against *Pseudomonas aeruginosa* (mean: 11.21 ± 0.57), *Staphylococcus aureus* (mean: 8.66 ± 0.57), and *Bacillus cereus* (mean: 7.22 ± 0.57). However, among the green bean extracts, Heirloom 3 and Heirloom 4 showed the highest activity against all bacterial strains tested (mean: 3.63 ± 0.21; 4.74 ± 0.28). Altogether in green and roasted beans extracts, the highest antibacterial activity was shown against *S. aureus* and *B. cereus*, and the lowest antibacterial activity was shown against *E. coli* (mean: 4.80 ± 0.28; 4.30 ± 0.23; 0.44 ± 0.04). These findings suggest that roasted bean extracts are a rich source of phenolic compounds and exhibit antibacterial activity against both gram-positive and gram-negative bacteria. The antibacterial activities of the flavonoid compounds could be attributed to their reaction with extracellular proteins resulting in the formation of complex compounds that damage the cell membrane and therefore, inhibit the use of oxygen by bacteria (Cushnie and Lamb 2005).

Table 9. Antibacterial activity of the roasted beans in four *Coffea arabica* varieties studied.

Variety	Concentration (g/mL)	Inhibition zone (mm)			
		Staphylococcus aureus	***Pseudomonas aeruginosa***	***Escherichia coli***	***Bacillus cereus***
Heirloom 1	0.1	6.33 ± 0.57^{e}	7.33 ± 0.57^{de}	9.00 ± 1.00^{abc}	7.33 ± 0.57^{de}
	0.2	10.00 ± 1.00^{ab}	7.66 ± 1.15^{cde}	0.00 ± 0.00^{g}	10.00 ± 1.00^{ab}
	0.4	10.00 ± 1.00^{ab}	9.66 ± 0.57^{ab}	5.33 ± 0.57^{f}	10.33 ± 1.15^{a}
Heirloom 2	0.1	6.33 ± 0.57^{g}	7.66 ± 0.57^{ef}	7.00 ± 1.00^{efg}	6.66 ± 0.57^{fg}
	0.2	10.00 ± 1.00^{b}	10.33 ± 0.57^{b}	8.00 ± 1.00^{de}	7.33 ± 0.57^{efg}
	0.4	10.00 ± 1.00^{b}	13.66 ± 0.57^{a}	10.66 ± 0.57^{b}	9.00 ± 0.00^{cd}
Heirloom 3	0.1	7.66 ± 0.57^{de}	8.66 ± 0.57^{cd}	0.00 ± 0.00^{f}	7.66 ± 0.57^{de}
	0.2	8.33 ± 0.57^{cd}	11.33 ± 0.57^{b}	0.00 ± 0.00^{f}	10.00 ± 1.00^{bc}
	0.4	10.00 ± 0.00^{bc}	13.66 ± 0.57^{a}	6.00 ± 0.00^{e}	11.66 ± 0.57^{b}
Heirloom 4	0.1	7.33 ± 0.57^{e}	6.00 ± 0.00^{f}	6.33 ± 0.57^{f}	6.66 ± 0.57^{ef}
	0.2	8.33 ± 0.57^{d}	6.33 ± 0.57^{f}	7.33 ± 0.57^{e}	10.33 ± 0.57^{ab}
	0.4	9.66 ± 0.57^{bc}	9.66 ± 0.57^{bc}	9.00 ± 0.00^{cd}	10.66 ± 0.57^{a}
Distilled water		0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
		0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
		0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Data are expressed as mean ± standard deviation (n = 3); Means with different letters within each variety denote significant differences according to Duncan's multiple range test ($p \leq 0.05$).

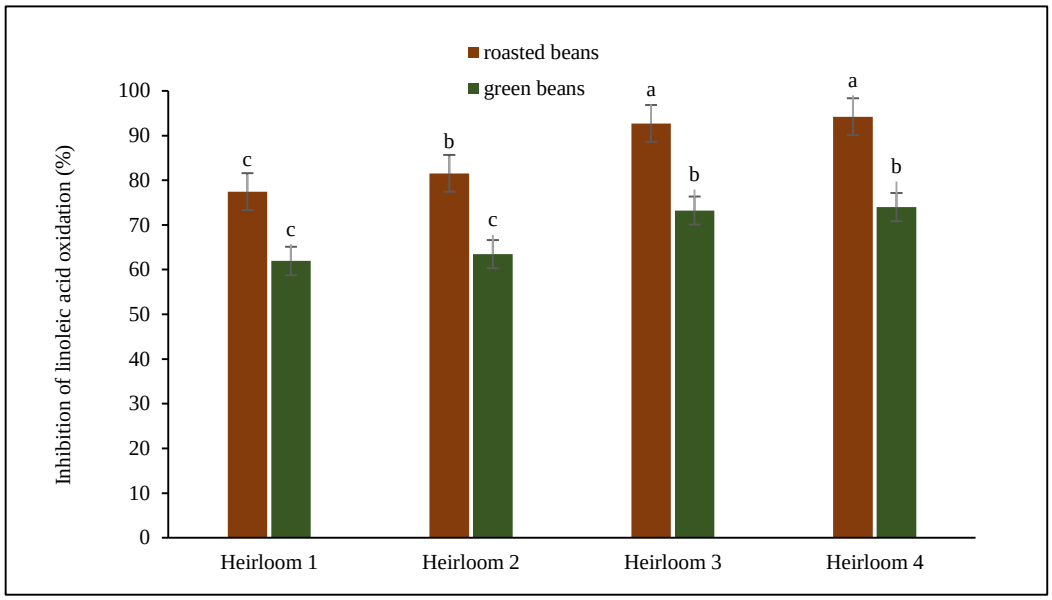


Figure 3. Inhibition of bleaching of ß-carotene/linoleic acid emulsion by the four *Coffea arabica* varieties studied in comparison with BHT as a synthetic antioxidant (82.06%); Means with different letters denote significant differences according to Duncan's multiple comparison test ($p \leq 0.05$).

Table 10. Antibacterial activity of the green beans in four *Coffea arabica* varieties studied.

Variety	Concentration (g/mL)	Inhibition zone (mm)			
		Staphylococcus aureus	***Pseudomonas aeruginosa***	***Escherichia coli***	***Bacillus cereus***
Heirloom 1	0.1	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	5.00 ± 0.00^{b}
	0.2	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	5.00 ± 0.00^{b}
	0.4	6.33 ± 0.57^{a}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	5.33 ± 0.57^{b}
Heirloom 2	0.1	6.00 ± 0.00^{b}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}
	0.2	6.33 ± 0.57^{b}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}
	0.4	6.33 ± 0.57^{b}	5.33 ± 0.57^{c}	0.00 ± 0.00^{d}	8.33 ± 0.57^{a}
Heirloom 3	0.1	0.00 ± 0.00^{d}	8.00 ± 0.00^{b}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}
	0.2	0.00 ± 0.00^{d}	10.33 ± 0.57^{a}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}
	0.4	6.66 ± 0.57^{c}	10.33 ± 0.57^{a}	0.00 ± 0.00^{d}	8.33 ± 0.57^{b}
Heirloom 4	0.1	7.66 ± 0.57^{bc}	0.00 ± 0.00^{g}	0.00 ± 0.00^{g}	6.33 ± 0.57^{de}
	0.2	8.00 ± 0.00^{b}	0.00 ± 0.00^{g}	0.00 ± 0.00^{g}	7.00 ± 0.00^{cd}
	0.4	10.33 ± 0.57^{a}	6.00 ± 1.00^{ef}	5.33 ± 0.57^{f}	6.33 ± 0.57^{de}
Distilled water		0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
		0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
		0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Data are expressed as mean ± standard deviation (n = 3). Means with different letters within each variety denote significant differences according to Duncan's multiple range test ($p \leq 0.05$).

Conclusions

In general, various factors including genetic factors affect the content of chemical compounds in coffee beans. In addition to these factors, environmental conditions like altitude (investigated in this study), extraction methods, and type of solvent used (ethanol/water), affect the final chemical composition and bioactivity of the extracts. All antioxidant activity assays showed a close relationship with the phenolic content in green and roasted samples. Our findings indicated that the extracts from coffee green or roasted beans have potent free radical scavenging activity in different antioxidant activity assays, such as DPPH-radical scavenging, metal chelating, and β-carotene/linoleic acid inhibition. Moreover, the differences in the antioxidant activity between green and roasted bean extracts in the same variety could be related to the roasting process. The antibacterial activity of aqueous extracts of the green and roasted beans may be partially attributed to the effect of phenolic compounds on the permeability of the cell wall and cytoplasmic membrane. Overall, it appears that the bioactivity of the coffee beans depends on the chemical composition of each variety, which requires further investigation.

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Ethical considerations

The authors avoided data fabrication and falsification.

Conflict of interest

The authors declare that they have no conflict of interest with any organization concerning the subject of the manuscript.

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Effects of drought stress on yield and morphophysiological traits of quinoa (*Chenopodium quinoa* Willd.) at different levels of nitrogen

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Abstract

Objective: This study aimed to investigate the effect of drought stress and nitrogen fertilizer on grain yield and some morphophysiological traits of quinoa.
Methods: A factorial experiment was performed for the quinoa Titikaka cultivar. The factors included three levels of water-deficit stress based on soil moisture depletion [45% (control), 65%, and 85%] and three levels of nitrogen fertilizer including 50 (control), 100, and 150 kg/ha.
Results: The highest leaf area index in the first year was obtained at 45% soil moisture depletion (without drought stress) and the highest chlorophyll index was obtained at 65% soil moisture depletion level. In the second year, the highest leaf area index (11.3) and the highest chlorophyll index (64.20) were obtained from 65% and 85% soil moisture depletion combined with 150 kg/ha of nitrogen fertilizer, respectively. In the first year of the experiment, the 45% soil moisture depletion with 150 kg/ha of nitrogen fertilizer had the highest number of panicles per plant (13.1). Also, the highest 1000-seed weight (2.47 g), grain yield (1926.63 kg/ha), and plant height (49.15 cm) were obtained from the 45% soil moisture discharge and 150 kg/ha of nitrogen fertilizer.
Conclusion: The population structure and genetic relationships of the Iranian bread wheat landraces presented here highlight their diverse genetic architecture. The results of this study provide valuable information for the utilization of landraces in the genetic improvement of bread wheat.

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Introduction

Quinoa (*Chenopodium quinoa* Willd) is a broadleaf plant of the Amaranthaceae family (Langeroodi *et al.* 2020). Recently, interest in quinoa seeds for human nutrition has increased worldwide (Goldberger and Detjens 2019; Langeroodi *et al.* 2020). From an agricultural point of view, this plant is important due to its resistance to abiotic stresses such as drought and salinity (Fathi and Kardoni 2020). Crop yield is limited by environmental stresses and therefore there is a significant difference between potential and actual crop yield. Lack of moisture at different stages of growth reduces nutrient uptake, water uptake, transfer of nutrients within the plant, and yield (Payandeh *et al.* 2018). Nitrogen is involved in forming amino acids, nucleic acids, enzymes, chlorophyll, vitamins, and hormones (Robertson and Vitousek 2009). If adequate nitrogen is available to the plant, it increases the growth of the plant and stores protein in the grain (An *et al.* 2024). It was found that N improves quinoa drought tolerance and improves seed yield and quality (Alandia *et al.* 2016). Due to the importance of using quinoa as a drought-tolerant plant, this study aimed to evaluate grain yield and some morphophysiological traits of quinoa under water-shortage stress at different levels of nitrogen fertilizer.

Materials and Methods

This study was conducted in 2017 and 2018 in the Sistan Agricultural and Natural Resources Research Center, Zahak, Iran. Zahak is located at an altitude of 481 m above sea level. According to Koppen's classification, the region's climate is classified as arid and very hot with hot and dry summers. According to the statistics of Zahak Meteorological Station, long-term (>75 years) average annual rainfall is approximately 53 mm and evaporation is 4000-4500 mm/year (Sistan and Baluchistan Provincial Portal). The meteorological information of the study area in 2017 and 2018 is given in Table 1.

Before starting the experiment, different parts of the field soil were randomly sampled from a depth of 0-30 cm. Then, a composite sample was selected, transferred to the laboratory, and subjected to chemical and physical analysis. Tables 2 and 3 show the results of the analysis of soil samples.

The experiment was performed as factorial based on a randomized complete block design with three replications. The factors were water-deficit stress (using TDR) at three levels of 45% (control), 65%, and 85% soil moisture depletion and nitrogen fertilizer (Urea) at three levels of 50 (control), 100, and 150 kg/ha.

Tillage operations included plowing and two vertical discs, followed by leveling and plotting. Each plot consisted of six planting rows at a distance of 25 cm with a length of 3 m and a plant-to-

Table 1. Meteorological information of the study area (www.timeanddate.com).

2017						2018					
Month	**Temperature (°C)**			**Precipitation**	**Wind**	**Month**	**Temperature (°C)**			**Precipitation**	**Wind**
	Max	**Min**	**Mean**	**(mm)**	**(km/h)**		**Max**	**Min**	**Mean**	**(mm)**	**(km/h)**
Oct.	30	-2	15	0	9	March	35	4	23	1.5	13
Nov.	26	-11	9	0.9	10	Apr.	36	11	26	0.2	12
Dec.	24	-4	9	4	12	May	42	17	31	0	13
Jan.	28	-7	13	2	15	June	42	18	33	0	14
Feb.	34	5	20	3.6	15	July	39	13	30	0	11

Table 2. Physical and chemical properties of the soil in the experimental site.

Texture	**Clay**	**Sand**	**Silt**	**Depth**	**EC**	**pH**	**TNV**	**Total N**	**P**	**K**	**OC**	**Fe**	**Zn**	**Cu**	Mn
	%	**%**	**%**	**Cm^3**	**ds/m**		**%**	**%**	**ppm**	**ppm**	**%**	**ppm**	**ppm**	**ppm**	**ppm**
Sandy loam	12	64	24	0-30	2.1	7.6	12.5	0.03	12	170	0.32	4.08	0.42	0.59	3.83

Table 3. Anions and cations (meq/l) in the soil samples of the experimental site.

Cd	**Bd**	**CEC**	**Plaster**	**K**	**ESP**	**SAR**	**Na**	**Mg**	**Ca**	**pH**	**EC**
ppm	**g/cm^3**	**cmol/kg**	**%**								**ds/m**
N.D	1.71	5.7	Less than 0.5	1.5	3.1	3.06	7.5	4	8	7.6	2.1

plant distance of 5 cm within a row. Before planting, furrow irrigation was conducted and according to the soil test (Table 2), 50 kg/ha of triple superphosphate was applied with the irrigation water. Sowing was done on November 10 in both years. The Titi-Kaka cultivar quinoa was obtained from Gorgan Agricultural and Natural Resources Research Center, Gorgan, Iran. The weeds were controlled by hand throughout the growing season. To control *Aphidoidea* and *Agrotis* spp., two grams per litter of diazinon were sprayed on the shoots three times in 7-day intervals. Water-deficit stress was applied at the stem elongation stage.

Irrigation regime time was determined by using the soil water curve with the application of digital hygrometer equipment of Time-Domain Reflectometry (TDR, Model Trim, FM3, Germany). Nitrogen fertilizer was applied with the irrigation water at two stages ½ of the fertilizer at 6-8 leaf stage and the remaining half before the flowering.

Plants were harvested manually on 20 June at physiological maturing when quinoa plants were completely yellow. The seeds within the inflorescence were easily separated by hand.

Measurements of the physiological traits were made at a fixed time of day between 11.00 to 13.00 h at 50% flowering stage. Three plants were randomly taken at the flowering stage and leaf area was measured using the Delta T Device. SPAD values or leaf chlorophyll index (SPAD-502, Minolta Co Ltd, Osaka, Japan), were measured as an average of 10 leaves. Also, canopy temperature (Tc), was measured by a portable infrared thermometer (USA), and air temperature (Ta) was measured by a hydrargyric thermometer. In each plot, plants of four central rows were harvested to determine seed yield.

Relative water content (RWC) was calculated according to the method of Weatherley (1950). Leaf samples were saturated in 100 ml of water for 4 h and their turgid weights were recorded. Then, they were rolled in dried butter paper and oven dried at 65 ^{o}C for 48 h, then, their dry weights were recorded. RWC was calculated as:

RWC= (fresh weight-dry weight)/(turgid weight- dry weight)

Canopy temperature was measured using a thermometer on sunny days, 11-13 hours, and 90 days after planting. At the blooming stage, three plants were randomly separated and leaf area was measured using the Delta T Device and then the leaf area index (LAI) was calculated.

Plant height was measured from the crown (ground connection) to the tip of the plant, using a calibrated wooden ruler. One thousand seeds were randomly selected from the threshed seeds and weighed using a digital scale with an accuracy of 0.01 g. A digital scale was used to quantify the biological yield per unit area after the plants were harvested and dried. The harvest index was computed by dividing the grain yield by the biological yield multiplied by 100 The number of seeds per panicle was acquired by counting the number of seeds per 10 panicles per plot. To determine the biological yield and grain yield of the crop, harvesting was done from the middle part of each plot by observing the marginal effects and removing half a meter from the sides and half a meter from the beginning and end of the plot. The experiment in the second year was performed similarly to the first year.

After collecting the data, the Bartlett test of homogeneity of variances between experimental errors of the years was performed for the studied traits. The chi-square statistic was not significant for the biological yield, the number of seeds per panicle, and relative leaf water content, so a two-year combined analysis was performed for these traits. Separate analyses for each year were performed for the other traits. After analysis of variance, the means were compared by Duncan's multiple range test at the 5% probability level. The data were analyzed using SAS v.9.4 statistical software and the diagrams were drawn using Excel software.

Results and Discussion

Leaf area index

The effect of nitrogen fertilizer in both years and water deficit stress in 2017 on the leaf area index was significant. However, the interaction of these factors for leaf area index was only significant in 2018 (Table 4). In the first year, the maximum leaf area index (4.38) was produced from 45 percent soil moisture depletion, and in the case of nitrogen fertilizer, the highest value (5) was obtained for 150 kg ha^{-1} (Table 5). In the second year, the mean comparison of treatments revealed that 65 percent soil moisture depletion with 150 kg ha^{-1} nitrogen fertilizer produced the greatest leaf area index (11.33) followed by 45 percent soil moisture depletion with 150 kg ha^{-1} nitrogen fertilizer (Table 6). The results of this study in the first year are consistent with the studies conducted by Ghassemi-Golezani and Mousavi (2022) on the reduction in leaf area index in corn in the face of high levels of drought stress. Also, the experimental results of Geren (2015) in quinoa and Bahamin *et al.* (2019) in corn showed that using nitrogen fertilizer increased the leaf area index in these plants.

Chlorophyll index (SPAD)

The first year's data analysis revealed that only the effect of drought stress on chlorophyll index was significant (Table 4). With 65 percent soil moisture depletion, the maximum chlorophyll index (54.40) was achieved but increasing the drought stress to 85 percent soil moisture depletion decreased it significantly (46.92) (Table 5). However, the analysis of variance of data in the second year showed that the effect of drought stress, nitrogen fertilizer, and their interactions was significant on the chlorophyll index (Table 4). With 45 percent soil moisture depletion and 150 kg ha^{-1} nitrogen fertilizer, the maximum chlorophyll index (64.20) was obtained. When compared to the control levels of drought stress and nitrogen fertilizer, this treatment increased by 24.29 percent. The lowest chlorophyll index (48.6) was attained by depleting 85 percent of soil moisture with 50 kg ha-*1* nitrogen fertilizer (Table 6). Fghire *et al.* (2015) stated that the application of drought stress reduced the pigmentation of quinoa leaves and, as a result, the content of chlorophyll in this plant. The reasons for reducing quinoa chlorophyll due to drought stress could be due to the degradation of chloroplast thylakoid membrane, chlorophyll optic oxidation because of increased activity of reactive oxygen species, and increased chlorophyllase activity (Hinojosa *et al*. 2018). Also, the reason for the superiority of 150 kg/ha of nitrogen fertilizer could be due to the adequate and gradual supply of nitrogen may have caused quinoa to produce chlorophyll and other elements required for photosynthesis. Others have also reported the significant effect of chemical fertilizers on increasing the chlorophyll content in quinoa (Gomaa 2013).

Table 4. Analysis of variance for physiological traits of quinoa under the influence of water-deficit stress and nitrogen fertilizer in two crop years.

Source of variation	df	Mean squares					
		Leaf area index		Chlorophyll (SPAD)		Canopy temperature	
		2017	2018	1st year	2nd year	1st year	2nd year
Replication	2	0.194	3.37	39.55	24.96*	53.72**	20.15
Water-deficit stress (S)	2	1.750**	3.70	132.11*	36.75**	1.068	2.46
Nitrogen fertilizer (N)	2	10.361**	9.93*	13.27	56.91**	1.748	39.85*
S × N	4	0.528	12.48**	30.65	90.89**	1.322	18.97
Error	16	0.278	1.954	34.35	5.790	2.416	7.21
Coefficient of variation (%)	-	12.99	15.09	11.68	4.54	10.04	9.63

* and **: Significant at the 5% and 1% probability levels, respectively.

Table 5. Means of quinoa physiological traits under drought stress and nitrogen fertilizer in two crop years.

Treatment	Leaf area index	Chlorophyll (SPAD)	Canopy temperature (ºC)	
	1st year	1st year	1st year	2nd year
Soil moisture depletion				
45%	4.38a	49.21ab	15.12a	27.30a
65%	4.22a	54.40a	15.81a	27.95a
85%	3.55b	46.92b	15.46a	28.33a
Nitrogen fertilizer				
50 kg / ha	2.88c	50.90a	15.83a	25.43b
100 kg / ha	4.27b	48.77a	14.97a	29.10a
150 kg / ha	5.00a	50.85a	15.58a	29.05a

According to Duncan's multiple range test, means with different letters for each factor in each column are significantly different at the 5% probability level.

Table 6. Means of quinoa leaf area index and chlorophyll (SPAD) for the combination of drought stress and nitrogen fertilizer factors in two crop years.

Treatment	Leaf area index	Chlorophyll (SPAD)
	2nd year	2nd year
S_1N_1	9.00^{ab}	49.2^{c}
S_1N_2	10.00^{a}	55.2^{b}
S_1N_3	11.00a	64.2^{a}
S_2N_1	9.33^{ab}	49.2^{c}
S_2N_2	6.00^{c}	51.9^{bc}
S_2N_3	11.33^{a}	54.0^{bc}
S_3N_1	$7.00b^{c}$	48.6^{c}
S_3N_2	10.67^{a}	51.3^{bc}
S_3N_3	9.00^{ab}	52.8^{bc}

According to Duncan's multiple range test, means with different letters in each column are significantly different at the 5% probability level; S1 (control), S2, and S3 are 45%, 65%, and 85% soil moisture depletion; N_1, N_2, and N_3 are 50, 100, and 150 kg/ha nitrogen fertilizer.

Canopy temperature

The analysis of variance in the first year showed no significant effects of drought stress, nitrogen fertilizer, and their interaction on canopy temperature in quinoa. But in the second year, this trait wasonly affected by nitrogen fertilizer (Table 4). According to Table 1, the maximum temperatures during the growth period of the first year were lower than the second year, and probably for this reason, no significant effect was observed in the first year.

Increasing the nitrogen fertilizer from 50 to 100 or 150 kg/ha, increased the canopy temperature significantly (Table 5). It seems that the presence of more nutrients due to nitrogen fertilizer and consequently, more plant growth has increased the plant's water requirement, however, the water absorbed and transferred by the roots did not compensate for the plant's transpiration and consequently, the canopy temperature rose. High temperatures often intensify and complicate the effects of drought and can sometimes affect plant survival under severe stress. Riahinia *et al.* (2006) reported a positive linear relationship between leaf water potential and canopy temperature in maize, sunflower, cotton, and beans.

Number of panicles per plant

Based on the analysis of variance the effect of nitrogen and its interactions with nitrogen fertilizer was significant on the number of panicles per plant in quinoa in the first year (Table 7). However, in the second year, none of the factors and their interaction had a significant effect on this trait (Table 7). According to Table 8, irrigation at 45 percent soil moisture depletion (no stress) with 100 kg/ha of nitrogen fertilizer produced the highest number of panicles (13.10), followed by 65% and 85% soil moisture depletion at this nitrogen level 12.88 and 12.42, respectively. However, the number of panicles decreased significantly with 150 kg/ha at all moisture levels. Basra *et al.* (2014) also reported the reduction of spike number in quinoa when 75 kg of nitrogen fertilizer was applied per hectare. It looks like that in our experiment the optimum nitrogen level for the number of panicles at all soil moisture levels is 100 kg/ha.

In this experiment, the soil moisture stress did not affect the number of panicles at all nitrogen levels. However, Jamali *et al*. (2018) indicated that reducing the amount of water had a negative impact on yield and yield components of quinoa.

1000-seed weight

According to the first year of the experiment, the effects of drought stress and nitrogen fertilizer and their interactions on the 1000-seed weight of quinoa were significant but in the second year, none of

these effects were significant (Table 7). With 45 percent soil moisture depletion (no stress) and 150 kg ha^{-1} nitrogen fertilizer, the maximum 1000-seed weight (2.47 g) was obtained followed by 65 percent soil moisture depletion at the same nitrogen fertilizer level. Increasing the use of nitrogen in quinoa reduces the physiological removal of flowers and increases the number of sub-branches in the plant, increases the production of photosynthetic materials, flower fertility, and flowering period, and thus 1000-seed weight (Saeedi *et al.* 2019). The flow of photosynthetic material and the re-transfer of carbon and nitrogen to the grain at the grain-filling stage depend on the source-reservoir ratio.

Table 7. Analysis of variance of quinoa agronomic traits under the influence of drought stress (S) and nitrogen fertilizer (N) in two crop years.

Source of variation	df	Panicle/ plant		1000-seed weight		Plant height		Grain yield		Harvest index	
		1st year	2nd year	1st year	2nd year	1st year	2nd year	1st year	2nd year	1st year	2nd year
Replication	2	0.091	1.149	0.00009	0.0209	0.58	7.09	625.4*	25998	1.28	3.8
S	2	0.030	2.301	0.2126**	0.0368	79.37**	50.61**	6064**	188677*	134.4**	120.2**
N	2	2.87**	0.515	0.017**	0.0164	20.70*	12.4*	17056**	93968	32.0**	312.2**
S × N	4	0.51**	2.011	0.4590**	0.0416	75.49**	1.73	23878**	236147	89.3**	70.6**
Error	16	0.101	0.778	0.0027	0.0184	5.37	3.54	157.1	36932	2.98	5.92
CV (%)	-	2.61	10.60	2.48	7.04	5.25	4.01	0.71	11.40	9.96	11.69

* and **: Significant at the of 5 and 1%probability levels, respectively.

Table 8. Means of quinoa agronomic traits under the influence of drought stress and nitrogen fertilizer in two crop years.

Treatment	Harvest index (%)		Grain yield (kg/ha)	Plant height (cm)	1000-seed weight (g)	Panicle/ plant
	1st year	2nd year	1st year	1st year	1st year	1st year
S_1N_1	14.08^{c}	25.97^{b}	1721.07^{de}	44.77^{bc}	2.22^{bc}	11.55^{d}
S_1N_2	12.60^{c}	15.35^{de}	1741.26^{cd}	47.80^{ab}	2.14^{c}	13.10^{a}
S_1N_3	11.90^{c}	22.69^{bc}	1926.63^{a}	49.15^{a}	2.47^{a}	11.92^{cd}
S_2N_1	19.60^{b}	18.64^{cd}	1681.05^{fg}	39.07^{ef}	1.71^{d}	11.73^{d}
S_2N_2	26.85^{a}	13.85^{e}	1781.41^{b}	47.63^{ab}	2.15^{c}	12.88^{ab}
S_2N_3	12.32^{c}	18.25^{de}	1718.72^{de}	48.40^{ab}	2.40^{a}	11.92^{cd}
S_3N_1	22.35^{b}	36.41^{a}	1670.96^{g}	36.48^{f}	1.69^{d}	12.33^{bc}
S_3N_2	14.91^{c}	16.67^{de}	1754.77^{c}	40.48^{de}	1.70^{d}	12.42^{bc}
S_3N_3	21.30^{b}	19.41^{cd}	1700.29^{ef}	43.52^{cd}	2.30^{b}	11.50^{d}

According to Duncan's multiple range test, means with different letters in each column are significantly different at the 5% probability level; S1 (control), S2, and S3 are 45%, 65%, and 85% soil moisture depletion; N1, N2, and N3 are 50, 100, and 150 kg/ha nitrogen fertilizer.

Higher drought stress (85 percent soil moisture depletion) decreased 1000-seed weight at all nitrogen levels, indicating the negative impact of severe drought stress on the seed weight of quinoa (Table 8). Drought stress reduces leaf gas exchange and thus the size of the source and reservoir in

the plant. In this case, the plant's discharge and distribution of photosynthetic material are impaired (Farooq *et al.* 2009). According to Elewa *et al.* (2017), drought stress reduces the 1000-seed weight of quinoa.

Plant height

The effects of drought stress and nitrogen fertilizer and their interactions in the first year were significant on the plant height of quinoa (Table 7). With 45 percent soil moisture depletion and 150 kg ha^{-1} nitrogen fertilizer, the maximum plant height (49.15 cm) was obtained (Table 8). Increasing nitrogen fertilizer at all soil moisture depletion levels increased the plant height of quinoa. However, drought stress decreased the plant height at all nitrogen levels.

In the second year, the effects of drought stress and nitrogen fertilizer were significant on plant height, but their interaction was not significant (Table 7). The maximum plant height was attained with 45 percent soil moisture depletion (48.90 cm) and 150 kg ha^{-1} nitrogen fertilizer (47.90 cm). The lowest plant height was attained with 85 percent soil moisture depletion (44.23 cm) and 50 kg ha^{-1} nitrogen fertilizer (45.56 cm) (Table 9).

The 85% soil moisture depletion may reduce the amount of non-structural carbohydrates stored in the stems, and leaf area, thereby reducing dry matter production. The reduction in the quinoa plant height in response to drought stress may be due to reduced cell length, turgor, and volume (Elewa *et al*. 2017).

It seems that increasing the use of nitrogen fertilizer in this study increased the photosynthetic capacity of quinoa and therefore, had a positive effect on plant height. The results of a study on quinoa in Pakistan showed that nitrogen fertilizer increased all growth-related traits such as crop growth rate, leaf area index, and plant height. It was stated that this increase is because nitrogen is an integral part of the photosynthetic system (chlorophyll and chloroplasts) (Basra *et al.* 2014).

Grain yield

The effects of drought stress and nitrogen fertilizer and their interactions were significant on grain yield in the first year, but in the second year, only the effect of drought stress was significant (Table 7). In the first year, the maximum grain production (1926.63 kg ha^{-1}) was observed with 45 percent soil moisture depletion (no stress) and 150 kg ha^{-1} nitrogen fertilizer (Table 8). At this nitrogen rate, the grain yield decreased under all drought-stress conditions. The lowest grain yield (1670.96 kg ha^{-1}) was obtained from the soil moisture depletion of 85 percent with 50 kg ha^{-1} nitrogen fertilizer

Table 9. Means for quinoa plant height and grain yield under the influence of drought stress and nitrogen fertilizer in two crop years.

Treatment	Plant height (cm)	Grain yield (kg/ha)
	2nd year	2nd year
Soil moisture depletion		
45%	48.90a	1802.81a
65%	47.30a	1730.04a
85%	44.23b	1523.69b
Nitrogen fertilizer		
50 kg/ha	45.56b	-
100 kg/ha	46.96ab	-
150 kg/ha	47.90a	-

According to Duncan's multiple range test, the means with different letters for each factor in each column are significantly different at the 5% probability level.

(Table 8). The positive interaction between nitrogen and water indicates that nitrogen uptake by plants is directly related to water availability (Ercoli *et al.* 2008).

In the second year, the 45 percent soil moisture depletion showed the highest grain yield (1802.81 kg ha^{-1}) and the 85 percent soil moisture depletion resulted in the lowest grain yield (1523.69 kg/ha) (Table 9). It seems that the reduction in yield compared to the control (no stress) was due to the reduction in photosynthetic pigments, plant height, and 1000-seed weight. Gomaa (2013) reported that nitrogen fertilizer increased vegetative growth, metabolic process, and the accumulation of dry matter in quinoa. The results of an experiment performed on the effect of nitrogen fertilizer levels (0, 50, 75, 100, 125, 150, and 175 kg ha^{-1}) on quinoa grain yield in the Mediterranean climate showed that the best amount of nitrogen for the highest grain yield, harvest index, and 1000-grain weight was 150 kg ha^{-1} (Geren 2015).

Harvest index

In the first and second years of the experiment, the effects of drought stress, nitrogen fertilizer, and their interaction were significant on the harvest index (Table 7). In the first year, with 65 percent soil moisture depletion (no stress) and 100 kg ha^{-1} nitrogen fertilizer, the maximum harvest index (26.85 percent) was obtained. When compared to the control treatment (no drought stress and 50 kg ha^{-1} of nitrogen fertilizer, it exhibited a 48.35 percent increase. The lowest harvest index (11.90%) was observed with 45 percent soil moisture depletion combined with 50 kg ha^{-1} nitrogen fertilizer (Table 8). In the second year, the maximum harvest index (36.41 percent) was produced from 85 percent soil moisture depletion with 50 kg ha^{-1} nitrogen fertilizer. When compared to the control, this treatment increased the harvest index by 24.42 percent (Table 8). The opposite results from the second year

compared to the first year, could be due to the more pronounced effect of the environmental conditions in the first year on grain yield than biological yield.

Biological yield

The combined analysis of variance of data showed that year × drought stress × nitrogen fertilizer interaction was significant on the biological yield of quinoa (Table 10). The highest biological yield (15319.1 kg ha^{-1}) in the first year was obtained from 45% soil moisture depletion (no stress) with 100 kg ha^{-1} of nitrogen fertilizer. When compared to the control treatment, this treatment increased by 22.41 percent. Drought stress normally reduces plant biological yield (Hirich *et al*. 2014; Elewa *et al.* 2017), attributed to reduced uptake of nutrients, stomatal conductance, and thus the photosynthetic capacity of quinoa (Yang *et al.* 2016). In a field experiment on canola with four levels of irrigation (Irrigation after 70, 100, 130, and 160 mm evaporation), it was found that water shortage increased leaf temperature and decreased leaf water content, chlorophyll content, and plant biological yield, which resulted in the reduction of the grain yield per unit area (Ghassemi-Golezani *et al.* 2023).

The lowest biological yield (3966.6 kg ha^{-1}) was observed from the soil moisture depletion of 85 percent with 50 kg ha^{-1} nitrogen fertilizer in the second year (Table 11). This could be the reason why the highest harvest index was observed for the combination of 85 percent soil moisture depletion with 50 kg ha^{-1} nitrogen fertilizer because it seems that drought stress in the second year has affected the vegetative organs more than the grain yield.

Number of seeds per panicle

The combined analysis of variance showed that year × drought stress × nitrogen fertilizer interaction was significant for the number of seeds per panicle in quinoa (Table 10). The largest number of seeds per spike (19.49) was produced in the first year from 45 percent soil moisture depletion (without stress) combined with 100 kg ha^{-1} nitrogen fertilizer. When compared to the control, there was a 32.22 percent increase in the number of seeds per panicle. The lowest number of seeds per spike (12.56) was obtained in the second year for the 85 percent soil moisture depletion combined with the 50 kg ha^{-1} nitrogen fertilizer (Table 11). In general, the number of seeds per panicle determines the capacity of plant reservoirs. The higher the number of seeds, the more reservoirs the plant has produced to store the photosynthetic material, and consequently will result in the yield increase.

The reduction in the number of seeds per panicle in the second year can be attributed to undesired temperature conditions at the flowering and pollination stages of the plant. Tavoosi and Sepahvand (2014) reported that due to the increase in mean air temperature at the flowering stage and decrease

at the seed formation stage, the number of seeds per plant of quinoa decreased. Co-occurrence of flowering, pollination, and grain filling stages with heat stress at the end of the season reduces grain yield by reducing the number of seeds and 1000-seed weight.

Relative leaf water content

The results of the combined analysis of variance showed that the interaction between year, drought stress, and nitrogen fertilizer for the relative leaf water content of quinoa was significant (Table 10). The maximum relative leaf water content (95.13 percent) was recorded from the first year, when the soil moisture was depleted by 45 percent (without drought stress), and 150 kg ha^{-1} of nitrogen fertilizer was applied. When compared to the control treatment this treatment exhibited a 22.05 percent increase in the relative water content. However, the lowest relative water content (57.62 percent) was produced in the second year of the experiment from 85 percent soil moisture depletion combined with 50 kg ha^{-1} nitrogen fertilizer (Table 11). Increasing the nitrogen fertilizer application in dehydrated conditions where grain yield decreases can increase water use efficiency. The interaction between water and nitrogen causes faster leaf growth at high levels of nitrogen due to the increase in stomatal closure and minimized ammonium evaporation. In an experiment on quinoa at different levels of irrigation (based on 50, 75, and 100% water requirements) it was found that the relative water content of the leaves decreased with the increase of the water deficit stress (Mostafaee et al. 2023). Also in research on pepper, the relative water content of leaves decreased with increasing drought stress (Khazaei and Estaji 2021).

Table 10. Combined analysis of variance of quinoa traits under the influence of drought stress and nitrogen fertilizer in two crop years.

Source of variation	df	Biological yield	Number of seeds per panicle	Leaf relative water content
Replication	2	224496.4	0.485	651.59**
Year (Y)	1	61587309.5**	79.267**	10.471
Replication /Y	2	33253.6	0.123	10.431
Drought stress (S)	2	49150984.2	2.282*	128.86**
S × Y	2	37185201.6**	0.622	45.671
Nitrogen fertilizer (N)	2	50995769.0	3.011	108.57
N × Y	2	16724123.8**	1.826*	80.159*
S × N	4	5643537.3	8.206	503.80
S × N × Y	4	24200788.8**	7.462**	1053.3**
Error	32	354178.2	0.491	18.978
CV (%)	-	6.01	4.82	5.72

* and **: Significant at the 5% and 1% probability levels, respectively.

Table 11. Means of quinoa traits for the combinations of drought stresses, nitrogen fertilizers, and years.

Treatment	Biological yield (kg/ha)	Number of seeds per panicle	Leaf relative water content (%)
$Y_1S_1N_1$	11885.8c	13.21h	71.97ef
$Y_1S_1N_2$	15319.1a	19.49a	75.26de
$Y_1S_1N_3$	14517.4ab	16.15bcd	95.13a
$Y_1S_2N_1$	8803.2de	16.37bc	70.49efg
$Y_1S_2N_2$	6608.2f	15.01def	73.50def
$Y_1S_2N_3$	14133.3b	15.14cde	94.13a
$Y_1S_3N_1$	8047.1e	16.46b	74.74def
$Y_1S_3N_2$	11399.4c	14.72efg	76.48de
$Y_1S_3N_3$	7924.5e	15.13cde	85.91bc
$Y_2S_1N_1$	6588.7f	13.09h	63.21ghi
$Y_2S_1N_2$	11098.0c	13.77fgh	74.71def
$Y_2S_1N_3$	8843.4de	13.71fgh	94.17a
$Y_2S_2N_1$	8592.8de	12.86h	59.27hi
$Y_2S_2N_2$	13761.9b	13.75fgh	66.82fgh
$Y_2S_2N_3$	9235.8d	13.83fgh	92.34ab
$Y_2S_3N_1$	3966.6g	12.56h	57.62i
$Y_2S_3N_2$	8734.8de	12.66h	62.05hi
$Y_2S_3N_3$	8592.8de	13.64gh	81.33cd

According to Duncan's multiple range test, means with different letters in each column are significantly different at the 5% probability level; Y1: 1st year, Y2: 2nd year; S1 (control), S2, and S3 are 45%, 65%, and 85% soil moisture depletion; N1, N2, and N3 are 50, 100, and 150 kg/ha nitrogen fertilizer.

Conclusion

According to the results of this research, it can be said that under drought stress, the use of nitrogen fertilizer reduced the effect of drought stress on the several morphophysiological traits of quinoa. This study showed that some quinoa traits, including the grain yield, responded better to the application of 150 kg/ha of nitrogen fertilizer than other levels of nitrogen in Sistan conditions of Iran. Also, quinoa yield components were enhanced by increasing the nitrogen fertilizer, especially under water availability conditions, which consequently resulted in higher grain yield.

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Ethical considerations

The authors avoided data fabrication and falsification.

Conflict of interest

The authors declare that they have no conflict of interest with any organization concerning the subject of the manuscript.

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Influence of Arbuscular mycorrhiza fungi and *Bradyrhizobium japonicum* on the fatty acids profile of soybean (*Glycine max* L.) under drought stress conditions

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Abstract

Objective: Water deficiency changes the composition of soybean fatty acids. Arbuscular mycorrhiza fungi and *Bradyrhizobium* play a vital role in the improvement of soybean protein and oil. Therefore, this study aimed to evaluate the effects of different drought levels and inoculation/non-inoculation with mycorrhiza fungi and *Bradyrhizobium* on the soybean oil, protein percentage, and fatty acid composition.

Methods: This experiment was conducted a split-plot factorial layout based on a randomized complete block design with three replications in 2017. The main factor was irrigation after 70, 110, and 150 mm of evaporation from a class A evaporation pan. Subplots included mycorrhiza fungal inoculations with *Funneliformis mosseae, Rhizophagus intraradices,* and without fungus, and bacterium inoculation at two levels *(Bradyrhizobium japonicum* and without bacteria).

Results: Drought stress significantly reduced the oil percentage. The highest oil content (21.94%) was observed in *F. mosseae* mycorrhiza inoculation. The highest amount of unsaturated fatty acid (74.75%) was observed after inoculation with *R. intraradices* and bacteria under full irrigation conditions. Inoculation with mycorrhiza fungi and bacteria increased the unsaturated fatty acids in some cases.

Conclusion: In conclusion, to improve the oil and protein percent, enhance unsaturated fatty acids, and reduce saturated fatty acids in the soybean, the application of mycorrhiza fungi and inoculation with *B. japonicum* may be beneficial.

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Introduction

Soybean (*Glycine max* L.) is the most important oilseed crop in the world. Soybean is a nitrogen-fixing legume that is usually grown in rotation with cereals. Its importance in seed production is increasing due to its high yield potential and lower harvest costs compared to other seeds (Mesquita *et al*. 2007). However, drought stress, as one of the critical abiotic factors, affects soybean production, leading to significant reductions in yield and quality (Lobell *et al.*, 2011; Le *et al*. 2012). Oil content and fatty acid composition are modified by physiological, ecological, and planting conditions (Srivastava and Yadav 2024). Under drought conditions, soybean plants often experience alterations in physiological processes, which can negatively affect their fatty acid composition, particularly the balance between saturated and unsaturated fatty acids. According to Bellaloui *et al*. (2013), drought stress changes soybean fatty acid composition, particularly during the seed-filling period (R5-R6). These fatty acids are essential not only for the nutritional quality of soybean seeds but also for their market value (Wang *et al*. 2022). Studies have shown that drought conditions tend to increase the levels of saturated fatty acids while decreasing the levels of unsaturated fatty acids, which can negatively affect the nutritional quality of the seeds (Boyer 1982; Wang *et al*. 2022). This shift in fatty acid composition is often associated with changes in plant metabolic pathways, particularly those involved in lipid biosynthesis (Ezzati Lotfabadi *et al*. 2022). In addition, drought stress significantly impacts fatty acid synthesis, leading to reduced oil content in plants (Rezvani Moghaddam *et al*. 2014). Under severe water deficiency, both the total fatty acid content and composition decrease (Laribi *et al*. 2009). For instance, drought conditions have been shown to lower seed oil percentages and levels of oleic and linoleic acids, while increasing protein, palmitic, and stearic acid content in safflower seeds (Mohsennia and Jalilian 2012; Sehgal *et al*. 2018). According to Liu *et al*. (2008), changes in seed fatty acids occurred due to drought stress and intolerant cultivars had higher palmitic acid levels and lower levels of linolenic acid. Divsalar *et al*. (2017) indicated that drought severity increases linoleic acid levels while oleic and palmitic acid levels decline. However, the application of some substances, such as humic acid, alleviate the adverse effects of drought stress (Abhari and Gholinezhad 2019). Heshmati *et al*. (2007) reported that under water deficiency conditions during the reproductive stage, applying bio-fertilizers such as phosphorus fertilizer, can enhance unsaturated fatty acids and oil yield. The use of 50 kg ha^{-1} phosphorus fertilizer along with Phosphate Barvar 2, a biological fertilizer, resulted in a significant reduction in palmitic and stearic acid content.

Recent studies have highlighted the roles of mycorrhiza fungi and beneficial bacteria enhancing plant resilience to drought stress. Vatan Doost *et al*. (2018) reported the lowest levels of linolenic acid

(7.12%) and oleic acid (61.08%) in non-inoculated seedlings exposed to drought stress during seed formation. Conversely, the highest levels of palmitic acid (4.56%) and erucic acid (2.89%) under drought conditions without bacterial treatment. However, by applying the beneficial bacteria plant growth and stress tolerance enhance through mechanisms such as phytohormone production and soil health improvement, whereas mycorrhiza fungi improve nutrient and water uptake through their extensive hyphal networks (Smith and Read 2010; Vessey 2003). However, the specific effects of these microbial inoculants on the fatty acid profiles of soybean under drought stress remain poorly explored. The inoculation of soybean with mycorrhiza fungi and beneficial bacteria mitigate some adverse effects of drought stress. For instance, mycorrhiza fungi enhance the synthesis of unsaturated fatty acids by improving the plant's nutrient uptake and overall health (Smith and Read 2010). Similarly, beneficial bacteria can influence the fatty acid composition leading to a higher proportion of unsaturated fatty acids in seeds (Vessey 2003). Recent findings suggest that the synergistic effects of mycorrhiza fungi and beneficial bacteria may lead to an even greater improvement in fatty acid profiles under drought conditions. Moreover, the combined use of Azotobacter and Azospirillum with nitrogen fertilizers can enhance oil content in canola by improving soil characteristics and nutrient absorption (Hasanzadeh Ghorttapeh and Javadi 2016).

Despite the promising results of previous studies, several challenges and research gaps remain. There is a need for comprehensive studies that simultaneously evaluate the effects of mycorrhiza fungi and beneficial bacteria on plant physiology and fatty acid composition under drought stress. In addition, the specific mechanisms by which these microbial partners influence fatty acid biosynthesis in soybean plants are not yet fully understood. Research is also needed to determine the optimal combinations and application methods of these inoculants to maximize benefits. This research aimed to evaluate the effect of inoculation/non-inoculation with mycorrhiza fungi and *Bradyrhizobium* bacterium on soybean oil content, protein percentage, and fatty acid composition under different levels of water deficit.

Materials and Methods

Geographical conditions of the experimental site

The experiment was carried out at the Research Station of the Agricultural High School of Urmia, Iran in 2017. The station is located 12 km away from Urmia city, Iran (longitude: 37° 32′ E; latitude: 45° 2′ N; altitude: 1332 m above sea level). According to meteorological statistics, the area is part of arid and semi-arid climatic conditions with 150 to 180 dry days, cold and humid winters, and hot and dry summers. Some important meteorological features are listed in Table 1.

Some soil properties at the test site

To determine some characteristics of the soil at the experimental site and estimate the soybean fertilizer requirement, five soil samples were taken from the 0-30 cm depth. The soil analyses were carried out according to the literature (Walkley and Black 1934; Olsen 1954; Bouyoucos 1962; Allison and Moodie 1965; Thomas and Hargrove 1984; Bremner 1996; Haluschak 2006), and the results are presented in Table 2.

Experimental design and treatments

The experiment was performed as a split-plot factorial layout based on a randomized complete block design with three replications. The soybean cultivar used was Kowsar. The main factor was irrigation at three levels: normal (irrigation after 70 mm evaporation from class A evaporation pan), moderate drought stress (irrigation after 110 mm evaporation), and severe drought stress (irrigation after 150 mm evaporation). Subplots included the factorial combinations of mycorrhiza fungus at three levels (without mycorrhiza, *Funneliformis mosseae*, and *Rhizophagus intraradices*) and bacterium *Bradyrhizobium japonicum* at two levels (with and without inoculation with *Bradyrhizobium*).

Table 1. The mean monthly temperature, precipitation, evaporation, and humidity during the growing season of soybean in the studied area.

Meteorological parameters	Month					
	March	**April**	**May**	**June**	**July**	**August**
Maximum temperature (°C)	16	23	29	33.3	34.7	32.5
Minimum temperature (°C)	2.9	8	11	16	17	13
Mean temperature (°C)	9.45	15.5	20	24.65	25.85	22.75
Total precipitation (mm)	70.5	16	0.8	0	0.7	0
Total evaporation (mm)	62	160	246	304	281	213
Average humidity (%)	54	54	35	38	36	34

Table 2. Some soil physical and chemical characteristics in the experimental site.

Soil depth (cm)	Soil texture	EC (ds m^{-1})	pH -	C.C.E -	Saturation moisture (%)	Clay (%)	Silt (%)	Sand (%)	Organic carbon (%)	Nitrogen (%)	Phosphorus (mg kg^{-1})	Potassium (mg kg^{-1})
0-30	Loamy	0.85	7.64	23.8	32	40	35	25	1.15	0.12	5.16	211

Planting operations

The field preparation operations included plowing by moldboard tillage and leveling by a tiller. At this stage, based on fertilizer recommendations, 120 kg ha^{-1} potassium sulfate and 100 kg ha^{-1} sulfur were mixed with the soil. Plots and furrows were prepared according to the planting map. The seeds were planted in the second half of May 2017. The row spacing and plant-to-plant spacing were 50 cm and 10 cm, respectively. Each plot consisted of four rows of 4 m long. The distance between sub-plots was 2 m and for the main plots was 4 m. Seeds were sown and covered with 3 cm soil. Mycorrhiza fungi were prepared from Turan Biotech Co. in Shahroud, Iran. Their spores were examined by microscope. The mycorrhiza fungi spores were spread on a sterile culture bed with no living organisms. Inoculum was produced by the trap cultivation on plants such as clover or corn. The mycorrhiza inoculum included sterile sand, mycorrhiza hyphae, spores, and colonized root fragments. Mycorrhiza fungi (10 g) were put in the planting holes in the corresponding plots and then covered with 2 cm soil. The special biological fertilizer for soybean (*Bradyrhizobium* bacterium) was purchased by Mehr Asia Co. There were 10^8 bacteria in each gram of fertilizer.

Seeds were inoculated according to the recommendations on the package of biological fertilizers. Irrigation levels were applied from 2-4 leaf stage (seedling establishment). Thinning was carried out by hand at the 3-4 leaf stage to achieve an optimum density. Weed control was performed manually and twice during the growing season.

Measured traits

Seed yield: After removing the border rows and 50 cm from the ends of each row, the seed yield was measured from a 2 m^2 area in each plot. Harvesting was performed in the second half of September. The seeds of each plot were dried and weighed at 70 °C until a constant weight was achieved.

Oil and protein percentage: The percentage of seed oil was determined by using a Soxhlet oil extractor (Soxtherm 2000 automatic, Germany). Protein content was determined using the Kjeldahl apparatus (VAP 50, Germany) according to the following equations (Fazlara 2009):

$$N\% = \frac{The\ amount\ of\ acid\ used\ in\ titration \times 0.0014}{Sample\ weight} \times 100$$

$$\text{Protein (\%)} = N\% \times 5.63 \text{ (Sosulski and Holt 1980)}$$

Oil and protein yield: By multiplying the oil percentage by the seed yield, the oil yield was calculated. Also, the protein yield was calculated by multiplying the protein percentage by the seed yield.

Fatty acids' profile: The oil samples were initially homogenized with Vertex and 100 mg of each sample was weighed carefully. The fat was then converted to methyl ester by adding 3 ml of methanol potassium hydroxide (2 mM) and then 5 ml of methanol sulfuric acid (12% v/v). The methyl ester was extracted with 1 ml of normal heptane and injected into a gas chromatography (GC) apparatus to analyze the 1 µl of the normal heptane phase of the fatty acid profile. Sigma Company standard fatty acid mixture was used to identify individual fatty acids by comparing the inhibition times. An Agilent 6890N GC apparatus (Agilent Technologies, Wilmington, DE, USA) equipped with an FID detector and split/splitless injector was used for sample analysis. Separations were performed using an HP-88 capillary column (88% - Cyanopropy) aryl-polysiloxane, 100 m length, 0.25 mm i.d., 0.2 µm film thickness (Agilent). ChemStation software was used to process the data. The oven temperature was programmed as follows: 5 min at 140 °C, subsequently increased 4 ˚C per minute to reach 240 ˚C and held for 15 min at 240 ˚C. Nitrogen was used as the carrier and make-up gas, and flow rates were 1 ml per min and 45 ml min, respectively. The temperatures of the injection port and detector were set at 260 ˚C and 280 ˚C, respectively. The injector was set in a split mode (split ratio of 1:30) (AOAC 2000; Barthet *et al*. 2002).

Analysis of variance was carried out after testing for the homoscedasticity of variances with the Bartlett test. Means were compared by the LSD method at the 5% probability level. Statistical analyses were done by SAS software (version 9.1).

Results

The results the analysis of variance revealed that oil and protein percentage, seed yield, oil yield, protein yield, and myristic, palmitic, arachidic, behenic, oleic, and linolenic acids were significantly affected by the irrigation. The effect of mycorrhiza fungi on oil and protein percentage, seed yield, oil yield, protein yield, and palmitic, stearic, arachidic, behenic, palmitoleic, oleic, linolenic, and total unsaturated fatty acids were significant. The effect of the bacterium significantly influenced oil and protein percentage, seed yield, oil yield, protein yield, and myristic, stearic, arachidic, behenic, palmitoleic, oleic, linoleic, linolenic, and total unsaturated fatty acids. The interaction of irrigation × mycorrhiza fungi was significant for protein percentage, protein yield, and myristic, palmitic, stearic, arachidic, behenic, oleic, total saturated, and total unsaturated fatty acids (Table 3). The interaction of irrigation × bacterium significantly influenced seed yield, oil yield, protein yield, and palmitic, stearic, arachidic, behenic, oleic, linoleic, linolenic, and total saturated and total unsaturated fatty acids. The interaction of bacterium × mycorrhiza fungi was significant on seed yield, oil yield, arachidic, behenic, oleic, and total unsaturated fatty acids. The irrigation × mycorrhiza fungi × bacterium interaction

significantly affected palmitic, arachidic, behenic, oleic, linoleic, linolenic, total saturated, and total unsaturated fatty acids (Table 3).

Table 3. Analysis of variance of irrigation and inoculation with mycorrhiza and bacteria for oil content, protein content, seed yield, oil yield, protein yield, and fatty acid composition in soybean.

Source of variation	Mean squares										
	df	% oil	% protein	Seed yield	Oil yield	Protein yield	Myristic acid methyl ester (C14:0)	Palmitic acid methyl ester (C16:0)	Stearic acid methyl ester (C18:0)	Arachidic acid methyl ester (C20:0)	Behenic acid methyl ester (C22:0)
Block	2	19.05	33.06	2960	384.1	1745	0.0008	4.03	3.14	0.14**	0.005
Irrigation (I)	2	5.05**	527.1**	37254**	1990**	17711**	0.0004*	10.88**	0.088	0.023**	0.034**
Error a	4	1.11	13.50	911.4	69.87	575.4	0.0003	1.65	0.48	0.001	0.003
Mycorrhiza (M)	2	12.66**	44.50**	117.24**	1309**	3774**	0.0001	1.46*	2.39**	0.058**	0.048**
Bacteria (B)	1	48.16**	78.62**	66704 **	6455**	14317**	0.001**	0.0001^{ns}	3.47**	0.11**	0.103**
I × M	4	0.88	3.25*	199.7	25.38	212.2**	0.0003*	2.45**	1.06**	0.008**	0.015**
I × B	2	0.38	0.75	628.5*	62.89*	236.9**	0.00001	1.70*	0.53*	0.008**	0.009**
B × M	2	0.22	1.29	1001**	82.2*	93.63	0.00002	0.17	0.39	0.014**	0.016**
I × M × B	4	0.61	0.71	301	33.77	55.48	0.0001	1.14*	0.30	0.002**	0.007**
Error b	30	0.60	1.13	177.3	15.39	32.04	0.0001	0.42	0.12	0.0004	0.001
C.V. (%)	-	3.67	3.23	3.55	4.91	4.51	13.53	6.30	11.09	5.80	13.59

*,**: Significant at 5% and 1% probability levels, respectively.

Table 3. Continued

Source of variation	df	Mean squares					
		Saturated fatty acids	Palmitoleic acid methyl ester (C16:1)	Oleic acid methyl ester (C18:1n9c)	Linoleic acid methyl ester (C18:2n6c)	Linolenic acid methyl ester (C18:3n3)	Unsaturated fatty acids
Block	2	15.12	0.001	2.08	297.2	5.17	349.6
Irrigation (I)	2	7.57	0.0007	70.01**	214.4	22.17*	327.0
Error a	4	2.50	0.0002	1.42	45.16	2.06	68.26
Mycorrhiza (M)	2	9.04**	0.0006**	7.52*	48.78^{ns}	3.93**	125.9**
Bacterium (B)	1	1.56	0.0015**	32.34**	264.3**	7.01**	175.8**
I × M	4	5.67**	0.00004	48.35**	31.52	0.78	146.6**
I × B	2	3.39*	0.00001	41.58**	69.48*	5.07**	254.9**
B × M	2	1.23	0.00001	47.63**	46.34	0.24	166.7**
I × M × B	4	2.20*	0.0001	31.33**	47.11*	1.56**	126.9**
Error b	30	0.78	0.0001	2.03	16.36	0.35	22.64
C.V. (%)	-	6.26	11.87	9.21	15.35	6.67	9.37

**,*, and ns; significant at 1% and 5% probability levels and non-significant, respectively.

Oil percentage and yield

Seed oil percentage decreased by drought stress. The highest (21.72%) and lowest amount (20.66%) of oil percent were obtained from normal irrigation and moderate drought stress, respectively. Inoculation with mycorrhiza fungi and bacterium enhanced the oil content. The maximum amount of oil (21.94%) was observed in plants inoculated with *F. mosseae*. The highest (22.11%) and lowest oil percentage (20.22%) were seen in bacterium inoculation and non-bacterium inoculation, respectively. Inoculation with *F. mosseae* compared to non-inoculation of mycorrhiza increased seed oil content by 8.24%. Inoculation with bacterium increased seed oil content by 9.35% compared to non-inoculation conditions (Figure 1A, 1B, 1C). Severe and moderate water deficit reduced oil yield by 24% and 9%, respectively, compared to optimal irrigation (Table 4).

The highest oil yield (100.95 g m^{-2}) was obtained from the interaction of optimal irrigation with inoculation with the bacterium. The lowest oil yield (59.77 g m^{-2}) was obtained from the combination of severe water deficit and no inoculation with bacterium (Table 4). In normal irrigation, moderate drought stress and severe drought stress conditions, inoculation with bacterium increased oil yield by 23%, 27%, and 23%, respectively (Table 4). Also, the highest oil yield (101.54 g m^{-2}) was obtained from *F. mosseae* and bacterium inoculation. The lowest oil yield (61.50 g m^{-2}) was seen in the treatment with no mycorrhizal and bacterial inoculation (Table 5). Inoculation with *F. mosseae* and *R. intraradices* enhanced oil yield by 20% and 12%, respectively, compared to lack of mycorrhiza inoculation (Table 5).

Protein percentage and yield

Inoculation with bacterium rose protein content by approximately 7.6% compared to non-inoculation conditions (Figure 1D). The combination of irrigation and mycorrhiza fungi indicated that mild drought stress increased the protein content, but severe drought stress decreased it. The maximum protein percentage (40.43%) was derived from the combination of moderate stress and inoculation with *F. mosseae*. The lowest protein content (26.71%) was obtained following the treatment with severe drought stress without mycorrhiza inoculation. Moderate drought stress increased protein content by 38.15% compared to optimum irrigation. Severe drought stress reduced protein content by 12.13% compared to optimum irrigation (Figure 2A). Also, severe drought stress reduced protein yield by 31% compared to optimal irrigation (Table 4). The highest protein yield (173.14 g m^{-2}) was obtained from the moderate water deficit under inoculation with F. mosseae (Table 4). The lowest protein yield (80.85 g m^{-2}) was also obtained from the treatment of severe drought stress without mycorrhiza

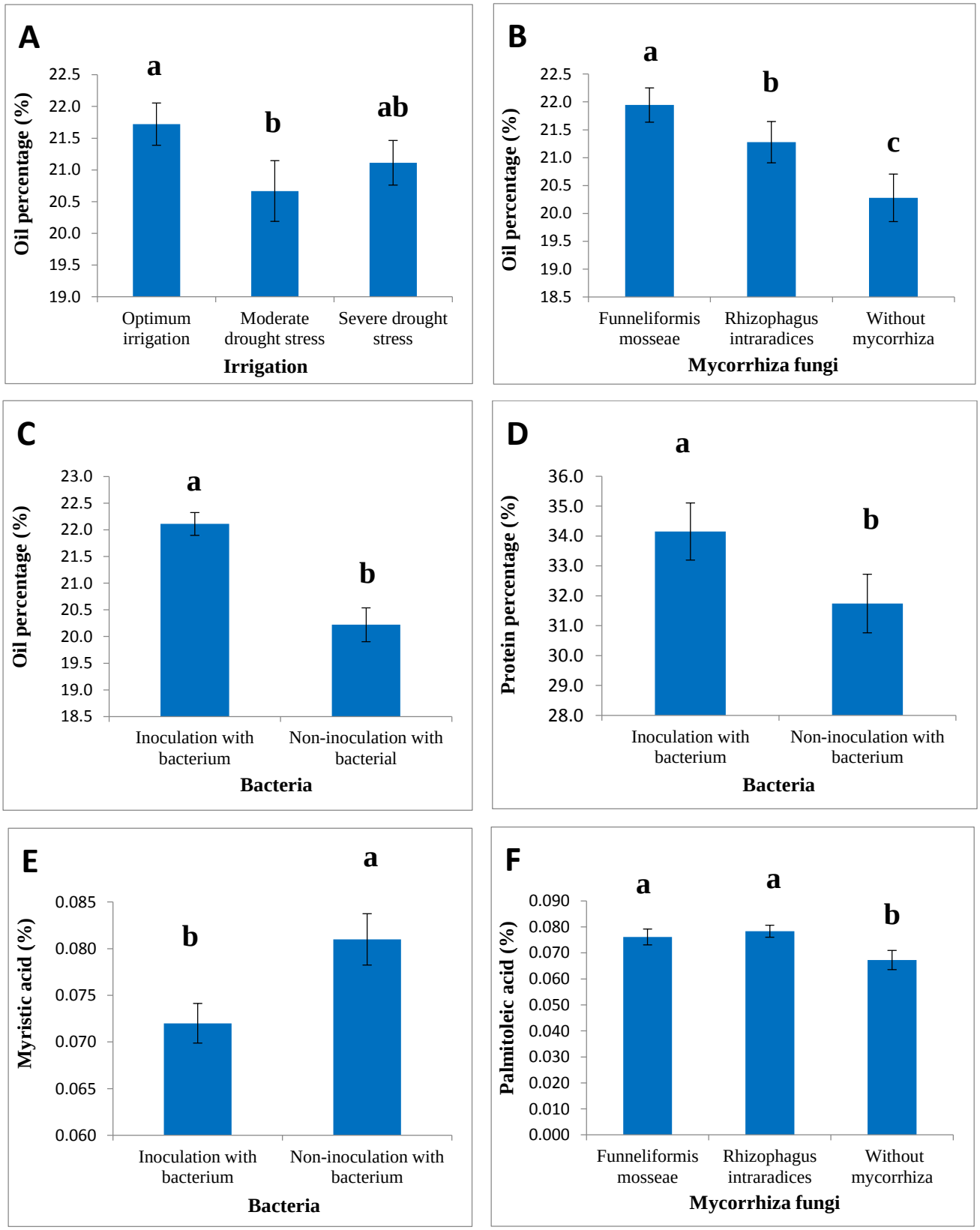


Figure 1. The effects of irrigation on oil percentage (A), mycorrhiza fungi on oil percentage (B) and palmitoleic acid (C), bacterium on oil percentage (D), protein percentage (E), and myristic acid (F); Means followed by the same letter are not significantly different at 5% probability level.

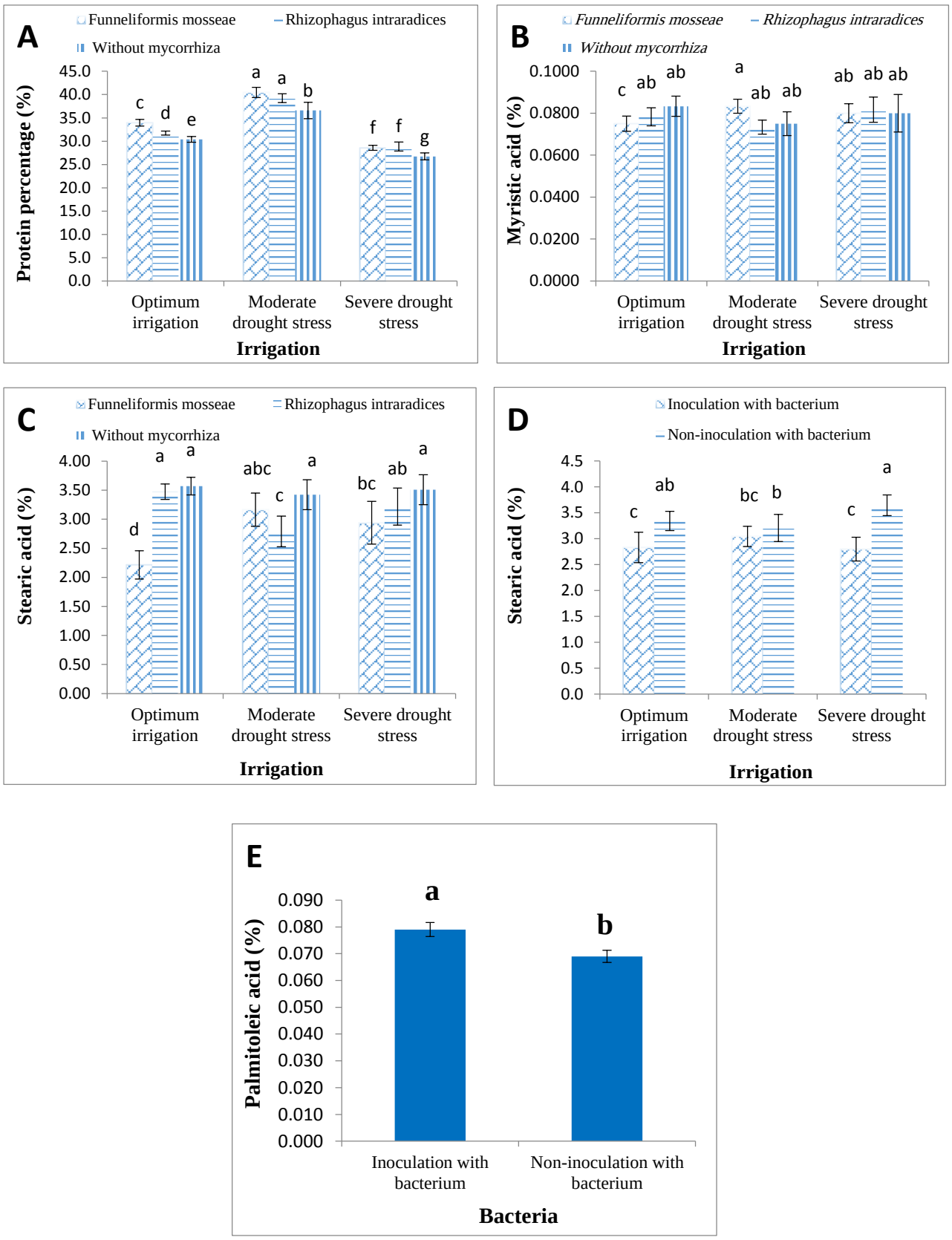


Figure 2. The effect of bacteria on palmitoleic acid (A), interaction effects of irrigation with mycorrhiza on protein percentage (B), myristic acid (C), stearic acid (D), and interaction effects of irrigation with bacterium on stearic acid (E); Means followed by the same letter are not significantly different at 5% probability level.

Table 4. Effects of combination of irrigation (I) with bacterium (B on seed yield, oil yield, and protein yield.

Treatment I×B	Seed yield (g m^{-2})	Oil yield (g m^{-2})	Protein yield (g m^{-2})
$I_1 \times B_1$	446.78 a	100.95 a	148.00 b
$I_1 \times B_2$	372.44 c	77.91 c	115.75 d
$I_2 \times B_1$	431.11 b	94.01 b	173.14 a
$I_2 \times B_2$	351.55 d	69.13 d	132.76 c
$I_3 \times B_1$	351.77 d	77.45 c	103.77 e
$I_3 \times B_2$	294.79 e	59.77 e	78.70 f
$LSD_{5\%}$	12.80	3.77	5.44

I_1= Optimum irrigation, I_2= Moderate drought stress, I_3= Severe drought stress; B_1= Inoculation with bacterium, B_2= Non-inoculation with bacterium; Means with the same letter(s) in each column are not significantly different based on the LSD test (5% probability level).

Table 5. Effects of combination of mycorrhiza fungi with bacterium on seed yield and oil yield.

Treatment M×B	Seed yield (g m^{-2})	Oil yield (g m^{-2})
$M_1 \times B_1$	443.23 a	101.54 a
$M_1 \times B_2$	355.80 d	74.74 d
$M_2 \times B_1$	407.77 b	90.16 b
$M_2 \times B_2$	344.58 d	70.58 e
$M_3 \times B_1$	378.66 c	80.71 c
$M_3 \times B_2$	318.40 e	61.50 f
$LSD_{5\%}$	12.80	3.77

M_1= *Funneliformis mosseae*, M_2= *Rhizophagus intraradices*, M_3= without mycorrhiza; B_1= Inoculation with bacterium, B_2= Non-inoculation with bacterium; Means with the same letter(s) in each column are not significantly different based on the LSD test (5% probability level).

(Table 4). In all three different irrigation conditions, bacterium inoculation increased protein yield compared to non-inoculation (Table 4). Under full irrigation, and moderate and severe drought stresses, bacterium inoculation increased protein yield by 22%, 25%, and 24%, respectively (Table 4).

Seed yield

Severe and moderate water stress decreased seed yield by 22% and 5%, respectively, compared to optimal irrigation. The highest seed yield (446.78 g m^{-2}) was obtained at optimal irrigation in the presence of bacterium inoculation. The lowest seed yield (294.79 g m^{-2}) was observed from the treatment of severe drought stress without bacterial inoculation (Table 4). In favorable irrigation conditions, and mild and severe drought stresses, bacterium inoculation enhanced seed yield by 17%,

19%, and 17%, respectively (Table 4). Also, according to Table 5, the highest seed yield (443.23 g m^{-2}) was obtained from the inoculation with *F. mosseae* and bacterium. The lowest seed yield (318.40 g m^{-2}) was associated with the absence of mycorrhiza and bacterium inoculation (Table 5). Inoculation with *F. mosseae* and *R. intraradices* enhanced seed yield by 13% and 8%, respectively, compared to the non-inoculation of mycorrhiza fungi (Table 5).

Myristic acid

Bacterium inoculation reduced myristic acid by 12.5% compared to its corresponding control (non-inoculation) (Figure 1E). Also, the maximum amount of myristic acid (0.083%) was related to the interaction between moderate stress and inoculation with *F. mosseae*. The lowest myristic acid (0.060%) was obtained at optimum irrigation together with *F. mosseae* inoculation (Figure 2C). With a rising water deficit, myristic acid increased significantly in the presence of *F. mosseae*. (Figure 2B).

Palmitic acid

The highest amount of palmitic acid (11.74%) was obtained from the interaction between moderate drought stress and inoculation with *F. mosseae* and bacterium. The lowest palmitic acid (8.75%) was related to the treatment of optimum irrigation together with the inoculation by *F. mosseae* and bacterium (Table 6). Under optimum irrigation conditions, inoculation of *F. mosseae* alone (without bacterium inoculation) led to a relative increase in palmitic acid content compared with the combination of inoculation by *F. mosseae* and bacterium. Under severe drought stress, there were no significant differences among all treatments in terms of palmitic acid (Table 6).

Stearic acid

The minimum and maximum stearic acid levels of 2.21% and 3.57% were obtained from optimum irrigation × *F. mosseae* and optimum irrigation × non-inoculation with mycorrhiza fungi, respectively (Figure 2C). The maximum amount of stearic acid (3.64%) was related to the non-inoculation of the bacterium under severe drought stress, whereas the lowest (2.79 %) was observed in the bacterium inoculation under severe drought stress (Figure 2D).

Arachidic acid

Our findings of this research showed that the maximum amount of arachidic acid (0.57%) was obtained from the treatment of severe drought stress × non-inoculation with mycorrhiza fungi × bacterium inoculation. The lowest arachidic acid content (0.25%) was associated with three treatments, such as

Table 6. Effects of combination of irrigation, inoculation with mycorrhiza fungi, and bacterium on fatty acid composition.

	Palmitic acid (%)			Arachidic acid (%)			Behenic acid (%)			Saturated fatty acid (%)		
M×B	**I1**	**I2**	**I3**	**I1**	**I2**	**I3**	**I1**	**I2**	**I3**	**I1**	**I2**	**I3**
$M_1 \times B_1$	8.75f	11.74a	9.36ef	0.41c	0.31fgh	0.37d	0.32bc	0.24efg	0.31cd	11.25i	15.48ab	12.38hi
$M_1 \times B_2$	9.95de	10.64bcd	9.92de	0.34def	0.26ij	0.28hij	0.26def	0.16hi	0.24efg	13.35fgh	14.39a-f	14.16b-f
$M_2 \times B_1$	10.82a-d	11.40ab	9.22ef	0.29ghi	0.28hij	0.32efg	0.20ghi	0.23efg	0.25efg	14.85a-e	14.69a-f	12.58ghi
$M_2 \times B_2$	10.79a-d	10.21cde	9.97de	0.25j	0.25j	0.25j	0.15hi	0.21fgh	0.21fgh	14.76a-f	13.62e-f	14.26b-f
$M_3 \times B_1$	11.58ab	11.13abc	9.26ef	0.47b	0.35de	0.57a	0.37b	0.25efg	0.55a	15.82a	15.14a-d	13.90c-g
$M_3 \times B_2$	10.90a-d	11.39ab	9.51ef	0.31fgh	0.29ghi	0.33ef	0.23efg	0.21fgh	0.27cde	15.36abc	15.49ab	13.77d-h
$LSD_{5\%}$	1.087			0.032			0.057			1.478		

M_1= *Funneliformis mosseae,* M_2= *Rhizophagus intraradices,* M_3= without mycorrhiza; B_1= Inoculation with bacterium Non-inoculation with bacterium*;* I1 = Optimum irrigation, I2= Moderate drought stress, I3= Severe drought stress; Means with the same letter(s) in each column are not significantly different based on the LSD test (5% probability level).

Table 6. Continued

	Oleic acid (%)			Linoleic acid (%)			Linolenic acid (%)			Unsaturated fatty acid (%)		
M×B	**I1**	**I2**	**I3**	**I1**	**I2**	**I3**	**I1**	**I2**	**I3**	**I1**	**I2**	**I3**
$M_1 \times B_1$	9.21d	14.79bcd	13.84cd	27.35bcd	31.56 b	24.21cd	9.98a	10.12a	8.32b	46.63cd	56.55b	46.45cd
$M_1 \times B_2$	15.25bcd	12.34cd	24.94a	26.86bcd	24.31cd	23.93cd	10.17a	7.17de	8.19bc	52.37bc	43.90d	57.13b
$M_2 \times B_1$	21.71ab	15.47bcd	13.93cd	42.78a	30.26bc	23.06d	10.17a	10.47a	8.17bc	74.75a	56.29b	45.25cd
$M_2 \times B_2$	17.56abc	11.26cd	17.48abc	24.88bcd	22.60d	24.24cd	10.20a	8.45b	8.08bcd	52.72bc	42.39d	49.88bcd
$M_3 \times B_1$	14.27bcd	12.04cd	17.22bc	30.51bc	25.47bcd	21.76d	10.38a	8.86b	6.55e	55.25b	46.45cd	45.61cd
$M_3 \times B_2$	16.26bcd	14.62bcd	16.68bcd	27.17bcd	22.08d	21.05d	8.94b	8.13bcd	7.22cde	52.45bc	44.90cd	45.02cd
$LSD_{5\%}$	2.373			6.737			0.985			7.925		

M_1= *Funneliformis mosseae,* M_2= *Rhizophagus intraradices,* M_3= without mycorrhiza; B_1= Inoculation with bacterium Non-inoculation with bacterium*;* I1 = Optimum irrigation, I2= Moderate drought stress, I3= Severe drought stress; Means with the same letter(s) in \each column are not significantly different based on the LSD test (5% probability level).

the combination of inoculation with *R. intraradices* fungi and non-inoculation with the bacterium under all irrigation conditions. In addition, under optimum irrigation conditions and moderate and severe drought stresses, bacterium inoculation reduced significantly arachidic acid content compared to non-inoculation with the bacteria (Table 6).

Behenic acid

The maximum amount of behenic acid (0.55%) was obtained from the interaction of severe drought stress × bacterium inoculation × non-inoculation with mycorrhiza fungi. The lowest behenic acid (0.15%) was derived from the treatment of optimum irrigation × inoculation with *R. intraradices* × non-inoculation with the bacterium. Under three irrigation levels, the rate of inoculation with *R. intraradices* × non-inoculation with the bacterium was lower than that of some other treatments. Under

optimum irrigation, moderate drought, and severe drought conditions; inoculation with the bacterium increased behenic acid levels in most cases compared with non-inoculation conditions (Table 6).

Saturated fatty acids

The maximum amount of saturated fatty acid was obtained from the interaction of full irrigation × non-inoculation with mycorrhiza fungi × bacterium inoculation (15.82%), but it was not significantly different from eight other treatments. The lowest saturated fatty acid was related to the treatment of optimum irrigation × inoculation with *F. mosseae* × bacterium inoculation (11.25%) followed by two other treatments. In most cases, non-inoculation with mycorrhiza fungi had higher values than other treatments under three levels of irrigation (Table 6).

Palmitoleic acid

Inoculation with mycorrhiza fungi significantly increased the palmitoleic acid. The maximum amount of palmitoleic acid (0.078% and 0.076%) was obtained from *R. intraradices* and *F. mosseae*, respectively. In these treatments, palmitoleic acid content was increased approximately 16.42 and 13.43%, respectively (Figure 1F). Palmitoleic acid content was higher (16.18%) in bacteria inoculation than in non-inoculation treatment (Figure 2E).

Oleic acid

The maximum amount of oleic acid (24.94%) was related to the interaction of severe drought stress × *F. mosseae* × non-inoculation with the bacterium. The lowest amount (9.21%) was obtained from the treatment of optimum irrigation × *F. mosseae* × bacterium inoculation. Moderate drought stress reduced oleic acid content compared to optimum irrigation, except for the *F. mosseae* × bacterium inoculation (Table 6).

Linoleic acid

Linoleic acid was higher (42.78%) in the treatment of optimum irrigation × inoculation with *R. intraradices* × bacterium inoculation than other treatments. The lowest linoleic acid (21.05%) was derived from the treatment of severe drought stress × non-inoculation with mycorrhiza fungi × non-inoculation with the bacterium, but it was not significant from 13 other treatments. In most cases, moderate and severe drought stresses reduced the linoleic acid content compared to optimum irrigation. Bacterium inoculation enhanced the linoleic acid compared to non-inoculation in most treatments.

Also, inoculation with *F. mosseae* and *R. intraradices* raised linoleic acid content in most instances (Table 6).

Linolenic acid

The results indicated that the maximum amount of linolenic acid (10.38%) was derived from the treatment of optimum irrigation × non-inoculation with mycorrhiza fungi × bacterium inoculation, which was not significantly different from six other treatments. The lowest level of linolenic acid (6.55%) was associated with severe drought stress × non-inoculation with mycorrhiza fungi × bacterium inoculation. With increasing drought stress, linolenic acid levels decreased. In most cases, inoculation with *F. mosseae* and *R. intraradices*, along with bacterium inoculation, enhanced the linolenic acid content compared with non-inoculation by mycorrhiza fungi and bacteria (Table 6).

Unsaturated fatty acids

The maximum amount of unsaturated fatty acid (74.75%) was obtained from the interaction of optimum irrigation × inoculation with *R. intraradices* × bacterium inoculation. The lowest unsaturated fatty acid (42.39%) was related to the treatment of moderate drought stress × inoculation with *R. intraradices* × non-inoculation with the bacterium. Moderate and severe drought stress reduced unsaturated fatty acid levels compared to optimum irrigation, except in the presence of *F. mosseae*. Inoculation with *F. mosseae* and *R. intraradices* increased unsaturated fatty acid content at moderate stress conditions under bacterium inoculation. However, at the severe drought stress, inoculation with *F. mosseae* and *R. intraradices* increased unsaturated fatty acid content under no bacterium inoculation. Bacterium inoculation enhanced unsaturated fatty acid content when accompanied with *R. intraradices* at the moderate drought stress conditions and with *F. mosseae* at the severe stress conditions, compared with the non-inoculation state (Table 6).

Discussion

Recently, inoculation with mycorrhiza fungi and bacteria has been indicated to have considerable advantages for soybean growth and yield in field trials under drought stress conditions (Igiehon *et al*. 2021). However, several studies have focused mostly on the evaluation of the quantity and quality of soybean yield. In the present study, not only yield but also the impact of inoculation with mycorrhiza fungi and bacteria on the oil, protein, and fatty acid profiles of soybean under drought stress conditions was investigated. In line with our research findings, other reports have also shown that treatments with arbuscular mycorrhiza fungi and *B. japonicum* improved the growth and yield of soybean plants under

both drought stress and normal water conditions compared with untreated plants (Sheteiwy *et al*. 2021). It was reported in a study that dual inoculation of soybean plants with beneficial microbes reduces water stress effects, thereby allowing normal plant growth under drought stress conditions (Ashwin *et al*. 2023).

Protein content is a quality trait that increases under water-deficit stress. Moderate water deficit stress enhanced protein content in mycorrhiza fungi, whereas severe drought stress reduced protein content. However, at all irrigation conditions, inoculation with mycorrhiza fungi increased the protein percentage. Drought stress caused by closing the stomata and reducing photosynthesis results in the transfer of less assimilates to the seeds and decreases seed yield. In addition, drought stress may have affected nitrogen fixation and eventually reduced seed yield and protein percentage. These findings are consistent with the results of Navabpour *et al*. (2017). In our study, under water-deficit stress conditions, an increase in protein percentage did not compensate for the decrease in seed yield, resulting in a decrease in protein yield. In the results of other researchers, the highest and lowest yields of seed protein were obtained under normal irrigation conditions and severe drought stress, respectively (Rostami Ajirloo *et al*. 2022).

Based on our research, oil content decreased under drought stress. Inoculation with mycorrhiza fungi and bacteria significantly enhanced oil content. Navabpour *et al*. (2017) also reported that the oil percentage decreased with increasing drought stress and reported a negative relationship between oil percentage and protein. The decrease in oil content and increase in protein content under drought stress (moderate drought stress in our experiment) can be explained by reducing the moisture requirement of the plant and shortening the filling period of the seeds. In addition, oil fills more in seeds under full irrigation conditions than compared to water-deficit conditions. The results of the present study and other research findings were coordinated (Purdehgan *et al*. 2015). In summary, drought stress reduced oil yield. The large decrease in oil yield was due to the effect of water-deficit stress on the capacity of seeds to accumulate oil, reducing the percentage of seed oil, and decreasing seed yield. Some studies have shown that oil yield decreases with increasing of drought stress (Ezzati Lotfabadi *et al*. 2022).

At the severe stress conditions, the palmitic acid content increased significantly or did not change significantly. In this study, severe drought stress enhanced behenic (only in the presence of bacterium), stearic, and myristic acid (only in the presence of *F. mosseae*) compared with optimum irrigation. In a study, drought stress was not found to have a significant effect on the fatty acid composition, except for palmitic acid (Zarei *et al*. 2010). According to researchers, changes in the enzyme activity's involved in the synthesis of fatty acids due to drought stress are the main cause of the changes in the amount of fatty acids (Bellaloui *et al*. 2013). Tohidi-Moghaddam *et al*. (2011) by applying drought

stress from the flowering stage showed a reduction in the percentage of saturated fatty acids in the seed oil (stearic acid and arachidic acid) in six rapeseed cultivars, which could be attributed to shortening the plant growth period under stress conditions

In this study, moderate drought stress increased saturated fatty acids (only in the presence of *F. mosseae* and bacterium, whereas severe drought stress decreased saturated fatty acids in some cases. Water deficit and irregular irrigation increase the percentage of saturated fatty acids but access to water increases the rate of unsaturated fatty acids (Seyed Sharifi 2016).

Based on this research, increasing drought stress reduced the linoleic, linolenic, and unsaturated fatty acid contents in most cases. At moderate drought stress, oleic acid level decreased, but at severe drought stress it increased in some cases. Tohidi Moghadam *et al*. (2011) reported that drought stress reduced rapeseed oil and linoleic acid content. In another study, drought stress increased unsaturated fatty acid content, such as linoleic, linolenic, and oleic acids (Ayoubizadeh *et al*. 2018). In addition, under drought stress conditions, the ratio of saturated to unsaturated fatty acids increased in the seed oil of canola cultivars (Tohidi-Moghaddam *et al*. 2011).

In our research, inoculation with mycorrhiza fungi compared with non-inoculation reduced myristic (only *F. mosseae*), behenic, and saturated fatty acids, while increased palmitoleic, oleic, linoleic, linolenic, and unsaturated fatty acids (in some cases). Inoculation with bacterium also reduced behenic and saturated fatty acids (in some cases), while increased myristic, palmitoleic, oleic, linoleic, linolenic, and unsaturated fatty acids (in some cases). In a study, the stearic acid content decreased under inoculation with *F. mosseae* but increased under non-inoculation conditions (Ghasemi *et al.* 2023). Growth-promoting bacteria reduces the amount of saturated fatty acids (palmitic and stearic acids) and increases unsaturated fatty acids (oleic, linoleic, and linolenic acids) (Seyed Sharifi 2016). Nosheen *et al*. (2013) reported that inoculation with *Azospirillum* bacterium increased oleic and linoleic acid levels but reduced erucic acid in rapeseed. Vatan Doost *et al*. (2018) indicated that the maximum amount of oleic acid and linolenic acid was observed in inoculation with *Azospirillum* and complete irrigation They stated that the application of biofertilizer with triple superphosphate fertilizer during the drought stress at the reproductive stage can be positive on unsaturated fatty acids. Drought stress and nutrient deficiencies in *Pseudomonas aureofaciens* and *Arthrobacter protophormiae* increased the ratio of saturated to unsaturated fatty acids, and raised the ratio of trans fatty acids to cis during 16 days of incubation (Kieft *et al*. 1997).

Conclusion

It can be concluded that seed yield, oil and protein content, and the composition of fatty acids in soybean were affected by water deficit and inoculation with mycorrhiza fungi and bacteria. In three different irrigation conditions, inoculation with *Bradyrhizobium japonicum* bacterium increased seed yield, protein and oil content, and palmitoleic acid and decreased stearic and myristic acids compared with non-inoculation conditions. Mycorrhiza fungi also enhanced seed yield, oil yield, oil content, and protein content. Under moderate drought stress protein content increased significantly, but at the severe drought stress conditions decreased significantly. Under water deficit stress conditions, the oil yield and percentage decreased. In all three irrigation conditions, using mycorrhiza fungi appeared to increase protein percentage and unsaturated fatty acids, and reduce saturated fatty acids in some cases in soybean.

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Ethical considerations

The authors avoided data fabrication and falsification.

Conflict of interest

The authors declare that they have no conflict of interest with any organization concerning the subject of the manuscript.

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