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Co-Inoculation of Bradyrhizobium and Arbuscular Mycorrhizal Fungus Alleviates the Effects of Drought Stress in Soybean (*Glycine max* L.)

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ABSTRACT

Legumes are sensitive to drought stress, which adversely affects their seed yield, protein and oil content. This was investigated in a two-year field experiment conducted using a split-plot design with three replications in the Mughan plain, Ardabil. The experimental factors included drought stress as the main plot at three levels (60, 100, and 140 mm of evaporation from a class A pan) and the co-inoculation of soybean symbiotic bacteria and arbuscular mycorrhizal fungus species across eight treatments (*Bradyrhizobium japonicum*, *Funneliformis mosseae*, *Rhizophagus irregularis*, *Glomus fasciculatum*, *B. japonicum* + *F. mosseae*, *B. japonicum* + *R. irregularis*, *B. japonicum* + *G. fasciculatum*, and control) as the subplot. The results revealed that heightened drought stress led to a reduction in plant dry weight, pod number, seed number per plant, and seed yield in all treatments in both study years. However, this reduction was less pronounced in some treatments, especially those involving co-inoculation with *B. japonicum* + *R. irregularis* and *B. japonicum* + *G. fasciculatum*. Conversely, all treatments exhibited an increase in stomatal resistance, chlorophyll *a* concentration, soluble sugars, malondialdehyde (MDA), peroxidase (POD), and superoxide dismutase (SOD) under drought conditions (100 and 140 mm) compared to the normal irrigation conditions (I_{60}). The saturated fatty acids (palmitic and stearic acids) declined in inoculated plants compared to the control, while the trend was the opposite for unsaturated fatty acids (linoleic, linoneic, and oleic acids). Drought stress increased palmitic acid content by up to 32.4% and reduced linolenic acid content by up to 13.4%. Among the treatments, co-inoculation with *B. japonicum* + *R. irregularis* and *B. japonicum* + *G. fasciculatum* demonstrated a more significant improvement in the soybean's drought tolerance compared to the others. Given these results, inoculating soybean plants with rhizobial bacteria and *R. irregularis* mycorrhizae can be recommended as a strategy to enhance their drought resistance and improve their seed yield and oil quality.

1. INTRODUCTION

Over the past two decades, climate change has emerged as a significant environmental concern, manifested as rising air temperatures and reduced rainfall in Iran, leading to diminished agricultural yields. Among the affected crops, soy production has notably declined. Soybeans are a vital source of protein and oil whose significance is underscored by their high unsaturated fatty acid content, oil digestibility, meal quality, ability to fix nitrogen through symbiosis with Rhizobium bacteria, and contribution to soil fertility enhancement (Carter & Tegeder, 2016; Israilov et al., 2023). Soybean oil is composed of 49% linoleic acid and 25% oleic acid. Soybeans contain more protein than other oilseeds (Ran et al., 2024). The global significance of soybeans is attributed to their high protein content, ranking them third in value within the typical human diet (Semba et al., 2021). Drought stress during the soybean growth cycle, particularly in the reproductive phase, adversely affects its quantity and quality. In this regard, water scarcity has been reported to reduce the number of pods and seeds per plant, thousand-seed weight, seed yield, and harvest index (Mondani et al., 2019). In addition, drought stress alters the synthesis pathways of various compounds and secondary metabolites by inducing secondary stresses like oxidative stress (Hasanuzzaman et al., 2022).

The application of cost-effective, stable, and eco-friendly agents, such as mycorrhizal fungi, is a beneficial mechanism to enhance plant tolerance to drought stress (Wang et al., 2017; Samsami et al., 2019). These fungi form symbiotic relationships with plant roots, thereby improving the uptake efficiency of macronutrients and even micronutrients. Legumes can also form symbiotic associations with mycorrhiza. This symbiosis aids the plant in adapting to environmental changes (Ashwin et al., 2022). In addition to mycorrhiza, a variety of soil microorganisms in the rhizosphere, known as plant growth-promoting rhizobacteria (PGPR), can bolster plant growth and yield through mechanisms like biological N_2 fixation, the synthesis of phytohormones (auxins, cytokinins, and gibberellins), the increased solubility of insoluble compounds like phosphorus and potassium through synthesizing organic acids, the production of siderophores, and the higher availability of micronutrients, especially Fe (Pathania et al., 2020). Also, they have particularly been interesting due to their ability to colonize the root area and produce a wide range of enzymes and metabolites, which can be beneficial in water-deficient conditions (Elabed et al., 2019). The colonization of mycorrhizal helper bacteria, such as rhizobial symbiotic bacteria, on the root area strengthens the symbiotic relationship and confers numerous advantages to the host plant (Bencherif et al., 2019; Gough et al., 2021).

Inoculating soybean plants with bacteria like Bradyrhizobium was reported to increase seed yield by up to 28%, oil content by up to 18%, and seed protein level by up to 37% under drought conditions compared to non-inoculated plants (Israilov et al., 2023). On the other hand, it has been reported that dual inoculation with mycorrhiza and Bradyrhizobium can mitigate drought stress damage by reducing lipid peroxidation and membrane permeability and boosting the accumulation of osmosis-regulating compounds and antioxidant enzyme activity (Mohammadi et al., 2019; Samsami et al., 2019). In addition, symbiotic N-fixing bacteria can synthesize and secrete biologically active compounds in the root zone, thereby contributing to the root system's development and mycorrhizal colonization. On the other hand, by producing biologically active compounds and providing nutrients, mycorrhizal fungi help plant growth-stimulating bacteria survive and multiply (Egamberdieva et al., 2015). The synergy escalates plant growth by producing beneficial compounds, improving nutrient availability, and inducing plant resistance to various stresses, including nutrient deficiency, salinity, drought, acidity, and soil temperature (Musyoka et al., 2020).

The present study investigated the effects of the combined application of Bradyrhizobium bacteria and mycorrhizal fungi on the ecophysiological traits of soybeans under drought stress in field conditions over two years in the Mughan plain, northwest Iran.

2. MATERIALS AND METHODS

The present field research was conducted in 2022 and 2023 as a split plot experiment based on a randomized complete block design with three replications in Parsabad, Mughan, Iran (longitude 47°30’ E., latitude 39°20’ N., elevation 78 m above sea level). Figure 1 depicts the average rainfall and monthly temperature for both years during the crop growth period. The study was implemented as a second crop following the wheat harvest. A soil sample was collected from a depth of 0-30 cm to ascertain the texture and chemical properties of the soil at the experimental site. The results are displayed in Table 1.

The experimental treatments included drought stress as the main plot, gauged by the amount of evaporation (mm) from a Class A pan, with three levels: normal irrigation (60 mm evaporation, I_{60}), moderate drought stress (100 mm evaporation, I_{100}), and severe drought stress (140 mm evaporation, I_{140}). The subplot was assigned to the combined inoculation of soybean symbiotic bacteria and mycorrhizal fungi at eight levels: *Bradyrhizobium japonicum*, *Funneliformis mosseae*, *Rhizophagus irregularis*, *Glomus fasciculatum*, *B. japonicum* + *F. mosseae*, *B. japonicum* + *R. irregularis*, *B. japonicum* + *G. fasciculatum*, and a control treatment.

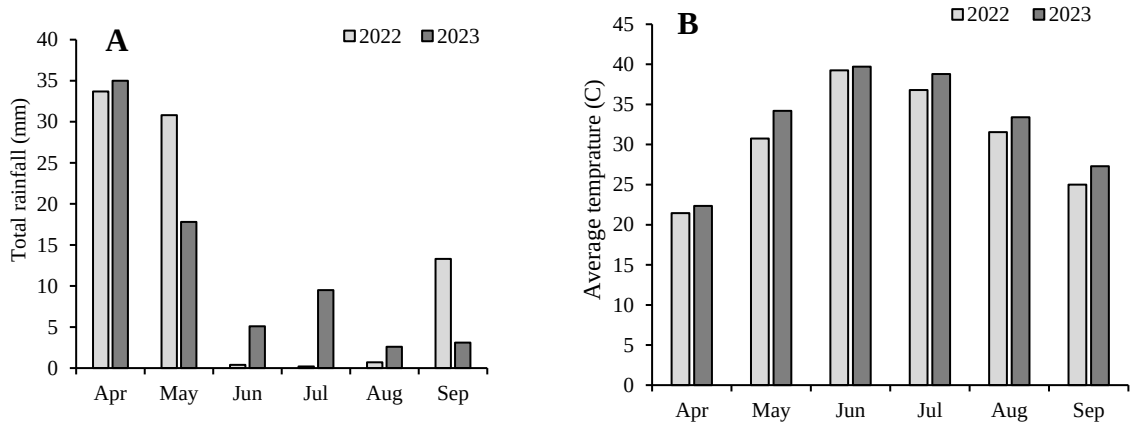

Figure 1. Total monthly rainfall (A) and average monthly temperature (B) during the soybean growth (Parsabad Weather Station).

Table 1. Results of physio-chemical analysis of the experimental soil

Year	Sand	Silt	Clay	EC	pH	OC	N	P	K
	(%)			(dS m^{-1})		(%)	(%)	(mg kg^{-1})	
2017	17.3	37.5	45.2	0.678	7.18	0.92	0.218	19.8	234
2018	18.4	37.0	44.6	0.509	6.91	0.88	0.241	17.5	251

Following the wheat harvest, the land was prepared by plowing and discing. Each experimental plot consisted of four 5 m long rows with a planting density of 8 × 30 cm. After plot preparation, seeds were coated with gum arabic and inoculated with bacteria (7 g of inoculum per kg of seeds, containing 10^7 live bacterial cells g^{-1}). The mycorrhizal fungi were applied to the seedbed. Then, the seeds were sown by hand. Soil moisture was adjusted to field capacity for each irrigation event. Irrigation for all treatments was uniform until the plants were fully established, indicated by the formation of 4-5 nodes on the main stem (V_4 growths stage). At the 50% flowering stage, three plants were randomly selected from the central rows of each plot and transported to the laboratory in a liquid nitrogen container to analyze their biochemical traits.

Chlorophyll extraction and measurement

Chlorophyll *a*, *b*, and total were quantified by Arnon's (1949) method. 100 mg (0.1 g) of fresh leaf tissue was ground in a mortar with 10 ml of 80% acetone. The resulting extract was then centrifuged at 6000 rpm for 10 min. The supernatant was transferred to a 25 ml volumetric flask, and its volume was made up to 25 ml with 80% acetone. Pigments were measured spectrophotometrically using a UV 160A SHIMADZU device, for which the absorbance of the solutions was read at 645 and 663 nm. Finally, considering a final volume of 25 ml, chlorophyll concentrations were expressed in mg g^{-1} of leaf tissue, and the amounts of chlorophyll *a*, *b*, and total were calculated.

Stomatal resistance

It was measured at the flowering stage on the youngest fully developed leaves between 06:00 and 08:00 AM before irrigation using an AP4 Delta-T Prometer device.

Concentration of soluble sugars

The concentration of soluble sugars was measured according to Irigoyen et al. (1992). So, 0.2 g of leaf samples were ground with 2 ml of sodium phosphate buffer (pH=7) and then centrifuged at 10000 rpm at 4 °C. Next, 990 µl of distilled water was added to 10 µl of the resulting supernatant. Thereafter, 5% phenolphthalein and 2.5 ml of 98% sulfuric acid were added to 0.5 ml of the resulting solution, and the absorbance was read at 490 nm.

Soluble protein

It was measured using Li's (2000) procedure. Fresh leaf samples (0.1 g) were weighed, a small amount of quartz sand was added to the homogenates, and 1 ml of distilled water was added to each before centrifuging at 12,000 rpm for 10 min. The sample extracts (0.2 ml) were placed in test tubes (two repeat tubes) and added with 1 ml of Coomassie brilliant blue G-250 reagent. The mixture was shaken vigorously, and after 2 min, absorbance was measured at 595 nm. The soluble protein content was calculated with the corresponding standard curve obtained using bovine serum albumin (BSA).

Proline

The proline content in leaf tissues was measured via reaction with ninhydrin (Bates et al., 1973). For colorimetric determinations, a solution of proline, ninhydrin acid, and glacial acetic acid (1:1:1) was incubated at 90 °C for 1 h. The chromophore was extracted using 2 ml of toluene, and its absorbance was read at 520 nm using a BioMate spectrophotometer (Shimadzu UV-160, Japan).

Malondialdehyde (MDA)

The MDA content, which is indicative of lipid peroxidation, was assessed following Boominathan & Doran's (2002) protocol. Leaf tissue was extracted in a 0.1% trichloroacetic acid (TCA) solution and centrifuged at 10,000 rpm for 5 min. The supernatant was mixed with 20% TCA solution containing 0.5% thiobarbituric acid at a ratio of 1:4, and then, it was placed in a water bath at 95 °C for 30 min. The tubes were then rapidly cooled in ice and centrifuged at 10,000 rpm for 15 min. Alongside the leaf extracts, standard solutions ranging from 0 to 100 nmol of 1,1,3,3-tetraethoxypropane were prepared, and the absorbance of the samples was read at 532 nm using a spectrophotometer. Finally, the MDA concentration in the samples was calculated in nmol g^{-1} FW.

Enzyme extraction and assay

Frozen leaves were ground in liquid nitrogen using mortar and pestle as much as possible. Then, 0.5 g of the ground sample was transferred to a 2 ml microtube and vortexed by adding 1 ml of phosphate buffer. It was then centrifuged at 14000 rpm at 4 °C for 15 min. After that, the supernatant was transferred to a 1.5 ml microtube using a micro-sampler and re-centrifuged at the same conditions for another 10 min. The resulting supernatant was transferred to another microtube of the same size. The microtubes containing residues of the sample ground and centrifuged were transferred to -80 °C for probable future measurements (Sairam et al., 1998).

Catalase (CAT) activity

The CAT activity was assayed according to Aebi (1971) by monitoring the rate of decomposition of H_2O_2 at 240 nm in the reaction mixture consisting of 50 mM potassium phosphate buffer, 10 mM H_2O_2, and the crude enzyme solution. The H_2O_2 content was determined according to Loreto and Velikova (2001).

Superoxide dismutase (SOD) activity

The SOD activity was determined by its capacity to inhibit the photoreduction of nitroblue tetrazolium chloride (NBT) using Giannopolitis & Ries's (1977) method. The reaction mixture, totaling 3 ml, contained 50 mM potassium phosphate buffer (pH = 7.8), 13 mM methionine, 75 µM NBT, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 360 µM riboflavin, and 30 µL of the crude enzyme extract. After thorough mixing, the spectrophotometer cells were exposed to light from a 15-W fluorescent lamp placed 35 cm away for 10 min. The reaction was halted by switching off the lamp, and the absorbance of the mixture was measured at 560 nm. One unit of SOD activity was defined as the amount of enzyme required to inhibit 50% of the NBT photoreduction.

Peroxidase (POD) activity

The POD activity was assessed following the method described by Plewa et al. (1991). To this end, 900 µL of 50 mM phosphate buffer (pH = 7.0) containing 0.1 mM EDTA, 0.1 mM H_2O_2, and 10 mM guaiacol (2-methoxyphenol) was mixed with 50 µL of the enzyme extract. The increase in absorbance due to tetraguaiacol formation caused by POD activity was monitored at 470 nm over 2 min. One unit of enzyme activity was defined as an increase of 0.01 in absorbance at 470 nm per minute.

Yield and yield components

Eight plants were randomly selected and harvested from each plot at the maturity phase, taking care of the margins. The number of pods, seeds per pod, seeds per plant, and 100-seed weight were recorded. Seed yield, adjusted to 14% moisture content, was calculated from a 5 m^2 area at the center of each plot. Seed moisture content was determined using a digital moisture meter (Model GMK-303R5, Korea). To ascertain shoot dry weight, plants harvested from a 1-m^2 area were enclosed in paper bags and oven-dried at 72 °C for 48 h, and the result was recorded as the biological yield per ha.

Oil determination

Seed oil was extracted using rotary evaporators based on Folch et al.'s (1957) protocol. The extracted fatty acids were transformed to their methyl esters (FAME) using Metcalf et al.'s (1966) method and were determined using a gas chromatography (Unicam 4600) equipped with an FID detector.

Data analysis

All measured traits were consistent across both years, except for fatty acid composition, which was only assessed in the second year. The results recorded for the comparison of means represent the average of the three replicates per

treatment. Data were analyzed using SAS software (version 9.2), and the means were compared using the LSD test. When a significant interaction was observed between traits, the means were compared for the interactive effects using the slice method and the procedure of L.S.Means.

3. RESULTS

Leaf soluble sugar and protein contents

The analysis of variance (ANOVA) revealed that the interaction of year × drought stress × microorganisms significantly influenced the soluble sugar and protein contents (Table 2). It was revealed that as drought stress intensity was increased from I_{60} to I_{140}, the concentration of soluble sugars in the leaves rose by 13-45% in the first year and by 10-34% in the second year in most treatments. The control and single inoculation treatments exhibited higher soluble sugar levels than the co-inoculation treatments (Table 4). The changes in leaf soluble proteins with increasing drought stress varied among the treatments. However, in both years and across all drought stress levels, the plants treated with *B. japonicum + F. mosseae* and those treated with *B. japonicum + R. irregularis* displayed higher soluble protein levels than most other treatments (Table 4).

Table 2. ANOVA of the effect of drought stress and microorganisms on content of chlorophyll a (Chl a), chlorophyll b (Chl b), total chlorophyll (Total chl), Stomatal resistance, Soluble sugars and Soluble proteins of soybean

Source of variance	Mean Square (MS)					
	Chl a	Chl b	Total chl	Stomatal resistance	Soluble sugars	Soluble proteins
Y	5.900**	2.34**	15.70**	1562**	372069**	89.4**
Y(R)	0.171	0.059	0.244	32.90	895	28.6
D	4.950**	1.636**	12.16**	3720**	52864**	17.2ns
Y × I	6.238**	2.188**	15.80**	1041**	5650*	13.2ns
D(Y× R)	0.069	0.015	0.084	32.30	1430	10.2
M	0.032ns	0.004ns	0.039ns	16.64ns	5471**	23.9*
Y × F	0.023ns	0.010ns	0.034ns	32.56ns	6456**	14.5ns
D × M	0.067**	0.007ns	0.072*	20.23ns	12667**	24.9**
Y × D × M	0.066**	0.014ns	0.093**	40.49ns	18763**	25.1**
Error	0.020	0.017	0.035	27.70	1727	9.3
CV (%)	5.75	8.53	4.67	16.8	21.27	20.4

** Significant in 0.01, * significant in 0.05, and ns: non-significant. Y: Year, R: Replication, D: Drought stress, M: Microorganisms

Table 3. ANOVA of the effect of drought stress and microorganisms on proline and MDA content and activity of SOD, CAT, and POD of soybean

Source of variance	Mean Square (MS)				
	Proline ($\mu g\ g^{-1}$)	MDA ($nmol\ g^{-1}$)	SOD ($U\ g^{-1}\ FW\ min^{-1}$)	CAT ($U\ g^{-1}\ FW\ min^{-1}$)	POD ($U\ g^{-1}\ FW\ min^{-1}$)
Y	45966**	152828**	202740**	529.7**	4.05*
Y(R)	6235ns	67.3ns	1115ns	8.74ns	1.32ns
D	5199ns	13326**	224503**	2861**	10.31**
Y × I	38105**	57103**	28557*	124.0ns	0.197ns
D(Y× R)	10241ns	1182ns	13981*	44.61ns	0.728ns
M	7094.ns	24820**	27431**	213.4**	2.86**
Y × F	23832**	11756**	20125**	77.1ns	1.425ns
D × M	16068**	22317**	46734**	36.6ns	2.24**
Y × D × M	22422**	12333**	46574**	45.2ns	3.21**
Error	6255	865.9	6263	52.86	0.808
CV (%)	23.2	20.92	19.86	16.46	23.31

** Significant in 0.01, * significant in 0.05, and ns: non-significant. Y: Year, R: Replication, D: Drought stress, M: Microorganisms

Stomatal resistance

Based on the results of ANOVA, stomatal resistance was influenced by the interactive effects of year × drought stress (Table 2). The comparison of means for this trait showed an increase from 11.3% to 34.6% in the first year and from 7.73% to 20.5% in the second year as drought stress intensified from I_{60} to I_{140}. However, no significant difference was observed between I_{100} and I_{140} in the first year (Figure 2A).

Leaf chlorophyll

The results demonstrated that the chlorophyll *a* level was significantly impacted by the interaction of year × drought stress × microorganisms, and the chlorophyll *b* content was influenced by the interaction of year × drought stress (Table 2). The comparison of means for chlorophyll *a* revealed a decrease in all treatments with increasing water deficit. The

decline was 17-31% in the first year and 20-33% in the second year at the 100 mm level, and it was 28-49% in the first year and 31-43% in the second year at the 140 mm level compared to the 60 mm level (no stress). Among the treatments, the co-inoculated plants with *B. japonicum* + *R. Irregularis* and *B. japonicum* + *G. fasciculatum* generally exhibited higher chlorophyll *a* levels under most water-deficit conditions (Table 4). The comparison of means for the chlorophyll *b* level indicated that, in both years, the 90 mm drought level exhibited the highest chlorophyll *b* content, while there was no significant difference between the 60 mm and 140 mm levels (Figure 2B). Moreover, the total chlorophyll content was affected by the interaction of year × drought stress and the simple effect of microorganisms (Table 2). As depicted in Figure 2C, the total chlorophyll content decreased with escalating stress intensity. Among the microorganisms, the inoculation treatments resulted in higher total chlorophyll content, with co-inoculation treatments (*B. japonicum* + *R. Irregularis* and *B. japonicum* + *G. fasciculatum*) outperforming other treatments (Figure 2D).

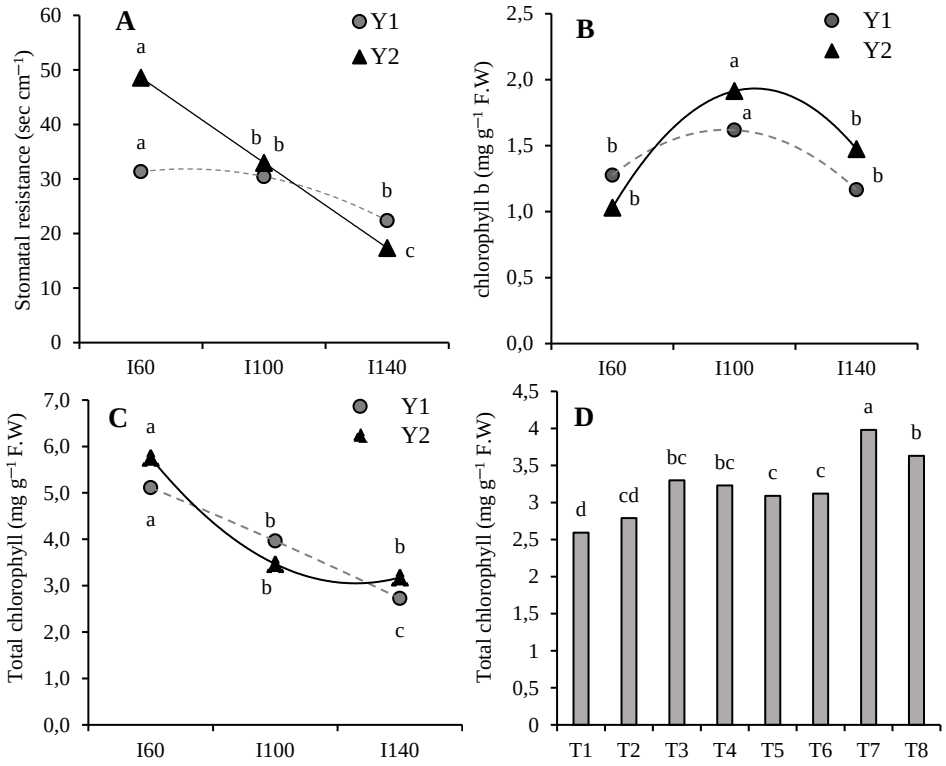

Figure 2. Mean comparison of the interaction effect of year × drought stress on stomatal resistance (A), chlorophyll b (B), and total chlorophyll (C), as well as the main effect of microorganism on total chlorophyll (D). I60, I100 and I140 (60, 100 and 140 mm evaporation from a class A pan, respectively). T1: control, T2: *B. japonicum*, T3: *F. mosseae*, T4: *R. irregularis*, T5: *G. fasciculatum*, T6: *B. japonicum* + *F. mosseae*, T7: *B. japonicum* + *R. irregularis*, T8: *B. japonicum* + *G. fasciculatum*.

Leaf proline and MDA contents

As the results of ANOVA indicated, the interaction between year, drought stress, and microorganisms was significant for the proline and MDA contents (Table 3). The comparison of means revealed that with increasing drought intensity, the proline and MDA contents in all treatments rose significantly in both years. Notably, plants with co-inoculation had higher proline levels and lower MDA levels than the control plants. Among the treatments, the plants treated with *B. japonicum* + *R. irregularis* produced the highest proline level under severe drought stress (I140) (510 and 580 μg g^{-1} in the first and second years, respectively). Conversely, the highest MDA levels were found in the control treatment, with 143 and 202 nmol g^{-1} in the first and second years, respectively (Table 4).

SOD, CAT, and POD activity

The results of ANOVA demonstrated that the interaction of year, drought stress, and microorganisms had a significant impact on SOD and POD activity (Table 3). Based on the comparison of means for SOD activity, it was higher at greater drought severity in both years. Specifically, SOD activity at I_{100} increased by 14.1-43.7% in the first year and 11.3-36.5% in the second year, while at I_{140}, it increased by 38.6-69.7% in the first year and 26.2-57.3% in the second year, compared to I_{60}. Although the highest SOD activity was observed in the control treatment in the first year, no significant difference was found in the second year compared to mycorrhizal treatments without *B. japonicum*, and SOD activity was lower in the co-inoculation treatments. In most conditions, the plants treated with *B. japonicum* + *R. irregularis* and *B. japonicum*

+ *F. mosseae* exhibited the lowest SOD activity (Table 4). POD activity patterns were similar to those of SOD. Based on the results, POD activity increased under I_{100} and I_{140} compared to I_{60} by 31.3-56.4% in the first year and 26.1-68.5% in the second year. Co-inoculated plants showed lower POD activity than the control and *B. japonicum* treatment (Table 4). However, CAT activity increased from I_{60} to I_{100} and then decreased from I_{100} to I_{140} (Figure 3A). The comparison of the treatments revealed that *B. japonicum* + *R. irregularis* and *B. japonicum* + *G. fasciculatum* had significantly higher CAT activity than the other treatments (Figure 3B).

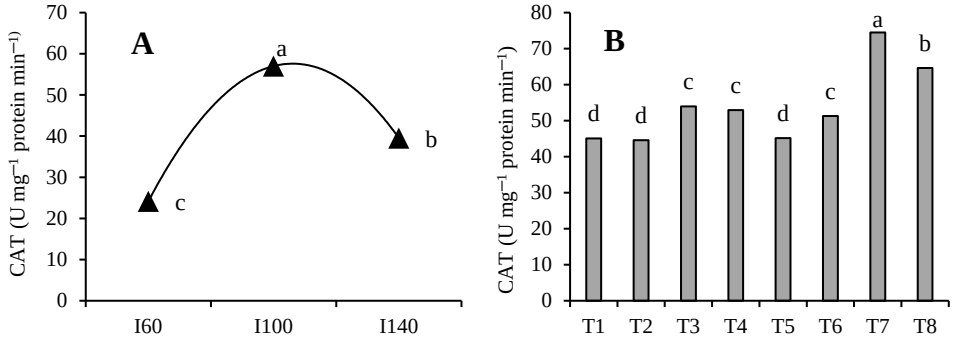

Figure 3. Mean comparison of the main effect of drought stress (A) and microorganisms (B) on CAT activity. I60, I100 and I140 (60, 100 and 140 mm evaporation. T1: control, T2: *B. japonicum*, T3: *F. mosseae*, T4: *R. irregularis*, T5: *G. fasciculatum*, T6: *B. japonicum* + *F. mosseae*, T7: *B. japonicum* + *R. irregularis*, T8: *B. japonicum* + *G. fasciculatum*.

Table 4: Mean comparison of the interaction effect of year × drought stress × microorganisms on chlorophyll a, soluble sugar, soluble protein, proline, and MDA contents and activity of SOD, and POD of soybean

Year	Drought stress	Microorganisms	chlorophyll a	Soluble sugar	Soluble protein	Proline	MDA	SOD	POD
				(mg g^{-1} FW)		(µg g^{-1})	(nmol g^{-1})	(U mg^{-1} protein min^{-1})	
		T1	4.45d	221a	15.7ab	208e	44.0a	257ab	2.761a
		T2	4.94a-d	182b	14.43ab	271b	33.1b	251b	2.73a
	I60	T3	4.57cd	140b-d	14.3ab	220d	29.0b	283ab	2.511ab
		T4	4.97a-c	103d	12.01b	253bc	41.0a	287a	2.328b
		T5	4.68cd	172bc	14.10ab	268b	30.4b	188b	2.672a
		T6	4.91b-d	101d	14.76ab	256bc	31.7b	261ab	2.561ab
		T7	5.52a	111d	16.5a	235cd	33.0b	166cd	2.370b
		T8	5.33ab	124cd	15.4ab	310a	28.0b	143d	2.411b
		T1	2.76d	329a	11.86b	301bc	142a	449a	4.16a
		T2	2.98a-d	299ab	12.81ab	273c	130bc	291e	4.188a
		T3	2.86a-d	180cd	11.93b	295bc	121d	358bc	3.473ab
2021	I100	T4	2.83b-d	135cd	12.76ab	288bc	126cd	334cd	3.473ab
		T5	3.08a-c	112d	13.16ab	322ab	119d	367b	3.926a
		T6	2.81cd	174cd	15.41a	335ab	136ab	356bc	3.173b
		T7	3.11ab	119d	15.83a	324ab	108e	295e	3.436ab
		T8	3.14a	225bc	13.8ab	366a	94.1f	316de	3.466ab
		T1	2.063c	338a	11.90c	346d	154a	665a	6.43a
		T2	2.25a-c	314a	13.23bc	340d	138b	611ab	5.72ab
		T3	2.136bc	274a	12.73bc	358c	135b	567b	5.193ab
		T4	2.386a	290a	14.05a-c	373bc	132b	468cd	5.42ab
	I140	T5	2.273ab	302a	13.11bc	376bc	131b	472c	5.46ab
		T6	2.350a	159b	18.06a	398b	129b	454cd	5.50ab
		T7	2.374a	278a	16.58ab	510a	122b	407d	4.613b
		T8	2.285ab	162b	14.9a-c	410b	105c	451cd	4.776b
		T1	3.52b	155a	13.43bc	238c	86.0b	377a	3.15a
		T2	4.07ab	140ab	13.75bc	278ab	84.7b	362a	3.05ab
	I60	T3	4.15ab	132bc	12.46c	241c	118a	314a-c	3.083a
		T4	3.94ab	125bc	12.58c	266ab	70.3c	347ab	2.81a-c
		T5	4.17ab	127bc	17.0ab	237c	54.1de	250c	2.58bc
		T6	3.95ab	104d	14.75b	281ab	45.7e	323ab	2.46bc
		T7	4.36a	118cd	18.89a	286ab	55.4d	291bc	2.40bc
		T8	4.32a	116cd	17.52ab	296a	72.7c	265c	2.03c
		T1	2.63d	242a	9.98c	284c	130b-d	472a	4.13a
		T2	2.91c	233a	10.25c	308bc	144a	471a	4.066a
		T3	2.74cd	127c	14.25bc	326b	140ab	391b	3.482bc
2022	I100	T4	2.96c	162b	14.88bc	336b	127cd	423b	3.80ab
		T5	2.91c	246a	17.52ab	276c	136a-c	420b	3.68ab
		T6	2.95c	120c	16.57ab	332b	132a-d	418b	3.666ab
		T7	3.35b	124c	22.85a	395a	134a-d	412b	3.23c
		T8	3.63a	82.6d	16.81ab	374ab	125d	398b	3.44c
		T1	2.096b	313a	10.27d	453b	203a	632a	6.072a
	I140	T2	2.28ab	328a	14.10c	513ab	198a	624a	5.647ab
		T3	2.24ab	254a-c	15.71bc	383c	182b	511cd	5.345ab
		T4	2.31ab	213bc	15.90bc	463b	152c	540bc	5.912a
		T5	2.201ab	259a-c	21.56a	479b	170b	572b	5.758ab
		T6	2.243ab	316a	22.08a	449b	177b	468e	5.216ab
		T7	2.43a	170c	21.45a	580a	150c	495de	5.180ab
		T8	2.57a	303ab	17.65b	566a	143c	507cd	4.759b

Similar letter(s) in each column show insignificance of the difference based on the LSD test at the $P < 0.05$ level.
I60, I100 and I140 (60, 100 and 140 mm evaporation from a class A pan, respectively). T1: control, T2: *B. japonicum*, T3: *F. mosseae*, T4: *R. irregularis*, T5: *G. fasciculatum*, T6: *B. japonicum* + *F. mosseae*, T7: *B. japonicum* + *R. irregularis*, T8: *B. japonicum* + *G. fasciculatum*.

Table 5. ANOVA of the effect of drought stress and microorganisms on pod number, grain number, grain yield, plant biomass, oil content, and oil yield of soybean (combined analysis of the two years)

Source of variance	MS (mean square)					
	Pod number	Grain number	Grain yield (g m^{-2})	Plant biomass (g $plant^{-1}$)	Oil content (%)	Oil yield (g m^{-1})
Y	18.21*	42.1ns	395661**	414**	2405**	0.653ns
Y(R)	2.55	102	7772ns	3.52	91.2	313**
D	524.2**	3006**	2181007**	1711**	2687**	2432**
Y × I	5.033 ns	66.7 ns	191249**	395**	2547**	224 ns
D(Y× R)	2.094 ns	49.1 ns	5982ns	2.47ns	48.13	237
M	4.26 ns	88.7 ns	39732**	2.53ns	21.1ns	428**
Y × F	6.89 ns	140.1**	64769**	6.71*	16.6ns	102ns
D × M	6.25 ns	73.2 ns	27296**	2.48ns	470**	244**
Y × D × M	15.07**	98.6*	42690**	6.34*	619**	55.9ns
Error	4.36	48.8	5500	3.339	20.6	83.9
CV (%)	25.9	35.6	22.5	10.5	9.35	20.3

** Significant in 0.01, * significant in 0.05, and ns: non-significant. Y: Year, R: Replication, D: Drought stress, M: Microorganisms

Table 6. Mean comparison of the interaction effect of year × drought stress × microorganisms on pod number, grain number, grain yield and plant biomass

Irrigation	Microorganism	Pod number		Grain number		Grain yield (g m^{-2})		Plant biomass (g $plant^{-1}$)	
		2021	**2022**	**2021**	**2022**	**2021**	**2022**	**2021**	**2022**
I60	T1	19.6e	19.7e	42.7d	19.7e	147d	163d	203d	198e
	T2	23.1c-e	16.4f	62.0bc	16.4f	211cd	227c	233cd	287c-e
	T3	25.5b-d	26.4bc	58.9bc	26.4bc	205cd	241bc	266c	260de
	T4	21.8de	23.2d	46.5cd	23.2d	189cd	259bc	261c	311cd
	T5	22.3de	24.1cd	47.1cd	24.1cd	195cd	239bc	265c	316cd
	T6	27.4bc	23.1d	68.6b	23.1d	235bc	268a-c	263c	350bc
	T7	33.7a	29.2a	84.9a	29.2a	309a	308a	435a	517a
	T8	29.1ab	26.7b	72.2ab	26.7b	281ab	271ab	348b	416b
I100	T1	14.6bc	10.36e	286.1c	10.36e	102c	84.1b	102d	127e
	T2	16.2b	14.57bc	31.8bc	14.57bc	109c	109.5ab	116d	134e
	T3	16.37b	13.07cd	33.6bc	13.07cd	107c	107ab	139cd	131e
	T4	13.12c	16.55a	33.4bc	16.55a	114bc	119.3a	132cd	153cd
	T5	13.70c	15.03ab	34.8bc	15.03ab	125bc	106.4ab	150b-d	162bc
	T6	14.92bc	13.23c	38.9b	13.23c	146ab	122.6a	187a-c	140de
	T7	18.65a	11.57de	52.0a	11.57de	162a	128.2a	228a	189a
	T8	19.60a	13.29c	48.1a	13.29c	173a	121a	199ab	170b
I140	T1	6.06b	7.46c	11.23d	7.46c	24.0e	30.55e	35.6c	32.2c
	T2	7.01b	9.03a-c	11.91d	9.03c	25.6de	37.0c-e	43.3bc	50.0b
	T3	7.63b	9.51a-c	19.12c	9.51c	43.4bc	50.1a-c	38.0bc	37.2c
	T4	8.12b	9.76a-c	18.16c	9.76c	37.1cd	47.6a-d	58.3c	51.6b
	T5	33.7b	8.35bc	17.04c	8.35bc	32.5de	35.1de	50.3bc	59.6b
	T6	11.76a	11.467a	28.0a	11.467a	57.3a	59.7a	58.0c	54.0b
	T7	11.0a	11.23ab	22.86b	11.23ab	49.3ab	51.3ab	80.3a	89.3a
	T8	11.2a	10.33ab	22.47b	10.33ab	46.0bc	39.2b-e	61.3ab	79.0a

Similar letter(s) in each column shows insignificance of the difference based on the LSD test at the P < 0.05 level.
I60, I100 and I140 (60, 100 and 140 mm evaporation from a class A pan, respectively). T1: control, T2: *B. japonicum*, T3: *F. mosseae*, T4: *R. irregularis*, T5: *G. fasciculatum*, T6: *B. japonicum* + *F. mosseae*, T7: *B. japonicum* + *R. irregularis*, T8: *B. japonicum* + *G. fasciculatum*.

Seed oil content

ANOVA indicated a significant interaction between drought stress and microorganisms for both seed oil content and oil yield (Table 5). The comparison of means for the seed oil content showed that, except for the control, *B. japonicum* + *R. irregularis*, and *B. japonicum* + *G. fasciculatum* treatments, the seed oil content decreased as stress intensity increased. The highest oil content was obtained from the *B. japonicum* + *F. mosseae* treatment (29.6%) under normal irrigation conditions (I_{60}) and from the *B. japonicum* + *R. irregularis* treatment under I_{100} and I_{140} (Figure 4A). Oil yield, which is a function of seed yield and seed oil concentration, was the highest in all treatments at the I_{60} level. However, drought stress (I_{100} and I_{140}) resulted in a reduction in oil yield, ranging from 13.13% to 60.59%, in all treatments. At all drought levels, the maximum oil yield was achieved with the co-inoculation treatments *B. japonicum* + *R. irregularis*, and *B.*

japonicum + *G. fasciculatum*, which resulted in an increase in oil yield by 34.3-61.7% and 29.2-52.3%, respectively, compared to the control (Figure 4B).

Seed oil quality

The results indicated that drought stress significantly affected the PA and LINLA levels (Table 7). As drought stress intensified, the PA level increased by 10.2-12.4% (Figure 4C), whereas the LINLA level decreased by 7.14-8.09% (Figure 4D). Microorganisms also had a significant impact on fatty acids (Table 7). The comparison of means revealed that the PA and SA levels were lower in mycorrhizal plants than in the control. However, the OA, LILA, and LINLA levels were higher in most inoculated plants than in the control. Co-inoculated plants showed a marked improvement over both the control and *B. rhizobium*. Overall, the combination of *B. japonicum* + *F. mosseae* exhibited the highest levels of OA and LA, while *B. japonicum* + *G. fasciculatum* had the highest LINLA levels (Table 8).

Table 7. ANOVA of the effect of drought stress and microorganisms on content of palmitic acid (PA), stearic acid (SA), oleic acid (OA), linoleic acid (LILA), and linolenic acid (LINLA)

Source of variance	MS (mean square)				
	PA (%)	SA (%)	OA (%)	LILA (%)	LINLA (%)
R	0.014ns	0.026ns	1.21ns	18.3**	0.881**
D	2.75**	0.0131ns	1.077ns	2.16ns	2.18**
Error1	0.0406	0.077	0.891	0.371	0.211
M	0.613*	0.151*	2.64**	10.6**	0.429**
D × M	0.073ns	0.005ns	0.049ns	0.8281ns	0.212ns
Error2	0.238	0.044	0.689	0.862	0.109
CV (%)	4.37	5.43	3.52	1.78	8.51

** Significant in 0.01, * significant in 0.05, and ns: non-significant. Y: Year, R: Replication, D: Drought stress, M: Microorganisms.

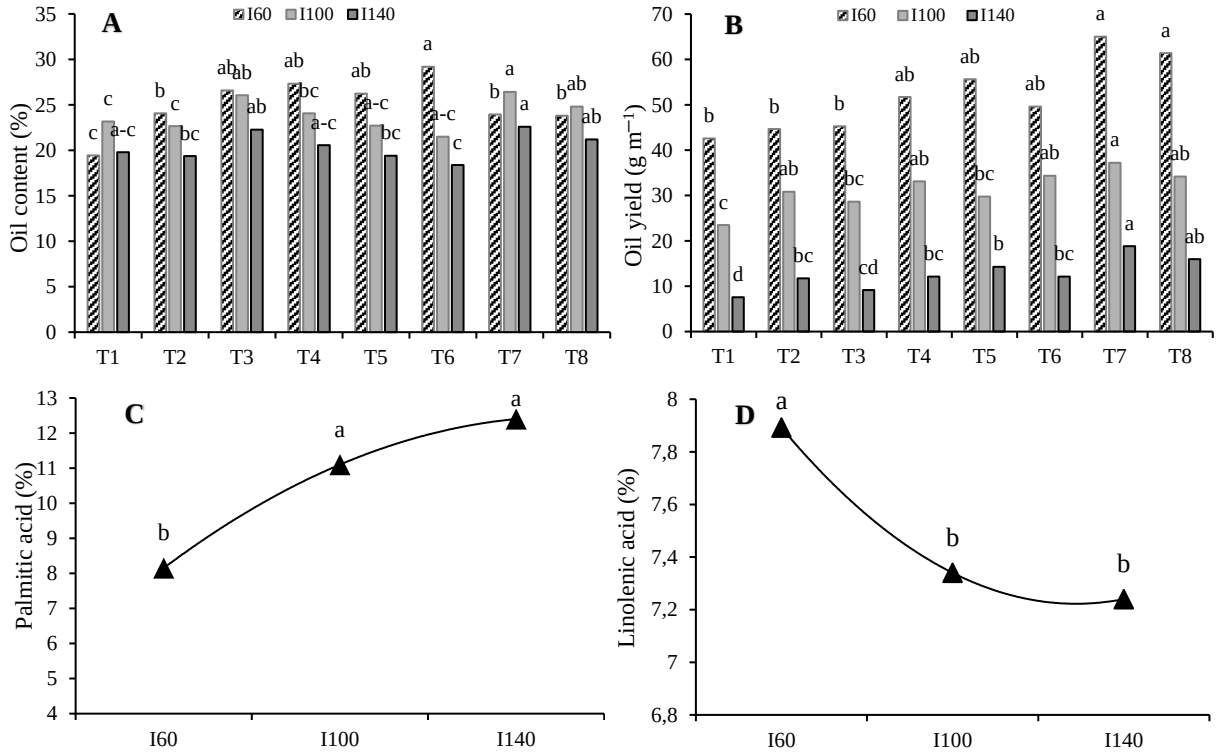

Figure 4. Mean comparison of the intraction effect of main effect of drought stress × microorganisms on oil content (A) and oil yield (B); main effect of drought stress on palmitic acid (A) and linolenic acid (B). I60, I100 and I140 (60, 100 and 140 mm evaporation from a class A pan, respectively). T1: control, T2: *B. japonicum*, T3: *F. mosseae*, T4: *R. irregularis*, T5: *G. fasciculatum*, T6: *B. japonicum* + *F. mosseae*, T7: *B. japonicum* + *R. irregularis*, T8: *B. japonicum* + *G. fasciculatum*.

Table 8: Mean comparison of the interaction effect of drought stress × microorganisms on content of palmitic acid (PA), stearic acid (SA), oleic acid (OA), linoleic acid (LILA), and linolenic acid (LINLA)

Microorganisms	PA (%)	SA (%)	OA (%)	LILA (%)	LINLA (%)
Control	11.6a	4.544a	22.13c	48.63c	7.295c
Brady rhizobium japonicum	11.56ab	4.453ab	23.03bc	50.4c	7.321c
Funneliformis mosseae	11.00b-d	4.484ab	23.95ab	51.8b	7.53bc
Rhizophagus irregularis	11.09a-d	4.378ab	23.78ab	52.58ab	7.558bc
Glomus fasciculatum	10.89cd	4.474ab	23.7ab	52.01b	7.63bc
B. japonicum + F. mosseae	11.33a-c	4.043c	24.21a	53.65a	7.498c
B. japonicum + R. irregularis	11.06a-d	4.055c	24.02a	52.88ab	7.918ab
B. japonicum + G. fasciculatum	10.69d	4.35ab	23.6ab	52.53ab	8.16a

Similar letter(s) in each column shows insignificance of the difference based on the LSD test at the $P < 0.05$ level.

4. DISCUSSION

The application of plant growth-stimulating microorganisms has recently emerged as an eco-friendly and cost-effective method for sustainable crop production in various conditions. Our findings indicated that inoculating soybean plants with certain mycorrhizal species and Bradyrhizobium enhanced their physiological traits, such as leaf chlorophyll, proline, MDA, and CAT, POD, and SOD activities, thereby increasing their drought tolerance, in both study years. It was observed that co-inoculation with *R. irregularis*, *G. fasciculatum*, and *B. japonicum* increased chlorophyll *a* content by 4.7-17.3% under I_{100} and 6.34-22.5% under I_{140} conditions compared to the control. Also, it increased the proline content by 13.2-39.4% in the I_{100} conditions and 6.8-19.5% in the I_{140} conditions versus the control. However, most treatments exhibited lower SOD activity than the control. The plants co-inoculated with *B. japonicum + R. irregularis* and *B. japonicum + F. mosseae* showed the least SOD activity under most conditions. These results are consistent with the findings of Musyoka et al. (2020) and Dabré et al. (2022). Gough et al. (2021) attributed the rise in leaf chlorophyll content and oxidative enzyme activity in mycorrhizal plants to enhanced water uptake by mycorrhizal fungi because the hyphae of the mycorrhizal fungi extended root reach in the soil and increased water availability (Mohammadi et al., 2019). The increase in SOD activity as a general response to water stress (Hasanuzzaman et al., 2022) could be due to inhibited enzyme synthesis, altered subunit enzyme assembly under stress (Fazeli et al., 2015), degradation by induced peroxisomal proteases, or photoinactivation of the enzyme (Liu et al., 2008). Severe drought stress leads to a reduction in chlorophyll *a*, *b*, and total, as well as carotenoids, due to increased production of oxygen free radicals, which cause lipid peroxidation and subsequent pigment breakdown (Jiménez et al., 2021). Under stress conditions, the moderate increase in leaf SOD activity in plants inoculated with Rhizobium and mycorrhiza suggests that inoculation can enhance this enzyme's activity to combat oxidative damage arising from water scarcity. Microorganisms can, therefore, modulate oxidative reactions and antioxidant defenses (Ortiz et al., 2015) such that co-inoculation with mycorrhizal fungi and Bradyrhizobium boosts antioxidant production, reducing reactive oxygen species (ROS) under stress and protecting cells from oxidative damage (Samsami et al., 2019). Andrade et al. (2010) reported increased SOD and POD activity in bean plants inoculated with mycorrhiza. Salloum et al. (2018) state that increased root colonization extends the host plant's root system, which allows for greater soil penetration by fungal hyphae, thus expanding the root's access to soil and improving water and nutrient uptake efficiency. Proline and MDA levels rose with increasing drought severity in all treatments. However, co-inoculated plants showed higher proline and lower MDA levels than the control. In this regard, Nath et al. (2016) asserted that high MDA concentrations in leaves under water stress could be accompanied by elevated H_2O_2 accumulation in plants, indicating extensive membrane lipid peroxidation. Nonetheless, inoculated plants exhibited lower MDA levels than non-inoculated control plants, suggesting that both microorganisms played a role in ROS metabolism (Ashwin et al., 2022). Water stress has been shown to decrease MDA concentrations in mycorrhizal species compared to the control, although the extent varies among symbiotic fungal species (Basyal & Walker, 2023).

The accumulation of proline and soluble proteins under stress may, on the other hand, result from reduced proline oxidation, its enhanced synthesis from glutamate, or increased protease activity (Jiménez et al., 2021). Proline serves to protect cytosolic enzymes (e.g., carboxylase) and cell structures, so it accumulates in cells during stress (Fang & Xiong, 2015). Water deficit conditions trigger an increase in proline within the plant by upregulating proline-synthesizing enzymes and reducing the activity of proline-degrading enzymes (Serraj & Sinclair, 2002). Mycorrhizal plants, benefiting from improved water relations and nutrition, can better withstand drought stress than non-mycorrhizal plants, so their proline content increases slightly versus non-mycorrhizal plants (Hashem et al., 2019; Oliveira et al., 2022). However, lower soluble sugar content in the leaves of mycorrhizal plants, compared to non-mycorrhizal ones, may be due to the increased translocation of these sugars to the roots (Garg & Cheema, 2021; Basyal & Walker, 2023).

The number of pods, a key determinant of legume yield, is highly reliant on the plant's nutrient supply (Carter & Tegeder, 2016; Ilker et al., 2018; Semba et al., 2021). Our findings indicated that while drought stress reduced yield and its components, microorganisms bolstered plant tolerance and increased the number of pods, seeds, and grain yield. This

enhancement is likely due to a gradual nutrient supply and improved physiological growth conditions. It can be inferred that the chemical and physical properties of humic acid surrounding mycorrhizae (Wang et al., 2017) may contribute to increasing the accumulation of nutrients required by the plant and enhancing the number of auxiliary branches, pods, and seed yield per plant (Al-Karaki & Williams, 2021) by improving water retention, nutrient storage capacity, growth-regulating hormones, and the activity of microorganisms (Mondani et al., 2019). Beneficial microorganisms, particularly mycorrhizal fungi, can boost plant biomass and biological yield by synthesizing phytohormones, enhancing nutrient availability, facilitating nutrient uptake, reducing heavy metal toxicity in the plant, deterring pathogens, and inducing systemic resistance to pathogens (Grümberg et al., 2015; Wang et al., 2017). Similarly, Samsami et al. (2019) observed a 43.8% increase in biological yield due to soybean seeds inoculated with Rhizobium and mycorrhiza, attributing this boost to the production of indole-3-acetic acid (IAA) by these microorganisms. On the other hand, Bharti et al. (2018) found that soybean plants inoculated with rhizobium exhibited a significant increase in auxiliary branches, seeds, and pods and ascribed this finding to the increase in nitrogen fixation, nutrient uptake, and the production of growth-stimulating hormones. In the same vein, Dabré et al. (2022) and Egamberdieva et al. (2015) reported that the rise in seed and pod numbers in plants might stem from the increased nutrient availability provided by mycorrhizae and Bradyrhizobium, which supports our findings. Musyoka et al. (2022) assessed the role of mycorrhiza in mung bean root development. They found that mycorrhiza enhanced nutrient uptake from the soil by boosting photosynthetic capacity and improving plant water relations. Some researchers believe that the combination of humic acid or amino acids as carbon sources can increase the performance of mycorrhizal fungi, so Torun & Toprak (2020) concluded that the combination of AMF and K-humate enhanced the antioxidant defense system by increasing antioxidant enzymes and antioxidant capacity. Basak et al. (2019) also reported that under salinity stress applying mycorrhiza showed a positive relationship to stem height, stem and root wet weight, and root amino acids.

The synergistic interaction between mycorrhizae and Bradyrhizobium with soybean has been reported as a crucial factor in helping plants protect themselves from various stresses. Gholinezhad et al., (2020) studied the impact of mycorrhizal species on the seed oil and yield of sesame cultivars and reported their positive influence on seed yield and oil quality under drought conditions. Furthermore, Rahimzadeh & Pirzad (2019) discovered that the seeds of mycorrhizal plants yielded a higher oil percentage and synthesized more α-linolenic, linoleic, and oleic acids. Our research revealed that drought stress led to an increase in palmitic acid (a saturated fatty acid) and a decrease in linolenic acid (an unsaturated fatty acid). Thus, it can be inferred that water stress significantly reduces unsaturated fatty acid content while increasing saturated fatty acid content. According to Tohidi Moghadam et al. (2011), water stress diminishes the sink capacity by shortening the growth period, thereby curtailing the synthesis time for unsaturated fatty acids in seeds. However, mycorrhizal inoculation, particularly along with Bradyrhizobium, reduced palmitic and stearic acids (saturated fatty acids) and elevated oleic, linoleic, and linolenic acids (unsaturated fatty acids). Based on Ghasemi et al. (2023), mycorrhiza application enhanced seed oil quality by lowering erucic acid and increasing linoleic acid in sesame seeds. *R. irregularis* and *G. fasciculatum* outperformed the other two species in most evaluated traits. Indeed, various studies on different plants across various regions of Iran, particularly in arid zones, have consistently identified *F. mosseae* and *R. irregularis* as dominant species (Zardak et al., 2018; Bazgir et al., 2020). However, in our study, *F. mosseae* had a lesser impact on the measured traits than the other two species, possibly due to its lower adaptability to the regional environmental and soil conditions or the host plant species and variety. Augé (2001) argues that the extent of colonization by a fungal species is influenced by the plant species, fungus type, and even isolates of the same species collected from different locations. Therefore, the symbiotic compatibility of fungal species with the host plant depends on the plant type, species, physiological state, soil conditions (e.g., pH and EC), and altitude (Wang et al., 2017).

5. CONCLUSION

The data indicated that both yield and yield components declined as the intensity of drought stress increased. The application of *R. irregularis* and *G. fasciculatum* mycorrhizae, particularly to the plants inoculated with *B. japonicum*, showed positive effects. This combination helped mitigate oxidative damage from drought, as evidenced by lower MDA levels. It also increased soluble proteins, proline, and POD and SOD activities, contributing to better drought tolerance and improved seed yield, oil content, and quality. Consequently, our findings suggest that symbiotic relationships of *R. irregularis* and *G. fasciculatum* mycorrhizae, along with *B. japonicum* bacteria, can alleviate drought stress damage by enhancing certain physiological characteristics of the soybean plant, thereby bolstering its stress tolerance. For subsequent research, it is recommended to assess the impact of drought stress at various growth stages of soybean inoculated with diverse mycorrhizal species and growth-promoting bacteria on physiological and agronomic traits.

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