Fathalipour, B., B., Ahadnezhad R., A., Abbasi, A. (2022): Changes in antioxidant enzymes of Hordeum vulgare L. using the endophytic fungus Piriformospora indica and the bacterium Azospirillum spp. under drought stress. Agriculture and Forestry, 68 (1): 261-283. doi:10.17707/AgricultForest.68.1.17

DOI: 10.17707/AgricultForest.68.1.17

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CHANGES IN ANTIOXIDANT ENZYMES OF BARLEY (*Hordeum vulgare* L.) USING THE ENDOPHYTIC FUNGUS *PIRIFORMOSPORA INDICA* AND THE BACTERIUM *AZOSPIRILLUM* SPP. UNDER DROUGHT STRESS

SUMMARY

Piriformospora indica and *Azospirillum* spp. due to great ability to effectively improve plant growth and stress tolerance, have been considered significantly in recent decades. The present study was conducted as factorial based on a randomized complete block with three replications in the research field of Plant Production Engineering and Genetics, Faculty of Agriculture, Maragheh University. The experimental treatments included three levels of stress (full irrigation, irrigation at 70% of field capacity, and irrigation at 50% of field capacity), endophytic fungus *Piriformospora indica* (non-use and use) and bacterium *Azospirillum* (non-use and use). The results of the present study showed that the interaction between drought stress and using fungi and bacteria had a significant effect at the probability level of 1% on leaf area, chlorophyll a, the activity of glutathione reductase, ascorbate peroxidase, and catalase; and the content of malondialdehyde, hydrogen peroxide, superoxide dismutase, Fe-SOD isozymes, Mn-SOD, and proline at the probability level of 5% on Cu/Zn-SOD isozyme. The simple effects of the studied treatments were also significant on the parameters of chlorophyll b and carotenoids at the statistical level of 1%. The highest content of superoxide dismutase, Cu/Zn-SOD, Fe-SOD, Mn-SOD isozymes, ascorbate peroxidase, catalase peroxidase and glutathione reductase was obtained from inoculation with bacteria and fungi in irrigation treatment at 50% of field capacity resulting in a reduction by 47.01 and 33.93% in the content of hydrogen peroxide and malondialdehyde in irrigation treatment at 50% of field capacity and the combined use of fungi and bacteria compared to the non-use of fungi and bacteria in the same treatment. So, it can be concluded that the combined use of *Piriformospora indica* and *Azospirillum* spp. under conditions of severe drought stress can play an important role in modulating Barley growth.

Keywords: Ascorbate peroxidase, catalase, hydrogen peroxide, lipid peroxidation, yield

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Notes: The authors declare that they have no conflicts of interest. Authorship Form signed online.
Recieved:09/08/2021 *Accepted:25/02/2022*

INTRODUCTION

Barley (*Hordeum vulgare* L.) is one of the main sources of animal nutrition. The plant is the fourth largest crop in the world after wheat, rice and maize (Biel and Jacyno, 2013). According to the World Food Organization (FAO), in 2019 the global cultivation area of Barley is 47 million hectares and the global mean production of this plant is 31,000 kg per hectare.

For the importance of water, it should be mentioned that out of 70% of the water that covers the surface of the earth, only 2.5% is sweet and can be involved in meeting the needs of plants and organisms (Gleick and Palaniappan, 2010). Drought stress is one of the most important environmental stresses that affects more than 45% of agricultural lands, about 38% of the world's population (Ashraf and Foolad, 2007). By 2030, nearly 2.8 billion people in 48 countries will face drought stress, according to the Food and Agriculture Organization of the United Nations (Fao, 2009). During drought stress, plants show different physiological and biochemical changes, the most important of which is the reduction in the production of pure photoassimilates due to the closure of the stomata (Ashraf and haris, 2004). Under such conditions, the reduction in carbon dioxide gas stabilization causes a severe reduction in the photosynthetic electron transfer chain, electron leakage to the oxygen molecule and the formation of reactive oxygen species (Anjum et al., 2003). Different types of reactive oxygen species include superoxide radical ($O2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$), hydroperoxyl radical ($HO2^{\cdot}$), hydrogen peroxide (H2O2), radical alcohol ($RO^{\cdot}$), radicalproxy ($ROO^{\cdot}$), single oxygen (1O2) and carbonyl excited (RO*) (Dismukes et al., 2001; Karuppanapandian et al., 2006a, 2006 b, 2006 c, 2008, 2011; Karuppan- apandian and Manoharan, 2008; Vellosillo *et al*., 2010). Under drought stress, increasing the production of reactive oxygen species causes oxidative degradation of lipids (Suzuki and Mittler, 2006), proteins (Waszczak *et al*., 2018) and nucleic acids (Karuppanapandian *et al*., 2011), which with the continuation of stress leads to planned death in the cell (PCD, Vranova *et al*., 2002; Manoharan *et al*., 2005; Karuppanapandian *et al*., 2011). The mechanisms for coping with drought stress in plants include a combination of stress prevention, tolerance, recovery and avoidance strategies (Malinowski and Belesky, 2000).

However, in crops, the main role is played by reducing the destructive effects of drought stress due to the presence of antioxidants and antioxidant enzymes (Cossania *et al*., 2012). Enzymes are essentially catalysts for physiological reactions in plants and are the most sensitive agents of physiological changes in plants under environmental stress (Foyer and Shigeoka, 2011). The antioxidant system formed in higher plants against these threats consists of several special enzymes, including glutathione peroxidase, catalase, superoxide dismutase, and ascorbate peroxidase (Lisar *et al*., 2012). This defense system is able to collect reactive oxygen radicals and minimize their adverse effects (Tan *et al*., 2006) that finally leads to plant tolerance to environmental stresses, including drought stress (Sharma *et al*., 2012).

Using biological methods based on potential of beneficial soil microorganisms for establishing symbiosis relationships with plants has been mentioned as an effective solution to increase the tolerance of crops to environmental stresses (Bacilio *et al*., 2004). Endomycorrhizal fungus *Piriformospora indica* belongs to the group of mycorrhizal fungi, the order Sebacinales, Hymenomycetes and the division basidiomycota (Kumar *et al*., 2011). Many researchers have reported a correlation between *P. indica* and the roots of different plant species and confirmed their positive effect on the yield of these plants (Dolatabadi *et al*., 2011; Oelmuller *et al*., 2009; Varma *et al*., 2012). Hosseini et al., (2017) reported that drought stress affected fresh weight of roots and stems, root volume, leaf area, relative water content, leaf water potential, and chlorophyll content severely. The presence of endophytic fungi can reduce the adverse effects of environmental stresses. In this regard, Yaghoubian et al., (2014) showed that drought stress caused increasing hydrogen peroxide content and lipid peroxidation in wheat, but inoculation of these plants with *G. mosseae* and *P. indica* increased the activity of antioxidant enzymes catalase, ascorbate peroxidase and peroxidase and finally reduced reactive oxygen species and plant growth.

Plant growth-promoting bacteria are beneficial bacteria that promote the rooting of crops and increase their growth and yield. One of the most important bacteria is *Azospirillum* spp., which in addition to stabilizing atmospheric nitrogen (Döbereiner and Day, 1976), also can mineralize nutrients from the soil (Bashan et al. 2004; Fibach-Paldi et al., 2012). In addition, several researchers report that *Azospirillum* spp.can play a positive role in reducing the negative impacts of abiotic stresses (Casanovas *et al*., 2003; Creus *et al*., 2004; Barassi *et al*., 2006; Pereyra *et al*., 2006; Creus *et al*., 2010). The most important effects of *Azospirillum* spp. under drought stress can be altered levels of some plant hormones such as abscisic acid, indoleacetic acid (Dobbelaere *et al*., 2003; Spaepen *et al*., 2007), direct absorption of water by hyphae and transfer to the host, increased leaf gas exchange, increased activity of antioxidant enzymes, assimilation of nitrate and phosphorus, increased hydraulic conductivity of leaf water, osmotic regulation and increased flexibility of cell membranes (Bashan and de-Bashan, 2010).

Drought stress in fields of Barley affects the quantitative and qualitative yield of this crop. It is obvious that reducing the effects of drought stress can play an effective role in improving the current situation. In the present study, it was attempted to investigate the effect of using the endophytic fungus *Piriformospora indica* and the bacterium *Azospirillum* spp. in order to increase the moderation of Barley and changes in the defense systems of this plant under different moisture conditions.

MATERIAL AND METHODS

The present study was conducted in the research field of the Faculty of Agriculture of Maragheh University with geographical coordiantes of latitude 37.37 ´ N, longitude 46/27 ´E and altitude 1552 m above sea level. The climate of Maragheh is temperate, cold and relatively humid. Also, the annual rainfall in this city is about 330 mm and its icy days are about 114 days per year. The present study was conducted as factorial based on a randomized complete block design with three replications. The studied factors are three levels of stress (complete irrigation, irrigation at 70% of field capacity, and irrigation at 50% of field capacity), endophytic fungus *Piriformospora indica* (non-use and use) and *Azospirillum* bacteria (non-use and use). Before performing the test, a sample of field soil was prepared from a depth of 0 to 30 cm and after mixing, a composite soil sample was transferred to the laboratory to determine some physical and chemical properties, the results of which are shown in the Table 1.

Table 1. Results of physical and chemical decomposition of soil used before planting

Properties measured in the tested soil	Amounts	Properties measured in the tested soil	Amounts
Soil texture	Lumi - Sandy	Total nitrogen (percentage)	0.06
The acidity of saturated mud	7.51	Absorbable phosphorus (mg / kg - Olson method)	3.81
Electrical conductivity (dS / m)	0.46	Absorbable potassium (mg / kg - ammonium acetate)	346
Equivalent calcium carbonate (percentage)	9.61	Absorbable zinc (mg / kg-DTPA)	0.38
Organic carbon (percentage)	0.32	Absorbable manganese (mg / kg-DTPA)	1.56
Saturation humidity (percentage)	46	Absorbable iron (mg / kg-DTPA)	3.26

In order to apply drought stress, all the treated treatments up to 4-6 leaf stage were fully irrigated. Then, irrigation times of the field were determined by measuring the soil moisture by weight method through soil sampling in the middle of each day from the depth of root development in different treatments and reaching the desired moisture (Martin *et al*., 1990). In order to apply drought stress, the amount of irrigation water for each plot was calculated taking into account the depth of root development (50 cm), plot area and soil moisture capacity in cubic meters and added to each plot in a certain amount (Rostamza *et al.*, 2011).

$$In = \frac{(Fci - \theta i) \times D \times A}{100}$$

In: Volume of water use, Fci: Soil moisture at field capacity, Θi: Soil moisture content during sampling, D: Appropriate depth of root penetration, A: Plot area used.

In order to extract superoxide dismutase (SOD), catalase (CAT) and guaiacol peroxidase (GPX), 0.5 g of each leaf sample was weighed with a 000 scale and homogenized using cold porcelain mortar and liquid nitrogen. The

prepared homogenate was centrifuged at 15000 rpm at 4 °C for 15 min after transfer to the microtube (Sairam *et al*., 1998).

Sairam *et al*., (2002) method was used to measure the activity of superoxide dismutase and its isozymes. According to this method, the reaction complex consisted of sodium carbonate, potassium phosphate buffer, distilled water, EDTA, methionine and extractive enzyme. By adding riboflavin, the reaction started and after stopping the reaction, the absorbance of the samples was read at the wavelength of 560 nm using a spectrophotometer (Sairam *et al*., 1998).

Catalase activity was measured according to Aebi (1984) method. The reaction complex consists of potassium phosphate buffer, hydrogen peroxide, double distilled water and enzymatic solution. The adsorption of the reaction complex was read at the wavelength of 290 nm and the enzyme activity was calculated using extinction coefficient of 36.16 $mmol^{-1}$ cm^{-1}. In order to measure the activity of ascorbate peroxidase, a reaction complex including phosphate buffer, double distilled water, hydrogen peroxide, EDTA, ascorbate and enzymatic solution extracted. The adsorption of the reaction complex was read at the wavelength of 290 nm using a spectrophotometer and the enzyme activity was calculated using extinction coefficient of 2.8 $mmol^{-1}$ cm^{-1} (Sairam *et al*., 1998).

In order to measure the activity of guaiacol peroxidase, the reaction complex consists of phosphate buffer, guaiacol, hydrogen peroxide, EDTA and the extracted enzyme solution. The increase in absorption was recorded using a spectrophotometer for 60 seconds at a wavelength of 470 nm and obtained using extinction coefficient of 26.6 $mmol^{-1}$ cm^{-1} (Yoshimura *et al*., 2000).

Malondialdehyde was measured by Stewart and Bewley (1980) method. Homogenized leaf samples were centrifuged at 15000 × g for 10 min. The resulting solution was mixed with trichloroacetic acid and thiobarbituric acid and the resulting complex was centrifuged again at 10000 × g for 10 min after transfer to a cold-water bath. The absorbance of the samples was recorded at wavelengths of 532 and 600 nm using a spectrophotometer. The content of malondialdehyde was obtained from the difference between the absorption waves and extinction coefficient 155 $mmol^{-1}$ cm^{-1}.

In order to estimate the content of hydrogen peroxide, the digested samples were centrifuged with trichloroacetic acid at 12000 × g for 15 min. The reaction complex containing supernatant, phosphate and potassium iodide was prepared and their adsorption was read at the wavelength of 390 nm using a spectrophotometer (Chen *et al*., 2000).

Before statistical analysis of data and analysis of variance, normal data distribution test and errors were performed. The mean comparison was performed by Duncan's multiple range at a probability level of 1%. GenStat 12, Excel and SPSS17 software were used for data analysis and diagram drawing.

RESULTS AND DISCUSSION

According to the analysis of variance, the interaction between drought stress and the use of fungi and bacteria had a significant effect on the probability level of 1% on leaf area, chlorophyll a, the activity of glutathione reductase, ascorbate peroxidase, and catalase, and the content of malondialdehyde, hydrogen peroxide, superoxide dismutase, Fe-SOD isozymes, Mn-SOD, and proline at the statistical level of 5% on Cu/Zn-SOD isozyme (Table 2). The simple effects of each treatment were significant on chlorophyll b and carotenoids at the probability level of 1% (Table 2).

Table 2. Analysis of variance of parameters measured using the endophyte fungus *Piriformospora indica* and *Azospirillum* spp. in different humidity conditions

Sources of changes	Degrees of freedom	average of squares						
		Lef area	Chlorophyll a	Chlorophyll b	Cartonoid	Superoxide dismutase	Cu/Zn-SOD	Mn-SOD
Repetition	2	2.389	2.389	98.53	15.5	3.3190	0.07381	0.3701
Drought stress	2	824.541**	824.541**	7591.69**	15792.1**	329.1600**	7.95363**	11.7005**
inoculated	3	137.116**	137.116**	5171.70**	18954.5**	53.1575**	1.06360**	2.6460**
Drought stress * Inoculation	6	34.768**	34.768**	60.06^{ns}	288.4^{ns}	12.1503**	20.237*	1.2533**
Error	22	1.633	1.633	29.07	201.1	0.4257	0.07446	0.1547
Coefficient of variation		3	3	5.9	8.3	5.7	18.3	12.3

Sources of changes	Degrees of freedom	average of squares						
		Fe-SOD	Catalase	Ascorbate Peroxidase	glutathione peroxidase	hydrogen peroxide	Malondialdehyde	Proline
Repetition	2	2.7191	0.12564	0.00259	0.03301	0.25617	25.514	7.926
Drought stress	2	2.45184**	2.58139**	2.45184**	8.90215**	11.90521**	802.273**	235.648**
inoculated	3	22.4641**	0.46357**	2.30755**	1.82285**	3.60833**	168.101**	110.025**
Drought stress * Inoculation	6	5.1014**	0.15018**	0.62351**	0.50145**	0.69514**	35.404**	31.494**
Error	22	0.2010	0.01460	0.03444	0.03181	0.05752	2.689	2.919
Coefficient of variation		6.6	20.5	14.3	9.9	9.8	5.2	5.7

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

The results of analysis of variance of the present study showed that the endophytic fungus *Piriformospora indica* and the bacterium *Azospirillum* spp. under drought stress had a significant effect on Hordeum vulgare L. leaf area (Table 2). According to the mean comparison (Table 3), the highest and lowest leaf area were obtained from irrigation treatment at 90% of field capacity and combined use of fungi and bacteria and irrigation treatment at 50% of field capacity and no inoculation, respectively. Using fungi and bacteria at 50, 75 and 90% of field capacity showed 12.72, 14.49, 40.75% increase compared to non-use of fungi and bacteria, respectively (Figure 1). Kim et al., (2017) also reported that leaf area index is the most morphologically sensitive trait to drought stress

that has a high correlation with biological and economic yield. Drought stress has the greatest effect on the optical part of photosynthesis. Liu and Stützel (2004) found that drought stress significantly reduced dry weight and leaf area in four genotypes of Amaranthus spp. In contrast, the colonization of maize roots by *P. indica* under drought stress increased maize leaf area (Xu et al., 2017).

Table 3. Mean comparisons of traits of antioxidant enzymes and malondialdehyde under the influence of endophytic fungus *Piriformospora indica* and *Azospirillum* spp. under drought stress conditions

Drought stress	Treatment	Superoxide dismutase	Cu/Zn-SOD	Mn-SOD	Fe-SOD	Glutathione peroxidase	Proline
Irrigation at 90% of field capacity	Lack of insemination	5.74h	0.623f	2.170c	2.943e	0.937g	24.94d
	Inoculation with bacteria	5.78h	0.623f	2.233c	2.927e	0.847g	24.69d
	Inoculation with mushrooms	6.00h	0.700ef	2.337c	2.960e	0.850g	25.86d
	Inoculation with fungi and bacteria	6.39h	0.723ef	2.473c	3.193e	0.883g	24.47d
Irrigation at 75% of field capacity	Lack of insemination	9.80g	1.113def	2.767bc	5.897d	1.123fg	36.33ab
	Inoculation with bacteria	9.91fg	1.230cdef	2.987bc	5.690d	1.687ef	31.88bc
	Inoculation with mushrooms	12.33de	1.500bcde	3.047bc	7.873c	2.103de	29.88cd
	Inoculation with fungi and bacteria	15.99bc	2.223ab	3.333bc	10.433b	2.787bc	25.19d
Irrigation at 50% of field capacity	Lack of insemination	11.97ef	1.860bcd	2.678bc	7.420c	1.853de	40.93a
	Inoculation with bacteria	14.29cd	2.077bc	3.757b	8.457c	2.260cd	36.20ab
	Inoculation with mushrooms	17.93b	2.247ab	5.103a	10.583b	2.857ab	29.68cd
	Inoculation with fungi and bacteria	21.45a	2.997a	5.483a	12.967a	3.380a	27.94cd

According to the results of the present study, drought stress caused a reduction in leaf area and the combined use of fungi and bacteria improved the leaf area compared to the non-use of fungi and bacteria under stress and even desired moisture conditions. According to the analysis of variance, using fungi and bacteria under different moisture conditions had a significant effect at the probability level of 1% on the content of chlorophyll a (Table 2). As shown in Figure 2, the highest content of chlorophyll a was recorded in irrigation treatment at 90% of field capacity and combined use of fungi and bacteria, respectively. Inoculation with fungi and bacteria at 50, 75 and 90% of field capacity showed an

increase in chlorophyll a of Hordeum vulgare L. by 12.72, 14.49 and 40.75%, respectively, compared to the control. In this regard, Ommen et al., (1999) in spring wheat, Manivannan et al., (2007) in sunflower, and li et al., (2006) in barley reported a reduction in chlorophyll a content due to drought stress. Yaghoubian et al., (2014) showed that inoculation with *G. mosseae* and *P. indica* increased chlorophyll a content of wheat under drought stress. Zarea et al., (2012) also reported that using *Piriformospora indica* and *Azospirillum* increased chlorophyll a content in wheat under salinity stress. The reason for this in subsequent reports was the positive relationship between phosphorus concentration and chlorophyll content in plants. On the one hand, the reason for the reduction in chlorophyll content under drought stress can be the degradation of chloroplast thylakoid membranes and chlorophyll optical oxidation due to increased activity of reactive oxygen species and high chlorophylase activity (Ashraf, 2009). Drought and salinity stresses increase the concentration of growth regulators such as abscisic acid and ethylene, which stimulate chlorophylase and cause the decomposition of chlorophyll (Orabi et al., 2010). The combined use of fungi and bacteria in Hordeum vulgare L. under drought stress improved water uptake of the plant compared to the non-use of fungi and bacteria under moisture stress, which in turn reduced the activity of reactive oxygen species and chlorophylase and prevent degradation of chloroplast thylakoid membranes and chlorophyll optical oxidation, which can also result in improved plant growth.

According to the analysis of variance, the simple effects of using fungi and bacteria and application of drought stress were significant at the statistical level of 1% on the content of chlorophyll b (Table 2). According to the mean comparison, the highest value of this parameter was recorded in irrigation treatment at 90% of field capacity (113.42 mmol / g fresh weight) and combined use of fungi and bacteria (122.11 mmol / g fresh weight) (Table 4). Many researchers have reported an increase in chlorophyll b content using fungi and bacteria in various plants (Yaghoubian et al., 2014; Hoseini et al., 2017). several researchers have reported a reduction in chlorophyll b content under drought stress in different plants (Ommen et al., 1999; Manivannan et al., 2007; Gregersen and holm, 2007; li et al., 2006). Drought stress causes oxidative stress, which in turn causes the production of active oxygen in chloroplasts, which are very harmful radicals and have adverse effects on the photosystem (Cruz de Carvalho, 2008). And by increasing amount and time the degradation process of chlorophyll pigments increases. Chlorophyll content in crops is one of the important factors in maintaining photosynthetic capacity. Agami et al., (2017) reported that chlorophyll content in wheat inoculated with *Azospirillum* spp. increased under stress. Barassi et al., (2000) reported that *Azospirillum* spp. is a growth-promoting bacterium under drought and salinity stress. Many reports have proven the ability of this bacterium to resolve drought stress (Casanovas et al., 2003). Symbiosis with mycorrhizal fungi can increase the biosynthesis of chlorophyll in crops by improving the absorption of magnesium and phosphorus (kadian et al., 2013). In this regard, researchers have found an increase in

chlorophyll a and b in plants inoculated with *P. indica* due to improved plant water status and absorption of minerals such as magnesium (Giri and Mukerji, 2004). Harman et al., (2021) stated that endophytic fungus *P. indica* played an effective role in maintaining and stabilizing photosynthesis by having a positive effect on proteins involved in the process of photosynthesis and Calvin cycle and increasing their expression. The degradation of and reduction in chlorophyll under drought stress can be compatible because by reducing chlorophyll, the electron excited during photosynthesis reduces and consequently the damage caused by the formation of oxygen free radicals is reduced (Kranner et al., 2002). Limited CO2 stabilization due to stress leads to a reduction in carbon by Calvin cycle and $NADP^+$ oxide as electron acceptor in photosynthesis. By reducing Ferredoxin (Fd) during electron transfer, the electron maybe transferred from PSI to O2 to form $O2^{\cdot-}$ by a process called the Mahler reaction. The environmental stress disturbs the balance between light absorption and energy use and increases reactive oxygen.

Table 4. Comparison of the mean simple effects of drought stress and lack of inoculation and inoculation with fungi and bacteria on chlorophyll b and carotenoids

Treatment	Chlorophyll b	carotenoid
Lack of insemination	65.67d	123.00d
Inoculation with bacteria	81.00c	149.60c
Inoculation with mushrooms	94.78b	182.80b
Inoculation with fungi and bacteria	122.11a	229.40a
Drought stress	Chlorophyll b	carotenoid
Irrigation at 90% of field capacity	113.42a	208.20a
Irrigation at 75% of field capacity	95.50b	169.60b
Irrigation at 50% of field capacity	63.75c	135.80c

According to the analysis of variance, the simple effects of using fungi and bacteria and application of drought stress were significant at the statistical level of 1% on the content of carotenoids (Table 2). According to the mean comparison, the highest value of this parameter was recorded in irrigation treatment at 90% of field capacity (208.2 mmol / g fresh weight) and the treatment of the combined use of fungi and bacteria (229.4 mmol / g fresh weight) (Table 4). As a non-enzymatic antioxidant, carotenoids stop the oxidation process through neutralizing free radicals and reduce the effects of stress. They are part of tetraterpene compounds and are considered as protectors for chlorophylls by converting single oxygen to ternary oxygen and reducing the damage caused by the presence of reactive oxygen species (Zur et al., 2000). Carotenoids protect the plant against oxidative stress by removing all types of

active oxygen (Mahajan and Tuteja, 2005). Carotenoids accumulate harmful oxygen species and protect chlorophyll through interfering with the xanthophyll cycle. In this regard, Abid et al., (2018) stated that drought stress caused a reduction in carotenoid concentration in wheat. On the one hand, inoculation with *P. indica* and *Azospirillium* in several reports reduced the effects of drought stress and moderated carotenoid levels (Basak et al., 2011).

According to the analysis of variance, using fungi and bacteria under different moisture conditions had a significant effect on the activity of superoxide dismutase at the probability level of 1% (Table 2). As shown in Table 3, the highest superoxide dismutase activity was recorded in irrigation treatment at 90% of field capacity and the combined use of fungi and bacteria. Inoculation with fungi and bacteria in irrigation treatments at 50 and 75% of field capacity showed an increase by 79.19 and 63.19%, respectively, compared to the control (Table 3). Superoxide dismutase combines two superoxide radical molecules with hydrogen and finally produces two molecules of water and oxygen, hence, reducing the adverse effects of superoxide radicals on plant cells (Wang *et al*., 2018). In a study by Chakraborty *et al*., (2013) a rapid reduction in the activity of superoxide dismutase was recorded after 3 days of applying drought stress, while using bacteria, the activity of this enzyme reduced much less rapidly. The activity of superoxide dismutase increased in plants inoculated with different fungi under various environmental stresses including drought, salinity and heavy metals (Gururani *et al*., 2013). According to analysis of variance, using the endophytic fungus *Piriformospora indica* and the bacterium *Azospirillum* spp. under different moisture conditions had a significant effect on the activity of Fe-SOD and Mn-SOD isozymes at the probability level of 1% and Cu/Zn-SOD isozyme at the statistical level of 5% (Table 2). The highest and lowest Fe-SOD isozyme activity was obtained from irrigation treatment at 50% of field capacity and the combined use of fungi and bacteria (12.967 enzymatic units per mg of protein) and irrigation treatment at 90% of field capacity and using fungi, respectively (2.927 enzyme units per mg of protein) (Table 3). The highest and lowest Cu/Zn-SOD isozyme activity was obtained from irrigation treatment at 50% of field capacity and the combined use of fungi and bacteria (2.997 enzyme units per mg of protein) and irrigation treatment at 90% of field capacity and using fungi (0.623 enzyme units per mg of protein), respectively (Table 3). The highest and lowest Mn-SOD isozyme activity was obtained from irrigation treatment at 50% of field capacity and the combined use of fungi and bacteria (5.483 enzymatic units per mg of protein) and irrigation treatment at 90% of field capacity and non-use of fungi and bacteria (2.170 enzyme units per mg of protein), respectively (Table 3). Simultaneously with the expression of Cu/Zn-SOD and ascorbate peroxidase genes in chloroplasts of transgenic plants, tolerance to abiotic stresses was observed (Lee et al., 2007). Superoxide dismutase is one of the key enzymes in the immune system of plant cells, which plays an important role in the oxidation of cellular biological matter, which converts superoxide radical into hydrogen peroxide (Alscher *et al*., 2002). Reducing superoxide dismutase leads to

superoxide radical increase, which in turn causes damage to plant cells, followed by metabolic disorders and finally programmed cell death (Breusegem *et al*., 2001). According to some reports, the activity of superoxide dismutase increases at the beginning of drought stress (Baisak *et al*., 1994), but by increasing drought stress, the activity of this enzyme also reduces. The combined use of fungi and bacteria, in other words, in addition to increasing the activity, increases the period of activity of this enzyme under stress. In the present study, it was found that the combined use of fungi and bacteria simultaneously by increasing levels of drought stress increases the activity of this enzyme to respond to the occurrence of stress. However, a reduction in the activity of superoxide dismutase under stress has also been recorded by Yong *et al*., (2006).

According to the analysis of variance, using fungi and bacteria under drought stress had a significant effect on the activity of ascorbate peroxidase at the probability level of 1% (Table 2). According to the mean comparison, inoculation with fungi and bacteria at 50 and 75% of field capacity showed an increase by 219.73 and 76.92%, respectively, compared to non-inoculation in the activity of this enzyme (Figure 3). The highest and lowest activity of ascorbate peroxidase was obtained from irrigation treatment at 50% of field capacity and the combined use of fungi and bacteria (2.900 enzyme units per mg of protein) and irrigation treatment at 90% of field capacity and no inoculation (0.787 enzyme unit per mg of protein), respectively (Figure 3). Ascorbate peroxidase, by combining hydrogen peroxide and ascorbic acid and producing water and dihydroascorbate in chloroplasts and cytosols of crops, causes the accumulation of hydrogen peroxide, especially under environmental stress (Asada, 2000). Reduced ascorbate peroxidase activity in wheat was reported by Sofo *et al*., (2015) The researchers stated that reducing this enzyme increased hydrogen peroxide. When the amount of hydrogen peroxide increases, the activity of some enzymes stops in Calvin cycle, such as ribulose-5-phosphate kinase and bisphosphatases and isozymes of superoxide dismutase. Heidari *et al.,* (2009) reported an increase in ascorbate peroxidase activity in sorghum under moderate stress, which was introduced as the main factor of plant tolerance to stress and reduction in its adverse effects. Further studies have shown that plant type and level of stress have a significant effect on the process of enzyme changes. Xu *et al*., (2018) stated that *P. indica* can increase the synthesis of auxin by coexisting with plant roots. Sarker *et al*., (2018) stated that wheat inoculated with this fungus modulated the enzymatic activity of ascorbate peroxidase due to increased water absorption and reduced effects of drought stress. Caverzan *et al*., (2012) showed that the presence of this fungus increased the expression of ascorbate peroxidase gene under salinity and drought stress. Vurukonda *et al*., (2016) reported that growth-promoting bacteria (PGPR) accumulated hydrogen peroxide and increased plant tolerance through increasing mRNA expression of ascorbate peroxidase, superoxide dismutase and catalase in potato.

According to analysis of variance, the interaction between the endophytic fungus *Piriformospora indica* and the bacterium *Azospirillum* spp. under different

moisture conditions had a significant effect on the level of catalase activity at the probability level of 1% (Table 2). According to the mean comparison, the highest and lowest activity of this enzyme was obtained from irrigation treatments at 50% of field capacity and the combined use of fungi and bacteria (1.4080 enzyme units per mg of protein) and irrigation treatment at 90% of field capacity, and using fungi (0.0520 units of enzyme per mg of protein), respectively (Figure 4). The combined use of fungi and bacteria at 50 and 75% of field capacity compared to non-inoculation treatment caused an increase by 219.73 and 197.15% in this enzyme, respectively (Figure 4). Catalase reduces the effects of oxidative stress on crops by removing hydrogen peroxide produced in peroxisome by oxidases involved in β fatty acid oxidation, cell respiration, purine catabolism (Mittler, 2002; Vellosillo *et al*., 2010). With the activity of this enzyme, hydrogen peroxide is converted into water and oxygen and the adverse effect of this harmful matter is reduced. Yamazaki *et al*., (2003) considered the high level of hydrogen peroxide as a direct factor in preventing carbon dioxide stabilization because some of the enzymes of Calvin cycle are highly sensitive to hydrogen peroxide. Shao et al., (2005) Heidari *et al*., (2009) and Srivall *et al*., (2004) considered the increase in catalase activity as effective on stress tolerance. Yaghoubian *et al*., (2014) found that using *G. mosseae* and *P. indica* increased the activity of wheat catalase and ascorbate peroxidase under drought stress. In general, bacterial residues depend on their contribution to reducing abiotic stress and plant growth (Dimkpa et al., 2009).

According to the analysis of variance, the interaction between fungi and bacteria and drought stress had a significant effect on the activity of glutathione Peroxidase at the probability level of 1% (Table 2). According to the mean comparison, the highest and lowest activity of this enzyme was obtained from irrigation treatment at 50% of field capacity and the combined use of fungi and bacteria (3.380 enzyme units per mg of protein) and irrigation treatment at 90% of field capacity and using fungi (0.847 enzyme units per mg of protein), respectively (Table 3). The combined use of fungi and bacteria at 50 and 75% of field capacity showed an increase by 82.40, 148.17% compared to non-use of fungi and bacteria, respectively, and irrigation treatment at 90% of field capacity with the combined use of fungi and bacteria reduced glutathione Peroxidase activity by 5.76% (Table 3). Gupta et al., (2021) showed that the activity of glutathione Peroxidase in roots and stems of rice inoculated with P. indica increased under salinity stress. Glutathione Peroxidase catalyzes the combination of oxidized glutathione with adenine nicotine dinucleotide phosphate, leading to the formation of glutathione. Glutathione is involved in the regeneration of ascorbate from dehydroascorbate by dehydroascorbate reductase. Therefore, it can be concluded that inoculation with fungi and bacteria through increasing the activity of glutathione reductase under drought stress causes glutathione accumulation and reduction in ascorbic acid in ascorbic acid-glutathione cycle, and adverse effects of superoxide radical, hydroxyl radical and hydrogen peroxide on plant cells.

The results of analysis of variance of the present study showed that using the endophytic fungus *Piriformospora indica* and the bacterium *Azospirillum* spp. under drought stress had a significant effect on the amount of hydrogen peroxide of Barley at the probability level of 1% (Table 2). According to the mean comparison, the highest and lowest accumulations of hydrogen peroxide (4.56 and 1.09 mmol / g, respectively) was obtained from irrigation treatment at 50% of field capacity and no inoculation of fungi and bacteria and irrigation treatment at 90% of field capacity and the combined use of fungi and bacteria, respectively (Figure 5). The amount of this matter in the treatment of the combined use of fungi and bacteria in irrigation treatment at 50% of field capacity was recorded about 2.42 mmol / g fresh weight (Figure 5). In other words, only with the combined use of fungi and bacteria in irrigation treatment at 50% of field capacity, the amount of hydrogen peroxide accumulation reduced by 46.92% (Figure 5). Hydrogen peroxide (H_2O_2) is one of the most toxic forms of depleted oxygen reduction, which has detrimental effects on plant cell metabolism, including the oxidation of thiol groups. Also, the activity of some Calvin cycle enzymes such as ribulose 5-phosphate kinase and bisphosphatases is reduced or stopped with high amount of hydrogen peroxide. As a result, due to the reduction in $NADP^+$ / NADPH, H^+ ratio, the production of reactive oxygen species also increases. In addition, Mn-SOD and Cu/Zn-SOD isozymes are sensitive to high amount of this matter and lose their activity. Costa et al., (2005) stated that accumulation of hydrogen peroxide in addition to damaging membranes causes damage to macromolecules such as DNA and proteins. According to reports by Sairam et al., (2002) drought and salinity stress cause a significant increase in the amount of hydrogen peroxide inside the cell. Sairam et al., (2002) stated that the accumulation of hydrogen peroxide increased the lipid peroxidation and reduced the membrane stability index in wheat leaf cells. Under normal conditions, plant cells continuously produce some hydrogen peroxide, which is decomposed by enzymatic antioxidants such as catalase and ascorbate peroxidase. Under drought stress, the production of hydrogen peroxide increases, plant cells are synthesized and activity of these enzymes increases to some extent to minimize the negative impacts of the production of this matter. In the present study, the activity of two enzymes ascorbate peroxidase and catalase under drought stress was higher than the control. Different species and strains of Azospirillium bacteria directly affect the activity of these enzymes by modulating the intensity of stress. These bacteria inhibit the production of ethylene by producing aminocyclopropane and carboxylic acid deaminase, and finally reduce the level of antioxidant enzymes. Reduced hydrogen peroxide accumulation, growth modulation of different plants and the activity of catalase and ascorbate peroxidase have also been reported by Sofa et al., 2015 Studies by Baltruschat et al., (2008) showed that P. indica stimulates the accumulation of ascorbate in the root cells of host plants. Ascobic acid acts as a raw material in glutathione ascorbate cycle to detoxify hydrogen peroxide and regulate the production of other types of reactive oxygen species.

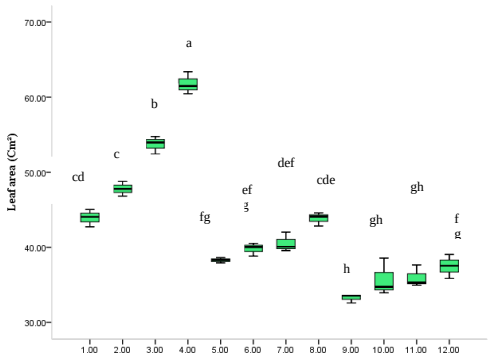

Figure 1. Changes in leaf area with inoculation of barley under drought stress

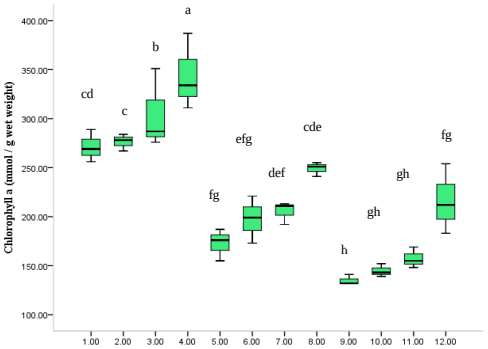

Figure 2. Changes in chlorophyll a by inoculation of barley under drought stress

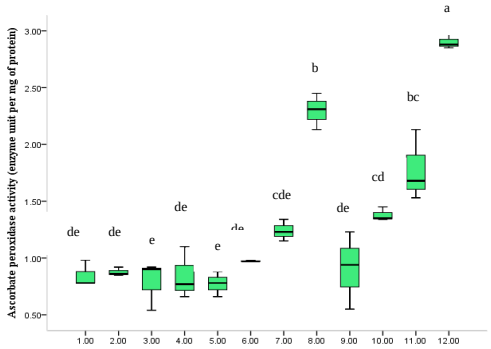

Figure 3. Changes in ascorbate peroxidase activity with barley inoculation under drought stress

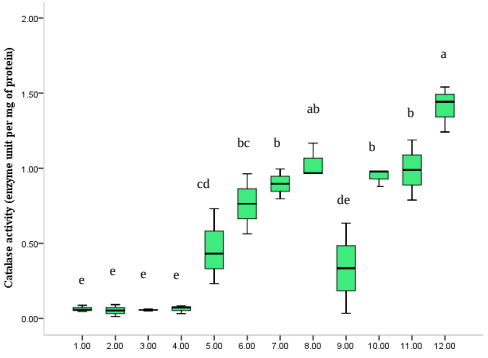

Figure 4. Changes in catalase activity with inoculation of barley under drought stress

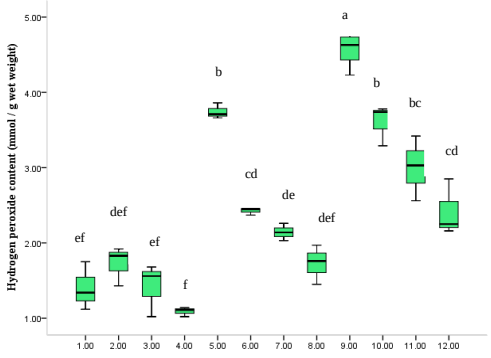

Figure 5. Changes in the amount of hydrogen peroxide with inoculation of barley under drought stress

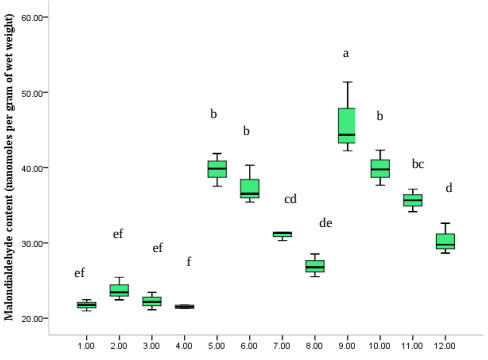

Figure 6. Changes in malondialdehyde content with barley inoculation under drought stress

The study results of the activity of superoxide dismutase, catalase and ascorbate can confirm this result.

The results of analysis of variance of the present study showed that the interaction between the endophytic fungus *Piriformospora indica* and the bacterium *Azospirillum* spp. under drought stress was significant on malondialdehyde accumulation at the probability level of 1% (Table 2). By increasing water shortage, the accumulation of malondialdehyde inside plant cells increased, so that the highest amount of this matter was observed in irrigation treatment at 50% of field capacity and no use of fungi and bacteria (45.98 nmol / mg fresh weight) (Figure 6). On the one hand, using fungi and bacteria in irrigation treatment at 50% of field capacity, the amount of malondialdehyde accumulation was significantly reduced. The amount of this matter in the combined use of fungi and bacteria in irrigation treatment at 50% of field capacity was recorded at about 30.35 nmol / g fresh weight. In other words, only with the simultaneous use of fungi and bacteria in irrigation conditions in 50% of field capacity, the amount of malondialdehyde accumulation reduced by 33.9% (Figure 6). It should be noted that the lowest accumulation of malondialdehyde was recorded in irrigation treatment at 90% of field capacity using bacteria (21.01 nmol/mg fresh weight) (Figure 6). Malondialdehyde is a biomarker used to study the effects of environmental stresses on lipid peroxidation and membrane damage (Gawel *et al*., 2004). Damage to membranes is one of the destructive effects of reactive oxygen species. It should be noted that the endophytic fungus *Piriformospora indica* and the bacterium *Azospirillum* spp. in various ways can increase the tolerance of plants to drought stress (Amina and Hanan, 2011) and by preventing oxidative stress and reducing the damage caused by free radicals, increase the tolerance of plants to drought stress. For non-use of fungi and bacteria, one of the reactions that accelerates with the production of reactive oxygen species is the peroxidation of membrane lipids, which produces aldehydes such as malondialdehyde and products such as ethylene (Srivall and Khanna, 2004). The reduction in this compound is due to the activity of antioxidant enzymes. By increasing the activity of these enzymes, the amount of active oxygen produced under drought stress is controlled and the severity of damage to vital biomolecules and metabolic disorders is reduced. According to the results of the present study on the accumulation of hydrogen peroxide, it was found that the highest accumulation of this matter as one of the types of active oxygen was obtained from irrigation treatment at 50% of field capacity and no inoculation of fungi and bacteria that can confirm high amount of malondialdehyde accumulation in this treatment.

The results of analysis of variance of the present study showed that using endophytic fungus *Piriformospora indica* and the bacterium *Azospirillum* spp. under drought stress had a significant effect on plant proline content (Table 2).

We obtained the highest and lowest proline content (40.92 and 24.47 mg / g fresh weight, respectively) from the treatment of irrigation at 90% of field capacity and non-inoculation of fungi and bacteria and treatment of irrigation at 50% of field capacity and the combined use of bacteria and fungi (Table 3). The accumulation of proline is directly related to plant drought tolerance. In this

regard, there are reports that proline modifies the negative impact of moisture stress on carbon stabilization and can moderate the reduction in rubisco activity under such conditions (Fendina *et al*., 1993). One of the evaluation indicators of plants under water shortage stress is the accumulation of proline in various plant organs (Zengin, 2006). The osmotic regulatory effects of proline on water balance and drought stress tolerance have been reported in various studies (Mohammadkhani and Heidary, 2008). Xu et al., 2017 found that colonization of roots with P. indica under drought stress increased maize leaf proline content. Camaille *et al*., 2021 showed that using bacteria, the content of proline in wheat increased significantly. It has also been reported that inoculation with Bacillus species increased proline content under drought stress in maize seedlings. This may be due to the high regulation of proline biosynthesis. Because proline helps maintain cellular water status and protect membranes and proteins from degradation. Ansary *et al*., (2012) also reported that drought stress increased leaf proline in maize, which is also found in plants inoculated with *P. fluorescens*. Although accumulation of proline during a short period after the end of stress helps the plant to regain its growth and therefore will have a positive effect on yield, but in various studies conducted under long-term drought stress, its effects on physiological and morphological properties were not significant and its accumulation will even have a negative impact on yield because it diverts plant photosynthetic resources to processes other than seed filling.

CONCLUSIONS

Today, using endophytic fungi has improved physiological and biochemical properties of various plants under drought stress. According to the results of the present study, inoculation with bacteria and fungi under drought stress improves leaf area, chlorophyll a, superoxide dismutase, Cu/Zn-SOD, Fe-SOD, Mn-SOD, ascorbate peroxidase isozymes, catalase peroxidase and glutathione reductase to the non-use of fungi and bacteria. So that we obtained the highest superoxide dismutase, Cu/Zn-SOD, Fe-SOD, Mn-SOD, ascorbate peroxidase, catalase, peroxidase and glutathione reductase isozymes from inoculation with bacteria and fungi with irrigation at 50% of the field capacity. Finally, it can be acknowledged that using fungi and bacteria through increasing the activity of the antioxidant system reduces the harmful effects of reactive oxygen species in Barley especially under stress. The result of these changes was a reduction in the content of hydrogen peroxide, malondialdehyde and proline compared to the treatment of non-use of fungi and bacteria. It should also be noted that inoculation with bacteria and fungi increased leaf area, chlorophyll b and carotenoids, the result of which can certainly be seen in modulating the growth of plants under stress and maintaining acceptable yield in these plants.

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