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PHYSIOLOGICAL AND BIOCHEMICAL RESPONSES OF BARLEY TO APPLICATION OF BIO-FERTILIZERS AND NANO IRON OXIDE UNDER SALINITY STRESS IN GREENHOUSE

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Abstract

To study the effects of bio-fertilizers and nano iron oxide on some physiological and biochemical traits of barley under salinity stress, a factorial experiment was conducted based on RCBD with three replications under greenhouse condition. Factors in the study, included four levels of salinity, four levels of nano iron oxide use and four preparations of bio-fertilizer application. Increasing salinity in the soil decreased chlorophyll content, quantum yield, relative water content and grain yield, whereas soluble sugars, proline content, electrical conductivity and the activities of catalase, peroxidase and polyphenol oxidase enzymes increased. Inoculation of plants with bio-fertilizers and nano iron oxide application improved these traits (except electrical conductivity) under salinity as well as normal conditions. Results showed that application of *Azospirillum* and mycorrhiza and 0.9 g/l nano iron oxide (B_3Fe_3) increased about 15.45% from grain yield in comparison with B_4Fe_4 under the highest salinity level. Based on the results, it seems that bio-fertilizers and nano iron oxide application can be useful in alleviating salinity stress in barley.

Introduction

Salinity is one of the important and adverse environmental constraints restricting growth and development of plant particularly in arid and semiarid regions. Soil salinity induces water stress, nutritional imbalance, hormonal imbalance and generation of reactive oxygen species (ROS) which may cause membrane destabilization (Omar *et al*. 2009). Moreover, it decreases the yield of many crops by inhibiting plant photosynthesis, photosystem II efficiency (Netondo *et al*. 2004), protein synthesis and lipid metabolism. Mittova *et al.* (2003) reported that the activities of the antioxidative enzymes such as CAT and SOD increased under salt stress in plants. One approach to solve the salt stress problem is the use of plant growth promoting rhizobacteria (PGPR) and mycorrhiza. The PGPR are a group of rhizosphere colonizing bacteria that produce substances to increase the growth of plants, synthesize different phytohormones, synthesize enzymes, including phosphatase, catalase that can modulate plant growth and development (Glick 2012). Wang *et al.* (2012) found that inoculation of PGPR strains improved plant enzyme activity, which alleviates the oxidative damage induced by drought and salinity. Seyed *et al.* (2016) reported that inoculation with PGPR enhanced proline content, relative water content, and photochemical efficiency of PSII and the activity of antioxidant enzymes of wheat under salinity stress. Large number of plant species are capable of forming symbiotic associations with arbuscular mycorrhizal fungi (Glassop *et al*. 2005). They also impart other benefits to them, including production/accumulation of secondary metabolites, osmotic adjustment under osmotic stress, enhanced photosynthesis rate and increased resistance against biotic and abiotic stresses (Willis *et al*. 2013). They are also interactive with different soil bacteria. AM hyphae are able to produce C products in a little amount as a source of energy for soil microbes in the mycorrhizosphere. Bacterial communities are able to promote germination of AM fungal spores and increase the rate

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and extent of root plant colonization by AM fungi. Also, soil microbes produce plant hormones, which can influence AM establishment as well as spore and hyphal growth (Miransari 2011). Qun *et al*. (2007) reported that AM-inoculated seedlings maintained higher activities of SOD, CAT and POD as compared to un-inoculated seedlings under salinity stress. Mycorrhizal fungi increase the sugar content of the host plant by hydrolysis of starch to sugars and preventing structural changes in soluble protein (Kapoor *et al*. 2013).

Iron is an important component of many vital enzymes such as CAT and SOD, and also participates in the synthesis of chlorophyll, electron transport and photosynthesis (Jeong and Connolly 2009). Babaei *et al.* (2017) reported that microelements application such as iron and zinc increased the proline content, chlorophyll content, soluble sugars, antioxidant enzyme activity and yield of wheat under salinity stress. The problem of soil salinization is a scource for agricultural productivity worldwide. Also iron deficiency is a common nutritional disorder in many crop plants, resulting in poor yields and reduced nutritional quality. Application of bio-fertilizers and iron is one of the most important strategies for alleviation of salinity stress effects. Therefore, the aim of this study was to evaluate the effects of bio-fertilizers and iron on some physiological and biochemical responses of barley under salinity stress conditions.

Materials and Methods

A factorial experiment based on RCBD with three replications was conducted under greenhouse condition in 2016**.** Experimental factors were salinity in four levels [no-salt (control or S_1), salinity 25 (S_2), 50 (S_3) and 75 (S_4) mM NaCl)], four bio-fertilizers levels [(*Azospirillum* (B_1), mycorrhiza (B_2), both application *Azospirillum* and mycorrhiza (B_3) and no bio-fertilizer (B_4)] and four nano iron oxide levels [application of 0.3 (Fe_1), 0.6 (Fe_2), 0.9 (Fe_3) g/l and without nano iron oxide as control (Fe_4)]. The studied area soil was silty loam, *Haplic Cambisol* according to World Reference Base (WRB 2014), with pH about 6.7, total organic C - 0.074%, Fe - 5.35 mg/kg soil. Air temperature ranged from 24 - 2°C during the day and 17 - 20°C during the night. Humidity ranged from 60-65% (Seyed Sharifi *et al*. 2016). The barley cultivar Valfajr' was used in the experiment. Optimal density of cultivar 'Valfajr' is 400 seeds/m^2, so 40 seeds were sown in each pot, filled approximately with 18 kg of above mentioned soil. Salt stress treatments were applied in two stages [immediately after planting and two weeks after planting (at 3 - 4 leaf stage)]. Foliar application with nano iron oxide was done in two stage (4 - 6 leaves stages and before of booting stages) of period growth. Mycorrhiza fungi (*Glomus intraradices)* was purchased from the Zist Fanavar Turan institute and soils were treated based on the manufacturer's protocol 10 g of inoculums per 1 kg soil, each pot containing approximately 865 spores. *Azospirillum lipoferum* strain of was isolated from the rhizospheres of wheat by Research Institute of Soil and Water, Tehran, Iran. The strains and cell densities of microorganisms used as PGPR in this experiment were 10^8 colony forming units (CFU). At the mid of booting stage, the flag leaves of plants were separated for measuring the following determinations (Zayed *et al*. 2014).

At the mid of the booting stage, the flag leaves of plants were separated. Samples were placed in aluminum foil and transported from the field on ice bath**.** Catalase, peroxidase and polyphenol oxidase activity was assayed according to Karo and Mishra (1976).

Method of Bates *et al*. (1973) was used to measure the proline. Soluble sugars were determined based on phenol sulphuric acid method (Dubois *et al*. 1956).

A portable chlorophyll meter (SPAD-502; Konica Minolta Sensing, Inc., Japan) was used to measure the leaf greenness of the barley plants. For each plant, measurements were taken at three locations on each leaf, and four plants per treatment were evaluated. The quantum yield was measured by the uppermost fool expanded leaf using a fluorometer (chlorophyll fluorometer;

Optic Science-OS-30 USA). For this purpose, the plants adapted to darkness for 20 minutes by using one special clamp then the fluorescence amounts were measured in 1000 (µM photon m^2s), and calculation was done using following formula (Kheirizadeh Arough *et al*. 2016): ØPSII = (Fm–F0)/Fm

Quantum yield amount of photosystem II, Fm or maximum fluorescence after a saturated light pulse on plants adapted to darkness and F_0, the minimal fluorescence in the light adapted were determined by illumination with far-red light.

Relative water content was estimated according to the method of Tambussi *et al*. (2005). Electrical conductivity was calculated according the method of Jodeh *et al*. (2015).

In order to measure grain yield, 10 plants of each pot randomly were harvested. Analysis of variance and mean comparisons were performed using $SAS_{9.1}$ computer software packages. The main effects and interactions were tested using LSD test at the 0.05 probability level.

Results and Discussion

Results showed that the activity of CAT, POD and PPO enzymes increased with the increase of salinity stress, application of bio fertilizers and nano iron oxide in comparison with control (Table 1). In the present results, increase in activities of CAT and POD as a result of salt stress is in concurrence with the findings of Kheirizadeh Arough *et al*. (2016) for triticale. The highest activity of CAT (193.35 OD µg protein min^{-1}), POD (80.49 OD µg protein min^{-1}) and PPO (65.83 OD µg protein/min) were observed in $S_4B_3Fe_3$ (Table 2). There were an increase about 4.71, 39.76 and 47.83% in activity of CAT, POD and PPO enzymes, respectively in the highest salinity level, application of bio fertilizers and nano iron oxide ($S_4B_3Fe_3$) in comparison with B_4Fe_4 in the same salinity level (Table 2). When plants are subjected to various abiotic stresses, some ROS such as superoxide (O^{2-}), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-) and singlet oxygen are produced (Bor *et al*. 2003). To be able to control the level of ROS and to protect cells under stress conditions, plant tissues have several enzymes scavenging ROS such as peroxidases (POD), polyphenol oxidase (PPO) and catalase (CAT) (Seyed Sharifi *et al*. 2016).

Enhancement in antioxidant enzyme activity under stress conditions helps in quick scavenging of ROS and maintaining their levels below the deleterious levels (Hashem *et al*. 2015). Our results showed that application of *Azospirillum* and mycorrhiza increased about 3.37% in CAT, 14.9% in POD and 19.24% in PPO activity, in comparison with no-bio fertilizer (Table 1). The impact of nano iron oxide on activity of CAT and PPO and POD were similar to bio-fertilizers. So, there was an increase about 0.23, 18.05 and 8.64% in activity of CAT, POD and PPO respectively by foliar spraying 0.9 g/l nano iron oxide in comparison to control (Table 1).

Hashem *et al.* (2015) reported that the activities of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) increased by salt stress and were further enhanced by AMF inoculation. Iron is an essential mineral for plants that is required for biological redox systems, and it is also a vital component of many enzymes that play important roles in the physiological and biochemical processes of plants. It acts as a cofactor of key enzymes involved in plant hormone synthesis (Jeong and Connolly 2009).

Proline content significantly increased under salinity stress. Inoculation with *Azospirillum*, mychorrhiza and both these bio-fertilizers under salinity stress increased proline content. In addition, the proline content significantly increased when nano iron oxide was applied. Interaction effect among salinity, bio-fertilizers and nano iron oxide showed that the highest content of proline (5.64 µg/g FW) was obtained in $S_4B_3Fe_3$ (Table 3). The minimum of proline (1.56 µg/g FW) was observed in $S_1B_4Fe_4$ (Table 3). Results showed that at the highest salinity level, application of bio-fertilizers as B_3 and nano iron oxide as Fe_3 increase about 37.22% in content of

Table 1. Variance analysis and means comparison of bio-fertilizers and nano iron oxide on some physiological and biochemical traits under salinity stress.

	CAT (OD µg protein/ min)	POD (OD µg protein/ min)	PPO (OD µg protein/ min)	Proline (µg/g FW)	Soluble sugars (mg/g FW)	Quantum yield	Electrical conductivity (µS/cm)	Chlorophyll content	Relative water content (%)	Grain yield (g/ plant)
Salinity										
S_1 = no-salt (control)	82.68 d	12.97 d	9.87 d	1.86 d	23.99 d	0.833 a	378.43 d	51.71 a	79.87 a	1.928 a
S_2 = 25 mM	107.56 c	21.43 c	17.82 c	2.89 c	35.67 c	0.767 b	402.66 c	47.78 b	68.78 b	1.827 b
S_3 = 50 mM	146.63 b	40.32 b	27.53 b	3.59 b	60.80 b	0.689 c	420.02 b	44.98 c	61.26 c	1.505 c
S_4 = 75 mM	188.22 a	65.98 a	50.65 a	4.81 a	97.07 a	0.615 d	442.17 a	41.28 d	55.36 d	1.424 d
Bio-fertilizers										
B_1 = *azospirillum*	132.83 a	34.70 c	26.09 b	3.28 c	54.92 b	0.727 c	402.01 d	46.67 b	65.80 c	1.672 b
B_2 = mycorrhiza	130.98 b	36.04 b	26.14 b	3.30 b	53.66 b	0.728 b	411.62 b	46.33 c	70.06 a	1.668 c
B_3 = *Azosp.*+ mycorrhiza	132.80 a	37.40 a	29.18 a	3.50 a	61.35 a	0.751 a	409.49 c	48.23 a	66.35 b	1.74 a
B_4 = no bio-fertilizer	128.47 c	32.55 d	24.47 c	3.07 d	47.61 c	0.698 d	420.16 a	44.52 d	63.06 d	1.604 d
Nano iron oxide (g/l)										
Fe_1 = 0.3	131.71 a	34.06 c	26.25 b	3.24 c	54.19 b	0.725 c	412.40 b	46.45 c	65.89 c	1.663 c
Fe_2 = 0.6	131.33 a	35.62 b	26.76 b	3.34 b	55.13 b	0.735 b	408.56 c	46.76 b	67.26 b	1.687 b
Fe_3 = 0.9	131.18 a	38.45 a	27.53 a	3.46 a	58.53 a	0.737 a	405.03 d	47.46 a	67.99 a	1.702 a
Fe_4 = 0 as control	130.87 a	32.57 d	25.34 c	3.11 d	49.69 c	0.707 d	417.28 a	45.10 d	64.12 d	1.633 d
S * B	**	**	**	**	**	**	**	**	**	**
S * Fe	**	**	**	**	*	**	**	**	**	**
B * Fe	**	**	ns	**	ns	**	**	ns	**	**
S * B * Fe	**	**	**	**	ns	**	**	**	**	**
C.V.	2.15	6.40	7.03	5.58	11.20	0.33	7.16	11.33	7.33	7.27

The same letters in each column show non-significant difference at $p \leq 0.05$ by LSD test. (ns) and (*, **) show no significant and significant differences at 0.05, 0.01 probability level, respectively. C.V: Coefficient of variation; CAT: Catalase; POD: Peroxidase; PPO: Polyphenol oxidase.

Table 2. Comparison of means for the experimental factors including salinity, bio-fertilizer and nano iron oxide on enzymes activity of barley.

Salinity	Bio-fertilizer	CAT (OD µg protein/min)				POD (OD µg protein/min)				PPO (OD µg protein/min)			
		Iron levels (g/l)				Iron levels (g/l)				Iron levels (g/l)			
		Fe_1	Fe_2	Fe_3	Fe_4	Fe_1	Fe_2	Fe_3	Fe_4	Fe_1	Fe_2	Fe_3	Fe_4
S_1	B_1	83.84±0.54	83.14±1.12	80.03±0.55	80.82±1.66	12.11±0.48	14.11±0.56	13.13±0.65	12.10±0.71	9.86±0.11	10.59±0.61	11.15±0.85	8.54±0.19
	B_2	86.24±0.85	85.66±2.84	84.42±.69	82.64±3.14	12.89±0.66	12.22±0.37	13.29±0.42	12.15±0.35	9.74±1.22	10.84±0.82	10.06±0.84	9.57±1.32
	B_3	83.37±0.83	81.48±1.41	81.04±0.32	85.31±0.78	14.27±0.94	14.05±1.39	15.46±0.46	13.14±0.13	10.71±0.47	10.49±0.81	11.73±0.05	10.76±0.61
	B_4	80.69±1.54	84.42±1.55	80.87±0.71	77.97±0.77	11.86±0.06	12.20±0.06	12.88±0.39	11.44±0.21	8.56±0.18	8.65±0.44	8.62±0.22	8.10±0.10
S_2	B_1	110.20±0.60	114.55±1.42	109.72±4.96	108.03±0.44	20.82±0.12	21.42±0.76	23.98±0.90	21.56±0.48	18.50±0.35	19.02±0.50	19.34±1.08	16.92±0.65
	B_2	108.66±5.12	106.80±2.10	106.77±1.12	102.33±4.01	21.96±0.44	21.08±2.36	23.32±0.72	19.03±0.57	17.48±0.02	16.76±0.05	18.18±0.15	16.42±0.69
	B_3	112.27±0.56	102.00±0.22	100.68±3.38	105.67±3.48	22.73±1.34	22.17±0.77	22.57±0.31	22.24±0.51	19.58±1.09	19.16±1.00	19.19±0.49	19.06±0.72
	B_4	104.73±5.82	111.71±1.74	108.94±2.15	107.85±5.10	19.17±1.44	19.50±1.16	22.88±1.05	18.42±0.69	17.27±0.68	16.57±0.02	16.19±0.40	15.53±0.14
S_3	B_1	146.85±4.80	145.69±0.91	155.69±5.16	152.53±2.42	36.55±0.90	36.71±1.06	43.76±2.86	38.44±2.41	28.58±0.08	26.89±0.06	28.03±1.47	27.45±1.25
	B_2	145.97±4.80	145.96±1.73	137.26±0.51	146.16±3.16	42.64±2.68	41.61±0.12	49.33±6.01	37.57±1.63	27.92±0.84	28.20±1.20	28.27±0.38	27.88±0.72
	B_3	137.85±2.90	129.88±0.30	139.06±3.39	143.41±0.90	36.73±0.42	40.74±3.67	52.04±5.61	37.75±2.83	27.35±0.51	26.24±0.15	26.76±1.04	27.58±1.17
	B_4	156.19±6.84	158.28±2.79	147.60±3.61	157.68±4.01	36.44±1.00	40.70±0.95	36.77±0.93	37.29±0.88	28.28±0.84	28.14±1.02	27.00±0.81	25.99±1.20
S_4	B_1	188.78±1.88	190.77±2.06	187.25±3.70	187.46±4.37	69.56±1.92	68.35±2.15	67.83±3.63	54.55±2.04	48.48±0.62	52.16±0.32	46.90±1.05	45.03±0.33
	B_2	189.62±3.59	190.16±2.16	189.50±2.78	187.52±0.33	60.75±0.51	71.70±9.91	76.43±2.05	60.65±1.58	46.12±1.47	50.88±6.73	54.10±0.22	45.77±0.65
	B_3	188.33±3.30	183.03±0.80	193.35±0.95	188.85±3.41	68.34±1.96	68.46±1.15	80.49±0.64	67.26±3.29	56.05±3.72	60.06±5.73	65.83±9.07	56.30±4.05
	B_4	183.77±6.77	187.78±2.38	190.73±1.04	184.65±3.26	58.11±2.47	64.85±7.62	60.80±0.79	57.59±1.96	45.53±0.32	43.53±0.26	49.14±2.96	44.53±0.75
$LSD_{0.05}$		4.57				3.63				3.008			

S_1, S_2, S_3 and S_4 indicate no-salt (control), salinity 25, 50 and 75 mM NaCl), respectively. B_1, B_2, B_3 and B_4 indicate *Azospirillum*, mycorrhiza, both application of *Azospirillum* and mycorrhiza, no bio-fertilizer, respectively. Fe_1, Fe_2, Fe_3 and Fe_4 indicate application of 0.3, 0.6, 0.9 g/l and without nano iron oxide as control, respectively.

proline in comparison to B_4 and Fe_4 in the same salinity level (Table 3). Increased accumulation of compatible organic solutes such as proline is one of the important traits determining the tolerance potential of plants. Studies showed that proline and soluble sugars accumulation in plants were performed by different methods including: stimulating of synthesis from pre-material, reducing of proline oxidasE activity, proteins destruction and reducing of proteins structure partnership (Yordanov Arough *et al*. 2003). Observation of increase in proline due to AMF is in agreement with the findings of Kheirizadeh *et al*. (2016) for *Triticale*. The highest content of soluble sugars (97.07, 61.35 and 58.53 mg/g FW) were observed in salinity of 75 mM NaCl, application of *Azospirillum* and mycorrhiza and 0.9 g/l nano iron oxide, respectively (Table 1). Interaction effect between salinity and nano iron oxide showed that the highest content of soluble sugars (105.39 mg/g FW) was obtained in severe salinity stress and 0.9 g/l nano iron oxide (Table 4). There were increases about 18.5% in content of soluble sugars content in the S_4Fe_3 in comparison to S_4Fe_4 (Table 4). Also interaction effect between salinity and bio-fertilizers indicate that the highest content of soluble sugars (110.59 mg/g FW) was observed in S_4B_3 (Table 4). The lowest of it (20.86 mg/g FW) was obtained in S_1B_4 (Table 4). On the other hand, at the highest salinity level, application of both *Azospirillum* and mycorrhiza increased 32.64% in soluble sugars content in comparison to control (Table 4). Increase of sugar under environmental stress was recognized as a result of starch degradation, sugar synthesis by non-photosynthesis pathways, non-converting of these components to other productions and decreasing of transporting from leaves (Premachandre *et al*. 1991). AMF fungi significantly increased photosynthetic of host plants and thereby caused an increase in sugar content (Feng *et al*. 2002). Babaei *et al.* (2017) reported that PGPR and nano iron oxide increased the proline and soluble sugars content of wheat under salinity stress.

Results showed that the quantum yield and chlorophyll content decreased under salinity stress. While application of bio-fertilizers and nano iron oxide increased these traits. The highest chlorophyll index (55.2) and quantum yield (0.870) were obtained in $S_1B_3Fe_3$, while the lowest (38.73 and 0.566, respectively) was obtained in $S_4B_4Fe_4$ (Table 5). Under the highest salinity level, application of bio-fertilizers as B_3 and foliar spraying as Fe_3 increased about 13.42% in chlorophyll index and 13.42% in quantum yield, in comparison to B_4 and Fe_4 in the same salinity level (Table 5). Reduction in chlorophyll index due to salinity stress is more or less similar to the findings of Seyed Sharifi *et al.* (2016) in wheat, and Kheirizadeh *et al*. (2016) in *Triticale*. The main reason for the decrease in chlorophyll might be due to degradation by reactive oxygen species (ROS) (Navari-Izzo *et al*. 1990). Increase in the synthesis of proline also led to a decrease in the chlorophyll content in salinity condition. Reduction of chlorophyll content finally resulted in the decrease in the efficiency of photosynthesis. A decrease in this ratio results from photosynthetic electron transport impairment (Pereira *et al*. 2000). Reduced chlorophyll content under stress is attributed to increased activity of chlorophyllase causing degradation of pigments (Hashem *et al*. 2015). Seyed Sharifi *et al.* (2007) reported that salt stress decreased chlorophyll content of wheat, but inoculation with bio-fertilizers increased the chlorophyll pigments.

Kaya *et al*. (2009) demonstrated that inoculation of AMF increased chlorophyll content under normal as well as salt-stressed conditions. Injury to PSII can lead to a change in chlorophyll fluorescence. Thus, chlorophyll fluorescence has been used as a powerful and reliable non-invasive method for assessing the changes in the function of PSII and for reflecting the primary photosynthetic processes under environmental stress conditions (Maxwell and Johnson 2000). Arbuscular mycorrhizal fungus plays a role in Fv/Fm increase by improving plants nutritional status and activating mediated genes (Sayar *et al*. 2008). Saito *et al.* (2014) demonstrate that Fe deficiency decreased the PSII content of barley.

Table 3. Comparison of means for the experimental factors including salinity stress, bio-fertilizers and nano iron oxide on proline content of barley.

Salinity	Bio-fertilizers	Proline (µg/g FW)			
		Iron levels (g/l)			
		Fe_1	Fe_2	Fe_3	Fe_4
S_1	B_1	1.88 ± 0.04	1.94 ± 0.02	1.93 ± 0.02	1.80 ± 0.02
	B_2	1.85 ± 0.01	1.93 ± 0.02	1.95 ± 0.02	1.79 ± 0.02
	B_3	2.00 ± 0.02	2.01 ± 0.03	2.02 ± 0.03	1.91 ± 0.02
	B_4	1.65 ± 0.02	1.69 ± 0.02	1.83 ± 0.02	1.56 ± 0.02
S_2	B_1	2.90 ± 0.04	2.92 ± 0.04	3.13 ± 0.04	2.83 ± 0.03
	B_2	2.72 ± 0.04	2.84 ± 0.04	3.06 ± 0.04	2.69 ± 0.04
	B_3	2.96 ± 0.03	3.12 ± 0.04	3.13 ± 0.04	2.87 ± 0.04
	B_4	2.81 ± 0.02	2.83 ± 0.05	2.84 ± 0.05	2.66 ± 0.03
S_3	B_1	3.58 ± 0.05	3.71 ± 0.06	3.72 ± 0.07	3.43 ± 0.04
	B_2	3.58 ± 0.05	3.62 ± 0.06	3.65 ± 0.05	3.57 ± 0.05
	B_3	3.69 ± 0.04	3.73 ± 0.06	3.72 ± 0.05	3.62 ± 0.06
	B_4	3.42 ± 0.07	3.47 ± 0.03	3.64 ± 0.06	3.28 ± 0.05
S_4	B_1	4.66 ± 0.07	4.65 ± 0.06	5.09 ± 0.08	4.27 ± 0.06
	B_2	4.80 ± 0.07	5.16 ± 0.06	5.34 ± 0.08	4.32 ± 0.06
	B_3	5.20 ± 0.08	5.39 ± 0.06	5.64 ± 0.08	4.99 ± 0.06
	B_4	4.19 ± 0.07	4.55 ± 0.08	4.64 ± 0.06	4.11 ± 0.06
$LSD_{0.05}$			0.03		

Table 4. Comparison of means for the experimental factors including salinity × nano iron oxide and salinity × bio-fertilizers on soluble sugars of barley.

Sali-nity	Soluble sugars (mg/g FW)							
	Iron levels (g/l)				Bio-fertilizers			
	Fe_1	Fe_2	Fe_3	Fe_4	B_1	B_2	B_3	B_4
S_1	24.03 ± 2.48	24.47 ± 2.46	25.12 ± 2.20	22.35 ± 2.09	24.17 ± 1.39	24.20 ± 1.79	26.75 ± 0.94	20.86 ± 1.00
S_2	35.70 ± 2.10	36.15 ± 2.97	37.59 ± 3.10	33.25 ± 3.31	36.01 ± 1.31	35.04 ± 2.52	38.74 ± 2.33	32.90 ± 3.41
S_3	59.90 ± 6.73	63.07 ± 7.67	66.03 ± 10.77	54.21 ± 7.36	59.32 ± 7.29	61.26 ± 7.99	69.31 ± 8.70	53.31 ± 4.76
S_4	97.13 ± 15.13	96.82 ± 14.51	105.39 ± 17.16	88.93 ± 12.23	100.18 ± 15.51	94.14 ± 9.32	110.59 ± 14.29	83.37 ± 8.69
$LSD_{0.05}$	6.94				5.79			

The RWC value decreased in barley plants when exposed to saline conditions, but EC content increased. The highest RWC (84.27%) was obtained at no salinity, application of mycorrhiza and 0.9 g/l nano iron oxide ($S_1B_2Fe_3$) (Table 6). Whereas, the lowest RWC (50.53%) was observed in salinity 75 mM and control treatment (B_4Fe_4) (Table 6). Application of bio- fertilizers as B_3 and foliar spraying as Fe_3 under salinity 75 mM increased about 14.09% in RWC, in comparison to B_4 and Fe_4 in the same salinity level (Table 6). The highest EC (469.14 µS /cm) was observed at the highest salinity level, no application of bio-fertilizer and nano iron oxide ($S_4B_4Fe_4$) (Table 6). There was a decrease of about 8.7% in content of electrical conductivity at the $S_4B_3Fe_3$ in comparison with $S_4B_4Fe_4$ (Table 6). Plant growth is dependent on water status of leaf, as salt and drought stress can create a water deficit inside plant tissues. The decrease in RWC could be related

Table 5. Comparison of means for the experimental factors including salinity stress, bio-fertilizers and nano iron oxide on quantum yield and chlorophyll content of barley.

Salinity	Bio-fertilizers	Chlorophyll index				Quantum yield			
		Iron levels (g/l)				Iron levels (g/l)			
		Fe_1	Fe_2	Fe_3	Fe_4	Fe_1	Fe_2	Fe_3	Fe_4
S_1	B_1	52.10 ± 1.10	53.16 ± 0.05	52.10 ± 0.40	50.46±1.15	0.831±0.010	0.842±0.013	0.833±0.013	0.816±0.011
	B_2	50.40 ± 1.20	51.36 ± 0.15	52.50 ± 0.10	49.90±0.90	0.826±0.012	0.825±0.014	0.853±0.011	0.820±0.012
	B_3	53.90 ± 0.45	54.66 ± 0.55	55.20 ± 0.30	52.20±1.00	0.841±0.013	0.866±0.012	0.870±0.013	0.833±0.011
	B_4	49.36 ± 0.65	50.50 ± 0.30	51.36 ± 0.95	48.20±0.30	0.822±0.012	0.815±0.010	0.834±0.012	0.806±0.007
S_2	B_1	48.50 ± 0.30	48.26 ± 0.75	49.06 ± 0.15	46.96±0.05	0.759±0.011	0.782±0.011	0.781±0.013	0.748±0.010
	B_2	48.60 ± 0.50	48.40 ± 0.40	47.00 ± 1.30	47.16 ± 0.15	0.764±0.009	0.777±0.007	0.771±0.013	0.744±0.011
	B_3	49.50 ± 0.10	49.10 ± 1.00	50.60±0.30	47.66 ± 0.15	0. 791 ± 0.01	0.801 ± 0.009	0.811 ± 0.010	0.772 ± 0.013
	B_4	46.00 ± 0.50	46.30 ± 0.30	46.10±0.50	45.36 ± 0.45	0.735 ± 0.010	0.758 ± 0.012	0.749 ± 0.015	0.733 ± 0.008
S_3	B_1	45.40 ± 0.20	45.26 ± 0.55	46.80±0.20	43.90 ± 0.80	0.665 ± 0.008	0.706 ± 0.006	0.718 ± 0.010	0.653 ± 0.012
	B_2	45.20 ± 0.10	44.06 ± 0.55	46.80±0.30	43.73 ± 0.45	0.715 ± 0.011	0.730 ± 0.007	0.716 ± 0.011	0.662 ± 0.011
	B_3	46.80 ± 0.50	47.50 ± 1.20	48.90±0.50	45.10 ± 0.80	0.724 ± 0.009	0.727 ± 0.011	0.729 ± 0.009	0.706 ± 0.011
	B_4	42.70 ± 0.50	43.23 ± 1.25	43.26±0.05	41.16 ± 0.15	0.646 ± 0.007	0.647 ± 0.006	0.660 ± 0.010	0.623 ± 0.008
S_4	B_1	40.93 ± 0.25	41.56 ± 0.55	42.30±0.90	40.06 ± 0.85	0.634 ± 0.006	0.636 ± 0.006	0.623 ± 0.010	0.607 ± 0.007
	B_2	41.26 ± 0.35	41.70 ± 0.90	43.60±0.40	39.70 ± 0.40	0.616 ± 0.010	0.611 ± 0.009	0.617 ± 0.008	0.605 ± 0.006
	B_3	42.40 ± 0.10	43.00 ± 0.20	43.93±0.25	41.26 ± 0.05	0.645 ± 0.010	0.646 ± 0.010	0.642 ± 0.007	0.619 ± 0.009
	B_4	40.20 ± 0.50	40.06 ± 0.15	39.83±0.85	38.73 ± 0.65	0.597 ± 0.006	0.592 ± 0.010	0.583 ± 0.005	0.566 ± 0.006
$LSD_{0.05}$			1.003				0.003		

S_1, S_2, S_3 and S_4 indicate without salt (control), salinity 25, 50 and 75 mM NaCl), respectively. B_1, B_2, B_3 and B_4 indicate *Azospirillum*, mycorrhiza, *Azospirillum* + mycorrhiza, no bio-fertilizer, respectively. Fe_1,Fe_2, Fe_3 and Fe_4 indicate application of 0.3, 0.6, 0.9 g/l and without nano iron oxide as control, respectively.

Table 6. Comparison of means for the experimental factors including salinity stress, bio-fertilizers and nano iron oxide on RWC and EC content of barley.

Salinity	Bio-fertilizers	RWC (%)				EC (µS/cm)			
		Iron levels (g/l)				Iron levels (g/l)			
		Fe_1	Fe_2	Fe_3	Fe_4	Fe_1	Fe_2	Fe_3	Fe_4
S_1	B_1	78.96±1.31	78.53±0.79	81.38±0.82	76.82±1.23	370.36±5.64	367.40±6.10	356.37±5.42	377.90±8.10
	B_2	81.47±0.82	83.69±0.64	84.27±0.42	81.08±0.82	383.86±5.34	374.70±4.50	379.22±5.77	387.09±6.91
	B_3	80.32±0.67	80.58±0.58	80.68±0.81	76.59±1.23	380.56±5.44	372.33±5.67	370.27±4.62	387.60±5.39
	B_4	77.47±0.53	79.20±0.80	79.75±0.25	77.13±0.63	386.22±5.37	384.15±5.85	383.36±5.84	393.51±5.48
S_2	B_1	68.83±0.31	69.30±0.70	72.74±0.59	64.67±0.65	396.76±6.04	396.02±5.98	392.81±6.49	401.88±6.12
	B_2	74.57±0.72	77.15±0.78	76.94±0.99	72.60±0.73	406.08±5.92	402.96±6.13	397.44±6.56	411.73±6.27
	B_3	66.74±0.76	70.46±0.36	67.77±0.68	64.27±0.50	400.92±5.08	397.73±6.56	396.95±6.04	406.08±5.92
	B_4	63.36±0.64	64.85±0.15	63.31±0.69	63.00±0.64	409.27±5.72	406.30±6.69	406.91±5.69	412.81±6.28
S_3	B_1	62.14±0.63	60.64±0.65	64.06±0.65	58.66±0.56	415.18±5.81	409.64±7.35	405.82±6.18	414.88±6.32
	B_2	64.73±0.65	65.27±0.51	65.38±0.66	59.53±0.68	423.56±5.43	418.62±6.37	411.74±5.25	423.15±6.44
	B_3	60.57±0.61	61.86±0.41	62.88±0.63	62.33±0.63	421.27±6.13	416.65±6.34	414.00±5.79	428.47±6.52
	B_4	57.57±0.49	58.25±0.35	59.40±0.60	56.92±0.62	429.92±7.68	426.01±6.48	426.02±5.98	435.37±6.63
S_4	B_1	53.22±0.53	53.67±0.42	57.29±0.58	51.94±0.56	433.44±7.55	430.34±6.55	425.03±5.96	438.32±6.67
	B_2	57.20±0.58	58.95±0.44	61.22±0.61	56.92±0.67	443.25±6.75	440.69±6.20	437.44±6.66	444.43±6.77
	B_3	55.40±0.56	60.59±0.59	57.65±0.58	52.95±0.53	442.95±6.74	440.38±7.21	431.44±5.55	444.23±6.76
	B_4	51.80±0.52	53.26±0.58	53.20±0.53	50.53±0.48	454.78±6.42	453.10±6.90	445.72±6.57	469.14±6.86
$LSD_{0.05}$		0.36				1.09			

to low water availability under stress conditions or to root systems, which are not able to compensate water loss by transpiration through a reduction of the absorbing surface (Gadallah 2000). The RWC of PGPR-treated plants were observed to be higher than that of control during salinity stress. The present findings are in confirmatory with other studies (Babaei *et al.* 2017). Previous studies have found that mycorrhizal plants often show higher leaf RWC compared to non-mycorrhizal plants. Guo *et al.* (2010) reported that mycorrhizal roots can explore more soil volume due to their extra matrical hyphae that facilitate them for absorption and translocation of more nutrients than by non-mycorrhizal plants. Moreover, better water status might result in the increased activity and hydraulic conductivity of the roots. Dröge (2002) has reported that salinity at high concentrations, is a major factor that enhances the oxidative damage of membrane components and cell structures, which in turn could explain a higher value of EC in the highest

Table 7. Comparison of means for the experimental factors including salinity stress, bio-fertilizers and nano iron oxide on grain yield of barley.

Salinity	Bio-fertilizers	Grain yield (g per plant)			
		Iron levels (g/l)			
		Fe_1	Fe_2	Fe_3	Fe_4
S_1	B_1	1.936±0.024	1.987±0.035	1.988±0.032	1.871±0.016
	B_2	1.872±0.03	1.937±0.022	1.931±0.031	1.855±0.018
	B_3	1.993±0.033	2.02±0.028	2.04±0.035	1.977±0.036
	B_4	1.853±0.025	1.848±0.028	1.915±0.025	1.831±0.028
S_2	B_1	1.842±0.032	1.823±0.028	1.844±0.029	1.786±0.026
	B_2	1.834±0.026	1.892±0.028	1.87±0.029	1.801±0.031
	B_3	1.868±0.031	1.904±0.031	1.914±0.03	1.833±0.035
	B_4	1.737±0.027	1.748±0.021	1.831±0.028	1.712±0.026
S_3	B_1	1.511±0.023	1.452±0.027	1.53±0.025	1.445±0.023
	B_2	1.486±0.022	1.522±0.027	1.532±0.026	1.484±0.021
	B_3	1.571±0.024	1.573±0.028	1.586±0.031	1.568±0.027
	B_4	1.44±0.018	1.425±0.022	1.459±0.025	1.408±0.022
S_4	B_1	1.382±0.02	1.438±0.024	1.47±0.024	1.365±0.025
	B_2	1.42±0.022	1.43±0.017	1.423±0.023	1.4±0.02
	B_3	1.505±0.025	1.494±0.026	1.524±0.026	1.477±0.026
	B_4	1.353±0.017	1.408±0.022	1.382±0.02	1.32±0.02
$LSD_{0.05}$		0.007			

salinity level. Bano and Fatima (2009) observed that the salt tolerance in *Zea mays* inoculated with rhizobium and *pseudomonas* was mediated by the decrease in electrolyte leakage, increase in proline production and maintenance of water content of leaves with selective uptake of K^+ ions. Zago and Oteiza (2001) stated that iron element by increasing the activity of antioxidant systems in plants decreased reactive oxygen species injuries and plays an important role in membrane stability.

The grain yield decreased in barley crop under salinity conditions. Results showed that inoculation with bio-fertilizers and foliar application of nano iron oxide under salinity stress significantly increased grain yield of barley. The highest grain yield (2.04 g per plant) was obtained in no-salinity, application of bio-fertilizer as B_3 and nano iron oxide as Fe_3 (Table 7). According to the results, the stimulatory effect of bio-fertilizer and nano iron oxide has been attributed to several mechanisms that increase plant yield, including enhanced RWC, proline, chlorophyll content, quantum yield and enhanced activity of PPO, POD and CAT in the leaves. Babaei *et al.* (2017) reported that salinity stress significantly decreased grain yield of wheat. One of the methods of increasing the plant growth and yield by PGPR is the ability to produce siderophore and increase the level of iron in the plant (Bhattacharyya and Jha 2012). Azcon and Barea (2010) have proposed co-inoculation PGPR and AM fungi as an efficient procedure to increase yield and plant growth.

The present research indicated that salinity stress reduced grain yield per plant, quantum yield and chlorophyll content of the plants. But antioxidant enzymes activity, soluble sugars and proline content increased. Also application of bio-fertilizer and nano iron oxide improved grain yield, RWC, quantum yield, chlorophyll content, antioxidant enzyme activity, proline and soluble sugars content under salinity condition. Application of bio-fertilizer and nano iron oxide can be recommended for profitable barley production under salinity condition.

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