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To cite this article: Khadijeh Babaei, Raouf Seyed Sharifi, Alireza Pirzad & Razieh Khalilzadeh (2017) Effects of bio fertilizer and nano Zn-Fe oxide on physiological traits, antioxidant enzymes activity and yield of wheat (*Triticum aestivum* L.) under salinity stress, Journal of Plant Interactions, 12:1, 381-389, DOI: [10.1080/17429145.2017.1371798](https://doi.org/10.1080/17429145.2017.1371798)

To link to this article: <https://doi.org/10.1080/17429145.2017.1371798>



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Published online: 18 Sep 2017.



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RESEARCH ARTICLE



Effects of bio fertilizer and nano Zn-Fe oxide on physiological traits, antioxidant enzymes activity and yield of wheat (*Triticum aestivum* L.) under salinity stress

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ABSTRACT

In order to evaluate the effects of nano Zn-Fe oxide and bio fertilizer on physiological traits, antioxidant activity and yield of wheat under salinity stress, a factorial experiment was conducted based on RCBD with three replications. Treatments were included salinity in three levels (no-salt, salinity 25 and 50 mM NaCl), four bio fertilizers levels (no bio fertilizer, seed inoculation by *Azotobacter*, *Azospirillum*, *Pseudomonas*) and nano oxide (without nano, application of nano Zn oxide, nano Fe oxide and nano Fe-Zn oxide 1.5 g/lit). Salinity stress decreased the chlorophyll-a, chlorophyll-b, total chlorophyll, photochemical efficiency of PSII and yield of wheat, whereas electrical conductivity, soluble sugars, proline content, and the activities of Catalase (CAT), Peroxidase (POD) and Polyphenol Oxidase (PPO) enzymes increased. Similar results were observed in CAT, POD and PPO activities due to inoculation by bio fertilizers and nano oxide. Maximum of soluble sugars and proline content were observed in the highest salinity level and application of *Pseudomonas*. Application of nano Zn-Fe oxide increased about 17.40% from grain yield in comparison with no application of nano oxide in the highest salinity level. Generally, it was conducted that bio fertilizer and nano oxide can be used as a proper tool for increasing wheat yield under salinity condition.

ARTICLE HISTORY

Received 6 February 2017
Accepted 24 July 2017

KEYWORDS

Enzyme activity; PGPR; proline; quantum yield

1. Introduction

Salinity is one of the most important abiotic stresses affecting yield and quality of agricultural plants worldwide. Salt stress limits plant growth by adversely affecting numerous physiological and biochemical processes, including photosynthesis, antioxidant capacity and ion homeostasis (Ashraf & Harris 2004). Relative water content (RWC), antioxidant enzymes activity, chlorophyll, proline content and stomata conductance have been used as indicators of plant stress (Ashraf & Parveen 2002; Maccaferri et al. 2011). Rodriguez et al. (2005) reported that chlorophyll content, leaf area and stomatal conductance declined under salinity stress. At the molecular level, one of the effects of salinity is impaired cellular function through the accelerated production of reactive oxygen species or ROS (Gao et al. 2008). ROS have reduced forms of atmospheric oxygen, which are produced in vital processes such as photosynthesis, photorespiration and respiration and can damage the cell membranes and other essential macromolecules such as photosynthetic pigments, proteins, DNA and lipids (Sairam et al. 2005).

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 superoxide dismutase (SOD), peroxidases (POX) and catalase (CAT) (Apel & Hirt 2004). The balance between ROS production and activities of antioxidative enzymes determines whether oxidative signaling or damage will occur (Moller et al. 2007). An increase in the activity of antioxidative enzymes under salinity could be indicative of an increased production of ROS and a buildup of a protective mechanism

to reduce oxidative damage triggered by stress experienced by plants (Meloni et al. 2003).

Several strategies have been developed in order to decrease the toxic effects caused by high salinity on plant growth. Among them, use of bio fertilizers such as plant growth promoting rhizobacteria (PGPR) plays a very important role in yield improvement. The PGPR synthesize different phytohormones, including auxins, cytokinins and gibberellins, which can enhance various stages of plant growth, and synthesize enzymes, including phosphatase, CAT, that can modulate plant growth and development (Glick 2012). Broetto et al. (2007) reported that salt stress decreased chlorophyll content of maize, but inoculation with bio fertilizers increased the chlorophyll pigments. Plants infected with IAA-overproducing PGPR strains showed high antioxidant enzyme activities that contribute to enhance plant protection against salt stress (Bianco & Defez 2009). Heidari and Golpayegani (2012) suggested that PGPR inoculation enhanced the proline, chlorophyll and RWC of basil (*Ocimum basilicum* L.) under stress conditions. Noorieh et al. (2013) have reported that PGPR species like *Azotobacter* and *Pseudomonas* increased the growth and biomass of canola (*Brassica napus* L.) by regulating the oxidative stress enzymes and essential nutrient under salinity stress. Proline is known to function as an osmoregulatory molecule that prevents cellular dehydration through osmotic adjustment. In addition, it may interact with crucial macromolecules of the cell to maintain their biological activity under stressful conditions. In a number of studies, increased proline biosynthesis was observed for various plant species inoculated with different PGPR under abiotic stress conditions (Hoque et al. 2007).

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Table 1. Soil physicochemical properties.

Characteristic	Saturation extract	pH	Fe	Zn	K	P (available P)	Total N	Organic carbon	Sand	Silt	Clay	CaCO ₃	Texture
			(mg/kg)					(%)					
Amount	47	6.9	5.5	0.97	275	8.4	0.12	0.62	35	42	23	15	Silt loam

Recent research has shown that a small amount of nutrients, particularly Zn and Fe applied by foliar spraying can affect the susceptibility of plants to stress (Sultana et al. 2001; Cakmak 2008). Zinc (Zn) and Iron (Fe) are known as important micronutrients and their deficiency is recognized as a critical problem in plants, especially grown on saline conditions with high pH values. It is well known that zinc and iron are important components of many vital enzymes such as glutamate dehydrogenase (GDH), CAT and SOD, and also participates in the synthesis of chlorophyll, indole-3-acetic acid (IAA) (Li et al. 2006; Jeong & Connolly 2009), and a structural stabilizer for proteins, membrane and DNA-binding proteins (Aravind and Prasad 2004). Zinc ions are also known to be strong inhibitors of enzymes generating oxygen radicals and protect salt-stressed plants from damaging attack of these compounds (Weisany et al. 2012).

In recent years, a considerable improvement in salinity tolerance has been achieved in some crop species by nanotechnology (Chen & Yada 2011). Nanoparticles (NPs) with small size and large surface area are expected to be the ideal material for use as a Zn/Fe fertilizer in plants. Currently, the use of nanomaterials provides an important route to release trace elements gradually and in a controlled manner and has found its position and functions in agriculture (Naderi et al. 2011). It was also shown that Zn concentration decreased with elevated soil salinity on wheat, rice and pepper plants (Gunes et al. 1996; Jamalomididi et al. 2006; Khoshgoftarmanesh et al. 2006). Cakmak (2008) speculated that Zn deficiency stress may inhibit the activities of a number of antioxidant enzymes.

Supplying the vegetative parts of crops with sufficient micronutrients during critical growth phases may be adequate to solve the immediate agronomic need, but to improve human nutrition, it is necessary to enrich the edible parts of the plants. Foliar applications of micronutrient sprays have been effective towards both agronomically beneficial and economical goals (Johnson et al., 2005). The obtained results on micronutrient foliar application were efficiency and economy, were reported by Johnson et al. (2005) and Sultana et al. (2001). A better understanding of wheat physiological responses under salinity may help in programs which the objective is to improve the grain yield under salinity levels. Therefore, the aim of this study was to evaluate the effects of bio fertilizers and micronutrient (Zn and Fe) on the physiological responses (i.e. antioxidant enzyme activity, chlorophyll, protein, soluble sugars and proline) of wheat under salinity stress conditions.

2. Material and methods

2.1. Materials used in experiment

A factorial experiment based on randomized complete block design with three replications was conducted under greenhouse condition in 2015. Experimental factors included salinity in three levels [no-salt (S₁) or control, salinity 25 (S₂) and 50 (S₃) equivalent of 2.3 and 4.6 dS m⁻¹ respectively], four bio fertilizers levels [(no bio fertilizer (F₁), seed

inoculation by *Azotobacter chroococum* strain 5 (F₂), *Azospirillum lipoferum* strain OF (F₃), and *Pseudomonas putida* strain 186 (F₄)] and nano particles [(without nano (N₁), application of 1.5 g L⁻¹ nano Zn oxide (N₂), 1.5 g L⁻¹ nano Fe oxide (N₃) and 1.5 g L⁻¹ nano Fe-Zn oxide (N₄)]. The studied area soil is an Entisol with a silty loam texture and pH about 6.9. Other physicochemical properties of soil are shown in Table 1. Air temperature ranged from 22°C to 27°C during the day and 18–21°C during the night. Humidity ranged from 60% to 65%. The wheat cultivar 'Attila 4' was used in the experiment. Optimal density of cultivar 'Attila 4' is 400 seeds m⁻², so 40 seeds were sown in each pot with 4 cm deep, filled approximately with 20 kg of above-mentioned soil. The pots were immediately irrigated after planting. Salt stress treatments were applied 18 days after planting (at 3–4 leaf stage). Foliar application of nano Fe-Zn oxide was conducted in two steps of vegetative growth (4–6 leaves stage and before of booting stage). *Azotobacter chroococum* strain 5, *Pseudomonas putida* strain 186 and *Azospirillum lipoferum* strain OF were isolated from the rhizospheres of wheat by Research Institute of Soil and Water, Tehran, Iran. For inoculation, seeds were coated with gum arabic as an adhesive and rolled into the suspension of bacteria until uniformly coated (Seyed Sharifi & Khavazi 2011). The strains and cell densities of microorganisms used as PGPR in this experiment were 10⁸ 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).

2.2. Catalase, peroxidase and polyphenol oxidase assay

At the mid of the booting stage, the flag leaves of plants were separated for measuring the CAT, POD and PPO activity. Samples were placed in aluminum foil and transported from the field on an ice bath.

To measure the enzyme activity, 0.2 g of fresh tissue of flag leaf was crushed by using liquid nitrogen and then one ml of buffer Tris-HCl (0.05 M, pH = 7.5) was added. Obtained mixture centrifuged for 20 min (13000 rpm and 4°C), then the supernatant was used for enzyme activity measurements. CAT, POD and PPO activity was assayed according to Karo and Mishra (1976). Also, the evaluation of protein carried out by Bradford (1976) method, 0.2 g of plant tissue was squashed with 0.6 ml extraction buffer and was centrifuged at 11500 rpm for 20 min at 4°C. The supernatant was transferred to the new tubes and centrifuged for 20 min at 4000 rpm. To measure the protein amount, 10 µl of obtained extract was added to 5 µl Bradford solution and 290 µl extraction buffer and the absorbance rate was read at 595 nm.

2.3. Proline and soluble sugars assay

Soluble sugars were determined based on phenol sulfuric acid method (Dubois et al. 1956). In this method, 0.5 g of

fresh weight of leaves was homogenized with ethanol. The extract was filtered and then treated with 5% phenol and 98% sulfuric acid. This mixture remained for 1 h and then its absorption at 485 nm was measured by the spectrophotometer. Soluble carbohydrate contents were shown as mg g^{-1} of fresh weight. Leaf proline content was measured according to Bates et al. (1973).

2.4. Chlorophyll content, maximum efficiency of PSII, RWC and electrolyte leakage

Photosynthetic pigment content: chlorophyll content measured in 0.2 g fresh leaf tissue, which gradually worn with 80% acetone and the solution volume was brought to 20 ml using acetone 80%. Then it was centrifuged for 10 min at 400 rpm and the absorbance at 645, 663 and 470 nm was recorded by a spectrophotometer. The chlorophyll a, chlorophyll b, total chlorophyll were obtained based on the following equations (Khalilzadeh et al. 2016):

$$\text{Chlorophyll } a (\text{Chl } a) = (19.3 \times A_{663} - 0.86 \times A_{645})V/100 W$$

$$\text{Chlorophyll } b (\text{Chl } b) = (19.3 \times A_{645} - 3.6 \times A_{663})V/100 W$$

$$\text{Total Chlorophyll} = \text{Chlorophyll } a + \text{Chlorophyll } b$$

The quantum yield was measured by the uppermost full expanded leaf using a fluorometer (chlorophyll fluorometer; Optic Science-OS-30 USA) (Moludi et al. 2014). Three measurements (non-dimensional) were made in each leaf. One leaf per plant and six plants per treatment were evaluated. RWC was estimated gravimetrically according to the method of Tambussi et al. (2005). Electrolyte leakage was calculated by following the standard method of Jodeh et al. (2015). EC values were measured at room temperature of $23 \pm 1^\circ\text{C}$ using an EC meter. In order to measure grain yield per plant, 10 plants of each pot randomly were harvested. Analysis of variance and means comparison were performed using SAS computer software packages. The main effects and interactions were tested using the least significant difference (LSD) test.

3. Results and discussion

Analysis of variance showed significant effects between salinity and bio fertilizer on proline, soluble sugars, CAT, POD, PPO, RWC and grain yield (Table 2). Interaction of salinity and nano oxide significantly affected CAT, PPO and grain yield (Table 2). Soluble sugars and POD also was affected by the interaction of nano oxide and bio fertilizer. Chlorophyll a, b, total chlorophyll content and F_v/F_m were affected by the interaction of nano oxide, bio fertilizer and salinity (Table 2).

3.1. The F_v/F_m ratio and chlorophyll content

The results showed that the chlorophyll content decreased under salinity stress. The highest of chlorophyll a, chlorophyll b and total chlorophyll content (7.13 , 2.2 and 9.33 mg g^{-1} FW, respectively) were obtained in no salinity stress, application bio fertilizers and nano oxide as F_4N_4

(Table 3). Also, the minimum of chlorophyll a, b and total chlorophyll content (2.26 , 0.95 and 3.21 mg g^{-1} , respectively) were obtained in application of bio fertilizer as F_1 and nano oxide as N_1 under severe salt stress (Table 3). Similar results were obtained for F_v/F_m ratio. Photosynthetic pigments and proline are both synthesized from the same substrate (Aspinall & Paleg 1981). Thus an increase in the synthesis of proline leads to a decrease in the chlorophyll content in salinity condition. Reduction of chlorophyll and other pigments 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). This indicates that in the plants that had salt stress, reaction centers are damaged (photochemically inactive), thus reducing electron transport capacity in PSII. In supporting our finding, Basra and Basra (1997) reported that reduction of chlorophyll and other pigments finally resulted in the decrease in the efficiency of photosynthesis.

Results showed that at the highest salinity level, application of bio fertilizers and nano oxide as F_4N_4 increased the chlorophyll a, chlorophyll b, and total chlorophyll (to about 53.24%, 26.01% and 45.43%, respectively) in comparison with F_1N_1 in the same salinity level (Table 3). The increased chlorophyll content in nano Zn and Fe and bio fertilizer-treated plants coincided with an increase in the maximum efficiency of PSII photochemistry by the greater F_v/F_m ratio. Low chlorophyll content under salinity stress was reported as a result of lower chlorophyll synthesis, destroy the PSII reaction center, inhibit carbonic anhydrase and nitrate reductase activities, an imbalance in the ion flux inside plants, affect membrane stability index and reduce RWC (Talaat & Shawky 2012). On the other hand, reduction of chlorophyll and other pigments finally resulted in the decrease in the efficiency of photosynthesis. Zarrouk et al. (2005) indicated a positive correlation of Zn concentrations with leaf chlorophyll content in plants. Rengel (1995) reported that application of Zn on wheat resulted in a decrease of the CA activity and in quantum yield. A Zn-enhancement CA activity is very beneficial for plants in order to facilitate the supply of CO_2 from the stomatal cavity to the site of CO_2 fixation (Sasaki et al. 1998). Jeong and Connolly (2009) reported that iron is essential for the proper functioning of multiple metabolic and enzymatic processes such as electron transport, chlorophyll biosynthesis and photosynthesis. Shaharoon et al. (2007) also reported that inoculation with PGPR significantly affected the pigments under salinity stress.

3.2. Proline and soluble sugars content

Proline and soluble sugars content contribute to osmotic adjustment during stress and protect the structure of macromolecules and membranes during extreme dehydration (Farhoudi et al. 2015). The beneficial effect of higher osmolyte concentration is reflected in the maintenance of higher RWC and stabilization of essential enzyme proteins such as CAT, POD and PPO resulting in higher activity under salinity stress (Sairam et al. 2005; Ashraf and Foolad 2007). Proline reduces cytoplasmic pH and maintains the proper ratio of $\text{NADP}^+/\text{NADPH}$ in metabolism and increase different enzymes activities (Szabados and Savoure 2010). The highest content of proline (7.26 mg g^{-1} FW) and soluble sugars (102.85 mg g^{-1} FW)

Table 2. Effects of bio fertilizers and nano oxide on activity of CAT, POD and PPO enzymes, chlorophyll index, F_v/F_m , proline and total soluble carbohydrate of wheat under salinity stress.

	Chlorophyll a (mg g ⁻¹ FW)	Chlorophyll b (mg g ⁻¹ FW)	Chlorophyll (mg g ⁻¹ FW)	F_v/F_m	Proline (mg g ⁻¹ FW)	Soluble sugars (mg g ⁻¹ FW)	CAT(OD µg protein min ⁻¹)	POD(OD µg protein min ⁻¹)	PPO(OD µg protein min ⁻¹)	Relative water content (%)	Electrical conductivity (µs.m ⁻¹)	Grain yield (g per plant)
Soil salinity (mM)												
S ₁ = tap water (control)	4.6a	1.76a	6.37a	0.72a	3.87c	65.86c	32.98c	100.18b	41.08c	68.79a	104.47c	2.49a
S ₂ = low salinity	3.8b	1.41b	5.22b	0.55b	4.61b	75.17b	41.47b	104.15a	47.04b	61.70b	122.31b	2.11b
S ₄ = high salinity	3.09c	1.18c	4.27c	0.44c	6.66a	92.93a	50.21a	104.15a	60.64a	59.78c	135.68a	1.94c
LSD (<i>p</i> < .05)	0.025	0.014		0.037	0.21	3.55	2.05	3.82	2.37	1.88	3.58	0.08
Bio fertilizers												
F ₁ = no inoculation as control	2.84d	1.22d	4.08d	0.49b	4.77b	74.25b	38.44b	95.34c	46.60b	60.56c	126.75a	2.08c
F ₂ = <i>Azotobacter</i>	3.42c	1.32c	4.7c	0.56a	4.66b	69.29b	37.21b	97.43c	44.20b	62.54bc	125.51a	2.17ab
F ₃ = <i>Azospirillum</i>	4.04b	1.55b	5.6b	0.62a	5.16ab	82.96a	45.01a	113.12a	53.16a	64.89ab	114.14b	2.21ab
F ₄ = <i>Pseudomonas</i>	5.03a	1.69a	6.73a	0.61a	5.59a	85.46a	45.55a	105.42b	54.38a	65.70a	116.87b	2.27a
LSD (<i>p</i> < .05)	0.029	0.017	0.035	0.066	0.58	5.99	3.69	4.11	4.30	2.69	4.13	0.14
Nano oxide												
N ₁ = without nano oxide	3.33d	1.29d	4.63d	0.52b	4.92c	72.61b	39.69b	98.55b	47.17b	60.6b	123.64a	2.05b
N ₂ = nano Zn oxide	3.65c	1.41c	5.07c	0.53b	5.03bc	77.58ab	40.53b	101.24b	47.85b	62.48b	122.39a	2.11b
N ₃ = nano Fe oxide	3.99b	1.52b	5.51b	0.57b	5.08ab	78.98ab	41.39ab	102.32b	49.64ab	63.56ab	120.53ab	2.17b
N ₄ = nano Zn + Fe oxide	4.35a	1.58a	5.93a	0.66a	5.16a	82.79a	44.61a	109.20a	53.68a	65.59a	116.71b	2.39a
LSD (<i>p</i> < .05)	0.029	0.017	0.035	0.065	0.12	6.52	4.00	4.11	4.61	2.78	4.13	0.13
S * F	**	*	**	**	**	**	**	*	**	**	ns	**
S * N	**	**	**	**	ns	ns	*	ns	**	ns	ns	**
F * N	**	**	*	**	ns	**	ns	**	ns	ns	ns	ns
S * F * N	**	**	**	**	ns	ns	ns	ns	ns	ns	ns	ns
C.V.	3.2	4.8	5.8	0.48	5.08	3.62	4.96	4.14	4.41	6.34	7.31	6.16

Notes: ns, * and ** show no significant and significant differences at 0.05, 0.01 probability level, respectively. CAT: catalase; POD: peroxidase; PPO: polyphenol oxidase.

Table 3. Comparison of means for the experimental factors including salinity stress, bio fertilizer and nano oxide on chlorophyll content and F_v/F_m of wheat under salinity stress.

Treatment		Chlorophyll a (mg g ⁻¹ FW)				Chlorophyll b (mg g ⁻¹ FW)			
Soil salinity	Bio fertilizers	N ₁	N ₂	N ₃	N ₄	N ₁	N ₂	N ₃	N ₄
S ₁	F ₁	2.93 ± 0.38	3.06 ± 0.37	3.23 ± 0.42	3.37 ± 0.41	1.23 ± 0.29	1.41 ± 0.33	1.48 ± 0.3	1.52 ± 0.3
	F ₂	3.62 ± 0.39	3.73 ± 0.37	3.83 ± 0.43	4.72 ± 0.41	1.56 ± 0.27	1.72 ± 0.29	1.81 ± 0.32	1.87 ± 0.25
	F ₃	4.1 ± 0.42	4.35 ± 0.4	5.64 ± 0.47	5.88 ± 0.42	1.78 ± 0.25	1.89 ± 0.3	1.99 ± 0.31	2.02 ± 0.3
	F ₄	5.02 ± 0.39	6.37 ± 0.39	6.75 ± 0.51	7.13 ± 0.4	1.79 ± 0.25	1.84 ± 0.25	2.1 ± 0.37	2.2 ± 0.29
S ₂	F ₁	2.68 ± 0.34	2.76 ± 0.35	2.77 ± 0.37	3.16 ± 0.41	1.11 ± 0.3	1.18 ± 0.29	1.29 ± 0.32	1.32 ± 0.33
	F ₂	3.3 ± 0.36	3.39 ± 0.35	3.73 ± 0.41	4.13 ± 0.47	1.06 ± 0.33	1.13 ± 0.35	1.16 ± 0.34	1.23 ± 0.34
	F ₃	3.38 ± 0.39	3.94 ± 0.77	4.34 ± 0.59	4.65 ± 0.65	1.43 ± 0.31	1.49 ± 0.28	1.59 ± 0.34	1.7 ± 0.29
	F ₄	4.08 ± 0.33	4.8 ± 0.38	4.84 ± 0.42	4.99 ± 0.36	1.57 ± 0.32	1.73 ± 0.29	1.78 ± 0.34	1.87 ± 0.32
S ₃	F ₁	2.26 ± 0.36	2.45 ± 0.37	2.58 ± 0.37	2.87 ± 0.37	0.95 ± 0.32	1 ± 0.31	1.1 ± 0.33	1.16 ± 0.34
	F ₂	2.47 ± 0.32	2.59 ± 0.41	2.67 ± 0.38	2.95 ± 0.57	0.86 ± 0.5	1.09 ± 0.35	1.19 ± 0.33	1.23 ± 0.29
	F ₃	2.46 ± 0.5	2.68 ± 0.4	3.25 ± 0.33	3.86 ± 0.48	0.98 ± 0.41	1.12 ± 0.4	1.31 ± 0.32	1.38 ± 0.32
	F ₄	3.69 ± 0.49	3.91 ± 0.37	4.28 ± 0.51	4.49 ± 0.57	1.21 ± 0.37	1.31 ± 0.43	1.43 ± 0.39	1.55 ± 0.36
LSD _{0.05}		0.24				0.14			
Treatment		Chlorophyll content (mg g ⁻¹ FW)				F_v/F_m			
Soil salinity	Bio fertilizers	N ₁	N ₂	N ₃	N ₄	N ₁	N ₂	N ₃	N ₄
S ₁	F ₁	4.16 ± 0.66	4.47 ± 0.7	4.71 ± 0.71	4.89 ± 0.71	0.58 ± 0.116	0.64 ± 0.128	0.66 ± 0.132	0.69 ± 0.130
	F ₂	5.18 ± 0.64	5.45 ± 0.67	5.64 ± 0.73	6.59 ± 0.65	0.71 ± 0.142	0.72 ± 0.144	0.72 ± 0.144	0.73 ± 0.146
	F ₃	5.88 ± 0.67	6.25 ± 0.68	7.63 ± 0.77	7.9 ± 0.67	0.74 ± 0.148	0.75 ± 0.150	0.76 ± 0.152	0.79 ± 0.158
	F ₄	6.81 ± 0.63	8.21 ± 0.61	8.84 ± 0.82	9.33 ± 0.68	0.75 ± 0.150	0.77 ± 0.154	0.78 ± 0.156	0.81 ± 0.162
S ₂	F ₁	3.78 ± 0.62	3.94 ± 0.6	4.06 ± 0.67	4.48 ± 0.72	0.38 ± 0.076	0.41 ± 0.082	0.48 ± 0.096	0.66 ± 0.132
	F ₂	4.36 ± 0.68	4.51 ± 0.69	4.9 ± 0.74	5.36 ± 0.8	0.46 ± 0.092	0.48 ± 0.096	0.55 ± 0.110	0.69 ± 0.138
	F ₃	4.81 ± 0.68	5.42 ± 1.02	5.93 ± 0.92	6.35 ± 0.93	0.57 ± 0.114	0.62 ± 0.124	0.67 ± 0.134	0.70 ± 0.140
	F ₄	5.65 ± 0.65	6.52 ± 0.67	6.62 ± 0.76	6.86 ± 0.68	0.45 ± 0.090	0.51 ± 0.102	0.59 ± 0.118	0.68 ± 0.136
S ₃	F ₁	3.21 ± 0.67	3.45 ± 0.66	3.68 ± 0.68	4.03 ± 0.67	0.32 ± 0.064	0.33 ± 0.066	0.36 ± 0.072	0.42 ± 0.084
	F ₂	3.33 ± 0.82	3.68 ± 0.73	3.85 ± 0.69	4.18 ± 0.82	0.37 ± 0.074	0.38 ± 0.076	0.47 ± 0.094	0.54 ± 0.108
	F ₃	3.44 ± 0.88	3.8 ± 0.74	4.56 ± 0.65	5.24 ± 0.75	0.56 ± 0.112	0.34 ± 0.068	0.35 ± 0.070	0.61 ± 0.122
	F ₄	4.9 ± 0.86	5.22 ± 0.79	5.71 ± 0.89	6.05 ± 0.92	0.40 ± 0.080	0.47 ± 0.094	0.49 ± 0.098	0.63 ± 0.012
LSD _{0.05}		0.29				0.048			

Notes: S₁, S₂ and S₃ indicate no salinity, 25 mM and 50 mM salinity, respectively. F₁, F₂, F₃ and F₄ indicate without inoculation, inoculation with *Azotobacter chroococcum* strain S, *Azospirillum lipoferum* strain OF, *Pseudomonas putida* strain 186, respectively. N₁, N₂, N₃ and N₄ are without nano as control, application of nano Zn oxide, nano Fe oxide, nano Zn + Fe oxide application, respectively.

was obtained in salinity of 50 mM and application of bio fertilizers as F₄ (Table 4). But the minimum of the mentioned osmolytes was observed in control treatment (S₀ and F₀) (Table 4). These results agree with Slama et al. (2007) who indicated that proline is regarded as a source of energy, carbon and nitrogen for recovering tissues under saline condition. There was an increase about 60.60% in soluble sugars content in the F₃N₄ application in comparison with F₁N₁ (Table 6). Alloway (2008) reported that Zinc is an essential micronutrient for carbohydrate and protein metabolisms, membrane integrity, auxin synthesis and reproduction. The higher accumulation of proline could be due to enhanced activities of ornithine aminotransferase (OAT), the enzymes involved in proline biosynthesis (Kohl et al., 1990), as well as due to inhibition of proline catabolizing enzymes, proline oxidase and proline dehydrogenase (PDH) (Kandpal et al. 1981). However, proline content increased with *Pseudomonas* application by 38.78% and 14.15% at 25 and 50 mM NaCl treatments, compared with salinity treatments without *Pseudomonas* application (Table 4), respectively. Qudsaia et al. (2013) reported that *Azospirillum lipoferum* increased maize growth, soluble sugars and proline during stress. One of the common hypotheses employed in most of the studies conducted under salinity stress was the lowering of ethylene level by the ACC-deaminase activities of PGPR (Nadeem et al. 2014). In general, very high accumulation of proline and total soluble carbohydrates might be due to increased synthesis and decreased degradation under stress conditions (Szabados & Savouré 2010). It seems that increase of proline and soluble sugars content under bio fertilizer application is probably due to the higher sensitivity of photosystem II, reduction in efficiency of photosynthesis, high level of O₂

evolution and high level of 3-phosphoglycerate (De Ridder and Salvucci 2007).

3.3. Activity of CAT, POD and PPO enzymes

Antioxidant enzymes play a key role in the defense system of the plant against oxidative stresses induced by salinity. Change in the activity of antioxidant enzymes is a defense mechanism of plants under oxidative stress induced by environmental stresses (Gao et al. 2008). Interaction effect between salinity and bio fertilizer showed that the highest activity of CAT (53.55 OD µg protein min⁻¹), POD (116.18 OD µg protein min⁻¹) and PPO (68.46 OD µg protein min⁻¹) were obtained at the highest salinity level and application of bio fertilizer as F₄ (Table 4) and the least activity of CAT (28.02 OD µg protein min⁻¹) POD (64.38 OD µg protein min⁻¹) and PPO (37.62 OD µg protein min⁻¹) were obtained in S₁F₁ (Table 4). Antioxidative enzymes like CAT, POD are the most important components in the scavenging system of ROS (Notor & Foyer 1998). Correlation between CAT activity and salt tolerance has been described by Apel and Hirt (2004). Mittova et al (2003) reported that the activities of the antioxidative enzymes such as CAT and SOD increase under salt stress in plants and a correlation of these enzyme levels and salt tolerance exist. It has been found that plants infected with PGPR strains showed high antioxidant enzymes activity which contributed to enhance plant protection against salt stress (Noorieh et al. 2013). These PGPR-induced antioxidative enzymes are believed to be contributing to the salt stress tolerance in plants also by eliminating hydrogen peroxide from salt-stressed roots (Noorieh et al. 2013).

The highest of CAT and PPO activity (52.62 and 66.69 OD µg protein min⁻¹, respectively) was observed in salinity

Table 4. Means comparison of salinity and bio fertilizers treatments on some physiological traits of wheat.

Treatment		Proline (mg g ⁻¹ FW)	Soluble sugars (mg g ⁻¹ FW)	CAT (OD µg protein min ⁻¹)	POD (OD µg protein min ⁻¹)	PPO (OD µg protein min ⁻¹)	Relative water content (%)	Grain yield (g per plant)
Soil salinity	Bio fertilizers							
S ₁	F ₁	3.84 ± 0.65	64.35 ± 11.09	28.02 ± 5.66	64.38 ± 11.26	37.62 ± 6.56	63.48 ± 10.85	2.34 ± 0.41
	F ₂	3.79 ± 0.65	57.95 ± 15.25	29.34 ± 5.82	76.30 ± 16.15	38.31 ± 6.62	67.04 ± 12.29	2.51 ± 0.47
	F ₃	3.83 ± 0.65	72.68 ± 13.07	39.32 ± 7.35	87.01 ± 14.03	45.68 ± 8.03	69.59 ± 12.85	2.44 ± 0.44
	F ₄	4.01 ± 0.68	68.46 ± 11.94	35.27 ± 6.70	93.02 ± 18.80	42.70 ± 7.79	75.03 ± 16.23	2.68 ± 0.55
S ₂	F ₁	4.10 ± 0.70	70.31 ± 12.07	37.90 ± 6.59	75.80 ± 14.52	44.26 ± 7.74	60.08 ± 10.50	2.02 ± 0.41
	F ₂	3.97 ± 0.68	66.08 ± 12.97	35.35 ± 6.87	91.00 ± 16.90	42.46 ± 7.79	61.43 ± 10.69	2.08 ± 0.43
	F ₃	4.69 ± 0.86	79.22 ± 14.30	44.82 ± 7.78	96.18 ± 20.54	49.45 ± 8.88	63.62 ± 10.91	2.24 ± 0.46
	F ₄	5.69 ± 0.99	85.09 ± 14.82	47.83 ± 8.31	99.62 ± 18.40	52.00 ± 9.35	61.68 ± 10.69	2.11 ± 0.43
S ₃	F ₁	6.36 ± 0.99	88.07 ± 15.08	49.42 ± 8.63	95.82 ± 16.52	57.91 ± 10.14	58.12 ± 9.89	1.87 ± 0.35
	F ₂	6.23 ± 1.06	83.84 ± 15.68	46.96 ± 8.66	98.00 ± 16.90	51.82 ± 10.21	59.16 ± 10.14	1.92 ± 0.34
	F ₃	6.77 ± 1.16	96.98 ± 17.20	50.90 ± 9.02	106.62 ± 19.40	64.36 ± 11.34	61.47 ± 13.73	1.95 ± 0.39
	F ₄	7.26 ± 1.24	102.85 ± 17.80	53.55 ± 9.17	116.18 ± 21.54	68.46 ± 12.71	60.38 ± 10.47	2.03 ± 0.38
LSD _{0.05}		0.20	4.52	2.34	5.01	2.87	3.18	0.15

Notes: S₁, S₂ and S₃ indicate no salinity, 25 mM and 50 mM salinity, respectively. F₁, F₂, F₃ and F₄ indicate without inoculation, inoculation with *Azotobacter chroococcum* strain 5, *Azospirillum lipoferum* strain OF, *Pseudomonas putida* strain 186, respectively.

of 50 mM, foliar application of nano oxide as N₄ (Table 5). The lowest of them (30.78 and 39.42 OD µg protein min⁻¹, respectively) was obtained at no salinity, application of nano oxide as N₁ (Table 5). On the other hand, there were an increase of 70.95% and 53.95% in the activity of CAT and PPO enzymes, respectively at the highest salinity level and application of nano oxide as N₄ (S₃N₄) in comparison with S₃N₁ (Table 5). There was an increase of 15.84% in the activity of POD enzyme in the F₄N₄ application in comparison with F₄N₁ (Table 6). Nano Zn-Fe oxide plays a significant role in activation of certain antioxidant enzymes that are of significant importance in alleviating salt stress oxidative damages on plant cells. The increase in the enzymes activities in relation to nano iron oxide might be due to triggering induction of CAT and POD genes expression by the iron application as suggested in wheat by Ghaffari and Razmjoo (2015).

Wang et al. (2012) found that inoculation of PGPR strains improves plant enzyme activity, which alleviates the oxidative damage induced by drought and salinity. Noorieh et al. (2013) have also reported PGPRs species like *Pseudomonas* sp. increased the growth and biomass of canola plants by regulating the oxidative stress enzymes and essential nutrient under water deficit. Kheirizadeh Arough et al. (2016) suggested that the positive effects of zinc application under salt stress are included protecting chlorophyll against free radicals, removing the reactive oxygen species, increasing of CAT and PPO activity.

3.4. RWC and EC

The RWC value was decreased in wheat plants exposed to saline conditions, which has been partly attributed to the impact of the salt on the electrical potential of the plasma membrane that affected not only the absorption of ions but also that of water, generating water stress (Munns, 2002). The highest RWC (75.03%) was obtained at no salinity condition and bio fertilizer application as F₄ (S₁F₄) (Table 4). Whereas, the lowest RWC (58.12%) was observed in salinity 50 mM in control treatment (S₄F₁) (Table 4). Under different salinity levels, increased ionic flux can damage the plant cellular membranes and affect water potential of the plant's cell (Hussain et al. 2008). Increased production of proline along with decreased electrolyte leakage, which may result in the higher RWC of leaves. Thus it can be assumed that increase in RWC has increased the chlorophyll content and F_v/F_m. High RWC is a resistant mechanism to stress and high RWC is the result of more osmotic regulation or less elasticity of tissue cell wall (Ritchie et al. 1990). It was also found that higher RWC in F₄ indicates a better plant water status. Higher RWC in bio fertilizer-treated plants may be beneficial for moving water through the plants to the evaporating surfaces and maintaining opened stomata in leaves (Nelsen & Safir 1982). It seems that the inhibitory and deleterious effects of salinity stress decreased by seed inoculation with plant regulation. Indeed, improving plant growth due to bio fertilizer application is contributed to produce hormones

Table 5. Means comparison of salinity and nano oxide treatments on CAT, PPO and yield per plant of wheat.

Treatment		CAT (OD µg protein min ⁻¹)	PPO (OD µg protein min ⁻¹)	Grain yield (g per plant)
Soil salinity	Nano oxide			
S ₁	N ₁	30.78 ± 6.92	39.42 ± 7.33	2.35 ± 0.49
	N ₂	32.09 ± 7.21	40.33 ± 7.51	2.49 ± 0.48
	N ₃	31.88 ± 8.12	40.90 ± 8.09	2.54 ± 0.47
	N ₄	37.20 ± 7.93	43.67 ± 8.53	2.59 ± 0.48
S ₂	N ₁	39.21 ± 9.07	44.70 ± 8.58	1.92 ± 0.35
	N ₂	40.89 ± 8.77	45.37 ± 8.56	2.01 ± 0.38
	N ₃	41.79 ± 8.94	47.33 ± 9.36	3.10 ± 0.41
	N ₄	44.01 ± 8.89	50.78 ± 9.61	2.43 ± 0.42
S ₃	N ₁	49.09 ± 9.26	57.39 ± 12.30	1.84 ± 0.32
	N ₂	48.62 ± 9.18	57.86 ± 12.35	1.89 ± 0.35
	N ₃	50.51 ± 9.07	60.69 ± 12.73	1.88 ± 0.33
	N ₄	52.62 ± 8.76	66.60 ± 12.13	2.16 ± 0.39
LSD _{0.05}		3.90	10.84	0.12

Notes: S₁, S₂ and S₃ indicate no salinity, 25 mM and 50 mM salinity, respectively. N₁, N₂, N₃ and N₄ are without nano as control, application of nano Zn oxide, nano Fe oxide, nano Zn + Fe oxide application, respectively.

Table 6. Comparison of means for the experimental factors including bio fertilizer and nano oxide on POD and soluble sugars of wheat.

Treatment Bio fertilizers	Soluble sugars (mg g ⁻¹ FW)				POD (OD µg protein min ⁻¹)			
	N ₁	N ₂	N ₃	N ₄	N ₁	N ₂	N ₃	N ₄
F ₁	56.19 ± 17.93	72.60 ± 16.75	74.87 ± 16.80	76.22 ± 17.07	93.42 ± 16.17	94.93 ± 16.74	94.38 ± 16.53	98.62 ± 18.22
F ₂	73.29 ± 16.61	72.44 ± 16.37	72.44 ± 16.68	76.08 ± 16.72	96.94 ± 16.92	96.87 ± 16.89	95.37 ± 16.32	100.57 ± 18.40
F ₃	79.39 ± 17.56	80.76 ± 17.71	81.43 ± 17.87	90.25 ± 19.32	105.71 ± 19.13	111.25 ± 21.41	111.57 ± 21.66	123.94 ± 26.98
F ₄	81.58 ± 20.32	84.51 ± 21.15	87.16 ± 21.49	88.60 ± 21.59	98.14 ± 17.19	101.90 ± 17.96	107.94 ± 19.62	113.69 ± 20.64
LSD _{0.05}	11.66				4.32			

F₁, F₂, F₃ and F₄ indicate without inoculation, inoculation with *Azotobacter chroococcum* strain 5, *Azospirillum lipoferum* strain OF and *Pseudomonas putida* strain 186, respectively.

N₁, N₂, N₃ and N₄ are without nano as control, application of nano Zn oxide, nano Fe oxide and nano Zn + Fe oxide application, respectively.

by these bacteria and improving root growth (Zahir et al. 2008).

The highest EC (135.68, 126.75 and 123.64 µSm⁻¹) were observed at the highest salinity level (S₄) and no application of bio fertilizer and nano oxide, respectively (Table 2). Dröge (2002) has reported 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 salinity level (Table 2). The higher stability of cellular membrane has been attributed to bio fertilizer application as a result of enhanced mineral uptake and increased antioxidant production (Evelin et al. 2012). Weisany et al. (2012) reported that the protective role of nano Zn was ascribed to its role in the maintenance of plasma membrane integrity and thus controlling the toxic ions uptake under salinity stress.

3.5. Grain yield

The highest grain yield (2.68 g per plant) was obtained in no salinity and bio fertilizer application as N₄ (Table 4). But the minimum yield (1.87 g per plant) was obtained at the highest salinity level and without application of bio fertilizers (Table 4). On the other hand, at the highest salinity level, nano Zn oxide, nano Fe oxide and nano Zn and Fe oxide increased yield by 2.71%, 2.17% and 17.39% in comparison with control. Salt stress affects plant metabolism, which results in decreased growth and yields. Based on these results, the stimulatory effect of bio fertilizer has been attributed to several mechanisms that increase plant yield, including enhanced RWC, proline, soluble sugars content and enhanced activity of PPO, POD and CAT in the leaves by plants. It has been suggested that improvement of the grain yield under bio fertilizer and nano oxide treatments might be associated with the enhanced chlorophyll content and F_v/F_m hereby improving the performance of the plants under suboptimal growth conditions. Vivas et al. (2003) suggested that there are synergistic effects on plant growth when PGPR are inoculated, particularly under growth limited conditions. Also, means comparison between salinity and nano oxide showed that the highest grain yield (2.59 g per plant) was obtained in nano oxide as N₄ in no salinity stress (Table 5). Kheirizadeh Arough et al. (2016) showed that salinity stress negatively affected growth, yield, antioxidant enzymes and ions accumulation in barley plants; however, some of these changes could be compensated by the nano oxide foliar application. Such effects of foliar application with micronutrients (Zn and Fe) might be due to their critical role in crop growth, involving in photosynthesis processes and other biochemical and physiological activates and thus their importance in achieving higher yields.

4. Conclusions

The present study indicated that salinity stress caused a number of physiological and biochemical changes in the wheat plants, including decreased grain yield, RWC, chlorophyll content and F_v/F_m and increased antioxidant enzymes activity, soluble sugars and proline. Also, the application of bio fertilizers and nano oxide improved grain yield, chlorophyll content, antioxidant enzyme activity, proline and soluble sugars under salinity condition. However, application of nano oxide and bio fertilizer reduced the negative effects at each level of salinity testing.

Disclosure statement

No potential conflict of interest was reported by the authors.

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