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Original Article

Effect of Zinc and Bio Fertilizers on Antioxidant Enzymes Activity, Chlorophyll Content, Soluble Sugars and Proline in *Triticale* Under Salinity Condition

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Abstract

In order to study the effects of bio fertilizers and zinc fertilizer on antioxidant enzymes activity, chlorophyll content, soluble sugars and proline in *triticale* under salinity condition, a factorial experiment was conducted based on randomized complete block design with three replications under greenhouse condition. Experiment factors were included salinity in four levels [no-salt (control or S_0), salinity 20 (S_1), 40 (S_2) and 60 (S_3) mM NaCl) equivalent of 1.85, 3.7 and 5.55 dS m^{-1} respectively], four bio fertilizers levels (no bio fertilizer (F_0), application of mycorrhiza (F_1), PGPR (F_2), both application PGPR and mycorrhiza (F_3) and three nano zinc oxide levels (without nano zinc oxide as control (Zn_0), application of 0.4 (Zn_1) and 0.8 (Zn_2) g lit^{-1}). Results showed that salinity severe stress (60 mM) decreased chlorophyll *a*, chlorophyll *b*, total chlorophyll, carotenoid and grain yield of *triticale*, whereas soluble sugars and proline content, the activities of Catalase (CAT), Peroxidase (POD) Polyphenol Oxidase (PPO) enzymes increased. Results showed that both application of bio fertilizer and 0.8 g lit^{-1} nano zinc oxide (F_3Zn_2) increased about 39% from grain yield in comparison with F_0Zn_0 under the highest salinity level. Based on the results, it was concluded that bio fertilizers and nano zinc oxide application can be recommended for profitable *triticale* production under salinity condition.

Keywords: catalase, grain yield, mycorrhiza, peroxidase, plant growth promoting rhizobacteria, polyphenol oxidase

Introduction

Triticale is a human-made crop, being a hybrid by cross-fertilization of wheat (*Triticum* spp.) and rye (*Secale* spp.). In general, *triticale* combines the high yield potential of wheat with the biotic and abiotic stress tolerance of rye, making it more suitable for the production in marginal areas (acidic, saline, or soils with heavy metal toxicity) (Cantale *et al.*, 2016).

Salinity is one of the major abiotic environmental stresses, which affect almost every aspect of plant life and significantly reduces crop yield in affected areas (Yamaguchi and Blumwald, 2005). Thus it is a serious threat to agricultural productivity especially in arid and semi-arid regions (Parvaiz and Satyawati, 2008). Salinity stress is known to affect many physiological activities related to the accumulation of ions and osmolytes such as proline (Lee *et al.*, 2008). The response of plants to salinity depends on several factors such as developmental stage, severity, duration of stress, and cultivar genetics. Salinity also causes oxidative damage as a consequence of producing large amounts of reactive oxygen species (ROS) in different cell organelles (Foyer and Shigeoka, 2011). The induction of ROS-scavenging enzymes, such as SOD, POD, APX, CAT (Mitter, 2002) and other compounds such as carotenoids (Burke and Mahan, 1991), soluble protein (Sinha *et al.*, 2005) is the most common mechanism for detoxifying ROS synthesized during stress responses. The antioxidant system plays an important role in plant tolerance against stress conditions and high concentrations of these antioxidative enzymes have been reported in tolerant species compared to sensitive ones (Gill and Tuteja, 2010). Jin *et al.* (2009) reported that salt stress increased POD activity in barley genotypes differing in salt tolerance. Nadeem *et al.* (2006) reported that salt stress decreased chlorophyll pigments (*a*, *b* and carotenoids contents) of maize, but inoculation with bio fertilizers increased the chlorophyll pigments. 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 mycorrhiza and plant growth promoting rhizobacteria (PGPR) plays important role in yield improvement of plants (Dimkpa *et al.*, 2009).

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Dimkpa *et al.* (2009) reported that rhizosphere microorganisms, exclusively beneficial bacteria and fungi, can improve plant performance under stress environments and enhance yield. The use of PGPR may be proper in developing strategies to facilitate plant growth in saline soils (Vessy, 2003). PGPR can facilitate plant growth indirectly by reducing plant pathogens, or directly by facilitating the uptake of nutrients from the environment, by influencing phytohormone production (e.g. auxin, cytokinin and gibberellins) and production of siderophores (Kohler *et al*., 2006). Mycorrhiza is a symbiotic association between plant roots and fungi. Arbuscular mycorrhizal fungi promote salinity tolerance by utilizing various mechanisms such as accumulation of compatible solutes (Evelin *et al*., 2013) and production of higher antioxidant enzymes (Manchanda and Garg, 2011). 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). Researchers have showed that AM fungi can improve plant tolerance to drought and salinity stress (Gamalero *et al*., 2009). Plants infected with IAA-overproducing PGPR strains showed high antioxidant enzyme activities that contribute to enhance plant protection against salt stress (Bianco and Defez, 2009). Inoculation barley plants with *Pseudomonas sp*. could compensate the salt effects and improve plant development through enhanced production of proline, chlorophyll pigment and soluble sugars and increase dry biomass (Hmaeid *et al*., 2014). Using biologic fertilizers such PGPR can increase quantity and quality of crop yield, efficiency of chemical fertilizers and tolerance of salt and drought stresses as one of the suitable ways to adapt to environment (Arzanesh *et al*., 2009).

Zinc is an essential micronutrient for humans, animals and plants, which act either as the metal component of enzymes or as a functional structural or a regulatory co-factor of a large number of enzymes. A number of researchers have reported the essentiality and role of zinc for plant growth and yield (Fageria *et al*., 2002). Zinc is required for chlorophyll production, pollen function, fertilization and germination and plays an important role in biomass production (Cakmak, 2008). Ebrahimian and Bybordi (2011) suggested that foliar application of zinc activated enzymes involved in reactive oxygen species detoxification and accumulation of proline in sunflower under salt stress conditions.

A better understanding of physiological responses under salinity may help in programs which the objective is to improve the salt tolerance of crop. During the course of these stresses, active solute accumulation of compatible solutes such as proline and the activities CAT, POD and PPO enzymes are claimed to be an effective stress tolerance mechanism. Therefore, the aim of this study was to evaluate the effects of bio fertilizers and zinc on some the physiological responses (i.e., antioxidant enzyme activity, chlorophyll, protein, soluble sugars and proline) of *triticale* under salinity stress conditions.

Materials and Methods

Experimental design

A factorial experiment based on randomized complete block design with three replications was conducted under greenhouse condition in 2014**.** Factors experiment were included salinity in four levels [no-salt (control or S_0), salinity 20 (S_1), 40 (S_2) and 60 (S_3) mM NaCl) equivalent of 1.85, 3.7 and 5.55 dS m^{-1} respectively], four bio fertilizers levels (no bio fertilizer (F_0), application of mycorrhiza (F_1), PGPR (F_2), both application PGPR and mycorrhiza (F_3) and three nano zinc oxide levels (without nano zinc oxide as control (Zn_0), application of 0.4 (Zn_1) and 0.8 (Zn_2) g lit^{-1}). Mycorrhiza fungi (*Glomus mosseae)* 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 790 spores.

Psedomunas putida strain 186 and *Azotobacter chrocoocum strain* 5 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 and Khavazi, 2011). The strains and cell densities of micro organisms used as PGPR in this experiment were 10^7 colony forming units (CFU).

The soil was silty loam, with pH about 6.9. Air temperature ranged from 23-26 °C during the day and 18-20 °C during the night. Humidity ranged from 60-65%. The *triticale* cultivar 'Joanilo' was used in the experiment. Optimal density of cultivar 'Joanilo' is 400 seeds m^{-2}, so forty seeds of *triticale* were sown in each pot with 4 cm deep. The pots were immediately irrigated after planting. Salt stress treatments were applied 18 days after planting (at 2-3 leaf stage). Nano zinc oxide was with the average of particle size less than 30 nm and special surface of particle more than 30 $m^2 g^{-1}$. Nano zinc oxide powder added to deionized water and was placed on ultra sonic equipment (100 w and 40 kHz) on a shaker for better solution (Prasad *et al*., 2012). Foliar application with nano zinc oxide was done in two stage of period growth (4-6 leaf stage and before of booting stage).

Catalase assay

To measure the enzyme activity, 0.2 g of fresh tissue was used. In order to extract protein, 0.2 g of plant fresh tissue 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 (13,000 rpm and 4 °C), then supernatant was used for enzyme activity measurements (Sudhakar *et al*., 2001). Catalase activity was assayed according to Karo and Mishra (1976). The 60 µL protein extract was added to Tris buffer (50 mM, pH = 7) containing 5 mM H_2O_2 on the ice bath, then the absorbance curve was plotted at a wavelength of 240 nm. Enzyme activity was obtained for OD µgprotein min^{-1} of fresh tissue.

Peroxidase assay

Peroxidase activity measured as explained by Karo and Mishra (1976): 50 µl protein extract was added to 2.5 ml extraction buffer containing 100 µM Tris buffer 100 mM and hydrogen peroxide 5 mM and 10 mM Pirogalol in the ice bath and absorbance changes was read at a wave length of 425 nm graph. Enzyme activity was obtained for OD µg protein min^{-1} of fresh tissue.

Polyphenol oxidase assay

Enzyme activity was measured by Karo and Mishra (1976) method: 100 µl protein extract was solved in 1.5 ml Tris 0.2 M and 0.3 ml Pirogalol 0.02 M and the resulting composition was placed in the bain marie bath at 25 °C for five minutes and then the absorbance at 420 nm was recorded. Enzyme activity was obtained for OD µg protein min^{-1} of fresh tissue. 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 11,500 rpm for 20 minutes at 4 °C. The

supernatant was transferred to the new tubes and centrifuged for 20 minutes at 4,000 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.

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 20 ml using acetone 80%. Then it was centrifuged for 10 minutes at 400 rpm and the absorbance at 645, 663 and 470 nm was recorded by a spectrophotometer. Chlorophyll and carotenoids were obtained based on the following equations (Arnon, 1949):

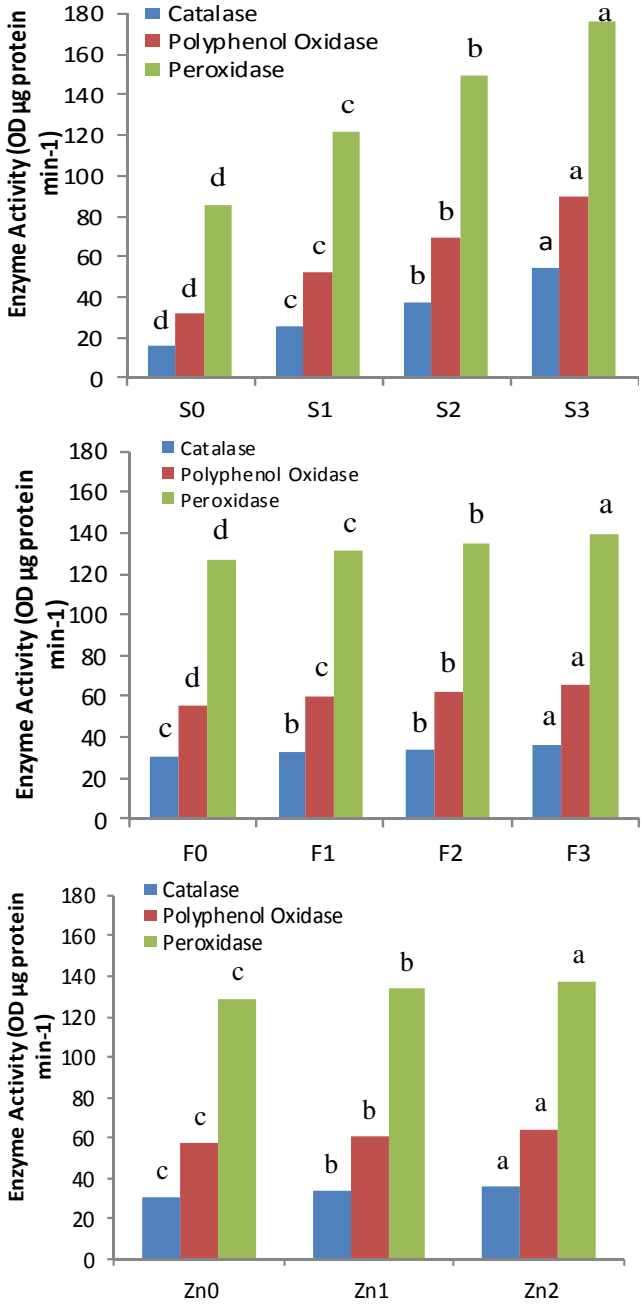

Fig. 1. Effect of salinity, bio fertilizers and nano zinc oxide on CAT, PPO and POD activity of *triticale*; S_0, S_1, S_2 and S_3 are no-salt (control), salinity 20, 40 and 60 mM NaCl), respectively. F_0, F_1, F_2 and F_3 are no bio fertilizer, application of mycorrhiza, PGPR, both applications PGPR and mycorrhiza respectively. Zn_0, Zn_1 and Zn_2 are without nano zinc oxide as control, application of 0.4 and 0.8 g lit^{-1} respectively

Chlorophyll a = (19.3 × A663 - 0.86 × A645) V/100 W
Chlorophyll b = (19.3 × A645 - 3.6 × A663) V/100 W
Total Chlorophyll = Chlorophyll a + Chlorophyll b
Carotenoid = (1000 A470 - 1.82 C_a - 85.02 C_b) /198

Proline assay

In order to measure proline, 0.5 g of plant fresh tissue was crushed in 10 ml sulpho acetic acid solution to obtain a homogeneous mixture. Then, the solution was smoothed using whit-man and 2 ml dimenhydrinate reagent and 2 ml glacial acetic acid were added. The extract was mixed and stirred on bain-marie at 100 °C for one hour and then 4 ml toluene added and the extract was vortexed to form two separate phases. The supernatant was read at 520 nm by a spectrophotometer (Bates *et al.*, 1973). Soluble sugars were extracted from flag leaf using the modified phenol-sulphuric acid method (Dubois *et al.*, 1956).

In order to measure grain yield per plant, 10 plants of each pot randomly were harvested.

Statistic analysis

Analysis of variance and mean comparisons were performed using SAS computer software packages. The main effects and interactions were tested using the least significant difference (LSD) test.

Results and Discussion

Activity of CAT, POD and PPO enzymes

Results indicated that salinity stress, bio fertilizers and nano zinc oxide had a significant effect on the activities antioxidant enzymes. The activity of CAT, POD and PPO enzymes were increased with the increase of salinity stress, application of bio fertilizers and nano zinc oxide in comparison with control. The highest activity of CAT (54.29, 36.55 and 35.64 OD µg protein min^{-1}), PPO (89.53, 65.16 and 64.23 OD µg protein min^{-1}) and POD (176.26, 139.83 and 137.54 OD µg protein min^{-1}) were observed in salinity of S_3, application bio fertilizers as F_3, nano zinc oxide as Zn_2 respectively (Fig. 1). The lowest of CAT (15.59, 30.83 and 30.91 OD µg protein min^{-1}), PPO (31.47, 54.91 and 57.28 OD µg protein min^{-1}) and POD activity (85.89, 127.31 and 128.79 OD µg protein min^{-1}) were obtained at no-salinity, no bio fertilizers and without nano zinc oxide (Fig. 1). Abdel Latef (2011) suggested that plants develop self defense mechanisms by producing antioxidant enzymes like superoxide dismutase, ascorbate peroxidase and catalase. A continued increase in CAT, PPO and POD activity might indicate that these enzymes are a major enzymes detoxifying hydrogen peroxide in *triticale* under salinity stress.

Our results dictated that there was an increase about 18.5%, 15.7% and 9.8% in activity of CAT, PPO and POD, respectively with bio fertilizer application as F_3 in comparison with F_0. Belimov *et al.* (2009) have reported beneficial effects of PGPR for improving plant growth under normal as well as stressful environment. Gamalero *et al.* (2009) showed that bio fertilizers such as mycorrhiza protect the plants from reactive oxygen species produced under stress conditions.

The impact of nano zinc oxide on activity of CAT and PPO and POD were similar to bio fertilizers. So, there was an increase about 15.3%, 12.1% and 6.7% in activity of CAT, PPO and POD, respectively by nano zinc oxide foliar spraying as Zn_2 in comparison with Zn_0 (Fig. 1). Zinc is known to have a stabilizing and protective

effect on bio membranes against oxidative and peroxidative damage (Bettger and O'Dell, 1981). Park *et al.* (2011) suggested that the positive effects of zinc application under salt stress is included protecting chlorophyll against free radicals, removing the reactive oxygen species, increasing of CAT and PPO activity. In this study, the activities of CAT, PPO and POD enzymes increased by application of zinc. Zinc ions bind to ligands containing sulfur, nitrogen, and to a lesser extent oxygen, and preferentially bind to the membrane proteins (Bettger and O'Dell, 1981). The balance between free radical generation and free radical defense determines the survival of the system. Therefore, Zn may have a role in modulating free radicals and their related damaging effects by enhancing plants antioxidant systems (Zago and Oteiza, 2001).

Interaction effect between salinity and bio fertilizers showed that the highest activity of CAT and PPO (58.02 and 94.65 OD µg protein min^{-1} respectively) were obtained in salinity 60 mM with bio fertilizer application as F_3 (Fig. 2) and the least activities of them (14.82 and 28.33 OD µg protein min^{-1} respectively) were obtained in control treatment or S_0F_0 (Fig. 2). On the other hand, there were an increase about 10.5% and 12.1% in activity of CAT and PPO enzymes, respectively in the highest salinity level and bio fertilizers (S_3F_3) in comparison with (S_3F_0) (Fig. 2).

Also interaction effect between salinity and nano zinc oxide showed that the highest of CAT and PPO activities (57.4 and 94.13 OD µg protein min^{-1} respectively) were obtained in S_3Zn_2 (Fig. 2). Also there were an increase about 14.5% and 9.9% in activity of CAT and PPO enzymes, respectively in the highest salinity level and nano zinc oxide (S_3Zn_2) in comparison with S_3Zn_0 (Fig. 2).

Plants develop self defense mechanisms by producing antioxidant enzymes like superoxide dismutase, ascorbate peroxidase and catalase (Abdel Latef, 2011). Inoculation with bio fertilizers under salinity stress, significantly increased CAT, POD and PPO enzymes activity. Similar results have been reported by Ma *et al.* (2011). They suggested that bio fertilizers can improve plant tolerance to salinity and drought and enable plants to survive under unfavourable environmental conditions. Belimov *et al.* (2009) have reported beneficial effects of bio fertilizers for improving plant growth under normal as well as stressful environment. Similar results have also been reported by Mar Vazquez *et al.* (2000). Antioxidative enzymes like catalase (CAT), peroxidase (POD) are the most important components in the scavenging system of ROS (Noctor and Foyer, 1998).

Proline content

Proline has significantly changed during salinity stress and application of bio fertilizers and nano zinc oxide. By increasing the salinity stress, proline content increased. The highest content of proline (9.18, 7.78 and 7.8 mg g^{-1} FW respectively) were obtained in the highest of salinity level, application of bio fertilizer as F_3 and nano zinc oxide as Zn_2 (Fig. 3). The minimum of these values (4.1, 6.41 and 6.59 mg g^{-1} FW) were obtained in S_0, F_0 and Zn_0 respectively (Fig. 3). Also interaction effect between salinity and bio fertilizers showed that the highest of proline (9.72 mg g^{-1} FW) was obtained in S_3F_3 and the lowest of it (3.82 mg g^{-1} FW) was observed in S_0F_0 (Fig. 4). There was an increase about 16.1% in content of proline in the highest salinity level and bio fertilizers (S_3F_3) in comparison with S_3F_0 (Fig. 4). proline is known to act as an osmo regulator under stress conditions (Ashraf and Foolad, 2007). Proline accumulation in stress condition is a defensive mechanism (Koocheki *et al.*, 2004). So, accumulation of proline in the cell protects the plant by adjusting osmotic pressure as well as by stabilizing many functional units like complex II of the electron transport system, removal of hydroxyl radicals (Mattioli *et al.*, 2009). Proline reduces cytoplasmic pH and maintains the proper ratio of $NADP^+$/NADPH in metabolism and increase different enzymes activities (Szabados and Savoure, 2009). Some studies demonstrated that AM association affects the physiological processes of plants by increasing proline contents (Ruiz-Lozano *et al.*, 1995). Proline accumulation was studied in resistant and non-resistant varieties of *Silen vulgaris* to increasing concentrations of zinc (Schat *et al.*, 1997).

Soluble sugars

The results of measurement of soluble sugars showed the concentration of soluble sugars increased under salinity stress. The highest content of soluble sugars (99.48 mg g^{-1} FW) was obtained in 60 mM, application bio fertilizers as F_3 nano zinc oxide as Zn_2. Also the minimum of it (25.11 mg g^{-1} FW) was observed in

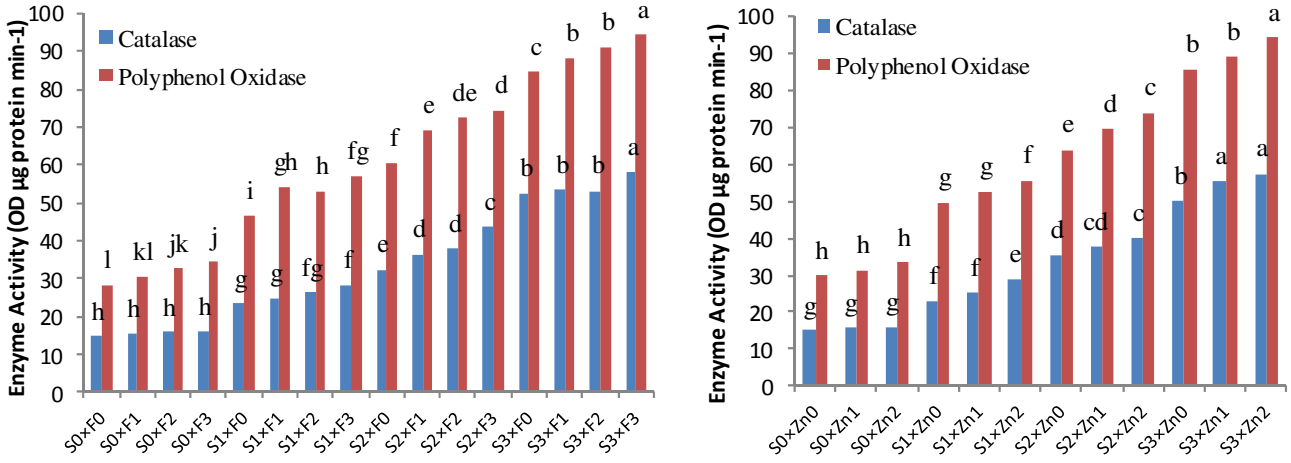

Fig. 2. Effect of salinity and bio fertilizers application, salinity and nano zinc oxide on CAT and PPO activity of *triticale*; S_0, S_1, S_2 and S_3 are no-salt (control), salinity 20, 40 and 60 mM NaCl), respectively. F_0, F_1, F_2 and F_3 are no bio fertilizer, application of mycorrhiza, PGPR, both applications PGPR and mycorrhiza respectively. Zn_0, Zn_1 and Zn_2 are without nano zinc oxide as control, application of 0.4 and 0.8 g lit^{-1} respectively

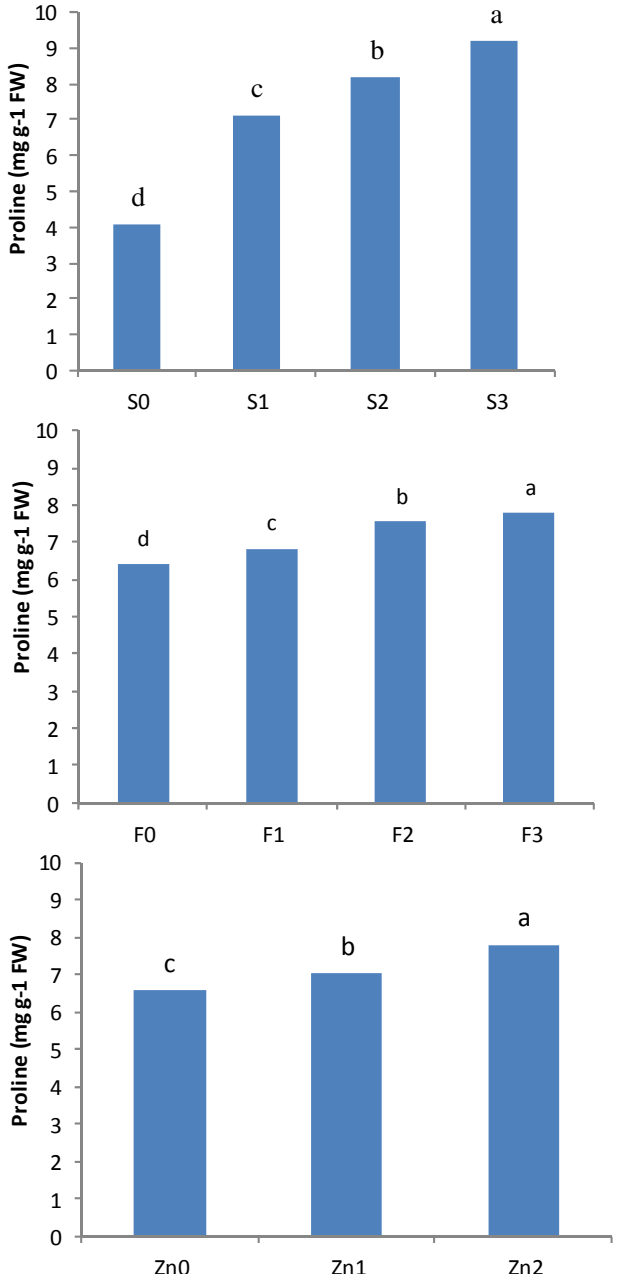

Fig. 3. Effect of salinity, bio fertilizers and nano zinc oxide on proline content of *triticale*; S_0, S_1, S_2 and S_3 are no-salt (control), salinity 20, 40 and 60 mM NaCl), respectively. F_0, F_1, F_2 and F_3 are no bio fertilizer, application of mycorrhiza, PGPR, both applications PGPR and mycorrhiza respectively. Zn_0, Zn_1 and Zn_2 are without nano zinc oxide as control, application of 0.4 and 0.8 g lit^{-1} respectively

control treatment (S_0, F_0 and Zn_0) (Table 1). Results showed that at the highest salinity level, application bio fertilizers as F_3 and nano zinc oxide as Zn_2 increase about 72.3% in content of soluble sugars in comparison with F_0 and Zn_0 in the same salinity level (Table 1). Van and Clijsters (1990) indicated that salinity increased soluble sugars. Accumulation of soluble sugars helps regulate osmotic in plant cells and leads to preservation of biological molecules and membranes and maintaining turgor pressure via osmotic regulation (Irannejad and Shahbazian, 2004). Concentration of sugars may increase photosynthesis of plants during stress and also prevent plasmolysis (Sato *et al*., 2004). In a saline environment

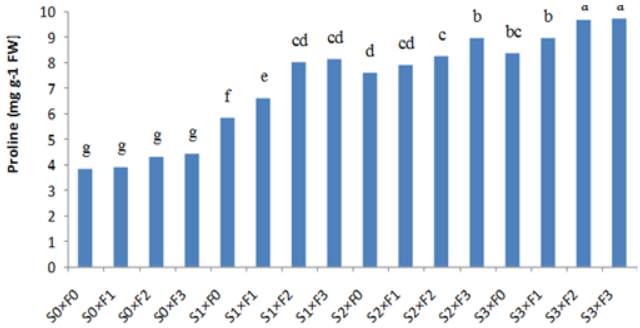

Fig. 4. Effect of salinity and bio fertilizers application on proline content of *triticale*; S_0, S_1, S_2 and S_3 are no-salt (control), salinity 20, 40 and 60 mM NaCl), respectively. F_0, F_1, F_2 and F_3 are no bio fertilizer, application of mycorrhiza, PGPR, both applications PGPR and mycorrhiza respectively

plant water uptake decreases due to changes in soil water potential. Under such conditions, accumulation of compatible solutes like soluble sugars, proline, glycine betaine and many other such organic solutes, takes place in the plant body that plays an important role to protect the plant from the stress induced deleterious effects by osmotic adjustment, limiting water loss and diluting the concentration of toxic ions (Slama *et al*., 2006). The plant with the increase in soluble sugar and maintaining the osmotic potential in stress conditions, will be able to store their carbohydrate metabolism of the cell is kept at an optimum level (Gibson, 2005). It was stated of VAM fungi significantly increasing photosynthetic of host plants and there by causing an increase in sugar content (Marschner and Dell, 1994).

Photosynthetic pigments

Salinity stress, bio fertilizers and nano zinc oxide application significantly affected the photosynthetic pigment content. The highest content of chlorophyll *a* (6.61 mg g^{-1} FW), chlorophyll *b* (1.84 mg g^{-1} FW), total chlorophyll (8.45 mg g^{-1} FW) and Carotenoid (0.88 mg g^{-1} FW) were obtained in $S_0F_3Zn_2$, while the lowest values (1.74 mg g^{-1} FW, 0.58 mg g^{-1} FW, 2.32 mg g^{-1} FW and 0.173 mg g^{-1} FW respectively) were determined in $S_3F_0Zn_0$ (Tables 2 and 3). Results showed that in the highest salinity level, application bio fertilizers as F_3 and nano zinc oxide as Zn_2 increased about 69.5%, 81%, 72.8% and 64.7% content of chlorophyll *a*, chlorophyll *b*, total chlorophyll and Carotenoid respectively, in comparison with F_0 and Zn_0 (Tables 2 and 3).

Salinity stress caused the reduction in chlorophyll content while the application of bio fertilizers and nano zinc oxide enhanced the chlorophyll content, which revealed the bio fertilizers and nano zinc oxide important in mitigating stress effect. Environmental stress reduced chlorophyll and carotenoids content. The main reason for the decrease in chlorophyll may be degradation by reactive oxygen species (ROS). Another reason for the decline in chlorophyll is the application of a glutamate precursor for the biosynthesis of proline (Navari-Izzo *et al*., 1990). Sultana *et al*. (1999) reported that decrease in carotenoids in salt stress is Beta carotene destruction and Zea xanthin formation. It was reported that total chlorophyll and carotenoids are decreased in tomato under salt stress (Parida and Das, 2005). Reduction of chlorophyll and other pigments finally resulted in decrease in the efficiency of photosynthesis (Basra and Basra, 1997). Giri *et al*. (2003) found that mixed inoculation of six arbuscular mycorrhizal fungi species enhanced the chlorophyll content in *Acacia auriculiformis* under salinity stress. Sannazzora *et al*. (2005)

Table 1. Interaction effect between salinity×biofertilizers×nano zinc oxide on soluble sugars of *triticale*

Treatment		Soluble Sugars (mg g^{-1} FW)		
Salinity Stress	Bio Fertilizers	Zinc levels (g lit^{-1})		
		0	0.4	0.8
	F_0	25.11±5.61	32.01±4.83	35.17±5.65
S_0	F_1	27.08±5.70	37.12±7.46	37.03±8.23
	F_2	34.29±4.27	38.68±7.19	40.37±6.78
	F_3	39.67±6.94	40.55±7.36	41.52±6.41
	F_0	42.58±5.66	43.98±4.87	45.75±3.43
S_1	F_1	40.47±0.77	46.59±4.84	50.67±8.04
	F_2	45.24±2.80	51.18±8.20	58.28±5.47
	F_3	51.98±2.84	55.97±6.01	63.01±5.10
	F_0	48.54±3.70	48.67±4.73	51.85±7.15
S_2	F_1	57.60±4.52	69.36±5.42	78.61±7.60
	F_2	56.06±2.49	63.47±3.04	61.44±6.90
	F_3	62.01±2.34	73.93±4.64	81.79±1.73
	F_0	57.72±1.44	62.43±2.68	66.44±5.37
S_3	F_1	65.75±3.23	71.28±3.09	94.29±3.10
	F_2	83.68±4.53	89.97±5.63	93.82±4.92
	F_3	87.59±3.48	97.18±1.17	99.48±1.44
$LSD_{0.05}$			4.42	

Difference between mean difference treatments significant differences (LSD test, $P < 0.05$); S_0, S_1, S_2 and S_3 are no-salt (control), salinity 20, 40 and 60 mM NaCl), respectively. F_0, F_1, F_2 and F_3 are no bio fertilizer, application of mycorrhiza, PGPR, both applications PGPR and mycorrhiza respectively.

Table 2. Interaction effect between salinity×biofertilizers×nano zinc oxide on chlorophyll *a* and chlorophyll *b* of *triticale*

Treatment		Chlorophyll *a* (mg g^{-1} FW)			Chlorophyll *b* (mg g^{-1} FW)		
Salinity Stress	Bio Fertilizers	Zinc levels (g lit^{-1})			Zinc levels (g lit^{-1})		
		0	0.4	0.8	0	0.4	0.8
	F_0	4.05±0.20	4.20±0.17	4.20±0.40	1.20±0.30	1.31±0.29	1.37±0.25
S_0	F_1	4.10±0.35	4.34±0.16	5.11±0.46	1.34±0.25	1.53±0.35	1.65±0.29
	F_2	4.35±0.04	5.35±0.41	6.22±0.51	1.33±0.29	1.46±0.33	1.73±0.36
	F_3	4.50±0.39	5.84±0.39	6.61±0.40	1.46±0.21	1.44±0.28	1.84±0.28
	F_0	3.34±0.26	3.60±0.09	3.84±0.08	0.86±0.33	0.89±0.37	0.92±0.36
S_1	F_1	3.39±0.17	3.65±0.08	3.73±0.56	0.97±0.36	1.16±0.37	1.19±0.28
	F_2	3.56±0.43	3.64±0.42	4.31±0.43	1.09±0.34	1.29±0.32	1.33±0.32
	F_3	3.56±0.32	4.28±0.36	4.45±0.34	1.20±0.31	1.41±0.33	1.47±0.26
	F_0	2.18±0.38	2.22±0.31	2.64±0.40	0.77±0.27	0.92±0.31	0.95±0.32
S_2	F_1	2.24±0.36	2.86±0.35	3.41±0.47	0.78±0.31	0.78±0.35	0.9±0.34
	F_2	2.81±0.36	3.21±0.40	3.57±0.50	0.69±0.32	0.77±0.33	1.04±0.38
	F_3	2.82±0.36	3.38±0.37	3.61±0.23	0.97±0.35	1.23±0.31	1.25±0.33
	F_0	1.74±0.36	2.05±0.36	2.28±0.36	0.58±0.31	0.73±0.32	0.8±0.33
S_3	F_1	1.93±0.37	2.16±0.34	2.36±0.35	0.64±0.30	0.73±0.34	0.86±0.32
	F_2	1.95±0.31	2.10±0.35	2.53±0.33	0.62±0.32	0.82±0.32	0.88±0.33
	F_3	2.04±0.32	2.17±0.36	2.95±0.34	0.68±0.32	0.92±0.34	1.05±0.34
$LSD_{0.05}$			0.23			0.09	

Difference between mean difference treatments significant differences (LSD test, $P < 0.05$);
S_0, S_1, S_2 and S_3 are no-salt (control), salinity 20, 40 and 60 mM NaCl), respectively. F_0, F_1, F_2 and F_3 are no bio fertilizer, application of mycorrhiza, PGPR, both applications PGPR and mycorrhiza respectively.

reported plants inoculated with *Glomus intraradices* had higher protein and chlorophyll density in comparison with non–mycorrhiza inoculated plants. In this study, photosynthetic pigments were increased under the effect of co-inoculation with PGPR and mycorrhizal. Giri and Mukerji (2004) reported that mycorrhiza and PGPR decrease effects of salinity in chlorophyll synthesis. Shaharoona *et al*. (2006) reported that inoculation with PGPR containing ACC-deaminase activity significantly affected the pigments under salinity stress. Sharma *et al*. (1994) reported that added zinc enhanced the growth of cabbage and improved the chlorophyll content and photosynthetic activity in the leaves. Zarrouk *et al*. (2005) indicated a positive correlation of Zn concentrations with leaf chlorophyll content in plants.

Grain yield

The salinity stress, bio fertilizers and nano zinc oxide foliar significantly affected the grain yield per plant. The highest grain yield (3.64 g per plant) was obtained in no-salinity, application of bio fertilizer as F_3 and nano zinc oxide as Zn_2 (Table 4). The lowest grain yield per plant (1.65 g) was determined in the highest salinity level and without application of bio fertilizers and nano zinc oxide (Table 4). Azcón and Barea (2010) has been proposed co-

Table 3. Interaction effect between salinity×biofertilizers×nano zinc oxide on total chlorophyll and carotenoid of *triticale*

Treatment		Total Chlorophyll (mg g^{-1} FW)			Carotenoid (mg g^{-1} FW)		
Salinity Stress	Bio Fertilizers	Zinc levels (g lit^{-1})			Zinc levels (g lit^{-1})		
		0	0.4	0.8	0	0.4	0.8
	F_0	5.26±0.48	5.52±0.45	5.57±0.65	0.526±0.008	0.549±0.008	0.593±0.041
S_0	F_1	5.45±0.61	5.88±0.44	6.77±0.76	0.543±0.009	0.544±0.005	0.542±0.047
	F_2	5.69±0.32	6.82±0.70	7.96±0.82	0.530±0.007	0.593±0.071	0.763±0.076
	F_3	5.96±0.58	7.29±0.65	8.45±0.67	0.559±0.045	0.751±0.044	0.880±0.036
	F_0	4.21±0.57	4.50±0.44	4.77±0.43	0.328±0.066	0.403±0.062	0.446±0.044
S_1	F_1	4.36±0.51	4.81±0.44	4.92±0.81	0.403±0.035	0.408±0.045	0.435±0.043
	F_2	4.66±0.73	4.94±0.75	5.65±0.76	0.404±0.083	0.439±0.034	0.434±0.046
	F_3	4.76±0.64	5.70±0.70	5.93±0.60	0.418±0.056	0.456±0.031	0.475±0.015
	F_0	2.95±0.62	3.14±0.60	3.59±0.72	0.252±0.060	0.326±0.080	0.353±0.028
S_2	F_1	2.96±0.55	3.65±0.69	4.31±0.79	0.389±0.002	0.382±0.073	0.388±0.034
	F_2	3.51±0.66	3.99±0.73	4.61±0.89	0.352±0.075	0.393±0.072	0.401±0.066
	F_3	3.8±0.68	4.61±0.68	4.86±0.55	0.346±0.075	0.371±0.033	0.410±0.036
	F_0	2.32±0.66	2.79±0.67	3.09±0.68	0.173±0.055	0.182±0.051	0.215±0.075
S_3	F_1	2.57±0.65	2.89±0.66	3.23±0.67	0.178±0.058	0.235±0.083	0.257±0.061
	F_2	2.58±0.62	2.92±0.67	3.41±0.67	0.262±0.060	0.226±0.094	0.272±0.069
	F_3	2.73±0.62	3.10±0.68	4.01±0.67	0.253±0.071	0.255±0.061	0.285±0.038
$LSD_{0.05}$			0.05			0.075	

Difference between mean difference treatments significant differences (LSD test, $P < 0.05$);
S_0, S_1, S_2 and S_3 are no-salt (control), salinity 20, 40 and 60 mM NaCl), respectively. F_0, F_1, F_2 and F_3 are no bio fertilizer, application of mycorrhiza, PGPR, both applications PGPR and mycorrhiza respectively

Table 4. Interaction effect between salinity×biofertilizers×nano zinc oxide on yield of *triticale*

Treatment		Yield (g per plant)		
Salinity Stress	Bio Fertilizers	Zinc levels (g lit^{-1})		
		0	0.4	0.8
	F_0	2.45±0.1	2.68±0.06	2.82±0.09
S_0	F_1	2.72±0.09	2.89±0.08	3.10±0.06
	F_2	3.10±0.01	3.10±0.10	3.39±0.06
	F_3	3.11±0.09	3.41±0.07	3.64±0.05
	F_0	2.34±0.05	2.43±0.08	2.6±0.09
S_1	F_1	2.49±0.01	2.6±0.09	2.86±0.07
	F_2	2.72±0.10	2.93±0.09	3.00±0.10
	F_3	2.91±0.06	3.01±0.06	3.28±0.13
	F_0	1.93±0.10	2.12±0.09	2.32±0.10
S_2	F_1	2.06±0.08	2.20±0.10	2.43±0.08
	F_2	2.21±0.08	2.32±0.11	2.54±0.08
	F_3	2.52±0.10	2.73±0.13	2.89±0.09
	F_0	1.65±0.07	1.82±0.09	2.08±0.10
S_3	F_1	1.82±0.11	1.96±0.11	2.12±0.11
	F_2	1.94±0.13	2.20±0.10	2.33±0.11
	F_3	2.13±0.11	2.23±0.12	2.31±0.09
$LSD_{0.05}$			0.04	

Difference between mean difference treatments significant differences (LSD test, $P < 0.05$); S_0, S_1, S_2 and S_3 are no-salt (control), salinity 20, 40 and 60 mM NaCl), respectively. F_0, F_1, F_2 and F_3 are no bio fertilizer, application of mycorrhiza, PGPR, both applications PGPR and mycorrhiza respectively.

inoculation with bio fertilizer as an efficient procedure to increase plant growth. Vivas *et al.* (2003) suggested that there are synergistic effects on plant growth when PGPR and mycorrhiza are inoculated, particularly under growth limited conditions.

Conclusion

The results showed that salinity stress reduced grain yield per plant and chlorophyll content of the plants. But antioxidant enzymes activity, soluble sugars and proline increased. Also application of bio fertilizer and nano zinc oxide improved of grain yield, chlorophyll content, antioxidant enzyme activity, proline and soluble sugars under salinity condition. Our results suggested that plants apply defensive mechanisms, such as syntheses of antioxidant enzymes, soluble sugars and proline to improvement effects of stress. It seems that application of bio fertilizer and nano zinc oxide can be recommended for profitable *triticale* production under salinity condition.

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