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Physiological Response of *Triticale* to Zinc Application and Biofertilizers under Various Water Limitation Treatments

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In order to evaluate the effects of biofertilizers and zinc on some physiological traits of *Triticale* under limited water conditions, a factorial experiment was conducted in randomized complete block design with three replications in 2014 and 2015. Experiment factors included water limitation at three levels [normal irrigation (W_0) as control; moderate water limitation (W_1) - irrigation withheld at 50% of heading stage; severe water limitation (W_2) - irrigation withheld at 50% of booting stage]; four biofertilizer levels: no biofertilizer (F_0), application of mycorrhiza (F_1), application of plant-growth promoting rhizobacteria (PGPR) (F_2), application of both PGPR and mycorrhiza (F_3); and four nano zinc oxide levels [(without nano zinc oxide (Zn_0) as control, application of 0.3 (Zn_1), 0.6 (Zn_2) and 0.9 (Zn_3) g L^{-1})]. Results showed that water limitation decreased chlorophyll *a*, chlorophyll *b*, total chlorophyll, carotenoid and yield of *Triticale*, while soluble sugars and proline content, and the activity of catalase (CAT), peroxidase (POD), and polyphenoloxidase (PPO) enzymes increased. However, inoculation of plants with biofertilizers and zinc application improved these traits under water limitation conditions and normal irrigation. Application of biofertilizer and nano zinc oxide as F_3Zn_3 increased grain yield by 87.5% when compared with F_0Zn_0 under severe water limitation. Based on the results, biofertilizers and nano zinc oxide application can be recommended for profitable *Triticale* production under water limitation conditions.

Key Words: antioxidant enzyme, mycorrhiza, PGPR, proline, *Triticale*, water deficit

Abbreviations: AM – arbuscular mycorrhizal, AMF – arbuscular mycorrhizal fungi, CAT – catalase, FW – fresh weight, OD – optical density, PGPR – plant-growth promoting rhizobacteria, POD – peroxidase, PPO – polyphenoloxidase, ROS – reactive oxygen species

INTRODUCTION

Triticale is a cereal crop cultivar obtained by cross-fertilization of wheat (*Triticum* spp.) and rye (*Secale* spp.). The importance of *Triticale* as cereal crop has increased in recent years because its tolerance to biotic and abiotic stresses is higher than that of wheat and because its grain yield is higher than that of rye (Tohver et al. 2005).

Drought is the most severe abiotic stress factor limiting plant growth and crop production. Many physiological processes in plants are impaired by drought stress, including photosynthesis, enzyme activity and membrane stability. It inhibits the photosynthesis of plants, causes changes in chlorophyll content and damages the photosynthetic apparatus. It also inhibits the photochemical activities and decreases the activities of enzymes in the Calvin cycle (Farooq et al. 2009). Drought stress breaks down the balance between the production of reactive oxygen species (ROS) and the antioxidant defense system, thus causing the accumulation of ROS, which induces oxidative stress to protein and membrane lipids (Farooq et al. 2009). Drought stress can damage pigments and plastids, reduce chlorophyll *a*, chlorophyll *b* and other carotenoids, and can also hydrolyze proteins and prevent photochemical reactions in most plants (Farooq et al. 2009).

Among the numerous microorganisms in the rhizosphere, some have positive effects on plant growth promotion. These microorganisms are biofertilizers, which colonize the rhizosphere and roots of many plant species and provide beneficial effects to plants (Gusain et al. 2015). Inoculation of plants with native suitable microorganisms may decrease the deleterious effects of environmental stresses and increase stress tolerance of plants by a

variety of mechanisms, including synthesis of phytohormones such as auxins, solubilization of minerals such as phosphorus, production of siderophores, and increase in nutrient uptake, chlorophyll, and soluble leaf protein content (Dobbelaere et al. 2003). Higher proline accumulation in inoculated plants indicates higher plant tolerance to water stress (Gusain et al. 2015). Inoculation of maize plant with the plant-growth promoting rhizobacterium (PGPR) *Pseudomonas putida* GAP-P45 (Sandhya et al. 2010) improved plant growth through accumulation of free amino acids and soluble sugars compared with non-treated plants under drought stress.

Mycorrhiza is a symbiotic association between a group of soil fungi called arbuscular mycorrhizal fungi (AMF) and plants. The AMF take carbohydrates compounds from their plant hosts, while the plants benefit from the association by the increased uptake of nutrients, which improve tolerance to abiotic stress (drought or salinity). A study conducted on wheat under water stress environment showed that mycorrhizal inoculation enhanced the activities of antioxidant enzymes such as peroxidase and catalase compared with those in uninoculated control plants (Khalafallah and Abo-Ghalia 2008). Mycorrhizal inoculation significantly increased the contents of proline, free amino acids, total soluble and crude proteins and total carbohydrates in wheat plants. The same study suggested that mycorrhizal association could improve the osmotic adjustment, enhance the defense system of plants, and alleviate oxidative damage caused by drought stress (Khalafallah and Abo-Ghalia 2008).

Zinc (Zn) is an essential micronutrient required for many enzymes involved in numerous physiological and metabolic processes of plants. Zn is also known to have a stabilizing and protective effect on the bio-membrane against oxidative and peroxidative damage, loss of plasma membrane integrity and also alteration of the permeability of the membrane (Cakmak 2000). Similarly, in Zn-treated *Solanum lycopersicum*, the superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) activities increased (Cherif et al. 2011). The total chlorophyll content improved in the leaves of snap beans under higher levels of Zn treatment (El-Tohamy and El-Greadly 2007).

This study investigated the effects of biofertilizers and nano zinc oxide on the physiological responses (i.e., antioxidant enzyme activity, chlorophyll, protein, soluble sugars and proline) and yield of *Triticale* under water limitation conditions.

MATERIALS AND METHODS

A factorial experiment based on randomized complete block design with three replications was conducted at the research farm of the University of Mohaghegh Ardabili, Ardabil, Iran in the 2014 and 2015 cropping seasons. The area is located at 38° 15′ N latitude and 48° 15′ E longitude with an elevation of 1350 m above mean sea level. Factors in the experiment included water limitation in three levels [normal irrigation (W_0) as control, moderate water limitation (W_1) or withholding irrigation at 50% of heading stage, and severe water limitation (W_2) or withholding irrigation at 50% of booting stage]; four biofertilizer levels: no biofertilizer (F_0), application of mycorrhiza (F_1), plant-growth promoting rhizobacteria (PGPR) (F_2), application of both PGPR and mycorrhiza (F_3) and four nano zinc oxide levels [(without nano zinc oxide as control (Zn_0), application of 0.3 (Zn_1), 0.6 (Zn_2) and 0.9 (Zn_3) g L^{-1})].

Mycorrhiza fungi (*mosseae)* was purchased from the Zist Fanavar Turan Corporation and soils were treated based on the method described by Gianinazzi et al. (2001). *Pseudomonas putida* strain 186 and *Azotobacter chrocoocucum* strain 5 were isolated from the rhizospheres of wheat at the Research Institute of Soil and Water, Tehran, Iran. Nano zinc oxide had an average particle size of less than 30 nm and the special surface of particle was more than 30 m^2 g^{-1}.

The *Triticale* cultivar 'Joanilo' was used in the experiment at a plant density of 400 seeds m^{-2}. For inoculation, seeds were coated with gum Arabic as an adhesive and rolled into the suspension of bacteria until the seeds were uniformly coated. The strains and cell densities of microorganisms used as PGPR in this experiment were 1×10^7 colony-forming units (CFU). In each plot, there were five rows that were 2 m long. Plots and blocks were separated by 1 m unplanted distances. All phosphorus (60 kg ha^{-1} in the form of super phosphate) and potassium (60 kg ha^{-1} in the form of potassium sulphate) fertilizers were applied as basal dose at the time of seedbed

preparation. Nitrogen fertilizer was applied as ½ at sowing, and ½ at the stage when 6–8 leaves had grown. The seeds were planted on the 16th of May 2014 and on the 23rd of May 2015. Mean temperature and precipitation for each cropping season are presented in Table 1.

The plots were immediately irrigated after planting. Nano zinc oxide powder was added to deionized water and was placed on ultrasonic equipment (100 w and 40 kHz) on a shaker for better solution. Foliar application with nano zinc oxide was done in two stages of growth (4–6 leaf stage and before 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 1 mL of buffer Tris-HCl (0.05 M, pH 7.5) was added. The mixture obtained was centrifuged for 20 min (13,000 rpm and 4 °C), then a supernatant was used for enzyme activity measurements (Sudhakar et al. 2001). Catalase activity was assayed according to the method described by 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 optical density (OD) µg protein min^{-1} of fresh tissue.

Peroxidase (POD) Assay

Measurement of peroxidase activity followed the method explained by Karo and Mishra (1976) thus: 50 µL of 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 ice bath, and change in absorbance was read at a wavelength of 425 nm graph. Enzyme activity was obtained for OD µg protein min^{-1} of fresh tissue.

Polyphenol Oxidase (PPO) Assay

Enzyme activity was measured by the method used by Karo and Mishra (1976) described as follows: 100 µL of protein extract was dissolved 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 5 min 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 was carried out by using the Bradford (1976) method as follows: 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 the 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 was measured in 0.2 g fresh leaf tissue, which was gradually worn with 80% acetone, and the volume of the solution was brought to 20 mL using 80% acetone. Then the solution was centrifuged for 10 min at 400 rpm and the absorbance at 645, 663 and 470 nm was recorded by using a spectrophotometer. Chlorophyll and carotenoids were obtained based on the following equations (Arnon 1949):

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 Ca − 85.02 Cb) /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 filter paper (Whatman) after which 2 mL dimenhydrinate reagent and 2 mL glacial acetic acid were added. The extract was mixed

Table 1. Minimum and maximum temperature and rainfall recorded during the period of *Triticale* growth.

Month	Mean of Maximum Temperature (°C)		Mean of Minimum Temperature (°C)		Mean of Monthly Temperature (°C)		Mean of Monthly Rainfall (mm)	
	2014	2015	2014	2015	2014	2015	2014	2015
May	22.4	21.2	8.1	4.6	15.3	12.9	35.4	35.7
June	25	26	10.5	9.7	17.8	17.9	24.5	26.5
July	25.5	26.7	13.3	13.1	19.4	19.9	12.2	7
August	26.4	29	13.2	11.8	19.8	20.4	0.4	3.6
September	25.8	23.4	11.8	10.4	18.8	16.9	0.6	0.4

and stirred on bain-marie at 100 °C for 1 h and then 4 mL toluene was added. 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 the flag leaf using the modified phenol-sulphuric acid method (Dubois et al. 1956).

Three central rows (1 m long each) were harvested from each plot. 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

Soil samples that were collected at a depth of 0–35 cm and analyzed for various physicochemical properties are presented in Table 2.

Activity of CAT, POD and PPO Enzymes

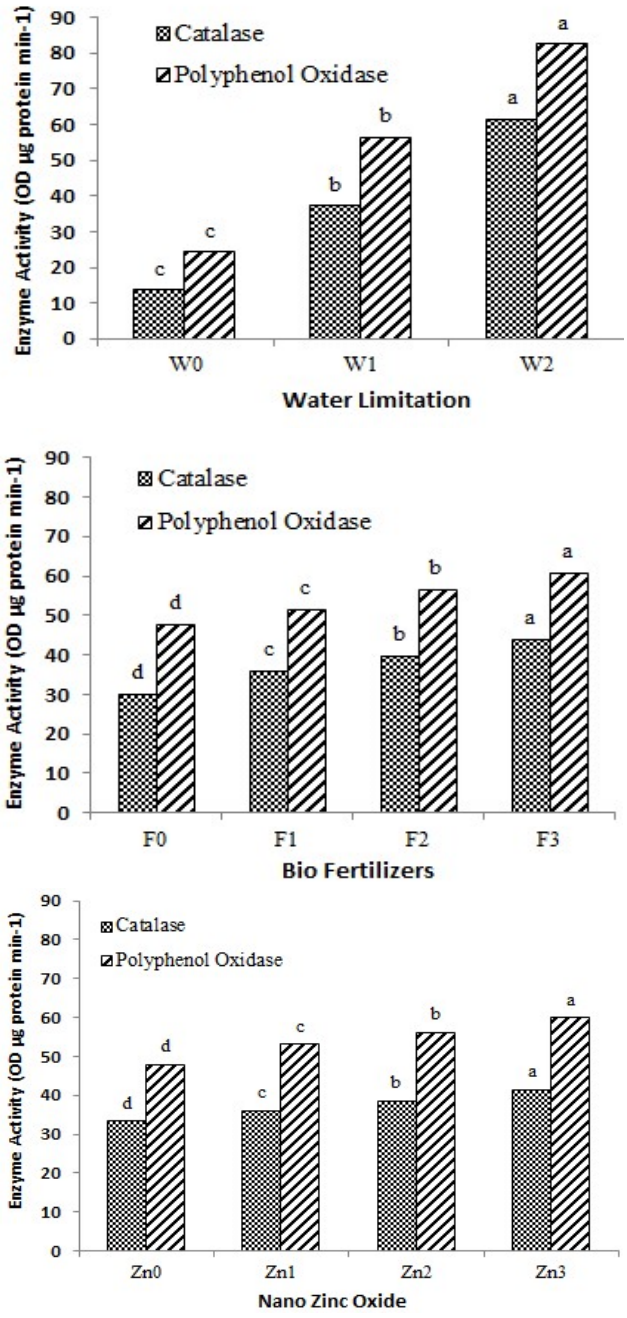
The water limitation treatments significantly increased activity of CAT, POD and PPO enzymes compared with the control plants. Results showed that the highest contents of CAT (61.23, 43.76 and 41.42 OD μg protein min^{-1}) and PPO (82.39, 60.92 and 59.9 OD μg protein min^{-1}) were observed in plants under severe water limitation, biofertilizer application as F_3 and nano zinc oxide as Zn_3, respectively (Fig. 1). The lowest CAT content (13.49, 29.88 and 33.29 OD μg protein min^{-1}) and PPO activity (24.01, 47.72 and 47.81 OD μg protein min^{-1}) were obtained at W_0, F_0 and Zn_0 (Fig. 1). It seems that when plants are subjected to various abiotic stresses, reactive oxygen species such as superoxide ($O2^{-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\cdot}OH$) and singlet oxygen ($^1O_2$) are produced. These Reactive Oxygen Species (ROS) may initiate destructive oxidative processes such as lipid peroxidation, chlorophyll bleaching, protein oxidation and damage to nucleic acids. In this regard, the balance between ROS production and activities of antioxidative enzymes determines whether or not oxidative signaling and/or damage will occur as observed by Moller et al. (2007) who reported that antioxidant enzyme activity was increased when plants were exposed to water stress. Plant growth improvement under stressful environments could be due to the significant role of the enzymatic antioxidant system in alleviating oxidative impact (Farooq et al. 2009).

Inoculation with PGPR, mycorrhiza, and aplication of both PGPR and mycorrhiza under water limitation significantly increased CAT, POD and PPO enzyme activity of *Triticale*. Our results indicated that there was an increase of 46.4%, 27.6% and 10.3% in the activity of CAT, PPO and POD, respectively, with biofertilizer application as F_3 in comparison with F_0 (Fig. 1). Ma et al. (2011) reported that biofertilizers can improve plant tolerance to salinity, drought, flooding, and heavy metal toxicity and can enable plants to survive under unfavorable environmental conditions. They also reported beneficial effects of these biofertilizers for improving plant growth under normal as well as stressful environmental conditions. There are other authors who have reported that AM fungi can increase enzyme activities and can protect plants from ROS produced under stress conditions (Khalafallah and Abo-Ghalia 2008).

Corroborating results in our study showed that antioxidant enzyme activity increased when nano zinc oxide was applied as Zn_3 in comparison with Zn_0. In this regard, there was an increase of about 24.4%, 25.2% and 10.7% in the activity of CAT, PPO and POD, respectively, as a result of nano zinc oxide foliar spraying as Zn_3 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 (Cakmak 2000). 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 plant antioxidant systems (Jain et al. 2010). In our study, application of Zn increased antioxidant enzyme activity, which indicates the impact of Zn in

Table 2. Soil physicochemical properties at a depth of 0–35 cm.

Texture	Sand (%)	Silt (%)	Clay (%)	Total N (%)	*Available* K (mg kg^{-1})	*Available* P (mg kg^{-1})	$CaCO_3$ (%)	*Available* Zn (mg kg^{-1})	Organic Carbon (%)	EC (dsm^{-1})	pH	SP (%)
Silt loam	35	45	26	0.058	219	8.29	14.4	28	0.62	2.68	7.8	49



W_0, W_1 and W_2 are normal irrigation (control), moderate and severe water limitation, respectively.

F_0, F_1, F_2 and F_3 are no biofertilizer, application of mycorrhiza, plant-growth promoting rhizobacteria (PGPR), and application of both PGPR and mycorrhiza, respectively.

Zn_0, Zn_1, Zn_2 and Zn_3 are without nano zinc oxide as control, application of 0.3, 0.6 and 0.9 g L^{-1}, respectively.
OD – optical density

Fig. 1. Effect of water limitation, application of biofertilizers and nano zinc oxide on catalase (CAT) and polyphenoloxidase (PPO) activity of *Triticale* (mean of 2 yr or combined analysis of the 2 yr, 2014–2015).

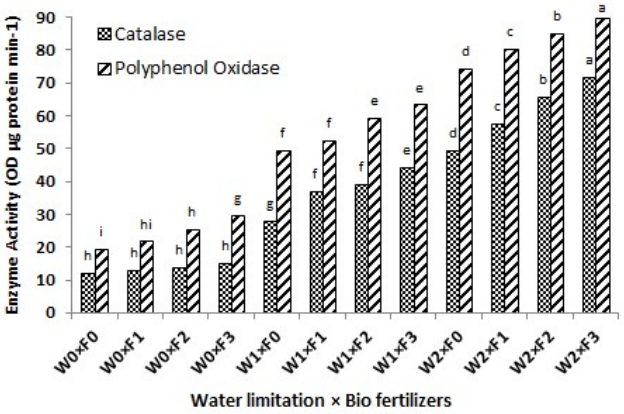
relieving the effect of stress. Jain et al. (2010) also reported that activity of POD and CAT enzymes increased under high levels of Zn application.

The interaction effect between water limitation and biofertilizers showed that the maximum of CAT (71.94 OD µg protein min^{-1}) and PPO (89.73 OD µg protein min^{-1}) activities were obtained in severe water limitation and biofertilizer application as F_3 (Fig. 2). The lowest CAT (12 OD µg protein min^{-1}) and PPO (19.32 OD µg protein min^{-1}) activity was obtained in W_0F_0 (Fig. 2). On the other hand, there was an increase of 45.1% and 20.6% in the activity of CAT and PPO enzymes, respectively, in the highest levels of water limitation and biofertilizers (W_2F_3) in comparison with W_2F_0 (Fig. 2).

Also, the interaction effect between water limitation and nano zinc oxide showed that the highest CAT enzyme activity (66.78 OD µg protein min^{-1}) was obtained in W_2Zn_3 (Fig. 3), while the minimum (12.13 OD µg protein min^{-1}) was observed in W_0Zn_0. There was an increase of 18.8% in the activity of CAT enzyme in W_2Zn_3 treatment compared with W_2Zn_0.

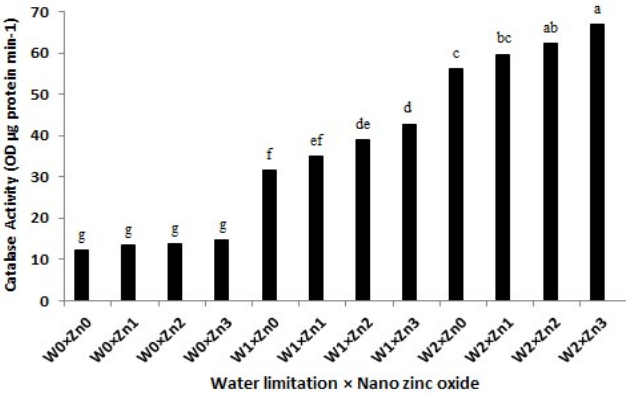
The highest POD activity (162.04 OD µg protein min^{-1}) was observed in severe water limitation, biofertilizer application as F_3 (130.67 OD µg protein min^{-1}) and nano zinc oxide application as Zn_3 (130.5 OD µg protein min^{-1}) (Fig. 4). The lowest of these activities (82.42, 118.37 and 117.79 OD µg protein min^{-1}, respectively) were obtained in normal irrigation, application of biofertilizer as F_0, and application of nano zinc oxide as Zn_0 (Fig. 4).

A continued increase in CAT, POD and PPO activity indicates that these enzymes are major enzymes detoxifying hydrogen peroxide in *Triticale* under drought stress. All plant species naturally have various defensive networks to protect their cells from the deleterious effects of ROS which include enzymatic and non-enzymatic antioxidants (Farooq et al. 2009). POD is the primary H_2O_2-scavenging enzyme that detoxifies H_2O_2 in the chloroplasts and cytosol of the plant cells, and CAT is frequently used by cells to rapidly catalyze the decomposition of H_2O_2 into less reactive gaseous oxygen and H_2O (Gusain et al. 2015). These two enzymes constitute the main safeguards of the H_2O_2-scavenging system in cells. Under stress conditions, the activity of these enzymes is altered and the degree of alteration may be linked to stress tolerance of the plant (Farooq et al. 2009).



W_0, W_1 and W_2 are normal irrigation (control), moderate and severe water limitation, respectively.
F_0, F_1, F_2 and F_3 are no biofertilizer, application of mycorrhiza, plant-growth promoting rhizobacteria (PGPR), and application of both PGPR and mycorrhiza, respectively.
OD – optical density

Fig. 2. Effect of water limitation and biofertilizers application on catalase (CAT) and polyphenoloxidase (PPO) activity (mean of 2 yr or combined analysis of the 2 yr, 2014–2015).

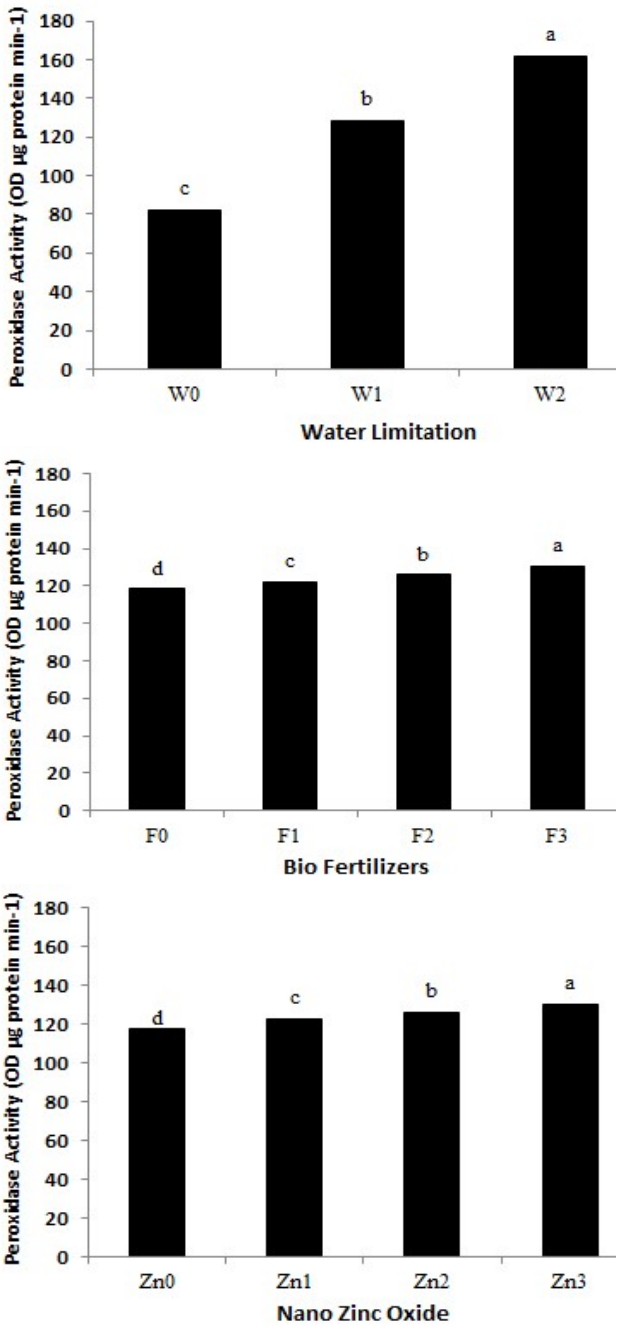


W_0, W_1 and W_2 are normal irrigation (control), moderate and severe water limitation, respectively. Zn_0, Zn_1, Zn_2 and Zn_3 are without nano zinc oxide as control, application of 0.3, 0.6 and 0.9 g L^{-1}, respectively.
OD – optical density

Fig. 3. Effect of water limitation and nano zinc oxide on catalase (CAT) activity (mean of 2 yr or combined analysis of the 2 yr, 2014–2015).

Proline and Soluble Sugars Content

The proline and soluble sugars content significantly increased under water limitation condition. These results are in accordance with those observed by Abass and Mohamed (2011) who reported that drought condition caused a significant increase in the proline and soluble sugars content in shoots of the common bean. Inoculation with PGPR, mycorrhiza



W_0, W_1 and W_2 are normal irrigation (control), moderate and severe water limitation, respectively.

F_0, F_1, F_2 and F_3 are no biofertilizer, application of mycorrhiza, plant-growth promoting rhizobacteria (PGPR), and application of both PGPR and mycorrhiza, respectively.

Zn_0, Zn_1, Zn_2 and Zn_3 are without nano zinc oxide as control, application of 0.3, 0.6 and 0.9 g L^{-1}, respectively.
OD – optical density

Fig. 4. Effect of water limitation, biofertilizers and nano zinc oxide on peroxidase (POD) activity of *Triticale* (means of 2 yr or combined analysis of the 2 yr, 2014–2015).

and both of these biofertilizers under water limitation increased proline and soluble sugars of *Triticale*. In addition, the proline and soluble sugars content significantly increased when nano zinc oxide was applied. Results showed that the highest content of proline (9.82, 7.71 and 7.94 mg g^{-1} FW) and that of soluble sugars (102.88, 81.2 and 82.78 mg g^{-1} FW) were observed in severe water limitation, biofertilizer application as F_3 and nano zinc oxide as Zn_3, respectively (Table 3). The lowest content of proline (4.71, 7.1 and 6.85 mg g^{-1} FW) and soluble sugars (41.64, 64.56 and 63.53 mg g^{-1} FW) were obtained at W_0, F_0 and Zn_0, respectively (Table 3). The interaction effect between water limitation × biofertilizers × nano zinc oxide showed that the highest content of soluble sugars (125.09 mg g^{-1} FW) and proline (10.89 mg g^{-1} FW) were obtained in severe water limitation, application of biofertilizer as F_3 and nano zinc oxide as Zn_3 (Table 4). The minimum of soluble sugars (36.44 mg g^{-1} FW) and that of proline (4.01 mg g^{-1} FW) were obtained in W_0, F_0 and Zn_0 (Table 4). There was an increase of 46.1% and 21.1% in the content of soluble sugars and proline in the $W_2F_3Zn_3$ treatment compared with $W_2F_0Zn_0$ (Table 4).

In response to different stresses, plants accumulate large quantities of different types of compatible solutes. Compatible solutes are low in molecular weight, but are highly soluble organic compounds that are usually non-toxic at high cellular concentrations. These solutes provide protection to plants from stress by contributing to cellular osmotic adjustment, ROS detoxification, protection of membrane integrity and enzyme/protein stabilization (Farooq et al. 2009). Proline accumulation is the most important contributing factor among osmolytes involved in osmoregulation in plants exposed to drought stress (Szabados and Savoure 2009). The maintenance of leaf turgor under water stress might be achieved through proline accumulation in the cytoplasm, thus improving water uptake from drying soil (Oraki et al. 2012). Moreover, proline has been demonstrated to provide wheat plants tolerance to drought stress by strengthening the antioxidant system rather than by increasing osmotic adjustment (Szabados and Savoure 2009). Also, proline can act as a signaling molecule to modulate mitochondrial functions, influence cell proliferation or cell death and trigger specific gene expression, which can be essential for plant recovery from stress (Szabados and Savoure 2009).

Water stress also increases the levels of soluble sugars. These compounds protect plants against stresses by cellular adjustment through the protection of membrane integrity and enzyme stability (Farooq et al. 2009). Concentration of sugars may increase photosynthesis of plants during stress and also prevent plasmolysis. However, under water deficit, accumulated soluble sugars and proline contribute to the turgor maintenance by osmotic adjustment (Farooq et al. 2009).

Table 3. Effect of water limitation, application of biofertilizers and nano zinc oxide on physiological traits of *Triticale* (means of 2 yr or combined analysis of the 2 yr, 2014–2015).

Treatment	Proline (mg g^{-1} FW)	Soluble Sugars (mg g^{-1} FW)	Chlorophyll *a* (mg g^{-1} FW)	Chlorophyll *b* (mg g^{-1} FW)	Total Chlorophyll (mg g^{-1} FW)	Carotenoid (mg g^{-1} FW)	Yield (g m^{-2})
W_0	4.71 c	41.64 c	5.71 a	1.9 a	7.62 a	0.708 a	465.1 a
W_1	7.66 b	72.32 b	3.65 b	1.28 b	4.94 b	0.471 b	363.7 b
W_2	9.82 a	102.88 a	2.92 c	1.11 c	4.03 c	0.325 c	270 c
$LSD_{0.05}$	0.08	1.17	0.06	0.03	0.06	0.015	0.47
F_0	7.1 d	64.56 d	3.55 d	1.31 c	4.86 d	0.439 d	309.2 d
F_1	7.3 c	68.91 c	3.95 c	1.39 b	5.35 c	0.474 c	378.7 b
F_2	7.48 b	74.46 b	4.31 b	1.42 b	5.74 b	0.519 b	343.6 c
F_3	7.71 a	81.2 a	4.56 a	1.6 a	6.17 a	0.572 a	433.7 a
$LSD_{0.05}$	0.1	1.35	0.06	0.03	0.07	0.017	0.55
Zn_0	6.85 d	63.53 d	3.62 d	1.2 d	4.82 d	0.458 d	303.1 d
Zn_1	7.22 c	67.55 c	3.93 c	1.35 c	5.29 c	0.477 c	348.5 c
Zn_2	7.58 b	76.28 b	4.29 b	1.5 b	5.8 b	0.521 b	379.3 b
Zn_3	7.94 a	82.78 a	4.53 a	1.68 a	6.22 a	0.549 a	434.3 a
$LSD_{0.05}$	0.1	1.35	0.06	0.03	0.07	0.017	0.55

Means with similar letters in each column are not significantly different at $p \leq 0.05$ by LSD test.
W_0, W_1 and W_2 are normal irrigation (control), moderate and severe water limitation, respectively.
F_0, F_1, F_2 and F_3 are no biofertilizer, application of mycorrhiza, application of plant-growth promoting rhizobacteria (PGPR), and application of both PGPR and mycorrhiza, respectively.
Zn_0, Zn_1, Zn_2 and Zn_3 are without nano zinc oxide as control, application of 0.3, 0.6 and 0.9 g L^{-1}, respectively.

Table 4. Interaction effect between water limitation × biofertilizers × nano zinc oxide on proline and soluble sugars of *Triticale* (mean of 2 yr or combined analysis of the 2 yr, 2014–2015).

Treatment		Proline (mg g^{-1} FW)				Soluble Sugars (mg g^{-1} FW)			
		Zinc Levels (g L^{-1})				Zinc Levels (g L^{-1})			
		0	0.3	0.6	0.9	0	0.3	0.6	0.9
W_0	F_0	4.01 ± 0.07	4.18 ± 0.18	4.44 ± 0.34	4.79 ± 0.27	36.44 ± 2.73	37.23 ± 2.63	38.37 ± 2.56	40.32 ± 0.94
	F_1	4.13 ± 0.21	4.43 ± 0.28	4.55 ± 0.31	4.96 ± 0.33	36.77 ± 2.54	37.88 ± 3.46	39.64 ± 3.74	43.13 ± 0.86
	F_2	4.21 ± 0.19	4.44 ± 0.4	4.87 ± 0.39	5.39 ± 0.46	38.27 ± 2.94	40.02 ± 3	45.12 ± 3.26	46.46 ± 2.49
	F_3	5 ± 0.38	5.06 ± 0.31	5.4 ± 0.26	5.61 ± 0.3	44.68 ± 1.6	45.32 ± 0.94	45.65 ± 1.2	48.05 ± 0.84
W_1	F_0	6.55 ± 0.42	7.54 ± 0.41	7.61 ± 0.51	7.97 ± 0.71	50.27 ± 2.62	52.63 ± 2.64	63.93 ± 4.87	80.64 ± 6.04
	F_1	6.83 ± 0.29	7.61 ± 0.51	7.87 ± 0.54	7.99 ± 0.71	51.04 ± 6	58.65 ± 4.99	80.11 ± 5.35	89.77 ± 5.87
	F_2	7.38 ± 0.43	7.86 ± 0.61	7.86 ± 0.47	8.12 ± 0.49	57.64 ± 4.2	66.74 ± 4.73	81.18 ± 4.72	92.18 ± 5.15
	F_3	7.53 ± 0.49	7.8 ± 0.59	7.75 ± 0.81	8.33 ± 0.54	70.62 ± 4.68	77.72 ± 2.93	89.92 ± 5.82	97.18 ± 3.33
W_2	F_0	8.99 ± 0.74	9.25 ± 0.49	9.67 ± 0.4	10.27 ± 0.4	85.59 ± 5.57	88.78 ± 5.35	99.68 ± 2.16	103.06 ± 2.48
	F_1	9.12 ± 0.51	9.32 ± 0.64	10.13 ± 0.67	10.3 ± 0.54	87.66 ± 5.33	92.48 ± 3.81	105.7 ± 3.41	104.94 ± 3.93
	F_2	9.11 ± 0.64	9.57 ± 0.56	10.28 ± 0.41	10.67 ± 0.46	92.41 ± 2.56	103.92 ± 1.1	110.06 ± 3.11	119.57 ± 1.18
	F_3	9.33 ± 0.64	9.66 ± 0.53	10.53 ± 0.45	10.89 ± 0.26	101.94 ± 1.67	109.27 ± 1.03	115.98 ± 3.65	125.09 ± 2.52
$LSD_{0.05}$				0.34				4.7	

W_0, W_1 and W_2 are normal irrigation (control), moderate and severe water limitation, respectively.
F_0, F_1, F_2 and F_3 are no biofertilizer, application of mycorrhiza, plant-growth promoting rhizobacteria (PGPR), and application of both PGPR and mycorrhiza, respectively. (LSD test, $P < 0.05$).

Mycorrhizae enhanced concentration of soluble sugars in host plants. For example, improved osmoregulation capacity in AM-inoculated maize was related to higher soluble sugar (Feng et al. 2002). It was stated that vesicular arbuscular mycorrhiza (VAM) fungi significantly increased photosynthesis of host plants and thereby caused an increase in sugar content (Feng et al. 2002). PGPR strain *Pseudomonas mendocina* Palleroni and an arbuscular mycorrhizal (AM) fungus (either *Glomus intraradices* or *Glomus mosseae*) significantly enhanced proline accumulation in lettuce leaves under moderate and severe drought stress (Kohler et al. 2008). PGPRs consortia alleviated drought stress in rice plants by enhancing accumulation of proline in plants grown under drought conditions, and thereby improved plant growth (Gusain et al. 2015). Zinc foliar application activated enzymes involved in ROS detoxification (Cakmak 2000).

Photosynthetic Pigments

Water limitation, biofertilizers and nano zinc oxide significantly affected photosynthetic pigment content. Means comparison showed that the highest content of chlorophyll *a* (5.71, 4.56 and 4.53 mg g^{-1} FW), chlorophyll *b* (1.9, 1.6 and 1.68 mg g^{-1} FW), total chlorophyll (7.62, 6.17 and 6.22 mg g^{-1} FW) and carotenoids (0.708, 0.572 and 0.549 mg g^{-1} FW) were observed in normal irrigation, biofertilizer application as F_3 and nano zinc oxide as Zn_3, respectively (Table 3). The minimum content of chlorophyll *a* (2.92, 3.55 and 3.62 mg g^{-1} FW), chlorophyll *b* (1.11, 1.31 and 1.2 mg g^{-1} FW), total chlorophyll (4.03, 4.86 and 4.82 mg g^{-1} FW) and carotenoids (0.325, 0.439 and 0.458 mg g^{-1} FW) were observed in W_2, F_0 and Zn_0, respectively (Table 3). Interaction effect between water limitation × biofertilizers × nano zinc oxide showed that the highest content of chlorophyll *a* (7 mg g^{-1} FW) and chlorophyll *b* (2.51 mg g^{-1} FW) were obtained in $W_0F_3Zn_3$, while the lowest rates (2.3 and 0.86 mg g^{-1}, FW respectively) were obtained in $W_2F_0Zn_0$ (Table 5). Furthermore, biofertilizers and nano zinc oxide foliar application enhanced chlorophyll content under stress condition. Water limitation caused reduction in carotenoids and total chlorophyll, while application of biofertilizers and nano zinc oxide increased values for these traits. Also, interaction effect between water limitation × biofertilizers × nano zinc oxide showed that the highest content of total chlorophyll (9.52 mg g^{-1} FW) and carotenoids (0.972 mg g^{-1} FW) were observed in normal irrigation, application of biofertilizers as F_3, and foliar spraying with Zn_3 (Table 6). The lowest amount of total chlorophyll (3.16 mg g^{-1} FW) and carotenoids (0.25 mg g^{-1} FW) was obtained in $W_2F_0Zn_0$ (Table 6). Results showed that under severe water limitation, application of biofertilizers as F_3 and foliar spraying as Zn_3 increased to 58.2%, 72%, 62.3% and 64.8% the content of chlorophyll *a*, chlorophyll *b*, total chlorophyll and carotenoid, respectively, in comparison with F_0 and Zn_0 in the same water limitation level (Tables 5 and 6).

Decrease in photosynthetic pigments under severe water limitation might be due to the reduction in the synthesis of essential compounds such as

Table 5. Interaction effect between water limitation × biofertilizers × nano zinc oxide on chlorophyll *a* and chlorophyll *b* of *Triticale* (mean of 2 yr or combined analysis of the 2 yr, 2014–2015).

Treatment		Chlorophyll *a* (mg g^{-1} FW)				Chlorophyll *b* (mg g^{-1} FW)			
		Zinc Levels (g L^{-1})				Zinc Levels (g L^{-1})			
		0	0.3	0.6	0.9	0	0.3	0.6	0.9
W_0	F_0	4.65 ± 0.23	4.8 ± 0.3	4.85 ± 0.25	5.02 ± 0.39	1.51 ± 0.22	1.61 ± 0.2	1.67 ± 0.21	2.06 ± 0.06
	F_1	4.82 ± 0.38	5.06 ± 0.22	5.71 ± 0.39	5.9 ± 0.43	1.62 ± 0.19	1.88 ± 0.35	1.95 ± 0.22	2.23 ± 0.18
	F_2	5.52 ± 0.49	5.95 ± 0.37	6.49 ± 0.15	6.67 ± 0.2	1.59 ± 0.26	1.76 ± 0.26	2.09 ± 0.32	2.3 ± 0.22
	F_3	5.93 ± 0.36	6.44 ± 0.32	6.62 ± 0.32	7 ± 0.34	1.61 ± 0.23	1.74 ± 0.2	2.25 ± 0.21	2.51 ± 0.13
W_1	F_0	2.84 ± 0.2	2.88 ± 0.16	3.23 ± 0.33	3.43 ± 0.34	1.06 ± 0.21	1.21 ± 0.26	1.25 ± 0.25	1.31 ± 0.17
	F_1	2.89 ± 0.19	3.46 ± 0.27	4.01 ± 0.39	4.16 ± 0.23	1.07 ± 0.25	1.09 ± 0.27	1.2 ± 0.26	1.33 ± 0.32
	F_2	3.4 ± 0.29	3.8 ± 0.33	4.03 ± 0.28	4.25 ± 0.22	1 ± 0.25	1.08 ± 0.26	1.3 ± 0.25	1.48 ± 0.23
	F_3	3.42 ± 0.3	3.97 ± 0.29	4.2 ± 0.18	4.48 ± 0.33	1.28 ± 0.27	1.53 ± 0.24	1.55 ± 0.26	1.78 ± 0.37
W_2	F_0	2.3 ± 0.28	2.62 ± 0.29	2.84 ± 0.28	3.12 ± 0.34	0.86 ± 0.25	1.01 ± 0.27	1.08 ± 0.27	1.1 ± 0.3
	F_1	2.49 ± 0.3	2.73 ± 0.27	2.92 ± 0.28	3.27 ± 0.33	0.91 ± 0.24	1 ± 0.28	1.14 ± 0.26	1.3 ± 0.14
	F_2	2.51 ± 0.23	2.66 ± 0.28	3.09 ± 0.26	3.39 ± 0.31	0.9 ± 0.26	1.1 ± 0.26	1.15 ± 0.27	1.32 ± 0.16
	F_3	2.7 ± 0.11	2.86 ± 0.09	3.51 ± 0.27	3.64 ± 0.18	0.94 ± 0.28	1.18 ± 0.31	1.33 ± 0.28	1.48 ± 0.28
$LSD_{0.05}$		0.24	0.13						

W_0, W_1 and W_2 are normal irrigation (control), moderate and severe water limitation, respectively.
F_0, F_1, F_2 and F_3 are no biofertilizer, application of mycorrhiza, plant-growth promoting rhizobacteria (PGPR), and application of both PGPR and mycorrhiza, respectively. (LSD test, $P < 0.05$).

Table 6. Interaction effect between water limitation × biofertilizers × nano zinc oxide on total chlorophyll and carotenoid of *Triticale* (mean of 2 yr or combined analysis of the 2 yr, 2014–2015).

Treatment		Total Chlorophyll (mg g^{-1} FW)				Carotenoid (mg g^{-1} FW)			
		Zinc Levels (g L^{-1})				Zinc Levels (g L^{-1})			
		0	0.3	0.6	0.9	0	0.3	0.6	0.9
W_0	F_0	6.16 ± 0.43	6.42 ± 0.5	6.52 ± 0.46	7.09 ± 0.35	0.601 ± 0.01	0.623 ± 0.01	0.672 ± 0.04	0.603 ± 0.06
	F_1	6.45 ± 0.55	6.94 ± 0.55	7.67 ± 0.6	8.13 ± 0.61	0.626 ± 0.02	0.6 ± 0.02	0.61 ± 0.04	0.637 ± 0.02
	F_2	7.12 ± 0.66	7.71 ± 0.57	8.59 ± 0.44	8.98 ± 0.42	0.618 ± 0.03	0.668 ± 0.06	0.815 ± 0.07	0.866 ± 0.05
	F_3	7.54 ± 0.2	8.18 ± 0.51	8.87 ± 0.52	9.52 ± 0.45	0.692 ± 0.07	0.826 ± 0.03	0.897 ± 0.05	0.972 ± 0.07
W_1	F_0	3.91 ± 0.39	4.09 ± 0.4	4.49 ± 0.57	4.75 ± 0.5	0.385 ± 0.05	0.405 ± 0.07	0.428 ± 0.02	0.465 ± 0.02
	F_1	3.97 ± 0.44	4.55 ± 0.53	5.21 ± 0.64	5.49 ± 0.55	0.467 ± 0.01	0.473 ± 0.03	0.488 ± 0.02	0.499 ± 0.03
	F_2	4.41 ± 0.52	4.88 ± 0.58	5.34 ± 0.51	5.74 ± 0.42	0.443 ± 0.03	0.468 ± 0.06	0.48 ± 0.05	0.506 ± 0.05
	F_3	4.7 ± 0.54	5.51 ± 0.53	5.76 ± 0.4	6.27 ± 0.58	0.455 ± 0.01	0.445 ± 0.02	0.515 ± 0.04	0.613 ± 0.04
W_2	F_0	3.16 ± 0.53	3.63 ± 0.54	3.93 ± 0.55	4.22 ± 0.64	0.25 ± 0.04	0.26 ± 0.04	0.293 ± 0.07	0.291 ± 0.04
	F_1	3.41 ± 0.52	3.73 ± 0.52	4.07 ± 0.54	4.58 ± 0.48	0.289 ± 0.08	0.312 ± 0.07	0.335 ± 0.05	0.344 ± 0.05
	F_2	3.42 ± 0.48	3.76 ± 0.54	4.25 ± 0.54	4.72 ± 0.48	0.34 ± 0.05	0.304 ± 0.08	0.349 ± 0.06	0.378 ± 0.04
	F_3	3.65 ± 0.39	4.05 ± 0.4	4.85 ± 0.54	5.13 ± 0.44	0.337 ± 0.07	0.342 ± 0.06	0.363 ± 0.03	0.412 ± 0.05
$LSD_{0.05}$					0.27	0.059			

W_0, W_1 and W_2 are normal irrigation (control), moderate and severe water limitation, respectively.
F_0, F_1, F_2 and F_3 are no biofertilizer, application of mycorrhiza, plant-growth promoting rhizobacteria (PGPR), and application of both PGPR and mycorrhiza, respectively. (LSD test, $P < 0.05$).

chlorophyll pigment. Degradation of pigment protein complex and oxidative complex causes damage to the chloroplast lipids, pigments and proteins (Tambussi et al. 2005). Application of biofertilizers and nano zinc oxide increased the chlorophyll and carotenoid contents, which indicates the impact of biofertilizers and zinc in relieving the effect of stress. It seems that the main reason for the decrease in chlorophyll may be degradation by ROS. Another reason for the decline in chlorophyll is the application of a glutamate precursor for the biosynthesis of proline. Also, the reduction in chlorophyll content under drought stress has been considered as a typical symptom of oxidative stress and may be the result of pigment photo-oxidation and chlorophyll degradation. El-Ghinbihi and Hassan (2007) found that drought stress caused reduction in photosynthetic pigments [(chl. *a*, chl. *b*, total chl (*a* + *b*) and carotenoids)] of pepper plants. Relative chlorophyll content has a positive relation with photosynthetic rate.

The decrease in chlorophyll content has been considered a typical symptom of oxidative stress and chlorophyll degradation under water stress condition (Oraki et al. 2012). Sannazzaro et al. (2005) reported that plants inoculated with *Glomus intraradices* had higher protein and chlorophyll density than plants without mycorrhiza inoculation. Vivas et al. (2003) showed that inoculation of bacterial strain increased chlorophyll content of lettuce compared with the control plants. Apparently, zinc is involved in the production of chlorophyll. Zinc is also considered as

an excellent protective agent against the oxidation of these vital cell components under water stress condition (Cakmak 2000). The study of Zarrouk et al. (2005) also indicated a positive correlation of Zn concentrations with leaf chlorophyll content in plants.

Grain Yield

Our results also indicated that there was a significant difference among irrigation regimes in terms of grain yield (Table 3). Grain yield decreased as a result of moderate and severe water limitation, when compared with data from normal irrigation treatment. Grain yield increased as a result of the application of biofertilizers and nano zinc oxide under normal irrigation and water limitation. Results showed that the highest grain yield (465.1, 433.7 and 434.3 g m^{-2}) was observed in normal irrigation, biofertilizer application as F_3 and nano zinc oxide as Zn_3, respectively (Table 3). The lowest grain yield (270, 309.2 and 303.1 g m^{-2}) was observed in W_2, F_0 and Zn_0, respectively (Table 3). Interaction effect between water limitation × biofertilizers × nano zinc oxide showed that the maximum grain yield (663.2 g m^{-2}) was observed in normal irrigation and application of biofertilizer as F_3 and nano zinc oxide as Zn_3 (Table 7). The lowest yield (198.46 g m^{-2}) was obtained in $W_2F_0Zn_0$ (Table 7).

Azcon and Barea (2010) proposed co-inoculation of PGPR and AM fungi as an efficient procedure to increase yield and plant growth. Vivas et al. (2003) suggested that there are synergistic effects on plant growth when bacteria (PGPR) and AM fungi are inoculated, particularly under limited growth conditions. Many researchers have indicated that biofertilizers can alleviate the unfavorable effects of drought stress on plant growth (Khalafallah and Abo-Ghalia 2008). Significant increases in growth and yield of agronomically important crops in response to inoculation with PGPR have been reported by Sandhya et al. (2010). Significant increases in grain yield with foliar Zn application have also been reported in other crops (Cakmak 2000).

CONCLUSION

The results showed that water limitation reduced yield and photosynthetic pigments of the plants. The activity of CAT, POD and PPO enzymes, and soluble sugars and proline content increased under limited water conditions. Application of biofertilizer and nano zinc oxide increased grain yield, photosynthetic pigments, the activity of antioxidant enzymes, and proline and soluble sugars content under water limitation conditions. Our results showed that the highest yield and photosynthetic pigments were observed in normal irrigation, biofertilizer application as F_3 and nano zinc oxide as Zn_3, while the highest proline and soluble sugars content and CAT, POD and PPO enzyme activity were obtained under severe water limitation, biofertilizer application as F_3 and nano zinc oxide as Zn_3. Effects of water limitation × biofertilizers × nano zinc oxide showed that the highest photosynthetic pigments

Table 7. Interaction effect between water limitation × biofertilizers × nano zinc oxide on yield of *Triticale* (mean of 2 yr or combined analysis of the 2 yr, 2014–2015).

Treatment		Yield (g m^{-2})			
		Zinc Levels (g L^{-1})			
		0	0.3	0.6	0.9
W_0	F_0	317.6 ± 6	326.1 ± 7.05	407.7 ± 7.01	462 ± 5.79
	F_1	410.4 ± 7.1	488.1 ± 8	492.7 ± 7.8	570.8 ± 7.6
	F_2	388.5 ± 6.15	398.4 ± 7.75	419.2 ± 5.39	512.3 ± 8.59
	F_3	450.6 ± 6.97	558.6 ± 8.45	576.4 ± 7.5	663.2 ± 6.55
W_1	F_0	255 ± 7.83	296.5 ± 6.38	334.6 ± 8.82	371.1 ± 7.99
	F_1	311.5 ± 6.95	363.2 ± 7.18	383.9 ± 7.75	418.9 ± 8.09
	F_2	292 ± 8.32	312.1 ± 9.7	380.6 ± 7.8	418.2 ± 7.2
	F_3	344.5 ± 7.61	399.8 ± 6.81	450.6 ± 7.61	487.1 ± 8.45
W_2	F_0	198.4 ± 6.26	219 ± 7.95	242.9 ± 7.3	280 ± 10.3
	F_1	219.1 ± 8.06	258 ± 8.96	264.2 ± 7.52	363.7 ± 7.12
	F_2	210 ± 7.21	241.6 ± 8.55	258.1 ± 7.6	292 ± 8.86
	F_3	239.4 ± 9.11	320.7 ± 7.46	341.3 ± 8.45	372. ± 8.01
$LSD_{0.05}$				17.42	

W_0, W_1 and W_2 are normal irrigation (control), moderate and severe water limitation, respectively.
F_0, F_1, F_2 and F_3 are no biofertilizer, application of mycorrhiza, plant-growth promoting rhizobacteria (PGPR), and application of both PGPR and mycorrhiza, respectively. (LSD test, $P < 0.05$).

and yield were observed in $W_0F_3Zn_3$, but the highest contents of proline and soluble sugars were observed in $W_2F_3Zn_3$. It seems that plants apply defensive mechanisms such as syntheses of antioxidant enzymes, soluble sugars and proline to alleviate damage caused by stress. Based on these results, application of biofertilizer and nano zinc oxide can be recommended for profitable *Triticale* production under water limitation conditions.

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