EXOGENOUS APPLICATION OF 24-EPIBRASSINOLIDE AND NANO-ZINC OXIDE AT FLOWERING IMPROVES OSMOTIC STRESS TOLERANCE IN HARVESTED TOMATO SEEDS

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Abstract. The favorable effects of exogenously applied brassinosteroids and zinc micronutrient on plant growth performance and seed development have been extensively outlined in many studies. Nevertheless, almost no published document is available on the effects of 24-epibrassinolide and nano-zinc oxide on tomato seed quality and osmotic stress tolerance of the resulted seedlings. In this study, the effects of 24-epibrassinolide (0, 15 and 30 $mg.ha^{-1}$ EBL) and nano-zinc oxide (0, 600 and 1200 ppm N-ZnO), which were exogenously applied on tomato mother plants, on seed germination parameters and stress tolerance capability of the produced seedlings under osmotic stress conditions (water potential of 0, -0.1 and -0.2 MPa stimulated by PEG-6000) were investigated. According to obtained results, the water potential of -0.2 MPa resulted in the lowest seed germinability and seedling growth performance with the highest amount of proline, hydrogen peroxide, malondialdehyde and activity of catalase and peroxidase, compared to non-stress control. All the foliar treatments resulted in decreased H_2O_2 and MDA content, especially EBL_2+N-ZnO_2. In addition, foliar application of EBL and N-ZnO enhanced the activity of antioxidative enzymes and ameliorated the inhibitory effects of osmotic stress on tomato seedling growth, except for percentage and rate of germination.
Keywords: *antioxidant system, brassinosteroids, micronutrients, seed quality, water potential*

Introduction

Tomato is the second most important vegetable crop next to potato worldwide (Eevera and Vanangamudi, 2006), and also it is highly popular in Northwest of Iran. According to the Food and Agriculture Organization (FAO), in 2016, around 4.7 million hectares of world farms allocated to tomatoes, which included about 177 million tons of annual production of fresh fruit (Anonymous, 2016). Tomato is mainly cultivated in tropical and sub-tropical countries, preferably in Mediterranean regions, which is mostly encountered by the varying environmental fluctuations. Among these adverse conditions, soil drying in seedbed is one of the critical problems that negatively influence seed germination and subsequent seedling growth (George, 2009; Chandrasekaran et al., 2017). The reduction in soil water potential results in disorder in seed germination by decreasing the imbibition rate (Eisvand et al., 2010). Individual seeds have to cross a threshold water potential in order to develop enzyme activity and lower their puncture force. The endosperm weakening opposite the radicle tip

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determines the threshold water potential for germination, which determines the rate and extent of germination (Finch-Savage, 2013).

In normal conditions, the reactive oxygen species (ROS) and antioxidant enzymes are produced in plant cells in a stable equilibrium (Gill and Tuteja, 2010), while under abiotic stresses, the balance can be overcome, mediating an accumulation of ROS and inducing oxidative damage (Mignolet-Spruyt et al., 2016). Lipid peroxidation as a main destructive event occurs due to increased ROS production and is detected by malondialdehyde accumulation in plant cells (Petrov et al., 2015). The antioxidant defense including the antioxidative enzymes (such as catalase and peroxidase) and metabolites is exploited by plant cells to cope with ROS attack (Gill and Tuteja, 2010). Catalase converts hydrogen peroxide (H_2O_2) into water and molecular oxygen (DeLay, 2017). Peroxidase can also detoxify H_2O_2 using various compounds as electron donor (Puthur, 2016). In addition, some researchers have introduced proline as an antioxidant molecule, proposing its role as ROS scavenger along with compatible osmolyte (Hossain et al., 2014; Rejeb et al., 2014). Under osmotic stress condition, the high capacity of antioxidant system is essential for plants to scavenge ROS and then avoid oxidative stress (Uzilday et al., 2014).

To ensure optimum stand establishment in soils with low water content, high-quality seeds must be used to achieve rapid and uniform seedling emergence, which may improve the resources use efficiency of established seedlings (Copeland and McDonald, 2012; Taiz et al., 2015). High-quality, also known as high-vigor seed lot has a great potential for rapid and uniform seed germination and successful seedling emergence under stressful conditions (Ellis and Roberts, 1981; Rehman et al., 1999; de Figueiredo et al., 2003). In all horticultural plants, including tomato, the high-quality seeds are the most important input in an efficient plant production system. So that, the low-quality seeds result in the poor stand establishment and consequently lower the crop productivity, especially under abiotic stresses (Rashid and Singh, 2000; Elias et al., 2006; George, 2009).

Seed quality consists of the physiological, physical and health attributes and is influenced by the growth conditions of the mother plant, which is well known as ‘maternal effect’ (Sangkram and Noomhorm, 2002; Pfeifer et al., 2011). In tomato plant, the fruit as a surrounding environment directly transfers the external conditions to the developing seeds. In this respect, the phytohormones and the mineral nutrients that reach the seeds play a key role in plant cell metabolism and ultimately improve the quality of produced seeds (Srivastava and Handa, 2005). Overall, the internal concentrations of plant growth regulators such as brassinosteroids and nutritional status of the plant, especially after adequate supply of micronutrients during the formation and development of seeds on the mother plant, are so vital. Some researchers have previously reported the production of high-quality seeds in plants exogenously treated with brassinosteroids and zinc (Biesaga-Koscielniak et al., 2014; Laware and Raskar, 2014).

Brassinosteroids are the sixth group of phytohormones, which have different physiological effects on seed and seedling tissues including the stimulation of cell elongation and division, the regulation of enzymes activity and the promotion of seed germination (Özdemir et al., 2004). Previous researchers have reported that these compounds alleviate the detrimental impacts of environmental hazards such as drought, on plant performance (Prusakova et al., 2000; Vardhini et al., 2010). In this regard, Yuan et al. (2010) demonstrated that the exogenous application of 24-epibrassinolide

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mediated drought tolerance in tomato plants through reducing malondialdehyde and hydrogen peroxide and increasing the activity of antioxidant enzymes. The micronutrient element of Zinc (Zn) plays a fundamental role in plant metabolism (Marschner, 2011) and biosynthesis of RNA, DNA and proteins (Welch, 2001). Although zinc is required in small amounts (5-100 ppm), its complete lack or insufficiency may influence the important physiological aspects of plant growth (Baybordi, 2006) including antioxidative enzymes and some regulatory metabolites (Cakmak, 2000). Supplemental zinc can alleviate the negative impacts of oxidative damage on plant metabolism (Tavallali et al., 2010). For example, zinc can help maintain sulfhydryl groups of cell membrane, which are easily oxidized by ROS (Rengel, 1995; Rengel and Wheal, 1997).

Already, some efforts have been made to produce high-quality seeds by employing certain agronomic techniques. The aim of the present research was to evaluate the osmotic stress tolerance capability (as an indicator of seed quality) of tomato seeds harvested from mother plants subjected to 24-epibrassinolide and nano-zinc oxide at the flowering stage. In the present experiment, seed germinability and seedling growth indices were measured and the antioxidative enzyme activities, hydrogen peroxide, malondialdehyde and proline accumulation in tomato seedlings were determined. This work is important as it demonstrates the collaborative role of brassinosteroid and zinc micro-element in providing osmotic stress tolerance and creating awareness of the potential for tomato plants to be cultivated in arid and semi-arid areas.

Materials and methods

The present experiment was conducted during 2015-2016 growing season in the Department of Agronomyand Plant Breeding, Faculty of Agriculture and Natural Resoursec at the University of Mohaghegh Ardabili, Ardabil, Iran. The seed lot of tomato (*Solanum lycopersicum* L.) cv. Y was obtained from SPCRI (Seed and Plant Certification and Registration Institute, Karaj). The viability and moisture content of prepared seeds were about 98% and 8%, respectively. All chemicals used in this research were purchased from Merck and Sigma-Aldrich.

Tomato seeds were sown in nursery beds of 0.5 m × 0.5 m, rich in loam and provided with shade and regular watering for seed germination. After 25 days of growth in the nursery, tomato plantlets were carefully transferred to the main field. The farm soil characteristics are listed in *Table 1*. The fertilizer application was 180 kg N, 150 kg P_2O_5 and 150 kg K per hectare. Tomato seedlings were transplanted in 3 rows in each plot at spacing of 45 cm × 120 cm, giving a total of 1500 plants per experimental unit. The final crop density was around 18,500 plants per hectare. A factorial experiment was conducted based on randomized complete block design (RCBD) with three replications. Experimental factors were foliar treatment combinations [control (only water), 15 $mg.ha^{-1}$ of 24-epibrassinolide (EBL_1), 30 $mg.ha^{-1}$ of 24-epibrassinolide (EBL_2), 600 ppm of nano-zinc oxide (N-ZnO_1), 1200 ppm of nano-zinc oxide (N-ZnO_2), EBL_1 + N-ZnO_1, EBL_1 + N-ZnO_2, EBL_2 + N-ZnO_1, EBL_2 + N-ZnO_2] and osmotic stress levels (0, -0.1 and -0.2 MPa). After complete vegetative growth in the field, foliar treatments with 24-epibrassinolide (EBL) and nano-zinc oxide (N-ZnO) were imposed at the flowering stage. Tween-20 (0.1%) was used as surfactant to enhance the effectiveness of foliar spray. The foliar applications were made with a portable field sprayer at 150 kPa pressure with 1000 l of liquid applied per hectare.

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Table 1. *Soil characteristics of the farm where the experiment was conducted*

Characteristics	Values
Soil texture	Loam
pH (1:1 H_2O)	7.4
EC_e ($dS.m^{-1}$)	3.8
Organic carbon (%)	0.9
Total N (%)	0.09
Available P ($mg.kg^{-1}$)	20
Available K ($mg.kg^{-1}$)	230
Zn ($mg.kg^{-1}$)	0.994

After harvesting the fully ripe fruits, the seeds were extracted by fermentation method (at 25 °C for 24 h) and then dried to moisture content of about 8%. The quality of obtained seeds was assayed under osmotic stress condition. In order to create the different water stress levels (0, -0.1 and -0.2 MPa) in seed germination mediums, PEG-6000 was used. The standard germination test of the processed seeds was conducted according to the rules approved by the International Seed Testing Association (ISTA, 2008). In brief, after 10 min of surface sterilization with sodium hypochlorite 1%, four replicates of 50 seeds were incubated in alternating temperatures (25/30 °C) for 14 days. The daily counting of the number of germinated seeds (>2 mm radicle protrusion) continued until the end of the 14th day. In each experimental unit, the standard germination percentage and dry weight and length of seedlings were measured. The germination rate was calculated using the formula of Ellis and Roberts (1981). The vigor index (VI) was calculated using the following equation: VI = seedling length × germination percentage.

Free proline content in tomato seedlings was determined using ninhydrin reagent according to the most used method described by Bates et al. (1973). Malondialdehyde (MDA) content was measured by thiobarbituric acid reaction (Heath and Packer, 1968), with slight modifications. Fresh seedlings (0.1 g each) were homogenized in 1 ml of 0.1% (w/v) trichloroacetic acid (TCA) and the homogenates were centrifuged at 5000 × g for 10 min at 4 °C. In the following, 400 µl of the supernatant was added into1 ml of 0.5% (w/v) thiobarbituric acid. The obtained mixture was incubated at a temperature of 95 °C in a water bath for 30 min. After centrifugation at 10000 × g for 10 min, the absorbance of the supernatant was measured at 532 and 600 nm in a spectrophotometer. To calculate the concentration of MDA, the related extinction coefficient (0.155 $\mu M^{-1}.cm^{-1}$) was used and the values were expressed as $\mu mol.g^{-1}$ F.W. Hydrogen peroxide was quantified by the method of Hung et al. (2005). Fresh seedlings (0.1 g each) were homogenized in 1 ml of 0.1% (w/v) TCA. The obtained mixture was centrifuged at 10000 × g for 10 min, at 4 °C, then 0.5 ml of supernatant was added to 0.5 ml of 10 mM potassium phosphate buffer (pH = 7) and 1 ml of 1 mM potassium iodide. The absorbance was read at a wavelength of 390 nm. The amount of hydrogen peroxide in each sample was calculated using the specific extinction coefficient (0.28 $\mu M^{-1}.cm^{-1}$) and the values were expressed in units of $\mu mol.g^{-1}$ F.W. Catalase (CAT) and peroxidase (POD) activities in tomato seedlings were assayed using the methods of Aebi (1984) and MacAdam et al. (1992), respectively.

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After conducting the normality test of obtained data, two-way analysis of variance (ANOVA) for randomized complete block design was done using SAS 9.4 software and the means were compared by least significant difference (LSD) test at 5% probability level and the figures were created with Microsoft Excel 2013.

Results

In the present study, tomato plants were exogenously treated with different combinations of 24-epibrassinolide (EBL) and nano-zinc oxide (N-ZnO) at the flowering stage and then the harvested seed lots were subjected to osmotic stress (OS). Finally, germination percentage and rate, seedling length, dry weight and vigor index, proline, malondialdehyde and H_2O_2 content and catalase and peroxidase activity in the seedlings were estimated. In general, the main effects and interaction of two experimental factors: (1) foliar application of EBL+N-ZnO and (2) osmotic stress were statistically significant ($P \leq 5\%$) in all measured characteristics, but germination percentage and rate which had the main effects, were significant (*Table 2*).

Table 2. *Analysis of variance of EBL+N-ZnO effects on some characteristics related to seed germination and seedling growth performance of tomato under drought stress condition*

S.O.V.	d.f.	Mean squares				
		Germination percentage	**Germination rate**	**Seedling length**	**Seedling weight**	**Vigour index**
Block	2	1.148^{ns}	0.0066**	0.031**	0.0002^{ns}	0.13^{ns}
EBL+N-ZnO	8	170.527**	0.0094**	4.714**	0.0538**	11.126**
Drought stress	2	148.111**	0.453**	96.123**	1.09**	176.553**
EBL+N-ZnO × drought stress	16	1.486^{ns}	0.00001^{ns}	1.008**	0.0113**	0.973**
Error	52	5.827	0.0005	0.0038	0.00006	0.135
C.V. (%)	-	*2.360*	*5.522*	*1.403*	*1.486*	*2.625*

Continuation

S.O.V.	d.f.	Mean squares				
		Proline content	**Hydrogen peroxide content**	**Malondialdehyde content**	**Catalase activity**	**Peroxidase activity**
Block	2	4733.405^{ns}	0.0009^{ns}	0.0008**	0.000002*	0.00001^{ns}
EBL+N-ZnO	8	152822.922**	0.157**	0.287**	0.00008**	0.0003**
Drought stress	2	2794500.611**	3.344**	1.216**	0.0012**	0.0150**
EBL+N-ZnO × drought stress	16	27339.950**	0.033**	0.075**	0.0002**	0.0001**
Error	52	5188.522	0.0004	0.050	0.000001	0.0002
C.V. (%)	-	*1.713*	*3.032*	*7.609*	*1.575*	*2.870*

The effect of osmotic stress on the germination percentage and rate of seeds produced from tomato mother plants exogenously treated with EBL and N-ZnO are shown in *Table 3*. Overall, the promotive influence of foliar application of EBL and N-ZnO on the germination percentage and rate were observed. The treatment, EBL_1 (15 mg.ha^{-1}), had no significant difference from control ($P > 5\%$) in percentage germination. The sole applications of EBL and N-ZnO were statistically the same in the rate of germination. The highest germination percentage and rate were recorded when EBL_2 + N-ZnO_2 was applied in the mother plants, compared to control in which only the

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distilled water was sprayed. The germination percentage and rate was significantly decreased with increased levels of osmotic stress. So that, the maximum reduction in germination percentage (about 5%) and rate (approximately 72%) were observed in water potential of -0.2 MPa compared with control.

Table 3. *Germination percentage and rate of seeds harvested from tomato plants subjected to exogenous EBL and N-ZnO in response to osmotic stress*

Experimental factors		**Germination percentage**	**Germination rate (d^{-1})**
Foliar application of EBL and N-ZnO	Control	84.0 ± 1.756 f	0.164 ± 0.039 e
	EBL_1 (15 $mg.ha^{-1}$)	85.8 ± 1.839 fe	0.178 ± 0.038 d
	EBL_2 (30 $mg.ha^{-1}$)	92.4 ± 1.732 c	0.185 ± 0.037 d
	N-ZnO_1 (600 ppm)	87.3 ±1.849 e	0.182 ± 0.037 d
	N-ZnO_2 (1200 ppm)	90.0 ± 1.636 d	0.181 ± 0.041 d
	EBL_1+N-ZnO_1	89.8 ± 1.632 d	0.197 ± 0.038 dc
	EBL_1+N-ZnO_2	93.7 ± 1.563 bc	0.221 ± 0.037 c
	EBL_2+N-ZnO_1	92.1 ± 1.501 b	0.244 ± 0.041 ab
	EBL_2+N-ZnO_2	97.1 ± 1.444 a	0.263 ± 0.038 a
LSD (5%)		**2.283**	**0.0346**
Osmotic stress	Control	93.1 ± 0.590 a	0.347 ± 0.009 a
	-0.1 MPa	89.9 ± 0.773 b	0.165 ± 0.008 b
	-0.2 MPa	88.1 ± 0.735 c	0.097 ± 0.008 c
LSD (5%)		**1.318**	**0.02**

Similar letters indicate no significant difference at $p \leq 5\%$

Changes in the length, dry weight and vigor index of tomato seedlings in response to treatment compounds almost followed the same trend (*Fig. 1 A-C*). Overall, the foliar treatments of EBL and N-ZnO significantly increased the seedling growth parameters, compared to untreated control (only water spray). On the other hand, the inhibitory effect of osmotic stress on the growth performance of tomato seedlings was intensified by decreasing the water potential. The highest values of seedling length (*Fig. 1A*), dry weight (*Fig. 1B*) and vigor index (*Fig. 1C*) were recorded in treatment combination of EBL_2+N-ZnO_2 under non-stress condition. The lowest length, dry weight and vigor index of tomato seedlings belonged to both N-ZnO_1 (600 ppm of nano-zinc oxide) and untreated control, when -0.2 MPa water potential was created in the growth medium. Notably, when the osmotic stress was amplified in seedling growth medium (-0.2 MPa water potential), the effectiveness of exogenously applied brassinosteroid and zinc was relatively maintained in comparison with the mild water deficit condition (-0.1 MPa water potential).

The free proline content in tomato seedlings was significantly influenced by osmotic stress levels ($P \leq 0.05$). In an osmotic potential of -0.2 MPa, the accumulation of proline was increased approximately by 15% compared to non-stress control. In general, the foliar treatments had negligible but significant positive effects on the proline amount ($P > 0.05$). On the other hand, the interaction between the two experimental factors was statistically significant. The proline content was considerably elevated in response to concomitant application of 24-epibrassinolide and nano-zinc oxide under both osmotic stress levels, so that the highest proline accumulation occurred in treatment combination of EBL_2+N-ZnO_2 under water potential of -0.2 MPa (*Fig. 2*).

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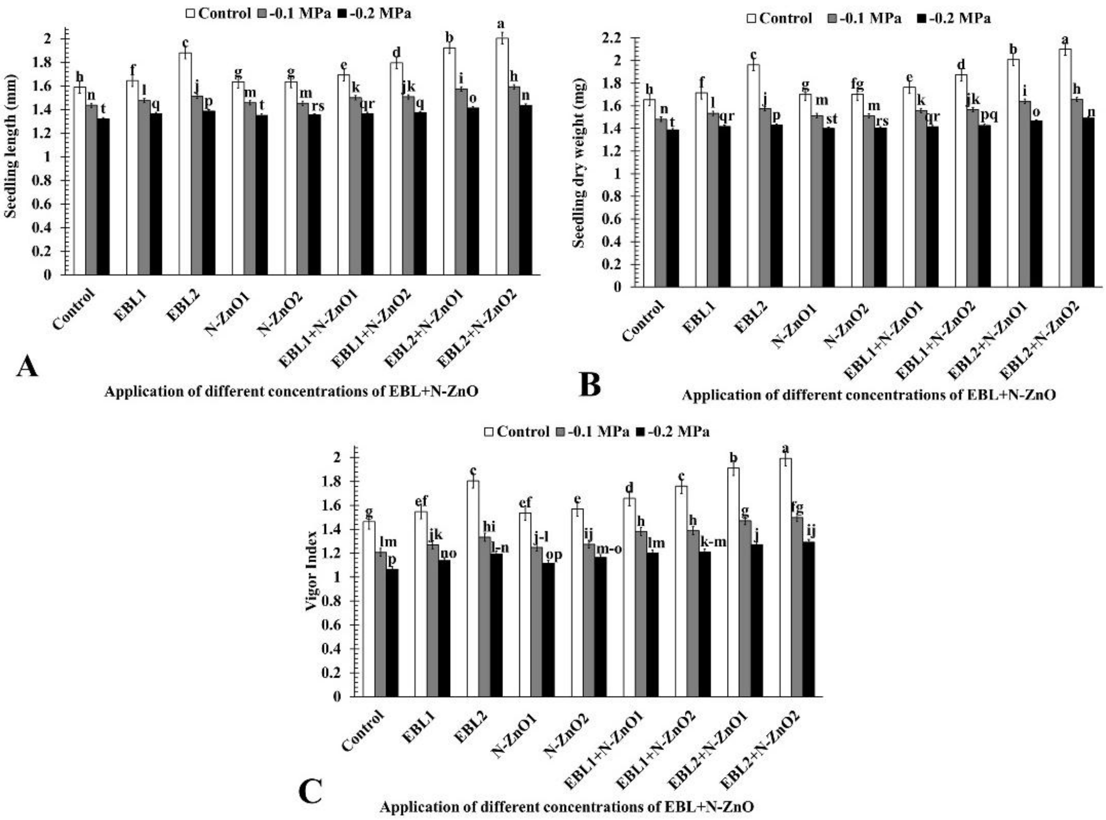

__Figure 1.__ Changes in the seedling length (A), dry weight (B) and vigor index (C) in response to foliar spray treatments (EBL and N-ZnO) applied on tomato mother plants and osmotic stress applied in the germination medium of produced seeds. Similar letters indicate no significant difference at $P \leq 5\%$. LSD values for the seedling length, dry weight and vigor index were 0.01, 0.0134 and 0.06, respectively

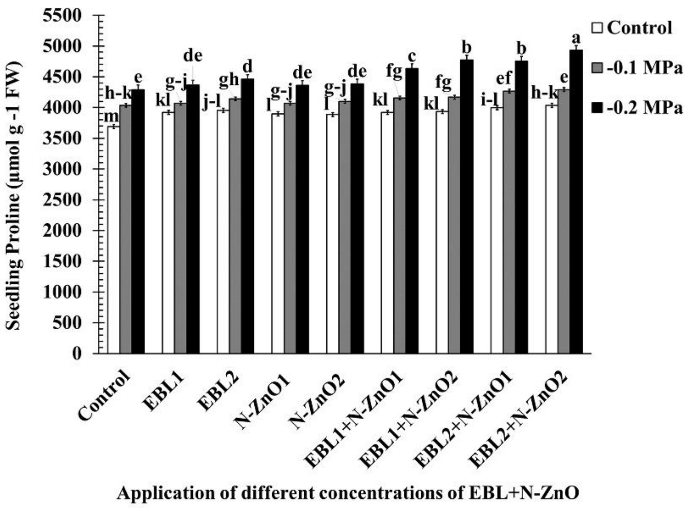

__Figure 2.__ Changes in the free proline content of seedling in response to foliar spray treatments (EBL and N-ZnO) applied on tomato mother plants and osmotic stress applied in the germination medium of produced seeds. Similar letters indicate no significant difference at $P \leq 5\%$. LSD value was 118.02

The tomato seedlings showed the higher H_2O_2 accumulation under osmotic stress compared to the corresponding control, especially when the osmotic potential of the culture medium was -0.2 MPa (*Fig. 3*). Under both non-stress and osmotic stress conditions, foliar sprayed EBL+N-ZnO combination had more significant reduction in the H_2O_2 accumulation compared to sole application of EBL and/or N-ZnO. The lowest H_2O_2 content was clearly observed in EBL_2+N-ZnO_2 (30 mg.ha^{-1} EBL and 1200 ppm N-ZnO) under non-stress condition. Among the sole applications of 24-epibrassinolide (EBL_1 and EBL_2) and nano-zinc oxide (N-ZnO_1 and N-ZnO_2), 30 mg.ha^{-1} brassinosteroid (EBL_2) showed more decrease in hydrogen peroxide amount.

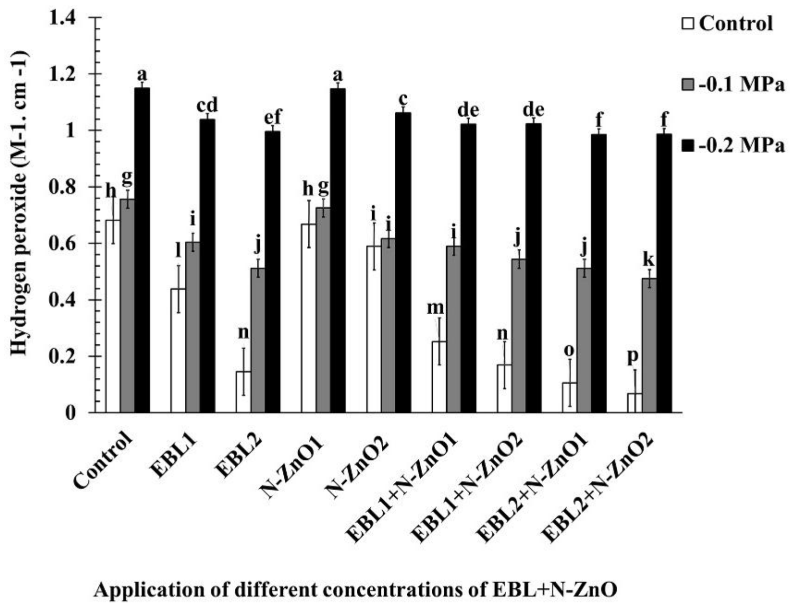

Figure 3. *Changes in the H_2O_2 content of seedling in response to foliar spray treatments (EBL and N-ZnO) applied on tomato mother plants and osmotic stress applied in the germination medium of produced seeds. Similar letters indicate no significant difference at $P \leq 5\%$. LSD value was 0.0328*

The osmotic stress led to a significant increase in malondialdehyde (MDA) content in tomato seedlings compared to corresponding control (*Fig. 4*). The greatest accumulation of MDA was recorded in tomato seedlings untreated with EBL and N-ZnO under osmotic stress level of -0.2 MPa. Among the sole applications of brassinosteroid and zinc, EBL_2 (30 mg.ha^{-1}) indicated more reduction in MDA content. Overall, the osmotic stress-induced MDA production was further restricted when EBL+N-ZnO combinations were exogenously applied, instead of using them alone. The lowest accumulation of malondialdehyde was observed in EBL_2 + N-ZnO_2 treatment under all three levels of osmotic stress.

As shown in *Figure 5,* the catalase activity in tomato seedlings was significantly increased under osmotic stress condition (-0.1 and -0.2 MPa) compared to control, and the foliar treatments relatively enhanced the CAT activity. The highest activity of catalase (about 3-fold of control) was observed in water potential of -0.2 MPa when 30 mg.ha^{-1} EBL and 1200 ppm N-ZnO were concomitantly applied.

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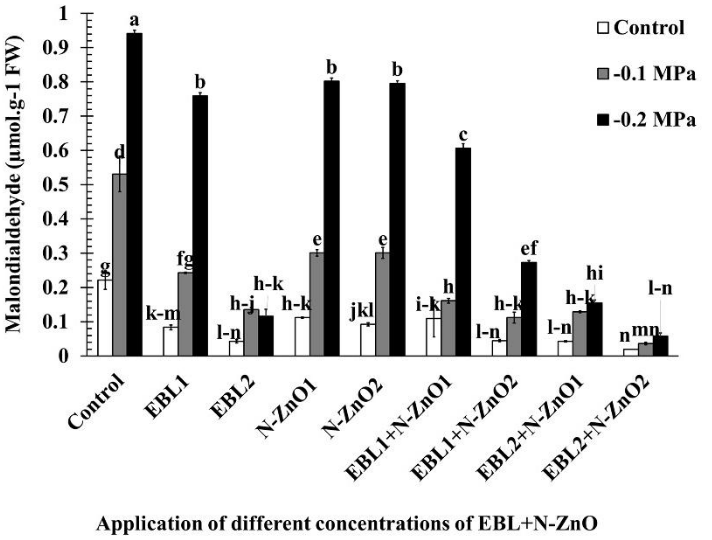

Figure 4. *Changes in the MDA content of seedling in response to foliar spray treatments (EBL and N-ZnO) applied on tomato mother plants and osmotic stress applied in the germination medium of produced seeds. Similar letters indicate no significant difference at P ≤ 5%. LSD value was 0.0509*

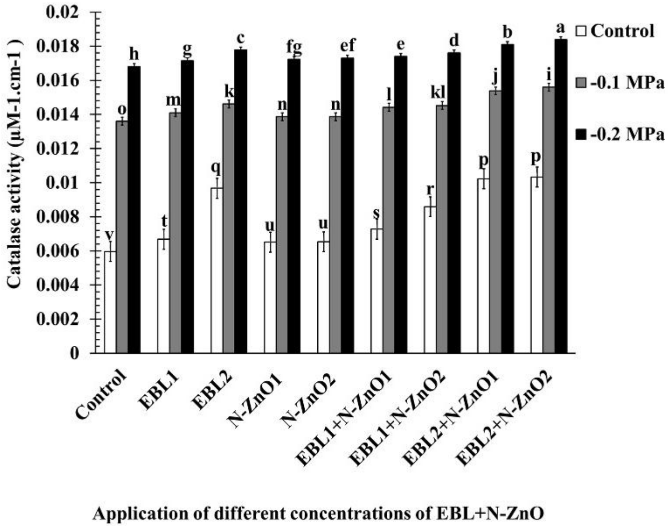

Figure 5. *Changes in the activity of CAT in response to foliar spray treatments (EBL and N-ZnO) applied on tomato mother plants and osmotic stress applied in the germination medium of produced seeds. Similar letters indicate no significant difference at P ≤ 5%. LSD value was 0.0001*

Under non-stress conditions, foliar sprayed EBL and N-ZnO did not have any significant effect on the peroxidase activities (*Fig. 6*). Under osmotic stress, the activities of POD in tomato seedlings were obviously increased, whereas the application of 24-epibrassinolide and nano-zinc oxide negligibly but significantly promoted the increase. Like the CAT enzyme, the highest activity of POD (more than two fold of control) was recorded in treatment combination of EBL_2 + N-ZnO_2 under water potential of -0.2 MPa.

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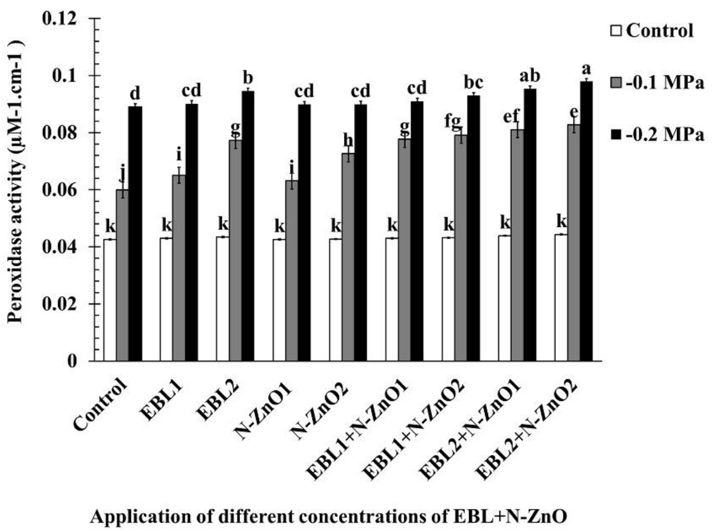

Figure 6. *Changes in the activity of POD in response to foliar spray treatments (EBL and N-ZnO) applied on tomato mother plants and osmotic stress applied in the germination medium of produced seeds. Similar letters indicate no significant difference at P ≤ 5%. LSD value was 0.0033*

Discussion

Promotive effects of brassinosteroid and zinc micronutrient on seed quantity and quality of field crops has already been mentioned by some authors (Hayat et al., 2001; Biesaga-Koscielniak et al., 2014; Laware and Raskar, 2014). According to previous studies, exogenous application of 24-epibrassinolide (Özdemir et al., 2004) and nano-zinc oxide (Prasad et al., 2012) at germination stage can lead to improved seed germinability and subsequent seedling growth. To the best of our knowledge, almost no research has been conducted to evaluate the effects of foliar spray of EBL and N-ZnO on seed germination and subsequent seedling performance under osmotic stress condition in seed lots produced from tomato mother plants. In the present research, the results showed that osmotic stress resulted in a delayed and decreased germination, which was not improve by foliar application of EBL and N-ZnO in tomato plants (*Table 2*). The results are in good agreement with previous investigations which reported osmotic stress-induced reduction in the seed germinability (PENG et al., 2013; Bhatt et al., 2015). Rajabi et al. (2013) stated that interruption in water absorption by seed could affect the metabolic activities related to germination process under osmotic stress condition. On the other hand, foliar spray of EBL and N-ZnO combinations at the flowering stage could enhance the percentage and rate of germination in the obtained seeds (*Table 2*). Brassinosteroids have a similar role as gibberellin in the germination process and enhances the germinability of seeds by reducing and/or increasing their biosynthesis and sensitivity to abscisic acid and/or gibberellin, respectively (Erik et al., 1996; Steber and McCourt, 2001; Zhang et al., 2009; Divi and Krishna, 2010). Similar results have reported that exogenous application of zinc in safflower (Movahhedy-Dehnavy et al., 2009) and soybean (Sedghi et al., 2013) increased the germination of produced seeds under drought stress conditions. Zinc micro-element is required for the biosynthesis of auxin (IAA) through the tryptamine pathway (Haslett et al., 2001), and has a promotive effect on the cell division (Alloway, 2004). Therefore, it can be

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concluded that Zn-mediated increase in the seed germination parameters can be originated from the signaling of IAA and the proliferation of cells.

According to the present results, the tomato seedling performance (measured as seedling length, dry weight and vigor index) was adversly affected when osmotic stress was intensified in the culture medium of harvested seeds. As seen in *Figure 1,* 30 mg.ha^{-1} EBL along with 1200 ppm N-ZnO (treatment combination of EBL_2 + N-ZnO_2) may to a great extent compensate for the drought-induced inhibition in tomato seedling growth. The results of the current study are consistent with the recent study (Khripach et al., 1998) in which the growth performance of seedlings was improved when the mother plants was treated with 24-epibrassinolide and the harvested seeds were exposed to low water potentials. Prasad et al. (2012) demonstrated that the foliar spray of nano-zinc oxide caused a significant increase in seedling length and dry weight, compared to the control. Boonchuay et al. (2013) found that foliar application of zinc resulted in higher seedling vigor index in seed lots obtained from exogenously treated mother plants. Overall, nanoparticles provide an efficient means to distribute agro-chemicals including fertilizers, in a controlled fashion (Agrawal and Rathore, 2014). So, plants can rapidly absorb slow-release nano-fertilizers and surely cause the saving of fertilizer consumption and minimize environmental pollution (Mura et al., 2013; Bindraban et al., 2015). It should be noted that the hormone (24-epibrassinolide) application was more efficient than the micronutrient (nano-zinc oxide), when only one of them was used alone, compared to EBL+N-ZnO co-application.

In consistency with the results of this experiment, many researchers reported that osmotic stress increases the accumulation of free proline in tomato seedlings (Calvo-Polanco et al., 2016; Sun et al., 2016). Kishor et al. (2014) stated that the proline accumulation has a positive and direct relationship with increasing the tolerance to water deficit stress in plants, as was evident in the current study (*Fig. 2*). Further increase in proline amount leads to preventing ROS attack and subsequently reducing the cell membrane damage. In addition, drought tolerance is increased by proline-induced osmotic adjustment (Szabados and Savouré, 2010). On the other hand, exogenous EBL and N-ZnO, exogenously applied in tomato mother plants during anthesis, promotes the production of free proline in seedlings under low water potential (*Fig. 2*). In this regard, Behnamnia et al. (2009) reported that 24-epibrassinolide increased the proline content in tomato leaves under limited water access. It has commonly been assumed that, EBL can promote free proline biosynthesis through inducing the expression of genes responsible for the related enzymes (Talaat and Shawky, 2013) and regulating the synthesis of nucleic acids (Bajgaz, 2000). As an important cofactor, Zinc has a key role in the production and activity of several enzymes (Bagci et al., 2007), particularly those involved in the main pathway of proline biosynthesis, i.e. glutamate pathway which is dominant in higher plants (Delaney et al., 1993). So, it seems that great increase in the proline content of tomato seedlings in response to exogenous application of EBL and N-ZnO at the flowering stage can help maintain an efficient osmotic homeostasis into the cell (by increasing proline and other compatible osmolytes), and therefore induce the osmotic stress tolerance in seedlings.

In this study, the hydrogen peroxide (*Fig. 3*) and malondialdehyde (*Fig. 4*) accumulation was clearly observed in leaves in response to low water potential imposed to seedlings from harvested tomato seeds. There is strong evidence that osmotic stress can result in the accumulation of ROS including hydrogen peroxide and MDA, as an indicator of lipid peroxidation, in different plant tissues (Todorova et al., 2016). As previous studies

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have emphasized, the overproduction of toxic ROS in plant cells under stress can mainly damage the lipid-containing components of cell (Tavallali et al., 2010) and then cause the plasma membrane disintegrity that increases the permeabilization (Anjum, 2015). As found in the present experiment, osmotic stress-induced accumulation of H_2O_2 and MDA in seedlings was significantly declined as a result of foliar treatment of tomato mother plants with 24-epibrassinolide and nano-zinc oxide (*Figs. 3* and *4*). Several researchers reported the decline in H_2O_2 and MDA content of leaves due to 24-epibrassinolide application (Yuan et al., 2010; Talaat and Shawky, 2013). Brassinosteroid hormones may prevent the membrane damage caused by lipid peroxidation through influencing fatty acid composition and regulating the expression of genes (Khripach et al., 1998; Cao et al., 2005). In addition, Zn reduced ROS generation by indirect activation of antioxidant system (Grewal and Wiliams, 2000; Bagci et al., 2007).

Higher plants evolutionary have an antioxidant defense mechanism for scavenging reactive oxygen species, and efficient modulation of this defense system is essential for plants to tolerate the stress-iduced oxidative damage (Mittova et al., 2015). In the present study, antioxidative enzymes including catalase (*Fig. 5*) and peroxidase (*Fig. 6*) showed a significant increase in tomato seedlings under osmotic stress conditions, which were effective in alleviating the adverse impact of ROS and subsequently preventing lipid peroxidation. Foliar application of EBL and N-ZnO resulted in increased activity of both CAT and POD, especially when no osmotic stress was imposed on tomato seedlings. This may also imply that CAT plays more important role than POD in scavenging excessive hydrogen peroxide in osmotic stress condition. This is in consistency with the same report in silicon-supplemented tomato seedlings exposed to water deficit stress (Shi et al., 2014). Yuan et al. (2010) reported that the use of epibrassinolide induced tolerance to drought in tomato via reducing malondialdehyde and hydrogen peroxide and increasing the activity of antioxidant enzymes. Researchers believe that brassinosteroids increase the resistance of plants to ROS-mediated oxidative damage through the modulation of expression of genes encoding antioxidative enzymes (Goda et al., 2002; Cao et al., 2005). Zinc is effective in the induction of gene expression responsible for the synthesis of proteins and antioxidant enzymes and in some cases, is also considred a cofactor for these enzymes (Grewal and Wiliams, 2000; Bagci et al., 2007; Alharby et al., 2016).

Conclusion

Finally, it could be concluded that seeds produced from tomato plants, which were treated with 24-epibrassinolide and nano-zinc oxide, showed higher tolerance to osmotic stress. So that seed germination indices, seedling growth performance and activity of antioxidative enzymes were significantly increased by preventing the accumulation of toxic ROS and MDA as the main peroxidative products. Although, both two applied concentrations of 24-epibrassinolide and nano-zinc oxide showed the promotive effects on osmotic stress tolerance in tomato seedlings, the combined treatment of 30 $mg.ha^{-1}$ 24-epibrassinolide and 1200 ppm nano-zinc oxide (EBL_2+N-ZnO_2), had higher tolerance capability. Based on the current findings, foliar application of 24-epibrassinolide and nano-zinc oxide with the optimal concentrations at the flowering stage is highly recommended. On the other hand, it is recommended that researchers investigate the effects of optimal concentrations of 24-epibrassinolide and nano-zinc oxide on different developmental stages of tomato seeds and report their results in order to complete the results of current study.

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