Zinc oxide nanoparticles alleviate drought stress effects on soybean antioxidant system during germination

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Abstract

In order to assess the effects of zinc oxide nanoparticles (ZO-NP) on soybean seed germination under drought stress induced by polyethylene glycol, a factorial experiment was conducted in a completely randomized design at the University of Mohaghegh Ardabili, Ardabil, Iran, in 2013. The experiment consisted of three levels of drought (0, -0.5, and -1 MPa) and various concentrations of ZO-NP (0, 0.5, and 1 g lit^{-1}). The soybean seeds used in this experiment were DPX prepared from Moghan Agricultural Research Center. Results showed that drought stress reduced the activity of peroxidase, superoxide dismutase, malate synthase, and isocitrate lyase enzymes together with the germination rate, ascorbate, and alpha-tocopherol concentrations. However, the use of ZO-NP could counteract the adverse effects of drought, so that non-enzymatic antioxidant levels and antioxidant enzyme activity were increased and this improved the germination rate. Applying ZO-NP increased the activity of isocitrate lyase and malate synthase enzymes. Based on these results, it seems that the use of ZN-OP can be one way to mitigate the adverse effects of drought stress during soybean germination.

Keywords: antioxidant, germination, drought, soybean, nano zinc oxide

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Introduction

Germination is the first stage of plant life cycle. It is of great importance and sensitivity and is considered as a key process of emergence and establishment. This step is strongly affected by drought (Bradford and Nonogaki 2008).

Drought stress is one of the environmental stresses that causes irreparable damage to the plants. One of its effects is the production of reactive oxygen species (ROS), which include superoxide radical (O^{2-}), hydroxyl radical (OH^{-}), and hydrogen peroxide (H_2O_2) (Yıldıztugay et al. 2011). Free radicals cause lipid peroxidation and produce active and toxic aldehydes (Sunkar et al. 2003). Research indicates that drought stress reduces germination rate and percentage (Hilhorst et al. 2007), and also has effects on the activity of antioxidant enzymes (Mohammadi et al. 2011)Plants have various defense mechanisms against oxidative stress which include antioxidant enzyme systems and

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non-enzymatic antioxidant systems (Gill and Tuteja 2010). The antioxidant enzyme system involves superoxide dismutase (Cu, Zn-SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX), polyphenol oxidase (PPO), and guaiacol peroxidase (GPX) while the non-enzymatic antioxidant system consists of ascorbate, alpha-tocopherol, carotenoids, anthocyanins, flavonoids, and glutathione (Apel and Hirt 2004). Superoxide dismutase is among the first enzymes that inhibit ROS. This enzyme is able to convert superoxide anion, which can be one of the most dangerous free radicals to H_2O_2 (Turkan 2011). H_2O_2 is also part of the ROS but is less toxic than superoxide radical (Bhattacharjee 2005). Some of the enzymes, including catalase and peroxidase, are able to convert H_2O_2 to non-toxic compounds (Turkan 2011).

Peroxidase in plants has multiple physiological and biochemical roles and is involved in linking with cell wall molecules, the oxidation of auxin, lignin production, and response to biotic and abiotic stresses (Quiroga et al. 2000). Peroxidase acts as an electron acceptor in a wide range of reactions and thus reduces the production of ROS (Raeesi Sadati et al. 2016).

Ascorbate (vitamin C) is one of the most important vitamins that is synthesized in plants and in addition to its role in cell division and metabolic processes, it inhibits hydrogen peroxide as a powerful antioxidant. Ascorbate is present in chloroplasts, the cytosol, the vacuole, and apoplastic area of the leaf cells, in high concentration (Anjum et al. 2014). Alpha-tocopherol (vitamin E) has been introduced as a major factor inhibiting lipid peroxidation in seeds during germination (Sattler et al. 2006).

Plant essential mineral elements which have specific physiological roles are divided into two groups of macro and micronutrients. Zinc is a micronutrient, also known as a heavy metal. High concentrations of heavy metals in plants have adverse effects on growth and yield (Varma 2021), including oxidative stress and ROS production (Turkan 2011). There are several ways to reduce the toxicity of heavy metals, one of them being the use of nanotechnology. The science of nanotechnology is now present in all areas of science, and agriculture is no exception. Taking advantage of nano-fertilizers causes the plant to absorb nutrients slowly and gradually (Iqbal 2019). In many plant enzymatic activities, zinc has a structural or catalytic activator role and participates in protein synthesis and degradation, saccharides metabolism, and photosynthesis to produce carbohydrates and reduce the damage caused by oxidative stress to cell membranes. It also plays a role in the processes related to adaptation (George et al. 1995). Many studies have shown that the use of zinc increased the activity of catalase, peroxidase, and superoxide dismutase (Waraich et al. 2011); (Poornima and Koti 2019). Also, it improves germination under stress (Latifzadeh et al. 2013). (Taran et al. 2017)concluded that Cu and Zn nanoparticles increase the antioxidant enzymes activity in wheat seedlings via reducing the level of accumulation of thiobarbituric acid reactive substances, stabilizing the content of photosynthetic pigments and increasing relative water content in leaves.

Soybean is a drought-sensitive plant and water deficit during germination has a negative effect on its seedling establishment. It is a species of legume native to East Asia, widely grown for its edible bean, which has numerous uses. Soybean seeds have 18% oil and their meal is used as a protein source for livestock and poultry feeds (Sheykhbaglou et al. 2018).

In this study, the effect of water deficit stress and the use of nano zinc oxide were investigated on the activity of antioxidant enzymes and germination of soybean. High concentrations of essential elements from heavy metals if prepared in nano-fertilizer forms can reduce the adverse effects of the elements and help to improve their beneficial effects. This was the motivation to investigate the impact of nano zinc oxide on the improvement of soybean seed germination under drought stress conditions.

Materials and Methods

In order to assess the effects of ZO-NP to reduce the damage caused by drought on the antioxidant enzyme activity and germination of soybeans, a factorial experiment was conducted based on a completely randomized design at the University of Mohaghegh Ardabili, Ardabil, Iran in 2017. Treatments consisted of three levels of drought (0,

- 0.5, and -1 MPa) and various concentrations of ZO-NP (0, 0.5, and 1 g lit^{-1}).

Soybean seeds used in this study were DPX prepared from Moghan Agricultural Research Center, Iran. Seed surfaces were sterilized with sodium hypochlorite 10% for 3 minutes and then washed with distilled water before they were transferred to a piece of filter paper. Then, a PEG 6000 solution was used for water stress at concentrations of -0.5, and -1 MPa. Michel and Kaufmann (1973) formula was used for calculating the required amount of PEG 6000 at 20 °C as follows:

$SP = -((1.18 * 10^{-2}) * E10) - ((1.18 * 10^{-4}) * E10\text{^}2) + ((2.67 * 10^{-4}) * E10 * D10) + ((8.39 * 10^{-7}) * ((E10\text{^}) * D10))$

in which SP is osmotic potential (MPa), E10 is the amount of PEG (g Kg^{-1}), and D10 is the temperature (°C).

ZO-NP solution was prepared and added to the cultures by different study concentrations and Petri dishes were sealed with Parafilm avoiding evaporation of water. ZO-NP was dissolved by an ultrasonic device (100 w and 40 kHz) on a shaker to prevent the deposition of solution. Also, for more confidence, several pieces of magnets were placed in the ZO-NP solution. Only distilled water was used in the control. The number of germinated seeds (sprouting radicles by two mm) was counted on a daily basis and this continued regularly until the eighth day. Germination rate was calculated using the following formula Scott et al. (1984):

$$\overline{D} = \sum_{t=1}^{t=10} \frac{n}{t}$$

where $\overline{D}$ is the germination rate, n is the number of germinated seeds per day, and t is the days after placing the seeds in Petri dishes.
Germination percentage was calculated using the following formula:

$G_P=100.\ (N_G/N_T)$

where G_P is the germination percentage, N_G is the number of seeds germinated, and N_T is the total number of seeds.

The antioxidant enzyme activities

In order to extract the enzyme, 0.5 g of the sample were homogenized using a mortar and liquid nitrogen, and then 5 ml of cold phosphate buffer (pH 7.5) containing 0.5 mM EDTA was added. The homogeneous solution was transferred to the test tube and then centrifuged for 15 minutes at 4 ℃ and 15,000 g. To prevent the harmful effects of consecutive freezing and melting of samples, the supernatant was divided into three parts and kept at 20 ℃ prior to the measurement of enzyme activities (Sairam and Srivastava 2002).

Measurement of catalase activity

This enzyme activity was measured according to the method of (Aebi 1984). The reaction mixture contained 1.5 ml of 100 mM potassium phosphate buffer, 0.5 ml of 7.5 mM hydrogen peroxide, and 50 µl of enzyme solution. The final volume was brought to 3 ml by adding distilled water. The reaction was started by adding hydrogen peroxide, and a decrease in the absorption of the samples at 240 nm was recorded within a minute. A blank solution was included in all of the aforementioned compounds except the enzyme extract. The amount of hydrogen peroxide decomposition was calculated using extinction coefficient (Ɛ = 39.4 $Mm^{-1}cm^{-1}$). The specific activity of the enzyme was expressed based on mM hydrogen peroxide per mg protein in a minute.

Measurement of peroxidase (POD) activity

POD activity was measured according to the method of (MacAdam et al. 1992). Accordingly, 450 µl of H_2O_2 solution (225 mM) was mixed together with 450 µl of guaiacol solution (45 mM) at low temperature and 100 µl of the enzyme extract was added; changes in absorption at 470 nm was read using a spectrophotometer. Enzyme activity was calculated using Lambert-Beer law and the extinction coefficient of the product of guaiacol peroxidase reaction (13.3 $\mu M^{-1}c^{-1}m$).

Finally, the enzyme activity was expressed in µM g^{-1} FW min^{-1}.

Table 1
Analysis of variance for the effect of ZO-NP and water stress on the activity of antioxidant enzymes and compounds in soybean

		MS						
SOV	df	Peroxidase	Superoxide dismutase	Ascorbate	Tocopherol	Isocitrate Lyase	Malate syntase	Germination rate
ZO-NP	2	1686.41**	1871.25**	32.98**	191.17**	0.72**	0.83**	0.45**
PEG	2	1154.76**	750.95**	5.34**	56.48**	0.2**	0.14**	5.11**
ZO-NP* PEG	4	9.41**	6.55**	0.31**	3.07**	0.012**	0.003**	0.008ns
Error	18	2.04	0.94	0.032	0.087	0.0061	0.0004	0.015
CV (%)	-	0.41	0.39	0.62	1.4	1.87	0.8	1.88

* and ** are significant at 5% and 1% probability levels, respectively.

Peroxidase activity (unit mg^{-1}) = $\frac{\mathrm{POD/min}}{13.3}$

Measurement of super oxide dismutase (SOD) activity

This enzyme assay was performed based on the method described by Giannopolitis and Ries (1977). Inhibition of superoxide radical reaction with nitro blue tetrazolium and prevention from the formation of superoxide-nitro blue tetrazolium complex are the basis for SOD assay. In other words, in the presence of light, reaction complex produces superoxide radicals that react with nitro blue tetrazolium. Enzyme samples were prepared by mixing 885 µl of the buffer 1, 15 µl of the buffer 2, and 100 µl of enzyme extract. Absorption was read at 560 nm with a spectrophotometer.

Superoxide dismutase activity (Unit.mg^{-1}) =

$$\frac{100\left[-\frac{(\text{OD Control}-\text{OD Sample})}{\text{OD Control}}\times 100\right]}{50}$$

Measurement of tocopherol content

Measurement of alpha-tocopherol in soybean seeds was performed by high performance liquid chromatography (HPLC) with Soxhlet extraction procedure (Lim and Traber 2007). A sample of 100 mg seeds was placed in a Soxhlet apparatus and extraction was done using 100 ml of hexane as a solvent and 0.1% butylated hydroxytoluene (BHT) as antioxidant. After six hours of extraction, samples were exposed for three hours inside an ultrasound device. Then, the extracts were filtered using a piece of filter paper. Again, the filtered extract was filtered using a 22 µm membrane and the extracts were immediately analyzed using chromatography (Varian model 210, optical density 200-800 nm, and C-18 reverse phase column). Washing was done with 50% methanol and 50% acetonitrile. Time of decomposition was 10 minutes, pump flow rate was 1.5 ml per minute and injection volume was 10 µl. Chromatography grade methanol and hexane were used with pure water as a solvent. Alpha-tocopherol content was expressed in µg g^{-1} of seed samples.

Measurement of ascorbate content

Total ascorbate content was measured according to the method of Murthy (Murthy 1996)with some modifications (De Pinto et al. 1999). The samples were homogenized using meta-phosphoric acid (5%) at 4 ℃. Then, they were centrifuged at 20,000 g for 15 minutes and the supernatant was used to measure the amount of ascorbate. Dihydroxy ascorbate was converted into ascorbate using dithiothreitol (DTT). The reaction mixture contained 0.1 ml of supernatant, 0.25 ml of 150 mM phosphate buffer (pH 7.4) with EDTA 5 mM, and 0.05 ml of 10 mM DTT. After 10 minutes, 0.05 ml of N-methylmaleimide 5% was added to wash DTT. By adding 0.2 ml of 10% Trichloroacetic acid (TCA), 0.2 mL of 44% ortho phosphoric acid, 0.2 ml of 4% α and α'- dipyridyl dissolved in 70% ethanol and 0.3 % iron chloride, the solution began to change color. The absorbance was measured at 525 nm after 40 minutes at 40 ℃.

Measurement of glyoxylate cycle enzymes activity

The samples were kept in liquid nitrogen. Then, they were homogenized by a mortar that had been pre-cooled manually and were mixed with 0.15 M Tris-HCl (pH 7.5) containing 1 mM EDTA, 2 mM DTT, 10 mM KCL, 10 mM $MgCl_2$, and 0.6 M sucrose. The homogenized solution was centrifuged for 20 minutes at 12,000 g. The supernatant as enzyme extract was used to determine the activity of the malate synthase and isocitrate lyase (Lin et al. 2011).

Statistical analysis of the data was performed using SAS 9.1 after normality test and means were compared by LSD at 5% probability level.

Results

Germination rate (GR)

Analysis of variance of data showed that the simple effect of drought and ZO-NP was significant on germination rate (Table 1). Comparison of means showed that GR significantly decreased with increasing severity of drought stress and reduction rate was 25% at -1 MP (Table 2). The highest GR (6.88 seeds per day) was obtained from the use of 1 g lit^{-1} ZO-NP (Table 2).

Peroxidase (POX) enzyme activity

Analysis of variance showed that the interaction of ZO-NP and drought stress on peroxidase activity was significant (Table 1). According to Fig. (I), peroxidase enzyme activity decreased due to the effect of drought stress and increased because of the use of ZO-NP.

Activity of superoxide dismutase (SOD)

The analysis of variance showed a significant interaction effect between drought and ZO-NP on the activity of SOD (Table 1). SOD activity increased with the use of ZO-NP under stress conditions (Fig. II), so that the highest activity of SOD (81.36-unit min^{-1} mg protein) was related to application of 1 g ZO-NP under non-stress conditions.

Treatment	Means	
	concentration	Germination Rate
ZO-NP (g lit^{-1})	0	6.43 c
	0.5	6.66 b
	1	6.88 a
PEG (MP)	0	7.43 a
	- 0.5	6.6 b
	- 1	5.93 c

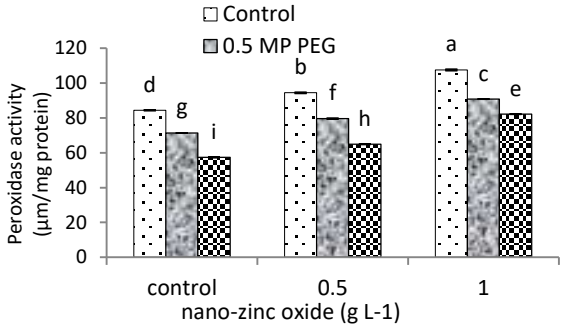

Fig. I. Effect of ZO-NP and water stress on the peroxidase activity in soybean seedlings

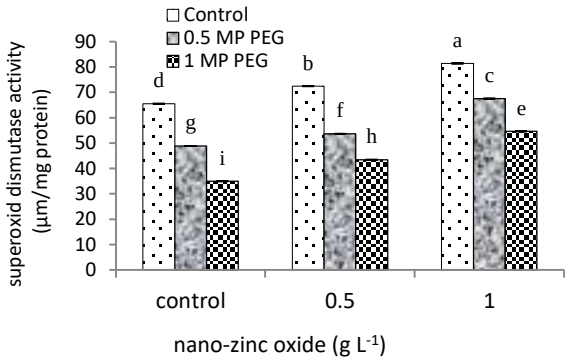

Fig. II. Effect of nano zinc oxide on the superoxide dismutase activity under water stress condition in soybean seedlings

Ascorbate content

The interaction of water stress induced by polyethylene glycol and ZO-NP was significant on the amount of ascorbate (Table 1). Drought stress reduced and ZO-NP increased the amount of ascorbate and the maximum amount of ascorbate (9.57g mg^{-1}) observed in non-stress conditions using 1 g lit^{-1} ZO-NP (Fig. III). Results showed that the application of nano zinc oxide increased the ascorbate content in soybean seedlings.

Tocopherol content

The results of analysis of variance showed that the interaction between stress and various concentrations of ZO-NP was significant on tocopherol content (Table 1). Also, application of nano zinc oxide increased tocopherol content under drought stress conditions (Fig. IV). The highest tocopherol content was observed in non-stress condition by using 1 g lit^{-1} nano zinc oxide.

Activity of glyoxylate cycle enzymes

Analysis of variance showed that the interaction of stress and ZO-NP was significant on the activity of malate synthase and isocitrate lyase (Table 1). With increasing concentration of zinc oxide nanoparticles under conditions of drought stress, the activity of these enzymes increased. The highest activity of isocitrate lyase was related to the application of 1 g lit^{-1} ZO-NP under non-stress conditions (Fig. V). The lowest activity of this enzyme was observed in -1 MPa stress and lack of nano zinc oxide. Malate synthase activity was also maximized using 1 g lit^{-1} zinc oxide under non-stress conditions and the lowest activity was observed in -1 MPa stress without using nano-oxide (Fig. VI).

Discussion

Under drought stress, water absorption reduces and slow physiological and metabolic processes lead to decrease in the seed GR. It may also slow the hydrolysis of metabolic compounds or transfer of the hydrolyzed materials which are required for the development and growth of the embryo (Turk et al. 2004). Researchers reported that GR decreased with increasing drought stress(Kaya et al. 2006). Due to the fact that Zn is involved as a cofactor in the activation of enzymes or is a part of the enzyme structure, it is expected to increase the rate and percentage of germination by improving the enzyme activity. Increased GR was reported in several studies, including the use of nutrients such as phosphorus and zinc (Abdolrahmani et al. 2009) and zinc sulphate and urea (Latifzadeh et al. 2013)under different stresses which correspond with the results of the present study. Also, increase in the activity of antioxidant enzymes that reduced the damage

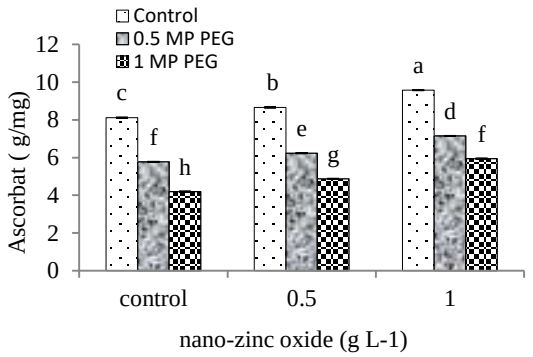

Fig. III. Effect of nano zinc oxide and water stress on the ascorbate content in soybean seedlings

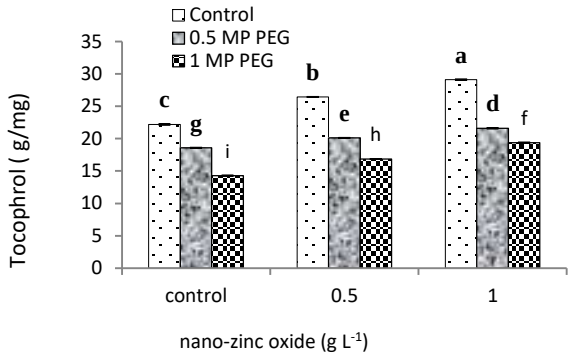

Fig. IV. Effect of water stress and nano zinc oxide on the tocopherol content in soybean seedlings

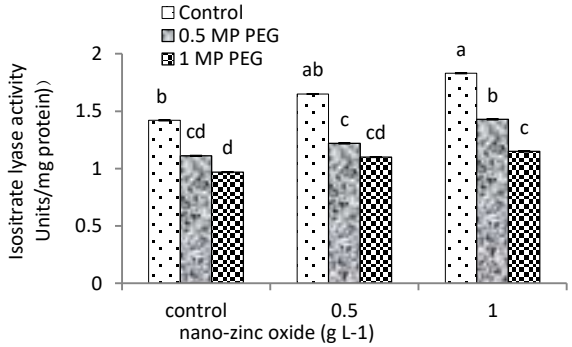

Fig. V. Effect of nano zinc oxide on the isocitrate lyase activity under water stress condition in soybean seeds

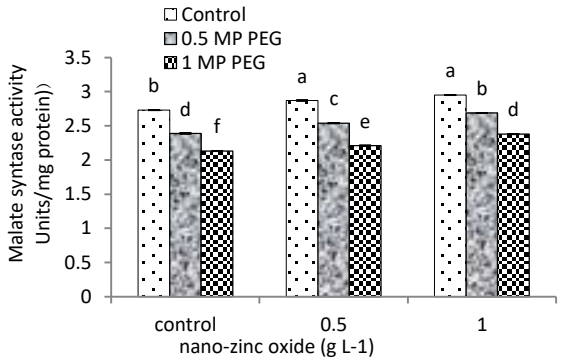

Fig. VI. Effect of nano zinc oxide and water stress on the malate syntase activity in soybean seeds

caused by the stress may be the reason for improvement in the germination rate.

Peroxidase in plants has multiple physiological and biochemical roles and is involved in the binding with cell wall molecules, oxidation of auxin, lignin production, and response to various stresses (Quiroga et al. 2000). It is an antioxidant enzyme that can break down and detoxify H_2O_2 (Zhang et al. 2009). Increase in peroxidase activity by applying Zn (Waraich et al. 2011)may be due to the impact of this element on the level of ROS. Weisany et al. (2012) studied the effect of Zn on the activity of catalase, peroxidase, and ascorbate peroxidase under stress conditions and found that the activity of these enzymes in soybean increased by the use of Zn, and Zn improved the damages caused by the stress. Also, Scientists reported that ROS concentration increased under Zn deficiency. Application of Zn may increase the stability and antioxidant enzyme activity, thus resulting in decreased ROS production(Anjum et al. 2014).

The results of Jin et al. (2008)showed that SOD activity increases with Zn application. Increase in SOD activity is important for plants to overcome water deficit under oxidative stress (McKersie et al. 2000)because the SOD enzyme is among the first group of plant cell protectants against oxidative stress (Balešević-Tubić et al. 2014). SOD traps free radicals and converts them to H_2O_2 (Turkan 2011). The toxicity of H_2O_2 is less than O_2^-, but it has more stability; however, catalase and peroxidase inhibit H_2O_2 and convert it to non-toxic materials (Gill and Tuteja 2010). As shown in Figs. (I) and (II), the activity of these enzymes increased as a result of using Zn. In many plant enzyme systems, Zn has catalytic or structural roles (Brown et al. 1995). It supports SOD as a cofactor (Turkan 2011) and is an essential element in increasing its activity. The use of ZO-NP can be one of the appropriate methods for reducing the effects of oxidative stress.

It has been reported that zinc plays an important role in protecting plant cells against reactive oxygen species (Marschner 2011). Various correlations have been reported between water deficit and the amount of soluble intracellular antioxidants (Agarwal and Pandey 2004). So increasing the activity of antioxidant enzymes and increase the intracellular antioxidants such as ascorbic acid, may be one way to increase resistance to drought.

Ascorbic acid is one of the powerful antioxidants which inhibit free radicals by reducing them and reducing drought induced damages (Rosales et al. 2006). Ascorbate can protect alpha-tocopherol against ROS and even contributes to the conversion of α-tocopheroxyl radical into Alpha-tocopherol (Veljovic-Jovanovic et al. 2001); (Pastori and Foyer 2002). Application of Zn can increase intracellular ascorbic acid (Jin et al. 2008). Besides the antioxidant role of ascorbate, it also protects other antioxidants (Anjum et al. 2014). Because of the increase in the amount of ascorbate with the use of Zn, it is possible to increase the antioxidant enzymes and prevent damage to the tocopherol.

Alpha-Tocopherol as a non-enzymatic antioxidant helps to inhibit ROS. It protects chloroplast membranes from damage caused by free radicals, and since alpha-tocopherol takes place in the phospholipid membranes (Burton and Ingold, 1989) it prevents lipid peroxidation (Anjum et al. 2014). The presence of ascorbate and alpha-tocopherol prevents lipid peroxidation by ROS (Munné-Bosch et al. 2001). However, lipid peroxidation can cause transformation of alpha-tocopherol to α-tocopheroxyl radical while the presence of ascorbate can return alpha-tocopherol (Anjum et al. 2014). Scientists reported that drought stress increases alpha-tocopherol content while heat stress reduces it(Keleş and Öncel 2002). Also, some researches (Munné-Bosch et al. 2001)showed that with increasing severity of drought stress the amount of alpha-tocopherol is reduced.

Reserches (Muscolo et al. 2007)showed that drought stress induced by polyethylene glycol decreased the activity of malate synthase and isocitrate lyase. These two enzymes are among the five enzymes involved in glyoxylate cycle (Hayashi et al. 1995). The presence of this cycle in oil seed plants has a key role in energy supply (Eastmond and Graham 2001). Drought stress reduces the activity of these two enzymes which leads to reduction in fatty acids consumption as

these enzymes cause breakdown of fatty acids in germinated seeds (Bradford and Nonogaki 2008). It is most likely that reductions in the breakdown of fatty acids that are the main soybean seed reservoirs reduce germination. Increases in the activity of these two enzymes were observed in the use of nano zinc oxide. Since Zn is one of the most important structural components of auxin and auxin plays a role in the catabolism of lipids, it seems that application of Zn affects auxin and increases the activity of malate synthase and isocitrate lyase (Sedghi et al. 2013).

Conclusion

The results of this research showed a decrease in germination rate under drought stress. The drought reduced antioxidant enzyme activity, non-enzymatic antioxidants, and activity of glyoxylate cycle enzymes in soybean seeds in which the highest decrease was obtained in -1 MPa. However, the use of nano zinc oxide improved seed germination of soybean under drought stress and increased antioxidant enzyme activity and non-enzymatic antioxidant contents under drought. Therefore, it can be concluded that the use of nano zinc oxide is likely to increase the tolerance of soybean seedlings to drought stress during germination and with increasing malate synthase and isocitrate lyase activity this causes the catabolism of lipids and thereby helps to provide energy for germination.

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