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Role of Zinc Oxide Nanoparticles in Ameliorating Salt Tolerance in Soybean

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AS LONG as we know, little attention has been given to evaluating roles of ZnO nanoparticles (ZnONPs) in plants grown under salinity stress. In the present study, biochemical and molecular responses of salt-stressed soybean cv. Giza111 under different ZnONPs were assessed. Treatment with a high NaCl concentration (250mM) alone caused a significant reduction (65%) in germination percentage as well as a significant decrease in all measured growth parameters (root length, shoot height, fresh and dry weight of roots and shoots) and photosynthetic performance (Fv/Fm) compared to control plants. In contrast, malondialdehyde (MDA) & proline contents, and activities of antioxidant enzymes (catalase CAT, peroxidase POX and superoxide dismutase SOD) were highly increased. In contrast, presoaking of soybean seeds with different concentrations of ZnONPs (25, 50, 100, 200mg/L) stimulated the growth of stressed plants which was exhibited by improved growth parameters and photosynthetic performance as well as lower levels of proline and MDA in stressed plants particularly at ZnONPs concentration of 50 mg/L compared to controls. Moreover, isozyme analysis of CAT, POX and SOD showed variable pattern of alleles at 50mg/L ZnONPs. Accordingly, these results recommend application of 50mg/L ZnONPs for improving the productivity of soybean cultivated in saline soils.

Keywords: Antioxidant enzymes, Fv/Fm, *Glycine max* L., Isozymes, Nanoparticles, Salinity.

Introduction

Salinity stress is one of the most considerable abiotic factors that limits crop production worldwide (Efisue & Dike, 2020); particularly in semi-arid zones like Egypt. It has been reported that 33% of the cultivated lands in Egypt are salinized, therefore it is important to develop new strategies to ameliorate the salinity tolerance of plants in such regions and produce salt tolerant crops to assure sustainable crop yield (Latef et al., 2017; Sharma & Singh, 2019).

It usually affects the ribulose biphosphate carboxylase-oxygenase activity, photosystem II light-capturing efficiency and electron transport ability (Yang et al., 2006). Recently, chlorophyll a fluorescence (ChlF) has been used to evaluate impacts of different stresses including salt stress on photosynthetic performance (Galić et al., 2020). ChlF is usually used to estimate the maximum quantum yield of primary PSII photochemistry (Fv/Fm) as well as the capability of electron transport (Kalaji et al., 2017; Galić et al., 2020). Fv/Fm is deemed a sensitive performance indicator of plant photosynthetic apparatus. The maximum Fv/Fm value of about 0.83 was observed with healthy plants (Björkman & Demmig, 1987). In contrast, lower Fv/Fm values were described with plant samples under abiotic or biotic stress (Kalaji et al., 2016).

Soybean (*Glycine max* L.) is one of the most important plants in the world. It is widely grown for its edible beans and as an industrial crop (Teixeira et al., 2019). It is considered as the second important crop for Egyptian oil industries (Hemeid, 2020). It was shown that the yield of soybean in Egypt is declined drastically due to several factors which are negatively affecting soybean production as well as the lack of soybean species with high productivity and tolerance to abiotic stresses (Latef et al., 2016).

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Recently, nanoparticles (NPs) are highly required in many fields including textile, cosmetic, electronic, agriculture and pharmaceutical industries (Siddiqui et al., 2015; Malhotra & Mandal, 2019). Generally, NPs are described to improve crop yields by preventing fertilizers loss via leaching, increase complete absorption by plant due to availability and controlled release during growth period (Kalteh et al., 2014).

Zinc oxide nanoparticles (ZnONPs) are one of the most regularly used nanoproducts in food preserving and drugs (Youssef & El-Sayed, 2018; Patel et al., 2020). ZnONPs are also being frequently used in sun-protective creams, wall paints, and ceramic manufactures (Ahmad & Akhtar, 2019). Zn is widely used in fertilizers due to its natural requirement as an essential element necessary for growth and development of plants (Faizan et al., 2020). In addition, it is an important cofactor in essential biocatalytic enzymes (Latef et al., 2017). Earlier studies have described both positive and negative effects of ZnONPs on the physio-biochemical characteristics of plants employing various model plant systems (Latef et al., 2017). Nevertheless, the effect of nanoparticles on plants differs with growth stage and species as well as attributes of used nanoparticles (Burman et al., 2013).

Interestingly, priming of seeds with ZnONPs positively affected the growth traits in salt-stressed plants whereas, ZnONPs stimulated natural auxin (Indole Acetic Acid) and thus activating cell division and enlargement (Ali & Mahmoud, 2013; Latef et al., 2017), maintaining the structural integrity of biomembranes (He et al., 2015), improvement of protein synthesis (Landa et al., 2015), phospholipids accumulation, scavenging free oxygen radicals, translocation of nutrients from the aged cells to newly formed cells and decreasing the uptake of excess of Na^+ and Cl^- (Farhangi-Abriz & Torabian, 2018) as well as augmentation of photosynthetic pigments, organic solutes, total phenols, ascorbic acid and Zn in stressed plants (Latef et al., 2017).

The current study was conducted to investigate the potential role of seed-soaking in ZnONPs for improving salt-tolerance in soybean.

Materials and Methods

Plant materials

Seeds of soybean cultivar (cv. Giza 111) were obtained from the Food and Legumes Research Department, Field Crops Research Institute, Agricultural Research Center, Giza, Egypt (March, 2018).

Synthesis and characterization of ZnO nanoparticles

ZnO nanoparticles were synthesized using the chemical bath deposition (CBD) method as described by El-Shaer et al. (2018). The synthesis was performed using 0.25M zinc nitrate hexahydrate and 2.13M of potassium hydroxide as precursors in 20mL of deionized water. Each solution was separately stirred for 10 min, then mixed and stirred again for 10 min. The final mixture was kept in the oil bath at 80°C for 4hrs. After that, the precipitated ZnO nanoparticles were rinsed several times with deionized water and ethanol and then dried at 105°C. The synthesized ZnO nanostructures were characterized using XRD (Shimadzu 6000), UV-vis spectrophotometer (JASCO V-630), and scanning electron microscope (JSM-651OLV).

The crystalline structure and optical properties of the prepared ZnO nanostructures were examined with X-ray Diffraction (XRD), while the samples morphology was investigated using scanning electron microscope (SEM). As presented in Fig. 1A, ZnO nanostructures are formed as Nano-rods with hexagonal quartzite crystal structure. These Nano-rods accumulated with each other to form surface morphology of grains like flower. The XRD pattern of ZnO Nano-rods is shown in Fig. 1B. The diffraction peaks at 31.8°, 34.4°, 36.3°, 47.5°, 57°, 62.9°, 66.4°, 67.9°, 69° and 77° correspond to the (100), (002), (101), (102), (110), (103), (200), (112), (201), and (202) lattice planes, respectively (Fig. 1B).

Plant growth conditions

Soybean seeds (cv. Giza 111) were sterilized using 70% ethanol for 5min and Sodium hypochlorite (10% w/v) for 10min, then washed several times with distilled water. Four concentrations of ZnONPs (25 (T_1), 50 (T_2), 100 (T_3) and 200 (T_4) mg/L were prepared and sterilized at 120ºC for 20min. Then, soybean seeds were presoaked with each concentration and with distilled water (control group) for 2hrs at room temperature.

After presoaking, the thoroughly washed seeds were sown (20seeds/ pot) in plastic pots filled with 24kg of 2:1 (clay: sandy) soil. Plastic pots (45cm /40cm^2) were divided into 5 groups (control and four ZnONPs concentrations). 250mM NaCl was chosen as a sub-lethal salinity

level based on the preliminary experiment results and used in this study. This experiment was carried out in the green house of Botany Dept., Faculty of Science Tanta University, Tanta, Egypt under natural conditions of temperature, light, and humidity during the growing season of soybean plant from 1st May to 30th Aug 2018. The pots were irrigated with tap water and NaCl solution for 21 days (seedling stage). The salt solution was added twice on the 7th and 14th day with 250mM to 80% field capacity, thereafter; it was irrigated with water till harvesting (21-day-old). Three pots were used as replica for each treatment. Then, 21-day-old soybean plants of all treatments were collected, washed and used for further analyses.

Growth traits

The root and shoot lengths were measured using a scale ruler. In addition, fresh weights of plant samples were documented, then they were dried at 105ºC for 48hrs in oven, and finally the dry weights of plant samples were determined. Moreover, leaf area of plant samples was also measured.

Photosynthetic performance

The photosynthetic performance was determined as fluorescence kinetic [variable fluorescence (Fv) / maximum fluorescence (Fm)] of dark-adapted leaves and measured using OS-30p Chlorophyll fluorometer (Hudson, NH03051, USA).

Photosynthetic pigments

The photosynthetic pigments Chlorophyll a, Chlorophyll b, and Carotenoids were determined using the spectrophotometric method as described by Metzner et al. (1965). Carotenoids were estimated according to Horvath et al. (1972) and adopted by Kissimon (1999).

Antioxidant enzymes activities

The activity of Catalase (CAT) (EC1.11.1.6) and Peroxidase (POX) (EC 1.11.1.7) was estimated according to Kato & Shimizu (1987), while superoxide dismutase (SOD) (EC 1.151.1) was determined according to the method of Beyer & Fridovich (1987).

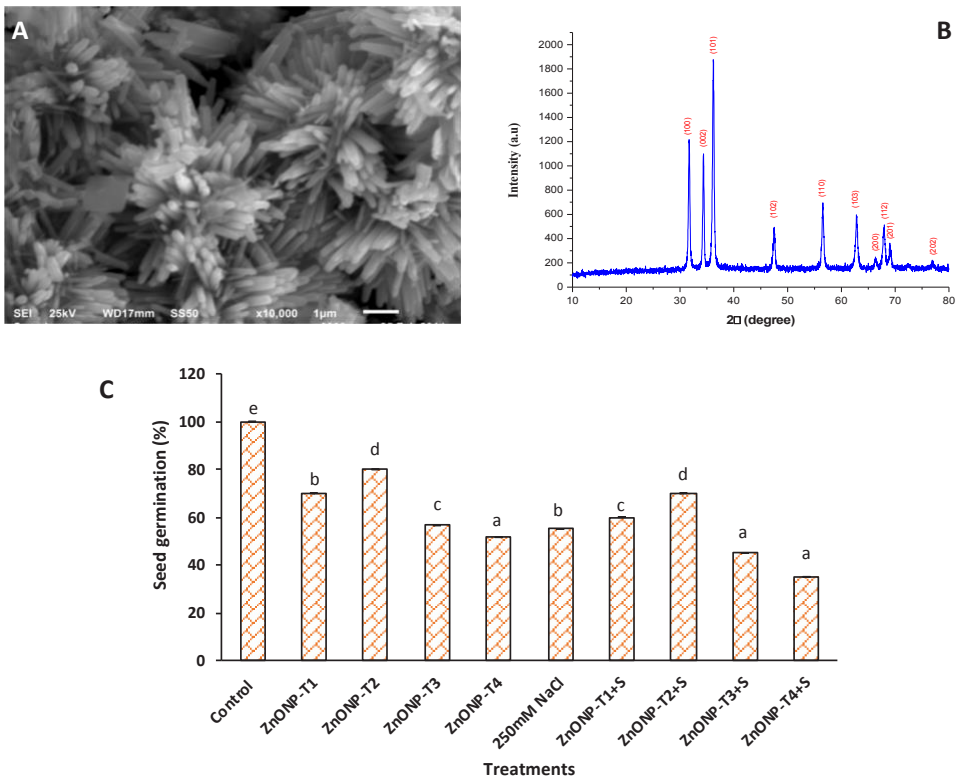

Fig. 1. (A) SEM image and (B) XRD of ZnO nanostructures prepared by CBD method. (C) Effect of NaCl (250mM) on the germination percentage of 7-day-old soybean (cv. Giza 111) seedlings after presoaking of soybean seeds in different concentrations of ZnONPs (ZnONP-T1= 25, ZnONP-T2= 50, ZnONP-T3= 100, and ZnONP-T4= 200mg/L) [Treatments with different letters are significant at P≤ 0.05, while treatments with identical letters are non-significant].

Malondialdehyde (MDA) content

Lipid peroxidation was measured in the leaves of seedlings by determining the amount of malondialdehyde (MDA). The MDA content was estimated using method of Heath & Packer (1968). The absorbance was determined at 532 and 600nm, then MDA content was calculated using the extinction coefficient ($155mM^{-1}$ cm^{-1}) and expressed as (µM/g FW).

Determination of proline content

The proline was determined according to the method described by Bates et al. (1973). The absorbance of the toluene layer was measured at 520nm using toluene as a blank.

Isozymes analysis of antioxidant enzymes

For antioxidant enzyme activity and isozymes analysis, leaf samples were collected on the 21st day of the experiment and were immediately frozen at -80ºC. Total crude protein extraction was performed using 2g of the frozen leaf samples after grinding up to fine powder in liquid nitrogen by using a mortar and pestle. Each sample was extracted in 0.5ml of 30% sucrose (w/v) at 4ºC for 2hrs, then total protein extracts were centrifuged at 15000 rpm for 5min at 4ºC using cooling centrifuge (Sigma 3-30KS, Germany). The supernatants were then stored at -20ºC until use.

Samples of soybean total protein extracts were electrophoresed in 10% (w/v) polyacrylamide slab gel at pH= 8.9 under nondenaturing conditions at 100V (Cleaver Scientific Ltd) as described by Laemmli (1970). The gels were stained for isoenzymes of three antioxidant enzymes (CAT, POX and SOD). Isoenzymes of antioxidant enzymes were visualized in gels by the methods of Beauchamp & Fridovich (1971) for SOD, Shaw & Prasad (1970) for CAT and Soltis et al. (1983) for POX.

For peroxidase (POX) isozymes staining, the gel was stained by incubating in 0.05 M acetate buffer (PH= 5) containing 65 mg Benzidine dissolved in 1mL ethanol. 2mL of 0.1M $CaCl_2$ were added as coenzyme. Finally, 2mL of H_2O_2 (3% v/v) were added as the substrate. Then, gel was incubated in refrigerator until brown bands appeared (Soltis et al., 1983).

For catalase (CAT) isozymes staining, the gel was incubated in 3% H_2O_2 at room temperature for 30min, rinsed several times with dist. water, then incubated in a solution of potassium ferrocyanide 2% and ferric chloride 2% (1:1) for about 30min. Catalase activity appeared as white bands on a dark blue background (Shaw & Prasad, 1970). After staining, the gel was photographed and scored.

Isozymes of superoxide dismutase were localized by soaking the gels in nitro blue tetrazolium (NBT) for 20min, followed by immersion for 15min in a solution containing 0.028M tetramethylethylenediamine (TMED), riboflavin, and 0.036M potassium phosphate at pH 7.8. The gels were illuminated for 5 to 15min. During illumination, the gels became uniformly blue except at positions containing superoxide dismutase. Illumination was discontinued when maximum contrast between the achromatic zones and the general blue color had been achieved (Beauchamp & Fridovich, 1971). Then, gels were photographed.

Determination of Zinc content

The determination of Zn^{+2} content was performed using dried leaf samples after acid digestion according to Allen et al. (1974). The concentration of Zn^{+2} (mg/g) was determined by Atomic Absorption Spectrophotometer (Perkin Elmer, 2380) at the Central Laboratory of Tanta University.

Statistical analyses

Statistical analyses were carried out according to the method of complete randomized blocks design using analysis of variance and the significance was determined using L.S.D values at P= 0.05 and 0.01 according to Bishop (1983). The results were analyzed statistically using one-way (ANOVA) test to determine the degree of significance. In addition, correlation coefficient (R^2) was applied for investigating the significance of the relationships between the studied variables. The statistical analyses were performed using CoStat Software ver. 6.311 (CoHort Software, CA, USA).

Results

Seed germination

Effect of different concentrations of ZnONPs (25, 50, 100, 200mg/L) on germination percentage of soybean Giza 111 seeds indicated that 25mg/L (ZnONP-T1) decreased the germination percentage by 30% compared to control. In addition, 100 and 200mg/L (ZnONP-T3 and ZnONP-T4) reduced the percentage by 43.4% and 48.4% respectively, compared to control (Fig. 1C). In contrast, 50mg/L (ZnONP-T2) significantly increased

germination percentage of the presoaked seeds by 20% compared to control. Under salinity stress (250mM NaCl), 25mg/L and 50mg/L ZnONPs increased germination percentage of the presoaked seeds compared to salt-stressed seeds (Fig. 1C). The maximum increase was found in seeds presoaked with 50mg/L (ZnONP-T2+S) where a significant increase in the germination percentage by 37% was recorded compared to salt-stressed seeds (250mM NaCl). However, a reduction in germination percentage was recorded with 100 and 200mg/L (ZnONP-T3+S and ZnONP-T4+S) by 18% and 36.3%, respectively.

Growth traits

The root and shoot lengths as well as fresh and dry weights of NaCl-stressed soybean (21-day-old) seedlings were estimated to assess the impact of seed soaking with different concentrations of ZnONPs on plant growth and tolerance. Results showed that high NaCl (250mM) produced a highly significant reduction in root length (59.8%), shoot length (54.1%), fresh weight (53.7%), and dry weight (62.7%).

On the other hand, seed-soaking with ZnONPs caused increase in most (all) growth parameters (Fig. 2A, B); a significant increase in root and shoot lengths of all ZnONPs concentrations except ZnONPs-T4+S which showed a non-significant increase and decrease, respectively. On the other hand, significant decrease in fresh weight was found with all ZnONPs concentrations except with ZnONPs-T2+S (Fig. 2B).

The maximum increase in studied growth parameters was recorded in stressed seedlings soaked with ZnONP-T2+S, where 79.5%, 72.7%, 58% and 25.2% in root length, shoot length, fresh weight, and dry weight, respectively were found compared to only salt-stressed seedlings (Fig. 2A, B). This result indicates that ZnONP-T2 (50mg/L) is very likely the most successful zinc oxide nanoparticles concentration that improves soybean plant growth.

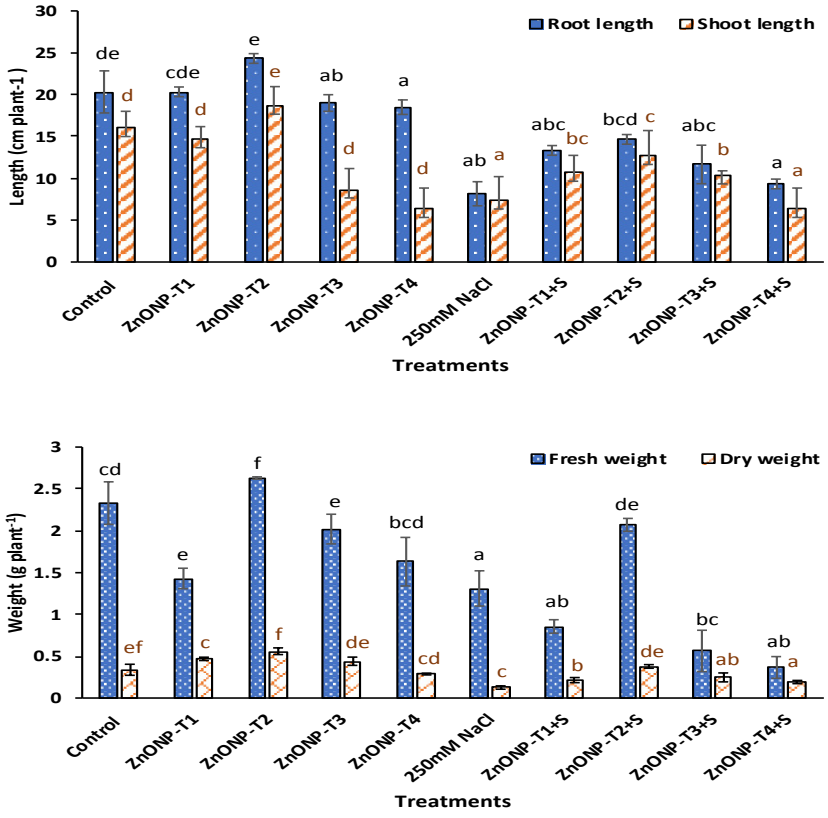

Fig. 2. Effect of NaCl (250mM) on the root length and shoot length of 21-day-old soybean (cv. Giza 111) seedlings after presoaking of soybean seeds in different concentrations of ZnONPs (ZnONP-T1= 25, ZnONP-T2= 50, ZnONP-T3= 100, and ZnONP-T4= 200mg/L) [Treatments with different letters are significant at P≤ 0.05, while treatments with identical letters are non-significant].

Photosynthetic pigments and photosynthetic performance

Results of the effect of different concentrations of ZnONPs (25, 50, 100, and 200mg/L) on photosynthetic performance and pigments (Chl. a, Chl. b and carotenoids) of (21-day-old) soybean seedlings are shown in Table 1. High concentration of NaCl (250mM) induced a significant reduction in the content of Chl. a and Chl. b by 47.7% and 47%, respectively compared to control. Meanwhile, there was a significant increase in carotenoids by 1.18-fold compared to control. In contrast, soaking soybean seeds with only ZnONPs or together with high NaCl (250mM) primarily increased photosynthetic pigments in unstressed as well as stressed seedlings.

Interestingly, seed presoaking with 50mg/L of ZnONPs (ZnONP-T2) significantly increased Chl. a and Chl. b and decreased carotenoids under salinity stress (250mM NaCl) by 53%, 42.3% and 27.3%, respectively compared with salt-stressed seedlings alone (Table 1).

Similarly, NaCl stress significantly reduced the photosynthetic performance (Fv/Fm= 0.34) by 46.8% compared to control (Fv/Fm= 0.64). In contrast, photosynthetic performance was significantly increased in ZnONP-T2 seedlings (Fv/Fm= 0.48) under salinity stress (250mM NaCl) by 41.1% compared to salt-stressed seedlings alone (Fv/Fm= 0.34). In addition, soybean plants treated with 50mg/L ZnO nanoparticles alone significantly elevated the Fv/Fm value (0.73) compared to control plants (0.64). This result indicates that 50mg/L of ZnONPs enhanced the photosynthetic performance of unstressed and salt-stressed soybean seedlings.

Antioxidant enzymes

Data in Fig. 3 showed that high-salt concentration (250mM NaCl) caused a high significant increase in the activity of antioxidant enzymes catalase (CAT), peroxidase (POX), and superoxide dismutase (SOD) by 56%, 54.3% and 94% respectively, compared to control (unstressed soybean seedlings). Seed-soaking with ZnONPs in combination with 250 mM NaCl reduced the activity of all tested antioxidant enzymes under high salinity stress. The most reduction was more obvious in ZnONP-2+S seedlings (Fig. 3 A-C).

TABLE 1. Effect of NaCl (250mM) on photosynthetic performance and photosynthetic pigments (Chl. a) (A), (Chl. b) (B), (Carotenoids) (C) of 21-day-old soybean (cv. Giza 111) seedlings after presoaking of soybean seeds in different concentrations of ZnONPs.

	Treatments	Photo. activity	Pigments (mg $^{-1}$ g DW)		
		Fv^{-1} Fm	Chl. a	Chl. b	Carotenoids
ZnO	Control	0.64±0.019 ^{d}	5.88±0.588 ^{d}	4.46±0.177 ^{d}	2.39±0.089 ^{c}
	ZnONP-T1 (25mg/L)	0.65±0.011 ^{d}	5.18±0.054 ^{cd}	3.40±0.188 ^{c}	2.10±0.082 ^{b}
	ZnONP-T2 (50mg/L)	0.73±0.018 ^{e}	5.37±0.053 ^{cd}	4.35±0.311 ^{d}	2.23±0.047 ^{bc}
	ZnONP-T3 (100mg/L)	0.51±0.008 ^{c}	5.18±0.106 ^{cd}	3.45±0.103 ^{c}	2.00±0.004 ^{b}
	ZnONP-T4 (200mg/L)	0.41±0.006 ^{b}	4.32±0.498 ^{bc}	3.34±0.051 ^{c}	1.98±0.057 ^{b}
ZnO and 250mM NaCl	Salt (250mM NaCl)	0.34±0.019 ^{b}	3.07±0.593 ^{a}	2.36±0.133 ^{b}	5.23±0.047 ^{g}
	ZnONP-T1+S (25mg/L)	0.38±0.065 ^{b}	4.11±0.093 ^{b}	2.36±0.141 ^{b}	4.29±0.049 ^{f}
	ZnONP-T2+S (50mg/L)	0.48±0.036 ^{c}	4.70±0.410 ^{bc}	3.36±0.141 ^{c}	3.80±0.081 ^{e}
	ZnONP-T3+S (100mg/L)	0.38±0.064 ^{b}	3.37±0.285 ^{a}	2.76±0.175 ^{b}	3.20±0.081 ^{d}
	ZnONP-T4+S (200mg/L)	0.19±0.025 ^{a}	3.15±0.133 ^{a}	1.28±0.173 ^{a}	1.53±0.286 ^{a}
	F-value	**56.5**	**16.01**	**66.9**	**241.8**
	L.S.D	**0.06**	**0.73**	**0.34**	**0.22**
	Significance		**		

- Values are the mean of three replicates ± SD. Values within the same column for each factor designated by different letters are significant at P≤ 0.05, while values with identical letters are non-significant.
- Fv: Variable fluorescence and Fm: maximum fluorescence.
- **: Highly significant.

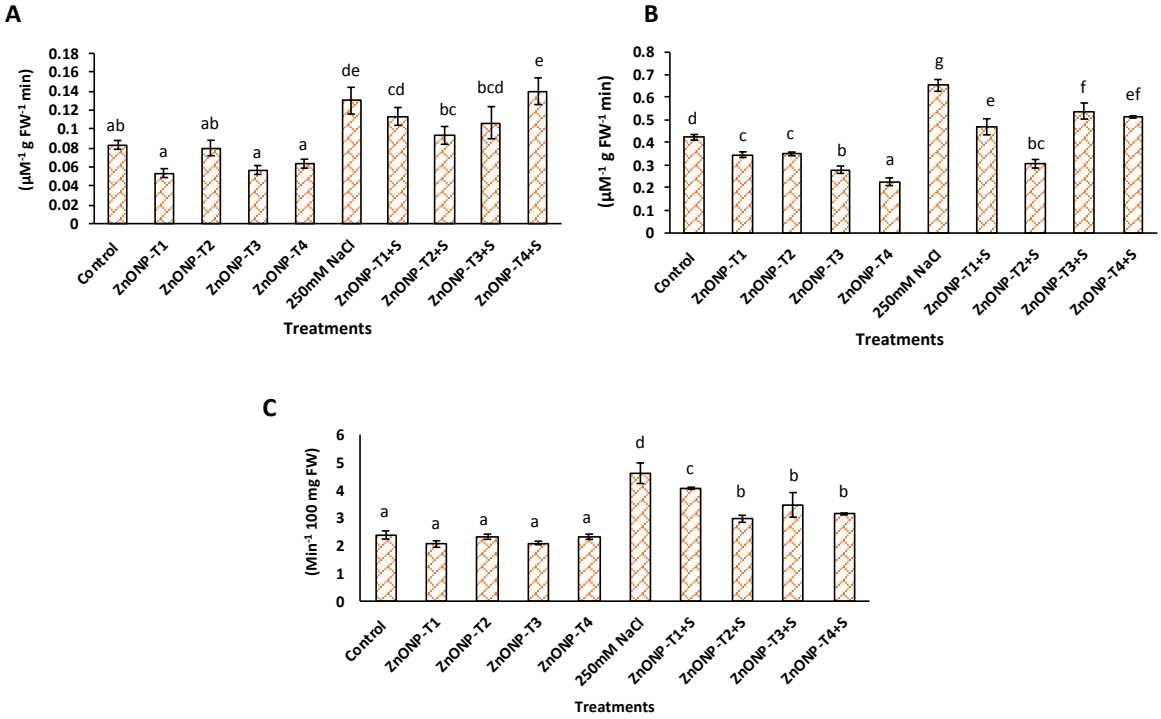

Fig. 3. Effect of NaCl (250mM) on the activity of peroxidase (A), catalase (B) and superoxide dismutase (C) of 21-day-old soybean (cv. Giza 111) seedlings after presoaking of soybean seeds in different concentrations of ZnONPs (ZnONP-T1=25, ZnONP-T2= 50, ZnONP-T3= 100, and ZnONP-T4= 200mg/L) [Treatments with different letters are significant at P≤ 0.05, while treatments with identical letters are non-significant].

Malondialdehyde (MDA) and Proline contents

When soybean seedlings were exposed to 250mM NaCl, malondialdehyde (MDA) content greatly accumulated by 1.19-fold compared to control plants (Fig. 4A). In contrast, soaking of soybean seeds with ZnONPs reduced MDA content. The maximum reduction (30.2%) was observed in ZnONP-T2+S seedlings compared to salinized seedlings alone (Fig. 4A).

The results represented in Figure 4B indicated that high salt concentration (250mM NaCl) caused a highly significant increase in proline content by 1.75-fold compared to control. Soaking soybean seeds with different ZnONPs concentrations generally decreased proline content in ZnONP-T1, ZnONP-T2, ZnONP-T3 and ZnONP-T4. However, the least reduction was observed in ZnONP-T2 seedlings by 5% compared to control (unstressed) seedlings. On the other hand, the maximum reduction (36.6%) in proline content was recorded in ZnONP-T2+S seedlings compared to salinized seedlings alone (Fig. 4B).

Antioxidant isozymes analysis

Isoenzyme patterns of antioxidant enzymes were analyzed (Fig. 5A-C). Results showed stress-dependent changes in isoenzyme patterns for catalase (CAT), peroxidase (POX) and superoxide dismutase (SOD).

CAT isozymes pattern showed only one locus of two allelic bands (Fig. 5A). Salinity stress (250mM NaCl) resulted in appearance of one isozyme band compared to control. Interestingly, in ZnONP-T2+S seedlings, this above-mentioned isozyme band disappeared compared to salinized seedlings alone (Fig. 5A).

On the other hand, POX isozymes pattern exhibited two loci with multi-allelic bands (locus 1 and locus 2, Fig. 5B). Locus 1 was monomorphic under different treatments while in locus 2 two allelic bands disappeared also in ZnONP-T2+S seedlings compared to other seedlings treated with nanoparticles under salt stress (Fig. 5B). Similarly, SOD isozymes pattern showed two loci with multi-allelic bands (locus 1 and locus 2, Fig. 5C). It is obvious that most of these isozyme bands disappeared in ZnONP-T2+S plants compared to salinity stressed plants (Fig. 5C).

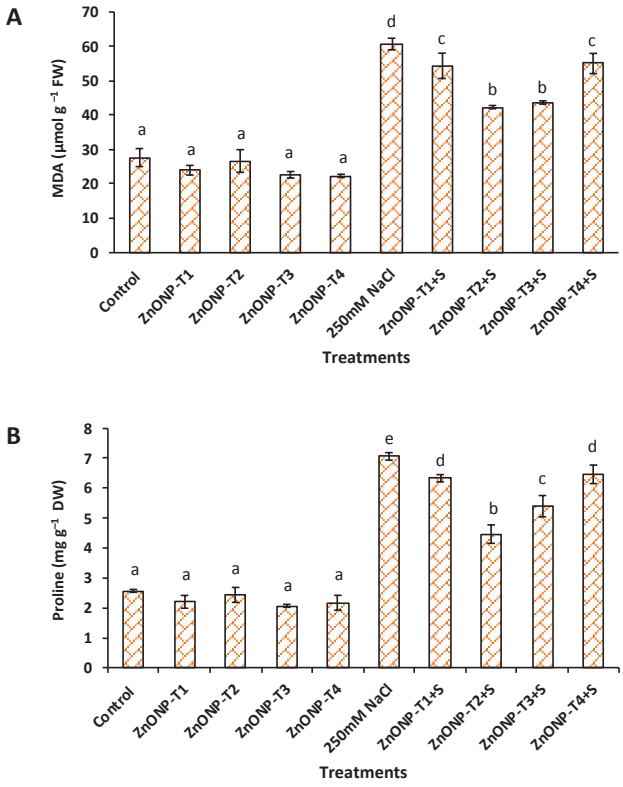

Fig. 4. Effect of NaCl (250mM) on malondialdehyde (MDA) (A) and Proline (B) contents of 21-day old soybean (cv. Giza 111) seedlings after presoaking of soybean seeds in different concentrations of ZnONPs (ZnONP-T1=25, ZnONP-T2= 50, ZnONP-T3= 100, and ZnONP-T4= 200mg/L) [Treatments with different letters are significant at P ≤ 0.05, while treatments with identical letters are non-significant].

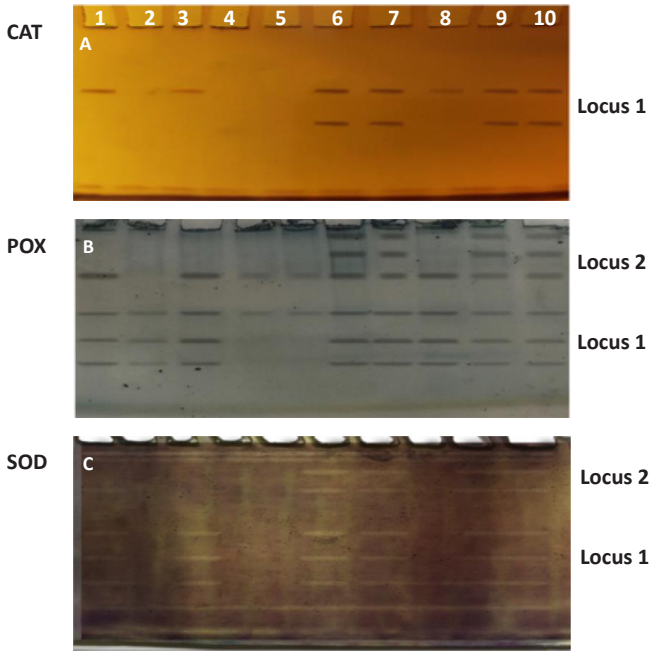

Fig. 5 . Isozyme patterns of CAT, POX, and SOD of soybean seedlings treated with NaCl (250mM) after presoaking in different concentrations of ZnONPs. 1: Control water, 2: 25mg/L, 3: 50mg/L, 4: 100mg/L, 5: 200mg/L, 6: 250mM NaCl, 7: 25mg/L & 250mM NaCl, 8: 50mg/L & 250mM NaCl, 9: 100mg/L & 250mM NaCl and 10: 200mg/L & 250mM NaCl.

Zinc content

Results showed that Zn^{+2} content was significantly decreased under salinity stress by 76% compared to control (Table 2). On the other hand, presoaking of soybean seeds with ZnONPs (50mg/L) increased Zn^{+2} content under salinity stress (ZnONP-T2+S plants) by 2.7-fold compared to salt treatment alone. This pointed to the ameliorating effect of this ZnONPs concentration. However, higher ZnONPs 100 (ZnONP-T3+S plants) and 200 (ZnONP-T4+S plants) mg/L inversely decreased Zn^{+2} content and the maximum decrease was recorded in ZnONP-T4+S plants.

TABLE 2. Effect of NaCl (250 mM) on the mineral content (Zn^{+2}) of 21-day-old soybean (cv. Giza 111) seedlings after presoaking of soybean seeds in different concentrations of ZnONPs.

	Treatments	Zn^{+2} content (mg/g)
ZnO	Control	0.480±0.005 [b]
	ZnONPT1 (25mg/L)	0.428±0.003 [b]
	ZnONPT2 (50mg/L)	0.435±0.003 [b]
	ZnONPT3 (100mg/L)	0.191±0.003 [a]
	ZnONPT4 (200mg/L)	0.126±0.002 [a]
	F-value	**189.2**
	L.S.D	**0.34**
ZnO and 250 mM NaCl	Salt (250mM NaCl)	0.089±0.001 [a]
	ZnONPT1+S (25mg/L)	0.178±0.001 [b]
	ZnONPT2+S (50mg/L)	0.338±0.002 [c]
	ZnONPT3+S (100mg/L)	0.182±0.002 [b]
	ZnONPT4+S (200mg/L)	0.081±0.004 [a]
	F-value	**278.1**
	L.S.D	**0.12**
	Significance	**

- Values are the mean of three replicates ± SD. Values within the same column for each factor designated by different letters are significant at P≤ 0.05, while values with identical letters are non-significant.
- ****:** Highly significant.

Discussion

Exposure of soybean seedlings to high salinity stress (250mM NaCl) imposed adverse impacts on germination percentage as well as plant growth traits which is known to arise from water deficit that cause abnormal changes in plant morphology osmotic stress, and biochemical discrepancy (Bali & Sidhu, 2020; Soliman et al., 2020).

In is study, soaking seeds of soybean with ZnONPs improved the germination percentage and other growth traits in the NaCl-stressed seedlings as Zn is a key element needed for plant growth and development (Ashraf et al., 2020). Zn plays an essential role in building natural auxin (Indole Acetic Acid) and consequently activating cell division and enlargement (Elsheery et al., 2020), maintenance of bio-membranes integrity (Weisany et al., 2012; He et al., 2015), accumulation of phospholipids (Jiang et al., 2014), improvement of protein synthesis (Ebrahimian & Bybordi, 2011; Landa et al., 2015), scavenging of oxygen free radicals, translocation of nutrients from the aged cells to newborn cells and decreasing the uptake of excess of Na and Cl^- (Jiang et al., 2014). This finding is consistent with the results procured by Prasad et al. (2012) on groundnut Raskar & Laware (2014) on onion, Mukherjee et al. (2014) on pea (*Pisum sativum*), Rezaei & Abbasi (2014) on cotton (*Gossypium hirsutum* L.), Soliman et al. (2015) on moringa (*Moringa peregrina*) and Latef et al. (2017) on lupine (*Lupinus termis*). Interestingly, the maximum increase in growth traits under normal and saline conditions was observed in seedlings previously soaked with 50mg/L ZnONPs, which signifies a superior promoting effect of such concentration in unstressed or stressed seedlings. It was suggested that higher concentrations of Zinc in plant seeds which were soaked with ZnO particles induced increment in germination percentage and growth traits (Prasad et al., 2012; Sheteiwy et al., 2016). It is likely that Zinc nanoparticles may increase turgor pressure and plant size by improving water use efficiency and leaf relative water content.

In addition, salinity stress interferes with plant growth due to reduction of osmotic potential and toxicity of Na^+ ions. Zinc nanoparticles reduce Na^+ toxicity by reducing Na^+ absorption resulting in the improvement of plant growth (Latef et al., 2017; Farhangi-Abriz & Torabian, 2018). Moreover, application of zinc nanoparticles generally increased shoot fresh and dry weight under saline condition (Gao et al., 2006; Latef et al., 2017; Abbasifar et al., 2020), which is in accordance with the findings of this study.

In this study, reduction of photosynthetic pigments and activity of soybean plants under salinity stress is in full agreement with the finding

of Sadak et al. (2020), where the reduction of pigment content was attributed to chloroplast damage, pigment-protein complex instability, and chlorophyllase activity (Dawood et al., 2016; Da-Silva et al., 2020). Total chlorophyll (a & b) contents in leaves of soybean plants were significantly increased as a result of seed soaking with ZnONPs (25, 50, 100, and 200gm/L). The most significant concentration of ZnONPs was 50mg/L. These findings were similar to the results of Singh et al. (2013) on vegetable crops, Farnia & Omidi (2015) on maize (*Zea mays* L.), Farrag (2015) on *Lemna gibba* (L.) and Latef et al. (2017) on lupine (*Lupinus termis* L.) who revealed that Nano-Zn application increased total chlorophyll contents as compared to control. These results may be due to the association of Zn with chlorophyll formation as suggested by Mazaherinia et al. (2010) and Rodrigues et al. (2020).

Moreover, in the present study soybean plants treated with 50mg/L ZnO nanoparticles alone or in a combination with high (250mM) NaCl significantly elevated Fv/Fm values (0.73 and 0.48, respectively) compared to control (0.64) and salt-stressed plants (0.34), which indicates that 50mg/L of ZnONPs enhanced the photosynthetic performance of unstressed as well as salt-stressed soybean seedlings.

The above-mentioned results are in accordance with Kalaji et al. (2016), who stated that salt stress is generally reducing the performance of the photosynthetic apparatus by disturbing of the electron transport system. It was demonstrated by Oukarroum et al. (2009) that the greater the plant stress, the lower the Fv/Fm ratio is, which is similar to the results obtained in this study. This finding indicates that Zinc oxide nanoparticles can minimize the hazardous effects of salinity on plant health, therefore can be used as Nano-fertilizer for improving soybean salt tolerance.

On the other hand, carotenoid contents in soybean leaves were significantly decreased as a result of ZnONPs treatments. The highest significant increase was in response to treatment with 50mg/L ZnONPs. This finding agreed with results of Singh et al. (2013) on vegetable crops cabbage (*Brassica oleracea* var. Capitata), cauliflower (*Brassica oleracea* var. Botrytis) and tomato (*Lycopersicon esculentum* L.) plants, Sanjay et al. (2015) on *Solanum melongena* (L.), Soliman et al. (2015) on moringa (*Moringa peregrina*) plants and Latef et al. (2017) on lupine (*Lupinus termis* L.).

In this study, results showed an increase in proline and MDA content in soybean plants exposed to salinity stress. Accumulation of soluble proteins under salinity stress may be the result of improved synthesis of specific salinity-related proteins (Ahmad et al., 2016). Free amino acids and proline are important organic solutes that assist in cell osmoregulation under salinity stress (Latef et al., 2017; Shafi et al., 2019). Soaking with ZnONPs resulted in a significant increment in organic solutes in unstressed and stressed soybean plants. This increment reached its maximum value in seedlings presoaked with 50mg/L ZnONPs. Zn also plays a main role in sugar formation and enzyme structure involved in the biosynthesis of amino acids (Soliman et al., 2015; Iqbal et al., 2018).

Membrane lipid peroxidation is an indication of membrane destruction under salinity stress (Samadi et al., 2019; Yilmaz et al., 2020). Results of the present study showed a high accumulation of MDA content under saline condition indicating integrity destruction of the cellular membrane and cellular compounds including proteins and lipids. Seed soaking with ZnONPs (particularly 50mg/L) decreased MDA, thus ameliorating the damage usually caused by salinity stress. This result is consistent with the results of Burman et al. (2013) who reported that ZnO induced defensive impacts on bio-membranes versus alternations of membrane permeability and oxidative stress in chickpea seedlings.

It has been reported in many studies that exposure to salt stress could induce ROS formation causing increased activity of antioxidant enzymes as a defense system (Ahmad et al., 2016; Latef et al., 2017; Al-Mahmud et al., 2020). This is compatible with the obtained results showing that growing soybean in saline conditions led to a marked increase in the antioxidant enzymes, which can be considered as good evidence of ROS production. ROS are considered mainly as dangerous molecules; however, it has been realized that ROS play vital roles in defense system of plants (Kononenko et al., 2020). Normally, plant cells are protected by a complex antioxidant system comprised of non-enzymic as well as enzymic antioxidants, such as superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX) (Dolatabadian et al., 2008). A close relationship between antioxidant activity and stress tolerance has been identified in several crops (Santander et al., 2020). High activity of SOD,

POX and CAT under salt stress is a good sign of soybean ability to adapt with ROS. Therefore, it is suggested that the increase in the activities of antioxidant enzymes may be attributed to the adaptive defense system of soybean against the harmful effects introduced by NaCl.

Depending on plant species and dose of the metal, studies indicated that ZnO nanoparticles can either stimulate or inhibit the activities of several antioxidant enzymes (Zhu et al., 2019; Husen, 2020). This is consistent with results which showed that seeds soaked with ZnONPs, especially ZnONP-T2, have low antioxidant enzymes activity when compared with salinized seedlings. However, the present finding was opposite to what have been found by Latef et al. (2017).

In this study, ZnO nanoparticles induced changes of the isoenzyme patterns of SOD, CAT and POX, especially in the salinity-stressed seedlings, which agrees with the finding of Abdelhamid et al. (2019) and Morsi et al. (2018). Therefore, it is suggested that the isozymes of SOD, CAT and POX present in soybean cells growing in the presence of 50mg/L of ZnO are required to remove ROS under unstressed condition. Under salinity condition, the level of ROS becomes too high to be dealt with by the existing antioxidant isoenzymes. Thus, soybean cells switch on additional antioxidant enzyme-dependent defense reactions, which involve novel isoenzymes. This was also observed with all ZnONPs concentrations except 50mg/L. It has been reported that induction of stress-related isoenzymes is probably related to the level of ROS, which causes oxidative damage of various cellular components, such as proteins, membrane lipids and nucleic acids (Asghar et al., 2016; Ahmed et al., 2017). Moreover, presoaking of soybean seeds with ZnONPs (50mg/L) increased Zn^{+2} content under salinity stress (ZnONP-T2+S plants) by 2.7-fold compared to salt treatment alone. This result agrees with the findings of Soliman et al. (2015) and Latef et al. (2017).

Conclusion

Salinity stress is one of the major limitations of soybean productivity worldwide. One of the most important strategies for increasing soybean cultivation is application of nanoparticles which play a key role in enhancing plant growth and maintaining the crop yields under salt stress. It can be concluded that applying of zinc oxide nanoparticles (ZnONP-T2 = 50mg/L) had a pronounced role in alleviating the adverse impacts of salinity in salt-stressed soybean plants by increasing photosynthetic performance and antioxidant enzyme activities leading to a balanced oxidative status. Therefore, it increases the adaptation capability of soybean to the saline environment.

Conflict of interests: The authors declare that they have no conflict of interest.

Authors contribution: R.M. Gaafar: Conceived and planned the experiments, took the lead in writing the manuscript. R.H. Diab: Carried out the experiments and performed the analysis. M.L. Halawa: Supervised the research experiments. A.R. El-Shanshory: provided critical feedback and revised MS. A. El-Shaer: Prepared and provided zinc oxide nanoparticles. M.M. Hamouda: Supervised the research experiments.

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دور الجسيمات النانومترية لأكسيد الزنك في تحسين تحمل الملوحة في فول الصويا

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على حسب ما نعلم فانه لم يتم اعطاء اهتمام كبير لتقييم دور الجسيمات النانومترية لأكسيد الزنك في النباتات التي تزرع تحت اجهاد الملوحة. في هذه الدراسة، قد تم تقييم الاستجابات الظاهرية والفسيولوجية والجزيئية لبادرات صنف فول الصويا جيزة 111 المجهدة بالملوحة تحت تركيزات مختلفة من الجسيمات النانومترية لأكسيد الزنك. حيث وجد ان المعاملة بتركيز مرتفع من الملح 250 ملي جرام وحده قد تتسبب في انخفاض كبير (65%) في النسبة المئوية للإنبات وكذلك انخفاضًا كبيرًا في جميع متغيرات النمو (طول الجذر، طول الساق والوزن الطازج والجاف للجذور والسيقان) ونشاط البناء الضوئي مقارنة مع العينات المرجعية. بالإضافة إلى ذلك، تم زيادة محتوى المالونداى الدهيد والبرولين، وأنشطة الإنزيمات المضادة للأكسدة (الكاتاليز CAT، بيراوكسيديز POX و سوبراوكسيد ديسميوتيز SOD). على النقيض من ذلك، فإن النقع المسبق لبذور فول الصويا بتركيزات مختلفة (25، 50، 100، 200 ملي جرام/ لتر) من الجسيمات النانومترية لأكسيد الزنك قد حفز نمو النباتات المعرضة للإجهاد الملحي حيث ظهر تحسن في معايير النمو، بالإضافة إلى انخفاض مستويات البرولين و المالونداى الدهيد في النباتات المجهدة خاصة عند تركيز 50 ملي جرام/ لتر مقارنة مع العينات المرجعية. علاوة على ذلك، أظهر تحليل مشابهات الانزيمات لكل من CAT و POX و SOD نمطًا متغيرًا من أليلات مشابهات الانزيمات خاصة عند 50 ملي جرام / لتر من الجسيمات النانومترية لأكسيد الزنك. واعتمادا على ذلك، يتضح أن استخدام 50 ملي جرام/ لتر من الجسيمات النانومترية لأكسيد الزنك يمكن ان يؤدي الى تحسين إنتاجية فول الصويا المزروع في الأراضي المالحة.