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Changes in antioxidant enzymes activity and plant performance by salinity stress and zinc application in soybean (*Glycine max* L.)

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Abstract

An experiment was conducted to investigate the effects of zinc application and salinity stress (0, 33, 66 and 99 mM NaCl) on some morphological and physiological parameters of soybean (*Glycine max* L., var. Williams). Results showed that zinc application improved shoot length, root fresh and dry weight and shoot fresh and dry weight under all salinity treatments. The catalase (CAT), ascorbate peroxidase (APX), polyphenoloxidase (PPO) and peroxidase activity (POD) and also proline content increased as a result of salinity stress. Lipid peroxidation and hydrogen peroxide concentration under the highest salinity were significantly greater than those under other treatments. However, lipid peroxidation and hydrogen peroxide concentration under salinity treatments significantly reduced as a result of zinc application. Zinc may act as a scavenger of ROS for mitigating the injury on biomembranes under salt stress. Adequate zinc also prevents uptake and accumulation of Na in shoot, by increasing membrane integrity of root cells.

Keywords: Antioxidant enzyme, Hydrogen peroxide, Lipid peroxidation, Salinity, Zinc.
Abbreviations: APX - Ascorbate peroxidase; CAT - Catalase; H_2O_2 - Hydrogen peroxide; MDA - Malondialdehyde; POD - Peroxidase activity; PPO - Polyphenoloxidase.

Introduction

Crops grown in arid and semi-arid regions are often exposed to adverse environmental factors such as drought or high soil salinity. Soil salinity is one of the major abiotic stresses that adversely affects plant productivity and quality (Zhu, 2001), due mainly to excess Cl^- and Na^+ ions in plants (Shilpim and Narendra, 2005). The production of activated oxygen species occurs when plants are subjected to stress conditions (Dionisio-Sese and Tobita, 1998). In plant cells chloroplasts, mitochondria and peroxisomes are important intracellular generators of ROS (Rich and Bonner, 1978). It is now widely accepted that reactive oxygen species (ROS) are responsible for various stress-induced damage to macromolecules and ultimately to cellular structure (Moftah and Michel, 1987; Kandpal *et al*., 1981), and needs to be scavenged for maintenance of normal growth. Ascorbate peroxidase (APX, EC 1.11.1.11), catalase (CAT, EC 1.11.1.6) and peroxidase (POD, EC 1.11.1.7), together with low-molecular mass scavengers such as ascorbate, glutathione and proline, act as the main defense against ROS produced in various parts of plant cells (Apel and Hirt, 2004). Catalase, which is located in peroxisomes, glyoxysomes and mitochondria, and is apparently absent in the chloroplast, dismutates mostly photorespiratory or respiratory H_2O_2 into water and molecular O_2 (Apel and Hirt, 2004), Peroxidases (POD) decomposes H_2O_2 by oxidation of co-substrates such as phenolic compounds and/or antioxidants. Polyphenol oxidase (PPO; EC 1.10.3.1) is an enzyme which oxidizes some phenols to chinone. This enzyme has been studied in a number of plants in relation to wounding, enzymatic browning (Demir and Kocacaliskan, 2001) and biosynthesis of alkaloids under biotic and abiotic stress conditions (Bilková *at al*., 2005). Changes in activities of various antioxidant enzymes under salinity stress have been reported (Koskeroglu and Tuna, 2008; Venkatesan and Sridevi, 2009; Hernández *et al*., 2000). However, little information is available on the effects of zinc on the activated oxygen species metabolism and antioxidant enzymes activity in soybean under saline stress. This knowledge can supply information on the possible involvement of antioxidants as a defense against reactive oxygen species in the mechanism of non-zinc applications plants and zinc applications plants under salinity stress. Salinity causes a range of deleterious effects such as inhibition of photosynthetic rate, chlorophyll content, damage to plasma membrane permeability and other metabolic disturbances (Ashraf and Parveen, 2002; Karimi *et al*., 2005). Proline accumulation is one of the most frequently reported modifications induced by salinity and water deficit in plants (Giridara Kumar *et al*., 2000; Ramanjulu and Sudhakar, 2001), and it is often considered to be involved in stress resistance mechanisms. Some workers did not observe any appreciable increase in free proline content (Koca *et al*., 2007; Kumar *et al*., 2003), whilst others consider enhanced proline level merely a stress effect, rather than a cause of stress tolerance (Moftah and Michel, 1987). The higher accumulation of proline could be due to enhanced activities of ornithine aminotransferase (OAT) and pyrroline -5-carboxylate reductase (P-5-CR), the enzymes involved in proline biosynthesis (Kohl *et al*., 1990), as well as due to

inhibition of proline oxidase, proline dehydrogenase (PDH) and proline catabolizing enzymes (Kandpal *et al*., 1981). It is well known that zinc is an important component of many vital enzymes, and a structural stabilizer for proteins, membrane and DNA-binding proteins (Aravind and Prasad, 2004). Zinc deficiency is now recognized as one of the most critical micronutrient deficiency in plants grown on calcareous, saline and sodic soils with high pH values. It is estimated that about 50% of soil used for plantations in the world have low levels of plant-available Zn (Graham and Welch, 1996). Zn deficiency is also recognized to cause higher levels of ROS in plants and relevant damages to plants (Marschner, 1995; Cakmak, 2000). Under Zn application, the activity of membrane-bound NADPH oxidase producing ROS decreases (Cakmak and Marschner, 1988a) and photooxidation process reduces (Marschner and Cakmak, 1989), while the activities of SOD, POD, and CAT enhance (Yu *et al*., 1998). Soybean is a major food and oil crop in the most countries where salinity problems exist or might develop. Large areas of formerly arable land are being removed from crop production every year due to increasing soil salinity. Soybean is classified as a salt-sensitive glycophyte (Launchli, 1984). Zinc plays a fundamental role in several critical cellular functions such as protein metabolism and IAA metabolism (Marschner, 1995). Furthermore, zinc supply could mitigate the adverse effects of NaCl (Marschner and Cakmak, 1989). However, the effects of zinc application on morphological, physiological and biochemical performance of soybean are poorly understood. Thus, this research was aimed to evaluate this subject with considerable details.

Results

Morphological parameters

NaCl treatment decreased the shoot length, root and shoot fresh and dry weights to a large extent. The shoot length decreased by 24%, 32% and 47% under salinity treatments of 33, 66 and 99 mM NaCl, respectively (significant at $P \leq 0.01$). Zinc application on NaCl stressed soybean plants increased the shoot length by 23% and 41% under both salinity levels at 66 and 99 mM (Table 2), respectively. The root length was not significantly affected by NaCl levels (Table 2). Whereas, under the high saline levels 66 and 99 mM NaCl the fresh weight of roots decreases by 23% and 20%, respectively (significant at $P \leq 0.01$). The root dry weight decreased by 53% under both salinity levels at 66 and 99 mM (Table 2). Whereas zinc application on plants exposed to salinity levels 66 and 99 mM caused a noticeable enhancement of root dry weight by 20% and 50% (Table 2), respectively (significant at $P \leq 0.001$). Results showed that shoot fresh and dry weights significantly influenced by salinity levels (significant at $P \leq 0.001$) (Table 2). Salinity levels 33, 66 and 99 mM NaCl, caused 46%, 61% and 80% and 44%, 65% and 83% decrease in shoot fresh and dry weight in non-zinc application plants (Table 2), respectively. Whereas, zinc application on plants exposed to salinity levels 33, 66 and 99 mM increased the shoot fresh weight by 13%, 42% and 137% and shoot dry weight by 7%, 88% and 250% (Table 2), respectively.

Antioxidant enzyme activities

CAT activity increased in both conditions (with and without zinc application) in root and leaf tissues by 150%, 352% and 597% and 188%, 740% and 547% under 33, 66 and 99 mM NaCl treatments respectively, compared with control (significant at $P \leq 0.05$) (Fig. 1 A). Constitutive activity levels were almost similar in both conditions in root and leaf tissues, but at 66 and 99 mM NaCl treated soybean plants, the highest activity was observed in both root and leaf tissues (Fig. 1 A). However, CAT activity was not significantly affected by zinc application. The values of APX activity in leaf tissues began to rise at mild salt stress and then showed a marked increase at severe salt stress. In particular, during severe salt stress, APX activity in leaves increased by 544% and 780% at 66 mM NaCl with zinc and 99 mM NaCl, respectively (significant at $P \leq 0.05$). Furthermore, APX activity in roots increased at 33 mM NaCl with zinc by 385% and 99 mM NaCl without zinc application by 464% (Fig. 1 B). There was no significant change in APX activity in response to zinc application conditions**.** Results showed that in both leaf and root, PPO activity was significantly influenced by salt stress ($P \leq 0.05$ and $P \leq 0.01$) (Fig. 1C). The PPO activity increased in both conditions (with and without zinc application) in leaf and root tissues under 33, 66 and 99 mM NaCl treatments, compared with control. The 33 and 66 mM NaCl treatments caused a 381% and 485% increases in PPO activity in leaves of non-zinc application plants (Fig. 1 C), respectively. However, there was no significant change in PPO activity in response to zinc application conditions, although a relative increase was observed in PPO activity in root at 66 and 99 mM NaCl with zinc application (Fig. 1 C). POD activity in zinc and non-zinc treated soybean root and leaf was significantly enhanced when the plants were grown under 99 mM NaCl, but there was no significant difference between 33 and 66 mM in both zinc and non-zinc treatments (significant at $P \leq 0.05$ and $P \leq 0.01$) (Fig. 1 D). Change in the enzyme activity for plants having zinc was only significant at 99 mM NaCl. The levels of POD activity in the leaves and roots were not altered in response to zinc application, and there were no significant differences between zinc and non-zinc treated plants at any NaCl levels (Fig. 1 D).

Lipid peroxidation

The oxidative damage was observed as malondialdehyde (MDA) content, which is a product of lipid peroxidation. Results showed that in both leaf and root, lipid peroxidation was significantly influenced by salt stress ($P \leq 0.001$ and $P \leq 0.05$) (Table 3). Leaf and root MDA was higher under saline conditions, compared with control. Salinity levels at 33, 66 and 99 mM NaCl, caused 248%, 429% and 422% and 87%, 150% and 233% increase in leaf and root MDA content in non-zinc application plants (Table 3), respectively. However, lipid peroxidation significantly reduced at 66 and 99 mM NaCl by 19% and 20% in leaf and 29% and 39% in root, compared with NaCl treatments without zinc application, respectively. The MDA accumulation was more pronounced in leaves than in roots (Table 3).

Proline content

The proline content leaves significantly influenced by salinity levels (significant at $P \leq 0.001$) (Table 3). The free proline content was significantly enhanced in the stressed plants over control plants in both conditions (with and without zinc application). There was a considerable increase in free proline accumulation with increasing salt stress. A more pronounced increase was observed under the 99 mM NaCl by 340% in comparison with the other treatments (Table 3). However, with zinc application proline content significantly reduced at 33 and 99 mM NaCl by 55% and 44% in leaves

Table 1. Some physical and chemical properties of the soil used in the experiment.

Texture	pH	ECe (dSm^{-1})	K	P	Mg	Zn	Mn	Fe	Cu
			($mg\ kg^{-1}$soil)						
Sandy clay loam	7.82	0.038	281	17.73	191.3	0.366	6.654	7.27	0.726

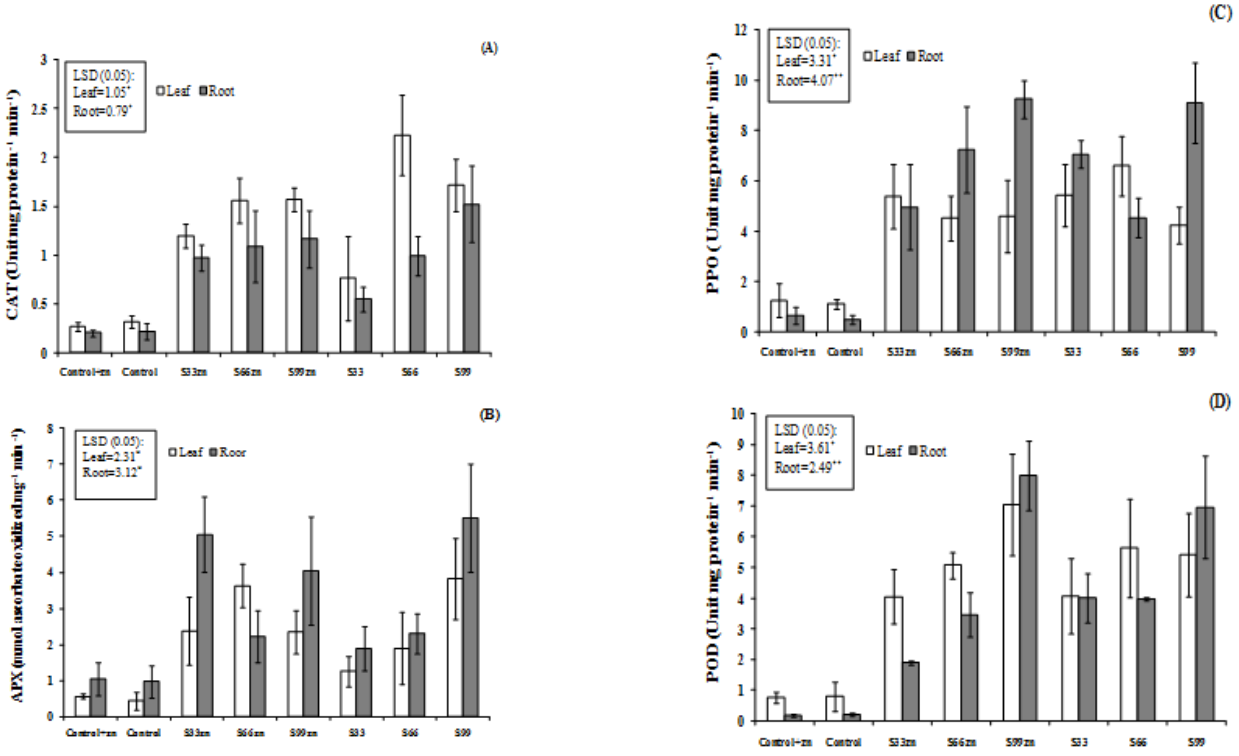

Fig 1. Effects of Zn on catalase (CAT) (A), ascorbate peroxidase (APX) (B), polyphenol oxidase (PPO) (C) and peroxidase (POD) (D) activities in leaves of soybean under NaCl stress: Control, S33, S66 and S99 refer to 0, 33, 66, and 99 Mm NaCl, respectively, and Control+Zn, S33Zn, S66Zn and S99Zn refer to 0, 33, 66, and 99 Mm NaCl + Zinc, respectively. Results are the mean of three replication ±SD. *, ** Significant at $P \leq 0.05$ and $P \leq 0.01$, respectively.

respectively, compared with NaCl treatments without zinc application.

Hydrogen peroxide concentration

The hydrogen peroxide concentration of leaf tissue was significantly increased with increasing salinity, so that the most hydrogen peroxide concentration was observed under the highest salinity level. The 33, 66 and 99 mM NaCl treatments caused a 150%, 189% and 223% increases in H_2O_2 concentration in leaves of non-zinc application plants (Fig. 1 C), respectively. However, H_2O_2 decreased with zinc application by 53%, 54% and 56% at 33, 66 and 99 mM NaCl treatments, compared with salinity treatments without zinc application (Table 3), respectively.

Discussion

A decrease in whole plant FW and DW was noted due to salinity stress (Table 2). Similarly, Aghaleh and Niknam (2009) observed that high salinity levels (100, 150 and 200 mM NaCl) decreased the growth parameters of soybean. Salinity stress limits plant growth by adversely affecting various physiological and biochemical processes like photosynthesis, antioxidant phenomena, nitrogen metabolism, ion homeostasis (Misra et al., 2006; Ashraf, 2004), proline metabolism and osmolytes accumulation (Misra and Gupta, 2005). Saline treatments increased Na+ and Cl− concentrations in root and leaf tissues of soybean. At the highest concentration of salinity, the enhancement of Na+ and Cl− concentrations led to a significantly reduction in plants growth. Reduction in both K+ and K/Na at high salinity is another opposing effect of salinity, which impairs the function of K in the salinised plants (data are not shown). A reduction in K+ concentration and K/Na ratio in saline conditions was reported by Liu and van Staden (2001). Zinc application on NaCl stressed soybean plants increased the shoot length, root and shoot fresh and dry weights (Table 2). Previously, it has been often hypothesized that improving the zinc nutritional status of plants growing in saline conditions

Table 2. The effect of zinc treatment on soybean growth parameters (root and shoot length and fresh and dry weights) under salinity stress.

Treatments	Root length (cm)	Shoot length (cm)	Root fresh weight (g)	Root dry weight (g)	Shoot fresh weight (g)	Shoot dry weight (g)
NaCl (mM) without zn						
0	42.06 ± 0.976	35.30 ± 2.154	25.27 ± 2.26	6.52 ± 0.090	47.92 ± 1.408	14.47 ± 0.078
33	39.68 ± 4.365	26.83 ± 3.382	19.28 ± 2.10	4.05 ± 0.066	25.53 ± 4.125	8.086 ± 0.170
66	33.96 ± 0.788	23.73 ± 1.467	19.92 ± 1.17	3.03 ± 0.164	18.52 ± 1.74	4.96 ± 0. 461
99	37.03 ± 1.140	18.66 ± 1.800	20.26 ± 1.44	3.08 ± 0.080	9.36 ± 0.396	2.44 ± 0.317
NaCl (mM) along with zn						
0	44.76 ± 0.266	36.5 ± 0.577	26.61 ± 0.36	6.02 ± 0.003	62.1 ± 0.305	19.98 ± 0.125
33	41.41 ± 3.260	27.73 ± 2.89	21.7 ± 1.78	4.06 ± .0290	28.87 ± 5.86	8.67 ± 0.127
66	40.66 ± 2.806	29.36 ± 2.45	20.44 ± 2.40	4.86 ± 0.011	26.307 ± 2.30	9.34 ± 0.698
99	40.93 ± 1.888	26.40 ± 2.62	20.43 ± 0.29	4.65 ± 0.069	22.24 ± 1.58	8.78 ± 0.135
F-test[+]	0.165	**	**	***	***	***
LSD (0.05)[+]	7.42	7.26	4.44	0.23	6.70	1.07

[+] *F*-test and LSD test (0.05) are to compare mean performances among salinity levels where the denoted symbols indicate significant difference at the 0.001 (***), 0.01 (**), and 0.05 (*) levels. Results are the mean of three replication ±SD.

Table 3. Mean proline content (mg g^{-1} DW), hydrogen peroxide concentration (H_2O_2) and malondialdehyde (nmolMDA g^{-1} FM) (leave and root) in soybean under different salinity levels with and without zinc application.

Treatments	Proline (mg g^{-1} DW)	H_2O_2 (µmol g^{-1} DW)	Malondialdehyde (nmol MDA g^{-1} FM)	
NaCl (mM) without zn			Leave	Root
0	13.77 ± 3.660	2.814 ± 0.212	2.85±1.211	1.39±0.410
33	40.10 ± 6.227	7.253 ± 0.444	9.93±2.421	2.60±0.269
66	41.37 ± 8.309	8.130 ± 1.292	15.1±0.167	3.48±0.619
99	60.69 ± 13.36	9.101 ± 1.137	14.9±0.359	4.64±1.391
NaCl (mM) along with zn				
0	12.48 ± 0.990	2.758 ± 0.426	3.72±1.183	0.86±0.279
33	17.86 ± 0.62	3.412 ± 0.563	9.52±1.442	2.75±0.570
66	26.72 ± 6.419	3.722 ± 0.452	12.2±1.418	2.45±0.170
99	33.67 ± 10.31	3.933 ±0.705	11.8±1.183	2.79±0.557
F-test[+]	***	***	***	*
LSD (0.05)[+]	18.49	1.94	3.16	1.95

[+] *F*-test and LSD test (0.05) are to compare mean performances among salinity levels where the denoted symbols indicate significant difference at the 0.001 (***), 0.01 (**), and 0.05 (*) levels. Results are the mean of three replication ±SD.

was critical for protection of plants against salt toxicity. This protective role of zinc was ascribed to its role in maintenance of the structural integrity of the plasma membrane and thus controlling the uptake of Na and other toxic ions (Cakmak and Marschner, 1988b). In several plant species, application of NaCl, even at a low concentration, stimulated the activities of antioxidative enzymes, which suggests a role of salt stress in ROS formation (Wang *et al*., 2005). CAT activity increased in root and leaf tissues of soybean under salinity stress with or without zinc application (Fig. 1 A). Reactive oxygen species include, for example, hydrogen peroxide, hydroxyl radicals and superoxide anions. ROS are usually generated by normal cellular activities such as photorespiration and *β*-oxidation of fatty acids, but their levels increase when plants are exposed to biotic or abiotic stress conditions. Elimination of ROS is mainly achieved by antioxidant compounds such as PPO, POD, CAT and APX. Under salinity stress conditions, a constitutively high antioxidant capacity can prevent damages due to ROS formation (Harinasut *et al*., 2003). Salinity stress led to a significant increase in APX activity in leaf tissues, which began to rise at mild salinity stress and then showed a marked increase at severe salinity stress (Fig. 1 B). APX activity, which is important component of the antioxidant system, plays a key role in eliminating H_2O_2 molecules and in the modulation of its steady-state levels in various plant subcellular compartments (Najami *et al.*, 2008). Moreover, high levels of intercellular H_2O_2 induced cytosolic APX activity under salinity stress (Lee *et al*., 2001). Our data show that the H_2O_2 concentration of leaf tissue was significantly enhanced with increasing salinity (Table 3). The increase in APX activity might be due to the high levels of intercellular H_2O_2 under salinity stress in leaf tissue of soybean. The PPO activity increased in leaf tissues under salinity stress with or without zinc application (Fig. 1 C). Increased polyphenol oxidase activity under stress indicates the ability to oxidize and degrade the toxic substance such as phenolic compounds which are generally accumulated during salt stress. Significant roles of POD have been suggested in plant development processes (Gaspar *et al*., 1985), which was involved in scavenging of H_2O_2 produced in chloroplasts (Asish and Anath, 2005). POD activity in zinc and non-zinc treated soybean root and leaf was significantly enhanced when plants were grown under severe salinity stress (99 mM NaCl) (Fig. 1 D). Aghaleh and Niknam (2009) found that salinity increased total POD activity in explants of both cultivars (Hack and Zan) of soybean under salinity stress. The enhancement of POD activity by salinity has also been observed in rice (Lee *et al*., 2001), pea (Shahid *et al*., 2011) and mulberry (Harinasut *et al*., 2003). In tolerant plants, POD activity was found to be higher to protect plants against the oxidative stresses (Sreenivasulu *et al*., 1999). Peroxidation of membrane lipids is an indication of membrane damage and leakage under salt stress conditions (Katsuhara *et al*., 2005).

Estimation of malondialdehyde (MDA) amount, which is a secondary end product of polyunsaturated fatty acid oxidation, is widely used to measure the extent of lipid peroxidation as indicator of oxidative stress (Lin and Kao, 2000). Peroxidation of lipids in plant cells appears to be initiated by a number of ROS. The rate of lipid peroxidation in terms of MDA can be used as an indication to evaluate the tolerance of plants to oxidative stress as well as the sensitivity of plants to salt stress (Jain *et al*., 2001). Our results showed that lipid peroxidation was influenced by salinity stress in both leaf and root of soybean. However, there was a significant decline in lipid peroxidation at NaCl+Zn treatments, compared with NaCl treatments. Increase in MDA contents under salt stress was also found in rice (Tijen and Ìsmail, 2005), alfalfa (Wang and Han, 2007), cotton (Diego *et al*., 2003) and wheat (Sairam and Srivastava, 2002). H_2O_2 concentration of soybean leaf tissue was significantly enhanced with increasing salinity (Table 3). Probably it was connected with the decrease in water potential just from the beginning of the severe salt stress, which might have limited H_2O_2 diffusion from the place of its generation. Together with higher hydration of tissues, H_2O_2 migrates more easily within a cell and reacts with some cell compounds resulting in lipid peroxides formation (Halliwell and Gutteridge, 1999). Adequate zinc also prevents uptake and accumulation of Na in shoot, by increasing membrane integrity of root cells (data are not shown). Increase in lipid peroxidation due to salt stress has been also reported by the other researchers (Gapinska *et al*., 2008; Radic *et al*., 2006; Sairam and Srivastava, 2002). Proline accumulation is one of the adaptations of plants to the salinity and water deficit (Giridara Kumar *et al*., 2000; Ramanjulu and Sudhakar, 2001). Earlier reports showed that proline accumulation may contribute to osmotic adjustment at the cellular level in many plants grown under elevated saline levels (Storey *et al*., 1977). Hence, these solutes play an important role in osmoregulation. Free proline content was significantly elevated in the stressed plants under salinity stress (Table 3), which suggests that proline may play an important role in the protection of soybean against salinity stress. Similarly, Li *et al*. (2010) observed that proline accumulation increased at middle salt level (200 mM) in castor bean seedlings. Proline content decreased with zinc application, compared with salinity treatments without zinc application (Table 3). These results are confirmed by Hendawy and Khalid (2005) and Cha-um *et al*. (2009), they demonstrated that, total carbohydrates and proline content were decreased with application of zinc in *Salvia Officinalis* L. plants**.** Because zinc application increased the shoot fresh and dry weight of the plants under salinity stress (Table 2), the decline in plant proline might be due to the dilution effect. One consequence of salt stress in plants is excessive generation of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide and hydroxyl radicals (Mitler, 2002). Hydrogen peroxide is a toxic ROS, which has deleterious effects in plant tissue (Asada and Takahashi, 1987). It has been suggested that decrease in membrane stability reflects the extent of lipid peroxidation caused by reactive oxygen species (Dhindsa *et al*., 1981). Higher H_2O_2 accumulation and lipid peroxidation in sensitive cultivars of wheat (Sairam *et al*., 2005) and pea (Hernandez *et al*., 1993) have been reported earlier. The hydrogen peroxide concentration of soybean leaf tissue was significantly enhanced with increasing salinity, so that the most hydrogen peroxide concentration was shown under the highest salinity level. However, H_2O_2 decreased with zinc application, compared with salinity treatments without zinc application (Table 3). Zinc plays a key role in controlling the generation and detoxicification of free oxygen radicals which can damage membrane lipids and sulphydryl groups (Alloway, 2004). Zinc may help to limit lipid peroxidation rate since it is a protective and stabilizing component of biomembranes against activated oxygen species (H_2O_2).

Materials and methods

Plant material and treatments

The experiments were conducted in 2009 at the greenhouse of the Faculty of Agriculture, University of Kurdistan, Iran. Some physical and chemical properties of the soil are given in Table 1. The soil samples were air-dried, crushed to pass through a 2-mm sieve, and mixed with sand at 2:1 ratio. Then zinc was combined thoroughly with soil at a rate of 10 mg kg^{-1} as $ZnSO_47H_2O$. Each 4-L plastic pot was filled with 3.5 kg zinc treated soil. Five replicates (pots) were prepared for each salt treatment. The certified seeds of soybean (cv. Williams) were obtained from Agricultural Research Center of Kurdistan, Iran. These seeds were surface-sterilized with 0.1% $MgCl_2$ solution for 5 min and washed thoroughly five times with distilled water. The experiment was carried out using a complete randomized design with three replications. Treatments applied in four NaCl levels (0, 33, 66, and 99 mM) with and without zinc application. Plants were kept under the natural light of day with supplementary light that was kept 13 h photoperiod with irradiance at plant level of 900–1200 µmol m^{-2} s^{-1} (PAR). Temperature and relative air humidity were 27 ± 3ºC and 60 ± 5% respectively. The salinity treatments were applied when plants were 3 weeks old (three nodes on the main stem with fully developed leaves beginning with the unifoliate nodes). At the end of experiment, electric conductivity of each pot was measured by using a digital conductivity meter (Inolab Model, Weilheim, Germany) to estimate final concentration of NaCl levels. Conductivity was conserved in a favorable amount by adding water or concentrated salt to pots as follows: 33 mM NaCl, 3~5 dS m^{-1}; 66 mM NaCl, 6~8 ds m^{-1}; 99 mM NaCl, 8~10 ds m^{-1}.

Growth parameters

Morphological parameters like root length and plant height were measured in fresh samples. Three to five plants were collected when plants were 6 weeks old for the measurement of fresh and dry weight. Plants were weighed individually for their fresh weight and kept in brown paper bags. Bags were then kept for 72 h in oven at 70°C. Finally dry weight was determined by weighing the dried roots, shoots, using precision balance.

Enzyme assays

Three weeks after salinization young leaves were collected and enzyme activities were assayed. Leaf samples were collected in an ice bucket and brought to the laboratory. Leaves were then washed with distilled water and surface moisture was wiped out. Leaf samples (0.5 g) were homogenized in ice cold 0.1 M phosphate buffer (pH 7.5) containing 0.5 mM EDTA with prechilled pestle and mortar. The homogenate was transferred to centrifuge tubes and was centrifuged at 4°C in Beckman refrigerated centrifuge at 15000 rpm for 15 min. The supernatant was transferred to 30 mL tubes and referred to enzyme extract. Protein Content in the enzyme extracts was determined according to Bradford

(1976) using Bovine Serum Albumin V as a standard. Catalase CAT (EC 1.11.1.6) activity was measured according to Beer and Sizer (1952), with minor modifications. The reaction mixture (1.5 mL) consisted of 100 mmol L^{-1} phosphate buffer (pH 7.0), 0.1 mmol L^{-1} EDTA, 20 mmol L^{-1} H_2O_2 and 20 µL enzyme extract. The reaction was started by addition of the extract. The decrease of H_2O_2 was monitored at 240 nm and quantified by its molar extinction coefficient (36 $M^{-1}cm^{-1}$) and the results expressed as CAT units mg^{-1} of protein (U = 1 mM of H_2O_2 reduction min^{-1} mg^{-1} protein).Ascorbate peroxidase (APX, EC 1.11.1.11) activity was determined according to Nakano and Asada (1981). The reaction mixture contained 50 mM potassium phosphate (pH 7.0), 0.2 mM EDTA, 0.5 mM ascorbic acid, 2% H_2O_2, and 0.1 mL enzyme extract in a final volume of 3 mL. The decrease in absorbance at 290 nm for 1 min was recorded and the amount of ascorbate oxidized was calculated using extinction coefficient (ε = 2.8 mM^{-1} APX was defined as 1 mmol mL^{-1} per min at 25°C. cm^{-1}). One unit of ascorbate oxidized as 1 mmol mL^{-1} ascorbate oxidized per min at 25°C. Polyphenol oxidase (PPO) (EC1.10.3.1) activity was assayed by the method of Kumar and Khan (1982). Assay mixture for PPO contained 2 mL of 0.1 M phosphate buffer (pH 6.0), 1 mL of 0.1 Mcatechol and 0.5 mL of enzyme extract. This was incubated for 5 min at 25°C, after which the reaction was stopped by adding 1 mL of 2.5 N H_2SO_4. The absorbance of the purpurogallin formed was read at 495 nm. To the blank 2.5 N H_2SO_4 was added of the zero time of the same assay mixture. PPO activity is expressed in Umg^{-1} protein (U=change in 0.1 absorbance min^{-1} mg^{-1} protein). Peroxidase POD (EC 1.11.1.7) activity was estimated according to Hemeda and Klein (1990). The reaction mixture contained 25 mmol L^{-1} phosphate buffer (pH 7.0), 0.05% guaiacol, 10 mmol L^{-1} H_2O_2 and enzyme. Activity was determined by the increase in absorbance at 470 nm due to guaiacol oxidation (E = 26.6 mM^{-1} cm^{-1}).

Proline content

For proline determination, the fifth fully expanded leaves were detached from the plants after the salinity treatment. Free proline content in the leaves was determined in accordance with the method of Bates *et al.* (1973). Leaf samples (0.5 g) were homogenized in 5 mL of sulphosalycylic acid (3%) using mortar and pestle. About 2 mL of extract was taken in test tube and 2 mL of glacial acetic acid and 2 mL of ninhydrin reagent were added to it. The reaction mixture was boiled in water bath at 100°C for 30 min. After cooling the reaction mixture, 6 mL of toluene was added and then transferred to a separating funnel. After thorough mixing, the chromophore containing toluene was separated and absorbance read at 520 nm in spectrophotometer against toluene blank. Concentration of proline was estimated by referring to a standard curve of proline.

Hydrogen peroxide concentration

Hydrogen peroxide (H_2O_2) concentration was determined according to Loreto and Velikova (2001). Leaf samples of 0.5 g were homogenized in 3 mL of 1% (w/v) tri-chloro-acetic acid (TCA). The homogenate was centrifuged at 10,000 rpm and 4°C for10 min. Subsequently, 0.75 mL of the supernatant was added to 0.75 mL of 10 mM K-phosphate buffer (pH 7.0) and 1.5 mL of 1M KI. H_2O_2 concentration of the supernatant was evaluated by comparing its absorbance at 390 nm to a standard calibration curve. The concentration of H_2O_2 was calculated from a standard curve plotted in the range from 100 to 1000 µmol mL^{-1}. H_2O_2 concentration was expressed as µmol g^{-1} DW.

Determination of lipid peroxidation rate

Oxidative damage to leaf lipids, resulting from salt stress, was estimated by the content of total 2-thiobarbituric acid reactive substances (TBARS) expressed as equivalents of malondialdehyde (MDA). TBARS content was estimated by the method of Cakmak and Horst (1991) with some modifications. Fresh leaf samples (0.2 g) were ground in 5ml of 0.1% (w/v) trichloroacetic acid (TCA), at 4 °C. Following the centrifugation at 12,000 × g for 5 min, an aliquot of 1ml from the supernatant was added to 4 mL of 0.5% (w/v) thiobarbituric acid (TBA) in 20% (w/v) TCA. Samples were heated at 90°C For 30 min. Thereafter, the reaction was stopped in ice bath. Centrifugation was performed at 10,000 × g for 5 min, and absorbance of the supernatant was recorded at 532 nm on a spectrophotometer (Model Camspec M330 UV/Vis) and corrected for non-specific turbidity by subtracting the absorbance at 600 nm. The following formula was applied to calculate malondialdehyde content using its absorption coefficient (ε) and expressed as nmolmalondialdehyde g^{-1} fresh mass following the formula:

MDA (nmol g^{-1} FM) = [(A532-A600) ×V×1000/ε] ×W

where ε is the specific extinction coefficient (=155 mM cm^{-1}), V is the volume of crushing medium, W is the fresh weight of leaf, A600 is the absorbance at 600 nmwavelength and A532 is the absorbance at 532nm wavelength.

Statistical analysis

Analysis of variance was performed using SAS version 9.1 (SAS Institute Inc., Cary, NC, USA). The data were presented as the means for each treatment. Means were compared using the LSD test at the 5% probability level.

Conclusion

The present study indicated that salinity stress caused a number of morphological and physiological changes in the soybean plants, including decreased shoot length, root fresh and dry weight and shoot fresh and dry weight and increased catalase (CAT), ascorbate peroxidase (APX), polyphenoloxidase (PPO) and peroxidase (POD) activities and free proline content as well as lipid peroxidation and hydrogen peroxide concentration. In contrast, treatment with zinc application decreased MDA and H_2O_2 content. Greater stimulation of total CAT, APX, PPO and POD activities under salinity treatments may be related to oxidative reactions corresponding to the higher level of MDA and H_2O_2 content in plant cells. The mechanism of zinc in alleviation of the severe salinity stress needs further investigations. Overall, the results indicated that treatment with zinc could reduce the effects of salinity stress in soybean plants.

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