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Physiological responses of soybean (*Glycine max* L.) to zinc application under salinity stress

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Abstract

A greenhouse research was conducted to evaluate the ameliorative effects of zinc application on soybean photosynthetic parameters, leaf relative water content (RWC), relative electrolytic leakage (REL), chlorophyll contents (Chl), and leaves and roots lipid peroxidation rate under salinity stress (0, 33, 66 and 99 mM NaCl). The results revealed that zinc application on plants exposed to salinity stress caused a noticeable enhancement of photosynthesis (*Pn*) by 110%, water use efficiency (WUE) by 54%, mesophyll efficiency (ME) by 98% and quantum yield (*Φ*) by 102% compared with plants exposed to salinity stress alone. The chlorophylls *a*, *b* and total chlorophyll content and relative water content were significantly reduced with increasing NaCl salinity. The highest REL and lipid peroxidation were occurred at the highest salinity level.

Keywords: Chlorophyll, photosynthesis, salinity stress, soybean, transpiration, zinc.

Abbreviations: *C*i: internal CO_2 concentration, *Tr:* transpiration rate, *Pn*: net photosynthetic rate, WUE: water use efficiency, ME: mesophyll efficiency, *Φ*: quantum yield, RWC: leaf relative water content, REL: relative electrolytic leakage, Chl: chlorophyll.

Introduction

Salinity is one of the major environmental stresses affecting the performance of many crop plants. Salinity has various effects on plant physiological processes such as increased respiration rate and ion toxicity, decreased leaf net CO_2 assimilation rate (Hajlaoui et al., 2006), efficiency of photosynthesis (Ashraf and Shahbaz, 2003; Kao et al., 2006; Sayed, 2003), and membrane disruption (Marschner, 1986; Gupta et al., 2002). Decreased photosynthetic rates may result from the closure of stomata and decreased mesophyll conductance, induced by osmotic stress, or from salt-induced damage to the photosynthetic apparatus (Flexas et al., 2004). The first step of photosynthetic CO_2 assimilation is catalyzed by ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO) in C_3 plants, and by phosphoenolpyruvate carboxylase (PEPC) in C_4 plants. Salinity enhances the oxygenase activity of RuBPCO, while it curtails its carboxylase activity (Sivakumar et al., 2000). Salinity often leads to decrease in chlorophyll contents and photosynthetic rates (Lee et al., 2004; Kao et al., 2006). It can seriously change the photosynthetic carbon metabolism, leaf-chlorophyll content, and photosynthetic efficiency (Seeman and Critchley, 1985; Sharkey et al., 1985). Zinc supply could mitigate the adverse effects of NaCl (Parker et al., 1992). Inside the chloroplasts proteolytic activities are dependent on zinc, for example, the repair processes of photosystem II through turning over photo-damaged protein (Bailey et al., 2002). The reduction in chlorophyll level and the destruction of chloroplast ultra structure led to decrease in photosynthesis in Zn-deficient plants. Zinc is a constituent of other enzymes involved in photosynthesis, including ribulose-1,5-bisphosphate carboxylase (RuBPC), which has been found to catalyse the initial step of carbon dioxide fixation in photosynthesis (Brown et al., 1993). 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 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). In addition, zinc plays a fundamental role in several critical cellular functions such as protein metabolism and IAA metabolism (Marschner, 1995).

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. Therefore, it is necessary to evaluate the physiological responses of crop plants to salt stress in order to develop appropriate strategies to sustain food production under adverse environmental conditions. Leaf photosynthetic capacity is suggested to be a key parameter determining crop yield (Jiang et al., 2002; Zhang et al., 2007). Furthermore, zinc can noticeably enhance photosynthesis parameters and chlorophyll content under saline conditions. However, the effects of zinc application on physiological performance of soybean are poorly understood. Thus, this research was aimed to evaluate this subject with considerable details.

Results and discussion

Photosynthesis parameters

According to the results of this study, net photosynthesis (*Pn*), internal CO_2 concentration (*Ci*), water use efficiency (WUE), mesophyll efficiency (ME), and quantum yield (*Φ*) significantly decreased with increasing salinity (Figure 1). Salinity also significantly reduced transpiration rate (*Tr*), compared with control (Figure 1B). But, during increasing salinity level, the differences between each level compared with the next one were mostly not significant (Figure 1B). Similar results were reported by Flexas et al. (2004) for C_3 plants. Salinity causes a range of deleterious effects such as inhibition of photosynthesis, pigment synthesis, damage to plasma membrane permeability, and other metabolic disturbances (Sasaki et al., 1998; Karimi et al., 2005). Reduction in *Pn* may result from the restriction on CO_2 diffusion into the chloroplast, via limitations on stomatal opening mediated by shoot and root-generated hormones, and on the mesophyll transport of CO_2, to alterations in leaf photochemistry and carbon metabolism (Flexas et al., 2004). Inhibition of photosynthetic capacity may also result from a reduced efficiency of ribulose-1,5-bisphosphate (RuBP) carboxylase, or a reduction of RuBP regeneration capacity, or from the sensitivity of PSII to NaCl (Ball and Anderson, 1986). The Zn addition under salt stress showed an obvious enhancement of *Pn*, WUE, ME, and *Φ* through increasing chlorophyll content of the soybean plants. Reduction of WUE under salinity stress without zinc application was due to a decline in *Pn* (Figure 1E). The reduction in *Tr* and *Ci* can be related to stomata closure (Lee et al., 2004). Reduction in ME may be also related to a decline in *Pn* (Figure 1A). Zn application on the plants exposed to salt stress caused noticeable enhancement of *Pn*, WUE, ME, and *Φ* compared with the plants exposed to salt stress alone (Figure 1A, D, E and F). Zn, acting as an inhibitor on hyperactive polarization-activated inward anion/Cl^- channels, may be beneficial for reducing the Cl^- absorption and enhancing the NO_3^- uptake to plants leaves exposed to salt stress (Yamaguchi and Blumwald, 2005). In photosynthesis, carbonic anhydrase (CA) is a Zn-containing enzyme that catalyzes the reversible conversion of carbon dioxide and water into carbonic acid, and requires Zn for its catalytic activity. Therefore, carbonic anhydrase employs a two-step mechanism: at in the first step, there is a nucleophilic attack of a zinc-bound hydroxide ion on carbon dioxide; at in the second step, the active site is regenerated by the ionization of the zinc-bound water molecule and the removal of a proton from the active site (Lindskog, 1997). Zn enhancement can be very beneficial for plants in order to facilitate the supply of CO_2 from the stomatal cavity to the site of CO_2 fixation (Sasaki et al., 1998). Furthermore, zinc is a constituent of other enzymes involved in photosynthesis, including ribulose-1, 5-biphosphate carboxylase (RuBPC), which has been found to catalyze the initial step of carbon dioxide fixation in photosynthesis and has been found in navy beans, barley, rice, and pearl millet (Brown et al., 1993).

Leaf chlorophyll content

Chlorophylls *a*, *b,* and *a*+*b* content of leaves were significantly decreased as NaCl salinity increase (Table 2). Similar result was reported by Hasaneen et al. (2009) for *Lactuca sativa* Plant. This deduction was greater in treatments without Zn application. There were no significant differences among the treatments in the ratio of Chl *a*/*b*. Salinity decreased nitrogen availability which could be one of the reasons for decreased chlorophyll content (Parashar and Verma, 1993). The reduction of total chlorophyll content was probably related to the enhanced activity of the enzyme chlorophyllase (Reddy and Vora, 1986) and inducing the destruction of chloroplast structure and the instability of pigment protein complex (Singh and Dubey, 1995). Plants treated with NaCl and Zn had significantly greater pigment contents than those exposed to salt stress alone. Zn probably maintains chlorophyll synthesis through sulphydryl group protection, a function primarily associated with Zn (Cakmak, 2000). Moreover, it participates in the synthesis of chlorophyll (Li et al., 2006).

Relative water content (RWC)

RWC significantly was decreased with increasing salinity. Nevertheless, when plants were subjected to different salt treatments along with zinc, the relative water content significantly improved (Table 3). The relative water content of non-salinized plants grown either in presence or in absence of zinc remained relatively at high levels (Table 3). The decrease in leaf RWC could be related to low water availability under stress conditions (Shalhevet, 1993), or to root systems, which are not able to compensate for water lost by transpiration through a reduction of the absorbing surface (Gadallah, 2000). Salt stress induced a reduction in the relative water content of the leaves, which indicates a loss of turgor that resulted in limited water availability for cell extension process (Katerji et al., 1997). Zinc may participate in stomatal regulation due to its role in maintaining membrane integrity. Sharma et al., (1995) observed a decrease in the K^+ content of guard cells in non-zinc application plants. This may be linked to enhanced K^+ efflux relative to influx, through leaky cell membranes, as absence zinc reduces membrane integrity. However, the specific role of zinc in stomata regulation requires further investigation. Generally, when stomatal closure is induced by salinity stress, there is a near constancy in leaf water use efficiency as the reduction in transpiration is slightly greater than a reduction in net photosynthesis (Figure 1A). However, no zinc application caused lower *Pn* and WUE. The data indicate that absence zinc and salinity stress, plants not only use less available water, but also the water transpired is used less efficiently.

Relative electrolytic leakage (REL) and Lipid peroxidation rate

The REL of the leaf tissue was significantly increased as salinity increased. The highest REL was observed under 99 mM NaCl conditions. REL was reduced with Zn application, compared to no Zn application treatments (Table 3). The oxidative damage was observed as MDA content, which is a product of lipid peroxidation increased during salinity treatment. Peroxidation of membrane lipids is an indication of membrane damage and leakage under salt stress conditions (Katsuhara et al., 2005). MDA is the decomposition product of polyunsaturated fatty acids of membranes under stress. The rate of lipid peroxidation level in terms of MDA can, therefore, be used as an indication to evaluate the tolerance of plants to oxidative stress as well as the sensitivity of plants to salinity stress (Jain et al., 2001). The results presented in Table 3 clearly show that in both leaf and root, MDA is influenced by salt stress as leaf and root MDA was higher under saline conditions, compared to control. Variations in MDA contents have been found in rice (Tijen and Ìsmail, 2005), cotton (Diego et al., 2003) cultivars differing in salt tolerance, and in two alfalfa cultivars under salt stress (Wang et al., 2005). 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

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

Texture	pH	EC (dSm^{-1})	K	P	Mg	Zn	Mn	Fe	Cu
			(mg kg^{-1}soil)						
Sandy clay loma	7.82	0.038	281	17.73	191.3	0.466	6.654	07.27	0.726

Table 2. Total chlorophyll concentration (T Chl), Chlorophyll *a* (Chl *a*), Chlorophyll *b* (Chl *b*) (mg g^{-1} FM) and Chl *a* /Chl *b* ratio in soybean leaves under different salinity levels with and without Zn application.

Treatments	Chl *a*	Chl *b*	Chl *a*/Chl *b* ratio	T Chl
NaCl levels (mM) without Zinc				
0	1.13 ± 0.19^{a}	0.52 ± 0.11^{a}	2.19 ± 0.09^{ab}	1.60 ± 0.30^{a}
33	0.36 ± 0.09^{bc}	0.17 ± 0.04^{b}	2.11 ± 0.22^{b}	0.54 ± 0.13^{c}
66	0.28 ± 0.02^{c}	0.11 ± 0.01^{b}	2.38 ± 0.04^{ab}	0.40 ± 0.03^{d}
99	0.22 ± 0.04^{c}	0.09 ± 0.008^{b}	2.31 ± 0.24^{ab}	0.32 ± 0.04^{e}
NaCl levels (mM) along with Zinc				
0	1.29 ± 0.10^{a}	0.59 ± 0.07^{a}	2.18 ± 0.09^{ab}	1.89 ± 0.17^{a}
33	0.85 ± 0.21^{ab}	0.36 ± 0.04^{ab}	2.37 ± 0.03^{ab}	1.21 ± 0.30^{b}
66	0.91 ± 0.06^{ab}	0.33 ± 0.04^{ab}	2.85 ± 0.42^{ab}	1.24 ± 0.04^{b}
99	0.86 ± 0.38^{ab}	042 ± 0.23^{ab}	2.31 ± 0.27^{ab}	1.29 ± 0.62^{ab}

Each value is the mean (± SE) of three replicates (Duncan's test, $P \leq 0.05$).

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). However, MDA was significantly reduced under NaCl+Zn treatments, compared with NaCl treatments without zinc application. In present work, zinc application compensated Zn shortage in plant (data are not shown) and reduced the deleterious effects of salinity on *Pn*, WUE, ME, *Φ*, Chl, RWC, REL, and MDA in soybean (Figure 1and Tables 2, 3). The principal role of zinc in preserving the integrity of cell membranes lay in its ability to protect membrane proteins and lipids from the destructive effects of superoxide radicals and their derivatives produced by redox reactions within the cell (Cakmak and Marschner, 1988). Zn can also interfere with reactive oxidative species (ROS) produced by the membrane-bound NADPH oxidase, and thus represents an excellent protective antioxidant against the oxidation of several vital cell components such as chlorophyll, membrane lipids, and proteins (Cakmak 2000).

Materials and methods

Plant material and growth conditions

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 mgkg^{-1} as $ZnSO_4.7H_2O$. Each 4-L plastic pot was filled with 3.5 kg zinc treated soil. 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. The pots were kept under natural photoperiod and watered regularly. Light duration was about 13 h. Temperature and relative air humidity were 27 ± 3ºC and 60 ± 5% respectively. The salinity treatments were applied when plants were 4 weeks old (three nodes on the main stem with fully developed leaves beginning with the unifoliate nodes) and maintained until final harvest. Ten leaves on the above one-half to one-third of the stem were harvested for the evaluation of the experimental parameters.

Net photosynthesis rate

Net photosynthesis (*Pn*) and transpiration (*Tr*) rates and internal CO_2 concentration (*Ci)* were measured on a fully expanded youngest leaf of each plant using an open system *LCA-4 ADC* portable infrared gas analyzer (Analytical Development Company, Hoddesdon, Herts, England), Measurements were performed from 09:30 to 11:30 A.M. with the following specifications and adjustments: leaf chamber area was fixed at 6.25 cm^2, ambient CO_2 concentration (C_{ref}) 295.35 µmol mol^{-1}, temperature in leaf chamber in the range of 28.67–30.24°C, leaf chamber gas flow rate (V) of 4.20–4.26 cm^3 s^{-1}, molar flow of air per unit leaf area (Us) of 404.8 mmol m^2 s^{-1}, ambient pressure (p) of 99.9 kPa and PAR at leaf surface (Q_{leaf}) of 1100-1453 µmol m^{-2} s $^{-1}$. All the analyses were carried out using the middle trifoliate of the third expanded trifoliate leaf from the apex. Water use efficiency and mesophyll efficiency were calculated using the formula of Ashraf et al., (2002). The value of quantum yield (*Φ*) was calculated according to de Palma (1996).

Leaf chlorophyll content

For chlorophyll determination, the fifth fully expanded leaves were detached from the plants after the salinity treatment. Prior to extraction, fresh leaf samples were cleaned with deionized water to remove any surface contamination. Fresh leaf samples (1 g) were ground in 90% acetone using a pestle

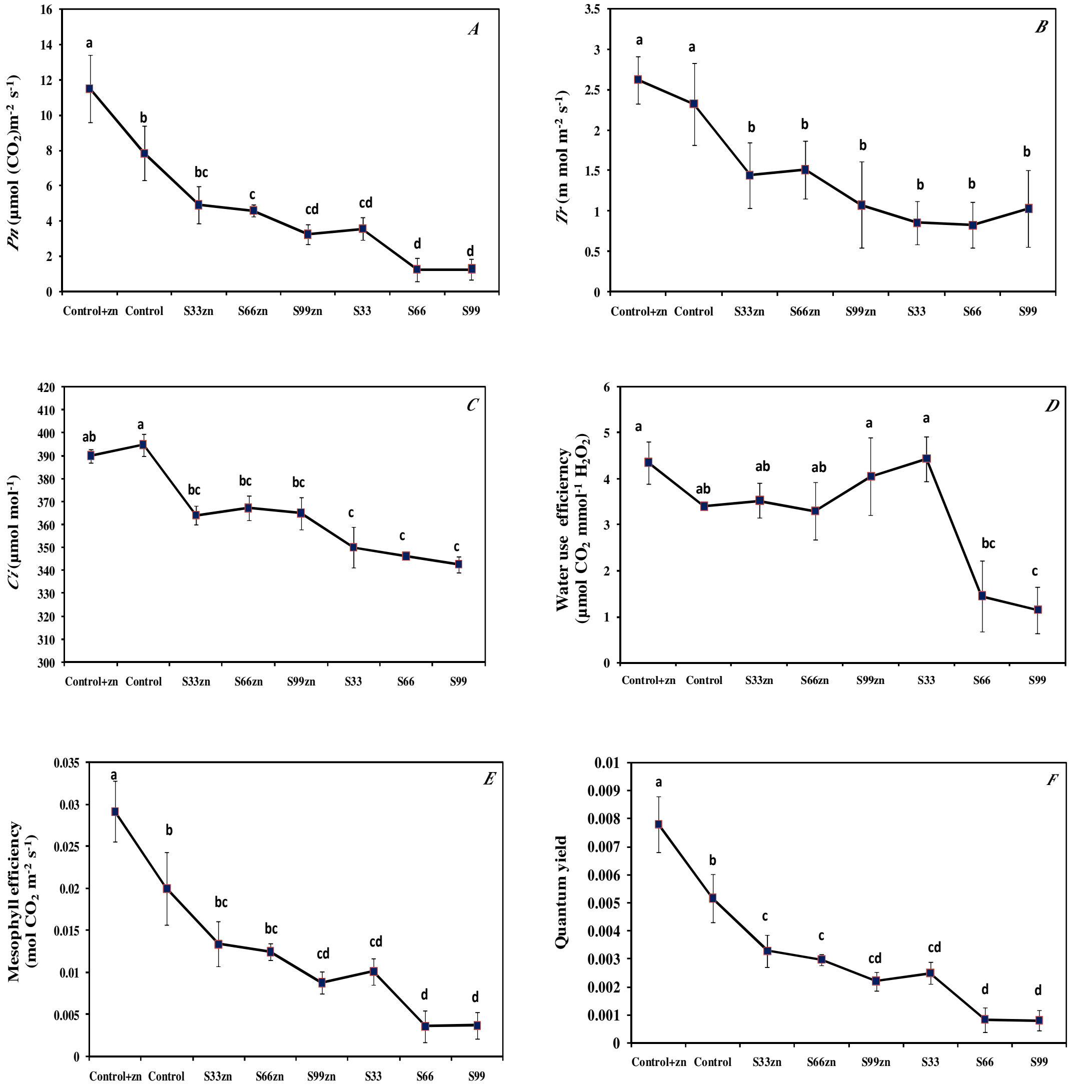

Fig 1. Means of A: photosynthesis rate (*Pn*), B: transpiration rate (*Tr*), C: internal CO_2 concentration (*Ci*), D: water use efficiency (WUE), E: mesophyll efficiency (ME) and F: Quantum yield (*Φ*) in leaves of soybean under NaCl stress (0, 33, 66, and 99 mM NaCl) with and without Zn application. Each value is the mean (± SE) of three replicates (Duncan's test, $P \leq 0.05$).

and mortar. The absorbance was measured using a UV/visible Shimadzu 160 A spectrophotometer, and chlorophyll content were calculated using the equation proposed by Strain and Svec (1966).

Relative water content (RWC)

Relative water content (RWC) of leaves was measured at stage of pod formation. Twenty healthy leaf discs of 1 cm diameter were cut from the plants using a leaf punch. RWC was calculated as: (FM - DM)/(TM - DM) × 100, where FM is the fresh mass, TM is the mass after rehydrating samples for 24 h by soaking the leaves in water, and DM is the dry mass obtained after oven-drying at 70°C for 36 h.

Relative electrolytic leakage (REL)

Ten leaf discs (5 mm^2) from the young fully expanded leaves were placed in 50 mL glass vials, rinsed with distilled water to remove electrolytes released during leaf disc excision. Vials were then filled with 30 mL of distilled water and allowed to stand in the dark for 24 h at room temperature. Electrical conductivity (EC1) of the bathing solution was determined at the end of the incubation period. Vials were heated in a temperature-controlled water bath at 95ºC for 20 min and then cooled to room temperature and the electrical conductivity (EC2) was again measured. The REL was calculated as REL = (EC1/EC2) × 100 (Shi et al., 2006).

Table 3. Amounts of MDA in leaves and roots, RWC, and REL of soybean subjected to different NaCl treatments (0, 33, 66 and 99 mM) with and without Zn application.

Treatments	Leaf MDA (nmol MDA g^{-1} FM)	Root MDA (nmol MDA g^{-1} FM)	RWC (%)	REL (%)
NaCl levels (mM) without Zinc				
0	2.58 ± 1.21^{d}	2.07 ± 0.61^{c}	70.4 ± 1.56^{b}	16.0 ± 2.92^{e}
33	9.93 ± 2.42^{bc}	8.09 ± 2.49^{ab}	57.3 ± 1.56^{d}	32.4 ± 4.26^{c}
66	15.3 ± 0.11^{a}	10.0 ± 0.42^{a}	49.5 ± 5.48^{e}	58.2 ± 2.04^{b}
99	16.0 ± 0.89^{a}	12.1 ± 4.22^{a}	38.9 ± 6.94^{e}	65.5 ± 4.92^{a}
NaCl levels (mM) along with Zinc				
0	3.72 ± 1.18^{d}	1.29 ± 0.41^{c}	76.4 ± 0.69^{a}	12.3 ± 0.47^{f}
33	6.30 ± 2.07^{cd}	4.12 ± 0.85^{bc}	69.1 ± 0.26^{b}	24.3 ± 1.15^{d}
66	19.1 ± 1.31^{bc}	3.67 ± 0.25^{bc}	68.8 ± 1.20^{b}	23.4 ± 2.91^{d}
99	11.8 ± 1.18^{ab}	4.19 ± 0.836^{bc}	63.9 ± 2.06^{c}	20.2 ± 5.69^{de}

Each value is the mean (± SE) of three replicates (Duncan's test, $P \leq 0.05$).

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 using 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 4ºC. Following the centrifugation at 12000×*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 10000×*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 MDA content using its absorption coefficient (ε) and expressed as nmol MDA g^{-1} fresh mass:

$$\text{MDA (nmol g}^{-1}\text{ FM)} = [(A532\text{-}A600) \times V \times 1000/\varepsilon] \times W$$

Where, ε is the specific extinction coefficient (=155mMcm^{-1}), V is the volume of crushing medium, W is the fresh weight of leaf, *A*600 and *A*532 are the absorbance at 600 nm and 532 nm wavelength respectively.

Statistical analysis

Analysis of variance was performed using the SAS software (Ver. 9.1). The data were presented as the means for each treatment (n = 8). Means were compared using the Duncan test at the 5% probability level.

Conclusion

In summary, these results demonstrated different roles of Zn in decreasing the effects of salt-stress on soybean. Zn may not act as its own direct nutritional function on soybean under salinity stress, but indirectly act as a scavenger of ROS for mitigating the injury on bio-membranes (including plasma membrane, chloroplast membrane, thylakoid membrane, and so on) (Cakmak 2000).

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