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ARTICLE

Exogenous ascorbate improves antioxidant defense system and induces salinity tolerance in soybean seedlings

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ABSTRACT Germination, growth and antioxidant defense system were investigated under salinity stress and pre-treatment with ascorbate (ASC) in two soybean cultivars SAHAR and DPX. Sterilized seeds were soaked in distilled water or ASC solution (0, 400 mg L^{-1}) for 4 hrs before they were sown in distilled water or NaCl solution (0, 12.5 and 50 mM). Salt stress reduced the growth of both cultivars through reduction in percentage of germination, shoot and root length and dry weight of seedling. ASC induced enhancement in growth of salt-stressed plants coupled with an increase in catalase and peroxidase activity in seedlings only in SAHAR cultivar, and an increase in superoxide dismutase activity in both cultivars. These findings led us to conclude that applied ASC counteracts the adverse effects of salt stress on growth of soybean; however, these effects were cultivar specific.
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KEY WORDS

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ascorbate
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Salinity is an increasing problem affecting 20% of the world's cultivated lands and nearly half of the area under irrigation, for that reason, genetic improvement of salt tolerance has become a critical need for the future of agriculture in arid and semi arid regions (Kaviani and Kharabian 2008). Excessive amounts of salt in the soil, most commonly NaCl, have detrimental effects on plant growth and productivity (Reynolds et al. 2005; Zilli et al. 2008; Sobhanian et al. 2010). Salt stress changes the morphological, physiological and biochemical responses of plants. Salinity causes both ionic and osmotic stresses and affects plant growth and development (Munns 2002; Benlloch-Gonzalez et al. 2005). One of the biochemical changes occurring when plants are subjected to salt stress is the production of reactive oxygen species (ROS). ROS causes cellular damage via membrane peroxidation, protein oxidation and DNA damage. To prevent such damages, plants have evolved an effective scavenging system involving antioxidant molecules like carotenoids, ascorbate, glutathione and tocopherols as well as antioxidant enzymes such as peroxidase (POD), superoxide dismutase (SOD) and catalase (CAT) (Bandeoglu et al. 2004). It has also been reported that plants with high levels of antioxidants, whether constitutive or induced, have a greater resistance to oxidative damages induced by ROS (Alqurainy 2007).

In many crop plants natural accumulation of osmoprotectants and other antioxidant compounds is very low and this deficiency can be overcome by their exogenous application (Makela et al. 1998). Exogenous application of plant growth regulators, fertilizers, osmoprotectants and antioxidants have been reported to successfully alleviate the adverse effects of salt stress on plants (Shalata and Neumann 2001; Khadri et al. 2007). ASC is a major water-soluble antioxidant, protecting biologically important macromolecules from oxidative damage caused by hydroxyl radicals, superoxide, and singlet oxygen. In addition to its importance in photoprotection and the regulation of photosynthesis (Smirnoff 2000; Foyer and Noctor 2000), ASC plays an important role in the regulation of cell cycle and several fundamental processes of plant growth and development.

Soybean (*Glycine max* L. Merr.), one of the most popular legumes, is one of the oldest cultivated crops. It is also considered as a good source of vegetable protein and oil since it has the highest level of protein in comparison with the other leguminous plants. It is necessary to investigate certain abiotic factors such as drought and salinity that may limit the soybean yield (Shilpi and Narendra 2005).

The purpose of this study was to determine the influence of ASC pretreatment on soybean seeds subjected to saline stress during germination and growth criteria through change in antioxidant capacity such as superoxide dismutase, catalase and peroxidase activity.

Materials and methods

Plant materials and treatments

Glycine max L. cv. SAHAR and cv. DPX seeds were obtained from Azarbaijan Agricultural Research Center and Natural Resources of Tabriz. Selected seeds were sterilized with 70% ethanol for 2 min and 0.5% sodium hypochlorite

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Table 1. Germination (%), shoot and root length (cm) in two soybean cultivars grown for 10 days under 0, 12.5 and 50 mM NaCl salinity and ASC at 0 or 400 mg l^{-1}. Values are the mean ± SD (n=4). Data of each column within each cultivar indicated by the same letters are not significantly different (P<0.05).

Parameters			Treatments		Cultivar
Root lenght	Shoot lenght	Germination %	NaCl	ASC	
12.9 ± 4.7 ^{a}	10.2 ± 1.1 ^{a}	95 ± 3.8 ^{a}	0	0	
12.7 ± 2.9 ^{ab}	9.4 ± 0.7 ^{ab}	83 ± 3.8 ^{bc}	12.5	0	
4.4 ± 1.6 ^{c}	6.0 ± 1.8 ^{c}	75 ± 6.0 ^{c}	50	0	SAHAR
13.0 ± 5.4 ^{a}	10.1 ±1.2 ^{ab}	93 ± 3.8 ^{ab}	0	400	
15.0 ± 3.9 ^{a}	10.2 ± 1.7 ^{a}	90 ± 2.3 ^{ab}	12.5	400	
7.50 ± 2.0 ^{ab}	7.3 ± 0.6 ^{bc}	85 ± 6.8 ^{bc}	50	400	
14.4 ± 1.2 ^{a}	8.9 ± 0.6 ^{a}	81 ± 8.2 ^{a}	0	0	
12.3 ± 4.2 ^{a}	4.4 ± 0.9 ^{b}	75 ± 8.8 ^{a}	12.5	0	
12.9 ± 4.3 ^{a}	4.0 ± 1.0 ^{b}	72 ± 12.6 ^{a}	50	0	DPX
17.3 ± 5.6 ^{a}	8.0 ± 1.5 ^{a}	86 ± 9.5 ^{a}	0	400	
13.6 ± 2.3 ^{a}	7.8 ± 1.5 ^{a}	80 ± 11.7 ^{a}	12.5	400	
15.0 ± 1.9 ^{a}	8.1 ± 0.8 ^{a}	78 ± 10.9 ^{a}	50	400	

solution for 15 min. Seeds were soaked in distilled water or ASC solution (0, 400 mg l^{-1}) for 4 hrs before they were sown in distilled water or NaCl solutions (0, 12.5, 50 mM). The seeds were allowed to germinate at 25°C in darkness. Seeds were considered to germinate after a radicle emerged from the testa. After 10 days, growth parameters were recorded in controlled growth chamber.

Preparation of extracts for enzyme assays

For shoot crude extract preparation, 1 g tissue was homogenized in 3 ml of 0.1 M phosphate buffer (pH 7.0) at 4°C. The homogenate were then transferred into eppendorf tubes and centrifuged at 14000 g for 20 minutes at 4°C. Supernatant was used for antioxidant enzyme and protein content assays.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was assayed as described by Winterbourn et al. (1976). The reaction mixture contained 0.05 ml of enzyme extract, 2.650 ml of 67 mM potassium phosphate buffer (pH 7.8), 0.2 ml of 100 mM EDTA/ 0.3 mM sodium cyanide (NaCN) and 0.1 ml of 1.5 mM nitroblue tetrazolium (NBT). One unit of enzyme activity was defined as the amount of SOD required to produce a 50% inhibition of reduction of NBT and the specific enzyme activity was expressed as units per milligram of total protein.

Peroxidase (POD, EC 1.11.1.7) was assayed as described by Ghamsari et al. (2007). Assays were carried out at room temperature (~ 20-25ºC) using T-60 spectrophotometer (PG Instrument, UK). Reaction mixture contained 3 ml of 0.1 M citrate-phosphate-borate buffer system (pH 7.0), 25 µl of 480 mM guaiacol, 25 µl of 96 mM H_2O_2 and 30 µl of extract. The reaction was started by the addition of extract. Activity of POD was calculated as enzyme protein required for the formation of 1 µmol tetraguaiacol per min.

Catalase (CAT, EC 1.11.1.6) activity was measured according to the method given by Obinger et al. (1997). The assay mixture contained 3 ml of 50 mM citrate-phosphate-borate buffer (pH 7.0), 26 µl of 11.8 mM H_2O_2 and 50 µl of extract. The decomposition of H_2O_2 was followed by the decline in absorbance at 240 nm. One unit of CAT activity represents one µmol of H_2O_2 decomposed per min.

Soluble protein was estimated spectrophotometrically by the dye-binding method (Bradford 1976).

Statistical Analysis

All the values in the figures and tables are the mean of four independent determinations. Differences between control and treated seeds were analyzed by one-way ANOVA followed by Tukey's multiple range test ($P \leq 0.05$).

Results

Effect of salinity and ACS on growth parameters

50 mM NaCl delayed germination and decreased final germination percentage in SAHAR cultivar, but in DPX cultivar there were no significant differences as compared with the control (Table 1). Pretreatment with ASC led to a significant increase in the percentage of germination in DPX cultivar. Increasing NaCl levels decreased shoot length of both cultivar's seedlings. In SAHAR cultivar 50 mM NaCl decreased shoot length by 41% and for DPX by 54.6%. Root length was decreased significantly (66%) in SAHAR cultivar under 50 mM NaCl treatment whereas this parameter remained unchanged in DPX. Salt stress significantly decreased shoot dry weight in both cultivars ($P<0.05$). Root dry weigth increased only in ASC treated DPX cultivar (Table 2).

Effect of salinity and ASC on protein content and antioxidant enzymes

50 mM NaCl decreased protein content of both cultivars seedlings (SAHAR, 41% and DPX, 49%) (Table 2). Activity of

Table 2. Protein content (mg g^{-1} FW), shoot and root dry weight (mg $plant^{-1}$) in two soybean cultivars grown for 10 days under 0, 12.5 and 50 mM NaCl salinity and ASC at 0 or 400 mg l^{-1}. Values are the mean ± SD (n=4). Data of each column within each cultivar indicated by the same letters are not significantly different (P<0.05).

Parameters			Treatments		Cultivar
Root dry weight	Shoot dry weight	Protein content	NaCl	ASC	
18.7 ± 1.50 [a]	87.7 ± 6.23 [a]	23.52 ± 0.61 [b]	0	0	
22.7 ± 1.25 [a]	89.2 ± 6.65 [a]	24.82 ± 0.22 [a]	12.5	0	
15.5 ± 4.04 [a]	15.7 ± 5.37 [b]	12.62 ± 0.30 [d]	50	0	SAHAR
19.5 ± 3.69 [a]	119 ± 5.47 [a]	21.22 ± 0.34 [c]	0	400	
28.5 ± 9.60 [a]	107 ± 34.7 [a]	24.12 ± 0.71 [ab]	12.5	400	
20.2 ± 6.55 [a]	104 ± 18.9 [a]	23.12 ± 0.30 [b]	50	400	
23.5 ± 1.29 [abc]	142 ± 7.20 [a]	21.82 ± 0.26 [d]	0	0	
21.7 ± 2.62 [bc]	115 ± 20.4 [a]	23.82 ± 0.34 [b]	12.5	0	
17.5 ± 5.19 [c]	16.7 ± 3.09 [b]	6.92 ± 0.35 [e]	50	0	DPX
30.5 ± 7.50 [ab]	139 ± 24.1 [a]	27.82 ± 0.26 [a]	0	400	
33.2 ± 5.85 [a]	123 ± 32.2 [a]	22.82 ± 0.18 [c]	12.5	400	
33.5 ± 3.10 [a]	127 ± 38.3 [a]	21.92 ± 0.42 [d]	50	400	

SOD in both cultivars increased in salt stress. In SAHAR and DPX cultivars, 50 mM of NaCl caused significant increase in activity of SOD (P<0.05) compared to control (Fig. 1). ASC increased POD activity rather than NaCl treatment in SAHAR cultivar. However, increasing NaCl concentration with or without ASC caused a significant decrease in POD activity (P<0.05) in comparison with control group of DPX (Fig. 1). CAT activity was decreased in both cultivars under the salt stress. However, applied ASC caused an increase in CAT activity of salt stressed seedlings of SAHAR in comparison with salt stressed group, whereas it decreased in all different treatments of DPX.

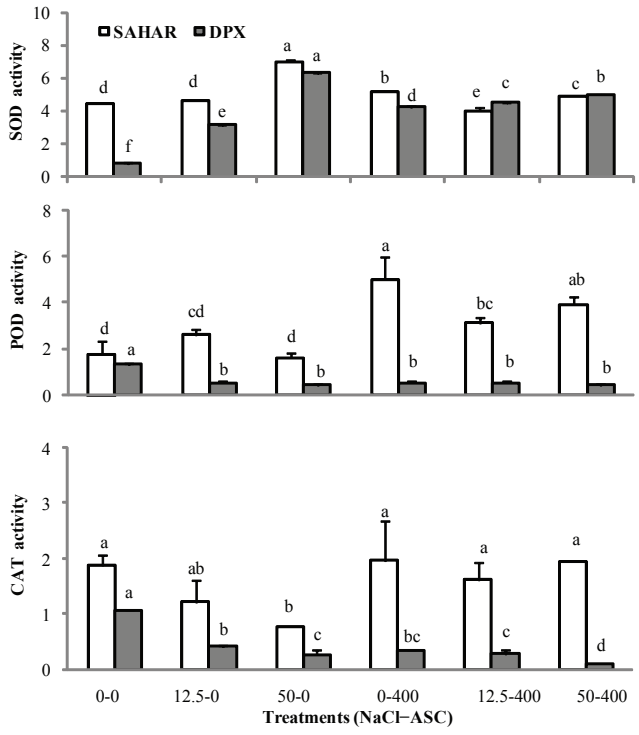

Figure 1. Specific activity of superoxide dismutase (SOD, U mg^{-1} protein min^{-1}), peroxidase (POD, nmol tetraguaiacol mg^{-1} protein min^{-1}) and catalase (CAT, µmol H_2O_2 mg^{-1} protein min^{-1}) in seedlings of two soybean cultivars grown for 10 days under 0, 12.5 and 50 mM NaCl salinity and ASC (ascorbate) at 0 or 400 mg l^{-1}. Data were compared within each cultivar. Values are the mean ± SD (n=4). Bars indicated by the same letters are not significantly different (P<0.05).

Discussion

Growth parameters and protein content

Plants under salt stress use a variety of strategies to neutralize the adverse effects of salt stress on their growth. In the present study growth was significantly decreased in salt-treated seedlings. Similar results on the reduction of soybean growth due to salt stress were reported by Amirjani (2010). Non-enzymatic antioxidants are more important due to their dominant role in plant growth and development in addition to their antioxidant capacity (Khan et al. 2006). Comparison of germination and root length under salinity showed that DPX is a rather more salt tolerant cv than SAHAR cv. There is no published work concerning the expression of salt tolerance in SAHAR and DPX cultivars. In this work, exogenously applied ASC, through pretreatment, caused enhancement in germination percentage and growth of salt stressed seedlings of soybean cultivars. There are several reports, which provide evidence that ASC accelerates cell division and cell enlargement as observed in different plants such as *Pisum* (De Cabo et al. 1996), and *Lupinus albus* (Citterio et al. 1994). These findings and the results of the present study suggest that the growth-promoting effect of ASC may have been due to enhanced antioxidant capacity under salt stress (Athar et al. 2008).

ROS are produced under salt stress that may cause protein denaturation and oxidation (Sajid and Aftab 2009). Soybean plants exposed to 100 and 200 mM NaCl show significant decrease in their protein content (Muthukumarasamy et al. 2000). A remarkable decrease in the protein content of salt stressed radish plants have been reported (Moussa 2004). Also, in this study soybean seedlings showed a significant decrease in protein content when stressed with 50 mM NaCl.

Antioxidant enzymes

It is now well-known that salt tolerance in most crop plants is associated with a more efficient antioxidant system including enzymes (SOD, APX, and CAT) and non-enzyme antioxidants (ASC, tocopherols, salicylic acid and carotenoids) (Athar et al. 2008). The results of this work suggest a protective role of ASC against salt-induced oxidative damage in soybean cultivars. Salinity caused an increase in SOD activity in both cultivars. Similar results of SOD activity reported in salt-tolerant cultivars of pea (Hernandez and Almansa 2002), sugar beet (Bor et al. 2003) and tomato (Koca et al. 2006) under salt stress. In this research the enzyme activity indicated that under saline condition, in both cultivars, only SOD activity increased, therefore we can say that in the seedling stage SOD play an important role in the free radical scavenging system.

The present study demonstrated that ASC may play an important role in salt stress by protecting soybean seedlings from salt-induced oxidative damage through the maintenance and/or increase of the activity of antioxidant enzymes. These findings led us to conclude that applied ASC counteracts the adverse effects of salt stress on growth of soybean; however, these effects were cultivar specific.

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