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Amelioration of salinity tolerance in *Solanum tuberosum* L. by exogenous application of ascorbic acid

Zahoor Ahmad Sajid · Faheem Aftab

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Abstract The present investigation envisaged revealing the role of exogenous application of ascorbic acid in increasing resistance against NaCl stress. Shoot apices from 60-d-old, *in vitro*-grown plants of two commercially important cultivars of *Solanum tuberosum* L., cvs. Desiree and Cardinal, were inoculated on Murashige and Skoog (MS) medium supplemented with 0.5 mM ascorbic acid for 72 h as a pretreatment. Pretreated and non-pretreated shoot apices were transferred to MS medium containing different concentrations of NaCl (0–140 mM; eight treatments). Results were recorded for morphological (shoot length, shoot number, root length, root number, and number of nodes) and biochemical features (protein, peroxidase, catalase, and superoxide dismutase activities) after 60 d of salt treatment. Similarly, 60-d-old, well-proliferated callus cultures were also pretreated with ascorbic acid for 24 h and transferred to an optimized callus proliferation medium containing different concentrations of salt. Results were recorded after 60 d of salt treatment for percentage relative fresh weight growth and biochemical parameters. Salinity severely inhibited all the growth parameters in both the cultivars. Pretreatment with ascorbic acid to both salt-treated plants and callus cultures showed significant differences with respect to almost all of the growth and biochemical parameters studied. Protein content as well as catalase and superoxide dismutase activities increased significantly in both the cultivars, although peroxidase activity showed a decreasing trend in ascorbic acid-pretreated plants as well as callus cultures.

Z. A. Sajid · F. Aftab (✉)
Department of Botany, University of the Punjab,
Quaid-e-Azam Campus,
Lahore 54590, Pakistan
e-mail: faheem@botany.pu.edu.pk

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Introduction

Salinity is one of the major causes of decreases in agricultural production. Nearly 20% of the world's cultivated area and half of the irrigated area are affected by salinity (FAO Statistics 2005). Higher concentration of salt in rooting medium exerts many effects, such as low osmotic potential, ion specificity, nutritional imbalance (Karimi et al. 2005), change in cell metabolism levels (Wahid and Ghazanfar 2006), and reduction in growth and yield (Vaidyanathan et al. 2003). While cells are under stress, certain reactive oxygen species (ROS) are produced that may cause membrane peroxidation, protein denaturation, DNA damage (Noctor and Foyer 1998), or show toxicity to metabolic functions after conversion to H_2O_2 (Hernandez et al. 1995). ROS are produced in all cellular compartments as a by-product of cellular metabolism. Plants possess both enzymatic and non-enzymatic mechanisms for scavenging ROS (Rahnama et al. 2003; Vaidyanathan et al. 2003). The overproduced ROS-scavenging enzymes associated with salinity reported so far include superoxide dismutase, peroxidase, catalase, glutathione reductase, and glutathione-synthesizing enzymes (Harinasut et al. 2003). Non-enzymatic factors include several small molecules that are antioxidant in nature, such as quaternary ammonium compounds, polyamines, polyols, alpha-tocopherol (vitamin E), ascorbic acid (vitamin C), and carotenoids (Ashraf and Harris 2004; Sairam et al. 2005).

Ascorbate, also known as vitamin C, is an important antioxidant molecule that acts as a primary substrate in the cyclic pathway for enzymatic detoxification of not only

hydrogen peroxide (H_2O_2) but also superoxide ($O_2^{\bullet-}$), hydroxyl radical (OH•), and lipid hydroperoxides (Yu 1994). Its role as an ascorbate peroxidase substrate that scavenges hydrogen peroxide in the chloroplast stroma has been well documented by Nakano and Asada (1981), Gadallah (2000), and Shigeoka et al. (2002). Ascorbic acid is water-soluble, so it has an additional role on the thylakoid surface in protecting or regenerating oxidized carotenes and α-tocopherols, a lipophilic antioxidant molecule (Noctor and Foyer 1998).

Potato is an important tuber crop all over the world (Chiru et al. 2008). Potato does not thrive well in soils heavily infested with sodium salts and has been classified as moderately salt-tolerant to moderately salt-sensitive (Mckenzie 1988). Exogenous application or pretreatment with different inorganic and organic compounds, such as NaCl, KCl, Na_2SO_4, H_2O_2, PEG, sorbitol, mannitol, glycine betaine, proline, IAA, gibberellic acid and ascorbic acid, has been considered an efficient method to enhance the tolerance of plants against abiotic stresses, perhaps more so than plant breeding and genetic engineering techniques (Ashraf and Foolad 2005; Wahid et al. 2007). Al-Hakimi and Hamada (2001), Shalata and Neumann (2001), and Khan et al. (2006) have reported the pretreatment of seeds with ascorbic acid.

Pretreatment against salt stress of *in vitro*-grown tissues with ascorbic acid has not been reported previously. The choice of explant in this study was shoot apices and callus cultures because earlier studies on salinity tolerance in potato (Sasikala and Prasad 1993; Martinez et al. 1996; Farhatullah et al. 2002) had shown that shoot apices were a good explant source to evaluate *in vitro* salinity tolerance. In the present study, the effect of ascorbic acid on enhancing salinity tolerance in potato was investigated on both *in vitro* plants as well as callus cultures.

Materials and Methods

Procurement and surface sterilization of plant material. Healthy tubers (without any visual symptoms of disease) of the potato cultivars Cardinal and Desiree were grown in sterile sand in a glasshouse at 26±2°C and 70% relative humidity. Shoots 1.0 cm long, obtained from these tubers, were used as an explant source. To surface disinfect tissues, the explants were first washed thoroughly with a household detergent (Unilever, Pakistan) and then placed in a 0.7% sodium hypochlorite (NaClO) solution containing 0.1% (*v*/*v*) Tween-20 for 5–10 min in an Erlenmeyer flask (250 mL, Pyrex) on an orbital shaker at 125 rpm. Explants were then washed three times with autoclaved distilled water to remove all traces of NaClO. Murashige and Skoog (MS; Murashige and Skoog 1962) basal medium supplemented with 30 gL^{-1} sucrose, solidified with 0.7% (*w*/*v*) agar (Oxoid; Hampshire, UK) and adjusted to pH5.7 prior to autoclaving was used for shoot induction and plant growth after sterilization by autoclaving at 121°C and 15 lbs $in.^{-2}$ for 15 min. Exactly 15 mL medium was dispensed into 25×150-mm culture tubes (Pyrex). A single nodal explant was inoculated in each culture vessel and all cultures were incubated at 26±2°C in 16 h continuous light (40 μmol $m^{-2}s^{-1}$) using cool white fluorescent tube lights (Philips Ltd., Karachi, Pakistan).

Ascorbic acid pretreatment of in vitro plants. For ascorbic acid pretreatment, 60-d-old shoot apices (1.0 cm long) were excised from *in vitro*-grown plants of both the cultivars and subcultured onto MS basal medium supplemented with 0.5 mM ascorbic acid (added directly in MS medium before pH adjustment and autoclaving) for 72 h. The dose and time for pretreatment in this experiment was based on a previous study (Shalata and Neumann 2001). Pretreated and non-pretreated shoot apices were then transferred to MS medium containing different concentrations of NaCl (0–140 mM; eight treatments), thus making an 8×2 factorial combination of media and pretreatment for each cultivar. Fifteen culture vessels (25×150 mm) were inoculated for each treatment. Cultures were maintained at 26±2°C in a 16-h photoperiod (40 μmol $m^{-2}s^{-1}$) using cool white fluorescent tube lights.

Results were recorded for shoot length, shoot number, root length, root number, number of nodes, protein content, peroxidase, catalase, and superoxide dismutase activities after 60 d of salt treatment. For this purpose, the plants were harvested prior to the recording of the shoot length with a ruler from the top of the medium to the tip of shoot minus 1.0 cm (size of the original explants at the time of explant inoculation). The root length was also measured using a ruler from the tip of the root up to the basal end of the shoot.

Ascorbic acid pretreatment of in vitro callus cultures. A medium previously optimized in our laboratory for callus induction and proliferation was used in this study. For callus induction, internodal explants from 60-d-old, *in vitro*-grown plants were inoculated on MS basal medium supplemented with 10.0 μM 6-benzylaminopurine (BAP) and 1.1 μM α-naphthaleneacetic acid (NAA). Because of excessive browning of callus cultures after 72 h of ascorbic acid pretreatment in preliminary experiments (data not shown), 60-d-old pre-weighed callus cultures (derived from the original explant) of both the cultivars were pretreated by ascorbic acid for 24 h. Before pretreatment of callus cultures to ascorbic acid, culture vessels were weighed with or without callus to determine the weight of callus. For

pretreatment, callus cultures were cultured for 24 h on optimized callus proliferation medium (as described above) supplemented with 0.5 mM ascorbic acid. After 24 h, the callus cultures were transferred to the same medium but containing different concentrations of NaCl (0–140 mM) as explained above. Callus cultures were maintained under dark conditions at 26±2°C. Data were recorded for percentage relative fresh weight growth (PRFWG) of callus cultures and their biochemical features after 60 d of salt treatment. The calluses were subcultured every 15 d to the respective salt-containing media. The PRFWG of calluses was calculated using the formula $W_1 - W_0/W_0 \times 100$ (where W_1 = weight after 60 d of salt treatment and W_0 = weight of callus culture before salt treatment).

Biochemical analysis of in vitro plants and callus cultures. For protein and enzyme assay, 1 g fresh plant material (leaves, shoot, or callus cultures) was ground in liquid nitrogen into a very fine powder using an ice-chilled pestle and mortar. The ground tissue was suspended in 2.0 mL of 0.1 M phosphate buffer, pH7.2 (13.6 g KH_2PO_4 and 17.4 g K_2HPO_4 in 1,000 mL of solution) containing 0.5% (*v*/*v*) Triton X-100 and 0.1 g of polyvinyl-pyrrolidone. The slurry was centrifuged at 14,000 rpm at 4°C for 30 min using a Sorval RB-5 refrigerated super speed centrifuge. The resultant supernatant was collected and stored at 0°C for further estimation of protein, peroxidase, catalase, and superoxide dismutase levels.

The Biuret method of Racusen and Johnstone (1961) was adopted for the estimation of soluble protein content. The reaction mixture consisted of 2.0 mL of Biuret reagent (3.8 g $CuSO_4 \cdot 5H_2O$, 1.0 g KI, 6.7 g Na-EDTA, 200 mL 5 N NaOH in 1,000 mL of solution) and 0.1 mL of supernatant. The control consisted of 0.1 mL of distilled water instead of supernatant. The optical density was measured at 545 nm using a Hitachi U-1100 spectrophotometer. The amount of protein was calculated from a standard curve of known protein concentrations, which was prepared from bovine serum albumin.

To determine the quantitative estimation of peroxidases (E.C 1.11.1.7), the "Guaiacol-H_2O_2" method of Luck (1974) was adopted with certain modifications. The assay mixture consisted of 3.0 mL 0.1 M phosphate buffer (pH 7.2), 0.05 mL of 20 mM guaiacol (2-methoxyphenol) solution, 0.1 mL crude enzyme extract, and 0.03 mL of 12.3 mM H_2O_2 solution. Peroxidase activity was calculated by time required to increase the absorbance 0.1 at 240 nm and expressed as units per milliliter enzyme.

Catalase (E.C 1.11.1.6) activity was assayed according to Beers and Sizer (1952) with certain modifications. The reaction was carried out using two buffer solutions (A and B). Buffer A consisted of 50 mM potassium phosphate (pH7.0), while buffer B was 0.036% H_2O_2 solution in 50 mM potassium phosphate buffer (pH7.0). The reaction mixture consisted of 2.9 mL buffer B and 0.1 mL of enzyme extract, while control consisted of only 3.0 mL of buffer A. The enzyme activity was measured by time required for the absorbance (at 240 nm) to decrease from 0.45 to 0.40 and expressed as units per milliliter of enzyme.

Superoxide dismutase (SOD; E.C 1.15.1.1) activity was assayed spectrophotometrically by measuring its ability to inhibit photochemical reduction of nitroblue tetrazolium (NBT) according to Maral et al. (1977). Two tubes were taken, each containing 2.0 mL of 1.0 mM sodium cyanide (NaCN), 13 mM methionine, 75 µM NBT, 0.1 mM EDTA, and 2.0 µM riboflavin as a substrate. One tube was used as sample containing reaction mixture + 5.0 µL enzyme extract, placed approximately 30 cm below the bank of two 30-W fluorescent tubes for 15 min. The other tube containing reaction mixture without enzyme extract was covered with black cloth at the same time. The absorbance of the illuminated tube was compared to non-illuminated mixture at 560 nm. SOD activity was expressed as units per milligram of protein.

Statistical analysis. Univariate analysis was applied to the data using *F* test (SPSS 12.0.0) for the interpretation of results. Data were transformed wherever required. Each experiment was repeated thrice.

Results

Morphological features. A statistically significant difference for shoot length in ascorbic acid pretreated as well as non-pretreated shoot apices of cv. Desiree was observed (Table 1). Explant growth was completely inhibited at 120 or 140 mM NaCl for the two cultivars Desiree and Cardinal, respectively. On the other hand, shoot apices treated with ascorbic acid not only showed an enhanced growth but also survived at both the highest NaCl levels. In cv. Cardinal, there was no statistically significant difference between pretreated and non-pretreated shoot apices, although pretreatment seemed to enhance the survival rate of plants at 140 mM NaCl.

Data in Table 1 also reveal that there was a significant difference in pretreated and non-pretreated shoot apices of cultivar Cardinal, but non-significant difference in Desiree in case of root length. In the case of salt-treated plants at concentrations greater than 80 and 100 mM, root formation completely ceased in cvs. Desiree and Cardinal, respectively. Application of ascorbic acid, however, resulted not only in root formation but also increased shoot/root growth at still higher NaCl levels (120 and 140 mM in cvs. Cardinal and Desiree, respectively).

Table 1. A comparison of morphological features of ascorbic acid (0.5 mM) pretreated or non-pretreated shoot apices of *S. tuberosum* cvs. Desiree and Cardinal maintained on MS medium supplemented with various NaCl levels at day 60

MS medium + NaCl (mM)	Ascorbic acid pretreated (T) or non-treated (NT)	Shoot length (cm)[y]		Root length (cm)		Number of nodes		Number of shoots		Number of roots	
Cultivars[z]		Des	Car	Des	Car	Des	Car	Des	Car	Des	Car
0	T	11.00±1.862	13.50±2.027	4.12±1.780	7.48±2.627	15.80±3.870	17.20±2.398	2.00±1.307	1.40±1.687	6.60±1.780	11.00±2.137
	NT	13.52±2.603	13.62±3.187	8.72±0.446	7.14±2.667	14.00±3.427	19.40±2.247	5.00±1.387	4.60±1.507	10.20±2.087	8.80±0.787
20	T	9.50±2.867	11.14±1.970	7.00±3.210	5.10±2.437	15.20±3.071	17.80±2.670	2.00±1.137	2.00±1.137	4.40±1.787	7.00±2.367
	NT	13.66±2.097	10.56±1.790	6.78±3.070	9.32±2.057	14.40±3.210	16.00±2.817	5.00±2.097	3.40±1.057	6.80±1.917	5.40±1.057
40	T	9.60±2..387	9.10±1.407	5.62±2.987	4.12±1.755	12.80±2.227	15.20±2.887	2.00±1.287	2.40±1.057	6.20±1.207	5.80±3.298
	NT	5.18±2.837	10.30±2.207	9.62±2.720	5.28±2.207	8.60±2.387	15.00±2.557	5.00±1.407	5.20±1.527	5.80±1.711	6.40±2.970
60	T	8.48±1.733	6.82±1.557	5.58±3.270	3.92±3.570	11.80±1.687	11.40±2.137	3.00±1.070	3.00±0.907	7.60±0.788	6.00±2.935
	NT	2.18±2.467	4.80±0.710	6.06±1.970	5.14±0.611	7.60±2.217	10.60±1.058	4.00±1.370	5.60±1.917	1.20±0.987	3.80±1.787
80	T	6.24±1.907	4.00±1.327	3.48±1.817	5.04±2.298	14.00±1.657	9.20±2.217	5.20±1.917	3.00±1.137	4.20±3.087	8.00±3.367
	NT	0.56±3.187	5.18±0.987	1.20±2.817	2.12±1.227	10.2±3.087	12.20±2.887	14.4±0.707	6.80±1.787	0.80±1.207	2.80±1.207
100	T	2.54±1.53	2.08±0.367	3.96±1.987	3.36±1.498	4.00±1.467	8.20±3.298	2.2±1.777	3.20±0.777	2.40±2.887	7.60±2.137
	NT	0.40±1.540	3.76±1.327	N.D	1.28±0.597	8.00±1.987	12.00±3.087	9.80±1.207	9.60±2.667	N.D	3.40±0.822
120	T	2.64±1.332	0.82±0.367	2.60±0.827	1.28±0.527	6.00±2.070	4.20±0.770	6.00±1.857	4.20±1.870	2.40±1.987	2.40±1.407
	NT	N.D	1.50±0.327	N.D	N.D	N.D	9.60±1.407	N.D	8.60±1.057	N.D	N.D
140	T	3.02±1.720	0.54±0.298	2.00±1.827	N.D	6.80±2.398	4.20±1.087	3.60±1.917	5.00±1.467	3.33±2.087	N.D
	NT	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Effect of medium with 7 and 196 *df* for cv. Des and with 7 and 210 *df* for Car		S	S	S	S	S	S	S	S	S	S
Effect of pretreatment with 1 and 196 *df* for cv. Des and with 1 and 210 *df* for Car		S	NS	NS	S	S	S	S	S	S	NS
Effect of medium and pretreatment with 5 and 196 *df* for cv. Des and with 6 and 210 *df* for Car		S	S	S	S	S	S	S	S	S	S

The data were recorded at day 60 of initial culture to respective media, and all the growth parameter values are means ± SD from 15 replicate cultures

Significant (S) or non-significant (NS) ($P<0.01$) according to F test with *df* mentioned against each

Transformation of data was carried out using ln(y) (where y is the value of root length, number of shoots and roots in cv. Des) and arcsin(y), log10 (y), $4\sqrt{y}$, $4\sqrt{y}$, $\sqrt{y}$ (where y is the value of shoot, root length, number of nodes, shoots, and roots in cv. Car) to normalize the data. Non-transformed mean values are presented

N.D not determined.

[z] Cultivars: *Des* Desiree, *Car* Cardinal

[y] Shoot apices (1.0 cm long) were used as an explant source. Unless otherwise mentioned, the same explant size was used throughout this study

As the concentration of NaCl increased from 20 to 140 mM, the number of nodes decreased significantly. In cv. Desiree, pretreatment with ascorbic acid generally resulted in a higher number of nodes (15.2, 12.8, 11.8, 14.0, 4.0, 6.0, and 6.8 at 20, 40, 60, 80, 100, 120, and 140 mM) as compared to the non-pretreated shoot apices (14.4, 8.6, 7.6, 10.2, and 8.0 at 20, 40, 60, 80, and 100 mM, respectively). Plants did not survive above 100 mM NaCl. In comparison to Desiree, not much of a difference in the number of nodes in cv. Cardinal was observed up to the 80 mM NaCl level (17.8, 15.2, 11.4, 9.2 *vs* 16.0, 15.0, 10.6, and 12.2, respectively, at 20, 40, 60, and 80 mM NaCl). However, ascorbic acid did not prove to be effective at still higher NaCl levels (100 mM and above).

A significant difference between pretreated and non-pretreated shoot apices of both the cultivars was also observed in the case of number of shoots. There were a greater number of shoots (more than six) in both the cultivars at higher NaCl levels (14.4 and 9.8 at 80 and 100 mM in Desiree and 6.8, 9.6, and 8.6 at 80, 100, and 120 mM, respectively, in Cardinal), albeit with stunted growth that resulted in a bunchy or rosette-type appearance.

Rosette or bunchy appearance was not observed at all at any of the above NaCl levels when supplemented with ascorbic acid.

Pretreatment of ascorbic acid considerably enhanced the number of roots (7.6, 4.2, 2.4, 2.4, and 3.3 at 60, 80, 100, 120, and 140 mM in Desiree and 6.0, 8.0, 7.6, and 2.4 at 60, 80 100, and 120 mM salt in cv. Cardinal) as compared to non-pretreated (1.2 and 0.8 at 60 and 80 mM in Desiree and 3.8, 2.8, and 3.4 at 60, 80, and 100 mM NaCl in Cardinal). Ascorbic acid pretreatment thus supported root formation even at higher NaCl levels (120 and 140 mM in Cardinal and Desiree, respectively), while root formation was not observed in the non-pretreated cultures at the same salt levels.

Figure 1 depicts that there was a significant difference with reference to PRFWG between ascorbic acid-pretreated and non-pretreated callus cultures of both the cultivars. Pretreated callus cultures at all salt concentration showed higher PRFWG as compared to non-pretreated calluses in both the cultivars. PRFWG in pretreated callus cultures was increased from 87%, 89%, 73%, and 57% to 92%, 90%, 83%, and 68% in Desiree and from 72%, 79%, 66%, and 62% to 84%, 81%, 72%, and 69% at 20, 40, 60, and 80 mM NaCl in Cardinal. However, at 100 mM salt concentration, ascorbic acid-pretreated callus culture showed relatively less PRFWG as compared to non-pretreated ones in both the cultivars. Callus cultures without ascorbic acid pretreatment were completely necrotic when media contained NaCl above 100 mM.

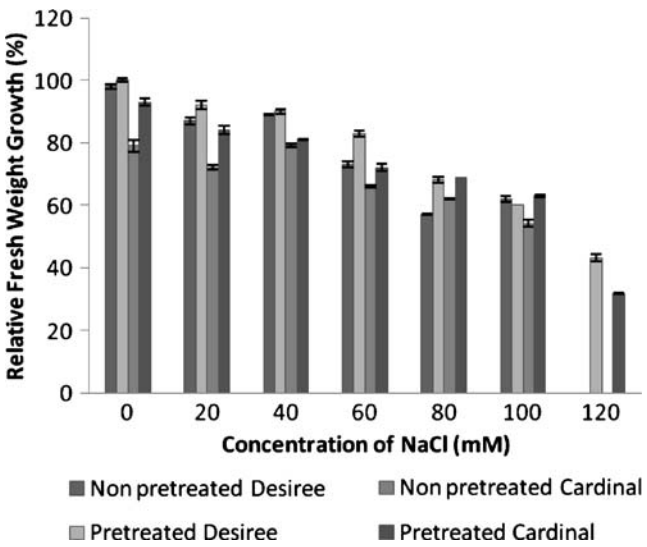

Figure 1. Percentage relative fresh weight growth of ascorbic acid-pretreated and non-pretreated callus cultures of *S. tuberosum* cvs. Desiree and Cardinal after 60 d of salt treatment. Values represent the mean ± SE from 15 replicate cultures for each salinity and ascorbic acid treatment for both the cultivars, and experiment was repeated thrice. Data were recorded for PRFWG of callus cultures after 60 d of salt treatment.

The interactive effect of medium and pretreatment was also significant in all the studied growth parameters (shoot/root length, number of nodes/shoots/roots and PRFWG) in both the cultivars (Table 1, Figs. 1 and 2).

Biochemical assays. In both the cultivars, ascorbic acid-pretreated plants showed an increase in protein content as compared to non-pretreated plants (Table 2). It was observed that protein content increased in ascorbic acid-pretreated plants of cv. Desiree from 0.89, 1.04, 1.16, 1.30, and 1.34 mg/g to 1.88, 1.84, 4.02, 3.03, and 3.17 mg/g at 20, 40, 60, 80, and 100 mM salt, respectively. In cv. Cardinal, this increase in protein contents was from 1.07, 1.32, 1.56, 1.03, 1.45, and 1.06 to 1.97, 3.41, 2.48, 1.44, 2.76, and 2.05 mg/g at 20, 40, 60, 80 100, and 120 mM NaCl.

Callus cultures also showed a similar behavior as in plants with reference to protein content. Protein content increased from 1.06, 1.34, 1.62, 1.40, and 1.01 mg/g to 1.99, 1.98, 2.17, 2.09, and 1.16 mg/g in cv. Desiree and 1.43, 1.23, 0.67, 0.48, and 0.27 to 1.80, 1.35, 0.88, 0.52, and 0.40 at 20, 40, 60, 80, and 100 mM NaCl (Table 3).

Generally, in both the cultivars, peroxidase activity increased in NaCl-treated plants without ascorbic acid pretreatment as compared to pretreated ones (1.76, 2.16, and 2.58 *vs* 1.6, 1.03, and 0.77 at 60, 80, and 100 mM NaCl in Desiree and 1.02, 1.78, 2.14, 1.83, 1.84, and 1.34 *vs* 0.69, 0.63, 0.72, 0.45, 0.97, and 0.58 U/mL of enzyme at 20, 40, 60, 80, 100, and 120 mM NaCl in Cardinal). However, in cv. Desiree, peroxidase activity showed an increasing trend at 0, 20, and 40 mM NaCl (Table 2). Peroxidase activity decreased significantly (by 29.05%, 59.64%, 63.63%, 51.87%, and 42.85% in Desiree and by 22.78%, 29.87%, 52.38%, 51.42%, and 20.40% at 20, 40, 60, 80, and 100 mM NaCl in Cardinal; $P<0.01$) in ascorbic acid-pretreated, NaCl-stressed callus cultures as compared to non-pretreated ones (Table 3).

Data presented in Tables 2 and 3 reveal that in both the cultivars, catalase activity increased significantly ($P<0.01$) in ascorbic acid-pretreated plants raised from shoot apices as well as in callus cultures as compared to non-pretreated ones.

A higher SOD activity (by 54.01%, 5.76%, 71.95%, 34.55%, and 17.01% in Desiree and 42.70%, 42.75%, 31.73%, 46.99%, and 16.50% in Cardinal at 20, 40, 60, 80 and 100 mM NaCl) was observed in ascorbic acid-pretreated plants raised from shoot apices as compared to non-pretreated ones. A similar statistically significant ($P<0.01$) trend of SOD activity was quite apparent in callus cultures as well. SOD activity was apparently more pronounced in cv. Cardinal as compared to cv. Desiree in both types of plant material (Tables 2 and 3). In interactive terms of medium and pretreatment, a significant difference ($P<0.01$) was recorded in the case of protein content and

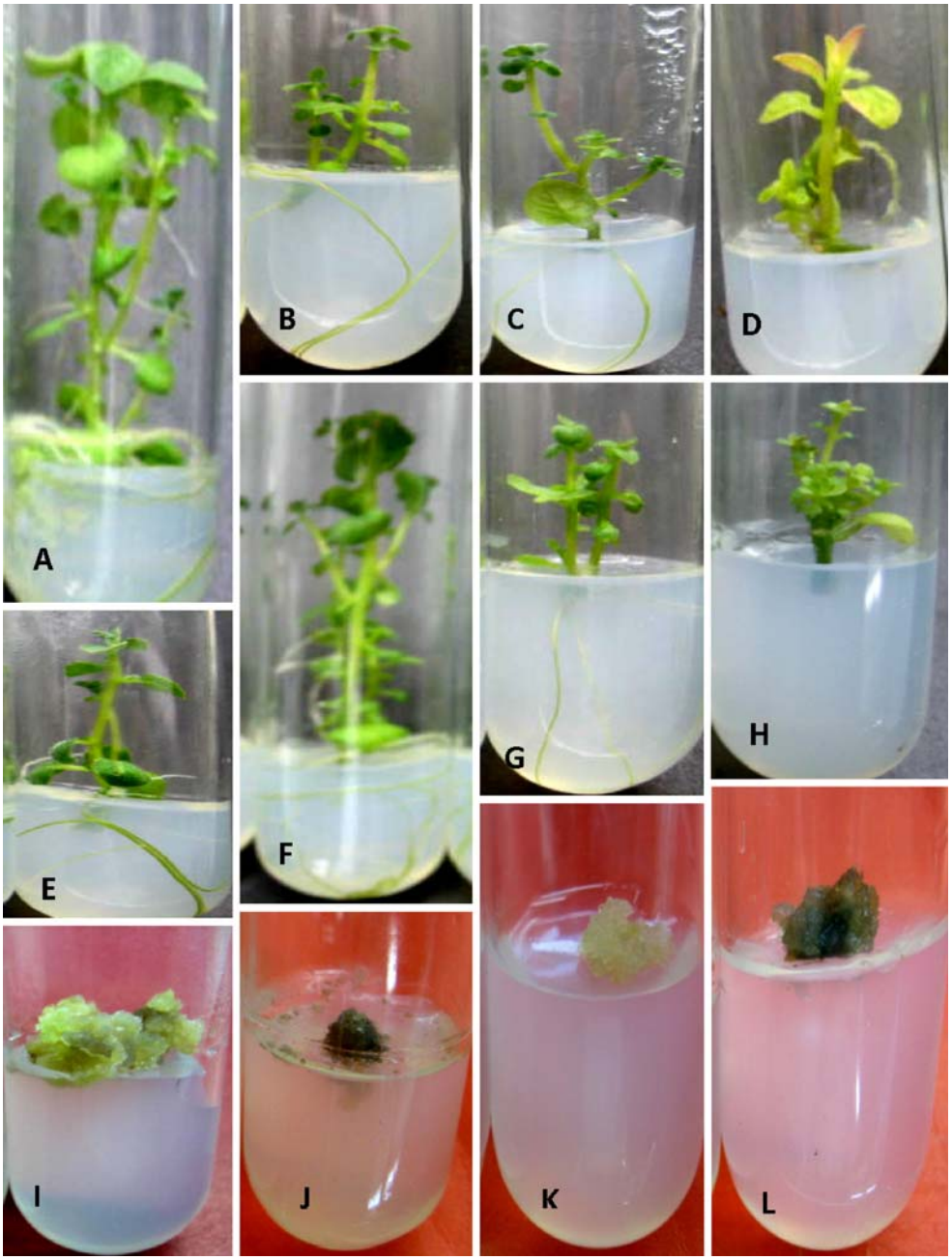

Figure 2. Selected photographs of ascorbic acid-pretreated and non-pretreated plants and callus cultures of potato cvs. Desiree and Cardinal at different NaCl levels at day 60. (*a*) Ascorbic acid-pretreated Desiree plants at 80 mM NaCl. (*b*) Non-pretreated plants of Desiree at 80 mM NaCl. (*c*) Pretreated Desiree plants at 100 mM NaCl. (*d*) Non-pretreated Desiree shoots at 80 mM NaCl without root formation. (*e*) Ascorbic acid-pretreated Cardinal plant at 100 mM NaCl. (*f*) Non-pretreated Cardinal plants at 100 mM NaCl. (*g*) Ascorbic acid-pretreated Cardinal plants at 120 mM NaCl. (*h*) Non-pretreated Cardinal plants at 120 mM NaCl showing rosette-type of plant growth without root formation. (*i*) Ascorbic acid-pretreated callus culture of Desiree at 120 mM NaCl. (*j*) Non-pretreated callus cultures of Desiree at 120 mM NaCl showing signs of necrosis. (*k*) Ascorbic acid-pretreated callus culture of Cardinal at 120 mM NaCl. (*l*) Non-pretreated callus cultures of Cardinal at 120 mM NaCl (2×).

peroxidase, catalase, and superoxide dismutase activity in both the cultivars.

Discussion

The present study highlights the role of ascorbic acid to enhance salinity tolerance in *in vitro*-grown potato. In this regard, several studies have indicated possible roles of ascorbic acid. Exogenously applied ascorbic acid was suggested to be utilized in cell metabolism and to enhance the cell division efficacy of competent cells (Citterio et al. 1994). The direct effect of salt was reported to alter the structure of photosynthetic membranes (Fidalgo et al. 2004). The same group also suggested that salt stress in potato lowers the capability of cells to remove ROS. Exogenously applied ascorbic acid is shown to have increased the ascorbic acid content in chloroplasts of leaves, which in turn results in the protection of chloroplast membrane integrity and chloroplast degradation (Gadallah 2000). Reactive oxygen species (superoxide, hydrogen peroxide, hydroxyl radical, and singlet oxygen) produced under NaCl stress readily oxidize proteins, unsaturated fatty acids, and DNA, thus resulting in damage to cellular function and growth of plants (Heber et al. 1996). Ascorbic acid is considered an important antioxidant, protecting

Table 2. A comparison of protein, peroxidase, catalase, and superoxide dismutase activities of ascorbic acid (0.5 mM) pretreated or non-pretreated shoot apices of *S. tuberosum* cvs. Desiree and Cardinal maintained on MS medium supplemented with various NaCl levels at day 60

MS medium + NaCl (mM)	Ascorbic acid pretreated (T) or non-treated (NT)	Protein contents (mg/g)		Peroxidase activity (U/mL)		Catalase activity (U/mL)		SOD activity (U/mg of protein)	
Cultivars[z]		Des	Car	Des	Car	Des	Car	Des	Car
0	T	1.92±0.027	3.58±0.028	1.90±0.034	0.58±0.019	14.58±0.026	16.96±0.047	18.58±0.058	51.94±0.048
	NT	0.77±0.136	0.77±0.037	1.34±0.215	1.82±0.125	9.95±0.045	17.45±0.278	12.94±0.038	16.72±0.011
20	T	1.88±0.059	1.97±0.072	1.72±0.017	0.69±0.018	14.31±0.567	26.56±0.038	36.60±0.987	31.12±0.066
	NT	0.89±0.080	1.07±0.057	1.36±0.218	1.02±0.048	8.28±0.028	12.83±0.211	16.83±0.927	17.83±0.022
40	T	1.84±0.036	3.41±0.057	0.84±0.059	0.63±0.018	12.03±0.018	18.52±0.137	20.83±0.028	32.21±0.587
	NT	1.04±0.104	1.32±0.756	0.80±0.113	1.78±0.028	11.33±0.066	4.13±0.069	19.63±0.228	18.44±0.033
60	T	4.02±0.058	2.48±0.108	1.60±0.128	0.72±0.018	5.44±0.108	13.25±0.018	29.17±0.028	24.20±0.228
	NT	1.16±0.369	1.56±0.018	1.76±0.098	2.14±0.098	4.56±0.048	7.84±0.158	8.18±0.108	16.52±0.038
80	T	3.03±0.028	1.44±0.048	1.03±0.012	0.45±0.028	7.04±0.186	14.57±0.084	34.90±0.058	31.60±0.058
	NT	1.30±0.108	1.03±0.028	2.16±0.099	1.83±0.058	5.48±0.387	5.36±0.077	22.84±0.048	16.75±0.028
100	T	3.17±0.987	2.76±0.079	0.77±0.917	0.97±0.287	7.00±0.397	13.64±0.088	14.52±0.017	13.27±0.038
	NT	1.34±0.027	1.45±0.041	2.58±0.987	1.84±.0387	4.56±0.117	11.14±0.037	12.05±0.087	11.08±0.078
120	T	0.95±0.104	2.05±0.047	1.31±0.056	0.58±0.987	9.89±0.258	15.23±0.080	31.55±0.058	32.54±0.058
	NT	N.D	1.06±0.037	N.D	1.34±0.028	N.D	7.80±0.080	N.D	30.24±0.457
140	T	1.61±0.103	0.65±0.044	1.71±0.028	0.95±0.068	10.76±0.198	0.036±0.038	15.54±0.038	34.32±0.038
	NT	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Effect of medium with 7 and 196 *df* for cv. Des and with 7 and 210 *df* for Car		S	S	S	S	S	S	S	S
Effect of pretreatment with 1 and 196 *df* for cv. Des and with 1 and 210 *df* for Car		S	S	S	S	S	S	S	S
Effect of medium and pretreatment with 5 and 196 *df* for cv. Des and with 6 and 210 *df* for Car		S	S	S	S	S	S	S	S

The data were recorded at day 60 of initial culture to respective media, and all the biochemical parameter values are means ± SD from 15 replicate cultures

Significant (S) or non-significant (NS) ($P<0.01$) according to F test with df mentioned against each

Transformation of data was done using log10(y) (where y is the value of protein and peroxidase activity in cv. Des) and log10(y), ln(y), $\sqrt{y}$, log10(y) (where y is the value of protein, peroxidase, catalase, and SOD activity in cv. Car) to normalize the data. Non-transformed mean values are presented

N.D not determined

[z] Cultivars: *Des* Desiree, *Car* Cardinal

plants from oxidative stress by eliminating several ROS (Smirnoff 2005). It accomplishes this either directly or indirectly, e. g., H_2O_2 is eliminated indirectly through the activity of ascorbate peroxidase (Asada 1992). Moreover, ascorbate has also been reported to be an important cofactor for a number of enzymes involved in hormone synthesis, e. g., gibberellins (Prescott and John 1996). This opens yet another possible explanation for better growth parameters of potato cultures as observed in our study.

Results of the present study indicate that all the morphological parameters (shoot and root length, number of nodes, roots and shoots) were significantly inhibited by the application of salt in the growth medium. At higher than 60 mM NaCl in cv. Desiree and 80 mM NaCl in cv. Cardinal, plantlets showed a bunchy appearance with little or no root formation. Plant growth was completely inhibited at 120 and 140 mM NaCl in cvs. Desiree and Cardinal, respectively. Many earlier works have also reported reduction in various plant growth parameters in response to NaCl stress. Sasikala and Prasad (1993) reported reduced *in vitro* growth in potato under 0.4–0.6% NaCl stress. Similarly, Ekanayake and Dodds (1993) reported a severe growth reduction of *Ipomoea batatas* cultures at higher NaCl level. Cherian et al. (1999) also reported that growth of *Avicennia marina* plants decreased progressively with an increase in salt concentrations. This

Table 3. A comparison of protein, peroxidase, catalase, and superoxide dismutase activities of ascorbic acid (0.5 mM) pretreated or non-pretreated callus cultures of *S. tuberosum* cvs. Desiree and Cardinal maintained on optimized callus proliferation medium supplemented with various NaCl levels at day 60

Optimized callus proliferation medium + NaCl (mM) Cultivars[z]	Ascorbic acid pretreated (T) or non- treated (NT)	Protein contents (mg/g)		Peroxidase activity (U/mL)		Catalase activity (U/mL)		SOD activity (U/mg of protein)	
		Des	Car	Des	Car	Des	Car	Des	Car
CIM[a] + 0	T	2.61±0.357	1.20±0.167	1.77±0.187	1.79±0.357	14.92±0.187	8.80±0.777	19.36±0.237	37.98±4.477
	NT	2.52±0.347	1.02±0.107	1.31±0.067	1.80±0.307	10.20±0.047	11.00±2.117	13.13±0.065	18.24±2.597
CIM + 20	T	1.99±0.267	1.80±0.307	0.83±0.087	0.61±0.087	14.44±0.467	6.60±2.467	23.57±0.498	27.65±4.987
	NT	1.06±0.097	1.43±0.287	1.17±0.070	0.79±0.127	7.66±0.267	6.18±1.870	15.01±0.167	15.31±2.987
CIM + 40	T	1.98±0.137	1.35±0.247	0.69±0.077	0.54±0.227	12.10±0.067	6.40±2.977	20.51±0.870	29.71±3.987
	NT	1.34±0.127	1.23±0.044	1.71±0.117	0.77±0.087	11.78±0.207	5.80±3.298	20.83±1.198	18.87±2.887
CIM + 60	T	2.17±0.398	0.88±0.137	0.92±0.087	0.40±0.117	5.77±0.187	5.26±1.970	25.72±0.700	23.50±4.907
	NT	1.62±0.157	0.67±0.317	2.53±0.228	0.84±0.177	4.90±0.307	4.50±1.557	24.98±0.427	17.75±2.987
CIM + 80	T	2.09±0.077	0.52±0.147	0.90±0.087	0.34±0.070	6.70±0.427	7.60±4.087	29.85±0.477	13.86±3.967
	NT	1.40±0.117	0.48±0.107	1.87±0.147	0.70±0.078	6.65±0.227	2.80±1.207	23.09±1.098	14.08±3.987
CIM + 100	T	1.16±0.027	0.40±0.127	1.08±0.047	0.39±0.117	7.48±0.247	7.60±2.137	14.48±0.547	31.44±3.987
	NT	1.01±0.057	0.27±0.087	1.89±0.007	0.49±0.067	4.92±0.087	3.80±0.417	12.60±0.697	13.24±1.987
CIM + 120	T	0.70±0.067	0.30±0.070	1.00±0.087	0.53±0.116	2.18±0.087	2.24±1.087	25.23±0.081	36.01±0.987
	NT	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
CIM + 140	T	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
	NT	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Effect of medium with 6 and 182 *df* for cv. Des and with 6 and 182 *df* for Car		S	S	S	S	S	S	S	S
Effect of pretreatment with 1 and 182 *df* for cv. Des and with 1 and 182 *df* for Car		S	S	S	S	S	S	S	S
Effect of medium and pretreatment with 5 and 182 *df* for cv. Des and with 5 and 182 *df* for Car		S	S	S	S	S	S	S	S

The data were recorded at day 60 of salt treatment, and all the biochemical parameters values are means ± SD from 15 replicate cultures

Significant (S) or non-significant (NS) ($P<0.01$) according to F test with *df* mentioned against each

Transformation of data was done using log10(y), log10(y), arcsin(y) (where y is the value of protein peroxidase and catalase activity in cv. Des) and log10(y), 1/y, 1/$\sqrt{y}$ (where y is the value of protein peroxidase and SOD activity in cv. Car) to normalize the data. Non-transformed mean values are presented

N.D not determined

[z] Cultivars: *Des* Desiree, *Car* Cardinal

[y] CIM: optimized callus induction and proliferation medium (MS+BAP 10 and NAA 1.1 μM)

growth inhibition might be due to the utilization of plant energy to maintain growth under stress conditions (Croughan et al. 1981). Furthermore, reports on salt stress influencing the metabolic processes occurring in the chloroplast and mitochondria (Cheeseman 1988) suggest the prevention of important physiological phenomena such as osmosis and diffusion (Azooz et al. 2004). Therefore, the reduced growth of potato plants under salt stress in this study might be due to one or a combination of the aforementioned factors.

When NaCl-stressed plants were pretreated with 0.5 mM ascorbic acid, it considerably helped plant growth at higher salt concentration (120 and 140 mM in cvs. Desiree and Cardinal, respectively). All the growth parameters in ascorbic acid-pretreated plants were significantly different than from non-pretreated plants except in the cases of shoot length and number of roots in cv. Cardinal and root length in cv. Desiree. Similar results were reported regarding the role of ascorbic acid to enhance the plant growth. Shalata and Neumann (2001) observed that additional supply of

ascorbic acid (0.5 mM) before salt treatment considerably helped the recovery and long-term survival of wilted tomato seedlings. Similarly, Al-Hakimi and Hamada (2001) studied the role of pretreatment of ascorbic acid on wheat seedling under salt stress. They found that ascorbic acid suppress the effect of salt by the accumulation of proline. Recently, Khan et al. (2006) suggested that pretreatment of ascorbic acid alleviated the adverse effects of sea salt on seed germination in halophytes. Although contrasting results demonstrating decrease (Sreenivasulu et al. 2000) or increase (Benavides et al. 2000) of ascorbate in salt-treated plants have been reported, a higher level of endogenous ascorbate is generally considered to be essential in maintaining the antioxidant defense system of various plants against oxidative damage caused by abiotic stresses (Shigeoka et al. 2002; Fidalgo et al. 2004). It was perhaps for the same reason that biochemical parameters tested in this study were so influenced by ascorbic acid pretreatment that they resulted in enhanced morphological parameters.

In this study, pretreatment of ascorbic acid to NaCl-treated plants increased the protein content in both the cultivars. Protein content in callus cultures of both the cultivars has also shown an increasing trend in ascorbic acid-pretreated calluses as compared to non-pretreated ones. This higher protein content might be attributed to their property as osmolytes in maintaining the osmotic imbalance. Earlier works suggested that increasing levels of protein help the plants to maintain their growth under stress conditions. Thus, our findings are in line with those of Agastian et al. (2000) and Fidalgo et al. (2004), which reported that stress-induced proteins play a major role in salt tolerance. These salt-responsive proteins were also suggested to be quite valuable for further analysis of general cellular adaptive mechanism to abiotic stress (Ashraf and Harris 2004).

In response to ROS, plants are reported to produce a high amount of enzymatic antioxidants, i.e., superoxide dismutase, catalase, and/or peroxidase (Azevedo-Neto et al. 2006). In the present investigation, SOD and catalase activity of both the cultivars increased substantially in ascorbic acid-pretreated plants as well as callus cultures as compared to non-pretreated ones. The plants with higher levels of antioxidant enzymes have been reported by several works to have greater resistance to withstand salt stress (Rahnama et al. 2003; Molassiotis et al. 2006). Mittova et al. (2003) suggested that the increase in the activity of antioxidant enzymes could be associated with a salt-tolerant behavior of the plants. Vaidyanathan et al. (2003) reported that a salt-tolerant rice cultivar had better growth and higher level of ROS-scavenging enzymes (catalase and superoxide dismutase) as compared to the sensitive cultivar of rice. They suggested that a combined action of both enzymatic and non-enzymatic ROS-scavenging machineries was vital to overcome salinity stress. In our study, however, peroxidase activity decreased in ascorbic acid-pretreated potato plants as well as in callus cultures. It has earlier been suggested that this enzyme perhaps does not take a central part in the defense mechanism against oxidative stress (Muthukumarasamy et al. 2000; Jaleel et al. 2007) or it may be the fact that it takes part in a different manner. Variation in peroxidase activity under different sets of stress conditions was suggested to be due to the species and the developmental and metabolic state of the plants (Reddy et al. 2004).

In conclusion, salinity is a serious constraint to potato growth, possible to reduce through ascorbic acid pretreatment to *in vitro* plants and callus cultures. Ascorbic acid probably minimized the oxidative damage by increasing the amount of antioxidant enzymes, which in turn was reflected in better growth parameters in the two tested potato cultivars. The information gathered from this study necessitates further work both under *in vitro* as well as greenhouse and field conditions to evaluate and harness the potential benefits it holds.

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