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ROLE OF SALICYLIC ACID IN AMELIORATION OF SALT TOLERANCE IN POTATO (*SOLANUM TUBEROSUM* L.) UNDER *IN VITRO* CONDITIONS

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Abstract

Salicylic acid (SA) has long been considered as signal molecule and is known to reduce the oxidative damage caused by salinity stress. The objective of this study was to work out a suitable methodology for improving salt tolerance in potato by the exogenous application of SA. For this purpose, *In vitro* plants of two economically important potato cultivars, i.e., Cardinal and Desiree were raised. Single nodal explants (1.0 cm long) from two-month-old *in vitro* potato plants were inoculated on MS medium with or without 60 m*M* NaCl supplemented with or without different concentrations (0.125, 0.25, 0.50 and 0.75 m*M*) of salicylic acid. Complete randomized experimental design was employed with 10 replicates and one nodal explant per treatment. After 60 days of inoculation, number of growth (root, shoot length, number of root, shoot and nodes, fresh/dry weight) and biochemical parameters (protein contents) were studied. Application of 60 m*M* NaCl to *In vitro*-grown potato plants in general adversely affected several growth as well as biochemical parameters. On the other hand, exogenously-applied SA enhanced the growth of both potato cultivars. It was observed that SA application at two higher tested levels (0.50 and 0.75 m*M*) did not confer much tolerance to NaCl stress in these potato cultivars in comparison with the lower concentrations (0.125, 0.25 m*M*) that proved quite effective in enhancing growth in Cardinal and Desiree, respectively. These results hint at a possibility that relatively low-to-moderate concentrations of salicylic acid may, in future, be useful in improving yield of potato plants under saline conditions.

Introduction

Soil salinity is one of the most important abiotic stresses that reduce growth and agricultural productivity more than many other similar stress factors. It is considered as a largest soil toxicity problem in tropical Asia (Greenland, 1984). The severity of this problem is gradually being aggravated by the build-up of salts in soils through common irrigation practices. According to an FAO statistics (2005), of the current 230 Mha of irrigated land on the globe, 45 Mha (19.5%) are salt-affected. In Pakistan, 16.72 Mha are being irrigated. Of this irrigated land, 6.3 Mha are affected by salinity. The magnitude of problem can be estimated from a fact that the area of productive land is being damaged by salinity at an alarming rate of about 40,000 ha annually (http://www.icid.org/cp_pakistan.html). Salinity affects plants in several ways such as osmotic stress, specific ion imbalance, ion toxicity, nutritional disparity and hormonal disturbance or a combination of all of these (Lauchli & Epstein, 1990; Ashraf, 1994; Wahid *et al*., 2007). All these factors badly affect plant growth and development at both physiological and biochemical levels (Munns, 2002; Munns & James, 2003; Tester & Devenport, 2003). In addition to these interrelated and co-existing impacts, salinity also produces an oxidative stress (Panda & Upadhyay, 2004) due to rapid and transient accumulation of reactive oxygen species (ROS) like superoxide radical, hydroxyl radical and singlet oxygen. These ROS cause pigment co-oxidation, lipid peroxidation, membrane destruction, protein denaturation and/or DNA mutation (Mittler, 2002). Plants have to opt for a specific protective mechanism like production of enzymatic (superoxide dismutase, catalase and peroxidase) and/or non-enzymatic (ascorbic acid, salicylic acid, α-tocopherol, carotenoids etc) antioxidants to lessen the harm initiated by these ROS.

Salicylic acid (SA) has been considered as a signal molecule (Horvath *et al*., 2007a) that may promote the generation of reactive oxygen species during salt stress thus playing an important role in stress tolerance (Borsani *et al*., 2001). SA treatments may enhance the production of H_2O_2 to levels that can intern reduce the oxidative damage caused by salinity stress in wheat plants (Wahid *et al*., 2007). Several developmental, physiological and biochemical functions of exogenously-applied salicylic acid in plants have been reported, e.g., enhancing the drought and salt stress resistance of plants (Senaratna *et al*., 2000; Tari *et al*., 2002), influencing seed germination and fruit yield (Cutt & Klessing, 1992; Raskin, 1992), transpiration rate, stomatal closure (Rai *et al*., 1986), membrane permeability (Barkosky & Einhellig, 1993), growth and photosynthesis (Khan *et al*., 2003; Khodary, 2004; El-Tayeb, 2005). Though some encouraging results have already been achieved using strategies like exogenous application of antioxidant molecules and compounds for the amelioration of salinity tolerance, such studies are still in their infancy. Many parameters like strength or duration of treatment along with treatment conditions are precisely not known for many plants including potato. The current research therefore was carried out in order to work out efficacy of salicylic acid for improving salt tolerance in two economically important potato cultivars (Cardinal and Desiree) in lab conditions.

Materials and Methods

Plant material and culture conditions: Apical shoot explants (ca. 1.0 cm) of 60-day-old *In vitro* plants of two cultivars (Cardinal and Desiree) of *Solanum tuberosum* L., were inoculated on Murashige & Skoog (1962) medium for further growth and establishment under various treatments during this study. The pH of the medium was adjusted to 5.7 and solidified with 0.7% agar (Oxoid, Hampshire, England). The medium in culture vessels (Pyrex 25 × 150 mm) was sterilized by autoclaving at 121°C and 15 lbs inch^{-2} for 15 minutes. The cultures were maintained in 16 h photoperiod (40 µmol m^{-2} s^{-1}; cool white fluorescent lights) at 25 ± 2°C.

Effect of salicylic acid treatment to *In vitro* salinized plants of *Solanum tuberosum* L: Single nodal explants (details as above) were inoculated on MS medium with or without 60 mM NaCl supplemented with or without different concentrations (0.125 m*M*, 0.25 m*M*, 0.50 m*M* and 0.75 m*M*) of salicylic acid. The dose and time for pretreatment was based on previous studies on different plant species (Senaratna *et al*., 2000; Arfan *et al*., 2007; Gunes *et al*., 2007). Three different treatment groups were formed, i.e., 1) control without NaCl and salicylic acid, 2) with NaCl and without salicylic acid, 3) with NaCl and salicylic acid. Ten culture vessels (25 × 150 mm) were inoculated for each treatment for both the cultivars. Experimental design was completely randomized with 10 replicate for each treatment (one nodal explant for each replicate). The cultures were maintained at 26 ± 2°C in 16 h photoperiod, 40 µmoles m^{-2} s^{-1} light intensity from cool white florescent tube light. After 60 days of inoculation, number of growth (average root, shoot length, number of root, shoot and nodes, fresh, dry weight) and biochemical parameters (protein contents) were studied.

Morphological and biochemical studies: For this purpose, plants were taken out of the culture vessels and after removing medium from the roots they were analyzed carefully for counting the number of shoots, roots and nodes. Shoot length was measured with the help of a ruler excluding 1.0 cm (initial explant size) and root length was measured from the tip of the root up to the shoot. Estimation of fresh and dry weight of plantlets was carried out respectively by using fresh plant samples and then after drying in an oven at 70 °C for 72 hours using fractional electric balance (Scientech 5220).

Extraction and estimation of total soluble protein contents: Fully expended 2-3 mature leaves were ground in liquid nitrogen into a fine powder using an ice-chilled pestle and mortar. One-g ground tissue was suspended in 2.0 ml of 0.1 M phosphate buffer, pH 7.2 (13.6 g KH_2PO_4 and 17.4 g K_2HPO_4 in 1L of solution) containing 0.5% (*v/v*) Triton X-100 and 0.1 g of polyvinylpyrrolidone (PVP). The slurry so obtained was centrifuged at 14,000 rpm at 4°C for 30 min. using Sorval RB-5 refrigerated super-speed centrifuge. The resultant supernatant was collected and stored at 0°C for further estimation of protein contents. Biuret method of Racusen & Johnstone (1961) was adopted for the estimation of soluble protein contents. The reaction mixture consisted of 2.0 ml of Biuret reagent (3.8 g $CuSO_4.5H_2O$, 1.0 g KI, 6.7 g Na-EDTA, 200 ml 5N NaOH in 1 L of solution) and 0.2 ml of supernatant. The control consisted of 0.2 ml of distilled water instead of supernatant. The optical density was measured at 545 nm using Hitachi U-1100 spectrophotometer. The amount of protein was calculated from a standard curve for protein that was prepared from bovine serum albumin.

Results

Effect of salicylic acid on growth characteristics of salinized potato plants (cvs. Cardinal and Desiree): In all the tested salicylic acid treatments, maximum shoot length (2.39 cm) was observed in medium M3 (MS + 60 m*M* NaCl + 0.125 m*M* SA) followed by M5 (medium containing 0.50 m*M* SA) where the shoot length was 2.26 cm in Cardinal. In case of Desiree, maximum shoot length (2.36 cm) was observed in M4 medium (medium containing 0.25 m*M* SA). Shoot length was increased at all salicylic acid levels in comparison with plants containing only NaCl in the medium (M2). The results were somewhat different in Cardinal where shoot length decreased to 1.66 and 1.69 cm in M4 and M6 media, respectively) in comparison with salt-stressed plants (Table 1).

Table 1. Effect of salicylic acid on different growth parameters in *Solanum tuberosum* L. cv. Cardinal.

Medium*	NaCl + SA (mM)	Shoot length (cm)	Number of shoots	Root length (cm)	Number of roots	Number of nodes	Fresh wt. (g)	Dry wt. (g)
M1	0 + 0	9.09 ± 2.09^a	1.40 ± 0.45^a	6.61 ± 1.61^a	6.40 ± 1.77^a	14.90 ± 3.31^a	0.45 ± 0.12^a	0.04 ± 0.010^a
M2	60 + 0	1.86 ± 0.70^b	2.80 ± 0.87^a	3.27 ± 1.71^b	0.90 ± 0.43^b	5.20 ± 1.60^b	0.11 ± 0.03^b	0.01 ± 0.003^b
M3	60 + 0.125	2.39 ± 0.43^b	2.80 ± 0.53^a	0.52 ± 0.34^b	0.70 ± 0.40^b	7.10 ± 0.98^b	0.13 ± 0.02^b	0.02 ± 0.002^b
M4	60 + 0.250	1.66 ± 0.28^b	2.70 ± 0.63^a	0.92 ± 0.45^b	1.20 ± 0.55^b	6.50 ± 0.82^b	0.10 ± 0.02^b	0.01 ± 0.002^b
M5	60 + 0.500	2.26 ± 0.36^b	2.20 ± 0.36^a	1.01 ± 0.43^b	0.80 ± 0.39^b	6.80 ± 0.93^b	0.10 ± 0.02^b	0.01± 0.002^b
M6	60 + 0.750	1.69 ± 0.14^b	2.70 ± 0.61^a	0.24 ± 0.18^b	0.30 ± 0.21^b	6.30 ± 0.52^b	0.07 ± 0.01^b	0.01 ± 0.001^b
Significance (p≤0.05)		**S**	**NS**	**S**	**S**	**S**	**S**	**S**

*M1 to M6 media designated for MS supplemented with NaCl and salicylic acid given against each
Values are mean ± S.E from 30 replicate cultures
Means followed by the same letter(s) are not significantly different at p≤0.05
Values are significant (S) or non-significant (NS) at p ≤0.05

There were significant differences in rooting behavior between the salicylic acid-treated or non-treated plants of Desiree (Table 2). Roots were generally absent in salt stressed Desiree plants. In salicylic acid-supplemented medium, maximum growth of roots was observed in M4 (0.25 m*M* SA) medium. However, this type of behavior was diametrically different in Cardinal where the root length was observed to have decreased in salicylic acid-treated plants as compared to salinized-plants without SA treatment. Salicylic acid treatment to salinized plants improved the rooting behavior, as an increase in root number (from 0.90 to 1.20) was observed using M4 medium (0.25 m*M* SA) in Cardinal (Table 1) and (0 to 1.30) in Desiree (Table 2). Salinized plants showed bunchy appearance due to the formation of more shoots with shorter internodal distances (Fig. 2 a, b). An application of salicylic acid in the medium resulted in decreased number of shoots in both the cultivars. The situation was different in case of number of nodes, where the treatment with SA increased the number of nodes. Maximum number of shoots (2.80 and 2.40) was observed on M2 medium (MS + 60 m*M* NaCl) in cultivar Cardinal and Desiree, respectively. Maximum number of nodes (7.10 and 7.50) was observed on M3 and M4 medium respectively in cvs. Cardinal and Desiree. Statistically a non-significant difference was observed in case of number of shoots (Cardinal) and number of nodes (Desiree) at different salicylic acid treatments.

Table 2. Effect of salicylic acid on different growth parameters in *Solanum tuberosum* L. cv. Desiree.

Medium*	NaCl + SA (mM)	Shoot length (cm)	Number of shoots	Root length (cm)	Number of roots	Number of nodes	Fresh wt. (g)	Dry wt. (g)
M1	0 + 0	6.03 ± 2.03[a]	0.90 ± 0.35[b]	4.97 ± 1.72[a]	3.60 ± 1.38[a]	6.60 ± 2.34[a]	0.40 ± 0.14[a]	0.03 ± 0.01[a]
M2	60 + 0	1.26 ± 0.28[b]	2.40 ± 0.63[a]	0.00 ± 0.00[b]	0.00 ± 0.00[b]	4.50 ± 1.10[a]	0.10 ± 0.02[b]	0.01 ± 0.00[b]
M3	60 + 0.125	1.49 ± 0.35[b]	0.90 ± 0.28[b]	0.66 ± 0.45[b]	0.20 ± 0.13[b]	3.80 ± 0.88[a]	0.05± 0.01[b]	0.01 ± 0.00[b]
M4	60 + 0.250	2.36 ± 0.28[b]	2.20 ± 0.29[a]	0.71 ± 0.28[b]	1.30 ± 0.47[b]	7.50 ± 0.64[a]	0.11 ± 0.01[b]	0.01 ± 0.00[b]
M5	60 + 0.500	1.68 ± 0.31[b]	1.60 ± 0.50[ab]	0.25 ± 0.18[b]	0.20 ± 0.13[b]	4.50 ± 0.78[a]	0.09 ± 0.02[b]	0.02 ± 0.01[ab]
M6	60 + 0.750	1.82 ± 0.29[b]	1.30 ± 0.26[ab]	0.61 ± 0.56[b]	0.30 ± 0.15[b]	5.60 ± 0.82[a]	0.08 ± 0.01[b]	0.01 ± 0.00[b]
Significance ($p \leq 0.05$)		**S**	**S**	**S**	**S**	**NS**	**S**	**NS**

*****M1 to M6 media designated for MS supplemented with NaCl and salicylic acid given against each
Values are mean ± S.E from 30 replicate cultures
Means followed by the same letter(s) are not significantly different at $p \leq 0.05$
Values are significant (S) or non-significant (NS) at $p \leq 0.05$

Maximum fresh and dry weight was observed on MS medium without NaCl and SA (M1) as in case of all the other growth parameters. When salicylic acid-treated salinized plants were compared with the plants treated with salt only, increase in fresh and dry weight (0.13 and 0.02g, respectively) was observed on M3 medium in Cardinal while the same trend was observed on M4 and M5 medium in Desiree (Table 1 and 2). Mean values were significantly different for fresh weights in both the cultivars and so was the case with the dry weights in the cultivar Cardinal. The situation was rather different in case of dry weights in cv. Desiree where the results were non-significant in statistical terms.

Effect of salicylic acid on protein contents of the salinized Cardinal and Desiree plants: Protein contents underwent an increasing trend in salicylic acid-treated salinized plants compared with only salt-treated potato plants in both the cultivars. Maximum protein accumulation (1.17 and 0.88 mg/g) was recorded at 0.75 and 0.50 m*M* salicylic acid treatment in cv. Cardinal and Desiree, respectively. Protein contents did not change at 0.125 and 0.50 m*M* concentration of SA in Cardinal. In Desiree, protein contents at 0.50 m*M* SA were maximum and then decreased sharply with further rise in SA concentration. Mean values were significantly different from each other for protein contents in both the cultivars (Fig. 1 A&B). For protein content, there was a statistically significant difference between salicylic acid-treated and non-treated potato plants of both the cultivars.

Discussion

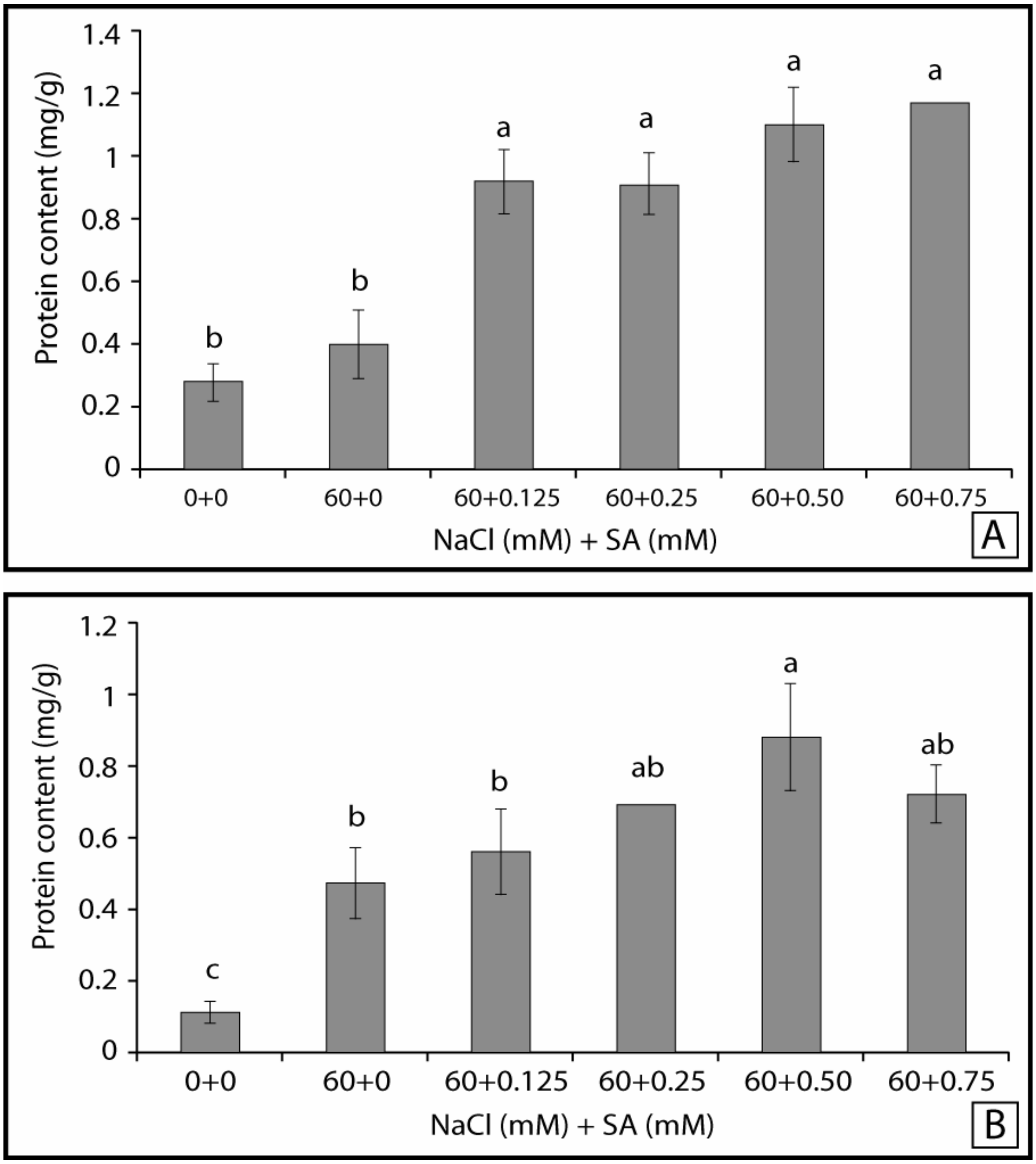
The present investigation showed an effect of salicylic acid on different growth and biochemical features of salt-stressed *In vitro* potato cultures. Salinity was found to strongly inhibit plant growth since higher concentrations of NaCl are known to cause ionic imbalance and osmotic stress in many plants (Maggio *et al*., 2000). These effects may lead to the development of other types of stresses such as oxidative damage to plants that may be responsible for reduced plant growth (Zhu, 2001). Similar results were noted in the present study where application of high concentration of NaCl (60 m*M*) to *In vitro*-grown potato plants adversely affected several of their growth (shoot/root length/number, number of nodes, fresh and dry weight) as well as biochemical (proteins) parameters. This general response to salt stress is also reported for other potato cultivars (Benavides *et al*., 2000), as well as for other plant species (Rodriguez *et al*., 1997; Hernandez *et al*., 1999; Rashid *et al*., 1999).

In the present study, treatment of salt-stressed Cardinal and Desiree plants with different concentrations (0.125, 0.25, 0.50 or 0.75 m*M*) of salicylic acid resulted in increased growth of both the tested potato cultivars. These results support the previous studies in which increase in salt tolerance in maize plants was observed by the application of salicylic acid. It enhanced the growth parameters (fresh, dry weight and length of shoots/roots) in plants as compared to only salt stressed-plants (Khodary, 2004). Similar results have also been reported earlier in salt-stressed cucumber plants where SA application resulted in higher values for above-mentioned growth parameters (Yildirim *et al*., 2008). Increase in shoot and root growth was observed by El-Tayeb *et al*., (2006) in case of copper-stressed plants of *Helianthus annuus* L. which were treated with salicylic acid. These ameliorative effects of salicylic acid on growth of stressed plants may be due to the fact that SA potentiates the generation of reactive oxygen species and increases the production of H_2O_2 in plants that in turn reduce the oxidative damage under saline stress, as described, for example, in case of wheat (Wahid *et al*., 2007).

Fig. 1. **(A)** Comparison of potato shoots (cv. Desiree) at various salicylic acid levels. Culture vessel at left is CONTROL (0 mM NaCl + 0 mM SA), whereas the rest of the five culture vessels from left to right are showing a comparison of shoot length at 0, 0.125, 0.25, 0.50 and 0.75 mM SA respectively in MS medium containing 60 mM NaCl. **(B)** Bunchy appearance of shoots (arrow) in potato plants (Desiree) exposed to salt stress (60 mM NaCl, 1.6x). **(C)** Bunchy appearance of shoots (arrow) in potato plants (Cardinal) exposed to salt stress (60 mM NaCl, 2.0x).

It was also observed in this study that moderate levels of SA (0.125 or 0.25 m*M*) resulted in an increase in the growth parameters of both the potato cultivars. Higher than 0.25 m*M* SA, however, had no significant protective effect against salinity stress under our experimental conditions. This might be due to the toxic effects of salicylic acid at higher concentrations. Previously, adverse effects of high SA concentrations (above 1.0 m*M*) were observed on bean and tomato plants when grown in high and low temperature stresses (Senaratna *et al*., 2000).

During this study, it was observed that the two tested cultivars of potato responded differently to specific salicylic acid treatments. In case of Cardinal, applications of 0.125-0.175 m*M* salicylic acid resulted in reduction of root length compared with salt stressed plants without salicylic acid treatment. However in Desiree, SA application showed a positive effect on root length (highest at 0.250 m*M*). Horvath *et al*., (2007b) has previously reported that salicylic acid pre-treatment decreased the drought tolerance of one wheat cultivar (Chinese spring) while increased in another (Cheyenne).



Values are mean (± S.E) from 30 replicate cultures
Cultivars: Cardinal (A) and Desiree (B)
Fig. 2. Effect of different SA concentrations on protein contents of *In vitro*-grown potato plants (cvs. Cardinal and Desiree)

The available literature reveals that SA induces abiotic stress tolerance in plants perhaps by regulating the expression of certain receptor protein kinases. These protein kinases have been found to initiate response to specific stress signals, as described, for example, after wounding in *Brassica oleracea* (Pastuglia *et al*., 1997) or in peaches (Bassett *et al*., 2005). In the present investigation, protein contents showed an increasing trend in both potato cultivars as compared to plants given only salinity stress. This increase in protein contents was more in Cardinal as compared to Desiree. The accumulation of protein in SA-treated plants is rather well documented in literature (Mc-Cue *et al*., 2000; Kang *et al*., 2003; El-Tayeb *et al*., 2006). This increase in protein contents by salicylic acid application was also previously reported in heat-stressed plants (Cronje and Bornman, 1999).

Conclusion

Overall, exogenously-applied SA enhanced the growth of both the tested cultivars of potato. This improvement in growth behavior might be due to its overall ameliorative effect since salicylic acid is known to promote seed germination, uptake of water and may also act as signal molecule under salt stress. It can be interpreted from the results that SA application with relatively higher concentrations did not confer much tolerance to NaCl stress in potato cultivars in comparison to moderate SA concentrations. In particular, 0.125 and 0.25 m*M* SA proved very effective in enhancing growth in Cardinal and Desiree, respectively. These results hint at a possibility that moderate concentrations of salicylic acid may, in future, be helpful in improving yield of plants under saline conditions.

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