Ameliorative role of salicylic acid and spermidine in the presence of excess salt in *Lycopersicon esculentum*

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Abstract

Salicylic acid (SA) and polyamines (PA) are widely used to overcome various abiotic stresses including salt (NaCl) stress in plants. In the present investigation, co-application efficacies of SA and PA on the salt stress (200 mM NaCl) were evaluated in *Lycopersicon esculentum*. After transplantation, at 10-d stage, seedlings were exposed to NaCl through soil and then allowed to grow till 30-d stage. At 31-d stage of growth, plants were sprayed with double distilled water (control) or spermidine (1.0 mM) and/or SA (10^{-5} M). The salt stress significantly reduced the growth, gas-exchange parameters, but increased antioxidant enzymes and proline content in the leaves. Moreover, the loss caused by salt stress was successfully restored by the following treatment of spermidine and SA.

Additional key words: abiotic stress; antioxidant; photosynthesis; proline.

Introduction

Salinity stress is a major limiting factor for sustainable productivity, especially of glycophytic plants, such as cucumber and tomato. Soil salinity is a universal problem that affects nearly 20% of flooded land and reduces crop yields significantly (Qadir *et al.* 2014). A high salt content in the soil affects the soil porosity, decreases the soil water potential (Hopkins and Huner 2009), and also affects physiology of plants at the cellular as well as the whole plant level (Murphy and Durako 2003). Plant growth is particularly affected by osmotic stress-specific ion toxicity, ion imbalance, and oxidative stress generated by salt stress (Li *et al.* 2010). Salt affects various morphological, physiological, and biochemical processes (Koca *et al.* 2007), such as photosynthesis through changes in gas exchange, pigment content, chloroplast development, and also decline in chlorophyll (Chl) fluorescence and accumulation of low-molecular-mass compounds, such as proline, glycine betaine (Mutlu and Bozcuk 2005, Yusuf *et al.* 2008, Mir *et al.* 2015), and proteins (Unni and Rao 2001). An excessive amount of sodium ions in the cells also causes the inhibition of enzymes, such as those for nitrogen metabolism and carbon fixation (Soussi *et al.* 1998). Ionic imbalance induced by salt stress causes high Na^+ concentration in plants and it also influences the uptake of other ions, particularly, K^+ and Ca^{2+}. The accumulation of toxic amounts of salts leads to the hyperosmotic stress in plants that ultimately stimulates the production of reactive oxygen species (ROS) (Ahmad *et al.* 2009), dehydration, and turgor loss in cells and tissues (Mittler 2002).

Salicylic acid (SA), a ubiquitous plant phenolic acid, is recognized as an endogenous regulator of plant metabolism. It has been found to generate a wide range of metabolic and physiological responses in plants by affecting their growth and development and it improves plant growth (Khodary 2004), transpiration rates, stomatal regulation, photosynthesis (Arfan *et al.* 2007), and ion uptake and transport (Manaa *et al.* 2014). The role of SA in defense mechanism under both biotic and abiotic stresses suggests that it also alleviates the salt stress in plants (Tissa *et al.* 2000, Al-Hakimi and Hamada 2001, Bosch *et al.* 2007, Jayakannan *et al.* 2015). Exogenous SA could regulate the activities of antioxidative enzymes and increase plant tolerance to abiotic stresses (He *et al.* 2002,

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Abbreviations: C_i – intercellular CO_2 concentration; CA – carbonic anhydrase; CAT – catalase; Chl – chlorophyll; DAS – days after sowing; DDW – double distilled water; DM – dry mass; FM – fresh mass; g_s – stomatal conductance; LSD – least significant difference; NR – nitrate reductase; PA – polyamines; POX – peroxidase; P_N – net photosynthetic rate; ROS – reactive oxygen species; SA – salicylic acid; Spd – spermidine; SOD – superoxide dismutase; SPAD – soil and plant analysis development.

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Yusuf *et al*. 2008, Parashar *et al.* 2014). It has a regulatory effect on activating biochemical pathways associated with tolerance mechanisms in plants (Najafian *et al.* 2009). Moreover, SA treatment supported tolerance against copper toxicity in cucumber and tobacco (Strobel and Kuc 1995). In *Cassia tora*, SA enhanced Al tolerance by increasing the citrate efflux of the roots and thus inhibiting Al uptake (Yang *et al*. 2003). It was revealed that Al increased the concentration of SA in the roots. Cd treatment increased the endogenous free SA content in barley (Metwally *et al*. 2003) and in maize (Pal *et al*. 2005). Polyamines (PA) are low-molecular-mass aliphatic polycations that are ubiquitously distributed in all living organisms (Hussain *et al.* 2011). They are known to be essential for growth and development in prokaryotes and eukaryotes (Tabor and Tabor 1984, Tiburcio *et al*. 1990). PAs have been implicated in various processes associated with plant growth and development, such as embryogenic competence (Silveira *et al.* 2013), programmed cell death (Kim *et al.* 2013), fruit ripening (Gil-Amado and Gomez-Jimenez 2012), xylem differentiation (Tisi *et al.* 2011), as well as biofilm formation (Lee *et al.* 2009). Besides this, PAs elicit diverse physiological responses, including tuber formation, root initiation, embryogenesis, flower development, fruit ripening, organogenesis, and biotic and abiotic stress responses (Gil-Amado and Gomez-Jimenez 2012, Silveira *et al.* 2013, Mir *et al.* 2015). PAs have been involved in various protective responses in the cell (Pothipongsa *et al.* 2012, Tanou *et al*. 2012, 2014), which include strengthening of membranes and other macromolecules, which are targets of ROS damage. It has been suggested that PAs play pivotal roles in modulating defense response of plants to diverse environmental stresses (Bouchereau *et al.* 1999), which include metal toxicity (Groppa *et al.* 2003), oxidative stress (Rider *et al.* 2007), drought (Yamaguchi *et al.* 2007), salinity (Duan *et al*. 2008, Mir *et al.* 2015), and chilling stress (Groppa and Benavides 2008). In addition to this, it has been reported that exogenous application of PA under various abiotic stresses is also an effective approach for enhancing stress tolerance and productivity of crops. The present study was executed with an objective to study the impact of combined application of salicylic acid and spermidine to the salt-stressed plants of *Lycopersicon esculentum* and to elucidate how these compounds trigger antioxidant system leading to tolerance against salinity.

Materials and methods

Hormone preparation: The stock solution (1 M) of salicylic acid (SA) or spermidine (Spd) were prepared by dissolving the 13.812 g(SA) and 14.525 g(Spd) in 5 mL of ethanol, in 100-mL volumetric flasks and the final volume was made up to the mark by using double distilled water (DDW). The required concentrations of SA (10^{-5} M) or Spd (1 mM) were prepared by the dilution of stock solution with DDW and these concentrations were selected on the basis of our previous studies by Fariduddin *et al.* (2003) and Mir *et al.* (2015), respectively. *Tween-20* (0.5%) was added to the solution prior to the treatment.

Experimental design: Surface-sterilized seeds were sown in nursery beds in a sandy-loam soil and cow manure (6:1, v/v) mixed under natural environmental conditions. Nursery beds were irrigated on alternate days with tap water. At 20-d stage, seedlings were uprooted and transplanted to earthen pots (25 cm in diameter) filled with sandy-loam soil and cow manure (6:1, v/v) under natural environmental conditions in the net house of Department of Botany, Aligarh Muslim University, Aligarh, India. After 30 d from sowing (DAS), plants were exposed to NaCl (200 mM) through soil and then allowed to grow till 45 DAS. These plants were sprayed with DDW (control), SA (10^{-5} M), Spd (1mM), or SA + Spd for three days. Each plant was sprinkled thrice and the nozzle of the sprayer was adjusted in such a way that it pumped out *ca.* 1 mL in one sprinkle. Each treatment included five pots (replicates) and in each pot three plants were maintained after thinning under a completely randomized block design. At 60 DAS, plants from each treatment were harvested to assess the various growth and biochemical parameters and photosynthetic attributes.

Plant growth analysis: The plants were removed from the pots along with the soil and were dipped in a bucket filled with water. The plants were moved smoothly to remove the adhering soil particles and the lengths of shoots and roots were measured by using a meter scale. The plants were blotted and weighed to record their shoot and root fresh mass (FM) and then placed in an oven at 70°C for 72 h. The samples were weighed again after allowing them to cool at room temperature to record their root and shoot dry mass (DM). The leaf area was measured by using a leaf area meter (*ADC Bioscientific*, UK).

Leaf water potential: The leaf water potential (LWP) was monitored with the *Psypro* water potential system (*Wescor, Inc.* South Logan, Utah, USA) in the third fully expanded leaves of the plant.

SPAD chlorophyll: The SPAD value of Chl was measured by using the SPAD Chl meter (*SPAD-502; Konica, Minolta Sensing Inc.,* Japan) in the third fully expanded leaves of the plant.

Gas-exchange parameters were determined on the third fully expanded leaves between 11:00 and 12:00 h by using an infrared gas analyzer portable photosynthetic system (*LI-COR 6400, LI-COR,* Lincoln, NE, USA). To measure net photosynthetic rate (P_N) and its related attributes [stomatal conductance (g_s), internal CO_2 concentration

(C_i), and transpiration rate (E)] the air temperature, relative humidity, CO_2 concentration, and PPFD were maintained at 25°C, 85%, 600 μmol mol^{-1}, and 800 μmol(photon) mol^{-2} s^{-1}, respectively.

Electrolyte leakage: The total inorganic ions leaked out from leaves were estimated by the method of Sullivan and Ross (1979). Twenty leaf discs were taken in boiling tube containing 10 mL of deionized water and electrical conductivity was measured (Ec_a) (*PC-700, Eutech Instruments, Thermo Scientific*, Mumbai, India). The contents were heated at 45°C and 55°C for 30 min each in water bath and electrical conductivity was measured (Ec_b). Later the content were again boiled at 100°C for 10 min and electrical conductivity (Ec_c) was recorded again. The electrolyte leakage was calculated by using the formula.

$$\text{Electrolyte leakage [\%]} = \frac{Ec_b - Ec_a}{Ec_c} \times 100$$

Carbonic anhydrase (CA): The activity of CA (EC 4.2.1.1) in the leaves was measured following the method described by Dwivedi and Randhawa (1974). The leaf samples were cut into small pieces in cysteine hydrochloride solution. These leaf samples were blotted and transferred in a test tube, followed by the addition of phosphate buffer (pH 6.8), 0.2 M $NaHCO_3$, bromothymol blue, and the methyl red indicator, at the last. The reaction was titrated against 0.05 N HCl. The activity of enzyme was expressed on the basis of fresh mass (FM) as mol (CO_2) g^{-1}(FM) s^{-1}.

Nitrate reductase (NR): The activity of NR (EC 1.7.1.1) was measured following the method of Jaworski (1971). The fresh leaf samples were cut into small pieces and transferred to plastic vials, containing phosphate buffer (pH 7.5), KNO_3, and isopropanol, and then they were incubated at 30°C for 2 h. After incubation, sulfanilamide and N-1-naphthylethylenediamine hydrochloride solutions were added. The absorbance was read at 540 nm on a spectrophotometer (*Spectronic 20D*, Milton Roy, USA). The activity of enzyme was expressed in on the basis of FM as nM(NO_2) g^{-1}(FM) s^{-1}.

Antioxidative enzymes: The leaf tissue (0.5 g) was homogenized in 50 mM phosphate buffer (pH 7.0) containing 1% (w/v) soluble polyvinylpyrrolidone. The homogenate was centrifuged at 27,600 × *g* for 10 min at 4°C and the supernatant was used as source of enzymes catalase, peroxidase, and superoxide dismutase.

Peroxidase (POX, EC 1.11.1.7) and catalase (CAT, EC 1.11.1.6) were assayed following the procedure described by Chance and Maehly (1955). CAT was estimated by titrating the reaction mixture, consisting of phosphate buffer (pH 6.8), 0.1 M H_2O_2, enzyme extract, and 2% H_2SO_4, against 0.1 N potassium permanganate solution. The activity of enzyme was expressed on the basis of FM as nM(H_2O_2 decomposed) g^{-1}(FM).

The reaction mixture for POX consisted of pyrogallol, phosphate buffer (pH 6.8), 1% H_2O_2, and enzyme extract. The change in absorbance due to catalytic conversion of pyrogallol to perpurogalline was noted at an interval of 20 s for 2 min, at 420 nm by a spectrophotometer (*Spectronic 20D*, Milton Roy, USA). A control set was prepared by using double distilled water instead of enzyme extract. The activity of enzyme was expressed on the FM basis as U g^{-1}(FM).

The activity of superoxide dismutase (SOD, EC 1.15.1.1) was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium using the method of Beauchamp and Fridovich (1971). The reaction mixture contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 mM nitroblue tetrazolium (NBT), 2 mM riboflavin, 0.1 mM EDTA, and 50 μL of enzyme extract and was placed under 15-W fluorescent lamp (*Philips Pvt Ltd*, India). The reaction was started by switching on the light and was allowed to run for 10 min. The reaction was stopped by switching off the light. Fifty percent inhibition under light was considered as one enzyme unit. The activity of enzyme was expressed on the basis of FM as U g^{-1}(FM).

Proline accumulation: The proline content in fresh leaf samples was determined by adopting the method of Bates *et al.* (1973). Proline was extracted in 3% sulphosalicylic acid. In the extract, an equal volume of glacial acetic acid and ninhydrin solutions were added. The sample was heated at 100°C, to which 5 mL of toluene was added after cooling. The absorbance of the toluene layer was read at 520 nm, by a spectrophotometer (*Spectronic 20D*, Milton Roy, USA).

Statistical analysis: Data were statistically analyzed using *SPSS, 17.0* for *Windows* (*SPSS,* Chicago, IL, USA). Analysis of variance (*ANOVA*) was performed on the data to determine the least significant difference (LSD) between treatment means with the level of significance at $p \leq 0.05$.

Results

Growth biomarkers: The plants raised in the soil supplemented with 200 mM of NaCl showed a significant decrease in the values of all the growth biomarkers (root and shoot length, root and shoot FM, and DM) in comparison to control plants (Fig. 1). The spray of Spd (1.0 mM) and/or SA (10^{-5} M) to the foliage of stress-free plants significantly increased all the growth markers. The combination of Spd and SA, increased the shoot and root

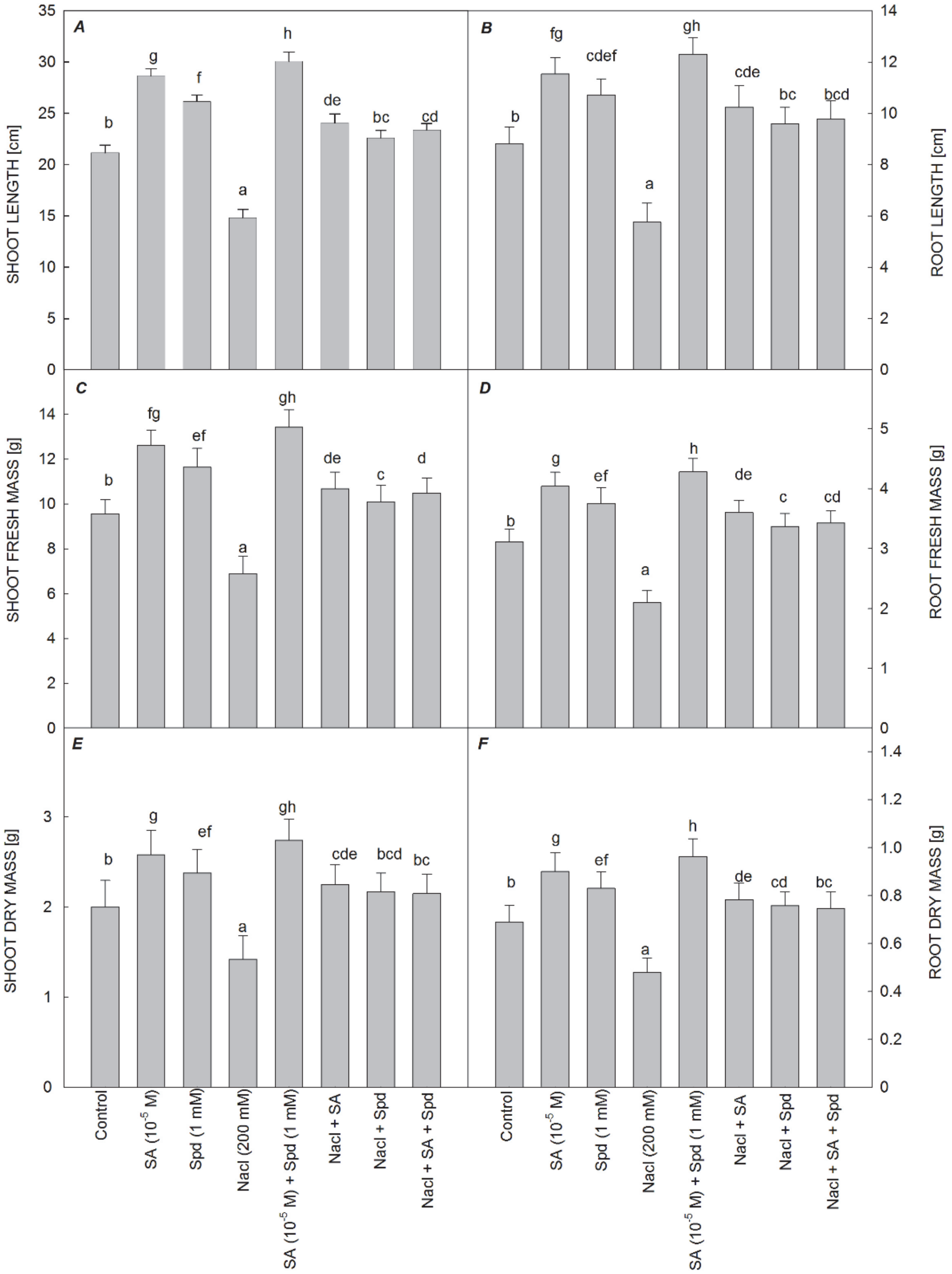

Fig. 1. Effect of salicylic acid (SA; 10^{-5} M) and/or spermidine (Spd; 1 mM) on the salinity (NaCl; 200 mM) induced changes in the (*A*) shoot length, (*B*) root length, (*C*) shoot fresh mass, (*D*) root fresh mass, (*E*) shoot dry mass, and (*F*) root dry mass in tomato at 60 days after sowing. All the data are the means of five replicates (*n* = 5); vertical bars show standard errors (± SE). Means with *different letters* above the bars are significantly different between the treatments.

length by 40 and 42%, respectively, DM of roots and shoots by 39 and 37%, respectively, compared with the control plants. Moreover, the stressed plants treated with SA+Spd completely recovered from the damaging effects generated by 200 mM of NaCl.

Leaf area: The leaf area of the plants was significantly affected by the presence of NaCl and a decrease (32% lesser area than that of the control) was noted at 60 DAS (Fig. 2*A*). The foliar application of SA and Spd alone improved the leaf area, compared with the control. The treatment of SA and Spd to the NaCl-treated plants completely recovered the loss of leaf area in comparison to stressed plants.

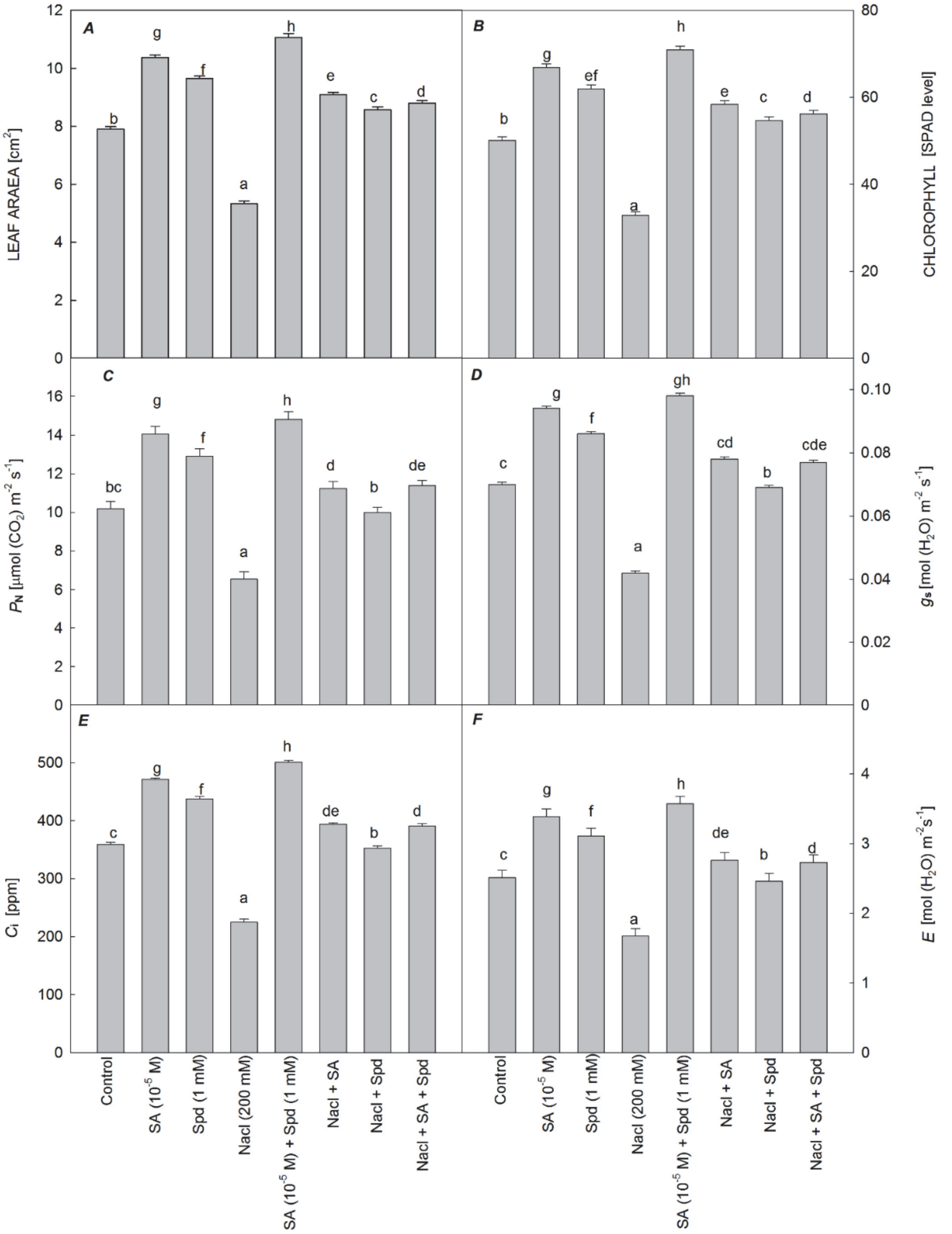

Fig. 2. Effect of salicylic acid (SA; 10^{-5} M) and/or spermidine (Spd; 1 mM) on the salinity (NaCl; 200 mM) induced changes in the (*A*) leaf area, (*B*) SPAD chlorophyll, (*C*) net photosynthetic rate (P_N), (*D*) stomatal conductance (g_s), (*E*) intercellular CO_2 concentration (C_i), and (*F*) transpiration rate (*E*) in tomato at 60 days after sowing. All the data are the means of five replicates ($n = 5$); vertical bars show standard errors (± SE). Means with *different letters* above the bars are significantly different between the treatments.

Leaf water potential increased significantly in the plants treated with Spd and/or SA at 60 DAS (Fig. 3*A*). However, the combined effect of Spd+SA proved to be more efficient and increased the LWP by 36% in plants at 60 DAS, when compared with their respective controls. Moreover, the loss of LWP caused by NaCl was successfully restored by the following treatment by Spd and SA at 60 DAS.

SPAD Chl: Among various treatments, foliar application of SA and Spd alone and in combination (SA+Spd) significantly increased the Chl content (SPAD value) by 33, 23, and 41%, respectively, compared with the control plants (Fig. 2*B*). However, the soil treated with NaCl significantly decreased the SPAD value of Chl in comparison with the control plants. Moreover, NaCl-induced decline was completely overcome with the combined SA+Spd treatment.

Photosynthesis and related attributes: P_N, g_s, C_i, and E were significantly reduced by the treatment of NaCl in comparison with the control plants (Fig. 2*C–F*). The the values of P_N were reduced by 36%, g_s by 43%, C_i by 37%, and E by 33% as compared with the control. The application of of both SA+Spd to the plants significantly increased the value of P_N (45%) and g_s (29%), C_i (39%), and E (42%) compared with the control. However, NaCl-stressed plants treated with the SA+Spd completely neutralized the toxic effect generated by NaCl.

Activities of NR and CA: The plants exposed to NaCl stress possessed significantly lower activities of CA and NR than their respective control plants (Fig. 3*B*,*C*). However, the application of Spd or SA alone or SA+Spd to the foliage of nonstressed plants significantly increased the activity of CA by 19, 27, and 32%, respectively, and NR by 25, 17, and 30%, respectively, at 60 DAS. Furthermore, the application of SA and Spd to the foliage of salt-stressed plants completely nullified the toxic effect of NaCl.

Antioxidant enzymes: The activities of antioxidant enzymes (CAT, POX, and SOD) significantly increased in response to various treatments, compared with the control plants (Fig. 3). The foliar application of SA to the NaCl-treated plants had an additive effect on the activities of these enzymes. The highest values for CAT, POX, and SOD were noted in the NaCl-treated plants and also after the treatment by SA and Spd; and their activities were higher by 72, 85, and 69%, respectively, compared with the control.

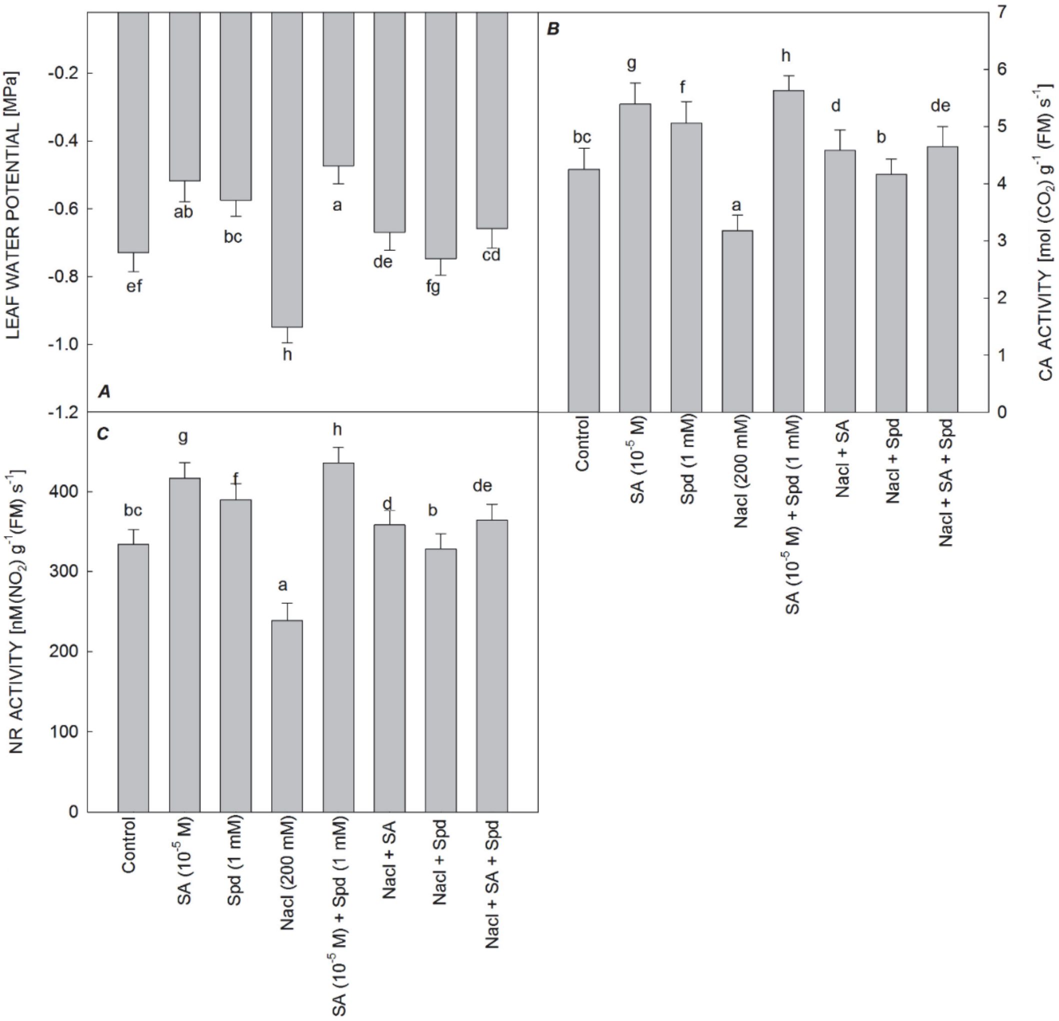

Fig. 3. Effect of salicylic acid (SA; 10^{-5} M) and/or spermidine (Spd; 1 mM) on the salinity (NaCl; 200 mM) induced changes in the (*A*) leaf water potential, (*B*) carbonic anhydrase (CA), and (*C*) nitrate reductase (NR) in tomato at 60 days after sowing. All the data are the means of five replicates ($n = 5$); vertical bars show standard errors (± SE). Means with *different letters* above the bars are significantly different between the treatments.

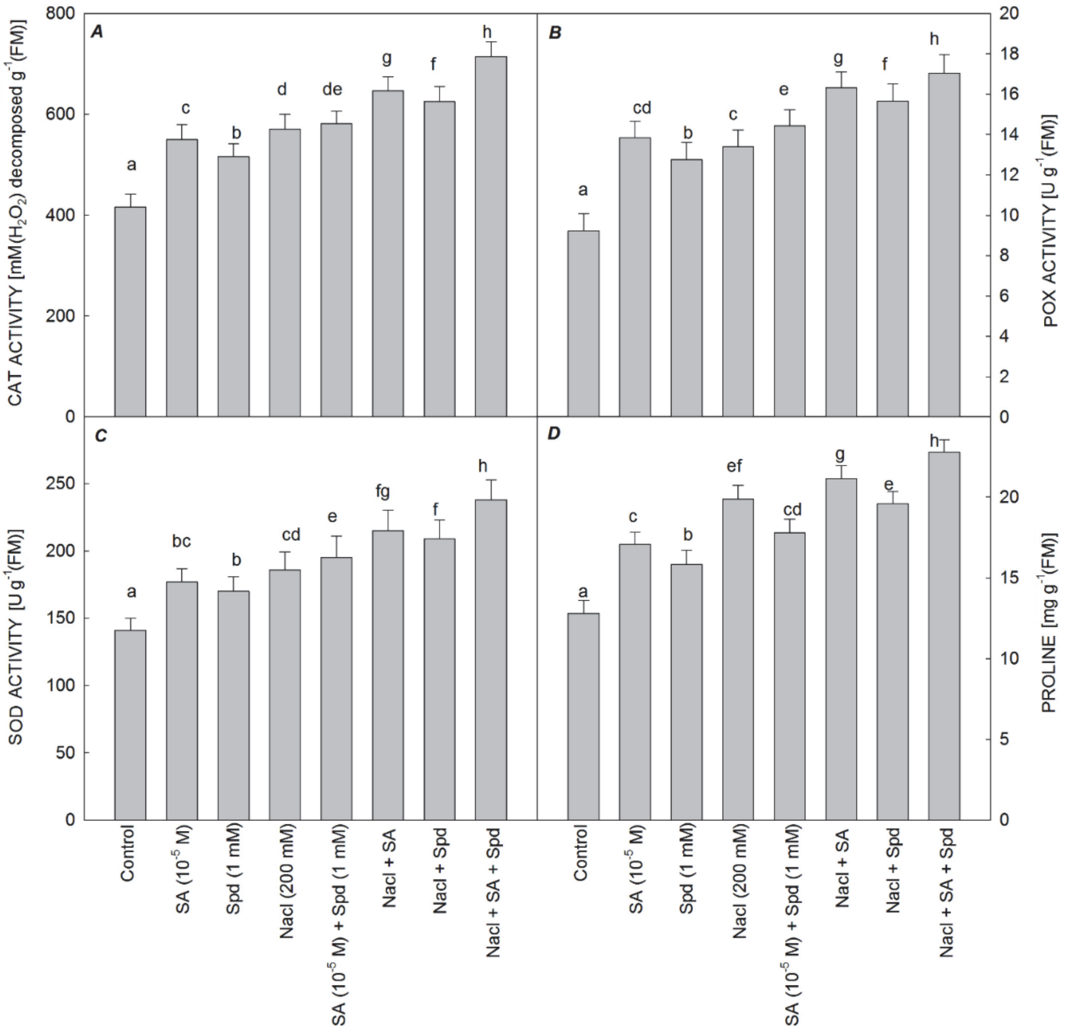

Fig. 4. Effect of salicylic acid (SA; 10^{-5} M) and/or spermidine (Spd; 1 mM) on the salinity (NaCl; 200 mM) induced changes in the (*A*) catalase (CAT), (*B*) peroxidase (POX), (*C*) superoxide dismutase (SOD), and (*D*) proline content in tomato at 60 days after sowing. All the data are the means of five replicates (*n* = 5); vertical bars show standard errors (± SE). Means with *different letters* above the bars are significantly different between the treatments.

Proline content in leaves was elevated by all the treatments as compared with their respective control (Fig. 3*D*). The values of proline increased by 33, 24, and 55%, respectively, in comparison with the respective control, in plants treated with SA, Spd or NaCl. The following treatment both SA+Spd further increased the proline content. The maximum accumulation of proline was 78% higher than that of the control in the plants grown in NaCl supplemented soil.

Discussion

Salinity limits crop production in arid and semiarid regions and under soil moisture deficiency. Salinity of soil or irrigation water is the major limiting factor for crop growth in many regions of the world (Ashraf *et al.* 2008, Kausar *et al.* 2013). Salt stress at any stage of crop growth can cause an irreversible loss in a yield potential (Hameed *et al.* 2013) of many crops including tomato. In the present study, all growth parameters were significantly lowered in the plants exposed to salinity stress. Our results were in agreement with that of Wani *et al*. (2016) and Pirasteh-Anosheh *et al.* (2014) who reported marked reduction in growth parameters of *Brassica juncea* and *Hordeum vulgare* subjected to NaCl stress. Growth inhibition by salinity has been attributed to the disturbance in water and osmotic potential, toxicity of excessive Na^+ and Cl^-, disturbance in the accumulation of nutrients, disruption in the structure and the activity of the enzymes, damage in cell organelles and plasma membrane, disturbances in photosynthesis, respiration, and protein synthesis (Feng *et al*. 2002, Munns 2005). Salt-stress tolerance in crop plants is generally accompanied by low uptake and accumulation of Na^+ (Hameed *et al.* 2013), a cation that has been shown to have antagonistic effects on crops due to its toxic effects (Ouerghi *et al.* 2000). Therefore, Na^+ concentration in plant tissues could be used as an important indicator for salinity tolerance (Tadayon and Emam 2007). However, the subsequent application of SA and/or Spd was beneficial to sustain the growth and reduce deleterious effects of salt stress (Fig. 1). The stressed plants treated with SA or Spd increased their FM and DM as well as root and shoot length and leaf area, compared with those grown without SA or Spd (Figs. 1, 2*A*). A similar increase by SA treatment has been reported by Yusuf *et al.* (2008). However, Mimouni *et al.* (2016) reported that SA-treated plants had greater shoot and root dry mass, leaf area compared with untreated plants when exposed to salt stress. The combination of SA (Parashar *et al.* 2014) and PA (Benavides *et al.* 1997, Duan *et al.* 2008, Mir *et al.* 2015) are known to improve the growth of root and/or shoot in various plant groups. The increase in the growth parameters caused by SA is due to the regulation of cell cycle (Carswell *et al.* 1989) and might be due to a negative balance of leaf xylem import and phloem export process after the modification of leaf soluble salt during recovery (DeLacerda *et al.* 2005). It appears that a large amount of ions absorbed during stress might be transferred to young leaves after recovery (Alarcon *et al.* 1993). Furthermore, the involvement of PA, in wide array of plant processes, such as DNA replication, transcription of genes, cell division, and root growth (Bais and Ravishankar 2002), are responsible for the growth improvement. The enhanced tolerance to salt stress may be due to involvement of PA in the maintenance of tonoplast H^+-ATPase, proton-translocating inorganic pyrophosphatases (H^+-PPase), and Na^+/H^+ transporter (Liu *et al.* 2006). Spd functions as membrane surface stabilizers through interaction with phospholipids or other negatively charged groups of membrane (Amri and Shahsavar 2010). It is interesting to observe that co-application of SA and Spd proved to be more effective in the improvement of growth parameters under salt stress, perhaps, it could be due to their synergistic or additive effects.

In addition to the primary effects, salinity inevitably leads to oxidative stress through an increase in ROS, such as superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^\bullet$) (Zheng *et al.* 2009). It is now widely accepted that these cytotoxic ROS are responsible for various stress-induced damages to macromolecules and ultimately to cellular structure (Amor *et al.* 2006, Sekmen *et al.* 2012). ROS detoxification by enzymatic (SOD, CAT, and POX) antioxidants is the effective defence mechanism against oxidative damage in plants (Verslues *et al.* 2006, Khan and Panda 2008). In the present study, we observed higher activities of CAT, POX, and SOD, as well as the proline content in the plants exposed to NaCl stress with or without SA and/or Spd treatments (Fig. 4). SA conferred protection against salinity in tomato plants probably by improving the activity of aldose reductase and APX enzymes and through the accumulation of osmolytes, *e.g.*, sugar alcohol or proline (Tari *et al.* 2002, Szepesi *et al.* 2005). Proteomic study also revealed that synthesis of antioxidant enzymes and the mobilization of seed storage proteins play pivotal role in SA-mediated salt tolerance (Rajjou *et al.* 2006). In addition to this, Chen and Dickman (2005) reported that proline is a potent scavenger of ROS and also a major component of structural proteins in plants, known to be osmoprotectant capable of mitigating the impacts of various abiotic stresses in plants (Szabados and Savowre 2009). Similarly, the application of Spd had been also shown to ameliorate NaCl stress in chickpea and *Vigna radiata* plants by elevating the activities of CAT, glutathione peroxidase, glutathione reductase, and SOD (Sheokand *et al.* 2008, Mir *et al.* 2015). Recently, Li *et al.* (2016) was reported that Spd also enhanced the CAT, POX, and SOD activity when exposed to salt stress in *Zoysia japonica*. Spd in particular increased the DNA-binding activity of transcription factors and promoted gene transcript levels (Childs *et al.* 2003). Sung *et al.* (2011) reported that Spd provided defence against hypersalinity through the upregulation of FeSOD gene, thereby improving oxidative stress damage. Our results validated the additive interactions of SA and Spd for amelioration of the oxidative stress generated by NaCl.

Salt stress damages the photosynthetic machinery at multiple levels, such as pigment content, structure and function of thylakoids, electron transport and enzymes, stomatal functioning, and gas exchange (Geissler *et al.* 2009). Salinity causes a decrease in Chl content *via* the acceleration of Chl degradation or inhibition of its biosynthesis (Xu *et al.* 2000). The effect of these processes resulted in the decreased SPAD value of Chl as it was found in our study (Fig. 2*B*). Similar decrease in Chl content was observed by Hayat *et al.* (2012). These results are further supported by Wu *et al.* (2012), Wang *et al.* (2010), Ahmad *et al.* (2012a), and Wani *et al.* (2013, 2016). However, the subsequent treatment by SA and/or Spd improved the SPAD values. In the present study, SA treatment of nonstressed plants increased the Chl content; it was also reported by others (Parashar *et al.* 2014) and also well supported by the earlier observations in wheat and/or mung bean seedlings under stress-free conditions (Moharekar *et al.* 2003, Hayat *et al.* 2005) as well as under water stress (Singh and Usha 2003). Moreover, Spd treatment also increased the SPAD value. It was reported that Spd also increased the Chl content in rice and *Vigna radiata* (Krishnamurthy 1991, Mir *et al.* 2015). It could be due to the elevated concentration of Mg^{2+} (Lakra *et al.* 2006), which is essential for Chl synthesis. Salt stress caused the closure of stomata (Bethkey and Drew 1992), therefore, it decreased partial CO_2 pressure and thus C_i, g_s, and consequently CA activity (Ali *et al.* 2008). Besides this, salinity impairs photosynthesis and the photosynthetic electron transport chain (Sudhir and Murthy 2004). All these impaired events finally resulted in a severe decline in P_N (Fig. 2*C*). The damage caused by salt stress could be also attributed to water stress or a kind of physiological drought generated by NaCl (Perez-López *et al.* 2009, Belkheiri and Mulas 2013) as evident from the decrease in leaf water potential in our present study (Fig. 3*A*). Decrease in all gas-exchange parameters (P_N, g_s, C_i, and E) due to salinity has also been reported in *Brassica juncea* (Wani *et al.* 2013, 2016), and *Vigna radiata* (Hayat *et al.* 2010, Mir *et al.* 2015). Other reasons for the decrease in P_N under NaCl are faster senescence and changes in enzyme activities induced by dysfunction of proteins and negative feedback by reduced sink activity (Iyengar and Reddy 1996). SA is well-known as an important plant growth regulator involved in regulating stomatal closure, Chl content (Hussein *et al.* 2007, Parashar *et al.* 2014), net photosynthesis, and other physiological parameters, such as transpiration, glycolysis, uptake and transport of nutrients, membrane permeability, flowering and thermogenesis, and growth rate (Ashraf *et al.* 2010). SA has been established as an important regulator of photosynthesis, water relations, and metabolic aspects of plants, depending on its analogues, concentrations, mode of application and plant type. SA is known to affect leaf and chloroplast structure (Uzunova and Popova 2000), stomatal closure (Mateo *et al.* 2004, Melotto *et al.* 2006), Chl and carotenoid contents (Chandra and Bhatt 1998, Fariduddin *et al.* 2003), and the activity of enzymes, such as Rubisco and CA (Slaymaker *et al.* 2002, Parashar *et al.* 2014). Moreover, treatment of SA and/or Spd improved the gas-exchange parameters. Therefore, the plants treated with SA, both in presence and absence of stress, exhibited the higher P_N and related attributes. PAs are well recognized for their positive effects on photosynthetic efficacy under various stress conditions. Spd comprises of extremely protonated amino and imino groups and could be conjugated with other negatively charged organic molecules, such as proteins, nucleic acids, and phospholipids. The binding is important for the maintenance of the thylakoid membranes and avoidance of the hydrolysis of photosynthetic proteins (Ahmad *et al.* 2012b). In our present study, foliar application of Spd also resulted in physiological damage related with salinity, thus confirming previous observations that exogenous PAs significantly improved the physiological status of stressed plants (Sen *et al.* 2014, Tanou *et al.* 2014). Moreover, PAs play a role in retarding the loss of D1*,* D2*,* and cytochrome *f* from the thylakoid membranes as well as large subunits of Rubisco and Chl from the leaf tissue (Besford *et al.* 1993). This could increase P_N in the present study. Spd application improved the CA activity by its involvement at the level of transcription and/or translation (Cohen 1998) that might generate a significant impact on CA activity (Fig. 3*B*). Plants exposed to salinity exhibited lower activity of NR (Fig. 3*C*). This might be an after-effect of the inhibition and/or metabolic dysfunction of NR (Hopkins and Huner 2009). Moreover, stress factors interfere with the structure and fluidity of the membrane (Alia-Mohanty and Saradhi 1992, Karim *et al.* 1999) on exposure to salt stress. It might restrict the uptake of nitrate, the inducer and substrate for the NR (Campbell 1999) resulting in the decreased NR activity (Fig. 3*C*). However, treatment of SA and/or Spd to both stressed and nonstressed plants enhanced the activity of NR. The most appropriate reason to explain the SA-mediated elevation in the activity of NR is that it corrects the stress-mediated damage to the plasma membrane, as evident from an increase in the membrane stability index in wheat (Agarwal *et al.* 2005). The membrane correction/stabilization could facilitate the increased uptake of nutrients including that of nitrate, which acts as an inducer of NR (Campbell 1999). The increase in the uptake of various nutrients, including NO_3 and activation of NR, under normal growth conditions is well established (Hayat *et al.* 2005) which strongly supports our present results. These results are further supported by Yusuf *et al.* (2008), Idrees *et al.* (2012), and Mir *et al.* (2015). Furthermore, Rosales *et al.* (2012) proposed that PAs could participate in the regulation of NR activity in a dual manner. At short time, PAs inhibit the NR activity by increasing NO production (a signaling molecule involved in the inhibition of NR activity) and 14-3-3 protein-master regulator of many signal transduction cascades and their interaction with NR (Athwal and Huber 2002). At longer time, PAs modulate the association of 14-3-3 proteins with the H^+-ATPase, thus activating NR activity, and this action could prevail over the effect of the increased NO concentration. The role of SA and PAs in restoring growth has been shown independently in several studies (Rady 2011, Parashar *et al.* 2014). It is apparent that both plant growth regulators (SA and PA) crosstalk to induce defensive genes countering stress conditions.

Conclusions: Salt stress decreased the growth, physiological, and biochemical parameters in *L. esculentum*. Application of both SA+Spd was more effective in alleviation of toxicity generated by excess NaCl than their individual effects. Our study further confirmed that treatment of plant with both SA and Spd at particular stage of growth could be exploited to overcome the loss of crop productivity due to presence of excess salt in soil

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