

Salicylic acid pre-treatment induced physiological and biochemical changes in *Solanum lycopersicum* L. under salinity stress

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Abstract

Agricultural crop production around the world is adversely affected by excess salt accumulation in the soil. Plants initiate broad range of signal transduction pathways to respond any stress. Salicylic acid (SA) is an endogenous plant growth regulator that acts as a signal molecule to modulate plant response by reducing the effects of abiotic stress on plants. The main objective of this study is to examine whether exogenous salicylic acid pre-treatment may reduce the adverse effects of salt stress and enhance salt tolerance in *Solanum lycopersicum*. For this experiment, two weeks old seedlings were subjected to salt stress by adding salt water (100 mM NaCl) for three days with or without salicylic acid pre-treatment. After salt stress exposure plant leaves were harvested and the various measures were recorded. Results of this study exhibited that salicylic acid pre-treatment mitigates various adverse effects of salt stress on plant growth by stimulating plant biomass, water relations, protein content, chlorophyll pigment, and inorganic osmolytes accumulation. Simultaneously, an increase in activity of antioxidant enzymes of SOD, CAT and POX were also triggered. This current study suggested that pre-treating of *Solanum lycopersicum* with salicylic acid attenuates the depressive effect of salinity by accelerating the osmolyte accumulation and triggering activity of free radical scavenging enzymes.

Keywords: antioxidant enzymes; salicylic acid; salt stress; tomato

Introduction

Agricultural crop production around the world is severely limited by a variety of abiotic and biotic stresses. One of the most important abiotic factors that inhibited crop production was salt in arid and semi-arid regions, where the salt content was naturally high and rainfall was insufficient to allow for leaching in soil (Zhao *et al.*, 2007). Increased salinity affected nearly one-third of cultivated land (Kaya *et al.*, 2002). Salt stress adversely affects the growth and productivity of cultivated plants. To cope with the altering climatic conditions, certain plants change their metabolism and activates variety of defence systems (Sticher *et al.*, 1997). Although, certain plants involve in the mechanism of accumulation of compatible osmolytes such as proline and soluble sugar to overcome the salt stress (Shahba *et al.*, 2010).

Plants respond to any stress by initiating a broad range of signal transduction pathways. Among the signalling molecules, salicylic acid (SA; o-hydroxybenzoic acid) is an endogenous plant growth substance

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worked as a signal molecule involving in modulating plant response (Sing and Usha, 2003) and signal transducer (Senaratna *et al.*, 2000). Through extensive signalling cross-talk with other growth hormones, salicylic acid also modulates different aspects of plant responses to abiotic stressors (Iqbal *et al.*, 2015).

Many plant species can benefit from exogenous administration of SA to reduce toxicity symptoms caused by salt stress. Exogenously applied SA's ability to boost photosynthetic activity, enhance antioxidant protection by changing antioxidant enzyme activities, increase soluble carbohydrate accumulation, raise ATP content, and maintain an optimal K⁺/Na⁺ ratio under saline circumstances has been proposed as potential mechanisms of salt tolerance in plants (Ashraf *et al.*, 2010; Hayat and Ali, 2010).

Hence, this study was designed to evaluate the salicylic acid influences on amelioration of salt tolerance of plants by studying some physiological and metabolic significance in tomato (*Solanum lycopersicum*).

Materials and Methods

Cultivation of plants

Tomato seeds (*Solanum lycopersicum*) were planted at 2 cm depth in black polythene covers filled with vermicompost, soil and sand (1:2:1) (Figure 1). Growing seedlings were daily sprinkled with distilled water and maintained in green house.



Figure 1. Plants under greenhouse condition

Cultural conditions and treatment to the plants

Two weeks old plants after the onset of third leaf, experiment was divided into four groups. Control: Control-Irrigated only with tap water for three days. SA: Salicylic acid treated-Irrigated with 80 mM of salicylic acid for 3 days. NaCl: Stressed- Plants subjected with salt water (100 mM NaCl) for three days. SA+NaCl: Pre-treated and stressed- Plants were treated with 80 mM SA for 3 days, after pre-treatment the same plants were again subjected with salt water for three days. Finally, after three days of salt exposure, all plants were harvested washed quickly with distilled water, blotted dried on filter papers and stored in deep freezer for future purpose.

Fresh and dry weight

The leaf and root part of the plants were immediately separated cautiously after harvesting and was washed with distilled water and dried on absorbing paper in order to eradicate any additional salt surface contamination. The fresh weights (FW) of the plants were calculated prior to oven drying at 70°C for 72 hrs and their dry weights were recorded later.

Relative Water Content (RWC)

After determining the fresh weights, the samples were immersed in petri dish with distilled water for 24 hrs at 4 °C in darkness and the turgid weight (TW) was determined. The petri dishes were oven dried (70 °C for 72 h) and the dry weight (DW) was calculated. The RWC was calculated by following formula of RWC (%) = $\frac{FW - RW}{TW - DW} \times 100$ (Silveira *et al.*, 2003).

Chlorophyll content

By following the method of Arnon (1949), Green pigment contents such as chlorophyll-a, chlorophyll-b and total chlorophyll were measured by taking 200 mg of fresh leaves were homogenized in 8 ml 80% acetone with homogenizer. Then the homogenates were centrifuged at 4 °C for 20 min at 3000 rpm. The separately collected supernatants used for the analysis of pigments. With spectrometer, the samples absorbance was determined at 645 nm, 652 nm and 663 nm respectively. The following equations were used for measuring the green pigment content (Litchenthaler and Wellburn, 1983).

Total Chl: $A_{652} \times 27.8 \times 20$ /mg leaf weight

Chl-a: $(11.75 \times A_{663} - 2.35 \times A_{645}) \times 20$ /mg leaf weight

Chl-b: $(18.61 \times A_{645} - 3.96 \times A_{663}) \times 20$ /mg leaf weight

Total free aminoacids

Method of Moore and Stein (1948) was followed to estimate the total free amino acid content in the plant samples. The leaf tissues were treated with 80 percent of boiling ethanol for the purpose of obtaining extracts (5 ml extract represents 1 g tissue). About one ml of the ethanol extract was taken in a 25 ml test tube and was neutralized with 0.1 N sodium hydroxide using methyl red indicator. Additionally, one ml of ninhydrin reagent was added (800 mg stannous chloride in 500 ml citrate buffer (pH 5.0), 20 g ninhydrin in 500 ml methylcellulose, both solutions were mixed). The mixture of above content was placed in a water bath and allowed to boil for 20 minutes and later 5ml of diluting solution (distilled water and n-propanol mixed in equal volume) was added. The sample was then cooled and diluted up to 25ml with distilled water. The absorbance was measured at 570 nm in a spectrophotometer.

Lipid peroxidation

Methodology of thio-barbituric acid (TBA) reaction was tracked by following Buege and Aust (1978) for determining the level of lipid peroxidation by measuring the amount of malondialdehyde (MDA) released as a by product of lipid peroxidation in the samples. For this experiment, about 500mg of leaf material was homogenized in 5 ml of 0.1% TCA and was centrifuged at 10,000 rpm for 5 minutes. For every 1ml of aliquot, 4 ml of 20% TCA containing 0.5% thio-barbituric acid was added. The mixtures were kept at water bath at 95 °C for 30 min. At last mixture was immediately transferred to ice bath for cooling. Finally, mixture was centrifuged at 10,000 rpm for 15 minutes. The amount of MDA in a supernatant was measured at 532 nm.

Proline content

For the estimation of proline method of Bates *et al.* (1973) was followed. The leaf tissues of 500 mg were taken and homogenised with 5 ml of 3% aqueous sulphosalicylic acid and the homogenate was centrifuged for 10 min at 1000 X g. One ml of collected extract supernatant was transferred into a fresh tube and 2 ml of ninhydrin acid was mixed with extract. In addition, 2 ml of glacial acetic acid was added and heated for 18 hours

at 100 °C. Later, 4 ml of toluene was used to extract the reaction mixture. The reaction mixture was well shaken and mixed by vortexing for 20-25 seconds. From the aqueous phase toluene-chromophore assortment was separated and the absorbance was measured at 520nm in a spectrophotometer against toluene blank.

Total protein extraction

About 500 mg of leaf was homogenized with 1ml of ice-cold extraction buffer which contains 100 mM EDTA, 100 mM tris (pH 8.0), 2% of β mercaptoethanol v/v, 50 mM ascorbic acid, 30% sucrose w/v, 50 mM borax, 1% pvpp w/v and 1% triton x-100 v/v. The above homogenate was vortexed at room temperature for 5 min. Further, two volumes of saturated phenol were added followed by gentle vortexing mixture was centrifuged for 10 min at 15000 rpm at 4 °C. Then the upper phase was transferred carefully into the fresh centrifuge tube and equal volume of ice-cold extraction buffer was added and centrifuged for 15000 rpm for 15 min at 4 °C. Again, the aqueous phase was transferred to fresh centrifuge tube and five volumes of methanol saturated with ammonium acetate was added and incubated at -20 °C for 6 hours for protein precipitation. After incubation the mixture was centrifuged, pellet was suspended and rinsed with ice cold methanol followed by ice cold acetone twice and spun down at 15000 rpm for 5 min at 4 °C. After each washing supernatant was decanted and Protein pellets were air dried. Finally, pellet retrieved with lysis buffer was maintained at -80 °C (Akshaya *et al.*, 2018).

Protein estimation

Total protein concentration of the sample was determined by referring Bradford (1976). Bovine Serum Albumin was used (1 $\mu\text{g}/\mu\text{l}$) as a standard and the absorbance was recorded at 562 nm.

Estimation of superoxide dismutase activity (SOD)

The SOD activity was determined by taking 550 μl of 50 mM phosphate buffer (pH 7.4), 38 μL of 20 mM L-methionine, 20 μl of Triton X-100, and 38 μl of 10 mM hydroxylamine hydrochloride, 50 μl of EDTA and 50 μl sample. This reaction mixture was incubated at 37 °C for 5 min and 50 μl of riboflavin was added into the mixture. Followed by the addition of riboflavin again it was incubated for 10 min under fluorescent lamp. After incubation mixture was finally added with 500 μl of Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% Naphthalene diamine dihydrochloride). The formation of colour was recorded by taking absorbance at 543 nm. One unit of the enzyme activity was defined as the amount of Superoxide dismutase required for inhibiting 50% of nitrate formation $\text{min}^{-1} \text{mg}^{-1}$ protein. The enzyme activities were measured twice for each sample replicate (Das *et al.*, 2000).

Estimation of catalase activity (CAT)

The activity of catalase was measured by following the procedure of Sinha (1972). About 0.9 ml of 0.01M potassium phosphate buffer (pH 7.0), 0.1ml of enzyme extract and 0.4 ml of 0.2M H_2O_2 were taken in the reaction tube. Enzyme-substrate reaction was stopped at 0 and after 60 seconds by the addition of 2 ml of dichromate acetic acid mixture. After addition of inhibitor solution tubes were immediately kept in boiling water bath for 10min and then cooled. Colour development in the reaction mixture was read at 530 nm. The catalase activity of the reaction mixture was calculated using the decreased in absorbance and molar extinction coefficient $\epsilon = 0.0436 \mu\text{M}^{-1} \text{cm}^{-1}$ and expressed as μM of H_2O_2 utilized $\text{g}^{-1} \text{min}^{-1} \text{mg}^{-1}$ protein. The enzyme activities were measured twice for each sample replicate.

Estimation of peroxidase activity (POX)

The enzyme activity of POX was carried out according to the method described by Neto *et al.* (2006). The reaction mixture for peroxidase assay consisted of 0.245 ml of 1M phosphate buffer (pH 6.5), 0.25 ml of 0.1M pyrogallol, 0.05 ml of 100 mM hydrogen peroxide, 0.01ml of enzyme extract and 1.36 ml of distilled water. The reaction mixture was kept for incubation at 25 °C for 5min and further reaction was stopped by

addition of 0.5 ml of 10% (v/v) sulphuric acid. Absorbance was measured at 420 nm using UV-VIS spectrophotometer. Reaction mixture without enzyme extract served as blank. Enzyme activity of POX was calculated using molar extinction coefficient $\epsilon = 2.47 \times 10^{-3} \mu\text{M}^{-1} \text{cm}^{-1}$ and expressed as $\mu\text{M min}^{-1} \text{mg}^{-1}$ protein. The enzyme activities were measured twice for each sample replicate.

Estimation of glutathione peroxidase activity (GPX)

The activity of Glutathione peroxidase was assayed by following the procedure of Rotruck *et al.* (1973). This experiment was initiated by taking 100 μl of 40 mM phosphate buffer (pH 7.4), 50 μl of 4 mM EDTA, 25 μl of 10 mM sodium azide, 50 μl of 4 mM reduced glutathione, 25 μl of 2.5 mM H_2O_2 and 50 μl of sample. This mixture was incubated at 37 °C for 10 min. Then 125 μl of 10% Trichloroacetic acid was added and incubated for 15 mins. After incubation 750 μl of 30 mM disodium hydrogen phosphate and 125 μl of Ellman's reagent was added. Finally, the colour developed was read at 412 nm and the enzyme activity was expressed in terms of μg of glutathione utilized $\text{min}^{-1} \text{mg}^{-1}$ protein. The enzyme activities were measured twice for each sample replicate.

Results and Discussion

Effects on fresh and dry weight

The fresh and dry weight of leaf's were measured after pre exposing and unimposing the plants to 80 mM SA for 3days (Figure 2a and Figure 2b). Fresh and dry weight of leaf was significantly affected by high salinity stress (100 mM NaCl) by causing 42% and 37% decrease in fresh and dry weight of leaf. Whereas, salicylic acid pre-treatment diminishes salinity affects by increasing 42% and 25% of leaf fresh and dry weight. Neelam and Rahul, 2012 reported that salicylic acid applied *Lycopersicon esculentum* showed increased dry weight under salinity stress. Furthermore, Singh and Usha (2003) suggested that increase in dry mass of water stressed plants in response to salicylic acid related to the induction of antioxidant responses that protect the plant from damage.

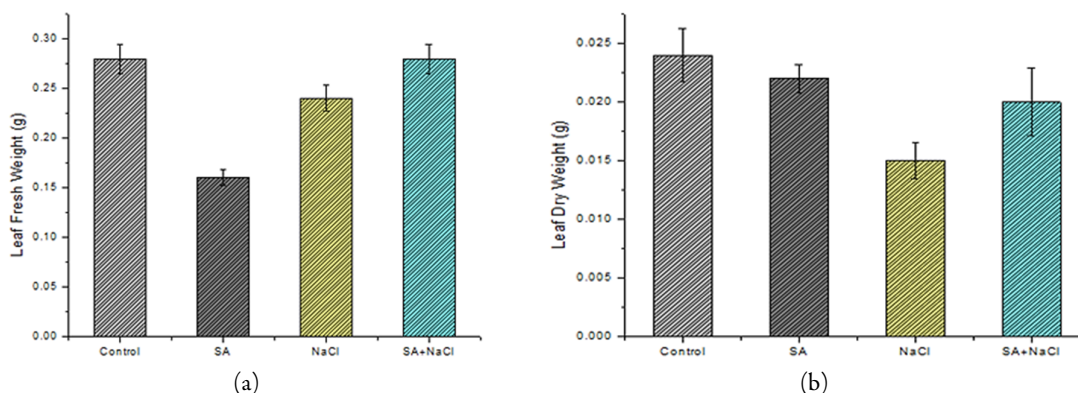


Figure 2. Leaf fresh weight (a) Leaf fresh weight of both individual and combined effect of salinity and SA. X axis-Treatment group; Y axis- fresh weight of leaves in gram. (b) Leaf dry weight of both individual and combined effect of salinity and SA. X axis-Treatment group; Y axis- Dry weight of leaves in gram. Values are stated as mean \pm SD, n=3 in each group.

Effects on relative water content

Relative Water Content (RWC) is a key pointer of the degree of cell and tissue hydration. In the present study leaves of *Solanum lycopersicum* were showed highest RWC content in SA (96%) followed by SA+NaCl treated samples (91%) whereas under salinity stress plant leaf showed less RWC content of 79%. Similar to

present study, Tari *et al.* (2002) stated that exogenous SA treatment enhances RWC in leaves of tomato under salt stress. Hence, SA application may help in maintaining water status of plants by enhancing RWC to a marked level under salt stress represented in Figure 3.

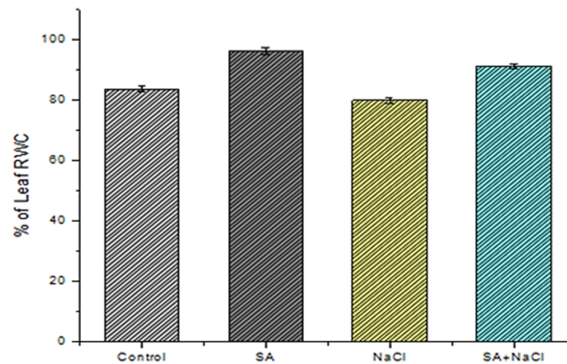


Figure 3. Relative Water Content (RWC): Relative water content of both individual and combined effect of salinity and SA. X axis-Treatment group; Y axis- Percentage of leaf RWC

Values are expressed as mean \pm SD, n=3 in each group.

Effects on chlorophyll content

The concentration of chlorophyll pigment 'a' was pointedly more in control (26.21 ± 1.54 mg/FW) sample followed by SA (23.44 ± 1.27 mg/FW) and SA+NaCl (22.51 ± 0.6 mg/FW). It was considerably found in low concentration in NaCl (16.51 ± 0.4 mg/FW) compared to other treatments and control. The concentration of chlorophyll 'b' was found to be more in control (55.52 ± 0.74 mg/FW) followed by SA (42.21 ± 1.01 mg/FW). While treatment with NaCl resulted in low concentration of chlorophyll 'b' such as 23.73 ± 1.42 mg/FW. There was a significant increase was observed in SA pre-treatment (42.42 ± 1.59 mg/FW). Total chlorophyll content found to be more in control followed by SA, NaCl and SA+NaCl as 82.51 ± 1.3 mg/FW, 64.53 ± 0.8 mg/FW, 39.89 ± 0.15 mg/FW 63.36 ± 0.673 mg/FW, respectively (Figure 4). Maryam *et al.* (2014) reported that pre-treatment of *Lycopersicon esculentum* with 1mM of salicylic acid following salinity stress caused significant increases in chlorophyll content. This current study results suggest SA treatment reduced the stress-induced loss in chlorophyll content.

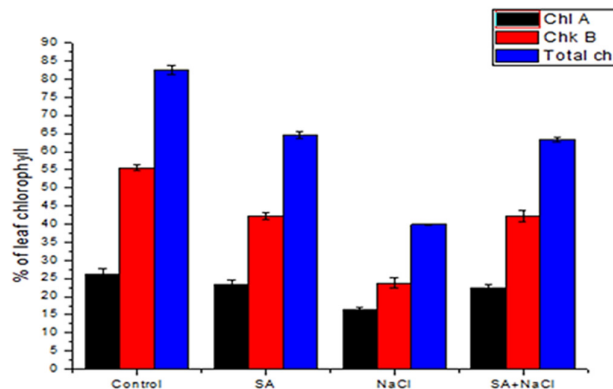


Figure 4. Chlorophyll content: individual and combined effect of salinity and SA on chlorophyll content.

X axis-Treatment group; Y axis- Percentage of leaf chlorophyll pigments

Values are expressed as mean \pm SD, n=3 in each group.

Effects on total free amino acid

Accumulation of free amino acids is considered as an imperative factor for the majority of changes that occurs in osmotic potential and due to the hydrolysis of proteins. When compared to other treatment marked increase of total free amino acid level was reported in NaCl treated plants as 88 $\mu\text{g/ml}$ FW (Figure 5) and negligible amount of free amino acid accumulation was observed in salicylic acid pre-treatment. Thus, this current result indicates that protein degradation due to salt stress might be overcome by pre-treatment of plants with salicylic acid.

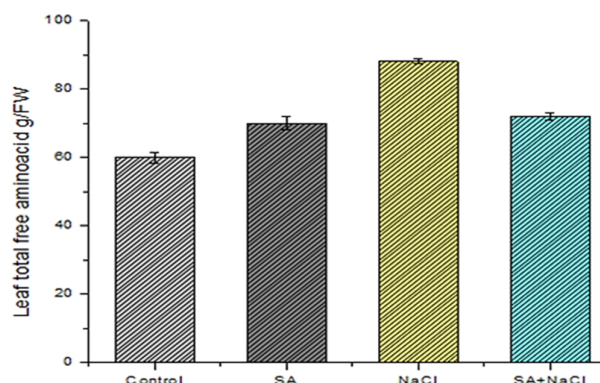


Figure 5. Leaf total free amino acid content: individual and combined effect of salinity and SA on leaf total free amino acid content. X axis -treatment group; Y axis - concentration of free amino acid (gram/ fresh weight)

Values are expressed as mean \pm SD, n=3 in each group.

Effects on lipid peroxidation

The malondialdehyde (MDA) production is considered as an indicator for membrane lipid peroxidation as a result of various stresses in plant membranes. A marked increase of MDA content was recorded in plants under salt stress. The leaves of *Solanum lycopersicum* treated with NaCl showed highest MDA content of 58 nmol/ml which showed 31% and 46% of increases than control and SA treated plants, respectively. Whereas, salicylic acid pre-treated plants exhibited 61% reduction of MDA content (Figure 6). It has been suggested that SA application declined the rate of H_2O_2 production caused by salt stress in tomato plants.

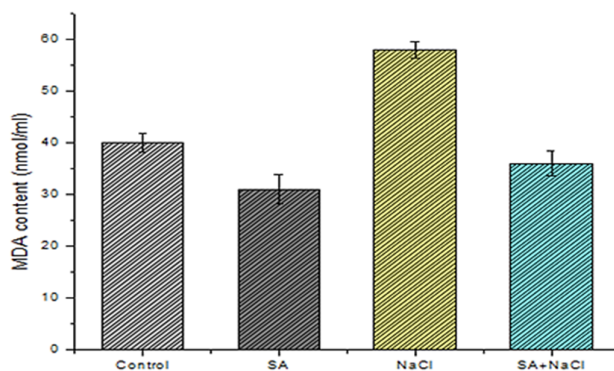


Figure 6. Leaf lipid peroxidation (MDA content): individual and combined effect of salinity and SA on leaf lipid peroxidation. X axis -treatment group; Y axis- concentration of MDA (nmol/ml)

Values are expressed as mean \pm SD, n=3 in each group.

Effects on proline content

Accumulation of proline level increased significantly in salt stressed plants compared to SA pre-treated and control plants (Figure 7). Kadioglu *et al.* (2011) reported that salicylic acid treatment substantively increases the proline content of tomato plant under salt stress. Generally, accumulation of proline and its concentration has been shown to be more in plants affected by salinity stress to influences protein solvation and preserves the quaternary structure of complex proteins, maintains membrane integrity and reduces oxidation of lipid membranes or photo-inhibition (Demiral and Turkan, 2006).

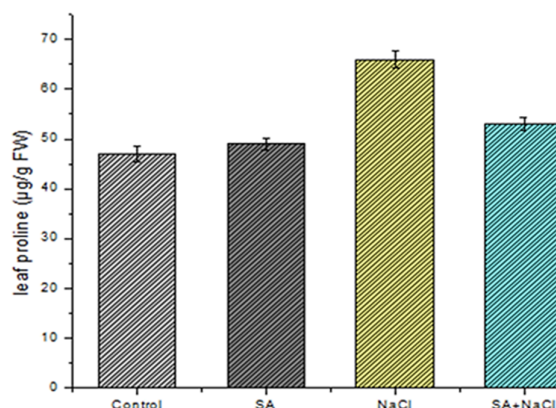


Figure 7. Leaf proline content: individual and combined effect of salinity and SA on leaf proline content.

X axis -treatment group; Y axis - concentration of leaf proline (µg/FW)

Values are expressed as mean \pm SD, n=3 in each group.

Effects on antioxidant enzymes

The salicylic acid pre-treatments effects on tomato plants under salinity were monitored by assaying the activity of free radical scavenging enzymes such as Catalase, Glutathione Peroxidase, Superoxide Dismutase and Peroxidase. As a result of phytohormone pre-treatment changes in enzyme activity on plants under salt stress were shown in the Table 1. Interaction of hormone and salinity on antioxidant enzymes were exhibited significant differences.

Table 1. Individual and combined effect of salinity and SA on antioxidant enzymes

Group	Control	SA	NaCl	SA+NaCl
SOD	0.23 \pm 0.03	0.27 \pm 0.02	0.15 \pm 0.01	0.36 \pm 0.02
CAT	0.09 \pm 0.002	0.07 \pm 0.003	0.04 \pm 0.002	0.08 \pm 0.002
POX	0.038 \pm 0.002	0.058 \pm 0.006	0.07 \pm 0.005	0.097 \pm 0.003
GPX	0.131 \pm 0.003	0.162 \pm 0.007	0.15 \pm 0.007	0.17 \pm 0.01

SOD

Activity of SOD in the leaf tissue of SA, SA+NaCl treated tomato plants were shown in Figure 8. The tomato leaf tissues treated with NaCl showed decreased SOD activity than SA treated and control plants. The salicylic acid pre-treated plants exhibited more SOD activity than NaCl treated plants. The above finding indicates that salicylic pre-treatment protects the plants from damaging effects salinity stress. Hayat *et al.* (2010) admitted that SA enhanced the SOD activity in tomato plants under salinity stress. Similar enhanced SOD activity by SA treatment has been recorded in tomato grown under saline induced oxidative stress (Krantev *et al.*, 2008).

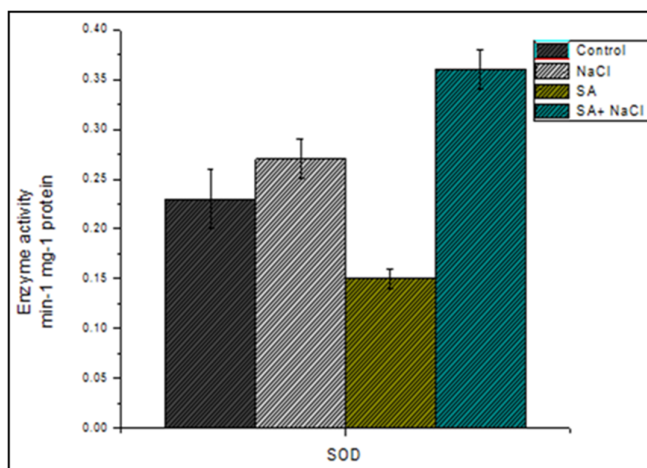


Figure 8. Superoxide dismutase activity: individual and combined effect of salinity and SA on leaf superoxide dismutase. X axis -treatment group; Y axis - enzyme activity ($\text{min}^{-1}\text{mg}^{-1}$ protein) Values are expressed as mean \pm SD, n=3 in each group.

CAT

The stimulation effect of catalase enzyme by the interaction between salicylic acid pre-treatment and salinity were shown in Figure 9. The highest value of catalase activity was recorded at control followed by salicylic acid treated leaves. The NaCl treated plants exhibited least catalase activity. Whereas, pre exposing the plant with salicylic acid before salinity resulted in increased CAT activity as compared with unexposed plants.

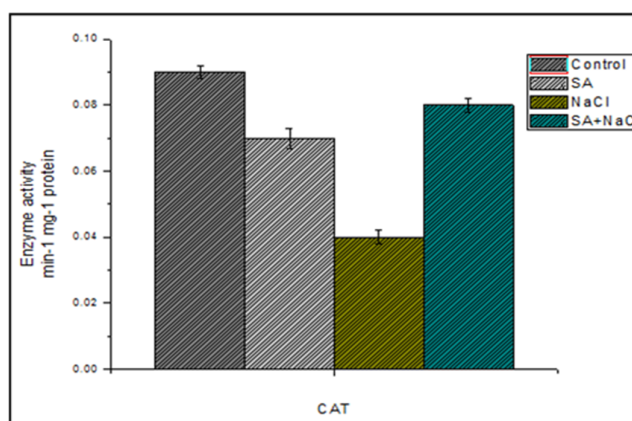


Figure 9. Catalase activity: individual and combined effect of salinity and SA on catalase activity. X axis - treatment group; Y axis - enzyme activity ($\text{min}^{-1}\text{mg}^{-1}$ protein) Values are expressed as mean \pm SD, n=3 in each group.

POX

Changes in the activity of peroxidase enzyme as the result of NaCl and SA+NaCl treatment on tomato plant were shown in Figure 10. Plants which are pre-treated with SA showed increased POX activity than the NaCl, SA and control. This study result indicates that plants treated with SA might be reducing the level oxidative damage caused by high salinity stress.

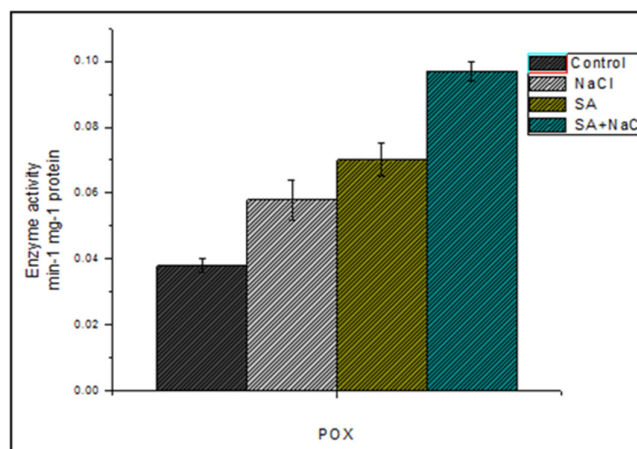


Figure 10. Peroxidase activity: individual and combined effect of salinity and SA on peroxidase activity. X axis -treatment group; Y axis - enzyme activity ($\text{min}^{-1}\text{mg}^{-1}$ protein)
Values are expressed as mean \pm SD, n=3 in each group.

GPX

Glutathione peroxidase enzyme level in the leaf tissue of Control, SA, NaCl and SA+NaCl treated tomato plants were shown in Figure11. There was a significant change in the enhancement of GPX enzyme was observed between salt stressed and SA pre-treated treated plants.

Overall salt induced negative effects were significantly diminished by enhanced GPX activity by salicylic acid pre-treatment.

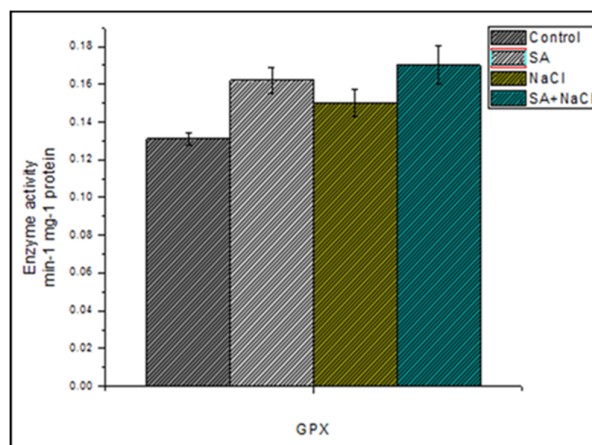


Figure 11. Glutathione peroxidase activity: individual and combined effect of salinity and SA on glutathione peroxidase activity. X axis - treatment group; Y axis- enzyme activity ($\text{min}^{-1}\text{mg}^{-1}$ protein)
Values are expressed as mean \pm SD, n=3 in each group.

Conclusions

Overall, this current study shows that pre-treating the Tomato (*Solanum lycopersicum* L) with exogenous pre-treatment of 80mM salicylic acid substantially influence considerable protection against salinity stress through balancing relative water content, free aminoacid and MDA content as well as proline and antioxidant enzymes. Salicylic acid's ability to protect plants against salt stress may be due to its effects on Na⁺ and K⁺ uptake, as well as an increase in the amount of chlorophyll, which is required for several metabolic processes. As a result, exogenously applied salicylic acid reduced external osmotic potential and ion toxicity, suggesting that it may be useful in reducing the negative consequences of salt stress. The findings show that the salt tolerance level of the plants was increased by pre-treatment of exogenous salicylic acid.

Authors' Contributions

Study conception, design and acquisition of data done by GA; Analysis and interpretation of data by AVS; Drafting of manuscript by GA, AVS, VB; Critical revision done by VB.

All authors read and approved the final manuscript.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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