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ORIGINAL ARTICLE

Effect of Short Term NaCl Stress on Cultivars of *S. lycopersicum*: A Comparative Biochemical Approach

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Tomato is a crop plant with high fruit nutritive value and other useful properties. The cultivation of this species is dependent on many environmental factors, e.g. temperature, salinity, nutrients etc, affecting the yield and reproductive potential of the plant. Salinity in soil or water is of increasing importance to agriculture because it causes stress to crop plants. Plants exposed to an excess amount of salts such as NaCl undergo osmotic stress, water deficit and ionic imbalances and can increase production of reactive oxygen species(ROS). Higher plants possess very efficient enzymatic and non-enzymatic antioxidative defense mechanisms that allow the scavenging of ROS and protection of cellular components from oxidative damage.

Studies were conducted to investigate the effect of short term salinity stress on some physiological alterations in three tomato cultivars Pusa Ruby(PR), Punjab Keshari (PK) and Ailsa Craig(AC). Some biochemical parameters (anthocyanin and carotenoeid content, polyamines, proline, cysteine, peroxidase and malondialdehyde) were set and applied at two month old stage of tomato plants. Three tomato cultivars were grown in 0.5xMS for 2 months and at this stage, they were treated with 0 and 200mM NaCl for a short period of six hours in hydroponic conditions. The genotypes exhibited different responses in terms of different osmoprotectant, antioxidant, and pigment level. The relationships among the salinity and accumulation of these compounds in leaf were then determined.

It was concluded that, tomato cultivars under study responded differently showing their sensitivity or tolerance to salinity stress. Among three cultivars PK appeared to be more tolerant genotype than the other two cultivars PR and AC. PK could rapidly evolve physiological and antioxidant mechanisms to adapt to salt and manage the oxidative stress. The research was conducted in a completely randomized design with three replications.

Key words: Antioxidant, Biochemical, Genotypes, NaCl, Tomato, Salt stress, Salt tolerance

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Salt stress is one of the main environmental factors interfering with the growth, metabolism of plants. High concentrations of salt in soils account for large decreases in the yield of a wide variety of crops all over the world (Tester & Davenport, 2003). Yield reductions induced by salinity may be due to both the osmotic stress that results from relatively high solute concentrations in the root growing medium, and specific toxicity due to the accumulation of high concentrations of Na and Cl in the plant, which provokes a wide variety of physiological and biochemical alterations that

inhibit plant growth and production (Maggio *et al.*, 2004; Munns, 2005). Reduced photosynthesis, growth, and development are found in plants growing under high salinity. All of these are associated with ionic/osmotic effects, nutritional imbalance, or oxidative stress (Ashraf & Foolad, 2007; Ahmad *et al.*, 2008; Ashraf, 2009; Lee *et al.*, 2008; Munns & Tester, 2008; Gill & Tuteja, 2010). It is a prerequisite to understand how plants respond and adapt to this stress to prevent crop yield losses. Tomato is one of the most important horticultural crops in the world, and tomato plant growth was shown to be moderately sensitive or moderately tolerant to salinity depending on cultivar or growth stage (Santa-Cruz *et al*, 2002; Fernandez-Garcia *et al.*, 2004; Estan *et al.*, 2005). Study on the physiological responses of tomato plants to salt stress could give novel insight into the planting and modifying of tomato cultivars.

Plants require biochemical and molecular strategies to survive the problem of salinity. Biochemical strategies used to enhance salt tolerance in plants include the control of ion transfer from roots to leaves. Under conditions of stress, plants adjust osmotically their cellular content by synthesizing amino acids such as proline (Ashraf, 2004). Proline oxidase is the main regulatory enzyme responsible for the accumulation of osmolytes; it converts proline into glutamate. The active accumulation of compatible solutes like polyamines and sugars also appears to be an effective mechanism of stress tolerance (Rosa-Ibarra, 1995). Plants have improved complex mechanisms for adaptation to osmotic and ionic stress caused by high salinity. Protection against various environmental stresses has been well documented (Khan and Singh, 2008; Gill *et al.*, 2011). Still understanding of these mechanisms remains incomplete (Fu *et al.*, 2013; Talei *et al.*, 2013). Some of these strategies of salt tolerance involve altering their germination and growth rates (Debez *et al.*, 2004), accumulating osmolytes, and increasing the anti-oxidant system's activity to protect plants against the oxidative stress induced by reactive oxygen species (ROS) etc. (Ashraf & Foolad, 2007; Fu *et al.*, 2013; Talei *et al.*, 2013). Enzymatic antioxidants (such as superoxide dismutase, SOD; catalase, CAT; ascorbate peroxidase, APX; glutathione reductase, GR) and non-enzymatic antioxidants (glutathione, GSH; and carotenoids) are the main components of antioxidants defence system (Mittler *et al.*, 2004). ROS include hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH·) that can directly attack membrane lipids, proteins, DNA and macromolecules (Bailey-Serres & Mittler, 2006). Furthermore, apart from the many harmful effects on plant growth and metabolism, if generated at low amounts, ROS have a positive role in signalling events (Van Breusegem *et al.*, 2008; Miller *et al.*, 2010). There is also evidence that ROS are essential signals in mediating stomatal closure through the regulatory action of the plant phytohormones such as abscisic acid (ABA), which is defined as a stress hormone (Huang *et al.*, 2008). Accumulation of ABA, a phytohormone, critical for plant growth and development, is induced by environmental stresses and mediates many stress responses that help plants to survive. The effects of ABA on ROS, antioxidant defence system and oxidative damage, calcium signalling and H_2O_2 accumulation have been examined in several studies. These studies showed that ABA increased the level of ROS and activity of antioxidant enzymes against oxidative stress (Jiang & Zhang, 2001, 2002, 2003; Xiong *et al.*, 2006; Hu *et al.*, 2008; Kumar *et al.*, 2008) and plays a pivotal

role in ABA-induced antioxidant defence mechanism.

While determining the role of various antioxidants in the salt tolerance of tomato, Mittova (2002) found that higher salt tolerance of wild tomato (*Lycopersicon pennellii*) as compared to cultivated tomato (*Lycopersicon esculentum*) was correlated with increased activities of SOD, APX, and POD (guiacol peroxidase). During the course of plant growth, the form and functions of various organs undergo significant change, and the ability of the plant to react to salinity stress depends on those genes that are expressed at the stage of development during which the stress is imposed (Epstein, 1987). Osmotic adjustment in plants subjected to salt stress can occur by the accumulation of high concentrations of either inorganic ions or low molecular weight organic solutes. Although both of these play a crucial role in higher plants grown under saline conditions, their relative contribution varies among species, among cultivars and even between different compartments within the same plant (Ashraf, 1994; Greenway, 1980). Tomato is a crop plant with high fruit nutritive value and other useful properties. The cultivation of this species is dependent on many environmental factors, e.g. temperature, salinity, nutrients etc, affecting the yield and reproductive potential of the plant.

The aim of this study was to compare the responses of 3 tomato cultivars to salinity. We cultivated plants in vitro and then in vivo. The plant growth parameters, pigments, antioxidant enzyme activities, lipid peroxidation activity levels, polyamines and proline accumulation were determined. The salt tolerances of the 3 varieties were compared according to the results of biochemical analyses. Such a comparison of different cultivars will make easy the evaluation of their relative performance and characterization of mechanisms which contribute to their salt tolerance.

MATERIALS AND METHODS

Plant material and growth conditions

Seeds of local cultivars like *Solanum lycopersicum* cv. Pusa Ruby, cv. Punjab Keshari were sold from Amtala Seed Centre, Amtala, West Bengal, India and used for our study. Seeds of *Solanum lycopersicum* cv. Ailsa Craig were obtained from Dr. D. Grierson (Nottingham University, UK).

Plants were grown in 0.25x Murashige & Skoog liquid basal medium (Sigma) and the experiments were done by treating them with or without salt (NaCl solution) for different time periods as described later. Seeds of different cultivars like Pusa Ruby (PR), Punjab Keshari (PK) and Ailsa Craig (AC) were surface sterilized in 0.1% $HgCl_2$ for 10 min, and then rinsed with water. Selected seeds were then germinated aseptically on petriplates containing moistened filter paper. The seeds germinated after 3 days. The obtained seedlings were transferred to glass bottles containing 0.25 x MS liquid media in aseptic condition for 15 days in tissue culture room (16 hrs dark and 8 hrs light period, 25-26°C). After 15 days, a set of three tomato cultivars were transferred to bottle containing 50ml of 0.25 x MS for in vitro growth and another set of three cultivars were transferred to tray containing enough fresh 0.25 x MS (Sigma) liquid media. The plants in bottle were grown for another 24 days supplemented with different level of NaCl (0, 50, 100, 150 and 200mM). Plants in tray were grown in vivo for another 45 days and after this time they were treated with or without 200 mM NaCl for 6 hrs. The media with or without supplements were refreshed weekly. At the end of the treatments,

plants were washed thoroughly with sterile de-ionized water and leaves were harvested and stored in -80 ˚C for investigating some biochemical indices.

Plant growth and estimation of chlorophyll content

We monitored the growth pattern of three cultivars carried out both in vitro and in vivo experiments with tomato plants. 15 days old plantlets were transferred and treated with 0.25xMS alone or with 0.25xMS supplemented with 0, 50, 100, 150 and 200mM NaCl in culture room condition for another 24 days. For chlorophyll estimation leaves from all these NaCl treated and untreated plants in bottles were taken. Chlorophyll content was estimated by following the method of Arnon (1949).

Biochemical methods:

Analysis of three tomato cultivars (PR, PK and AC) through biochemical investigations

Estimation of Anthocyanin Content (Reddy *et al.*, 1995; Liu *et al.*, 2002; Harborne, 2000). Leaf samples (3 gm) were properly crushed with liquid nitrogen and they were extracted with acidified methanol (1% v/v). Extracts were then kept at 4°C for continuous shaking for 24 hours. Supernatants were taken from each after 10 mins of high speed centrifugation and their absorbance were observed at 525 nm and the anthocyanin concentrations were calculated in mg/gm of fresh weight using the millimolar extinction coefficient of 31.6.

Estimation of β-carotene content: (Nagata and Yamashita, 1992, with modification). Leaf samples (3 gm) were ground in liquid nitrogen and extracted with 10 ml of acetone: hexane mixture (4:6), and filtered through Whatman No.4 filter paper. The absorbance of the filtrate was measured at 453, 505, 663 nm in the Shimadzu UV-Vis spectrophotometer. β-carotene was calculated according to the following equation.

β-Carotene (mg/100ml) = 0.216 A_{663} – 0.304 A_{505} + 0.452 A_{453}

The assays were carried out in triplicate. The β-carotene content was cross checked from the standard curve prepared by the purified β-carotene from Sigma.

Determination of Lycopene Content: (Concepcion *et al.*, 1999). Leaf samples (3 gm) were ground in liquid nitrogen and extracted with 10 ml of acetone: hexane (2:1) solution. The suspension was centrifuged at 5,000g for 10 min in 50 ml corex tubes. The upper hexane layer was removed and 1:10 dilution of this extract was determined in the Shimadzu UV-Vis spectrophotometer ($A_{453,}$ $A_{505,}$ $A_{663)}$. The amount of lycopene was calculated from the standard curve prepared by the supplied lycopene from Sigma. Lycopene was calculated according to the following equation.

Lycopene (mg/100ml) = -0.0458 A_{663} + 0.372 A_{505} – 0.0806 A_{453}

Extraction of Polyamines: (Reggiani *et al.*, 1989). 1 gm of leaf samples were extracted with 10% (w/v) cold perchloric acid, PCA (300mg/ml) in a mortar and pestle for extraction of PCA soluble polyamine. The homogenate was placed in ice for 30 min and then centrifuged at 15,000 rpm at 4 ºC for 20 min in cold centrifuge. The 0.1 ml supernatant was dansylated according to the method of Reggiani *et al.* 1989. To the 0.1 ml supernatant 0.1 ml of saturated sodium carbonate and 0.2 ml of dansyl chloride (10 mg/ml acetone) were added and stored overnight in the dark at 25ºC. Next day, the dansylated amines were extracted in 0.2 ml toluene by vortex mixing. When

the two phases were well separated then the supernatant were collected and 100 µl was loaded on an activated (80°C for 1hr) HPTLC plate (E-merck, F.R.G) of silica gel with concentration zone using a microsyringe. The plates were run with cyclohexane: ethyl acetate (3:2, v/v) as a solvent in a glass chamber in a saturated atmosphere. After complete upward running of solvent through the TLC plate, the plate was dried at room temperature. The spots were marked under UV lamp with reference to the standard used (as standardized in our lab). Each spot was scraped out in a tube and mixed with 4 ml acetone by vortex mixing. A Fluorescence Spectrofluorometer (Perkin-Elmer, MPF 44B) quantified the putrescine, spermidine and spermine at excitation and emission wavelength of 360 and 506 nm, respectively.

H_2O_2 Content: (Velikova *et al.,* 2000). 1 gm of leaf samples were homogenized in an ice bath with 0.1% trichloroacetic acid and centrifuged at 12,000 x g for 15 mins. Then 0.5ml of each supernatant was added to 0.5 ml of 10mM phosphate buffer, pH 7.0 and 1 ml of 1M potassium iodide (KI). The absorbances of the supernatants were measured at 390 nm. The content of H_2O_2 was determined using the standard curve.

Lipid Peroxidation Assay: (Fu and Huang, 2001). Malondialdehyde (MDA) is the end product of lipid peroxidation. So for the assay of malondialdehyde 1 gm of each leaf samples were properly homogenized with 50mM sodium phosphate buffer pH 7.5 and then centrifuged at 20,000 x g for 25 mins at 4°C. 4 ml of 20% trichloroacetic acid containing 0.5% thiobarbituric acid was added to 1 ml aliquot of each supernatant. The mixtures were heated at 95°C for 30 mins, quickly cooled on ice and centrifuged for 10 mins. The absorbances of the supernatants were read at A_{532} and the value of non specific absorbance at A_{600} was subtracted from A_{532} reading. The MDA concentration of each case was calculated using the MDA extinction coefficient 155mM $^{-1}$cm^{-1} (Fu and Huang, 2001).

Estimation of cysteine content. The cysteine content was determined according to Gaitonde (1967) method. 0.5gm of control and salt treated plant materials were homogenized in 3ml of 10% PCA (perchloric acid), and centrifuged at 10,000 rpm for 10 mins at 4°C. 0.5ml of supernatant was mixed with 0.5ml of ninhydrin and 50 µl of DTT at room temperature and kept at incubation for 30 mins. The samples were all heated in boiling water bath for 10 mins and the reactions were terminated by placing the tubes in ice bath. Then 1ml of 95% ethanol was added, vortexed, and absorbance was taken at 560 nm against blank of PCA.

Estimation of proline content. Free proline content was determined according to the procedure of Bates *et al.* (1973). 0.5gm of control and salt treated leaf materials were homogenized in 10ml of 3% aqueous sulphosalicyclic acid. The homogenate was filtered through whatman No.1 filter paper and then 2ml of the filterate was mixed with 2ml of glacial acetic acid and 2ml of freshly prepared acid ninhydrin (1.25g Ninhydrin warmed in 30ml glacial acetic acid and 20ml 6M phosphoric acid, with agitation until dissolved). The samples were all heated in boiling water bath for 1hr and the reactions were terminated by placing the tubes in ice bath. 4 ml of toluene was added to the reaction mixture and stirred well for 30 seconds. The toluene layer was separated and warmed to room temperature. The red color intensity was measured at 520 nm. A standard curve was prepared with pure proline, and the amount of proline in the test samples was estimated with respect to the standard curve.

RESULTS

Effect of salinity stress on growth and Chlorophyll

In the treatment of different levels (0, 50, 100, 150, 200mM) of NaCl stress, the growth of tomato plants varied and suppressed by salt stress. Overall growth showed that among the 3 tomato cultivars, PK was less affected by salinity stress followed by PR and AC so far their root/shoot growth and chlorophyll content are concerned. Figure1 shows that the different level of salt treatment reduces the chlorophyll content of the leaves in PR and AC but the chlorophyll content in PK was increased slowly (up to 20%) with the increasing salinity stress(0, 50, 100, 150, 200mM NaCl).The sharp decline in PR and AC was statistically significant though. at low concentration of NaCl, chlorophyll content increased in AC but after which it declined.

Effect of salinity stress on pigment (flavonoids) contents of the leaves (200mM NaCl treated and untreated for 6 hours)

β-Carotene concentration was determined from leaves of 2 month old control (0mM NaCl) and salt treated (200mM NaCl treated) plants and calculated on the basis of standard curve and amounts were expressed in terms of mg g^{-1} FW of leaves. In PK β-Carotene level was increased 1.5 fold after salinity stress where as in PR and AC it was more or less same or unaffected (Figure 2B).

Lycopene (an intermediate or precursor of beta-carotene) content of the leaves from control (0mM NaCl) and salt treated (200mM NaCl treated) plants was found to be similar or almost unaffected in all three cv. PR, PK and AC. Lycopene concentration was determined and calculated on the basis of standard curve and amounts were expressed in terms of mg g^{-1} FW of leaves (Figure 2C).

Anthocyanins are a large class of water soluble pigments in the flavonoid group, found in all plant tissues throughout the plant kingdom. Anthocyanin was measured in the leaves of untreated and treated (200mM NaCl) plants of PR, PK & AC. Anthocyanin concentration was determined and calculated on the basis of standard curve and expressed as mg g^{-1} FW of leaves. Only the cv. PK had higher anthocyanin contents in the stressed condition in comparison to the other two cultivars (Figure 2A), which is significant. In **t**he other two cultivars PR & AC, the anthocyanin content was almost unaffected by 6hrs of short term salinity stress.

Effect of salinity stress on Proline and Polyamines

It is well established that osmotic stress results in an increase of proline biosynthesis rate. The proline content was measured from treated and untreated leaves of PR, PK and AC. Proline concentration as μg g^{-1} FW of leaves determined by using a standard curve of L-Proline. Results represented in Figure 3A shows that, proline content increased upon salinization only in PK by more than 3 fold in comparison to its control state. In the other two cultivars no such increase in the proline level was noticed after 6 hrs of stress.

Polyamines isolated from treated and untreated leaves of PR, PK and AC measured, as shown in Figure 3C as putrescine, spermidine and spermine. Figure 3B presents the profile of total polyamine. Polyamine profile by thin layer chromatography HPTLC plates of silica gel 60 (E Merck, Germany) has been depicted in Figure 4. Equal volume from each sample was spotted on the TLC plate. Authentic PAs i.e., putrescine, spermidine and spermine (Sigma) were spotted respectively on the three extreme right lanes to compare the mobility with the polyamines isolated from the leaves. The dansylated PAs were photographed by exposing the TLC plate to UV light. Lanes 1, 3 and 5 contained

control samples of PR, PK and AC, whereas lanes 2,4, and 6 contained NaCl treated samples of PR, PK and AC. Lanes 7,8 and 9 contained standards of spermine, spermidine and putrescine. The changes in the individual polyamine like putrescine, spermidine and spermine content varied significantly. Salinity stress was found to enhance spermidine and putrescine level maximum in PK which is about 2-3 fold. Within the three cultivars AC was found to trigger lesser enhancement in the level of putrescine, spermidine and spermine after NaCl stress. The results show that the PK produced higher level of PAs (putreseine and spermidine) when treated with 200 mM NaCl for 6 hrs followed by PR and AC (Figure 3B)**.** The summation of all individual PAs, putreseine, spermidine and spermine (total polyamines) showed a 3 fold enhancement in PK, 2 fold in PR after salinity stress whereas in AC salinity stress could not induce total polyamine content similarly. This salinity stress induced enhancement in PAs was prominent as visible from the TLC Plate-photograph as shown in Figure 4.

Effect of salinity stress on the level of antioxidants

Malondialdehyde (MDA), a product of lipid peroxidation, has been considered an indicator of oxidative damage. Here we estimated the level of MDA from leaves of control (0mM NaCl) and treated (200mM NaCl) PR, PK and AC tomato plants and the data has been shown in Figure 5A**.** The exposure to NaCl stress for 6 hrs, induced very low level of MDA in PR and AC whereas this was found to be almost unaltered in PK.

Hydrogen peroxide (H_2O_2) is a well known reactive oxygen species. The Figure 5B shows the H_2O_2 level in leaves of control (0mM NaCl) and treated (200mM NaCl, 6hrs) of PR, PK & AC. The H_2O_2 content was determined and calculated on the basis of standard curve. The exposure to 200mM NaCl for 6hrs on 2month old plants showed that the H_2O_2 generation was 2 fold in PR over their control but in PK and AC, H_2O_2 remained same as in control plants.

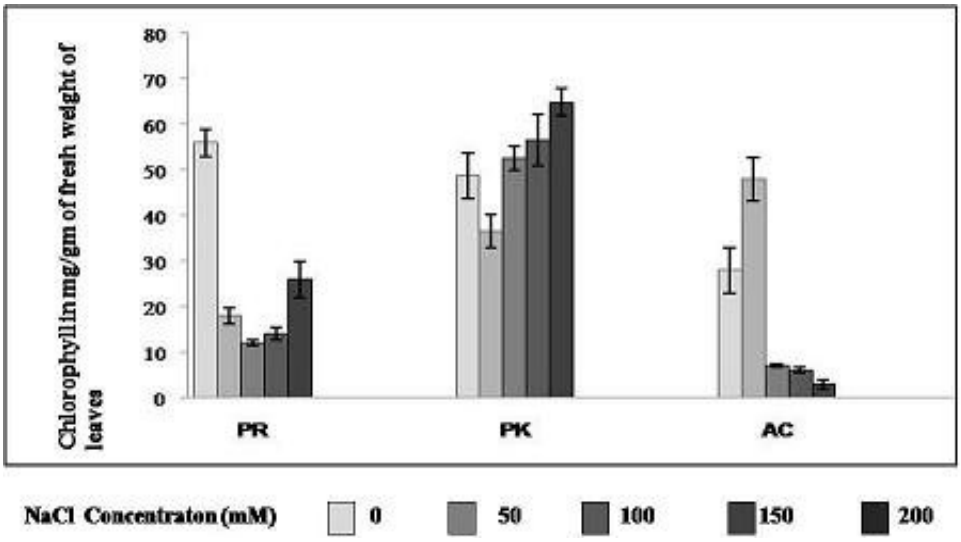

Figure 1 : Effect of salinity stress on the level of Chlorophyll in leaves of different tomato cultivars Pusa Ruby(PR), Punjab Keshari(PK) and Ailsa Craig(AC). Chlorophyll content from leaves of different cultivars of tomato grown against different concentration of NaCl (0, 50 100, 150 & 200mM), was measured according to method of Arnon, (1949). Data points and vertical bars represent means of triplicates and SE respectively.

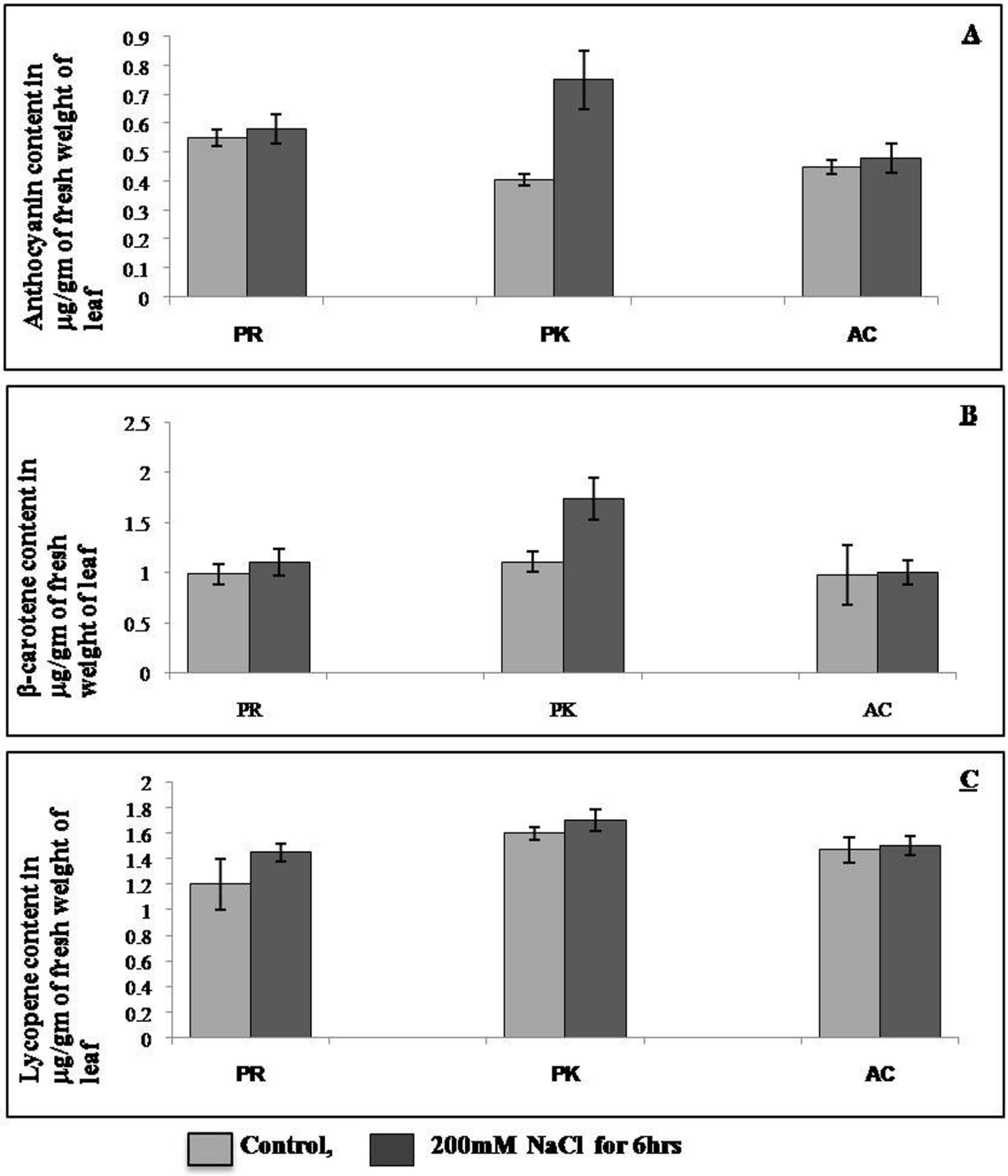

Figure 2 : Effect of salinity stress (200mM NaCl for 6hrs) on the level of Anthocyanin(A), β-carotene(B), and Lycopene(C) in leaves of 2 month old different tomato cultivars Pusa Ruby(PR), Punjab Keshari(PK) and Ailsa Craig(AC). Anthocyanin concentration was determined following the protocol of Liu *et al*., 2002, and calculated as mg g^{-1} FW of leaves. β-Carotene concentration was determined following the protocol of Nagata *et al*., (1992) with modification and calculated on the basis of standard curve and amounts were expressed as mg g^{-1} FW of leaves. Lycopene concentration was determined following the protocol of Concepcion *et al*., (1999) and calculated on the basis of standard curve and amounts were expressed as mg g^{-1} FW of leaves. Data points and vertical bars represent means of triplicates and SE, respectively.

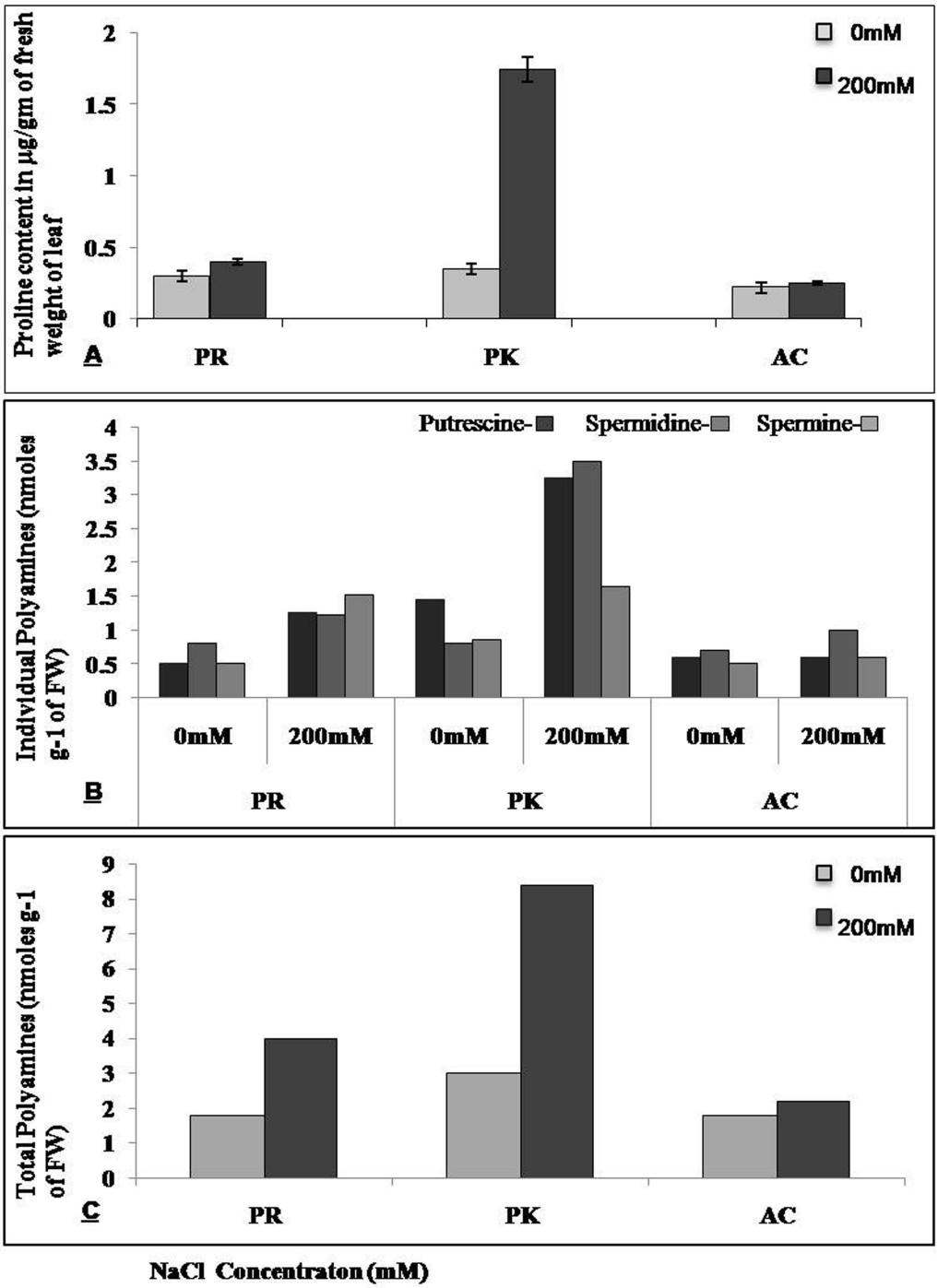

Figure 3 : Effect of salinity stress (200mM NaCl for 6hrs) on the levels of Proline(A), individual polyamine like Putrescine, Spermidine and Spermine (B), and total polyamine (C) in leaves of 2 month old different tomato cultivars Pusa Ruby(PR), Punjab Keshari(PK) and Ailsa Craig(AC) (measured from the plates as shown in the Figure 4). Proline(A) concentration was determined following the protocol of Bates *et al.*, (1973) and calculated on the basis of standard curve of L-Proline. Proline amounts were expressed in terms of µg g^{-1} FW of leaves and Data points and vertical bars represent means of triplicates and SE respectively. Polyamine levels from leaves of control and induced PR, PK and AC, represented in form of histogram. The spots corresponding to Putrescine, Spermidine and Spermine were scrapped from the TLC plate, dissolved in acetone and quantified by using UV Spectrofluorometer at excitation and emission wavelengths of 360nm and 506nm, respectively. The amounts were calculated in nmoles g^{-1} FW by comparing with standard Putrescine, Spermidine and Spermine from Sigma.

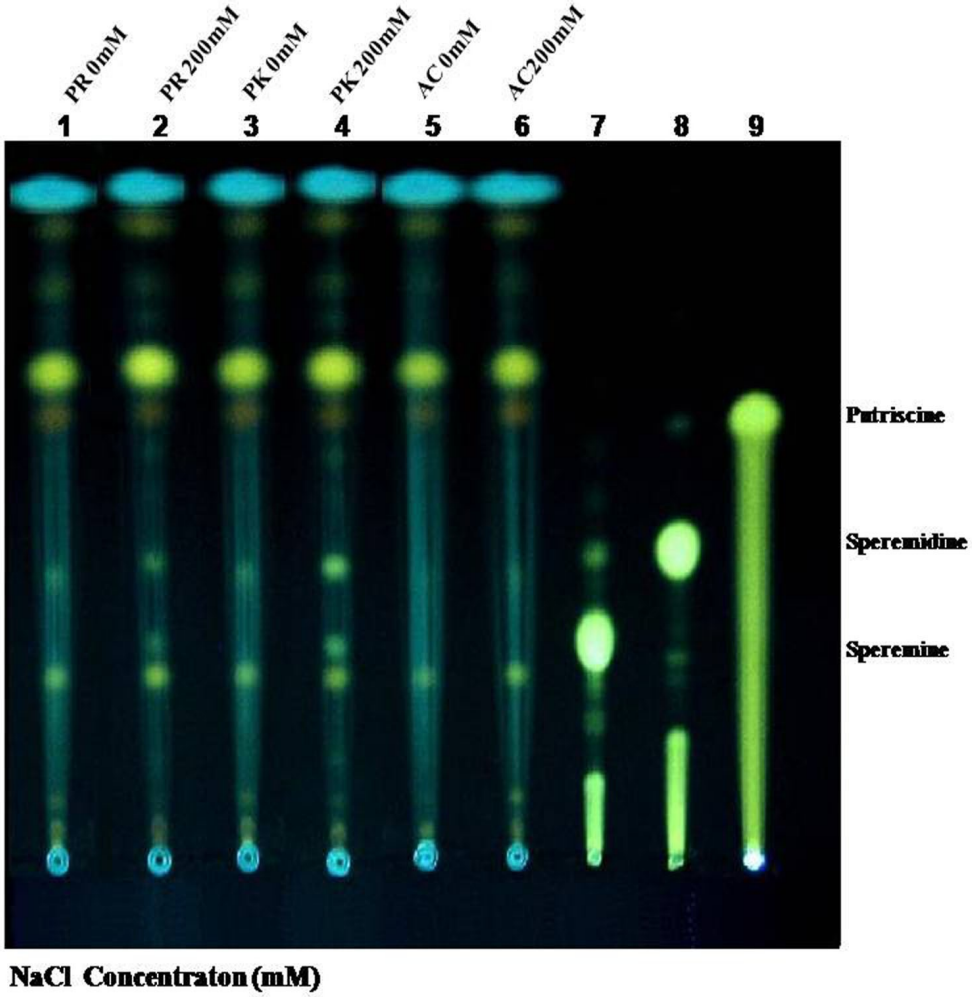

Figure 4 : Variation of polyamine profile in the leaves of control (0mM NaCl) and salt treated (200mM NaCl) leaves of tomato cultivars Pusa Ruby (PR), Punjab Keshari(PK) & Ailsa Craig (AC). The figure shows the polyamine profile through Thin Layer Chromatography on HPTLC plates of Silica Gel 60. Equal volume from each sample was spotted on the TLC plate (E Merck Germany). Authentic polyamines (Sigma, USA) i.e., Putrescine, Spermidine and Spermine were spotted on the three extreme right lanes to compare the mobility with the polyamines isolated from leaves. The dansylated polyamines were photographed by exposing the TLC plate to UV light. Lanes 1, 3 and 5 contain control samples of PR, PK and AC, whereas lanes 2, 4, and 6 contain NaCl treated samples of PR, PK and AC. Lanes 7, 8 and 9 contain standards of spermine, spermidine and putrescine.

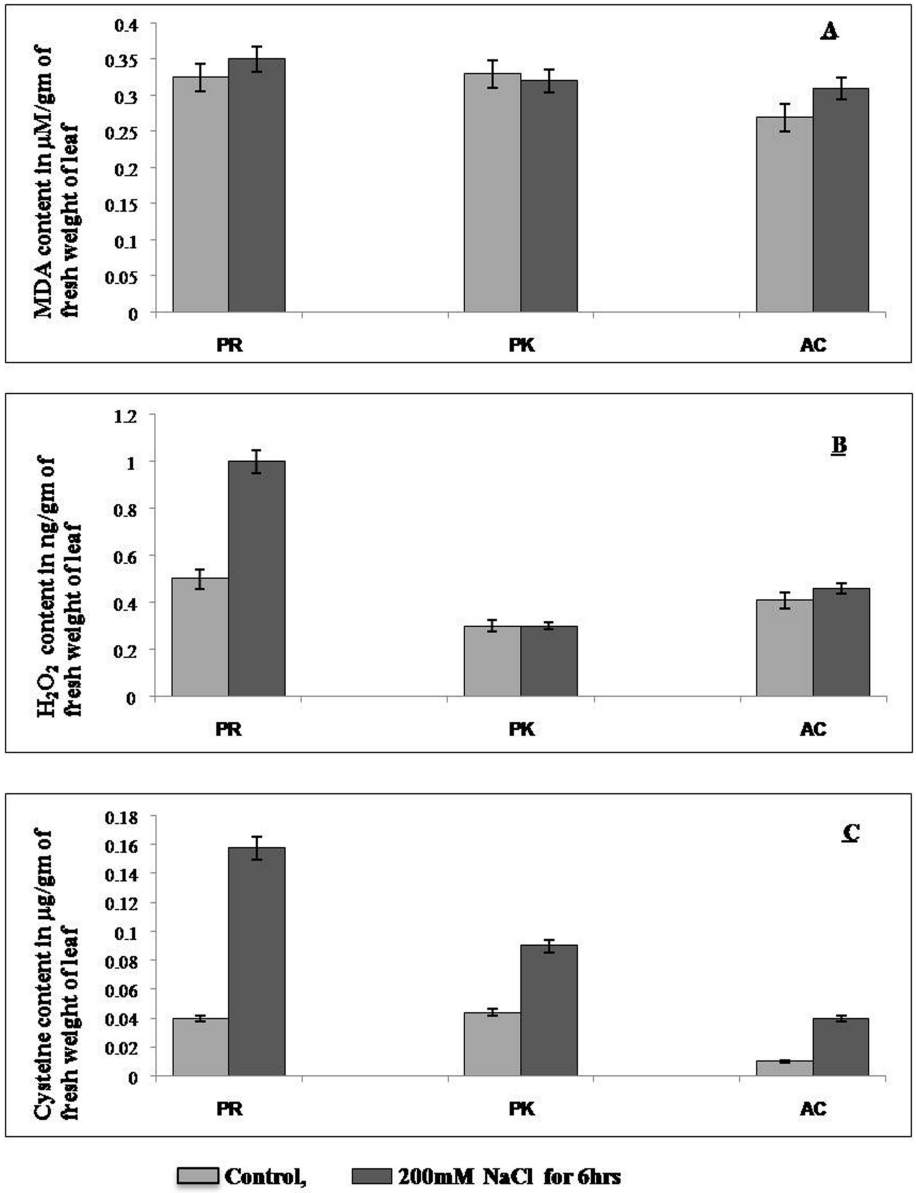

Figure 5 : Effect of salinity stress (200mM NaCl for 6hrs) on the level H_2O_2 (A), Malondialdehyde (B), Cysteine (C) in leaves of 2 month old tomato cultivars Pusa Ruby(PR), Punjab Keshari(PK) and Ailsa Craig(AC). H_2O_2 Content was determined following the protocol of Velikova *et al.*, (2000) and calculated on the basis of standard curve and were expressed in terms of ng g^{-1} FW of leaves. Malonaldialdehyde content was determined following the protocol of Fu and Huang, (2001). Cysteine concentration was determined following the protocol of Gaitonde (1967) and calculated on the basis of standard curve of cysteine. Cysteine amounts were expressed in terms of µg g^{-1} FW of leaves. Data points and vertical bars represent means of triplicates and SE respectively.

Figure 5C shows that the level of cysteine was induced by salinity stress in all the three cultivars, PR, PK, and AC. It was extracted and calculated on the basis of standard curve of cysteine and expressed in terms of µg g-1 FW of leaves. The result shows an enhancement in all three cultivars upon salinization, 2 fold in PK and 3 fold in PR and AC over their respective controls.

DISCUSSION

Our study showed that NaCl treatment caused reduction in the overall growth of all tomato plants as compared to their control plants. The growth of PK was found better among the three cultivars under long or short term salt exposure. Chlorophyll

content becomes a first indication of responses in different plants under salinity stress. When the plants were subjected to 24 days exposure to different level of NaCl (0, 100, 150 and 200mM), chlorophyll content varied in three cultivars PR, PK and AC (Figure 1). The cultivar PK showed enhancement of their chlorophyll content whereas in other cultivars (PR and AC), the inhibition was clearly visible. The result may be due to PK's better adaptation and resistance to salinity stress. Although photosynthesis has long been known to be partially or completely suppressed by sufficiently severe water stress (Boyer, 1976), studies on the effect of salinity on photosynthesis have produced contradictory conclusions. Some investigators have shown that photosynthesis was hardly slowed down by salinity and was sometimes even stimulated by low salt concentrations (Boyer, 1976; Heuer, 1981; Downton, 1983; Rawat, 1998; Yeo, 1985). Others have shown a significant decrease in photosynthesis in plants exposed to salinity (Kapulnik, 1991; Leidi, 1991; Francois, 1994). Tantawy (2009) also observed the decrease in total chlorophyll content in tomato with the increasing level of salinity. Dogan (2010) reported that chlorophyll concentration was lesser in salt-sensitive cultivars than in salt-resistant cultivars of tomato as we also evidenced in our studies. Genotypic differences in the response of plant photosynthesis to salinity have previously been reported (Heuer, 1984, 1989).

Few reports are there for 6 hrs of short-term salt treatment for measuring physiological parameters. In our study of biochemical changes due to short-term NaCl treatment has shown alterations thus showing their genotypic variations. Here we could indeed demonstrate that the level of β-carotene as unaffected by salinity stress in PR and AC may be because of such short term stress whereas in PK enhancement was upto 1.5 fold over its control (Figure 2B) may be due to its better tolerance to salinity. Carotenoids are the pigments in plants which has antioxidative function to protect plants in oxidative stress. Carotenoids are present in all photosynthetic organism and are integral constituents of the thylakoid membrane in chloroplasts. The light energy absorbed by the carotenoids is transferred to chlorophyll for photosynthesis. Because of this role they are called accessory pigments. In addition carotenoids also help protect the plants from damage caused by light (Taiz and Zeiger, 2010). According to Armstrong (1996), carotenoids have two major functions in photosynthesis. They protect chloroplast from photo-oxidative damage and they also act as accessory light harvesting pigments because they absorb the light energy in the range of 400-500nm (blue), and pass this excitation energy to chlorophyll molecules. They are also one of the non-enzymatic antioxidants which play an important role in the protection against oxidative stress (Kojo, 2004). So higher carotenoids in PK must be an attributor in this regard. In plants, carotenoids are synthesized from geranylgeranyldiphosphate (GGPP) in plastids. Lycopene β-cyclase is one of the crucial enzymes for carotenoid biosynthesis. Tomato genome contains two types of lycopene β-cyclase genes, LCY-B and CYC-B, encoding chloroplast- and chromoplast-specific lycopene β-cyclase enzymes, respectively. LCY-B is expressed in leaves, flowers and in fruits until breaker-stage of fruit ripening (Pecker, 1996). Activities of both of these enzymes together make α-carotene, while activity of Lycopene β-cyclase alone leads to formation of β-carotene (Giuliano, 2008).

Interestingly anthocyanin level was enhanced in PK after salinity stress whereas in PR and AC no

such changes occured. The effects of salinity on the anthocyanin contents were almost similar in the PR and AC cultivars. Figure 2A shows that anthocyanin content was increased by nearly 45% in the PK tomato plants compared with statistically insignificant increase in PR and AC plants at 200 mM NaCl over the control plants. Such increase in anthocyanin also could have contributed to PK's tolerance for salinity stress. Anthocyanins are a large class of water soluble pigments in the flavonoid group found in all plant tissues throughout the plant kingdom, are largely responsible for coloration in flowers and fruits of higher plants (Harborne, 2000). Flavonoids protect cells from excessive UV-B radiation (280nm), because they accumulate in the epidermal layers of leaves and stems and absorb light strongly in the UV-B region while allowing the visible light (photosynthetically active) wavelengths to pass through uninterrupted (Taiz and Zeiger, 2010). In other reports where researchers found that, low molecular weight antioxidants like anthocyanins (Tsuda *et al.*, 1996; Chalker-Scott, 1999), polyphenols (Sgherri *et al.*, 2004), flavonoids (Hernandez *et al.*, 2004) and carotenoids (Strz-alka *et al.*, 2003) can effectively scavenge harmful radicals and stabilize lipid oxidation. In our experiment higher accumulation of anthocyanin in PK genotype is certainly pointing towards such defence mechanisms.

Many plants accumulate high levels of proline in response to osmotic stress, and proline is thought to play an adaptive role during osmotic stress (Delauney and Verma, 1993). It is one of the most important osmoprotectant in plants. Under salt stress most plant species exhibit a remarkable increase in their proline content (Patel and Pandey 2008, Dasgan *et al.*, 2009). In our experiment where comparison was done among the 3 cultivars, (keeping the treatment condition as 200mM NaCl treated and untreated for 6hrs, two month old plants) a short term NaCl induction failed to produce higher proline in PR and AC but despite such a short term of salinization, proline accumulated hugely (about 4 fold) in cv. PK over their control (Figure 3A). Many reports are there in support to our findings like when exposed to high salt content in the soil (leading to water stress), many plants accumulate high amounts of proline, in some cases several times the sum of all the other amino acids (Mansour, 2004). The biosynthesis of proline appears as a common response of the plant to stressful environments (Claussen *et al.*, 2005). Proline accumulates in plants under a broad range of stress conditions such as water shortage, salinity, extreme temperatures, and high light intensity (Aspinall and Paleg, 1981; Mansour, 2000) and is believed to play a major role in plants osmotic adjustment. Veeranjaneyulu and Kumari, (1989) reported that proline content in leaves and roots of mulberry plants increased when water stress increased from mild to severe. In our experiments we also observed a similar behaviour in the leaves of tomato plants. Likewise, in tomato salt tolerance was attributed to the degree of plant to accumulate osmoprotectants, like proline. Proline is generally assumed to serve as a physiologically compatible solute that increases as needed to maintain a favourable osmotic potential between the cell and its surroundings (Patel and Pandey, 2008; Dasgan *et al.*, 2009). Other authors repoted that accumulation of proline in two sorghum genotypes contrasting in salt tolerance suggested that proline accumulation was a reaction to salt stress and not a plant response associated with tolerance (Parvaiz and Satyawati, 2008). In another experiment,

researchers also showed that under salt stress, higher concentration of proline was accumulated in sensitive rice cultivars than in tolerant genotypes (Parvaiz and Satyawati, 2008).

It is well known that plants are capable of synthesizing polyamines (PAs) and due to their polycationic nature they are likely to interact with polyanions like plasmamembrane (Roy *et al.*, 2005), other membranes, nucleic acids and polyanionic proteins. These findings have been discussed in several review articles (Tiburcio *et al.*, 1993; Galston *et al.*, 1997; Bais and Ravishankar, 2002). Even a very short term NaCl stress could induce enhanced level of total polyamines in only cv. PK which must have played a crucial role in combating stress condition. From the Figure 3B, 3C and 4**,** it is clear that the PK produced higher level of polyamines (putrescine and spermidine) when treated with 200Mm NaCl than PR and AC which is similar to the reports where salt tolerant rice cultivars were found to contain higher level of spermine and spermidine than in salt sensitive rice cultivars (Roy *et al.*, 2005; Chattopadhyay *et al.*, 2002). Polyamines (PAs) are low molecular weight polycations found in all living organisms (Cohen, 1998). They are known to be essential for growth and development in prokaryotes and eukaryotes (Tabor and Tabor, 1984; Tiburcio *et al*., 1990). In plant cells, the diamine putrescine, triamine spermidine and tetramine spermine constitute the major PAs. PAs are synthesized from arginine and ornithine by arginine decarboxylase (ADC) and ornithine decarboxylase (ODC). The intermediate agmatine, synthesized from arginine, is converted to putrescine which is further transformed to spermidine and spermine. In other reports, exogenously supplied spermidine and spermine has been shown to enhance membrane integrity and thereby reduces salinity stress induced rupture of membrane as it binds to the plasmalema (Chattopadhyay *et al.*, 2002). Therefore enhancement of PA-level in probably provides support to PK's efficiency in maintaining cellular integrity. This could be a way to make PK more salt tolerant than PR and AC.

Malondialdehyde (MDA) content, a product of lipid peroxidation, has been considered an indicator of oxidative damage (Shalata *et al.*, 2001). The lipid peroxidation levels in leaves of PR, PK and AC tomato cultivars, measured as the content of MDA, exhibited in Figure 5A. After the 6 hrs exposure to NaCl there is no significant change in the MDA level in any of the three cultivars may be due to short-term exposure to NaCl stress. Though the changes were at low level, it was near about 7% increase in PR and AC but almost unaltered in PK. The level of MDA produced during peroxidation of membrane lipids, is often used as an indicator of oxidative damage. The controlled lipid peroxidation in PK (Figure 5A) must have given it a better protection against oxidative damage under salt stress. There are reports of enhancement in the amount of MDA initially with the increase in salt stress in the salt-sensitive cultivar as compared to tolerant cultivar of rice (Roychowdhury *et al.*, 2011) and in roots of rice varieties (Khan and Panda, 2008).

Salt stress causes stomatal closure which reduces the carbon dioxide/oxygen ratio in plant cells. The excess oxygen in the plant is then used in the formation of reactive oxygen species (ROS). ROS production increases to dangerous levels when a plant is under abiotic stress (Xiong, 2002). In our experiment of 6hrs stress, the H_2O_2 generation was found to be elevated by about 80% only in PR but low and no marked difference in PK and AC. May be 6hrs stress with 200mM NaCl on 2 month old plants

did not affect PK and AC that much (Figure 5B) but as it was doubled in PR, so it may be interpreted that cultivars PK and AC have better adaptive mechanism in scavenging H_2O_2. As reported earlier, under stressful condition H_2O_2 accumulation and lipid peroxidation in sensitive cultivars of pea and rice were higher (Hernandez *et al.*, 2002, Lee *et al.*, 2001). Plants under salt stress displayed an increase in the generation of H_2O_2 (Gueta-Dahan *et al.*, 1997; Roxas *et al.*, 2000). Excessive amounts of highly reactive ROS can damage proteins, lipids and nucleic acids by oxidation (Halliwell, 1985). Therefore, it is critical that the plant counteract the production of reactive oxygen species with mechanisms for neutralizing them. However, hydrogen peroxide can be decomposed by the activity of catalases and several classes of peroxidases which act as important antioxidants. As may be expected, expression of the genes for ROS scavenging enzymes is upregulated in plants under abiotic stress (Zhu and Chinnusamy, 2005). Moreover, the ability of certain species to increase production of antioxidant compounds and enzymes in response to salinity (ROS production) has been correlated with salt tolerance (Shalata *et al.*, 2001).

Cysteine is an important constituent of glutathione (GSH) and therefore glutathione level is dependent on the supply of cysteine. GSH plays a pivotal role in detoxification of ROS (Noctor and Foyer, 1998). The induction of cysteine and glutathione synthesis during salt stress in plants suggests a possible protective mechanism against salt-induced oxidative damage. As in this study the cysteine content in all the three cultivars increased uder NaCl stress over their control ones (Figure 5C). It was almost 3 fold increase in PR, highest among the three. The glutathione level also must be higher in PR. Our report (Figure 5B) has also showed a considerable accumulation of H_2O_2 in PR under stress, and this 3 times higher cysteine in PR may be in order to mitigate the damaging effect of H_2O_2. The major substrate for the reductive detoxification of H_2O_2 is ascorbate, which must be continuously regenerated from its oxidized form. A major function of GSH in protection against oxidative stress is the reduction of ascorbate via the ascorbate-glutathione cycle, where GSH acts as a recycled intermediate in the reduction of H_2O_2 (Foyer and Halliwell, 1976; Noctor *et al.*, 1998). As reported by Ruiz and Blumwald (2002), that salt stress induced a sulfur demand for cysteine biosynthesis. Since cysteine is required for GSH (Glutathione) synthesis, the activation of GSH synthesis in the salt tolerant plants would be expected. Moreover, elevated GSH levels, mediated by increased γ-ECS (glutamylcysteine synthetase) activities, have been measured in extracts of pea, tobacco, maize, and tomato subjected to different stresses (Rennenberg *et al.*, 1982; Chen and Goldsbrough, 1994; Kocsy *et al.*, 1996). Studies support the notion that cysteine availability in plants also plays an important role in determining cellular GSH concentrations (Noctor *et al.*, 1997). So antioxidant enzymes and metabolites increase under various environmental stresses, with their comparatively higher activity in stress-tolerant cultivars, suggesting that higher antioxidant activity imparts tolerance (Polle, 1997; Sairam and Saxena, 2000).

CONCLUSIONS

In conclusion, data presented here suggest that even in very short term of salt stress can bring about changes at the biohemical level that can help in the identification of those that are most likely to play a role in salt tolerance in tomato and useful as screening criteria for different tomato genotypes

for higher salinity. These genetic differences present a good basis to provide information about genotypes that could be grown in salt-affected areas to chance crop productivity. Polyamines as evidenced, elevated in all the three cultivars may have prepared the cell to meet and combat stress by stabilizing membranes and forming a potential of higher buffering and antioxidant capacity. Particularly in PK, under unfavourable condition, significant accumulation of osmoprotectants and antioxidants prove that the defence system of PK is much stronger than PR and AC. PK also regulated the ROS production more efficiently. Taken together, our result show that the salt tolerance in tomato depend greatly on the osmotic adjustment (proline, PAs), keeping reactive oxygen species and MDA under control. Non enzymatic antioxidants also played constantly in encountering adverse condition.

Together with conventional plant physiology, genetics and biochemical approaches to studying plant responses to abiotic stresses have begun to bear fruit recently. Relevant information on biochemical indicators at the cellular level may serve as selection criteria for tolerance of salts in agricultural crops. There are numerous reports in the literature that show that plants containing high concentrations of antioxidants or flavonoids show considerable resistance to salinity as well as other abiotic stresses.

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