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Effects of Salinity on Growth, Activity of Antioxidant Enzymes and Sucrose Content in Tomato (*Lycopersicon esculentum* Mill.) at the Reproductive Stage

Sumalee Chookhampaeng, Wattana Pattanagul and Piyada Theerakulpisut*

Applied Taxonomic Research Center, Department of Biology, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand.

* Corresponding author, E-mail: piythe@kku.ac.th

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ABSTRACT: The effects of NaCl on the growth and physiology of tomato (*Lycopesicon esculentum* Mill.) cultivar Cln 1463 x Ace 55VF were studied. The plants were hydroponically grown in half-strength Hoagland solution for 45 days followed by treatments with 0, 25, 50 and 100 mM NaCl for 75 days. Growth parameters of 120-day-old plants were recorded. Mature leaves were harvested and analyzed for the amount of proline, protein, sucrose, activity of sucrose phosphate synthase (SPS), ascorbate peroxidase (APX), superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD). Mature fruits were analyzed for the amount of sucrose and activity of SPS. A low (25 mM NaCl) level of salinity treatment had no deleterious effects on vegetative growth parameters, but caused significant decreases in the number and average weight of mature fruits. At higher concentrations of NaCl (50 and 100 mM), both vegetative and fruit growth parameters were drastically reduced. Salinity treatments caused a reduction in protein content, accumulation of proline and enhancement of APX activity in leaves. In mature fruits and leaves, the amount of sucrose and the activity of SPS, the key enzyme in the sucrose synthesis pathway, increased with salinity treatment. Although salinity treatment caused significant reduction in fruit yield, it could improve fruit quality by increasing sucrose content.

KEYWORDS: Salinity, Growth, Activity, Antioxidant enzymes, Sucrose content, Tomato (L*ycopersicon esculentum* Mill.), Reproductive stage.

INTRODUCTION

Tomatoes are the world's most widely grown vegetable, other than the white potato. The tomato is grown widely in Thailand and one of the most popular crops. The main production areas of tomato are in the northeast and north of Thailand. The yield of tomato in Thailand is about 2,849 kg/ha, which is very low compared with that in other countries in the tropics[1]. It is well known that salinity reduces plant growth and that there are differences in tolerance to salinity among species and among cultivars[2]. Salt causes significant reduction in all growth parameters although the relative effects vary and the classification of the cultivars for their salt tolerance would vary according to the parameters used[3].

Salinity increases the osmotic pressure in the root environment and significantly decreases fresh yield of tomato. It is known that salinity (high EC) reduces yield. Uptake of water into the fruits is reduced by a high osmotic pressure of the irrigation water, and as a result the fruit size is smaller. On the benefit side, mild saline irrigation water may improve the quality of horticultural products by increasing dry matter content and sugar concentration in the fruit[4,5].

The production of activated oxygen species (AOS), which can damage DNA, proteins, chlorophyll and membrane function, is a by-product of oxidative metabolism in chloroplasts, mitochondria and peroxisomes. AOS production is further enhanced in response to various abiotic stresses, such as drought, salt, extreme temperatures and herbicides. Numerous studies showed that the level of antioxidative enzymes increases when plants are exposed to biotic or abiotic stresses including salinity. Comparison of the responses of cultivars and/or related species that exhibit differential sensitivity to salt stress showed a correlation between salt tolerance and increased activity of the antioxidant system[6].

Although soil salinisation in the northeastern region of Thailand is a severe problem for agriculture, tomatoes can grow well in this area. Therefore, tomato provides us as a good model for studying the mechanisms of adaptation in plants to salt concentration. In the present work, tomato plants had been treated with NaCl during reproductive growth stages and the effects of salinity on some aspects of growth and physiology, including protein content, proline content, antioxidant enzyme

activity, sucrose content and sucrose phosphate synthase activity, were examined.

Materials and methods

Plant Materials

Seeds of tomato cultivar Cln 1463 x Ace 55VF, a high-yielding cultivar which has been bred and field-tested by Srisaket Horticutural Research Station, Srisaket, Thailand, were germinated in distilled water for seven days in a culture room at the Biology Department, Khon Kaen University, under artificial light (200 µmol m^{-1} sec^{-1}, 16-hr photoperiod). Seven-day-old seedlings were transferred to half-strength Hoagland solution[7] and grown hydroponically in the culture room until the plants were 30 days old. Sodium chloride was then added in small increments until the final concentrations of 0, 25, 50 and 100 mM were reached when the plants were 45-days-old. The plants were then grown for the next 75 days during which the salinized solutions were changed every 5 days under glasshouse conditions. Four plants were grown for each salinity treatment. After 75 days of salinity treatment, the growth, yields, activity of antioxidant enzymes: APX, SOD, CAT and POD in leaves, sucrose phosphate synthase (SPS) in leaves and fruits, sucrose contents in leaves and fruits, and protein and proline content in leaves were determined for the 120-day-old plants.

Growth and Yield

One hundred and twenty days old plants were harvested, and plant height and growth and yield parameters were determined as follows: number of lateral shoots, fresh and dry weight of shoots and roots, basal diameter of stem, the days of first flowering, and the number and weight of mature fruits.

Protein Content

Plants were analyzed at 75 days after exposure to 0, 25, 50 and 100 mM NaCl. Fully expanded mature leaves at the 6th node from the shoot apex were harvested and frozen in liquid N_2 prior to storage at –80 °C. Sample tissue (0.5g) was ground on ice with a mortar and pestle with 5 ml of 10 mM potassium phosphate buffer (pH 7.0) containing 4% (w/v) polyvinylpyrrolidone. Crude extract was centrifuged at 12000 x g for 30 min at 4 °C and the supernatant was used as enzyme extract. The amount of protein in the enzyme extract was determined by the Bradford method[8].

Proline Content

Total proline was extracted by the method of Bates et al. (1973)[9]. Leaf samples (0.1 g) were homogenized in 10 ml of 3% aqueous sulfosalicylic acid and the homogenate was filtered through Whatman # 2 filter paper. Two milliliters of the filtered extract was reacted with 2 ml acid ninhydrin and 2 ml of glacial acetic acid in a test tube for 1 hour at 100 °C, and the reaction terminated by placing it on ice. The reaction mixture was extracted with 4 ml toluene and vortex. The chromophore containing toluene was aspirated from the aqueous phase, warmed to room temperature and the absorbance was read at 520 nm using toluene as a blank.

Antioxidant Enzyme Activity

Ascorbate peroxidase (APX) activity was determined by measuring a decrease in optical density at the wavelength of 290 nm as ascorbate was oxidized, as described by Prochazhava et al. (2001)[10]. The reaction mixture (3 ml) contained 50 mM ascorbic acid, 0.1 mM EDTA, and 0.1 ml enzyme extract and the reaction was started by adding hydrogen peroxide to 1.5 mM . The non- enzyme extract mixture served as a blank. The ascorbate peroxidase activity was calculated using the extinction coefficient of 2.8 $mM^{-1}cm^{-1}$ (Nakano and Asada, 1981)[11] and the activity was expressed as mmol ascorbate oxidized mg $protein^{-1}$ min^{-1}.

Superoxide dismutase (SOD) activity was determined by measuring the ability of SOD to inhibit the photochemical reduction of nitroblue tetrazolium chloride (NBT) according to the method of Dhindsa et al. (1981)[12] with some modifications. The reaction mixture (3 ml) contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 mM NBT, 0.1 mM EDTA and 0.05 ml enzyme extract. The reaction was started by adding 2 mM riboflavin and the reaction tube was placed under two 18-watt fluorescent lamps for 10 min. A complete reaction mixture without enzyme extract served as a control. The reaction was stopped by switching off the light and the tube was transferred into the dark. A non-irradiated reaction mixture served as a blank. The reaction mixture lacking enzyme gave maximum color and the intensity of the color decreased in the mixtures with enzyme extract. The absorbance was recorded at the wavelength of 560 nm in a spectrophotometer. One unit of enzyme was determined as the amount of enzyme reducing 50% of the absorbance reading compared with the non-enzyme tube. The activity was expressed as unit mg $protein^{-1}$ min^{-1}.

Catalase activity was determined by measuring the initial rate of disappearance of hydrogen peroxide as described by Velikova et al. (2000)[13]. The reaction mixture (3 ml) contained 10 mM potassium phosphate buffer (pH 7.0) and 0.1 ml enzyme extract and the reaction was started by adding 0.035 ml of 3% hydrogen peroxide. A decrease in hydrogen peroxide concentration was followed by a decline in optical

Table 1. Effects of NaCl on vegetative parameters of 120-day-old tomato plants after 75 days salinity treatment: plant height (cm), fresh and dry weight of shoot and root (g/plant), number of lateral shoots and diameter of stem (cm). [% = percentage reduction (negative values) or increase (positive values) compared with the control].

NaCl (mM)	Plant height	%	FW of shoot	%	DW of shoot	%	FW of root	%	DW of root	%	Number of lateral shoot	%	Diameter of stem	%
0	173.3±4.1a	0	483±23a	0	77.6±2.2a	0	95.1±15.6a	0	13.5±2.4a	0	15.5±1.0a	0	1.72±0.03a	0
25	165.5±4.6a	-4.47	509±28a	+5.21	83.5±2.2a	+7.7	95.2±11.4a	+0.20	13.4±3.6a	-0.74	15.0±1.0a	-3.26	1.74±0.04a	+1.16
50	129.8±5.0b	-25.1	374±25b	-22.6	63.7±3.4b	-13.9	54.4±9.1b	-42.8	12.5±0.7b	-7.41	7.5±0.6b	-51.61	1.55±0.02b	-9.88
100	119.8±5.9b	-30.9	372±24b	-23.1	61.8±2.6b	-20.4	49.2±6.6b	-48.3	11.6±1.1b	-14.07	5.75±0.8b	-62.90	1.44±0.04c	-16.3

Means in the same column followed by different letters differ significantly at $P<0.05$.

density at the wavelength of 240 nm. The non-enzyme extract mixture served as a blank. The catalase activity was calculated using the extinction coefficient of 40 $mM^{-1}cm^{-1}$ and the activity was expressed as µmol H_2O_2 reduced mg $protein^{-1}min^{-1}$.

Peroxidase activity was determined as an increase in optical density due to the formation of guaiacol dehydrogenation product according to Velikova et al. (2000)[13]. The reaction mixture (3 ml) contained 10 mM potassium phosphate buffer (pH 7.0), 0.04 ml enzyme extract, 0.6 ml guaiacol, and 1% (w/v) aqueous solution, and the reaction was started by adding 0.15 ml of 100 mM hydrogen peroxide. The absorbance was recorded at the wavelength of 470 nm in a spectrophotometer. The non-enzyme extract mixture served as a blank. The peroxidase activity was determined using the extinction coefficient of 26.6 $mM^{-1}cm^{-1}$ and the activity was expressed as µmol GDHP mg $protein^{-1}min^{-1}$.

Sucrose Content

Samples for sucrose determination consisted of 0.5 g FW of the pericarp excised from freshly harvested mature fruits, extracted with 5 ml of 90% (v/v) ethanol at 60 °C and filtered through cellulose nitrate membrane. The resulting extract was analyzed by high performance liquid chromatography (HPLC) on a Zorbax Carbohydrate Anlys. Col 4.6 x 150 mm, Hewlett-Packard, USA.

Sucrose Phosphate Synthase (SPS)

SPS activity was estimated by the fructose-6-P dependent formation of sucrose (+sucrose-P) from UDP-glucose at saturating substrate concentration[14]. The extraction buffer for SPS contained 100 mM Tris buffer (pH 7.5), 10 mM $MgCl_2$, 1 mM EDTA, 2.5 mM dithiothreitol (DTT), 1 mM glutathione (GSH), 10% (v/v) glycerol, 1% (w/v) bovine serum albumin (BSA) with 0.2 g polyvinylpolypyrrolidone (PVP) for each extraction (4 ml). Activity was assayed in a reaction mixture (0.3 ml) containing 7.5 mM UDP-glucose, 7.5 mM fructose-6-P, 10 mM glucose-6-P, 1.5 mM $MgCl_2$, 100 mM Tris-HCL (pH 7.5) and an aliquot of the desalted extract at 30 °C. The reaction was terminated after 20 min by addition of 4 M NaOH to 75 mM. Each assay had a control containing boiled enzyme. Unreacted fructose-6-P was removed by placing the reaction mixture in a boiling water bath for 10 min. After cooling, 0.25 ml of 0.1% (v/v) resorcinol in 95% (v/v) ethanol and 0.75 ml of 30% (w/v) HCl were added. The mixture was then incubated at 80 °C for 8 min, allowed to cool and absorption was determined at 540 nm.

Statistical Analysis

The experimental design was a randomized complete block. The data are presented with the respective standard errors of means and the least significant difference (LSD 0.05) between treatments, derived from an analysis of variance.

Results

Growth Parameters at the Reproductive Stage

Salinity treatments caused the retardation in growth and development of tomato plants during both vegetative and reproductive phases. Stressed plants produced flowers and fruits more slowly and the fruits are smaller compared to the non-stressed plants. The effects of salinity treatments on the vegetative growth parameters of 120-day-old plants after 75 days of NaCl treatment are summarized in Table 1. Treatment with 25 mM NaCl resulted in a non-significant reduction in plant height and increase in stem diameter. Higher concentrations

Table 2. Effects of NaCl on reproductive parameters of 120-day-old tomato plants after 75 days salinity treatment; Days of first flowering, number of mature fruits per plant and weight of fruits (g/fruit). [% = percentage reduction (negative values) or increase (positive values) compared with the control].

NaCl (mM)	Days until first flowering	%	Number of mature fruits	%	Weight of fruits	%
0	52.75±1.0a	0	16.5±1.6a	0	45.6±0.8a	0
25	55.75±1.1a	-5.69	10.25±0.85b	-37.9	38.9±2.1b	-14.5
50	64.75±0.95b	-22.74	7.75±1.8b	-53.0	20.9±1.0c	-54.2
100	65.00±1.8b	-23.22	8.00±0.70b	-51.5	18.8±1.4c	-58.7

Means in the same column followed by different letters differ significantly at P<0.05.

of 50 and 100 mM NaCl caused 25.1% and 30.9% reductions in plant height and 9.88% and 16.3% reductions in stem diameter respectively. The number of lateral shoots was drastically reduced from 15.5 to 7.5 (51.6%) and 5.75 (62.9%) shoots per plant when treated with 50 and 100 mM NaCl respectively. Salinity treatment at 50 and 100 mM had less effect on the fresh weight of shoots (22.6% and 23.1% reduction, respectively) than on that of roots (42.8% and 48.3% reduction, respectively), whereas the reverse was observed with the dry weight. On a dry weight basis, NaCl at 50 and 100 mM had more deleterious effects on shoot growth (13.9% and 20.4% reduction, respectively) than root growth (7.4% and 14.1% reduction, respectively).

The effects of salinity treatments on the reproductive growth parameters of 120-day-old plants after 75 days of full NaCl treatment are summarized in Table 2. In nonstressed condition, the plants flowered at the age of 52.75 days. Under 50 and 100 mM NaCl stress, flowering was significantly delayed for 12 days. Although 25 mM NaCl had no significant deleterious effects on any of the vegetative parameters and the time of flowering, it did cause significant reduction in mature fruit size (Fig 1). At the low level of NaCl stress (25 mM), the number of mature fruits was reduced from 16.5 to 10.25 per plant (37.9% reduction) and the average fruit weight was reduced from 45.56 g to 38.94 g (14.5% reduction). At 50 and 100 mM NaCl, the number of mature fruits per plant was drastically reduced by 53.0% and 51.5%, and the average fruit weight was reduced by 54.2% and 58.7%, respectively.

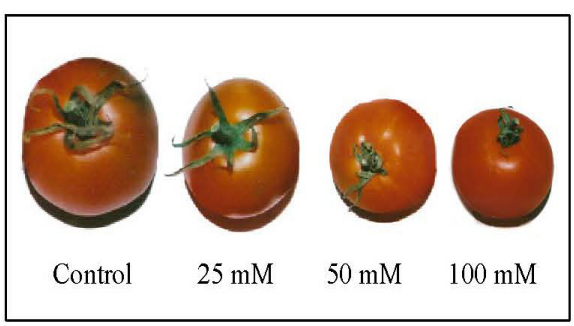

Fig 1. The effect of NaCl concentration on the size of mature fruits.

Some Physiological Parameters of Mature Leaves at the Reproductive Stage

Salinity stress induced changes in several physiological parameters in mature leaves of 120-days-old plants after 75 days of NaCl treatment. Leaf protein was significantly reduced in stressed plants subjected to 25, 50 and 100 mM NaCl compared to the non-stressed plants (Fig 2a). On the other hand, accumulation of proline in the mature leaves of the plants treated with 25, 50 and 100 mM NaCl was 2.55, 2.78 and 11.4 times the level found in the controlled plants, respectively (Fig 2b).

In the investigation of the activity of antioxidative enzymes, APX and SOD, it was shown that NaCl affected

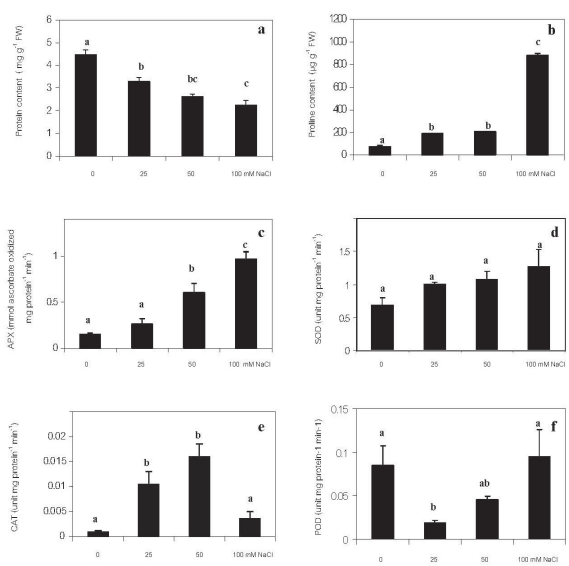

Fig 2. Effects of NaCl on (a) protein content, (b) proline content (c) activity of ascorbate peroxidase APX, (d) superoxide dismutase SOD, (e) catalase CAT and (f) peroxidase POD, in mature leaves of 120-day-old tomato plant, after 75 days of salinization. Different letters indicate that the mean values are significantly different (P<0.05).

the activities of both of these enzymes (Fig 2c and Fig 2d). It is apparent that in tomato leaves the activity of APX was enhanced to a higher extent than SOD when the plants are subjected to NaCl stress. The activity of APX in the plants treated with 50 and 100 mM NaCl was dramatically increased to 4.0 and 6.4 times the level found in the control. The activity of SOD, however, was not significantly increased. The responses of CAT (Fig 2e) and POD (Fig 2f) to NaCl were somewhat different from those of APX and SOD. The activity of CAT was 11.6 and 17.5 folds enhanced when the plants were treated with 25 and 50 mM NaCl, respectively, but sharply decreased when treated with higher salinity. The constitutive activity of POD was higher than that of CAT, and POD activity changed in the opposite direction to that of CAT in response to increasing salinity. When the plants were treated with low concentrations of NaCl, POD activity was significantly decreased and returned to the normal level when treated with higher NaCl concentration.

Sucrose and Sucrose Phosphate Synthase Activity in Mature Leaves and Fruits

The measurement of sucrose content in mature fruits and leaves (Fig 3) showed that, in fruits, sucrose increased with increasing concentrations of NaCl reaching the highest level at 100 mM NaCl (2.3 times increase from control). While the amount of sucrose in mature leaves was significantly higher than the control when treated with 25 mM NaCl, it reduced to similar levels as the control when treated with higher NaCl concentrations. The greater amount of sucrose in the fruits will increase the sweetness and therefore the quality of the fruits. The increased sucrose levels seen in the fruits treated with different levels of NaCl conformed with the activity of SPS. The activity of SPS in mature fruits showed significant increases (30.5% and 49.6%) from control plants, when treated with 50 and 100 mM NaCl, respectively. Similar trends were also observed for the activity of SPS in mature leaves, which showed significant increases (51.6%, 53.9% and 57.0%) from controlled plants when treated with 25, 50 and 100 mM NaCl, respectively.

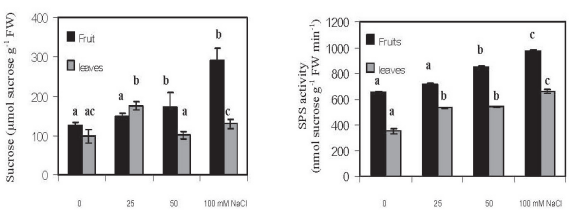

Fig 3. Changes in total sucrose and SPS activity in mature leaves and fruits of 120-day-old tomato plants after 75 days salinity treatment. Different letters indicate that the mean values of each tissue are significantly different ($P<0.05$).

Discussion

Long term treatment (75 days) of NaCl at low concentration (25 mM NaCl) to tomato plants cv Cln 1463 x Ace 55VF had little effect on the vegetative growth of 120-day-old tomato plants. Some parameters, such as fresh and dry weights of shoots were slightly enhanced by this low level of NaCl. Higher concentrations of NaCl (50 and 100 mM) resulted in significant reduction in all vegetative growth parameters, including number of lateral shoots, fresh and dry weight of shoots and roots and stem diameter. Shoot growth, as indicated by percentage reduction in dry weight, tended to be more affected than root growth. Similar observations were reported by several authors and reviewed by Cuartero and Fernández-MuHoz (1999)[15].

The amount of proline in the mature leaves of 45-day-old tomato plants prior to NaCl treatment was approximately 50 mg g^{-1} FW (result not shown) and that of 120-day-old control plants was 74 mg g^{-1} FW. It was found that after 75 days of salinization, proline accumulated at a much faster rate in the mature leaves and reached the levels of 188 and 205 mg g^{-1} FW in 25 and 50 mM NaCl treatment, respectively. Moreover, at 100 mM NaCl, the amount of proline was dramatically increased to 840 mg g^{-1} FW (16.5 folds increase from the original level in 45-day-old plants). The accumulation of nitrogen-containing compatible solutes including proline is known to function in osmotic adjustment, protection of cellular macromolecules from damage by salts, storage of nitrogen and scavenging of free radicals. Many plants, both halophytes and glycophytes, accumulate proline as a non-toxic and protective osmolyte under saline conditions, including mangrove[16], maize[17], sorghum[18] and mulberry[19]. Some authors have, however, argued that excessively high levels of proline accumulation may be a response to leaf damage when exposed to high salt concentrations and that a higher level of proline accumulation is associated with salt sensitive traits in tomato[20] and sorghum[18]. Proline accumulation in response to lower salt concentrations may contribute positively to salt tolerance, whereas the extremely high concentration in leaf tissues under high salinity treatment may be partly due to leaf damage. Moreover, the higher growth reduction in high-salt treatment may be related to the higher partitioning of metabolic energy needed for synthesis of proline, which costs a much larger amount of ATP than accumulation of inorganic solutes[21].

An important consequence of salinity stress is the generation of excessive reactive oxygen species (ROS) which leads to cell toxicity, membrane dysfunction and cell death. Tomato plants have been reported to defend

against the ROS by enhancement of antioxidative enzymes including SOD, APX, CAT and glutathione reductase[22]. In this tomato cultivar under our experimental condition, APX, CAT and SOD played more active roles than POD in alleviating the leaf cells from oxidative stress. Similarly, Chaparzadeh et al. (2004)[23] also observed enhanced activities of APX and CAT, but lowered activity of POD in the leaves of *Calendula officinalis* L. in response to salt stress. The rise in CAT activity at low and moderate salt concentrations correlated with cellular defense against salt-induced photorespiration in peroxisomes. Under high salt stress, however, CAT is inhibited and the leaf cells are protected from oxidative damage largely by APX, SOD and POD. In leaves of salt-stressed wild tomato (*L. pennellii*), Mittova et al. (2002)[6] found that the activity of APX was most enhanced (4.7 folds over that of control), followed by SOD (2.7 folds) and POD (1.9 folds). Greater enhancement of the activities of these antioxidative enzymes in the wild tomato resulted in less oxidative stress compared to the cultivated tomato. Enhancement of antioxidative enzymes has been observed in several cultivated plants. Harinasut et al (2003)[19] reported the enhanced activity of antioxidative enzymes (APX and SOD) in leaves of mulberry grown under salt stress (150 mM NaCl) conditions. Rajguru et al (1999)[24] studied ovule growth and activity of antioxidative enzymes in four cotton cultivars subjected to salt stress and found a relationship between salt tolerance and enhanced activity of SOD and APX.

Sucrose concentration in mature fruits increased when plants were treated with NaCl during the reproductive growth period. The result agreed with the observation that sucrose concentration in tomato leaves is increased in plants exposed to 50 or 100 mM NaCl as compared to the control plants[14]. Balibrea et al. (1996)[25] reported that moderate salinity provoked the highest hexose concentration along the growing period of the fruit. Soluble carbohydrates concentrations increased in the leaves of salt stressed sorghum seedlings, especially in the older leaves[18]. Total soluble solids (TSS) content is the most important quality criterion for tomato paste processing. TSS in ripe fruit increases with salinity and hence the use of moderately saline irrigation water (3-6 dS m^{-1}) is recommended to improve fruit quality[15]. SPS activity in both mature leaves and fruits increased in plants exposed to 25, 50 and 100 mM NaCl as compared to the control plants. Gao et al (1998)[14] similarly reported the enhancement of SPS in the leaves of plants receiving 50 mM NaCl, in which SPS activity was enhanced by nearly twofold over its level in control plants. Our results showed that the concentration of sucrose in fruits correlates with the activity of SPS, a key enzyme for sucrose synthesis. Treatment of this tomato cultivar with 100 mM NaCl resulted in a 2.3 fold higher amount of sucrose in the fruits, although they suffered from more than 50% yield reduction. Further studies are required to adjust the level, period of treatment and conditions of salinity stress application to obtain the suitable balance between fruit yield and quality.

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