Roles of ascorbic acid on physiological, biochemical, and molecular system of *Lycopersicon esculentum* Mill. against salt stress

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Abstract

Tomato is an important plant grown for food and medicine, which is sensitive to abiotic stresses. On the other hand, ascorbic acid (AsA) is a compound used as the plants' universal defense mechanism against stresses specially salinity and drought to scavenge reactive oxygen species. Due to global warming and subsequent scarcity of water resources, drought, and salinization of soils, there is a need to investigate AsA effects on tomato plants under salinity stress. In this context, the effects of applying ascorbic acid and salinity stress on some physiological parameters, key enzymes, namely superoxide dismutase (SOD) and ascorbate peroxidase (APX), and expression of *LeNHX1* and *SlERF16* genes involved in antioxidant system were studied in L*ycopersicon esculentum* Mill. plants in hydroponic conditions. Results showed that ascorbic acid treatments increased activity of SOD and APX and improved physiological parameters such as lipid peroxidation and Na^+/K^+ contents of tomato under salt stress via increases in *LeNHX1* and *SlERF16* genes expression.

Keywords: ascorbic acid; genes expression; salinity; *LeNHX1; SlERF16*

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Introduction

Tomato (*Lycopersicon esculentum* Mill.) is a genus of Solanum that Philipe Miller in 1768 moved it to its own genus and before that, it was named as *solanum lycopersicum* L. by Linnaeus in 1753 (Valdes and Gray, 1998; Perlata and Spooner, 2007). Today, because of its flavor, minerals, vitamins, and antioxidant compounds, tomato is a popular food item worldwide (Elizondo and Oyanede, 2010).

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Vitamin C or ascorbic acid (AsA) has critical role in stress condition, especially under water stress such as drought and salinity. In fact, by applying ascorbic acid in plants such as tomato, in addition to making the plant resistant to stresses, the resulting fruit can help us resist against illnesses such as cold, influenza, SARS, Covid-19, etc. (Khan et al., 2020).

Environmental stresses are a limiting factor for the growth and yield of plants by reducing the osmotic potential and disrupting the absorption of certain nutrients (El-Tayeb, 2005). Salinity stress disrupts cell division, growth, membrane lipids, proteins, nucleic acids, and all metabolic reactions of the plants as a result of accumulation of active oxygen species in the cell

(Kaya et al., 2006; Agarwal and Shaheen, 2007). Ion channels play crucial roles in plant resistance and overcoming stresses. Cation transporters such as *LeNHX* regulate the Na^+ contents of cells and their homeostasis (He and Huang, 2012). There are 4 isoforms of *LeNHX* in tomato's vacuolar cells that contribute to K^+ accumulation (Abedlaziz et al., 2019). Also, ERF family genes have a role in response to biotic and abiotic stresses. Both of these genes are responsible for encoding ion channels and its accumulation (Gharsallah et al., 2016). Antioxidant enzymes such as superoxide dismutase (SOD) and ascorbate peroxidase (APX) are among the factors that neutralize the toxic effects of reactive oxygen species. Some metabolites such as ascorbic acid can increase the activity of these enzymes to mitigate the effects of Malondialdehyde level that is a sign of lipid peroxidation in cell membranes and is used as a factor to measure the rate of membrane damage under stress conditions (Beltagi, 2008; Jaleel et al., 2007). The activity of antioxidant enzymes is correlated with detoxification and elimination of ROS. Ascorbic acid, as an antioxidant, can reduce the ROS produced by stress (Ansari and Sharif-Zadeh, 2012). Additionally, it is involved in the purification of active species in chloroplasts and cytosol in ascorbate glutathione way and acts as a key ingredient (Asada, 1999). In plants that are not exposed to salt stress, ascorbic acid does not affect the content of their soluble proteins, because the effect of ascorbic acid is indirect and by eliminating the ROS it prevents the destruction of proteins and lipid peroxidation (Upadhyaya and Panda, 2004). Considering the importance of tomato plant across the world and also the role of AsA in stress conditions, we decided to investigate the effects of AsA on some physiological, biochemical, and molecular characteristics of tomato under salinity stress. Our findings will help identify genes for genetic improvement of tomato characteristics to resist abiotic stresses specially salinity conditions.

Material and Methods

Tomato seeds (*L. esculentum* Mill.) were obtained from the Seed and Plant Improvement Institute, Karaj, Iran. They were surface sterilized with 2.5% sodium hypochlorite for 10 min and then rinsed with distilled water. Ascorbic acid (AsA: 0, 5, and 10 mM) was used as donor and sodium chloride (NaCl: 0, 50, 100, and 150 mM) was used to apply salt stress. They were germinated after 9 days and then, were transferred to half-strength Hoagland medium. After 20 days, samples were ready for physiological parameter measurements and genes expression.

Determination of K^+ and Na^+

Samples were dried in an oven set at about 65 ℃ for 48 h before they were ground in a grinding machine to pass through a 20-mesh sieve.

Malondialdehyde (MDA)

Frozen leaf samples (0.25g) were homogenized in a pre-chilled mortar with 5 ml ice-cold trichloroacetic acid (TCA) 5% (w/v) and centrifuged at 12000 rpm for 15 min at 4 ℃. The assay mixture containing 2 ml aliquot of supernatant and 2 ml of thiobarbituric acid 0.67% (w/v) was heated to 100 ℃ for 30 min. and then rapidly cooled to 4 ℃ in an ice bath. After centrifugation (10000 rpm for 1min at 4 ℃), the supernatant absorbance was read (532 nm) and values corresponding to non-specific absorption (600 nm) were subtracted. The final concentration of MDA was expressed in µM/L of MDA g/FW.

Extraction of the enzymes

Leaf sample (10 g) was homogenized in 50 volumes of 100 mM Tris-HCl (pH 7.5) containing 5 mM DTT (Dithiothreitol), 10 mM $MgCl_2$, 1 mM EDTA (Ethylene di amine tetra acetic acid), 5 mM magnesium acetate, 1.5% PVP-40 (Polyvinylpyrrolidone), 1 mM PMSF (phenyl methane sulfonyl fluoride), and 1 µg ml^{-1} aproptinin. After the filtration, the samples were centrifuged at 12,000 rpm for 10 min. The supernatant harvested was used as enzyme source (Ahmad et al., 2015).

RNA extraction, cDNA synthesis, and qRT-PCR

Total RNA was isolated from root and leaf tissues using the RNA kit (RNA Biotechnology Co. Iran) according to the manufacturer's instructions. First-strand cDNA was synthesized from 2 mg of total RNA with oligo (dT) and MMLV reverse transcriptase (200U/ll, Invitrogen) according to the manufacturer's instructions. ABI A Prism 7000 sequence detection system (Applied Biosystems) was used for quantitative real-time PCR (qPCR) under the following cycle conditions: 10 min. at 95 ℃ followed by 40 cycles of 15s at 95 ℃, 1 min. at 60 ℃. The ACTIN tomato gene (ACT) was used as internal reference gene (Lovdal and Lillo, 2009). Genes and their corresponding primers are shown in Table 1. Template of PCR reaction included 50 ng of cDNA sample, 400 nM forward and reverse primers, and SYBR green qPCR master Mix-Rox.

Measurements

The K^+ and Na^+ contents were determined using a flame photometer (flame photometer, corning M410, UK) following Brown and Lilleland (1946). The level of lipid peroxidation was measured as 2-thiobarbituric acid-reactive substances (TBARs), mainly (MDA), following the modified method of Heath and Parker (1968). APX (EC 1.11.1.11) activity was determined following the method of Nakano and Asada (1981). The absorbance was read at 290 nm by a spectrophotometer (S 2100 S UV, USA). $EUmg^{-1}$ protein expresses the APX activity. Estimation of SOD (EC1.15.1.1) activity was carried out following the photo reduction of nitro blue tetrazolium (NBT) (Van Rossum et al., 1997). The OD was taken at 560 nm by the spectrophotometer (S 2100 S UV, USA). SOD activity is inversely proportional to the NBT reduction. SOD unit is the amount of protein that restricts 50% photo-reduction of NBT. SOD activity was expressed as enzyme unit (EU) mg^{-1} protein. Finally, the Relative quantification was performed by applying the 2DDCt method (Livak and Schmittgen, 2001).

Statistical Analysis

Completely randomized design with factorial arrangements was used for the experiment. Data were subjected to statistical analyses using SAS 9.1 statistical software. Analysis of variance (ANOVA) and comparison of means (Duncan test) were at 5% level.

Table 1
Primer sequence used for gene expression

Genes names		Primer sequence
LeNHX1	Forward	GACAGTCCTGGAAAATCT
	Reverse	GGTTATCAGCCCAAACACC
SIERF16	Forward	GCGAATAATACAGAACCCGAACTT
	Reverse	TGAGGAAGAAGAAAGATCCGAATT
ACTIN	Forward	GAAATAGCATAAGATGGCAGACG
	Reverse	ATACCCACCATCACACCAGTAT

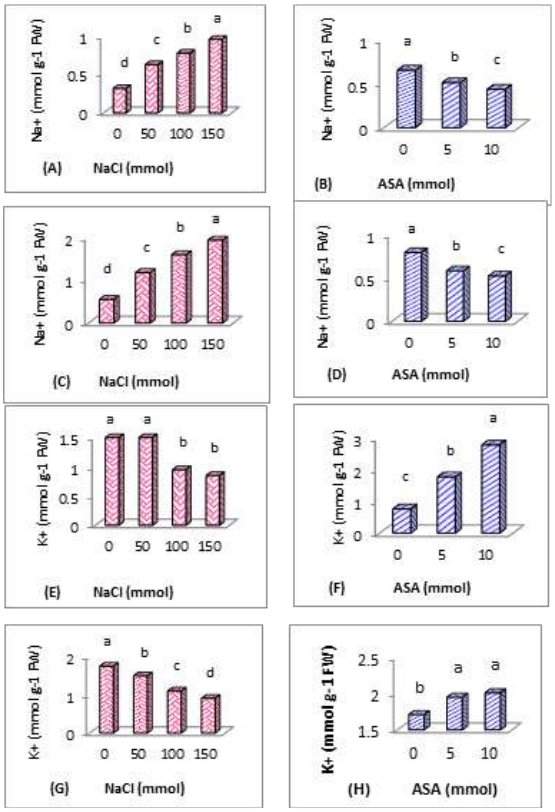

Fig. I. (A and B): effects of NaCl and AsA on Na^+ contents of leaves; (C and D): effects of NaCl and AsA on Na^+ contents of roots; (E and F): effects of NaCl and AsA on K^+ contents of leaves; (G and H): effects of NaCl and AsA on K^+ contents of roots.

Results

Na+ and K+ contents

After 20 days' post stress application, we analyzed the distribution of Na^+ and K^+ in both

Table 2
Analyses of variance (ANOVA) of the effects of salt and AsA treatments on physiological and biochemical parameters of tomato leaves

Mean Differences	K^+		Na^+		MDA	SOD	APX
	Roots	leaves	Roots	leaves			
Salt	18.88 **	15.37 **	19.35**	12.05**	7.03**	727.96**	17.53**
AsA	9.74 **	7.55 **	11.79 **	8.31 **	4.51 **	19.24 ns	1.01 ns
Salt*AsA	8.14 **	1.09 ns	1.57 ns	5.23 **	1.43**	160.81**	3.89 **
Confidence of Variation	2.12	1.26	1.85	1.44	11.07	7.09	4.25

ns: non-significant ** significant at p<0.01

Table 3
Effects of salinity stress, ASA concentrations and interaction between them on measured parameters of tomato

Salt (mM)	AsA (mM)	MDA	SOD	APX	SIERF9	LeNHX4
0	0	0.65 j	19.11 h	0.65 f	14.21 h	1.45 i
	5	0.45 k	21.35 g	0.62 ef	19.35 ef	2.55 h
	10	0.41 k	23.85 f	0.65 ef	21.30 de	3.35 g
50	0	2.40 d	27.65 e	1.62 d	16.90 fg	2.75 gh
	5	1.82 f	34.80 c	2.34 c	27.00 c	5.65 e
	10	1.15 i	35.00 c	2.35 c	48.54 a	8.55 d
100	0	2.95 b	32.60 d	1.86 cd	16.35 gh	4.85 f
	5	1.97 e	34.11 c	3.38 b	23.25 d	8.70 d
	10	1.39 h	42.22 b	3.65 b	35.50 b	13.17 c
150	0	3.84 a	34.05 c	2.11 cd	20.35 e	8.70 d
	5	2.61 c	42.35 b	4.53 a	36.90 b	14.82 b
	10	1.62 g	47.90 a	4.56 a	49.62 a	16.65 a

Conforming to Duncan test, means with similar letters (lower case letters for interactions and capital letters for means) are not significantly different at 5% level. Dash is an untreated sign.

leaves and roots. As expected, with increasing salt stress, the sodium content increased in leaves and roots (0.96 and 1.94 mM g^{-1} FW, respectively). But the use of AsA in 10 mM could reduce this parameter by 0.43 and 0.52 mM g^{-1} FW in leaf and root, respectively) (Fig. I. A-D), (Table 2). One-way ANOVA ($p<0.01$) carried out for the effect of different concentrations of NaCl on potassium in plants under stress showed that the lowest and highest K^+ contents belonged to 150 mM (0.85 and 0.91 mM g^{-1} FW in leaf and root and 2.88 and 2.01 mM g^{-1} FW in leaf and root) in AsA 10 mM treatment, respectively (Fig. I. E-H).

Lipid peroxidation and MDA production

In roots of plants under salt stress that were treated with AsA, lipid peroxidation significantly decreased (Tables 2 and 3). Results indicated that the trends of MDA changes due to the increase in AsA contents at each salinity level were similar. Maximum level of MDA (3.84 µM g^{-1} FW) was observed in 150 mM salinity and non-AsA. But AsA in 10 mM treatment decreased lipid peroxidation of plants to 1.62 µm/g^{-1} FW under high level of salinity stress (150 mM).

Antioxidant enzymes activity

To decrease the ROSs produced, plant cells increase the enzyme activity such as SOD and APX to scavenge ROSs. In this study, the effect of salinity on oxidative parameters were measured. One-way ANOVA followed by Duncan comparisons test showed significant differences in the activities of APX and SOD (Tables 2 and 3). Maximum activity of APX was recorded under 150 mM salinity and 10 mM AsA. Increased enzyme activities is an adaptive way to conquer salinity effects by decreasing H_2O_2 levels. Decrease in levels of H_2O_2 and MDA in peroxisomes (data was not shown) was the result of increased enzymes activities (Mittova et al., 2004; Gharsallah et al., 2016; Sofo et al., 2015) (Fig. II).

Genes expression

Many plants have the ability to store Na^+ at low level in their cytoplasm (Gupta and Huang, 2014). The data of genes expression involved in stress were influenced by different treatments in this experiment. Results indicated that the treatments had significant increasing effects on *LeNHX1* and *SlERF16* genes expression (Table 3). Increasing the amount of AsA led to the increased expression of *LeNHX1* and *SlERF16* genes in leaves and roots, respectively under salinity stress conditions. Plants under salt stress and treated with 10 mg AsA showed the highest genes expression. Maximum rate of *LeNHX1* gene expression was observed in salinity treatment with 100 mM NaCl (Fig. III. A). Expression of *SlERF16* gene increased significantly in 100 and 150 mM NaCl treatments (Fig. III- B).

Discussion

Na^+ and K^+ contents

Findings indicated that the Na^+ and K^+ contents were affected by salt and differences between them maybe due to the antagonism between absorption positions of these ions in plasma membrane. Increase and decrease in Na^+ accumulation in roots and in leaves, respectively maybe attributed to the exclusion mechanism of this cultivar. This is similar to findings of other studies reported on tomato and potato genotypes (Gharsallah et al., 2016; Dasgan et al., 2002). AsA treatments could maintain K^+ ions and homeostasis of cells during the stress stages; therefore, it is a key ion to regulation of metabolic process by promoting the exclusion of Na^+ (Chakraborty et al., 2016).

Lipid peroxidation and MDA production

Peroxidation of unsaturated fatty acids produces aldehyde which is expressed as an oxidative stress index (Torres-Franklin et al., 2008). Also, peroxidation of lipids is a natural metabolic process under normal conditions but intensifies under stress conditions (Gill and Tuteja, 2010). Increased lipid peroxidation in salinity stress conditions in other plants has also been reported (Beltagi, 2008). Decreased lipid

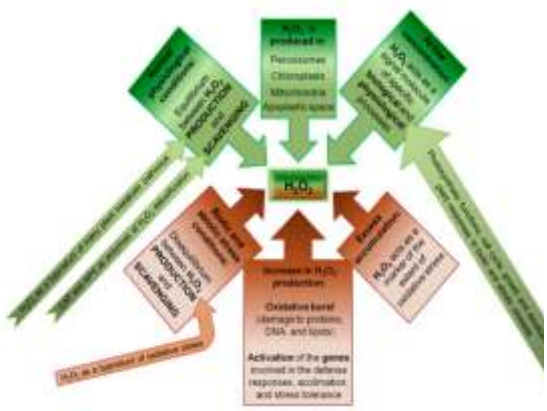

Fig. II. The role of H_2O_2 in normal and stress conditions (Sofo et al., 2015)

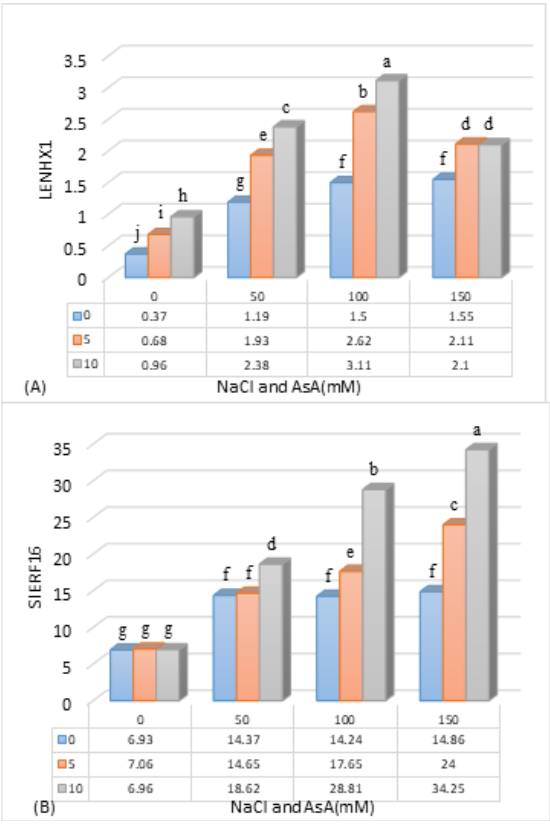

Fig. III. A: Interaction of salinity and AsA on LeNHX1; B: SlERF16 genes expression of tomato

peroxidation with ascorbic acid application was also observed in Cicer arietinum L. plants under salt stress (Beltagi, 2008). The results of this study showed that lipid peroxidation decreased in plants treated with salinity and ascorbic acid compared to salinity treatments without AsA. Ascorbic acid

by neutralizing the oxygen radicals through the use of ROS and mono de-hydro ascorbate production prevents damage to the cells and lipids in the membranes and reduces lipid peroxidation (Taqi et al., 2011).

Antioxidant enzymes activity

In the present study, increasing the level of salinity stress in different treatments increased antioxidant enzymes. Increases in APX activity were reported in salt-stressed Panax ginseng and L. pennellii plants (Kim et al., 2014; Mittova et al., 2004). Researchers reported that the salinity increased the activity of SOD, APX, CAT, and GR in wheat genotypes (Sairam et al., 2001). Similar to the results of this experiment, the use of exogenous ascorbic acid improved the growth characteristics and increased activity of the antioxidant enzymes of bean plants under salt stress (Younis et al., 2010). It has been shown that in normal conditions, ascorbic acid can increase plant resistance and decrease the activity of antioxidant enzymes by eliminating active oxygen species. But under salinity stress conditions, ascorbic acid can increase the plant tolerance by strengthening the antioxidant system (enzymatic and non-enzymatic) and through preventing the accumulation of free radicals. SOD transforms the anion superoxide into hydrogen peroxide. Also, AsA acts as a cofactor for enzymes such as APX which is involved in the destruction of hydrogen peroxide (Upadhyaya et al., 2010).

Genes expression

Application of ascorbic acid increased the expression of *LeNHX* and *SIERF* genes by reducing the absorption of sodium, increasing potassium uptake, and subsequently increasing osmolytes. Previous studies reported that *LeNHX* is essential for K^+ uptake at the tonoplast, turgor regulation, and stomatal function. The roles of NHX isoforms have been known in ions (Na^+, K^+, and H^+) homeostasis and regulation of functions in plants (Almeida et al., 2014). Also, Sharma et al (2010) and Gharsallah et al (2016) announced that ERF gene has direct relationship with salt tolerance during salinity stress. In fact, these genes are responsible for reducing the negative effects of stress and adjusting the plants' internal conditions. Salt tolerance through stress-reducing compounds such as AsA is achieved by retrieval of Na^+ from the xylem vessels to xylem parenchyma cells, promoting vacuolar accumulation and thus protecting photosynthetic leaf tissues from the adverse effects of Na^+ (Xue et al., 2011; Munns et al., 2012). Sharma et al (2010) reported the induction of ERF family genes in response to various stress treatments (salt, cold, heat, dehydration, mechanical stress, oxidative stress, and submergence stress), suggesting a crosstalk between different stress-signaling pathways. All of these are interrelated in an intricate way that ultimately lead to modification of target proteins that may have enzymatic or structural function and activate the cellular responses at the physiological, biochemical, and molecular levels. Our study indicated that rapid and high expression of *SIERF* and *LeNHX* by AsA treatment is closely related to salinity stress tolerance. *LeNHX1* cation/H^+ antiporter keeps the sodium in vacuoles and as a results leads to regulation of the homeostasis of potassium and internal pH in saline and normal terms (Barragan et al., 2012; Leidi et al., 2010). Beside members of *LeNHX* gene family, ERF as a transcription factor is critical in regulation of genes expression. Researchers reported that the SIERF80 and SIERF5 increased in saline and drought conditions in transgenic tomatoes (Sharma et al., 2010).

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