



## Molecular and biochemical protective roles of sodium nitroprusside in tomato (*Lycopersicon esculentum* Mill.) under salt stress

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### Abstract

Salinity stresses act as inhibitor factors of plant growth. They can change the physiological characteristics and limit the production of crops. Sodium nitroprusside (SNP) is a stable free radical which is used as a signaling molecule in plants and participates in various plants' physiological, biochemical, and molecular processes and also in plants' responses to environmental stresses. We investigated the effect of SNP on physiological parameters such as photosynthetic and non-enzymatic pigments, biochemical parameters like APX and SOD enzymes and *HKT1.2* and *SLWRKY 8* genes expression as a molecular section on tomato under salt stress. In this study, SNP was used as nitric oxide (NO) donor. Tomato seedling roots were subjected to various levels of salinity including 0, 40, 80, and 120 mM and SNP (0, 50 and 100 mM) for 20 days. The SNP had protective effects on photosynthetic parameters by increase in non-enzymatic and enzymatic antioxidants. It had also reducing and increasing effect on *HKT1.2* and *SLWRKY 8* gene expressions, respectively.

**Keywords:** *HKT1.2*; *SLWRKY 8*; salinity; SNP; tomato

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### Introduction

Solanaceae family consists of about 100 genera and 2500 species of flowering plants that includes shrubs, lianas, trees, and agriculture crops such as tomato (*Lycopersicon esculentum* Mill), eggplant (*Solanum melongena* L.), potato (*Solanum tuberosum* L.), chili pepper (*Capsicum annuum* L.), etc. The name solanum means nightshade plant (Olmstead and Bohs 2007; Quinn et al., 1989). Tomatoes are dicots and they have regular leaves and yellow flowers with five lobes on corolla, clustered on stem between two nodes. Each

cluster has 4-8 flowers (Gupta and Huang, 2014; Halfacre, 1979). It is distributed worldwide in regions and countries as diverse as Spain, China, Italy, Britain, India, north America, Middle East, North Africa, and Iran (Schroeder et al., 2013; Talbi et al., 2015; Olmstead and Bohs, 2007). Tomatoes are known as rich in vitamins C, A, E, K, and B groups, antioxidants such as lycopene and flavonoids, and minerals like magnesium, manganese, phosphorus, potassium, etc. This is why they are considered a useful for treating cancer, cardiovascular diseases, cold, SARS, influenza, and Covid-19 (Wilcox et al., 2003; Khan et al., 2020). Tomato plants are sensitive to environmental stresses such as temperature,

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salinity, drought etc. Therefore, the crop's contents and yields are affected by these abiotic stresses (Naz et al., 2020). The role of nitric oxide as a molecule involved in regulating multiple plant responses to varieties of biotic and abiotic stresses and reducing the damage caused by oxidative stresses has been confirmed by these researchers. Nitric oxide may act as an antioxidant and signaling molecule; therefore, it mitigates cell damage by producing proxy nitrite and altering the expression of some defense genes that may increase plant tolerance to salinity stress (Lamattina et al., 2003). Ion channels play critical roles in adaptation to salt stress situations (Gharsallah et al., 2016). Histidine kinase transporter (*HKT*) is one of the cation transporters that regulate the concentration of sodium ( $\text{Na}^+$ ). Other studies reported that the increase and decrease of the *HKT* expression levels were related to salinity. The *HKT1.2* is a tomato sodium transporter that has a selective function to homeostasis regulation of  $\text{Na}^+$  and  $\text{K}^+$  ions (Hauser and Horie, 2010). Studies show that the *HKT*s can eliminate the excess  $\text{Na}^+$  and photosynthetic protection against it. Stress situations induce biochemical ways by molecular regulations. Researchers revealed about 18 tomato *SIWRKY* genes at the salt stress some of them being involved in adaptation of plants. *SIWRKY* is a transcription factor of *WRKY* family that is related to photosynthesis, enzymes and oxidative pathways in cells (Gharsallah et al., 2016; Huang et al., 2012; Sun et al., 2014). Biochemical mechanisms like antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POX), catalase (CAT), etc. and non-enzymatic compounds such as anthocyanins, flavonoids and phenols are synthesized in stress conditions and they can change the ROSs to water and oxygen and modify cell homeostasis (Chawla et al., 2013; Talbi et al., 2015). Many species of plants cannot grow in saline soils and there is a need to improve ways to increase the plant tolerance. The use of mitigation agents such as sodium nitroprusside can be used as an effective solution in managing the use of saline water and soil resources for many important horticultural crops (Klessig et al., 2000). In this study, we investigated the tomato's *HKT* and *SIWRKY* genes and some of its physiological properties under salt stress, particularly its

tolerance to saline soil when treated with sodium nitroprusside.

## Material and Methods

### Plant section

Tomato seeds (*L. esculentum* Mill. 'Super Chef') were obtained from the Seed and Plant Improvement institute, Karaj, Iran. Their surfaces were sterilized by 2.5% sodium hypochlorite sodium for 10 min and rinsed with distilled water. SNP was used as a donor and NaCl was used to apply salt stress. Twenty (20) seeds were planted into culture trays containing peat moss and soil medium (1:1 and with EC 0.3  $\text{ds.m}^{-1}$ ) and kept in a growth chamber for 10 days. After germination and at the four-leaf growth stage the seedlings were transferred to half-strength Hoagland medium (for 10 days). Different levels of salinity stress (0, 40, 80, and 120 mM) and SNP (0, 50, and 100 mM) were added. The mediums of roots were regularly aerated by a pump and the Hoagland solutions were changed every 48 hours (Fig. 1).

### Evaluation of chlorophyll content

Fresh samples of apical leaves of a similar age were collected and washed thoroughly with distilled water. Approximately, 0.1 g of leaves was weighed and placed in a mortar; then, 2 ml of 80% acetone was added to the samples and the leaves were gently crushed till the mixture was formed in a uniform state. The samples were centrifuged at 6000 rpm for 15 min. A spectrophotometer device (UV-1201, Japan) with wavelength of 663 and 645 nm was set to read the absorption of chlorophyll.

### Flavonoid assay

Flavonoid contents were determined following the method described by Zhishen et al (1999).

### Total soluble sugars content (TSS)

The total soluble sugars content was determined according to Dubois et al., (1956). The fresh leaves (100 mg) were incubated in 2 mL of 80% ethanol for 48 h in the dark. Thereafter, all the alcohol was evaporated by putting the test

tubes in a water bath at 70 °C. After cooling, 20 mL of distilled water was added and 1 mL of the solution was used to react with 1 mL of phenol 5%. Then 2 mL concentrated sulfuric acid was added and the test tubes were placed in a bath ice for 25 min.

### Antioxidant enzyme activity

Leaf extract was used to measure enzymes activities as described by Ahmad et al (2015). Briefly, fresh leaves (10 g) were crushed in 50 volumes of 100 mM Tris-HCl (pH 7.5) containing 5 mM DTT (Dithiothreitol), 10 mM MgCl<sub>2</sub>, 1 mM EDTA (ethylene diamine tetra acetic acid), 5 mM magnesium acetate, 1.5% PVP-40, 1 mM PMSF and 1 µg.ml<sup>-1</sup> aproptinin. The homogenate was filtered using a cheese cloth and centrifuged for 15 min. at 10,000 rpm. The enzyme-containing supernatant was collected after centrifugation.

### RNA extraction, cDNA synthesis, and qRT-PCR

Total RNA was isolated from root and leaf tissues using the RNA kit (RNA BIOTECHNOLOGY CO, Iran). First-strand cDNA was synthesized from 2 mg of total RNA with oligo (dT) and MMLV reverse transcriptase (200U.l<sup>-1</sup>, Invitrogen) according to the manufacturer's instructions. Quantitative real-time PCR (qPCR) was done under the following cycle conditions: 10 min at 95 °C followed by 40 cycles of 15s at 95 °C, and 1 min at 60 °C. The *ACTIN* tomato gene (*ACT*) was used as an internal reference gene. Genes and their corresponding primers are shown in Table 1. PCR reactions were carried out in 96-well optical reaction plates (Applied Biosystems). Reaction included 50 ng of cDNA sample as a template, 400 nM forward and reverse primers, and SYBR green qPCR master Mix-Rox.

### Statistical Analysis

A completely randomized design with factorial arrangements and three replications was used for each treatment. Data were subjected to statistical analyses using SAS 9.1 statistical software and comparison of means was based on Duncan's test at 5%.

Table 1  
Primer sequences used for genes expression

Genes names	Primer sequence	
<i>SIWRKY39</i>	Forward	GCGGTAATGCCAAGACAAAC
	Reverse	TCAGTTCCTGGTGATTTACGC
<i>HKT1.1</i>	Forward	TCTAGCCCAAGAACTCAAT
	Reverse	CTAATGTTACAACCTCAAGGAATT
<i>ACTIN</i>	Forward	GAAATAGCATAAGATGGCAGACG
	Reverse	ATACCCACCATCACCCAGTAT



Fig. 1. Effect of NaCl and SNP (mM) treatments on tomato plants after 20 days (Left to Right); Top row: control, 40 mM NaCl, 80 mM NaCl, 120 mM NaCl, 50 mM SNP, 50 mM SNP, and 40 mM NaCl; Bottom row: 50 mM SNP and 80 mM NaCl, 50 mM SNP and 120 mM NaCl, 100 mM SNP, 100 mM SNP and 40 mM NaCl, 100 mM SNP and 80 mM NaCl, and 100 mM SNP and 120 mM NaCl

### Measurements

Amounts of chlorophyll a and b were calculated using the following formulas (Arnon, 1949):

$$1) \text{ Chlorophyll a} = 12.25(A663) - 2.55(A645) \times V/W$$

$$2) \text{ Chlorophyll b} = 20.31(A645) - 4.91(A663) \times V/W$$

where V = volume of extract (ml) and W = weight of tissue (mg).

Table 2  
Effects of salinity stress, SNP concentrations, and interaction between them on measured parameters of tomato

NaCl (mM)	SNP (mM)	Chl. total (mg g <sup>-1</sup> )	Flavonoid (Quercetin equivalent) (μmol g <sup>-1</sup> FW)	TSS (mg g <sup>-1</sup> FW)	APX (Units mg <sup>-1</sup> protein)	SOD (Units mg <sup>-1</sup> protein)	<i>HKT1.2</i>	<i>SIWRKY8</i>
0	0	1.373 d	0.40 i	31.8 j	0.35 i	20.45 h	3.65 g	0.85 i
	50	1.651 b	0.74h	31.9 j	0.42 hi	21.11 h	1.75 h	1.59 h
	100	1.776 a	0.99 g	31.9 j	0.65 h	20.81 h	1.75 h	1.60 h
40	0	1.165 f	1.07g	72.4 f	1.35 g	25.85 f	18.85 c	1.40 h
	50	1.431 c	1.82e	63.8 g	1.80 f	32.12 d	10.05 e	3.84 e
	100	1.440 c	2.05 d	47.6 i	2.15 e	36.25 c	7.71 f	5.36 c
80	0	0.946 g	1.20 f	97.7 c	1.55 fg	29.49 e	24.85 a	2.31 g
	50	1.276 e	2.05 d	75.1 e	2.75 d	36.10 c	15.85 d	4.50 d
	100	1.306 e	2.86 b	60.0 h	3.21 c	43.65 a	9.81 e	7.35 b
120	0	0.811 h	1.78e	127.9 a	1.75 f	28.95 e	25.05 a	3.31 f
	50	1.186 f	2.65 c	113.1 b	3.75 b	40.85 b	20.41 b	8.65 a
	100	1.258 e	3.51 a	84.8 d	4.20 a	43.85 a	19.50 bc	8.75 a

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

The absorbance of flavonoids was measured at 510 nm (spectrophotometer, S 2100 S UV, USA), and the amount of flavonoids was expressed in equivalents of catechin (mg eq. C g<sup>-1</sup> DW), used as standard. The measure of total soluble sugars content was at 490 nm (spectrophotometer, S 2100 S UV, USA), and L-glucose used as a standard. Moreover, the activity of SOD was expressed as enzyme unit (EU) mg<sup>-1</sup> protein. One unit of SOD was defined as the amount of protein causing 50% decrease in the SOD-inhibitable NBT reduction. The method of Nakano and Asada (1981) was followed to determine the ascorbate peroxidase (APX) activity, with the absorbance read at 290 nm (spectrophotometer, S 2100 S UV, USA). The APX activity was expressed as EU mg<sup>-1</sup> protein. Also, relative quantification was performed by applying the 2DDCt method (Valverde et al., 2019; Livak and Schmittgen, 2001).

## Results

### Chlorophyll a, b and total

The results of this study showed that different levels of salinity stress and SNP application affected the amounts of chlorophyll a and b, so that the lowest amount of chlorophyll a and b was observed under 120 mM salt and the

highest amount of these pigments were obtained under 100 mM of SNP (Fig. II), (Table 2).

### Flavonoid contents

The flavonoid content increased significantly with increasing NaCl concentrations compared to control. The lowest amount of flavonoid (40.21 μM g<sup>-1</sup> FW) was obtained in the control plant in non-stress conditions (Table 2).

### Total soluble sugars content (TSS)

Salt stress caused a significant rise in soluble sugars contents (Table 2). The highest soluble sugars content was related to 120 mM of NaCl. In interaction of salinity and SNP, soluble sugars contents had significantly decreased and maximum soluble sugars contents were observed in high level of salinity and 0 mM SNP (Table 2).

### Antioxidant enzymes activities (APX and SOD)

In this experiment, application of SNP increased the activity of APX in tomato plants and as a result, improved the negative effects of salinity stress so that the highest APX enzyme content (4.20 Units mg<sup>-1</sup> protein) was observed in 120 mM salt and 100 mM SNP and the lowest APX

enzyme (0.35 Units  $\text{mg}^{-1}$  protein) was found in control plants (Table 2). Moreover, SOD activities increased significantly in salinity stress with application of SNP. The highest SOD activities (43.85- and 43.65-Units  $\text{mg}^{-1}$  protein) were obtained in 120 mM salt and 100 mM SNP, and 80 mM salt and 100 mM SNP, respectively (Table 2).

### Genes expression

Our results indicated that increasing the amount of SNP affected expression of the *SLWRKY 8* gene. The highest expression of the *SLWRKY 8* genes was recorded in seedlings treated with 100 Mm SNP and 120 Mm NaCl (Table 2), (Fig. III).

### Discussion

#### Chlorophyll a, b and total

Significant decreases in chlorophyll a, b, and total chlorophyll under salt stress observed in this study were in agreement with findings of previous studies (Turan et al., 2007; Taffouo et al., 2010). Salinity causes metabolic disorders in plants such as a decrease in chloroplast activity and photosynthesis, increase in chlorophyllase enzyme activity, and an increase in respiration followed by reduction in chlorophyll contents (Taibi et al., 2016; Parida and Das, 2005; Blunden et al., 1996). The use of SNP increased the chlorophyll contents. SNP prevents intercostal chlorosis in leaves and increases the chlorophyll contents. NO enhances the iron absorption by plants under stress conditions; iron plays an important role in the structure of chloroplast protein units (Ashraf and Bashir, 2003).

### Flavonoid

In addition to enzymatic antioxidants, plants possess a variety of non-enzymatic molecules which play a substantial role in counteracting oxidative stress. They include flavonoids, ascorbic acid, carotenoids, and phenolic compounds (Schafer et al., 2002). Flavonoids are frequently induced by abiotic stress and have roles in promoting the plant protection mechanisms (Grace and Logan, 2000). Several

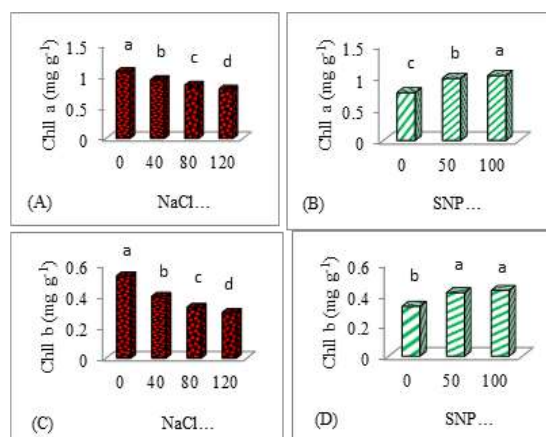


Fig. II. (A): Effects of NaCl on Chl. A; (B): SNP effects on Chl. a; (C) effect of NaCl on Chl. b; (D): SNP effects on Chl. B

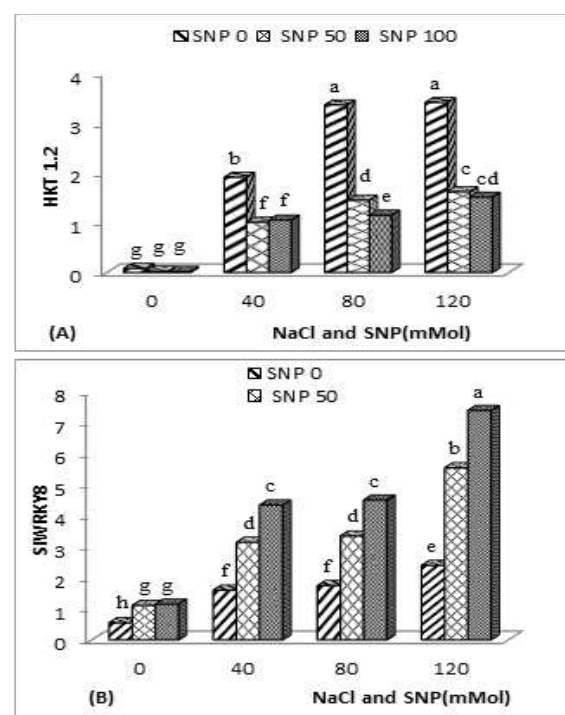


Fig. III. (A): Interaction of salinity and SNP on HKT 1.2 (A) and SLWRKY8 genes expression of tomato (B).

flavonoids act as potential inhibitors of the enzyme lipoxygenase, which converts the polyunsaturated fatty acids to oxygen-containing derivatives (Nijveldt et al., 2001). These compounds accumulate in plant tissue and could help to protected them from damaging effects and may help to inhibit lipid peroxidation in plants under stress (Potapovich and Kostyuk, 2003).

### Total soluble sugars content (TSS)

Salinity significantly increased the total soluble sugar while SNP application reduced it as compared with control samples. Sugars from organic solutes involved in osmotic adjustment maintain cells turgidity and sustain the stability of proteins and cell membranes (Ashraf and Harris, 2004).

Accumulation of soluble sugars such as sucrose, glucose and fructose are closely related to stress tolerance in plants. SNP application reduced the total soluble sugar compared to the control in salinity and non-salinity conditions. NO has been shown to increase the photosynthetic function and stimulate the metabolism of carbohydrates. The reduction of soluble sugar contents in stress through applying SNP can be due to SNP's ability to improve the plant growth and soluble-sugar intake (Saed-Moucheshi et al., 2014).

### Antioxidant enzymes activities (APX and SOD)

Plants use a number of enzymatic and non-enzymatic antioxidants such as APX, SOD etc. to prevent oxidative damage and reduce the level of ROSs in response to salt stress (Kaymakanova et al., 2008). These enzymes are highly important in regulating H<sub>2</sub>O<sub>2</sub> intracellular levels (Azevedo-Neto et al., 2006). It is striking that the salt induced APX activation in the SNP treatment, and this was accompanied by a higher increase in SOD activity. For that reason, it may be supposed that the SOD and APX have probably the same roles in the detoxification of H<sub>2</sub>O<sub>2</sub> under salinity (Taïbi et al., 2012; Ozgur et al., 2013). Results of this experiment are in agreement with reports suggesting that APX activity, coordinated with SOD activity and plays a critical protective role under salinity (Taïbi et al., 2012; Demirel and Turkan, 2005).

### Genes expression

Involvement of *WRKY* factors in plant salt adaptation was shown for *WRKY* which increased salt tolerance in many plant species (Huang et al., 2012; Sun et al., 2014; Bakshi et al., 2014). These

transcription factors are well interconnected with other complex signaling pathways corresponding to cell homeostasis, photosynthesis, oxidative pathway, and enzyme activity (Bakshi et al., 2014). Reports have shown that *SLWRKY* genes acts as an agent for adjusting the plants' traits in stress condition (Campbell et al., 2017).

In this study the highest *WRKY* gene expression was observed under the highest SNP concentration. Salinity stress also increased the expression of *HKT1.2* gene in roots. With an increase in the SNP concentration, expression of the *HKT1.2* gene significantly reduced in comparison with the control. The use of SNP reduces the expression of some genes (*HKT*) while it increases plant pigmentation and antioxidant enzymes activities to mitigate stress conditions. The *HKT* gene family improves salt tolerance by regulating ion transport (Gupta and Huang, 2014).

In tomato, *HKT1.1* and *HKT1.2* are responsible for the major QTL involved in Na<sup>+</sup> and K<sup>+</sup> homeostasis (Asins et al., 2012). In Arabidopsis, *HKT* transporters protect the plant from the adverse effects of salinity by preventing excess Na<sup>+</sup> accumulation in leaves. Schroeder et al. (2013) suggested that *HKT* class I transporters remove the excess Na<sup>+</sup> from xylem, protecting the photosynthetic leaf tissues from the toxic effect of Na<sup>+</sup> (Chawla et al., 2013). But, unlike the *WRKY* gene, *HKT1.2* gene showed a high expression in the absent of SNP, and SNP treatment reduced its expression. Transcription factors such as *WRKYs* family are really important in tolerance to salinity stress. They have a role in expression activation of downstream genes and protection of plants in stress conditions (Wang et al., 2018). Researchers reported that the *GmWRKY21*, *TaWRKY2* and 19, *WRKY8*, *ZmWRKY58* increased under salt and drought stress in soybean, wheat, Arabidopsis, and *Zea mays*, respectively (Zhou et al., 2008; Niu et al., 2012; Hu et al., 2013; Cai et al., 2014).

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