

Interaction effects of nitric oxide and salicylic acid in alleviating salt stress of *Gossypium hirsutum* L.

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Abstract

The effects of sodium nitroprusside (SNP, a nitric oxide donor), salicylic acid (SA), and their combination on some physiological parameters of cotton (*Gossypium hirsutum* L.) seedlings grown under saline conditions were studied. 100 mM NaCl resulted in a decrease in photosynthetic attributes and chlorophyll content, and induced an increase in the level of electrolyte leakage, malondialdehyde (MDA) content, $O_2^{\cdot-}$ generation rate, H_2O_2 and proline contents. 0.1 mM SNP decreased Na^+ and Cl^- accumulation as well as the lipid peroxidation level and $O_2^{\cdot-}$ accumulation in leaves, which contributed to alleviate oxidative stress in cotton leaves. 0.1 mM SA alleviated the oxidative stress by increasing K^+ and Mg^{2+} absorption and osmotic regulators accumulation, and decreasing the H_2O_2 content. However, the protective action of NO and SA (especially 0.05 mM SNP and 0.05 mM SA) against salt-induced oxidative damage was more efficient than the separate effects of SA and SNP. We observed that the accumulation of osmotic regulators was apparently accelerated by 0.05 mM SNP and 0.05 mM SA. As well, it was observed that they had synergistic effects in antioxidant system and ion absorption. Thus, the combination of SNP and SA can be a new indicator of a protective mechanism to lower lipid peroxidation, improve ion absorption, activate the metabolic of osmotic-regulated substances and facilitate the membrane transport to detoxify reactive oxygen species (ROS).

Keywords: Antioxidant enzymes, cotton, nitric oxide, NaCl, oxidative stress, salicylic acid

Abbreviations: CAT-Catalase; DW-Dry weight; FW-Fresh weight; H_2O_2 -Hydrogen peroxide; MDA-Malondialdehyde; NaCl-Sodium chloride; NO-Nitric oxide; $O_2^{\cdot-}$ -Superoxide anion; POD-Peroxidases; ROS-Reactive oxygen species; SNP-Sodium nitroprusside; SA -Salicylic acid; SOD-Superoxide dismutase

1. Introduction

Salinity is one of the important factors affecting growth and metabolic responses of crops throughout the world (Deinlein *et al.*, 2014). Salt-affected plants deteriorate as a result of ROS production such as the superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$) and singlet oxygen (1O_2) that can damage mitochondria and chloroplasts by disrupting cellular structures (Tariq *et al.*, 2011). Excess of ROS triggers phytotoxic reactions such as lipid peroxidation, enzymes inactivation and protein degradation. These changes lead to osmotic and ion-specific effects as well as to imbalances in plant nutrition, which may range from deficiencies in several nutrients (Qadir *et al.*, 2006). To mitigate and repair the damage, plant cells detoxify ROS by the synchronous action of various antioxidants constituted of non-enzymatic and enzymatic components. Superoxide dismutase (SOD), peroxidase (POX), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) are the enzymatic antioxidants, while water soluble components (sugars and proteins) and the lipid-soluble components (carotenoids and α -tocopherol) are the non-enzymatic antioxidants (Sheetal *et al.*, 2012). Hence, the alleviation of the oxidative damage and increased resistance to salinity and other environmental stresses are often correlated with an efficient antioxidative system and an accumulation of water-soluble component.

Nitric oxide (NO) is a small, highly diffusible gas and an ubiquitous bioactive molecule. Its chemical properties make NO a versatile signal molecule that functions through the interactions with cellular targets via either redox or additive chemistry (Lamattina *et al.*, 2003). Undoubtedly, there are many studies show that NO itself is the plant signaling molecule, and today it is known to play a crucial role in regulating many key physiological processes in different plants, such as in

rice, cucumber, mung bean and so on (Panda *et al.*, 2011). Various mechanisms contribute to NO-induced increase of salt tolerance. First, NO might be involved in increasing the antioxidant content and antioxidant enzyme activity to scavenge ROS. Second, NO might increase risk element accumulation in root cell walls and decrease the risk element accumulation in the soluble cell fraction to maintain the cellular redox homeostasis and regulation of the leaves (Panda *et al.*, 2011; Manai *et al.* 2014). Finally, NO could function as a signaling molecule in the cascade of events leading to changes in gene expression under risk element stress (Xiong *et al.*, 2009). However, an excess amount of NO may interact with $O_2^{\cdot-}$ to form peroxynitrite (ONOO \cdot), which has been reported to damage lipids, proteins and nucleic acids. Nevertheless, $O_2^{\cdot-}$ and H_2O_2 are more toxic than NO and ONOO \cdot . This type of dual roles of NO as a potent oxidant or effective antioxidant generally depends on the status of the environments (Panda *et al.*, 2011) and also contents.

Salicylic acid (SA) is another signaling molecule or chemical messenger and its role in the defense mechanism is well-established under stress conditions (Gunes *et al.*, 2005). A large number of studies have reported the effects of exogenous SA on alleviating environment stress, including salt (Misra *et al.*, 2009), heavy metal (Sanjib *et al.*, 2007), water and chilling, by the stimulation of growth. SA has been reported to ameliorate the adverse effects of salinity by improving the functioning of ion uptake and transport, membrane permeability, and antioxidative enzymes (Gunes *et al.*, 2005). Furthermore, SA is also considered to be a signaling molecule that plays a key role in growth, development, and defense responses in plants under stress conditions. Although a few authors have reported the effects of exogenous NO and SA alleviating salt toxicity in different plants by various

mechanisms, information is still scant regarding the influences of NO and SA on salt tolerance improvement in cotton seedlings.

Cotton (*Gossypium hirsutum* L.) has been considered as an important crop for fibre production in several countries. This species can grow well in saline areas, indicating tolerance to salt stresses (Silva *et al.*, 2010; Wang *et al.* 2014); however, it is sensitive to salt in the seedling stage (Liu *et al.*, 2013). Therefore in the present study, we suppose that NO and SA may ameliorate toxic effects of excess Na on cotton. Furthermore, it is also of a great interest to know whether exogenous NO and SA could act as a regulator of antioxidant intervention strategy in preventing leaf oxidative stress in response to NaCl stress to improve the oxidation resistance capacity, and to get a better understanding of how plants adjust to an adverse environment.

2. Materials and Methods

2.1. Plant materials and experimental design

Before sowing, healthy seeds of cotton (*Gossypium hirsutum* L.) were surface-sterilized with 1% sodium hypochlorite for 10 min, and then vigorously rinsed with distilled water. The seeds were sown in plastic pots filled with matrix. The pots were arranged in a growth chamber (28/20 °C; day/night, light intensity 150 $\mu\text{mol}/\text{m}^2/\text{s}$, 14-hour photoperiod, 60 % relative humidity) to cultivate. After germination, 7-day-old seedlings were cultivated in a hydroponics system in the growth chamber. 100 mM NaCl (Sigma, USA) without or with SNP (0.05 or 0.1 mM, Sigma, USA) or SA (0.05 or 0.1 mM, Sigma, USA) were added to Hoagland solution during the third week of seedling growth (We determine the concentrations used for SNP, SA and NaCl in this study according to the experiment of ourselves (Liu *et al.*, 2013) and the experiment of other person (Dong *et al.*, 2014).

SNP was used as a donor of NO. The Hoagland solution was daily renewed.

2.2. Chemical treatment

NaCl, SNP and SA treatments were as follows: (1) control, CK; (2) 100 mM NaCl (T1); (3) 100 mM NaCl+0.1 mM SNP (NO, T2); (4) 100 mM NaCl+0.1 mM SA (SA, T3); (5) 100 mM NaCl+0.05 mM SNP+0.05 mM SA (1/2(NO+SA, T4); (6) 100 mM NaCl+0.1 mM SNP+0.1 mM SA (NO+SA, T5). The plants were sampled at 15 days after the treatment. The nutrient solution pH was adjusted to 6.8. Each of the glassware included 5 seedlings and represented one replicate, and there were three replicates per treatment. The treatment solution was changed everyday to maintain constant NaCl concentration.

2.3. Determination of NO levels

NO levels were determined according to the method described by Chen *et al.*, (2010). Fresh samples were cut into approximately 5-mm segments and immediately incubated with 100 U CAT and 100 U SOD for 5 min to remove endogenous ROS before addition of oxyhemoglobin (5 mM). After 3 min of incubation, NO was quantified by spectrophotometric measurement of the conversion of oxyhemoglobin to methemoglobin.

2.4. Determination of SPAD reading

The portable chlorophyll meter (SPAD-502, Minolta Camera Co., Japan) can estimate total chlorophyll amounts in the leaves of a variety of species with a high degree of accuracy, which is a non-destructive method. Measurements were taken at 4 locations on each leaf; two on each side of the midrib on all fully expanded leaves and then averaged (Khan *et al.*, 2003).

2.5. Determination of ion concentration

For determination of ion contents, 100 mg of ground dry material of the leaf, stem and roots were digested by adding 4 mL HClO_4 at 200 °C until a colorless liquid was formed. After digestion, each sample was brought up to a 25 mL final volume with distilled-deionized water. The Na^+ and K^+ contents were determined with a flame photometer. The Ca^{2+} and Mg^{2+} contents were measured by flame atomic absorbance spectrometry (SHIMADZU AA-6300, Kyoto, Japan). The Cl^- content was measured in accordance with Tsukada (2005).

2.6. Determination of electrolyte leakage and lipid peroxidation

Electrolyte leakage was used to assess the cell membrane permeability as described by Tariq *et al.*, (2011). The leaves were rinsed three times with distilled water. Plant material (5-mm leaf discs) was transferred to each test vial containing 10 mL of double-distilled water. The vials were sealed and then placed on a rotary shaker for 24 h. The electric conductivity (EC_1) of the content of the vial was determined, using an electrical conductivity meter (Metzer Optical Instruments, India). The vials were again sealed and autoclaved at 120 °C for 20 min. The contents of the vials were cooled at room temperature, followed by recording the electric conductivity (EC_2) again. Membrane damage was calculated using the equation: $\text{MD} (\%) = (\text{EC}_1/\text{EC}_2) \times 100$.

Malondialdehyde (MDA) content uses the thiobarbituric acid reaction method (Heath & Packer, 1968). Fresh samples (0.2 g) were ground in 3 mL of trichloroacetic acid (0.1 %, w/v). The homogenate was centrifuged at 10,000×g for 10 min and 1 ml of the supernatant fraction was mixed with 4 mL of 0.5 % thiobarbituric acid (TBA) in 20 % TCA. The mixture

was heated at 95 °C for 30 min, chilled on ice, and then centrifuged at 10,000×g for 5 min. The absorbance of the supernatant was measured at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The amount of MDA was calculated using the extinction coefficient of 155 $\text{mM}^{-1} \text{cm}^{-1}$ and expressed as $\text{nmol g}^{-1} \text{FW}$.

2.7. Determination of endogenous ROS production

The second fully expanded leaves of the plants were sampled for enzymatic analysis. Samples were homogenized in 0.05 M phosphate buffer (pH 7.8) by grinding with a mortar and pestle under ice-cold condition. The homogenate was filtered through four layers of muslin cloth and centrifuged at 12,000×g for 10 min at 4 °C, and the supernatants were used for measurements of O_2^- generation rate according to Sui *et al.* (2007). Fresh samples (1 g) were homogenized in 2 ml ice-cold acetone. Titanium reagent (2 % TiCl_2 in conc. HCl) was added to one milliliter of extract supernatant to give a Ti (IV) concentration of 2 %. The Ti- H_2O_2 complex, together with unreacted Ti, was then precipitated by adding 0.2 ml 17 M ammonia solution for each 1 ml of extract. The precipitate was washed five times with ice acetone by resuspension, drained, and dissolved in 1 M H_2SO_4 (3 ml). The absorbance of the solution was measured at 410 nm against blanks prepared similarly, however without plant tissue (Sui *et al.*, 2007).

2.8. Determination of proline content

Proline was determined as described by Simaei *et al.*, (2011). Fresh leaf tissues (0.5 g) from each treatment were homogenized in 10 mL of 3 % w / v sulphosalicylic acid and the homogenate was filtrated. The resulting solution was treated with 2.5 % ninhydrine solution and glacial acetic acid. In test tubes, the reac-

tion mixtures were kept in a water bath at 100 °C for 60 min to develop the colors. Soon after removal from the water bath, the test tubes were cooled in ice bath and toluene was added to separate chromophores. Optical density was read at 520 nm using UV-VIS spectrophotometer (Shimadzu UV-2210).

2.9. Determination of soluble sugar content

Plants from each treatment/control were washed thoroughly with distilled water, cut into small pieces, weighed, placed separately in glass vials containing 10 mL of 80 % (v / v) ethanol, and heated at 60 °C for 30 min. The extract was then filtered and diluted with 80 % (v / v) ethanol up to 20 ml. The concentrations of soluble sugar were determined in this extract as described by Giannakoula *et al.* (2008).

2.10. Enzyme extraction and enzyme assays

For determination antioxidant enzymes, 0.5 g powder of freeze-dried leaves were homogenized with 50 mM $\text{Na}_2\text{HPO}_4\text{-NaH}_2\text{PO}_4$ buffer (pH 7.8) containing 0.2 mM EDTA and 2 % insoluble polyvinylpyrrolidone in a chilled pestle and mortar. The slurry was centrifuged at $12,000\times g$ for 20 min and the supernatant was used for enzyme activities assay. The experiment was carried out at 4 °C.

Total SOD activity was assayed by the photochemical method (Rao and Sresty 2000). The 3 mL reaction mixture contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 μM nitroblue tetrazolium, 2 mM riboflavin, 10 mM EDTA and 0.1 mL enzyme extract. One unit of the enzyme activity was defined as the amount of enzyme required to result in a 50 % inhibition of the rate of nitro blue tetrazolium (NBT) reduction measured at 560 nm. SOD activity was expressed as $\text{U g}^{-1} \text{FW min}^{-1}$. Guaiacol peroxidase (POD) activity was estimated after Nickel

and Cunningham (1969) method. Activity was measured by the increase in absorbance at 470 nm due to guaiacol oxidation. The activity was expressed as $\text{U g}^{-1} \text{FW min}^{-1}$. CAT activity was determined by following the consumption of H_2O_2 at 240 nm according to the method of Cakmak and Marschner (1992). CAT activity was expressed as $\text{nmol H}_2\text{O}_2 \text{ mg}^{-1} \text{FW min}^{-1}$.

2.11. Statistical analysis

Each pot was treated as one replicate, and all the treatments were repeated three times. Excel 2003 software to process data and draw the table, the DPS software (DPS 7.05) for statistical analysis, and the least significant difference (LSD) was calculated to compare the differences between means in each treatment.

3. Results

3.1. NO Production

NO concentration in the leaves and roots increased dramatically after 5 days of NaCl treatment relative to the control (Figure 1). T1 increased leaves NO content by 37.86 % compared to CK. T2 and T4 also increased leaves NO content by 98.92 and 109.49 %, respectively. Root NO concentration was similar to that of leaves of NaCl exposure. The NO content was significantly increased in roots under NaCl stress, but this increase was also effectively enhanced by application of exogenous SNP or/and SA, especially T4 treatment. To prove that the SNP-released NO, rather than other substances associated with SNP decomposition (such as cyanide and Fe), was responsible for the SNP-induced alleviation effect on NaCl toxicity, NaCl-treated cotton seedlings were treated with $100\mu\text{M K}_4\text{Fe}(\text{CN})_6$ and $100 \mu\text{M SNP} + 100 \mu\text{M cPTIO}$ separately. And the plant fresh weight and dry weight were measured. The results indicated that treatment with neither $100\mu\text{M SNP} + 100\mu\text{M}$

cPTIO nor $100\mu\text{M K}_4\text{Fe(CN)}_6$ had an ameliorating effect on NaCl-induced inhibition of cotton seedlings growth (Figure 2). This evidence proves the hypothesis that SNP-release NO, rather than other substances of SNP decomposition, accounts for the alleviation effect on NaCl toxicity.

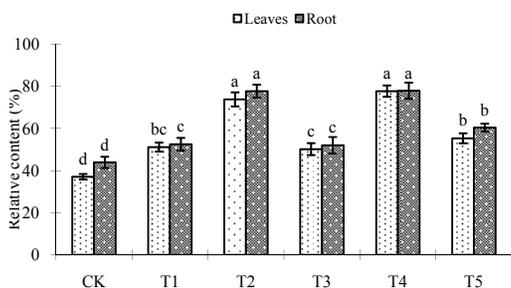


Figure 1. NO content in leaves and roots of cotton seedlings exposed to NaCl and as affected by SNP and SA. Values are the mean of three replicates.

Note: Values are the mean of three replicates. The values followed by the different letter show statistically significant differences at $P < 0.05$. CK-control, T1-100 mM NaCl, T2-0.1 mM SNP, T3-0.1 mM SA, T4-0.05 mM SNP + 0.05 mM SA, T5-0.1 mM SNP + 0.1 mM SA.

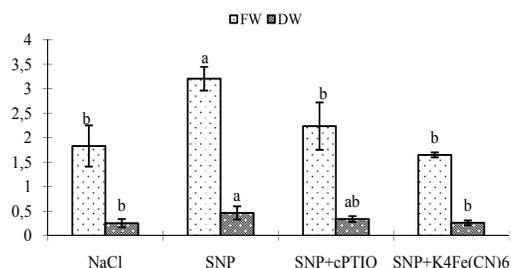


Figure 2. Effects of SNP, cPTIO and $\text{K}_4\text{Fe(CN)}_6$ on FW and DW in cotton seedlings subjected to NaCl stress. FW, fresh weight (g plant^{-1}); DW, dry weight (g plant^{-1}).

Note: Values are the mean of three replicates. The values followed by the different letter show statistically significant differences at $P < 0.05$. CK-control, T1-100 mM NaCl, T2-0.1 mM SNP, T3-0.1 mM SA, T4-0.05 mM SNP + 0.05 mM SA, T5-0.1 mM SNP + 0.1 mM SA.

3.2. SPAD reading

As shown in Figure 3A, the trend of SPAD reading change was determined during ten days. The SPAD reading was significantly decreased in T1 after 6 days, SNP or/and SA treatments can alleviate the symptoms. T1 treatment sharply decreased the SPAD reading by 32.9 % with CK on the tenth day. T2 elevated the decreased the reading by 21.34 % compared with T1 treatment, whereas T4 substantially enhanced concentration of chlorophyll on the tenth day, which was enhanced 36.67 % compared with T1 treatment.

3.3. Na, K, Ca, Mg, and Cl concentrations

As shown in Table 1, in NaCl-treated plants, Na accumulated more in shoots than in roots. In the presence of SNP or/and SA, Na content decreased in roots and shoots, as compared to T1 treatment. Cl concentration was similar to that of Na in NaCl exposure. However, in contrast to Na accumulation, salinity significantly decreased K, Ca and Mg content of cotton plants. Conversely, there was a slight rise in salt-stressed plants supplemented with SNP or/and SA than NaCl treated only (Table 1).

3.4. Membrane damages and electrolyte leakage

For the control plants, the level of MDA was low, but under T1 treatment, cotton seedlings showed higher concentrations of MDA. Both SNP and SA used in this study caused a decrease in the MDA contents (Table 2). Simultaneously, T4 (the combination of SNP and SA) is remaining comparable to CK.

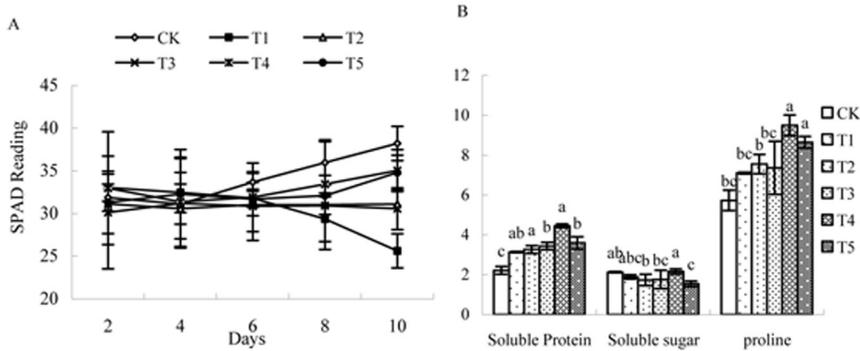


Figure 3. Effects of SNP and SA on SPAD reading (A) in cotton seedlings subjected to NaCl stress in ten days; Effects of SNP and SA for NaCl on proline, soluble protein and soluble sugar contents (B) in cotton seedlings subjected to NaCl stress. proline ($\mu\text{mol g}^{-1}$, FW); soluble protein (mg g^{-1} , FW); soluble sugar (mg g^{-1} , FW). Values are the mean of three replicates.

Note: Values are the mean of three replicates. The values followed by the different letter show statistically significant differences at $P < 0.05$. CK-control, T1-100 mM NaCl, T2-0.1 mM SNP, T3-0.1 mM SA, T4-0.05 mM SNP + 0.05 mM SA, T5-0.1 mM SNP + 0.1 mM SA.

NaCl treated plants exhibited a significant increase in electrolyte leakage. Application of SNP or/and SA decreased the level of electrolyte leakage in salt stressed plants (Table 2). T1 resulted in a 44.92 % increase in electrolyte leakage as compared with CK whereas the electrolyte leakage was decreased by 24.87 % and 13.85 % in T4 and T5 treated plants as compared with T1.

3.5. Endogenous ROS production and osmotic regulators accumulation

The results show that excess NaCl led to an enhancement in H_2O_2 content in leaves, up to 0.95 times ($p < 0.05$) higher than those of CK plants, indicating an evident oxidative stress occurred in the NaCl-stressed cotton leaves (Table 2). Application of SA or SNP, especially T4 treatment, significantly reduced H_2O_2

accumulation in NaCl-stressed plants. Similar to the moderating effect of H_2O_2 , the SNP or SA could significantly decrease the $\text{O}_2^{\cdot -}$ generation rate that induced by salt.

Soluble protein in cotton leaves increased with 100 mM NaCl levels (Figure 3B). And it increased in SNP or SA addition more sharply. T4 caused the most accumulation which was especially significant of CK. In addition, total proline showed a similar trend. T1 increased the content of soluble sugar measured in the leaves of salt-treated cotton plants. When compared to CK, salt stress increased soluble sugar of cotton plants by 2.15-fold. When SNP or SA was applied to the plants, soluble sugar in the leaves showed a little decreased. Moreover, T4 almost nullified the effect of stress caused by NaCl and improved the soluble sugar significantly.

Table 1. Effects of SNP and SA on Na (%), K (%), Ca (g kg⁻¹), Mg (g kg⁻¹), and Cl contents (mg g⁻¹) in cotton seedlings subjected to NaCl stress.

Element/Type of plant tissue	Treatments						
	CK	T1	T2	T3	T4	T5	
Na	Leaves	0.13±0.02c	1.43±0.12a	1.05±0.04b	1.34±0.04b	1.08±0.06b	1.38±0.11a
	Stem	0.43±0.02c	2.79±0.47a	2.62±0.23a	2.69±0.24a	2.18±0.08b	2.54±0.09ab
	Root	0.43±0.03c	2.63±0.29a	1.61±0.02b	2.32±0.07ab	1.88±0.12b	1.94±0.06ab
K	Leaves	2.27±0.35a	1.83±0.83b	1.99±0.36ab	2.25±0.27ab	2.22±0.06ab	2.14±0.11ab
	Stem	3.71±0.36a	1.81±0.26c	1.95±0.21c	1.92±0.10c	2.23±0.21bc	2.44±0.36b
	Root	4.27±0.09a	2.64±0.17c	3.75±0.08b	3.03±0.11c	4.08±0.51ab	4.11±0.22ab
Ca	Leaves	150.22±11.22ab	124.43±9.93c	128.43±17.92c	138.01±15.87bc	156.13±22.07a	150.12±26.14ab
	Stem	102.45±23.46a	86.60±12.45b	90.02±3.39b	94.57±14.21ab	100.62±23.32a	95.92±10.97ab
	Root	84.46±14.97a	45.09±15.42b	56.38±5.65b	51.18±3.33b	75.39±13.64a	74.56±8.90a
Mg	Leaves	2.45±0.22b	1.52±0.12d	2.10±0.19c	2.21±0.08bc	2.84±0.09a	2.82±0.17a
	Stem	2.80±0.42a	1.65±0.03c	2.12±0.04b	1.70±0.03c	1.68±0.08c	1.66±0.03c
	Root	3.83±0.12b	2.98±0.18f	3.67±0.28c	3.03±0.41e	3.96±0.64a	3.62±0.19d
Cl	Leaves	0.12±0.02e	1.63±0.10a	0.93±0.06c	1.28±0.05b	0.74±0.04d	0.87±0.06c
	Stem	0.41±0.13c	2.47±0.07a	1.92±0.03b	1.77±0.07bc	1.35±0.05d	1.67±0.18c
	Root	0.35±0.04c	1.57±0.22a	1.61±0.42a	1.55±0.05a	1.25±0.14b	1.63±0.22a

Notes: Values are the means ± SD of three replicates. The values followed by the different letter show statistically significant differences at $P < 0.05$. CK-control, T1-100 mM NaCl, T2-0.1 mM SNP, T3-0.1 mM SA, T4-0.05 mM SNP + 0.05 mM SA, T5-0.1 mM SNP + 0.1 mM SA.

Table 2. Effects of SNP and SA on MDA content, electrolyte leakage and antioxidant enzymes in cotton seedlings subjected to NaCl stress. MDA content ($\mu\text{mol g}^{-1}$ FW); Electrolyte leakage (%); $\text{O}_2^{\cdot -}$ generation rate ($\mu\text{mol g}^{-1} \text{h}^{-1}$); H_2O_2 ($\mu\text{mol g}^{-1}$ FW); CAT, catalase, ($\mu\text{mol H}_2\text{O}_2 \text{mg}^{-1} \text{min}^{-1}$ FW); POD, peroxidase, ($\text{U mg}^{-1} \text{min}^{-1}$ FW); SOD, superoxide dismutase (units g^{-1} FW).

	Treatments					
	CK	T1	T2	T3	T4	T5
MDA	10.40±0.14e	28.97±1.63a	19.59±0.13b	18.01±0.89c	11.98±0.68d	17.64±0.55c
Electrolyte leakage	40.62±1.33d	58.86±6.74a	45.78±2.38c	53.44±5.36b	44.22±2.44c	50.71±2.36b
$\text{O}_2^{\cdot -}$ generation rate	17.17±1.80b	26.40±2.52a	18.13±1.44b	19.06±2.62b	16.82±0.45b	18.37±1.12b
H_2O_2	1.38±0.76c	4.59±0.35a	2.30±0.68b	2.36±0.28b	1.71±0.45bc	1.91±0.37bc
CAT	3.90±0.26a	2.38±0.11b	2.64±0.10b	3.70±0.59a	3.72±0.41a	4.00±0.06a
POD	590.11±86.01a	496.16±33.61b	541.97±45.65ab	521.51±34.25ab	582.66±32.70a	547.30±26.86ab
SOD	92.51±3.46a	72.31±0.80b	81.72±5.69ab	79.38±18.65ab	85.09±2.91ab	88.02±4.91a

Notes: Values are the mean of three replicates. The values followed by the different letter show statistically significant differences at $P < 0.05$. CK-control, T1-100 mM NaCl, T2-0.1 mM SNP, T3-0.1 mM SA, T4-0.05 mM SNP + 0.05 mM SA, T5-0.1 mM SNP + 0.1 mM SA

3.6. Antioxidant enzymes

Since NaCl treatment caused oxidative stress in cotton plants, we examined the behavior of the antioxidant enzymes next. SOD activity was inhibited by 17.85 % in leaves of cotton plants treated with T1 as compared with CK. When SNP and SA were added to nutrient solution containing NaCl, SOD activity increased by 17.67 % and 27.94 % respectively in leaves. The POD activity in leaves of T1-treated plants decreased by 15.92 % as compared with CK. When NaCl-stressed plants were treated with SA or SNP, POD activity significantly increased in leaves. Whereas treated with T4, in NaCl-treated plants, the POD activity was increased by 17.43 % compared with NaCl-treated alone. The CAT activity in leaves of T1-treated plants was decreased by 38.94 % as compared with CK. When SA and SNP were added to nutrient solution containing NaCl, the CAT activity of leaves and roots increased. In NaCl-treated plants, SNP or SA, especially T4 improved the enzyme activity in comparison to NaCl -treated plants.

4. Discussion

Nitric oxide (NO) as a ubiquitous signal molecule participates in major physiological processes in plant growth and development, and in response to biotic and abiotic stresses (Wilson, 2008). A notable increase of NO content was detected in the cotton seedlings exposed to 100 mM NaCl, whereas there was more increase in that of the SNP treatment under NaCl-stress (Figure 1). NaCl-induced inhibition of fresh and dry weight was significantly reversed when cotton seedlings were treated with SNP (Figure 2), compared with the treatment of the cPTIO and $K_4Fe(CN)_6$. These evidences proved the hypothesis that SNP-released NO, rather than other substances of SNP decomposition, accounts for the alleviation effect on NaCl toxicity.

In this work, we analyzed the possible role of exogenous SNP and SA in the modulation of the antioxidant defense system against NaCl stress in cotton seedlings. Evidences have been demonstrated that the interaction between NO and SA had synergistic effects in decreasing of the damages induced by all kinds of abiotic stress (Simaei *et al.*, 2011; Gil'vanova *et al.*, 2012). In the present study, we were able to increase the cotton antioxidant capacity and its components under salinity by applying NO and SA. Measurement of SPAD reading under salinity conditions explained that NaCl has the negative effects of chlorophyll contents. Treated with NO or / and SA, especially the combination of SNP and SA, decreased these damages induced by salt (Figure 3A). The decrease in SPAD reading in salt-affected plants might be attributed to the possible oxidation of chlorophyll and other chloroplast pigments coupled with instability of the pigment protein complex under salt stress (Tariq *et al.*, 2003). Exogenous SNP and SA lead to the promotion of protective reactions to the photosynthetic pigments and induce a pre-adaptive response to salt stress (Simaei *et al.*, 2011), which confirms our results.

In addition, the correlations between and among various non-enzyme indices (e.g., ions content, leaf electrolyte leakage, MDA content, H_2O_2 content, O_2^- generation rate, proline contents, and soluble sugar) and enzyme indices (e.g., CAT, POD, and SOD) in cotton seedlings subjected to salt stress were analyzed. Results showed that significant correlations existed between these non-enzyme indices and enzyme parameters. The contents of Na and Cl increased in leaves, stems and roots of the cotton under NaCl treatment, while an opposite trend for K, Ca and Mg content was obtained (Table 1). Na and Cl, entering into the cytoplasm through the cell membrane, lead to their accumulation in the cytoplasm, decreasing the availability of some of the essential nutrients as well (Deinlein, 2014). On the other hand, the excess of Na in the nutrient media led to the

accumulation of H_2O_2 in cotton leaves and SNP/SA led to eliminating the accumulation (Table 2). High concentration of Na caused membrane disorganization. As the second messenger, Ca in cytosolic was increased, might induce further physiological response including the expression of osmotic responsive genes for salt adaptation in plants. This facilitation could be attributed to the induction of antioxidant responses and elevated Ca uptake that protects the plants from the oxidative damage by SNP and SA (El-tayeb *et al.*, 2005). In addition, alteration of mineral uptake from NO and SA applications may be one mechanism for the alleviation of salt stress.

Also, excessive accumulation of Na in the cytosol has an adverse effect on maintaining a stable electrical conductivity of solution. Electrolyte leakage indicates cell membrane injury when plants are subject to salinity stress. The leaves electrolyte leakage was significantly affected by the salt stress treatments, but not the NO/SA concentrations and their interactions (Table 2). Maintenance of the integrity of cellular membranes under salt stress is considered an integral part of the mechanism of salinity tolerance (Tariq *et al.*, 2003). Studies suggested the SA facilitated maintenance of membrane functions through induction of antioxidant mechanisms and elevated ion uptake, thereby protecting the plants against the oxidative damage (El-tayeb *et al.*, 2005). While, NO effectively reduce the level of ROS generated during stress, and thus, limit oxidative damage in plant cells (Arasimowicz *et al.*, 2007). According to the cited authors, the application of NO and SA, which facilitated maintenance of membrane functions under salt stress, could be an indicator of the build-up of a protective mechanism to reduce cytoplasm fluid leakage induced by salt stress.

Application of NO and/or SA reduced NaCl-induced increase in the content of MDA (Table 2). It was suggested that superoxide anion ($O_2^{\cdot-}$), H_2O_2 , radicals of hydroxyl ($OH\cdot$), lipid alcoxyl ($LO\cdot$), and lipid peroxy

($LOO\cdot$), which are produced during salinity, can be the major factors responsible for lipid peroxidation. The level of MDA, product of lipid peroxidation, has been considered an indicator of oxidative damage (Li *et al.*, 2008). Decreased lipid peroxidation against oxidative stress, may assist in overcoming severe stresses and providing a rapid recovery. Li (2008) also indicated that salt stress could cause damages to the integrity of the cellular membrane such as lipids that was sensitive to oxidative stress. SA was reported to inhibit production of hydroxyl radical and to decrease the content of MDA (Gunes *et al.*, 2005). Proline is able to scavenge hydroxyl radical and stabilize the structure and function of macromolecules and structures, such as DNA, protein, and membranes (Misra *et al.*, 2009). It can be clearly seen that NaCl induced proline accumulation (Figure 3B). It has been reported that SA, through the induction of ABA-mediated proline production, can decrease the deleterious effects of salt stress and water deficit in wheat seedlings (Sakhabutdinova *et al.*, 2003). Because MDA and proline are often considered a compatible solute involved in osmotic adjustment under stress, reduction in the level of MDA and increase in the level of proline in (NO+SA)-treated plants indicate the stress amelioration role of NO and SA that might be responsible for maintenance of plant growth under stress.

Salinity has been shown to reduce water uptake by roots because of lowered osmotic potentials of the substrate, and to cause changes in metabolic activities leading to the reduction in plant growth (Jaleel *et al.* 2007). The overall reduction in the growth of cotton seedlings might be ascribed to the ill effect of salt stress on the various physiological processes such as photosynthesis, ion homeostasis, accumulation of compatible solutes, and activities of antioxidant enzymes. The content of soluble protein is significantly increased in the leaves at the NaCl-treated (Figure 3B). With respect to the salinized plants, soluble protein content increased

significantly in leaves with T4 treatment than other treatments. SA treatment induced more accumulation of soluble sugar of the stressed plants (Khodary *et al.*, 2009). Soluble sugar can be involved in, or related to, ROS-producing metabolic pathways. It might either directly detoxify ROS in chloroplasts and vacuoles or indirectly stimulate the classic antioxidative defense systems (Khodary *et al.*, 2009). In this regard, it is assumed that, 1/2 (SNP+SA) may stimulate hydrolysis of proteins, providing a pool of compatible osmolyte, which is important in osmotic adjustment in the presence of sodium.

Furthermore, electron transport processes, such as photosynthesis and respiration, generate basal levels of H_2O_2 , which is increased in response to stress (Simaei *et al.*, 2011). Under salt stress, plants are overloaded with ROS, which inhibit several plant processes and cause damage to plants in different ways. Thus, to maintain metabolic functions under stress, scavenging of ROS is essential. ROS scavenging depends on detoxification mechanisms provided by antioxidant enzymes. The increase in the activities of CAT and SOD in NaCl-treated plants has been documented by various workers investigating salinity stress in several plant species (Tariq *et al.*, 2011). In this regard, CAT appears to play an essential protective role in scavenging ROS in coordination with SOD. SOD initiates detoxification of singlet oxygen by producing H_2O_2 , which is eliminated by its conversion to H_2O in subsequent reactions. It was suggested that H_2O_2 was key regulators of plant responses to a range of endogenous signals, which are involved in developmental processes, such as seed germination, and root development (Khan *et al.*, 2003). SNP and SA-induced CAT and SOD activities increase indicate that they can play a critical role in modulating the cell redox balance, thereby protecting plants against oxidative damage. In our study, the CAT and SOD activities were higher in T5 (0.1mM NO+0.1mM SA) were higher than T4 (0.05 mM SNP + 0.05 mM

SA), which maybe associated with promoting the absorption of K, but the reason need further research. Contents of endogenous H_2O_2 and $O_2^{\cdot-}$ were measured to determine the internal ROS status induced in cotton exposure to salinity treatments. Contents of H_2O_2 and $O_2^{\cdot-}$ were higher in plants treated with NaCl, indicating an increase in ROS level of the cells in salt-treated plants. Among the antioxidant enzymes, CAT and SOD are well known ROS scavengers. When the ROS contents exceed their threshold level under stress, they are scavenged by these enzymes. SNP and SA treatment increased CAT and SOD activities to scavenge the elevated level of ROS in this study.

5. Conclusion

In summary, SNP combined with SA (0.05 mM SNP+0.05 mM SA) could play a better role to reduce oxidative stress unlike them alone, improve Ca^{2+}/Mg^{2+} and decrease Na^+ absorption, enhance the activity of antioxidant enzymes, trigger the accumulation of endogenous osmotic regulators for the facilitation of the membrane transport and ROS detoxifying. The data also showed only the optimum concentration of SNP and SA could act directly on oxidative stress to promote salt-tolerance and plant growth.

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