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Effects of exogenous nitric oxide on growth of cotton seedlings under NaCl stress

Y.J. Dong[1*,2], S.S. Jinc[3], S. Liu[1], L.L. Xu[1], J. Kong[1]

[1]College of Resources and Environment, Shandong Agricultural University, Tai'an 271018, P.R.China. [2]Shandong Provincial Key Laboratory of Eco-Environmental Science for Yellow River Delta (Binzhou University), Binzhou 256600, P.R.China. [3] Sate Key Laboratory of Crop Biology,College of Life Sciences,Shandong Agricultural University ,Tai'an 271018 P.R.China. [*]Corresponding author: yuanjiedong@163.com

Abstract

In the present investigation, the role of sodium nitroprusside (SNP, a donor of NO) in inducing salinity tolerance (100 mM NaCl) in cotton was studied. Salt stress reduced the values of photosynthetic attributes and total chlorophyll content and inhibited the activities of nitrate reductase. Furthermore, salt stress also induced oxidative stress as indicated by the elevated levels of lipid peroxidation compared to CK. The application of SNP at 1.00 mM promoted the growth and restrained superoxide anions ($O_2.^-$) generation rate. And activities of antioxidant enzymes, namely, catalase (CAT) and superoxide dismutase (SOD), were enhanced by SNP treatment. On the other hand, an increase in the K^+ content, antioxidant enzyme activities, along with a decrease in the Na^+/K^+ ratio, the contents of thiobarbituric acid reactive substances (TBARS) and malondialdehyde (MDA) were observed in the NaCl-stressed seedlings subjected to the low level (0.1 mM) SNP. These results indicated that the application of moderate SNP can be used to protect plants growth and induce its antioxidant defense system under salt stress.

Keywords: Antioxidant enzymes, cotton seedlings, mineral elements, reactive oxygen species, SNP; salt-tolerance

Abbreviations: CAT, catalase; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; NO, nitric oxide; $O_2.^-$, superoxide radical; POD, peroxidase; ROS, reactive oxygen species; SNP, sodium nitroprusside; SOD, superoxide dismutase; TCA, trichloroacetic acid

1. Introduction

NaCl stress has recently gained interest in the study of environmental stress on non-halophytic plants. NaCl stress causes a number of changes in plant metabolism, (1) low water potential, (2) osmotic stress, (3) active oxygen radicals generation ($O_2.^-$, OH^- and H_2O_2) and antioxidant enzyme inactivation, (4) ion toxicity (Ons *et al*., 2012). Reactive oxygen species (ROS) are known to serve as signaling intermediates during biotic and abiotic stresses. ROS can seriously cripple normal metabolism through oxidative damage to lipids, proteins and nucleic acids. Taken together, proline and carbohydrates have accumulated in plant tissues under saline stress, and these substances are suspected of contributing to osmotic adjustment. Recent studies had demonstrated that accumulations of ROS were associated with the antioxidant enzyme system, which has generally been considered to be an adaptive response to the stress condition.

Antioxidant enzymes, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), peroxidase (POD) and catalase (CAT) can limit or scavenge the generation of ROS. However, high concentrations also cause an imbalance of the cellular ions resulting in ion toxicity, osmotic stress and production of ROS (Cramer *et al*., 1994). The modulation of the activities of these enzymes may be important in the resistance of plant to environmental stress.

Nitric oxide (NO), a reactive nitrogen species, acts as a signaling molecule with multiple biological functions in plants. NO had been reported to exert a protective effect in response to heavy metal stress (Singh *et al*., 2008), UV radiation stress (Shi *et al*., 2005) and disease resistance. Recent data by Zheng *et al*. (2009) indicated that NO serves as a signal in inducing salt tolerance by increasing the activities of SOD and CAT, decreasing the contents of MDA and the $O_2^{\cdot -}$ generation rate in the mitochondria. For instance, under salinity condition, the exogenous NO can enhance salt tolerance by stimulating proton-pump activities and Na^+/H^+ antiport in the tonoplast, and increasing the K^+/Na^+ ratio (Wang *et al*., 2009). Although the relationship between NO and ROS has been revealed, it is not a straightforward positive correlation. Therefore, it's urgent to further understand the physiological mechanisms between NO and NaCl tolerance, and probe into the methods increasing salinity stress tolerance in plants.

Cotton (*Gossypium hirsutum* L.) has been considered an important crop for fibre production in several countries. This species can grow well in saline areas, indicating tolerance to salt stresses; however, it is sensitive to salt in the seedling stage (Liu *et al*., 2013). For taking full advantage of saline soils, the first imperative thing is to enhance the cotton seedling salt-tolerance (Zhang *et al*., 2011). The aim of this study was to test the hypothesis exogenous application of NO was involved in the acclimation of cotton seedlings to salt stress, by quenching ROS, maintaining ion homeostasis in cells, thus helping to overcome the oxidative damage caused by salt stress.

2. Materials and Methods

2.1. Plant materials and treatment

Cotton seeds (*Gossypium hirsutum* L.) were surface sterilized with 2.5% sodium hypochlorite for 10 min and rinsed thoroughly with distilled water, then germinated on moist filter paper in an incubator at 30 °C. The germinated seeds were sown in the washed matrix in the growth chamber (28/20 °C; day/night, light intensity 150 μmol m^{-2} s^{-1}, 14 hours photoperiod, 60% relative humidity). The cotton seedlings at the second-true leaf stage were watered with one quarter-strength Hoagland nutrient solution. After one week, the seedlings were watered with half-strength Hoagland nutrient solution. Uniformly growing cotton seedlings at the 4-6 true leaf stage were transferred to glassware (Diameter of 15.5 cm, Height of 14 cm) filled with Hoagland nutrient solution, and the roots were rinsed with distilled water. At the 6-8 true leaf stage, salinity and NO treatment were started by adding NaCl and SNP to the nutrient solution. Culture solution devoid of NaCl and SNP was served as control. The nutrient solution was adjusted to pH 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 concentrations. The plants were harvested after 15 days of treatment. The experimental design is provided in Table 1.

2.2. Plant growth parameters

The plants were washed with tap water to remove adhering foreign particles. The plants from each treatment were carefully uprooted, and fresh weight (FW), stem height and root length were recorded. The roots were removed, and the individual shoot fresh weight was recorded. The shoots were dried at 80 °C for 48 h, and their dry weights (DW) were recorded.

The relative growth rate (RGR) was determined using the following formula: [ln (final FW) - ln (initial FW)]/ days (Baligar *et al*., 1993).

Table 1. The experimental design

Code name	Processing code	Content
CK	CK	Hoagland nutrient solution contains neither NaCl nor SNP
T1	NaCl	Hoagland nutrient solution contains 100 mM NaCl
T2	SNP_1	Hoagland nutrient solution contains 0.1 mM SNP
T3	SNP_2	Hoagland nutrient solution contains 0.25 mM SNP
T4	NaCl +SNP_1	Hoagland nutrient solution contains both 100 mM NaCl and 0.1 mM SNP
T5	NaCl +SNP_2	Hoagland nutrient solution contains both 100 mM NaCl and 0.25 mM SNP

Note: All the treatments in other Tables and Figures (CK and T1 to T5) are in accordance with the descriptions in Table 1

2.3. Fluorescence parameters, photosynthetic parameters and chlorophyll content

Young leaves were selected to measure chlorophyll fluorescence by using the pulse amplitude modulated system (model FMS2. Hansatech Instruments. UK) and to measure photosynthetic parameters by using the photosynthesis system (CIRAS-2, UK). They were done between 10:00-11:30 AM.

Young leaves (0.5 g of fresh weight) were powdered with liquid nitrogen, and pigments were extracted with 4 volumes of 80% (v/v) acetone until complete bleaching. The content of chlorophyll was determined according to Arnon. (1949).

2.4. Antioxidant enzymes and $O_2^{\cdot-}$ generation rate extraction and assay

To extract antioxidant enzymes, leaves were homogenized with 50 mM Na_2HPO_4-NaH_2PO_4 buffer (pH 7.8) contains 0.2 mM EDTA and 2% insoluble polyvinylpyrrolidone (PVP) using a chilled mortar and pestle. The homogenate was centrifuged at 12 000 × g for 20 min, and the resulting supernatant was used for determination of enzyme activities. The entire extraction procedure was carried out at 4 °C. All spectrophotometric analysis was conducted using a SHIMADZU UV-2450 spectrophotometer (Kyoto, Japan). SOD activity was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium following the method of Tariq *et al.* (2011). CAT activity was measured as the decline in absorbance at 240 nm due to the decrease in H_2O_2 extinction according to the method of Tariq *et al*. (2011). POD activity was measured by the increase in absorbance at 470 nm due to guaiacol oxidation (Zhang *et al*., 2012).

To measure the $O_2^{\cdot-}$ generation rate, 0.3 g of fresh leaves were ground in liquid N_2 and extracted in 3 mL of ice-cold 50 mM phosphate buffer solution (PBS) (pH 7.0). The $O_2^{\cdot-}$ generation rate was determined by monitoring the A_{530} of the hydroxylamine reaction following a modified method described by He *et al*. (2005). A 1-mL aliquot of the supernatant of a fresh leaf extract was added to 0.9 mL of 65 mM PBS (pH 7.8) and 0.1 mL of 10 mM hydroxylammonium chloride. The reaction was incubated at 25 °C for 35 min. A 0.5-mL aliquot of the solution from the reaction mixture described above was then added to 0.5 mL of 17 mM sulfonic acid and 0.5 mL of 7.8 mM α-naphthylamine solution. After a 20-min reaction, 2 mL of ether was added and mixed well. The solution was centrifuged at 1500 × g at 4 °C for 5 min. The absorbance of the pink supernatant was measured at 530 nm with a spectrophotometer. The absorbance values were calibrated to a standard curve generated with known concentrations of HNO_2.

2.5. Determination of lipid peroxidation

Lipid peroxidation was determined by measuring MDA, a major TBARS and products of lipid peroxidation. 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 5min. The absorbance of the supernatant was measured at 532 nm. The value for non-specific absorption at 600 nm was subtracted.

2.6. Proline content assay

Proline accumulation was determined as described by Bates *et al.* (1973). 0.5 g of fresh leaf tissues 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% ninhidrine solution and glacial acetic acid. In test tubes, the reaction 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. The proline concentration was determined from a standard curve and calculated on a fresh weight basis (μg proline g^{-1} of fresh weight material).

2.7. Nitrate reductase (NR) activity assay

NR activity was measured according to Yaneva *et al*. (2002). Leaf segments were homogenized in a medium containing 5 mM EDTA, 5 mM GSH, 1% (w/v) casein, 0.1% (w/v) insoluble PVP and 50 mM HEPES pH 7.5 and centrifuged for 15 min at 17 000 × g. The assay mixture for measuring NR activity contained 200 μmol KNO_3, 0.2 μmol NADH and 100 μL of the homogenate. After incubation at 30°C for 20 min, the reaction was stopped by the addition of 50 μL 1 M zinc acetate. The mixture was centrifuged 5 min at 7 000 × g and the supernatant was used to determine nitrite production by reading the absorbance at 540 nm after the addition of 1% sulphanilamide in 1.5 M HCl and 0.01% N-(1-naphthyl)-ethylenediammonium dichloride.

2.8. Na^+, K^+, Ca^{2+} and Mg^{2+} contents assay

For the determination of Na^+, K^+, Ca^{2+} and Mg^{2+} concentrations, powdered dried sample mixtures were digested in an acid mixture (HNO_3-$HClO_4$ [3:1]) and briefly centrifuged. Na^+, K^+, Ca^{2+} and Mg^{2+} concentrations were determined using an atomic absorption spectrophotometer (SHIMADZU AA-6300, Kyoto, Japan).

2.9. Statistical analysis

Excel 2003 software was used to process data and construct the tables, the SPSS software (SPSS 17.0) was used for statistical analysis, and the least significant difference (LSD) was calculated to compare the differences between means in each treatment. Means followed by different letters are statistically significant at $p \leq 0.05$.

3. Results

3.1. Plant growth parameters

NaCl stress had a strong impact on plant growth. RGR, stem height and root length exhibited significant decreases in the cotton treated with NaCl (Table 2). However, root/shoot had no significantly difference. Compared with CK and SNP treatment, RGR, stem length and root length decreased (by 30.02%, 11.28%, 10.79%), and their decrement extent under 0.1 mM concentration were less than under 0.25 mM concentration.

3.2. Fluorescence parameters, photosynthetic parameters and chlorophyll Content

Photosynthesis system is sensitive to environmental stress. The chlorophyll content and stomatal conductance had been proved to be key limiting factors on photosynthesis. Both Pn and Tr exhibited amelioration treated with SNP in the presence or absence of NaCl stress (Table 3). Increases in Pn and Tr (52.12%, 14.29%) occurred in T2. But a decrease

in Pn and Tr (13.60%, 4.76%) occurred in T4 (Table 3). The decrease in Ci (2.26%) occurred in the NaCl stress.Treatment of 0.1mM SNP and NaCl stress caused a significant increase in Ci (1.66%).

Table 2. Effects of NaCl and SNP on the growth attributes in cotton seedlings.

Treatment	RGR (g day^{-1})	Stem height (cm $plant^{-1}$)	Root length (cm $plant^{-1}$)	Root/shoot
CK	0.35±0.04a	12.77±0.25a	11.77±0.25b	0.15±0.02c
T1	0.19±0.22c	9.67±0.35c	10.43±0.40c	0.29±0.01a
T2	0.41±0.05a	13.33±0.29a	8.17±0.29e	0.19±0.01b
T3	0.28±0.03b	11.33±0.29b	9.40±0.40d	0.21±0.01b
T4	0.27±0.41b	11.33±0.76b	10.50±0.87c	0.28±0.02a
T5	0.15±0.08c	7.67±0.29d	14.23±0.64a	0.21±0.03b

Note: Values represent the mean ± S.D. (n = 3). Different lowercase letters indicate significant differences at p <0.05.

Table 3. Effects of NaCl and SNP treatments on fluorescence parameters and photosynthetic parameters in cotton leaves.

Treatment	Net photosynthetic rate *Pn* (μmol m^{-2} s^{-1})	Intercellular CO_2 concentration (Ci) (μmol mol^{-1})	Transpiration rate *Tr* (mmol m^{-2} s^{-1})	Photosystem II (PS II)	PSII maximum light energy transformation (Fv/Fm)	PSII Activity (Fv/Fo)
CK	2.59±0.05b	699.13±2.84e	0.21±0.01b	0.35±0.02b	0.81±0.01bc	4.32±0.02d
T1	0.83±0.01f	732.23±2.03b	0.16±0.01e	0.29±0.01c	0.74±0.14d	3.28±0.11f
T2	3.94±0.06a	683.33±2.49f	0.24±0.01a	0.53± 0.02a	0.82±0.01b	4.41±0.01c
T3	2.06±0.04d	714.80±0.56c	0.18±0.02d	0.52± 0.01a	0.81±0.02bc	4.55±0.01b
T4	2.28±0.04c	710.73±0.38d	0.20±0.01c	0.36± 0.01b	0.84±0.02a	5.03±0.01a
T5	1.38±0.24e	744.87±1.07a	0.16±0.02e	0.31± 0.01c	0.80±0.20c	4.07±0.02e

Note: Values represent the mean ± S.D. (n = 3). Different lowercase letters indicate significant differences at p <0.05.

As shown in Table 3, compared with CK, PSII, Fv/Fm and Fv/Fo exposed NaCl were reduced significantly (by 17.14%, 8.64% and 24.07%). Different concentrations of SNP under normal growth condition could improve PSII, Fv/Fm and Fv/Fo.

NaCl stress resulted in the usually observed decrease in chlorophyll content (Table 4). However, a significant increase in chlorophyll content was seen in the leaves treated with SNP and NaCl. Treatments with 100 mM NaCl, 0.1mM SNP, 100 mM NaCl and 0.1mM SNP all resulted in significant increases in carotenoids. However, the increase observed in T5 was lower (8.47%) than CK.

3.3. Antioxidative enzyme activities

Results showed that NaCl stress could be an influential component of environmental stress on cotton (Table 5).

Table 4. Effects of NaCl and SNP treatments on chlorophyll contents in cotton leaves.

Treatment	*Chl* a (mg kg^{-1})	*Chl* b (mg kg^{-1})	*Chl* a/ b	*Chl* a+b (mg kg^{-1})	Carotenoids (mg kg^{-1})
CK	0.85±0.15b	0.41±0.11ab	2.12±0.21c	1.25±0.04b	0.14±0.02b
T1	0.68±0.23b	0.30±0.11bc	2.30±0.04bc	0.95±0.01d	0.14±0.03b
T2	1.23±0.16a	0.52±0.07a	2.36±0.02b	1.68±0.15a	0.22±0.04a
T3	0.82±0.09b	0.35±0.03bc	2.33±0.01bc	1.10±0.04c	0.15±0.02b
T4	0.80±0.20b	0.33±0.07bc	2.45±0.10ab	1.13±0.02c	0.16±0.04b
T5	0.64±0.02b	0.24±0.03c	2.67±0.23a	0.87±0.01e	0.13±0.05b

Note: Values represent the mean ± S.D. (n = 3). Different lowercase letters indicate significant differences at $p < 0.05$.

Table 5. Effects of NaCl and SNP treatments on the SOD activity, POD activity, CAT activity, $O_2^{\cdot-}$ generation rate, the content of MDA and proline content in leaves of cotton seedlings.

Treatment	SOD activity (U g^{-1} FW)	POD activity (U g^{-1} min^{-1} FW)	CAT activity (μmol H_2O_2 mg^{-1} min^{-1} FW)	$O_2^{\cdot-}$ generation rate (μmol·g^{-1}·h^{-1} FW)	MDA content (μmol·g^{-1} FW)	Proline content (μg g^{-1} FW)
CK	257.36±13.38d	255.33±18.63c	1.13±0.12e	0.83±0.01c	0.0024±0.0002d	9.64±1.48d
T1	362.26±8.44b	510.17±21.77a	3.51±0.12bc	1.21±0.12b	0.0059±0.0012b	14.39±0.68c
T2	384.42±12.13b	396.93±24.08b	1.76±0.08d	0.95±0.04c	0.0033±0.0003c	16.86±0.66b
T3	328.08±2.60c	407.86±60.51b	2.52±0.73c	1.14±0.10b	0.0062±0.0010b	6.08±0.52e
T4	419.9720.14a	396.91±45.53b	4.11±0.18a	0.91±0.01c	0.0045±0.0005c	32.03±1.78a
T5	260.90±22.18d	459.87±36.88ab	2.69±0.19c	1.39±0.04a	0.0075±0.0012a	16.77±0.82b

Note: Values represent the mean ± S.D. (n = 3). Different lowercase letters indicate significant differences at $p < 0.05$.

An increase in SOD activity was observed under NaCl stress. Adding SNP further increased the SOD activity. Compared with CK, the SOD activity of T1 was increased by 40.76%. Table 5 showed that SOD activity was increased by 63.18% and 1.38% under T4 and T5, respectively. POD activity also showed an increase under NaCl treatment (Table 5). 65.46% and 93.13% increase was observed with T2 and T4. However, 0.25 mM SNP caused a decrease in activity under NaCl stress. NaCl stress also decreased CAT activity. A 210.62% decline was observed under NaCl stress compared with CK. SNP treatment could partially ameliorate the toxic effect of NaCl treatment on CAT activity. 0.1mM SNP was more effective in increasing the CAT activity.

3.4. Superoxide anions ($O_2^{\cdot-}$) generation rate and lipid peroxidation

Whether NO is protective or toxic to plants is found to

be quite concentration dependent. Low concentration of SNP (0.1mM) could inhibit the $O_2^{\cdot -}$ generation rate and reduce MDA content, while high concentration (0.25 mM) of SNP had opposite effects (Table 5). The $O_2^{\cdot -}$ generation rate under NaCl stress increased to 45.78% compared with CK while it increased to 14.60% and 36.68% in the presence of the two concentrations of SNP alone. But there was no pronounced difference between CK and 0.1mM SNP under NaCl stress.

MDA is an indicator of lipid peroxidation and oxidative damage to membrane. The results reported here (Table 5) showed that the accumulation of MDA was higher in NaCl treatment in cotton leaves, and the increase was remarkably prevented in the 0.1 mM SNP treatment. However, increments of MDA were more severe under 0.25 mM SNP treatment no matter under normal growth condition or under NaCl stress condition.

3.5. Proline content

Table 5 showed that a large number of proline would be accumulated under NaCl stress. Meanwhile, proline of 0.1 mM SNP and NaCl treatment accumulated more than others. Compared with CK, proline content in T1 was increased by 49.31%. Under normal condition, by adding 0.1 mM SNP, the content increased by 74.96% of CK. While T5 increased proline accumulation in leaves by 122.57% than only NaCl treated.

3.6. Nitrate reductase activity

In cotton plants grown with T4, NR activity showed the maximum activity (Figure 1). In addition, SNP supply affected leaf NR activity since leaf NR activity in cotton plants treated with 100 mM NaCl was lower than that in plants treated with 0.1mM and 0.25 mM SNP. These results supported the idea that NaCl was negative for NR activity and the SNP was positive for NR activity.

3.7. Na^+, K^+, Ca^{2+}, and Mg^{2+} contents

As shown in Figure 2, Na^+ concentration in dry leaves was increased under NaCl stress. Conversely, K^+ content in CK was relatively high, but decreased under NaCl stress.

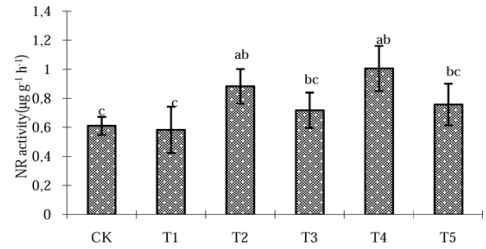

Figure 1. Effects of NaCl and SNP treatments on the nitrate reductase activity in cotton seedlings. Note: Values represent the mean ± S.D. (n = 3). Different lowercase letters indicate significant differences at $p < 0.05$.

SNP treatment decreased Na^+ concentration, but increased K^+ concentration under NaCl stress in cotton leaves. Na^+ concentrations in the root and stem under the 0.1mM SNP treatment were decreased by 62.17% and 9.17% (Figure 2a). K^+ concentration in the leaf under T3 was increased by 95.61% (Figure 2b), compared with T1. After 15 days of 100 mM NaCl stress, Ca^{2+} and Mg^{2+} contents decreased significantly in both, but the cotton treated with T5 accumulated more Ca^{2+} and Mg^{2+} than treated with NaCl (Figure 2c-d).

As shown in Table 6, the seedlings with root, stem and leaves exposed to 100 mM NaCl had a marked increase in Na^+/K^+ ratio (by 12.42, 11.60, 19.64 times, respectively) and had a signally decrease in Ca^{2+}/Na^+ ratio (by 94.55%, 75.62%, 95.21%, respectively). Therefore, we examined the effects of SNP on the content of the Na^+/K^+ and Ca^{2+}/Na^+ ratio under NaCl stress. We found SNP led to a lower Na^+/K^+ ratio and an upper Ca^{2+}/Na^+ ratio under NaCl stress. These results further suggest that the SNP has an effectively function in the regulation of ionic balance under NaCl stress.

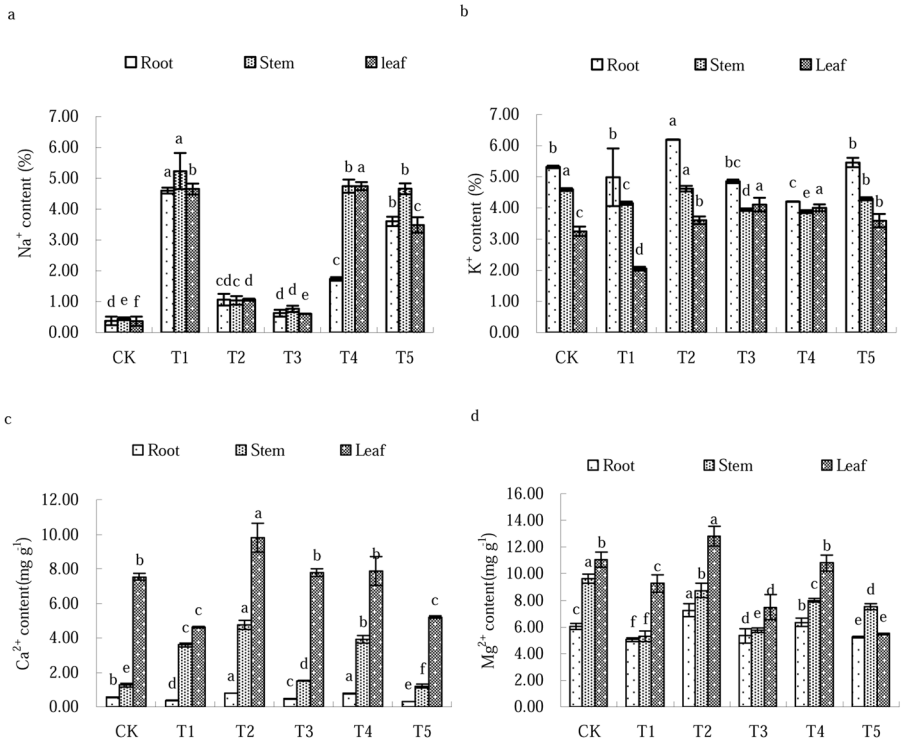

Figure 2. Effects of NaCl and SNP treatments on the content of Na^+ (a), K^+ (b), Ca^{2+} (c) and Mg^{2+} (d) in cotton seedlings. Note: Values represent the mean ± S.D. (n = 3). Different lowercase letters indicate significant differences at $p < 0.05$.

4. Discussion

The results showed that the ROS level increased significantly when the NaCl-tolerant cotton was subjected to NaCl and the level of ROS generated was limited in the presence of NO. Under NaCl stress, the RGR of cotton seedlings suffered different degrees of inhibition under different treatment (Table 2). Under salt condition, we not only found that SNP caused enhancement in plant growth, but also found enhancement in stem and root length. Hence, it may be the strong evidence that NO is involved in the plant growth increasing osmotic pressure of the cell and improving the cytoplasmics viscosity. However, high concentration of SNP inhibited the growth of cotton seedlings.

Most recently, it was reported that an appropriate concentration of NO could improve photosynthesis, fluorescence parameters and chlorophyll content. In this research, 100 mM NaCl could restrain chlorophyll synthetic, reduce photosynthetic efficiency and transpiration rate. However, SNP supplementation could alleviate the adverse effects (Table 3, Table 4).

Table 6. Effects of NaCl and SNP treatments on the Na^+/K^+, $Ca2^+/Na^+$ of root, stem and leaf in cotton seedlings.

Treatment	Na^+/K			Ca^{2+}/Na		
	Root	Shoot	Leaf	Root	Shoot	Leaf
CK	0.07±0.01d	0.10±0.01f	0.11±0.01e	1.47±0.05a	2.83±0.30b	20.65±0.36a
T1	0.94±0.17a	1.26±0.01a	2.27±0.07a	0.08±0.01c	0.69±0.01de	0.99±0.01d
T2	0.17±0.01d	0.23±0.03e	0.30±0.02d	0.74±0.01bc	4.62±0.66a	9.17±0.80c
T3	0.13±0.01d	0.19±0.01b	0.15±0.01e	0.74±0.01bc	1.97±0.03c	12.90±0.34b
T4	0.41±0.28c	1.22±0.02b	1.18±0.04b	0.89±1.00ab	0.83±0.01d	1.66±0.18d
T5	0.66±0.01b	1.09±0.07c	0.97±0.01c	0.08±0.01c	0.26±0.01e	1.49±0.03d

Note: Values represent the mean ± S.D. (n = 3). Different lowercase letters indicate significant differences at $p < 0.05$.

In addition, NaCl also increased stomatal resistance and Ci, and decreased Pn. Low photosynthetic pigment or high Na^+ content could inhibit Mg^{2+} absorbing and restrain chlorophyll synthesis (Li *et al.*, 2011), it possibly due to too much Na^+ inhibited protein synthesis, weakened the links of chlorophyll and chloroplastin, and led to chlorophyll decomposing. (Corpas *et al*., 2006) suggested that adding appropriate SNP could really alleviate salt toxicity though improving the plant photosynthesis.

The ratio Fv/Fm is proportional within a similar range, 0.76 to 0.85, to normal conditions. Yet the ratio decreased under stress (Liang *et al*., 2010). Thus, this parameter was often used to evaluate the photoinhibition and the damage of PSII complex. Results showed that NaCl stress induced a decrease of Fv/Fm and Fv/Fo, which indicated the destruction or inactivation of PSII reaction center. However, SNP supplementation alleviated the effect. PSII plays an especially important role in the response of photosynthesis in higher plants to environmental perturbations and stresses. The relationship between PSII and photosynthetic CO_2 assimilation is examined and factors identified that may modulate PSII activity in vivo. Thus, it can be inferred that apart from a signaling molecule, SNP supplement may act as a stress factor in promoting photosynthesis.

As for the possible role of NO in the NaCl-induced upregulation of antioxidant enzyme activity, the addition of NO to the media in the absence of salt stress resulted in an increase in antioxidant enzyme activity. The same results were observed in the present studies point towards its induction to quench higher levels of superoxide radical generated due to NaCl stress. Salt stress, like another abiotic stress, can lead to oxidative stress through the increase in ROS, which can potentiate the accumulation of MDA, an indicator of salt-induced oxidative damage to the membranes. Therefore, we deduced that the protective role of SNP might be related to its effects on the elimination of MDA. As such, this experiment suggested SNP could partially alleviate the toxic effect of NaCl on $O_2^{\cdot -}$ generation rate. However, the high concentration of SNP could make SOD, POD and CAT activity decrease, and MDA content increase, which may be due to high concentration of SNP interactions with $O_2^{\cdot -}$, and generate a large number of peroxynitrite anion. The peroxynitrite anion form peroxynitrite with strong oxidizing, which can destroy the structure and function of biological macromolecules (Zhang *et al*., 2004).

Proline plays important roles on clearance of ROS, improvement of the antioxidant capacity, adjustment of osmotic substances and stability of the structure of biological macromolecules.

This study showed that NaCl stress increased the proline accumulation, and SNP supplement could further induce the proline accumulation. Proline can protect plants from free-radical induced damage by quenching of singlet oxygen (Matysik *et al*., 2002). SNP treatment increased proline content to scavenge the elevated level of ROS and increased the antioxidase activity in this study.

A key step in nitrate assimilation is the reduction of this anion to nitrite in the reaction catalyzed by NR, an enzyme that is highly regulated at the transcriptional and post-transcriptional levels (Kaiser, 2001). NR has been studied extensively as a key enzyme of nitrogen metabolism. The involvement of NO in the activation of NR activity has been reported recently in leaves of wheat (Rosales *et al*., 2012), where NR activity was significantly enhanced by the addition of SNP. In concordance with these results, NR activity was significantly stimulated by 0.1mM SNP in our experimental conditions. All these results indicate that endogenous NO is indispensable and important for enhancing NR activity. But Xiong (2009) study reported that treatment with SNP had no observable effect on NR activity. In order to prove the true theory, further research is required.

Ionic imbalance in plants is mainly caused by the influx of excess Na^+. It is the essential condition of maintaining the cytoplasm enzyme activity through maintaining low Na^+ and high K^+ (Wang *et al*., 2011). Yamamoto (2011) showed that salt-tolerance of plant is concerned with low absorption and accumulation in Na. So if growing in Na^+ excessive environment, the plant could lack K^+, and restrain the absorption of Ca^{2+}, Mg^{2+} as well. Under NaCl stress, the content of inorganic ions in cotton seedlings changed markedly (Figure 2), with Na^+ showing increased levels and K^+, $Ca2^+$, Mg^{2+} showing decreased levels. The result was same with Kapila's result (Kapila *et al.*, 2010). However, adding the appropriate concentration of SNP could reduce Na^+ absorption and promote K^+, Mg^{2+}, Ca^{2+} absorption significantly, which may be the reason that NO serves as a signal in inducing NaCl tolerance by increasing K^+/Na^+ through increasing expression of PM H^+-ATPase activity. In addition, the protective effect of SNP on NaCl stress might be related to its increase osmotic regulation substances accumulation and its increase "rejecting-salt" capacity of its root. Furthermore, NO can simultaneously increase the absorption of Ca^{2+} by increasing the cell permeability. Dogan *et al.* (2010) showed that only the salt-tolerant cultivars maintained higher Ca^{2+} contents in all tissues of the plant, so promoting absorption of Ca^{2+} is one way of improving salt tolerance. SNP-induced K^+, Ca^{2+}, and Mg^{2+} contents in NaCl-treated cotton plants indicates that SNP can play a critical role in modulating cell ion balance, thereby protecting plants against oxidative damage.

An optimum Na^+/K^+ ratio is an important characteristic of salt tolerant plants. NaCl stress will greatly affect the selective absorption of K^+ and Na^+, and break the ionic balance (Wang *et al*., 2011). Some researches showed that NO signaled the increased expression of PM H^+-ATPase which increased salt tolerance by increasing the K^+ to Na^+ ratio. In the present study, the treatments with SNP showed that NO actually inhibited Na^+ absorption and maintained a higher Na^+/K^+ and Ca^{2+}/Na^+ ratio. This strongly suggests that NO is involved in "turning off" the antioxidant component of the environmental stress response, perhaps after other acclimation mechanisms such as the accumulation of proline or other low molecular weight compounds that may serve as osmoprotectancts and the adjustment of the K^+ to Na^+ ratio are induced (Shantel *et al*., 2008).

5. Conclusion

This study showed that, NaCl stress restrained the growth and enzyme activity, and disequilibrated ionic homeostasis. While low levels of SNP performed advantageous effects on attenuation of inhibition of seedling growth. Low concentration of NO alleviates NaCl toxicity and scavenges the ROS by: (1) promoting photosynthetic rate, fluorescence parameters and

accelerating the growth rate, (2) inducing a better antioxidant system in plants, (3) adjusting cations absorption and membrane permeability. The alleviating effects on the various organs are root > stem > leaf. However, high concentration SNP reduced the cotton salt resistance. All of them were strong evidence to support the physiological role of clearance oxidative stress for proper concentration of exogenous NO in cotton salt-tolerance.

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