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Roles of exogenous nitric oxide in regulating ionic equilibrium and moderating oxidative stress in cotton seedlings during salt stress

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Abstract

Using a potted experiment, we studied the effect of slow-release nitric oxide (NO) on the physiological characteristics of cotton seedlings subjected to salt stress (585 mg/kg NaCl). Sodium nitroprusside (SNP, an NO donor, 2.62 mg) was applied either directly to the soil, via slow release methods (slow-release bags, slow-release capsules, or slow-release particles), or via foliar application. NaCl decreased plant weight and chlorophyll content and increased electrolyte leakage and the contents of proline, ascorbic acid (ASA), and Na. NaCl also induced oxidative stress, as indicated by elevated levels of lipid peroxidation and ROS production. Foliar spray of SNP (0.09 mM) enhanced plant growth, promoted ion absorption and transport, and increased enzyme activity. Slow-released NO increased the levels of chlorophyll, ASA, and proline; promoted the uptake of K, Ca and Mg; and decreased Na. Moreover, increases in SOD and CAT activity were demonstrated to counter oxidative stress. However, different methods of SNP application have different effects on salt-tolerance. Foliar application was optimal. Slow-release NO, especially slow-release particles, was better able to alleviate NaCl toxicity compared with direct application of NO to the soil.

Keywords: Cotton, ionic equilibrium, NaCl, oxidative stress, slow-release NO

Abbreviations: ASA, ascorbic acid; CAT, catalase; CRF, controlled release fertilizer; H_2O_2 , hydrogen peroxide; LRWC, leaf relative water content; MDA, malondialdehyde; NO, nitric oxide; POD, peroxidase; PBS, phosphate buffered saline; ROS, reactive oxygen species; SNP, sodium nitroprusside; SOD, superoxide dismutase; TCA, trichloroacetic acid

1. Introduction

Na^+ in saline soils is toxic to plants and exerts adverse effects on K nutrition, enzyme activity, and metabolism. Several mechanisms function cooperatively to prevent the accumulation of Na^+ in the cytoplasm, including active Na^+ efflux, restriction of Na^+ influx, and compartmentalization of Na^+ in the vacuole (Shi *et al*., 2000). These changes lead to osmotic and ion-specific effects as well as imbalances in plant nutrition, which may involve deficiencies in several nutrients, such as K, Ca, and Mg (Qadir *et al*., 2006). To mitigate and repair the damage, plant cells perform detoxification via the synchronous action of various antioxidants composed of non-enzymatic and enzymatic components. Enzymatic antioxidants

include superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX), whereas non-enzymatic antioxidants include water-soluble components, such as soluble sugar, and lipid-soluble components, such as ascorbic acid (ASA) and protein (Sheetal *et al.*, 2012). Thus, compatible solutes and antioxidants may constitute a strategy for enhancing salt tolerance in plants.

Nitric oxide (NO) is a small, highly diffusible gas and a ubiquitous bioactive molecule. Its chemical properties make NO a versatile signaling molecule that functions through interactions with cellular targets via either redox or additive chemistry. In recent years, increasing evidence has indicated that NO is involved many physiological processes of plants, such as germination (Beligni and Lamattina, 2000), mitochondrial functionality (Zottini *et al*., 2002) and enzymatic activity (Zeng *et al*., 2011). Taken together, these reports reveal the importance of exogenous NO in the protection against deleterious effects and suggest that one of the mechanisms of NO is to help plants resist stress. First, NO might be involved in increasing the antioxidant content and antioxidant enzyme activity to scavenge reactive oxygen species (ROS). Second, NO might increase excess Na accumulation in root cell walls and decrease its accumulation in the soluble cellular fraction of the leaves. Lastly, NO could function as a signaling molecule in the cascade of events leading to changes in gene expression under risk element stress (Procházková *et al*., 2012; Xiong *et al.*, 2010). Moreover, previous evidence indicates that sodium nitroprusside (SNP) was reversed by the addition of an NO scavenger, 2-(4-carboxy-2-phenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (c-PTIO), suggesting that the ameliorating effect of SNP is due to release of NO (Srivastava *et al*., 2012). In our previous study, we added exogenous SNP to controlled release fertilizer (CRF) during the process of CRF production. The application of this mixture to the roots of peanut plants had beneficial effects during iron-deficiency stress (Zhang *et al*., 2012). However, it remains unclear whether slow-release NO can alleviate salt stress. Based on the known chemical properties of SNP, we choose the SNP as an NO donor and examined the ability of slow-release NO to alleviate salt stress in cotton.

Cotton is an important industrial crop and is a source of fiber. However, the susceptibility of cotton seedlings to salt stress is a major factor in reduced growth and productivity. Information on the tolerance of cotton seedlings to salinity is lacking. Therefore, enhancing the salinity tolerance of cotton by some means would be an important strategy for improving the productivity of this crop.

The potential for exogenous NO to alleviate NaCl toxicity in plants has mainly been studied via hydroponic experiments. Studies utilizing exogenous NO to explore NaCl toxicity in cotton grown on NaCl-contaminated soil are limited. In the present work, we utilized several new approaches to supply NO to cotton grown in NaCl-contaminated soil. SNP was either applied directly to the soil, via slow release methods (slow-release bag, slow-release capsule, or slow-release particle), or via foliar application. The overall objectives of the present study were to investigate whether slow-release NO can increase salt tolerance in cotton seedlings and to select a better means of alleviating salt toxicity in cotton seedlings.

2. Materials and Methods

2.1. Plant materials and treatments

A pot experiment was conducted using saline soil. The soil type was brown soil with the following properties: total nitrogen (N), 0.59 g kg^{-1}; Olsen phosphorus (P), 55.06 mg kg^{-1}; available potassium (K), 78.64 mg kg^{-1}; and pH (H_2O) 7.79. The salt-stressed environment was created by adding 585 mg of NaCl to 1 kg of soil.

Cotton (*Gossypium hirsutum* L.) seeds were seeded on May 15, 2012. The plants were grown in plastic pots (four plants per pot) with a capacity of 2.5 kg of air-dried soil. The pots were arranged in a randomized block design with three replicates.

During the growing season, the plants were managed under commonly used agronomic and irrigation practices. The experimental design is provided in Table 1. The fertilizer was supplied to the soil directly. The nutrient content of the fertilizer was 15% N, 15% P_2O_5, and 15% K_2O. SNP was supplied to the soil directly, via or by the slow-release way, or via foliar application. The slow-release SNP was provided by the Chinese National Engineering Research Center for Slow/Controlled Release Fertilizers. The amount of SNP added directly to the soil was 2.62 mg. The same quantity of SNP was added to a small paper bag and a capsule or developed into a slow-release particle. The three slow-release materials were used for root application. SNP solution was sprayed onto the leaves when the seedlings were 10 days old. Every pot was sprayed once per day with 10-mL in the evening. The total quantity of foliar SNP application was consistent with that added into the soil.

2.2. Plant growth and chlorophyll content analysis

The plants from each treatment were carefully uprooted, and the stem height was recorded. The plants were washed with tap water to remove adhering foreign particles. The roots were removed, and the individual shoot FW was recorded. The shoots weredried at 80 ^oC for 48 h, and their DW were recorded. A portable chlorophyll meter (SPAD-502, Konica Minolta Sensing, Inc., Japan) was used to measure changes in leaf greenness during processing (Neufeld *et al*., 2006).

2.3. Plant biochemical analysis

Leaf relative water content (LRWC) was measured by adopting the method of Yamasaki and Dillenburg (1999). Ten leaves were obtained for each treatment. To minimize the age effect, leaves were collected from the mid-section of the plant. To obtain their FW, the leaves were weighed immediately following removal from the stem. To determine turgid weight (TW), the leaves were kept in distilled water inside a Petri dish covered with its lid for 4 h. Subsequently, the water was gently wiped from the leaf surface with tissue paper, and the leaves were weighed. To determine DW, the leaf samples were dried at 80 °C for 24 h. FW, TW and DW values were used to calculate LRWC using the following equation: LRWC (%) = (FW-DW)/(TW-DW) ×100.

To assess membrane permeability, electrolyte leakage was determined using the method of Tariq *et al*. (2011). Leaf samples were washed three times with distilled water to remove surface contaminants. Young leaf discs were placed in a vial containing 10 mL of distilled water which was closed and incubated on a rotatory shaker for 24 h. Subsequently, the electrical conductivity (EC_1) of the solution was determined. The samples were then autoclaved at 120 °C for 20 min, and the final electrical conductivity (EC_2) was noted after cooling the solution at room temperature. Electrolyte leakage was calculated as follows: Electrolyte leakage (%) = (EC_1/EC_2) ×100.

Table 1. The experimental design

No.	Code	Treatment
CK	CK	0.27 g kg^{-1} fertilizer alone
T1	NaCl	0.27 g kg^{-1} fertilizer and 585 mg kg^{-1} NaCl
T2	Slow-release bag	0.27 g kg^{-1} fertilizer, 585 mg kg^{-1} NaCl, and SNP added to a paper bag
T3	Slow-release capsule	0.27 g kg^{-1} fertilizer, 585 mg kg^{-1} NaCl, and SNP added to a capsule
T4	Slow-release particle	0.27 g kg^{-1} fertilizer, 585 mg kg^{-1} NaCl, and slow release particles containing SNP
T5	Foliar application	0.27 g kg^{-1} fertilizer, 585 mg kg^{-1} NaCl, and foliar application of 0.09 mM SNP
T6	SNP applied into soil directly	0.27 g kg^{-1} fertilizer, 585 mg kg^{-1} NaCl, and direct application of SNP into the soil

Note: All the treatments in other Tables and Figures (CK and T1 to T6) are in accordance with the descriptions in Table 1

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 saline (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 hydroxyl ammonium chloride. The reaction was incubated at 25°C for 35 min. Solution (0.5 mL) from above 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.

For the measurement of hydrogen peroxide (H_2O_2) concentration, 1 g of a fresh sample was homogenized in 2 mL of ice-cold acetone. Titanium reagent (2% $TiCl_2$ in conc. HCl) was added to a known volume of the 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 of a 17 M ammonia solution for each 1 mL of extract. The precipitate was washed five times with ice-cold 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 that had been prepared similarly but lacked plant tissue (Tariq *et al.*, 2011).

Malondialdehyde (MDA) content is determined via the thiobarbituric acid reaction method (Heath and Packer, 1968). The supernatant from the antioxidant enzyme extraction was used for an MDA content assay. The MDA content was expressed as μmol g^{-1} FW.

Proline was determined as described by Kojić *et al*. (2012). Briefly, plant material was homogenized in 3% aqueous sulfosalicylic acid and the resulting homogenate was centrifuged for 10 min at 10 000 rpm. The supernatant was then used to estimate proline content. A reaction mixture consisting of 2 mL of supernatant, 2 mL of acid ninhydrin (1.25 g of ninhydrin in 30 mL of glacial acetic acid and 60 mL of 6 M phosphoric acid) and 2 mL of glacial acetic acid was boiled at 100 °C for 1 h. The reactions were then terminated in an ice bath. The resulting reaction mixture was extracted with 4 mL of toluene, and the absorbance of the proline-ninhydrin chromophore was measured at 520 nm. The proline concentration was determined from a standard curve and calculated on a fresh weight basis (μg proline g-1 of FW material).

ASA was determined spectrophotometrically following the 2,4-dinitrophenylhydrazine colorimetry method described by Bao (2002). ASA was assayed in a similar manner except that 200 μL of deionized H_2O was substituted for DTT and N-ethylmaleimide. Color was developed in both series of reaction mixtures with the addition of 400 μL of 10% (w/v) trichloroacetic acid (TCA), 400 μL of 44% (v/v) o-phosphoric acid, 400 μL α-α'dipyridyl in 70% (v/v) ethanol and 200μL of 30 g l^{-1} $FeCl_3$. The reaction mixtures were incubated at 40 °C for 1 h and quantified spectrophotometrically at 525 nm.

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

To extract antioxidant enzymes, leaves and roots were homogenized with 50 mM Na_2HPO_4-NaH_2PO_4 buffer (pH 7.8) containing 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).

2.4. Statistical analysis

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

Table 2. Effects of salinity (NaCl) and sodium nitroprusside (SNP) supplied via different application methods on the growth attributes and chlorophyll content of cotton seedlings grown in saline soil.

Treatment	Plant height (cm)	Root length per plant (cm)	Dry weight per plant (g)	Fresh weight per plant (g)	SPAD reading
CK	17.67±1.53ab	9.50±0.051c	0.44±0.11ab	3.94±0.69ab	45.13±2.20ab
T1	12.68±2.08c	5.67±0.58d	0.25±0.08c	1.83±0.42d	37.53±6.22d
T2	17.33±1.52ab	5.90±0.85c	0.47±0.04ab	3.40±0.42ab	42.37±2.27bcd
T3	16.96±1.02ab	13.17±4.19b	0.35±0.05bc	2.53±0.55cd	39.03±1.60cd
T4	17.83±1.26ab	18.83±2.57a	0.46±0.14ab	3.21±0.24bc	45.60±2.10ab
T5	19.33±2.89a	17.27±0.75a	0.56±0.08a	4.08±0.36a	49.47±3.30a
T6	15.67±3.21bc	6.90±0.36cd	0.34±0.06bc	2.23±0.48d	44.47±3.35abc

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

3. Results

3.1. Growth parameters and chlorophyll content

NaCl exposure significantly decreased the plant height and root length of cotton seedlings (Table 2); however, this inhibition was alleviated by SNP addition, especially in the T4 and T5 treatments. Compared with NaCl treatment, the height and root length of plants subjected to T4 treatment were increased by 40.62% and 232.10%, respectively; T5 treatment increased these values by 52.44% and 204.59%, respectively. NaCl treatment significantly decreased the FW and DW of cotton plants; however, this was significantly ameliorated by SNP addition ($p<0.05$). Chlorophyll content was also reduced in salt-stressed plants, with the most severe effects noted in the T4 and T5 treatments (Table 2).

3.2. Leaf relative water content (LRWC)

The effects of salt stress and SNP on LRWC are shown in Table 3. Treatment with excess NaCl significantly decreased the LRWC in the leaves, and the addition of SNP alleviated the decreases, especially when the slow-release particle and foliar application methods were employed.

3.3. Electrolyte leakage

As shown in Table 3, plants supplemented with NaCl exhibited a significant increase in electrolyte leakage compared with the plants grown in non-saline soil.

NO decreased electrolyte leakage in the plants grown with NaCl. Slow-release particle and foliar application methods were found to be more effective in the

amelioration of NaCl stress and caused a significant decrease in electrolyte leakage.

3.4. Lipid peroxidation

Oxidative damage to tissue lipids was assessed by determining total MDA content. NaCl-generated stress induced MDA production (Table 3). The application of SNP to NaCl-treated plants decreased the MDA content. The maximal alleviation of salt stress was recorded in the plants treated with slow-release particles, as reflected by 26.92% and 41.67% decreased in leaf and root MDA compared with salt-stressed plants.

Table 3. Effects of salinity (NaCl) and sodium nitroprusside (SNP) supplied via different application methods on leaf relative water content, electrolyte leakage, lipid peroxidation and endogenous ROS production of cotton seedlings grown in saline soil.

Treatments	LRWC (%)	Electrolyte leakage (%)	Lipid peroxidation (μmol·g^{-1} FW)		$O_2^{\cdot-}$ generation rate (μmol·g^{-1}·h^{-1} FW)		H_2O_2 (μmol·g^{-1} FW)
			Leaves	Root	Leaves	Root	
CK	0.96±0.03a	0.30±0.04c	0.017±0.002e	0.008±0.004c	1.33±0.26c	4.96±0.95e	0.91±0.13d
T1	0.82±0.02c	0.69±0.03a	0.026±0.004a	0.012±0.008a	2.33±0.59a	19.80±0.42a	3.01±0.23a
T2	0.92±0.02ab	0.64±0.12a	0.022±0.001c	0.011±0.015b	2.03±0.28ab	11.02±0.65c	2.31±0.06b
T3	0.91±0.04b	0.66±0.05a	0.022±0.007c	0.005±0.002e	1.86±0.15abc	13.96±0.84b	2.22±0.31b
T4	0.93±0.01ab	0.29±0.08c	0.019±0.003d	0.007±0.001d	1.78±0.03bc	9.51±0.71d	2.02±0.02bc
T5	0.95±0.07ab	0.53±0.08b	0.019±0.011d	0.010±0.001b	1.77±0.39bc	12.23±1.00c	1.78±0.39c
T6	0.84±0.12c	0.69±0.16a	0.023±0.006b	0.011±0.010a	2.19±0.03ab	13.67±0.24b	2.81±0.09a

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

3.5. Endogenous ROS production

Overaccumulation of ROS in plant cells is the main effect of salt stress. As shown in Table 3, NaCl stress induced a dramatic increase in $O_2^{\cdot-}$ and H_2O_2 production in the roots and leaves of cotton seedlings compared with the CK. SNP applied directly into soil had no effect on $O_2^{\cdot-}$ content in the leaves but markedly decreased $O_2^{\cdot-}$ accumulation in the root. SNP added to a capsule, a paper bag and slow-release particles or sprayed onto leaves markedly diminished NaCl-induced $O_2^{\cdot-}$ accumulation in the roots and leaves; this effect was especially obvious with slow-release particles.

The lowest values for H_2O_2 content were recorded in the non-stressed plants, and plants grown with NaCl accumulated the maximum H_2O_2 content. The application of NO to salt-stressed plants reduced the H_2O_2 content, and T5 treatment effectively alleviated the adverse effect of NaCl on this parameter. Used of slow-release particles arrested the effect of salt stress up to a considerable limit and caused a 32.89% decrease in H_2O_2 content compared with the plants grown with NaCl only.

3.6. ASA and proline content

Proline levels were higher in NaCl-treated plants compared with the control (Figure 1A). The application of NO to stressed plants could significantly alter the proline level when compared with control plants. The lowest level of proline was observed in the salt-stressed plants, which were subjected to NO spraying. ASA content significantly increased in NaCl-stressed plants compared to the control (Figure 1B). The ASA level was higher in stressed plants supplied with NO

compared with salt-stressed plants. In addition, slow-release particle and foliar application methods triggered the plants to accumulate additional ASA content.

3.7. Na, K, Ca, Mg, and Fe contents

NaCl treatment dramatically increased the Na concentration in the leaves, stems, and roots. However, addition of NO greatly decreased the Na concentration in the leaves and stems and increased Na concentration in the roots under excess NaCl.

Treatment with excess NaCl did not significantly influence the Mg concentration in the leaves; however, excess NaCl decreased the Mg concentration in the stems and roots. Excess NaCl significantly decreased the Ca concentration in the leaves, stems, and roots; however, the addition of NO, especially the T4 and T5 treatments, significantly increased the Ca and Mg concentration under excess NaCl. As shown in Table 4, NaCl treatment significantly decreased the Fe concentration in the leaves, stems, and roots, and the addition of NO alleviated the decreases in the leaves and stem. The T5 treatment increased the Fe concentration in the leaves, and the T4 treatment significantly increased the Fe concentration in the stem and root.

3.8. Antioxidant enzymes

The activities of several representative antioxidant enzymes, including SOD, POD, and CAT, were determined in cotton seedlings to assess how SNP application affects the regulation of these antioxidant enzymes upon NaCl stress. As shown in Figure 2, NaCl increased SOD, POD, and CAT activity in the leaves, and a combined treatment including excess Na and SNP stimulated enzyme activity to a much greater extent. Unlike in leaves, NaCl decreased the SOD and POD activity in the root; however, use of slow-release capsule, slow-release bag and slow-release particle increased the activity compared with NaCl treatment. Foliar SNP application was optimal for increasing CAT activities in leaves, and slow-release particles were optimal for increasing SOD activities in roots.

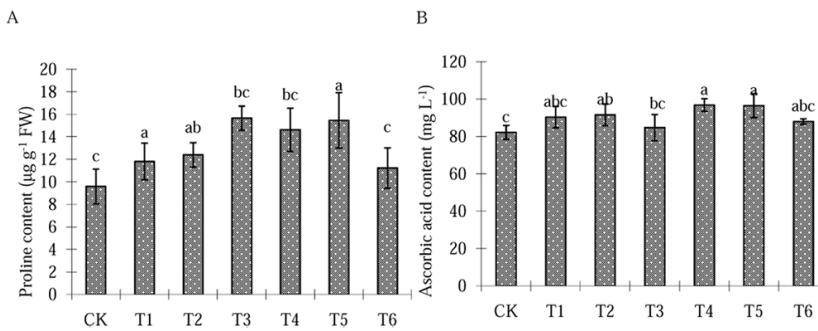

Figure 1. Effects of salinity (NaCl) and sodium nitroprusside (SNP) supplied via different application methods on the proline content (A) and ascorbic acid content (B) of cotton seedlings grown in saline soil. Note: Values represent the mean ± S.D. (n = 3). Different lowercase letters indicate significant differences at $p<0.05$.

Table 4. Effects of salinity (NaCl) and sodium nitroprusside (SNP) supplied via different application methods on the Na (%), K (%), Ca (g kg^{-1}), Mg (g kg^{-1}) and Fe (mg kg^{-1}) contents of cotton seedlings grown in saline soil.

Element/Type of plant tissue		Treatments						
		CK	T1	T2	T3	T4	T5	T6
Na	Leaves	0.016±0.01c	0.168±0.05a	0.131±0.06b	0.179±0.02a	0.135±0.07b	0.141±0.01b	0.142±0.03b
	Stem	0.016±0.02c	0.167±0.05a	0.162±0.01a	0.150±0.08ab	0.136±0.13b	0.151±0.22a	0.160±0.06a
	Root	0.053±0.02c	0.328±0.03a	0.349±0.06a	0.336±0.03b	0.273±0.01b	0.331±0.04a	0.349±0.02a
K	Leaves	0.37±0.06b	0.19±0.07c	0.20±0.07c	0.50±0.03a	0.50±0.04a	0.51±0.03a	0.30±0.11bc
	Stem	0.36±0.09cd	0.22±0.02e	0.47±0.08bc	0.65±0.06a	0.27±0.03de	0.31±0.10de	0.50±0.10b
	Root	1.05±0.07b	0.83±0.02c	1.28±0.07a	0.85±0.06c	0.98±0.05b	0.66±0.09d	0.57±0.09d
Mg	Leaves	1.25±0.20ab	1.31±0.12a	0.64±0.25d	1.27±0.13ab	0.97±0.08bc	0.67±0.17d	0.78±0.17cd
	Stem	1.61±0.25a	0.82±0.08bc	0.77±0.04c	0.74±0.04c	0.96±0.10b	0.90±0.05bc	0.72±0.02c
	Root	1.50±0.02a	0.78±0.12c	1.27±0.23ab	1.08±0.05b	1.28±0.06ab	1.43±0.18a	1.50±0.28a
Ca	Leaves	8.14±0.41a	5.11±0.93cd	5.12±0.20cd	4.67±0.39d	6.49±0.53b	6.41±0.12b	5.69±0.20bc
	Stem	9.76±0.92a	5.97±0.88d	6.29±1.09cd	7.54±0.13bcd	8.84±0.73ab	7.68±0.30bc	6.93±1.53cd
	Root	3.34±0.15a	1.93±0.07c	2.32±0.41c	2.42±0.11bc	3.11±0.09ab	3.14±0.23ab	3.15±1.05ab
Fe	Leaves	1.30±0.22a	0.44±0.12c	0.68±0.19bc	0.94±0.076bc	0.79±069abc	1.03±0.18ab	0.80±0.31abc
	Stem	2.94±0.11b	1.37±0.11c	1.16±0.20c	1.27±0.18c	3.55±1.07a	2.64±0.44b	1.34±0.49c
	Root	1.98±0.04a	1.05±0.18c	1.62±0.38b	1.61±0.06b	1.70±0.15ab	1.69±0.16ab	1.55±0.11b

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

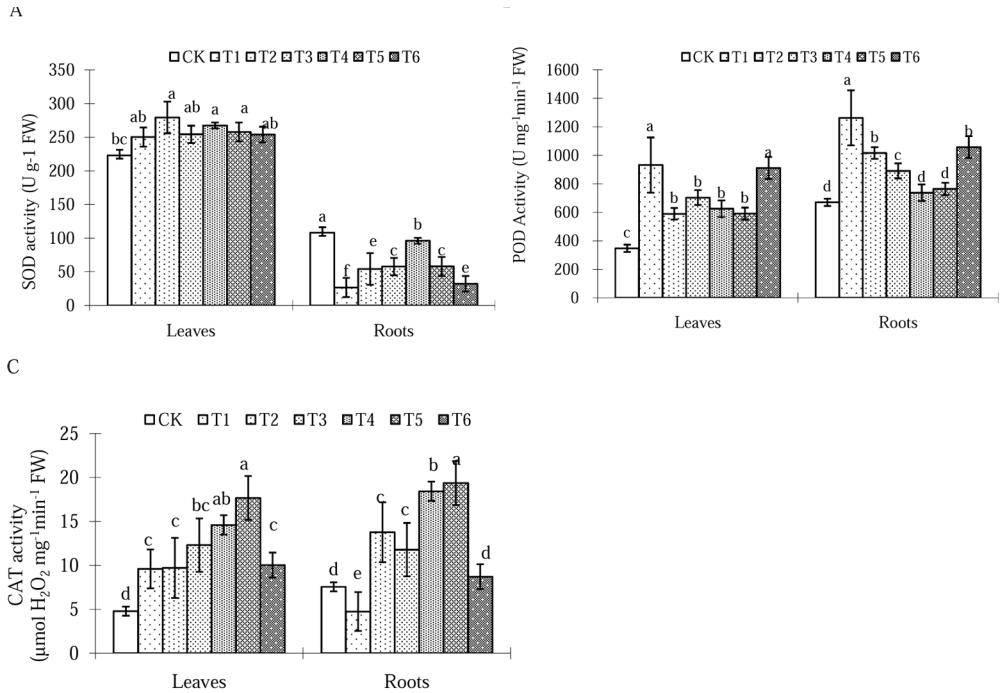

Figure 2. Effects of salinity (NaCl) and sodium nitroprusside (SNP) supplied via different application methods on the activities of SOD (A), POD (B), and CAT (C) in the leaves and roots of cotton plants grown in saline soil. Values represent the mean ± S.D. (n = 3). Different lowercase letters indicate significant differences at $p<0.05$.

4. Discussion

In the present work, we provided evidence that different SNP application methods have different effects in salt tolerance. The results from the current experiments clearly indicated that the growth and quality of cotton seedlings grown in NaCl-contaminated soil were inhibited significantly (Table 2). The suppression of growth and quality might be due to enhanced production and accumulation of ROS, which damaged photosynthetic activity by decreasing chloroplastic pigments levels (Table 2). Similar results were reported in NaCl-treated linseed and cotton (Khan *et al.*, 2010; Liu *et al.*, 2013). The decline in chlorophyll content in cotton leaves is believed to be due to impairment in the supply of Mg^{2+} required for the synthesis of chlorophylls (Table 4). NaCl-induced chlorophyll synthesis inhibition was significantly reversed when cotton seedlings were treated with exogenous SNP. However, the application of SNP by various methods alleviated the inhibitory effects. Slow-release or foliar application of SNP improved the vegetative growth of plants, thereby increasing the fresh weight under saline conditions. The SNP-induced increase in the dry matter of cotton seedlings could be due to its membrane-protective role (Liu *et al*., 2013) and antioxidant activity (Gunes *et al*., 2007). Controlled release fertilizers are used to reduce fertilizer application frequency and reduce fertilizer waste when compared with other fertilizer systems (Ruter *et al*., 1992). Slow-release NO may reduce NO application frequency and NO waste to regulate ionic equilibrium and membrane function, thereby promoting growth.

External NaCl lowered the LRWC of cotton seedlings (Table 3). Similar results were reported in linseed and Brassica juncea seedlings (Khan *et al*., 2010d; Zeng *et al*., 2011). However, NaCl stress in association with SNP significantly enhanced LRWC. Parida and Das (2005) reported that the relative water content, water potential, and osmotic potential of plants become more negative with an increase in salinity. Treatment with SNP reduced membrane injury by dehydration and improved the water status of plants, which may be the reason for improved seedling growth under SNP treatments. Use of slow-release particles may improve water availability and uptake and weaken the ability of Na^+ to compete for membrane binding sites, resulting in enhanced uptake of other ions under saline conditions, as exhibited by increases in the LRWC level and leaf nutrient content.

Electrolyte leakage indicates cell membrane injury when plants subjected to salinity stress. NO application completely abolished the effects of salinity in plants grown with NaCl (Table 3). Similar findings were noted by Zeng *et al*. (2011) in Brassica juncea and by El-Tayeb *et al*. (2005) in barley. Both studies suggested that SNP facilitated the maintenance of membrane functions through induction of antioxidant mechanisms and elevated ion uptake, thereby protecting the plants against oxidative damage. It is well known that the degree of lipid peroxidation is closely related to the accumulation of ROS, and the lipid peroxidation process is one important factor that exerts an effect on ATPase under changing environmental conditions. Therefore, the ability of exogenous NO to decrease lipid peroxidation should be considered as one factor that maintains higher ATPase activity (Shi *et al*., 2007). In the present study, slow-release NO slows down the NO release rate and continuously delivered NO to cotton, thus sustained regulating the activity of ATPase to decrease lipid peroxidation.

Proline and ASA contents were increased in plants grown with NaCl alone and were further increased by SNP application (Figure 1). The accumulation of osmotic regulation substances facilitates the osmotic adjustment of plants. ASA plays an important role in the protection of plants against membrane lipid peroxidation. Proline is the major source of energy and nitrogen during immediate post-stress metabolism, and accumulated proline apparently supplies energy for growth and survival, thereby inducing salinity tolerance (Zeng *et al*., 2011). The increased accumulation of proline and ASA in SNP-treated plants counteracted the negative effects of salinity and

might have aided the osmotic adjustment of plants. Thus, slow-release NO application improved plant salt tolerance, which was observed by an increase in contents of proline and ASA.

Exogenous SNP exerts various effects on the uptake of K, Ca, Mg, and Fe. A decrease in seedling micronutrient content (K, Ca, Mg, and Fe) and a significant increase in Na content were recorded in the plants grown with NaCl alone (Table 4). Under NaCl stress, higher concentrations of K, Ca, Mg, and Fe were recorded when the plants were provided with slow-release NO compared with NaCl alone. Similar results were reported by Khan *et al.* (2010). Increased K and Ca contents due to the application of slow-release NO may be explained based on (1) the ability of K and Ca to replace Na as a result of mutual competition between the two ions for a transport site on a carrier protein or (2) a decrease in the magnitude of the pH gradient across the plasma membrane, resulting in reduced net influx of Na and inhibition of membrane-associated carrier proteins, thereby maintaining the balance of K and Ca (Khan *et al*., 2010). It is expected that cotton seedlings transported micronutrient contents to shoots, and maintained low Na^+ concentrations; these traits may help the plant adapt to saline environments. Also, the increased in leaf Fe content due to the foliar application of NO to salinity-affected plants may be explained based on Fe transfer to leaves, which may have occurred as a result of increased chlorophyll content due to an increase in the number of chloroplasts per cell.

ROS such as O_2^- and H_2O_2 are often produced in large quantities by plants during various stress responses. The MDA content was measured as an index of lipid peroxidation. We investigated the involvement of these molecules in our experiments. The results showed that NaCl-induced overaccumulation of O_2^-, H_2O_2, and MDA in the roots and leaves of cotton was eliminated by the addition of SNP (Table 3). NO inhibits oxidation damage by regulating general mechanisms for cellular redox homeostasis, and promoting the transformation of O_2^- to H_2O and O_2, and enhancing the activity of H_2O_2-scavenging enzymes (Shi *et al*., 2007; Fan *et al*., 2013). Slow-release bags, slow-release capsules and slow-release particles were all able to inhibit oxidative damage. Foliar SNP application also decreased the damage from reactive oxygen and inhibited MDA accumulation. ROS generated hydroxyl radicals and other destructive species such as lipid peroxides (Vaidyanathan *et al.*, 2003), which leads to the destruction of the cell membrane as reflected by increased MDA levels under NaCl stress. Thus, scavenging ROS to maintain metabolic functions under salt stress is essential. Scavenging ROS depends on detoxification via the action of SOD and CAT. The results of the current study demonstrated that NO supplementation alleviates the NaCl-induced inhibition of SOD, POD and CAT activities, especially when supplied via the three slow-release materials or by foliar application (Figure 2). Zeng *et al*. (2011) and Nounjan *et al.* (2012) also reported similar effects on the levels of antioxidants. In this regard, CAT and SOD appear to play an essential protective role in ROS scavenging (Tariq *et al*., 2011). SOD initiates detoxification of O_2^- by producing H_2O_2, which is eliminated by its conversion to H_2O in subsequent reactions. Furthermore, it is well known that the accumulated Ca might be responsible for the decreased MDA and H_2O_2 contents because Ca has a unique role in membrane stabilization (Hirschi *et al*., 2004). Thus, the NO-enhanced uptake of Ca into the cytoplasm in this experiment may decrease MDA and H_2O_2 contents and reduce oxidative stress. The induction of CAT and SOD activities by NO in salt-stressed cotton plants indicates that slow-release NO can play an important role in persistently modulating the cellular redox balance, thereby protecting plants against oxidative damage.

5. Conclusion

The present work revealed the effect of various methods of SNP application on the alleviation of NaCl toxicity in cotton seedlings. Foliar application

can regulate ion absorption and maintaining normal metabolism and growth. However, foliar application is time-consuming because spraying must be performed every day. SNP release occurs differently in soil versus foliar application. The physiological mechanisms by which slow-release NO improves cotton salt tolerance involve the regulation of antioxidant system establishment and the re-establishment of the cyto-balance system, including the synthesis of osmotic protection components and restoration of ionic equilibrium. Significantly, it was found that slow-release particles released the highest amount of osmotic protection components. Thus, it might be concluded that SNP contained within the particle structure was slowly released into the soil and had long-lasting effect against oxidative stress during salt stress. Thus, SNP contained within slow-release particles may be a new preparation that can be used to improve salt tolerance. However, further research is required to determine the changes in these physiological parameters at the molecular level.

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