

# Effects of nitric oxide on salt stress tolerance in *Kosteletzkya virginica*<sup>☆</sup>

Yuqi Guo<sup>1,2</sup>, Zengyuan Tian<sup>1</sup>, Daoliang Yan<sup>2</sup>, Jie Zhang<sup>2</sup>, Pei Qin<sup>2,\*</sup>

<sup>1</sup>Bio-engineering Department, Zhengzhou University, Zhengzhou, Henan 450001, China; <sup>2</sup>Halophyte Research Lab., Nanjing University, Nanjing 210093, China

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## Abstract

Salt stress is considered to be a major limiting factor for plant growth and crop productivity. Our previous study showed that exogenous application of sodium nitroprusside (60  $\mu$ M SNP), a nitric oxide (NO) donor, could attenuate salt injuries in *Kosteletzkya virginica*. To further understand such protective roles of NO against salt stress, the effects of exogenous SNP on dry weight, activities of major antioxidant enzymes, proline accumulation, lipid peroxidation and distribution of sodium in *K. virginica* under salt stress were investigated. Application of SNP can increase dry weight, activities of catalase, peroxidase and superoxide dismutase, proline accumulation and decrease MDA contents in the presence of SNP under salt stress. Treatment with NaCl at 100, 200, 300 and 400 mM and SNP reduced Na<sup>+</sup> levels but increased K<sup>+</sup> levels in roots and shoots in comparison with the NaCl-treated plants. Correspondingly, the plants treated with exogenous SNP and NaCl maintained a lower ratio of [Na<sup>+</sup>]/[K<sup>+</sup>] in NaCl-stressed plants. These data suggest that NO might confer salt tolerance in *K. virginica* by preventing both oxidative membrane damage and translocation of Na<sup>+</sup> from root to shoots. [Life Science Journal. 2009; 6(1): 67 – 75] (ISSN: 1097 – 8135).

**Keywords:** *Kosteletzkya virginica*; salt stress; NO; physiological effects

## 1 Introduction

*Kosteletzkya virginica* (*K. virginica*) is an obligate wetland species native in the southeastern US. It is a salt tolerant hardy perennial herb that performs well in salt marsh and freshwater restoration and mitigation projects as well as in storm water treatment ponds<sup>[1]</sup>. Its niche in salt marshes and above mean high tide zones foretells its tolerance for salt stress.

Leakage of electrons to the cell milieu occurs during normal electron transport in the mitochondria and chloroplasts. These leaked electrons react with O<sub>2</sub> during aerobic metabolism to produce activated oxygen species such as superoxide, hydrogen peroxide and hydroxyl radical<sup>[2]</sup>. These activated oxygen species can seriously disrupt normal metabolism through oxidative damage to lipids, proteins and nucleic acids<sup>[3]</sup>. The enzyme super-

oxide dismutase (SOD) converts (O<sub>2</sub><sup>-·</sup>) into H<sub>2</sub>O<sub>2</sub>. Catalase (CAT) and a variety of peroxidases (POD) catalyze the breakdown of H<sub>2</sub>O<sub>2</sub><sup>[4]</sup>. Although, CAT is apparently absent in the chloroplasts, H<sub>2</sub>O<sub>2</sub> could be detoxified through ascorbate (Asc)-glutathione cycle in a reaction catalyzed by an As-specific peroxidase often present in high levels in this organelle<sup>[5]</sup>. Both As and glutathione were reported in millimolar concentrations within the chloroplasts<sup>[2]</sup>. The balance between the production of reactive oxygen species and the quenching activity of antioxidants becomes upset when plants are subjected to environmental salt stresses, often resulting in oxidative damage.

Nitric oxide (NO) is a small, highly diffusible gas and a ubiquitous bioactive molecule. Its chemical properties make NO a versatile signal molecule that functions through interactions with cellular targets via either redox or additive chemistry<sup>[6]</sup>. It was found to regulate the expression of mitogen activated protein (MAP) kinases<sup>[7]</sup>, pathogenesis related protein (PR-1), which are proteins involved in programmed cell death and plant-pathogen

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\*Corresponding author. Tel: 86-371-67767675; Email: guoyuqi@zzu.edu.cn.

responses<sup>[8]</sup> and can counteract other phenomena such as cell death, ion leakage, and DNA fragmentation. It was reported to counteract the toxicity of ROS generated by diquat or paraquat (PQ) in potato and rice<sup>[9,10]</sup>.

In recent years there has been increasing evidence that NO is involved in many key physiological processes in plants under normal and salt stress conditions. NO can mediate plant regulators and ROS metabolism<sup>[11,12]</sup>. SOD rapidly converts  $O_2^-$  to  $H_2O_2$  and an oxygen molecule under an ordinary physiological condition. However, a large amount of NO may combine with  $O_2^-$  to form peroxynitrite ( $ONOO^-$ ), which has been reported to damage lipids, proteins and nucleic acids<sup>[13]</sup>. Nevertheless,  $O_2^-$  and  $H_2O_2$  are more toxic than NO and  $ONOO^-$ . Therefore, NO may protect cells from destruction<sup>[14]</sup>. Furthermore, in plants, NO is also used for other intercellular and intracellular signaling functions such as stomatal closure, germination. NO can promote germination of plant seeds without or in the presence of sodium chloride and reduce the detrimental effect of the salt stress on root growth efficiently<sup>[15,16]</sup>. The protective effect of NO in stressed roots may be at least partly due to the stimulation of SOD activity or direct scavenging of the superoxide anion. And NO was found to increase accumulation of ABA<sup>[17]</sup>. ABA accumulation is one of the most important responses of a plant to water stress and it plays a key role in stomatal closure and water maintenance of plants under osmotic stress.

NO has been suggested to have dual roles, either toxic or protective, depending on its environments<sup>[18]</sup>. The protective role of NO during osmotic stress or as a secondary messenger is in a dosage-dependent manner<sup>[15]</sup>. At low concentrations, the mechanism of NO in leaf water control is ABA-dependent. But at high concentrations, NO can maintain leaf water by inducing stomatal closure independent of ABA accumulation which might act on gene expression<sup>[17]</sup>.

In previous studies, it was reported that exogenous NO stimulated the expression of plasma membrane (PM)  $H^+$ -ATPase in plant under salt stress, which is involved in responses to biotic and abiotic stresses<sup>[12]</sup> and dramatically improved antioxidant capacity<sup>[19]</sup>. On the other hand, NO was found to serve as a signal for inducing salt resistance by increasing the ratio of  $K^+$  to  $Na^+$  in the calluses of reed<sup>[12]</sup>.

There is no report on the effects of NO on antioxidative enzymes in *K. virginica* under NaCl stress, and this paper was to investigate whether NO is involved in the regulation of ROS metabolism, and whether exogenous NO can increase the halophyte *K. virginica* tolerance to salt stress by increasing the ratio of  $K^+$  to

$Na^+$ .

## 2 Materials and Methods

### 2.1 Plant materials and salt treatments

Sterilized *K. virginica* seeds were placed in Petri dishes containing  $H_2O$ , and kept at 26 °C. When seeds germinated, 15-day-old seedlings were cultivated in a hydroponic solution in a growth chamber (12 hours light periods, 25.8 °C, humidity 70%). All the measurements were carried out during the stage of three leaves. Sodium nitroprusside (SNP) was used as NO donors. Different concentrations of NaCl (0 mM, 100 mM, 200 mM, 300 mM, 400 mM) without or with SNP (0.06 mM), were added on the surface of the 1/2 Hogland. Controls were treated with 1/2 Hogland solutions. After 5 days of treatment, the samples of roots and shoots were collected, washed for 2 minutes by distilled water, and used immediately to examine dry weight, activities of CAT, POD, SOD, accumulation of proline, the content of malondialdehyde (MDA), and distribution of  $Na^+$  and  $K^+$  in plant.

### 2.2 Assay of enzyme activity

**2.2.1 CAT activity.** Root and leaf tissues were grounded into fine powder in liquid  $N_2$  and then dissolved in 2 ml of 50 mM potassium phosphate buffer (pH 7.0), 1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM Disoascorbic acid (AA), 2% (w/v) polyvinylpyrrolidone (PVP) and 0.05% (w/v) Triton X-100 using a chilled pestle and mortar. The homogenate was centrifuged at  $10,000 \times g$  for 10 minutes at 4 °C and the supernatants were collected and used for the assays of CAT.

CAT activity was determined spectrophotometrically by measuring the rate of  $H_2O_2$  disappearance at 240 nm, taking  $\Delta\epsilon$  at 240 nm as  $43.6 / M \cdot cm$ <sup>[20]</sup>. The reaction mixture contained 50 mM potassium phosphate (pH 7.0) and 10.5 mM  $H_2O_2$ . The reaction was run at 25 °C for 2 minutes, after adding the enzyme extract containing 20  $\mu g$  of protein and the initial linear rate of decrease in absorbance at 240 nm was used to calculate the activity.

**2.2.2 POD activity.** The measurement of total POD activity was carried out based on the determination of guaiacol oxidation [extinction coefficient 26.6  $mmol / (L \cdot cm)$ ] at 470 nm by  $H_2O_2$ <sup>[21]</sup>. In brief, approximately 0.50 g (fresh weight) of root or leaf tissue was homogenized on ice in 2 ml of 0.05 mM sodium phosphate buffer (pH 5.5) containing 1% PVP-40. The homogenate was centrifuged at  $1,500 \times g$  for 10 minutes at 4 °C, and then the supernatant was diluted to 25 ml for

assays. We added 300  $\mu$ l of the diluted enzyme extract into a 5-ml reaction system that contained 2.7 ml of 0.05 mM sodium phosphate buffer (pH 5.5), 1.0 ml of 2% H<sub>2</sub>O<sub>2</sub>, and 1.0 ml of 0.05 M guaiacol that had been incubated at 34 °C (the optimum temperature for enzyme reaction). Immediately after the addition of enzyme extracts, the absorbency of mixture was measured at 470 nm and recorded every 30 seconds during a 150-second observation period. One unit of POD was defined as the amount of enzyme that causes a 0.01 absorbency increase at 470 nm in comparison with a blank control. In blank controls, the same amount of 0.05 M sodium phosphate buffer (pH 5.5, 300  $\mu$ l) was added into the reaction system instead of the enzyme extract.

**2.2.3 SOD activity.** For assay of SOD, fresh roots or leaves (1 g) were homogenized in 8 mL potassium phosphate buffer (50 mM, pH 7.8) containing 0.1 mM Na<sub>2</sub>-EDTA and 1% insoluble PVP with a chilled pestle and mortar. The homogenate was centrifuged at 20,000  $\times$  g for 20 minutes. The supernatant was collected and used for the assay of SOD following the method of Beyer and Fridovich (1987)<sup>[22]</sup>. Reaction mixture was prepared by mixing 27 ml of 50 mM potassium phosphate, pH 7.8, 1.5 ml of L-methionine (300 mg/10 ml), 1 ml of nitroblue tetrazolium salt (NBT) (14.4 mg/10 ml) and 0.75 ml of Triton X-100. Aliquots (1 ml) of this mixture were delivered into small glass tubes, followed by 20  $\mu$ l of enzyme extract and 10  $\mu$ l of riboflavin (4.4 mg/100 ml). The cocktail was mixed and then illuminated for 7 minutes in an aluminum foil-lined box, containing two 20 W florescent tubes. A control tube in which the sample was replaced by 20  $\mu$ l of buffer was run in parallel and the absorbance A<sub>560</sub> was measured in all tubes. The test tubes containing the reaction mixtures were exposed to light immersing the glass tubes in a cylindrical glass container three fourth filled with clean water maintained at 25 °C, and placed in between two 20 W florescent tubes. The increase in absorbance due to formazan formation was read at 560 nm. Under the described conditions, the increase in absorbance without the enzyme extract was taken as 100% and the enzyme activity was calculated by determining the percentage inhibition per minute. About 50% inhibition was taken as equivalent to 1 unit of SOD activity. The determination of SOD activity was performed by using SOD-dependent inhibition of the reduction of NBT to purple formazan by superoxide.

**2.2.4 MDA content.** Standard procedures required for MDA measurement were following as Heath and Pacher<sup>[23]</sup>. Approximately 0.50 g (fresh weight) of root and leaf tissue was homogenized in 1.5 ml of 5%

trichloroacetic acid (TCA, w/v). The homogenate was centrifuged at 1,500  $\times$  g for 10 minutes, and then the supernatant was diluted to 10 ml. 2 ml of the diluted extract were mixed with 2 ml of 0.67% 2-thiobarbituric acid (TBA, w/v). The mixture was incubated in boiled water (95 – 100 °C) for 30 minutes, and then centrifuged at 1,500  $\times$  g for 10 minutes. Absorbencies of the aqueous phase at 450 nm, 532 nm, and 600 nm were measured respectively. MDA content in the aqueous phase was calculated according to the following formula: C ( $\mu$ mol/L) = 6.45  $\times$  (A<sub>532</sub> – A<sub>600</sub>) – 0.56  $\times$  A<sub>450</sub>.

**2.2.5 Estimation of proline.** The concentration of proline was estimated according to the method of Bates<sup>[24]</sup>. Five grams of root or leaf tissue were homogenized with 30% sulphosalicylic acid and filtered through a Whatman No.1 filter paper. A volume of 2 ml of glacial acetic acid and 2 ml acid ninhydrin were added to 2 ml of filtrate and incubated for 1 hour in a boiling water bath followed by cooling in ice bath. About 4 ml of toluene was then added and mixed vigorously. The chromophore containing toluene was aspirated from aqueous phase and the absorbance was measured at 520 nm.

**2.2.6 Distribution of Na<sup>+</sup>/K<sup>+</sup>.** The roots, shoots and leaves of the plants were rinsed with deionized water three times, and then dried at 80 °C to a constant weight after filtration with Whatman paper. 0.1 g dry powder samples were then extracted with 5 ml 4 M HNO<sub>3</sub> at 37 °C overnight to release the free cations and centrifuged at 10,000  $\times$  g for 10 minutes. The resulting supernatants of the extracts were diluted and Na<sup>+</sup> and K<sup>+</sup> determined with a Shimadzu AA-680 atomic absorption/flame spectrophotometer.

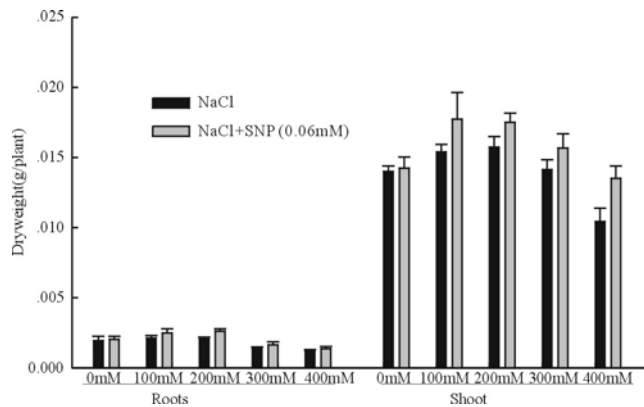
**2.2.7 Statistical analysis.** Values presented were means  $\pm$  one standard deviation (SD) of three replicates. Statistical analysis of the results was carried out according to two-sample paired t-test for means at a 0.05 probability level using SAS software.

## 3 Results

### 3.1 Shoot and root dry weights

After plants were treated for five days, they were separated into shoot and root. The shoots and roots were washed with deionized water and were then dried for dry weight determination and subsequently for analysis. The dry weight of both shoot and root of plants was slightly increased when *K. virginica* seedlings were treated with 100 and 200 mM NaCl, while slightly decreased with 300 and 400 mM NaCl stress compared to control (0 mM

NaCl). Shoot and root dry weights of plants grown under salt stress are shown in Figure 1. The dry weights of both roots and leaves were significantly increased by the NO treatments ( $P < 0.05$ ) (Figure 1). Without salt stress, NO treatment slightly increased the dry weight of both shoots and roots.



**Figure 1.** Effect of SNP supply on root and shoot dry weight of 5 days treatment in *K. virginica* plants grown in half Hogland solution with NaCl without or with SNP (60  $\mu$ M). Data are means  $\pm$  SD of three replicates. Roots:  $P = 0.0265$ ; leaves:  $P = 0.0191$ .

### 3.2 Antioxidant enzyme activity

The activities of three antioxidative enzymes (CAT, POD and SOD) in *K. virginica* under different levels of salt treatment without or with SNP were assayed. When *K. virginica* seedlings were treated with 100, 200, 300 and 400 mM NaCl without SNP, CAT activities in leaves showed significant decrease at 200 mM NaCl while decrease roots at 100 mM NaCl in comparison with control. In contrast, plants grown at 200 mM NaCl showed significant increases in SOD, POD activities of roots and leaves compared to that grown at 0 mM NaCl.

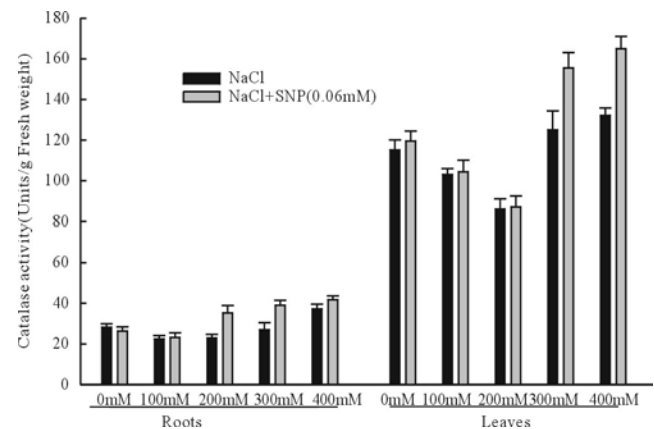
To determine SNP effects on plant growth and antioxidant activity, *K. virginica* seedlings were also treated with 0, 100, 200, 300 and 400 mM NaCl with SNP, the results also indicated the activities of antioxidant enzymes in roots and leaves of *K. virginica* seedlings increased when treated with SNP and salt stress in comparison with salt stress treated only (Figure 2, Figure 3, Figure 4). Treatment with SNP (60  $\mu$ M) resulted in remarkable increase in the activities of POD (Figure 3) in roots and leaves and SOD (Figure 4) in leaves of *K. virginica* seedlings ( $P < 0.05$ ) and slight increase in the roots and leaves of the activities of CAT ( $P > 0.05$ ) (Figure 2).

### 3.3 Content of proline

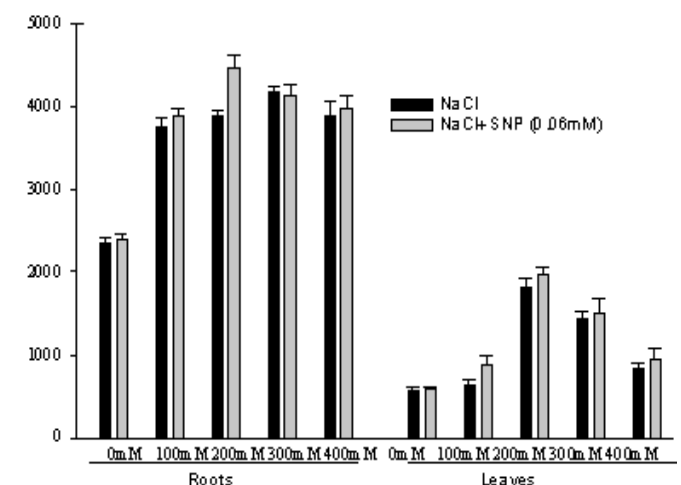
There was an increase in proline content with increase in salinity in the roots of *K. virginica* seedlings. Moreover, application of SNP exogenously enhanced this remarkable increase in roots and leaves compared with treatment of salt stress alone ( $P < 0.05$ ) (Figure 5).

### 3.4 Analysis of lipid peroxidation

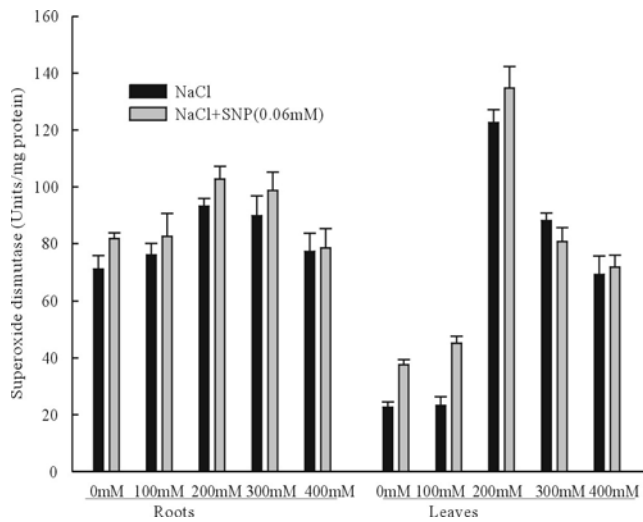
The content of MDA is an indicator of lipid peroxidation and oxidative damage to membrane. Figure 6 showed that salt treatment caused a significant increase in comparison with the control in MDA content whereas SNP treatment slightly inhibited the increase in MDA contents in shoots and roots of *K. virginica* seedlings



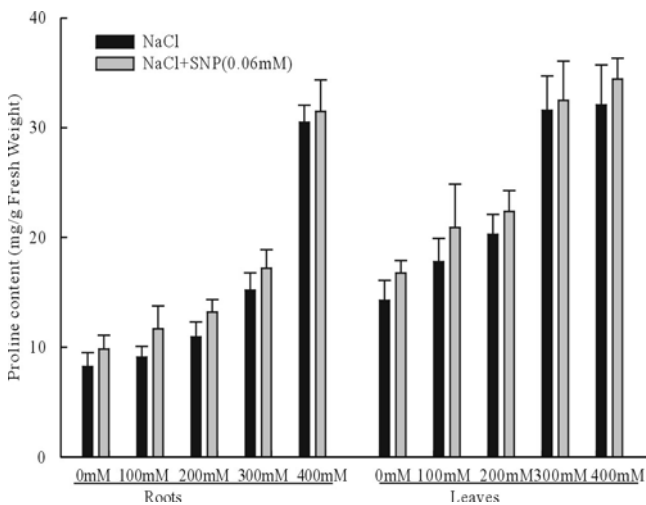
**Figure 2.** CAT activity (units/g fresh weight) of roots and leaves in *K. virginica* seedlings 5 days after being subjected to the salt stress treatment without or with SNP (60  $\mu$ M). Data are means  $\pm$  SD of three replicates. Roots:  $P = 0.1234$ ; leaves:  $P = 0.1249$ .



**Figure 3.** POD activity (units/g fresh weight) of roots and leaves in *K. virginica* seedlings 5 days after being subjected to the salt stress treatment without or with SNP (60  $\mu$ M). Data are means  $\pm$  SD of three replicates. Roots:  $P = 0.0240$ ; leaves:  $P = 0.0348$ .



**Figure 4.** SOD activity (units/mg protein) of roots and leaves in *K. virginica* seedlings 5 days after being subjected to the salt stress treatment without or with SNP (60  $\mu$ M). Data are means  $\pm$  SD of three replicates. Roots:  $P = 0.0116$ ; leaves:  $P = 0.1601$ .



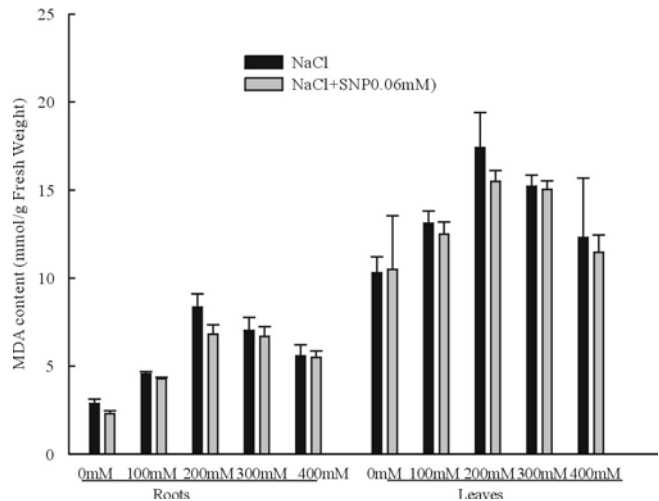
**Figure 5.** Proline content (mg/g fresh weight) of roots and leaves in *K. virginica* seedlings 5 days after being subjected to the salt stress treatment without or with SNP (60  $\mu$ M). Data are means  $\pm$  SD of three replicates. Roots:  $P = 0.0024$ ; leaves:  $P = 0.0038$ .

induced by salt stress in roots and leaves ( $P > 0.05$ ) (Figure 6).

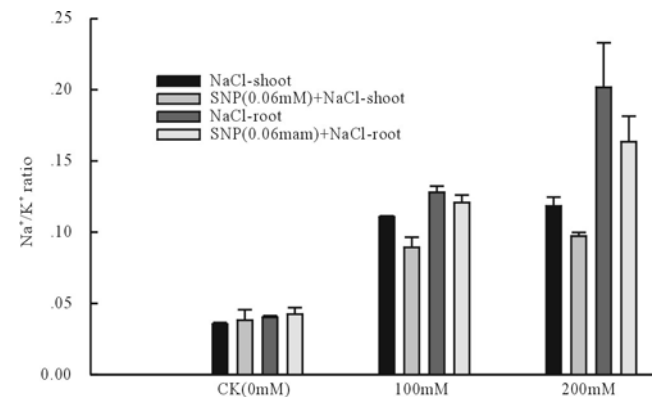
### 3.5 Ratio of $\text{Na}^+/\text{K}^+$

Figures 7 – 9 showed the influence of salinity stress on  $\text{Na}^+$  contents,  $\text{K}^+$  contents and  $\text{Na}^+/\text{K}^+$  ratio in the roots and shoots of *K. virginica* at different concentrations of NaCl (0, 100 and 200 mM) without or with SNP (60  $\mu$ M). *K. virginica* plants accumulated  $\text{Na}^+$  mainly in the roots under salinity conditions (Figure 8). Treatment

with SNP significantly decreased the  $\text{Na}^+/\text{K}^+$  ratio and  $\text{Na}^+$  contents (Figure 7 and Figure 8), increased the  $\text{K}^+$  contents (Figure 9) of *K. virginica* seedlings in both shoots and roots under salt stress. Maybe exogenous NO limited  $\text{Na}^+$  absorption and transport, while promoted  $\text{K}^+$  absorption. Our experiments showed that NO might confer salt tolerance on *K. virginica* seedlings.



**Figure 6.** MDA content (mmol/g Fresh Weight) of roots and leaves in *K. virginica* seedlings 5 days after being subjected to the salt stress treatment without or with SNP (60  $\mu$ M). Data are means  $\pm$  SD of three replicates. Roots:  $P = 0.0964$ ; leaves:  $P = 0.1346$ .

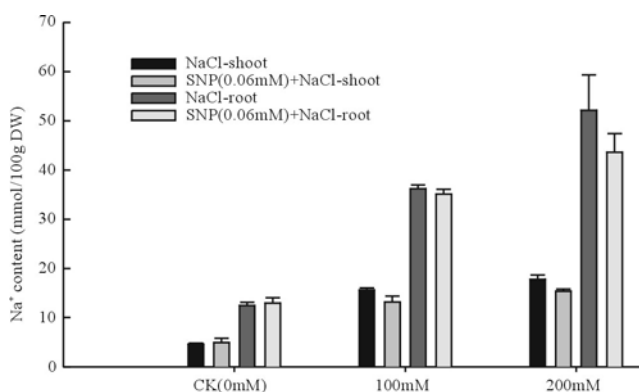


**Figure 7.** Effect of SNP (0.06 mM) on the  $\text{Na}^+/\text{K}^+$  ratio of *K. virginica* seedlings under non-stress or salt stress.

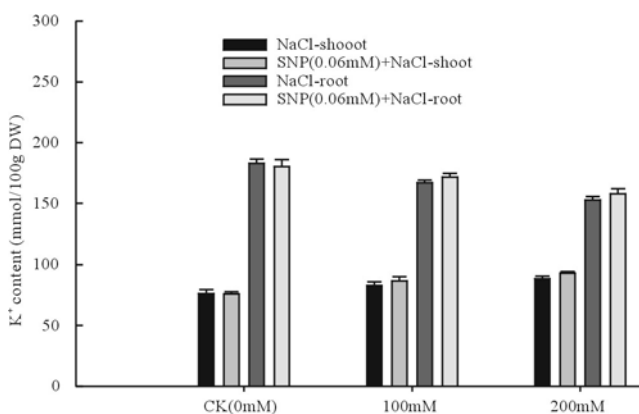
## 4 Discussion

NO, an endogenous signaling molecule in plants, mediates responses to abiotic and biotic stresses. It was reported to be involved in the responses to drought stress<sup>[25-28]</sup>, heat stress<sup>[29]</sup>, disease resistance<sup>[30,31]</sup>, apoptosis<sup>[32]</sup>, and formation of lateral root<sup>[33]</sup>. In our

studies, the dry weights of both roots and shoots of *K. virginica* seedlings were significantly increased by exogenous NO treatment. Previous studies have indicated that salt stress induced oxidative stress, which resulted in cellular membrane injuries. Plant tolerance to salt stress should partly depend on the enhancement of antioxidant defense systems including enzymatic and non-enzymatic. Antioxidant enzymes can protect the cell structure against the ROS generated by stress condition. In this work, a significant increase in the activities of POD, SOD in roots and leaves of *K. virginica* seedlings in response to salt stress was apparent when compared with control. Increased activities of POD, SOD play a crucial role in scavenging ROS during salinity. Interestingly, SNP can improve this role.



**Figure 8.** Effect of SNP (0.06 mM) on Na<sup>+</sup> contents in *K. virginica* seedlings under non-stress or salt stress.



**Figure 9.** Effect of SNP (0.06 mM) on K<sup>+</sup> contents in *K. virginica* seedlings under non-stress or salt stress.

Here we demonstrate that SNP, when applied exogenously, eliminated the oxidative stress in *K. virginica* imposed by salt stress. Moreover, the activities of CAT, POD and SOD in the presence of SNP under

salt stress were much higher than those under salt stress alone. This finding was in agreement with the previous study carried out by Akio Uchida (2002)<sup>[28]</sup>, who found NO induced AOS (active oxygen scavenging) activities in rice under salt stress. Our results were not consistent with the results of wheat roots<sup>[8]</sup>, or were unaffected as in the case of SOD in cucumber<sup>[34]</sup>. Such difference indicates that the influence of salt stress on the antioxidant enzymes is complex and related to the plant treatment time, plant tissues, plant species and genotypes.

Our experiments demonstrated that *K. virginica* seedlings accumulate proline in response to salt stress and that application of SNP exogenously enhances the increase of proline significantly under salt stress. It seems that increased level of proline has an important role in protecting enzymes involved in the antioxidant system against damaging effects of salt stress. Recently Hoque's research suggested that the concentration of proline is not high enough to adjust the osmotic potential in some plants under stress conditions<sup>[35]</sup>, and that proline can mitigate the inhibition of growth of tobacco cells due to its role in antioxidant defense systems against oxidative damage of ROS. Exogenous proline increased the activities of all enzymes except MDHAR involved in NaCl-induced ASC-GSH cycle which were reduced under salt stress. It is possible that application of SNP exogenously enhanced protecting enzymes involved in the antioxidant system indirectly by the increase in proline contents.

NO may serve as a signal molecular in growth and development of plants<sup>[36,37]</sup>. However, in response to salt stress, NO can cause protection against salt stress in young rice seedlings<sup>[28]</sup> and maize seedlings<sup>[34]</sup>. A key factor limiting plant growth is excessive Na<sup>+</sup>, a harmful mineral element not required by most plants. High Na<sup>+</sup> tissue content is often considered as the most critical factor responsible for salt toxicity. A possible survival strategy of plants under saline conditions was to sequester absorbed Na<sup>+</sup> in roots. In our work, with 100 and 200 mM NaCl treatment for 5 days, *K. virginica* plants accumulated Na<sup>+</sup> mainly in roots, and maintained lower [Na<sup>+</sup>]/[K<sup>+</sup>]. Treatment with SNP significantly decreased the Na<sup>+</sup> contents and Na<sup>+</sup>/K<sup>+</sup> ratio, increased the K<sup>+</sup> contents of *K. virginica* seedlings in both shoots and roots under salt stress. Previous studies showed that NaCl induced a transient increase in the NO level in maize leaves. Both NO and NaCl treatment stimulated vacuolar H<sup>+</sup>-ATPase and H<sup>+</sup>-PPase activities, resulting in increased H<sup>+</sup>-translocation and Na<sup>+</sup>/H<sup>+</sup> exchange<sup>[38]</sup>. NO stimulated by NaCl may stimulate

plasma membrane associated PLD, which hydrolyzes membrane phospholipid and generates PA (polyamine). PA may stimulate tonoplast  $H^+$ -ATPase activity through other signaling cascades<sup>[39]</sup>. NO could be acting upstream of PA. Moreover, the downstream targets of PA have been identified in plants, including protein phosphatase and protein kinase<sup>[40]</sup>. Atnoa1 (nitric oxide synthetase gene) mutant plants displayed a greater  $Na^+$  to  $K^+$  ratio in shoots than wild-type plants due to enhanced accumulation of  $Na^+$  and reduced accumulation of  $K^+$  when exposed to NaCl. Treatment of Atnoa1 plants with SNP attenuated the NaCl-induced increase in  $Na^+$  to  $K^+$  ratio. NOA1-dependent NO production in Atnoa1 plants is related to its enhanced sensitivity to salt stress<sup>[41]</sup>. Our results and previous studies indicate that NO may serve as a signal in inducing salt resistance by increasing the  $K^+$  to  $Na^+$  ratio, which may be dependent on the increased PM  $H^+$ -ATPase activity.

Changes in protein expression and gene expression profiles of the SNP treated plants were analyzed in mung bean<sup>[42]</sup> and tobacco<sup>[7,8,43]</sup>, and gene or protein expression induced by NO is involved in photosynthesis and the programmed cell death respectively. NO maybe also paradoxically act as an antioxidant and an antiapoptotic modulator that prevent cell death<sup>[44]</sup>. These cytotoxic and protective effects of NO are often concentration dependent<sup>[45]</sup>. Beside, studies on gene expression carried out by Polverari *et al*<sup>[31]</sup>, who used cDNA-AFLP to profile the transcriptional changes induced by SNP and found many NO responsive genes, were previously reported to be modulated in disease-related experiments. Recently, NtGRAS1 expression was studied by treating cells with SNP, and NtGRAS1 was demonstrated to play a critical role in the transcriptional regulation of genes involved in the plant stress response<sup>[46]</sup>. In the present study, exogenous NO greatly elevated activities of antioxidant enzymes, and alleviated oxidative stress to *K. virginica* seedlings induced by salt stress. NO also decreased  $Na^+/K^+$  ratio and enhanced accumulation of proline concentration as adaptive mechanisms. Whether or not these gene expressions of *K. virginica* seedlings subjected to salt and SNP treatment were induced remains unclear. Here, our findings provide a perspective on protective roles of NO against salt stress, gene expression of *K. virginica* seedlings subjected to salt and SNP treatment await further elucidation.

Previous studies show that NO affects mitochondrial functionality in plant cells and reduces total cell respiration due to strong inhibition of the cytochrome pathway. Nevertheless, mitochondria from all plants contains a cyanide-resistant, alternative oxidase that

functions in parallel with cytochrome c oxidase as the terminal oxidase on the electron transfer chain<sup>[47]</sup>. In our experiment, whether SNP modulates mitochondrial respiration, activities of CuZn-SODs (SOD isozymes) involving in ROS metabolism, and cytochrome c of *K. virginica* seedlings under non-salt or salt stress is uncertain.

Three different enzymatic pathways and a few non-enzymatic reactions have been proposed for the generation of NO in plant roots. In addition to enzymatic NO formation by the plant, there are sources of NO in the soil and in the rhizosphere from bacterial nitrification and denitrification. NO is an uncharged lipophilic gas with a diffusion coefficient close to that of  $O_2$  in aqueous solution. Transport of NO across membranes with lipophilic layers or that from root system to the shoot may function as a gaseous signal to send information. But NO can also lead to a high reactivity with  $O_2$  and  $O_2^-$  and with several N compounds. In addition, dinitrogen trioxide and nitrogen dioxide are formed and represent the reactive N-nitrosating and S-nitrosating species produced during autoxidation of NO. Salinity not only induces oxidative stress but also nitrosative stress in olive leaves<sup>[48]</sup>. Salt stress caused an increase of the L-arginine-dependent production of NO, total S-nitrosothiols (RSNO) and reactive nitrogen species (RSN) occurred mainly in the vascular tissue during nitrosative stress. Thus, NO in excess is toxic to higher organisms<sup>[49]</sup>. NO has either protective roles or toxic damage depending on its environments. Moreover, the effect of cytoprotective or cytotoxic action of NO on plant metabolism depends to a large extent on the local concentration of the molecule and is affected by the rate of synthesis, displacement and efficiency of removal of this reactive nitrogen species. It may be speculated that concentration and distribution of NO is important for its either positive role or negative damage in the plants. As a consequence, it is certainly worthwhile to focus further research on the formation rates of NO and relation of NO with salt tolerance.

At present, there are at least three distinct types of haemoglobins (Hbs) in plants that have been classified as symbiotic, non-symbiotic Hbs (nsHbs) and truncated Hbs. The nsHbs appear to be ubiquitous in the plant kingdom, of which class-1 Hbs have an extremely high affinity for  $O_2$  and can be induced during stress and its activity is involved in NO degradation in a NAD(P)H-dependent manner. NO is an effective inhibitor of cytochrome oxidase in the mitochondrial electron transport chain and may further reduce cell respiration and energy production. Scavenging NO of nsHbs helps

in maintaining the energy status of plant cells. The effect of nSHbs in defence against nitrosative stress is observed during treatment with NO donors<sup>[50]</sup>. In addition to Hb-based NO detoxification, some enzymes such as xanthine oxidase, glutathione peroxidase and GSNO reductase are reported to break down NO-related species<sup>[51]</sup>. All these suggested that plants may be able to control the level of NO under different conditions. A fine-tuning of NO detoxification may exist when plants are under stress conditions.

Taken together, our study showed that exogenous application of SNP (60  $\mu$ M) could attenuate salt injuries in *K. virginica* plants by acting as an efficient scavenger breaking the oxidative chain. NO imposes not only the alterations in antioxidative metabolism in *K. virginica* under NaCl stress, decrease  $\text{Na}^+/\text{K}^+$  ratio as adaptive measures but also accumulation of osmolytes such as proline.

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