African Journal of Biotechnology Vol. 9(11), pp. 1619-1627, 15 March, 2010
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.1442
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

Protective effect of nitric oxide against arsenic-induced oxidative damage in tall fescue leaves

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Accepted 31 December, 2009

Nitric oxide (NO) is a key molecule involved in many physiology processes. The effects of NO on alleviating arsenic-induced oxidative damage in tall fescue leaves were investigated. Arsenic (25 µM) treatment induced significantly accumulation of reactive oxygen species (ROS) and led to serious lipid peroxidation in tall fescue leaves and the application of 100 µM SNP before arsenic stress resulted in alleviated arsenic-induced electrolyte leakage and malondiadehyde (MDA) content in tall fescue leaves, the levels of hydrogen peroxide (H_2O_2) and superoxide radical ($O_2^{\cdot -}$) were reduced as well. Moreover, the activities of superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) increased in tall fescue leaves in presence of SNP under arsenic stress. This pattern was reversed by application of NO scavenger, 2-(4-carboxy-2-phenyl)-4,4,5,5-tetramethy-limidazoline-1-oxyl-3-oxide (PTIO) before arsenic treatment. Pronounced increases in endogenous NO production was found in plants after exposure to arsenic stress. The results suggested that arsenic stress elevated endogenous NO level and that NO might act as a signaling molecule to enhance antioxidant enzyme activities, further protecting against injuries caused by arsenic toxicity.

Key words: Antioxidant enzymes, arsenic stress, nitric oxide, oxidative stress, tall fescue.

INTRODUCTION

Arsenic (As) is one of the five kinds of toxic heavy metals and it is distributed widely in natural environment. It occurred in a various chemical forms in soil, water, air and food and came from mining, heavy industry, semi-conductor manufacturing, forest products, landfill leachates, fertilizers, pesticides and sewage (Roberto et al., 2002). Arsenic contamination caused a major environmental and human disaster and effected the plant growth, development and yield (Li et al., 2006). Under a variety of abiotic stresses (drought, heat, salinity, UV-B radiation and heavy metals) generation of reactive oxygen species (ROS: H_2O_2, hydrogen peroxide; $O_2^{\cdot -}$, superoxide radical, $HO^{\bullet}$, hydroxyl radical; O_2^1, singlet oxygen) increased and resulted in oxidative stress in plant cells (Mittler, 2002; Schützendübel and Polle, 2002; Apel and Hirt, 2004). If these ROS are not removed immediately, it can cause damage to the cell membrane and even death (Gratao et al., 2005). To avoid ROS induced cellular injury, plants employ various antioxidative enzymes such as superoxide dismutase (SOD: EC 1.15.1.1), catalase (CAT: EC 1.11.1.6), peroxidase (POD: EC 1.11.1.7), ascorbate peroxidase (APX: EC 1.11.1.11), glutathione reductase (GR: EC 1.6.4.2) (Apel and Hirt, 2004).

Nitric oxide (NO) is a signaling molecule involved in many plant physiological and metabolic processes and it is acting as an intercellular and intracellular signal (Lamattina et al., 2003; Lamotte et al., 2005). NO itself is a reactive nitrogen species and its effects on different types of cells were proven to be either protective or toxic,

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Abbreviations: NO, Nitric oxide; **ROS,** reactive oxygen species; **MDA,** malondiadehyde; **SOD,** superoxide dismutase; **CAT,** Catalase; **APX,** ascorbate peroxidase; **POD,** peroxidase; **GR,** glutathione reductase; **PTIO,** 2-(4-carboxy-2-phenyl)-4,4,5,5-tetramethylimidazoline--oxyl-3-oxide; **REL,** relative electrolyte leakage; **As,** arsenic; **PPFD,** photosynthetic photo flux density.

depending on its applied concentration and location (Beligni and Lamattina, 1999). Recent researches indicated that NO were involved in plant growth and development, such as seed germination, de-etiolation, cell senescence and programmed cell death (Beligni and Lamattina, 2000; Neill et al., 2003). Moreover, NO was found to mediate plants responses to abiotic stresses, such as heat stress, drought stress, salinity, UV-B radiation and heavy metal toxicity (Laspina et al., 2005; Song et al., 2006; Shi et al., 2007; Vital et al., 2008; Zhao et al., 2008; Singh et al., 2009; Zhang et al., 2009).

Previous studies have demonstrated that exogenous NO provide resistance to plant against As-toxicity and an ameliorating effect (Singh et al., 2009), but no report was available on the relationship between endogenous NO and antioxidant systems when plants were exposed to arsenic stress. The objective of this study is to elucidate the role of NO (applied exogenous NO or depleted endogenous NO) in alleviating arsenic-induced oxidative damage in tall fescue leaves.

MATERIALS AND METHODS

Plant materials

Tall fescue seeds (*Festuca arundinacea* cv. Arid3) were obtained from Beijing Clover Seed and Turf CO., Ltd., China. Seeds were surface sterilized in 0.1% (w/v) sodium hypochlorite, rinsed several times in distilled water and germinated on moist filter paper room temperature for 7 days. Uniform seedlings were selected and placed into 5 L black plastic containers containing 4 L of nutrient solution. Each plastic container contained six plants. Seedlings cultured hydroponically in a continuously aerated nutrient solution containing: 4 mM $Ca(NO_3)_2$; 4 mM KNO_3; 1 mM KH_2PO_4; 2 mM $MgSO_4$; 46 µM H_3BO_3; 10 µM $MnSO_4$; 50 µM Fe-EDTA; 1.0 µM $ZnSO_4$; 0.05 µM H_2MoO_4; 0.95 µM $CuSO_4$. Nutrient solution was renewed once a week.

As-treatment and growth conditions

Sodium arsenate (molecular weight 312.01 g/mol) of technical grade (purity = 98.5%) was purchased from Tiangen Biotech (Beijing) CO., Ltd., China. Sodium nitroprusside (SNP; Sigma, USA) was used as NO donor. NaCN, ferrocyanide (Fe(II)CN), ferricyanide (Fe(III)CN), sodium nitrite ($NaNO_2$) and sodium nitrate ($NaNO_3$) were used as the controls of SNP decomposition. The potassium salt of 2-(4-carboxy-2-phenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO; Sigma, USA) was used as NO scavenger.

After 21 days of pre-culture, the treatments were started. Different treatments include: (a) water alone (control); (b) 25 µM sodium arsenate (As); (c) 25 µM As + 200 µM PTIO (As + PTIO); (d) 25 µM As + 100 µM SNP (As + SNP); (e) 25 µM As + 100 µM SNP + 200 µM PTIO (As + SNP+ PTIO). In all, there were five treatments and each treatment was replicated three times. The used concentration of SNP was based on an earlier experiment using a wide range of concentration (from 50 to 500 µM) on the tall fescue under hydroponic conditions. To further verify the SNP protective roles on antioxidant systems in tall fescue under arsenic stress, 100 µM NaCN, sodium ferrocyanide (Fe(II)CN), sodium ferricyanide (Fe(III) CN), sodium nitrite ($NaNO_2$) and sodium nitrate ($NaNO_3$) were as additional controls. The experiment was carried out in a completely randomized block design manner in a plant incubator at a day/night temperature 25/20°C, a relative humidity of 70%, a day/night regime of 14/10 h and a photosynthetic photo flux density (PPFD) at the height of the plants of 100 µmol m^{-2} s^{-1}. Light was provided by a fluorescent lamp. After 4 and 8 days of treatment, plants were harvested and frozen in liquid nitrogen and then stored at -80°C for further analysis.

Membrane permeability measurement

Membrane permeability was determined by the modified method according to Song et al. (2006). The fresh leaves (0.5 g) were washed in deionized water and placed in petri dishes with 5 ml of deionized water at 25°C for 2 h. After the incubation, the conductivity was measured (C_1). Then, the samples were boiled for 20 min and conductivity was read again (C_2). Relative electrolyte leakage (REL) was expressed as a percentage of the total conductivity after boiling (REL % $= C_1/C_2 \times 100$).

Analysis of lipid peroxidation

The level of lipid peroxidation was expressed as the amount of malondialdehyde (MDA) production with a slight modification of the thiobarbituric acid method described by Buege and Aust (1978). Leaves (0.5 g) were homogenized with a mortar and pestle in 10% trichloroacetic acid and then the homogenate was centrifuged at 4000×g for 30 min. A 2 ml aliquot of supernatant was mixed with 2 ml of 10% trichloroacetic acid containing 0.5% thiobarbituric acid. The mixture was heated at 100°C for 30 min. The absorbance of the supernatant was measured at 532 nm, with a reading at 600 nm subtracted from it to account for non-specific turbidity.

Determination of hydrogen peroxide and superoxide radical

Hydrogen peroxide content was measured according to Veljovic-Jovanovic et al. (2002). Leaves (0.5 g) were ground in liquid N_2 and the powder was extracted in 2 ml 1 M $HClO_4$ in the presence of 5% polyvinylpyrrolidone. The homogenate was centrifuged at 12000×g for 10 min and the supernatant was neutralized with 5 M K_2CO_3 to pH 5.6 in the presence of 0.1 ml of 0.3 M phosphate buffer (pH 5.6). The solution was centrifuged at 12000×g for 1 min and the sample was incubated for 10 min with 1 U ascorbate oxidase to oxidize ascorbate prior to assay. The reaction mixture contained 0.1 M phosphate buffer (pH 6.5), 3.3 mM 3-(dimethylamino) benzoic acid, 0.07 mM 3 -methy- 2 -benzothiazoline hydrazone, 0.3 U POD and 200 µl supernatant. Changes in absorbance at 590 nm were monitored at 25°C.

Superoxide radical production rate was determined by the modified method according to Elstner and Heupel (1976). Leaves (1.0 g) were homogenized in 3 ml of 50 mM potassium phosphate buffer (pH 7.8) and centrifuged at 12000×g for 20 min. The incubation mixture contained 1 ml of supernatant, 1 ml of 50 mM potassium phosphate buffer (pH 7.8) and 1 ml of 1 mM hydroxylaminonium chloride and the mixture was incubated in 25°C for 20 min. The mixture was subsequently incubated with 2 ml of 17 mM sulphanilic acid and 2 ml of 7 mM α-naphthyl amine at 25°C for 20 min. The final solution was mixed with an equal volume of ethyl ether and the absorbance of the pink phase was read at 530 nm. The production rate of $O_2^{\cdot -}$ was calculated based on a standard curve.

Antioxidant enzyme activity

Leaves (1.0 g) were homogenized with a mortar and pestle at 4°C in 5 ml 50 mM phosphate buffer (pH 7.0) containing 1 mM ethylene

diamine tetra acetic acid, 1% polyvinylpyrrolidone. The homogenate was centrifuged at 12000×g for 30 min at 4℃ and the supernatant was collected for enzyme assays.

The activity of SOD was measured by nitroblue tetrazolium method of Beauchamp and Fridovich (1971). One unit of SOD was defined as the amount of enzyme required to cause 50% inhibition of the reduction of nitroblue tetrazolium as monitored at 560 nm. The activity of CAT was determined by following the consumption of H_2O_2 at 240 nm (E = 39.4 mM^{-1} cm^{-1}) by the method of Aebi (1984). The activity of APX was measured according to Nakano and Asada (1981) by monitoring the rate of ascorbate oxidation at 290 nm (E = 2.8 mM^{-1} cm^{-1}). Enzyme activities were expressed on the basis of per unit protein weight. Protein content in the supernatant was determined using bovine serum albumin (BSA) as a standard (Bradford, 1976).

NO content determination

NO content determination was performed according to Murphy and Noack (1994) with some modifications. Fresh leaves (0.5 g) were incubated with 100 units of catalase and 100 units of superoxide dismutase for 5 min to remove endogenous ROS before addition of 5 ml oxyhaemoglobin (5 mM). After 2 min incubation, NO concentrations were estimated by following the conversion of oxyhaemoglobin to methaemoglobin spectrophotometrically at 577 and 591 nm.

Statistical analysis

Each experiment was repeated at least three times. Values were expressed as means ± standard deviation (SD). Statistical analyses were performed by analysis of variance (ANOVA). Means were separated using Duncan's multiple range test at 5% level of significance.

RESULTS

Effect of NO on electrolyte leakage under arsenic stress

Different SNP concentrations (50 to 500 μM) were applied, since the effects of NO on plants are concentration dependent. SNP concentrations from 50 to 300 μM alleviated electrolyte leakage, with 100 μM being most effective under arsenic stress. However, 500 μM SNP was found to be toxic, leading to a great increase of electrolyte leakage in tall fescue leaves under arsenic stress (Figure 1A). As a result, in the following experiments, we used 100 μM SNP as the NO donor to study NO responses to arsenic stress.

Apart from NO, SNP may also generate other residual products, such as sodium cyanide (NaCN), ferrocyanide (Fe(II)CN), ferricyanide (Fe(III)CN), sodium nitrite ($NaNO_2$) and sodium nitrate ($NaNO_3$). To determine which product functions in reducing electrolyte leakage, 100 μM NaCN, Fe(II)CN, Fe(III)CN, $NaNO_2$ and $NaNO_3$ were added separately. The results showed that supplementation of these residual products had little effect on electrolyte leakage in tall fescue leaves under arsenic stress (Figure 1B).

Arsenic stress caused a significant rapid increase in electrolyte leakage (at $P < 0.05$). After 4 days treatment, electrolyte leakage remarkably increased by 51.7% and then showed little increase after 8 days treatment (Figure 2A). Treatment of plant leaves with NO donor, SNP before arsenic stress resulted in significant decrease of electrolyte leakage in tall fescue leaves (at $P < 0.05$), especially 8 days after treatment. NO scavenger PTIO were utilized to further clarify the role of SNP, the results showed that addition of PTIO enhanced electrolyte leakage to similar levels in tall fescue leaves under arsenic stress, indicating that PTIO scavenged endogenous NO as well as exogenous NO supplied by SNP.

Effect of NO on plant growth under arsenic stress

As shown in Figure 2B, arsenic stress significantly decreased dry weight in tall fescue plants both 4 and 8 days after treatment (at $P < 0.05$) and the inhibition was significantly alleviated by exogenous NO treatment (at $P < 0.05$). The alleviating arsenic stress of exogenous NO was blocked by PTIO (an NO scavenger).

Effect of NO on lipid peroxidation under arsenic stress

Induction of the arsenic stress resulted in a significant increase in MDA contents in tall fescue leaves both 4 and 8 days after treatment (Figure 3A). Supplementation with NO donor, SNP before arsenic stress remarkably (at $P < 0.05$) reduced MDA contents in tall fescue leaves (at $P < 0.05$). However, when NO was removed (PTIO or SNP + PTIO addition), MDA content rose evidently, indicating that severe lipid peroxidation was caused (Figure 3A).

Effect of NO on H_2O_2 and $O_2^{\cdot-}$ production under arsenic stress

Compared to control leaves, arsenic stress caused significant accumulations (at $P<0.05$) of H_2O_2 and $O_2^{\cdot-}$ production in tall fescue leaves (Figures 3B and C). Application of NO significantly reduced the accumulation of H_2O_2 and $O_2^{\cdot-}$ (at $P < 0.05$). Addition of PTIO or SNP+PTIO remarkably increased H_2O_2 levels and $O_2^{\cdot-}$ production rate in tall fescue leaves under arsenic stress (Figures 3B and C).

Effect of NO on antioxidant enzyme activity under arsenic stress

All antioxidant enzymes measured have showed increased activities after arsenic treatments, especially enzymes in 4 days (Figures 4A - C). Arsenic stress had different effects on SOD, CAT and APX activity both 4 and 8 days

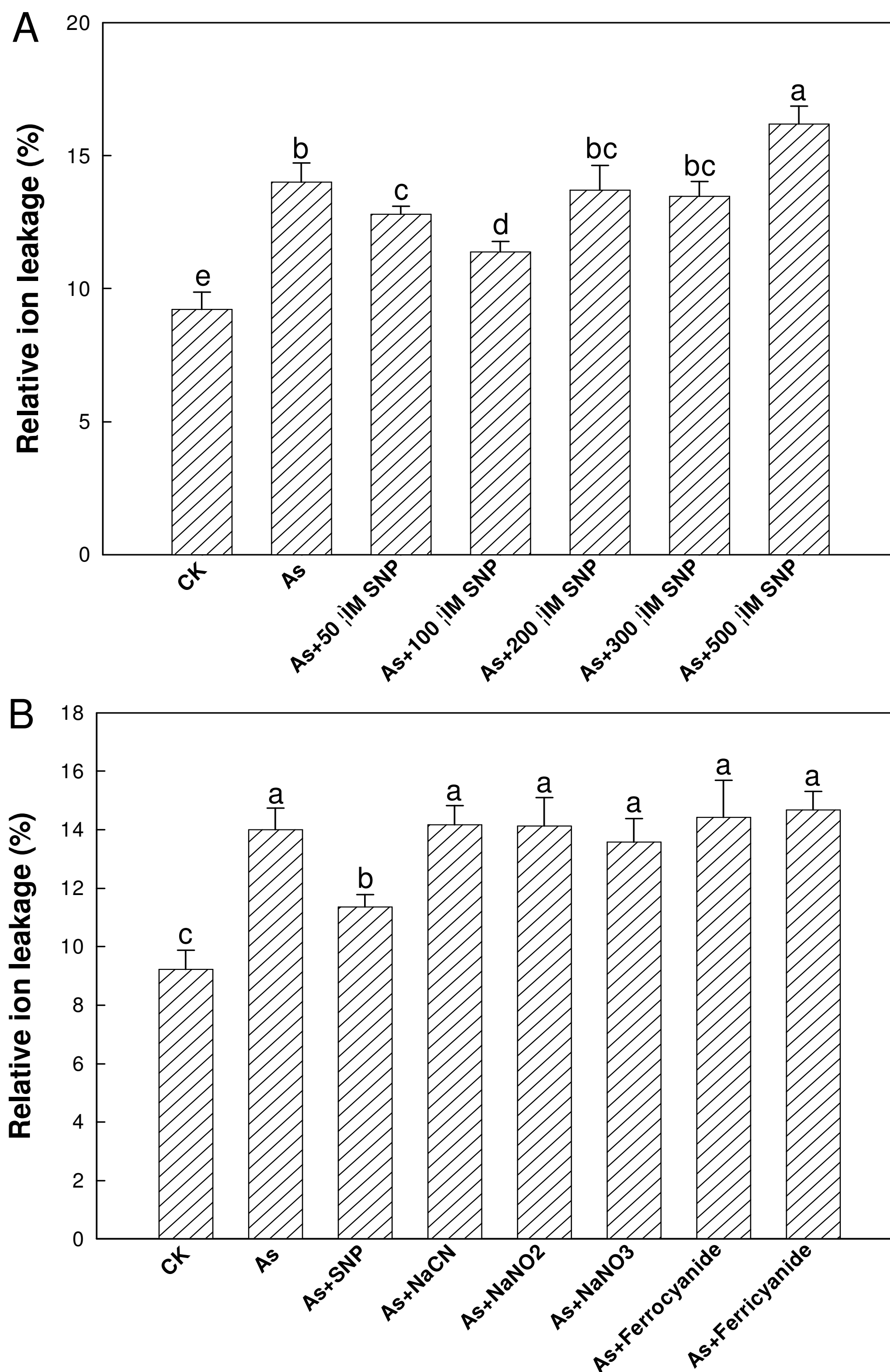

Figure 1. A) Changes of REL in the presence of different SNP concentration in tall fescue leaves under arsenic stress, after 4 days treatment. **B**) The effect of residual products of NO decomposition on REL of tall fescue leaves under arsenic stress, after 4 days treatment. Mean value ± SD (n = 3). Bars with different letters are significantly different at the 5% level.

after treatment in tall fescue leaves. On the 8th day of treatment, arsenic stress slightly induced SOD, CAT and APX activity (at $P < 0.05$). Under arsenic stress, application of NO kept SOD, CAT and APX relativity high activity on the 4th day of treatment but slightly decreased APX activity on the 8th day treatment. While PTIO or SNP+PTIO addition greatly reduced these antioxidant enzymes activities in tall fescue leaves (Figures 4A - C).

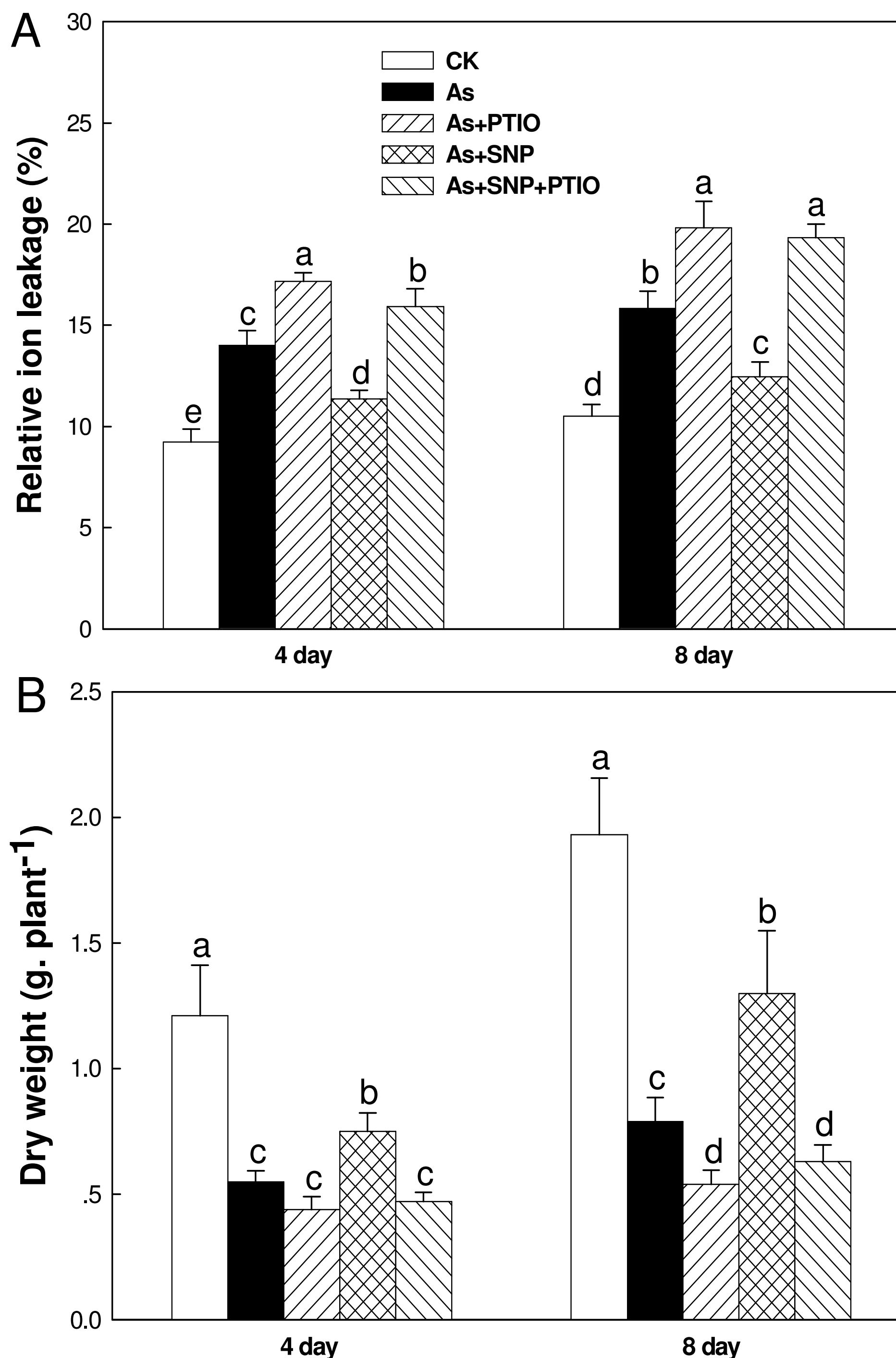

Figure 2. The influence of NO on relative electrolyte leakage (A) and biomass (B) of tall fescue leaves under arsenic stress. Mean value ± SD (n = 3). Bars with different letters are significantly different at the 5% level. CK, water alone; As, 25 µM sodium arsenate; As + PTIO, 25 µM As + 200 µM PTIO; As + SNP, 25 µM As + 100 µM SNP; As + SNP+ PTIO, 25 µM As + 100 µM SNP + 200 µM PTIO.

NO production under arsenic stress

To further reveal the relationship between NO accumulation and arsenic stress, NO production was measured. Arsenic stress caused significantly increased NO production both 4 and 8 days after treatment (Figure 4D). NO production increased by 160.9 and 70.8% in tall fescue leaves, respectively. Application of NO scavenger

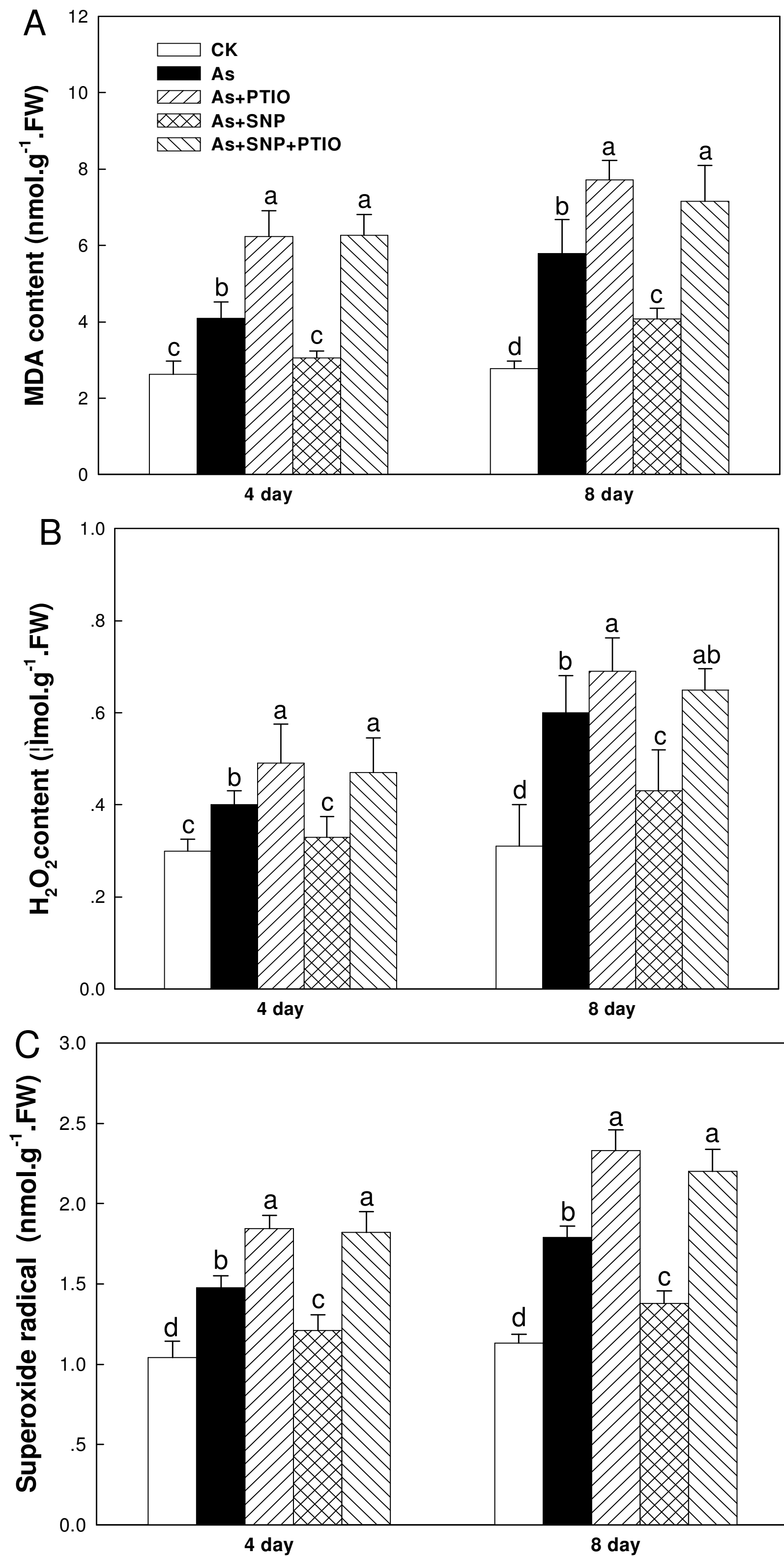

Figure 3. The influence of NO on the levels of (A) MDA, (B) H_2O_2 and (C) $O_2^{\cdot -}$ of tall fescue leaves under arsenic stress. Mean value ± SD (n = 3). Bars with different letters are significantly different at the 5% level. CK, water alone; As, 25 µM sodium arsenate; As + PTIO, 25 µM As + 200 µM PTIO; As + SNP, 25 µM As + 100 µM SNP; As + SNP+ PTIO, 25 µM As + 100 µM SNP + 200 µM PTIO.

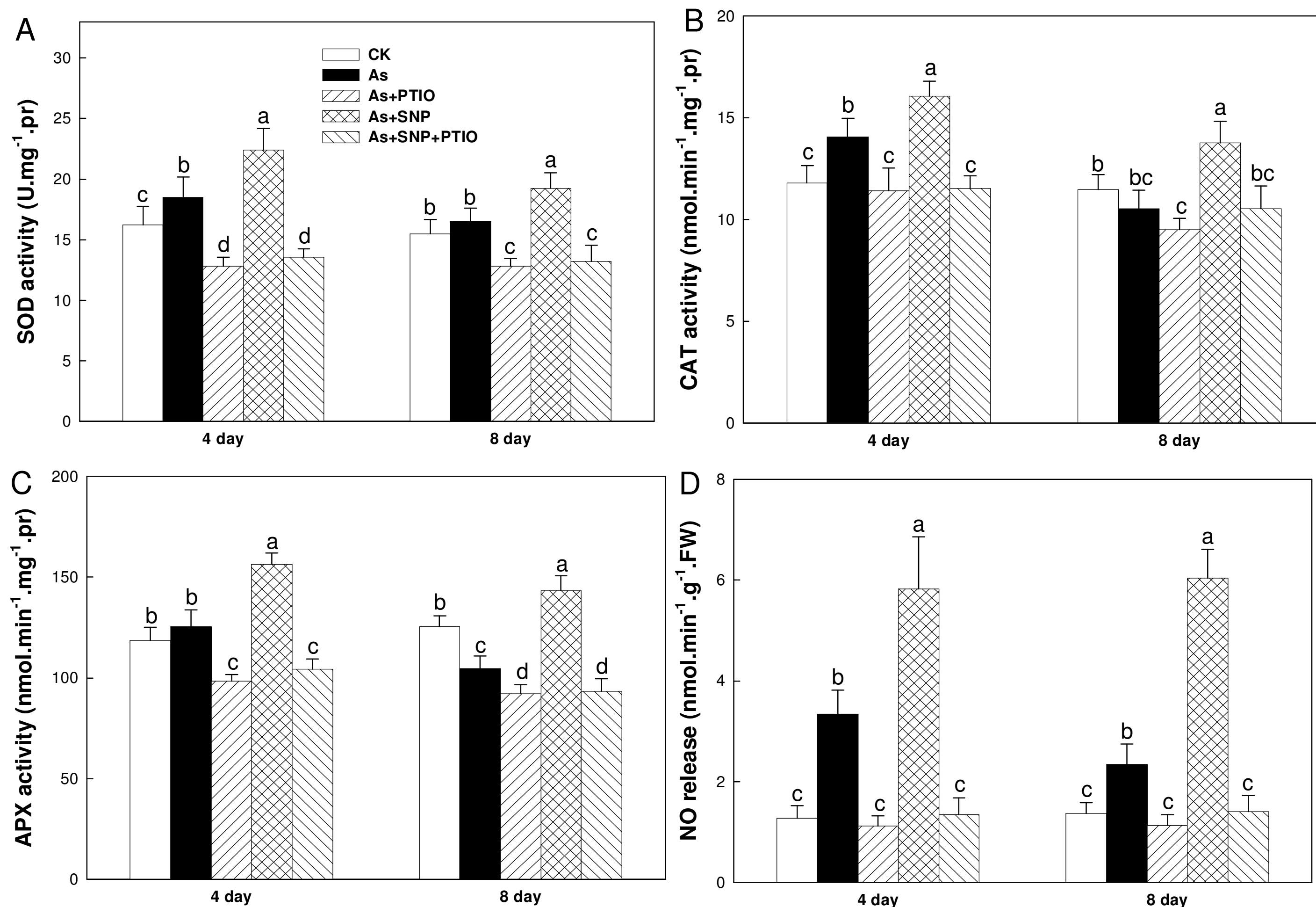

Figure 4. The influence of NO on the activities of antioxidant enzymes of **(A)** SOD, **(B)** CAT, **(C)** activity and **(D)** NO release of tall fescue leaves under arsenic stress. Bars with different letters are significantly different at the 5% level. CK, water alone; As, 25 µM sodium arsenate; As + PTIO, 25 µM As + 200 µM PTIO; As + SNP, 25 µM As + 100 µM SNP; As + SNP+ PTIO, 25 µM As + 100 µM SNP + 200 µM PTIO.

PTIO merely reduced NO content (Figure 4D).

DISCUSSION

Increased ROS (H_2O_2 and $O_2^{\cdot -}$) and lipid peroxidation has been obtained in plants under arsenic stress (Mascher et al., 2002; Stoeva et al., 2005; Shaibur et al., 2006; Singh et al., 2007; Singh et al., 2009). In the present study, the production of H_2O_2 and $O_2^{\cdot -}$ increased in tall fescue leaves (Figures 3B and C).The increased SOD activity may account for the increased accumulation of $O_2^{\cdot -}$ in tall fescue leaves (Figure 3A). Over production of ROS caused the oxidation of membrane lipids, proteins and enzymes necessary for the proper functioning of the chloroplasts and cells as a whole (Mittler, 2002). The increase in membrane permeability and MDA under arsenic stress indicated that arsenic (As) induced oxidative damage on membrane lipid and proteins (Figures 2A and 3A).

Previous studies have indicated that enhancements in the activities of ROS scavenging enzymes generally accompany exposure to arsenic stress. For example, it has been reported that ROS-scavenging enzymes active-ties increased under arsenic stress in red clover (Mascher et al., 2002) and mung bean (Singh et al., 2007). Our results indicated that tall fescue leaves exhibited increases in the activities of antioxidant enzymes (SOD, CAT and APX) both 4 and 8 days after arsenic treatment (Figures 4A - C). SOD was a major scavenger of $O_2^{\cdot -}$, catalyzing the dismutation of superoxide to H_2O_2 and O_2. CAT and APX were important H_2O_2 detoxifying enzymes. Increases in the activities of all anti-oxidant enzymes suggest that generation of ROS induced increased activities of antioxidant enzymes located in different cellular compartments (Logan et al., 1998; Apel and Hirt, 2004). Pronounced increase in anti-oxidant enzymes activities and the relatively low level of ROS in tall fescue leaves indicated that tall fescue alle-viated oxidative injuries through raising antioxidant

enzymes activities to scavenge newly-produced ROS.

Nitric oxide (NO) could counteract oxidative damage and had protective effect against various stressful conditions (Song et al., 2006; Arasimowicz and Floryszak-Wieczorek, 2007; Shi et al., 2007; Sun et al., 2007; Vital et al., 2008; Zhao et al., 2008; Singh et al., 2009). Addition of exogenous NO with SNP significantly enhanced antioxidant enzymes activities and reduced ROS levels, prevented lipid peroxidation and membrane damage, whereas a reversed pattern was found with the supplementation of NO savenger PTIO (Figures 2 and 3). This was consistent with postulated role of NO as a signalling molecule to induce increases in the activities of antioxidant enzymes (Laspina et al., 2005; Shi et al., 2007; Zhao et al., 2008; Zhang et al., 2009) to inhibit lipid peroxidation and membrane damage caused by environmental stress. Additionally, NO itself can detoxify ROS, such as reacting with $O_2^{\cdot -}$ and generating peroxynitrite ion ($ONOO^-$). In the physiological pH range, $ONOO^-$ is an unstable product. However, $ONOO^-$ can be protonated and decomposed to a nitrate anion and a proton, or it can react with hydrogen peroxide to yield a nitrite anion and oxygen. Thus, NO might provide protection against oxidative stress (Martinez et al., 2000; Wendehenne et al., 2001). Recent research also revealed that NO can induce APX and CAT antioxidant genes in *Arabidopsis* suspension cells (Huang et al., 2002). Tall fescue suffered severe peroxidative damage when endogenous NO was removed by PTIO, which further proved that NO plays an important role in protection effect against arsenic-induced stress.

In summary, ROS metabolism is clearly important for tall fescue during acclimatization to arsenic stress. The acquisition of tolerance to arsenic stress tall fescue leaves, are owed to the significantly increased ROS scavenging enzymes activities. As a bioactive antioxidant, NO protects tall fescue leaves against arsenic-induced oxidative damage by reacting with ROS directly or inducing activities of ROS-scavenging enzymes.

ACKNOWLEDGEMENT

This work was supported by the National Natural Science Foundation of China (No.40671084), the National Key Technologies R & D Program (No.2006BAD10A01), Beijing Natural Science Foundation (No.6072017), the Program for the New Century Excellent Talents in University of China (No.NCET-06-0107) and by the Program for Changjiang Scholars and Innovative Research Team (IRT0412).

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