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ORIGINAL PAPER

Effects of nitric oxide on alleviating cadmium stress in *Typha angustifolia*

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Abstract Nitric oxide (NO) is a key signal molecule that is involved in plant response to various abiotic stresses. The present study was conducted to investigate the effects of NO on *Typha angustifolia* which has been subjected to cadmium stress. Our results showed that sodium nitroprusside (SNP, a NO donor) treatment could significantly mitigate both the Cd ($CdCl_2 \cdot 2.5H_2O$)-induced increase of malondialdehyde content in the root and relative electrolyte leakage in the root and leaf. NO demonstrated a reduced or counteractive effect on the Cd-induced increase in the activities of some typical antioxidant enzymes. The Cd-induced changes on the contents of non-protein thiol and phytochelatins in the root could also be reversed by NO. The protected effect of NO is likely achieved through trapping Cd in the root and decreasing Cd accumulation in the leaf and sheath. Furthermore, NO enhanced the allocation of Cd in the cell wall and reduced the distribution of Cd in the soluble fraction of the root and leaf. Differential biosynthesis of ascorbic acid content in some tissues might also be responsible for the protected effect of NO. This study concludes that NO counteracts Cd toxicity strongly in *T. angustifolia* via regulating antioxidant metabolism and enhancing Cd accumulation in the cell wall of the root.

Keywords *Typha angustifolia* · Cadmium content · Nitric oxide · Subcellular distribution · Antioxidant enzymes

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Introduction

Cadmium (Cd) is a highly toxic and persistent environmental poison for plants and animals. It can be found in soil and water, and is readily uptaken by plants. Thus, with regards to agricultural plants growing in these affected areas, the sustentative consumption of high-Cd plant tissue constitutes a significant threat to human health (Wang et al. 2013; Wu et al. 2014). As a non-essential nutrient, Cd has been shown to influence the absorption, transportation, and usage of both water and essential nutrients while also causing various visible symptoms including chlorosis, wilting and root browning (Ding et al. 2013; Gallego et al. 2012; Garcia et al. 2006; Gratao et al. 2012). To cope with Cd stress, plants adopt many ways to avoid Cd stress or enhance tolerance to Cd toxicity (Baker 1987). The cell wall, which is the first barrier against Cd stress, could provide important ligands to immobilize Cd when it enters plants (Huguet et al. 2012). Moreover, cell wall deposition and vacuolar compartmentalization also play an important role in the detoxifying of metals (Verbruggen et al. 2009). When the cell wall is saturated with heavy metals, the excess of heavy metal ions could be transferred into the vacuole storage (Krämer 2000; Przymusinski and Wozny 1985; Wierzbicka 1987). Cd stress could cause the overproduction of reactive oxygen species (ROS) which subsequently leads to lipid peroxidation (Smeets et al. 2008). Excess ROS could be cleared by antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX) and non-enzymatic antioxidant such as ascorbic acid (AsA), which reduce glutathione and other molecules capable of quenching ROS. In addition, the excess of heavy metal ions can be chelated by some peptide substances, such as the non-protein thiol (NPT), phytochelatins (PCs) which can be induced by Cd stress (Cobbett 2000; Xiang and Oliver 1998).

Nitric oxide (NO) is a bioactive molecule that functions in numerous physiological processes in plants including plant growth and development (Durner et al. 1998). Besides its role, as an important signaling molecule, NO has been shown to be involved in the physiological responses of various plants to biotic and abiotic stresses such as drought, salt, heavy metal and heat (Arasimowicz and Floryszak-Wieczorek 2007). Two main mechanisms were proposed to be responsible for the protected effect of NO. First of all, NO could directly scavenge the overproduced ROS in cells. Secondly, NO might function as a signaling molecule to modulate antioxidative gene expression (Verma et al. 2013). However, accumulating evidence shows that NO is a double edged sword in plants. Low and high content of NO could accelerate and inhibit growth, respectively (Wink and Mitchell 1998). Apparently conflicting results have also been published on NO physiology. For example, the Cd-mediated induction (Bartha et al. 2005) or inhibition (Rodríguez-Serrano et al. 2009) of NO production has been found to be involved in the regulation of Cd cytotoxicity (Luo et al. 2012).

The Typha genus, important aquatic plants widely distributed in wetlands, show great capacity to absorb heavy metal so that it can be used in phytoremediation. Mojiri et al. (2013) suggests that *Typha domingensis* is an effective accumulator plant for phytoremediation of some heavy metals (Pb, Ni and Cd). *Typha angustifolia* has been proven to be strongly tolerant to Cd stress, and adding Cd could even increase its height and biomass (Bah et al. 2011). Therefore, Typha plants can be used in phytoremediation for Cd-polluted water. Our previous experiments also demonstrated that *T. angustifolia* was a metal-hyperaccumulator which showed high heavy metal tolerance (Wu et al. 2007, 2008). Therefore, *T. angustifolia* could be an excellent model system to explore the mechanisms involved in its high Cd tolerance. However, related studies were lacking. The effect of NO on plant tolerance to Cd stress is conflicted in different species and there is little information about it in *T. angustifolia*. Hence, we studied the role of NO in plant response to Cd stress by evaluating changes in various metabolic processes via application of a NO donor in *T. angustifolia*. This present study will be of significance as an elucidator of the functionality of NO in *T. angustifolia*, which has not yet been explored. As a result, this research may deepen our understanding of the mechanisms of NO-mediated amelioration of Cd toxicity in plants and can provide a theoretical basis for ameliorating phytoremediation.

Materials and methods

Plant materials and treatment

Typha angustifolia seedlings were grown in the growth chamber at 30/25 °C (day/night), with a light intensity of 200 μmol m^{-2} s^{-1} and a 12 h photoperiod. The Hoagland nutrient solution (pH 6.0) was refreshed every 2 d. After growing for 20 d, seedlings were incubated in six groups as follows: (1) CK; (2) 444.8 μM Cd ($CdCl_2 \cdot 2.5H_2O$); (3) 444.8 μM Cd + 100 μM sodium nitroprusside (SNP, a NO donor); (4) 444.8 μM Cd + OLD SNP; (5) 444.8 μM Cd + 100 μM SNP + 0.1 % Hemoglobin bovine (Hb, a NO scavenger, w:v); (6) 444.8 μM Cd + 0.1 % Hb (w:v). Cd concentration had been screened in preliminary experiments. An old SNP solution (presumably containing equimolar amounts of ferrocyanide, nitrate and nitrite, two normal products of NO decomposition) was obtained as a negative control by maintaining a 100 μM SNP solution for 10 days in the light to eliminate NO before its application (Tossi et al. 2009). After 4 days, root, sheath and leaf were harvested separately according to various physiological indexes. After 7 days, we took a photo for four groups which including CK, Cd, Cd + SNP and Cd + Hb. The concentrations of above chemicals used in this study were determined in pilot experiments from where the effective responses were obtained.

Assay of antioxidant enzyme

0.2 g fresh plant tissue (root, leaf or sheath) was homogenized in 1.6 mL 50 mM sodium phosphate buffer (PBS, pH 7.8) using a pre-chilled (4 °C) mortar and pestle. Extractions were centrifuged at 12,000×*g* for 20 min at 4 °C. The supernatant was used for enzyme activity assay. SOD activity was determined as previously described by Zhou et al. (1997). CAT activity was determined by measuring the changes in absorbance at 470, 240 and 290 nm, respectively (Aebi 1984).

Determination of malondialdehyde (MDA) content and electrolyte leakage

MDA content was measured on the basis of Devi and Prasad (1998) with minor modifications. Plant tissues (0.2 g) were homogenized in 3 mL of 0.5 % (w/v) Trichloroacetic acid (TCA) and then centrifuged at 3,000×*g* for 10 min. 2 mL of supernatant was mixed with 2 mL 0.67 % (w/v) 2-Thiobarbituric acid (TBA). The mixture was heated at 100 °C for 30 min, quickly cooled on ice, and then centrifuged at 3,000×*g* for 10 min. The absorbance of the supernatant was read at 450, 532 and 600 nm.

To measure electrolyte leakage, 0.2 g fresh tissues were floated on 50 mL of deionized water with continuous shaking for 24 h at room temperature. The electrolyte content in the solution was measured immediately (C0). Then samples were boiling for 20 min and electrolyte content was determined (C1). Results were expressed as

percentage of electrolyte leakage: relative electrolyte leakage = (C0/C1) × 100 % (Blum and Ebercon 1981).

Determination of Cd content

After 4 days of Cd treatment, seedlings were rinsed thrice with deionized water. Seedlings of each treatment were divided into two groups, one of which was dried at 105 °C for 30 min and then at 80 °C for 24 h. The plant material was digested with HNO_3:$HClO_4$ (87:13, v:v). The other group was used for the determination of subcellular Cd distribution, with some modification (Wang et al. 2008). 1 g sample was homogenized in 30 mL of extraction medium containing 250 mM sucrose, 50 mM Tris(hydroxymethyl)aminomethane(Tris–HCl) (pH 7.5) and 1 mM DL-Dithiothreitol (DTT). The homogenate was centrifuged at 300×*g* for 5 min to isolate the cell wall. The supernatant was centrifuged at 20,000×*g* for 45 min. The precipitate was cell organelles and the supernatant was the soluble fraction. All aforementioned steps were carried out at 4 °C. Cd content was determined by ICP (ICP-AES, Thermo Elemental, USA).

Determination of plant metabolites

Plant tissues (0.2 g) were homogenized in 2 mL of 5 % 5-Sulfosalicylic acid dehydrate and then centrifuged at 8000×*g* for 10 min. The NPT content was measured in the supernatant. The reaction mixture contained 200 μL of the supernatant, 2 mL of 0.2 M Tris–HCl (pH 8.2) and 0.15 mL of 10 mM 5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB) and was incubated for 20 min. After incubation, the absorbance was measured at 412 nm. An aliquot without DTNB was used to adjust the spectrophotometer to zero absorbance and GSH was used as standard (Devi and Prasad 1998).

Statistical analysis

The resulting data were analyzed through analysis of variance (ANOVA) while the difference (Duncan) test was employed to determine significant differences among the treatments at the $P < 0.05$ level.

Results

NO protects *T. angustifolia* against Cd toxicity

Under Cd stress, *T. angustifolia* seedlings showed symptoms of Cd toxicity: growth inhibition and severe oxidative damage (Fig. 1). Lipid peroxidation caused by Cd stress was monitored via measuring MDA, a product of peroxidation. After 4 days' treatment, Cd exposure caused significant increases in the MDA content of the leaf and sheath but it seemed to have little effect on the root (Fig. 2a). On the contrary, exogenous NO treatment significantly reduced Cd-induced MDA accumulation in root but caused slightly decreased MDA accumulation in the leaf and sheath. When combining with Cd, SNP and Hb demonstrated opposing tendencies in the sheath and root. The MDA content of the Cd + OLD SNP (old SNP containing no NO but ferrocyanide, nitrate and nitrite) and Cd + Hb treatment were close to the Cd treatment alone. This observation implies that NO can alleviate the damage of Cd by reducing the content of MDA in the root.

Relative electrolyte leakage was measured to evaluate the degree of Cd injury in *T. angustifolia*. Under Cd stress, relative electrolyte leakage was significantly higher than the control in the root and leaf of *T. angustifolia* while there seemed to be little effect in the sheath (Fig. 2b). SNP treatment significantly blocked the increase of electrical conductivity triggered by Cd stress in the leaf while adding Hb significantly relieved the effect of NO. Our data shows that the relative electrolyte leakage levels of plants treated with OLD SNP or Hb was not significantly different than Cd-exposed plants. In short, the aforementioned results proved that the application of exogenous NO exhibited protective effects against Cd-induced oxidative damage in *T. angustifolia*.

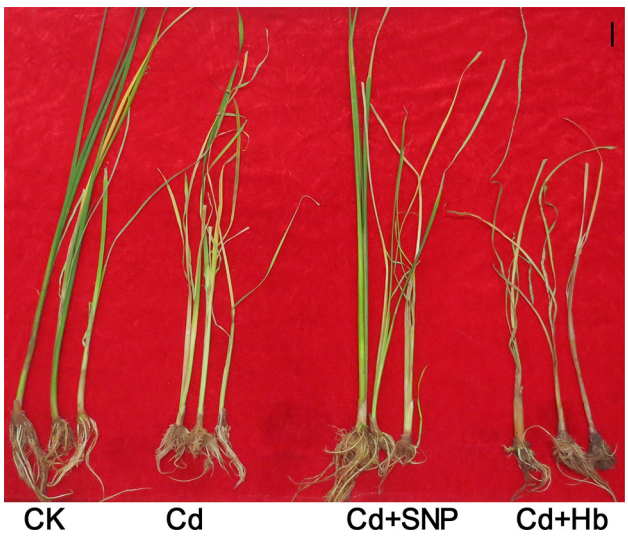


Fig. 1 Effect of Nitric oxide (NO) on seedling growth in the leaf, sheath and root of *T. angustifolia*. Plants were treated with Hoagland nutrient solution (control, CK), Cadmium (Cd, 444.8 μM), Cd (444.8 μM) + sodium nitroprusside (SNP, a NO donor, 100 μM) and Cd (444.8 μM) + 0.1 % Hemoglobin bovine (Hb, a NO scavenger, w:v) for 4 days. Photo was taken 7 days after treatment. *Vertical bars* represent standard error of the mean (n = 3). *Vertical bars* represent standard error of the mean (n = 3). *Bars* with *different letters* are significantly different within each group at $P < 0.05$ according to Duncan's multiple test

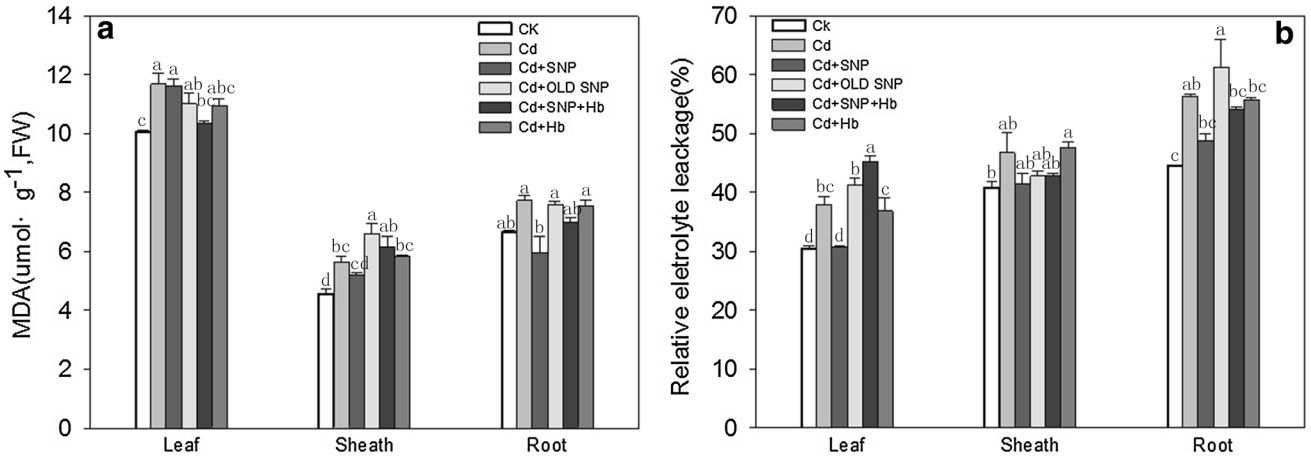


Fig. 2 Effect of sodium nitroprusside (SNP, a NO donor) on malondialdehyde (MDA) **a** content and relative electrolyte leakage **b** in the leaf, sheath and root of *T. angustifolia.* Plants were treated with Hoagland nutrient solution (control, CK), Cd (444.8 μM), Cd (444.8 μM) + SNP (100 μM), Cd (444.8 μM) + OLD SNP, Cd (444.8 μM) + SNP (100 μM) + 0.1 % Hb (w:v), Cd (444.8 μM) + 0.1 % Hb (w:v). *Vertical bars* represent standard error of the mean (n = 3). *Vertical bars* represent standard error of the mean (n = 3). *Bars* with *different letters* are significantly different within each group at $P < 0.05$ according to Duncan's multiple test

Effects of NO on activities of antioxidant enzymes and AsA content

The combined results from our lipid peroxidation and electrical conductivity assays suggested that SNP treatment enhanced plant tolerance to Cd stress probably through reducing ROS accumulation and subsequent oxidative damage. Thus, it was necessary to examine the antioxidant responsible for ROS scavenging. Subsequent analyses were carried out to investigate SOD and CAT in *T. angustifolia* plants which were subjected to Cd stress, with or without the SNP or Hb treatments. Our results showed that Cd treatment significantly increased SOD and CAT activities in *T. angustifolia* compared with CK (Fig. 3). However, we observed that SNP counteracted the Cd-induced increases to the activities of SOD and CAT. Likewise, adding Hb counteracted the effect of SNP on the activities of SOD and CAT. Among the two antioxidant enzymes studied, SNP treatment had insignificant or diametric effects on enzyme activities in *T. angustifolia* under Cd stress.

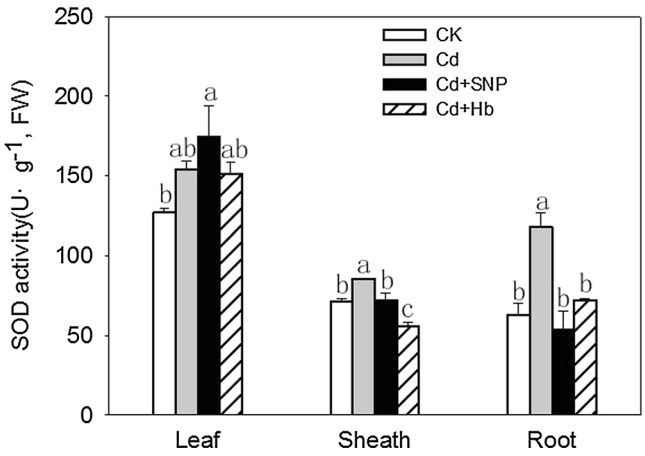
Ascorbic acid (AsA) is one of the most important water-soluble non-enzymatic antioxidants in plants, which are related to the detoxification of reactive oxygen species (Chen and Gallie 2012). In *T. angustifolia*, the content of AsA was induced by SNP treatment compared with Cd treatment, while Hb treatment reversed counteracted this increase (Fig. 4).

Effect of NO on the accumulation of metabolites

Both PCs and NPT are peptides which can relieve Cd stress by chelating Cd in cells. As shown in Fig. 5, Cd treatment significantly increased the contents of PCs and NPT in the root, while it had only slight effects on the leaf and sheath. SNP treatment was found to significantly counteract Cd-induced increases to PCs and NPT in the root. After application of Hb to plants under Cd stress, PCs and NPT content had only slight changes in the leaf and sheath, but there was an obvious reduction in the root compared with Cd stress. These results suggest that the protected effect of SNP might not work through chelating Cd ions in cells by PCs or NPT.

NO increase Cd accumulation in cell walls

Analysis of Cd content revealed that additional Cd in nutrient solution significantly increased Cd accumulation in different tissues of *T. angustifolia*. The uptake of Cd in the SNP-treated seedlings exhibited a significant decrease in the leaf and sheath (Table 1). However the accumulation of Cd in the root was significantly increased. When applied together with Cd and Hb, the accumulation of Cd had a crosscurrent with SNP treatment, but close to Cd stress. We further determined Cd content in the subcellular fractions of different tissues (Table 2). As expected, in Cd + SNP treated leaves, Cd content in different subcellular fractions decreased when compared with samples subjected to Cd stress alone. Adding SNP under Cd treatment, the accumulation of Cd had slightly increased in cell wall and organelles, but it was obviously reduced in the soluble fraction of the sheath when compared with plants that wrer only subjected to Cd stress. In the root, supplying SNP resulted in increased Cd accumulation in the cell wall and decreased Cd content in the soluble fraction of cells. The Cd content in different subcellular fractions of the leaf increased under Cd + Hb treatment when compared with



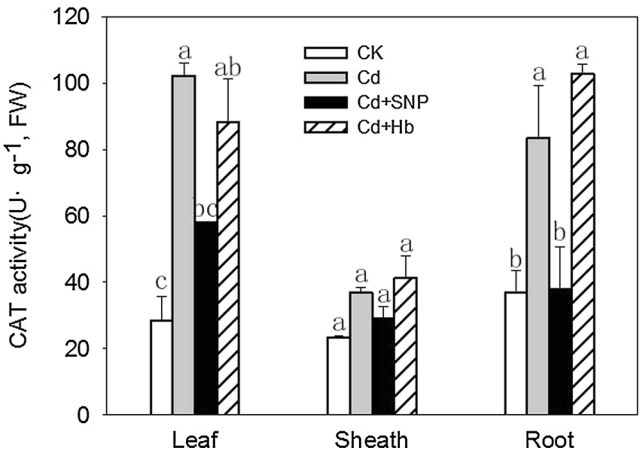


Fig. 3 Effect of sodium nitroprusside (SNP, a NO donor) on SOD and CAT activities in the leaf, sheath and root of *T. angustifolia* exposed to Cd in the presence of SNP. Plants were treated with Hoagland nutrient solution (control, CK), Cd (444.8 μM), Cd (444.8 μM) + SNP (100 μM) and Cd (444.8 μM) + 0.1 % Hb (w:v). *Vertical bars* represent standard error of the mean (n = 3). *Bars* with *different letters* are significantly different within each group at $P < 0.05$ according to Duncan's multiple test

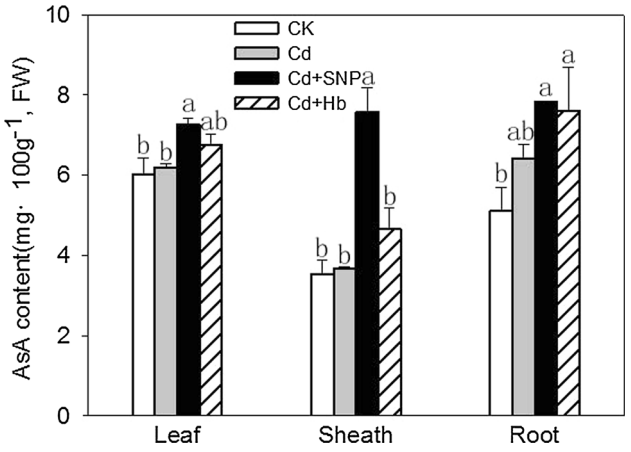


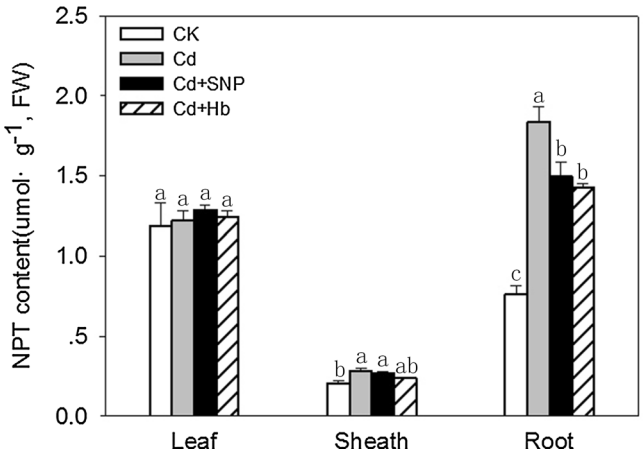
Fig. 4 Effect of sodium nitroprusside (SNP, a NO donor) on ascorbic acid (AsA) content in the leaf, sheath and root of *T. angustifolia*. Plants were treated with Hoagland nutrient solution (control, CK), Cd (444.8 μM), Cd (444.8 μM) + SNP (100 μM) and Cd (444.8 μM) + 0.1 % Hb (w:v). *Vertical bars* represent standard error of the mean (n = 3). *Bars* with *different letters* are significantly different within each group at $P < 0.05$ according to Duncan's multiple test

the Cd + SNP treated, and the Cd content in the organelles of Cd + Hb treated was significantly higher than it was in the leaf under Cd stress alone. Hb promoted the accumulation of Cd in the soluble fraction of root samples compared with SNP treatment.

Discussion

Cd is one of the toxic heavy metals and has become a widespread pollutant in the environment. Cd is easily accumulated in higher plants and causes severe inhibition of plant growth (Wagner 1993). It has been well documented that Cd stress can indirectly cause the increased generation of ROS, which subsequently results in oxidative damage to plant tissues (De Michele et al. 2009). MDA, a product of lipid peroxidation, is often measured as an indicator of oxidative stress. It has been reported in wheat root that MDA content increases under Cd stress and supplementary SNP could decrease MDA content and thus alleviate Cd stress (Singh et al. 2008). When applied together with Cd, SNP and Hb had an opposite tendency compared to Cd + SNP treatment. Consistent with this, our results showed that under Cd stress, adding SNP relieved Cd stress via decreasing MDA content in the root (Fig. 2a). An electrolyte leakage assay is frequently used to measure the degree of cell damage. Our data show that Cd exposure caused significant changes in REL in different tissues. We estimated the electrolyte leakage of the different tissues and found that SNP treatment could significantly block the increase of electrical conductivity triggered by Cd stress (Fig. 2b). However, application of Cd + SNP + Hb treatment increased levels of REL when compared with Cd + SNP treatment. Additionally, plants subjected to the Cd + OLD SNP treatment yielded opposing results compared with the Cd + SNP treatment. The increased levels of REL indicated that Cd exposure induced the generation of ROS, causing cellular damage in plants (Apel and Hirt 2004). This study found that SNP-treatment relieved the increase of MDA content and levels of REL, and it showed that exogenous NO could protect or repair effect of the plant cell membrane.

In normal conditions, production and scavenging of ROS are in equilibrium. Adversity stress causes ROS metabolic imbalance and leads to excessive accumulation of reactive oxygen species which thereby cause oxidative



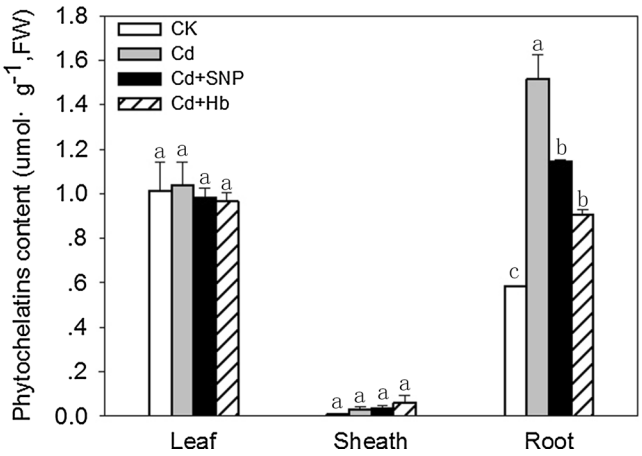


Fig. 5 Effect of sodium nitroprusside (SNP, a NO donor) on non-protein thiol (NPT) and phytochelatins (PCs) in the leaf, sheath and root of *T. angustifolia*. Plants were treated with Hoagland nutrient solution (control, CK), Cd (444.8 μM), Cd (444.8 μM) + SNP (100 μM) and Cd (444.8 μM) + 0.1 % Hb (w:v). *Vertical bars* represent standard error of the mean (n = 3). *Bars* with *different letters* are significantly different within each group at $P < 0.05$ according to Duncan's multiple test

Table 1 Effects of SNP on the distribution of Cd in root, sheath and leaf of *T. angustifolia*

Organ	Treatment	Cd content (mg·kg^{-1}, DW)
Leaf	CK	3.10 ± 0.46c
	Cd	63.72 ± 8.79b
	Cd + SNP	6.74 ± 0.02c
	Cd + Hb	88.96 ± 1.93a
Sheath	CK	9.17 ± 2.74b
	Cd	478.83 ± 134.33a
	Cd + SNP	85.99 ± 3.00b
	Cd + Hb	434.85 ± 34.06a
Root	CK	184.30 ± 18.04c
	Cd	9738.26 ± 249.35b
	Cd + SNP	15300.43 ± 1500.00a
	Cd + Hb	8265.86 ± 2065.77b

Vertical bars represent standard error of the mean (n = 3). Plants were treated with Hoagland nutrient solution (control, CK), Cd (444.8 μM), Cd (444.8 μM) + SNP (100 μM) and Cd (444.8 μM) + 0.1 % Hb (w:v) for 4 d. Vertical bars represent standard error of the mean (n = 3). Bars with different letters are significantly different within each group at $P < 0.05$ according to Duncan's multiple test

damage. Under Cd stress, plants scavenge excessive accumulation of ROS through the products of antioxidant enzymes and non-enzymatic antioxidants, thereby mitigating cell injury (Schützendübel et al. 2001; Wu et al. 2003). In this work, the activities of antioxidant enzymes including SOD and CAT increased in different tissues of *T. angustifolia* following Cd exposure, which illustrates that Cd stress leads to oxidative stress and which implies that the *T. angustifolia* is able to better tolerate Cd treatment by enhancing the activities of antioxidant enzymes (Fig. 3). However, the activities of SOD and CAT were decreased by Cd + SNP treatment, compared with plants which were subjected to Cd exposure alone. Similarly, it has been reported in wheat root that SNP supply resulted in a reduction in Cd-induced increased activities of SOD and CAT (Singh et al. 2008). Laspina et al. (2005) found that NO could prevent Cd-induced by increasing the activity of SOD in sunflower leaf. Under Cd exposure, Hb treatment could reverse the effect of SNP on the activities of SOD and CAT, and even higher than Cd exposure alone in the root. This effect might be ascribed to the capability of NO to scavenge Cd toxicity in other ways but antioxidant enzymes, which then hindered the increase in the activities of the antioxidant enzymes SOD and CAT.

We subsequently analyzed the changes of non-enzymatic antioxidants in *T. angustifolia*. The content of AsA, one of the most abundant and important non-enzyme antioxidants, was obviously induced by NO application in Cd exposed plants. However, the content of AsA had little increased by Hb treatment upon Cd exposure while comparing with Cd stress only (Fig. 4). These results indicated that the reduction of Cd toxicity might be aroused by antioxidant properties of NO, and SNP-induced AsA content might be involved in maintaining the balance of ROS.

Apart from scavenging of Cd-induced overproduced ROS, chelation of metals in the cytosol by high-affinity ligands is potentially a rather important mechanism of heavy-metal detoxification and tolerance. Some NPT compounds, comprised of several acid-soluble sulfhydryl components, such as cysteine and PCs, are known to be important for the detoxification or homeostasis of heavy metals (Clemens 2001). The PCs are the most widely studied high-affinity compounds in plants, especially in relation to Cd tolerance (Zenk 1996). The detoxification role of PCs has been supported by a range of biochemical

Table 2 Effects of SNP on subcellular distribution of Cd in *T. angustifolia*

Organ	Treatment	Cd content ($mg \cdot kg^{-1}$, FW)		
		Cell wall	Organelles	Soluble fraction
Leaf	CK	0.40 ± 0.01c	0.10 ± 0.01d	0.13 ± 0.03c
	Cd	5.38 ± 0.30a	0.76 ± 0.08b	3.51 ± 0.28a
	Cd + SNP	3.12 ± 0.83b	0.32 ± 0.02c	1.86 ± 0.47b
	Cd + Hb	4.67 ± 0.42a	0.94 ± 0.06a	3.27 ± 0.15a
Sheath	CK	0.60 ± 0.12c	0.25 ± 0.04c	0.27 ± 0.02c
	Cd	13.13 ± 0.52a	1.22 ± 0.16ab	10.49 ± 0.58a
	Cd + SNP	14.84 ± 2.13a	1.46 ± 0.04a	6.86 ± 0.37b
	Cd + Hb	7.24 ± 0.11b	1.02 ± 0.01b	6.17 ± 0.79b
Root	CK	6.80 ± 1.63c	0.40 ± 0.05d	3.53 ± 0.17c
	Cd	233.11 ± 3.81b	23.08 ± 0.16b	124.10 ± 6.56a
	Cd + SNP	761.38 ± 4.99a	32.69 ± 2.58a	81.28 ± 0.88b
	Cd + Hb	227.49 ± 5.30b	9.73 ± 0.45c	94.47 ± 2.63b

Vertical bars represent standard error of the mean (n = 3). Plants were treated with Hoagland nutrient solution (control, CK), Cd (444.8 μM), Cd (444.8 μM) + SNP (100 μM) and Cd (444.8 μM) + 0.1 % Hb (w:v) for 4 d. Bars with different letters are significantly different within each group at $P < 0.05$ according to Duncan's multiple test

and genetic evidence (Maier et al. 2003; Cobbett and Goldsbrough 2002). Once PCs are synthesized, they can be transported into the vacuole, where they then form high molecular weight complexes. These complexes are the ultimate and more stable storage form of cadmium (Mendoza-Cozatl and Moreno-Sanchez 2006). In this study, NPT and PCs contents in the root were significantly increased in plants exposed to Cd. However, the addition of SNP with Cd resulted in diametrically opposed changes in the NPT and PCs contents when compared to Cd exposure alone (Fig. 5). Application of Hb upon Cd stress significantly decreased PCs content in the root compared with simply Cd exposed plants.

We further analyzed Cd content in seedlings upon Cd stress. Our results showed that the highest content of Cd was accumulated in the root (Table 1). This is consistent with previous studies, which conclude that Cd mainly gathered in the root and accumulated lower Cd in ground (Küpper et al. 2000). Exogenous NO application increased Cd accumulation in the root and decreased its content in the leaf and sheath (Table 1). Namdjoyan and Kermanian (2013) found that treatment with 100 μM SNP considerably reduced root-to-shoot translocation of arsenic in watercress plants. Cd + Hb treatment resulted in increased accumulation of Cd in the leaf and sheath while decreasing Cd content in the root. Our results indicated that exogenous NO relieved Cd toxicity by reducing the transportation of Cd to the ground. The accumulation of Cd in different subcellular tissues was also analyzed. Table 2 showed that exogenous NO treatment enhanced Cd accumulation in the cell walls of the root in *T. angustifolia* seedlings, while it decreased Cd accumulation in the cell walls, organelles and the soluble fraction of the leaf. This decreased Cd content within cells resulted in less NPT and PCs being induced, which is responsible for the reduction of endogenous NPT and PCs contents upon Cd + SNP treatment. As might be expected, Cd + Hb treatment increased the Cd content in the soluble fraction of the root and leaf. Recalling basic botany, the cell wall is the first barrier to prevent Cd from entering into plant cells. Therefore, the accumulation of Cd in cell walls is one of the important mechanisms which plants use to mitigate Cd toxicity. Bringezu et al. (1999) indicated that a protein with oxalate oxidase activity in the cell walls could bind heavy metals. Thus, the uptake into the cells is restricted as only very small amounts were detected in the cytoplasm and in the organelles. It has also been previously reported that adding exogenous NO could relieve Cd stress by increasing pectin and hemicellulose contents in the root cell wall, increasing Cd accumulation in the rice root, and decreasing Cd accumulation in the rice leaf (Xiong et al. 2009). These results showed that exogenous NO could enhance cell wall holding functions to lessen Cd content in the soluble fraction which could ensure the normal order of various physiological and biochemical metabolism in the cytoplasm under Cd stress. We speculate that there might be an NO-dependent mechanism to exclude heavy metals from the cell and to prevent toxic concentrations from accumulating in the cytoplasm in *T. angustifolia*, but this model would need further investigation before it can be validated.

In summary, our study suggests that NO alleviates Cadmium toxicity in *T. angustifolia* mainly by enhancing the inherent ability of cell walls to chelate Cd. This study shows that the induced effect of NO on antioxidant enzymes may

also play a role in maintaining ROS level balance. As a signaling molecule, NO could also change enzyme activity of cell wall and thus affect the chemical properties of the cell wall (An et al. 2005). We speculate that NO might clearly regulate excess ROS by changing antioxidant enzyme activities of the cell wall; however, this theory requires further investigation. Collectively, our data imply that NO functions as a protector in *T. angustifolia* under Cd stress primarily via regulating antioxidant metabolism and enhancing Cd accumulation in the root cell wall.

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