Effects of exogenous nitric oxide on cadmium toxicity and antioxidative system in perennial ryegrass

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Abstract

The effects of sodium nitroprusside (SNP, a donor of NO) on cadmium (Cd) toxicity in ryegrass seedlings (*Lolium perenne* L.) were studied. 100 and 150 μM Cd stress had a detrimental effect on ryegrass seedlings. Exposure of 100 and 150 μM Cd inhibited plant growth, decreased chlorophyll concentration, and reduced the absorption of Fe, Cu and Zn. Excess Cd also altered the activities of antioxidant enzymes, and increased the accumulation of reactive oxygen species (ROS). Exogenous NO alleviated Cd toxicity of ryegrass plants, especially under the stress of 150 μM Cd, as evidenced by improved plant growth and increased concentrations of chlorophyll and mineral nutrients. Exogenous NO also mitigated oxidative stress by regulating the activities of antioxidant enzymes and the contents of non-antioxidants. Moreover, the absorption of Fe, Cu and Zn was increased, indicating that exogenous NO stimulated H^+ -ATPase activity to promote sequestration or uptake of ions. The applications of NO also reduced the translocation of Cd from roots to the leaves. These results indicate that the mechanisms of NO for mitigating Cd toxicity may be associated with reduced root-to-shoot translocation of Cd and enhanced capacity of antioxidative systems to protect plants from oxidative stress.

Keywords: Ryegrass, cadmium, sodium nitroprusside, antioxidative systems, phytochelatin, glutathione, ion accumulation

1. Introduction

Cadmium (Cd) is a widespread heavy metal pollutant that is extremely toxic to both plants and animals (Xu *et al.*, 2011, Retamal-Salgado *et al.*, 2017). It is released into the environment by heating systems, metallurgical industries, waste incinerators, urban traffic, cement factories, and as a contaminant of phosphate

fertilizers (Gallego *et al.*, 2012). Although Cd is not essential for plant nutrition, it can be easily taken up by roots and accumulated in all plant tissues, from roots to the above-ground organs (Tezotto *et al.*, 2012). The main symptoms of Cd-induced toxicity in plants are stunted growth, chlorosis, leaf epinasty,

altered chloroplast ultrastructure, photosynthesis inhibition, inactivation of enzymes in CO₂ fixation, induced lipid peroxidation, disturbance of the nitrogen (N) and sulfur (S) metabolism (Gill and Tuteja, 2011). At a cellular level, Cd toxicity enhanced oxidative stress by increasing levels of reactive oxygen species (ROS) such as the O₂^{•-}, hydroxyl. Furthermore, the increased ROS production induced by Cd can trigger lipid peroxidation, DNA damage and oxidative modifications of proteins, which can eventually lead to cellular dysfunction and necrotic cell death (Valko *et al.*, 2007).

However, plants in response to Cd stress are manifested by the activation of various strategies to cope with its toxicity. Plant cells are normally protected against oxidative damage by a broad spectrum of radical-scavenger systems (Wang *et al.*, 2013). To avoid Cd toxicity plants also has developed other mechanisms for Cd tolerance, which includes cell wall binding, chelation with phytochelatin (PCs), Cd compartmentation in vacuole, or enrichment in leaf trichomes (Verbruggen *et al.*, 2009). Earlier studies showed that the vacuole is the site for the accumulation of a number of heavy metals, including Cd (De, 2000), which acts as the subdominant site of preferential Cd binding in all test tissues. Other detoxification mechanisms that plants have developed to cope with damages caused by Cd are related to some stress signaling molecules, such as nitric oxide (NO) and salicylic acid.

NO is an important gaseous molecule, serving as important secondary messengers in plant response to various biotic and abiotic stresses. Since the discovery in 1998 that NO has a role in plants, its functions in plant development, metabolism and disease responses have been extensively studied. Processes were shown to be regulated by NO include seed germination, root growth, respiration, stomatal closure and adaptive responses to biotic and abiotic stresses (Moreau *et al.*,

2010). However, the effects of NO on different types of cells have been proved to be either protective or toxic, depending on the concentration and location of NO in plant cells (Lamattina *et al.* 2003). Saxena *et al.* (2013) indicated that preservation of suitable ROS levels enhanced under metal stress might correspond to survival response and NO interacts with ROS in different ways and serves as an antioxidant during various stresses. Two interrelated mechanisms by which NO may abate stress have been proposed. First, NO may function as an antioxidant, directly scavenging the ROS that is generated by most of the stressors (Hsu and Kao, 2004). Second, NO may function as a signaling molecule in plant stress responses, leading to alterations of antioxidative gene expression (Wendehenne *et al.*, 2001). Although several lines of evidence have highlighted the role of NO in the modulation of Cd-induced growth inhibition in perennial ryegrass (Wang *et al.*, 2013), but exactly how NO alleviates plant growth in intact plants exposed to heavy metals is still not yet known.

Perennial ryegrass, an important forage grass and cool-season turfgrass, is known for its rapid establishment rate and good wear tolerance (Xiong *et al.*, 2009). It is widely used for livestock, fiber products, improving soil contaminated with heavy metal, habitats for wildlife populations, recreation, and beautification. It can accumulate metals in its biomass, and commonly used as a suitable species for revegetation of metalliferous wastes (Arienzo *et al.*, 2004). Our previous studies showed that most Cd accumulated in the roots of perennial ryegrass, and the application of exogenous NO inhibited Cd translocation from roots to the shoots (Wang *et al.*, 2013). Therefore, perennial ryegrass can serve as a useful model for studying the physiological basis of Cd tolerance in perennial grass species. The object of this study was to investigate the mechanism and approaches of SNP for mitigating Cd stress by determining uptake and translocation of Cd,

oxidative damages and responses of antioxidative systems in both leaves and roots of ryegrass seedlings. This study focused on the impact of different Cd loadings on the growth of ryegrass seedlings and the alleviating effect of exogenous NO on the damages induced by different Cd concentrations in ryegrass seedlings.

2. Materials and Methods

2.1. Plant material and culture conditions

Ryegrass seeds were first sterilized with 5% sodium hypochlorite for 15 min and washed extensively with distilled water, then germinated on moist filter paper in the dark at 26°C for 3 days. Initially, seedlings of uniform size were transferred to plastic pots (volume 500 mL) filled with perlite (50 plants per pot) and watered with half-strength Hoagland nutrition solution for 7 days. The seedlings were then watered with full-strength Hoagland solution. Three-week-old uniform seedlings were transferred into 1,000 mL black plastic containers with 50 seedlings per container. The nutrient solution was renewed every 2 days. These treatments contain: CK: Hoagland's solution; SNP: 100 µM SNP-treated nutrient solutions; Cd1: 100 µM Cd-treated nutrient solutions; Cd2: 150 µM Cd-treated nutrient solutions; Cd1+SNP: 100 µM SNP added into 100 µM Cd-treated nutrient solutions; Cd2 + SNP: 100 µM SNP added into 150 µM Cd-treated nutrient solutions. Cd was given as CdCl₂.

The treatments were arranged in a randomized block design with four replicates. The experiment was carried out under a controlled-environment chamber at 14/10 light/dark photoperiod and photon flux density 150 µmol m⁻²s⁻¹ at the leaf level, day/night temperature of 25/18°C and 65±5% relative humidity. After 2 weeks of growth with the above conditions, the plants were harvested and the roots and leaves were sepa-

rated and washed with 5 mM CaCl₂ (only the roots) first and then repeatedly washed with deionized distilled water. For the estimation of plant dry matter, Cd and mineral nutrients content, the plants were dried at 80°C for 48 h. For the enzyme determination, fresh plant material was frozen in liquid nitrogen and stored at -70°C until use.

2.2. Determination of plant growth

Plant height was determined immediately after harvesting. At harvest, the roots and leaves were separated and oven-dried for 30 min at 105°C, then at 70°C till the materials reach their constant weight. Fresh weight and dry weight were measured.

2.3. Determination of chlorophyll content

The chlorophyll content was determined according to the method of Dong *et al.* (2015). 0.5 g fresh ryegrass leaf was extracted in 2 mL 95 % ethanol for 24 h in the dark, and the extract was analyzed. The amounts of chlorophyll a, b and carotenoid were determined using a spectrophotometer (SHIMADZU UV-2450, Kyoto, Japan) by reading the absorbance at 665, 649 and 470 nm wavelength. The chlorophyll content is expressed as mg per gram-dry weight (mg g⁻¹ DW).

2.4. Determination of O₂⁻ generation rate

For the measurement of O₂⁻ generation rate, 0.3 g fresh leaves were ground in liquid N₂ and extracted in 3mL of ice cold 50 mM sodium phosphate buffer (PBS) (pH 7.0). One milliliter of the supernatant extract was transferred to a clean test tube and added with 0.9 mL 65 mM phosphate buffer solution (pH 7.8) and 0.1 mL 10 mM hydroxylammonium chloride. The reaction was incubated at 25 °C for 35 min. 0.5 mL solution from the above reaction

mixture was then transferred to another test tube containing 0.5 mL 17 mM sulfanic acid and 0.5 mL 7.8 mM α -naphthylamine solution. After 20 min of reaction, 2 mL of ether was added into the above solution, and then mixed well. The solution was centrifuged at $1500 \times g$ at 4°C for 5 min. The absorbance of the pink supernatant was measured at 530 nm with the spectrophotometer. Absorbance values were calibrated to a standard curve generated with known concentrations of HNO_2 (Wang *et al.* 2013).

2.5. Determination of H_2O_2 concentration

For determination H_2O_2 concentration, leaf tissue (0.2 g) was extracted with 3 mL of 0.1% (w/v) trichloroacetic acid (TCA) in an ice bath and centrifuged at $12,000 \times g$ for 15 min (Velikova *et al.* 2000). An aliquot (0.5 mL) of the extract was transferred to a test tube containing 0.5 mL of phosphate buffer (pH 7.0) and 1 mL of 1 M KI. The absorbance of the mixture was read at 390 nm. H_2O_2 content was determined using the extinction coefficient $0.28 \text{ M}^{-1} \text{ cm}^{-1}$ and the amount expressed as $\mu\text{mol g}^{-1} \text{ DW}$.

2.6. Determination of lipid peroxidation and soluble protein

The level of lipid peroxidation in fresh leaf was measured in terms of malondialdehyde (MDA) concentration by the thiobarbituric acid reaction method (Wang *et al.*, 2016). The MDA concentration was expressed as $\text{nmol g}^{-1} \text{ DW}$. Proteins were estimated by the method of Xu (2014). Fresh leaves (0.5 g) were homogenized in 1 mL phosphate buffer (pH 7.0). The crude homogenate was centrifuged at $5,000 \times g$ for 10 min. Half mL of freshly prepared trichloroacetic acid (TCA) was added,

mixed well, and then centrifuged at $8,000 \times g$ for 15 min. After separating from the supernatant solution, the precipitate was dissolved in 1 mL of 0.1N NaOH and added with 5 mL Bradford reagent. The absorbance of the final solution was measured at 595 nm using a spectrophotometer (SHIMADZU UV-2450, Japan).

2.7. Determination of antioxidant enzyme activities

For extraction of antioxidative 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 in a chilled pestle and mortar. The homogenate was centrifuged at $12,000 \times g$ for 20 min and the supernatant was separated and used for determination of enzyme activities. The whole extraction procedure was carried out at 4°C . All spectrophotometric analysis was conducted on a SHIMADZU UV-2450 spectrophotometer (Kyoto, Japan).

Superoxide dismutase (SOD) activity was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium following the method of Xu *et al.*, (2014). One unit of SOD activity (U) was defined as the amount of crude enzyme extract that is required for inhibiting the reduction rate of nitro-blue tetrazolium by 50%. Guaiacol peroxidase (POD) activity was measured by the increase in absorbance at 470 nm due to guaiacol oxidation (Wang *et al.*, 2013). Variation of absorbance per minute per milligram protein ($\Delta\text{A}470 \text{ min}^{-1} \text{ mg}^{-1} \text{ protein}$) stands for enzymes activity. Catalase (CAT) activity was measured as the decline in absorbance at 240 nm due to the decrease in the extinction of H_2O_2 according to the method of Xu *et al.* (2014). Unit of CAT activity ($\Delta\text{A}240 \text{ min}^{-1} \text{ mg}^{-1} \text{ protein}$) was defined as variation of absorbance per minute per milligram protein.

2.8. Determination of cadmium and mineral element concentrations

The dried plant tissues were weighed and ground into powder for the determination of Cd and other mineral elements using flame atomic absorbance spectrometry (SHIMADZU AA-6300, Kyoto, Japan) after digested with mixed acids [$\text{HNO}_3 + \text{HClO}_4$ (3:1, v/v)] (Ali *et al.*, 2002).

2.9. Plasma membrane preparation

A membrane fraction enriched in plasma membrane vesicles was prepared as described by Wang *et al.* (2013a) with minor modifications. Excised samples were homogenized (1/2, w/v) with a mortar and pestle in a cold medium containing: 25 mM HEPES-Tris (pH 7.2), 250 mM mannitol, 5 mM EDTA, 1 mM DTT and 1.5% (w/v) PVP. The whole isolation procedures were carried out at 4 °C. The homogenate was filtered through four layers of cheesecloth and centrifuged at $560 \times g$ for 12 min, then the supernatant was centrifuged at $10,000 \times g$ for 15 min, and after separating from the precipitate, the supernatant was centrifuged at $60,000 \times g$ for 30 min to yield a crude membrane fraction. The resulted pellet was resuspended with 1 mL in a gradient buffer containing: 20 mM HEPES-Tris (pH 7.5), 5 mM EDTA, and 0.5 mM EGTA. The supernatant was layered on top of a step gradient consisting of 1 mL of 45%, 33%, and 15% (w/w) sucrose, respectively, and then centrifuged for 2 h at $70,000 \times g$ force.

2.10. Measurement of H^+ -ATPase in plasma membrane (PM) vesicles

ATP hydrolysis assays were performed on plasma membrane (PM) vesicles as described by Wang *et*

al. (2013). 0.5 mL of the reaction medium containing: 36 mM Tris-Mes (pH 6.5), 30 mM ATP- Na_2 , 3 mM MgSO_4 , 1 mM NaN_3 , 50 mM KNO_3 , 1 mM Na_2MoO_4 , 0.02% (v/v) Triton X-100 was used in the presence or absence of 2.5 mM Na_3VO_4 . The reaction was triggered by adding 50 μL PM vesicles. After 30 min incubation at 37°C, the reaction was quenched by the addition of 55% (w/v) TCA. The H^+ -ATPase activity was determined by measuring the release of Pi (Dong *et al.*, 2015).

2.11. Statistical analysis

The experiment was a completely random design with three replications. Statistical analyses were carried out by analysis of variance (ANOVA) using SAS software (SAS Institute, Cary NC). Differences between treatments were separated by the least significant difference (LSD) test at a 0.05 probability level.

3. Results

3.1. Plant growth

After 14 d of Cd exposure, ryegrass seedlings showed severe morphological disturbances, including stunted growth, brownish roots, chlorosis and necrosis on the leaves, especially under 150 μM Cd stress (Table 1). SNP treatment alone did not affect seedlings growth, as compared to CK. Cd exposure significantly decreased the growth of ryegrass seedlings. However, addition of 100 μM SNP substantially alleviated Cd toxicity of plants especially under the 150 μM Cd stress. For example, exogenous NO increased shoot height by 14.81% under 100 μM Cd stress and by 16.26% under 150 μM Cd stress. Similar trends were noted in the root length, fresh weight, dry weight, and root/shoot ratio and root volume.

Table 1. Effects of SNP on biomass yield of ryegrass plants after 14 days treatment of Cd stress .

Treatments	Shoot height (cm/plant)	Root length (cm/plant)	Fresh weight (g/50 plant)	Dry weight (g/50 plant)	Root/shoot ratio	root volume (mL/50 plant)
CK	31.09±0.50 a	16.20±0.24 a	9.61±0.53 a	1.37±0.07 a	0.14±0.01 a	3.88±0.04 a
SNP	30.41±0.59 a	15.58±1.10 a	9.21±0.74 a	1.30±0.06 a	0.15±0.01 ab	3.82±0.08 a
Cd1	25.26±0.53 d	7.93±0.09 c	6.87±0.17 c	0.98±0.02 c	0.11±0.01 c	2.52±0.04 d
Cd2	23.01±0.19 e	7.00±0.42 d	5.78±0.25 d	0.86±0.02 d	0.10±0.00 d	2.27±0.03 e
Cd1+ SNP	29.00±0.71 b	9.73±0.33 b	8.43±0.22 b	1.20±0.02 b	0.13±0.00 b	3.37±0.04 b
Cd2+ SNP	26.75±0.27 c	8.99±0.21 b	8.13±0.22 b	1.15±0.07 c	0.11±0.00 c	3.22±0.04 c

Values are the mean of four replicates. Each replicate has 50 plants. Means followed by different letters within the same column are significantly different at $P < 0.05$

3.2. Chlorophyll content

Ryegrass plants treated with both 100 and 150 μM Cd showed a significant decrease in total chlorophyll, chl a, chl b, chl a + b, and car content as compared to the control (Table 2). However, these

inhibitions were alleviated by addition of SNP. Cd1+SNP treatment increased total chlorophyll, chl a, chl b and car content by 17.92, 16.29, 27.78, and 28.56 %, respectively. And the corresponding values for Cd2 + SNP treatment were 30.75, 27.00, 38.14 and 59.63 %.

Table 2. Effects of SNP on the chlorophyll contents of ryegrass plants after 14 days treatment of Cd stress.

Treatments	Shoot height (cm/plant)	Root length (cm/plant)	Fresh weight (g/50 plant)	Dry weight (g/50 plant)	Root/shoot ratio	root volume (mL/50 plant)
CK	31.09±0.50 a	16.20±0.24 a	9.61±0.53 a	1.37±0.07 a	0.14±0.01 a	3.88±0.04 a
SNP	30.41±0.59 a	15.58±1.10 a	9.21±0.74 a	1.30±0.06 a	0.15±0.01 ab	3.82±0.08 a
Cd1	25.26±0.53 d	7.93±0.09 c	6.87±0.17 c	0.98±0.02 c	0.11±0.01 c	2.52±0.04 d
Cd2	23.01±0.19 e	7.00±0.42 d	5.78±0.25 d	0.86±0.02 d	0.10±0.00 d	2.27±0.03 e
Cd1+ SNP	29.00±0.71 b	9.73±0.33 b	8.43±0.22 b	1.20±0.02 b	0.13±0.00 b	3.37±0.04 b
Cd2+ SNP	26.75±0.27 c	8.99±0.21 b	8.13±0.22 b	1.15±0.07 c	0.11±0.00 c	3.22±0.04 c

Values are the mean of four replicates. Each replicate has 50 plants. Means followed by different letters within the same column are significantly different at $P < 0.05$

3.3. O_2^- generation rate and hydrogen peroxide concentration

O_2^- generation rate and H_2O_2 concentration showed significant rises in leaves and roots under both 100 and 150 μM Cd stress, as compared to the control (Figure 1). O_2^- generation rate and H_2O_2 concentration in leaves and roots were not

affected by SNP application alone. Seedlings subjected to 150 μM Cd exhibited substantial O_2^- generation rate acceleration in leaves and roots, which were increased by 76.76 % and 285 %. Addition of SNP significantly decreased the O_2^- generation rate and H_2O_2 concentration in leaves and roots of plants under Cd stress, particularly 150 μM Cd exposure.

Figure 1. Effects of SNP on O_2^- generation rate (a) and H_2O_2 content (b) in leaves and roots of ryegrass plants after 14 days treatment of Cd stress. Values are the mean of four replicates. Each replicate has 50 plants. Bars with different letters are significantly different at $P < 0.05$.

3.4. MDA and soluble protein contents

When plants were subjected to environmental stress, oxidative damage resulted in membrane lipid peroxidation, which could be estimated by MDA levels. Figure 2a showed that both 100 and 150 μ M Cd exposure increased MDA concentration in plants significantly. However, addition of SNP to the 100 and 150 μ M Cd treatments decreased MDA concentration by 18.60

%, 20.41 % in leaves and 24.72%, 22.57% in roots, respectively.

Ryegrass seedlings treated with Cd (100 and 150 μ M) showed significant declines in soluble protein content in both leaves and roots (Figure 2b). However, this decline was alleviated by the addition of SNP. SNP treatment increased soluble protein content by 95.1% in leaves and 58.19% in roots, as compared to Cd2 treatment.

Figure 2. Effects of SNP on MDA (a) and soluble protein content (b) in leaves and roots of ryegrass plants after 14 days treatment of Cd stress. Values are the mean of four replicates. Each replicate has 50 plants. Bars with different letters are significantly different at $P < 0.05$.

3.5. Antioxidant enzymes activities

The effects of Cd and SNP on activities of antioxidant enzymes were presented in Figure 3. Cd treatments (100 and 150 μM Cd) increased

the activities of SOD, POD and CAT in leaves, but reduced them in roots. Addition of SNP alleviated Cd stresses by increasing the activities of SOD, POD and CAT in leaves and restored them in roots.

Figure 3. Effects of SNP on SOD (a), CAT (b) and POD (c) content in leaves and roots of ryegrass plants after 14 days treatment of Cd stress. Values are the mean of four replicates. Each replicate has 50 plants. Bars with different letters are significantly different at $P < 0.05$.

3.6. Ca, Fe, Cu, Zn and Mg concentrations

Cd1 and Cd2 treatments significantly decreased Fe, Cu and Zn concentration in both leaves and roots, but Ca concentration was increased in leaves while decreased in root. (Table 3). In contrast with Ca, Mg concentration was decreased in leaves but increased in roots. The reduction of Fe, Cu and Zn concentration was more obvious at the higher Cd treatment

level (150 μM). Addition of SNP alleviated Cd stress by raising Fe, Cu and Zn concentration in plant. For instance, Cd2 + SNP treatment increased Fe, Cu and Zn concentrations by 39.77%, 29.56% and 39.61%, respectively in leaves and by 76.71%, 51.89% and 26.86%, respectively in roots, as compared to Cd2 treatment alone. Moreover, SNP addition increased Mg concentration in leaves and Ca concentration in roots, particularly under 150 μM Cd stress.

Table 3. Effects of SNP on the mineral contents (mg kg⁻¹ DW) in shoots and roots of ryegrass plants after 14 days treatment of Cd stress.

Items		Treatments					
		CK	SNP	Cd1	Cd2	Cd1+ SNP	Cd2+ SNP
Ca	shoots	327.9±10.48 d	399.30±5.77 b	426.16±4.58 a	416.14±9.59 a	366.55±13.29 c	336.16±2.83 d
	roots	143.6±10.60 a	110.13±3.03 cd	118.22±5.92 c	106.73±0.78 d	132.36±3.41 b	134.11±4.20 ab
Fe	shoots	360.4±17.40 a	338.20±7.99 a	241.34±13.77 d	195.09±22.45 e	306.83±3.45 b	272.68±9.59 c
	roots	4949±40.41 a	3669.13±43.87 d	2938.72±33.70 e	2399.95±92.17 f	4512.41±74.11 b	4241.05±40.17 c
Cu	shoots	8.46±0.05 a	7.06±0.10 c	6.00±0.15 d	5.48±0.09 e	7.54±0.04 b	7.09±0.06 c
	roots	44.93±0.67 a	37.85±0.33 b	31.23±0.35 d	23.76±0.62 e	38.45±1.45 b	36.09±1.28 c
Zn	shoots	811.5±7.22 a	571.01±10.64 d	495.10±1.45 e	462.75±4.89 f	691.35±5.66 b	646.06±8.28 c
	roots	1038±29.69 a	887.18±8.49 c	830.94±3.37 d	747.00±18.85 e	975.54±12.10 b	947.61±7.02 b
Mg	shoots	1755±6.68 a	1711.79±7.93 b	1591.71±3.47 e	1502.00±6.66 f	1650.46±4.23 c	1629.34±6.09 d
	roots	2202±2.37 d	2206.23±2.78 d	2678.19±2.24 c	3273.79±2.92 a	2154.31±3.39 e	2753.93±6.15 b

Values are the mean of four replicates. Each replicate has 50 plants. Means followed by different letters within the same column are significantly different at $P < 0.05$.

3.7. Cd concentration in leaves and roots

Cd concentrations were significantly higher in roots than in leaves at both the Cd1 and Cd2 treatments (Figure 4), although Cd concentration in both leaves and roots was elevated with increasing Cd concentration in nutrient solution.

Addition of SNP decreased Cd concentration in leaves but increased it in roots, especially at the Cd2 treatment. Addition of SNP to the 100 and 150 μM Cd solutions decreased Cd concentration by 21.04 % and 21.55 %, respectively in leaves, but increased by 24.80 % and 38.06 % respectively, in roots.

Figure 4. Effects of SNP on Cd content in leaves and roots of ryegrass plants after 14 days treatment of Cd stress. The values of control and SNP treatments were 0 mg kg⁻¹ DW. Values are the mean of four replicates. Each replicate has 50 plants. Bars with different letters are significantly different at $P < 0.05$.

3.8. H^+ -ATPase activity in plasma membrane

As shown in Figure 5, Cd stresses decreased H^+ -ATPase activity in leaves and roots, especially at the 150 μM Cd. In plants with Cd1 and Cd2 treatments, H^+ -ATPase activity was decreased by

56.89% and 67.08%, respectively in leaves and by 52.37 % and 62.38 %, respectively in roots, as compared to CK. However, exogenous NO application restored H^+ -ATPase activity in both leaves and roots, especially with the Cd2 + SNP treatment.

Figure 5. Effects of SNP on H^+ -ATPase activity in leaves and roots of ryegrass plants after 14 days treatment of Cd stress. Values are the mean of four replicates. Each replicate has 50 plants. Bars with different letters are significantly different at $P < 0.05$.

4. Discussion

The mechanisms of NO as a signaling regulatory molecule and reactive oxygen scavenger in improving plant tolerance to Cd stress were poorly understood. In the present study, the growth of ryegrass plants was significantly affected by Cd stress, including reduced plant height, root length, fresh weight, dry weight, root/shoot ratio and root volume (Table 1). Kovaik *et al.* (2014a) also noted that Mn excess depressed plant growth (but not germination). The inhibition of growth in ryegrass might be resulted from the alteration of fundamental metabolic processes, chlorophyll content (Table 2), antioxidative system (Figure 3) and uptake of mineral elements (Table 3) in leaves and roots under Cd stress. However, the inhibitory effects were significantly alleviated by exogenous NO, and the mitigation effect of NO on ryegrass under 150

μM Cd stress was greater than 100 μM Cd stress. The stimulation of plant growth by NO has also been reported in ryegrass (Wang *et al.*, 2013), barley (Chen *et al.*, 2010) and peanut (Xu *et al.*, 2014). The alleviation of Cd stress by NO may be related to increased chlorophyll content (Table 2), improved nutrient balance (Table 3), better regulated activities of antioxidant enzymes (Figure 3), and inhibited Cd translocation from roots to the leaves (Figure 4), thus enhancing the tolerance of ryegrass plants to Cd toxicity.

A notable reduction of chlorophyll parameters occurred in ryegrass seedlings exposed to 100 μM and 150 μM Cd stress (Table 2). Several authors reported a decreased chlorophyll content in the leaves of Cd-treated plants (Belkhadi *et al.*, 2010). The decrease in chlorophyll content in Cd-affected ryegrass seedlings might be attributed to the possible oxidation of chlorophyll and the damaged ultrastructure of chloroplasts

(Chen *et al.*, 2010). In addition, the decline in chlorophyll content may be due to the decreased Mg content in leaves under Cd stress (Table 3), which is an essential element in the synthesis of chlorophyll. However, these effects were reverted by NO, suggesting its protecting role against Cd induced toxicity which is corroborated with previous reports (Ekmekçi *et al.*, 2008). Our previous study also noted that increased uptake of Fe and Mg by NO application was responsible for improving chlorophyll synthesis (Wang *et al.*, 2013). Furthermore, treatment with Cd plus SNP induced a substantial increase of PCs, which might protect photosynthesis (Xiong *et al.*, 2009). In addition, NO effectively reduced the level of ROS generation during Cd stress, and thus resulted in alleviation of the oxidative negative effects of ROS on growth and chlorophyll content, thus improving the tolerance of ryegrass seedlings to Cd stress.

To maintain metabolic functions under stress conditions, the balance between generation and degradation of ROS is essential, otherwise oxidative injuries may occur. In this study, we observed that the treatment of 100 μM and 150 μM Cd enhanced the accumulation of $\text{O}_2^{\cdot-}$, H_2O_2 (Figure 1) and MDA (Figure 2a), especially with the 150 μM Cd. The increased ROS production may trigger lipid peroxidation, DNA damage and oxidative modifications of proteins, which can eventually lead to cellular dysfunction and necrotic cell death. The growth inhibition might be partly due to enhanced production and accumulation of ROS. The level of ROS in plant tissues is controlled by an antioxidant system that consists of antioxidant enzymes (SOD, POD, CAT and glutathione reductase) (Schutzendubel and Polle, 2002) and nonenzymatic low molecular weight antioxidants (glutathione and ascorbic acid etc.). Therefore, it was expected that the exposure of ryegrass seedlings to Cd could elevate the level of antioxidant enzymes. So, the interesting thing that emerged in the present

study was that the treatment of plants with Cd increased the activities of SOD, POD and CAT at a degree in shoots while inhibited these enzymes in roots (Figure 3). Kovacik *et al.* (2015) also indicated that antioxidative enzyme activities showed significantly higher values in 10 μM treatments of both Cr oxidation states. This may be due to the fact that plants have evolved a complex antioxidant system to avoid the harmful effects of ROS. However, exogenous NO prevented Cd-induced increase in the activities of antioxidant enzymes (SOD, POD and CAT) in leaves but enhanced these activities of antioxidant enzymes in roots. The regulation in the activities of antioxidant enzymes by SNP alleviated the stress of ryegrass and scavenged the $\text{O}_2^{\cdot-}$ and H_2O_2 (Figure 1), as well as MDA (Figure 2a). So the stimulation of antioxidant production may suggest that NO can stabilize the cell membranes, counteract oxidative damages and protect ryegrass against stressful condition. Soluble protein content in organisms is an important indicator of reversible and irreversible changes in metabolism and responds to a wide variety of stressors (Singh and Tewari, 2003). In the present study, the soluble protein content in leaves and roots declined under Cd stress, especially at 150 μM Cd (Figure 2b). Our studies coincide with Wang *et al.* (2013) who reported that Cd stress caused a decrease in soluble protein content in isolated mitochondria. Generation of H_2O_2 , and other ROS may directly correlate with damage to proteins. Moreover, Cd forms disulfide bridges within proteins leading to distorted membrane ion channels and leakage of ions (Verma *et al.*, 2013). Interestingly, NO-treatment caused a marked enhancement of Cd-induced soluble protein content in leaves and roots, which indicated that the seedlings were partially relieved from Cd stress. And the relief effect of NO was greater under 150 μM Cd than 100 μM Cd stress.

Metal excess typically depresses accumulation of mineral nutrients in plants (Kovacik *et al.*, 2014a). Our study indicated a marked change in mineral nutrient concentration occurred in ryegrass leaves and roots under Cd stress, and this effect was almost completely regulated when plants were treated with NO (Table 3). Previous studies have reported excess of Cd usually affects accumulation of essential mineral nutrients (Kovacik *et al.*, 2009), which may account for the reduction of Fe, Mg, Cu, and Zn concentration in leaves and Fe, Ca, Cu, and Zn concentration in roots (Table 3). On the contrary to these elements, Ca concentrations in leaves were increased under Cd toxicity, which may be due to the fact that cell wall is the main store for Ca in plant and the accumulation of Ca may facilitate to reduce the detrimental effect of Cd. Changes of mineral concentration indicated that Cd disturbed ionic homeostasis and NO stimulated its maintenance, especially under the 150 μ M Cd stress. In addition, H⁺-ATPase in plasma membrane plays an important role in the transport of multiple ions (Wang *et al.*, 2016), and this study indicated that NO treatment induced the absorption of plasma membrane H⁺-ATPase activity under Cd toxicity (Figure 5), which might be responsible for NO to adjust ion balance by increasing Fe, Cu, and Zn concentrations (Table 3). Moreover, an increase in H⁺-ATPase activity is the mechanism of protecting the integrity of plasma membrane, which can improve the resistance to Cd toxicity. So the plants can absorb more mineral nutrients and maintain ionic homeostasis.

In plants, the root is in direct contact with Cd and the cell walls of roots play a significant role in heavy metal tolerance (Xiong *et al.* 2009). In the present study, Cd concentrations in different plant tissues of ryegrass decreased in the order of roots > leaves (Figure 4), which implies that the translocation of

Cd from roots to the shoots is restricted by internal barriers to defend the shoots. In addition, treatment with 150 μ M Cd induced a more obvious increase of Cd concentrations in both leaves and roots. This result is similar to that reported for wheat (Singh *et al.*, 2008). However, NO elevated Cd uptake in plants while inhibited the root-to-shoot translocation of Cd (Figure 4), thus resulting in lower Cd accumulation in leaves, this may be an important mechanism for NO-increased Cd tolerance. Our data agree with those reported in chamomile where SNP enhanced Cd uptake (Kovacik *et al.*, 2014b). Therefore, this may be an important tolerance mechanism of metal exclusion. Concurred to our findings, exogenous NO reduced the root-to-shoot translocation of Cd in ryegrass plants under Cd stress (Wang *et al.*, 2013). This may be due to the fact that the exogenous NO increased Fe, Mg, Cu, and Zn concentrations in leaves, and thus reduce Cd accumulation in seedlings. Moreover, previous reports also indicated that SNP induced an increase in Cd accumulation in the cell wall of roots, which may in turn decreased Cd translocation from roots into the leaves (Xiong *et al.*, 2009).

5. Conclusion

The present study demonstrated that exogenous NO could alleviate Cd toxicity to ryegrass plants and the ameliorated effect was more evident under the high Cd stress. The relief mechanisms of exogenous NO in alleviating Cd toxicity in ryegrass may be related to: (1) increased chlorophyll concentration; (2) reduced oxidative stress and improved antioxidative system; (3) regulated mineral nutrient balance in leaves and roots; and (4) decreased Cd translocation from roots to the leaves. These results may be applicable to other plant species under different heavy metal stress.

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