Effects of application of exogenous NO on the physiological characteristics of perennial ryegrass grown in Cd-contaminated soil

W.W. Wang, X.Y. Bai, Y.J. Dong*, W.F. Chen, Y.L. Song, X.Y. Tian

College of Resources and Environment, Shandong Agricultural University, Tai'an 271018, P.R.China

**Corresponding author: yjdong@sdau.edu.cn*

Abstract

A pot experiment was conducted to study the effects of sodium nitroprusside (SNP, a nitric oxide [NO] donor) on cadmium (Cd) toxicity in ryegrass plants. In the present study, ryegrass plants subjected to Cd toxicity were treated with different concentrations of SNP (50, 100, 300 and 500 μM), SNP solution was poured into Cd-contaminated soil every seven days. Cd treatment inhibited the plants growth and the absorption of micronutrients, increased accumulation of Cd in both shoots and roots. In Cd-treated plants, the activities of antioxidant enzymes and the contents of proline, soluble protein and ascorbic acid (AsA) decreased dramatically, whereas the accumulation of superoxide anion ($\text{O}_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) increased significantly. Addition of SNP significantly decreased $\text{O}_2^{\cdot-}$, H_2O_2 and MDA accumulation in both shoots and roots of Cd-stressed plants, decreased the transport of Cd from roots to shoots, alleviated the inhibition of Ca, Fe, Mg and Zn absorption induced by Cd and promoted the plant growth. In SNP-treated plants, the chlorophyll content, the contents of proline, soluble protein and ascorbic acid, and the activities of superoxide dismutase, peroxidase and catalase increased considerably. Addition of 300 μM SNP had the most significant alleviating effect against Cd toxicity while the addition of 500 μM SNP had no significant effect with Cd treatment. Based on these results, we concluded that the optimal concentrations of exogenous SNP could alleviate Cd induced stress and promote ryegrass plant growth.

Keywords: Cadmium, nitric oxide, ryegrass, antioxidative systems, ion accumulation

Abbreviations: SNP, sodium nitroprusside, NO, nitric oxide, Cd, cadmium, SOD, superoxide dismutase, POD, peroxidase, CAT, catalase, ROS, reactive oxygen species, $\text{O}_2^{\cdot-}$, superoxide anion radical, H_2O_2 , hydrogen peroxide; MDA, malondialdehyde, AsA, ascorbic acid

1. Introduction

In recent years, soil pollution was becoming more and more severe due to the increased using in mining, manufacturing and of synthetic products, such as pesticides, paints, batteries, industrial waste and land application of industrial or domestic sludge (Fang and Wang, 2000). And contamination of the environment with toxic heavy metals, such as cadmium (Cd), is an important environmental and human health problem. Cd is rather mobile in soil and contaminates food and fodder crops even at low soil levels. Since Cd is also not naturally biodegradable, it is persistent once released into the environment and hence Cd removal from both soil and water is an important environmental protection issue (Chen *et al.*, 2013). Cadmium can interfere with enzymatic activities, membrane functions, and hormone balance. One of the main toxic effects of Cd is oxidative stress. Oxidative stress is defined as all of the effects such as cellular damage, caused by the active forms of oxygen such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^\cdot$) and singlet oxygen (1O_2). As the results of oxidative stress, lipid is peroxidized, protein synthesis is inhibited, enzyme is inactivated, and membrane systems are damaged.

Nitric oxide (NO) exists in plant cells and intercellular spaces widely and it is a ubiquitous signal molecule involved in multiple plant responses to environmental stress (He *et al.*, 2012). Under stress conditions, such as salinity (Dong *et al.*, 2015), drought (Durán *et al.*, 2016), heavy metal toxicity (Rady and Hemida, 2015), plants produce active oxygen species, which are harmful to plant growth and productivity. Different studies have particularly demonstrated NO signalling in the induction of cell death, defense genes and interaction with reactive oxygen species (ROS) during plant defense against pathogen attack (Wendehenne *et al.*, 2001; Neill *et al.*, 2003). These studies

suggest that NO might have acted as an antioxidant for scavenging reactive oxygen species (ROS) generated under the abiotic stress conditions. Plants have evolved a complex antioxidant system (*i.e.*, enzymatic and nonenzymatic detoxification mechanisms) for protecting potential cell injury against tissue dysfunction (Srivastava *et al.*, 2004). The antioxidant enzymes, such as catalase (CAT), peroxidases (POD), superoxide dismutases (SOD), etc., can scavenge different types of ROS. NO also might be involved in increasing the antioxidant content and antioxidative enzymes activity in plants. In recent decade, the effects of exogenous NO on alleviating heavy metal toxicity in plants were reported by many authors (Khairy *et al.*, 2016; Saxena and Shekhawat, 2013). Much of the published research concerned with NO has important roles in modulating the plant responses to Cd toxicity (Liu *et al.*, 2015; Kováčik *et al.*, 2015). However, little knowledge is reported regarding the effects of application of exogenous NO in Cd-contaminated soil on plant growth and development in the whole growth period, and further, or how the growing plants cope with Cd toxicity through physiological and biochemical adaptations.

Perennial ryegrass is one of the most widely used perennial grasses in temperate regions around the world. Perennial ryegrass can accumulate metals in its biomass, and commonly used as a suitable species for revegetation of metalliferous wastes (Arienzo *et al.*, 2004). Previous researches demonstrated that perennial ryegrass has potential for rehabilitation of Cd-contaminated soils (Arienzo *et al.*, 2004; Lou *et al.*, 2013). In addition, better understanding of Cd-induced oxidative stress and Cd-accumulation in perennial ryegrass can provide important information for phytoremediation. Our previous studies have demonstrated that NO may ameliorate toxic effects of excess

Cd on lettuce seedlings (Xu *et al.*, 2014) and on ryegrass seedlings grown in Cd-treated nutrient solutions (Wang *et al.*, 2013). Based on the above studies, the objective of this study was to determine the application of exogenous NO could alleviate Cd toxicity, increase Cd-tolerance of perennial ryegrass grown in Cd-contaminated soil, and then improve potential in phytoremediation of cadmium polluted soils.

2. Materials and Methods

2.1. Plant material and culture conditions

A pot experiment was conducted on a brown clay loam soils (a soil group in Chinese genetic soil classification; named Hapli-Udic Argosols in Chinese soil taxonomy), at College of Resources and Environment, Shandong Agricultural University, Shandong, China. The topsoil (0–20 cm) had a EC of 197.1 $\mu\text{S cm}^{-1}$ (soil:water ratio 1:5) and contained 14.1 g kg^{-1} organic matter, 0.622 g kg^{-1} total nitrogen, 0.0465 g kg^{-1} available nitrogen, 0.0225 g kg^{-1} available phosphorus, and 0.1061 g kg^{-1} available potassium. Soil subsamples were mixed with appropriate amounts of analytical grade solid CdCl_2 in order to add 50 mg kg^{-1} Cd of soil (oven-dry basis). Basal fertilizers applied were compound fertilizer. The nutrient content of compound fertilizer was 15% N, 15% P_2O_5 , and 15% K_2O . The mass of the fertilizer applied in each pot was 667.5 mg, and the fertilizer was thoroughly mixed with the soil. Seeds of ryegrass (*Lolium perenne* L.) were sown on April 27, 2013. Plants were grown in plastic pots (fifty plants per container) with a capacity of 2.5 kg airdried soil. Pots were arranged in randomized block designs with three replicates. During the growing season, plants were managed under commonly used agronomic and irrigation practices. The experimental design is given as follows: contrast check (CK): no Cd-contaminated soil, Cd: 50

mg kg^{-1} Cd-contaminated soil, T1: SNP solutions (50 μM , 100 mL) were poured into 50 mg kg^{-1} Cd-contaminated soil, T2: SNP solutions (100 μM , 100 mL) were poured into 50 mg kg^{-1} Cd-contaminated soil, T3: SNP solutions (300 μM , 100 mL) were poured into 50 mg kg^{-1} Cd-contaminated soil, T4: SNP solutions (500 μM , 100 mL) were poured into 50 mg kg^{-1} Cd-contaminated soil. The SNP solution was poured into soil after a 1-month growth period, and the time interval of twice pour actions was seven days. Three successive harvests were taken. Before the SNP solution was poured into soil, it was stored in the dark. The first was taken after a 2-month growth period, the second after a 3-month growth period, and the third after a 4-month growth period. The ryegrass was clipped with a stainless steel shears at each harvest to leave a 2.5 cm stubble.

2.2. Determination of plant growth and root activity

After 30, 60 and 90 days treatment, above-ground fresh and dry biomasses were measured. The samples were separated and oven-dried for 30 min at 105 °C, then at 70 °C till the materials reach their constant weights. Root activity was determined by the TTC method according to Zhang and Di (2003). It was expressed as absorbance per unit gram root fresh weight.

2.3. Determination of chlorophyll content

The chlorophyll content was determined according to the method of Knudson *et al.* (1977). Fresh ryegrass leaf (0.5 g) was extracted in 2 mL 95 % ethanol for 24 h in the dark, and the extracted solution 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. The chlorophyll content results are expressed as unit's mg per gram-fresh weight (mg g^{-1} FW).

2.4. Determination of $O_2^{\cdot-}$ generation rate and MDA content

The production rate of $O_2^{\cdot-}$ was measured as described by Elstner and Heupel (1976). Fresh leaves (0.2 g) were homogenized in 1 mL of 50 mM phosphate buffer (pH 7.8), and the homogenate was centrifuged at 10,000g for 10 min.

Then 0.5 mL of the supernatant was added to 0.5 mL 50 mM phosphate buffer (pH 7.8) and 0.1 mL of 10 mM hydroxylamine hydrochloride. After 1 h reaction at 25 °C, the mixture was added to 1 mL 17 mM sulfanilamide and 1 mL 7 mM α -naphthylamine at 25 °C for 20 min. The specific absorbance at 530 nm was determined. Sodium nitrite was used as a standard solution to calculate the production rate of $O_2^{\cdot-}$. The level of lipid peroxidation in fresh leaf was measured in terms of malondialdehyde (MDA) concentration by the thiobarbituric acid reaction method (Heath and Packer 1968). MDA concentration was expressed as nmol g⁻¹ FW.

2.5. Determination of H_2O_2 concentration

Fresh samples (1.0 g) were homogenized in 2 mL ice-cold acetone. Titanium reagent (2% $TiCl_2$ in conc. HCl) was added to a known volume of extract supernatant to give a Ti (IV) concentration of 2%. The Ti- H_2O_2 complex, together with unreacted Ti, was then precipitated by adding 0.2 mL 17 M ammonia solution for each 1 mL of extract. The precipitate was washed five times with ice acetone by resuspension, drained, and dissolved in 1 M H_2SO_4 (3 mL). The absorbance of the solution was measured at 410 nm against blanks, which had been prepared similarly but without plant tissue (Patterson *et al.*, 1984).

2.6. Determination of antioxidant enzyme activities

For extraction of antioxidative enzymes, samples 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×g for 20 min and the resulted supernatant was 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). SOD activity was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium following the method of Stewart and Bewley (1980). CAT activity was measured as the decline in absorbance at 240 nm due to the decrease of extinction of H_2O_2 according to the method of Patra *et al.* (1978). POD activity was measured by the increase in absorbance at 470 nm due to guaiacol oxidation (Nickel and Cunningham, 1969).

2.7. Determination of proline content

Proline concentration was determined using the method of Bates *et al.* (1973). After extraction at room temperature with 3% 5-sulfosalicylic acid solution, the proline content was determined from a standard curve and calculated on fresh weight basis.

2.8. Determination of soluble protein and ascorbic acid (AsA) content

Protein was estimated by the method of Bradford (1976). Fresh leaves (0.5 g) were homogenized in 1 mL phosphate buffer (pH 7.0). The crude homogenate was centrifuged at 5,000 × g for 10 min.

Half milliliter of freshly prepared trichloroacetic acid (TCA) was added and centrifuged at $8,000 \times g$ for 15 min. The debris was dissolved in 1 mL of 0.1N NaOH and 5 mL Bradford reagent was added.

Absorbance was recorded at 595 nm using a spectrophotometer (SHIMADZU UV-2450, Japan).

Ascorbic acid (AsA) concentration was measured according to Tonamura (1978). Fresh leaves (0.2 g) were homogenized in ice-cold 2 mL 10% metaphosphoric acid. After centrifugation at $15,000 \times g$ for 10 min, to 0.5 mL of supernatant was added 1 mL citric acid-phosphoric acid buffer (pH 2.3) and 1 mL 2, 6-dichlorophenol indophenol (30 mg L^{-1}). After 30 s, the absorbance was determined at 524 nm. AsA concentration was expressed as mg g^{-1} FW.

2.9. Determination cadmium and mineral element concentrations

The dried tissues were weighed and grinded into powder for the determination of cadmium and mineral element concentrations, which was measured by flame atomic absorbance spectrometry (SHIMADZU AA-6300, Kyoto, Japan) after digested with mixed acid [$\text{HNO}_3 + \text{HClO}_4$ (3:1, v/v)] (Ali *et al.*, 2002).

2.10. 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 and root activity

Cd exposure significantly decreased fresh weight, dry weight and root activity of ryegrass plants (Table 1). However, this inhibition was significantly alleviated by 100 and 300 μM SNP, especially 300 μM SNP. After 30, 60 and 90 days treatment, the fresh weights of T3 treatment were increased by 39.74, 59.26 and 45.11% compared with Cd-alone treatment, the dry weights were increased by 46.15, 56.00 and 37.04%. But T1 and T4 treatments had no significant effect. Furthermore, the root activity of ryegrass under Cd stress also increased markedly with the addition of SNP. Taken together, the alleviating effect of SNP was found in a general trend of $\text{T3} > \text{T2} > \text{T1} > \text{T4}$.

Table 1. Effects of application of different concentrations of NO every seven days on fresh weight, dry weight in leaves and root activity of ryegrass plants grown in Cd-contaminated soil.

Treatments	Fresh weight [g (10 plants) $^{-1}$]			Dry weight [g (10 plants) $^{-1}$]			Root activity [μg (g h) $^{-1}$ FW]		
	30 Days	60 Days	90 Days	30 Days	60 Days	90 Days	30 Days	60 Days	90 Days
CK	1.09 $\pm$ 0.02a	1.88 $\pm$ 0.01a	2.22 $\pm$ 0.19a	0.21 $\pm$ 0.01a	0.39 $\pm$ 0.03a	0.39 $\pm$ 0.01a	58.83 $\pm$ 2.09b	73.62 $\pm$ 1.99a	88.07 $\pm$ 1.16a
Cd	0.78 $\pm$ 0.03d	1.08 $\pm$ 0.03d	1.33 $\pm$ 0.02d	0.13 $\pm$ 0.01d	0.25 $\pm$ 0.01c	0.27 $\pm$ 0.01d	37.53 $\pm$ 0.96e	31.71 $\pm$ 1.63f	47.01 $\pm$ 1.94f
T1	0.86 $\pm$ 0.02c	1.16 $\pm$ 0.05d	1.42 $\pm$ 0.01cd	0.16 $\pm$ 0.02c	0.29 $\pm$ 0.02c	0.30 $\pm$ 0.02c	43.40 $\pm$ 1.29d	37.13 $\pm$ 1.58e	52.71 $\pm$ 1.37e
T2	0.98 $\pm$ 0.06b	1.40 $\pm$ 0.07c	1.57 $\pm$ 0.02c	0.17 $\pm$ 0.02bc	0.34 $\pm$ 0.03b	0.34 $\pm$ 0.02b	48.89 $\pm$ 2.29c	46.34 $\pm$ 1.22d	68.52 $\pm$ 1.96c
T3	1.09 $\pm$ 0.04a	1.72 $\pm$ 0.06b	1.93 $\pm$ 0.11b	0.19 $\pm$ 0.01ab	0.39 $\pm$ 0.02a	0.37 $\pm$ 0.00a	61.55 $\pm$ 0.93a	65.68 $\pm$ 0.48b	79.92 $\pm$ 1.23b
T4	0.84 $\pm$ 0.04cd	1.16 $\pm$ 0.04d	1.39 $\pm$ 0.01b	0.16 $\pm$ 0.02cd	0.28 $\pm$ 0.02c	0.31 $\pm$ 0.01c	43.06 $\pm$ 0.80d	57.72 $\pm$ 0.66c	64.98 $\pm$ 1.51d

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

3.2. Chlorophyll content

After 30, 60 or 90 days treatment, ryegrass plants under Cd stress showed a significant decrease in chlorophyll content, chlorophyll a, chlorophyll b and carotenoids (Table 2) concentrations compared to CK. However, When treated with SNP (50, 100, 300 or 500 μM) in the presence of Cd-contaminated soil, this inhibition was alleviated, especially 300 μM SNP. In the three consecutive collections of samples, the chlorophyll contents of T3 were increased by 128.31, 76.34 and 76.78% compared with Cd-alone treatment, the chlorophyll a were increased by 110.70, 83.07 and 51.13%, the chlorophyll b were increased by 188.16, 66.28 and 190.73%, the carotenoids were increased by 68.91, 92.71 and 62.77%.

3.3. $\text{O}_2^{\cdot-}$ generation rate, H_2O_2 and MDA content

Compared to the CK, Cd treatment significantly increased $\text{O}_2^{\cdot-}$ generation rate (Table 3) by 208.49% after

30 days, by 334.69% after 60 days and by 184.25% after 90 days. The application of exogenous NO could decrease $\text{O}_2^{\cdot-}$ generation rate under Cd stress. The T1, T2, T3 and T4 treatments decreased $\text{O}_2^{\cdot-}$ generation rate by 15.15, 24.21, 48.52, 37.55% after 30 days, by 18.37, 32.75, 56.89, 42.04% after 60 days and by 17.74, 32.72, 45.74, 27.11% after 90 days than Cd treatment. The T3 treatment had the lowest $\text{O}_2^{\cdot-}$ generation rate in leaves of ryegrass plants. Similar findings were found for the H_2O_2 content (Table 3).

When plants were subjected to environmental stress, oxidative damage resulted in membrane lipid peroxidation, which could be estimated by MDA content (Table 3). Similar to $\text{O}_2^{\cdot-}$ and H_2O_2 change, Cd treatment significantly increased MDA contents in ryegrass leaves after 30, 60 or 90 days, T1, T2, T3 and T4 significantly reduced MDA contents in leaves. The T3 treatment had the best effect of alleviation.

Table 2. Effects of application of different concentrations of NO every seven days on the chlorophyll contents (mg g^{-1} FW) in leaves of ryegrass plants grown in Cd-contaminated soil.

Items		Treatments					
		CK	Cd	T1	T2	T3	T4
Total Chl contents	30 days	2.42±0.14a	0.99±0.03e	1.34±0.03d	1.78±0.03c	2.27±0.05b	1.76±0.03c
	60 days	2.84±0.06a	1.42±0.07e	1.72±0.06d	2.10±0.01c	2.51±0.05b	2.03±0.09c
	90 days	2.74±0.01a	1.39±0.02e	1.62±0.10d	2.10±0.08c	2.46 ±0.01b	1.66±0.08a
Chl <i>a</i> content	30 days	1.59±0.05a	0.77±0.03d	1.04±0.03c	1.38±0.09b	1.62±0.03a	1.33±0.03b
	60 days	1.67±0.05a	0.85±0.01d	1.05±0.10c	1.26±0.05b	1.56±0.12a	1.36±0.09b
	90 days	1.78±0.02a	1.14±0.01d	1.29±0.08c	1.59±0.04b	1.72±0.03a	1.30±0.10c
Chl <i>b</i> content	30 days	0.83±0.10a	0.23±0.01e	0.31±0.01de	0.40±0.07cd	0.65±0.05b	0.42±0.03c
	60 days	1.16±0.04a	0.57±0.07e	0.66±0.05de	0.84±0.05c	0.95±0.07b	0.67±0.04d
	90 days	0.96±0.01a	0.26±0.02e	0.33±0.02d	0.51±0.04c	0.74±0.02b	0.36±0.02a
Carotenoids content	30 days	0.39±0.03a	0.24±0.02d	0.29±0.04c	0.33±0.03d	0.40±0.02a	0.26±0.02cd
	60 days	0.40±0.02a	0.21±0.01d	0.26±0.02bc	0.31±0.03b	0.40±0.02a	0.23±0.05cd
	90 days	0.39±0.03a	0.22±0.00c	0.25±0.01bc	0.29±0.01b	0.36±0.04a	0.28±0.02b

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

Table 3. Effects of application of different concentrations of NO every seven days on O₂⁻ generation rate, H₂O₂ and MDA content in leaves of ryegrass plants grown in Cd-contaminated soil.

Treatments	The O ₂ ⁻ generation rate ($\mu\text{mol g}^{-1} \text{h}^{-1} \text{FW}$)			H ₂ O ₂ concentration ($\mu\text{mol g}^{-1} \text{FW}$)			MDA concentration ($\text{nmol g}^{-1} \text{FW}$)		
	30 Days	60 Days	90 Days	30 Days	60 Days	90 Days	30 Days	60 Days	90 Days
CK	15.19±0.15f	12.89±0.52f	17.50±0.36f	34.53±0.98a	33.26±0.25a	27.02±0.51b	15.45±0.70d	21.91±0.78f	14.11±0.68f
Cd	46.87±0.80a	56.04±0.06a	49.75±1.28a	22.34±0.48e	21.15±0.10d	16.85±0.60e	26.61±0.46a	37.06±0.67a	28.17±0.93a
T1	39.77±0.54b	45.75±0.32b	40.92±0.63b	24.60±0.79d	26.21±0.40c	22.18±1.11d	23.33±0.64b	31.65±1.22c	24.69±0.43b
T2	35.52±0.80c	37.69±0.42c	33.47±0.65d	27.68±0.41c	27.83±0.52c	27.57±0.86b	18.57±0.76c	28.14±0.74d	21.17±0.70c
T3	24.13±0.62e	24.16±0.63e	26.99±1.09e	30.24±0.13b	31.00±1.87b	31.61±1.07a	13.13±0.64e	25.03±0.65e	15.55±0.54e
T4	29.27±0.34d	32.48±1.00d	36.26±1.02c	23.73±0.71d	26.84±1.04c	25.51±0.46c	17.70±1.01c	33.44±0.54b	17.21±0.39d

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

Table 4. Effects of application of different concentrations of NO every seven days on SOD, POD and CAT content in leaves of ryegrass plants grown in Cd-contaminated soil.

Treatments	SOD activity ($\text{U g}^{-1} \text{FW}$)			POD activity ($\text{U mg}^{-1} \text{min}^{-1} \text{FW}$)			CAT activity ($\mu\text{mol H}_2\text{O}_2 \text{mg}^{-1} \text{min}^{-1} \text{FW}$)		
	30 Days	60 Days	90 Days	30 Days	60 Days	90 Days	30 Days	60 Days	90 Days
CK	112.50±3.40a	97.52±1.56a	168.59±3.76a	97.56±3.26a	203.76±1.42b	228.85±1.50a	8.13±0.12a	8.28±0.02a	8.45±0.07a
Cd	59.64±1.18e	44.29±3.70f	96.63±1.87e	51.68±4.21e	154.26±0.60e	161.98±1.77f	5.93±0.08e	6.30±0.06e	5.76±0.05f
T1	64.62±0.42d	54.76±1.92e	106.14±2.33d	64.59±1.78d	166.85±1.05d	180.86±1.17e	6.22±0.05d	6.58±0.12d	6.24±0.10e
T2	74.03±1.25c	66.10±1.90c	112.77±2.39c	76.44±2.00c	197.37±1.62c	209.92±0.97c	6.79±0.14c	7.27±0.02c	7.34±0.12c
T3	86.09±1.93b	83.22±2.30b	138.43±3.44b	90.28±1.53b	246.80±2.19a	221.66±1.35b	8.13±0.09a	7.55±0.13b	8.18±0.08b
T4	68.06±2.04a	59.60±2.78d	111.15±3.04cd	65.17±1.03d	203.53±1.91b	185.68±2.43d	7.54±0.02b	6.18±0.06e	6.95±0.10d

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

3.4. Antioxidant enzymes

As shown in Table 4, Superoxide dismutase activity decreased markedly under Cd stress in leaves of ryegrass plants. After 30, 60 or 90 days treatment, Cd treatment significantly decreased SOD activity by 46.99, 54.59 and 42.69% in leaves. The T1, T2, T3 and T4 treatments increased SOD activity by 8.35, 24.13, 44.36, 14.13% after 30 days, by 23.64, 49.26, 87.90, 34.57% after 60 days and by 9.84, 16.71, 43.27, 15.03% after 90 days than Cd treatment. It was similar in activities of POD and CAT. All of antioxidant enzyme activities were found to be in a general trend of CK > T3 > T2 > T1, or T4 > Cd.

3.5. Proline content

To investigate the contributions of non enzymatic antioxidants to the response of ryegrass to Cd toxicity, proline content was examined. After 30, 60 and 90 days treatment, Proline content in leaves of ryegrass plants (Figure 1) decreased by 50.57, 34.82 and 45.51% in response to Cd application. The T1, T2, T4 and especially T3 treatments significantly increased proline content in Cd-treated plants. Compared to the Cd alone treatment, after 30, 60 and 90 days treatment, T3 treatment increased proline content by 105.63, 54.49 and 106.52%.

Figure 1. Effects of application of different concentrations of NO every seven days on proline content in leaves of ryegrass plants grown in Cd-contaminated soil. Values are the mean of three replicates. Values are the mean of three replicates. Each replicate has 50 plants. Bars with different letters are significantly different at $P<0.05$.

Table 5. Effects of application of different concentrations of NO every seven days on soluble protein and AsA content in leaves of ryegrass plants grown in Cd-contaminated soil.

Treatments	Soluble protein content (mg g ⁻¹ FW)			AsA content (nmol g ⁻¹ FW)		
	30 Days	60 Days	90 Days	30 Days	60 Days	90 Days
CK	21.13±0.48b	29.33±0.41b	23.09±0.38c	22.95±0.14a	12.45±0.37a	12.82±0.19a
Cd	12.52±0.58e	20.85±0.47e	15.41±0.08f	13.50±0.75e	3.81±0.05f	6.21±0.12f
T1	16.23±0.71d	25.28±0.56d	17.68±0.94e	14.86±0.30d	4.47±0.06e	7.10±0.03e
T2	18.16±0.50c	27.69±0.72c	21.05±0.95d	16.43±0.35c	6.90±0.04c	7.99±0.06d
T3	22.90±1.45a	32.75±0.90a	32.09±0.05a	17.95±0.35b	8.81±0.05b	10.55±0.19b
T4	18.68±0.47c	28.49±0.34bc	25.81±0.59b	16.45±0.69c	5.85±0.13d	8.70±0.10c

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

3.6. Soluble protein and AsA content

Cd treatment decreased soluble protein content and AsA content in leaves of ryegrass plants dramatically (Table 5). However, these inhibitions were significantly alleviated by 50, 100, 300 and 500 μ M SNP, especially 300 μ M SNP. Compared with Cd treatment, the soluble protein and AsA content of T3 treatment were increased by 82.95, 32.96% after 30 days, by 57.08, 131.03 % after 60 days and by 108.31, 70.00% after 90 days.

3.7. Cd concentrations

Cadmium accumulation was significantly higher in roots than in shoots (Table 6) and Cd concentrations in shoots and roots of Cd-treated plants were accumulated at a high level. However, after 30, 60 and 90 days treatment, Cd accumulation was decreased not only in shoots but also in roots with addition of SNP in Cd-contaminated soil, and T3 treatment had the best effect on inhibiting Cd accumulation in shoots and roots of ryegrass plants.

Table 6. Effects of application of different concentrations of NO every seven days on Cd content (mg kg⁻¹ DW) in shoots and roots of ryegrass plants grown in Cd-contaminated soil

Treatments	30 Days		60 Days		90 Days	
	Shoots	Roots	Shoots	Roots	Shoots	Roots
CK	0.00±0.00e	0.00±0.00e	0.00±0.00e	0.00±0.00f	0.00±0.00f	0.00±0.00e
Cd	142.40±5.14a	1039.51±33.30a	117.92±4.41a	871.23±8.49a	131.09±4.87a	819.38±4.97a
T1	132.51±1.26b	898.82±25.93b	108.25±2.36b	734.01±8.59c	113.15±3.94c	760.77±25.96b
T2	121.78±3.22c	835.16±22.55c	102.70±2.65c	560.30±20.83d	101.54±4.42d	666.83±3.18c
T3	114.57±1.41d	750.43±7.58d	95.26±1.53d	485.58±13.84e	87.44±2.26e	579.09±18.93d
T4	144.39±7.28a	851.22±34.65c	103.28±0.78c	771.46±20.23b	124.18±4.48b	818.92±11.11a

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

Table 7. Effects of application of different concentrations of NO every seven days on mineral content (g kg⁻¹ DW) in leaves and roots of ryegrass plants grown in Cd-contaminated soil.

Items		Treatments					
		CK	Cd	T1	T2	T3	T4
30 days							
Ca	Shoots	0.19ab	0.13d	0.16c	0.17b	0.19a	0.16c
	Roots	0.16a	0.09b	0.10b	0.15a	0.16a	0.10b
Fe	Shoots	0.96a	0.36e	0.39d	0.45c	0.61b	0.34e
	Roots	1.73a	0.74e	0.91d	1.03c	1.52b	0.98cd
Mg	Shoots	3.11c	2.67e	2.86d	3.30b	3.56a	2.79d
	Roots	5.15a	2.57d	2.78d	3.24c	4.42b	3.25c
Zn	Shoots	3.56a	2.57e	2.85c	3.04b	3.59a	2.74d
	Roots	6.01a	3.64e	3.97d	4.35c	5.39b	3.93d
60 days							
Ca	Shoots	0.33a	0.16e	0.19d	0.21c	0.28b	0.22c
	Roots	0.15a	0.05c	0.10b	0.11b	0.14a	0.10b
Fe	Shoots	0.46a	0.23e	0.26d	0.32c	0.39b	0.25d
	Roots	1.12a	0.68d	0.81c	0.90b	1.07a	0.85bc
Mg	Shoots	3.02a	1.77f	1.93e	2.14c	2.41b	2.01a
	Roots	2.84a	1.98f	2.17e	2.37c	2.54b	2.26d
Zn	Shoots	4.28a	2.22f	2.46d	2.96c	3.45b	2.33e
	Roots	5.20b	3.98e	4.15d	4.57c	5.39a	4.22d
90 days							
Ca	Shoots	0.10a	0.04d	0.05cd	0.06bc	0.10a	0.07b
	Roots	0.06b	0.04d	0.05cd	0.06bc	0.07a	0.06bc
Fe	Shoots	0.43a	0.22d	0.32c	0.37b	0.43a	0.36b
	Roots	1.50a	0.64e	1.06d	1.14c	1.33b	1.10cd
Mg	Shoots	2.75a	2.06c	2.27b	2.37b	2.65a	2.02c
	Roots	3.68a	2.56e	2.86d	3.25c	3.53b	2.92a
Zn	Shoots	4.35a	2.63e	3.06d	3.43c	3.70b	3.36c
	Roots	4.00a	2.77e	2.97d	3.26c	3.71b	3.18c

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

3.8. Ca, Fe, Mg and Zn contents

Table 7 showed Ca, Fe, Mg and Zn contents in shoots and roots of ryegrass plants. Compared with CK, Cd treatment significantly decreased Ca, Fe, Mg and Zn contents in shoots and in roots. However, the application of SNP increased Ca, Fe, Mg and Zn contents in shoots and in roots, and 300 μM SNP had the most obvious promotion on mineral concentrations. Taken together, the alleviating effect of SNP was found in a general trend of $\text{T3} > \text{T2} > \text{T4} > \text{T1}$.

4. Discussion

In the recent decades, an increasing number of studies have reported the effect of SNP on alleviating heavy metal toxicity in plants (Chen *et al.*, 2010; Xu *et al.*, 2010; Wang *et al.*, 2013). In the present experiment, we investigated the effects of application of different concentrations of NO on ryegrass plants under Cd stress grown in 50 mg kg^{-1} Cd-contaminated soil and analyzed the possible roles of exogenous NO in mitigating Cd toxicity. After 30, 60 and 90 days treatment, compared to CK, the growth of ryegrass plants was inhibited significantly under Cd stress (Table 1). The inhibition of growth in ryegrass might be resulted from Cd-induced alteration of fundamental metabolic process, photosynthesis and uptake of nutrient elements. Cadmium is phytotoxic, as it can interfere with photosynthetic and respiratory activities, mineral nutrition, enzymatic activities, membrane functions, and hormone balance. Cd may lead to changes in plant nutrient concentration and composition. However, the application of SNP had a promoting effect on plant growth under Cd stress (Table 1). The mitigation effect may be due to the fact that the optimum concentration of NO can act directly on the cell wall components and plasma membrane bilayers, resulting in enhancing membrane fluidity and promoting

cell expansion and growth (Xiong *et al.*, 2009; Wang *et al.*, 2013).

A notable reduction of chlorophyll concentration was detected under Cd stress in ryegrass leaves (Table 2). Chen *et al.* (2008) suggested that decreasing leaf chlorophyll content was one of the most general toxicity effects of Cd on plants, which was confirmed again in the present study. The decrease in chlorophyll content in Cd-affected ryegrass plants might be attributed to the possible oxidation of chlorophyll and the damaged ultrastructure of chloroplasts under Cd stress (Chen *et al.*, 2010). However, the addition of SNP increased chlorophyll content dramatically, and the best of alleviating effect was T3 treatment. In this experiment, the application of SNP and especially 300 μM SNP significantly increased Fe concentration in both the shoots and roots of ryegrass and then increased chlorophyll content. In addition, SNP may effectively reduce the level of ROS generated during stress resulting in alleviating the oxidative negative effects of ROS on growth and chlorophyll content.

It has been shown that Cd leads to an increased production of H_2O_2 and induces lipid peroxidation (Tamás *et al.*, 2015) in plants. MDA content was measured as an index of lipid peroxidation. In our experiment, we investigated the involvement of these molecules. The markedly increases in contents of H_2O_2 , $\text{O}_2^{\cdot-}$ and MDA under Cd stress were observed (Table 3). Similar results have been observed in ryegrass and lettuce (Wang *et al.*, 2013; Xu *et al.*, 2014).

It is also widely agreed that plants resist the stress induced production of ROS by increasing components of their intrinsic defense system including altered activities of enzyme proteins (Manai *et al.*, 2014). SOD constitutes the first line of defense against ROS, which is crucial for the removal of $\text{O}_2^{\cdot-}$ in the compartments where $\text{O}_2^{\cdot-}$ radicals formed. POD catalyses H_2O_2 -dependent oxidation of substrate, while CAT and APX eliminate H_2O_2 by breaking it down directly to form

water and oxygen. In the present study, Cd stress led to decreases in activities of SOD, POD and CAT (Table 4). This result was in agreement with Tamás *et al.* (2015) who reported that exposure to Cd stress significantly decreased activities of SOD, CAT and POD. However, exogenous NO improved activities of SOD, POD and CAT (Table 4). The similar results have been observed in rice under Cd stress (Xiong *et al.*, 2009). SNP-induced antioxidant enzyme activities increase indicated that they can play a critical role in modulating the cell redox balance, thereby protecting plants against oxidative damage. Osmotic adjustment is the main part of the physiological machinery by which plants respond to heavy metals toxicity. Proline plays important roles on clearance of ROS and adjustment of osmotic substances (Dong *et al.*, 2014). There are recent reports suggesting that NO improve Cd tolerance in *M. truncatula* roots (Xu *et al.*, 2010) and rice seedlings (He *et al.*, 2014) by an increase in the production of proline. As shown in Figure 1, proline content decreased in ryegrass plants grown with Cd stress alone. However, the addition of SNP and especially 300 μ M SNP significantly increased proline content in Cd-treated plants. In fact, proline has multiple functions under stressful conditions, such as osmotic pressure regulation, protection of membrane integrity, stabilization of enzymes/proteins, maintaining appropriate NADP⁺/NADPH ratios, and scavenger of free radicals (Idrees *et al.*, 2012).

Results of our study showed that Cd decreased the content of soluble protein in ryegrass plants significantly, and treatments with SNP increased the level of soluble protein (Table 5). This study coincided with Ericson and Alfinito (1984) who reported that abiotic stress may inhibit a synthesis of some proteins and promote others with a general trend of decline in the overall content. However, SNP supplementation improved Cd-decreased soluble protein content (Table 5). It is likely that nitric oxide (NO)

could alleviate the heavy metal-induced phytotoxicity and had very important role in protein nitration (Saxena and Shekhawat, 2013). Ascorbic acid (ASA) is the most abundant, powerful and water soluble antioxidant acts preventing or at least alleviating deleterious effects caused by ROS in plants, thus providing membrane protection. However, in this present study, Cd decreased the content of ASA in ryegrass plants significantly, and the treatments with SNP increased the ASA content (Table 5). This suggested SNP mediated the enhancement of AsA levels in ryegrass plants. Moreover, ASA can participate in detoxifying ROS directly or through certain enzymes restricting lipid peroxidation and oxidative stress, and then improved the tolerance of Cd toxicity.

Tight control of Cd in sensitive tissues may act as the first line of defense in plants to counteract Cd toxicity. In plants, the root is in direct contact with Cd and the cell walls of roots play a significant role in heavy metal tolerance. A principal defense step to counteract Cd toxicity in plants is to prevent Cd accumulation in shoot tissues. It was documented by Xiong *et al.* (2009) that exogenous NO alleviated Cd toxicity in rice by increasing pectin and hemicellulose contents in root cell walls, increasing Cd deposition in root cells and decreasing Cd accumulation in soluble fractions of leaves. Previous reports also indicated SNP supplementation at low concentrations reduced the transport of Cd from roots to shoots in perennial ryegrass (Wang *et al.*, 2013). In this work, increased uptake of Cd in both roots and shoots as compared to control is noted in Cd-treated plants. And exposure of plants to Cd stress resulted in that Cd accumulated at higher concentrations in roots than in shoots (Table 6). This suggests that the ability of ryegrass plants to prevent excessive accumulation of Cd in shoots in the presence of Cd stress may at least partially account for the tolerance to Cd. However, addition of SNP in the growth medium lowered the accumulation of Cd

and the root-to-shoot translocation of Cd in Cd + SNP treatment than that in plants grown under Cd alone (Table 6). This result is consistent with the findings of Wang *et al.* (2013).

Cadmium toxicity has been related to interactions between uptake and translocation of mineral nutrients in plants. In our study, Cd toxicity led to a deficiency of Ca, Mg, Fe and Zn in the ryegrass plants (Table 7), disturbed intracellular ion homeostasis, and then result in growth inhibition. Similar results were reported in our previous study (Wang *et al.*, 2013). These results demonstrated that Cd interfered with nutrient uptake and with nutrient distribution into the different plant parts. However, the SNP treatment was found to promote the uptake of these mineral elements (Table 7). This could be explained by SNP increasing the activity of H⁺-ATPase, which plays an important role in the transport of multiple ions. Moreover, Ca provides intermolecular linkages and is thought to play a crucial role in the stabilization of cell wall and membrane. Neill *et al.* (2002) demonstrated that NO could interact with Ca²⁺ channel in plants. In addition, the addition of SNP can promote the uptake of Ca in plants under Cd stress. For these reasons, the uptake of Mg, Fe and Zn from medium to plant was promoted by the addition of SNP. These results indicated that SNP could ameliorate ion equilibrium in ryegrass cells under Cd stress and promoted plant growth.

5. Conclusion

This study demonstrated that, Cd stress depressed plant growth, reflected by oxidative stress and the inhibition of chlorophyll synthesis and nutrient elements uptake. However, application of SNP at a proper concentration could significantly alleviate the effect of Cd toxicity in ryegrass plants exposed to Cd stress. The potential mechanisms include: (1) promoted chlorophyll synthesis; (2) induced a better

antioxidant system in plants; (3) reduced Cd uptake, inhibited Cd transport from roots to shoots and enhanced the absorption of nutrient elements.

And 300 µM SNP had the best of alleviating effect against Cd toxicity, while high concentrations of SNP had no significant effect. Our results may have a potential value on repair of heavy metal contaminated areas, but further study is required.

Acknowledgement

This research work was financially supported by a Project of Shandong Province Higher Educational Science and Technology Program (J14LF08) and the Shandong Provincial Natural Science Foundation of China (ZR2013CM003). Special acknowledgements are given to the editors and reviewers.

Reference

- Ali, N.A., Bernal, M.P., Ater, M. 2002. Tolerance and bioaccumulation of copper in *Phragmites australis* and *Zea mays*. *Plant Soil*. 239, 103–111.
- Arienzo, M., Adamo, P., Cozzolino, V. 2004. The potential of *Lolium perenne* for revegetation of contaminated soil from a metallurgical site. *Sci. Total Environ*. 319, 13–25.
- Bates, L.S., Waldern, S.P., Teare, I.D. 1973. Rapid determination of free proline for water-stress studies. *Plant Soil*. 39, 205–207.
- Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dyes binding. *Anal. Biochem*. 72, 248–254.
- Chen, F., Wang, F., Sun, H.Y., Cai, Y., Miao, W.H., Zhang, G.P., Vincze, E., Wu, F.B. 2010. Genotype-dependent effect of exogenous nitric oxide on Cd-induced changes in antioxidative metabolism,

- ultrastructure, and photosynthetic performance in barley seedlings (*Hordeum vulgare*). J. Plant Growth Regul. 29, 394–408.
- Chen, F., Wang, F., Zhang, G.P., Wu, F.B. 2008. Identification of barley varieties tolerant to cadmium toxicity. Biol. Trace Elem. Res. 121, 171–179.
- Chen, G.C., Liu, Y.Q., Wang, R.M., Zhang, J.F., Owens, G. 2013. Cadmium adsorption by willow root: the role of cell walls and their subfractions. Environ. Sci. Pollut. Res. 20, 5665–5672.
- Dong, Y., Jinc, S., Liu, S., Xu, L., Kong, J. 2014. Effects of exogenous nitric oxide on growth of cotton seedlings under NaCl stress. Journal of Soil Science and Plant Nutrition. 14, 1–13.
- Dong, Y.J., Wang, Z.L., Zhang, J.W., Liu, S., He, Z.L., He, M.R. 2015. Interaction effects of nitric oxide and salicylic acid in alleviating salt stress of *Gossypium hirsutum* L.. Journal of Soil Science and Plant Nutrition. 15, 561–573.
- Durán, P., Acuña, J.J., Armada, E., López-Castillo, O.M., Cornejo, P., Mora, M.L., Azcón, R. 2016. Inoculation with selenobacteria and arbuscular mycorrhizal fungi to enhance selenium content in lettuce plants and improve tolerance against drought stress. Journal of Soil Science and Plant Nutrition. 16, 201–225.
- Elstner, E.F., Heupel, A. 1976. Inhibition of nitrite formation from hydroxyl ammonium-chloride: a simple assay for superoxide dismutase. Anal. Biochem. 70, 616–620.
- Ericson, M.C., Alfinito, A.E. 1984. Proteins produced during salt stress in tobacco cell cultures. Plant Physiol. 74, 506–509.
- Fang, F.M., Wang, Q.C. 2000. Research progress on mercury pollution in soil. Soil Environ. 9, 326–329.
- Foy, C.D., Chaney, R.L., White, M.C. 1978. The physiology of metal toxicity in plants. Annu. Rev. Plant Physiol. 29, 511–566.
- Heath, R.L., Packer, L. 1968. Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. Arch. Biochem. Biophys. 125, 189–198.
- He, H.Y., Zhan, J., He, L.F., Gu, M.H. 2012. Nitric oxide signaling in aluminum stress in plants. Protoplasma. 249, 483–492.
- He, J.Y., Ren, Y.F., Chen, X.L., Chen, H. 2014. Protective roles of nitric oxide on seed germination and seedling growth of rice (*Oryza sativa* L.) under cadmium stress. Ecotoxicology and Environmental Safety. 108, 114–119.
- Idrees, M., Naeem, M., Nasir Khan, M., Aftab, T., Khan, M.M.A., Moinuddin, 2012. Alleviation of salt stress in lemongrass by salicylic acid. Protoplasma. 249, 709–720.
- Khairy, A.I.H., Oh, M.J., Lee, S.M., Kim, D.S., Roh, K.S. 2016. Nitric oxide overcomes Cd and Cu toxicity in *in vitro*-grown tobacco plants through increasing contents and activities of rubisco and rubisco activase. Biochimie Open. 2, 41–51.
- Knudson, L.L., Tibbitts, T.W., Edwards, G.E. 1977. Measurement of ozone injury by determination of leaf chlorophyll concentration. Plant Physiol. 60, 606–608.
- Kováčik, J., Klejdus, B., Babula, P., Hedbavny, J. 2015. Nitric oxide donor modulates cadmium-induced physiological and metabolic changes in the green alga *Coccomyxa subellipsoidea*. Algal Research. 8, 45–52.
- Liu, S.L., Yang, R.J., Pan, Y.Z., Ma, M.D., Pan, J., Zhao, Y., Cheng, Q.S., Wu, M.X., Wang, M.H., Zhang, L. 2015. Nitric oxide contributes to minerals absorption, proton pumps and hormone equilibrium under cadmium excess in *Trifolium repens* L. plants. Ecotoxicology and Environmental Safety. 119, 35–46.
- Lou, Y.H., Luo, H.J., Hu, T., Li, H.Y., Fu, J.M. 2013. Toxic effects, uptake, and translocation of Cd

- and Pb in perennial ryegrass. *Ecotoxicology*. 22, 207–214.
- Manai, J., Kalai, T., Gouia, H., Corpas, F.J. 2014. Exogenous nitric oxide (NO) ameliorates salinity-induced oxidative stress in tomato (*Solanum lycopersicum*) plants. *Journal of Soil Science and Plant Nutrition*. 14, 433–446.
- Neill, S.J., Desikan, R., Clarke, A., Hurst, R.D., Hancock, J.T. 2002. Hydrogen peroxide and nitric oxide as signalling molecules in plants. *J Exp Bot*. 53, 1237–1247.
- Neill, S.J., Desikan, R., Hancock, J.T. 2003. Nitric oxide signalling in plants. *New Phytology*. 159, 11–35.
- Nickel, K.S., Cunningham, B.A. 1969. Improved peroxidase assay method using leuco 2,3', 6-trichloroindophenol and application to comparative measurements of peroxidatic catalysis. *Anal. Biochem*. 27, 292–299.
- Patra, H.L., Kar, M., Mishre, D. 1978. Catalase activity in leaves and cotyledons during plant development and senescence. *Biochem. Pharmacol*. 172, 385–390.
- Patterson, B.D., MacRae, E.A., Ferguson, I.B. 1984. Estimation of hydrogen peroxide in plant extracts using titanium (IV). *Anal. Biochem*. 139, 487–492.
- Rady, M.M., Hemida, K.A. 2015. Modulation of cadmium toxicity and enhancing cadmium-tolerance in wheat seedlings by exogenous application of polyamines. *Ecotoxicology and Environmental Safety*. 119, 178–185.
- Saxena, I., Shekhawat, G.S. 2013. Nitric oxide (NO) in alleviation of heavy metal induced phytotoxicity and its role in protein nitration. *Nitric Oxide*. 32, 13–20.
- Stewart, R.C., Bewley, J.D. 1980. Lipid peroxidation associated with accelerated aging of soybean axes. *Plant Physiol*. 65, 245–248.
- Tamás, L., Mistrík, I., Alemayehu, A., Zelinová, V., Bořcová, B., Huttová, J. 2015. Salicylic acid alleviates cadmium-induced stress responses through the inhibition of Cd-induced auxin-mediated reactive oxygen species production in barley root tips. *Journal of Plant Physiology*. 173, 1–8.
- Tonamura, B. 1978. Test reactions for a stopped flow apparatus regulation of 2, 6-D and potassium ferricyanide by L-ascorbic acid. *Anal. Biochem*. 84, 370–383.
- Wang, Q.H., Liang, X., Dong, Y.J., Xu, L.L., Zhang, X.W., Hou, J., Fan, Z.Y. 2013. Effects of exogenous nitric oxide on cadmium toxicity, element contents and antioxidative system in perennial ryegrass. *Plant Growth Regul*. 69, 11–20.
- Wendehenne, D., Pugin, A., Klessig, D.F., Durner, J. 2001. Nitric oxide: comparative synthesis and signalling in animal and plant cells. *Trends in Plant Sciences*. 6, 177–183.
- Xiong, J., An, L.Y., Lu, H., Zhu, C. 2009. Exogenous nitric oxide enhances cadmium tolerance of rice by increasing pectin and hemicellulose contents in root cell wall. *Planta*. 230, 755–765.
- Xu, J., Wang, W.Y., Yin, H.X., Liu, X.J., Sun, H., Mi, Q. 2010. Exogenous nitric oxide improves antioxidative capacity and reduces auxin degradation in roots of *Medicago truncatula* seedlings under cadmium stress. *Plant Soil*. 326, 321–330.
- Xu, L.L., Dong, Y.J., Kong, J., Liu, S. 2014. Effects of root and foliar applications of exogenous NO on alleviating cadmium toxicity in lettuce seedlings. *Plant Growth Regul*. 72, 39–50.
- Zhang, Z.L., Di, W.J. 2003. Laboratory guide for plant physiology [M]. Higher Education Press, Beijing.