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Effects of lead and nitric oxide on photosynthesis, antioxidative ability, and mineral element content of perennial ryegrass

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Abstract

Hydroponics experiments were conducted to study the effects of sodium nitroprusside (SNP, a donor of NO) on lead toxicity in ryegrass (*Lolium perenne* L.) seedlings. When the ryegrass seedlings were grown in a nutrient solution containing 500 μM Pb^{2+} for two weeks, the plant biomass as well as net photosynthetic rate, transpiration rate, chlorophyll and carotenoid content of leaves decreased. The Pb stress also induced the production of superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), leading to malondialdehyde (MDA) accumulation. Furthermore, the activities of superoxide dismutase (SOD), peroxidase (POD), and ascorbate peroxidase (APX) decreased in the Pb-treated seedlings, but the catalase (CAT) activity increased. Additionally, the content of Cu in shoots and the content of K, Mg, Fe, and Zn in both shoots and roots decreased, but the content of Ca in shoots and roots increased under the Pb stress. Moreover, Pb accumulated mostly in roots, whereas a small quantity was translocated to shoots. However, the addition of 50, 100, and 200 μM SNP into the solution containing Pb increased the chlorophyll content and net photosynthetic rate, reduced Pb-induced oxidative damages, improved antioxidant enzyme activities, and inhibited translocation of Pb from roots to shoots. In particular, 100 μM SNP had the best effect on promoting growth of the ryegrass seedlings under the Pb toxicity. However, the application of 400 μM SNP had no obvious alleviating effect on Pb toxicity in the ryegrass seedlings.

Additional key words: ascorbate peroxidase, carotenoids, catalase, chlorophyll, hydrogen peroxide, *Lolium perenne*, malondialdehyde, peroxidase, superoxide dismutase.

Introduction

Lead is highly toxic to plants, animals, and humans. As non-essential element for plants, Pb becomes phytotoxic mostly by influencing cell division (Samardakiewicz and Woźny 2005), mineral nutrition (Kopittke *et al.* 2007, Liu *et al.* 2009), by inducing oxidative stress (Verma and Dubey 2003, Zhou *et al.* 2010), and by inhibiting photosynthesis (Gopal and Rizvi 2008). Toxic concentrations of Pb lead to the disruption of proteins and thylakoid membranes and to the inhibition of enzymes activities (Alkhatib *et al.* 2011). To survive oxidative stress, plants activate effective defense systems which are associated with metal tolerance (Mittler *et al.* 2004, Jiang and Liu 2010). These include antioxidants glutathione and ascorbic acid (AsA), and enzymes, such as catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX).

Nitric oxide, well known naturally occurring signaling molecule, has various effects on plants and may play a key role in regulating their growth and productivity (Xiong *et al.* 2009, Xu *et al.* 2010). Recently, an increasing number of articles have reported the role of exogenous NO released by an NO donor, such as sodium nitroprusside (SNP) in alleviating the negative effects of heavy metals, *e.g.*, Cu, Ni, and Cd (Zhang *et al.* 2009,

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Abbreviations: APX - ascorbate peroxidase; CAT - catalase; MDA - malondialdehyde; $O_2^{\bullet-}$ - superoxide anion; POD - peroxidase; ROS - reactive oxygen species; SNP - sodium nitroprusside; SOD - superoxide dismutase.

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Mihailovic and Drazic 2011, Wang *et al.* 2013a). Added NO also reduces Pb uptake leading to fewer toxic symptoms (Phang *et al.* 2011a). Besides, Hermes *et al.* (2013) indicated that NO can affect gene expression related to oxidative stress response. But, many studies have demonstrated that effects of NO depends on its concentration and the type of cells (Lamattina *et al.* 2003). The possible alleviation of Pb stress by NO has been rarely studied so far. Therefore, a further analysis is required to give an insight into the signal mechanism of NO protection of plants against Pb stress.

Perennial ryegrass is an important and widespread perennial plant (Hannaway *et al.* 1999). It can accumulate metals in its biomass and is commonly used as suitable species for revegetation of metalliferous wastes (Arienzo *et al.* 2004). Our previous studies showed that most Cd accumulates in the roots of perennial ryegrass, and the application of exogenous NO inhibits Cd translocation from roots to shoots (Wang *et al.* 2013a,b). Based on the above studies, we hypothesize that SNP may ameliorate Pb-induced toxic effects in this species. The influence of different concentrations of SNP on Pb-induced changes in growth and photosynthesis as well as on the antioxidant system and mineral distribution in ryegrass seedlings has been investigated.

Materials and methods

Ryegrass seeds were sterilized with 5 % (m/v) sodium hypochlorite for 15 min and washed extensively with distilled water, then germinated on moist filter paper in the dark at 26 °C for three days. Initially, seedlings of uniform size were transferred to plastic pots (volume 500 cm^3) filled with *Perlite* (50 plants per pot) and watered with a half-strength Hoagland nutrition solution for 7 d. The seedlings were then watered with a full-strength Hoagland solution. Three-week-old uniform seedlings were transferred into 1 000 cm^3 black plastic containers (20 seedlings per container). The nutrient solution was renewed every two days. The treatments were: CK - Hoagland's solution; Pb - nutrient solution with 500 μM Pb given as $Pb(NO_3)_2$; Pb+SNP1, Pb+SNP2, Pb+SNP3, or Pb+SNP4 - nutrient solution with 500 μM Pb plus 50, 100, 200, or 400 μM SNP, respectively. The containers were arranged in a randomized block design with four replicates, giving a total of 24 containers. The experiment was carried out under a 14-h photoperiod, a photon flux density (PFD) of 150 μmol m^{-2} s^{-1} at the leaf level, day/night temperatures of 25/18 °C, and a 65 ± 5 % relative humidity. After two weeks, the plants were harvested and roots and leaves were separated and washed with 5 mM $CaCl_2$ first and then repeatedly with deionized distilled water. Plant height, fresh and dry masses, root volume, and root/shoot ratio were measured. The root volume was determined by a water displacement. For the estimation of plant dry matter and minerals, the plants were dried at 80 °C for 48 h. The dried tissues were weighed, grinded into powder, and digested with mixed acids [HNO_3 + $HClO_4$ (3:1, v/v)]. Mineral element concentrations were determined by flame atomic absorbance spectrometry (*Shimadzu AA-6300*, Kyoto, Japan) (Ali *et al.* 2002). For the determination of enzyme activities, fresh plant material was frozen in liquid nitrogen and stored at -70 °C until use.

The chlorophyll content was determined according to the method of Knudson *et al.* (1977). Fresh ryegrass leaves (0.5 g) were extracted in 2 cm^3 of 95 % (v/v) ethanol for 24 h in the dark. The amounts of chlorophyll (Chl) *a*, Chl *b* and carotenoids (Car) were determined spectrophotometrically (*Shimadzu UV-2450*, Kyoto, Japan), by reading the absorbance at 665, 649, and 470 nm. The net photosynthetic rate (P_N) and transpiration rate (E) were monitored *in vivo* with a portable photosynthesis system (*LI-6400*, *LI-COR*, Lincoln, NE, USA). Measurements were made on four plants, four adult leaves per plant oriented toward the south. Measurements were done between 09:00 and 11:00 at a constant airflow rate of 500 μmol s^{-1}, saturation PFD of 1 200 μmol m^{-2} s^{-1}, an ambient CO_2 concentration of 350 cm^3 m^{-3}, and a temperature of 25 °C.

The production rate of $O_2^{\bullet-}$ was measured as described by Elstner and Heupel (1976). Fresh leaves (0.2 g) were homogenized in 1 cm^3 of a 50 mM phosphate buffer (pH 7.8), and the homogenate was centrifuged at 10 000 *g* for 10 min. Then 0.5 cm^3 of the supernatant was added to 0.5 cm^3 of a 50 mM phosphate buffer (pH 7.8) and 0.1 cm^3 of 10 mM hydroxylamine hydrochloride. After a 1-h reaction at 25 °C, the mixture was added to 1 cm^3 of 17 mM sulfanilamide and 1 cm^3 of 7 mM α-naphthyl-amine and let at 25 °C for 20 min. The absorbance at 530 nm was determined. Sodium nitrite was used as standard. For the determination of H_2O_2 content, leaf tissue (0.2 g) was homogenized with 3 cm^3 of 0.1 % (m/v) trichloroacetic acid (TCA) in an ice bath and centrifuged at 12 000 *g* for 15 min (Velikova *et al.* 2000). An aliquot (0.5 cm^3) of the supernatant was added to 0.5 cm^3 of a phosphate buffer (pH 7.0) and 1 cm^3 of 1 M KI. The absorbance of the mixture was read at 390 nm. The H_2O_2 content was determined using the coefficient of absorbance (ε) of 0.28 μM^{-1} cm^{-1}.

Lipid peroxidation was determined by measuring malondialdehyde (MDA), a major thiobarbituric acid reactive species (TBARS) and the product of lipid peroxidation (Heath and Packer 1968). Samples (0.2 g) were ground in 3 cm^3 of TCA (0.1 %, m/v). The

homogenate was centrifuged at 10 000 *g* for 10 min and 1 cm^3 of the supernatant fraction was mixed with 4 cm^3 of 0.5 % (m/v) thiobarbituric acid in 20 % TCA. The mixture was heated at 95 °C for 30 min, chilled on ice, and then centrifuged at 10 000 *g* for 5 min. The absorbance of the supernatant was measured at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The amount of MDA was calculated using ε of 155 $mM^{-1}cm^{-1}$.

For the extraction of antioxidative enzymes, leaves and roots were homogenized with a 50 mM Na_2HPO_4 - NaH_2PO_4 buffer (pH 7.8) containing 0.2 mM EDTA and 2 % (m/v) 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 the spectrophotometric analyses were conducted on a *Shimadzu UV-2450* spectrophotometer. SOD activity was assayed by measuring SOD ability to inhibit the photochemical reduction of nitroblue tetrazolium following the method of Stewart and Bewley (1980). One unit of SOD is defined as the amount of enzyme required to cause a 50 % inhibition of the NBT photoreduction rate. CAT activity was measured as the decline in absorbance at 240 nm due to the decrease of H_2O_2 content according to the method of Patra *et al.* (1978). POD activity was measured based on the increase in absorbance at 470 nm due to guaiacol oxidation (Nickel and Cunningham 1969). One unit of POD is quantified by the amount of tetraguaiacol formed using ε of 26.6 mM^{-1} cm^{-1}. Activity of ascorbate peroxidase (APX) was measured according to Nakano and Asada (1981) by monitoring the rate of ascorbate oxidation at 290 nm. The assay mixture contained 0.25 mM AsA, 1.0 mM H_2O_2, 0.1 mM EDTA, and 0.1 cm^3 of the enzyme extract in a 25 mM phosphate buffer (pH 7.0).

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

Results

The Pb exposure inhibited the growth of ryegrass seedlings compared with CK, and the reductions of plant height, fresh mass, dry mass, root volume, and root/shoot ratio was 35.77, 18.28, 55.18, 54.12, and 34.62 %, respectively. However, this inhibition was alleviated by the additions of 50, 100, and 200 μM SNP, especially by 100 μM SNP. Compared to Pb alone, the addition of 100 μM SNP increased these parameters by 51.53, 19.24, 79.59, 42.00, and 29.41 %. However, the Pb-induced inhibition of plant growth was not mitigated by adding 400 μM SNP (Table 1).

The content of total Chl, Chl *a*, Chl *b*, and Car decreased markedly under the Pb treatment (by 40.50, 44.16, 28.25, and 51.11 %, respectively, as compared with CK). However, this inhibition was alleviated by the additions of 50, 100, and 200 μM but not by 400 μM SNP. Compared to the Pb treatment, the addition of 100 μM SNP increased the total Chl, Chl *a*, Chl *b*, and Car content by 52.26, 59.37, 35.00, and 109.09 %, respectively (Table 2).

In the Pb-treated plants, P_N and E significantly decreased by 52.88 and 39.37 %, respectively, compared to CK. The application of 100 μM SNP increased P_N significantly, although the additions of 50, 200, and 400 μM SNP had no effect. The inhibition of E was alleviated by the additions of 50, 100, and 200 μM SNP, especially 100 μM SNP was very effective (Table 2).

Compared to CK, the Pb treatment significantly increased the $O_2^{\bullet-}$ generation rate by 44.95 % in shoots and by 147.06 % in roots. However, the additions of 50, 100, 200, and 400 μM SNP significantly decreased the $O_2^{\bullet-}$ generation rate both in shoots and in roots under

Table 1. Effects of different concentrations of SNP on the growth of ryegrass seedlings in nutrient solutions with 500 μM Pb for 14 d. CK - control, Hoagland's solution; Pb - nutrient solution with 500 μM Pb given as $Pb(NO_3)_2$; Pb+SNP1, Pb+SNP2, Pb+SNP3, or Pb+SNP4 - nutrient solution with 500 μM Pb plus 50, 100, 200, or 400 μM SNP, respectively. Means ± SD of four replicates with 20 plants. The values followed by different letters are significantly different at $P < 0.05$.

Treatments	Plant height [cm]	Fresh mass [g $plant^{-1}$]	Dry mass [g $plant^{-1}$]	Root volume [cm^3 $plant^{-1}$]	Root/shoot ratio
CK	29.49 ± 1.67a	6.68 ± 0.14a	0.341 ± 0.010a	1.92 ± 0.04a	0.26 ± 0.01a
Pb	20.39 ± 0.36b	5.68 ± 0.24b	0.160 ± 0.005e	1.37 ± 0.03c	0.18 ± 0.01d
Pb+SNP1	22.53 ± 1.27b	6.33 ± 0.21a	0.205 ± 0.005d	1.61 ± 0.03b	0.19 ± 0.01cd
Pb+SNP2	28.83 ± 0.96a	6.54 ± 0.16a	0.276 ± 0.007b	1.88 ± 0.03a	0.23 ± 0.01b
Pb+SNP3	22.43 ± 1.38b	6.34 ± 0.21a	0.227 ± 0.005c	1.81 ± 0.02a	0.20 ± 0.01c
Pb+SNP4	21.43 ± 0.45b	5.65 ± 0.12b	0.170 ± 0.008e	1.36 ± 0.03c	0.19 ± 0.01d

Table 2. Effects of different concentrations of SNP on the Chl and Car content, P_N, E, $O_2^{\bullet-}$ generation rate, H_2O_2 and MDA content, and on the activities of SOD, POD, CAT, and APX in shoots and roots of ryegrass plants grown in nutrient solutions without or with 500 μM $Pb(NO_3)_2$ for 14 d. Means ± SD of four replicates with 20 plants. The values followed by different letters are significantly different at $P < 0.05$. See Table 1 for other details.

Parameters		CK	Pb	Pb+SNP1	Pb+SNP2	Pb+SNP3	Pb+SNP4
Total Chl content [mg g^{-1}(f.m.)]	shoots	3.23a	1.92d	2.56c	2.92b	2.85b	1.86d
Chl *a* content [mg g^{-1}(f.m.)]		2.48a	1.39d	1.92c	2.21bc	2.13b	1.35d
Chl *b* content [mg g^{-1}(f.m.)]		0.74a	0.53c	0.64b	0.71a	0.72a	0.51c
Car content [mg g^{-1}(f.m.)]		0.45a	0.22b	0.37a	0.46a	0.40a	0.26b
P_N [μmol(CO_2) $m^{-2}s^{-1}$]		49.92a	23.52d	34.18bc	39.62b	31.67bcd	25.94cd
E [mmol(H_2O) $m^{-2}s^{-1}$]		7.29a	4.42d	5.62c	6.82b	5.47c	4.40d
$O_2^{\bullet-}$ generation [nmol g^{-1}(f.m.) min^{-1}]		2.14d	3.11a	2.75b	2.16d	2.61c	3.02a
H_2O_2 content [μmol g^{-1}(f.m.)]		0.86e	2.43b	1.86c	1.47d	1.86c	2.72a
MDA content [nmol g^{-1}(f.m.)]		14.13c	26.83a	19.33b	17.24bc	18.98bc	29.20a
SOD activity [U g^{-1}(f.m.]		176.42a	90.22c	150.04b	166.29ab	153.11b	94.17c
POD activity [U mg^{-1}(f.m.]		6.06a	3.89c	5.11b	6.11a	5.28b	3.50d
CAT activity [μmol(H_2O_2) mg^{-1}(f.m.) min^{-1}]		6.99c	9.20a	8.19b	7.16c	8.45b	9.61d
APX activity [μmol(ASA mg^{-1}(f.m.) min^{-1}]		0.67a	0.24f	0.33d	0.65b	0.39c	0.27e
$O_2^{\bullet-}$ generation [nmol g^{-1}(f.m.) min^{-1}]	roots	1.36d	3.36a	2.29b	1.98c	2.22b	3.32a
H_2O_2 content [μmol g^{-1}(f.m.)]		1.40d	4.70a	3.44b	2.98c	3.46b	4.93a
MDA content [nmol g^{-1}(f.m.)]		12.45c	25.14a	19.14b	15.72bc	18.49b	26.45a
SOD activity [U g^{-1}(f.m.]		92.61a	40.76d	61.72c	83.22b	70.31c	42.43d
POD activity [U mg^{-1}(f.m.]		17.42a	8.38d	13.95c	15.63b	15.24bc	8.40d
CAT activity [μmol(H_2O_2) mg^{-1}(f.m.) min^{-1}]		1.88d	2.66b	2.41c	1.56e	2.38c	2.93a
APX activity [μmol(ASA mg^{-1}(f.m.) min^{-1}]		4.72a	2.43d	3.41b	4.78a	3.49b	2.97c

the Pb stress. The 100 μM SNP supply had the best alleviating effect both in shoots and roots (Table 2). Compared with CK, the Pb stress also increased the H_2O_2 content by 181.86 % in leaves and by 235.95 % in roots. Te addition of 50, 100, and 200 but not 400 μM SNP reduced the Pb-caused H_2O_2 accumulation in leaves and roots. The addition of 100 μM SNP decreased the H_2O_2 content by 39.72 % in leaves and by 36.57 % in roots compared with Pb alone. Correspondingly, MDA in leaves and roots accumulated significantly due to the Pb treatment. Compared to Pb alone, the additions of 50, 100, and 200 μM SNP decreased the MDA content by 27.95, 35.74, and 29.27 % in shoots and by 23.89, 37.48, and 26.46 % in roots, respectively.

The Pb treatment decreased the SOD activity by 46.74 % in shoots and by 55.99 % in roots. When

Table 3 Effects of different concentrations of SNP on the content of mineral elements in shoots and roots of ryegrass plants grown in nutrient solutions without or with 500 μM $Pb(NO_3)_2$ for 14 d. Means ± SD of four replicates with 20 plants. The values followed by different letters are significantly different at $P < 0.05$. See Table 1 for other details.

Minerals		CK	Pb	Pb+SNP1	Pb+SNP2	Pb+SNP3	Pb+SNP4
Pb [mg kg^{-1}(d.m.)]	shoots	0.00f	0.21a	0.19c	0.16e	0.17d	0.19b
K [g kg^{-1}(d.m.)]		38.05 a	15.41d	31.28c	35.72ab	32.37bc	18.39d
Ca [g kg^{-1}(d.m.)]		5.26d	10.14a	8.67b	6.04c	8.77b	10.14a
Mg [g kg^{-1}(d.m.)]		8.21a	6.69f	7.35d	7.81b	7.59c	7.07e
Fe [mg kg^{-1}(d.m.)]		366.11a	208.44c	350.11a	366.85a	351.40a	229.12b
Zn [mg kg^{-1}(d.m.)]		92.41ab	65.86d	85.88bc	93.87a	84.62c	72.23d
Cu [mg kg^{-1}(d.m.)]		38.23a	20.32d	30.53bc	30.92b	28.91bc	23.20cd
Pb [mg kg^{-1}(d.m.)]	roots	0.00e	1.49d	1.58c	1.79a	1.66b	1.54cd
K [g kg^{-1}(d.m.)]		50.37a	37.85b	24.00de	19.78e	27.94cd	28.78c
Ca [g kg^{-1}(d.m.)]		1.32c	2.82ab	2.51ab	1.74c	2.38b	2.87a
Mg [g kg^{-1}(d.m.)]		6.62b	6.45c	6.77a	6.69ab	6.63b	6.66ab
Fe [mg kg^{-1}(d.m.)]		405.05c	178.56e	447.96b	472.45a	452.89ab	226.53d
Zn [mg kg^{-1}(d.m.)]		70.27a	43.59c	60.33b	65.43ab	59.77b	48.38c
Cu [mg kg^{-1}(d.m.)]		68.33c	76.69c	92.58b	113.19a	90.92b	71.14c

50, 100, and 200 μM SNP was added, the inhibiion of the SOD activity was alleviated, in particular in the case of 100 μM SNP. Similar patterns were found for the activities of POD and APX (Table 2). However, the CAT activity in both shoots and roots of the Pb-treated plants increased significantly compared to CK but less after the additions of 50, 100, and 200 μM SNP. The application of 400 μM SNP further increased the CAT activity in shoots and roots (Table 2).

The Pb exposure increased the Pb content in shoots and roots significantly and most Pb was located in roots. The 100 μM SNP treatment led to the lowest Pb content in shoots and the highest Pb content in roots, but the application of 400 μM SNP did not decrease the Pb accumulation in shoots and increased the Pb accumulation in roots.

In shoots, the Pb exposure decreased the K, Mg, Fe, Zn, and Cu content by 59.50, 18.51, 43.07, 28.73, and 46.85 %, whereas increased the Ca content by 92.78 % compared to CK. The applications of 50, 100, and 200 μM SNP alleviated these changes. The positive effect of the SNP treatments was in general: Pb+SNP2 > Pb+SNP3 > Pb+SNP1 > Pb+SNP4. In roots, the Pb stress inhibited the uptake of K, Mg, Fe, and Zn significantly and had no obvious effect on the Cu uptake. The applications of 50, 100, and 200 μM SNP inhibited the Pb-increased Ca content, whereas the application of 400 μM SNP had no effect on the Pb-induced uptake of Ca. The SNP supplementation increased the uptake of Fe, Zn, and Cu, especially at the 100 μM concentration.

Discussion

Nitric oxide has achieved huge attention in recent years mainly due to its function in plant growth and development under biotic and abiotic stresses. NO counteracts metal toxicity in soybean (Orozco-Cardenas and Ryan 2002), sunflower (Uchida *et al.* 2002), and rice (Zhang *et al.* 2011). However, the effects of NO could be either protective or toxic, depending on its concentration and situation (Wink and Mitchell 1998, Beligni and Lamattina 1999, Lamattina *et al.* 2003, Tu *et al.* 2003, Saxena and Shekhawat 2013). In this experiment, different concentrations of SNP were applied to the growth medium with Pb. We investigated the physiological characteristics of ryegrass under the Pb stress together with different concentrations of SNP, aiming at searching for a proper concentration of SNP for alleviating Pb toxicity.

In the present experiment, the growth of the ryegrass seedlings was delayed significantly in the presence of Pb compared to CK. This result is in agreement with Islam *et al.* (2008) who indicated that an exposure to Pb cause an inhibition of leaf growth of *Elsholtzia argyi*. However, the inhibitory effects were alleviated by low concentrations of SNP (50, 100, and 200 μM) significantly, but not by the higher concentration of SNP (400 μM) (Table 1). Therefore, the alleviation of inhibitory growth by SNP may depend on improving Chl content (Table 2), counteracting oxidative damage (Table 2), and increasing the uptake of mineral content (Table 3) under Pb stress. These results are in conformity with the observations of alleviation of Cd toxicity in ryegrass seedlings (Wang *et al.* 2013a) and Cu toxicity (Dong *et al.* 2014) by SNP.

Notable reductions of Chl content, P_N, and E were detected in the ryegrass seedlings exposed to Pb (Table 2). Several authors also reported a decreased chlorophyll content in leaves of Pb-treated plants (Burzynski 1987, Yan *et al.* 1998, Pal *et al.* 2013); such kind of chlorosis can result from Fe deficiency and from the inhibition of chlorophyll synthesis (Ahamed *et al.* 2007). Pb causes an impaired uptake of essential elements, such as Mg and Fe by plants (Burzynski 1987). However, the addition of lower concentrations of SNP increased the Chl content under the Pb stress, but the higher concentration did not. These results are in agreement with the effect of SNP in ryegrass seedlings grown under a Cd stress (Wang *et al.* 2013a). In this experiment, 50, and 200 μM SNP, and especially 100 μM SNP increased the Fe content in both shoots and roots. In addition, NO might effectively reduce the accumulation of ROS generated during the stress, thus alleviating the oxidative effects of ROS on the chlorophyll content.

Pb has been reported to cause oxidative damage due to the production of ROS (Verma and Dubey 2003) including superoxide radical ($O_2^{\bullet -}$) and H_2O_2, which was confirmed in our study. The MDA content was measured as index of lipid peroxidation. Its increased content in plants grown in the nutrient solution with Pb confirmed the Pb-induced oxidative stress. Similarly, Ahamed *et al.* (2007) demonstrated that Pb toxicity may generate ROS, disrupt tissue oxidant/antioxidant balance, and alter lipid metabolism. However, the SNP-treated plants counteracted oxidative damage due to the decreased production rate of $O_2^{\cdot -}$ and the accumulation of H_2O_2 (Table 2) leading to a less accumulation of MDA (Table 2). This could be due to enhancing activities of H_2O_2-scavenging enzymes. Previously, it has been found that NO can promote cellular antioxidant systems under heavy metal stress (Kopyra and Gwozdz 2003, Laspina *et al.* 2005, Xiao *et al.* 2007).

In the present study, the excess of Pb reduced the activities of SOD, POD, and APX and increased the activity of CAT (Table 2). These results are in conformity with Islam *et al.* (2008) who reported that a Pb treatment decreases the activities of SOD and POD in *Elsholtzia argyi*, but increases the activity of CAT. The increase in CAT activity can be an adaptive mechanism to maintain a low content of H_2O_2 (Reddy *et al.* 2005, Tao *et al.* 2012).

However, when SNP was applied to the nutrient solution, the SOD, POD and APX activities increased, whereas the CAT activity decreased (Table 2). The stimulation of some antioxidative enzymes might suggest that NO participated in the stabilization of cell membranes. But the activities of the enzymes did not change significantly when the higher concentrations of SNP were applied. The difference indicates that the influence of NO on the antioxidant enzymes under the Pb stress was very complex and may be related to the plant species, tissues, and treatment time.

Plant root is a main tissue directly contacting with heavy-metal ions in soil. A majority of Pb accumulated in roots and a small amount in shoots (Table 3) may imply that the translocation of Pb from roots to shoots is restricted by internal barriers to defend the above-ground part. Generally, it has been thought that Pb accumulation in the cell wall and vacuoles is an adaptation that protects the plant cell cytoplasm and organelles from Pb toxicity (Phang *et al.* 2011b). The addition of low concentrations of SNP effectively increased the Pb accumulation in roots and decreased its content in shoots (Table 3), which indicates that NO inhibited the translocation of Pb from roots to shoots, thereby contributing to improve the resistance to the Pb stress and explaining the ameliorating effect of NO on the growth. This is in agreement with the participation of NO in resistance of plants to different heavy metals (Hsu and Kao 2004, Hu *et al.* 2007, Dong *et al.* 2014, Wang *et al.* 2013a).

The beneficial effect of SNP on plant growth could also be attributed to the maintenance of optimal mineral nutrition. In the ryegrass plants, Pb toxicity led to a deficiency of K, Mg, Fe, Zn, and Cu in shoots, and K, Mg, Fe, and Zn in roots (Table 3), disturbing intracellular ion homeostasis and exerting a toxic effect on plants, which may be a cause of growth inhibition. These results demonstrate that Pb interfered not only with nutrient uptake but also with nutrient distribution into the different plant parts. However, the SNP treatment was found to promote the uptake and translocation of these mineral elements (Table 3). It is well known that Zn is required for the synthesis of auxin (IAA), and exogenous NO promotes Zn uptake, which might account for the promotion of plant growth under Pb stress. In addition, Fe is involved in the chlorophyll synthesis, thylakoid synthesis, and chloroplast development, so the deficiency of Fe led to a reduction of the chlorophyll content (Table 2) and finally caused chlorosis. Moreover, H^+-ATPase in the plasma membrane plays an important role in the transport of multiple ions (Palmgren and Harper, 1999), and SNP may induce H^+-ATPase activity, which might be responsible for NO increased absorptions of K, Mg, Fe, Zn, and Cu under Pb toxicity.

In conclusions, the low concentrations of SNP might attenuate Pb toxicity in the ryegrass plants exposed to the Pb stress, but the high concentration of SNP did not alleviate Pb toxicity. The mechanism of increased Pb tolerance by exogenous NO at low concentrations included the regulation of chlorophyll content and photosynthesis, the improvement of antioxidant system, maintenance of intracellular ion equilibrium and the reduction of Pb translocation from roots to shoots.

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