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Exogenous spermidine enhanced Pb tolerance in *Salix matsudana* by promoting Pb accumulation in roots and spermidine, nitric oxide, and antioxidant system levels in leaves

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ABSTRACT

The protective role of spermidine addition in media to *Salix matsudana* exposed to Pb was investigated by a hydroponic experiment in this study. The application of spermidine enhanced the biomass of *S. matsudana* exposed to Pb. Pb induced an increase in Pb levels in all organs of *S. matsudana* with the order of roots (maximum of 27616.0 mg/kg DW) > cuttings > twigs > leaves. Exogenous spermidine promoted the accumulation of Pb in the roots, but inhibited the Pb translocation to the shoots and thus the Pb content in the twigs and leaves. The Pb-induced decrease in the concentrations of spermidine, soluble protein, and nitric oxide was elevated by the application of spermidine in the leaves of *S. matsudana*. Exogenous spermidine could also effectively up-regulate the antioxidative capacity and reduced the accumulation of superoxide anion, hydrogen peroxide, malondialdehyde, and putrescine. The results suggested that *S. matsudana* could accumulate a high level of Pb in the roots, and exogenous spermidine could enhance *S. matsudana* tolerance to Pb by the synergistic promotion of Pb accumulation in the roots and the levels of spermidine, nitric oxide, and antioxidants in the leaves.

1. Introduction

The phytoremediation of heavy metal polluted environment has received much concern due to a green and effective technique (Gao et al., 2015; Harguinteguy et al., 2015). However the remediation efficiency reckons heavily on the full understanding of the tolerance mechanisms of plants to heavy metals pollutants, including the accumulation of heavy metals and the antioxidative capacity of plants. The excessive accumulation of reactive oxygen species (ROS) induced by heavy metal contamination can cause the disorder of antioxidant system, and thus the lipid peroxidation, degradation of protein, adverse effects on plant growth, and even the death of plants (Zhang et al., 2013; Xu et al., 2015). However, plants are equipped with various defense mechanisms to cope with heavy metal stress during their evolution process. Metal accumulation in non-sensitive tissues is one of the metal detoxification pathways in plants (Sun et al., 2016). Antioxidant system consisting of both enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), ascorbate peroxidase (APX), and glutathione reductase (GR), and low molecular weight non-enzyme antioxidants, like reductive ascorbate (ASA) and glutathione (GSH), can also protect plants against metal-induced oxidative stress to some extent (Xu et al., 2015). SOD catalyzes the conversion of $O_2^{\bullet -}$ to O_2 and H_2O_2 by dismutation reaction. CAT, a key enzyme in C3 plants, can catalyze high level of H_2O_2 to H_2O without needing any substrates. ASA-GSH cycle is an important component of antioxidative system in plants. With the catalysis of GPX and APX, GSH and ASA can react with H_2O_2 to form H_2O and oxidative glutathione (GSSG) and monodehydroascorbate acid (MDHA) and dehydroascorbate acid (DHA), respectively. GSH and ASA can be formed by the reaction between oxidative gluthaione and ascorbate acid under the catalysis of GR and dehydroascorbate

Abbreviations: APX, ascorbate peroxidase; ASA, reductive ascorbate; BAFs, bioaccumulation factors; CAT, catalase; GPX, glutathione peroxidase; GR, glutathione reductase; GSH, reductive glutathione; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; NO, nitric oxide; Put, putrescine; $O_2^{\bullet -}$, superoxide anion; SOD, superoxide dismutase; Spm, sperdimine; TFs, translocation factors; TIs, tolerance indexes.

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reductase (DHAR), respectively. GR catalyzes the reduction of GSSH to GSH and plays an important role in maintaining the homeostasis between GSH and GSSG (Wu et al., 2015).

Recently, considerable attention has been focused on the regulation behavior of exogenous polyamines (PAs) in plants exposed to heavy metal stress (Wang et al., 2007; Choudhary et al., 2012a, 2012b; Liu et al., 2015; Nahar et al., 2016). PAs are low-molecular-weight aliphatic amines existing in almost all organisms and have been proven to participate in specific roles in plants, including the regulation of DNA replication, cell division, organ development, fruit ripening, leaf senescence, and responses to abiotic and biotic stresses. The three main PAs, putrescine (Put), spermidine (Spd), and spermine (Spm), are positively charged at physiological pH value and can affect physiological activities by strongly binding to anionic sites in vitro, including membrane phospholipids, proteins, and nucleic acids. Moreover, PAs are easily induced in response to oxidative damage, and the biosynthesis and degradation of PAs are of significant importance in maintaining the normal growth of plants under environmental stresses (Kusano et al., 2008). Put has been suggested as a physiological marker of stress situations in plants, and the antioxidative properties of Spd and Spm responding to heavy metal stress have been reported in wheat (Groppa et al., 2008), rice (Hsu and Kao, 2007), pear (Wen et al., 2011), *Malus hupehensis* (Zhao and Yang, 2008), and *Raphanus sativus* (Choudhary et al., 2012a, 2012b).

However, how PAs elevate plant tolerance to heavy metal pollutants is still a challenge and requires more research to reveal. Some willow plants might be suitable candidates for phytoremediation species due to their high genetic variability, biomass production, metal tolerance, deep-rooting ability, and landscape restoration (Utmazian et al., 2007; Salam et al., 2016). Although the Cd accumulation in *Salix matsudana* has been investigated recently (Wu et al., 2016), currently, the research of *S. matsudana* tolerance to Pb and especially the mitigation of the exogenous application of Spd on Pb stress is rare. Exogenous Spd is presumed to alleviate the toxicity of Pb to *S. matsudana* in this work. In a quest for the tolerance mechanisms of Spd to Pb in *S. matsudana*, this study aimed to measure the biomass of *S. matsudana*, analyze the accumulation and translocation of Pb, and assay the oxidative damage and antioxidative ability.

2. Materials and methods

2.1. Plant material and pretreatment

One-year-old branches of *S. matsudana* were collected from Forestry Center of Dongting Lake Area, Hunan Province, China. Leaves were removed, and the similar cuttings of 15 cm long were fixed with styrofoam plates and placed in a big plastic container. All the cuttings were cultivated in 10% Hoagland solution under natural sunlight. After 7 weeks of preincubation, the cuttings with uniform twigs were selected for further experiments. Every three seedlings were cultivated in a 4 L plastic pot. Treatments based on 25% Hoagland solution were as follows: (1) Control: 25% Hoagland solution; (2) Spd: 0.1 mmol/L Spd; (3) Pb 25: 25 mg/L $PbCl_2$; (4) Pb 25 + Spd: 25 mg/L $PbCl_2$ + 0.1 mmol/L Spd; (5) Pb 50: 50 mg/L $PbCl_2$; and (6) Pb 50 + Spd: 50 mg/L $PbCl_2$ + 0.1 mmol/L Spd. All the treatments were performed in triplicate, and all the solutions were refreshed once a week. In order to prevent the precipitation reaction between PO_4^{3-} and Pb^{2+} ions, KH_2PO_4 was not added into the Hoagland solution, but sprayed (0.025% KH_2PO_4 containing 0.1% Tween-80, v/v) on leaves twice a day at 07:00 and 19:00, respectively. Pb and Spd concentrations used in this experiment were selected based on our preliminary experiments and the concentrations reported in Zheng et al. (2012) and Harguinteguy et al. (2015).

After 3 days of treatment, fresh leaves were picked for the evaluation of the levels of oxidative stress, antioxidants, NO, and PAs. The whole plants were harvested after Pb treatment for 23 days, and the fresh weight (FW) and root length were recorded. Harvested fresh plants were separated into leaves, twigs, cuttings, and roots, immersed in 50 mmol/L of Na_2EDTA for 5 min to resolve the surface-bound Pb, and rinsed with distilled water. All the samples were dried to a constant dry weight (DW) at 75 °C for 4 days, ground to pass a sieve with 20 mesh, and stored for further analysis.

2.2. Extraction and assay of oxidants, antioxidants, and soluble protein of leaves

All the extraction and analysis of $O_2^{\bullet -}$, H_2O_2, SOD, CAT, GPX, GR, GSH, NO, and soluble protein adopted the methods described by Nanjing Jiancheng Biological Engineering Institute (2015) using multiskan (Thermo Scientific Multiskan GO 1510, 96 plates, America).

MDA and ASA were extracted as followings: 0.5000 g fresh leaves were ground with liquid nitrogen and 5 mL of pre-cold trichloroacetic acid (10% v/v), centrifuged at 10000*g* at 4 °C for 15 min, and the supernatant was kept at −70 °C. The extraction of APX was the same as that of GPX. The levels of ASA and APX were measured according to ASA-Fe^{3+} redox reaction as described by Chen and Wang (2006) with ultraviolet spectrophotometer (UV-1700, Shimadzu, Japan). The MDA content was analyzed by the method of thiobarbituric acid (TBA) reaction described in Heath and Parker (1968) by an ultraviolet spectrophotometer (UV-1700, Shimadzu, Japan) at 450, 532, and 600 nm, respectively.

2.3. PAs extraction and analysis

Free PAs were extracted according to the modified method described by Flores and Galston (1982). Fresh leaves of 1.0000 g were broken by liquid nitrogen and homogenized with 5 mL of 5% (v/v) pre-cold perchloric acid, kept on ice for 1 h, and centrifuged at 15 000*g* for 30 min.

500 μL supernate was put in a 10 mL plastic centrifuge tube added with 10 μL benzoyl chloride and 1 mL of 2 mol/L NaOH. After vortexing for 20 s and incubation at 37 °C for 20 min, 2 mL saturated NaCl solution and 2 mL ether were added as extraction reagents and then centrifuged at 1500*g* for 5 min. 1 mL of the ether phase was collected in a 2 mL centrifuge tube, vacuum dried, and redissolved by 200 μL methanol (HPLC grade). Standard Put, Spd (Sigma) with concentration of 10, 50, 100, 150, 200, and 250 nmol/L, respectively, were treated in a similar way. The benzoylated samples were stored at −78 °C.

Samples were ultrafiltered by 0.45 μm filter membranes before analysis by high performance liquid chromatograph (Aglient 1200, America). Chromatographic condition: C18 reverse phase column (4 × 250 mm, 5 μm particle size); column temperature: 30 °C; mobile phase: 64% methanol + 36% redistilled water; flow rate: 0.7 mL/min; detection wavelength: 230 nm.

2.4. Measurement of Pb concentrations

0.5000 g dry weight (DW) samples were digested with HNO_3—$HClO_4$ (3:1, v/v) and properly diluted to a constant volume. The concentrations of Pb (mg/kg DW) in different plant organs were analyzed by inductively coupled plasma atomic emission spectrometry (Thermo iCAP 6000, USA).

Tolerance indexes (TIs) were calculated as the ratio of dry weight of plants treated with Pb to dry weight in the control plant. Bioaccumulation factors (BAFs, expressed as L/kg) were equal to the Pb