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Effects of Pb on the oxidative stress and antioxidant response in a Pb bioaccumulator plant *Vallisneria natans*

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ABSTRACT

The effects of Pb on photosynthetic pigments, oxidative stress and antioxidant response were assayed using biochemical and histochemical methods in leaves of *Vallisneria natans* (Lour.) Hara treated with 0–100 μM Pb^{2+} for 0–6 d. The Pb content increased with the increase of exposure duration and a highest Pb uptake value (about 9.4 mg Pb g^{-1} dry weight) was obtained at 6 d. Pb induced the accumulation of H_2O_2 and O_2^-. The increase of malondialdehyde content and the decrease of total chlorophyll and carotenoids were detected in *V. natans* under Pb stress. Activities of NAD(P)H oxidase, guaiacol peroxidase, glutathione reductase and ascorbate peroxidase increased at 75 μM Pb^{2+} for 2–6 days, while activities of superoxide dismutase and catalase and the content of ascorbic acid increased within two days in plants exposed to 75 μM Pb^{2+} and decreased thereafter. The Pb uptake and accumulation mechanism were discussed.

1. Introduction

Lead (Pb) is recalcitrant in the environment and can cause damage to biota (Zeng et al., 2006). Unfortunately, excessive amounts of Pb are released continually into the environment due to human activities including mining and smelting of Pb-ores, storage battery industries, automobile exhausts and finishing operations (Eick et al., 1999). Pb can be accumulated by plants although it is not necessary for plants. Therefore, phytoremediation using plants that remove metals from the environment or reduce their toxicity has made rapid progress in the clean-up of metal-polluted areas in a cost-effective and environmentally friendly manner (Lee et al., 2003). However, Pb phytotoxicity is one of the major limitations for the application of phytoremediation. For example, more than 30 μg g^{-1} dry weight of Pb in tissues is considered toxic to plants (Prasad, 1999). Consequently, studies on the Pb uptake and endurance mechanisms of plants are needed for the requirements in the improvement of Pb accumulative capacity through genetic manipulation.

Pb has adverse effects on plants. For example, Pb toxicity can result in changes in the catalytic activities of various enzymes, the increases in membrane permeability, the decreases in the content of photosynthetic pigments and the disturbance of mineral nutrition balance (Sharma and Dubey, 2005). These result in the inhibition on biomass production and plant growth. In addition, Pb stress can alter the expression of genes. For example over 1310 genes in *Arabidopsis* seedlings have been shown to be responsive to Pb treatment (Liu et al., 2009). Moreover, Pb-induced excess reactive oxygen species (ROS) has been detected in plants (Reddy et al., 2005), although Pb is a non-redox-metal and cannot generate reactive oxygen species (ROS) through Haber–Weiss reactions. ROS including superoxide radical (O_2^-), hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2) are toxic to plant cell, because they can rapidly attack nucleic acid, proteins and membrane lipids (Apel and Hirt, 2004; Gopal and Rizvi, 2008).

ROS are continuously produced in metabolically active cells, but their cellular concentrations are normally strictly regulated by the action of antioxidant systems that help to maintain a balance between the production and scavenging of ROS (Apel and Hirt, 2004). ROS can also serve as signal molecules for the induction of the antioxidant response (Apel and Hirt, 2004). These include the enhanced activity of antioxidant enzymes such as superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), glutathione reductase (GR; EC 1.6.4.2), ascorbate peroxidase (APX; EC 1.11.1.11) and guaiacol peroxidase (POD; EC 1.11.1.7), and the increase of low-molecular mass antioxidants including carotenoids, non-protein thiols (NP-SH) and ascorbate (Blikhina et al., 2003; Gupta et al., 2010; Singh et al., 2010).

Submerged plants play important roles in maintaining the health of aquatic ecosystems through the accumulation and/or decomposition of toxins (Rai et al., 1995). *Vallisneria natans* (Lour.) Hara is a fully submerged, perennial, rooted plant and is widely distributed in freshwater in China. In previous report, we found that the leaves of *V. natans* accumulated high levels of Pb and that

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Pb stress altered the nutrient uptake and induced secondary metabolism (Wang et al., 2011). The aims of this study were to investigate (i) the effects of Pb on the alterations in the level of photosynthetic pigments, malondialdehyde (MDA) and ROS, (ii) the Pb stress on the activities of antioxidant enzymes and (iii) the relationship of Pb with antioxidant response in leaves of *V. natans* treated with 0–100 μM Pb^{2+} for 0–6 d.

2. Materials and methods

2.1. Plant cultures and treatments

V. natans seedlings at similar stages of growth were purchased from a market in Hangzhou, Zhejiang Province, China. Plants were acclimatized for at least two weeks under laboratory conditions (100 μmol m^{-2} s^{-1} light with 14 h photoperiod at 25 ± 2 °C) in adapted Hoagland's solution (53 μM KNO_3, 78 μM $Ca(NO_3)_2$, 10 μM KH_2PO_4, 32.2 μM $MgSO_4$, 3.7 μM H_3BO_3, 0.8 μM $ZnSO_4$, 0.03 μM $CuSO_4$, 0.01 μM Na_2MoO_4, 0.9 μM $MnCl_2$, 5 μM $FeSO_4$ and 5.5 μM Na_2EDTA.) and irradiance of 100 μE m^2 s^{-1}. The bottom of the 70 L tanks was covered with a 5 cm layer of quartz sand, and roots of *V. natans* were embedded in the sand. For duration-dependent experiments, acclimatized plants were grown in the primary medium alone or in the same medium supplemented with 75 μM Pb for 0 (control), 0.5, 1, 2, 4 and 6 d. For concentration-dependent experiments, plants were grown in adapted Hoagland's solution containing 0 (control), 10, 25, 50, 75 and 100 μM Pb for 4 d. Pb stock solutions were prepared with $Pb(NO_3)_2$. The NO_3^- concentration was normalized by diluting the mixture of NO_3^- salt (4:1:1 of HNO_3, KNO_3 and $NaNO_3$) among all culture solutions. Experiments were done in quadruple and each replicate contained 35 plants of comparable sizes. pH values were monitored twice everyday and maintained at 6.5 in all treatments.

All culture solutions were refreshed in static way every 2 d throughout the acclimation and experimental periods. After the experimental treatments, leaves were harvested, washed with distilled water, blotted, well mixed and frozen immediately in liquid nitrogen for storage at −80 °C.

2.2. Determination of content of photosynthetic pigments and lipid peroxidation

Photosynthetic pigments were analyzed using standard spectrophotometric methods as described previously (Wang et al., 2009). The level of lipid peroxidation in plant leaves was determined by estimation of the MDA content according to the method of Hodges et al. (1999).

2.3. Histochemical detection of O_2^- and H_2O_2

Histochemical detection of O_2^- and H_2O_2 was performed according to the method described previously (Wang et al., 2010). Plants were washed with ddH_2O, and then grown in 10 mM Na-citrate buffer (pH 6.0) containing 6 mM nitroblue tetrazolium (NBT) or in 1percent 3,3′-diaminobenzidine (DAB, pH 3.8) for 8 h at 25 °C. After the treatments, the leaves were immersed in boiling ethanol (96percent) for 10 min. NBT and DAB can react with O^{2-} and H_2O_2 forming the insoluble dark blue insoluble formazan compound and deep brown polymerization, respectively.

2.4. Determination of catalytic activities of antioxidant enzymes

A 0.5 g sample was ground in liquid N_2 and homogenized with 3 mL of 50 mM potassium phosphate buffer (PBS, pH 7.0) containing 1 mM EDTA and 1percent polyvinylpyrrolidone, with the addition of 5 mM ascorbate for the APX assay. The homogenate was centrifuged at 15000*g* for 20 min at 4 °C, and the supernatant used for the enzyme assays. Protein contents were determined following the methods described by Bradford (Bradford, 1976), using bovine serum albumin as standard.

SOD activity was detected based on the method described by Giannoplities and Ries (1977). One unit of SOD activity was defined as the amount of enzyme required to cause a 50percent inhibition of the rate of NBT as monitored spectrophotometrically at 560 nm. POD activity was determined in terms of oxidation of guaiacol by H_2O_2 (Upadhyaya et al., 1985) as measured by the increase in absorbance at 420 nm (E=26.6 mM^{-1} cm^{-1}). APX activity was determined essentially as described by Nakano and Asada (1981) but with a few modifications. One unit of APX activity (ε=2.8 mM^{-1} cm^{-1}) was defined as the amount required to decompose 1 μmol ascorbic acid oxidized min^{-1} mg^{-1} protein. GR activity was assayed by following the reduction of nicotinamide adenine dinucleotide phosphate (NADPH) reflected as a change in the absorbance at 340 nm, as described by Carlberg and Mannervik (1985). One unit of GR activity (ε=6.22 mM^{-1} cm^{-1}) was defined as the amount required to decompose 1 nmol NADPH min^{-1} mg^{-1} protein.

Table 1
Alteration of Pb concentration (μM) in Pb^{2+} spiked solutions at 0 h and 2 d.

The desired Pb concentration	0 h	2 d
	Mean ± std. dev.	Mean ± std. dev.
0	NT	NT
10	11.29 ± 1.04	7.11 ± 2.14
25	25.94 ± 2.81	17.12 ± 3.44
50	51.11 ± 4.98	41.79 ± 5.66
75	74.97 ± 6.07	61.75 ± 8.77
100	95.21 ± 8.42	81.24 ± 9.21

2.5. Determination of low-molecular antioxidants

For determining the non-protein thiols' (NP-SH) content, 0.3 g of a powdered leaf sample was resuspended in 1.5 mL of ice-cold 5percent (w/v) sulfosalicylic acid solution. The mixture was centrifuged at 10,000*g* at 4 °C for 30 min, and the supernatant was collected and immediately assayed using Ellman's reagent (1959). Ascorbic acid (AsA) and total ascorbate (AsA+dehydroascorbate (DHA)) were determined according to the method of Hodges et al. (1996). The content of glutathione was determined by the method of (Gupta et al., 2010) using reduced glutathione (GSH) as a standard.

2.6. Determination of Pb content in cultural solution and plants

The concentrations of Pb in leaves of *V. natans* were determined according to the method described previously (Wang et al., 2009). After being washed with a 10 mM EDTA solution and three times with ddH_2O in sequence, the harvested samples were immediately blotted and oven-dried at 105 °C for 20 min and then kept at 80 °C for 72 h. The dried material was ground. Of the powdered sample, 0.2 g was digested with 10 mL of 10:1 HNO_3–$HClO_4$ solution and heated at 100–200 °C until near dryness. The cooled residue was dissolved in 5 mL of 5percent HNO_3 and ddH_2O was added up to 25 mL of total volume. The contents of Pb in cultural solution (Table 1) and extracts from samples were determined by inductively-coupled plasma atomic emission (Leeman-LABS, Prodigy, USA).

2.7. Gel electrophoresis

Isoenzymes of SOD and POD were identified by separation on discontinuous polyacrylamide gels (stacking gel 5percent and separating gel 10percent) under non-denaturing conditions. After electrophoresis, activity of SOD and POD was estimated by zymogram staining according to the method described by Ádám et al. (1995). Gel electrophoresis was performed in triplicate and similar results were obtained.

2.8. Statistical analysis

Sample variability is defined as the standard error of the mean values of replicates (n=4) in figures. Data of duration-dependent experiments were analyzed using independent samples by Student's t-test in order to examine the differences between treatments and controls ($p < 0.05$). Data from concentration-dependent experiments were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc analysis at $p < 0.05$. The correlation coefficients (R) between the Pb content and other parameters were calculated using the data from duration-dependent experiments. The values of R have been given within text at relevant places ($^{\Psi\Psi}$ indicates the $p < 0.01$; $^{\Psi}$ indicates the $p < 0.05$).

3. Results

3.1. Pb and ROS accumulation in leaves of plants.

The Pb decreased obviously in cultural solutions at 2 d compared with that at 0 h. In *V. natans* plants exposed to 75 μM Pb for 12 h, 1, 2, 4 and 6 d there was a duration-dependent increase in Pb accumulation with 3.1, 4.9, 6.8, 8.4 and 9.4 mg Pb g^{-1} DW, respectively (Fig. 1A).

NBT and DAB were, respectively, used to localize O_2^- (indicated by the dark blue color) and H_2O_2 (indicated by the deep brown color) in *V. natans* leaves. Fig. 1B–E illustrate that there was an increase of O_2^- and H_2O_2 levels in leaves exposed to 75 μM Pb for 4 d compared with their control.