Journal of Hazardous Materials 177 (2010) 437–444

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

The detoxification of lead in *Sedum alfredii* H. is not related to phytochelatins but the glutathione

D.K. Gupta[a,*], H.G. Huang[a], X.E. Yang[a], B.H.N. Razafindrabe[c], M. Inouhe[b]

[a] Ministry of Education Key Laboratory of Environmental Remediation and Ecosystem Health, Zhejiang University, Hua-jian-chi Campus, Hangzhou 310029, China
[b] Department of Biology and Environmental Sciences, Graduate School of Science and Engineering, Ehime University, Matsuyama, Ehime 790-8577, Japan
[c] Graduate School of Environment and Information Sciences, Yokohama National University, 79-7 Tokiwadai, Hodogaya Ku, 247-8501 Yokohama, Japan

ARTICLE INFO

Article history:
Received 30 September 2009
Received in revised form 9 December 2009
Accepted 9 December 2009
Available online 21 December 2009

Keywords:
Ascorbate peroxidase
Glutathione
Lead
Phytochelatins
Superoxide dismutase
Sedum alfredii

ABSTRACT

Two ecotypes of *S. alfredii* [Pb accumulating (AE) and Pb non-accumulating (NAE)] differing in their ability in accumulating Pb were exposed to different Pb levels to evaluate the effects on plant length, photosynthetic pigments, antioxidant enzymes (SOD and APX), cysteine, non-protein thiols (NP-SH), phytochelatins (PCs) and glutathione (GSH) vis-à-vis Pb accumulation. Both ecotypes showed significant Pb accumulation in roots, however only the AE showed significant Pb accumulation in shoots. We found that both AE and NAE of *S. alfredii*-induced biosynthesis of GSH rather than phytochelatins in their tissue upon addition of even high Pb levels (200 μM). Root and shoot length were mostly affected in both ecotypes after addition of higher Pb concentrations and on longer durations, however photosynthetic pigments did not alter upon addition of any Pb treatment. Both superoxide dismutase (SOD) and ascorbate peroxidase (APX) activities of AE were higher than NAE. The levels of cysteine and NP-SH were also higher in AE than in NAE. Hence, the characteristic Pb accumulation of ecotypes differed presumably in relation to their capacity for detoxification of Pb. These results suggest that enzymatic and non-enzymatic antioxidants play a key role in the detoxification of Pb-induced toxic effects in *Sedum alfredii*. This plant can be used as an indicator species for Pb contamination.

1. Introduction

Lead (Pb) is a hazardous heavy metal pollutant that originates from various sources, which include paints, gasoline additives, Pb smelting and refining, pesticide production, etc. [1,2]. Many studies have been conducted on the use of plants for the removal of Pb from the environment in an ecological and cost-effective way. This includes phytoextraction, which is a type of phytoremediation that involves the removal of heavy metals from contaminated soils/ground water with plants that accumulate large amounts of heavy metals in their shoot part [3,4]. Heavy metals can cause serious damages-even at very low doses by replacing essential elements involved in biological reactions [5,6]. Despite the significant problems caused by Pb for agriculture, Pb tolerance mechanisms of plants are not well understood to date.

Metal detoxification and tolerance in plants can be achieved by numerous mechanisms, such as chelation by metal-binding compounds, metal deposition in vacuoles, alterations of membrane structures, and synthesis of stress metabolites [7]. Phytochelatins (PCs), a class of small thiol (SH)-rich peptides, bind heavy metals and metalloid through thiolate coordination [8,9]. Due to their ability to bind metals, PCs are generally considered to be an important cellular chelating agent. However, there is now considerable debate on the role of PCs in metal detoxification and tolerance of higher plants [10,11]; since recent studies have demonstrated that metal hyperaccumulation phenotype of the plants does not correlate with PCs [12].

Sedum alfredii H., grown in old Pb/Zn mined area of southeast China, has been identified as a Zn/Cd hyperaccumulator [13], and is also recognized as a Pb-accumulating species. It could accumulate up to 514 mg kg^{-1} and 13,922 mg kg^{-1} DW of Pb in shoot and root, respectively under hydroponic conditions [14]. Sun et al. [12,15,16] reported that they could not find PCs in the leaf, stem and root tissues of the mine population of *S. alfredii* in the presence of Cd, Zn or Pb at certain concentrations. However, Zhang et al. [17] confirmed PC formation could be induced only in the stem and root when exposed to 700 μM Pb. The main objective of our present study was to investigate, whether PCs in different plant tissues can be induced in Pb accumulating ecotype (AE) of *S. alfredii*, and also whether they can play a role in Pb detoxification and tolerance or not.

* Corresponding author. Present address: Departamento de Bioquimica, Biologia Cellular y Molicular de Plantas, Estacion Experimental Del Zaidin, CSIC, Apartado 419, C/Profesor Albareda No 1, E-18080 Granada, Spain.
Tel.: +86 571 86971907/+34 958181600x299; fax: +86 571 86971907.
E-mail address: guptadk1971@gmail.com (D.K. Gupta).

0304-3894/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jhazmat.2009.12.052

In order to validate previous outcome in more detail, we used different Pb concentrations and time durations to re-examine PC induction in *S. alfredii*. In our present study, we investigated cysteine, non-protein thiols (NP-SH), glutathione (GSH), and PC synthesis in AE and non-lead accumulating ecotype (NAE) of *S. alfredii*.

2. Materials and methods

2.1. Plant material, growth condition and treatment

The Pb accumulating ecotype (AE) of *S. alfredii* was collected from an old Pb/Zn mined area and the non-accumulating ecotype (NAE) was obtained from a tea garden of Hangzhou in Zhejiang province of China. Healthy and equal-sized shoots of *Sedum alfredii* H. were chosen and grown in 2.5 L pots for 2 weeks in distilled water for the initiation of new roots. Plants were grown in a glasshouse with natural light, day/night temperature of 30/25 °C and day/night humidity of 70/90%. The pH of nutrient medium was adjusted to 5.5 using 0.1 M NaOH or 0.1 M HCl and was continuously aerated with an aquarium pump and renewed after every fourth day during the experiment. The composition of the nutrient medium used for Pb treatment was as follows: 2000 μM KNO_3, 50 μM KCl, 500 μM $Ca(NO_3)_2{\cdot}4H_2O$, 200 μM $MgSO_4{\cdot}7H_2O$, 100 μM NH_4NO_3, 25 μM KH_2PO_4, 12 μM H_3BO_3, 2.0 μM $MnSO_4{\cdot}H_2O$, 0.5 μM $ZnSO_4{\cdot}7H_2O$, 0.2 μM $CuSO_4{\cdot}5H_2O$, 0.1 μM Na_2MoO_4, 0.1 μM $NiSO_4$, 20 μM Fe-EDTA.

After 14 d of pre-culture, the young plants (fourteen) of both populations were exposed to different concentrations of Pb (0, 5, 25, 100, 200 μM, as $Pb(NO_3)_2$) and maintained in 100% Hoagland's solution [18] in 2.5 L black plastic pots under above mentioned laboratory conditions for a period of 1, 3 and 5 d. KH_2PO_4 concentration was adjusted to 0.025 mM in order to prevent precipitation of Pb [14]. The experiment was randomly arranged with each treatment in triplicate. Pots without Pb served as control. After harvesting, plants were washed with double distilled water, blotted dry and used for the study of various parameters. All chemicals used were of analytical grade purchased from Sigma Chemical Company (USA) and Chemical Factory, Shanghai (China).

2.2. Plant growth analysis

Plants were harvested after 1, 3 and 5 d of treatment, and morphological parameters (root and shoot length) were measured by Vernier calipers; both expressed in cm plant^{-1}. After that, plants were washed thrice with distilled water and finally with de-ionized water. Roots and primary leaves were collected for enzyme analysis and 1 g of fresh samples were frozen in liquid nitrogen and stored at −80 °C.

2.3. Metal estimation

At the time of harvest, roots of intact plants were washed with distilled water for metal analysis, and immersed in 20 mM Na-EDTA for 15–20 min to remove adsorbed Pb adhering to the root surface. Then, plants were washed thrice with distilled water and finally with de-ionized water, and oven-dried at 70 °C for approximately 72 h. Pb concentration was estimated in both roots and shoots of both varieties (AE/NAE). 0.1 g of plant sample was digested with 5 ml HNO_3 and 1 ml $HClO_4$ in closed teflon vessels until a clear digest was obtained. The digested material was washed into 50 ml flask and made up to 50 ml volume using de-ionized water. Metal concentrations in plant samples were determined using Integrated Couple Plasma Mass Spectrophotometer (Agilent 7500a). The tissue Pb concentration was expressed as mg kg^{-1} DW.

2.4. Determination of total chlorophyll and carotenoid concentration

Plant material (100 mg) was ground in chilled 80% acetone in dark conditions. After centrifugation at 10,000 × *g* for 10 min at 4 °C, absorbance of the supernatant was taken at 663, 645, 510 and 480 nm. The concentration of chlorophylls was estimated by the method of [19] and that of carotenoid concentration by using the formula given by [20].

2.5. Activities of antioxidant enzymes

For enzyme assays, 0.3 g leaves were ground with 3 ml ice-cold 25 mM Hepes buffer (pH 7.8) containing 0.2 mM EDTA, 2 mM AsA and 2% PVP. The homogenate was centrifuged at 4 °C for 20 min at 12,000 × *g* and the resulting supernatants were used for determination of enzyme activity. All spectrophotometer analyses were conducted on a SHIMADZU UV-2410 PC spectrophotometer.

2.5.1. Assay of SOD activity (EC 1.15.1.1)

The activity of superoxide dismutase (SOD) was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) [21]. 3 ml reaction mixture contained 50 mM phosphate buffer (pH 7.8), 130 mM methionine, 750 μM NBT, 20 μM riboflavin, 0.1 mM EDTA and 0.1 ml of plant extract. Reaction was started by adding 20 μM riboflavin and placing the tubes under 15 W fluorescent lamps for 15 min. A complete reaction mixture without enzyme, which gave the maximal colour, served as control. Reaction was stopped by switching off the light, and the tubes were covered with a black cloth. A non-irradiated complete reaction mixture served as a blank. The absorbance was recorded at 560 nm, and one unit of SOD was defined as being present in the volume of extract that caused inhibition of the photo-reduction of NBT by 50%.

2.5.2. Assay of APX activity (EC 1.11.1.11)

Ascorbate peroxidase (APX) activity was measured according to [22]. The assay depends on the decrease in absorbance at 290 nm as ascorbate was oxidized. 2 ml reaction mixture contained 25 mM sodium phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.1 mM hydrogen peroxide, 0.1 mM EDTA and 0.1 ml supernatant. The reaction was started by adding hydrogen peroxide (H_2O_2).

2.6. Total cysteine concentration

Plant material (800 mg) was homogenized in 5% chilled perchloric acid and centrifuged at 10,000 × *g* for 10 min at 4 °C. Cysteine concentration was measured in supernatant using acid ninhydrin reagent at 560 nm according to the method of Gaitonde [23].

2.7. Non-protein thiols (NP-SH) concentration

NP-SH concentration was measured following the method of Ellman [24]. Plant material (100 mg) was homogenized in 5% sulfosalicylic acid. After centrifugation at 10,000 × *g* for 15 min at 4 °C, NP-SH concentration was measured in the supernatant by reaction with Ellman reagent and absorbance was recorded at 412 nm.

2.8. HPLC analysis of phytochelatins (PCs) and glutathione (GSH)

Plant tissues were extracted with an equal volume (1 ml g^{-1} FW) of 10% (w/v) of 5-sulfosalicylic acid (SSA) at 0 °C, as described previously [25]. The extracts were centrifuged at 10,000 × *g* for 1 min and the supernatants were kept at 0 °C for 30 min just before HPLC analysis. The separation of PCs was carried out by the post-column method of Mendum et al. [26], with some modifications