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Phytochemistry 66 (2005) 2549–2556
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Increase of glutathione in mine population of *Sedum alfredii*: A Zn hyperaccumulator and Pb accumulator

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Received 22 November 2004; received in revised form 20 April 2005
Available online 11 October 2005

Abstract

Phytochelatins (PCs) have been induced in a large range of plant species, but their role in heavy metal tolerance is unclear. *Sedum alfredii* is a new zinc (Zn) hyperaccumulator and lead (Pb) accumulator found in an old Pb/Zn mine in the Zhejiang Province of China. Until now, the mechanisms of its hyperaccumulation/accumulation and tolerance were poorly understood. The aim of this work was to investigate whether PCs were differentially produced in mine populations of *S. alfredii* compared with a non-mine control of the same species. The results showed that plants from the mine site were more tolerant to increasing Zn and Pb concentrations than those from the control site. No PCs and cysteine (Cys) were detected by pre-column derivatization with HPLC fluorescence in any tissues of two populations at any treatment, which in turn indicated they were not responsible for Zn and Pb tolerance in the mine population. Instead, Zn and Pb treatments resulted in the increase of glutathione (GSH) for both populations in a tissue-dependent manner. Significant increases were observed in leaf, stem and root tissues of plants grown on the mine site. The results suggest that GSH, rather man PCs, may be involved in Zn and Pb transport, hyperaccumulation/accumulation and tolerance in mine population of *S. alfredii*.

Keywords: *Sedum alfredii*; Glutathione; Phytochelatins; Zinc hyperaccumulator; Lead accumulator

1. Introduction

Sedum alfredii is a new zinc (Zn) hyperaccumulator and lead (Pb) accumulator, which is found at an old Pb/Zn mine in the Zhejiang Province (ZJ) of China (Yang et al., 2002; He et al., 2002, 2003). The species is a perennial herb, which grows fast with a high biomass, and can reproduce asexually (Yang et al., 2002). It is, therefore, apparently an ideal plant material to decontaminate Zn/Pb from metal-polluted soil. The use of higher plants in phytoextraction of heavy metals from polluted soil is not only based on their ability to take up, translocate, and accumulate metals, but also on mechanisms able to alleviate their toxic effects (Salt et al., 1998). Therefore, investigation of metal hyperaccumulation/accumulation and tolerance in mine populations of *S. alfredii* and its survival mechanism in a severe environment could provide important information on the utility and potential of this plant for effective soil remediation. To date, the mechanisms of Zn/Pb hyperaccumulation/accumulation and tolerance in this plant are not fully understood.

Physiological studies indicate that heavy metal tolerance is one of the prerequisites of heavy metal hyperaccumulation in plants (Raskin et al., 1997). Higher plants have acquired various mechanisms to tolerate heavy metals (Gülriz, 2002). The mechanism on which most information is available is chelation. Of potential ligands, phytochelatins (PCs) have been the most widely studied in plants (Rauser, 1999; Goldsbrough, 2000). Due to their ability to bind metals by thiolate coordination, PCs are generally

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doi:10.1016/j.phytochem.2005.08.012

considered to be important cellular chelating agents, which function as heavy metal detoxification and/or homeostasis agents (Zenk, 1996; Goldsbrough, 2000).

Several inhibitor (Grill et al., 1987; Gussarsson et al., 1996), biochemical (Kneer and Zenk, 1992) and mutant studies (Howden et al., 1995; Ha et al., 1999) showed that Cd complexation by PCs is an important detoxification mechanism in higher plants. It has been observed that naturally selected As hypertolerance in *Holcus lanatus* is related to enhanced rates of PCs and increased PC-thiol to As molar ratios in roots, suggesting that PC synthesis may be essential for hypertolerance to As (Hartley-Whitaker et al., 2001). PCs might also be required for Hg and Cu detoxification and tolerance by plants (Grill et al., 1988; Howden and Cobbett, 1992). However, until now no convincing evidence is in favor of the role of PCs in Cd, Cu and Zn detoxification system in naturally selected metal-tolerant plants. *Silene vulgaris* has been widely used as a metal tolerant-plant in many studies of elemental pollution and has served as a model system for studies on PCs as a general detoxifying agent. Most results revealed that metal tolerance of this plant does not rely on differential phytochelatin production (De Knecht et al., 1992, 1994, 1995; Schat and Kalff, 1992; Harmens et al., 1993; De Vos et al., 1992). It has also been concluded that PCs are not responsible for Cd/Zn and As hyperaccumulation and detoxification in a Cd/Zn hyperaccumulator *Thlaspi caerulescens* (Ebbs et al., 2002; Schat et al., 2002), and in an As hyperaccumulator *Pteris vittata* (Zhao et al., 2003).

Although Cd is a more effective inducer of PC synthesis than Zn in higher plants (Zenk, 1996), it was unexpectedly observed that the levels of PC synthesis in a marine green alga, *Dunaliella tertiolecta*, treated with Zn are significantly higher than Cd-treated cells (Hirata et al., 2001). The pretreatment of the alga, *D. tertiolecta* with Zn enhanced the tolerance toward toxic heavy metals such as Cd, Hg, Cu and Pb by Zn-induced PC synthesis (Tsuji et al., 2002). Scarano and Morelli (2002) provided the first evidence of the separation of natural Pb-PCs complexes in the marine diatom *Phaeodactylum tricornutum*. These new findings strengthen the possibility of PCs in metal tolerance.

In view of the common occurrence of PCs in fungi, plants, and some animals (Vatamaniuk et al., 2001), it remained to be determined if PCs could be induced in mine populations of *S. alfredii* in response to Zn and Pb. Given that different detoxification mechanisms exist among plant species and the uncertainty of the role of PCs in metal tolerance, the major objective of the present study is to assess whether PCs can be responsible for in Zn and Pb detoxification and tolerance in this plant.

2. Results

2.1. Metal tolerance by plant

Compared with plants from the GD control ('clean') site, fresh weights of leaf, stem and root of plants from the ZJ mine site were largely unaffected by increasing Zn and Pb in treatment solutions, although a slight decrease at higher concentrations of Zn and Pb was observed (Figs. 1(a) and 2(a)). Under appropriate concentrations (100–400 μM), Zn stimulated the growth of this plant, as manifested by increasing fresh weights of leaf, stem and root tissues. However, Zn and Pb treatments showed a significantly ($P < 0.01$) inhibitory effect on the growth of plants from the GD control site (Figs. 1(b) and 2(b)). Fresh weights of leaf, stem and root tissues were decreased by 17–45%, 16–48% and 30–78%, respectively, within the range of Zn concentrations investigated. Similarly, increasing Pb exposure led to greater reduction of each of the three tissue parts of the GD control plants.

2.2. Metal accumulation by plant

Concentrations of Zn and Pb were calculated based on tissue fresh weights, where it was shown that concentrations of Zn and Pb in leaf, stem and root of plants both from the mine site and control site increased with an increase of Zn and Pb in treatment solutions (Tables 1 and 2). Compared with those of the GD control plants, most Zn (>60%) and a substantial amount of Pb accumulated in leaf and stem tissues of the ZJ mine plants, indicating that too much Zn and Pb in mine populations of *S. alfredii* were mobile and easily transported to the shoots.

2.3. Relationship between metal accumulation and non-protein thiols

No PCs and Cys were detected in leaf, stem and root tissues of plants from both the mine and control sites at any Zn and Pb treatment using HPLC fluorescence analysis. In-

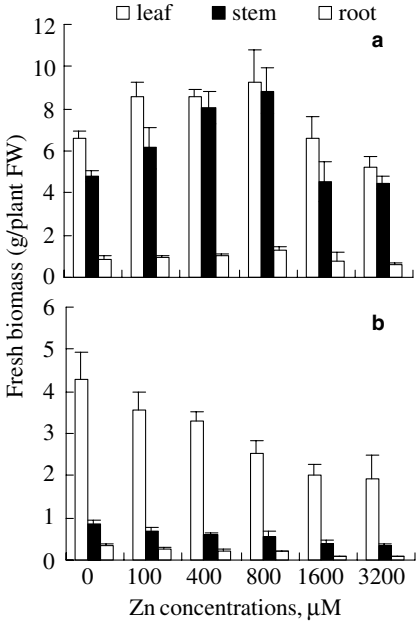

Fig. 1. Fresh weights of leaf, stem and root of the ZJ mine plants (a) and the GD control plants (b) of *Sedum alfredii* exposed to a range of Zn concentrations for 7 d (means ± SD, $n = 3$).

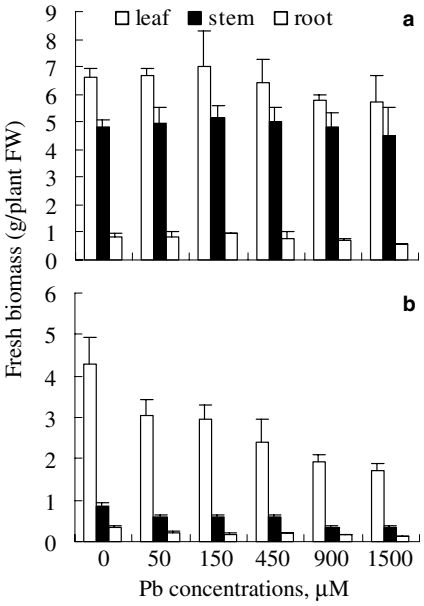

Fig. 2. Fresh weights of leaf, stem and root of the ZJ mine plants (a) and the GD control plants (b) of *Sedum alfredii* exposed to a range of Pb concentrations for 7 d (means ± SD, $n = 3$).

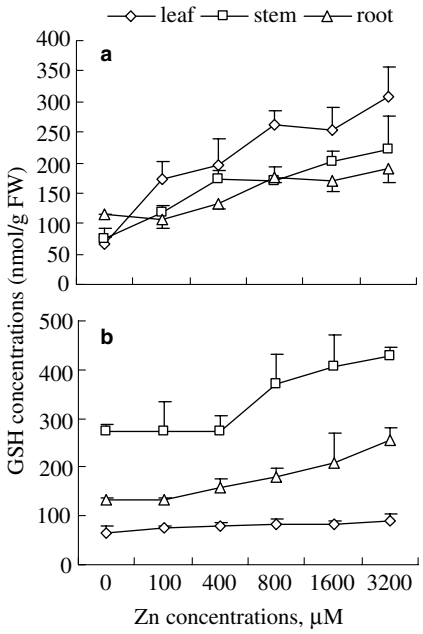

Fig. 3. Concentrations of GSH in leaf, stem and root of the ZJ mine plants (a) and the GD control plants (b) of *Sedum alfredii* exposed to a range Zn concentrations for 7 d (means ± SD, $n = 3$).

stead of PCs and Cys, a considerable amount of GSH was detected in these plants (Figs. 3 and 4). After exposure to increasing Zn, the concentrations of GSH significantly ($P < 0.01$) increased in these plants, whereas a marked increase was observed in each of the three tissue parts of the ZJ mine plants (Fig. 3(a) and (b)). The increase of GSH was almost linearly consistent with Zn accumulation in leaf ($R^2 = 0.853$, $P < 0.05$), stem ($R^2 = 0.828$, $P < 0.05$) and root ($R^2 = 0.887$, $P < 0.01$) of the ZJ mine plant. However, this relationship was weaker in different tissue parts of

Table 1
Zinc concentrations in leaf, stem and root of *Sedum alfredii* from the ZJ mine site and the GD control site exposed to a range of Zn concentrations for 7 d (mg/kg FW) (means ± SD, $n = 3$)

Zn in solution (μM)	ZJ mine plants			GD control plants		
	Leaf	Stem	Root	Leaf	Stem	Root
0	75 (4)^{d}	84 (10)^{d}	36 (4)^{f}	1.3 (0.32)^{c}	2 (2)^{d}	1 (0)^{d}
100	154 (18)^{d}	273 (79)^{c}	129 (16)^{e}	12 (1.32)^{b}	28 (4)^{d}	349 (45)^{c}
400	267 (37)^{c}	266 (70)^{c}	212 (11)^{d}	29 (12)^{a}	77 (2)^{cd}	511 (65)^{b}
800	357 (23)^{b}	535 (89)^{b}	382 (13)^{c}	36 (3.7)^{a}	146 (12)^{bc}	536 (54)^{b}
1600	515 (69)^{a}	693 (100)^{a}	489 (91)^{b}	10 (1.0)^{b}	181 (37)^{b}	622 (97)^{ab}
3200	547 (85)^{a}	723 (16)^{a}	1067 (22)^{a}	101 (0.32)^{b}	275 (39)^{a}	638 (104)^{a}

Data in the same column with the same letters are not significantly different from each other at the level of $P < 0.05$. The standard deviation (SD) is given in parentheses.

Table 2
Lead concentrations in leaf, stem and root of *Sedum alfredii* from the ZJ mine site and the GD control site exposed to a range of Pb concentrations for 7 d (mg/kg FW) (means ± SD, $n = 3$)

Pb in solution (μM)	ZJ mine plants			GD control plants		
	Leaf	Stem	Root	Leaf	Stem	Root
0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
50	3.4 (0.44)^{c}	11 (1.55)^{d}	549 (114)^{c}	0.66 (0.12)^{c}	6.1 (1.1)^{d}	2177 (318)^{d}
150	4.8 (0.84)^{b}	37 (3.83)^{c}	4637 (596)^{b}	0.70 (0.18)^{c}	17 (1.9)^{c}	3411 (515)^{d}
450	4.8 (0.44)^{b}	40 (4.44)^{c}	5680 (1080)^{b}	0.98 (0.15)^{b}	21 (3.5)^{bc}	7149 (380)^{c}
900	5.5 (0.75)^{b}	61 (6.05)^{b}	13514 (2417)^{a}	1.3 (0.21)^{b}	27 (5.8)^{b}	15687 (1756)^{b}
1500	7.6 (0.66)^{a}	81 (17)^{a}	14480 (1887)^{a}	2.0 (0.25)^{a}	58 (8.2)^{a}	18449 (2406)^{a}

Data in the same column with the same letters are not significantly different from each other at the level of $P < 0.05$. The standard deviation (SD) is given in parentheses. n.d., not detection.

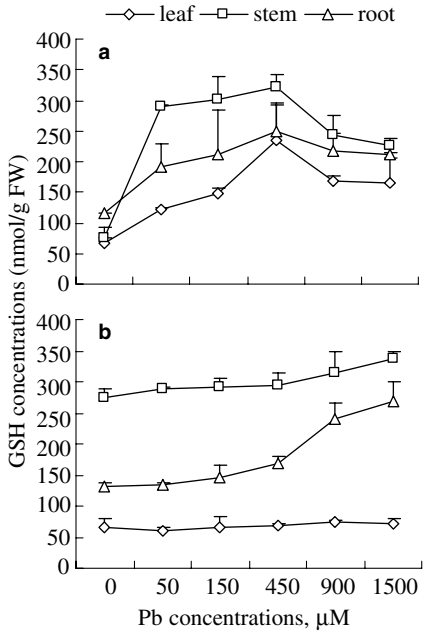

Fig. 4. Concentrations of GSH in leaf, stem and root of the ZJ mine plants (a) and the GD control plants (b) of *Sedum alfredii* exposed to a range of Pb concentrations for 7 d (means ± SD, $n = 3$).

the GD control plants, except the stem ($R^2 = 0.895$, $P < 0.01$).

Lead increased the concentrations of GSH in each of the three tissues of the ZJ mine plants in the descending order of stem > root > leaf (Fig. 4(a)). Conversely, the concentrations of GSH showed a slight increase in stem and leaf tissues in the control plants, though significantly ($P < 0.05$) increased in the roots (Fig. 4(b)). A mild chlorosis of leaf tissue was observed in the ZJ plants at 900 and 1500 μM Pb.

3. Discussion

Consistent with previous reports (Yang et al., 2002, 2004; He et al., 2002, 2003), the Zn hyperaccumulation/Pb accumulation trait of *S. alfredii* from the mine site was further confirmed in this study, showing much more tolerance to increasing Zn hyperaccumulation/Pb accumulation. Bert et al. (2002) reported that *Arabidopsis halleri* from non-metallicolous populations aside from metallicolous populations is also a Zn and Cd hyperaccumulator. However, the non-mine population of *S. alfredii* was highly sensitive to increasing Zn and Pb treatments and did not have the same ability to accumulate Zn and Pb as the mine population of *S. alfredii*. The results implied that the Zn hyperaccumulation/Pb accumulation to very high concentrations is a constitutive property of the mine population of *S. alfredii*.

Two methods are commonly used for measuring PCs: (1) separation with RP–HPLC, using post-column derivatization with Ellman's reagent [DTNB, 5,5′-dithiobis (2-nitrobenzoic acid)] (Grill et al., 1987; De Knecht et al., 1994) and (2) pre-column derivatization with monobromobimane (mBBr), followed by separation with RP–HPLC fluorescence (Newton et al., 1981; Ahner et al., 1994; Rijstenbil and Wijnholds, 1996). The second method (chosen for this study) has a higher sensitivity with a minimum concentration for detection of 0.3 pmol for SH per injection and also a stronger stability of the mBBr derivates, with no loss of fluorescence even over 20 months (Fahey and Newton, 1987; Sneller et al., 2000). The results clearly demonstrated that no PCs were induced in any tissue part of plants from the mine and control sites at any Zn and Pb treatments. Similar results were also reported in various clones of *Salix viminalis*, with different metal tolerance (Landberg and Greger, 2004), Ni hyperaccumulating tree *Sebertia acuminate* (Sagner et al., 1998) and Co hyperaccumulator *Crotalaria cobalticola* (Oven et al., 2002). The present data indicated that PCs-dependent constitutive metal tolerance did not occur in the mine population of *S. alfredii*.

The present results support the idea that PCs are not involved in metal hyperaccumulation and tolerance in plants. Schat et al. (2002) revealed that PCs synthesis is not responsible for Cd, Zn, Cu, Ni and Co tolerance in the Zn/Cd hyperaccumulator *T. caerulescens*, as also observed by Ebbs et al. (2002). Similarly, De Knecht et al. (1992) reported that sensitive plants of *S. vulgaris* produce more PCs than tolerant plants when exposed to the same external Cd concentration. The in vivo acid-labile sulphide contents of the Cd-PC complexes and PC chain length distributions are identical (De Knecht et al., 1994). Furthermore, De Knecht et al. (1995) obtained equal capacities and activation constants for Cd-induced PCs synthesis in crude protein extracts prepared from roots of Cd-hypertolerant and non-metallicolous *S. vulgaris*. Moreover, sensitive lines of this plant produced more PCs than tolerant lines that were exposed to Cu and Zn (De Vos et al., 1992; Harmens et al., 1993). In fact, the role of PCs in adaptive tolerance has been questioned (Meharg, 1994; Schat et al., 2000). It was indicated that an over-production of PCs in plants chronically exposed to heavy metals, would cost such a large amount of energy required for sulphate reduction, that a mechanism of this kind of increased tolerance is unlikely to have evolved (Steffens, 1990).

Since PCs are not induced in the ZJ mine plants of *S.alfredii* in response to Zn and Pb, it presumably means that PC-independent mechanisms have emerged to deal with increasing Zn and Pb accumulation and tolerance. The results of the present study reveal that Zn significantly stimulated an increase in GSH levels for each of the three tissue parts of the ZJ mine plant, with only a slight increase in the control plant, which cannot prevent Zn toxicity. GSH is the most abundant cellular thiol in most living organisms and involved in metal stress in a number of ways. Firstly, GSH is a well-known antioxidant playing a prominent role in defense against radicals caused by metal stress in plants. Secondly, GSH is a potential cytosolic chelator of metal and further reduces metal toxicity in a less

toxic form (Vögeli-Lange and Wagner, 1996). Mehra et al. (1995) presented the role of GSH as an effective donor of heavy metal ion, and it is probably the in vivo donor of heavy metals to PCs (potentially toxic heavy metal ions are firstly chelated by GSH and then transferred to PCs for eventual sequestration) (Mehra and Mulchandani, 1995). Thirdly, It was widely suggested that GSH serves as a precursor in PC biosynthesis. PC synthesis induced by metals is accompanied by a rapid depletion of total GSH in plant cell suspensions and intact plants (Jackson et al., 1992; Gupta et al., 1998). In this experiment, no PCs were induced in the mine population of *S. alfredii*. It was easily conceived that GSH might serve as an antioxidant or a metal chelator involved in Zn/Pb detoxification in this plant. He (2003) has reported that the chlorophyll content and MDA content was not affected in mine population of *S. alfredii* by Pb treatments up to 320 mg/L. It was also verified that Cu exerted little physiological damage to Cu hyperaccumulator of *Commelina communis* at >1000 μg/g in dry leaf tissues, while in a non-accumulator of *C. communis* superoxide dismutase, guaiacol peroxidase, and ascorbate peroxidase were activated, and MDA was increased (Wang et al., 2004). Similar results have also been found in Cd-resistant type seedlings of *A. thaliana* (Cho and Sohn, 2004). Based on these data, it can be further speculated that GSH might largely serve as a metal chelator involved in Zn transport, hyperaccumulation and detoxification in the mine population of *S. alfredii*.

Long (2002) showed that most Zn is accumulated in the leaf and stem cell walls of the ZJ mine plants of *S. alfredii*, while a substantial amount of Zn is also present in the soluble fraction of cells. For example, at 500 μM Zn, 38.09% and 20.13% Zn are distributed in the soluble fraction of leaf and stem cells, respectively. Additionally, the distributed ratio of Zn in the soluble fraction of leaf cells increased with an increase of Zn in treatment solutions. These results indicate that this part of Zn should be presented in Zn complexation or stored in the vacuole. The ratio of GSH to Zn in the soluble fraction of leaf and stem cells in a rough estimation is found to be very low. This low ratio suggests that GSH is not the only compound involved in Zn complexation in the soluble fraction of cells. Therefore, Zn hyperaccumulation and tolerance experienced by the ZJ mine plant of *S. alfredii* would also depend on activity of additional detoxification mechanisms. It has been suggested that increased Zn tolerance may be due to an increase in the activity of a Zn-malate shuttle system that would transport Zn into the vacuole (Ernst, 1975; Mathys, 1977). Investigation of frozen roots and shoots of *T. caerulescens* showed that 70% of the Zn in roots is associated with histidine while 16,38, and 9% of the Zn in shoots are associated with histidine, citrate and oxalate, respectively (Salt et al., 1999). These ligands may facilitate metal loading into xylem, translocation to shoot, and presumably metal storage in cell vacuoles. Whether such mechanism of chelation would operate in the ZJ mine plant of *S. alfredii* needs to be further investigated.

Similar to Zn, most Pb is distributed in the cell wall of each of the three tissue cells of the ZJ mine plant of *S. alfredii*, while a considerable amount of Pb is present in the soluble fraction of cells compared with legumes investigated by Piechalak et al. (2002). For example, at the concentration of 160 mg/L Pb, 11.1%, 40.8% and 20% Pb are localized in the soluble fraction of leaf, stem and root cells of the mine plants of *S. alfredii*, respectively (He, 2003). The present study indicated that Pb stimulated the increase of GSH concentrations in each of the three tissues of the ZJ mine plants, especially in stem, while in the GD control plants GSH concentrations only showed a slight increase in leaf and stem. According to the above reasoning, GSH may serve as a potential metal chelator involved in Pb accumulation and tolerance in mine populations of *S. alfredii*. The estimated ratio of GSH to Pb in the soluble fractions was significantly higher in leaf and stem cells than in root cells, indicating that GSH might be involved in Pb accumulation and tolerance in the above-ground parts of the mine plant, while Pb tolerance in root may be manipulated by other mechanisms. The exact role played by GSH, however, is not clear based on the current results. Salt et al. (1999) indicated that different ligands in different tissues of *T. caerulescens* are responsible for Zn complexation and hyperaccumulation. It was also reported that in tomato and *S. vulgaris* cell cultures exposed to Pb and Zn ions, heavy metals are bound to lower molecular weight ligands, but not phytochelatins (Piechalak et al., 2002). It was very likely that Pb in the shoots and roots of the ZJ mine plants have been complexed by different unknown ligands. It is thus necessary to conduct future research in order to better understand the mechanisms of Pb accumulation and tolerance in mine populations of *S. alfredii*.

4. Experimental

4.1. Plant materials and chemicals

Plant samples of *S. alfredii* were collected from an old Pb/Zn mine site in Quzhou, Zhejiang Province (ZJ mine plant), and a 'clean' site in Guangzhou, Guangdong Province (GD control plant), China, respectively.

The following chemicals were obtained: *N*-[2-hydroxyethyl] piperazine-*N*′-[3-propane sulfonic acid] (HEPPS, >99.5%), glutathione (GSH, >98%), L-cysteine (Cys, >98%) and trifluoroacetic acid (TFA, 99%) from Sigma; monobromobimane (mBBr, >95%), methanesulfonic acid (MSA, >99%) and diethylenetriamine-pentaccetic acid (DTPA, >99%) from Fluka; CH_3CN (ACN, >99.9%, HPLC grade) from Lab-Scan; PCn ($n = 2, 3, 4$, >95%) standards from Biopeptide Co., LLC. USA. Other chemicals were of reagent grade or of the commercially highest grade available. Milli-Q water (18.3 MΩ) was used to prepare aqueous solutions for the phases of this study. mBBr was prepared daily, where other solutions were prepared weekly and stored in the dark at 4 °C.

4.2. Plant culture conditions

Healthy and uniform-sized shoots of *S. alfredii* collected from mine and 'clean' sites were chosen and transplanted in 2.5 L containers (6 plants per pot) containing nutrient solution, which was a modified Hoagland solution (Hewitt, 1966) with half-strength macronutrients and full-strength micronutrients (Fe was supplied as Fe-EDTA). The solution pH's were adjusted to 5.8 (pH of the Pb/Zn mine substrate) with 0.1 M HCl or NaOH once every 3 d. The nutrient solution was aerated continuously and renewed once every 6 d. Plants were grown in a greenhouse (with a natural photoperiod, with temperature controlled at 20–25 °C). After 30 d, plants (with new roots) were exposed to varying concentrations of Zn (0–3200 μM) using $ZnSO_4 \cdot 7H_2O$ and Pb (0–1500 μM) using $Pb(NO_3)_2$, respectively. There were three replicates for each treatment. Considering the precipitation of Pb in the nutrient solution, P was removed from the nutrient solution for all Pb treatments. The treated solution was renewed once every 2 d. After 7 d, the roots of all plants were immersed in ice-cold 10 mM $CaCl_2$ for 10 min to remove adhering metals from root surfaces. After washing with tap water and deionized water, plants were separated into roots, stems arid leaves. Each plant tissue was subdivided into two halves, one was immediately weighed, frozen in liquid nitrogen (−196 °C) and stored at −80 °C for analysis of PCs and other low molecular weight thiols, while the other was used for analysis of metal concentrations.

4.3. Analytical methods

4.3.1. Determination of PCs and other low molecular weight thiols

Step 1 (extraction): Extraction and analysis of PCs and other low molecular weight thiols were performed according to the method described by Sneller et al. (2000) with a slight modification. Frozen plant tissues were homogenized in a mortar and a pestle with quartz sand in 2 ml of 6.3 mM DTPA with 0.1% TFA at 4 °C. The homogenate was centrifuged at 14,000*g* at 4 °C for 12 min. The clear supernatants were collected and immediately used for the assay of PCs and other low molecular weight thiols by high-performance liquid chromatography (HPLC), using pre-column derivatization with a fluorescent probe mBBr.

Step 2 (derivatization): 250 μl of supernatant was mixed with 450 μl of 200 mM HEPPS buffer, at pH 8.2, with 6.3 mM DTPA, and 10 μl of 25 mM mBBr. Derivatization was carried out in the dark at 45 °C for 30 min. The reaction was terminated by adding 300 μl of 1 M MSA. The samples were stored in the dark at 4 °C until HPLC analysis. Blank samples were used to identify the reagent peaks.

Step 3 (detection with HPLC fluorescence): The bimane derivatives were separated using a binary gradient of mobile phase A (0.1% TFA) and B (100% ACN) at room temperature (22 ± 2 °C). Fluorescence was detected at 380 nm excitation and 470 nm emission wavelengths. The flow rate was 0.5 mL min^{-1}. Fifty μl of the derivatized sample was run in a linear gradient from 12% to 25% B for 15 min, then 25% to 35% B for 14 min, and next 35% to 50% B for 21 min. Before injecting a new sample, the column was cleaned (5 min, 100% B) and equilibrated (10 min, 12% B) and post-time was 5 min. Total analysis time was 70 min. All solvents were degassed before use. Analytical data were integrated by using the HP Chemstation. Retention times of PCs and other low molecular weight thiols (GSH, Cys) in biological samples were checked with PC_2, PC_3, PC_4, GSH and Cys standards, respectively. Individual PC subtypes were quantified by using the relationship peak vs. concentrations of GSH standard solutions. PCs were expressed as γ-Glu-Cys units. Corrections for differential derivatization efficiencies were made according to the method stated by Sneller et al. (2000).

4.3.2. Determination of total Zn and Pb

Fresh tissues were weighed accurately to 3 g for leaves and stems, and 1 g for roots and then oven-dried (70 °C) to a constant weight. The dried tissues were digested by mixed acid [15.4 M HNO_3/11.6 M $HClO_4$ (85/15, v/v)] and tested for Zn and Pb using an Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES, Perkin–Elmer, Model 3000DV). A standard reference plant material (GBW07602) from the Department of Earth and Mine, China, was used to verify the accuracy of metal determination. The recovery rates for all metals were within 90 ± 10%.

4.3.3. Statistical analyses

Statistical analyses were performed using the SPSS statistical package (version 10.0 for windows). Data were tested at significant levels of $P < 0.05$ or $P < 0.01$ by one-way ANOVA. The subsequent multiple comparisons among means were examined based on the Least Significance Deviation (LSD) (Little and Hills, 1978).

Acknowledgements

This work was supported by the Research Council of University Grants Committee, Hong Kong (RGC: HKBU-2072/01M and HKBU-2181/03M), the National Natural Science Foundation of China (No. 20237010) and Innovation Foundation of Advanced Universities' Graduate of Jiangsu Province, China.

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