Available online at www.sciencedirect.com
ScienceDirect
Phytochemistry 69 (2008) 911–918
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Detection of phytochelatins in the hyperaccumulator *Sedum alfredii* exposed to cadmium and lead

Zhongchun Zhang, Xiang Gao, Baosheng Qiu *

College of Life Sciences, Central China Normal University, Wuhan, 430079 Hubei, PR China

Received 1 May 2007; received in revised form 5 October 2007
Available online 26 November 2007

Abstract

Phytochelatins (PCs) are known to play an essential role in the heavy metal detoxification of some higher plants and fungi by chelating heavy metals. However, three recent papers reported that no PCs could be detected in the hyperaccumulator *Sedum alfredii* Hance upon cadmium, lead or zinc treatment, respectively. In this paper, PC synthesis was assayed again in the mine population of *S. alfredii* with the help of reversed phase high-performance liquid chromatography (HPLC), HPLC-mass spectrometry, and HPLC-tandem mass spectrometry. Our data showed that PC formation could be induced in the leaf, stem and root tissues of *S. alfredii* upon exposure to 400 μM cadmium, and only in the stem and root when exposed to 700 μM lead. However, no PCs were found in any part of *S. alfredii* when it was subjected to exposure to 1600 μM zinc.

Keywords: *Sedum alfredii*; Crassulaceae; Hyperaccumulator; Phytochelatins; Cadmium; Lead; Zinc

1. Introduction

Phytochelatins (PCs) are enzymatically synthesized peptides produced in some higher plants and fungi when exposed to heavy metals, such as cadmium, lead, zinc and so on (Zenk, 1996; Cobbett, 2000). They are rich in the -SH group and have the structure of $(\gamma\text{-Glu-Cys})_n$-Gly, where n is generally varied from two to five (Cobbett, 2000). In addition, it exists in other forms of PCs, namely, *iso*-phytochelatins: $(\gamma\text{-Glu-Cys})_n$, $(\gamma\text{-Glu-Cys})_n$-Glu, $(\gamma\text{-Glu-Cys})_n$-Gln, $(\gamma\text{-Glu-Cys})_n$-Ser, $(\gamma\text{-Glu-Cys})_n$-$\beta$-Ala (or called hPCs) (Rauser, 1995). Heavy metals can be chelated by PCs, and the resulting ligand–metal complexes are usually compartmentalized in the vacuole (Zenk, 1996; Cobbett, 2000). PCs could also play an important role in regulation of homeostasis of heavy metals within plants (Zenk, 1996).

PCs have been detected in *Thlaspi caerulescens* when exposed to Cd (Ebbs et al., 2002; Wójcik et al., 2005). Like *T. caerulescens*, *Sedum alfredii* Hance was a newly identified hyperaccumulator of Zn and Cd and an accumulator of Pb (He et al., 2002; Yang et al., 2002, 2004). However, Sun et al. (2005, 2006, 2007) 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 either Cd, Zn or Pb, respectively. The goal of our study was to re-examine PC induction in *S. alfredii* by optimizing the experimental protocol developed previously.

2. Results and discussion

According to the retention time of standard PCs, they were detected in leaf, stem and root tissues of *S. alfredii* exposed to 400 μM Cd for 5 d (Fig. 1), which is different from the results reported by Sun et al. (2006, 2007). Sun et al. (2005, 2006, 2007) and our group have adopted the same mine population of *S. alfredii* which was collected at an old Pb/Zn mining area in Quzhou City, Zhejiang Province. Our previous study had shown that its growth was not affected at the Cd level $\leqslant$200 μM in the nutrient solution and it could accumulate over 10000 and

* Corresponding author. Tel.: +86 27 67861514.
E-mail address: bsqiu@public.wh.hb.cn (B. Qiu).

0031-9422/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2007.10.012

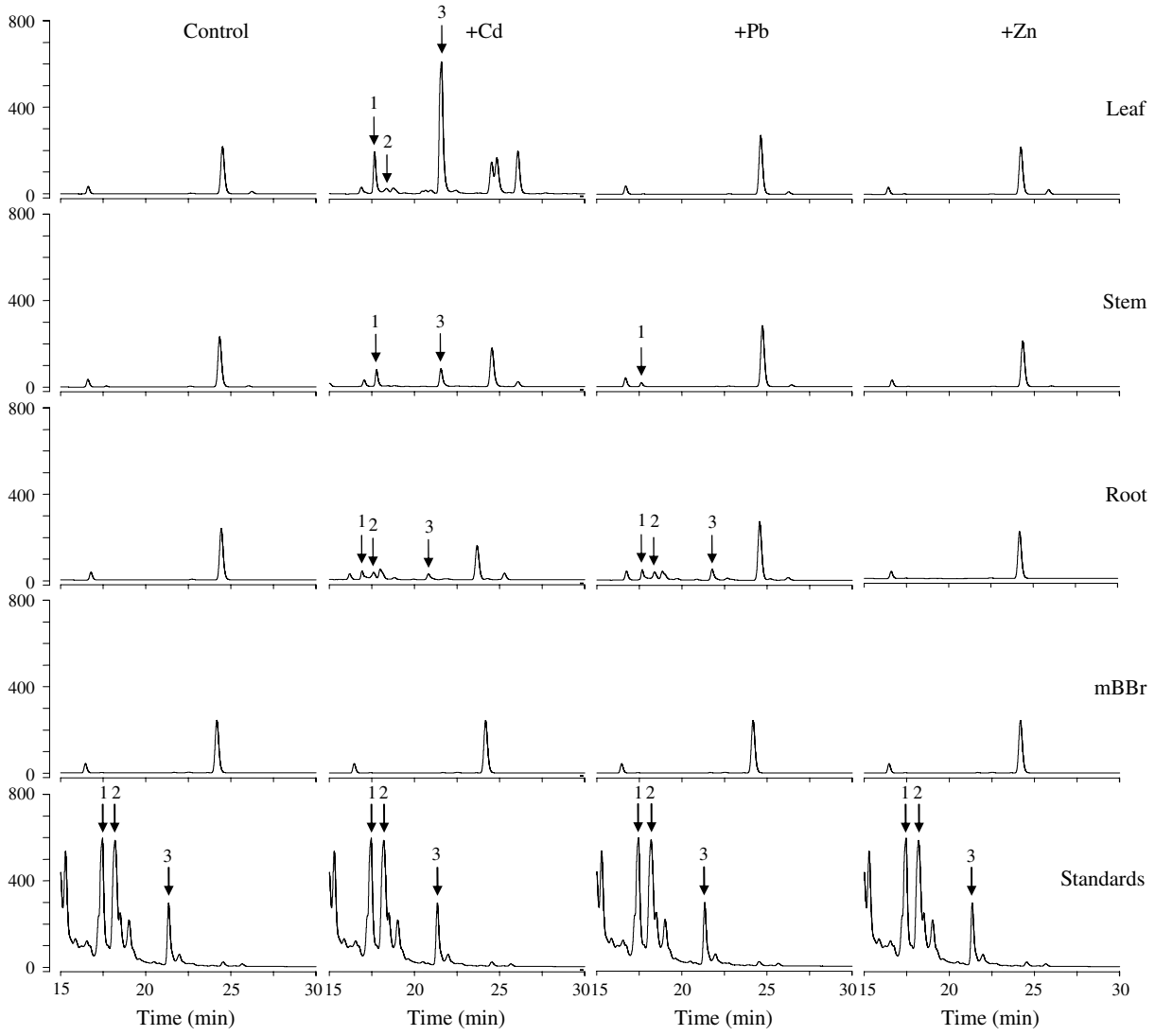

Fig. 1. PC profiles in leaf, stem and root tissues of the mine population of *Sedum alfredii* when exposed to 400 μM Cd for 5 d, 700 μM Pb for 7 d or 1600 μM Zn for 7 d, respectively. Samples grown in the absence of the above heavy metals were used as controls. The injection of mBBr alone was used to identify reagent peaks. 1, PC_2; 2, PC_3; 3, PC_4.

5000 mg kg^{-1} Cd in the leaf and stem tissues, respectively (Zhou and Qiu, 2005). This ecotype of *S. alfredii* has recently been identified as the hyperaccumulator of Cd (Yang et al., 2004), and the PC assaying method in this study was similar to Sun et al. (2005, 2006, 2007). Our two groups adopted the pre-column derivatization with monobromobimane (mBBr) and separation by reversed phase high-performance liquid chromatography (RP-HPLC); this is one of the most widely used methods for PC detection (Ebbs et al., 2002; Stolt et al., 2003; Landberg and Greger, 2004).

We further identified the HPLC fluorescent peaks with high-performance liquid chromatography-mass spectrometry (HPLC-MS). The reliability of this method was checked with standard PCs. As reported by Chen et al. (2006), the PC_2 standard labeled with two mBBr molecules showed the predicted mass of the +1 ion at *m/z* 920 (Fig. 2a), the PC_3 standard labeled with three mBBr molecules showed the predicted mass of the +1 ion at *m/z* 1342 (Fig. 2c), and the PC_4 standard labeled with four mBBr molecules showed the predicted mass of the +2 ion at *m/z* 883 (Fig. 2e). The derivatized leaf extract of *S. alfredi* treated with 400 μM Cd for 5 d were then analyzed using HPLC-MS (Fig. 2b, d, f). Its HPLC fluorescent peaks appeared at the same retention time as those of PCs standards (Data not shown). Furthermore, the masses of the $M^+ + 1$ ion at *m/z* 920 (Fig. 2b), the $M^+ + 1$ ion at *m/z* 1342 and the $M^+ + 2$ ion at *m/z* 672 (Fig. 2d), and the $M^+ + 2$ ion at *m/z* 883 (Fig. 2f) were observed in the derivatized leaf extract of *S. alfredi* upon Cd exposure.

In order to further characterize the three Cd-induced thiol-containing peptides in 400 μM Cd treated samples, their fragments of protonated molecules were obtained by HPLC-tandem mass spectrometry (HPLC-MS/MS) (Fig. 3). Several expected ion fragments arose from the cleavage of peptide bonds. The charges could be retained

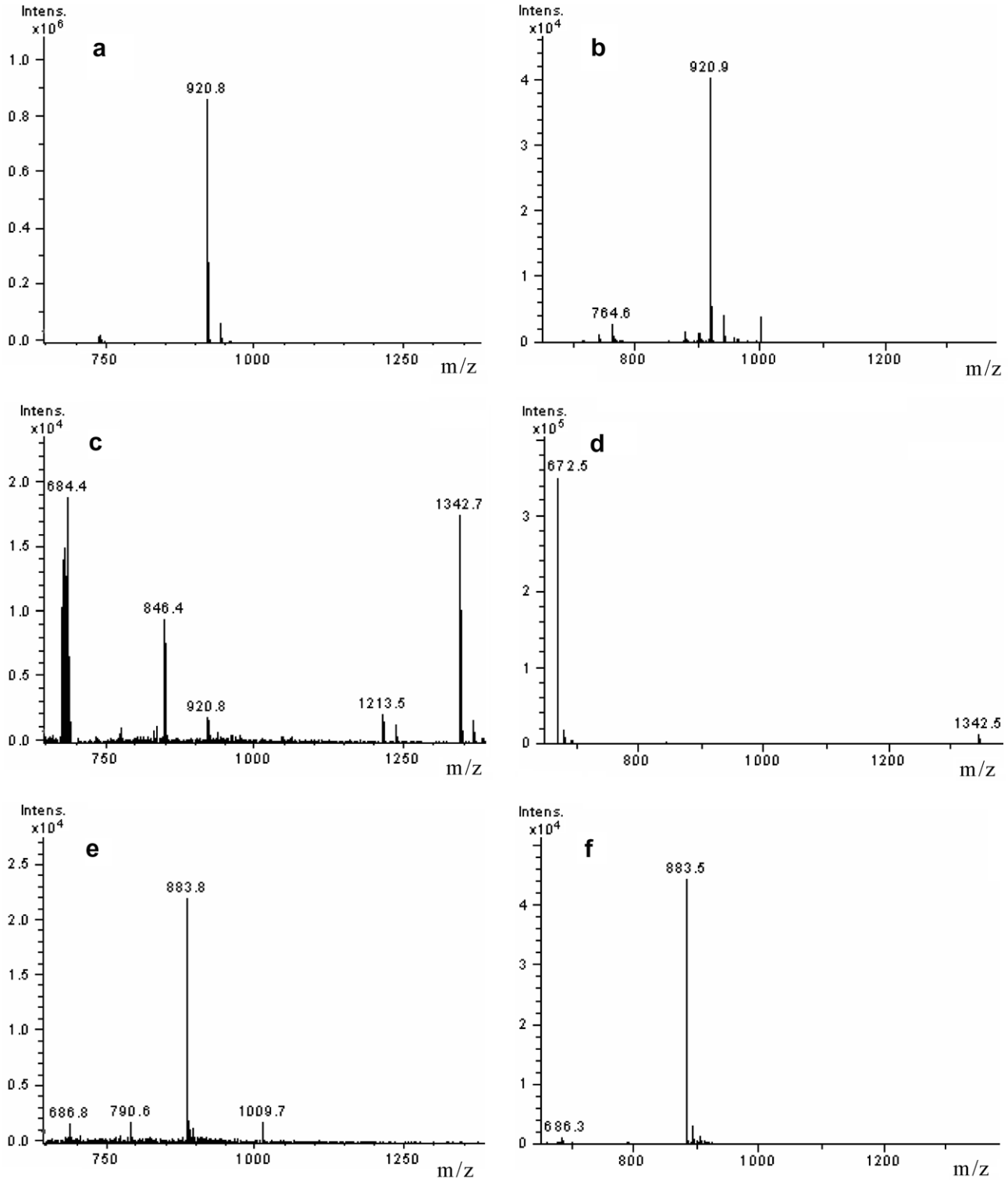

Fig. 2. PC confirmation by the electrospray mass spectrum run concurrently with fluorescence RP-HPLC. a, c, e were mass spectra of PC standards. b, d, f were the mass spectra of the derivatized leaf extract of *Sedum alfredii* upon exposure to 400 μM Cd for 5 d. PC_2 was indicated as the $M^+ + 1$ ion at *m/z* 920 (a and b), PC_3 the $M^+ + 1$ ion at *m/z* 1342 or the $M^+ + 2$ ion at *m/z* 672 (c and d), PC_4 the $M^+ + 2$ ion at *m/z* 883 (e and f).

either on the amino terminus or on the carboxy terminus. For example, the molecule of *m/z* 920 at the +1 ion state could result in the ion fragments of *m/z* 845, 552 and 423 for the amino terminus but 498 and 369 for the carboxy terminus, respectively (Fig. 3a and b). Similar results were observed for the molecules of *m/z* 672 and *m/z* 883 at +2 ions state (Fig. 3c and d). These ion fragments were also detected for PCs standards (PC_2, PC_3 and PC_4) by the

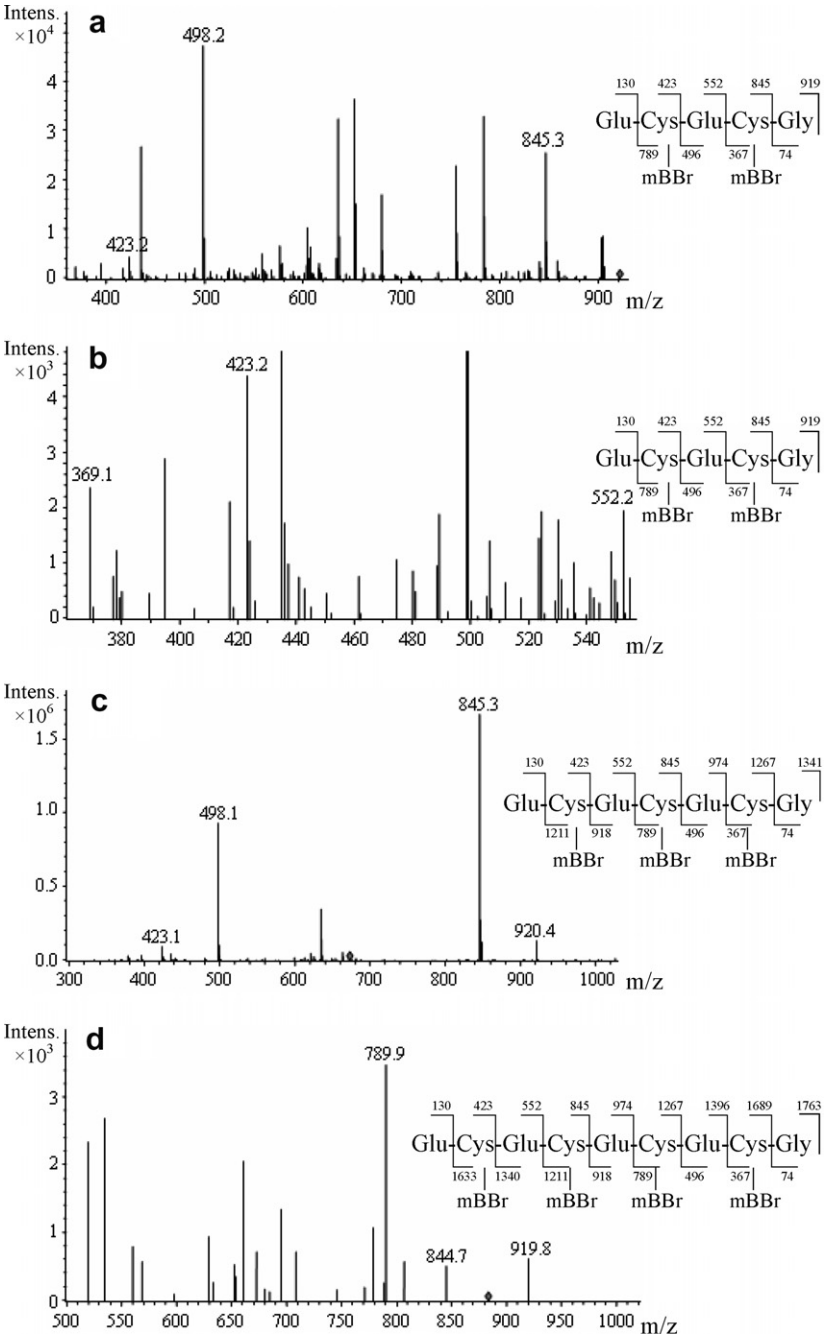

Fig. 3. HPLC-MS/MS spectra of fluorescent peaks corresponding to the $M^+ + 1$ ion at *m/z* 920 (a and b), the $M^+ + 2$ ion at *m/z* 672 (c), and the $M^+ + 2$ ion at *m/z* 883 (d) in the leaf extract of *Sedum alfredii* upon exposure to 400 μM Cd for 7 d.

HPLC-MS/MS (Data not shown). Considering several cleavage possibilities, other ion fragments were produced. However, these results confirmed the existence of Cd-induced PCs in *S. alfredii*.

The PC contents were 122 nmol g (fr. wt)$^{-1}$ in the leaf of *S. alfredii* exposed to 400 μM Cd for 7 d (Table 1). This was close to that in the leaf of Cd-hyperaccumulator *T. caerulescens* (ecotype, Prayon) exposed to 50 μM Cd for 4 d (Ebbs et al., 2002). However, PC contents in the shoot of *S. alfredii* were much less than that in Cd-tolerant *T. caerulescens* (ecotype, Plombières) treated with 200–500 μM Cd for 14 days (Wójcik et al., 2005). Furthermore,

Table 1
PC contents in leaf, stem and root tissues of *Sedum alfredii* when exposed to 400 μM Cd for 7 d or 700 μM Pb for 7 d, respectively

Treatment	PCs content (nmol g (fr. wt)$^{-1}$)		
	Leaf	Stem	Root
400 μM Cd	122.51 ± 2.16	51.52 ± 2.22	49.98 ± 5.85
700 μM Pb	ND	6.72 ± 1.06	43.20 ± 29.14

Data are the mean ± SD ($n = 2$–3). ND, not detected.

PC contents in the roots of *S. alfredii* were quite less compared to those in roots of the above two ecotypes of *T. caerulescens* (Ebbs et al., 2002; Wójcik et al., 2005). Cd-

induced PCs had been detected in other plants such as *Arabidopsis*, *Brassica juncea*, chickpea, tomato and wheat (Scheller et al., 1987; Gupta et al., 2002; Stolt et al., 2003; Wójcik and Tukiendorf, 2004; Gadapati and Macfie, 2006). Owing to the lack of phytochelatin synthase, hPCs, rather than PCs, were observed in Azuki bean when subjected to Cd (Inouhe et al., 2000; Oven et al., 2001). In contrast, PCs had not been found in Cd-tolerant plant *Salix viminalis* (Landberg and Greger, 2004).

We have shown that when *S. alfredii* was exposed to 700 μM Pb for 7 d, PC_2 and PCs (PC_2, PC_3 and PC_4) could be detected in the stem and root, respectively (Fig. 1). However, contrary to what we found for Cd, no PCs were detected in the leaf of *S. alfredii* when exposed to Pb. Sun et al. (2005) reported no PCs in these three parts of the mine population of *S. alfredii* after 7 d exposure to Pb concentrations up to 1500 μM. PCs mainly accumulated in the roots of *S. alfredii* subjected to 700 μM Pb for 7 d, and PC contents were 43 and 6 nmol g (fr. wt)$^{-1}$, respectively, in the root and stem tissues (Table 1). The distribution pattern of PCs within *S. alfredii* was consistent with the Pb content in the soluble fraction of different tissues, and the latter had already been shown to follow the order: root > stem ≫ leaf (He et al., 2003). The content of Pb-induced PCs can vary a lot in different higher plants. It was 2727 nmol g (fr. wt)$^{-1}$ in the roots of *Pisum sativum* exposed to 1 mM Pb for 48 h, while only 16 nmol g (fr. wt)$^{-1}$ PCs were induced in the root of *Vicia faba* exposed to 1 mM Pb for 48 h (Piechalak et al., 2002). The PC-Pb complex has also been found in *Hydrilla verticillata* and *Phaeodactylum tricornutum* (Gupta et al., 1995; Scarano and Morelli, 2002). Although PCs have not been detected in *Phaseolus vulgaris* and *Salix viminalis* upon exposure to Pb, hPCs (hPC_2, hPC_3 and hPC_4) have been reported present in the former species (Piechalak et al., 2002; Landberg and Greger, 2004).

It is known that zinc ions can strongly induce PC synthesis in *Dunaliella tertiolecta* and *Stigeoclonium tenue* (Hirata et al., 2001; Pawlik-Skowrońska, 2003). However, in our work, PCs were not observed in any part of *S. alfredii* when subjected to 1600 μM Zn for 7 d (Fig. 1). These results were in agreement with those obtained by Sun et al. (2005), in which no PCs were detected in the mine population of *S. alfredii* in the presence of 0–3200 μM Zn. In two other recent reports, PCs were also not detected in *T. caerulescens* (ecotype, Plombières) exposed to 250–2500 μM Zn (Wójcik et al., 2006), or in *Salix viminalis* exposed to 10–100 μM Zn (Landberg and Greger, 2004). It had been suggested that accumulation of malate and citrate was responsible for Zn tolerance in *T. caerulescens* (ecotype, Plombières) (Wójcik et al., 2006). In addition, the majority of intracellular Zn was found to be coordinated with histidine in the root of *T. caerulescens* (ecotype, Prayon) (Salt et al., 1999). Thus, Zn was perhaps chelated by some unknown ligands and could not activate phytochelatin synthase in *S. alfredii*.

3. Concluding remarks

We may possibly explain the differences between our results and those from Sun et al. (2005, 2006, 2007) by various reasons. It is known that PCs were prone to rapid oxidation after they were isolated from the cells (Kawakami et al., 2006). Therefore, to avoid PC degradation, the tissues should be fully ground and the process of sample preparation should be rapid. The content of PCs was low in the root when *S. alfredii* was exposed to Cd or Pb (Table 1). The protocol of sample preparation might be one reason that very low concentration of PCs cannot be detected in the roots of the mine population of *S. alfredii*, when treated with Cd or Pb in Sun et al. (2005, 2006, 2007). Thus, we followed their preparation protocol and about 0.3 g frozen roots were homogenized using a mortar and pestle with 1.2 ml extraction buffer at 0 °C. We have also shown that no PCs could be detected in the root of *S. alfredii* treated with 400 μM Cd for 20 d (Fig. 4). However, PCs could be detected in less than 0.2 g frozen roots with the procedure developed in our study (Fig. 4). Another explanation of the differences between results of our study and the ones of Sun et al. (2005, 2006, 2007) may be linked to the derivatization protocol. Samples were extracted according to Sun et al. (2005, 2006), derivatized with mBBr at room temperature, and immediately analyzed by RP-HPLC with the same working conditions of mobile phases as those in Sun et al. (2005, 2006, 2007). However, PCs were observed in the leaf tissues of *S. alfredii* when exposed to 400 μM Cd for 5 d (Fig. 5). PCs derivatives with mBBr would slightly be degraded after 30 d at 4 °C (Kawakami et al., 2006).

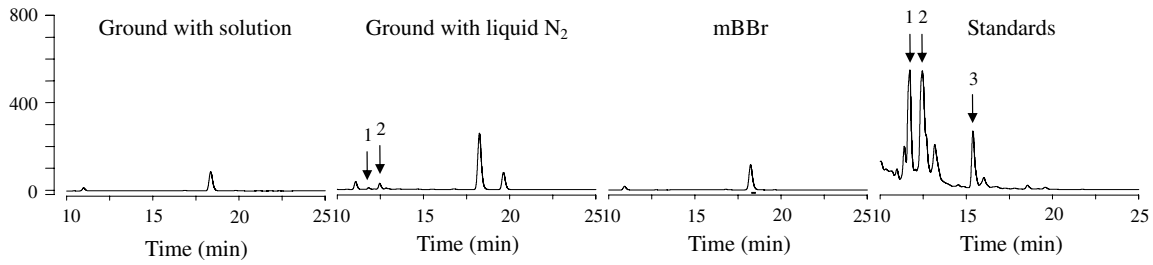

Fig. 4. Effects of different extracting protocols on PC detection in the roots of *Sedum alfredii* when exposed to 400 μM Cd for 20 d. 1, PC_2; 2, PC_3; 3, PC_4.

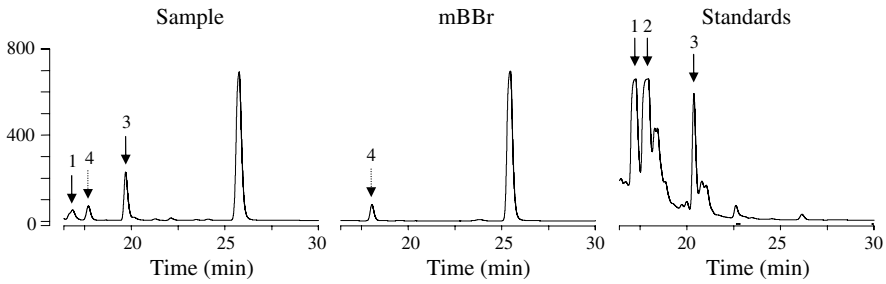

Fig. 5. PC profiles in the leaf of *Sedum alfredii* detected according to the protocol of Sun et al. (2005, 2006, 2007) except for derivatization at room temperature. Samples had been exposed to 400 μM Cd for 5 d. 1, PC_2; 2, PC_3; 3, PC_4; 4, unidentified peak.

Thus, the derivatized samples should not be stored for a long time before assay as it was done for part of the experiments by Sun et al. (2006, 2007). In the latter case, no PCs were detected in the mine population of *S. alfredii* exposed to Cd. Therefore, in our study, all samples were analyzed within two or three days after derivatization, which seemed to have increased the detection level of PCs.

4. Experimental

4.1. Chemicals and the treatment of experimental wares

The chemicals methanesulfonic acid (MSA), monobromobimane (mBBr), diethylenetriamine-pentaccetic acid (DTPA) and glutathione (GSH) were purchased from Sigma, and 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) from Boehringer Mannheim. All other chemicals were of reagent grade. The mBBr, dissolved in CH_3CN, was stored in the dark at −20 °C, and other solutions were stored at 4 °C.

All laboratoryware were metal- and organic-free during PC extracting and analyzing, because their contamination could promote the degradation of reagents and thiol compounds (Kawakami et al., 2006). The glassware and plastic bottles were carefully washed, soaked in 10% HCl for 24 h, and then rinsed four times with Milli-Q (18.2 MΩ) H_2O. The tips and Eppendorf tubes were also soaked in 10% HCl overnight before use.

4.2. Plant material, cultivation and treatment

Sedum alfredii Hance was collected from an old Pb/Zn mining area in Quzhou City, Zhejiang Province, PR China where Sun et al. (2005, 2006, 2007) collected the mine population of *S. alfredii*. Young shoots were chosen and transplanted into 2.5 l plastic containers. The nutrient solution consisted of 2 mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.1 mM KH_2PO_4, 0.5 mM $MgSO_4 \cdot 7H_2O$, 0.1 mM KCl, 0.7 mM K_2SO_4, 10 μM H_3BO_3, 0.5 μM $MnSO_4 \cdot H_2O$, 0.5 μM $ZnSO_4 \cdot 7H_2O$, 0.2 μM $CuSO_4 \cdot 5H_2O$, 0.01 μM $(NH_4)_6Mo_7O_{24}$ and 100 μM Fe-EDTA, pH 5.8. The nutrient solution was renewed every four days and continuously aerated with an aquarium pump. Plants were grown at 25 °C and 200 μmol m^{-2} s^{-1} with a 14 h light/10 h dark cycle.

After 14 d cultivation, *S. alfredii* was treated with 400 μM $CdCl_2$ for 5 d, 7 d or 20 d, 700 μM $Pb(NO_3)_2$ for 7 d or 1600 μM $ZnSO_4 \cdot 7H_2O$ for 7 d. Phosphorus was removed from the nutrient solution before lead treatment to avoid the occurrence of lead precipitation (Sun et al., 2005). All nutrient solutions were renewed every two days. The intact plants were rinsed with tap water for several minutes and the roots were completely immersed in 20 mM Na_2-EDTA for 15 min after treatment of heavy metals (Zhou and Qiu, 2005). Then, the intact plants were washed thoroughly with deionized H_2O. Finally, root, stem and leaf tissues were separated, frozen in liquid N_2 and stored at −80 °C for the analysis of PCs.

4.3. PC analysis

PC detection was performed according to Sneller et al. (2000) with some minor modifications. The tissues were ground in liquid N_2 and the powdered samples (0.20 g leaf, 0.15 g stem or 0.15 g root) were used for PC extraction in 1.2 ml of 0.1% (w/v) CF_3CO_2H (TFA) with 6.3 mM DTPA. The homogenate was centrifuged at 10000*g* for 20 min at 4 °C. The supernatant (250 μl) was mixed with 200 mM HEPES buffer containing 6.3 mM DTPA (pH 8.2, 450 μl) and 25 mM mBBr (10 μl). Derivatization was then carried out for 30 min in the dark at room temperature. The reaction was terminated by adding 300 μl of 1 M MSA. Then, the samples were stored in the dark at 4 °C for HPLC analysis within the next two or three days. The injection of mBBr alone was used to identify the reagent peaks.

By using a binary gradient of mobile phase A (0.1% TFA) and B (100% CH_3CN) at room temperature, the samples were analyzed on a Varian HPLC system and the derivatized thiols were detected by a Varian fluorescence detector, with the excitation wavelength at 380 nm and emission at 470 nm. The C18 column (Varian C18, 5 μm, 4.6 × 150 mm) was adopted, and the flow rate was set at 1 ml min^{-1}. Derivatized samples (20 μl) were run with a linear gradient (0–10 min, 12–20% B; 10–40 min, 20–35% B; 40–50 min, 35–100% B; 50–55 min, 100% B; 55–65 min, 100–10% B) and then equilibrated for 10 min

with 12% B. Total analysis time was 75 min. All the solvents were filtered through 0.7 μm Whatman G/F filter and degassed before use. Retention time of phytochelatin oligomers was checked with PC_2, PC_3 and PC_4 standards. PCs standards were run after every twenty samples to monitor the slight shift of PCs peaks in the retention time. The concentrations of PCs were quantified by using the relationship peak area vs. concentrations of GSH standard solutions. PCs were expressed as γ-Glu-Cys units.

4.4. PCs characterized by HPLC-MS and HPLC-MS/MS

The mBBr derivatized PCs were separated and identified by RP-HPLC coupled with a fluorescence detector and a mass spectrometry instrument with electrospray interface (ESI) source (Agilent 1100 LC/MSD-Trap-XCT). The RP-HPLC conditions were the same as the above-mentioned method of PCs analysis. Positive ion mode was used. The working conditions for ESI were as follows: dry temperature 350 °C, dry gas 10 l min^{-1}, nebulizer 40 psi, HV capillary 3.5 kV, capillary exit 158.5 V, trap drive 74.5. For HPLC-MS/MS to characterize Cd-induced thiol-containing peptides, the following conditions were adopted: HV capillary 4.5 kV, capillary exit 80 V, Frag Ampl 0.5 V and others were the same as for HPLC-MS.

Acknowledgements

We thank Professor Meinhart H. Zenk (Donald Danforth Plant Science Center, St. Louis, Missouri, USA) for kindly providing PC_2, PC_3 and PC_4 standards. We are also grateful to Professor Philippe Juneau (Université du Québec à Montréal, Canada) for his helpful revision of our manuscript. This work was supported by Wuhan Chenguang Project for Youth Scholar (No. 20045006071-24), and the "211 Project" of Educational Ministry of China.

References

Chen, A., Komives, E.A., Schroeder, J.I., 2006. An improved grafting technique for mature *Arabidopsis* plants demonstrates long-distance shoot-to-root transport of phytochelatins in *Arabidopsis*. Plant Physiol. 141, 108–120.

Cobbett, C.S., 2000. Phytochelatins and their roles in heavy metal detoxification. Plant Physiol. 123, 825–832.

Ebbs, S., Lau, I., Ahner, B., Kochian, L., 2002. Phytochelatin synthesis is not responsible for Cd tolerance in the Zn/Cd hyperaccumulator *Thlaspi caerulescens* (J. & C. Presl). Planta 214, 635–640.

Gadapati, W.R., Macfie, S.M., 2006. Phytochelatins are only partially correlated with Cd-stress in two species of *Brassica*. Plant Sci. 170, 471–480.

Gupta, M., Rai, U.N., Tripathi, R.D., Chandra, P., 1995. Lead induced changes in glutathione and phytochelatin in *Hydrilla verticillata* (I.f.) Royle. Chemosphere 30, 2011–2020.

Gupta, D.K., Tohoyama, H., Joho, M., Inouhe, M., 2002. Possible roles of phytochelatins and glutathione metabolism in cadmium tolerance in chickpea roots. J. Plant Res. 115, 429–437.

He, B., Yang, X.E., Ni, W.Z., Wei, Y.Z., Long, X.X., Ye, Z.Q., 2002. *Sedum alfredii*: a new lead-accumulating ecotype. Acta Bot. Sin. 44, 1365–1370.

He, B., Yang, X.E., Ni, W.Z., Wei, Y.Z., Ye, H.B., 2003. Pb uptake, accumulation, subcellular distribution in a Pb-accumulating ecotype of *Sedum alfredii* Hance. J. Zhejiang Univ. Sci. 4, 474–479.

Hirata, K., Tsujimoto, Y., Namba, T., Ohta, T., Hirayanagi, N., Miyasaka, H., Zenk, M.H., Miyamoto, K., 2001. Strong induction of phytochelatin synthesis by zinc in marine green alga, *Dunaliella tertiolecta*. J. Biosci. Bioeng. 92, 24–29.

Inouhe, M., Ito, R., Ito, S., Sasada, N., Tohoyama, H., Joho, M., 2000. Azuki bean cells are hypersensitive to cadmium and do not synthesize phytochelatins. Plant Physiol. 123, 1029–1036.

Kawakami, S.K., Gledhill, M., Achterberg, E.P., 2006. Determination of phytochelatins and glutathione in phytoplankton from natural waters using HPLC with fluorescence detection. Trends Anal. Chem. 25, 133–142.

Landberg, T., Greger, M., 2004. No phytochelatin (PC_2 and PC_3) detected in *Salix viminalis*. Physiol. Plant. 121, 481–487.

Oven, M., Raith, K., Neubert, R.H.H., Kutchan, T.M., Zenk, M.H., 2001. Homo-phytochelatins are synthesized in response to cadmium in Azuki beans. Plant Physiol. 126, 1275–1280.

Pawlik-Skowrońska, B., 2003. When adapted to high zinc concentrations the periphytic green alga *Stigeoclonium tenue* produces high amounts of novel phytochelatin-related peptides. Aquat. Toxicol. 62, 155–163.

Piechalak, A., Tomaszewska, B., Baralkiewicz, D., Malecka, A., 2002. Accumulation and detoxification of lead ions in legumes. Phytochemistry 60, 153–162.

Rauser, W.E., 1995. Phytochelatins and related peptides: structure, biosynthesis, and function. Plant Physiol. 109, 1141–1149.

Salt, D.E., Prince, R.C., Baker, A.J.M., Raskin, I., Pickering, I.J., 1999. Zinc ligands in the metal hyperaccumulator *Thlaspi caerulescens* as determined using X-ray absorption spectroscopy. Environ. Sci. Technol. 33, 713–717.

Scarano, G., Morelli, E., 2002. Characterization of cadmium- and lead-phytochelatin complex formed in a marine microalga in response to metal exposure. Biometals 15, 145–151.

Scheller, H.V., Huang, B., Hatch, E., Goldsbrough, P.B., 1987. Phytochelatin synthesis and glutathione levels in response to heavy metals in tomato cells. Plant Physiol. 85, 1031–1035.

Sneller, F.E.C., Van Heerwaarden, L.M., Koevoets, P.L.M., Vooijs, R., Schat, H., Verkleij, J.A.C., 2000. Derivatization of phytochelatins from *Silene vulgaris*, induced upon exposure to arsenate and cadmium: comparison of derivatization with Ellman's reagent and monobromobimane. J. Agri. Food Chem. 48, 4014–4019.

Stolt, J.P., Sneller, F.E.C., Bryngelsson, T., Lundborg, T., Schat, H., 2003. Phytochelatin and cadmium accumulation in wheat. Environ. Exp. Bot. 49, 21–28.

Sun, Q., Ye, Z.H., Wang, X.R., Wong, M.H., 2005. Increase of glutathione in mine population of *Sedum alfredii*: A Zn hyperaccumulator and Pb accumulator. Phytochemistry 66, 2549–2556.

Sun, Q., Ye, Z.H., Wang, X.R., Wong, M.H., 2006. Analysis of phytochelatins and other thiol-containing compounds by RP-HPLC with monobromobimane pre-column derivatization. Front. Chem. China 1, 54–58.

Sun, Q., Ye, Z.H., Wang, X.R., Wong, M.H., 2007. Cadmium hyperaccumulation leads to an increase of glutathione rather than phytochelatins in the cadmium hyperaccumulator *Sedum alfredii*. J. Plant Physiol. 164, 1489–1498.

Wójcik, M., Tukiendorf, A., 2004. Phytochelatin synthesis and cadmium localization in wild type of *Arabidopsis thaliana*. Plant Growth Regul. 44, 71–80.

Wójcik, M., Vangronsveld, J., Tukiendorf, A., 2005. Cadmium tolerance in *Thlaspi caerulescens* I. Growth parameters, metal accumulation and phytochelatin synthesis in response to cadmium. Environ. Exp. Bot. 53, 151–161.

Wójcik, M., Skórzyńska-Polit, E., Tukiendorf, A., 2006. Organic acids accumulation and antioxidant enzyme activities in *Thlaspi caerulescens* under Zn and Cd stress. Plant Growth Regul. 48, 145–155.

Yang, X.E., Long, X.X., Ni, W.Z., Fu, C.X., 2002. *Sedum alfredii* H: a new Zn hyperaccumulating plant first found in China. Chin. Sci. Bull. 47, 1634–1637.

Yang, X.E., Long, X.X., Ye, H.B., He, Z.L., Calvert, D.V., Stoffella, P.J., 2004. Cadmium tolerance and hyperaccumulation in a new Zn-hyperaccumulating plant species (*Sedum alfredii* Hance). Plant Soil 259, 181–189.

Zenk, M.H., 1996. Heavy metal detoxification in higher plants – a review. Gene 179, 21–30.

Zhou, W.B., Qiu, B.S., 2005. Effects of cadmium hyperaccumulation on physiological characteristics of *Sedum alfredii* Hance (Crassulaceae). Plant Sci. 169, 737–745.