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Subcellular distribution and chemical forms of cadmium in *Phytolacca americana* L.

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ABSTRACT

Phytolacca americana L. (pokeweed) is a promising species for Cd phytoextraction with large biomass and fast growth rate. To further understand the mechanisms involved in Cd tolerance and detoxification, the present study investigated subcellular distribution and chemical forms of Cd in pokeweed. Subcellular fractionation of Cd-containing tissues indicated that both in root and leaves, the majority of the element was located in soluble fraction and cell walls. Meanwhile, Cd taken up by pokeweed existed in different chemical forms. Results showed that the greatest amount of Cd was found in the extraction of 80% ethanol in roots, followed by 1 M NaCl, d-H_2O and 2% HAc, while in leaves and stems, most of the Cd was extracted by 1 M NaCl, and the subdominant amount of Cd was extracted by 80% ethanol. It could be suggested that Cd compartmentation with organo-ligands in vacuole or integrated with pectates and proteins in cell wall might be responsible for the adaptation of pokeweed to Cd stress.

1. Introduction

Cadmium (Cd) is a widespread heavy metal, released to the environment as a consequence of industrial and agricultural activities [1]. Cd accumulation in soil is becoming a major environmental problem, due to its great toxicity and high mobility from soil to plants and further to the food chain [2,3]. Excess Cd in plants can profoundly interfere with a series of physiological processes such as enzyme activity, respiration, photosynthesis [3], and nutrient element assimilation [4,5]. In order to avoid Cd toxicity, plants have developed intra and extra cellular mechanisms for metal detoxification, such as binding and precipitation in the cell wall and/or compartmentalization in vacuoles [6,7]. However, the mechanisms have not been well elucidated for the great variation in Cd tolerance among plant species or genotypes within a species.

There is some evidence that subcellular distribution and chemical forms of heavy metal may be associated with metal tolerance and detoxification in plants. Ramos et al. [8] observed that in lettuce most of Cd was present in the cell wall fraction, and similar subcellular distribution pattern has been reported in ramie [9]. Meanwhile, Wu et al. [10] compared Cd distribution and chemical form between Cd-resistant and Cd-sensitive barley genotypes, and found that the former had a larger concentration of pectates and protein integrated Cd, most of which was distributed in the soluble and cell wall containing fractions. However, the studies to date have not yet provided consistent results. For example, Wang et al. [9] found that the greatest amount of Cd was in the form of pectates/protein integrated Cd and insoluble Cd–phosphate complexes.

Phytolacca americana (pokeweed) is a vigorous, high yielding species and grown widely in China. It was found to have a high potential in co-accumulating high concentrations of Mn and Cd in shoots at the contaminated sites in Xiangxi area, China [11,12]. Meanwhile, the interaction between Mn and Cd in plant uptake indicated that Mn and Cd ions might use the same transport system for metal uptake by pokeweed [13]. Furthermore, we investigated Mn accumulation, subcellular distribution, chemical speciation and interactions with calcium in the plant, and concluded that excess Mn in soluble fraction of leaf cells (most likely in vacuoles) could contribute to Mn hyperaccumulation and detoxification in pokeweed in previous studies [14,15]. However, to our best knowledge, little information is available on Cd distribution pattern in response to Cd stress in the plant species concerned. Therefore, the aims of this study were to investigate the characteristics of Cd subcellular distribution and chemical forms in pokeweed and their implication in Cd tolerance.

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2. Materials and methods

2.1. Plant materials and growth conditions

The seeds of pokeweed were obtained from the tailing wastelands at Xiangtan mine areas of Hunan Province, China. After germinated in a plastic basin filled with sand, the seedlings were transplanted and grown hydroponically in 3.5 L containers filled with Hoagland nutrient solution with 3 plants per container. The solutions were adjusted to pH 6.0 with 0.1 M NaOH or 0.1 M HCl, and renewed every three days. Plant culture was performed in a greenhouse (25/20 °C day/night; 16 h/d light; 70–75% relative humidity). After 14 d growth, plants were subjected to different treatments. The $Cd(NO_3)_2$ was added into solutions at the concentration of 0, 25, 50 and 100 μM. Each treatment was conducted with three replicates. At the end of the experiment (after 7 d exposure to Cd), plants were harvested and separated into roots, stems and leaves, and immediately frozen in liquid N_2 and kept frozen until use.

2.2. Tissue fractionation

Frozen materials were pretreated according to the method described by Lozano-Rodriguez et al. [16]. In brief, plant tissues were homogenized in extraction buffer (50 mM HEPES, 1.0 mM DTT, 500 mM sucrose, 5.0 mM ascorbic acid, 1.0% (w/v) Polyclar AT PVPP, adjusted to pH 7.5 with NaOH). The homogenate was sieved through a nylon cloth (100 μm mesh size) and the residue constituted the cell wall-containing fraction. The filtrate was centrifuged at 10,000 × *g* for 30 min and the pellet retained was the organelle-rich fraction. The supernatant was then centrifuged at 100,000 × *g* for 30 min and the pellet designated as the membrane-containing fraction and the supernatant as the soluble fraction. The resultant pellets were resuspended in extraction buffer. All steps were performed at 4 °C. The fractions were dried and wet digested separately, and then Cd concentrations in the digests were determined by M6 Thermo flame atomic absorption spectrometry (AAS).

2.3. Chemical forms extraction

To determine chemical forms of Cd in pokeweed, the experiment was carried out by the designated solutions in the following order [17]: (1) 80% ethanol, extracting inorganic Cd giving priority to nitrate/nitrite, chloride, and aminophenol cadmium. (2) Deionized water (d-H_2O), extracting water soluble Cd-organic acid complexes and $Cd(H_2PO_4)_2$. (3) 1 M NaCl, extracting Pectates and protein integrated Cd. (4) 2% Acetic acid (HAc), extracting undissolved cadmium phosphate including $CdHPO_4$ and $Cd_3(PO_4)_2$ and other Cd–phosphate complexes. (5) 0.6 M HCl, extracting cadmium oxalate.

Frozen tissues were homogenized in extraction solution with a mortar and a pestle, diluted at the ratio of 1:100 (w/v), and shaked for 22 h at 25 °C. After that, the homogenate was centrifuged at 5000 × *g* for 10 min, obtaining the first supernatant solution in a conical beaker. The sedimentation was re-suspended twice in extraction solution and shaked 2 h at 25 °C, centrifuged at 5000 × *g* for 10 min, then pooled the supernatant of the three suspending and centrifuge steps for each of the five extraction solutions. Each of the pooled supernatant solution was then evaporated on an electric plate at 70 °C to constant weight, and digested at 145 °C with an acid oxidative mixture of HNO_3:$HClO_4$ (2:1, v/v).

2.4. QA/QC control and statistic analysis

Quality assurance and quality control (QA/QC) for Cd in plants were conducted by using the Merk cadmium atomic spectroscopy standard solution which was traceable to standard reference material (SRM) from National Institute of Standards and Technology (NIST), USA. Reagent blank and analytical duplicates were also used where appropriate to ensure accuracy and precision in the analysis. Data were expressed as means and standard deviations (SDs). The data were statistically analyzed with one-way analysis of variance using the SPSS 16.0 program. Least significant difference (LSD) was used for multiple comparisons between different treatment means.

Table 1
Subcellular distribution of Cd in pokeweed leaves.

Cd in solution (μM)	Cd in subcellular fractions (mg/kg, FW)			
	Cell wall	Organelles	Membranes	Soluble fraction
25	6.40 ± 1.39a*	1.04 ± 0.11a	0.57 ± 0.03a	10.51 ± 1.37a
50	6.36 ± 0.42a	0.94 ± 0.22a	0.68 ± 0.12a	13.29 ± 2.92a
100	13.95 ± 1.85b	2.10 ± 0.88b	0.98 ± 0.15b	19.59 ± 1.32b

* Different letters in the same column indicate a significant difference at the 5% level.

3. Results and discussion

3.1. Subcellular distribution of Cd

We investigated the subcellular distribution of Cd in pokeweed leaves and roots, which are shown in Tables 1 and 2, respectively. Both in leaves and roots, most of the Cd was present in the soluble and cell wall containing fraction, while a minor part of this element associated with the organelle and membrane fraction. Moreover, compared with 25 and 50 μM Cd treatments, Cd concentration in the different subcellular fractions was markedly increased in the plants treated with 100 μM Cd. Meanwhile, the proportion of Cd in different subcellular fractions remained fairly constant for all treatments in leaves. However, in roots, with increasing Cd supply in the medium, relative accumulation of Cd in soluble fraction decreased and the proportion of Cd in organelles increased, respectively (Fig. 1).

In our study, Cd analysis at the subcellular level of plant tissue demonstrated that large proportion of Cd (53.7–68.3%) was stored in the soluble fraction (Fig. 1). As the vacuole is a dynamic organelle that comprises as much as 90% of the total cell volume in some cell types [18], we may deduce that the vacuole was the predominant sink for Cd. Complexation of metals with organo-ligands within the storage sites results in decreased free ion activity and thus reduced toxicity, and these organo-ligands for Cd compartmentation in vacuole are mainly sulfur-rich peptides and organic acids [2,19]. In addition, pokeweed has an intrinsically high content of oxalate in leaves [15], chelation of Cd by oxalate could be an essential detoxification mechanism to render excess Cd inactive, and therefore we may think that oxalic acid could play an important role in Cd accumulation and detoxification in pokeweed.

For plant cell walls, which function as the first barrier protecting the protoplast from Cd toxicity, are mainly composed of polyose (including cellulose, hemicellulose and pectin) and protein, providing negative charge sites on their surfaces, and so can bind Cd ions and restrict their transportation across cytomembrane. In our

Table 2
Subcellular distribution of Cd in pokeweed roots.

Cd in solution (μM)	Cd in subcellular fractions (mg/kg, FW)			
	Cell wall	Organelles	Membranes	Soluble fraction
25	16.6 ± 2.53a*	4.36 ± 0.68a	2.12 ± 0.12a	53.36 ± 1.87a
50	19.66 ± 2.15a	4.18 ± 0.39a	2.02 ± 0.24a	42.99 ± 2.99b
100	26.18 ± 1.81b	6.98 ± 0.92b	3.21 ± 0.21b	53.92 ± 7.35a

* Different letters in the same column indicate a significant difference at the 5% level.

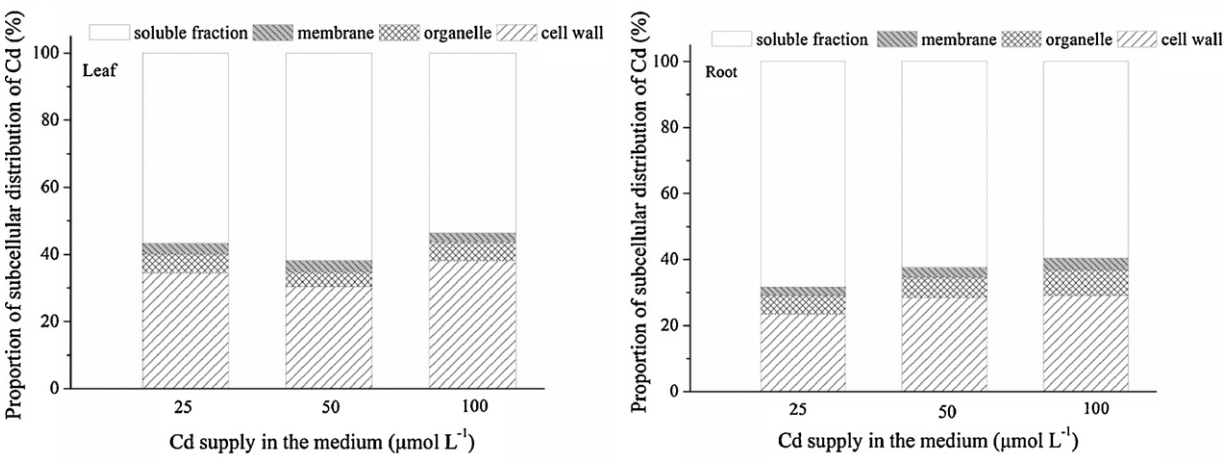

Fig. 1. Proportion of subcellular distribution of Cd in organs of pokeweed.

study, a less proportion (23.4–29.1%) of Cd was bound to the cell wall fraction, suggesting that the cell wall is another large buffer which can accumulate heavy metals and therefore is believed to play a role in metal tolerance. Meanwhile, we found that in plant root, following the increase of Cd concentration in solution, the proportion of Cd in the cell wall fraction increased while in the soluble fractions decreased (Fig. 1), indicating the limitation for root cell walls to subsequently translocate Cd to shoots. These results were in line with those presented for Cd-treated barley [10] and soybean [20] plants. In contrast to the present results, several studies found that the main site of Cd accumulation in plant roots is the apoplast, particularly cell walls [8,9,21]. These differences may be attributed to the distinct Cd concentrations used by the different authors and also to the variable levels of Cd tolerance of the plants.

Meanwhile, the mechanisms of Cd storage and detoxification in different organs of plants seem to be quite different. Though both in leaves and roots, the majority of the element was present in the soluble and cell wall fraction, the proportion of Cd in different subcellular fractions acted inconsistently to different level of Cd addition. These results are to some extent in line with those found by Wójcik et al. [21], for they detected Cd almost in all root tissues and different cell compartments in hyperaccumulator *Thlaspi caerulescens* using energy-dispersive X-ray microanalysis, while in leaves Cd was found only in electron-dense deposits inside vacuoles. It could be suggested that in hyperaccumulators, the cell wall plays a significant role in Cd retention in roots, while in leaves the main mechanisms of Cd detoxification are located inside cells, in vacuoles.

3.2. Chemical forms of Cd

In the present study, we failed to detect any significant differences between 25 and 50 μM Cd treatments; therefore we did not examine chemical speciation of Cd under 50 μM Cd treatments. We found that Cd concentrations bound to different chemical forms in the plant increased in a concentration-dependent manner following Cd exposure (Figs. 2–4). In roots, the Cd forms extracted by 80% ethanol and 1 M NaCl were predominant, representing 67% and 28% of the total amount, averaged over 2 Cd levels, while the forms extracted by other solutions were rather low. In leaves and stems, the extraction of 1 M NaCl occupied the largest proportion of Cd (75% in leaves and 77% in stems), followed by extraction of 80% ethanol (14% in leaves and 10% in stems), extraction of d-H_2O (5% in leaves and 6% in stems) and extraction of 2% HAc (5% in leaves and 6% in stems), extraction of 0.6 M HCl had the lowest Cd.

Chemical speciation of heavy metals is closely related to their biological function, and different chemical forms of Cd extracted by different designated extraction solutions have distinct toxicity degree and migration of Cd. For instance, inorganic and organic water-soluble Cd (extracted by 80% ethanol and d-H_2O, respectively), with higher capacity to migrate, is more deleterious to plant cells than the undissolved Cd phosphate (extracted by 2% HAc) and cadmium oxalate (extracted by 0.6 M HCl) [9]. Meanwhile, for plants containing high concentration of Cd that showed no or little toxicity, Cd should be in a chemical form that causes low or no

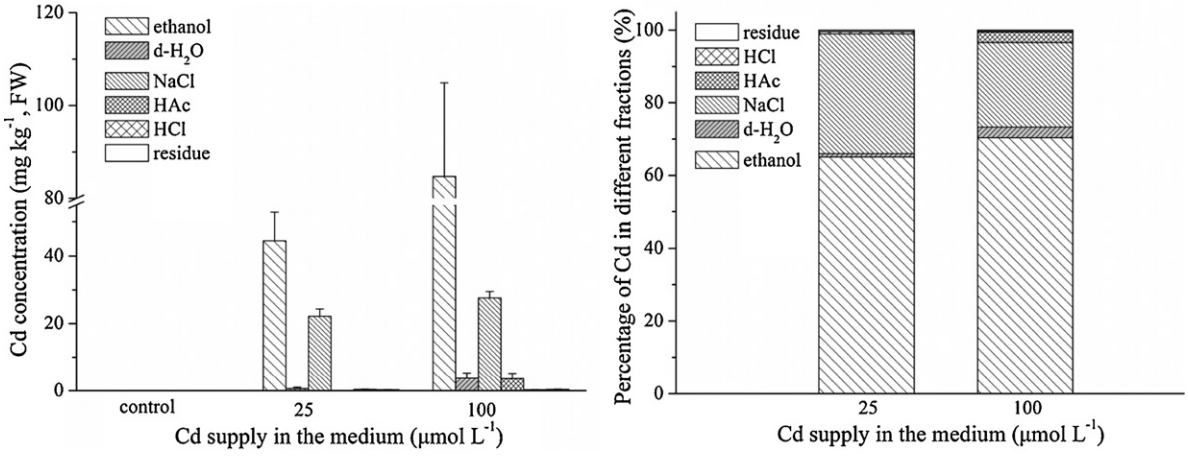

Fig. 2. Different chemical forms of Cd and its proportion in roots of pokeweed.

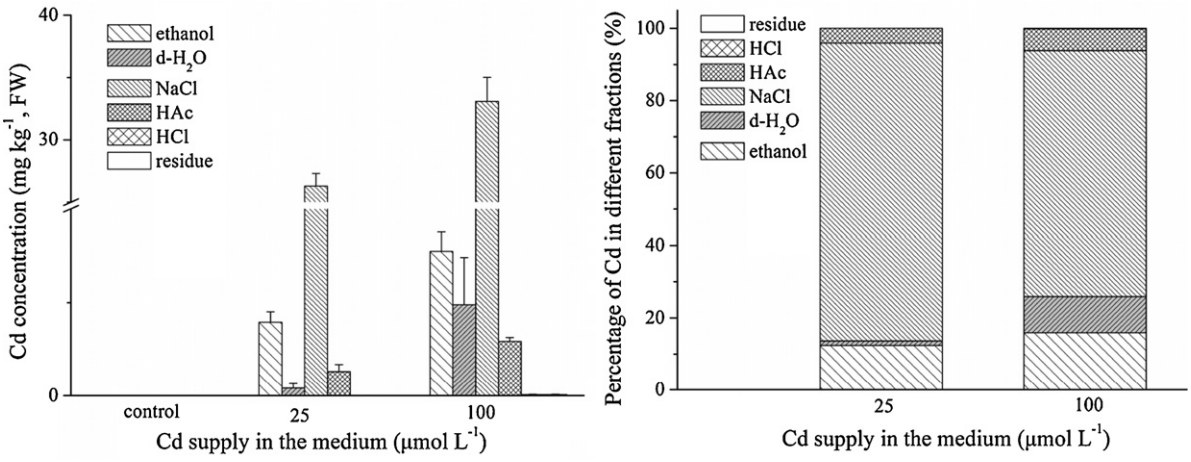

Fig. 3. Different chemical forms of Cd and its proportion in leaves of pokeweed.

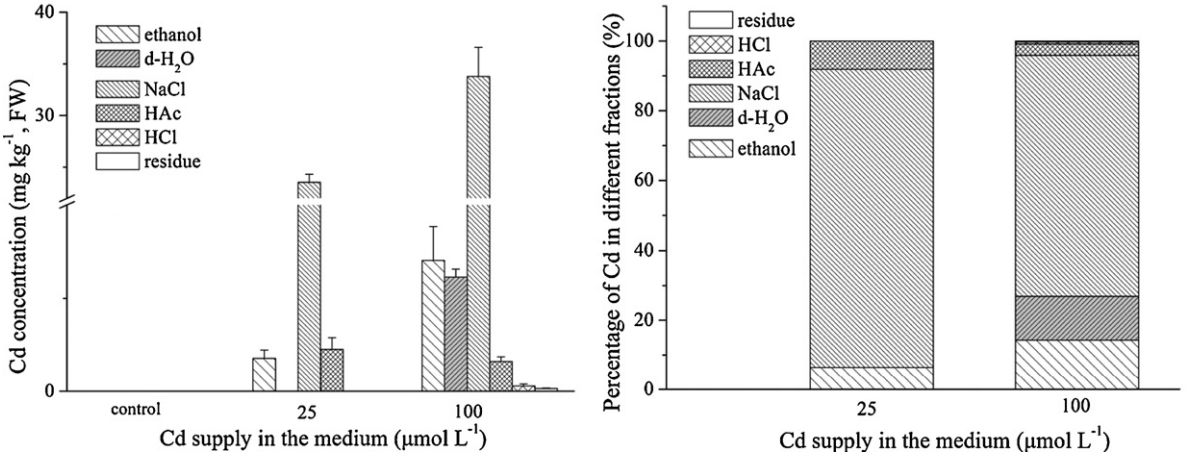

Fig. 4. Different chemical forms of Cd and its proportion in stems of pokeweed.

phytotoxicity. In our study, Cd was existed in different chemical forms among different pokeweed tissues. In roots, the majority of Cd was in inorganic form (Fig. 2), suggesting high capacity to be transported to aboveground tissues, while in stems and leaves (Figs. 3 and 4), most Cd was integrated with pectates and protein (extracted by 1 M NaCl). Therefore, Cd was hypothesized to be chelated by some specific polar material, such as hydroxyl or carboxyl, to form a non-toxic complex. It may also be assumed that larger percentages of 80% ethanol-extractable Cd in roots and NaCl-extractable Cd in shoots are responsible for the adaptation of pokeweed to Cd high accumulation and stress, which stands for the point of view that compartmentation of vacuolar and sequestration in cell wall may be crucial for the detoxification of Cd and thus tolerance to metal stress.

It has been well documented that one of the strategies for plants to tolerate Cd is the complexation with strong ligands such as thiol groups provided by phytochelatins and glutathione [22]. Nevertheless, Isaure et al. [23] found that the majority of Cd was bound to O/N ligands likely provided by the cell wall, and a minor fraction could be bound to S-containing ligands in *Arabidopsis thaliana* using micro X-ray absorption near edge structure (μ-XANES) spectroscopy. From our analysis, we could not conclude about the occurrence of Cd complexes with pectate or organic acids (mostly oxalate) other than phytochelatins through successive extraction. Therefore, further studies should be conducted using micro X-ray absorption analysis or other advanced techniques to determine the direct complexes of Cd in plants.

Furthermore, genotypic difference may exist in heavy metal tolerance and detoxification. A Cd-sensitive barley genotype was found to have higher Cd concentration in inorganic and water-soluble forms, while lower in pectates/protein integrated Cd in comparison with the other three Cd-resistant genotypes [10]. However, Cd accumulation seems to be a constitutive trait of pokeweed. Not only our former studies (data not shown) but also several other studies have shown that different ecotypes of pokeweed from uncontaminated or contaminated site could accumulate similar level of Cd [24]. Since pokeweed is grown widely in China, the lack of Cd sensitive ecotypes may be due to insufficient surveys. So, more field studies are needed to seek Cd sensitive ecotype to well document the mechanisms of Cd tolerance and detoxification.

This work is still at a preliminary stage, and further analyses are required to examine distribution of Cd and its bonding state at the ultrastructural level, and to improve our understanding of the effects of Cd on cell ultrastructure within the plant. Meanwhile, we also plan to investigate interaction mechanisms between Cd and Mn in pokeweed. A better understanding of metal sequestration in plants may eventually contribute towards the development of biorecovery techniques for the remediation of soils contaminated with these two metals.

4. Conclusions

In summary, Cd analysis at the subcellular level of plant tissue demonstrated that large proportion of Cd was stored in the sol-

uble fraction or bound to the cell wall fraction. Besides, Cd must be existed in different chemical forms among different tissues and Cd concentrations bound to each form in the plant increased in a concentration-dependent manner following Cd exposure. In roots, the majority of Cd was in inorganic form, while in stems and leaves most of the Cd was integrated with pectates and protein. From our study, we deduce that both the vacuoles and the cell walls might be involved in the Cd tolerance mechanisms to protect metabolically active cellular compartments from toxic Cd concentrations.

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