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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.