Isolation of Mercury-binding Peptides in Vegetative Parts of *Chromolaena odorata*

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Mercury-binding peptides from roots, stems, and leaves of Hg-treated *Chromolaena odorata* plants were isolated and partially characterized using RP-HPLC and ESI-MS. Upon exposure of *C. odorata* plants to high concentrations of 1.0 and 2.0 μM $Hg(NO_3)_2$ treatments from 0–28 days, they accumulated as much as 125 mg/g (dry wt) Hg in the roots, 15.280 mg/g (dry wt) Hg in the stems, and 0.800 mg/g (dry wt) Hg in the leaves indicating that *C. odorata* has a high potential as a phytoremediation agent of inorganic mercury. The plant's ability to accumulate and sequester Hg ions was primarily attributed to the production of Hg-binding peptides, which were initially detected through the use of Ellman's reagent. Isolation techniques using RP-HPLC equipped with a C18 column manifested a single prominent peak consistently appearing at a retention time of 2.6–2.8 min in all the plant samples treated with different Hg concentrations at varying lengths of exposure. Further characterization of this prominent peak using electrospray ionization mass spectrometry revealed the presence of a peptide containing several cysteine residues with the highest peak concentration recorded at 91 mV and 89 mV in roots and stems of plants treated with 2.0 μM $Hg(NO_3)_2$ for 4 wk ($P < 0.05$) and 85 mV in leaves treated with 1.0 μM $Hg(NO_3)_2$ for 1 wk.

Key words: Chromolaena odorata, Phytoremediation, Phytochelatin

Introduction

In a Hg-contaminated mine tailings dumpsite in Itogon, Benguet, Philippines, the noxious weed *Chromolaena odorata* (L.*f.*) R. M. King et H. Robinson was surveyed to be one of the most dominant in the area accumulating highly toxic levels of Hg concentrations. The high tolerance of *C. odorata* plants to phytotoxic concentrations of Hg can be attributed to the presence of Hg-binding peptides which function to scavenge intracellular Hg ions translocated by the plant into its system away from its essential metabolic activities. However, the chelation of the Hg ion by a ligand may come in the form of organic acids, amino acids, or Cys-containing peptides (Briat and Lebrun, 1999).

Metal induced, sulfhydryl-rich peptides known as phytochelatins (PCs) have been described as effective chelators and detoxifiers of heavy metal ions. Several types of phytochelatins which are produced by varying types of plants with different heavy metal (Cd, Pb, Cu) treatments have been successfully isolated and sequenced. The differences are primarily due to variants of GSH, the precursor of phytochelatins, with different C-terminal residues found in some plants. The most prevalent phytochelatins synthesized from GSH in most plants have a glycine C-terminus (γ-Glu-Cys)n–Gly (n = 2 to 11) (Grill *et al.*, 1987; Hayashi and Mutoh, 1994; Mehra, 1998; Ohtake *et al.*, 1990; Zenk, 1996). In other plants, however, no glycine C-terminus was found producing repeating sequences of (γ-Glu-Cys)n (Grill *et al.*, 1986). Plants synthesizing homoglutathione in place of GSH produce phytochelatins with Ala at its C-terminus (γ-Glu-Cys)n–Ala (Grill *et al.*, 1987). Moreover, four sets of phytochelatin peptides have recently been discovered; all are homologous to GSH but with different C-terminal residues. One contains β-alanine instead of glycine thus named homophy-

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tochelatin (h-PC) with the following sequence: (γ-Glu-Cys)*n*-β-Ala (*n* = 2 to 7) (Grill *et al.*, 1987). These are prevalent in order Fabales, family Phaseoleae (Zenk, 1996). However, plants in the Poaceae family contain hydroxymethyl-GSH synthesizing hydroxymethyl-PCs with the following sequence: (γ-Glu-Cys)*n*-Ser (Klaphek *et al.*, 1994). Another set of peptides, (γ-Glu-Cys)*n*-Glu, is also induced by Cd in maize (Meuwly *et al.*, 1995). And more recently, roots of Cd-treated horseradishes were detected to have (γ-Glu-Cys)*n*-Gln using electrospray ionization mass spectroscopy (Kubota *et al.*, 2000).

Unfortunately, no plant has yet been identified to naturally hyperaccumulate mercury (Henry, 2000; Raskin and Ensley, 2000). Only transgenic plants such as *Arabidopsis thaliana*, *Liriodendron tulipifera*, and *Nicotiana tabacum* which were inserted with bacterial *mer* A and *mer* B genes to convert Hg^{2+} to Hg^0 and methyl mercury to Hg^{2+} were developed to provide potential phytoremediation steps in alleviating Hg-polluted areas (Bizily *et al.*, 1997; Rugh *et al.*, 1996, 1998).

Moreover, studies involved in extracting and isolating phytochelatins induced by Hg treatments in higher plants have not been well elucidated. In a study conducted by Satoh *et al.* (1999), high levels of nonproteinaceous thiol-containing compounds were exhibited by a unicellular green algae, *Tetraselmis tetrethele* (Prasinophyceae), upon mercury and cadmium exposure. However, no phytochelatins have been detected. Further identification of these thiol-containing compounds using HPLC reveals the presence of glutathione and a tripeptide, Arg-Arg-Glu, which appears to bind and scavenge Hg and Cd ions. In higher plants, further isolation and characterization of Hg-binding peptides remain to be explored. Thus, it was the objective of this study to isolate, identify, and partially characterize the Hg-binding peptides involved in *C. odorata.*

Materials and Methods

Plant material and Hg treatment

Cuttings of *Chromolaena odorata* plants measuring 10–12 cm in height were surface sterilized for 5 s using hydrogen peroxide, then washed with sterilized water three times. Plants were grown in 10-l basins (12 × 6 × 4) filled with ½ strength Hoagland's solution with 0.01% indole acetic acid (IAA) to induce root formation. After 2 wk of acclimatization and root formation, these seedlings were subjected to Hg treatments with modified ½ Hoagland's solution. Inorganic mercury in the form of $Hg(NO_3)_2$ (Merck Brand, 99.9%–100.5%) was used to prepare 2 concentrations, *i.e.*, 1.0 and 2.0 μM $Hg(NO_3)_2$, which were added to the nutrient solution. 30 ml of deionized water were added everyday to maintain solution volume. Nutrient solution was maintained at pH 6.5. Harvests were done after 1 wk every wk for 4 consecutive wks.

Determination of total Hg content

Total Hg content was determined using CV-AAS (thermo Jarrel Ash Atomic Absorption Spectro Video 11e) as subscribed from the methods of Kloke (1981). Harvested plant samples were initially sprayed with 0.1 M EDTA at the roots. Plants were then separated into roots, stems, and leaves, air-dried for 24 h, digested overnight in conc. HNO_3, then submitted to the Institute of Chemistry for CV-AAS analyses.

Harvested plants from each Hg concentration were segregated into roots, stems, and leaves. 2-cm samples from each of the plant parts were specifically cut from the following: (1) root tip, 0.5 cm from tip; (2) stem, 1 cm above base of stem cutting; (3) petiole of leaf at the 2nd node from stem base; and (4) base of leaf from the 2nd node. These were preserved in FAA then subjected to microtechnique using saffranin red and fast green as stains.

Extraction, fractionation, and isolation of Hg-binding biomolecules

Extraction methods used to isolate the Hg-binding peptides were adopted from Grill *et al.* (1986) and Keltjens and van Beusichem (1998) with some modifications. 1 g of the plant material was washed with deionized water, freeze-dried with liquid nitrogen, and homogenized for 5 min in 10 ml of N_2-saturated extraction solution (5% w/v sulfosalicylic acid added to 6.3 nM diethylenetriaminepentaacetic acid) using a mortar and pestle. The homogenate was centrifuged for 45 min at 3,000 rpm at 4 °C. The supernatant was subjected immediately to gel filtration using Sephadex G-50 (1.5 × 92 cm), equilibrated with 0.05 M sodium phosphate buffer (pH 7.6). The plant extract was eluted at a flow rate of 30 ml/h and each of the fifteen 10-ml fractions collected was spectrophotometrically

monitored for SH-containing compounds. A 200-μl aliquot from each of the 15 fractions was mixed with 1 ml of 0.2 M Tris-HCl (pH 8.2) and 0.15 ml of 10 mM 5′,5′-dithiobis-nitrobenzoic acid (DTNB) were added. These were incubated in ice for 20 min then spectrophotometrically read at 412 nm. Peak fractions detected to contain 0.200 OD and higher amounts of SH-containing compounds were pooled and lyophilized prior to RP-HPLC analysis.

Isolation of Hg-binding peptides was subscribed from the methods of Grill *et al.* (1987) with some modifications from Bruns *et al.* (1999), Kubota *et al.* (1995), and Vogeli-Lange and Wagner (1990). Approx. 0.1 mg of the precipitates pooled from the lyophilized fractions was dissolved in 0.5 ml of 20% acetonitrile in 0.1% TFA. 25 μl of the sample was injected into a C18 analytical column (Inertsil ODS-80A, 4.6 mm i.d. × 250 mm) using reverse phase high performance liquid chromatography (Shimadzu LC-10AD) for optimization and detection purposes. The gradient conditions were controlled by a system controller (Shimadzu SCL-10A) with 20% acetonitrile in 0.1% (v/v) TFA as solvent with a flow rate of 1 ml/min. Detection was set at 214 nm. Prominent peaks were collected separately and lyophilized for further analyses.

Partial characterization of the Hg-binding peptides

Partial characterization of the Hg-binding peptides was subscribed from the methods of Kubota *et al.* (2000) with some modifications. Lyophilized samples isolated as prominent peaks from RP-HPLC were dissolved in pure methanol (Merck, HPLC grade) and subsequently introduced into an electrospray ionization mass spectrometer (Thermo Quest LCQ) by infusion. In addition, approx. 0.1 g of the lyophilized peak sample was digested with conc. HNO_3 and subjected to CV-AAS analyses to confirm if these peaks isolated from RP-HPLC were bound to mercury.

Results and Discussion

Total Hg content

Chromolaena odorata plants generally exhibited high Hg uptake in the different Hg treatments at varying lengths of exposure ($P < 0.001$). Among the three vegetative organs, the roots accumulated the highest levels of Hg compared to stems and leaves ($P < 0.001$). Total Hg levels were measured per dry wt of samples. As presented in Fig. 1A, the roots of *C. odorata* plants contained the highest amounts of total Hg accumulating as much as 125 mg/g Hg after 4 wk of 2.0 μM $Hg(NO_3)_2$ treatment ($P < 0.05$) compared to the stems (Fig. 1B) which localized 15 mg/g Hg after 4 wk of 2.0 μM $Hg(NO_3)_2$ ($P < 0.05$) and finally, the leaves (Fig. 1C) translocated as much as 880 μg/g Hg (dry wt) after 3 wk of 2.0 μM $Hg(NO_3)_2$ ($P < 0.05$).

Mercury treatments of 1.0 and 2.0 μM $Hg(NO_3)_2$ after 1 to 4 wk induced highly significant levels of Hg in the roots ($P < 0.001$). At 1.0 μM $Hg(NO_3)_2$ concentration, the roots accumulated 3,000 μg/g Hg after 1 wk of Hg exposure ($P < 0.05$) which radically increased to 16,500 μg/g Hg after 2 wk of the same treatment ($P < 0.05$). However, after 3 wk of treatment, Hg levels dramatically decreased to 4,200 μg/g ($P < 0.05$). Although at 4 wk of Hg exposure, Hg levels drastically escalated to 42,700 μg/g Hg ($P < 0.05$) with the plant showing no signs of stress morphologically. Similarly, at 2.0 μM $Hg(NO_3)_2$ treatment, the roots translocated higher amounts of intracellular Hg contents of 14,800 μg/g Hg after 1 wk ($P < 0.05$) but Hg levels significantly dropped to 3,800 μg/g Hg after 2 wk of the same treatment ($P < 0.05$). However, the mercury contents gradually increased after 3 wk of 2.0 μM $Hg(NO_3)_2$ treatment to 4,200 μg/g Hg ($P < 0.05$) then peaked to 125,600 μg/g Hg after 4 wk of the same Hg treatment ($P < 0.05$).

The stems similarly accumulated significant amounts of Hg after 4 wk of 1.0 ($P < 0.001$) and 2.0 μM $Hg(NO_3)_2$ treatment ($P < 0.001$) as exhibited in Fig. 1B. At 1.0 μM $Hg(NO_3)_2$ treatment, 800 μg/g Hg were localized in the stem after 1 wk ($P < 0.05$) which increased to 1,000 μg/g Hg after 2 wk ($P < 0.05$). Amounts of Hg further escalated to 1,200 μg/g ($P < 0.05$) and doubled to 2,400 μg/g after 4 wk of Hg treatment in the stem ($P < 0.05$). Moreover, *C. odorata* plants treated with 2.0 μM $Hg(NO_3)_2$ concentrated relatively significant amounts of Hg in the stem ($P < 0.001$). An estimated 120 μg/g Hg were localized in the stem after 1 wk of 2.0 μM $Hg(NO_3)_2$. This increased to 2,100 μg/g Hg after 2 wk ($P < 0.05$) but declined rapidly to 860 μg/g Hg after 3 wk ($P < 0.05$). However, total Hg content was recorded to be at its highest (15,300 μg/g) ($P < 0.05$) in the stems after 4 wk of 2.0 μM $Hg(NO_3)_2$ treatment.

In addition, as manifested in Fig. 1C, the different Hg treatments at varying lengths of exposure generally did not cause any significant in-

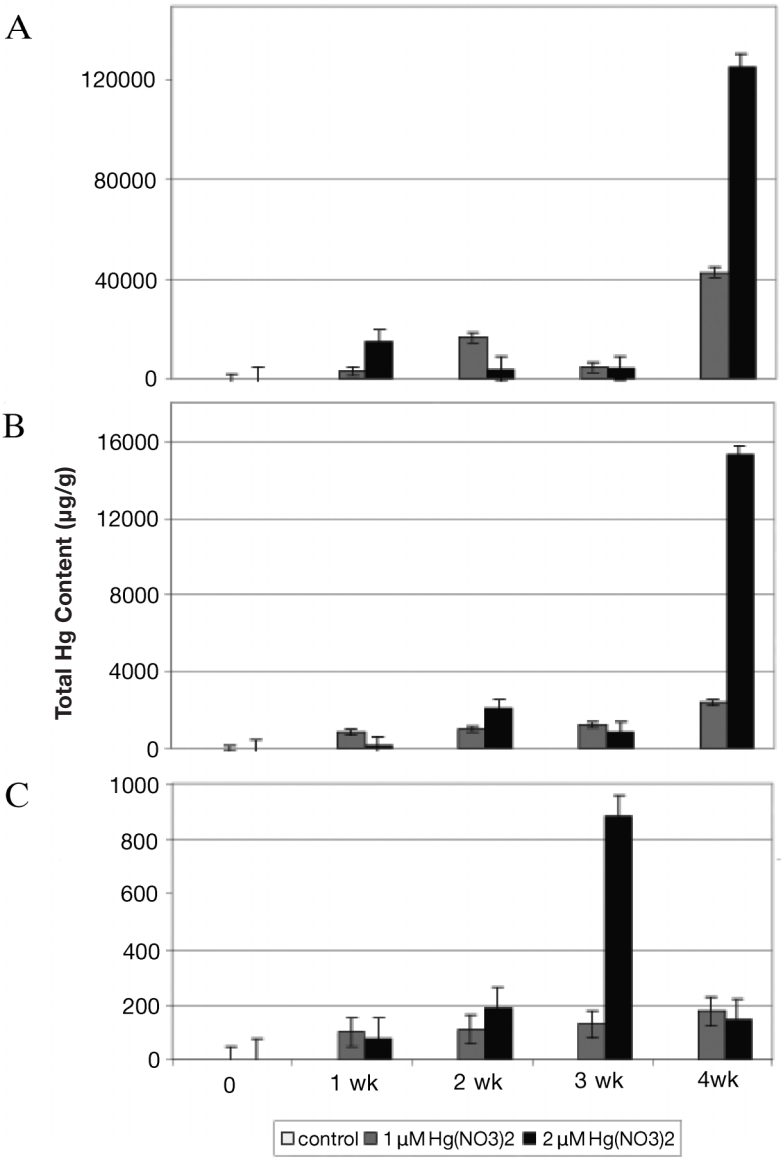

Fig. 1. Total Hg content in (A) roots, (B) stems, and (C) leaves of *Chromolaena odorata* after Hg treatments. Values are means with standard error ($n = 3$).

creases in the total Hg content in the leaves ($P = 0.986$) except after 3 wk of 2.0 μM $Hg(NO_3)_2$ treatment wherein there was a significant increase of 880 μg/g Hg ($P < 0.05$).

Tissue localization of Hg

Cross-sections of roots, stems, and leaves of Hg-treated plants generally exhibited an aggregation of dark, granular deposits that were totally absent in untreated plants. These dark clusters appeared to increase in size with higher Hg treatments and longer exposure periods to the treatments. In roots (Fig. 2A) of plants treated with 1 μM $Hg(NO_3)_2$ after 1 and 2 wk, black granular aggregations were found at the root epidermal layers as well as cortical tissues. However, after 2 to 4 wk of 1.0 μM $Hg(NO_3)_2$ and 1 to 4 wk of 2.0 μM $Hg(NO_3)_2$ treat-

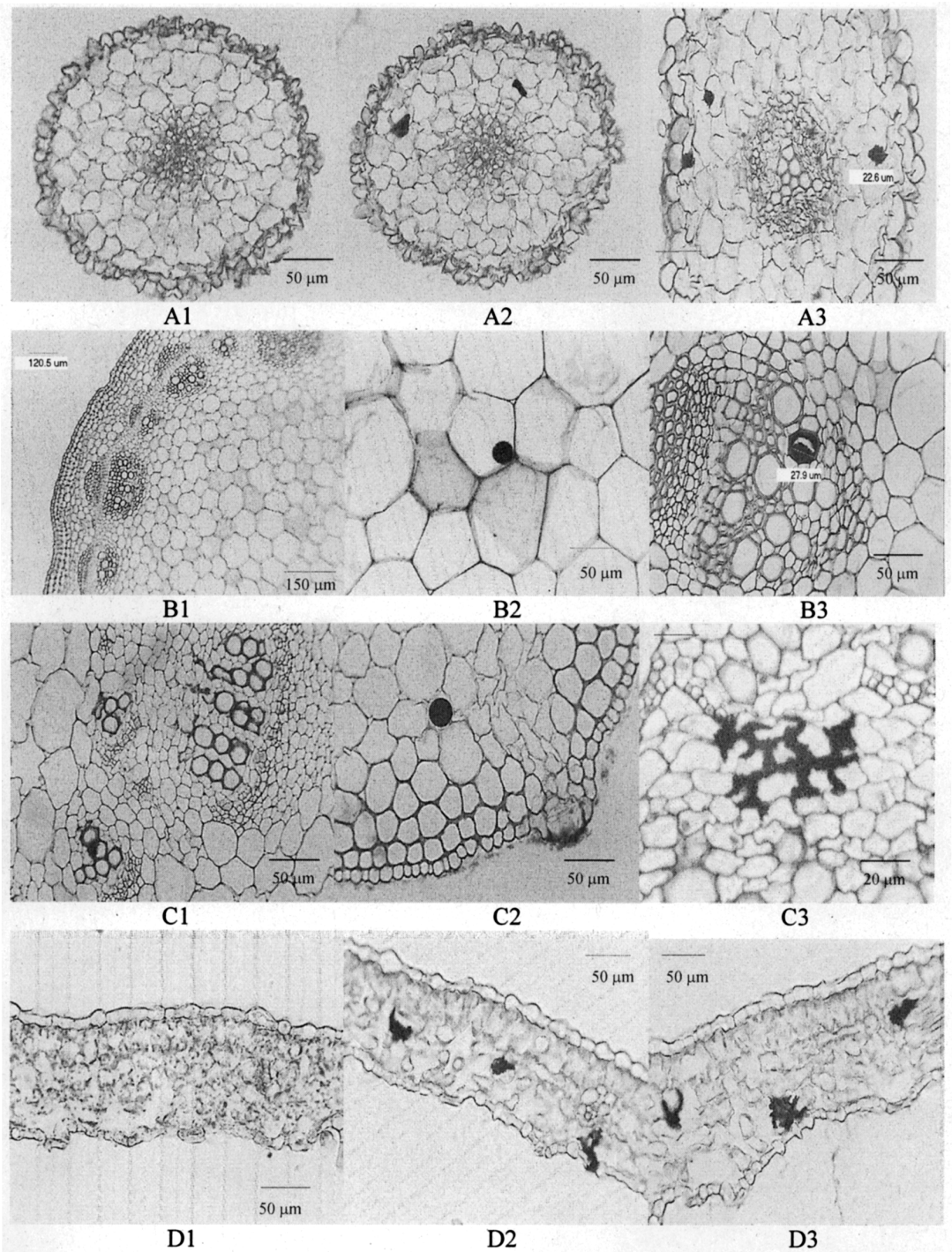

Fig. 2. Cross sections of roots at (A1) control (400x), (A2) 1.0 μM $Hg(NO_3)_2$ for 2 wk (400x), (A3) 2.0 μM $Hg(NO_3)_2$ for 4 wk (400x); stems at (B1) control (100x), (B2) 1.0 μM $Hg(NO_3)_2$ for 2 wk (400x), (B3) 2.0 μM $Hg(NO_3)_2$ for 4 wk (400x); petiole with (C1) 1.0 μM $Hg(NO_3)_2$ for 2 wk (400x), (C2) 1.0 μM $Hg(NO_3)_2$ for 3 wk (400x), (C3) 2.0 μM $Hg(NO_3)_2$ for 4 wk (1,000x); and leaves at (D1) control (400x), (D2) 1.0 μM $Hg(NO_3)_2$ for 2 wk (400x), (D3) 2.0 μM $Hg(NO_3)_2$ for 2 wk (400x). Diameters (μm) of granular deposits were measured in A3 and B3.

ment, these dark aggregations were found to penetrate through the endodermis moving further into the xylem vessels while some were still confined at the epidermal and cortical tissues. In stems, as exhibited in Fig. 2B, these black granular deposits were localized in the stem cortical parenchyma cells after 1 wk then at the xylem and pith after 2–4 wk of 1.0 μM $Hg(NO_3)_2$ treatment. At 2.0 μM $Hg(NO_3)_2$ treatment, these deposits were more pronounced in the xylem vessels after 1 wk exposure, then became visible at the stem pith parenchyma cells after 2 to 4 wk exposure. Figs. 2C and 2D, on the other hand, present cross-sections of petiole and leaves of treated and untreated plants. Cross-sections of petiole and leaves of control were totally devoid of dark aggregates; however, these black aggregates were detected at epidermal outgrowths such as trichomes and epidermal layer of petioles from all the treated plants. Moreover, leaf cross-sections of Hg-treated plants from 1 to 4 wk treatments similarly exhibited dark granular deposits in both the spongy and mesophyll layer and xylem vessels.

However, deposits of electron dense dark, granular spots, which we believe are the accumulated Hg because of its absence in all the control plants, appeared to be small in the root epidermal layer. These black granules clustered into bigger aggregates and localized in the parenchyma cells of the cortex. However, the biggest dark granular spots were found in the dead cells of the xylem illustrating further that the accumulated Hg was translocated upstream. The roots contained the highest Hg levels simply because they have a greater surface area to absorb and localize Hg into their epidermis, cortex, and xylem. These are then loaded in the xylem sap and translocated to the aerial parts of the plants through transpirational stream and stomatal conductance. Thus, high levels of Hg content were similarly found in stems and leaves. Dark aggregations of granular spots were confined in parenchymatous cells of the cortex and pith, which renders the Hg ions less toxic and away from the plant's basic metabolic activities. Moreover, since there were no electron dense, dark granular deposits found in the phloem, there was only an upward transport of mercury and no redistribution from leaves to roots. Thus, the leaves may have served as a waste depot of mercury particularly in the trichomes and mesophyll layer of the leaves in both the spongy and palisade cells.

Hg-binding peptides

Detection and isolation techniques conducted using RP-HPLC revealed only one prominent peak, which appeared at 2.6–2.8 min in all the Hg-treated plant samples corresponding to the elution time of cysteine. This was similarly one of the findings of Kubota *et al.* (1995) wherein cysteine, GSH, and phytochelatins were identified as the SH-containing compounds involved in Cd detoxification in normal root cultures of *Rubia tinctorum.* Cysteine particularly eluted at a retention time of 2.8 min. However, the relative concentration of this peak varied significantly which increased at higher Hg treatments ($P < 0.001$) and at longer Hg exposures ($P < 0.001$). All the control samples from the vegetative parts generally did not exhibit any peak at 214 nm. Mercury-treated roots, stems, and leaves were observed to contain relatively high amounts of this single prominent peak. Moreover, root concentrations of this peak appeared to increase at longer Hg treatment indicating that prolonged Hg exposure induces the synthesis of cysteine to deal with the increased Hg uptake of the plant. The highest peak concentration was recorded at 91 mV in roots and 89 mV in stems of plants treated with 2.0 μM $Hg(NO_3)_2$ for 4 wk ($P < 0.05$). However, in leaves, the highest peak concentration was exhibited by 1.0 μM $Hg(NO_3)_2$ treated plants for 1 wk at 85 mV. Moreover, after 2 wk of varying Hg treatments, lyophilized samples from leaf extracts started to exhibit 2 prominent peaks. The first peak eluted at an earlier retention time of 1.663 min and the second peak at 2.796 min. It was also observed that treatments with higher Hg concentrations and longer Hg exposures for two to four wk induced the synthesis of one to two more prominent peaks.

Based on the ESI-MS chromatogram of the isolated peak samples, the similarity between the peaks isolated from the roots, stems, and leaves in the first five ion peaks (*m/z* 142.5, 183, 262, 381, and 403) indicates that the Hg-binding peptides found in all the vegetative parts of the Hg-treated plant may have the same basic component or parent material. The repeating sequences of *m/z* 119 observed in all the samples corresponds to the exact mass of cysteine. However, the amino acid cysteine was not detected in mass spectrometry because the molecular weight of cysteine is below limitation for detection assuming that there are individual amino acids of cysteine. It appears, however, that a series of five to nine cysteine residues

are repetitively attached to this long chain of Hg-binding peptide. Based on their respective mass spectra, the Hg-binding peptide isolated from the roots exhibits five cysteine residues while those isolated from the stems and leaves contain as much as nine cysteine residues which may affect their binding capacities with Hg. Moreover, the molecular ion peaks exhibited by the stem and leaf isolates were highly similar differing only in terms of the relative abundance of the individual monomers. In addition, the molecular ion base peak in the stems and leaves were relatively similar (*i.e.*, *m/z* 619.9 and 620.1, respectively) indicating further that the Hg-binding peptides isolated from these vegetative parts may be one and the same and functionally localized in the shoots to translocate Hg ions upstream. Although the molecular ion base peak exhibited by the roots was relatively lighter (*m/z* 522.5), this ion peak was also found in high quantities in both stem and leaf isolates.

In addition, some of the ion peaks corresponding with (γ-Glu-Cys)2 (desGly-PC2) at *m/z* 500.6 and (γ-Glu-Cys)3–Gly at *m/z* 804.9 were also detected. Moreover, an ion peak at *m/z* 946.1 also appeared in all the samples analyzed which corresponds to an ion peak of (γ-Glu-Cys)4 (desGly-PC4) found in the roots of *Rubia tinctorum* L. by Kubota *et al.* (2000). However, an ion peak, which corresponded with the exact mass of γ-Glu-Cys-Gly, commonly known as glutathione, was particularly found only in the root isolates.

CV-AAS analyses of peaks isolated from the roots contained a total Hg content of 1.26–2.23 μg/g. However, purified stem and leaf samples contained significantly ($P < 0.05$) higher Hg levels of 21–23 μg/g and 30 μg/g, respectively. It is likely that the Hg ion forms a double bond with the sulfur of cysteine producing cinnabar, which is quite stable in the environment. This implies a promising phytoremediation agent for Hg which is cost-effective and low maintenance performing both phytoextraction and phytostabilization. *Chromolaena odorata* is a perennial plant that persists all throughout the year and tolerates a variety of harsh conditions without the application of fertilizers. Although it is known as a noxious weed, its efficacy and potential to alleviate Hg-contaminated areas would far outweigh its disadvantages. Thus, even when the plant sheds off its leaves, dies and decomposes in the natural environment, the Hg (in the form of cinnabar) will be stable and unavailable for further uptake and leaching into the groundwater.

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