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Subcellular Localization, Isolation, and Partial Purification of Mercury-binding Biomolecules in *Chromolaena odorata* (L.*f.*) R.M. King *et* H. Robinson

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ABSTRACT

Chromolaena odorata (L.*f.*) R.M. King *et* H. Robinson plants were grown in Hoagland's solution modified with 1.00 ppm $Hg(NO_3)_2$. Cold Vapor-Atomic Absorption Spectrophotometry (CV-AAS) analyses for Hg^{2+} contents established the presence of Hg^{2+} in 3 out of 4 of the subcellular components obtained from the leaves of the Hg-treated *C. odorata* plants. Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) analyses of the isolated protoplasts and vacuoles revealed that the ultimate localization of Hg^{2+} was in the vacuoles.

The Hg-binding, SH-containing biomolecules, which were initially detected through the 5,5'-dithiobis(2-nitro-benzoic acid) (DTNB) assay, manifested as a predominant peak in the chromatographs of both the control and Hg-treated plants, obtained through Reverse Phase-High Performance Liquid Chromatography (RP-HPLC), with their retention times falling within the ranges of reduced glutathione, metallothionein, and cysteine standards. However, the concentrations of the glutathione- and/or metallothionein-like, cysteine-containing biomolecules detected in the leaves of Hg-treated *C. odorata* plants were ten-fold higher than those detected in the control.

The findings of this study provided evidence that the enhanced production of Hg-binding biomolecules and the localization of Hg^{2+} ions are ultimately in the vacuoles of the leaves and that these are the mechanisms which bring about Hg^{2+} tolerance and homeostasis in *C. odorata* plants exposed to mercury. These results indicate that *C. odorata* is a hyperaccumulator and hence, a potentially effective phytoremediator for Hg^{2+} ions.

Keywords: *Chromolaena odorata*, mercury, phytoremediation, phytochelatin, glutathione, metallothionein, cysteine, subcellular localization, DTNB assay, RP-HPLC

INTRODUCTION

Mercury contamination in the environment comes from both natural and anthropogenic sources. Agricultural sources include fertilizers, pesticides, and fungicides (Patra and Sharma, 2000), which have been extensively used to control diseases affecting economically important food crops. Mercury has low bioavailability in soils but excessive amounts combined with a low soil pH and high cation exchange capacity can promote increased plant uptake. The ability of plants to take up and sequester exceptionally high concentrations of the heavy metal in the aboveground harvestable parts (Pollard *et al.*, 2002) is known as hyperaccumulation. Plants with this special ability tend to display heavy metal tolerance, wherein their roots absorb high levels of the heavy metals and concentrate these either in their root or shoot systems without significantly affecting metabolic functions. Plants known or suspected to demonstrate hyperaccumulating abilities for heavy metals would necessarily be tolerant to these pollutants. Together, hyperaccumulation and heavy metal tolerance form the basis for an emerging technology known as phytoremediation. This involves the use of plants and their root-associated microbes to remove, detoxify, and concentrate heavy metals and other pollutants (Chaney *et al.*, 1997). It offers a biological alternative to tackling the world's pollution problem and has gained popularity towards the end of the 1990s (Salt *et al.*, 1998). It is more inexpensive than the conventional methods, mainly because plants do not need additional external energy input. Phytoremediation is also non-destructive and non-invasive, and may beautify the contaminated site. In addition, metals that had been accumulated by the plants or plant parts may be recovered and recycled.

Plants possess several mechanisms for coping with stress brought about by the presence of heavy metals, ranging from morphological modifications to enhanced synthesis of defense substances (Peuke and Rennenberg, 2005). Exposure of plants to heavy metals induces synthesis of compounds that chelate these metals and thus, contribute to their detoxification (Rauser, 1999). These chelators, *viz.*, glutathione, phytochelatins, and metallothioneins, are well-studied and well-documented for a number of plant species (Grill *et al.*, 1987; Alloway and Ayres, 1993; Subhadra *et al.*, 1993; Maitani *et al.*, 1996; Mehra *et al.*, 1996; Zenk, 1996; Gupta *et al.*, 1998; Cobbett and Goldsbrough, 2002; Ortega-Villasante *et al.*, 2005; Iglesia-Turiño *et al.*, 2006).

Chromolaena odorata (L.*f.*) R.M. King *et* H. Robinson, locally known as "hagonoy", is a perennial 1-3-m-tall shrub belonging to the Family Asteraceae. Although a noxious weed, this plant has been valued for restoring soil fertility and is considered as main fallow species by most farmers (Vanoordwijk *et al.*, 1996; Roder *et al.*, 1997). Its leaves have been reported to have medicinal properties and may be applied as poultice for wounds (Phan *et al.*, 1996). The leaf extracts of *C. odorata* contain antioxidant activity against hydrogen peroxide and hypoxanthine-xanthine-oxidase-induced damage (Phan *et al.*, 2001).

Recently, *C. odorata* plants were found to abound and dominate in a dumpsite for mine tailings in Benguet Mines (Phase II) in Itogon, Benguet (Velasco-Alinsug *et al.*, 2005a). This earlier study reported that C. odorata accumulated high amounts of mercury in its vegetative tissues, without exhibiting any toxicity symptoms. This study determined the biomolecules involved when *C. odorata* plants were exposed to mercury, results herein, may help provide an understanding of the efficacy by which *C. odorata* accumulates high levels of Hg, making it a potentially effective phytoremediator.

The objectives of this research were (1) to determine the subcellular localization site(s) of absorbed Hg in the leaf tissues of *C. odorata*; (2) to assess for the presence of Hg-binding biomolecules; (3) to determine changes in total protein content; and, (4) to extract, isolate, and partially purify the Hg-binding biomolecules.

MATERIALS AND METHODS

Plant Material, Culture, Exposure to Mercury, and Harvest

C. odorata cuttings were collected from the backyard of Sampaguita Residence Hall, U.P. Diliman and transferred to two plots located at the Botanical Gardens II, Institute of Biology, College of Science, U.P. Diliman. The plants maintained in these plots were cultivated, propagated and served as the source plants for all the *C. odorata* cuttings utilized for the duration of the study.

C. odorata cuttings of relatively the same age and morphological vigor, *i.e.,* 10 in long, with 4-5 nodes from the terminal bud, were collected and transferred to the Institute of Biology Plant Physiology Laboratory (Rm. 4106-4108, Pavilion IV). The cuttings were carefully and thoroughly washed with running water and surface-sterilized with 0.10% commercial sodium hypochlorite solution for 1 min. Root induction was promoted by dipping the cuttings in a root growth stimulant (Hormix B-1®) for 10 min. These were then planted in plastic pots (9.0 cm x 11.0 cm x 3.0 cm) containing a mixture of equal amounts of coarse vermiculite (Buhaghag®) and perlite (HortiPerl®). Sixteen 2-week old rooted cuttings of similar vigor were transferred to each of 6 basins (7.0 in x 15.0 in x 13.0 in). Three basins contained Hoagland's solution (Hoagland and Arnon, 1938) modified with $Hg(NO_3)_2$ H_2O (UNIVAR AR $\geq$ 98.0%) to obtain a final concentration of 1.0 ppm $Hg(NO_3)_2$ for mercury exposure The unmodified half-strength Hoagland's solution in the remaining 3 basins served as the control. Each of the 6 basins was filled with 4.5 L of either the Hg-modified or unmodified Hoagland's solution. The control and Hg-treated plants were prepared in triplicates. The plants were maintained for 7 d under continuous illumination (40-W fluorescent tubes, 30μ $Em^{-2}s^{-1}$) (Josue *et al.*, 2004) at ambient temperatures with constant aeration. The basins were arranged in a complete randomized block design. The plants were harvested at the end of the 7 d-treatment. Each of the plants was carefully and thoroughly washed with deionized water to remove any adsorbed mercury.

Subcellular Localization of Mercury

Differential Centrifugation

The method of Hernandez *et al.* (1998) as modified by Josue *et al.* (2004) was adopted for the differential centrifugation of the crude subcellular fractions. Previous work showed that the leaves from *C. odorata* plants had the highest accumulation of Hg-binding biomolecules (Velasco-Alinsug *et al.*, 2005b). A gram of FW tissue samples from the leaves of control and Hg-exposed *C. odorata* plants were separately homogenized in 20 ml extraction buffer pH 7.5. The homogenate was sieved through a nylon cloth (mesh size, 240 μm) and the residue constituted the crude cell wall-containing fraction and was designated as Fraction 1. The filtrate underwent differential centrifugation at 10,000 x *g* for 15 min. The resulting pellet represented the crude organelle-containing fraction (Fraction II), with the nuclei, mitochondria, and chloroplasts. The supernatant on the other hand, was further centrifuged at 100, 000 x *g* for 30 min to obtain the pellet with the crude membrane-containing fraction (Fraction III). The supernatant from this centrifugation represented the crude soluble fraction (Fraction IV), with the ribosomes and large molecules. All centrifugation processes were performed at 4°C. The crude fractions were immediately subjected to Hg content analyses through CV-AAS (Bouchard, 1973). Each of the samples was transferred into separate reaction flasks. Five ml stannous chloride solution was added and each flask was immediately attached to the aeration apparatus. After the absorbance has been read, a standard curve was made by plotting the absorbance readings versus μg Hg. The value was obtained and calculated from the prepared standard curve applying the formula as follows:

$$\mu g\ Hg / ml = W / Ws$$

where: W = μg Hg in the sample obtained from the standard curve
Ws = sample weighting

Isolation and Purification of the Protoplasts and Vacuoles

The method of Vogeli-Lange and Wagner (1990) was adopted to isolate and purify the protoplasts in *C. odorata* leaves. The laminae were cut parallel to the midrib into approximately 1 mm^2 longitudinal pieces. The protoplasts were liberated by placing 1.0 g (FW) leaf material abaxial side down in Petri plates (diam 9.0 cm) containing 10 ml 2.0% (w/v) cellulase (Sigma® C-1794) and 0.5% (v/v) pectinase (Sigma® P-4716), incubated for 1.5 h at 29°C with gentle shaking. The suspension was then filtered through a nylon cloth (mesh size 100m), rinsed with 3.0 ml 0.3 M mannitol, and was layered onto 4.0 ml 15% (w/v) Ficoll in 0.3 M mannitol. After centrifugation for 4 min at 150 x *g*, intact protoplasts were collected from the interphase and were diluted with 8 ml 0.3 M mannitol. The protoplast suspension was layered again onto a cushion of 15% (w/v) Ficoll in 0.3 M mannitol and after centrifugation under the same conditions, purified protoplasts were recovered from the interphase. Aliquots of these protoplasts were used for ICP-AES Hg analysis, isolation of vacuoles, and acid phosphatase assay.

The vacuoles were isolated using the method of Matoh *et al.* (1987) with modifications from Vogeli-Lange and Wagner (1990). The lysis of protoplasts was induced by mixing 0.5 ml protoplasts (1.0×10^6 protoplasts /ml) with 10 ml vacuole isolation medium. The mixture was gently stirred with a wooden dowel for 5-10 min. At about this time, at least 90% of the protoplasts had lysed. The lysate was divided into two portions and were each separately mixed with equal volumes of 20 % (w/v) Ficoll in 0.24 M mannitol, 1.0 mM EGTA, 0.5 mM CHAPS, and 20 mM HEPES-Tris (pH 8.0) to come up with a 10% protoplast content in the lysate. This was overlayered with 3.0 ml 3.9% (w/v) Ficoll solution, followed by 1.5 ml 0% (w/v) Ficoll in 0.24 M mannitol, 0.5 mM CHAPS, and 20 mM HEPES-Tris (pH 8.0). After centrifugation at 150 x *g* for 15 min, most of the vacuoles floated in the 0/3.9% Ficoll interphase and were collected with a Pasteur pipette. Aliquots of these vacuoles were used for ICP-AES Hg analysis and for acid phosphatase assay.

The isolated protoplasts and vacuoles were immediately subjected to Hg content analyses through ICP-AES (Varian Liberty Series II serial no.EL98023600). The concentrations of Hg and S of the various samples were determined using ICP-AES.

Test for the Purity of Vacuoles Using Acid Phosphatase Assay

The method of Puzon *et al.* (2008) as modified from the method of Josue *et al.* (2004) was adopted based on the recommended diagnostic test and reagents (colorimetric/endpoint method)for acid phosphatase activity. A 200 µL-volume of sample was added to 5.5 ml of reagents containing 0.1 M citrate-NaOH, pH 5.0 (Sigma® C0759) and 5 mM ρ-nitrophenyl phosphate (Sigma® N-3129). Incubation was at 37°C for 30-45 min. The assay was stopped by adding 0.5 ml 0.2 M borate-NaOH (ph 9.8) and read spectrophotometrically at 400 nm (Beckman, DU60 Spectrophotometer). The % activity of the enzyme in the vacuole was then computed as follows:

$$\%\ activity_{acid\ phosphatase} = \frac{absorbance\ readings_{vacuoles}}{absorbance\ readings_{protoplasts}} \times 100$$

Detection and Partial Characterization of the Mercury-Binding Biomolecules

Gel Filtration and Determination of Total Protein Content

The method of Grill *et al.* (1987) for gel filtration chromatography as modified by Kubota *et al.* (2000) and Josue *et al.* (2004) was adopted. A 1-g frozen plant sample was homogenized in liquid N_2 with 20 ml of 10 mM Tris-HCl buffer solution (pH 7.4) containing 10 mM KCl (Univar AR) and 1 M $MgCl_2$ (HiMedia AR RM 728). The homogenate was filtered through nylon cloth (mesh size, 100μm) and was centrifuged (Hitachi micro ultracentrifuge CS150GXL/CS120GXL) at 13,400 x *g* for 60 min at 4°C. Five-ml aliquots of the different supernatant were each subjected to gel filtration chromatography using Sephadex G-50 (Sigma-Aldrich® G50150) column (1.5 cm x 60 cm) equilibrated and developed with 10 mM Tris-HCl (pH 7.4) with KCl, and ran at a flow rate of 5 ml/h. Fifteen 5-ml fractions were collected from each plant sample. Two-ml aliquots of each of the gel filtration fractions were read spectrophotometrically at 280 nm for total protein content determination. One-ml aliquots were prepared from each of the 5-ml fractions and were subsequently lyophilized (Lobronco Freeze dry system/freezone 4.5) and frozen for all subsequent assays.

DTNB Assay

One-ml aliquot from each gel filtration fraction was analyzed using Ellman's reagent to detect the presence of non-protein, SH-containing substances through the DTNB assay. Two hundred μL of 10 mM EDTA was mixed with the aliquot, followed by the addition of 200μL of 75μM DTNB. The mixture was chilled in a tray of ice for 30 min before its absorbance was read at 412 nm under UV-VIS mode (Hitachi U-2000 Spectrophotometer).

RP-HPLC

The evaluation for Hg-binding biomolecules was accomplished using the method of Grill *et al.* (1987) as modified by Kubota *et al.* (2000) and Josue *et al.* (2004; 2006). The frozen lyophilized 1-ml aliquots of the gel filtration fractions which showed the highest detected Hg-binding biomolecules were used. One mg of each lyophilized fraction was dissolved in 1 ml 0.1% (w/v) $NaBH_4$ (in 1 M NaOH) acidified with 20 μL 3.6 M HCl. The resultant mixture was subsequently filtered using an interchangeable syringe (MicroLiter™ # 702 Hamilton Co., Reno, Nevada) equipped with mixed cellulose ester microfilter (0.45 μm pore size Nylon Acrodisc 13, Advantec Membrane Filter Systems, Inc.). Twenty μL of the fraction was subjected to a RP-HPLC (Agilent Technologies 1200 Series Quaternary with Perkin Elmer Series 200 Refractive Index Detector) equipped with a C_{18} column (Restek Flow Pinnacle II C18 , 5 μm, 250 mm x 4.6 mm). The gradient conditions set and determined by a system controller were as follows: 0-20 min, linear gradient from 0 to 20% CH_3CN in 0.1% trifluoroacetic acid; 20-40 min, 20% CH_3CN in 0.1% trifluoroacetic acid.

The reduced glutathione (GSH) standard (Sigma-Aldrich® G4251), the metallothionein (MT) standard (Sigma®), the cysteine standard (Sigma®), and the eluents were separately allowed to flow at a rate of 1ml/min and were monitored for characteristic peaks at 220 nm. Chromatograms of extracts from control and Hg-exposed leaves were compared with those of the standards.

Data Analysis

The data were analyzed statistically using SPSS® version 12.0 (Apache Software Foundation, 2000). The results were expressed as means of triplicates ± SD unless indicated otherwise. The differences at $\alpha = 0.05$ were considered significant.

RESULTS AND DISCUSSION

Subcellular Localization of Mercury

The results of CV-AAS analyses of Hg^{2+} (Table 1) revealed that mercury was detected in 3 out of the 4 subcellular components derived from the leaves of Hg-exposed *C. odorata* plants. The combined Hg^{2+} contents of the protoplasmic fractions of the treated leaves (*i.e.,* fractions II and III) point to the localization of Hg^{2+} in these fractions than in the cell walls (fraction I). The non-detection of Hg^{2+} in the cytosolic fraction (fraction IV) indicates that these toxic ions are readily transported into the organelles once they have passed through the cell wall. The detection of Hg^{2+} in the control plants however was observed only at the cell wall fraction. These represent the surface-adsorbed amounts of Hg^{2+}. The Hg^{2+} ions ultimately accumulated in the vacuoles, as evidenced by the detection of the highest concentrations of Hg^{2+} in the organelle, *i.e.,* 66.2% of the total protoplasmic content (Table 2). Vacuoles were isolated earlier from the protoplasts of control and Hg-exposed plants and their purity were confirmed from the results of the acid phosphatase assay (Table 3). The high percentage of accumulated Hg^{2+} in the vacuoles point to its role in the chelation, sequestration, and detoxification of Hg^{2+} in *Chromolaena odorata* leaves.

Table 1. Mercury contents of the crude subcellular fractions of *Chromolaena odorata* leaves exposed to 0.00 ppm and 1.00 ppm $Hg(NO_3)_2$ after 7d-treatment.

Treatment pmm $Hg(NO_3)_2$ / Crude Subcellular Fractions	Mercury (pmm)[a,b]	
	0.00	1.00
I. cell wall materials	0.05 (LOQ[c])	0.150
II. nuclei, mitochondria, chloroplasts	<MDL[d]	0.162
III. Microsomes	<MDL[d]	0.136
IV. Cytosol	<MDL[d]	<MDL[d]

[a]n = 3

[b]The individual effect of treatment is significant but the interaction between the crude subcellular fraction and Hg treatment is not significant by two-way ANOVA at 5% level of significance.

[c]LOQ = Limit of Quantitation (LOQ = 0.06)

[d]MDL = Method Detection Limit (MDL = 0.02)

Table 2. Mercury content of the isolated protoplasts and vacuoles from *Chromolaena odorata* leaves exposed to 0.00 and 1.00 ppm $Hg(NO_3)_2$ after 7d-treatment.

Treatment pmm $Hg(NO_3)_2$ / Subcellular Components	Mercury (pmm) [a,b]	
	0.00	1.00
Protoplasts	0.095 ± 0.006	0.296 ± 0.194
Vacuoles	0.102 ± 0.017	0.196 ± 0.020

[a]n = 3

[b]The individual effect of treatment is significant but the interaction between the specific subcellular component and Hg treatment with respect to Hg content is not significant by two-way ANOVA at 5% level of significance.

[b]The individual effect of treatment as well as the interaction between the specific subcellular component and Hg treatment with respect to S contents are significant by two-way ANOVA at 5% level of significance.

Table 3. Absorbance and percentage phosphatase activity of isolated protoplasts and vacuoles from *Chromolaena odorata* leaves exposed to 0.00 ppm and 1.00 ppm $Hg(NO_3)_2$ after 7d-treatment.

Treatment pmm Hg $(NO_3)_2$	Absorbance at 400nm[a]		% Phosphatase Activity of Vacuoles[a]	
Subcellular Components	0.00	1.00	0.00	1.00
Protoplasts	0.4351	0.6886	-	-
Vacuoles	0.2662	0.5311	61.18%	77.12%

[a]n=3

Partial Characterization of the Mercury-Binding Biomolecules

The fractions obtained by gel filtration from the leaves of Hg-treated plants that exhibited significantly higher absorbance readings at 280 nm (Figure 1) indicated the presence of some peptides in *C. odorata* as a response to increased internal Hg^{2+} levels, eluting out in fractions 7-11. The Hg^{2+} ions which accumulated in the protoplasm of the Hg-exposed plants had thus, influenced the production of SH-containing biomolecules in *C. odorata.* Several higher plants synthesize special organic compounds that provide protection to plant cells against toxic heavy metals. In particular, SH-containing biomolecules, like glutathione and phytochelatins, protect plant cell and its components from the toxic Hg^{2+} ions which were transported into the protoplasm (Josue *et al.*, 2004; 2006). In this study, significantly higher amounts of SH-containing biomolecules were detected in the fractions obtained from the leaves of Hg-treated plants, compared to those from the control (Figure 2). These results strongly suggest that more SH-containing biomolecules were synthesized when the plants were exposed to Hg^{2+}. In plant species that take up and accumulate Hg^{2+}, the toxic ions are detoxified by phytochelatins or by their precursor glutathione, or by glutathione-like biomolecules (Zenk, 1996). These molecules are rich in Cys residues that provide thiols for binding to metals, rendering them harmless. GSH was reported to be the primary line of defense against metal toxicity in most organisms (Singhal *et al.*, 1987; Mehra *et al.*, 1996). Similarly, glutathione-like biomolecules detoxified the otherwise deleterious effects of Hg^{2+} ions in *C. odorata* cells.

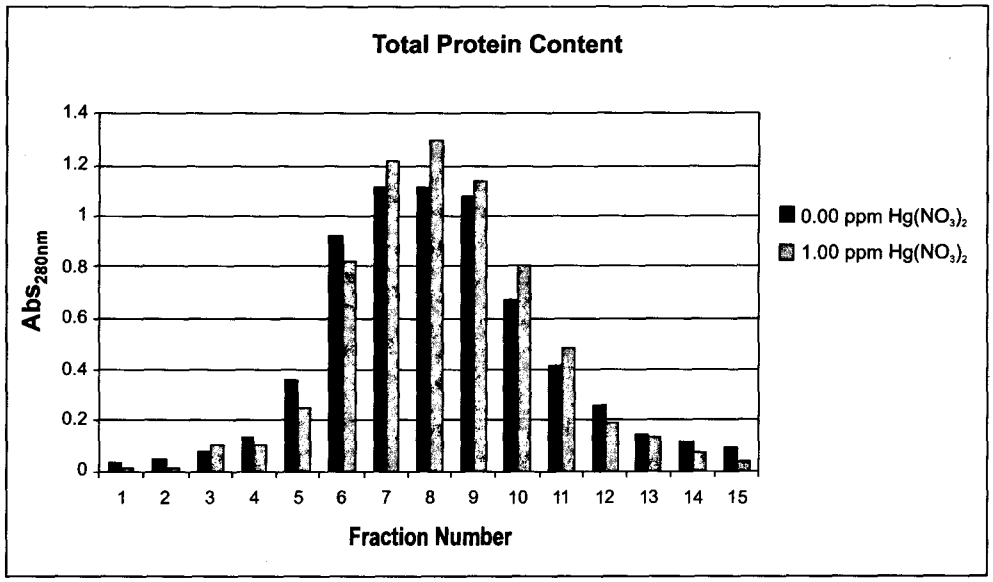

Figure 1. Absorbance profile (280 nm) of gel filtration extracts of *C. odorata* plants exposed to 0.00 and 1.00 ppm $Hg(NO_3)_2$ for 7d. Data are means of three replicates (±SD).

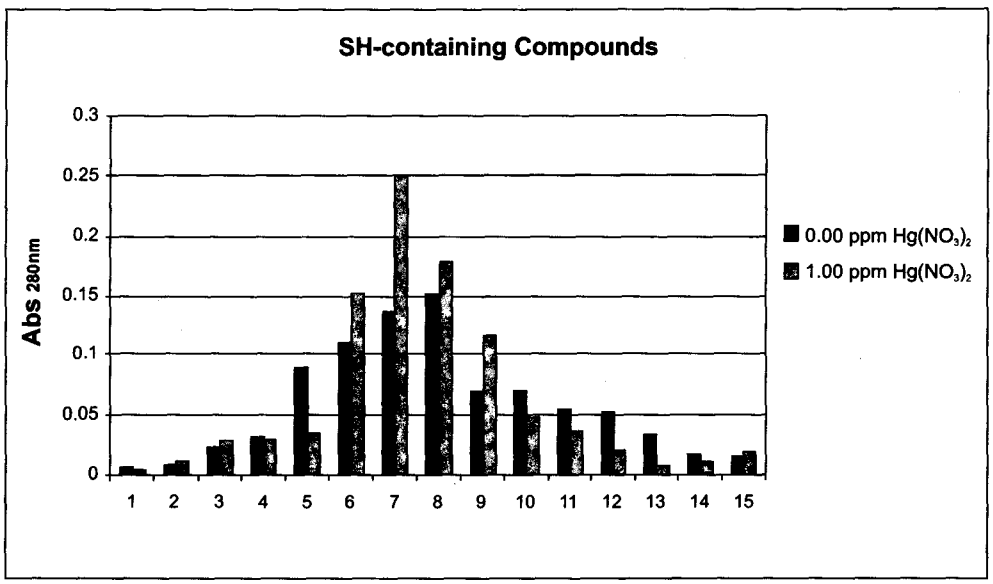

Figure 2. Gel filtration chromatography elution profile of SH-containing compounds in *C. odorata* plants exposed to 0.00 and 1.00 ppm $Hg(NO_3)_2$ for 7d. Data are means of three replicates (±SD).

The results on the absorbance readings (254 nm) of the fractions eluted from gel filtration (Figure 3) suggested that the leaves from the Hg-exposed plants contained more S-metal complexes, compared to those from the control. The Hg^{2+} ions bind with high affinity to S-rich peptides and form insoluble compounds (Kabata-Pendias and Pendias, 1986). The presence of more S-metal complexes in the Hg-treated leaves may thus, be due to the binding of Hg^{2+} to these S-rich biomolecules, like phytochelatins, glutathione, metallothioneins, and other glutathione-like and metallothionein-like substances, as was the case in *C. Odorata*.

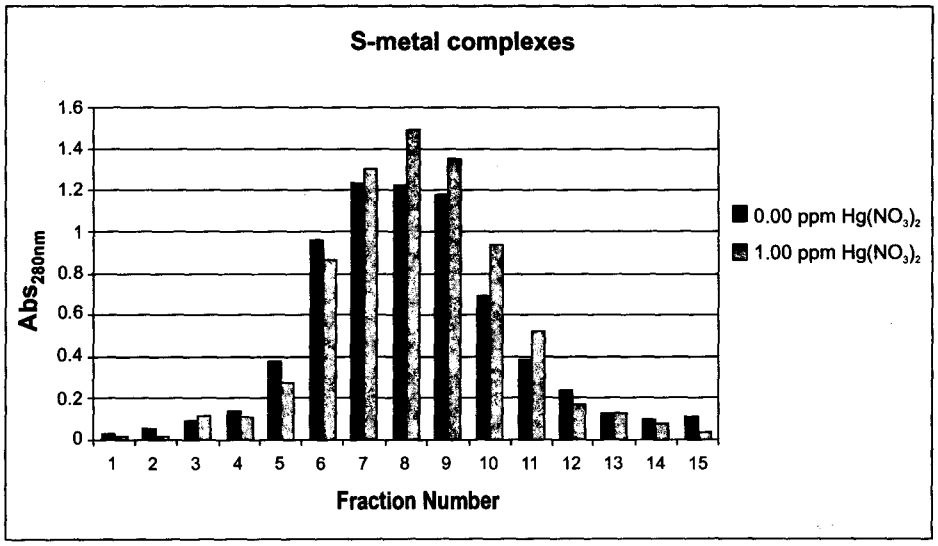

Figure 3. Absorbance profile (254 nm) of gel filtration extracts of *C. odorata* plants exposed to 0.00 and 1.00 ppm $Hg(NO_3)_2$ for 7 days. Data are means of three replicates (±SD).

The profiles of the gel filtration fractions from the leaves of both control and Hg-exposed plants reflected a prominent peak within the range of 2.400 - 2.500 min retention time. This range overlaps with the respective retention time for the GSH, MT, and cysteine standards (Figures 4-6), indicating the presence of GSH-like and/or MT-like, Cys-containing biomolecules (Figures 7 and 8). Moreover, it was revealed that there was a dramatic 10-fold increase in the concentration of GSH- or MT-like, Cys-containing biomolecules in the Hg-treated plants (Table 4). This 10-fold increase in the synthesis of GSH- and MT-like, Cys-containing biomolecules in the Hg-exposed plants is due to the presence of Hg^{2+} ions. These results strongly suggest a Hg-enhanced synthesis of GSH- and MT-like Cys-containing biomolecules. Similarly, GSH-like substances were also produced in the leaves of *Ipomoea aquatica* exposed to 1.0 ppm $Hg(NO_3)_2$ and were reported to complex with Hg^{2+} ions and bring about detoxification in this plant (Josue *et al.*, 2004; 2006). In this study, the amplified production of SH-containing biomolecules is the first line of defense of *C. odorata* against Hg^{2+} toxicity.

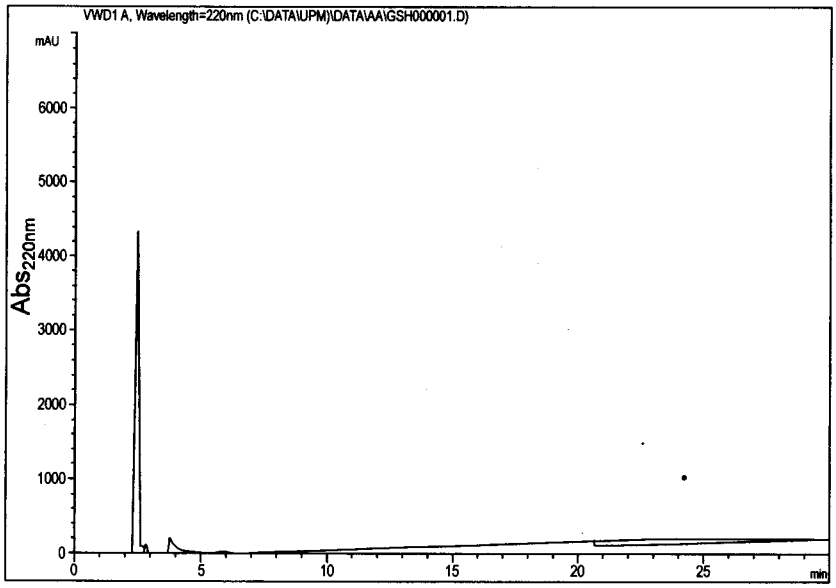

Figure 4. RP-HPLC chromatograph of glutathione (GSH) as a reference substance.

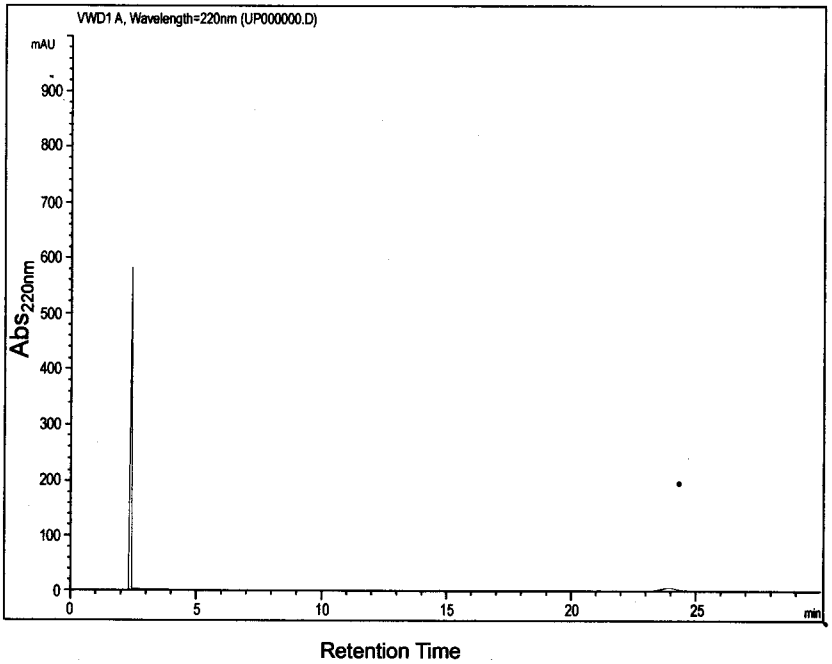

Figure 5. RP-HPLC chromatograph of metallothionein (MT) as a reference substance.

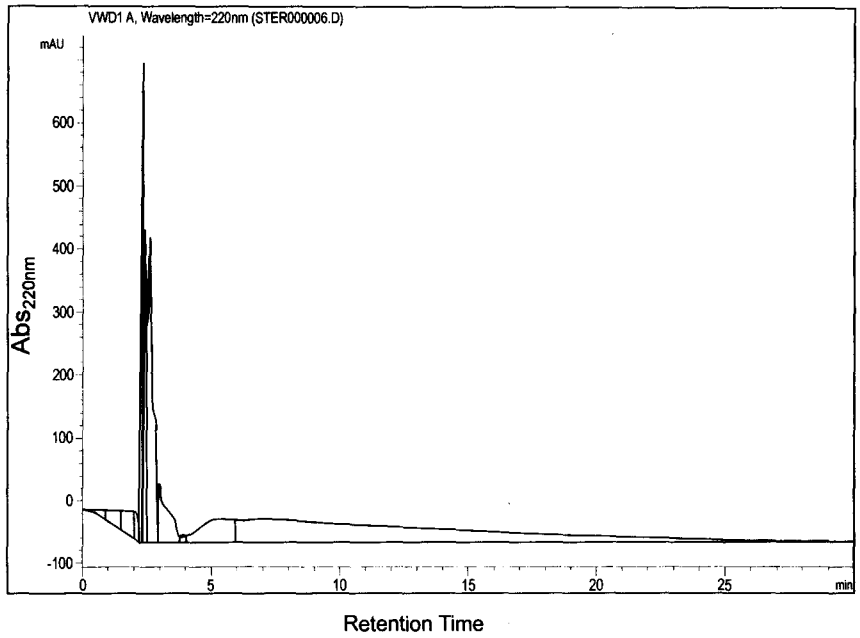

Figure 6. RP-HPLC chromatograph of cysteine as a reference substance.

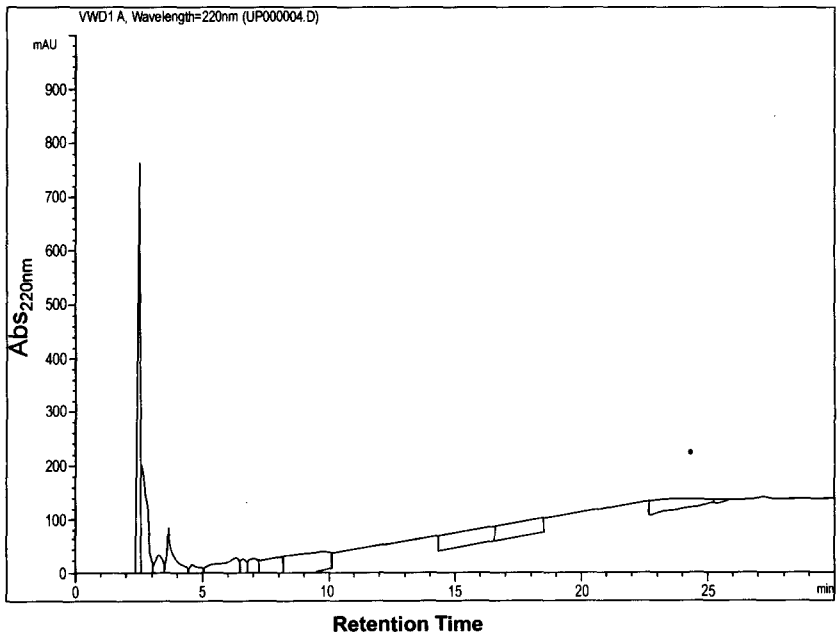

Figure 7. RP-HPLC chromatograph of GSH- and/or MT-like, cysteine-containing biomolecules from the gel filtration eluted fractions of leaves from control *C. odorata* plants.

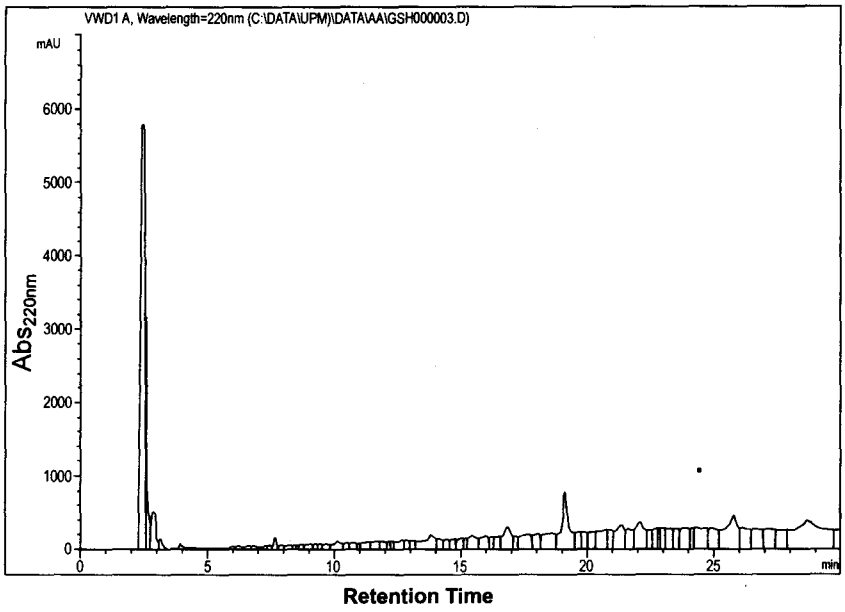

Figure 8. RP-HPLC chromatograph of GSH- and/or MT-like, cysteine-containing biomolecules from the gel filtration eluted fractions of leaves from Hg-exposed *C. odorata* plants.

Table 4. RP-HPLC analyses of Hg-binding biomolecules from *Chromolaena odorata* leaves exposed to 0.00 and 1.00 ppm $Hg(NO_3)_2$ after 7 d-treatment.

Standards and Samples	Retention Time (min)	Area (m AU*s)	Height (mAU)	GSH conc. (mg/ml))	MT conc. (mg/ml)	Cysteine conc. (mg/ml)
GSH standard	2.415	2.790588 x10^4	4398.00879	1.00	---	---
MT standard	2.408	2661.46387	722.93915	---	1.00	---
Cys standard	2.289	3023.47095	772.21692	---	---	1.00
Control 0.00 ppm $Hg(NO_3)_2$	2.515	6642.02051	818.1554	0.238	2.496	2.197
Hg-treated 1.00 ppm $Hg(NO_3)_2$	2.437	6.63089 x10^4	5793.87402	2.38	24.91	21.92

SUMMARY AND CONCLUSIONS

The findings of the study revealed that *C. odorata* was capable of taking up and accumulating the heavy metal, Hg. The results of the CV-AAS and ICP-AES revealed the mobility and accumulation of Hg^{2+} in all the subcellular fractions of the leaves from the Hg-exposed *C. odorata* plants and the ultimate sequestration of Hg^{2+} in the vacuoles of these leaves. The SH-containing biomolecules detected through DTNB assay manifested a predominant peak in the RP-HPLC chromatographs of both the control and Hg-treated plants, with their respective retention time falling within the ranges for those of the GSH, MT, and cysteine standards. However, the concentrations of the GSH- and/or MT-like cysteine-containing biomolecules detected in the leaves of Hg-treated *C. odorata* plants were ten times higher than those detected in the leaves from the control.

The results of this study confirm *C. odorata* as a potential phytoremediator agent. The enhanced production of SH-containing, Hg-binding biomolecules, as well as the compartmentalization of Hg^{2+} ions in the vacuoles, constitute the cellular mechanisms for Hg^{2+} tolerance and homeostasis in Hg-exposed *C. odorata* plants. With what *C. odorata* had exhibited as demonstrated by the results of this study, further evaluation methods may be applied to test the capacity of this plant species as a phytoremediation agent of Hg^{2+}.

It is recommended that further studies be conducted on the molecular characterization and structure elucidation of the molecule/s responsible for the sequestration and detoxification of mercury.

REFERENCES

Alloway, B. and D. Ayres. 1993. Heavy metals. **IN**: Chemical Principles of Environmental Pollution. Chapman and Hall, Great Britain. 140-164.

Chaney, R., M. Malik, Y. Li, S. Brown, E. Brewer, J. Angle, and A. Baker. 1997. Phytoremediation of Soil Metals. *Current Opinion in Biotechnology*, **8**: 279-284.

Cobbett, C. and P. Goldsbrough. 2002. Phytochelatins and metallothioneins: Roles in heavy metal detoxification and homeostasis. *Annual Review of Plant Biology*, **53**: 159-182.

Grill, E., E. Winnacker and M. Zenk. 1987. Phytochelatins, a class of heavy-metal-binding peptides from plants, are functionally analogous to metallothioneins. *Proceedings of the National Academy of Sciences*, U.S.A., **84**: 439-443.

Gupta, M., R. Tripathi, U. Rai, and P. Chandra. 1998. Role of glutathione and phytochelatin in *Hydrilla verticillata* (L.*f.*) Royle and *Vallisneria spiralis* L. under mercury stress. *Chemosphere*, **37**: 785-800.

Hernandez, L., E. Lozano-Rodriguez, A. Garate, and R. Carpena-Ruiz. 1998. Influence of cadmium on the uptake, tissue accumulation, and subcellular distribution of manganese in pea seedlings. *Plant Science*, **132**: 139-151.

Hoagland, D.R. and D.I. Arnon. 1938. The water-culture method of growing plants without soil. *University of California Agri Exptl Sta Circ*, **347**: 1-39.

Iglesia-Turiño, S., A. Febrero, O. Jauregui, C. Caldelas, J. Araus, and J. Bort. 2006. Detection and Quantification of Unbound Phytochelatin 2 in Plant Extracts of *Brassica napus* Grown with Different Levels of Mercury. *Plant Physiology,* **142**:742-749.

Josue, R., J. Villanueva, and G. Rivero. 2004. Mercury uptake and phytochelatin production in *Ipomoea aquatica* Forsk. *The Philippine Agricultural Scientist, 87*(3): 312-321.

Josue, R., J. Puzon, G. Rivero, and J. Villanueva. 2006. *Glutathione Mediates Mercury Binding and Detoxification in Ipomoea aquatica Forsk. Proceedings of the 8th International Conference on Mercury as a Global Pollutant,* Madison, Wisconsin, U.S.A. p.115.

Kabata-Pendias, A. and H. Pendias. 1986. Mercury. IN: Trace Elements in Soils and Plants. CRC Press, Inc.: Boca Raton, Florida, USA. 116-125.

Kubota, H., K. Sato, T. Yamada, and T. Maitani. 2000. Phytochelatin homologs induced in hairy roots of horseradish. *Phytochemistry*, **53**: 239-245.

Maitani, T., H. Kubota, K. Saio, and T. Yamada. 1996. The composition of metals bound to class III metallothionein (phytochelatin and its desglycyl peptide) induced by various metals in root cultures of *Rubia tinctorum. PIant Physiology,* **110**: 1145-1150.

Matoh, T., J. Watanabe, and E. Takahashi. 1987. Sodium, potassium, chloride, and betaine concentrations in isolated vacuoles from salt-grown *Atriplex gmelini* leaves. *Plant Physiology,* **84**: 173-177.

Mehra, R., J. Miclat, V. Kodati, R. Abdullah, T. Hunter and P. Mulchandani. 1996. Optical spectroscopic and reverse-phase HPLC analyses of Hg(II) binding to phytochelatins. *Biochemical Journal,* **314**: 73-82.

Ortega-Villasante, C., R. Rellán-Alvarez, F. Del Campo, R. Carpena-Ruiz, and L. Hernández. 2005. Cellular damage induced by cadmium and mercury in Medicago sativa. *Journal of Experimental Botany,* **56**(418):2239-51.

Patra, M., and A. Sharma. 2000. Mercury toxicity in plants. *The Botanical Review,* **66(3):379-421.**

Peuke, A. and H. Rennenberg. 2005. Phytoremediation with transgenic trees. *Zeitschrift für Naturforschung,* **60**: 199-207.

Phan, T., M. Hughes, G. Cherry, T. Le, and H. Pham. 1996. An aqueous extract from the leaves of *Chromolaena odorata* (Formerly *Eupatorium odoratum*) (Eupolin) inhibits collagen lattice contraction by human dermal fibroblasts. *The Journal of Alternative and Complementary Medicine,* **2**: 335-343.

Phan,T., P. See, S. Lee, and S. Chan. 2001. Anti-oxidant effects of the extracts from the leaves of *Chromolaena odorata* on human dermal fibroblasts and epidermal keratinocytes against hydrogen peroxide and hypoxanthine-xanthine oxidase induced damage. *Burns,* **27**: 319-327.

Pollard A., K. Powell, F. Harper, and J. Smith. 2002. The genetic basis of metal hyperaccumulation in plants. *Critical Reviews in Plant Sciences,* **21**(6):539-66.

Puzon, J., G. Rivero, and M. Montaño. 2008. Vacuolar sequestration as a coping mechanism in cadmium-treated *Eichhornia crassipes* (Mart.) Solms. *The Philippine Agricultural Scientist,* **91**: 123-133.

Rauser, W.E. 1999. Structure and function of metal chelators produced by plants: the case for organic acids, amino acids, phytin, and metallothioneins. *Cell Biochemistry and Biophysics,* **31**: 19-48.

Roder, W., S. Phengchanh, and B. Keobulapha. 1997. Weeds in slash-and-burn rice fields in Northern Laos. *Weed Research,* **37**(2): 111-119.

Salt, D., R. Smith, and I. Raskin. 1998. Phytoremediation. *Annual Review of Plant Physiology and Plant Molecular Biology,* **49**: 643-668.

Singhal, R.K., M.E. Anderson, and A. Meister. 1987. Glutathione, a first line of defense against cadmium toxicity. *FASEB J,* **1**:220-223.

Subhadra, A., K. Panda, and B. Panda. 1993. Residual Mercury in seed of Barley of methanesulfate, maleic hydrazide, methyl mercury chloride and mercury-contaminated soil. *Mutation Research,* **300** (34): 141-149.

Vanoordwijk, M., K. Hairiah, S. Partoharjan, R. Labios, and D. Garrity. 1996. Food crop-based production systems as sustainable alternatives for *Imperata* grasslands. *Agroforestry systems,* **36**(1): 5-82.

Velasco-Alinsug M.P., J.A. Namocatcat, and G.C. Rivero. 2005a. Bioavailability of mercury to plants thriving in a mine tailings dumpsite. Swiss Federal Institute for Forest, Snow and Landscape Research WSL (ed.) 2005: Metal fluxes and stresses in terrestrial ecosystems. Abstracts. Birmensdorf, Swiss Federal Institute for Forest, Snow and Landscape Research WSL.

Velasco-Alinsug, M.P., G.C. Rivero, and T.O. Quibuyen. 2005b. Isolation of Mercury-binding Peptides in Vegetative Parts of *Chromolaena odorata. Biosciences,* **60**: 252-259.

Vogeli-Lange, R. and G. Wagner. 1990. Subcellular localization of cadmium and cadmium-binding peptides in tobacco leaves. *Plant Physiology,* **92**: 1086- 1093.

Zenk, M.H. 1996. Heavy metal detoxification in higher plants A review. *Gene,* **179**(1):21-30.