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Research Article

Tolerance mechanisms in mercury-exposed *Chromolaena odorata* (l.*f.*) R.M. King *et* H. Robinson, a potential phytoremediator

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Abstract : *Chromolaena odorata* (L.*f.*) R.M. King *et* H. Robinson plants were grown in Hoagland's solutions with 0.00 ppm and 1.00 ppm $Hg(NO_3)_2$. The calcium, magnesium, iron, and sulfur levels in the leaves were found to be not significantly affected by presence of the uptaken Hg^{2+}. The chlorophyll *a*, chlorophyll *b*, and total chlorophyll contents of its leaves also remained within normal levels, which may indicate that the photosynthetic machinery of the Hg-exposed *C. odorata* was unaffected by the presence of Hg^{2+}. The results of the ICP-AES analyses of the Hg^{2+} contents established the presence of Hg^{2+} in all the subcellular components obtained from the leaves of the Hg-treated *C. odorata* plants, and that the ultimate localization of Hg^{2+} is in the vacuoles. The findings revealed no significant differences in the degree of oxidative injury between the cells from the control and Hg-treated plants, as evidenced by the low lipid peroxidation levels obtained with the TBARS assay. The SH-containing biomolecules that were initially detected through DTNB assay manifested a predominant peak in the RP-HPLC chromatographs of both the control and Hg-treated plants, with their retention times falling within the ranges of GSH, MT, and cysteine standards. However, the concentrations of the GSH- and/or MT-like, Cys-containing biomolecules detected in the leaves of Hg-treated *C. odorata* plants were ten times higher than those of the control. The findings of this study suggest that the enhanced antioxidative capacity, the production of Hg-binding biomolecules, and the localization of Hg^{2+} ions ultimately in the vacuoles of the leaves are the mechanisms which bring about Hg^{2+} tolerance and homeostasis in *C. odorata* plant. These results indicate that *C. odorata* is a potentially effective phytoremediator for Hg^{2+}.

Keywords*: antioxidants, Chromolaena odorata, mercury, phytoremediation*

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), *e.g.,* antioxidants and metal

chelators. 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). However, the specific mechanism and role of various antioxidants in controlling heavy metal tolerance and detoxification within the plant system remain unclear, since heavy metal exposure results in either increased or decreased antioxidant activities depending on the plant species as well as the kind and concentration of metals in the substrate (De Vos et al*.,* 1992; Gallego et al*.,* 1996; Prasad et al*.,* 1999; Cho and Park, 2000; Rao and Stresty, 2000; Schützendubel et al., 2001).

Chromolaena odorata, commonly known as bitter bush, Siam weed, or Christmas bush, and 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 (Van Noordwijk 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).

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 sites(s) of absorbed Hg in the leaf tissues of *C. odorata*; (2) to compare levels of antioxidant production in the leaves of control and mercury-exposed *C. odorata* plants; and, (3) to detect and partially characterize the Hg-binding biomolecules from the leaves of mercury-exposed *C. odorata* plants.

Materials and Methods

Plant material, culture, exposure to mercury, and harvest

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. 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 $Hg(NO_3)_2 \cdot H_2O$ (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, 30 $\mu Em^{-2}s^{-1}$) (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 and S content analyses through Inductively Coupled Plasma – Atomic Emission Spectrometry (ICP-AES, Varian Liberty Series II serial no. EL98023600).

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 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^{o}C with gentle shaking. The suspension was then filtered through a nylon cloth (mesh size 100 μm), 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 x 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^{o}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 a ratio between the absorbance readings of the fractions representing the vacuoles and the absorbance readings of the fractions representing the protoplasts, multiplied by 100.

Antioxidant evaluation

Chlorophyll content

The procedure by Moore (1974) for total chlorophyll content was adopted. The total chlorophyll content of the leaves was determined after 7 days of Hg exposure. A 0.25-g FW lamina was ground with 20 ml 80% acetone using mortar and pestle. The slurry was then passed through a Whatman No. 42 filter paper. The residue was again ground in 15 ml 80% acetone (Merck K36818614) and filtered. These steps were repeated, this time with only 10 ml 80% acetone. All filtrates were pooled. The resulting solution was again filtered into a 50-ml volumetric flask and diluted to volume using 80% acetone. This was then subjected to UV-Vis Absorption Spectrophotometry (Beckman, DU60 Spectrophotometer) at 645 nm and 663 nm wavelengths. The 80% acetone served as blank. Total chlorophyll of the lamina was calculated using Arnon's formula (Arnon, 1949):

Chlorophyll a (μg/L) = $12.21(Abs_{663}) – 2.81(Abs_{645})$
Chlorophyll b (μg/L) = $20.19(Abs_{645}) – 5.03(Abs_{663})$
Total chlorophyll (μg/L) = $20.2(Abs_{645})+ 8.02(Abs_{663})$

Extraction of enzymes and protein content determination

One g leaves of C. odorata was ground in 20 ml Tris-HCl buffer and centrifuged at 12, 000 x g for 20 mins

at 4°C. The supernatant was used to measure the protein content and the subsequent enzyme assays.

a. Ascorbate peroxidase

Ascorbate peroxidase (APX) activity was measured according to Rama Devi and Prasad (1998) with some modifications. The supernatant was mixed with 50 mM sodium phosphate buffer (pH 7.0), 0.2 mM EDTA (Univar AR A663), 0.5 mM ascorbic acid, and 250 mM H_2O_2. The decrease in the absorbance at 290 nm (SPECTRONIC® GENESYS 2) was measured after 3 min in order to estimate the oxidation of ascorbic acid. Enzyme activity was calculated using the absorbance coefficient of ascorbic acid (ε = 2.6 mM^{-1} cm^{-1}).

b. Catalase activity and H_2O_2 scavenging assay

Catalase (CAT) activity was measured according to Rama Devi and Prasad (1998) with some modifications. The supernatant was mixed with 50 mM sodium phosphate buffer (pH 7.0) and 2 mM H_2O_2 (Merck ZU60063009). Oxidation of H_2O_2 was measured at 240 nm (SPECTRONIC® GENESYS 2) for 5 min. The change in absorbance of 1 OD represented the oxidation of 25 µmol H_2O_2. Moreover, catalase units were determined based on the absorbance of catalase (A_{240nm} = 0.071).

Lipid peroxidation

Lipid peroxidation was measured according to Rama Devi and Prasad (1998) with some modifications. A 0.3-g leaf sample from the control and Hg-exposed C. odorata plants were separately ground in 4 ml of 0.25% TBA in 10% TCA and were incubated at 95°C for 30 min. These were cooled to room temperature and were centrifuged at 12, 000 x g (International Equipment Company B-22M Model 3495) for 10 min. The absorbance of the supernatant was measured at 560 and 600 nm (SPECTRONIC® GENESYS 2). The absorbance at 600 nm was subtracted from the 560 nm absorbance to correct the non-specific turbidity. The absorbance coefficient of malondialdehyde (MDA) (ε = 1.56 x 10^5 M cm^{-1}) was used in the calculation.

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, 100 µm) 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 200 µL of 75 µM 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), Josue et al. (2004), and Josue et al. (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.

Results and Discussion

Background levels of mercury

There was no Hg^{2+} detected in the soil onto which the source plants were cultivated. The stock plants did not also show any detectable levels of Hg^{2+} (Table 1). The Hg^{2+} concentration in the nutrient solution during the 7-d period was determined to initially evaluate the ability of *Chromolaena odorata* plants to take up Hg^{2+} from the nutrient solution. The decrease from 1.0 ppm $Hg(NO_3)_2$ to 0.0028 ppm Hg in the Hg-amended Hoagland's solution (Table 1) indicated that much of the Hg^{2+} in the original solution, i.e., 0.9972 ppm Hg, was taken up by the *C. odorata* plants exposed for a week to 1.0 ppm $Hg(NO_3)_2$. The Hg^{2+} ions detected in the treated plants were thus, derived from the 1.0 ppm $Hg(NO_3)_2$-amended Hoagland solution since the soil and source plants were all free of detectable levels of Hg (Table 1).

Subcellular Localization of Mercury and Sulfur

The results of ICP-AES analyses of Hg^{2+} (Table 2) revealed the presence of variable levels of Hg^{2+} in all subcellular components of the cells derived from the leaves of Hg-exposed *C. odorata* plants. In contrast, no detectable levels of Hg^{2+} were recorded in any of the subcellular fractions from the control plants. The combined Hg^{2+} contents of the protoplasmic fractions of the treated leaves (*i.e.,* fractions II, III, and IV) point to the localization of Hg^{2+} in these fractions than in the cell walls (Table 2).

Table 1. The Hg^{2+} contents of the study plots, source plants, and hydroponic solutions after 7 d-treatment of *Chromolaena odorata* plants.

Sample	Total Hg content (ppm)[a]
soil (rhizosphere)	< LLD[b]
source plant	< LLD[c]
Hoagland's solution with 0.00 ppm $Hg(NO_3)_2$	< LLD[c]
Hoagland's solution with 1.00 ppm $Hg(NO_3)_2$	0.0028

[a]n = 3
[b]LLD = lower limit of detection for soil samples (0.010 ppm).
[c]LLD = lower limit of detection for water and plant samples (0.002 ppm).

Table 2. Mercury and sulfur contents of the crude subcellular fractions of *Chromolaena odorata* leaves exposed to 0.00 ppm and 1.00 ppm $Hg(NO_3)_2$ after 7 d-treatment.

Treatment / ppm $Hg(NO_3)_2$ / Crude Subcellular Fractions	Mercury (ppm)[a,b]		Sulfur (ppm)[a,b]	
	0.00	1.00	0.00	1.00
I. cell wall materials	< LLD[c]	0.163± 0.05	1, 440.00± 0.00	3, 870.00± 0.05
II. nuclei, mitochondria, chloroplasts	< LLD[c]	0.038± 0.05	1, 760.00± 0.00	4, 020.00± 0.07
III. microsomes	< LLD[c]	0.051± 0.004	2, 050.00± 0.00	3, 250.00± 0.06
IV. cytosol	< LLD[c]	0.130± 0.12	2, 070.00± 0.00	4, 440.00± 0.06

[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]LLD = lower limit of detection (0.002 ppm)

Moreover, the observed Hg^{2+} in the cell wall level would only represent the adsorbed amounts of Hg^{2+}. The protoplasts with 0.296 ppm Hg and vacuoles with 0.196 ppm Hg from the fresh leaves of the Hg-exposed plants contained significantly higher Hg^{2+} ions than the same subcellular components obtained from the control plants with 0.095 ppm Hg and 0.102 ppm Hg, respectively. Ultimately, the Hg^{2+} ions were

localized in the vacuoles, as evidenced by the accumulation of 66.2% of the total protoplasmic Hg^{2+} content (Table 4). Isolated vacuoles from the protoplasts of control and Hg-exposed plants were pure, as revealed by the acid phosphatase assay (Table 3). Similarly, S was present in all the subcellular components of the leaves from control and Hg-treated plants, the levels of which were 2-3 folds higher in those from the Hg-exposed plants (Table 2). Moreover, high amounts of S were also detected in the isolated vacuoles of the leaves from the Hg-treated plants (Table 4). The high percentages of accumulated Hg^{2+} and S_2 in the vacuoles point to the role of the vacuoles and S-containing compounds in the chelation, sequestration, and detoxification of Hg^{2+} in *Chromolaena odorata* leaves.

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 7 d-treatment.

Treatment ppm $Hg(NO_3)_2$ Subcellular Components	Absorbance at 400nm[a]		% Phosphatase Activity of Vacuoles[a]	
	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

Table 4. Mercury and sulfur contents of the isolated protoplasts and vacuoles from *Chromolaena odorata* leaves exposed to 0.00 and 1.00 ppm $Hg(NO_3)_2$ after 7 d-treatment.

Treatment ppm $Hg(NO_3)_2$ Subcellular Components	Mercury (ppm)[a,b]		Sulfur (ppm)[a,c]	
	0.00	**1.00**	**0.00**	**1.00**
Protoplasts	0.095± 0.006	0.296± 0.194	1520.00± 0.015	1920.00± 0.015
Vacuoles	0.102± 0.017	0.196± 0.020	220.00± 0.002	310.00± 0.002

[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.

Antioxidant levels and state of lipid peroxidation

The protective function of chlorophyll against heavy metal toxicity has initially been reported in *Chlorella vulgaris* exposed to Cd^{2+} ions (Lintongan et al*.,* 2004). Similarly, Hg^{2+} did not cause any significant effect in the chl *a*, chl *b*, and total chl of Hg-exposed *C. odorata* plants. The protective mechanisms adapted by plants to scavenge reactive oxygen species include a number of antioxidants and antioxidant enzymes. In this study, Hg^{2+} treatment did not cause any dramatic changes in the ascorbate peroxidase activity (APX) (Figure 1). On the other hand, Hg^{2+} brought about a significant increase in the catalase activity as indicated by the amount of oxidized H_2O_2 (Figure 2). Moreover, the actual catalase units in the Hg-treated plants were also significantly higher than those in the control (Figure 3). Thus, this study indicates that catalase could be one of the main antioxidative protection systems working to bring about tolerance of *C. odorata* to the high Hg^{2+} ions in their vacuoles.

It is known that both APX and CAT play important roles in reducing oxidative injury by catalyzing the oxidation of H_2O_2 (Shohael et al*.,* 2007). This was indeed the case in the Hg-exposed *C. odorata* plants, as evidenced by the pertinent APX and CAT levels analyzed. However, it was only CAT which apparently appeared to be significantly activated upon exposure of the *C. odorata* plants to Hg^{2+} ions. The insignificant difference in the MDA contents between the Hg-treated and the control plants (Figure 4) further indicated that Hg^{2+} did not bring about any significant oxidative injury during Hg^{2+} exposure.

In addition to these enzymes, other biomolecules play a role in protecting the different macromolecules

against the deleterious effects of heavy metals, like mercury. Among these are glutathione (GSH) and other non-protein thiols, both of which are well-documented for other plant species (Noctor and Foyer, 1998; Schützendubel and Polle, 2002). In this study, significantly higher amounts of non-protein thiols were detected in the leaves obtained from the Hg-treated plants than those from the control plants (Figure 5). The increase was determined to be two-fold. Increased amounts of thiol-enriched molecules were also reported in *H. verticillata* and *V. spiralis* as a strategy to alleviate Hg^{2+} toxicity (Gupta et al., 1998).

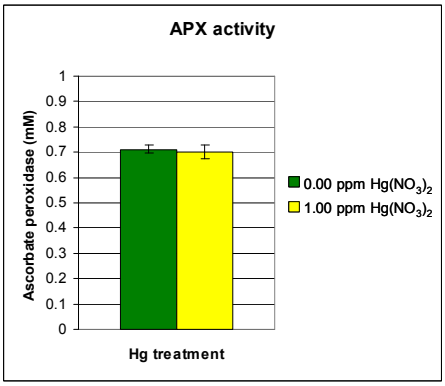

Figure 1. Levels of ascorbate peroxidase activity measured as mM APX in *C. odorata* plants exposed to 0.00 and 1.00 ppm $Hg(NO_3)_2$ for 7 d. Data are means of three replicates (±SD).

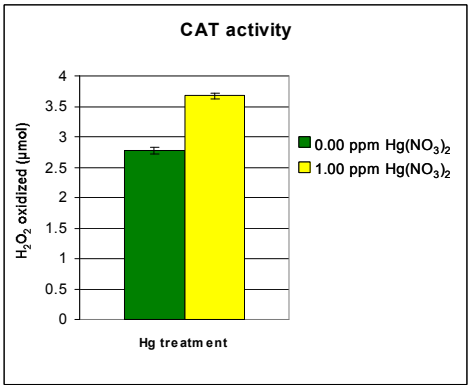

Figure 2. Levels of catalase activity measured as µmol oxidized H_2O_2 in *C. odorata* plants exposed to 0.00 and 1.00 ppm $Hg(NO_3)_2$ for 7 d. Decrease of 1 OD indicates 25 µmol H_2O_2 oxidized. Data are means of three replicates (±SD).

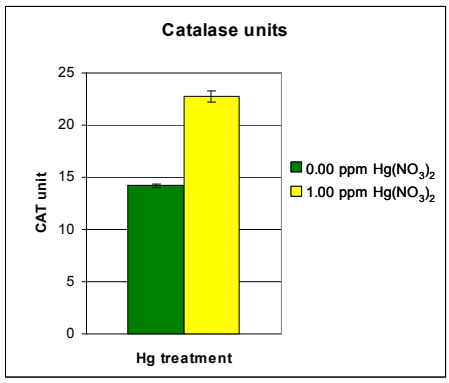

Figure 3. Catalase units in C. odorata plants exposed to 0.00 and 1.00 ppm Hg(NO3)2 for 7 d. CAT units are measured based on the change in absorbance of 1 mM catalase for 1 min. Data are means of three replicates (±SD).

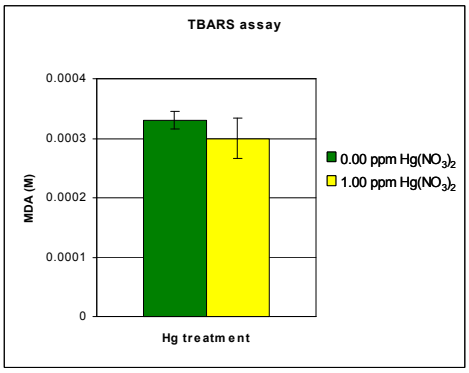

Figure 4. Levels of lipid peroxidation products measured as thiobarbituric acid reactive substances (malondialdehyde) in *C. odorata* plants exposed to 0.00 and 1.00 ppm $Hg(NO_3)_2$ for 7 d.

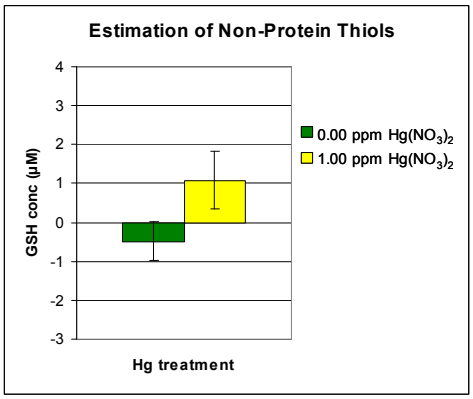

Figure 5. GSH concentration in *C. odorata* plants exposed to 0.00 and 1.00 ppm $Hg(NO_3)_2$ for 7 d. Data are means of three replicates (±SD).

Partial characterization of the mercury-binding biomolecules

The fractions obtained from the leaves of Hg-treated plants that exhibited significantly higher absorbance readings at 280 nm (Figure 6) indicated the presence of some peptides in *C. odorata* as a response to Hg^{2+} stress, eluting out as fractions 7-11. 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; Josue et al., 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 7). 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). Similarly, glutathione-like biomolecules detoxified the otherwise deleterious effects of Hg^{2+} ions in *C. odorata* cells.

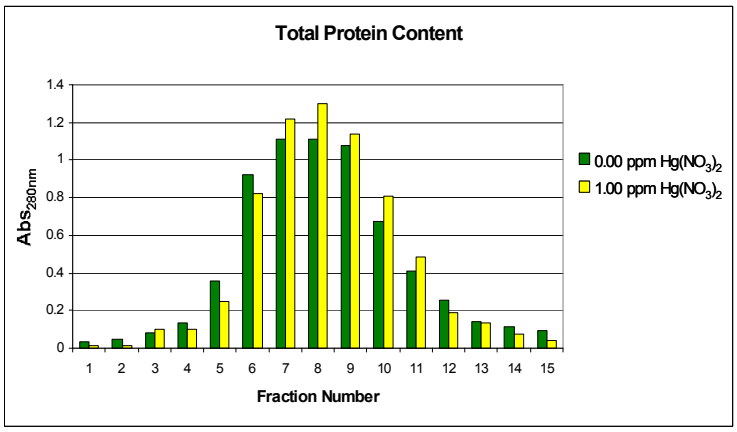

Figure 6. Absorbance profile (280 nm) of gel filtration extracts of *C. odorata* plants exposed to 0.00 and 1.00 ppm $Hg(NO_3)_2$ for 7 d. Data are means of three replicates (±SD).

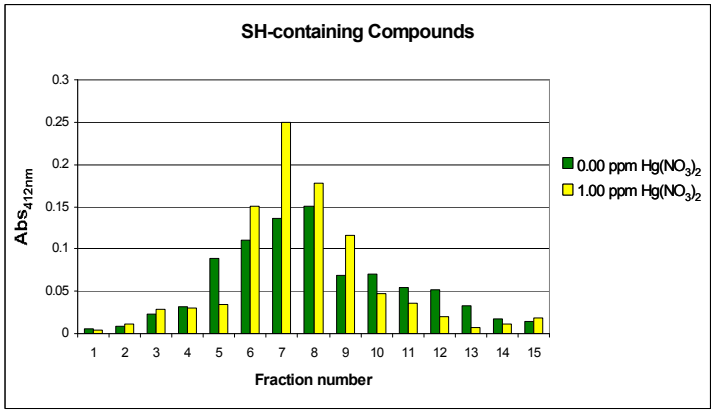

Figure 7. Gel filtration chromatography elution profile of SH-containing compounds in *C. odorata* plants exposed to 0.00 and 1.00 ppm $Hg(NO_3)_2$ for 7 d. Data are means of three replicates (±SD).

The results on the absorbance readings at 254 nm of the fractions eluted from gel filtration (Figure 8) 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, 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 in the case of *C. odorata.*

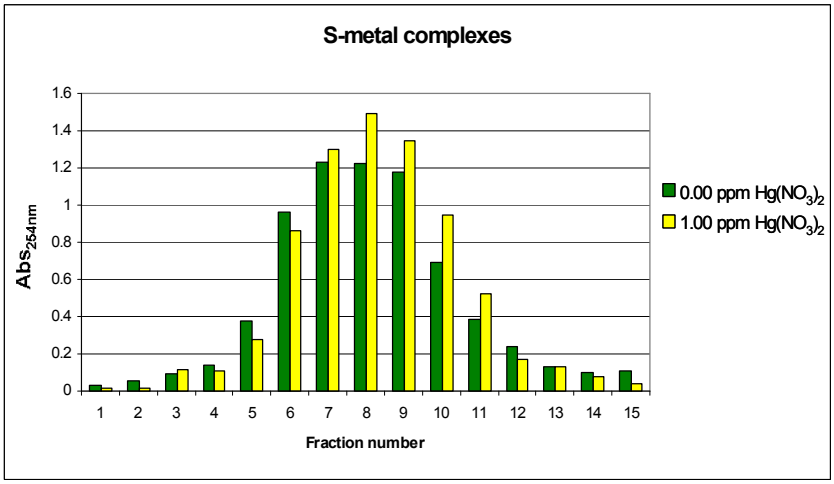

Figure 8. Absorbance profile (254 nm) of gel filtration extracts of *C. odorata* plants exposed to 0.00 and 1.00 ppm $Hg(NO_3)_2$ for 7 days. Data are means of three replicates (±SD).

The RP-HPLC 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 9a-c), indicating the presence of GSH-like and/or MT-like, Cys-containing biomolecules (Figures 9d-e). 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 5). 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; Josue et al., 2006). In this study, the amplified production of SH-containing biomolecules is the first line of defense of *C. odorata* against Hg^{2+} toxicity.

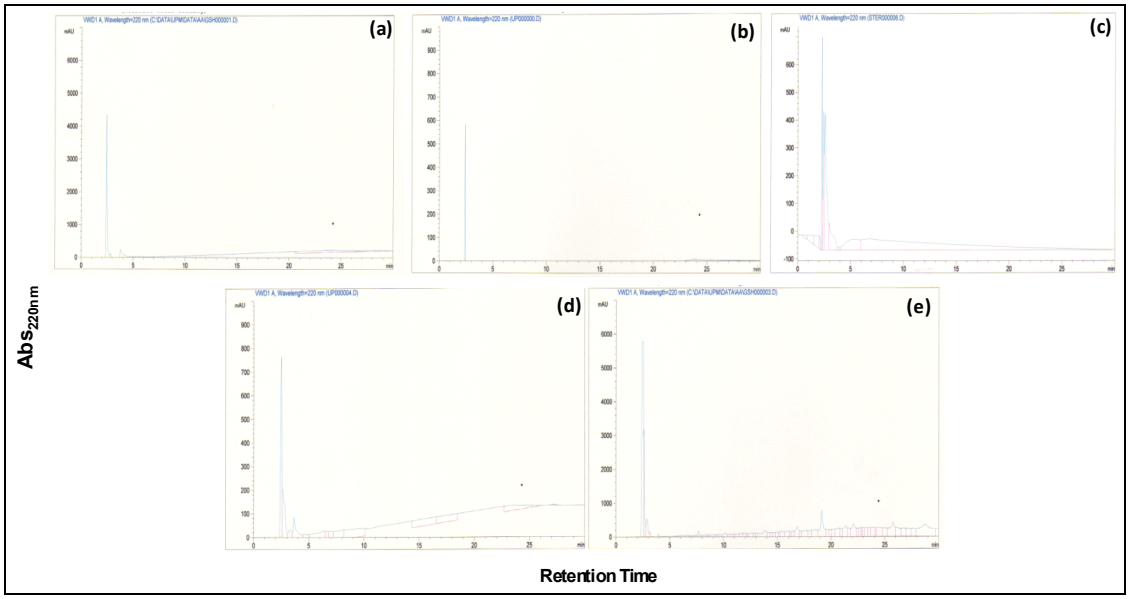

Figure 9. RP-HPLC chromatographs of glutathione (GSH). (a) standard GSH; (b) standard metallothionein (MT); standard cysteine; (d) control *C. odorata* plants; (e) treated *C. odorata* plants.

Table 5. 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 (mAU*s)	Height (mAU)	GSH conc. (mg/ml)	MT conc. (mg/ml)	Cysteine conc. (mg/ml)
GSH standard	2.415	2.790588×10^4	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	6.63089×10^4	5793.87402	2.38	24.91	21.92

Conclusion

The findings of the study suggest that *C. odorata* exhibits the criteria for a Hg phytoremediation agent. It was able to uptake, accumulate, and tolerate the heavy metal Hg. The total Ca, Mg, Fe, and S levels in the leaves were not found to be significantly affected by Hg^{2+} treatment. The chlorophyll contents of leaves from the Hg-exposed *C. odorata* plants remained at normal levels, demonstrating that its photosynthetic machinery was functioning normally. The results of the 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 the leaves. The increased S levels in the vacuoles isolated from the leaves of the Hg-treated plants also indicated that the S-containing biomolecules in the vacuoles are involved in the chelation, sequestration, and detoxification of Hg^{2+}. Various antioxidant systems possibly operate as protective and tolerance mechanisms in *C. odorata* against the otherwise oxidative injury caused by Hg^{2+}. Specifically, the metal tolerance exhibited by *C. odorata* plants can be attributed to increased CAT activity, increased catalase units, and increased production of non-protein thiols. Moreover, the APX activity and chlorophyll contents were at normal levels despite the Hg^{2+} treatments. 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 retention times falling within the ranges 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 from the control. The results of this study confirm *Chromolaena odorata* as a potential phytoremediator agent. The enhanced antioxidative capacity and 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 *Chromolaena odorata* plants.

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