Phytotoxicity and Phytoremediation Potential of Mercury in Indian Mustard and Two Ferns with Mercury Contaminated Water and Oak Ridge Soil - 9241

Y. Su, F.X. Han, J. Chen, S. Shiyab, D.L.Monts
Institute for Clean Energy Technology (ICET), Mississippi State University, Mississippi State, MS 39762

D.L.Monts
Department of Physics and Astronomy, Mississippi State University, Mississippi State, MS 39762

ABSTRACT

Phytoremediation is an emerging technology that uses various plants to degrade, extract, contain, or immobilize contaminants from soil and water. Certain fern and Indian mustard species have been suggested as candidates for phytoremediation of heavy metal-contaminated soil and water because of their high efficiency of accumulating metals in shoots and their high biomass production. Currently, no known hyperaccumulator plants for mercury have been found. Here we report the Hg uptake and phytotoxicity by two varieties of fern and Indian mustard. Their potential for Hg phytoremediation application was also investigated. Anatomical, histochemical and biochemical approaches were used to study mercury phytotoxicity as well as antioxidative responses in ferns [Chinese brake fern (*P. vittata*) and Boston fern (*N. exaltata*)] and Indian mustard (Florida broadleaf and longstanding) (*Brassica juncea* L.) grown in a hydroponic system. Phytoremediation potentials of these plant species were estimated based on their Hg uptake performance with contaminated soils from Oak Ridge (TN, USA). Our results show that mercury exposure led to severe phytotoxicity accompanied by lipid peroxidation and rapid accumulation of hydrogen peroxide (H_2O_2) in *P. vittata*, but not in *N. exaltata*. The two cultivars of fern responded differently to mercury exposure in terms of antioxidative enzymes (superoxide dimutase, SOD; catalase, CAT; peroxidase, POD; glutathione redutase, GR). Mercury exposure resulted in the accumulation of ascorbic acid (ASA) and glutathione (GSH) in the shoots of both cultivars of fern. On the other hand, Indian mustard effectively generated an enzymatic antioxidant defense system (especially CAT) to scavenge H_2O_2, resulting in lower H_2O_2 in shoots with higher mercury concentrations. These two cultivars of Indian mustard demonstrated an efficient metabolic defense and adaptation system to mercury-induced oxidative stress. In both varieties of fern and Indian mustard, a majority of Hg was accumulated in the roots and low translocations of Hg from roots to shoots were found in two cultivars of Indian mustard. Experiments with aged soil indicate that fern can accumulate up to 1700-2000 mg/kg Hg in roots and 60-170 mg/kg in shoots, while two varieties of Indian mustard accumulated up to 2000-4000 mg/kg Hg in roots and 260-430 mg/kg Hg in shoots from contaminated Oak Ridge soil. Thus, these varieties of Indian mustard and *N. exaltata* (fern) might be potential candidate plants for phytoremediation of mercury-contaminated water and soils. More field studies are planned for further evaluation.

INTRODUCTION

Mercury (Hg) is one of the major toxic metals because it bio-accumulates and bio-magnifies in animal and human bodies through the food chain. Millions of tons of mercury have been released and accumulated in the global environment since the beginning of the Industrial Revolution (Han et al., 2002, 2006). The main sources of mercury pollution include mining of gold and silver, the coal industry, untreated discarded batteries and industrial waste disposal. Mercury can also enter into agricultural soil by anthropogenic activities, such as from fertilizers, sludge, pesticides, lime, and manure.

Merury in soil can be in many forms, such as elemental mercury (Hg^0), ionic mercury (Hg^{2+}), methyl mercury (MeHg), mercury hydroxide ($Hg(OH)_2$), and mercury sulfide (HgS). Hg^{2+} is the predominant toxic forms of mercury. It can be directly taken up by plants and leads to mercury toxicity to plants. Phytoremediation is novel technology that utilizes plants to remove, transform, or stabilize contaminants, including organic pollutants and toxic metals, located in water, sediments, or soils. Phytoremediation has been accepted and utilized widely because of cost-effectiveness, permanent removal and protection of nature. There are mainly five types of phytoremedation: phytoextration, phytostabilization, rhizofiltration, phytoreduction, and phytovolatilization (Raskin and Ensley, 2000). Over 400 plant species have been identified as natural metal hyperaccumulators (Raskin and Ensley, 2000). For example, *Alyssum murale*, *Thlaspi rotundifolium*, *Dicoma niccolifera*, *Pteris vittata* and *Sesbania drummondii* are famous for their ability to hyperaccumulate Ni, Zn, Cr, As and Pb, respectively (Ma et al., 2001). But no hyperaccumulator for mercury has been found to our knowledge.

Although the detailed mechanism of oxidative stress in plants resulting from heavy metal exposure is not yet fully understood, it is recognized that the antioxidant system in plants is an important mechanism for plants to respond to heavy metal stress. Mercury triggers oxidative stress by inducing production of H_2O_2, lipid peroxides, and reactive oxygen species (ROS) in many plants, such as tomato, cucumber and alfalfa (Shiyab et al., 2009; Chen et al., 2009). Plant cells have antioxidants like α-tocopherol, glutathione, ascorbate and antioxidative enzymes, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), guaiacol peroxidase (POD), catalase (CAT) and glutathione reductase (GR), that participate in scavenging reactive oxygen species. Plants can adapt to oxidative stress through these antioxidative enzymes and antioxidants. For example, mercury stress induced a significant increase in the activity of antioxidative enzymes in *Sesbania drummondii* and *Zea mays*. Compared to arsenic-sensitive fern species, there were greater activity of antioxidative enzymes and more antioxidants in arsenic-hyperaccumulative fern species, such as *P. vittata*;

The physiological and molecular mechanisms responsible for metal hyperaccumulation and tolerance in plants have been extensively studied (McGrath et al., 2002; 2003). As an arsenic hyperaccumulator, a considerable number of studies have been performed to investigate the resistance mechanism of Chinese brake fern (*Pteris vittata*) to arsenic stress (Ma et al., 2001), but not for mercury. On the other hand, Indian mustard plants have higher biomass and faster growth rates compared to the other hyperaccumulator plants for heavy metals, it is recommended as a candidate for phytoremediation of metals- (such as Pb, Zn, Cd and Cr) polluted soils (Han *et al.,* 2004). Few studies have been conducted on the phytotoxicity of mercury to Indian mustard (Su et al., 2008). The objectives of this prelimary study were to investigate the uptake of mercury by two common cultivars of Indian mustard (*Brassica Juncea* L.) and two fern cultivars [Chinese brake fern (*P. vittata*) and Boston fern (*N. exaltata*)] and its phytotoxicity to these plants. The overall purpose of our study was to seek the potential application of Indian mustard and ferns to phytoremediation of mercury-contaminated soils, sediments and water. However, this will require a series of long period experiments with contamianted field soils and waste water before its application.

MATERIALS AND METHODS

Plant growth and experiment design

Two major commercial cultivars of Indian mustard were selected for the experiment. The cultivar Long standing, a southern giant curled, has frilled leaves with maturation usually at 56 days while cultivar Florida Broad leaf has large, broad, rich green leaves and a mild-flavor with maturation usually at 45 days after seed sowing. The seeds were obtained locally. The seeds were sown in commercial potting mix (organic residue, peat, manure and nutrients) in plastic trays and each tray contained approximately 30 seeds. The plants were transferred into hydroponic solutions after two weeks (2-week-old) and one month

(one-month-old) of growth, respectively. Plants were grown for 8 days for 2-week-old young seedlings and for 2 weeks for 1-month-old plants in the hydroponics system, respectively (Fig. 1). In one study, 20 seedlings (two week-old) were grown in one tray containing 4000 mL of modified Hoagland's solution. In another study, one plant (1 month-old) was grown in each container, which contained 1000 mL of modified Hoagland's solution (Chen et al., 2009).

Two fern species were transferred into a modified full-strength Hoagland nutritant solution containing 33.95 mg l^{-1} KH_2PO_4, 19.93 mg l^{-1} NH_4NO_3, 126.44 mg l^{-1} KNO_3, 60.19 mg l^{-1} $MgSO_4$, 164.1 mg l^{-1} $Ca(NO_3)_2$, 0.71 mg l^{-1} H_3BO_3, 0.02 mg l^{-1} $CuSO_4$, 1.33 mg l^{-1} $FeSO_4 \cdot 7H_2O$, 0.604 mg l^{-1} $MnSO_4 \cdot H_2O$, 0.004 mg l^{-1} MoO_3, and 0.055 mg l^{-1} $ZnSO_4$. The pH of the nutrient solution is 5.5, which is suitable for fern growth. $HgNO_3 \cdot H_2O$ with different Hg concentrations (1, 5 and 20 mg l^{-1}), was added to the solution after transferring the plants for three days for plants to adapt to the new growth medium. After treatment with mercury for three and for seven days, fresh leaf samples were collected and weighed for microscopy study and biochemical measurements. After exposure to mercury for 10 days, leaf and root samples were collected, dried for 48h at 80ºC, and then weighed for mercury content analysis. Plant roots were washed by 1% (v/v) HCl to remove the mercury adhering to the surface of the roots.

For aged Oak Ridge soil experiment, two varieties of Indian mustard were grown on aged Oak Ridge soils for 53-58 days and for a Chinese bake fern (*P. vittata*) for 43 days. Oak Ridge soils were contaminated with various mercury forms (Hg nitrate, chloride, and sulfide) (Han et al., 2006).

Determination of lipid peroxidation, H_2O_2, and Enzyme assay and non-enzyme and protein

The content of thiobarbituric acid reactive substances (TBARS) was determined as an indicator of the level of lipid peroxidation in the leaves (Chen et al., 2009; Shiyab et al., 2009a, b). About 0.05 g of fresh leaf sample was ground with 1.5 ml of 0.1 % (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 10000 g for 10 min and 0.5 ml of the supernatant was mixed with 2 mL of 20% (w/v) TCA containing 0.5% (w/v) 1,3-diethyl-2-thiobarbituric acid (TBA). The mixture was heated at 90 °C for 20 min and then cooled in ice. The mixture was then centrifuged at 10000 g for 5 min. The absorbance of the supernatant was measured at 532 nm and at 600 nm. The TBARS content was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

Approximate 0.1 g of leaf sample was homogenized with a mortar and pestle in 1.5 ml phosphate buffer (50 mM, pH 6.5) containing 1 mM hydroxylamine. After centrifugation at 10000 g for 10 min, 0.5 ml supernatant was mixed with 1.5 ml 20% H_2SO_4 containing 0.1% (v/v) $TiCl_4$. After through mixing, the mixture was centrifuged at 10000 g for 10 min. Then the absorbance of the supernatant was measured at 410 nm. The content of H_2O_2 was calculated using an extinction coefficient of 0.28 μM^{-1} cm^{-1}.

About 0.05 g of leaf tissue was homogenized in 1.5 ml of ice-cooled phosphate buffer (50 mM, pH 7.0, containing 1 mM EDTA and 1% w/v insoluble ployvinylpyrrolidone). The homogenate was centrifuged at 15000 g for 10 min at 4°C. The supernatant was used as the crude extract for the assay of enzyme activities. Activity of guaiacol peroxidase (POD, EC 1.11.1.7) was assayed by measuring the oxidation of guaiacol using hydrogen peroxide (Chen et al., 2009; Shiyab et al., 2009). The reaction mixture contained 1 ml of 100 mM potassium phosphate buffer (pH 5.8), 50 ml of 1.5% hydrogen peroxide, 1 ml of 0.25% guaiacol and 50 ml enzyme extract. The increase in absorbance at 470 nm was recorded. Activity was calculated using an extinction coefficient of 26.6 mM^{-1} cm^{-1}. One unit of POD activity is defined as the amount required to decompose 1 μmol of hydrogen peroxide min^{-1} mg^{-1} protein under assay conditions.

Activity of superoxide dismutase (SOD, EC 1.1.5.1.1) was assayed by measuring the inhibition of photochemical reduction of nitro-blue tetrazolium (NBT) (Chen et al., 2009; Shiyab et al., 2009). The reaction mixture (3 ml) contained 50 mM potassium phosphate buffer (pH 7.8), 13 m*M* methionine, 2 μM

riboflavin and 75 μM NBT, 0.1 mM EDTA and 30 ml enzyme extract. The absorbance of the solution was measured at 560 nm. One unit of SOD is defined as the amount of enzyme that causes half-maximal inhibition of the NBT reduction under assay conditions.

Activity of catalase (CAT, EC 1.11.1.6) was assayed by measuring the decomposition of hydrogen peroxide. About 50 ml enzyme extract was added to the reaction mixture containing 1 ml phosphate buffer solution (50 mM, pH 7.0) and 0.1% H_2O_2. The decrease of the absorbance at 240 nm was recorded. Activity was calculated using an extinction coefficient of 0.036 mM^{-1} cm^{-1}. One unit of CAT activity is defined as the amount required to decompose 1 μmol of hydrogen peroxide min^{-1} $\cdot mg^{-1}$ protein under assay conditions.

Glutathione reductase (GR, EC 1.8.5.1) activity was determined by the transformation of oxidized glutathione (GSSG) to glutathione (GSH) (Chen et al., 2009; Shiyab et al., 2009a, b). One ml assay mixture contained 200 mM potassium phosphate buffer (pH 7.5), 0.2 mM Na_2EDTA, 1.5 mM $MgCl_2$, 0.5 mM GSSG, 50 μM NADPH, and enzyme extract. The reaction was initiated by addition of NADPH and the decrease in absorbance at 340 nm was recorded. Corrections were made for nonenzymatic oxidation of NADPH by recording the decrease at 340 nm without adding GSSG to the assay mixture. The enzyme activity was calculated from the initial rate of the reaction after subtracting non-enzymatic oxidation. Activity was calculated using an extinction coefficient of 6.2 nM^{-1} cm^{-1}. One unit of GR activity is defined as the amount required to decompose 1 μmol of NADPH $min^{-1}\cdot mg^{-1}$ protein under assay conditions.

Leaf samples were prepared for ascorbic acid (ASA) and glutathion (GSH) analyses by homogenizing 1 g of leaf material (fresh weight) in 10 ml of cold 5 % meta-phosphoric acid. The homogenate was centrifuged at 22000 g for 15 min at 4 °C, and the supernatant was collected for analyses of ascorbate and glutathione.

For ASA assay, the reaction mixture for ascorbic acid contained 0.3 ml aliquot of the supernatant, 0.75 ml of 150 mM phosphate buffer (pH 7.4) containing 5 mM EDTA, and 0.3 mL H_2O. Color was developed in reaction mixtures after addition of the following reagents: 0.6 ml of 10% TCA, 0.6 ml of 44% ortho-phosphoric acid, 0.6 ml 4% α,α'-dipyridyl in 70% ethanol, and 0.3% $FeCl_3$. After vortex-mixing, the mixture was incubated at 40°C for 40 min and the absorbance at 525 nm was read. A standard curve ASA in the range 0-100μg ml^{-1} was prepared (Chen et al., 2009; Shiyab et al., 2009a, b).

For GSH assay, one ml aliquot of the supernatant was neutralized with 1.5 mL of 0.5 M phosphate buffer (pH 7.5), then 50μl H_2O was added; this sample was used for the assay of total glutathione. Glutathione content was measured in a 3 ml reaction mixture containing 0.2 mM NADPH, 100 mM phosphate buffer (pH 7.5), 5 mM EDTA, 0.6 mM DTNB and 3 units of GR. The reaction was started by adding 0.l ml of extract sample obtained as described above. The reaction rate was monitored by measuring the change in absorbance at 412 nm for 1 min. A standard curve was developed based on GSH in the range 0-50 μM.

Protein in samples was quantified by the Bradford method (Chen et al., 2009; Shiyab et al., 2009a, b), using bovine serum albumin as standard.

Procedure for microscopic study

Leaf samples of 5 mm in length were excised from the middle portion of the lamina between the second and the third vein from the leaf midrib. The samples were collected from the third leaf from the stem root intersection. The leaf samples were prepared for light microscopy (LM, 510 Confocal laser scanning microscope, Carl Zeiss SMT, Cambridge, UK), scanning electron microscopy (LEO SEM, Carl Zeiss SMT, Cambridge, UK), and transmission electron microscopy (JEM-100CX II TEM, JEOL, Tokyo, Japan). LM samples were immediately fixed in formaldehyde-acetic acid (FAA) and the plant samples were alcohol dehydrated, ultramicrotomed and subjected to 1% toluidine blue staining for further

observation. The procedure for collection of leaf samples for SEM was the same as described for LM. All the samples were immediately fixed in 2.5 % glutaraldehyde in 0.05 M potassium phosphate buffer (pH 7.1) for eight hours (Su et al., 2005) and then dehydrated in an ethanol series. The samples were sealed in Parafilm, frozen in liquid nitrogen and fractured transversely using a pre-cooled knife. The cryofractured specimens were critical point dried through carbon dioxide, mounted on stubs and coated with gold-palladium. All materials were observed with a LEO SEM. The samples for TEM were fixed in 2.5% glutaraldehyde in 0.05 M potassium phosphate buffer (pH 7.1) for eight hours and post fixed with OsO_4. The samples were dehydrated in an ethanol series and embedded in Spurs epoxy resin. Ultrathin sections were obtained, ultramicrotomed, and stained with uranyl acetate and basic lead citrate for observation with a JEOL TEM .

RESULTS AND DISCUSSION

Uptake of Mercury by Indian mustard and Ferns

Mercury concentrations in both shoots and roots of two ferns and Indian mustard increased with mercury concentrations in solution and soils. Majority of mercury was accumulated in roots of two plants which grown in either hydroponic solution or contaminated Oak Ridge soils (Fig. 1 and Table 1).

P. vittata and *N. exaltata* were exposed to solutions with 0, 1, 5 and 20 mg l^{-1} mercury for up to 10 days. Mercury concentrations increased in both shoots and roots of these two varieties with an increase in mercury concentration in growth medium. More significant amounts of mercury accumulated in the roots than in the shoots of both varieties, especially after exposure to 20 mg l^{-1} mercury. Compared to N. exaltata, *P. vittata* accumulated more mercury in both shoots and roots (Chen et al., 2009).

Similarly, uptake of mercury by the two cultivars of Indian mustard increased as a function of mercury concentration and mercury exposure periods in hydroponic solutions (Fig. 1). The highest mercury concentrations were found in one-month-old Broad leaf cultivar after two weeks of mercury treatment (16.7 mg L^{-1}), which were 2570 and 27900 mg kg^{-1} in shoots and roots, respectively. However, mercury concentrations in shoots and roots of one-month-old Long standing cultivar after two weeks of treatment were 2240 and 18400 mg kg^{-1}, respectively. A majority of the mercury in plants remained in roots. Ratios of mercury concentrations in roots/shoots for all the Hg treatments were in the range of 8-100 and decreased with Hg concentrations in solution. (Shiyab et al., 2009b).

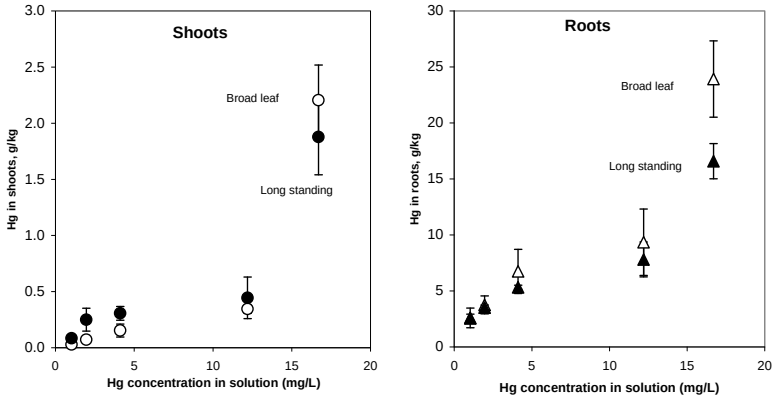

Fig. 1. Plant (Indian mustard) uptake of mercury from hydroponic solutions (1-month-old plants after 2 weeks of Hg treatment) as a function of mercury in solution actually measured by ICP-AES/CVAAS.

Table 1. Hg uptake by two varieties of Indian mustard and a fern grown in aged Oak Ridge (TN) contaminated soils with various Hg forms.

Growing			CK	Hg(NO3)2	HgCl2			HgS	
Season			0	43 ± 6	37 ± 28	47 ± 18	128 ± 52	1000	2000
						mg/kg Hg			
Indian mustard: Broadleaf									
Spring	Shoots	Min	0	5	1	35	243	11	69
		Max	2	9	3	62	778	58	151
		Avg	1	7	2	50	434	39	103
		Stdev	1	1	1	10	211	18	30
	Roots	Min	2	36	39	82	1619	59	188
		Max	3	61	76	137	1937	63	312
		Avg	3	46	50	112	1790	62	258
		Stdev	1	11	17	26	152	2	60
Fall	Shoots	Min	0	5	1	13	288	11	62
		Max	1	9	3	35	388	51	105
		Avg	1	7	2	22	323	34	79
		Stdev	1	2	1	11	56	21	23
	Roots	Min	2	17	14	97	1619	59	175
		Max	2	34	41	120	1998	63	202
		Avg	2	23	27	110	1774	61	187
		Stdev	0	10	14	11	198	2	14
Indian Mustard: Long standing									
Spring	Shoots	Min	0	2	1	9	174	14	11
		Max	0	4	3	19	324	49	21
		Avg	0	3	2	13	262	26	15
		Stdev	0	1	1	4	64	15	4
	Roots	Min	2	23	18	69	783	29	140
		Max	3	35	65	106	4637	62	204
		Avg	2	28	36	87	2593	49	159
		Stdev	1	5	21	18	1878	15	30
Fall	Shoots	Min	0	11	13	85	262	21	23
		Max	1	19	23	164	388	39	40
		Avg	1	14	16	134	325	28	31
		Stdev	0	4	6	43	63	9	9
	Roots	Min	2	25	25	97	783	49	189
		Max	3	35	55	139	3721	92	290
		Avg	2	29	38	114	1912	73	248
		Stdev	0	5	16	22	1583	22	53
Fern: Pteris Vittata L									
Spring	Shoots	Avg	0	15	2	15	148	28	41
		Stdev	0	5	1	3	23	4	13
	Roots	Avg	2	37	25	91	1584	73	146
		Stdev	0	7	5	11	261	6	25

Experiments with aged soil indicate that fern can accumulate up to 1700-2000 mg/kg Hg in roots and 60-170 mg/kg in shoots, while two varieties of Indian mustard accumulated up to 2000-4000 mg/kg Hg in roots and 260-430 mg/kg Hg in shoots from contaminated Oak Ridge soil (Table 1).

Mercury induced changes in leave structure

Indian mustard grown in solutions with 12.2 and 16.7 mg/L of mercury showed cellular structural changes. Compared to the control, thickly stained areas surrounding the vascular bundles in leaves of Indian mustard are observed in LM micrographs. A decrease in the number of palisade and spongy parenchyma cells of the two cultivars of Indian mustard grown in mercury-treated solutions (4.11 and 16.7 mg/L) was observed in the SEM micrographs. SEM micrographs showed reduced cell size and clotted depositions compared to the control. These vascular depositions are more prominent in shoots of plants in mercury-treated solution as compared with the control. The leaves from plants in solutions with 20 mg/L mercury showed a decrease in size of vacuoles along the cell walls and decreased number of vacuoles and electron-dense material along the walls of the xylem and phloem vessels. TEM micrographs showed significant changes in chloroplasts in both palisade and spongy mesophyll cells (Fig. 2A). The

palisade chloroplasts exhibited a loss of spindle shape and decreases in their amounts and starch grains compared to their respective controls (Shiyab et al., 2009b).

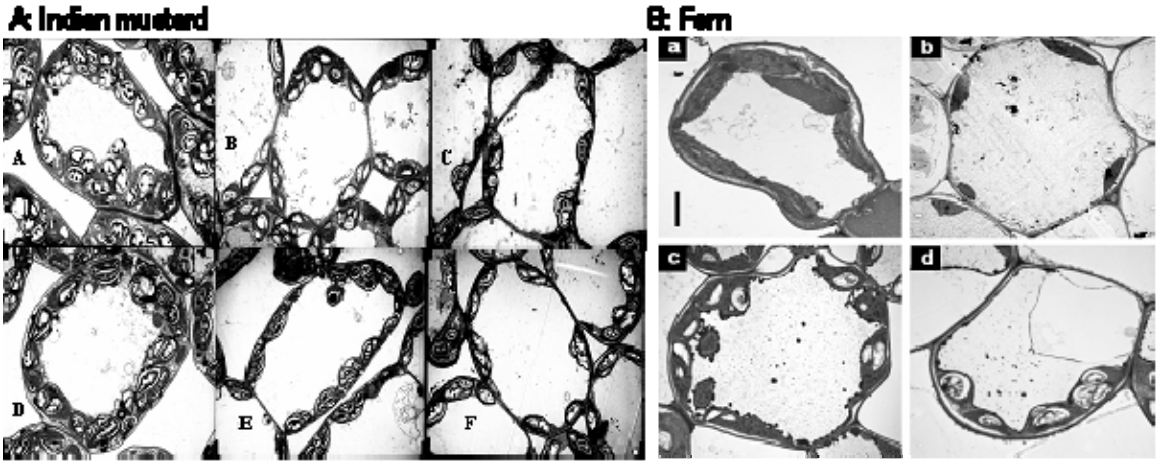

Fig. 2. A. TEM micrographs showing the chloroplast of lower palisade parenchyma cells of Hg-treated plants (1-month-old) of cultivar Broad leaf and Long standing after 2 weeks exposure to mercury solution (A, B and C were the control, 4.11, and 16.7 mg L^{-1} mercury treatment for the cultivar Broad leaf, respectively; D, E and F the control, 4.11, and 16.6 mg L^{-1} mercury treatment for the cultivar Long standing, respectively). B. TEM micrographs of the leaf cells of ferns. Plants were exposed to 20 mg l^{-1} mercury for 10 days. (a) the control of *P. vittata*; (b) 20 mg l^{-1} Hg treatment of *P. vittata*; (c) the control of *N. exaltata*; (d) 20 mg l^{-1} Hg treatment of *N. exaltata*. Bar indicates 20 µm.

Electron microscopy study indicates different toxic effects of mercury on the cellular structure in leaves of *P. vittata* and *N. exaltata*. The SEM micrographs show changes of the vascular cells in the leaf samples. Exposure to 20 mg l^{-1} mercury for 10 days resulted in a loss of cell shape and decreases the intercellular spaces in *P. vittata* compared to the control. Mercury exposure also led to the shrinkage of vascular bundle in the leaves of *P. vittata*. Some precipitates filled in the intercellular spaces in leaves of *P. vittata* after mercury treatment. For *N. exaltata*, changes in vascular cell structure were not significant for mercury treatment compared to the control. Furthermore, after exposure to 20 mg l^{-1} mercury for 10 days, the TEM micrographs showed a significant break down of chloroplast in leaves of *P. vittata* (Fig. 2B) compared to the control. There were some precipitates adhering to the cell walls in the leaves of *P. vittata* after mercury treatment. For *N. exaltata*, the TEM micrographs showed no significant changes in the leaf cell, expect for a slight break down of chloroplast after mercury exposure (Chen et al., 2009). Compared to the control, exposure of mercury for 7 days significantly decreased the relative water content (RWC) in the shoots of *P. vittata*, especially at 5 and 20 mg l^{-1} mercury.

Phytotoxicity of mercury

Phytotoxicity of ionic mercury to Indian mustard resulted in the reduction of biomass and relative water contents of both shoots and roots in two cultivars (Fig. 3) (Shiyab et al., 2009b). Biomass of both shoots and roots of two cultivars of Indian mustard followed a logarithmic decrease with mercury concentrations in both shoots and roots. Initially, biomass of both shoots and roots quickly decreased with mercury concentration in plants, followed by a very slow change at higher mercury concentration (Fig. 3). The critical mercury concentrations in roots causing 25% or 50% decrease in biomass were significantly higher (20-30 times) than those in shoots. Moreover, these critical mercury concentrations in both shoots

and roots increased with the age of plants. Mature Indian mustard plants (30/45-day-old) had significantly higher critical mercury concentrations in both shoots and roots than younger plants (15-23 day-old). Mercury concentrations in younger plants (15/23-day-old) causing 25% biomass decrease in shoots and roots were 30 and 740 mg/kg, respectively. But the critical mercury concentrations in the mature plants (30/45-day-old) causing 25% reduction of biomass in shoots and roots increased to 168 and 3250 mg/kg, respectively. Similar trends were observed for the critical mercury concentrations in plants causing 50% biomass decreases. This indicates that the two cultivars of Indian mustard significantly increased their tolerance to mercury and the amount of mercury translocated to shoots with age. Another important indicator of mercury toxicity is relative moisture (water) contents (RWC). RWC (%) of shoots significantly decreased with mercury concentrations in plants (Fig. 4). Compared to the control, exposure of mercury for 7 days significantly decreased the relative water content (RWC) in the shoots of *P. vittata*, especially at 5 and 20 mg l^{-1} mercury. But RWC in the shoots of *N. exaltata* were not affected by the mercury exposure. After 7 days of mercury exposure, obvious toxic symptoms (such as withering, chlorosis and falling of leaves) appeared in *P. vittata*, especially at high mercury concentration; in contrast, no obvious toxic symptoms were found in *N. exaltata* (Chen et al., 2009) .

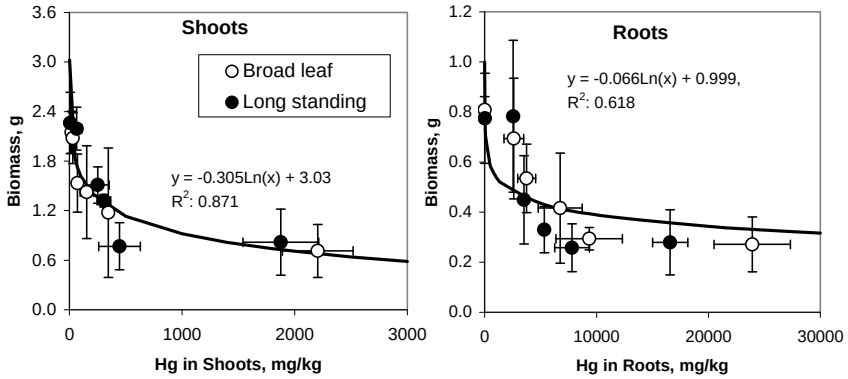

Fig. 3. Effect of mercury exposure duration on plant uptake of mercury (for 2-week-old and 1-month-old plants at 12.2 mg L^{-1} Hg solutions). Mercury uptake increased with mercury exposure in both early and later growth periods. There was no significant statistic difference between two cultivars.

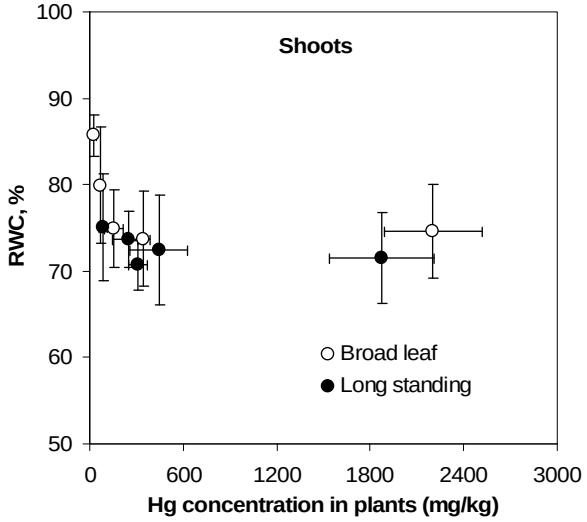

Fig. 4. Effect of mercury on leaf relative moisture content (%) of two cultivars of Indian mustard (1-month-old plants after 2 weeks of mercury treatment). The relative moisture contents in shoots initially

significantly decreased with mercury concentrations in shoots from about 90% to 70%, followed by a slow change with further increases in mercury concentrations.

Effect of mercury on lipid peroxidation

The toxic effects of mercury on the damage of cell membrane in tissues were examined by evaluating the content of thiobarbituric acid-reactive substances (TBARS), an indicator of lipid peroxidation. After exposure to mercury for 3 days and 7 days, the content of TBARS in the shoots of *P. vittata* increased with an increase in mercury concentration in growth medium, reached a maximum value at 5 mg l^{-1} mercury, and then decreased at 20 mg l^{-1} mercury . The content of TBARS in the shoots of *N. exaltata* was less than that of *P. vittata*. No significant differences were found in *N. exaltata* between the treatments with mercury and the control after mercury exposure (Chen et al., 2009b).
Concentrations of TBARS in leaves of Indian mustard increased with mercury concentration in solutions (Shiyab et al., 2009a). In young plants (two-week-old) after 8 days of mercury exposure, TBARS concentrations in shoots of both varieties increased with mercury concentration, but started to decrease when mercury concentrations in solutions were higher than 1 mg/L. However, TBARS contents in all mercury-exposed plants were higher than those in the control. In mature plants (1-month-old) after 2 weeks of mercury treatment, TBARS contents increased with mercury concentration in solutions as well as the mercury concentrations in shoots. When mercury concentrations in solution were higher than 4-10 mg/L and mercury concentration in shoots higher than approximately 200 mg/kg, TBARS concentrations plateaued. The concentrations of TBARS were also found to increase with mercury exposure duration in both Indian mustard cultivars. Increased TBARS contents in plants indicate that the mustard plants experienced substantial oxidative damage when exposed to high concentrations of mercury. Destruction of lipid components of membrane by lipid peroxidation causes membrane impairment and leakage. This may result in the potassium leakages.

Effect of mercury on hydrogen peroxide accumulation

Exposure to mercury induced a significant accumulation of hydrogen peroxide (H_2O_2) in the shoots of *P. vittata.* There was a mercury concentration-dependent change in H_2O_2 content in the shoot of *P. vittata.* The maximum formation of H_2O_2 was found with 20 mg l^{-1} mercury after exposure for 3 and 7 days; these H_2O_2 concentrations were 1.26 and 2.90 times higher than their controls, respectively. Histochemical staining also gave an indication of H_2O_2 accumulation *in vivo.* H_2O_2 can react with 3,3-diaminobenzidine (DAB) to appear pink, which can be used as an indicator of H_2O_2 production *in vivo*. Compared to the control, more pink color appeared inside of nervation in leaves of *P. vittata* after exposure to 20 mg l^{-1} mercury for 7 days. *N. exaltata* did not show significant changes in H_2O_2 content in the shoots, even after exposure to mercury for 7 days (Chen et al., 2009b).

In Indian mustard, contents of H_2O_2 in shoots linearly decreased with an increase in mercury concentration in hydroponic solutions. Concentrations of H_2O_2 in shoots initially linearly decreased with increasing mercury concentration in shoots at low mercury concentrations, but H_2O_2 concentrations did not change significantly with mercury in shoots at higher mercury concentrations. The decrease in H_2O_2 in plants at mercury stress was obviously related to the elevated H_2O_2-scavenging enzymes, especially CAT (Fig. 5). CAT mediates the cleavage of H_2O_2 evolving O_2 (Scandalios, 1993). Mercury treatment significantly increased concentrations of CAT. The concentrations of H_2O_2 linearly decreased with an increase in CAT activities in shoots of both cultivars of Indian mustard (Fig. 5). This indicates that Hg-induced CAT in Indian mustard effectively scavenges the mercury-induced H_2O_2. To reduce 1 unit of H_2O_2 caused by mercury stress required 5.6 units of CAT for two week-old plants after 4 days of exposure (Fig. 5). However, 7-7.7 units of CAT were required to decrease 1 unit of H_2O_2 for both two week- and one month-old plants after 1 week of mercury exposure. Furthermore, 39 units of CAT were

required to scavenge 1 unit of H_2O_2 for one-month-old plants after 2 weeks of mercury exposure (Shiyab et al., 2009a).

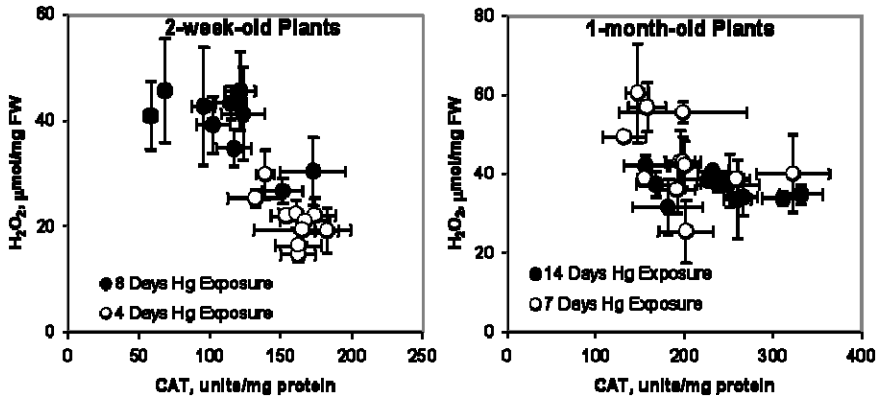

Fig. 5. Relationship between H_2O_2 production and catalase (CAT) in shoots of Indian mustard (expressed as an average and standard deviation of each treatment level).

Our results also suggest that a shorter mercury exposure of plants (both younger and mature age) provide better protection capability against mercury-induced oxidative stress. A shorter period of mercury exposure induced plants to generate a lower level of CAT to scavenge the generated H_2O_2, resulting in lower levels of H_2O_2 in cells. However, prolonged mercury exposure maintained a higher level of H_2O_2 in leaves, even though plants generated more elevated H_2O_2-scalvenging CAT than during a shorter exposure period (Shiyab et al., 2009a).

Effect of mercury on antioxidant enzymes

All of the mercury treatments induced significant increase in SOD activity in *P. vittata*. 5 mg l^{-1} mercury exposure induced greatest SOD activity in *P. vittata*, which were 52% and 199% higher than control after exposure to mercury for 3 and 7 days, respectively. On the contrary, there were no significant differences in the SOD activity in the shoots of *N. exaltata* between the mercury treatments and the control after exposure to mercury for 3 days. But exposure to mercury for 7 days at both the 5 mg l^{-1} and the 20 mg l^{-1} mercury levels significantly increased the SOD activity in the shoots of *N. exaltata*; these were 85% and 76% higher than the control, respectively. The activity of CAT in *P. vittata* and *N. exaltata* remained stable in responses to mercury exposure for 3 days, except for a lower CAT activity in *N. exaltata* at 20 mg l^{-1} mercury compared to the control. Exposure to mercury for 7 days resulted in a decrease in the activity of CAT in *N. exaltata* at 1 mg l^{-1} mercury and a significant increase at 5 and 20 mg l^{-1} mercury. In *P. vittata*, 5 mg l^{-1} mercury treatment induced a maximum CAT activity compared to the other treatments that were not response to mercury in CAT activity. The activity of POD in *P. vittata* decreased significantly after exposure to mercury. Compared with the control, exposure to mercury at 1, 5 and 20 mg l^{-1} for 3 days caused 66%, 59% and 73% decreases in POD activity in *P. vittata,* respectively. There were no significant changes in the POD activity in *N. exaltata* after exposure to mercury. Enhancement of glutathione reductase (GR) in the shoots of *P. vittata* and *N. exaltata* was significantly induced by mercury. After exposure to mercury for 3 days, the activity of GR in *P. vittata* increased significantly at 5 and 20 mg l^{-1} mercury compared with the control. *N. exaltata* only showed a significant increase in GR activity at 20 mg l^{-1} mercury level. Exposure to mercury at all mercury treatments for 7 days significantly increased GR activity in both *P. vittata* and *N. exaltata*. The highest POD activities appeared at 5 mg l^{-1}

mercury treatment. For both control and mercury treatments, *P. vittata* maintained much higher POD activity than did *N. exaltata* (Chen et al., 2009).

Concentrations of CAT in shoots of the two varieties of Indian mustard increased with mercury concentration in solutions and shoots (Shiyab et al., 2009a). In a similar manner, SOD and POD increased with mercury concentration in both shoots and hydroponic solutions. When mercury concentrations in shoots were approximately 200-250 mg/kg, which was equivalent to 2-5 mg/L in hydroponic solutions, CAT, SOD and POD in shoots all reached a plateau. In other words, at and above these critical concentrations of mercury, plant cells would not generate greater amounts of these enzymes to counter the ROS generated by mercury stress, resulting in toxicity to plants. Toxic concentrations of mercury cause oxidative stress, as demonstrated by the increased lipid peroxidation and H_2O_2 formation in leaves and roots of plants. The established inductions of a particular group of enzymes, including peroxides and super-oxides, are considered to play an important role in heavy metal stress. Catalases and peroxides remove the bulk of H_2O_2 in cells at the lower levels of H_2O_2 in plants observed in the present study, whereas the enzyme metabolites of the ascorbate-glutathione cycle are also involved in the fine regulation of the H_2O_2 level. Higher activity of catalase during a short exposure time was suggested to be related to low levels of H_2O_2 as discussed earlier.

However, these three major enzymes responded differently to mercury stress. In shoots, CAT increased with the duration of mercury treatments. However, SOD seemed to decrease with duration of mercury treatment. For POD, mature plants seemed to decrease POD production, but it also increased with mercury treatment duration.

Effect of mercury on non-enzymatic antioxidants

Compared to the controls, exposure to mercury for 3 and 7 days at 1 and 5 mg l^{-1} levels induced significant increases in ASA content in the shoots of both *P. vittata* and *N. exaltata* . Exposure of mercury for 7 days, the mercury treatment at 1 mg l^{-1} level began to induce an increase in ASA content in both *P. vittata* and *N. exaltata* and peaked at 5 mg l^{-1} mercury. ASA content also showed significant increase in *P. vittata* at 20 mg l^{-1} mercury, but no noteworthy change appeared in *N. exaltata* at this point. Mercury induced production of gluthione (GSH) in both *P. vittata* and *N. exaltata*. Exposure to mercury for 3 days at all mercury levels induced significant increases in GSH content in *P. vittata.* Mercury treatment at 5 mg l^{-1} level caused a maximum production of GSH in *P. vittata*. For *N. exaltata*, only 1 mg l^{-1} of mercury induced a significant increase in GSH content. Exposure to mercury for 7 days increased the production of GSH in *P. vittata* with the peak also at 5 mg l^{-1} mercury. GSH in *N. exaltata* began to increase at 1 mg l^{-1} mercury and peaked at 5 mg l^{-1}, followed by a significant decrease at 20 mg l^{-1} mercury (Chen et al., 2009).

Conclusions

Mercury exposure triggered strong oxidative stress and phytotoxicity in both ferns and both Indian mustard. Mercury had a stronger toxicity in *P. vittata* than *N. exaltata*. *N. exaltata* appears more resistant to mercury stress than *P. vittata*.

Uptake of mercury by the two cultivars of Indian mustard and ferns increased as a function of mercury concentration in hydroponic solutions and soils. The highest mercury concentrations (0.26% and 2.8% on the dry base) were found in shoots and roots of Indian mustard, respectively, among all mercury treatments employed in this study. Mercury uptake induced a significant reduction in both biomass and leaf relative water contents of two cultivars of Indian mustard. Elevated mercury in plants led to significant cellular structural changes in leaves. The changes include thickly stained areas surrounding the vascular bundles, decreases in the number of palisade and spongy parenchyma cells, reduced cell size and

clotted depositions, and decreases in size and number of vacuoles along the cell walls. The palisade chloroplasts exhibited a loss of spindle shape and decreased in starch content. These two cultivars of Indian mustard demonstrated an efficient metabolic defense and adaptation system to mercury-induced oxidative stress. Similarly, the relatively higher enhancements in the activities of anti-oxidative enzymes were found in *N. exaltata* than in *P. vittata* under mercury stress. Due to high accumulation of mercury in both shoots and roots, Indian mustard and fern *N. exaltata* might be a potential candidate plant for phytoremediation of mercury contaminated soils and waters despite of high mercury phototoxicity. More field studies are planned for further evaluation.

REFERENCES

J. Chen, S. Shiyab, F.X. Han, D. L. Monts, C. A. Waggoner, Z. M. Yang, Y. Su."Bioaccumulation and physiological effects of mercury in *Pteris vittata* and *Nephrolepis exaltata"*. *Ecotoxicology* DOI 10.1007/s10646-008-0264-3, (2009).

F.X. Han,Y. Su, D.L. Monts, C.A. Waggoner, and M. J. Plodinec, "Binding, distribution, and plant uptake of mercury in a soil from Oak Ridge, Tennessee, USA". *Science of Total Environment* 368: 753-768 (2006).

F.X.Han, Banin, A, Su, Y., Monts, D.L., Plodinec, M.J., Kingery, W.L., Triplett, G.B. "Industrial age anthropogenic inputs of heavy metals into the pedosphere". Naturwissenschaften 89, 497-504. (2002).

F.X. Han, B.B.M. Sridhar, D.L. Monts, Y.Su, "Phytoavailability and toxicity of trivalent and hexavalent chromium to Brassica juncea L. Czern". New Phytologist. 162, 489-499 (2004).

L.Q. Ma, K.M. Komar, C. Tu, W.H. Zhang, Y. Cai, E.D. Kennelley, "A fern that hyperaccumulates arsenic: a hardy versatile, fast growing plant helps to remove arsenic from contaminated soils". Nature 409, 579 (2001).

S.P McGrath, F.J. Zhao, "Phytoextraction of metals and metalloids from contaminated soils". Current Opinion in Biotechnology 14, 277–282 (2003).

S.P McGrath, F.J. Zhao, E. Lombi, "Phytoremediation of metals, metalloids, and radionuclides". Advance in Agronomy 75, 1–56 (2002).

I. Raskin, B.D. Ensley, "Phytoremediation of toxic metals: using plants to clean up the environment". John Wiley & Sons, New York, pp. 193–229 (2000).

S. Shiyab, J. Chen, F.X. Han, D.L. Monts, F.B. Matta, M. Gu, Y.. Su. "Phytotoxicity of mercury in Indian mustard (*Brassica juncea L.*)". Ecotoxicology and Environmental Safety. DOI:10.1016/j.ecoenv.2008.06.002. 72: 619– 625 (2009b).

S. Shiyab, J. Chen, F.X. Han, D.L. Monts, F.B. Matta, M. Gu, Y. Su, M.A. Masad. "Mercury-induced oxidative stress in Indian mustard (*Brassica juncea* L.)". Environmental Toxicology (in press) (2009a).

B.B.M. Sridhar, S.V. Diehl, F.X. Han, D.L. Monts, Y. Su, "Anatomical changes due to uptake and accumulation of zinc and cadmium in Indian mustard (Brassica juncea)". Environmental and Experimental Botany 54, 131-141 (2005).

Y. Su, F.X. Han, J. Chen, B.B. M. Sridhar, D.L. Monts, "Phytoextraction and accumulation of mercury in three plant species: Indian mustard (*Brassica juncea*), Beard grass (*Polypogon monospeliensis*), and Chinese brake fern (*Pteris vittata)"*. International Journal of Phytoremediation 10:547-560 (2008).

Y. Su, F.X. Han, B.B.M. Sridhar, D.L. Monts, "Phytotoxicity and phytoaccumulation of trivalent and hexavalent chromium in brake fern". Environmental Toxicology and Chemistry. 24, 2019-2026 (2005).