Antioxidative mechanisms on selenium accumulation in *Pteris vittata* L., a potential selenium phytoremediation plant

R.W. Feng[1,2], C.Y. Wei[3]

[1]*Centre for Research in Ecotoxicology and Environmental Remediation, Institute of Agro-Environmental Protection of the Ministry of Agriculture, Tianjin, P.R. China*
[2]*Open Key Laboratory of Agro-environment and Food Safety of the Ministry of Agriculture, Tianjin, P.R. China*
[3]*Institute of Geographic Sciences and Natural Resources Research, Chinese Academy of Sciences, Beijing, P.R. China*

ABSTRACT

Selenium (Se) contamination due to industrial activities has received increasing concerns. Phytoremediation has been suggested to be an efficient and feasible way to remove Se from Se-contaminated environment. Recently, an arsenic (As) hyperaccumulator *Pteris vittata* L. (Chinese Brake fern) was found to be a Se accumulator. This study was carried out to investigate Se accumulation mechanisms concentrating on antioxidant responses of this plant to six levels of selenite (0, 1, 2, 5, 10, and 20 mg/L). The results showed that Chinese Brake fern can accumulate a large amount of Se without any visible toxic symptoms and significant decreases in its biomass. However, the root took up more Se than the fronds. The highest concentration of Se in the roots and fronds was 1.536 mg/kg and 242 mg/kg, respectively, demonstrating a typical accumulation character to Se. Addition of 2 mg/L Se decreased, but ≥ 5 mg/L Se enhanced the production of malondialdehyde (MDA), suggesting an antioxidant role of low dosages of Se. The enzymes of catalase (CAT), ascorbate peroxidase (APX) and peroxidase (POD) contributed their anti-oxidative functions only under low dosages of Se, as shown by their increased activities at Se levels ≤ 5 mg/L and lowered activities at Se levels > 5 mg/L. The concentration of glutathione (GSH) and enzyme activity of glutathione reductase (GR) were stimulated by ≥ 5 mg/L Se. Superoxide dismutase (SOD) activity was also enhanced by 20 mg/L Se. Our results suggest that SOD, GSH and GR were likely responsible for, but enzymes of POD, APX, and CAT have limited roles in Se accumulation in Chinese Brake fern.

Keywords: SOD; GSH; GR; antioxidants; Chinese Brake

Selenium (Se) contamination resulting from anthropogenic activities has received increasing concerns, especially in water system (Lemly 2004). In China, high levels of Se have been found in the soils and waters in some regions, such as the EnShi county in the HuBei province. Phytoremediation was proven to be a promising technology for the removal of Se from contaminated environments (Zayed et al. 2000). Se-accumulators, which can tolerate and accumulate high concentrations of Se, were explored for their accumulation potential as well as the tolerance mechanisms (Pickering et al. 2003). To date, most identified Se-accumulating plants are found to be included with the genera of *Brassica*, *Aster*, *Atriplex*, *Astragalus*, and *Melilotus* (Pezzarossa et al. 2007). Recently, a fern plant belonging to the genus of *Pteris*, named Chinese Brake fern (*Pteris vittata* L.), was found to be a possible selenite-accumulator (Feng et al. 2009a). This fern was also identified as an arsenic (As)

Supported by the National Science Foundation of China, Project No. 41103075; by the Central Public Research Institute Basic Funds for Research and Development (Agro-Environmental Protection Institute, Ministry of Agriculture, Project No. 11-szjj-frw), and by the President Funds of Chinese Academy of Agricultural Sciences, Project No. 2011–frw–11.

hyperaccumulator (Ma et al. 2001). It was found that this fern could accumulate 1028 mg Se/kg in the fronds in hydroponic conditions, and potentially can be used to remediate the Se-contaminated environment (Srivastava et al. 2005). Chinese Brake fern distributes over a considerable extent in the south of China. To better practise Se phytoremediation, endemic plants are preferential and the studies on the accumulation mechanisms of Se in these plants are thus needed. It is believed that Se can substitute sulfur to form various proteins, which is regarded as a major mechanism of Se toxicity in common plants (Terry et al. 2000). Furthermore, glutathione (GSH) synthesis in plants can be interfered by all kinds of Se compounds, which may result in oxidative stress and produce damages to plants. Previous reports showed that in Se-tolerant plants, the avoidance of forming Se-containing proteins is considered as the main mechanism of Se tolerance (Terry et al. 2000). Since the interference of GSH synthesis will produce oxidative stress, the investigations on the responses of antioxidative enzymes to high Se exposure in Se tolerant plants are thus desired. However, such information is scarce until now. Physiological responses to Se in non-accumulator plants are well documented. Generally, Se exhibits its functions in two opposite ways: at trace levels, selenium acts as an antioxidant to decrease the formation of malondialdehyde (MDA), an indicator of lipid peroxidation intensity and the grade of oxidation stress (Djanaguiraman et al. 2005), resulting in increased plant biomass (Djanaguiraman et al. 2005); in elevated levels, selenium can produce oxidative stress in plants, resulting in reduced plant biomass and enhanced formation of MDA (Hartikainen et al. 2000). To eliminate excess of reactive oxidative species (ROS), many reducers are synthesized in plants as part of the antioxidative defense system, which include low molecular weight antioxidants (e.g. GSH) and enzymatic antioxidants (such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX)) (Meharg and Hartley-Whitaker 2002, Cao et al. 2004). These antioxidants can react directly or via enzyme catalysis with ROS. GSH, an important intracellular peptide, serves several vital functions, including scavenging free radicals and keeping essential thiol status (Lu 1999). SOD can catalyze the one-electron dismutation of superoxide (O_2^-) into O_2 and H_2O_2 (Thompson 1987). The production of H_2O_2 then can be reduced to water by CAT, APX and POD, or through the ascorbate (AsA)-GSH cycle (Asada 2006). High capability of scavenging ROS, indicating high activities of antioxidative enzymes and/or high contents of low molecular weight antioxidants, is thought to be one mechanism of high As tolerance in Chinese Brake fern (Cao et al. 2004). However, whether this is the case for Se in Chinese Brake fern remains unclear. The primary objectives of this study were to (1) further investigate the ability of Chinese Brake fern to accumulate Se so as to preliminarily assess its potential phytoremediation use; (2) explore the accumulation mechanisms focusing on antioxidative responses to Se stress, especially at high dosages.

MATERIAL AND METHODS

Experimental design. The spores were sown in pottery pots that were filled with fine sandy soil without amending with Se fertilizers. Methods for germination and cultivation of Chinese Brake fern are according to the methods described in Feng et al. (2009a). Healthy and uniform ferns with 8–12 fronds were selected for experiments. The controlled conditions of the greenhouse were as follows: the temperatures ranged from 25°C to 28°C, relative humidity was ca. 75%. A fourteen-hour photoperiod was employed with an average photon flux density of 820 μmol/m^2/s supplied by cool-white fluorescent lamps. Ferns were firstly rinsed thoroughly with tap water and then de-ionized water. They were then transplanted to the hydroponic systems containing 20% strength Hoagland-Arnon nutrient solution to acclimate for 2 weeks with vigorous aeration (Hoagland and Arnon 1938). The treatment solution was replaced once a week. Two weeks later, the plants were transplanted to an opaque plastic pot containing one liter of treatment solution, in which Se was added in the form of Na_2SeO_3. One plant was placed in a hole on a styrofoam sheet as the cover of each pot and each treatment was replicated 3 times. The Se treatment solution was replaced every 3 days. The pH of the solution was adjusted to 6.5 with diluted HCl or NaOH. After 14 days growth under Se exposure, the plants were harvested. Some fresh fronds in each pots were sampled, washed and stored in a refrigerator for immediate antioxidant analysis, the remaining fern samples were rinsed with tap water and then with de-ionized water. Subsequently, the plants were separated into above-ground (fronds) and below-ground (roots plus rhizomes) portions, dried and pulverized for Se determination.

Extractions and assays of enzymes and non-enzymes. Methods for extractions of enzymes, activity assays of enzymes (SOD, POD, CAT, APX,

and glutathione reductase (GR)) and determination of MDA content are described in Feng et al. (2009b), briefly as follows: Approximately 0.2 g of fresh tissue was homogenized in a pre-cooled mortar with 5 mL 50 mmol/L phosphate buffer (pH 7.8) solution. The homogenate was centrifuged at 11 000 g for 20 min at 4°C. The supernatant was used for determinations of enzyme activities (SOD, CAT, POD) and the content of MDA. For SOD assay, 3 mL reaction mixtures contained 0.3 mL of each: 750 μmol/L nitroblue tetrazolium, 20 μmol/L riboflavin, 130 mmol/L methionine, and 100 μmol/L EDTA-Na_2, 1.5 mL of 50 mmol/L phosphate buffer (pH 7.8), 0.25 mL of deionized water, and 0.05 mL of enzyme extract. One unit of enzyme activity was defined as the amount of the enzyme that resulted in 50% inhibition of the rate of Nitroblue tetrazolium (NBT) reduction. For CAT assay, 0.1 mL enzyme extract was added to mixture solution of 1 mL 0.3% H_2O_2 and 1.9 mL 50 mmol/L phosphate buffer (pH 7.0). The activity of CAT was measured by determining the rate change of H_2O_2 absorbance in 60 s at 240 nm. For POD, 3 mL of reaction solution contained 1 mL 0.3% H_2O_2, 0.95 mL 0.2% guaiacol, 1 mL 50 mmol/L phosphate buffer (pH 7.0) and 0.05 mL enzyme extract. One unit of enzyme activity was defined as the amount of the enzyme that resulted in 1% absorbance increase in 60 s at 470 nm. For MDA assay, a solution containing 2.5 mL of 20% (w/v) trichloroacetic acid, 0.5% (w/v) thiobarbituric acid and 1.5 mL enzyme extract. After boiling water bath, quickly cooled and refrigerated, the homogenate was centrifuged at 5.000 g for 10 min at 25°C. The absorbance of the supernatant was recorded at 532 nm and 600 nm, respectively.

To assay APX, about 0.2 g of fresh fronds was homogenized with 5 mL potassium phosphate buffer (50 mmol/L). The homogenate was passed through two layers of cheesecloth and the filtrate was centrifuged for 10 min at 11 000 g at 4°C. The reaction mixture (3 mL) contained 0.1 mL supernatant, 1.8 mL 50 mmol/L potassium phosphate (pH 7.0), 0.1 mL 15 mmol/L ascorbic acid and 1 mL 0.3 mmol/L hydrogen peroxide. One unit of enzyme activity was defined as the amount of the enzyme resulting in 1% absorbance variety in 60 s at 290 nm at 25°C.

Glutathione reductase (GR) activity was measured by decreasing the rate of NADPH oxidation at A340 nm. Fresh tissues (0.2 g) were homogenized in 5 mL Tricine-NaOH buffer (pH 7.8, 0.1 mol/L) and the homogenate was centrifuged (11 000 g, 4°C) for 20 min. One mL of assay mixture contained Tricine-NaOH buffer (pH 7.8, 0.6 mL), NADPH (1 mmol/L, 0.1 mL), GSSG (5 mmol/L, 0.1 mL) and enzyme extract (0.2 mL). One unit of enzyme activity was defined as the amount of the enzyme that resulted in 1% absorbance variety in 60 s at 340 nm.

Method for assay of total protein contents was carried out following that of Bradford (Bradford 1976) using bovine serum albumin as the standard.

For GSH, about 0.2 g of fresh fronds was homogenized with 5.0 mL trichloracetic acid (5% (w/v)). The homogenate was centrifuged at 11 000 g for 20 min. The assay system contained 0.25 mL of the supernatant, 2.6 mL of 150 mmol/L phosphate buffer (pH 7.7) and 0.18 mL 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) solution (75.3 mg DTNB dissolved in 30 mL phosphate buffer (pH 6.8, 100 mmol/L)). After incubation at 30°C for 5 min, the absorbance of the solution was measured at 412 nm.

Determination of Se. The ground-up samples were digested with concentrated HNO_3-$HClO_4$, and the concentrations of Se were determined by a hydride generation atomic fluorescence spectrometer (AFS820, Beijing Titan Instruments Co., China). Accuracy of the elemental analysis was verified using standard reference material from the Center for Standard Reference of China.

Data analysis. One-way ANOVA with multiple comparisons by the Tukey's test at $P < 0.05$ was used to compare means among different treatments. All results in this paper are presented as means with corresponding standard errors. All statistical analyses were completed using the SPSS software.

RESULTS AND DISCUSSION

After 14 days of exposure to selenite, the fern grew well without any visual toxic symptoms. Its biomass was not remarkably affected by Se (data not shown). Selenium concentrations in both fronds and roots increased steadily with the addition of Se with more Se accumulated in roots than in fronds. The highest concentration of Se recorded in the roots was 1.536 mg/kg and in the fronds it was 242 mg/kg. Translocation factors (TFs, defined as the ratio of Se in fronds to that in roots) ranged from 0.07 to 0.40 (Table 1). The very large Se concentration in the roots confirmed that this fern is a Se-accumulator, but not a Se-hyperaccumulator because of the frond Se concentration < 1000 mg/kg and a TF < 1. This result is in accordance with the result of our previous report (Feng et al. 2009a), but does not agree with that of Srivastava et al.

Table 1. Selenium concentrations in the fronds and roots of Chinese brake fern (*Pteris vittata* L.)

Treatments (mg Se/L)	Fronds (mg/kg)	Roots (mg/kg)	Translocation factor (TFs)
0	0.8 ± 0.1$^{e(1)}$	2.0 ± 0.3^{d}	0.40
1	40.0 ± 2.5^{d}	155 ± 18.9^{d}	0.26
2	47.0 ± 5.5^{d}	663 ± 14.8^{c}	0.07
5	104 ± 2.6^{c}	991± 44.5^{b}	0.10
10	178 ± 1.6^{b}	1.131 ± 128^{b}	0.16
20	242 ± 21^{a}	1.536 ± 131^{a}	0.16

$^{(1)}$Values are means ± SE (n = 3). Means followed by different letters in the same column indicate significant differences using one way ANOVA with Tukey's multiple range test (P < 0.05)

(2005) who reported that Chinese Brake fern exe posed to 20 mg/L selenite accumulated more Se in the fronds than that in the roots. Whether the disagreement is due to the genotypic difference in this fern would be a subject of further investigation. In this study, this fern showed relatively low frond Se concentration as 242 mg/kg, however, it is still an excellent phytoremediation material for Se removal from Se-contaminated water system owing to its high root Se concentration.

Selenium addition of 2 mg/L remarkably decreased MDA content in the fronds. However, with Se addition over 2 mg/L, MDA content began to increase rapidly (Figure 1a). Above results suggested that low dosages of Se can alleviate oxidative stress and protect the lipid membrane in Chinese Brake fern. This was in agreement with previous reports in other common plants exposed to selenate or selenite (Hartikainen et al. 2000).

GSH content in the fronds was suppressed by 5 mg/L Se, however, 20 mg/L Se remarkably enhanced its production (Figure 1b). The decreases in GSH are probably related to the depression of thiol, because in plants exposed to selenite GSH may directly react with Se to form Se-containing cysteine and methionine, and finally synthesize Se-containing proteins (Terry et al. 2000). Similar decreases in GSH content were observed by De Kok and Kuiper (1986) in a spinach (*Spinacia oleracea* L., cv. Estivato) leaf disk trial treated with selenate. Reports have shown that H_2O_2, *in vitro* trials, was predominantly produced at low, whereas O_2^- was mainly produced at high thiol concentrations (Kramer and Ames 1988). Therefore, the decreased GSH content in this study might indicate higher production of H_2O_2 than that of O_2^-. The activities of enzyme GR followed the variation trends as GSH content, however, it was not significantly affected by the various Se dosages unlike GSH (Figure 1c). Since GR can reduce oxidized glutathione to regenerate reduced

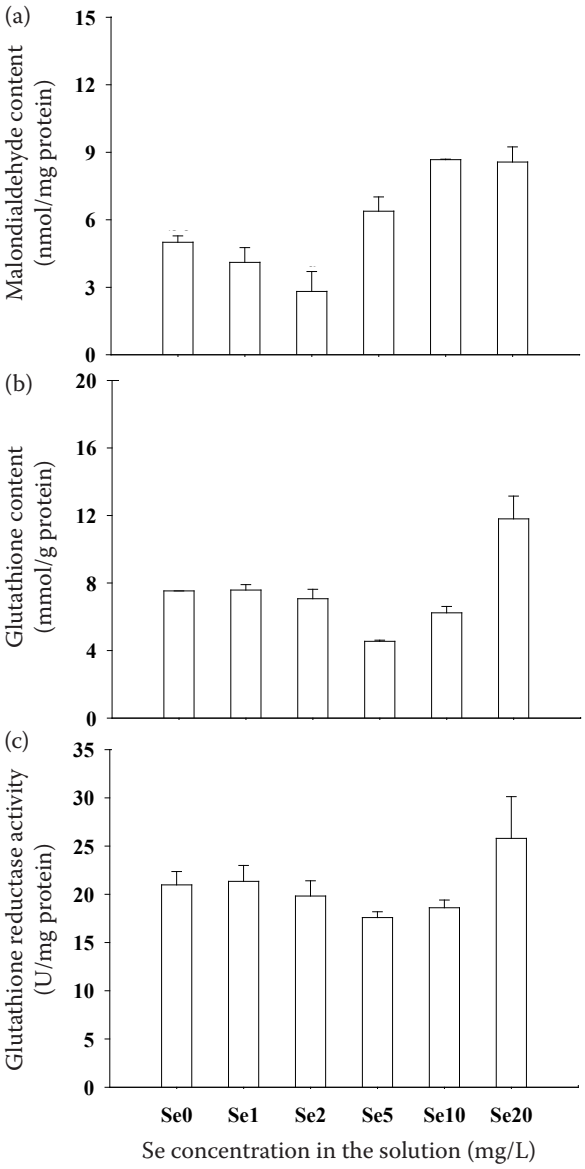

Figure 1. Contents of malondialdehyde (MDA), glutathione (GSH) and activity of glutathione reductase (GR) in the fronds of Chinese brake fern grown in hydroponic conditions under Se stress for 2 weeks. The bars on the curves are standard errors for the mean of three replications (P < 0.05)

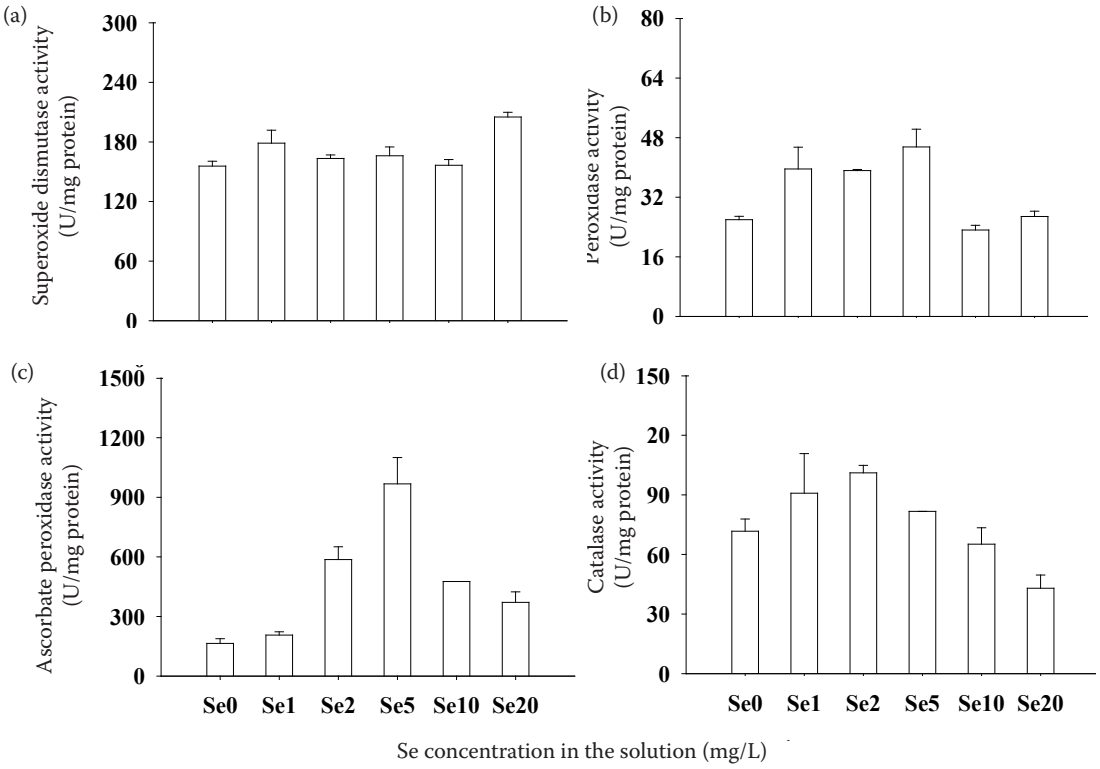

Figure 2. Activities of peroxidase (POD), ascorbate peroxidase (APX), superoxide dismutase (SOD) and catalase (CAT) in the fronds of Chinese brake fern grown in hydroponic conditions under Se stress for 2 weeks. The bars on the curves are standard errors for the mean of three replications ($P < 0.05$)

GSH (Lu 1999), this explains the same pattern for variation of GR activities and GSH content in the fronds of Chinese Brake fern.

It was established that ROS will be produced during the reduction processes of selenite by sulfhydryl groups like GSH (Kramer and Ames 1988). In this study, no significant changes in the activity of SOD were observed between the controls and Se treatments, except a significant increase of activity at 20 mg Se/L (Figure 2a). The activities of POD increased with Se addition from 0 to 5 mg/L; however, with Se addition greater than 5 mg/L, they were found to be in the same level as compared with the control (Figure 2b). The variation of APX followed the same trends with that of POD, but with a much rapid increase upon Se addition from 0 to 5 mg/L (Figure 2c). The enhancement of CAT activity was only found with Se addition from 0 to 2 mg/L, while more addition of Se caused a constant decrease of CAT activities (Figure 2d). The enhanced activities of POD, APX, and CAT but unchanged SOD activity at low Se levels suggest more production of H_2O_2 than O_2^- which was in good agreement with the decreased GSH content. Reports suggested that in the presence of Se, external H_2O_2 was primarily removed by glutathione peroxidase (GSH-PX) (Hartikainen et al. 2000). Regardless of the role of GSH-PX in this study, the increasing activities of POD, APX and CAT clearly show their participations in alleviating lipid per-oxidation to quench H_2O_2 below 5 mg/L Se exposure. Similar increases in POD and APX activities were also observed in white clover (*Trifolium repens* L.) treated with selenite (Mora et al. 2008).

When Se addition was up to 20 mg/L, selenium enhanced MDA production. However, this fern had no remarkable reduction in its biomass, indicating a high tolerance to high Se exposure. The significant increases in GSH content at 20 mg Se/L perhaps suggested the involvement of GSH in the tolerance mechanism, in which this fern synthesized more GSH to maintain the balance of thiol to maintain protein thiols at their reduced state so as to protect membranes from peroxidation by ROS. The increase in GSH content observed in this study was not seen in non-hyperaccumulating plants treated with selenate (De Kok and Kuiper 1986), but observed in Chinese Brake fern under high As exposure (Cao et al. 2004), suggesting important roles of GSH in the tolerance of this fern to As and Se stress. Additionally, reports pointed out that toxic doses of Se salts is due to the excess production of O_2^- (Bébien et al. 2002) and O_2^- was mainly produced at high thiol concentrations as described above. Thus, the increased GSH content

might indicate the up-regulated thiol concentration and O_2^- production. Exceeded O_2^- then need more SOD to scavenge itself, which was in this study indirectly supported by the increased SOD activity and down-regulated POD, APX, and CAT activities at the highest Se exposure. The lowered activities of POD, APX, and CAT also suggest their limited roles in the tolerance of this fern to high Se levels.

In summary, Chinese Brake fern can take up a large amount of selenium under hydroponic condition which can possibly be used to remove Se from Se-contaminated water, but further studies must be conducted to assess its remediation efficiency and the influence factors of Se removal.

REFERENCES

Asada K. (2006): Production and scavenging of reactive oxygen species in chloroplasts and their functions. Plant Physiology, *141*: 391–396.

Bébien M., Lagniel G., Garin J., Touati D., Verméglio A., Labarre J. (2002): Involvement of superoxide dismutases in the response of *Escherichia coli* to selenium oxides. Journal of Bacteriology, *184*: 1556–1564.

Bradford M.M. (1976): A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, *72*: 248–254.

Cao X.D., Ma L.Q., Tu C. (2004): Antioxidative responses to arsenic in the arsenic-hyperaccumulator Chinese brake fern (*Pteris vittata* L.). Environmental Pollution, *128*: 317–325.

De Kok L.J., Kuiper P.J.C. (1986): Effect of short-term dark incubation with sulfate, chloride and selenate on the glutathione content of spinach leaf discs. Physiologia Plantarum, *68*: 477–482.

Djanaguiraman M., Durga D.D., Shanker A.K., Sheeba J.A., Bangarusamy U. (2005): Selenium – an antioxidative protectant in soybean during senescence. Plant and Soil, *272*: 77–86.

Feng R.W., Wei C.Y., Tu S.X., Sun X. (2009a): Interactive effects of selenium and arsenic on their uptake by *Pteris vittata* L. under hydroponic conditions. Environmental and Exprimental Botany, *65*: 363–368.

Feng R.W., Wei C.Y., Tu S.X., Wu F.C., Yang L.S. (2009b): Antimony accumulation and antioxidative responses in four fern plants. Plant and Soil, *317*: 93–101.

Hartikainen H., Xue T.L., Piironen V. (2000): Selenium as an anti-oxidant and pro-oxidant in ryegrass. Plant and Soil, *225*: 193–200.

Hoagland D.R., Arnon D.I. (1938): The water-culture method for growing plants without soil. California Agricultural Experiment Station Bulletin, *347*: 1–39.

Kramer G.F., Ames B.N. (1988): Mechanisms of mutagenicity and toxicity of sodium selenite (Na_2SeO_3) in *Salmonella typhimurium*. Mutation Research, *201*: 169–180.

Lemly A.D. (2004): Aquatic selenium pollution is a global environmental safety issue. Ecotoxicology and Environmental Safety, *59*: 44–56.

Lu S.C. (1999): Regulation of hepatic glutathione synthesis: current concepts and controversies. The Federation of American Societies for Experimental Biology Journal, *13*: 1169–1183.

Ma L.Q., Komar K.M., Tu C., Zhang W.H., Cai Y., Kennelley E.D. (2001): A fern that hyperaccumulates arsenic. Nature, *409*: 579.

Meharg A.A., Hartley-Whitaker J. (2002): Arsenic uptake and metabolism in arsenic resistant and nonresistant plant species. New Phytologist, *154*: 29–43.

Mora M., Pinilla L., Rosas A., Cartes P. (2008): Selenium uptake and its influence on the antioxidative system of white clover as affected by lime and phosphorus fertilization. Plant and Soil, *303*: 139–149.

Pezzarossa B., Petruzzelli G., Petacco F., Malorgio F., Ferri T. (2007): Absorption of selenium by *Lactuca sativa* as affected by carboxymethylcellulose. Chemosphere, *67*: 322–329.

Pickering I.J., Wright C., Bubner B., Ellis D., Persans M.W., Yu E.Y., George G.N., Prince R.C., Salt D.E. (2003): Chemical form and distribution of selenium and sulfur in the selenium hyperaccumulator *Astragalus bisulcatus*. Plant Physiology, *131*: 1460–1467.

Srivastava M., Ma L.Q., Cotruvo J.A. (2005): Uptake and distribution of selenium in different fern species. International Journal of Phytoremediation, *7*: 33–42.

Terry N., Zayed A.M., de Souza M.P., Tarun A.S. (2000): Selenium in higher plants. Annual Review of Plant Physiology and Plant Molecular Biology, *51*: 401–432.

Thompson J.E., Legge R.L., Barber R.F. (1987): The role of free radicals in senescence and wounding. New Phytologist, *105*: 317–344.

Zayed A., Pilon-Smits E., de Souza M., Lin Z.Q., Terry N. (2000): Remediation of selenium-polluted soils and waters by phytovolatilization. In: Terry N., Bañuelos S.G. (eds): Phytoremediation of Contaminated Soil and Water. Lewis Publishes, Boca Raton, 61–78.

Received on May 29, 2011

Corresponding author:

Renwei Feng, Centre for Research in Ecotoxicology and Environmental Remediation, Institute of Agro-Environmental Protection, the Ministry of Agriculture, Tianjin 300191, P.R. China
Open Key Laboratory of Agro-environment and Food Safety of the Ministry of Agriculture, Tianjin, P.R. China
phone: + 86 22 2300 3713, e-mail: frw1_79_79@yahoo.com.cn