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Effects of Zinc on Root Morphology and Antioxidant Adaptations of Cadmium-Treated *Sedum alfredii* H.

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ABSTRACT

The study demonstrated *S. alfredii* is an excellent cadmium (Cd)/zinc (Zn) hyperaccumulator as Cd and Zn concentrations in leaves reached 2,183 and 13,799 mg kg^{-1} DW, respectively. There was a significant increase in root morphological parameters induced by 50 and 500 μM Zn supplement; however, a sharp decrease in these parameters occurred when treated with 100 μM Cd +1000 μM Zn. The inhibited root dehydrogenase activity in 100 μM Cd treated plants was restored to control levels when supplemented with 500 μM Zn. Moderate Zn supplement did not produce significant changes in (malondialdehyde) MDA concentrations as compared with those treated with Cd alone. Variations of the antioxidative enzymes proved an ineffective role in coping with metal-stress in *S. alfredii*. Combined Cd and Zn treatment significantly enhanced ascorbic acid (AsA) and glutathione (GSH) contents in leaves of *S. alfredii*, as compared with those treated with Cd alone. Thus, Zn may rely on the involvement of GSH in detoxification and tolerance.

Keywords: antioxidatant, ascorbate, glutathione, hyperaccumulation, zinc-cadmium interaction

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INTRODUCTION

Higher cadmium (Cd) concentrations are generally deleterious for plants, affecting various physiological and biochemical processes (Sanita di Toppi and Gabbrielli, 1999). Additionally, due to a high solubility, Cd can easily enter food chain. Thus, Cd is one of the most dangerous environmental pollutants. Although the mechanisms of heavy metal hyperaccumulation are still not fully understood, terrestrial plants have gained considerable interest for their use in phytoremediation studies for reclamation of surface soils contaminated with toxic Cd.

Cadmium can elicit oxidative stress by promoting the generation of reactive oxygen species (ROS) and lipid peroxidation in plant cells (Aravind and Prasad 2005a; Chaoui et al., 1997; Dixit et al., 2001; Romero-Puertas et al., 2004; Zhang et al., 2007). Irrespective of the production pathways, ROS are highly cytotoxic and their level within plant cells must be controlled by antioxidant defense systems. Plants cope with oxidative stress by using antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), guaiacol peroxidase (GPX), catalase (CAT), and the low molecular weight antioxidants such as ascorbic acid (AsA) and glutathione (GSH) (Semane et al., 2007; Noctor and Foyer, 1998).

Sedum alfredii H. is a Chinese native hyperaccumulator perennial plant species of zinc (Zn) and Cd which develops a relatively large biomass quickly and can be propagated asexually. It is therefore an ideal plant material for studying mechanisms of hyperaccumulation and employing it for phytoremediation of contaminated soils (Yang et al., 2006; 2004). Zinc is an essential plant microelement, regulating many biological functions. *S. afredii*, particularly growing in Zn/lead (Pb) mined sites, has the ability to acclimate to higher Zn concentration, and even displays growth inhibition under Zn deficient conditions (data not shown). Such results may imply that Zn facilitates growth in *S. alfredii* under Cd stress. The aim of this work was to investigate effects of Zinc on Cd-induced changes in plant growth and concentration patterns of antioxidant systems.

MATERIALS AND METHODS

Plant Collection and Culture

Seedlings of *S. alfredii* were collected from the mining site on 5 April 2007. Plants with healthy and uniform shoots were selected and precultured for four weeks in the basic nutrient solution containing (mmol L^{-1}): calcium nitrate [$Ca(NO_3)_2{\cdot}4H_2O$] (2.00); potassium phosphate (KH_2PO_4) (0.10); magnesium sulphate ($MgSO_4{\cdot}7H_2O$) (0.50); potassium chloride (KCl) (0.10); potassium sulphate (K_2SO_4) (0.70); and (in μmol L^{-1}) boric acid (H_3BO_3)

(10.00); manganese sulfate ($MnSO_4 \cdot H_2O$) (0.50); zinc sulfate ($ZnSO_4 \cdot 7H_2O$) (1.00); copper sulfate ($CuSO_4 \cdot 5H_2O$) (0.20); ammonium molybdate [$(NH_4)_6 Mo_7O_{24} \cdot 4H_2O$] (0.01); Fe–ethylenediaminetetraacetic acid (EDTA) (100). The nutrient solution pH was adjusted to 5.5 daily using 0.1 mol L^{-1} of either sodium hydroxide (NaOH) or hydrochloric acid (HCl). Plants were grown in the greenhouse of Huajiachi campus, Zhejiang University, Hangzhou, China, located at 30°161N latitude and 120°121E longitude, of southeast China, with day/night temperature and relative air humidity of 26/20°C and 70/85%, respectively. The nutrient solution was continuously aerated and renewed after every three days.

Cadmium Treatment and Sample Preparations

After preculturing, healthy and uniform seedlings were selected for various Cd and Zn treatments. A Cd treatment of 100 μM was applied to the plants using cadmium chloride ($CdCl_2$) Zn supplements (50,500 and 1000 μM) to the Cd (100 μM) treated plants were given using zinc chloride ($ZnCl_2$). Each treatment was applied in triplicate using a randomized complete block design. Plants were harvested after eight days of treatment. At the time of harvest, roots were soaked in 20 mM Na_2-EDTA for 15 min to remove excess metal ions adhering to the root surface.

Fresh samples were immediately frozen in liquid nitrogen and stored at −80°C for analysis of antioxidants, hydrogen peroxide (H_2O_2), and malondialdehyde (MDA).

Elemental Analysis

Harvested plant materials were thoroughly washed in distilled water and separated into leaves, stem and roots(after Na-EDTA treatment), and finally were oven dried at 65°C. Dried plant materials were powdered (100 mg) and wet digested in a 10: 1 mixture of nitric acid (HNO_3): perchloric acid ($HClO_4$) at 160°C. Digested material was diluted with de-ionized water and Cd and Zn concentrations were determined using an Inductively Coupled Plasma Mass Spectrometer (ICP/MS, Agilent 7500a, Wilmington, DE, USA).

Measurement of Root Morphological Parameters and Activity

Root morphological parameters were measured after plants were exposed to different metal concentrations for eight days. Root length, root surface area, root diameter, root volume and count of lateral roots were carried out using root

automated scan apparatus (MIN MAC, STD1600$^+$) equipped with WinRHIZO software (Regent Instruments Co., Canada).

A fraction of 0.5 g fresh root sample was placed in a scaled tube and 5 ml 0.4 % Triphenyl Tetrazolium Chloride (TTC) and 5 mL 0.1 mM phospatic buffer solution (pH 7.0) were added and allowed to react for 2 hr at 37°C. Then, 2 mL of 1.0 M sulfuric acid (H_2SO_4) was added to the tube to terminate the reaction. The root activity was expressed by the amount of triphenyl formazan (TPF) deoxidized by TTC (Jiang, 1999).

MDA and H_2O_2 Assays

Leaf and root MDA and H_2O_2 contents were determined as previously described (Velikova et al., 2000). Each sample 1 g (FW) was homogenized with 4 mL of 0.1% (w/v) TCA in an ice bath. The homogenate was centrifuged at 12,000 g_n for 20 min and supernatant was used for both MDA and H_2O_2 analysis. The 2-thiobarbituric acid (TBA) test, that determines MDA as an end product of lipid peroxidation, was used for the determination of lipid peroxidation in the samples. For a 1 mL aliquot, 1 mL of 20% (w/v) TCA comprised of 0.5% (w/v) TBA was added. The mixture was heated at 95°C for 30 min and then cooled in an ice bath to terminate the reaction. The tubes were centrifuged at 10,000 g_n for 10 min, and the absorbance of the supernatant was noted at the wavelength of 532 nm using a spectrophotometer. The value for non-specific absorption at 600 nm was subtracted. The amount of MDA-TBA complex was calculated from the extinction coefficient, i.e., 155 mM^{-1} cm^{-1}. For the H_2O_2 assay, 1 mL of the supernatant was added to a mixture containing 1 mL of 10 mM potassium phosphate buffer (pH 7.0) and 2 mL of 1 M KI. Hydrogen peroxide concentration was estimated based on the absorbance of the supernatant at 390 nm using a spectrophotometer.

Antioxidant Enzyme Assays

Plant samples (1 g FW) were homogenized in 6 ml pre-cooled 50 mM potassium phosphate buffer (pH 7.0) containing 0.2 mM EDTA and 2%(w/v) polyvinylpyrrolidone (PVP) in an ice bath using a prechilled mortar and pestle. The homogenate was centrifuged for 20 min at 12,000 g_n at 4°C and the supernatant was used for enzyme analysis. An aliquot of the extract was used to determine total soluble protein content following previously described method (Bradford, 1976) using bovine serum albumin as standard.

Superoxide dismutase (EC 1.15.1.1) was determined by the photochemical method (Giannopolitis and Ries, 1977). One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the

nitroblue tetrazolium chloride (NBT) reduction rate at 560 nm. Catalase (EC 1.11.1.6) activity was assayed in a reaction mixture containing 25 mM potassium phosphate buffer (pH 7.0, containing 0.1 mM EDTA), 10 mM H_2O_2, and the enzyme. The decrease in absorbance of H_2O_2 within 1 min at 240 nm ($E = 39.4\ mM^{-1}cm^{-1}$) was recorded (Cakmak et al., 1993). Guaiacol peroxidase (GPX, EC 1.11.1.7) was also assayed (Cakmak et al., 1993). The reaction mixture contained 25 mM potassium phosphate buffer (pH 7.0, containing 0.1 mM EDTA), 0.05% guaiacol, 10 mM H_2O_2, and the enzyme activity was measured by the increase in absorbance at 470 nm caused by guaiacol oxidation ($E = 26.6\ mM^{-1}cm^{-1}$). Ascorbate peroxidase (EC 1.11.1.11) was measured according the method devised by Nakano and Asada (1981) by monitoring the rate of ascorbate oxidation at 290 nm ($E = 2.8\ mM^{-1}cm^{-1}$). The reaction mixture contained 25 mM potassium phosphate buffer (pH 7.0, containing 0.1 mM EDTA), 100 mM H_2O_2, and 0.25 mM AsA, and the enzyme aliquot. Glutathione reductase (EC 1.6.4.2) was assayed following the method of Foyer and Halliwell (1976) by monitoring the decrease in absorbance at 340 nm caused by NADPH oxidation ($E = 6.2\ mM^{-1}cm^{-1}$). The reaction mixture contained 25 mM potassium phosphate buffer (pH 7.8, containing 0.2 mM EDTA), 0.5 mM oxidized glutathione (GSSG), 0.12 mM NADPH, and the enzyme aliquot.

Antioxidant (Glutathione and Ascorbic Acid) Assays

The GSH concentrations were estimated fluorimetrically following a previously described method (Hissin and Hilf, 1976) with some modifications. The sample was extracted in an ice bath with 3 mL of 100 mM phosphate buffer (pH 8.0, containing 5 mM EDTA) and 1 mL of 25% meta-phosphoric acid and then centrifuged at 10,000 g_n for 30 min. The supernatant was further diluted five times with phosphate-EDTA buffer (pH 8.0). The final assay mixture (2 ml) contained 100 all of the diluted tissue supernatant, 1.8 mL of phosphate-EDTA buffer, and 100 μL of *o*-phthaladehyde (OPT) solution. After thorough mixing and incubation at room temperature for 15 min, fluorescence intensity was recorded at 420 nm after excitation at 350 nm using a fluorescence spectrophotometer.

Determination of AsA levels followed the previous procedure (Singh et al., 2006) with few modifications. A known weight (1 g FW) of sample was extracted with 3 mL of 5% (w/v) TCA and centrifuged at 18,000 g_n for 15 min. The AsA was determined in a reaction mixture consisting of 0.2 mL of supernatant, 0.5 mL of 150 mM phosphate buffer (pH 7.4, containing 5 mM EDTA) and 0.2 ml of de-ionized water, and the color was developed in both reaction mixtures with the addition of 0.4 ml of 10% (w/v) TCA, 0.4 ml of 44% (v/v) phosphoric acid, 0.4 ml of α,α'-dipyridyl in 70% (v/v) ethanol, and 0.2 mL of 3% (w/v) iron chloride ($FeCl_3$). The reaction mixtures were

incubated at 40°C for 40 min and the absorbance was read at 532 nm using AsA as a standard.

Statistical Analysis

Statistical analysis was performed using the SPSS statistical software (version 11.0). All reported values were the means of three replicates. Data were tested at significant levels of $P < 0.05$ using one way ANOVA. Graphical work was carried out using Sigma Plot™ v.10.

RESULTS

Metal Concentrations in Plant Tissues

Cadmium concentrations in leaves of *S. alfredii* reached 2,183 mg kg^{-1} DW when the plants were exposed to 100 μM Cd for eight days. Zinc supplements (50 and 500 μM) to Cd-treated plants had no significant effects on the accumulation of Cd. However, in comparison to 100 μM Cd treatment alone, the Cd concentration in roots and leaves was reduced 9.1% and 30.0%, respectively, when Cd-treated plants were supplemented with 1,000 μM Zn (Figure 1A). Zinc concentrations in the plant tissues increased with increasing Zn levels in the solution, whereas Zn concentration in leaves of *S. alfredii*

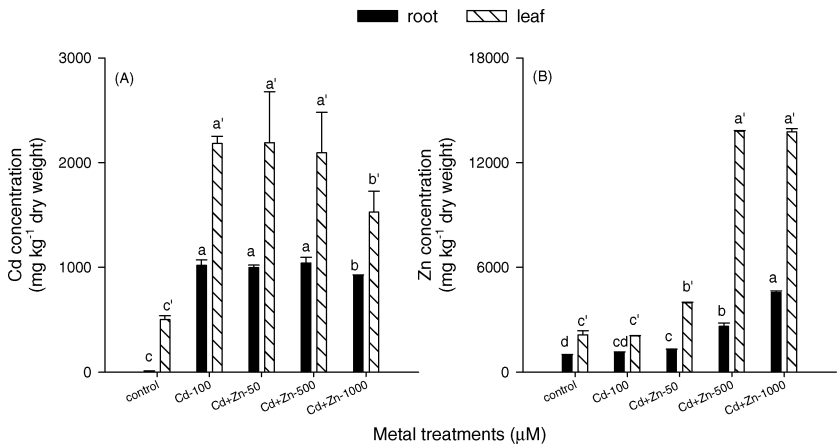

Figure 1. (A) Cadmium and (B) zinc concentrations in the leaves and roots of *Sedum alfredii* H. Each value is the mean of three individual replicates (±SD). Means followed by the same letter were not significantly different at $P < 0.05$.

reached 13,799 mg kg^{-1} DW when the plants were exposed to 500 μM Zn combined with 100 μM Cd. However, Zn levels of 1000 μM Zn showed insignificant increases in Zn accumulation in the leaves of the Cd-treated plants (Figure 1B).

Effect of Zn and Cd on Root Morphology

The root morphological parameters of *S. alfredii* showed variable response to different Zn and Cd treatments (Figure 2). Total root length and surface area were not affected when treated with 100 μM Cd for 8 days, but significantly increased after combination of same treatments with 50, 500 μM Zn, respectively, as compared with control (Figures 2A and 2B). However, it was revealed that both root length and surface decreased by 54.3% and 35.1%, respectively, when treating with 100 μM Cd +1000 μM Zn, as compared with its combination with 500 μM Zn. Similar treatments produced identical trend for root volume and the numbers of root tips (Figures 2D and 2E), while no significant differences between the treatment and control for the average root diameter were observed (Figure 2C). For the root dehydrogenase activity, it could be seen that it activity decreased by 27.2% and 21.6%, respectively, as compared with the control when treating with 100 μM Cd and 100 μM Cd + 50 μM Zn. While combined treatment of Cd with 500 μM Zn, root dehydrogenase activity recovered to the control level. However, when treated with 100 μM Cd + 1000 μM Zn, root dehydrogenase activity decreased significantly by 62% over control (Figure 2F).

Effect of Zn on Lipid Peroxidation and H_2O_2 Contents of Cd-Treated Plants

Exposure to heavy metals caused membrane damage and lipid peroxidation in plants under investigation, such damage was obvious from significant increase in the H_2O_2 and MDA contents (Figure 3). After treating with 100 μM Cd, H_2O_2 content both in roots and leaves increased by 1.4- and 13.3-fold, as compared with control. While the same concentrations of Cd were applied in combination with Zn, the H_2O_2 content increased gradually, as compared with those treated with Cd alone (Figure 3A). As for MDA content, it increased by 3.3- and 4.4-fold under same treatments, both in roots and leaves of *S. alfredii* as compared with control. While in combination with 50 and 500 μM Zn, there were no significant changes as compared with those treated with Cd alone. However, for the Zn treatment level of 1,000 μM, MDA contents in roots and leaves increased by 19.8% and 26%, respectively, as compared to the treatment of 100 μM Cd + 500 μM Zn (Figure 3B).

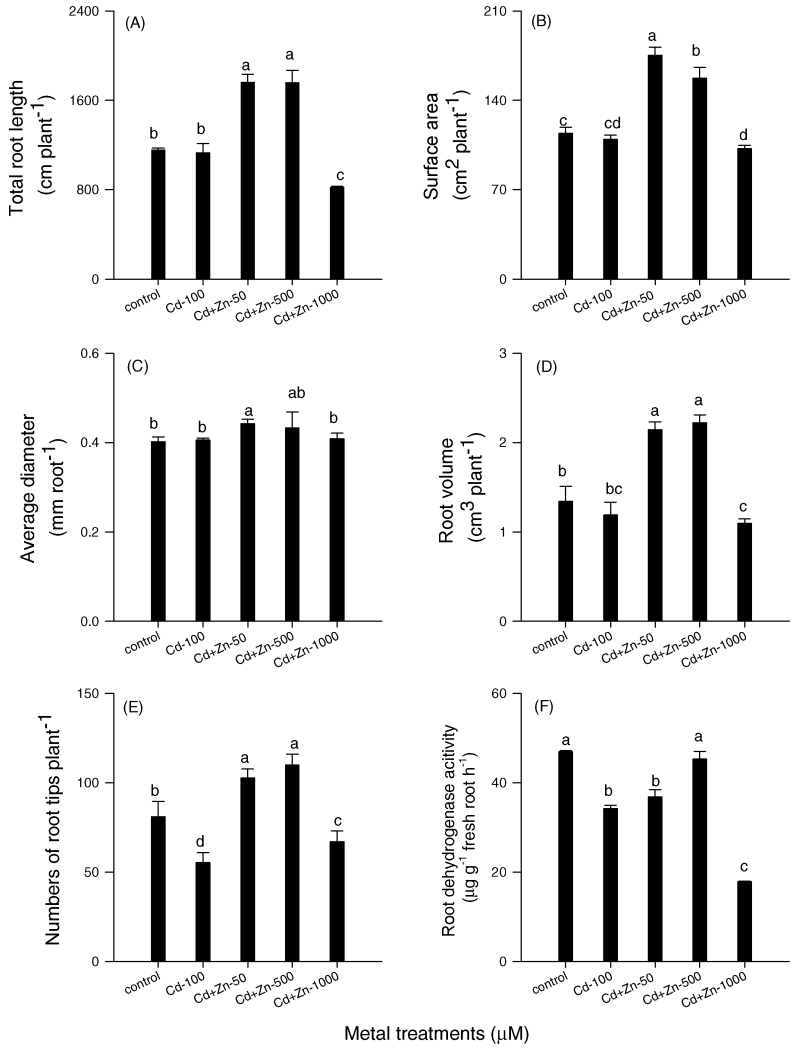

Figure 2. Effects of metal levels on (A) total root lengths, (B) surface area, (C) average diameter, (D) root volume, (E) number of root tips and (F) root dehydrogenase activity of S. alfredii Values are mean of three individual replicates (±SD). Means followed by the same letter were not significantly different at $P < 0.05$.

Effect of Zn on Activities of Antioxidant Enzymes of Cd-Treated Plants

Application of various Zn and Cd treatments brought significant changes in the total soluble proteins and antioxidant enzymes activities in both roots and leaves of *S. alfredii* (Figure 4). After treating with 100 μM Cd + 50 μM

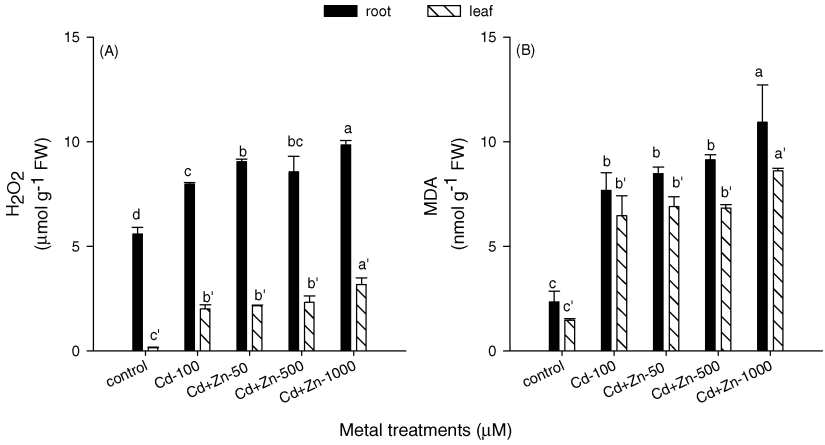

Figure 3. Effects of metal levels on (A) H_2O_2 concentrations and (B) lipid peroxidation production (expressed in terms of MDA concentration) in leaves and roots of *Sedum alfredii* H. Values are mean of three individual replicates (±SD). Means followed by the same letter were not significantly different at $P < 0.05$.

Zn and 100 μM Cd + 500 μM Zn, the total soluble proteins (TSP) in leaves increased by 47.9% and 88.5%, respectively; the same trend was also traced for roots. However, for the treatments of 100 μM Cd and 100 μM Cd + 1000 μM Zn, no significant changes were noted compared with control (Figure 4A). It was seen that the Cd and Zn treatments significantly stimulated SOD activity (Figure 4B). However, there were no significant changes in CAT activity in both roots and leaves of the metal-treated *S. alfredii* over the control (Figure 4C). The treatments levels of 100 μM Cd + 50 μM Zn significantly decreased the GPX activity in roots of *S. alfredii* by 56.9%, while its activity in leaves increased by 3.1-fold after treating with 100 μM Cd, as compared with control (Figure 4D). The APX activity in root of *S. alfredii* decreased significantly after treating with Zn and Cd, as compared with control; in contrast, the treatment levels of 100 μM Cd, 100 μM Cd + 50 μM Zn, 100 μM Cd + 500 μM Zn had no significant effects on the APX activities in leaves of *S. alfredii*, while the treatment of 100 μM Cd + 1000 μM Zn decreased significantly by 53.1% as compared with the control (Figure 4E). The GR activity in roots of *S. alfredii* significantly decreased by 34.8%, 32.1%, and 34.3%, respectively, as compared with the control when treated with 100 μM Cd, 100 μM Cd + 50 μM Zn, 100 μM Cd + 500 μM Zn. As for leaves, GR activity decreased sharply by 56.2% when treating with 100 μM Cd alone, and while combination with 50 μM Zn GR activity increased significantly by 77.9% as compared with the control (Figure 4F).

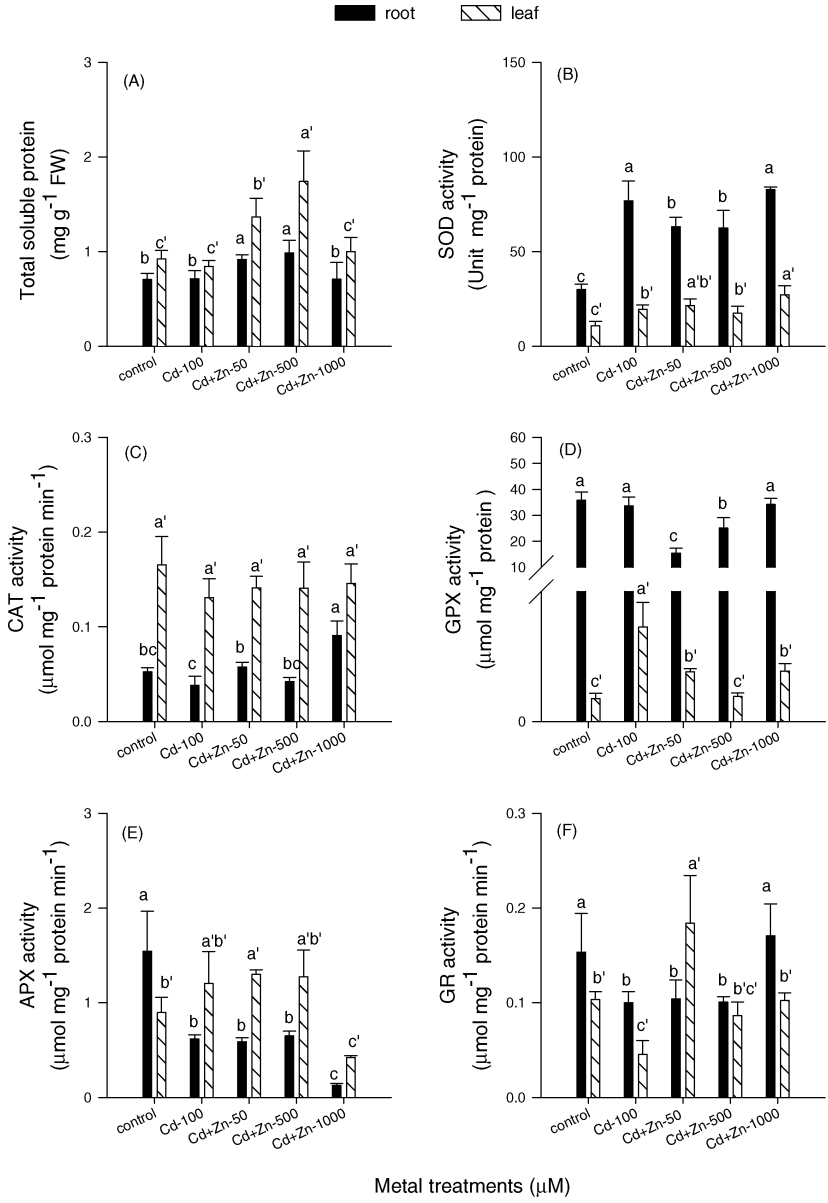

Figure 4. Effects of metal levels on (A) total soluble protein contents and (B) SOD, (C) CAT, (D) GPX, (E) APX and (F) GR activities in leaves and roots of S*edum alfredii* H. Values are mean of three individual replicates (±SD). Means followed by the same letter were not significantly different at $P < 0.05$.

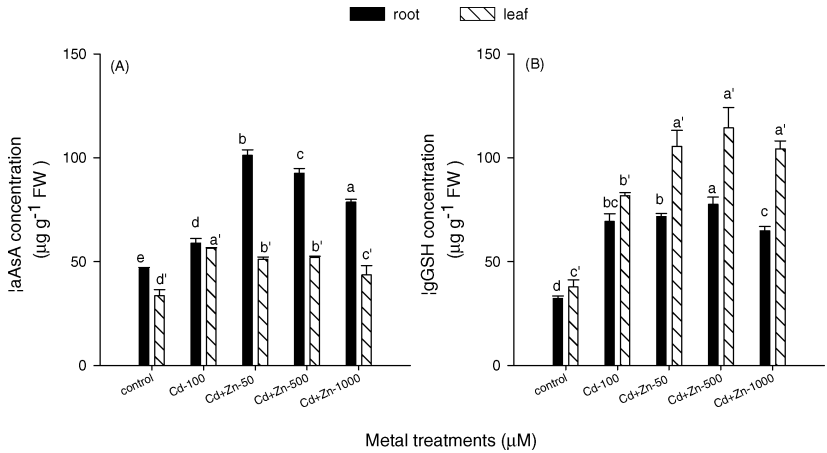

Figure 5. Effects of metal levels on reduced (A) AsA and (B) GSH contents in leaves and roots of S*edum alfredii* H. Values are mean of three individual replicates (±SD). Means followed by the same letter were not significantly different at $P < 0.05$.

Effect of Zn and Cd on Ascorbic Acid (AsA) and Glutathione (GSH)

The AsA and GSH contents in both roots and leaves of *S. alfredii* varied with various Cd and Zn treatments (Figure 5). The AsA contents in roots of *S. alfredii* increased sharply when Cd was applied in combination with Zn, as compared to those treated with Cd alone where slight changes in AsA of leaves were noted (Figure 5A). The GSH contents in both roots and leaves increased significantly after combined treatment of Cd and Zn; additionally, the Zn combinations significantly increased GSH contents in leaves of *S. alfredii* as compared with those treated with Cd alone (Figure 5B).

DISCUSSION

The present study demonstrated that *S. alfredii* H. is an excellent Cd/Zn hyperaccumulator. As evident from Figure 1, Cd and Zn concentrations in leaves of *S. alfredii* reached 2,183 and 13,799 mg kg^{-1} DW, respectively, which greatly surpassed the generally accepted threshold concentration for 100 and 10,000 mg kg^{-1} DW for Cd hyperaccumulators and Zn hyperaccumulators, respectively (Baker and Brooks, 1989). It is a well-established fact that Cd alone can be highly toxic to plant species causing oxidative stress. However, appropriate Zn concentrations (50 and 500 μM) alleviated Cd toxicity by maintaining plant growth. Some inhibitory effects of higher Zn supply were also observed in plants, which affected Cd uptake; such inhibitory effects of Zn were only

observed when very high Zn concentrations (1,000 μM) were supplemented to Cd-treated plants (Figure 1). This is in agreement with our study on root morphology, where the root morphological parameters of *S. alfredii* were significantly induced by 50 and 500 μM Zn, but sharply decreased when treating with 100 μM Cd +1,000 μM Zn (Figures 2A, 2B, 2D, and 2E). Particularly, the root dehydrogenase activity was inhibited in plants treated with 100 μM Cd alone, but it was restored to control levels when Cd-treated plants were supplemented with 500 μM Zn (Figure 2F). It has been well documented that Cd toxicity symptoms can be clearly alleviated by Zn in *Ceratophyllum demersu*, *Phaseolus vulgaris* (Aravind and Prasad, 2005a, 2005b, 2005c; Chaoui et al., 1997). Moreover, *S. alfredii* H. is a newly identified Zn hyperaccumulator originally growing in Zn/Pb mined sites, which has a greater ability to adapt to high Zn (Yang et al., 2002, 2006).

Though Cd does not appear to directly generate free radicals, Cd accumulation causes oxidative stress through free radicals and H_2O_2 and elevate lipid peroxidation (Shaw et al., 2004; Chaoui et al., 1997; Gallego et al., 1996; Audet and Charest, 2007; Ünyayar et al., 2006; Zhang et al., 2007). Oxidative stress in *S. alfredii* H. caused by Cd was shown by significant increases in H_2O_2 and MDA contents (Figure 3). The elevated MDA content is considered to be a general indicator of lipid peroxidation (Chaoui et al., 1997). Supplementation with moderate Zn levels (50 and 500 uM Zn) caused no significant changes in MDA concentrations, as compared with those treated with Cd alone (Figure 3B), which confirmed that *S. alfredii* could acclimatize itself to Zn.

Variations in the responses of antioxidant enzymes, i.e., SOD, CAT, GPX, APX, and GR, to Cd toxicity and Zn supplements (Figure 4) revealed that the antioxidant enzyme systems did not play an effective role in coping with metal-stress in *S. alfredii.* Nevertheless, Cd and Zn treatments significantly induced the appearance of AsA and GSH contents in *S. alfredii* (Figure 5). Particularly, Zn supplement was found to significantly elevate GSH contents in leaves of *S. alfredii* as compared with those treated with Cd alone (Figure 5B). This observation demonstrated the ability of *S. alfredii* to adapt to high Zn concentrations. The endogenous non-enzymatic antioxidants such as AsA and GSH are effectively free radical scavengers (Noctor and Foyer, 1998). Apart from signal transduction (Noctor et al., 2002), GSH is a substrate for several reductive enzymes including enzymes that reduce peroxides (Foyer and Noctor, 2005). In metabolically active tissues, millimolar concentrations of GSH act as a key redox buffer, forming a barrier between protein Cys groups and ROS (Foyer and Noctor, 2005). Our previous work has proved preferential Cd-stress response in *S. alfredii* was suggested to create changes in the GSH pool, where acclimation was manifested by increased GSH concentrations, and that results on buthionine sulfoximine (BSO, an inhibitor of glutathione synthesis) and Cd/Zn treatment indicated that GSH biosynthesis may play an important role as a signal of the stress regulation (data not shown).

CONCLUSIONS

It was demonstrated that the *S. alfredii* from a Zn/Pb old mined site has a greater ability to acclimatize Cd/Zn toxicity and Cd/Zn hyperaccumulation. Cadmium induced distinct enhancement in lipid peroxidation levels and overproduction of H_2O_2, but supplement with moderate Zn levels (50 and 500 uM Zn) did not aggravate its oxidative stress. Consistently, Cd-treated *S. alfredi* supplemented with appropriate Zn showed notable rise in root morphological parameters and root dehydrogenase activity. These results confirmed that *S. alfredii* has greater competence to tolerate Zn. The tolerance of *S. alfredii* to heavy metal stress, i.e., Cd and Zn, may rely on the involvement of GSH in detoxification and tolerance.

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