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ScienceDirect
Journal of Hazardous Materials 156 (2008) 387–397
Journal of Hazardous Materials
www.elsevier.com/locate/jhazmat

Effects of cadmium on ultrastructure and antioxidative defense system in hyperaccumulator and non-hyperaccumulator ecotypes of *Sedum alfredii* Hance

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Received 23 August 2007; received in revised form 10 December 2007; accepted 10 December 2007
Available online 1 February 2008

Abstract

Plant growth, ultrastructural and antioxidant adaptations and glutathione biosynthesis in Cd-hyperaccumulating ecotype *Sedum alfredii* Hance (HE) countering high Cd environment were investigated and compared with its non Cd-hyperaccumulating ecotype (NHE). Cadmium exposure resulted in significant ultrastructural changes in root meristem and leaf mesophyll cells of *S. alfredii*, but damage was more pronounced in NHE even when Cd concentrations were one-tenth of those applied to HE. Cadmium stress damaged chloroplasts causing imbalanced lamellae formation coupled with early leaf senescence. Histochemical results revealed that glutathione (GSH) biosynthesis inhibition led to overproduction of hydrogen peroxide (H_2O_2) and superoxide radical ($O_2^{\bullet -}$) in HE but not in NHE. Differences were noted in both HE and NHE for catalase (CAT), guaiacol peroxidase (GPX), ascorbate peroxidase (APX) and glutathione reductase (GR) activities under various Cd stress levels. No relationship was found between antioxidative defense capacity including activities of superoxide dismutase (SOD), CAT, GPX, APX and GR as well as ascorbic acid (AsA) contents and Cd tolerance in the two ecotypes of *S. alfredii*. The GSH biosynthesis induction in root and shoot exposed to elevated Cd conditions may be involved in Cd tolerance and hyperaccumulation in HE of *S. alfredii* H.

Keywords: Antioxidants; Glutathione; Hyperaccumulation; *Sedum alfredii* Hance; Cellular ultrastructure

1. Introduction

Consequent to increased environmental burdens from industrial, agricultural, energy and municipal sources, cadmium (Cd) contamination of soils has become a severe global issue. Moreover, due to neurotoxic, mutagenic and carcinogenic effects, high water solubility and thereby easier entry into human body via food chain render Cd a dangerous environmental pollutant [1,2]. Cadmium is a strong phytotoxic heavy metal causing growth inhibition with concomitant visible symptoms such as necrosis, leaf curling, brown stunted roots and severely affects cell division and ultrastructure [1,3,4].

Plant species differ significantly in capacity for uptake and tolerance to Cd and other heavy metals [5,6]. Metal-hyperaccumulating plants have the additional ability of storing large amounts of metals in their aerial parts [7]. This feature makes hyperaccumulators highly suitable for phytoremediation, *i.e.*using plants to cleanup metal-polluted soils. During the last decade, a number of studies have been conducted to investigate the mechanisms responsible for enhanced metal uptake and tolerance using natural hyperaccumulators as model plant species [8–10]. Metal hyperaccumulation in plants is generally accepted as a combination of higher metal uptake coupled with

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doi:10.1016/j.jhazmat.2007.12.064

a better tissue tolerance to damaging effects of elevated metal concentrations by means of sequestration at the cellular level and an enhanced antioxidative response [11,12].

Sedum alfredii H., a natural inhabitant of old Pb/Zn mined sites in Zhejiang province of China (where soil total Cd and diethylene triamine pentaacetic acid (DTPA)–Cd were 40.4 and 2.77 mg kg^{-1}, respectively), has been identified as a new Cd/Zn hyperaccumulator, with a shoot concentration of 456 mg kg^{-1} dry wt. under field condition [13]. This extraordinary higher Cd concentration has largely surpassed the generally accepted threshold concentration of 100 mg kg^{-1} dry wt. for Cd hyperaccumulators [14]. As a perennial species, *S. alfredii* has the ability to grow fast by developing a relatively large biomass and can propagate asexually. It has been reported that *S. alfredii* is capable of accumulating more than 9000 mg kg^{-1} Cd when grown hydroponically without showing toxicity symptoms [13,15]. Such properties of *S. alfredii* and its vigorous growth under heavy metal stress have attracted considerable scientific attention recently.

High Cd levels can cause oxidative stress by favoring the production of reactive oxygen species and lipid peroxidation [16–18]. Cd is known to disrupt the plant defense system against naturally occurring reactive oxygen species. This antioxidant defense system mainly includes the antioxidative enzymes such as superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), ascorbate peroxidase (APX) and glutathione reductase (GR) and non-enzymatic antioxidant compounds such as glutathione (GSH), ascorbic acid (AsA), carotenoids, cysteine, etc. SOD is the key enzyme responsible for catalyzing the dismutation of highly reactive $O_2^{\bullet -}$ to O_2 and H_2O_2. The resulting H_2O_2 is further decomposed to water and oxygen either by APX of the ascorbate–glutathione cycle or by GPX and CAT localized in the cytoplasm and other cellular compartments. GR is the complementary enzyme of the ascorbate–glutathione cycle, maintaining a high GSH/oxidized glutathione (GSSG) ratio for protection against oxidative damage [19–21]. GSH (γ-glutamylcysteinyl glycine) is a major non-enzymatic scavenger of reactive oxygen species (ROS) due to its unique structural properties, broader redox potential, abundance and wide distribution in plants.

The main objective of this study was to investigate the role of antioxidative defense systems in Cd tolerance of hyperaccumulator and non-hyperaccumulator ecotypes of *S. alfredii* by following the changes in biomass, ultrastructure, antioxidative enzymes (*i.e.* SOD, GPX, CAT, APX and GR) and low molecular weight antioxidants (GSH and AsA) under low, moderate and high Cd treatments.

2. Materials and methods

2.1. Plant collection and culture

Seedlings of the Cd-hyperaccumulating ecotype of *S. alfredii* (HE) were collected from an old Pb/Zn mined site in Zhejiang province of China, while those of non-hyperaccumulating ecotype (NHE) were obtained from a tea garden near Hangzhou, Zhejiang province of China. Plants with healthy and uniform shoots were selected and precultured for 4 weeks in the basic nutrient solution containing (in mmol L^{-1}) $Ca(NO_3)_2{\cdot}4H_2O$ 2.00, KH_2PO_4 0.10, $MgSO_4{\cdot}7H_2O$ 0.50, KCl 0.10, K_2SO_4 0.70; and (in μmol L^{-1}) H_3BO_3 10.00, $MnSO_4{\cdot}H_2O$ 0.50, $ZnSO_4{\cdot}7H_2O$ 1.0, $CuSO_4{\cdot}5H_2O$ 0.20, $(NH_4)_6$ $Mo_7O_{24}{\cdot}4H_2O$ 0.01, Fe–ethylene diamine tetraacetic acid (EDTA) 100. The nutrient solution pH was adjusted to 5.5 daily by 0.1 mol L^{-1} NaOH or HCl. Plants were grown under glasshouse conditions with natural light, day/night temperature of 26/20 °C and relative air humidity of 70/85%. The nutrient solution was continuously aerated and renewed after every 3 days.

2.2. Cd treatment and sample preparations

After preculturing for 4 weeks, healthy and uniform seedlings were selected for various Cd treatments. NHE plants were exposed to different Cd concentrations, *i.e.* control (0 μM Cd), 1, 5, 10 and 40 μM Cd, whereas, Cd treatments for HE were control (0 μM Cd), 10, 50, 100 and 400 μM Cd. Levels of Cd treatments for NHE were one-tenth of those applied to HE due to its sensitivity to Cd. Each treatment was applied in triplicates in a completely randomized design. Cadmium was applied as $CdCl_2$. Nutrient solution was aerated continuously and renewed after every 3 days with the pH maintained at 5.5. Plants were harvested after 7 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 surfaces.

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

2.3. Elemental analysis

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

2.4. Transmission electron microscopy

Fresh root tips (about 1–3 mm in length) and leaf sections (∼1 mm^2) from the middle section of fifth leaf from top of both ecotypes treated with different Cd concentrations were selected for TEM studies. Root and leaf sections were fixed in 4% glutaraldehyde (v/v) in 0.2 M sodium phosphate buffer (pH 7.2) for 6–8 h, post-fixed in 1% osmium tetroxide (OsO_4) for 1 h and washed in 0.2 M sodium phosphate buffer (pH 7.2) for 1–2 h. Dehydration was carried out in a graded ethanol series (50, 60, 70, 80, 90, 95, and 100%) followed by acetone (100%), then samples were infiltrated and embedded in Spurr's resin. Ultra-thin sections (80 nm) were prepared and mounted on copper grids and viewed under transmission electron micro-

scope (JEOL TEM-1200EX, Japan) at an accelerating voltage of 60.0 kV.

2.5. MDA and H_2O_2 content assays

Leaf and root MDA and H_2O_2 contents were determined by the previously described method of Velikova et al. [22]. Each fresh sample (about 1 g) was homogenized with 4 ml of 0.1% (w/v) TCA in ice bath. The homogenate was centrifuged at 12,000 × *g* for 20 min and supernatant was used for both MDA and H_2O_2 analysis. The 2-thiobarbituric acid (TBA) test, which determines MDA as an end product of lipid peroxidation was used for the lipid peroxidation determination in samples. For every 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 stop the reaction. The tubes were centrifuged at 10,000 × *g* for 10 min, and the absorbance of the supernatant was noted at 532 nm. 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}. With respect to the H_2O_2 assay, 1 ml of the supernatant was added to 1 ml of 10 mM potassium phosphate buffer (pH 7.0) and 2 ml of 1 M KI. H_2O_2 contents were estimated based on the absorbance of the supernatant at 390 nm.

2.6. In situ localization of H_2O_2 and $O_2^{\bullet -}$

In situ H_2O_2 and $O_2^{\bullet -}$ accumulations were detected by the method described by Romero-Puertas et al. [18]. For H_2O_2 detection, the excised leaves were immersed in 10 mM 4-morpholineethanesulfonic acid (MES) buffer (pH 6.5, containing 1% 3,3′-diaminobenzidine (DAB)), vacuum-filtered for 5 min and then incubated for 8 h in the dark at 25 °C. The leaves were illuminated until appearance of brown spots, characteristic of the reaction of DAB with H_2O_2. To verify the specificity of precipitates, before staining with DAB Cd-exposed leaves were immersed in 1 mM AsA (H_2O_2 scavenger) for 2 h.

For $O_2^{\bullet -}$ detection, leaves were immersed in 0.1% nitroblue tetrazolium (NBT) solution in 50 mM potassium phosphate buffer (pH 6.4) containing 10 mM Na-azide, and were vacuum-filtered for 10 min and finally illuminated until appearance of dark spots, characteristic of blue formazan precipitates. Negative controls were immersed in 1 mM tetramethylpiperidinooxy (TMP), an $O_2^{\bullet -}$ scavenger, for 3 h prior to staining with NBT.

The excised Cd-exposed leaves were also incubated with 10 μM diphenylene iodonium (DPI, oxidase inhibitor) and 100 μM buthionine sulfoximine (BSO, glutathione synthesis inhibitor) for 18 h at 25 °C. Then, the leaves were either immersed in DAB solutions or NBT solution to localize H_2O_2 and $O_2^{\bullet -}$ accumulations, respectively.

Bleaching of leaves was performed by immersing them in boiling ethanol to visualize the spots. H_2O_2 and $O_2^{\bullet -}$ deposits were quantified by scanning the spots from treated leaves and the numbers of pixels were quantified with PHOTOSHOP 7.0 software (Adobe Systems, USA). The results were expressed as percentage of spot area in pixels, versus total leaf area [(spot area/total leaf area) × 100] to compensate for differences in leaf size.

2.7. Antioxidant enzymes assays

Plant samples of a known weight (1 g fresh weight) 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* at 4 °C and the supernatant obtained was used for enzyme analysis. An aliquot of the extract was used to determine protein content following the previously described method of Bradford [23], using bovine serum albumin as standard.

SOD (EC 1.15.1.1) was determined by the photochemical method [24]. One unit of SOD activity was defined as the amount of enzyme required to cause a 50% inhibition of the NBT reduction rate at 560 nm. CAT (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 [25]. The GPX activity (EC 1.11.1.7) was assayed following the previously described method of Cakmak et al. [25]. 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}). APX (EC 1.11.1.11) was measured according to the method of Nakano and Asada [26] 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. GR (EC 1.6.4.2) was assayed following the method of Foyer and Halliwell [27] 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 GSSG, 0.12 mM NADPH, and the enzyme aliquot.

2.8. Antioxidant (glutathione and ascorbic acid) assays

The GSH contents were estimated fluorimetrically based on a previous method by Hissin and Hilf [28] 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* 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 μl 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.

Determination of AsA levels followed the previous procedure of Singh et al. [29] with few modifications. A known weight (1 g fresh weight) of sample was extracted with 3 ml of 5% (w/v) trichloroacetic acid (TCA) and centrifuged at 18,000 × *g* for 15 min. AsA was determined in a reaction mixture consisting of 0.2 ml of supernatant, 0.5 ml of 150 mM phosphate buffer

Table 1
Changes in dry weight, Cd concentrations and contents in the tissues of two ecotypes of *S. alfredii*

Ecotype	Cd levels (μM)	Dry weight (mg plant^{-1})		Cd concentration (mg kg^{-1})		Cd content (μg plant^{-1})		
		Shoot	Root	Shoot	Root	Shoot	Root	Shoot/root
NHE	Control	925 a	77 a	1 d	2 d	0.4 d	0.2 e	2.8 a
	1	945 a	86 a	52 c	101 d	18 d	9 d	2.1 ab
	5	858 a	64 b	147 b	338 c	41 b	22 c	1.9 bc
	10	618 b	53 c	219 a	696 b	54 a	37 b	1.4 bc
	40	518 b	41 d	203 a	2580 a	41 b	104 a	0.4 c
HE	Control	578 b	89 b	321 e	46 d	99 e	4 c	24 bc
	10	613 ab	93 b	1592 d	123 d	496 d	12 c	45 a
	50	619 ab	102 ab	2981 c	278 c	903 c	28 c	33 ab
	100	691 a	123 a	4264 b	593 b	1370 b	72 b	19 cd
	400	669 ab	115 ab	5482 a	1708 a	1672 a	197 a	9 d

Each value is the mean of three individual replicates. Means followed by the same letter are not significantly different at $P < 0.05$.

(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) $FeCl_3$. The reaction mixtures were incubated at 40 °C for 40 min and the absorbance was read at 532 nm using ascorbic acid as a standard.

2.9. Statistical analysis

Statistical analysis was carried out using the SPSS statistical software (version 11.0). All values reported are 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^{TM} v.10.

3. Results and discussion

3.1. Cd tolerance of S. alfredii

3.1.1. Effect of Cd on plant biomass and Cd concentrations in plant tissues

Both ecotypes of *S. alfredii* (HE and NHE) were exposed to different cadmium concentrations for 7 days. Cadmium treatments up to 100 μM did not cause visible phytotoxicity to the growth of HE; however, some visual Cd toxicity symptoms with necrosis and browned root tips were observed at 400 μM by the end of experiment. On the other hand NHE displayed obvious toxicity symptoms such as stunted roots, thickened cuticle on the root epidermis, cracked and brownish stem formation and wilted leaves upon exposure to Cd over 10 μM. Also in NHE treated with 40 μM Cd, older leaves started to fall at the end of experiment.

Exposure to different Cd concentrations caused variable effects on the biomass of both ecotypes (Table 1). In NHE, shoot and root biomass production (expressed as dry weight) was reduced significantly ($P < 0.05$) with ≥10 μM Cd (Table 1). However, shoot and root biomass of HE increased significantly ($P < 0.05$) with Cd treatments up to 100 μM Cd (Table 1). Cadmium concentrations (on dry weight basis) and its accumulation (content) in shoots and roots of both ecotypes revealed a linear increase in response to increasing external Cd supply levels. Shoot Cd concentration and contents of HE treated with 100 μM Cd for 7 days reached to 4246 mg kg^{-1} dry wt. and 1370 μg plant^{-1}, respectively (Table 1). In addition, distribution of Cd in shoot and root of both ecotypes differed significantly ($P < 0.05$). The shoot/root ratio of total Cd accumulated in HE ranged from 9 to 45 and shoot/root ratios were far greater than those of NHE (Table 1), confirming that HE of *S. alfredii* can effectively transport Cd from roots to shoots. Previously, HE of *S. alfredii* was reported to have a high tolerance to Cd as well as Zn and Pb toxicity, and only HE of *S. alfredii* from mined sites possessed Cd and Zn hyperaccumulation characteristics [13,15,30]. Such a characteristic can be regarded as consequence of a strong selection pressure under high soil heavy metal concentrations. Ultimately, it becomes a promising criterion to select *S. alfredii* ecotypes to be utilized in the phytoremediation of heavy metal-contaminated areas. The distinct intraspecific variations in heavy metal accumulation and tolerance of *S. alfredii* could be exploited to find a better understanding of mechanisms involved in hyperaccumulation.

3.1.2. Effect of Cd on ultrastructure

The effect of heavy metals on cellular organization is an important factor in understanding the physiological alterations induced by heavy metals due to complimentarity of structure and function.

Ultrastructural observations on root meristematic cells of both NHE (Fig. 1a) and HE (Fig. 1b) shows that these cells possessed a granular cytoplasm densely filled with numerous organelles that seemed to be without any obvious damage under control conditions. Cells possessed smooth, clean and continuous cell membranes and cell walls.

Electron microscopic studies showed that root meristematic cells in both ecotypes exposed to different Cd concentrations exhibited obvious ultrastructural changes over control, but cellular damage (*i.e.* vacuolation, damaged membrane systems, swollen mitochondria, rough endoplasmic membrane and cracked root epidermis) was more pronounced in NHE and the intensity increased with the increasing the metal concentrations. In root meristematic cells of NHE exposed to 10 μM

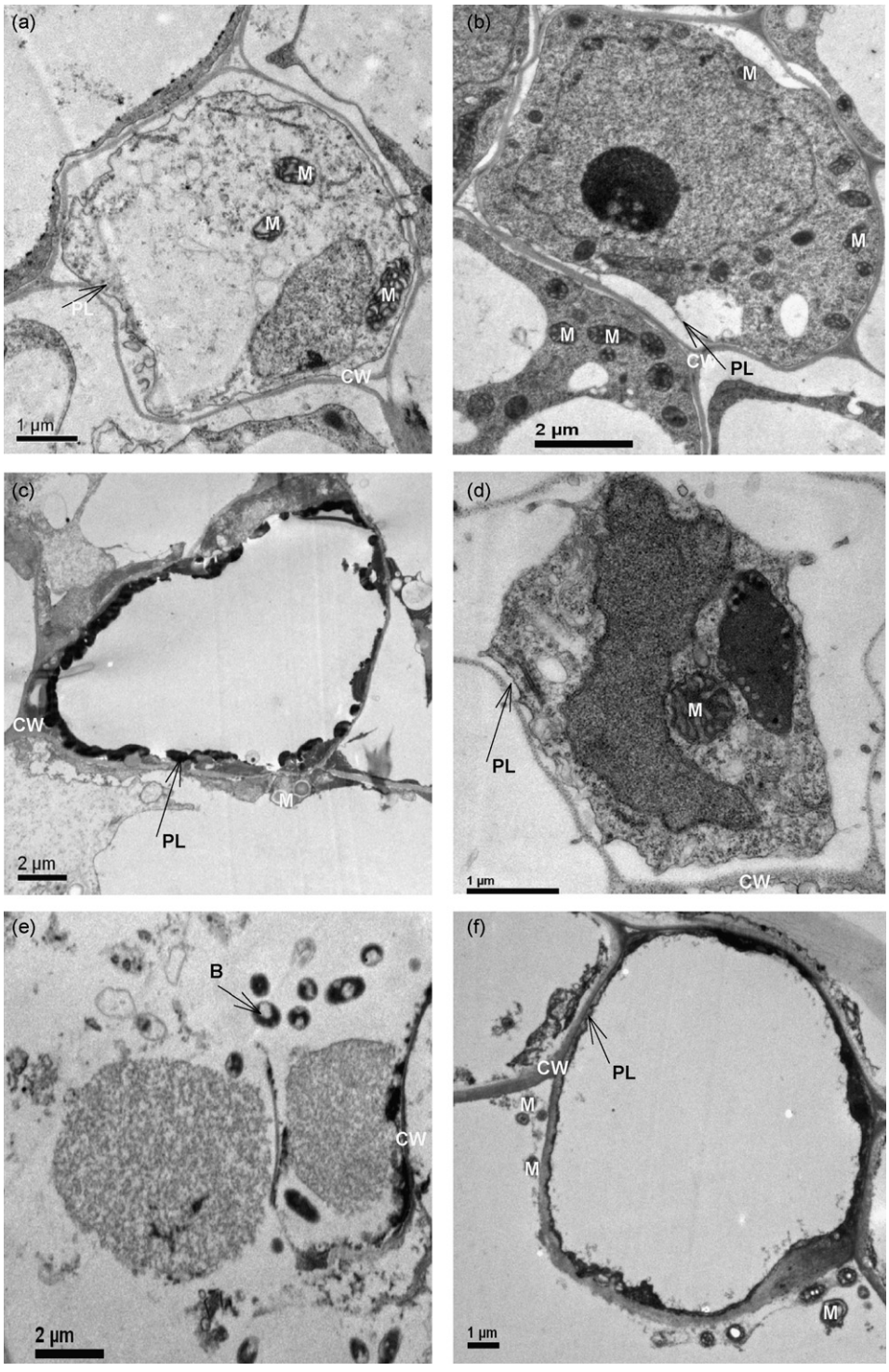

Fig. 1. Transmission electron micrograph of root meristematic cells from NHE *S. alfredii* (left panel) and HE *S. alfredii* (right panel). NHE was exposed to 0 μM (control, a), 10 μM (c) and 40 μM (e) Cd for 7 days, respectively while HE was exposed to 0 μM (control, b), 100 μM (d) and 400 μM (f) Cd for 7 days, respectively. Bars (a, d and f) = 1 μm and (b, c and e) = 2 μm, respectively. Labels: CW, cell wall; B, bacteria; M, mitochondria; PL, plasmalemma.

Cd, increased vacuolation, damaged membrane systems and swollen mitochondria were observed. Severely damaged cellular structures were noted in NHE roots exposed to 40 μM Cd, where root epidermis was cracked with bacterial infection and integral cellular organization was lost (Fig. 1e). For HE at 100 μM Cd, root cells still had a better cellular organization with distinct integrity of plasma membrane and cell wall (Fig. 1d). However, at 400 μM Cd treatment, advanced vacuolation and rough endoplasmic membrane were the main toxicity symptoms in root cells (Fig. 1f). Nevertheless the overall damage was much less compared to that of NHE at 10 μM Cd.

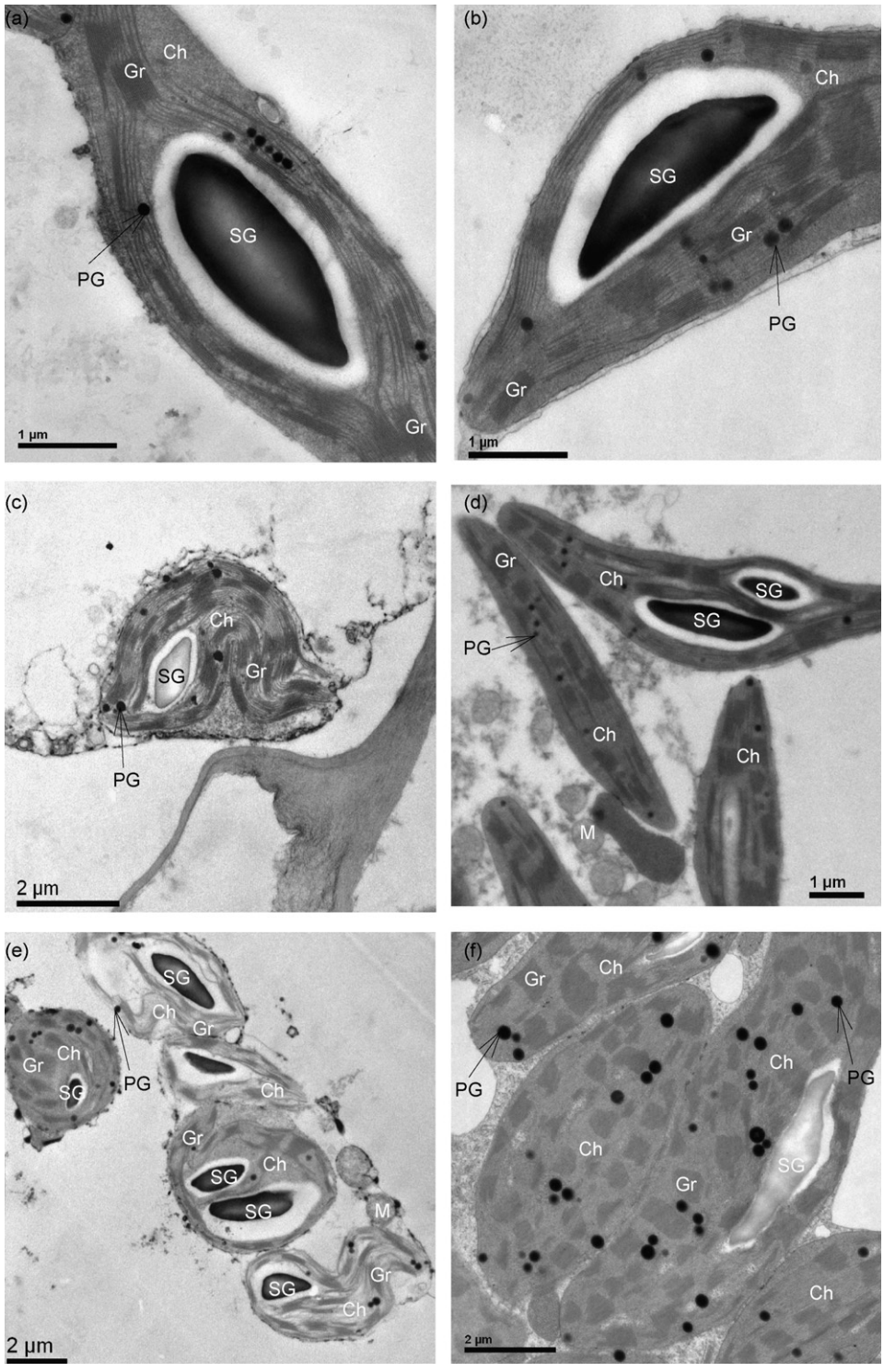

Fig. 2. Transmission electron micrograph of leaf mesophyll cells from NHE *S. alfredii* (left panel) and HE *S. alfredii* (right panel). NHE was exposed to 0 μM (control, a), 10 μM (c) and 40 μM (e) Cd for 7 days, respectively while HE was exposed to 0 μM (control, b), 100 μM (d) and 400 μM (f) Cd for 7 days, respectively. Bars (a, b and d) = 1 μm and (c, e and f) = 2 μm, respectively. Labels: Ch, chloroplast; SG, starch grain; PG, plastoglobule; M, mitochondrion; Gr, granum.

Transmission electron micrographs of the leaf mesophyll cells of both ecotypes are shown in Fig. 2. In mesophyll cells of NHE (Fig. 2a) and HE (Fig. 2b) grown without Cd, oblong chloroplasts were observed with regular thylakoid membrane arrangement of the stroma.

Ultrastructural studies revealed that chloroplast was the obvious site of damage caused by Cd stress. In NHE plants exposed high external Cd concentrations of 10–40 μM, disorganization of chloroplasts was more common, *i.e.* chloroplasts were rounded with a variable degree of internal-structural disruption

with a decrease in number of compact grana and light-colored thylakoid membranes (Fig. 2c and e). In case of HE at 100 μM Cd, the chloroplasts and mitochondria were still relatively in better shape with clear and regular thylakoid membranes but showing a reduction in chloroplast size (Fig. 2d). For HE at 400 μM Cd, the notable change in chloroplasts was only a considerable increase in the number and size of plastoglobuli. At larger magnification, swollen chloroplasts and loose thylakoid membranes were observed (Fig. 2f). These results indicated that Cd stress caused imbalanced synthesis of chloroplast lamellae leading to the senescence of leaves. Other events that may have taken place during the senescence included a reduction in the size of chloroplasts and loss of cytoplasmic components in the mesophyll cells of *S. alfredii* under high external Cd concentrations.

It has been reported that Cd induces premature senescence in leaves of several plant species including wheat [31], pea [16,32] and *Elodea canadensis* [33]. The senescence of leaves is mainly characterized by cessation of photosynthesis, disintegration of organelle structures, increase in lipid peroxidation and membrane damage [34].

3.2. Cd-induced oxidative stress in S. alfredii

3.2.1. Effects of Cd on lipid peroxidation and H_2O_2 concentrations

Lipid peroxidation can be defined as the oxidative deterioration of lipids containing any number of carbon–carbon double bonds. Lipid peroxidation in the leaves and roots of the two contrasting ecotypes of *S. alfredii* was estimated by measuring the contents of MDA. For both NHE and HE, Cd addition remarkably increased MDA contents in leaves and roots, whereas the concentrations of MDA were 1.22–11.87 times higher as compared to control. In both NHE and HE subjected to high Cd treatments, levels of MDA in leaves were much greater than those in roots (Fig. 3a and b). Interestingly, Cd exposure had a considerable effect on H_2O_2 accumulation in both leaves and roots of the two *S. alfredii* ecotypes and this was more pronounced in roots than in leaves (Fig. 3c and d).

A concentration-dependent increase in the levels of lipid peroxidation and H_2O_2 in plant tissues implied that Cd caused oxidative stress in both ecotypes of *S. alfredii*. The oxidative deterioration is considered as an intrinsic feature of senescence process in plants [35].

3.2.2. Histochemical detection of H_2O_2 and $O_2\cdot^-$ in leaves under Cd stress

Cd-stimulated distinct overproduction of H_2O_2 (Fig. 4a and b) and $O_2^{\bullet-}$ (Fig. 4c and d) in leaves of both *S. alfredii* ecotypes, which was verified by a histochemical method with DAB and NBT, respectively. Accumulation of H_2O_2 or $O_2^{\bullet-}$ could be eliminated by infiltration of AsA (a H_2O_2 scavenger) and TMP (an $O_2^{\bullet-}$ scavenger), respectively. Infiltration with DPI (an oxidase inhibitor) largely prevented H_2O_2 and $O_2^{\bullet-}$ accumulation, revealing the involvement of NADPH oxidase in both NHE and HE. Treatment with BSO (a glutathione synthesis inhibitor) brought about significant ($P < 0.05$) damage in leaves of HE with

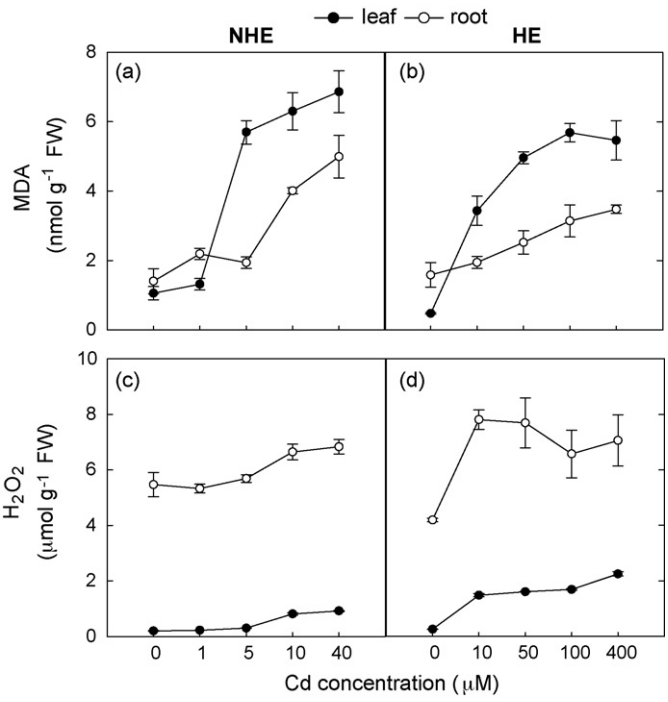

Fig. 3. Effects of Cd on lipid peroxidation expressed in terms of MDA and H_2O_2 contents in leaves and roots of NHE *S. alfredii* (a and c) and HE *S. alfredii* (b and d). Error bars represent standard errors from three individual replicates.

concomitant increases in H_2O_2 (31%) and $O_2^{\bullet-}$ (13%) production (Fig. 4) suggesting that glutathione biosynthesis may contribute to counter the Cd-induced ROS production and help to cope the Cd toxicity.

3.2.3. Antioxidative responses under Cd treatment

SOD activity, responsible for the elimination of superoxide radicals in cells, showed a remarkable increase in both HE and NHE grown under Cd stress (Fig. 5a and b). However, at 40 μM Cd treatment, SOD activity sharply declined in both roots and leaves of NHE (Fig. 5a). It is well known that CAT, localized in peroxisomes can decompose H_2O_2 into H_2O and O_2. However, a considerable decrease in CAT activity was observed in leaves of both NHE and HE as well as in roots of HE (Fig. 5c and d), whereas high Cd treatments brought 1.27–2.90-fold induction in CAT activity in roots of NHE (Fig. 5c).

APX and GPX have high affinity for H_2O_2. Upon Cd exposure, APX and GPX activities followed a similar pattern in the leaf tissue with no significant change as compared to control (Fig. 5e, f, i and j). Increasing Cd treatments up to 100 μM caused a marked reduction in both APX and GPX activities in roots of HE (Fig. 5f and j), but recovered partly at 400 μM Cd. This recovery in root enzymatic activity of HE at 400 μM was also pronounced in CAT and GR activities. In the case of NHE, root GPX activity increased linearly under Cd treatments. This increase in root GPX activity was accompanied by APX and GR to some extent. Similar to SOD, APX and GR activities also declined sharply in NHE at the highest Cd treatment.

Low molecular antioxidants such as GSH and AsA, which are found in chloroplasts and other cellular compartments, are

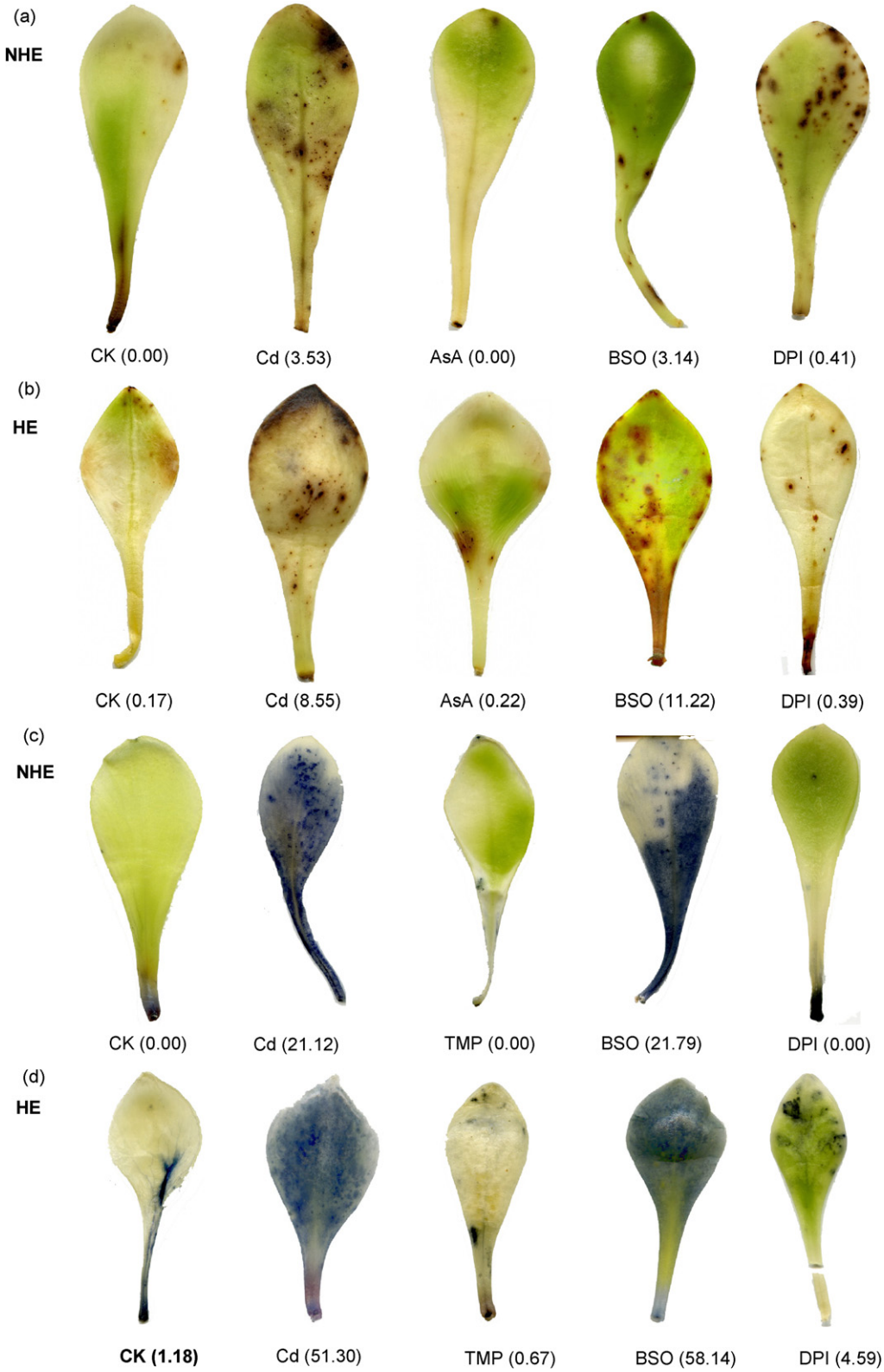

Fig. 4. Histochemical detection of H_2O_2 (a: NHE; b: HE) and $O_2^{\bullet -}$ (c: NHE; d: HE) in leaves of *S. alfredii* H. Excised leaves from both control and Cd-treated plants (NHE and HE were grown in 10 μM and 100 μM Cd for 7d, respectively) were immersed in DAB or NBT solution to visualize brown or blue spots characteristic of DAB or NBT reaction with H_2O_2 and $O_2^{\bullet -}$, respectively. And then leaves were bleached by immersing them in boiling ethanol to visualize the spots. Treatment symbols: AsA-H_2O_2 scavenger, TMP-$O_2^{\bullet -}$ scavenger, BSO- glutathione synthesis inhibitor, DPI-oxidase inhibitor. Scanned spots from treated leaves quantified H_2O_2 deposits and the number of pixels was quantified with PHOTOSHOP 7.0 software (Adobe Systems, USA). The results were expressed as percentage of spot area in pixels, vs. total leaf area [(spot area/total leaf area) × 100]. Leaves were taken from control and Cd exposed plants, plus from Cd exposed plants treated with other compounds. The effect of AsA, TMP, BSO, and DPI on control plants is excluded; the percentage effect in treated plants is indicated in brackets. Results are reported from five representative individual experiments.

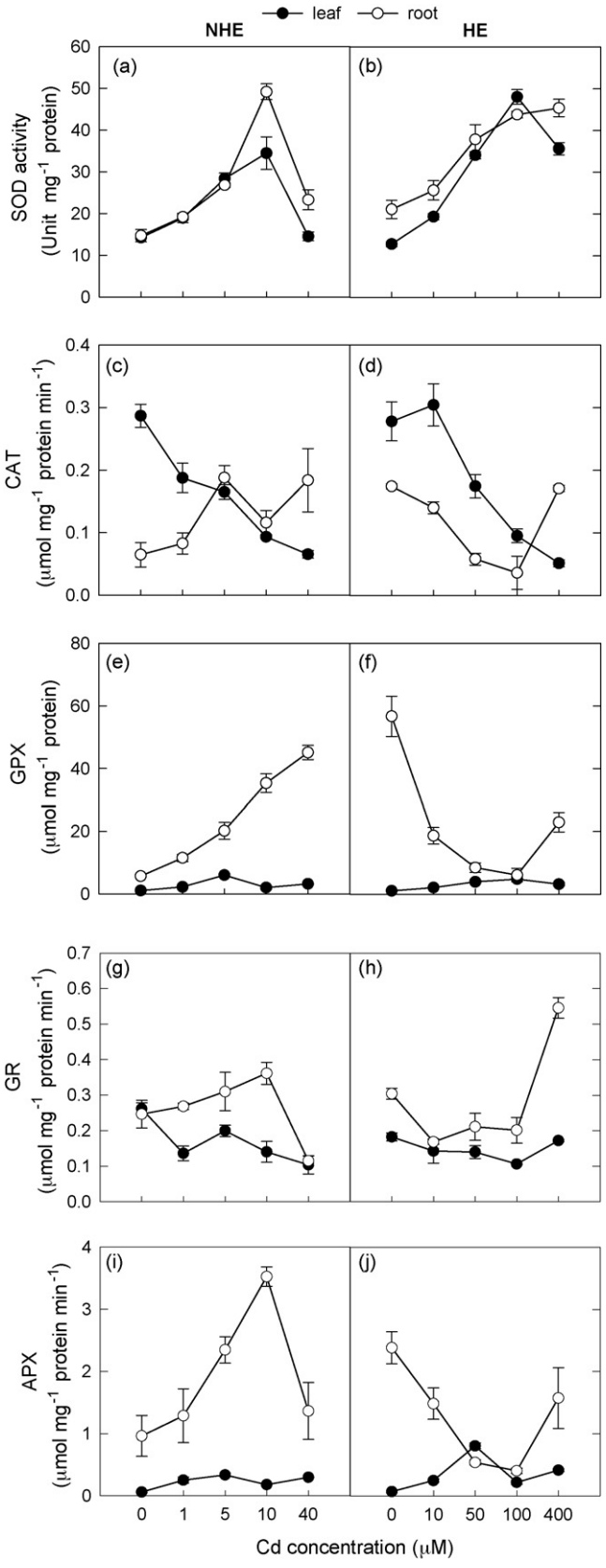

Fig. 5. Response of superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), glutathione reductase (GR) and ascorbate peroxidase (APX) antioxidant enzymes under Cd treatments in leaves and roots of NHE *S. alfredii* (a, c, e, g, and i) and HE S. *alfredii* (b, d, f, h, and j). Error bars represent standard errors from three individual replicates.

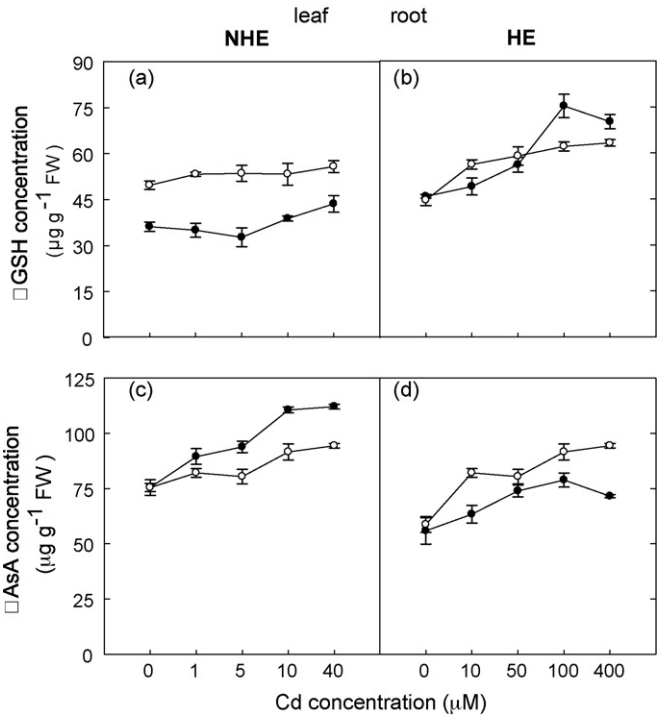

Fig. 6. Levels of glutathione (GSH) and ascorbic acid (AsA) in leaves and roots of NHE *S. alfredii* (a and c) and HE S. *alfredii* (b and d) exposed to various concentrations of Cd. Error bars represent standard errors from three individual replicates.

crucial for plant defense against oxidative stress [20]. On average, GSH contents were obviously higher in the HE tissues than in NHE (Fig. 6a and b). On the contrary, AsA pool was higher in NHE tissues (Fig. 6c and d). Application of Cd resulted in a significant ($P < 0.05$) enhancement in GSH levels in the leaves and roots of HE; however, GSH contents in the NHE changed slightly and did not correlate to Cd tolerance (Fig. 6a and b). Total AsA contents displayed a remarkable increase in leaves and roots of both HE and NHE with varying Cd treatments (Fig. 6c and d).

In present study, from the data of enzymes and AsA, it can be concluded that there is no relationship between antioxidative defense and Cd tolerance of the two ecotypes of *S. alfredii*. Particularly, increased GSH levels in HE plants may account for its greater ability to tolerate Cd toxicity. It has been suggested that GSH serves as a precursor in phytochelatin (PC) biosynthesis, therefore, the intracellular GSH level is thought to regulate PC synthesis [36]. However, Sun et al. reported that no PCs were induced in the mined population of *S. alfredii*, *i.e.* HE and thereby PCs were not involved in its Cd transport, hyperaccumulation and tolerance [37]. Moreover, GSH is not only known as an antioxidant playing a prominent role in defense system by maintaining the redox status, but also it has a relatively higher affinity ($Kd_{Cd} > 10^{10}$) to bind with Cd^{2+} [38] which makes it a potential cytosolic chelator of this metal and helps in reducing the acute metal toxicity [39]. So, it could be speculated that the elevated GSH in HE plants may serve as an antioxidant or metal chelator involved in Cd tolerance and hyperaccumulation. Consistently, our present study also ruled out the possibility that GSH biosyn-

thesis may play an important role as the signals for the stress regulation.

4. Conclusions

It can be concluded that the *S. alfredii* ecotype collected from the old-mined site has a greater ability to adapt Cd toxicity and Cd hyperaccumulation. Cadmium-induced severe ultrastructural changes in root meristematic and leaf mesophyll cells of *S. alfredii*, but damage was more pronounced in NHE when exposed to much lower Cd concentrations compared to HE. Severity of damage increased with increasing Cd concentrations. Chloroplast was an important site of damage induced by Cd stress, which may have caused imbalanced lamellae formation leading to early senescence of leaves.

A concentration-dependent enhancement in levels of lipid peroxidation and distinct overproduction of H_2O_2 and $O_2^{\bullet -}$ implied that Cd caused oxidative stress in both ecotypes of *S. alfredii*. It is suggested that there is no relationship between antioxidative defense and Cd tolerance of the two ecotypes of *S. alfredii*. Elevated GSH levels in plant tissues and GSH biosynthesis seemed to be more important and the most promising reply to the higher Cd tolerance and accumulation in HE of *S. alfredii*. However, further research is needed to clarify the possible involvement of GSH in Cd detoxification and tolerance in HE of *S. alfredii*.

Acknowledgements

The work was financially supported by a key project from the National Natural Science Foundation of China (#30630046), "973" project from the Science and Technology Ministry of China (#2002CB410804) and Program for Changjiang scholars and Innovative Research Team in University (#IRT0536). Authors are grateful to Dr. Levent Ozturk, Sabanci University, Turkey for his critical linguistic and pre-review of the MS. Authors are also thankful to Dr. Junying Li Zhejiang University, China for their kind help in Electron Microscopy.

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