



Calcium protects roots of *Sedum alfredii* H. against cadmium-induced oxidative stress

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ABSTRACT

Sedum alfredii is a well-known Cd (cadmium) hyperaccumulator native to China. The impacts of exogenous Ca on Cd-induced oxidative stress and antioxidant systems in roots of *S. alfredii* were investigated by using cellular and biochemical approaches. Supplementation of the medium with higher Ca levels resulted in alleviated growth inhibition and decreased Cd concentration, as well as increased Ca concentration in roots. Cadmium induced lipid peroxidation and loss of plasma membrane integrity, reactive oxygen species overproduction, as well as ultrastructural changes of root cells were largely reversed by Ca supplementation in the medium. Calcium application significantly altered the Cd effects on antioxidant enzymes and non-enzyme antioxidants (non-protein thiols), and significantly increased glutathione (GSH) biosynthesis. The results suggest that Ca is able to protect the roots of *S. alfredii* against Cd toxicity by restoration of Cd-displaced Ca, alleviation of the metal induced oxidative stress, as well as promotion of GSH biosynthesis.

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1. Introduction

Industrial processes and widespread use of phosphate fertilizers have resulted in an increase in cadmium (Cd) in the environment over the past decades (Pinto et al., 2004). Due to its high mobility and toxicity increased environmental Cd represents a threat to plant and animal health (Bertin and Averbek, 2006). Cadmium is a potent toxic element for plants, causing root tip damage, reduced photosynthesis, induced antioxidant responses in all plant organs, and growth inhibition (Das et al., 1997). A rare class of plants named “hyperaccumulator” (Baker et al., 2000), however, can tolerate and accumulate an extremely high concentration of Cd in the aboveground tissues and have potential for use in phytoremediation of metal-polluted soils (Pilon-Smits, 2005), and constitute an exceptional biological material for understanding mechanisms regulating plant metal homeostasis as well as plant adaptation to

extreme metallic environments (Verbruggen et al., 2009). *Sedum alfredii* is a fast growing, high-biomass Zn/Cd cohyperaccumulator and Pb accumulator native to China (Lu et al., 2008; Tian et al., 2009, 2010). The mechanisms involved in the metal accumulation by this plant species, however, are not well understood.

Metal hyperaccumulation in plants is generally achieved by a combination of enhanced metal uptake and translocation, coupled with a better tissue tolerance to the phytotoxicity of the elevated metals (Jin et al., 2008). Cadmium is a non-essential element for plants and its uptake is thought to occur primarily through transporters for essential elements as a consequence of a lack of specificity of the transporters (Welch and Norvell, 1999). Calcium shares many physical similarities with Cd and has similar charge and ionic radius at physiological pH values, and antagonism between the two elements has been frequently reported in plants. Cadmium competes with Ca for uptake through ion channels in root cells and guard cells of plants (Clemens et al., 1998; White, 2000; Perfus-Barbeoch et al., 2002). In previous studies, we found that patterns of Cd and Ca uptake, translocation and distribution in the hyperaccumulator *S. alfredii* were similar (Lu et al., 2008, 2010) and supplementation of Ca in the growth medium resulted in elevated Cd accumulation in shoots and improved plant growth of *S. alfredii* (Lu et al., 2010). The mechanisms involved in the Ca alleviation of Cd toxicity, however, are largely unknown.

Abbreviations: CAT, catalase; DHE, dihydroethidium; DTNB, 5,5'-dithiobis-2-nitrobenzoic acid; GSH, glutathione; G-POD, glutathione peroxidase; MDA, malondialdehyde; NPT, non-protein thiols; ROS, reactive oxygen species; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; TCA, trichloroacetic acid; TMP, tetramethyl piperidinoxy.

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The present investigation attempts to identify the mechanisms by which Ca protects *S. alfredii* from Cd stress. It is well known that Cd causes disturbances to plant antioxidant defenses, and indirectly triggers the production of various reactive oxygen species (ROS) by unknown mechanisms, giving rise to an oxidative burst in plants (Olmos et al., 2003; Romero-Puertas et al., 2004; Garnier et al., 2006; Rodriguez-Serrano et al., 2009). Calcium was shown to be involved in the signal transduction of environmental stimuli and related gene expression in plants, increasing the tolerance of plants against Cd and other stresses (Wang and Song, 2009). The protective effects of Ca against Cd-induced oxidative stress have now been reported in several plant species (Rodriguez-Serrano et al., 2009; Wang and Song, 2009). The present investigation was performed to determine if supplemental Ca protects the roots of *S. alfredii* against Cd-induced oxidative stress and to determine if this occurs through effects on ROS activity (H_2O_2 and O_2^-), glutathione metabolism, lipid peroxidation and the subsequent loss of plasma integrity. The study will focus on root tips comprising meristem, elongation and differentiating cells within a short distance of the root tip. Root tips are very metabolically active and extremely sensitive to biotic or abiotic stress.

2. Materials and methods

2.1. Plant culture

The hyperaccumulator *S. alfredii* was obtained from an old Pb/Zn mine area in Zhejiang Province, China. Plants were grown in non-contaminated soil for several generations to minimize the internal metal contents, uniform and healthy shoots were then selected, rooted and cultivated in the basal nutrient solution containing: 2 mM Ca^{2+} , 4 mM NO_3^- , 1.6 mM K^+ , 0.1 mM H_2PO_4^- , 0.5 mM Mg^{2+} , 1.2 mM SO_4^{2-} , 0.1 mM Cl^- , 10 μM H_3BO_3 , 0.5 μM MnSO_4 , 1 μM ZnSO_4 , 0.2 μM CuSO_4 , 0.01 μM $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$, 100 μM Fe-EDTA. Nutrient solution pH was adjusted daily to 5.8 with 0.1 N NaOH or 0.1 N HCl. Plants were grown under glasshouse conditions with natural light, day/night temperature of 26/20 °C and day/night humidity of 70/85%. The nutrient solution was aerated continuously and renewed every 3 d.

After growing for 14 d, plants of *S. alfredii* were subjected to treatments including (1) control, (2) 6.0 mM Ca [$\text{Ca}(\text{NO}_3)_2$], (3) 400 μM Cd, and (4) 6.0 mM Ca + 400 μM Cd, in nutrition solution (pH 5.8) for 24 h. Three replicate were used and analyzed independently for each treatment.

2.2. Root elongation inhibition

Root length measurements were performed according to Schutzenobel et al. (2001). Roots were marked 5 mm behind the tips with water-resistant ink 2 d before treatments. Root lengths were measured daily on 20 plants per treatment until harvest. The degree of the root elongation (percentage of control) was estimated as: $(B/A) \times 100$, where A was the root length elongated during treatment without Cd and B was the root length elongated during treatments with Cd.

2.3. Determination of cadmium content

At harvest, intact roots were soaked in 20 mM $\text{Na}_2\text{-EDTA}$ for 15 min to desorb Cd^{2+} from the root surfaces. After rinsing and blotting dry, root tips were cut 10 mm behind the apex, weighed, and digested with $\text{HNO}_3\text{-HClO}_4$. The digest was made to volume and filtered. Concentrations of Cd and Ca in the filtrates were analysed using Inductively Coupled Plasma Mass Spectroscopy (ICP-MS) (Agilent 7500a, USA).

2.4. Loss of plasma membrane integrity and lipid peroxidation

Plasma membrane integrity was measured spectrophotometrically with Evans blue according to Yamamoto et al. (2001). After treatment application, thirty root tips (10 mm) were incubated in Evans blue solution (0.025% [w/v] in 100 μM CaCl_2 (pH 5.6) solution) for 30 min. After washing the roots for 15 min with water, roots tips were homogenized with a microhomogenizer in 2 mL of a measuring solution (50% [v/v] MeOH and 1% [w/v] SDS). The homogenate was incubated for 15 min in a water bath at 50 °C and centrifuged at 14 000g for 15 min. The optical density of the supernatant was determined at 600 nm.

The level of lipid peroxidation in root tips was determined as thiobarbituric acid reactive substances (TBARS), and malondialdehyde (MDA), based on the method of Dixit et al. (2001). Briefly, approximately thirty root tips (weighed) were ground with 5 mL of 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 15 000g for 10 min and 0.5 mL of the supernatant was mixed with 2 mL of 0.5% TBA in 20% TCA. The mixture was heated at 90 °C for 20 min. After the reaction was stopped, the resultant mixture was centrifuged at 10 000g for 5 min. The absorbance of the supernatant was measured at 532 nm. The values were corrected for non-specific absorption by subtracting absorbance read at 600 nm. The amount of TBARS was calculated by using the extinction coefficient of 155 $\text{mM}^{-1} \text{cm}^{-1}$.

Histochemical detection of loss of plasma membrane integrity and lipid peroxidation in root apices was performed as described by Yamamoto et al. (2001). Intact roots of *S. alfredii* seedlings after different treatments were rinsed several times with 0.5 mM CaCl_2 (pH 4.5), dried with filter papers. For plasma membrane integrity determination, roots were incubated in 4 mL of Evans blue solutions (0.025% (w/v) prepared in 100 μM CaCl_2 (pH 5.6) for 30 min. For lipid peroxidation analysis, roots were incubated in Schiff's reagent for 60 min, rinsed with a solution containing 0.5% (w/v) $\text{K}_2\text{S}_2\text{O}_5$ (prepared in 0.05 M HCl) until the root color became light red. All of the roots, stained with the specific reagents as indicated above, were washed three times with water and observed under a light microscope (model SZH-ILLD; Nikon, Japan).

2.5. ROS (H_2O_2 and O_2^-) content assay and fluorescence imaging

The content of H_2O_2 was measured according to the method of Jin et al. (2008) with small modifications. Root tips were ground in 50 mM K-phosphate buffer (pH 7.8). To the homogenate, 5% TCA was added (TCA: mixture/1:0.7). The mixture was centrifuged at 10 000g for 10 min. and the supernatant was collected. One 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 concentration was estimated based on the absorbance of the supernatant at 390 nm. Determination of O_2^- production in root tips of plants were measured as described by Huang et al. (2008). Root tips samples were ground with ice-cold sodium phosphate buffer (pH 7.8, 50 mM). The extracts were centrifuged at 13 000g for 20 min at 4 °C. The supernatant (0.5 mL) was collected and incubated at 25 °C for 60 min in the presence of 1 mM hydroxylamine hydrochloride in 50 mM sodium phosphate buffer (pH 7.8). The reaction mixture was then incubated with 1 mL of 17 mM P-aminobenzene sulphonic acid anhydrous (dissolved with acetic acid: $\text{H}_2\text{O}_2 = 3:1$) and 1 mL of 7 mM α -naphthylamine (dissolved with acetic acid: $\text{H}_2\text{O}_2 = 3:1$) at 25 °C for 30 min. The absorbance was measured at 530 nm. A calibration curve was established using sodium nitrite.

Intact roots of *S. alfredii* seedlings after different treatments were rinsed several times with 0.5 mM CaCl_2 (pH 4.5), dried with filter papers, and immediately immersed into the following specific reagents. Superoxide radicals were detected by staining with 10 μM dihydroethidium (DHE) (Yamamoto et al., 2002), and H_2O_2

was localized using with 10 μM carboxy- H_2DCFDA (Molecular Probes, Eugene, OR) for 15 min and then rinsed (Freeman et al., 2004). The segments were washed twice in the same buffer for 15 min each and were then embedded in 30% polyacrylamide blocks (Rodriguez-Serrano et al., 2006). A Nikon Eclipse 3000 epifluorescent microscope (Melville, NY) equipped with a green fluorescent protein filter (excitation 450–490 nm, emission 500–530 nm) was used for epifluorescence images. Exposure times were equal for all samples. Autofluorescence was not observed in unstained controls at the exposure time used. Images were captured with a SPOT camera (Nikon).

2.6. Determination of non-protein thiols and glutathione

Non-protein thiols (NPT) were extracted by homogenizing approximately thirty root tips (weighed) in 2 mL ice-cold 5% (w/v) sulfosalicylic acid solution. After centrifugation at 10 000g at 4 °C for 30 min, the supernatant was collected and immediately assayed. NPT was measured with Ellman's reagent (Devos et al., 1992). Briefly, 300 μL of the supernatant was added to 1.2 mL 0.1 M K-phosphate buffer (pH 7.6). After obtaining a stable absorbance at 412 nm, 25 μL of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) solution (6 mM DTNB dissolved in 5 mM EDTA and 0.1 M phosphate buffer solution (pH 7.6) was added and the increase in absorbance at 412 nm was read. GSH was assayed by the GSSG-recycling method according to Devos et al. (1992).

2.7. Antioxidant enzymes assays

Antioxidant enzymes were determined according to the methods described by Jin et al. (2008). Root 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 pre-chilled mortar and pestle. The homogenate was centrifuged for 20 min at 12 000g at 4 °C and the supernatant obtained was used for enzyme analysis. An aliquot of the extract was used to determine the enzymes contents. The activities of SOD isoenzymes were determined according to Xu et al. (2010). The absorbance was recorded at 560 nm. One unit of SOD activity was defined as the amount of enzyme required to inhibit the reduction rate of NBT by 50%. Catalase (CAT) 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 ($E = 39.4 \text{ mM}^{-1} \text{ cm}^{-1}$) was recorded. The glutathione peroxidase (G-POD) activity was assayed in a 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 ($E = 26.6 \text{ mM}^{-1} \text{ cm}^{-1}$).

2.8. Transmission electron microscopy

Fresh root tips (about 1–3 mm in length) with different treatments were selected and 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 microscope (JEOL TEM-1200EX, Japan) at an accelerating voltage of 60.0 kV.

2.9. Statistical analysis of data

All data were statistically analyzed using the SPSS package (Version #11.0), analysis of variance (ANOVA) was performed on the data sets, with the mean and SE of each treatment calculated.

3. Results

3.1. Root growth and Cd concentration in root tips

Inhibition of root elongation is usually the first visual symptom of Cd toxicity in plants. Treatment of roots with 400 μM Cd for 24 h had a significantly inhibitory effect on the root growth of *S. alfredii* ($P < 0.05$), Fig. 1A. The addition of Ca to the Cd medium significantly improved root elongation while the application of Ca separately in the culture medium did not notably affect root growth (Fig. 1A). Calcium addition to medium significantly increased Ca content ($P < 0.05$, Fig. 1B) and reduced Cd accumulation by 42.2% ($P < 0.05$, Fig. 1C) in root tips of *S. alfredii*.

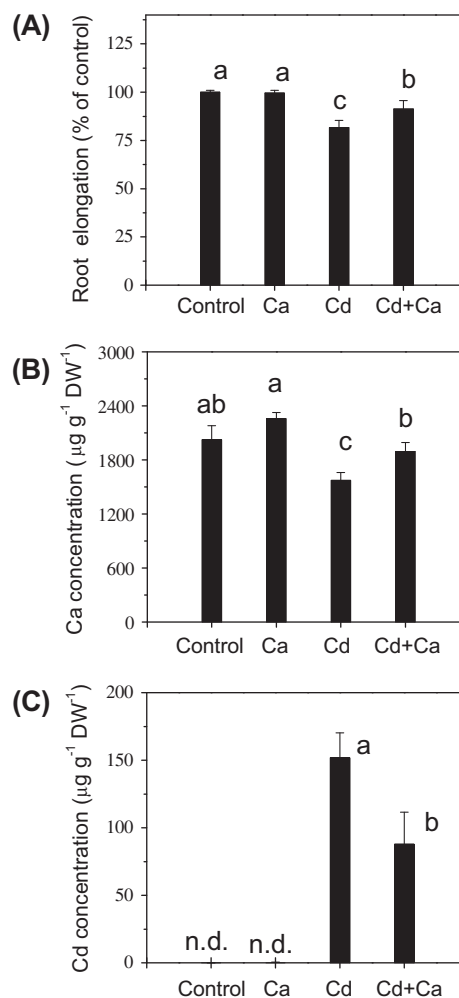


Fig. 1. Cadmium concentration (A) and root elongation inhibition (B) in roots of *S. alfredii*. Seedlings growing in nutrient solution were exposed to four treatments (1) control, (2) 6.0 mM Ca, (3) 400 μM Cd, and (4) 6.0 mM Ca + 400 μM Cd (pH 5.8) for 24 h. Inhibition of root elongation (percentage of control) was calculated as $(B/A) \times 100$, where A was the root length elongated in the control plants, and B was the root length in treated plants. All data show the means \pm SE of three replicates. n.d., not detected.

3.2. Loss of plasma membrane integrity and lipid peroxidation

The effect of Cd exposure on plasma membrane integrity and lipid peroxidation in roots of *S. alfredii*, was determined with histochemical staining with Evans blue (Fig. 2A) and Schiff's reagent (Fig. 2B). The roots of *S. alfredii* treated with Cd alone were heavily stained by both reagents while those treated with supplemental Ca had significantly lighter staining.

Quantitative determination of Evans blue accumulation indicated that the loss of plasma membrane integrity in root tips of *S. alfredii* was reduced significantly ($P < 0.05$) in the presence of Ca in Cd medium. Calcium addition to the treatment medium resulted in Evans blue accumulation 1.89 times that of the control, as compared to 3.15 times of control in the absence of Ca (Fig. 2C). Quantitative determination of lipid peroxidation in root tips of the plants was measured as the content of MDA, a thiobarbituric acid reactive metabolite. MDA content increased significantly in root tips of *S. alfredii* when the plants were exposed to 400 μM Cd, while the effect of Cd was significantly alleviated by addition of Ca in the medium (Fig. 2D, $P < 0.05$).

3.3. Reactive oxygen species (H_2O_2 and O_2^-)

Cd-induced oxidative damage in plant cells has been linked to the enhanced production of reactive oxygen species (ROS) (Gechev and Hille, 2005; Zhu et al., 2005). The concentration of both H_2O_2 and O_2^- in root tips of *S. alfredii* treated with Cd increased significantly ($P < 0.05$), as compared with the controls (Fig. 3). Application of Ca separately in the culture solution in the absence of Cd did not notably affect ROS productions in roots of *S. alfredii* how-

ever, addition of Ca to the Cd medium significantly reduced the production of O_2^- ($P < 0.05$), and there was a non-significant reduction in H_2O_2 accumulation in root tips of *S. alfredii*.

To visualize H_2O_2 and O_2^- production, carboxy- H_2DCFDA (Freeman et al., 2004) (Fig. 3C) and dihydroethidium (DHE) (Yamamoto et al., 2002) (Fig. 3D) was used, respectively. The specificity of both probes was checked by using specific ROS scavengers (Rodriguez-Serrano et al., 2006), ASC for H_2O_2 , and TMP for O_2^- (data not shown). Under exposure of 400 μM Cd, the overproduction of both H_2O_2 and O_2^- was observed *in vivo* in root tips by fluorescence imaging. However, the production of both H_2O_2 and O_2^- were prevented by exogenous Ca to the Cd medium. A slight fluorescence of H_2O_2 , but no fluorescence of O_2^- , were observed in the root tips from the control plants, both with or without application of exogenous Ca (Fig. 3C and D).

3.4. Antioxidative enzymes (SOD, CAT, G-POD) and antioxidants (GSH and NPT)

Regulation of ROS levels can be achieved by the antioxidative system composed of enzymatic scavengers such as SOD, CAT and G-POD (Schutzendubel and Polle, 2002). As shown in Table 1, total activities of both SOD and CAT increased significantly upon the exposure to 400 μM Cd ($P < 0.05$). However, SOD and CAT activities were notably decreased in the roots of *S. alfredii* treated with Cd when Ca was present ($P < 0.05$). Analysis of SOD isoenzymes indicated that addition of Ca significantly decreased Cd-induced FeSOD activity ($P < 0.05$), but has no such effect on either MnSOD or Cu/ZnSOD (Table 1). Treatment with Cd strongly inhibited activities of G-POD in the roots of *S. alfredii* and enzyme activities

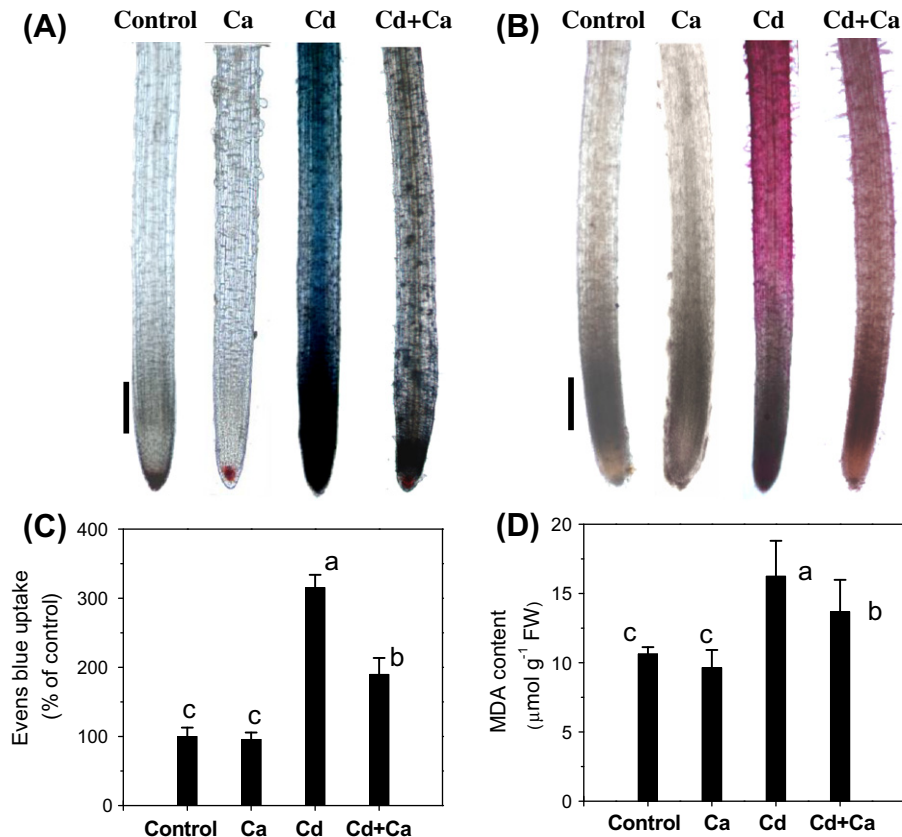


Fig. 2. Cadmium-induced loss of plasma membrane integrity and lipid peroxidation in the root tips of *S. alfredii*. Seedlings growing in nutrient solution were exposed to four treatments (1) control, (2) 6.0 mM Ca, (3) 400 μM Cd, and (4) 6.0 mM Ca + 400 μM Cd (pH 5.8) for 24 h. [A] microscope images of Evans blue uptake in roots tips, [B] microscope images of lipid peroxidation in roots tips of *S. alfredii*; [C] Evans blue uptake (% of control) in roots tips; [D] MDA content in roots tips. Bar represents 1 mm; All data show the means \pm SE of three replicates.

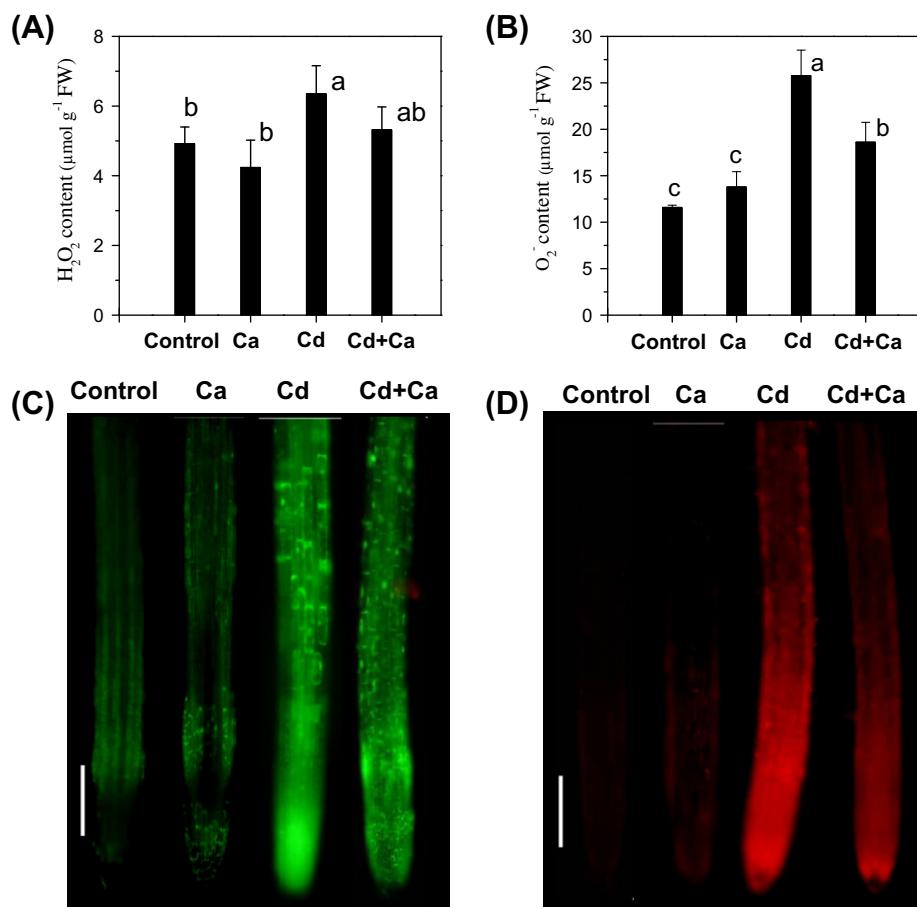


Fig. 3. Cd-induced reactive oxygen species in the root tips of *S. alfredii*. Seedlings growing in nutrient solution were exposed to four treatments (1) control, (2) 6.0 mM Ca, (3) 400 μM Cd, and (4) 6.0 mM Ca + 400 μM Cd (pH 5.8) for 24 h. [A] H₂O₂ contents in root tips of *S. alfredii*, and [B] O₂⁻ contents in root tips of *S. alfredii*, data show the means ± SE of three replicates; [C] and [D] represent fluorescence imaging of H₂O₂ and O₂⁻ production in the root tips of *S. alfredii*, respectively. Bar represents 1 mm.

Table 1

Response of superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (G-POD), non-protein thiols (NPT), and glutathione (GSH) in roots tips of *S. alfredii*.

Enzyme	Treatments			
	Control	+Ca	+Cd	+Ca + Cd
<i>Unit mg⁻¹ protein</i>				
TotalSOD	39.6 ± 3.6 c	40.2 ± 6.8 c	102.7 ± 7.2 a	85.1 ± 4.8 b
MnSOD	16.5 ± 2.1 b	17.4 ± 1.7 b	35.4 ± 4.1 a	36.7 ± 3.4 a
FeSOD	7.2 ± 1.5 c	9.3 ± 2.8 c	31.1 ± 3.6 a	17.7 ± 2.0 b
Cu/ZnSOD	16.0 ± 2.3 b	13.5 ± 1.8 b	36.2 ± 4.9 a	30.8 ± 2.4 a
<i>μmol mg⁻¹ protein</i>				
CAT	0.32 ± 0.03 c	0.33 ± 0.02 c	0.46 ± 0.04 a	0.39 ± 0.02 b
G-POD	42.8 ± 3.9 a	38.8 ± 2.8 ab	32.9 ± 2.2 b	37.8 ± 3.7 ab
<i>μmol g⁻¹ FW</i>				
NPT	0.52 ± 0.07 c	0.63 ± 0.10 c	1.50 ± 0.18 a	0.86 ± 0.12 b
GSH	0.20 ± 0.02 c	0.22 ± 0.03 c	0.32 ± 0.05 b	0.42 ± 0.04 a

Seedlings growing in nutrient solution were exposed to four treatments (1) control, (2) 6.0 mM Ca, (3) 400 μM Cd, and (4) 6.0 mM Ca + 400 μM Cd (pH 5.8) for 24 h. All data show the means ± SE of three replicates. Different letters in the same line indicate significance at $P < 0.05$.

largely recovered with the addition of Ca to the Cd-stressed roots (Table 1).

Levels of the most common non-enzymatic antioxidants (NPT and GSH) were measured in roots of *S. alfredii*. Treatment of seedlings with 400 μM Cd for 24 h significantly increased the accumulation of both NPT and GSH in the roots of *S. alfredii* (Table 1). Application of exogenous Ca in the Cd medium significantly

reduced the Cd-induced overproduction of NPT though levels were not reduced to those in the unstressed control plants (Table 1). Interestingly Ca supplementation in the Cd treatment increased the accumulation of GSH (Table 1).

3.5. Transmission electron microscopy

A visual comparison of representative cells viewed under transmission electron microscopy suggests that Cd causes significant ultrastructural damage to root tip cells. Under control conditions, the root cells of *S. alfredii* (Fig. 4A) exhibited a qualitatively 'richer' cytoplasm with abundant organelles, small vacuoles, smooth and continuous cell walls, with large nucleus and nucleolus. Clearly delineated cell walls, numerous endoplasmic reticulum, mitochondria and plasmodesmata were visible (Fig. 4A). Visual comparison with cells from Cd treated roots suggested an adverse effect on cell quality and ultrastructural integrity (Fig. 4B). Calcium supplementation considerably alleviated visual cell damage (Fig. 4C). At 400 μM Cd, the meristematic cells of roots of *S. alfredii* exhibited sparse cytoplasm and organelles including some swollen mitochondria, generally larger and more abundant vacuoles, and indistinct nucleus and nucleolus (Fig. 4B). Cell walls appeared less smooth and had many attachments, and possibly damaged membrane systems (Fig. 4B). For the seedlings exposed to 400 μM Cd with addition of Ca, root cells had better visual integrity including rich endoplasmic reticulum, plasma membrane and cell walls that resembled control cells and clear nucleus and nucleolus which were however, smaller than in control plants (Fig. 4C).

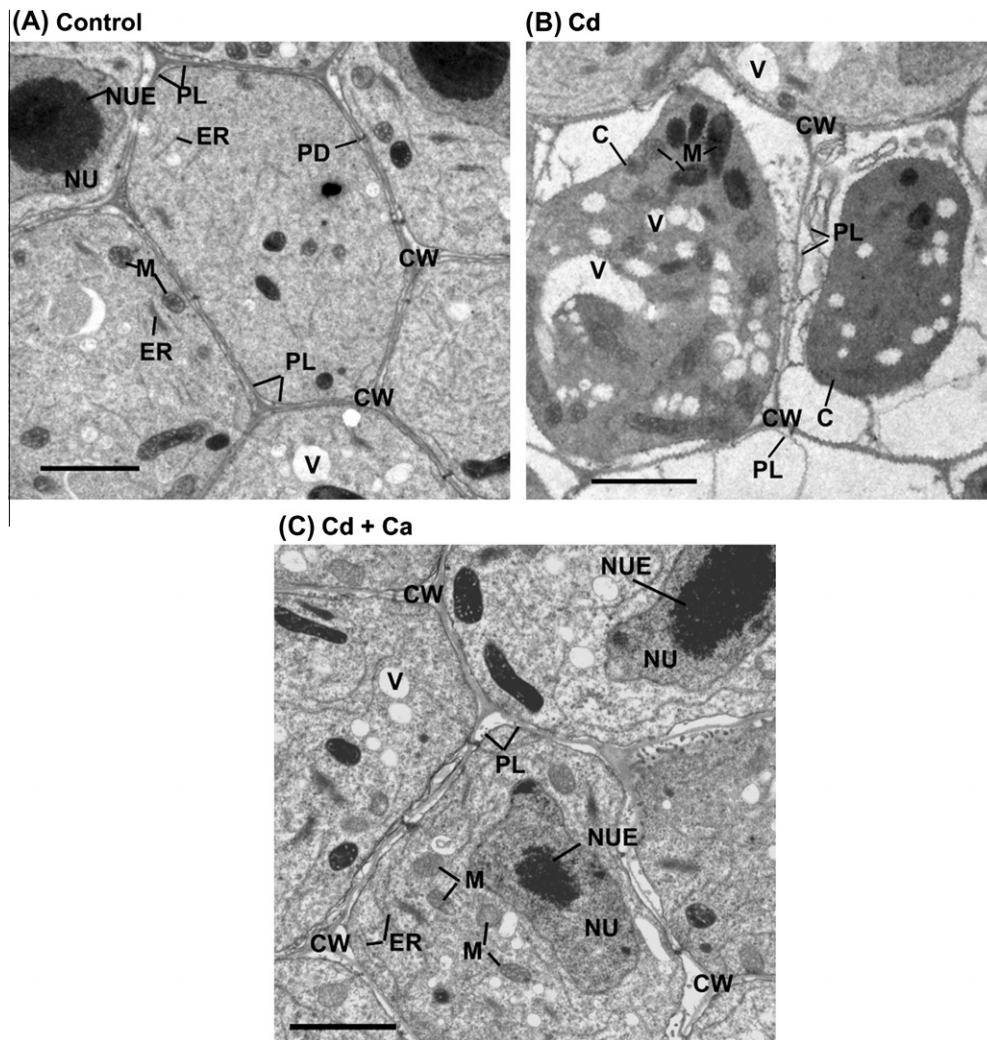


Fig. 4. Transmission electron micrograph of root meristematic cells from *S. alfredii*. Seedlings growing in nutrient solution were exposed to four treatments (1) control, (2) 6.0 mM Ca, (3) 400 μ M Cd, and (4) 6.0 mM Ca + 400 μ M Cd (pH 5.8) for 24 h. Bars = 2 μ m. Labels: C, cytoplasm; CW, cell wall; ER, endoplasmic reticulum; M, mitochondria; NU, nucleus; NUE, nucleolus; PD, plasmodesmata; PL, plasma lemma; V, vacuole.

4. Discussion

In the presence of rhizotoxic levels of mineral toxicants, supplementation of the medium with higher levels of Ca has been shown to alleviate growth inhibition (Kinraide, 1998; Rodriguez-Serrano et al., 2009; Wang and Song, 2009). In the present study, we confirmed that supplementation of the medium with higher levels of Ca alleviates growth inhibition and oxidative stress of roots of *S. alfredii* under the exposure of rhizotoxic levels of Cd.

Several mechanisms for the Ca alleviation of mineral toxicities have been suggested (Kinraide, 1998), one of which is the restoration of toxicant-displaced Ca in plant organelles and cell surfaces (Kinraide, 1998; Wang and Song, 2009). It has been reported that Cd application resulted in a decrease of Ca content in various plant species (Gussarsson et al., 1996; Sandalio et al., 2001; Rodriguez-Serrano et al., 2009; Wang and Song, 2009). The results of the present investigation confirm this observation as exposure to 400 μ M Cd significantly reduced Ca content in the root tips of *S. alfredii*, as compared with the controls, and the application of Ca in the solution significantly reversed this effect (Fig. 1B). In the present study, Ca application reduced the Cd content in the roots of *S. alfredii* seedlings (Fig. 1C). Similar ameliorative effects of exogenous Ca on Cd accumulation in plants has been reported in *Trifolium repens*

L. seedlings (Wang and Song, 2009). These results suggest that Ca competes with Cd for uptake through Ca transporters (Lu et al., 2008, 2010).

Calcium and Cd likely compete not only for the root plasma membrane transporters but also for intracellular binding sites within plants and in Ca dependent metabolic function (Rodriguez-Serrano et al., 2009; Lu et al., 2010). Calcium is involved in the regulation of plant cell metabolism and signal transduction and modulates cellular processes by binding proteins such as calmodulin (CaM), which in turn regulates the activity of target proteins (Rodriguez-Serrano et al., 2009). In this study, we provide evidence that Ca supplementation is able to reduce Cd-induced oxidative stress in roots of *S. alfredii* and to reduce lipid peroxidation and loss of plasma membrane integrity (Fig. 2), prevent ROS (H_2O_2 and O_2^-) over production (Fig. 3), and maintain ultrastructural integrity of root cells (Fig. 4) in the presence of otherwise toxic levels of Cd.

Plants have well-developed defense systems to maintain metabolically compatible levels of H_2O_2 and O_2^- (Alscher et al., 2002). The enzymes SOD and CAT are involved in the detoxification of O_2^- and H_2O_2 , respectively. Within a cell, SOD constitutes the first line of defense against O_2^- by rapidly converting it to O_2 and H_2O_2 (Alscher et al., 2002). It has been suggested that external Ca could

alleviate oxidative stress by improving antioxidant enzyme (SOD and CAT) activity in Cd-stressed plants (Rodriguez-Serrano et al., 2009; Wang and Song, 2009). In this study, however, a reduced activity of SOD and CAT was detected in the Ca-treated Cd challenged roots (Table 1), suggesting that Ca depressed generation of ROS that trigger the SOD, most likely FeSOD, and CAT activity. This suggests that some other mechanism may account for the Ca alleviation of ROS overproduction in *S. alfredii*. GSH is well characterized antioxidant that plays a prominent role in defense system of plants (Clemens, 2006). In these experiments we observed an increase in GSH concentrations in the Cd treated roots and observed a further stimulation of GSH production with the application of supplemental Ca (Table 1), suggesting that Ca alleviation for antioxidative stress of Cd is probably related to GSH biosynthesis. A stimulation of GSH biosynthesis with Cd exposure has been observed previously in this species (Sun et al., 2007; Jin et al., 2008). The exact mechanism of Cd-induced GSH biosynthesis and Ca alleviation of Cd toxicity in the hyperaccumulator *S. alfredii* needs to be further investigated.

5. Conclusion

The results of this study demonstrate that exogenous Ca alleviated Cd-induced growth inhibition and decreased Cd concentration and increased Ca concentration in roots of *S. alfredii*. Lipid peroxidation and loss of plasma membrane integrity, ROS (H_2O_2 and O_2^-) over production, as well as ultrastructural changes in root cells induced by Cd were largely reversed by Ca supplementation. Calcium application considerably alleviated the Cd effects on antioxidant enzymes and NPT, but significantly increased GSH biosynthesis. These results suggest that Ca protects the roots of *S. alfredii* against Cd toxicity by restoration of Cd-displaced Ca, alleviation of the metal induced oxidative stress, as well as promoted GSH biosynthesis.

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References

Alscher, R.G., Erturk, N., Heath, L.S., 2002. Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. *J. Exp. Bot.* 53, 1331–1341.

Baker, A.J.M., McGrath, S.P., Reeves, R.D., Smith, J.A.C., 2000. Metal hyperaccumulator plants: a review of the ecology and physiology of a biological resource for phytoremediation of metal-polluted soils. *Phytoremediation Contamin. Soil Water* 85, 107.

Bertin, G., Averbeck, D., 2006. Cadmium: cellular effects, modifications of biomolecules, modulation of DNA repair and genotoxic consequences (a review). *Biochimie* 88, 1549–1559.

Clemens, S., 2006. Toxic metal accumulation, responses to exposure and mechanisms of tolerance in plants. *Biochimie* 88, 1707–1719.

Clemens, S., Antosiewicz, D.M., Ward, J.M., Schachtman, D.P., Schroeder, J.L., 1998. The plant cDNA *LCT1* mediates the uptake of calcium and cadmium in yeast. *Proc. Natl. Acad. Sci. USA* 95, 12043–12048.

Das, P., Samantaray, S., Rout, G.R., 1997. Studies on cadmium toxicity in plants: a review. *Environ. Pollut.* 98, 29–36.

Devos, C.H.R., Vonk, M.J., Vooijs, R., Schat, H., 1992. Glutathione depletion due to copper-induced phytochelatin synthesis causes oxidative stress in *silene cucubalus*. *Plant Physiol.* 98, 853–858.

Dixit, V., Pandey, V., Shyam, R., 2001. Differential antioxidative responses to cadmium in roots and leaves of pea (*Pisum sativum* L. Cv. Azad). *J. Exp. Bot.* 52, 1101–1109.

Freeman, J.L., Persans, M.W., Nieman, K., Albrecht, C., Peer, W., Pickering, I.J., Salt, D.E., 2004. Increased glutathione biosynthesis plays a role in nickel tolerance in *Thlaspi* nickel hyperaccumulators. *Plant Cell* 16, 2176–2191.

Garnier, L., Simon-Plas, F., Thuleau, P., Agnel, J.P., Blein, J.P., Ranjeva, R., Montillet, J.L., 2006. Cadmium affects tobacco cells by a series of three waves of reactive oxygen species that contribute to cytotoxicity. *Plant Cell Environ.* 29, 1956–1969.

Gechev, T.S., Hille, J., 2005. Hydrogen peroxide as a signal controlling plant programmed cell death. *J. Cell Biol.* 168, 17–20.

Gussarsson, M., Asp, H., Adalsteinsson, S., Jensen, P., 1996. Enhancement of cadmium effects on growth and nutrient composition of birch (*Betula pendula*) by buthionine sulphoximine (BSO). *J. Exp. Bot.* 47, 211–215.

Huang, H.G., Li, T.X., Tian, S.K., Gupta, D.K., Zhang, X.Z., Yang, X.E., 2008. Role of EDTA in alleviating lead toxicity in accumulator species of *Sedum alfredii* H. *Bioresour. Technol.* 99, 6088–6096.

Jin, X.F., Yang, X.E., Islam, M., Liu, D., Mahmood, Q., 2008. Effects of cadmium on ultrastructure and antioxidative defense system in hyperaccumulator and non-hyperaccumulator ecotypes of *Sedum alfredii* Hance. *J. Hazard. Mater.* 156, 387–397.

Kinraide, T.B., 1998. Three mechanisms for the calcium alleviation of mineral toxicities. *Plant Physiol.* 118, 513–520.

Lu, L.L., Tian, S.K., Yang, X.E., Wang, X.C., Brown, P., Li, T.Q., He, Z.L., 2008. Enhanced root-to-shoot translocation of cadmium in the hyperaccumulating ecotype of *Sedum alfredii*. *J. Exp. Bot.* 59, 3203–3213.

Lu, L.L., Tian, S.K., Zhang, M., Zhang, J., Yang, X.E., Jiang, H., 2010. The role of Ca pathway in Cd uptake and translocation by the hyperaccumulator *Sedum alfredii*. *J. Hazard. Mater.* 183, 22–28.

Olmos, E., Martinez-Solano, J.R., Piqueras, A., Hellin, E., 2003. Early steps in the oxidative burst induced by cadmium in cultured tobacco cells (BY-2 line). *J. Exp. Bot.* 54, 291–301.

Perfus-Barbeoch, L., Leonhardt, N., Vavasseur, A., Forestier, C., 2002. Heavy metal toxicity: cadmium permeates through calcium channels and disturbs the plant water status. *Plant J.* 32, 539–548.

Pilon-Smits, E., 2005. Phytoremediation. *Ann. Rev. Plant Biol.* 56, 15–39.

Pinto, A.P., Mota, A.M., de Varennes, A., Pinto, F.C., 2004. Influence of organic matter on the uptake of cadmium, zinc, copper and iron by sorghum plants. *Sci. Total Environ.* 326, 239–247.

Rodriguez-Serrano, M., Romero-Puertas, M.C., Pazmino, D.M., Testillano, P.S., Risueno, M.C., del Rio, L.A., Sandalio, L.M., 2009. Cellular response of pea plants to cadmium toxicity: cross talk between reactive oxygen species, nitric oxide, and calcium. *Plant Physiol.* 150, 229–243.

Rodriguez-Serrano, M., Romero-Puertas, M.C., Zabalza, A., Corpas, F.J., Gomez, M., Del Rio, L.A., Sandalio, L.M., 2006. Cadmium effect on oxidative metabolism of pea (*Pisum sativum* L.) roots, imaging of reactive oxygen species and nitric oxide accumulation in vivo. *Plant Cell Environ.* 29, 1532–1544.

Romero-Puertas, M.C., Rodriguez-Serrano, M., Corpas, F.J., Gomez, M., Del Rio, L.A., Sandalio, L.M., 2004. Cadmium-induced subcellular accumulation of O_2^- and H_2O_2 in pea leaves. *Plant Cell Environ.* 27, 1122–1134.

Sandalio, L.M., Dalurzo, H.C., Gomez, M., Romero-Puertas, M.C., del Rio, L.A., 2001. Cadmium-induced changes in the growth and oxidative metabolism of pea plants. *J. Exp. Bot.* 52, 2115–2126.

Schutzenbeutel, A., Polle, A., 2002. Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization. *J. Exp. Bot.* 53, 1351–1365.

Schutzenbeutel, A., Schwanz, P., Teichmann, T., Gross, K., Langenfeld-Heyser, R., Godbold, D.L., Polle, A., 2001. Cadmium-induced changes in antioxidative systems, hydrogen peroxide content, and differentiation in Scots pine roots. *Plant Physiol.* 127, 887–898.

Sun, Q., Ye, Z.H., Wang, X.R., Wong, M.H., 2007. Cadmium hyperaccumulation leads to an increase of glutathione rather than phytochelatin in the cadmium hyperaccumulator *Sedum alfredii*. *J. Plant Physiol.* 164, 1489–1498.

Tian, S.K., Lu, L.L., Yang, X.E., Labavitch, J.M., Huang, Y.Y., Brown, P., 2009. Stem and leaf sequestration of zinc at the cellular level in the hyperaccumulator *Sedum alfredii*. *New Phytol.* 182, 116–126.

Tian, S.K., Lu, L.L., Yang, X.E., Webb, S.M., Du, Y.H., Brown, P.H., 2010. Spatial imaging and speciation of lead in the accumulator plant *Sedum alfredii* by microscopically focused synchrotron X-ray investigation. *Environ. Sci. Technol.* 44, 5920–5926.

Verbruggen, N., Hermans, C., Schat, H., 2009. Molecular mechanisms of metal hyperaccumulation in plants. *New Phytol.* 181, 759–776.

Wang, C.Q., Song, H., 2009. Calcium protects *Trifolium repens* L. Seedlings against cadmium stress. *Plant Cell Rep.* 28, 1341–1349.

Welch, R.M., Norvell, W.A., 1999. Mechanisms of cadmium uptake, translocation and deposition in plants. In: McLaughlin, M.J., Singh, B.R. (Eds.), *Cadmium in Soils and Plants*. Kluwer Academic Publishers, Dordrecht, pp. 125–150.

White, P.J., 2000. Calcium channels in higher plants. *Biochim. Biophys. Acta-Biomebr.* 1465, 171–189.

Xu, J., Yin, H.X., Li, Y.L., Liu, X.J., 2010. Nitric oxide is associated with long-term zinc tolerance in *Solanum nigrum*. *Plant Physiol.* 154, 1319–1334.

Yamamoto, Y., Kobayashi, Y., Devi, S.R., Rikiishi, S., Matsumoto, H., 2002. Aluminum toxicity is associated with mitochondrial dysfunction and the production of reactive oxygen species in plant cells. *Plant Physiol.* 128, 63–72.

Yamamoto, Y., Kobayashi, Y., Matsumoto, H., 2001. Lipid peroxidation is an early symptom triggered by aluminum, but not the primary cause of elongation inhibition in pea roots. *Plant Physiol.* 125, 199–208.

Zhu, R.K., Macfie, S.M., Ding, Z.F., 2005. Cadmium-induced plant stress investigated by scanning electrochemical microscopy. *J. Exp. Bot.* 56, 2831–2838.