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Differential generation of hydrogen peroxide upon exposure to zinc and cadmium in the hyperaccumulating plant specie (*Sedum alfredii* Hance)*

Yue-en CHAO, Min ZHANG, Sheng-ke TIAN, Ling-li LU, Xiao-e YANG†‡

(*MOE Key Laboratory of Environmental Remediation and Ecosystem Health, College of Environmental and Resource Sciences, Zhejiang University, Hangzhou 310029, China*)

†E-mail: xyang@zju.edu.cn

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Abstract: *Sedum alfredii* Hance has been identified as zinc (Zn) and cadmium (Cd) co-hyperaccumulator. In this paper the relationships of Zn or Cd hyperaccumulation to the generation and the role of H_2O_2 in *Sedum alfredii* H. were examined. The results show that Zn and Cd contents in the shoots of *Sedum alfredii* H. treated with 1000 μmol/L Zn^{2+} and/or 200 μmol/L Cd^{2+} increased linearly within 15 d. Contents of total S, glutathione (GSH) and H_2O_2 in shoots also increased within 15 d, and then decreased. Total S and GSH contents in shoots were higher under Cd^{2+} treatment than under Zn^{2+} treatment. However, reverse trends of H_2O_2 content in shoots were obtained, in which much higher H_2O_2 content was observed in Zn^{2+}-treated shoots than in Cd^{2+}-treated shoots. Similarly, the microscopic imaging of H_2O_2 accumulation in leaves using H_2O_2 probe technique showed that much higher H_2O_2 accumulation was observed in the Zn^{2+}-treated leaf than in the Cd^{2+}-treated one. These results suggest that there are different responses in the generation of H_2O_2 upon exposure to Zn^{2+} and Cd^{2+} for the hyperaccumulator *Sedum alfredii* H. And this is the first report that the generation of H_2O_2 may play an important role in Zn hyperaccumulation in the leaves. Our results also imply that GSH may play an important role in the detoxification of dissociated Zn/Cd and the generation of H_2O_2.

Key words: Hydrogen peroxide (H_2O_2), Glutathione (GSH), *Sedum alfredii* Hance, Zinc (Zn), Cadmium (Cd), Hyperaccumulator
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INTRODUCTION

Successful implementation of phytoremediation depends on identification of suitable plant species that are not only capable of growing on soils containing high levels of metals, but also accumulating much higher concentrations of metals in their shoots than normal species do. To date, many plant species, usually those found in heavy metal contaminated areas, have been identified as hyperaccumulator, i.e., they have the ability to accumulate unusually high concentrations of heavy metals without impact on their growth and development (Baker and Brooks, 1989; Xiong, 1997). However, most hyperaccumulators identified so far are not suitable for phytoremediation applications (in the field) due to slow growth and production of small biomass of shoots (Salt *et al*., 1998; Shen *et al*., 2002).

Deep understanding of physiological mechanisms of metal tolerance and hyperaccumulation is necessary for exploring heavy metal hyperaccumulating plant species. In recent years, numeral researches have been reported that in response to toxicity of heavy metals plants could produce many kinds of reactive oxygen species, antioxidant enzyme (Rodriguez-Serrano *et al*., 2006; Stohs and Bagchi, 1995), glutathione (GSH) (Howden *et al*., 1995; Nocito *et al*., 2006; Sun *et al*., 2005), phytochelatins

‡ Corresponding author
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(PCs) (Cobbett, 2000; Grill *et al*., 1985; Mishra *et al*., 2006; Stolt *et al*., 2003), and other complex ligands (de la Rosa *et al*., 2004; Isaure *et al*., 2006; Zhang *et al*., 2004). The detoxification ways of heavy metals in plant tissues include speciation of metal with organic ligands and elimination of oxidative stress through the function of antioxidant reaction. Hydrogen peroxide (H_2O_2), a kind of natural reactive oxygen species, is generated with various environmental and developmental stimuli. It has been approved to act as a new signal molecule and played important roles in many physiological process such as cell expansion, development, stomatal closure and programmed cell death (Bienert *et al*., 2006; Cheng and Song, 2006; Laloi *et al*., 2004). GSH could eliminate toxicity of heavy metals in the cells by chelating with itself -SH in addition to clean up substance of active oxygen (Freeman *et al*., 2004; Jumarie *et al*., 2001; Mendoza-Cozatl *et al*., 2005). It is also the main antioxidation matter which could clean H_2O_2 under the action of GSH peroxidase in cells (Aravind and Prasad, 2005; Elia *et al*., 2003).

Sedum alfredii Hance (*S. alfredii* H.) has been identified as a new Zn and Cd co-hyperaccumulator plant species native to China (Yang *et al*., 2002; 2004). It has an extraordinary ability to tolerate and accumulate high concentrations of Zn and Cd, with the characteristics of large biomass, fast growth speed, asexual propagation and perenniality (Ni *et al*., 2004; Yang *et al*., 2002; 2004; 2006). Therefore, it is an ideal plant for studying mechanisms which are responsible for hyperaccumulation and phytoremediation practice. Comparison between the hyperaccumulating ecotype (HE) and the non-hyperaccumulating ecotype (NHE) of *S. alfredii* H. under nutrient solution culture conditions showed that Zn concentration was over 19-fold higher in leaves and stems of the HE than that of the NHE. When grown in media contained Zn level of 50~1000 μmol/L, Zn uptake by shoots of the HE was over 20-fold higher than that of the NHE (Yang *et al*., 2001; 2004). *S. alfredii* could also tolerate Cd level up to 200 μmol/L in nutrient solution, and the Cd content in leaves reached a maximum of approximately 9000 mg/kg (Yang *et al*., 2004). The optimal growth of the hyperaccumulating species of *S. alfredii* was found at external Zn level of 500 μmol/L, and Cd level of 50 μmol/L (Yang *et al*., 2002; 2004).

However, the mechanisms behind tolerance and hyperaccumulation of Zn and Cd in *S. alfredii* H. are still not fully understood. To date, the changes of the contents of H_2O_2, GSH and the relations to heavy metals accumulation in *S. alfredii* H. have not been revealed. In this paper, the relationships of Zn and/or Cd hyperaccumulation to the generation of H_2O_2 in *S. alfredii* H. were examined, and the roles of GSH in detoxification of Zn/Cd in the hyperaccumulator were discussed.

MATERIALS AND METHODS

Plant culture

The HE of *S. alfredii* H. was obtained from an old Pb/Zn mine area in Zhejiang Province in China. Healthy and uniform shoots of *S. alfredii* H. were chosen and grown for 2 weeks in the basal nutrient solution, the composition of nutrients in solution according to description of Yang *et al*.(2004). The pH of nutrient solution was adjusted daily to 5.5 with NaOH or 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 4 d. Seedlings of *S. alfredii* H. were precultured for 20 d (for the initiation of new roots) prior to exposure. We choose the metal treatment levels of 1000 μmol/L Zn^{2+} as $ZnNO_3$ and 200 μmol/L Cd^{2+} as $CdCl_2$, because our repeated experiments showed that shoot growth of the hyperaccumulator *S. alfredi* H. did not decline when exposed to 1000 μmol/L Zn^{2+} and/or 200 μmol/L Cd^{2+} for more than 15 d (Yang *et al*., 2002; 2004). That means that treatment with 1000 μmol/L Zn^{2+} or 200 μmol/L Cd^{2+} produced similar degree of metal toxic stress to the hyperaccumulator *S. alfredi* H. as they are the maximum tolerable levels. Plants were harvested after exposure to metal treatments every 5 d. The whole treated time was 25 d.

Zinc, cadmium and total sulphur determination

The dried plant materials were then ground using a stainless steel mill and passed through a 0.25-mm sieve for elemental analysis. Dry plant samples (0.1 g) of each treatment were digested with HNO_3-$HClO_4$ (v/v, 4/1) at 180 °C. The digest solution was trans-

ferred to a 1000-ml volumetric flask, made up to volume and filtered. Contents of Zn, Cd and total S in the filtrate were analyzed using ICP-MS (Agilent 7500a, USA).

GSH and H_2O_2 analysis

Portions of 1 g fresh sample of shoots frozen in liquid nitrogen were ground. The GSH and H_2O_2 contents were analyzed by chemical colorimetry as specifically described in the GSH and the H_2O_2 Assay Kits (Nanjing Jiancheng Bioengineering Institute, China), respectively. The GSH and H_2O_2 levels were calculated from the standard curve.

Microscopic imaging of H_2O_2 accumulation in leaves

After plants were grown with and without 1000 μmol/L Zn^{2+} or 200 μmol/L Cd^{2+} for 15 d, leaf segments of the plants were incubated with 10 μmol/L carboxy-H_2DCFDA (Molecular Probes, Eugene, OR, USA) for 15 min in darkness and then were rinsed. A Nikon Eclipse 3000 epifluorescent microscope (Melville, NY, USA) equipped with a green fluorescent protein filter (excitation 450 to 490 nm, emission 500 to 530 nm) was used for fluorescence detection. Exposure time was equal for all samples. Auto fluorescence was not observed in unstained controls at the exposure time used. Fluorescence and concurrent differential interference contrast images were taken and captured with a spot camera (Nikon).

RESULTS AND DISCUSSION

Kinetics of Zn and Cd accumulation in shoots

HE of *S. alfredii* H. has been shown to have an extraordinary ability to tolerate and hyperaccumulate Zn and Cd. Our former studies proved that the maximum accumulation of Zn and Cd in shoots of the HE of *S. alfredii* H. occurred at 1000 μmol/L Zn^{2+} and 200 μmol/L Cd^{2+}, respectively (Ni *et al*., 2004; Yang *et al*., 2002). In this study, we observed that the plants of HE *S. alfredii* H. grew healthily and showed no significant toxic symptoms under the treatments of 1000 μmol/L Zn^{2+} and 200 μmol/L Cd^{2+} for 25 d. Both Zn and Cd contents increased in shoots of HE *S. alfredii* H. in response to different Zn^{2+} or Cd^{2+} treatment time. Linearly increase in Zn and Cd contents in the shoots was observed within the treatment time of 20 d, and afterwards the changes of Zn and Cd contents with treatment time were leveled off (Fig.1). After exposure to 1000 μmol/L Zn^{2+} and 200 μmol/L Cd^{2+} for 25 d, accumulation of Zn and Cd in shoots reached the highest at 32280 mg/kg and 9380 mg/kg, respectively. Results indicated the great ability of this plant to take up large amount of Zn and Cd in its shoot parts and the toleration ability for long time toxic treatments. This hyperaccumulator has great potential use in the phytoextraction of heavy metals in polluted soils.

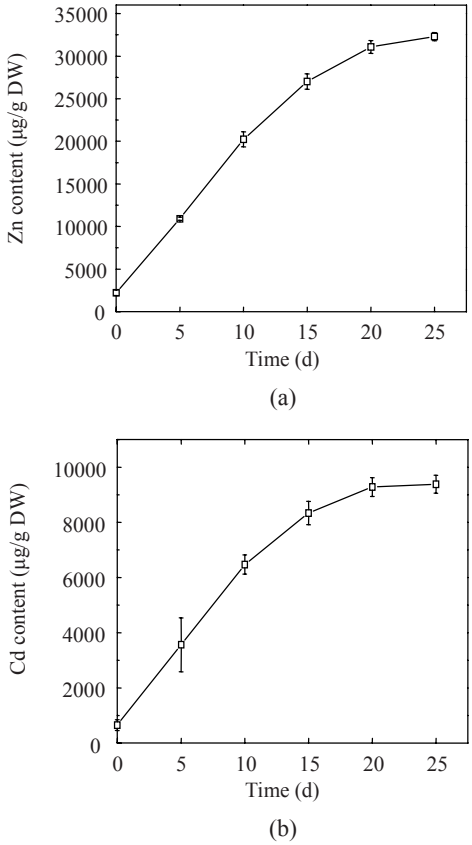

Fig.1 Time-course variations of Zn/Cd accumulation in shoots of *Sedum alfredii* Hance upon exposure to Zn and Cd levels. (a) Zn treatment of 1000 μmol/L; (b) Cd treatment of 200 μmol/L

Kinetics of GSH accumulation in shoots

The detoxification of heavy metals in plant cell through antioxidant system has been investigated, and GSH has been approved to play an important role in detoxifying heavy metals like nickel (Ni) and Cd (Howden *et al*., 1995; Mendoza-Cozatl *et al*., 2005).

Sun *et al*.(2005; 2007) reported that GSH concentration in shoots of *S. alfredii* H. increased with increasing of Zn, Pb and Cd supply levels.

In this study we also found that the GSH content in the shoots of *S. alfredii* H. increased rapidly with metal treatment time within 15 d and then decreased with further advance of treatment time. Shoot GSH content reached as high as 1610.5 μmol/kg FW (fresh weight) after exposure to 200 μmol/L Cd^{2+}, whereas only 277 μmol/kg FW after exposure to 1000 μmol/L Zn^{2+} for 15 d (Fig.2). After treatment for 25 d, GSH content in shoots of *S. alfredii* H. dropped to 1010.5 μmol/kg FW at the Zn treatment, and 98.3 μmol/kg FW at the Cd treatment, respectively, which was approximately equal to the contents with treatment for 5 d. The content of GSH in shoots induced by Cd^{2+} was up to 7 times higher than that induced by Zn^{2+}, indicating that GSH may play more important role in detoxifying Cd than Zn in the hyperaccumulator *Sedum alfredii* H.

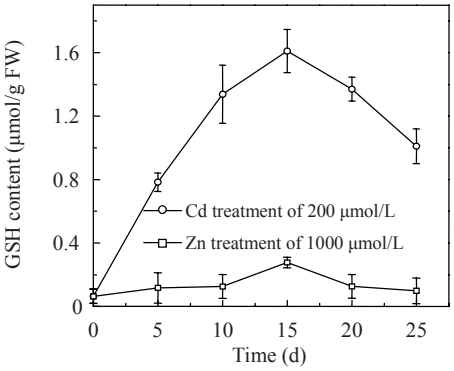

Fig.2 Time-course variations of glutathione (GSH) accumulation in shoots of *Sedum alfredii* Hance upon exposure to Zn and Cd levels

Kinetics of H_2O_2 accumulation in shoots

As a signal in plants, the H_2O_2 could be induced by many environmental stresses and was also an important reactor in the resistance of plants to environmental stresses (Moloi and van der Westhuizen, 2006; Sun *et al*., 2007). The action of H_2O_2 in stress resistance of plants was involved in many regulation processes (Baysal *et al*., 2007; Bright *et al*., 2006; de Pinto *et al*., 2002; Shin and Schachtman, 2004). The accumulation of H_2O_2 could reflect the oxidative stress and the changes of antioxidants in plant.

In this study, we found that the H_2O_2 content in shoots of *S. alfredii* H. increased by treatment of 1000 μmol/L Zn^{2+} and 200 μmol/L Cd^{2+} within 15 d (Fig.3). The H_2O_2 content increased with increasing of treatment time and reached to the highest on Day 15 of treatments, afterwards the H_2O_2 content decreased rapidly with further treatment time, especially in Cd^{2+} treated plants (Fig.3). The content of H_2O_2 with Zn^{2+} treatment was much higher than that with Cd^{2+} treatment. This is probably due to the smaller increase of GSH with Zn^{2+} treatment as compared with Cd^{2+} treatment (Fig.3). These results suggested a close relation between the GSH content and the H_2O_2 content in the shoots of the hyperaccumulator *S. alfredii* H. The results also imply that H_2O_2 may not have toxic effect, but acts as a positive signal for the hyperaccumulation of Zn in *S. alfredii* H. Because both 1000 μmol/L Zn^{2+} and 200 μmol/L Cd^{2+} produced similar extents of toxic stress to the hyperaccumulator *S. alfredii* H., it could be deduced that H_2O_2 may play an important role in Zn hyperaccumulation for *S. alfredii* H. More experimental evidences are needed to verify the relationship of H_2O_2 generation and Zn hyperaccumulation.

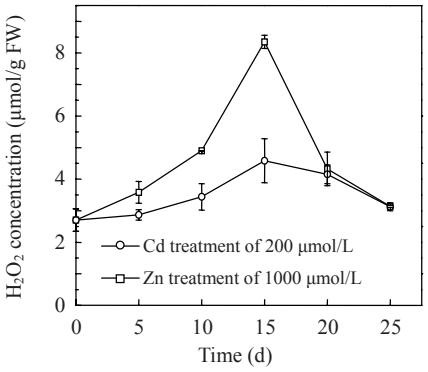

Fig.3 Time-course variations of H_2O_2 content in shoots of *Sedum alfredii* Hance upon exposure to Zn and Cd levels

Cellular distribution of H_2O_2 in leaves

Preferential accumulation of Zn in the leaf epidermis has previously been observed in the Zn hyperaccumulator *Thlaspi caerulescens* (Küpper *et al*., 1999; Vázquez *et al*., 1994). Our studies demonstrated that preferential distribution of Zn and Cd was also observed in epidermal cells of stem or leaf in *S. alfredii* (unpublished data). Cellular distribution of H_2O_2 was investigated using fluorescence probe. The

results showed that high concentration of H_2O_2 was observed within the epidermis cells of the leaves with both Zn^{2+} and Cd^{2+} treatment (Fig.4), and the cellular distribution pattern for H_2O_2 was quite similar to those of Zn and Cd. After exposure to 1000 μmol/L Zn^{2+} or 200 μmol/L Cd^{2+} for 15 d, the highest H_2O_2 concentration was observed in the epidermis cells of the leaf of *S. alfredii*, followed by the vascular bundle (Fig.4). But the accumulation of H_2O_2 under Zn exposure was much higher than that under Cd exposure. However, there was very rare of green fluorescence in the leaves of control plants (without metal treatment). These results indicated that low H_2O_2 concentration was generated in the leaves of *S. alfredii* without any treatments of Zn or Cd (Fig.4). The results imply that H_2O_2 may play a special signal role in Zn hyperaccumulation.

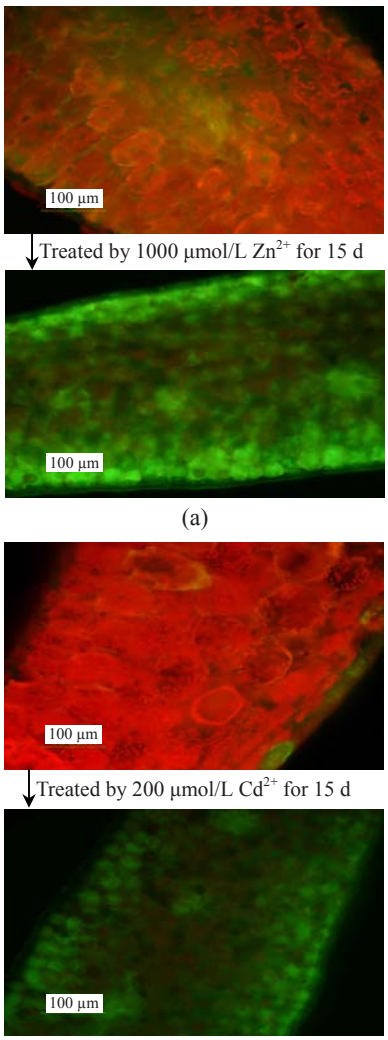

Fig.4 Cellular distribution of H_2O_2 in leaves of *Sedum alfredii* Hance. (a) Zn^{2+} treatment of 1000 μmol/L for 15 d; (b) Cd^{2+} treatment of 200 μmol/L for 15 d

Total sulfur accumulation in shoots

Total S content in shoots of *S. alfredii* H. increased gradually with advance of treatment time by 1000 μmol/L Zn^{2+} and 200 μmol/L Cd^{2+} within 15 d. The maximum content of S was obtained on Day 15 of the treatment (Fig.5), after then the S content slightly decreased with further increasing of treatment time. Interestingly, higher shoot S content was noted with Cd treatment than with Zn treatment. The time-course kinetic pattern of total S accumulation showed a similar trend to that of GSH accumulation, implying that GSH may be the main form of S-containing compounds in the hyperaccumulator *S. alfredii* H. The increase of total S and GSH reflect the antioxidant action in the plant under metal toxicity and the powerful chelating ability to the metals. The decline of both S and GSH after 15 d treatment indicated that SH- containing compounds were not the only and final associated ligands for Cd/Zn in the hyperaccumulating plant of *S. alfredii* H.

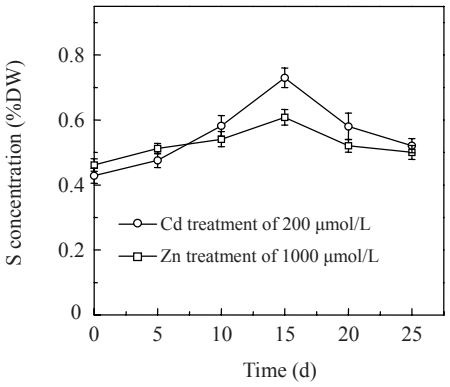

Fig.5 Time-course variations of total S contents in shoots of *Sedum alfredii* Hance

Much higher contents of both GSH and total S were found in shoots treated with 200 μmol/L Cd^{2+} than those with 1000 μmol/L Zn^{2+}, suggesting that the SH- containing compounds may play more important roles in Cd hyperaccumulation than in Zn hyperaccumulation for *S. alfredii* H. The contents of GSH and H_2O_2 decreased after 15 d treatment (Figs.2 and 3), whereas the contents of Zn and Cd in shoots still kept increasing with metal treatment time from 15 to 20 d

(Fig.1). These results imply that roles of GSH and H_2O_2 cannot totally account for long-term hyperaccumulation of Zn and Cd in the shoots of *S. alfredii* H.

CONCLUSION

Our results revealed the different responses in generation of H_2O_2 between Zn and Cd treatments in the hyperaccumulator *Sedum alfredii* H. Much higher contents of H_2O_2 were observed in leaves with Zn^{2+} treatment at 1000 μmol/L than with Cd^{2+} treatment at 200 μmol/L, whereas the reverse trend was found for GSH accumulation. The generated H_2O_2 is mainly distributed within the epidermis cells and vascular bundle, which is similar to the distribution of Zn in the hyperaccumulator *S. alfredii* H. The results suggest that H_2O_2 may play a special signal role in Zn hyperaccumulation of *S. alfredii* H. More researches are needed to further verify the role of H_2O_2 in Zn hyperaccumulation.

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