Full Length Research Paper

Effect of cadmium hyperaccumulation on antioxidative defense and proline accumulation of *Solanum nigrum* L.

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Changes in cadmium (Cd) accumulation, the activity of antioxidant enzymes including superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and the concentrations of malondialdehyde (MDA), chlorophyll and free proline in *Solanum nigrum*, Cd-hyperaccumulator were examined and compared with a non-hyperaccumulator, *Solanum lycopersicum* L. It was indicated that the root and leaf SOD, POD and CAT activities of *S. nigrum* were significantly higher than that of *S. lycopersicum*. In comparison with *S. nigrum*, there was a decrease in the growth and chlorophyll content, and an increase in MDA concentrations in the roots and leaves of *S. lycopersicum*. Although lipid peroxidation was promoted in both *S. nigrum* and *S. lycopersicum* by high Cd stress, higher increase occurred in the tissues of *S. lycopersicum*. The concentration of free proline in the leaves and roots of *S. nigrum* was higher than those in *S. lycopersicum* across all the Cd treatments. These results showed that the Cd-hyperaccumulator, *S. nigrum* had a greater capacity than *S. lycopersicum* to adapt to oxidative stress caused by Cd.

Key words: *Solanum nigrum* L., hyperaccumulator, antioxidative defense, proline, cadmium.

INTRODUCTION

Nearly 400 species of terrestrial plants have been identified as hyperaccumulators of various heavy metals in the world (Baker et al., 2000), which are capable of accumulating high levels of heavy metals without suffering metal toxicity or cell damage (Boominathan and Doran, 2003). The exploitation of plants to remove toxic heavy metals from the environment is being given more attention due to considerable commercial interest. So far, numerous studies have focused on the mechanisms of hyperaccumulators to tolerate and accumulate metals within their tissues (Wei et al., 2006). Although phytoextraction using hyperaccumulating plants is seen as a promising technique, a lack of understanding of the basic physiological, biochemical and molecular mechanisms involved in heavy metal hyperaccumulation prevents the optimization of the phytoextraction technique and its further commercial application.

Cadmium is not essential to plant growth, and it can interfere with physiological processes including carbon assimilation decrease, chlorophyll synthesis inhibition, oxidative stress generation, etc. (Benavides et al., 2005; Gratão et al., 2008). Hyperaccumulators have been known to accumulate Cd above 0.01% dry tissue (100 $\mu g g^{-1}$), whereas the normal range of Cd concentrations in leaf tissue (dry weight) of some species is 0.05 to 0.2 μg g^{-1}. Cadmium toxicity causes oxidative stress, which can take place possibly by generating reactive oxygen species (ROS) such as superoxide radicals (O_2^-), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^*) (Gratão et al., 2005). To scavenge ROS and avoid oxidative damage, plants possess the antioxidative enzymes superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD), glutathione peroxidase, ascorbate peroxidase and glutathione reductase, as well as nonenzyme antioxidants such as ascorbic acid and glutathione (Kanazawa et al., 2000).

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Regulation of antioxidative enzymes can provide plants with an additional protective ability against oxidative stress (Sun et al., 2007). Accordingly, hyperaccumulators should have an effective Cd tolerance strategy related to the expression of antioxidative enzymes under Cd stress (Boominathan and Doran, 2003). Free proline has been found to chelate Cd in plants and form a nontoxic Cd-proline complex (Sharma et al., 1998).

Solanum nigrum L. species are worldwide weeds of arable land, but in many developing countries, they constitute a minor food crop, with the shoots and berries not only being used as vegetables and fruits, but also for various medical and local uses. A cadmium-hyperaccumulator, *S. nigrum* species was first discovered in areas contaminated with heavy metal (Wei et al., 2006). The objectives of the present study were to investigate the changes in lipid peroxidation, antioxidative enzyme activities and free proline accumulation in *S. nigrum*, and to evaluate the role of antioxidative metabolism and proline accumulation in Cd tolerance by *S. nigrum*, in comparison with a closely botanically-related species, *Solanum lycopersicum* L.

MATERIALS AND METHODS

Plant culture and hydroponic preparation

Seeds of *S. nigrum* were collected from a non-contaminated field in the Shenyang Station of Experimental Ecology, Chinese Academy of Sciences and germinated on filter paper moistened with distilled water. After 5 weeks, seedlings with similar biomass were transferred to a 450 ml plastic jar containing 400 ml solution. The nutrient solution comprised of 3 mM KNO_3, 0.5 mM $NH_4H_2PO_4$, 2 mM $Ca(NO_3)_2$, 1 mM $MgSO_4 \cdot 7H_2O$, 4.5 µM $MnCl_2 \cdot 4H_2O$, 23 µM H_3BO_3, 0.4 µM $ZnSO_4 \cdot 7H_2O$, 0.15 µM $CuSO_4 \cdot 5H_2O$, 0.05 µM $H_2MoO_4.H_2O$, and 22 µM EDTA-Fe (Hoagland and Arnon, 1938).

The nutrient medium was continuously aerated with an aquarium air pump, renewed every 3 days, and all solutions were adjusted to pH 6.0 to 6.2. These plants were grown in 50% nutrient solution for 1 week, and then cultures were changed to 100% nutrient solution for the following two weeks. Then the seedlings were exposed for one week to the different Cd concentrations. $CdCl_2 \cdot 2.5H_2O$ (analytical purity) was separately diluted in deionized water and added to hydroponic cultures. Treatments were prepared at concentrations of Cd; 0 mg·L^{-1} (control), 0.01, 0.1 and 1 mg·L^{-1}. Plants were arranged in a completely randomized design and the experiment was repeated three times. Plants were grown in a controlled-environment growth chamber with a 16 h light period (light intensity of 350 µmol m^{-2} s^{-1}), a 25/15°C light/dark temperature regime and 60% relative humidity.

Analysis of biomass and contents of cadmium

The harvested plants were rinsed with distilled water and shoots and roots were separated. The plant samples were dried in oven at 70°C for 48 h to a constant weight after which dry weight of shoots and roots were determined. Dried plant materials were ground and the powders were digested with a concentrated acid mixture of $HNO_3/HClO_4$ (3:1, v/v) (Liu et al., 2009). The Cd concentration in the plant tissues was determined by flame atomic absorption spectroscopy (Spectra AA220, Varian).

Assays of enzyme activity and metabolite

Samples of 0.5 g fresh weight of leaves and roots were homogenized in a pre-chilled mortar under ice-cold conditions in 5.0 ml 50 mM cold Na-phosphate buffer (pH 7.8), with 0.1 mM EDTA and 1% (w/v) polyvinylpyrrolidone (PVP). After centrifuging at 13,000 rpm for 30 min at 4°C, the supernatant was used for further analyses. MDA was measured as described by Liu et al. (2004) and expressed as µmol g^{-1} fresh weight. The activity of SOD was measured as described by Krivosheeva et al. (1996). The activities of POD and CAT were determined using guaiacol and H_2O_2 substrates, respectively, as described previously (Wu and von, 2002; Pinhero et al., 1997).

The content of chlorophyll was determined in an 80% acetone extract of 0.1 g leaf (Hegedüs et al., 2001) and expressed as mg g^{-1} fresh weight. For the analysis of free proline, 0.5 g fresh weight of leaves and roots was homogenized with 5 ml of 3% sulfosalicylic acid, and the homogenate was cooled after heating for 10 min at 100°C. After centrifugation at 3000 rpm for 10 min, the content of free proline in the supernatant was measured using ninhydrin reagent at 520 nm (Zhang et al., 1990) and expressed as µg g^{-1} fresh weight.

Statistical analysis

Controls and treatments were performed in triplicates. Data were tested for statistical significance using the software SPSS13.0 for Windows. The difference was considered significant at the $P < 0.05$ and $P < 0.01$ levels.

RESULTS

It was a typical indication that chlorophyll content in the leaves decreased with increasing Cd accumulation in the leaves (Figure 1). The chlorophyll content of *S. nigrum* was decreased by Cd treatments. However, the effect was not significant from 0 to 0.01 mg L^{-1} of Cd ($P > 0.05$). In contrast, the content of chlorophyll in *S. lycopersicum* was gradually reduced with increasing Cd concentrations in the solution. The chlorophyll content in *S. nigrum* leaves were significantly higher than that of *S. lycopersicum* from 0.01 to 1 mg L^{-1} of Cd ($P < 0.05$).

Decreases in shoot and root biomasses of *S. nigrum* and *S. lycopersicum* were observed with increasing Cd concentrations in the solutions (Figure 2), however, the shoot and root growth of *S. nigrum* was generally unaffected by the Cd 0.01 mg L^{-1} treatment when compared with the control. The increased Cd in the solution reduced shoot and root biomass of *S. nigrum* by 44.46 to 54.55% and 38.10 to 64.76% in the treatments, Cd 0.1 and Cd 1 mg L^{-1}, respectively. In comparison with *S. nigrum*, the shoot and root biomasses of *S. lycopersicum* were lower than the corresponding *S. nigrum* tissues across all the Cd treatments ($P < 0.05$). The shoot and root biomasses of *S. lycopersicum* were significantly decreased by Cd treatments at an average of 33.07 to 70.9% and 24.86 to 79.71% when compared with the control ($P < 0.05$).

The Cd accumulation in the shoots and roots of *S. nigrum* and *S. lycopersicum* grown at different Cd
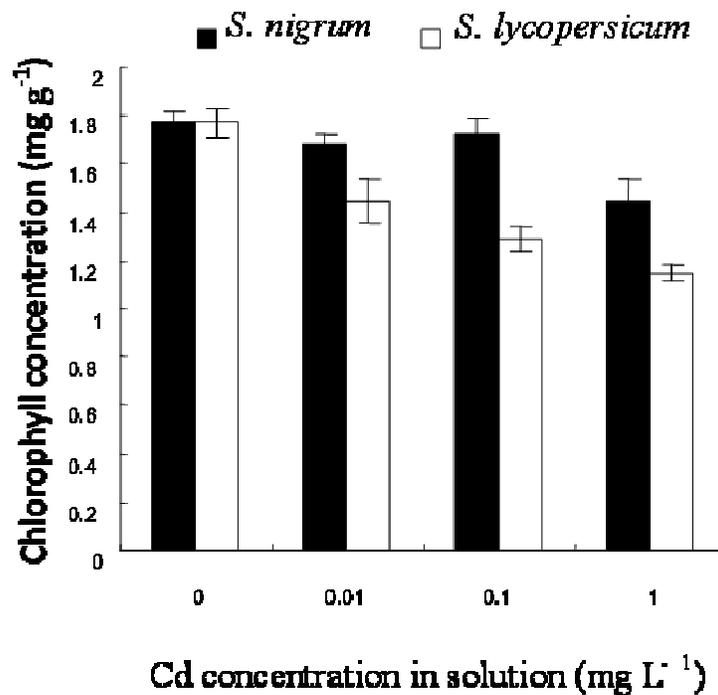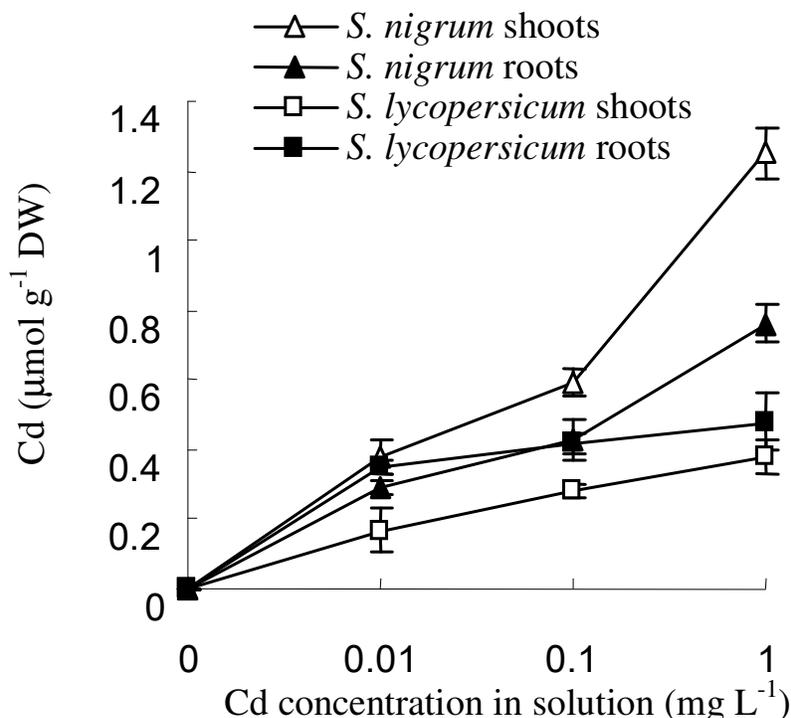
Figure 1. Chlorophyll concentrations in the leaves of *S. nigrum* and *S. lycopersicum* exposed to different Cd concentrations.
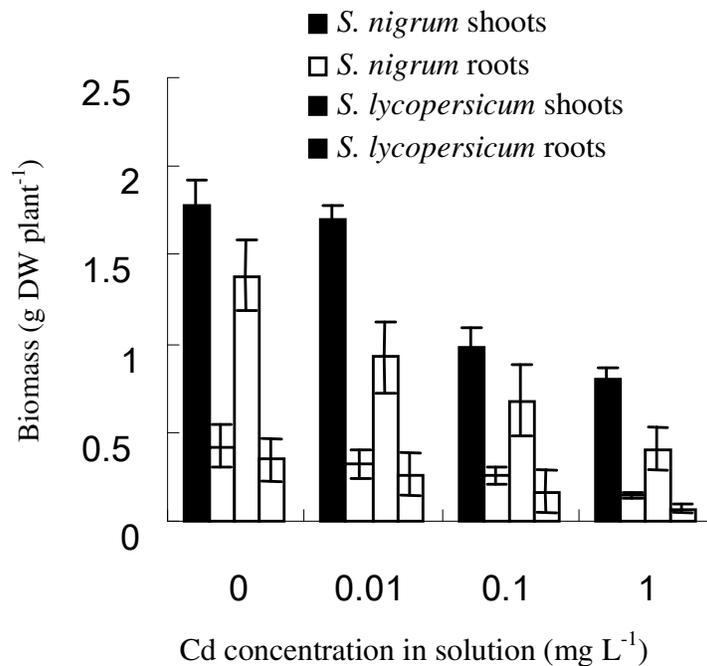
Figure 2. Shoot and root biomass of *S. nigrum* and *S. lycopersicum* exposed to different Cd concentrations.

concentrations is shown in Figure 3. The accumulation of Cd in the shoots of *S. nigrum* was always higher than that in the roots, with the translocation factor (Mattina et al. 003) varying between 1.31 and 1.64. Furthermore, the

Figure 3. Cadmium concentrations in the shoots and roots of *S. nigrum* and *S. lycopersicum* exposed to different Cd concentrations.

Cd accumulation in shoots reached a maximum of 1.25 µmol g^{-1}DW at 1 mg L^{-1} Cd. The concentration of Cd also exceeded the threshold value of 100 µg Cd g^{-1} DW in the shoots, which is used to define a Cd-hyperaccumulator (Baker et al., 1994). On the contrary, the *S. lycopersicum* had higher Cd concentrations in the roots than those in the shoots, which is not consistent with a hyper-accumulating species. In relation to Cd uptake, the concentration of Cd in the shoots of *S. nigrum* was two to three fold higher than that in *S. lycopersicum*.

The elevation in MDA content in the leaves and roots of *S. nigrum* and *S. lycopersicum* indicated that the two species were subject to Cd-induced oxidative stress (Figure 4). In general, the MDA levels in the leaves of *S. lycopersicum* were about 1.09 to 1.44 fold higher than that in the leaves of *S. nigrum* across all the Cd treatments. In addition, the MDA levels in the roots of *S. lycopersicum* were about 1.21 to 1.79 fold higher than that in the roots of *S. nigrum* across all the Cd treatments. The MDA levels in the leaves of *S. nigrum* and *S. lycopersicum* were significantly higher than in the roots ($P < 0.05$).

As shown in Figure 5, the SOD activities in the leaves of *S. nigrum* were about 1.67 to 3.11 fold higher than that in the leaves of *S. lycopersicum*. The difference in SOD activities was statistically significant between the two species across all the Cd treatments ($P < 0.05$). When compared with the control, the activities of SOD in the leaves of *S. nigrum* and *S. lycopersicum* significantly

decreased with an increase in Cd concentration ($P < 0.05$). Cadmium treatments significantly effected SOD activities in the leaves of *S. nigrum* and *S. lycopersicum*. The activity of SOD was much lower in the roots than in the leaves of both *S. nigrum* and *S. lycopersicum*, however, SOD activities in the roots of *S. nigrum* were higher than that of *S. lycopersicum* ($P < 0.05$). The POD activities of *S. nigrum* and *S. lycopersicum* decreased gradually with the different treatments of Cd, however, when compared with the control, the changes were not always significant ($P < 0.05$) (Figure 6). The POD activities in the leaves and roots of *S. nigrum* were approximately 2 fold higher than that in *S. lycopersicum* across all the Cd treatments.

Changes in the activities of CAT in *S. nigrum* and *S. lycopersicum* are shown in Figure 7. The activities of CAT in *S. nigrum* and *S. lycopersicum* leaves significantly increased with an increase in Cd concentration ($P < 0.05$). The CAT activities in the leaves of *S. nigrum* and *S. lycopersicum* were significantly enhanced by Cd treatments at an average of 52 to 88% and an average of 25 to 133.33% when compared with the control. The CAT activities in the leaves of *S. nigrum* were about 1.68 to 2.53 fold higher than in the leaves of *S. lycopersicum*. In general, the CAT activities in the roots of *S. nigrum* and *S. lycopersicum* gradually increased with increasing Cd concentrations in the solution, however the difference was not always significant for both *S. nigrum* and *S. lycopersicum* when compared with the control ($P < 0.05$).
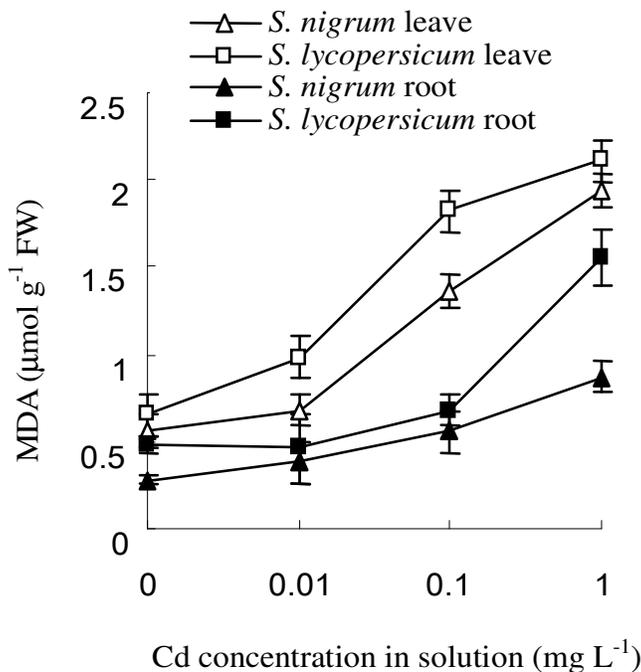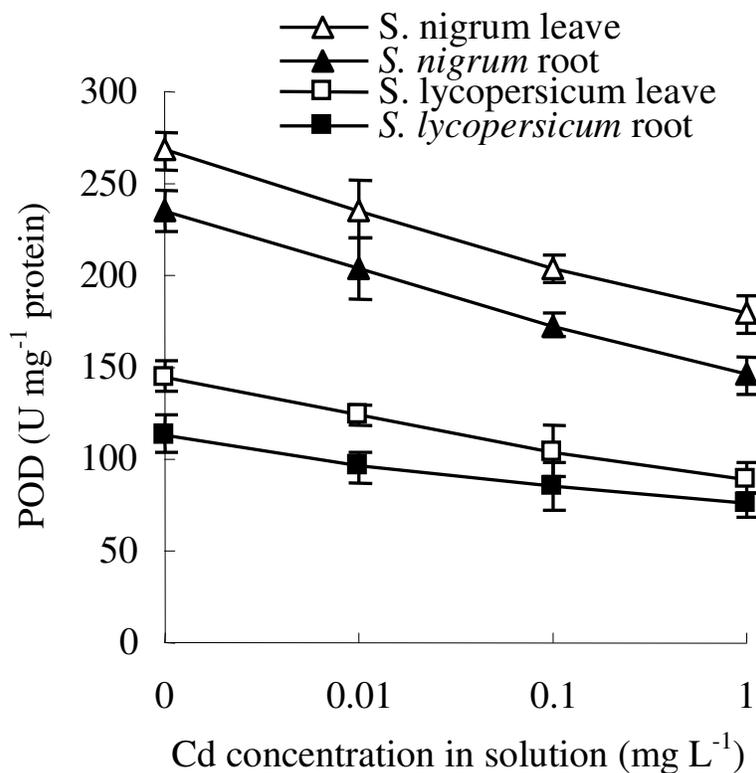
Figure 4. MDA concentrations in the leaves and roots of *S. nigrum* and *S. lycopersicum* exposed to different Cd concentrations.
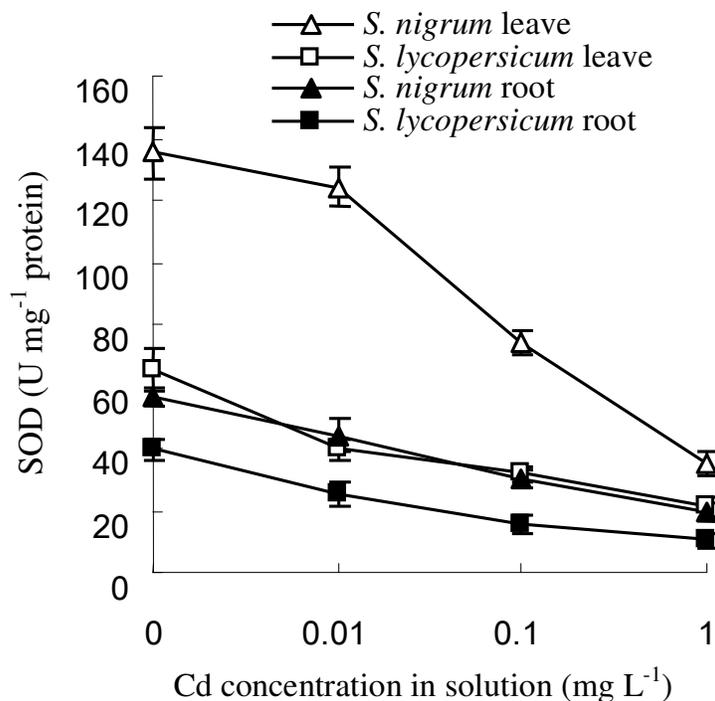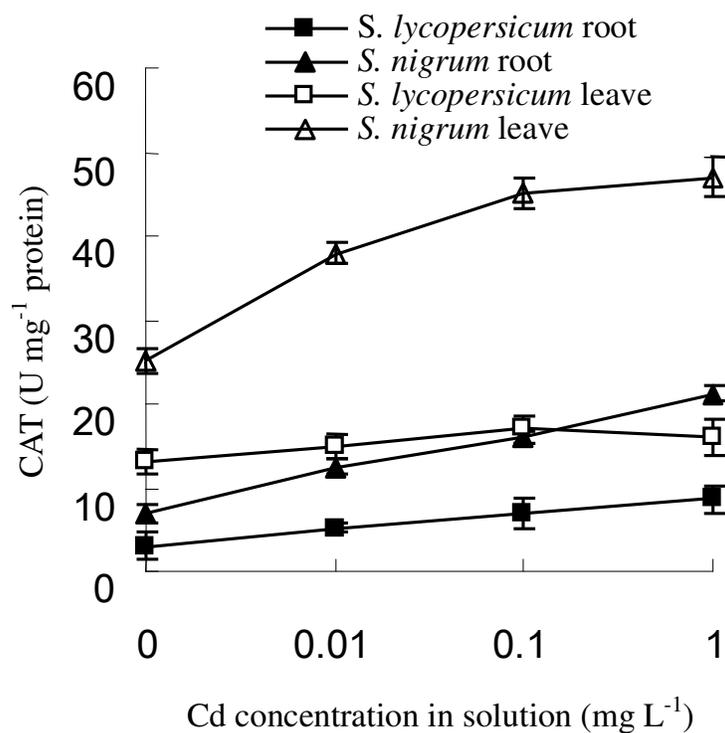

Figure 5. SOD activities in the leaves and roots of *S. nigrum* and *S. lycopersicum* exposed to different Cd concentrations.

The CAT activities in the roots of *S. nigrum* were about 1.42 to 2.29 fold higher than that in the roots of *S. lycopersicum*. On the whole, the application of Cd increased CAT activities in the leaves and roots of *S*. The
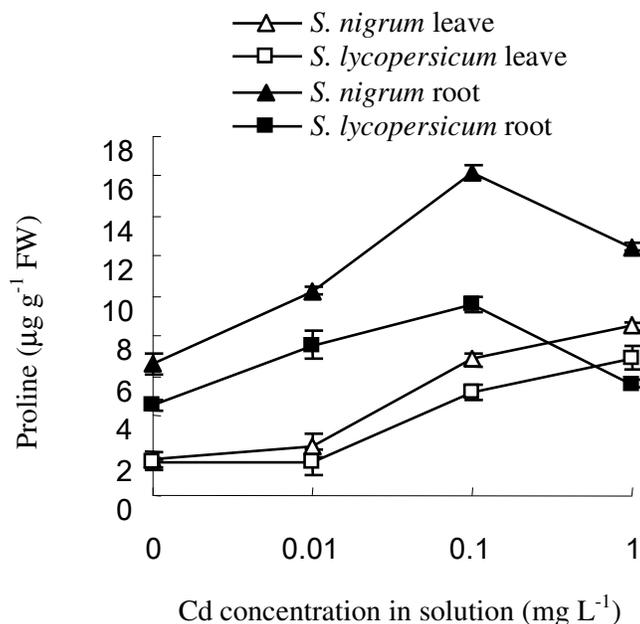
Figure 6. POD activities in the leaves and roots of *S. nigrum* and *S. lycopersicum* exposed to different Cd concentrations.

Figure 7. CAT activities in the leaves and roots of *S. nigrum* and *S. lycopersicum* exposed to different Cd concentrations.

Figure 8. Proline concentrations in the leaves and roots of *S. nigrum* and *S. lycopersicum* exposed to different Cd concentrations.

free proline contents in the leaves and roots of *S nigrum* and *S. lycopersicum* are shown in Figure 8. The concentrations of free proline in *S. nigrum* leaves were *nigrum* and *S. lycopersicum*. higher than that of *S. lycopersicum* across all the Cd treatments, however only from Cd 0.1 to Cd 1 mg L^{-1} was there significant difference (P < 0.05). Cadmium treatments increased free proline levels in the leaves of *S. nigrum* and *S. lycopersicum* by 42.86 to 385.71% and 0.6 to 313.17% (compared to the control), respectively. The concentrations of free proline in the roots of *S. nigrum* were approximately 1.36 to 2.23 fold higher than that of *S. lycopersicum*. The concentration of free proline in the roots of *S. nigrum* and *S. lycopersicum* increased from Cd 0 to Cd 0.01 mg L^{-1} and then decreased; it reached the highest level of 16.25 and 9.58 µg/g, respectively.

DISCUSSION

Cadmium is an important pollutant with high toxicity towards plants and is expected to negatively affect plant growth (Benavides et al., 2005). In the present study, with increasing Cd solution concentrations, chlorophyll accumulation was inhibited in *S. nigrum* and *S. lycopersicum,* but the chlorophyll content of *S. nigrum* was significantly higher than that of *S. lycopersicum* (P < 0.05). The inhibition of chlorophyll content in *Vigna mungo* leaves exposed to Cd stress have also been reported (Singh et al., 2008). It has been suggested that Cd interferes with chlorophyll biosynthesis and the reduction of chlorophyll

content could in turn lead to a decrease in shoot length and biomass at least in part (Orcutt and Nilsen, 2000). *S. nigrum* was able to accumulate Cd up to 1.25 µmol g^{-1} DW in shoots when the plants were exposed to Cd 1 mg L^{-1} (Figure 2). The Cd concentrations in the shoots of Cd-treated *S. nigrum* greatly exceeded the threshold value of 100 µg Cd g^{-1} DW, which is used to define a Cd-hyperaccumulator (Baker et al., 1994). On the contrary, *S. lycopersicum* had higher Cd concentrations in the roots than in the shoots, which is not consistent with a hyperaccumulating species. The Cd concentration in the shoots of S. nigrum was 2.11 to 3.29 fold higher than that of *S. lycopersicum.*

The high amount of metals in hyperaccumulator tissues suggests the existence of defense mechanisms to avoid the harmful effects caused by the metals. This study showed that the antioxidative defense system plays an important role in Cd tolerance and accumulation in the hyperaccumulator, *S. nigrum*. SOD is one of the stress-resistant enzymes and can catalyze the disproportionation of two $O_2^{\bullet-}$ radicals to H_2O_2 and O_2 (Gratão et al., 2005). H_2O_2 is also toxic to plant cells and CAT can eliminate H_2O_2 by decomposition to water and oxygen. Therefore, the combination of SOD and CAT plays an important role in the resistance of a plant to environmental stress (Sun et al., 2007). In this study, exposure of *S. nigrum* and *S. lycopersicum* to Cd resulted in a reduction of SOD activities in the leaves and roots, however, the SOD activities of the leaves and roots in *S. nigrum* were significantly higher than that in *S. lycopersicum*. The decline in SOD activities in the leaves

and roots of *S. nigrum* and *S. lycopersicum* after Cd exposure, especially at the Cd 1 mg L^{-1} treatment, indicated that the scavenging function of SOD was impaired by severe Cd stress. But *S. nigrum* resistance to Cd was still higher as compared to *S. lycopersicum*.

Simultaneously, increased CAT activities were observed in the leaves and roots of *S. nigrum* with increasing Cd concentrations. But, the Cd treatments had a negligible effect on CAT activities in the leaves and roots of *S. lycopersicum*. It has been suggested that the Cd-induced reduction of SOD could be responsible for an inactivation of the enzyme by H_2O_2 produced in different compartments, where SOD catalyses the dispropor-tionation of superoxide radicals (Vitoria et al., 2001). When SOD in the leaves and roots were inactivated, the enhanced CAT activities may promote the removal of H_2O_2. The results here indicated that the SOD and CAT enzymes in the leaves and roots of the Cd-hyper-accumulator, *S. nigrum* represented an effective defense strategy. Either an ionic microenvironment or tissue specific gene expression in plants could enhance the POD activity (Dong et al., 2006). Moreover, POD participation in lignin biosynthesis could build up a physical barrier against toxic heavy metals (Hegedüs et al., 2001). These results suggest that Cd induced physiological and biochemical changes in these plants. The activities of antioxidative enzymes could serve as important components of antioxidative defence mecha-nisms against oxidative injury.

Antioxidant enzymes and certain metabolites play an important role in adaptation and ultimate survival of plants during periods of stress (Dinakar et al., 2008; Gratão et al., 2008). Cadmium has been reported to enhance lipid peroxidation in many plant species, resulting in AOS formation (Sun et al., 2009). The oxidative stress induced by non-redox reactive heavy metals can be demonstrated by MDA formation (Gratão et al., 2005). In this study, the MDA contents in the leaves and roots of *S. nigrum* were observed to be lower than that in *S. lycopersicum*. This showed that the tissues of *S. lycopersicum* were more severely damaged than those of *S. nigrum* and that the antioxidative defense in *S. nigrum* might play an important role in Cd tolerance.

The important role of proline in the response of plants to heavy metal toxicity may be related to its antioxidative properties (Siripornadulsil et al., 2002), functioning as metal chelator (Sharma et al., 1998) and the ability to protect enzymes (Maheshwari and Dubey, 2007). Balestrasse et al. (2005) reported that proline levels increased in the roots of soybean plants with Cd stress. Dinakar et al. (2008) also reported that Cd treated plant tissues showed a significant increase in proline as compared to the control samples. In this work, *S. nigrum* plants with high Cd concentrations had more proline than *S. lycopersicum* in the leaves and roots. Therefore, this suggests that free proline might play an important protective role against Cd stress and that the hyperaccumulator, *S. nigrum* has stronger self-protection ability than *S. lycopersicum*.

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